THE EFFECTS OF SALINITY AND SMOLTIFICATION ON THE TOXICOKINETICS OF BENZO[A]PYRENE IN JUVENILE COHO SALMON (*ONCORHYNCHUS KISUTCH*) AND ADULT RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

John Matthew Seubert

B.Sc., Simon Fraser University

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ABSTRACT

The parr-smolt transformation, a developmental process, occurs in juvenile anadromous salmonids as they preadapt for a marine existence. This adaptive process may compromise an organisms ability to deal with the many potentially toxic chemicals in the environment. In addition to developmental factors, such as smoltification, the fish often encounter environmental parameters which may modify the toxicokinetics of chemicals within the organism. For example, migrating fish will confront changing salinities as it moves from freshwater streams and rivers to the marine environment. The effects of the smoltification process and salinity on the toxicokinetics of benzo[a]pyrene (B[a]P) in anadromous salmonids were investigated in this study.

Juvenile coho salmon, obtained from Capilano hatchery, showed characteristic freshwater adapted (parr) traits at the beginning of the experiment followed by a characteristic transformation into seawater adapted fish (smolts) by the end of the experiment. The baseline activities and levels of biotransformation enzymes and the tissue distribution of B[a]P were significantly altered in the fish. Juvenile coho metabolized B[a]P to both phase I and II metabolites with a high percentage of organic soluble compounds. B[a]P was eliminated via the bile within 24 hours and not altered during the transformation process. Environmental salinity had no influence on the toxicokinetics of B[a]P in the juvenile coho.

Rainbow trout subjected to either an acute increase or decrease in salinity had alterations in tissue distribution of B[a]P from fish in acclimation treatments. Rainbow trout metabolized B[a]P to both phase I and II metabolites with a higher percentage of

organic soluble metabolites in fish acclimated to saltwater than freshwater. Fish acclimated to freshwater produced larger quantities of urine compared to saltwater but no differences in the renal processing of B[a]P were observed. The majority of B[a]P was eliminated via the bile from the organism 48 h after administration. The fish exposed to B[a]P following an acute salinity change had a lower elimination rate than fish exposed in acclimated salinities. Toxicokinetic data indicated fish subjected to an acute salinity change had slower movement of B[a]P from the blood to the tissues as well as slower body clearances.

The results of this study indicate that the mechanisms and alterations involved in adapting to an environment with different osmotic demands alters the toxicokinetics of B[a]P in both juvenile coho and mature rainbow trout. This research illustrates the modulatory effects of developmental processes and environmental factors on the toxicokinetics of chemicals in aquatic organisms.

DEDICATION

This thesis is dedicated to my parents, Diane and Paul.

ACKNOWLEDGEMENTS

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AIC	Akaike's information criterion
BAN	benzanthracene
B[a]P	benzo[a]pyrene
BSA	bovine serum albumin
$CaCO_3$	calcium carbonate
CNDB	1-chloro-2,4-dinitrobenzene
CO	carbon monoxide
Ci	curie
Cyt P450	cytochrome P450
Q_b	total body clearance
dpms	disintegrations per minute
EDTA	ethylenediamine-tetraacetic acid
EROD	ethoxyresorufin-O-deethylase
g	acceleration of gravity
g	gram
GST	glutathione-S-transferase
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
i.a.	intraarterial
i.d.	inside diameter
i.p.	intraperitoneal
KCl	potassium chloride
L	litre
LSC	liquid scintillation counting
M	molar
MFO	mixed function oxidase
min	minute
MS 222	3-aminobenzoic acid ethyl ester methane sulphonate
$MgSO_4$	magnesium sulfate
N	normal
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometer
o.d.	outside diameter
O_2	oxygen

PAH	polycyclic aromatic hydrocarbon
ppt	parts per thousand
rpm	revolutions per minute
S.E.	standard error
U	units
S2	supernatant
UV	ultraviolet
Vc	volume of central compartment
v/v	volume to volume
WSSR	weighted sum of squares residuals

Part I: General Introduction

The coho salmon (*Oncorhynchus kisutch*), found distributed in the North Pacific Ocean and Bering Sea, is important to the biological and economic resources of the countries of the North Pacific rim (Croot and Margolis, 1991). The life history of the salmon has three main characteristics which include anadromy, homing, and semelparity- all of which are key to the fate of the organism. The life cycle of this anadromous fish begins as adults migrate from the sea into freshwater rivers and streams to deposit their eggs in the gravel beds. Emerging from eggs that incubated during winter months in the gravel, fry take up residency in freshwater systems for approximately one year (Sandercock, 1991). The yearling fish will naturally migrate out to sea were they have a rapid growth phase and remain until returning to spawn (Sandercock, 1991). Individuals that survive a marine existence return almost invariably to the stream or river in which they began their lives. Such homing mechanisms allow fish to return to their place of origin resulting in differentiation of populations into distinct units that are reproductively isolated (NRC, 1996). These anadromous salmon die after their first spawning season and hence only reproduce once during their lifetime.

Prior to, or accompanying the seawater migration of juvenile coho, a period of development known as smoltification occurs, which consists of a spectrum of simultaneous or consecutive morphological, physiological and biochemical changes which culminate in the adaptations necessary for a marine existence (Hoar, 1963). The process of 'parr-smolt transformation' is under both endogenous (i.e., nervous and endocrine system) and exogenous (i.e., photoperiod and temperature) control (Beouf, et al., 1992). This

transition has profound effects on, and leads to substantial adaptations of, the physiology and metabolism of anadromous salmonids (Wedemeyer, Saunders and Clark, 1979; Folmar and Dickhoff, 1980). Perhaps some of the more significant changes associated with the parr (freshwater adapted) to smolt (seawater adapted) transition, include an increase in $Na+/ K+$ ATPase activity in the gill, changes in the water permeability of osmoregulatory membranes and adjustments in water and ion movement in the intestine and urinary bladder (Barron, 1986). Other changes include reduced glycogen and elevated glucose levels in the liver (Blake and Roberts, 1984), a shift in metabolism from an anabolic to catabolic function (Wedemeyer, Saunders and Clarke, 1979), a shift to lipids as a major energy resource and an increase in metabolic oxygen demand (Sheridan, 1989; Boeuf, 1993). Smoltification is an unique phenomenon that differs from behaviors observed in other salmonids which will migrate to coastal zones to avoid unfavorable conditions (such as; flooding, pollution, lack of water) and readily return to their freshwater habitats (Beouf, 1993). The anadromous fish, while still residing in freshwater, in fact are transformed into seawater salmon before actually entering a saline environment (Beouf, 1993).

Freshwater and marine environments act as sinks for the deposition of numerous chemicals of natural and anthropogenic origin (Buhler and Williams, 1989). The detrimental impact of human activity on the environment include forestry practices, agriculture, industrial activities and urbanization which produce substances that pollute aquatic systems. There are many different compounds which are harmful to aquatic life, ranging from organic materials, excessive nutrition, suspended solids, toxic chemicals to thermal pollution (Heath, 1995). These detrimental impacts may be exacerbated by the unusual life cycle of the Pacific anadromous salmon, which require high quality

environments beginning with freshwater streams and rivers, through major rivers and estuaries to the ocean (NRC, 1996). Salmon will encounter a wide range of differing habitats during their life and poor environmental conditions may affect growth, reproductive potential or survival. During smoltification, the juvenile fish must deal with developmental changes and migrating out into drastically different environments, while coping with pollution which may ultimately affect their survival.

Early developmental stages in fish tend to be more sensitive to the toxic effects of xenobiotics than are juveniles or adults (Stegeman and Hahn, 1994). During outmigration, salmonids often encounter contaminated environments, and it has been indicated that fish are more sensitive to certain xenobiotics during smoltification or when exposed to chemicals in seawater which may effect marine survival. Exposure of smolts to water polluted from sulfite pulp mills, municipal sewage and agricultural runoff have reduced seawater adaptability, increased infestation of trematode parasites, and lowered disease resistance in the Upper Grays Harbor estuary of the Chehalis River in Washington State (NRC, 1996). In coho salmon, the toxicity of copper was found to increase as they entered the final stages of this development (Lorz and McPherson, 1976; and, Beckman and Zaugg, 1988). Outmigrants of pink salmon, sockeye salmon, and dolly varden acclimated to seawater were approximately twice as sensitive to aromatic hydrocarbons and the water soluble fraction of Prudhoe Bay crude oil as outmigrants tested in freshwater (Moles, Rice, and Korn, 1979) which may be related to an altered disposition of these compounds and a greater accumulation in target tissues (Thomas and Rice, 1981, 1986).

Water pollution encompasses a wide range of human activities which alter the chemical composition, temperature, or microbial composition to such an extent that harm

occurs to resident organisms (Lloyd, 1992). Presently, there are some 65,000 industrial chemicals in use in the marketplace and many of the toxic chemicals enter the waterways (Heath, 1995). For example, in the Fraser River, the volume of wastewater discharged into it had tripled since 1965 (Servisi, 1989). The major classes of concern to fish are metals, chlorine, cyanides, ammonia, detergents, acids, pesticides, polychlorinated biphenyls, petroleum hydrocarbons and pulpmill effluents (Heath, 1995). Polycyclic hydrocarbons (PAHs) constitute a small fraction of the complex mixture of chemicals known as petroleum hydrocarbons. They are released by natural processes such as marine seeps and forest and grass fires however the major source is from anthropogenic activities such as the pyrolysis of fossil fuels (McElroy et al., 1989). It was originally believed that the larger 4 to 6 ring PAHs, such as benzo[a]pyrene (B[a]P), were not readily available to fish once they entered the aquatic environment because they would be too tightly bound to particulate matter (Varanasi et al., 1989b). However, research found neoplasms in benthic fish, as well PAHs (i.e., B[a]P) in the sediment of the polluted areas. Concurrent research with rodents demonstrated that these compounds exert their toxic effect only after metabolic activation, which led to studies of bioavailability and toxicokinetics of carcinogenic PAHs in fish (Varanasi et al., 1989b).

The exposure concentration, duration and type of chemical influence the effect of a xenobiotic on an organism. The degree of toxicity from exposure ranges from sublethal effects to mortality to delayed effects like carcinogenicity, teratogenicity, mutagenicity and reproductive impairment (Buhler and Williams, 1991). Fish, like mammalian species, are equipped with detoxification systems to help in the removal of foreign chemicals from the body. The detoxification of the chemical is the result of biotransformation reactions,

performed by enzymes, that will alter the chemical structure of the xenobiotic, therefore enhancing its removal from the organism. This is accomplished mainly through catalytic reactions that involve oxidation, reduction, hydrolysis and conjugation of the foreign chemicals (Sipes and Gandolfi, 1986). The main consequences of biotransformation include the modulation of the concentration in the blood and tissues, bioconcentration and half-life and the conversion of toxic compounds to less toxic metabolites (Lech and Vodicnik, 1984).

Biotransformation of xenobiotic chemicals has been intensively studied in mammalian species for many years (Lech and Vodicnik, 1984) and an increasing amount of knowledge about biotransformation in fish is being gathered. Biotransformation reactions are usually classified into two categories: phase I and phase II reactions. Phase I reactions introduce polar groups into the xenobiotic molecule through oxidative, hydrolytic or reductive processes. Phase II reactions involve the conjugation of parent compound or phase I metabolite(s) with polar cellular constituents such as glucuronic acid, sulfate, or glutathione to form highly water-soluble conjugates which are easily excreted via the liver and kidney (Bend and James, 1979; Stegeman et al., 1979; Lindstrom-Seppa et al. 1981; and Buhler and Williams, 1989).

Excretion of a chemical, whether it be the parent molecule or its metabolite, reduces the potential of toxic effects to an organism. The rates and routes of excretion are dependent upon the physico-chemical properties of the chemical or metabolite as well as biotic and environmental parameters (Kennedy,1993). The excretion of xenobiotics can occur via several routes, including the gills, skin, mucous, bile, feces, and urine (Heath, 1987). Excretion appears to be partitioned on the basis of molecular size and lipid

solubility with molecules larger that 500 daltons, and more lipophilic chemicals being excreted through the liver and the smaller, more water-soluble chemicals excreted by the kidney (Kennedy, 1993; Hirom et al., 1972; Pritchard et al., 1980).

There is a growing body of evidence suggesting that environmental (Kennedy, Gill and Walsh 1993) and physiological factors (Koivussaari, 1983) can effect the toxicokinetics (including absorption, distribution and excretion) of xenobiotics in fish. Such alterations may have a major effect on tissue levels of toxicants, metabolite patterns and xenobiotic half-life in an organism. Variables such as an organisms health, nutritional, reproductive or developmental status can influence the metabolic system such that it will affect the expression of the type and quantity of enzymes present (Stegeman and Hahn, 1994). Current research indicates that hormonal status related to reproduction, development or disease can affect biotransformational systems, however the mechanisms and degree of effect are presently unknown (Stegeman and Hahn, 1994). Early life stages show that the rate of elimination of highly lipophilic PAHs is dependent on the state of development, for example in cod and killifish the larvae stages have greater elimination capacities than eggs due to a higher activity of xenobiotic metabolizing enzymes (Varanasi et al., 1989b).

The overall aim of this study was to determine the effects of the developmental process smoltification and the environmental factor salinity on the toxicokinetics of the model xenobiotic, benzo[a]pyrene, in fish. The first specific objective of the present study was to determine the approximate status of juvenile coho salmon with regards to the stage of smoltification. The second objective was to determine the effects of smoltification on the distribution, metabolism and elimination of benzo[a]pyrene in juvenile coho salmon.

The third objective of the study was to examine the effects of salinity on the distribution, metabolism and elimination of benzo[a]pyrene in juvenile coho and adult rainbow trout.

Part II: SMOLT STATUS

Introduction

All of the Pacific salmonids hatch in freshwater streams and rivers before migrating to the ocean. Before entry into the ocean, a specific stage of development referred to the 'parr-smolt transformation' or 'smoltification', occurs were the young salmonid undergoes external and internal transformations to prepare for growth and survival in a marine environment (Boeuf, 1993). The migration of coho downstream towards the sea begins in the spring after the first year in freshwater. The seasonal timing of migration is influenced by factors such as; the size of the fish, flow conditions, water temperature, dissolved oxygen levels, day length and food availability (McKeown, 1984; Sandercock, 1991). However, changes and differences in environmental conditions which affect their growth results in different migration and development times (McKeown, 1984; Sandercock, 1991). In general, peaks in outmigration of coho differ from California to those in the Gulf of Alaska by about one month, with the higher latitude stocks migrating later (Sandercock, 1991). Hatchery fish are affected by factors such as the physical and biological conditions of the facilities which differ from those observed in the natural environments. For example, hatchery rearing systems lack diversity in habitat structure, cover, diversity and temperature regimes, and exposure to natural prey and predators (NRC, 1996). These differences reveal the importance in understanding the status of the salmons preparedness for a marine existence and its migration timing.

Researchers have elucidated many changes associated with parr-smolt transformation, which is a progressive sequence of gradual events preparing the organism to enter the marine environment (McKeown, 1984; Boeuf, 1993). Various aspects of the transformation period have been studied and monitored from the early stages (parr) to late stages (smolt) in the different salmon species. External parameters have proven to be useful indices of smolting status, such as changes in coloration from the typical parr marks to the silvery appearance of a smolt, emergence and growth of teeth and a change in body shape (Boeuf, 1993). These morphological and growth aspects however are not sufficient by themselves to determine true smolt status (Folmar and Dickhoff, 1980; Zuagg, 1982).

There are marked metabolic and biochemical changes including increased oxygen consumption rates in smolts (Baraduc and Fontaine, 1956) and depletion of energy reserves during the transformation period (Sheridan, 1989). Increases in tissue lipolytic rates and decreases in lipid synthesis have been observed (Sheridan et al., 1985b) as well as a shift to a higher proportion of long-chain polyunsaturated fatty acids in smolts (Sheridan et al., 1985a). Major changes in hormone production and use are involved in the transformation of the smolts which appear closer to saltwater fish than freshwater salmon in certain aspects, such as the fatty acid composition of the membrane systems (Sheridan, 1989; Boeuf, 1993).

The ability of the anadromous salmon to control its internal osmolarity in the face of a changing osmotic environment is crucial for survival as it moves from freshwater to saltwater. The fish move from an environment which is hypotonic (freshwater) to their body to one that is hypertonic (saltwater), thus they have to alter their osmoregulatory ability (Kirschner, 1991; Evans, 1993). The organisms have to maintain appropriate

quantities of water, as well as various solutes, to remain at a homeostatic level of water and solutes. In the freshwater streams and rivers the fish are faced with eliminating excess water and retaining salts, whereas in the marine environment, the organism has the reverse problem and must retain water and deal with excess salts. Changes in water permeability of osmoregulatory membranes and adjustments in water and ion movements are observed. As the salmon reach the end of the transformation they remain in the estuary, brackish water, for a short period of time to progressively adapt to increased salinity (Zuagg et al., 1985).

The main organs involved in osmoregulation in coho are the skin, intestine, kidney, urinary bladder and the gills. Examples of physiological and biochemical changes during smoltification which preadapt fish to a seawater existence in terms of osmoregulation include increases in gill Na+/K+-ATPase activity, increased gill chloride cell density, a rise in fluid found in the intestinal tract and decreases in kidney glomular filtration rates and urinary bladder ion absorption in saltwater (Boeuf, 1993).

The capacity of an organism to utilize Na+/K+-ATPase for uptake and extrusion of NaCl in the environment is easily monitored by sampling from the gills. The Na+/K+-ATPase activity is found in the chloride cells of the gills were the basolateral infoldings develop tubular reticulum whose membrane contains the enzyme (Pagliarani et al., 1991). In seawater, fish have numerous, large and mitochondrial rich chloride cells spanning the whole gill epithelium in clustered bunches, separated by accessory cells which are connected by permeable leaky junctions (Pagliarani et al., 1991). The freshwater fish have rare and generally isolated chloride cells, poor mitochondria, few ATPase enzymes and tight junctions (Zuagg, 1982; Pagliarani et al., 1991). The increase in gill Na+/K+-

ATPase activity reflects the change in gill epithelium and increase in chloride cell content, complexity of basolateral membranes and density of enzyme units (Eddy, 1982; Pagliarani et al., 1991), as well differing isoforms of the enzyme in freshwater adapted organisms compared to saltwater adapted (Pagliarani et al., 1991).

There are many other changes associated with smoltification which can be used together for indicating the fishes preparedness for living in the ocean. For example, the levels of gill Na+/K+- ATPase activity can be used as a valuable indicator of saltwater adaptability in migratory salmonids (Wedemeyer et al., 1979; Hoar, 1988; Boeuf, 1993). Thus, since the objective of this section was to determine the approximate status of juvenile coho salmon with regards to a seawater existence, or stage of smoltification, the monitoring of both gill Na+/K+- ATPase activity and gross morphology were utilized.

Materials and Methods

A) Fish

Yearling coho salmon, *Oncorhynchus kisutch*, were obtained from Capilano Hatchery in North Vancouver, B.C. Fish from the same cohort were transported each month from the hatchery to the laboratory starting in February until June when the fish were released from the hatchery. Fish were maintained at seasonal temperatures (4-8°C) and under natural photo periods in 500 L fiberglass tanks supplied with flowing decholrinated water at pH 6.4, O_2 saturation >95% and hardness 5.2 to 6.0 mg/L $CaCO_3$ for several days until an experiment was started. Fish were fed 48 hours prior to any experiments.

B) Chemicals

All chemicals (reagent grade) and biochemicals were purchased from Sigma Chemicals (St. Louis, Mo.).

C) Determination of Gill Na+/K+-ATPase and Morphological Changes

Gill tissue was analyzed for Na+/K+-ATPase activity using methods of Flick et al., (1983), Madsen and Naamansen (1989), Fiske and Subbarow (1925) and Peterson (1978). Gills were removed from the arches, placed on ice and homogenized in 1 ml of buffer (0.3M sucrose, 0.2M imidazole and 0.7M Na_2EDTA, pH 7.2) at 4°C. The homogenate was centrifuged for 16 minutes at 5000 rpm, in a Model L7 Beckman ultracentrifuge. The resulting supernatant was centrifuged at 21 000 rpm at 4°C for 21 minutes. The S2 supernatant was centrifuged at 42 000 rpm at 4°C for 65 minutes The S3 supernatant was removed and the microsomal pellet was resuspended into 1 ml homogenization buffer. Membrane fractions were isolated and stored at -80°C in 0.5 ml of a medium containing

0.3M sucrose, 0.2M imidazole and 0.7M Na_2EDTA, pH 7.2. The membrane protein content was determined using the spectrophotometric method of Bradford (1976). An assay was started by the addition of a 25µl sample of the membrane preparation containing 10 to 40 µg/ml protein to 500µl medium A and B, and incubated for 15 to 20 minutes at 30°C. Medium A (ouabain-sensitive) contained 0.14M NaCl, 0.03M KCl, 0.005M $MgCl_2$, 0.03M imidazole, 0.01M Na_2EDTA and 0.05M Na_2ATP; medium B (ouabain-insensitive) contained 5mM ouabain added to medium A. The incubation was stopped by the addition of 1 ml of ice cold 1.25% acid ammonium molybdate, 0.5 ml of 5% SDS and 0.1 ml of 0.025% 1-amino-2-napthol-4-sulfonic acid (ANSA). These solutions were further incubated at room temperature for 30-45 minutes, upon which they were spectrophotometrically read at 700 nm against a blank of incubation reagents and 25µl of doubled distilled H_2O. A standard curve for inorganic phosphate was established.

One of the morphological changes associated with smoltification is a change in body coloration. Freshwater parr are characterized by dark pigmented melanin bars on the lateral surface, perpendicular to the lateral line which become less visible during smoltification (Fessler and Wagner, 1969; Folmar and Dickhoff, 1980). Fish were monitored from February to June and these coloration changes were recorded.

D) Mathematical Analysis

One-factor ANOVA and Student-Newman-Keuls multiple comparison test were used to establish differences between Na+/K+-ATPase activities on the different sample dates at $p<0.05$ (Zar, 1974).

Results

Gill Na+/K+-ATPase activities (Figure 1) are represented as means and standard errors of five fish. Na+/K+-ATPase activities showed an initial significant peak at the end of March and then increased by approximately 3 fold by the end of May which continued through the last sample date in June. The activities ranged from Feb. 03: 0.97±0.13 to May 28: 4.31±0.54 μmoles/ mg protein/hour.

The characteristic freshwater parr marks (dark pigmented bars on the lateral surface, perpendicular to the lateral line) were present at the start of the sample period. They remained distinct until May when there was a progressive disappearance until the characteristic silver appearance of the transformed smolt appeared at the end of the sample period in June.

Figure 2.1. Na+/K+-ATPase activities in the gills of yearling coho salmon from February to June. Values are means ± SE for six fish. Values with common symbols are not significantly different at p< 0.05.
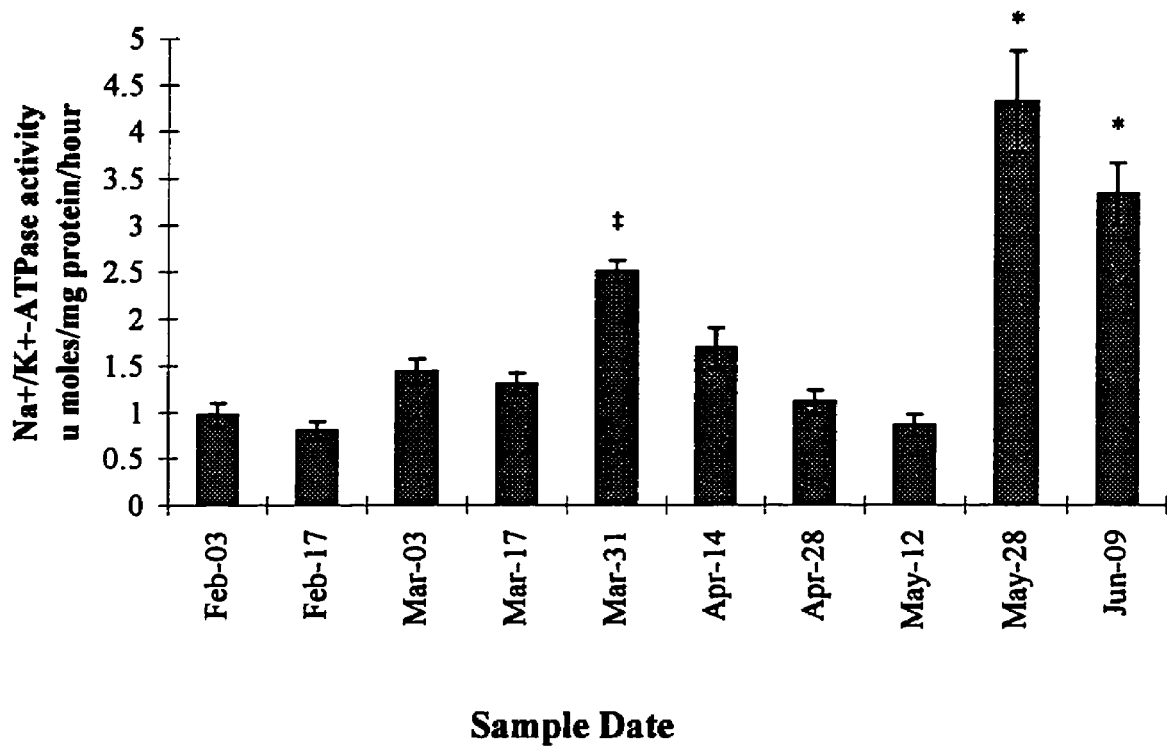
Discussion

The objective in this chapter was to determine the parr-smolt status of coho from February to June. Two parameters were used to monitor the transformation: morphological changes and gill Na+/K+-ATPase activity.

The morphological changes in colouration from the distinctive dark 'parr' marks to the silvery 'smolt appearance' were the same as indicated by Folmar and Dickhoff (1980). The present results of a progressive disappearance in parr marks and the characteristic silver appearance (owing to purine deposition on the scales) at the end of the sample period in June, are consistent with other results measured in juvenile coho salmon of a steady increase in skin guanine (a purine) levels peaking in June (Rogers et al., 1987). As well, the observable changes in body shape, such as emergence and growth of teeth and more slender streamline appearance were consistent with the literature (Fessler and Wagner, 1969; Folmar and Dickhoff, 1980; Boeuf, 1993).

Gill Na+/K+-ATPase is the key branchial enzyme for salt extrusion in seawater adapted salmonids (Madsen and Bern, 1992) and is used as an indicator of the progression of the parr-smolt transformation and the general level of hypoosmoregulatory ability (Zaugg and McLain, 1972; Folmar and Dickhoff, 1980; Madsen and Bern, 1992). The biochemical and physiological events in the gills of fish adapting to seawater involve the capacity of the organism to uptake and extrude NaCl in the environment. A seasonal rhythm of the branchial Na+/K+-ATPase activity associated with smoltification is influenced by external factors such as photoperiod and temperature (Lasserre et al., 1978; Boeuf, 1993). The early peak in March roughly coincides with the spring equinox when

daylight represents about 12 hours per day (Lasserre et al., 1978). The present study showed a cyclic change in gill Na+/K+-ATPase activity, peaking first in mid-late March and then in late May reaching a maximum, similar to that reported in coho salmon by Zaugg (1982) and Rodgers et al. (1987). This cyclic change was found in hatchery-reared fish but was not evident in the juvenile coho reared in the wild (Zaugg, 1982; Rodgers et al., 1987).

It can be concluded from the results that the hatchery fish utilized in this study showed characteristic freshwater adapted (parr) traits at the beginning of the experiment followed by a characteristic transformation into seawater adapted (smolt) fish by the end of the experimental period.

Part III: Developmental Modulators: Effects of Smoltification on the Distribution, Metabolism and Elimination of Benzo[a]pyrene in Juvenile Coho Salmon

Introduction

Knowledge of the effects of xenobiotics on molecular, subcellular and cellular systems must be attained to better understand the effects of xenobiotics on aquatic organisms.   Comprehension of the unique and special adaptations to life in aquatic environments and their interactions with foreign chemicals will provide knowledge useful in understanding the biological processes that govern the fate and effects of the chemical in aquatic animals.   The parr-smolt transformation process is one of the many unique adaptations and developmental stages seen in fish and involves fundamental morphological, biochemical and physiological alterations which may influence the distribution, metabolism and elimination, and hence fate, of xenobiotics.

Both freshwater and marine environments act as sinks for the deposition of numerous xenobiotics of natural and anthropogenic origin.   Xenobiotic compounds may be readily available in the aquatic environment and depending upon various factors, such as the metabolism and elimination capabilities of an organism, compounds can have detrimental effects on them (Heath, 1995).   The physico-chemical properties of the chemical such as lipophilicity, presence of acidic or basic groups and their pKa, and the size of the molecule, can separately and together influence the fate and effects of compounds on organisms (James and Kleinow, 1994).

Once absorbed by an organism, a xenobiotic becomes available for distribution throughout the body. The movement of the chemical to the tissues is determined by the

blood flow through the organ and the ease with which it crosses the cells of the capillary bed and penetrate the cells of the particular tissue (Klaassen, 1986). Various factors affect the fate of the xenobiotic within an organ, particularly the chemicals hydrophobicity, dissociation tendency and size, all of which influence its ability to pass through the cell membrane and affinity for the tissue (Klaassen, 1986). Some chemicals readily pass cell membranes and accumulate within a tissue as a result of protein binding, active transport, or high solubility, whereas other chemicals lacking these capabilities can be restricted in their distribution through the body. Once a xenobiotic reaches a specific tissue it may be stored until eliminated from the body. The concentration of toxicants at storage sites, remaining at equilibrium with free toxicant in the plasma, deplete as chemicals are biotransformed or excreted from the body (Klaassen, 1986). Some of the major storage sites for xenobiotics include plasma proteins that bind compounds, fat, bone, liver and kidney. The accumulation of xenobiotics in the liver and kidney relate to their importance in detoxication and elimination of chemicals from the body (Klaassen, 1986).

Lipophilic compounds which are readily absorbed and poorly excreted, have to be chemically changed to a more hydrophilic form for excretion out of the organism. The biochemical processes which convert xenobiotics to forms that are more readily excreted from the body are termed biotransformations (Sipes and Gandolfi; 1986). There are a large number of different enzymes which act on diverse types of substrates involved in the metabolic conversions of foreign compounds. Many of these enzymes and systems are under modification by internal variables, such as sex, developmental and reproductive status (Stegeman and Hahn; 1994).

The biotransformation of xenobiotics enzymatically converts a chemical from one structure into another, thereby altering its biological activity and potential interaction with the organism (Stegeman and Hahn, 1994). There are a large number of enzymes involved in the transformation of foreign compounds which can convert toxic structures into non-toxic forms, non-toxic structures into toxic forms and lipid soluble compounds into more water soluble compounds (Bend and James, 1978; Gelboin, 1980; Tan and Melius, 1986; Stegeman and Hahn, 1994). Early researchers believed that aquatic species did not metabolize xenobiotics, and that the elimination of unmetabolized xenobiotics across epithelial surfaces was a dominant process (Brodie and Maickel, 1962). Research has now shown an appreciable ability of fish to metabolize both natural and anthropogenic chemicals.

There are two general types of transformation reactions, phase I and phase II reactions. (Sipes and Gandolfi, 1986). The ultimate goal of biotransformation is to convert the xenobiotic into a structure that is more water-soluble molecule and thus more readily excreted.

Phase I reactions involve oxidations, reductions and hydrolyses which serve to add or expose functional groups (e.g., -OH, -SH, -NH$_2$, -COOH). The oxidative metabolism of xenobiotics, involving molecular oxygen, is the predominant pathway of a majority of lipophilic compounds (Stegeman and Lech, 1991). Two major groups of oxidative enzymes, the cytochrome P450 system (P450 or mixed function oxidase system) and the mixed function amine oxidases (flavin monooxygenases) are involved in the reactions (Sipes and Gandolfi; 1989). The cytochrome P450 system, being the most important, comprises a superfamily of related hemoproteins, that, in conjunction with several other

enzymes serve as an electron transport system to catalyze both endogenous and exogenous monooxygenase reactions of biological significance (Collier et al., 1995). They can be divided largely into synthesis and degradation reactions of endogenous substrates, such as steroids and fatty acids, and the metabolism of foreign compounds (Buhler and Williams; 1989). When a P450 enzyme becomes reduced (Fe^{2+}) it may form a ligand with carbon monoxide that will have a maximal absorbance of at 450 nm. This spectral property, present only when the enzyme is intact and functional, can provide a measure of the total P450 content in a microsomal fraction (Omura and Sato, 1964). However, measurement of the total P450 content of a microsomal fraction does not reveal the identity or function of any specific isoform present (Okey, 1990). In the current classification system, there are 36 gene families identified; the enzymes found in the first four families are the most predominant in the metabolism of xenobiotics (Stegeman and Hahn, 1994). The first gene family (CYP 1A) has been shown to include substrates such as 7-ethoxyresorufin and PAHs such as B[a]P, hence reactions involving these compounds are strongly influenced by the degree of expression of the particular isozymes (Buhler and Williams, 1989; Stegeman and Hahn, 1994; Heath, 1995). As well as detoxification of xenobiotics, CYP 1A can also play a role in the activation of xenobiotics such as aromatic hydrocarbon carcinogens. The measurement of molecular and catalytic properties of the teleost ethoxyresorufin O-deethylase (EROD) enzyme has been used as a diagnostic tool to indicate the presence of CYP 1A (Burke and Mayer; 1974; Stegeman, 1991; Stegeman and Hahn, 1994).

The phase II system involves biosynthetic or conjugation reactions which covalently link endogenous molecules (e.g., glucuronic acid, sulfate) to foreign compounds or phase I metabolites, thereby, increasing their water-solubility. These reactions require energy,

which is accomplished by activating cofactors or substrates to higher energy intermediates, thus the energy status of the organ and organism is important (Sipes and Gandolfi, 1986). The major pathways involve glucuronidation, sulfation and glutathione conjugation of compounds and the route depends upon both the nature of the chemical and characteristics of the enzymes (George, 1994). In fish, the major pathway for electrophilic compounds is conjugation with glutathione, and glucuronic acid conjugation with nucleophilic compounds (George, 1994). The UDP-glucuronosyl transferases catalyze the transfer of glucuronic acid to wide variety of substrates forming glucuronides, thereby enhancing inactivation and subsequent excretion of both endogenous and xenobiotic compounds in an organism (Sipes and Gandolfi, 1986). For example, the formation of glucuronides are important in the termination of thyroid hormone action; biliary T4 (thyroxine) glucuronide has been identified in some FW teleosts (Sinclair and Eales, 1972). The sulfotransferases conjugate hydroxyl groups of polyaromatic hydrocarbons to form sulfate monoesters. Phase I metabolites of B[a]P have the potential for either route (sulfation or glucuronidation); in rat hepatocytes at low doses sulfation is preferred but at high doses glucuronidation is preferred (Foureman 1989; George, 1994). The glutathione S-transferases, are a multigene superfamily of enzymes that provide protection from peroxidative damage, enhance cellular transport and enhance the detoxication of compounds (Foureman 1989; George, 1994). Glutathione S-transferases constitutes up to 10 % of the soluble cytosolic protein of the liver and are active towards metabolites of B[a]P such as B[a]P quinones, diol epoxides and the ultimate carcinogen 9,10-epoxy-7,8-dihydroxy benzo[a]pyrene (Foureman 1989; George, 1994). Presently, little is known about the enzymology and molecular biology of fish phase II systems. The mammalian

system has been studied in greater detail and provides a framework for interpretation of piscine systems (George, 1994)

Not all of the metabolic reactions performed by the biotransformation system results in detoxification of the xenobiotic (Jimenez and Stegeman, 1990). Sometimes the transformation can result in metabolites that are highly reactive compared to their parent compounds and these may be carcinogenic or toxic to the organism. An example is the metabolism of benzo[a]pyrene, which can be activated upon biotransformation to a diol epoxide (Gelboin, 1980; Stegeman and Lech, 1991; and Buhler and Williams, 1989). The formation of this B[a]P metabolite results in a product that can bind to DNA, forming a protein adduct which may lead to cancerous effects (Jimenez and Stegeman, 1990). This activation pathway has been confirmed for other polynuclear aromatic structures, leading to the bay region theory of carcinogenesis involving these compounds (Stegeman and Lech, 1991).

Recent advances have led to discovery of the large number and diversity of biotransformation enzymes, receptor mechanisms for their control and linkages with endogenous functions (Stegeman and Hahn, 1994). Of importance to understanding the full role of these enzyme systems in toxic mechanisms, is the linkages, temporally, physiologically and biochemically, between their function and regulation and other molecular events or processes (Stegeman and Hahn, 1994). Many of the enzyme systems involved in biotransformation are also engaged in critical physiological functions such as steroid hormone biosynthesis and inactivation, and fatty acid metabolism (Goksøyr and Förlin, 1992). Alterations in these systems may profoundly influence the activation and

detoxification of xenobiotics in organisms.   The unique process of smoltification, a developmental process which may modulate these systems may provide insights into early life-stage changes which can alter the susceptibility of aquatic organisms to xenobiotics.

Generalizations regarding xenobiotic excretion in fish is difficult because of the diversity of xenobiotics encountered and the multitude of excretory modes in fish (Heath, 1995).   Excretion of a chemical, occurs via various routes and mechanisms from fish, although the predominant organs include the skin, liver, kidney and gills.   The excretion of a chemical depends on its availability in plasma (which is influenced by many factors such as chemical or physical association with plasma proteins), sequestration in cells and tissues due to high lipophilicity, and circulation through an organ (Pritchard and Renfro, 1984). The metabolism of xenobiotics stimulates excretion by biotransforming the compound into a more polar and more water-soluble molecule, thereby making it available in the plasma for excretion.   Gills are an important route of excretion for smaller hydrocarbon compounds, such as naphthalene, toluene and pristane (Thomas and Rice, 1981; Le Bon et al., 1987).   Excretion of xenobiotics via the kidney depends primarily on its size, molecules larger than 500 daltons tend to excreted via the liver; and its availability because both filtration and secretion act only on chemicals present in plasma (Pritchard and Renfro, 1984).   Metabolites of benzo[a]pyrene may be actively secreted via the kidney against a concentration gradient but high lipid solubility tends to reduce the organs ability to excrete organic xenobiotics (Pritchard and Bend, 1984).   However, excretion of larger organic molecules, like benzo[a]pyrene, occurs predominantly through the bile (Thomas and Rice, 1982; Varanasi et. al., 1989; Kennedy, 1993).

The process of smoltification, in anadromous salmonids, can be considered a combination of cytological, morphological, behavioral, biochemical, physiological and endocrinological changes in the fish. This dynamic process, under both internal and external control, involves many metabolic and biochemical changes (Boeuf, 1992). Together these alterations within the organism, may effect the processes which distribute, metabolize and eliminate foreign compounds. Thus, there may be major effects on toxicant levels found in the tissues, patterns of metabolite formation, and the half-life of xenobiotics in an organism.

The first objective of this section was to determine the effects of smoltification on the baseline activities and levels of biotransformation enzymes during smoltification in the livers of juvenile coho salmon. The second objective was to determine if alterations in the distribution, metabolism and elimination of benzo[a]pyrene occurred in the coho salmon during smoltification. Benzo[a]pyrene used as a model xenobiotic in this study of the fate of chemicals in a biological systems because much research has been performed examining fate and effects in mammals and fish (Varanasi et al., 1989).

Materials and Methods

A) Fish

Yearling coho salmon, *Oncorhynchus kisutch*, were obtained from Capilano Hatchery in North Vancouver, B.C. The fish, from the same cohort, were transported each month from the hatchery to the laboratory starting in February until June when the fish were released from the hatchery. Fish were maintained at seasonal temperatures (4-8°C) and under natural photo periods in 500 L fiberglass tanks supplied with flowing decholrinated water at pH 6.4, O_2 saturation >95% and hardness 5.2 to 6.0 mg/L $CaCO_3$ for several days until an experiment was started. Fish were fed ad libum until 48 hours prior to an experiment.

B) Chemicals

Unlabelled benzo[a]pyrene (B[a]P) (>99% purity) was purchased from Sigma Chemicals (St. Louis, MO.) and [1,3,6-^{3}H]- benzo[a]pyrene (52 Ci/mmol) was purchased from NEN Research Products (DuPont, Canada Ltd., Mississauga, ON). HPLC analysis of labelled and unlabelled B[a]P according to the method of Elnenaey and Schoor (1981) revealed no detectable metabolites. B[a]P metabolite standards were obtained from the National Cancer Institute Chemical Repository, Midwest Research Institute (Kansas City, MO.). All other chemicals (reagent grade) and biochemicals were purchased from Sigma Chemicals (St. Louis, MO.).

C) Biotransformation Enzyme Analysis

The baseline activities and levels of several Phase I and II biotransformation enzymes of coho were measured biweekly starting in February until June from the same cohort as in other experiments. Six fish were sacrificed by anesthetization with 0.5 g/l MS

222 buffered with 1.0 g/l sodium bicarbonate and a blow to the head. Microsomal fractions were prepared according to Förlin and Andersson (1985) and Kennedy, Gill and Walsh (1990). The livers were removed, weighed and homogenized with a glass-teflon homogenizer in ice-cold buffer (0.15 M KCl, 0.2 M HEPES, pH 7.4) and centrifuged at 10 000g for 20 minutes in a Model L7 Beckman ultracentrifuge. The resulting supernatant was centrifuged at 100 000g at 4°C for 70 minutes. The S2 supernatant was removed and the microsomal pellet was resuspended in homogenization buffer with 20% glycerol (v/v). The supernatant and microsomal fractions were stored at -80°C until assays were run.

Microsomal and cytosolic protein concentrations of liver were measured by the method of Bradford (1976). A protein assay kit from BioRad was utilized (BioRad, Life Science Group, Hercules, California, USA) for determination of microsomal and cytosolic protein concentration. The addition of an acidic dye to the protein solution was subsequently measured at 595 nm with a spectrophotometer. The differential color change observed relates to various concentrations of protein. A standard curve was established with bovine serum albumin as a protein standard. Samples were diluted by the addition of 990 μl of ddH$_2$0 to 10 μl of either microsomal or cytosolic fractions. A 50 μl of the diluted sample was added to 1 ml of dye reagent which was vortexed and incubated at room temperature for approximately 30 minutes. The absorbance, compared to ddH$_2$0 blank, was read at 595 nm and values were extrapolated from the standard curve to determine relative protein concentration.

Total cytochrome P450 content was determined by the method of Omura and Sato (1964) which utilizes the difference between the absorption of dithionite-reduced cytochrome P450 with that of CO-bound reduced cytochrome P450. A 100 μl sample of

microsomal suspension was added to 2 ml of 0.1 M phosphate buffer and a few crystals of

sodium dithionite ($Na_2S_2O_4$).   The glass cuvette was covered with parafilm and gently

inverted while on ice (at 0 - 4°C).   A baseline absorbance spectrum for the dithionite

reduced micromes was determined between 400 and 500 nm.   The sample cuvette was

placed on ice, covered with parafilm and gently saturated with carbon monoxide gas for

approximately 1-2 minutes.   A new spectrum between 400 and 500 nm was run for the CO-

bound reduced cytochrome P450.   The difference between the absorption maximum at 450

nm (CO-bound reduced cyt P450) and the baseline at 490 nm (serving as a reference point)

was used.   An extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used to calculate the P450

content.

Ethoxyresorufin O-deethylase (EROD) activity was measured using the method of

Burke and Mayer (1974) which fluorometrically determined the formation of resorufin

from the O-deethylation of 7-ethoxyresorufin on a Perkin Elmer fluorescence

spectrophotometer (LS 50) linked to an IBM 50 Z computer.   A standard curve was

established with known concentrations of resorufin (ranging from 0.0005 to 0.05 mg/ml) in

methanol.   Standard curve determination was done exactly as the reaction assay except

10μl of resorufin replaced 10μl of 7-ethoxyresorufin.   The reaction mixture consisted of

1100μl of 0.1M HEPES (N-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid) buffer

solution pH 7.8, 50μl microsomal suspension, 10μl of 2.57 M $MgSO_4$, 50μl of 40mg/ml

BSA stock and 30μl of freshly prepared 0.5mM NADPH.   The reaction vials were

incubated for 5 minutes at 25°C followed by the addition of   10μl of 0.19mM 7-

ethoxyresorufin and incubated for a further 2 minutes.   The reaction was stopped by the

addition of 2.5 ml methanol.   In the control vials, methanol was added before the

ethoxyresorufin. The reaction vials were then centrifuged at 4400 rpm for 5 minutes at 4°C to remove proteins precipitated by methanol. The fluorescence of the reaction vials was measured at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Enzyme activity was determined by extrapolation from the resorufin standard curve.

Glutathione S-transferase (GST) in cytosol was measured spectrophotometrically as described by Habig, Pabst and Jakoby (1974). The reaction mixture consisted of 1.95 ml of 50mM HEPES, pH 7.5, 40µl of 1-chloro-2,4-dinitrobenzene (CDNB) and 25µl of 100 000g supernatant (S2; cytosolic suspension). The reaction was started by adding 25µl of 50mM reduced glutathione in 50mM HEPES. The change in absorbance at 340nm was monitored for 1-2 minutes. Controls were performed both without supernatant or glutathione in the reaction mixture.

D) Exposure to Benzo[a]pyrene

Each month from February to June, twenty fish from the hatchery were injected intraperitoneally (i.p.) with 10 mg (1 µCi)/kg of [^{3}H]-B[a]P to determine the effects of smoltification on chemical disposition, metabolism and elimination. Potential confounding effects on B[a]P uptake may have occurred if fish were exposed to B[a]P in water, since smoltification can affect respiration through an increased oxygen demand. Therefore, fish were exposed to the chemical by i.p. injection to ensure that all fish through the study received the same dose. Fish were then transferred to 120L tanks supplied with flowing dechlorinated water maintained at seasonal temperatures under a natural photo period, as described previously.

E) Tissue Sampling and Radioactivity Determination

Five fish were sampled each at 1, 2 ,4 and 7 days following injection with ^{3}H-B[a]P. The liver, kidney, visceral fat, gallbladder, and carcass were dissected, weighed, homogenized in 0.9% saline and then oxidized using an R.J. Harvey OX-100 biological oxidizer (R.J. Harvey Instruments Corp., Hillside, N.J.). The oxidized samples were dark adapted for 24 hours before being counted on a Beckman LS 3801 liquid scintillation counter (Beckman Instruments, Irvine, CA.) to determine the amount of ^{3}H-B[a]P derived radioactivity. Bile was released from excised gall bladders and rinsed with 1.0 ml sodium citrate buffer (pH 7.0). An aliquot was removed and counted for total radioactivity by LSC.

F) Biliary Metabolite Analysis

Bile from fish sampled 1 and 7 days following an i.p injection of B[a]P was released from excised gall bladders and rinsed with 1.0 ml sodium citrate buffer (pH 7.0). The method used by Kennedy, Gill and Walsh (1991) was followed for the quantitation of B[a]P and its metabolites in bile. Bile was extracted three times with ethyl acetate (5:1) to separate Phase I metabolites. The remaining aqueous layer then underwent a series of incubations and extractions; it was first adjusted to pH 5.0 and incubated for 24h at 37°C with 60U of ß-glucuronidase to hydrolyze glucuronic acid conjugates; it was then adjusted to pH 7.0 and incubated for 24h at 37°C with 20U of sulfatase to hydrolyze sulfate conjugates; and finally it was adjusted to pH 2.0 and incubated for 24h at 80°C with 1N H_2SO_4 to acid hydrolyze any remaining conjugated metabolites. After each step, the

aqueous layer was extracted three times with ethyl acetate (5:1). Aliquots of each ethyl

acetate extraction were removed, dried with nitrogen gas, resuspended in 1 ml of

methanol and added to liquid scintillation cocktail (Amersham, Oakville, ON, Canada) and

counted for radioactivity at each step by LSC.

B[a]P and phase I metabolites in the first organic extracts of bile from fish sampled

on day 7 were separated by HPLC using a Hewlett Packard 1050 series liquid

chromatograph equipped with a Perkin-Elmer HC-ODS SIL-X reverse phase column

(0.26 x 25 cm), a HP 1046A fluorescence detector and an HP Integrator (excitation 263

nm and emission 370 nm). For metabolite separation, the methods of Elnenaey and

Schoor (1981) were followed; a solvent system with a concave gradient starting with

100% Solvent B (30/70 methanol/water) as the eluting solvent and Solvent A (90/10

methanol/water) increasing in amount of methanol in a stepwise pattern. A flow rate of

0.6 ml/minute was used as in Elnenaey and Schoor (1981). Fractions were collected every

2 min using a BioRad Fraction collector. A volume of 10 ml of liquid scintillation cocktail

(Amersham, Oakville, ON, Canada) was added to each fraction, allowed to dark adapt for

at least 24 hours and counted for radioactivity at by LSC. B[a]P and its metabolites were

tentatively identified and quantified by comparison of peak retention times and dpms

recovered with retention times of known standard metabolites which included: r-7,t-8,9,c-

10-tetrahdroxytetrahdyrobenzo[a]pyrene (7,8,9,10-tetrol); 7,8-dihydroxybenzo[a]pyrene(-

)-trans-7,8-diol (t-7,8-dihydrodiol); 9,10-dihydroxybenzo[a]pyrene-(-)-trans-9,10-diol (t-

9,10-dihydrodiol); 1,6-benzo[a]pyrenequinone (1,3-dione); 3,6- benzo[a]pyrenequinone

(3,6-dione); 6,12-benzo[a]pyrenequinone (6,12-dione); 9-hydroxybenzo[a]pyrene (9-OH);

1-hydroxybenzo[a]pyrene (1-OH); and 3-hydroxybenzo[a]pyrene (3-OH).

G) Elimination of Benzo[a]pyrene

The radioactivity recovered from the oxidized tissues; carcass, liver, kidney and fat were totaled for each fish to obtain the amount dpms remaining in each organism. The elimination of [^{3}H]-B[a]P was calculated by subtracted the recovered radioactivity in the organism from the amount injected.

H) Mathematical Analysis

All percent data were arcsin transformed before statistics were performed. One-factor ANOVA and Student-Newman-Keuls multiple comparison tests were used to establish differences between tissue body burdens, B[a]P elimination, metabolite classes and biotransformation enzyme levels and activities at a significance level of $p < 0.05$ (Zar, 1974).

Results

A) Biotransformation Enzymes

Liver microsomal and cytosolic protein, cytochrome P450, EROD, glutathione S-transferase activities and levels in fish sampled from February to June are shown in Figure 3.1. Microsomal and cytosolic protein concentrations followed similar patterns, which increased two to three fold from February 3 to February 17 and then decreased to March 3. A second increasing trend towards the end of April and beginning portion of May was also observed. Cytochrome P450 levels, EROD and glutathione S-transferase activities are shown in Figure 3.2. Cytochrome P450 showed statistically significant peak activities in February and March and at the end of the sampling period in June.   EROD activity

followed a similar trend to the cytochrome P450 and protein levels showing statistically significant peak activities in February and March. Glutathione S-transferase activity rapidly decreased (approximately three fold) in activity from February to March and then remained constant until the end of the sampling period.

B) Tissue Distribution of Benzo[a]pyrene

The average weight of twenty fish sampled each month were: Feb.: 11.4 ± 0.5g, March: $11.0 \pm 0.0.4$g, April: 10.5 ± 0.5g, May 12.5 ± 0.7g, June: 12.7 ± 0.7g. There were no statistical differences observed between the months in fish weights. Visceral fat content was monitored during the sampling period because of its potential importance in the storage of lipophilic xenobiotics such as B[a]P and because of the reported changes in lipid content of salmonids during smoltification content (Fessler and Wagner, 1969; Malikova, 1959). The average weight of visceral fat dissected from twenty fish each month were: Feb.: 0.323 ± 0.016g. March: 0.389 ± 0.021g, April: 0.313 ± 0.019g, May: 0.303 ± 0.019g, June: 0.173 ± 0.015g. In March, a statistically significant increase in the weight of visceral fat was observed and in June a statistically significant decrease from the other sample periods was noted.

Following an i.p. injection of $[^3H]$-B[a]P, B[a]P-derived radioactivity was found in all tissues sampled. Figure 3.3 shows the percent body burden of B[a]P derived radioactivity in tissues sampled from February to June (1, 2, 4 and 7 days) after receiving an i.p. injection. The general trend of the body burden of B[a]P derived radioactivity in tissues over the experimental period was liver>fat>kidney>carcass. The highest levels of recovered radioactivity were found in the liver on all the sample days for every month over the experimental period, except in the month of June where fat had the higher percent body

burden. The percent body burden in the carcass remained relatively constant from February to June on all four sample days and were consistently the lowest. Kidney tended to have higher burden levels in February which decreased in June. When comparing individual tissue burdens over the experimental period, the statistically significant alterations in burdens of radioactivity in tissues occurred only on day 1. By days 2, 4 and 7 there were no statistically significant alterations in distribution patterns.

C) Biliary Metabolites

Juvenile coho salmon metabolized B[a]P to both Phase I and II metabolites (Table 3.1). HPLC analysis of bile collected at each sampling period revealed that <10% of the radioactivity recovered was parent compound. At each sample period, 55 to 60% of the recovered radioactivity was extractable Phase I metabolites, 16 to 24% were glucuronic acid conjugates, 8 to 10% were sulfate conjugates, 5 to 9% were unknown conjugates and the remaining 3 to 7% of the radioactivity recovered was an unknown water soluble metabolite(s). There were no significant differences in the proportions of these metabolite groups from February to June.

Chromatographic separation of B[a]P and organic soluble metabolites by HPLC revealed a variety of tentatively identified Phase I metabolites which included the following classes of metabolites triols/tetrols, diols, quinones and phenols (Table 3.2). When the metabolites were grouped according to major type and expressed as a percent of radioactivity in the organic soluble fraction of bile, quinones ranging from 20-40% and phenols ranging from 20-30% showed the highest accumulation. The radioactivity collected in HPLC fractions coeluted with standard metabolites accounted for 30-40% of

the organic soluble fractions. Approximately 10-20%of the organic soluble fraction eluted within the first 20 minutes of the separation which were the most polar metabolites and an indication of the presence of triols and tetrols (Varanasi et al., 1986; Varanasi et al., 1989). The remaining radioactivity did not coelute with known standard metabolites. The identified phase I metabolite which accumulated in the greatest quantity was r-7,t-8,9,c-10-tetrahydrotetrol.

D) Elimination of Benzo[a]pyrene

The elimination of B[a]P derived radioactivity from juvenile coho salmon is shown in Table 3.3. The majority of the injected B[a]P was eliminated 24 hours following i.p. injection and ranged from 71 to 94% of the total injected dose. The lowest amount eliminated (71% of the total dose) occurred in March. By day 2, all fish had excreted more than 94% of the injected dose at every sample period.

Figure 3.1. Microsomal and cytosolic protein levels in the livers of yearling coho salmon through smoltification from February to June. Values are means ± SEM for 6 fish. Values with common symbols are not statistically different at p< 0.05.
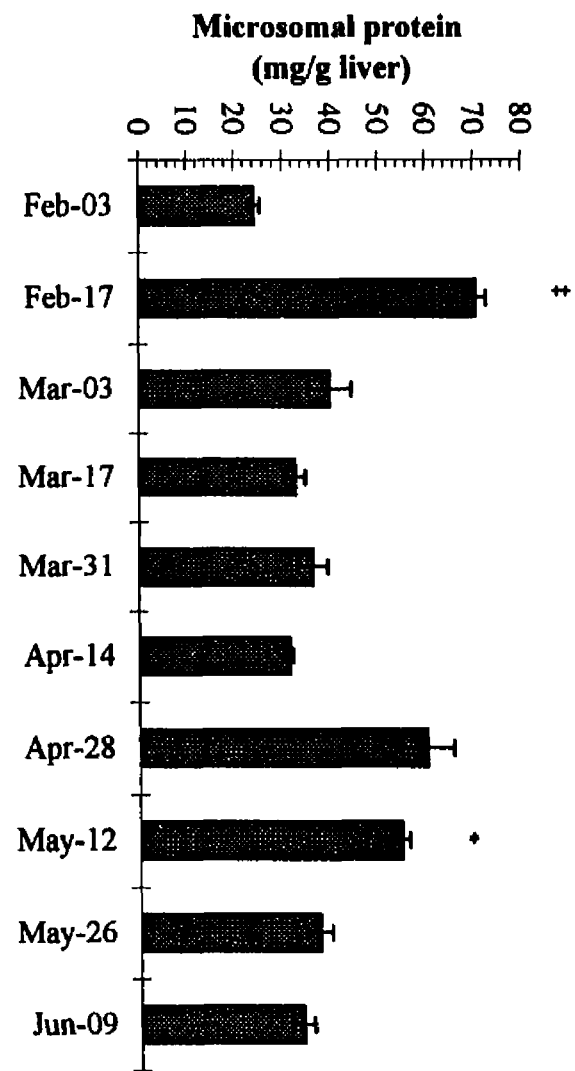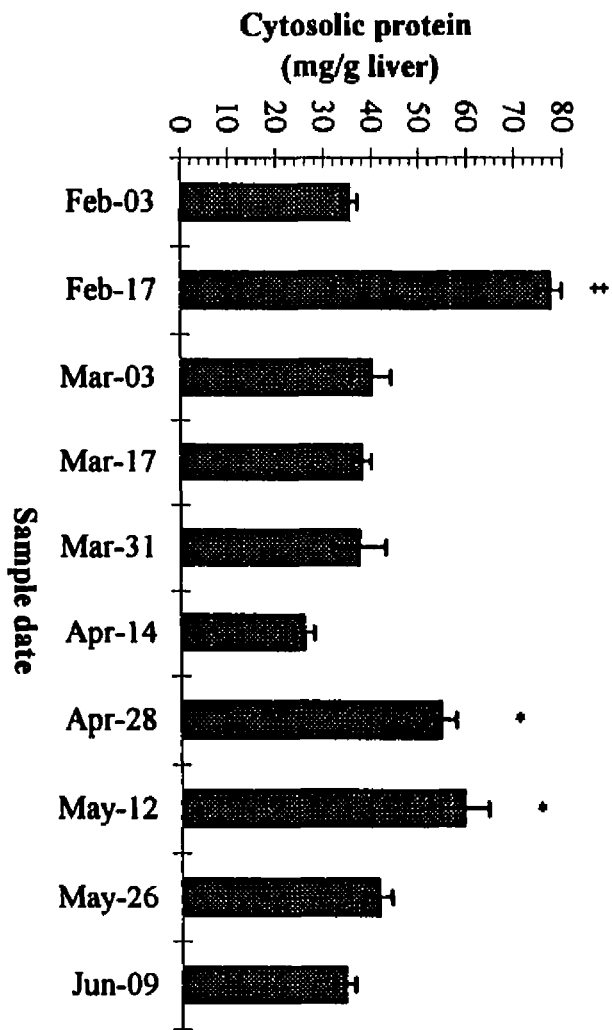
Figure 3.2. Cytochrome P450 levels and the activities of EROD and GST in the livers of yearling coho salmon through smoltification from February to June. Values are means ± SEM for 6 fish. Values with common symbols are not statistically different at $p < 0.05$.
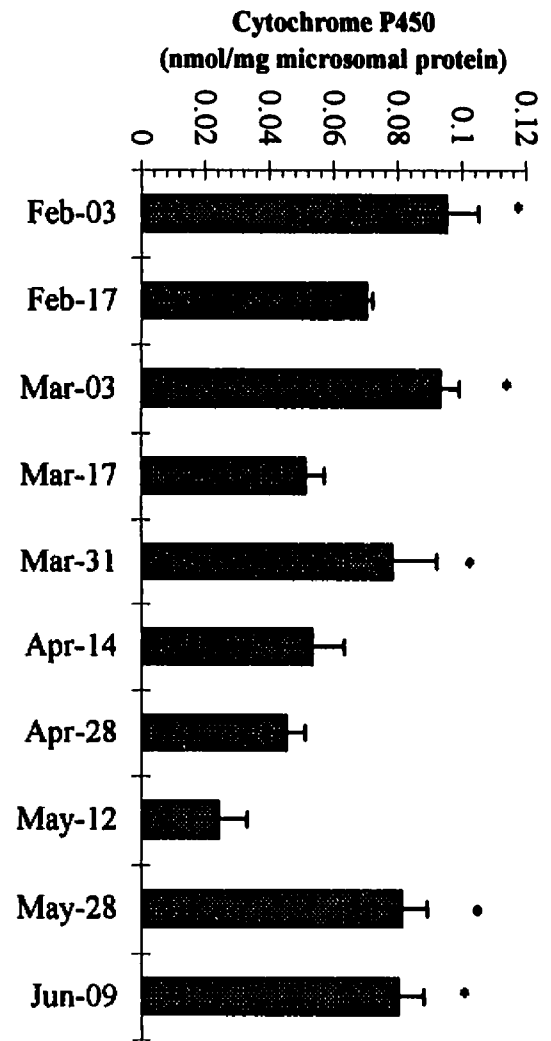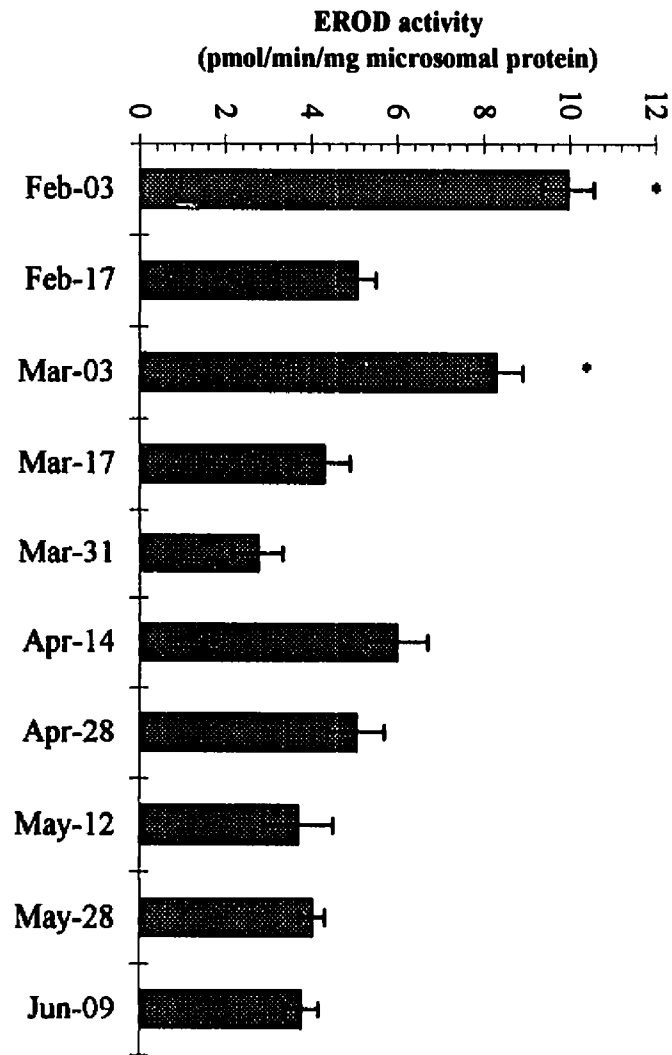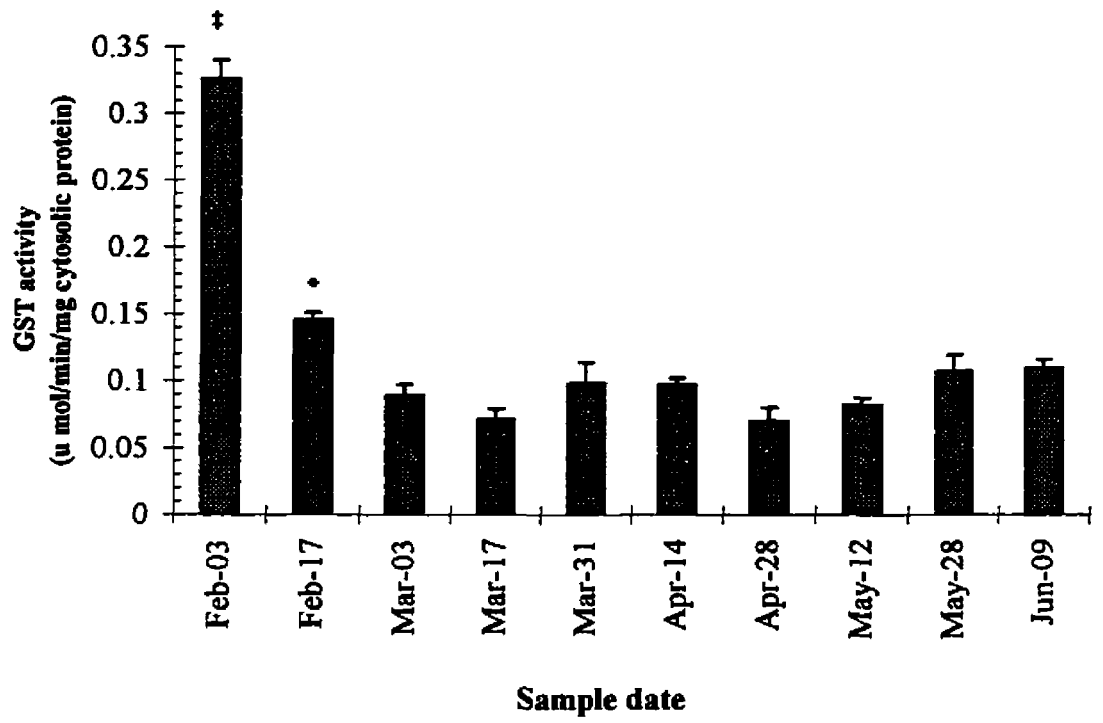
Figure 3.3: The percent body burden of [^{3}H]-B[a]P-derived radioactivity in the bile, kidney, visceral fat, liver and carcass of coho salmon, following an i.p injection of 10 mg (1μCi)/kg B[a]P. Sample dates were a) February b) March c) April d) May e) June. Values are means ± SE of five fish.

a)

February

b)

March

c)

April

d)

May

e)
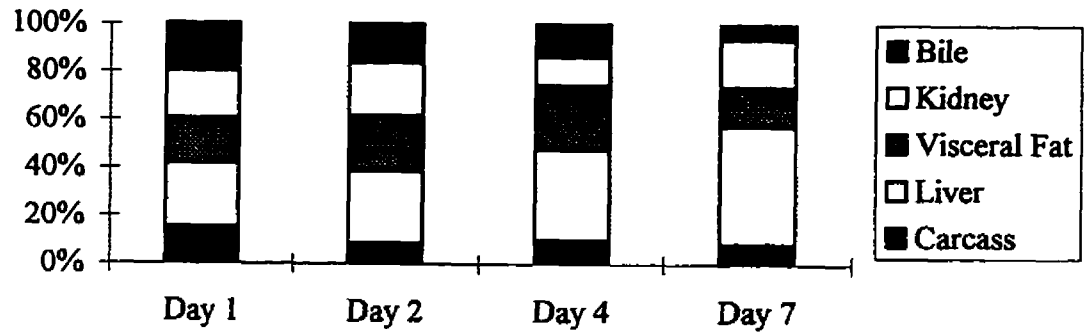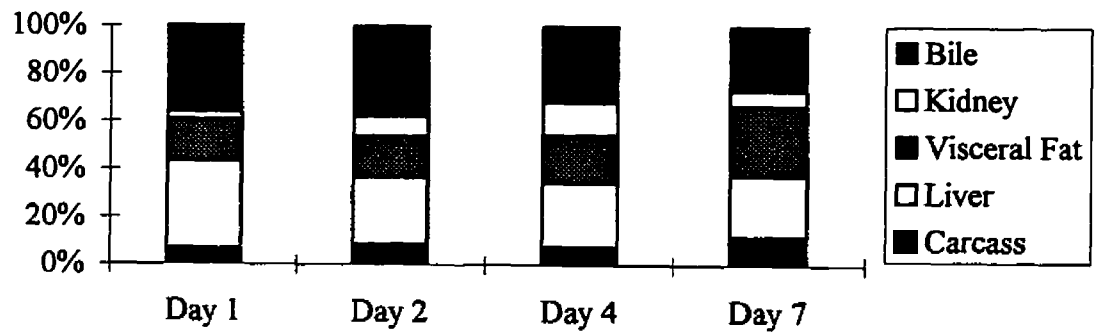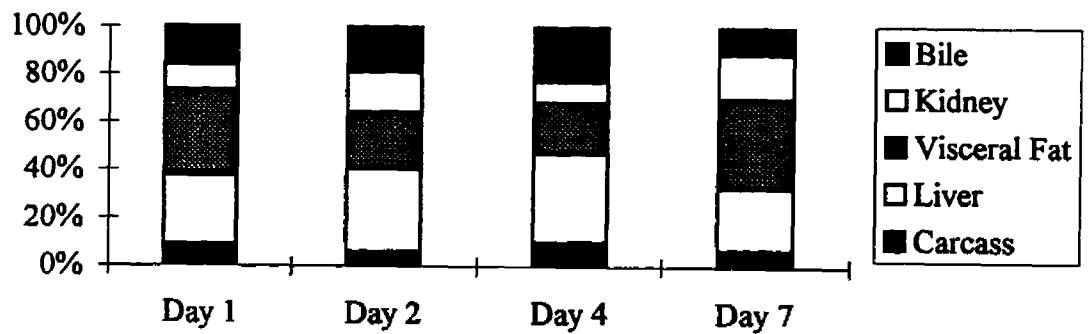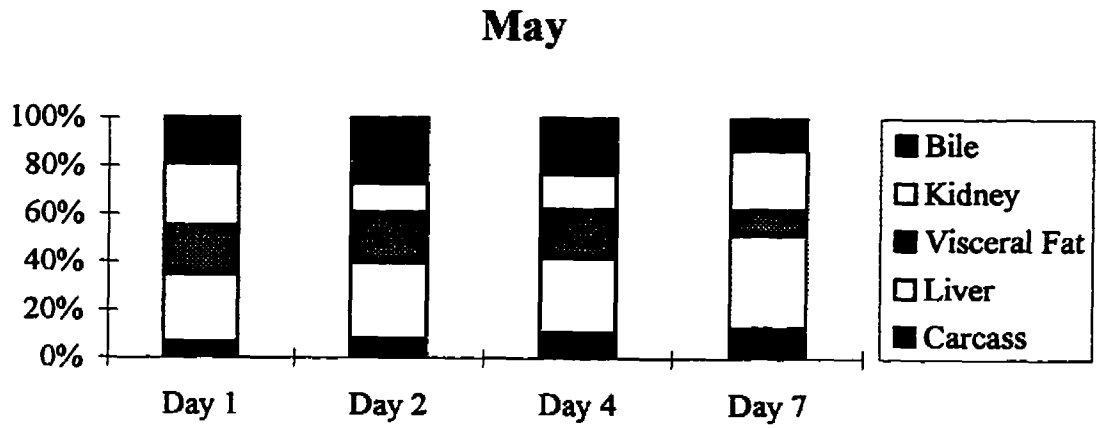
June
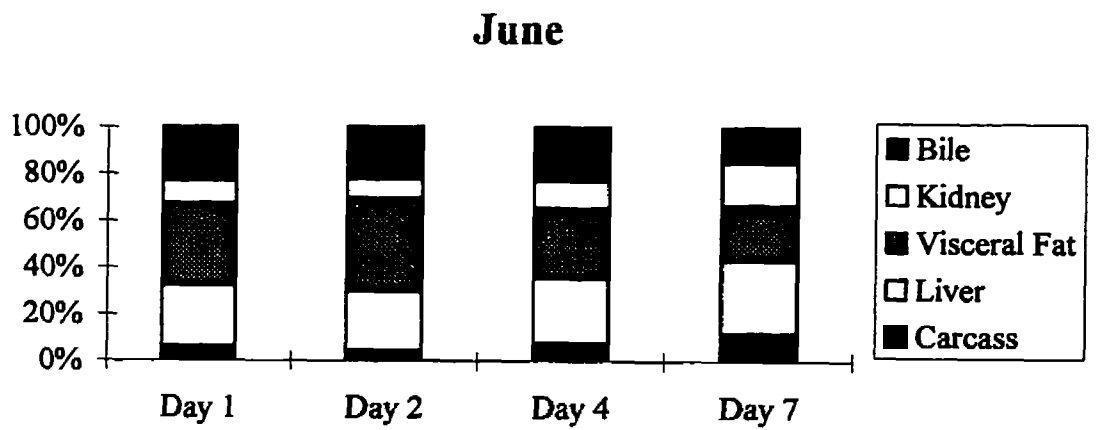
Table 3.1. The percent of total ^{3}H-radioactivity as organic soluble metabolites, glucuronide and sulfate conjugates and other aqueous soluble metabolites in the bile of coho salmon, 168 h (7 days) following an intraperitoneal injection with $10mg(1\mu Ci)/kg$ of $[^3H]$-B[a]P. Values are the means ± SE for 5 fish. There were no significant differences between months in the percents of classes of metabolites at a significance level of $p < 0.05$.

Metabolite groups (percent of total metabolites)

Bile	Organic soluble	Glucuronide	Sulfate	Unknown conjugates	Unknown aqueous soluble
February	62.9±2.8	16.2±2.7	8.2±0.3	7.1±0.4	5.7±0.7
March	64.5±4.5	18.5±3.4	8.5±1.2	5.5±0.5	3.1±0.5
April	57.2±3.7	20.0±0.8	9.9±1.3	6.0±1.4	7.0±1.7
May	59.4±1.9	18.0±0.7	8.3±0.7	8.7±0.8	5.6±0.2
June	54.7±1.3	23.7±0.9	8.3±1.9	7.8±0.6	5.4±0.8

Figure 3.4: A typical high performance liquid chromatograph of B[a]P and metabolites.
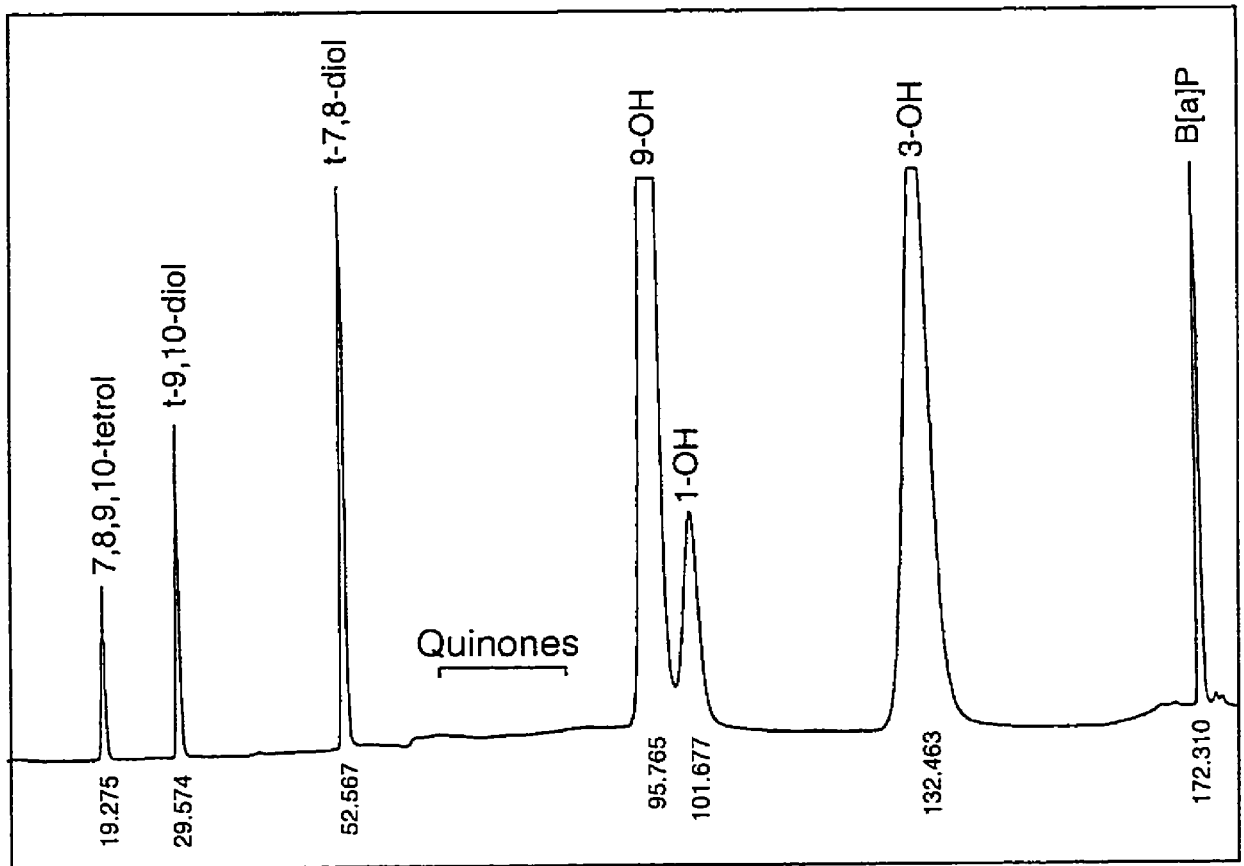
Table 3.2:   Individual metabolites as a percent of the total identified organic soluble metabolites in the bile of juvenile coho salmon exposed to intraperitoneal injection with 10mg(1μCi)/kg of [3H]-B(a)P. Values are means ± SE of five fish. Quinones were not adequately separated by this HPLC method and so include: 1,6-dione, 3,6-dione and 6,12-dione.

Percent organic soluble metabolites

Biliary metabolites	February	March	April	May	June
7,8,9,10 tetrol	21.2±9.2	15.7±7.2	21.3±5.9	17.5±5.5	24.8±5.7
t-9,10-diol	19.9±9.7	2	3.3±2.2	4.7±1.7	5.2±1.1
t-7,8-diol	17.6±9.9	12.9±8.1	4.3±1.2	21.0±9.5	13.5
Quinones	19.7±9.0	34.7±5.4	40.0±7.0	37.9±10.6	37.9±5.1
9-OH	16.6±8.8	10.9±5.5	9.2±1.0	20.5±6.6	16.1±4.5
1-OH	6.6±4.6	15.5±11.0	22.8±10.8	2.3±1.1	2.5
3-OH	16.5±9.9	10.0±3.3	4.0±1.5	5.4±2.2	5.6±1.0
B[a]P	2.9±1.8	5.5±2.7	8.9±2.2	6.2±1.5	10.3±2.6

Table 3.3:   Percent of [^{3}H]-B(a)P derived radioactivity eliminated from juvenile coho salmon (1, 2, 4 and 7 days) following an i.p injection of 10 mg (1µCi)/kg B[a]P. Values are means ± SE of five fish.

Percent of total dose eliminated

Month	D1	D2	D4	D7
February	86.1±6.3	93.13±0.5	94.0±1.3	94.9±0.6
March	71.3±3.4	93.9±0.8	94.6±0.6	94.9±0.7
April	93.6±0.8	94.1±0.3	94.6±0.6	95.6±0.9
May	94.0±0.6	95.1±0.3	95.1±0.4	95.1±0.8
June	93.9±0.7	94.5±0.7	95.5±0.7	97.0±0.6

Discussion

Due to the profound effects and substantial adaptations of the physiology and metabolism of anadromous salmonids involved in smoltification (Wedemeyer et al., 1979; Boeuf, 1993) it is important to understand the possible effects that this process may have on the toxicokinetics of xenobiotics to order to aid in the assessment of risks associated with environmental contamination to salmonids. A better understanding of altered pollutant kinetics during fish development may also aid in the determination of susceptible life stages in salmonids. The objective of this chapter was to determine the effects of smoltification on the distribution, metabolism and elimination of the model pollutant benzo[a]pyrene in juvenile coho salmon.

Various factors, including pre-exposure to xenobiotics, seasonal variations, reproductive status, developmental stage, species differences and fish migration can modulate the biotransformation system (Collier et al., 1995). As previously discussed, smoltification has pervasive effects on metabolic processes in salmonids, and in conjunction with the alterations in xenobiotic toxicity to fish during smoltification reported in the literature (Moles, Rice, and Korn, 1979; Thomas and Rice, 1981, 1986) it is suggested in this thesis that xenobiotic metabolism may also be affected. Therefore, alterations in basal levels and activities of xenobiotic metabolizing enzymes were monitored in juvenile coho to provide a framework for natural changes during the parr-smolt transformation period.

Measurement of detoxification enzymes provides pre-exposure activities and levels and may help define the susceptibility of the organism to xenobiotic exposure. Several xenobiotic metabolizing enzymes have been detected in salmonids (Schnell et al., 1980; Buhler and Williams, 1989), however, there have been no studies published which have

examined levels or activities of these enzymes during the coho 'parr-smolt' transformation. In the present study, activities of phase I and phase II biotransformation enzymes in the livers of yearling coho salmon, were measured biweekly from February until the release date from the hatchery in mid-June, in order to observe any alterations in baseline levels during smoltification. In general, with all of the enzymes examined, peak enzyme activities occurred in February and March and then declined through to June.

The cytochrome P450-dependent monooxygenases function in the oxidative metabolism of both endogenous and exogenous compounds and measurement of the total microsomal cytochrome P450 content in the liver provide an indication of the overall amount. Total cytochrome P450 levels, in the present study, ranged from 0.024 ± 0.009 to 0.095 ± 0.010 nmol/mg microsomal protein. The observed levels and activities were lower than those reported for other teleost species in the literature where cytochrome P450 levels generally clustered from 0.1-0.5 nmol/mg microsomal protein; for example rainbow trout showed activities around 0.22 nmol/mg microsomal protein (Bend and James, 1978; Bend et al., 1978; Buhler and Williams, 1989; Kennish, 1992; Haasch et al., 1993).

The extent to which an organism metabolizes an xenobiotic depends to a large degree on the complement of different P450 proteins present, their catalytic function and their regulation (Stegeman, 1989). The diversity of catalytic functions of fish liver P450 is extensive and measurement of ethoxyresorufin O-deethylase activity represents an O-dealkylation reaction which has been shown to play a role in the detoxification of xenobiotic compounds and activation of chemical carcinogens (Stegeman and Hahn, 1994). The ethoxyresorufin O-deethylase activity in juvenile coho salmon sampled ranged from 2.74 ± 0.75 to 9.94 ± 0.85 pmol/min/mg microsomal protein. The values for other teleosts

species reported in the literature had a wide range from 0.95 - 131 pmol/min/mg microsomal protein(Bend and James, 1978; Bend et al., 1978; Buhler and Williams, 1989; Kennish, 1992; Haasch et al., 1993).

The glutathione S-transferases are an important part of the phase II biotransformation system which conjugate many toxic compounds with glutathione rendering them more water-soluble. The glutathione S-transferase activities in juvenile coho salmon in this study ranged from 0.07 ± 0.01 to 0.33 ± 0.01 μmol/min/mg cytosolic protein in the present study. Values in the reported in the literature in other teleosts glutathione S-transferase activities ranged from 0.3-1.5 μmol/min/mg cytosolic protein (Gregus et al., 1983; Bauermeister et al., 1983; Foureman, 1989, Kennish, 1992).

The low activities and levels of detoxification enzymes in juvenile coho in the present study relative to other teleosts in the literature might have adverse consequences such as an increased sensitivity to pollutants. For example, the organism may not be able to adequately detoxify a xenobiotic upon an acute exposure or must utilize energy which may be required elsewhere to produce more quantities of the necessary detoxification enzymes.

The observed changes in biotransformation enzyme activities through the smoltification process may have several explanations, one of which is hormonal modulation. In mammalian systems, hormones appear to affect many of the hepatic mixed function oxidase activities *in vivo*, through complex and distinct regulatory controls. The pituitary growth hormone and thyroid hormones are potential candidates for interactions and modulation of hepatic mixed function oxidase activities by hormones (Skett, 1990; Waxman and Chang, 1995). Various hormones appear to affect hepatic mixed-function

oxidase activity in mammals, such as pituitary, gonadal, adrenal, thyroid and pancreatic hormones with many potential interactions, however, the mechanisms are still unknown (Skett, 1990). For example in mammalian systems, insulin was shown to be a general stimulator of the monooxygenase system and glucagon had a depressive effect (Skett, 1990). Reports concerning hormonal regulation of biotransformational enzymes in fish are sparse, however, hormonal regulation of cytochrome P450 enzymes has been observed (Stegeman and Hahn, 1994; Cravedi et al., 1995). Estradiol and testosterone are regulators of monooxygenase activity, presumably by altering the content and/or activity of various P450s, the exact mechanism however is still unknown ((Hansson and Gustafsson, 1981; Pajor et al., 1990; Stegeman and Hahn, 1994; Cravedi et al., 1995). Gonadal hormones appear to exert their action via the hypothalamo-pituitary axis and growth hormone being the factor mediating the effect (Cravedi et al., 1995). In juvenile rainbow trout, growth hormone tended to reduce total P450 content and UDP-glucuronyl transferases, by an unknown mechanism, but had no observable effect on GST activity (Stegeman and Hahn, 1994; Cravedi et al., 1995). There are several endocrine glands activated during smoltification, the pituitary-thyroid axis and pituitary-interrenal axis appearing to be the principal systems involved (Barron, 1986). Changes in the levels of growth hormone, insulin and circulating levels of thyroid hormones (Beouf, 1992; Barron, 1986) might be involved in the alterations of enzyme activity and levels, but which systems and the mechanisms might affect these enzymes are not known. The modulation and regulation of biotransformational enzymes by hormones occurs, but further research is required to elucidate the complex interactions.

The development of hepatic enzymes is under complex ontogenic control and generally do not develop gradually (Ronis and Cunny, 1994). In most mammals, levels of monooxygenase systems are low in the fetus and increase rapidly following birth, reaching a peak during early adulthood (Varanasi et al., 1989b). The monooxygenase system in fish has been shown to begin functioning as early as the embryo stage (Stegeman and Hahn, 1994). In mammals, there is a gradual development of some phase II enzyme activities in rats and humans which can have deleterious consequences. The relative ease by which phase I enzyme systems are induced compared to the refractory response of phase II enzymes could result in an imbalance in the metabolism of compounds producing a higher proportion of toxic, electrophilic intermediates due to insufficient or underdeveloped phase II activities (Varanasi et al., 1989b). In mammalian systems, clusters of different enzymes appear to develop during critical periods in the organism's life depending on the physiological needs required in a changing environment. Physiological demands which influence intracellular concentrations of necessary cofactors such as NADPH, GSH and UDPGA will affect the expression and activity of hepatic enzymes (Ronis and Cunny, 1994). As well, a in shift energy utilization may play a crucial role as energy is required for the synthesis of new enzymes and many biotransformational reactions (Sipes and Gandolfi, 1986). There are several changes in energetics during the 'parr-smolt' transformation process, which results in a shift from anabolic to catabolic metabolism (Wedemeyer, Saunders and Clark, 1979; Folmar and Dickhoff, 1980; McKeown, 1984). The depletion of body energy reserves represented by an active catabolism of stored lipids indicates the organism is mobilizing energy for biological processes. The exact biological processes supported by the mobilization of energy are not known, however they may be channeled

into hypo-osmoregulatory adjustments (Sheridan, 1989). This suggests the utilization of energy towards necessary adjustments required for a marine existence may limit the availability of energy for other demands such as the production detoxification enzymes.

A combination of biochemical mechanisms may be utilized by the coho for altering the activities and levels of the biotransformation enzymes during the transformation period such as, changing enzyme quantities (Hazel and Prosser, 1974), minor changes in isozymes (Hazel and Prosser, 1974; Hochachka and Somero, 1984), and changes in the microenvironment surrounding the enzymes (e.g., degree of membrane organization (Hazel, 1984)) and changes in intracellular pH (Walsh and Moon, 1983). The reasons and mechanisms for the observed changes in biotransformational enyzme activities and levels may be very complex and due to various factors yet to be elucidated in the future.

The effects of smoltification on the distribution of B[a]P was determined by analyzing tissue samples from February to June in juvenile coho following an i.p. injection of [^{3}H]-B[a]P. Upon entry into the circulatory system, a chemical may be distributed throughout the body to a site of toxic action, storage depot, organ of detoxication or interaction and eventual elimination (Klaassen, 1986). The distribution of a xenobiotic throughout the body depends upon the physico-chemical properties of the compound, the concentration gradient established between the blood and tissue, the ratio of blood flow to tissue mass, and the affinity of the chemical for the tissue (Riviere, 1994). The primary factors influencing the distribution of chemicals are the physico-chemical properties (e.g., pKa, lipid solubility, molecular weight) which determine a xenobiotics ability to cross membranes, bind to proteins and deposit at storage sites (Gumbleton and Forbes, 1994; Godin, 1995). Therefore the fate of an xenobiotic within an organism depends on the

amounts and types blood macromolecules, route of administration, rate of metabolism, polarity of the parent toxicant and metabolic products and rate of elimination (Riviere, 1994).

In the present experiment, [³H]-B[a]P (or its metabolites) were found in all tissues of the juvenile coho salmon. Studies on other teleost fish such as English sole, starry flounder, common carp and gulf toadfish using different routes of exposure show similar results in the distribution of B[a]P and its metabolites in the tissues and fluids (Varanasi and Gmur, 1981; Varanasi et al., 1989; Steward et al., 1991; Kennedy et al., 1989a). It appears that B[a]P and its metabolites remain in the circulatory system long enough to distributed throughout the organism to allow for accumulation in tissues with low systemic perfusion such as muscle, adipose tissue and bone (Riviere, 1994). The majority of recovered radioactivity was found in the liver and bile which is consistent with other teleosts exposed to B[a]P; such as trout, English sole, and starry flounder (Varanasi and Gmur, 1981; Thomas and Rice, 1982, Varanasi et al., 1989). The high level of radioactivity in the bile and liver above all other tissues reflects this organs function in the storage and metabolism of PAHs (Klaassen 1986; Godin, 1995).

The largest shifts in tissue distribution of B[a]P derived radioactivity occurred within the first two months of the experiment coinciding with significant changes in protein and enzyme activities and levels. There are several possible reasons which may cause an altered distribution of B[a]P which include alterations in detoxification rates, changes in the amounts of tissue lipids and osmoregulatory adjustments involving maintenance of water and ion balance. It is conceivable that the changing levels and activities in biotransformational enzymes during smoltification led to alterations in the distribution of

the [^{3}H]-B[a]P by altering the quantity of metabolizing enzymes. Alterations in detoxification enzymes will effect the metabolites produced by the fish and influence its distribution. For example, in Dolly Varden char, the metabolism B[a]P is not as efficient as in English sole and therefore was more available for accumulation by extrahepatic tissues (Varanasi et al., 1989). The extent of metabolism and the types of metabolites produced will affect both accumulation and excretion (Varanasi et al., 1989). It has been shown that induction of biotransformational enzymes often results in changes in tissue concentrations due to an increased metabolism which enhances excretory processes (Payne et al., 1987; Stegeman and Lech, 1991; Heath, 1995).

B[a]P is a very lipophilic compound with an octanol-water partition coefficient (log Kow) of 6.5 which predicts the partitioning of the chemical out of water and into lipids or binding to proteins (Varanasi et al., 1989). In the present study, significant portions of B[a]P-derived radioactivity were recovered in the fat of juvenile coho salmon reflecting B[a]P's lipophilicity. Any alterations in tissue lipid content or lipid constituents may influence the solubility and hence the distribution of B[a]P. Such changes may affect the distribution of a xenobiotic through the organism by affecting the availability and movement through the membranes. Smolting salmon characteristically undergo a transition from an anabolic to catabolic metabolism which includes glycogenolysis and lipolysis (Plisetskaya et al., 1988). Combined with a decrease in feeding, smolts typically deplete their total body lipids and have a decrease in body fat content (Wagner, 1969; Malikova, 1959). In this study, there was a statistically significant decrease in visceral fat content from the start of the experimental period to the end in June which may have contributed to the altered disposition of B[a]P in these fish. Sheridan *et al.* (1985a,b; 1989) showed that

an alteration in tissue fatty acids (mesenteric fat, dark muscle, light muscle and liver) from the predominance of saturated fatty acids in freshwater forms to polyunsaturated fatty acids in marine forms occurs before actual seawater entry. The change in tissue fatty acid composition and depletion of lipids together may play an influential role in the tissue distribution of lipophilic xenobiotics such as B[a]P in fish. The high burden of radioactivity in visceral fat in June over individual tissues reflects the organisms depletion of body lipids (Plisetskaya et al., 1988; Wagner, 1969; Malikova, 1959) indicated by the decrease in fat content and therefore a subsequent increase in burden. Based on the lipophilicity of B[a]P, changes in lipid distribution and content will likely result in alterations in the distribution throughout the organism.

The movement of xenobiotics through the circulatory system may occur in the aqueous portion of the blood, but the primary transport mode for many organic and inorganic compounds is in association with plasma proteins, such as lipoproteins and albumins (Riviere, 1994). Binding of xenobiotics to plasma proteins can reduce the toxic action of the compound and effect its distribution and elimination. The binding of chemicals to plasma proteins occurs by a variety of mechanisms involving both covalent and noncovalent binding including ionic binding, hydrophobic interactions, hydrogen bonds and Van de Waals forces (Klaassen, 1986). Studies with gulf toadfish and trout indicate that B[a]P and PCB's bind more to plasma proteins than red blood cells (Kennedy and Walsh, 1991; McKim and Heath, 1983). Chemicals have been shown to bind to different proteins of the blood plasma, for example, hydroxylated metabolites of PAHs bind primarily to plasma albumin, whereas unmetabolized PAHs associate more with plasma lipoproteins (Heath, 1995). Competition for similar binding sites on the plasma proteins by

endogenous and exogenous compounds results in displacement on one compound from the protein increasing the unbound fraction in the blood. Dissociation may also occur when the affinity of another biomolecule or tissue component exceeds that of the plasma protein to which the xenobiotic was originally attached (Riviere, 1994). The unbound fraction of xenobiotics in plasma exists in a dynamic equilibrium, involving binding to plasma proteins and uptake by extravascular tissues (Godin, 1995). The endothelial cells in certain organs, such as the liver, have receptors for the albumin-ligand complex, thereby preferentially absorbing such complexes, whereas chemicals bound to lipoproteins merely diffuse into endothelial cells of essentially all tissues (Heath, 1995). The primary distribution mechanism for water insoluble toxicants is with an association with plasma proteins (Riviere, 1994) therefore alterations in plasma proteins may effect the distribution of xenobiotics in the circulatory system. Changes in plasma proteins have been observed during smoltification, for example in Atlantic salmon alterations in the quantities and relative proportions of low molecular weight and several larger proteins have been observed (Johanning and Bradley, 1989).

Another possible factor which may influence the distribution of B[a]P include the physiological adjustments required for a changing osmotic environment. For example, a reduction in glomerular filtration rates occurs in organisms preparing for a seawater existence to compensate for water loss in a hypertonic environment (Evans, 1993; Kirschner, 1991). In the present study, the amount of radioactivity recovered in the kidney, although not statistically significant at p<0.05, had highest burden levels in February and an increase in May but lowest in June.

A characteristic increase in the metabolic rate, concomitant with an increase in oxygen consumption in smolts compared to parr (Baraduc and Fontaine, 1955; Wiggs et al., 1989; Higgins, 1985) may effect chemical uptake rates and the amounts of xenobiotics in the organism, therefore changing the distribution by altering the levels found in the circulatory system at any given time. The route of chemical exposure can also affect the distribution of an xenobiotic. For example, an i.p. injection provides immediate passage of the compound to the liver via the portal system, whereas dermal or respiratory routes provide at least one passage through the systemic circulation, before reaching the liver (Riviere, 1994), thus altering distribution in the organism.

The analysis of bile from juvenile coho after 7 days following an i.p injection of [^{3}H] B[a]P was undertaken to determine the effects of smoltification on metabolism. The metabolism of xenobiotics serves as a pathway of detoxication and ultimately elimination from the organism by conversion of the chemical into a more hydrophilic form. B[a]P is metabolized similarly by many fish species, including rainbow trout, winter flounder, gulf toadfish, and juvenile English sole (Gmur and Varanasi et al., 1982; Stegeman et al., 1984; Varanasi et al., 1989; and Kennedy et al., 1991). The general pathways of metabolism have been found for many species, however, due to the multiple metabolic steps involved and difficulty in obtaining a quantitative recovery of all metabolites, it is difficult to evaluate the overall pathways of PAH metabolism (Varanasi et al., 1989). The major pathways of B[a]P metabolism (Figure 3.5) begin with oxidation of B[a]P catalyzed by the cytochrome P450 system, the usual initial step, resulting in the formation of epoxides or arene oxides. These unstable metabolites may undergo three major reactions either, spontaneous rearrangement to form phenols; hydration reactions to form trans-dihydroxydihydro

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Figure 3.5: Schematic of the biotransformation and metabolic activation of benzo[a]pyrene. (Gelboin, 1980; Varanasi et al., 1989)
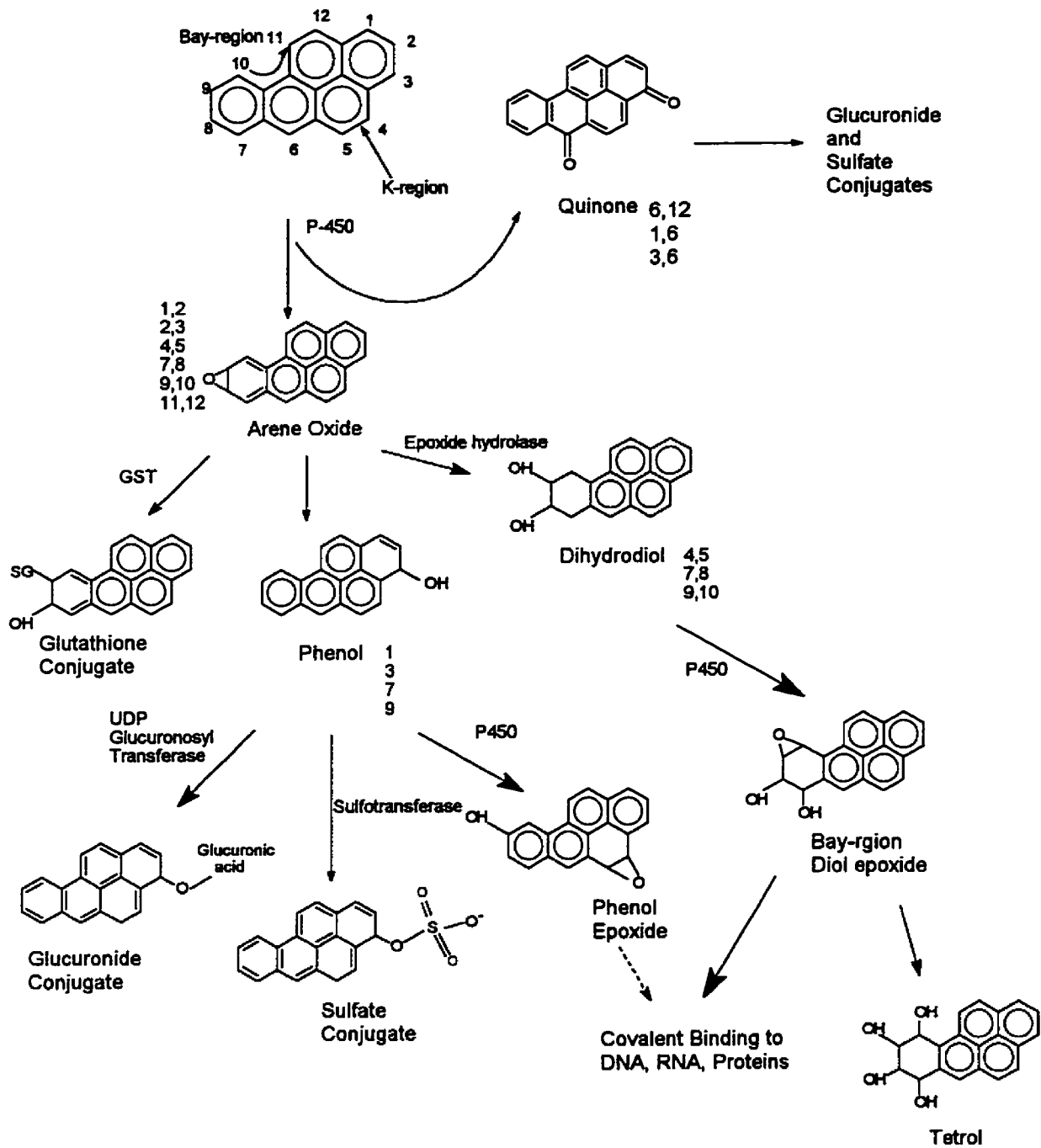
compounds (diols); or, conjugation with glutathione which is readily eliminated. The phenols and diols may be further metabolized through other phase II enyzme reactions to form glucuronic acid or sulfate conjugates. As well, phenols and diols may undergo further oxidation reactions to form multiple hydroxylated derivatives such as triols and tetrols, which are generally considered detoxication products. Certain metabolic intermediates in the formation of triols and tetrols, the bay-region diol-epoxides, are reactive metabolites which readily bind to DNA and induce tumor formation (Gelboin, 1980; Varanasi et al., 1989). Juvenile coho salmon were capable of metabolizing [^{3}H]-B[a]P to both unconjugated (Phase I) and conjugated (Phase II) forms. As well, the fast elimination of [^{3}H]-B[a]P and its metabolites occurring within the first two days reflects a rapid metabolism. Analysis of the bile revealed that 55 to 63% of the radioactivity was phase I metabolites, 16 to 24% glucuronide conjugates, 8% sulfate conjugates, 7% other conjugates and 6% aqueous-soluble metabolites. The major metabolites of English sole, starry flounder and carp exposed to B[a]P were glucuronide conjugates of B[a]P phenols and B[a]P -7,8-diol (Varanasi et al., 1989; Steward et al., 1991). The levels of glucuronide and sulfate conjugates in the bile of juvenile coho (about 24-34% of recovered metabolites) are similar to those found in English sole exposed to PAHs (Stein et al., 1984) but higher than those reported for gulf toadfish, English sole and starry flounder (6-7 % of recovered metabolites) exposed to B[a]P (Kennedy et al., 1989b; Varanasi et al., 1989). The glucuronide metabolites were 2 to 3 times more prevalent than sulfate metabolites in the juvenile coho, suggesting that glucuronidation is more predominant than sulfation reactions. Similar patterns were observed in English sole, starry flounder and carp exposed to B[a]P where more glucuronide than sulfate metabolites were recovered, however, in gulf

toadfish equal proportions of glucuronide and sulfate conjugates were recovered (Varanasi et al., 1989; Steward et al., 1991; Kennedy et al., 1989b). The aqueous soluble radioactivity present in the bile not conjugated to sulfate or glucuronic acid is probably a glutathione conjugate (Stein et al., 1987; Varanasi et al., 1989; Kennedy et al., 1989b). The low levels recovered in coho are well below other teleosts, such as gulf toadfish which where about 85% of radioactivity recovered in the aqueous soluble fraction of the bile after B[a]P exposure (Kennedy et al., 1989b). Similarly, in common carp, GSH conjugation did not appear to have as large a role in metabolism, accounting for approximately 16% after 72h (Steward et al., 1991). The corresponding low activities of GST measured in the livers of juvenile coho, compared to other teleost species, may be a reflection of the low levels of radioactivity recovered in the aqueous soluble fraction.

The amount of accumulated phase I metabolites in the bile of the juvenile coho was higher than that found in other species. For example, in gulf toadfish phase I metabolites accounted for less than 10 % of the total metabolites eliminated in the bile (Kennedy et al., 1989b). The chromatographic separation of B[a]P and organic soluble metabolites by HPLC revealed a variety of metabolites such as, tetrols, diols, quinones and phenols, and the major identified phase I metabolite of B[a]P in juvenile coho was 7,8,9,10-tetrol. This differs from saltwater species, such as the English sole and gulf toadfish, were the 7,8-dihydrodiol is the major metabolite (Gmur et al., 1982; Kennedy et al., 1989b). In freshwater species such as bullhead, 9,10-dihydrodiol is a predominate metabolite (Tan and Melius, 1986). The predominance of 7,8,9,10-tetrol is consistent with previously reported data on the relative regioselectivity of fish liver monooxygenases for metabolizing B[a]P at the bay-region (i.e., the 7-,8-,9- and 10 positions) rather than at the K-region (i.e., the 4,5-

positions) as in mammals (Tan and Melius, 1986; Varanasi et al., 1989; Steward et al., 1991). The conversion of 7,8-dihydrodiol to the reactive metabolite 7,8-diol-9,10 epoxide occurs via the MFO system, which may bind to DNA or RNA, or undergo further metabolism to the 7,8,9,10-tetrol metabolite (Gelboin, 1980). The presence of B[a]P quinones in the organic soluble extraction in juvenile coho were similar to those found in the common carp (Steward et al., 1991). B[a]P quinones are usually further metabolized into phase II conjugates of glucuronic acid and sulfates, rendering them none organic-solvent extractable (Tan and Melius, 1986). The juvenile coho appears to be unable to metabolize quinones further which may be reflective of the high organic extractable portion recovered.

The metabolites recovered from the bile of juvenile coho salmon indicate a metabolic pathway in coho towards the metabolic activation of B[a]P to carcinogenic metabolites capable of inducing cytotoxicity and tumor production (Varanasi et al, 1989). The production of both 7,8-dihydrodiol and 7,8,9,10-tetrol indicate the presence of this pathway. In mammals, B[a]P triols and tetrols are formed non-enzymatically from diol-epoxides indicating the presence of a similar pathway (Gelboin, 1980). The large percentage of triols and tetrols in organic soluble extractions in the present study, provides further support of the presence of the metabolic pathway leading to bioactivation of B[a]P. The implication of this is an increased rate of production of potentially carcinogenic metabolites, such as 7,8-dihydrodiol-9,10-epoxide (Gmur and Varanasi, 1982). The larger percentage of organic soluble metabolites over other metabolites extracted suggest the phase II system of juvenile coho salmon is operating at lower capacity than other teleost species, possibly attributed to the developmental state of the organism rendering them

unable to conjugate compounds effectively during this period of their life cycle. The hepatocytes of juvenile rainbow trout have been shown to have a reduced presence of secretory and biosynthetic structures, such as Golgi and rough endoplasmic reticulum (Chapman, 1981; Leland, 1982), which might impose a limitation on biotransformation and excretion of xenobiotics in juvenile fish (Heath, 1995). Although biotransformation enzyme activities were altered during smoltification, there were few significant changes in metabolite groups produced over this period. Further detailed research is needed to determine if the reported changes in enzyme activities in this study can result in altered production rates or accumulation of specific metabolites with possible consequence to the organism.

The elimination of a xenobiotic, whether it be the parent compound or its metabolite, reduces the potential toxic effects to the organism. Depending upon the physico-chemical properties of the chemical, biotic and environmental parameters, a xenobiotic may be excreted via the skin, mucus, bile, feces, urine or gills (Kennedy, 1995, Heath, 1995). The excretion of larger organic molecules, such as anthracene and B[a]P, via the gills is of little significance compared to the bile (Thomas and Rice, 1982). Several studies with teleosts, such as the rainbow trout, carp, Dolly varden, English sole, starry flounder, and gulf toadfish indicate B[a]P is primarily excreted via the biliary route (Thomas and Rice, 1982; Varanasi et al., 1989; Kennedy et al., 1989a,b; Steward et al., 1991; Heath 1995). However, the urinary excretion of B[a]P metabolites was found to be a significant pathway of elimination in southern flounder (Pritchard and Bend, 1984;1991). Despite the few studies on the mechanisms of biliary excretion in fish, similarities with mammalian systems suggest that the route of elimination depends on the molecular size and

lipid solubility of the chemical. Molecules larger than 300 daltons and the more lipophilic molecules are excreted by the liver and the smaller, more water-soluble chemicals being eliminated through the kidney (Pritchard and Bend, 1984;1991; Klaassen, 1986; Gumbleton and Forbes, 1994; Kennedy, 1995). The molecular weight of B[a]P is 252.3 therefore its excretion appears to be determined more by its lipophilicity than molecular weight.

In the present study, the vast majority of the injected dose of [^{3}H]-B[a]P was excreted into the bile of the fish 24 hours following chemical administration. The high percentage of B[a]P derived radioactivity and the phase I and phase II metabolites recovered in the bile of the coho indicate the importance of the heptaobiliary route for its excretion. This finding is consistent with those other studies which indicate that B[a]P is primarily excreted *via* the biliary route (Kotin et al., 1959; Varanasi and Gmur, 1981; and Thomas and Rice, 1982). For example, in gulf toadfish approximately 75% of the injected dose of B[a]P was found in the bile by 24 hours (Kennedy et al., 1989 a, b). The rate of excretion of B[a]P into the bile tends to be slower than other aromatic hydrocarbons, for example, in Dolly varden sampled 24 h after chemical administration, only 4% of the injected dose of B[a]P was recovered in the bile, whereas 66% of phenol, 30% of toluene and 10.8% of naphthalene were recovered in the bile (Thomas and Rice, 1982). The relatively delayed elimination of B[a]P compared to the other compounds was attributed to the required metabolism of the parent compound. The relatively fast elimination of B[a]P into the bile of juvenile coho may be a reflection of the biotransformational capabilities of these fish which possess an efficient excretion system for B[a]P. It appears from the higher portion of phase I metabolites compared to phase II metabolites in the bile of coho, that the compound was metabolized sufficiently eliminate B[a]P. Hence, even with the relatively

low capacity to metabolize the compounds further to conjugated metabolites, a relatively rapid movement of the metabolites into the bile of the coho was still obtained.

Depuration of aromatic hydrocarbons such as B[a]P is relatively insignificant via the gills and urine but predominates through the biliary mechanism (Heath, 1995; Thomas and Rice,1982). However, the importance of elimination of B[a]P (and its metabolites) via the renal system was shown in southern flounder (Pritchard and Bend, 1991). There was a significant amount radioactivity recovered in the kidneys of juvenile coho during the experimental period. Although no conclusive evidence of the importance of the urinary route for the excretion of xenobiotics in coho salmon could be established the relative importance may be reflected by the recovery of B[a]P-derived radioactivity in the kidney. No direct relationship between biotransformation enzyme activities and the elimination of B[a]P could be made because the majority of the chemical had been eliminated by the first sample period. Future research should focus on studies investigating the interactions and modulation of the biotransformation enzymes by hormones, the importance of urinary excretion for B[a]P and elimination of B[a]P within the first 24 hours after an administration.

This study begins to assess the effects of smoltification on the toxicokinetics of the model carcinogen B[a]P and to determine the causes of altered susceptibility to chemicals during this developmental period in juvenile coho. Significant changes in xenobiotic metabolizing enzyme activities and chemical distribution occurred during this period, however, there were no significant changes in the classes of metabolites produced or in the total elimination of the chemical. The metabolites recovered from the bile of juvenile coho salmon indicate metabolic pathways leading towards both detoxification and the metabolic

activation of B[a]P to potentially carcinogenic metabolites which may be capable of inducing cytotoxicity and tumor production. This research illustrates the complex modulatory effects of developmental processes on chemical toxicokinetics in aquatic organisms.

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Part IV: Environmental Modulators - Salinity Effects on the Toxicokinetics of Benzo[a]pyrene

Introduction

Organisms must often deal with natural stressors, which may compromise effective defense against impingements of anthropogenic origin. Therefore, in order to successfully use aquatic toxicology research in biomonitoring programs, predictive modeling, or in ecological risk assessment, an understanding of the possible effects of environmental parameters on the toxicokinetics of xenobiotics is important. One such stressor may be alterations in environmental salinity which requires significant alterations in the biochemistry and physiology that may affect the toxicokinetics of xenobiotics in organisms such as fish. It has been extensively shown that fish in early stages of life are sensitive to environmental stress, in particular, during the parr-smolt transformation (Peters et al., 1996; Cameron et al., 1992; Lorz and McPherson, 1976; Beckman and Zaugg, 1988; and, Moles, Rice, and Korn, 1979). Salmon preparing for a marine existence must cope with a variety of environmental stress factors in the rivers and estuaries. For example, mortality and physiological problems may be caused by stress due to unfavorable water flows and temperatures, scale loss, the activation of latent infections due to the stress of seawater conversion and gill parasite infestations which reduce osmoregulatory competence (Wedemeyer et al., 1980). As well, contaminant exposure can have deleterious effects on smoltification and early marine survival, such as copper exposure during the parr-smolt transformation, partially or completely inactivates the gill ATPase system effecting the fishes ability to survive in seawater (Lorz and McPherson, 1976).

Migrating teleosts, such as salmon, undergo changes in osmoregulatory ability as they prepare to move from a freshwater to a saltwater environment. Juvenile coho salmon preparing for a marine existence undergo changes in water permeability of membranes and adjustments in water and ion movement (Beouf, 1992). Being an osmoregulator, coho maintain an internal homeostatic osmolarity in face of different environmental electrolyte concentrations, therefore must sustain appropriate quantities of water and concentrations of various solutes for survival (Eckert, Randall, and Augustine, 1988). The blood of a teleost has an approximate osmolarity of 200 to 300 milliosmoles, however a migrating coho will encounter a large variation in environmental concentrations, from several milliosmoles/L in freshwater to about 1000 milliosmoles/L in seawater (Eckert, Randall, and Augustine, 1988). Thus while in freshwater, the organism is hyperosmotic to the surrounding environment and must deal with a net water gain and net salt loss. Upon migration into the marine environment, the organism becomes hypoosmotic relative to the surrounding water and is faced with water loss and salt gains (Evans, 1993). The 'parr-smolt' transformation allows the coho to migrate from an environment which is hypotonic relative to their internal osmolarity to a one that is hypertonic. Examples of physiological and biochemical changes during smoltification which enable the juvenile fish to adapt are increases in gill Na+/K+-ATPase activity and in gill chloride cell density as well as decreases in kidney glomular filtration rates and urinary bladder ion absorption in seawater (Beouf, 1992).

The various changes associated with the preparation and survival in a marine environment may effect the kinetics of foreign compounds in aquatic organisms. As previously discussed, this transformation has pervasive effects the on metabolic processes

within the fish, which suggests that metabolism of xenobiotics and patterns of metabolites produced may be changed.   As well, the alterations in physiology associated with osmoregulatory adjustments may alter the disposition and excretion of xenobiotics.

The first part of this section of the study investigated the effects of salinity on the toxicokinetics of B[a]P in juvenile salmon.   Experiments were similar to those described previously in Part III involving juvenile coho salmon, except that fish were placed into 5, 10 and 20 ppt saltwater following i.p injection of [^{3}H]-B[a]P and examined for changes in distribution, metabolism and elimination of benzo[a]pyrene.

The second part of this section of the study used mature rainbow trout to further investigate the effects of salinity on the kinetics of B[a]P in salmonids.   The rates and amounts of excreted B[a]P and its metabolites can occur via several routes and the utilization of mature salmonids

information regarding tissue accumulation, or the formation of chemically reactive metabolites (Renwick, 1989). Physiologically-based and compartmental models have been routinely employed to describe the changes in blood plasma levels of foreign compounds and to provide an understanding of the processes which are in involved in the fate of the chemical in the organism (Renwick, 1989).

Section 1: Salinity Effects on the Disposition, Metabolism and Elimination of Benzo[a]Pyrene in Juvenile Coho Salmon

Materials and Methods

A) Fish

Yearling coho salmon, *Oncorhynchus kisutch*, were obtained from Capilano Hatchery in North Vancouver, B.C. The fish, from the same cohort, were transported each month from the hatchery to the laboratory starting in February until June when the fish were released from the hatchery. Fish were maintained at seasonal temperatures (4-8°C) and natural photo period in 500 L fiberglass tanks supplied with flowing decholrinated water at pH 6.4, O_2 saturation >95% and hardness 5.2 to 6.0 mg/L $CaCO_3$ for several days until an experiment was started. Fish were fed ad libum until 48 hours prior to an experiment.

B) Chemicals

Unlabelled benzo[a]pyrene (>99% purity) was purchased from Sigma Chemicals (St. Louis, Mo.) and $[1,3,6-^3H]$- benzo[a]pyrene (52 Ci/mmol) was purchased from NEN Research Products (DuPont, Canada Ltd.). HPLC analysis of labelled and unlabelled B[a]P according to the method of Elnenaey and Schoor (1981) revealed no detectable B[a]P metabolites. B[a]P metabolites were obtained from the National Cancer Institute Chemical Repository, Midwest Research Institute (Kansas City, MO.). All other chemicals (reagent grade) and biochemicals were purchased from Sigma Chemicals (St. Louis, Mo.).

C) Exposure

Each month from February to June, five fish from the hatchery were injected intraperitoneally (i.p.) with 10 mg (1 µCi)/kg of [^{3}H]-B[a]P. They were then transferred into 120L tanks at 0, 5, 10, and 20 ppt saltwater for 1, 2, 4, and 7 days at seasonal temperatures under natural photo periods to determine the effects of smoltification and salinity on B[a]P disposition, metabolism and elimination.

D) Distribution, Metabolism and Elimination of Benzo[a]pyrene in Coho Salmon at Differing Salinities

The procedures for determining the disposition, metabolism and elimination of benzo[a]pyrene in smolting coho salmon were as described in the materials and methods section in Part III. The analysis of bile for B[a]P and its metabolites was only done at 0 and 20 ppt saltwater on day 7 after B[a]P administration.

E) Mathematical Analysis

All percent data were arcsin transformed before statistics were performed. One-factor Anova and Student-Newman-Keuls multiple comparison test was used to establish differences between tissue body burdens, B[a]P elimination, metabolite classes and biotransformation enzyme levels and activities in different salinities and between different months at a significance level of $p < 0.05$ (Zar, 1974).

Results

A) Tissue Distribution of Benzo[a]pyrene

Following an i.p. injection of [^{3}H]-B[a]P, B[a]P-derived radioactivity was found in all tissues sampled in the juvenile coho salmon. The percent body burden of B[a]P-derived radioactivity in fish tissues 1, 2, 4, and 7 days following chemical administration and

exposed to 5, 10 and 20 ppt saltwater are shown for in Figure 4.1. The tissue distribution of B[a]P at 0 ppt was done in Part III and are shown in Figure 3.3.

In order to test for a change in tissue distribution in fish placed in salinities of 0, 5, 10 and 20 ppt, an analysis of the individual tissue burden of B[a]P-derived radioactivity was done on a monthly and daily basis (e.g., comparison of liver on February day 1: at 0, 5, 10 and 20 ppt). There were no statistically significant changes in tissue distribution when fish were placed in any of the salinities.

An analysis to observe changes in tissue distribution through the smoltification process (February to June) involved comparing individual tissue burdens of B[a]P-derived radioactivity. In February the general tissue distribution pattern was liver>fat/kidney>bile>carcass on each day. In March, the general tissue distribution pattern was liver/bile>fat>kidney/carcass. The highest amount of radioactivity recovered in the bile and liver occurred in March. The lowest amounts of radioactivity recovered in the kidney occurred in March. In April and May, the general tissue distribution pattern was carcass<kidney<bile<fat<liver. In June the general tissue distribution pattern was carcass<kidney<bile<liver<fat. The highest amounts of radioactivity recovered in fat occurred in June.

Changes in tissue distribution were examined over the seven days following an injection which involved comparison of individual tissue burdens of B[a]P-derived radioactivity. There were a few significant trends observed in tissue distribution between the sample days. The general tissue distribution patterns observed on day 1 was the same for the remaining sample days and similar to the pattern observed for the month as previously described.

B) Biliary Metabolites

The analysis of bile was only done at 0 ppt (Tables 3.1 and 3.2) and 20 ppt to observe any effects of salinity change on the metabolism of B[a]P. As well, only bile from fish sampled 7 days following B[a]P administration were used because it was assumed that the fish would have completed the majority the biotransformation of B[a]P by this point in time. The results of metabolism of B[a]P in 0 ppt were discussed in Part III.

Juvenile coho salmon at 20 ppt metabolized B[a]P to both Phase I and II metabolites (Table 4.1). At 20 ppt in each sample period, 55 to 60% of the recovered radioactivity was extractable Phase I metabolites, 15 to 23% were glucuronic acid conjugates, 8 to 12% were sulfate conjugates, 5 to 9% were unknown water soluble metabolite(s). At each sampling period, <10% of the radioactivity was parent compound. There were no significant differences in the proportions of these metabolite groups in fish sampled from February to June at 20 ppt saltwater. No statistically significant differences in the amounts and patterns of metabolites produced were observed from fish exposed to B[a]P at either 0 or 20 ppt saltwater.

Figure 4.1: The percent body burden of ^{3}H-B[a]P-derived radioactivity in the bile, kidney, visceral fat, liver and carcass of coho salmon through smoltification from a) February b) March c) April d) May e) June. Following exposure to an i.p injection of 10 mg (1μCi)/kg B[a]P for one, two, four and seven days at 5, 10 and 20 ppt saltwater. Values are means ± SEM of five fish.

a)

February 5 ppt
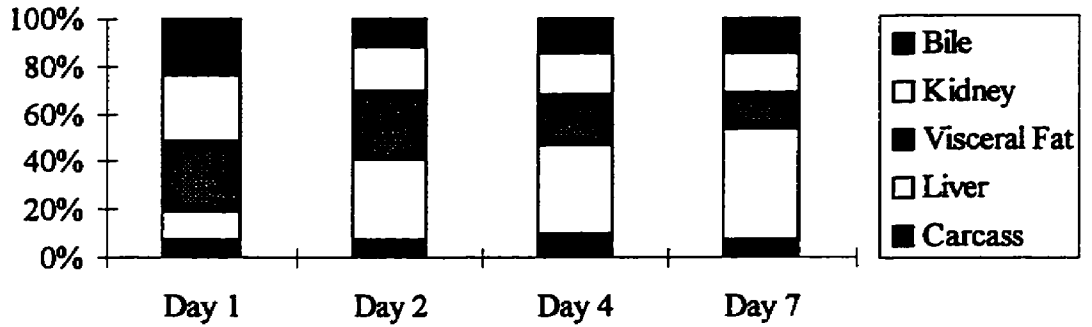
February 10 ppt
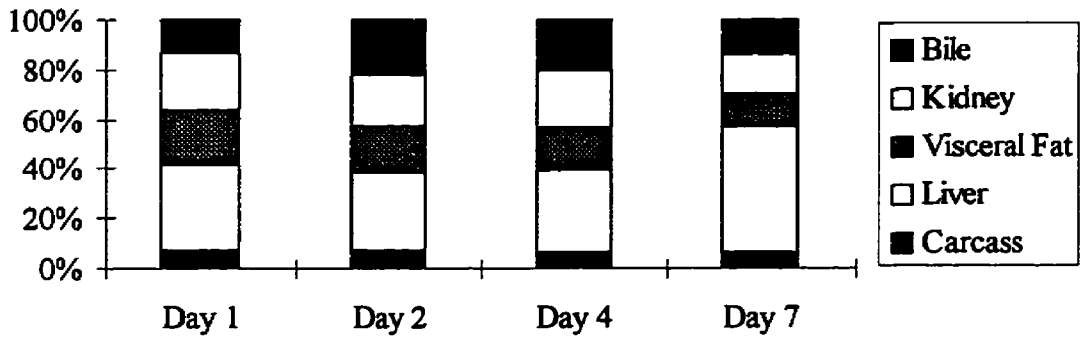
February 20 ppt
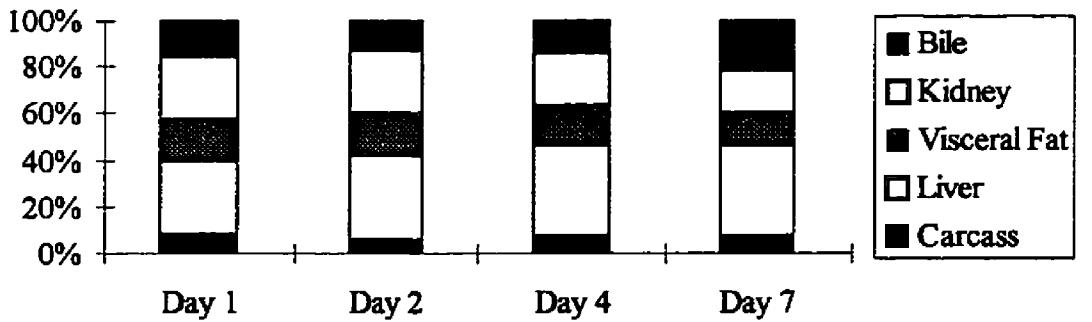
b)

March 5 ppt
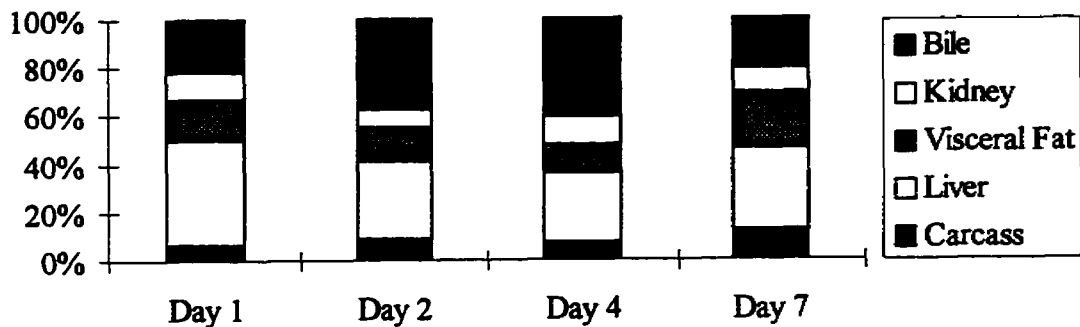
March 10 ppt
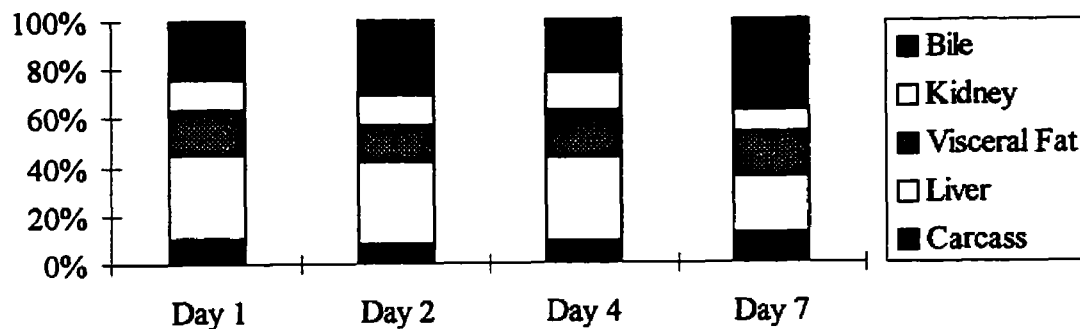
March 20 ppt
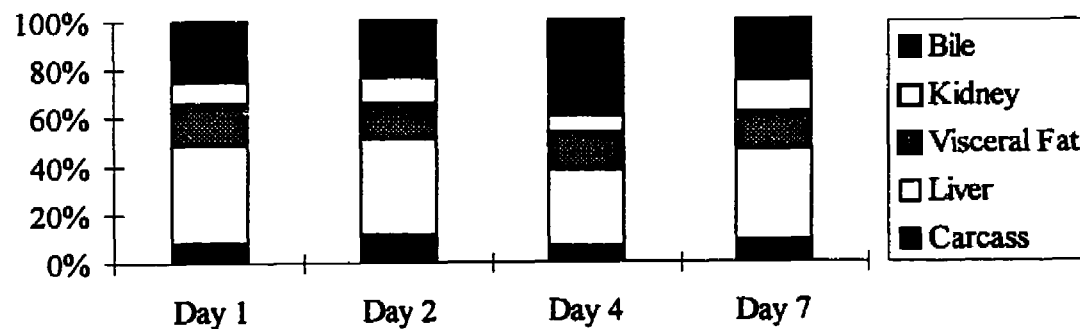
c)

April 5 ppt
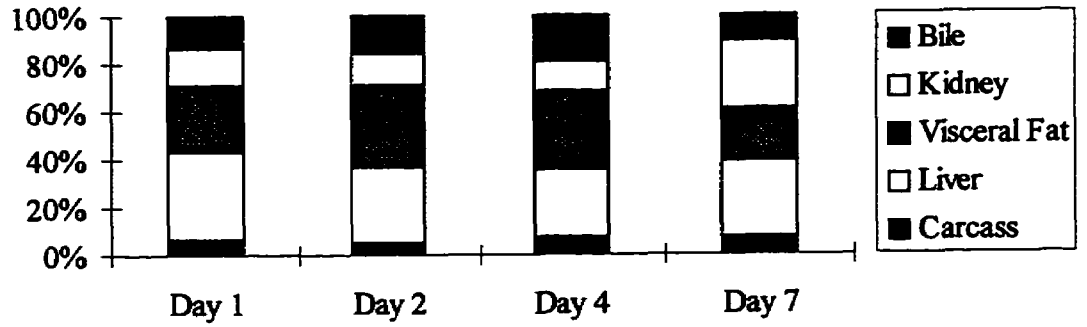
April 10 ppt
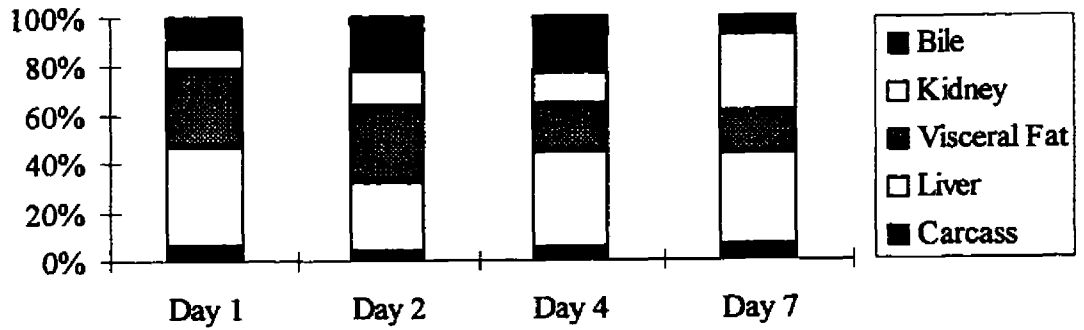
April 20 ppt
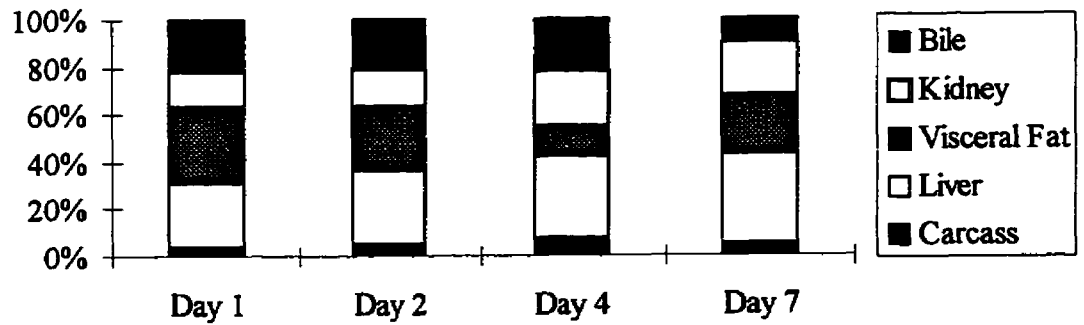
d)

May 5 ppt
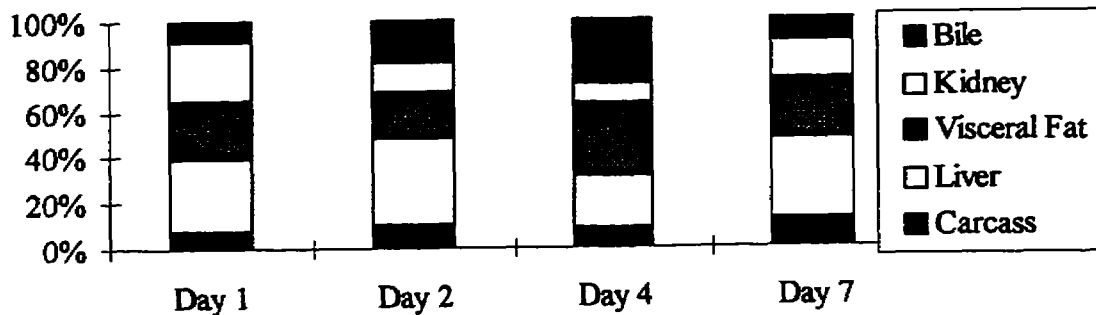
May 10 ppt
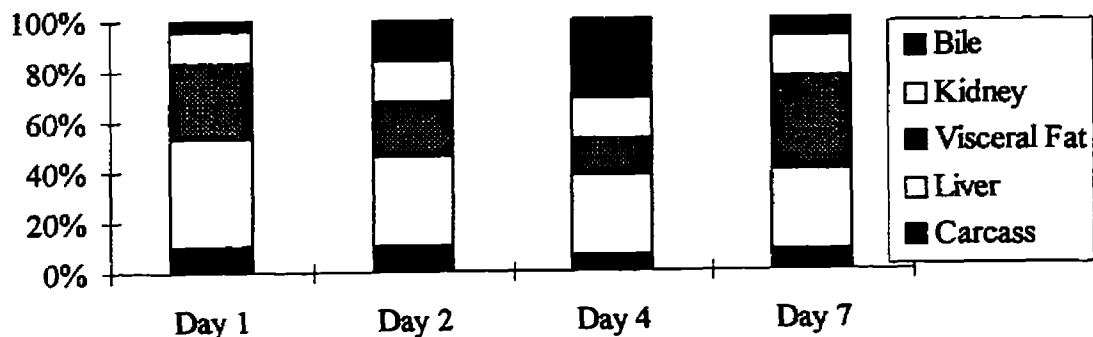
May 20 ppt
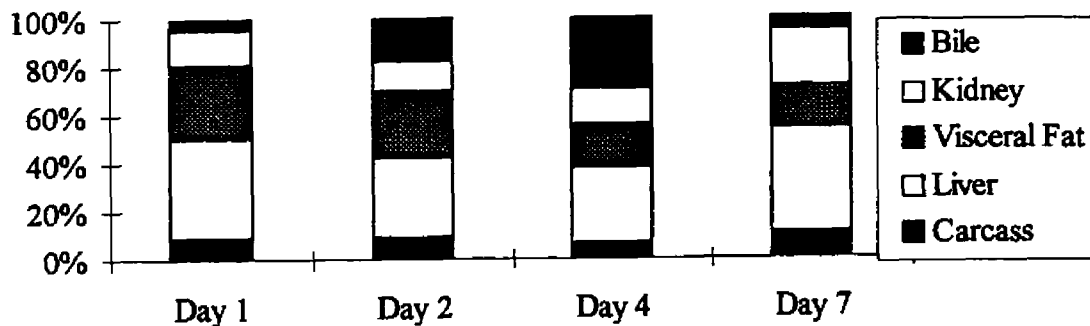
e)

June 5 ppt
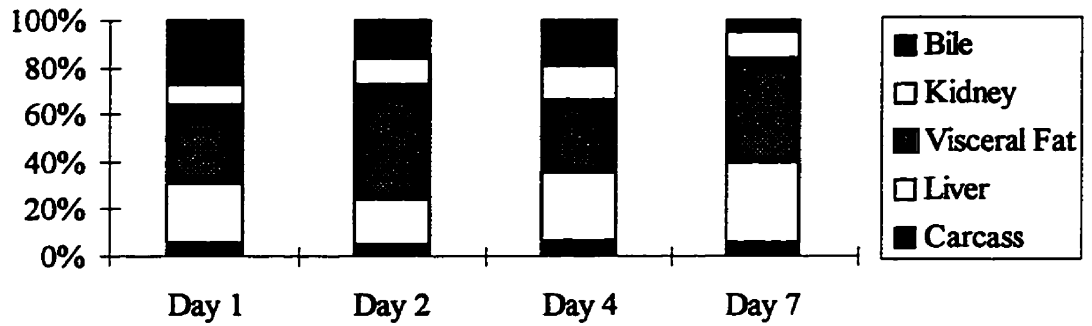
June 10 ppt
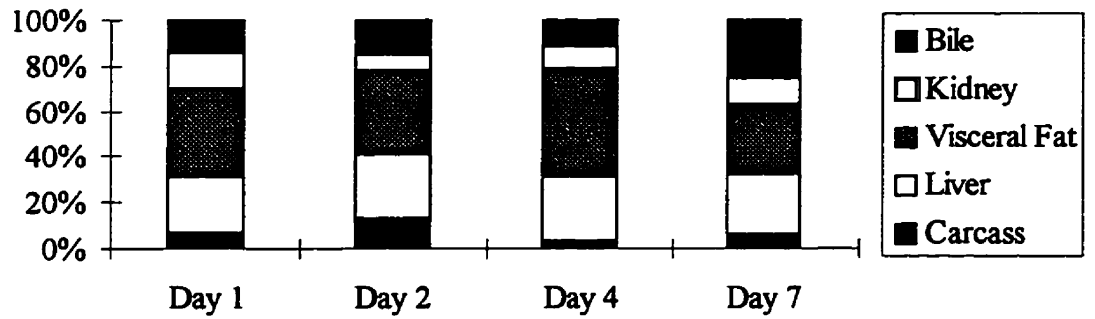
June 20 ppt
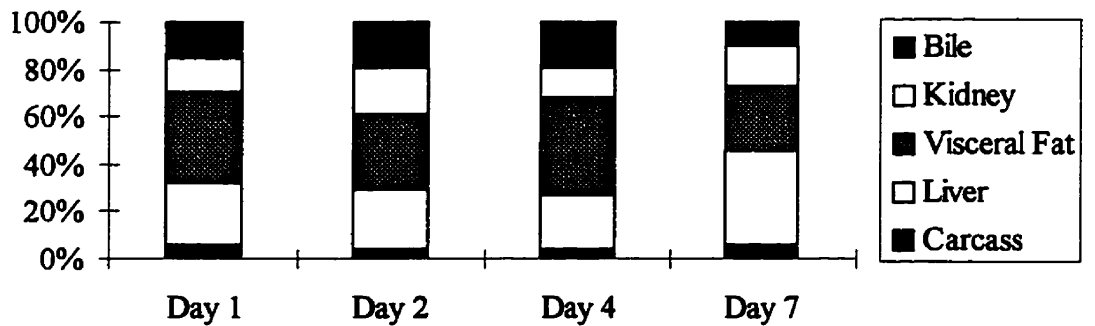
Table 4.1: The percent of total ^{3}H-radioactivity as organic soluble metabolites, glucuronide and sulfate conjugates and other aqueous soluble metabolites in the bile of coho salmon, following an intraperitoneal injection with 10 mg(1µCi)/kg of B[a]P at 20 ppt saltwater. Values are the means ± SE for 5 fish. There were no significant differences between months or salinities in the percents within classes of metabolites at a significance level of $p < 0.05$.

<u>Metabolite Groups (percent of total metabolites)</u>

Bile (20 ppt)	Organic Soluble	Glucuronide	Sulfate	Unknown conjugates	Unknown aqueous soluble
February	63.9±3.8	15.6±0.7	8.7±1.6	6.7±1.3	5.3±0.9
March	66.4±1.6	13.6±1.4	7.6±0.5	8.2±0.7	4.2±0.4
April	58.9±3.1	20.8±1.9	9.5±0.7	7.3±0.3	3.5±1.5
May	53.1±4.5	22.9±5.1	8.8±0.7	8.6±0.8	6.6±0.6
June	56.6±2.1	17.30±1.6	12.1±3.2	8.3±0.5	5.7±0.9

Table 4.2: Individual metabolites as a percent of the total identified organic soluble metabolites in the bile of juvenile coho salmon exposed to intraperitoneal injection with 10mg(1μCi)/kg of [^{3}H]-B(a)P at 20 ppt saltwater. Values are means ± SE of five fish. Quinones include: 1,6-dione, 3,6-dione and 6,12-dione.

Metabolites (20 ppt)	February	March	April	May	June
7,8,9,10 tetrol	16.9±5.8	23.8±5.0	18.5±6.8	13.7±5.6	20.5±5.5
t-9,10-diol	15.8±8.4	4.6	1.9±0.9	N.A.	1.4
t-7,8-diol	6.4±4.4	6.9±2.1	29.0±10.6	8.4±2.2	11.4±6.9
Quinones	30.7±7.9	33.6±7.8	35.2±6.4	16.3±5.1	28.9±5.1
9-OH	15.8±7.1	14.3±5.0	9.9±1.6	28.5±5.7	15.9±5.1
1-OH	2.5±0.4	N.A.	N.A.	9.5±3.8	N.A.
3-OH	3.2±1.0	9.1±1.7	4.2±1.4	16.3±7.9	4.2±0.6
B[a]P	5.5±1.9	4.5±0.8	7.5±2.7	23.4±4.5	8.7±2.3

Chromatographic separation of B[a]P and organic soluble metabolites from bile of fish at 20 ppt saltwater by HPLC revealed a similar pattern as observed at 0 ppt (Table 3.2) with a variety of Phase I metabolites; tetrols, diols, quinones and phenols (Table 4.2). Thirty to forty percent of the radioactivity collected in HPLC fractions coeluted with standard metabolites. Approximately 10 to 18 percent of the organic soluble fraction eluted within the first 20 minutes of the separation. When the metabolites were grouped according to major type and expressed as a percent of radioactivity in the organic soluble fraction of bile, quinones showed the highest accumulation ranging from 16 to 35 percent. The remaining radioactivity did not coelute with known metabolites. The identified Phase I metabolite accumulated in the greatest quantity was 7,8,9,10-tetrol. There was a reduction in t-9,10-dihydrodiol and 1-hydroxyB[a]P produced in the bile of fish at a salinity of 20 ppt compared to 0 ppt.

C) Elimination of Benzo[a]pyrene

The elimination of B[a]P derived radioactivity from juvenile coho salmon is shown in Table 4.3. The majority of the injected B[a]P (ranging from 93-96%) was eliminated from fish 24 hours at 5, 10 and 20 ppt seawater following i.p. injection. There were no significant differences in total elimination of B[a]P at the different salinities.

Table 4.3: Percent of [^{3}H]-B(a)P derived radioactivity eliminated from juvenile coho salmon following a single i.p. injection. Following exposure to an i.p injection of 10 mg (1μCi)/kg B[a]P for one, two, four and seven days at 5, 10 and 20 ppt saltwater. Values are means ± SE of five fish.

	Percent of total dose eliminated			
Salinity 5 ppt	Day 1	Day 2	Day 4	Day 7
February	94.3±1.0	94.8±0.6	95.2±0.5	95.7±0.4
March	94.8±0.6	94.7±0.4	95.0±0.6	97.0±0.2
April	93.4±0.4	94.7±0.4	95.6±0.3	96.4±0.8
May	95.6±0.7	94.4±0.4	94.4±0.5	95.9±0.6
June	95.4±0.4	94.6±1.3	97.1±0.5	98.5±0.1
Salinity 10 PPT	Day 1	Day 2	Day 4	Day 7
February	95.1±0.9	95.8±0.2	96.7±0.4	97.2±0.3
March	95.7±0.5	94.5±0.8	96.4±0.7	96.8±0..6
April	95.6±0.2	95.4±0.7	96.4±0.2	97.3±0.6
May	96.1±0.2	96.2±0.6	96.9±0.6	98.2±0.2
June	93.9±0.6	95.2±0.9	98.3±0.2	95.0±3.9
Salinity 20 PPT	Day 1	Day 2	Day 4	Day 7
February	93.4±2.3	97.4±0.6	97.4±0.4	97.1±0.4
March	96.5±0.4	95.2±0.4	97.2±0.2	98.2±0.2
April	95.5±0.5	95.7±0.3	96.7±0.5	97.9±0.1
May	96.9±0.3	97.0±0.2	97.4±0.3	97.7±0.5
June	94.6±0.5	96.4±0.6	97.4±0.4	98.5±0.2

Section 2: Salinity Effects on the Toxicokinetics of Benzo[a]pyrene in Mature Rainbow Trout

Materials and Methods

Part A: Distribution, Metabolism and Excretion of B[a]P in Trout

A) Fish

Rainbow trout, *Oncorhynchus mykiss*, weighing approximately 300 g, were obtained from West Creek Trout Farm in Aldergrove, B.C. Fish were maintained at seasonal temperatures (4-8°C) and natural photo period in 500 L fiberglass tanks supplied with flowing decholrinated water at pH 6.4, O_2 saturation >95% and hardness 5.2 to 6.0 mg/L $CaCO_3$ for several days until an experiment was started. Fish were fed ad libum until 48 hours prior to an experiment.

B) Salinity Regimes

Rainbow trout were separated into two groups and acclimated for three weeks at 0 or 20 ppt saltwater, seasonal temperature and photo period. Fish were fed *ad libitum* until 48 hours prior to surgery. A dorsal aortic cannula and urinogenital catheter were implanted into each fish. Fish were then allowed a minimum of 24 hours to recover from the surgery in dark plexiglass chambers supplied with fresh flowing water.

Fish from each of the two acclimation groups (0 ppt and 20 ppt) were exposed to B[a]P at both test salinities (0 ppt and 20 ppt). Thus there were four experimental exposure groups (acclimation salinity : test salinity): 0:0, 0:20, 20:20, 20:0 as described in the following table. Fish were not subjected to acute salinity changes until a minimum of 24 h post surgery.

Treatment Groups:	Acclimation Salinity:	Exposure Salinity:
FW:FW	0 ppt	0 ppt
FW:SW	0 ppt	20 ppt (acute salinity increase)
SW:SW	20 ppt	20 ppt
SW:FW	20 ppt	0 ppt (acute salinity decrease)

C) Cannulation of the Dorsal Aorta of Trout

The dorsal aorta of trout were cannulated using the modified procedures of Smith and Bell (1964) one day before the experiment. Trout were anaesthetized with 0.2 g/l MS 222 (ethyl-N-aminobenzoate methane sulfonic acid, Sigma Chemical Co., St. Louis, MO.) and 0.2 g/l sodium bicarbonate as a buffer (Wedemeyer, 1970) and placed on an operating table when all opercular movement had stopped. Recirculating water containing 0.1 g/l MS 222 and 0.1 g/l sodium bicarbonate was continuously passed over the gill of the trout to maintain anaesthesia during the operation. The water was kept cool and aerated with pure oxygen. A small puncture was made at the first gill arch using a needle. Then guitar string wire, inserted into PE50 tubing (0.58 mm i.d., 0.965 mm o.d.,), was inserted into the dorsal aorta of the trout at the first gill arch. When the tubing had entered the dorsal aorta and blood filled tube the guitar string wire was removed. The cannula was sutured at two points to the along the roof of the mouth with silk sutures (size 3.0, Ethicon, Inc., Somerville, NJ.) to secure it in place. The cannula was passed through a hole in the side of the mouth and a stopper - bulb had been attached to cannula to prevent it being pulled out. The cannula was further secured to the dorsal of the fish. It was filled with heparinized saline (5 I.U./ml) and sealed with tack. The entire procedure took approximately 20 minutes. Fish were placed into darkened plexiglass boxes supplied with

fresh water and allowed one day to recover before the experiment. The cannula was washed twice daily with heparinized saline to prevent clotting.

D) Catheterization of the Urinogenital Tract in Trout

An internal catheter was inserted (at the same time as dorsal aorta cannula) into the urinogenital tract of trout using the modified procedures of Wood and Patrick (1994) two days before the experiment. Trout were anaesthetized with 0.2 g/l MS 222 (ethyl-N-aminobenzoate methane sulfonic acid, Sigma Chemical Co., St. Louis, MO.) and 0.2 g/l sodium bicarbonate as a buffer (Wedemeyer, 1970) and placed on an operating table when all opercular movement had stopped. Recirculating water containing 0.1 g/l MS 222 and 0.1 g/l sodium bicarbonate was continuously passed over the gill of the trout to maintain anaesthesia during the operation. The water was kept cool and aerated with pure oxygen. A heat-molded catheter made of polyethylene tubing was prepared ahead of time. The end of the catheter was heat-polished to prevent tissue damage. PE 50 tubing was used as the catheter, a heat-molded PE 120 piece (bend at a 30° angle with flared ends) was cemented over top (providing stability, a tighter fit in the urinary papillae and a reference for extent of penetration) and about two cm from the end that was inserted into urinary bladder. The catheter was pre-filled with 0.9% saline solution and inserted into the urinary tract. The flow of urine was ensured before the catheter was secured by three sutures to the body and anal fin. It was anchored so a direct pull on the catheter would exert tension on the sutures, as well heat molded bubbles were previously added to the catheter to prevent slipping through sutures. At the end of the operation 0.5 ml of saline was gently injected into the catheter and then plugged to prevent air bubble from forming. Fish were placed into darkened plexiglass boxes supplied with fresh water and ensuring

that the catheter was never raised above the elevation of the trout. The fish were allowed to recover from the surgery for at least 24 hours and urine collection was monitored. The catheter was placed 2-8 cm below the water-level and allowed to collect into graduated cylinders that were kept in a dark, ice cooled box. . Urine volumes were taken and then frozen at -80 °C until metabolite analysis.

Exposure

E) Chemicals

Unlabelled benzo[a]pyrene (>99% purity) was purchased from Sigma Chemicals (St. Louis, Mo.) and [1,3,6-^{3}H]- benzo[a]pyrene (52 Ci/mmol) was purchased from NEN Research Products (DuPont, Canada Ltd.). HPLC analysis of labelled and unlabelled B[a]P according to the method of Elnenaey and Schoor (1981) revealed no detectable metabolites. B[a]P metabolite standards were obtained from the National Cancer Institute Chemical Repository, Midwest Research Institute (Kansas City, MO.) All other chemicals (reagent grade) and biochemicals were purchased from Sigma Chemicals (St. Louis, Mo.).

F) Intraarterial Administration

The injection bolus was prepared by dissolving benzo[a]pyrene (10 mg (1 μCi)/kg of [^{3}H]-B[a]P) in a few drops of Mulgofen EL 719 (GAF Ltd., Wythenshawe, Manchester, UK) and mixed into a homogenous solution with 0.9% saline heparin (5 I.U./ml). The chemical was injected as a bolus through the cannula into the dorsal aorta. This was followed by an injection of 0.2 ml of 0.9% saline with heparin through the cannula to ensure that the entire dose of B[a]P entered the circulatory system.

G) Distribution of Benzo[a]pyrene in Trout

Fish from each salinity group were sampled at 48 hours following the intraaterial administration of ^{3}H-B[a]P. The liver, gall bladder, spleen, kidney, visceral fat, intestine, stomach, gill muscle, urinary tract and skin were dissected, weighed, homogenized in 0.9% saline and then oxidized using an R.J. Harvey OX-100 biological oxidizer (R.J. Harvey Instruments Corp., Hillside, NJ.). The oxidized samples were dark adapted for 24 hours before being counted on a Beckman LS 3801 liquid scintillation counter (Beckman Instruments, Irvine, CA.) to determine the amount of ^{3}H-B[a]P derived radioactivity. Bile was released from excised gall bladders and rinsed with 1.0 ml sodium citrate buffer (pH 7.0). An aliquot was removed and counted for total radioactivity by LSC.

H) Metabolism of Benzo[a]pyrene in Trout

Bile, as collected above, was released from excised gall bladders and rinsed with 1.0 ml sodium citrate buffer (pH 7.0). The method used by Kennedy, Gill and Walsh (1991) was followed for the quantitation of B[a]P and its metabolites in bile and urine. Bile and urine were extracted three times with ethyl acetate (5:1) to separate Phase I metabolites. The remaining aqueous layer then underwent a series of incubations and extractions; it was first adjusted to pH 5.0 and incubated for 24h at 37°C with 60U of ß-glucuronidase to hydrolyze glucuronic acid conjugates; it was then adjusted to pH 7.0 and incubated for 24h at 37°C with 20U of sulfatase to hydrolyze sulfate conjugates; and finally it was adjusted to pH 2.0 and incubated for 24h at 80°C with 1N H_2SO_4 to acid hydrolyze any remaining conjugated metabolites. After each step, the aqueous layer was extracted three times with ethyl acetate (5:1). Aliquots of each ethyl acetate extraction were removed, dried with nitrogen gas, resuspended in 1 ml of methonol and added to

liquid scintillation cocktail (Amersham, Oakville, ON, Canada) and counted for radioactivity at each step by LSC.

B[a]P and Phase I metabolites in the first organic extracts of bile and urine, were separated by HPLC using a Hewlett Packard 1050 series liquid chromatograph equipped with a Phenomenex Primesphere 5µm C18 reverse phase column (250 x 4.6 mm), a HP 1046A fluorescence detector and an HP 3396 Series II Integrator (excitation 340-380 nm and emission >430 nm). The methods of Varanasi et al. (1986) were followed; using a nonlinear gradient starting at 80% Solvent A (0.005% glacial acetic acid in water, v/v) and 20% Solvent B (methanol) that was first held isocratically for 1 minute and then changed: 20-60% Solvent B in 12 minutes; 60-70% Solvent B in 12 minutes; 70-100 % Solvent B in 10 minutes; and then held at 100% for 10 minutes. The gradient was returned stepwise to starting proportions over a 10 minute period. The flow rate was 1.0 ml/min. Fractions were collected every 30 seconds using a BioRad Fraction collector. A volume of 10 ml of liquid scintillation cocktail (Amersham, Oakville, ON, Canada) was added to each fraction, allowed to dark adapt for at least 24 hours and counted for radioactivity at by LSC. B[a]P and its metabolites were determined by comparison of peaks and dpms recovered with known standard metabolites: r-7,t-8,9,c-10-tetrahdroxytetrahdyrobenzo[a]pyrene (7,8,9,10-tetrol); 7,8-dihydroxybenzo[a]pyrene(-)-trans-7,8-diol (t-7,8-dihydrodiol); 9,10-dihydroxybenzo[a]pyrene-(-)-trans-9,10-diol (t-9,10-dihydrodiol); 1,6-benzo[a]pyrenequinone (1,3-dione); 3,6- benzo[a]pyrenequinone (3,6-dione); 6,12-benzo[a]pyrenequinone (6,12-dione); 9-hydroxybenzo[a]pyrene (9-OH); 1-hydroxybenzo[a]pyrene (1-OH); and 3-hydroxybenzo[a]pyrene (3-OH)..

I) Elimination of Benzo[a]pyrene in Trout

The total elimination of [³H]-B[a]P was calculated by subtracting the amount radioactivity remaining in the organism after 48 hours from the amount injected.

Urine was collected into graduated cylinders and sampled 6, 12, 24 and 48 hours following intraaterial administration of [³H]-B[a]P. Urine volumes were noted and the urine frozen at -80 °C until analyzed for metabolites and parent compound by the above methods.

J) Mathematical Analysis

All percent data were arcsine transformed before statistics were performed. One-factor ANOVA and Student-Newman-Keuls multiple comparison test was used to establish differences between tissue body burdens, metabolite classes and B[a]P elimination between the different treatment groups at a significance level of $p < 0.05$ (Zar, 1974).

Part B: Toxicokinetics Modeling of B[a]P in the Blood of Trout

A) Fish

Rainbow trout, *Oncorhynchus mykiss*, weighing approximately 300 g, were obtained from West Creek Trout Farm in Aldergrove, B.C. Fish were maintained at seasonal temperatures (4-8°C) and natural photo period in 500 L fiberglass tanks supplied with flowing decholrinated water at pH 6.4, O_2 saturation >95% and hardness 5.2 to 6.0 mg/L $CaCO_3$ for several days until an experiment was started. Fish were fed ad libum until 48 hours prior to an experiment.

B) Salinity Regimes

Rainbow trout were separated into two groups and acclimated for three weeks at 0 or 20 ppt saltwater, seasonal temperature and photo period. Fish were fed *ad libitum* until 48 hours prior to surgery. A dorsal aortic cannula was implanted into each fish as described previously. Fish were then allowed a minimum of 24 hours to recover from the surgery in plexiglass chambers. Fish from each of the two acclimation groups (0 ppt and 20 ppt) were exposed to B[a]P at both test salinities (0 ppt and 20 ppt). Thus there were four experimental exposure groups (acclimation salinity : test salinity): 0:0, 0:20, 20:20, 20:0. Fish were not subjected to acute salinity changes for a minimum of 24 h post surgery. A bolus injection of B[a]P was given *via* the dorsal aorta cannula.

C) Chemicals

Unlabelled benzo[a]pyrene (>99% purity) was purchased from Sigma Chemicals (St. Louis, Mo.). HPLC analysis of unlabelled B[a]P according to the method of Elnenaey and Schoor (1981) revealed no detectable metabolites. B[a]P metabolite standards were obtained from the National Cancer Institute Chemical Repository, Midwest Research Institute (Kansas City, MO.) All other chemicals (reagent grade) and biochemicals were purchased from Sigma Chemicals (St. Louis, Mo.).

D) Exposure to benzo[a]pyrene

The injection was prepared by dissolving benzo[a]pyrene (10 mg (1 µCi)/kg of [^{3}H]-B[a]P) in a few drops of Mulgofen EL 719 (GAF Ltd., Wythenshawe, Manchester, UK) and mixed into a homogenous solution with 0.9% saline heparin (5 I.U./ml). The chemical was injected as a bolus through the cannula into the dorsal aorta. This was

followed by an injection of 0.2 ml of 0.9% saline containing heparin through the cannula to ensure that the entire dose of 10 mg/kg entered the circulatory system.

E) Blood Sampling

A control blood sample was withdrawn from all trout prior to chemical administration. This 0.2 ml blood sample was withdrawn through the cannula and subsequently replaced with an equal volume of heparinized solution. The cannula was filled with heparinized saline to prevent blood clotting in the cannula.

F) Analysis of Blood

A modified method of Kennedy and Law (1990) was used to extract blood samples for B[a]P analysis. Distilled water (0.5 ml) and 0.1 N sulfuric acid were added to deproteinize the blood sample (0.2 ml) it in a centrifuge tube. The mixture was vortexed and extracted three times with ethyl acetate in a 5:1 proportion (ethyl acetate: aqueous). After each addition of ethyl acetate the tubes were vortexed, shaken on a reciprocating shaker for 30 minutes and centrifuged at 3500 rpm for 15 minutes on a low speed centrifuge to separate the aqueous and organic phases. The organic extracts were combined and evaporated to dryness under nitrogen. The residue was redissloved in 1 ml of methanol containing benzanthracene (1 μg/ml) as an internal standard. The sample in methanol was analyzed by a Hewlett Packard 1050 series liquid chromatograph equipped with Phenomenex Primesphere 5μm C18 reverse phase column (250 x 4.6 mm), a HP 1046A fluorescence detector and an HP 3396 Series II Integrator (Kennedy and Law, 1990). Benzo[a]pyrene was eluted from the column isocratically with methanol : water (80%:20%). The fluorescence detector was set at an excitation 340-380 nm and emission >430 nm (Varanasi et al., 1986).

G) Pharmacokinetic/Toxicokinetic Analysis

The time course of B[a]P in the blood of trout after intraarterial administration were analyzed by a nonlinear least squares regression program (PCNONLIN) (Metzler et al., 1974; Metzler and Weiner, 1986, Kennedy and Law, 1990). The time course of B[a]P in the blood of trout after intraarterial administration was described by the following equation (Gibaldi and Perrier, 1975):

$$C_B = \sum_{i=1}^{n} A_i e^{-X_i t}$$

where C_B is the blood concentration of B[a]P at time t and A_i and X_i are the coefficients and constants of the exponential components, respectively. The parameters of the equation were estimates from the nonlinear least squares regression program (PCNONLIN). The statistical weighting factor in the least-square procedure was the inverse of the observed blood concentrations (Ottaway, 1973). The overall goodness of fit was determined by comparing the sum of the squared deviations and by scatter of the actual points around the fitted function. Akaike's information criterion was used to select the most appropriate model (Yamaoka et al., 1978). Calculation of model dependent parameters were derived from estimates of parametres from the nonlinear regression analysis (Gibaldi and Perrier, 1975).

Results

Part A: Distribution, Metabolism and Excretion of B[a]P in Trout

A) Distribution of B[a]P in Trout

Radioactivity was found in all tissues examined after a bolus injection of 10 mg/kg [^{3}H]-B[a]P *via* the dorsal aorta. The percent body burden in each tissue is shown in Table 4.4. In the four salinity treatments (acclimation : test salinities), FW:FW (0:0 ppt), FW:SW (0:20 ppt), SW:SW (20:20 ppt), SW:FW (20:0 ppt), the total injected body burden of radioactivity recovered after 48 hours in the sampled tissues was 34.5±1.7, 45.0±8.2, 37.9±8.1, 38.5±3.3%, respectively (Table 4.10). The distribution of the B[a]P-derived radioactivity recovered 48 hours following intraarterial injection in the urine, bile and tissues sampled, is shown in Table 4.10. Statistically significant differences in the percent body burden for specific tissues between salinity treatment groups were found for the kidney, spleen, intestine, stomach and gill (Table 4.4). The liver had the highest burden of radioactivity in all treatment groups, followed by the gall bladder and kidney. Urinary tract and skin showed the lowest burden of radioactivity in all treatment groups. Fish exposed to B[a]P in the SW:SW treatment had higher tissue burdens of radioactivity in the intestine, stomach and visceral fat than fish exposed in the FW:FW treatment group. Fish that were exposed to an acute salinity change (FW:SW or SW:FW) showed an increase in radioactivity in the spleen, kidney, and muscle and a decrease in radioactivity in the gall bladder compared to the respective acclimation treatment groups (FW:FW vs. FW:SW or SW:SW vs. SW:FW).

Table 4.4: The percent body burden distribution of [^{3}H]-B(a)P derived radioactivity in mature rainbow trout. Trout were sacrificed 48h after receiving a single intraaortal injection of 10 mg(1μCi)/kg -[^{3}H]-B(a)P. The values are mean ± SE for three trout. Values with common letters are not significantly different within the same salinity treatment at p<0.05. Values with common symbols are not significantly different for the same tissue between salinity treatments at p<0.05.

Organs and Tissues	Radioactivity (% body burden)			
	FW:FW	FW:SW	SW:SW	SW:FW
liver	46.0±7.7[a]	48.7±8.8[a]	52.1±0.9[a]	42.4±3.3[a]
gall bladder	15.0±4.6[b]	5.8±2.9[c,d]	11.4±0.9[c]	7.6±2.6[b,c]
spleen	1.4±0.2[t,d]	1.8±0.2[*,c,d]	1.2±0.08[t]	6.4±3.7[*,d]
kidney	9.2±2.7[c]	21.5±7.5[*,b]	9.2±0.8[c]	14.8±3.7[*,b]
visceral fat	5.9±2.6[c]	2.5±1.1[c,d]	8.7±0.9[c]	8.8±1.9[c,d]
intestine	2.1±1.4[d]	3.1±1.4[c,d]	10.1±1.4[*,c]	6.9±2.8[*,c,d]
stomach	0.8±0.07[d]	1.1±0.5[d]	3.7±0.27[*,d]	0.7±0.2
gill	3.7±0.2[c,d]	3.7±1.5[c,d]	2.6±0.4[d]	2.1±0.5[*]
muscle	2.4±0.7[c,d]	9.6±5.0[c]	1.5±1.1	2.3±0.9[d,8]
urinary tract	0.2±0.1[d]	0.2±0.03[d]	0.6±0.4	0.5±0.2
skin	0.5±0.1[d]	2.0±1.1[c,d]	0.7±0.4	0.7±0.1

B) Metabolism of Benzo[a]pyrene in Trout

The separation of B[a]P and its metabolites by organic solvent extraction, hydrolytic enzyme incubations and subsequent HPLC analysis, revealed that most of the radioactivity in the bile and urine was conjugated or unconjugated metabolites of B[a]P. The proportion of unmetabolized B[a]P after 48h was less than 1% of the recovered radioactivity. The percentages of total B[a]P-derived radioactivity recovered in the bile as organic soluble, glucuronide, sulfate or unknown conjugates and other aqueous soluble metabolites are listed in Table 4.5. There was a significant difference in the proportion of organic soluble metabolites between the fish which were acclimatized to different salinities, FW:FW 2.3±0.4% and SW:SW 14.1±5.9%. Whereas fish subjected to acute salinity changes (FW:SW 2.54±0.6%; SW:FW 11.0±3.0%) did not differ from their respective acclimated treatment. There were no significant differences between different salinity treatments in glucuronic acid conjugates (SW:SW 18.9±0.5%; FW:FW 23.4±4.9%; FW:SW 24.4±1.6%; SW:FW 27.4±4.6%) or sulfate conjugates (SW:FW 28.5±5.1%; SW:SW 29.2±5.6%; FW:FW 32.2±1.7%; FW:SW 37.1±0.8%). The amount of unknown conjugates recovered from fish acclimated and exposed in FW was significantly higher than fish acclimatized and exposed in SW (FW:FW 28.6±2.6% vs. SW:SW 11.6±3.9%). Fish subjected to an acute salinity change had a decrease in unknown conjugates when moved into SW and had an increase when moved into FW (FW:FW 28.6±2.6% vs. FW:SW 17.2±0.5%; and SW:SW 11.6±3.9% vs. SW:FW 13.9±2.8%). The reverse was observed in the proportions of aqueous soluble metabolites, fish acclimated to SW had significantly higher amounts than FW acclimated fish (FW:FW

13.0±0.9% vs. SW:SW 26.3±3.6%). Fish subjected to acute salinity change had an increase in aqueous soluble metabolites when moved into SW and had a decrease when moved into FW (FW:FW 13.0±0.9% vs. FW:SW 18.7±0.4%; SW:SW 26.3±3.6% vs. SW:FW 19.3±2.8%).

Chromatographic separation of B[a]P and organic soluble metabolites from the bile of fish revealed a variety of Phase I metabolites which included tetrols, diols, quinones and phenols (Table 4.6). Although there were no statistically significant differences observed between treatment groups in the identified metabolites, the identified phase I metabolites which accumulated in the greatest quantities were 7,8,9,10-tetrahydroB[a]P, 3-hydroxyB[a]P and t-9,10-dihydrodiol. Fish which underwent an acute salinity change (FW:SW and SW:FW) showed an increased trend in 7,8,9,10-tetrahydroB[a]P accumulation compared to the respective acclimation salinity treatment groups (FW:FW and SW:SW). Fish acclimated to FW had a higher accumulation of t-9,10-dihydrodiol whereas fish acclimated to SW had a higher accumulation of 3-hydroxyB[a]P. When the metabolites were grouped according to major type and expressed as a percent of radioactivity in the organic soluble fraction of the bile the following trends were observed. Fish exposed to B[a]P in the FW:FW treatment had lower proportions of phenols but higher proportions of quinones than fish exposed in the SW:SW treatment group (phenols: FW:FW 33.4±8.4% vs. SW:SW 45.1±10.4%; quinones: FW:FW 34.9±8.8% vs. SW:SW 15.4±0.5%). Acute salinity increases showed increase in phenols (FW:FW 33.4±8.4% vs. FW:SW 47.9±7.7%) and decreases in quinones (FW:FW 34.9±8.8% vs. FW:SW 18.0±4.4%), whereas acute salinity decreases showed decreases in phenols (SW:SW

45.1±10.4% vs. SW:FW 17.9±3.9%) and increases in quinones (SW:SW 15.4±0.5% vs. SW:FW 24.3±5.2%) compared to the respective acclimation treatment groups.

Urine sampled 6, 12, 24 and 48 h after administration of B[a]P contained less than 2% as unmetabolized B[a]P. The percentages of total B[a]P-derived radioactivity recovered in the urine sampled 6, 12, 24,and 48h as organic soluble, glucuronide, sulfate, unknown conjugates and other aqueous soluble metabolites are listed in Table 4.6. Fish which were acclimated to FW higher proportion of organic soluble metabolites (FW:FW 32.2±6.9% vs. SW:SW 15.1±6.9%) and lower proportions of both unknown conjugates (FW:FW 12.4±4.6% vs. SW:SW 27.5±8.6%) and aqueous soluble metabolites (FW:FW 7.8±2.6% vs. SW:SW 17.8±0.8%) compared to fish acclimated to SW. Fish that were exposed to an acute salinity increase showed a decrease in organic soluble metabolites (FW:FW 32.2±6.9% vs. FW:SW 22.2±1.7%) and an increase in both unknown conjugates (FW:FW 12.4±4.6% vs. FW:SW 34.6±10.7%) and aqueous soluble metabolites (FW:FW 7.8±2.6% vs. FW:SW 12.2±6.8%) compared to the acclimation treatment groups. Fish that were exposed to an acute salinity decrease showed an increase in organic soluble metabolites (SW:SW 15.1±6.9% vs. SW:FW 21.1±4.5%) and a decrease in both unknown conjugates (SW:SW 27.5±8.6% vs. SW:FW 15.7±8.1%) and aqueous soluble metabolites (SW:SW 17.8±0.8% vs. SW:FW 15.9±3.5%) compared to the acclimation treatment groups. There were no significant difference between salinity treatments in glucuronic acid conjugates (range of 21.2±1.3 to 25.5±4.1%) and sulfate conjugates (range of 9.8±7.1 to 23.3±7.9%).

Chromatographic separation of B[a]P and organic soluble metabolites from the urine of fish revealed a variety of phase I metabolites which included tetrols, diols, quinones and phenols (Table 4.7). The identified phase I metabolites which accumulated in the greatest quantities were 7,8,9,10-tetrahydroB[a]P and 3-hydroxyB[a]P. Fish acclimatized to SW had a significantly higher accumulation of 3-hydroxy, t-9,10 dihydrodiol and t-7,8 dihydrodiol as well had a lower accumulation of quinones than fish acclimatized to FW. Fish which underwent an acute salinity change (FW:SW and SW:FW) showed an increase in 7,8,9,10-tetrahydroB[a]P accumulation compared to the respective acclimation salinity treatment groups (FW:FW and SW:SW).

Table 4.5: The percent of total [^{3}H]-B(a)P derived-radioactivity as organic soluble metabolites, glucuronide and sulfate and other aqueous soluble metabolites in the bile of rainbow trout, 48h following intraaortal injections with 10mg(1μCi)/kg of B[a]P. The values are mean ± SEM for three fish. Values with common symbols are not significantly different within the same salinity treatment at p<0.005. Values with common letters are not significantly different for the same metabolite group between salinity treatments at p<0.05.

Metabolite Groups (percent of total metabolites) in the bile

Salinity treatment	Organic soluble	Glucuronide	Sulfate	Unknown conjugates	Unknown aqueous soluble
FW:FW	2.33±0.37†ª	23.89±4.78	32.23±1.67	28.59±2.55ᶜ	12.97±0.88*ᶠ
FW:SW	2.54±0.56ª	24.43±1.55	37.13±0.82	17.23±0.54†ᵈ	18.69±0.37†ᶠˑᵍ
SW:SW	14.1±5.9*ᵇ	18.91±0.5*	29.17±5.6*	11.58±3.9*	26.25±3.6*ᵉˑᵍ
SW:FW	11.04±3.0*ᵇ	27.37±4.55†	28.49±5.14†	13.85±2.82*ᵈ	19.25±2.79*ᶠᵍ

Figure 4.2: A typical high-performance liquid chromatograph of B[a]P and its metabolite standards using method 2.
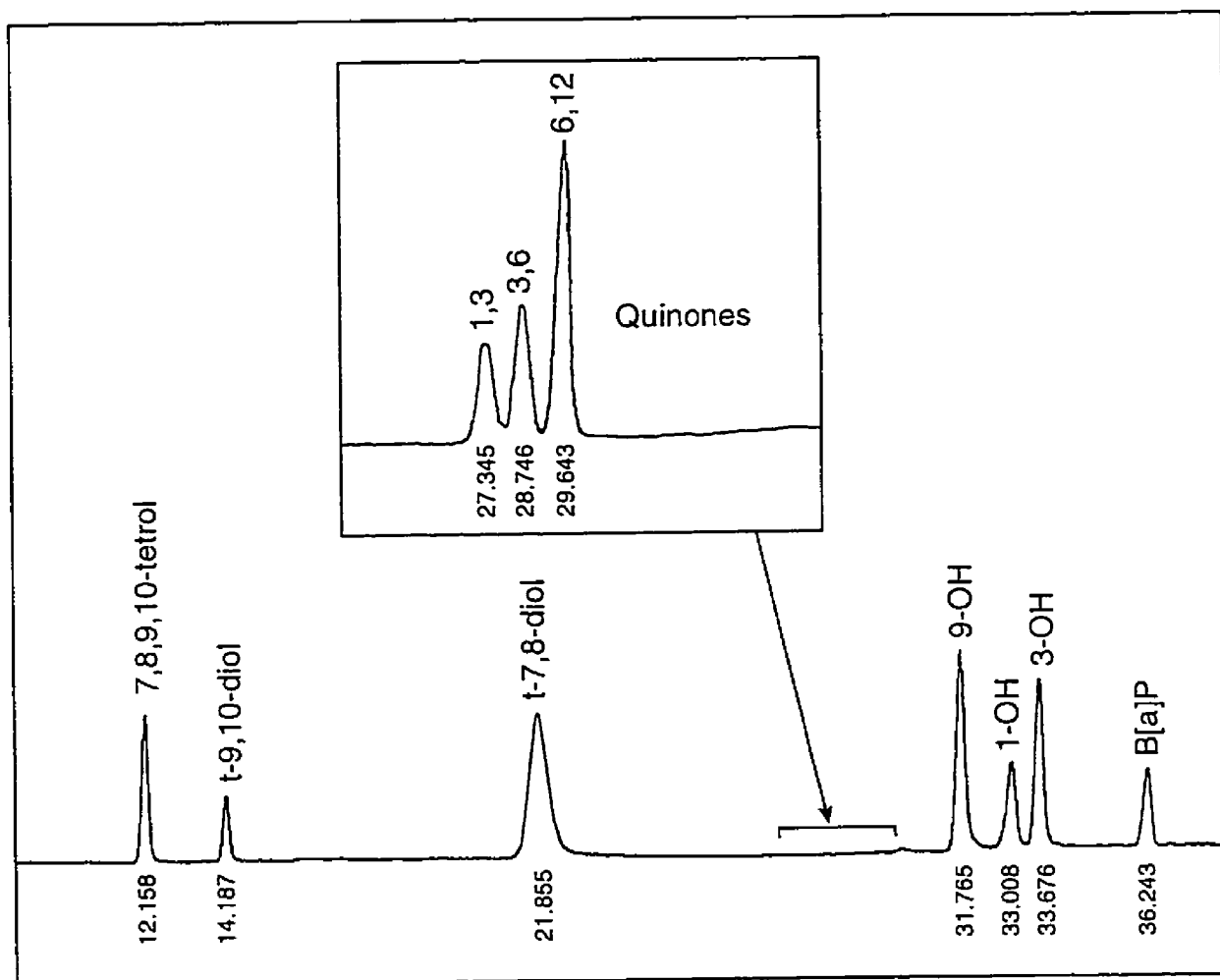
Table 4.6: The percent of individual metabolites as a percentage of identified organic soluble metabolites in the bile of rainbow trout exposed for 48h via intraaortal injection of 10 mg (1 μCi)/kg [^{3}H] B(a)P. Values are means ± SEM for three trout for each group.

Biliary metabolites	Percent organic soluble metabolites Salinity treatments			
	FW:FW	FW:SW	SW:SW	SW:FW
7,8,9,10 tetrahydroB[a]P	10.2±6.2	16.6±0.1	13.7±4.0	31.5±9.6
t 9,10 dihydrodiol	25.2±17.7	12.4±2.3	12.4±6.3	10.6±1.0
t 7,8 dihydrodiol	6.3±2.8	10.7±1.3	6.6±2.8	3.1±1.3
1,3 dione	10.9±8.6	11.2±1.2	7.4±1.0	7.8±2.6
3,6 dione	6.7±2.1	8.6	3.3±0.5	10.7±1.9
6,12 dione	10.7±5.8	5.1	4.7±1.2	12.1±3.6
9-hydroxy	6.7±1.9	10.9±3.0	8.4±6.3	4.6±0.6
1-hydroxy	8.3±2.3	19.9±3.0	4.5±1.6	7.4±1.4
3-hydroxy	12.7±6.7	17.0±7.7	32.1±19.4	7.7±1.9
B[a]P	2.4±0.9	2.8±0.2	6.9±2.3	4.8±1.2

Table 4.7: The percent of total [^{3}H]-B(a)P derived-radioactivity as organic soluble metabolites, glucuronide and sulfate and other aqueous soluble metabolites in the urine of rainbow trout, 48h following intraaortal injections with 10mg(1μCi)/kg of B[a]P. The values are mean ± SEM of the urine sampled 6, 12, 24 and 48h for three fish. Values with common symbols are not significantly different within the same salinity treatment at p<0.005. Values with common letters are not significantly different for the same metabolite group between salinity treatments at p<0.05.

<u>Metabolite Groups (percent of total metabolites) in the urine</u>

Salinity treatment	Organic soluble	Glucuronide	Sulfate	Unknown conjugates	Unknown aqueous soluble
FW:FW	32.2±6.9	24.3±5.3	23.3±7.9	12.4±4.6*	7.8±2.6*
FW:SW	22.2±1.7	21.2±1.3	9.8±7.1	34.6±10.8	12.2±6.8
SW:SW	15.1±6.9	21.2±3.3	18.5±0.8	27.5±8.6	17.8±0.8
SW:FW	21.1±4.5	25.5±4.1	21.8±5.9	15.7±8.1	15.9±3.5

Table 4.8: The percent of individual metabolites as a percentage of identified organic soluble metabolites in the urine of rainbow trout exposed for 48h via intraaortal injection of 10 mg (1 µCi)/kg [^{3}H] B(a)P. Values are means ± SEM of the urine sampled 6, 12, 24 and 48h for three trout for each group.

Urinary metabolites	Percent of organic soluble metabolites Salinity treatment			
	FW:FW	FW:SW	SW:SW	SW:FW
7,8,9,10 tetrahydroxyB[a]P	16.8±4.0	24.1±1.6[*]	9.6±1.9	23.1±4.2[*]
t 9,10 dihydrodiol	7.4±2.6	9.3±2.3	15.7±4.6	17.0±4.9
t 7,8 dihydrodiol	6.8±1.1	11.4±1.5	12.4±4.5	13.7±4.7
1,3 dione	18.7±7.9	12.2±3.7	5.0±1.1	5.2±1.6
3,6 dione	6.5±1.8	9.1±2.4	3.7±0.7	5.3±0.8
6,12 dione	13.3±5.9	12.2±0.7	4.6±1.2	4.9±1.2
9-hydroxy	14.9±3.9[a]	6.9±4.3	7.4±1.9	5.9±1.1
1-hydroxy	6.7±0.6	9.6±2.6	5.3±1.1	5.6±1.3
3-hydroxy	13.9±3.9	6.2±1.6	25.0±10.4[a]	14.7±4.8
B[a]P	4.2±0.6	4.5±1.3	3.5±0.9	4.5±0.7

C) Elimination of Benzo[a]pyrene in Trout

The percent of the injected dose of [^{3}H]-B[a]P and its metabolites eliminated from trout in 48 hours is shown Table 4.9. Fish injected in the FW:FW treatment had eliminated similar amounts of [^{3}H]-B[a]P (and its metabolites) as fish injected in the SW:SW treatment group (FW:FW 98.8±0.6% vs. SW:SW 98.1±1.0%) by 48 hours. Fish that were exposed to an acute salinity change (FW:SW 90.4±3.7% or SW:FW 93.1±1.4%) had eliminated less [^{3}H]-B[a]P (and its metabolites) than the respective acclimation treatment groups (FW:FW or SW:SW) by 48 hours.

Urine volumes collected at 6, 12, 24 and 48 hours from all treatment groups are shown in Table 4.11. Fish acclimated to FW showed a higher production of urine over fish acclimated to SW. There was a decrease in urine production in the FW:SW treatment group and an increase in production in the SW:FW treatment group. No significant difference between any treatment group was found in the percent of radioactivity recovered in urine once all collected urine was added together (6, 12, 24 and 48 hour samples).

The hepatobiliary system (liver, gall bladder and bile) contained majority of the radioactivity recovered 48 hours following an intraarterial administration of [^{3}H]-B[a]P (Tables 4.4 and 4.10). Greater than 50% of the remaining radioactivity in the tissues after 48 hours was recovered in the liver and gall bladder in all salinity treatments.

Table 4.9: The percent of the injected dose of 10mg(1μCi)/kg of B[a]P eliminated from rainbow trout in 48h following intraarterial injections. The values are means ± SE for three trout. Common symbols represent values not significantly different at $p < 0.05$.

Percent of Injected Dose Excreted
Salinity treatment

FW:FW	FW:SW	SW:SW	SW:FW
98.8±0.6*	90.4±3.7	98.1±1.0*	93.1±1.4

Table 4.10:   The distribution of total [³H]-B(a)P derived-radioactivity recovered 48h following intraarterial injections with 10mg(1μCi)/kg of B[a]P, between the urine, bile and tissues in rainbow trout. The values are means ± SE for three trout.

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	Distribution of Radioactivity at 48 Hours **Salinity treatment**			
	FW:FW	**FW:SW**	**SW:SW**	**SW:FW**
Urine	4.8±2.3	6.1±3.8	8.7±2.6	12.2±3.2
Bile	55.0±1.7	44.0±7.4	50.6±8.8	48.5±4.2
Tissue	34.5±1.7	45.0±8.2	37.9±8.1	38.5±3.3

Table 4.11: Urine volumes (ml) collected from rainbow trout, 6h, 12h, 24h and 48h following intraaortal injections with 10mg(1μCi)/kg of B[a]P. Exposed to an acute or acclimated salinity treatment. Values are means ± SEM for three rainbow trout.

		<u>Urine Production</u> <u>Salinity treatment</u>		
	FW:FW	**FW:SW**	**SW:SW**	**SW:FW**
Time	**Urine Volume (ml)**			
6h	4.4±1.9	5.8±4.2	0.75±0.2	2.3±0.8
12h	4.5±2.8	0.38±0.1	0.5±0.2	4.3±1.5
24h	5±3.5	0.6±0.1	2.4±1.3	5.3±1.2
48h	18.9±2.4	1.5±0.8	2.8±1.0	14.7±2.0
Total (ml)	32.8±5.6	7.9±2.7	6.4±1.9	25±7.7

RESULTS

Part B: Toxicokinetics of B[a]P in the Blood of Trout

A) Toxicokinetics of Benzo[a]pyrene in the Blood of Trout

A typical chromatograph of B[a]P and the internal standard benzanthracene is shown in figure 4.3. The time course of B[a]P in the blood of trout following an intraarterial administration are shown in figure 4.4. The concentration of B[a]P in the blood declined biexponentially with time. The mean data from three trout were fitted to a two-compartment and three compartment open toxicokinetic model using a nonlinear least-squares regression analysis program. The weighted sum of squares residuals (WSSR) and Akaike's information criterion (AIC) values for the two-compartment model were in all cases lower than the three-compartment model. The scatter of observed values about the predicted values in the two-compartment model were random. Therefore, the two compartment model was considered the best interpretation of the data. The two-compartment model chosen to describe the disposition of B[a]P in the trout is shown in figure 4.5.

The parameter estimates of the exponential equation fitted to these data are shown in Table 4.12. The terminal half-lives of decay from the blood ($b2_{HL}$) for fish acclimated and exposed to B[a]P in FW were similar to that from fish acclimated and exposed in SW (FW:FW 2.9 d vs. SW:SW 2.1 d). Fish that were exposed to an acute salinity increase (FW:SW) had a longer terminal half-life ($b2_{HL}$) compared to fish acclimated and exposed to B[a]P in the FW (FW:FW 2.9 d vs. FW:SW 4.0 d). Fish exposed to an acute salinity

decrease (SW:FW) had a longer terminal half-life ($b2_{HL}$) compared to fish acclimated and exposed to B[a]P in SW (SW:SW 2.1 d vs. SW:FW 4.7d).

The total body clearance (Q_b) of B[a]P from trout acclimated and exposed in the FW had the same value as fish acclimated and exposed in SW (FW:FW 0.2 ml/min vs. SW:SW 0.2 ml/min). Fish that were exposed to an acute salinity increase (FW:SW) had a slower total body clearance (Q_b) of B[a]P compared to fish acclimated and exposed in the FW (FW:FW 0.2 ml/min vs. FW:SW 0.09 ml/min). Fish exposed to an acute salinity decrease (SW:FW) slower total body clearance (Q_b) of B[a]P compared to fish acclimated and exposed in SW (SW:SW 0.2 ml/min vs. SW:FW 0.05 ml/min).

Figure 4.3: A typical high-performance chromatograph of B[a]P and internal standard BAN.
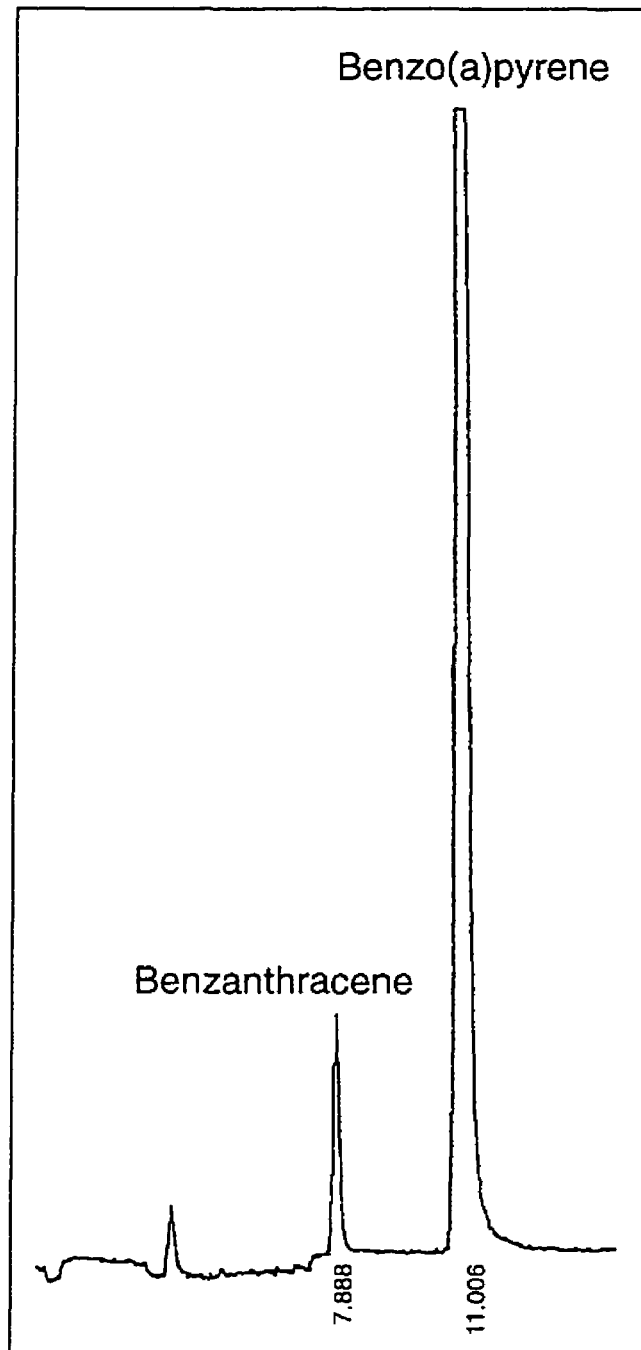
Figures 4.4: Time course of unchanged B[a]P in the blood of trout following a single intraarterial administration of 10 mg/kg and different salinity regimes a) FW:FW, b) FW:SW, c) SW:SW and d) SW:FW. The curves represent the two-compartment model prediction of the data. The values are the means ± SEM of three fish.

a) **FW:FW**
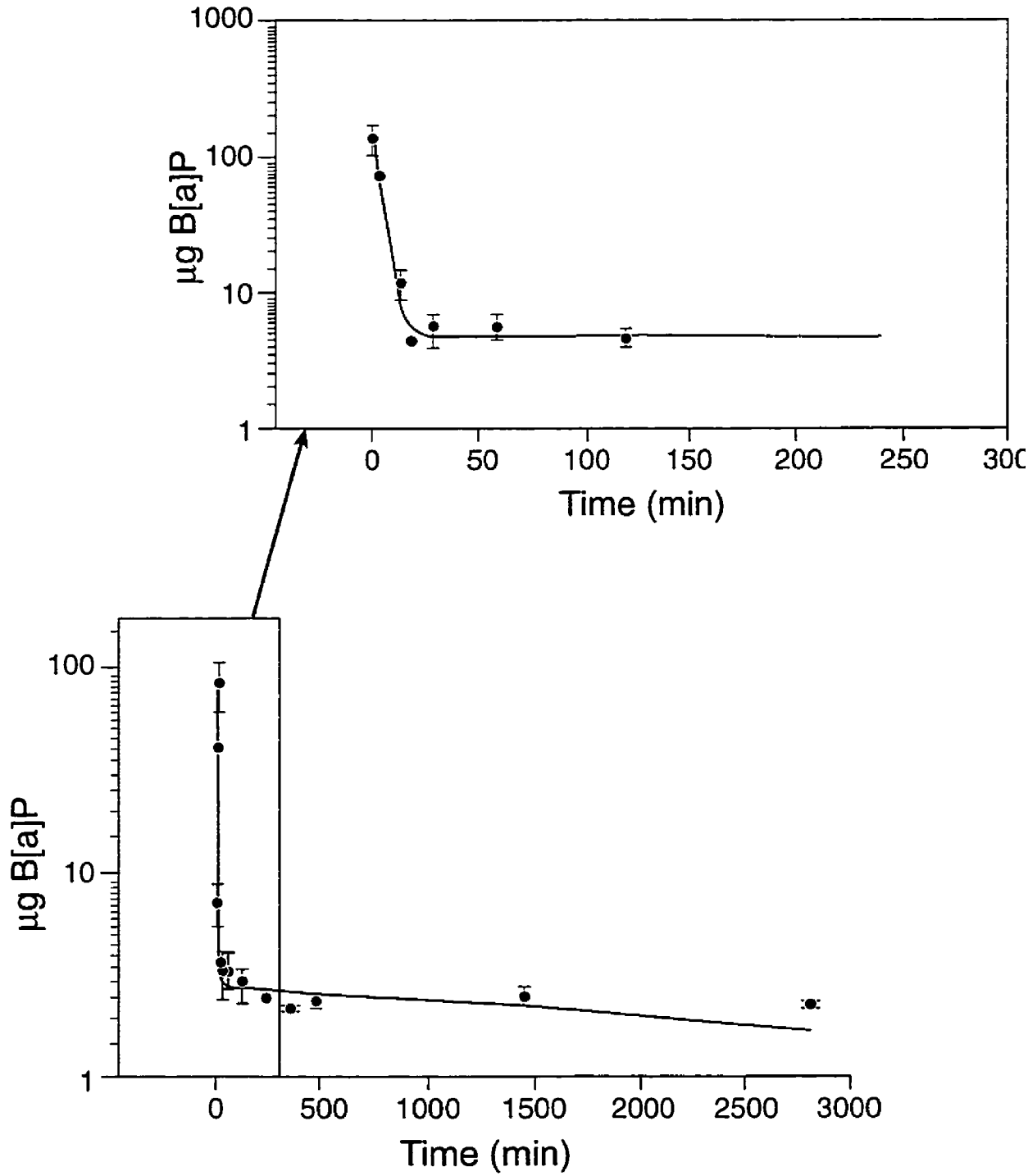
b)   FW:SW
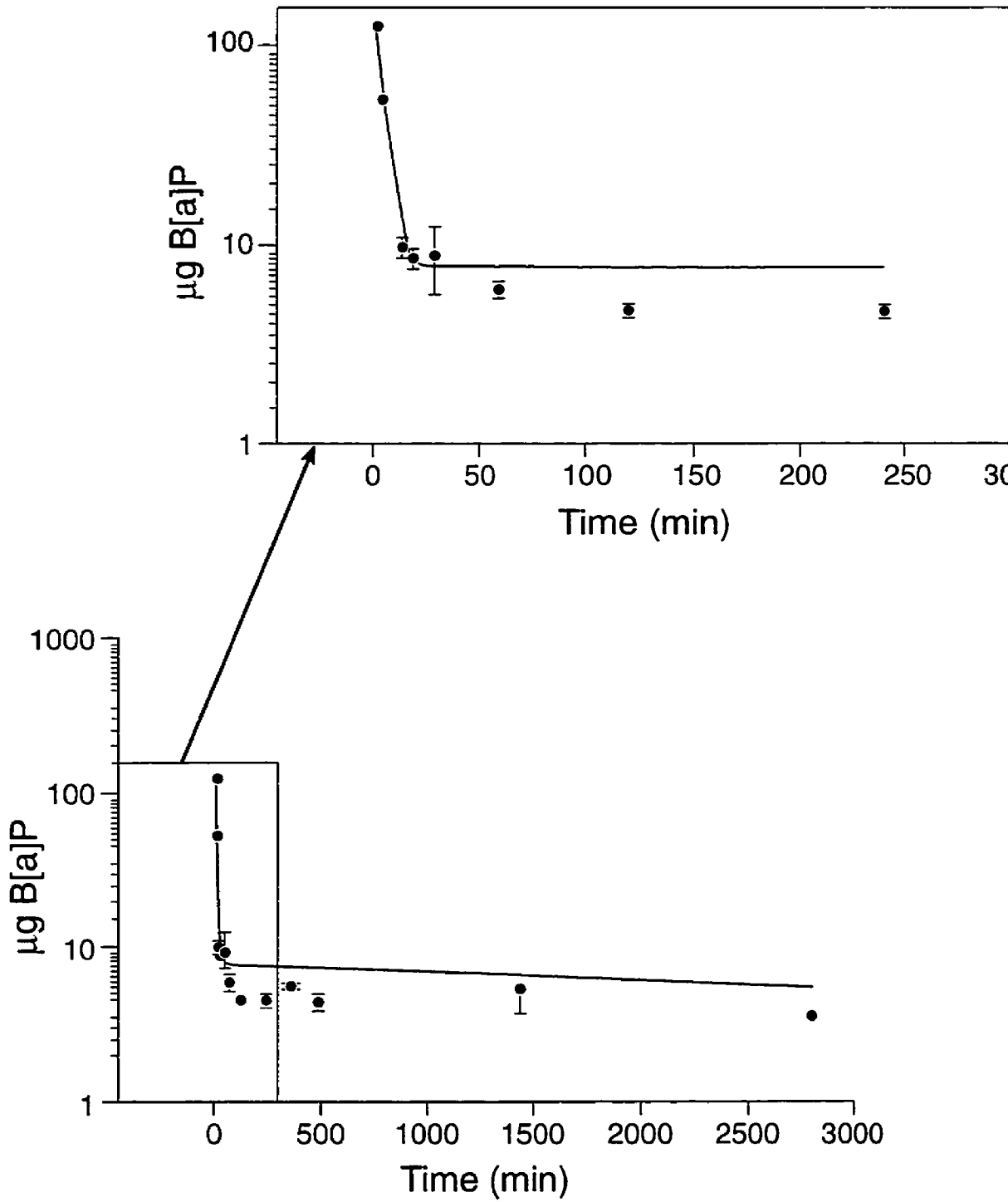
c) **SW:SW**
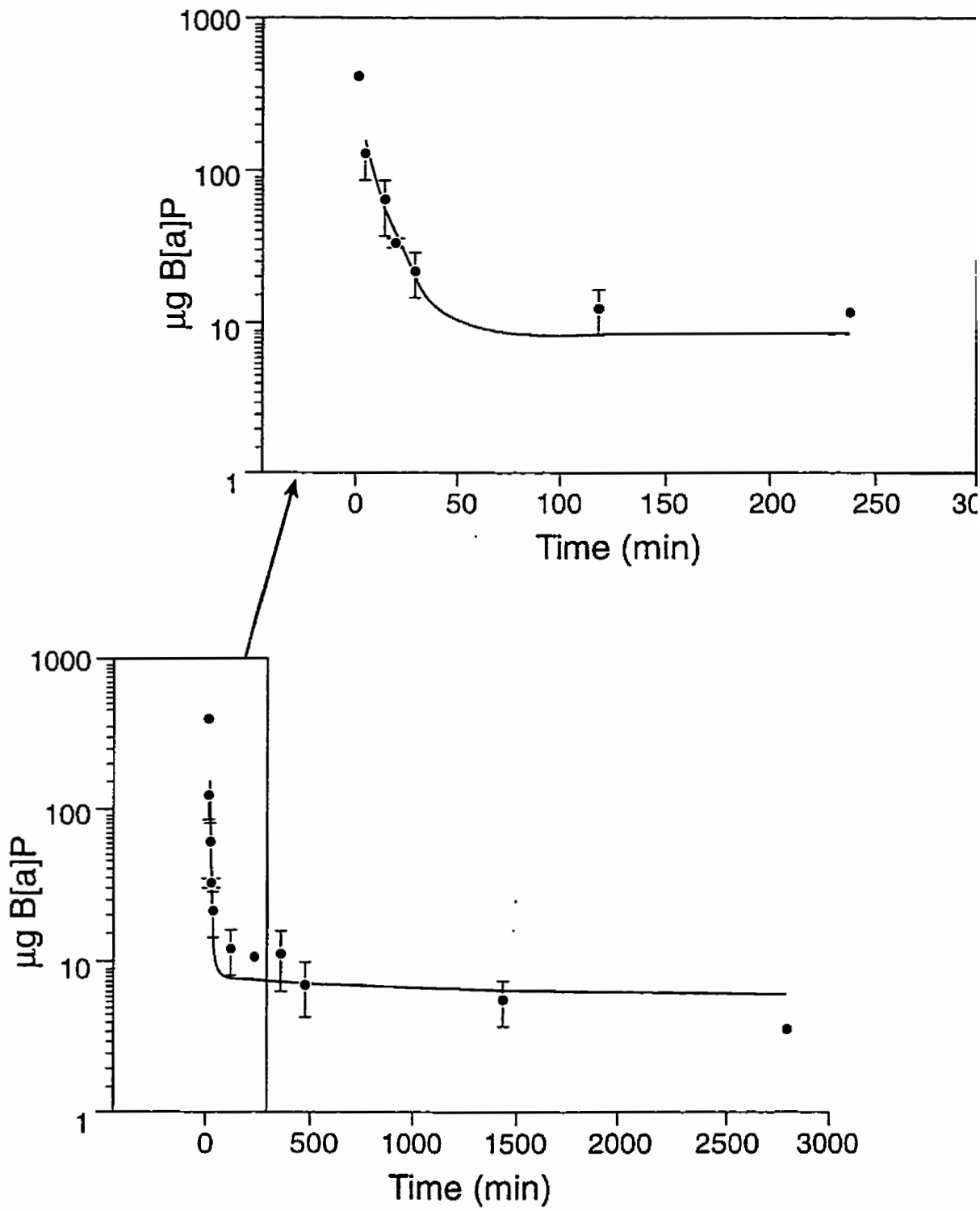
d)   SW:FW
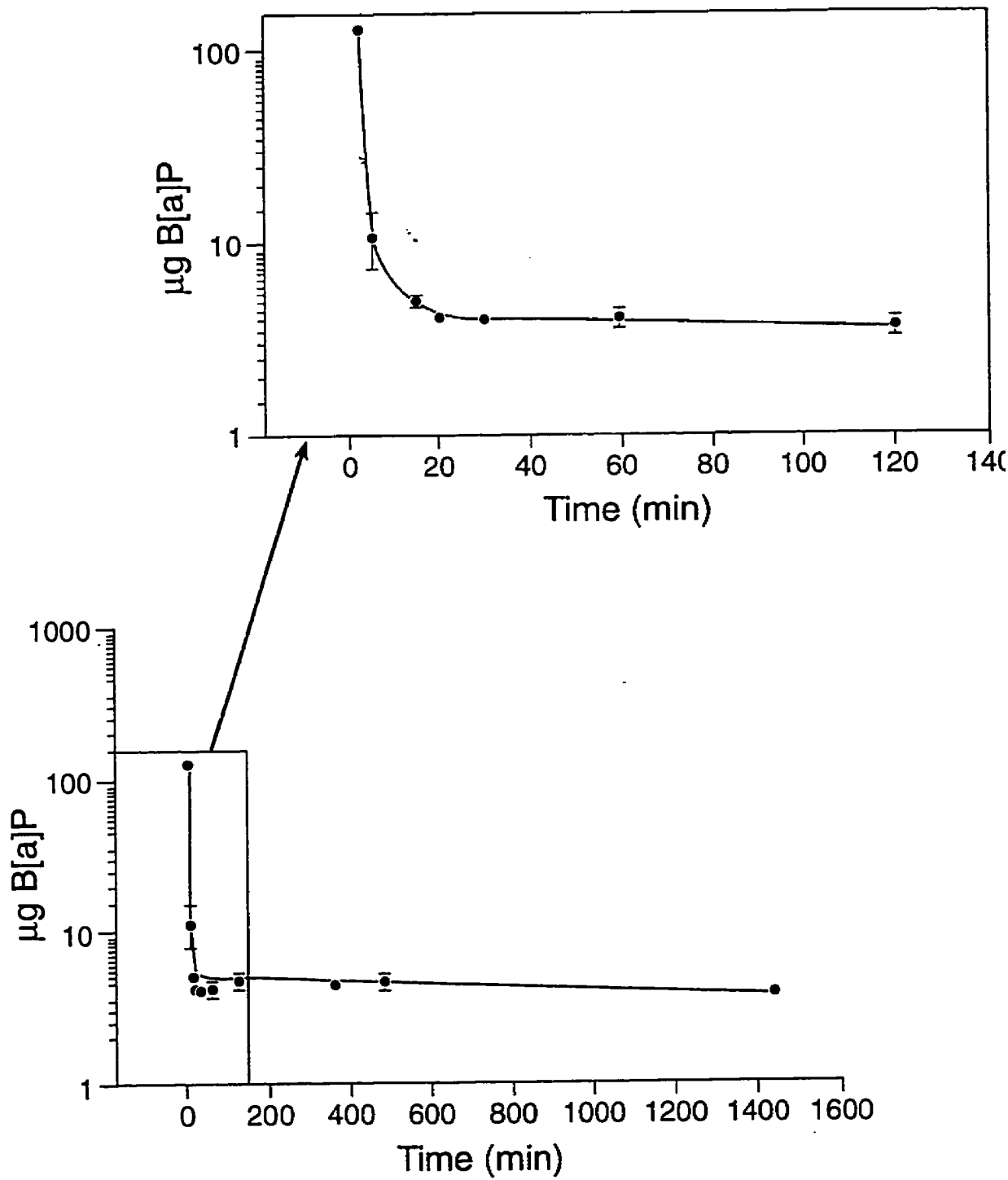
Figure 4.5: Schematic representation of a two-compartment model consisting of a central and peripheral compartment used to describe the disposition of B[a]P in rainbow trout after intraarterial administration (Gibaldi. and Perrier, 1975).
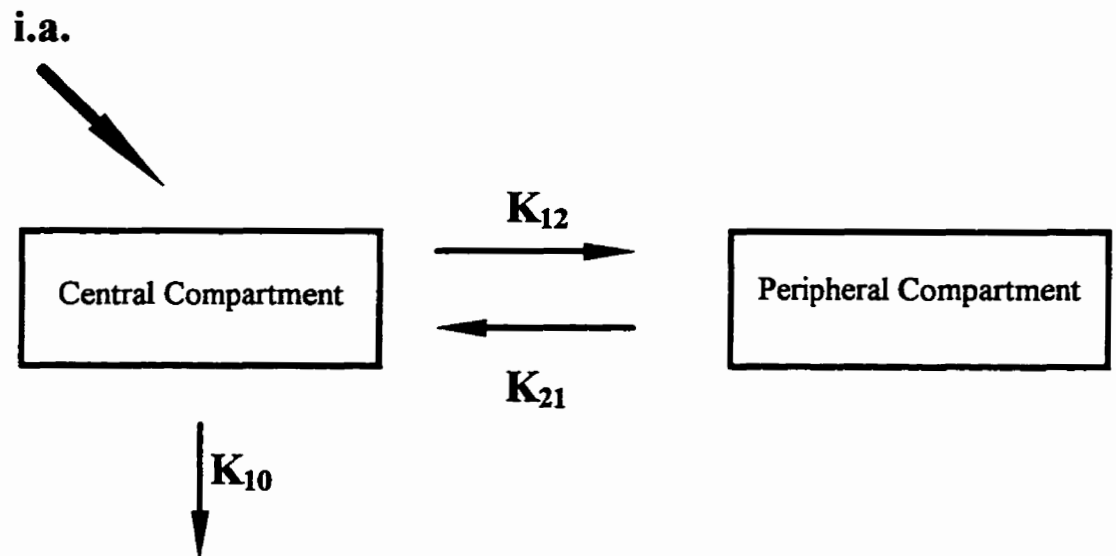
Table 4.12: Model parametres describing blood concentrations of B[a]P in trout following a single intraarterial administration of 10 mg/kg.

Parametres	Fw:Fw	Fw:Sw	Sw:Sw	Sw:Fw
A1 (ug/ml)	253.9	218.3	226.8	110.7
A2 (ug/ml)	5	7.7	5	7.7
b1 (min -1)	0.292	0.305	0.305	0.1
b2 (min -1)	0.00017	0.0012	0.000237	0.0001
b1 HL (min)	2.37	2.3	2.27	9
b2 HL (min)	4105.9	5708.6	3015.19	6804.3
K12 (min-1)	0.278	0.263	0.288	0.100
K21 (min-1)	0.006	0.012	0.006	0.010
K10 (min-1)	0.010	0.032	0.011	0.002
AUC (ug*min/ml)	30378.2	63945	21291.342	77174.1
Vc (ml)	22.8	24.3	17.51	29.5
Qb (ml/min)	0.2	0.09	0.2	0.05
dose (ug)	5900	5487	4060	3487

Discussion

In part IV, the effects of environmental salinity on the distribution, metabolism and elimination of B[a]P were examined. Three main experiments were undertaken to determine the above objective. First, the effects of an acute salinity change on B[a]P kinetics in juvenile coho during smoltification were determined. Second, the effects of salinity acclimatization and acute salinity changes on B[a]P kinetics in adult rainbow trout were determined. And thirdly, the effects of salinity acclimatization and acute salinity changes on the toxicokinetics of B[a]P in the blood of rainbow trout was determined.

Salinity Effects on the Distribution, Metabolism and Elimination of Benzo[a]Pyrene in Juvenile Coho Salmon

Juvenile coho salmon were injected i.p each month, from February to June, with [^{3}H]-B[a]P and then transferred into either 0, 5, 10 or 20 ppt saltwater. The effects of salinity on the distribution of B[a]P was determined by analyzing tissue samples for B[a]P-derived radioactivity. In the present experiment, [^{3}H]-B[a]P (or its metabolites) were found in all tissues of the fish in each salinity treatment. PAHs such as B[a]P are widely distributed in organs and tissues of fish once they enter the organism (Kennedy et al., 1991; Steward et al., 1991), as the present results indicate. There were no statistically significant differences in the distribution of B[a]P in fish between the different salinity treatments, however, the distribution between tissues changed significantly from month to month.

The distribution of a chemical within an organism depends upon the blood circulatory system, blood flow through the organs, and the physico-chemical

characteristics of the compound (Klaassen, 1986; Godin, 1995). The present results indicate that environmental salinity had no major effect on the processes or factors that influence the distribution of B[a]P. The fact that B[a]P was i.p injected into the juvenile coho eliminated the effect of altered respiratory rates which occur during smoltification which may have influenced uptake rates and therefore possible distribution patterns (Boeuf, 1993).

The patterns of distribution observed at 5, 10 and 20 ppt saltwater were not significantly different than the results observed at 0 ppt in Part III, thus indicating that the altered distribution of i.p injected B[a]P may be attributed to changes the organism undergoes during smoltification and not due to environmental salinity. The majority of the recovered radioactivity was found in the bile and liver above all other tissues, at all tested salinities, which is consistent with the results at 0 ppt and reflects the organs function in the storage and metabolism of PAHs (Kotin et al., 1959; Varanasi and Gmur, 1981; Thomas and Rice, 1982; and Kennedy et al., 1989 a, b; Klaassen, 1986; Godin, 1995).

The metabolism of xenobiotics serves as a pathway of detoxication and ultimately elimination from the organism by conversion of the chemical into a more hydrophilic form. B[a]P is metabolized similarly by many fish species, including rainbow trout, winter flounder, gulf toadfish, and juvenile English sole (Gmur and Varanasi et al., 1982; Stegeman et al., 1984; Varanasi et al., 1989; and Kennedy et al., 1991). In order to observe the effects of salinity on the metabolism in juvenile coho, bile from fish 168h after transfer to 20 ppt saltwater and an i.p injection of [^{3}H] B[a]P was analyzed to determine metabolites produced. Juvenile coho salmon were capable of metabolizing [^{3}H]-B[a]P to both unconjugated and conjugated forms while in 20 ppt saltwater.

The predominance of phase I metabolites in the bile of the juvenile coho occurred at both 0 ppt and 20 ppt saltwater which is higher than that found in other species. For example, in gulf toadfish Phase I metabolites accounted for less than 10 % of the total metabolites eliminated in the bile (Kennedy et al., 1989b). The chromatographic separation of B[a]P and organic soluble metabolites from fish at 20 ppt by HPLC revealed a variety of metabolites such as, tetrols, diols, quinones and phenols, and the major identified phase I metabolite of B[a]P in juvenile coho was 7,8,9,10-tetrahydroB[a]P, similar to fish at 0 ppt. There were increases in some metabolites produced in fish at higher salinities. Similar to the results at 0 ppt, the predominance of 7,8,9,10-tetrahydroB[a]P is consistent with previously reported data on the relative regioselectivity of fish liver monooxygenases for metabolizing B[a]P at the bay-region (i.e., the 7-,8-,9- and 10 positions) rather than at the K-region (i.e., the 4,5-positions) as in mammals (Tan and Melius, 1986; Varanasi et al., 1989; Steward et al., 1991). The conversion of 7,8-dihydrodiol to the reactive metabolite 7,8-diol-9,10 epoxide occurs via the MFO system, which may bind to DNA or RNA, or undergo further metabolism to the 7,8,9,10-tetrol metabolite (Gelboin, 1980). There was no significant difference in the accumulation of B[a]P quinones in the organic soluble extraction between 0 ppt and 20ppt saltwater treatments.

The metabolites recovered from the bile of juvenile coho salmon at 20 ppt indicate a similar metabolic pathway as observed at 0 ppt, leading towards metabolic activation of B[a]P to carcinogenic metabolites capable of inducing cytotoxicity and tumor production (Varanasi et al, 1989). The production both 7,8-dihydrdiol and 7,8,9,10-tetrahydroB[a]P indicate the presence of this pathway, as well, the indication of a large percentage of triols and tetrols in organic soluble extractions.

The elimination of an xenobiotic, whether it be the parent compound or its metabolite, reduces the potential toxic effects to the organism. The physiological processes involved in adapting to survival in a marine existence, such as increase in Na+/K+-ATPase activity in the gill, changes in water permeability of osmoregulatory membranes and adjustments in water and ion movement in the intestine and kidney may alter the excretion of xenobiotics. Associated with survival in seawater is a decrease in urine production, for example seawater smolts produced 1-5% the amount of urine compared to freshwater levels in salmonids within 18h of seawater transfer (Holmes and Stainer, 1966). All of the above changes may be important implications for xenobiotic excretion. To date there have been no studies published examining the effects of environmental salinity on xenobiotic excretion.

In the present study the vast majority of the injected dose of $[^3H]$-B[a]P was excreted into the bile of the fish 24 hours following chemical administration at all salinities tested. There were no significant differences in the amounts $[^3H]$-B[a]P and its metabolites eliminated at the different salinities. The high percentage of B[a]P derived radioactivity and the phase I and phase II metabolites recovered in the bile of the coho at 20 ppt saltwater indicate the importance of the heptaobiliary route for excretion similar to fish at 0 ppt. This finding is consistent with previous results in Part III and other studies which indicate that B[a]P is primarily excreted *via* the biliary route, such as in gulf toadfish were approximately 75% of the injected dose of B[a]P was found in the bile (Kotin et al., 1959; Varanasi and Gmur, 1981; Thomas and Rice, 1982; and Kennedy et al., 1989 a, b). The similar excretion of B[a]P and its metabolites at different environmental salinities in juvenile

coho salmon, suggest the rate and route of elimination by the organism is not significantly effected by a change in salinity.

The purpose of the present section was to investigate the effects of environmental salinity on the toxicokinetics of B[a]P in juvenile coho. The results indicated that there were no changes in the distribution of B[a]P within the fish due to an alterations in environmental salinity. Fish exposed to B[a]P after being placed into saltwater produced a different profile of unconjugated metabolites, but still contained a metabolic pathway which may lead towards the metabolic activation of B[a]P. Environmental salinity had no effect on the amounts and rates of B[a]P eliminated from the juvenile coho.

Salinity Effects on the Distribution, Metabolism and Elimination of Benzo[a]Pyrene in Adult Rainbow Trout

The second portion of this section utilized mature rainbow trout to study the effects of salinity on the kinetics of B[a]P in salmonids. Trout were acclimatized to either 0 ppt or 20 ppt saltwater and received a single intraarterial injection of [^{3}H]-B[a]P after then transferred to the acclimation salinity or an acute salinity change.

The distribution of a chemical within an organism depends upon the blood circulatory system and blood flow through the organs. The recovery of B[a]P-derived radioactivity throughout the rainbow trout was consistent with the juvenile coho salmon and other aquatic species (Kennedy et al., 1991; Steward et al., 1991). Tissue distribution were relatively similar between fish acclimated to either FW or SW. However, fish subjected to an acute salinity change (FW:SW or SW:FW) had increased amounts of radioactivity recovered in kidney, spleen and decreased amounts in the gall bladder compared to the respective acclimation treatment groups (FW:FW or SW:SW).

The altered amounts of B[a]P-derived radioactivity recovered in tissues of fish subjected to an acute salinity change might be a reflection in the alteration in blood and circulatory system. Research on osmoregulatory challenges in euryhaline fish species have shown that acute salinity changes can lead to alterations in blood and circulatory patterns (Miles, 1971; Pritchard and Renfro, 1984; Bath and Eddy, 1979; Maxime et al., 1991; Larsen and Jensen 1993; Madsen et al., 1996). Rainbow trout subjected to an acute salinity increase showed an interarterial hypoxia caused by a rapid dehydration of gill tissue attributed to a redistribution of gill blood flow (Larsen and Jensen 1993; Madsen et al., 1996). Adult migrating coho transferred into freshwater showed an increase in dorsal

aortic pulse pressure indicating the redistribution of branchial blood flow (Miles, 1971). As well, teleost fish subjected to acute salinity changes have shown initial decreases in blood hematocrit levels and percent plasma total solids which may alter the vascular systems blood flow resistance (Miles, 1971; Maxime et al., 1991; Larsen and Jensen 1993). The redistribution of blood, specifically to the organs responsible for osmoregulation, may serve to limit water loss and ion gain following an acute salinity change (Larsen and Jensen 1993).

Following an intraaortal administration, the rainbow trout metabolized B[a]P to both Phase I and II metabolites. The high percentage of B[a]P derived radioactivity and the phase I and phase II metabolites recovered in the bile of rainbow trout in all salinity treatments reflects the heptaobiliary route for excretion. This finding is consistent with the juvenile coho in the present study and other studies were B[a]P is primarily excreted via the biliary route. Analysis of the bile showed that >97% of the radioactivity was present as metabolites of B[a]P. The major differences between freshwater acclimated fish and saltwater acclimated fish were changes in the amounts of organic soluble metabolites and phase II conjugates. In fish acclimated to FW, about 2-3% of the radioactivity was organic soluble metabolites (phase I) which is similar to other species (Varanasi et al., 1986; Kennedy et al., 1989b; Steward et al., 1991). Fish acclimated to SW had a statistically significant increase in organic soluble metabolites (11-14%). However, fish subjected to an acute salinity change (FW:SW or SW:FW) did not differ from the respective acclimatization treatment group. There were no significant differences in the levels of glucuronide and sulfate conjugates in the bile (Table 4.5), however values in this study were higher than those reported for gulf toadfish (Kennedy et al., 1989b) and

English Sole (Gmur et al., 1982), although similar to the common carp (Steward et al., 1991). The data showed sulfate to be the predominant phase II conjugate over glucuronide conjugation, which is the reverse of results shown for the carp (Steward et al., 1991). The aqueous soluble radioactivity in the bile which was not conjugated to sulfate or glucuronic acid is probably a glutathione conjugate (Stein et al., 1987; Varanasi et al., 1986; Kennedy et al., 1989b). The freshwater acclimated fish had significantly lower amount of radioactivity recovered in the aqueous soluble fractions compared to saltwater acclimated fish. This result appears consistent with high levels of aqueous soluble fractions recovered in marine species, such as gulf toad fish, and English sole (Varanasi et al., 1989; Kennedy et al., 1989b). The freshwater acclimated fish also had higher levels of unknown conjugates suggesting the involvement of different biotransformation enzyme(s). Fish in the FW:SW treatment had an increase in aqueous soluble metabolites compared to fish in the FW:FW treatment, whereas fish in the SW:FW treatment had a decrease in aqueous soluble metabolites compared to fish in the SW:SW treatment. These results suggest the involvement of different phase II biotransformation enzymes in freshwater and saltwater acclimated fish.

The major identified phase I metabolites of B[a]P in rainbow trout acclimated to freshwater was t-9,10-dihydrodiolB[a]P, consistent with freshwater species where t-9,10-dihydrodiolB[a]P is the major metabolite produced (Gmur et al., 1982; Kennedy et al., 1989b; Varanasi et al, 1989). The major identified phase I metabolite of B[a]P in the rainbow trout acclimated to saltwater was 3-hydroxyB[a]P which differs from saltwater species, such as the English sole and gulf toadfish, where t-7,8-dihydrodiol is the major metabolite (Gmur et al., 1982; Kennedy et al., 1989b; Varanasi et al, 1989). In fish

subjected to an acute salinity change 7,8,9,10-tetrahydroB[a]P was the major identified phase I metabolite. Unlike saltwater species such as, English sole, Starry flounder and gulf toadfish, t-7,8-dihydrodiol did not contribute to a significant proportion of the metabolites identified (Varanasi et al., 1986; Kennedy et al., 1989b). Again, the metabolites identified in the organic soluble fraction are consistent with other fish species for metabolizing B[a]P at the bay-region (Tan and Melius, 1986; Varanasi et al., 1989; Steward et al., 1991).

Analysis of the urine collected indicated that B[a]P and its metabolites are excreted via the kidney. Both phase I and II metabolites were identified in the urine and eliminated in all treatments. The freshwater acclimated fish had a higher percentage of organic soluble metabolites recovered than saltwater acclimated fish. As well, a significantly lower percentage of unknown conjugates and aqueous soluble fractions were found in fish acclimated to freshwater. There is little research done on the renal excretion of PAHs and their metabolites in aquatic species. However, the large amounts of glucuronide and sulfate conjugates are consistent with results from southern flounder injected with metabolites of B[a]P (Pritchard and Bend, 1991). As well, conjugated metabolites of pyrene in rainbow trout (Law et al., 1994) and of the lampricide 3-trifluoromethyl-4-nitrophenol in adult coho salmon (Hunn and Allen, 1975) were the predominant metabolites recovered in the urine. In the present study, differences in the percentages of metabolites recovered in freshwater and saltwater acclimated fish suggests a difference in phase II enzymes.

The major metabolite identified in fish subjected to an acute salinity change (FW:SW or SW:FW) was 7,8,9,10-tetrahydroB[a]P which increased significantly

compared to acclimated treatments. The major metabolite identified in fish acclimated to saltwater was 3-hydroxyB[a]P. In southern flounder the bulk of the metabolites recovered in the urine were believed to be produced outside the kidneys (Pritchard and Bend, 1991). The major identified metabolites in the urine of rainbow trout, in the present study, reflect the metabolites recovered in the bile, suggesting the production of metabolites outside the kidney as well as similar enzymes in the kidney and liver.

The metabolites identified from the urine and bile in the present study indicate a difference in the biotransformation of B[a]P in freshwater and saltwater acclimated fish. Various factors may influence the biotransformation of xenobiotics, such as physiological and biochemical adjustments required for osmoregulation (as previously discussed in Part III), changes in membrane lipids and hormonal regulation. For optimal membrane function, in the prevailing environmental media, utilization of appropriate lipids for a homeostatic state are required. The shift from saturated fatty acids to polyunsaturated fatty acids is an adaptive mechanism seen in aquatic organisms occupying a freshwater versus saltwater environment (Sheridan, 1989) and warm versus cold water environments (Hazel, 1995). Lipid and fatty acid compositional changes have been associated with environmental salinity changes as well as preadaptive processes such as smoltification (Tocher et al, 1995). The higher production of phase I metabolites in saltwater adapted rainbow trout and in the juvenile coho salmon undergoing smoltification may result from a difference in MFO function and activity with different types of membrane composition.

The regulation of various osmoregulatory processes are under hormonal control and the mechanisms are only beginning to be understood (Bern and Madsen, 1992; Heath, 1995). In teleosts, prolactin is the primary hormone controlling hyperosmoregulation

(freshwater adaptation), whereas cortisol and growth hormone are involved in hypoosmoregulation (saltwater adaptation) (Bern and Madsen, 1992). The MFO system has a very broad substrate base due to the presence of multiple isozymes (Andersson and Förlin, 1992) which is under hormonal control (Heath, 1995). For example, cortisol has been found to elevate the activity of the MFO system (Celander et al., 1989; Devaux et al., 1992) and growth hormone has been shown to be involved in the regulation of hepatic cytochrome P450 dependent monooxygenases and UDP glucuronyl transferases in rainbow trout (Cravedi et al., 1995). There are pronounced sex differences in liver microsomal P450 content and activities in fish which has been shown to be closely related to the reproductive status of the organism (Pajor et al., 1990). Experimental evidence from immature rainbow trout and brook trout showed that treatment with estradiol decreased levels of total hepatic P450, whereas testosterone elevated the amount of total P450 content (Hansson and Gustafsson, 1981; Hansson, 1982; Stegeman et al., 1982; Pajor et al., 1990). The complex and unclear mechanism in the regulation and control appear to be interrelated, involving the endocrine and MFO system in maintaining osmo-ionic homeostasis and biotransformation in fish. The literature and data do not provide conclusive connection but supports this hypothesis which requires further research.

In the present study the vast majority of the injected dose of $[^3H]$-B[a]P was excreted into the bile and urine of the fish 48 hours following chemical administration. Fish which were acclimated to either freshwater or saltwater had eliminated >98% of the injected dose by 48h, however fish subjected to an acute salinity change eliminated a statistically significant less amount of the injected dose, 90-93% by 48h.

There are various explanations for the decrease in elimination of B[a]P and its metabolites when fish were subjected to an acute salinity change. First, the elimination may be a reflection of an alter blood circulation (Miles, 1971; Bath and Eddy, 1979; Maxime et al., 1991; Larsen and Jensen, 1993; Madsen et al., 1996) resulting in a slower distribution of the chemical to detoxification and excretory organs. A second explanation for the decreased excretion may be the result of an altered blood pH due an acute salinity change (Larsen and Jensen, 1993; and Madsen et al., 1996). A change in pH may alter the proportion of compounds which are in the ionized state to those in their unionized state, therefore hindering the permeability of compounds through membranes, thus slowing the movement into the detoxification and excretion organs. A third explanation may relate to the secretion systems for organic anion and cations and neutral organic compounds found in the kidney and liver (Pritchard and Miller, 1993). Although little information is available on transport protein structure-function relationships and the physiological and pharmacological regulation of transport mechanisms (Pritchard and Miller, 1993), an alteration in pH will effect the critical pH and membrane electrical potential gradients which are important for these secretion systems thus possibly hindering excretion.

Radioactivity was recovered in the urine (Table 4.10) indicating the renal excretion of B[a]P (and its metabolites), as found in southern flounder (Pritchard and Bend, 1991). Fish exposed to B[a]P in saltwater (FW:SW and SW:SW) had significantly lower production of urine than fish exposed in freshwater (FW:FW and SW:FW) (Table 4.11), a reflection of decreased urination in fish living in saline environments (Kirschner, 1991; Evans, 1993). The urine production in the freshwater treatments, 3-4 fold higher than saltwater treatments, was similar to the difference in urination rates observed in adult coho

transferred from saltwater into freshwater (Miles, 1971). The data showed no statistically significant alteration in radioactivity recovered in urine between treatment groups thus indicating the organism is still eliminating the B[a]P at a similar rate even with the reduced urination via the kidney. The data supports earlier work performed on the excretion of naphthalene and toluene in freshwater versus saltwater adapted dolly varden (Thomas and Rice, 1981). Little difference was observed in amount eliminated via the urine. Marine teleosts have an extensive renal portal system, were the anatomical arrangements allow for maximal excretion of solutes from the circulatory system via the kidney by bathing tubules in a blood flow that approaches the entire cardiac output (Pritchard and Miller, 1980; Pritchard and Bend, 1991). It may provide an explanation for the equal burden of radioactivity detected in urine at differing salinities and urination rates. As saltwater adapted organisms have a decreased glomular filtration rate and decreased urination (Kirschner, 1991; Evans, 1993) as well, an increased access to blood flow may provide the necessary means of eliminating metabolites. In spite of theoretical consideration salinity appears to have little effect on renal excretion (Heath, 1995).

The present section gives further evidence that environmental salinity can affect the kinetics of B[a]P in fish. Fish subjected to either an increase or decrease in salinity underwent alterations in the kinetics of B[a]P compared to acclimated fish. The metabolites identified indicate a difference in biotransformation of B[a]P between freshwater and saltwater acclimated fish possibly due to a difference in enzyme(s) activities or levels, presence of different enzymes or isozymes, or a difference in the modulation of the biotransformation enzymes. The elimination of B[a]P was similar when fish were exposed at their acclimation salinity, however, there was a decrease in

elimination when fish were subjected to an acute salinity change. The present results provide evidence for the effects of an environmental modulator on the kinetics of xenobiotics in fish. The effects of salinity on the toxicokinetics reflect a complex system yet to be fully understood.

Salinity Effects on the Toxicokinetics of Benzo[a]Pyrene in the Blood of Adult Rainbow Trout

Toxicokinetic modeling was used to determine the fate of B[a]P in fish acclimated to freshwater, saltwater or following an acute salinity change. The results indicate that the toxicokinetics of intraaerterially administered B[a]P can be described adequately by a two-compartment open toxicokinetic model which consists of a central and peripheral compartment. The central compartment represents the vascular system of the fish since the calculated volume of the central compartment (Vc) (Table 4.12) was similar to the blood volume of trout (Gibaldi and Perrier, 1975). The blood volume of rainbow trout is estimated at 5.58% of its body weight (Milligan and Wood, 1982). The average weight of the rainbow trout used in the experiment averaged 432± 29.5g and the calculated blood volume (24.1±1.6 ml) is close to the Vc (23.5 ± 2.5 ml) obtained from the model.

The biphasic decrease in B[a]P concentration with respect to time (Figure 4.4) is divided into an alpha phase which represents the net loss from the first compartment to the second and rapid decline in plasma concentration. The beta (terminal) phase begins once an equilibrium is reached between the two compartments and represents the slowly declining blood levels (Welling, 1986). The large rate constants describing the distribution of B[a]P from the central into the peripheral compartment (k_{12}) and small rate constants describing the redistribution back from the peripheral to central compartment (k_{21}) indicate, an overall fast disposition of B[a]P to the tissues where it remains slowly moving back into the blood for elimination. The data indicates that the initial movement from the central to the peripheral compartments in fish acclimated and exposed in FW were similar to fish acclimated and exposed in SW. Fish subjected to an acute salinity change (FW:SW

or SW:FW) showed slower initial movement from the central to the peripheral compartments compared to the respective acclimation treatment groups (FW:FW or SW:SW). Other studies done with freshwater rainbow trout and PAHs, such as 2-methylnaphthalene, fluorene and pyrene, also showed the fast disposition into the tissues and slow movement back into the blood for elimination (Kennedy and Law, 1990). During an acute salinity change (FW:SW; SW:FW) there was a slower distribution from central to peripheral compartments and reduced total body clearances (Q_b). Successful movement between FW and SW requires an organism to reverse its osmoregulatory mechanisms, to either hypoosmotic or hyperosmotic regulation. The effects of an acute salinity transfer on the redistribution of blood flows which serve to limit water and ion losses and gains (Larsen and Jensen, 1993), may play an important role in the latent movement of B[a]P from the central to peripheral compartments.

The long terminal half lives for B[a]P salinity and small body clearances for the four treatment regimes are consistent with the lipophilic characteristics of PAHs. However, the fish subjected to an acute salinity change had longer terminal half lives than the respective acclimation groups which is reflected by the smaller body clearances. These results indicate that fish subjected to an acute salinity change do not eliminate B[a]P as readily than fish exposed to B[a]P at an acclimation salinity. Whole body biological half-lives for B[a]P have been reported to be approximately 3 days for other aquatic organisms (Dunn and Fee, 1979; Spacie et al., 1983; Shugart et al., 1987), for example zebrafish exposed to B[a]P in sediment had a depuration half-life of 5.75 days (Djomo et al., 1996).

Part V Summary and Conclusions

Salmon are of great biological, economic, recreational and symbolic importance to the Pacific Northwest. Coho, one of the seven species of Pacific salmon, are often described as 'opportunistic' which may be attributed to its aggressiveness and determination to reach small headwaters, creeks and tributaries of larger rivers to spawn (Sandercock, 1991). During outmigration to the ocean the juvenile coho encounters many hazards, such as water pollution which originates from a wide range of human activities including agricultural, forestry and heavy industry.

A well balanced assessment of the adverse effects of chemical pollutants, should be based on studies integrating analytical, toxicological and ecological information. However, limitations such as uncertainty in linking cause and effect, difficulty in extrapolating between individual organism and population responses, complex chemical mixtures and extrapolation between field and laboratory testing inhibit impact assessment and management of natural ecosystems (Munkittrick and McCarty, 1995). The better understanding of the integration of relevant physiological processes and the mode of action and fate of chemicals may help reduce these limitations (Brouwer et al., 1991). The overall objective of this study was to determine the effects of the developmental process smoltification and the environmental factor salinity on the toxicokinetics of the model xenobiotic, benzo[a]pyrene, in fish.

The results indicated that the juvenile coho used in this study were undergoing the 'parr' to 'smolt' transformation from February to June. Fish showed characteristic 'parr' traits in February which progressively changed to characteristic 'smolt' traits by June. During smoltification, baseline activities and levels of biotransformational enzymes in the

liver of the juvenile coho changed significantly. The low activities and levels imply that coho may be vulnerable to exposure to chemicals during smoltification and migration through polluted environments. The baseline activities and levels of the biotransformation enzymes tested provide groundwork for environmental monitoring using biochemical indicators for pollutant exposure in juvenile coho. However, the results indicate the importance of understanding biological systems as changing activities and levels of biotransformation enzymes could be associated with a variety of factors and not simply due to chemical exposure.

The effects of the developmental process smoltification on the toxicokinetics of B[a]P in the juvenile coho salmon was investigated. Xenobiotic tissue distribution patterns changed slightly through smoltification. The hepatobiliary system was the primary route of metabolism and elimination for B[a]P in coho as in other fish species. Juvenile coho metabolized B[a]P to both phase I and II metabolites with a relatively higher percentage of phase I metabolites compared to other species. The predominance of phase I metabolites produced from B[a]P implies the organism may be vulnerable to a prolonged exposure by a xenobiotic during a developmental period which may increase the susceptibility to development of cancer in the juvenile coho. No differences were seen in the elimination of B[a]P during smoltification.

The effect of environmental salinity on the toxicokinetics of B[a]P in juvenile coho revealed no difference in the tissue distribution. Juvenile coho metabolized B[a]P to both phase I and II metabolites, however there were different unconjugated metabolites. No differences were seen in the elimination of B[a]P between the different salinities. The fact that juvenile coho were undergoing a developmental process which involved physiological

and biochemical adaptations for a marine existence may explain the lack of effect of a salinity change on the toxicokinetics of B[a]P in the fish. Thus suggesting that the susceptibility to xenobiotics after absorption into the body of the juvenile coho will be similar throughout the smoltification process.

The modulator effects of environmental factors, such as salinity, on the toxicokinetics of B[a]P was shown in the adult rainbow trout. The results showed a difference in the distribution and metabolism of B[a]P in freshwater and saltwater acclimated fish. The distribution of B[a]P, overall, had a fast disposition to the tissues where it remained until slowly moving back into the blood for elimination, similar to other PAHs in fish. No difference were seen in the elimination of B[a]P in freshwater and saltwater acclimated fish. Trout acclimated and exposed to B[a]P in freshwater had similar half-lives and body clearances of the chemical to fish acclimated and exposed in saltwater. However, fish subjected to an acute salinity change showed differences in the distribution, metabolism and elimination of B[a]P from those fish exposed in their acclimated salinity. Fish subjected to acute salinity changes had longer terminal half-lives and smaller body clearances than acclimated fish confirming the observation of a slower elimination of B[a]P. These results suggest the susceptibility to xenobiotics by the trout, in the present study, may be the result of an initial transfer to a different salinity and the adaptations required for a rapidly changing osmotic environment.

The juvenile coho migrate through a wide range of different habitats beginning in small rivers beds and streams to the large rivers into estuaries and final into the ocean. Fish have to contend with habitat destruction and loss, such as removal of riparian vegetation, loss of ground cover, excessive sediment input, creation of migration

blockages, high water temperatures and toxic discharges. The present study provides information about the ability of juvenile coho salmon to deal with xenobiotic exposure during smoltification. The coho metabolized and eliminated B[a]P efficiently, however, the results indicate a susceptibility to xenobiotics during smoltification whether in freshwater or saltwater. The results may assist in risk assessments by providing knowledge about the baseline activities and levels of biotransformation enzymes, production of a higher percentage of phase I metabolites and a metabolic pathway leading to the bioactivation of xenobiotics in juvenile coho during smoltification and outmigration. Thus an exposure to a xenobiotic or a mixture of chemicals for an extended period of time may prove detrimental to the fish. For example, renal excretion of B[a]P conjugates by southern flounder was markedly reduced by pretreatment with the herbicide 2,4-dichlorophenoxyacetic acid (Pritchard and Renfro, 1984).

The difficulty in environmental protection or risk assessment involves trying to resolve the risks on an individual to effects on a population. This research illustrates the complex modulatory effects of developmental processes and environmental parameters on chemical toxicokinetics in aquatic organisms. The juvenile coho were naturally undergoing the adaptations required for a marine existence which reduced the effect of the environmental perturbation of salinity to further alter the toxicokinetics of B[a]P.

Part VI Appendix

Table A1. Basal enzyme levels and activities of yearling coho salmon through smoltification from February to June. Values are means ± SEM for 6 fish. Values with common symbols are not statistically different at p < 0.05.

	Feb-03	Feb-17	Mar-03	Mar-17	Mar-31	Apr-14	Apr-28	May-12	May-28	Jun-09
Protein microsomal (mg/g liver)	24.26±1.16	70.60±2.16†	34.18±4.63	32.66±2.21	34.13±4.41	36.82±6.64	56.23±5.9*	54.66±1.83*	36.89±3.55	33.86±2.26
Protein cytosolic (mg/g liver)	35.33±1.68	77.30±2.48†	39.73±4.16	37.85±1.97	37.22±5.65	25.68±2.17	54.37±3.15*	59.12±5.22*	38.85±4.47	33.95±2.11
Na+/K+ ATPase umol/mg/hour	0.97±0.13	0.799±0.09	1.43±0.14	1.3±0.11	2.51±0.12†	1.68±0.21	1.11±0.13	0.85±0.12	4.31±0.54*	3.33±0.33*
Cytochrome P450 nmol/mg microsomal protein	0.095±0.01†	0.07±0.002	0.093±0.006 †	0.051±0.006	0.078±0.014 †	0.053±0.01	0.045±0.006	0.024±0.009	0.081±0.008	0.08±0.008† †
EROD pmol/min/ mg microsomal protein	9.94±0.85†	5.04±0.46	8.27±0.89†	4.28±0.82	2.74±0.75	5.97±1.15	5.02±0.98	3.87±1.46	4.58±0.15	4.64±1.67
GST umol/min/mg cytosolic protein	0.326±0.014†	0.145±0.006 *	0.089±0.008	0.071±0.008	0.098±0.016	0.097±0.005	0.07±0.01	0.082±0.005	0.107±0.012	0.109±0.007

Table A2: Values from blood toxicokinetics of B[a]P versus time and 2 compartmental model

time (min)	FW:FW ug B[a]P	model	FW:SW ug B[a]P	model	SW:SW ug B[a]P	model	SW:FW ug B[a]P	model
2	125.6±37.4	146.5	125.9±6.6	126.7	128.8±2.0	128.3	391.8±1.7	
5	70.0±3.42	63.83	77.8±0.7	55.1	13.7±3.6	10.9	123.1±37.9	149.3
15	10.4±2.82	8.132	12.2±1.1	13.29	7.1±0.4	7.332	56.1±22.9	51.65
20	5.9±0.2	5.698	9.9±1.0	8.149	6.1±0.1	5.489	33.1±3.5	31.44
30	6.9±1.4	4.996	13.0±3.4	7.673	6.0±0.2	4.99	22.5±6.7	18.69
60	6.9±1.1	4.932	6.7±0.7	7.622	6.0±0.5	4.932	7.8±1.5	8.76
120	6.3±0.8	4.882	6.5±0.3	7.566	6.7±0.6	4.864	10.7±3.8	7.632
240	5.6±0.1	4.784	6.3±0.4	7.457	9.8±0.1		12.3±0.4	7.529
360	5.5±0.2	4.688	7.5±0.4	7.349	6.3±0.2	4.603	12.4±4.5	7.437
480	5.6±0.3	4.594	6.3±0.5	7.243	6.6±0.7	4.478	8.6±2.6	7.347
1440	5.7±0.4	3.907	7.2±1.6	6.446	5.6±0.2	3.591	7.3±1.8	5.737
2880	5.5±0.2	3.105	5.4±0.1	5.465	6.4±0.9		5.3±0.1	3.207

Tables A3: Percent body burden values of $[^3H]$-B(a)P derived radioactivity in juvenile coho salmon exposed to a single i.p. injection 10 mg/kg for 1, 2, 4 and 7 days at 0, 5, 10 and 20 ppt saltwater, a) February b) March c) April d) May and e) June.

a) February

Organs and Tissues	Day 1			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	21.6±5.2	15.5±1.1	15.9±0.7	16.6±1.9
Liver	31.1±4.3	20.3±2.3	35.8±2.4	34.1±1.4
Visceral Fat	25.2±4.3	32.0±4.8	27.8±2.1	24.5±2.4
Kidney	26.4±3.2	30.8±4.7	28.6±1.8	31.1±1.9
Bile	25.7±5.7	29.0±1.0	20.4±2.7	20.5±5.6

Organs and Tissues	Day 2			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.6±2.0	15.3±0.8	15.3±1.0	11.7±2.3
Liver	32.0±5.4	34.2±3.1	32.2±2.5	32.3±2.0
Visceral Fat	27.3±4.3	31.1±1.8	24.3±2.7	18.9±5.7
Kidney	26.5±5.4	24.7±1.2	26.4±1.7	25.2±7.0
Bile	22.6±3.6	18.7±2.7	26.5±2.6	15.8±5.6

Organs and Tissues	Day 4			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	15.1±2.1	15.6±2.1	14.1±0.9	14.0±0.8
Liver	31.7±4.6	31.2±2.9	33.4±2.9	32.3±2.0
Visceral Fat	31.4±6.6	23.3±2.2	23.3±2.9	20.6±2.4
Kidney	25.5±8.3	20.3±3.3	26.9±4.5	24.3±1.8
Bile	18.3±1.8	18.4±3.2	21.7±9.1	18.6±1.0

Organs and Tissues	Day 7			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	12.7±2.1	13.9±0.8	12.5±1.3	12.8±2.3
Liver	32.8±4.8	37.0±3.2	36.0±5.8	32.3±2.0
Visceral Fat	16.5±4.4	20.5±1.0	15.4±4.1	17.9±3.0
Kidney	19.7±3.3	20.6±3.3	19.7±2.2	20.0±4.1
Bile	10.8±1.8	19.3±2.3	16.9±3.3	21.6±5.7

b) March

Organs and	Day 1			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	6.93+/-1.23	15.1±1.2	18.9±1.0	16.4±0.8
Liver	39.17+/-4.33	41.3±1.6	35.8±3.4	39.5±2.4
Visceral Fat	18.36+/-2.02	23.9±2.1	28.7±1.4	24.2±2.0
Kidney	3.34+/-0.23	19.6±1.2	20.0±1.8	17.1±2.0
Bile	38.63+/-1.22	27.5±1.3	22.5±7.5	29.4±4.3

Organs and	Day 2			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.9±0.5	18.4±1.6	16.7±1.0	19.9±0.9
Liver	31.9±2.1	35.6±3.0	36.8±2.9	39.1±1.3
Visceral Fat	24.3±1.1	22.6±2.9	23.2±3.1	22.7±1.3
Kidney	16.6±1.9	16.2±0.4	20.7±2.2	18.8±0.6
Bile	37.8±2.4	31.5±8.2	27.8±7.2	28.6±1.8

Organs and	Day 4			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.3±2.0	15.3±1.9	17.5±1.7	14.7±1.0
Liver	32.2±4.1	32.1±3.9	35.8±2.0	34.0±1.5
Visceral Fat	21.3±1.9	19.7±2.5	25.3±3.1	23.1±1.5
Kidney	20.9±5.4	19.3±2.5	22.8±3.1	15.1±0.8
Bile	35.5±3.9	38.7±6.0	26.8±4.2	38.8±2.8

Organs and	Day 7			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	19.9±1.3	20.2±1.0	20.3±3.4	17.3±0.7
Liver	30.6±1.4	35.9±1.4	23.5±6.1	37.6±1.3
Visceral Fat	31.2±6.0	27.9±3.2	25.1±3.6	23.5±1.7
Kidney	14.6±1.4	18.3±1.0	17.4±2.0	20.8±1.5
Bile	30.3±4.7	26.7±3.3	38.3±5.5	29.8±2.1

c) April

Organs and	Day 1			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.3±3.4	14.7±0.5	15.1±0.6	11.8±0.8
Liver	32.2±2.4	37.3±2.8	39.4±3.2	31.2±0.9
Visceral Fat	36.7±2.5	31.4±1.9	34.5±3.3	34.4±4.1
Kidney	18.5±1.3	23.1±2.4	16.4±3.0	22.0±1.3
Bile	23.5±2.3	20.5±3.2	18.8±6.0	26.6±5.1

Organs and	Day 2			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	14.4±0.6	13.0±0.7	12.0±2.8	12.9±0.6
Liver	35.8±1.8	34.2±2.2	32.7±3.6	35.1±1.1
Visceral Fat	28.6±3.3	35.3±2.0	36.1±3.3	31.5±3.2
Kidney	24.0±2.2	21.5±1.6	22.2±0.9	23.25±1.2
Bile	25.4±2.5	22.8±3.1	21.9±7.7	24.0±4.6

Organs and	Day 4			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	18.4±1.2	15.2±2.3	14.0±0.6	15.8±1.6
Liver	37.2±3.1	31.8±2.5	40.1±2.8	36.8±2.2
Visceral Fat	26.6±4.1	34.4±3.1	28.1±1.9	20.3±2.6
Kidney	17.3±0.4	20.7±2.5	21.4±1.0	28.4±3.4
Bile	28.2±2.9	24.4±5.1	30.0±0.5	27.6±4.3

Organs and	Day 7			
Tissues	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	15.3±1.0	15.7±1.4	14.5±2.3	13.2±0.6
Liver	30.4±1.5	33.8±2.3	33.0±7.6	38.5±0.8
Visceral Fat	37.3±4.2	27.5±2.3	26.1±3.2	29.8±1.5
Kidney	25.3±2.7	32.4±2.4	34.6±6.7	28.4±1.8
Bile	17.9±4.3	18.4±2.2	14.3±3.7	18.5±2.1

d) May

Organs and Tissues	Day 1			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	14.8±0.8	16.1±1.0	18.9±0.7	16.8±0.6
Liver	31.8±2.8	33.9±3.6	41.4±2.0	42.2±1.5
Visceral Fat	26.5±1.7	29.6±4.7	32.8±2.4	32.0±1.5
Kidney	29.5±5.1	29.8±4.6	21.0±1.1	22.2±1.3
Bile	25.4±3.1	16.7±2.3	10.6±2.0	10.6±1.8

Organs and Tissues	Day 2			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.3±1.0	19.3±0.7	18.9±1.2	17.4±0.8
Liver	34.5±1.0	37.5±1.5	36.6±1.6	35.0±2.9
Visceral Fat	26.8±3.0	26.1±3.3	27.9±1.6	31.3±3.7
Kidney	19.9±0.8	21.3±0.5	23.2±1.3	20.5±2.0
Bile	30.7±4.4	23.8±5.3	21.4±5.3	23.0±4.8

Organs and Tissues	Day 4			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	19.1±0.8	17.3±1.3	14.8±0.8	15.3±0.8
Liver	33.8±2.5	26.9±5.0	35.4±3.0	34.2±1.3
Visceral Fat	26.3±2.2	33.3±6.6	21.4±5.4	24.5±2.0
Kidney	21.6±3.4	17.3±0.7	24.3±1.3	22.3±1.5
Bile	27.6±5.3	30.7±5.9	34.3±5.5	32.3±3.7

Organs and Tissues	Day 7			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	20.9±1.2	21.2±2.8	15.7±1.6	18.8±1.2
Liver	38.3±3.1	38.8±2.5	34.4±2.4	41.1±1.5
Visceral Fat	17.0±4.1	32.3±2.9	37.4±3.6	24.9±1.2
Kidney	29.6±3.9	25.5±2.3	23.4±1.1	28.5±1.1
Bile	19.5±4.5	18.5±2.0	15.0±1.4	13.2±1.6

e) June

Organs and Tissues	Day 1			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	14.3±1.4	14.8±0.8	15.6±0.4	13.9±0.8
Liver	30.5±1.7	31.0±2.3	30.6±2.8	30.6±2.3
Visceral Fat	36.1±1.7	36.3±2.4	36.2±2.5	38.0±4.1
Kidney	17.5±2.2	18.0±1.2	23.8±2.2	22.3±1.9
Bile	28.5±2.3	32.3±1.8	21.7±3.3	21.4±4.2

Organs and Tissues	Day 2			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	11.6±1.8	12.8±1.5	28.0±13.5	11.7±0.7
Liver	29.7±3.0	26.8±1.7	26.0±6.9	30.0±1.2
Visceral Fat	39.0±5.1	44.8±2.8	32.4±8.3	33.1±5.9
Kidney	16.2±2.2	19.4±2.4	14.0±3.0	25.7±4.0
Bile	27.6±3.6	22.2±5.1	17.5±6.6	24.4±5.2

Organs and Tissues	Day 4			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	16.2±1.9	15.0±1.1	10.7±1.2	12.0±1.1
Liver	33.1±2.8	33.7±3.8	31.3±5.0	28.0±3.1
Visceral Fat	33.3±4.2	34.3±4.7	43.3±4.5	39.4±5.4
Kidney	19.5±3.6	22.6±4.7	17.8±2.1	20.6±1.6
Bile	28.9±4.7	25.7±5.5	19.1±2.5	23.4±6.8

Organs and Tissues	Day 7			
	0 ppt	5 ppt	10 ppt	20 ppt
Carcass	20.2±1.7	14.0±0.9	12.6±3.1	14.3±0.5
Liver	34.0±1.1	35.2±2.6	28.0±7.8	38.8±3.2
Visceral Fat	28.2±3.7	42.0±4.4	32.4±10.3	30.3±5.6
Kidney	24.7±3.1	18.1±3.2	18.2±4.4	24.0±2.2
Bile	22.1±3.7	12.3±2.4	27.3±12.4	18.2±1.9

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TEST TARGET (QA-3)

1.0
2.8 2.5
3.2 2.2
3.6
4.0 2.0
1.6
1.1 1.8

1.25 1.4 1.6

150mm

6"
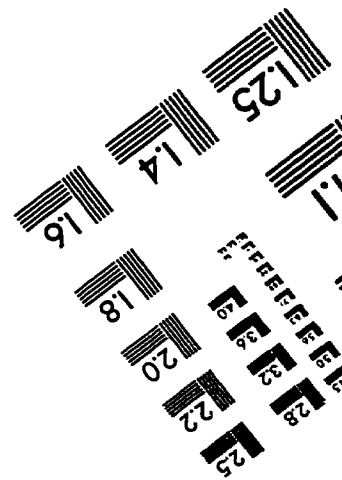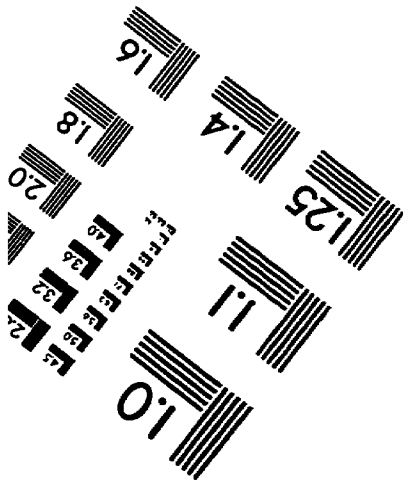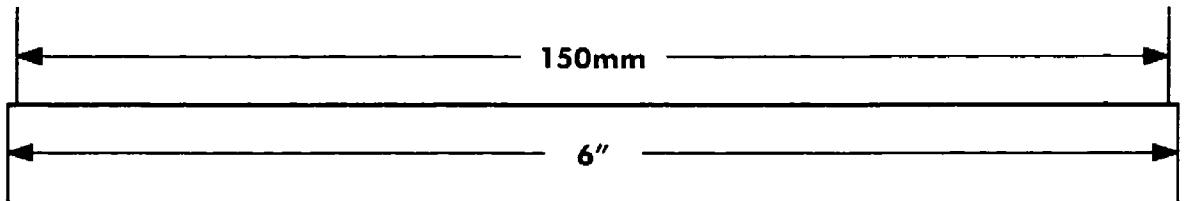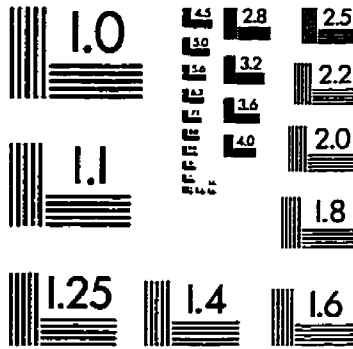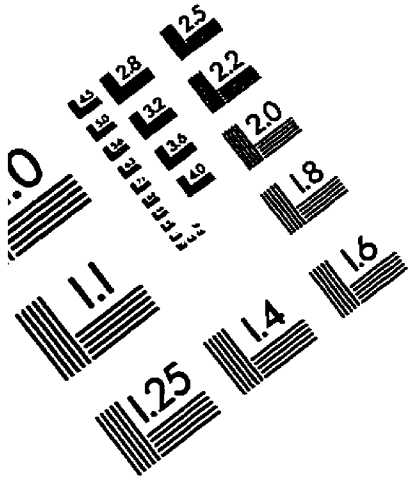
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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