AN ABSTRACT OF THE DISSERTATION OF

Claudia F. Bravo for the degree of Doctor of Philosophy in Toxicology presented on December 9, 2005.
Title: Assessing Mechanisms of Immunotoxicity for Polycyclic Aromatic Hydrocarbons in Rainbow Trout (*Oncorhynchus mykiss*)

Abstract approved: Redacted for privacy

Lawrence R. Curtis

During the past 30 years, numerous studies have focused on the toxicities of polycyclic aromatic hydrocarbons (PAH). Laboratory and field studies have helped elucidate the detrimental effects of these chemicals on growth, reproduction and immune response. Polycyclic aromatic hydrocarbons are in the priority list of chemicals to be studied by different governmental agencies and universities and understanding their mechanisms of action is the focus of the current research. The manuscripts presented in this dissertation are focused on the effects and mechanism of action of PAH on disease susceptibility.

After a dietary exposure to PAH for up to 50 days (chapter II) and samplings after 3, 7, 14, 28 and 50 days, a number of biomarkers of PAH exposure were measured: Fluorescent aromatic compounds (FACs) in bile, ethoxyresorufin-o-deethylase (EROD) in liver microsomes, cytochrome P450 1A immunohistochemistry in liver and kidney and adduct formation in liver. Additionally markers of oxidative stress were measured: comet assay in blood, protein nitration in kidney and F2-isoprostanes in kidney. Oxidative stress was a probable factor in PAH induced responses in fish

adapted to long-term PAH exposures and aryl hydrocarbon activation was not necessarily involved in this process. Disease challenge with *Aeromonas salmonicida* (chapter III) resulted in differences in mortalities that demonstrated that fish exposed to PAH were more susceptible to disease than fish not exposed to PAH. Determination of gene expression in head kidney of fish exposed and not exposed to PAH challenged with *A. salmonicida* using microarray and RT-PCR technologies 2, 4, 10 and 20 days after challenge (chapter IV), suggested that PAH exposure was associated with down regulation of interleukin 8, transport associated protein 1, NF-kB modulator, recombination activating gene and major histocompatibility complex II two days after challenge in fish exposed to PAH. The transcript levels were closer to control levels 20 days after challenge, this indicated a recovery from the effect of PAH exposure.

Assessing Mechanisms of Immunotoxicity for Polycyclic Aromatic Hydrocarbons in Rainbow Trout (*Oncorhynchus mykiss*)

by

Claudia F. Bravo

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented December 9, 2005

Commencement June 2006

Doctor of Philosophy dissertation of Claudia F. Bravo

Presented on December 9, 2005.

APPROVED:

Redacted for privacy

Major Professor, representing Toxicology

Redacted for privacy

Head of the Department of Environmental and Molecular Toxicology

Redacted for privacy

Dean of the Graduate School

I understand that my dissertation will become part of permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request

Redacted for privacy

Claudia F. Bravo, Author

ACKNOWLEDGMENTS

I would like to thank my six committee members Dr(s): Lawrence Curtis, Mary Arkoosh, William Baird, Nancy Kerkvliet, Gary DeLander and Tracy Collier for taking the time to serve in my committee, attending meetings, providing helpful comments during the development of this project and reviewing this dissertation. In particular I wish to thank my dissertation advisor Dr. Lawrence Curtis, for introducing me to new techniques in toxicology, different views and ideas, providing me excellent opportunities for participation in different laboratories around campus, teaching me valuable skills for proposal and dissertation writing and excellent discussion for the research presented here. Also, Dr. Tracy Collier for supporting this research and providing me the example and opportunity to develop this work at the different facilities in the National Marine Fisheries Service in Newport OR, Seattle WA, and Mukilteo WA, a forever valuable experience that will shape the way I do research from now on. To Dr. Mary Arkoosh, thank you for offering me personal advice and encouragement to complete this dissertation, also for all the collaboration to develop the experiment and for offering me your expertise and experience in the disease challenge experiment part of the project. I wish to thank Dr. Christopher Bayne, Dr. Lena Gerwick, Jana Brooks, Cheri Goodall and Randy Bender for their collaboration and valuable advice in the design and execution of the microarray and real-time PCR part of this project. I learned basic skills for molecular biology work that I will take with me to the next step. I also thank Dr. James Meador from the National Marine Fisheries Service for his valuable advice in the beginnings of the project and for

allowing me to participate in some of his experiments that served as pillars for the development of this research.

I also wish to acknowledge the following individuals who contributed with helpful professional and technical advice and for their scientific insight during the course of this research: Dr. Frank Loge, Elisabetta Lambertini at U. C Davis, thank you for your support and advice with the statistical part of this project. Mark Myers, Gina Ylitalo, Jon Buzitis, Dr. Don Brown, Dr. Margaret Krahn, Cathy Laetz, William Reichert, Bernadita Anulacion, Maryjean Willis, Lyndal Johnson, Jana Labenia, Carta Stehr, Dr. John Incardona, Frank Sommers from National Marine Fisheries Service in Seattle, WA thank you for teaching me the techniques that allowed the successful completion of this project. Coral Stafford, Ethan Clemons, Deborah Boylen, Dr. Paul Reno, Paul Lang and the technical support group in Newport OR. Dr. David Yu, Eric Johnson, Dr. Brinda Mahadevan, Tamara Musafia, Lauren Courter, Dr. Joseph Beckman, Kay Fisher, Dr. Richard Holt and Caprice Rosato in Oregon State University thank you for your advice and help with different techniques in the various stages of this project.

I thank Dr. Jason Morrow from the Vanderbilt University School of Medicine in Nashville Tennessee, Dr (s): Mark Hahn and John Stegeman from Woods Hole Oceanographic Institution in Massachusetts for their valuable collaboration with lipid peroxidation analysis, AHR binding assay and CYP1A1 antibody for immunohistochemistry respectively.

To my friends, Junga Lee, Lauren Courter, Kerri Stanley, Jana Brooks and to my peers Dr. Margaret Pratt, Angie Perez, Mauricio Rodriguez, Tracey Momoda, Castle Funatake Dr. Wendy Walker thank you for making my experience at OSU enjoyable

and unforgettable both in the lab and outside, thank you for your advice and help in the different stages of this process. Finally I offer sincere and special acknowledgement and thanks to my husband Francisco Madriñán. His endless encouragement, support, patience, love, understanding and advice throughout the completion of this research will always be cherished and appreciated and to my mother, Eugenia Garzon de Bravo thank you for her prayers, support and love at the beginning, middle and end of this process.

These studies were funded in part by the Northwest Fisheries Science Center, NOAA Fisheries and through funding provided by the Endangered Species Act Recovery and Research Program. I would also like to acknowledge partial funding for the DNA microarray work from NOAA's West Coast Center for Oceans and Human Health and the Cooperative Institute for Marine Resources Studies (CIMRS) that fosters collaborative research between the National Oceanic and Atmospheric Administration (NOAA) and Oregon State University (OSU). I also wish to thank the Environmental and Molecular Toxicology departmental funding and The Oregon Agricultural Experimental Station at OSU.

CONTRIBUTION OF AUTHORS

Dr (s): Lawrence Curtis, Mary Arkoosh, Tracy K. Collier and Christopher Bayne were the principal or co-principal investigators of the research projects that resulted in the manuscripts presented here. Their collective contributions included: assistance with initial development of the research questions, guidance with methodology and interpretation of the results. Mark Myers and Dr. Mark Hahn contributed with immunohistochemistry data collection and interpretation and aryl hydrocarbon binding assay data collection in their respective laboratories that were presented in chapter II. Dr. James Meador collaborated with diet preparation, dosage and disease challenge pilot studies that although not presented in chapter III, provided important insight into the design and response of fish exposed to PAH and challenged with *Listonella anguillarum* and helped us improve the design of the study presented in chapter IV. Dr. Frank Loge collaborated with statistical analysis of the data presented in Chapter II, III and IV. Elisabetta Lambertini helped with the analysis of the microarray data presented in chapter IV.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

DEDICATION

This work is dedicated to my mother, Eugenia Garzon de Bravo, my sister, Pilar Bravo, my husband Francisco Madriñán and to the memory of my father in law, Fernando Madriñán Micolta.

Assessing Mechanisms of Immunotoxicity for Polycyclic Aromatic Hydrocarbons in Rainbow Trout (*Oncorhynchus mykiss*)

Chapter I

INTRODUCTION

Background

Pacific salmon are listed as threatened or endangered species under the endangered species act (NRC, 1996). Their current status is attributed to the cumulative effects of natural and anthropogenic factors (Arkoosh and Collier, 2002). Most of the research efforts are focused on identifying the factors affecting survival and reproduction of salmon in fresh water (Arkoosh et al, 1998a). Watershed land use and activities such as agriculture, dams, livestock, road construction and urbanization are known to have impacts on salmon (Arkoosh and Collier, 2002).

Little effort has focused on determining the risk to survival of juvenile salmon in estuarine and oceanic environments (Arkoosh et al, 1998a). Estuarine habitats are transitional areas for anadromous salmonids that spent part of their life cycle in fresh water and part at sea (Crisp, 1999). These habitats provide refuge from predation, feeding grounds and facilitate physiological transition from fresh water to sea water (Thorpe, 1994).

Factors that affect the health of juvenile fish during this critical period have a negative effect during this stage or later in their marine life (Parker, 1962). Estuaries are affected by human activities such as land use, physical alterations and introduction of toxic materials (Day et al, 1989). Chemical exposure produces one of the most

devastating environmental changes that impact upon aquatic animal health (Zelikoff et al, 2002). Fish exposed to toxic chemicals from discharges to lakes, rivers and oceans are sensitive to the toxic effects of many of these compounds (Zelikoff et al, 2002). Polluted estuaries are potentially one of the factors contributing to the decline of wild Pacific salmon (Arkoosh and Collier, 2002). Polycyclic aromatic hydrocarbons (PAH) have been observed to be the most concentrated group of chemicals in estuaries and coastal environments near urban centers (Latimer and Zheng, 2003). The major sources of PAH in the coastal marine environment include urban run-off, wastewater effluents, industrial outfalls and spills during the transport of fossil fuels (Latimer and Zheng, 2003). PAH compounds are considered to be one of the most toxic chemicals found in a contaminated estuary or watershed (Arkoosh and Collier, 2002). Previous studies have been conducted to determine the effects of PAH in salmon from the different contaminated sites in the Pacific Northwest such as Puget Sound, WA as described in Johnson et al (unpublished). Juvenile salmon from polluted waterways in the Puget Sound had increased PAH metabolites in bile, elevated PAH concentrations in stomach contents and suppressed immunocompentence (Arkoosh et al, 1991, 1998).

Disease in fish is linked to environmental stress (Munro and Roberts, 2001). Disruption of homeostasis beyond tolerable limits by a stressor may predispose the fish to disease (Munro and Roberts, 2001). Disease occurrence in fish depends on three variables that are controlled by biotic, abiotic and genetic factors (Sniezko, 1973). These variables are the following: the quality of the environment, differential susceptibility of individuals to pathogens and virulence of the pathogen (Arkoosh et al, 1998b). Any of these variables alter the dynamics between pathogen-host and the

susceptibility of fish to disease (Arkoosh et al, 1998b). Juvenile salmon are exposed to PAH in their short residence time in the estuary, primarily through the diet. Arkoosh (1998b) demonstrated that salmon exposed to a contaminated estuary are more susceptible to disease. This increased predisposition may reduce the size of a chronically exposed fish population. If pathogens are present, perturbations in the environment such as poor water quality may increase the potential of disease in fish populations (Sniezko, 1973; Loge et al, 2005).

Polycyclic aromatic hydrocarbons are immunosuppressive in mammals (Blanton et al, 1986) and in fish (Arkoosh et al, 1998a; Carlson and Zelokiff, 2002). Most of these studies have focused on benzo(a)pyrene or 7, 12 dimethylbenzanthracene (DMBA) as model compounds for PAH exposure. However because PAH are found in the environment as mixtures and fish are mainly exposed in the diet, additional studies are necessary to better characterize the effects of PAH on immunocompentence of juvenile salmon. Toward this effort, the three manuscripts that comprise this dissertation examine the mechanism of action of PAH through the aryl hydrocarbon receptor or oxidative stress to help explain the toxicity of these compounds. Specifically examining how markers of oxidative stress (F2-isoprostanes, comet assay and protein nitration) measured in different rainbow trout target organs (liver and kidney) respond during a chronic exposure in the diet to PAH at environmentally relevant concentrations that mimic the residence time that chinook salmon spends in the estuary.

Oxidative stress

PAH require metabolic activation to exert their toxic activity (McLeod et al, 1980). They can be activated to electrophilic metabolites to exert their carcinogenic or mutagenic effects (Warshawsky and Warshawsky, 2005) and cause oxidative damage. There are currently three accepted pathways for the transformation of PAH to their most toxic metabolites: activation to diol epoxides, activation to radical cations and activation to O-quinones (Warshawsky and Warshawsky, 2005). This dissertation is focused on the third mechanism of PAH activation, which induces the formation of O-quinones catalyzed by dihydrodiol dehydrogenases (Penning et al, 1999). All PAH O-quinones can undergo non-enzymatic redox cycling with the production of reactive oxygen species and cause oxidative stress (Penning et al, 1999). Oxidative stress can be defined as the damage to biomolecules from free oxyradicals such as superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) (Newman and Unger, 2003). Free radicals generated by redox cycling of PAH metabolites can react with a variety of macromolecules. They can oxidate membrane lipids, proteins and nucleic acids and alter the cell redox status (Di Giulio et al, 1995).

Markers of oxidative stress are utilized to implicate free radicals in patophysiological processes to assess effects in lipids, DNA and proteins. Lipid peroxidation is considered an endpoint of oxidative stress. These measurements include the quantification of short chain alkanes, malondialdehide or conjugated dienes (Morrow and Roberts, 1992). F2-isprostanes produced from the peroxidation of arachidonic

acid is use to assess lipid peroxidation and oxidative stress *in vivo*. To date this is the most specific method to assess lipid peroxidation.

There are different alterations that can be produced in DNA by oxidative damage. Reactive oxygen species can directly damage DNA through the production of strands scissions and chromosomal breakage. These effects are not specific for free radicals, because binding of metabolites to DNA (adduct formation) can also cause strand breaks in DNA (Di Giulio et al, 1995). DNA strand breaks can be determined by the alkaline unwinding assay (comet assay) (Fig 1.1). In this assay, a change in doubled stranded DNA during alkaline denaturation is determined under conditions in which the rate of unwinding is proportional to the number of breaks in the phosphodiester backbone of DNA. This technique was modified (Shugart, 1988) to allow the estimation of DNA strand breaks from tissue (Shugart, 1995).

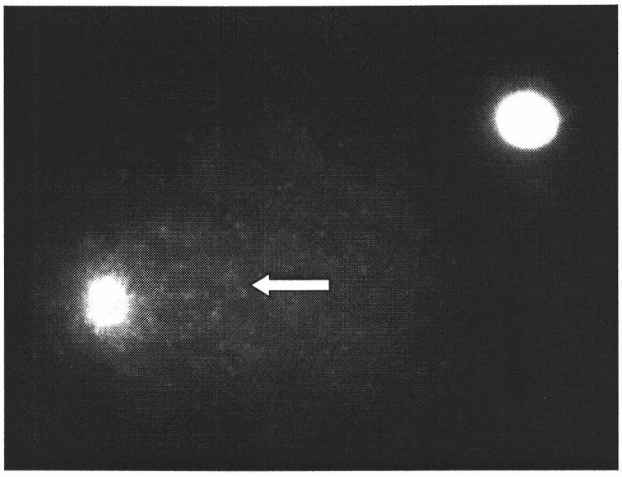


Figure 1.1 Normal and damage (indicated) DNA in nucleus of rainbow trout blood treated with PAH measured by comet assay.

Effects of radical oxygen species in proteins result in the inactivation and acceleration of its degradation. Currently there are no markers that measure protein oxidation in fish. In this dissertation, we propose the use of the 3-nytrotyrosine antibody by immunohistochemistry that allows the visualization and quantification of proteins modified by peroxynitrite. Peroxynitrite is a potent oxidizing agent that has been previously identified (MacMillan-Crow et al, 1996). To nitrate tyrosine residues in proteins generating a permanent modification that can be detected with the use of 3-nytrotyrosine antibody by immunological methods (MacMillan-Crow et al, 1996). To our knowledge, this is the first time that this antibody is successfully used in rainbow trout as marker of protein nitration (Chapter II).

Additionally to these selected markers of oxidative stress measured in chapter II, biomarkers of PAH exposure (fluorescent aromatic compounds in bile, ethoxyresorufin-O-deethylase induction, cytochrome 1A1 immunohistochemistry and DNA adduct formation) were measured during the feeding experiment to assess the response to chronic exposure of rainbow to different PAH treatments in the diet.

Model species

We selected juvenile rainbow trout (*Oncorhynchus mykiss*) as model organism for salmonid exposures because it belongs to the family salmonidae (Quinn, 2005). All salmonids spawn in fresh water, some spend their entire lives living in streams, some stocks of rainbow trout and cutthroat, while others typically migrate to sea to grow and then return to fresh water to reproduce (chinook, coho, pink, chum and sockeye). This life-history pattern is known as anadromy (Quinn, 2005). Rainbow trout, one fresh

water form of salmonids, coexists with the anadromous forms (e. chinook) at the beginning of their life cycle. Rainbow trout are also of high commercial value, and are widely used for experimental purposes. They are easy to handle, maintain and manipulate in the laboratory.

Summary of findings

The major results of chapter II indicate that a mixture of ten high molecular weight PAH induced CYP1A1 in liver and kidney and increased oxidative damage as indicated by DNA strands breaks measured in blood by comet assay, protein nitration measured in kidney by immunohistochemistry and lipid peroxidation in kidney by F2-isoprostanes. After longer periods of exposure to PAH, fish tend to recover but responses were still significantly different from control after 50 days. Benzo(e)pyrene exhibited neglible affinity for AHR but CYP1A1 was induced in liver indicating that other mechanisms such as 4s, 8s protein might be involved.

Chapter III examined disease susceptibility of rainbow trout after 50 days of dietary PAH exposure. Rainbow trout exposed to an environmentally relevant mixture of PAH at 400 ppm had 10% increased mortality after *Aeromonas salmonicida* compared to control when exposed to LC_{20} and LC_{30} of the bacterium.

Chapter IV addresses the mechanism by which PAH decreases host resistance in fish. The results suggest that PAH decreases transcription of genes involved in the immune response. These genes participate in innate and adaptive immunity and some are key regulators of immune response such as NF-kB modulator.

Future Publications

Chapter II entitled: Recovery from oxidative stress during sub-chronic exposures to high molecular weight polycyclic aromatic hydrocarbons in rainbow trout (*Oncorhynchus mykiss*), will be submitted to the journal Toxicology and Applied Pharmacology. Chapter III entitled: Increased susceptibility of rainbow trout (*Oncorhynchus mykiss*) to *Aeromonas salmonicida* after exposure to PAH in the diet will be submitted to the Journal of Aquatic Animal Health. Chapter IV entitled: Transcriptional Patterns in head kidney of polyaromatic hydrocarbon mixture pretreated rainbow trout (*Oncorhynchus mykiss*) challenged with *Aeromonas salmonicida* will be submitted to Toxicological Sciences.

Chapter II

Recovery from oxidative stress during subchronic exposures to high molecular weight polycyclic hydrocarbons in rainbow trout (*Oncorhynchus mykiss*)

Claudia Bravo[1], Lawrence Curtis[1], Mary Arkoosh[2], Mark Myers[3] Mark Hahn[4] and Tracy Collier [3]

1. *Department of Environmental and Molecular Toxicology, Oregon State University, ALS 1007, Corvallis OR 97331 USA.*
2. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2030 South Marine Science Drive, Newport OR 97365, USA*
3. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Boulevard East, Seattle, Washington, 98112, USA*
4. *Woods Hole Oceanographic Institution Woods Hole, MA 02543, USA*
   Department of Environmental and Molecular Toxicology and Department of Biochemistry & Biophysics. Oregon State University, 1158 Ag Life Sciences Bldg, Corvallis OR 97331 USA

To be submitted to *Toxicology and Applied Pharmacology*

ABSTRACT

Rainbow trout were fed a diet that contained a mixture of ten high molecular weight polycyclic aromatic hydrocarbons (PAH) at 40 or 400 parts per million (ppm). Diets that contained 160 ppm of benzo(a)pyrene (BAP) or benzo(e)pyrene (BEP) were fed as respective positive and negative controls for aryl hydrocarbon receptor (AHR) agonism. After 3, 7, 14, 28 and 50 days of exposure, 18 fish per treatment were sacrificed: bile, blood, liver and kidney were sampled. Various biomarkers of PAH exposures were measured: fluorescent aromatic compounds (FACs) in bile, ethoxyresorufin-O-deethlylase (EROD) activity in liver microsomes, CYP1A immunohistochemistry in liver and kidney, and DNA adducts in liver. After 50 days of exposure to PAH, biliary FACs metabolites were significantly higher ($p>0.05$) in all treatments compared to levels measured after 3 days of exposure (except for 40 ppm PAH mixture). EROD activity was induced in all treatments in liver microsomes and the same trends were observed for CYP1A stain intensity in tissue. No DNA adducts were found at any time point. Markers of oxidative stress showed that prevalence of DNA damage in blood cells increased over time measured by the comet assay. The highest levels of comets occurred at 14 days and decreased after 50 days of exposure. Increased nitrotyrosine immunostaining was observed in kidneys after 7 days of exposure to the 400 ppm PAH mixture but decreased at later times. F2-isoprostanes in kidney, a measure of lipid peroxidation, were significantly higher in fish fed 160 ppm BAP and 400 ppm PAH mixture for 50 days.

All measures of oxidative stress (except F2-isoprostanes that was only measured at 50 days of exposure) were reduced by 50 days. This indicated an adaptive response to PAH exposures. Additionally, a competitive binding assay with AHR enriched fish hepatoma cells, demonstrated BAP but not BEP displaced [^{3}H] 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin suggesting that BEP is not a ligand for AHR. Oxidative stress was a probable factor in PAH-induced responses and AHR activation was probably not necessary for initiating the toxic effects of PAH.

Keywords: rainbow trout, PAH, oxidative stress, AHR, CYP1A, recovery

INTRODUCTION

Polyaromatic hydrocarbons (PAH) contain two or more fused benzene rings (McElroy et al, 1989) that are derived from incomplete combustion of fossil fuels (Burns-Naas et al, 2001). There are more than 100 different PAH (Research Triangle Institute, 1995). The Environmental Protection Agency (EPA) considers 17 PAH to be of environmental concern, including the following: fluoranthene, pyrene, benz(a)anthracene, chrysene, benz(b)flouranthene, benz(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene among others. These PAH are at the highest concentrations on the National Priority List (NPL) for hazardous waste sites. They generally are present in the environment as complex mixtures not as single compounds (Canadian Environmental Protection Act, 1994). PAH occur naturally and are manufactured individually for research purposes. PAH are widespread pollutants in the aquatic environment (Faisal et al,1993) and can reach this compartment through the air, runoff

and wastewater from industrial and municipal sources (Kirso et al, 2001). Polycyclic aromatic hydrocarbons have low vapor pressure and high octanol-water partition coefficients. The later reflects the hydrophobic properties of these compounds. They occur in water, particulate matter and living organisms (low and high concentrations respectively) (Kirso et al, 2001).

The metabolism of PAH plays a role in the detoxification, excretion and formation of toxic metabolites responsible for carcinogenicity and immunotoxicity (Willet et al, 1995). Cytochrome P450 (CYP) dependent-mixed function oxidase (MFO) initiates PAH metabolism (Neff, 1979). There are several possible fates for PAH in vertebrates, the major elimination pathway requires the activity of MFO system. These enzymes initiate the biotransformation of PAH to more polar, water-soluble metabolites that can be excreted (Hellou, 1996). Oxidation can yield multiple metabolites per PAH while conjugation can take place with several types of molecules (amino acids, sulfate groups, glucuronic acid) (Hellou, 1996).

It is well recognized that PAH cause immunotoxicity (Silkworth et al, 1995, Arkoosh et al, 1994, 1998; Grinwis et al, 2000; Arkoosh et al, 2001; Carlson and Zelikoff, 2002a, 2002b; Jacobson et al, 2003). They suppress both cell mediated and humoral immune reactions (Anon, 1981). For example, a reduction in macrophage phagocytosis occurs in fish collected from environments with high PAH contamination (Weeks et al, 1986).

There are consistent findings that PAH with different structures that contain the same number of rings act through different mechanisms to exert their toxic effects

(Incardona et al, 2004). Few studies report altered biological responses to PAH due to long term exposures at environmentally relevant dietary doses. Most studies with long term exposure focus on carcinogenic and immunological effects of PAH. The work presented here assesses biological response to PAH that involve two different mechanisms of action: aryl hydrocarbon receptor (AHR) activation and oxidative stress. This work compares and contrasts exposures to a model high molecular weight PAH mixture, benzo(e)pyrene (BEP) and benzo(a)pyrene (BAP) in juvenile rainbow trout. Several validated biomarkers of PAH exposures were measured: bile metabolites, ethoxyresorufin-O-deethylase (EROD), CYP1A immunohistochemistry and DNA adducts. DNA strand breaks in blood cells (comet assay), tyrosine nitration by immunohistochemistry in trunk kidney and lipid peroxidation (F2-isoprostanes) in kidney were measured to assess oxidative stress. Additionally BEP and BAP affinity for AHR was assessed with a binding assay.

MATERIALS AND METHODS

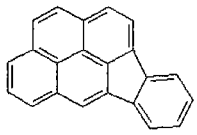
Chemicals

Pyrene (purity>99%), chrysene (purity>99%), benz[b]fluoranthene (purity>99%), benz[k]fluoranthene (purity>99%), benzo(a)pyrene (purity>99%), dibenz[a,h]anthracene (purity>99%), fluoranthene (purity>99%) and benzo(e)pyrene (purity>99%) were purchased from Sigma-Aldrich (Milwaukee, WI). Benz[a]anthracene (purity>98%), indeno[1,2,3-cd]pyrene (purity>98%) and benzo[g,h,i]perylene (purity>98%) were purchased from Cambridge Isotopes

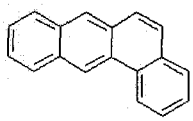
Laboratories Inc, (Andover, MA). Stock PAH solutions were made with HPLC grade dichloromethane (methylene chloride) (Budick and Jackson, Muskegon MI).

Preparation of the PAH model mixture

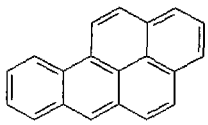
A PAH model mixture containing 10 high molecular weight compounds was prepared at the same concentrations described in Arkoosh et al (2001) (Fig 2.1). Additionally diets containing either BAP or BEP were prepared. The stock solutions were made by weighing the PAH in 100 ml volumetric flasks and adding methylene chloride to the mark. Solutions were sonicated using a Branson 1210 sonicator in water bath for 30 min and stored at 4°C in the dark for future use.



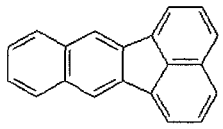
Indeno (1,2,3-cd)perylene
(4%) MW 273.3



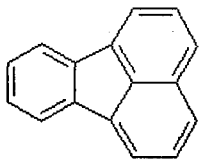
Benz(a)anthracene
(7%) MW 228.9



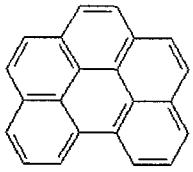
Benzo(a)pyrene
(6%) MW 252.32



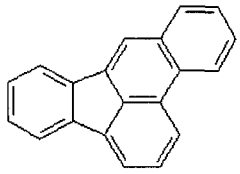
Benzo(k)fluoranthene
(6%) MW 252.32



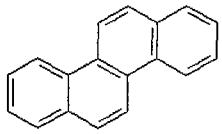
Fluoranthene
(21%) MW 202.26



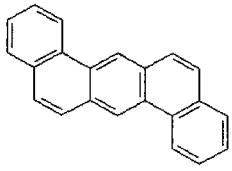
Benzo(g,h,i)perylene
(5%) MW 276.3



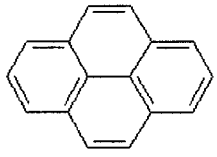
Benzo(b)fluoranthene
(17%) MW 252.32



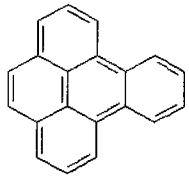
Chrysene
(13%) MW 228.29



Dibenzo(a,h)anthracene
(1%) MW 278.35



Pyrene
(20%) MW 102.26



*Benzo(e)pyrene
MW 252.32

Figure 2.1 Structures and percentages of the PAH used in the mixture.
*Benzo(e)pyrene was not used in the mixture. It was used as individual treatment.

To prepare the experimental diets, 3000 g of food were weighted in stainless steel bowls pre-rinsed with methylene chloride. The desired amounts of the stock were diluted to a total volume of 2 L methylene chloride (vehicle control methylene chloride contained no PAH, BAP at 160 ppm, BEP at 160 ppm, high molecular weight PAH mixture at 40 ppm, high molecular weight PAH mixture at 400 ppm).

Two liters of the diluted stock were added to the food and constantly stirred with a stainless steel spoon for 1 h. The treated food was left under the hood away from light for a week and stirred occasionally this assured that the methylene chloride was completely evaporated. All the stock-food preparation was performed under yellow light since PAH are light sensitive. A color-coded system was used to avoid mistakes in the preparation of the stock solutions and diets throughout the feeding process. Food was stored in 1L glass canning jars and kept frozen at -80°C until use. A sample of 30 g from each diet was taken for verification of PAH concentrations in each treatment by gas chromatography analysis/ mass spectrometry.

Animals

Laboratory bred rainbow trout (*Oncorhynchus mykiss*) were obtained from the Sinnhuber Aquatic Research Laboratory Oregon State University, Corvallis OR. Fish (7 g) were transported to the NWFSC Fish Disease Laboratory at the Hatfield Marine Science Center, Newport OR using a live transport insulated container (DX333 Aquatic Ecosystems, Apopka, FL) with gassed oxygen. Oxygen levels were kept at 9.7 mg/L during the 1 h trip. In the laboratory, 4000 fish were placed in duplicate 2000 L fiberglass circular tanks, maintained at 10°C and pH 7 for about a month. Water temperature, chlorine and pH were checked daily with a thermometer (Aquatic

Ecosystems, Apopka, FL) chlorine test strips (Corning®, waterworks™.,St Louis, MO) and a pH-meter, respectively. During this period, fish were fed 2% body weight (dry weight basis) with Trout Diet No.4 (Rangen Inc, Angleton, TX), with the following characteristics: 11% crude fat content, 45% crude protein, 9-10% water, fish meal protein 30%, phosphorus 1%, 1.5 mm pellet.

A month later, groups of ten fish were transferred to 12 gallon buckets equipped with battery powered air stones and randomly located into circular fiber glass 400 L tanks. Six replicate tanks per treatment with 90 fish each per tank were distributed among 36 tanks for this study. Ten fish were collected per tank to obtain weight and length weekly. Fish averaged 10 g at the start of the experiment. Fish were fed 2 % body weight (six times a week) with treated diets or control for a total of 50 days (feeding time). After 3, 7, 14, 28 and 50 days of feeding, three fish per tank were collected from all the treatments. Fish were sacrificed by blow to the head. Length and weight were recorded on individuals and sex was determined by inspection of reproductive organs during necropsy.

Blood, liver, kidney and bile were collected for various analyses (comet assay, EROD-CYP1A immunohistochemistry, DNA adducts, protein nitration, lipid peroxidation and PAH metabolites respectively). Comet analyses in the blood samples were performed as described in Shugart (1998) the day of sampling. The remaining samples were stored at -80°C until analysis. Liver and kidney samples collected for immunohistochemistry were fixed in neutral buffered formalin for 24 h, washed in running water and stored in 95% ethanol at 4°C for further analysis.

Chemical analyses of Diet

The diet (BEP, BAP, 40 ppm PAH mixture, 400 ppm PAH mixture, vehicle control and untreated food) was analyzed by gas chromatography/ mass spectrometry (GC/MS) for PAH. The process involved three steps: 1) extraction with high purity methanol and acetic acid (Budick and Jackson, Muskegon MI) 2) clean-up with silica/alumina columns and size exclusion high performance liquid chromatography and 3) quantitation of PAH by GC/MS as described in Sloan et al. (2004). These analytes were quantitated and reported as the sum of their concentrations because some co-eluted during GC/MS analysis (Sloan et al, 2004).

Biomarkers of PAH exposure

PAH metabolites in the bile

A total of 200 individual bile samples were processed (10 fish per treatment, per time point) from fish after 3, 7, 14, 28 and 50 days of feeding. High performance liquid chromatography (HPLC) for PAH metabolites was performed as described by Krahn et al (1984, 1986, 1993). Briefly, bile was injected directly onto a C_{18} reverse phase column (Phenomenex® Synergi Hydro, Torrance, CA) and eluted with a linear gradient from 100% water (with a trace amount of acetic acid) to 100% methanol at a flow of 1.0 ml using an automatic sampler (Millipore-Waters, Wisp model 710A). Chromatograms were recorded at the following wavelengths pairs: 260/380 nm (3-4 ring compounds fluoresce) and 380/430 nm (4-5 ring compounds fluoresce). Peaks eluting after 5 min were integrated and the areas of the peaks summed. The concentrations of fluorescent PAH metabolites in bile were determined using phenanthrene (PHN) and BAP as external standards and converting the fluorescence

response of bile to phenanthrene (ng PHN equivalents/ g bile), and BAP (ng BAP equivalents/ g bile) equivalents. Bile metabolites fluorescing at phenanthrene wavelengths indicated exposure to lower molecular weight PAH (3-4 rings), while metabolites fluorescing at BAP (4-6 rings) wavelengths indicated exposure to higher molecular weight PAH. Enough bile (>20 μL) was collected from all fish in all treatments to perform the assay. Bile protein was measured as described by Lowry (1951). Bile PAH equivalents were calculated as ng/g and ng/mg of protein.

EROD activity in microsomes

The liver was placed in a 1.5 ml tube (Eppendorf, Hamburg, Germany), snap frozen in liquid nitrogen and stored at -80 °C. Composite samples of liver 0.45-0.6 g (3-4 fish per composite) were processed. A total of four composites of liver per treatment were used. Microsomes were prepared as described by Collier et al (1986) and Collier and Varanasi (1991). Briefly, composite livers were weighted in 10 ml glass tubes and homogenized in three volumes of ice cold buffer (10 mM potassium phosphate buffer (Sigma-Aldrich, Milwaukee, WI) pH 7.5, 0.15 M KCl (Sigma-Aldrich, Milwaukee, WI), 1mM EDTA (Sigma-Aldrich, Milwaukee, WI) and 0.1mM PMSF) using a polytron homogenizer for ten seconds, homogenates were then centrifuged (Beckman Optima ^{TM} LE-80K ultracentrifuge rotor type 50, Fullerton, CA) at 10,000 x g for 23 min at 4°C, supernatant was removed and placed into new tubes and pellet was discarded. A second centrifugation at 100,000 x g for 93 min at 4°C was performed in a Beckman L7-55 ultracentrifuge rotor type 70 (Fullerton, CA) and the pellet was kept and supernatant discarded. EROD activity was measured according to the method described in Eggens and Galgani (1992). The fluorescence of the product, resorufin,

was measured at excitation-emission wavelengths of 535 and 580 nm, respectively, with a Molecular Devices fluorescence plate reader (SpectraMax Genomics, Molecular Devices, Sunnyvale, CA). The EROD activity was calculated with a SOFTmax program (Molecular Devices Co, Sunnyvale, CA). Activity as fluorescence units per min was converted to specific molar activity (pmol resorufin/min/mg protein) by reference to a standard curve of resorufin (2.5, 5, 7.5, 10 and 12.5 pmol resorufin) included in each plate as described in Hawkins et al. (2002). Protein concentrations in liver samples were measured in triplicate according to the method described by Lowry (1951).

CYP1A Immunohistochemistry

Head and trunk kidney and liver tissue samples for immunohistochemistry were processed individually, three fish per treatment and per time point after 3, 7, 14, 28 and 50 days. Neutral buffered formalin fixed tissues were embedded paraffin blocks. Sections were cut at 4 microns, mounted on slides (Biotech Probe on plus) and air dried overnight. Sections were deparaffinized and hydrated as described in Luna (1968). Bovine serum albumin (Sigma-Aldrich, Milwaukee, WI) in phosphate buffered saline (pH 7.2) and 10% normal goat serum (Sigma-Aldrich, Milwaukee, WI) blocked non-specific binding (Lester et al, 1992). Lyophilized Mab 1-12-3 antibody (150 ug) against scup cytochrome P4501A kindly supplied by J. Stegeman and B. Woodin from Wood Hole Oceanographic Institute was reconstituted by adding 10 ul of distilled water. Sections were incubated using the Shandon^{TM} coverslip system (Pittsburg, PA) for 2 h with 150 ul aliquots of 0.6 ug/ml Mab 1-12-3. Antibody reaction was visualized using avidin-biotin reaction kit (Biogenex streptavidin mouse,

BioGenex, San Ramon, CA) and DAB tablets with metal enhancer (Sigma-Aldrich, Milwaukee, WI). A negative control for each slide was used by omitting primary antibody from each duplicate. An ordinal system scored stain intensity. Staining was ranked from 0-7 (0=absent, 1=minimal, 2=minimal-mild, 3=mild, 4= mild-moderate, 5=moderate, 6=moderate-strong and 7=strong). Distribution was scored in the range 1-5 (1=focal, 3=multifocal and 5=diffuse) as described in (Hyyti et al, 2001).

DNA adducts

Composite samples of liver (four livers per composite) (0.12-0.25 g) collected after 3, 7, 14, 28 and 50 days of exposure to PAH in the diet were analyzed using the ^{32}P-postlabeling protocol for assaying levels of hydrophobic DNA adducts in fish as described in Reichert and French (1994). DNA extraction was based on the protocol described by Reddy and Randerath (1987) that used a nuclei precipitation step that minimized RNA contamination. Once DNA was extracted, the pellet was resuspended in 850 ul of 1% SDS/ 20 mM EDTA (Sigma-Aldrich, Milwaukee, WI) pH 7.4 solution containing RNAses and α-amylase and incubated for 30 min at 37°C. The RNAses and α-amylase solution were prepared as described in Reichert and French (1994). To further purify the DNA, proteins were digested by the addition of 0.4 mg of proteinase K in 40 ul of 1 M Tris-HCl (Sigma-Aldrich, Milwaukee, WI) pH 7.4 per sample. Proteins were removed by using solvent extraction with phenol:chloroform (Reichert and French, 1994). DNA quality and concentration in the extracted samples were evaluated with UV absorbance at 230/260 nm and 260/280 nm respectively in a Shimadzu UV 2100U reader. Radioactive adenosine-5'-triphosphate (gamma-^{32}P) (MP Biomedicals Inc, Irvine, CA) phosphorylated the deoxyribose of the adduct

(Reichert and French, 1994). Results were visualized using storage phosphor imaging technology (Molecular Dynamics Sunnyvale, CA) that located radioactivity associated with DNA adducts on the chromatograms and quantified using Image Quant Software (Molecular Dynamics version 3.3, Molecular Dynamics Sunnyvale, CA).

Markers of oxidative stress

Comet assay in blood

Individual blood samples were analyzed on the day of sampling. Blood from a non-treated fish was left under the hood overnight under UV light as positive control. Blood from a healthy non-treated fish was used as negative control. A total of 4 fish per treatment group were processed for every feeding treatment time-point. Blood cells were collected by cutting the fish tail with a sterile No. 11 blade and drawing the blood in pre-heparinized capillary glass tubes (Corning, Corning, NY). Blood was diluted 1:1000 in phosphate buffered saline solution (PBS): NaCl 1.37 M (Sigma-Aldrich, Milwaukee, WI) KCl 0.026 M (Fisher Scientific, Fairlawn, NJ), Na_2HPO_4 X $2H_2O$ 0.08 M (Fisher Scientific Fairlawn, NJ), KH_2PO_4 0.01 M (Sigma-Aldrich, Milwaukee, WI) diluted ten times with distilled water at final pH 7.3-7.4. The single cell method (SGC) as described in Singh et al. (1988) and Anderson et al. (1996) was used. Briefly, frosted microscope slides (Surgipath, Richmond, IL) were each covered in molten 0.5% low melting point agarose (LMPA) and placed in a slide warmer for 10 min to allow agarose to solidify. This layer promoted the attachment of the second layer of LMPA as described in Anderson et al. (1996). Around 30,000 cells were mixed with 75 ul of 0.5% LMPA at 37°C. Cells were then immersed in freshly prepared cold lysing solution for 1 h at 4°C, 0.01 M Tris (Sigma-Aldrich, Milwaukee,

WI), 0.18 M of Na_2EDTA x 2 H_2O (Sigma-Aldrich, Milwaukee, WI), 5.03 M NaCl (Sigma-Aldrich, Milwaukee, WI). The solution was adjusted to pH 10 with NaOH pellets (Sigma-Aldrich, Milwaukee, WI). Triton X-100 (1%) and DMSO (10%) were added to the solution before use. The slides were then placed in an electrophoresis tank (Fisher Scientific) in 1.6 L of electrophoresis solution for 30 min to allow DNA unwinding and expression of alkali-labile damage. Electrophoresis was conducted at 4°C using 40 V and 400 mA current by adjusting the power supply (Thermo EC 1000-90). All of these steps were conducted under dim light to prevent further DNA damage as described in Anderson (1992); after electrophoresis, slides were rinsed with Tris buffer (0.4 M Tris pH 7.5) drop wise to neutralize alkali. Ethidium bromide (20 ug/ml) (Sigma-Aldrich, Milwaukee, WI), was added to each slide before they were coverslipped. Slides were scored within 4 h of staining. Fifty cells per slide were scored using the Comet Assay Image Analysis software III (Perceptive Instruments version 3.0, Suffolk, UK) under a fluorescent microscope (Nikon Eclipse E40, Lake Forrest, CA).

Protein nitration

The presence of protein nitration in rainbow trout trunk kidney and liver was evaluated immunohistochemically. Tissue was formalin-fixed, paraffin embedded and sectioned at 4-5 microns and placed on Microprobe slides (Fisher Scientific). Following rehydration, slides underwent high temperature antigen retrieval using citrate buffer, pH 6.0 (Dakocytomation, Glostrup, Denmark) for 5 min in a microwave pressure cooker (Tendercooker) and rested at room temperature for 20 min. Slides were washed in distilled water (dH_2O) followed by endogenous peroxide blocking in 3% H_2O_2 in

Tris buffered saline with Tween (TBST) (Dakocytomation, Dako, Denmark) for 10 min. After washing in dH_2O, slides were placed in the Microprobe capillary gap slide holder (Fisher Scientific) and repeatedly washed and blotted to insure good capillary flow. Slides were blocked in Serum Free Blocking Solution (Dakocytomation Dako, Denmark) for 10 min, blotted and primary antibody was applied for 1 h at room temperature. Nitrotyrosine antibody was from UpstateCell Signaling Solutions (Lake Placid, NY) (lot #29502) at a dilution of 1:500 in antibody diluent with background reducing compounds (Dakocytomation Dako, Denmark). As a control, the antibody was blocked by incubating with 3-nitro-p-tyrosine 10 mM in PBS for 1 h prior to reaction with slides. Negative control was Universal Negative Control Rabbit (Dakocytomation Dako, Denmark). Slides were blotted and washed six times in TBST followed by Envision anti-rabbit polymer (Dakocytomation Dako, Denmark) for 30 min and again washed and blotted in TBST. Slides were loaded on a Dakocytomation Autostainer and the chromagen Nova Red (Vector Laboratories, Burlingame, CA) was applied for 5 min. Slides were counterstained in hematoxiline Shandon (Thermo Electron Co, Houston, TX) cleared in xylene and coverslipped.

F2-isoprostanes

After 50 days of feeding, kidney was collected to determine lipid peroxidation measured by F2-isoprostanes. A total of 6 individual fish kidney samples per treatment were taken after 50 days of feeding. Samples were sent to Vanderbilt University to be analyzed as described in Morrow et al (1992).

Other assays

Ah receptor binding assay with BAP and BEP

DMSO (Sigma-Aldrich, Milwaukee, WI) solutions that contained 10 μM of BAP and BEP were prepared for the AHR binding assay. PLHC-1 fish hepatoma cells were used as described by Hahn et al (1993). Rainbow trout AHR2a was expressed by in vitro transcription and translation and incubated with [3H] 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (2 nM) with DMSO, BAP or BEP (1, 10 or 100 nM final concentration) overnight on ice. The incubations were then layered on sucrose gradients, centrifuged, and fractionated as described (Hahn et al 1993). UPL (unprogrammed TNT lysate) was used as a control for non-specific binding). The AHR peak of specific binding was measured in fractions 10-20.

Statistical Analysis

Statistical analyses were conducted with NCSS statistical analysis and data analysis software (Kaysville, UT) and Stat View statistical package (Kari, NC). Differences between treatments for Fulton condition index, environmental parameters (water temperature, pH), bile contaminant concentrations and EROD activity were determined by ANOVA. For DNA adducts, comet assay and F2-isoprostanes data analysis, one way ANOVA was employed. Lower and upper 95% confidence limits were established. The significance level for all analysis was set at $\alpha = 0.05$. For immunohistochemistry (CYP1A and protein nitration), data was analyzed with Kruskal-Wallis non parametric test (Sokal and Rohlf, 1995).

RESULTS

Environmental variables and fish condition

Average water temperature throughout the dietary exposure was 11.5±2 with a minimum of 9.4°C and a maximum of 12.6°C. The pH averaged 7.12±0.09 with a minimum of 7.03 and a maximum of 7.29.

Condition index (k) was calculated (k=weight x 100/length^3) and expressed as g/cm^3. No significant differences in condition index between treatments were detected during this feeding study ($p>0.05$), male (1.024 g/cm^3±0.0249) and female (1.073 g/cm^3±0.0249). Indices were compared between male and females, no differences were found at $p>0.05$.

Chemical analyses of Diet

We measured the PAH concentrations in the treatment diets and verified concentrations were close to the nominal concentrations: 160 ppm BAP and BEP, 40 ppm mixture, 400 ppm mixture and control. There was no statistical difference between nominal and actual concentrations ($p>0.05$). Untreated and vehicle control diet treated with methylene chloride tested negative for PAH.

Biomarkers of PAH exposure

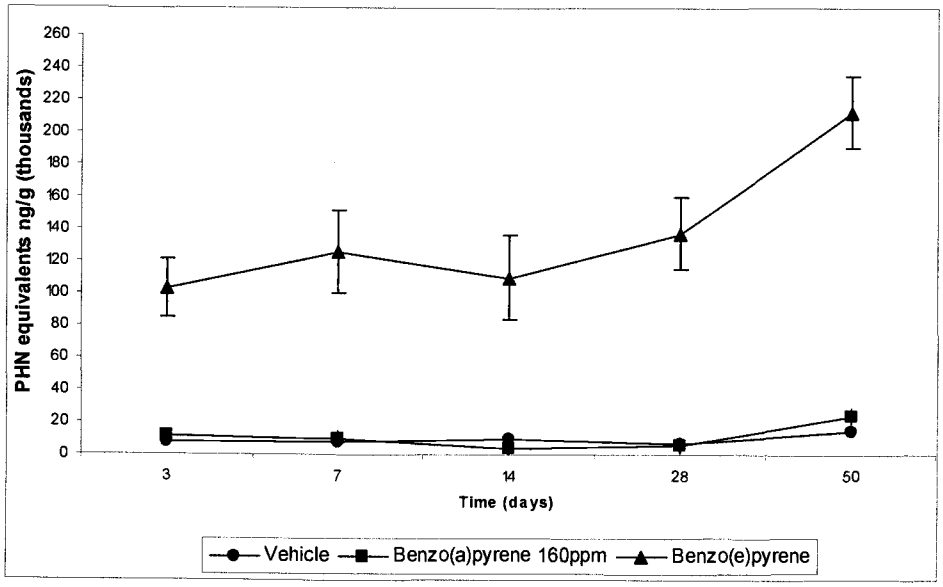
Bile analysis

Equivalents of PAH metabolites in bile were calculated as ng/g and ng/g protein corrected for all treatments. It was suggested that biliary fluorescence data should be normalized to protein concentration (Collier and Varanasi, 1991).

To our knowledge, BEP metabolites have not been previously analyzed by this method after long-term dietary exposure. Emission maxima for BEP were 330 nm and 390 nm (Osborne and Crosby, 1987); emission maxima for BAP were 406 nm and 386 nm (Fig 2.2b). The BEP metabolites fluoresced at phenanthrene wavelengths 290/335, 341/383 and not at BAP wavelengths 380/430 nm. The BAP metabolites only fluoresced at BAP wavelength pair but not at phenanthrene wavelength pair.

The levels of PAH metabolites in bile increased rapidly during the first three days of exposure followed by a slower increase during the end of the exposure (after 28 days), a similar pattern has been observed in previous studies (Aas et al, 2000). After 28 days, BAP equivalents in bile were not significantly different than after 3 days ($p>0.05$), similar results were obtained for BEP phenanthrene wavelength pairs (Fig 2.2a). This pattern was also observed in the mixture fed 40 and 400 ppm mixture treatments ($p>0.05$) (Fig 2.3 a, b) the range for protein normalized bile data was 133 ng/g to 1206 ng/g bile protein for BAP wavelength pair equivalents and 13067 to 32166 ng/g bile protein in phenanthrene wavelength pair equivalents. Metabolite concentrations in the bile increased or remained constant over time in all treatments and were significantly higher than control ($p<0.05$) throughout the exposure.

(a)



(b)

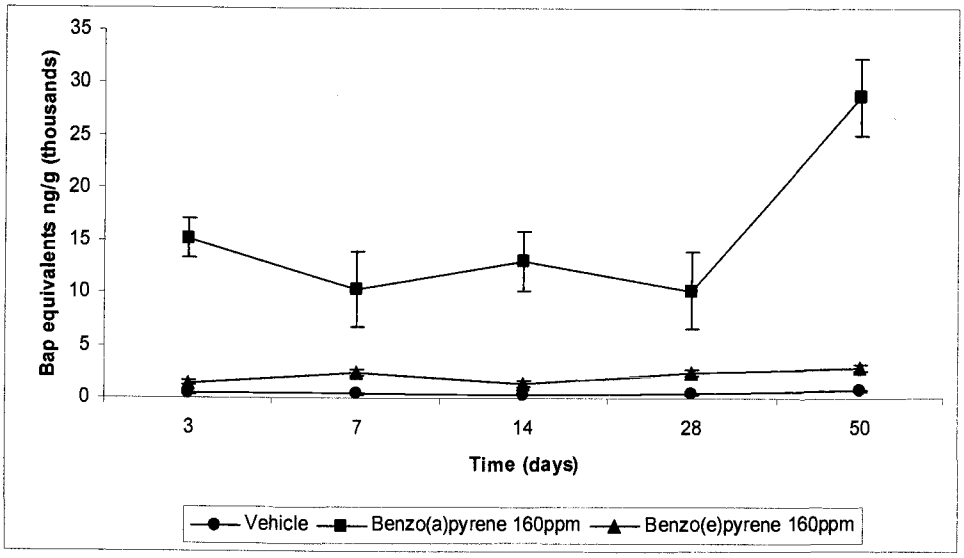
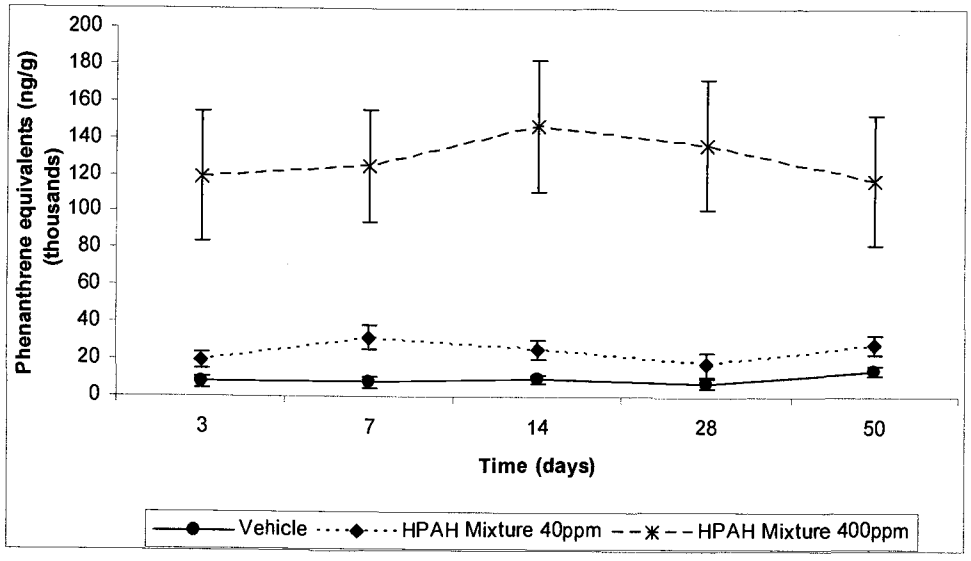


Figure 2.2 (a) Phenanthrene equivalents in bile from rainbow trout exposed to 160 ppm of benzo(e)pyrene and benzo(a)pyrene in the diet for 50 days. Only BEP fluoresces at this wavelength. (b) BAP equivalents in bile from rainbow trout exposed to 160 ppm of benzo(e)pyrene and benzo(a)pyrene in the diet for 50 days. Only BAP fluoresces at this wavelength.

(a)



(b)

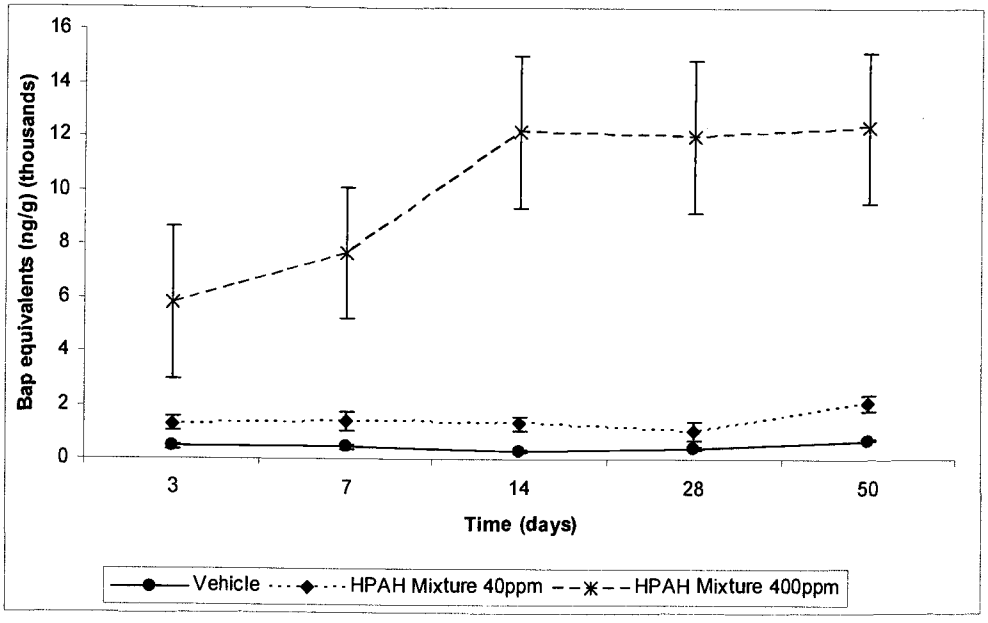


Figure 2.3 (a) Phenanthrene equivalents in bile from rainbow trout exposed to 40 or 400 ppm PAH mixture in the diet for 50 days. (b)BAP equivalents in bile of rainbow trout exposed to a PAH mixture in the diet for 50 days.

EROD induction

There were significant differences in EROD induction for fish fed 400 ppm mixture ($p<0.05$) and all other treatments at all sampled times. After 3 days EROD activity in 160 ppm BAP fed fish was significantly higher than in 160 ppm BEP fed fish. This difference was not statistically significant after 14 days (Fig 2.4a).

There was no significant difference ($p>0.05$) in EROD induction in 40 ppm PAH mixture fed-fish at any time-point.

Histology

No histopathological lesions in kidney or liver were observed at any time in any of the treatments. Morphology was normal in all sections observed.

CYP1A Immunohistochemistry

The tissue content of CYP1A protein was examined immunohistochemically in liver and kidney after 3, 7, 14 and 28 days of dietary exposures with PAH. Cellular localization of CYP1A was identified using a monoclonal antibody against scup P4501A1 (Mab 1-12-3).

For the liver, staining was present mainly in hepatocytes or parenchyma cells (Fig 2.5 a, b) and to a lesser extent in the endothelial cells of the larger blood vessels, the sinusoids and the bile canaliculi (not shown).

For head kidney, CYP1A staining was present in endothelial cells and most commonly in the smaller blood vessels or sinuses as well as in the endothelium of the larger blood vessels (Fig 2.5 c, d)

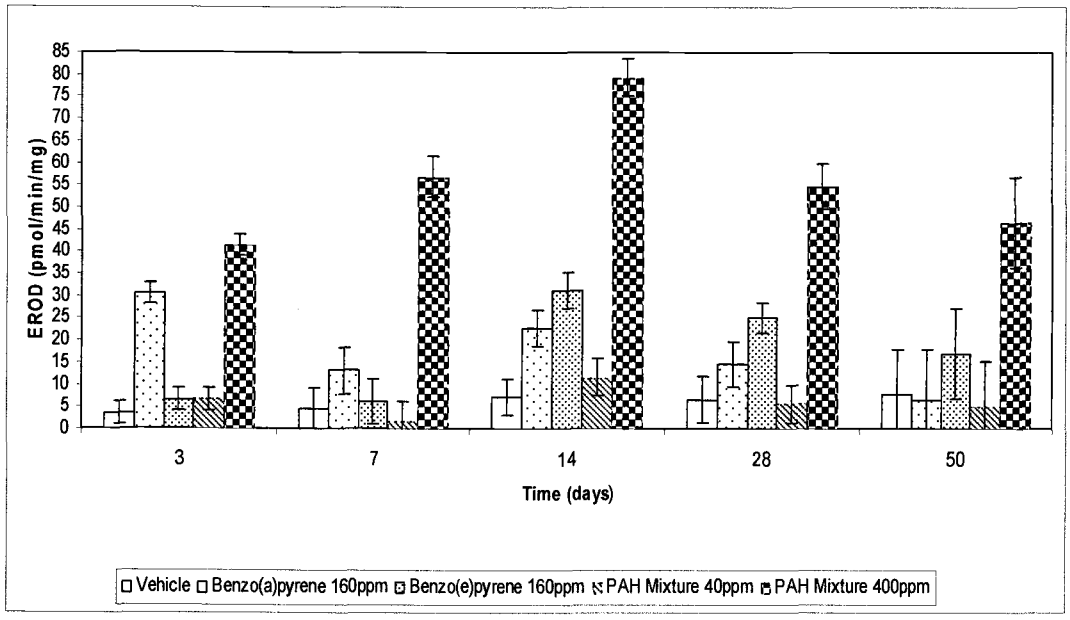
For the trunk kidney CYP1A staining was present in the tubular epithelium of the second proximal segment of the nephron, in the endothelium of the glomerular capillaries and in the endothelium of the blood vessels within the renal interstitium (Fig 2.5 e, f)

Significant differences in stain intensity between vehicle and 400 ppm mixture PAH were detected in kidney after 3, 14 and 28 days (Table 1.1). No abnormal morphological changes were present in any of the groups.

Comparison CYP1A immunohistochemistry and EROD induction in liver

A direct comparison of CYP1A content quantified by immunohistochemical staining and CYP1A-mediated EROD induction (Hyyti et al, 2001) was not possible because EROD was measured using composite samples whereas immunohistochemistry was evaluated individually. Therefore we visually compared the trend of CYP1A expression after 3, 7, 14 and 28 days using both techniques (Figs 2.4 a, b) and found that in both cases 400 ppm PAH mixture treatment exhibited higher staining of CYP1A followed by BEP, BAP, 40 ppm PAH mixture and control. The peak of CYP1A staining was obtained 14 days after exposure and the recovery was seen 28 days after exposure for all treatments except for PAH mixture 40 ppm. The magnitude of CYP1A elevation differed between the two methods.

(a)



(b)

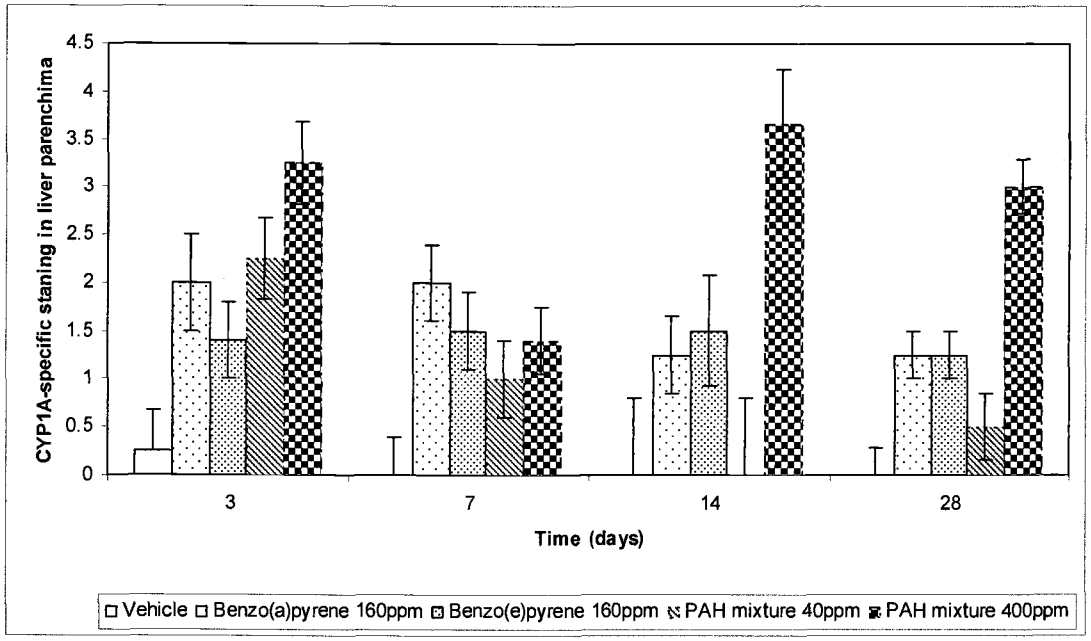


Figure 2.4 (a) EROD induction expressed as pmol/min/mg in rainbow trout liver exposed to PAH in the diet for 50 days. (b) CYP1A immunohistochemistry expressed as stain intensity in rainbow trout exposed to PAH in the diet for 28 days. Samples for day 50 immunohistochemistry were not available.

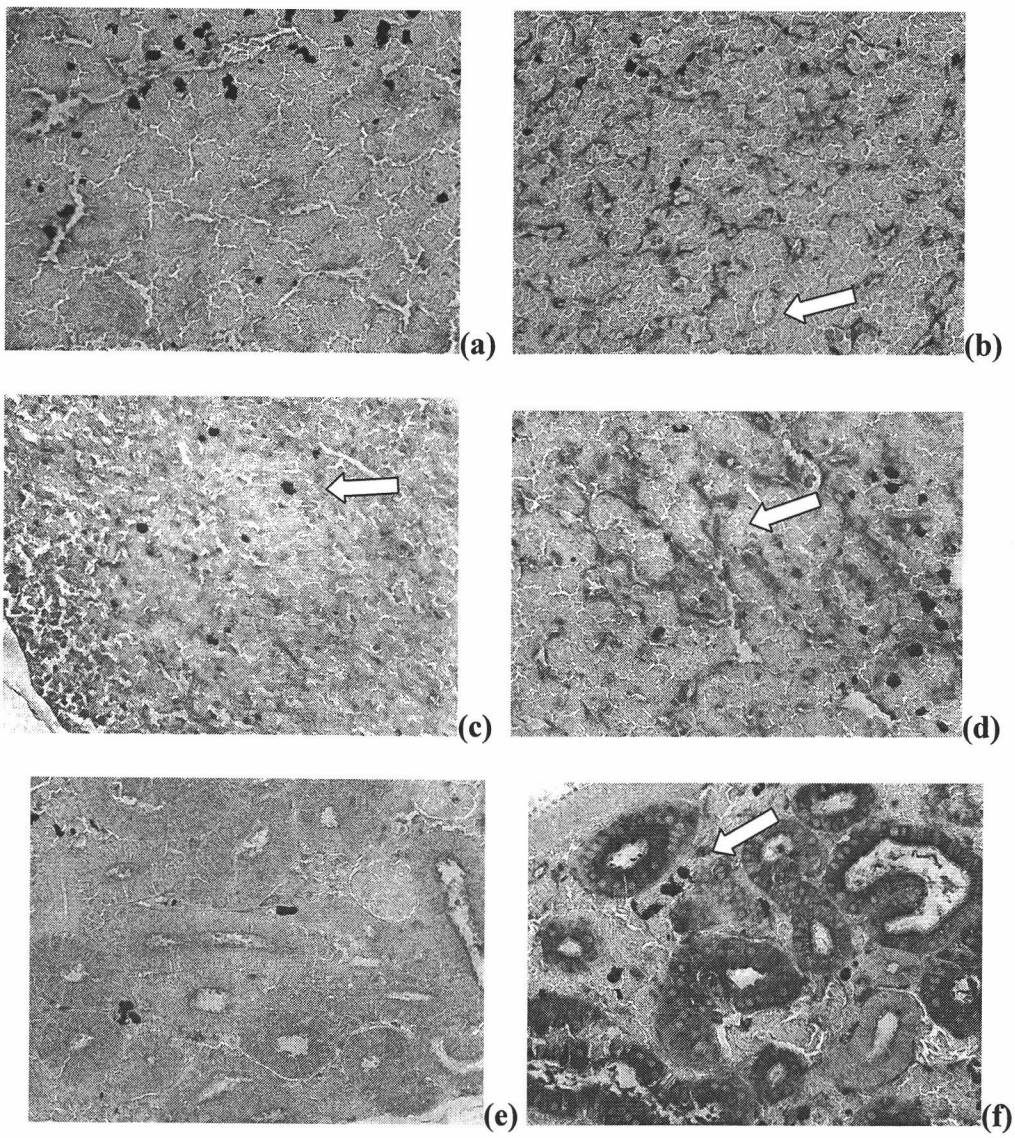


Figure 2.5 Distribution and localization of CYP1A in rainbow trout liver, head kidney and trunk kidney (a) control Rainbow liver. Very low CYP1A-associated staining is evident (40X). (b) 400 ppm High molecular weight mixture exposed rainbow trout liver after 14 days. Section showing positive CYP1A-strong specific staining in parenchyma and endothelial cells of the larger blood vessels. Darker brown spots are macrophage aggregates. (c) Control Rainbow trout head kidney. (d) Rainbow trout head kidney exposed to 400 ppm PAH mixture after 14 days. Section showing positive CYP1A-strong specific staining in endothelium cells of the vasculature is indicated. Darker brown spots are macrophage aggregates. (e) Control rainbow trout trunk kidney. Very low CYP1A-associated staining is evident (40X). Darker spots are macrophage aggregates (f) Rainbow trout trunk kidney after 14 days of exposure to 400 ppm PAH. Section showing positive multifocal CYP1A-strong specific staining in proximal tubules and endothelium cells of the vasculature is shown.

Treatment	Sampling day	Head Kidney Endothelial cells	Trunk Kidney tubules	Trunk Kidney Endothelium of glomerular capillaries	Trunk Kidney Endothelium of blood vessels within renal interstitium
BAP 160 ppm	3	1.5±0.74	4±0.82	1.33±0.59	1.333±0.55
	7	0.5±0.56	1.5±0.64	0±0.37	0±0.43
	14	0±0.68	0.25±0.14	0±0.12	0±0.44
	28	0±0.13	0.5±0.31	0.25±0.12	0±0.43
BEP 160 ppm	3	0±0.60	0.25±0.15	0.75±0.12	0±0.34
	7	0±0.46	0±0.14	1±0.51	0±0.43
	14	0.5±0.60	0±0.14	0.5±0.31	0.75±0.43
	28	0.25±0.13	0.25±0.20	0.5±0.31	0±..31
PAH mixture 40 ppm	3	1.5±0.74	1.25±0.71	0.25±0.12	1±0.48
	7	0.25±0.39	0±0.14	0.33±0.12	0±0.5
	14	1±0.85	0±0.14	0±0.36	0±0.48
	28	0±0.15	0±0.35	0±0.45	0±0.51
PAH Mixture 400 ppm	3	5.5±0.52	5.25±0.71	3.5±0.51	4.25±0.48
	7	4.5±0.56	3.6±0.58	4±0.33	3.8±0.49
	14	2.75±0.60	1±0.28	3±0.62	3±0.51
	28	2±0.15	3±0.35	2.33±0.45	2.33±0.55
Vehicle control	3	0±0.60	0±0.14	0.66±0.21	0.33±0.55
	7	0±0.46	0±0.20	1±0.52	0±0.61
	14	0±0.70	0±0.20	0.5±0.31	0±0.62
	28	0±0.27	0.5±0.43	0.5±0.31	0±0.51

Table 1.1 Stain intensity (mean±SE) of CYP1A associated expression in head kidney and trunk kidney of rainbow trout after 3,7,14 and 28 days of dietary exposure to PAH

DNA Adducts

DNA adducts were calculated in liver of rainbow trout exposed to PAH in the diet. Numbers obtained for adducts were close to background (1-30 nmol adducts/mol bases) (data not shown). During the procedure, ATP excess was not found for all samples. Only the ^{32}P images obtained from samples that had excess ATP were analyzed and non-detectable level of adducts were found (data not shown). Previous work demonstrated low adduct formation in similar experiments with chinook salmon fed different concentrations (20 ppm- 1620 ppm) of a PAH mixture containing high as well as low molecular weight PAH in the diet for 60 days (Meador 2001, 2003 unpublished data). Our experiment confirmed those findings.

Markers of oxidative stress

Comet assay

DNA damage was analyzed as tail moment (tail length fraction of nuclear DNA in tail) and DNA percent damage (percent of broken strands present in the tail of nucleus). The former is preferentially used in the literature (Andersson et al, 1994). In this study, both parameters yielded identical trends. Only percent DNA damage results were reported (Fig 2.6).

There was a significant difference ($p < 0.05$) between all the treatments and the control on day 14. Trout blood tested displayed significant ($p<0.05$) and extensive DNA damage.

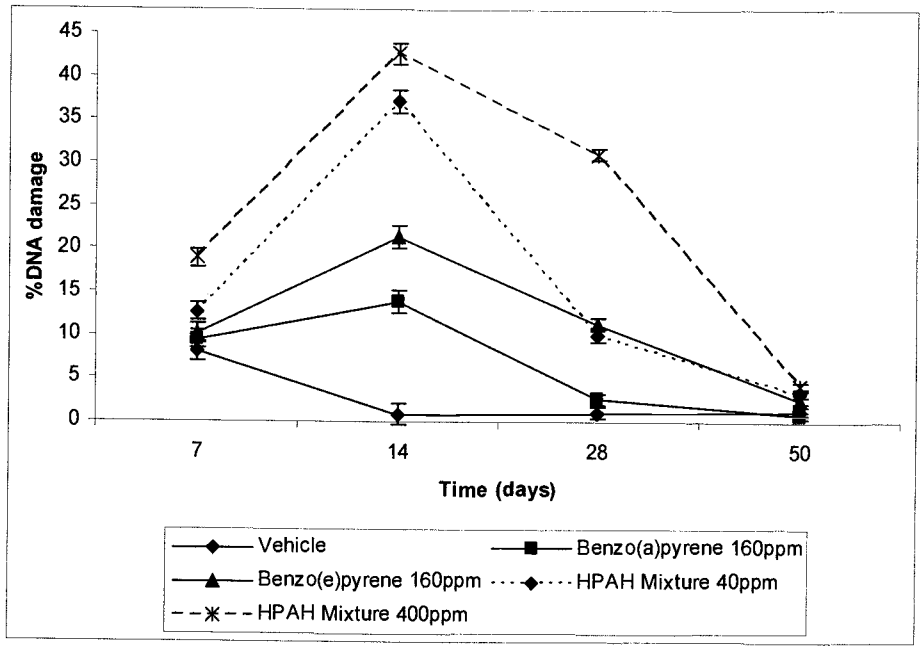


Figure 2.6 Percent DNA damage in blood cells of rainbow trout exposed to PAH in the diet for 7, 14, 28 and 50 days.

after 7 days of feeding. This damage was further increased after 14 days but decreased by 50 days. This pattern was observed in all treatments.

Percent DNA damage after 14 days for individual fish ranged from 0-5% for control, 10-15% for 160 ppm benzo(a)pyrene, 20-25% 160 ppm for benzo(e)pyrene, 35-40% for 40 ppm PAH mixture and 40-45% for 400 ppm PAH.

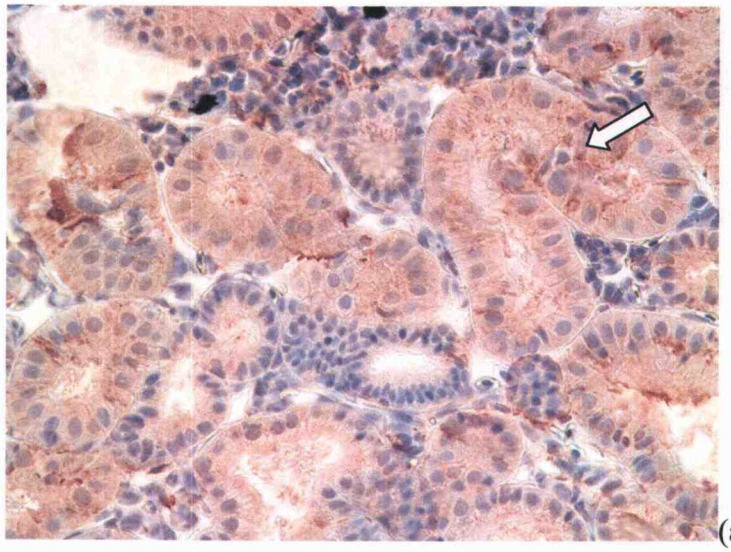
Protein nitration

Nitrotyrosine staining was evaluated in liver and trunk kidney. In liver, no significant differences ($p>0.05$) in stain intensity were observed in any of the treatments at any time-point relative to control. However, immunodetection with polyclonal anti-nitrotyrosine antibody demonstrated low but detectable levels of tyrosine nitrated in trunk kidney in all treatments evaluated. Stain intensity in vehicle diet controls was quite similar at all sampling times (Table 1.2). After 7 days of treatment, trunk kidney from fish fed 400 ppm PAH mixture stained more intensely and that from fish fed 160 ppm BEP stained less intensely than control tissue.

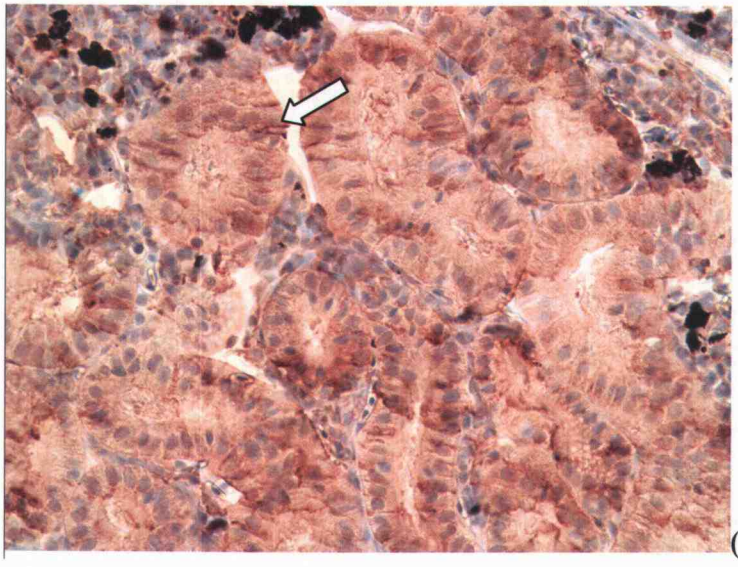
Staining was mainly present in the tubular epithelium of the second proximal segment of the nephron (Figure 2.7). Stain intensity was very similar in all groups after 28 days of treatment.

TREATMENT	SAMPLING DAY	TUBULES (TRUNK KIDNEY)
BAP 160 ppm	3	3.5± 0.79
	7	1.5± 0.91
	14	2 0±0.64
	28	3.0 ±1.03
BEP 160 ppm	3	3.3± 0.79
	7	0.7± 0.12
	14	1.7± 0.64
	28	3.0 ± 1.19
PAH Mixture 40 ppm	3	3.3± 0.91
	7	4.5± 0.91
	14	1.3± 0.64
	28	3.8± 1.03
PAH Mixture 400 ppm	3	3.2± 0.79
	7	5.5± 0.91
	14	2.5± 0.64
	28	2.8± 1.03
Vehicle control	3	3.0±0.79
	7	3 .0±0.91
	14	2.7±0.74
	28	3.0± 1.03

Table 1.2 Stain intensity (mean±SE) of nitrotyrosine antibody associated expression in trunk kidney of rainbow trout after 3,7,14 and 28 days of dietary exposure to PAH.



(a)



(b)

Figure 2.7 Evidence of protein nitration examined with polyclonal anti-nitrotyrosine antibody (40x) (a) Section showing control rainbow trout kidney, very low nitrotyrosine-associated staining is evident. (b) Section showing positive multifocal staining in endothelial cells (darker pink) is indicated after 7 days of exposure to 400 ppm PAH.

F2 Isoprostanes

BAP 160 ppm and 400 ppm mixture presented significantly higher levels of F2-isoprostanes in head kidney than the control ($P<0.05$) (Fig 2.8). Isoprostanes in rainbow trout kidney tissue were expressed as bound isoprostanes since this was related to lipid oxidation in the tissue (Fig 2.8).

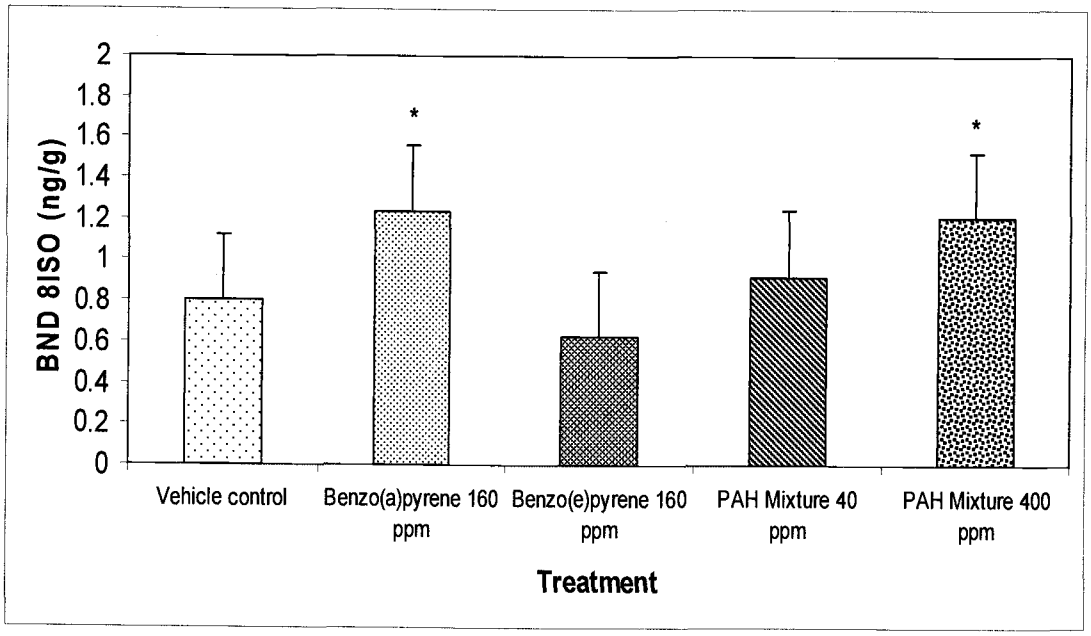


Figure 2.8 Lipid peroxidation expressed as bound (BND 8ISO ng/g) F2-isoprostanes in rainbow trout head kidney after 50 days of dietary exposure to PAH.
* Significantly different than control ($p<0.05$)

Other assays

AHR binding BEP and BAP

This experiment was performed twice with similar results. BAP competed with TCDD for binding as expected; competition was not apparent with BEP. There was a slight decrease at 1 nM BEP (82% control specific binding). Similarly the apparent increase in TCDD binding at higher concentrations of BEP was not deemed significant (Fig 2.9).

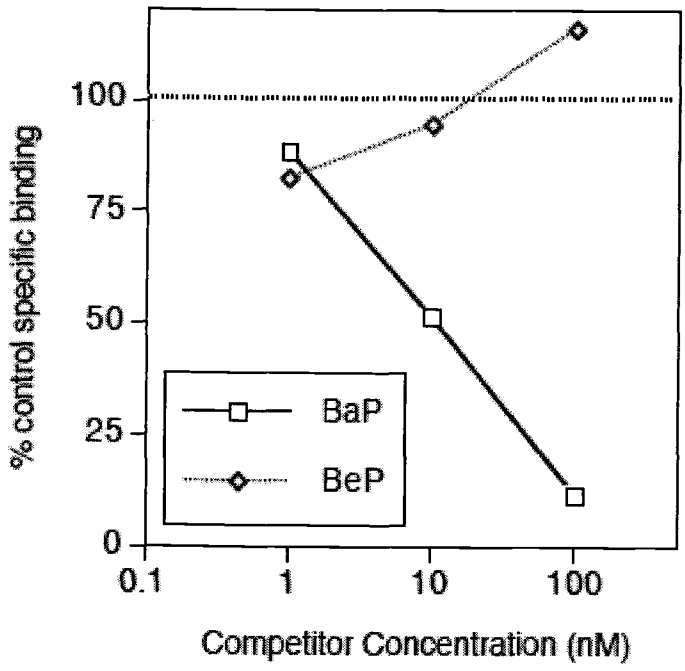


Figure 2.9. AHR binding assay with BEP and BAP

DISCUSSION

The PAH selected for inclusion in the dietary test mixture are found in different compartments in the environment (sediment, water and air). The sources of these compounds were the following: exhaust of diesel and gasoline engines, cigarette smoke condensate, different crude oils, distillate fuel oils, coke oven emissions in air and coal tar emissions from municipal incinerations (Neff, 1979). Feeding was identified as an important route for fish exposed to PAH mixtures in the environment. Rainbow trout were fed an environmentally relevant PAH mixture at concentrations based on stomach contents of other salmonids collected in the Puget Sound area (WA) (Varanasi et al, 1993). The BAP, BEP and PAH mixture concentrations fed (Fig 2.1) in this study were not overtly toxic to rainbow trout. Hendricks et al (1985) reported no direct mortalities in rainbow trout fed 1000 ppm of BAP for up to 12 months.

Bile

Measurement of PAH metabolites in bile were previously used as biomarker of exposure (Krahn et al, 1984, 1986; Willet et al, 1995; Aas et al, 2000; Aas and Goksoyr, 2000). This method allowed the quantitation of polar biliary metabolites by integrating the intensity of eluted peaks at different wavelength pairs (Fig 2.2 and 2.3). Biotransformation of BAP in English sole (*Parophrys vetulus)* produced various primary metabolites: 7,8-dihydrodiol, 9,10-dihydrodiol, 1-hydroxy-benzo(a)pyrene and 3-hydroxybenzo(a)pyrene which were metabolized by phase II enzymes to more soluble conjugates and excreted in the bile (Altenbulger et al, 2003). This and other studies demonstrated that PAH were metabolized before biliary excretion (Hendricks

et al, 1985; Hawkins et al, 2002). PAH metabolite concentrations in the bile of rainbow trout in this laboratory study, compared to bile protein normalized values to values for field collected in juvenile chinook salmon, assessed relevance of dietary exposure concentrations.

BAP bile metabolites in rainbow trout were 1206 ng/g bile protein to 1106 ng/g bile protein after 3 and 28 days of exposure, respectively. Phenanthrene (PHN) wavelength pair equivalents ranged from 32,166 ng/g bile protein to 13,067 ng/g bile protein after 3 and 28 days of exposure to 400 ppm PAH mixture, respectively. For 40 ppm PAH mixture, values ranged from 113 ng/g bile protein to 223 ng/g bile protein in BAP wavelength pair equivalents. PHN equivalents from 1633 ng/g bile protein to 3766 ng/g bile protein after 3 and 28 days of feeding respectively. These values were within the range of the concentrations found in previous field studies (unpublished data). The limitation of this technique was that specific PAH metabolites were not identified.

EROD induction

Metabolic activation of PAH by fish liver CYP1A was reported earlier (Ahokas, 1984) specifically for BAP (Au et al, 1999). Andersson (1992) reported that purified CYP protein from trunk kidney in juvenile rainbow trout catalyzed certain CYP1A mediated reactions at similar rates as that from the liver. Normal levels of CYP1A type activities in liver microsomes from trout were about 0.2 nmol/mg microsomal proteins (Pesonen et al, 1987). In the liver microsomes, we found 2-5 pmol/min/mg of EROD activity for control fish. The high rate of BAP metabolism by trout liver microsomes was associated with the formation of reactive intermediates which bind covalently to protein and DNA (Ahokas, 1984).

Our findings indicated that EROD was significantly induced by day 3 in BAP-treated animals (30-35 pmol/min/mg) compared to control (2-5 pmol/min/mg) (Fig 2.4a). This confirmed earlier findings that showed a marked induction of hepatic EROD activity in rainbow trout two days after the start of a waterborne exposure at 5 mg/kg (Sandvik et al, 1998).

Even though specific metabolic activities of other PAH metabolizing organs were lower than liver the local generation of reactive metabolites was of potential high toxicological relevance. It was proposed that CYP1A was an important source of catalytic iron that participated in redox-cycling and contributed to toxicity (Zangar et al, 2004).

To confirm biochemical findings, relative staining and intensity were determined subjectively by light microscopy.

Histology

No histopathological lesions in kidney or liver were observed at any time. Previous studies reported histological lesions in livers of rainbow trout exposed chronically to 1000 ppm of BAP for 18 months. These lesions were observed as early as after 12 months of exposure (Hendricks et al. 1985). BAP was carcinogenic in rainbow trout after dietary exposures or intraperitoneal injections (Hendricks et al, 1985). Long term exposures (up to 10 months) were required to detect tumors in liver tissue therefore it was not surprising that no lesions were detected in the present study.

CYP1A immunohistochemistry

Immunohistochemistry in rainbow trout liver showed the highest level of the CYP1A protein in hepatocytes, consistent with previous findings (Hyyti et al, 2001).

The induction of CYP1A stain intensity with immunohistochemistry followed a similar pattern of EROD activity in the liver microsomes (Fig 2.4 a, b). However in CYP1A immunohistochemistry we observed that after 3 days of exposure the highest intensity was achieved in all treatments and decreased by 28 days close to control levels for all treatments except for 400 ppm PAH mixture (Fig 2.4 b). Activity of EROD was not measured in any extra-hepatic organ but immunohistochemistry in trunk kidney indicated that CYP1A was present as previously described (Stegeman et al, 1991; Myers et al, 1996; Hyyti et al, 2001). The presence and induction of CYP1A indicated PAH exposure. Increased CYP1A probably increased oxidative stress and perhaps contributed to increased DNA strand break detected in the comet assay (Fig 2.6). Induction of CYP1A in kidney was indicative that this tissue was a potential target for oxidative stress (Hyyti et al, 2001)

Previous studies indicated that the localization of elevated CYP1A expression in extra hepatic organs was limited to endothelial cells of the vasculature and no organ-specific cell types. Our study demonstrated that it was also present in endothelial cells of kidney but also in smaller blood vessels (Fig 2.5). Previous findings reported by Hyyti et al (2001) confirmed that CYP1A inducing agents were distributed via the blood.

DNA adducts

No adducts were detected in any of the treatments in the present study. This suggested that rainbow trout were perhaps less susceptible to tumor formation than other species like English sole reported as sensitive to liver abnormalities after exposure to BAP (Varanasi et al, 1986)

Liver microsomes from different fish species catalyzed production of BAP intermediates that bound to DNA at very different rates (Varanasi et al, 1989) factors like detoxication potential will influence the activation of PAH *in vivo* (Varanasi et al,1989). Stein et al (1995) reported adduct formation in English sole were 8 to 22 times greater than in salmon collected from contaminated estuaries in the Puget Sound. This likely indicated species differences in exposure and perhaps in uptake and metabolism of PAH.

Comet assay

PAH were reported as carcinogenic and genotoxic in experimental animals and humans. In this study the comet assay detected DNA damage in blood cells from rainbow trout after 7, 14, 28 and 50 days of dietary exposure to PAH (Fig 2.6). Comet assays have previously detected DNA damage in fish models (Gamal, 1999; Hayasaki et al, 1998; Mitchelmore and Chipman, 1998b). The DNA damage observed in trout blood cells indicated that PAH were metabolized in the blood or PAH metabolites were released from tissue such as liver or a combination of both. Fish blood cells metabolized BAP at approximately 1% of hepatic rates (Mitchelmore and Chipman 1998a; Kennedy et al, 1991). Mitchelmore and Chipman (1998a) reported that DNA damage in blood cells and hepatocytes from brown trout incubated with BAP (0-200 μM) increased in a dose-dependent manner and was statistically significant $P<0.05$ in hepatocytes but not in blood cells. The contribution of metabolites produced in blood and those released from tissues to comet assay results were estimated. DNA single and double strand breaks probably occurred via a number of mechanisms (Mitchelmore et

al, 1998). This assay was not specific because the nature of strands breaks were not indicated (Mitchelmore and Chipman, 1998a).

Perhaps PAH metabolites or reactive oxygen species (ROS) formed in liver or other tissues reached the blood and caused DNA damage. Alternatively, this DNA damage was perhaps caused by direct metabolism of these compounds by CYP1A in the blood. In this study CYP1A induction in the blood was not measured. ROS induced significant oxidative DNA damage. DNA strand breaks were potentially produced by direct interaction of the hydroxyl radical ($OH^{\cdot}$) with chromatin. Other cellular and physiological processes mediated by $OH^{\cdot}$ radical released from redox cycling of PAH metabolites (especially quinones) was likely (Fairbain et al, 1995).

Shugart (1998) chronically exposed blue gill sunfish (*Lepomis macrochinus*) to BAP. The effect of chronic exposure to BAP at a concentration of 1 μg/l for 30 days produced chemically induced damage to liver DNA. DNA damage decreased after 15 days of exposure.

Dietary exposure to 160 ppm of BAP or 160 ppm of BEP and a high and low concentration mixture of 10 high molecular weight PAH (40 ppm and 400 ppm respectively) increased strand breaks in the blood cells. Comet results were concentration-dependent for the mixture and equivalent for BEP and BAP on day 14. After 14 days, DNA damage decreased in all treatments and returned towards control levels after 50 days. This indicated that animals adapted to the chemical insult and perhaps an increased ability to repair damage.

We measured DNA damage in whole blood cells rather than a subset of cells because procedures for cell type isolation such as density gradient increased DNA damage and

introduced artifacts. Giovannely et al (2003) reported that in humans, comet assay of peripheral leukocytes was simpler and involved less cell manipulation than the gradient isolation of all cell subtypes. Different from human red blood cells, fish red blood cells were nucleated this greatly increased the number of nucleated cells in a sample and required dilution of blood.

Mitchelmore and Chipman (1998a) found no significant ($p>0.05$) effect with any comet assay parameter in blood with *in vitro* exposures was found. In a different study, brown trout were given a single intraperitoneal injection of BAP 50 mg/kg, highly significant increases in strand breaks were seen in hepatocytes and blood cells analyzed 24 h after injection (Mitchelmore and Chipman, 1998b).

Protein nitration

Chronic inflammation released several active oxygen species (Lewis et al, 1986). ROS readily modified cellular macromolecules leading to a variety of toxic effects including lipid peroxidation, protein dysfunction, nucleic acid oxidation, cell death and cancer (Zangar et al, 2004). Results demonstrated that endogenous tyrosine nitration was detected and increased in fish fed 400 ppm PAH mixture after 7 days exposure (Table 1.2). Absence of increased tyrosine nitration at later times suggested recovery from protein nitration. This was consistent with comet assay results (Fig 2.6). ROS modified macromolecules such as proteins leading to protein dysfunction (Zangar et al, 2004).

F2-isoprostanes

We examined lipid peroxidation in head kidney with the F2-isoprostanes technique after 50 days of feeding (fig 2.8). Our results showed that BAP 160 ppm and 400 ppm

PAH mixture were significantly higher than control ($p<0.05$). Teleost kidney has an important role in defense mechanism as lymphoid organ (Zapata et al, 1996). Damage to kidney potentially impaired the immune response of the fish.

Xenobiotic transformation in rainbow trout kidney was previously described (Pesonen et al, 1987). It was possible that PAH parent compounds were accumulated via the gills and gastrointestinal tract by blood and passed through the dorsal aorta to the kidney were they were metabolized to quinones and generated oxidative stress (Petersen et al, 1985). Reactive oxygen species (ROS) generation in kidney and liver likely contributed to the damage observed in kidney and blood. Here the highest dose 400 ppm PAH mixture, yielded the strongest effect. Metabolism of PAH in kidney was through CYP1A that was possibly activated through the AHR. Immunohistochemistry detected no increase in CYP1A after 3, 7, 14 and 28 days of feeding BEP 160 ppm (Fig 2.4 b). This perhaps explained why BEP 160 ppm caused no damage in kidney (Fig 2.8). Characterization of CYP1A in rainbow trout kidney determined that induction of activity was 5-fold lower than that of the liver (Petersen et al, 1985). This low level in BAP and 400 ppm PAH mixture perhaps stimulated metabolism in kidney and contributed to increased F2-isoprostanes. Pesonen et al (1987) found that kidney was less effective than liver in conjugation. UDP-glucuronosyl transferases were 11% of that in liver, this indicated that kidney was more susceptible to the reactive metabolites of PAH and this was potentially detrimental to the fish.

Evidence of oxidative stress

We reported an adaptive response after a 50 day exposure to PAH. The highest response in all biomarkers was obtained after 3-14 days of exposure. After 50 days DNA strand breaks in blood and EROD activity in liver microsomes declined. Immunohistochemistry for CYP1A1 and nitration of tyrosine residues in trunk kidney were consistent with these results after 28 days of PAH exposure. Although these responses were lower at 28 and 50 days than at peaks at earlier times, they were significantly higher than control ($P<0.05$). In the present study, after 50 days of exposure to PAH, the effects of PAH were consistently significantly higher in 400 ppm PAH treatment compared to control.

PAH mechanisms of toxicity were studied previously. Two pathways were proposed: PAH such as BAP were metabolized by CYP1A enzymes and formed reactive epoxides such as *trans*-7,8 diol-9, 10 epoxide, adducted DNA (Mitchelmore et al, 1998). A second mechanism involved a one electron ($1e^-$) oxidation step catalyzed by CYP1A and P450 peroxidase and formed cation radicals that bind to macromolecules such as DNA (Mitchelmore et al, 1998; Warshawsky and Warshawsky, 2005). Recently, Warshawsky and Warshawsky (2005) accepted a third pathway: the ortho-quinone pathway catalyzed by dihydrodiol dehydrogenase. This pathway ultimately formed quinones involved in ROS production.

Based on previous (Au et al, 1999) and present findings a relationship between increased DNA damage due to PAH exposure and formation of toxic metabolites like quinones was suggested. Redox cycling of quinones and ROS generation likely contributed to DNA damage detected in blood. DNA damage in blood was greater in

the 40 ppm PAH mixture compared to BAP or BEP alone. The highest mixture concentration indicated consistently the strongest effect in all biomarkers measured.

Our results indicated that rainbow trout adapted to PAH insult after 14 days of exposure. Control levels in EROD activity and comet assay recovered after 50 days of exposure for all treatments except the highest concentration of the PAH mixture. It was unclear whether recovery from continuous feeding of 400 ppm was possible after longer exposure periods. Reducing CYP1A activity probably contributed to decreased ROS in cells; as previously proposed (Zangar et al, 1994). We observed that CYP1A levels decreased after 14 days and DNA strands breaks in blood and protein nitration in kidney was closer to control levels after 50 days. Another possible explanation for the adaptive response observed in this study was the activation of the antioxidant response element (ARE), a cis-acting enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress (Nguyen et al, 2003). Many of the proteins with expression mediated by ARE participate in the regulation of cellular redox status and cellular protection from oxidative damage such as glutathione-S-peroxidase (Nguyen et al, 2003). Perhaps oxidative stress caused by the PAH metabolites in liver, kidney and possibly blood activated ARE and reduced the oxidative damage through increased levels of phase II enzymes. The activity of phase II enzymes was not measured in this study. Roberts et al. (1987) addressed antioxidant responses to contaminants in free living spot (*Leistomus xanthurus*) from a PAH-polluted site. Higher activities of superoxide dismutase in hepatic S-9 fractions were found in fish from contaminated sites. Ongoing work in this laboratory was

recently initiated aimed at understanding the mechanism for the adaptive response in rainbow trout.

BEP/BAP binding assay

Previous studies (Zelikoff et al, 2004) reported intraperitoneal injection of BEP produced no change in CYP1A protein or EROD activity in hepatic microsomes 48 h after injection in medaka. Our results supported those findings. EROD was not induced after 3 days of feeding 160 ppm BEP. EROD was induced after 14 days of feeding (Fig 2.4a). The role of AHR in CYP1A induction was investigated by a binding assay with PLCH-1 fish hepatoma cells (Hahn et al, 1993). BAP competed with TCDD, a known AHR ligand (Fig 2.9) (Hahn et al, 1998). There was not concentration-dependent competition of BEP for TCDD binding (Fig 2.9). This suggested that mechanisms other than AHR binding were involved in the induction of CYP1A by BEP. It was possible that PAH binding proteins 4S and 8S were involved in CYP1A induction by BEP. Earlier studies suggested that BEP 4S binding protein complex played a role in the regulation of CYP1A. BEP bound exclusively to the 4S receptor in mice but this was not investigated in fish (Sterling et al, 1994).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Frank Loge (UC Davis, CA) for his guidance and assistance with statistical advice, Dr. Margaret Krahn, Gina Ylitalo and Jon Buzitis (NWFS/NOAA) for providing useful information and training for the bile work. Monoclonal antibody (Mab) 1-12-3 against scup CYP1A1 was generously provided by John J. Stegeman (WHOI, MA). We also thank Bruce Woodin (WHOI, MA) for providing information about the use of the antibody. We are grateful with Dr. James

Meador for his assistance with diet preparation and Maryjean Willis for her support with the histology and immunohistochemistry. We thank Dr. Joseph Beckman for providing the nitrotyrosine antibody and Kay Fisher for providing the immunohistochemical analysis for protein nitration. The authors wish to thank Eric Johnson and Greg Gonnerman for care and maintenance of fish. This work was funded by CIMRS-NMFS (NOAA) and OSU- Environmental Toxicology and Chemistry departmental grant ATX 506.

REFERENCES

Aas E, Baussant T, Balk L, Liewenborg B, Anderssen O, 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory exposure with Atlantic cod. Aquatic Toxicology 51: 241-258.

Aas B and Goksoyr A, 2000. Fixed wavelength fluorescent (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filters effect and signal interpretation. Biomarkers 5: 9-23.

Ahokas I. 1984. Metabolic activation of PAH by fish liver CYP450. Marine Environmental research 14: 59-69.

Andersson T. 1992. Purification, characterization and regulation of male-specific cytochrome P450 in the rainbow trout kidney. Marine Environmental Research 34: 109-112.

Anderson D, Yu D, Phillips B and Schmezer, 1994. The effects of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in the COMET assay. Mutation Research 307: 261-271.

Anderson D, Dhawan A, Yu T, Plewa M, 1996. An investigation of bone marrow and testicular cell *in vivo* using the comet assay. Mutation Research 370: 159-174.

Anon. 1981. Effects on the Immune Systems. Environmental Pathology Toxicology, 5(1):222-223.

Arkoosh M, Clemons E, Myers M, Casillas E. 1994. Suppression of B-cell mediated Immunity in Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) After Exposure

either to a Polycyclic Aromatic Hydrocarbon or to Polychlorinated Biphenyls. Immunopharmacology and Immunotoxicology 16(2):293-314.

Arkoosh M, Casillas E, Clemons E, Kagley A, Olson R, Reno P, Stein J. 1998. Effect of Pollution on Fish Diseases: Potential Impacts on Salmonid Populations. Journal of Aquatic Animal Health 10:182-190.

Arkoosh M, Clemons E. Huffman P, Kagley A. 2001. Increased Susceptibility of Juvenile Chinook salmon to Vibriosis after Exposure to Chlorinated and Aromatic Compounds Found in Contaminated Urban Estuaries. Journal of Aquatic Animal Health 13:257-268.

Au D.W.T, R.S.S.W Wu, B.S Zhou, P.K.S Lam, 1999. Relationship between ultra structural changes and EROD activities in liver of fish exposed to benzo(a)pyrene. Environmental Pollution 104: 235-247.

Bailey S, Williams D and Hendricks J, 1996. Fish Models for Environmental carcinogenesis : The rainbow trout. Environmental Health Perspectives Vol 104 Suppl 1: 5-21.

Burns-Naas L, Meade J, Munson A: Toxic Responses of the Immune System in: Curtis D. Klaassen (ed): Cassarett and Douls Toxicology the Basic Science of Poisons. McGraw-Hill 6th edition. 2001. pp 419-460.

Canadian Environmental Protection Act. 1994. Priority Substances List Assessment Report. Polycyclic Aromatic Hydrocarbons. Government of Canada. Canadian cataloguing in Publication Data (ed). pp 10-25

Carlson D, Zelikoff J. 2002a. Exposure to Japanese Medaka (*Oryzias latipes*) to Benzo(a)pyrene Suppresses Immune Function and Host Resistance Against Bacterial Challenge. Aquatic Toxicology, 56:289-301.

Carlson D, Zelikoff J. 2002b. The Japanese Medaka (*Oryzias latipes*) Model: Applicability for Investigating the Immunosuppressive Effects of the Aquatic Pollutant Benzo(a)pyrene (BaP). Marine Environmental Research, 54:565-568

Collier T.K, Stein J, Wallace R and Varanasi U. 1986. Xenobiotic metabolizing enzymes in Spawning English sole (*Parophrys vetulus*) exposed to organic solvent extracts of marine sediments from contaminated and reference areas. Comparative Biochemistry and Physiology C:84:291-298

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

Collier T.K, L.L Johnson, M.S Myers, C.M Stehr, M.M Krahn and J. E Stein. 1998. Fish Injury in the Hylebos Waterway in Commencement Bay, Washington. U.S Department of Commerce, NOAA Technical Memo, NMFS-NWFSC-36, Washington D.C (available at www.nwfsc.noaa.gov/pubsl).

Eggens M and Galgani F. 1992. Ethoxyresorufin-O-deethylase (EROD) Activity in Flatfish: Fast Determination with a Fluorescent Plate-Reader. Marine Environmental Research Vol 3 No3: 213-221

Fairbairn D, Olive P, O'Neil K, 1995. The comet assay: a comparative review. Mutation Research 339: 37-59.

Faisal M, Hugget R. 1993. Effects of Polycyclic Aromatic Hydrocarbons on the Lymphocite Mitogenic Responses in Spot, *Leiostomus xanthurus* . Marine Environmental Research 35:121-124

Gamal A, Abd-Allah,Refaat I, Micah S, Heckman R and O'Neill K. 1999. A comparative Evaluation of Aflatoxin B1 genotoxicity in fish models using the comet assay. Mutation research 446: 181-188

Giovanneli L, Pitozzi V, Riolo S and Dolara P. 2003. Measurement of DNA strand breaks and oxidative damage in polymorphonuclear and mononuclear and mononuclear white blood cells: a novel approach using the comet assay. Mutation Research 538: 71-80.

Grinwis G, Vetaak A, Wester P, Vos J. 2000. Toxicology of Environmental Chemicals in the Flounder (*Platichthys flesus*) with Emphasis on the Immune System: Field, Semi-field (mesocosm) and Laboratory Studies. Toxicology Letters (112-113): 289-301.

Hahn M, Lamb T, Schultz M. Smolowitz R and Stegeman J. 1993. Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in a Ah receptor-containing fish hepatoma cell line (PLHC-1). Aquatic Toxicology 26:185-208

Hahn M. 1998. The aryl hydrocarbon receptor a comparative perspective. Comparative Biochemistry and Physiology Part C 121:23-53.

Hawkins A, Billiard S, Samir P, Brown S and Hodson P. 2002. Altering cytochrome P4501A activity affects PAH metabolism and toxicity in rainbow trout *O.mykiss.* Environmental Toxicology and Chemistry Vol 21 No 9:1845-1853.

Hayashi H, Veda T, Uyeno K, Wada K, Kinae N, Saotore K, Tanaka N, Takai A, Fsusaki Y, Asaro N, Sofuni T and Ojima Y. 1998. Development of genotoxicity assay systems that use aquatic organisms. Mutation Research. 399: 125-133.

Hellou J. 1996. Polycyclic aromatic hydrocarbons in marine mammals, finfish and mollusk in: Beyer N, Heinz G and Norwood A Eds. Environmental contaminants in wildlife, interpreting tissue concentrations. Lewis Publishers.

Hendricks J, Meyers T, Shelton D, Casteel J and Bailey G. 1985. Hepatocarcinigenicity of BAP to rainbow trout by dietary exposure and intraperitoneal injection. Journal of the National Cancer Institute.74: 839-851.

Hyyti O, Nyman M, Willis M.L, Raunio H, Pelkoen O, 2001. Distribution of cytochrome P450 (CYP1A) in the tissues of Baltic ringed and gray seals. Marine Environmental Research 51: 465-485.

Jacobson K, Arkoosh M, Kagley A, Clemons E, Collier T, Casillas E. 2003. Cumulative Effects of Natural and Anthropogenic Stress on Immune Function and Stress Resistance in Juvenile Chinook Salmon. Journal of Aquatic Animal Health, 15:1-12.

Kennedy C, Gill K and Walsh P. 1991. In vitro metabolism of benzo(a)pyrene in the blood of the gulf toad fish *Opsanus beta.* Marine Environmental Research 31:37-53.

Krahn M, Myers M, Burrows D and Mallins D, 1984. Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. Xenobiotica Vol 14 No8: 633-646.

Krahn M, Moore L, and MacLeod J. 1986. Standard analytical procedures of the NOAA National Analytical Facility, 1986. Metabolites of Aromatic Compounds in Fish Bile. NOAA Tech Memo. NMFS-F/NWC-102

Krahn M, Ylitalo G, Buzitis J, Chan S and Varanasi U. 1993. Rapid high-performance liquid chromatography methods that screen for aromatic compounds in environmental samples. Journal of chromatography 642:15-32.

Kirso L, Voll M, Irha N, Urbas E. 2001. Distribution of the Persistent Organic Pollutants, Polycyclic Aromatic Hydrocarbons, Between Water, Sediments and Biota. Aquatic Ecosystem Health and Management 4:151-163

Lewis J, Hamilton T and Adams O. 1986. The effect of macrophage development on the release of reactive oxygen intermediates and lipid peroxidation products and their ability to induce oxidative DNA damage in mammalian cells. Carcinogenesis 7: 813-818.

Lowry O, Rosenbrough N, Farr A and Randall R. 1951. Protein Measurements with the Folin phenol reagent. Journal of Biological Chemistry 193:265-275

Luna C. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Third Edition. Pp 12-47. McGraw Hill.

Mitchelmore C and Chipman J. 1998 (a). Detection of DNA strand breaks in brown trout (*salmo trutta*) hepatocytes and blood cells using the single cell electrophoresis (comet) assay. Aquatic Toxicology 41: 161-182.

Mitchelmore C and Chipman J. 1998 (b). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Research 399: 135-147.

Mitchelmore C, Birmelin C, Chipman K and Livingstone D, 1998. Evidence for cytochrome P-450 catalysis and the free radical involvement in the production of DNA strand breaks by benzo(a)pyrene and nitroaromatics in mussel (*Mytilus edulis L.*) digestive gland cells. Aquatic Toxicology 41: 193-212.

Morrow JD, Awad JA, Kato T, Takahashi K, Badr KF, Roberts LJ 2nd, Burk R. 1992. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. Journal of Clinical Investigation 90 (6) 2502-7.

Myers M, Willis M, Mette A, Anders G and Collier T, 1996. Immunohistochemical localization of CYP1A in multiple types of contaminant- associated hepatic lesions in English sole (*Pleuronectes vetulus*). Marine Environmental Research 39: 283-288.

Neff J. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment sources fates and biological effects. Applied sciences publishers Ltd London. Pp 7-42.
Osborne M, Crosby N. 1987. Benzopyrenenes. Cambridge University Press, London. Pp 28 and 234.

Nguyen T, Sherratt P and Pickett C. 2003. Regulatory Mechanisms Controlling Gene Expression Mediated by the Antioxidant Response Element. Annual Review Pharmacology and Toxicology 43: 233-260.

Pesonen M, Celander M, Forlin L and Andersson T, 1987. Comparison of xenobiotic biotransformation enzymes in kidney and liver of rainbow trout (*Salmo gairdneri*) Toxicology and Applied Pharmacology 91: 75-84

Petersen D, Keinow K, Kraska C and Lench J, 1985. Uptake, disposition and elimination of acrylamine in rainbow trout. Toxicology Applied Pharmacology. 80: 58-65.

Reddy M and Randerath K. 1987. ^{32}P-analysis of DNA adducts in somatic and reproductive tissues of rats treated with the anticancer antibiotic, mitomycin C. Mutation Research 179:75-88.

Reichert W and French B. 1994. The ^{32}P-Postlabeling Protocols for Assaying Levels of Hydrophobic DNA Adducts in Fish. NOAA Technical Memorandum NMFS-NWFSC-14.

Research Triangle Institute. 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons. U.S Department of Health and Human Services. pp 1-10

Roberts M.H, D.W Sved and S.P Felton. 1987. Temporal changes in AHH and SOD activities in feral spot from the Elizabeth River, a polluted sub-estuary. Marine Environmental Research 23:89-101.

Sandvik M, Horsberg T, Skaale U and Ingebringtsten K.1998. Comparison of dietary and waterborne exposure to benzo(a)pyrene availability, tissue disposition and CYP1A induction in rainbow trout *Oncorhynchus mykiss* Biomarkers Vol 3 No 6: 399-410.

Silkworth J, Lipinskas T, Stoner C. 1995. Immunosuppressive Potential of Several Polycyclic Aromatic Hydrocarbons (PAHs) Found at a Superfund Site: New Model Used to Evaluate Additive Interactions Between Benzo(a)pyrene and TCDD. Toxicology 105:375-386.

Shugart L,1998. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Toxicology 13:43-52

Sokal R and Rohlf J. 1995. Biometry. Third edition. Freeman and Company ed. Pp 423.

Singh N, Danner D, Tia R, Brant L and Schneider E, 1990. DNA damage and repair with age in individual human lymphocytes. Mutation Research 237: 123-130.

Sloan C.A, D.M Brown, Pearce R.W, Boyer R.H, Bolton J.L, Burrows D.G, Herman D.P. and Khran M.M 2004. Extraction, cleanup and gas chromatography/mass spectrometry analysis of sediments and tissues for organic contaminants. U.S Department of Commerce NOAA Tech Memo. NMFS-NWFSC-59. P47.

Stegeman J, Smolowithz R and Hahn M. 1991. Immunohistochemical localization of environmentally induced CYP1A in multiple organs of the marine teleost *Stenotomus chrysops* (scup). Toxicology and applied pharmacology 110:1-7.

Stegeman J and Hahn M, 1994. Biochemistry and molecular biology of monooxygenases current perspectives on the forms, functions and regulation of cytochrome P450 in aquatic species in: D.C Malins and G.K Ostrander, Aquatic Toxicology Molecular Biochemical and Cellular Perspectives Pp 87-206. Boca Raton: Lewis Publishers.

Stein E.J, Hom T, Coller K. T, Brown D and Varanasi U, 1995. Contaminant exposure and biochemical effects of out-migrant Chinook salmon from urban and non-urban estuaries of Puget Sound WA. Environmental Toxicology and Chemistry Vol 14 No 6: 1019-1029.

Sterling K, Raha A, Bresnick E. 1994. Induction of CYP1A gene expression in mouse hepatoma cells by benzo(e)pyrene, a ligand of the 4s polycyclic hydrocarbon biding protein. Toxicology and Applied pharmacology Vol 12B No1 Pp 18-24.

Varanasi U, Nishimoto M, Reichert W and Eberhart B. 1986. Comparative metabolism of benzo(a)pyrene and covalent binding to hepatic DNA to English sole, starry flounder and rat. Cancer research 46: 3817-3824.

Varanasi U, Nishimoto M, Baird W and Smolarek T. 1989. Metabolic activation of PAH subcellular fractions and cell cultures from aquatic and terrestrial species. In: Varanasi U Editor. Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC press Boca Raton FL. Pp 203-245.

Varanasi U, Casillas E, Arkoosh M, Hom T, Misitiano D, Brown D, Chan S, Collier T, McCain B and Stein J. 1993. Contaminant Exposure and Associated Biological Effects in Juvenile Chinook Salmon (Oncorhynchus tshawytscha) from Urban and Nourban Estuaries of Puget Soud. NOAA Technical Memorandum NMFS-NWFSC-8 pp5-25.

Weeks B, Warinner J, Manson P, McGinnis D. 1986. Influence of Toxic Chemicals on the Chemotactic Response of Fish Macrophages. Journal of Fish Biology, 28:653-658.

Weiling X and Warshawsky D. 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicology and Applied Pharmacology 206: 73-93.

Willet K, Steinberg M, Thomsen J, Narasimhan T, Safe S, McDonald S, Beatty K and Kennicutt M, 1995. Exposure of killing fish to benzo(a)pyrene: comparative metabolism, DNA adduct formation and aryl hydrocarbon (Ah) receptor agonist activities. Comparative Biochemistry and Physiology Vol 112B N° 1: 93-103.

Zapata A, Chiba A and Varas A. 1996. Cells and Tissues of the Immune system of fish in: Iwana G, Nakanishi T (eds). The fish Immune system: Organism, pathogen and environment. Academic press Inc. Pp 1-62.

Zangar R, Davydov R and Seema V.2004. Mechanism that regulate production of reactive oxygen species by cytochrome P450. Toxicology and Applied Pharmacology 199:316-331.

Zelikoff J and Carlson E. 2004. Benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*): relationship between lymphoid CYP1A activity and humoral immune suppression. Toxicology and Applied Pharmacology 201: 40-52.

Chapter III

Increased susceptibility of rainbow trout *Oncorhynchus mykiss* to *Aeromonas salmonicida* after exposure to PAH in the diet

Claudia F. Bravo[1], Mary Arkoosh[2], Lawrence R. Curtis[1], James Meador[3], Frank Loge[4] and Tracy K. Collier[3]

1. *Department of Environmental and Molecular Toxicology, Oregon State University, ALS 1007, Corvallis OR 97331 USA.*
2. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2030 South Marine Science Drive, Newport OR 97365, USA*
3. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Boulevard East, Seattle, Washington, 98112, USA*
4. *Department of Civil and Environmental Engineering, University of California Davis, 1 Shields Avenue, Davis, CA 95616, USA.*

To be submitted to *Journal of Aquatic Animal Health*

ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss)* exposed to polycyclic aromatic hydrocarbons (PAH) in the diet at environmentally relevant concentrations under controlled laboratory conditions exhibited higher susceptibility to *A. salmonicida* than fish exposed only to control diet. Disease susceptibility was assessed by examining the percent cumulative mortality of the trout after exposure to the common fresh water pathogen *Aeromonas salmonicida.* Polycyclic aromatic hydrocarbons were used in the same concentrations as those previously found in chinook salmon (*Oncorhynchus tshawytsha*) stomach contents from contaminated estuaries in the Pacific Northwest. A mixture containing ten high molecular weight PAH was used at 40 ppm and 400 ppm. Additionally, a group of fish were fed either benzo(a)pyrene (BAP) or benzo(e)pyrene (BEP) at 160 ppm. The lethal concentrations (LC) of bacteria selected for the exposure were LC_{20} and LC_{30}. The peak of mortalities for all treatments occurred at 4 to day 7 post-challenge. Mortalities in rainbow trout after exposure to 40 and 400 ppm of PAH mixture and a LC_{20} of *A.salmonicida* was 30% for both concentrations of PAH compared with the 18% observed in the control (exposed to bacteria but not to PAH). Twenty percent cumulative mortality occurred in fish fed with BEP and a total of 18% mortality was observed in BAP treated animals. At LC_{30}, 38% cumulative mortality occurred in fish fed with 400 ppm PAH mixture and 160 ppm BEP compared to 28% in control, 32% and 24% cumulative mortality occurred in animals fed with 40 ppm PAH mixture and 24% for 160 ppm BAP respectively. There were significant differences in mortality between control fish and those treated

with 40 and 400 ppm PAH mixture after challenges with the bacterial LC_{20}. The fish treated with the 400 ppm PAH mixture and 160 ppm BEP experience significantly higher mortality than controls after the LC_{30} bacterial challenge. These findings demonstrated that feeding PAH to rainbow trout at environmentally relevant concentrations increased susceptibility to disease.

Keywords: PAH, disease susceptibility, *Aeromonas salmonicida*, rainbow trout.

INTRODUCTION

One of the most important points in salmon life cycle is the time spent in inshore estuaries, where they physiologically adapt for transition from fresh water to sea water (Thorpe. 1994), feed and grow before migrating to sea. Human impacts upon estuaries include drainage, stream channeling and obstruction of fish movements by dams (Crisp, 2000). Changes in water temperature due to hydropower complexes on rivers and streams, and contamination appear to contribute to salmon decline (Nehlsen et al, 1991).

Estuaries are usually heavily urbanized areas and receive chemical contaminants via direct pipeline discharges from coastal communities and from ships, rivers and atmospheric deposition (Johnson et al, 1998). Polycyclic aromatic hydrocarbons (PAH) are worldwide contaminants that are most heavily concentrated in urbanized coastal areas (Latimer and Zheng, 2003; Meador, 2003)). PAH are considered the major pollutants of the estuarine and coastal ecosystems (Meador et al, 1995). Municipal wastewater facilities and industrial outfalls are the major point sources of PAH for most estuaries (Latimer and Zheng, 2003). Factors that affect the health of

juvenile fish during this critical period may have subsequent negative effects during this stage or later in their marine life (Arkoosh et al, 2001).

Previous laboratory studies demonstrated suppression in salmon collected from a contaminated estuary in the Pacific Northwest. Field monitoring showed suppressed immunological memory (Arkoosh et al, 1991). Different groups of chemicals were present: Polychlorinated Biphenyls (PCBs), dichloro-diphenyl-trichloro*ethane* (DDT) and PAH. Therefore it was difficult to link a particular chemical group with the effect observed. To determine the effects of PAH on immune response, Arkoosh et al, (1994) injected intraperitoneally (i.p) juvenile chinook salmon with either 7,12-dimethylbenz[a]anthracene (DMBA), a PAH, or with a commercial PCB, Aroclor 1254 and assessed the effects on B-cell mediated immune response. This study showed a causal relationship between impaired secondary plaque forming cell on B-cell response (PFC) response and chemical exposure. These studies demonstrated that juvenile chinook salmon from contaminated estuaries in the Puget Sound WA were immunosuppressed. Fish in these conditions were potentially more susceptible to disease and ultimately mortality. Differences in susceptibility to a marine pathogen in out-migrant chinook salmon from an urban estuary in Puget Sound, WA were assessed by Arkoosh et al, (1998). This two year study confirmed that salmon exposed to chemical contaminants were immunosuppressed. This condition was associated with increased disease susceptibility and mortality.

In summary these studies showed that chinook salmon were more susceptible to disease after exposure to contaminants such as PAH and PCB found in the estuaries in the Pacific Northwest. The mechanism of immunosupressant action by these

chemicals remained unknown and the action on the immune system of each specific group of chemical individually required study.

Although these studies established a causal relationship between exposure to chemicals and increased disease susceptibility, chinook salmon in the field bioaccumulated significant concentrations of chemical contaminants during their relative short residence time in the estuary primarily from the diet. Therefore it was important to determine disease susceptibility in fish fed environmentally relevant PAH concentrations for a period that mimicked the residence time in the estuary.

Various fish species are considered models for environmental effects of chemicals Japanese Medaka (*Oryzias* latipes), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss)* are the common examples. Rainbow trout belong to the family salmonidae and the genus *Oncorhynchus*. Rainbow trout life history shares characteristics with pacific salmon especially in the early stages where both salmonids reside in streams. Chinook salmon move downstream to estuaries and their residence time varies from a few days up to six months before migrating to sea (Healey, 2003). Rainbow trout are widely distributed and has a native range from northwest Mexico to Alaska (Quinn, 2005). Attention to this species as a model for carcinogen compound exposure was drawn in the early 1960s (Bailey et al, 1996). Since then, rainbow trout has been used in the laboratory specifically in carcinogenesis research. Its metabolic response to a wide range of PAH is well known (Hendricks et al 1985; Bailey et al, 1996). Xenobiotic biotransformation enzymes important for the metabolism of foreign compounds have been characterized in rainbow trout kidney and liver (Pesonen et al, 1987). Rainbow trout are the model organism in the present study,

because they are readily available, easy to handle and they are representative of salmonid species.

The aim of the present study was to determine host resistance of rainbow trout chronically exposed to PAH in the diet at environmentally relevant concentrations under controlled laboratory conditions after challenge with *A. salmonicida,* a common fresh water pathogen and the causative agent of furunculosis. This study confirmed that fish exposed to PAH in the diet were more susceptible to disease.

MATERIALS AND METHODS

Experimental time-line

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Sinnhuber Aquatic Research Laboratory Oregon State University, Corvallis OR. Fish were transported at 7 g with oxygen to the Hatfield Marine Science Center, Newport OR using a live transport insulated container (DX333 Aquatic Ecosystems, Apopka, FL). Oxygen levels were kept at 9.7 mg/L during the 1 h trip to reduce stress. In the laboratory, 4000 fish were placed in duplicate 2000L fiber glass circular tanks in fresh water at 10°C for about a month; water temperature, chlorine and pH were checked daily with a thermometer (Aquatic Ecosystems, Apopka, FL) chlorine test strips (Corning®, waterworks™,St Louis, MO) and a pH-meter, respectively. During this period, fish were fed 2% of their body weight (dry weight basis) with a low fat diet Trout No.4 (Rangen Inc, Angleton, TX), with the following characteristics: 11% crude fat content, 45% crude protein, 9-10% water, fish meal protein 30%, phosphorus 1%, 1.5 mm pellet.

A month later, ten fish were transferred to 12 gallon buckets equipped with battery powered air stones and randomly located into circular fiber glass 400 L tanks. Fish were fed 2% body weight (six times a week) with PAH-treated diets or control for a total of 50 days (feeding time). Diets were prepared using methylene chloride as solvent as described in chapter II: an environmentally relevant model mixture of 10 high molecular weigh PAH was used at 40 ppm and 400 ppm the same proportions described in Arkoosh et al (1998). Two additional treatments, benzo(e)pyrene (BEP) or benzo(a)pyrene (BAP) at 160 ppm were employed. BAP was demonstrated as immunosuppressive in fish and was therefore selected as a positive control (Carlson and Zelikoff, 2002). BEP was selected as negative control because it appeared non-immunosuppressive in fish (Carlson and Zelikoff, 2002)

A week before the last day of feeding, water level was lowered to 50 L in all tanks. After 50 days, feeding was stopped and fish were bath-exposed to *A. salmonicida* at LC_{20}, LC_{30}. The control group for the disease challenge consisted of duplicate tanks with 75 fish per tank that were previously fed 160 ppm BAP or BEP, high molecular weight PAH mixture at 40 or 400 ppm or vehicle control diet. The fish in these tanks were not exposed to bacterium but to 25 ml of sterile trypticase soy broth (TSB) for 24 h. The experimental treatments consisted of same number of tanks as control (a total of 10) but were exposed to LC_{20} of *A. salmonicida* ($10^{5.5}$cfu/ml= 15.8ml from stock) and LC_{30} of *A. salmonicida* ($10^{5.7}$ cfu/ml=25 ml of broth from stock). For the present manuscript control referred to fish that received no bacteria during the challenge and experimental treatments were fish that received bacteria after feeding with PAH at the different concentrations.

Chemistry

Food analysis

The food prepared with either: 160 ppm BEP, 160 ppm BAP, 40 ppm PAH mixture, 400 ppm PAH mixture or vehicle control was analyzed by gas chromatography/ mass spectrometry (GC/MS). The process involved three steps: 1) extraction with high purity methanol and acetic acid (Budick and Jackson. Muskegon MI) 2) clean-up with silica/alumina columns and size exclusion high performance liquid chromatography and 3) quantization of aromatic hydrocarbons by GC/MS as described in Sloan et al. (2004). These analytes were quantitated and reported as the sum of their concentrations because they co-eluted during GC/MS analysis (Sloan et al, 2004).

PAH metabolites in the bile

A total of 60 individual bile samples were collected (12 fish per treatment). Bile was analyzed for PAH metabolites three days before challenge and 21 days post-challenge by high performance liquid chromatography (HPLC) as described by Krahn et al (1984, 1986 and 1993). Briefly, bile was injected directly onto a C_{18} reverse phase column (Phenomenex® synergi Hydro, Torrance, CA) and eluted with a linear gradient from 100% water (with a trace amount of acetic acid) to 100% methanol at a flow of 1.0 ml using an automatic auto sampler (Millipore-Waters, Wisp model 710A). Chromatograms were recorded at the following wavelengths pairs: (1). 260/380 nm (3-4 ring compounds fluoresce) and (2). 380/430 nm 4-5 ring compounds fluoresce. Peaks eluting after 5 min were integrated and the areas of the peaks summed. The concentrations of fluorescent PAH in bile were determined using phenanthrene (PHN) and (BAP) as external standards and converting the fluorescence

response of bile to phenanthrene (ng PHN equivalents/ g bile), and BAP (ng BAP equivalents/ bile) equivalents. Bile metabolites fluorescing at phenanthrene wavelengths indicated exposure to lower molecular weight PAH (3-4 rings), while metabolites fluorescing at BAP (4-6 rings) wavelengths indicated exposure to higher molecular weight PAH. Enough bile >20 μL was collected from all fish in all treatments to perform the assay. Bile protein was measured as described in Lowry (1951). Bile was reported as ng/g protein.

Fish health

Thirty fish sacrificed prior to *A. salmonicida* challenge were analyzed for overall health status. Gill, liver, kidney and spleen smears were observed under the microscope and cultured on trypticase soy agar (TSA) (Sigma-Aldrich). After incubation at 37 °C or 25 °C for 24 h, colony growth was examined. No bacteria were isolated from the fish. No antibiotics or any other treatment was given to the fish used in this experiment. Mortalities during acclimatization period/ feeding period were <1%.

Water quality

During the disease challenge, fresh water was kept at a temperature of 11.9°C±0.13°C, pH 7.0±0.05 (average±SD n=21) in a flow through system. Oxygen content was kept above a minimum of 8 mg/L. Water quality was monitored daily with an pH meter (Toledo MP 125), ammonia levels were checked with ammonia test (AquaChek Hach ® Elkhart, IN) for chlorine measurements total chlorine and free chlorine test strips were used (Aquatic Ecosystems, Apopka, FL) as described in Nordmo et al (1998).

Aeromonas salmonicida

A culture of *A. salmonicida* isolated from a natural furunculosis outbreak at a Rock Creek Hatchery in Oregon was generously donated by Dr. Richard Holt from the Department of Microbiology at Oregon State University. An inoculum of the bacterium was grown in trypticase soy broth (TSB) (Sigma-Aldrich) at 20◦C at constant agitation on a digital oscillator (Thermoline Scientific, Smithfield, Australia) at speed setting 130. To avoid loss of pathogen virulence, *A. salmonicida* was grown in TSB until the early stationary phase (optical density =1) 20% vol/vol of sterile glycerol was added (Bricknell, 1995). Aliquots of 2 ml were stored in Eppendorf vials and frozen at -80◦C for future use. The bacterium was reconstituted by adding 2 ml of the thawed preparation to 4 L of TSB freshly prepared. The purity of the culture was determined by Gram stain, cell morphology and agglutination test. The agglutination test was performed with BioNor mono-aqua test kit (BioNor Skien, Norway) specific for *A. salmonicida.* A culture from previous pure culture passage was used as positive control. To further test for purity, an aliquot was cultured on 2 TSA plates; one was incubated at 20◦C and the other at 37◦C. *A. salmonicida* is a non-motile, gram negative rod which produces a brown water soluble pigment on trypticase soy agar (TSA), does not grow at 37°C and produces catalase and oxidase (Austin and Adams, 1996). After 48 h, colonies from the 20◦C incubated plate were inoculated onto comassie blue agar as a differential medium for *A. salmonicida* (Markward et al, 1989).

To determine the rate of growth of the bacterium, aliquots of 1 ml were taken at 2 h intervals and optical density was measured at 540 nm in a single cell module

spectrophotometer (Life Science UV/Vis Beckman DU® 530), optical densities were measured at 540 nm until the period near to the end of the exponential growth phase. Additionally 1 ml was taken and several dilutions 10^{-1}-10^{-7}in TSB broth were made, 0.1ml of the dilution 10^{-4} to 10^{-7} was plated on triplicate TSA for bacterial count. At 24 h colonies were visible. The colonies in the plates were counted after a 48 h incubation period and the bacteria concentration was expressed as CFU/ml.

Lethal concentration curve with A. salmonicida

A lethal concentration curve for juvenile rainbow trout was determined with *A. salmonicida*. Bacterial concentration was measured at 540 nm until an optical density of 1 was achieved during the period near the end of the exponential growth phase as described in Arkoosh et al, (1998).

Pilot disease challenges with *A. salmonicida* were performed with healthy rainbow trout from the same stock as the experimental fish. A total of 1000 fish were challenged in different pilot tests that determined the optimal bacterial concentrations and conditions that generated acute furunculosis. The same number of fish (75 per tank) in duplicate tanks per dose was used during pilot challenge and mimicked those for the actual experiment. A total of 5 disease challenges of 7-14 days duration order determined the concentrations for 20, 30, 50, 80 and 100 percent mortality and optimized the conditions for acute mortality. This information provided a baseline mortality for healthy fish, that determined the challenge concentrations for fish fed with the treated diets. Pilot challenges were performed every two weeks in separate tanks while feeding with the experimental diet occurred.

For the first challenge five log 10 dilutions (10^{-1}-10^{-5}) and zero bacteria culture per ml fresh water of the stock growth culture of *A. salmonicida* determined the lethal concentration response curve. The fish were bath exposed to *A. salmonicida* for 12 h in the tanks under static conditions after which fish were monitored morning and afternoon to collect mortalities for 7 days. During this time the fish were not fed. Five fish were sampled per tank and kidney samples were stroked and plated on TSA after 24 h-48 h incubation at 20°C. Fifty percent of the fish were positive for *A. salmonicida* but no physical evidence of infection was observed. This indicated that fish were exposed and infected but the conditions were not optimal for an outbreak of acute furunculosis.

For the second challenge the volume of the tanks was reduced from 400 L to 200 L to increase packing density and promote an acute mortality. The fish were bathed with the same concentrations and for the same time-period (12 h) as in the first pilot. No mortalities were observed on the first 4 days after challenge. However, 6 days post-challenge physical effects of the disease such as bloody fins and darkening of the skin were evident in at least 2% of the fish.

For the third challenge, we tested if the time of exposure to the bacterium was a major factor to generate an acute outbreak with a volume of 100 L. Eight tanks with 75 fish each was used. Duplicate tanks were exposed to 10^{6} CFU/ml water of *A. salmonicida* (previously reported as LC_{50} for rainbow trout) for 12 h, 18 h and 24 h under static conditions. The control was exposed to sterile TSB. Fish exposed to the bacterium for 12 h and 18 h started dying after 8 days post-challenge. Fish exposed for 24 h started

dying after 5 days. Accordingly the time of exposure was an important factor in acute furunculosis.

Given the results of the third pilot, the fish were exposed for 24 h to pathogen; the water level was lowered to 50 L. Additionally for this experiment we decreased the water flow of the tanks after challenge from 1 gpm (20 water changes per day) to 0.15 gpm (3 water changes per day). A total of 8 tanks with 75 fish each were used with the following bacteria concentrations: $10^{5.5}$ $10^{5.7}$ and 10^{6} or 0 CFU/ml fresh water in tanks. We observed mortalities after 3 days post-challenge. Positive isolation and identification of *A. salmonicida* was obtained from all mortalities collected. After 14 days 20%, 30% and 50% and 0% mortalities were obtained.

The fifth and final challenge, a lethal concentration response curve was obtained under the optimized conditions determined above. The water level of the tanks was 50 L and the water flow set to 0.15 gpm one week before challenge to allow the fish to acclimatize to the new conditions. No mortalities occurred during this time in any of the tanks. We grew *A. salmonicida* in TSB for 24 h to an optical density of 1 and then added the following volumes of the pathogen to the tanks: $10^{5.5}$=15.8 ml, $10^{5.7}$=25 ml, 10^{6}=50 ml, $10^{6.5}$=158 ml or $10^{6.7}$ 250 ml. The control tank received 250 ml of sterile TSB. The experiment was terminated after 14 days.

Disease challenge of PAH exposed fish

A total of 36 tanks with 75 fish each were used. The LC_{20} and LC_{30} of *A. salmonicida* were selected for disease challenge on PAH-treated fish. These concentrations produced different levels of lethality. Water level and flow was lowered from 400 L to 50 L and 1 gpm-0.15 gpm respectively seven days before the challenge and kept at

this level throughout the exposure. Feeding was interrupted three days before challenge and food was not provided during the remaining of the experiment. The day before the challenge a 2 ml aliquot of *A. salmonicida* was reconstituted in 4 L of TSB and incubated under constant shaking at 20°C for 24 h.

On the day of the challenge the water supply was turned off. Aeration was kept constant. The pathogen was added at an OD of 1.024 (the final concentration of bacterium was 1 X $10^{5.5}$ LC_{20} (15.8ml) and 1 X $10^{5.7}$ cfu/ml of tank water LC_{30} (25ml)). Control tanks received 25ml of sterile TSB. Fish were exposed under static conditions for 24 h with constant aeration. No mortalities occurred during this time. After challenge, flow was resumed at 0.15 gpm. The first mortalities occurred on day 2 after exposure. Fish were monitored twice a day for 21 days. Mortalities were collected daily; kidneys were struck and cultured to test for *A. salmonicida.* Additionally, dead fish were counted and examined for external conditions characteristic of infection (darker skin and bloody fins). Kidney was struck on trypticase soy agar (TSA) and incubated at 20°C for 48 h. When growth was present, a colony was cultured on comassie blue agar (Markwardt et al, 1989) which confirmed the pathogen presence. A colony was also sampled and analyzed for a positive reaction with an agglutination test BioNor mono-aqua (BioNor Skien, Norway) specific for *A. salmonicida.*

The experiment was terminated 21 days post-challenge. Three days had passed with no mortalities in any of the treatments. Survivors were sampled and bile was collected from 3 fish/ tank to determine PAH metabolites during this period. The remaining fish

were sacrificed with an overdose of MS-222 (Sigma-Aldrich) and counted before disposal.

Statistical analysis

The cumulative incidence of mortality and associated error bars were estimated using Number Cruncher Statistical Software (Kaysville, Utah) based on a non-parametric approach outlined in Marubini and Valsecchi (1995) at an alpha value of 0.05 with appropriate background mortalities (control fish not exposed to *A. salmonicida*) subtracted prior to analyses. Statistical differences in the incidence in mortality between animals exposed to PAH and pathogen or no PAH and pathogen were assessed on day 21 using a two sided t-test.

RESULTS

Fish health inspection

Skin scrape, gill biopsy, fin clip, liver, spleen and kidney smear preparations microscopically observed showed no signs of disease in the experimental fish before challenge. No bacteria growth was present after 48 h at 25°C and 37 °C in TSA in any of the tissues sampled.

Water quality

Water quality for all challenge tests was at 11°C, pH 7.1, supplied at a rate of 0.15 gpm in a full flow through system. Oxygen was kept at a minimum 9 mg/L. All effluent water was treated with UV to break down residual PAH and to kill the bacterium.

Chemistry

Food chemistry

We tested the effects of ten high molecular weight PAH compounds containing 4-6 rings (chapter II) on chronic dietary exposure in juvenile rainbow trout (see chapter II for preparation of the diet). We measured the PAH concentrations in the treated diets and verified concentrations were close to the nominal concentrations: 160 ppm BAP and BEP, 40 ppm PAH mixture, 400 ppm PAH mixture and control. There was no statistical difference between nominal and actual concentrations ($p>0.05$). The vehicle control diet was treated with methylene chloride and tested negative for PAH.

Bile chemistry

Equivalents of PAH metabolites in bile were calculated as ng/g. BEP metabolites fluoresced at different wavelengths than BAP (chapter II). Emission maxima for BEP were 330 nm and 390 nm (Osborne and Crosby, 1987), emission maxima for BAP were 406 nm and 386 nm. The levels of PAH in the bile measured three days before challenge decreased after 21 days post-challenge. After 21 days, BAP and BEP equivalents in bile were significantly lower compared to the levels three days before challenge ($p<0.05$), similar results were obtained for BEP phenanthrene wavelength pairs (Fig 3.1). For the mixture low and high doses the differences were not significant ($p>0.05$) (Fig 3.1). Metabolite concentration in the bile decreased or was remained constant after 21 days post-challenge in all treatments and were significantly higher than control ($p<0.05$) throughout the challenge.

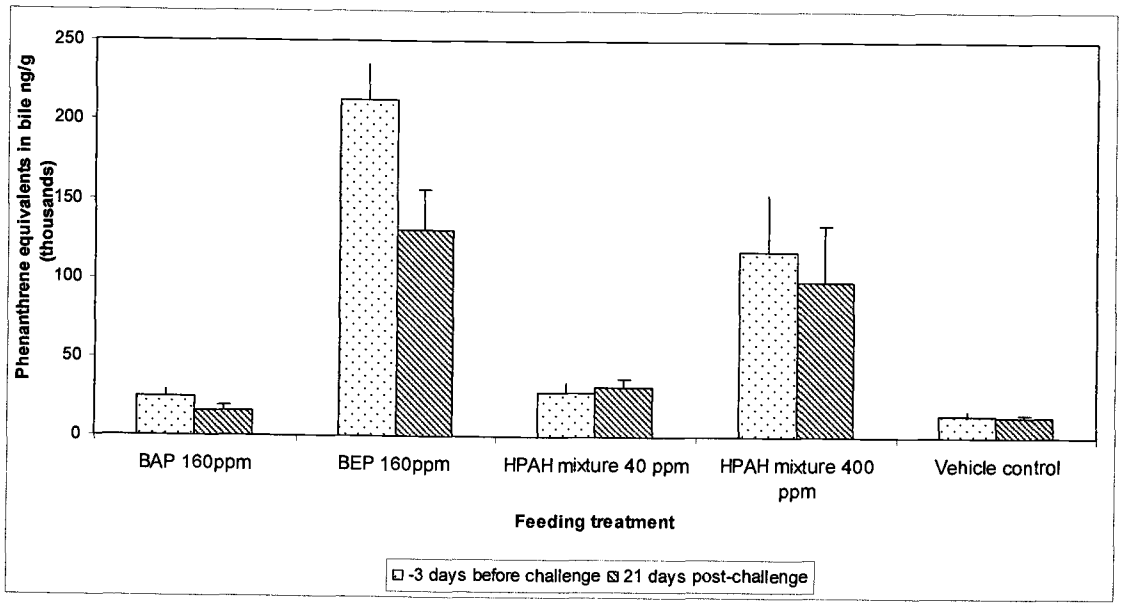
A. salmonicida growth curve

The peak of exponential of *A. salmonicida* growth phase grown in 4L TSB constantly agitated at 130 at 20°C was reached after approximately 24 h of incubation. Plates were counted and we found that at the end of the exponential phase (O.D= 1) the concentration of bacteria was 1.2 X 10^9 cfu/ml (Fig 2.2). Pilot and final challenge experiments with *A. salmonicida* cultures were grown to this O.D.

Lethal concentration curve of *A. salmonicida*

After five pilot disease challenges we found that the optimal conditions to produce acute furunculosis in juvenile rainbow trout held at 11◦C and at 15-20 g weight were as follows: water level of the tanks were lowered to 50 L with 75 fish per tank, water flow of 0.15 gpm and constant aeration provided throughout the experiment. Pathogen exposure was under static conditions for 24 h.

(a)



(b)

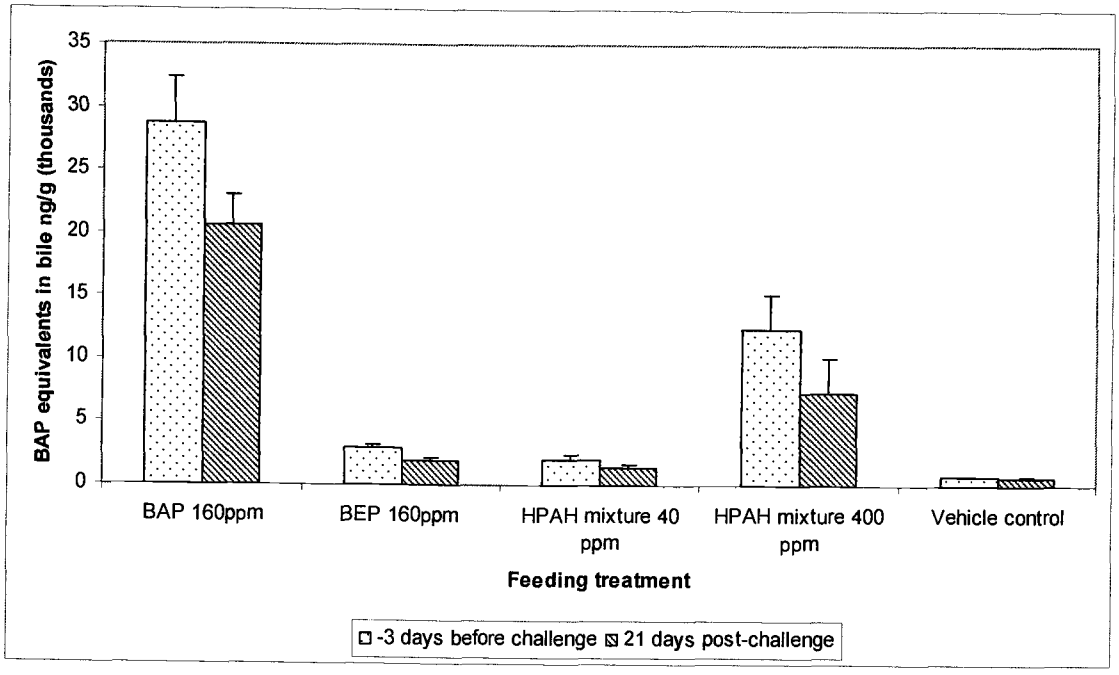


Figure 3.1 (a) Phenanthrene equivalents in bile from rainbow trout exposed to 160 ppm of benzo(e)pyrene and benzo(a)pyrene, 40 and 400 ppm of a PAH mixture for 50 days in the diet (b) BAP equivalents in bile from rainbow trout exposed to 160 ppm of benzo(e)pyrene and benzo(a)pyrene, 40 and 400 ppm of PAH mixture after 50 days of feeding (-3 days before challenge) and 21 days post-challenge.

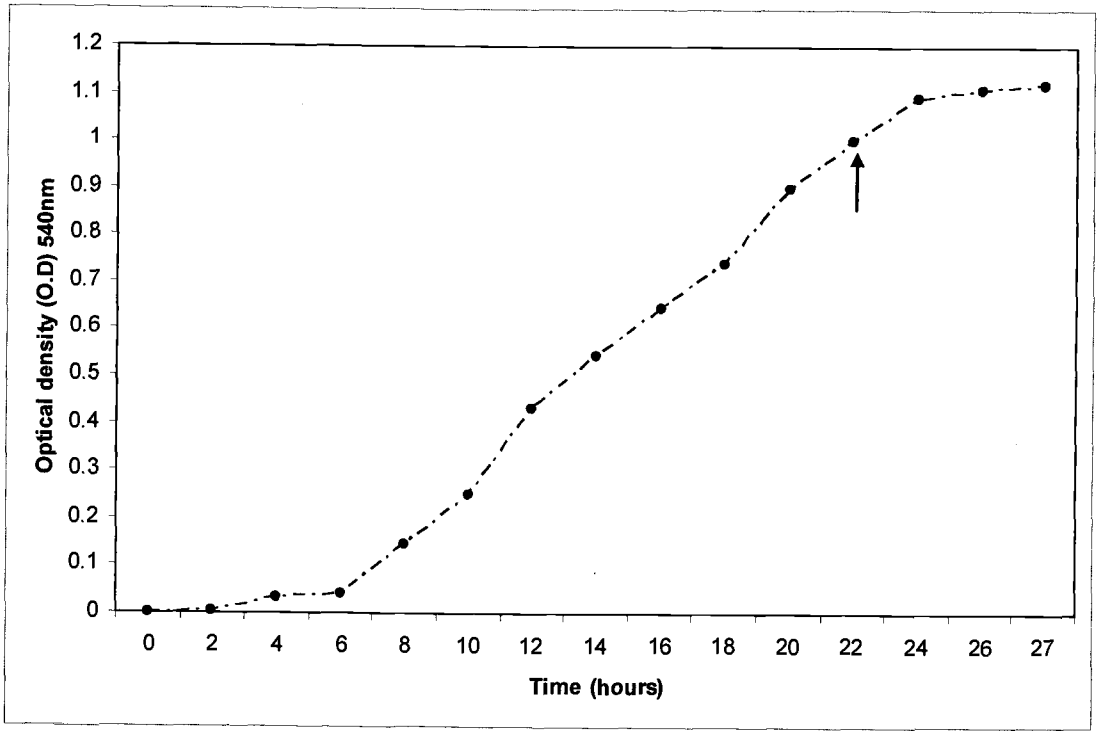


Figure 3.2 Growth curve of *Aeromonas salmonicida* measured as optical density at 540 nm. The arrow indicates the end of exponential phase of the bacteria.

We found that LC_{50}= 10^6 cfu/ml fresh water in tanks was also reported elsewhere (Adams et al, 1987).

Two of the five pilot challenges did not cause acute furunculosis outbreak because the volume of water in tanks was too high (100-340 L) and the time of exposure too short (10-16 h). Mortalities in these two challenges were less than 20% after 2 weeks of exposure with 10^6 cfu/ml. Based on the three remaining pilot challenges performed we found that the lethal concentration curve for juvenile rainbow trout shows that the stock culture O.D=1 diluted in 50 L of tank fresh water causes $10^{5.7}$ cfu/ml =LC_{30}, $10^{5.5}$ cfu/ml =LC_{20}, 10^6 cfu/ml= LC_{50}, $10^{6.5}$ cfu/ml =LC_{80} and $10^{6.7}$ cfu/ml =LC_{100} (Fig 3.3). The peak of mortality in all challenges was reached between 4-7 days. All fish that died showed signs of furunculosis. The acute form of furunculosis, which is most common particularly in growing fish and adults, is characterized by general septicemia, small hemorrhages in the base of the fins and in some cases darkening in color (Austin and Austin , 1987). Acute furunculosis is fatal in 2-3 days and because the short duration of the disease, furuncle development is unusual (Mc Carthy, 1980; Hiney and Oliver, 1999). No furuncles were observed in the fish at any of the doses. The test organism *A. salmonicida* was re-isolated from 96% of the samples of dead fish which we examined bacteriologically.

In summary, we could generate an acute furunculosis infection in juvenile rainbow trout by decreasing the water level to 50 L, decreasing the water flow to 3 complete changes per day (0.15 gpm per tank) and exposing the fish to the optimized concentrations of bacteria for 24 h.

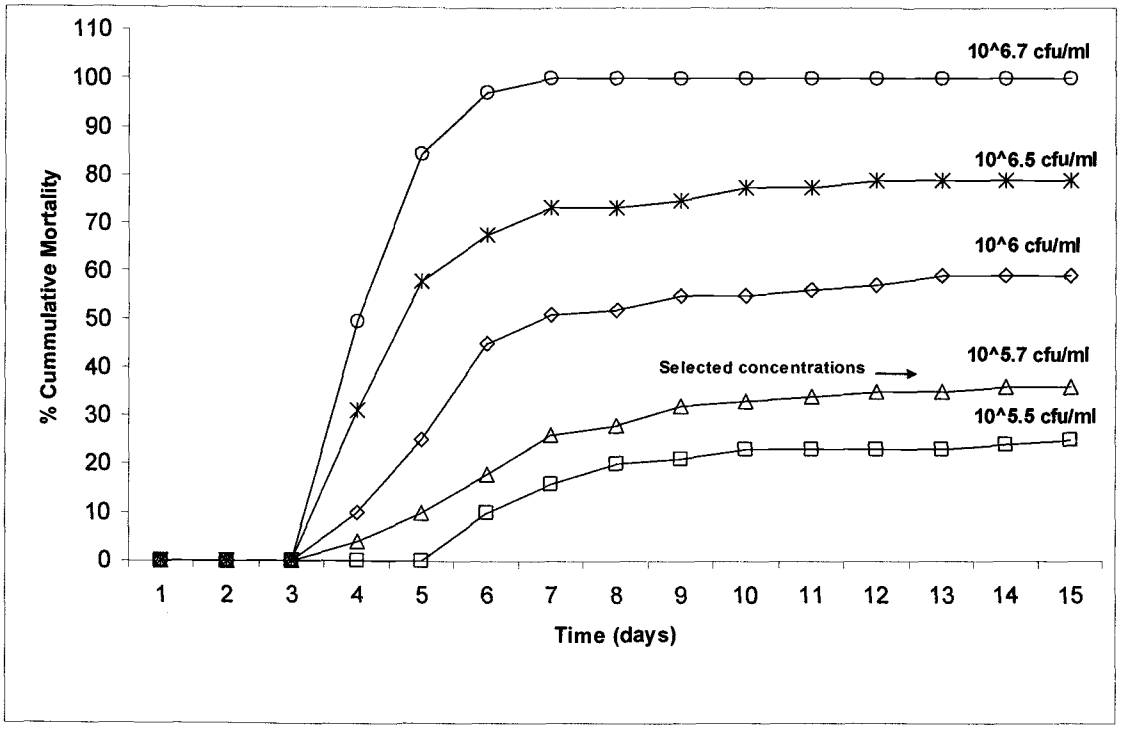


Figure 3.3 *A. salmonicida* lethal concentration curve for acute furunculosis obtained in pilot experiments.

Disease challenge of PAH exposed fish

To evaluate the effects of contaminant exposure on disease susceptibility bacterial challenges were conducted with LC_{20} and LC_{30}. Mortalities in tanks not exposed to *A. salmonicida* were less than 2 %, samples were taken from these fish to test for bacteria; no *A. salmonicida* was detected in all cases. No significant difference in mortalities ($p>0.05$) were present in control tanks not exposed to the pathogen (Fig 3.4).

The percent cumulative mortality of the different groups of juvenile rainbow trout exposed to LC_{20} and LC_{30} of *A. salmonicida* is shown is Figs 3.5 and 3.6, respectively. These results represent the net cumulative mortality attributed to exposure to the pathogen after subtraction of the background mortality observed in the control groups (Arkoosh et al, 2001).

Statistical testing was performed beginning at day 2 post-exposure to determine differences between the treatments. In the lower bacteria concentration (LC_{20}) the net cumulative mortality of rainbow trout after receiving a high molecular weight PAH mixture at 40 ppm and 400 ppm for 50 days was 30% for both doses compared to 18% in fish fed vehicle control diet at 21 days post-challenge. The net cumulative mortality of juvenile rainbow trout exposed to the bacteria after receiving BAP and BEP at 160 ppm ranged from 18% to 20% respectively compared to 18% in the control diet at 21 days post-challenge. Therefore, the cumulative mortality was significantly higher in fish exposed to 40 ppm and 400 ppm of the PAH mixture but not in the BAP, BEP treated animals relative to fish receiving only methylene chloride treated diet.

At higher bacteria concentration (LC_{30}), the net cumulative mortality of juvenile rainbow trout exposed to the bacteria was 38% and 32% for 400 ppm and 40 ppm mixture

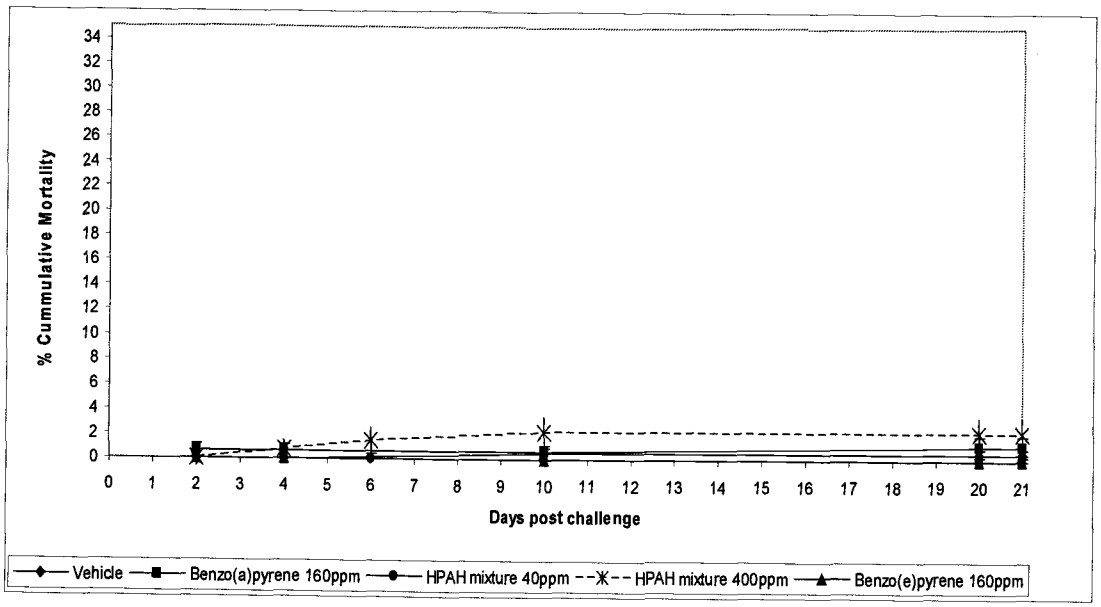


Figure 3.4 Percent cumulative mortality in juvenile rainbow trout fed either with benzo(a)pyrene 160ppm, benzo(a)pyrene 160 ppm, a PAH mixture 40 ppm or 400 ppm and vehicle control for 50 days not exposed to *A. salmonicida* during the disease challenge experiment

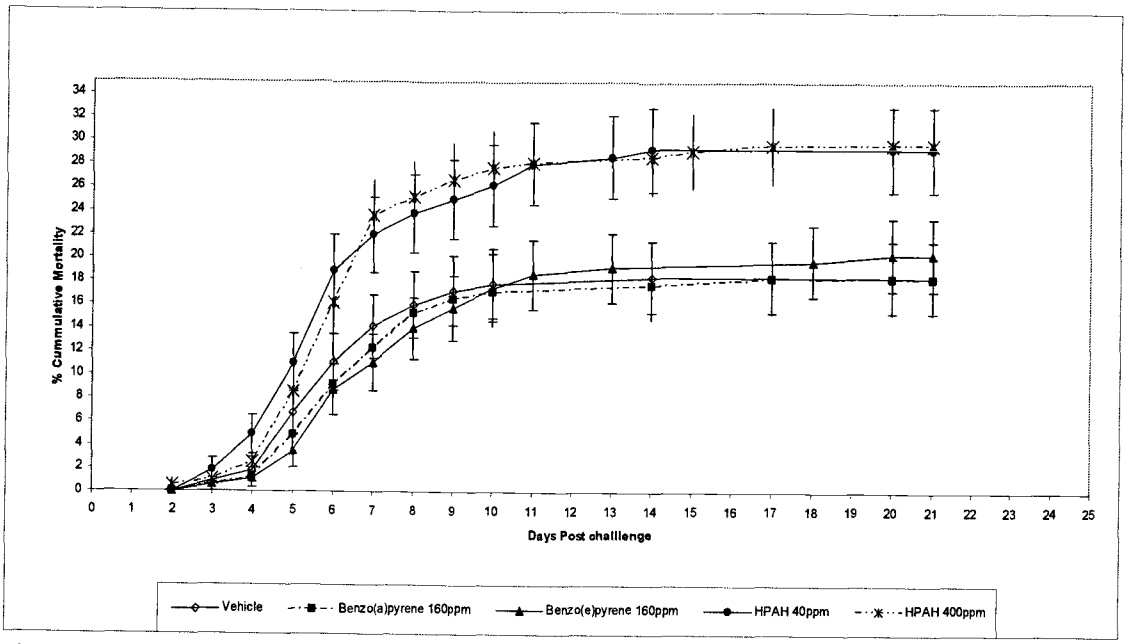


Figure 3.5 Percent cumulative mortality of juvenile rainbow trout fed with either benzo(a)pyrene 160 ppm, benzo(e)pyrene 160 ppm, a PAH mixture at 40 ppm or 400ppm and vehicle control treated diet with methylene chloride after exposure to LC_{20} of *A. salmonicida*

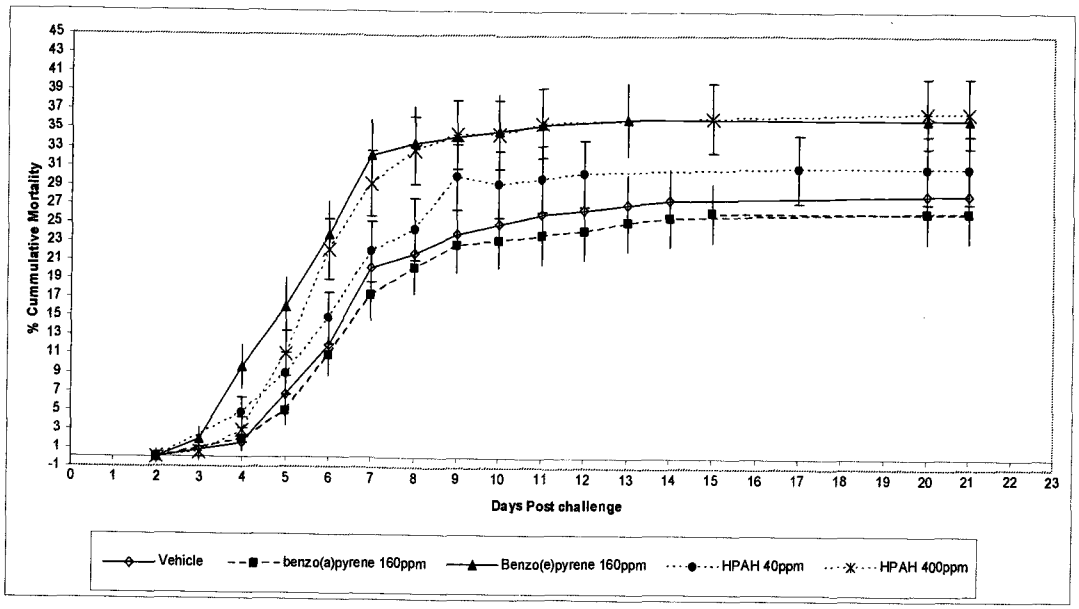


Figure 3.6 Percent cumulative mortality of juvenile rainbow trout fed with either benzo(a)pyrene 160 ppm, benzo(e)pyrene 160 ppm, a PAH mixture at 40 ppm or 400ppm and vehicle control treated diet with methylene chloride after exposure to LC_{30} of *A. salmonicida.*

respectively. For BEP, the net cumulative mortality was 38% and 24% for BAP treated animals compared to 28% in methylene chloride treated diet. Therefore, the cumulative mortality was significantly higher in fish exposed to 400 ppm of PAH mixture and 160 ppm of BEP. In fish exposed to 40 ppm or BAP, no significantly differences in mortalities were present at LC_{30} of *A. salmonicida*. Significant differences in the net mortality between rainbow trout exposed 40, 400 ppm PAH mixture, BEP, BAP and control was detected as early as 5 days post-challenge these differences were observed throughout the experiment.

We examined 100% of the carcasses collected during disease challenge, 96% were infected with *A. salmonicida*. Mortality was very low (< 2%) among toxicant-exposed fish not challenged with pathogen. Therefore mortalities in the challenge disease groups were due to acute furunculosis. Subtracting the low background mortalities from experimental groups allowed us to use exact numbers for the statistical analysis.

DISCUSSION

Bile chemistry

The dose and route of exposure to PAH significantly influenced accumulation and disposition of these compounds in fish. Studies in disposition of radioactive BAP in English sole (*Parophrys vetulus*) after dietary exposure showed that the relative ranking of BAP-derived radioactivity was: bile> liver> gill> kidney> blood> skin> muscle (Stein et al, 1987). Bile concentrations of PAH in the different treatments were measured on the last day of feeding (3 days before challenge) and after 21 days post-challenge. We found that the levels of PAH after 21 days of no feeding were significantly lower $p<0.05$ than the levels 3 days before challenge but the levels were

still significantly elevated compared to controls with no PAH in their diet (Fig 3.1). This demonstrates that although juvenile rainbow trout were exposed for 50 days to PAH in the diet and feeding was interrupted during the 21 days of the duration of the challenge, PAH metabolites in bile remained after a period of no chemical exposure. This indicated that since fish were not fed the gallbladder was not emptied often or perhaps at all.

Pilot challenges

Acute furunculosis in juvenile fish is difficult to generate in the laboratory. Factors including fish size, water temperature, and stress and species susceptibility affect the outcome of the disease (larger trout (50 g) takes over 10 days to show signs of disease whereas smaller trout (8.5 g) can present mortalities after day 1 of challenge (Adams et al, 1987)). There is a high degree of temperature dependency for a furunculosis outbreak. High temperature (20◦C) will generate a faster development of furunculosis. Groberg et al (1978) reported that the percent mortality decreased from 100% at 20◦C to 12% at 6.7◦C. the development of the disease is suppressed at water temperature lower than 6.7◦C and it is progressively enhanced at 9.4◦C and above. Stress decreases resistance to *A. salmonicida*. Fevolden et al (1992) found that mortality of stressed rainbow trout was higher than mortality in non-stressed fish. Rainbow trout are reportedly more resistant to *A. salmonicida* and therefore require higher doses of bacteria to cause 60% mortality than other salmonid species (Adams et al, 1987).

Reproducing the bath challenges in different laboratories with *A. salmonicida* was a major problem (Nordmo et al, 1998). Our test system reproduced acute furunculosis after two pilot experiments and in our final challenge with fish fed with different PAH

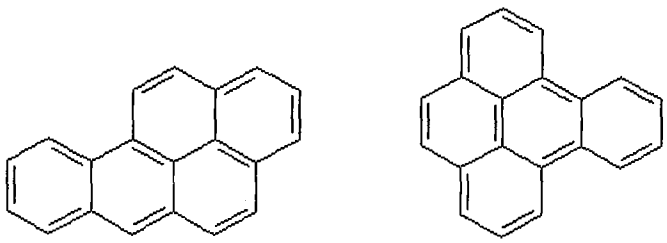
treatments. The pathogen was also isolated from 96% of the carcasses and positive results in all the re-isolates tested with the agglutination test were obtained. The pilot challenge was reproducible in the final experiment because we used the same stock of fish as our experimental fish, also the pilot studies were performed at the same facility simultaneously with the feeding of the experimental fish (Fig 3.3). We recommend the practice of performing pilot studies for future studies to optimize the conditions to generate an acute furunculosis using fish from the same stock as the experimental fish to account for the different factors that seem to affect the outcome of the disease challenge as reported in Nordmo et al (1998) and Nordmo and Ramstad (1999). We selected doses below LC_{50} since PAH were previously know as immunosuppressant compounds and high challenge level decreased the probability of detecting differences in mortality between PAH-exposed not exposed animals (White et al, 1992).

BAP and BEP effects on disease susceptibility

In our study we found that rainbow trout exposed to *A. salmonicida* LC_{30} (Fig 3.6) a higher cumulative mortality was seen in BEP exposed animals (38%) compared to BAP exposed animals (24%). BAP cumulative mortality was not significantly different than the control $P>0.05$. Our results showed that BEP causes higher disease susceptibility than BAP at LC_{30} dose of *A. salmonicida* in rainbow trout. We believe that our results cannot be directly compared from previous studies because the method and length of exposure to the chemical and pathogen were different.

BAP (Fig 3.7) is the most studied PAH. Its immunotoxic effects in mammals include immunosupression of antibody production and alteration of B-cell maturation (O'Halloren et al, 1998). In mammals BAP has not been shown to compromise host

survival after infection with a pathogen. Additionally early mice studies have reported that BAP had no effect on the resistance against infection with *Listeria monocytogenes* (Ward et al, 1985). There is less literature concerning the immunomodulating potential of BAP in fish. Limited data suggest that fish are also affected by the immunosuppressive effects of BAP. Carlson et al (2002) exposed medaka to BAP i.p to 2, 20 and 200 μg BAP/ g body weight to determine the effects upon immune function; fish were injected with *Yersinia rockery* 48h after chemical exposure. Mortalities were obtained after 14 days of injection with pathogen at the two highest doses BAP compare to vehicle control.



Benzo(a)pyrene Benzo(e)pyrene

Figure 3.7 Benzo(a)pyrene and benzo(e)pyrene structures

In a later study, Carlson et al (2004) exposed medaka (*Oryzias latipes*) to BAP with a single i.p injection and monitored its effects for 48 h, and found that BAP was shown to alter humoral immune function in fish. The results obtained in mammals supports our findings that host survival seems not to be compromised by BAP but contradict the results obtained in fish studies. During infection with *A. salmonicida* significant changes occur in non-humoral factors, especially in fish that succumb to acute furunculosis. Acute furunculosis that occurs in juvenile fish is characterized by generalized bacteria septicemia (Carnahan and Altwegg, 1996; Hiney and Oliver, 1999).

Innate immunity appears to be sensitive to the effects of BAP but not to the same extent as humoral related immunity (Carlson et al, 2002, 2004). This could explain why BAP had little or no effect on host resistance. In our study, BEP showed a higher effect on cumulative mortality after exposure to LC_{30} of *A. salmonicida*. Carlson and Zelikoff (2004) reported BEP apparent lack of effect on reduction in lymphocyte proliferation, phagocyte mediated superoxide generation and antibody forming cell numbers. BEP does not affect humoral mediated immunity in mice (Luster et al, 1983) or fish Carlson and Zelikoff (2004) but its effect on innate immune response remains to be elucidated. In fish alterations in immune function include reduction of phagocytosis by macrophages (Faisal and Hugget, 1993).

In the present study, mortality in BAP exposed animals is not different from the controls (Fig 3.5 and 3.6). We believe that 160 ppm BAP does not compromise host resistance but this does not exclude the possibility that BAP is immunosuppressive in rainbow trout. This indicates that the concentration of BAP selected in our study had

no effect upon host survival but this same concentration for BEP affected host survival when animals received a higher challenge concentration (Fig 3.6) suggesting that BEP might be immunosuppressive at doses that BAP is not when administered in the diet for long periods of time. BEP has a similar structure to BAP (Fig 3.7) but it is known to be a non carcinogenic compound. Its ability to suppress the humoral immune response in fish has been reported elsewhere (Carlson et al, 2002).

The immunosuppressive effects of BAP and BEP have been compared. In most cases, BAP was clearly immunosuppressive whereas BEP was inactive (Word Health Organization, 1994). Studies comparing these two compounds have used mice and administered the chemicals intraperitoneally (i.p). Dean et al (1983) found that after subcutaneous injections with BAP and BEP at 5, 20 and 40 mg/kg body weight, BAP altered differentiation and antibody production in immature B-cells but BEP did not have the same effect. This was later confirmed by White and Holsapple (1984).

In vitro studies have shown that both BEP and BAP caused a significant dose-dependent suppression of T-dependent and polyclonal antibody responses (Blanton et al, 1986).

BAP suppresses *in vivo* and in vitro measures of T-dependent B cells antibody responses (Ladics et al, 1992). BEP added to *in vitro* cell cultures does not suppress B-cell responses to either a T-cell dependent antigen or a polyclonal mitogen (White and Holsapple, 1984). In general it appears that the primary immunosuppressive effect of BAP is on humoral immunity, however BAP is also capable of suppressing certain aspects of the cell-mediated response (Davila et al, 1995). BEP has no major effects

upon cell-mediated responses (Davila et al, 1995) but BEP given *in vivo* was shown to be moderately suppressive to CTL activity (Wojdjani and Alfred, 1984).

Experimental infections can be induced by a variety of routes: intraperitoneal (i.p), intramuscular (i.m) injections, bath and cohabitation (Nordmo, 1997). Bacterial virulence is known to be multifactorial and it is important to take into account all aspects of virulence. Bath and cohabitation are the challenge methods that most closely mimic natural exposure (Nordmo, 1997). The i.p and i.m methods are reproducible and highly reliable but a large number of microorganisms are introduced without exposure to natural resistance factors and external immunity is not all accounted for.

Earlier studies examined immunotoxicity of BAP after single i.p injection (Carlson et al, 2002) or multiple i.p injections (Holladay et al, 1998). In our study we administered the chemicals in the diet and exposed the fish to the pathogen with water bath therefore it is difficult to compare the current findings with those previously reported. The route of exposure of the chemical and the pathogen affects the final outcome. Nordmo and Ramstad (1999) demonstrated that i.p injection challenge with *Vibrio salmonicida* produces cumulative mortality of almost 100% within 15 days post-challenge whereas a bath-challenge model with same bacterium was incapable of producing cumulative mortality above 70%. Bath challenge is regarded as a more natural challenge than i.p (Nordmo and Ramstad, 1997).

PAH mixture effects on disease susceptibility

There is a large amount of literature in mammals (Blanton et al, 1986, Dean et al, 1983, Hardin et al, 1992) and in fish (Durnier and Siwicki, 1995; Carlson et al, 2002;

Arkoosh et al 1998, 2001; Mohamed and Hugget, 1993; Grinwis et al, 2000) that suggest that PAH are immunotoxic compounds.

In the present study juvenile rainbow trout exposed to two concentrations of a PAH mixture have significantly higher ($p<0.05$) predisposition to the pathogen *A. salmonicida* than the fish not exposed to PAH. This supported by other studies that showed that PAH are immunosuppressant (Arkoosh et al, 2001). The proportions and concentrations of the PAH tested represent specific subsets of predominant estuarine chemical pollutants (Arkoosh et al, 2001). The present study together with previous work Arkoosh (1994, 1998 and 2001) support the hypothesis that juvenile fish exposed to PAH may be immunocompromised and their potential for survival may be reduced.

The greatest effect on host resistance was observed in animals exposed to 400 ppm PAH mixture (Fig 3.6). An increase in 10% mortalities was observed between the 400 ppm dose and the control for both bacteria concentrations. Compared to BAP, very little information is available on the immunological effects of the other PAH included in the mixture. Few experiments in mice have shown that dibenz[a,h]anthracene reduces serum antibody levels in response to antigenic challenge compared to control (Malmgren et al, 1952). Hinoshita et al (1992) found that fluoranthene slowed the rate of B-cell precursor growth when given at 5 μg/ml or altered cell survival. In our mixture dibenz[a,h]anthracene was 1% of the mixture whereas fluoranthene was 20% (Arkoosh et al, 2001; chapter II).

Immunotoxicity in fish of the PAH mixture used in this study has been demonstrated elsewhere. Arkoosh et al (2001) exposed juvenile salmon to a PAH mixture with the

same compounds and concentrations employed in the present study. The results showed a 10% increased mortality in the PAH-treated group respect to control after 7 days of challenge with *Listonella anguillarum.* The fish were in the range of 15-20 g of body weight as in the present study. The decreased host resistance of juvenile rainbow trout to *A. salmonicida* exposed to estuarine-levels of a PAH mixture is consistent with these previous findings that demonstrate the effects of a specific group of chemical contaminants on immunocompentence and disease susceptibility in the laboratory.

To our knowledge this is the first study that chronically exposes rainbow trout to different PAH in the diet and uses a bath challenge with *A. salmonicida* to measure host resistance.

Our findings contradict the results in Palm et al (2003). In their study, chinook salmon were fed for 28 days with a PAH mixture similar to the one selected in our study. Fish were then challenged with *Listonella anguillarum* a common marine pathogen. Their results suggested that exposure to an environmentally relevant mixture of PAH compounds produced no loss of immunocompentence of juvenile chinook salmon. To our knowledge this was the first study to report negative effects of PAH on disease susceptibility.

Most studies used injections for chemical and bacterial exposure in mice and fish experiments and therefore we are unable to directly compare our results to these previous findings. To determine the effects of PAH in the immune system the route of exposure of the chemical and the pathogen should mimic as closely as possible natural conditions. Additionally it is necessary to use preliminary challenges to optimize the

disease challenges conditions when *A. salmonicida* is used since different factors affect the outcome of the experiment. The disadvantage of this approach is that is difficult to reproduce but very valuable information is obtained that can be comparable to natural conditions.

In conclusion, our current findings suggest that juvenile rainbow trout exposed to PAH characteristic of those found in stomach content of salmonids from contaminated estuaries, are more susceptible to disease than salmon not exposed to contaminants. This study shows that PAH with similar structures might have different mechanisms of action. BEP and BAP, appear to have a different mechanism of action that decreases disease susceptibility. BAP is known to affect the humoral immune response and BEP might affect innate immune response and decrease overall survival. However the exact mechanism of action remains unknown. An environmentally relevant mixture of PAH showed the highest effects on disease susceptibility clearly demonstrating that these chemicals are immunosuppressive and can potentially affect the survival of populations.

ACKNOWLEDGEMENTS

The authors wish to thank Gina Ylitalo and Jon Buzitis (NWFSC/NOAA) for providing useful information and training for the bile work. Dr. Kim Anderson for her collaboration with the analysis of the effluent water samples (OSU), Dr. Tim Miller Morgan (OSU) for diagnosing fish health, Dr. Paul Reno for his advice with the disease challenge and Dr. Richard Holt for providing the microorganism (OSU). We also wish to thank Coral Stafford for help with fish care and sampling. Ethan Clemons for helping with fish transportation. Hatfield Marine Science Center FDL maintenance

personnel for their collaboration in all the aspects water treatment and tank care. This study was funded by CIMRS-NMFS and the Oregon Agricultural Experimental Station

REFERENCES

Adams A, Leschen W, Wilson A and Horne M.T. 1987. A bath challenge model for furunculosis in rainbow trout, *Salmo gairdneri* Richardson and Atlantic salmon *Salmo salar L.* Journal of Fish Diseases 10: 495-504.

Arkoosh M, Casillas E, Clemons E, McCain B and Varanasi U. 1991. Suppression of Immunological Memory in Juvenile Chinook salmon (*Oncorhynchus tshawytsha*) from an urban estuary. Fish and Shellfish Immunology 1: 126-277.

Arkoosh M, Clemons E, Myers M and Casillas E. 1994. Suppression of B-cell Mediated Immunity in juvenile chinook salmon (*Oncorhynchus tshawytsha*) after exposure to either polycyclic aromatic hydrocarbon or polychlorinated byphenyls. Immunopharmacology and immunotoxicology 16(2) 293-314.

Arkoosh M, Casillas E, Huffman P, Clemons E, Evered J, Stein J and Varanasi U. 1998. Increased susceptibility of juvenile Chinook salmon from a contaminated estuary to *vibrio anguillarum.* Transactions of the American Fisheries Society. 127: 360-374.

Arkoosh M, Clemons E, Huffman P and Kagley A. 2001. Increased Susceptibility of juvenile chinook salmon to vibriosis after exposure to chlorinated and aromatic compounds found in contaminated urban estuaries. Journal of Aquatic Animal Health 13: 257-268.

Austin B and Adams C. 1996. Fish pathogens Ch 8 in: Austin B, Altwegg M, Goslin P and Joseph S. The genus Aeromonas. John Wiley and sons. Chinchester west Sussex England. Pp 197-243.

Austin B and Austin A. Bacterial Fish pathogens: disease in farmed and wild fish. Ellis Horwood Limited publishers Pp 154.

Bailey S, Williams D and Hendricks J,1996. Fish models for environmental carcinogenesis : The rainbow trout. Environmental Health Perspectives Vol 104 Suppl 1: 5-21.

Blanton H. Lyte M, Myers M and Bick P. 1986. Immunomodulation by polyaromatic hydrocarbons in mice and murine cells. Cancer research 46: 2735-2739.

Bricknell I. 1995. A reliable method for the induction of experimental furunculosis. Journal of fish diseases. 18: 127-133.

Carlson E and Zelikoff J. 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo(a)pyrene suppresses immune function and host resistance against bacterial challenge. Aquatic Toxicology 56: 289-301.

Carlson E and Zelikoff J. 2004. benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*) relationship between lymphoid CYP1A1 activity and humoral immunesuppression. Toxicol and Applied Pharmacology 201: 40-52.

Carnahan A and Altwegg M.1996. Taxonomy in: Austin B, Altwegg M, Goslin P and Joseph S. The genus Aeromonas. John Wiley and sons. Chinchester west Sussex England. Pp 1-38.

Davila DR, Davis De Ann, Campbell K, Cambier J, Zigmong L, Burchiel S.W. 1995. Role of alterations in Ca^{+2}-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. Journal of Toxicology and Environmental health 45: 101-126.

Dean J, Luster M, Boorman G, Lauer L, Leubke R and Lawson L. 1983. selective immunosupression resulting from exposure to the carcinogenic congener of benzopyrene in BGC3F1 mice. Clinical and Experimental Immunology Apr 52: 199-206.

Dunier M and Siwicki A. 1995. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish and shellfish immunology 3: 423-438.

Faisal M and Hugget R. 1993. Effects of polycyclic Aromatic hydrocarbons on the lymphocyte mitogenic responses in spot *Leistomus xanturus*. Marine Environmental Research 35: 121-124.

Fevolden S, Kefstic T and Roed K. 1992. Disease resistance in rainbow trout (*Oncorhynchus mykiss*) selected for stress response. Aquaculture 104: 19-29.

Grinwis G, Vethaak A, Wester P and Vos J. 2000. Toxicology of environmental chemicals in the flounder *platichthys flesus* with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies. Toxicology letters 112-113: 289-301.

Groberg W.J Jr, R.H Mc Coy, K.S Pilcher and J.L Fryer. 1978. Relation of water temperature to infections of coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*) and steelhead trout (*Salmo gairdneri*) with Aeromonas salmonicida and A. Hydrophyla. Journal of the fisheries Research Board of Canada. Vol 35 No1 Pp 1-7

Hardin J, Hinoshita F and Sherr D. 1992. Mechanisms by which benzo(a)pyrene, an environmental carcinogen, suppresses B-cell lymphopoiesis. Toxicology and Applied Pharmacology. 117: 155-164.

Healey M.C. 2003. Life History of Chinook salmon *Oncorhynchus tshawytsha* in: Groot C and Margolis L. Pacific Salmon Life Histories UBS press, Vancouver, Canada. Pp 313-383.

Hendricks J, Meyers T, Shelton D, Casteel J and Bailey G. 1985. Hepatocarcinigenicity of BAP to rainbow trout by dietary exposure and intraperitoneal injection. Journal of the National Cancer Instute.74: 839-851.

Hiney M and Oliver G. 1999. Furunculosis (*Aeromonas salmonicida*) in: Woo P and Bruno D eds. Fish diseases and disorders, Vol 3 viral, bacterial and fungal infections. CABI publishing. Pp341-425

Hinoshita F, Hardin JA, Sherr DH. 1992. Fluoranthene induces programmed cell death and alters growth of immature B-cells populations in bone marrow cultures. Toxicology 73(2): 203-218.

Holladay S, Beteman D, Gogal R, Hrubec R, Ahmeds R. 1998. benzo(a)pyrene induced hypocellularity of the pronephros in tilapia (*Oreochromis niloticus*) is accompanied by alterations in stromal and parenchimal cells by enhanced immune cell apoptosis. Veterinary Immunology, Immunopathology. 64: 69-82.

Johnson S.L 1988. The effects of the 1983 el niño on Oregon's Coho (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawitscha*). Fisheries Research 6: 105-123.

Krahn M, Myers M, Burrows D and Mallins D, 1984. Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. Xenobiotica Vol 14 No8: 633-646.

Krahn M, Moore L, and MacLeod J. 1986. Standard analytical procedures of the NOAA National Analytical Facility, 1986. Metabolites of Aromatic Compounds in Fish Bile. NOAA Technical Memorandum. NMFS-F/NWC-102

Krahn M, Ylitalo G, Buzitis J, Chan S and Varanasi U. 1993. Rapid high-performance liquid chromatography methods that screen for aromatic compounds in environmental samples. Journal of chromatography 642:15-32.

Latimer J and Zheng J. 2003. The Sources, transport and fate of PAHs in the marine environment, in: Douben P. PAHs an Ecotoxicological Perspective. John Wiley and Sons Ltd. Pp 9-33.

Ladics G, Kawabata T, Manson A.E and White K.L. 1992. Evaluation of Murine Splenic cell type metabolism of BAP and functionality in vitro following repeated in vivo exposure to Bap. Toxicology and Applied Pharmacology. 116: 248-257

Lowry O, Rosenbrough N, Farr A and Randall R. 1951. Protein Measurements with the Folin phenol reagent. Journal Biological Chemistry. 193:265-275

Malmgren RA. 1952. Reduced antibody titers in mice treated with carcinogenic and cancer chemotherapeutic agents. Proceedings Society Biological Medicine 79: 484.

Markwardt N. Yvonne G and Klontz G. 1989. A new application for comassie brilliant blue agar: detection of A.salmonicida in clinical samples. Diseases of Aquatic organisms. Vol 6: 231-233.

Marubini, E, and Valsecchi. M.G. 1995. Analyzing survival data from clinical trials and observational studies. John Wiley, Chichester, N.Y. 424 pp.

Mc Carthy.1980. Furunculosis of fish the present state of our knowledge in Droop M and Jannasch H eds. Advances in Aquatic Microbiology. London, Academy Press P293-341.

Meador J. 2003. Bioaccumulation of PAHs in Marine Invertebrates in: Douben P. PAHs an Ecotoxicological Perspective. John Wiley and Sons Pp 147-171.

Meador JP, Stein JE, Reichert WL and Varanasi U (1995). A Review of bioaccumulation of polycyclic aromatic hydrocarbons by marine organism. Reviews in Environmental Contamination and Toxicology, 143: 79-165.

Nehlsen W, Williams J.E and Lichatowitch J.A. 1991. Pacific salmon at the croosroads; stocks at risk from California, Oregon, Idaho and Washington. Fisheries 16(2) 4-21

Nordmo R, Ramstad A. 1997. Comparison of different challenge methods to evaluate efficacy of furunculosis vaccines in Atlantic salmon *Salmo salar* L. Journal of Fish Disease 20: 119-126

Nordmo R. 1997. Strenghts and weaknesses of different challenge methods. In: Gudding R, Lillenhaug A, Midtlyng PJ and Brown F (eds): Fish vaccinology. Developments in Biological Standarization. Basel, Karger Vol 90 PP 303-309.

Nordmo R, Ramstad A and Holth R. 1998. Induction of experimental furunculosis in heterogeneous test populations of Atlantic salmon (*salmo salar L.*) by use of a cohabitation method. Aquaculture 162:11-21.

Nordmo R and Ramstad A. 1999. Variables affecting the challenge pressure of *A. salmonicida* and *V. salmonicida* in Atlantic salmon (*salmo salar L*). Aquaculture 171: 1-12.

O'Halloran K, Ahokas N and Wright P. 1998. the adverse effects of aquatic contaminants on fish immune responses. Australassian Journal of Ecotoxicology. 4: 9-28.

Osborne M, Crosby N. 1987. Benzopyrenenes. Cambridge University Press, London. Pp 28 and 234.

Parker N and Munn C. Cell surface properties of virulent and attenuated strains of *A. salmonicida* in Ellis AE (ed) Fish and Shellfish pathology. London Academy press 97-105.

Pesonen M, Celander M, Forlin L and Andersson T. 1987. Comparison of xenobiotics biotransformation enzymes in kidney and liver of rainbow trout (*Salmo gairdneri*). Toxicology and Applied pharmacology 91: 75-84.

Palm R, Powell D, Skillman A and Godtfredsen K. 2003. Immunocompentence of juvenile chinook salmon against Listonella anguillarum following dietary exposure to polycyclic aromatic hydrocarbons. Environmental Toxicology and Chemistry Vol 22, No 2 Pp 2986-2994.

Quinn T.2005. The behavior and ecology of Pacific salmon and trout. American fisheries Society. Bethesda, Maryland. Pp 3-36 and 37-53.

Secombes C and Olivier G. 1997. Host-pathogen interactions in salmonids. In: Bernoth E, Ellis A, Midtlyng P, Olivier G and Smith P. Furunculosis Multidisciplinary Fish disease research. Academy press San Diego Pp 269-321.

Sloan C.A, D.M Brown, Pearce R.W, Boyer R.H, Bolton J.L, Burrows D.G, Herman D.P. and Khran M.M 2004. Extraction, cleanup and gas chromatography/mass spectrometry analysis of sediments and tissues for organic contaminants. U.S Department of Commerce NOAA Tech Memo. NMFS-NWFSC-59. P47.

Stein J.E, Hom T, Casillas E, Friedman A and Varanasi U. 1987. Simultaneous exposure of English sole (*Parophrys vetulus*) to sediment-associated xenobiotics II Chronic exposure to an urban estuarine sediment, with added ^{3}H-Benzo(a)pyrene and ^{14}C-polychlorinated biphenyls. Marine Environmental Research 22: 123.

Thorpe J. 1994. Salmonid Fishes and the Estuarine Environment. Estuaries 17: 76-93.

Walczak B.Z, Blunt B.R and Hodson P.V. 1987. Phagocytic function of monocytes and haematological changes in rainbow trout injected intraperitoneally with

Benzo(a)pyrene (B(a)p) and benzo(a)anthracene (B(a)A). Journal of Fish Biology 31 supplement A Pp: 251-253

Ward E, Murray M and Dean J. 1985. immunotoxicity on non halogenated polycyclic aromatic hydrocarbons. In: Dean J (ed) immunotoxicology and immunopharmacology. Raven press, New York Pp 291-304.

White K and Holsapple M.P. 1984. Direct Suppression of in vitro antibody production by mouse spleen cells by the carcinogen Benzo(a)pyrene but not but the non-carcinogenic congener Benzo(e)pyrene. Cancer Research 44: 3388-3393

White K.L Jr. 1992. Specific Immune Function Assays. Pp 304-323 in: K. Miller, J. Turk and S. Nicklin (eds). Principles and Practice of Immunotoxicology. Blackwell Scientific Publications, Cambridge, Massachussets.

Wojdani A, Attarzadeh M, Wode-Tsadik G and Alfred L.J. 1984. Immunotoxicity Effects of polycyclic aromatic hydrocarbons on mouse lymphocytes. Toxicology 31: 181-189.

Wojdani A and Alfred L.J. 1984. Alterations in cell-mediated immune functions induced in mouse splenic lymphocytes by polycyclic aromatic hydrocarbons. Cancer Research 44: 942-945.

World Health Organization. 1998. Environmental Health Criteria 202. Selected non-heterocyclic polycyclic aromatic hydrocarbons: a structure activity relationship in B6C3F1 and DBA/2 mice. Immunopharmacology 9: 155-164.

Chapter IV

Transcriptional patterns in head kidney of polyaromatic hydrocarbon mixture pretreated rainbow trout (*Oncorhynchus mykiss*) challenged with *Aeromonas salmonicida*

Claudia F. Bravo[1], Lawrence R. Curts[1], Christopher Bayne[2], Mary Arkoosh[3], Elisabetta Lambertini[4], Frank Loge[4] and Tracy K. Collier[5]

1. *Department of Environmental and Molecular Toxicology, Oregon State University, ALS 1007, Corvallis OR 97331 USA.*
2. *Department of Zoology, Oregon State University, Cordley 3029, Corvallis, Oregon 97331 USA.*
3. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2030 South Marine Science Drive, Newport OR 97365, USA*
4. *Department of Civil and Environmental Engineering, University of California Davis, 1 Shields Avenue, Davis, CA 95616, USA.*
5. *Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Boulevard East, Seattle, Washington, 98112, USA*

To be submitted to *Journal of Toxicological sciences*

ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) occur as complex mixtures in aquatic ecosystems. Rainbow trout (*Oncorhynchus mykiss*) were fed a mixture of ten high molecular weight PAH at an environmentally relevant concentration (400 ppm) for 50 days. After 50 days of PAH exposure, fish were challenged with *Aeromonas salmonicida* the causative agent of furunculosis at lethal concentration 30 (LC_{30}). Rainbow trout head kidney was collected after 2, 4, 10 and 20 days post-challenge. RNA was extracted and reverse transcribed to cDNA and hybridized to the customized rainbow trout array version 2.0 developed at Oregon State University. Over 20 immunologically relevant genes differentially expressed after pathogen challenge were identified. We selected 5 important immune genes that were differentially expressed in PAH exposed fish, relative to control diet fed fish after pathogen challenge: Interleukin 8 (IL 8), transport associated protein 1 (TAP 1), NF-kB essential modulator (NEMO), recombination activating gene 2 (RAG 2), major histocompatibility complex II (MHC II) and a toxicologically relevant gene: cytochrome P450 1A (CYP1A1). Selected gene transcripts were measured by RT-PCR at the time points examined by microarray. The expression profile of all selected genes indicated that they were down regulated after 2 days post-challenge, this response was constant over time and significantly different than the control ($P<0.05$). These genes were associated with oxidative stress and immune function by other investigators.

Keywords: *Disease susceptibility, PAH, Microarray, rainbow trout, RT-PCR.*

INTRODUCTION

Pacific salmon have disappeared from approximately 40% of their historical Pacific Northwest habitat (Nehlsen et al, 1991). Since the nineteenth century, diverse human activities have depleted salmon populations (Quinn, 2005). Factors that can affect salmon decline include habitat degradation, dam passage, predation and disease (Loge et al, 2005). In the Columbia River basin, disease is a spectrum of responses depending upon the intensity of the interaction of variables defined for the host, pathogen and environment (Hedrick, 1998). Disease is an important component influencing population dynamics (Loge et al, 2005). Diseases are a natural phenomena and important controlling factors of wild fish populations (Sindermann, 1990; Hedrick, 1998). They are inherent to aquatic ecosystems. The impact of infection in the host will depend on resistance to infection (Gullard, 1995). The development of immunity can be modulated by extrinsic factors such as stress, changes in nutrition and ingestion of chemicals that can alter levels of infection with a variety of pathogens, usually resulting in the suppression of the immune response (Loyd, 1995). The dose or numbers of pathogens, route of host delivery and the duration of the exposure determine the severity of infection (La Patra et al, 1989). Significant alterations of habitat due to human intervention (power generation, mining, contamination, etc) have affected wild populations in a negative way (Hedrick, 1998). A number of chemicals are considered environmental contaminants (pesticides, heavy metals, polycyclic aromatic hydrocarbons). Polycyclic aromatic hydrocarbons (PAH) are ubiquitous in nature and in the aquatic environment that primarily originate from anthropogenic sources (Hellou et al, 1996). Chronic exposure to these compounds at

sublethal doses can cause immunosupression. PAH are immunotoxic compounds (Davila et al, 1995) they also increase disease susceptibility (Arkoosh et al, 1998, 2001, chapter III). The mechanism of action in the immune system remains to be elucidated. Benzo(a)pyrene (BAP) is the most studied PAH. BAP is mutagenic, carcinogenic and immunotoxic. In mammals both innate and acquired immune responses are affected by BAP exposure (White et al, 1994). The mechanism of action seems to be T-lymphocyte dependent (Silkworth et al, 1995). Page et al (2004) demonstrate that although metabolism of dimethylbenz[a]anthracene (DMBA) via CYP1A1 in mice is essential to decrease bone marrow cellularity, and production of reactive metabolites alone is not sufficient and other mechanism such as down regulation of cytokines may play a role in PAH immunotoxicity. This goes beyond the classic model of PAH toxicity that involves metabolite generation, DNA adduct formation and apoptosis (Page et al, 2004). Davila et al (1995) propose that PAH may exert some of their immunomodulatory effect by interfering with calcium homeostasis and signal transduction. In fish, BAP decreases host resistance against *Listonella anguillarum* (Arkoosh, 2001), *Yersinia ruckery* (Carlson and Zelikoff, 2002) and *A. salmonicida* (chapter III). BAP also suppresses T and B- lymphocyte proliferation (Carlson and Zelikoff, 2002).

Earlier studies demonstrated reduced immunological memory in salmon from PAH contaminated sites in the Pacific Northwest (Puget Sound, WA) (Arkoosh et al, 1991, 1996). PAH seemed to lower the mitogen-induced lymphocyte proliferation in rainbow trout (Karrow et al, 1999). Because of the sensitivity of the immune response, immunodysfunction was perhaps predictive of the toxicological hazards/ risk

associated with chemical contamination. Biomarkers that detected immunosupression served as rapid indicators of the direction of a change in the toxic exposure and effects at a particular monitoring site (Zelikoff et al, 2000). Increased disease susceptibility after chemical exposure was of most concern (Payne et al, 2003). The immune system responded to chemicals present in the environment at low levels, but any one immune assay inadequately described xenobiotics-induced immunotoxicity in fish. The need remained for new biomarkers applicable for field work that detected early immunotoxicity of chemicals such as PAH.

In the present study we fed rainbow trout to an environmentally relevant mixture of PAH that mimicked the concentrations and proportions found in stomach contents in chinook salmon from the Pacific Northwest. After 50 days, we challenged the fish with *Aeromonas salmonicida* and collected head kidney after 2, 4, 10 and 20 days post-challenge. To help understand the mechanism of action of these chemicals in the immune system, we studied the gene expression profile in head kidney by microarray and selected genes transcripts by real time PCR (RT-PCR). The selected genes were potential biomarkers of exposure to this group of compounds. We concluded that PAH were immunotoxic compounds that down regulated key genes of the immune response.

MATERIALS AND METHODS

Animals and treatment

Shasta rainbow trout (*Oncorhynchus mykiss*) were bred and raised at the Sinnhuber Aquatic Research laboratory at Oregon State University and transported at 7 g to the Hatfield Marine Science Center in Newport, OR as previously described (chapter II).

Fish were fed 2% body weight (six times a week) with treated or control diets for a total of 50 days. Diets were prepared using methylene chloride as solvent as described in chapter II: an environmentally relevant model mixture of 10 high molecular weight PAH was used at 40 and 400 parts per million (ppm) at the same proportions described in Arkoosh et al. (1998), two additional treatments of 160 ppm benzo(e)pyrene (BEP) or benzo(a)pyrene (BAP) was fed to the fish. A week before the last day of feeding water level was lowered from 400 to 50L in all tanks. On day 50, feeding was interrupted and 2 days later fish were bath exposed to *Aeromonas salmonicida* at lethal concentration $(LC)_{30}$ (chapter III).

Sampling for microarray analysis

At 2, 4, 10 and 20 days after bacterial challenge, three fish per tank were sampled from each of the 36 tanks used in this experiment and taken in 20 L buckets with constant aeration to the laboratory for sample collection. Fish were then sacrificed by blow to the head; kidney samples were collected and stored in liquid nitrogen (LN2) until RNA extraction. Control and experimental samples were obtained at the same time to avoid circadian effects on gene transcription. The response obtained in the biomarkers measured (chapter II). Only fish from the 400 ppm PAH mixture treatment and vehicle control (feeding experiment) (chapter II) exposed and not exposed to *A. salmonicida* at LC_{30} (disease challenge experiment) (chapter III) were selected for microarray analysis for their consistent response in the biomarkers measured during feeding (chapter II) and disease susceptibility to *A. salmonicida* (chapter III).

Microarray and quality control

An oligonucleotide array (OSUrbt version 2.0) containing 1672 features representing approximately 1400 genes was designed at Oregon State University (Tilton et al, 2005). Briefly, for each gene an at least 70-mer oligonucleotide was selected and printed in duplicate and a set of ten SportReport Alien Oligos (Stratagene, La Jolla, CA) were printed 16 times and included as internal positive controls. Buffer only spots were included as negative controls. To assess for printing quality and oligonucleotide retention, all slides were scanned for red reflectance using a Scan 4000 scanner (PerkinElmer, Boston, MA) (Tilton et al, 2005). At least one slide per printing batch was stained with Syto 61 (Molecular Probes, Eugene OR) and scanned at 633 nm. Slides were stored under desiccation for two months prior to use.

RNA extraction and quality control

Total RNA was extracted by adding 1 ml Trizol solution (Invitrogen, Carlsbad, CA) to the kidney samples (0.5-1 g). Pestles pre-treated with RNAse Away (Molecular Bio Products, San Diego, CA) and washed with diethyl pyrocarbonate (DEPC) treated water (Sigma- Aldrich) were used for tissue homogenization. Trizol reagent was used with the Invitrogen protocol for extraction. Total RNA was then dissolved in 50- 150 ul of DEPC-treated water (depending upon pellet size). RNA concentrations were measured spectophotometrically at 260 nm by adding 2 μl of sample in a Nanodrop ND-1000 spectrophotometer (NanDrop Technologies, Wilmington, DE). Measurements were calculated in Nanodrop 3.1.0 software (NanDrop Technologies, Wilmington, DE). The quality of the RNA was determined in RNA 6000 Nano Labchip ® (Agilent technologies, Waldbronn, Germany) in Agilent 2100 bioanalyzer

(Agilent technologies, Waldbronn, Germany). All the samples met the quality criteria with RNA integrity number (RIN) between 9-10 (ten being the highest) indicative of clean intact RNA. We extracted RNA from 6 individual fish per condition: vehicle control no *A. salmonicida*, vehicle control/ *A. salmonicida*, 400 ppm PAH/ no *A. salmonicida* and 400 ppm/ *A. salmonicida*. We selected 4 high quality extracts for a total of 16 samples per time point.

A reference RNA sample was made by combining 21 kidneys and 15 livers pooled from the different conditions described above at a total concentration of 987.8 µg/ µl. The pooled RNA was aliquoted and stored at -80 °C until use.

Reverse transcription to cDNA

Total RNA (2.5 µg) from samples and the reference were used for each reaction (Gerwick et al, unpublished) and reverse transcribed using 200 units of Superscript III® (Invitrogen) that contained specific primers with tails that complemented secondary binding with either CY3 or CY5 labeled fluorophore (Genisphere 900™). To each reaction 1 µl of Alien, 1 µl of primer, 2-4 µl of extracted RNA and RNAse free water was added for a final volume of 6 µl. Aliens oligos® (Stratagene, La Joya, CA) were used as a positive control for reverse transcription quality. A master mix with standard amounts recommended by Genisphere 900™ of 5X Superscript III first strand buffer, 0.1 M DTT, Superase-in RNAse inhibitor, dNTP mix and Superscript III enzyme (Invitrogen) was prepared in one stock and 4.5 µl added to the RNA reverse transcription master mix. cDNA was stored at -80 °C until use.

First hybridization to microarray

We used a standard reference design with dye swapping in individual fish. For each slide, hybridization consisted of 6.5 µl of sample cDNA from a fish labeled CY5 mixed with 6.5 µl of reference cDNA labeled CY3 and 17 ml of 2X formamide buffer for a total volume of 30 ml. There were a total of 32 slides per time point. A Genisphere 900™ protocol was followed with modifications (Gerwick et al, unpublished). Hybridization temperature was 49 °C for 24 h in a humidified chamber. Before use, slides were pre-washed under static conditions with the following reagents: 2 SSC 0.2% SDS for 20 min at 49 °C, 0.2 SSC at 18-21 °C for 5 min and DEPC water for 3 min also at 18-21°C. After incubation, the slides were washed as described in Gerwick et al (unpublished), briefly all washes were performed at constant agitation under low intensity light with 2X SSC 0.2% SDS at 49 °C for 12 min, 2X SSC at 18-21 °C for 12 min and 0.2 SSC for 12 min also at 18-21 °C. The slides were dried by centrifugation at 1000 x g for 3 min.

Second hybridization to microarray

Tagged CY3 and CY5 dyes from Genisphere 900™ were mixed with 2X formamide buffer (Gerwick et al, unpublished) and anti-fade reagent (Genisphere 900™ Hatfield, PA). Hybridization was done in the dark for 4 h at 49°C in a humidified chamber. Slides were washed as described above and dried again by centrifugation 1000 x g for 3 min. To protect dyes from fading, slides were dipped for 2 s in dye saver™ 2 anti-fade coating (Genisphere 900™) followed by another centrifugation 100 x g for 3 min. Slides were dried for 10 min and immediately scanned.

Scanning and quality control

Slides images were acquired with Axon 4200A (Molecular Devices, Union City, CA) at an excitation of 532 nm for CY3 and 635 nm for CY5. The photomultiplier tube (PMT) settings and percent power of the laser for each fluorophore was set based on intensity of spiked internal Alien controls, so that the Alien control showed an overall ratio of means (CY5 to CY3 signal of the entire array) close to one. This corrected for differences in intensity between arrays due to technical errors, and normalized between arrays, aimed at increased array comparability. The scanned array files were examined individually for spots that were saturated, yielded weak signals or were artifacts and were flagged as "bad spots". The GenePix Pro 6.0 files were imported into S-plus array analyzer program (Lucent technologies, Inc Seattle, WA).

Analysis of data

A quality diagnostics step was performed at the beginning of the analysis that assessed the quality of the data a second time. Data distribution and trend determined whether normalization was necessary. For this step the data was visualized in image plots, MvA plots and intensity box plots (not shown). The intensity values used as input were the mean of the foreground (spot) and background (area around spot) of the green and red channels. The background value was subtracted from the foreground and corrected intensity. The intensity files were analyzed for differential gene expression with the S+Array Analyzer 2.0 software in S+ environment (S+® 6.2 for Windows Professional Edition, Lucent Technologies, Inc, Seattle, WA). The analysis considered the following comparison repeated for each time point (2, 4, 10 and 20 days post-challenge): differential expression between individuals exposed and not

exposed to PAH challenge with *A. salmonicida*. In summary, we performed a pair-wise comparison (unpaired t-test) with dye swap and reference design. We used the Bonferoni method at 95% confidence level.

Filtering

A gene filtering step eliminated genes that corresponded with any corrupted spot on the array from the image analysis. All the faulted spots were eliminated, but the corresponding gene was not eliminated. A gene was represented by 16 "combined" spots (each one, the combination of two spots on the same array). Thus even if some of the spots were faulted and eliminated there was other information that quantified expression of that gene. Different principles were adopted to filtered genes, here; the criterion was that a gene was retained for analysis if at least half of the spots exhibited no quality faults (8 out of 16).

Normalization

The quality diagnostic step showed a systematic intensity-dependent bias as well as differences due to the use of different print-tips to print the different blocks of spots on the array (Tilton et al, 2005). In order to correct this bias, the intensity data was normalized using the print-tip loess method within arrays. The Loess procedure (S+® 6.2 for Windows Professional Edition, Lucent Technologies, Inc, Seattle, WA) was applied as an intensity-based procedure while the print-tip loess accounted for location of the spot on the array.

Result interpretation

Transcripts that met the criteria, and were considered differentially expressed were categorized based on function using TIGR (www.tigr.org), Gene Ontology

(www.geneontology.org) and Pub Med (www.ncbi.nlm.nih.gov) databases. In some cases gene function was unknown, otherwise TIGR allowed derived and probable gene function that followed similarity with genes of other organisms.

Gene selection for Real time PCR

Microarray results were validated and more exact copy number of gene transcripts selected from the microarray experiment was determined by real time PCR (RT-PCR). A total of 6 genes (5 immunological and 1 toxicologically relevant genes) that were differentially up-regulated or down-regulated in any of the time points were selected: Interleukin 8 (IL 8), transport associated protein 1 (TAP 1), NF-kB essential modulator (NEMO), recombination activating gene 2 (RAG 2), major histocompatibility complex II (MHC II) and cytochrome P450 1A (CYP1A1). The first five genes were chosen because each represented an important component of the immune system. Selected genes were measured for RT-PCR confirmation at the time point that they appeared differentially expressed on microarray. Gene expression over time was followed with measured copy numbers at all time points in the groups exposed to bacterium and 2 and 20 days after challenge in the fish not exposed to *A. salmonicida.*

RT-PCR

Primer pairs were designed for each targeted gene by using the program Primer 3 (Whitehead Institute for Biomedical Research, Totowa, NJ). Amplicons of 60-120 base pairs were generated. Primer sequences were as follows: 5'-CCTGTGTGTCCTTCACATCG-3' and 5'- TGACTGAAGCCAATCACGAG-3' for interleukin 8 (IL8); 5'- GCATCACCACAGACACCAAC-3' and 5'-

GTGACCCGCATGAAGTACCA-3' for transport associated protein (TAP1); 5'-CAGGACCGCAACATACTGGAC-3' and 5'-GCTGCTTCCTCTGTTGTTCCA-3' for NF-kB essential modulator (NEMO); 5'- GCGCTGTCAGGAGAGAGAGT-3' and 5'- GCGCTGTCAGGAGAGAGAGT-3' for recombination activating gene 2 (RAG) 2; 5'- GTTCCTGGCCAACCTACAGA-3' and 5'-CCAATTACGTGCCCAAGTCT-3' for Major histocompatibility complex class II (MHC II); 5'- GATGTCAGTGGCAGCTTTGA-3' and 5'-CCTGGTCATCATGGCTGTA-3' for cytochrome P450 1A1 (CYP1A1). Primers were purified by using agarose gel electrophoresis. PCR conditions were optimized. To confirm that PCR yielded a pure product of the predicted size, we used Eppendorf master mix 2.5x (Eppendorf North America Inc, Westbury, NY) for a total reaction volume of 20 µl. We used Min Elute gel extraction kit protocol to extract and purify DNA from agarose gel. We quantitated the amplified cDNA of known size spectophotometrically in Nanodrop ND-1000 spectrophotometer (NanDrop Technologies, Wilmington, DE). A standard curve of cycle number to threshold vs. cDNA copy number was generated for each target gene (Gerwick et al, unpublished). The ng/µl value obtained in the spectrophotometer was converted to molecules per µl. we diluted to yield 10^1 to 10^6 molecules/ µl to be use as standard curve. We used SYBR® premix ex Taq™ (Takara Minus Bio Inc, Madison, WI) with primers at 20 µM final concentration, 0.4 µl of a 50X Rox™ solution (Invitrogen), 1µl of cDNA template diluted 1:200 (Gerwick et al, unpublished) for a final volume of 20 µl/ well. Samples were run in triplicate. An ABI 7000™ (Applied Biosystems, Foster City, CA) was programmed for 95°C x 30 s, 95 °C x 5 s, annealing and extension at 58-62 °C

(depending upon primer pair for 32 s). For comparison purposes with the microarray results, the reference sample was also measured.

RT-PCR data analysis

A one way ANOVA was performed for each gene selected. Significance was established at 95% confidence. We used Statgraphics (StatPoint Inc. Herndon Virginia) and Number Cruncher Statistical Software (Kaysville, Utah) for statistical analysis.

RESULTS

Gene expression analysis

All biomarkers of PAH effect and exposures increased after 50 days of pretreatment with 400 ppm PAH mixture (chapter II); therefore, this treatment was selected for comparison with control in microarray analysis.

The microarray transcript analysis detected as significantly altered 2, 4, 10 and 20 days after challenge with *A. salmonicida* were listed in Appendix 1. Head kidney gene expression was considered differentially expressed after a multiple comparison analysis and Bonferoni correction with a significance level of 95% among biological replicates (n=4) and after dye swapping. Overall 36 genes, 2 genes, 113 genes and 26 genes were differentially regulated between animals exposed and not exposed to PAH after 2, 4 10 and 20 days after challenge with *A. salmonicida* respectively.

In the microarray experiment the selected genes had the following fold change (PAH Vs. no PAH exposed to bacterium): IL8 was up regulated 2.45 fold 10 days after challenge; TAP 1 was down regulated -2.73 fold 2 days after challenge NEMO was

found -2.20 down regulated after 2 and 10 days post-challenge; RAG 2 was found -1.93 fold 10 days after challenge and MHC class II invariant chain that appeared -4.67 fold 10 days after challenge.

RT-PCR results

RT-PCR validated the microarray results and quantified gene transcript changes over time for the selected genes. The results showed that IL8 was significantly down regulated in 400 ppm PAH pretreated fish animals after 2 days ($p<0.05$), after 4 days no differences were observed between treatment and control ($p<0.05$); 10 and 20 days after challenge the gene was significantly down regulated ($p<0.05$). No recovery was observed after 20 days (Fig 4.1). The TAP 1 gene was only significantly down regulated 2 days after challenge ($p<0.05$), no statistically significant differences were observed after 4, 10 or 20 days. But the number of transcripts per 2.5 μg total RNA increased significantly in 400 ppm PAH pretreated animals after 4, 10 and 20 days post-challenge compared to the number of transcripts obtained 2 days after challenge (Fig 4.2). NEMO was down regulated 2 and 10 days after challenge ($p<0.05$) respectively. After 4 days no statistically significant differences were observed and after 20 days post-challenge NEMO was significantly higher in PAH-exposed fish than control (Fig 4.3). RAG 2 differences between treatment and control were significantly different at all time-points except 4 days after challenge ($p<0.05$); after 2 days, RAG 2 was down regulated significantly -3.47 fold, in PAH treated animals compared to control. After 10 days the genes were up regulated 0.3 fold and this difference is also significant ($p<0.05$), after 20 days is only 0.5 fold up regulated. This indicated that PAH down regulated RAG 2 and as the disease progressed the levels of

gene expression were increased. MHC II presented the same response as RAG 2 (Figs 4.4 and 4.5).

In summary, 10 days after challenge IL8, NEMO, RAG 2 and MHC II were differentially expressed in the microarray experiment these results were later confirmed with the RT-PCR. Pretreatment with 400 ppm PAH decreased the response of all genes 2 days after challenge. At 20 days after challenge differences were observed in IL8 and RAG 2 the transcript number was significantly higher than control and this indicated that the genes were close to control levels.

Additional to these immune genes, CYP1A1 response was determined by RT-PCR. The gene was significantly down regulated 2 days after challenge. Significant up regulation ($p<0.05$) was observed 10 and 20 days after challenge respectively in 400 ppm PAH pretreated fish compared to control (Fig 4.6).

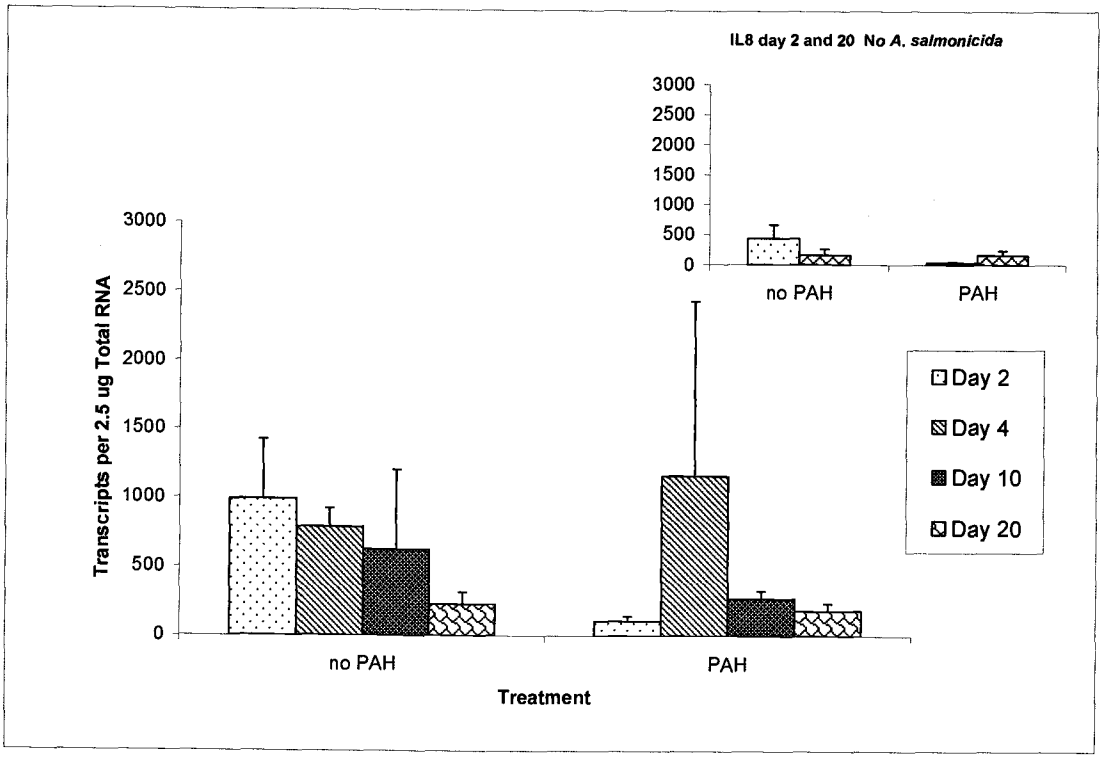


Figure 4.1 IL8 transcripts per 2.5 ug total RNA calculated in RT-PCR

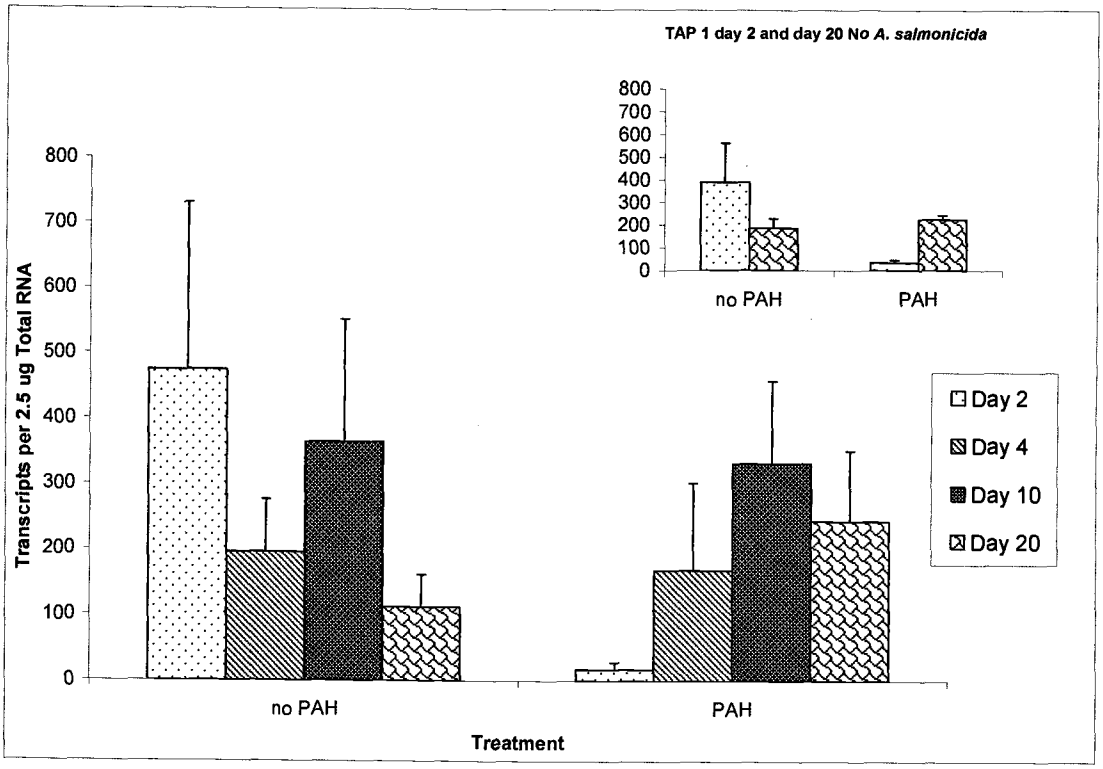


Figure 4.2 TAP 1 transcripts per 2.5 ug total RNA calculated in RT-PCR

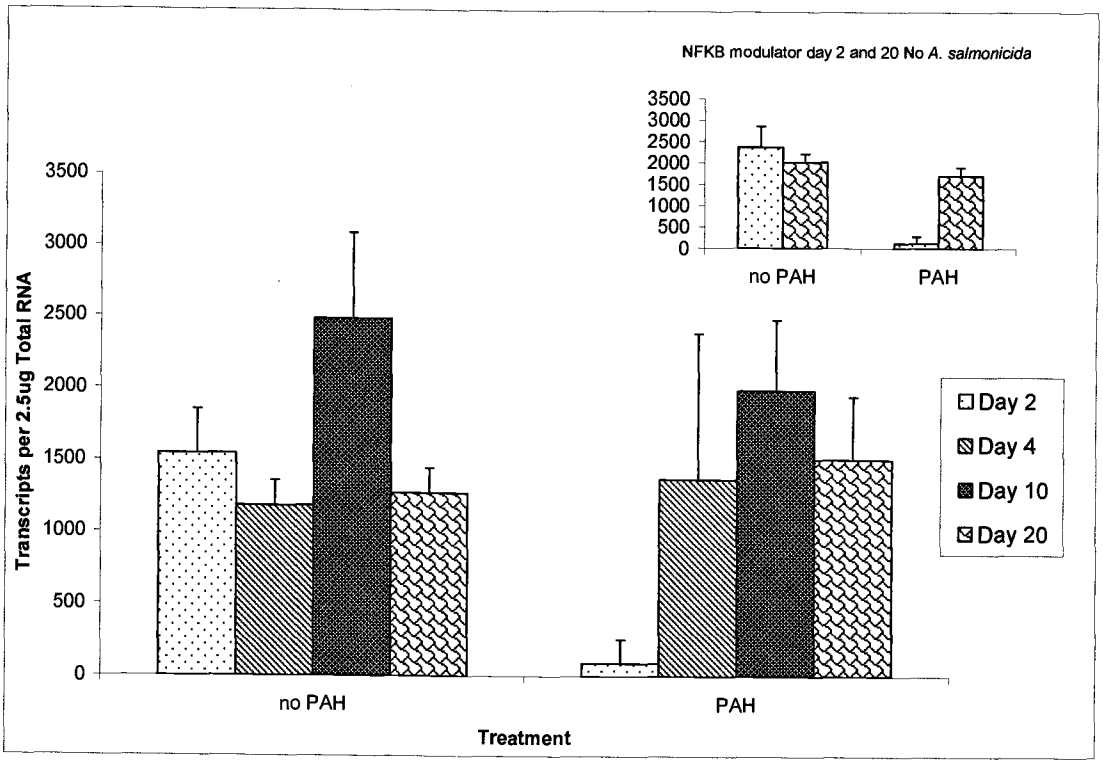


Figure 4.3 NF-kB modulator (NEMO) transcripts per 2.5 ug total RNA calculated in RT-PCR

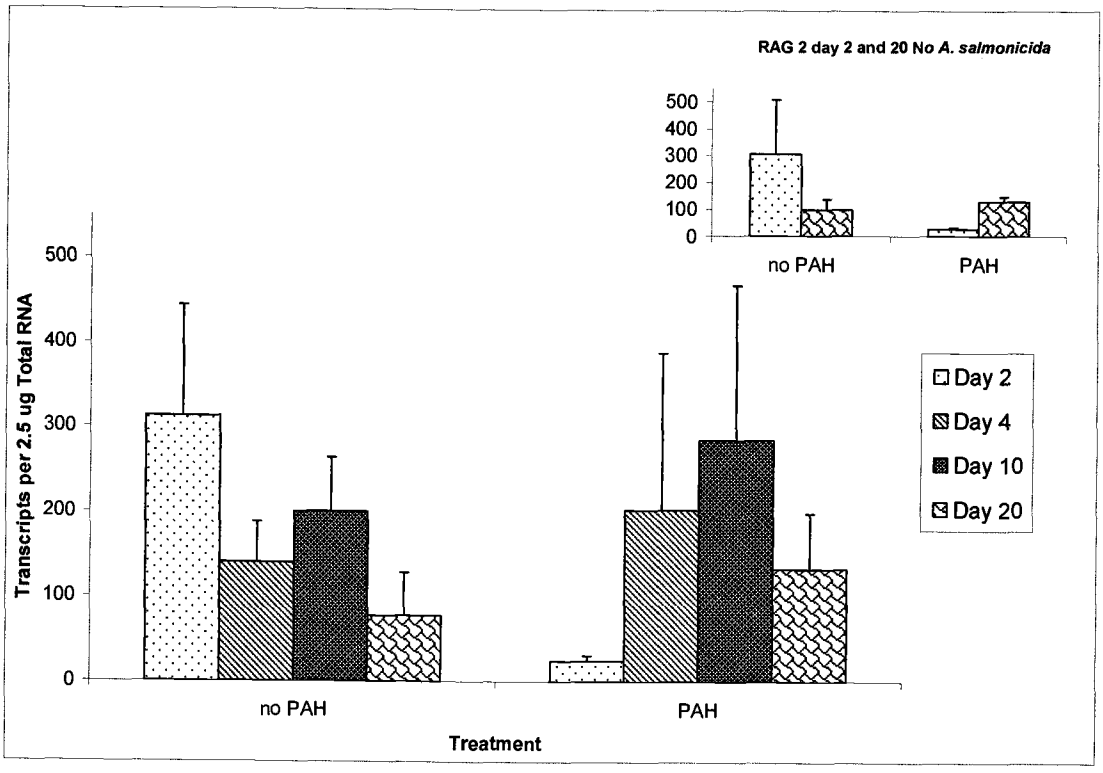


Figure 4.4 RAG 2 transcripts per 2.5 ug total RNA calculated in RT-PCR

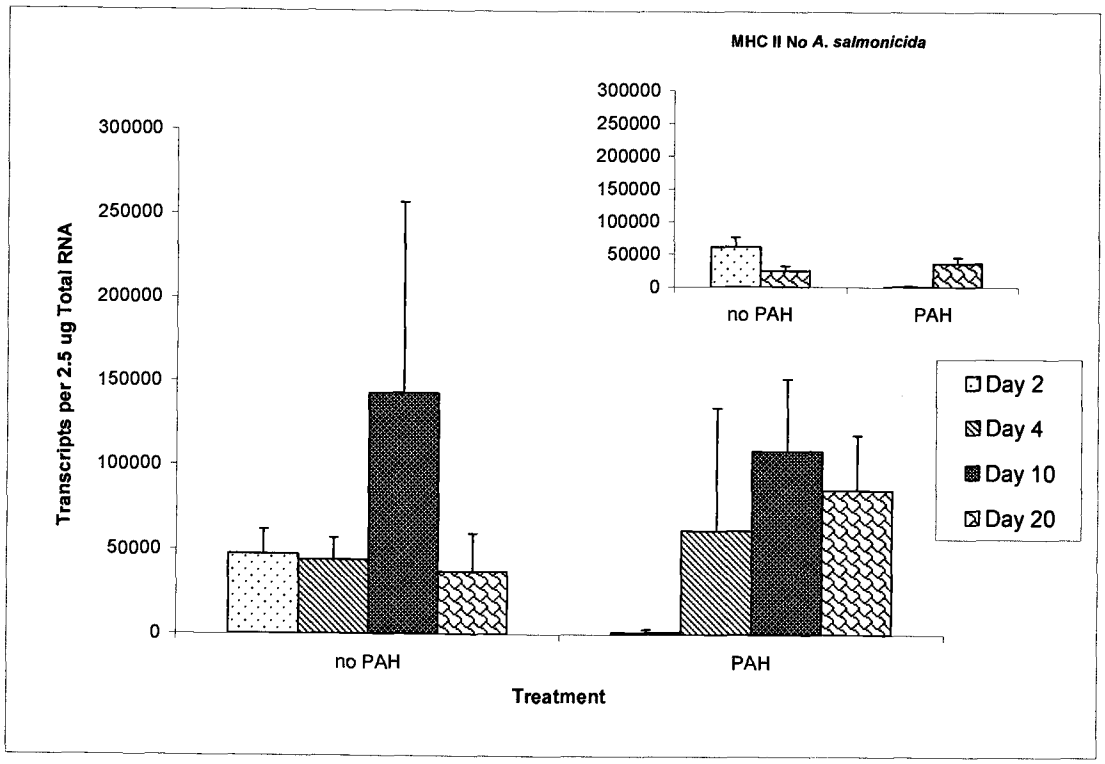


Figure 4.5 MHC II transcripts per 2.5 ug total RNA calculated in RT-PCR

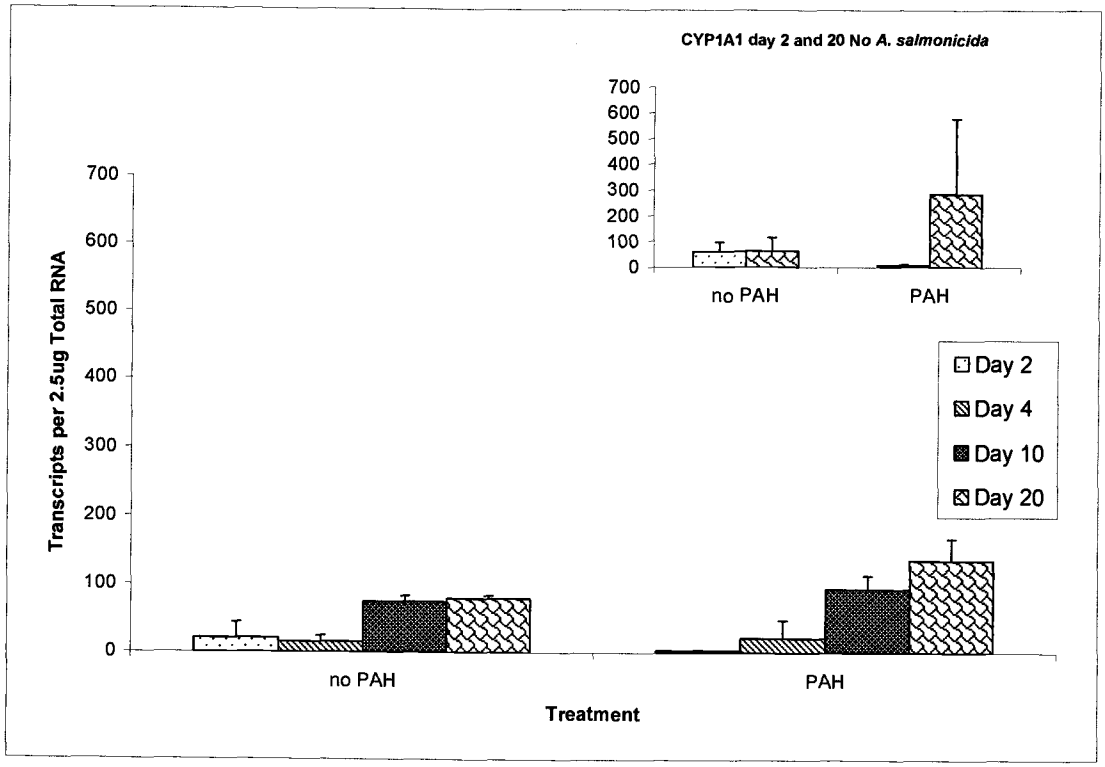


Figure 4.6 CYP1A1 transcripts per 2.5 ug total RNA calculated in RT-PCR

DISCUSSION

Gene expression profile

Several studies evaluated microarray analysis for chemical risk assessment for elucidation of mechanism of toxicity for specific compounds (Krasnov et al, 2005) and to search for new biomarkers. Gene expression profiling with microarray identified multiple candidate genes associated with different conditions such as immunosupression. This study was undertaken for increased understanding of the mechanism of toxicity of PAH and processes related to suppressed immune response. PAH were found in the environment as mixtures and fish were usually exposed to these compounds in the diet. Threatened species such as Pacific salmon bioaccumulated chemical contaminants during relative shortly time in the estuary (Arkoosh and Collier, 2002). In these same estuaries there was a widespread occurrence of pathogens in wild salmon (Arkoosh et al 2004). Previous studies demonstrated that disease appeared an important factor in population numbers of salmon in the Pacific Northwest (Arkoosh et al 2004). PAH were identified as immunosuppressant compounds most heavily concentrated in the estuary (Latimer and Zheng, 2003). Rainbow trout were fed PAH for 50 days to mimic the time that other salmonids spend in the estuary and then challenged with *A. salmonicida.* Gene expression profile was evaluated 2, 4, 10 and 20 days after challenge and candidate genes were selected as biomarkers of immunosupression and toxicity.

Different probes for the same gene spotted independently in different sites on the array and analyzed separately yielded consistent results indicating reproducibility (Vanya et

al, 1995). Among these genes MHC I appeared down regulated after 2 days with -3.34 and -2.06 fold change in a different probed oligos 10 days after challenge (Appendix 1). The majority of genes more highly up regulated or down regulated in head kidney were those involved in immune response and detoxication. Some of the genes in Appendix 1 increased during bacterial infection as expected. For example: precerebellin like protein previously reported as part of the trout acute phase serum was usually up regulated during bacterial infection (Gerwick et al, 2000). In our microarray experiment we detected precerebellin at -3.21 fold 10 days after challenge this indicated that PAH negatively affected genes that participated in acute phase response of rainbow trout to *A. salmonicida* infection.

The majority of the genes related to the immune response were down regulated. Genes that participated in innate immune response were important in response against infections (Vanya et al, 2005). There were not previous studies that measured transcriptome responses to chemical during bacterial infection. In this study, we observed down regulation of several classical markers of immunosupression and toxicity.

Overall more genes were down regulated than up regulated in response to infection after PAH exposure. Vanya et al (2005) found that fish challenged with *A. salmonicida* by cohabitation without previous chemical exposure most of the genes were up regulated and this was explained by innate immune response during development of bacterial infection. In this experiment NF-kB was up regulated and perhaps resulted in the induction of inflammatory cytokines important to mount an innate immune response. In our experiment NEMO, the modulator of NF-kB was

down regulated in PAH exposed fish and perhaps as consequence higher mortalities were obtained in fish exposed to PAH compare to control (chapter III).

Vanya et al (2005) reported c- type lectin as an immunologically relevant gene that was strongly up regulated during infection in salmon. Its presence was previously reported in kidney of Atlantic salmon infected with *A. salmonicida* (Tsoi et al, 2004). In the present study, it was -3.78 fold down regulated in PAH exposed fish 10 days after challenge (Appendix 1). The microarray experiment indicated that nuclear factor of activated T-cells (NFAT) was repressed -2.76 fold 10 days after challenge. This gene product was identified as a transcription factor that played an important role in immune and inflammatory response by regulating the expression of genes encoding cytokines and immunoregulatory proteins (Pan et al, 1997; Rao et al, 1997)). This protein was initially considered a T-cell specific transcription factor but later was detected in other immune system cells, like mast cells (Sherman et al, 1999). Studies with NFAT deficient mice demonstrated that this factor was responsible for IL4 production in activated T cells (de la Pompa et al,1998).

Functional genomic analyses of bacterial infection typically detected fewer down regulated genes than up regulated genes (Vanya et al, 2005). The current work indicated that PAH depressed immune gene expression in head kidney. More down regulated genes than up regulated and overall more mortality occurred in PAH pretreated compared to control indicating that PAH were immunosuppressant. The 400 ppm PAH mixture was selected for microarray analysis because the phenotypic anchoring responses were consistently significantly higher compared to control and other PAH treatments (chapter II). The 400 ppm PAH mixture treated animals also

experienced 10% increased mortality after exposure to LC_{20} and LC_{30} *A. salmonicida* compared to control (chapter III).

The water bath challenge of pathogen mimicked the natural route of infection and therefore the transcriptional responses were relevant to host defense. The advantage of this method was that all fish were exposed to pathogen at the same time and with the same bacterial concentration. This created a somewhat uniform response to infection that was evident in the lower variability present at the individual response. The majority of the mortalities occurred between 4-7 days post-challenge. The microarray experiment detected fewer genes differentially expressed 4 days after challenge compared to the other time points. This likely occurred at this time-point due to the individual differences in activation of the immune system of the fish. These events were important determinants of fish surviving or succumbing to infection at 10 days post-challenge. The fish that mounted an adequate innate immune response survived the mortality window. This was confirmed by the RT-PCR results that demonstrated high variability between individuals in selected genes 4 days after challenge.

RT-PCR

A group of 5 genes previously identified as important components of the immune system and that appeared differentially regulated at different time-points during the disease challenge were selected: IL8, TAP1, NEMO, RAG 2 and MHC II. IL8 and TAP 1 were associated with the innate immune response, NEMO participated in innate and adaptive immune response and RAG2 and MHC II were components of the adaptive immune response.

IL 8 was identified as a multifactorial cytokine that activated and attracted migratory neutrophils to an inflammatory site (Zhang et al, 2002). IL8 was down regulated significantly 2 days after bacterial challenge and remained down regulated in the PAH-treated fish 20 days after challenge (Fig 4.1). This was consistent with the results from the microarray experiment. The transcript of a similar gene that participated in the inflammatory response, CXC chemokine receptor gene was down regulated -2.28 fold in microarray experiments. Chemokine receptors were identified as G proteins-linked receptors that possess seven transmembrane domains; and were previously reported as expressed in trout kidney (Secombes et al, 1998; Sangrador-Vegas et al, 2001) with a proposed role in inflammatory response.

The MHC complex included a pair of related genes (TAP 1 and TAP 2), that encoded a large super family of proteins of similar structure and function (Grimholt, 1997). TAP 1 transported peptides to the endoplasmic reticulum for loading onto MHC class I molecules. Once loaded, class I molecules traveled to the cell surface and presented these peptides to T-cells, specifically, $CD8^+$ T cells (Grimholt, 1997; Elliot, 1997). TAP 1 and 2 genes in Atlantic salmon showed high sequence similarity with human, mouse and rat (Grimholt, 1997). In mammals, TAP 1 and 2 were required for transfer of peptides from cytosol to endoplasmic reticulum where class I molecules bind to the peptide TAP (Ohta et a. 1999). TAP genes map to the MHC in teleost fish (Grimholt, 1997). TAP 1 and 2 expression occurred in Atlantic salmon head kidney (Grimholt, 1997). TAP 1 mRNA was found in all tissues with its highest expression in spleen, intestine and liver (Ohta et al, 1999). In our study, TAP1 down regulation occurred 2 days after challenge in PAH treated animals with and without *A. salmonicida* (Fig

4.2). In fish fed control and then challenged with bacteria, the number of transcripts decreased from almost 500 at day 2 to 100 at day 20. The opposite effect is seen in fish fed PAH prior to bacteria challenge, transcript number increases from 20 at day 2 to 250 at day 20 (Fig 4.2).

The NF-kB transcription factor was an important regulator of genes involved in inflammation, cell proliferation and apoptosis (Barnes and Kann, 1997; May and Ghosh et al, 1998). NF-kB activation was achieved through the signal induced proteolytic degradation of IKB in the cytoplasm (Mercurio et al, 1999). Bacterial and viral products were potent inducers of NF-kB (Sangrador-Vegas et al, 2005). NF-kB was characterized in rainbow trout kidney. It induced the expression of genes involved in the immune response of fish (Beg and Baldwin, 1993; Sangrador-Vegas et al, 2005). Earlier studies (Mann et al, 2001) assessed the role of NF-kB in PAH induced pre B cell apoptosis. The results showed that BAP and 7, 12-dimethylbenz(a)anthracene (DMBA) down regulated NF-kB. The authors suggested that NF-kB down regulation was part of the molecular mechanism resulting in PAH-induced pre-B cell apoptosis. NF-kB was usually located in the cytoplasm bound to inhibitory proteins called IkB-α, IkB-β and IkB-ε. NF-kB essential modulator (NEMO) was required for bacterial lipopolyssacharide stimulation of NF-kB activity (Yamaoka et al, 1998). IKKγ/ NEMO were proteins of the IkB complex. NEMO was an essential factor required for NF-kB activation (Li et al, 2001; Li and Stank, 2002). NEMO binds to IKKα and IKKβ. Upon cell stimulation these two proteins degrade the inhibitors of NF-kB, a transcription factor that traslocates into the nucleus where it mediated the expression of various inflammatory and stress response genes (Ghosh et

al, 1998; Sangrador-Vegas et al, 2005). NEMO was significantly down regulated ($P<0.05$) 2 days after challenge in fish exposed to PAH compared to control. The NEMO mRNA levels returned close to control levels 4 days after challenge (Fig 4.3). Recombinase activating gene, formed part of a recombinase that consisted of RAG 1 and RAG 2. This complex was lymphoid specific and cleared DNA to form a double strand break that ubiquitous non homologous and joining proteins (NHEJ) repair (Hansen and Kaatari, 1996). V(D)J recombination was important in generation of a diverse immune repertoire during B and T cell development. Different loci rearranged and recombined by a common recombinase. The primary immunoglobulins (Ig) and antigen-receptor repertoire for B and T cells were generated by site-specific recombination events of variable region termed V(D)J recombination (Schutz et al, 1992) within the primary lymphoid organs. V(D)J recombination was the combinational process by which developing lymphocytes generated their enormous range of binding specificities from a limited amount of genetic information (Oettinger et al, 1990). The combination and stable expression of recombinase activity was dependent of two closely linked genes RAG-1 and RAG-2 (Oettinger et al, 1990; Hansen and Kaattari, 1996). The activation of V(D)J recombination, required both activated RAG genes in mice and in human (Oettinger et al, 1990). Mice that lacked either RAG 1 or RAG 2 were unable to initiate V(D)J recombination and produced no mature B and T lymphocytes (Lewis, 1994). Strong expression of RAG 1 and RAG 2 was previously found in the kidney of trout (Schutz et al, 1992; Hansen and Kaattari, 1996). The highest signal intensities for both genes occurred in thymus and kidney suggesting that these organs were the primary location for V(D)J recombination of

lymphocyte receptors in trout, similar results have been found in zebra fish (*Danio rerio*). This supports the idea that the thymus and the kidney are the major lymphoid organs in teleost (Hansen and Kaattari, 1996; Willet et al, 1997). The periodic expression of these two genes indicate that this locus is tightly regulated (Peixoto et al, 2000) if one of the genes is down regulated by PAH it is possible that the other gene is down regulated as well, they are closely linked and convergently transcribed (Peixoto et al, 2000). The distance between RAG 1 and RAG 2 is 2.4 kb in rainbow trout (Hansen and Kaatari, 1996). RAG 2 was down regulated 2 days after challenge and this occurred after PAH without exposure to bacterium (Fig 4.4). This suggests that PAH suppresses RAG 2 in trout.

Deficiencies in RAG 1 or 2 lead to a weakened immune system (Hansen and Kaatari, 1996).

The major histocompatibility complex (MHC) class II molecules in mammals present peptides derived from foreign proteins to CD4 T cells and activate the specific immune response (Mc Connell et al, 1998; Hansen et al, 1999; Fujiki et al, 2003). MHC down regulation occurs 2 days after challenge; transcript number however increases 4 days after challenge in PAH exposed animals. This down regulation is statistically significant ($P<0.05$) (Fig 4.5).

CYP1A down regulation in fish exposed to PAH occur 2 days after challenge. Down regulation of CYP1A by bacteria and their products has been previously reported in carp (Chambras et al, 1999 and Vanya et al, 2005) (Fig 4.6).

All genes evaluated by RT-PCR after identification of repression by microarray were significantly down regulated ($P<0.05$) 2 days after challenge. This was also observed

in fish not exposed to bacterium. This confirmed that PAH down regulated the selected genes. Mechanisms of this down regulation were not resolved but our results confirmed that PAH were immunosuppressant compounds (Mann et al, 2000).

The transcripts levels of the selected genes increased 4 days after challenge in fish exposed to PAH, the opposite was true for animals not exposed to PAH. This suggested that PAH exposed animals recovered from exposure 20 days after challenge. The microarray results showed that the effects of exposure to PAH 20 days after challenge caused the down regulation of other immune related genes that were not differentially expressed in animals not exposed to *A. salmonicida*. This indicates that even though there is a recovery from exposure at this time point in the selected genes, there are some other immune genes that remain affected at the end of the challenge.

Application of RT-PCR for microarray validation of differentially expressed genes improved quantification. In several cases RT-PCR showed a higher down regulation or up regulation compared to the microarray but the orientation was similar for both techniques. This indicated that RT-PCR was more sensitive than microarray. We also observed in some cases 20 days after challenge where gene expression changed less than 2 fold in microarray and was not significantly different, RT-PCR detected differential expression. This indicated that the non- statistical criteria of selecting the genes that are 2.0 or more fold changed as significant in microarray was not always accurate and this increased the chance of false negatives. Bonferoni correction used in our study increased the number of false negatives. This argued against use of the fold

change criteria to select genes as differentially expressed in the microarray as previously described (Tilton et al, 2005).

In summary, these data indicated that microarray was a valuable and reliable tool for broadly scanning genes of interest and that the method of analysis eliminated false positives. It allowed the selection of meaningful genes quantified with RT-PCR which supported the microarray results.

The change in gene transcription profile in response to *A. salmonicida* infection in fish with or without prior PAH exposure is of special interest. Furunculosis is a systemic infection. The successful pathogen avoids, withstand or overcome the non-specific and specific defense mechanisms of the host (Evenberg et al, 1985). The information reported here shows that PAH are immunosuppressant to fish at environmentally relevant concentrations. We observed that the genes differentially expressed in all time points corresponded to innate and acquired immunity genes in fish that have been previously been described as important components of resistance of salmon to *A. salmonicida* (Gerwick et al, 2002). This study is one of the first to report gene expression profile in fish challenged to *A. salmonicida* after PAH exposure.

This study provided a comprehensive profile of transcriptional response in rainbow trout after exposure to PAH during *A. salmonicida* challenge. Several genes that participated in immune response regulation were uncovered; this provided further knowledge in to the mechanism of immunosupression of PAH. Some of the genes revealed to be differentially expressed were unknown. The salmon / *A. salmonicida* model was highly informative because the biology and immunology of salmon was

well understood (Vanya et al, 2005). Additionally rainbow trout response to PAH was previously studied and characterized (Hendricks et al, 1985)

AKNOWLEDGMENTS

The authors wish to thank Dr. Lena Gerwick for her advice and training on the microarray technique, to Caprice Rosato for the printing of the slides, Cheri Goodall, and Jana Brooks for advice in the design of primers and training on the RT-PCR technique and technical as well as scientific advice in the gene selection for RT-PCR.

REFERENCES

Arkoosh M , Casillas E, Clemons E, McCain B and Varanasi U, 1991. Suppression of Immunological memory in Juvenile chinook salmon (*Oncorhynchus tshawytsha*) from an urban estuary. Fish and Shellfish Immunology 1: 261-277.

Arkoosh M, Clemons E, Huffman P, Sanborn R, Casillas E and Stein J. 1996. Leukoproliferative response of splenocytes from English sole (*Pleuronectes vetulus*) exposed to chemical contaminants. Environmental Toxicology and Chemistry 15(7): 1154-1162.

Arkoosh M, Casillas E, Huffman P, Clemons E, Evered J, Stein J and Varanasi U. 1998. Increased susceptibility of juvenile Chinook salmon from a contaminated estuary to *vibrio anguillarum*. Transactions of the American Fisheries Society. 127: 360-374.

Arkoosh M, Clemons E, Huffman P and Kagley A. 2001. Increased Susceptibility of juvenile Chinook salmon to vibriosis after exposure to chlorinated and aromatic compounds found in contaminated urban estuaries. Journal of Aquatic Animal Health 13: 257-268.

Arkoosh M and Collier T, 2002. Ecological Risk Assessment Paradigm for salmon: Analyzing Immune Function to Evaluate Risk. Human and Ecological Risk Assessment: Vol 8 (2): 265-276.

Arkoosh M, Clemons E, Kagley A, Stafford C, Glass A, Jacobson K, Reno P, Myers M, Casillas E, Loge F, Johnson L and Collier T. 2004. Survey of Pathogens in Juvenile salmon *Oncorhynchus spp* migrating through Pacific Northwest estuaries. Journal of Aquatic Animal Health 16: 186-196.

Barnes P and Kann M, 1997. Nuclear Factor-kappa B: a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine. 336: 1066-1071.

Beg A, Baldwin A, 1993. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev 7: 2064-2070.

Carlson E and Zelikoff J. 2004. benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*) relationship between lymphoid CYP1A1 activity and humoral immunosupression. Toxicology and Applied Pharmacology 201: 40-52.

Carlson E and Zelikoff J. 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo(a)pyrene suppresses immune function and host resistance against bacterial challenge. Aquatic Toxicology 56: 289-301.

Chambras C, Manonet D, Tayssel D, Deschauux P, Moreau J and Bosgraud C, 1999. Xenobiotic-metabolizing enzymesnin carp (*Cyprinus carpio*) liver, spleen and head kidney following experimental *Listeria monocytogenes* infection. Journal of Toxicology and Environmental Health A: 56: 205-219.

Davila D, Davis D, Campbell K, Cambier J, Zigmond L, Burchiel S. 1995. Role of alterations of Ca^{2+} associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. Journal of Toxicology and Environmental Health 45:101-126.

De la Pompa J, Timmerman L, Takimoto H, Yoshida A, Elia J, Samper E, Potter A, Wakeham L, Marengere B, Lagille L. 1998. Role of NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature 392: 182.

Elliot T, 1997. Transporter associated with antigen processing. Advance Immunology 65:47

Evenberg D, Verslius B and Lugtenberg B, 1985. Biochemical and immunological characterization of the cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. Biochemica et Biophysica Acta 815: 233-234.

Fujiki K, Smith C, Liu L, Sandick R and Dixon B, 2003. Alternative forms of MHC class II associated invariant chain are not produced by alternative splicing in rainbow trout (*Oncorhynchus mykiss*) but are encoded by separate genes. Developmental and Comparative Immunology 27: 377-391.

Gerwick L, Reynolds W, Bayne C. 2000. A precerebellin-like protein is part of the acute phase response in rainbow trout. Developmental and Comparative Immunology 24: 597-607.

Gerwick L, Steinhauer R, Lapatra S, Sandell T, Otuno J, Hajiseyedjavadi N, Bayne C. 2002. Changes in Plasma proteins of rainbow trout in response to inflammatory agents. Fish and Shellfish Immunology. 12: 229-242.

Ghosh S, May M and Koop E. 1998. NF-kB and rel proteins: evolutionarily conserved mediators of immune responses. Annual Review Immunology 16: 225-260.

Grimholt U. 1997. Transport-associated proteins in Atlantic salmon (*Salmo salar*). Immunogenetics 46: 213-221.

Gulland F, 1995. The impact of infectious diseases on wild animal populations-a review in: Grantal B and Dobson A (eds). Ecology of infectious diseases in natural populations. Cambridge University press Pp 21-51.

Hansen J, Strassburger P, Thorgaard G, Young W and Pasquier L. 1999. Expression, linkage and polymorphism of MHC- related genes in rainbow trout, *Oncorhynchus mykiss*. The Journal of Immunology 774-786.

HansenJ, Kaattari S, 1996. The recombination activating gene 2 (RAG 2) of the rainbow trout *Oncorhynchus mykiss*. Immunogenetics 44: 203-211.

Hedrick R, 1998. Relationship of the Host pathogen and environment: Implications for diseases in cultured and wild fish populations. Journal of Aquatic Animal health 10: 107-111.

Hendricks J, Meyers T, Shelton D, Casteel J and Bailey G. 1985. Hepatocarcinigenicity of BAP to rainbow trout by dietary exposure and intraperitoneal injection. Journal National Cancer Institute.74: 839-851.

Hellou J, 1996. Polycyclic Aromatic Hydrocarbons in Marine Mammals, finfish and mollusk in: Beyer N, Heinz G, Redmon-Norwood A. Environmental contaminants in wildlife interpreting tissue concentrations CRC Lewis Publishers Pp 229-250.

Karrow N, Buermans D, Dixon G, Honfella K, Solomin J, White J and Bols N. 1999. Characterizing the immunotoxicity of creosote to rainbow trout *Oncorhynchus mykiss*: A microcosm study. Aquatic Toxicology 45: 223-239.

Krasnov A, Koskinen H, Rexroad C, Afanasyev S, Molsa H, Oikari A 2005. Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology. Aug 15; 74(1):70-81.

La Patra S, Fryer J, Wingfield W and Hedrick R. 1989. Infectious hepatopoyetic necrosis virus (IHNV) in coho salmon. Journal of Aquatic Animal Health 1:277-280.

Lang D, Becker S, Devlin R, Koren H. 1998. Cell specific differences in the susceptibility of potential cellular targets of human origin derived from blood and lung following treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Cell Biology Toxicology 14: 23-38.

Latimer J and Zheng. 2003. The sources, transport and fate of PAHs in the marine environment. In: Douben P (ed). PAHs an Ecotoxicological perspective. John Wiley and sons Ltd. Pp 9-32

Lewis S. 1994. The mechanism of V(D)J joining: Lessons from Molecular Immunological and Comparative Analysis. Advances Immunology 56: 27-50.

Li X and Stank G, 2002. NF-kappa B-dependent signaling pathways. Experimental Hematology 30: 285-296.

Li X, Fang X and Gaynor R, 2001. Role if Ikkγ/ NEMO in assembly of the IKb kinase complex. The Journal of Biological Chemistry. Vol 276 No 6: 4494-4500.

Lloyd S, 1995. Environmental Influences on host immunity in: Grentell B and Dobson A. Ecology of infectious diseases in natural populations. Cambridge University press. Pp 327-361.

Loge F, Arkoosh M, Ginn T, Johnson L and Collier T. 2005. Impact of Environmental stressors on the dynamics of disease transmission. Environmental Science and Technology Sep 15;39(18):7329-36.

Mann K, Doerre S, Sclezinger J, Sherr D and Quadri S. 2001. The role of NK-kB as survival factor in Environmental chemical-induced pre-B cell apoptosis. Molecular pharmacology Vol 59: No2: 302-309.

May M and Gosh S, 1998. Signal Transduction through NF-kB. Immunology Today 19: 80-88.

Mc Connel T, Godwin U and Cutriberson B. 1998. Expressed Major Histocompatibility Complex class II loci in Fishes. Immunological Reviews. Vol 166: 294-300.

Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M, Manning AM. 1999. IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Molecular Cell Biology19(2):1526-38.

Nehlsen W, Williams J and Lichatowich J. 1991. Pacific salmon at the crossroads: Stocks at risk from California, Oregon, Idaho and Washington. Fisheries 16: 4-21.

Oettinger M, Schutz D, Gorka C and Baltimore D, 1990. RAG-1 and RAG-2 adjacent genes that synergistically activate V(D)J recombination. Science Vol 248:1517-1522.

Ohta V, Haliniewski D, Hansen J and Flajnik M, 1999. Isolation of transporter associated with antigen processing genes TAP 1 and TAP 2, from the horned shark *Heterodontus francisci*. Immunogenetics 49: 981-986.

Page T, Mac Williams P, Suresh M, Jefcoate C and Czuprynski C. 2004. 7-12 dimethylbenz[a]anthracene induced bone marrow hypocellularity is dependent on signaling through both the TNFR and PKR. Toxicology and Applied Pharmacology 198: 21-28.

Pallardy N, House R and Dean J. 1989. Molecular mechanism of 7,12-dimethylbenz[a]anthracene-induced immunosupression: evidence for action via the interleukin-2-pathway. Molecular Pharmacology 36(1) 128-133.

Pan S, Kayano-Nakagawa N, Tsuruta L, Amasaki Y, Yokota T, Mori S, Arai N and Arai K. 1997. Molecular cloning and functional characterization of murine cDNA encoding transcription factor NFATc. Biochemical and Biophysical research communications 240: 314-323.

Payne J, Matnieu A and Collier T, 2003. Ecotoxicological Studies focusing on marine and freshwater fish in: Douben P (ed). PAH an Ecotoxicological perspective. John Wiley and Sons LTD. Pp 101-224.

Peixoto B, Mikawa Y, Brenner S. 2000. Characterization of the recombinase activating gene-1 and 2 locus in the Japanese pufferfish *Fugu rubripes*. Gene 246:275-283.

Quinn T, 2005. The behavior and Ecology of Pacific Salmon and trout. Unv of Washington press. Pp 3-27

Rao A, Luo C and Hogan P, 1997. Transcription factors of the NFAT family regulation and function. Annual Reviews in Immunology. 15:707-47. Review

Sangrador-Vegas A. Smith T and Cairns M. 2005. Cloning and Characterization of a homologue of the alpha inhibitor of NF-kB in rainbow trout *Oncorhynchus mykiss*. Veterinary Immunology and Immunopathology. 103: 1-7.

Sangrador-Vegas A, Leanington J, Smith T. 2001. Molecular Cloning of a IL8 like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression substractive hybridization. Cytokine 17: 66-70.

Schutz D, Oettinger M and Schlissel M. 1992. V(D)J recombination: Molecular Biology and Regulation. Annual Reviews in Immunology. 10: 359-383.

Secombes C, Zou J, Daniels C, Cunningham C, Koussounandis A and Kemp G. 1998. Rainbow trout cytokine and cytokine receptor genes. Immunological Reviews Vol 166:333-340.

Sherman M, Powell D, Weiss D and Brown M, 1999. NF-ATc isoforms are differentially expressed and regulated in murine T and mast cells. The Journal of Immunology. 162: 2820-2828.

Silkworth JB, Lipinskas T, Stoner CR. 1995. Immunosuppressive potential of several polycyclic aromatic hydrocarbons (PAHs) found at a Superfund site: new model used to evaluate additive interactions between benzo[a]pyrene and TCDD. Toxicology.105(2-3):375-86.

Sinndermann C, 1990. Principal Diseases of Marine fish and shellfish. Academic press New York.

Tilton S, Gerwick L, HendricksJ, Rosato C, Smith G, Givan S, Bailey G, Bayne C and Williams D, 2005. Use of rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B1 induced hepatocellular carcinoma compared to adjacent liver. Toxicological Sciences advance Access Published Sep 1-2005.

Tsoi S, Ewart K, Penny S, Melville K, Liebscher R, Brown L. 2004. Identification of Immune relevant genes from Atlantic salmon using suppression subtractive hybridization. Marine Biotechnology 6: 199-214.

Vanya E, Belanger J, Williams J, Karakach T, Penny S, Tsoy S, Richards R and Douglas S, 2005. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Developmental and Comparative Immunology 29: 333-347.

White L, Kawabata T and Ladics G, 1994. Mechanisms of polycyclic aromatic hydrocarbon Immunotoxicity. In: Dean J, Luster M, Munson A and Kimber E (Eds). Immunotoxicology and Immunopharmacology Second Ed. Raven Press Ltd. New York. Pp 123-142.

Willet C, Cherri J, Steiner L, 1997. Characterization and Expression of the Recombination Activating Gene (RAG1 and RAG2 of Zebrafish. Immunogenetics 45: 394-404.

Yamaoka S, Courtois G, Bessia C, Whiteside S, Weil R, Ayat F, Kirk H and Israel A. 1998. Complementation cloning of NEMO, a component of the IkB kinase complex essential for NF-kB activation. Cell Vol 93: 1231-1240.

Zelikoff J, Raymond A, Carlson E, Li Y, Beaman J and Anderson M, 2000. Biomarkers of Immunotoxicity in Fish: from the lab to the ocean. Toxicology letters 112-113: 235-331.

Zhang H, Thorgaard G, Ristow S. 2002. Molecular Cloning and genomic Structure of an Interleukin-8 receptor-like gene from homozygous clones of rainbow trout *Oncorhynchus mykiss*. Fish Shellfish Immunology. Sep;13(3):251-8.

Chapter V

CONCLUSIONS

The research presented in this dissertation describes some of the effects on immune response after polycyclic aromatic hydrocarbon exposures in juvenile rainbow trout. Induction of cytochrome P4501A (CYP1A) occurred and probably contributed to oxidative stress. Aryl hydrocarbon receptor was likely involved in many but not all instances. BEP induced CYP1A as demonstrated by EROD and immunohistochemistry in liver. BEP AHR affinity was weak indicating that other mechanisms such as 4s, 8s protein might be involved. The remaining high molecular weight PAH selected for this study probably induced CYP1A through the AHR pathway that metabolized PAH in liver and kidney tissues. These metabolites were perhaps further metabolized to other compounds such as quinones that generated reactive oxygen species causing oxidative damage observed in blood, kidney and liver tissues of rainbow trout. Effects increased after 3 days of exposure, peaked at 14 days and were reduced close to control levels by 50 days indicating that rainbow trout was able to adapt to insult by using its repair mechanisms. We conclude that for high molecular weight PAH a combination of AHR and oxidative stress are responsible for the harmful effects observed in the organs evaluated. There was an adaptive response to PAH by these fish after long-term exposure (chapter II).

The disease challenge experiment (chapter III) suggests that rainbow trout exposed to PAH are more susceptible to disease than controls. PAH with similar chemical structures such as benzo(e)pyrene (BEP) and benzo(a)pyrene (BAP) appear to have

different mechanism of action to increase disease susceptibility. The environmentally relevant mixture of PAH showed the highest effect on disease susceptibility overall. PAH affect the transcription of important immune genes (chapter IV). Understanding this as a potential mechanism of immunosupression is important and it may help explain increased disease susceptibility in PAH-exposed fish. PAH down-regulated genes that participate in innate (interleukin 8, transport associated protein 1) and adaptive (recombination activating gene 2 and major histocompatibility complex) as well as genes that have an important role in innate and adaptive immune response (NF-kB modulator) two days after challenge. This could mean that during the time that fish are exposed to PAH their immune response is affected and disease susceptibility is increased.

Although the above research has significantly contributed to our understanding of PAH mechanisms of action in immune response in fish, there is always more insight that can be gained through continuous research efforts. Additional more candidate genes should be investigated with RT-PCR to identify potential biomarkers of PAH exposure in diseased organisms. We believe that future research should include the study of these candidate genes at the protein level and measure these potential biomarkers at different time points after stopped PAH exposure.

REFERENCES

Aas B and Goksoyr A, 2000. Fixed wavelength fluorescent (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filters effect and signal interpretation. Biomarkers 5: 9-23.

Aas E, Baussant T, Balk L, Liewenborg B, Anderssen O, 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory exposure with Atlantic cod. Aquatic Toxicology 51: 241-258.

Adams A, Leschen W, Wilson A and Horne M.T. 1987. A bath challenge model for furunculosis in rainbow trout, *Salmo gairdneri* Richardson and Atlantic salmon *Salmo salar L.* Journal of Fish Diseases 10: 495-504.

Ahokas I. 1984. Metabolic activation of PAH by fish liver CYP450. Marine Environmental research 14: 59-69.

Anderson D, Dhawan A, Yu T, Plewa M, 1996. An investigation of bone marrow and testicular cell *in vivo* using the comet assay. Mutation Research 370: 159-174.

Anderson D, Yu D, Phillips B and Schmezer, 1994. The effects of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in the COMET assay. Mutation Research 307: 261-271.

Anderson T. 1992. Purification, characterization and regulation of male-specific cytochrome P450 in the rainbow trout kidney. Marine Environmental Research 34: 109-112.

Anon. 1981. Effects on the Immune Systems. Environmental Pathology Toxicology, 5(1):222-223.

Arkoosh M , Casillas E, Clemons E, McCain B and Varanasi U, 1991. Suppression of Immunological memory in Juvenile chinook salmon (*Oncorhynchus tshawytsha*) from an urban estuary. Fish and Shellfish Immunology 1: 261-277.

Arkoosh M, Clemons E, Myers M and Casillas E. 1994. Suppression of B-cell Mediated Immunity in juvenile chinook salmon (*Oncorhynchus tshawytsha*) after exposure to either polycyclic aromatic hydrocarbon or polychlorinated byphenyls. Immunopharmacology and immunotoxicology 16(2) 293-314.

Arkoosh M, Clemons E, Huffman P, Sanborn R, Casillas E and Stein J. 1996. Leukoproliferative response of splenocytes from English sole (*Pleuronectes vetulus*) exposed to chemical contaminants. Environmental Toxicology and Chemistry 15(7): 1154-1162.

Arkoosh M, Casillas E, Huffman P, Clemons E, Evered J, Stein J and Varanasi U. 1998a. Increased susceptibility of juvenile Chinook salmon from a contaminated estuary to *vibrio anguillarum*. Transactions of the American Fisheries Society. 127: 360-374.

Arkoosh M, Casillas E, Clemons E, Kagley A, Olson R, Reno P, Stein J. 1998b. Effect of Pollution on Fish Diseases: Potential Impacts on Salmonid Populations. Journal of Aquatic Animal Health 10:182-190.

Arkoosh M, Clemons E, Huffman P and Kagley A. 2001. Increased Susceptibility of juvenile chinook salmon to vibriosis after exposure to chlorinated and aromatic compounds found in contaminated urban estuaries. Journal of Aquatic Animal Health 13: 257-268.

Arkoosh M and Collier T, 2002. Ecological Risk Assessment Paradigm for salmon: Analyzing Immune Function to Evaluate Risk. Human and Ecological Risk Assessment: Vol 8 (2): 265-276.

Arkoosh M, Clemons E, Kagley A, Stafford C, Glass A, Jacobson K, Reno P, Myers M, Casillas E, Loge F, Johnson L and Collier T. 2004. Survey of Pathogens in Juvenile salmon *Oncorhynchus spp* migrating through Pacific Northwest estuaries. Journal of Aquatic Animal Health 16: 186-196.

Austin B and Adams C. 1996. Fish pathogens Ch 8 in: Austin B, Altwegg M, Goslin P and Joseph S. The genus Aeromonas. John Wiley and sons. Chinchester west Sussex England. Pp 197-243.

Austin B and Austin A. Bacterial Fish pathogens: disease in farmed and wild fish. Ellis Horwood Limited publishers Pp 154.

Bailey S, Williams D and Hendricks J, 1996. Fish Models for Environmental carcinogenesis : The rainbow trout. Environmental Health Perspectives Vol 104 Suppl 1: 5-21.

Barnes P and Kann M, 1997. Nuclear Factor-kappa B: a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine. 336: 1066-1071.

Beg A, Baldwin A, 1993. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev 7: 2064-2070.

Blanton H. Lyte M, Myers M and Bick P. 1986. Immunomodulation by polyaromatic hydrocarbons in mice and murine cells. Cancer research 46: 2735-2739.

Bricknell I. 1995. A reliable method for the induction of experimental furunculosis. Journal of fish diseases. 18: 127-133.

Burns-Naas L, Meade J, Munson A: Toxic Responses of the Immune System in: Curtis D. Klaassen (ed): Cassarett and Douls Toxicology the Basic Science of Poisons. McGraw-Hill 6th edition. 2001. pp 419-460.

Canadian Environmental Protection Act. 1994. Priority Substances List Assessment Report. Polycyclic Aromatic Hydrocarbons. Government of Canada. Canadian cataloguing in Publication Data (ed). pp 10-25

Carlson D, Zelikoff J. 2002a. Exposure to Japanese Medaka (*Oryzias latipes*) to Benzo(a)pyrene Suppresses Immune Function and Host Resistance Against Bacterial Challenge. Aquatic Toxicology, 56:289-301.

Carlson D, Zelikoff J. 2002b. The Japanese Medaka (*Oryzias latipes*) Model: Applicability for Investigating the Immunosuppressive Effects of the Aquatic Pollutant Benzo(a)pyrene (BaP). Marine Environmental Research, 54:565-568

Carlson E and Zelikoff J. 2004. benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*) relationship between lymphoid CYP1A1 activity and humoral immunesuppression. Toxicol and Applied Pharmacology 201: 40-52.

Carnahan A and Altwegg M.1996. Taxonomy in: Austin B, Altwegg M, Goslin P and Joseph S. The genus Aeromonas. John Wiley and sons. Chinchester west Sussex England. Pp 1-38.

Chambras C, Manonet D, Tayssel D, Deschauux P, Moreau J and Bosgraud C, 1999. Xenobiotic-metabolizing enzymesnin carp (*Cyprinus carpio*) liver, spleen and head kidney following experimental *Listeria monocytogenes* infection. Journal of Toxicology and Environmental Health A: 56: 205-219.

Collier TK, Stein J, Wallace R and Varanasi U. 1986. Xenobiotic metabolizing enzymes in Spawning English sole (*Parophrys vetulus*) exposed to organic solvent extracts of marine sediments from contaminated and reference areas. Comparative Biochemistry and Physiology C:84:291-298

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

Collier T.K, L.L Johnson, M.S Myers, C.M Stehr, M.M Krahn and J. E Stein. 1998. Fish Injury in the Hylebos Waterway in Commencement Bay, Washington. U.S Department of Commerce, NOAA Technical Memo, NMFS-NWFSC-36, Washington D.C (available at www.nwfsc.noaa.gov/pubsl).

Crisp D. 1999. Trout and salmon: ecology conservation and rehabilitation. Blackwell Science. Pp 88-89.

Davila D, Davis D, Campbell K, Cambier J, Zigmond L, Burchiel S. 1995. Role of alterations of Ca^{2+} associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. Journal of Toxicology and Environmental Health 45:101-126.

Day J, Hall C, Kemp W and Yanez-Aracibia A. 1989. Estuarine Ecology. Wilet NY.

De la Pompa J, Timmerman L, Takimoto H, Yoshida A, Elia J, Samper E, Potter A, Wakeham L, Marengere B, Lagille L. 1998. Role of NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature 392: 182.

Dean J, Luster M, Boorman G, Lauer L, Leubke R and Lawson L. 1983. selective immunosupression resulting from exposure to the carcinogenic congener of benzopyrene in BGC3F1 mice. Clinical and Experimental Immunology Apr 52: 199-206.

Di Giulio R.T, Benson W.H, Sanders B.M and Van Veld P.A. 1995. Biochemical Mechanisms: Metabolism, Adaptation and Toxicity in: Rand G.M Ed. Fundamentals of Aquatic Toxicology: effects, environment and risk assessment. Second Edition. Taylor and Francis Publishers. Pp 523-561.

Dunier M and Siwicki A. 1995. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish and shellfish immunology 3: 423-438.

D.W.T Au, R.S.S.W Wu, B.S Zhou, P.K.S Lam, 1999. Relationship between ultra structural changes and EROD activities in liver of fish exposed to Benzo(a)pyrene. Environmental Pollution 104: 235-247.

Eggens M and Galgani F. 1992. Ethoxyresorufin-O-deethylase (EROD) Activity in Flatfish: Fast Determination with a Fluorescent Plate-Reader. Marine Environmental Research Vol 3 No3: 213-221

Elliot T, 1997. Transporter associated with antigen processing. Advance Immunology 65:47

Evenberg D, Verslius B and Lugtenberg B, 1985. Biochemical and immunological characterization of the cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. Biochemica et Biophysica Acta 815: 233-234.

Fairbairn D, Olive P, O'Neil K, 1995. The comet assay: a comparative review. Mutation Research 339: 37-59.

Faisal M and Hugget R. 1993. Effects of polycyclic Aromatic hydrocarbons on the lymphocyte mitogenic responses in spot *Leistomus xanturus*. Marine Environmental Research 35: 121-124.

Fevolden S, Kefstic T and Roed K. 1992. Disease resistance in rainbow trout (*Oncorhynchus mykiss*) selected for stress response. Aquaculture 104: 19-29.

Fujiki K, Smith C, Liu L, Sandick R and Dixon B, 2003. Alternative forms of MHC class II associated invariant chain are not produced by alternative splicing in rainbow trout (*Oncorhynchus mykiss*) but are encoded by separate genes. Developmental and Comparative Immunology 27: 377-391.

Gamal A, Abd-Allah,Refaat I, Micah S, Heckman R and O'Neill K. 1999. A comparative Evaluation of Aflatoxin B1 genotoxicity in fish models using the comet assay. Mutation research 446: 181-188

Gerwick L, Reynolds W, Bayne C. 2000. A precerebellin-like protein is part of the acute phase response in rainbow trout. Developmental and Comparative Immunology 24: 597-607.

Gerwick L, Steinhauer R, Lapatra S, Sandell T, Otuno J, Hajiseyedjavadi N, Bayne C. 2002. Changes in Plasma proteins of rainbow trout in response to inflammatory agents. Fish and Shellfish Immunology. 12: 229-242.

Ghosh S, May M and Koop E. 1998. NF-kB and rel proteins: evolutionarily conserved mediators of immune responses. Annual Review Immunology 16: 225-260.

Giovanneli L, Pitozzi V, Riolo S and Dolara P. 2003. Measurement of DNA strand breaks and oxidative damage in polymorphonuclear and mononuclear and mononuclear white blood cells: a novel approach using the comet assay. Mutation Research 538: 71-80.

Grimholt U. 1997. Transport-associated proteins in Atlantic salmon (*Salmo salar*). Immunogenetics 46: 213-221.

Grinwis G, Vethaak A, Wester P and Vos J. 2000. Toxicology of environmental chemicals in the flounder *platichthys flesus* with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies. Toxicology letters 112-113: 289-301.

Groberg W.J Jr, R.H Mc Coy, K.S Pilcher and J.L Fryer. 1978. Relation of water temperature to infections of coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*) and steelhead trout (*Salmo gairdneri*) with Aeromonas salmonicida and *A. Hydrophyla*. Journal of the fisheries Research Board of Canada. Vol 35 No1 Pp 1-7

Gulland F, 1995. The impact of infectious diseases on wild animal populations-a review in: Grantal B and Dobson A (eds). Ecology of infectious diseases in natural populations. Cambridge University press Pp 21-51.

Hahn M. 1998. The aryl hydrocarbon receptor a comparative perspective. Comparative Biochemistry and Physiology Part C 121:23-53.

Hahn M, Lamb T, Schultz M. Smolowitz R and Stegeman J. 1993. Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in a Ah receptor-containing fish hepatoma cell line (PLHC-1). Aquatic Toxicology 26:185-208

Hansen J, Kaattari S, 1996. The recombination activating gene 2 (RAG 2) of the rainbow trout *Oncorhynchus mykiss*. Immunogenetics 44: 203-211.

Hansen J, Strassburger P, Thorgaard G, Young W and Pasquier L. 1999. Expression, linkage and polymorphism of MHC- related genes in rainbow trout, *Oncorhynchus mykiss*. The Journal of Immunology 774-786.

Hardin J, Hinoshita F and Sherr D. 1992. Mechanisms by which benzo(a)pyrene, an environmental carcinogen, suppresses B-cell lymphopoiesis. Toxicology and Applied Pharmacology. 117: 155-164.

Hawkins A, Billiard S, Samir P, Brown S and Hodson P. 2002. Altering cytochrome P4501A activity affects PAH metabolism and toxicity in rainbow trout *O.mykiss.* Environmental Toxicology and Chemistry Vol 21 No 9:1845-1853.

Hayashi H, Veda T, Uyeno K, Wada K, Kinae N, Saotore K, Tanaka N, Takai A, Fsusaki Y, Asaro N, Sofuni T and Ojima Y. 1998. Development of genotoxicity assay systems that use aquatic organisms. Mutation Research. 399: 125-133.

Healey M.C. 2003. Life History of Chinook salmon *Oncorhynchus tshawytsha* in: Groot C and Margolis L. Pacific Salmon Life Histories UBS press, Vancouver, Canada. Pp 313-383.

Hedrick R, 1998. Relationship of the Host pathogen and environment: Implications for diseases in cultured and wild fish populations. Journal of Aquatic Animal health 10: 107-111.

Hellou J. 1996. Polycyclic aromatic hydrocarbons in marine mammals, finfish and mollusk in: Beyer N, Heinz G and Norwood A Eds. Environmental contaminants in wildlife, interpreting tissue concentrations. Lewis Publishers. Pp 229-250.

Hendricks J, Meyers T, Shelton D, Casteel J and Bailey G. 1985. Hepatocarcinigenicity of BAP to rainbow trout by dietary exposure and intraperitoneal injection. Journal of the National Cancer Institute.74: 839-851.

Hiney M and Oliver G. 1999. Furunculosis (*Aeromonas salmonicida*) in: Woo P and Bruno D eds. Fish diseases and disorders, Vol 3 viral, bacterial and fungal infections. CABI publishing. Pp341-425

Hinoshita F, Hardin JA, Sherr DH. 1992. Fluoranthene induces programmed cell death and alters growth of immature B-cells populations in bone marrow cultures. Toxicology 73(2): 203-218.

Holladay S, Beteman D, Gogal R, Hrubec R, Ahmeds R. 1998. benzo(a)pyrene induced hypocellularity of the pronephros in tilapia (*Oreochromis niloticus*) is accompanied by alterations in stromal and parenchimal cells by enhanced immune cell apoptosis. Veterinary Immunology, Immunopathology. 64: 69-82.

Hyyti O, Nyman M, Willis M, Raunio H, Pelkoen O, 2001. Distribution of cytochrome P450 (CYP1A) in the tissues of Baltic ringed and gray seals. Marine Environmental Research 51: 465-485.

Jacobson K, Arkoosh M, Kagley A, Clemons E, Collier T, Casillas E. 2003. Cumulative Effects of Natural and Anthropogenic Stress on Immune Function and Stress Resistance in Juvenile Chinook Salmon. Journal of Aquatic Animal Health, 15:1-12.

Johnson S.L 1988. The effects of the 1983 el niño on Oregon's Coho (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawitscha*). Fisheries Research 6: 105-123.

Karrow N, Buermans D, Dixon G, Honfella K, Solomin J, White J and Bols N. 1999. Characterizing the immunotoxicity of creosote to rainbow trout *Oncorhynchus mykiss*: A microcosm study. Aquatic Toxicology 45: 223-239.

Kennedy C, Gill K and Walsh P. 1991. In vitro metabolism of benzo(a)pyrene in the blood of the gulf toad fish *Opsanus beta.* Marine Environmental Research 31:37-53.

Krahn M, Myers M, Burrows D and Mallins D, 1984. Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. Xenobiotica Vol 14 No8: 633-646.

Krahn M, Moore L, and MacLeod J. 1986. Standard analytical procedures of the NOAA National Analytical Facility, 1986. Metabolites of Aromatic Compounds in Fish Bile. NOAA Technical Memorandum. NMFS-F/NWC-102

Krahn M, Ylitalo G, Buzitis J, Chan S and Varanasi U. 1993. Rapid high-performance liquid chromatography methods that screen for aromatic compounds in environmental samples. Journal of chromatography 642:15-32.

Kirso L, Voll M, Irha N, Urbas E. 2001. Distribution of the Persistent Organic Pollutants, Polycyclic Aromatic Hydrocarbons, Between Water, Sediments and Biota. Aquatic Ecosystem Health and Management 4:151-163

Krasnov A, Koskinen H, Rexroad C, Afanasyev S, Molsa H, Oikari A 2005. Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (*Oncorhynchus mykiss*).Aquatic Toxicology. Aug 15; 74(1):70-81.

La Patra S, Fryer J, Wingfield W and Hedrick R. 1989. Infectious hepatopoyetic necrosis virus (IHNV) in coho salmon. Journal of Aquatic Animal Health 1:277-280.

Ladics G, Kawabata T, Manson A.E and White K.L. 1992. Evaluation of Murine Splenic cell type metabolism of BAP and functionality in vitro following repeated in vivo exposure to Bap. Toxicology and Applied Pharmacology. 116: 248-257

Lang D, Becker S, Devlin R, Koren H. 1998. Cell specific differences in the susceptibility of potential cellular targets of human origin derived from blood and lung following treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Cell Biology Toxicology 14: 23-38.

Latimer J and Zheng J. 2003. The Sources, transport and fate of PAHs in the marine environment, in: Douben P. PAHs an Ecotoxicological Perspective. John Wiley and Sons Ltd. Pp 9-33.

Lewis J, Hamilton T and Adams O. 1986. The effect of macrophage development on the release of reactive oxygen intermediates and lipid peroxidation products and their ability to induce oxidative DNA damage in mammalian cells. Carcinogenesis 7: 813-818.

Lewis S. 1994. The mechanism of V(D)J joining: Lessons from Molecular Immunological and Comparative Analysis. Advances Immunology 56: 27-50.

Li X and Stank G, 2002. NF-kappa B-dependent signaling pathways. Experimental Hematology 30: 285-296.

Li X, Fang X and Gaynor R, 2001. Role if Ikkγ/ NEMO in assembly of the IKb kinase complex. The Journal of Biological Chemistry. Vol 276 No 6: 4494-4500.

Lloyd S, 1995. Environmental Influences on host immunity in: Grentell B and Dobson A. Ecology of infectious diseases in natural populations. Cambridge University press. Pp 327-361.

Loge F, Arkoosh M, Ginn T, Johnson L and Collier T. 2005. Impact of Environmental stressors on the dynamics of disease transmission. Environmental Science and Technology Sep 15;39(18):7329-36.

Lowry O, Rosenbrough N, Farr A and Randall R. 1951. Protein Measurements with the Folin phenol reagent. Journal Biological Chemistry. 193:265-275

Luna C. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Third Edition. Pp 12-47. McGraw Hill.

Malmgren RA. 1952. Reduced antibody titers in mice treated with carcinogenic and cancer chemotherapeutic agents. Proceedings Society Biological Medicine 79: 484.

Mann K, Doerre S, Sclezinger J, Sherr D and Quadri S. 2001. The role of NK-kB as survival factor in Environmental chemical-induced pre-B cell apoptosis. Molecular pharmacology Vol 59: No2: 302-309.

Markwardt N. Yvonne G and Klontz G. 1989. A new application for comassie brilliant blue agar: detection of A.salmonicida in clinical samples. Diseases of Aquatic organisms. Vol 6: 231-233.

Marubini, E, and Valsecchi. M.G. 1995. Analyzing survival data from clinical trials and observational studies. John Wiley, Chichester, N.Y. Pp 424

May M and Gosh S, 1998. Signal Transduction through NF-kB. Immunology Today 19: 80-88.

Mc Carthy.1980. Furunculosis of fish the present state of our knowledge in Droop M and Jannasch H eds. Advances in Aquatic Microbiology. London, Academy Press P293-341.

Mc Connel T, Godwin U and Cutriberson B. 1998. Expressed Major Histocompatibility Complex class II loci in Fishes. Immunological Reviews. Vol 166: 294-300.

MacLeod M.C, Levin W, Conrey H.A, Lehr R.E, Mansfield B.K, Jerina D.M and Selkirk J.K. 1980. Metabolism of benzo(e)pyrene by rat liver microsomal enzymes. Carcinogenesis Vol 1: 165-173.

Mac Millan-Crow L.A, Crow J.P, Kerby J.D, Beckman J.S and Thompson J.A. 1996. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proceedings of the National Academy of Sciences USA. Vol 93: 11853-11858

Meador J. 2003. Bioaccumulation of PAHs in Marine Invertebrates in: Douben P. PAHs an Ecotoxicological Perspective. John Wiley and Sons Pp 147-171.

Meador JP, Stein JE, Reichert WL and Varanasi U (1995). A Review of bioaccumulation of polycyclic aromatic hydrocarbons by marine organism. Reviews in Environmental Contamination and Toxicology, 143: 79-165.

Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M, Manning AM. 1999. IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Molecular Cell Biology19(2):1526-38.

Morrow JD, Awad JA, Kato T, Takahashi K, Badr KF, Roberts LJ 2nd, Burk R. 1992. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. Journal of Clinical Investigation 90 (6) 2502-7.

Morrow J.D, Roberts J. 1992. Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress. Methods in Enzymology Vol 300: 3-12.

Mitchelmore C and Chipman J. 1998 (a). Detection of DNA strand breaks in brown trout (*salmo trutta*) hepatocytes and blood cells using the single cell electrophoresis (comet) assay. Aquatic Toxicology 41: 161-182.

Mitchelmore C and Chipman J. 1998 (b). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Research 399: 135-147.

Mitchelmore C, Birmelin C, Chipman K and Livingstone D, 1998. Evidence for cytochrome P-450 catalysis and the free radical involvement in the production of DNA strand breaks by benzo(a)pyrene and nitroaromatics in mussel (*Mytilus edulis L.*) digestive gland cells. Aquatic Toxicology 41: 193-212.

Munro A and Roberts R. 2001. The aquatic environment in: Roberts R (ed). Fish Pathology Third Ed.W.B Saunders, London Pp 1-11.

Myers M, Willis M, Mette A, Anders G and Collier T, 1996. Immunohistochemical localization of CYP1A in multiple types of contaminant- associated hepatic lesions in English sole (*Pleuronectes vetulus*). Marine Environmental Research 39: 283-288.

Neff J. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment sources fates and biological effects. Applied sciences publishers Ltd London. Pp 7-42.

Nehlsen W, Williams J.E and Lichatowitch J.A. 1991. Pacific salmon at the croosroads; stocks at risk from California, Oregon, Idaho and Washington. Fisheries 16(2) 4-21

Newman C.M and Unger A.M. 2003. Fundamentals of Ecotoxicology. Second Edition. Lewis Publishers. Bocan Raton. John Wiley and sons Ltd. Pp 9-32

Nguyen T, Sherratt P and Pickett C. 2003. Regulatory Mechanisms Controlling Gene Expression Mediated by the Antioxidant Response Element. Annual Review Pharmacology and Toxicology 43: 233-260.

Nordmo R. 1997. Strenghts and weaknesses of different challenge methods. In: Gudding R, Lillenhaug A, Midtlyng PJ and Brown F (eds): Fish vaccinology. Developments in Biological Standarization. Basel, Karger Vol 90 PP 303-309.

Nordmo R, Ramstad A. 1997. Comparison of different challenge methods to evaluate efficacy of furunculosis vaccines in Atlantic salmon *Salmo salar* L. Journal of Fish Disease 20: 119-126

Nordmo R, Ramstad A and Holth R. 1998. Induction of experimental furunculosis in heterogeneous test populations of Atlantic salmon (*salmo salar L.*) by use of a cohabitation method. Aquaculture 162:11-21.

Nordmo R and Ramstad A. 1999. Variables affecting the challenge pressure of *A. salmonicida* and *V. salmonicida* in Atlantic salmon (*salmo salar L*). Aquaculture 171: 1-12.

NRC (National Research Council) 1996. Upstream, salmon and society in the Pacific Northwest. National Academy Press, Washington, DC, USA.

O'Halloran K, Ahokas N and Wright P. 1998. the adverse effects of aquatic contaminants on fish immune responses. Australassian Journal of Ecotoxicology. 4: 9-28.

Oettinger M, Schutz D, Gorka C and Baltimore D, 1990. RAG-1 and RAG-2 adjacent genes that synergistically activate V(D)J recombination. Science Vol 248:1517-1522.

Ohta V, Haliniewski D, Hansen J and Flajnik M, 1999. Isolation of transporter associated with antigen processing genes TAP 1 and TAP 2, from the horned shark *Heterodontus francisci*. Immunogenetics 49: 981-986.

Osborne M, Crosby N. 1987. Benzopyrenenes. Cambridge University Press, London. Pp 28 and 234.

Page T, Mac Williams P, Suresh M, Jefcoate C and Czuprynski C. 2004. 7-12 dimethylbenz[a]anthracene induced bone marrow hypocellularity is dependent on signaling through both the TNFR and PKR. Toxicology and Applied Pharmacology 198: 21-28.

Pallardy N, House R and Dean J. 1989. Molecular mechanism of 7,12-dimethylbenz[a]anthracene-induced immunosupression: evidence for action via the interleukin-2-pathway. Molecular Pharmacology 36(1) 128-133.

Palm R, Powell D, Skillman A and Godtfredsen K. 2003. Immunocompentence of juvenile chinook salmon against Listonella anguillarum following dietary exposure to polycyclic aromatic hydrocarbons. Environmental Toxicology and Chemistry Vol 22, No 2 Pp 2986-2994.

Pan S, Kayano-Nakagawa N, Tsuruta L, Amasaki Y, Yokota T, Mori S, Arai N and Arai K. 1997. Molecular cloning and functional characterization of murine cDNA encoding transcription factor NFATc. Biochemical and Biophysical research communications 240: 314-323.

Parker R.R. 1962. Estimates of ocean mortality rates for Pacific salmon (Oncorhynchus). Journal of the Fisheries Research Board of Canada. 19: 561-589.

Parker N and Munn C. Cell surface properties of virulent and attenuated strains of *A. salmonicida* in Ellis AE (ed) Fish and Shellfish pathology. London Academy press 97-105.

Payne J, Matnieu A and Collier T, 2003. Ecotoxicological Studies focusing on marine and freshwater fish in: Douben P (ed). PAH an Ecotoxicological perspective. John Wiley and Sons LTD. Pp 101-224.

Peixoto B, Mikawa Y, Brenner S. 2000. Characterization of the recombinase activating gene-1 and 2 locus in the Japanese pufferfish *Fugu rubripes*. Gene 246:275-283.

Penning T.M, Burczynski M, Hung C.H, Mc Coull K.D, Palackal N.T and Tsuruda L.S. 1999. Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active O-quinones. Chemical research in Toxicology Vol 12(1): 1-18.

Pesonen M, Celander M, Forlin L and Andersson T. 1987. Comparison of xenobiotics biotransformation enzymes in kidney and liver of rainbow trout (*Salmo gairdneri*). Toxicology and applied pharmacology 91: 75-84.

Petersen D, Keinow K, Kraska C and Lench J, 1985. Uptake, disposition and elimination of acrylamine in rainbow trout. Toxicology Applied Pharmacology. 80: 58-65.

Quinn T.2005. The behavior and ecology of Pacific salmon and trout. American fisheries Society. Bethesda, Maryland. Pp 3-36 and 37-53.

Rao A, Luo C and Hogan P, 1997. Transcription factors of the NFAT family regulation and function. Annual Reviews in Immunology. 15:707-47. Review

Reddy M and Randerath K. 1987. ^{32}P-analysis of DNA adducts in somatic and reproductive tissues of rats treated with the anticancer antibiotic, mitomycin C. Mutation Research 179:75-88.

Reichert W and French B. 1994. The ^{32}P-Postlabeling Protocols for Assaying Levels of Hydrophobic DNA Adducts in Fish. NOAA Technical Memorandum NMFS-NWFSC-14.

Research Triangle Institute. 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons. U.S Department of Health and Human Services. pp 1-10

Roberts M.H, D.W Sved and S.P Felton. 1987. Temporal changes in AHH and SOD activities in feral spot from the Elizabeth River, a polluted sub-estuary. Marine Environmental Research 23:89-101.

Sandvik M, Horsberg T, Skaale U and Ingebringtsten K.1998. Comparison of dietary and waterborne exposure to benzo(a)pyrene availability, tissue disposition and CYP1A induction in rainbow trout *Oncorhynchus mykiss* Biomarkers Vol 3 No 6: 399-410.

Sangrador-Vegas A, Leanington J, Smith T. 2001. Molecular Cloning of a IL8 like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression substractive hybridization. Cytokine 17: 66-70.

Sangrador-Vegas A. Smith T and Cairns M. 2005. Cloning and Characterization of a homologue of the alpha inhibitor of NF-kB in rainbow trout *Oncorhynchus mykiss*. Veterinary Immunology and Immunopathology. 103: 1-7.

Schutz D, Oettinger M and Schlissel M. 1992. V(D)J recombination: Molecular Biology and Regulation. Annual Reviews in Immunology. 10: 359-383.

Secombes C and Olivier G. 1997. Host-pathogen interactions in salmonids. In: Bernoth E, Ellis A, Midtlyng P, Olivier G and Smith P. Furunculosis Multidisciplinary Fish disease research. Academy press San Diego Pp 269-321.

Secombes C, Zou J, Daniels C, Cunningham C, Koussounandis A and Kemp G. 1998. Rainbow trout cytokine and cytokine receptor genes. Immunological Reviews Vol 166:333-340.

Sherman M, Powell D, Weiss D and Brown M, 1999. NF-ATc isoforms are differentially expressed and regulated in murine T and mast cells. The Journal of Immunology. 162: 2820-2828.

Shugart L.R, 1988. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Toxicology 13:43-52

Shugart L.R, 1995. Environmental Genotoxicology. In: Rand G.M ed. Fundamentals of Aquatic Toxicology: effects, environmental fate and risk assessment. Second Edition. Taylor and Francis Publishers. Pp 405-419.

Shugart L,1998. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Toxicology 13:43-52

Silkworth JB, Lipinskas T, Stoner CR. 1995. Immunosuppressive potential of several polycyclic aromatic hydrocarbons (PAH) found at a Superfund site: new model used to evaluate additive interactions between benzo[a]pyrene and TCDD. Toxicology.105(2-3):375-86.

Singh N, Danner D, Tia R, Brant L and Schneider E, 1990. DNA damage and repair with age in individual human lymphocytes. Mutation Research 237: 123-130.
Sinndermann C, 1990. Principal Diseases of Marine fish and shellfish. Academic press New York.

Sloan C.A, D.M Brown, Pearce R.W, Boyer R.H, Bolton J.L, Burrows D.G, Herman D.P. and Khran M.M 2004. Extraction, cleanup and gas chromatography/mass spectrometry analysis of sediments and tissues for organic contaminants. U.S Department of Commerce NOAA Tech Memo. NMFS-NWFSC-59. P47.

Snieszko S. 1973. Recent Advances in scientific knowledge and developments pertaining to diseases of fishes. Advances in Veterinary Sciences and Comparative Medicine. 17: 291-314.

Sokal R and Rohlf J. 1995. Biometry. Third edition. Freeman and Company ed. Pp 423.
Stegeman J and Hahn M, 1994. Biochemistry and molecular biology of monooxygenases current perspectives on the forms, functions and regulation of cytochrome P450 in aquatic species in: D.C Malins and G.K Ostrander, Aquatic Toxicology Molecular Biochemical and Cellular Perspectives Pp 87-206. Boca Raton: Lewis Publishers.

Stegeman J, Smolowit R and Hahn M. 1991. Immunohistochemical localization of environmentally induced CYP1A in multiple organs of the marine teleost *Stenotomus chrysops* (scup). Toxicology and applied pharmacology 110:1-7.

Stein J.E, Hom T, Casillas E, Friedman A and Varanasi U. 1987. Simultaneous exposure of English sole (*Parophrys vetulus*) to sediment-associated xenobiotics II Chronic exposure to an urban estuarine sediment, with added ^{3}H-Benzo(a)pyrene and ^{14}C-polychlorinated biphenyls. Marine Environmental Research 22: 123.

Stein E.J, Hom T, Coller K. T, Brown D and Varanasi U, 1995. Contaminant exposure and biochemical effects of out-migrant Chinook salmon from urban and non-urban estuaries of Puget Sound WA. Environmental Toxicology and Chemistry Vol 14 No 6: 1019-1029.

Sterling K, Raha A, Bresnick E. 1994. Induction of CYP1A gene expression in mouse hepatoma cells by benzo(e)pyrene, a ligand of the 4s polycyclic hydrocarbon biding protein. Toxicology and Applied pharmacology Vol 12B No1 Pp 18-24.

Thorpe J. 1994. Salmonid Fishes and the Estuarine Environment. Estuaries 17: 76-93.

Tilton S, Gerwick L, HendricksJ, Rosato C, Smith G, Givan S, Bailey G, Bayne C and Williams D, 2005. Use of rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B1 induced hepatocellular carcinoma compared to adjacent liver. Toxicological Sciences advance Access Published Sep 1-2005.

Tsoi S, Ewart K, Penny S, Melville K, Liebscher R, Brown L. 2004. Identification of Immune relevant genes from Atlantic salmon using suppression subtractive hybridization. Marine Biotechnology 6: 199-214.

Vanya E, Belanger J, Williams J, Karakach T, Penny S, Tsoy S, Richards R and Douglas S, 2005. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Developmental and Comparative Immunology 29: 333-347.

Varanasi U, Nishimoto M, Reichert W and Eberhart B. 1986. Comparative metabolism of benzo(a)pyrene and covalent binding to hepatic DNA to English sole, starry flounder and rat. Cancer research 46: 3817-3824.

Varanasi U, Nishimoto M, Baird W and Smolarek T. 1989. Metabolic activation of PAH subcellular fractions and cell cultures from aquatic and terrestrial species. In: Varanasi U Editor. Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC press Boca Raton FL. Pp 203-245.

Varanasi U, Casillas E, Arkoosh M, Hom T, Misitiano D, Brown D, Chan S, Collier T, McCain B and Stein J. 1993. Contaminant Exposure and Associated Biological Effects in Juvenile Chinook Salmon (Oncorhynchus tshawytscha) from Urban and Nourban Estuaries of Puget Soud. NOAA Technical Memorandum NMFS-NWFSC-8 pp5-25.

Walczak B.Z, Blunt B.R and Hodson P.V. 1987. Phagocytic function of monocytes and haematological changes in rainbow trout injected intraperitoneally with Benzo(a)pyrene (B(a)p) and benzo(a)anthracene (B(a)A). Journal of Fish Biology 31 supplement A Pp: 251-253

Ward E, Murray M and Dean J. 1985. immunotoxicity on non halogenated polycyclic aromatic hydrocarbons. In: Dean J (ed) immunotoxicology and immunopharmacology. Raven press, New York Pp 291-304.

Weeks B, Warinner J, Manson P, McGinnis D. 1986. Influence of Toxic Chemicals on the Chemotactic Response of Fish Macrophages. Journal of Fish Biology, 28:653-658.

Warshawsky W and Warshawsky D. 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicology and Applied Pharmacology 206: 73-93.

White K and Holsapple M.P. 1984. Direct Suppression of in vitro antibody production by mouse spleen cells by the carcinogen Benzo(a)pyrene but not but the non-carcinogenic congener Benzo(e)pyrene. Cancer Research 44: 3388-3393

White K.L Jr. 1992. Specific Immune Function Assays. Pp 304-323 in: K. Miller, J. Turk and S. Nicklin (eds). Principles and Practice of Immunotoxicology. Blackwell Scientific Publications, Cambridge, Massachussets.

White L, Kawabata T and Ladics G, 1994. Mechanisms of polycyclic aromatic hydrocarbon Immunotoxicity. In: Dean J, Luster M, Munson A and Kimber E (Eds). Immunotoxicology and Immunopharmacology Second Ed. Raven Press Ltd. New York. Pp 123-142.

Willet K, Steinberg M, Thomsen J, Narasimhan T, Safe S, McDonald S, Beatty K and Kennicutt M, 1995. Exposure of killing fish to benzo(a)pyrene: comparative metabolism, DNA adduct formation and aryl hydrocarbon (Ah) receptor agonist activities. Comparative Biochemistry and Physiology Vol 112B N° 1: 93-103.

Willet C, Cherri J, Steiner L, 1997. Characterization and Expression of the Recombination Activating Gene (RAG1 and RAG2 of Zebrafish. Immunogenetics 45: 394-404.

Wojdani A and Alfred L.J. 1984. Alterations in cell-mediated immune functions induced in mouse splenic lymphocytes by polycyclic aromatic hydrocarbons. Cancer Research 44: 942-945.

Wojdani A, Attarzadeh M, Wode-Tsadik G and Alfred L.J. 1984. Immunotoxicity Effects of polycyclic aromatic hydrocarbons on mouse lymphocytes. Toxicology 31: 181-189.

World Health Organization. 1998. Environmental Health Criteria 202. Selected non-heterocyclic polycyclic aromatic hydrocarbons: a structure activity relationship in B6C3F1 and DBA/2 mice. Immunopharmacology 9: 155-164.

Yamaoka S, Courtois G, Bessia C, Whiteside S, Weil R, Ayat F, Kirk H and Israel A. 1998. Complementation cloning of NEMO, a component of the IkB kinase complex essential for NF-kB activation. Cell Vol 93: 1231-1240.

Zangar R, Davydov R and Seema V.2004. Mechanism that regulate production of reactive oxygen species by cytochrome P450. Toxicology and Applied Pharmacology 199:316-331.

Zapata A, Chiba A and Varas A. 1996. Cells and Tissues of the Immune system of fish in: Iwana G, Nakanishi T (eds). The fish Immune system: Organism, pathogen and environment. Academic press Inc. Pp 1-62.

Zelikoff J, Raymond A, Carlson E, Li Y, Beaman J and Anderson M, 2000. Biomarkers of Immunotoxicity in Fish: from the lab to the ocean. Toxicology letters 112-113: 235-331.

Zelikoff J, Carlson E, Raymond A, Duffy J, Beaman J and Anderson M. 2002. Immunotoxicity Biomarkers in fish development, validation and application for field studies and risk assessment. Human and Ecological Risk Assessment Vol 8 No 2 Pp 253-263.

Zelikoff J and Carlson E. 2004. Benzo(a)pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*): relationship between lymphoid CYP1A activity and humoral immune suppression. Toxicology and Applied Pharmacology 201: 40-52.

Zhang H, Thorgaard G, Ristow S. 2002. Molecular Cloning and genomic Structure of an Interleukin-8 receptor-like gene from homozygous clones of rainbow trout *Oncorhynchus mykiss*. Fish Shellfish Immunology. Sep;13(3):251-8.

APPENDIX

Differentially expressed genes between fish pretreated to a PAH mixture in the diet and challenged to *Aeromonas salmonicida*

DAY 2

Immune related Genes Rainbow trout (all fold changes are significant P<0.05)

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_212_CD3epsil/gamadelta	CA357253	T-cell surface glycoprotein CD3 epsilon chain precursor (T-cell surface antigen T3/Leu-4 epsilon chain).	**3.75**
OSUrbt2_711_MHC1AF104582	TC13263	MHC class I alpha 2 antigen gene	**-3.34**
OSUrbt2_289_CTL	TC22403	VHSV-induced C-lectin-like protein [Oncorhynchus mykiss]	**-2.08**
OSUrbt2_735_MICA	TC13215	MHC class I heavy chain [Oncorhynchus mykiss]	**-2.36**
OSUrbt2_713_MHC1AF287485	TC13253	Oncorhynchus mykiss MHC class I heavy chain precursor(Onmy-UBA) mRNA.	**-2.06**
OSUrbt2_819_NFKBIB	BX320957	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, betA	**-2.07**
OSUrbt2_275_CR2	TC68550	complement receptor	**-1.90**
OSUrbt2_283_CSF1	CA369458	Homo sapiens colony stimulating factor 1 (macrophage)	**2.93**
OSUrbt2_615_IRF1	TC11489	Oncorhynchus mykiss interferon regulatory factor 1 (IRF-1)mRNA,complete cds. interferon regulatory factor 1 [Oncorhynchus mykiss]	**-1.62**

OSUrbt2_1101_TAP1	TC27069	TAP1 protein {Oncorhynchus mykiss}, complete.Transporter associated with antigen processing	**-2.73**

DAY 2
Toxicology-related genes

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_19_ABCG2	BC021281	ATP-binding cassette, sub-family G (WHITE), member 2, mRNA weakly similar to ABC transporter ABCG2 {Homo sapiens}, partial (18%)	**3.81**
OSUrbt2_697_Metallothionein2	TC9381	Metallothionein 2	**-2.08**
OSUrbt2_386_EPHNB2	TC45159	A gene expressed in all stem cells and concerned with cell death.	**5.32**
OSUrbt2_773_MT1H	TC9381	metallothionein B; rainbow trout (*Oncorhynchus mykiss)*	**-1.70**
OSUrbt2_8_AANAT	TC22535	Homo sapiens arylalkylamine N-acetyltransferase	**-3.18**
OSUrbt2_317_CYP2A6	TC8588	cytochrome P-450 *O.mykiss*	**-2.43**

DAY 4
Immunologically relevant genes

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_1416_VSHVB51	TC25842	Oncorhynchus mykiss clone B32 VHSV-induced protein mRNA, complete cds. intracellular protein transport, antimicrobial humoral response	**1.04**
COLEC12_Omy_C6888	TC25842	Collectin sub-family member 12. This protein is a scavenger receptor, a cell surface glycoprotein that can bind to carbohydrate antigens on microorganisms facilitating their recognition and removal. In addition, these receptors can recognize oxidized phospholipids so they may also participate in removing oxidatively damaged or apoptotic cells.	**2.27**

DAY 10
Immune related genes

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_815_NFAT	TC23795	Similar to NFC1_PIG Nuclear factor of activated T-cells cytoplasmic 1 (NFAT transcription complex cytosolic component), partial (6%)	**-2.76**
OSUrbt2_819_NFKBIB	BX320957	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB), mRNA	**-2.12**
OSUrbt2_724_MHC1AF441856	AF441856	Oncorhynchus mykiss MHC class I antigen (Onmy-UBA) gene, Onmy-UBA*501 allele, upstream region and partial cds.	**2.73**
OSUrbt2_205_CD23	TC23186	Homo sapiens Fc fragment of IgE, low affinity II, receptor for(CD23A) (FCER2), mRNA. weakly similar to PIR JC7595 scavenger receptor C-type lectin type I - human, partial (9%)	**-3.78**
OSUrbt2_738_MIF	BX314384	Macrophage migration inhibitory factor (MIF)	**1.98**
OSUrbt2_578_IKBKG	TC38615	NF-kappa B essential modulator	**-2.20**
OSUrbt2_707_MHC1AF091779	TC11968	Oncorhynchus mykiss non-classical MHC class I antigen(Onmy-UAA) gene, Onmy-UAA*0101 allele, complete cds.	**3.68**

OSUrbt2_981_RAG2	TC44479	V(D)J recombination activating protein 2 (RAG-2). [Rainbow trout, Salmo gairdneri]	**-1.93**
OSUrbt2_723_Mhc1AF318190	TC23087	Oncorhynchus mykiss MHC class I heavy chain (Onmy-UBA)	**3.23**
LAMP1_Omy_C4375	TC49600	lysosomal-associated membrane protein 1 (LAMP1)	**4.97**
OSUrbt2_167_CBLNLTC40649	TC43973	Mus musculus cerebellin 1 precursor protein, partial cds Precerebellin is a large protein with distant homology to the noncollagen domain of complement component C1qB	**-3.21**
OSUrbt2_719_MHC1AF318187	TC21722	Oncorhynchus mykiss MHC class I heavy chain (Onmy-UBA) mRNA, Onmy-UBA*5001 allele, partial cds. MHC class I heavy chain [Oncorhynchus mykiss]	**-2.22**
OSUrbt2_470_GRO		Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2),mRNA. putative interleukin 8	**-1.73**
OSUrbt2_220_CD7	TC16886	Homo sapiens CD7 antigen (p41), mRNA immunoglobulin light chain	**-1.78**
OSUrbt2_709_MHC1AF104579	TC9287	Oncorhynchus mykiss isolate MHC class I alpha 2antigen gene.	**-3.85**
OSUrbt2_345_DAF	TC19090	Homo sapiens decay accelerating factor for complement (CD55)	**-1.54**

OSUrbt2_1271_TC8507	TC8507	(antigen binding activity) (extracellular) (immune response) weakly similar to immunoglobulin light chain {Cyprinus carpio}, partial (95%)	**-2.62**
OSUrbt2_729_MHCIIAY081776	TC9478	Oncorhynchus mykiss MHC class II invariant chain-like protein 1 invariant chain S25-7	**-4.67**
OSUrbt2_721_MHC1AF318188	TC21575	Oncorhynchus mykiss MHC class I heavy chain	**-2.36**
OSUrbt2_597_IL2R	TC55220	Cytokine receptor common gamma chain	**-1.38**
OSUrbt2_559_ICE	BX300532	caspase 1 isoform alpha precursor; interleukin 1-beta convertase;interleukin 1-B converting enzyme; IL1B-convertase [Homo sapiens].	**-2.05**
PIGT_Omy_C11484		phosphatidyl inositol glycan class T (PIGT); transferrin, MHC Class I, microsatellit, Tc-like transposon sequence, transposone, comprising a jumble of things. Oncorhynchus mykiss Mx1 gene, promoter and partial sequence.	**6.01**
OSUrbt2_1356_TRIF	TC38948	Homo sapiens TIR domain containing adaptor inducing interferon-beta (TRIF), mRNA	**-1.46**
MHC1AF104582	TC13263	Oncorhynchus mykiss isolate Onmy-UA*1 MHC class I alpha 2 antigen gene	**-3.25**
IL8RA_Omy_C11254	TC63288	interleukin 8 receptor, alpha (IL8RA	**2.45**
OSUrbt2_1040_SELE/SELL	TC43279	E-selectin {Mus musculus}, partial (25%);;	**-1.96**
OSUrbt2_1328_TNFS13B	CA341875	B-lymphocyte stimulator	**-1.74**

DAY 20
Immune related genes

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_202_CD161	TC10619	Homo sapiens killer cell lectin-like receptor subfamily B, member 1(KLRB1), CD209b antigen [Mus musculus]. CD209b antigen. weakly similar to antifreeze protein{Clupea harengus}, partial (34%)	**-1.36**
OSUrbt2_293_CTLTC27883	TC27883	weakly similar to C-type lectin{Cyprinus carpio}, partial (30%).	**-1.33**
OSUrbt2_300_CX3CR1/CXCR1	TC17580	Chemokine (C-X3-C motif) receptor 1 [Homo sapiens].	**-2.28**
OSUrbt2_1215_TC8358	TC8358	(antigen binding activity) (extracellular) (immune response) similar to immunoglobulin light chain precursor {Anarhichas minor}, partial (91%)	**-1.38**
OSUrbt2_12_ABCB3B	TC19240	MHC class I heavy chain [Oncorhynchus mykiss]; MHC class Iheavy chain precursor [Oncorhynchus mykiss]Length	**-2.06**
OSUrbt2_204_CD16A	TC8435	Macaca fascicularis Fc gamma receptor IIIa mRNA,	**-1.87**
OSUrbt2_106_BLK	TC8971	Homo sapiens B lymphoid tyrosine kinase (BLK)	**-1.21**

DAY 20
Toxicology-related genes

Array ID + common name	Accession number	Gene function	Fold change
OSUrbt2_318_CYP2K1v2	TC20294	cytochrome P-450; cytochrome P450 monooxygenase CYP2K1v2 [Oncorhynchusmykiss];	**5.05**
OSUrbt2_48_APAF1	TC13272	Homo sapiens apoptotic protease activating factor (APAF1), transcript variant 1, mRNA. {Mus musculus}, partial (76%)	**-1.34**
OSUrbt2_10_ABCB2	TC17733	ABCB2 Homo sapiens transporter 1, ATP-binding cassette, sub-family B(MDR/TAP). TAP1 protein [Oncorhynchus mykiss]	**-1.27**
ESR1_Omy_C10805_O4		estrogen receptor 2 aka ESRB	**-1.30**