Methodologies for evaluating planar chlorinated hydrocarbon (PCH) and polycyclic aromatic hydrocarbon (PAH) exposure and bioconcentration in fish

by

Jeffrey J. Whyte

A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 1998

© Jeffrey John Whyte, 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-30659-3

Canada

The University of Waterloo requires the signatures of all persons using or photocopying this thesis.

Please sign below, and give address and date.

Abstract

The accumulation of complex chemical mixtures in aquatic organisms and their resultant effects are difficult to estimate due the wide range of physical and biological properties of the chemicals and the potential interactions among them. In this thesis several techniques were integrated and compared to determine their utility in assessing chemical mixture exposure and effects. Two specific families of chemicals were examined, the planar halogenated hydrocarbons (PCHs) and polycyclic aromatic hydrocarbons (PAHs).

Chapter 1 describes the determination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-equivalent concentrations (TECs) in four ways in livers of lake trout from three Great Lakes sites. Bioassay-derived (BD) TECs were measured with rat (H4IIE) and rainbow trout (RTL-W1) cell lines and chemistry-derived (CD) TECs were calculated with TCDD equivalency factors (TEFs) derived with H4IIE and RTL-W1. Generally, BD-TECs and CD-TECs for individual samples did not differ significantly for either H4IIE or RTL-W1 indicating that all of the AhR-active compounds in a sample were accounted for by the congener analysis and that these compounds acted in an additive fashion in both mammalian and piscine systems. However, contributions of individual PCHs to overall CD-TECs differed with H4IIE and RTL-W1 TEFs, and for some individual samples, TECs did depend on the method used. Hepatic TECs and ethoxyresorufin-O-deethylase (EROD) levels differed significantly between sites. For TECs the order was Glenora > Jackfish Bay > Black Bay; for EROD activity the order was Jackfish Bay > Glenora = Black Bay. No correlation was found between hepatic TECs and EROD activity, which suggests that the two measurements are evaluating different but related consequences of contaminant exposure.

In Chapter 2, a three-step approach was employed to assess the exposure of rainbow trout to polycyclic aromatic hydrocarbons (PAHs) in standardized creosote contaminated microcosms. Bioconcentration of PAHs was approximated using semipermeable membrane devices (SPMDs) and compared to levels seen in the trout. Large differences in concentrations of PPPAHs in the two matrices were seen, with SPMDs accumulating higher levels of analytes compared to the fish. Increases in hepatic EROD levels and bile metabolites with creosote dose indicate the difference is mainly due to biotransformation of accumulated PPPAHs in the trout. Species-specific response to PPPAHs extracted from trout and SPMDs was assessed by calculating 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalent concentrations (TECs) using mammalian and piscine cell line bioassays. The use of RTL-W1 or H4IIE did lead to different TECs, but the magnitude of the differences was not great and depended on whether SPMD or liver extracts were being examined. Possible reasons for the disparity include subtle differences in the AhR signal transduction system, PAH metabolism between fish and mammals or TEF derivation methods. This dissimilarity may indicate that RTL-W1 is more appropriate than H4IIE for assessing the toxic impact of PAHs to rainbow trout. The integration of the exposure assessment methodologies provided evidence of concentration-dependent PPPAH uptake in rainbow trout, despite low levels detected in the liver.

Chapter 3 examined the time-dependent uptake of priority pollutant polycyclic aromatic hydrocarbons (PPPAHs) in both semipermeable membrane devices (SPMDs) and rainbow trout exposed to creosote-dosed microcosms. SPMDs rapidly accumulated PPPAHs over time while rainbow trout maintained steady, low levels throughout the exposure period. In contrast, rainbow trout hepatic EROD was elevated by the first collection point, indicating

exposure to CYP1A inducing compounds. It is hypothesized that this elevated activity lead to the metabolism and elimination of PPPAHs from the rainbow trout livers. A rainbow trout liver cell line, RTL-W1, was exposed to PPPAH extracts from both SPMDs and trout livers to determine bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations (BD-TECs). BD-TECs followed the levels of chemical concentrations measured in both matrices. Using RTL-W1 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalency factors (TEFs), chemically-derived (CD) TECs were determined for SPMDs and trout livers. Elevated BD-TECs compared to CD-TECs suggests the presence of an unknown EROD inducing compound(s) in the microcosms. This study demonstrates the importance of examining PPPAH uptake over time, its relationship to biological response, and the utility of a multilevel analysis for a more complete representation of the actual events that occur upon chemical exposure.

Acknowledgments

To properly thank everyone who has assisted me in the preparation of this thesis would practically require a document equal in size to the one you are reading now. Due to University regulations, I'll try to be somewhat more brief.

First thanks go to my supervisors, Dr. Niels Bols and Dr. George Dixon. George, I'm sure you regretted telling me 'your door was always open', since every time I walked through it I had a new catastrophe for you to solve. I appreciate the guidance you gave me, and the freedom to apply it. Niels, during the best times of my degree, your enthusiasm for our work inspired me. During the worst times, your humor helped me weather the storm. I'm grateful to you for your dedication, for sacrificing your evenings and weekends, and for introducing me to "The King's English". Thanks for making it fun, and I look forward to our continued conversations in the future.

If there's one thing I learned from this endeavor, it's that science is not a solitary effort. The list of people I'm grateful to is a long one, so forgive me if I miss anyone. I thank everyone in the Bols Lab for making my stay in Waterloo a good one. Thanks to Denise Tom for teaching me the ins and outs of tissue culture while simultaneously reintroducing me to the pop culture of the early eighties; Kristin Schirmer for answering all of my PAH questions and for showing Nicole and I the real meaning of adventure, although we'll probably plan our next backpacking trip for the summer (brrrr!); Rosemarie (Rm) Ganassin for repeatedly lending me the software that helped me get through my thesis; Janine Clemons for introducing me to the EROD assay and the concept of dioxin-equivalents (why didn't you just tell me to kick a hornet's nest?); Liz Heikkila for all of the bioassays you did, and for the parenting advice on the side; John Brubacher for being one of the smartest, most likable guys I know. You deserve a medal (oh, that's right - you got one). Special thanks to Janet Turner and Anita Mirabelli for their assistance in the lab and the field. I'd also like to thank all of the Fisheads down in Dixon's Lab, especially Niel Karrow for introducing me to rainbow trout ice fishing out at the creosote-dosed microcosms. Now that's good eatin'! I'm also

indebted to Brendan McConkey for showing me how an HPLC works, and how an HPLC doesn't work.

I wouldn't be at this point in my life if it wasn't for the people most important to me. Although I'll never be able to completely express my gratitude to my parents, I'd like to thank you anyway. Dad, thanks for introducing me to science in the first place. Ever since I saw the science room at Dougall Avenue School, I knew where I was heading. Mom, thanks for teaching me that the only way to get anywhere in life is one step at a time. It's helped me more times than you realize. I'm also grateful to Todd, for teaching me the importance of the past, and to Jennifer and Rich, for reminding me that life is always full of nice surprises. During the last twelve years, I've also been very fortunate to have the support of a whole other family. Nick and Mary, I thank you for all you've done for me, especially for having a daughter named Nicole. And thanks to the rest of the Gavrelets: Tanya, Greg and Kristyn (the Adams), Stephan, Rachel, Christina, Patricia, and Sara. During the writing of my thesis, I needed lots of laughs, and everyone more than delivered.

Finally, I owe so much to my wonderful wife Nicole and beautiful daughter Sophie. This thesis is dedicated to both of you, for during the time that I was writing it, I realized how much I appreciate my time spent with you. Nicole, you more than anyone, were there with me through the best times and the hardest times. Thank you for believing in me.

Table of Contents

GENERAL INTRODUCTION 1

A.1 HAZARD ASSESSMENT OF CHEMICAL MIXTURES 1

A.2 SOURCE DEFINITION: PLANAR CHLORINATED HYDROCARBONS 2

A. General Chemistry 2

B. General Sources 4

C. General Toxicity 5

A.3 SOURCE DEFINITION: POLYCYCLIC AROMATIC HYDROCARBONS 8

A. General Chemistry 8

B. General Sources 8

C. General Toxicity 9

A.4 ENVIRONMENTAL DESCRIPTION 10

A.5 PCH AND PAH EXPOSURE AND EFFECTS ASSESSMENT: ESTIMATING BIOCONCENTRATION AND BIOLOGICAL POTENCY IN FISH 10

A. Semipermeable Membrane Devices (SPMDs) 11

B. 2,3,7,8-TCDD Equivalent Concentrations (TECs) 12

C. Hepatic EROD Activity 13

A.4 OVERALL GOAL 14

A.6 REFERENCES 15

CHAPTER 1. COMPARISON OF MAMMALIAN AND TELEOST CELL LINE BIOASSAY AND CHEMICALLY DERIVED TCDD-EQUIVALENT CONCENTRATIONS IN HEPATIC TISSUE OF LAKE TROUT (*SALVELINUS NAMAYCUSH*) FROM LAKE SUPERIOR AND LAKE ONTARIO ... 24

1.1 ABSTRACT 24

1.2 INTRODUCTION 25

1.3 MATERIALS AND METHODS 29

A. Lake trout sampling 29

B. Isolation of PCHs from liver tissue 30

C. RTL-W1 and H4IIE cell maintenance 31

D. RTL-W1 and H4IIE bioassay procedure 32

E. Calculation of BD-TECs in lake trout livers 33

F. PCB, dioxin and furan analysis and CD-TEC determination 34

G. Hepatic Enzyme Analysis 36

H. Statistics 36

1.4 RESULTS 37

A. Whole fish parameters 37

B. Coplanar PCB, chlorinated dioxin furan congener determination 39
C. Bioassay-derived 2,3,7,8-TCDD equivalent concentrations 44
D. Chemically-derived 2,3,7,8-TCDD equivalent concentrations 44
E. Bioassay-derived vs chemically-derived 2,3,7,8-TCDD equivalent concentrations 51
F. Hepatic Enzyme Analysis 54
1.5 DISCUSSION 54
A. Coplanar PCB, chlorinated dioxin furan congener determination 55
B. Bioassay-derived 2,3,7,8-TCDD equivalent concentrations 57
C. Chemically-derived 2,3,7,8-TCDD equivalent concentrations 59
D. Chemically-derived versus bioassay-derived 2,3,7,8-TCDD equivalent concentrations 61
E. Hepatic Enzyme Analysis 61
F. Summary 63
1.6 ACKNOWLEDGEMENTS 64
1.7 REFERENCES 64

CHAPTER 2. THE USE OF MICROCOSMS TO EVALUATE METHODOLOGIES FOR ASSESSING THE EXPOSURE OF RAINBOW TROUT TO POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN CREOSOTE CONTAMINATED ENVIRONMENTS 76

2.1 ABSTRACT 76
2.2 INTRODUCTION 77
2.3 METHODS 82
A. Experimental Microcosms 82
B. SPMD Exposure and Collection 83
C. Rainbow Trout Exposure and Collection 85
D. PAH Isolation from SPMDs and Rainbow Trout Liver 86
E. HPLC Analysis and Preparation of Dilution Series For Cell Line Bioassays 87
F. RTL-W1 and H4IIE cell maintenance 88
G. RTL-W1 and H4IIE bioassay procedure 89
H. Calculation of BD-TECs in SPMDs and Hepatic Tissue 90
I. Calculation of CD-TECs in SPMDs and Hepatic Tissue 91
J. Hepatic Enzyme Analysis 91
K. Estimation of Water Concentrations from SPMDs 92
L. Statistics 93
2.4 RESULTS 94
A. PPPAHs in SPMDs 94
B. PPPAH concentrations in water estimated by SPMDs 96

C. PPPAHs in rainbow trout liver 97
D. TCDD equivalent concentrations (TECs) in SPMDs 102
E. TCDD equivalent concentrations (TECs) in liver extracts 109
F. SPMD TECs vs liver TECs 110
G. Hepatic EROD activity 115
2.5 DISCUSSION 115
A. SPMDs and PPPAH concentrations in water 116
B. PPPAHs in SPMDs and rainbow trout liver 118
C. Estimating TECs with RTL-W1 vs H4IIE 121
D. SPMD TECs versus rainbow trout liver TECs 122
E. BD-TECs versus CD-TECs 123
F. Assessment strategy 124
2.6 ACKNOWLEDGMENTS 126
2.7 REFERENCES 127

CHAPTER 3. THE USE OF CREOSOTE CONTAMINATED MICROCOSMS AND SEMIPERMEABLE MEMBRANE DEVICES (SPMDS) TO EVALUATE THE TIME-DEPENDENT UPTAKE AND POTENTIAL EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH) IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*). **138**

3.1 ABSTRACT 138
3.2 INTRODUCTION 139
3.3 MATERIALS AND METHODS 143
A. Experimental Microcosms 143
B. Rainbow Trout Exposure to Creosote Microcosms 144
C. SPMD Specifications and Exposure 145
D. Collection of Water Samples from Microcosms 146
E. PAH Isolation from SPMDs and Rainbow Trout Liver 146
F. GC Analysis and Preparation of Dilution Series For Cell Line Bioassays 149
G. RTL-W1 Maintenance and Bioassay Procedure 149
H. Calculation of CD- and BD-TECs in SPMDs and Hepatic Tissue 150
I. Estimation of Aqueous Concentrations of PPPAHs from SPMD Concentrations 151
J. Hepatic Enzyme Analysis 152
K. Statistical Analysis 153
3.4 RESULTS 154
A. Chemical Uptake by SPMDs 154
B. Estimated Aqueous Concentrations of PPPAHs from SPMD Concentrations 163

C. Chemical Uptake by Rainbow Trout *164*
D. CD- and BD-TECs in SPMDs and Trout *167*
E. Hepatic EROD in Trout *173*
3.5 DISCUSSION 176
A. PPPAH Accumulation in SPMDs and Rainbow Trout *176*
B. Non-priority Aromatic Compound Accumulation in SPMDs and Rainbow Trout *179*
C. Aqueous Concentrations of PPPAHs *180*
D. CD- and BD-TECs in SPMDs and Trout *181*
E. Hepatic EROD in Trout *183*
G. Summary *185*
3.7 REFERENCES 186

GENERAL CONCLUSIONS **194**

A. Additive Versus Non-additive Responses to PCH and PAH Mixtures *194*
B. Species Specificity in Response to PCHs and PAHs *195*
C. Hepatic EROD Activity as an Indicator of Exposure to PCHs and PAHs *195*
D. Use of SPMDs as Predictors of Exposure to PPPAHs *196*
E. Summary *197*

APPENDIX I **198**

APPENDIX II **201**

List of Tables

Chapter 1:

Table 1.1. RTL-W1, H4IIE and ELS TCDD equivalency factors for the PCHs measured in lake trout livers during this study.....35

Table 1.2. Data summary for lake trout collected from Black Bay, Jackfish Bay and Lake Ontario.....38

Table 1.3. PCH congener concentrations (pg/g wet wt.) detected in lake trout sampled from three Great Lakes sites.....40

Table 1.4. Effect of TEF source on the contribution of different PCHs to the CD-TECs in individual lake trout.....50

Chapter 2:

Table 2.1. Concentrations (µg/g lipid) of priority pollutant PAHs detected in lipid extracted from SPMDs exposed for 28 days in creosote dosed microcosms.....95

Table 2.2. SPMD estimated average water concentrations (ng/L) of selected PPPAH analytes sequestered over a 28 day exposure.....97

Table 2.3. Mean concentrations of priority pollutant PAHs detected in livers of rainbow trout exposed for 28 days in creosote dosed microcosms.....98

Table 2.4. PPPAHs found in extracts of this study and their 2,3,7,8-TCDD equivalent factors (TEFs) in RTL-W1 and H4IIE.....103

Chapter 3:

Table 3.1. Properties of analytes examined in extracts from SPMDs and rainbow trout exposed to creosote dosed microcosms over a 28 day period.....148

Table 3.2. Aqueous concentrations of selected PAHs in creosote dosed microcosms measured by conventional water sampling.....156

Table 3.3. Average aqueous concentrations of selected PPPAHs in creosote dosed microcosms over 28 days estimated using SPMD concentrations and a mathematical model and conventional water sampling.....164

Table 3.4. Mean concentrations of PPPAHs extracted from livers of rainbow trout exposed to creosote dosed microcosms over a 28 day period.....166

List of Figures

General Introduction:

Figure A.1. Paradigm for predictive ecological risk assessments.....2

Figure A.2. Representative planar chlorinated hydrocarbon (PCH) and polycyclic aromatic hydrocarbon (PAH) congeners.....4

Figure A.3. Proposed mechanism of AhR-mediated toxicity.....6

Chapter 1:

Figure 1.1. Locations of lake trout sampling sites examined in this study.....30

Figure 1.2. Comparison of mean hepatic PCDD and PCDF concentrations in white sucker from Jackfish Bay and lake trout from Jackfish Bay and Lake Ontario.....42

Figure 1.3. RTL-W1 and H4IIE bioassay-derived 2,3,7,8-TCDD equivalent concentrations (BD-TECs) determined for individual lake trout livers sampled from three sites in the Great Lakes.....45

Figure 1.4. Percent contributions of PCH congener classes to CD-TECs in lake trout livers from three sites in the Great Lakes.....48

Figure 1.5. Bioassay-derived (BD-) TECs relative to chemistry-derived (CD-) TECs in lake trout livers.....52

Chapter 2:

Figure 2.1. Effect of nominal creosote dose in microcosms on the amount of total PPPAHs accumulated by SPMDs and by rainbow trout liver during a 28 day exposure in the microcosms and on the total PPPAH levels in the water and sediment of the microcosms.....99

Figure 2.2. Mean concentrations of individual PPPAHs expressed as a percentage of the total PPPAH concentrations for both SPMDs and trout liver across all creosote doses.....100

Figure 2.3. Effect of nominal creosote dose in microcosms on the RTL-W1 CD and BD-TECs accumulated by SPMDs and by rainbow trout liver.....104

Figure 2.4. Effect of nominal creosote dose in microcosms on the H4IIE CD and BD-TECs accumulated by SPMDs and by rainbow trout liver.....106

Figure 2.5. A comparison of the effects of a 28 day exposure to microcosms of differing nominal creosote doses on rainbow trout hepatic EROD activity and on RTL-W1 BD-TECs accumulated by rainbow trout liver and SPMDs.....111
Figure 2.6. A comparison of the effects of a 28 day exposure to microcosms of differing nominal creosote doses on rainbow trout hepatic EROD activity and on H4IIE BD-TECs accumulated by rainbow trout liver and SPMDs.....113

Chapter 3:

Figure 3.1. Uptake with time of total PPPAHs by SPMDs from creosote microcosms.....155
Figure 3.2. Uptake with time of individual PPPAHs by SPMDs from creosote microcosms.....158
Figure 3.3. Uptake with time of individual non-priority aromatic hydrocarbons by SPMDs from creosote microcosms.....161
Figure 3.4. EROD dose-response curves in RTL-W1 for 2,3,7,8-TCDD and selected extracts.....171
Figure 3.5. Uptake with time of total 2,3,7,8-TCDD equivalent concentrations by SPMDs from creosote microcosms as determined by the RTL-W1 bioassay.....174
Figure 3.6. Hepatic EROD activity in rainbow trout exposed to creosote microcosms for varying periods of time.....175

List of Abbreviations

AHH	Arylhydrocarbon hydroxylase
AhR	Arylhydrocarbon receptor
BD-TEC	Bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentration
CD-TEC	Chemically-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentration
CYP1A	Cytochrome P4501A
DMSO	Dimethylsufoxide
ELS	Early Life Stage Mortality
EROD	Ethoxyresorufin-*O*-deethylase
GC/MS	Gas chromatography/mass spectrometry
H4IIE	Rat hepatoma cell line
HPLC	High performance liquid chromatography
MEM	Modified Eagle's medium
MS222	methane tricainesulfonate
OCDD	Octachlorodibenzo-*p*-dioxin
OCDF	Octachlorodibenzofuran
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
PCH	Planar chlorinated hydrocarbon
PPPAH	Priority pollutant polycyclic aromatic hydrocarbon
RTL-W1	Rainbow trout liver cell line
SPMD	Semipermeable membrane device
TEC or TEQ	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin equivalent concentration
TEF	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin equivalency factor
1,2,3,4,6,7,8-HpCDD	1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin
1,2,3,4,6,7,8-HpCDF	1,2,3,4,6,7,8-Heptachlorodibenzofuran
1,2,3,4,7,8-HxCDD	1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin
1,2,3,4,7,8-HxCDF	1,2,3,4,7,8-Hexachlorodibenzofuran
1,2,3,6,7,8-HxCDD	1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin
1,2,3,6,7,8-HxCDF	1,2,3,6,7,8-Hexachlorodibenzofuran
1,2,3,7,8,9-HxCDD	1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin
1,2,3,7,8,9-HxCDF	1,2,3,7,8,9-Hexachlorodibenzofuran
1,2,3,7,8-PeCDD	1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin
1,2,3,7,8-PeCDF	1,2,3,7,8- Pentachlorodibenzofuran
2,3,4,6,7,8-HxCDF	2,3,4,6,7,8-Hexachlorodibenzofuran
2,3,4,7,8-PeCDF	2,3,4,7,8- Pentachlorodibenzofuran
2,3,7,8-TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
2,3,7,8-TCDF	2,3,7,8-Tetrachlorodibenzofuran
3,3',4,4',5,5'-HxCB	3,3',4,4',5,5'-Hexachlorobiphenyl (PCB 169)
3,3',4,4',5-PeCB	3,3',4,4',5-Pentachlorobiphenyl (PCB 126)
3,3',4,4'-TCB	3,3',4,4'-Tetrachlorobiphenyl (PCB 77)
3,4,4',5-TCB	3,4,4',5-Tetrachlorobiphenyl (PCB 81)

GENERAL INTRODUCTION

A.1 HAZARD ASSESSMENT OF CHEMICAL MIXTURES

For several decades the public has become increasingly aware of the potential threat posed by contamination of the environment with toxic chemicals. A primary goal of toxicological studies is to assess whether or not exposure to these contaminants will put populations of organisms at risk. To deal with the complexities of evaluating the hazard associated with chemical exposure, a general model for assessing risk has been devised (Suter 1992). This paradigm for risk assessment of chemical exposure is presented in Figure A.1. It defines the hazard to be assessed, followed by estimation of exposure and relationship of effects to exposure, characterizes the risk resulting from the combination of estimated exposure and exposure-response relationship, and finally conveys the results to a risk manager (Suter 1992).

Although most toxicological studies have assessed the biological effects of individual compounds, environmental contaminants generally exist as complex mixtures. To confront the problems associated with the differing bioavailabilities, toxic potencies and interactive effects of combinations of chemicals, a variety of assessment tools have been proposed. In this thesis, the risk assessment paradigm was employed as a framework for the integration and critical assessment of several of these tools. Two specific families of compounds, the planar chlorinated hydrocarbons (PCHs) and the polycyclic aromatic hydrocarbons (PAHs), were examined. The main hypothesis was that the use of a multilevel approach to estimate both chemical uptake and biological effect in fish can provide more realistic and effective results than any single assessment

tool. By providing several lines of evidence for exposure and effect, decisions based on risk assessment procedures can be made with an increased level of confidence.

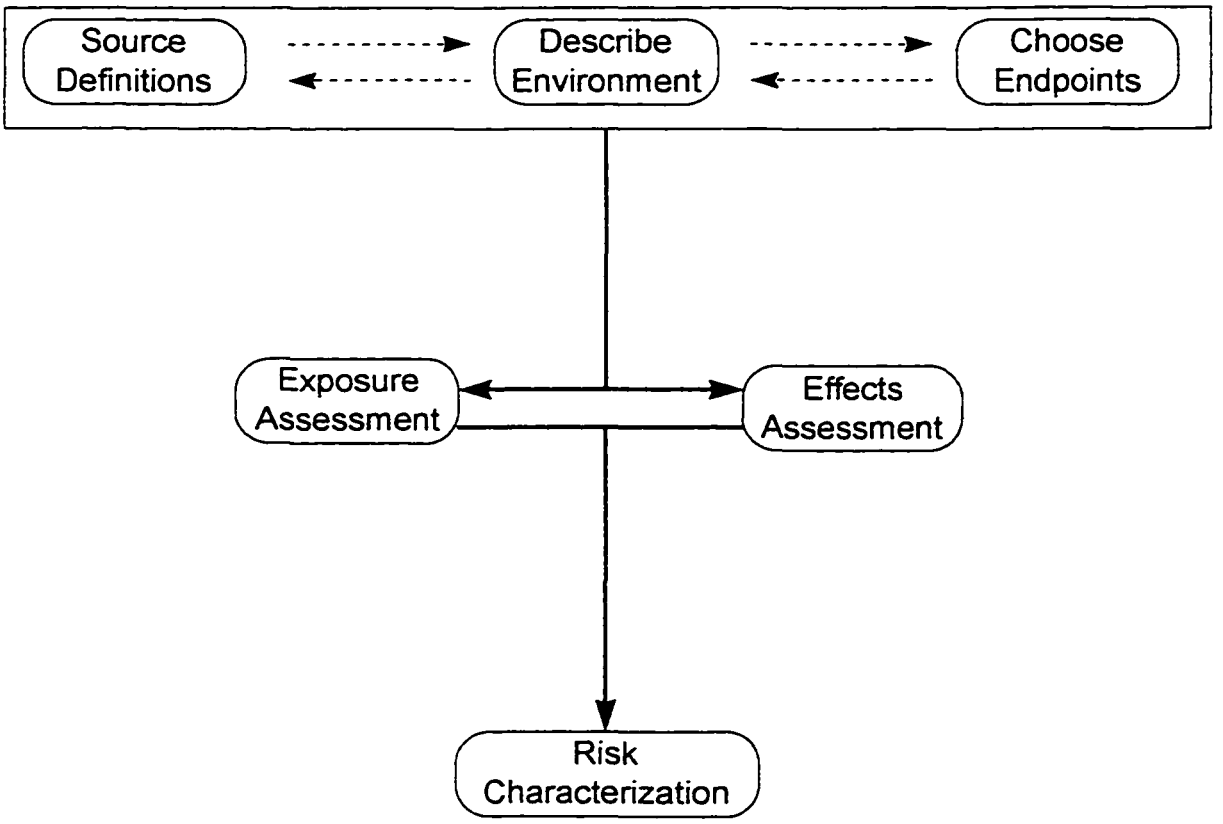


Figure A.1. Paradigm for predictive ecological risk assessments. The solid arrows represent the sequential flow of the procedure. The dashed arrows represent feedback and other constraints on one assessment process by other processes (Suter 1995).

A.2 SOURCE DEFINITION: PLANAR CHLORINATED HYDROCARBONS

A. General Chemistry

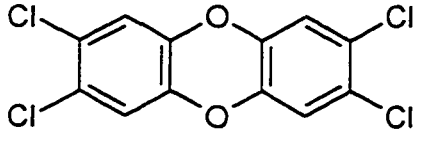
Planar chlorinated hydrocarbons (PCHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are

a group of environmental pollutants that have been identified in a variety of environmental compartments worldwide (Safe 1994; Grimwood and Dobbs 1995). The PCDDs and PCDFs are planar, chloro-substituted polycyclic aromatic compounds, all with similar chemical characteristics. There are 75 PCDDs and 135 PCDFs based on varying patterns of ring chlorination, and the name of each compound is based on the positions of these substituted chlorine atoms (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 2,3,7,8-TCDD, Figure A.2.a). PCDDs and PCDFs are very insoluble in water (10^{-7} - 10^{-10} g L^{-1}) and have correspondingly high octanol-water partition coefficients (log K_{OW} 5.04-10.32). These properties result in the association of PCDDs and PCDFs with organic matter in aquatic environments, and they are readily accumulated in organisms. Because these compounds are highly resistant to chemical and biological breakdown they persist for long periods of time adsorbed to sediments (Grimwood and Dobbs 1995; Devito and Birnbaum 1995; Safe 1990).

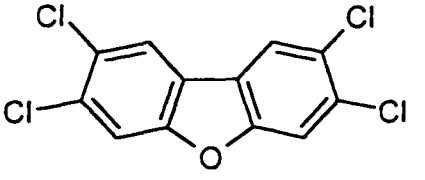
PCBs are a group of aromatic organic chemicals consisting of 209 congeners composed of a basic two-ring structure (Figure A.2.c). They are highly stable, inflammable, and hydrophobic. Like PCDDs and PCDFs, these properties cause PCBs to persist in environments and bioaccumulate in aquatic organisms. The naming system of PCBs is based on the placement of chlorine atoms on the molecule (e.g. 3,3′,4,4′,5-pentachlorobiphenyl) but to simplify references to PCBs, a single number nomenclature system was introduced, each congener being assigned a number from 1 to 209 (Metcalfe and Haffner 1995; Safe 1994; Kimbrough 1995).

B. General Sources

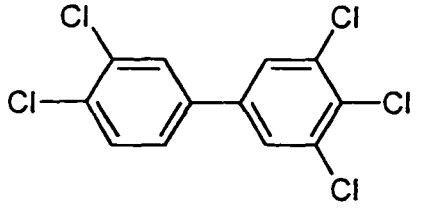
PCDDs and PCDFs are not intentionally produced, but rather result as trace impurities during the manufacture of chlorophenols and herbicides (e.g. 2,4,5-trichlorophenoxyacetic acid). Other sources of these contaminants include chlorine bleaching of paper products, incineration of municipal waste, and the production of steel and copper. In contrast, the production or PCBs was deliberate. The industrial manufacture of PCBs began in 1929 and continued until quite recently in some countries. The properties of PCBs that make them persistent environmental contaminants were quite desirable in industrial applications.



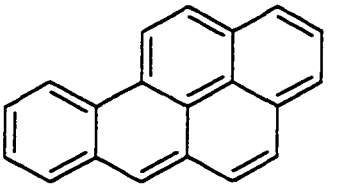
(a) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin



(b) 2,3,7,8-Tetrachlorodibenzofuran



(c) 3,3′,4,4′,5-Pentachlorobiphenyl (PCB 126)



(d) Benzo[a]pyrene

Figure A.2. Representative planar chlorinated hydrocarbon (PCH) and polycyclic aromatic hydrocarbon (PAH) congeners. The molecules demonstrate the general structure of compounds in the following classes: (a) polychlorinated dibenzo-*p*-dioxins (PCDDs), (b) polychlorinated dibenzofurans (PCDFs), (c) polychlorinated biphenyls (PCBs) and (d) polycyclic aromatic hydrocarbons (PAHs).

Their stability made PCBs ideal as flame retardants, hydraulic lubricants, sealants and dielectric fluids in transformers and capacitors. A significant portion of the introduction of PCBs into the environment has been due to improper disposal practices, leakage from chemical waste disposal sites and industrial accidents. Production of PCBs worldwide is estimated to be 1.5 million tonnes (Safe 1994; De Voogt and Brinkman 1989).

C. General Toxicity

The major cellular mechanism of PCH toxicity is believed to occur through the binding of these compounds to a cytosolic aryl hydrocarbon receptor (AhR) (Nebert et al. 1993). A variety of AhR-mediated responses to PCHs include severe wasting syndrome, epidermal hyperplasia and metaplasia, tumor promotion, and thymic involution (Poland and Knutson 1982). Figure A.3. reveals the sequence of events that occur following cellular exposure to PCHs.

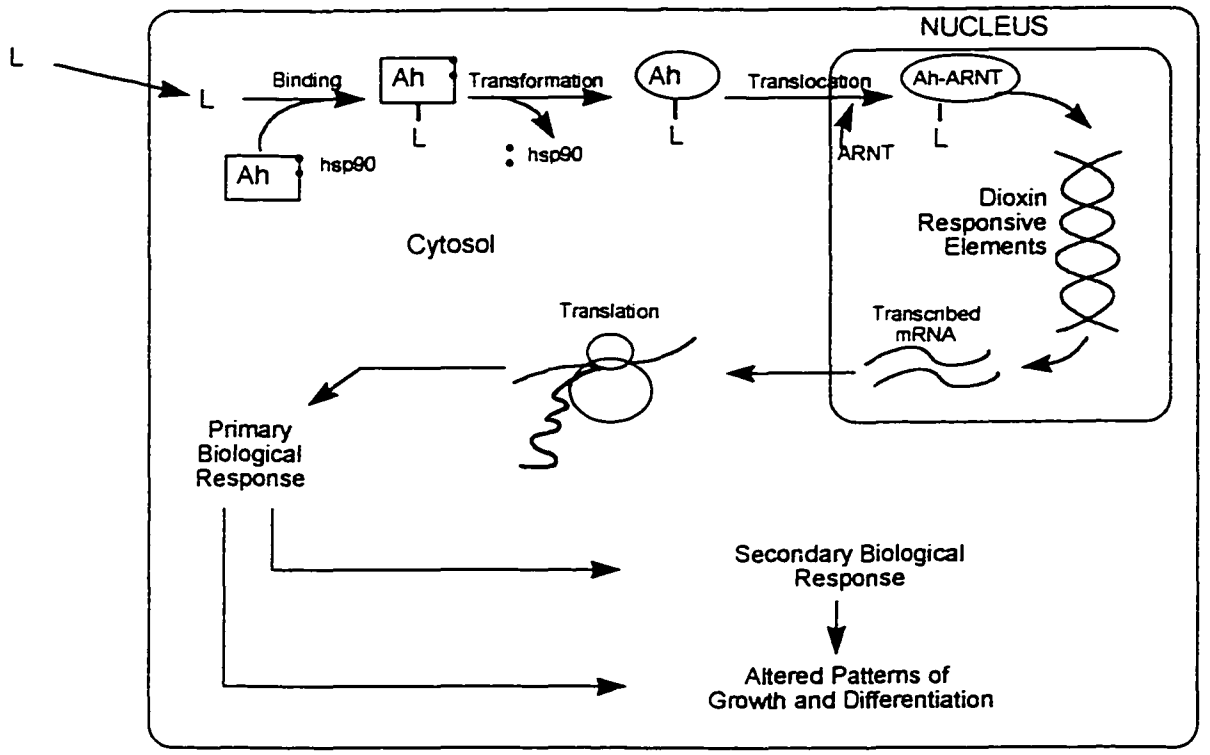


Figure A.3. Proposed mechanism of AhR-mediated toxicity. Signal transduction by PCH ligands (L) is mediated by the AhR, which forms a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT). This heterodimer recognizes a specific DNA sequence, the dioxin responsive element (DRE) and leads to the induction of several genes (the Ah gene battery). The elevated levels of the protein products are thought to be involved in the toxic action of PCHs (modified and redrawn from Okey et al., 1994).

PCHs enter the cell through passive diffusion and bind to the AhR in the cytosol. An activation step involving the release of heat shock proteins (hsp90) is thought to allow the AhR to bind to an aryl hydrocarbon nuclear translocation (ARNT) protein. In the nucleus, this complex binds to specific regions of DNA, designated as dioxin responsive elements, due to the fact that 2,3,7,8-TCDD (dioxin) is the most potent ligand for the AhR. Upon binding, several genes are induced, which have been designated the Ah-gene battery (Nebert et al. 1993). These include the phase I drug metabolizing cytochrome P450 enzymes, CYP1A1 and 1A2, as well as several phase II drug metabolizing enzymes. The resultant proteins are thought to be involved in the toxic action of PCH, causing alterations in cellular homeostasis. An alternative hypothesis has been suggested that upon binding to the AhR, PCHs may inhibit the action of a yet unidentified natural AhR ligand, such that a critical life function cannot be carried out (Nebert et al. 1993).

It is currently thought that PCH contamination may pose a threat to the rehabilitation of lake trout populations in the Great Lakes (Zint et al. 1995; Mac 1988). Although other causes of lake trout population decline have been identified, such as sea lamprey (*Petromyzon marinus*) predation and overfishing (Eshenroder 1987), stocking efforts, reduction in commercial fishing and sea lamprey control have only resulted in viable population establishment in Lake Superior (Hansen et al. 1995). The early life stages of lake trout have been suggested as the most sensitive target for PCH toxicity (Walker et al. 1996), and elevated levels of these contaminants have been detected in lake trout eggs from Lake Ontario (Guiney et al. 1996). Lake trout eggs receive PCHs by maternal transfer (Miller 1993) so knowing the concentration of biologically-active chemicals in the adult fish is important. As top predators, lake trout tend to accumulate high concentrations of PCHs

(Mac and Schwartz 1991). Other factors that lead to increased levels of contaminants in adult lake trout are their large size, long life span, high lipid levels and their position at the top of the food chain (Miller 1993).

A.3 SOURCE DEFINITION: POLYCYCLIC AROMATIC HYDROCARBONS

A. General Chemistry

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous environmental pollutants containing carbon and hydrogen atoms with more than one benzene ring in their structure (Figure A.2.d). PAHs are hydrophobic, and this property generally increases with molecular weight (log K_{OW} 3.3 - 7.5). As a result, thermodynamic equilibrium tends to favour partitioning of larger PAHs to more nonpolar environments, including organism lipid. The degradation of PAHs in aquatic environments can be affected by several factors, such as redox state, temperature, and biological activity (biodegradation). In addition, several PAHs are susceptible to photolytic degradation. Combined, these factors can result in rapid reductions of PAHs in aquatic environments, with only small percentages of original concentrations remaining after periods of a few months (Meador et al. 1995; Hellou 1996; Lee et al. 1978).

B. General Sources

Although natural sources of PAHs exist (e.g. forest fires), contamination of aquatic environments originates mainly from anthropogenic sources such as incomplete combustion of fossil fuels, petroleum spills, discharges from ships, industrial effluents, highway runoff,

seepage from creosote treated wood products and creosote spills (the main source of PAHs examined in this thesis). Creosote, composed of roughly 85% PAHs, is used in Canada as a lasting wood preservative for railway ties, bridge timbers and marine pilings (Government of Canada et al. 1993). Waste creosote is known to be entering groundwaters and surface waters at 24 sites across Canada, and there is an estimated 256 000 m^3 of creosote contaminated soil from creosote treatment facilities.

C. General Toxicity

Like PCHs, PAHs can exert biological effects through the AhR. In addition to this common mechanism of action, the metabolism of PAHs by induced P450 enzymes can result in the formation of reactive intermediates, such as the diol epoxides (Ralston et al. 1997). These intermediates can bind to DNA and have demonstrated carcinogenic and mutagenic potential. Several examples of PAH toxicity to aquatic organisms exist, most of which involve these compounds in the formation of hepatic neoplasms in fish (Collier et al. 1992; Malins et al. 1985; Vogelbein et al. 1990). Aside from carcinogenesis, PAHs also have immunosuppressive and estrogenic activities (Davila et al. 1997; Santodonato 1997). Because they are often readily and extensively metabolized in fish, PAHs frequently only leave a trace of the parent compound in the tissue (Meador et al. 1995). This metabolism can mask the link between exposure, uptake and biological effect of PAHs.

A.4 ENVIRONMENTAL DESCRIPTION

For several decades, evaluation of the biological effects of chemicals on aquatic organisms were generally based on the results of laboratory toxicity tests. Although easily standardized and cost efficient, simple laboratory tests often overestimate the magnitude and duration of exposure relative to that observed in natural field systems (Pratt et al. 1990). One method of harmonizing the advantages of laboratory and field studies is through the use of simulated field environments such as outdoor microcosms (Graney et al. 1995). Microcosms simulate some of the complexities of a natural ecosystem while maintaining a degree of control close to that of laboratory exposures. Defined concentrations of contaminants can be added to experimental ponds and the chemical proportions of the mixture should remain comparable due to equal volumes, water chemistry and sediment composition in each microcosm. Exposure and collection of test organisms can be standardized and simplified, which is not always the case in field studies (Bankey et al. 1994). Microcosms do have limitations in that the impact of the physical environment on large scale aquatic ecosystems in nature can differ greatly from that represented in a microcosm (Graney et al. 1995).

A.5 PCH AND PAH EXPOSURE AND EFFECTS ASSESSMENT: ESTIMATING BIOCONCENTRATION AND BIOLOGICAL POTENCY IN FISH

Assessing the degree of uptake and resultant effects of PCHs and PAHs is complicated due to the vast number of these chemicals in the environment, each with varying degrees of bioavailability and toxicity. Although it is possible to determine the

identities and concentrations of organic contaminants in water, sediments and biological tissues through analytical chemistry techniques (Zitko 1993; Huestis et al. 1996; Huestis et al. 1995; Mátlová et al. 1995), to estimate biological effects, it is more appropriate to measure only the truly dissolved, bioavailable compounds present. Because many PAHs and some PCHs are rapidly metabolized in fish, a direct measurement of chemical concentrations in tissue can result in an underestimation of toxicant exposure.

A. Semipermeable Membrane Devices (SPMDs)

In an attempt to predict the uptake of bioavailable compounds in an aquatic environment, a device that mimics the bioconcentration process by passively sampling dissolved hydrophobic contaminants has been developed (Huckins et al. 1996). The semipermeable membrane device (SPMD) is composed of a thin film of neutral lipid (triolein) sealed in a section of lay-flat polyethylene tubing (Prest et al. 1992). Hydrophobic contaminants move through the polyethylene membrane via passive diffusion and partition into the lipid where they are concentrated. In contrast to gill membranes, SPMD polyethylene membranes are not altered in response to stress (Gale et al. 1997) and contaminants sequestered in the triolein phase are not subject to metabolic transformations that occur in fish (Moring and Rose 1997). SPMDs have advantages over conventional methods of contaminant measurement. Firstly, SPMDs provide an estimation of the average contaminant uptake over time, integrating both chronic and episodic contamination events. Secondly, only truly dissolved chemicals are sampled, eliminating the inclusion of organically-bound chemicals.

B. 2,3,7,8-TCDD Equivalent Concentrations (TECs)

Although SPMDs are useful in determining the presence of bioavailable contaminants, they do not clearly indicate the effects of the chemicals present in an organism, or the potential for certain contaminants to interact with one another to alter the overall effect of the mixture. In order for the toxicity of a complex PCH and PAH mixture to be evaluated, the toxic equivalent approach has been developed (Eadon et al. 1986; Barnes 1991). This requires two data sets. The first is a congener analysis and quantification, which can be done by such analytical chemistry techniques as GC-MS (Huestis et al. 1995). The second is a toxicological data base that allows the assignment to each congener of a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent factor (TEF) or induction equivalency factor (IEF) (Kennedy et al. 1996). TEFs have been derived from a variety of in vivo and in vitro toxicity tests (Kutz et al. 1990; Safe 1990; Safe 1994; Ahlborg et al. 1994; Parrott et al. 1995; Kennedy et al. 1996; Clemons et al. 1997) and convert the dose of the congener to the dose of TCDD that would produce the same response. TCDD is chosen as the reference because it is usually the most potent congener, and the mechanistic basis of its toxicity is best understood (i.e. AhR-mediated toxicity) (Okey et al. 1994). The application of the two data sets in the toxic equivalent approach requires the assumption that the congeners in a sample act in an additive manner. Therefore, the toxic potency of a complex chemical mixture is estimated by summing the TCDD-equivalent concentration of each congener present to give the TCDD-equivalent quantity (TEQ) or concentration (TEC). The more recent term TEC will be used throughout this thesis. Due to the fact that this type of TEC calculation depends on knowing the congeners present, this is sometimes called the chemistry-derived TEC (CD-TEC) (Bols et al. 1997).

As well as analytical methods, the measurement of contaminants in biological tissues can be done with bioassays (Firestone 1991). The most frequent bioassay has been the induction in liver cell lines of CYP1A, which has been measured as aryl hydrocarbon hydroxylase (AHH) or 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Tillitt et al. 1991). This is the same response that has been used to derive TEFs in vitro, and for the bioassays, usually the same cell lines are used that have been used for TEF derivation. Most commonly, the cell line is the rat hepatoma H4IIE (Safe 1987; Tillitt et al. 1991). Recently, questions have been raised as to the applicability of mammalian systems for predicting toxic effects in aquatic species (Bols et al. 1997; Clemons et al. 1997). To address this issue, use has been made of two rainbow trout (*Oncorhynchus mykiss*) cell lines: hepatic RTL-W1 (Bols et al. 1997) and gonadal RTG-2 (Zabel et al. 1996). For individual compounds, subtle species-specific differences exist (Bols et al. 1997). Cell bioassays integrate the potency of all the compounds in the sample. As well as PCHs and PAHs, this includes any other compound(s) that might interact with the AhR to induce CYP1A (i.e. "AhR-active" compounds). The potency of an extract is expressed relative to TCDD. Since the identity of the congeners in the sample will be unknown by this method, the measurement is sometimes referred to as the bioassay-derived TCDD equivalent concentration (BD-TEC) (Bols et al. 1997).

C. Hepatic EROD Activity

In addition to *in vitro* induction of CYP1A enzymes, measurement of EROD activity in the tissues of PCH and PAH exposed fish can serve as a potential link between

contamination and biological effect. By integrating exposure to the entire bulk of contaminants present, EROD activity can reflect the cumulative, synergistic, or antagonistic effects of complex chemical mixtures. Furthermore, EROD activity can persist after the inducing chemicals have been metabolized and/or eliminated from an organism, leaving a "biochemical fingerprint" of exposure. It should be noted, however, that although several studies have correlated EROD induction with deleterious effects in fish, a causal link between increased EROD activity and these effects has yet to be demonstrated (Bucheli and Fent 1995). Because of the sensitivity of EROD as an indicator of exposure, it has been referred to as an early warning system for assessing environmental health (Payne et al. 1987).

A.4 OVERALL GOAL

The overall goal of this thesis is to combine the techniques described in order to evaluate the strengths and weaknesses of individual procedures and combinations of procedures in estimating the exposure of fish to PCH and PAH mixtures. Demonstration of the utility of multilevel assessments will provide risk managers with a means of achieving more useful and congruent data sets for decision making. In chapter one, TECs and hepatic EROD are combined and used to examine PCH contamination in lake trout from the Great Lakes. In chapters two and three, microcosms, SPMDs and TECs are used to study the exposure of rainbow trout to PAHs in creosote-contaminated environments.

A.6 REFERENCES

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

Barnes, D.G. 1991. Toxicity equivalents and EPA's risk assessment of 2,3,7,8-TCDD. The Science of the Total Environment. 104: 73-86.

Bols, N.C., Whyte, J.J., Clemons, J.H., Tom, D.J., van den Heuvel, M.R., and Dixon, D.G. 1997. Use of liver cell lines to develop TCDD equivalency factors and TCDD equivalent concentrations in environmental samples. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment. J.T. Zelikoff, ed. SOS Publishing, Fair Haven, NJ, 1st ed.: pp. 329-350.

Bucheli, T.D. and Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25: 201-268.

Clemons, J.H., Dixon, D.G., and Bols, N.C. 1997. Derivation of 2,3,7,8-TCDD toxic equivalent factors (TEFs) for selected dioxins, furans, and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. Chemosphere. 34: 1105-1119.

Collier, T.K., Singh, S.V., Awasthi, Y.C., and Varanasi, U. 1992. Hepatic xenobiotic metabolizing enzymes in two species of benthic fish showing different prevalences of contaminant-associated liver neoplasms. Toxicology and Applied Pharmacology. 113: 319-324.

Davila, D.R., Mounho, B.J., and Burchiel, S.W. 1997. Toxicity of polycyclic aromatic hydrocarbons to the human immune system: models and mechanisms. Toxicology and Ecotoxicology News. 4: 5-9.

De Voogt, P. and Brinkman, U.A.T. 1989. Production, properties and usage of polychlorinated biphenyls. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. R.D. Kimbrough and A.A. Jensen, eds. Elsevier Science Publishers, Amsterdam, 2nd ed.

Devito, M.J. and Birnbaum, L.S. 1995. Dioxins: model chemicals for assessing receptor-mediated toxicity. Toxicology. 102: 115-123.

Eadon, G., Kaminsky, L., Silkworth, J., Aldous, K., Hilker, D., O'Keefe, P., Smith, R., Gierthy, J., Hawley, J., Kim, N., and DeCaprio, A. 1986. Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. Environmental Health Perspectives. 70: 221-227.

Eshenroder, R.L. 1987. Socioeconomic aspects of lake trout rehabilitation in the Great Lakes. Transactions of the American Fisheries Society. 116: 309-313.

Firestone, D. 1991. Determination of dioxins and furans in foods and biological tissues: review and update. Journal of the Association of Official Analytical Chemists. 74: 375-384.

Gale, R.W., Huckins, J.N., Petty, J.D., Peterman, P.H., Williams, L.L., Morse, D., Schwartz, T.R., and Tillitt, D.E. 1997. Comparison of the uptake of dioxin-like compounds by caged channel catfish and semipermeable membrane devices in the Saginaw River, Michigan. Environmental Science and Technology. 31: 178-187.

Government of Canada, Environment Canada, and Health and Welfare Canada. 1993. Creosote-impregnated waste materials. In: Canadian Environmental Protection Act. Minister of Supply and Services, Canada, pp. 1-25.

Graney, R.L., Giesy, J.P., and Clark, J.R. 1995. Field studies. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 493-521.

Grimwood, M.J. and Dobbs, T.J. 1995. A review of the aquatic ecotoxicology of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Environmental Toxicology and Water Quality. 10: 57-75.

Guiney, P.D., Cook, P.M., Casselman, J.M., Fitzsimmons, J.D., Simonin, H.A., Zabel, E.W., and Peterson, R.E. 1996. Assessment of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced sac fry mortality in lake trout (*Salvelinus namaycush*) from different regions of the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences. 53: 2080-2092.

Hansen, M.J., Peck, J.W., Schorfhaar, R.G., Selgby, J.H., Shreiner, D.R., Schram, S.T., Burnham-Curtis, M.K., Curtis, G.L., Swanson, B.L., Heinrich, J.W., and Young, R.J. 1995. Lake trout (*Salvelinus namaycush*) populations in Lake Superior and their restoration in 1959-1993. Journal of Great Lakes Research. 21 (supplement 1): 152-175.

Hellou, J. 1996. Polycyclic aromatic hydrocarbons in marine mammals, finfish, and molluscs. In: Environmental contaminants in wildlife: interpreting tissue concentrations. W.N. Beyer, G.H. Heinz, and A.W. Redmon-Norwood, eds. CRC Press, Boca Raton, Florida, 1st ed.: pp. 229-250.

Huckins, J.N., Petty, J.D., Lebo, J.A., Orazio, C.E., Prest, H.F., Tillitt, D.E., Ellis, G.S., Johnson, B.T., and Manuweera, G.K. 1996. Semipermeable membrane devices (SPMDs) for the concentration and assessment of bioavailable organic contaminants in aquatic environments. In: Techniques in Aquatic Toxicology. G.K. Ostrander, ed. CRC Press, Boca Raton, Florida, 1st ed.: pp. 625-655.

Huestis, S.Y., Servos, M.R., Sergeant, D.B., Leggett, M., and Dixon, D.G. 1995. Methods for determination of organochlorine pesticides, polychlorinated biphenyl congeners and chlorinated dibenzo-*p*-dioxins and furans in fish. Canadian Technical Report on Fisheries and Aquatic Sciences. 2044: 1-30.

Huestis, S.Y., Servos, M.R., Whittle, D.M., and Dixon, D.G. 1996. Temporal and age-related trends in levels of polychlorinated biphenyl congeners and organochlorine contaminants in lake ontario lake trout (*Salvelinus namaycush*). Journal of Great Lakes Research. 22: 310-330.

Janz, D.M. and Metcalfe, C.D. 1991. Nonadditive interactions of mixtures of 2,3,7,8-TCDD and 3,3',4,4'-tetrachlorobiphenyl on aryl hydrocarbon hydroxylase induction in rainbow trout (*Oncorhynchus mykiss*). Chemosphere. 23: 467-472.

Kennedy, S.W., Lorenzen, A., and Norstrom, R.J. 1996. Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. Environmental Science and Technology. 30: 706-715.

Kimbrough, R.D. 1995. Polychlorinated biphenyls (PCBs) and human health: an update. Critical Reviews in Toxicology. 25: 133-163.

Kutz, F.W., Barnes, D.G., Bottimore, D.P., Greim, H., and Bretthauer, E.W. 1990. The international toxicity equivalency factors (I-TEF) method of risk assessment for complex mixtures of dioxins and related compounds. Chemosphere. 20: 751-757.

Lee, R.F., Gardner, W.S., Anderson, J.W., Blaylock, J.W., and Barwell-Clarke, J. 1978. Fate of polycyclic aromatic hydrocarbons in controlled ecosystems enclosures. Environmental Science and Technology. 7: 832-838.

Mac, M.J. 1988. Toxic substances and survival of Lake Michigan salmonids: field and laboratory approaches. In: Toxic contaminants and ecosystem health; a Great Lakes focus. M.S. Evans, ed. John Wiley and Sons, Inc, pp. 389-401.

Mac, M.J. and Schwartz, T.R. 1991. Investigation in to the effects of PCB congener on reproduction of lake trout from the Great Lakes. In: Cause-Effect Linkages II Symposium Abstracts. R.S. Schneider and R. Campbell, eds. Michigan Audubon Society, pp. 19-20.

Malins, D.C., Krahn, M.M., Myers, M.S., Rhodes, L.D., Brown, D.W., Krone, C.A., McCain, B.B., and Chan, S.L. 1985. Toxic chemicals in sediments and biota from a creosote-polluted harbor: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). Carcinogenesis. 6: 1463-1469.

Mátlová, L., Machala, M., Nezveda, K., Granátová, M., and Nevoránková, Z. 1995. Biochemical screening of highly toxic aromatic contaminants in river sediment and comparison of sensitivity of biological model systems. Chemosphere. 30: 1363-1371.

Meador, J.P., Stein, J.E., Reichert, W.L., and Varanasi, U. 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Reviews in Environmental Contamination and Toxicology. 143:79-165: 79-165.

Metcalfe, C.D. and Haffner, G.D. 1995. The ecotoxicology of coplanar polychlorinated biphenyls. Environmental Reviews. 3: 171-190.

Miller, M.A. 1993. Maternal transfer of organochlorine compounds in salmonines to their eggs. Canadian Journal of Fisheries and Aquatic Sciences. 50: 1405-1413.

Moring, J.B. and Rose, D.R. 1997. Occurrence of polycyclic aromatic hydrocarbons in semipermeable membrane devices and clams in three urban streams of the Dallas-Forth Worth Metropolitan Area, Texas. Chemosphere. 34: 551-566.

Munkittrick, K.R., Blunt, B.R., Leggett, M., Huestis, S., and McCarthy, L.H. 1995. Development of a sediment bioassay to determine bioavailability of PAHs to fish. Journal of Aquatic Ecosystem Health. 4: 169-181.

Nebert, D.W., Puga, A., and Vasiliou, V. 1993. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. Ann. N. Y. Acad. Sci. 685:624-40: 624-640.

Okey, A.B., Riddick, D.S., and Harper, P.A. 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Toxicology Letters. 70: 1-22.

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

Payne, J.F., Fancey, L.L., Rahimtula, A.D., and Porter, E.L. 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. Comparative Biochemistry and Physiology.C. 86: 233-245.

Poland, A. and Knutson, J.C. 1982. 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharmacol. Toxicol. 22: 517-554.

Pratt, J.R., Bowers, J. and Cairns, J. 1990. Effect of sediment on estimates of diquat toxicity in laboratory microcosms. Water Res. 24: 51-57.

Prest, H.F., Jarman, W.M., Burns, S.A., Weismuller, T., Martin, M., and Huckins, J.N. 1992. Passive water sampling via semipermeable membrane devices (SPMDs) in concert with bivalves in the Sacramento/San Joaquin River Delta. Chemosphere. 25: 1811-1823.

Ralston, S.L., Coffing, S.L., Seidel, A., Luch, A., Platt, K.L., and Baird, W.M. 1997. Stereoselective activation of dibenzo[a,l]pyrene and its trans-11,12- dihydrodiol to fjord region 11,12-diol 13,14-epoxides in a human mammary carcinoma MCF-7 cell-mediated V79 cell mutation assay. Chem. Res. Toxicol. 10: 687-693.

Safe, S. 1987. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. Chemosphere. 16: 791-802.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology. 21: 51-88.

Safe, S.H. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Reviews in Toxicology. 24: 87-149.

Santodonato, J. 1997. Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. Chemosphere. 34: 835-848.

Suter, G.W. 1995. Introduction to ecological risk assessment for aquatic toxic effects. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 803-816.

Tillitt, D.E., Giesy, J.P., and Ankley, G.T. 1991. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environmental Science and Technology. 25: 87-92.

Vogelbein, W.K., Fournie, J.W., Van Veld, P.A., and Huggett, R.J. 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. Cancer Research. 50: 5978-5986.

Walker, M.K., Cook, P.M., Butterworth, B.C., Zabel, E.W., and Peterson, R.E. 1996. Potency of a complex mixture of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in causing fish early life stage mortality. Fundamental and Applied Toxicology. 30: 178-186.

Zabel, E.W., Pollenz, R., and Peterson, R.E. 1996. Relative potencies of individual polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners and congener mixtures based on induction of cytochrome P4501A mRNA in a rainbow trout gonadal cell line (RTG-2). Environmental Toxicology and Chemistry. 15: 2310-2318.

Zint, M.T., Taylor, W.W., Carl, L., Edsall, C.C., Heinrich, J., Sippel, A., Lavis, D., and Schaner, T. 1995. Do toxic substances pose a threat to rehabilitation of lake trout in the Great Lakes? A review of the literature. Journal of Great Lakes Research. 21 (supplement 1): 530-546.

Zitko, V. 1993. Patterns of polynuclear aromatic hydrocarbons (PAH) in sediments. Central European Journal of Public Health. 1: 125-129.

CHAPTER 1

COMPARISON OF MAMMALIAN AND TELEOST CELL LINE BIOASSAY AND CHEMICALLY DERIVED TCDD-EQUIVALENT CONCENTRATIONS IN HEPATIC TISSUE OF LAKE TROUT (*SALVELINUS NAMAYCUSH*) FROM LAKE SUPERIOR AND LAKE ONTARIO.

1.1 ABSTRACT

For livers of lake trout from three Great Lakes sites, the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-equivalent concentrations (TECs) were determined in four ways. Bioassay-derived (BD) TECs were measured with rat (H4IIE) and rainbow trout (RTL-W1) cell lines and chemistry-derived (CD) TECs were calculated with TCDD equivalency factors (TEFs) derived with H4IIE and RTL-W1. Generally, BD-TECs and CD-TECs for individual samples did not differ significantly for either H4IIE or RTL-W1 indicating that all of the AhR-active compounds in a sample were accounted for by the congener analysis and that these compounds acted in an additive fashion in both mammalian and piscine systems. However, contributions of individual PCHs to overall CD-TECs differed with H4IIE and RTL-W1 TEFs, and for some individual samples, TECs did depend on the method used. Hepatic TECs and ethoxyresorufin-*O*-deethylase (EROD) levels differed significantly between sites. For TECs the order was Glenora > Jackfish Bay > Black Bay; for EROD activity the order was Jackfish Bay > Glenora = Black Bay. No correlation was found between hepatic TECs and EROD activity, which suggests that the two measurements are evaluating different but related consequences of contaminant exposure.

1.2 INTRODUCTION

Planar chlorinated hydrocarbons (PCHs) are a group of structurally similar, environmentally ubiquitous chemicals. Included are polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). These contaminants can enter aquatic environments through both point sources and atmospheric deposition (Niimi 1996), and have been detected at various trophic levels in the Great Lakes ecosystem (Environment Canada et al. 1991). A variety of human activities, such as the incineration of municipal solid waste, generation of byproducts during production of industrial compounds, and the operation of bleached kraft pulp mills, have resulted in the introduction of PCDDs and PCDFs into aquatic environments (Vanden Heuvel and Lucier 1993). These contaminants have been detected in tissues of fish from various regions in the Great Lakes (Zacharewski et al. 1989). Contamination of aquatic systems with PCBs has been caused mainly through their use in commercial mixtures for industrial applications (now discontinued). Elevated levels of PCBs in various species from the Great Lakes has been well documented (Ankley et al. 1991; Janz et al. 1992; Williams et al. 1992).

PCH levels in fish tissues can be estimated by analytical chemistry methods to give a chemistry-derived (CD) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent concentration (TEC) or CD-TEC and by bioassays to give a bioassay-derived (BD) TCDD equivalent concentration (TEC) or BD-TEC (Firestone 1991). For the CD-TEC, analytical chemistry techniques, such as GC-MS, are used to identify and quantify individual PCH congeners (Huestis et al. 1995). The concentration values for individual congeners are converted to a single value, the CD-TEC, in two steps. Firstly each congener is assigned a

TCDD equivalent factor (TEF) (Eadon et al. 1986; Barnes 1991; Ahlborg et al. 1994). TEFs have been derived from a variety of in vivo and in vitro toxicity tests (Kutz et al. 1990; Safe 1990; Safe 1994; Ahlborg et al. 1994; Parrott et al. 1995; Kennedy et al. 1996; Clemons et al. 1997) and convert the dose of the congener to the dose of TCDD that would produce the same response. TCDD is chosen as the reference because it is usually the most potent PCH congener, and the mechanistic basis of its toxic actions is thought to be through a common receptor, the aryl hydrocarbon receptor (AhR) (Okey et al. 1994). The second step is to assume that the congeners in a sample act in an additive manner. Therefore, the CD-TEC is calculated by summing the TCDD-equivalent concentration or quotient of each congener present in the fish tissue extract. For the BD-TEC, the most common biological response has been the ability of tissue extracts to induce in liver cell lines CYP1A1, which has been measured as aryl hydrocarbon hydroxylase (AHH) or 7-ethoxyresorufin-O-deethylase (EROD) activity (Tillitt et al. 1991b), and to express this ability relative to an equivalent TCDD concentration. EROD induction is the same response that has been used to derive TEFs in vitro, and usually the same cell lines that have been used for TEF derivation in vitro are used in bioassays. Most commonly, the cell line is the rat hepatoma H4IIE (Safe 1987; Tillitt et al. 1991b), but recently, use has been made of two rainbow trout (Oncorhynchus mykiss) cell lines: hepatic RTL-W1 (Bols et al. 1997) and gonadal RTG-2 (Zabel et al. 1996). Cell bioassays integrate the potency of all the compounds in the sample. As well as PCHs, this includes any other compound(s) that might interact with the Ah receptor to induce CYP1A1 (i.e. "AhR-active" compounds).

A commonly used in vivo indicator of PCH exposure in fish populations is elevated levels of CYP1A1, typically inferred from AHH or EROD activity (Bucheli and Fent 1995).

Several mammalian studies have demonstrated that the toxicological effects of PCHs can be mediated through binding of the PCHs to the Ah receptor, which can be identified by increased levels of CYP1A1 (Goldstien and Safe 1989). Currently, EROD induction is used as an indicator of PCH exposure, which may be indirectly related to toxicological effects, but a direct mechanistic link between EROD activity and deleterious consequences has not been established (Bucheli and Fent 1995).

Contamination by PCHs in the Great Lakes is widespread, with varying patterns of specific congener concentrations in different areas (Environment Canada et al. 1991). In the present study, PCH concentrations, TECs and in vivo EROD activity were examined in lake trout (*Salvelinus namaycush*) collected from two regions of the Great Lakes with known PCH contamination, Jackfish Bay, Lake Superior and the Lake Ontario waters offshore of Glenora, Ontario. The Jackfish Bay region of Lake Superior has been classified as an area of concern by the International Joint Commission (IJC) due to the discharge of large volumes of bleached kraft mill effluent (BKME) (Great Lakes Water Quality Board of the International Joint Commission 1987). The chemistry of BKME is complex but it can contain PCHs such as PCDDs and PCDFs as byproducts of the bleaching process (Owens 1991). Lake Ontario has been contaminated by industrial, agricultural and municipal sources, although concentrations of chemicals have declined and are lower than they were two decades ago (Environment Canada et al. 1991; Huestis et al. 1996). Despite the decrease in levels of contaminants, including PCHs, concentrations have leveled off suggesting continued cycling of persistent toxic substances in Lake Ontario (Zint et al. 1995).

A concern about the toxic potencies of PCHs in fish (salmonids particularly) is that certain PCH congeners differ in their potencies when compared between mammalian and

teleost systems. This has lead to the derivation of TEFs in fish toxicity assays. The most complete set of fish TEFs has been derived with rainbow trout systems. These have been the early life stage mortality (ELS) (Walker and Peterson 1991; Zabel et al. 1995), EROD or AHH in vivo (Parrott et al. 1995; Newsted et al. 1995; Metcalfe and Haffner 1995), and EROD induction in vitro with RTL-W1 (Clemons et al. 1994; Clemons et al. 1996; Bols et al. 1997). Among the 15 PCHs that have been examined, some differences have been found between rainbow trout and mammalian TEFs. Generally, PCDDs and PCDFs are slightly more potent in the RTL-W1 in vitro system; the PCBs more potent in H4IIE.

In this paper, our goal has been to examine the livers of lake trout from the eastern basin of Lake Ontario, Jackfish Bay and a reference site for differences in TCDD equivalent concentrations (TECs) and to determine whether the outcome is influenced by the fish and mammalian cell lines that have been used either to measure TECs in bioassays (BD-TECs) or to derive TEFs for the conversion of PCH congener concentrations to TECs (CD-TECs). Concentrations of selected PCHs in lake trout livers were compared to those in livers from white sucker (*Catostomus commersoni*), a philopatric (site tenacious) species, collected from Jackfish Bay to see if similar contaminant patterns were present between the two types of fish. Additionally, the levels of EROD activity in lake trout livers have been determined and compared with TECs as indicators of PCH contamination.

1.3 MATERIALS AND METHODS

A. Lake trout sampling

Two sites in Lake Superior, Jackfish Bay (48°50'N, 86°58'W) and Black Bay (48°72'N, 88°44W), and one site in Lake Ontario near Glenora, Ontario (44.05N 76.89W) were sampled in the spring of 1993 (Figure 1.1). Lake trout were captured in four inch gill nets at all sampling sites. Captured fish were rendered unconscious by a blow to the head. Fish weight, length, sex and sexual maturity were recorded. Livers were excised, weighed and divided into two subsamples. Ten to fifteen grams of the first subsample were wrapped in aluminium foil (previously heated to 400°C), placed in a plastic bag and put in ice. These samples remained frozen at -20°C pending contaminant extraction. For hepatic EROD analysis, approximately 1 g of tissue was placed in a 2 mL cryovial, frozen in liquid nitrogen, and stored at -80°C pending analysis. White sucker were collected in an adjacent stream to Jackfish Bay during the fall of 1993. To determine the likelihood that the PCH accumulation is primarily from the Jackfish Bay region, PCDD and PCDF concentration patterns were compared between white sucker, which are more stationary and primarily resident in Jackfish Bay, and lake trout captured from both Jackfish Bay and Black Bay.

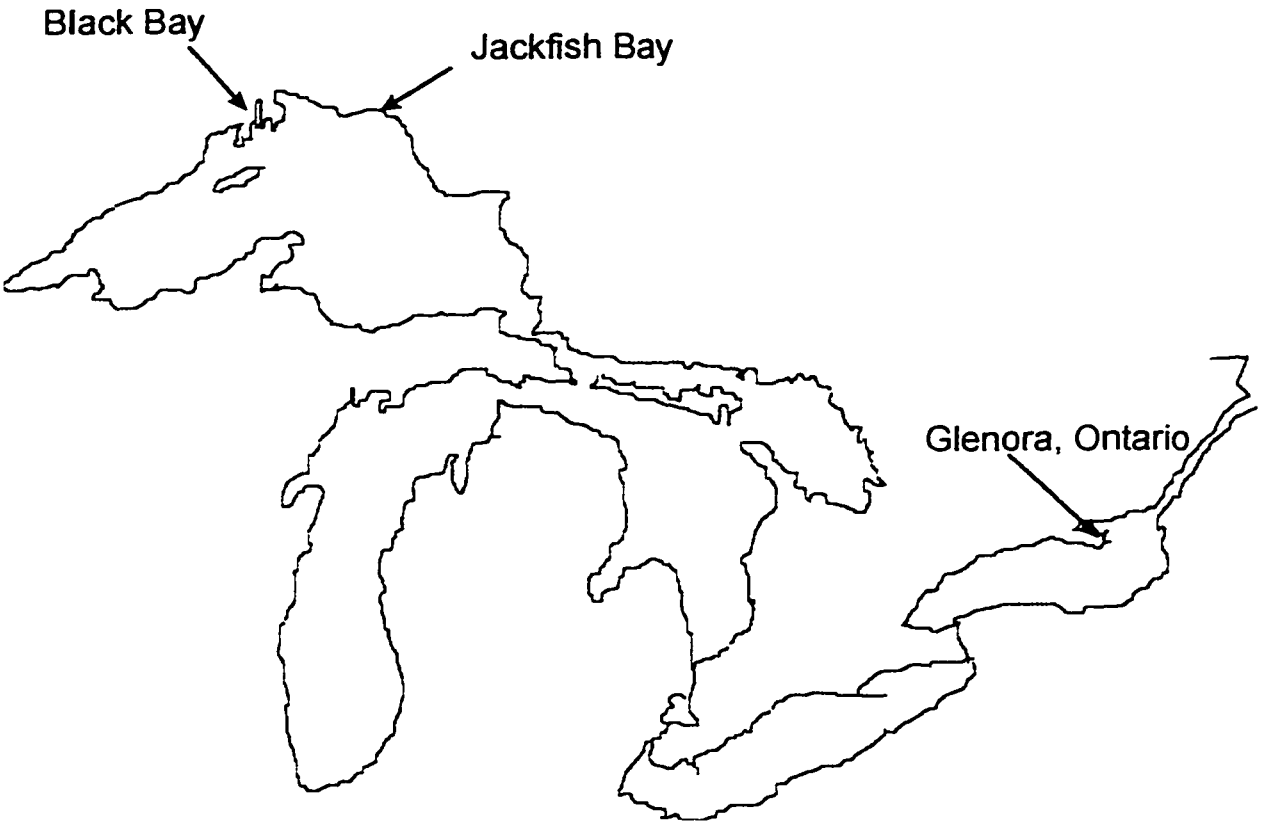


Figure 1.1. Locations of lake trout sampling sites examined in this study. Jackfish Bay receives effluent from a bleached kraft mill located at Terrace Bay, Ontario (Robinson et al. 1994). The reference site, Black Bay, receives no industrial or significant domestic effluent. The waters of Lake Ontario have significant levels PCH contamination from various sources (Environment Canada et al. 1991).

B. Isolation of PCHs from liver tissue

Solvent extraction of PCHs from tissue and subsequent cleanup were performed according to Huestis et al. (1995). Liver samples were weighed and ground with anhydrous Na_2SO_4 (previously fired at 400°C). The tissue-Na_2SO_4 mixture was divided into two fractions: one for the bioassay analysis and one for determination of congener concentrations (see below). PCH extraction was conducted using methylene chloride in a pre-rinsed soxhlet apparatus. Extracted lipids and other hydrophobic co-extractives were removed by gel

permeation chromatography (GPC, Biorad SX-3 GPC resin, Richmond, CA) followed by basic alumina (activity I) chromatography (Fisher, Fairlawn, NJ). Lipid content was estimated gravimetrically by evaporating the solvent phase of the first GPC fraction and weighing the residual lipid. The second GPC fraction, containing the PCHs of interest, was solvent-exchanged into 80 μL of dimethylsulfoxide (DMSO, BDH, Darmstadt, W. Germany). A geometric dilution series of seven doses was prepared from the original extract. A procedural blank was run with every set of extractions to indicate possible contamination. No significant contamination occurred , and no correction factor was applied. The recovery using this methodology has been estimated to be >96% for the compounds of interest in this study (van den Heuvel et al. 1995).

C. RTL-W1 and H4IIE cell maintenance

Rainbow trout liver (RTL-W1) cells were maintained as previously described in Lee et al. (1993). The growth medium used was Leibovitz's L-15 (Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and penicillin/streptomycin (100 U penicillin, 10 μg streptomycin·mL^{-1}, ICN/Flow, Costa Mesa, CA). Rat hepatoma (H4IIE) cells (American Type Culture Collection, CRL 1548) were maintained as previously described (Tillitt et al. 1991b). Cells were grown in Dulbecco's modified Eagle's medium (MEM, Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS, Gibco, Gaithersburg, MD), L-glutamine, gentamycin sulphate (50 μg·mL^{-1}), MEM essential amino acids (1.5×), MEM non-essential amino acids (2×) and

MEM vitamins (1.5×). RTL-W1 cells were maintained at 22°C ± 1 in a pure air environment, while H4IIE were maintained at 37°C ± 1 in a humidified 5% CO_2 atmosphere.

D. RTL-W1 and H4IIE bioassay procedure

The cell culture assay for EROD activity was performed using live cells and is a modification of Clemons et al. (1996) and Kennedy et al. (1993). Both cell lines were seeded in 48-well culture clusters (Costar, Cambridge, MA) at a density of 40,000 cells·well^{-1}. After a 24 h period, cells were dosed with the PCH extract dilutions. A 2,3,7,8-TCDD dilution series was run with every three extracts to serve as a positive control and standard for TEC determination. Cells were incubated for 24 h prior to the EROD bioassay.

For the enzymatic assay, the overlying contaminated media was removed by plate inversion to reduce damage to the cell monolayer. Each well was rinsed with 100 µL phosphate buffered saline (PBS) then received 250 µL 7-ethoxyresorufin-MEM solution (4.87 µM for H4IIE, 0.825 uM for RTL-W1). Dicumarol (Sigma, St. Louis, MO) was added to the H4IIE reaction mixture at a concentration of 0.29 mM to prevent reduction of the resorufin product by diaphorase, as previously described by Donato et al. (1992). This reagent was not added to the RTL-W1 reaction mixture, as it was found to have no effect on resorufin fluorescence for this cell line (Clemons et al. 1996). Reactions took place for 15 min at 22 and 37°C for RTL-W1 and H4IIE, respectively. Resorufin production was measured fluorometrically using a Cytofluor 2350 plate reading fluorometer (Perspective Biosystems, Framingham, MA). Excitation and emission filters were set at 530 nm and 590 nm respectively. A resorufin standard curve was run with each assay to allow conversion of

fluorescent units to pmoles resorufin. Cellular protein was determined as described by Lorenzen and Kennedy (Lorenzen and Kennedy 1993). A bovine serum albumin (BSA, Sigma, St. Louis, MO) standard curve was run with each protein assay to allow conversion of fluorescent units to mg of protein.

E. Calculation of BD-TECs in lake trout livers

Fluorometric readings were plotted as concentration response relationships in order to determine median effective concentrations for half maximal EROD induction in cells (EC_{50}). EC_{50}s from PCH extracts and 2,3,7,8-TCDD standard curves were used to determine BD-TECs in lake trout livers using a formula similar to that derived by Tillitt et al. (1991a):

$$\text{BD-TEC (pg TCDD/g lipid)} = \frac{EC_{50}\ \text{TCDD (pg/well)}}{EC_{50}\ \text{extract (dilution·well}^{-1})} \times \frac{\text{extract volume (}\mu\text{L)}}{\text{dose volume (}\mu\text{L)}} \times \text{lipid mass}^{-1}\ \text{(g)}$$

The majority of Jackfish Bay extract curves and all Lake Ontario extract curves did reach a plateau, but for some Jackfish Bay and Black Bay liver extracts, concentration-response curves did not achieve maximal levels of EROD activity. Since an EC_{50} could not be determined for these samples, a TEC was determined by matching the highest level of EROD activity induced by the extract to the corresponding level of EROD activity in the TCDD standard, and taking that concentration of TCDD to be the TEC (corrected for dosing volume of extract). This technique follows the basic assumption of the TEC model that the concentration-response curves of the extract and 2,3,7,8-TCDD are parallel. As a test of the

validity of this approach, TECs were calculated from those extract dose-response curves that were very similar to TCDD dose-response curves in two different ways, the EC_{10} versus the EC_{50}. The two different methods yielded TECs that were within 10 to 15% of each other.

F. PCB, dioxin and furan analysis and CD-TEC determination

Lake trout and white sucker liver samples were provided to Susan Huestis (Canada Centre for Inland Waters, Environment Canada, Burlington, Ontario) for PCH congener analysis. Analytical methodologies are described in Huestis et al. (1995). Five samples from each site were randomly selected for examination. Liver was spiked with a mixture of ^{13}C dioxin and furan internal standards, extracted with methylene chloride in a pre-rinsed soxhlet apparatus, and cleaned up using GPC and alumina chromatography. Isolation of planar compounds was accomplished using carbon fibre chromatography. Samples were quantified using gas chromatography with high resolution mass-spectroscopy (VG Autospec). Concentrations were corrected for the recovery of the ^{13}C labelled congeners.

Three sets of TEFs were used to determine CD-TECs. TEFs for H4IIE and RTL-W1 (Table 1.1) were derived using a similar bioassay systems described above by Bols et al. (1997). It has been suggested that the PCHs may increase the risk of early life stage (ELS) mortality in lake trout (Walker and Peterson 1991; Walker et al. 1996). To examine the risk of potential effects of the compounds in the lake trout liver extracts on trout ELS, CD-TECs were determined using a third set of TEFs derived by Zabel et al. (1995). CD-TECs were calculated by multiplying the measured concentrations of the PCB, dioxin and furan congeners by their respective TEFs and summing (Eadon et al. 1986).

Table 1.1. RTL-W1, H4IIE and rainbow trout early life stage mortality (ELS) TCDD equivalency factors for the PCHs measured in lake trout livers during this study.

Congeners Analyzed	RTL-W1[1] TEF	H4IIE[1] TEF	ELS[2] TEF
PCBs			
3,3',4,4'-TCB (PCB 77)	0.0053	0.0069	0.00016
3,4,4',5-TCB (PCB 81)	0.0064	0.0072	0.00056
3,3',4,4',5-PeCB (PCB 126)	0.023	0.1	0.005
3,3',4,4',5,5'-HxCB (PCB 169)	0.00016	0.0014	0.000041
Dioxins			
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	2.6	1.1	0.73
1,2,3,4,7,8-HxCDD	1.1	0.5	0.319
1,2,3,6,7,8-HxCDD	0.2	0.2	0.024
1,2,3,7,8,9-HxCDD	n.a.	n.a.	n.a.
1,2,3,4,6,7,8-HpCDD	0.2	0.1	0.002
OCDD	n.a.	n.a.	n.a.
Furans			
2,3,7,8-TCDF	0.2	0.4	0.028
1,2,3,7,8-PeCDF	0.2	0.2	0.034
2,3,4,7,8-PeCDF	1.9	0.4	0.359
1,2,3,4,7,8-HxCDF	1.1	0.3	0.28
2,3,4,6,7,8-HxCDF	n.a.	n.a.	n.a.
1,2,3,6,7,8-HxCDF	n.a.	n.a.	n.a.
1,2,3,7,8,9-HxCDF	n.a.	n.a.	n.a.
1,2,3,4,6,7,8-HpCDF	n.a.	n.a.	n.a.
OCDF	n.a.	n.a.	n.a.

[1] (Bols et al. 1997).

[2] (Zabel et al. 1995).

n.a. = Rainbow trout TEF not available for use in calculations, although H4IIE TEFs have been published for all PCDFs listed (Tysklind et al. 1994)

G. Hepatic Enzyme Analysis

Hepatic CYP1A activity was quantified as EROD activity using a modified method of Kennedy and Jones (Kennedy and Jones 1994). Postmitochondrial supernatant was isolated from the lake trout livers by grinding tissue in ice-cold dithiothreitol buffer, followed by centrifugation (9000 g). The following reagent concentrations were used in the reaction mixture: 7-ethoxyresorufin, 1.7 μM (Sigma, St. Louis, MO); NADPH, 0.5 mM (Boehringer Mannheim, Germany); $MgSO_4$, 17 mM; and HEPES, 0.1 M, pH 7.8 M (Sigma, St. Louis, MO). EROD activity was determined in 48-well culture plates (Costar, Cambridge, MA) using a Cytofluor 2350 plate reading flourometer (Perspective Biosystems, Framingham, MA). Excitation and emission filters were set at 530 nm and 590 nm respectively. Activity was determined at 25°C using 25 μL postmitochondrial supernatant in the reaction mixture. Simultaneous termination of the reaction and protein determination were achieved by addition of ice cold fluorescamine in acetonitrile (1.08 mM). The protein assay is a modification of Lorenzen and Kennedy (Lorenzen and Kennedy 1993).

H. Statistics

Calculation of EC_{50}s used in determination of bioassay derived TECs was accomplished using the SigmaPlot non-linear curve fitting module (Jandel Scientific, San Rafael, CA). Data were fitted to a four parameter logistic function:

$$f(x) = \frac{a - d}{1 + e^{b(x-c)}} + d$$

where $f(x)$ is the EROD response, x is the contaminant concentration or dilution, a is the EROD minimum to maximum range, b is the slope coefficient, c is the EC_{50}, and d is the minimum EROD response. Weight, and length at the two sites were tested for normality using the Kolmogorov-Smirnov test (with Lilliefors' correction), and for equal variance using the Levene Median test. Differences between the TECs (both BD and CD) due to factors of site and cell line (species) were determined using two way analysis of variance (ANOVA, $p<0.05$) or Friedman repeated measures ANOVA on ranks. This was followed by multiple comparison analysis using the Student-Newman-Keuls test. Normality and equal variance were also tested for these data. Strength of association between variables was determined using Pearson product moment correlation.

1.4 RESULTS

A. Whole fish parameters

Table 1.2 presents a summary of the data for lake trout collected from the three sites. Analysis of variance revealed no significant differences in mean weight ($p = 0.326$) or length ($p = 0.421$) among lake trout collected at Jackfish Bay, Black Bay or Lake Ontario. Fin clips on Black Bay trout indicated that most of the fish were stocked, while no fin clips were seen on Jackfish Bay trout. It is assumed that fish from Lake Ontario were stocked, as efforts have been ineffective in re-establishing self-sustaining lake trout stocks in inshore waters of the Great Lakes, except in Lake Superior (Evans and Olver 1995). Equal numbers

Table 1.2. Data summary for lake trout collected from Black Bay, Jackfish Bay and Lake Ontario.

							TCDD Equivalent Concentration (TEC, pg/g)[1]				
Sampling	Sample		Body	Body	Liver	Percent	Bioassay-Derived (BD-TEC)		Chemistry-Derived (CD-TEC)		
Site	I.D.	Sex	Length (cm)	Mass (g)	Mass (g)	Lipid	H4IIE	RTL-W1	H4IIE	RTL-W1	ELS
Black Bay	BB1	F	50.9	1747	33.7	26.9%	1.2	5.2	8.1	3.8	0.3
(Lake	BB3	F	46.5	1427	24.8	36.9%	6.6	5.7			
Superior)	BB4	F	61.6	3273	47.5	17.5%	20.2	4.7			
	BB9	F	59.7	2994	45.3	13.3%	1.0	4.8	4.3	3.7	0.6
	BB2	M	47.8	1449	30.5	40.5%	4.7	4.2	21.0	19.6	4.1
	BB5	M	54.9	2088	47.5	27.0%	47.0	18.3	36.1	32.8	6.9
	BB6	M	53.2	1987	37.8	42.5%	0.6	8.2	7.1	3.2	0.4
	BB7	M	53.2	1979	36.0	32.2%	8.4	3.2			
Jackfish Bay	JB2	F	51	—	41.2	22.1%	22.8	28.5			
(Lake	JB3	F	46	1240	27.3	21.7%	14.7	12.2			
Superior)	JB6	F	52	1755	45.2	31.1%	4.3	9.6	26.9	23.9	7.0
	JB9	F	62.8	3212	74.0	16.1%	77.6	72.4	49.3	45.2	18.2
	JB10	F	49	1275	21.5	14.6%	27.1	17.5			
	JB11	F	48.5	1312	16.8	8.0%	8.6	7.1			
	JB12	F	52.2	1882	48.9	11.0%	49.5	20.4	30.1	25.8	10.0
	JB7	I F	46	1195	33.9	31.0%	4.2	7.8	36.2	34.2	11.8
	JB8	I F	45	1068	17.2	22.0%	10.4	49.5			
	JB4	I M	44	946	18.4	27.1%	4.5	10.6	39.1	33.2	8.5
Glenora	LO1	F	47.3	1380	19.0	20.9%	76.1	66.6			
(Lake	LO4	F	67.4	4703	73.0	17.7%	16.8	23.4	32.9	29.9	5.7
Ontario)	LO5	F	59.8	2945	42.0	24.0%	128.0	80.0	169.4	166.3	41.1
	LO2	I M	47.6	1387	18.0	11.7%	51.1	36.6	54.1	66.0	18.9
	LO8	I M	21.4	953	11.0	31.6%	176.0	180.9			
	LO9	I M	39.4	773	6.0	21.2%	239.9	146.1			
	LO7	I M	45.7	980	13.0	29.5%	129.1	119.6			
	LO3	M	61.9	3397	33.0	22.0%	214.0	71.7	134.6	101.7	23.0
	LO6	M	63.8	3514	39.0	20.8%	31.1	43.4	29.8	23.5	6.9
Black Bay	**Mean**		**53.5**	**2118.0**	**37.9**	**29.6%**	**11.2**	**6.8**	**15.3**	**12.6**	**2.5**
	S.E.		1.9	239.1	2.9	0.04	5.6	1.7	5.9	5.9	1.3
Jackfish Bay	**Mean**		**49.7**	**1542.8**	**34.4**	**20.5%**	**22.4**	**23.6**	**36.3**	**32.5**	**11.1**
	S.E.		1.7	231.7	5.8	0.03	7.6	6.8	3.9	3.8	1.9
Glenora	**Mean**		**50.5**	**2225.8**	**28.2**	**22.1%**	**118.0**	**85.4**	**84.1**	**77.5**	**19.1**
	S.E.		4.8	477.1	7.0	0.02	26.8	17.7	28.5	26.3	6.4

[1] pg TCDD/g tissue

of male and female lake trout were captured at Black Bay, and ratios of males:females:immature were 0:7:3 and 2:3:4 for Jackfish Bay and Lake Ontario respectively. Although Black Bay trout had the highest mean percent lipid for livers, no significant difference in lipid level was found between the sites ($p = 0.07$).

B. Coplanar PCB, chlorinated dioxin furan congener determination

Concentrations of selected PCB, PCDD and PCDF congeners were determined in liver samples from individual fish (Table 1.3). Of the coplanar PCB congeners examined, number 77 (3,3',4,4'-TCB) was the most prominent congener seen in the samples from each site. In Jackfish Bay and Black Bay, PCB 81 (3,4,4',5-TCB) was detected in the lowest concentrations, while in Lake Ontario, the lowest concentrations were for PCB 81 and 169 (3,3',4,4',5,5'-HxCB). Of the four coplanar PCBs analyzed, only the concentration of PCB 77 was found to be differ significantly among the sites, with concentrations in Lake Ontario being higher than both Jackfish Bay and Black Bay ($p < 0.01$).

For PCDD congeners, 1,2,3,4,6,7,8-HpCDD and OCDD were found in all samples. Significant differences were detected among the sites for OCDD concentrations ($p = 0.01$), but no differences were seen in the concentrations of 1,2,3,4,6,7,8-HpCDD. The most potent CYP1A inducing PCH congener, 2,3,7,8-TCDD, was detected in significantly higher concentrations in both Jackfish Bay and Lake Ontario samples than in Black Bay samples ($p = 0.01$). No significant difference in 2,3,7,8-TCDD concentration was found between Jackfish Bay and Lake Ontario.

For Black Bay trout, concentrations of 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF were the only prominent PCDFs, while most other congeners were below instrumental detection limits. The mean concentration of 2,3,7,8-TCDF in Jackfish Bay samples was significantly

Table 1.3: PCH congener concentrations (pg/g wet wt.) detected in lake trout livers sampled from three Great Lake sites.

	Black Bay, Lake Superior					Jackfish Bay, Lake Superior					Glenora, Lake Ontario				
	BB 1	**BB 2**	**BB 5**	**BB 6**	**BB 9**	**JB 4**	**JB 6**	**JB 7**	**JB 9**	**JB 12**	**LO 2**	**LO 3**	**LO 4**	**LO 5**	**LO 6**
Coplanar PCBs															
PCB 77	277	308	470	122	70	165	106	146	210	156	1870	6690	1750	6880	2950
PCB 81	<0.09	<0.09	18	18	<0.09	29.2	5.6	8	28.8	7.2	92.8	76.8	103	301	<0.09
PCB 126	56.1	78	122	27.6	16	112	41.6	58	93.2	47.6	168	582	117	546	<0.44
PCB 169	55.3	80	114	32.4	21.6	80.4	30.4	54.4	60.4	41.6	76.6	279	82.4	258	<0.10
Dioxins															
2,3,7,8-TCDD	<0.23	0.72	1.8	<0.23	<0.23	4.44	4.12	7.08	12.7	7.04	8.55	11.5	1.8	16.8	6.24
1,2,3,7,8-PeCDD	<0.33	1.6	<0.33	<0.33	<0.33	<0.33	<0.33	1.08	<0.33	<0.33	3.06	<0.33	<0.33	6.68	<0.33
1,2,3,4,7,8-HxCDD	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
1,2,3,6,7,8-HxCDD	<0.07	2	1.24	<0.07	<0.07	2.28	1.24	0.88	<0.07	<0.07	2.47	<0.07	1.24	4.88	<0.07
1,2,3,7,8,9-HxCDD	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	0.56	<0.07
1,2,3,4,6,7,8-HpCDD	5.26	4.68	1	0.84	0.84	23.3	11.7	3.36	0.84	4.72	1.06	0.76	1	1.12	0.56
OCDD	5.84	4.36	1.84	1.96	2.48	42.6	22.7	4.08	2.6	5.12	2	1.72	1.84	1.96	1
Furans															
2,3,7,8-TCDF	<0.13	14.6	36.2	8.2	4.28	42	35.8	43.5	54	34.9	8.21	25.4	5.6	57.6	7.88
1,2,3,7,8-PeCDF	<0.14	1.72	<0.14	<0.14	<0.14	<0.14	2	2.44	<0.14	5.32	3.36	<0.14	5.56	8.84	<0.14
2,3,4,7,8-PeCDF	<0.14	3.52	9.44	<0.14	1.04	6.08	4.28	6.2	9.48	3.96	13.4	18.7	5.6	30	<0.14
1,2,3,4,7,8-HxCDF	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	0.32	<0.06	0.24	5.91	<0.06	2	10.7	<0.06
2,3,4,6,7,8-HxCDF	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	0.16	<0.06	0.2	<0.06	<0.06	<0.06	<0.06	<0.06
1,2,3,6,7,8-HxCDF	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	0.24	<0.06	0.24	<0.06	<0.06	<0.06	<0.06	<0.06
1,2,3,7,8,9-HxCDF	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07
1,2,3,4,6,7,8-HpCDF	<0.25	0.6	<0.25	<0.25	<0.25	1.76	0.48	0.28	<0.25	0.48	<0.25	<0.25	<0.25	<0.25	<0.25
OCDF	0.157	0.88	<0.09	<0.09	<0.09	6.52	3.12	0.6	0.24	0.8	<0.09	<0.09	<0.09	<0.09	<0.09

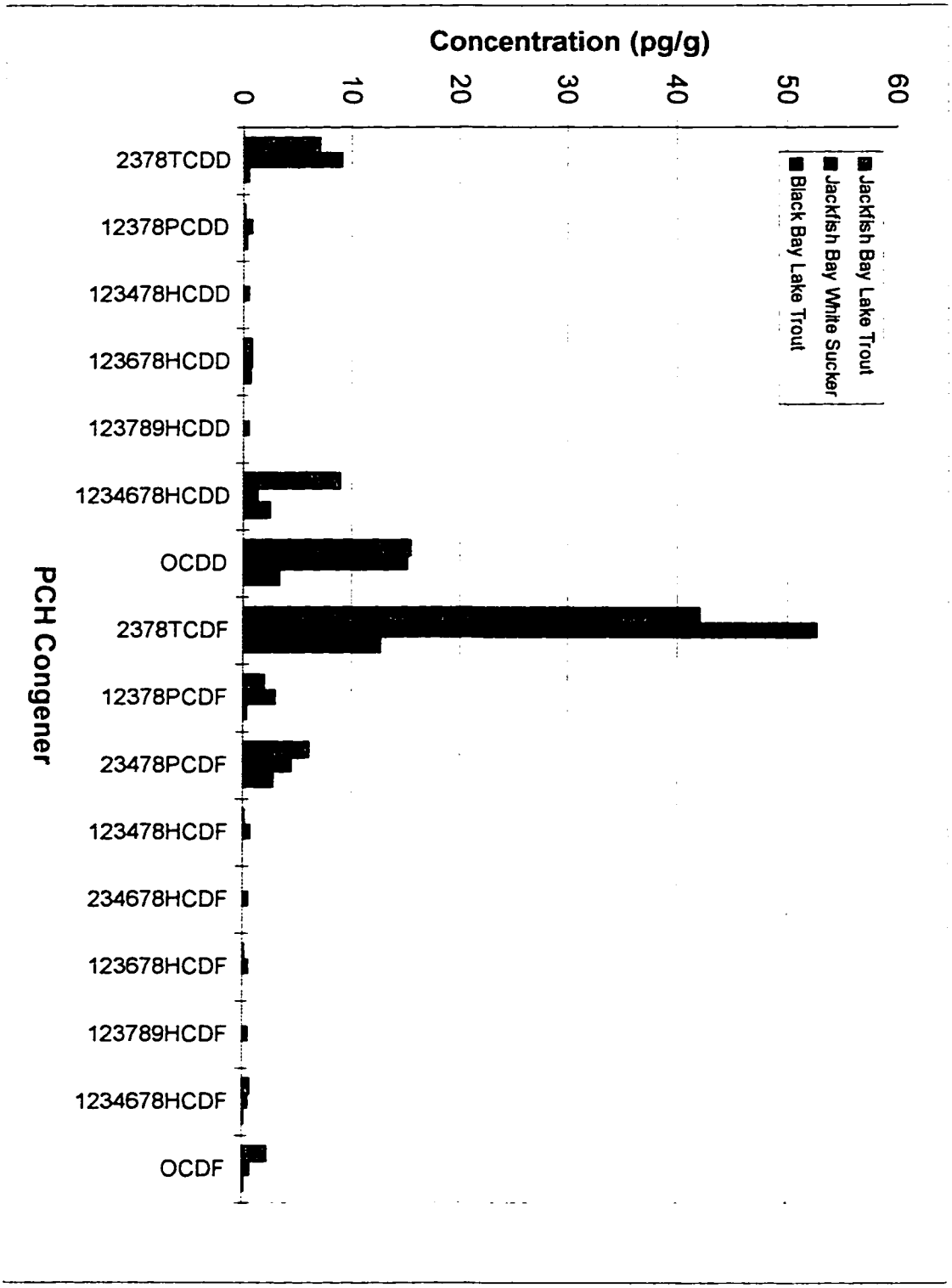
higher than Black Bay samples ($p < 0.05$), but not Lake Ontario samples. In addition, Jackfish Bay trout had significantly higher concentrations of OCDF than both Black Bay and Lake Ontario samples ($p < 0.01$).

Although concentrations of OCDD, 1,2,3,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, and OCDF were detected in some liver samples, TEFs for RTL-W1 have not yet been calculated for these congeners; they were not used in the calculation of CD-TECs in this study. In order

to justify this omission, H4IIE TEFs for these congeners (Safe et al. 1987) were used to generate a second set of CD-TECs in the lake trout livers. No significant increase in TEC (>1% in each sample) was found when these TEFs were added compared to TECs generated without them, indicating that these congeners do not play as significant a role as other, more potent, PCH congeners.

Figure 1.2 compares mean PCDD and PCDF congener concentrations in white sucker collected from Jackfish Bay with lake trout samples from Jackfish Bay and Black Bay. A significant correlation was seen between the concentrations seen in Jackfish Bay lake trout and white sucker ($r = 0.972$, $p < 0.01$). Black Bay lake trout concentrations did not have the same strength of association with the white sucker, although a significant relationship was seen ($r = 0.800$, $p < 0.01$), indicating a difference in the pattern of PCH contamination in that region. The fact that Jackfish Bay PCH levels were more highly correlated with the white sucker samples than they were with Black Bay samples ($r = 0.798$, $p < 0.01$) indicates that they were likely accumulating contaminants from the same source as the white sucker.

Figure 1.2. Comparison of mean hepatic PCDD and PCDF concentrations in white sucker from Jackfish Bay and lake trout from Jackfish Bay and Lake Ontario.


Concentration (pg/g)
0
10
20
30
40
50
60
Jackfish Bay Lake Trout
Jackfish Bay White Sucker
Black Bay Lake Trout
PCH Congener
2378TCDD
12378PCDD
123478HCDD
123678HCDD
123789HCDD
1234678HCDD
OCDD
2378TCDF
12378PCDF
23478PCDF
123478HCDF
234678HCDF
123678HCDF
123789HCDF
1234678HCDF
OCDF

C. Bioassay-derived 2,3,7,8-TCDD equivalent concentrations

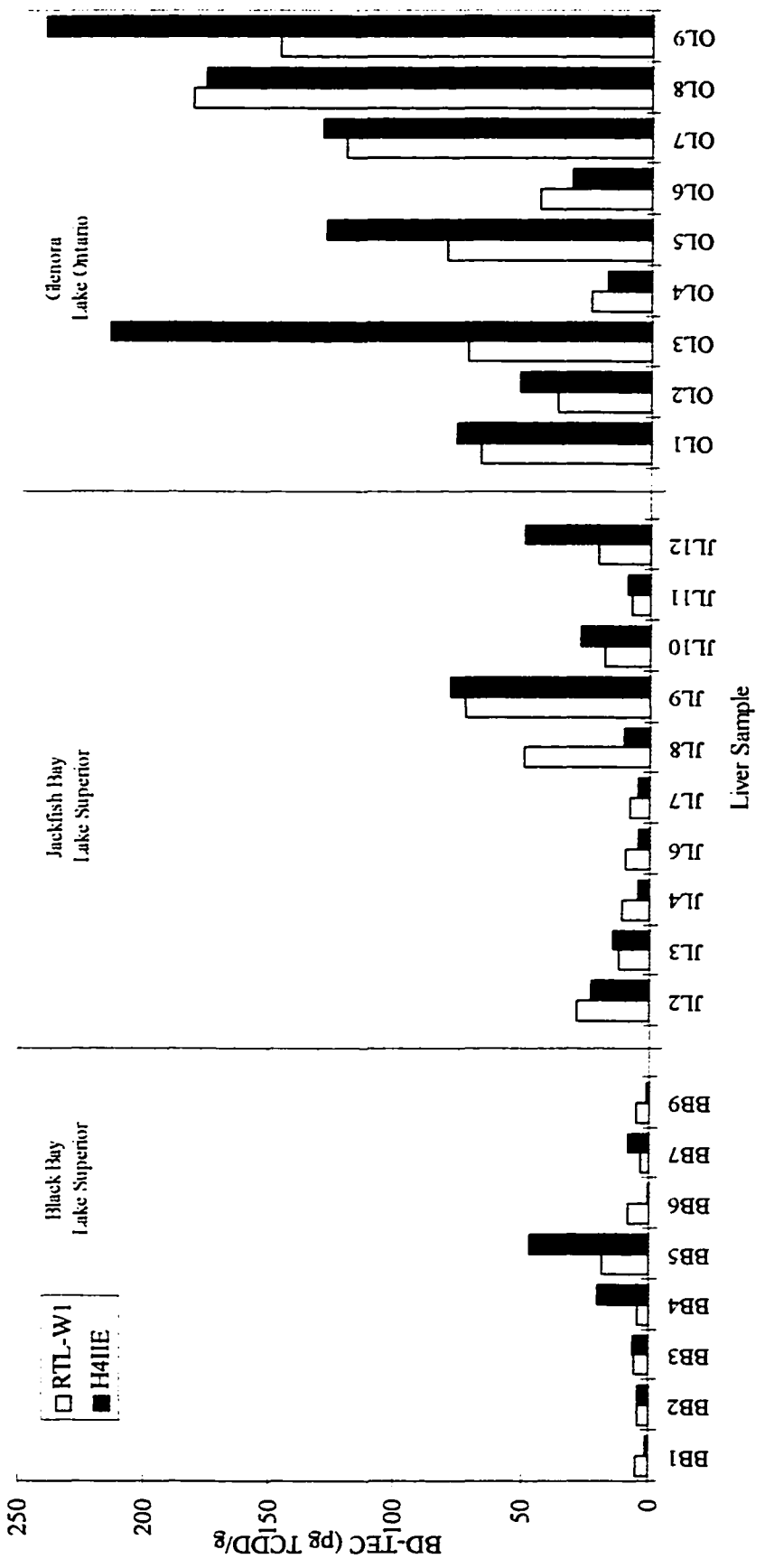
The highest BD-TECs were found in Lake Ontario samples with a mean of 85.4 pg/g when derived with RTL-W1 and 118.0 pg/g when derived with H4IIE (Table 1.2). Jackfish Bay trout had mean BD-TECs of 23.6 pg/g and 22.4 pg/g determined using the RTL-W1 and H4IIE bioassays respectively. Trout collected from the reference site, Black Bay, yielded mean BD-TECs of 6.8 pg/g (RTL-W1) and 11.2 pg/g (H4IIE). Mean BD-TECs from the three sites were significantly different ($p < 0.01$), regardless of which cell line was employed. BD-TECs were highly variable within each site (Figure 1.3), including high TECs seen in the reference site (BB4, BB5) and low TECs seen in Jackfish Bay (JB6, JB7) and Lake Ontario (LO4) livers. The RTL-W1 derived concentration ranges were as follows; Black Bay: 3.2 pg/g to 18.3 pg/g, Jackfish Bay 7.1 pg/g to 72.4 pg/g and Lake Ontario 23.4 pg/g to 180.9 pg/g. The ranges in the H4IIE derived concentrations for Black Bay, Jackfish Bay and Lake Ontario were 0.6 pg/g to 47.0 pg/g, 4.2 pg/g to 77.6 pg/g and 16.8 pg/g to 239.9 pg/g respectively.

The BD-TECs for individual fish were not significantly different when derived using either the RTL-W1 or the H4IIE bioassay (Figure 1.3). This was true for all three sites, resulting in a high correlation between H4IIE and RTL-W1 BD-TECs ($r^2 = 0.768$). No correlation was found between fish weight and BD-TECs.

D. Chemically-derived 2,3,7,8-TCDD equivalent concentrations

Tissue concentrations of PCHs in lake trout livers (Table 1.3) were used to calculate CD-TECs using RTL-W1, H4IIE and ELS TEFs. When all samples are

Figure 1.3. RTL-W1 and H4IIE bioassay-derived 2,3,7,8-TCDD equivalent concentrations (BD-TECs) determined for individual lake trout livers sampled from three sites in the Great Lakes.


RTL-W1
H4IIE
Black Bay
Lake Superior
Jackfish Bay
Lake Superior
Glenora
Lake Ontario
BD-TEC (pg TCDD/g
0
50
100
150
200
250
BB1
BB2
BB3
BB4
BB5
BB6
BB7
BB9
JL2
JL3
JL4
JL6
JL7
JL8
JL9
JL10
JL11
JL12
OL1
OL2
OL3
OL4
OL5
OL6
OL7
OL8
OL9
Liver Sample

considered (Table 1.2), CD-TECs varied considerably between individual lake trout. However, excellent correlations were found between the CD-TECs calculated with the three different sets of TEFs (H4IIE vs RTL-W1 r^2 =0.964; RTL-W1 vs ELS r^2 =0.948; H4IIE vs ELS r^2 =0.887). H4IIE CD-TECs were significantly higher than both RTL-W1 and ELS CD-TECs, while RTL-W1 was significantly higher than ELS CD-TECs ($p < 0.01$). It was found that lake trout livers from Lake Ontario and Jackfish Bay had elevated levels of CYP1A inducing compounds compared to Black Bay, as is demonstrated by the significantly higher mean TEC concentrations at these sites. This was true for all three sets of TEFs. CD-TECs generated using early life stage mortality TEFs were considerably lower compared to RTL-W1 and H4IIE CD-TECs. Several early life stage mortality TEFs are considerably lower than the corresponding RTL-W1 or H4IIE TEFs (Table 1.1) leading to a decreased CD-TEC.

Contributions of PCH congener classes to total CD-TECs in the lake trout livers are shown in Figure 1.4. Comparison of samples from Lake Superior show that Jackfish Bay livers received a greater proportion of total CD-TEC from PCDD and PCDF congeners, while Black Bay CD-TECs were composed mainly of PCB and PCDF contributions. Lake Ontario CD-TECs were comprised of PCB and PCDF contributions, and had a higher PCB ratio than did Jackfish Bay samples. If the TEF source is examined, it is shown that H4IIE CD-TECs had higher percentages of PCB contributions at each site compared to the two teleost systems. Conversely, PCDDs and PCDFs made up a greater amount of the total CD-TECs for both the RTL-W1 and ELS systems.

A closer examination of the major components making up the individual CD-TECs is seen in Table 1.4. At first glance it is obvious that the different TEF sets do not give similar patterns of congener contribution to the final CD-TECs. In Jackfish Bay, it

Figure 1.4. Percent contributions of PCH congener classes to CD-TECs in lake trout livers from three sites in the Great Lakes. At each site, CD-TECs were calculated from TEFs that had been derived either from EROD induction in RTL-W1 (rainbow trout liver) or H4IIE (rat hepatoma) or from early life stage (ELS) mortality in rainbow trout.

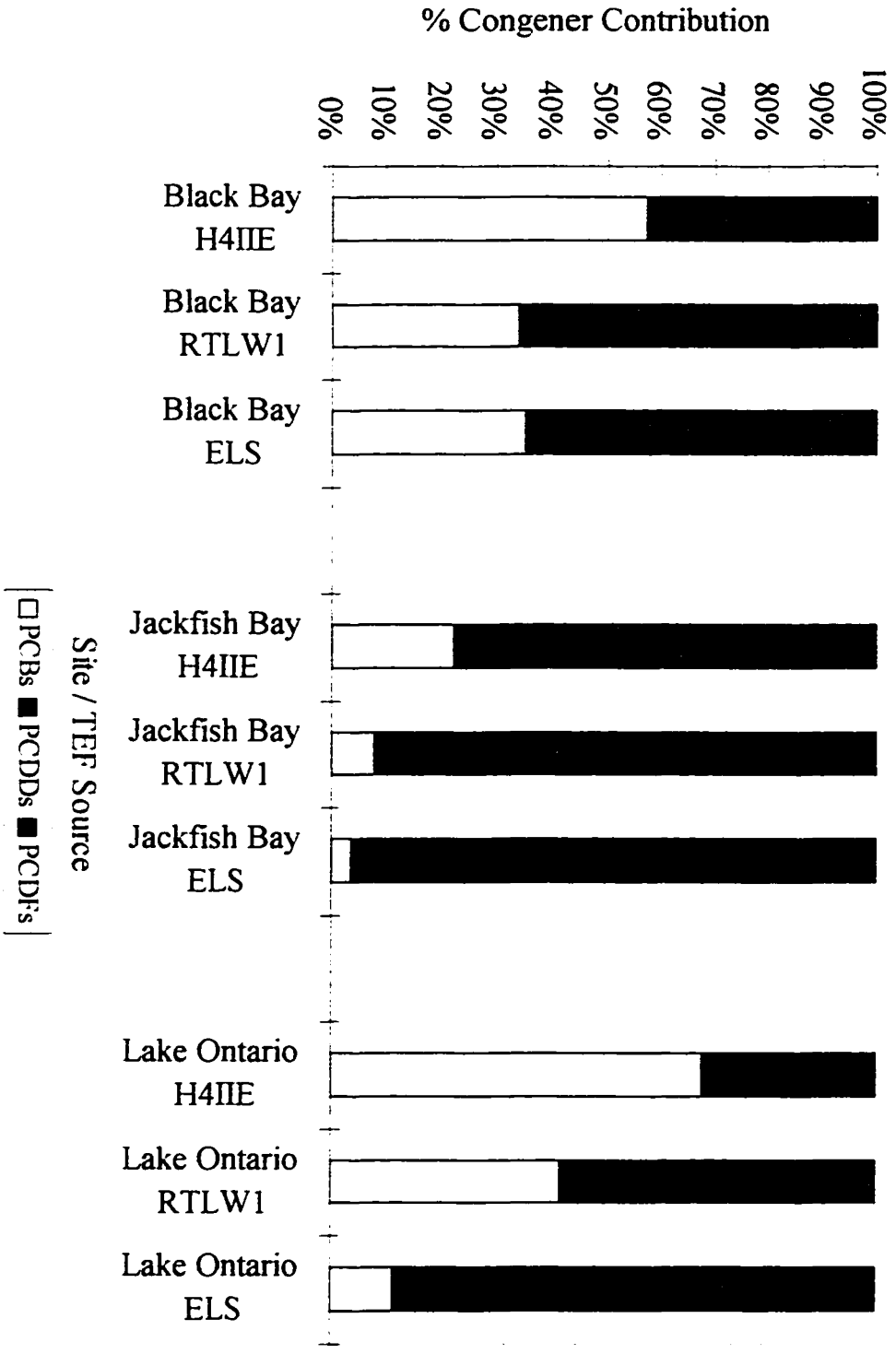

% Congener Contribution
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Black Bay H4IIE
Black Bay RTLW1
Black Bay ELS
Jackfish Bay H4IIE
Jackfish Bay RTLW1
Jackfish Bay ELS
Lake Ontario H4IIE
Lake Ontario RTLW1
Lake Ontario ELS
Site / TEF Source
□PCBs ■PCDDs ■PCDFs

Table 1.4. Effect of TEF source on the contribution of different PCHs to the CD-TECs in individual lake trout.

	TEF Source					
	H4IIE		RTL-W1		ELS	
LD [1]	Primary Contributor	Secondary Contributor	Primary Contributor	Secondary Contributor	Primary Contributor	Secondary Contributor
BB1	PCB 126 (69%)	PCB 77 (24%)	PCB 77 (39%)	PCB 126 (34%)	PCB 126 (83%)	PCB 77 (13%)
BB2	PCB 126 (37%)	2,3,7,8-TCDF (28%)	2,3,4,7,8-PeCDF (34%)	1,2,3,7,8-PeCDD (21%)	2,3,4,7,8-PeCDF (31%)	1,2,3,7,8-PeCDD (28%)
BB5	2,3,7,8-TCDF (40%)	PCB 126 (34%)	2,3,4,7,8-PeCDF (55%)	2,3,7,8-TCDF (22%)	2,3,4,7,8-PeCDF (49%)	2,3,7,8-TCDD (26%)
BB6	2,3,7,8-TCDF (46%)	PCB 126 (39%)	2,3,7,8-TCDF (51%)	PCB 126 (20%)	2,3,7,8-TCDF (57%)	PCB 126 (34%)
BB9	2,3,7,8-TCDF (40%)	PCB 126 (37%)	2,3,4,7,8-PeCDF (53%)	2,3,7,8-TCDF (23%)	2,3,4,7,8-PeCDF (64%)	2,3,7,8-TCDF (20%)
JB4	2,3,7,8-TCDF (43%)	PCB 126 (29%)	2,3,4,7,8-PeCDF (35%)	2,3,7,8-TCDF (25%)	2,3,7,8-TCDD (52%)	2,3,4,7,8-PeCDF (26%)
JB6	2,3,7,8-TCDF (53%)	PCB 126 (15%)	2,3,4,7,8-PeCDF (34%)	2,3,7,8-TCDF (30%)	2,3,7,8-TCDD (59%)	2,3,4,7,8-PeCDF (22%)
JB7	2,3,7,8-TCDF (48%)	2,3,7,8-TCDD (20%)	2,3,4,7,8-PeCDF (34%)	2,3,7,8-TCDF (25%)	2,3,7,8-TCDD (60%)	2,3,4,7,8-PeCDF (19%)
JB9	2,3,7,8-TCDF (44%)	2,3,7,8-TCDD (26%)	2,3,4,7,8-PeCDF (40%)	2,3,7,8-TCDD (28%)	2,3,7,8-TCDD (70%)	2,3,4,7,8-PeCDF (19%)
JB12	2,3,7,8-TCDF (46%)	2,3,7,8-TCDD (23%)	2,3,4,7,8-PeCDF (29%)	2,3,7,8-TCDF (27%)	2,3,7,8-TCDD (71%)	2,3,4,7,8-PeCDF (14%)
LO2	PCB 126 (31%)	PCB 77 (24%)	2,3,4,7,8-PeCDF (39%)	PCB 77 (15%)	2,3,7,8-TCDD (45%)	2,3,4,7,8-PeCDF (26%)
LO3	PCB 126 (43%)	PCB 77 (34%)	2,3,4,7,8-PeCDF (35%)	PCB 77 (35%)	2,3,7,8-TCDD (50%)	2,3,4,7,8-PeCDF (29%)
LO4	PCB 77 (37%)	PCB 126 (35%)	2,3,4,7,8-PeCDF (36%)	PCB 77 (31%)	2,3,4,7,8-PeCDF (35%)	2,3,7,8-TCDD (32%)
LO5	PCB 126 (32%)	PCB 77 (28%)	2,3,4,7,8-PeCDF (33%)	PCB 77 (22%)	2,3,7,8-TCDD (41%)	2,3,4,7,8-PeCDF (25%)
LO6	PCB 77 (68%)	2,3,7,8-TCDD (21%)	PCB 77 (66%)	2,3,7,8-TCDD (27%)	2,3,7,8-TCDD (90%)	PCB 77 (7%)

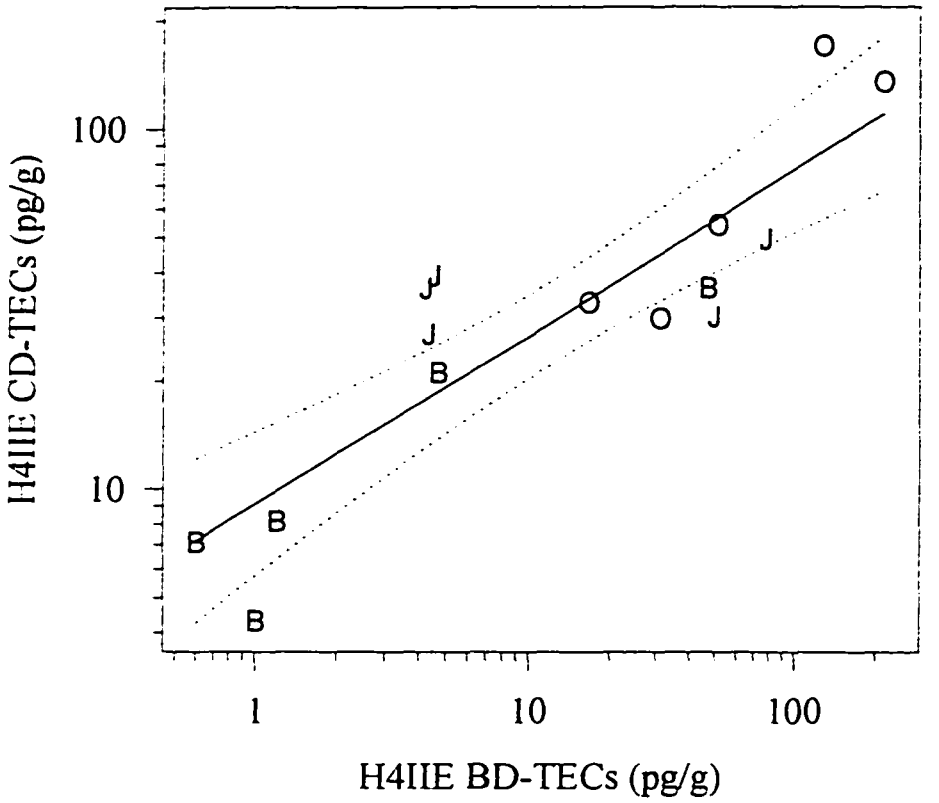
[1] BB - Black Bay, Lake Superior; JB - Jackfish Bay, Lake Superior; LO - Lake Ontario

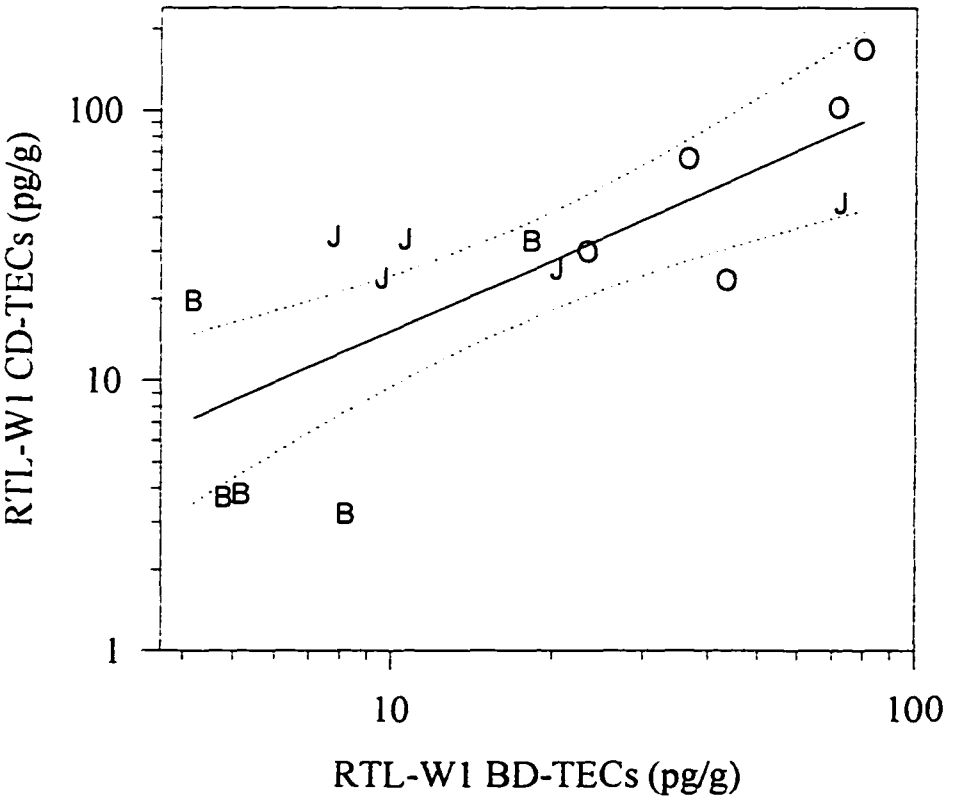
can be seen that RTL-W1 CD-TECs are composed mainly of 2,3,4,7,8-PCDF and 2,3,7,8-TCDF, while H4IIE CD-TECs receive the highest contributions from 2,3,7,8-TCDF, 2,3,7,8-TCDD and PCB 126. When the same samples are analyzed using ELS TEFs, over 50% of the total CD-TEC is derived from 2,3,7,8-TCDD contributions in each case. In Lake Ontario samples, RTL-W1 CD-TECs still receive the highest contribution from 2,3,4,7,8-PCDF. For H4IIE CD-TECs from this site, PCBs 77 and 126 comprise over 50% of the total CD-TEC in each case. As was the case in Jackfish Bay, ELS CD-TECs mainly received the largest contribution from 2,3,7,8-TCDD. Patterns of CD-TEC contributions in samples from the reference site, Black Bay, were not as clear. RTL-W1 CD-TECs for these samples were composed mainly of PCDFs while H4IIE CD-TECs had a combination of PCB 126 and 2,3,7,8-TCDF as the largest portions. For ELS CD-TECs in Black Bay, aside from sample BB1, which received major contributions from PCB 77 and 126, the largest percentage of the CD-TECs were made up of PCDFs.

E. Bioassay-derived vs chemically-derived 2,3,7,8-TCDD equivalent concentrations

When compared on an individual sample basis, RTL-W1 BD-TECs were not significantly different than RTL-W1 CD-TECs (Table 1.2). This was true for all sampling sites. Similarly, H4IIE BD-TECs and CD-TECs for individual trout liver samples at each site did not differ significantly . Scatterplots of BD-TECs versus CD-TECs for both H4IIE and RTL-W1 are shown in Figure 1.5. The following correlations were seen between BD-TEC and CD-TEC for each cell line: H4IIE $r = 0.871$, $p < 0.05$; RTL-W1 $r = 0.810$, $p < 0.05$.

Figure 1.5. Bioassay-derived (BD-) TECs relative to chemistry-derived (CD-) TECs in lake trout livers from three sites in the Great Lakes determined using H4IIE (top) and RTL-W1 (bottom) cell lines. The following symbols represent the different sites: B - Black Bay, Lake Superior, J - Jackfish Bay, Lake Superior, and O - Lake Ontario.


H4IIE CD-TECs (pg/g)
100
10
1
10
100
H4IIE BD-TECs (pg/g)


RTL-W1 CD-TECs (pg/g)
100
10
1
10
100
RTL-W1 BD-TECs (pg/g)

F. Hepatic Enzyme Analysis

Mean EROD activity was highest in livers of trout collected from Jackfish Bay ($p < 0.01$, Table 1.2). Lake Ontario and Black Bay trout had lower levels of hepatic EROD activity, and the levels from these two sites did not differ significantly from one another. Overall, hepatic EROD was not found to be significantly different between male and female trout at each site. When all samples were examined, hepatic EROD was inversely correlated with trout body mass ($r = -0.562$, $p < 0.05$) and liver mass ($r = -0.424$, $p < 0.05$).

When the in vivo EROD activity in individual trout is compared to their corresponding BD-TECs, no significant relationship was seen for RTL-W1 ($r = -0.095$, $p > 0.05$) or H4IIE ($r = -0.181$, $p > 0.05$). Examination within sites revealed no correlations between BD-TECs and hepatic EROD. Similarly, no significant relationship was found between CD-TECs and hepatic EROD.

1.5 DISCUSSION

Liver cell lines from a rat (H4IIE) and a rainbow trout (RTL-W1) have been used as tools to express the contaminant content in the liver of individual lake trout from three Great Lakes sites as TCDD equivalent concentrations (TECs). As direct bioassay tools, H4IIE and RTL-W1 gave similar values (BD-TECs), regardless of the large differences in BD-TECs between sites. This suggests that the combinations of contaminants at each site are equally active in mammalian and piscine systems. As a source of TCDD equivalent factors (TEFs) for use in converting chemical data to TCDD equivalent concentrations,

H4IIE gave slightly higher values (CD-TECs) than RTL-W1 and quite different contributions to the CD-TECs by separate PCH classes. However, these differences between cell lines were small compared to the CD-TECs calculated with TEFs derived from rainbow early life stage mortality. Despite the discrepancies in CD-TECs due to differences in vitro and in vivo TEFs, the complexity of the contaminant chemistry between individuals and between different sites warrants several bioassay approaches, both direct and indirect, in order to evaluate the potential potencies of different contaminant combinations.

A. Coplanar PCB, chlorinated dioxin/furan congener determination

For the congeners measured in this study, the coplanar (non-ortho substituted) PCBs were the most abundant PCHs detected in lake trout livers at all three sites. The four PCBs assessed were congeners 77, 81, 126, and 169. The majority of samples from Lake Ontario had detectable levels of these compounds. The ranking and concentrations in these samples are consistent with values reported by Huestis et al. (1996) in Lake Ontario lake trout and by Janz et al. (1992) in Lake Ontario rainbow trout. The levels also followed the rank order of concentrations detected in lake trout eggs (Guiney et al. 1996) from the eastern basin of Lake Ontario. PCB 77 was the dominant congener in all Lake Ontario samples, followed by PCB 126. A notable exception to the pattern was sample LO6 where only PCB 77 was above the instrumental detection limits. No physical differences were evident between this sample and the others collected from this site. Relative proportions of non-ortho PCBs seen in Jackfish Bay and Black Bay corresponded to Lake Ontario

samples, but concentrations were considerably lower. The similarities between concentrations of PCBs in samples from Jackfish Bay and Black Bay are likely due to the fact that atmospheric transport is the major source of PCB contamination in Lake Superior (Young et al. 1987). In Lake Ontario, both point source contamination and atmospheric deposition of PCBs contribute to the higher levels accumulated in samples from this region.

PCDD and PCDF concentrations determined in this study were generally in accordance with previous studies on fish from the Great Lakes (Huestis et al. 1996; Servos et al. 1994; Zacharewski et al. 1989). In all three sites, 2,3,7,8-TCDF was the dominant PCDF congener, with highest concentrations seen in Jackfish Bay trout. In Lake Ontario samples, the PCDD congener detected at the highest concentrations was 2,3,7,8-TCDD. In Jackfish Bay, high levels of both 2,3,7,8-TCDD and OCDD were seen in liver extracts, while the reference site, Black Bay, had the lowest concentrations of PCDDs/PCDFs.

It has been shown that congeners with the 2,3,7,8- substitution pattern are the most readily bioaccumulated (Grimwood and Dobbs 1995). Octochloro-substituted congeners are thought not to be readily bioaccumulated in aquatic organisms due to membrane permeation limitations in the gill and gut (Muir et al. 1985). The high levels of OCDD detected in the liver samples from Jackfish Bay are probably due to two factors: first, inputs of OCDD tend to be higher relative to other congeners, and second, OCDD is resistant to degradation due to the lack of unsubstituted carbons necessary for metabolism (Servos et al. 1989; Servos et al. 1992).

B. Bioassay-derived 2,3,7,8-TCDD equivalent concentrations

The highest concentrations of BD-TECs were found in Lake Ontario trout livers, indicating elevated concentrations of CYP1A1 inducers in the extracts. This corresponds to the analytical chemistry data, which shows high levels of coplanar PCBs relative to Jackfish Bay and the reference site samples. Jackfish Bay BD-TECs were significantly higher than those from Black Bay, most likely due to the greater concentration of 2,3,7,8-TCDD in the samples from Jackfish Bay. Previous studies on white sucker from the Jackfish Bay region have found elevated levels of BD-TECs attributable to bioaccumulation of PCHs from contaminated food sources near BKME inputs (van den Heuvel et al. 1995). Although Black Bay had the lowest BD-TECs, detectable levels of CYP1A-inducing compounds were found in samples from this site, as demonstrated by both the analytical chemistry data and BD-TECs. Atmospheric transport of PCHs and food chain accumulation are the probable sources of the BD-TECs in samples from this site.

Although significant differences were found in mean BD-TECs at each site, individual within-site samples varied considerably (Figure 1.3). Very few studies have looked at the variation in TECs between individuals of a species within the same site. Several modifying factors can contribute to differing levels of accumulation of PCHs within an aquatic species (Spacie et al. 1995). These can include chemical and physical characteristics of the surrounding environment, and biological factors related to the organism itself. Two interrelated factors could be the diet and movement. Lake trout are known to take advantage of a variety of food sources, including crustaceans, insects, many species of fish (including lake trout), and even small mammals (Scott and Crossman 1973).

Coupled with the fact that lake trout tend to move freely over distances of several miles, it is clear that these organisms can accumulate varying amounts of PCHs depending on available food and location. Lake trout at each site also varied by sex and sexual maturity which may have altered uptake of PCHs and contributed to the wide range of BD-TECs in the sites. The BD-TECs showed no correlation with sex and body weight.

Comparison of the two bioassays employed in this study indicate that both the mammalian and teleost cell systems responded in a similar manner to the lake trout liver extracts. Overall, this suggests that the extracts are equally active on the Ah receptor-signal transduction system of rainbow trout and rat. It has been suggested that the response of mammalian cell systems to PCH mixtures may not accurately predict the response of teleost systems when exposed to the same mixture (Zabel et al. 1996), and differences in responses to individual congeners by the two cell systems have been demonstrated (Clemons et al. 1996; Clemons et al. 1997). For the PCH extracts in this study, this was not the case. Regardless of site and PCH congener patterns, the use of either H4IIE or RTL-W1 did not result in statistically significant differences in the overall BD-TECs, although in a few individual samples the H4IIE and RTL-W1 BD-TECs differed (Figure 1.3). H4IIE and RTL-W1 gave similar BD-TECs for liver extracts from white sucker caught at two different Lake Superior sites, but for liver extracts from white sucker that had been caged in BKME effluent for 8 days, the two cell lines yielded different BD-TECs. In this case the H4IIE BD-TEC was higher, suggesting that H4IIE was more responsive to compound(s) in the BKME effluent than RTL-W1 (van den Heuvel et al. 1996). Zabel et al. (1996) found that Lake Ontario salmon and Saginaw Bay carp BD-TECs determined using a rainbow

trout gonadal cell line (RTG-2) were similar to those determined using H4IIE, but that BD-TECs for sediment samples were one order of magnitude higher when derived using RTG-2. Although these studies seem to give conflicting results, an examination of the use of CD-TECs in conjunction with BD-TECs for environmental extracts may help explain the differences.

C. Chemically-derived 2,3,7,8-TCDD equivalent concentrations

Differences in the congener concentrations in the liver of fish from the three sites led to differences in the CD-TECs calculated. Black Bay had the lowest CD-TECs, which corresponds to the reduced concentrations of the AhR-active PCHs measured in the trout tissue at this site. As was the case with the BD-TECs, high CD-TECs were determined in lake trout livers from Lake Ontario and Jackfish Bay, where elevated levels of coplanar PCBs and PCDD/Fs were detected respectively.

Of the three sets of TEFs used in this study, CD-TECs derived using the mammalian (H4IIE) TEFs yielded the highest values, followed by RTL-W1 and ELS CD-TECs. Although the difference between the RTL-W1 and H4IIE CD-TECs was not large, it was statistically significant. Therefore, for the combination of PCHs in these extracts, H4IIE were slightly more sensitive than RTL-W1. This is in contrast to the findings for the BD-TECs, where the two cell systems predicted equal values. The reduced sample size available for the CD-TEC determination may have contributed to this difference. More striking were the low CD-TECs determined using the ELS TEFs. The difference in mean CD-TEC between the in vitro and in vivo teleost systems was three-fold or greater at each

site. The possibility exists that the differences between mammalian and teleost species may be more pronounced if in vivo systems are employed. Despite this potential drawback of in vitro assays, they still have an advantage in that they can be more easily developed from a range of species and performed more rapidly than most in vivo assays.

The fact that all three sets of TEFs produced the same ranking of the sites in terms of mean CD-TEC seems to indicate equal predictive ability of each system. However, examination of the contribution of congeners in each system indicates that this may not always be the case, depending on variations in patterns of individual PCH concentrations. As Figure 1.4 shows, at each site, PCBs contribute more to H4IIE CD-TECs than to RTL-W1 or ELS CD-TECs. The H4IIE TEFs for PCBs are generally higher than in both of the rainbow trout systems, with PCB 126 in particular several orders of magnitude higher. As a result, in sites where PCBs are the predominant source of PCH contamination, the mammalian system will typically predict higher CD-TECs than the trout counterparts. The situation is reversed when PCDD/Fs are considered. The RTL-W1 TEFs for 2,3,4,7,8-PeCDF and 1,2,3,4,7,8-HxCDF are high compared to H4IIE. The consequence of the differing TEFs is clearly seen in samples from Lake Ontario (Table 1.4), where the major contributors to the CD-TECs for H4IIE are PCB 77 and 126, while RTL-W1 CD-TECs receive the greatest percentage of the CD-TECs from 2,3,4,7,8-PeCDF and 2,3,7,8-TCDD respectively. Consequently, the similarity in the total CD-TECs (and BD-TECs) for RTL-W1 and H4IIE may be illusory. PCBs in Lake Ontario samples that contribute to H4IIE CD-TECs are counterbalanced by 2,3,4,7,8-PeCDF that contribute to RTL-W1 CD-TECs. It is evident that if a chemical mixture contains a high proportion of a single congener, other than

2,3,7,8-TCDD, differences in CD-TECs calculated using various TEF systems will diverge. In general, the majority of the contribution to ELS CD-TECs comes from 2,3,7,8-TCDD whenever it is present in a sample, as its TEF effectively dwarfs other ELS TEFs.

D. Chemically-derived versus bioassay-derived 2,3,7,8-TCDD equivalent concentrations

The fact that the BD-TECs and CD-TECs for individual samples did not differ significantly for either the H4IIE or RTL-W1 cell systems indicates that, in general, all of the AhR-active compounds in a sample were accounted for by the congener analysis and that these compounds are acting in an additive fashion. In spite of this overall similarity between BD-TECs and CD-TECs, there were individual samples that appeared to have distinct differences in TECs depending on which method was used to determine them. An example of this can be seen with the Lake Ontario sample, LO5 (Table 1.2). For RTL-W1, the CD-TEC is twofold higher than the BD-TEC. This emphasizes the potential differences between strict congener analysis and biological response to a mixture of undefined compounds. Further studies on an individual sample basis are probably warranted to appreciate the complexity of contaminant burdens and the range of responses possible in a population.

E. Hepatic Enzyme Analysis

The lack of correlation between TECs and hepatic EROD activity likely indicates that the two measurements are evaluating different but related consequences of

contaminant exposure. The compounds that make up the TECs have half lives of weeks, months and years in fish (van den Berg et al. 1994), and once accumulated, only a fraction of their concentration might be physiologically active. Therefore, TECs would appear to measure the store of EROD-inducing compounds accumulated over a relatively long time period. By contrast, the induction and decay of EROD activity in fish is generally on the order of days (Bucheli and Fent 1995). Therefore, EROD activity likely reflects a more recent exposure to EROD inducing compounds. This can be particularly important in migratory fish such as lake trout that can be exposed to variable contamination levels in different regions. Three additional causes of the poor correlation between TECs and hepatic EROD activity exist. The first two pertain to loss of EROD inducing compounds prior to cell line exposure. The metabolism of EROD-inducing compounds in vivo or the loss of compounds during the extraction procedure can both affect the level of contaminant in an extract and hence the derived TECs (van den Berg et al. 1994; Low et al. 1987). Finally, the complex array of factors that regulate CYP1A expression (Bucheli and Fent 1995) could confound the correlation.

The higher EROD activity in the livers of lake trout at Jackfish Bay has several possible interpretations. The most probable is the presence at this site of an EROD inducer(s) that does not bioaccumulate in the liver of fish, and therefore, is not measured in TECs. A possible source of this inducer(s) is BKME. Recent work supports the presence in BKME of non-PCH EROD inducers that are water soluble and appear not to bioaccumulate in fish (Burnison et al. 1996; Hodson et al. 1997; Parrott and Tillitt 1997). Other likely possibilities for this higher level of EROD activity are that the fish at Jackfish Bay had

been more recently exposed to inducing levels of PCHs than had the fish at Black Bay and Glenora, or that factors that can influence the P450 enzyme system in fish such as temperature and/or nutrition (Bucheli and Fent 1995), differ between these sites.

F. Summary

In this study, the livers of lake trout from three Great Lakes sites showed evidence of PCH contamination but the types of PCHs and the PCH levels expressed as TCDD equivalent concentrations (TECs) varied between sites. Livers of lake trout collected from Lake Ontario were found consistently to have higher levels of PCB 77 than the livers from two sites (Jackfish Bay and Black Bay) in Lake Superior, and this contributed to higher liver TECs for these fish. This probably reflects the greater levels of PCB congeners in Lake Ontario due to historical sources of local PCB contamination (Oliver and Niimi 1988; Niimi and Oliver 1989). Lake trout from Jackfish Bay had significantly higher liver TECs as compared to the reference site, Black Bay, and these differences are presumably due to food chain accumulation of CYP1A-inducing compounds present in BKME at Jackfish Bay. Fish from Black Bay had the lowest levels of PCHs and TECs. Atmospheric transport of CYP1A-inducing compounds are the likely source of these compounds at this reference site. When all of the individual samples are considered together, differences between BD-TECs and CD-TECs or between H4IIE and RTL-W1 TECs were minor. This suggests that an additive mechanism of CYP1A induction occurred in both the mammalian and piscine systems and that all of the AhR-active compounds in a sample were accounted for by the congener analysis. For certain samples though, distinct differences existed between BD-

TECs and CD-TECs depending on which method was used to determine them, indicating the possibility of subtle variance in species-specific responses to PCH mixtures. Although liver EROD activity was not correlated with TECs at any of the three sites, hepatic EROD activity was significantly elevated at Jackfish Bay as compared to the other two sites. A possible explanation for this is the presence at Jackfish Bay of an CYP1A-inducing compound(s) that does not bioaccumulate.

1.6 ACKNOWLEDGEMENTS

This research was funded in part by the Great Lakes University Research Fund (GLURF), Natural Sciences and Engineering Research Council (NSERC) operating grants to D.G.D. and N.C.B., and a NSERC postgraduate scholarship to J.J.W. Assistance with lake trout sampling by Andrea Farwell, Mark McMaster, Kelly Munkittrick, Leo and Mae Marchand, and the fisheries staff at Glenora MNR Research Centre is greatly appreciated.

Susan Huestis died suddenly in April 1997 at the age of 34. She was an excellent person to work with and a dedicated and insightful environmental scientist. She is missed by all of us.

1.7 REFERENCES

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

Ankley, G.T., Tillitt, D.E., Giesy, J.P., Jones, P.D., and Verbrugge, D.A. 1991. Bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in PCB-containing extracts from the flesh and eggs for Lake Michigan Chinook Salmon (*Oncorhynchus tshawytscha*) and possible implications for reproduction. Canadian Journal of Fisheries and Aquatic Sciences. 48: 1685-1690.

Barnes, D.G. 1991. Toxicity equivalents and EPA's risk assessment of 2,3,7,8-TCDD. The Science of the Total Environment. 104: 73-86.

Bols, N.C., Whyte, J.J., Clemons, J.H., Tom, D.J., van den Heuvel, M.R., and Dixon, D.G. 1997. Use of liver cell lines to develop TCDD equivalency factors and TCDD equivalent concentrations in environmental samples. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment. J.T. Zelikoff, ed. SOS Publishing, Fair Haven, NJ, 1st ed.: pp. 329-350.

Bucheli, T.D. and Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25: 201-268.

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

Clemons, J.H., van den Heuvel, M.R., Stegeman, J.J., Dixon, D.G., and Bols, N.C. 1994. Comparison of toxic equivalent factors for selected dioxin and furan congeners derived using fish and mammalian liver cell lines. Canadian Journal of Fisheries and Aquatic Sciences. 51: 1577-1584.

Clemons, J.H., Lee, L.E.J., Myers, C.R., Dixon, D.G., and Bols, N.C. 1996. Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines from rat and trout and the derivation of toxic equivalency factors. Canadian Journal of Fisheries and Aquatic Sciences. 53: 1177-1185.

Clemons, J.H., Dixon, D.G., and Bols, N.C. 1997. Derivation of 2,3,7,8-TCDD toxic equivalent factors (TEFs) for selected dioxins, furans, and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. Chemosphere. 34: 1105-1119.

Donato, T.M., Castell, J.V., and Gómez-Lechón, M.J. 1992. A rapid and sensitive method for measuring monooxygenase activities in hepatocytes cultured in 96-well plates. Journal of Tissue Culture Methods. 14: 153-158.

Eadon, G., Kaminsky, L., Silkworth, J., Aldous, K., Hilker, D., O'Keefe, P., Smith, R., Gierthy, J., Hawley, J., Kim, N., and DeCaprio, A. 1986. Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. Environmental Health Perspectives. 70: 221-227.

Environment Canada, Department of Fisheries and Oceans, and Health and Welfare Canada. 1991. Toxic chemicals in the Great Lakes and associated effects: volume 1 - contaminant levels and trends. 1-488.

Evans, D.O. and Olver, C.H. 1995. Introduction of lake trout (*Salvelinus namaycush*) to inland lakes of Ontario, Canada: factors contributing to successful colonization. Journal of Great Lakes Research. 21 (Supplement 1): 30-53.

Firestone, D. 1991. Determination of dioxins and furans in foods and biological tissues: review and update. Journal of the Association of Official Analytical Chemists. 74: 375-384.

Goldstien, J.A. and Safe, S. 1989. Mechanism of action and structure-activity relationships for the chlorinated dibenzo-*p*-dioxins and related compounds. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. R.D. Kimbrough and A.A. Jensen, eds. Elsevier Science Publishers, Amsterdam, 2nd ed.: pp. 239-293.

Great Lakes Water Quality Board of the International Joint Commission. 1987. Report on Great Lakes Water Quality.

Grimwood, M.J. and Dobbs, T.J. 1995. A review of the aquatic ecotoxicology of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Environmental Toxicology and Water Quality. 10: 57-75.

Guiney, P.D., Cook, P.M., Casselman, J.M., Fitzsimmons, J.D., Simonin, H.A., Zabel, E.W., and Peterson, R.E. 1996. Assessment of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced sac fry mortality in lake trout (*Salvelinus namaycush*) from different regions of the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences. 53: 2080-2092.

Hodson, P.V., Maj, M.K., Efler, S., Burnison, B.K., Van Heiningen, A.R.P., Girard, R., and Carey, J.H. 1997. MFO induction in fish by spent cooking liquors from kraft pulp mills. Environmental Toxicology and Chemistry. 16: 908-916.

Huestis, S.Y., Servos, M.R., Sergeant, D.B., Leggett, M., and Dixon, D.G. 1995. Methods for determination of organochlorine pesticides, polychlorinated biphenyl congeners and chlorinated dibenzo-*p*-dioxins and furans in fish. Canadian Technical Report on Fisheries and Aquatic Sciences. 2044: 1-30.

Huestis, S.Y., Servos, M.R., Whittle, D.M., and Dixon, D.G. 1996. Temporal and age-related trends in levels of polychlorinated biphenyl congeners and organochlorine contaminants in lake ontario lake trout (*Salvelinus namaycush*). Journal of Great Lakes Research. 22: 310-330.

Janz, D.M., Metcalfe, T.L., Metcalfe, C.D., and Haffner, G.D. 1992. Relative concentrations of cytochrome P450-active organochlorine compounds in liver and muscle of rainbow trout from Lake Ontario. Journal of Great Lakes Research. 18: 759-765.

Kennedy, S.W., Lorenzen, A., James, C.A., and Collins, B.T. 1993. Ethoxyresorufin-*0*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. Analytical Biochemistry. 211: 102-112.

Kennedy, S.W., Lorenzen, A., and Norstrom, R.J. 1996. Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. Environmental Science and Technology. 30: 706-715.

Kennedy, S.W. and Jones, S.P. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Analytical Biochemistry. 222: 217-223.

Kutz, F.W., Barnes, D.G., Bottimore, D.P., Greim, H., and Bretthauer, E.W. 1990. The international toxicity equivalency factors (I-TEF) method of risk assessment for complex mixtures of dioxins and related compounds. Chemosphere. 20: 751-757.

Lee, L.E.J., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K., Pasitschniak-Arts, M., Mosser, D.D., and Bols, N.C. 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biology and Toxicology. 9: 279-294.

Lorenzen, A. and Kennedy, S.W. 1993. A fluorescence-based protein assay for use with a microplate reader. Analytical Biochemistry. 214: 346-348.

Low, G.K., Batley, G.E., and Brockbank, C.I. 1987. Solvent-induced photodegradation as a source of error in the analysis of polycyclic aromatic hydrocarbons. Journal of Chromatography. 392:199-210: 199-210.

Metcalfe, C.D. and Haffner, G.D. 1995. The ecotoxicology of coplanar polychlorinated biphenyls. Environmental Reviews. 3: 171-190.

Muir, D.C.G., Marshall, W.K., and Webster, G.R.B. 1985. Bioconcentration of PCDDs by fish: effects of molecular structure and water chemistry. Chemosphere. 14: 829-833.

Newsted, J.L., Giesy, J.P., Ankley, G.T., Tillitt, D.E., Crawford, R.A., Gooch, J.W., Jones, P.D., and Denison, M.S. 1995. Development of toxic equivalency factors for PCB congeners and the assessment of TCDD and PCB mixtures in rainbow trout. Environmental Toxicology and Chemistry. 14: 861-871.

Niimi, A.J. 1996. PCBs in aquatic organisms. In: Environmental contaminants in wildlife: interpreting tissue concentrations. W.N. Beyer, G.H. Heinz, and A.W. Redmon-Norwood, eds. CRC Press, Boca Raton, Florida, 1st ed.: pp. 117-152.

Niimi, A.J. and Oliver, B.G. 1989. Distribution of polychlorinated biphenyl congeners and other halocarbons in whole fish and muscle among Lake Ontario salmonids. Environmental Science and Technology. 23: 83-88.

Okey, A.B., Riddick, D.S., and Harper, P.A. 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Toxicology Letters. 70: 1-22.

Oliver, B.G. and Niimi, A.J. 1988. Trophodynamic analysis of polychlorinated biphenyl congeners and other chlorinated hydrocarbons in the Lake Ontario ecosystem. Environmental Science and Technology. 22: 388-397.

Owens, W.J. 1991. The hazard assessment of pulp and paper effluents in the aquatic environment: a review. Environmental Toxicology and Chemistry. 10: 1511-1540.

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

Parrott, J.L. and Tillitt, D.E. 1997. The use of semipermeable membrane devices (SPMDs) to concentrate inducers of fish hepatic mixed function oxygenase (MFO). In: Ecotoxicology: Responses, Biomarkers and Risk Assessment. J.T. Zelikoff, ed. SOS Publishing, Fair Haven, NJ, pp. 185-196.

Robinson, R.D., Carey, J.H., Solomon, K.R., Smith, I.R., Servos, M.R., and Munkittrick, K.R. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 1. mill characteristics, receiving-water chemical profiles and lab toxicity tests. Environmental Toxicology and Chemistry. 13: 1075-1088.

Safe, S. 1987. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. Chemosphere. 16: 791-802.

Safe, S., Mason, G., Farrell, K., Keys, B., Piskorska-Pliszczynska, J., Madge, J.A., and Chittim, B. 1987. Validation of in vitro bioassays for 2,3,7,8-TCDD equivalents. Chemosphere. 16: 1723-1728.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology. 21: 51-88.

Safe, S.H. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Reviews in Toxicology. 24: 87-149.

Scott, W.B. and Crossman, E.J. 1973. Freshwater fishes of Canada. Bulletin of the Fisheries Research Board of Canada. Ottawa, Ontario. 966 pages.

Servos, M.R., Muir, D.C.G., Whittle, D.M., Sergeant, D.B., and Webster, G.R.B. 1989. Bioavailability of octachlorodibenzo-*p*-dioxin in aquatic ecosystems. Chemosphere. 19: 969-972.

Servos, M.R., Muir, D.C.G., and Webster, G.R.B. 1992. Bioavailability of polychlorinated dibenzo-*p*-dioxins in lake enclosures. Canadian Journal of Fisheries and Aquatic Sciences. 49: 735-742.

Servos, M.R., Huestis, S.Y., Whittle, D.M., Van Der Kraak, G.J., and Munkittrick, K.R. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 3. polychlorinated dioxins and furans in muscle and liver of white sucker (*Catostomus commersoni*). Environmental Toxicology and Chemistry. 13: 1103-1115.

Spacie, A., McCarty, L.S., and Rand, G.M. 1995. Bioaccumulation and bioavailability in multiphase systems. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 493-521.

Tillitt, D.E., Ankley, G.T., Verbrugge, D.A., Giesy, J.P., Ludwig, J.P., and Kubiak, T.J. 1991a. H4IIE rat hepatoma cell bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in colonial fish-eating waterbird eggs from the Great Lakes. Archives of Environmental Contamination and Toxicology. 21: 91-101.

Tillitt, D.E., Giesy, J.P., and Ankley, G.T. 1991b. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environmental Science and Technology. 25: 87-92.

Tysklind, M., Tillitt, D., Eriksson, L., Lundgren, K., and Rappe, C. 1994. A toxic equivalency factor scale for polychlorinated dibenzofurans. Fundamental and Applied Toxicology. 22: 277-285.

van den Berg, M., De Jongh, J., Poiger, H., and Olson, J.R. 1994. The toxicokinetics and metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. Critical Reviews in Toxicology. 24: 1-74.

van den Heuvel, M.R., Munkittrick, K.R., Van Der Kraak, G.J., Servos, M.R., and Dixon, D.G. 1995. Hepatic 7-ethoxyresorufin-O-deethylase activity, plasma steroid hormone concentrations, and liver bioassay-derived 2,3,7,8-TCDD toxic equivalent concentrations in wild white sucker (*Catostomus commersoni*) caged in bleached kraft pulp mill effluent. Canadian Journal of Fisheries and Aquatic Sciences. 52: 1339-1350.

van den Heuvel, M.R., Servos, M.R., Munkittrick, K.R., Bols, N.C., and Dixon, D.G. 1996. Evidence for a reduction of 2,3,7,8-TCDD toxic equivalent concentrations in white sucker (*Catostomus commersoni*) exposed to bleached kraft mill effluent, following process and treatment improvements. Journal of Great Lakes Research. 22: 264-279.

Vanden Heuvel, J.P. and Lucier, G. 1993. Environmental toxicology of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans. Environmental Health Perspectives. 100: 189-200.

Walker, M.K., Cook, P.M., Butterworth, B.C., Zabel, E.W., and Peterson, R.E. 1996. Potency of a complex mixture of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in causing fish early life stage mortality. Fundamental and Applied Toxicology. 30: 178-186.

Walker, M.K. and Peterson, R.E. 1991. Potencies of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology. 21: 219-238.

Williams, L.L., DeGalan, N., Verbrugge, D.A., Tillitt, D.E., Ankley, G.T., and Welch, R.L. 1992. Prediction of concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents from total concentrations of polychlorinated biphenyls in fish fillets. Environmental Science and Technology. 26: 1151-1159.

Young, J.W., Allan, R.J., Machta, L., Wagner, T., and Wise, P.L. 1987. Summary report of the workshop on Great Lakes atmospheric deposition. International Joint Commission, Windsor, ON.

Zabel, E.W., Cook, P.M., and Peterson, R.E. 1995. Toxic equivalency factors of polychlorinated dibenzo-*p*-dioxin, dibenzofuran and biphenyl congeners based on early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology. 31: 315-328.

Zabel, E.W., Pollenz, R., and Peterson, R.E. 1996. Relative potencies of individual polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners and congener mixtures based on induction of cytochrome P4501A mRNA in a rainbow trout gonadal cell line (RTG-2). Environmental Toxicology and Chemistry. 15: 2310-2318.

Zacharewski, T., Safe, L., Safe, S., Chittim, B., DeVault, D., Wilberg, K., Bergqvist, P.A., and Rappe, C. 1989. Comparative analysis of polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners in Great Lakes fish by gas chromatography-mass spectrometry and in vitro enzyme induction activities. Environmental Science and Technology. 23: 730-735.

Zint, M.T., Taylor, W.W., Carl, L., Edsall, C.C., Heinrich, J., Sippel, A., Lavis, D., and Schaner, T. 1995. Do toxic substances pose a threat to rehabilitation of lake trout in the Great Lakes? A review of the literature. Journal of Great Lakes Research. 21 (supplement 1): 530-546.

CHAPTER 2

THE USE OF MICROCOSMS TO EVALUATE METHODOLOGIES FOR ASSESSING THE EXPOSURE OF RAINBOW TROUT TO POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN CREOSOTE CONTAMINATED ENVIRONMENTS.

2.1 ABSTRACT

A three-step approach was employed to assess the exposure of rainbow trout to polycyclic aromatic hydrocarbons (PAHs) in standardized creosote contaminated microcosms. Bioconcentration of PAHs was approximated using semipermeable membrane devices (SPMDs) and compared to levels seen in the trout. Large differences in concentrations of PPPAHs in the two matrices were seen, with SPMDs accumulating higher levels of analytes compared to the fish. Increases in hepatic EROD levels and bile metabolites with creosote dose suggest the difference in bioconcentration between trout and SPMDs is mainly due to biotransformation of accumulated PPPAHs in the trout. Species-specific response to PPPAHs extracted from trout and SPMDs was assessed by calculating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations (TECs) using mammalian and piscine cell line bioassays. The use of RTL-W1 or H4IIE did lead to different TECs, but the magnitude of the differences was not great and depended on whether SPMD or liver extracts were being examined. Possible reasons for the disparity include subtle differences in the AhR signal transduction system, PAH metabolism between fish and mammals or

TEF derivation methods. This dissimilarity may indicate that RTL-W1 is more appropriate than H4IIE for assessing the toxic impact of PAHs to rainbow trout. The integration of the exposure assessment methodologies provided evidence of concentration-dependent PPPAH uptake in rainbow trout, despite low levels detected in the liver.

2.2 INTRODUCTION

Creosote, produced through the distillation of coal tar, is a complex mixture composed of approximately 85% polycyclic aromatic hydrocarbons (PAHs), 10% phenolic compounds and 5% N-, S-, and O- heterocyclics (Mueller et al. 1989). In Canada, creosote is used as a lasting wood preservative for railway ties, bridge timbers, marine pilings, and large-sized lumber (Government of Canada et al. 1993). When properly used and disposed of, creosote does not appear to pose a significant threat to ecosystem health (Mueller et al. 1989). Contamination of sites by creosote can occur through improper removal and storage of creosote-treated wood products, spillage of creosote during manufacture and transportation, and from wood treatment plant wastewater (Government of Canada et al. 1993; Borthwick and Patrick, Jr. 1982).

The effects of creosote on fish have been studied in a variety of ways. English sole (*Pleuronectes vetulus*) from Puget Sound, a region contaminated by creosote waste, showed hepatic lesions such as neoplasms and unique degenerative conditions (Myers et al. 1990). Similar effects were found to occur in the laboratory environment in English sole injected with a PAH fraction isolated from the sediments collected at the same site. Mummichog (*Fundulus heteroclitus*) captured from the Elizabeth River, VA, had a high

prevalence of hepatic cancers that were associated with PAH contamination of creosote origin (Van Veld et al. 1992). Likewise, in an 8-day laboratory exposure of spot (*Leiostomus xanthurus*) to creosote-contaminated sediments collected from Elizabeth River, VA, fish developed skin lesions, changes in hepatic and pancreatic function, reduced weight gain and increased mortality (Hargis et al. 1984; Roberts et al. 1989). In fish, exposure to creosote and PAHs induces cytochrome P450 forms (Stegeman and Lech 1991; Collier and Varanasi 1991; Munkittrick et al. 1995; Schoor et al. 1991). The metabolism of many PAHs to carcinogenic and/or more toxic compounds involves cytochrome P4501A enzymes (CYP1A), and therefore the activity of these enzymes is a factor that can place cells at risk (Smolowitz et al. 1992; Schmalix et al. 1993).

One potential approach to the study of creosote effects on fish is the use of microcosms, or "model ecosystems" (Graney et al. 1995). Microcosms simulate some of the complexities of a natural ecosystem while maintaining a degree of control close to that of laboratory exposures. Defined concentrations of creosote can be added to experimental ponds and the chemical proportions of the mixture should remain comparable due to equal volumes, water chemistry and sediment composition in each microcosm. Exposure and collection of test organisms can be standardized and simplified, which is not always the case in field studies (Bankey et al. 1994). Microcosms do have limitations in that the impact of the physical environment on large scale aquatic ecosystems in nature can differ greatly from that represented in a microcosm (Graney et al. 1995).

Regardless of the exposure method, two major problems exist in the estimation of risk associated with creosote exposure. Firstly, because creosote is a mixture of hundreds

of different chemicals, it is important to know the concentrations of bioavailable compounds present in a creosote-contaminated environment in order to estimate its potential toxicity. Determination of the concentrations and types of organic contaminants can be made by measuring specific compounds in the water column or sediment cores (Zitko 1993; Munkittrick et al. 1995; Mátlová et al. 1995), but in order to predict biological effects, it is more appropriate to measure only the truly dissolved, bioavailable compounds present. Although it is possible to determine the levels of contaminants bioaccumulated in the tissues of fish, many PAHs are rapidly metabolized in the liver (Meador et al. 1995). A direct measurement of chemical concentrations in tissue can therefore result in an underestimation of toxicant exposure.

To assess the level of bioavailable contaminants present in an aquatic environment, Huckins and coworkers designed a lipid-containing semipermeable membrane device (SPMD) that would mimic the bioconcentration process by passively sampling truly dissolved hydrophobic contaminants (Huckins et al. 1996). The device consists of a thin film of neutral lipid (triolein) sealed in a section of lay-flat polyethylene tubing (Prest et al. 1992). Hydrophobic contaminants move through the polyethylene membrane via passive diffusion and partition into the lipid where they are concentrated. In contrast to gill membranes, SPMD polyethylene membranes are not altered in response to stress (Gale et al. 1997) and PAHs sequestered in the triolein phase are not subject to metabolic transformations that occur in fish (Moring and Rose 1997). Comparisons of the bioconcentration of hydrophobic contaminants by SPMDs versus living organisms have begun to be made. Moring and Rose (1997) compared uptake of PAHs in clams and

SPMDs and observed distinct differences in the degree of accumulation between the two matrices. Twenty PAHs were detected in SPMDs, whereas only three were detected at considerably lower levels in the clams. These results were interesting, since PAH metabolism in invertebrates is substantially less than in vertebrates, which can lead to the accumulation of higher steady state concentrations of these compounds (Stegeman and Hahn 1994). The authors attributed the difference to reduced filtering rates caused by stress in the clams. In a recent comparison of channel catfish (*Icatalurus punctatus*) to SPMDs for uptake of dioxin-like compounds (Gale et al. 1997), markedly different levels of total polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) were seen in the two matrices, with concentrations being generally lower in the fish. The two main hypotheses proposed to explain the difference were metabolism of congeners with lower chlorination patterns and reduced uptake due to the stress of caging on the fish. Planar chlorinated hydrocarbons (PCHs), including PCBs, dioxins and others are metabolized slowly in fish (Stegeman and Hahn 1994), lending more support to the second hypothesis. By comparison, the importance of the present study in relation to previous experiments is that it is the first to contrast patterns of accumulation of compounds known to be rapidly metabolized in fish to those seen in SPMDs.

Although a measure of the bioavailable contaminants in a creosote-contaminated environment is useful, a second issue complicating the risk assessment of creosote exposure is the number (>200) of individual compounds (mainly PAHs) in creosote. To estimate the potential toxicity of a mixture of compounds, the toxic equivalent approach

has been developed (Eadon et al. 1986; Barnes 1991). This technique requires information from two sources. The first is an analysis and quantification of the chemicals present in a mixture extracted from an environmental sample. The second is a toxicological data base that allows the assignment to each chemical of a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalent factor (TEF) or induction equivalency factor (IEF) (Kennedy et al. 1996). TEFs convert the dose of the compound to the dose of TCDD that would produce the same biological response and have been derived from a variety of *in vivo* and *in vitro* toxicity tests (Kutz et al. 1990; Safe 1990; Safe 1994; Ahlborg et al. 1994; Parrott et al. 1995; Kennedy et al. 1996; Clemons et al. 1997). The most common *in vitro* response is the induction of aryl hydrocarbon hydroxylase (AHH) or 7-ethoxyresorufin-*O*-deethylase (EROD) activity in liver cell lines, which are measures of CYP1A induction (Tillitt et al. 1991b). Liver cell lines from a rat hepatoma (H4IIE) and a normal rainbow trout liver (RTL-W1) have been found to yield different TEF values for some planar chlorinated hydrocarbons (PCHs) (Clemons et al. 1997; Bols et al. 1997b), which suggests that TEFs might have to be matched with the organism under evaluation. TCDD is chosen as the reference due to its biological potency and the fact that the mechanistic basis of its toxicity is best understood (Okey et al. 1994). TCDD and structurally related compounds are thought to mediate most of their actions, including the induction of AHH and EROD, by activating the aryl hydrocarbon receptor (AhR) and can be described as AhR-active compounds. The application of the two data sets in the toxic equivalent approach requires the assumption that the compounds in a sample act in an additive manner. Therefore, the toxic potency of a complex chemical mixture is estimated by summing the TCDD-

equivalent concentration of each chemical present to give the TCDD-equivalent (TEQ) or concentration (TEC). Due to the fact that this depends on knowing the compounds present, this is sometimes called the chemistry-derived TEC (CD-TEC) (Bols et al. 1997b).

As well as analytical methods, estimation of a chemical mixture's potential toxicity can be done with bioassays (Firestone 1991). The most frequent bioassay has been the induction in liver cell lines of AHH or EROD activities. This is the same response that has been used to derive TEFs *in vitro*, and for the bioassays, usually the same cell lines are used that have been used for TEF derivation. Cell bioassays integrate the potency of all the compounds in the sample that might interact with the AhR to induce CYP1A (i.e. AhR-active compounds). The potency of an extract is expressed relative to TCDD. Since the identity of the compounds in the sample will be unknown by this method, the measurement is sometimes referred to as the bioassay-derived TEC (BD-TEC) (Bols et al. 1997b).

This paper uses microcosms dosed with creosote to evaluate the use of SPMDs, piscine versus mammalian TEFs and cell bioassays, and hepatic EROD activity as assessment tools for evaluating the impact on fish of PAHs in creosote-contaminated environments.

2.3 METHODS

A. Experimental Microcosms

The Canadian Network of Toxicology Centres' (CNTC) outdoor microcosm facility, located in Guelph, Ontario, consists of 30 freshwater ponds used for testing the

impact and effects of chemicals and substances on fresh-water organisms. They consist of steel ponds lined with PVC, are 1.05 m deep with a surface area of 11.95 m^2 and contain 12000 L of freshwater. To control fluctuations in water temperature, the ponds are constructed in gravel bedding. Plastic trays of sifted sediment were added to the microcosms, providing 50% coverage of the floor surface area.

In the spring 1995, as part of a larger study on the effects of creosote at various trophic levels, the microcosms were dosed with sub-surface injections of liquid creosote into a stream of water pumped at a rate of 1360 L/h. The dosing regime was based on a series of graded creosote doses with no replication, a protocol commonly referred to as the regression approach. The benefits of this design include cost savings relative to replication, interpolative capability inherent in the regression model and the fundamental relation of regression to the concentration-response relationship (Thompson et al. 1994). Prior to the addition of creosote, water in the ponds was circulated to a large freshwater holding pond for a four week period. Following creosote addition, water circulation ceased, creating a static exposure system. In October 1995, six ponds of the ponds were chosen for the present study. These static system ponds had received original doses to yield nominal creosote concentrations in the ponds of 0, 5, 9, 17, 31, and 56 µL/L. Temperature, pH, and dissolved oxygen were monitored routinely throughout the study.

B. SPMD Exposure and Collection

Ten SPMDs were obtained from Environmental Sampling Technologies (St. Joseph, Missouri). The devices were composed of low-density polyethylene lay-flat tubing

(2.5 cm width, 0.91 cm length, 89 μm membrane thickness), contained a thin film of triolein (1 g, 95% purity) and were heat sealed at both ends. SPMDs were shipped at -20°C to the study site in sealed tins containing pure nitrogen gas and were not removed from the tins until the initiation of the study.

Fifteen weeks after the initial dosing of the microcosms with creosote, six SPMDs were randomly assigned to the ponds, one SPMD for each concentration. Due to financial reasons, replication of samplers in ponds was not performed, although previous studies have shown excellent reproducibility among SPMDs (Lebo et al. 1992; Lebo et al. 1996; Huckins et al. 1993). SPMDs were suspended in the water column by nylon fishing line to a depth of 50 cm and weighted at the bottom with a 3 g galvanized anchor. To protect against possible photodegradation of accumulated PAHs, SPMDs were shaded from direct sunlight with 3 cm thick, 0.35 m^2 polystyrene surface floats. These floats also served as a suspension point for the SPMDs. To estimate PAH uptake rate over the 28 day exposure period, three of the remaining SPMDs were deployed in the same manner in the 31 μL/L creosote-dosed pond. The final SPMD served as a trip blank/day 0 time point for the PAH uptake rate experiment.

During the 28 day exposure period, a single SPMD was removed from the 31 μL/L pond at each of the following time points: day 3, 7, 14, and 28. Upon removal from the microcosm, SPMDs were rinsed with ethanol and wiped with paper towels to remove any biofouling on the polyethylene surface, such as algae and insect larvae (Lebo et al. 1992). The devices were then wrapped in hexane-rinsed aluminum foil and placed on dry ice.

After transport to the lab, SPMDs were stored at -80°C pending analysis. All other SPMDs were collected on day 28 from their respective ponds and processed in the same manner.

C. Rainbow Trout Exposure and Collection

Two weeks prior to the initiation of the study, an acclimation pond adjacent to the microcosm facility was stocked with female rainbow trout obtained from Rainbow Springs Fish Hatchery (Thamesford, ON). After this period, fish were weighed, tagged and randomly assigned to the various experimental ponds. Three trout were added to each of the six ponds on the same day as the addition of the SPMDs, and three more trout were added per day for the following four days to give a total of fifteen trout at each concentration. The control pond received a total of 30 fish in order to characterize natural population variability. Trout were housed in nylon mesh bags supported by three 40 cm diameter circular plastic rings. The bags were suspended in the pond so that the trout could migrate in the water column through a range of 10 to 100 cm below the water surface. Fish were fed a diet of commercial trout chow (2% body weight/day) during the study.

After the 28 day exposure period, trout were sampled over five days in the same order in which they had been exposed. Recorded mortalities in the control, 5, 9, 17, 31, and 56 µL dosed ponds were 5, 3, 4, 3, 2, and 5 respectively. Upon removal from the pond, fish were anaesthetized with methane tricainesulfonate (MS-222) and weighed. Following cervical dislocation, livers were excised, weighed and divided into two subsamples. The first portion of the liver was wrapped in hexane-rinsed aluminium foil and stored on ice. These samples remained frozen at -20°C pending PAH extraction. The second portion was

placed in a 2 mL cryovial (NUNC, Roskilde, Denmark), frozen in liquid nitrogen, and stored at -80°C until hepatic EROD analysis. Samples of bile collected from rainbow trout were supplied to J. Lewis (Department of Biology, University of Guelph, Guelph, ON, Canada) for determination of biliary PAH metabolites (Lewis 1997).

D. PAH Isolation from SPMDs and Rainbow Trout Liver

Isolation and cleanup of PAH extracts from trout hepatic tissue and SPMDs were performed using modified methods of Huestis et al. (1995), Lebo et al. (1996) and P. Helm (personal communication). Although hexane dialysis is an effective method of isolating hydrophobic contaminants from SPMDs (Lebo et al. 1992; Meadows et al. 1993; Huckins et al. 1993), in this study the devices were cut open at both ends using hexane rinsed scissors and lipid was washed into a flask with multiple rinses of methylene chloride (40 mL) (Prest et al. 1992). The solvent-lipid mixture was transferred to a 1 cm diameter column containing Na_2SO_4 (previously fired at 400°C) which was then washed with 300 mL methylene chloride. Using a similar method, trout liver samples (approximately 1 g) were weighed and ground in anhydrous Na_2SO_4. The homogenate was added to 1 cm diameter glass columns and solvent extracted with 300 mL methylene chloride. For both SPMD and tissue samples, gel permeation chromatography (GPC, Biorad SX-3 GPC resin, Richmond, CA) was used to remove lipids and other hydrophobic co-extractives. Lipid content in this fraction was estimated gravimetrically by evaporating the extract to a constant mass. The second GPC fraction, containing the PAHs of interest, was cleaned up by SPE with Florisil microcolumns (VWR Canlab, Mississauga, ON). The eluate was

evaporated with N_2 gas and dissolved in 1.00 mL acetonitrile (VWR Canlab, Mississauga, ON).

E. HPLC Analysis and Preparation of Dilution Series For Cell Line Bioassays

Concentrations of 16 priority pollutant PAHs (PPPAHs) in SPMD and trout hepatic extracts were determined by Brendan McConkey (Department of Biology, University of Waterloo, Waterloo, Ontario) using HPLC. This analysis was performed with a dual pump chromatography system equipped with a diode array detector (Shimadzu Scientific Instruments Inc., Columbia, MD) and a 25 cm × 4.6 mm i.d. Supelcosil C_{18} reverse-phase column with 5 μm packing (Supelco Inc., Bellefonte, PA) (McConkey et al. 1997). Extract volumes of 100 μL were applied to the column with an autosampler. Water, adjusted to pH 3.0 with phosphoric acid, and acetonitrile were used as elution solvents at a flow rate of 1 mL/min. Both solvents were degassed with helium prior to use. The chromatography solvent composition was 1% acetonitrile for 2 min, then a linear gradient to 90% acetonitrile over 30 min. Detection limits were 5-15 $ng \cdot mL^{-1}$ for the analytes measured. Appropriate standard curves were used to determine concentrations. Percent recovery was determined by extracting 1 mL triolein (95%, Sigma, St. Louis, MO) samples spiked with a certified standard containing the PPPAHs of interest (Supelco, Bellefonte, PA). No correction was made for procedural losses incurred during the extraction procedure. Procedural blanks did not indicate the occurrence any significant contamination during the extractions.

Following HPLC analysis, the remaining acetonitrile was evaporated with N_2 gas and dissolved in 50 µL of dimethylsulfoxide (DMSO, BDH, Darmstadt, W. Germany). A geometric dilution series of seven doses was prepared from the original extract for the cell line bioassays.

F. RTL-W1 and H4IIE cell maintenance

Rainbow trout liver (RTL-W1) cells were maintained as previously described (Lee et al. 1993). Leibovitz's L-15 (Gibco BRL, Gaithersburg, MD) growth medium was supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and penicillin/streptomycin (100 U penicillin, 10 µg streptomycin·mL^{-1}, ICN/Flow, Costa Mesa, CA). Cells were grown at 22°C ± 1 in a pure air environment.

Rat hepatoma (H4IIE) cells (American Type Culture Collection, CRL 1548) were maintained as previously described by Tillitt et al. (1991b). Dulbecco's modified Eagle's medium (MEM, Sigma, St. Louis, MO) was supplemented with 15% fetal bovine serum (FBS, Gibco, Gaithersburg, MD), L-glutamine, gentamycin sulphate (50 µg·mL^{-1}), MEM essential amino acids (1.5×), MEM non-essential amino acids (2×) and MEM vitamins (1.5×) (Sigma, St. Louis, MO). H4IIE were maintained at 37°C ± 1 in a humidified 5% CO_2 atmosphere.

G. RTL-W1 and H4IIE bioassay procedure

Determination of EROD activity in cultured cells dosed with PAH extracts was a modification of Clemons et al. (1996) and Kennedy et al. (1993). RTL-W1 and H4IIE were plated in 48-well culture clusters (Costar, Cambridge, MA) at an initial density of 40,000 cells·well^{-1}. Cells were allowed to grow for 24 h prior to dosing with the extracts. A dilution series of 2,3,7,8-TCDD in DMSO was run with every three extracts to serve as a positive control and standard for TEC determination.

Following a 24 h incubation period, the contaminated media was removed from the cells by plate inversion and the wells were rinsed with 100 µL phosphate buffered saline (PBS). The substrate, 7-ethoxyresorufin in methanol, was diluted in MEM to give a final concentration of 4.87 µM for H4IIE and 0.825 uM for RTL-W1. To prevent conjugation of the resorufin product in H4IIE by diaphorase (Donato et al. 1992), dicumarol (Sigma, St. Louis, MO) was added to the substrate-MEM mixture at a concentration of 0.29 mM. This reagent was not added to the RTL-W1 reaction mixture, as it was found to have no effect on resorufin fluorescence for this cell line (Clemons et al. 1996). Cells received 250 µL of the 7-ethoxyresorufin-MEM mixture and reactions were allowed to proceed for 15 min at 22 and 37°C for RTL-W1 and H4IIE, respectively. Resorufin production was measured fluorometrically (excitation 530 nm, emission 590 nm) using a Cytofluor 2350 plate reading fluorometer (Perspective Biosystems, Framingham, MA). A resorufin standard curve was run with each assay to allow conversion of fluorescent units to pmoles resorufin. Cellular protein was determined as described by Lorenzen and Kennedy (1993). A bovine

serum albumin (BSA, Sigma, St. Louis, MO) standard curve was run with each protein assay to allow conversion of fluorescent units to mg of protein.

H. Calculation of BD-TECs in SPMDs and Hepatic Tissue

Median effective concentrations of the extracts and 2,3,7,8-TCDD yielding one half maximal EROD activity in the cells (EC_{50}) were determined from concentration-response curves generated using the cell line bioassay. To calculate BD-TECs in trout liver and SPMDs, a ratio of the 2,3,7,8-TCDD EC_{50} to the extract EC_{50} was determined as described by Tillitt et al. (1991a):

$$\text{BD-TEC}_{(\text{pg TCDD/g lipid})} = \frac{EC_{50}\ \text{TCDD (pg/well)}}{EC_{50}\ \text{extract (dilution·well}^{-1})} \times \frac{\text{extract volume (µL)}}{\text{dose volume (µL)}} \times \text{lipid mass}^{-1}\ \text{(g)}$$

TECs were divided by 0.9 to correct for the volume of extract removed for HPLC analysis. Although Equation 1 could be used to determine BD-TECs in all SPMD extracts, rainbow trout liver extract concentration-response curves did not achieve high levels of EROD activity and failed to plateau at maximal levels. Since an EC_{50} could not be determined for these samples, a TEC was determined by matching the highest level of EROD activity induced by the extract to the corresponding level of EROD activity in the TCDD standard, and taking that concentration of TCDD to be the TEC (corrected for dosing volume of extract). As with other methods of TEC determination, this technique assumes that the concentration-response curves of the extract and 2,3,7,8-TCDD are parallel (Neubert et al. 1992).

I. Calculation of CD-TECs in SPMDs and Hepatic Tissue

RTL-W1 TEFs for EROD inducing PPPAHs were derived using the bioassay systems described above (Bols et al. 1997a). H4IIE TEFs used for CD-TEC calculation were determined for the same PPPAHs by Willett et al. (1997). Concentrations of individual PPPAHs in an extract were multiplied by their respective TEFs and summed to yield a CD-TEC (Eadon et al. 1986).

J. Hepatic Enzyme Analysis

Hepatic CYP1A activity in rainbow trout was quantified as EROD activity using a modification of the multiwell plate method of Kennedy and Jones (1994). Postmitochondrial supernatant was isolated from the livers by homogenizing tissue in ice-cold dithiothreitol buffer, followed by centrifugation at 9000 g. The following reagent concentrations were used in the EROD reaction mixture: 7-ethoxyresorufin, 1.7 μM (Sigma, St. Louis, MO); NADPH, 0.5 mM (Boehringer Mannheim, Germany); $MgSO_4$, 17 mM; and HEPES, 0.1 M, pH 7.8 M (Sigma, St. Louis, MO). EROD activity was determined in 48-well culture plates (Costar, Cambridge, MA) using a Cytofluor 2350 plate reading flourometer (Perspective Biosystems, Framingham, MA). Excitation and emission filters were set at 530 nm and 590 nm respectively. Activity was determined at 25°C using 25 μL postmitochondrial supernatant in the reaction mixture. Simultaneous termination of the reaction and protein determination were achieved by addition of ice cold fluorescamine

in acetonitrile (1.08 mM). Hepatic protein was estimated using a modification of Lorenzen and Kennedy (1993).

K. Estimation of Water Concentrations from SPMDs

To estimate aqueous concentrations of bioavailable PPPAHs in the microcosms, SPMD sampling rate data and the linear model developed by Huckins and co-workers (1993) were used in conjunction with the measured PPPAH concentrations in the lipid of the SPMDs from this study. Four compounds that were in the linear phase of the SPMD uptake by sampling day 7 were chosen for this analysis: fluorene, phenanthrene, pyrene and benz[a]anthracene. When the uptake rate is linear for these compounds, the following model can be used to estimate water concentrations:

$$C_W = C_L V_L / R_S B t$$

where C_W is the water (aqueous) concentration of the analyte, C_L is the concentration of the analyte in the SPMD lipid, V_L is the volume of triolein in the SPMD, R_S is the sampling rate in L/d, t is the time in days, and B is the correction factor for estimated uptake hindrance due to biofouling. In studies at the National Biological Service's Midwest Science Center, sampling rate data (R_S) have been determined for the PPPAHs examined in this study (J.N. Huckins, National Biological Service, personal communication). Values of R_S used were determined at 10°C, while the temperature in the present study declined

steadily from 12°C to 3°C (mean = 7.2°C ± 3.0°C, n=14). No correction of R_S values were made for temperature differences.

Water concentrations of the four PPPAHs selected were also estimated through conventional water sampling. Sample volumes of 1L were taken from each microcosm on day 10 and preserved with 80 g/L sodium thiosulphate. Compounds of interest were isolated using liquid-liquid extraction with methylene chloride. Samples were concentrated under a stream of purified N_2 and resuspended in 2 mL isooctane. Percent recovery was determined with an internal standard of bromonaphthalene with each sample. PPPAH concentrations were determined with a Varian 3400 gas chromatograph equipped with a Varian Saturn II ion trap mass spectrometer. Samples were injected onto a 30 m × 0.25 mm SPB-5 column with a stationary phase thickness of 0.25 μm at 300°C under splitless conditions. The transfer line and manifold temperatures were held constant at 260 and 250°C, respectively. Compounds were mass scanned at 45-550 m/z.

In addition to these measurements, results from this study were compared to concentrations of total PPPAHs determined in microcosm sediment 84 days after initial dosing (Figure 2.1, Bestari et al. 1996).

L. Statistics

Calculation of EC_{50}s used in determination of bioassay derived TECs was accomplished using the SigmaPlot non-linear curve fitting module (Jandel Scientific, San Rafael, CA). Trout body weight and hepatic percent lipid were tested for normality using

the Kolmogorov-Smirnov test (with Lilliefors' correction), and for equal variance using the Levene Median test. ANOVA was used to determine significant differences in body weight among microcosms. Dose response relationships for creosote effects on different variables were determined with linear regression. Differences between BD-TECs due to cell line (species) and nominal creosote dose were determined using two way repeated measures (RM) ANOVA. Differences between CD-TECs due to cell line and between BD- and CD-TECs within cell lines were determined by comparison of matched samples (paired comparison t-test) due to reduced sample size. Strength of association between variables was determined using Pearson product moment correlation. A level of significance of $p<0.05$ was used for all tests.

2.4 RESULTS

A. PPPAHs in SPMDs

PPPAH concentrations in SPMDs determined by HPLC were generally found to increase with increasing nominal creosote dose (Table 2.1). Mean percent recoveries of the 16 PPPAH external standards indicated good recovery for most compounds, although some procedural loss was observed for compounds of lower molecular weights. Gravimetric analysis of GPC lipid fraction revealed 87.9% ± 2% recovery (mean, S.D., $n=10$) of the original lipid within the SPMD. Concentrations of PPPAHs were not adjusted for lipid loss since it was not known if this occurred during the extraction procedure or the microcosm exposure. Although the SPMD trip blank did not contain detectable levels of any PPPAH,

the SPMD sampled from the control microcosm accumulated low levels of phenanthrene, fluoranthene and chrysene. The total PPPAHs in the remaining SPMDs followed a trend of non-linear increasing concentration with higher nominal creosote dose ($y = 0.84x - 5.3$ for log transformed data), and the values were significantly correlated ($r^2 = 0.91$, $p<0.01$). In terms of individual PPPAHs, pyrene and fluoranthene were the most abundant compounds detected in the devices. It was observed that for certain analytes, concentration did not

Table 2.1. Concentrations (ug/g lipid) of priority pollutant PAHs detected in lipid extracted from SPMDs exposed for 28 days in creosote dosed microcosms.

			Nominal Creosote Concentration in Microcosm					
PPPAH	MW	% Recovery [1]	Control	5 uL/L	9 uL/L	17 uL/L	31 uL/L	56 uL/L
Naphthalene	3.34	35.7 (4.2)	--[2]	--	--	--	--	--
Acenaphthylene	4.08	41.7 (2.5)	--	--	--	--	--	--
Acenaphthene	4.08	34.6 (3.5)	--	0.098	0.266	0.672	0.333	0.342
Fluorene	4.22	36.7 (4.8)	--	0.072	0.304	0.464	0.312	0.284
Phenanthrene	4.53	67.6 (9.6)	0.060	0.155	0.615	0.898	0.378	0.151
Anthracene	4.53	62.6 (6.4)	--	--	--	--	--	--
Fluoranthene	5.24	97.1 (25.3)	0.111	0.262	0.432	0.731	5.016	26.745
Pyrene	5.07	101.3 (16.0)	--	0.071	0.140	0.338	6.233	17.948
Benz [a] anthracene	5.90	81.5 (8.7)	--	--	--	--	0.424	0.625
Chrysene	5.77	83.0 (8.2)	0.021	0.070	0.093	0.129	0.383	0.556
Benzo [b] fluoranthene	6.52	81.5 (4.9)	--	0.019	0.067	0.179	0.288	0.302
Benzo [k] fluoranthene	6.73	82.1 (7.0)	--	0.015	0.031	0.078	0.107	0.071
Benzo [a] pyrene	6.23	86.7 (2.2)	--	--	--	0.035	0.054	0.048
Dibenz [a,h] anthracene	6.47	79.9 (9.5)	--	--	--	--	--	--
Benzo [g,h,i] perylene	7.03	79.5 (2.8)	--	--	--	--	--	--
Indeno [1,2,3-cd] pyrene	7.43	79.4 (1.1)	--	--	--	--	--	--
Total PPPAHs (µg/g)			0.191	0.761	1.948	3.524	13.528	47.072

[1] Percent recovery of external PPPAH standard (S.E., $n = 3$).

[2] -- indicates that the compound was not present at quantifiable concentration.

increase steadily with creosote dose. These included the lower molecular weight PAHs (acenaphthene, fluorene, phenanthrene) and higher molecular weight PAHs (benzo[k]fluoranthene, benzo[a]pyrene).

B. PPPAH concentrations in water estimated by SPMDs

The data from Table 2.1 and concentrations from SPMDs collected at the various time points were used to estimate water concentrations of four PPPAH analytes in the 31 μL/L microcosm. Due to some biofouling of SPMDs, a correction factor (B) of 0.75, based on surface area coverage of the growth, was used in the calculations. These estimates were compared to concentrations determined through conventional water sampling (Table 2.2). All four concentrations calculated with the linear model were lower than the measured values. Fluorene, phenanthrene, and pyrene were each approximately 2-fold lower than the corresponding measured water concentrations, and benz[a]anthracene was more than 40-fold lower.

Table 2.2. SPMD estimated average water concentrations (ng/L) of selected PPPAH analytes sequestered over a 28 day exposure in a 31 µL/L nominal creosote dosed microcosm.

Analyte	Log K_{OW}	Water conc. estimated from model (ng/L)	Water conc. measured in water (ng/L)
Fluorene	4.22	43.70	81.60
Phenanthrene	4.53	31.43	79.00
Pyrene	5.07	195.87	420.50
Benz[a]anthracene	5.90	11.49	505.60

C. PPPAHs in rainbow trout liver

PPPAHs and their concentrations in rainbow trout liver extracts were determined by HPLC and concentrations were found not to increase with increasing nominal creosote dose (Table 2.3). Lipid levels in the trout liver showed low variability and thus chemical concentrations were expressed on a lipid weight basis without introducing excessive bias. Unlike SPMDs, concentrations of total PPPAHs in tissue did not follow a significant dose-response and were below those detected in SPMDs (Figure 2.1). Also shown in this figure are concentrations of total PPPAHs measured in water and sediment collected from the mesocosms. In addition to the differences observed in concentration, the specific PPPAHs present in the liver did not follow the pattern seen in the SPMD. Figure 2.2 compares the types of PPPAHs present in SPMDs and trout liver averaged over all of the ponds and expressed as a percentage of total PPPAH. Fluoranthene and pyrene, the most highly concentrated compounds in the SPMDs, were below quantifiable levels in rainbow trout. Conversely, anthracene was detected in rainbow trout liver, a compound noticeably absent in SPMDs. The largest proportion of total PPPAH in the trout liver, 52.7%, was

from phenanthrene, while in SPMDs this compound contributed only 18.6%, although actual concentrations were comparable between the two matrices. One similarity between the SPMDs and the tissue was the lack of accumulation of low and high molecular weight PAHs. Of CYP1A inducing PAHs analyzed for in the tissue samples, only benz[a]anthracene was detected in some samples, but no relationship between the concentration of this compound and creosote dose was found.

Table 2.3. Mean concentrations (S.E.n =5, ug/g lipid) of priority pollutant PAHs detected in livers of rainbow trout exposed for 28 days in creosote dosed microcosms.

	Nominal Creosote Concentration in Microcosm					
PPPAH	Control	5 uL/L	9 uL/L	17 uL/L	31 uL/L	56 uL/L
Naphthalene	--[1]	--	--	--	--	--
Acenaphthylene	--	--	--	--	--	--
Acenaphthene	--	--	--	--	--	--
Fluorene	0.200 (0.049)	0.063 (0.047)	0.082 (0.034)	0.022 (0.022)	--	0.096
Phenanthrene	0.109 (0.029)	0.264 (0.120)	0.317 (0.080)	0.181 (0.040)	0.125 (0.032)	0.500
Anthracene	0.072 (0.044)	0.102 (0.054)	0.131 (0.033)	0.115 (0.016)	0.059 (0.010)	0.197
Fluoranthene	--	--	--	--	--	--
Pyrene	--	--	--	--	--	--
Benz [a] anthracene	0.094 (0.046)	0.048 (0.048)	0.063 (0.047)	--	--	0.044
Chrysene	--	--	--	--	--	--
Benzo [b] fluoranthene	--	--	--	--	--	--
Benzo [k] fluoranthene	--	--	--	--	--	--
Benzo [a] pyrene	--	--	--	--	--	--
Dibenz [a,h] anthracene	--	--	--	--	--	--
Benzo [g,h,i] perylene	--	--	--	--	--	--
Indeno [1,2,3-cd] pyrene	--	--	--	--	--	--
Total PPPAHs (µg/g)	0.474 (0.106)	0.477 (0.266)	0.593 (0.133)	0.318 (0.065)	0.184 (0.035)	0.836
Mean trout weight (g)	105.4 8.3	120.8 6.7	106.5 3.6	115.1 4.8	115.8 1.3	113.0
Mean % lipid	2.982 (0.20)	3.825 (0.53)	3.364 (0.61)	3.056 (0.08)	4.081 (0.37)	2.775

[1] -- indicates that the compound was not present at quantifiable concentration.

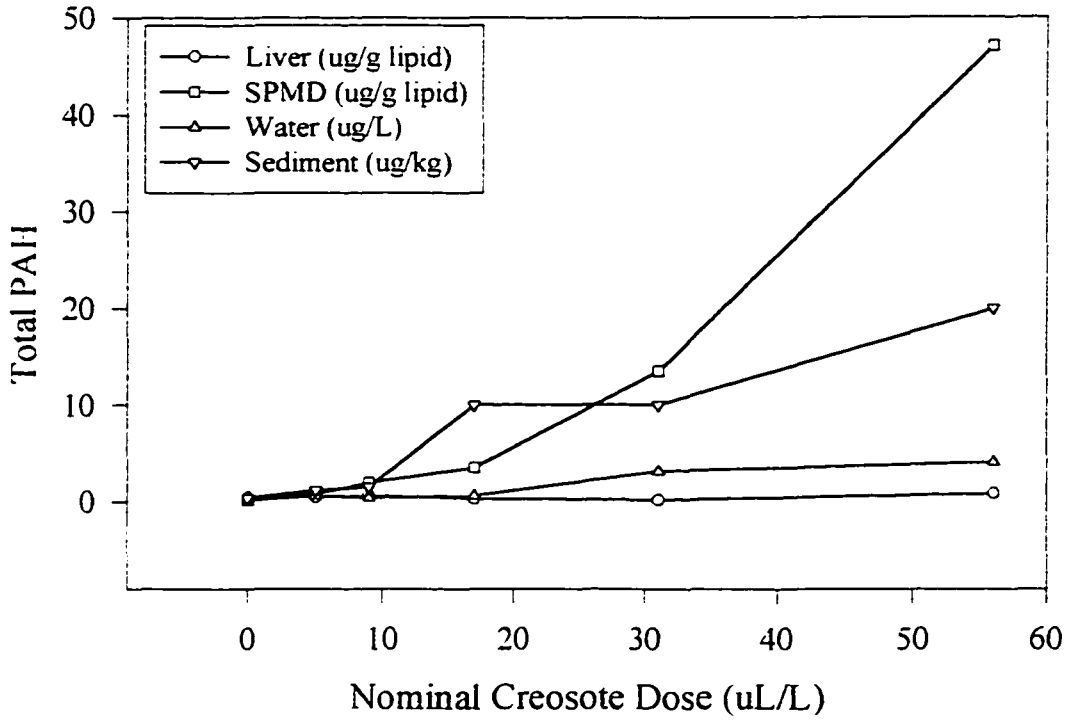
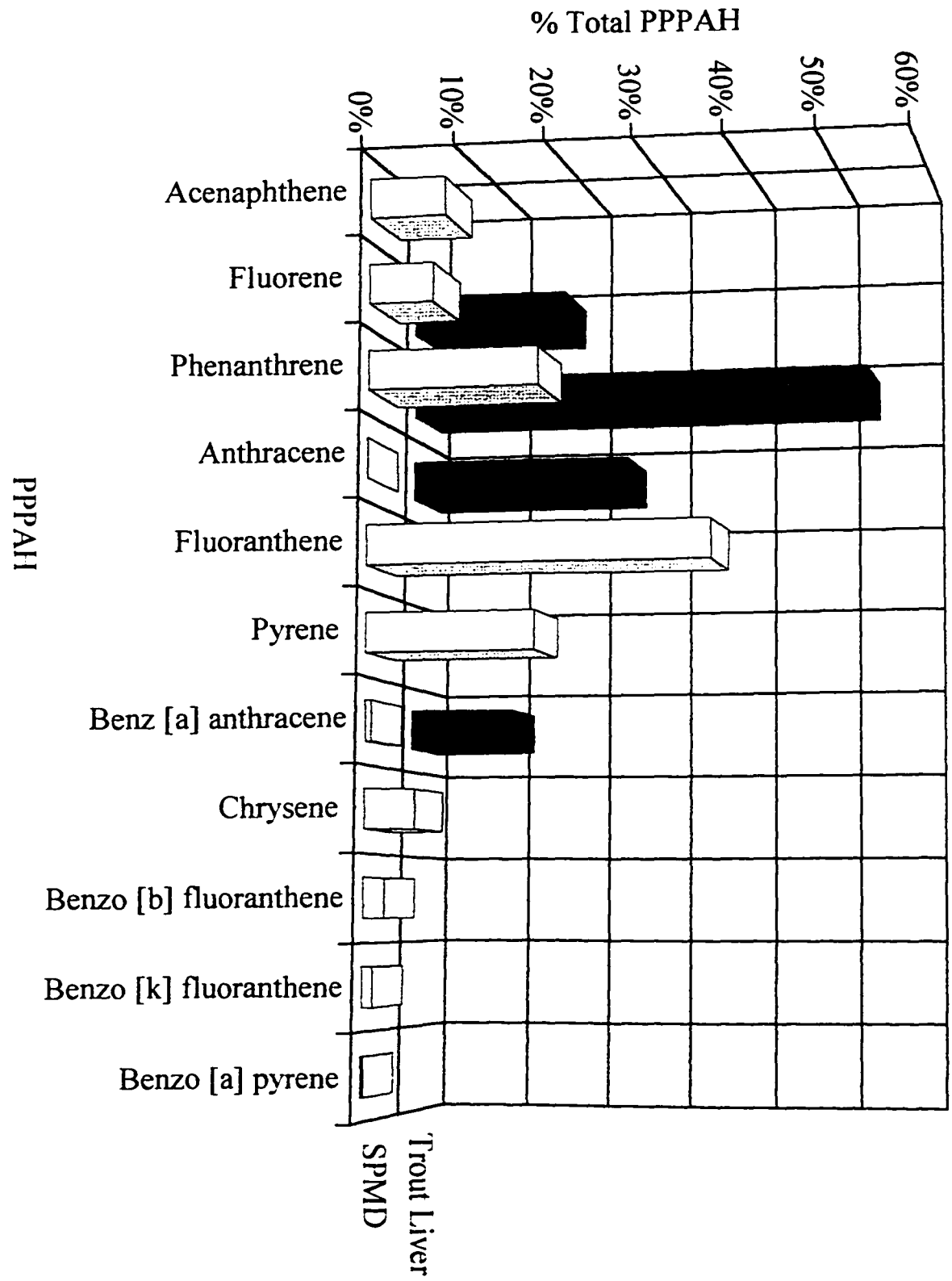


Figure 2.1. Effect of nominal creosote dose in microcosms on the amount of total PPPAHs accumulated by semi-permeable membrane devices (SPMDs) (—) and by rainbow trout liver (‿) during a 28 day exposure in the microcosms and on the total PPPAH levels in the water (Δ) and sediment (▽) of the microcosms. SPMDs and fish were added to the microcosms 103 days after creosote dosing of the microcosms. Water and sediment samples were collected from microcosms at 84 days after creosote dosing.

Figure 2.2. Mean concentrations of individual PPPAHs expressed as a percentage of the total PPPAH concentrations for both SPMDs (white bars) and trout liver (black bars) across all creosote doses accumulated over a 28 day exposure period. PPPAHs that were not detected in either matrix are not shown.


% Total PPPAH
0%
10%
20%
30%
40%
50%
60%
Acenaphthene
Fluorene
Phenanthrene
Anthracene
Fluoranthene
Pyrene
Benz [a] anthracene
Chrysene
Benzo [b] fluoranthene
Benzo [k] fluoranthene
Benzo [a] pyrene
PPAH
Trout Liver SPMD

In contrast to parent compounds, levels of biliary PAH metabolites (estimated as 1-pyrenol equivalents) in the trout liver were found to increase with nominal creosote dose (Lewis 1997). In addition, these metabolite concentrations were correlated with hepatic EROD activity (See below).

D. TCDD equivalent concentrations (TECs) in SPMDs

The PPPAH concentrations in SPMDs were expressed as TCDD equivalent concentrations (TECs) by using TEFs derived from EROD induction in liver cell lines from either rainbow trout (RTL-W1) or rat (H4IIE). For the eleven PPPAHs found in the extracts of this study, TEFs were available for only five, which are listed in Table 2.4. The remaining six PAHs appear not to be EROD inducers in either rainbow trout or rat (Bols et al. 1997a; Willett et al. 1997; Piskorska-Pliszczynska et al. 1986) and are not accounted for in the calculation of the CD-TECs for the samples in this study. As nominal creosote dose increased, CD-TECs increased, whether RTL-W1 TEFs (Figure 2.3a) or H4IIE TEFs (Figure 2.4a) were used to calculate the CD-TECs. A high degree of correlation was seen between the data sets ($r = 0.998$, $p<0.05$). However, H4IIE CD-TECs were significantly higher than RTL-W1 CD-TECs in comparisons of matched samples ($p<0.05$, $n=6$), which is explained in the next paragraph.

Table 2.4. PPPAHs found in extracts of this study and their 2,3,7,8-TCDD equivalent factors (TEFs) in RTL-W1 and H4IIE.

	RTL-W1 Bioassay	H4IIE Bioassay
2,3,7,8-TCDD	1.0	1.0
Benzo[k]fluoranthene	0.001048	0.00478
Benzo[a]pyrene	0.000329	0.000354
Benzo[b]fluoranthene	0.000265	0.00253
Benz[a]anthracene	0.000067	0.000025
Chrysene	0.000052	0.0002
Acenaphthene	NI[1]	NA[2]
Fluorene	NI	NA
Phenanthrene	NI	NA
Anthracene	NI	NA
Fluoranthene	NI	NA
Pyrene	NI	NA

[1] NI = Compound not shown to induce EROD activity (Bols et al. 1997).

[2] NA = Compound TEF not available (Willett et al. 1997).

Figure 2.3. Effect of nominal creosote dose in microcosms on the RTL-W1 chemistry-derived (CD) and bioassay-derived (BD) 2,3,7,8-TCDD equivalent concentrations (TECs) accumulated by semipermeable membrane devices (SPMDs) (a) and by rainbow trout liver (b) during a 28 day exposure in the microcosms. Bars represent single TECs for SPMDs or means ± S.E. for livers (n = 5 for each microcosm).

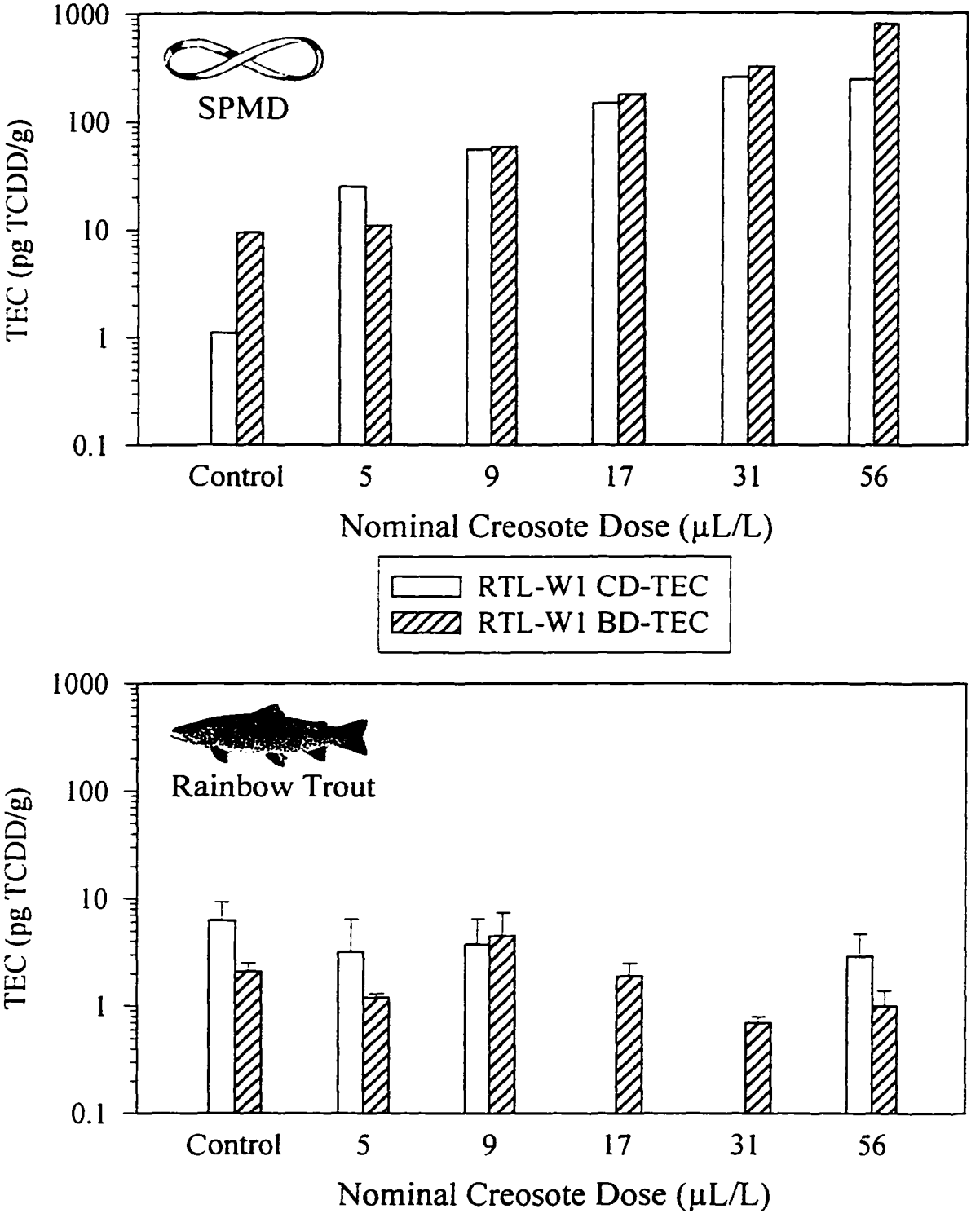
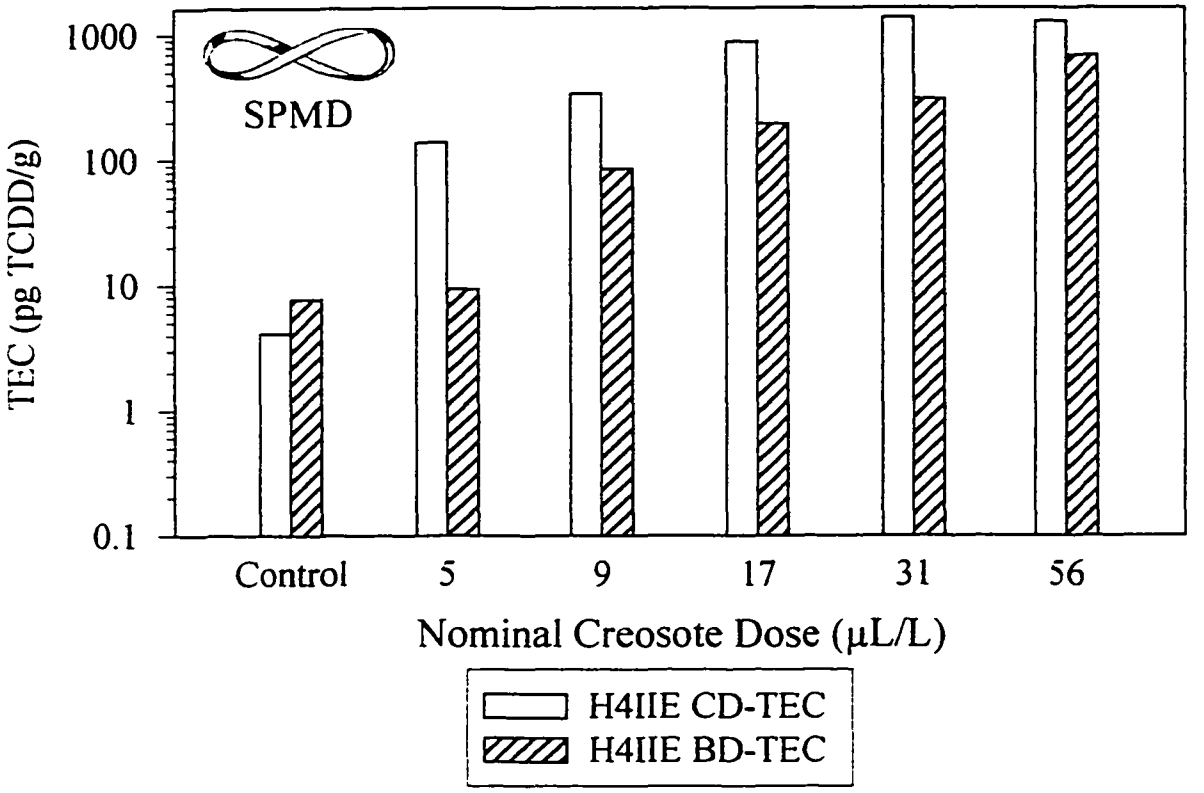
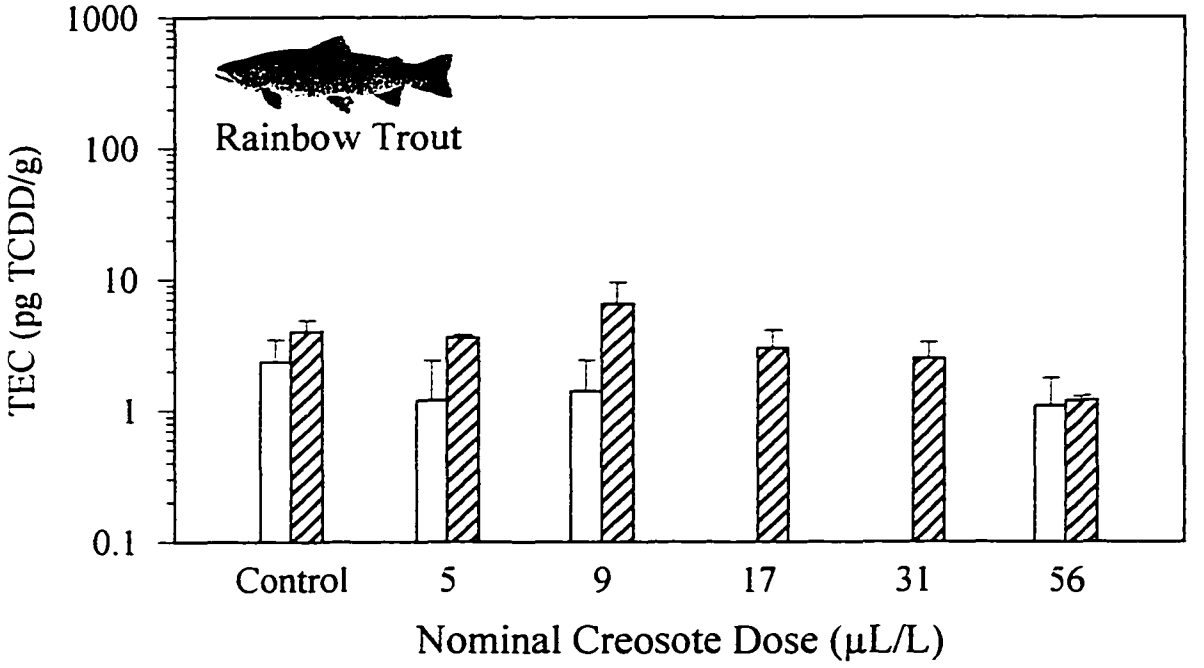

1000
100
10
1
0.1
TEC (pg TCDD/g)
SPMD
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)
RTL-W1 CD-TEC
RTL-W1 BD-TEC
1000
100
10
1
0.1
TEC (pg TCDD/g)
Rainbow Trout
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)

Figure 2.4. Effect of nominal creosote dose in microcosms on the H4IIE chemistry-derived (CD) and bioassay-derived (BD) 2,3,7,8-TCDD equivalent concentrations (TECs) accumulated by semipermeable membrane devices (SPMDs) (a) and by rainbow trout liver (b) during a 28 day exposure in the microcosms. Bars represent single TECs for SPMDs or means ± S.E. for livers (n = 5 for each microcosm).


SPMD
TEC (pg TCDD/g)
1000
100
10
1
0.1
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)
H4IIE CD-TEC
H4IIE BD-TEC


Rainbow Trout
TEC (pg TCDD/g)
1000
100
10
1
0.1
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)

For CD-TECs, it was possible to calculate the contribution of specific PAHs to the SPMD TECs. If analyte percent contributions were averaged over all of the ponds (excluding control, which had only one EROD inducer present) and expressed as a percentage of total RTL-W1 CD-TEC, the bulk of the total CD-TEC came from two analytes, benzo[b]fluoranthene (30% ± 2%) and benzo[k] fluoranthene (51% ± 6%). For H4IIE TECs, benzo[b]fluoranthene and benzo[k] fluoranthene were also the greatest contributors to total CD-TEC, but in opposite proportions to RTL-W1 (51% ± 4% and 41% ± 4% respectively). The remaining analytes contributed 10% or less to the average CD-TECs in both cell lines. The higher CD-TEC with H4IIE is due to the TEFs for benzo[b]fluoranthene and benzo[k]fluoranthene being higher with this cell line.

As an alternative to the chemistry-derived method, the complete set of compounds extracted from the SPMDs were expressed as TCDD equivalent concentrations (TECs) by assessing their ability to induce EROD activity in liver cell lines from either rainbow trout (RTL-W1) or rat (H4IIE). This is the bioassay derived-TCDD equivalent concentration (BD-TEC). As nominal creosote dose increased, RTL-W1 BD-TECs increased ($y = 14.22x - 53.17$, $r^2 = 0.973$, $p<0.05$), as did H4IIE BD-TECs ($y = 11.96x - 24.99$, $r^2 = 0.985$) (Figures 2.3a & 2.4a). The two sets of BD-TECs for SPMDs were very similar, and a significant relationship between the two was seen ($r^2 = 0.993$, $n=6$).

The two methods of TEC determination are compared in Figures 2.3a & 2.4a. With RTL-W1, a significant correlation existed between the BD- and CD-TECs ($r^2 = 0.674$, $p<0.05$, $n=6$), and a paired comparison of log transformed values from each pond revealed

no significant difference between the values for the two types of TECs. With H4IIE, BD-TECs were found to rise with CD-TECs ($r^2 = 0.698$, $p<0.05$, $n=6$), but the CD-TECs were significantly higher than the corresponding BD-TECs ($p<0.05$, $n=6$). CD- and BD-TECs both increased with an increase in nominal creosote dose, but at the highest creosote doses, the CD-TECs reached a plateau.

E. TCDD equivalent concentrations (TECs) in liver extracts

The PPPAH concentrations in rainbow trout liver extracts were expressed as TCDD equivalent concentrations (TECs) by using TEFs derived from EROD induction in liver cell lines from either rainbow trout (RTL-W1) (Bols et al. 1997a) or rat (H4IIE) (Willett et al. 1997). Two-thirds of the liver extracts did not contain detectable quantities of EROD inducing PPPAHs. As a result, the CD-TECs for these samples had values of zero (or below detection). In particular, no samples from the microcosms dosed with 17 and 31 μL creosote contained detectable quantities of EROD inducing PPPAHs, as is indicated by the absence of bars in Figures 2.3b & 2.4b. In the other ponds, the only EROD inducing PPPAH was benz[a]anthracene, so that in all cases, 100% of the CD-TEC was due to the contribution from this single analyte. Because this PAH was more potent in RTL-W1 (Table 2.4), each RTL-W1 TEC was higher than the H4IIE TEC when this compound was present. However, in neither case did these CD-TECs increase with an increase in nominal creosote dose (Figure 2.3b & 2.4b).

In contrast to the absence of *measured* EROD inducers, all trout liver extracts induced EROD activity to some degree in the RTL-W1 and H4IIE bioassays, which

allowed the calculation of BD-TECs for all samples. The BD-TECs derived with H4IIE were significantly higher than those derived with RTL-W1 at each creosote dose (RM ANOVA, $p<0.05$, $n=30$), but the two sets of values were significantly correlated ($r^2 = 0.669$, $p<0.05$, $n=30$). The BD-TECs for liver extracts did not change as the nominal creosote dose increased (Figure 2.3b & 2.4b).

CD-TECs and BD-TECs in the same liver extracts were compared (Figures 2.3b & 2.4b). To reduce bias imposed by the large number of trout liver CD-TECs with values below detection limits, only samples with detectable CD- and BD-TECs were compared. In paired-comparisons between BD- and CD-TECs with values greater than zero, CD-TECs derived with RTL-W1 TEFs were significantly higher than their corresponding BD-TECs determined with RTL-W1 ($p<0.05$, $n=8$). However, no significant correlation existed between CD-TECs and BD-TECs. H4IIE BD- and CD-TECs for liver extracts did not differ significantly, but the concentrations determined using the two methods were not correlated.

F. SPMD TECs vs liver TECs

Regardless of how they are derived, the TECs for the SPMDs were profoundly higher than the TECs for the rainbow trout liver extracts (Figures 2.5 & 2.6). For example, in the microcosm with the nominal creosote dose of 17 µL/L, the RTL-W1 and H4IIE SPMD TECs were approximately 90 and 60 times higher than the liver TECs, respectively. The disparity between the two was magnified with increasing nominal creosote dose. The

Figure 2.5. A comparison of the effects of a 28 day exposure to microcosms of differing nominal creosote doses on rainbow trout hepatic EROD activity (cross-hatched bar) and on RTL-W1 bioassay-derived (BD) 2,3,7,8-TCDD equivalent concentrations (TECs) accumulated by rainbow trout liver (open bar) and semipermeable membrane devices (SPMDs) (dark bar). Values represent means ± S.E. with n=5 for trout and n=1 for SPMDs.

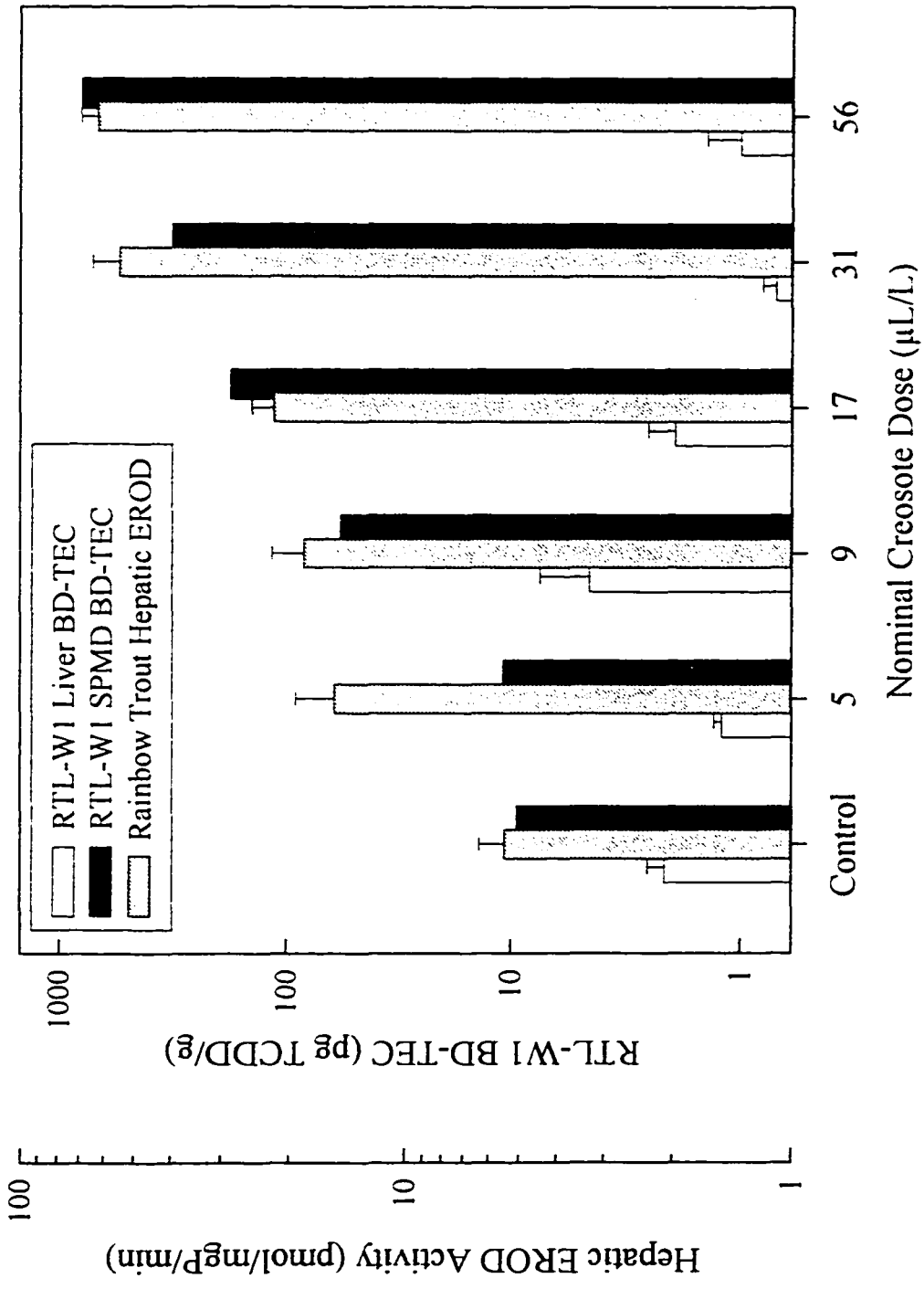
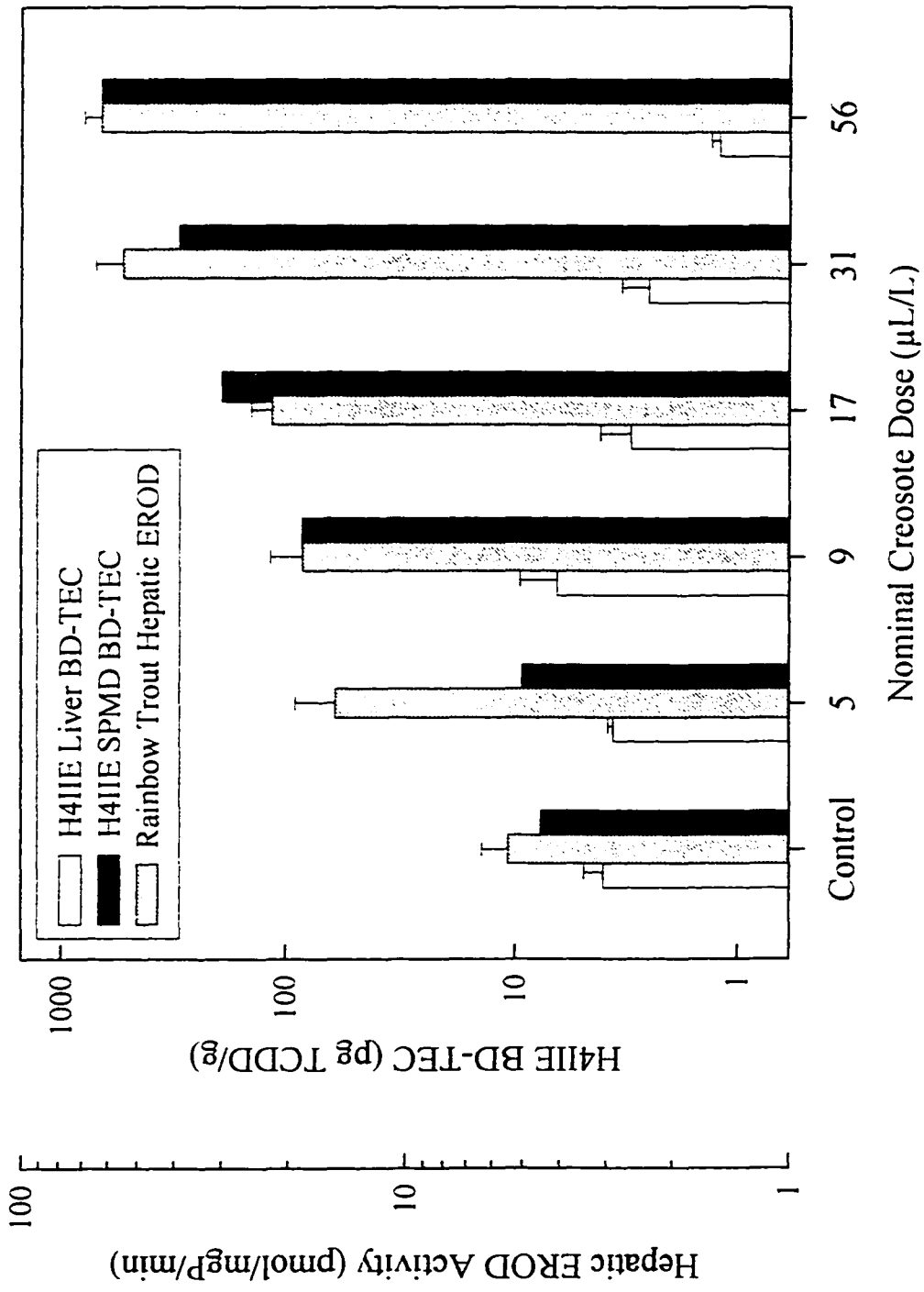

RTL-W1 Liver BD-TEC
RTL-W1 SPMD BD-TEC
Rainbow Trout Hepatic EROD
RTL-W1 BD-TEC (pg TCDD/g)
1000
100
10
1
Hepatic EROD Activity (pmol/mgP/min)
100
10
1
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)

Figure 2.6. A comparison of the effects of a 28 day exposure to microcosms of differing nominal creosote doses on rainbow trout hepatic EROD activity (cross-hatched bar) and on H4IIE bioassay-derived (BD) 2,3,7,8-TCDD equivalent concentrations (TECs) accumulated by rainbow trout liver (open bar) and semipermeable membrane devices (SPMDs) (dark bar). Values represent means ± S.E. with n=5 for trout and n=1 for SPMDs.


H4IIE Liver BD-TEC
H4IIE SPMD BD-TEC
Rainbow Trout Hepatic EROD
1000
100
10
1
H4IIE BD-TEC (pg TCDD/g)
100
10
1
Hepatic EROD Activity (pmol/mgP/min)
Control
5
9
17
31
56
Nominal Creosote Dose (µL/L)

only exception to this pattern occurred in the control pond where the mean liver CD-TEC was higher than the SPMD CD-TEC.

G. Hepatic EROD activity

EROD activity in rainbow trout liver extracts was determined and related to different measures of PPPAH exposure. Because BD-TECs encompass all extracted EROD inducers, it was felt that these values would most closely resemble the *in vivo* response. Hepatic EROD activity was compared to BD-TECs that had been derived with either RTL-W1 in Figure 2.5 or H4IIE in Figure 2.6. In both cases mean levels of hepatic EROD showed a relationship with BD-TECs in SPMDs (RTL-W1 $r^2 = 0.811$, $p<0.05$; H4IIE $r^2 = 0.836$, $p<0.05$). In contrast, no correlation was seen between RTL-W1 BD-TECs in trout liver and hepatic EROD, and a weak negative correlation was determined when hepatic EROD was compared to H4IIE trout liver BD-TECs ($r^2 = 0.159$, $p<0.05$). Hepatic EROD was found to rise significantly as nominal creosote dose increased ($y = 0.97x + 7.27$, $r^2 = 0.827$, $p<0.05$, $n=30$). One way ANOVA revealed that all treatments were significantly different from the control pond ($p<0.05$, $n=30$), and that only ponds 5 versus 9 and 9 versus 17 did not differ significantly.

2.5 DISCUSSION

This study compares the uptake of PPPAHs from creosote-dosed microcosms by SPMDS and rainbow trout, and uses mammalian and teleost liver cell lines to estimate the toxic potential of these bioavailable PAHs. The results indicate dramatic differences

in both the types and concentrations of PPPAHs sequestered in the two matrices, with SPMDs accumulating higher levels of analytes compared to the fish. The difference is mainly due to biotransformation of accumulated PPPAHs in the trout, as is inferred from elevated levels of trout hepatic EROD activity with increasing creosote dose. TECs in SPMDs derived using both rat and trout cell cultures indicate the presence of potentially harmful PPPAH mixtures concentrated at high creosote doses. Despite increased EROD activity *in vivo*, rainbow trout liver TECs measured using the cell lines showed no relationship to creosote dose. The results demonstrate the potential of SPMDs for assessing exposure to potentially harmful bioavailable compounds that are not detected in organisms due to metabolic transformations.

A. SPMDs and PPPAH concentrations in water

Estimating water concentrations of selected PPPAHs through SPMD concentrations and a linear uptake model yielded lower values than those determined through conventional water sampling. Each method has limitations that may result in deviations from the true analyte concentration in the water column. Conventional water sample extraction using solvents does not distinguish between analytes that are truly dissolved in water, and those that are bound to colloidal particles. The high log K_{ow}s of the four PPPAHs examined increases the likelihood of their being associated with organic material in the water column, rather than existing in the dissolved form. This may explain the elevated concentration estimates determined using conventional water sampling. For instance, the high log K_{ow} of benz[a]anthracene would cause it to be preferentially associated with organic matter. If this compound dissociated from the

organic matter during the solvent extraction process of conventional water sampling, it would lead to an erroneously high dissolved concentration estimate. By excluding chemicals bound to organic matter, SPMDs can provide a better estimate of the concentration of chemical that is actually dissolved, and therefore bioavailable (Lebo et al. 1992). The other three compounds (fluorene, phenanthrene and pyrene) have lower hydrophobicities, which would reduce the degree of difference between the two modes of estimating dissolved water concentrations.

For SPMDs, the accuracy of concentrations estimated from the model will depend heavily on the accuracy of the parameters included in the model. Fluctuations in biofouling and water temperature can affect uptake of analytes into SPMDs (Huckins et al. 1993). A factor of 0.75 was used to approximate the effect of biofouling impedance. Growth of aufwuch colonies (periphyton, etc.) reduces uptake of PAHs (Lebo et al. 1992), but it is difficult to accurately predict how much of an effect this will have on the final concentration in the SPMD. In the present study, it may be that biofouling was a more significant hindrance to uptake than was approximated in the model, resulting in a lower water concentration estimate.

Temperature may also have led to differences between the two methods of estimating water concentrations. The effect of temperature on analyte sampling rate is mainly due to increased polyethylene membrane permeability to chemicals at higher temperatures (Huckins et al. 1993). Sampling rates (R_S) used for PPPAHs were derived at 10°C, while the mean water temperature in this study was 7.2°C. Examining Equation 2, it is clear that higher values for R_S at 10°C could lead to a reduced estimate of the water concentrations. Finally, the SPMD linear model predicts the average analyte

concentration over the 28 day period (Huckins et al. 1993). In contrast, water sampling in this study provided a single time point estimate of the analyte concentration. This type of measurement does not account for changes in analyte concentrations over time, unless multiple samples are taken.

The toxicological significance of the water concentration estimates can be examined by comparing them to PPPAH water quality guidelines set for protection of aquatic organisms. For example, the Canadian interim guideline for the protection of aquatic life set for phenanthrene is 0.8 μg/L (Environment Canada 1987). The conventional water sampling estimate for phenanthrene in this study is below this value by a factor of 10, while the SPMD estimate was even lower. In contrast, the concentration of pyrene estimated by conventional water analysis was above the Canadian interim guideline of 0.25 μg/L (Environment Canada 1987), but the SPMD estimate was slightly below this value. Because SPMDs can give a time-weighted estimate of water concentrations of contaminants, they may better predict the true exposure level to aquatic organisms. Future use of SPMDs in conjunction with microcosm-based toxicology studies may lead to revised water quality guidelines that are standardized based on exposure time and bioavailability of contaminants.

B. PPPAHs in SPMDs and rainbow trout liver

The total PAH concentrations were much higher in SPMDs than in rainbow trout liver extracts, and several factors likely for account for this. Metabolism is one. In the current study, metabolism did occur because PAH metabolites were detected in the bile of the rainbow trout (Lewis 1997). Metabolism would be the most likely explanation for

the higher levels in SPMDs of chrysene, benzo [b] fluoranthene, benzo [k] fluoranthene, and benzo [a] pyrene, which are PAHs whose metabolism in fish has been demonstrated or is likely (Pangrekar et al. 1995; Varanasi et al. 1989). Gale et al. (1997) observed that catfish accumulated generally lower levels of PCHs than did SPMDs, and the main differences were in lower levels of PCB 77 and 2,3,7,8-tetrachlorodibenzofuran, compounds known to be susceptible to metabolic breakdown. For other PCHs in catfish, the residue patterns in the two matrices remained very similar. The situation with PAHs is more complex as the accumulation of specific PPPAHs differed substantially, with the concentrations of some being higher in SPMDs and of others being lower.

Fluoranthene and pyrene concentrations were much higher in SPMDs than in rainbow trout livers. As well as metabolism, poor absorption of these PAHs could account for this. Niimi and Palazzo (1986) demonstrated that fluoranthene is rapidly eliminated from rainbow trout under dietary exposure, with a half-life of six days. In the same study, no reliable estimate for the half life of pyrene could be derived, although it was inferred from the response to other PAHs that it would be in the order of one or two days. For other PPPAHs, estimated half-lives are only slightly higher for fluorene and phenanthrene (7-9 days), but they were detected in hepatic tissue in this study, albeit at low levels.

Two PAHs, anthracene and benz[a]anthracene, were extremely low or absent in SPMDs but were found consistently in liver. A possible explanation for this is photodegradation, which has been well documented for PAHs (Low et al. 1987; Muel and Saguem 1985). Anthracene and benz[a]anthracene have short half lives under exposure to sunlight (Lyman 1995) and the transparent nature of SPMDs could leave

compounds susceptible to photolysis. Although precautions were taken to guard the devices from light exposure, penetration did occur during early morning and late afternoon hours. By contrast, once taken up by fish, these compounds are effectively shielded from photolytic degradation. This may explain the lack of relationship between the concentrations of anthracene and benz[a]anthracene in liver and the creosote dose to which the fish were exposed.

Generally, the smallest (naphthalene and acenaphthylene) and the largest (dibenz [a,h] anthracene, benzo [a,h] anthracene and benzo [g,h,i] perylene) PPPAHs did not accumulate in either matrix. Reduced uptake of higher molecular weight compounds is likely due to negligible movement across gill/polyethylene membranes caused by steric hindrance of multiple benzene rings (Meador et al. 1995; Lebo et al. 1992). In addition, the low water solubility and high K_{ow}s for these compounds indicate that they were likely strongly bound to the microcosm sediment. The absence of low molecular weight compounds in samples may be due to their high volatility, effectively eliminating them from the microcosms before the experiment began. Studies with fish have shown that elimination through gills is possible for PAHs possessing two or three aromatic rings (Thomas and Rice 1992), which may explain the absence of naphthalene, acenaphthylene, and acenaphthene in rainbow trout liver. Alternatively, potential loss may have occurred during the analytical procedures. Samples were stored frozen for a period of three months prior to chemical analysis. Lengthy cold storage of tissue can result in significant loss of more volatile PAHs (Moring and Rose 1997).

C. Estimating TECs with RTL-W1 vs H4IIE

For the SPMD extracts, the application of H4IIE TEFs yielded higher CD-TECs than did RTL-W1 TEFs, but the BD-TECs were similar with the use of either H4IIE or RTL-W1 cells in the bioassay. At least two very different explanations could account for H4IIE and RTL-W1 CD-TECs being different, and the H4IIE and RL-W1 BD-TECs being similar. One is that the difference between H4IIE and RTL-W1 CD-TECs is methodological. The higher H4IIE CD-TECs is due to the relatively large amounts of benzo[b]fluoranthene and benzo[k]fluoranthene in the SPMD extracts and the greater potency (TEFs) of these two PAHs in H4IIE than in RTL-W1. However, the difference between RTL-W1 and H4IIE TEFs for these two PAHs could have arisen from different methodologies rather than a fundamental difference in the response of RTL-W1 and H4IIE to these two PAHs. This is because the methodology for TEF derivation with RTL-W1 (Bols et al. 1997a) and for the bioassay (this study) with RTL-W1 and H4IIE was the same but different from the TEF derivation with H4IIE (Willett et al. 1997). A second possibility is that H4IIE and RTL-W1 respond differently to PAH mixtures and that during the bioassay of SPMD extracts, a less-than-additive response occurs with H4IIE.

For liver extracts, the CD-TECs were higher with RTL-W1 TEFs than with H4IIE TEFs, but the BD-TECs were higher with H4IIE than RTL-W1. Among the CD-TECs, the difference was entirely due to the TEF for benz[a]anthracene being larger in RTL-W1, as this was the only AhR-active PAH in the liver extracts. Among the BD-TECs, the higher values with H4IIE are open to speculation. One possibility is the presence in the

rainbow trout liver extracts of compound(s) that inhibit EROD induction preferentially in RTL-W1.

D. SPMD TECs versus rainbow trout liver TECs

SPMDs accumulated a significantly higher level of potentially toxic chemicals (AhR-active) than the rainbow trout, as is indicated by the higher BD- and CD-TECs. The failure of the rainbow trout livers to accumulate significant amounts of AhR-active compounds was likely due to the metabolism of these compounds by the fish rather than the failure of the livers to be exposed to AhR-active compounds. Three lines of evidence indicate that the AhR-active compounds were taken up by the fish in a dose-dependent manner. As the nominal creosote dose increased, hepatic EROD activity in the trout rose dramatically. Increased CYP1A activity in the liver has been established as a reliable indicator of exposure to increasing levels of PAHs in aquatic environments (Stegeman and Lech 1991; Munkittrick et al. 1995). The second indicator of PAH accumulation in the trout were increased levels of biliary PAH metabolites detected with higher doses of creosote. Because the metabolites were measured as 1-pyrenol equivalents, it cannot be conclusively stated that the parent compounds were all EROD inducers, but many AhR-active PAHs can serve as substrates for CYP1A metabolism (Smolowitz et al. 1992). This is supported in this study by the fact that biliary metabolite concentration and hepatic EROD activity were significantly correlated (Lewis 1997). Finally, as nominal creosote dose increased, both hepatic EROD activity in the trout and BD-TECs in the SPMDs increased and were strongly correlated with each other, which suggests that the

fish were responding to a suite of EROD-inducing chemicals similar to the suite accumulated by the SPMDs.

E. BD-TECs versus CD-TECs

For the SPMD extracts, the RTL-W1 BD-TECs were similar to the CD-TECs that had been calculated with RTL-W1 TEFs, but the H4IIE BD-TECs usually were lower than the CD-TECs that had been calculated with H4IIE TEFs. The similarity of the RTL-W1 BD-TECs and CD-TECs implies that most of the AhR-active compounds have been accounted for in these extracts and that these PAHs act in additive manner to induce EROD activity in RTL-W1. For H4IIE, the fact that CD-TECs were higher than the BD-TECs was possibly due to differences in the methodology of H4IIE TEF determination, as done by Willett et al. (1997), and of the H4IIE bioassay, as done in this study.

For the liver extracts, two different comparisons arose. In liver extracts from fish that had been in mesocosms with nominal creosote doses of 17 and 30 μL, a profound difference was found between BD-TECs and CD-TECs. No AhR-active PAH was identified in these extracts, yet the extracts induced EROD activity in both H4IIE and RTL-W1 bioassays. This has a number of possible interpretations. One is that the levels of individual AhR-active PAHs is too low to be detected chemically but when summed together, are adequate to induce EROD activity. Another is that liver of these fish had accumulated an AhR-active compound(s), which was not identified in the HPLC analysis of this study. This possibility is difficult to rule out due to the large number of compounds in creosote and differences in composition between commercial mixtures (Mueller et al. 1989). Although the exact composition of the creosote mixture employed

in this study is not known, classes of contaminants such as PCDDs and PCDFs have been detected at creosote contaminated sites (Copeland et al. 1993). Other potential EROD inducing compounds may have entered the microcosms from atmospheric sources during the months prior to the initiation of the present study. The strength of comparing TECs determined through chemical and biological analysis is that deviations from additivity can be detected, but whether the deviation is due to the presence of unidentified CYP1A inducers and the possible sources of these compounds deserve further exploration.

In liver extracts from fish that had been in the other microcosms, the RTL-W1 CD-TECs were higher than the RTL-W1 BD-TECs. As mentioned previously, this could have been due to compounds in the liver extract that impaired EROD induction in RTL-W1.

F. Assessment strategy

The results of this study support an integrated, three step approach for assessing the exposure of fish to PAHs in creosote contaminated environments. The steps were the use of SPMDs and chemical analysis to measure the PAHs that could be potentially accumulated by fish, a cell bioassay to evaluate the toxic potential of the accumulated PAHs to fish, and hepatic EROD activity in fish to indicate that fish actually had been exposed to potentially toxic PAHs. Each step alone could be suggestive, but together they provide strong evidence that the PAHs of a creosote contaminated environment had an impact on fish. The individual steps had strengths and weaknesses.

The strength of SPMDs was that they accumulated increasing amounts of PAHs from microcosms dosed with increasing levels of creosote, whereas the amounts

accumulated by fish in the same environment were low and showed little change with creosote dose. As well, SPMDs allowed water concentrations to be calculated. Despite these benefits, SPMDs did have limitations. For example, SPMDs do not mimic bioaccumulation, where contaminants are accumulated by ingestion of food and organic matter (Lebo et al. 1992). Feeding in fish can influence uptake of PAHs (Collier and Varanasi 1991) and will depend on the concentration of contaminant in the food source and the efficiency of absorption for individual chemicals. In the present study, uptake in trout via this method is assumed to be negligible, because trout consumed food rapidly during feeding periods, not allowing PAHs to partition into the food source. In fish collected from the wild, feeding histories are hard to define and may contribute significantly to PPPAH body burdens.

Either piscine (RTL-W1) or mammalian (H4IIE) liver cell lines could be used to evaluate the toxic potentials of accumulated PAHs, which were expressed as both CD-TECs and BD-TECs. The use of RTL-W1 or H4IIE did lead to different TECs. However, the magnitude of the differences was not great, and the occurrence of differences depended on whether SPMD or liver extracts were being examined. At this time, the dependence of these differences on subtle differences in the AhR signal transduction system and/or PAH metabolism between fish and mammals is unknown. However, RTL-W1 might be more appropriate than H4IIE for assessing the toxic impact of PAHs to rainbow trout because potentially the different TECs might reflect an underlying mechanistic difference between fish and mammals. Comparing CD-TECs and BD-TECs revealed whether all the AhR-active compounds had been identified in SPMD and liver extracts.

Hepatic EROD activity provided a quick, convenient indication that the fish had been exposed to AhR-active chemicals. The levels of hepatic EROD activity were progressively elevated in fish from microcosms dosed with increasing amounts of creosote and correlated with SPMD TECs from the same microcosms. The expression of EROD activity can be modified by a variety of factors, including temperature, nutrition and age (Bucheli and Fent 1995). The use in this study of standardized microcosms and fish of similar size, age and sex eliminated many of these variables. Thus interpretation of EROD activity in field studies likely would be more complex than in microcosms.

Time is likely a critical parameter for this three step approach. The current study has involved a fixed exposure period of 28 days. However, PAH accumulation in SPMDs and hepatic EROD activity would be expected to vary with exposure period. Thus the true utility of the approach will require establishing a time frame for these events, and this is currently under investigation.

2.6 ACKNOWLEDGMENTS

Funding for this study was provided by the Canadian Network of Toxicology Centres (CNTC) and the Natural Sciences and Engineering Research Council of Canada (NSERC). The authors wish to thank Sonia Magdic, Brendan McConkey, and Janet Turner for their technical assistance and Jim Huckins for providing SPMD sampling rate data for PPPAHs. Special thanks to Dr. K.R. Solomon and the CNTC for consenting to our use of the microcosm study area.

2.7 REFERENCES

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

Bankey, L.A., Van Veld, P.A., Borton, D.L., LaFleur, L., and Stegeman, J.J. 1994. Responses of cytochrome P4501A in freshwater fish exposed to bleached kraft mill effluent in experimental stream channels. Canadian Journal of Fisheries and Aquatic Sciences. 52: 439-447.

Barnes, D.G. 1991. Toxicity equivalents and EPA's risk assessment of 2,3,7,8-TCDD. The Science of the Total Environment. 104: 73-86.

Bestari, K.T., Robinson, R.D., Solomon, K.R., and Day, K.E. 1996. Quantification of 15 priority PAHs in creosote contaminated water and sediments. SETAC 17th Annual Meeting, Washington, D.C. 1996.

Bols, N.C., Schirmer, K., Heikkila, E.M., Greenberg, B.M., and Dixon, D.G. 1997a. Ability of intact and photomodified PAHs to induce 7-ethoxyresorufin-*O*-deethylase (EROD) activity in a rainbow trout liver cell line. Annual Research Symposium of the Canadian Network of Toxicology Centers, Toronto, ON, Canada, March 23-25, 1997.

Bols, N.C., Whyte, J.J., Clemons, J.H., Tom, D.J., van den Heuvel, M.R., and Dixon, D.G. 1997b. Use of liver cell lines to develop TCDD equivalency factors and TCDD equivalent concentrations in environmental samples. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment. J.T. Zelikoff, ed. SOS Publishing, Fair Haven, NJ, 1st ed.: pp. 329-350.

Borthwick, P.W. and Patrick, J.M., Jr. 1982. Use of aquatic toxicology and quantitative chemistry to estimate environmental deactivation of marine-grade creosote in seawater. Environmental Toxicology and Chemistry. 1: 281-288.

Bucheli, T.D. and Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25: 201-268.

Clemons, J.H., Lee, L.E.J., Myers, C.R., Dixon, D.G., and Bols, N.C. 1996. Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines from rat and trout and the derivation of toxic equivalency factors. Canadian Journal of Fisheries and Aquatic Sciences. 53: 1177-1185.

Clemons, J.H., Dixon, D.G., and Bols, N.C. 1997. Derivation of 2,3,7,8-TCDD toxic equivalent factors (TEFs) for selected dioxins, furans, and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. Chemosphere. 34: 1105-1119.

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

Copeland, T.L., Paustenbach, D.J., Harris, M.A., and Otani, J. 1993. Comparing the results of a Monte Carlo analysis with EPA's reasonable maximum exposed individual (RMEI): a case study of a former wood treatment site. Regulatory Toxicology and Pharmacology. 18: 275-312.

Donato, T.M., Castell, J.V., and Gómez-Lechón, M.J. 1992. A rapid and sensitive method for measuring monooxygenase activities in hepatocytes cultured in 96-well plates. Journal of Tissue Culture Methods. 14: 153-158.

Eadon, G., Kaminsky, L., Silkworth, J., Aldous, K., Hilker, D., O'Keefe, P., Smith, R., Gierthy, J., Hawley, J., Kim, N., and DeCaprio, A. 1986. Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. Environmental Health Perspectives. 70: 221-227.

Environment Canada. 1987. Task force on water quality guidelines of the Canadian Council of Resource and Environment Ministers. Ottawa, Canada.

Firestone, D. 1991. Determination of dioxins and furans in foods and biological tissues: review and update. Journal of the Association of Official Analytical Chemists. 74: 375-384.

Gale, R.W., Huckins, J.N., Petty, J.D., Peterman, P.H., Williams, L.L., Morse, D., Schwartz, T.R., and Tillitt, D.E. 1997. Comparison of the uptake of dioxin-like compounds by caged channel catfish and semipermeable membrane devices in the Saginaw River, Michigan. Environmental Science and Technology. 31: 178-187.

Government of Canada, Environment Canada, and Health and Welfare Canada. 1993. Creosote-impregnated waste materials. In: Canadian Environmental Protection Act. AnonymousMinister of Supply and Services, Canada, pp. 1-25.

Graney, R.L., Giesy, J.P., and Clark, J.R. 1995. Field studies. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 493-521.

Hargis, W.J., Roberts, M.R., and Zwerner, D.E. 1984. Effects of contaminated sediments and sediment-exposed effluent water on an estuarine fish: acute toxicity. Marine Environmental Research. 14: 337-354.

Huckins, J.N., Manuweera, G.K., Petty, J.D., Mackay, D., and Lebo, J.A. 1993. Lipid-containing semipermeable membrane devices for monitoring organic contaminants in water. Environmental Science and Technology. 27: 2489-2496.

Huckins, J.N., Petty, J.D., Lebo, J.A., Orazio, C.E., Prest, H.F., Tillitt, D.E., Ellis, G.S., Johnson, B.T., and Manuweera, G.K. 1996. Semipermeable membrane devices (SPMDs) for the concentration and assessment of bioavailable organic contaminants in aquatic environments. In: Techniques in Aquatic Toxicology. G.K. Ostrander, ed. CRC Press, Boca Raton, Florida, 1st ed.: pp. 625-655.

Huestis, S.Y., Servos, M.R., Sergeant, D.B., Leggett, M., and Dixon, D.G. 1995. Methods for determination of organochlorine pesticides, polychlorinated biphenyl congeners and chlorinated dibenzo-*p*-dioxins and furans in fish. Canadian Technical Report on Fisheries and Aquatic Sciences. 2044: 1-30.

Kennedy, S.W., Lorenzen, A., James, C.A., and Collins, B.T. 1993. Ethoxyresorufin-*0*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. Analytical Biochemistry. 211: 102-112.

Kennedy, S.W., Lorenzen, A., and Norstrom, R.J. 1996. Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. Environmental Science and Technology. 30: 706-715.

Kennedy, S.W. and Jones, S.P. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Analytical Biochemistry. 222: 217-223.

Kutz, F.W., Barnes, D.G., Bottimore, D.P., Greim, H., and Bretthauer, E.W. 1990. The international toxicity equivalency factors (I-TEF) method of risk assessment for complex mixtures of dioxins and related compounds. Chemosphere. 20: 751-757.

Lebo, J.A., Zajicek, J.L., Huckins, J.N., Petty, J.D., and Peterman, P.H. 1992. Use of semipermeable membrane devices for in situ monitoring of polycyclic aromatic hydrocarbons in aquatic environments. Chemosphere. 25: 697-718.

Lebo, J.A., Zajicek, J.L., Orazio, C.E., Petty, J.D., Huckins, J.N., and Douglas, E.H. 1996. Use of semipermeable membrane device (SPMD) to sample polycyclic aromatic hydrocarbon pollution in a lotic system. Polycyclic Aromatic Compounds. 8: 53-65.

Lee, L.E.J., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K., Pasitschniak-Arts, M., Mosser, D.D., and Bols, N.C. 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biology and Toxicology. 9: 279-294.

Lewis, J. 1997. Bioindicators of polycyclic aromatic hydrocarbon exposure in rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*). M.Sc. Thesis. University of Guelph. Guelph, Ontario, Canada. 1-81.

Lorenzen, A. and Kennedy, S.W. 1993. A fluorescence-based protein assay for use with a microplate reader. Analytical Biochemistry. 214: 346-348.

Low, G.K., Batley, G.E., and Brockbank, C.I. 1987. Solvent-induced photodegradation as a source of error in the analysis of polycyclic aromatic hydrocarbons. Journal of Chromatography. 392:199-210: 199-210.

Lyman, W.J. 1995. Transport and transformation processes. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 449-492.

Mátlová, L., Machala, M., Nezveda, K., Granátová, M., and Nevoránková, Z. 1995. Biochemical screening of highly toxic aromatic contaminants in river sediment and comparison of sensitivity of biological model systems. Chemosphere. 30: 1363-1371.

McConkey, B.J., Duxbury, C.L., Dixon, D.G., and Greenberg, B.M. 1997. Toxicity of a PAH photooxidation product to the bacteria *photobacterium phosphoreum* and the duckweed *lemna gibba*: effects of phenanthrene and its primary photoproduct, phenathrenequinone. Environmental Toxicology and Chemistry. 16: 892-899.

Meador, J.P., Stein, J.E., Reichert, W.L., and Varanasi, U. 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Reviews in Environmental Contamination and Toxicology. 143:79-165: 79-165.

Meadows, J., Tillitt, D., Huckins, J., and Schroeder, D. 1993. Large-scale dialysis of sample lipids using a semipermeable membrane device. Chemosphere. 26: 1993-2006.

Moring, J.B. and Rose, D.R. 1997. Occurrence of polycyclic aromatic hydrocarbons in semipermeable membrane devices and clams in three urban streams of the Dallas-Forth Worth Metropolitan Area, Texas. Chemosphere. 34: 551-566.

Muel, B. and Saguem, S. 1985. Determination of 23 polycyclic aromatic hydrocarbons in atmospheric particulate matter of the Paris area and photolysis by sunlight. International Journal of Environmental Analytical Chemistry. 19: 111-131.

Mueller, J.G., Chapman, P.J., and Pritchard, P.H. 1989. Creosote-contaminated sites: their potential for bioremediation. Environmental Science and Technology. 23: 1197-1201.

Munkittrick, K.R., Blunt, B.R., Leggett, M., Huestis, S., and McCarthy, L.H. 1995. Development of a sediment bioassay to determine bioavailability of PAHs to fish. Journal of Aquatic Ecosystem Health. 4: 169-181.

Myers, M.S., Landahl, J.T., Krahn, M.M., Johnson, L.L., and McCain, B.B. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology. I: Pathology and epizootiology. The Science of the Total Environment. 94: 33-50.

Neubert, D., Golor, G., and Neubert, R. 1992. TCDD-toxicity equivalencies for PCDD/PCDF congeners: prerequisites and limitations. Chemosphere. 25: 65-70.

Niimi, A.J. and Palazzo, V. 1986. Biological half-lives of eight polycyclic aromatic hydrocarbons (PAHs) in rainbow trout (*Salmo gairdneri*). Water Research. 20: 503-507.

Okey, A.B., Riddick, D.S., and Harper, P.A. 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Toxicology Letters. 70: 1-22.

Pangrekar, J., Kandaswami, C., Kole, P., Kumar, S., and Sikka, H.C. 1995. Comparative metabolism of benzo[a]pyrene, chrysene and phenanthrene by brown bullhead liver microsomes. Marine Environmental Research. 39: 51-55.

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

Piskorska-Pliszczynska, J., Keys, B., Safe, S., and Newman, M.S. 1986. The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. Toxicology Letters. 34: 67-74.

Prest, H.F., Jarman, W.M., Burns, S.A., Weismuller, T., Martin, M., and Huckins, J.N. 1992. Passive water sampling via semipermeable membrane devices (SPMDs) in concert with bivalves in the Sacramento/San Joaquin River Delta. Chemosphere. 25: 1811-1823.

Roberts, M.H., Hargis, W.J., Jr., Strobel, C.J., and DeLisle, P.F. 1989. Acute toxicity of PAH contaminated sediments to the estuarine fish, *Leiostomus xanthurus*. Bulletin of Environmental Contamination and Toxicology. 42: 142-149.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology. 21: 51-88.

Safe, S.H. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Reviews in Toxicology. 24: 87-149.

Schmalix, W.A., Maser, H., Kiefer, F., Reen, R., Wiebel, F.J., Gonzalez, F., Seidel, A., Glatt, H., Greim, H., and Doehmer, J. 1993. Stable expression of human cytochrome P450 1A1 cDNA in V79 Chinese hamster cells and metabolic activation of benzo[a]pyrene. European Journal of Pharmacology. 248: 251-261.

Schoor, W.P., Williams, D.E., and Takahashi, N. 1991. The induction of cytochrome P-450-IA1 in juvenile fish by creosote-contaminated sediment. Archives of Environmental Contamination and Toxicology. 20: 497-504.

Smolowitz, R.M., Schultz, M.E., and Stegeman, J.J. 1992. Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis* spp.) by waterborne benzo[a]pyrene. Carcinogenesis. 13: 2395-2402.

Stegeman, J.J. and Hahn, M.E. 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. CRC Press, Boca Raton, Florida, pp. 87-206.

Stegeman, J.J. and Lech, J.J. 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environmental Health Perspectives. 90: 101-109.

Thomas, R.E. and Rice, S.D. 1992. Metabolism and clearance of phenolic and mono- di-, and polynuclear aromatic hydrocarbons by Dolly Varden char. In: Physiological mechanisms of marine pollutant toxicity. J. Vernberg, A. Calabrese, and T.P. Thurberg, eds. Academic Press, New York, pp. 161-176.

Thompson, D.G., Holmes, S.B., Pitt, D.G., Solomon, K.R., and Wainio-Keizer, K.L. 1994. Applying concentration-response theory to aquatic enclosure studies. In: Aquatic mesocosm studies in ecological risk assessment. R.L. Graney, J.H. Kennedy, and J.H. Rodgers, Jr. eds. Lewis Publishers, Boca Raton, Fl. pp. 129-156.

Tillitt, D.E., Ankley, G.T., Verbrugge, D.A., Giesy, J.P., Ludwig, J.P., and Kubiak, T.J. 1991a. H4IIE rat hepatoma cell bioassay-derived 2,3,7,8- tetrachlorodibenzo-*p*-dioxin equivalents in colonial fish-eating waterbird eggs from the Great Lakes. Archives of Environmental Contamination and Toxicology. 21: 91-101.

Tillitt, D.E., Giesy, J.P., and Ankley, G.T. 1991b. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environmental Science and Technology. 25: 87-92.

Van Veld, P.A., Vogelbein, W.K., Smolowitz, R., Woodin, B.R., and Stegeman, J.J. 1992. Cytochrome P450IA1 in hepatic lesions of a teleost fish (*Fundulus heteroclitus*) collected from a polycyclic aromatic hydrocarbon-contaminated site. Carcinogenesis. 13: 505-507.

Varanasi, U., Stein, J.E., and Nishimoto, M. 1989. Biotransformation and disposition of polycyclic aromatic hydrocarbons (PAH) in fish. In: Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. U. Varanasi, ed. CRC Press, Boca Raton, FL,

Willett, K.L., Gardinali, P.R., Sericano, J.L., Wade, T.L., and Safe, S.H. 1997. Characterization of the H4IIE rat hepatoma cell bioassay for evaluation of environmental samples containing polynuclear aromatic hydrocarbons (PAHs). Archives of Environmental Contamination and Toxicology. 32: 442-448.

Zitko, V. 1993. Patterns of polynuclear aromatic hydrocarbons (PAH) in sediments. Central European Journal of Public Health. 1: 125-129.

CHAPTER 3

THE USE OF CREOSOTE CONTAMINATED MICROCOSMS AND SEMIPERMEABLE MEMBRANE DEVICES (SPMDs) TO EVALUATE THE TIME-DEPENDENT UPTAKE AND POTENTIAL EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH) IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

3.1 ABSTRACT

This study examined the time-dependent uptake of priority pollutant polycyclic aromatic hydrocarbons (PPPAHs) in both semipermeable membrane devices (SPMDs) and rainbow trout exposed to creosote-dosed microcosms. SPMDs rapidly accumulated PPPAHs over time while rainbow trout maintained steady, low levels throughout the exposure period. In contrast, rainbow trout hepatic EROD was elevated by the first collection point, indicating exposure to CYP1A inducing compounds. It is hypothesized that this elevated activity lead to the metabolism and elimination of PPPAHs from the rainbow trout livers. A rainbow trout liver cell line, RTL-W1, was exposed to PPPAH extracts from both SPMDs and trout livers to determine bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations (BD-TECs). BD-TECs followed the levels of chemical concentrations measured in both matrices. Using RTL-W1 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalency factors (TEFs), chemically-derived (CD) TECs were determined for SPMDs and trout livers. Elevated BD-TECs compared to CD-TECs suggests the presence of an unknown EROD inducing compound(s) in the

microcosms. This study demonstrates the importance of examining PPPAH uptake over time, its relationship to biological response, and the utility of a multilevel analysis for a more complete representation of the actual events that occur upon chemical exposure.

3.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a diverse class of compounds with two or more fused benzene rings, and often contain alkyl-side groups (Meador et al. 1995). Due to their hydrophobicity, they are readily bioaccumulated, and have demonstrated toxic effects on aquatic organisms (Malins et al. 1985; Van Veld et al. 1992; Hargis et al. 1984; Roberts et al. 1989). In vertebrate species, some PAHs can bind to a cytosolic arylhydrocarbon receptor (AhR) and induce cytochrome P450 monooxygenases such as CYP1A (Stegeman and Hahn 1994). The rapid metabolism of several PAHs by these enzymes can make estimating the degree of contaminant exposure and accumulation difficult (Bucheli and Fent 1995). In the present study, rather than simply taking a "chemical snapshot" of PAH accumulation in fish, a three step approach is used to examine the effect of exposure period on uptake of PAHs by rainbow trout (*Oncorhynchus mykiss*). These three steps include estimations of the degree of PAH bioconcentration by the fish, the potential toxicity of the accumulated chemical mixture, and the *in vivo* response to PAH exposure.

Creosote is a coal distillation product consisting of a complex mixture of non-, alkyl- and/or alkoxy-substituted phenolic compounds, a major component (85%) being PAHs (Ogata et al. 1995). Creosote is widely used as a wood preservative and currently, 90% of the production of creosote goes to pressure treatment of railroad ties, poles and marine pilings (Von Burg and Stout 1992). As a result, the leaching of creosote has resulted in its

discovery in soil, groundwater and some aquatic habitats (Mueller et al. 1989). Based on the available data, it is not clear whether PAHs in creosote are entering these environments in quantities sufficient to cause harmful effects (Government of Canada et al. 1993). In this study, an exposure of rainbow trout to creosote contaminated waters was conducted in experimental microcosms, or "model ecosystems" (Graney et al. 1995). Microcosms simulate some of the complexities of a natural ecosystem while maintaining a degree of control close to that of laboratory exposures.

Because PAHs in creosote can be quickly metabolized by trout upon their uptake (Chapter 2), other methods of estimating the degree of chemical accumulation are needed. To assess the level of bioavailable contaminants present in an aquatic environment, Huckins and coworkers designed a lipid-containing semipermeable membrane device (SPMD) that would mimic the bioconcentration process by passively sampling truly dissolved hydrophobic contaminants (Huckins et al. 1996). The device consists of a thin film of neutral lipid (triolein) sealed in a section of lay-flat polyethylene tubing (Prest et al. 1992). Hydrophobic contaminants move through the polyethylene membrane via passive diffusion and partition into the lipid where they are concentrated. In contrast to gill membranes, SPMD polyethylene membranes are not altered in response to stress (Gale et al. 1997) and PAHs sequestered in the triolein phase are not subject to metabolic transformations that occur in fish (Moring and Rose 1997). SPMDs are ideal for use in time-course studies since they give an estimate of the *average* level of contaminants concentrated over the period of exposure. Conventional water sampling methods only indicate the concentration of chemicals in the water at a specific point in time, and in addition, they may include

measurements of chemicals which are not in a truly dissolved form (e.g. bound to particulate matter) (Huckins et al. 1993).

Although a measure of the bioavailable PAHs in a creosote-contaminated environment is useful, a second issue complicating the risk assessment of exposure is the large number of individual PAHs in creosote, each with differing toxic potencies. One potential method of addressing the problem of complex chemical mixtures is the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalent factor (TEF) approach (Eadon et al. 1986). TEFs estimate the potency of a compound relative to the TCDD, which is the most toxic planar chlorinated hydrocarbon (PCH) and is assigned a TEF value of one. A TEF converts the concentration of the compound to the concentration of TCDD that would produce the same response (Safe 1990). Thus, for a complex environmental mixture such as creosote, the concentrations of the individual PAHs can be expressed as TCDD equivalent concentrations (TEC) and added together to give a single TEC for the mixture. Because the TECs determined using this method rely on analyte measurements in samples, they are sometimes referred to as chemistry-derived TECs (CD-TECs). The derivation of TEFs has been done through toxicity tests that monitor responses usually mediated by the AhR. A common response has been the induction of ethoxyresorufin-*O*-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) activity in mammalian liver cell lines (Safe 1987; Tillitt et al. 1991b). Recently, TEFs for five EROD inducing PAHs have been derived with a rainbow trout liver cell line, RTL-W1 (Bols et al. 1997a). Subtle differences in EROD induction by AhR-active contaminants has been shown between mammalian and teleost cell lines (Chapter 1 and 2). As a result, RTL-W1 was chosen as the cell line that would best represent the *in vivo* response to EROD inducing chemicals in rainbow trout for this study.

EROD induction in RTL-W1 also can be used to evaluate the level of AhR-active compounds in environmental samples (Bols et al. 1997b). Compounds of interest are extracted from samples and applied to cell cultures. Their ability to induce EROD activity is measured and expressed as TCDD equivalent concentrations. Such cell bioassays integrate the potency of all the compounds in the sample. Although the chemical identity of the compounds in the sample is unknown, knowledge of the compounds that are potentially capable of inducing EROD activity in the cells aids in the application and interpretation of the cell bioassays. For this reason, TECs determined in this manner are called bioassay-derived TECs (BD-TECs).

In addition to *in vitro* measurements, knowledge of whether a chemical contaminant induces EROD activity *in vivo* can be used in environmental toxicology in a number of ways. In fish, EROD has been shown to be a catalytic measure of CYP1A1 (Stegeman 1989), and recently, also of CYP1A2 (Gooneratne et al. 1997). In rainbow trout, these two proteins, which have been renamed CYP1A3 and CYP1A1 (Nelson et al. 1996), appear to be so closely related that they are difficult to distinguish catalytically or physically (Gooneratne et al. 1997). As in higher vertebrates, induction of CYP1A in fish involves the aryl-hydrocarbon receptor (AhR) (Sadar et al. 1996), which is thought to mediate the toxicity of dioxin-like compounds to animals in general (Okey et al. 1994). Thus, at the very minimum, induction indicates one pathway of action at the cellular level and a possible route to the expression of toxicity.

By integrating the use of SPMDs, the TEC model, and *in vivo* EROD measurements in rainbow trout, this study examines the importance of exposure time on the uptake and biological activity of compounds known to be rapidly metabolized. This was accomplished

by co-exposing rainbow trout and SPMDs in standardized creosote contaminated microcosms for 0, 7, 14, 21 and 28 day periods followed by chemical analysis of trout livers and SPMD lipid for 16 priority pollutant PAHs (PPPAHs) (Callahan et al. 1979) and five non-priority aromatic compounds. These extracts were also used to derive and compare RTL-W1 CD- and BD-TECs in both matrices. Finally, hepatic EROD activity was determined in the trout at each time point to compare the chemical and *in vitro* measurements with the *in vivo* response to PAH exposure.

3.3 MATERIALS AND METHODS

A. Experimental Microcosms

The Canadian Network of Toxicology Centres' (CNTC) outdoor microcosm facility, located in Guelph, Ontario, consists of 30 freshwater ponds used for testing the impact and effects of chemicals and substances on fresh-water organisms. They consist of steel ponds lined with PVC are 1.05 m deep with a surface area of 11.95 m^2 and contain 12000 L of freshwater. To control fluctuations in water temperature, the ponds are constructed in gravel bedding. Plastic trays of sifted sediment were added to the microcosms that provided 50% coverage of the floor surface area.

In the spring 1996, liquid creosote was added to the microcosms incrementally over a period of several days. The dosing regime was based on a series of graded creosote doses with no replication, a protocol commonly referred to as the regression approach (Thompson et al. 1994). Prior to the addition of creosote, water in the ponds was circulated to a large freshwater holding pond for a four week period. Following creosote addition, water

circulation ceased, creating a static exposure system. In October 1996, three ponds of the ponds were chosen for the present study. These static system ponds had received original doses to yield final creosote concentrations in the ponds of 0, 3, and 10 µL/L. Temperature, pH, and dissolved oxygen were monitored routinely throughout the study.

B. Rainbow Trout Exposure to Creosote Microcosms

One hundred and thirty female rainbow trout obtained from Rainbow Springs Fish Hatchery (Thamesford, ON) were placed in an acclimation pond adjacent to the microcosm facility for a two week period. Trout were then weighed, tagged and randomly assigned to wooden partitioned cages with mesh screening. Ten of the trout were randomly selected for the day 0 collection point and hepatic samples were excised and preserved as is described in the next paragraph. The cages containing the remaining trout were positioned in the exposure ponds so that fish could migrate from the surface to a depth of approximately 1 m. Each partitioned section of the cages contained a maximum of two trout. Fish were fed a diet of commercial trout chow (2% body weight/day) during the study.

On the 7th day of the study, 10 trout from each of the three ponds were collected. Following removal from the pond, fish were anaesthetized with methane tricainesulfonate (MS-222) and weighed. Trout were sacrificed by cervical dislocation and livers were excised, weighed and divided into two subsamples. The first portion of the liver was wrapped in hexane-rinsed aluminium foil and stored on ice. These samples remained frozen at -20°C pending PAH extraction. The second portion was placed in a 2 mL cryovial (NUNC, Roskilde, Denmark), frozen in liquid nitrogen, and stored at -80°C until hepatic

EROD analysis. This procedure was repeated for 10 trout from each pond on the 14th, 21st, and 28th days of the study.

C. SPMD Specifications and Exposure

A total of thirty nine SPMDs composed of low-density polyethylene were obtained from Environmental Sampling Technologies (St. Joseph, Missouri). The devices contained a thin film of triolein (0.25 g, 95% purity) and had the following dimensions: width: 2.5 cm, length: 25 cm and membrane thickness: 89 μm. SPMDs were shipped to the study site in sealed tins containing pure nitrogen gas at -20°C.

Concurrent with the addition of the trout to the microcosms, SPMDs were randomly assigned to the three ponds, 12 SPMDs for each pond. The remaining three SPMDs served as the day 0 sample point and were transported back to the lab for analysis. Microcosm-exposed SPMDs were suspended equidistant from each other in the water column by nylon fishing line to a depth of 50 cm and weighted at the bottom with a 3 g galvanized anchor. Wooden extensions from the trout cages served as a suspension points for the SPMDs. The cages also provided some sunlight shading for the SPMDs to protect against possible photodegradation of accumulated PAHs. To estimate PAH uptake rate over the 28 day exposure period, three SPMDs were collected from each of the microcosms on days 7, 14, 21 and 28. Upon removal from the microcosms, SPMDs were rinsed with ethanol and wiped with paper towels to remove any aufwuch fouling on the polyethylene surface, such as algae and insect larvae (Lebo et al. 1992). The devices were then wrapped in hexane-rinsed

aluminum foil and placed on dry ice. After transport to the lab, SPMDs were stored at -80°C pending analysis.

D. Collection of Water Samples from Microcosms

Water concentrations of acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[b]fluoranthene and benzo[a]pyrene were estimated through conventional water sampling. Sample volumes of 1L were taken from microcosms on each sampling day and preserved with 80 g/L sodium thiosulphate. Compounds of interest were isolated using liquid-liquid extraction with methylene chloride. Samples were concentrated under a stream of purified N_2 and resuspended in 2 mL isooctane. Percent recovery was determined with an internal standard of bromonaphthalene with each sample. PPPAH concentrations were determined with a Varian 3400 gas chromatograph equipped with a Varian Saturn II ion trap mass spectrometer. Samples were injected onto a 30 m × 0.25 mm SPB-5 column with a stationary phase thickness of 0.25 μm at 300°C under splitless conditions. The transfer line and manifold temperatures were held constant at 260 and 250°C, respectively. Compounds were mass scanned at 45-550 m/z.

E. PAH Isolation from SPMDs and Rainbow Trout Liver

Some properties of the compounds examined in this study are shown in Table 3.1. Isolation and cleanup of chemical extracts from trout hepatic tissue and SPMDs were performed using modified methods of Huestis *et al.* (1995), Lebo *et al.* (1996) and P. Helm

(personal communication). Although hexane dialysis is an effective method of isolating hydrophobic contaminants from SPMDs (Lebo et al. 1992; Meadows et al. 1993; Huckins et al. 1993), in this study the devices were cut open at both ends using hexane rinsed scissors and lipid was washed into a flask with multiple rinses of methylene chloride (40 mL) (Prest et al. 1992). The solvent-lipid mixture was transferred to a 1 cm diameter column containing Na_2SO_4 (previously fired at 400°C) which was then washed with 300 mL methylene chloride. Using a similar method, trout liver samples (1-2 g) were weighed and ground in anhydrous Na_2SO_4. The homogenate was added to 1 cm diameter glass columns and solvent extracted with 300 mL methylene chloride. For both SPMD and tissue samples, gel permeation chromatography (GPC, Biorad SX-3 GPC resin, Richmond, CA) was used to remove lipids and other hydrophobic co-extractives. Lipid content in this fraction was estimated gravimetrically by evaporating the extract to a constant mass. The second GPC fraction, containing the PAHs of interest, was cleaned up by SPE with Florisil microcolumns (VWR Canlab, Mississauga, ON). The eluate was evaporated with N_2 gas and dissolved in 1.00 mL methylene chloride (VWR Canlab, Mississauga, ON).

Table 3.1. Properties of analytes examined in extracts from SPMDs and rainbow trout exposed to creosote dosed microcosms over a 28 day period.

Compound				SPMD sampling	RTL-W1
Priority Pollutant PAHs	MW	Rings	log Kow[1]	rate (L/d)[2]	TEF[3]
naphthalene	128.2	2	3.34	0.1	---
acenaphthylene	152.2	3	4.08	0.5	---
acenaphthene	154.2	3	4.08	0.8	---
fluorene	166.2	2	4.22	1.3	---
phenanthrene	178.2	3	4.53	2.2	---
anthracene	178.2	3	4.53	1.7	---
fluoranthene	202.3	4	5.24	nd[4]	---
pyrene	202.3	4	5.07	3.8	---
benz[a]anthracene	228.3	4	5.9	2.4	0.000067
chrysene	228.3	4	5.77	2.5	0.000052
benzo[b]fluoranthene[a]	252.3	5	6.52	1.9	0.000265
benzo[k]fluoranthene[a]	252.3	5	6.73	1.8	0.001048
benzo[a]pyrene	252.3	5	6.23	2.1	0.000329
dibenzo[a,h]anthracene	278.4	5	6.47	1.1	---
indeno[1,2,3-cd]pyrene	276.3	6	7.43	1.7	---
benzo[g,h,i]perylene	276.3	6	7.03	1.2	---
Other Aromatics					
2-methyl-naphthalene[b]	142.2	2	3.91	nd	---
indole[b]	117.2	2	2.14	nd	---
1-methyl-naphthalene	142.2	2	3.88	nd	---
biphenyl	154.2	2	3.98	nd	---
Heterocyclics					
dibenzofuran	168.2	3	3.65	nd	---
carbazole	167.2	3	3.72	nd	---

[1] log Kow (octanol-water partition coefficient) for PPPAHs from Meador *et al.* 1995; for Other Aromatics and Heterocyclics from Mueller et al. 1989.

[2] J.N. Huckins, personal communication 1997.

[3] Bols et al. 1997.

[4] nd indicates value has not been determined.

[a, b] compounds co-eluted in the applied GC method.

F. GC Analysis and Preparation of Dilution Series For Cell Line Bioassays

For determination of the concentrations of 16 PPPAHs in SPMD and trout hepatic extracts, samples were provided to Kimberly Hamilton (Department of Earth Sciences, University of Waterloo, Waterloo, Ontario). Aliquots of 3 µl were injected into a Hewlett Packard 5890 Gas Chromatograph (GC) equipped with an HP7673A autosampler and Flame Ionization Detector (FID). The temperature was held at 35°C for 2 min, followed by a 15°C/min increase to 165°C, followed by a 30°C/min increase to 300°C and a constant temperature of 300°C for 10 min. The carrier gas was helium with a flow rate of 30 ml/min. The injector temperature of the GC was 200°C, and the FID temperature was 300°C. Percent recovery was determined by extracting 0.25 g triolein (95%, Sigma, St. Louis, MO) samples spiked with a certified standard containing the PPPAHs of interest (Supelco, Bellefonte, PA). No significant contamination was detected in the procedural blanks. The limits of detection for analytes ranged from 2 to 7 ng/g lipid.

Following GC analysis, the remaining methylene chloride was evaporated with N_2 gas and dissolved in 50 µL of dimethylsulfoxide (DMSO, BDH, Darmstadt, W. Germany). A geometric dilution series of seven doses was prepared from each original extract for the cell line bioassay.

G. RTL-W1 Maintenance and Bioassay Procedure

Rainbow trout liver (RTL-W1) cells were maintained as described by Lee *et al.* (1993). Briefly, Leibovitz's L-15 (Gibco BRL, Gaithersburg, MD) was supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and penicillin/streptomycin

(100 U penicillin, 10 μg streptomycin·mL^{-1}, ICN/Flow, Costa Mesa, CA). Cells were grown at 22°C ± 1 in a pure air environment. Using a modification of the EROD bioassay of Clemons *et al.* (1996) and Kennedy *et al.* (1993), cells were plated in 48-well culture clusters (Costar, Cambridge, MA) at an initial density of 40,000 cells·$well^{-1}$ and allowed to grow for 24 h prior to dosing with the extracts. A dilution series of 2,3,7,8-TCDD in DMSO was run with every three extracts to serve as a positive control and standard for TEC determination.

Following a 24 h incubation period, the contaminated media was removed from the cells by plate inversion and the wells were rinsed with 100 μL phosphate buffered saline (PBS). The substrate, 7-ethoxyresorufin in methanol, was diluted in MEM to give a final concentration of 0.825 uM. Cells received 250 μL of the 7-ethoxyresorufin-MEM mixture and reactions were allowed to proceed for 15 min at 22 °C. Resorufin production was measured fluorometrically (excitation 530 nm, emission 590 nm) using a Cytofluor 2350 plate reading fluorometer (Perspective Biosystems, Framingham, MA). A resorufin standard curve was run with each assay to allow conversion of fluorescent units to pmoles resorufin. Cellular protein was determined as described by Lorenzen and Kennedy (1993). A bovine serum albumin (BSA, Sigma, St. Louis, MO) standard curve was run with each protein assay to allow conversion of fluorescent units to mg of protein.

H. Calculation of CD- and BD-TECs in SPMDs and Hepatic Tissue

2,3,7,8-TCDD and sample extract median effective concentrations yielding one half maximal EROD activity in the cells (EC_{50}) were determined from concentration-response curves generated using the cell line bioassay. To calculate BD-TECs in trout liver and

SPMDs, a ratio of the 2,3,7,8-TCDD EC_{50} to the extract EC_{50} was determined as described by Tillitt *et al.* (1991a):

$$\text{BD-TEC (pg TCDD/g lipid)} = \frac{EC_{50}\ \text{TCDD (pg/well)}}{EC_{50}\ \text{extract (dilution·well}^{-1}\text{)}} \times \frac{\text{extract volume (}\mu\text{L)}}{\text{dose volume (}\mu\text{L)}} \times \text{lipid mass}^{-1}\ \text{(g)}$$

For a few SPMD extracts and all rainbow trout liver extracts, concentration-response curves did not achieve the levels of EROD activity seen with 2,3,7,8-TCDD and several failed to plateau at maximal levels. For these samples, a TEC was determined by matching the highest level of EROD activity induced by the extract to the corresponding level of EROD activity in the TCDD standard, and taking that concentration of TCDD to be the TEC (corrected for dosing volume of extract). This technique follows the basic assumption of the TEC model that the concentration-response curves of the extract and 2,3,7,8-TCDD are parallel (Neubert et al. 1992).

RTL-W1 TEFs for EROD inducing PPPAHs were derived using the bioassay systems described above (Bols et al. 1997a). Concentrations of individual PPPAHs in an extract were multiplied by their respective TEFs and summed to yield a CD-TEC (Eadon et al. 1986).

I. Estimation of Aqueous Concentrations of PPPAHs from SPMD Concentrations

Huckins *et al.* (Huckins et al. 1993) have developed mathematical models and derived PAH sampling rate (R_S) values for SPMDs that allow for the estimation of water concentrations of analytes from their concentrations in SPMD triolein. Assuming that PAH

equilibrium between the lipid in SPMDs and the microcosm water is not approached (linear uptake) and constant analyte concentration in water, then for a constant temperature, the following model can be used to estimate water concentrations:

$$C_W = C_L V_L / R_S B t$$

where C_W is the aqueous concentration of the analyte, C_L is the concentration of the analyte in the SPMD triolein, V_L is the volume of lipid in the SPMD, R_S is the sampling rate in L/d, t is the time in days, and B is the correction factor for estimated uptake hindrance due to biofouling. Values of R_S determined at 10°C for the PPPAHs examined in this study were generously provided by J.N. Huckins (National Biological Service's Midwest Science Center, Columbia, MO).

J. Hepatic Enzyme Analysis

Hepatic EROD activity in rainbow trout was determined using a modification of the multiwell plate method of Kennedy and Jones (1994). Postmitochondrial supernatant was isolated from the livers by homogenizing tissue in ice-cold dithiothreitol buffer, followed by centrifugation at 9000 g. The following reagent concentrations were used in the EROD reaction mixture: 7-ethoxyresorufin, 1.7 μM (Sigma, St. Louis, MO); NADPH, 0.5 mM (Boehringer Mannheim, Germany); $MgSO_4$, 17 mM; and HEPES, 0.1 M, pH 7.8 M (Sigma, St. Louis, MO). EROD activity was determined in 48-well culture plates (Costar, Cambridge, MA) using a Cytofluor 2350 plate reading flourometer (Perspective Biosystems,

Framingham, MA). Excitation and emission filters were set at 530 nm and 590 nm respectively. Activity was determined at 25°C using 25 μL postmitochondrial supernatant in the reaction mixture. Simultaneous termination of the reaction and protein determination were achieved by addition of ice cold fluorescamine in acetonitrile (1.08 mM). Hepatic protein was estimated using a modification of Lorenzen and Kennedy (Lorenzen and Kennedy 1993).

K. Statistical Analysis

Measurements were tested for normality using the Kolmogorov-Smirnov test (with Lilliefors' correction), and for equal variance using the Levene Median test. Two-way ANOVA was used to determine significant differences in parameters among microcosms and sampling days. Determination of EC_{50}s from bioassay concentration response curves was accomplished using the SigmaPlot non-linear curve fitting module (Jandel Scientific, San Rafael, CA). Dose response relationships for creosote effects on different variables were determined with linear regression. Differences between BD-TECs and CD-TECs due to nominal creosote dose and sampling day were determined using two way repeated measures (RM) ANOVA. Strength of association between variables was determined using Pearson product moment correlation. All test were based on a 0.05 level of significance.

3.4 RESULTS

A. Chemical Uptake by SPMDs

SPMDs were analyzed for time-dependent uptake of priority pollutant PAHs, members of the family of compounds making up the major fraction of most creosote formulations (Von Burg and Stout 1992). Concentrations of total PPPAHs in SPMDs rapidly increased with time in the 3 and 10 μL/L ponds, reaching a maximum on day 21, followed by a decline on day 28 (Figure 3.1). SPMDs in the control pond indicated accumulation of PPPAHs over time, albeit at low levels compared to the treatment ponds. Though concentrations of total PPPAHs in both treatment ponds were significantly higher than the control pond at each collection point (excluding time 0, ANOVA, $p<0.05$), the degree of PAH accumulation in SPMDs from the 3 and 10 μL/L ponds were very similar. Significantly higher concentrations of total PPPAHs in SPMDs from the 10 μL/L microcosm were observed on days 14 and 28 (ANOVA, $p<0.05$), but no significant difference was detected on the other three collection days. Measured water concentrations of PPPAHs from the ponds (Table 3.2) indicated that the total PPPAH concentration differed between the two treatment ponds, but that not all congeners followed this trend. In particular, it was noted that other than chrysene, compounds known to induce EROD activity in RTL-W1 did not differ greatly between the two ponds.

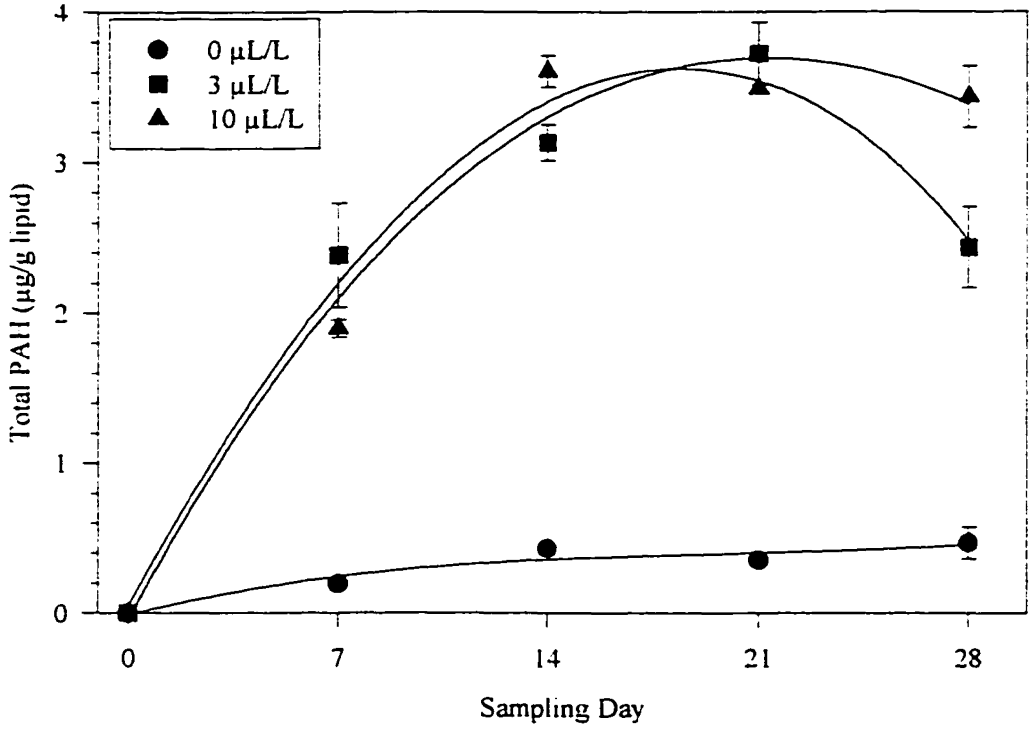


Figure 3.1. Uptake with time of total PPPAHs by SPMDs from creosote microcosms. The microcosms were dosed with 0, 3 and 10 µL/L creosote 103 days prior to the addition of SPMDs. Three SPMDs were removed from each microcosm at 7, 14, 21 and 28 days after their addition. These were subsequently extracted and analyzed for PPPAHs. The results were expressed as mean (±S.E.) total PPPAH concentration (µg/g of lipid).

Table 3.2. Aqueous concentrations of selected PAHs in creosote dosed microcosms measured by conventional water sampling.[1]

PPPAH	Microcosm Creosote Dose		
	0 µL/L	3 µL/L	10 µL/L
	ng/L	ng/L	ng/L
acenaphthene	nd[2]	69.14	230.83
fluorene	nd	103.84	213.87
phenanthrene	69.14	133.04	190.37
anthracene	nd	19.88	20.42
fluoranthene	nd	465.61	571.46
pyrene	nd	297.69	437.71
benz[a]anthracene*	nd	8.48	12.93
chrysene*	nd	nd	293.96
benzo[b]fluoranthene*	nd	45.96	47.09
benzo[k]fluoranthene*	4.99	15.23	11.92
benzo[a]pyrene*	nd	nd	nd
Σ PAH	**74.13**	**1158.87**	**2030.56**

[1] Water samples were collected 103 days after creosote dosing.

[2] Indicates that the compound was not detectable at quantifiable concentration.

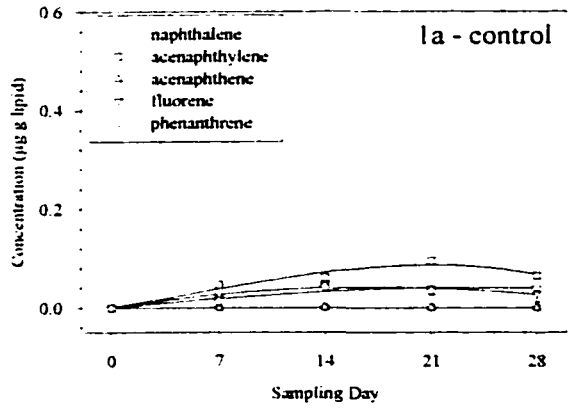
* Compound induces EROD activity in RTL-W1.

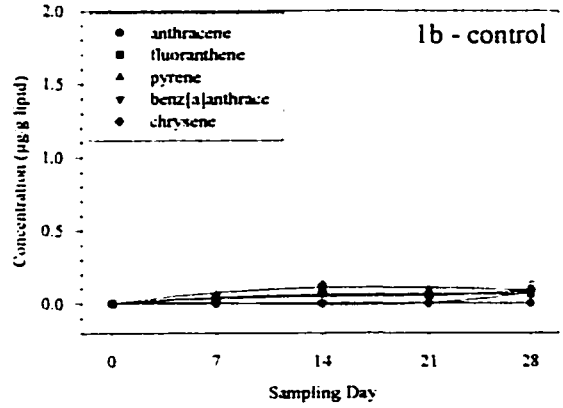
Concentrations of individual PPPAHs in measured in SPMD lipid are shown in Table B.1 (Appendix I). Percent recoveries of the PPPAH external standards were generally high, with the exception of some of the lower molecular weight compounds, which are susceptible to loss during the evaporation stages of the extraction procedure. PPPAH concentrations

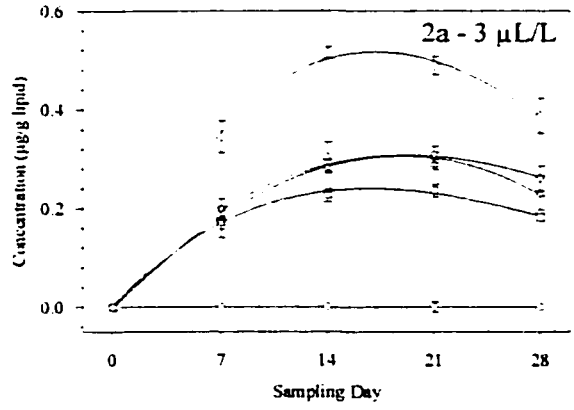
reported were not adjusted according to the recoveries of the external standards. Mean percent recovery of the lipid mass originally contained in the SPMDs during the extraction procedure was 96.6 ± 0.7%. Since lipid loss was minimal, no adjustments were made on the concentrations of PPPAHs.

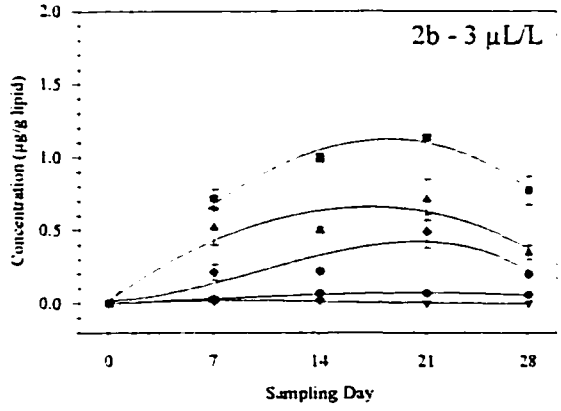
Fluoranthene and pyrene were the most highly concentrated compounds in SPMDs from the treatment ponds. This finding was reflected in the measured water concentrations of these compounds (Table 3.2). Pyrene is rapidly concentrated in SPMDs, having a sampling rate (R_s) of 3.8 L/d, the highest of all the PPPAHs (Table 3.1). At the present time, insufficient data exists to determine an accurate sampling rate of fluoranthene (J.N. Huckins, National Biological Service, personal communication, 1997). Acenaphthylene, fluorene, phenanthrene and chrysene were also concentrated in SPMDs, with highest concentrations generally seen in the 3 µL/L microcosm. In particular, phenanthrene was approximately 2 fold higher in this pond as compared to the 10 µL/L pond at each time point. Other analytes sequestered included anthracene and benz[a]anthracene, but at low levels compared to other PPPAHs detected. High molecular weight PPPAHs were not concentrated or were below the methodological limits of detection. Certain analytes, such as anthracene and pyrene did demonstrate an increase in concentration with higher creosote dose. In the majority of cases though,

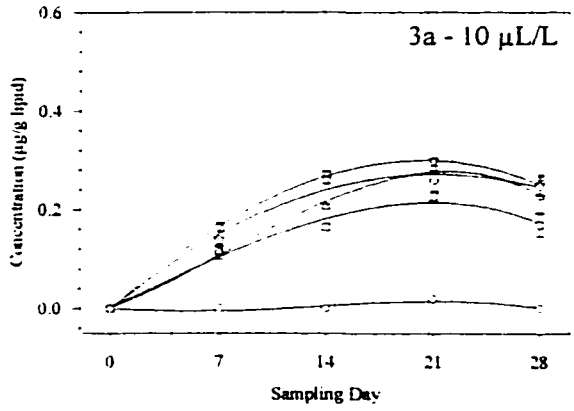
Figure 3.2. Uptake with time of individual PPPAHs by SPMDs from creosote microcosms. The microcosms were dosed with 0, 3 and 10 μL/L creosote 103 days prior to the addition of SPMDs. Three SPMDs were removed from each microcosm at 7, 14, 21 and 28 days after their addition. These were subsequently extracted and analyzed for individual PPPAHs. The results were expressed as mean (±S.E.) concentration (μg/g of lipid). The 10 PPPAHs detected have been arbitrarily grouped into two groups (panel a and panel b) of five.

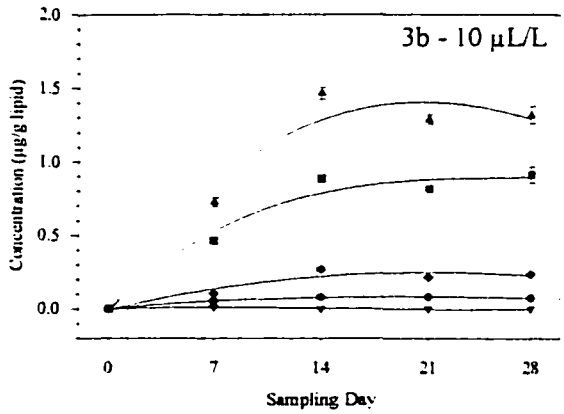

1a - control
naphthalene
acenaphthylene
acenaphthene
fluorene
phenanthrene
Concentration (µg/g lipid)
Sampling Day


1b - control
anthracene
fluoranthene
pyrene
benz[a]anthrace
chrysene
Concentration (µg/g lipid)
Sampling Day


2a - 3 µL/L
Concentration (µg/g lipid)
Sampling Day


2b - 3 µL/L
Concentration (µg/g lipid)
Sampling Day


3a - 10 µL/L
Concentration (µg/g lipid)
Sampling Day


3b - 10 µL/L
Concentration (µg/g lipid)
Sampling Day

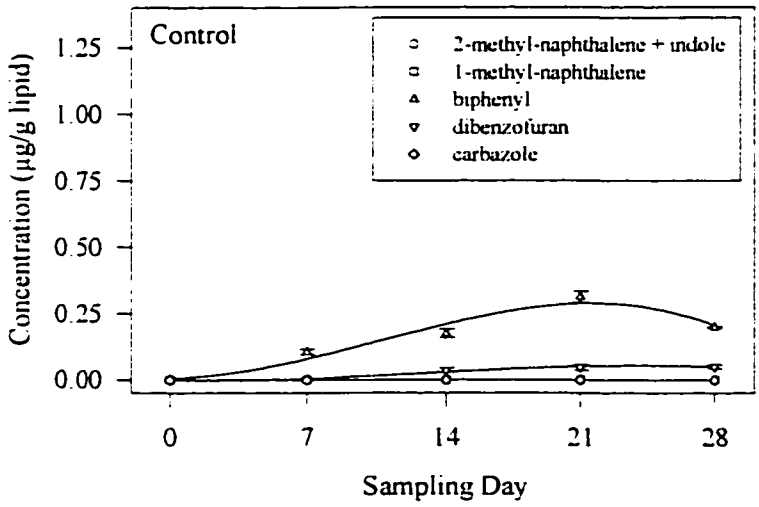
analyte concentrations did not differ greatly between the two creosote dosings, or were higher in the 3 μL/L pond.

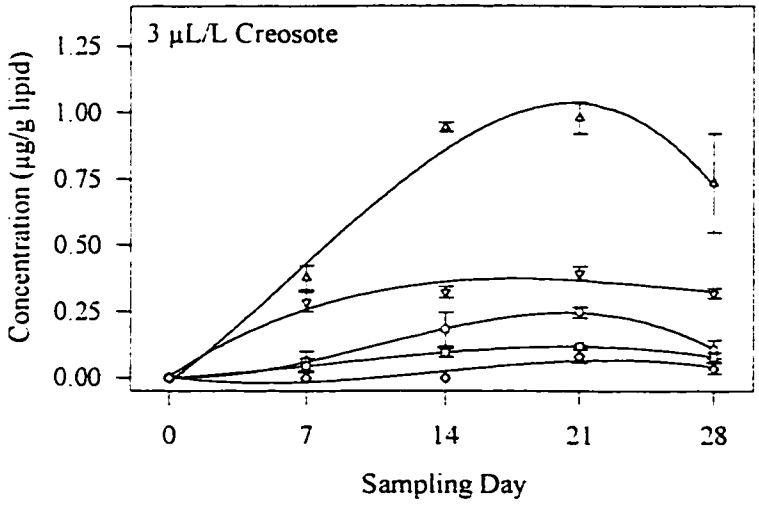
SPMD accumulation of PPPAHs over time is shown in Figure 3.2. Most compounds were rapidly taken up by SPMDs, reaching maximal concentrations by day 21, and declining by day 28. This was similar to the pattern seen with total PPPAHs. In general, analytes appeared to demonstrate linear kinetic uptake up to about day 14. In all three ponds, contaminant equilibrium between the lipid and water seemed to be reached, although the drop in concentration on day 28 indicates that other factors are likely influencing the accumulation and retention of PPPAHs.

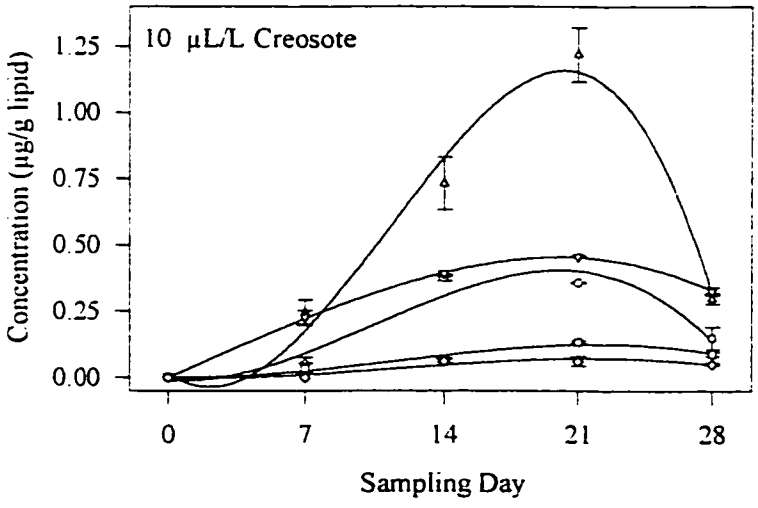
In addition to the 16 PAHs, the accumulation of five additional non-priority aromatic compounds in SPMDs was examined (Figure 3.3). As was the case with PPPAHs, analyte concentrations peaked at 21 days and generally declined following this time point. Measurable concentrations of biphenyl and dibenzofuran were present in SPMDs from the control pond, but these values were significantly lower than concentrations observed in the treatment pond SPMDs (ANOVA, $p<0.05$). In SPMDs from the treatment ponds, concentrations of the selected analytes were very similar between the 3 and 10 μL/L dosings (Appendix I, Table B.2). As in the control pond, biphenyl and dibenzofuran were the most highly concentrated of the five non-priority aromatic hydrocarbons in SPMDs from creosote dosed ponds. The rapid accumulation of these two compounds is evident in Figure 3.3. Possible causes for the dramatic decline of analyte concentrations on day 28 will be discussed.

In regards to the biological potency of the PPPAHs detected in SPMDs, only chrysene and benz[a]anthracene are demonstrated inducers of EROD activity in RTL-

Figure 3.3. Uptake with time of individual non-priority aromatic hydrocarbons by SPMDs from creosote microcosms. The microcosms were dosed with 0, 3 and 10 μL/L creosote 103 days prior to the addition of SPMDs. Three SPMDs were removed from each microcosm at 7, 14, 21 and 28 days after their addition. These were subsequently extracted and analyzed for individual compounds. The results were expressed as mean (±S.E.) concentration (μg/g of lipid).


Control
2-methyl-naphthalene + indole
1-methyl-naphthalene
biphenyl
dibenzofuran
carbazole
Concentration (µg/g lipid)
1.25
1.00
0.75
0.50
0.25
0.00
0
7
14
21
28
Sampling Day


3 µL/L Creosote
Concentration (µg/g lipid)
1.25
1.00
0.75
0.50
0.25
0.00
0
7
14
21
28
Sampling Day


10 µL/L Creosote
Concentration (µg/g lipid)
1.25
1.00
0.75
0.50
0.25
0.00
0
7
14
21
28
Sampling Day

W1, although the other PPPAHs detected are toxicologically significant due to their mutagenic and carcinogenic potential (Callahan et al. 1979). No information is currently available on the CYP1A inducing potential of the non-priority aromatic hydrocarbons examined in this study.

B. Estimated Aqueous Concentrations of PPPAHs from SPMD Concentrations

Average concentrations throughout the 28 day exposure period of six PPPAHs predicted using the model of Huckins et al. (1993) were, on the whole, lower than measured concentrations (Table 3.3). Despite this, it was observed that the values determined using each method were significantly correlated ($r^2 = 0.50$, $p<0.05$). Measured concentrations were typically two to ten fold higher than the estimated aqueous concentrations. Moderate biofouling was observed on SPMDs, and thus, a correction factor (B) of 0.75 was applied to the model to account for the reduced uptake of contaminant. Although there was a steady drop in temperature during the study (range = 14 to 4°C), the mean water temperature measured throughout the 28 day period was 9.1 ± 2.8°C. R_S values used in the model were determined at 10°C.

Table 3.3 Average aqueous concentrations of selected priority pollutant PAHs (PPPAHs) in creosote dosed microcosms over 28 days estimated using SPMD concentrations and a mathematical model (Huckins *et al.* 1993) and conventional water sampling.

	Control		3 µL/L		10 µL/L	
Analyte	Estimate (ng/L)	Measured (ng/L)	Estimate (ng/L)	Measured (ng/L)	Estimate (ng/L)	Measured (ng/L)
Acenaphthene	nd[1]	nd	40.5	69.1	27.4	230.8
Fluorene	3.4	nd	29.2	103.8	24.0	213.9
Phenanthrene	1.2	69.1	29.9	133.0	12.0	190.7
Anthracene	nd	nd	3.0	19.9	5.9	20.4
Pyrene	4.5	nd	39.0	297.7	54.5	437.7
Chrysene	nd	nd	16.8	nd	8.3	294.0

[1] Indicates that the compound was not detectable at quantifiable concentration.

C. Chemical Uptake by Rainbow Trout

Rainbow trout collected from the creosote dosed microcosms had a mean body weight of 128.8 ± 25.7 g and no significant difference was determined between ponds or collection day (two way ANOVA). Liver mass was significantly higher in trout collected on day 28 as compared to trout collected on day 0, but no significant difference was seen between creosote doses (two way ANOVA, $p<0.05$), indicating that the increase in mass is likely the result of trout growth over the 28 days. An examination of percent lipid in the excised livers revealed significantly higher lipid levels in fish from all collection days as compared to trout sacrificed on day 0 ponds (two way ANOVA, $p<0.05$).

Far fewer PPPAHs accumulated in rainbow trout liver tissue as compared to SPMDs during the 28 day exposure period (Table 3.4). Levels of individual PPPAHs and total PPPAHs in rainbow trout did not indicate increased uptake of contaminants with time of exposure as was seen with compounds in SPMDs. When present, the concentrations of individual PPPAHs in trout were generally in the same order of magnitude as was seen in SPMDs, although in some cases, trout liver analyte concentrations were considerably lower.

Acenaphthylene and acenaphthene were present in several fish, including trout collected from the control pond. Acenaphthylene was also detected in three of the six fish sacrificed on day 0, indicating the possibility of acenaphthylene contamination of the acclimation pond. Large differences in PPPAH concentrations in livers between the 3 and 10 µL/L ponds were not evident, but on several sampling days, concentrations of acenaphthylene and acenaphthene were higher in the control ponds than in the creosote dosed ponds. This may be an artifact caused by lower percent lipid levels in livers from control fish on certain sampling days. For example, fish collected on day 14 from the control pond had lipid levels of 3.7% while the 3 and 10 µL/L ponds were 5.9 and 5.6%, respectively. This could result in misleadingly elevated contaminant levels when concentrations are expressed per gram lipid. A similar situation existed on day 28, but since the percent lipid values in the control pond fish were not significantly lower than fish from the treatment ponds on both of these days, other reasons for the concentration differences may exist.

Table 3 4. Mean concentrations (S.E., n=6, µg/g) of priority pollutant PAHs (PPPAHs) extracted from livers of rainbow trout exposed to creosote dosed microcosms (0, 3, and 10 µL/L creosote) over a 28 day period.

		Creosote Dose Applied To Microcosms	
PPPAH[1]	Control	3 uL/L	10 uL/L
Day 0			
acenaphthylene	0.177 (0.092)	na[2]	na
acenapthene	nd[3]	na	na
fluorene	nd	na	na
anthracene	nd	na	na
Σ PAH	**0.177**		
Day 7			
acenaphthylene	0.755 (0.171)	nd	0.341 (0.039)
acenapthene	nd	0.034 (0.022)	0.108 (0.023)
fluorene	nd	nd	nd
anthracene	nd	nd	nd
Σ PAH	**0.755**	**0.034**	**0.449**
Day 14			
acenaphthylene	0.333 (0.081)	0.203 (0.052)	0.223 (0.021)
acenapthene	0.050 (0.050)	nd	0.025 (0.016)
fluorene	nd	nd	nd
anthracene	nd	nd	nd
Σ PAH	**0.382**	**0.203**	**0.248**
Day 21			
acenaphthylene	nd	0.280 (0.031)	0.369 (0.022)
acenapthene	0.599 (0.042)	nd	nd
fluorene	nd	nd	nd
anthracene	nd	nd	nd
Σ PAH	**0.599**	**0.280**	**0.369**
Day 28			
acenaphthylene	0.263 (0.059)	0.157 (0.030)	0.134 (0.020)
acenapthene	0.104 (0.104)	nd	0.014 (0.014)
fluorene	nd	nd	0.005 (0.005)
anthracene	nd	nd	0.027 (0.027)
Σ PAH	**0.367**	**0.157**	**0.181**

[1] The following PPPAHs were analyzed for and were not detected in any of the extracts at quantifiable concentration: phenanthrene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b+k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene, benzo[g,h,i]perylene

[2] Not applicable. Day 0 fish were not exposed to microcosms, but were sacrificed immediately upon removal from acclimation pond.

[3] Indicates that the compound was not detectable at quantifiable concentration.

Concentrations of non-priority aromatic hydrocarbons in rainbow trout livers were higher than those in SPMDs (Appendix I, Table B.2). Similar to SPMDs, biphenyl was generally present at high levels in the trout, but in contrast to SPMDs, the co-eluted compounds 2-methylnaphthalene and indole yielded the highest concentrations in most liver samples. Three of the five aromatic hydrocarbons were present in fish sacrificed on day 0, with 2-methylnaphthalene/indole levels higher than those seen in any fish exposed to the microcosms. Significantly lower lipid levels in livers excised from day 0 fish as compared to fish from other sampling days may have elevated the levels of contaminants in these trout. No relationship between creosote dose and concentration of compounds accumulated in trout was evident in any of the microcosms. In addition, chemical concentrations in the livers did not increase over the 28 day exposure period.

D. CD- and BD-TECs in SPMDs and Trout

Using TEFs derived with the RTL-W1 bioassay, CD-TECs for both sampling matrices were determined (Table 3.5). Of the five PPPAHs with TEFs in RTL-W1, SPMDs accumulated only two, benz[a]anthracene and chrysene, while rainbow trout livers were devoid of detectable levels of any EROD inducing PPPAHs. The sum concentrations of the EROD inducing compounds accumulated are shown in Table 3.5. Although the TEFs for benz[a]anthracene and chrysene are similar, the concentrations of chrysene detected in SPMDs were far greater than benz[a]anthracene. As a result, chrysene generally contributed 80 - 100% to the total CD-TEC for most SPMDs. Because CD-TECs are based on the measured chemical concentrations in the SPMDs, a similar relationship to creosote dose was seen with CD-TECS as was seen with the analyte concentrations. The two treatment ponds

generally had significantly higher CD-TECs than the control on each sampling day, with sampling day 28 being an exception (two way ANOVA, $p<0.05$). Due to the similar chemical concentrations in the 3 and 10 μL/L microcosms, other than on day 21, the CD-TECs in SPMDs from those ponds did not differ significantly (two way ANOVA, $p<0.05$). Increases in CD-TECs with time generally followed the pattern of chrysene accumulation in the SPMDs (see Figure 3.2, plots 2b and 3b), since the contributions of benz[a]anthracene were frequently so low. The rainbow trout liver CD-TECs were all zero due to the lack of detectable concentrations of EROD inducers in the samples.

Table 3.5. Total concentrations of measured EROD inducing PAHs and chemistry (CD-) and bioassay (BD-) derived TECs (mean, S.E.) in livers from rainbow trout and SPMDs and hepatic EROD activity in rainbow trout exposed to creosote dosed microcosms for 28 days.

Sampling Day	Creosote Dose (µL/L)	Total EROD Inducing PAHs (µg/g lipid)[1] Trout	SPMD	CD-TECs (pg TCDD/g lipid) Trout	SPMD	BD-TECs (pg TCDD/g lipid) Trout	SPMD	Hepatic EROD activity (pmol/mg/min)
0	0	nd[2]	nd	nd	0.0 (0.0)	0.9 (0.9)	0.0 (0.0)	0.2 (0.1)
7	0	nd	0.02 (0.01)	nd	1.1 (0.3)	1.3 (0.8)	0.0 (0.0)	0.0 (0.0)
	3	nd	0.23 (0.05)	nd	12.1 (2.3)	1.7 (0.5)	16.5 (2.8)	3.5 (1.0)
	10	nd	0.12 (0.01)	nd	6.4 (0.6)	3.2 (0.7)	18.0 (0.3)	13.2 (2.0)
14	0	nd	0.07 (0.01)	nd	3.6 (0.6)	1.8 (0.4)	1.8 (0.1)	0.6 (0.1)
	3	nd	0.24 (0.01)	nd	12.7 (0.3)	2.1 (0.3)	25.6 (3.7)	3.0 (0.6)
	10	nd	0.27 (0.01)	nd	14.0 (0.6)	1.1 (0.4)	39.2 (3.0)	10.0 (0.7)
21	0	nd	0.04 (0.01)	nd	2.2 (0.3)	0.9 (0.4)	1.6 (0.0)	0.0 (0.0)
	3	nd	0.49 (0.11)	nd	25.4 (5.8)	3.1 (0.6)	34.0 (2.1)	1.7 (0.3)
	10	nd	0.22 (0.00)	nd	11.3 (0.1)	1.5 (0.4)	35.2 (2.6)	4.1 (0.5)
28	0	nd	0.17 (0.04)	nd	10.3 (2.4)	1.7 (0.5)	1.9 (0.0)	0.6 (0.1)
	3	nd	0.20 (0.02)	nd	10.4 (0.9)	1.3 (0.3)	17.9 (1.9)	3.1 (0.7)
	10	nd	0.24 (0.01)	nd	12.3 (0.7)	1.1 (0.5)	30.3 (3.2)	5.4 (0.6)

[1] Sum concentrations of RTL-W1 EROD inducers (benz[a]anthracene, chrysene, benzo[b+k]fluoranthene and benzo[a]pyrene).

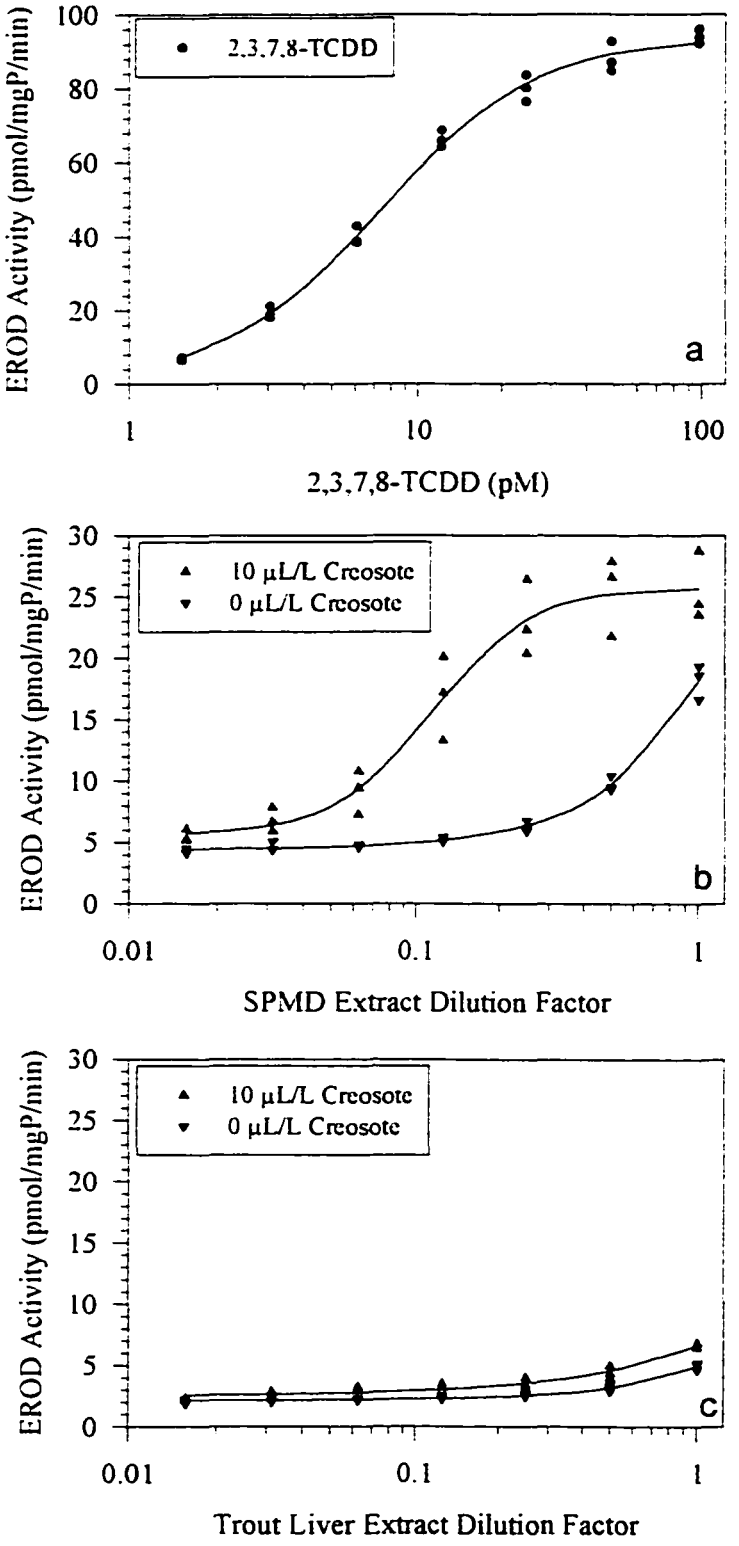
[2] Indicates that the compound was not detectable at quantifiable concentration.

In contrast to the chemical analysis, both rainbow trout and SPMD extracts induced EROD activity in RTL-W1, resulting in measurable BD-TECs for both matrices. Examples of the wide variety of concentration-response curves observed in RTL-W1 are shown in Figure 3.4. Levels of EROD induction were generally higher with SPMD extracts, while rainbow trout extracts did not produce high levels of activity. Over the 28 day exposure period, no time-related increase in rainbow trout BD-TECs in any of the three microcosms was observed.

SPMD BD-TECs from the 10 μL/L ponds were significantly higher than BD-TECs in the 3 μL/L pond at all sampling days except day 7, indicating a that a concentration response relationship with one or more AhR-active creosote components may exist. When rainbow trout and SPMD BD-TECs were compared, no correlation between the two matrices was detected. Levels of SPMD BD-TECs over the 28 day period closely followed the pattern of SPMD PPPAH accumulation (Figure 3.5) with a rise in BD-TECs to day 21 followed by lower values seen on day 28.

Comparison of BD-TECs and CD-TECs determined in SPMDs revealed a significant correlation between the two variables ($r^2 = 0.496$, $p<0.05$) despite observed differences in patterns of days 7 and 21. Paired comparisons of individual SPMD extracts indicated that BD-TECs were significantly higher than the corresponding CD-TECs (paired t-test, $p<0.05$). No relationship existed between BD and CD-TECs in rainbow trout due to the lack of values for liver CD-TECs.

Figure 3.4. EROD dose-response curves in RTL-W1 for 2,3,7,8-TCDD and selected extracts. EROD activity (pmol/mgP/min) was measured in RTL-W1 cultures 24 h after they been exposed to varying concentrations of either 2,3,7,8-TCDD (panel a) . SPMD extracts (panel b) and rainbow trout liver extracts (panel c). The extracts were prepared from SPMDs and rainbow trout that had been exposed to either the control microcosm (0 μL/L creosote) or 10 uL creosote dosed microcosm for 7 days.


EROD Activity (pmol/mgP/min)
2,3,7,8-TCDD
a
2,3,7,8-TCDD (pM)
10 µL/L Creosote
0 µL/L Creosote
b
SPMD Extract Dilution Factor
c
Trout Liver Extract Dilution Factor

E. Hepatic EROD in Trout

Induction of EROD activity in trout exposed to waterborne creosote occurred rapidly, reaching a maximum in the 3 and 10 µL/L microcosms by day 7 (Figure 3.6). Over days 14 and 21 there was a decline activity with time and a slight, but statistically insignificant, rise in EROD on the final sampling day. Trout from the control pond displayed the lowest levels of EROD activity. At all sampling points, EROD activity was highest in the 10 µL/L pond, followed by the 3 µL/L and control ponds (two way ANOVA, $p<0.05$).

No relationships between hepatic EROD activity and either BD- or CD-TECs in trout liver samples were observed. In addition, hepatic EROD activity was not correlated with SPMD CD-TECs or total concentrations of EROD inducing PPPAHs accumulated in SPMDs. However, SPMD BD-TECs were significantly correlated with the levels of hepatic EROD activity in rainbow trout ($r^2 = 0.330$, $p<0.05$).

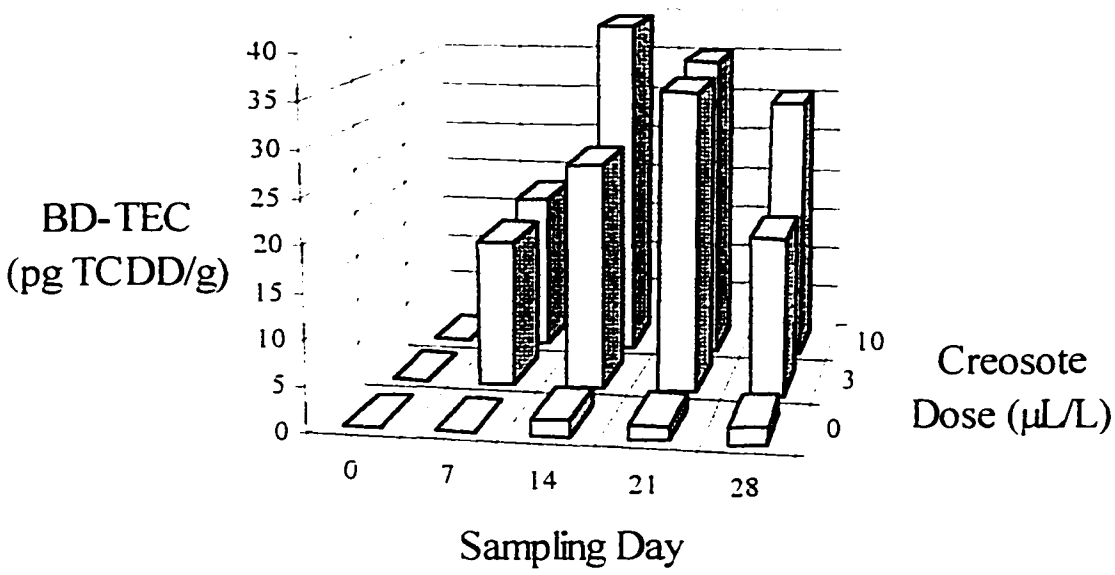


Figure 3.5. Uptake with time of total 2,3,7,8-TCDD equivalent concentrations by SPMDs from creosote microcosms as determined by the RTL-W1 bioassay . The microcosms were dosed with 0, 3 and 10 µL/L creosote 103 days prior to the addition of SPMDs. Three SPMDs were removed from each microcosm at 7, 14, 21 and 28 days after their addition. These were subsequently extracted and exposed to RTL-W1 to determine bioassay-derived 2,3,7,8-TCDD equivalent concentrations (BD-TECs).

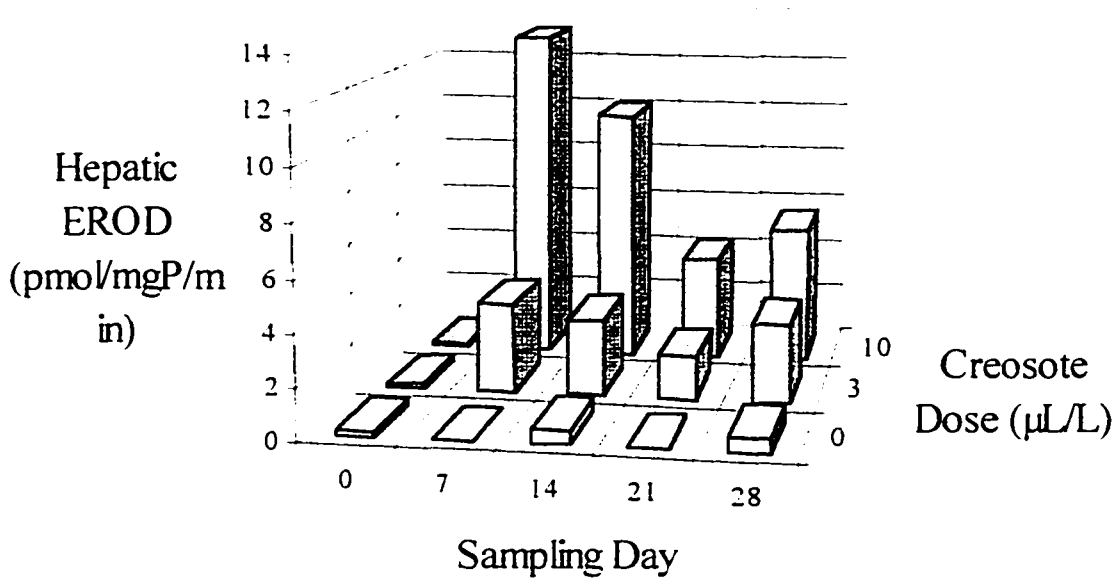


Figure 3.6. Hepatic EROD activity in rainbow trout exposed to creosote microcosms for varying periods of time. The microcosms were dosed with 0, 3 and 10 µL/L creosote 103 days prior to the addition of rainbow trout. Ten fish were removed from each microcosm at 7, 14, 21 and 28 days after their addition. Livers were removed, frozen, and subsequently assayed for EROD activity (pmol/mgP/min).

3.5 DISCUSSION

A. PPPAH Accumulation in SPMDs and Rainbow Trout

Comparing the uptake of specific components of creosote in rainbow trout and SPMDs over time provided valuable information regarding the accumulation of compounds that can be rapidly metabolized in biological systems. Distinct differences in the concentrations of analytes sequestered in SPMDs and trout existed, with SPMDs accumulating higher concentrations of a greater number of PPPAHs. Disparity in contaminant burdens between the two matrices increased with time of exposure, indicating that PPPAHs were rapidly transformed and/or eliminated from the trout while SPMDs continued to accumulate PPPAHs until equilibrium was reached between the water and triolein. Because potentially toxic chemicals such as PAHs are biotransformed in trout (Niimi and Palazzo 1986) (Chapter 2), the use of SPMDs as a tool for estimating the bioconcentration of these types of compounds is particularly advantageous. Examining uptake over time, it was shown that the majority of PPPAHs in trout were not detected throughout the period of exposure. It has been demonstrated that the stimulation of CYP1A activity in the trout can occur rapidly upon exposure to PAHs (Munkittrick et al. 1995), so it is likely that any accumulated PPPAHs were transformed in the trout and therefore not detected in liver samples. In contrast, analyte levels in SPMDs from treatment ponds were significantly higher than from the control pond by sampling day 7, demonstrating that these compounds are rapidly bioconcentrated in the absence of metabolic transformation processes.

Although it was clearly evident that PPPAH concentrations in SPMDs from creosote dosed ponds did differ from the control pond, a difference in accumulated analytes in

SPMDs between the 3 μL/L and 10 μL/L creosote doses was not apparent. The similarity in PPPAH patterns and concentrations between the two treatments may in part be due to the elapsed time between initial dosing and the start of this study. In a similar CNTC microcosm study in 1995, Bestari et al. (1996) showed that after initial creosote dosing, the concentration of total PAHs in various ponds declined asymptotically with time, and that after an 84 day period, differences in PAH concentrations between several ponds was reduced or ceased to exist. In the present study, a period of 103 days elapsed between the initial creosote dosing and the beginning of the SPMD/trout exposures. The fact that certain analytes in SPMDs, such as pyrene and anthracene, increased with higher creosote dose in this study where others did not is likely due to the unique half lives of different PAHs in aquatic environments (Lee et al. 1978).

The relationship between creosote dose and PPPAH concentration in trout did not reflect the observations in SPMDs. This was expected because of the metabolism of PPPAHs by rainbow trout (Chapter 2). What was not expected were the elevated levels of certain contaminants in rainbow trout collected from the control pond as compared to the treatment. An interesting explanation for this is that the higher levels of EROD inducing compounds in the creosote treated ponds stimulated a "metabolic threshold" in the trout. It is generally held that a specific concentration of AhR-active compounds is needed before the induction of CYP1A can occur (Hellou 1996). It may be that this threshold was not reached in control fish, resulting in the detection of higher concentrations of certain analytes in these fish as compared to trout from the treatment ponds. Alternatively, analytical error may have resulted in elevated detection of certain compounds in the control microcosms.

Analysis of time-based PPPAH uptake revealed that most analytes achieved equilibrium by day 21, followed by a precipitous drop in the chemical concentrations measured in SPMDs on day 28. One possible reason for this deals with the closed nature of the microcosm environment. Due to the number of sampling devices and trout in the pond, it is possible that PPPAHs were removed (bioconcentrated) from the water column at a higher rate than they were released from the sediment. By the end of the study, the water concentrations of PPPAHs may have been lower than at the outset of the study. This could result in disruption of contaminant equilibrium between the triolein and the water, with analytes moving back into the water phase. Huckins *et al.* (1996) demonstrated that in static exposure settings with single applications of four chemicals, water concentrations fell as lipid concentrations rose, resulting in a much more rapid approach to equilibrium than in flow-through exposure settings where chemical concentrations are constant. Alternatively, SPMD biofouling may have played a role in the reduced levels of analytes on day 28. In a study by Huckins et al. (1994), accumulation of phenanthrene in biofouled SPMDs over 28 days followed an uptake curve over time similar to those observed in this study, with reduced concentrations seen in SPMDs from the final collection day. When SPMDs were treated weekly with Sanaqua (didecyldimethylammonium chloride), an aufwuch inhibitor, this reduction in concentration was not observed. It is possible that with increased organic matter on the exterior surface of the SPMD that contaminants would partition into this phase from the internal triolein, thereby reducing the concentration in the lipid. Although the observations from this study correspond with those seen in the Sanaqua study, it is not known if the biofouling observed in this study was extensive enough to produce such an effect.

In general, PPPAHs with high molecular weights were not sequestered in SPMDs or rainbow trout. The large molecular size and hydrophobicity of these compounds were likely factors that reduced their bioavailability in this study. The high log K_{ow}s for these compounds would cause them to associate with sediment or organic matter in the water column. Although benzo[k]fluoranthene and benzo[b]fluoranthene were detected in the microcosms using conventional water sampling, it is possible that they were associated with dissolved organic matter (non-bioavailable) in the water samples, and that they were released upon solvent agitation in the extraction procedure. If these compounds were truly dissolved, their large molecular size would have reduced the probability of their passage through the polyethylene/gill membranes (Huckins et al. 1993). The PPPAHs accumulated in this study generally had lower log K_{ow}s and four aromatic rings or less, making them comparatively more bioavailable the larger PPPAHs examined.

B. Non-priority Aromatic Compound Accumulation in SPMDs and Rainbow Trout

In contrast to PPPAH concentrations, non-priority aromatic compounds measured in trout were generally detected at higher levels in trout than in SPMDs. To date, no studies have examined the behavior of these analytes in SPMDs. If the physical properties of these compounds are examined (Table 3.1), it can be seen that they are slightly less hydrophobic than most PPPAHs examined and have three or fewer rings. These are conceivable reasons that these compounds may not have accumulated to a high degree in SPMDs, but they do not explain the elevated levels in the trout. It is possible that these compounds are not metabolized to the same extent as the PPPAHs in trout, resulting in their greater accumulation. These results could indicate that SPMDs do not accurately predict the

accumulation of certain analytes in biota, but without further information on the behavior of these compounds in both aquatic samplers and organism, a definitive conclusion cannot be made.

Detection of non-priority analytes in fish sacrificed on day 0 indicate that either the acclimation pond was contaminated with these compounds or that the fish had accumulated them prior to delivery from the hatchery. The similarity in proportions of the detected analytes in the day 0 trout to fish from the treatment ponds indicates that there was likely contamination of the acclimation pond from the microcosms, possibly due to leaching. The fact that the levels of these analytes were high compared to the levels in fish exposed to the microcosms could be an artifact caused by the significantly higher levels of lipids in livers of day 0 trout, which would result in disproportionately high analyte concentrations. Notably, hepatic EROD activity was not elevated in the day 0 fish, indicating that these compounds likely do not contribute to induction of CYP1A in trout (see below).

C. Aqueous Concentrations of PPPAHs

Although estimates of aqueous PPPAH concentrations were lower when derived using the SPMD uptake model as compared to conventionally measured concentrations, the significant relationship between the values derived using the two methods indicates that the model has fairly accurate predictive ability. In fact, most estimates of the analyte concentrations in the treatment ponds were in the same order of magnitude as the measured concentrations. The difference between SPMD and conventional estimates may indicate that the SPMD measures truly dissolved, bioavailable concentrations of analytes in the water column. The SPMD model is subject to drawbacks. The two main factors that could

confound SPMD approximations of dissolved chemical concentrations are aufwuch growth (biofouling) and the assumptions of the SPMD uptake model (Gale et al. 1997). A biofouling correction factor was applied in the model, but this estimate is somewhat subjective, since it is based on the author's interpretation of the degree of surface area coverage and density of growth on the polyethylene surface. Other variables in the model are based on assumptions of constant chemical concentration and temperature in the water during the exposure period, both of which can vary during non-laboratory experiments. Despite the potential of these factors to reduce the accuracy of SPMD concentration estimates, the values determined in this study appear to reflect what is seen with the values determined with the traditional water analysis. It is likely that the lower values determined in the SPMDs in this study are due to the elimination of non-bioavailable analytes, supporting the use of these devices for the prediction of concentrations of chemicals that can be readily accumulated in organisms.

An additional benefit of SPMDs for estimating concentrations of analytes in water is their ability to concentrate chemicals that are present at very low levels, allowing for their detection which would be missed by methods that only sample a single time point (Huckins et al. 1993). This may be the case in the control pond, where concentrations of fluorene and pyrene were estimated by the SPMD model but were not detected by the conventional water sampling method.

D. CD- and BD-TECs in SPMDs and Trout

Distinct differences in the levels of accumulated AhR-active compounds were seen between SPMDs and rainbow trout, and this was reflected in both methods of TEC

determination. As expected, both CD- and BD-TECs were higher in SPMDs compared to trout, and the difference increased with time of exposure. This is likely due to the biotransformation and/or elimination of EROD inducing compounds in the trout. This was supported by the elevated levels of hepatic EROD activity in the trout (see below), which indicates that metabolism was induced.

Although no known RTL-W1 EROD inducing PPPAHs were detected in liver samples, BD-TECs were measured in these samples. Two possible reasons exist for this. First, the lack of AhR-active PPPAHs in the trout livers does not preclude their presence at concentrations below the methodological limits of detection in this study. Combined together, low concentrations of AhR-active analytes could result in EROD induction in RTL-W1. Even if this is true, the absence of a relationship between trout liver BD-TECs and creosote dose or exposure time supports the metabolism of the majority of accumulated inducers. Results from Chapter 2 provide additional support for this argument. The second possibility is the bioconcentration in trout of compounds capable of inducing CYP1A activity in RTL-W1 other than the suite of chemicals measured. If SPMDs accurately predict the bioconcentration of contaminants in trout, then the TECs in these devices may lend support to this hypothesis. SPMD BD-TECs were higher than CD-TECs indicating the possibility that not all EROD inducing compounds were accounted for in the chemical analysis. As described in Chapter 2, AhR-active compounds other than PAHs have been detected at sites contaminated with creosote (Copeland et al. 1993). Determining which compounds in creosote are capable of inducing CYP1A activity is inherently difficult due to the complexity of the mixture and the variation between different creosote formulations (Mueller et al. 1989). The same unknown EROD inducer(s) in SPMDs, if accumulated in

trout, could explain the difference between the trout liver BD and CD-TECs. Finally, it may be that both of these explanations play a role in explaining the detection of BD-TECs in the trout livers.

SPMD CD-TECs were mainly the result of contributions from chrysene and to a lesser extent, benz[a]anthracene. As described above, these two compounds did not appear to account for the total EROD induction in RTL-W1, as is indicated by the significantly higher BD-TECs in the same SPMD extracts. The presence of an AhR-active compound not measured in SPMDs is a possible explanation. An alternative reason for the difference between BD- and CD-TECs in SPMDs may be synergistic, or greater than additive, interactions between chemicals in the extract that resulted in higher than predicted EROD activity in RTL-W1, although such interactions have yet to be explored in this *in vitro* system. In Chapter 2, differences between RTL-W1 BD- and CD-TECs in SPMDs were not significant. Dissimilar rates of degradation of certain inducers in the microcosms may have occurred in two study years. Factors such as sunlight, temperature and bacterial activity in the microcosms were likely different in 1995 and 1996, leading to distinct contaminant patterns and concentrations.

E. Hepatic EROD in Trout

Induction of EROD activity in rainbow trout livers suggests there is a rapid asymptote in induction, and that once the trout are capable of metabolizing the accumulated chemicals, induction declines until a steady state is reached. These results are supported by Munkittrick et al. (1995), who observed similar patterns of induction in rainbow trout exposed to PAH contaminated sediment. They suggested that the higher early induction represents a response

to a higher early internal body burden of chemicals, which accumulate prior to the induction and full operation of excretory mechanisms. In the present study, concentrations of chemicals showed no increase over the time of exposure, indicating that the metabolism of accumulated EROD inducers occurred prior to the day 7 sampling point. This finding demonstrates the rapid nature of the biotransformation of compounds in rainbow trout. Time course studies examining AHH induction in English Sole (*Parophrys vetulus*) also revealed rapid induction of metabolic activity followed by a decline after 3-5 days (Collier and Varanasi 1991). The importance of the findings of the present study are revealed upon examination of the creosote dose-response relationship with hepatic EROD. If trout were only collected on day 28 of this study, EROD activity would have been shown to increase with creosote dose, as was observed in Chapter 2. But the levels of EROD are significantly lower at this time point compared to earlier in the time course. Induction of CYP1A in aquatic organisms has been correlated with different modes of toxicity (Bucheli and Fent 1995), and therefore, the high levels of EROD that occur in fish upon initial exposure to AhR-active compounds may be related to a correspondingly high level of toxicity. Examination of time point far removed from the initial contaminant exposure may not accurately reflect the potential toxicity to aquatic organisms. This finding could have particular relevance for chemical spills or rapid pulses of contaminants into an aquatic environment.

Hepatic EROD was found to be correlated with SPMD BD-TECs, but not SPMD CD-TECs or concentrations of measured EROD-inducing PPPAHs in SPMDs. This finding supports the hypothesis that another EROD inducer is accumulated in SPMDs and fish. As

stated above, pyrene may be a potential candidate as is indicated by the positive correlation between hepatic EROD and pyrene concentrations in SPMDs.

G. Summary

Using microcosm-based exposures, this study examined the time-dependent uptake of the dominant creosote components, PPPAHs, in both living and artificial systems. SPMD estimates of PPPAH bioconcentration in trout suggest a rapid uptake of analytes over time. This was reflected in the increased BD-TECs in SPMD extracts over the 28 days and their correlation to rainbow trout hepatic EROD activity. Rapid induction of hepatic EROD over time likely lead to the biotransformation of accumulated PPPAHs. This was supported by the lack of time-dependent increases in measured chemicals and TECs in the trout. The lack of relationship between SPMD BD- and CD-TECs suggests the presence of an unknown EROD inducing compound(s) in the microcosms. Measured chemical concentrations, in conjunction with *in vivo* and *in vitro* estimates of their CYP1A inducing potency provided insight to the way in which PAHs are accumulated and metabolized in rainbow trout. Each of the techniques employed have individual strengths, but when used together, a clearer picture of the uptake and elimination of PPPAHs by rainbow trout was obtained.

3.6 ACKNOWLEDGMENTS

The authors are grateful to the Canadian Network of Toxicology Centres (CNTC) and the Natural Sciences and Engineering Research Council of Canada (NSERC) for the funding provided for this study. We wish to thank Elizabeth Heikkila, Kimberly Hamilton and Anita Mirabelli for their technical assistance and Jim Huckins for providing SPMD sampling rate

data for PPPAHs. Special thanks to Dr. K.R. Solomon and the CNTC for consenting to our use of the microcosm study area.

3.7 REFERENCES

Bestari, K.T., Robinson, R.D., Solomon, K.R., and Day, K.E. 1996. Quantification of 15 priority PAHs in creosote contaminated water and sediments. SETAC 17th Annual Meeting, Washington, D.C. November, 1996.

Bols, N.C., Schirmer, K., Heikkila, E.M., Greenberg, B.M., and Dixon, D.G. 1997a. Ability of intact and photomodified PAHs to induce 7-ethoxyresorufin-*O*-deethylase (EROD) activity in a rainbow trout liver cell line. Annual Research Symposium of the Canadian Network of Toxicology Centers, Toronto, ON, Canada, March 23-25, 1997.

Bols, N.C., Whyte, J.J., Clemons, J.H., Tom, D.J., van den Heuvel, M.R., and Dixon, D.G. 1997b. Use of liver cell lines to develop TCDD equivalency factors and TCDD equivalent concentrations in environmental samples. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment. J.T. Zelikoff, ed. SOS Publishing, Fair Haven, NJ, 1st ed.: pp. 329-350.

Bucheli, T.D. and Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25: 201-268.

Callahan, M.A., Slimak, M., Gbel, N., May, I., Flower, C., Freed, R., Jennings, P., DuPree, R., Whitmore, F., Maestri, B., Holt, B., and Gould, C. 1979. Water-related environmental fate of 129 priority pollutants. EPA-44014-79-029a,b, NTIS.

Clemons, J.H., Lee, L.E.J., Myers, C.R., Dixon, D.G., and Bols, N.C. 1996. Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines from rat and trout and the derivation of toxic equivalency factors. Canadian Journal of Fisheries and Aquatic Sciences. 53: 1177-1185.

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

Copeland, T.L., Paustenbach, D.J., Harris, M.A., and Otani, J. 1993. Comparing the results of a Monte Carlo analysis with EPA's reasonable maximum exposed individual (RMEI): a case study of a former wood treatment site. Regulatory Toxicology and Pharmacology. 18: 275-312.

Eadon, G., Kaminsky, L., Silkworth, J., Aldous, K., Hilker, D., O'Keefe, P., Smith, R., Gierthy, J., Hawley, J., Kim, N., and DeCaprio, A. 1986. Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. Environmental Health Perspectives. 70: 221-227.

Gale, R.W., Huckins, J.N., Petty, J.D., Peterman, P.H., Williams, L.L., Morse, D., Schwartz, T.R., and Tillitt, D.E. 1997. Comparison of the uptake of dioxin-like compounds by caged channel catfish and semipermeable membrane devices in the Saginaw River, Michigan. Environmental Science and Technology. 31: 178-187.

Gooneratne, R., Miranda, C.L., Henderson, M.C., and Buhler, D.R. 1997. Beta-naphthoflavone induced CYP1A1 and 1A3 proteins in the liver of rainbow trout (Oncorhynchus mykiss). Xenobiotica. 27: 175-187.

Government of Canada, Environment Canada, and Health and Welfare Canada. 1993. Creosote-impregnated waste materials. In: Canadian Environmental Protection Act. Minister of Supply and Services, Canada, pp. 1-25.

Graney, R.L., Giesy, J.P., and Clark, J.R. 1995. Field studies. In: Fundamentals of Aquatic Toxicology; Effects, Environmental Fate, and Risk Assessment. G.M. Rand, ed. Taylor & Francis, Bristol, PA, 2nd ed.: pp. 493-521.

Hargis, W.J., Roberts, M.R., and Zwerner, D.E. 1984. Effects of contaminated sediments and sediment-exposed effluent water on an estuarine fish: acute toxicity. Marine Environmental Research. 14: 337-354.

Hellou, J. 1996. Polycyclic aromatic hydrocarbons in marine mammals, finfish, and molluscs. In: Environmental contaminants in wildlife: interpreting tissue concentrations. W.N. Beyer, G.H. Heinz, and A.W. Redmon-Norwood, eds. CRC Press, Boca Raton, Florida, 1st ed.: pp. 229-250.

Huckins, J.N., Manuweera, G.K., Petty, J.D., Mackay, D., and Lebo, J.A. 1993. Lipid-containing semipermeable membrane devices for monitoring organic contaminants in water. Environmental Science and Technology. 27: 2489-2496.

Huckins, J.N., Petty, J.D., Lebo, J.A., Orazio, C.E., Prest, H.F., Tillitt, D.E., Ellis, G.S., Johnson, B.T., and Manuweera, G.K. 1996. Semipermeable membrane devices (SPMDs) for the concentration and assessment of bioavailable organic contaminants in aquatic environments. In: Techniques in Aquatic Toxicology. G.K. Ostrander, ed. CRC Press, Boca Raton, Florida, 1st ed.: pp. 625-655.

Huestis, S.Y., Servos, M.R., Sergeant, D.B., Leggett, M., and Dixon, D.G. 1995. Methods for determination of organochlorine pesticides, polychlorinated biphenyl congeners and chlorinated dibenzo-*p*-dioxins and furans in fish. Canadian Technical Report on Fisheries and Aquatic Sciences. 2044: 1-30.

Kennedy, S.W., Lorenzen, A., James, C.A., and Collins, B.T. 1993. Ethoxyresorufin-*O*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. Analytical Biochemistry. 211: 102-112.

Kennedy, S.W. and Jones, S.P. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Analytical Biochemistry. 222: 217-223.

Lebo, J.A., Zajicek, J.L., Huckins, J.N., Petty, J.D., and Peterman, P.H. 1992. Use of semipermeable membrane devices for in situ monitoring of polycyclic aromatic hydrocarbons in aquatic environments. Chemosphere. 25: 697-718.

Lebo, J.A., Zajicek, J.L., Orazio, C.E., Petty, J.D., Huckins, J.N., and Douglas, E.H. 1996. Use of semipermeable membrane device (SPMD) to sample polycyclic aromatic hydrocarbon pollution in a lotic system. Polycyclic Aromatic Compounds. 8: 53-65.

Lee, L.E.J., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K., Pasitschniak-Arts, M., Mosser, D.D., and Bols, N.C. 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biology and Toxicology. 9: 279-294.

Lee, R.F., Gardner, W.S., Anderson, J.W., Blaylock, J.W., and Barwell-Clarke, J. 1978. Fate of polycyclic aromatic hydrocarbons in controlled ecosystems enclosures. Environmental Science and Technology. 7: 832-838.

Lorenzen, A. and Kennedy, S.W. 1993. A fluorescence-based protein assay for use with a microplate reader. Analytical Biochemistry. 214: 346-348.

Malins, D.C., Krahn, M.M., Myers, M.S., Rhodes, L.D., Brown, D.W., Krone, C.A., McCain, B.B., and Chan, S.L. 1985. Toxic chemicals in sediments and biota from a creosote-polluted harbor: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). Carcinogenesis. 6: 1463-1469.

Meador, J.P., Stein, J.E., Reichert, W.L., and Varanasi, U. 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Reviews in Environmental Contamination and Toxicology. 143:79-165: 79-165.

Meadows, J., Tillitt, D., Huckins, J., and Schroeder, D. 1993. Large-scale dialysis of sample lipids using a semipermeable membrane device. Chemosphere. 26: 1993-2006.

Moring, J.B. and Rose, D.R. 1997. Occurrence of polycyclic aromatic hydrocarbons in semipermeable membrane devices and clams in three urban streams of the Dallas-Forth Worth Metropolitan Area, Texas. Chemosphere. 34: 551-566.

Mueller, J.G., Chapman, P.J., and Pritchard, P.H. 1989. Creosote-contaminated sites: their potential for bioremediation. Environmental Science and Technology. 23: 1197-1201.

Munkittrick, K.R., Blunt, B.R., Leggett, M., Huestis, S., and McCarthy, L.H. 1995. Development of a sediment bioassay to determine bioavailability of PAHs to fish. Journal of Aquatic Ecosystem Health. 4: 169-181.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics. 6: 1-42.

Neubert, D., Golor, G., and Neubert, R. 1992. TCDD-toxicity equivalencies for PCDD/PCDF congeners: prerequisites and limitations. Chemosphere. 25: 65-70.

Niimi, A.J. and Palazzo, V. 1986. Biological half-lives of eight polycyclic aromatic hydrocarbons (PAHs) in rainbow trout (*Salmo gairdneri*). Water Research. 20: 503-507.

Ogata, N., Matsushima, N., and Shibata, T. 1995. Pharmacokinetics of wood creosote: glucuronic acid and sulfate conjugation of phenolic compounds. Pharmacology. 51: 195-204.

Okey, A.B., Riddick, D.S., and Harper, P.A. 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Toxicology Letters. 70: 1-22.

Petty, J.D. and Huckins, J. 1994. SPMD Technology Workshop. Columbia, Missouri.

Prest, H.F., Jarman, W.M., Burns, S.A., Weismuller, T., Martin, M., and Huckins, J.N. 1992. Passive water sampling via semipermeable membrane devices (SPMDs) in concert with bivalves in the Sacramento/San Joaquin River Delta. Chemosphere. 25: 1811-1823.

Roberts, M.H., Hargis, W.J., Jr., Strobel, C.J., and DeLisle, P.F. 1989. Acute toxicity of PAH contaminated sediments to the estuarine fish, *Leiostomus xanthurus*. Bulletin of Environmental Contamination and Toxicology. 42: 142-149.

Sadar, M.D., Ash, R., Sundqvist, J., Olsson, P.E., and Andersson, T.B. 1996. Phenobarbital induction of CYP1A1 gene expression in a primary culture of rainbow trout hepatocytes. The Journal of Biological Chemistry. 271: 17635-17643.

Safe, S. 1987. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. Chemosphere. 16: 791-802.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology. 21: 51-88.

Stegeman, J.J. 1989. Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. Xenobiotica. 19: 1093-1110.

Stegeman, J.J. and Hahn, M.E. 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: CRC Press, Boca Raton, Florida, pp. 87-206.

Thompson, D.G., Holmes, S.B., Pitt, D.G., Solomon, K.R., and Wainio-Keizer, K.L. 1994. Applying concentration-response theory to aquatic enclosure studies. In: Aquatic mesocosm studies in ecological risk assessment. R.L. Graney, J.H. Kennedy, and J.H. Rodgers, Jr. eds. Lewis Publishers, Boca Raton, Fl. pp. 129-156.

Tillitt, D.E., Ankley, G.T., Verbrugge, D.A., Giesy, J.P., Ludwig, J.P., and Kubiak, T.J. 1991a. H4IIE rat hepatoma cell bioassay-derived 2,3,7,8- tetrachlorodibenzo-*p*-dioxin equivalents in colonial fish-eating waterbird eggs from the Great Lakes. Archives of Environmental Contamination and Toxicology. 21: 91-101.

Tillitt, D.E., Giesy, J.P., and Ankley, G.T. 1991b. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environmental Science and Technology. 25: 87-92.

U.S.Environmental Protection Agency. 1988. Health Effects Assessment for Dibenzofuran. EPA/600/8-88/026.

Van Veld, P.A., Vogelbein, W.K., Smolowitz, R., Woodin, B.R., and Stegeman, J.J. 1992. Cytochrome P450IA1 in hepatic lesions of a teleost fish (*Fundulus heteroclitus*) collected from a polycyclic aromatic hydrocarbon-contaminated site. Carcinogenesis. 13: 505-507.

Von Burg, R. and Stout, T. 1992. Creosote. Journal of Applied Toxicology. 12: 153-156.

GENERAL CONCLUSIONS

Estimating the uptake and biological effect of chemical mixtures on aquatic organisms is complicated due the wide range of physical and biological properties of each chemical and the potential interactions among them. The goal of this thesis is to combine a variety of assessment techniques in order to evaluate the strengths and weaknesses of individual procedures and combinations of procedures in estimating the exposure of fish to contaminant mixtures. Two specific families of chemicals were examined: the planar halogenated hydrocarbons (PCHs) and polycyclic aromatic hydrocarbons (PAHs).

A. Additive Versus Non-additive Responses to PCH and PAH Mixtures

Comparison of bioassay and chemically derived (BD and CD) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations (TECs) revealed that in most cases, both methods produced similar results, indicating that most of the AhR-active compounds in the samples were accounted for by the congener analysis and that these compounds acted in an additive fashion (no synergism and antagonism). Using both techniques did prove important in cases where values did differ, for instance in revealing the presence of unknown EROD inducers, or inducers that are below the detection limits of analytical chemistry techniques. The conclusion from this portion of the thesis is that one should still perform BD- and CD-TEC comparisons because each new sample raises the possibility that one or more non-identified inducers are present.

B. Species Specificity in Response to PCHs and PAHs

At first examination, the choice of mammalian versus piscine cell line did not appear to have a large impact on the TECs determined for samples. However, this similarity is misleading. For PCHs, generally the TEFs from RTL-W1 are higher with furans (PCDFs), and the TEFs from H4IIE are higher with PCBs. When mixtures of chemicals are examined, the contributions of congeners to the total TEC will vary between cell lines, and can potentially mask differences between the two. For PPPAH extracts, differences existed between H4IIE and RTL-W1 TECs, although it was not clear if this was due to the methodology of TEF determination or subtle differences in the mammalian and piscine AhR signal transduction systems. The results of these studies indicate that RTL-W1 may be more appropriate than H4IIE for assessing the toxic impact of chemical mixtures to rainbow trout. A closer examination of the molecular mechanisms underlying PCH and PAH induction of CYP1A in mammals and fish is warranted. Regardless of which cell line is used, the ease and rapidness with which in vitro assays can be performed make them convenient tools for determining the potencies of environmental samples.

C. Hepatic EROD Activity as an Indicator of Exposure to PCHs and PAHs

This thesis demonstrated the complexity of hepatic EROD activity as an indicator of PCH and PAH exposure. Under relatively controlled conditions (microcosm exposures), hepatic EROD was closely related to creosote exposure in both concentration- and time-based studies. But examination of EROD in trout collected from the field (Chapter 1) did not correlate well with PCH burden in the fish. This study confirmed the importance of the effect of modifying factors, both physical and biological, on hepatic CYP1A activity.

Hepatic EROD was especially important in the examination of PPPAH exposure, by providing an indication of chemical exposure even though the chemical analysis did not. In addition, the effect of exposure time on EROD activity (Chapter 3) has important implications for biomonitoring studies. For example, if trout are exposed to a pulse of chemical, the response within the first 7 days is critical and may be missed if only one measurement is taken. This rapid induction may also prove important if the pulse occurs at a critical time in development (e.g. early life stage). It is concluded that although hepatic EROD induction does not imply toxicity, its value as an indicator of exposure should not be overlooked, especially in light of its ease of measurement.

D. Use of SPMDs as Predictors of Exposure to PPPAHs

The value of SPMDs in predicting uptake of bioavailable contaminants was found to be particularly well suited for studying PPPAH contamination. The metabolism of these compounds in living organisms makes estimating the degree of exposure difficult. When used in conjunction with biological indicators of exposure (hepatic EROD and BD-TECs) SPMDs provided valuable information on the types and concentrations of chemicals accumulated in trout. Although SPMDs have demonstrated utility, it is important to recognize their limitations and distinct differences from biological systems. The use of SPMDs in conjunction with co-exposed organisms appears to provide the most complete representation of chemical exposure.

E. Summary

The contamination of the environment with anthropogenic compounds has necessitated the development of tools to assess the exposure and effects of diverse combinations of chemicals on aquatic species. Although these methods can supply valuable information, no single technique can provide a comprehensive solution to this complex problem. The use of a variety of approaches together can give a more well-rounded appraisal of chemical uptake and effects. The continued study and improvement of the methods employed in this thesis will provide even greater insight to the toxicological significance of chemical mixtures.

APPENDIX I

Tables B.1 and B.2. Priority pollutant polycyclic aromatic hydrocarbon and non-priority aromatic compound concentrations in SPMDs and trout tissue.

Table B 1. Mean concentrations (S E , n=3, µg/g) of priority pollutant PAHs (PPPAHs) in lipid extracted from semipermeable membrane devices exposed to creosote dosed microcosms (0, 3, and 10 µL/L creosote) over a 28 day period

PAH	Percent Recovery	Day 0	Day 7			Day 14		
			0 µL/L	3 µL/L	10 µL/L	0 µL/L	3 µL/L	10 µL/L
naphthalene	41 7%	nd[1]	nd	nd	nd	nd	nd	nd
acenaphthylene	58 2%	nd	0 047 (0 002)	0 177 (0 019)	0 117 (0 010)	0 061 (0 004)	0 227 (0 013)	0 164 (0 006)
acenaphthene	68 3%	nd	nd	0 170 (0 030)	0 115 (0 015)	nd	0 304 (0 029)	0 206 (0 005)
fluorene	42 0%	nd	0 023 (0 002)	0 199 (0 020)	0 164 (0 009)	0 048 (0 002)	0 284 (0 014)	0 272 (0 007)
phenanthrene	85 6%	nd	0 014 (0 002)	0 345 (0 032)	0 139 (0 004)	0 043 (0 003)	0 515 (0 012)	0 261 (0 008)
anthracene	82 5%	nd	nd	0 027 (0 016)	0 053 (0 002)	nd	0 066 (0 005)	0 084 (0 003)
fluoranthene	86 2%	nd	0 032 (0 005)	0 717 (0 064)	0 464 (0 018)	0 077 (0 008)	0 995 (0 028)	0 885 (0 024)
pyrene	100 6%	nd	0 060 (0 004)	0 519 (0 119)	0 725 (0 033)	0 131 (0 007)	0 501 (0 021)	1 466 (0 042)
benz[a]anthracene*	89 6%	nd	nd	0 016 (0 009)	0 014 (0 008)	nd	0 020 (0 011)	nd
chrysene*	85 9%	nd	0 022 (0 005)	0 212 (0 055)	0 105 (0 003)	0 068 (0 011)	0 220 (0 018)	0 269 (0 012)
benzo[b+k]fluoranthene*	92 0%	nd	nd	nd	nd	nd	nd	nd
benzo[a]pyrene*	95 4%	nd	nd	nd	nd	nd	nd	nd
dibenzo[a,h]anthracene	92 5%	nd	nd	nd	nd	nd	nd	nd
indeno[1,2,3-cd]pyrene	87 2%	nd	nd	nd	nd	nd	nd	nd
benzo[g,h,i]perylene	86 2%	nd	nd	nd	nd	nd	nd	nd
Σ PAH		0 000	0 196	2 382	1 896	0 429	3 132	3 606

	Day 21			Day 28		
	0 µL/L	3 µL/L	10 µL/L	0 µL/L	3 µL/L	10 µL/L
naphthalene	nd	nd	0 019 (0 011)	nd	nd	nd
acenaphthylene	0 095 (0 006)	0 236 (0 013)	0 227 (0 011)	0 065 (0 007)	0 182 (0 008)	0 169 (0 023)
acenaphthene	nd	0 289 (0 011)	0 284 (0 011)	nd	0 229 (0 033)	0 227 (0 033)
fluorene	0 035 (0 009)	0 309 (0 017)	0 297 (0 008)	0 028 (0 016)	0 261 (0 025)	0 250 (0 021)
phenanthrene	0 035 (0 003)	0 489 (0 019)	0 259 (0 003)	0 041 (0 001)	0 387 (0 036)	0 248 (0 018)
anthracene	nd	0 067 (0 001)	0 083 (0 001)	nd	0 057 (0 003)	0 074 (0 003)
fluoranthene	0 054 (0 006)	1 134 (0 014)	0 817 (0 010)	0 069 (0 004)	0 773 (0 099)	0 912 (0 056)
pyrene	0 092 (0 012)	0 710 (0 142)	1 288 (0 033)	0 093 (0 052)	0 346 (0 047)	1 319 (0 059)
benz[a]anthracene*	nd	nd	nd	0 082 (0 016)	nd	nd
chrysene*	0 042 (0 006)	0 489 (0 112)	0 217 (0 002)	0 092 (0 025)	0 199 (0 017)	0 236 (0 013)
benzo[b+k]fluoranthene*	nd	nd	nd	nd	nd	nd
benzo[a]pyrene*	nd	nd	nd	nd	nd	nd
dibenzo[a,h]anthracene	nd	nd	nd	nd	nd	nd
indeno[1,2,3-cd]pyrene	nd	nd	nd	nd	nd	nd
benzo[g,h,i]perylene	nd	nd	nd	nd	nd	nd
Σ PAH	0 354	3 723	3 491	0 469	2 434	3 435

* Compound induces EROD activity in RTL-W1

[1] Indicates that the compound was not detectable at quantifiable concentration

Table B 2. Mean concentrations of selected non-priority aromatic hydrocarbons in lipid extracted from semipermeable membrane devices (S.E., n=3, μg/g) and livers of rainbow trout (S.E., n=6, μg/g) exposed to creosote dosed microcosms (0, 3, and 10 μL/L creosote) over a 28 day period

	Day 0	Day 7			Day 14		
SPMD		0 μL/L	3 μL/L	10 μL/L	0 μL/L	3 μL/L	10 μL/L
Aromatics							
2-methyl-naphthalene + indole	nd[1]	nd	0.063 (0.037)	0.047 (0.028)	nd	0.181 (0.064)	0.376 (0.012)
1-methyl-naphthalene	nd	nd	0.045 (0.026)	0.033 (0.019)	nd	0.094 (0.016)	0.069 (0.003)
biphenyl	nd	0.105 (0.010)	0.378 (0.046)	0.244 (0.049)	0.174 (0.016)	0.945 (0.017)	0.733 (0.099)
Heterocyclics							
dibenzofuran	nd	nd	0.286 (0.037)	0.227 (0.025)	0.035 (0.008)	0.322 (0.022)	0.392 (0.010)
carbazole	nd	nd	nd	nd	nd	nd	0.062 (0.007)
Rainbow Trout							
Aromatics							
2-methyl-naphthalene + indole	3.586 (0.544)	2.115 (0.250)	1.392 (0.102)	1.510 (0.113)	2.424 (0.429)	0.903 (0.103)	0.965 (0.062)
1-methyl-naphthalene	nd	0.420 (0.091)	0.288 (0.060)	0.233 (0.057)	0.569 (0.096)	0.215 (0.083)	0.386 (0.057)
biphenyl	1.829 (0.557)	2.749 (0.261)	1.001 (0.128)	0.910 (0.083)	1.205 (0.217)	0.633 (0.171)	0.972 (0.082)
Heterocyclics							
dibenzofuran	0.272 (0.133)	0.447 (0.157)	0.640 (0.055)	0.483 (0.135)	0.817 (0.188)	0.408 (0.053)	0.500 (0.037)
carbazole	nd	nd	nd	nd	nd	nd	nd

	Day 21			Day 28		
SPMD	0 μL/L	3 μL/L	10 μL/L	0 μL/L	3 μL/L	10 μL/L
Aromatics						
2-methyl-naphthalene + indole	nd	0.246 (0.021)	0.358 (0.002)	nd	0.107 (0.035)	0.149 (0.042)
1-methyl-naphthalene	nd	0.118 (0.002)	0.133 (0.006)	nd	0.078 (0.018)	0.089 (0.008)
biphenyl	0.312 (0.022)	0.978 (0.059)	1.219 (0.102)	0.199 (0.003)	0.734 (0.186)	0.300 (0.021)
Heterocyclics						
dibenzofuran	0.047 (0.010)	0.394 (0.026)	0.457 (0.002)	0.049 (0.008)	0.318 (0.018)	0.327 (0.015)
carbazole	nd	0.080 (0.025)	0.061 (0.018)	nd	0.036 (0.020)	0.050 (0.004)
Rainbow Trout						
Aromatics						
2-methyl-naphthalene + indole	0.655 (0.147)	1.447 (0.144)	1.093 (0.111)	1.567 (0.160)	0.811 (0.108)	1.054 (0.120)
1-methyl-naphthalene	nd	0.475 (0.029)	0.312 (0.045)	0.538 (0.172)	0.147 (0.047)	0.271 (0.064)
biphenyl	nd	1.476 (0.054)	0.858 (0.124)	1.017 (0.246)	0.548 (0.106)	0.838 (0.093)
Heterocyclics						
dibenzofuran	0.053 (0.053)	0.802 (0.075)	0.581 (0.059)	0.885 (0.133)	0.344 (0.036)	0.590 (0.086)
carbazole	nd	nd	nd	nd	nd	nd

[1] Indicates that the compound was not detectable at quantifiable concentration

APPENDIX II

H4IIE and RTL-W1
7-ethoxyresorufin-*O*-deethylase (EROD) Cell Line Bioassay
Standard Operating Procedure

I. Introduction

The deethylation of 7-ethoxyresorufin to resorufin is catalyzed by the major form of an arylhydrocarbon-inducible cytochrome P450 enzyme. The formal name for this enzyme is CYP1A. Of the many substrates used for the measurement of CYP1A activity, 7-ethoxyresorufin is becoming the method of choice. Advantages include: 1) high sensitivity; 2) simple to perform; 3) high specificity for the CYP1A enzyme; 4) the substrate and product pose little occupational hazard; 5) the reaction has a single metabolite and 6) the product can be differentiated from the substrate by a simple fluorometric analysis that has minimal interference.

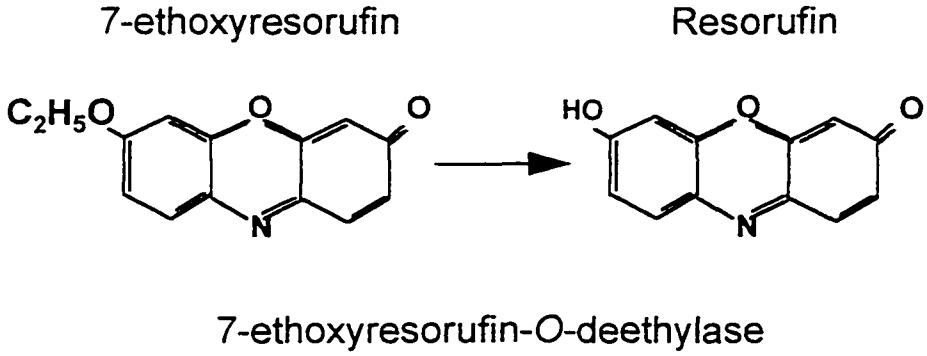


Figure C.1. Structural formulae for 7-ethoxyresorufin and resorufin.

The EROD analysis was first published by Burke and Mayer (1974). The method used in this operating procedure should be quoted as a modification of Clemons et al. (1995) and Kennedy et al. (1993).

II. Materials

A. Chemicals

1. 7-Ethoxyresorufin (MW = 241.2, Sigma, E-3763, 1 g)
2. Dicumarol (3,3'-methylene-bis(4-hydroxy coumarin) (MW = 336.3, Sigma M-1390, 1 g)
3. Dulbecco's phosphate buffered saline (PBS) (Sigma, D-5527, 500 mL, case of 6)
4. Resorufin (MW = 235.2, Sigma R-3257, 1g)
5. Fluorescamine (MW = 278.27, Aldrich, 20-165-0, 500mg, case of 4)
6. Bovine serum albumin (fraction V, Sigma A-3675, 500 mL, case of 2)
7. Potassium phosphate, monobasic (MW = 136.1, Sigma, P-0662, 500 g)
8. Potassium phosphate, dibasic (MW = 174.2, Sigma, P-3786, 500 g)
9. Dulbecco's modified Eagle's medium (Sigma, 5286, case of 12)
10. Leibovitz's L-15 medium (Gibco BRL)
11. Penicillin/Streptomycin (ICN/Flow)
12. Gentamycin sulfate (Sigma G-1397, case of 10)
13. Fetal bovine serum (Gibco, 26140-079, 500 mL, case of 3) [keep at -20°C]
14. Minimum essential medium essential amino acids (Sigma, M-7020, 100 mL, case of 6)
15. Minimum essential medium non-essential amino acids (Sigma, M-7145, 100 mL, case of 6)
16. L-glutamine (G-6392, powder in vial, case of 6)
17. Minimum essential medium vitamins (M-6895, case of 6) [keep at -20°C]
18. Methanol
19. Ethanol
20. Dimethylsulfoxide (DMSO) (MW = 78.13, BDH Assured ACS, 306-76, 1L)
21. Trypsin-EDTA 1x (Sigma, T-3924, 100 mL, case of 6)
22. Carbon dioxide gas (Praxair)
23. Nitrogen gas (Praxair, UHP)
24. Acetone
25. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (500 ng/mL in nonane, 100 mL, Cambridge Isotope Laboratory, ED-901)

B. Plastics and Glassware

1. 48-Well culture clusters (Costar, CS003548, Case of 100)
2. Sterile pipettes (10 mL, Baxter, case of 500)
3. Sterile centrifuge tubes (10 and 50 mL, case of 100 each)
4. Haemacytometer (1)
5. Combitips® (100, 250, 500 µL, Eppendorf, case of 100 each)
6. Sterile micropipette tips (for 1000 and 50 µL micropipettor, Eppendorf, case of 100 each)
7. Sterile Pasteur pipettes and bulbs
8. Plastic squeeze bottle (for ethanol)
9. Plastic weigh pans and weigh paper (Baxter)
10. Cryovials (Nunc, 66021-986, pack of 450)

11. Culture flasks (75 cm^2, Costar, CS003375, case of 100)
12. Parafilm
13. mL syringe (pack of 20)
14. Amber vials and caps (4 mL, Aldrich, 16161-064, 60815-1545 case of 100 each)

C. Equipment

15. Repeating pipettor (Eppendorf)
16. 37°C Cell incubator (Ultratech, W5 301T)
17. Centrifuge
18. pH meter
19. Cytofluor® 2350 fluorescence measurement system (Perspective Biosystems)
20. Laminar flow hood (SterilGARD, Class II Type A/B3, Baker Co. Ph. 207-324-8773)
21. Pipette-aid® (Fisher, 13-681-19)
22. Aspirator
23. Microscope
24. Micropipettor (1000 and 50 µL, Eppendorf)
25. Digital balance (Sartorius)
26. Freezer (-80°C, Sanyo)
27. Water bath (37°C)
28. Bunsen burner
29. Orbital shaking incubator (Thermolyne, ROSI 1000)
30. Vortex
31. Positive displacement microcapillary pipette (Nichiryo, Model 800)
32. Microcapillary pipette capillary tubes (Nichiryo, CAP-9, 5 µL volume, pack of 100)

III. Procedure

Section 1. Cell Line Maintenance

A. Preparation of H4IIE Cell Media

1. Obtain the following: 500 mL Dulbecco's modified Eagle's medium (hereafter referred to only as "medium"), gentamycin sulfate, L-glutamine, MEM non-essential amino acids, MEM essential amino acids, MEM vitamins, and 75 mL fetal bovine serum (FBS).
2. Allow MEM vitamins and FBS to thaw.
3. Turn on airflow in laminar hood and wipe the interior with 70% ethanol.
4. Wipe reagent bottles with ethanol and place under the hood.
5. Ignite Bunsen burner under hood.
6. Flame 10 mL pipette box to sterilize and open under hood.
7. Flame the necks of the reagent bottles.
8. Using a 1.0 mL syringe, add 0.50 mL gentamycin sulfate to the medium.
9. Using a flamed 10 mL pipette, transfer 10 mL medium to the L-glutamine powder and pour the dissolved solution into the medium bottle.
10. Using a flamed 10 mL pipette, transfer 10 mL MEM non-essential amino acids to the medium.
11. Using a flamed 10 mL pipette, transfer 15 mL MEM essential amino acids to the medium.
12. Using a flamed 10 mL pipette, transfer 7.5 mL MEM vitamins to the medium.
13. Flame the cap of the FBS tubes and transfer their contents to the medium.
14. Gently rock the medium bottle to mix the contents.
15. Label the media bottle (cell type, 15% FBS, date and name), flame the neck and cover with parafilm.
16. The media can be refrigerated until needed.
17. Clean up: Flame the mouths of the reagent bottles, reseal with parafilm and place in refrigerator/freezer, flame the pipette box and close, turn off the Bunsen burner, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

B. Preparation of RTL-W1 Cell Media

1. Obtain the following: 500 mL Lebovitz's L-15 medium, 10 mL penicillin/streptomycin, and 25 mL fetal bovine serum (FBS).
2. Allow FBS to thaw.
3. Turn on airflow in laminar hood and wipe the interior with 70% ethanol.
4. Wipe reagent bottles with ethanol and place under the hood.
5. Ignite Bunsen burner under hood.
6. Flame 10 mL pipette box to sterilize and open under hood.
7. Flame the necks of the reagent bottles.
8. Flame the cap of the penicillin/streptomycin tube and add 10 mL to the medium.
9. Flame the cap of the FBS tube and transfer its contents to the medium.
10. Gently rock the medium bottle to mix the contents.

11. Label the media bottle (cell type, 5% FBS, date and name), flame the neck and cover with parafilm.
12. The media can be refrigerated until needed.
13. Clean up: Flame the mouths of the reagent bottles, reseal with parafilm and place in refrigerator/freezer, flame the pipette box and close, turn off the Bunsen burner, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

C. Thawing of Frozen Cells

Day 1

1. Place media in a 37°C water bath for 1 hour.
2. Remove a cyrovial of cells from the -80°C freezer and place in a 37°C water bath.
3. Once cells have thawed, transfer to a beaker of 70% ethanol to sterilize.
4. Turn on airflow in laminar hood and wipe the interior with 70% ethanol.
5. Ignite Bunsen burner under hood.
6. Flame 10 mL pipette box to sterilize and open under hood.
7. Label a culture flask with cell type, passage number and date and place under the hood.
8. Remove media from water bath, wipe with ethanol and place under hood.
9. Remove cryovial from ethanol, wipe dry and place under the hood.
10. Using a 1000 µL micropipettor and sterile tip, transfer the contents of the cryovial to the culture flask.
11. Remove the parafilm from the media bottle, flame the neck and open the bottle.
12. Using a flamed 10 mL pipette, transfer 10 mL of media to the culture flask and cap the flask.
13. Place the culture flask in an incubator and grow H4IIE at 37°C in 5% CO_2 atmosphere, RTL-W1 at 22°C ± 1 in a pure air environment.
14. Clean up: Flame the mouth of the media, reseal with parafilm and place in refrigerator, flame the pipette box and close, turn off the Bunsen burner, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

Day 2

1. Warm media and phosphate buffered saline (PBS) and sterilize hood as is described in the day 1 procedure.
2. Flame Pasteur pipette box and 10 mL pipette box to sterilize and open under hood.
3. Start aspirator by turning on water.
4. Sterilize media and PBS bottles and open under the hood.
5. Remove cells in culture flask from incubator and place under the hood.
6. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the media from the culture flask.
7. Flame a 10 mL pipette and transfer 2 mL PBS to the culture flask. Cap the flask and gently rock it back and forth to rinse it.
8. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the PBS from the culture flask.
9. Flame a 10 mL pipette and transfer 10 mL media to the culture flask and cap it.
10. Label the flask with the symbol "Δ" and the date to indicate a media change.
11. Place the culture flask in an incubator and grow H4IIE at 37°C in 5% CO_2 atmosphere, RTL-W1 at 22°C ± 1 in a pure air environment.
12. Clean up: Flame the mouth of the media, reseal with parafilm and place in refrigerator, flame the pipette box and close, turn off the Bunsen burner, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

13. Cells should be ready for their first passage in about 3-4 days. If they are not confluent by this point, repeat this media change procedure and let cells grow another 3 days.

D. Passaging of Cells

1. For H4IIE, place media, PBS and trypsin-EDTA in a 37°C water bath for 1 hour. For RTL-W1, allow reagents to reach room temperature.
2. When solutions are warmed, turn on airflow in laminar hood and wipe the interior with 70% ethanol.
3. Ignite Bunsen burner under hood, start water for aspirator and turn on pipette-aid.
4. Flame Pasteur pipette box and 10 mL pipette box to sterilize and open under hood.
5. Place a sterile 15 mL centrifuge tube under the hood.
6. Wipe solutions with 70% ethanol and place under hood. Remove parafilm from bottles and flame bottle necks.
7. Remove culture flask from incubator and place under the hood.
8. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the media from the culture flask.
9. Flame a 10 mL pipette and transfer 2 mL PBS to the culture flask. Cap the flask and gently rock it back and forth to rinse it.
10. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the PBS from the culture flask.
11. Flame a 10 mL pipette and transfer 2 mL trypsin-EDTA to the culture flask. Cap the flask and gently rock it back and forth. Allow it to sit for 10-15 min until trypsin-EDTA has dislodged the cells from the culture flask surface. This can be checked under the microscope (10x magnification).
12. Flame a 10 mL pipette and add 8 mL media to the culture flask to bring the volume in the flask to 10 mL. Using the pipette, draw up the 10 mL solution and expel it back into the flask 2 times to fully dislodge the cells, then draw up the cells suspension and transfer it to a sterile 15 mL centrifuge tube.
13. Cap the tube and centrifuge it at 500g for 5 min.
14. Under the hood, flame a Pasteur pipette and aspirate the media from the centrifuge tube, taking care not to aspirate the cell pellet.
15. Using a flamed 10 mL pipette, gently resuspend the pellet in 5 mL media.
16. Transfer 1 mL of the cell suspension to the culture flask. If you want to increase the number of flasks you are maintaining, label new culture flasks and transfer 1 mL to them as well. Otherwise, the remaining suspension can be discarded.
17. Add 9 mL of media to the culture flask and recap it. Label the flask with the new passage number.
18. Place the culture flask in an incubator and grow H4IIE at 37°C in 5% CO_2 atmosphere, RTL-W1 at 22°C ± 1 in a pure air environment.
19. Clean up: Flame the mouths of the bottles, reseal with parafilm and place in refrigerator, flame the pipette box and close, turn off the Bunsen burner and aspirator, wipe the bench down with 70% ethanol and turn off the laminar flow hood.
20. To maintain healthy cultures, passage the cells every 3-4 days.

E. Cryopreservation of Cells

Note: Every few weeks, to guard against loss of your culture due to contamination, you should freeze some cells for later use.

1. For H4IIE, place media, PBS and trypsin-EDTA in a 37°C water bath for 1 hour. For RTL-W1, allow reagents to reach room temperature.
2. When solutions are warmed, turn on airflow in laminar hood and wipe the interior with 70% ethanol.
3. Ignite Bunsen burner under hood, start water for aspirator and turn on pipette-aid.
4. Flame Pasteur pipette box and 10 mL pipette box to sterilize and open under hood.
5. Place a sterile 15 mL centrifuge tube and sterile cryovial under the hood.
6. Wipe solutions with 70% ethanol and place under hood. Remove parafilm from bottles and flame bottle necks.
7. Wearing gloves, place a small tube of sterile DMSO (500 µL) under the hood.
8. Remove one of your culture flasks from incubator and place under the hood.
9. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the media from the culture flask.
10. Flame a 10 mL pipette and transfer 2 mL PBS to the culture flask. Cap the flask and gently rock it back and forth to rinse it.
11. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the PBS from the culture flask.
12. Flame a 10 mL pipette and transfer 2 mL trypsin-EDTA to the culture flask. Cap the flask and gently rock it back and forth. Allow it to sit for 10-15 min until trypsin-EDTA has dislodged the cells from the culture flask surface. This can be checked under the microscope (10x magnification).
13. Flame a 10 mL pipette and add 8 mL media to the culture flask to bring the volume in the flask to 10 mL. Using the pipette, draw up the 10 mL solution and expel it back into the flask 2 times to fully dislodge the cells, then draw up the cells suspension and transfer it to a sterile 15 mL centrifuge tube.
14. Cap the tube and centrifuge it at 500g for 5 min.
15. Under the hood, flame a Pasteur pipette and aspirate the media from the centrifuge tube, taking care not to aspirate the cell pellet.
16. Using a 1000 µL micropipettor, transfer 900 µL media to the centrifuge tube and gently resuspend the pellet.
17. Wearing gloves, add 100 µL DMSO to the centrifuge tube. Set micropipettor to 1000 µL and gently mix the solution in the tube.
18. Transfer the cell suspension to a labeled cryovial (cell type, last passage number and date) and cap it.
19. Place the cells in a Styrofoam container and put in a -80°C freezer for 24 h. After this period, the cells can be placed in a cryovial holder and stored at -80°C until needed.
20. Clean up: Flame the mouths of the bottles, reseal with parafilm and place in refrigerator, flame the pipette box and close, turn off the Bunsen burner and aspirator, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

Section 2. Plating of Cells for Toxicant Exposure

Note: Cells are plated in multiwell plates at a density of 40,000 cells per well.

1. For H4IIE, place media, PBS and trypsin-EDTA in a 37°C water bath for 1 hour. For RTL-W1, allow reagents to reach room temperature.
2. When solutions are warmed, turn on airflow in laminar hood and wipe the interior with 70% ethanol.
3. Ignite Bunsen burner under hood, start water for aspirator and turn on pipette-aid.
4. Flame Pasteur pipette box and 10 mL pipette box to sterilize and open under hood.
5. Place a sterile 15 mL centrifuge tube under the hood.
6. Wipe solutions with 70% ethanol and place under hood. Remove parafilm from bottles and flame bottle necks.
7. Remove culture flask from incubator and place under the hood.
8. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the media from the culture flask.
9. Flame a 10 mL pipette and transfer 2 mL PBS to the culture flask. Cap the flask and gently rock it back and forth to rinse it.
10. Flame a Pasteur pipette, bending the tip with the flame, and aspirate the PBS from the culture flask.
11. Flame a 10 mL pipette and transfer 2 mL trypsin-EDTA to the culture flask. Cap the flask and gently rock it back and forth. Allow it to sit for 10-15 min until trypsin-EDTA has dislodged the cells from the culture flask surface. This can be checked under the microscope (10x magnification).
12. Flame a 10 mL pipette and add 8 mL media to the culture flask to bring the volume in the flask to 10 mL. Using the pipette, draw up the 10 mL solution and expel it back into the flask 2 times to fully dislodge the cells, then draw up the cells suspension and transfer it to a sterile 15 mL centrifuge tube.
13. Cap the tube and centrifuge it at 500g for 5 min.
14. While the centrifuge is running, clean the haemacytometer and its associated cover slip with ethanol and allow to air dry under the hood.
15. Under the hood, flame a Pasteur pipette and aspirate the media from the centrifuge tube, taking care not to aspirate the cell pellet.
16. Using a flamed 10 mL pipette, gently resuspend the pellet in 5 mL media.
17. Using a flamed Pasteur pipette and sterile bulb, transfer a drop of the cell suspension to one end of the haemacytometer, and then repeat for the other end.
18. Under the microscope, count the cells in 5 of the 25 squares in the double-lined box. Multiply this number by 50,000 and by the volume of the cell suspension. For example for a count of 150 cells: 150 cells x 50,000 x 5 mL = 37,500,000 cells.
19. Repeat this for the other end of the haemacytometer and average the 2 counts.
20. To determine the number of cells needed for the assay, multiply the number of plates by 48 wells/plate and by 40,000 cells/well. For example, if 4 plates were going to be used for the assay: 4 plates x 48 wells/plate x 40,000 cells/well = 7,680,000 cells. Note: with each assay, a resorufin standard curve plate will be prepared. Include this plate when

performing these calculations (for example, if you need 3 plates for your assay, you will actually prepare 4 plates).

21. The volume of cells needed from the cell suspension to give us the desired number of cells to be plated is calculated by dividing the number of cells needed by the number of cells in the suspension and then multiplied by the volume of the suspension. For our example above: (7,680,000 cells / 37,500,000 cells) x 5 mL = 1.024 mL.
22. To determine the volume of media needed, multiply the number of plates by 48 wells/plate and then by 0.5 mL/well (e.g. 4 plates x 48 wells/plate x 0.5 mL/well = 96 mL).
23. Using a micropipettor and sterile tip, transfer the calculated volume of the cell suspension and necessary media to an autoclaved beaker. For our example above, 1.024 mL of cell suspension would be added to ≈ 96 mL of media in an autoclaved beaker.
24. One mL of the remaining cell suspension can be place back in the culture flask with 9 mL of media, labeled with the new passage number and placed in the 37°C incubator.
25. Using a repeating pipettor and a sterile 250 µL combitip, add 500 µL of the cell suspension to each well in a 48 well plate. To keep the cells from settling in the beaker, only add the suspension to 16 wells at a time, then expel the contents back into the beaker to resuspend the cells. Then continue with the next 16 wells.
26. Repeat this for each plate to be used.
27. For the resorufin standard curve plate, add 500 µL of the cell suspension to each of the wells in the top half of the plate (i.e. the top 24 wells). To the bottom 24 wells, add 500 µL of media (no cells) for the protein standard curve.
28. Label the plates (Cell type and date) and place in 37°C incubator for 24 h.
29. Clean up: Flame the mouths of the bottles, reseal with parafilm and place in refrigerator, flame the pipette box and close, turn off the Bunsen burner and aspirator, wipe the bench down with 70% ethanol and turn off the laminar flow hood.

Section 3. Toxicant Exposure

A. Preparation of 2,3,7,8-TCDD Standards

Note: This work should be done under the fume hood and wearing gloves.

1. Using a 10-100 µL micropipettor, transfer 50.0 µL of the 500 ng/mL 2,3,7,8-TCDD stock solution to a 2 mL amber vial. Re-seal the TCDD stock solution, cover in foil and store at 4°C.
2. Using a Pasteur pipette connected to a nitrogen gas cylinder, direct a steady, but gentle stream of nitrogen into the vial to evaporate the nonane to near dryness.
3. Using a 100-1000 µL micropipettor, transfer 1 mL DMSO to the amber vial containing 2,3,7,8-TCDD. Cap the vial and vortex for 60 s. Label this vial as 25 ng/mL 2,3,7,8-TCDD.
4. Obtain 7 clean amber vials and caps. Using a 100-1000 µL micropipettor, transfer 500 µL DMSO to each of the 7 vials and cap them.
5. Label the vials with the following concentrations: 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 ng/mL 2,3,7,8-TCDD and the final vial is labeled DMSO control.
6. Using a 100-1000 µL micropipettor and a clean pipette tip, transfer 500 µL from the 25 ng/mL solution to the vial labeled 12.5 ng/mL 2,3,7,8-TCDD. Cap both vials and vortex the 12.5 ng/mL 2,3,7,8-TCDD solution for 60 s.
7. Using a 100-1000 µL micropipettor and a clean pipette tip, transfer 500 µL from the 12.5 ng/mL solution to the vial labeled 6.25 ng/mL 2,3,7,8-TCDD. Cap both vials and vortex the 6.25 ng/mL 2,3,7,8-TCDD solution for 60 s.
8. Continue the above process for each concentration. The DMSO control has nothing added to it.
9. Take care that pipette tips are properly disposed of in the hazardous waste disposal container.
10. 2,3,7,8-TCDD standards can be stored at room temperature in the dark.

B. Dosing of Cells with Standard and Toxicant

1. Turn on airflow in a vertical flow hood and wipe the interior with 70% ethanol.
2. After 24 h of incubation, plated cells can be transferred to the vertical flow hood for toxicant dosing.
3. Obtain a capillary pipettor and set the volume to 2.5 µL.
4. Wearing gloves, place 2,3,7,8-TCDD standards and test toxicants in hood.
5. See figure 2 for plate dosing layout.
6. Using a capillary pipettor, transfer 2.5 µL from the DMSO control vial to each well in the first column of wells in plate #1. Note: Dose should be added to the centre of the well.
7. Working from lowest concentration to highest, transfer 2.5 µL from each 2,3,7,8-TCDD standard to the wells of the plate indicated in figure 2.
8. Remove the capillary tip form the pipettor and wipe the piston with a Kimwipe. Attach a new capillary tip to the pipettor.

9. Working from lowest concentration to highest, transfer 2.5 µL from each of the test toxicant concentrations to the wells of the plate indicated in figure 2.
10. Label the plate with the date of dosing, 2,3,7,8-TCDD standard and the test toxicant I.D.
11. Repeat the above procedure for each toxicant tested, using a new capillary tip for each sample. A 2,3,7,8-TCDD standard curve should be run with each plate until the assay has been fully established as reproducible in your lab.
12. For the resorufin and protein standard curve plate, transfer 2.5 µL from the DMSO control vial to each well of the plate.
13. When dosing is completed, plates are returned to a 37°C incubator for 24 h.
14. Clean up: Replace standards and toxicants in designated area, wipe hood with 70% ethanol and turn off air flow.

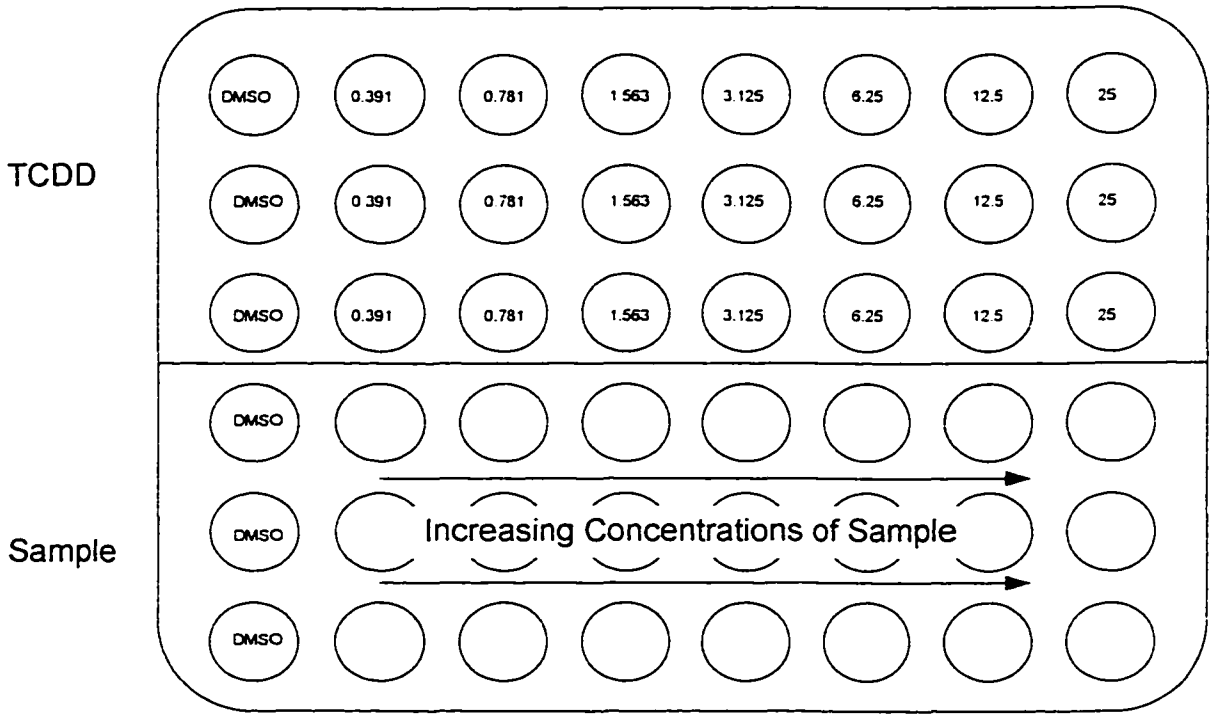


Figure 2. Plate layout for dosing H4IIE with toxicant (TCDD concentrations in ng/mL).

Section 4. EROD Activity Bioassay

A. Preparation of Stock Solutions

I. 7-Ethoxyresorufin solution
   A. Dissolve 2.0 mg of 7-ethoxyresorufin in 50 mL methanol.
   B. Label bottle as 40 µg/mL 7-ER and store in dark at 4°C.

II. Dicumarol solution
   A. Dissolve 165 mg of dicumarol in 50 mL methanol.
   B. Label bottle as 3.3 mg/mL Dicumarol and store in dark at 4°C.

III. Resorufin stock solution
   A. Dissolve 4.7 mg of resorufin in 100 mL methanol.
   B. Label bottle as 47 µg/mL resorufin and store in dark at 4°C.

B. Preparation of Resorufin Standards

1. Obtain 7 glass test tubes, label #1 to #7 and add 5.0 mL methanol to each.
2. Using a 10 mL pipette, transfer 5.0 mL of the resorufin stock solution (47 µg/mL) to tube #1 and vortex.
3. Using a *clean* 10 mL pipette, transfer 5.0 mL from tube #1 to tube #2 and vortex.
4. Using a *clean* 10 mL pipette, transfer 5.0 mL from tube #2 to tube #3 and vortex.
5. Repeat the above step until 5.0 mL has been added to tube #6. Do not add resorufin to tube #7, it will be used as a methanol control.
6. Obtain 7 15 mL amber vials and caps and label them as #1 - 100 µM resorufin in methanol, #2 - 50 µM resorufin in methanol, #3 - 25 µM resorufin in methanol, and so on. Label vial #7 as Methanol Control.
7. Store vials in dark at 4°C.

Resorufin Standard Concentrations

Vial	Concentration (µM)	Final concentration in Well (nM)
#1	100	497.1
#2	50.0	248.5
#3	25.0	124.3
#4	12.5	62.1
#5	6.2	31.1
#6	3.1	15.5
#7	0	0

C. Enzymatic Assay

a. Preparation of Solutions

I. For H4IIE, all solutions should be warmed to 37°C. For RTL-W1, all solutions should be warmed to 22°C.

II. Prepare the substrate-medium solution.
   A. Determine how much media you will need for the assay by multiplying 15 mL/plate x the number of plates dosed. Add 35 mL for the resorufin standard plate. Example: For 4 plates you would need 15 mL x 4 + 35 mL = 95 mL. Round up to 100 mL to make some excess.
   B. Under a flow hood, add the volume of media (MEM with no added solutions, no FBS) calculated to a foil covered beaker.
   C. For H4IIE, using a 100-1000 µL micropipettor, add a 333 x dilution of dicumarol stock to the media. For example, 300 µL of dicumarol would be added to 100 mL of media.
   D. For H4IIE, using a 100-1000 µL micropipettor, add a 33 x dilution of 7-ethoxyresorufin stock to the media. For example, 3.03 mL of 7-ethoxyresorufin would be added to 100 mL of media. For RTL-W1, using a 100-1000 µL micropipettor, add a 200 x dilution of 7-ethoxyresorufin stock to the media.
   E. Mix well and cover with foil.

III. Prepare the resorufin standards.
   A. Add 5 mL of the substrate-dicumarol medium solution to seven 25 mL beakers.
   B. Label the beakers from #1 to # 7.
   C. Using 10 - 100 µL micropipettor, transfer 25 µL of each resorufin standard in methanol to its corresponding beaker.
   D. Mix each beaker well and cover with foil.

IV. To determine how much PBS is needed for the assay, multiply the number of plates by 10 mL and then add 10 mL to this value for excess. Add the calculated volume of PBS to a beaker.

V. To determine how much deionized water is needed for the assay, multiply the number of plates by 15 mL and then add 10 mL to this value for excess. Add the calculated volume of deionized water to a beaker.

VI. For H4IIE, place all solutions in a 37°C orbital shaker. For RTL-W1, keep orbital shaker at room temperature.

b. EROD Measurement

b. i) Cytofluor Preparation

I. Turn on Cytofluor 15 min before running the assay and remove cells from incubator.

II. Start the Cytofluor 2350 software and prepare a file for each plate you will be using.
   A. Click on File, New

B. Add a filename in the designated box.
C. Set the plate type to "Costar 48-well".
D. Set the filters to excitation C (530 nm), emission C (595 nm) and sensitivity 3.
E. Click on Save

III. Repeat this for each plate used.

b. ii) Resorufin Standard Plate

1. Begin the assay with the resorufin standard plate.
2. Remove the culture medium by inverting the plate over a foil lined catch basin and gently shaking it. Keep an aspirator in the waste basin to empty it.
3. Using a 100 µL Combitip and repeating pipettor, add 100 µL of PBS to each well of the plate to rinse it.
4. Remove the PBS by inverting the plate over a foil lined catch basin and gently shaking it.
5. Begin timing the reaction with a stopwatch.
6. Using a 250 µL Combitip, add 250 µL of the substrate-dicumarol medium solution to the first column of wells in the plate.
7. Using the same Combitip, add 250 µL of the methanol control standard (beaker #7) to the second column of wells.
8. Using the same Combitip, add 250 µL of the resorufin standard in beaker #6 to the second column of wells.
9. Repeat this the above procedure until 250 µL from beaker #1 has been added to each well in column #8.
10. Open the designated file for the resorufin standard plate.
11. Remove the lid from the resorufin standard plate and place the plate in the Cytofluor for scanning.
12. Click on the Scan button. The plate scan will take about 30 s.
13. When the scan has ended, remove the plate from the Cytofluor.
14. Click on File, Export .CSV to export the data file to your home directory. For simplicity, the same Cytofluor file name is given to the .CSV file.
15. Click on Close and answer 'yes' to the question "do you want to save the file?".
16. Remove the reaction mixture from the plate by inverting the plate over a foil lined catch basin and gently shaking it.
17. Using a 100 µL Combitip and repeating pipette, add 100 µL of PBS to each well of the plate to rinse it.
18. Remove the PBS by inverting the plate over a foil lined catch basin and gently shaking it.
19. Using a 250 µL Combitip, add 250 µL of deionized water to each of the wells in the plate.
20. Let the plate sit for 5 min, then transfer to a -80°C freezer. Cells will remain frozen until the protein assay.

b. iii.) Toxicant Dosed Plates

For each plate, the following procedure is used:

1. Remove the culture medium by inverting the plate over a foil lined catch basin and gently shaking it. Keep an aspirator in the waste basin to empty it.
2. Using a 100 µL Combitip and repeating pipette, add 100 µL of PBS to each well of the plate to rinse it.
3. Remove the PBS by inverting the plate over a foil lined catch basin and gently shaking it.
4. Begin timing the reaction with a stopwatch.
5. Using a 250 µL Combitip, add 250 µL of the substrate-dicumarol medium solution to each of the wells in the plate. Add the mixture column by column from left to right.
6. Place the plate in the orbital shaker at the appropriate temperature for each cell line until the timer has reached 15 min.
7. During this time, open the designated file for the plate.
8. When 15 min has elapsed, remove the lid from the plate and place the plate in the Cytofluor for scanning.
9. Click on the Scan button. The plate scan will take about 30 s.
10. When the scan has ended, remove the plate from the Cytofluor.
11. Click on File, Export .CSV to export the data file to your home directory. For simplicity, the same Cytofluor file name is given to the .CSV file.
12. Click on Close and answer 'yes' to the question "do you want to save the file?".
13. Remove the reaction mixture from the plate by inverting it over a foil lined catch basin and gently shaking it.
14. Using a 100 µL Combitip and repeating pipette, add 100 µL of PBS to each well of the plate to rinse it.
15. Remove the PBS by inverting the plate over a foil lined catch basin and gently shaking it.
16. Using a 250 µL Combitip, add 250 µL of deionized water to each of the wells in the plate.
17. Let the plate sit for 5 min, then transfer to a -80°C freezer. Cells will remain frozen until the protein assay.

D. Protein Assay

Note: 30 min before the assay is to begin, remove the plates from the -80°C freezer to thaw.

a. Preparation of BSA Standards

1. Obtain a small beaker and fill with 25 mL deionized water and add a stir bar to it. Label the beaker as #4
2. Measure out approximately 20 mg BSA and record the exact mass. Add the BSA to the beaker and place on a magnetic stir plate for 10 min.
3. Obtain three small beakers and label them #1 to #3. Using a 10 mL pipette, add 10 mL deionized water to each.
4. Using a 10 mL pipette, transfer 10 mL from beaker #4 to beaker #3. Using the pipette, mix the solution well by drawing up and expelling it.
5. Using a *clean* 10 mL pipette, transfer 10 mL from beaker #3 to beaker #2. Using the pipette, mix the solution well by drawing up and expelling it.

BSA Standard Concentrations

Beaker	BSA Concentration in Plate Well
#1	0 µg
#2	50ug
#3	100 µg
#4	200 µg

b. Preparation of Solutions

I. Phosphate buffer solution
   - A. Add 7.49 g potassium phosphate, dibasic and 0.925 g potassium phosphate, monobasic to 1.0 L of distilled water.
   - B. Adjust pH to 8.0 and store at room temperature.

II. Fluorescamine solution (made fresh each assay)
   - A. Determine the volume of acetone needed by multiplying the number of plates x 15 mL. For example 4 plates would use 60 mL acetone.
   - B. In a foil covered beaker, make up a 0.3 mg/mL solution of fluorescamine in the volume of acetone calculated.

c. Cytofluor Preparation

I. Turn on Cytofluor 15 min before running the assay and remove cells from incubator.

II. Start the Cytofluor 2350 software and prepare a file for each plate you will be using.
   - A. Click on File, New

B. Add a filename in the designated box.
C. Set the plate type to "Costar 48-well".
D. Set the filters to excitation A (360 nm), emission A (460 nm) and sensitivity 4.
E. Click on Save

III. Repeat this for each plate used.

d. BSA Standard Plate

1. Using a 250 µL combitip, add 250 µL from beaker #1 to the empty wells in column 1 and column 5 (see figure 3).
2. Using a 250 µL combitip, add 250 µL from beaker #2 to the empty wells in column 2 and column 6 (see figure 3).
3. Using a 250 µL combitip, add 250 µL from beaker #3 to the empty wells in column 3 and column 7 (see figure 3).
4. Using a 250 µL combitip, add 250 µL from beaker #4 to the empty wells in column 4 and column 8 (see figure 3).
5. Using a 500 µL combitip, add 500 µL phosphate buffer to each well of the plate.
6. Begin timing the reaction with a stopwatch.
7. Using a 250 µL combitip, add 250 µL fluorescamine to each well of the plate.
8. Place the plate in a 37°C orbital shaker until the timer has reached 5 min.
9. During this time, open the designated file for the plate.
10. When 5 min has elapsed, remove the lid from the plate and place the plate in the Cytofluor for scanning.
11. Click on the Scan button. The plate scan will take about 30 s.
12. When the scan has ended, remove the plate from the Cytofluor.
13. Click on File, Export .CSV to export the data file to your home directory. For simplicity, the same Cytofluor file name is given to the .CSV file.
14. Click on Close and answer "yes" to the question "do you want to save the file?".
15. Remove the reaction mixture from the plate by inverting it over a foil lined catch basin and gently shaking it. The plate can then be disposed of in the hazardous waste container.

e. Toxicant Dosed Plates

1. Using a 500 µL combitip, add 500 µL phosphate buffer to each well of the plate.
2. Begin timing the reaction with a stopwatch.
3. Using a 250 µL combitip, add 250 µL fluorescamine to each well of the plate.
4. Place the plate in an orbital shaker until the timer has reached 5 min.
5. During this time, open the designated file for the plate.
6. When 5 min has elapsed, remove the lid from the plate and place the plate in the Cytofluor for scanning.
7. Click on the Scan button. The plate scan will take about 30 s.
8. When the scan has ended, remove the plate from the Cytofluor.

9. Click on File, Export .CSV to export the data file to your home directory. For simplicity, the same Cytofluor file name is given to the .CSV file.
10. Click on Close and answer "yes" to the question "do you want to save the file?".
11. Remove the reaction mixture from the plate by inverting it over a foil lined catch basin and gently shaking it. The plate can then be disposed of in the hazardous waste container.
12. Repeat the procedure for each plate in the assay.

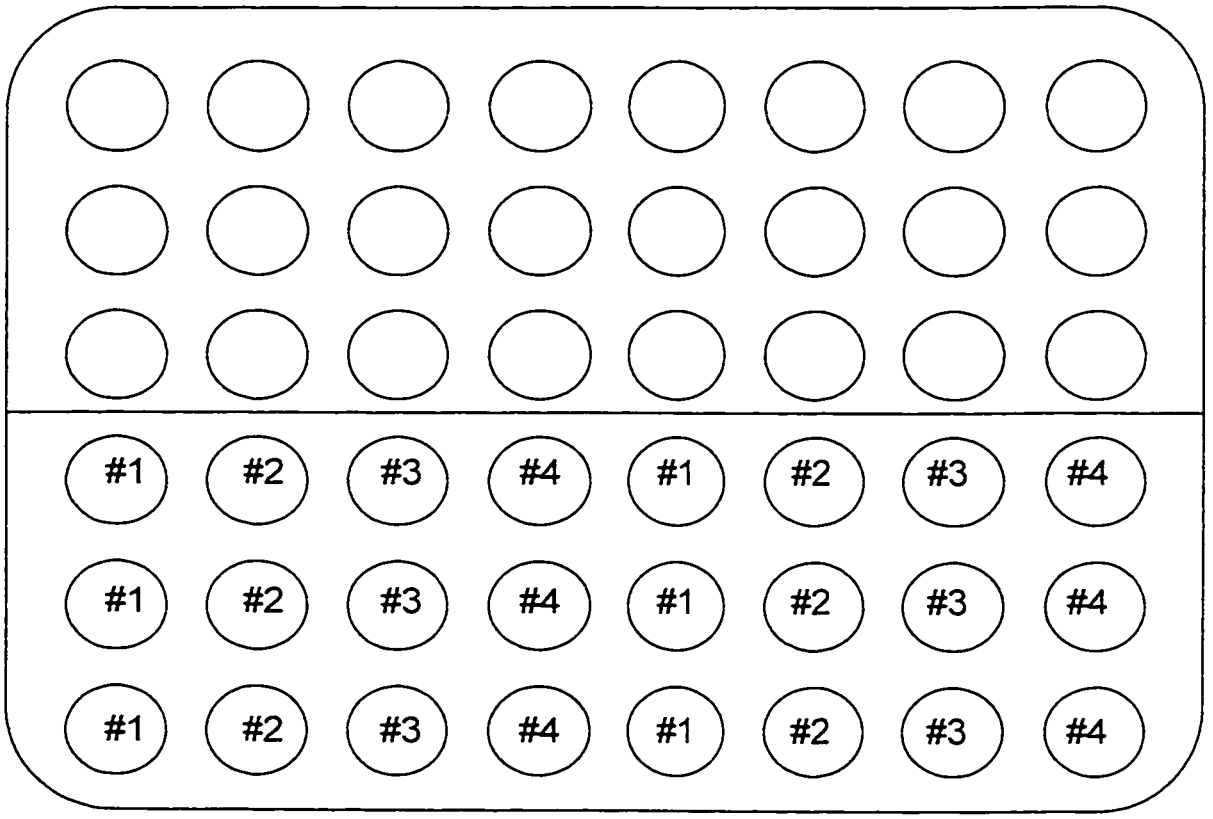


Figure 3. Plate layout for protein standard curve.

E. Calculations

Calculations for the EROD activity are based on the following formula:

$$\text{EROD Activity} = \frac{\text{pmoles of resorufin produced}}{\text{mg protein} \times \text{reaction time}}$$

$$= \frac{[(\text{resorufin F.U.} - \text{resorufin standard y-intercept})/\text{resorufin standard slope})]}{[(\text{protein F.U.} - \text{BSA standard y-intercept})/\text{BSA standard slope})] \times \frac{1000\ \mu g}{mg} \times \text{reaction time}}$$

a. EROD Determination Using the Microsoft Excel Template File

I. Copy the your .CSV files to your excel directory.
II. Open the EROD template file called **bioassay.xls** and click on the tab labeled Resorufin Standard.

Resorufin Standard

A. Open your resorufin standard curve .CSV data file and copy the data column containing the fluorescent units of interest to the designated column (Resorufin Standard F.U.) in the excel file.
B. Enter the date, excitation, emmission and sensitivity in the designated spot in the excel file.
C. Perform a linear regression analysis on the resorufin F.U. vs concentration, *making sure the summary output goes into* **cell G11.**

BSA Standard

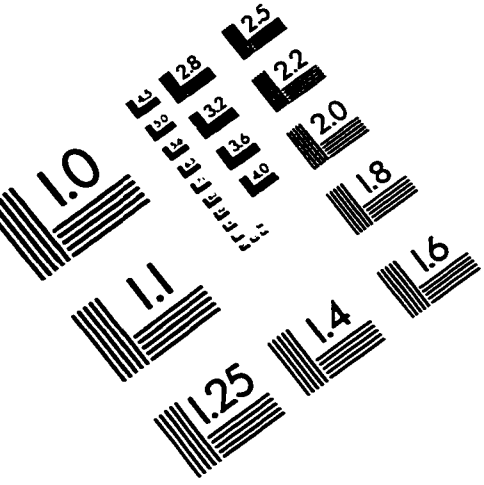
A. Click on the tab labeled BSA Standard.
B. Enter the mass of BSA measured out for the standard in the designated spot in the excel file.
C. Open your BSA standard curve .CSV data file and copy the data column containing the fluorescent units of interest to the designated column file (BSA Standard F.U.) in the excel file.
D. Enter the date, excitation, emission and sensitivity in the designated spot in the excel file.
E. Perform a linear regression analysis on the BSA F.U. vs concentration, *making sure the summary output goes into* **cell G11.**

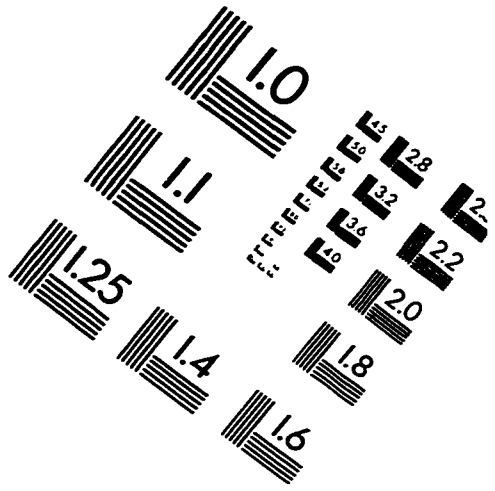
EROD Activity in Toxicant Dosed Plates

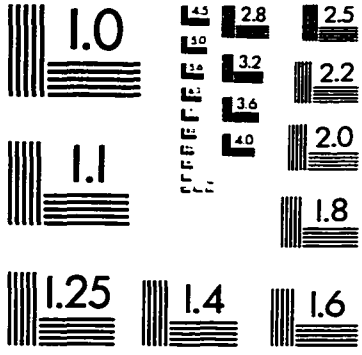
A. Click on the tab labeled Bioassay Results.

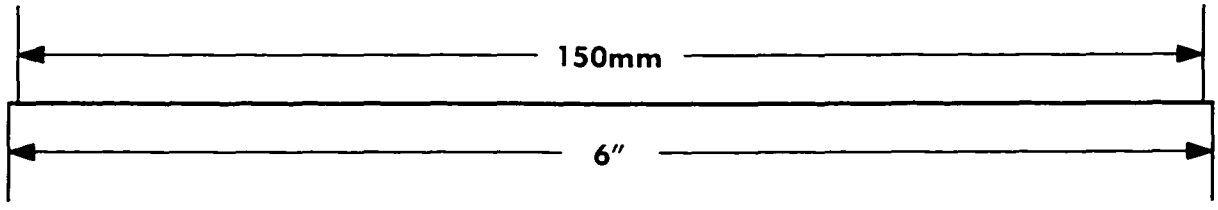
B. Open your .CSV data file containing the resorufin fluorescence of your dosed plate and copy the data column containing the fluorescent units of interest to the designated column file (Resorufin F.U.) in the excel file.
C. Open your .CSV data file containing the protein fluorescence of your dosed plate and copy the data column containing the fluorescent units of interest to the designated column file (Protein F.U.) in the excel file.
D. Enter the date, excitation, emission and sensitivity in the designated spot in the excel file.
E. Save the file under a new file name (do not overwrite your template file).
F. Determine EC_{50} using Jandel Scientific's SigmaPlot or similar software.

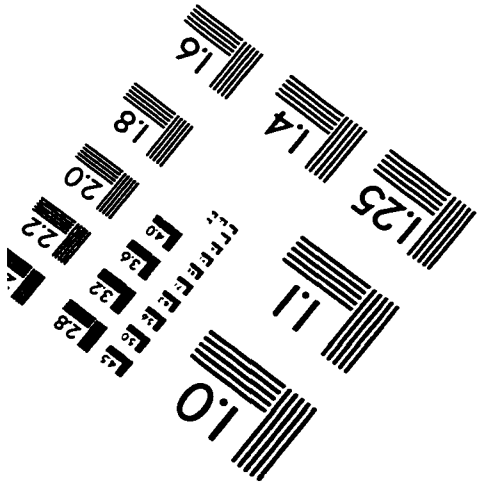
IMAGE EVALUATION
TEST TARGET (QA–3)

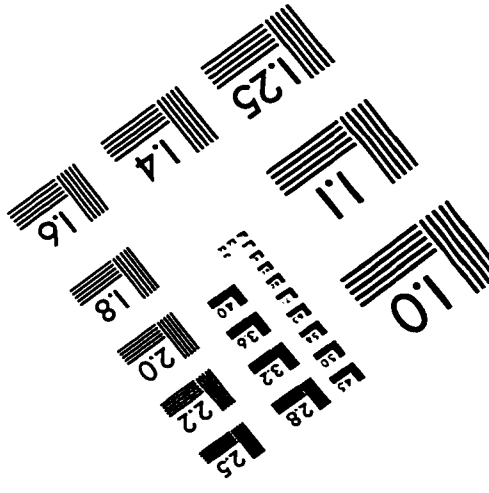












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved