Effect of Pre-exposure to Treated Wastewater Effluent on *in vitro* Biotransformation Rates of Hydrophobic Chemicals in Rainbow Trout (*Oncorhynchus mykiss*)

by

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Abstract

Efforts have been made to develop extrapolation methods of *in vitro* biotransformation data to improve chemical bioaccumulation assessment. A criticism of these methods is that animals used for *in vitro* studies may not represent animals in environments where contaminants are present. The effect of municipal wastewater effluent exposure on biotransformation rates of benzo(a)pyrene, pyrene, chrysene, and 9-methylanthracene in a rainbow trout liver preparation was examined. Results were extrapolated to organism level and modeled bioconcentration factors (BCFs). *In vitro* biotransformation rates (k_r) for benzo(a)pyrene and pyrene were elevated in one experiment following exposure to 10% effluent and when extrapolated, respective BCFs decreased. In a second experiment, exposure to 20% effluent had no significant effect on the mean k_r values for the test chemicals. Variability of k_r between exposures could be attributed to differences in effluent composition between experiments. This research highlights the importance of considering environmental factors in chemical bioaccumulation assessment.

Keywords: bioaccumulation; biotransformation; rainbow trout; pre-exposure; wastewater effluent

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List of Acronyms

A	Substrate
AHH	Aryl hydrocarbon hydroxylase
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APND	Aminopyrine N-demethylase
ARNT	Aryl hydrocarbon nuclear translocator protein
B	Bioaccumulative
B(a)P	Benzo(a)pyrene
BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
BNF	B-Naphthoflavone
BSA	Bovine Serum Albumin
CAR	Constitutive androstane receptor
CEPA	Canadian Environmental Protection Act
CL_H	Hepatic clearance
CL_{INT}	Intrinsic clearance
CYP	Cytochrome P450
d12-chrysene	Deuterated Chrysene
DSL	Domestic Substances List
EROD	Ethoxyresorufin-O-deethylase
GC/MS	Gas Chromatography-Mass Spectometry
GST	Gluatathione S-Transferases
GTF	Glycosyltransferase
i.p.	Intraperitoneal
I.S.	Internal standard
Ln	Natural logarithm
IVIVE	*In vitro-in vivo* extrapolation
k_{cat}	Catalytic constant
K_m	Michaelis-Menten Constant
k_{MET}	Whole body biotransformation rate constant
K_{ow}	Octanol-water partition coefficient

k_r	*In vitro* substrate depletion rate constant
m/z	Mass-to-charge Ratio
MFO	Mixed-function oxidation
MS-222	Tricaine methanesulfonate
OECD	Organisation of Economic Cooperation and Development
P	Persistent
PAH	Polycyclic aromatic hydrocarbon
PB	Phenobarbital
PBT	Persistent, Bioaccumulative, and/or Toxic
PBTK	Physiologically Based Toxicokinetic
PCB	Polychlorinated Biphenyl
PCDD	Dioxin
PCDF	Polychlorinated dibenzo-furan
PXR	Pregnane X receptor
QSAR	Quantitative Structure-Activity Relationship
REACh	Registration, Evaluation, Authorisation, and Registration of Chemical Substances
RXR	Retinoid X receptor
S9	Supernatant fraction obtained from liver homogenate by centrifuging at 9000 g for 20 min in a suitable medium
SD	Standard deviation
SE	Standard error
SFU	Simon Fraser University
ST	Sulfotransferase
T	Inherently toxic
TCBT	Tetrachlorobenzyltoluene
TRI	Toxic Release Inventory Program
TSCA	Toxic Substances Control Act
Tukey's HSD	Tukey's Honestly Significant Difference test
UDP	Uridine Diphosphate
UGT	Glucuronosyltransferase
V	Reaction rate
V_{max}	Theoretical maximum of the metabolic rate

1. Introduction

The annual release of several hundred thousand tonnes of anthropogenic chemical compounds has become a global cause for concern. These substances include approximately 100,000 commercial chemicals with additional substances being produced each year (Arnot & Gobas, 2006). Toxic substances that have the potential to persist in the environment for long periods of time (months to years) or to bioaccumulate in organisms in higher trophic levels are of particular concern as they can cause serious harm to human health and wildlife. Many of these compounds are ubiquitous in the environment and can be found far from their point of origin in the mid-latitudes in the Arctic and Antarctic (Gouin et al., 2004). Anthropogenic chemicals therefore need to be rigorously examined for their potential to be persistent (P), bioaccumulative (B) and inherently toxic (T) in order to be properly regulated and reduce risks to human health and the environment.

1.1. Regulation of Anthropogenic Compounds

In 1994, Environment Canada published the Domestic Substances List (DSL). The DSL is an inventory of approximately 23,000 substances that were manufactured in or imported into Canada in amounts exceeding 100 kilograms between January 1, 1984 and December 31, 1986 (Government of Canada, 1999). In 2009, the Canadian Environmental Protection Act (CEPA) was amended to add 500 more substances to the inventory (CEPA, 1999, Section 71 Notice). With a few exemptions, all substances not on the DSL are considered new and must be reported prior to their import or manufacture to be assessed if they are toxic or may become toxic to the environment or human health. Environment Canada and Health Canada are the agencies responsible for completing the regulatory assessment of P, B, and T criteria and set endpoint values to determine if a chemical has the potential to be a hazard to the environment or human health (Table 1-1).

Table 1-1. CEPA's criteria for assessing persistence, bioaccumulation, and toxicity of commercial chemicals on the DSL.

Persistence (P)		Bioaccumulation (B)	Toxicity (T)
Environmental Medium	Half-life ($t_{1/2}$)		
Air	≥ 2 days	BAF ≥ 5,000	CEPA-toxic or CEPA-toxic equivalent
Water	≥ 60 days	BCF ≥ 5,000	
Soil	≥ 182 days	log K_{OW} ≥ 5.0	
Sediment	≥ 365 days		

A substance is determined to be persistent based on the length of time it remains in the environment before being broken down. P is defined based on a substance's half-life, which is the time required for a substance to degrade in the environment by 50%. A substance is considered persistent when the criterion is met for any one medium (Table 1-1). B refers to processes that cause the chemical concentration in living organisms to exceed the chemical concentration(s) in their environment (e.g. water, air, food). Inherent toxicity refers the hazard a substance presents to the environment and human health and is determined by Environment Canada as having toxic effects at concentrations < 1mg/L based on its toxicity to aquatic organisms (Government of Canada, 1999). Chemicals that exceed T guidelines in addition to exceeding P or B criteria are subjected to phase 2 screening level assessments to prevent and manage risks posed to the environment and human health.

One of the challenges of this evaluation process is that the empirical data required for chemical evaluation are scarce. This is particularly true for B endpoints. When Canada began to assess the 23,000 substances on the DSL according to P, B and T criteria it became apparent that few empirical data (<4% of registered organic compounds) were available for B endpoints in comparison to endpoints related to P and T (Arnot and Gobas, 2006). In response, Canada began to rely on the application of methods aimed at both rapidly and accurately assessing the bioaccumulation potential of these substances (Weisbrod et al., 2009). The current methods used to screen chemicals for bioaccumulation are described in Section 1.2, along with the advantages and limitations of using each test.

1.2. Current Bioaccumulation Screening Criteria

Various assays are used by regulatory agencies to measure bioaccumulation. Common *in vivo* measurements include the bioaccumulation factor (BAF) and the bioconcentration factor (BCF) test. The BCF is a measurement of the ratio of the concentration of a substance in an organism to the concentration in water, based only on uptake from the surrounding medium (it does not include dietary exposure). The BAF is a measurement of the ratio of the concentration of a substance in an organism to the concentration in water, based on uptake all possible routes of chemical exposure (e.g., diet, dermal, respiratory). The octanol-water partition coefficient (K_{OW}) is another commonly used approach that does not include any biological factors contributing to bioaccumulation. K_{OW} is the ratio of the concentration of a substance in an octanol phase to the concentration of the substance in the water phase in a mixture of water and octanol at equilibrium. K_{OW} describes how a chemical thermodynamically distributes between water and the lipids of organisms as octanol is generally considered to be a reasonable surrogate phase for lipids in organisms (Mackay, 1982). National and international chemical management programs have developed criteria based on these bioaccumulation measures to evaluate and assess the bioaccumulation potential of chemicals (Table 1-2).

Although these regulatory bioaccumulation endpoints are commonly used, they do not come without limitations. Major disadvantages of BCF testing include the length of time required to complete the tests, the large number of animals the tests require, and the monetary costs of testing (Dyer et al., 2008). Specifically, the Organization for Economic Cooperation and Development (OECD) test No. 305 is the Bioconcentration Flow-through Fish Test is a BCF test is conducted over a 3-6 month period, uses a minimum of 108 fish, and costs approximately $125,000 per chemical due to extensive analytical measurements and/or synthesis of radiolabeled test substance (Weisbrod et al., 2007). More importantly BCF methods are based on aqueous exposure protocols and could underestimate chemical accumulation in the environment if dietary uptake is an important exposure route (Nichols et al., 2007). BAF values are typically measured from chemical concentrations in field-collected animals and media. While BAF values are the most environmentally relevant because all routes of exposure are considered, the costs associated with measuring environmental contaminants in the field may be

high. The results may also vary substantially due to environmental or site-specific factors (Nichols et al., 2007). Additionally analytical limitations and the lack of guidelines for field assessments make BAF values difficult to generate and less reliable (Burkhard, 2003).

Table 1-2. An overview of regulatory bioaccumulation endpoints and criteria used by various agencies around the world (Adapted from Arnot & Gobas, 2006)

Regulatory agency	Bioaccumulation endpoint	Criteria (log-transformed values)	Program
Environment Canada	K_{OW}	≥ 100,000 (5.0)	CEPA (1999)*
Environment Canada	BCF	≥ 5,000 (3.7)	CEPA (1999)
Environment Canada	BAF	≥ 5,000 (3.7)	CEPA (1999)
European Union 'Bioaccumulative'	BCF	≥ 2,000 (3.3)	REACh†
European Union 'Very Bioaccumulative'	BCF	≥ 5,000 (3.7)	REACh
United States 'Bioaccumulative'	BCF	1,000 - 5,000 (3.3 - 3.7)	TSCA, TRI‡
United States 'Very Bioaccumulative'	BCF	≥ 5,000 (3.7)	TSCA, TRI
United Nations Environment Programme	K_{OW}	≥ 100,000 (5.0)	Stockholm Convention§
United Nations Environment Programme	BCF	≥ 5,000 (3.7)	Stockholm Convention

* CEPA, Canadian Environmental Protection Act, 1999 (Government of Canada, 1999; Government of Canada 2000).
† Registration, Evaluation and Authorization of Chemicals (REACH) Annex XII (European Commission 2001).
‡ Currently being used by the US Environmental Protection Agency in its Toxic Substance Control Act (TSCA) and Toxic Release Inventory (TRI) programs (USEPA 1976).
§ Stockholm Convention on Persistent Organic Pollutants (UNEP 2001).

Although BAF and BCF testing have limitations, these values are considered to be reliable indicators of a substance's ability to bioaccumulate. BAF and BCF values are obtained from directly measuring the substance in organism (usually fish) tissue and account for physiological processes of the test animal. The problem is that these data are only available for less than 4% of substances on the DSL (Arnot and Gobas, 2006). When BCF and BAF values have not been measured for a particular chemical, the chemical's K_{OW} is used in B determination. Although K_{OW} values of neutral organic

materials are relatively inexpensive and may be easily measured or predicted using specialized software, relying solely on K_{OW} values in the determination of the chemical bioaccumulation potential may be inadequate because these values only reflect passive chemical partitioning (Weisbrod et al., 2009).

There are several physiological processes in fish that are not represented by using K_{OW} alone. These include active uptake/loss of chemicals via gills, chemical loss via fecal egestion, and biotransformation (Dyer et al., 2008). When a chemical is biotransformed it may be less likely to bioaccumulate in an organism or biomagnify in the food chain due to enzymatic conversion of hydrophobic parent compounds to more water-soluble metabolites. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 1,3,6,8-TCDD both have high and comparable K_{OW} values (log K_{OW} = 6.8), but the former is bioaccumulative in fish whereas the latter is not due to its rapid biotransformation (Hu & Bunce, 1999).

Due to the lack of empirical BCF and BAF values for many chemicals and the limitations associated with using K_{OW} as the sole B criterion, it is important to determine a chemical's bioaccumulation potential using other methods. Quantitative structure-activity relationships (QSARs) and other computer models have become more extensively employed to predict bioaccumulation potential (Weisbrod et al., 2007). Empirical models demonstrating linear correlations between measured BCF values in fish and $K_{OW,}$ have been proved (Veith et al., 1979; Mackay et al., 1982). Mass-balance models have also been adopted. For example, physiologically based toxicokinetic (PBTK) models have been used to provide insight to fish bioaccumulation potential. PBTK models incorporate knowledge of fish tissue physiology, interactions of chemicals between tissue compartments and fish responses to chemicals (Nichols et al., 1990; Law et al., 1991). Fugacity models are used to reflect processes that affect bioconcentration, bioaccumulation, and biomagnification in aquatic organisms and food webs. Estimates of the contribution of dietary and gill uptake of hydrophobic compounds may also be included in these models (Arnot & Gobas, 2003; 2004).

One of the common limitations of using a computational model is the absence of information on biotransformation. Biotransformation can reduce the extent of bioaccumulation and omitting this process can result in model over-prediction. Arnot &

Gobas (2003, 2004) acknowledge that biotransformation plays an important role in a chemical's bioaccumulative potential although most models assume that the biotransformation rate constant (k_{MET}) is equal to zero. This is because biotransformation data are available for only a limited number of chemicals. Inclusion of biotransformation information into bioaccumulation models appears to more accurately reflect a chemical's true bioaccumulative potential. Arnot and Gobas (2003) demonstrated that fitting their model with quality empirical BCF data for a group of PAHs resulted in a simulated k_{MET} value of 0.05 d^{-1}, which was comparable to values in the literature. This suggests that the inclusion of the metabolic transformation information in computational modeling can produce more precise BCF estimates by reducing the incidence of BCF overestimates. Therefore, it is important to develop rapid, cost-effective, and easily standardized methods to determine the biotransformation rate constants of organic chemicals, so they can be employed to improve the current practice of bioaccumulation assessment. Knowledge and data of chemical biotransformation rates in fish and other organisms have been identified as key requirements for the environmental evaluation of commercial chemicals (Weisbrod et al. 2009) because without this information, chemicals can be incorrectly categorized as bioaccumulative.

Using *in vitro* systems to investigate biotransformation has been proposed. The potential of these systems needs to be urgently evaluated as there are increasing regulatory needs to assess biotransformation and bioaccumulation (Dyer et al., 2008). *In vitro* hepatic metabolic transformation tests have been shown to provide an effective measurement of fish metabolic potential. These tests also require significantly fewer animals and allows for a reduction in monetary costs and time spent conducting the tests. *In vitro* testing additionally allows for several test chemicals to be tested simultaneously (Nichols et al., 2007). Extrapolating *in vitro* biotransformation rates to the *in vivo* level to assess the metabolic clearance of chemicals from the liver and from the whole body has been used extensively in the pharmaceutical field. *In vitro* studies involving freshly isolated fish hepatocytes, liver microsomes, S9 fractions, perfused fish liver preparations and extrapolation to the *in vivo* level (Nichols et al., 2007; Cowan-Ellsberry et al., 2008; Han et al., 2009) demonstrate that these methods may also be useful for determining the biotransformation rate of potentially bioaccumulative substances.

1.3. Xenobiotic Biotransformation in Fish

Physiological processes that determine whether a chemical will bioaccumulate in fish include uptake from the environment (water or diet) into fish tissues, biotransformation to water-soluble metabolites, and chemical elimination (Han et al., 2007; Weisbrod et al., 2009). Biotransformation enzymes are involved in the metabolism of both exogenous chemicals (e.g. drugs, chemical carcinogens, environmental pollutants) and endogenous chemicals (e.g. steroids, fatty acids, vitamins) (Uno et al., 2012). For protection against xenobiotics, enzymes with broad substrate specificities convert nonpolar lipophilic chemicals into polar water-soluble metabolites, which generally lead to their detoxification and excretion from the organism (Greim & Snyder, 2008). A two-phase process is responsible for the biotransformation of hydrophobic chemicals to more polar metabolites: phase I and phase II reactions, although it is common for chemicals to undergo only phase I-type reactions or phase II reactions.

1.3.1. Phase I Biotransformation

Phase I reactions result in the addition of a functional group that produces a small increase in hydrophilicity. Phase I reactions include three major types of reactions: oxidation, reduction, hydrolysis and most of the final products of these reactions contain chemically reactive functional groups, such as –OH, -NH_2, -SH and –COOH (Gibson & Skett, 1986). The vast majority of compounds metabolized in Phase I reactions are processed by the cytochrome P450 (CYP) mixed-function oxidation (MFO) system. CYP enzymes have low substrate specificity so they are able to convert thousands of hydrophobic compounds into more hydrophilic, readily excreted metabolites (Berg et al., 2002). The MFO system performs many different functionalization reactions, including hydroxylation of aromatics and aliphatics, epoxidation, dealkylation, oxidation (e.g. oxidative deamination, N & S-oxidation, thiophosphate oxidation), and dehalogenation.

1.3.2. Cytochrome P450 Enzyme System

The most important enzyme system catalyzing phase I reactions is the CYP monooxygenase system. CYPs are the most numerous of all xenobiotic-metabolizing enzymes and have the broadest substrate specificities. The CYP superfamily of enzymes has existed for over 3.5 billion years (Nelson et al., 1993) and are found in both

eukaryotic and prokaryotic organisms including vertebrates, invertebrates, plants, fungi, yeast and bacteria (Guengerich, 2008). The origin of the name cytochrome P450 is based on the enzyme spectrophotometric characteristics. When the reduced protein forms adducts with carbon monoxide, CYP enzymes absorb light at a wavelength at 450 nm. The standard nomenclature is CYP followed by a number indicating the gene family, a capital letter indicating the subfamily, and another number indicating the individual gene. Thus, CYP1A1 is encoded by the gene for the P450 in family 1, subfamily A, subfamily member 1. CYPs considered in the same family display 40% or more amino acid sequence similarity, and those within a subfamily are more than 55% similar (Greim & Snyder, 2008). In most animals, the liver is the richest source of these enzymes followed by the kidney and the gastrointestinal tract. In the cell, CYPs are located predominantly in the smooth endoplasmic reticulum (Klaassen & Watkins, 2003).

The general CYP-mediated reaction is described below. RH represents the xenobiotic, which is converted to the oxidized product, ROH:

$$\mathbf{NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH}$$

CYP activity is dependent upon the availability of molecular oxygen and NADPH (Klaassen & Watkins, 2003; Greim & Snyder, 2008). The reaction cycle (Figure 1-1) is initiated when the substrate binds to the oxidized (Fe^{3+}) CYP complex and facilitates electron transfer from NADPH to the complex (steps 1 through 2, Figure 1-1). Oxygen binds to the reduced (Fe^{2+}) CYP-substrate complex (step 3, Figure 1-1). A second electron is donated by NADPH-cytochrome P450 reductase or from cytochrome b_5 reductase, resulting in a rearrangement of the ternary complex (step 4, Figure 1-1). In step 5, proton addition causes the oxygen-oxygen bond to cleave followed by the release of water (step 6, Figure 1-1). The complex becomes electron deficient and either abstracts hydrogen atom or an electron from substrate to form $FeO^{3+}R\bullet$. Subsequent collapse of the intermediate generates the product "oxygen rebound" $FeO^{3+}ROH$ (steps 7 and 8, Figure 1-1). In step 9, product (ROH) dissociation restores CYP to the initial ferric state (Di Giulio & Hinton, 2008).

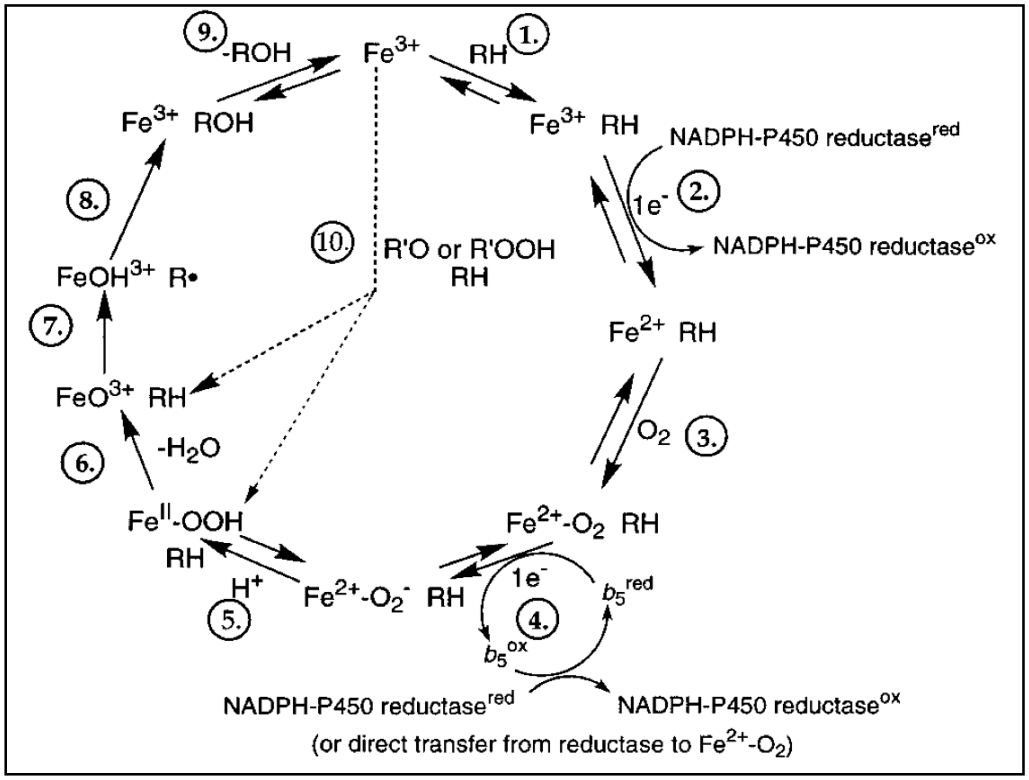

Figure 1-1. Generalized Cytochrome P450 Catalytic Cycle (Di Giulio & Hinton, 2008)

Research on CYPs in fish is more limited than mammals but research shows CYPs may be similar to those reported in mammalian species. Multiple CYP forms have been detected in fish in almost every tissue examined, with the majority being present in the liver (Uno et al., 2012). A sequence analysis showed up to 59% and 53% homology for CYP1A1 and CYP 1A2 genes, respectively between fish and mammals (Di Giulio & Hinton, 2008). CYP-mediated metabolism of some substrates can be highly complex. There are many oxidative metabolites that can form from one substrate. Metabolic profiles are variable and dependent on the species differences in CYP expression, catalytic activity, and the tissue or organ being investigated (Di Giulio & Hinton, 2008). Across all species of fish, 137 genes encoding P450s have been identified and have been classified into 18 CYP families. In Japanese pufferfish (*Takifugu rubripes*) 54 CYP genes have been identified (Uno et al., 2012). CYP families that are well established to be part of xenobiotic metabolism in the majority of fish are CYP1, CYP2, and CYP3 (Di Giulio & Hinton, 2008).

1.3.3. Phase II Biotransformation

The functional groups which are introduced or exposed in Phase I biotransformation can react with cofactors of Phase II enzymes and are conjugated with endogenous molecules. Phase II is often the true 'detoxification' of chemicals as these metabolites are more water-soluble and have a higher molecular weight than phase I metabolites and thus greatly promoting metabolite transport and elimination (Behrens & Segner, 2001; Di Giulio & Hinton, 2008). Phase II enzymes are located primarily within the cytosolic portion of the cell (Klaassen & Watkins, 2003). Glutathione is recognized as a protective device for the removal of potentially toxic compounds/phase I metabolites. Many of the compounds metabolized via phase I reactions become strong electrophiles and they can react with glutathione to form non-toxic conjugates. Glucuronidation is a major pathway of phase II biotransformation for exogenous and endogenous compounds. Sulfation, acetylation, methylation, and conjugation with amino acids (e.g. glycine, taurine, and glutamic acid) are also examples of other phase II biotransformation reactions. Specific transferases such as uridine diphosphate-glucuronosyltransferase (UGT), sulfotransferases (ST), glutathione S-transferases (GSTs), and several others catalyze these processes. Table 1-3 shows common phase II metabolism and conjugation reactions.

Table 1-3. Phase II Metabolism and conjugation reactions (Gibson & Skett, 1986)

Phase II Metabolism		
Reaction	Enzyme	Functional Group(s)
Glucuronide Conjugation	UDPGlucuronyltransferase (UGT)	-OH; -COOH; -NH_2; -SH
Glycosidation	UDP-Glycosyltransferase (GTF)	-OH; -COOH; -SH
Sulfate Conjugation	Sulfotransferase (ST)	-NH_2; -SO_2NH_2; -OH
Gluthione Conjugation	Gluthione-S-transferase (GST)	Epoxide; Organic halide
Amino acid Conjugation		-COOH
Acetylation	Acetyltransferase	-OH; -NH_2; -SO_2NH_2
Methylation	Methyltransferase	-OH; -NH_2

1.4. Enzymatic Reactions and Michaelis-Menten Kinetics

Enzymes are catalysts that considerably increase the rate of reaction without being consumed in the process. To achieve this effect they temporarily bind to the substrate which lowers the activation energy needed to convert the substrate into its final product. Many enzymatic reactions involve time-dependent reactions outside of equilibrium, as the reaction strives to achieve equilibrium state (Bisswanger, 2008). Enzyme kinetics involves the measurement and mathematical description of the rate of reaction and its associated constants (Rogers & Gibon, 2009). The reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics can reveal the catalytic mechanism of an enzyme, its role in metabolism, how its activity is controlled, and how a xenobiotic might inhibit or induce enzyme activity.

The Michaelis-Menten equation is a fundamental equation describing enzyme kinetics. Conventionally the equation relates the rate of an enzymatic reaction (v) at various concentrations of a substrate. Although the Michaelis-Menten equation is derived from a simple, single-substrate, irreversible reaction, it remains valid for more complex reactions. The simple conversion of substrate (A) into product (P) catalyzed by the enzyme (E) is described below (Equation 1.1). The first step is substrate binding (k_1 and k_{-1}) followed by the second step, which is the catalytic step (k_2):

$$\mathrm{E+A} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{EA} \xrightarrow{k_2} \mathrm{E+P} \qquad (1.1)$$

The formation of the product in terms of the dissociation rate (k_2) of the enzyme substrate complex denotes the catalytic constant (k_{cat}), and the concentration of the enzyme-substrate complex (EA):

$$k_2 = k_{cat}\,[EA] \qquad (1.2)$$

Michaelis-Menten kinetics assumes that the dissociation rate (k_2 in equation 1.1) of the enzyme–substrate complex (EA) is slow compared to association (k_1) and re-dissociation (k_{-1}) reactions (Rogers & Gibon, 2009), so that the first step (formation of EA) can be treated as a rapid equilibrium process.

For a given enzyme concentration and for relatively low substrate concentrations, the reaction rate increases linearly with substrate concentration; the enzyme molecules are largely free to catalyze the reaction, and increasing substrate concentration corresponds with an increased rate at which the enzyme and substrate molecules encounter one another. However as the substrate concentrations increases, all the available enzyme involved in catalysis (k_{cat}) becomes bound to the substrate (i.e. [EA]=[E_t], where [E_t] is total available enzyme) and the reaction rate reaches V_{max}, the enzyme's maximum rate:

(1.3) $$V_{max} = k_{cat} [E_t]$$

The Michaelis-Menten formula is given by:

(1.4) $$V = \frac{V_{max} [A]}{K_m + [A]}$$

The substrate concentration midway between the two limiting cases of high and low levels of substrate is denoted by K_m, the Michaelis-Menten constant, at which the reaction rate is half of V_{max} (Figure 1-2). The K_m of a reaction is a primary descriptor of the enzymatic kinetic behaviour of a biotransformation reaction (Obach & Reed-Hagen, 2002).

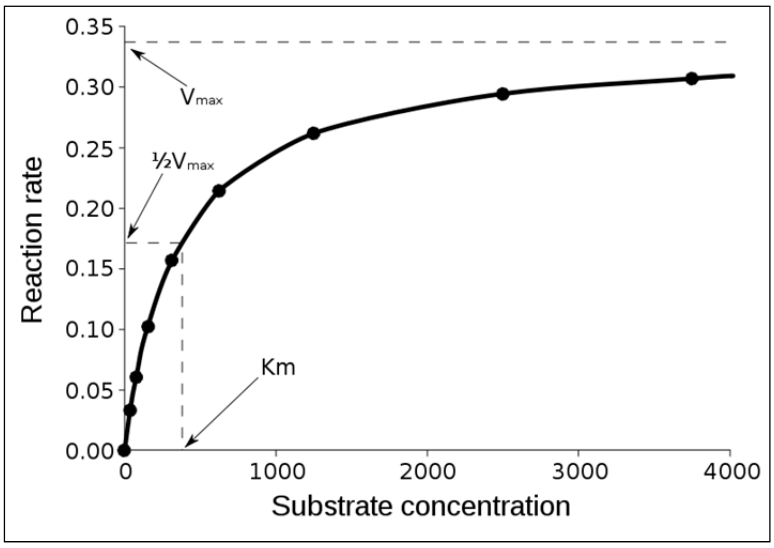

Figure 1-2. Michaelis-Menten saturation curve of an enzyme-catalyzed reaction.

Other determinations of K_m include assessing the rate of substrate depletion at several substrate concentrations. K_m values from substrate depletion experiments have been determined by plotting the substrate depletion rate constants (k_r) versus the substrate concentration on a linear-log plot using the following equation:

$$k_r = k_{r([A]=0)} \bullet (1 - [A] / [A] + K_m) \quad (1.5)$$

in which [A] is the substrate concentration; $k_{r\ ([A]=0)}$ represents the theoretical maximum depletion rate constant at a very low substrate concentration, and K_m is the Michaelis-Menten constant.

This method may be preferred over product formation methods because analytical methods require that the metabolites of the reaction be identified and have valid standards prepared. Nath and Atkins (2006) reported that both substrate depletion and traditional product formation approaches predicted kinetic parameters with about the same degree of accuracy.

1.4.1. Enzyme Induction

Induction is the process of increasing the amount of the enzyme activity through *de novo* production of the enzyme protein. In most cases, the mechanism by which CYP is induced is by transcriptional activation of an appropriate gene. In other cases it may be by protein and mRNA stabilization. For example, induction of human CYP2E1 by ethanol is not transcriptional and results from protein stabilization or increased protein translation (Fuhr, 2000). Enzyme induction is the homeostatic mechanism for regulating enzyme production in an organ, such as the liver. Induction can cause marked increases in some CYP enzymes resulting in enhanced biotransformation and increased chemical elimination (Ciccotelli et al., 1998). The onset and duration of induction depends on the kinetics of the inducing substance and the half-life of the CYP enzyme (Fuhr, 2000). Due to the potential contribution of induction to cause xenobiotic interactions and increase the proportion of toxic metabolites, it is important to understand induction mechanisms.

Induction mediated by the aryl hydrocarbon receptor (AhR) is the most well studied type of CYP induction. This type of induction is environmentally significant because it is seen as one of the first steps of the toxic response of dioxin-like

compounds. Inducers of the AhR include co-planar polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-furans (PCDFs) and dioxins (PCDDs) (Di Giulio & Hinton, 2008). The AhR is responsible for induction of CYP1A, particularly the CYP1A1 enzyme. AhR mediated enzyme induction commences when a ligand enters cell, and binds with AhR protein in the cytosol. This binding creates a receptor-ligand complex and leads to conformational change in the receptor which forms a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT), which recognizes specific DNA sequences and leads to the induction of several genes. This results in enhanced gene transcription followed by translation of transcribed mRNA in cytosol resulting in synthesis of new CYP protein. Then there is post-translational modification to give the catalytically active enzyme (Lee & Anderson, 2005). The elevated levels of the protein products are thought to be involved in the toxic action of AhR ligands (Whyte et al., 2000). Figure 1-3 shows the proposed mechanism of AhR mediated enzyme induction.

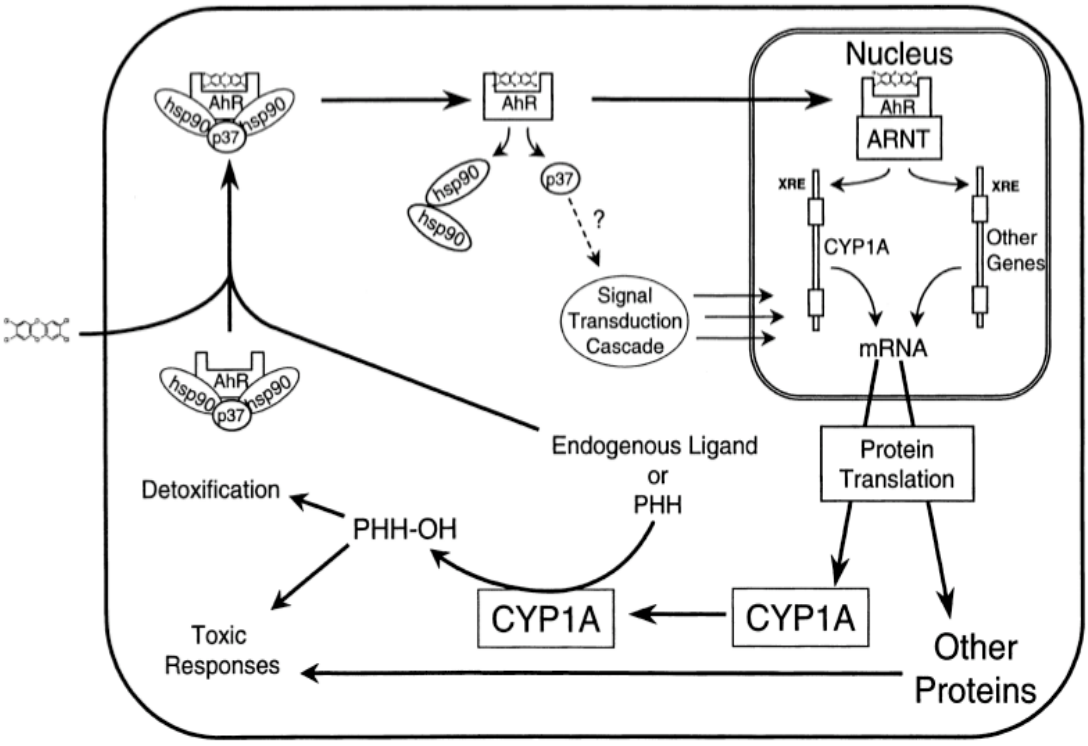

Figure 1-3. Proposed mechanism of AhR mediated toxicity of planar halogenated hydrocarbons (PHH) and PAHs (Whyte et al., 2000).

While the mechanism of AhR activation is mostly based on mammalian studies, the AhR has also been found in fish and induction of CYP1A-mediated activities has been observed in fish (Bucheli & Fent, 1995). CYP1A induction time courses have been reported in fish and studies tend to show similar results of significant induction occurring 3 to 7 days following exposure. After juvenile rainbow trout were treated with β-naphthoflavone (BNF) and PCB mixture (Clophen A50) hepatic Ethoxyresorufin-O-deethylase (EROD) activities were increased 172-and 49-fold, respectively following 3 to 7 days of treatment. (Anderson et al., 1985). Celander et al. (1990) reported similar results following exposure of rainbow trout to BNF via i.p. injection. CYP1A1 mRNA levels in microsomes were elevated 2-fold after 3 days exposure and EROD activities were increased 70-fold 3 to 7 days following BNF treatment. Zhang et al., 1990 reported EROD activity to be significantly elevated in rainbow trout at 12, 24, 48 and 96 hours following treatment with a single injection of a low dose of BNF (50 µg/kg). After a recovery period of 59 days, BNF-induced xenobiotic biotransformation enzymes in fish returned to basal levels (Zhang et al., 1990).

Less is known about the induction mechanisms of CYP2 and CYP3 isoforms in fish although they appear to be less sensitive in comparison to mammalian isoforms. For example, phenobarbital (PB) is a strong inducer of CYP2B genes in mammals but some studies have shown a low or diminished induction response some fish species (Di Giulio & Hinton, 2008). Functional differences in receptor activation or translocation between fish and mammals has been suggested to account for these differences (Di Giulio & Hinton, 2008). In humans, the nuclear pregnane X receptor (PXR) and constitutive androstane receptor (CAR) regulate several members of CYP families 1 through 3. Induction mechanisms by CAR and PXR are essentially similar to AhR induction. An inducer binds to CAR or PXR, and the inducer-receptor complex forms a heterodimer with the retinoid X receptor (RXR). This heterodimer binds to a DNA response element, enhancing DNA transcription and eventually protein synthesis (Fuhr, 2000). Induction of CYP3A and CYP2B have been studied and used as a biomarker of PXR and CAR activation (Kretschmer & Baldwin, 2005). Xenobiotics have been demonstrated to alter CYP2/3A gene transcription in mammals via binding and transactivation of PXR and CAR (Pascussi et al., 2003; Plant and Gibson, 2003).

Differences in CYP2 and CYP3 induction mechanisms among fish species have also been reported. In fish, expression of CYP2 family mRNA is not induced by PB-type inducers nor do they cause nuclear translocation of CAR. Although the CYP2 family catalyzes xenobiotics in fish, the gene regulatory systems may be different than those of mammals (Uno et al., 2012). In a review by Finn (2007) the control of CYP3A expression is suggested to be mediated by the AHR/ARNT pathway in zebrafish (*Danio rerio*). However in Atlantic salmon (*Salmo salar*) the PXR has been suggested to activate CYP3A genes and the AHR/ARNT pathway activates CYP1A (Finn, 2007). Recently, a single piscine CAR/PXR gene was identified when searching the pufferfish genome; however, this receptor was more related to PXR family members and hence a probable functional analog of PXR (Maglich et al., 2003). PB-type inducers proceed through activation of the CAR and binding to PB response elements in the promoter region of the CYP2B genes (Di Giulio & Hinton, 2008). The apparent lack of a piscine CAR may be an explanation for diminished CYP3A and CYP2B (PB-type) induction in fish (Maglich et al., 2003; Di Giulio & Hinton, 2008).

A classic toxicological concern with enzyme induction is the increased formation of toxic metabolites. Induction of CYP may increase the activation of pro-carcinogens to DNA-reactive metabolites, leading to increased tumour formation. Benzo(a)pyrene (B(a)P) can induce its own metabolism via CYP1A1 which may lead to increased formation of carcinogenic metabolite (+)benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (Figure 1-4).

For bioaccumulative substances, induction of xenobiotic metabolizing enzymes may increase biotransformation rates, which decreases the bioaccumulation of the parent chemical substances (Van der Oost et al., 2003). Reduced bioaccumulation of a chemical may result in a lower risk of toxicological impact to an organism.

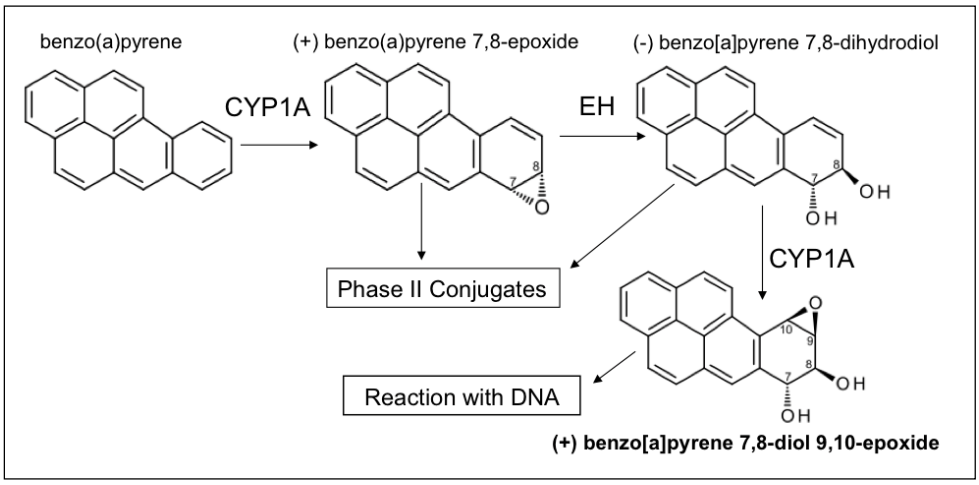

Figure 1-4. Biotransformation of B(a)P to carcinogenic metabolite, (+)benzo(a)pyrene 7,8 diol 9,10-epoxide (Adapted from Di Giulio & Hinton, 2008).

1.4.2. Enzyme Inhibition

Enzyme inhibition occurs when there is a decrease in enzyme activity due to the binding of an inhibitor to a catalytic site on the enzyme. The binding of an inhibitor can impede a substrate from entering an enzyme's active site and/or hinder the enzyme from catalyzing its reaction (Rogers & Gibon, 2009). Inhibitory effects of some chemicals on CYPs, such as piperonylbutoxide (PBO) (Sijm et al., 1993) and antifungal imidazoles, such as ketoconazole and miconazole (Miranda et al., 1998) are well known. Inhibition of biotransformation enzymes in an organism may potentiate the effect of toxic chemicals via increased bioaccumulation to levels that approach toxicological thresholds. Enzyme inhibition may also occur when two compounds share a similar biotransformation pathway which results in decreases in activity due to competition. Enzyme inhibition may be irreversible and involve the covalent binding of a toxic substance, causing permanent damage to the enzyme. Inhibition may also be reversible and affects the fine control of the enzyme's activity. Reversible inhibition involves non-covalent binding of an inhibitor to the enzyme, resulting in a temporary reduction of activity. There are three different types of reversible inhibition and they differ in mechanism to decrease enzyme activity (Rogers & Gibon, 2009).

Competitive inhibitors often resemble the structure of the enzyme's substrate and will bind to an enzyme's active site and either prevent or decrease the enzymes ability to bind with the substrate. Competitive inhibitors prevent the substrate from binding but are

not changed by the enzyme (Rogers & Gibon, 2009). The degree of inhibition depends on the concentrations of inhibitor and substrate present in addition to the affinity of the enzyme for the inhibitor and the substrate. Competitive inhibition increases K_m as it takes a higher concentration of the substrate to reach the K_m point because the inhibitor interferes with substrate binding. V_{max} remains constant since an infinite substrate concentration will exclude the inhibitor. Competitive inhibition can be overcome by sufficiently high concentrations of substrate by out-competing the inhibitor (Berg et al., 2002).

Non-competitive inhibitors do not bind to the catalytic site of the enzyme, but bind to a second site on the enzyme so the binding of either one is independent of the other. The extent of inhibition depends only on the concentration of the inhibitor. V_{max} decreases because the binding of the inhibitor hampers catalysis and the reaction cannot proceed as efficiently. K_m remains the same as the actual binding of the substrate still functions properly (Berg et al., 2002; Rogers & Gibon, 2009).

In uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex. This inhibition prevents normal behaviour of the enzyme-substrate complex because the inhibitor reduces the apparent k_{cat}, and ultimately V_{max}. This reduction in V_{max} is the result of the inhibited enzyme being less catalytically effective as it takes longer for the substrate to leave the active site of the enzyme. K_m also decreases with and indicates a higher binding affinity of the substrate to the enzyme (Berg et al., 2002). This is because the substrate has now been taken up to form the substrate-enzyme and the substrate-enzyme-inhibitor complexes, effectively consuming more substrate.

1.5. Environmental Factors Affecting Biotransformation

An organism's environment can have a significant role in modifying enzyme activity and consequently rates of biotransformation which may ultimately affect a substance's bioaccumulation potential. Environmental factors that may alter biotransformation enzymes include salinity (Bawardii et al., 2007), temperature (Carpenter et al., 1990; Trowell, 2010), water hardness (Tolls et al., 2000), and pH (Laitinen et al., 1984). At the cellular level sudden changes in these environmental factors may result in immediate reaction rate compensations resulting in changes in the

K_m or V_{max} of enzymes to prevent significant changes to metabolic pathways (Carpenter et al., 1990; Bisswanger, 2008).

Exposure to chemical substances may also result in enzyme induction (Behrens et al. 2001; Buckman et al. 2007) or inhibition (Miranda et al. 1998, Sijm et al. 1993; Lewis et al., 2006). Aquatic environments in particular are reservoirs for many chemicals that can enter the environment in the form of wastewater effluents from variety of industrial, agricultural, or municipal sources (Matthiessen et al., 1999). It is possible that these effluents contain chemicals that may either induce or inhibit enzymes involved in xenobiotic metabolism. Chemical mixtures may cause complex interactions on these enzyme systems and identifying their potential biological impact by measuring exposure via chemical analyses alone is virtually impossible due to the complexity of these mixtures (Ciccotelli et al., 1998).

1.5.1. Effects of Exposure to Chemical Mixtures

Continuous releases of chemical mixtures into the environment make both enzyme inhibition and induction environmentally relevant processes. Internal biochemical adaptations such as the induction of biotransformation enzymes in the presence of xenobiotic substances enable the organism to survive in polluted environments (Schmidt et al., 2004). The presence of additional enzymes attempts to protect the organism against chemical exposure. In environmental management scenarios, enzyme induction responses are commonly used as a biomonitoring tool to evaluate early signs of exposure to pollutants (de Maagd et al., 1998; Melanson et al., 2004).

Industrial effluents are major sources of mixed contaminants to aquatic ecosystems and are strong candidates for induction of CYP enzymes, particularly CYP1A, because they often contain well known ligands of the AhR such as PCDDs and PCBs (Kosmala et al., 1998; van Veld et al., 1990). Fish collected from contaminated areas have been reported have elevated CYP1A levels relative to nearby reference sites. There are numerous field studies which use CYP1A to biochemically measure the effects of inducing chemicals in the vicinity of kraft pulp and paper mill effluents (Lindstrom-Seppa & Oikari, 1990; Hodson et al., 1992; Forlin et al., 1995; Soimasuo et

al., 1995; Whatley et al., 2010). Additional literature for other point sources commonly evaluated by CYP1A induction include oil spills (Woodin et al., 1997; Kirby et al., 1999; Stagg et al., 2000), and industrial areas with chemical manufacturing (Vindimian et al., 1991; Behrens & Segner, 2001) or landfills (Gallagher & Di Giulio, 1989; Ueng et al., 1992). Significant CYP elevations in the livers of field collected fish (Lindstrom-Seppa & Oikari, 1990), fish placed in cages at near point sources (Soimasuo et al., 1995; Tuvikene et al., 1999; Melanson et al., 2004), and laboratory exposed of fish (Gagne & Blaise, 1993; Whatley et al., 2010) have all been reported for various chemical mixtures relative to control groups or reference sites.

Some anthropogenic compounds have also been found to act as inhibitors of CYP. Organotin compounds were shown to reduce both content and activity of CYP in marine and freshwater fish *in vivo* and *in vitro* (Bucheli & Fent, 1995). Burton et al. (2002) reported that tributyl tin potentiated PCB-126-associated CYP1A toxicity in channel catfish (*Ictalurus punctatus*). Similar effects were observed with benzene (Arinc and Sen, 1993) and cadmium chloride (Forlin et al., 1985; Lewis et al., 2006). It also has been shown that fish liver microsomal EROD activity may be inhibited by heavy metals (Viarengo et al., 1997; Oliveira et al., 2004). Additionally PAHs have been reported to inhibit CYP1A and include naphthalene (McKee et al., 1983) and fluoranthene (Wills et al., 2009).

Elevations of phase II biotransformation enzymes have even been reported following exposure to industrial chemicals, however the responsiveness is lower than that for CYP1A (Behrens & Segner, 2009). Other studies have failed to detect significant elevations of GST activities in fish following laboratory exposure to industrial chemicals (Bucheli & Fent, 1995). In a review by Van der Oost et al. (2003) hepatic UDPGT activities were reported to be increased in most laboratory experiments and field studies with fish exposed to PAHs, PCBs, PCDDs. An increase in hepatic GST activity has been reported in several studies after exposure of fish to PAHs, PCBs, and PCDDs, but most studies did not demonstrate any significant alterations. GST induction in field collected fish also yielded conflicting results. Several studies reported GST activities to be significantly increased, but in most cases no significant differences were observed between fish from control and polluted sites. In contrast, the same review reported significant decrease in GST activities was observed in rainbow trout, sea bass,

seabream, and sunfish exposed to PCDDs, pesticides, and PAHs (Van der Oost et al., 2003).

The use of CYP biomarkers integrates exposure to the entire bulk of pollutants present in the environment and will reflect any cumulative, synergistic, or antagonistic effect of complex mixtures (Bucheli & Fent, 1995). Till et al. (1999) reported that the inducing potency of a complex mixture of 16 PAHs was about two times higher than predicted from their individual induction equivalency factors (Grung et al., 2007). CYP1A can be measured at three levels of induction: at the mRNA level, at the protein level, and at the functional activity level (Behrens & Segner, 2001). Changes in gene expression may lead to alteration of protein content and enzyme activity, which may ultimately influence biotransformation processes.

Evaluation of the alteration of xenobiotic biotransformation reactions has been studied and compared between organisms from contaminated areas to reference areas. Hepatic microsomes in mallards (*Anas platyrhynchos*) and common mergansers (*mergus merganser americanus*) were collected in ducks from high activity industrial areas and control areas. Rates of microsomal metabolism for B(a)P were 7-fold higher in ducks collected from the polluted areas compared to non-polluted areas (Honey et al., 2000). Mosquitofish and snails were exposed *in vivo* to B(a)P concurrently with enzyme inhibitor, piperonyl butoxide (PBO). After 33 days exposure, BAF values were obtained for both B(a)P and B(a)P + PBO. BAF values increased with the addition of PBO for both mosquitofish (30 to 140 units) and the snail (4860 to 7520 units) showing the importance of biotransformation on the bioaccumulation of substances (Lu et al., 1977).

The influence of CYP-induction on PCB biotransformation rates was studied when rainbow trout were exposed to dietary concentrations of a mixture of three Aroclors (1248, 1254 and 1260). Pre-exposure was found to significantly influence the biotransformation rates of the PCBs studied. The addition of CYP2B-inducing PCB congeners to Aroclor dosed food resulted in elevated biotransformation rates of PCBs and an increase in the number of hydroxlated PCB metabolites. This trend was not observed when CYP1A-inducing congeners were added to the Aroclor dosed food suggesting that isoforms other than CYP1As are responsible for the majority of PCB biotransformation observed in rainbow trout (Buckman et al., 2007).

Other studies have been done where substrate depletion rates have been compared between chemicals incubated individually and in mixtures in hepatic *in vitro* systems. Incubated mixtures of tetrachlorobenzyltoluenes (TCBTs) (isomers 87, 88, and 97) and PCBs (congeners 15 and 153) in rainbow trout microsomes were not significantly different from the incubation of the single isomers and no substrate competition was suggested to occur in the mixture (Kramer et al., 2000). Biotransformation rates of PAHs (9-methylanthracene, B(a)P, chrysene) and PCB-153 as individual chemicals or as a mixture were measured using rainbow trout hepatocytes. Chemical mixture incubations resulted significantly lower depletion rate constants for B(a)P and chrysene in chemical mixtures than for individual incubations for B(a)P and chrysene (Trowell, 2010). This reduction may have been the result of competitive inhibition because both B(a)P and chrysene are known substrates for CYP1A (Stegeman et al., 1998).

1.5.2. Municipal Wastewater Effluents

Municipal wastewater effluents are recognized as a major environmental contamination source and contain many contaminants derived from both domestic and industrial sources. They may contain complex mixtures of PAHs, pesticides, surfactants, steroids, metals, pharmaceuticals, cosmetics and personal care products. The influence of municipal effluents, containing primarily pharmaceuticals and personal care products, on enzyme activities and biotransformation processes has not been as extensively explored. This is likely because classic CYP inducers are thought to be at low levels (ng/L) or removed following effluent treatment at wastewater treatment plants. Although many chemicals are removed with treatment, pharmaceuticals, cosmetics and personal care products are of great concern and it has been noted that these compounds are not efficiently removed during treatment and may persist in the environment (He et al., 2011). These chemicals are not persistent in the environment by the conventional definition but are “pseudo-persistent” due to their continuous discharge via wastewater outfalls.

Molecular analyses have been performed on fish following exposure to wastewater effluents and differences in CYP gene expression following exposure to treated effluents have been reported. Cuklev et al. (2012) observed that genes

associated with xenobiotic metabolism, including CYP1A, were differentially expressed in rainbow trout exposed to effluents that underwent conventional activated sludge treatment. Ings et al. (2011) reported significant CYP1A a mean 8.6 fold change difference in rainbow trout hepatic transcripts following exposure to 10% tertiary-treated municipal wastewater effluents relative to an upstream control. Gagne et al. (2012) reported increases in the expression of CYP1A1 and GST and decreases in CYP3A4 expression in rainbow trout hepatocytes following exposure to treated urban municipal wastewater effluents. Significant elevations in EROD were noted in Chinese gobiid fish, abehaze (*Mugilogobius abei*) following exposure to effluents containing personal care products and pharmaceuticals. Elevations of aminopyrine N-demethylase (APND) were also observed; aminopyrine is a substrate which is partially linked to CYP2B and CYP3A induction (He et al., 2011). A strained cyclopropane ring on some pharmaceuticals was suggested to provide a site for CYP1A induction. Quinolones and synthetic broad-spectrum antibiotics may also cause an induction response (He et al., 2011).

Individual substances commonly detected in municipal wastewater effluents have also been shown to influence xenobiotic metabolism. In particular, pharmaceuticals may cause side effects in non-target organisms through enzyme induction or inhibition because they are metabolized primarily by CYP enzyme families in humans (Thibault and Porte, 2008). In rainbow trout hepatocytes, pharmaceuticals diclofenac, carbamazepine, sulfamethoxazole, and, to a lesser extent, fenofibrate and clofibrate inhibited the basal EROD activity at sublethal concentrations. The same study reported that propranolol significantly induced EROD activity (Laville et al., 2004).

Widely used pharmaceuticals belonging to a variety of therapeutic classes were able to inhibit the catalytic activity of different CYP enzymes in carp liver (Thibaut et al., 2006). Anti-depressive drugs were strong inhibitors of CYP1A, CYP3A-like, and CYP2K-like catalyzed activities, while anti-inflammatory drugs were potent CYP2M- like inhibitors (Thibaut et al., 2006). Fibrate lipid regulators (clofibrate, bezafibrate), anti-inflammatory drugs (naproxen, ibuprofen) and anti-depressants (fluoxetine, fluvoxamine, paroxetine) were low to moderate inducers of EROD activity with potencies between 13% and 35% that of BNF. These chemicals have been detected in surface waters at the nanogram to microgram per litre level (Thibault and Porte, 2008).

Besse and Garric (2008) identified several enzyme inducing and inhibiting human pharmaceuticals that are commonly found in wastewater effluents. This study also prioritized pharmaceuticals to be monitored in environmental risk assessment and management scenarios involving wastewater effluents. Table 1-4 lists four compounds that were on this priority list that have the ability to modulate CYP activity (Besse & Garric, 2008) and may be present in municipal wastewaters.

Table 1-4. Human pharmaceuticals and their therapeutic use, and their effect on CYP enzyme activity. These pharmaceuticals are prioritized to be monitored in environmental risk assessment.

Pharmaceutical	Therapeutic use	CYP Inducer/Inhibitor	Enzyme or protein
Carbamazepine	Anti-convulsive	Inducer	CYP 3A4 CYP 2C9 CYP 1A2
Clarithromycin	Antibiotic	Inhibitor	CYP 1A2 CYP 3A4
Sertraline	Antidepressant	Inhibitor	CYP 2D6
Valporic acid	Anti-convulsive	Inhibitor	CYP 3A4 CYP 2C9

Exposure to several ionic detergents, Brij 35, CHAPS, cholate, deoxycholate, Lubrol, SDS, Triton X-100, Tween 20, caused varying levels of inhibition of EROD and GST activities in microsomes of leaping mullet (*Liza saliens*) (Sen and Semiz, 2007). Nicotine and phenobarbital are compounds that have both been identified in wastewater effluents and are suspected to have contributed to observed elevated EROD activity in rainbow trout hepatocytes (Grung et al., 2007). Triclosan is a widely used antimicrobial agent found in personal care products and is often detected in municipal wastewater effluents. It has also been reported to induce EROD activity in rat hepatocytes (Jinno et al., 1997). Estradiol significantly suppresses CYP1A activity in fish although little is known about the effects of xenoestrogens on CYP1A (McArdle et al., 2000; Williams et al., 1998). Nonylphenol has been detected widely in wastewater streams and exposure to nonylphenol reduced levels of CYP2K, CYP3A, and CYP1A proteins in juvenile Atlantic salmon (Wassmur et al., 2010).

Effluents contain a variety of chemicals that may induce or inhibit enzyme activities in fish and other aquatic organisms. The effect of exposure to chemical mixtures on xenobiotic biotransformation remains important, as exposure of aquatic organisms and fish to these mixtures are common. This is particularly important as municipal effluents are a major contamination source containing pharmaceuticals and personal care products that may also influence xenobiotic metabolism because they may not be removed following treatment processes. However the ability of these types of effluents to influence biotransformation processes is not as well understood as it is for industrial effluents containing well-established ligands of the AhR (i.e. PCBs, PAHs, PCDDs).

1.6. Hypothesis and Research Objectives

Exposure to environmental contaminants has the ability to alter biotransformation processes via inhibition or induction of xenobiotic metabolizing enzymes. Real world scenarios regularly involve exposure to various chemical mixtures, which may impact hepatic enzyme activities in organisms in the field. Exposure to chemical mixtures may impact biotransformation enzymes in organisms in a variety of ways and should be considered when biotransformation rates of substances are being measured in chemical evaluation. When the biotransformation rate of a substance is measured in laboratory-raised organisms it is not clear whether the biotransformation rate obtained in the lab is representative of rates obtained in field-derived organisms. Rates of biotransformation that are measured in organisms found in more representative real world exposure scenarios may offer more accurate assessment of bioaccumulation and consequently be more protective of fish and other organisms.

The objective of this research was to determine if exposure to environmentally relevant chemical mixtures of treated municipal wastewater effluent had a significant effect on the *in vitro* substrate depletion rate constants of hydrophobic chemicals in rainbow trout. The *in vitro* depletion rate constant data generated in the S9 *in vitro* system were employed in an *in vitro-in vivo* extrapolation (IVIVE) hepatic clearance model (Nichols et al., 2006) and then in a fish bioaccumulation model (Arnot & Gobas 2003) to extrapolate BCF values. Modeled BCFs were compared among exposure

groups to see if whether pre-exposure to municipal wastewater effluents have an effect on the BCF values in fish.

As was outlined in this review, when regulatory agencies are assessing chemicals for their bioaccumulative properties, chemicals may be more accurately evaluated if rates of biotransformation are available. Further understanding may be achieved with knowledge of how biotransformation rates are affected following exposure to low levels of chemical mixtures that are representative of those found in the environment. This may assist in understanding whether biotransformation rates obtained in the lab are representative of rates in the field. If there is no effect of pre-exposure then *in vitro* biotransformation rates obtained in the lab may be used with greater confidence. If there is an effect then evaluators need to be cautious when using *in vitro* substrate depletion rate data to evaluate chemicals for their bioaccumulation potential.

2. Materials and Methods

2.1. Chemicals

Pyrene (log K_{ow} 4.88), benzo(a)pyrene (log K_{ow} 6.04), chrysene (log K_{ow} 5.81), 9-methylanthracene (log K_{ow} 5.07) and deuterated (d-12) chrysene were obtained from Sigma-Aldrich (St Louis, MO, USA). Chemical purities were always >98%. Acetonitrile-190, methanol and hexane were obtained from Caledon Laboratories (Georgetown, ON, CAN).

2.2. Fish

Adult male rainbow trout (*Oncorhynchus mykiss*) were purchased from Miracle Springs Trout Farm (Mission, BC). Average fish weights and loading densities are for the 2011 and 2012 pre-exposure experiments are listed in sections 2.4.1 and 2.4.2, respectively. Fish were housed at Simon Fraser University (SFU) in 500-L flow-through tanks supplied with de-chlorinated municipal water at a temperature of 13°C ± 1°C under a light:dark photoperiod of 12:12. Fish were fed commercial salmon pellets (Ewos Pacifica Ltd., Surrey, BC) and were acclimated for at least two weeks at SFU in standard holding tanks prior to exposure to effluent.

2.3. Wastewater Effluent

Organism handling, facilities, general laboratory and sampling procedures followed Environment Canada's 'Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout', where appropriate (Environment Canada, 2007). Secondary treated municipal wastewater effluent was collected from the Chilliwack wastewater treatment plant. Plant influent comes from primarily municipal households with no industrial or agricultural sources in the area. The plant treats an estimated 17,000 residential properties, which is equivalent to approximately 55-60,000 people (personal communication with Plant Operator, June 30th

2012). Influent receives secondary treatment with ultraviolet radiation prior to release its release as effluent.

2.4. Pre-exposure Experiments

Effluent was collected for two separate pre-exposure experiments: (1) the 2011 experiment and (2) the 2012 experiment. The details of each experiment and liver S9 collection procedures are outlined in the following sections.

2.4.1. Fish Exposure and Sampling (2011 Experiment)

Effluent was collected on August 29th, 2011 and was transported to the laboratory in previously-unused high-grade polyethylene carboys (Reliance Products, # 00500013). Carboys containing the effluent were stored in the dark at 4 ^{o}C until the day before effluent was required for use when it was moved into the room where the pre-exposure experiment occurred and acclimated for 24 hours at 13 ^{o}C.

Pre-exposure took place over a 7 day period. Fish were exposed under static renewal conditions to 0, 0.1, 1 and 10% concentrations of effluent diluted in de-chlorinated water. There were four replications of each concentration with two fish per 175L tank. Mean fish weight was 160 ± 25 grams (n=32) with an average loading density in the experimental tanks of 1.83 ± 0.15 g fish/L water. After 84 hours exposure, fish were fed 1% of their body weight (~3 grams of food per tank for two fish) two hours prior to test renewal with fresh test solution (static, >90% replacement). Water quality measurements of temperature, pH, dissolved oxygen, and conductivity were taken daily for the entire duration of exposure (Section 3.1). On day 7 the experiment was terminated and sampling for liver S9 sub-cellular fraction began immediately after. Following euthanasia with MS-222, livers were immediately excised and pooled within treatment groups (Figure 2-1) with livers from eight fish per pool.

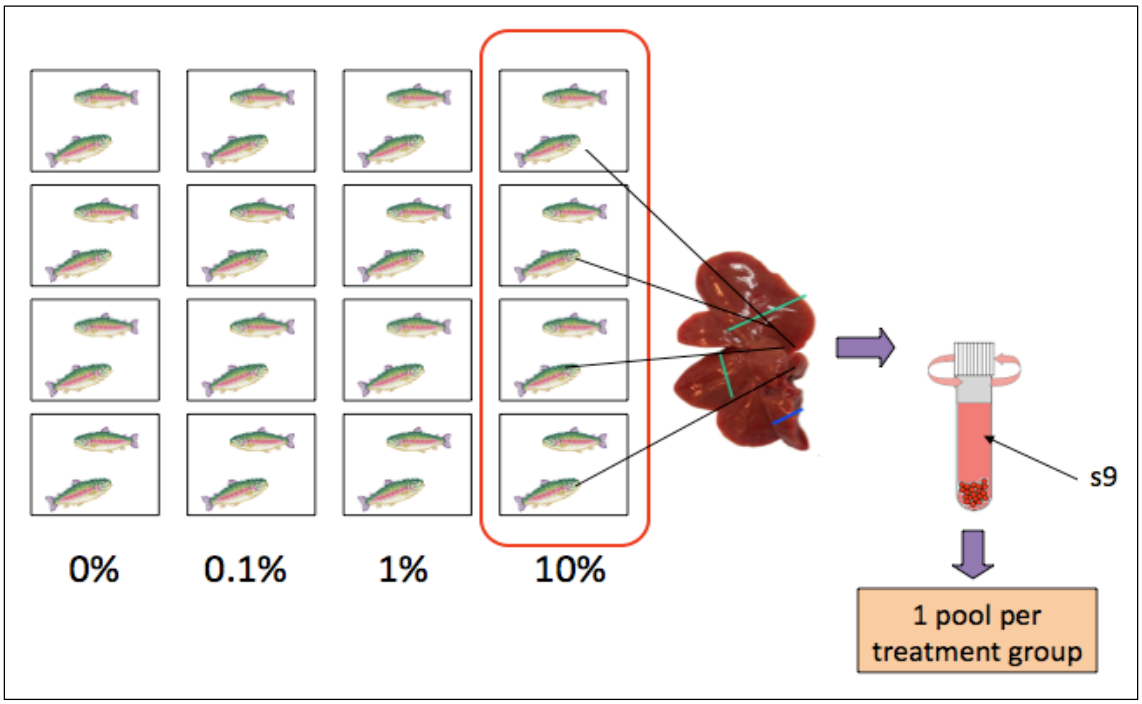

Figure 2-1. Following euthanasia, trout livers were excised and pooled together per treatment group to create pooled sub-cellular S9 fractions.

2.4.2. Fish Exposure and Sampling (2012 Experiment)

Effluent was collected on June 25th, 2012 and was transported to the laboratory in previously-unused high-grade polyethylene carboys (Reliance Products, # 00500013). Carboys were stored in the dark at 4 °C until the day before effluent was required for use where it was moved into the room where the pre-exposure experiment occurred and acclimated for 24 hours at 13 °C.

Pre-exposure took place over a 7 day period. Fish were exposed under static conditions to diluted effluent concentrations of 0 and 20%. There were nine tanks of each exposure concentration with two fish per 175L tank. Fish were randomly distributed among the eighteen tanks. Mean fish weight was 195.70 ± 21.30 grams (n=36) with an average loading density of 2.24 ± 0.24 g/L. After 84 hours exposure, fish were fed 1% of their body weight (~4 grams of food per tank for two fish) two hours prior to test renewal with fresh test solution (static, >90% replacement). Water quality measurements of temperature, pH, dissolved oxygen, and conductivity were taken daily for the entire duration of exposure. On day 7 the experiment was terminated and sampling for liver S9 sub-cellular fraction began immediately after. Following euthanasia, livers were

immediately excised and pooled together into three pools per treatment group (Figure 2-2) with livers from six fish per pool.

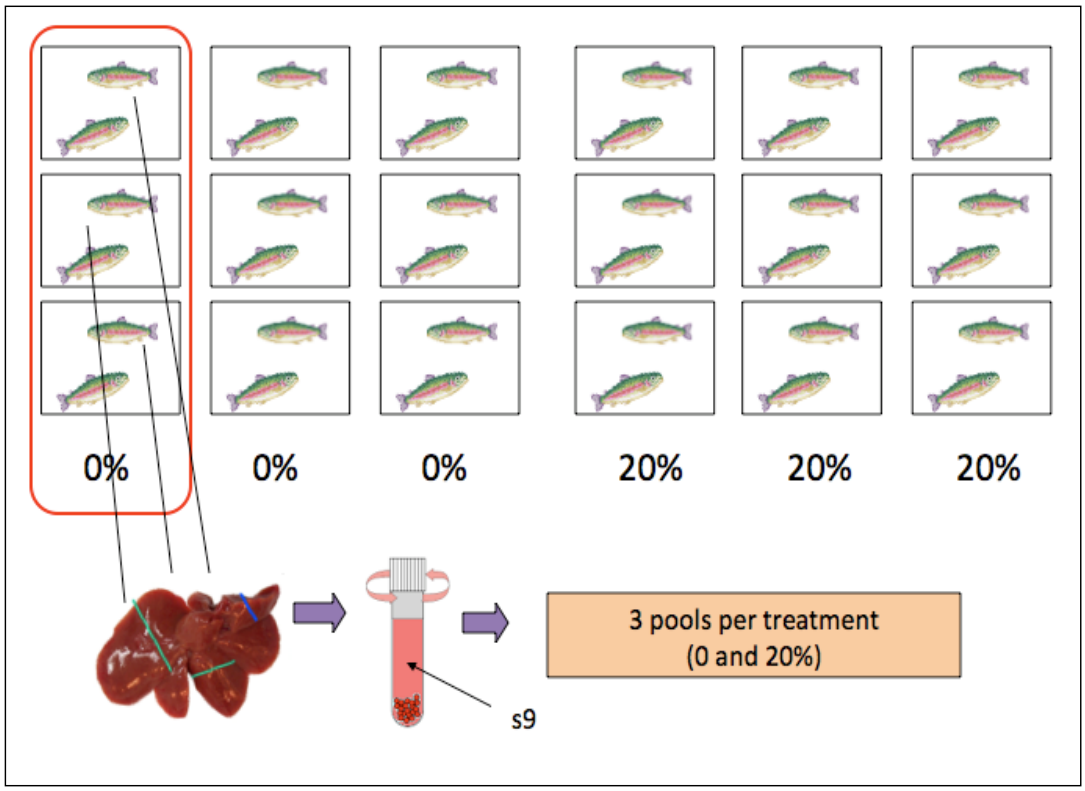

Figure 2-2. Following euthanasia, trout livers were excised and pooled into three replicates per treatment group to create pooled sub-cellular S9 fractions.

2.5. S9 Sub-cellular Fraction Preparation

Liver S9 sub-cellular fraction is obtained from centrifugation of homogenized liver at 9,000g. Centrifugation precipitates the nuclei and mitochondria and separates the supernatant for collection. The supernatant fraction contains the microsomal and cytosolic portions of the cell. The microsomal component of the S9 fraction contains CYP isoforms and other phase I biotransformation enzymes and the cytosolic portion contains phase II enzymes.

S9 preparation procedures were adopted from Han *et al*. 2008 with some modifications. Following the exposure, fish were humanely euthanized by anaesthetic overdose using a solution of 0.3 g/L MS-222 and 0.3 g/L sodium bicarbonate in de-chlorinated water. It has been reported elsewhere that MS-222 does not affect liver

biotransformation enzyme activity in rainbow trout (Kolanczyk et al., 2003). Livers were then immediately excised and pooled together per treatment group (as described in Section 2.4). The livers were rinsed in 4°C 1.15% KCl solution and then minced on an ice-cold Kimax pyrex glass Petri dish cover (approximate diameter of 100 mm) with a razor blade. Subsequently, they were homogenized in one volume of homogenization buffer (0.2 M phosphate buffer containing 1.15% KCl, at a pH of 7.4) using a Potter-Elvehjem tissue homogeniser with Teflon tipped pestle (Kimble tissue grind comp, size 22; Vineland, NJ, USA) and glass mortar (Kimble tissue grind tube, size 24; Vineland, NJ, USA) on ice. The speed of VWR Canlab homogenizer (West Chester, PA, USA) was set at approximately 1000 r.p.m., and the entire homogenizing process involved approximately fifteen passes.

The pooled homogenates were placed in 50 mL-Oak Ridge centrifuge tubes (Nalgene Labware; Rochester, NY, USA), balanced, capped, and centrifuged (Hermle Model Z 360 K; Wehingen, BW, Germany) at 9,000 g for 20 minutes at 4°C. One millilitre of the S9 homogenates were collected from the pool for protein analysis and the rest was transferred to multiple ice-cold 20 mL glass scintillation vials with foil-lined caps (VWR Canlab). All S9 samples were immediately stored in a freezer (Sanyo V.I.P. series -86°C; Moriguchi, Osaka, Japan) at -80°C until the day of the experiment.

2.6. Chemical Exposure and Incubation

The active rainbow trout liver S9 incubation mixtures contained 0.1 mL NADPH regenerating system (8 µmol of glucose-6-phosphate, 0.8 µmol of NADP, 4 µmol of $MgCl_2$, and 1.6 units of glucose-6-phosphate dehydrogenase), 0.2 ml phosphate buffer (0.2 M at pH 7.4), and 0.2 mL defrosted S9 fraction. Incubation mixtures containing 0.3 ml of the same phosphate buffer and 0.2 mL inactivated trout liver S9 served as negative controls. Inactivation was achieved via the omission of NADPH and cofactors from the incubation medium. The S9 fraction was also pre-incubated at ambient temperature 24 hours prior to the experiment.

Each incubation mixture was introduced in a 2 mL amber autosampler vial (Agilent; Mississauga, ON, Canada), capped with a screw cap with a Teflon/rubber/Teflon septum, and pre-incubated in a 13.5°C water bath for 5 minutes.

The reaction was initiated by adding 2.4 µL of a solution of 104.2 µM of test chemical in acetonitrile-190 (Caledon). The final concentration of test chemical in the incubation vial was 0.5 µM. Test chemicals concentrations in the S9 were well below reported Michaelis-Menten constants of 15.1 µM for pyrene hydroxylation measured by isolated hepatocytes from rainbow trout (Law et al., 1991) and 33-125 µM for benzo(a)pyrene hydroxylation measured in liver microsomes from rainbow trout (Carpenter et al., 1990). This suggests that enzyme saturation was not likely to occur and the first order enzyme kinetics was expected to be maintained in the experiments for these chemicals.

The reaction was carried out in a Grant OLS 200 water bath with CS 200G refrigerated immersion cooler (Figure 2), at 13.5°C. Vials were rolled at a speed of 60 r.p.m., approximately, throughout the incubation, and reactions were terminated at ten minute time intervals between 0 and 90 minutes by adding 0.2 mL of ice-cold methanol followed by 1 mL hexane. The vials were inverted multiple times and then placed on ice to ensure the reaction was terminated.

The internal standard, deuterated chrysene, was dissolved in hexane and 10 µL was added to each vial (final concentration of 0.25µM), followed by a 90-second vigorous shaking (SIP ® vortex mixer, Baxter Scientific Products, USA) at setting #6 and then a 10-minute centrifugation (Centra CL2 bench top centrifuge, Thermo IEC, USA) at 1,300 g to separate the two phases. The hexane supernatant (approximately 0.6 mL) was transferred to clean 2 mL amber autosampler vial (Agilent) and analyzed by GC MS.

2.7. Chemical Analysis

The extract was analyzed using an Agilent 6890 gas chromatograph (GC) in conjunction with an Agilent 5973 mass spectrometry (MS) detector (Agilent, Mississauga, ON). The column was an HP-5M5 5% phenyl methyl siloxane-coated capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) protected by a fused-silica deactivated guard column (5 m x 0.530 mm i.d.) (Agilent, Mississauga, ON). The injection volume was 1 µL, and the helium carrier gas flow rate was 1 mL/min. The GC was programmed with an injection temperature of 60°C, followed by a temperature ramp of 20°C/min to a temperature of 200°C. The temperature was held at 200°C for 1 min, after which a temperature ramp of 15°C/min was initiated to a maximum temperature of

285°C. The MS quantified the target compounds at select ions (m/z 212 for pyrene, m/z 252 for B(a)P, m/z 192 for 9-MA, m/z 228 for chrysene, and m/z 240 for d-12 chrysene) using an ion energy of 70eV and an ion source temperature of 230°C.

Calibration curves for test chemicals constructed in hexane. Calibration standards were run simultaneously with every analysis to account for instrument fluctuation. Calibration concentrations for all test chemicals were: 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 μM in hexane. The concentration of d12-chrysene was 0.25 μM. Linear regression of the data points was used to calculate the concentration of the dosing chemicals that were detected by the GC/MS. The formula of the linear regression is in the form “y = mx + b”, where “y” was the area ratio (test chemical/internal standard) and “x” is the value of the concentration of the test chemical remaining in the experimental vials in μM.

2.8. Determination of Extraction Efficiency

Extraction efficiency assays were conducted. Test chemicals (2.4 μL, final concentration 104.2 μM in acetonitrile-190) were added into the incubation mixtures under the same condition as the no-cofactor control. The incubation was terminated after 0, 10, 30, 60, 90, 120 minutes by adding 0.2 mL of methanol followed by 1 mL of hexane for extraction. After shaking on the vortex mixer and centrifugation, the supernatant was transferred to clean 2 mL amber autosampler vials and 20 μL of the d12-chrysene internal standard was added. The samples were then analyzed by GC/MS. Test chemicals (2.4 μL, final concentration 104.2 μM in acetonitrile-190) introduced into 1 ml of hexane served as the standard of 100% extraction efficiency (n=3). Extraction efficiencies for pyrene, B(a)P, chrysene, and 9-MA were not significantly different from 100%, therefore no correction factors were required when determining measured chemical concentrations in incubations (Appendix C).

2.9. Determination of Protein Content

The Bradford protein assay (Bradford, 1976) was used to determine the protein content of the trout liver S9 samples. A standard curve was made using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0, 0.005, 0.01, 0.015,

0.020, and 0.025 mg BSA/mL. A Pharmacia LKB Ultrospec III UV/Vis spectrophotometer (Creve Coeur, MO, USA) was used to record the absorbance of the BSA standards and trout liver S9 samples at a 595 nm wavelength.

2.10. Data Analysis and Statistical Design

The concentration of each chemical in incubation media was well below their published Michaelis-Menten constants (K_m), hence substrate depletion rate constants were assumed to follow first-order kinetics (0.5 μM << K_m), and were fit to the equation:

$$\text{Ln}C_t = \text{Ln}C_0 - k_r t \quad (2.1)$$

where C_0 and C_t are the concentrations of compound (μM) at time 0 and time t (min), and k_r is the first order substrate depletion rate constant (per minute) (Bisswanger, 2008).

Experiments were conducted in triplicate for the treatment groups. Measured concentrations of test chemicals were transformed by taking the natural logarithm (Ln) and plotted against reaction time. Data for active and in-active S9 samples were evaluated using linear regression. Slopes from each regression were compared for significant differences ($p<0.05$) using a Student's t test. There were no significant losses of the parent test chemicals in any of inactive S9 control treatment groups in both the 2011 and 2012 exposure studies ($p>0.05$). Therefore, the *in vitro* substrate depletion rate constant (k_r; per minute) was calculated from the slope of the linear regression line in the active S9.

All statistical analyses were conducted in JMP 9.0.2 (2010). In the 2011 experiment, an analysis of variance (ANOVA) was used to compare the *in vitro* substrate depletion constants (k_r values) for all effluent treatments (0, 0.1, 1 and 10%). A Tukey's Honestly Significant Difference (HSD) test was used to determine where significant differences between treatments were present ($p<0.05$). In the 2012 experiment, comparison of the *in vitro* depletion rate constants (k_r values) between effluent treatments (0 and 20%) were performed using a Student's t test ($p<0.05$).

2.11. Model Calculations

In vitro substrate depletion rate constants were extrapolated to the *in vivo* level using an *in vitro-in vivo* extrapolation (IVIVE) model. This approach is based extensively on the IVIVE model described in Nichols et al. (2006) and has been cited elsewhere (Cowan-Ellsberry et al., 2008; Han et al., 2007, 2009; Nichols et al. 2013). *In vitro* rate constants were divided by S9 protein concentration in the incubation medium to calculate intrinsic *in vitro* clearance ($CL_{IN\ VITRO,\ INT}$) and were extrapolated to intrinsic clearance in units of flow per gram of liver ($CL_{IN\ VIVO,\ INT}$; mL/hr/g liver tissue). Trout hepatic clearance (CL_H) was estimated from $CL_{IN\ VIVO,\ INT}$ based on a well-stirred liver model established by Wilkinson and Shand (1975). CL_H was used to determine the *in vivo* biotransformation rate constant (k_{MET}) (Cowan-Ellsberry et al., 2008; Han et al., 2007, 2009; Nichols et al. 2013). k_{MET} was then incorporated into the calculation of the BCF. BCF values were predicted based on the mass-balance fish bioaccumulation model described by Arnot and Gobas (2003, 2004). Model parameters for the IVIVE model and the fish bioaccumulation model are described in Appendix D.

3. Results

3.1. Water Quality in Exposure Tanks

The daily measurements of temperature, pH, conductivity, and dissolved oxygen in the water remained constant for all treatments through the experiment for both the 2011 and 2012 experiments. Water quality measurements were also taken for 100% effluent (Tables 3-1 and 3-2).

Table 3-1. Physical chemical parameters measurements in the water of the control and treatment tanks measured over the duration of the 2011 exposure. Values presented are the mean (± standard deviation).

Parameter	Effluent Concentrations (%v/v)				
Effluent (%)	0%	0.10%	1%	10%	100%
Temperature (°C)	13.6 ± 0.71	13.5 ± 0.62	13.4 ± 0.74	13.2 ± 0.45	11.9 ± 0.73
pH	6.98 ± 0.29	6.82 ± 0.14	6.95 ± 0.14	7.27 ± 0.20	88.3 ± 4.49
Dissolved Oxygen (%)	93.80 ± 9.72	87.80 ± 5.12	89.50 ± 9.80	88.40 ± 11.87	78.30 ± 0.49
Conductivity (µS/cm)	32.03 ± 2.17	34.26 ± 2.20	37.93 ± 2.75	90.38 ± 3.55	569.1 ± 34.56

Table 3-2. Physical chemical parameters measurements in the water of the control and treatment tanks measured over the duration of the 2012 exposure. Values presented are the mean (± standard deviation).

Parameter	Effluent Concentrations (% v/v)		
Effluent (%)	0%	20%	100%
Temperature (°C)	12.10 ± 0.71	12.5 ± 0.68	13.1 ± 0.64
pH	6.97 ± 0.53	7.59 ± 0.59	7.20 ± 0.18
Dissolved Oxygen (%)	92.80 ± 6.01	91.90 ± 4.53	73.60 ± 5.14
Conductivity (µS/cm)	22.66 ± 2.32	125.9 ± 8.07	566.6 ± 28.24

3.2. Calibration Curves

Calibration Curves for B(a)P, chrysene, 9-methylanthracene, and pyrene are displayed in Appendix A. R^2 values were greater than 0.98 for all calibration curves.

3.3. Protein Content of Trout Liver S9 Samples

3.3.1. Trout Liver Samples (2011 Experiment)

The mean protein contents of the pooled trout liver S9 samples from the four exposure groups are displayed in Table 3-3. These concentrations were diluted and were within the linear range of the standard curve. The standard curve had an R^2 of 0.9568. The standard curve and dilution calculations are displayed in Appendix B.

Table 3-3. Mean protein content (± standard deviation) of the pooled trout liver S9 samples for all wastewater exposure scenarios.

Effluent Exposure (% v/v)	mg protein/mL S9 (mean ± SD)
0%	57.97 ± 5.67
0.10%	57.70 ± 2.03
1%	57.09 ± 8.60
10%	67.30 ± 1.43

3.3.2. Trout Liver Samples (2012 Experiment)

The mean protein contents of the three replicates of the trout liver S9 samples from both 0 and 20% exposure groups are displayed in Table 3-4. These concentrations were diluted and were within the linear range of the standard curve. The standard curve had an R^2 of 0.9638. The standard curve and dilution calculations are displayed in Appendix B.

Table 3-4. Mean protein content (± standard deviation) of three replicates of the trout liver S9 samples for fish exposed to 0 and 20% wastewater effluent.

Effluent Exposure (%v/v)	Replicate/Batch #	mg protein/mL S9 (mean ± SD)
0%	1	45.44 ± 4.54
	2	44.05 ± 4.40
	3	42.66 ± 4.27
20%	1	43.21 ± 4.32
	2	45.20 ± 4.52
	3	44.72 ± 4.47

3.4. Effect of Pre-exposure on *in vitro* Substrate Depletion Rates

3.4.1. Effect of Pre-exposure on in vitro Substrate Depletion Rates (2011 Experiment)

Figures 3-1 through 3-4 show the concentration of test chemicals pyrene and B(a)P over time in each replicate (n=3) for all treatments (0, 0.1, 1, and 10%). The substrate depletion rate constants (k_r) were determined from the slope of the substrate concentration depletion curves and are presented in Table 3-5 and Figure 3-5.

An ANOVA (p= 0.0016) and the Tukey's HSD test revealed the mean B(a)P k_r value was significantly elevated in S9 collected from fish receiving 10% effluent treatment relative to the S9 collected from fish receiving the control (0% effluent) treatment. The k_r values collected from S9 in fish from the 0.1 and 1% groups were not significantly different from the k_r value measured in S9 in fish from the control treatment (Figure 3-2). The combination of ANOVA (p = 0.0083) and Tukey's HSD test revealed that mean k_r for pyrene was significantly lower in S9 collected from fish receiving 1% effluent treatment relative to the S9 collected from fish receiving the 0.1% effluent treatment. The mean k_r obtained from fish from the control treatment (0% effluent) was similar to mean k_r values from fish from all other treatments. The mean k_r for pyrene was 3.10 times higher in S9 collected from fish in the 10% effluent treatment but this observation was not statistically significant (p>0.05) due to variability in k_r measurement.

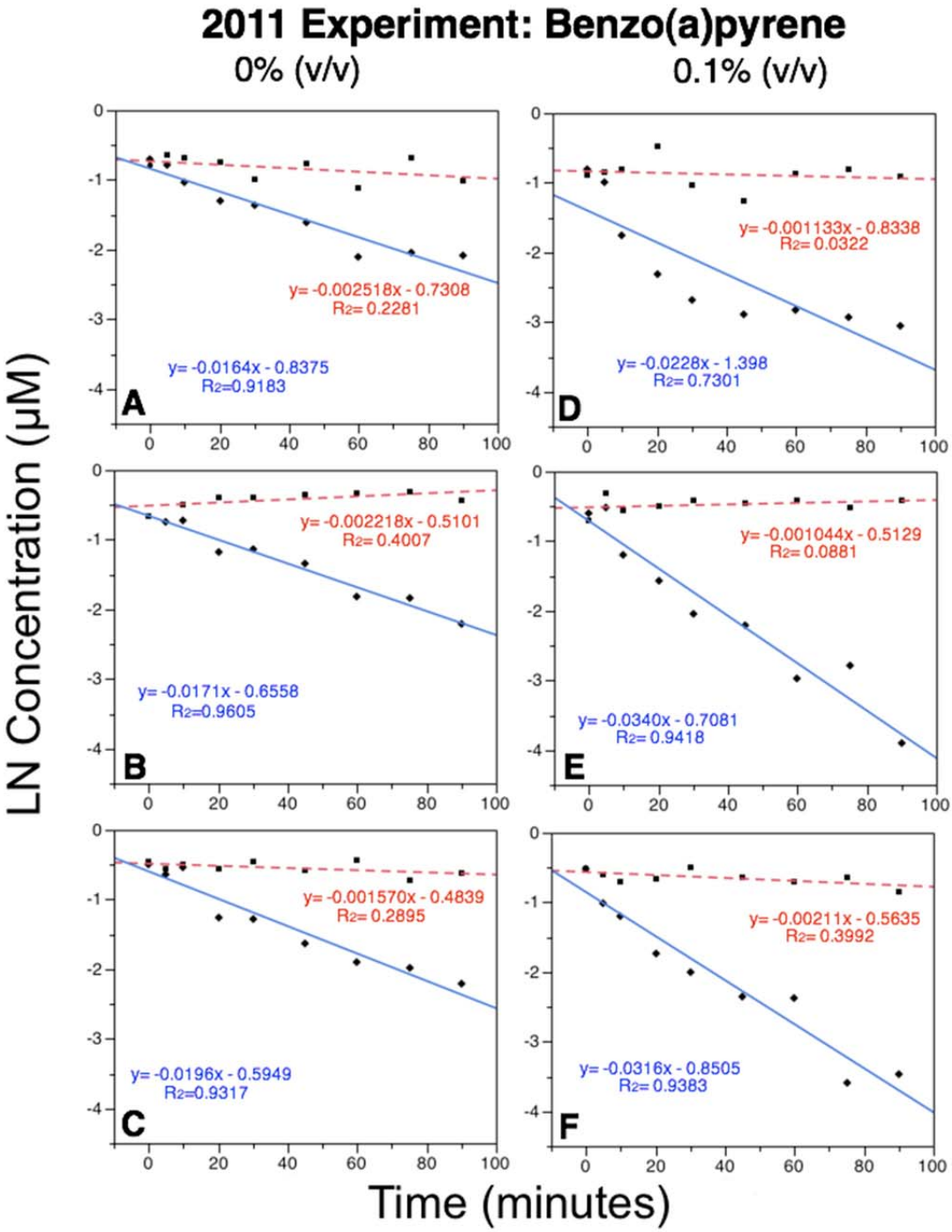

Figure 3-1. Natural logarithm of B(a)P concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 0.1% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 0.1% treatment (D, E and F).

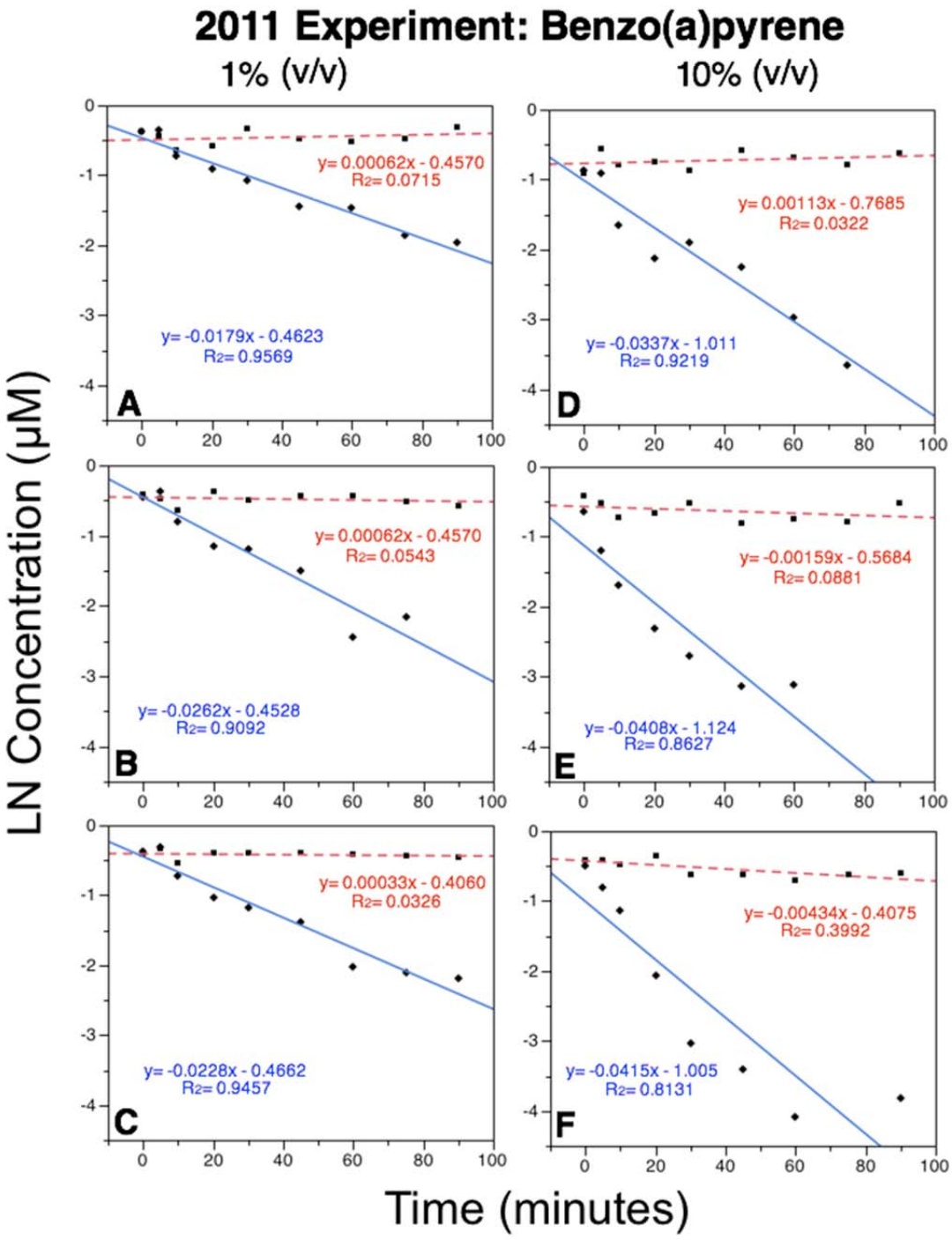

Figure 3-2. Natural logarithm of B(a)P concentration in the incubation as a function of the incubation time for effluent treatments of 1 and 10% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 1% treatment (A, B and C) and of the three replicate incubations for the 10% treatment (D, E and F).

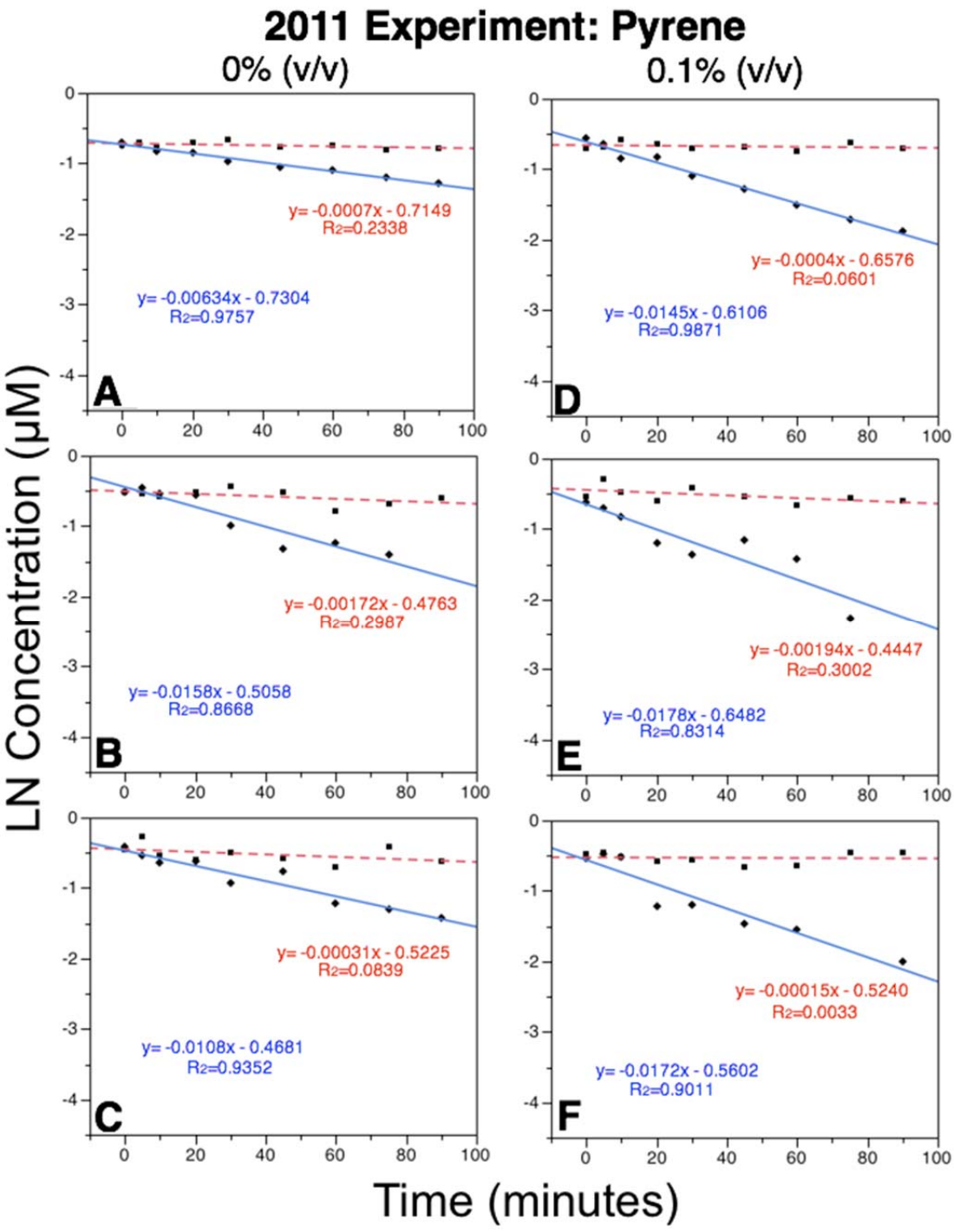

Figure 3-3. Natural logarithm of pyrene concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 0.1% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 0.1% treatment (D, E and F).

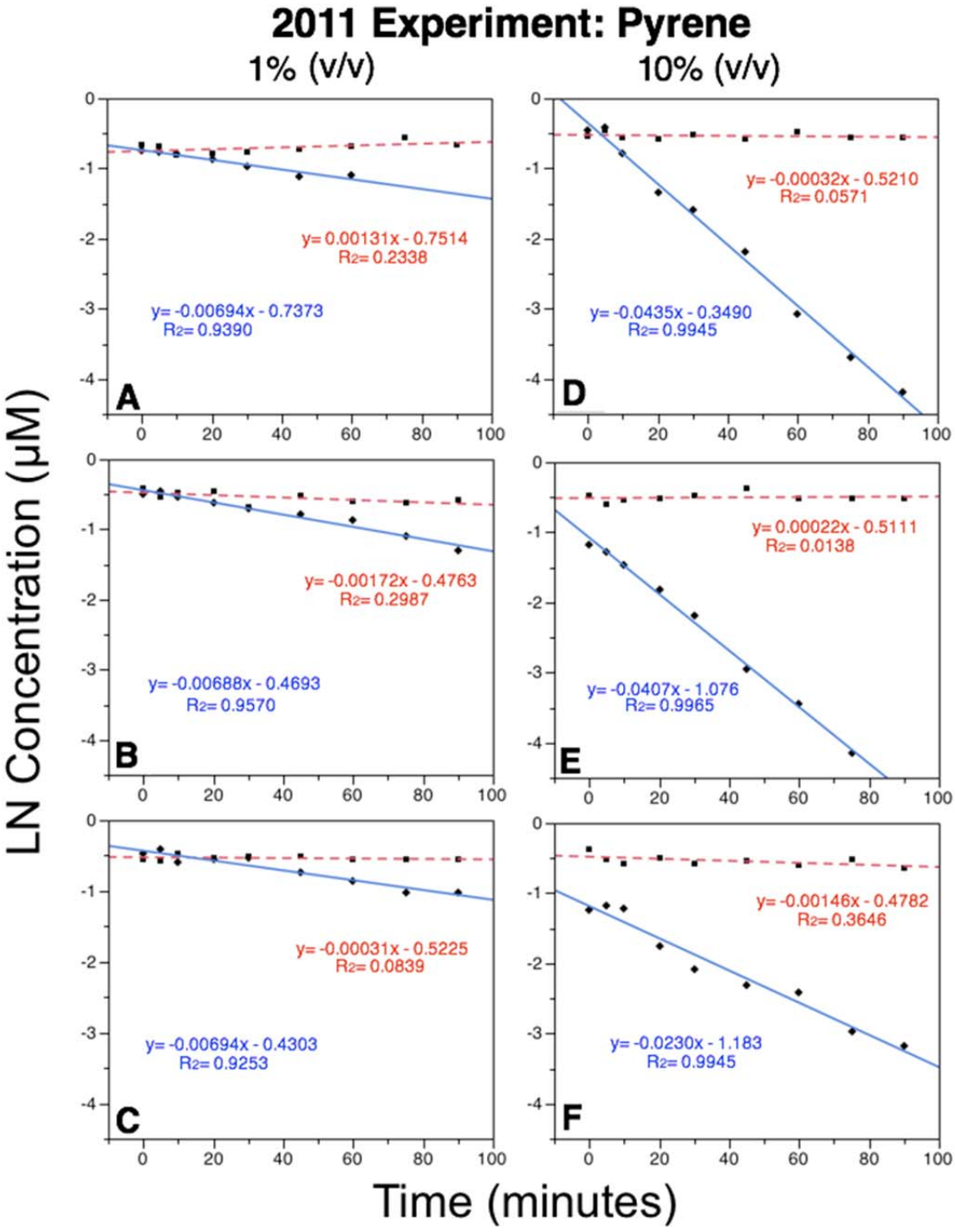

Figure 3-4. Natural logarithm of pyrene concentration in the incubation as a function of the incubation time for effluent treatments of 1 and 10% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 1% treatment (A, B, and C) and of the three replicate incubations for the 10% treatment (D, E and F).

Table 3-5. *In vitro* substrate depletion rate constants (k_r; 10^{-3} min^{-1}) for benzo(a)pyrene and pyrene in triplicate experiments for the four treatments. The 95% confidence intervals of the mean depletion rate constants are displayed in brackets.

Substrate	0%	0.10%	1%	10%
Benzo(a)pyrene				
Replicate 1	16.42	22.87	17.96	33.70
Replicate 2	17.10	34.06	21.62	40.8
Replicate 3	19.66	31.63	22.81	41.53
Mean	17.70 (14.61 – 20.79)	29.47 (18.63 – 40.30)	22.30 (14.63 – 29.97)	38.67 (30.74 – 46.59)
Pyrene				
Replicate 1	6.34	14.55	6.92	43.48
Replicate 2	15.79	17.80	6.88	40.70
Replicate 3	10.82	17.20	6.94	22.97
Mean	10.98 (2.28 – 19.76)	16.50 (13.27 – 19.73)	6.91 (6.85 – 6.97)	33.71 (17.73 – 53.73)

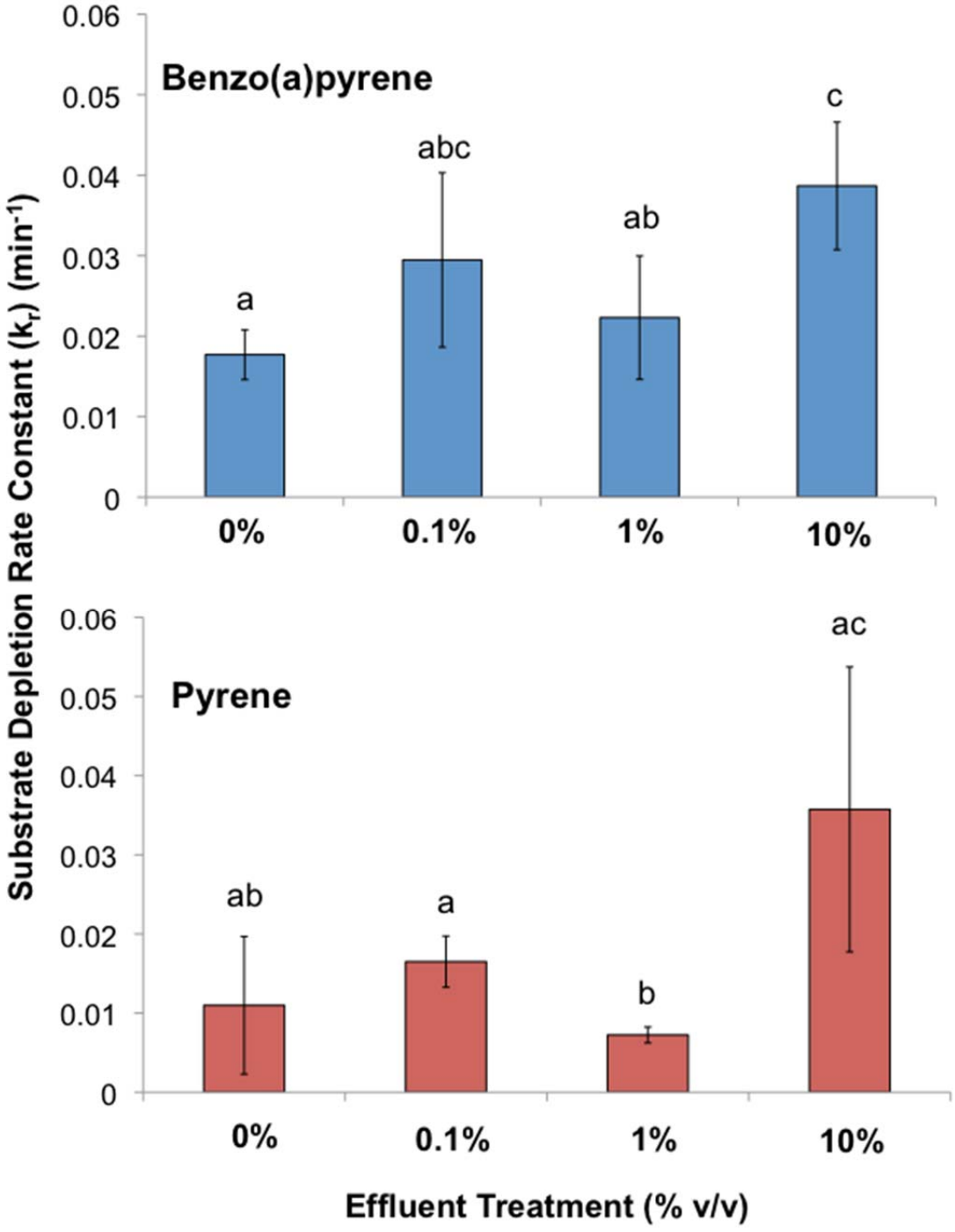

Figure 3-5. Mean depletion rate constants (k_r) of B(a)P and pyrene from fish exposed to 0, 0.1, 1, and 10% dilutions of secondary treated wastewater effluent. Error bars are the 95% confidence intervals of the mean. Different letters denote significant differences between effluent treatments (ANOVA and Tukey's HSD, $p<0.05$).

3.4.2. Effect of Pre-exposure on in vitro Substrate Depletion Rates (2012 Experiment)

Figures 3-6 through 3-9 show the concentration of test chemicals pyrene, B(a)P, 9-MA, and chrysene over time in each replicate (n=3) for all treatments (0 and 20%). The substrate depletion rate constants (k_r) were determined from the slope of the substrate concentration depletion curves and are presented in Table 3-6 and Figure 3-10. The results of a student's t-test showed no significant differences ($p > 0.05$) between mean k_r values from the 0 and 20% effluent treatments for the for any test chemical (i.e. pyrene, B(a)P, chrysene, and 9-MA).

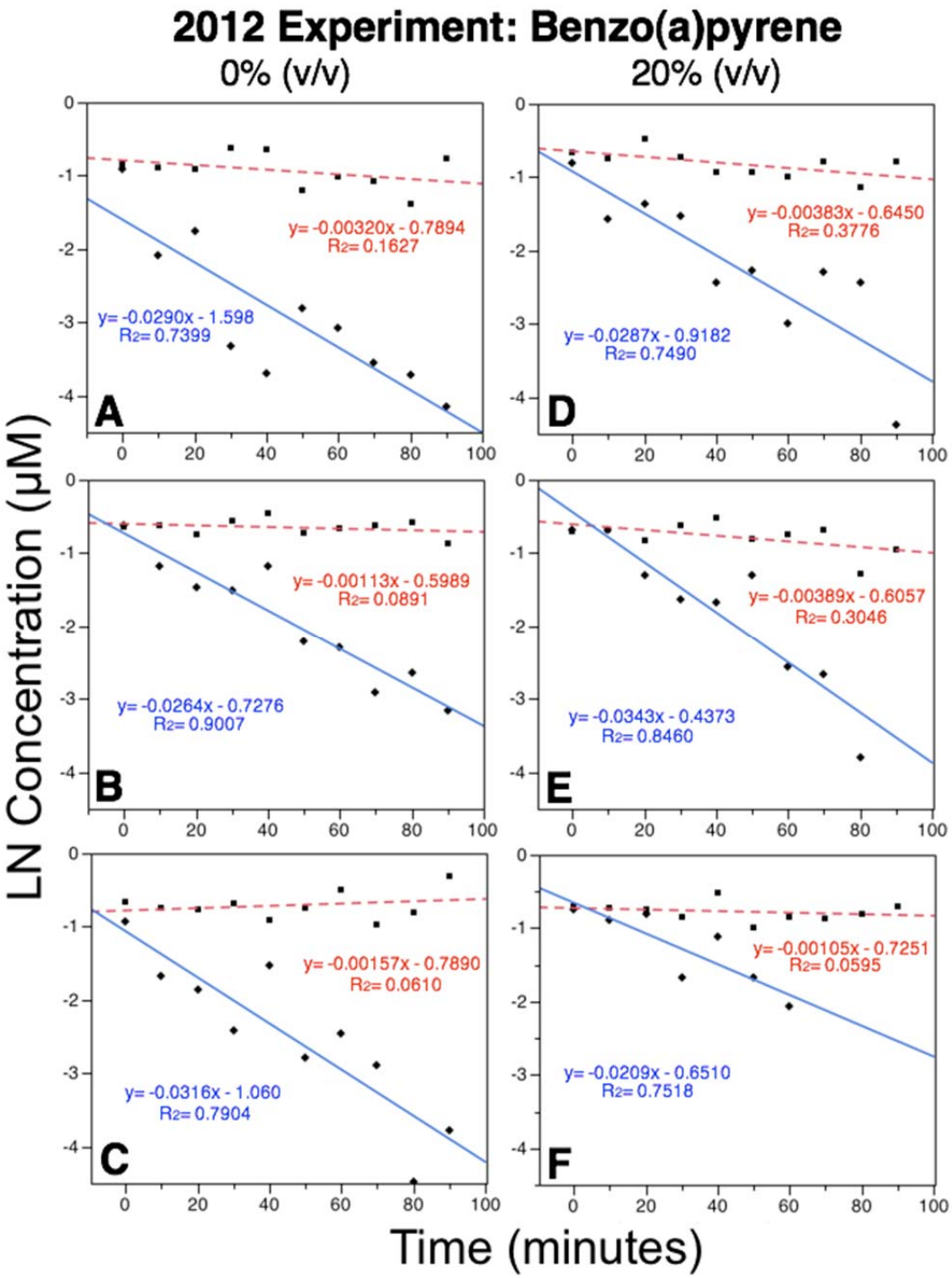

Figure 3-6. Natural logarithm of B(a)P concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 20% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 20% treatment (D, E and F).

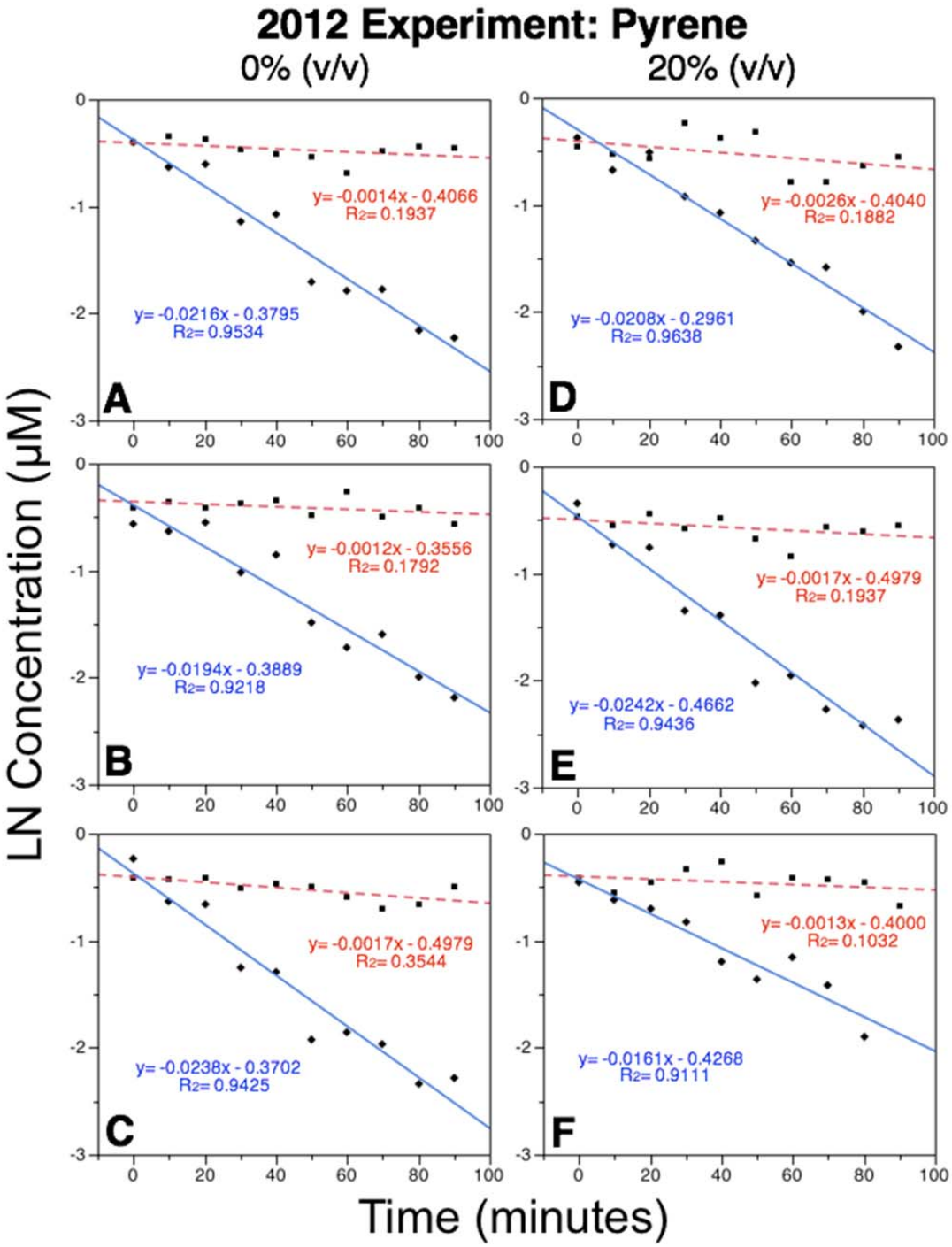

Figure 3-7. Natural logarithm of pyrene concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 20% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 20% treatment (D, E and F).

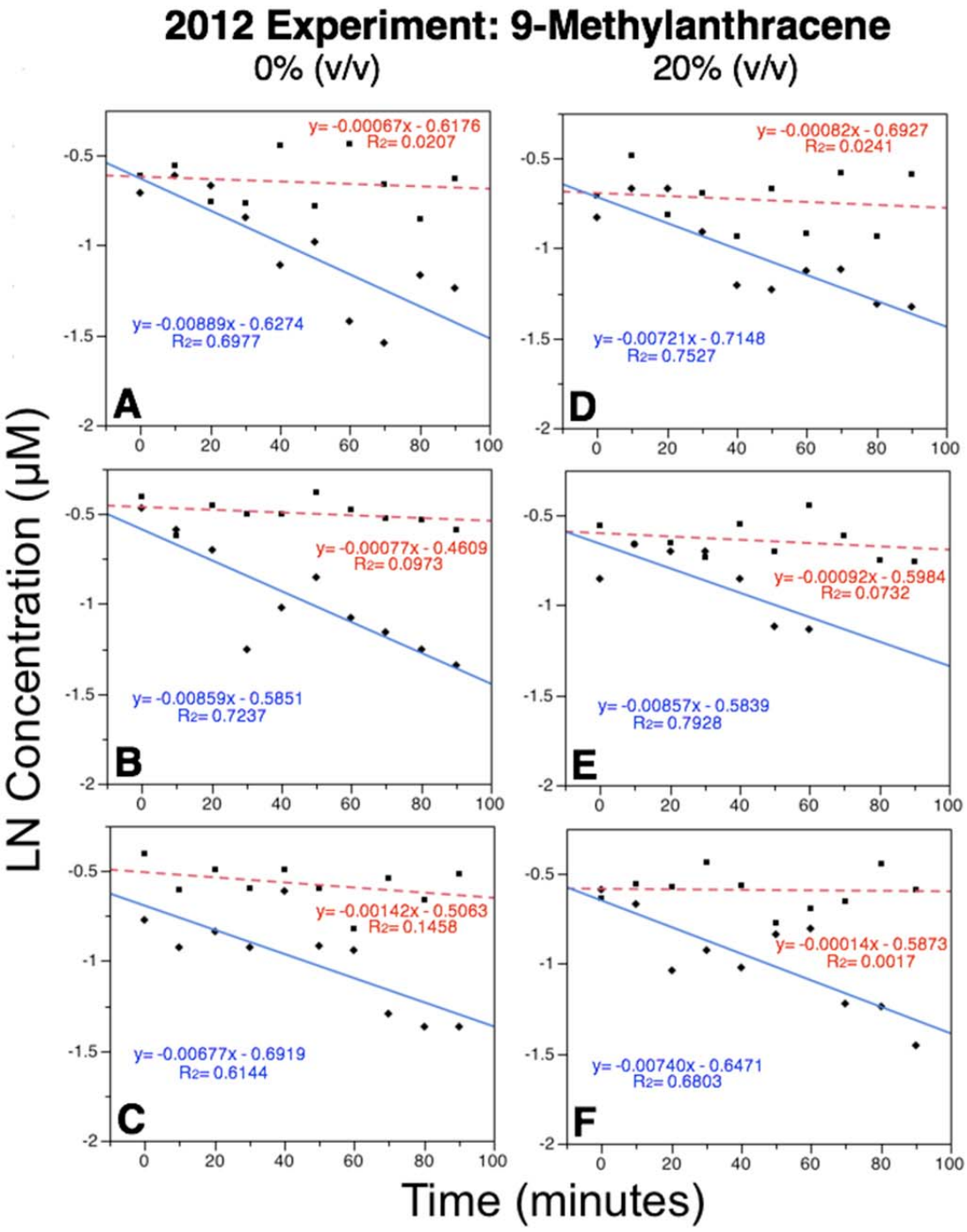

Figure 3-8. Natural logarithm of 9-methylanthracene concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 20% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 20% treatment (D, E and F).

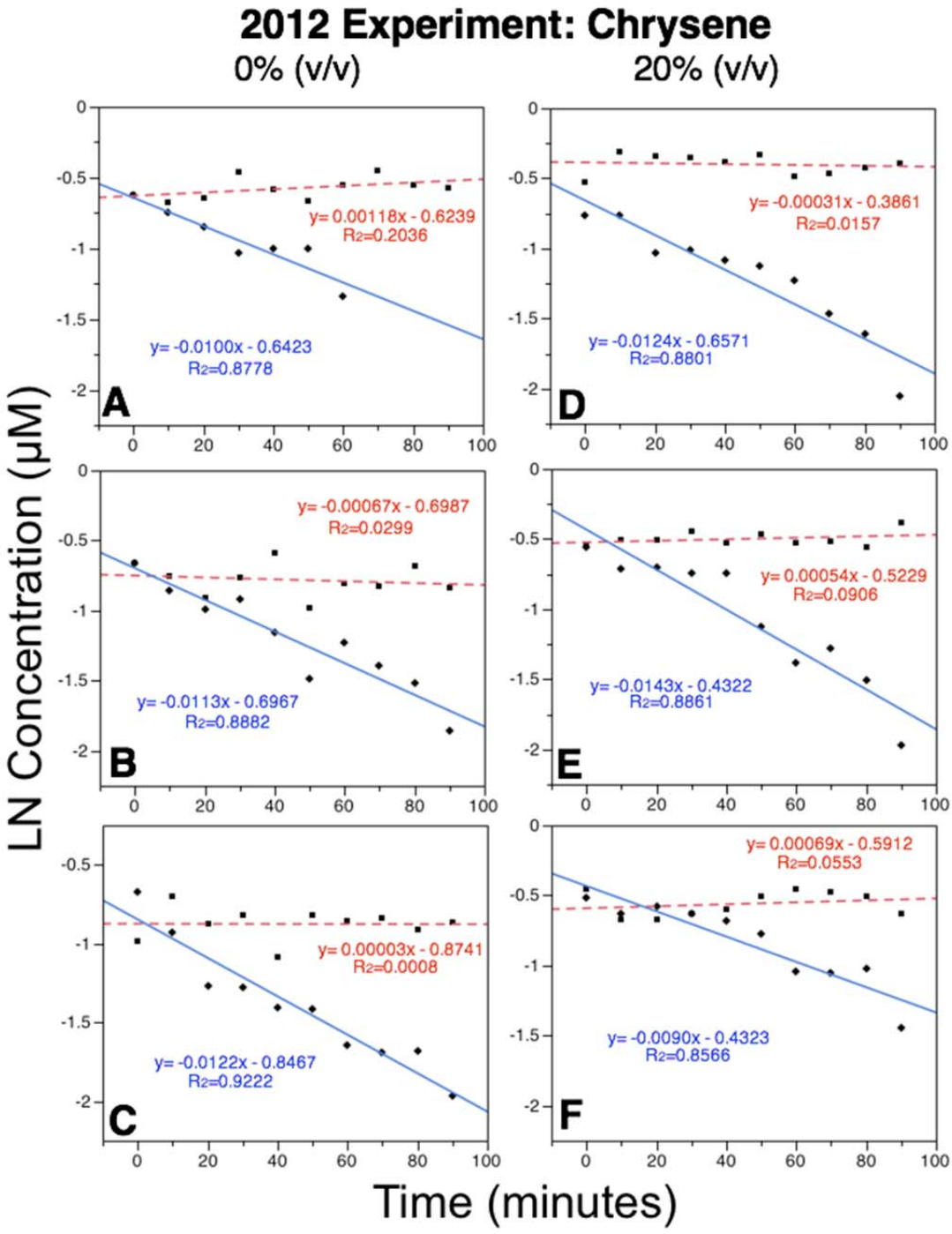

Figure 3-9. Natural logarithm of chrysene concentration in the incubation as a function of the incubation time for effluent treatments of 0 and 20% (v/v). Data for enzyme inactive control S9 (■, dashed red line) and active S9 (♦, solid blue line) are displayed. Plots shown are the results of the three replicate incubations for 0% treatment (A, B and C) and of the three replicate incubations for the 20% treatment (D, E and F).

Table 3-6. *In vitro* substrate depletion rate constants (k_r; 10^{-3} min^{-1}) for pyrene, benzo(a)pyrene, 9-methylanthracene, and chrysene in triplicate experiments for the two effluent treatments (0% and 20%). The 95% confidence intervals of the mean depletion rate constants are displayed in brackets

Substrate	0% Exposure	20% Exposure
Benzo(a)pyrene		
Replicate 1	29.04	28.64
Replicate 2	26.41	34.3
Replicate 3	31.6	20.9
Mean	29 (24.22 – 33.78)	27.97 (15.60 – 40.33)
Pyrene		
Replicate 1	21.62	20.8
Replicate 2	19.4	24.26
Replicate 3	23.8	16.1
Mean	21.6 (17.56 – 25.64)	20.37 (12.89 – 27.84)
9-Methylanthracene		
Replicate 1	8.89	7.21
Replicate 2	8.59	8.57
Replicate 3	6.77	7.4
Mean	8.08 (5.97 – 10.19)	7.72 (6.37 - 9.08)
Chrysene		
Replicate 1	10	11.23
Replicate 2	11.31	12.4
Replicate 3	12.19	9.03
Mean	11.17 (7.68 – 14.79)	11.23 (9.13 – 13.19)

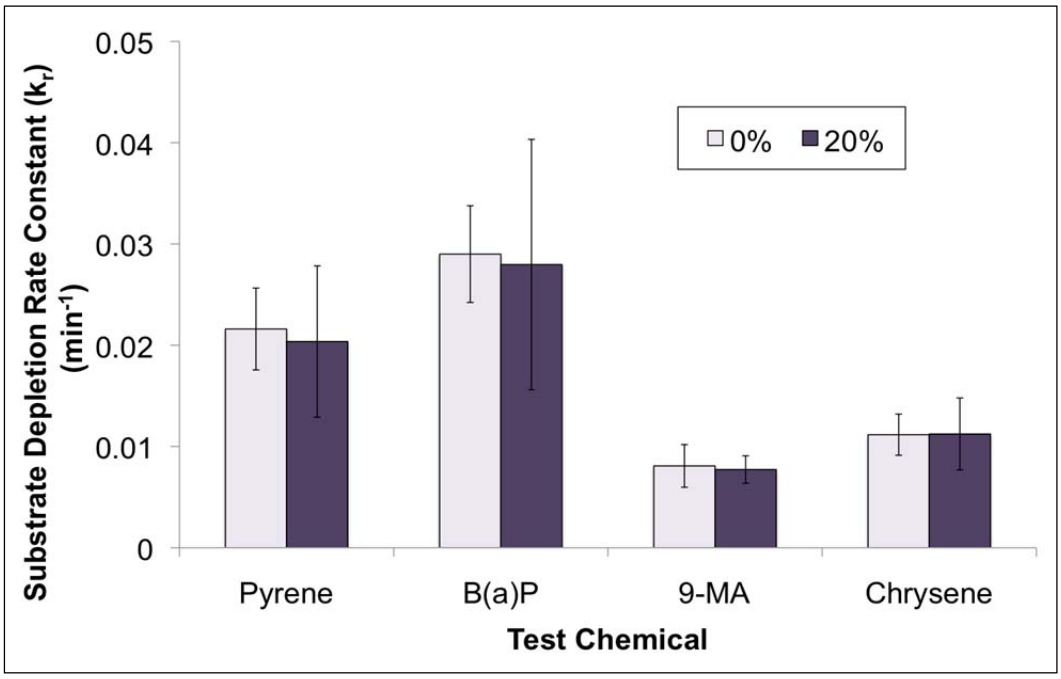

Figure 3-10. Mean depletion rate constants (k_r) of B(a)P, 9-MA chrysene, and pyrene following exposure to 0 and 20% wastewater effluent. Error bars represent the 95% confidence intervals of the mean. There was no significant difference ($p > 0.05$) between the depletion rate constants for any test chemicals between S9 prepared from control (0%) and exposed (20%) fish.

3.5. Modeled Bioconcentration Factors

Using an *in vitro-in vivo* extrapolation (IVIVE) model (Nichols et al., 2006) and a fish bioaccumulation model (Arnot & Gobas, 2003) *in vitro* substrate depletion rate constants were extrapolated to estimate whole organism k_{MET} and BCF values. The k_r values at the upper and lower extremes of the 95% confidence intervals were considered in the model calculations to generate corresponding ranges of BCF values for each test chemical however uncertainties in the models were not included in the calculations. Differences in calculated intrinsic clearance (CL_{INT}), hepatic clearance (CL_H) and the whole-body biotransformation rate constant (k_{MET}) values corresponded to differences in *in vitro* depletion rate constants with regards to differences with exposure treatments. Values of k_{MET} and BCF values for all chemicals calculated from *in vitro* biotransformation data from the 2011 and 2012 experiments are displayed in the following sections.

3.5.1. Effect of Pre-exposure on Modeled BCFs (2011 Experiment)

Similar to reports by other authors (Cowan and Ellsberry et al., 2008; Han et al., 2007, 2009; Trowell, 2010) the incorporation of modeled k_{MET} data into BCF calculations resulted in modeled BCF values that were significantly lower than modeled BCFs which did not consider biotransformation (k_{MET}=0) (Tables 3-7 and 3-8). Mean B(a)P and pyrene BCF values followed the same trends as the *in vitro* depletion rate constants following effluent exposure: increased mean k_r values resulted in increased extrapolated k_{MET} and decreased modeled BCFs.

For pyrene, the mean BCF calculated from the mean k_r value obtained in S9 collected from fish exposed to 10% diluted effluent was 2.16-fold lower relative to the mean k_r measured in S9 fish in the control group. For B(a)P, the mean BCF calculated from mean k_r value obtained in S9 collected in fish exposed to 10% diluted effluent was 1.96-fold lower than the mean k_r measured in S9 fish from the control group.

Table 3-7. Biotransformation rate constant (k_{MET}) in rainbow trout, modeled BCF values, and the percent reduction in modeled BCF values (compared to BCF k_{MET}=0) for pyrene generated using in vitro depletion rate constants observed after each exposure scenarios. The 95% confidence intervals are displayed in brackets.

Pyrene				
BCF k_{MET}=0	4,741			
	0%	0.1%	1%	10%
k_{MET} ($x10^{-2}$ day^{-1})	6.99 (1.49 – 12.21)	10.30 (8.38 – 12.91)	4.67 (4.03 – 5.30)	19.90 (11.03 – 29..84)
BCF	1,766 (1202 – 3485)	1,361 (1204 – 1570)	2,230 (2082 – 2405)	818 (579 – 1296)
% BCF reduction	63	71	53	83

Table 3-8. Biotransformation rate constant (k_{MET}) in fish, modeled bioconcentration factors, and the percent reduction in modeled BCF values (compared to BCF k_{MET}=0) for B(a)P generated using in vitro depletion rate constants for each exposure scenarios. The 95% confidence intervals are displayed in brackets.

Benzo(a)pyrene				
BCF k_{MET}=0	31,839			
	0%	0.10%	1%	10%
k_{MET} ($x10^{-2}$ day^{-1})	3.42 (2.83 – 4.01)	5.63 (3.60 – 7.61)	4.29 (2.84 – 5.72)	7.32 (5.86 – 8.74)
BCF	4,874 (4253 – 5705)	3,152 (2393 – 4671)	4,012 (3106 – 5699)	2,482 (2103 – 3038)
% BCF reduction	85	90	87	92

3.5.2. Effect of Pre-exposure on Modeled BCFs (2012 Experiment)

Table 3-9 gives both extrapolated k_{MET} values and corresponding BCF values for pyrene, B(a)P, 9-MA, and chrysene for fish collected from the 0% and 20% treatment groups. All modeled BCFs derived from both groups were significantly reduced relative to the modeled BCF where biotransformation was not considered (k_{MET}=0). The extrapolated k_{MET} data and modeled BCF values are similar to the results obtained from the *in vitro* assays: there were no differences in modeled BCF values between the 0% and 20% treatment groups.

Table 3-9. Biotransformation rate constant (k_{MET}) in fish, modeled bioconcentration factors, and the percent reduction in modeled BCF values (compared to BCF k_{MET}=0) for pyrene, B(a)P, 9-MA, and chrysene generated using in vitro depletion rate constants for all exposure scenarios. The 95% confidence intervals are displayed in brackets.

Pyrene			9-Methylanthracene		
BCF k_{MET}=0	4,741		BCF k_{MET}=0	7,007	
	0%	20%		0%	20%
k_{MET} ($x10^{-2}$ day^{-1})	13.27 (10.93 – 15.54)	12.56 (8.15 – 15.54)	k_{MET} ($x10^{-2}$ day^{-1})	4.26 (4.06 – 5.34)	4.08 (3.38 – 4.78)
BCF	1,129 (999 – 1305)	1,177 (999 – 1599)	BCF	2,783 (1219 – 2866)	2,858 (2594 – 3181)
% BCF reduction	76	75	% BCF reduction	60	59
Benzo(a)pyrene			Chrysene		
BCF k_{MET}=0	31,839		BCF k_{MET}=0	24,470	
	0%	20%		0%	20%
k_{MET} ($x10^{-2}$ day^{-1})	5.54 (4.65 – 6.42)	5.35 (3.02 – 7.62)	k_{MET} ($x10^{-2}$ day^{-1})	2.75 (1.90 – 3.62)	2.76 (2.25 – 3.23)
BCF	3,197 (2797 – 3737)	3,299 (1219 – 5409)	BCF	5,546 (4452 – 7290)	5,523 (4876 – 6445)
% BCF reduction	90	91	% BCF reduction	77	77

4. Discussion

4.1. *in vitro* Substrate Depletion Rates

In the 2011 experiment, the mean k_r value for pyrene observed in liver S9 from fish exposed to 10% diluted effluent was 3.1-fold higher than control, although the difference was not statistically significant. When B(a)P was used as the test substrate in the same liver S9 preparation, a 2.2-fold increase in the mean k_r value was observed over the control in the 10% exposure group, and this increase was statistically significant ($p<0.05$). Mean k_r values for B(a)P and pyrene in liver S9 from fish exposed to lower effluent concentrations of 0.1 and 1% were not significantly different from the control group. No concentration-response relationship between effluent concentration and k_r values was observed for either test chemical. The more rapid depletion of test substrates in S9 collected from fish exposed to 10% effluent may be caused by CYP enzyme inducing chemicals contained in the effluent. By contrast, in the 2012 experiment, no significant differences were observed between control and treatment groups despite the higher effluent exposure concentration (20% v/v) compared to the experiment done the previous year.

Different effects of pre-exposure to wastewater effluents on mean k_r values between the 2011 and 2012 experiments may reflect that real world exposure scenarios are highly complex. Chemicals in the environment may affect biotransformation via inhibitory, potentiating, or competitive processes. Aquatic organisms may live in a range of habitats from pristine areas to areas with frequent contaminant releases. The differences in mean k_r values between the 2011 and 2012 experiments may be due to differences in effluent composition between the two experiments. Effluents are complex mixtures with multiple combinations of chemicals. It may be that the chemical mixture varied between the two exposure studies, with perhaps a higher concentration of CYP1A-inducing chemicals in the 2011 experiment which induced enzymes, leading to the observation of more rapid *in vitro* B(a)P and pyrene biotransformation.

Temporal differences between the effluents could have influenced the mean k_r values between the 2011 and 2012 experiments. In year 2011, the effluent was collected in the summer in late August but the following year the effluent was collected in late June following a period of heavy rainfall. The heavy rainfall may have diluted the influent entering the sewage treatment plan, resulting in lower concentrations of chemicals in the effluents leaving the treatment plant (personal communication with plant operator, June 30th 2012). In addition to seasonal variation, other authors have stated that wastewaters are heterogeneous and that there may be significant short term variations in chemical concentrations in effluents (Ort et al., 2010; ter Laak et al., 2010). Ort et al. (2010) reported that sampling intervals as short as 5 minutes may be necessary to assess true temporal variation in chemical concentrations in effluents. These variations should be considered when assessing the effect of pre-exposure to effluents on *in vitro* biotransformation rates.

Another obstacle in interpreting the effect of effluents on biotransformation rates lies in the fact that these effluents are complex mixtures of chemicals, some of which may cause induction of CYP enzymes while others may cause inhibition. In such situations, it may not be possible to relate enzymatic activity (Gallagher & Di Giulio, 1989) or the biotransformation of substances, to contaminants present in a polluted environment. Thus the biological and environmental consequences of exposure to low level mixtures of anthropogenic chemicals can be very challenging to detect and assess. Additionally direct cause and effect relations that can be attributed to individual compounds common in municipal wastewater effluents, such as personal care products and some pharmaceuticals, are still lacking in the literature (Sen & Semiz, 2007).

Reported concentrations of pharmaceuticals in the environment may be 1000 times lower when compared to concentrations administered in *in vitro* toxicity tests (Laville et al., 2004). Low environmental concentrations may indicate that pharmaceutical interference with fish metabolic pathways may be unlikely, although it has been recommended that the potential adverse effects of pharmaceuticals to fish *in vivo* under chronic exposure be assessed (Laville et al., 2004). Other studies in the literature have reported increased CYP1A gene expression following exposure to municipal wastewater effluents (Ings et al., 2011; Cuklev et al., 2012; Gagne et al.,

2012). While these studies show induction of CYP1A mRNA it may not necessarily mean that this expression translates to increased CYP1A protein or enzymatic activity.

4.2. Modeled Bioconcentration Factors

Calculated whole-body biotransformation rate constant (k_{MET}) values corresponded to differences in *in vitro* depletion rate constants with regards to differences with exposure treatments. In the 2011 experiment, extrapolated mean k_{MET} values from the 10% effluent treatment increased in a similar proportion in comparison to the *in vitro* k_r values (2.14 and 2.85-fold relative to the control treatments for B(a)P and pyrene, respectively). The mean modeled B(a)P BCF from the 10% treatment (BCF = 2,482) was 49% lower than the BCF from the control treatment (BCF = 4,874). Similarly the mean BCF value (BCF = 818) for pyrene calculated from fish exposed to 10% diluted effluent was 54% lower than the BCF from the control treatment (BCF= 1,766). BCF reductions at a magnitude of 50% may have consequences when chemicals are being screened for their bioaccumulation potential. For example, if these pre-exposure conditions were considered for a bioaccumulative substance with a BCF value of 5,000, the value would fall to 2,500 and would no longer be categorized as bioaccumulative under CEPA. For all test chemicals from the 2012 experiment modeled BCF values did not differ between exposure treatments to 0 and 20% diluted effluent.

Although the effect of pre-exposure differed between experiments, the results in the 2011 experiment did show some evidence of a reduction in BCF. However only statistically significant reductions were only detected for B(a)P ($p<0.05$). The 2 to 3 fold increases in k_r values for observed in the 2011 experiment resulted in a 50% reduction in BCF for both pyrene and B(a)P. To explore these observation further, a literature review was conducted to determine what the magnitude of enzyme induction in fish may be and how it may influence the biotransformation of the test chemicals used in the present study. The extent of liver enzyme induction in fish is discussed in Section 4.3.

In a regulatory context, when chemicals are undergoing a screening assessment for their bioaccumulative potential, *in vitro* biotransformation assays may provide reasonable measurements in chemical screening assessments. Emphasis is placed on the use of these tests for screening purposes as the data are extrapolated using models

for predictive purposes and do not provide a comprehensive risk assessment. For chemicals with modeled BCFs that have predicted values around bioaccumulation criteria (e.g. BCF of ~5,000) evaluators may consider *in situ* scenarios where organisms may have induced or inhibited biotransformation enzymes which may alter bioaccumulation predictions. This would also warrant the use of additional tools such as *in vivo* testing or comprehensive risk assessment to further evaluate and confirm bioaccumulation potential (Nichols et al., 2006).

In the case of situations where there may be enzyme induction, chemical regulators may wish to adopt the precautionary principle by ignoring these scenarios, as they would result in reduced BCF values. This approach may be warranted to avoid underestimation of the BCF until other pre-exposure scenarios and their effects on biotransformation capacity are explored. The present study showed no evidence of significantly reduced mean k_r values due to potential enzyme inhibition caused by wastewater effluents. This is important because regulatory agencies want to be conservative and avoid underestimation of the BCF. Reduced k_r values would result in higher modeled BCFs, giving a substance a higher bioaccumulation potential.

The modeled BCF values generated in the present study were within range of reported empirical BCF values in the literature (Appendix F). It was challenging to attain a range BCF values for the four test chemicals in this study exclusively from fish and therefore other aquatic organisms were considered. Empirical BCF values for pyrene ranged from as low as 457 in goldfish (*Carassius auratus auratus*) (Ogata et al., 1984) to as high as 3,500 in *Daphnia magna* (Akkanen et al., 2000). Reported B(a)P BCF values were between 220 and 3,208 in bluegill sunfish (McCarthy & Jimenez, 1985) Only one BCF value of 4,538 in *Daphnia pulex* (Southworth et al., 1978) was found for 9-MA. The BCF range for chrysene was from 1,560 in amphipods (*Rhepoxynius abronius*) (Boese et al., 1999) to 6,088 in *Daphnia magna* (Newsted & Giesy, 1987). Biotransformation may vary significantly between fish species so caution should be taken when comparing modeled BCFs to empirical BCFs derived from aquatic organisms other than rainbow trout (Han et al., 2007).

Calculated BCFs from the present study were compared to extrapolated BCF values in the literature (Figure 4-1). Han et al. (2009) reported a predicted BCF value

from rainbow trout liver S9 for B(a)P to be 3,276. This BCF is almost identical to the BCF values calculated based on the 2012 experiment results for B(a)P (BCF values= 3,197 and 3,299 for 0 and 20% effluent treatments, respectively) and is within the 95% confidence interval range of the modelled BCFs for all effluent treatment groups except

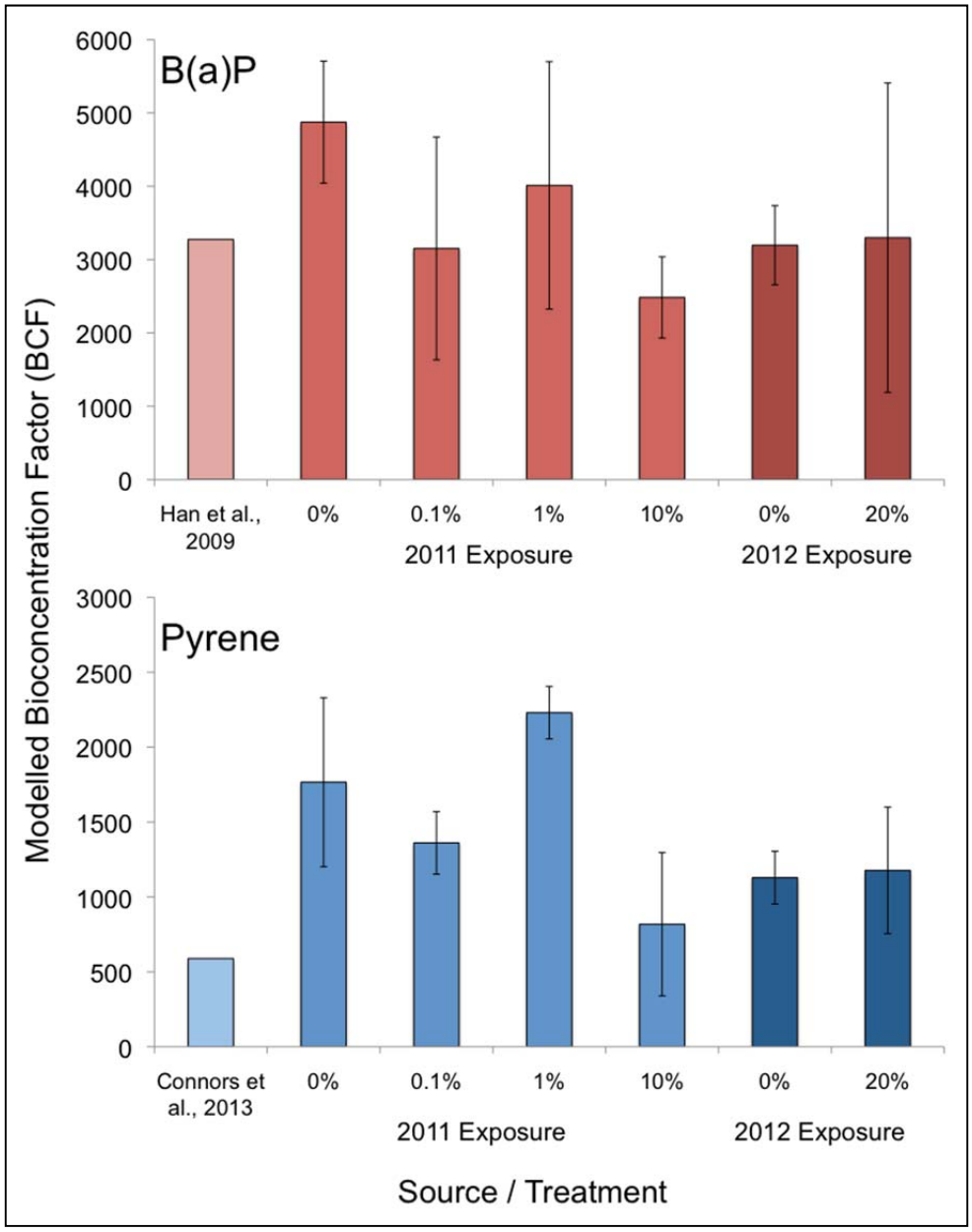

Figure 4-1. Comparison of modeled BCF values of B(a)P and pyrene calculated in the present study to extrapolated BCF values from the literature (Han et al., 2009; Connors et al., 2013). Error bars represent the 95% confidence intervals extrapolated from the mean k_r values.

the control group in the 2011 experiment (BCF=4,874). No statistical error margins were given on the BCFs modeled in Han et al. (2009) making detailed comparisons difficult. Connors et al. (2013) reported an intrinsic clearance value for pyrene of 472 mL/h/g liver in rainbow trout S9. This value was extrapolated to generate k_{MET} and input into the fish bioaccumulation model to provide a modelled BCF of 589. The modelled BCF extrapolated from Connors et al. (2013) was lower than the 95% confidence intervals generated for the modelled mean BCFs for pyrene except for the BCF obtained from S9 collected from fish exposed to 10% diluted effluent in the 2011 experiment (Figure 4-1).

It is noted that although the 95% confidence intervals of the mean k_r values were extrapolated to obtain a range of modeled BCFs for each effluent treatment groups, caution should be taken in the discussion of statistical significance as there are additional error associated with the extrapolations and calculations. The confidence intervals of the BCF values do not take into account the model error associated with extrapolation of the k_r values to k_{MET} and modeled BCF values. Uncertainty associated with the assumptions and parameters in these models are described below

In the present study, model assumptions were based on parameters already set in the IVIVE (Nichols et al., 1990; Nichols et al., 2006) and fish bioaccumulation (Arnot & Gobas, 2003) models unless they could be otherwise measured in the lab. The models are flexible and contain algorithms that adjust for user-specific parameters such as temperature, lipid content, fish mass, S9 protein content. In the IVIVE model, the assumed extrapolation factors and physiological and physical inputs may be quite variable as these parameters are derived via experimentation (Han et al., 2009). Additional work to validate and understand various model assumptions is on-going (Nichols et al., 2013) to determine which model inputs contribute the greatest amount of variability and uncertainty in predicted levels of accumulation (Johanning et al., 2012b).

Model parameters may be based on relatively variable data because they are experimentally derived (Han et al., 2009). Values of cardiac output in rainbow trout from the primary literature range between 1.85 L/h/kg fish (Brodeur et al., 2001) to as high as 4.31 L/h/kg fish (Neumann et al., 1983). Nichols et al. (2006) assumes cardiac output to be 2.07 L/h/kg fish and hepatic blood flow (arterial and portal) to be 25.9% of cardiac output. Although there is variation in reported values for cardiac output in fish, sensitivity

of the IVIVE model to changes in this parameter has been assessed and was reported to be minimal for chemicals with high K_{OW} (Nichols et al., 2013).

Other artefacts of *in vitro* testing include non-specific binding of hydrophobic chemicals to S9 or vials. This would result in decreased bioavailability of the chemical to the enzymes present in the *in vitro* system and would underestimate the rate of intrinsic clearance and ultimately k_{MET}. In the present study, the fraction unbound in the incubation medium was calculated based on the algorithm presented by Austin et al. (2002) however methods to measure the fraction unbound and *in vitro* biotransformation rates concurrently have been developed for rats (Lee et al., 2012) and are being developed for fish (Lee, et al., *in preparation*).

Nichols et al. (2006) stated that there may be incomplete recovery of metabolizing enzymes within S9 fractions when compared to the amount that may be present in intact tissue. Schulz and Hayton (1999) reported the recovery of *N*-depropylase activity using glucose-6-phosphate as a standard substrate to vary between 35% to 60% in liver S10 fractions in fish. Recovery may vary among S9 pools due to variation in preparation or handling (Nichols et a., 2006). Although enzyme recovery was not measured in the present study, it has been recommended that enzyme recovery be characterized for standardization of *in vitro* methods (Johanning et al., 2012a). Incorrectly characterizing enzyme recovery would either over or underestimate the *in vitro* biotransformation rate constant. Additionally contribution from other organs was not considered and the liver was assumed to be the dominant organ for biotransformation in fish. Other organs such as the intestine and the kidneys may also contribute to whole-organism biotransformation (Klaassen & Watkins, 2003). The IVIVE model provides a conservative estimate by only considering biotransformation in the liver.

Fish physiological and *in vitro* assay parameters were not the only variable experimental data used in the models in the present study. Reported log K_{OW} values for the four test chemicals vary as well (Table 4-2), providing additional uncertainty to the model predictions of k_{MET} and BCF. Nichols et al. (2013) reported that overall IVIVE model behaviour is shown to be strongly dependent on the relative hydrophobicity of the test chemical, so errors in K_{OW} can greatly influence modeled k_{MET} values.

Table 4-1. Reported logK_{OW} values from the literature (compiled by Mackay et al., 2006).

Test chemical	logK_{OW} values
Benzo(a)pyrene	5.78 to 7.99
Pyrene	4.77 to 5.52
Chrysene	5.61 to 5.91
9-Methylanthracene	5.07 to 5.61

K_{OW} error may also influence the fish bioaccumulation model's prediction of BCF. The BCF is more sensitive to biotransformation at high K_{OW} because other rates of elimination are slower (i.e. respiratory exchange) for high K_{OW} compounds than for low K_{OW} compounds (Arnot & Gobas, 2006). The calculation of the BCF (equation 4.1) shows that k_{MET} competes with other elimination constants (i.e. fecal egestion (k_E), growth dilution (k_G) chemical, elimination across gills (k_2)).

$$BCF = k_1 \Phi / (k_2 + k_E + k_G + k_{MET}) \quad (4.1)$$

Therefore selection of larger K_{OW} values in the range provided by Mackay et al. (2006) may result in BCF predictions that are more greatly influenced by biotransformation (k_{MET}) in comparison other elimination rate constants.

4.3. Extent of Liver Enzyme Induction in Fish

Pre-exposure to chemicals may affect the biotransformation and bioaccumulation of xenobiotics. Induction of CYP enzymes resulting from pre-exposure to chemical mixtures or known CYP inducers has been extensively studied. In particular, the extent of CYP1A enzyme induction has been assessed using assays of enzyme activity (e.g. aryl hydrocarbon hydroxylase (AHH), EROD) in subcellular liver preparations. The oxidative reactions of B(a)P (used in the AHH assay) and the O-demethylation of ethoxyresorufin (used in the EROD assay) are mediated largely by CYP1A and so are sensitive markers of this enzyme's activity. Bucheli and Fent (1995) reported that EROD activities in fish are usually increased up to 30-fold in contaminated areas compared to reference sites. Van der Oost et al. (2003) reported that 93% of laboratory studies and 79% of field studies showed significant increases in EROD relative to control or

reference sites. In the present study, there was evidence of potential enzyme induction with the observation of increased k_r values in the 2011 experiment. Therefore for a greater understanding of the magnitude of the effect of exposure of fish to CYP1A-inducing chemicals, a review of the literature was conducted and the results are given in Table 4-2. The literature review was restricted to the chemicals examined in the current work (B(a)P, pyrene, chrysene, and 9-MA).

For B(a)P there was a wide range of data to compare rates of biotransformation in induced and un-induced fish. Evaluation of rates of chrysene biotransformation following exposure to enzyme inducers was limited to only one study (Pangkregar et al., 2003). Studies comparing biotransformation of pyrene and 9-MA to induced and un-induced fish were not found.

Table 4-2. Literature values of the extent of induction of B(a)P and chrysene biotransformation measured in liver preparations of fish exposed to known CYP inducers. Reported error measurements are described in the footnotes following the table.

Fish species	Inducer: dose and duration	Enzymatic activity	Fold increase relative to control	Reference
Rainbow Trout *(Oncorhynchus mykiss)* liver microsomes	Control	B(a)P hydroxylation 0.31 ± 0.10[a]	1.6	van Veld et al., 1987
	B(a)P: 16mg/kg diet for 4 days	B(a)P hydroxylation 0.49 ± 0.24[a]		
Brown Bullhead (*Ictalurus nebulasus)* liver microsomes	Control	B(a)P oxidation: 38.2[b]	16.5	Pangkregar et al., 1995
	3MC: 20mg/kg bw i.p., single dose; enzyme activity tested 5 days later	B(a)P oxidation: 361.4[b]		
Tilapia (*Oreochromis niloticus x Oreochromis aureus*) liver microsomes	Control	B(a)P 3-hydroxylation 56 ± 14[c]	8.7	Ueng & Ueng, 1995
	3-MC: 20mg/kg bw/day i.p. for 4 days	B(a)P 3-hydroxylation 489 ± 24[c]		

Fish species	Inducer: dose and duration	Enzymatic activity	Fold increase relative to control	Reference
Rainbow Trout *(Oncorhynchus mykiss)* liver microsomes	Control	B(a)P hydroxylation 7.9 ± 5.3 [c]	15.6	Huuskonen et al., 1996
	BNF: 50 mg/kg bw i.p.; single dose; enzyme activity tested 6 days later	B(a)P hydroxylation 123 ± 10 [c]		
Safi fish *(Siganus canaliculatus)* liver microsomes	Control	B(a)P hydroxylation 6.15 ± 1.30[f]	2.2	Raza et al., 1995
	BNF: 40 mg/kg bw/day i.p.for 2 days	B(a)P hydroxylation 13.25 ± 1.25[f]		
Rainbow Trout *(Oncorhynchus mykiss)* liver microsome	Control	B(a)P hydroxylation 11.3 ± 0.08[a]	27.2	Vigano et al., 1993
	BNF: 50 mg/kg bw i.p.; single dose; enzyme activity tested one week later	B(a)P hydroxylation 307.8 ± 33.5[a]		
Brook Trout (*Salvelinus fontinalis*) liver microsomes	Control	B(a)P hydroxylation 0.59 ± 0.07[g]	2.0	Law & Addison, 1981
	Fed Aroclor 5460 every 2 days for 18 days. Concentrations in feed chosen to produce whole-body concentrations of 200 µg/g	B(a)P hydroxylation 1.18 ± 0.14[g]		
Trout (*Salvelinus fontinalis*) liver microsomes	Control	B(a)P hydroxylation 0.48 ± 0.05[g]	2.9	Law & Addison, 1981
	Fed FireMaster BP-6 every 2 days for 18 days. Concentrations in feed chosen to produce whole-body concentrations of 200 µg/g	B(a)P hydroxylation 1.39 ± 0.26[g]		
Rainbow Trout *(Salmo gairdneri)* liver microsomes	Control	B(a)P hydroxylation 0.03[e]	10.0	Elcombe & Lech, 1978
	Arochlor 1254	B(a)P hydroxylation 0.30[e]		

Fish species	Inducer: dose and duration	Enzymatic activity	Fold increase relative to control	Reference
European Sea Bass (*Dicentrarchus labrax*) liver microsomes	Control	B(a)P oxidation 7.64 ± 0.52 [d]	2.3	Lemaire et al., 1992
	B(a)P: 20mg/kg bw i.p., single dose; enzyme activity tested 14 hours later	B(a)P oxidation 17.42 ± 1.59 [d]		
Carp (*Cyprinus carpio*) Liver S9	Control	B(a)P hydroxylation 3.3 ± 1.2 [c]	11.8	Britvic et al., 1993
	3-MC: single injection of 50 mg/kg bw i.p;	B(a)P hydroxylation 38.8 ± 2.6[c]		
Brown Bullhead (*Ictalurus nebulasus)* liver microsomes	Control	Chrysene oxidation 30.1± 2.53[b]	2.7	Pangkregar et al., 2003
	3-MC: 20mg/kg bw i.p., single dose; enzyme activity tested 5 days later	Chrysene oxidation 82.2 ± 0.71[b]		

a. nmol B(a)P metabolites/min/mg; values represent mean ± SE
b. pmol metabolites formed/min/mg protein; values represent means of triplicate samples
c. pmol/min/mg protein; values represent mean ± SE
d. nmol B(a)P metabolites/min/mg microsomal protein; values represent mean ± SD
e. nmoles/min/mg protein. Measurement of error not provided (values taken from Figure 4)
f. fluorescent units/min/mg protein; values represent mean ± SD or SE (info not provided)
g. nmol 3-OH B(a)P formed/h/mg microsomal protein; values represent mean ± SE

The effect of enzyme induction by known inducers on rates of B(a)P biotransformation is variable, but is reported to generally be in the order of a 2 to 20-fold increase relative to controls (Table 4-2). For chrysene, a 2.3 fold increase in the rate of oxidation was reported in brown bullhead (*Ictalurus nebulasus*) microsomes following i.p. injection with 3-MC (Pangkregar et al., 2003). In the present study, the 2 to 3 fold increase in substrate depletion rate constants following exposure of fish to 10% diluted effluent in the 2011 experiment are consistent with the majority of studies from the literature that measure the biotransformation of PAHs using induced and un-induced fish (Table 4-2).

In the environment, animals living in contaminated areas are exposed to xenobiotics regularly and enzyme activity is potentially induced by a wide variety of compounds. Therefore in situations where chemicals may bioaccumulate in organisms,

using induced specimens in bioaccumulation tests may be more reflective of the metabolic potential of fish living in polluted areas. In the present study, the 2 to 3-fold increase in k_r values in fish exposed to 10% diluted effluents relative to controls resulted in modeled BCFs that were reduced by 50%. Fold increases in biotransformation rates in the higher end of the range reported (i.e. 5 to 20-fold increases) in Table 4-2 would likely have a greater influence on the bioaccumulation behaviour of substances. This could result in even more greatly reduced BCFs that may decrease at a similar magnitude to effect of induction observed in *in vitro* biotransformation assays.

One caveat to consider when comparing results generated by measuring metabolite formation to results using the substrate depletion method is that metabolite formation commonly measures the rate of production of only one or a few metabolites. It is possible that any effect of CYP1A enzyme induction on k_r values in the current study was attenuated because substrate depletion was monitored instead of the rate of a specific oxidation reaction catalyzed by this enzyme. It is likely that a number of enzymes contribute to the depletion of the parent compound in an *in vitro* liver incubation; thus, the ability to detect increased activity of one of these enzymes could depend on the relative contribution of that enzyme to the overall biotransformation.

Further studies should be conducted to confirm the effect of pre-exposure on *in vitro* biotransformation rate constants. This is important because the influence of pre-exposure/enzyme induction may vary depending on the inducing agent and its exposure route. It is recognized that the studies mentioned in Table 4-2 describe different routes of exposure (diet and i.p. injection) than the present study (aqueous exposure) and also that reported biotransformation rates are measured in different *in vitro* systems (primarily microsomes). Additionally biotransformation capacity may also vary between fish species.

In vitro enzyme assays are sensitive tools to measure biochemical responses in organisms to pollutants. Modeling responses based on the extent of enzyme induction reported in the literature may provide additional insight on the bioaccumulation potential of contaminants in the presence of enzyme inducers. The extent of induction reported for B(a)P and chrysene in Table 4-2 in addition to other chemicals could be incorporated IVIVE and fish bioaccumulation models to observe how they influence the resultant k_{MET}

and BCF values. A sensitivity analysis of k_{MET} and modeled BCFs to changes in the rate *in vitro* activity could be performed to evaluate the extent of enzyme induction on these parameters. A sensitivity analysis is beyond the scope of this project but may be worth exploring in future studies.

4.4. Study Limitations and Future Directions

The present study had a number of limitations that should be considered in future work. First, there was a difference in pooling methodologies between the two exposure studies. In the 2011 experiment, livers from fish in each of the four treatment groups were pooled, giving one S9 preparation per treatment group. This approach was used to reduce the biological variability that may be introduced with multiple pools of S9. This type of experimental design eliminates the confounding effect of biological variability in enzyme activity in multiple pools of S9 when assessing the biotransformation of multiple chemicals and using models to extrapolate to the *in vivo* level. However the experimental design of the 2011 experiment limits the ability to interpret the experimental variability of effluent treatment on multiple pools of S9 and must be considered in the interpretation of the results presented in this research project.

Second, the source of intra-lab variability between k_r values remains unknown. For example, the mean k_r values for B(a)P and pyrene in S9 collected from the control group in the 2011 experiment were 40-50% lower than the mean k_r value from the control group the 2012 experiment. This difference may be due to differences between the batches of fish. The fish from the 2011 experiment were ordered from the same fish supplier as the fish from the 2012 experiment (Miracle Springs, Mission BC). However the fish used for the 2011 experiment were housed at SFU for one year prior to the experiment. It may be speculated that the housing conditions at SFU (e.g. water quality parameters, diet) could possibly explain these differences between batches. Additionally, it is likely that individual fish have variable biotransformation capacity, which could have also resulted in enzyme differences between the 2011 and 2012 experiment S9 pools. Differences in enzyme recovery between S9 pools may also explain for the difference in mean k_r values between the control groups of the 2011 and 2012 experiments.

Furthermore, experimental artifacts such as investigator variability in liver handling and preparation of S9 could also contribute to differences in k_r values between the control groups between the 2011 and 2012 experiments (Johanning et al., 2012a). To examine this in more detail, *in vitro* data was extrapolated to generate intrinsic clearance values for B(a)P and pyrene (Figure 4-2). Mean $CL_{INT,\ in\ vitro}$ values for pyrene from the 2011 experiment for treatment groups 0, 0.1, and 1% were significantly lower than the $CL_{INT,\ in\ vitro}$ value reported in the 2012 experiment. For B(a)P, the mean $CL_{INT,\ in\ vitro}$ value from the control group in the 2011 experiment was significantly lower than the values generated for all $CL_{INT,\ in\ vitro}$ values calculated in the present study except the 0.1% treatment group from the 2011 experiment.

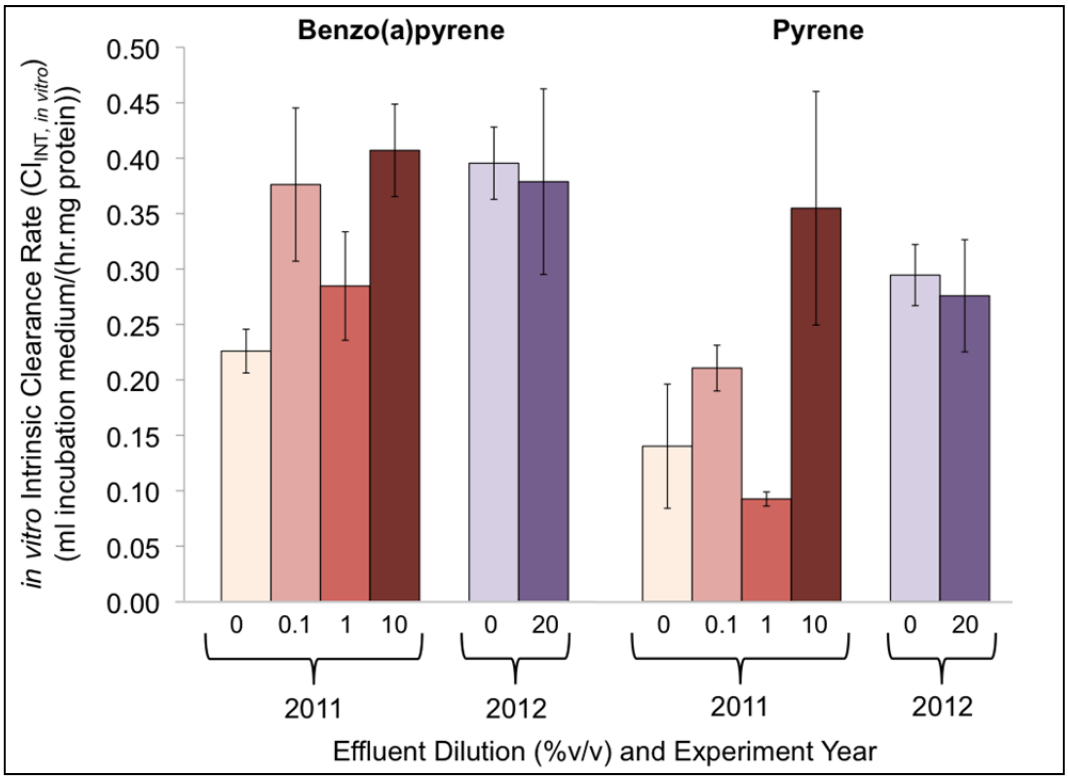

Figure 4-2. Comparison of *in vitro* rainbow trout liver S9 intrinsic clearance rates of B(a)P and pyrene between the 2011 and 2012 experiments. Error bars represent the 95% confidence intervals of the mean.

Mean $CL_{INT,\ in\ vitro}$ values (± 95% CI) measured in the present study were compared to other studies conducted in the Gobas Lab (Adekola, 2009; Lee et al., *in preparation*) These values are presented in Table 4-3.

Table 4-3. Mean $CL_{INT,\ in\ vitro}$ values (± 95% CI) obtained in the Gobas Lab

Test Chemical	Adekola, 2009	Lee et al. (*in preparation*)
B(a)P	--	0.49 (± 0.08)
Pyrene	0.40 (± 0.02)	0.29 (± 0.07)
Chrysene	--	0.20 (± 0.08)
9-MA	--	0.03 (± 0.02)

For most test chemicals, there were variations in reported $CL_{INT,\ in\ vitro}$ values obtained between the present study and studies previously conducted in the Gobas Lab. $CL_{INT,\ in\ vitro}$ values for 9-MA obtained in the 2012 experiment (0.11 mL/h/mg for both 0 and 20% treatments) were significantly higher than the $CL_{INT,\ in\ vitro}$ value in Lee et al. (*in preparation*). For pyrene, the mean $CL_{INT,\ in\ vitro}$ value obtained in Lee et al (in preparation) was not significantly different from pyrene CL_{INT} values obtained in the 2012 experiment of the present study. Adekola (2009) reported a pyrene $CL_{INT,\ in\ vitro}$ value of 0.40 mL/h/mg which is significantly higher in than the values reported in the present study (Figure 4-2) and by Lee et al. (*in preparation*). Mean $CL_{INT,\ in\ vitro}$ values for B(a)P from the 2011 experiment for treatment groups 0 and 1% were significantly lower (Figure 4-2) than the $CL_{INT,\ in\ vitro}$ value reported by Lee et al (*in preparation*). No significant differences were reported between the chrysene $CL_{INT,\ in\ vitro}$ values from the 2012 experiment (0.15 mL/h/mg for both 0 and 20% treatments) to the value reported by Lee et al. (*in preparation*). Intra-lab variation in the Gobas Lab may be attributed to differences in investigator handling, enzyme recovery in S9, and differences in fish weights (life stage).

Johanning et al. (2012b) reported a high degree of inter- and intra-laboratory variability when assessing chemicals for their *in vitro* biotransformation in S9. For example, *in vitro* substrate depletion rates of dibutyl phthalate showed high degree of intra-lab variability (mean k_r value of 2.58 h^{-1} ± 1.29 (SD)) in addition to high inter-lab variability (mean k_r values of 2.58, 5.15, and 1.00 h^{-1} measured from 3 different laboratories). No single factor was identified to contribute to this variability although it was suggested that S9 handling and preparation in addition to differences in analytical methodologies were likely contributing factors (Johanning et al., 2012b). Intra- and inter-laboratory variation increases the difficulty to compare data between studies. Further refinement of the experimental conditions is necessary and additionally important in the

evaluation of the effects of environmental factors on *in vitro* generated data, such as pre-exposure to contaminants.

Additional testing to confirm if increases in depletion rate constant for B(a)P and pyrene were attributed to increased enzymatic activity should be conducted with the use of enzyme activity assays (e.g. EROD). This step was omitted from the 2011 experiment due to too few S9 samples available for analysis. In the present study protein content was measured and compared between pools for standardization purposes and to allow for comparison with other *in vitro* data in the literature. The enzyme activity toward standard substrates has been suggested to further standardize *in vitro* biotransformation measurements so they may be used for regulatory purposes and to make comparisons with values in the literature (Johanning et al., 2012a). This would also be advantageous when studying the influence of environmental factors, such as pre-exposure, on *in vitro* biotransformation rates.

In future work, additional experiments should be performed using effluents from a more urban area. Larger cities have a higher number of industrial/manufacturing facilities and hospitals than smaller cities. We might expect that large cities release a more complex mixture of contaminants such as metals, PAHs, pharmaceuticals, and personal care products (Gange et al., 2012). In the present study, the plant treated an estimated 17,000 residential properties (~60,000 people) and influent came primarily from municipal households and no industrial or agricultural sources are in the area. Therefore the effluent used in this study may contain less enzyme inducing or inhibiting chemicals compared to urban areas such as Metro Vancouver. Other types of effluents (e.g. pulp and paper, industrial effluents, oil spills) may also contain contaminant constituents that differ from the effluents collected in the present study and this in turn may have different effects on biotransformation enzymes in aquatic organisms.

Analytical measurements of the chemical concentrations in the effluent would provide information to assist making more concrete conclusions on the effect of pre-exposure in the present study. This however is difficult as the constituents in the effluent are unknown and analytical measurements to attempt to scan for several chemical classes are costly. Additionally there may be complex interactions of many mixture components in the environment and identification of their potential biological impact via

chemical analyses alone is virtually impossible (Ciccotelli et al., 1998; Van der Oost et al., 2003). This has been the reason for the adoption of biochemical enzyme activity assays to assess the pollution. However due to the unknown effects of pharmaceuticals and personal care products on biotransformation enzymes and processes, exploration via chemical analysis may be warranted to establish a more cause-and-effect relationship and enhance current knowledge about these specific classes of pollutants.

"Homemade" effluent mixtures containing different types/classes of chemicals could also be used in a future experiment to better characterize a cause and effect relationship between pre-exposure and the potential impact on *in vitro* substrate depletion rates and modeled BCF values. Longer exposure durations with synthetic or collected effluents (from the same source or others) may also be conducted to ensure enough of an induction time course. Although significant induction of CYP1A activities has been reported to occur within 7 days (Anderson et al., 1985; Celander et al., 1990; Zhang et al., 1990) it may be possible that induction of other CYP isoenzymes may have a longer time course. Additionally the extent of inducability between CYP isoenzymes may differ. The use of positive controls for enzyme induction (e.g. BNF, 3-MC) in addition to enzyme inhibition (e.g. α-Naphthoflavone) would assist with the characterization of the extent of induction/inhibition caused by exposure to effluent mixtures.

Theoretical approaches may be taken to determine how changes to the *in vitro* substrate depletion rate constants would influence modeled BCF values. For example, a sensitivity analysis could be performed on *in vitro* rate constants for a range of log K_{OW} values to determine how decreases or increases in the *in vitro* rates would influence extrapolated k_{MET} values and ultimately modeled BCF values. As discussed in Sections 1.5 and 4.3 of this report, the primary literature contains a variety of studies that compare *in vitro* enzyme activities in fish from polluted and non-polluted areas. The magnitude of reported enzyme induction or inhibition relative to reference sites could be incorporated into IVIVE models. Although *in vitro* enzymatic responses are sensitive tools to measure biochemical responses to pollutants, modeling the effect of these alterations at the *in vivo* level would provide additional insight on if these changes influence modeled bioaccumulation endpoints in a sensitivity analysis.

5. Conclusion

Pre-exposure to 10% diluted wastewater effluent in the 2011 experiment resulted in mean k_r values that were 2 to 3-fold elevated relative to control treatments for both pyrene and B(a)P, however only statistically significant elevations were only detected for B(a)P ($p<0.05$). In the 2012 experiment, there was no influence of pre-exposure to wastewater effluent. These results provide some evidence that laboratory measured *in vitro* biotransformation rate constants may differ between those measured in pre-exposed fish. Increases of this magnitude were similar to other studies that evaluated the *in vitro* biotransformation of PAHs in induced and un-induced fish. When k_r values were extrapolated, proportional increases in k_{MET} and decreases in BCF were observed. Extrapolated BCFs for B(a)P and pyrene were reduced by 50%. Reductions of this magnitude could result in a substance being no longer classified as bioaccumulative if the BCF value were to fall below regulatory bioaccumulation criteria. Additionally modeled BCFs that incorporate *in vitro* biotransformation rates fit well with empirical BCF values.

As a potential substitute for empirical BCF data, *in vitro* biotransformation tests allow for rapid, cost-effective, and more accurate assessments of chemicals than relying on only log K_{OW} or computer models which do not consider biotransformation. These methods also reduce whole-animal testing. By increasing our knowledge of biotransformation and bioaccumulation of xenobiotics, the protection of the environment and human health may be improved. Understanding the effects of exposure to chemical mixtures like wastewater effluents on these processes is important as increases or decreases in biotransformation enzymes can subsequently decrease or increase the bioaccumulation potential of substances. This consequently may have toxicological implications which should be considered by regulatory agencies for the protection of the environment and human health.

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Appendices

Appendix A. GC/MS Calibration Curves

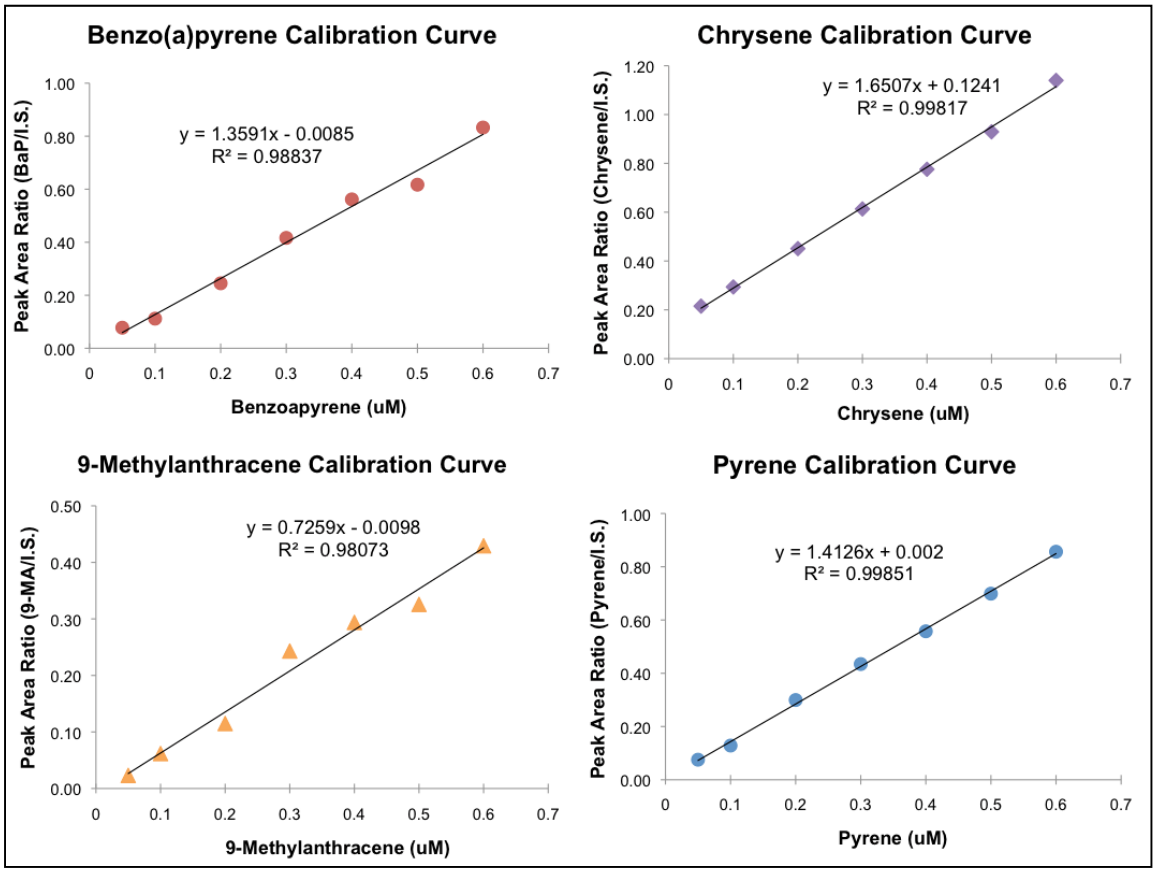

Figure A-1. Test chemical calibration curves for pyrene, B(a)P, 9-MA, and chrysene showing response, measured in terms of peak area, of test chemical, relative to the internal standard (d12-chrysene) as a function of test chemical concentration.

Appendix B. Protein Content of Trout Liver S9 Samples

2011 Exposure Data: Trout Liver S9 Samples

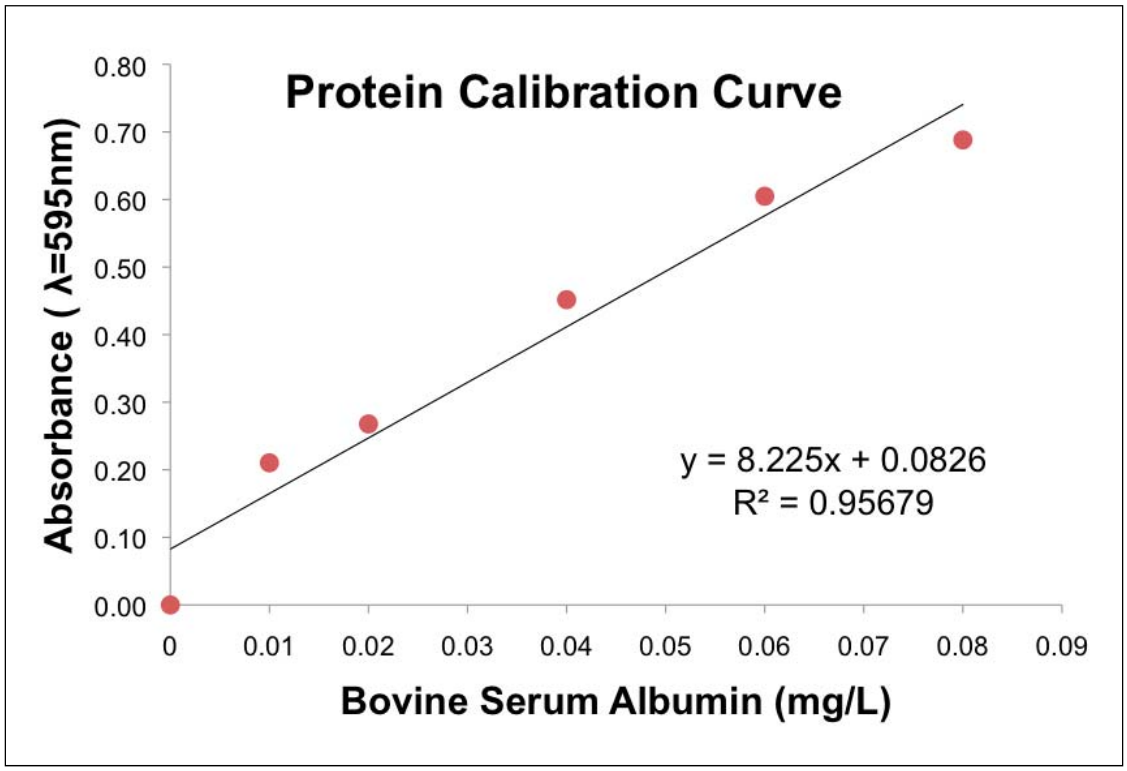

Figure B-2. Bovine serum albumin (BSA) calibration curve (blank corrected) showing response, measure in terms of absorbance (λ= 595nm) at various BSA concentrations.

Table B-1. Dilution Calculations used to calculate the mean protein concentration of S9 from trout livers from various exposure scenarios.

[Protein] (mg/mL)	Std. Curve Absorbance (λ=595)	Std. Curve - Blank Absorbance (λ=595)
0	0.4277	0
0.01	0.6380	0.2103
0.02	0.6956	0.2679
0.04	0.8795	0.4518
0.06	1.0325	0.6048
0.08	1.1158	0.6881

Table B-2. Dilution Calculations used to calculate the mean protein concentration of S9 from trout livers from various exposure scenarios. All concentrations are in mg/mL. Absorbance measurements are blank corrected.

Exposure Treatment	Absorbance (λ=595)	Mean [Protein] corresponding to standard curve	1000x dilution (Calculated [Protein] in S9)
0%	0.5594	0.0580	57.97
0.10%	0.5572	0.0577	57.70
1%	0.5522	0.0571	57.09
10%	0.6361	0.0673	67.29

2012 Exposure Data: Trout Liver S9 Samples

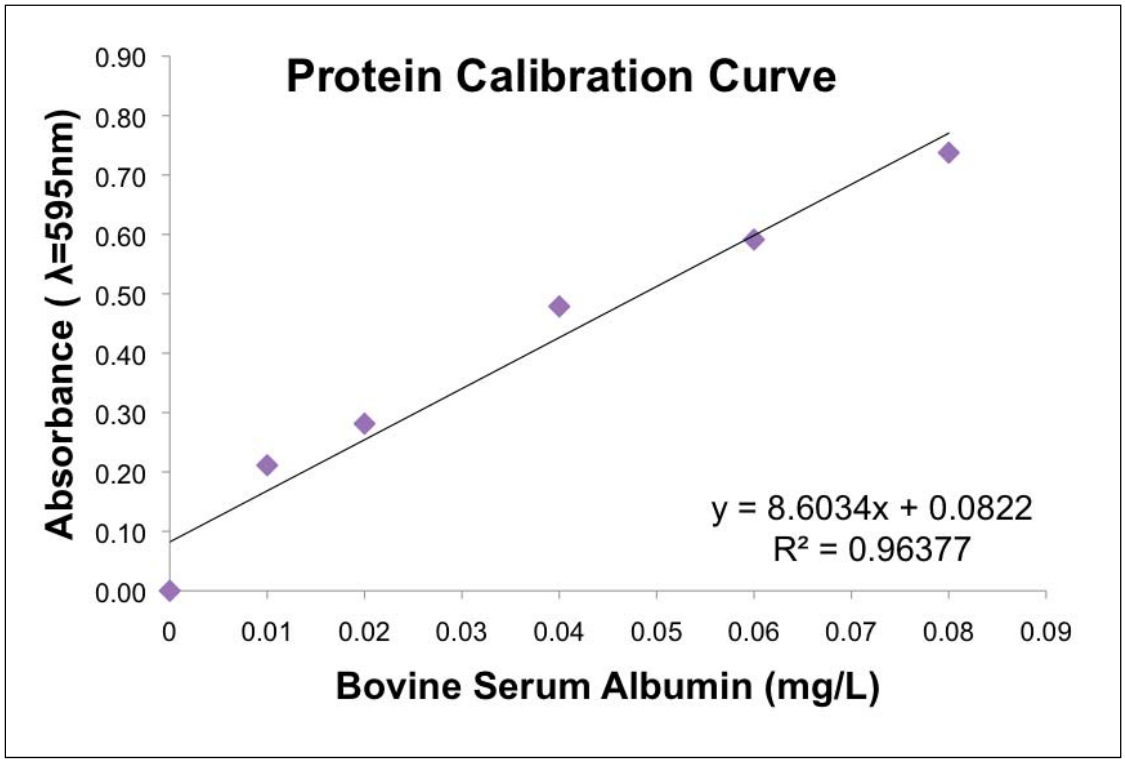

Figure B-2. Bovine serum albumin (BSA) calibration curve (blank corrected) showing response, measure in terms of absorbance (λ= 595nm) at various BSA concentrations.

Table B-1. Dilution Calculations used to calculate the mean protein concentration of S9 from trout livers from various exposure scenarios. All concentrations are in mg/mL.

[Protein] (mg/mL)	Std. Curve Absorbance (λ=595)	Std. Curve – Blank Absorbance (λ=595)
0	0.4790	0.0000
0.01	0.6903	0.2113
0.02	0.7603	0.2813
0.04	0.9577	0.4787
0.06	1.0703	0.5913
0.08	1.2166	0.7376

Table B-2. Dilution Calculations used to calculate the mean protein concentration of S9 from trout livers from various exposure scenarios. All concentrations are in mg/mL. Absorbance measurements are blank corrected.

Exposure Treatment	Replicate	Absorbance (λ=595)	Mean [Protein] corresponding to standard curve	12.5x dilution	100x dilution ([Protein] in S9)
0%	1	0.3950	0.0364	0.454	45.44
	2	0.3854	0.0352	0.440	44.05
	3	0.3758	0.0341	0.427	42.66
20%	1	0.3796	0.0346	0.432	43.21
	2	0.3933	0.0362	0.452	45.20
	3	0.3900	0.0358	0.447	44.72

Appendix C. Analyte Extraction Efficiency

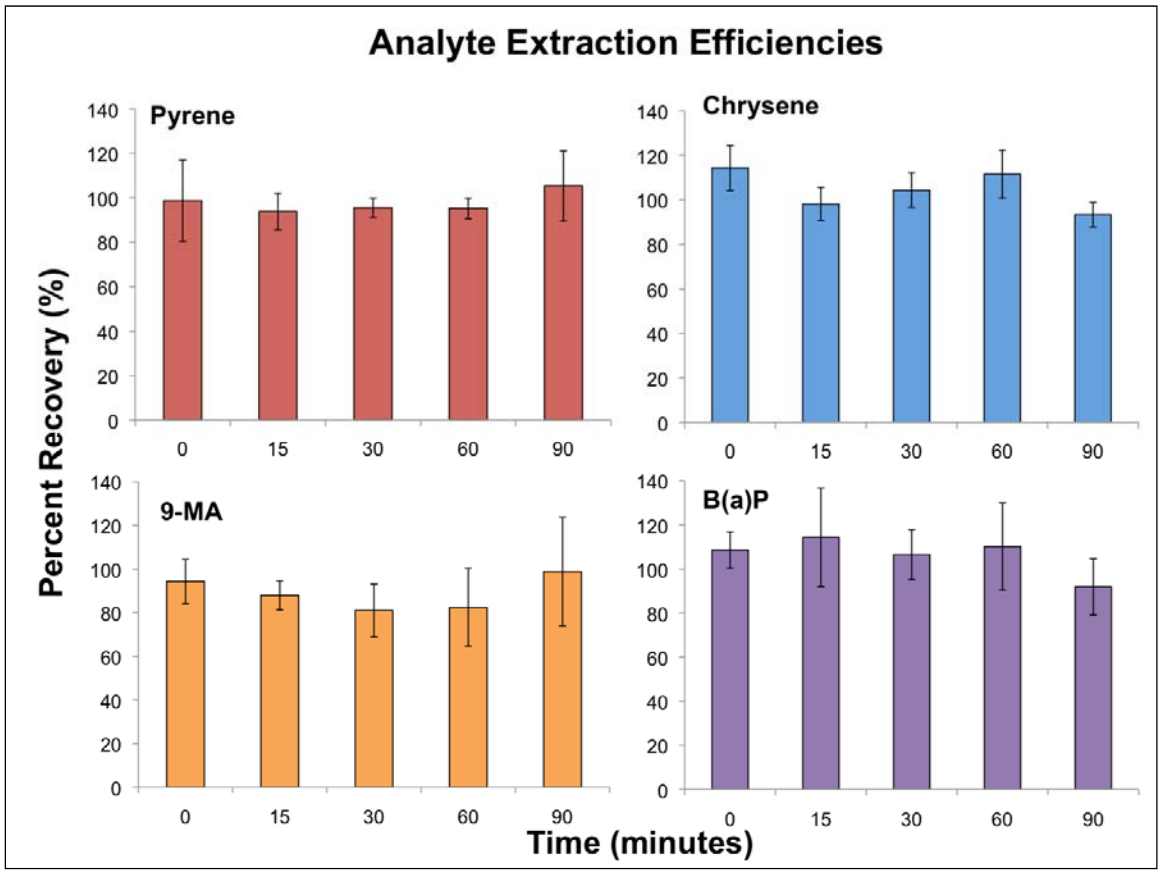

Figure C-1. Mean extraction efficiencies of pryene, chrysene, benzo(a)pyrene, and 9-methylanthracene from inactive S9 liver homogenates as a function of the incubation time. Error bars are the standard deviations of the mean (n=3).

Appendix D. Model Parameters

Table D-1. Parameters contained within the in vitro-in vivo extrapolation model (Nichols et al., 1990) and fish bioaccumulation model (Arnot & Gobas, 2003).

Parameter	Value	Units	Reference
In Vitro In Vivo Extrapolation (IVIVE) Model Parameters			
Organism Weight (W)	0.2	Kg	--
Fish Acclimation Temperature (T)	13	°C	--
Octanol-Water Partition Coefficient (K_{OW})		Unitless	--
Rate Substrate Depletion rate constant (k_r)	$LnC_t = LnC_0 - kt$	1/ time	Bisswanger et al., 2008
S9 Protein concentration (C_{S9})	40-60	mg/mL	Measured
S9 Protein concentration in incubation vial ($C_{S9,INC}$)	C_{S9} * (1/4 S9 dilution in PO_4 buffer) * (0.2 mL diluted S9 ÷ 0.5mL total incubation volume)	4 - 6	mg/mL	--
in vitro Intrinsic Clearance ($CL_{IN\ VITRO,INT}$)	kr (hour^{-1}) * C_{S9}		mL/h/mg protein	Nichols et al., 1990
Liver S9 protein content (L_{S9})	75	mg/g liver	Nichols et al., 1990
Liver weight as a fraction of whole-body weight (L_{FBW})	0.013	g/g	Nichols et al., 1990
in vivo Intrinsic Clearance ($CL_{IN\ VIVO,INT}$)	$CL_{IN\ VITRO,INT}$ * L_{S9} * L_{FBW} * 24		L/d/kg fish	Nichols et al., 1990
Unbound fraction in S9 system ($f_{U,S9}$)	1 / ($C_{S9,INC}$ * $10^{0.56\ logKow\ -1.41}$ + 1.0)		Unitless	Austin et al., 2002
Blood:water partition coefficient (P_{BW})	($10^{0.73\ logKow}$ *0.16) + v_{WBL})		Unitless	Nichols et al., 1990
Fractional water content of blood (v_{WBL})	0.84	Unitless	Bertelsen et al., 1998
Unbound fraction in blood plasma ($f_{U,P}$)	v_{WBL}/P_{BW}		Unitless	Nichols et al., 2006
Hepatic clearance binding term (f_U)	$f_{U,P}/f_{U,S9}$		Unitless	Nichols et al., 2006
Cardiac output (Q_C)	2.07	L/h/kg fish	Nichols et al., 1990
Liver Blood flow as a fraction of cardiac output (Q_{HFRAC})	0.259	Unitless	Nichols et al., 1990
Hepatic Blood Flow (Q_H)	Q_C * Q_{HFRAC}	536.1	mL/h/kg fish	Nichols et al., 1990
Hepatic Clearance (CL_H)	$Q_H f_U CL_{IN\ VIVO,\ INT}$ / (Q_H + f_U CL_{IN}		L/h/kg liver	Wilkinson & Shand,

Parameter		Value	Units	Reference
	$_{VIVO,INT}$)			1975
Lipid-Water Partition Coefficient (K_{LW})	Assume $K_{LW} = K_{OW}$		Unitless	--
Protein-Water Partition Coefficient (K_{PW})	0.035 * K_{LW}		Unitless	Gobas, 1993
Fractional whole-body lipid content ($v_{L,WB}$)		0.1	Unitless	Nichols et al., 1990
Fractional whole-body protein content ($v_{P,WB}$)		0.2	Unitless	Arnot & Gobas, 2004
Fractional whole-body water content ($v_{W,WB}$)	1.0- $v_{L,WB} - v_{P,WB}$	0.7	Unitless	--
Partition-based BCF (BCF_P)	($v_{L,WB}$ * K_{LW} + $v_{P,WB}$* K_{PW} + $v_{P,WB}$)		Unitless	Arnot & Gobas, 2004
Apparent volume of distribution ($V_{D,BL}$)	BCF_P/P_{BW}		mL	Nichols et al., 1990
Whole-body metabolic rate constant (k_{MET})	CL_H / $V_{D,BL}$		1/day	Nichols et al., 2006
Fish Bioaccumulation Model Parameters				
$BCF = k_1 \Phi / (k_2 + k_E + k_G + k_{MET})$				
Gill Uptake Rate Constant (k_1)	1/((0.01 + 1/Kow) * $W^{0.4}$		L/kg*d	Arnot & Gobas, 2003
Gill Elimination Rate Constant (k_2)	k_1/BCF_P		1/day	Arnot & Gobas, 2003
Fraction of freely dissolved chemical in the water (Φ)	1 / (1+ C_{POC} α_{POC} Kow + C_{DOC} α_{DOC} * Kow)			Arnot & Gobas, 2003
Concentration of particulate organic carbon (C_{POC})		5.0 * 10^{-7}	kg/L	Arnot & Gobas, 2003
Particulate organic carbon binding constant (α_{POC})		0.35	Unitless	Seth et al., 1999
Concentration of dissolved organic carbon (C_{DOC})		5.0 * 10^{7}	kg/L	Arnot & Gobas, 2003
Dissolved organic carbon affinity constant (α_{DOC})		0.08	Unitless	Burkhard, 2000
Dietary Uptake Rate Constant (k_D)	0.02 * $W^{-0.15}$ * $e^{(0.06-T)}$ / (5.1 *310^{-8} * Kow + 2)		kg/kg*d	Arnot & Gobas, 2003
Fecal Elimination Rate Constant (k_E)	0.125 * k_D		1/day	Arnot & Gobas, 2003
Growth Rate Constant (k_G)	0.0005 * $W^{-0.2}$		1/day	Arnot & Gobas, 2003

Appendix E. Substrate Depletion Data

Table E-1. Numerical data showing natural logarithm transformed benzo(a)pyrene concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated slopes (m), and R^2 values are also included. Shaded cells indicate data that was omitted in the calculation of m, and R^2 due to the possibility of enzyme attenuation or because benzo(a)pyrene was not detected (ND).

Benzo(a)pyrene - Substrate depletion data (2011 Exposure)

0% Effluent (v/v)									0.1% Effluent (v/v)								
Time (min)	Replicate 1		Replicate 2		Replicate 3		Time (min)		Replicate 1		Replicate 2		Replicate 3				
	C	T	C	T	C	T			C	T	C	T	C	T			
0	-0.790	-0.700	-0.657	-0.756	-0.459	-0.496	0		-0.889	-0.801	-0.711	-0.596	-0.520	-0.513			
5	-0.646	-0.779	-0.519	-0.735	-0.566	-0.641	5		-0.848	-0.999	-0.310	-0.514	-0.594	-1.007			
10	-0.688	-1.038	-0.491	-0.719	-0.492	-0.527	10		-0.807	-1.750	-0.548	-1.192	-0.693	-1.191			
20	-0.750	-1.303	-0.383	-1.178	-0.550	-1.265	20		-0.485	-2.317	-0.505	-1.569	-0.661	-1.739			
30	-0.995	-1.355	-0.390	-1.132	-0.457	-1.285	30		-1.033	-2.679	-0.404	-2.041	-0.486	-2.007			
45	-0.759	-1.620	-0.356	-1.339	-0.586	-1.624	45		-1.251	-2.880	-0.445	-2.213	-0.634	-2.357			
60	-1.111	-2.116	-0.322	-1.822	-0.426	-1.901	60		-0.866	-2.826	-0.414	-2.979	-0.710	-2.384			
75	-0.672	-2.037	-0.318	-1.846	-0.717	-1.989	75		-0.797	-2.939	-0.510	-2.780	-0.630	-3.586			
90	-1.011	-2.089	-0.432	-2.210	-0.629	-2.213	90		-0.910	-3.051	-0.420	-3.899	-0.851	-3.468			
Slope (m)	-0.0025	-0.0164	0.0022	-0.0196	-0.0016	-0.0197	Slope (m)		-0.0228	-0.0229	0.00104	-0.03406	-0.00212	-0.0316			
R^2	0.2281	0.9183	0.4007	0.9254	0.1988	0.9254	R^2		0.0322	0.7301	0.0881	0.9418	0.3992	0.9383			
1% Effluent (v/v)									10% Effluent (v/v)								
Time (min)	Replicate 1		Replicate 2		Replicate 3		Time (min)		Replicate 1		Replicate 2		Replicate 3				
	C	T	C	T	C	T			C	T	C	T	C	T			
0	-0.376	-0.375	-0.408	-0.448	-0.414	-0.373	0		-0.905	-0.858	-0.408	-0.648	-0.407	-0.488			
5	-0.434	-0.360	-0.475	-0.372	-0.334	-0.312	5		-0.564	-0.909	-0.511	-1.191	-0.408	-0.811			
10	-0.644	-0.728	-0.634	-0.810	-0.544	-0.726	10		-0.787	-1.660	-0.717	-1.688	-0.474	-1.125			
20	-0.578	-0.915	-0.362	-1.150	-0.386	-1.039	20		-0.737	-2.128	-0.662	-2.313	-0.346	-2.058			
30	-0.324	-1.078	-0.495	-1.187	-0.392	-1.175	30		-0.864	-1.899	-0.516	-2.704	-0.670	-3.035			
45	-0.469	-1.449	-0.430	-1.496	-0.388	-1.393	45		-0.586	-2.251	-0.805	-3.142	-0.625	-3.403			
60	-0.522	-1.456	-0.423	-2.448	-0.411	-2.026	60		-0.688	-2.982	-0.740	-3.118	-0.704	-4.093			
75	-0.477	-1.854	-0.505	-2.148	-0.434	-2.099	75		-0.779	-3.646	-0.779	-2.993	-0.760	ND			
90	-0.313	-1.963	-0.581	-2.086	-0.463	-2.178	90		-0.628	-3.674	-0.512	-2.995	-0.729	-3.826			
Slope (m)	*0.0009*	*-0.0262*	*0.0006*	*-0.0263*	*0.0003*	*-0.0218*	*slope (m)*		*0.0011*	*-0.0337*	*-0.0016*	*-0.0408*	*-0.00434*	*-0.0415*			
R^2	*0.0714*	*0.9092*	*0.0543*	*0.9092*	*0.0326*	*0.9457*	*R^2*		*0.0932*	*0.9219*	*0.1288*	*0.8627*	*0.5431*	*0.8131*			

Table E-2. Numerical data showing natural logarithm transformed pyrene concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated slopes (m), and R2 values are also included. Shaded cells indicate data that was omitted in the calculation of m, and R2 due to the possibility of enzyme attenuation or because pyrene was not detected (ND).

Pyrene - Substrate depletion data (2011 Exposure)

0% Effluent (v/v)							0.1% Effluent (v/v)						
Time (min)	Replicate 1		Replicate 2		Replicate 3		Time (min)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test		Control	Test	Control	Test	Control	Test
0	-0.751	-0.703	-0.511	-0.51	-0.461	-0.413	0	-0.694	-0.564	-0.535	-0.615	-0.479	-0.531
5	-0.693	-0.715	-0.535	-0.447	-0.264	-0.545	5	-0.688	-0.646	-0.281	-0.696	-0.444	-0.476
10	-0.770	-0.827	-0.569	-0.539	-0.532	-0.644	10	-0.578	-0.852	-0.472	-0.828	-0.506	-0.524
20	-0.711	-0.854	-0.525	-0.551	-0.597	-0.609	20	-0.632	-0.832	-0.602	-1.199	-0.570	-1.216
30	-0.652	-0.969	-0.442	-0.995	-0.499	-0.93	30	-0.710	-1.101	-0.418	-1.357	-0.551	-1.206
45	-0.774	-1.052	-0.506	-1.324	-0.571	-0.766	45	-0.675	-1.275	-0.534	-1.157	-0.664	-1.47
60	-0.746	-1.100	-0.788	-1.234	-0.699	-1.212	60	-0.742	-1.506	-0.667	-1.419	-0.646	-1.543
75	-0.797	-1.193	-0.679	-1.300	-0.407	-1.3	75	-0.624	-1.714	-0.552	-2.280	-0.45	ND
90	-0.775	-1.284	-0.59	-1.418	-0.629	-1.418	90	-0.706	-1.878	-0.590	-1.843	-0.456	-2.003
Slope (m)	*-0.0007*	*-0.0063*	*-0.0018*	*-0.0158*	*-0.0018*	*-0.0069*	*slope (m)*	*-0.0004*	*-0.0145*	*-0.0019*	*-0.0178*	*-0.0015*	*-0.0173*
R^2	*0.2338*	*-0.9757*	*0.2987*	*0.9254*	*0.0836*	*0.939*	*R2*	*0.06014*	*0.987*	*0.00331*	*0.8314*	*0.00331*	*0.9011*

1% Effluent (v/v)							10% Effluent (v/v)						
Time (min)	Replicate 1		Replicate 2		Replicate 3		Time (min)	Replicate 1		Replicate 2		Replicate 3	
	C	T	C	T	C	T		C	T	C	T	C	T
0	-0.661	-0.7485	-0.407	-0.486	-0.546	-0.478	0	-0.538	-0.444	-0.478	-1.168	-0.362	-1.235
5	-0.684	-0.761	-0.533	-0.459	-0.564	-0.401	5	-0.455	-0.419	-0.589	-1.280	-0.524	-1.178
10	-0.809	-0.776	-0.474	-0.545	-0.475	-0.585	10	-0.548	-0.79	-0.545	-1.471	-0.57	-1.219
20	-0.788	-0.875	-0.45	-0.621	-0.529	-0.557	20	-0.569	-1.336	-0.509	-1.808	-0.488	-1.751
30	-0.768	-0.961	-0.674	-0.711	-0.518	-0.54	30	-0.525	-1.587	-0.476	-2.193	-0.571	-2.086
45	-0.715	-1.119	-0.521	-0.792	-0.513	-0.729	45	-0.572	-2.19	-0.368	-2.956	-0.534	-2.304
60	-0.684	-1.120	-0.592	-0.863	-0.553	-0.864	60	-0.468	-3.066	-0.513	-3.455	-0.602	-2.409
75	-0.557	-1.063	-0.627	-0.893	-0.548	-1.032	75	-0.561	-3.689	-0.523	-4.141	-0.513	-2.977
90	-0.658	-1.128	-0.586	-1.158	-0.56	-1.013	90	-0.56	-4.185	-0.5241	-3.820	-0.632	-3.18
Slope (m)	*-0.0013*	*-0.0069*	*-0.0017*	*-0.0069*	*-0.0003*	*-0.0069*	*slope (m)*	*-0.0003*	*-0.0435*	*0.00022*	*-0.0407*	*-0.00147*	*-0.0229*
R^2	*0.2926*	*0.939*	*0.4046*	*0.957*	*0.1222*	*0.9259*	*R2*	*0.05707*	*0.9945*	*0.0138*	*0.9964*	*0.3646*	*0.9663*

Table E-3. Numerical data showing natural logarithm transformed pyrene concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated y intercepts (b), slopes (m), and R2 values are also included. Shaded cells indicate data that was omitted in the calculation of b, m, and R2 (Figure E-5) due to the possibility of enzyme attenuation.

Pyrene – Substrate depletion data						
0% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.405	-0.402	-0.418	-0.568	-0.413	-0.236
10	-0.350	-0.631	-0.352	-0.634	-0.429	-0.628
20	-0.372	-0.606	-0.406	-0.554	-0.415	-0.658
30	-0.464	-1.139	-0.371	-1.025	-0.504	-1.253
40	-0.508	-1.068	-0.344	-0.849	-0.463	-1.288
50	-0.535	-1.710	-0.475	-1.492	-0.489	-1.928
60	-0.688	-1.792	-0.260	-1.724	-0.595	-1.860
70	-0.479	-1.778	-0.490	-1.593	-0.702	-1.963
80	-0.443	-2.166	-0.416	-1.994	-0.659	-2.337
90	-0.450	-2.232	-0.564	-2.184	-0.489	-2.279
intercept (b)	*-0.4066*	*-0.3795*	*-0.3556*	*-0.3889*	*-0.4058*	*-0.3702*
slope (m)	*-0.0014*	*-0.0216*	*-0.0012*	*-0.0194*	*-0.0024*	*-0.0238*
R2	*0.1937*	*0.9534*	*0.1792*	*0.9218*	*0.5228*	*0.9425*
20% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.451	-0.367	-0.471	-0.338	-0.414	-0.455
10	-0.528	-0.675	-0.548	-0.729	-0.552	-0.615
20	-0.565	-0.514	-0.434	-0.759	-0.459	-0.696
30	-0.239	-0.921	-0.572	-1.350	-0.328	-0.828
40	-0.366	-1.075	-0.479	-1.384	-0.265	-1.199
50	-0.320	-1.330	-0.673	-2.017	-0.578	-1.357
60	-0.791	-1.539	-0.834	-1.950	-0.416	-1.157
70	-0.786	-1.577	-0.565	-2.271	-0.427	-1.419
80	-0.630	-1.995	-0.609	-2.418	-0.461	-1.900
90	-0.555	-2.328	-0.548	-2.361	-0.670	-1.765
intercept (b)	*-0.4040*	*-0.2961*	*-0.4979*	*-0.4662*	*-0.4000*	*-0.4268*
slope (m)	*-0.0026*	*-0.0208*	*-0.0017*	*-0.0243*	*-0.0013*	*-0.0161*
R2	*0.1882*	*0.9638*	*0.1937*	*0.9436*	*0.1032*	*0.9111*

Table E-4. Numerical data showing natural logarithm transformed 9-methylanthracene concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated y intercepts (b), slopes (m), and R2 values are also included. Shaded cells indicate data that was omitted in the calculation of b, m, and R2 (Figure E-6) due to the possibility of enzyme attenuation.

9-Methylanthracene - Substrate depletion data						
0% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.609	-0.707	-0.401	-0.466	-0.405	-0.769
10	-0.557	-0.611	-0.617	-0.590	-0.603	-0.928
20	-0.755	-0.664	-0.448	-0.697	-0.494	-0.835
30	-0.762	-0.841	-0.496	-1.256	-0.592	-0.921
40	-0.441	-1.107	-0.497	-1.020	-0.494	-0.613
50	-0.777	-0.980	-0.382	-0.849	-0.592	-0.913
60	-0.438	-1.423	-0.475	-1.080	-0.817	-0.940
70	-0.661	-1.539	-0.519	-1.157	-0.535	-1.293
80	-0.851	-1.163	-0.530	-1.256	-0.660	-1.366
90	-0.627	-1.240	-0.590	-1.346	-0.512	-1.366
intercept (b)	*-0.6176*	*-0.6274*	*-0.4609*	*-0.5851*	*-0.5064*	*-0.6919*
slope (m)	*-0.0007*	*-0.0089*	*-0.0008*	*-0.0086*	*-0.0014*	*-0.0068*
R2	*0.0207*	*0.6977*	*0.0973*	*0.7237*	*0.1458*	*0.6144*
20% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.705	-0.830	-0.552	-0.856	-0.633	-0.586
10	-0.482	-0.667	-0.657	-0.662	-0.555	-0.664
20	-0.812	-0.667	-0.654	-0.703	-0.567	-1.037
30	-0.689	-0.912	-0.733	-0.700	-0.432	-0.928
40	-0.929	-1.206	-0.547	-0.854	-0.562	-1.024
50	-0.666	-1.226	-0.703	-1.121	-0.771	-0.839
60	-0.916	-1.129	-0.441	-1.134	-0.689	-0.805
70	-0.576	-1.118	-0.608	-1.102	-0.649	-1.222
80	-0.932	-1.312	-0.749	-0.803	-0.445	-1.240
90	-0.590	-1.327	-0.754	-1.099	-0.590	-1.453
intercept (b)	*-0.6927*	*-0.7148*	*-0.5984*	*-0.5839*	*-0.5873*	*-0.6471*
slope (m)	*-0.0008*	*-0.0072*	*-0.0009*	*-0.0086*	*-0.0001*	*-0.0074*
R2	*0.0241*	*0.7527*	*0.0732*	*0.7976*	*0.0017*	*0.6803*

Table E-5. Numerical data showing natural logarithm transformed B(a)P concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated y intercepts (b), slopes (m), and R2 values are also included. Shaded cells indicate data that was omitted in the calculation of b, m, and R2 (Figure-E-7) due to the possibility of enzyme attenuation.

Benzo(a)pyrene - Substrate depletion data						
0% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.844	-0.912	-0.635	-0.622	-0.651	-0.919
10	-0.893	-2.077	-0.625	-1.178	-0.750	-1.663
20	-0.904	-1.763	-0.747	-1.471	-0.773	-1.862
30	-0.621	-3.314	-0.548	-1.507	-0.691	-2.411
40	-0.642	-3.692	-0.461	-1.181	-0.909	-1.527
50	-1.192	-2.808	-0.727	-2.214	-0.747	-2.795
60	-1.013	-3.068	-0.671	-2.287	-0.500	-2.446
70	-1.083	-3.557	-0.622	-2.905	-0.962	-2.882
80	-1.380	-3.718	-0.587	-2.640	-0.800	-4.474
90	-0.760	-4.143	-0.867	-3.156	-0.300	-3.781
intercept (b)	*-0.7894*	*-1.5985*	*-0.5989*	*-0.7276*	*-0.7790*	*-1.0601*
Slope (m)	*-0.0032*	*-0.0290*	*-0.0011*	*-0.0264*	*-0.0016*	*-0.0316*
R2	*0.1627*	*0.7399*	*0.0891*	*0.9007*	*0.0610*	*0.7904*
20% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.660	-0.801	-0.702	-0.688	-0.701	-0.737
10	-0.740	-1.568	-0.691	-0.688	-0.726	-0.897
20	-0.477	-1.366	-0.821	-1.292	-0.740	-0.797
30	-0.728	-1.521	-0.610	-1.626	-0.837	-1.673
40	-0.931	-2.435	-0.518	-1.668	-0.507	-1.113
50	-0.931	-2.263	-0.800	-1.296	-0.989	-1.674
60	-0.990	-3.000	-0.740	-2.567	-0.839	-2.072
70	-0.776	-2.294	-0.688	-2.671	-0.873	-1.285
80	-1.145	-2.445	-1.287	-3.793	-0.813	-1.832
90	-0.793	-4.382	-0.951	-3.242	-0.697	-2.084
intercept (b)	*-0.6449*	*-0.9182*	*-0.6057*	*-0.4373*	*-0.7251*	*-0.6510*
Slope (m)	*-0.0038*	*-0.0287*	*-0.0039*	*-0.0343*	*-0.0010*	*-0.0210*
R2	*0.3776*	*0.7490*	*0.3046*	*0.8460*	*0.0595*	*0.7518*

Table E-6. Numerical data showing natural logarithm transformed chrysene concentration time profiles in the active and inactive trout liver S9 for each replicate per effluent treatment. Calculated y intercepts (b), slopes (m), and R2 values are also included. Shaded cells indicate data that was omitted in the calculation of b, m, and R2 (Figure-E-7) due to the possibility of enzyme attenuation.

Chrysene – Substrate depletion data						
0% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.619	-0.624	-0.661	-0.659	-0.982	-0.672
10	-0.679	-0.746	-0.753	-0.858	-0.704	-0.925
20	-0.642	-0.850	-0.912	-0.988	-0.877	-1.272
30	-0.457	-1.036	-0.768	-0.918	-0.822	-1.278
40	-0.582	-1.002	-0.590	-1.153	-1.082	-1.411
50	-0.665	-1.002	-0.977	-1.490	-0.815	-1.418
60	-0.550	-1.335	-0.808	-1.231	-0.856	-1.647
70	-0.451	-1.085	-0.822	-1.389	-0.841	-1.687
80	-0.551	-1.314	-0.680	-1.514	-0.910	-1.677
90	-0.569	-1.318	-0.834	-1.857	-0.866	-1.967
intercept (b)	*-0.6293*	*-0.6423*	*-0.7502*	*-0.6967*	*-0.8741*	*-0.8467*
Slope (m)	*0.0012*	*-0.0100*	*-0.0007*	*-0.0113*	*0.0000*	*-0.0122*
R2	*0.2036*	*0.8778*	*0.0300*	*0.8882*	*0.0001*	*0.9222*
20% Effluent (v/v)						
Time (minutes)	Replicate 1		Replicate 2		Replicate 3	
	Control	Test	Control	Test	Control	Test
0	-0.530	-0.763	-0.547	-0.557	-0.452	-0.517
10	-0.307	-0.768	-0.508	-0.711	-0.668	-0.625
20	-0.338	-1.028	-0.509	-0.705	-0.674	-0.583
30	-0.356	-1.012	-0.446	-0.742	-0.631	-0.631
40	-0.377	-1.083	-0.525	-0.745	-0.603	-0.682
50	-0.330	-1.121	-0.464	-1.124	-0.507	-0.773
60	-0.489	-1.226	-0.526	-1.387	-0.451	-1.045
70	-0.463	-1.469	-0.520	-1.282	-0.476	-1.055
80	-0.421	-1.606	-0.562	-1.511	-0.507	-1.025
90	-0.389	-2.055	-0.382	-1.970	-0.630	-1.450
intercept (b)	*-0.3861*	*-0.6571*	*-0.5229*	*-0.4322*	*-0.5912*	*-0.4323*
slope (m)	*-0.0003*	*-0.0124*	*0.0005*	*-0.0143*	*0.0007*	*-0.0090*

Appendix F. Empirical BCF Values

Table F-1. BCF values derived *in vivo*, compiled from primary literature

Chemical	BCF Value	Test Species	Reference
B(a)P	224, 377, 608, 2657, 3208	Bluegill sunfish (*Lepomis macrochirus*)	McCarthy & Jimenez, 1985
	480	Bluegill sunfish (*Lepomis macrochirus*)	Jimenez et al., 1987
	930	Western Mosquitofish (*Gambusia affinis*)	Lu et al., 1977
	2310	Atlantic Salmon (*Salmo salar*)	Johnsen et al., 1989
	770, 1600	Bluegill sunfish (*Lepomis macrochirus*)	Spacie et al., 1983
	1160	Atlantic Salmon (*Salmo salar*)	Cohen et al., 1994
	380 – 2600	Water Flea (*Daphnia magna*)	Granier et al., 1999
	920	Rainbow Trout (*Oncorhynchus mykiss*)	Gerhart & Carlson, 1978
Pyrene	457	Goldfish (*Carassius auratus auratus*)	Ogata et al., 1984
	1000 - 1495	Sheepshead Minnow (*Cyorinodon variegates*)	Jonsson et al., 2004
	1560	Pacific Oyster (*Crassostrea gigas*)	Bustamante et al., 2012
	2512	Rainbow Trout (*Oncorhynchus mykiss*)	Johanning et al., 2012b
	1700, 3500	Water Flea (*Daphnia magna*)	Akkanen et al., 2000
	2691	Water Flea (*Daphnia magna*)	Axelman et al., 1995
9-MA	4583	Water Flea (*Daphnia pulex*)	Southworth et al., 1978
Chrysene	1560	Amphipod (*Rhepoxynius abronius*)	Boese et al., 1999
	1865	Juvenile Turbot (*Scophthalmus maximus*)	Baussant et al., 2001
	6088	Water Flea (*Daphnia magna*)	Newsted & Giesy, 1987