AN *IN VITRO* METHOD FOR MEASURING BIOTRANSFORMATION RATES OF CHEMICALS IN FISH: A STUDY WITH PYRENE

By

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ABSTRACT

To protect the health of humans and the environment, regulations in many jurisdictions require data on persistence, bioaccumulation and toxicity of commercial chemicals. Measuring bioconcentration factors (BCF) using *in vivo* testing to assess the bioaccumulative behaviour of chemicals can be expensive and slow. Therefore, an alternative approach for estimating *in vivo* BCF values using an *in vitro* rainbow trout (*Onchorhynchus mykiss*) hepatic S9 metabolism assay was tested. Depletion rates of pyrene were measured using trout hepatic S9 and GC-MS detection. These measured rates can be used to refine computer model predictions of BCFs. The measured biotransformation rate constant was 1.655±0.090 hr^{-1} and the Michaelis-Menten constant (K_m) for pyrene metabolism in trout S9 fraction was determined to be 0.23 ± 0.12μM. This study demonstrates that the *in vitro* hepatic S9 metabolism assay is a feasible and reproducible method for the measurement of hepatic biotransformation rates.

Keywords: pyrene, S9, biotransformation, rainbow trout, BCF, metabolism, depletion, *in vitro*, bioaccumulation

DEDICATION

I dedicate this project to God. He is my source and my rock.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and excretion
BCF	Bioconcentration factor
ºC	Degrees Celsius
Conc.	Concentration
DSL	Domestic Substance List
BMRL	Battelle Marine Research Laboratory in Sequim, WA, USA
C_p	Pyrene concentration
g	Acceleration of gravity
g	Gram
GC-MS	Gas chromatography-Mass spectroscopy
HESI	Health and Environmental Sciences Institute
hr	Hour
HPLC	High performance liquid chromatography
HT	Heat-treated
Kg	Kilogram
k_{dep}	depletion rate constant
K_m	Michaelis Constant. The substrate concentration required to reach half of the maximum reaction rate. ($V_{max}/2$).
K_{ow}	Octanol:water partition coefficient

L	Litre
ln	Natural logarithm
Log	Logarithm
M	Molar
Max	Maximum
min	Minutes
ml	Millilitre
MS222	3 – Amino benzoic acid ethyl ester
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced.
n	Nano
º	Degrees
PAH	Polycyclic Aromatic Hydrocarbon
PAPS	3’-Phosphoadenosine 5’-phosphosulfate
REACH	Registration, Evaluation and Authorization of chemicals
UDPGA	Uridine diphosphate glucuronic acid
V_{max}	Maximum reaction rate
µ	Micro

INTRODUCTION

Government organizations and scientists are working to assess the potential risk of chemicals to human and environmental health (Han *et al*, 2009). In Europe, one of such initiatives is REACH (Dyer *et al*, 2008), which requires that chemical substances with a log $K_{ow} \geq 3$, and produced at quantities ≥ 100 tonnes per year (Knight 2005) be examined for their potential to bioaccumulate. Canada has implemented a two-phased approach of the 1999 Canadian Environmental Protection Act. Phase one requires Environment Canada to assess 23,000 chemicals on the Canadian Domestic Substance List (DSL) for persistence (P), bioaccumulation (B) and toxicity (T) (CEPA 1999). Out of 11,300 organic chemicals reviewed on the Canadian Domestic Substance List, less than 5% have published empirical bioaccumulation values (Arnot and Gobas 2004; Arnot and Gobas 2006; Environment Canada 2006; Weisbrod *et al* 2007).

To measure the bioaccumulative nature of chemicals, the bioconcentration factor (BCF) value is used. Bioconcentration is the accumulation of a chemical in an aquatic organism by means other than dietary exposure (Meylan *et al.* 1999; Arnot and Gobas 2006). It can also be described as a measure used to express the ability of a chemical to accumulate in aquatic organisms (Meylan *et al*. 1999; Dyer *et al*. 2008; Han *et al*. 2009) and it is measured in live animals under controlled laboratory conditions (Arnot and Gobas, 2006). As the BCF value of a chemical increases, its potential to bioaccumulate increases. It has been

estimated that less than 4% of commercially available organic chemicals in Canada have BCF values considered by Arnot and Gobas 2006 as dependable values (Dyer *et al*., 2008) for categorization of the chemicals. The knowledge of the metabolic biotransformation capacity of chemicals is essential in estimating the bioconcentration potential of chemicals in biota. Biotransformation rates of chemicals are essential because without this data, estimated BCF values may be much higher than the true value when estimates are made using BCF computer model predictions based on log Kow, molecular weight and structure alone. Table 1 shows regulatory categorization of chemicals for bioaccumulation. REACH considers a BCF value greater than 5000 as very bioaccumulative (European commission, 2003), whereas Canada uses the Stockholm Convention Criteria, which considers a BCF equal to or greater than 5000 as bioaccumulative (UNEP, 2006).

BCF is a product of several processes, which includes the chemical uptake, chemical depletion, fecal egestion, growth dilution and metabolic transformation (Arnot and Gobas, 2006). One method to determine the BCF values of chemicals is using the log K_{ow} values, which is the octanol-water partition coefficient of chemicals. This approach is computer based and makes use of the physiochemical parameters of the chemical alone. If the chemical has a high affinity for fatty tissues, it will have a high log K_{ow} value and hence a higher BCF value but this is not always representative of true values because it considers only the physiochemical properties of the compound.

Table 1: Regulatory criteria for bioaccumulation categorization of chemicals

Program	**Categorization**	**Value**
CEPA	Bioaccumulative	BCF ≥ 5000
	Bioaccumulative	Log K_{ow} ≥ 5
REACH	Bioaccumulative	BCF ≥ 2000
	Very bioaccumulative	BCF ≥ 5000
TSCA	Bioaccumulative	BCF of 1000 - 5000
	Very bioaccumulative	BCF ≥ 5000

CEPA, Canadian Environmental Protection Act, 1999 (Government of Canada 1999, 2000). REACH: Registration, Evaluation, and Authorization of chemicals (European Commission 2001). TSCA: Toxic Substance Control Act (USEPA 1976).

Nevertheless, log K_{ow} values are important in determining the potential of a chemical to bioaccumulate. *In vivo* methods for estimating BCF values are expensive, use large numbers of animals and are labour intensive (OECD 305., 1996; Meylan *et al*., 1999). *In vivo* methods estimate BCF by finding the ratio of the concentration of the chemical in the organism and the chemical concentration in water at a steady state (Arnot and Gobas, 2006); using large numbers of test organisms over a long period of time. BCF values can also be calculated from the ratio of the chemical uptake rate constant from water and the depuration rate constant (Arnot and Gobas, 2006). An alternative approach to estimating BCF is the use of mathematical models (Nichols *et al*. 2007; Arnot and Gobas 2004); one such model is the Arnot and Gobas 2004 model used by Environment Canada. However, the lack of empirical data for biotransformation rates of chemicals in fish poses a limitation on such models. Scientists agree that one of the greatest sources of uncertainty in bioaccumulation models for fish is information on metabolic biotransformation rates (Nichols *et al*. 2007).

Biotransformation by enzymes

Testing the use of *in vitro* methods for analyzing the metabolic stability of environmental chemicals has been an area of interest in several toxicology laboratories. The *in vitro* methods aim to estimate *in vitro* metabolic biotransformation rates for environmental chemicals, to generate data for thousands of commercial chemicals. The output from these *in vitro* methods can be fed into extrapolation models like the recently developed model by Cowan-Ellsberry *et al*. (2008), which extrapolates the *in silico* biotransformation loss rate

(K_{met}) per day from the biotransformation rates of chemicals measured *in vitro*. These extrapolated *in silico* K_{met} values are also useful as a parameter in BCF models such as those developed by Arnot and Gobas (2004). The BCF values that are generated incorporate data on the rate of metabolism of the chemical. The present study is one of such efforts to assess and test the use of *in vitro* methods to estimate biotransformation rates of environmental chemicals.

There are drawbacks associated with using *in vitro* methods to analyze biotransformation rates of environmental chemicals. Common challenges include accurate extrapolation to *in vivo* metabolic rates, simulating and maintaining *in vivo* conditions in an *in vitro* system, and selecting the appropriate biological system (Coecke *et al*., 2006). However, there are advantages of *in vitro* methods, which include the use of fewer animals and the fact that it is less expensive and labour intensive than *in vivo* methods (Brandon *et al*., 2003).

Chemical biotransformation is a process of modification from one chemical form to another and is almost entirely dependent on enzymes. The liver plays a vital role in biotransformation because it contains a large number of known enzymes e.g. cytochrome P450 as well as unknown enzymes responsible for catalyzing the reactions involved in biotransformation of chemicals (Kulkarni 2001). The liver is generally found to have 5-100 fold cytochrome P450 activity compared to other tissues (Coecke *et al* 2006). Other tissues that are involved in biotransformation of environmental chemicals include the kidney, lungs, skin, and intestines. In general, the biotransformation potential of the liver > kidney > lung > gastrointestinal tract> nasal epithelium > placenta > brain (Coecke *et al* 2006).

However, this hierarchy could be altered depending on the substrate and route of exposure (Coecke *et al* 2006). Enzymes are biological catalysts that speed up the rate of a chemical reaction without being altered themselves and for enzymes to function favourably, various conditions have to be optimized. These conditions include: the time of contact between the enzyme and the substrate, the concentration ratio of substrates to enzymes, pH, temperature, and presence of co-factors (Ionescu and Caira. 2005).

Xenobiotics are foreign substances that are not produced nor expected to be found in the body. The metabolism of xenobiotics involves mainly two enzyme-catalyzed phases known as the phase I and phase II enzymes. Phase I reactions are usually referred to as functionalization reactions and are mostly oxidation reactions (e.g hydroxylation, dealkylation, deamination, and sulfoxide formation). Other phase I reactions are reduction (e.g azo reduction and addition of hydrogen) and hydrolysis (e.g; splitting of ester and amide bonds) (deBethizy and Hayes 1989). In phase I, most compounds become more water-soluble by acquiring polar functional groups such as - OH, - NH_2, - COOH or - SH (deBethizy and Hayes 1989). Phase II reactions are generally referred to as conjugation reactions. Conjugation either occurs by direct combination of the xenobiotic to water soluble endogenous substances such as Glycine, cysteine, glutathione (GSH), glucuronic acid and sulphates, or after the xenobiotic has been altered by a phase I reaction.

The main enzymes involved in phase I functionization reactions are known as the cytochrome P-450 system, the mixed function oxygenase (MFO) system

and the mixed-function amine oxidase (deBethizy and Hayes 1989). MFOs are localized in the smooth endoplasmic reticulum whereas the enzymes responsible for most Phase II conjugation reactions are localized in the cytoplasm (deBethizy and Hayes 1989). Non-cytochrome P450 phase I enzymes include microsomal flavin-containing monoxygenase (FMO), xanthine-dehydrogenase and aldehyde oxidase (Ionescu and Caira. 2005). Cytochrome P-450 enzymes are a diverse multigene family of heme-containing proteins found in all organisms so far examined and have extensive abilities to metabolize xenobiotics (Whyte *et al* 2000).

Table 2 shows various biological systems used to measure biotransformation rates of chemicals *in vitro*. Each system has its advantages and disadvantages and scientist use different systems to study specific areas of interest. The closeness of the biological system to *in vivo* conditions, cost of method, metabolic enzymes present, and preservation availability are major points of concern in choosing an *in vitro* assay system (Brandon *et al* 2003). Examples of *in vitro* biological systems used in studying biotransformation of chemicals are baculovirus insect cell expressed (trademark name is supersomes), liver cell lines, hepatocytes, liver slices,

Table 2: Biological *in vitro* systems for studying biotransformation of xenobiotics.

	Description	**Advantages**	**Disadvantages**
Supersomes	Transfection of insect cells with microsomes of human cytochrome P450 and uridine diphosphoglucuronosyl transferase (UGT) [1].	Can be used to study specific isoezymes and xenobiotic interactions.	Latency of glucuronidation in UGT supersomes
Liver cell lines	Isolated from primary tumors of liver parenchyma[1]. PLHC-1, Poeciliopsis lucida hepato-carcinoma cells[2].	Easier to culture compared to hepatocytes[2], Stable enzyme concentration[2].	Minimal or absence of some vital phase I and phase II metabolizing enzymes[1].
Hepatocytes	Liver cells isolated fresh from fish liver. They posses both phase I and phase II metabolizing enzymes.	Functionality is similar to whole liver in vivo, Can estimate cell BCF, Cryopreservation is possible[1].	Short life span, enzyme activity drops with time, Variable enzyme concentration
Liver slices	This is slices of isolated liver.	Intact cellular tissue[1], well established and characterized[1].	Inadequate penetration of mediums and damaged cells on the sliced outer edges[1], short viability time[1].
Isolated Perfused liver	Liver perfused with buffer and isolated from the animal.	Intact cellular structure and morphological studies are possible[1].	Expensive equipments needed, inadequate penetration of medium, Loss of viability is rapid, damages cells
Microsomes	9000 x g of hepatocytes (endoplasmic reticulum), and contains only phase I enzymes[2].	Can be cryopreserved, can be used to study chemicals at high concentrations, easily applicable and affordable[1].	Has only phase I enzymes, limited to loss rate[2], unsuitable for quantification measurements[1].
Post mitochondrial S9 supernatent	It is a fraction obtained from centrifugation of whole liver homogenate. Centifugation is usually at $9000g_{max}$. In this study it was centrifuged at $13000g_{max}$.	Has both phase I and phase II metabolizing enzymes, ease of preparation, can be cryopreserved	Can only be used to study loss rate, Lower enzyme activity[1].

[1]Brandon *et al* 2003; [2]Dyer *et al.* 2003

isolated perfused liver and subcellular systems (Brandon *et al* 2003). In this study, a subcellular biological system was used.

Subcellular systems are cell homogenates which may include purified enzymes, cytosolic soluble enzyme fractions (S100 supernatant), microsomal particulate enzyme fractions (S100 pellet) and the post-mitochondrial S9 supernatant which consist both of the cytosol and the microsome (Coecke *et al* 2006). The xenobiotic metabolizing property of these fractions is limited to the type of enzymes present in them. For example, the cytosol has only phase II enzymes while microsomes have only phase I enzymes. The S9 fraction contains both phase I and phase II enzymes. A major advantage of using microsomes alone is low cost, simplicity of approach and well characterized *in vitro* systems for xenobiotic biotransformation research (Brandon *et al* 2003). However, because CYP and UGTs are enriched in microsomal fractions, they are not useful for quantitative estimations of *in vivo* biotransformation (Brandon *et al* 2003). Thus, the S9 fraction offers a more complete representation of the metabolic profile (Brandon *et al* 2003).

Aims and objectives of study

This study is a part of a larger study by the HESI (Health and Environmental Sciences Institute) bioaccumation group. The goals of the group are to test the use of a combined approach for estimating trout BCF values for environmentally relevant chemicals. This approach combines an *in vitro* fish S9 metabolism assay and a predictive model (Cowan-Ellsberry *et al*., 2008). An objective of this group is to assess inter- and intra- laboratory repeatability of the

method, which involves at least five laboratories. In the first phase of this study, the *in vitro* metabolism of six environmentally relevant chemicals were measured based on the following criteria: the chemical should have environmental and commercial relevance, the chemical should have quality *in vivo* BCF data for trout, analytical methods should exist and analytical determination should be straight forward, the chemical have log K_{ow} values between 3.5 and 6.5, the metabolism should involve Phase I and Phase II biotransformation enzymes and the chemical should contain various functional groups. The commercial chemicals selected based on these criteria are 4-nonyl phenol, methoxychlor, dibenzyl ether, chloropyrifos, fluoroxypyr 1- methylheptylester and pyrene.

In the present study, the focus was to evaluate the potential of using one *in vitro* method to estimate biotransformation rate constants (k_m or k_{dep}). To assess and validate the *in vitro* approach, I incubated two sources of rainbow trout liver S9 fraction with one of the selected test chemicals, pyrene. I used two sources of trout liver S9 fraction in order to assess differences in estimated biotransformation rate constants due to different sources and preparations of the trout liver S9 fraction. I examined experimental variability by repeating metabolic incubation experiments within and between days.

The S9 subcellular fraction

A S9 fraction is the supernatant from centrifugation of tissue homogenates at 9000g_{max} and it contains both phase I and phase II metabolizing enzymes (Coecke *et al.* 2006). S9 fractions may be from different tissues that have

metabolizing enzymes such as the kidney, and liver. In this study, the trout liver was used to make the S9 fraction.

The liver is the organ of choice in this study because the liver is the major organ in which metabolism of many xenobiotics occurs in fish (Kennedy *et al*. 1991; Namdari and Law 1996; Namdari 1994, 1998). The use of the trout liver S9 as a biological system is attractive due to the use of fewer animals, ease of storage, low cost, and the presence of phase I and phase II enzymes (Brandon *et al*., 2003; Han *et al*, 2009).

It is important to carry out the study of bioconcentration of chemicals in fish especially because fish is a major part of the human diet. The choice of trout is because its metabolic potential has been studied extensively both *in vivo* and *in vitro* (Kennedy *et al*. 1991; Namdari and Law 1996; Namdari 1994, 1998) and because it is a commercially and economically relevant species of fish.

Pyrene as a test chemical

As the test chemical, pyrene was chosen based on the availability of the reported metabolic fate of pyrene in intact fish (Kennedy *et al*, 1991;Namdari 1994, 1998), availability of an analytical method, its environmental relevance, and pyrene having a log K_{ow} >3. The fact that pyrene's metabolic pathway is catalyzed by both phase I (Varanasi *et al.* 1989; Kennedy *et al.* 1991) and phase II enzymes (Shailaja and D'Silva 2003; Foureman., 1989) was also a determining factor.

Pyrene is a polyaromatic hydrocarbon (PAH) with four fused benzene rings (Howsam and Van Straalen., 2003) (see Figure 1). PAHs form through natural processes (McElroy *et al*., 1989) and originate from anthropogenic sources such as the incomplete combustion of organic matter (Hecht 2002; Elovaara 2007) including forest fires, fossil fuel combustion, industrial combustions, residential heating, vehicle emissions, and grilling (Luthe *et al.* 2002; Howsam and Van Straalen., 2003). Because of the many sources of PAHs, they are ubiquitous in the environment (Jones *et al*. 2008; Luthe *et al,* 2002; Zapata-Perez., 2004; Howsam and Van Straalen., 2003). Many high molecular weight PAHs are pro-carcinogens (Chou *et al*. 1985; Christensen *et al*. 2002; Kennedy and Law 1990; Varanasi and Stein 1991; Collier and Varanasi 1991), and metabolize readily to their carcinogenic metabolites in several organisms including fish (Buhler and Williams 1989; Varanasi *et al*. 1989). Pyrene is not a pro-carcinogen because its metabolites are not carcinogenic (Zapata-Perez, 2004). Pyrene is readily metabolized by polychaetes (Joorgensen *et al*., 2005), bacteria (Walter *et al*., 1991; Kazunga and Aitken, 2000), *Daphnia magna* (Ikenaka *et al*., 2006), fungus (Wunder *et al*., 1991), rodents (Dyer *et al*., 2008; Fitzsimmons *et al*., 2007), crabs (Eickhoff., 2004) and various species of fish including trout (Fitzsimmons, 2007; Namdari., 1994, 1998; Kennedy and Law., 1990).

Reported metabolic pathway of pyrene

The metabolic pathway of pyrene is well known and found to involve both phase I and phase II enzymatic reactions (see Figure 1). The major phase I

product of pyrene metabolism in fish is 1-hydroxypyrene (Luthe *et al*., 2002; Zapata-Perez *et al*., 2004; Namdari., 1994, 1998). 1-Hydroxypyrene has also been reported as the major phase I metabolite in microbial studies (Wunder, 1994) and in studies that involved other organisms (Jongeneelen, 2001; Howsam and Van Straalen., 2003). Viau *et al*. found that 60% of pyrene administered to rats was excreted through the urine as 1-hydroxypyrene after 24 hours.

Figure 1 shows that enzymes involved in phase II metabolism of PAHs are glutathione-S-transferase (GST), UDP-glucuronosyltransferase and sulfotransferases (Foureman, 1989; Kennedy *et al*, 1991). *In vivo* studies have shown that pyrene's phase II metabolites are conjugated glucuronides and sulphates (Kennedy and Law 1990; Luthe *et al*. 2002; Eickhoff 2004). These conjugated metabolites are polar and can easily be excreted (Williams 1959).

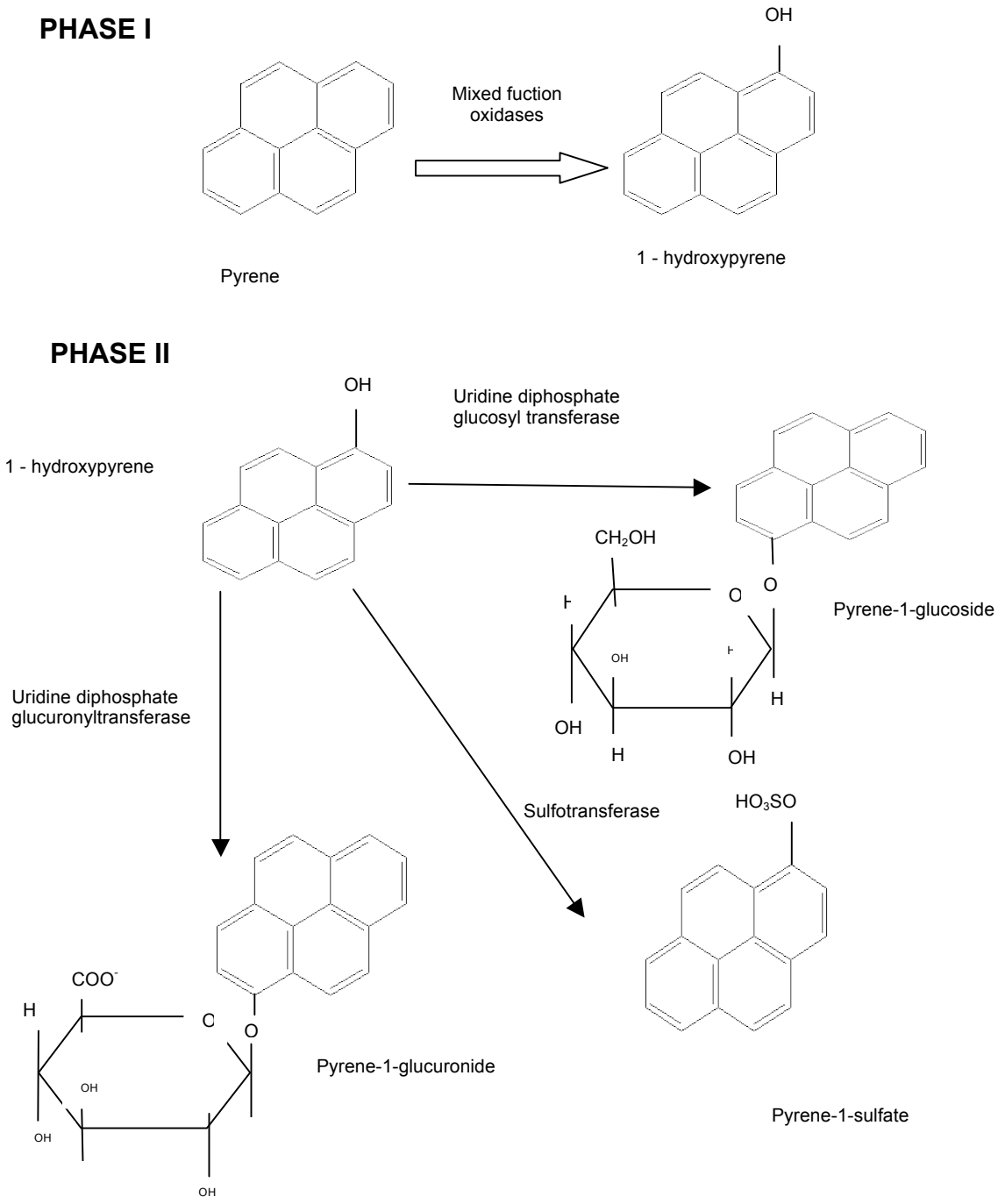

Figure 1: Reported metabolic pathway (Phase I and Phase II reactions) of pyrene in fish (Namdari 1994, 1998; Kennedy 1991).

MATERIALS AND METHODS

Test chemicals

Pyrene was purchased from Sigma Chemical company. It had a percentage purity of 98.4% and a molecular weight of 202.25 g/mol. The internal standard, Pyrene-d10, was purchased from Cambridge Isotope Laboratories Inc. Pyrene-d10 has a molecular weight of 212.31 g/mol and a purity of 100%. The solvents (methanol, hexane, acetonitrile) were all HPLC grade.

S9 Fraction Preparation

Trout liver S9 fractions used in this project originated from two sources. The HESI bioaccumulation group provided the first hepatic S9 pool. This pool of hepatic S9 fraction was prepared from 44 fish livers, taken from fish obtained from Battelle Marine Research Laboratory in Sequim, WA, USA. The fish weighed between 600 and 800 grams and were approximately 24 months old at the time of sacrifice. The fish spawned in the fall and were a hybrid of Shasta & Kamloop strains of rainbow trout. The fish were kept at 11.8 ± 0.8°C in a tank with a flow rate of 20L per minute. After the livers were excised at Batelle, the fish livers were frozen in liquid nitrogen and stored at -80ºC before they were transported to Invitrogen Inc. where they were used to prepare hepatic S9 fraction. The livers were stored at -80ºC for a few weeks at invitrogen Inc. before being used to prepare the trout hepatic S9 fraction. For the rest of this report, this

pool of S9 will be referred to as BMRL. It was prepared in April 2008 and stored at -80ºC until delivered on dry ice to CanTest Ltd in May 2008.

A second pool of S9 was prepared from five fishes collected from the animal care unit at Simon Fraser University on November 13, 2008. The supplier was Miracle Springs Trout farms in Mission, BC. Canada. At Miracle Springs Farm, the trout culture was kept for over 40 years and various strains of rainbow trout had been introduced including Steelhead, Kamloops and another unidentified strain from Washington State. The fish were kept at 10-10.5°C at SFU with a flow rate of 20L/min. All the fish used were male and weighed between 600-800 grams. They were fed EWOS Pacifica feed ad libitum but were starved for 24 hours prior to euthanizing with MS222 and an additional blow to the head. The body cavity of the fish was then dissected to expose the liver and the hepatic portal vein was isolated. A 25 G needle attached to a 10mL syringe was used to cannulate the hepatic portal vein and the liver was flushed with ice cold 0.9% NaCl until the liver turned pale. The liver was then separated from the rest of the fish and the gall bladder carefully cut off from the liver. The liver was then rinsed in cold homogenization buffer (See Appendix A for components of the buffer) and weighed (see Table 3).

Table 3: Body and liver weights of SFU fish

Fish number	Body weight (g)	Liver wet weight after perfusion (g)
1	609.62	6.26
2	661.76	6.87
3	760.00	6.14
4	759.83	6.90
5	637.23	6.48

The livers extracted from SFU fish were used immediately after extraction to prepare the SFU hepatic S9 fraction as described below.

Preparation of the liver S9 fraction from SFU fish

Where possible, all instruments and containers used in the preparation of the hepatic S9 fraction were pre-chilled on ice. In preparing the SFU S9, the homogenizing glass ware was kept in ice during the homogenization of the tissues and the S9 fraction was kept on dry ice or in a freezer at ~ -70±10°C during transportation. See Appendix A for details about the reagents, and buffers used for the preparation of the S9 fraction.

Using scissors, the livers were cut into small pieces (< 0.3 cm^2) on ice and the tissues were then homogenized at a ratio of 1:1 weight to volume in the homogenization buffer (50 mM Tris, pH 7.8 @ 4°C, 150 mM KCl, 2 mM EDTA, 1 mM DTT) at ~540 rpm with a motorized Potter-Elvehjem Teflon tissue grinder. The resulting homogenate was then transferred into pre-chilled 50 mL centrifuge tubes and centrifuged with Hermle Z360K centrifuge at ~ 13000g_{max} for 20 minutes at 4°C. Visible fat on the surface of the supernatant was removed with the use of a pipette and a cheese cloth was used to strain out any fat that might be left in the supernatant. The supernatant was then carefully decanted to prevent pellets from being included in the S9 pool and an aliquot of the decanted supernatant was taken for protein analysis. The S9 fraction was diluted to achieve a desired protein concentration of 20mg protein/mL and transferred into

1ml cryo tubes. The cryo tubes were placed in dry ice until the S9 fraction was completely frozen before transferring for storage to the ~ -70°C freezer.

The protein determination was carried out using the QuantiPro BCA Assay kit from Sigma. The bicinchoninic acid (BCA) assay is based on formation of a Cu^{2+}- protein complex, followed by the reduction of Cu^{2+} to Cu^{1+} under alkaline conditions. Absorbance of the standard protein solutions provided with the kit and the unknown samples was measured with ELx 800 universal microplate reader (Bio-Tek instrumentals Inc.) at 590 nm. The set up of the assay is presented in Appendix B. The S9 aliquot used for the protein analysis was first diluted 1000x to ensure that the protein concentration range was in the linear scale. Three other dilutions (dilution factors of 1, 2, and 10) were carried out. At the three dilutions and the protein concentration of the S9 fraction was found to be 22.59 ± 2.64 mg protein/mL. The S9 fraction was diluted further prior to use to achieve the desired protein concentration of 2 mg/ml in the test system. Appendix B presents the results of the protein determinations and the standard curve used to calibrate the protein assay.

Incubation procedure

To measure the biotransformation rate of pyrene, and to determine the K_m (the substrate concentration required to reach half of the maximum reaction rate $V_{max}/2$) and V_{max} (maximum reaction rate) in the trout hepatic S9 fraction, pyrene was incubated along with trout hepatic S9 fraction and selected cofactors in a buffered system. The experiments were carried out in a temperature-controlled room at 12 ± 1ºC. To stop the enzymatic reaction at each time point of interest,

200µL of 2.5µM of pyrene-d10 (Figure 2) dissolved in methanol was added to each 2.0mL GC amber vials, which were used as the test vials. Twenty

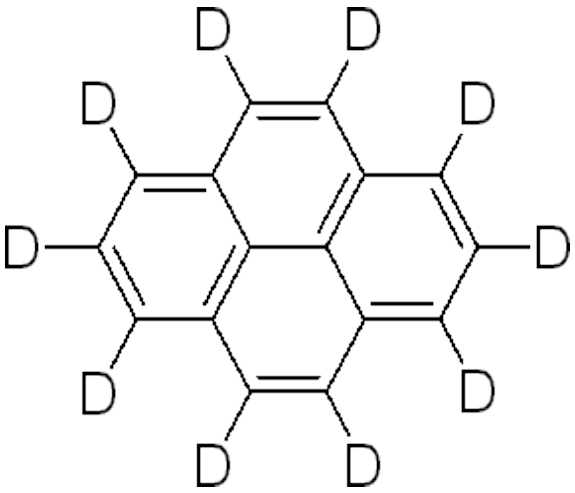

Figure 2: Structural Formula of Pyrene-d10

D represents deuterium atoms.

microlitres of 20mg of protein /mL of trout liver S9 fraction was added to a test volume of 200µl to achieve a protein concentration of 2mg protein/mL in each test vial.

Equipment and solutions were chilled on ice. To make sure the observed depletion was caused by enzymatic depletion of pyrene, incubation with heat-treated S9 fraction was ran simultaneously with the active S9 fraction at each concentration (0.1µM, 0.3µM, 0.5µM, 1.0µM, 1.5µM and 5.0µM). The heat treated hepatic S9 fraction (also referred to as the deactivated S9 fraction in this report), was prepared by heat treating the active S9 fraction at 100 ± 5°C for a minimum of 10 minutes. Apart from deactivated S9 control, there were also solvent controls, no-cofactor controls (samples that contained every other component of the test system except for the co-factors) and no-S9 fraction controls. The solvent control was used to observe how well the experiment performed by observing the amount of cross contamination of samples. The solvent control was also used to determine the method detection limit. The no-cofactor control was present to observe if there was any depletion in the test item due to pathways that we did not account for in our choice of cofactors. The no-S9 fraction control was present to observe any depletion in the test item that was non-enzymatic.

The total test volume was 200 µL consisting of 98 µL of 100mM phosphate buffer at pH 7.8, 20 µL active or deactivated fish liver S9, 20 µL alamethicin, 60 µL co-factors and 2 µL of pyrene in acetonitrile at 100X the desired concentration (see below).

A pool of active or deactivated S9 fraction was made by mixing 4.9ml of pre-chilled 100 mM phosphate buffer (pH 7.8 ± 0.1) with 1 mL of active or deactivated S9 fraction at protein concentration of 20 mg protein/mL. When necessary, a different volume of this mixture, using the same ratio of the phosphate buffer and the active or deactivated S9 was prepared. The phosphate buffer containing the active or deactivated S9 fraction was vortexed and 118 µL of the mixture was then dispensed into each test vial. The test vials were then placed in the water bath and allowed to pre-incubate at 12 ± 1°C for at least 5 minutes. Twenty microlitres of 250 µg/mL alamethicin was then added into each test vial and allowed to incubate for an additional 5 minutes in the shaking water bath at 12 ± 1°C. During the pre-incubation, the co-factor solution containing 10 mM NADPH (Nicotinamide adenine dinucleotide 2'-phosphate, reduced), 20 mM UDPGA (Uridine 5'-phosphoglucuronic acid), and 1 mM PAPS (3'-Phosphoadenosine 5'-phosphosulfate) was prepared by dissolving appropriate weights of the cofactors in 100mM phosphate buffer at pH 7.8 (see Appendix C for preparation of cofactors). The three co-factors were combined by mixing 10 mM NADPH, 20 mM UDPGA, and 1 mM PAPS solutions at a ratio of 1:1:1 (v/v/v). Sixty microlitres of this mixture was dispensed into each test vial with the exception of the no-cofactor control test vials (60µl of 100mM phosphate buffer was added in place of the cofactors in this control). The incubations began with the addition of 2µL of pyrene dissolved in acetonitrile at 100X the desired concentrations of pyrene. Thus, the total volume of acetonitrile in the test system did not exceed 1% of the total test volume (200µl). It is necessary to keep the

percentage of acetonitrile in the system to a minimal level because studies have shown that samples containing a higher percentage of organic solvents resulted in lower enzyme activity: 10% ACN reduced enzyme activity while complete inactivity of cytochrome c was observed at 50% ACN (Borole *et al*. 2004).

At the desired time points (0 min, 20mins, 40mins, 60mins, 90 mins and 120 mins), the enzyme activity in each vial was terminated by addition of 200µL of ice-cold methanol containing the internal standard (pyrene-d10) at 2.5µM. Hexane (1ml) was then added to each test vial and samples were vortexed for at least 10 seconds. The samples were then centrifuged at 3000g_{max} for 5 minutes. The supernatant (700 to 800µl) was then transferred into labeled 2ml amber GC vials and analyzed using the GCMS as described below.

Rationale for Choice of Co-factors and Alamethicin

Alamethicin is a peptide antibiotic derived from the fungus,*Trichoderma viride* . In whole cell incubations (e.g; hepatocytes), alamethicin has ion channel/pore forming abilities on cell membranes which enhances the accessibility of enzyme systems to UDP (Vedovato *et al*. 2007; Woolley 2007). Alamethicin contains 2-aminoisobutyric acid which induces helical peptide structures (Jones *et al*. 1980). Furthermore, its antibiotic properties are of importance in the S9 incubation studies to prevent bacterial metabolism of pyrene.

It was important to include both Phase I and Phase II cofactors in this study in order to ensure that both pathways were available for pyrene

metabolism. NADPH was necessary in this study because it is the reductant that is required by the main Phase I xenobiotic metabolizing enzymes, CYP450 (Pederson *et al*. 1974). UDPGA provides the test system with actived glucuronic acid. This is important for glucuronyltransferase-catalyzed conjugation reactions. Finally, PAPS served as the sulfuryl group donor for sulfotransferase catalyzed reactions in the test system (Klaassen and Boles 1997).

Sample Replication

Incubations were carried out with active and deactivated S9 (heat-treated S9). The first sets of incubations were carried out on two separate pools of S9 fraction I.e., BMRL S9 and SFU S9. There were nine replicate samples for both active and heat treated S9 fractions done over six days with pyrene concentrations of 0.5μM, 1.5μM and 5.0μM at six time points.

Based on observations made from the first set of incubations, further incubations at 0.1μM, 0.3μM and 1.0μM pyrene concentrations were performed in triplicate for 6 time points of 0, 20, 40, 60, 90 and 120 minutes. The heat-treated S9 reactions were incubated for 0, 60 and 120 minutes. Each incubant had solvent controls containing phosphate buffer, active S9, Alamethicin, NADPH, PAPS, UDPGA and acetonitrile , no-S9 controls containing phosphate buffer, Alamethicin, NADPH, PAPS, UDPGA and pyrene and no- cofactor controls containing phosphate buffer, active S9, alamethicin and pyrene.

Analytical Criteria and Approach

The solubility of pyrene in acetonitrile is 9.5020mM (Borole *et al*., 2004). The stock solutions used to make all the standards, QCs and spiking solutions was 5mM in ACN; hence, the solubility of pyrene was not exceeded in acetonitrile.

A calibration curve that covered the expected concentration range of pyrene used for the incubation was required at the beginning and end of each analytical run. The response from each calibration point was used to calculate the least square linear regression with the linear line not forced through zero. Acceptable standard points had percentage recovery ≤ 20% except for the lowest standard concentration where percentage recovery ≤ 25 was acceptable. A minimum of five concentrations was used for each calibration curve and the accuracy of the calibration curve was crosschecked with quality control samples. Quality control (QC) samples were done using three concentrations (low, medium and high of the test concentration of pyrene) in triplicate. QC samples were made from a weighing of pyrene separate from the weighing of pyrene that was used to make the standard solutions. QC samples were placed within runs to check for any changes in instrument performance over the period of the run. Both the calibration standards and QC (quality control) samples were prepared in 100mM phosphate buffer containing 2mg protein/mL of deactivated S9 fraction and the co-factors (NADPH, UDPGA, and PAPS), and alamethicin and at the same volumes as that of the incubation samples.

Prior to carrying out the incubations with pyrene, the GC-MS analytical method for analyzing the parent compound and the internal standard was validated by preparing the standard solutions and quality control samples in the matrix of the incubation samples. These samples (standard calibration curve samples and QC samples) were run on the GC-MS to optimize the conditions for analyzing for pyrene and pyrene-d10 on the GC-MS. After optimization of the method, it was used to analyze three separate batches of the desired standard calibration curve samples and QC samples to ascertain the ability of the method to analyze for pyrene and pyrene-d10.

All samples including incubation, quality control, and standard curve samples were extracted using hexane as the solvent. The hexane extract from each sample was injected directly into the GC-MS when samples from experiments with 0.5, 1.5 and 5μM of pyrene were analyzed. Samples generated from experiments done at 0.1, 0.3 and 1.0μM pyrene were extracted with 1ml hexane but they were then dried down with nitrogen and reconstituted into 50μl of acetonitrile before injecting into the GC-MS. The same volume and concentration of the internal standard, pyrene-d10, was added to all samples, standards and QCs before the extraction solvent was added to each sample.

Two GCMS machines was used at CANTEST Ltd and one at Simon Fraser University (SFU) for the analysis of the samples. The models of GCMS used at CANTEST were Hewlett Packard 6890 GC/5973 MSD and Agilent 6890 5985 MSD with 5% Phenyl Methyl Siloxane (30m x 0.25mm, 0.25μm) column and an HP 5MS Capillary column (30m x 0.25 mm., i.d, 0.25μm film thickness),

respectively. The model of the GC-MS used at SFU is Agilent 6890 with Agilent 7683 Series automatic liquid sampler attached to an Agilent 5973N mass spectrometer. The gas chromatograph is fitted with a programmable cool on-column capillary inlet attached to a HP-5MS 5% phenyl methyl siloxane-coated capillary column (30m x 0.25 mm i.d., 0.25 μm film thickness) and a fused-silica deactivated guard column (5 m x 0.530 mm i.d.). The same run conditions were used for each GC-MS. The injector port temperature was set at 230°C, the MS transfer line temperature was 280°C, the injection volume was 1μL, and the carrier gas was helium at 1mL/min. The oven temperature was programmed to 60°C for 1 min then 30°C/min to 300°C and held for 1 min.

The method detection limit (MDL) was derived from the mean value of the non-zero response values observed when the solvent control sample was analyzed. The reported MDL is the mean of the non-zero responses plus three standard deviations while the limit of detection (LOD) is the mean of the background noise from the GC-MS. The reported LOD was determined as the mean of the noise measured at the expected retention time for pyrene, plus 3 times the standard deviation (Frank Gobas, *personal communication*).

The precision of the standard curve and quality control samples was analyzed using percentage relative error and percentage recovery to measure the accuracy of the measurements.

$$\%\,\mathrm{RE\ (percentage\ \ relative\ error)} = \frac{\mathrm{Measured\ \ PC\ -\ Expected\ \ PC}}{\mathrm{Expected\ \ PC}} \times 100$$

$$\text{Percentage recovery} = \frac{\text{Measured PC}}{\text{Expected PC}} \times 100$$

“Measured PC” is the observed pyrene concentration following GC-MS analysis. “Expected PC” is the concentration of pyrene that was expected to be in the hexane extract.

Normalization

The measured rates were normalized to protein concentration of the hepatic S9 fraction used in the test system. A scaling factor of 95.9mg protein in S9 fraction per gram of liver as reported by Han *et al*., 2009, was used to convert the S9 protein concentrations to values in gram of liver.

Enzyme Kinetics

To estimate the V_{max} and K_M values of pyrene in trout liver S9 fraction, the Michaelis-Menten equation was linearized as follows:

The slope of the plot of natural logarithm of pyrene concentration at each time point against time (in hours) (see equation (III)) is the depletion first order rate constant (k_{dep}). Equation (I) is the first order rate equation. The rate law, Equation (II), is from the integration of equation (I) over the period of incubation (Ionescu & Caira 2005). Taking the natural logarithm of both sides of equation (II) gives a linear form seen in equation (III).

$$-dC_t / dt = k_{dep} * C_t \qquad \text{(I)}$$

$$C_t = C_o \exp^{(-k_{dep} * t)} \qquad \text{(II)}$$

$$\ln C_t = - k_{dep}\, t + \ln C_o \qquad \text{(III)}$$

C_t is concentration of pyrene at a specific time point. C_o is the concentration of pyrene at time zero. k_{dep} is the depletion rate constant and t is time.

V_{max} and K_m were estimated based on the Michaelis Menten equation;

$$-dC/dt = V_{max}*C_o/ (K_m+C_o) \quad \text{(IV)}$$

Substituting equation (I) into equation (IV) gives equation (V).

$$k_{dep} = V_{max}/(K_m+C_o) \quad \text{(V)}$$

Taking the reciprocal of both sides of equation (V) gives equation (VI).

$$1/ k_{dep} = (K_m + C_o) / V_{max} \quad \text{(VI)}$$

$$\text{Hence: } 1/ k_{dep} = (K_m/V_{max}) + (1/V_{max}) \times C_t \quad \text{(VII)}$$

Equation (VII) is in the form of y = mx + c, which is the general equation for a straight line (Engel 1981). From equation (VII), y is $1/ k_{dep}$ and x is C_t. So, $1/V_{max}$ is the slope of the plot of $1/ k_{dep}$ against C_t while K_m/V_{max} is the intercept on the y axis. The depletion rate constants estimated at all test concentrations were derived based on a first order rate reaction using equation (III). These k_{dep} values were used to derive the V_{max} and K_m of pyrene in the trout hepatic S9 fraction.

In order to validate the integration of Michaelis-Menten equation, a nonlinear regression plot of initial velocity against initial pyrene concentrations was used for estimating the Michaelis constants Vmax and K_M. The statistical software tool used was Prism Graphpad (version 5). The nonlinear regression is

based on the Michaelis Menten equation with the initial velocity at each incubation concentration determined by estimating the change in concentration (subtracting the concentration of pyrene in the test system at 20min from the concentration of pyrene in the test system at time zero) over the change in time.The change in time was 20min or 0.33hr.

The half-life of pyrene ($t_{½}$) was estimated using equation V (Ionescu & Caira 2005):

$$t_{½} = 0.693/k_{dep} \qquad \text{(XII)}$$

Statistical Evaluation

The *in vitro* k_{dep} values was estimated using the linear regression function in Microsoft Excel. k_{dep} is the slope of the graph of natural log (ln) of pyrene concentration per gram of protein against time in hours. The derived k_{dep} value could be used to estimate the BCF value of pyrene using an extrapolation model, e.g. the model developed for trout by Cowan-Elsberry *et al*; 2008.

The depletion of pyrene was only considered significant if the loss of pyrene was statistically significant with a P value less than 0.05. This statistical analysis were performed by comparing the loss of pyrene in the activated S9 and deactivated S9 treatments at time zero and point 120minutes in the incubation using ANOVA: single factor function in Excel. Where the loss of pyrene was significant in the deactivated S9 samples, the k_{dep} values derived for the

deactivated S9 was subtracted from the k_{dep} values derived for the active S9. This was to correct for non-enzymatic loss of pyrene in the test system.

RESULTS AND DISCUSSION

Chromatographic response of pyrene

The retention time of pyrene (M_2^+ = 202) and the internal standard, deuterated pyrene (d10-pyrene: M_2 = 212), were 9.69 mins and 9.68 mins, respectively. Figure 3 shows a mass spec and chromatogram of pyrene-d10 and pyrene. All standards used to calibrate the standard curves passed set quality control and quality assurance criteria detailed in the materials and methods. Figure 4 shows one of the standard curves used in estimating the concentration of pyrene in the test samples. All the quality control samples were within the set limits. The method detection limit (MDL) was 0.0175µM (3.5ng/mL). The limit of detection (LOD) was 0.00696µM (1.4ng/mL).

Depletion of pyrene

Table 4 shows the measured pyrene in the no-cofactor control, and no-S9 fraction control after two hours of incubation. There was no loss of pyrene in the no-cofactor controls, but there was a slightly higher concentration of pyrene observed in the no-cofactor controls when compared to pyrene concentration at time zero. The no-S9 control showed similar results as the no-cofactor control; however, depletion of pyrene was observed at 0.095µM and 0.543µM pyrene. The observed depletion of pyrene at these concentrations was not statistically significant, with a P value greater than 0.05.

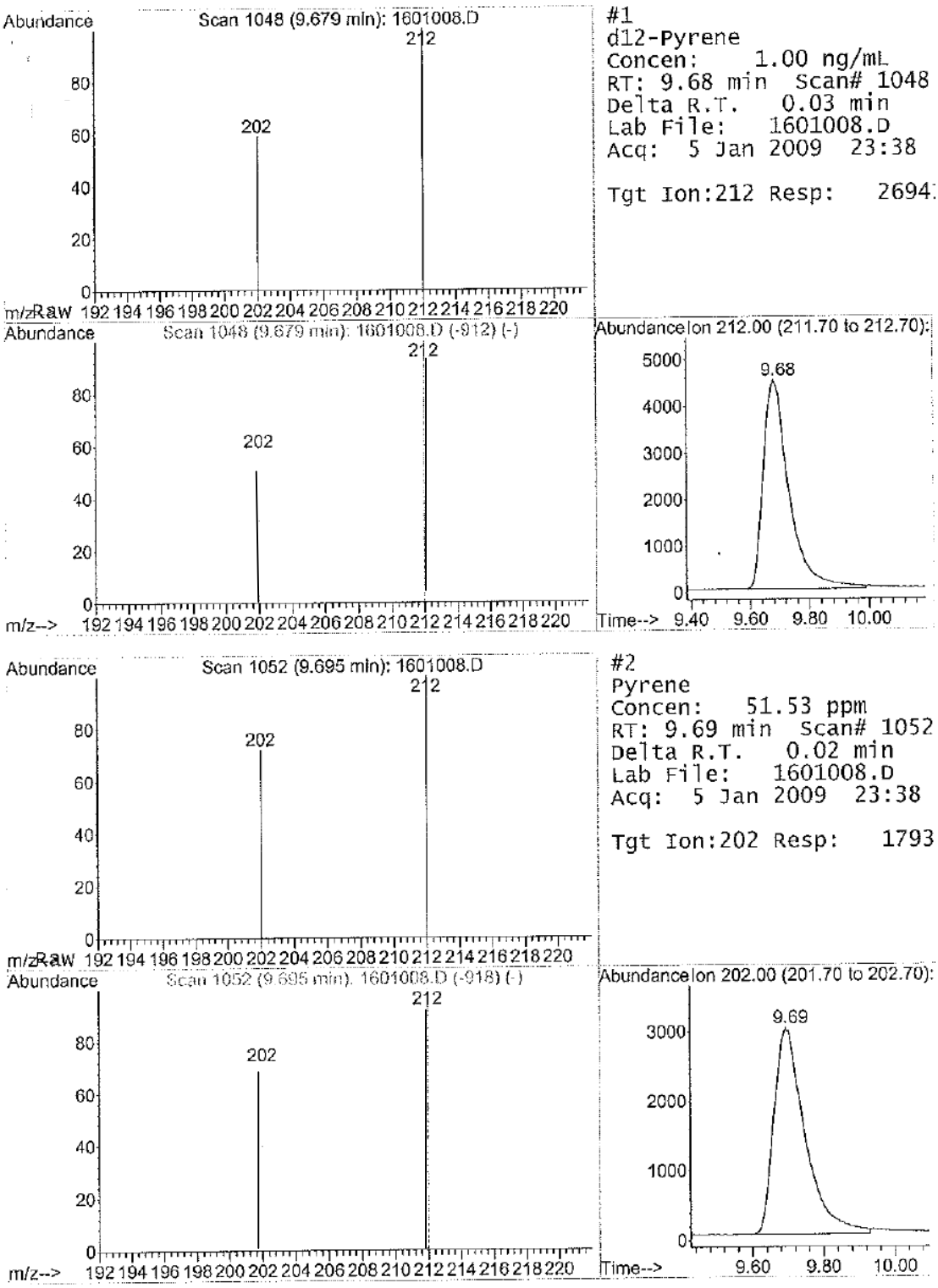

Figure 3: Chromatograms of pyrene-d10 (#1) and pyrene (#2) (right) and mass spectrometry of peaks from GC (left).

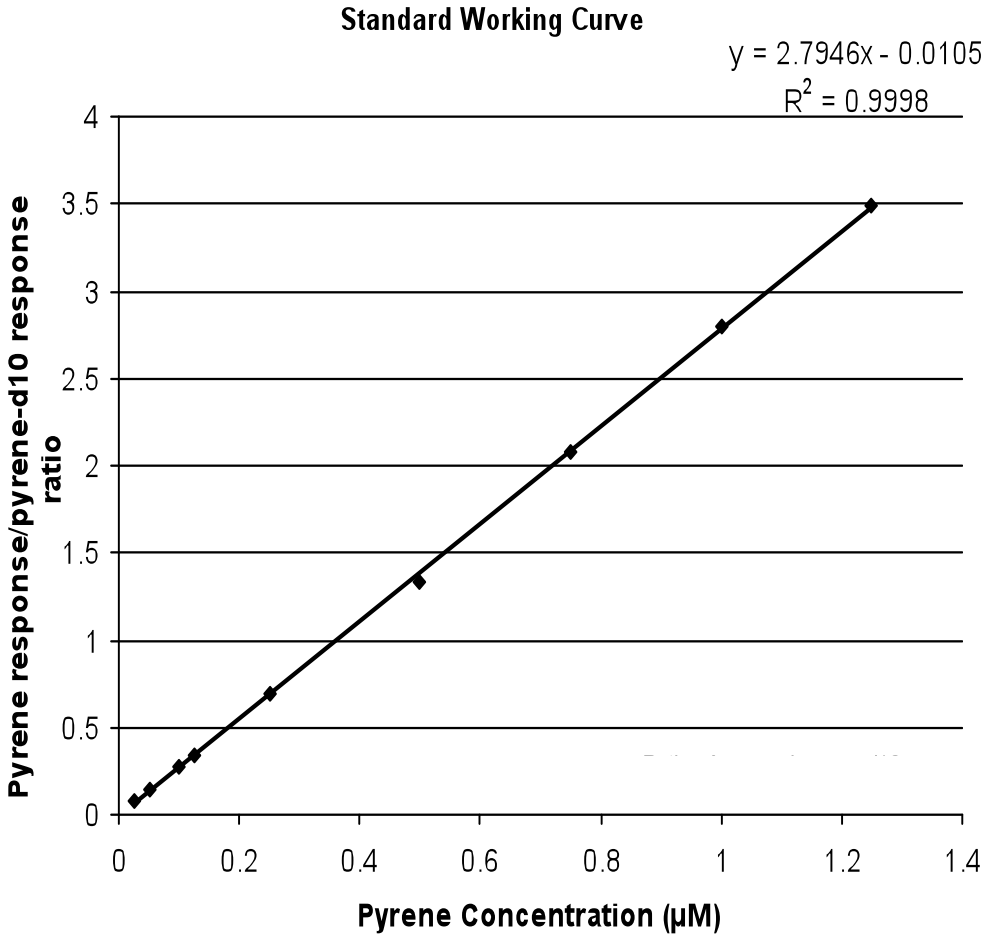

Figure 4: Sample of Standard Curve used for sample analysis

The ratio of the response (abundance) of pyrene and pyrene-d10 was used to measure the amount of pyrene in each sample.

Table 4: Pyrene concentration in No-S9 fraction control and No-cofactor controls after two hours of incubation

Pyrene concentration (µM) at time = 0	No- cofactor Control (µM)	No- S9 fraction control (µM)	P Value for No-S9 fraction control
0.095±0.005	0.098 ± 0.008	0.094 ± 0.001	0.22
0.284±0.006	0.298 ± 0.002	0.295 ± 0.002	N/A
0.543±0.036	0.506 ± 0.026	0.539 ± 0.022	0.43
0.926±0.031	0.984 ± 0.035	0.970 ± 0.014	N/A
1.510±0.059	1.550 ± 0.082	1.590 ± 0.015	N/A
5.220±0.163	5.820 ± 0.701	5.800 ± 0.626	N/A

N/A stands for not applicable. The P value was estimated when depletion of the test item in the controls was lower than the pyrene concentration at time = 0 minutes. The concentrations with N/A means that their concentration were higher or the same as the concentration of the test item in the active S9 test vials at time = 0 minutes.

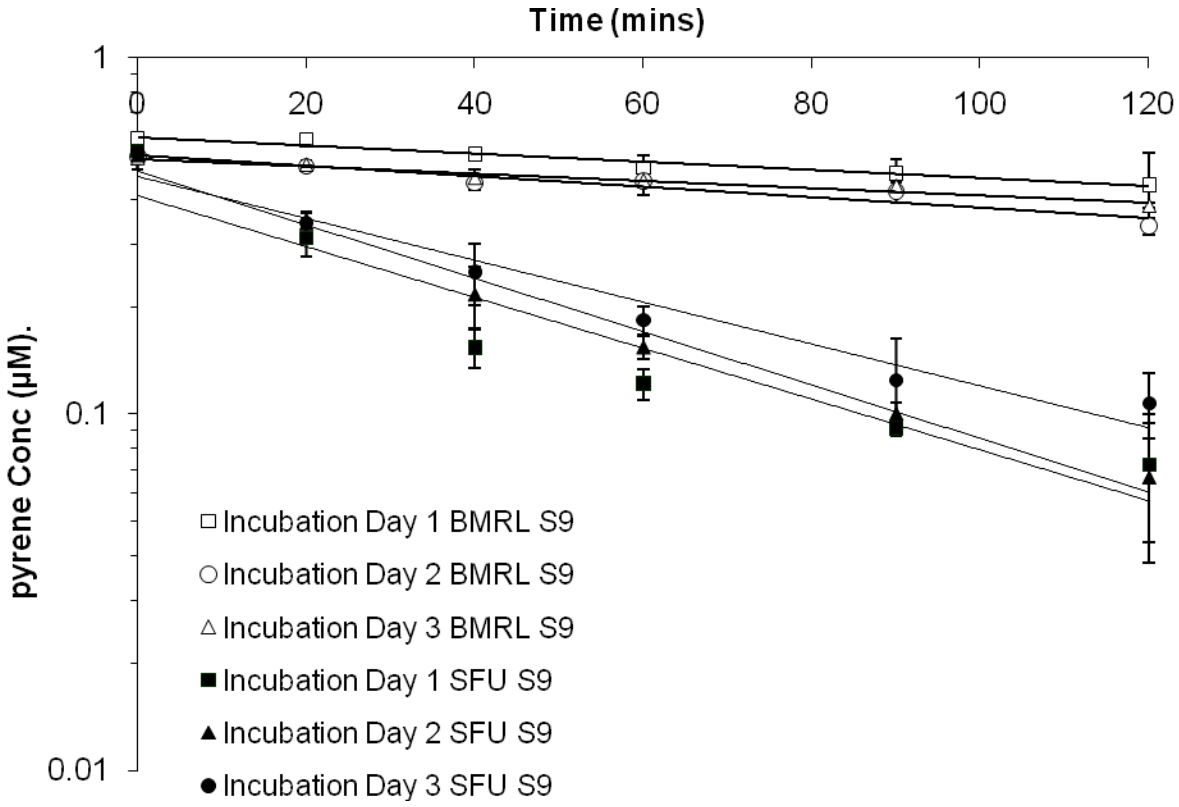

Figure 5: **Depletion of pyrene at expected concentration of 0.5µM in 2mg/mL protein concentration of BMRLS9 (S9 fraction made from livers extracted from fish from Battelle Marine Research Laboratory) and SFU S9 (S9 pool prepared from livers from Simon Fraser University fish stock). Incubation day refers to the mean of three replicates carried out on the same day.**

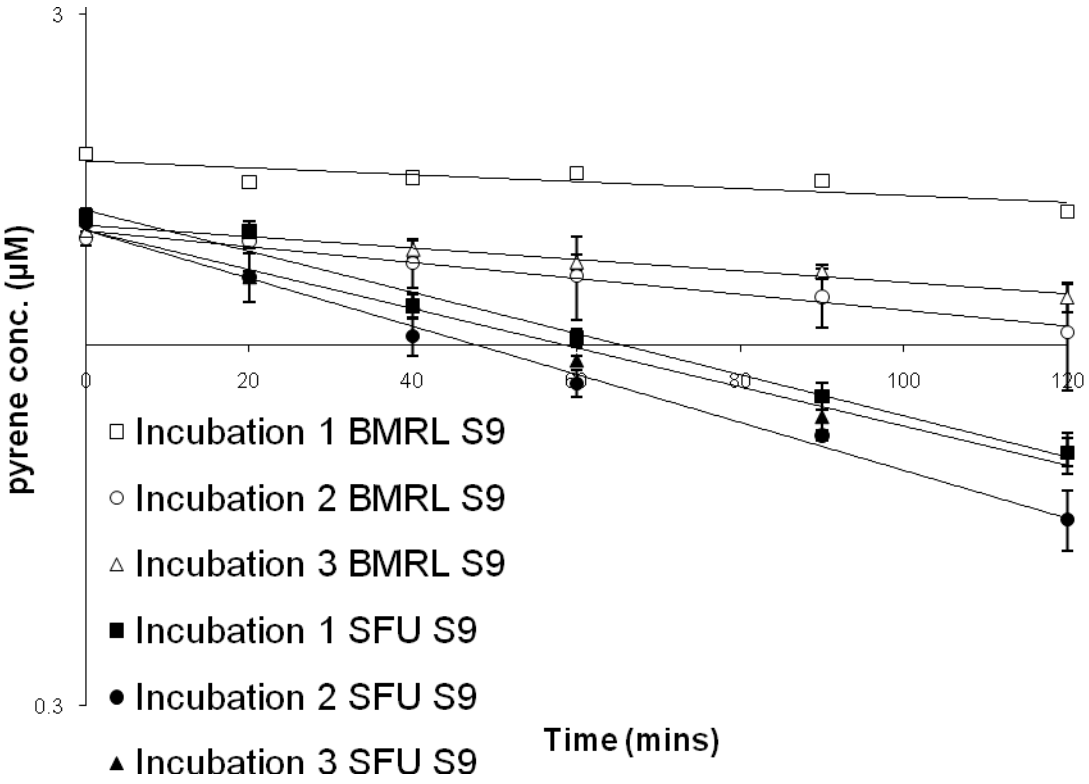

Figure 6: **Depletion of pyrene at an expected concentration of 1.5µM in 2mg/mL protein BMRL S9 (S9 fraction made from livers extracted from fish from Battelle Marine Research Laboratory) and SFU S9 (S9 pool prepared from livers extracted from fish obtained from Simon Fraser University).**

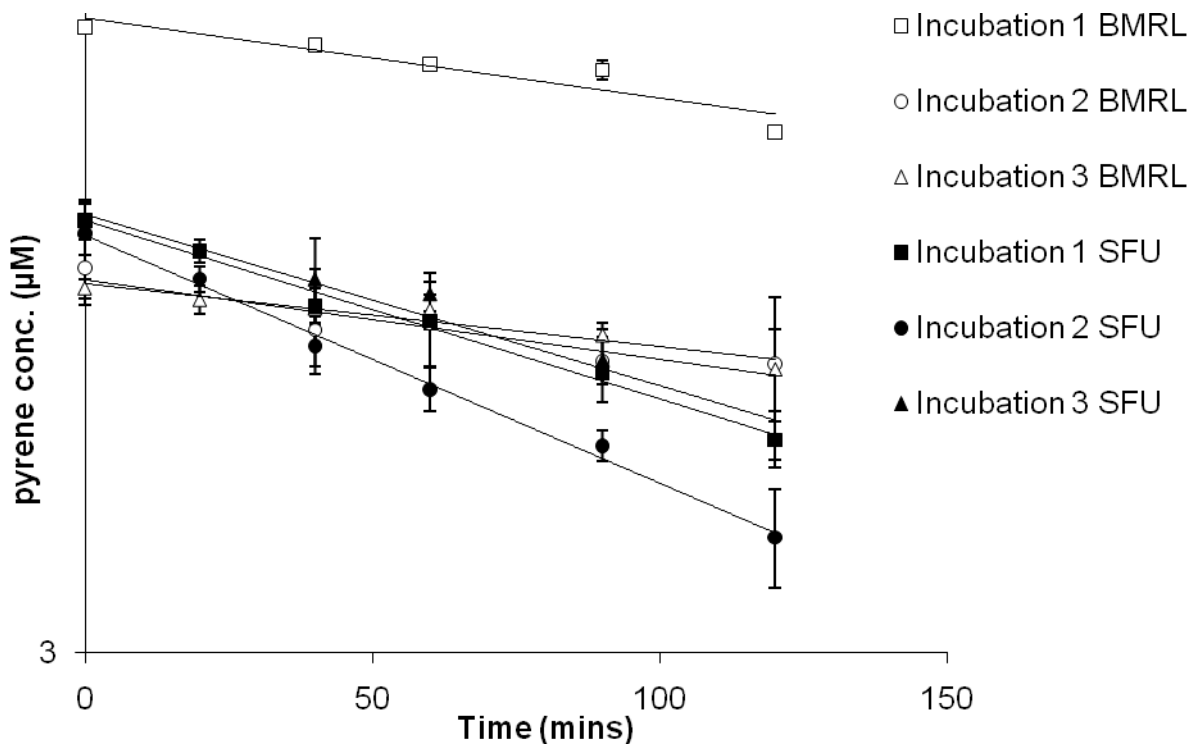

Figure 7: **Depletion of pyrene at an expected concentration of 5.0µM in 2mg/mL protein of BMRL S9 (S9 fraction made from livers extracted from fish from Battelle Marine Research Laboratory) and SFU S9 (S9 pool prepared from livers extracted from fish obtained from Simon Fraser University).**

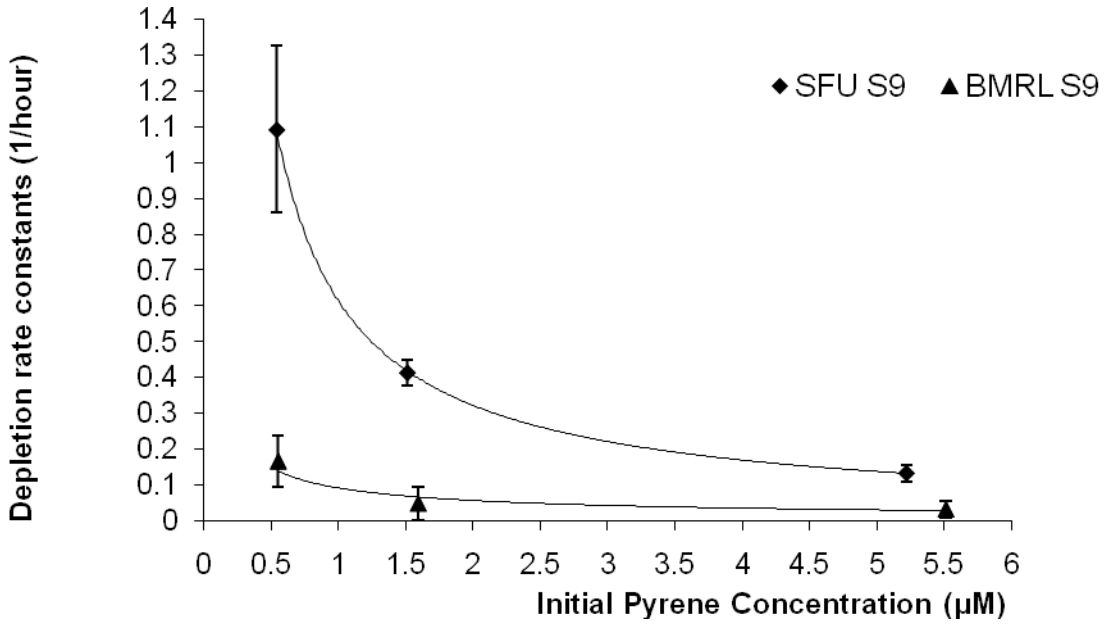

Figure 8: Depletion rate constants of pyrene in SFU S9 fraction and BMRL S9 fraction.

Figures 5 –7 show that the depletion of pyrene over two hours in the BMRL hepatic S9 fraction liver homogenate was about 2.5 - 6 fold lower than the fish hepatic S9 fraction made from fish from SFU. The SFU trout liver S9 fraction metabolized pyrene readily. After two hours, at an expected spiking concentration of 0.5μM, approximately 90% of the spiked pyrene was no longer present in the hepatic SFU S9 while approximately 20% of the spiked pyrene remained present in the BMRL S9 liver homogenate (Figure 5). Figure 8 shows the depletion rate constants of the SFU S9 fraction and the BMRL S9 fraction at three concentrations of pyrene in 2mg protein/ml of each S9 fraction. The *in vitro* k_{dep} values were estimated using only the linear part of the curve of the natural log of concentration versus time in hours. The *in vitro* k_{dep} value of pyrene in BMRL hepatic S9 fraction at 0.55μM pyrene was found to be 0.17 ± 0.03 hr^{-1} while the k_{dep} value for the SFU hepatic S9 was found to be 1.092 ± 0.233 hr^{-1} at 0.54μM of pyrene. The rapid depletion of pyrene observed in SFU S9 liver homogenate agrees with the results of an *in vivo* study by Namdari (1994) in which intact rainbow trout metabolized pyrene readily in blood, gut, liver, and kidneys.

The lower depletion rate of pyrene observed in BMRL hepatic S9 compared with the SFU liver S9 could be the result of several factors including stability of prepared S9 fraction at -70^{O}C, strain differences, and health and age-specific differences between the fish used to prepare the two batches of liver S9 fraction (Coecke *et al* 2006). A third batch of hepatic S9 fraction was prepared from the same source as BMRL. Incubation of this third liver S9 fraction with

pyrene gave similar results to the earlier BMRL liver S9 (results not shown). The fact that the livers excised from the BMRL fish (in both cases) were frozen at ca. -70ºC for a couple of days before been used to prepare the BMRL trout liver S9 fraction, might have contributed to the lower activity of BMRL liver S9 fraction. The SFU liver homogenates was prepared fresh on the same day the livers were extracted. The BMRL livers were isolated in Washington State, frozen with liquid nitrogen and stored at - 80°C, and transported to the lab at Invitrogen where the S9 pool was prepared. Transportation of the isolated livers could also have contributed to the lower activity observed in the BMRL pool. This suggests that freezing the liver before using it to prepare S9 fraction lowers the activity of the enzymes in the S9 fraction, and is therefore not recommended.

Both groups of fish (BMRL and SFU) used to prepare the trout liver S9 fraction spawn in the fall, and are about the same age. Other possible explanations for the lower enzyme activity in the BMRL liver S9 fraction is strain differences, health of the fish and, or induction of cytochrome P450 enzymes (Collier and Varanasi, 1991). The health of the fish cannot be addressed in this report because there were no set benchmarks to which the fish could have been classified as healthy or unhealthy. However, there were no observed/reported health problems for both sets of fish. A difference in strain could have contributed to the observed difference in the observed depletion of pyrene in the *in vitro* system. In fish and other species of organisms, studies have shown that different strains have varied metabolic capacity for the same xenobiotics (Fitzsimmons *et al*. 2007). The two pools of S9 were from two different strains of rainbow trout

and this could have attributed to the observed difference in the depletion rate of pyrene between BMRL hepatic S9 and SFU hepatic S9.

Due to low depletion rate of pyrene in the BMRL S9 fraction and the likelihood that prior freezing of the fish livers used in preparing the BMRL S9 could have compromised the viability of the enzymes in the BMRL S9 fraction, incubations with the BMRL S9 were discontinued after the incubations with the first three concentrations of pyrene were completed.

Figure 8 shows that there is an estimated 2.5 and 9 fold difference in the depletion rate constants between initial concentration of 0.54μM pyrene and the two higher pyrene concentrations of 1.51μM and 5.22μM. This suggests that some, if not all, of the pyrene concentrations used in incubating trout hepatic S9 fraction at this point in the study saturated one or more enzymes involved in the biotransformation of pyrene. Therefore, all other further incubations were carried out with the SFU trout hepatic S9 fraction at lower pyrene concentrations of 0.1μM, 0.3μM and 1.0μM to ascertain pyrene concentrations that followed first order rate reactions and to be able to estimate the kinetic constants (see figure 9-14).

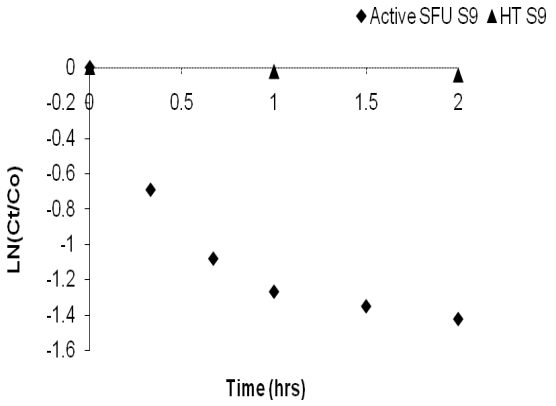

Figure 9: Depletion of 0.1µM pyrene (Replicate 1 of 3)

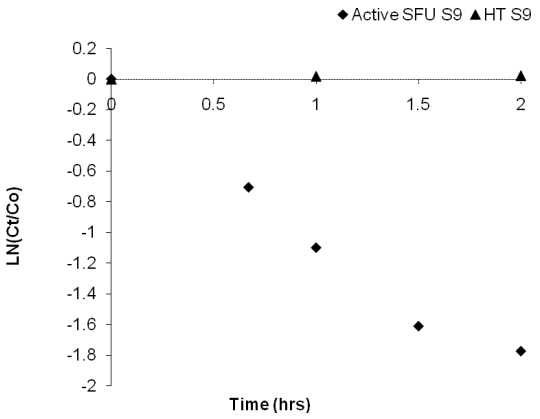

Figure 10: Depletion of 0.3µM pyrene (Replicate 1 of 3)

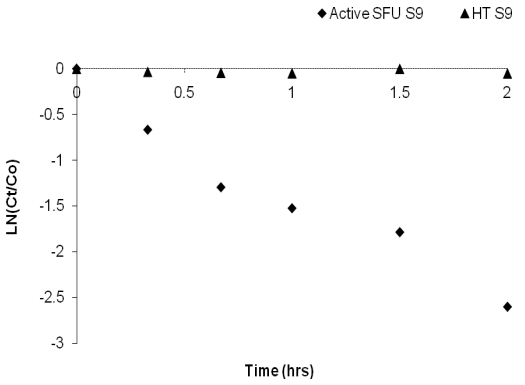

Figure 11: Depletion of 0.5µM pyrene (Replicate 1 of 9)

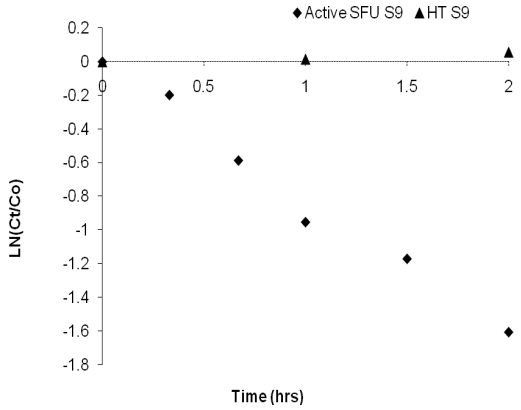

Figure 12: Depletion of 1.0µM pyrene (Replicate 1 of 3)

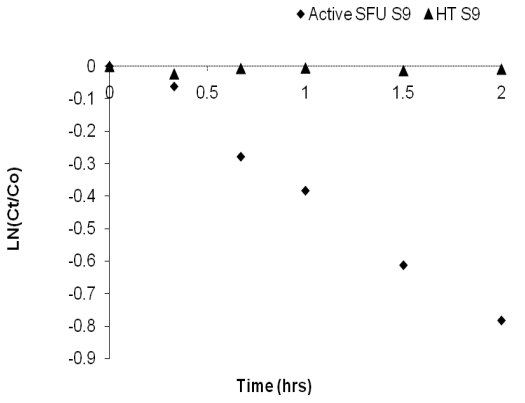

Figure 13: Depletion of 1.5µM pyrene (Replicate 2 of 9)

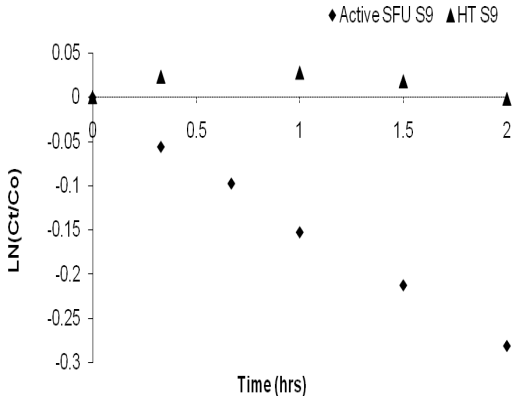

Figure 14: Depletion of 5.0µM pyrene (Replicate 3 of 9)

Incubations were carried out in 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

Table 5: **Depletion rate contants k_{dep} (1/hour) and pyrene half-lives assuming first order rate reaction.**

Nominal Pyrene conc. (µM)	k_{dep} values (Active S9)	Mean k_{dep} values (HT S9)	Half life (hour) (active S9)
0.095	- 1.655 ± 0.090	- 0.014 ± 0.018	0.42
0.284	- 1.440 ± 0.238	0.026 ± 0.033	0.48
0.543	- 1.092 ± 0.233	- 0.012 ± 0.036	0.63
0.926	- 0.789 ± 0.043	0.032 ± 0.006	0.88
1.510	- 0.413 ± 0.037	- 0.009 ± 0.018	1.68
5.220	- 0.132 ± 0.025	- 0.023 ± 0.017	5.25

Table 3 presents the depletion rate constants determined at the six initial pyrene concentrations used in the incubation of pyrene with SFU trout hepatic S9 fraction. The estimated half-lives observed increased with substrate concentration. In Table 3, the negative values of the depletion rate constants signify depletion of pyrene over the period of the test. The depletion rate constants were measured with only the points that were linear in the plots of natural log of pyrene concentration against time. At all initial test concentrations, substrate depletion was linear up to 40 min. At the three highest concentrations, the substrate depletion was linear over the two hours of incubation. Kennedy (1990) observed linearity up to 90minutes in the product formation of pyrene at all measured pyrene concentrations that ranged from 0.5μM to 200μM in trout liver hepatocytes. In the present study, the depletion of 0.52μM pyrene was also observed to be linear up to 90 minutes. The depletion rate constant values for the HT samples that had negative values were subtracted from the k_{dep} values for active hepatic S9 fraction that had similar initial pyrene concentration. The *in vitro* k_{dep} (same as k_m (biotransformation rate constant)) values for active S9 reported in Table 3 were corrected where applicable with HT k_{dep} values. The loss of pyrene in the active SFU trout hepatic S9 fraction at all test concentrations was statistically significant with a P value less than 0.0001.

In the heat treated S9 fraction (HT); at 0.095 μM initial pyrene concentration, the depletion of pyrene was statistically insignificant with a P value of 0.09. k_{dep} for HT was subtracted from the k_{dep} for the active S9 fraction. Loss of pyrene was statistically insignificant in the HT S9 samples at initial pyrene

concentration of 0.28μM. P value was 0.18. i.e P > 0.05. The depletion rate constant of 0.0193 per hour, was not subtracted from the k_{dep} for active S9 because the depletion rate constant is a positive value. Loss of pyrene is statistically insignificant in the HT S9 samples at 0.54μM with a P value of 0.40. However, the depletion rate constant (-0.0001 per hour) was subtracted from the depletion rate constant for the active S9 fraction. The depletion of pyrene was statistically significant in the HT S9 samples at initial pyrene concentration of 0.93μM. The P value for HT was 0.0001 (P < 0.05) and the k_{dep} value for the HT samples at this concentration is 0.032 per hour. However, because this value is positive, it is indicative of a slight increase in the pyrene concentration in the HT samples and not a depletion of the parent chemical. Thus, the k_{dep} was not subtracted from the k_{dep} for the active S9. At an initial pyrene concentration of 1.52μM, the depletion of pyrene was not statistically significant in the HT S9 samples with a P value of 0.068, (P > 0.05). The k_{dep} (-0.0087 per hour) was subtracted from the k_{dep} for the active S9 fraction. At 5.0μM initial pyrene concentration, depletion of pyrene was also not statistically significant in the HT S9 samples (P value was 0.13). The k_{dep} value (-0.0228 per hour) of the HT samples was subtracted from the k_{dep} value obtained for the active S9 fraction.

Enzyme Kinetics

Figure 15 shows the derived depletion rate constant (k_{dep}) plotted against initial pyrene concentration. The depletion rate constant was derived from the slope of the plots of the natural logarithm of depletion of pyrene against time, assuming a first order rate reaction.

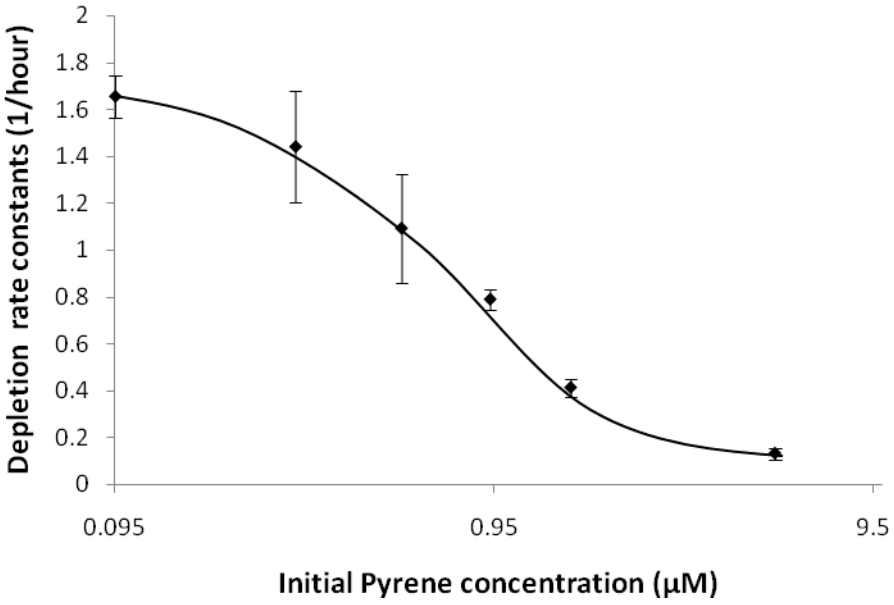

Figure 15: Depletion rate constants of pyrene in SFU trout hepatic S9 fraction at six pyrene concentrations (0.095µM, 0.284µM, 0.543µM, 0.926µM, 1.510µM and 5.220µM).

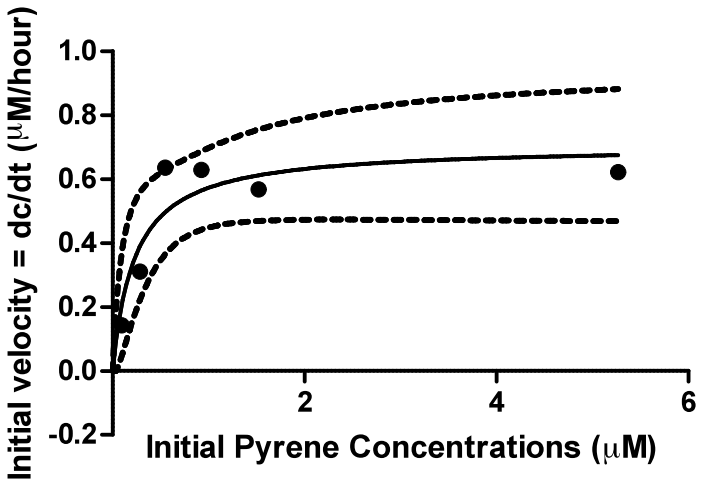

Figure 16: **V_{max} and K_M estimation plotting initial velocity againt pyrene initial concentration using non-linear regression in GraphPad prism. The dotted lines are showing 95% confidence interval.**

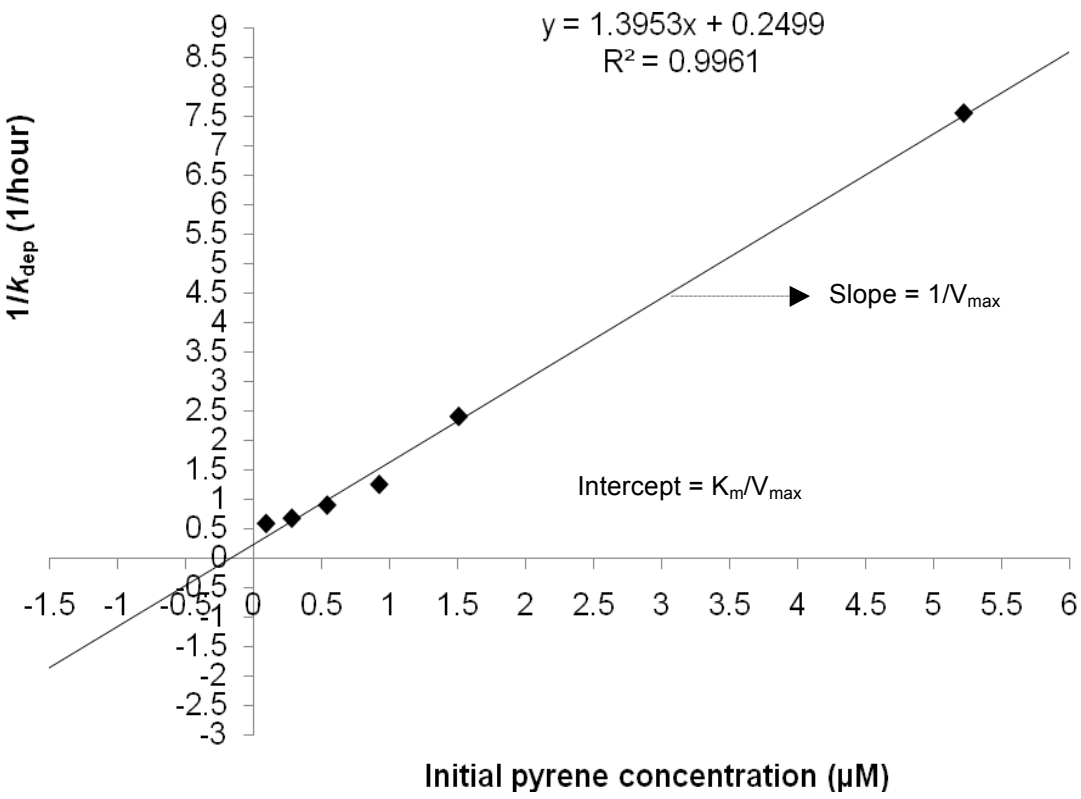

Figure 17: K_m and V_{max} estimation using the derivation method

Table 6: Summary of derived V_{max} and K_m from two methods of estimations.

	Estimated V_{max}		Estimated K_M	
	μM/hr in 2mg protein/ml S9 fraction	μg/min/g liver	μM	μg/ml
Graph Pad Prism non-linear regression Initial velocity method (Fig 16)	0.70 ± 0.09	0.56 ± 0.07	0.23 ± 0.12	0.046 ± 0.02
Linearization of Michaelis-Menten (Fig 17)	0.72	0.56	0.18	0.036
Flounder liver S9 fraction[1]	-	0.254 ± 0.099	-	9.63 ± 3.58

[1] Namdari (1998).

Extrapolation of S9 protein concentration to gram liver was done using the scaling factor described by Han *et al* (2009).

The depletion rate constants (k_{dep}) decreased significantly at the two highest initial concentrations (1.5μM and 5.0μM) of pyrene. The k_{dep} value at the two highest initial pyrene concentrations are approximately 4 to 14 folds lower than the k_{dep} derived at 0.095μM pyrene concentration, this signifies the possibility that there is a degree of saturation of one or more enzymes at these concentrations (Ionescu and Caira, 2005).

Figure 16 shows the nonlinear regression plot of initial velocity against initial pyrene concentrations using Prism graphpad version 5. The estimated values for V_{max} was 0.70 ± 0.09 μM/hour with a 95% confidence interval of 0.46 to 0.95 μM/hour and K_M was 0.23 ± 0.12 μM with a 95% confidence interval of 0.15 to 0.89μM in 2mg protein/ml of SFU S9 fraction.

The linearized form of the Michaelis-Menten equation that was used to estimate the V_{max} and K_M (details of the derivation is in Materials and Methods under the Enzyme kinetics section) gave similar results as the initial velocity approach. Assuming a first order rate reaction, the k_{dep} values from the six meassured incubation concentrations of pyrene were used to derive V_{max} and K_M values by plotting $1/k_{dep}$ against pyrene concentration (see Figure 18). V_{max} and K_m were found to be 0.72 μM/hour and 0.18 μM respectively in 2mg protein S9 fraction.

Table 6 shows the summary of the V_{max} and K_M values derived from the linearized approach and Initial velocity method used to estimate the Michaelis-Menten constants of pyrene in trout liver S9 fraction. The differences in the estimated values are statistically insignificant with P values greater than 0.05

between the methods. The V_{max} and K_M values estimated from the linearized approach and the initial velocity method were within 1.02 to 1.28 fold.

The values estimated using these methods are also in agreement with observations made in the test. The depletion rates measured from change in concentration over time (20 minutes) gave reaction rates of 0.1420 μM/hour, 0.3110 μM/hour, 0.6360 μM/hour, 0.6290 μM/hour, 0.5678 μM/hour and 0.6212 μM/hour at pyrene concentrations of 0.095 μM, 0.284 μM, 0.547 μM, 0.926 μM, 1.521 μM and 5.267 μM respectively. Therefore, the depletion rate reached its maximum rate at 0.547 μM initial pyrene concentration at a rate of 0.6360 μM/hour in 2mg protein/ml of SFU trout S9.

K_m and V_{max} values for pyrene in the SFU trout hepatic S9 fraction were 0.23 ± 0.12 μM/hour in 2mg protein/ml of SFU S9 fraction (0.046 ± 0.02μg/ml) and 0.70 ± 0.09μM/hr/2mg protein/ml of SFU S9 fraction (0.56 ± 0.07μg/min/g of liver).

Namdari (1998) reported K_m and V_{max} values of 9.63 ± 3.58 μg/ml and 0.254 ± 0.099 μg/min/g liver respectively for pyrene metabolism by hepatic S9 fraction from flounders. Though the estimated V_{max} reported in the present study is approximately 2 fold higher than what Namdari (1998) observed in the flounder, the K_m estimated in the present study were much lower than the values reported in Namdari's study. The lower K_m observed in the present study suggests that the rainbow trout enzymes involved in metabolizing pyrene may have a higher affinity for pyrene than the enzymes in flounder.

Kennedy (1990) reported a K_m of 2.52 – 2.9 µg/ml, and Law *et al* (1991) a V_{max} of 0.255 µg/min/g liver for metabolism of pyrene in trout liver hepatocytes. The V_{max} (maximum reaction rate) observed in the present study is about 2.5 fold faster than reported for the trout hepatocyte; this might be due to the fact that the enzymes in the S9 fraction are not membrane bound and hence have higher accessibility to the substrate. The K_m estimated for the hepatic S9 fraction is significantly lower than what was reported by Kennedy 1990 indicating that the trout hepatic S9 fraction has a higher affinity for pyrene than hepatocytes. Carpenter *et al* 1990 reported K_m values of 2.2 - 64µM for Benzo(a)pyrene in trout microsomes. These values are much higher than what was observed in the present study. Fitzsimmons *et al*, 2007 found that the K_m values for the same substrate varied among different fish species and strains. The K_m measured with this method is a combination of the K_m of different enzymes present in the hepatic S9 fraction.

The reaction rates measured between 0 and 20 minutes were 0.114µg/min/g liver, 0.249 µg/min/g liver, 0.508 µg/min/g liver, 0.503 µg/min/g liver, 0.454 µg/min/g liver and 0.4965 µg/min/g liver at initial pyrene concentrations of 0.095 µM, 0.284 µM, 0.547 µM, 0.926 µM, 1.521 µM and 5.267 µM respectively. The fastest reaction rate was reached at 0.547µM. In a first order reaction, the reaction rate is expected to increase with increase in substrate concentration i.e., when substrate concentration is doubled, the reaction rate is doubled (Ionescu and Caira, 2005). In the present study, doubling was observed from initial pyrene concentration of 0.095 ± 0.005 µM to 0.547 ±

0.036 μM. However, saturation of one or more enzymes involved in the biotransformation of pyrene occurred somewhere between initial pyrene concentration of 0.547μM and 5.2μM. It is also likely that mass transfer is the cause of the lower biotransformation rates observed at the higher concentrations of pyrene studied in the present study. An increase in the enzyme concentration at the same pyrene concentrations will bring more clarity to the observations made at the higher concentrations of pyrene used in this study.

Repeatability of the *in vitro* method in estimating k_{dep}

The inter-day and intra-day repeatability of this *in vitro* method was investigated by comparing the k_{dep} values between replicate incubations performed the same day and incubations carried out on different days.

Figures 19, 20 and 21 show the plot of the k_{dep} values of replicate 1 incubations against replicate 2 incubation samples, replicate 1 incubations against replicate 3 incubation samples, and replicate 2 incubations against replicate 3 incubation samples. The regression coefficients were 0.94, 0.90 and 0.96 between replicates 1 & 2, replicates1 &3, and replicates 2 & 3 respectively. These regression coefficients indicate that there is a high correlation between the k_{dep} values estimated for different incubation replications.

To assess the repeatability of the method intra-day; an analysis of variance (ANOVA) of the k_{dep} values for replicates at the six concentration of pyrene (0.095μM, 0.284μM, 0.543μM, 0.926μM, 1.51μM and 5.22μM pyrene) were analyzed using the k_{dep} values estimated for three replicates performed on

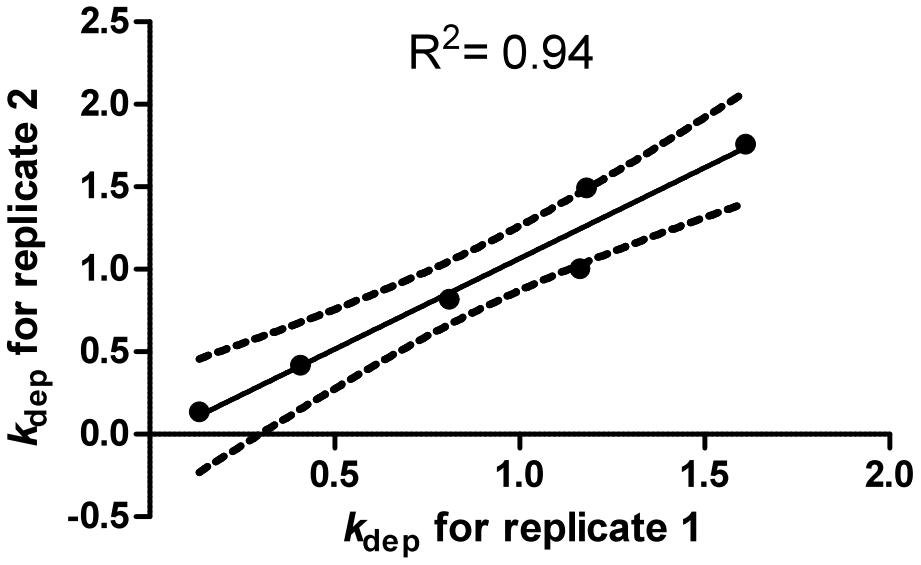

Figure 18: Comparison of k_{dep} values between Rep 1 and Rep 2 at six concentrations (0.1µM, 0.3µM, 0.5µM, 1.0µM, 1.5µM and 5µM pyrene) with the SFU hepatic S9 fraction. Dotted lines represent 95% confidence interval.

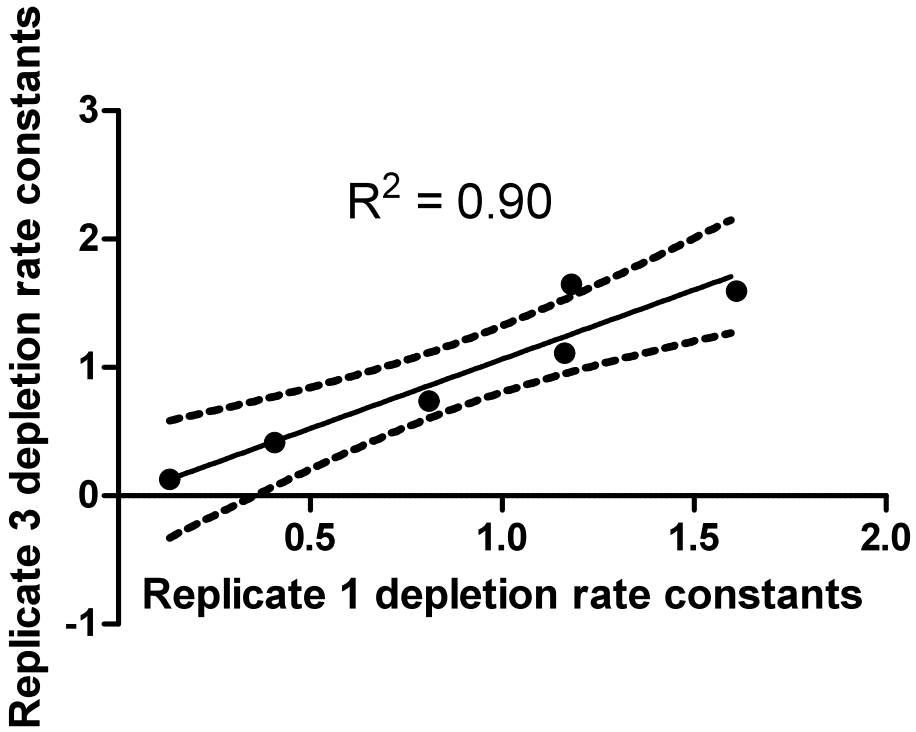

Figure 19: **Comparison of k_{dep} values between Rep 1 and Rep 3 at six concentrations (0.1µM, 0.3µM, 0.5µM, 1.0µM, 1.5µM and 5µM pyrene) with the SFU trout hepatic S9 fraction. Dotted lines represent 95% confidence interval.**

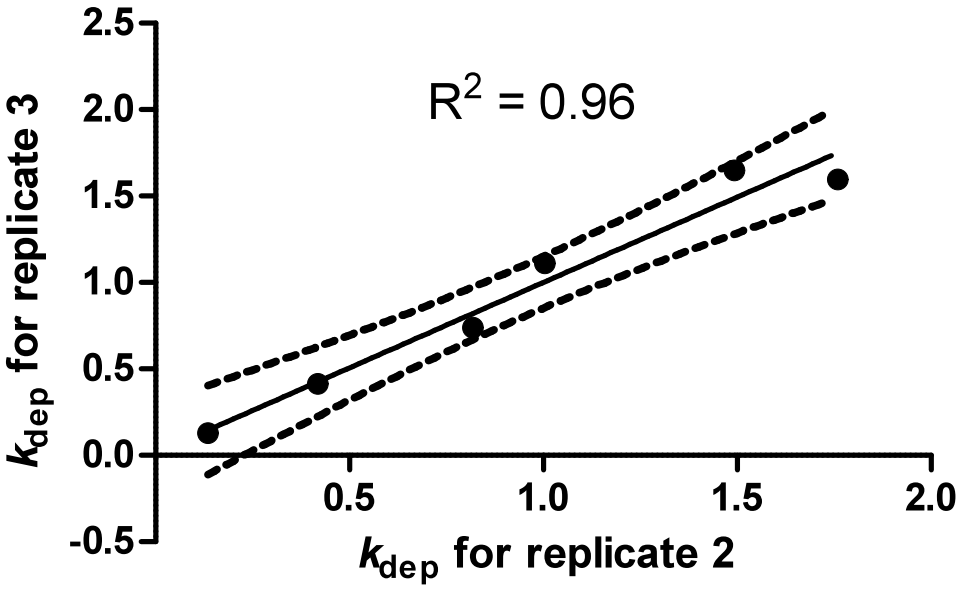

Figure 20: **Comparison of k_{dep} values between Rep 2 and Rep 3 at six concentrations (0.1µM, 0.3µM, 0.5µM, 1.0µM, 1.5µM and 5µM pyrene) with the SFU trout liver S9 fraction. Dotted lines represent 95% confidence interval.**

the same day. The P value was 0.99; hence, there was no difference in replicate incubations performed on the same day from the same defrosting of S9 fraction.

The assessment of the inter-day repeatability of the *in vitro* method was analyzed using the mean k_{dep} values from incubations carried out on three independent incubation days (each incubation day has replicate samples) with different defrosting of the same pool of trout liver S9 fraction. An analysis of variance gave a P value of 0.95, which implies that the differences in the estimated k_{dep} values are not statistically significant on different incubation days. However, it should be noted that that variability in the estimated k_{dep} values increase as the depletion rate constant increases. It appears that the variability in the estimated k_{dep} values between replicate incubations increases once k_{dep} exceeds 1.0 per hour.

Application of the *in vitro* method

REACH considers a BCF value ≥ 5000 as very bioaccumulative (European Commission 2003). Canada uses the Stockholm Convention Criteria, which considers a BCF value ≥ 5000 as bioaccumulative (UNEP, 2006). Using the Arnot and Gobas (2004) BCF model (see Figure 21) without incorporating *in vivo* metabolic transformation rate constants, the calculated BCF value of pyrene is 6745. In the case of pyrene, extrapolation of the depletion rate constants measured in this study to *in vivo* rates using extrapolation models, and then incorporating these values into predictive models will reduce the estimated BCF values lower than 5000. The results from this study suggest that pyrene will likely

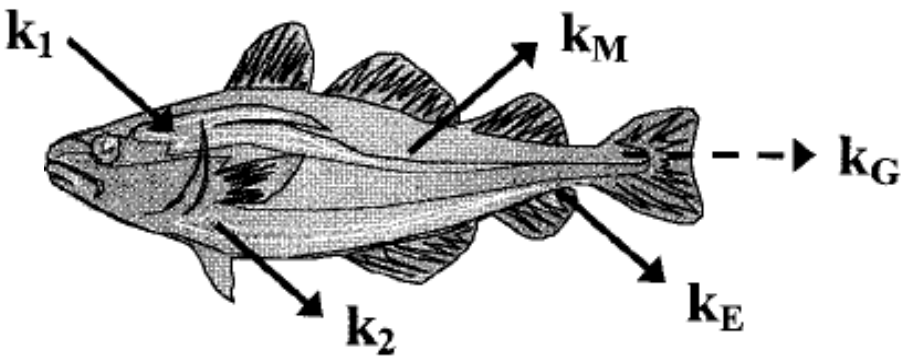

Figure 21: Application of depletion rate constants

- BCF = k_1 / (k_2 + k_E + k_G + k_M)
    - k_1= gill uptake rate constant
    - k_2= gill elimination rate constant
    - k_E = elimination rate through fecal egestion
    - k_G = growth dilution rate constant
    - ***k_m = in vivo metabolic transformation rate constant***

Arnot and Gobas (2004).

not bioaccumulate in trout because of its rapid hepatic biotransformation. If the *in vitro* depletion rate constants are extrapolated into *in vivo* biotransformation rates, and used to estimate the BCF value of pyrene, I anticipate that pyrene will have a much lower BCF value compared to the BCF value of 6745 when K_{met} has a value of zero. This assumption agrees with *in vivo* BCF values of 457 and 50 – 1479 estimated for pyrene by Ogata *et al* (1984) in gold fish and Jonsson *et al* (2004) in sheephead minnow, respectively.

This method can be useful in providing information on the metabolic biotransformation potentials of chemicals in a short period. It requires a short incubation time, is less labour and cost intensive, and hepatic S9 fraction can be cryopreserved.

Merits of the *in vitro* approach

Table 7 shows the differences between OECD 305, an *in vivo* method, and the S9 fraction *in vitro* approach. The approach of measuring biotransformation rates using hepatic S9 fraction includes both phase I and phase II enzymes and uses less animals than required by OECD 305 method (OECD 305 1996). The S9 fraction can easily be preserved, and the results compared to wildlife sources (Brandon *et al*, 2003).

Fish as the source of the livers used in this test is important because fish are a vital component of human diet and hence are a potential means through which humans can be exposed to environmental chemicals (Wolf *et al*, 2007).

Table 7: Comparison of OECD 305 method and liver S9 approach

	OECD 305 method (***in vivo*** **approach)**	**S9 approach** (***in vitro*** **method)**
Number of fish required per study.	Hundreds of fish is needed per study	As low as one fish can be used but to minimize individual variability effect, five fish were used for the SFU S9.
Average cost per study	$80,000 to $120,000	$30,000. Becomes cheaper as the number of chemicals to be studied increases.
Duration of test	Average of 40 days	A day
Labour need	Labour intensive	Less labour intensive

The use of fish as the test organism gives information about how mammals will metabolise the chemical since fish have similar metabolizing enzymes as mammals despite the lower activity (Kennedy *et al*. 1991; Foureman. 1989). Phase II enzymes of fish have lower activity when compared in general with terrestrial species (Gregus *et al* 1983), and their activities are substrate dependent (Gregus *et al*. 1983).

Though Dyer *et al* (2003) reported inconsistency in the results in incubations performed with hepatic S9 fraction; the present study observed a high degree of consistency in the metabolic rates measured inter-day and intra-day. However, the study by Dyer *et al* did not indicate if all their studies were performed with a single pool of S9 fraction as done in this study.

Limitations of approach

Some uncertainties are associated with the *in vitro* estimation of whole animal metabolic rates (Coecke *et al* 2006). A limitation of this approach is the lack of measurement of the metabolites of pyrene. Measurements of metabolites are important for *in vitro* studies because some metabolites of xenobiotics are known at times to be more harmful than the parent compound (Varanasi and Stein 1991). Though not as important with pyrene, because the metabolism of pyrene has been previously well documented in a variety of organisms, measuring metabolites can be very important when studying novel compounds. Measurement of metabolites is not necessary for the estimation of BCF values,

the measurement of metabolites is time consuming, and expensive. However, it has to be done for toxicity assessment.

Fish hepatic S9 has been found not metabolizing of some chemicals that are metabolized by hepatocytes and microsomes. For example, Dyer *et al.* (2003) studied Octaethylene glycol monotridecyl ether; and observed depletion of the substrate in hepatocytes but found no loss in the active S9 fraction. Hepatocytes and microsomes are generally reported to have higher specific enzyme activity than S9 fractions (Dyer *et al.* 2003, Fitzsimmons *et al* 2007, Han *et al.* 2009). Uptake rate is difficult to measure in both S9 fraction and microsomes but microsomes also limited to phase I metabolizing enzymes, which limits their use to the study of only phase I metabolic reactions (Dyer *et al.* 2003). Hepatocytes are limited by their short life span and a rapid loss of enzymatic activity (Dyer *et al.* 2003).

Recommendation and Conclusion

The approach used in this study is promising, but more chemicals with varied chemical characteristics still need to be analyzed to validate the method. There is also a need to investigate variability in S9 activity associated with different trout strains and different preparations of S9 to establish benchmarks that can guide the use of this method. A guideline needs to be in place that clearly states criteria that need to be met before hepatic S9 system is used for metabolic studies. This criteria should include but not be limited to the characterization of S9 fraction with established methods, inclusion of positive controls (using a known substrate with known activity), and establishing

standardized methods and procedures for excising livers and preparing, quantifying and storing the hepatic S9 fraction. If standardized procedures are not established, differences in metabolic activities observed at different locations with the same or different substrates, might arise due to differences in preparations and experimental conditions.

From the present studies, it appears that freezing fish livers before preparing hepatic S9 fraction reduces the activity of key enzymes involved in the biotransformation of certain substrates such as pyrene. As observed by Pederson *et al* (1974), the optimal experimental conditions suitable for *in vitro* studies with hepatic S9 fractions for mammals may be quite different for fish. Hence, care must be taken when designing fish in vitro experiments based on mammalian studies.

The present study is a part of a larger project between various labs in and outside Canada. The project as a whole is studying 21 diverse chemicals with pools of S9 prepared at different labs involved in the study. The results from these studies will ultimately shed more light on the acceptability of this method of estimating metabolic transformation rates of environmentally relevant chemicals.

APPENDICES

APPENDIX A

Preparation of the homogenization buffer for S9 fraction preparation.

To make the homogenization buffer (50 mM Tris, pH 7.8 @ 4°C, 150 mM KCl, 2 mM EDTA, 1 mM DTT);1M Tris-HCl pH 7.8, 1M potassium chloride, 1M potassium hydroxide, and 100mM DTT (dithiothreitol) were first prepared.

100mL 1M Tris-HCl (molecular weight = 157.60g/mol, purity is ≥ 99%) was prepared by diluting 15.76g of Tris-HCL in Type I water. 1N potassium hydroxide was then used to bring the solution to a pH of 7.8. To achieve 50 mM Tris, 50 mL of the 1M Tris-HCl was diluted in 950 mL of ultrapure water (Type I water). 1M potassium chloride (Molecular weight: 74.55g/mol, Purity is ≥ 99%) was then prepared by weighing 14.91g potassium chloride into 200mL of ultrapure water. To make 1M potassium hydroxide (74.55g/mol, Purity is ≥ 85%), 5.62g of potassium hydroxide was dissolved in 100mL of ultrapure water. The next solution that was made was 100mM dithiothreitol (molecular weight: 154.25 g/mol, purity was ≥ 99%) by dissolving 0.15425g in 10mL of ultra pure water. 0.5M ethylenediaminetetraacetic acid (molecular weight*:* 336.21) solution was purchased from Sigma chemical company. To make 1 litre of the homogenization buffer, 800 mL of 50 mM Tris was added to a 1 Litre volumetric flask followed by 150 mL of 1 M KCl, 4 mL of 0.5 M EDTA, 10 mL of 100 mM DTT and it was then adjusted to pH 7.8 with 1 M KOH. The buffer was then brought up to volume (1 litre) with 50mM Tris.

APPENDIX B.

Table 8: Protein determination analysis using the Sigma BCA kit (catalogue reference: QPBCA). The volume of each reagent added to each test vial for protein analysis is shown.

	Water	Std. Protein Soln.	S9 fraction	BCA Reagent	Protein conc.
Sample name	Type I (µl)	(50µg/ml) (µl)	(µl)	(µl)	(µg/ml)
Std 0	1000	0	0	1000	0
Std 0.5 µg/ml	990	10	0	1000	0.5
Std 5 µg/ml	900	100	0	1000	5
std 10 µg/ml	800	200	0	1000	10
std 20 µg/ml	600	400	0	1000	20
std 30 µg/ml	400	600	0	1000	30
S9 dilution x1	0	0	1000	1000	unknown
S9 dilution x2	500	0	500	1000	unknown
S9 dilution x10	900	0	100	1000	unknown

“Std protein soln” is the standard protein solution supplied by Sigma to derive a standard curve from which the protein concentration of the S9 fraction could be compared.

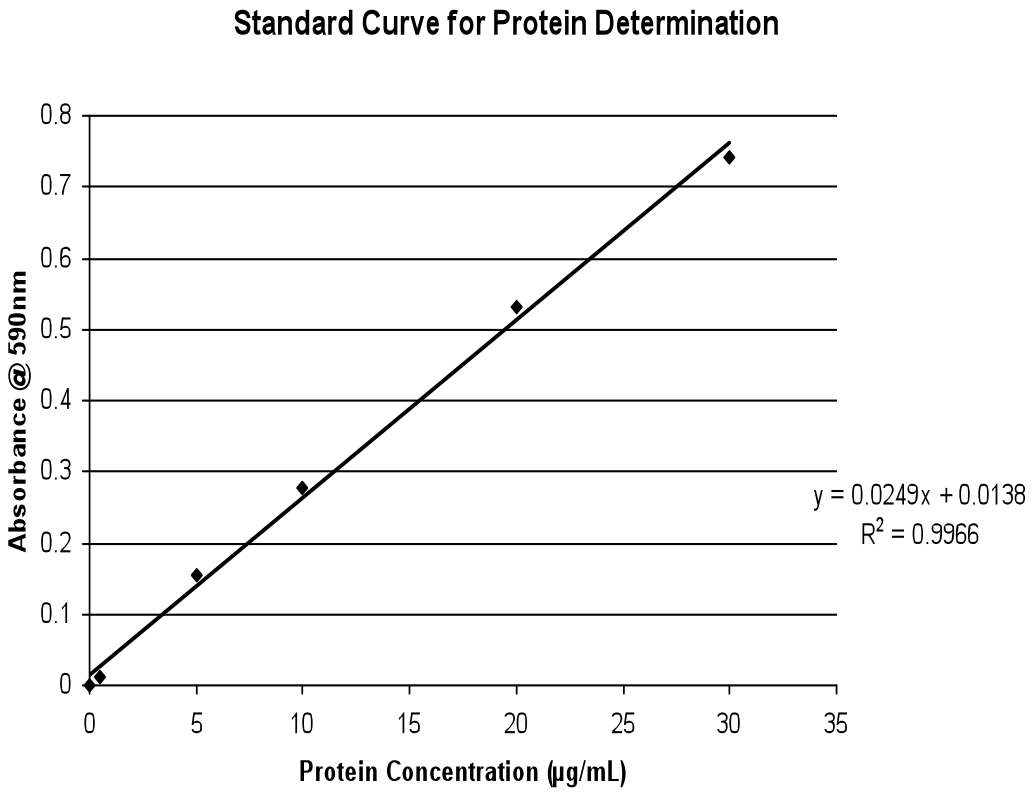

Figure 22: Standard Curve for protein determination of SFU S9 using the Sigma BCA kit.

APPENDIX C

Preparation of solutions for chemical incubation with the S9 fraction.

The volumes listed below were used; however, the recipes were scaled where and when necessary. Appropriate weight to volume of the reagent and solvent that achieved the desired concentration was also used.

100 mM potassium phosphate monobasic:

One hundred millimolar potassium phosphate monobasic was prepared by dissolving 2.72 g of potassium phosphate monobasic in 200 mL of ultra pure water.

100 mM potassium phosphate dibasic:

One hundred millimolar potassium phosphate dibasic was prepared by dissolving 3.48 g in 200mL of ultra pure water.

Phosphate Buffer:

To prepare 100 mM of the phosphate buffer at a pH of 7.8, appropriate volumes of 100 mM potassium phosphate monobasic and 100 mM potassium phosphate dibasic were mixed. The ratio was usually approximately 9:1; dibasic: monobasic. For example, 900 mL of 100 mM potassium phosphate dibasic and 100 mL of 100 mM potassium phosphate monobasic in a 1 L volumetric flask. This ratio was not fixed, it varied. The pH of the mixture was confirmed with a pH meter.

250 µg/ml Alamethicin:

0.5 mg of alamethicin (purchased from Sigma-aldrich) was dissolved in 0.5 mL of methanol. After dissolution, 1.5 mL of phosphate buffer was added to make 2 mL of 250 µg/mL alamethicin. The ratio of methanol to phosphate was 1:3.

10mM Nicotinamide adenine dinucleotide 2'-phosphate, reduced (NADPH):

NADPH was purchased from Sigma-aldrich. NADPH (1.67mg) was dissolved in 2 mL phosphate buffer.

20mM Uridine 5'-phosphoglucuronic acid (UDPGA): UDPGA (25.85mg) was weighed out and dissolved in 2 mL of phosphate buffer. UDPGA was purchased from Sigma-Aldrich.

1mM 3'-Phosphoadenosine 5'-phosphosulfate (PAPS): PAPS (1.02mg) was dissolved in 2 mL of phosphate buffer. PAPS was purchased from Sigma-Aldrich.

Deactivated S9 fraction (heat treated):

S9 fraction heated in boiling water @ 100 ± 5°C for a minimum of 10 minutes.

APPENDIX D

Table 9: **The concentration of pyrene remaining in test sample at different sampling times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 0.095µM pyrene.**

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	0.0948 ± 0.0053	0.0942 ± 0.0029
20	0.0480 ± 0.0048	Not done
40	0.0313 ± 0.0033	Not done
60	0.0254 ± 0.0028	0.0929 ± 0.0017
90	0.0239 ± 0.0023	Not done
120	0.0219 ± 0.0022	0.0930 ± 0.0009

Values represent mean ± standard deviation of n = 3

“Not done” means that there were no HT samples at the time points.

Table 10: The concentration of pyrene remaining in test sample at different samplying times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 0.284µM pyrene.

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	0.2839 ± 0.0064	0.2902 ± 0.0063
20	0.1814 ± 0.0124	Not done
40	0.1114 ± 0.0283	Not done
60	0.0693 ± 0.0237	0.2981 ± 0.0105
90	0.0459 ± 0.0124	Not done
120	0.0335 ± 0.0136	0.3018 ± 0.0125

Values represent mean ± standard deviation of n = 3

“Not done” means that there were no HT samples at the time points.

Table 11: The concentration of pyrene remaining in test sample at different samplying times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 0.547µM pyrene.

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	0.5470 ± 0.0355	0.5600 ± 0.0172
20	0.3370 ± 0.0298	0.5480 ± 0.0225
40	0.2091 ± 0.0551	0.5561 ± 0.0267
60	0.1556 ± 0.0295	0.5471 ± 0.0256
90	0.1064 ± 0.0247	0.5583 ± 0.0246
120	0.0828 ± 0.0303	0.5541 ± 0.0239

Values represent mean ± standard deviation of n = 9

Table 12: The concentration of pyrene remaining in test sample at different samplying times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 0.926µM pyrene.

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	0.9257 ± 0.0312	0.9557 ± 0.0273
20	0.7181 ± 0.0441	Not done
40	0.5064 ± 0.0079	Not done
60	0.3822 ± 0.0279	0.9937 ± 0.0031
90	0.2687 ± 0.0149	Not done
120	0.1962 ± 0.0230	1.0183 ± 0.0170

Values represent mean ± standard deviation of n = 3
“Not done” means that there were no HT samples at the time points.

Table 13: The concentration of pyrene remaining in test sample at different samplying times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 1.52 µM pyrene.

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	1.5212 ± 0.0589	1.6011 ± 0.0230
20	1.3338 ± 0.1190	1.6065 ± 0.0544
40	1.1158 ± 0.0731	1.5800 ± 0.0540
60	0.9589 ± 0.0678	1.6053 ± 0.0526
90	0.7959 ± 0.0531	1.5973 ± 0.1085
120	0.6584 ± 0.0812	1.5674 ± 0.0536

Values represent mean ± standard deviation of n = 9

Table 14: The concentration of pyrene remaining in test sample at different sampling times over 2 hours incubation with SFU S9 at 12ºC with 2mg protein/ml trout liver S9 fraction. Initial pyrene concentration was 5.27 µM pyrene.

Time (mins)	Active S9 (µM)	Heat treated S9 (µM)
0	5.2667 ± 0.1629	5.5497 ± 0.2101
20	5.0617 ± 0.0983	5.4587 ± 0.1321
40	4.7311 ± 0.2587	5.6048 ± 0.2361
60	4.5577 ± 0.2640	5.4152 ± 0.1799
90	4.2524 ± 0.2314	5.4975 ± 0.3084
120	3.8391 ± 0.2774	5.2674 ± 0.2540

Values represent mean ± standard deviation of n = 9

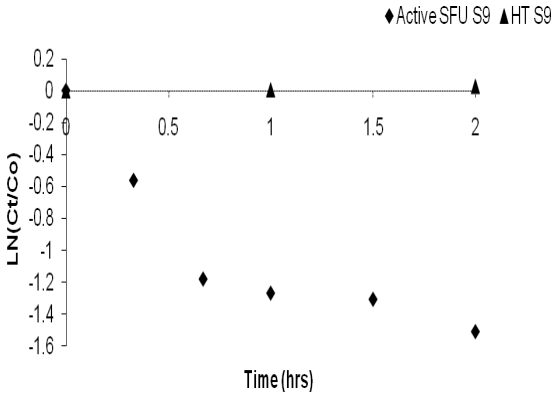

Figure 23: Depletion of 0.1 µM pyrene (Replicate 2 of 3)

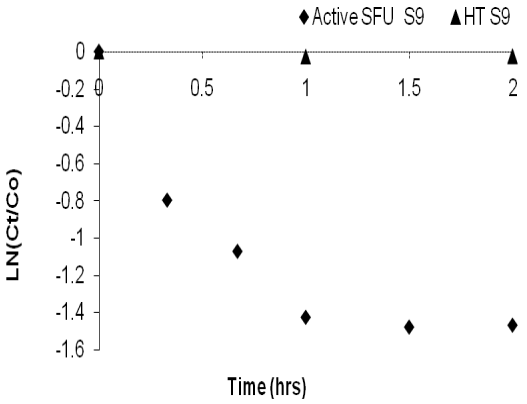

Figure 24: Depletion of 0.1 µM pyrene (Replicate 3 of 3)

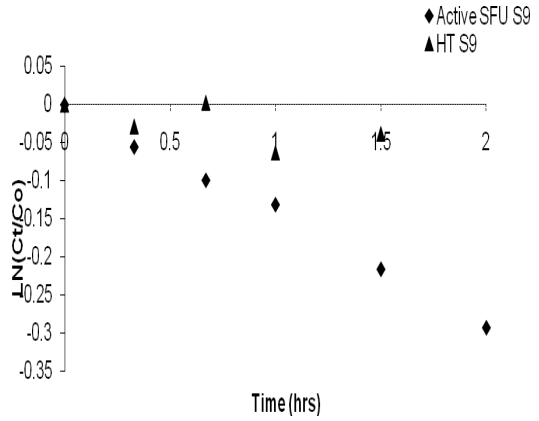

Figure 25: Depletion of 0.3µM pyrene (Replicate 2 of 3)

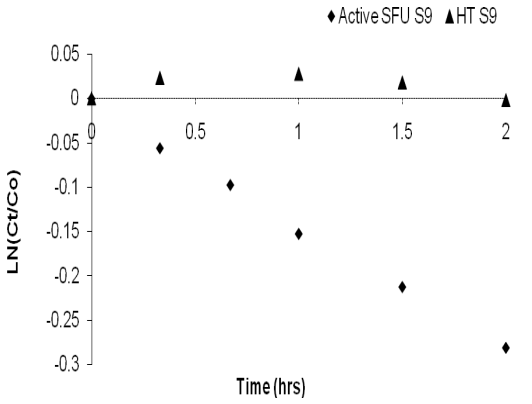

Figure 26: Depletion of 0.3µM pyrene (Replicate 3 of 3)

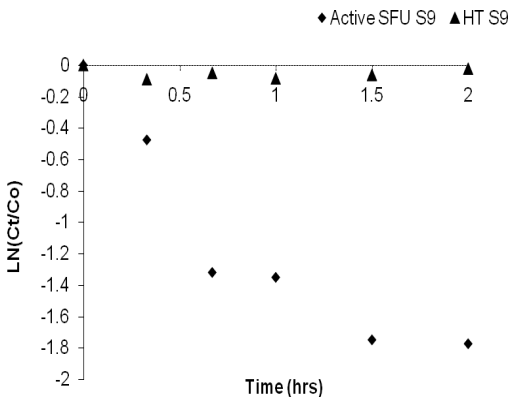

Figure 27: Depletion of 0.5µM pyrene (Replicate 2 of 9)

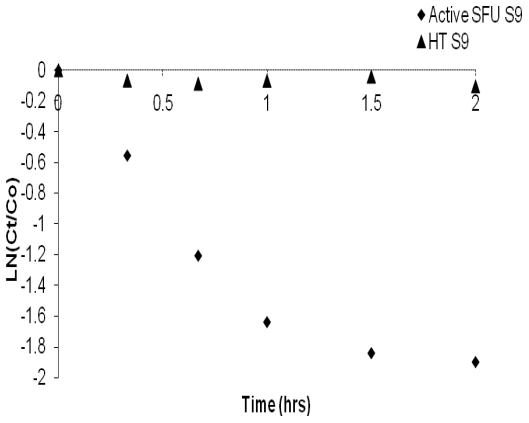

Figure 28: Depletion of 0.5µM pyrene (Replicate 3 of 9)

The above Figures represent replicate runs of the data presented in the text, the means are shown in Tables 9-14. Incubations were performed with 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

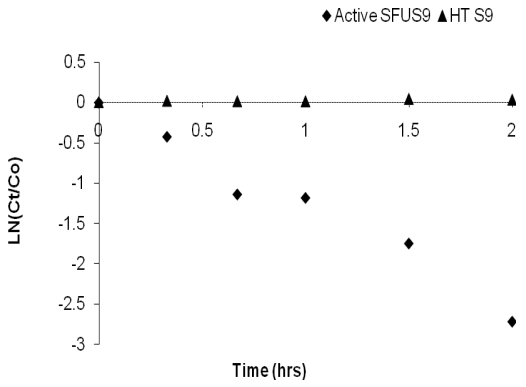

Figure 29: Depletion of 0.5µM pyrene (Replicate 4 of 9)

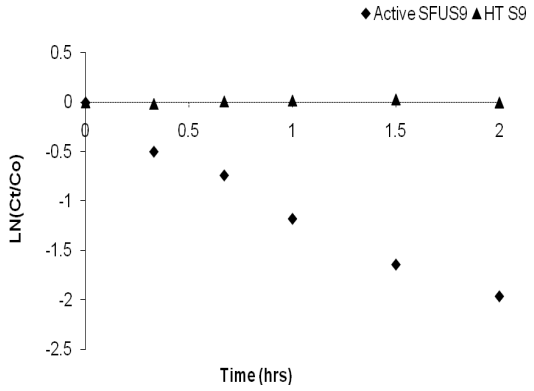

Figure 30: Depletion of 0.5µM pyrene (Replicate 5 of 9)

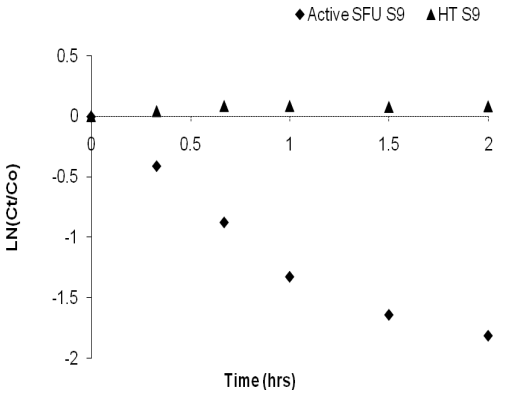

Figure 31: Depletion of 0.5µM pyrene (Replicate 6 of 9)

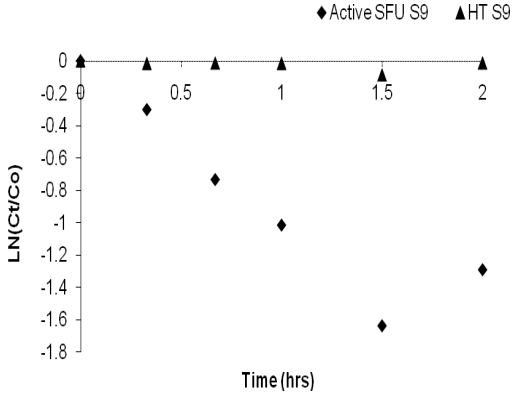

Figure 32: Depletion of 0.5µM pyrene (Replicate 7 of 9)

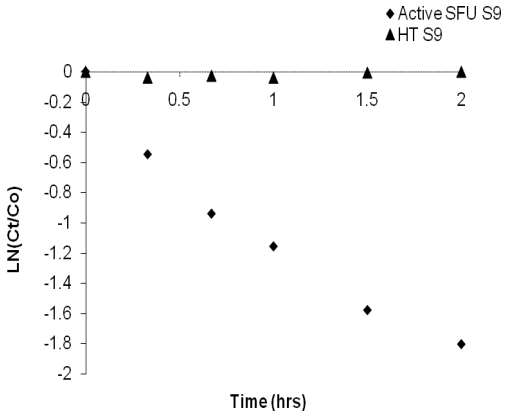

Figure 33: Depletion of 0.5µM pyrene (Replicate 8 of 9)

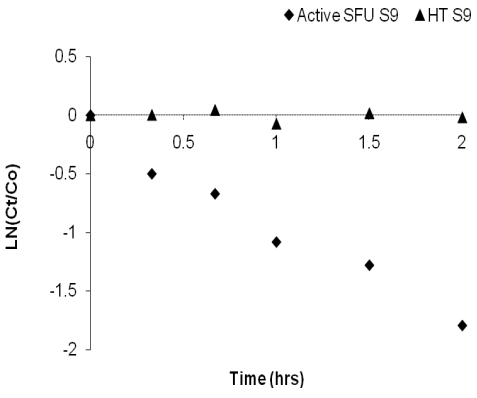

Figure 34: Depletion of 0.5µM pyrene (Replicate 9 of 9)

The above Figures represent replicate runs of the data presented in the text, the means are shown in Tables 9-14. Incubations were performed with 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

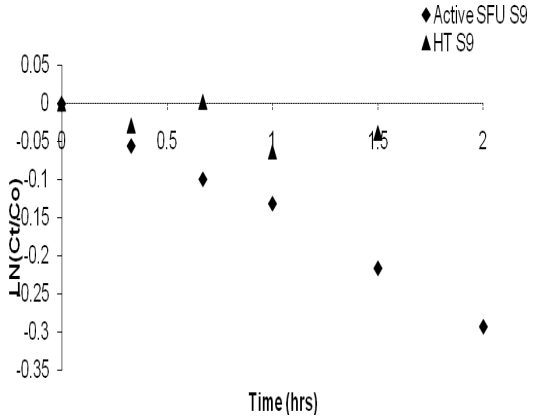

Figure 35: Depletion of 1.0µM pyrene (Replicate 2 of 3)

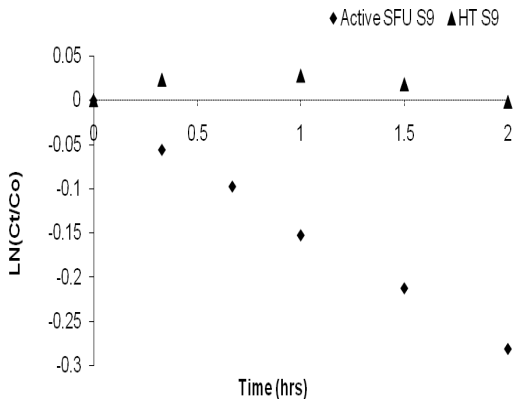

Figure 36: Depletion of 1.0µM pyrene (Replicate 3 of 3)

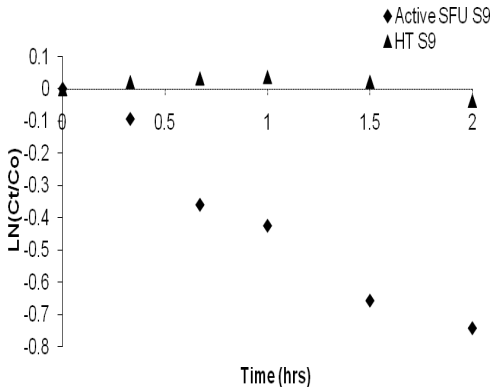

Figure 37: Depletion of 1.5µM pyrene (Replicate 1 of 9)

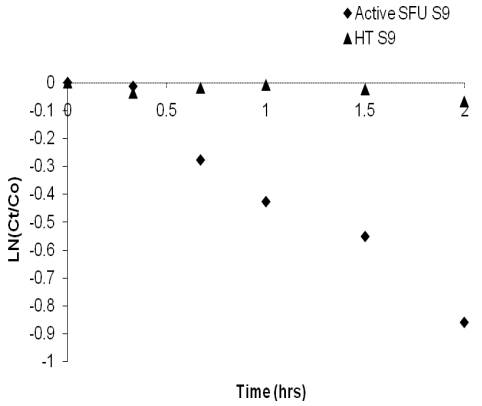

Figure 38: Depletion of 1.5µM pyrene (Replicate 3 of 9)

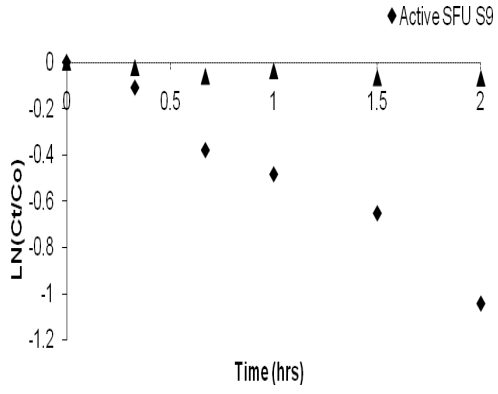

Figure 39: Depletion of 1.5µM pyrene (Replicate 4 of 9)

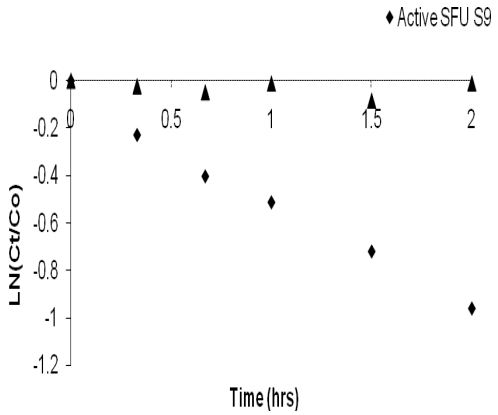

Figure 40: Depletion of 1.5µM pyrene (Replicate 5 of 9)

The above Figures represent replicate runs of the data presented in the text, the means are shown in Tables 9-14. Incubations were performed with 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

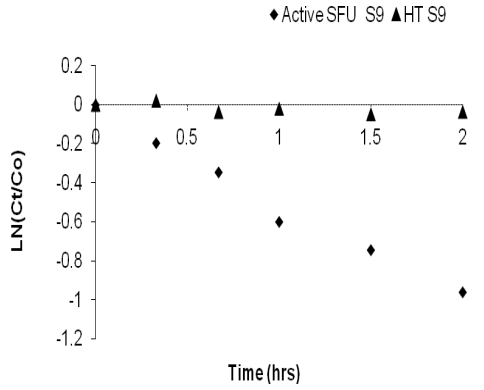

Figure 41: Depletion of 1.5µM pyrene (Replicate 6 of 9)

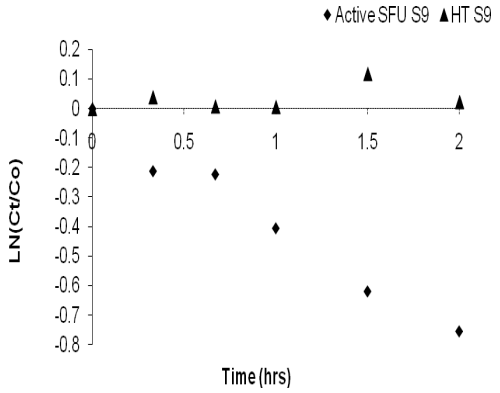

Figure 42: Depletion of 1.5µM pyrene (Replicate 7 of 9)

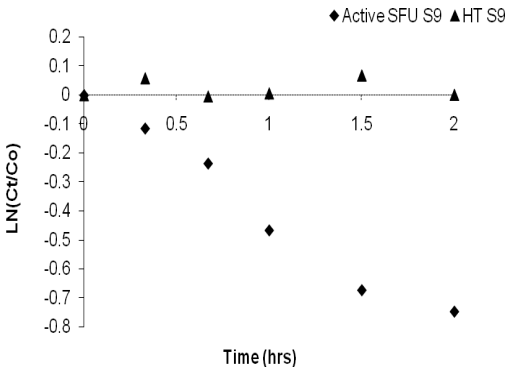

Figure 43: Depletion of 1.5µM pyrene (Replicate 8 of 9)

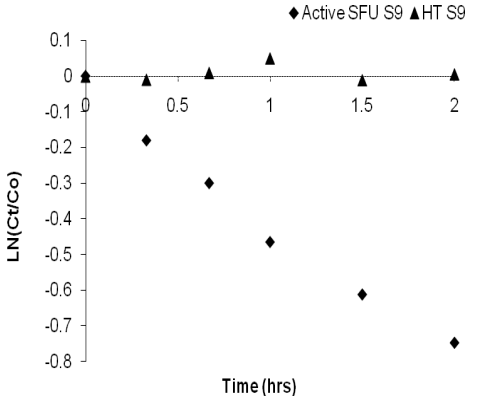

Figure 44: Depletion of 1.5µM pyrene (Replicate 9 of 9)

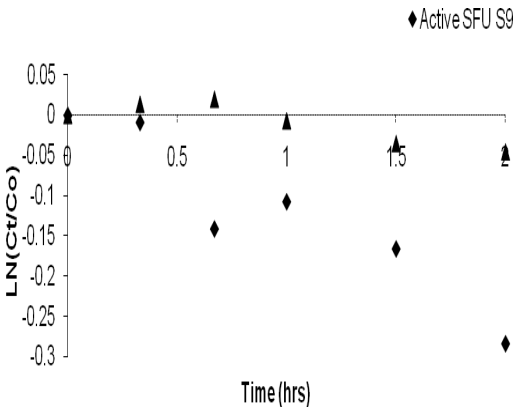

Figure 45: Depletion of 5.0µM pyrene (Replicate 1 of 9)

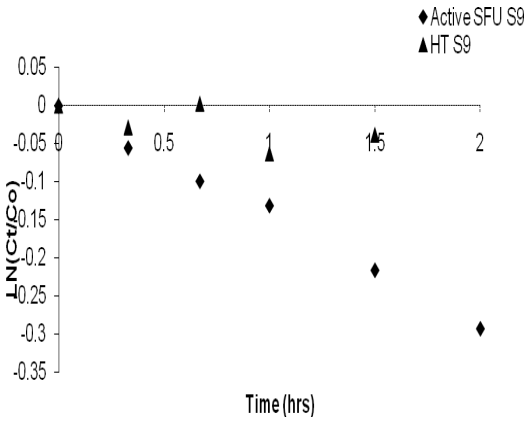

Figure 46: Depletion of 5.0µM pyrene (Replicate 2 of 9)

The above Figures represent replicate runs of the data presented in the text, the means are shown in Tables 9-14. Incubations were performed with 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

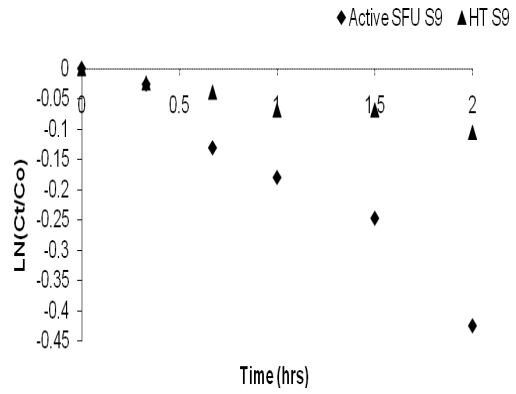

Figure 47: Depletion of 5.0µM pyrene (Replicate 4 of 9)

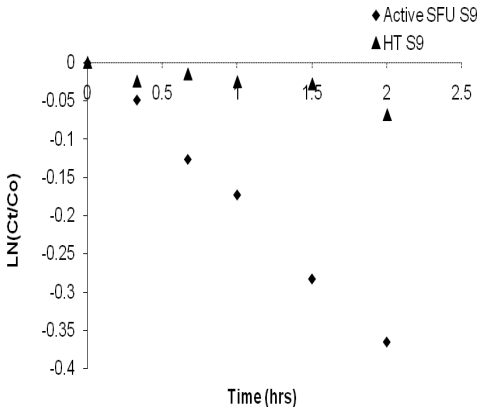

Figure 48: Depletion of 5.0µM pyrene (Replicate 5 of 9)

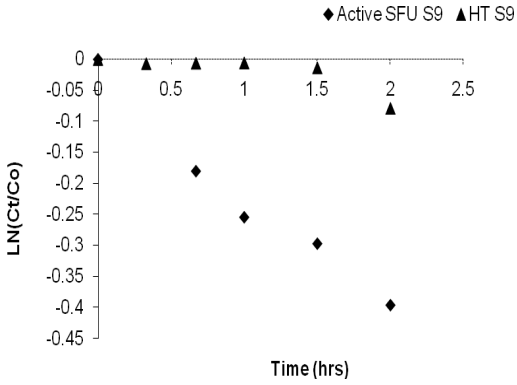

Figure 49: Depletion of 5.0µM pyrene (Replicate 6 of 9)

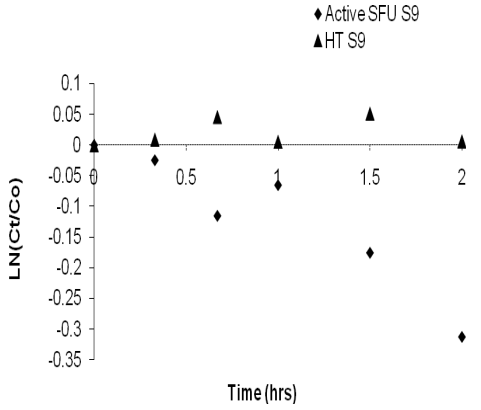

Figure 50: Depletion of 5.0µM pyrene (Replicate 7 of 9)

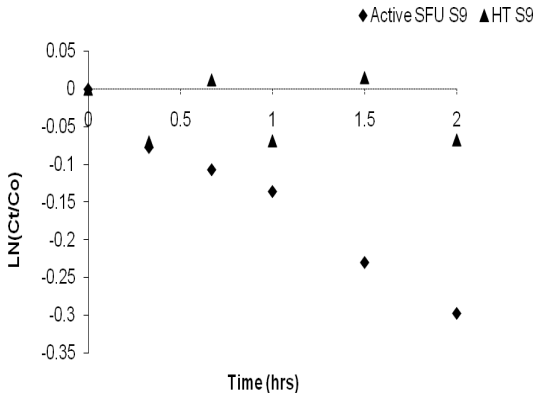

Figure 51: Depletion of 5.0µM pyrene (Replicate 8 of 9)

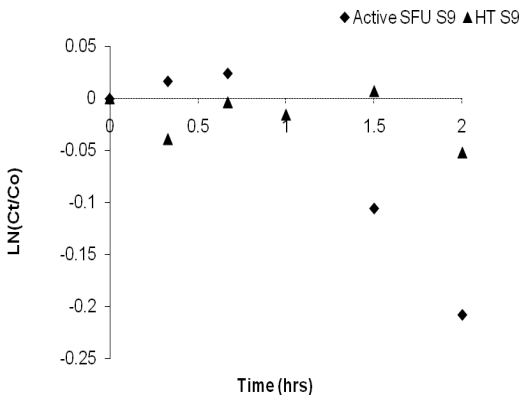

Figure 52: Depletion of 5.0µM pyrene (Replicate 9 of 9)

The above Figures represent replicate runs of the data presented in the text, the means are shown in Tables 9-14. Incubations were performed with 2mg protein/ml SFU S9 fraction at 12^{o}C for two hours. Active SFU S9 is freshly defrosted SFU S9 in a 12°C temperature controlled room. HT S9 is heat treated S9 at 100°C for 10 minutes.

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