BIOTRANSFORMATION AND MODELLED BIOCONCENTRATION FACTORS (BCFS) OF SELECT HYDROPHOBIC ORGANIC COMPOUNDS USING RAINBOW TROUT HEPATOCYTES

by

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Abstract

Biotransformation is an important factor in determining the extent that chemicals bioaccumulate. Since most anthropogenic chemicals lack data on biotransformation, this research used rainbow trout isolated hepatocytes to determine the depletion rates of several hydrophobic chemicals (benzo(a)pyrene, chrysene, 9-methylanthracene, polychlorinatedbiphenyl-153). These results were extrapolated to the organism level and bioconcentration factors (BCFs) modelled.

Since concurrent chemical exposure and temperature modify biotransformation, they were investigated for effects on modelled BCF values. Depletion rate constants were generally lower for chemical mixture than for individual incubations. At acclimation temperatures, chrysene biotransformation exhibited thermal compensation; for benzo(a)pyrene and 9-methylanthracene, lower acclimation temperature resulted in lower rate constants and increased BCFs. Acute temperature increases significantly increased depletion rate constants for benzo(a)pyrene and chrysene, and decreased BCF values. Acute temperature decreases had no effect. This research highlights the importance of considering environmental factors in evaluating the bioaccumulative potential of chemicals.

Keywords: biotransformation; bioconcentration factor (BCF); temperature; hepatocytes; mixture

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Glossary

9-MA	9-methylanthracene
B	Bioaccumulation
B[a]P	Benzo[a]pyrene
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
CEPA	Canadian Environmental Protection Act
CL_H	Hepatic clearance (ml/h/kg)
Cl_{int}	Intrinsic clearance (ml/h/10^6cells)
CYP	Cytochrome P450
GC-MS	Gas chromatography – mass spectrometry
HBSS	Hank's Balanced Salts Solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
K_m	Michaelis-Menten constant
k_{MET}	Metabolic rate constant
K_{ow}	Octanol-water partition coefficient
k_r	Depletion rate constant
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
Q_{10}	Temperature coefficient

1: Introduction

Anthropogenic chemical contamination is now a global cause for concern. Many of these compounds are ubiquitous in the environment being found as far away for their point of origin in the mid-latitudes as the Arctic and Antarctic (Jones & de Voogt 1999; Schwarzenbach et al. 2006; MacDonald 2007). These chemicals can also persist in the environment by being resistant to biotic or abiotic degradation for months or even years; consequently, they may accumulating within biota, resulting in elevated, possibly toxic body burdens. Therefore, anthropogenic xenobiotics need to be rigorously examined for their potential to be persistent (P), bioaccumulative (B) and inherently toxic (T) in order to be properly regulated, with the goal of reducing harm to both wildlife and humans.

1.1 Regulation of Anthropogenic Compounds

Acts and regulations regarding chemical management in Canada (Canadian Environmental Protection Act [CEPA], 1999) the United States (Toxic Substance Control Act [TSCA], 1976) and the European Union (Registration, Evaluation, and Authorization of Chemical substances Regulation [REACh], UNEP 2006) all mandate an assessment of anthropogenic chemicals in order to determine their P B and T status. For regulatory purposes, agencies set endpoint values to determine if a chemical has the potential to be an environmental hazard.

If a chemical exists unchanged in the air for $\geq$ 2days, in the water or soil for $\geq$ 6 months, or in the sediment for $\geq$ 1 year, it is considered to be persistent (P) by Health Canada and Environment Canada as per CEPA (1999). Inherent toxicity (T) is defined

as the hazard a substance presents to the environment (Government of Canada 1999) and is determined by Environment Canada as having toxic effects at concentrations < 1mg/L based on its toxicity to non-human, aquatic organisms. Bioaccumulation refers to the concentration of compound accumulated within an organism by all routes of chemical uptake (gastrointestinal, respiratory, and dermal) measured in field-caught organisms and compared to site water (Gobas & Morrison 2000). Bioaccumulation is measured by the ratio of the concentration of chemical in biota (C_B) to the fraction freely dissolved in water (C_{WD}) to determine the bioaccumulation factor ($BAF = C_B/C_{WD}$) (Gobas & Morrison 2000). When the BAF of a chemical exceeds 5000, it is considered to be bioaccumulative (B) according to the CEPA (1999). Chemicals that exceed P, B, and T guidelines subjected to phase 2 screening assessments.

1.2 Measurement of Bioconcentration and Bioaccumulation

Currently, P, B and T assessments (CEPA 1999) focus on assessing chemicals on Canada's Domestic Substances List (DSL (1991)); however, P B and T information on the 23,000 substances on this list are sparse. In fact, only 0.2% have empirically measured, publically available, bioaccumulation factor (BAF) values (Arnot & Gobas 2006). If field derived, empirical BAF values are not available, lab-derived bioconcentration factors (BCF) are used by Environment Canada (CEPA 1999) in B assessments as well. Bioconcentration is defined as the ratio of the concentration of a chemical that is absorbed via respiratory and dermal surfaces into the organism over the amount freely dissolved in water (Morrison & Gobas, 2000). BCF values can only be determined in controlled laboratory studies (Arnot & Gobas 2006) as exposure to the chemical must be via water, and concentrations in the water must be kept constant in

order to reach steady state within the organism and the water. Unfortunately, only 4% of chemicals on the DSL have BCF values (Arnot & Gobas, 2006). Due to the prohibitive cost and the tens of thousands of animals required to determine BCF values for all 23,000 chemicals on the DSL (as well as new chemicals requiring registration), it is not feasible to determine bioaccumulation *in vivo* for all chemicals; therefore other means of assessing bioaccumulation are required.

In lieu of BAF or BCF values, physico-chemical parameters such as log K_{ow}, the octanol-water partition coefficient, can be used B assessments. K_{ow} is an important factor in the uptake of chemicals; the process of partitioning between lipid (octanol) and water phases is thermodynamically driven by repulsive hydrophobic free energy resulting from the disintegration of the hydration shell when the hydrophobic compounds pass from the aqueous phase to the lipid phase (Connell & Schuurmann 1988; Meylan et al. 1999).

In a phase 1 B assessment, a chemical is considered bioaccumulative by Environment Canada if the BAF or BCF >5000, or if the log K_{ow} is > 5 (Government of Canada 1999; 2000). The Toxic Substances Control Act (US EPA 1976) states that a chemical is considered bioaccumulative if it has a BCF between 1000 and 5000, while BCF values >5000 are defined as "very bioaccumulative". In the European Union, a chemical is considered bioaccumulative if its BCF > 2000, and "very bioaccumulative" when >5000 (REACh regulations; European Commission, 2001). Regardless of the endpoint chosen, the bioaccumulative ability of an anthropogenic chemical must first be measured (either empirically or in the field) or modelled for the chemical to be categorized.

Bioaccumulation (BAF) assessments are measured in the field by taking many samples, over an extended time, of both biota and water and comparing between the concentration of chemical in the organisms and the concentration of chemical freely dissolved in the water (Burkhard 2003). These requirements for BAF assessments result in greater expense and complexity compared to laboratory-derived BCF tests. While these assessments are more indicative of the conditions found in the environment, there are difficulties involved, e.g. representative sampling in the field and labour required for extensive sampling. Furthermore, if the analytical methods of measuring chemicals that are not sufficiently sensitive, this can increase the uncertainty in the measured BAF values (Arnot & Gobas 2006). Nevertheless, BAF values are preferred over BCF or K_{ow} values in B assessments, and more work needs to be done to expand the database of available BAF values.

In order to measure the BCF, laboratory tests such as the 'Bioaccumulation: Flow-through Fish Test' (Organization for Economic Co-operation and Development (OECD) 305E, 1996) are employed. Fish are exposed to various concentrations of a chemical for a duration of time, and the concentration that is attained within the fish at steady state is measured and compared to the concentration freely available in the water. While this test is the standard method of determining BCF values, there are drawbacks. Prior knowledge of the compounds' kinetics are required in order to determine how long the exposure phase should be to reach steady state. Furthermore, the tests can be drawn out by lengthy depuration periods for high log K_{ow} compounds. Other difficulties with this test include expense, difficulty in measuring the freely dissolved chemical concentrations in the water, and the variability of chemical concentrations in the water over the duration of the

test through loss (volatilization or sorption to test apparatus or organic carbon such as faecal matter) (Gobas & Morrison 2000).

1.3 Modelling Bioaccumulation

Modelling bioaccumulation allows for an assessment of compounds using data on chemical properties and chemical kinetics within an organism. Basic parameters such as a compound's tendency to migrate into the lipid phase (expressed as K_{ow} or K_{oa} [the octanol:air coefficient]), can provide a preliminary estimate of regard to a compound's ability to bioaccumulate. While K_{ow} can be useful in assessing bioaccumulation, it does not take into account the potential for biotransformation of the chemical within the organism. Quantitative structure activity relationships (QSARs) incorporate a compound's K_{ow} value into a regression equation that predicts the compound's bioaccumulative abilities (Hawker & Connell 1986; Meylan et al. 1999). Other models employ factors such as the kinetics of absorption, distribution, metabolism and elimination within an organism. Kinetic models (Nichols et al. 1990; 2004; 2006) and physiologically-based toxicokinetic models (PBTK) (Law et al 1991; Nichols et al. 2007) use measures of chemical concentration in different tissues over time, integrating these concentrations with known kinetic parameters, to explain the behaviour of a particular compound within the organism. Fugacity-based, thermodynamic models describe chemical accumulation using fugacity ("escaping tendency") terminology (Gobas & Mackay 1987; Camphens & Mackay 1997; Mackay & Fraser 2000; Powell et al. 2009). One of the major drawbacks of the majority of models developed to date is their inability to incorporate the biotransformation of xenobiotics into models; without this data, BCF predictions may be overestimated.

Physical-chemical properties such as log K_{ow} are integral to many bioaccumulation models (QSARs, mass-balance models [Arnot & Gobas 2003; 2004], fugacity models), but without data on biotransformation, these models tend to overestimate BCF values. Biotransformation is critically important to determining whether a chemical will bioaccumulate; studies have shown that chemicals with similar K_{ow}s but slightly different chemicals structures have BCF values that are orders of magnitude different (Kapoor et al. 1973, Buckman et al. 2006). When modelling metabolisable chemicals, refinements are required to account for the increase in elimination due to metabolism and subsequent excretion (Mathew et al. 2008). Models have been developed to estimate biotransformation rates in fish from *in vivo* studies of chemical uptake and elimination (van der Linde et al. 2001, Powell et al. 2009, Arnot et al. 2008; 2009), but without this *a priori in vivo* data, biotransformation predictions remain inexact.

1.4 Biotransformation of Xenobiotics

A xenobiotic is a chemical that is foreign to a living organism (Murphy 2001), and is likely undesirable and possibly toxic. Biotransformation of xenobiotics into more hydrophilic compounds allows for their elimination, thereby preventing bioaccumulation (McCarty & Mackay 1993). Biotransformation is usually through oxidation, reduction, hydrolysis, hydration and/or conjugation reactions, and the end result is a metabolic product that can be more easily eliminated (Gibson & Skett 2001) due to its increased solubility in water and the addition of functional groups that can be recognized by efflux pumps. Biotransformation is usually divided into two categories of reactions, phase I and phase II. Phase I reactions which include oxidation, reduction and hydrolysis reactions

(among others). Phase II reactions are conjugation reactions which result in the addition of a chemical compounds such as glucuronic acid, glutathione, and others, that results in a more hydrophilic compound. The end result of phase I and II reactions is usually detoxification via the production of metabolites that can be eliminated across the gills (in fish) or by faecal excretion.

1.5 Effects of Temperature Change on Biotransformation and Bioaccumulation

An organism's environment can have a significant role in modifying chemical biotransformation rates and hence affect bioaccumulation potential. Environmental factors such as salinity (Bawardi et al. 2007), water hardness (Tolls et al. 2000) and pH (Laitinen et al. 1982; 1984) have all been shown to affect biotransformation. As well, prior exposure to pollutants can result in induction (Behrens et al. 2001; Buckman et al. 2007) or inhibition (Miranda et al. 2006, Sijm et al. 1993) of certain biotransformation enzymes. Temperature is a significant factor in overall metabolism in ectothermic organisms and has been shown to have a dramatic effect on the extent to which a chemical is biotransformed and bioaccumulated as well (Hochachka & Somero 2002).

Biotransformation is an enzymatic process that is significantly affected by temperature at all levels of biological organization. At the interface between the enzyme and its substrate, temperature plays an important role. Studies have shown that change in temperature has an effect on the formation rate of the enzyme-substrate complex, and their conversion to active complexes (Hochachka & Somero 2002). In order to generate the active complex, the free energy of activation "barrier" ($\Delta G^{\ddagger}$) must be exceeded. An increase in temperature increases the free energy, overcoming the barrier quicker,

allowing for reaction to occur more rapidly (Low et al. 1973). As well, higher temperatures result in a greater proportion of the enzymatic population having the activation energy (E_a) that is required for the reaction to take place (Hochachka & Somero 2002).

Changes in biotransformation rates due to acute change in temperature can be quantified using the van't Hoff temperature coefficient, $Q_{10} = (k_1/k_2)^{10/(T1-T2)}$. This equation takes the ratio of the biotransformation rate constants k_1 and k_2 determined at the high and low temperatures, $t_1$and t_2 respectively (Hochachka & Somero 2002). Generally, Q_{10} values of approximately 2.25 indicate that the biotransformation rates have doubled with a 10°C increase in temperature (Smirnov et al. 1987; Mustav 1994; Koivusaari & Andersson 1984). As a measure of the effect of temperature, Q_{10} represents the culmination of a suite of complex enzymatic events that result in the biotransformation of xenobiotics.

Phase I enzymes are highly sensitive to acute changes in temperature. With acute increases in temperature, increases in biotransformation rates and a consequent increase in phase II metabolites generally increase the elimination of the chemical from isolated cell preparations (Johnston et al 1999), and the converse is true with acute decreases in temperature. Membrane-bound phase I enzymes such as cytochrome P450 (CYP450) are also affected by the fluidity of the plasma membrane in the endoplasmic reticulum (ER) that are the result of acute changes in temperature. Acute temperature change also induces compensation in the CYP450 system through changes in the CYP450 reductase: CYP450 ratio (Blanck et al 1989). This correlation of changes in biotransformation enzyme activity to changes in temperature appears to be conserved across fish species, as

studies with rainbow trout (Koivusaari 1983; Carpenter et al. 1990; Blanck et al. 1989; Egaas & Varanasi, 1982), toadfish (*Opsanus beta*; Kennedy et al, 1991; Gill & Walsh, 1990), bluegill (*Lepomis macrochirus*; Ankley et al, 1985; Karr et al, 1985; Jimenez et al. 1987), dab (*Limanda limanda;* (Sleiderink & Boon 1996), sablefish (*Anoplopma fimbria*), black rockfish (*Sebastes melanops*), and chub mackerel (*Scomber japonicius*) (Johnston et al. 1999) have found similar changes in biotransformation with changes in temperature.

While there have been fewer studies examining the effects of acute temperature change on phase II enzymes, it is known that temperature change affects the different phase II enzymes differently. Both UDP-glucuronosyltransferase (UDPGT) and sulfotransferase (SFT) show sensitivity to acute changes in temperature, whereas glutathione *S*-transferase (GST) activity does not change with acute temperature change (Kennedy et al. 1991). As enzymatic activities of phase II enzymes are less well studied, less is known about how their role in xenobiotic biotransformation is affected by acute temperature change.

When cells are incubated at temperatures that match the fish's acclimation temperature, net baseline activities are expected for phase I enzymes (Karr et al. 1985; Blanck et al. 1989). Within cells, enzymes (including enzymes that biotransform xenobiotics) show adaptation to changes in acclimation temperature through changes in activity and/or abundance. These adaptations to change in temperature are referred to as thermal compensation (Gill & Walsh 1990; Hochachka & Somero 2002). With higher acclimation temperatures, enzyme affinities for substrates (such as xenobiotics) may be increased, and ectotherms may take advantage of this by reducing enzyme concentrations

thereby maintaining a constant level of overall activity (Hochachka & Somero 2002; Gill & Walsh 1990). When exposed to colder temperatures for longer periods of time, changes in enzyme concentration and activity are required to maintain constant biotransformation rates. The change in temperature and resulting adaptations in the lipid composition of the ER plasma membrane affect the CYP450 activity levels (Karr et al. 1985; Carpenter et al. 1990; Andersson & Koivusaari 1986) increasing activity with increased temperature. Among phase I enzymes, aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase (EH) showed increased enzymatic activity with decreasing acclimation temperature (James & Bend 1980; Kennedy et al. 1991; Egaas& Varanasi 1982) to compensate for decrease in kinetic energy with cooler temperatures (Hochachka & Somero 2002). Ankley and co-workers (1985) found corroborating results determining that B[a]P hydroxylase activity was more active in cold acclimated fish. Reports on changes in CYP450 concentrations with decreasing acclimation temperature are contradictory, as some researchers report no change in CYP450 concentration (Ankley et al. 1985) increases in enzyme concentration with decreasing temperature (Karr et al. 1985), and others report no sum change in concentration, but changes in the ratio of isozymes (a proportionate increase in CYP1A1) with changes in acclimation temperature (Carpenter et al. 1990).

While there is general agreement that phase I enzymes compensate for acclimation temperature changes, phase II enzyme alterations are not as consistent. For example, the Phase II conjugative enzyme UDPGT shows inverse compensation, resulting in decreased activity with decreased temperature (Koivusaari 1983; Kennedy et al. 1991). The evolutionary advantage for this inverse compensation is believed to limit

glucuronidation of sex hormones during winter spawning in order to maintain high levels of estradiol and testosterone (Andersson & Koivusaari 1986). Sulfotransferase (SFT) is markedly sensitive to changes in temperature, showing perfect compensation in enzymatic activity with acclimation (Kennedy et al. 1991). In contrast, while other detoxification enzymes undergo thermal adaptation, glutathione *S*-transferases (GST) maintain constant activity and concentration levels with changing temperature, appearing to be "temperature insensitive" (Kennedy et al. 1991). This phenomenon is likely due to the conservation of the enzyme's binding affinity at temperatures within the range that is normally experienced by the organism (Hochachka & Somero 2002).

There is some literature regarding the effect of temperature on bioaccumulation in fish; some authors have shown that increases in temperature increase the bioaccumulation of xenobiotics within an organism. BCF values reflect this increase in bioaccumulation. Karara and Hayton (1989) found that with an increase in temperature from 10°C to 30°C, the BCF for di(2-ethylhexyl) phthalate (DEHP) increased from 45 to 6510 in sheepshead minnows. Edgren and co-workers (1979) found that a 10°C increase in water temperature resulted in a doubling of the concentration of DDT, tetrachlorobiphenyl and hexachlorobipheyl in perch. Using fathead minnow, green sunfish and rainbow trout, Veith and co-workers (1979) found that the BCF of Aroclor 1254 increased by an order of magnitude with an increase in temperature (5°C to 25°C). Increased BCF values for bisphenol A with increased temperatures were found in freshwater clams, salmon eggs and tadpoles (Heinonen et al. 2002; Honkanen et al. 2001;Honkanen & Kukkonen 2006). Dichlorodiphenyldichloroethene (DDE) was also determined to increase in bioaccumulation (based on BCF values) with increased temperature (Nawaz & Kirk

1996) . Jimenez and colleagues (1987) found that BCF values for B[a]P, modelled from uptake and elimination rates, doubled when temperature increased 10°C. In general, the effect of increased temperature on bioaccumulation resulted in increased BCF values, due to changes in uptake, biotransformation and elimination.

Aside from the effect temperature has on biotransformation, other physiological parameters involved in bioaccumulation of xenobiotics are also affected. Rates of metabolism increase, resulting in increased feeding rates (Winslade 1974), increased oxygen demand and increased gill ventilation rates (Reid et al. 1996; Black et al. 1991); all are processes that increase the uptake of xenobiotics from the environment. Temperature increases can also result in an increase in absorption of chemicals at the gill, due to an increase in gill blood flow (Karara & William L. Hayton 1989). Increases in temperature generally increase the net rate of xenobiotic biotransformation within organisms (Jimenez et al. 1987; Kennedy et al. 1989b). The effect of temperature on elimination is equivocal; some studies have shown that elimination was increased in summer and significantly reduced in winter (Paterson et al. 2007), whereas other studies have found that the rate of xenobiotic excretion is dependent on the initial rate of uptake (Kennedy et al. 1989a; 1989b). The balance between uptake, biotransformation and elimination with change in temperature determine the extent of bioaccumulation.

1.6 Overview of Research

As a critical part of persistence (P), bioaccumulative (B) and inherent toxicity (T) assessments, BCF values need to be derived for many of the 23,000 anthropogenic chemicals on Canada's Domestic Substances List (DSL, 1999). As currently approved methods (such as the OECD 305E bioconcentration test) are costly and time consuming,

a means to rapid screen chemicals for bioaccumulative potential is required. Hepatocytes (isolated liver parenchymal cells) have been used as a screening tool, to determine which chemicals can be rapidly biotransformed, and hence are less likely to bioaccumulate. These cells are isolated from rainbow trout (*Oncorhynchus mykiss*); this species was chosen as it is recommended for xenobiotic testing by Environment Canada (2007) as a suitable representative for all fish species, as well as being extensively tested in primary literature. Extrapolating from biotransformation *in vitro* to whole organism bioconcentration factors (BCF) allows for comparison of the chemical's bioaccumulation potential to current guidelines. Work by previous researchers (Cowan-Ellsberry et al. 2008; Han et al. 2007, 2008) have integrated *in vitro* data into liver clearance models (such as the "well-stirred" liver model [Rowland et al. 1973; Jones & Houston 2004]) and extrapolated to bioconcentration factors using a fish bioaccumulation model (Arnot &Gobas 2003; 2004). The ability to estimate bioaccumulation using *in vitro* methods allows for more cost-efficient assessments, resulting in rapid phase I screening assessments of anthropogenic chemicals.

The goal of this research was to investigate the use of isolated hepatocytes to measure the rate of biotransformation of very hydrophobic organic chemicals. To assess the capacity of isolated hepatocytes for xenobiotic biotransformation, chemicals with a range of metabolism patterns were selected. The PAHs benzo(a)pyrene, 9-methylanthracene, and chrysene were chosen for their range in rates of metabolic breakdown, where as the polychlorinated biphenyl, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) is generally non-metabolizable and likely to bioaccumulate. All have a log K_{ow} of 5 or greater, are prevalent in the environment, and

are anthropogenically sourced, either through manufacturing or unintentionally. In addition, all are part of the DSL, and subject to P, B, and T assessments. To measure biotransformation within the hepatocyte suspension, the substrate depletion method was utilized to derive the depletion rate constant of the test chemical. By measuring the rate of disappearance of the parent compound over time, all metabolic pathways are included. Incorporating the substrate depletion rate constants measured in the hepatocyte suspensions into calculations to model the bioaccumulation of the chemical within biota, comparison to empirically-derived *in vivo* BCF values was possible.

Secondary objectives of this research were to utilize the framework of assessing depletion rate constants of xenobiotics to ascertain the effect of temperature change on the rate of metabolic breakdown in hepatocyte suspensions. As well, the effect that incubation with a mixture of chemicals has on depletion rate constants was measured, both in conjunction with temperature change and at constant temperature. The xenobiotic depletion rate constant data generated using the hepatocyte system were utilized in hepatic clearance models (the "well-stirred" liver model [Jones & Houston 2004]) and in fish bioaccumulation models (Arnot & Gobas 2003, 2004) to extrapolate BCF values. Modelled BCF values were compared to empirically-derived BCF values, in order to demonstrate the comparability of the hepatocyte test system, and compared to guidelines in acts and regulations (CEPA, 1999; TSCA, 1976; REACh, 2006).

2: Biotransformation of selected hydrophobic organic compounds assessed using Rainbow Trout hepatocytes, and extrapolation to estimate bioconcentration factors

2.1 Abstract

The parenchymal cells of the liver (hepatocytes) act as the primary site of biotransformation of environmental contaminants in many fish species, including rainbow trout (*Oncorhynchus mykiss*). Hepatocytes were used as an *in vitro* model system to extrapolate the effects of xenobiotic exposure to the whole organism. Of particular interest are organic chemicals with high octanol-water partition coefficient (K_{ow}) values, as these chemicals are likely to accumulate in the lipid tissues of aquatic organisms in an unmetabolized and possibly toxic form. This study examined the rate of biotransformation of the polycyclic aromatic hydrocarbons (9-methylanthracene, benzo(a)pyrene, chrysene) as well as PCB-153 as individual chemicals or within a mixture of four chemicals. Benzo(a)pyrene showed rapid biotransformation in the independent incubation, but when incubated in the mixture, the depletion rate constant (k_r) declined almost 30-fold. Chrysene also showed significant k_r, which declined by half in the mixture incubation. No metabolism of PCB-153 was detected. Using published models, these results were extrapolated to calculate the clearance of each chemical from rainbow trout liver. Finally, the bioconcentration factors (BCF) were estimated using the Arnot-Gobas bioaccumulation model.

2.2 Introduction

The potential negative impacts of the approximately 100 000 existing chemicals and 1000 – 2000 new substances developed each year (UNEP 2001; Walker et al. 2002) on both the environment and human health have been the impetus for several countries and international organizations to develop chemical management programs such as the Stockholm Convention on Persistent Organic Pollutants (POPs) (UNEP 2001) and Environment Canada's Existing Substances Program (Government of Canada 1999). These programs have initiated the creation or refinement of laws and regulations regarding new and existing chemicals, such that screening level risk or hazard assessments to determine a chemical's potential for harm are required (US EPA 1978; Government of Canada 1999; Brodar et al. 2002). Chemical regulatory programs in Canada (under CEPA), the United States (under TSCA), and the European Union (under the REACh program) are presently evaluating potential harm through the assessment of a chemical's environmental persistence (P), toxicity (T), and bioaccumulation (B).

The process of bioaccumulation occurs when a chemical is absorbed in an organism through all routes of exposure (dietary and abiotic sources), as would occur in the natural environment (Arnot & Gobas 2006). The extent to which a substance will bioaccumulate depends on a complex and dynamic interplay between environmental bioavailability, toxicokinetic processes (uptake, biotransformation, elimination), and species-specific parameters (e.g. growth, lipid content). Understanding the bioaccumulation potential of a chemical is important for P, T and B assessments, but this data has other important applications such as: determining environmental quality guidelines, establishing total maximum daily loadings, categorizing chemicals that are potential hazards, quantifying

risk to ecosystems and human health (Arnot & Gobas 2004; Government of Canada 1999; US EPA 2000; OECD 2001; Burkhard 2003). In addition, product classification and labelling, priority setting for chemical management, pollution prevention initiatives, and pre-manufacture notifications are also dependant on accurate information regarding bioaccumulation (European Commission 1979, 2003; UN Economic Commission For Europe 2005; Parkerton et al. 2008; UNEP 2006; US EPA 1999a, 1999b, 2000; OSPAR Commission 2004; UNEP 2006).

The criteria for assessing the bioaccumulative properties of a chemical in wildlife or humans include measures such as the logarithm_{10} of the octanol-water partition coefficient ($\log K_{ow}$) and the biota-sediment accumulation factor (BSAF). However, the bioconcentration factor (BCF) and the bioaccumulation factor (BAF) are currently the preferred measures of a chemical's bioaccumulation potential. The criteria for evaluating whether a chemical is bioaccumulative can vary between countries and regulatory programs; for example, under the REACh regulatory framework, a chemical is considered bioaccumulative if BCF values are >2000, and very bioaccumulative if BCF values are >5000, whereas other countries using criteria from the Stockholm Convention (UNEP 2006) consider that a chemical is bioaccumulative if its BCF value is >5000 (Cowan-Ellsberry et al. 2008). In Canada, in the absence of BCF or BAF values, $\log K_{ow}$ values >5 are considered to have bioaccumulative potential (Arnot & Gobas 2003, Government of Canada 1995).

Data used for B assessments can be obtained from empirical measurements or can be generated through various mathematical models. Currently, one of the primary empirical data sources used to determine the potential for a chemical to bioaccumulate is the

laboratory-derived fish BCF using the OECD TG 305 BCF flow-through bioconcentration test (Organization for Economic Co-operation and Development 1996). Good quality empirical data is preferable, but the lack of data has been cited as a critical need for conducting B assessments (Cowan-Ellsberry et al. 2008). In this regard, Arnot and Gobas (2006) found that <4% of commercial organic compounds had BCF values of reliable quality, and noted that guidance for critically reviewing BCF studies required standardization. Recently, key criteria for reviews of BCF values have been identified (Parkerton et al. 2008).

Field BAFs tend to be greater than laboratory-derived BCF values, which emphasizes the importance of environmental measurement for reliable assessment; however, only 0.2% of current-use organic chemicals have BAF measurements (Arnot & Gobas 2006). Despite the valuable nature of field BAF values, guidelines for conducting field programs are still lacking. Burkhard (2003) has identified several important factors that contribute to the uncertainty in data collected in field bioaccumulation studies, and provided several recommendations for the improvement of sampling designs intended to gather data on bioaccumulation endpoints.

When empirical fish BCF data are not available in the literature, computer models are the most common method for assessing bioaccumulation. These evolved from simple equilibrium partitioning models (Veith et al.1979; Neely et al. 1974), to kinetic (Gobas 1993) and fugacity-based food web bioaccumulation models (Camphens & Mackay 1997). Bioaccumulation and bioconcentration values can be estimated using quantitative structure-activity relationships (QSARs) (US EPA 2004; Arnot & Gobas, 2003), empirical models (Mackay 1982; Dimitrov et al. 2005), and mass balance models

(Nortstrom et al. 1976; Gobas 1993; Law et al. 1991; Camphens and Mackay 1997; Arnot & Gobas 2004).

The primary characteristic factored into the majority of these computer models is chemical partitioning behaviour, as expressed by the log K_{ow} value. Present models now also include uptake, elimination and metabolism processes (e.g., Arnot & Gobas 2003, 2004). However, due to limited empirical information on biotransformation and an inability to predict a-priori metabolic transformation rates (using computational molecular models [QSARs]), these models have met with limited success in predicting BCF values for compounds that are extensively metabolized (Dimitrov et al. 2005; Weisbrod et al. 2007). Hence, such models have been restricted to predicting the bioaccumulation of non-biotransformable compounds. Metabolic transformation rates are only available for a very small number of xenobiotics (i.e., non-pharmaceutical commercial chemicals) and are generally speaking "absent" for the chemicals that need to be assessed. Information regarding metabolic transformation rates would increase the range of chemicals for which accurate assessments of bioaccumulation behaviour could be made and would significantly improve the ability of computer models to predict realistic BCF values. At present, standardized, reliable and acceptable experimental methods of determining metabolism rates of commercial chemicals do not exist (Cowan-Ellsberry et al. 2008).

Biotransformation rates in fish have been assessed empirically *in vivo*, or through various *in vitro* methods such as isolated perfused livers, liver slices, isolated hepatocyte suspensions, and subcellular fractions including S9 fractions and microsomes. The suggestion that *in vitro* biotransformation tests can provide rapid, reliable and cost-

effective data to be used in computer model predictions of BCF (Nichols et al 2007; Cowan-Ellsberry et al. 2008) and recent work on the development of a hepatocyte clearance model (Han et al. 2007) prompted this investigation on an assessment of the bioaccumulative potential of several compounds using isolated trout hepatocytes as a model metabolic system. Hepatocytes are frequently used in pharmacology (Hewitt et al. 2007) and toxicology (Baksi & Frazier 1990; Pesonen & Andersson 1997) to assess drug and xenobiotic toxicokinetics and toxicodynamics. Unlike sub-cellular fractions (S9, liver microsomes), hepatocytes are highly representative of the *in vivo* situation with an intact cellular structure and a full complement of enzyme activities; their use avoids confounding factors associated with whole animal work by allowing greater control of the environmental conditions.

The overall objective of the present study was to measure the biotransformation of highly hydrophobic organic chemicals using an isolated trout hepatocyte preparation and determine whether the predicted BCFs were reasonable estimates of the empirically-derived values. In this study, the biotransformation rates of the following compounds with log K_{ow} values >5 were determined using a substrate depletion method: benzo(a)pyrene (B[a]P; log K_{ow}=5.97), chrysene (log K_{ow}=5.81), 9-methylanthracene (9-MA; log K_{ow}=5.07) and 2,2',4,4',5,5'-hexachloro-1,1'-biphenyl (PCB-153; log K_{ow}=7.75). Based on the literature, these chemicals also represent a wide range of biotransformation potential in fish from very low (PCB-153) to rapidly metabolizable (B[a]P). Depletion rate constants were determined and these were used to extrapolate whole organism clearance rates and finally, BCFs values were predicted using the model of Arnot and Gobas (2003, 2004). *In vitro* metabolism data has the potential to fulfil the urgent need

for methods to measure metabolic transformation rates to be used in regulatory assessments of the bioaccumulation behaviour of anthropogenic chemicals.

2.3 Materials and Methods

2.3.1 Chemicals

PCB-153 and PCB-209 were obtained from AccuStandard (New Haven, CT). B[a]P, chrysene, 9-MA, and deuterated chrysene were obtained from Sigma-Aldrich (St Louis, MO). Chemical purities were always >98%. Acetonitrile and hexane were obtained from Caledon Laboratories (Georgetown, ON, CAN).

2.3.2 Fish

Adult male rainbow trout (*Oncorhynchus mykiss*) with an average weight of 908 g ± 77g were purchased from Miracle Springs Trout Farm (Mission, BC). Fish were housed in 500-L flow-through tanks supplied with dechlorinated municipal water at a temperature of 13°C ± 1°C, under a light:dark photoperiod of 12:12. Fish were fed commercial salmon pellets (Ewos Pacifica Ltd., Surrey, BC) and were acclimated for at least two weeks prior to each experiment.

2.3.3 Hepatocyte isolation

Hepatocytes were isolated according to Moon *et al* (1985) with modifications (Gourley & Kennedy 2009). Briefly, following euthanasia (0.5 g/L buffered MS222), livers were perfused *in situ* with a solution of Hanks balanced salts solution (HBSS; Ca^{2+} and Mg^{2+}-free, Sigma) containing 10mM HEPES (BioShop, Burlington, ON), 0.81 mM $MgSO_4$, 5.95mM $NaHCO_3$ (pH=7.7) at a rate of 2.0 mL/min/g liver until cleared of blood.

Perfusion continued with HBSS containing collagenase (0.6 g/L Type IV, Sigma), until the hepatocytes began to disaggregate. The liver was then removed, minced, and filtered consecutively through 250 µm and 75 µm nylon mesh. The hepatocyte suspension was spun twice at low speed centrifugation (42 x *g* for 4 min at 4°C) in a Beckman Coulter GS-15R centrifuge and washed using the initial perfusion solution containing 0.99 mM $CaCl_2$ (Fisher Scientific, Fairlawn, NJ), 3 mM D-glucose (Sigma), and 20 g/L fatty acid-free bovine serum albumin (Sigma). The cell solution concentration was adjusted to 25 mg/ml (Bains & Kennedy 2004) and acclimated for at least 1 h at 13°C before use. Cell viability was determined by trypan blue exclusion (Gill & Walsh 1990) and was always >85% for use in any experiment.

2.3.4 Chemical exposure incubations

Aliquots (500 µL) of hepatocyte suspension were placed into 2 mL amber vials (Aglient Technologies, Santa Clara, CA) and incubated following the addition of 5 µl of an individual test compound solution. Final concentrations of each chemical in each vial were 0.5 µM (0.5 µM each of B[a]P, chrysene, 9-MA and PCB-153 in mixture incubations) dissolved in acetonitrile (final acetonitrile concentrations in incubations were 1% v/v). Reactions were terminated at 0, 10, 30, 60, 180 and 300 min by the addition of 1 mL ice-cold hexanes and brief vortexing. For heat-treated controls, aliquots of 500 µL of hepatocyte suspensions in amber vials were incubated at 100°C for 10 min, cooled, and incubated with chemicals as described above.

2.3.5 Chemical analysis

The internal standard, deuterated chrysene, was dissolved in acetonitrile to a final concentration of 5µM and was added to each vial (5 µL) containing the incubation matrix, which was vortexed for 1 min to completely resuspend the pellet (Y-S Lee, personal communication). Vials were centrifuged at 800 x *g* for 10 min, after which the hexane supernatant was removed and placed into a clean 2 mL amber vial. The extract was analysed using an Agilent 6890 gas chromatograph (GC) in conjunction with an Agilent 5973 mass spectrometry (MS) detector (Agilent, Mississauga, ON). The column was an HP-5M5 5% phenyl methyl siloxane-coated capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) protected by a fused-silica deactivated guard column (5 m x 0.530 mm i.d.) (Agilent, Mississauga, ON). The injection volume was 1 µL, and the helium carrier gas flow rate was 1 mL/min. The GC was programmed with an injection temperature of 63°C, followed by a temperature ramp of 20°C/min to a temperature of 200°C. The temperature was held at 200°C for 1 min, after which a temperature ramp of 15°C/min was initiated to a maximum temperature of 285°C. The MS quantified the target compounds at select ions (*m/z* 192 for 9-MA, *m/z* 228 for chrysene, *m/z* 252 for B(a)P, *m/z* 360 for PCB-153 and *m/z* 240 for deuterated chrysene) using an ion energy of 70eV and an ion source temperature of 230°C.

Extraction efficiency assays were conducted in order to determine the loss of parent compound due to non-specific binding. Hepatocytes were incubated as above for 1 h at 13°C with 30 mM sodium azide in order to inhibit oxidative metabolism (Zuckerbraun et al. 2007). All four test compounds were assessed simultaneously, at all time points, as well as PCB-209 as a negative control since it is not metabolized (Buckman et al. 2006). Incubations were terminated by the addition of 1 mL of hexane. Sample analysis differed

from that previously described: the hexane supernatant (0.6 mL) was extracted prior to addition of the internal standard. Analysis of the extract by GC-MS was as described previously.

2.3.6 Calculations

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

$$\mathrm{Ln}C_t = \mathrm{Ln}C_0 - kt \quad (1)$$

Where C_0 and C_t are the concentrations of compound (µM) per milligram of cells at time 0 and time t (min), and k is the first order depletion rate constant (per min) (Bisswanger 2008).

To incorporate substrate depletion rate constant data into existing models, the depletion rate constant (k) was converted from min^{-1} to mL/h/10^6cells, resulting in the rate of intrinsic clearance (Han et al. 2007). Using the conversion factor in Bains and Kennedy (2004) (25 mg of cells/ml = 2.8 x 10^6cells), intrinsic clearance (Cl_{int}) was calculated using the following equation (Han et al. 2007):

$$Cl_{int} = k\ (h^{-1}) / \text{concentration of cells } (10^6 \text{cells/mL}) \quad (2)$$

Intrinsic clearance was then used to calculate the clearance of chemical from the liver (CL_H) using the "well stirred" liver model (Rowland et al. 1973; Jones & Houston 2004).

$$CL_H = \frac{Q_H \times (CL_{int} H_T W_L) \times (f_{u,b} / f_{u,h})}{Q_H + (CL_{int} H_T W_L) \times (f_{u,b} / f_{u,h})} \quad (3)$$

This model incorporates several physiological parameters including hepatic blood flow (Q_H; 536.1 mL/h/lg body weight (Nichols et al. 1990)), liver weight (W_L; 12.7g/kg body weight (Nichols et al. 1990)), and hepatocellularity (H_T; 510 x 10^6 cells/g liver weight

(Hampton et al. 1989)), as well as the fraction of chemical unbound in hepatocyte suspension ($f_{u,h}$) and blood plasma ($f_{u,b}$) which were determined as in Han et al. (2007). Once hepatic clearance was determined, this was used for the parameter of total clearance in the calculation of k_{MET} in the BCF calculation.

The integration of metabolism data (k_{MET}) into bioconcentration calculations was conducted as per Han et al. (2007), based on the equations of Arnot and Gobas (2003, 2004). Hepatic clearance calculated in equation (3) above functions as the total clearance (CL_T) in the following equation,

$$k_{MET} = 0.024 CL_T / V_{ss} \quad (4)$$

where clearance is divided by the volume of distribution at steady state (V_{ss}) as described in Arnot and Gobas (2004). k_{MET} was then incorporated into the bioconcentration factor equation,

$$BCF = k_1 \Phi / (k_2 + k_e + k_G + k_{MET}) \quad (5)$$

which provides the basis for the bioaccumulation model. Inputs, such as gill uptake (k_1) of bioavailable chemicals (Φ, the ratio of freely dissolved to total concentration in the water), are offset by factors such as gill elimination (k_2), excretion (k_e), growth (k_G), and metabolism (k_{MET}). Inputs for all rate constants (except k_{MET}) exist as standard parameters for rainbow trout residing in typical temperate lakes (Arnot & Gobas 2004); other inputs include acclimation temperature, weight of fish (kg), as well as chemical log K_{ow} values.

2.3.7 Statistical Analysis

Dunnett's test was employed to compare chemical depletion rate constants to its respective control ($p<0.05$). If no significant difference between the control and that

chemical's depletion rate constant were found, k_{MET} values for that chemical were considered to be zero. A Student's *t* test was used to compare depletion rate constants of compounds when incubated individually *v.* in a mixture ($p<0.05$). Comparison of the depletion rate constants between chemicals when incubated either individually or in a mixture were performed using ANOVA followed by Tukey's Honestly Significant Difference test ($p<0.05$). All statistical analysis was conducted using JMP 7.0.2 (2007).

2.4 Results

2.4.1 Substrate depletion and depletion rate constants

Extraction efficiencies were not significantly different from 100% for any of the compounds (PCB-153, B[a]P, chrysene, and 9-MA) used; therefore no correction factors were required when determining measured chemical concentrations in incubations (see Appendix B). Moreover, there were no significant losses of the parent molecule in any of the heat-treated control treatment groups, and the control depletion rate constants calculated from natural logarithm of concentration over time curves (substrate depletion curves) were not significantly different from zero ($p<0.05$). Substrate depletion curves for B[a]P, chrysene, 9-MA, and PCB-153 individually and when combined in a mixture are shown in Figures 2-1 and 2-2.

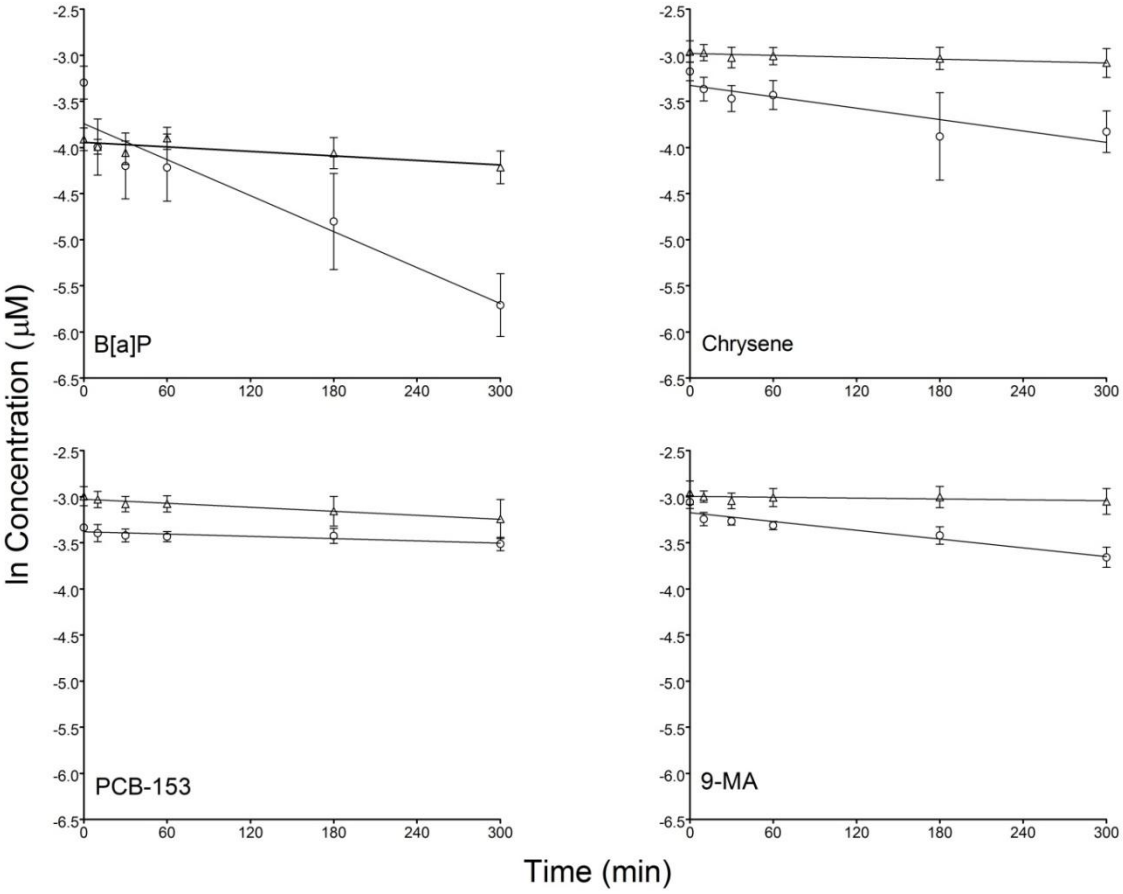

Figure 1: Substrate depletion graphs of B[a]P, chrysene, PCB-153 and 9-MA, incubated individually with 25mg isolated hepatocytes/ml. Control slopes, obtained using heated-treated hepatocytes, (Δ) were not significantly different from zero ($p<0.05$; $n=3$). The slope for PCB-153 was not significantly different from the control slope ($p<0.05$), i.e., no metabolism was observed.

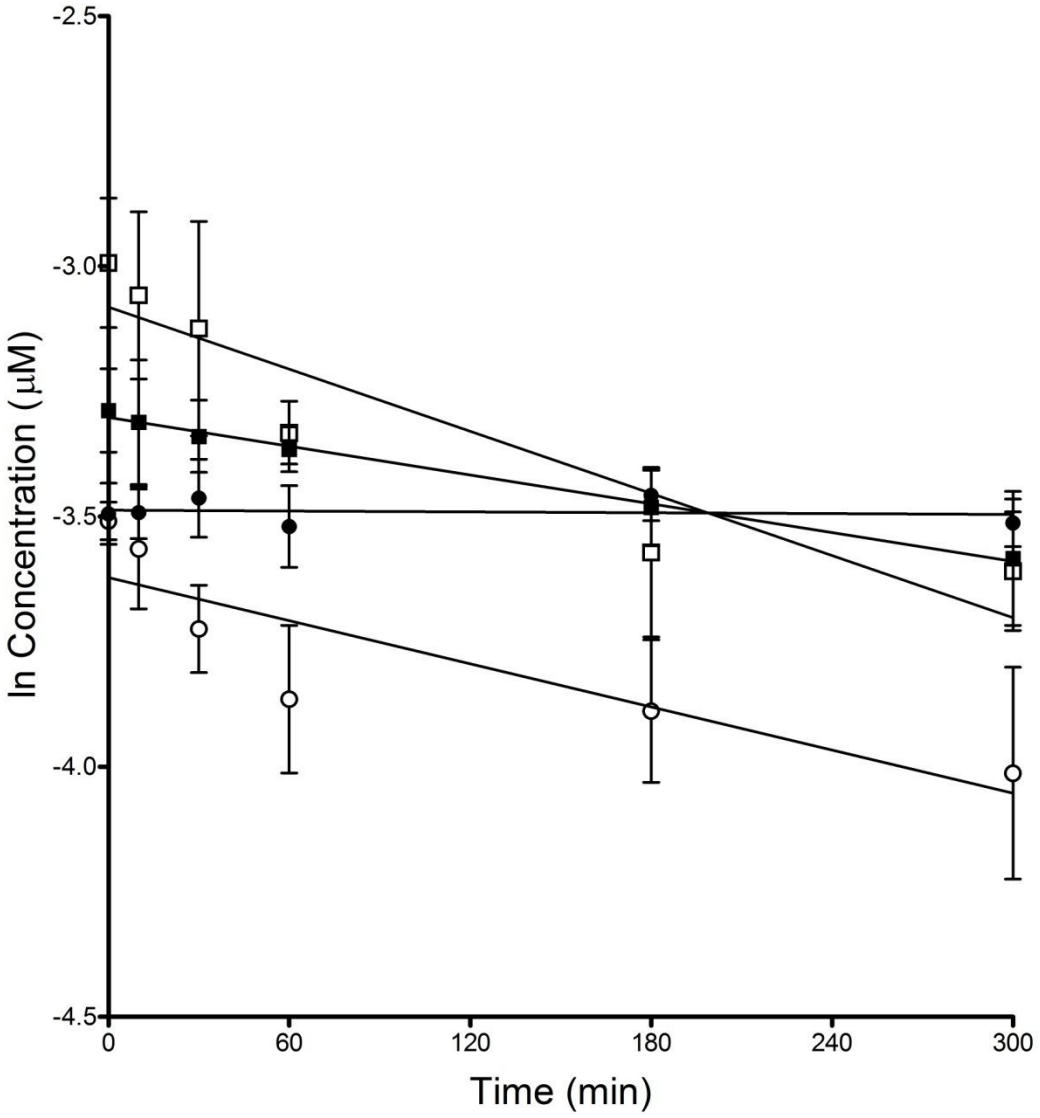

Figure 2: Substrate depletion graphs of 9-MA (□) B[a]P (○), chrysene (■) and PCB-153 (●) in mixture incubations with 25mg isolated trout hepatocytes/ml. Control slopes were omitted for clarity, as the slopes of the control were the same as those displayed in Figure 2-1 and were not significantly different from zero, ($p<0.05$). The slope for PCB-153 was not significantly different from the control ($p<0.05$).

Depletion of all compounds singly and in a mixture was linear for the duration of the assay and followed first order kinetics through the entire incubation period and calculated depletion rate constants are shown in Table 2-1. Incubated singly, in order of increasing value, depletion rate constants were: PCB-153 (not significantly different from control), 9-MA, chrysene, and B[a]P. The depletion rate constant for B[a]P (k_r = 6.51 x 10^{-3} min^{-1}) was significantly greater than the others (p=0.006). In the mixture incubation, the order of values for depletion rate constants was slightly different in that 9-MA was biotransformed more rapidly in this experiment. In increasing value, the results were: PCB-153, chrysene, B[a]P, and 9-MA. The differences in depletion rate constant for each chemical when incubated individually and when incubated in a mixture are shown in Figure 2-3. The depletion rate constant for B[a]P was significantly decreased (by 72%) when the chemical was incubated in a mixture compared to individually. (p=0.0131). No significant changes were observed with rates for chrysene or 9-MA incubated individually or in a mixture.

2.4.2 Intrinsic and hepatic clearances, and biotransformation rate constants

Depletion rate constants were normalized to number of cells (Bains & Kennedy, 2004) to calculate the rate of intrinsic clearance (Cl_{int}) (equation 2) and further extrapolated to total hepatic clearance (CL_H) using a 'well stirred' liver model (Jones & Houston 2004) which takes into account such parameters as hepatic blood flow, liver weight, hepatocellularity, and the unbound fractions of the chemical in blood and in the hepatocyte incubation. The biotransformation rate constant (k_{MET}) was calculated using total clearance in the fish (assumed to be CL_H since the majority of metabolism in trout occurs in the liver (Kennedy 1995)) and the volume of distribution at steady state (Arnot & Gobas 2004).

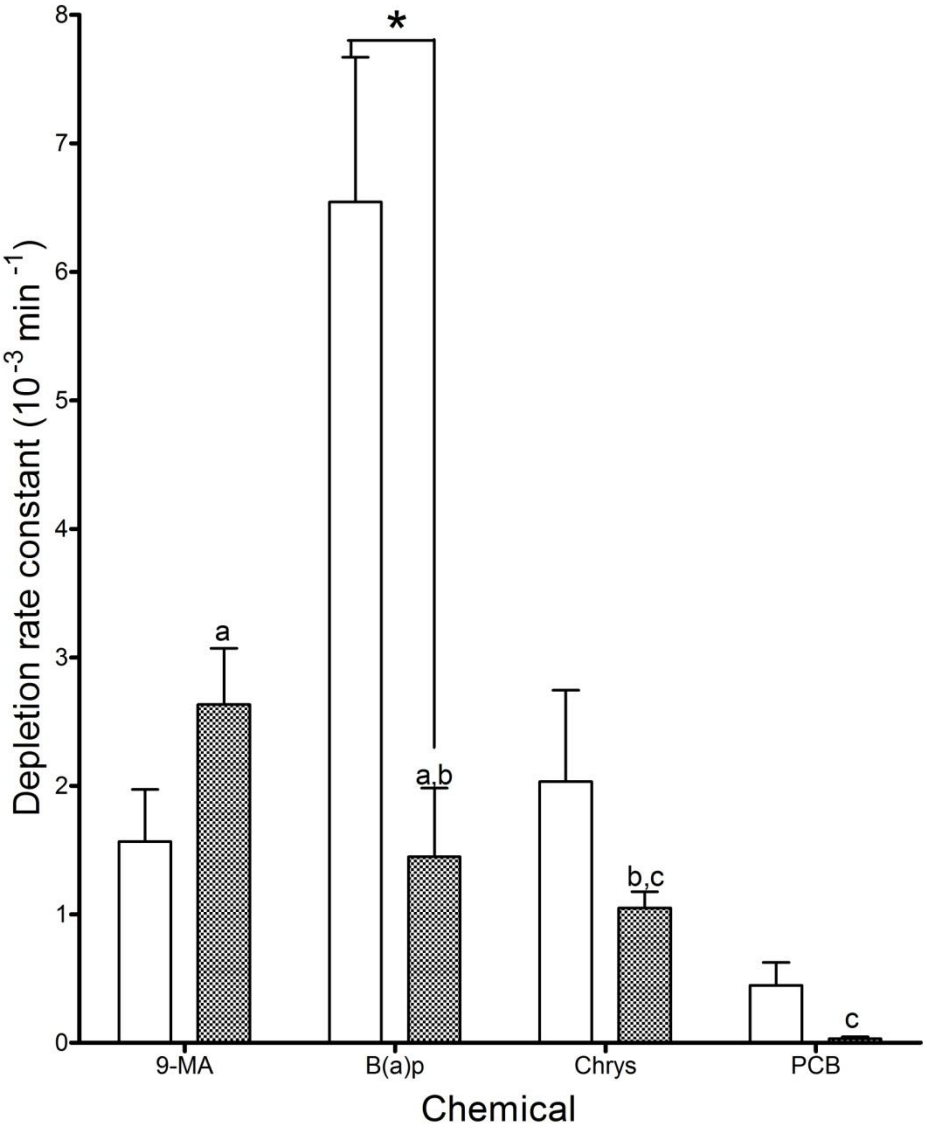

Figure 3: Comparison of depletion rate constants (k_r) of the compounds when incubated in mixture and between each compound, incubated alone (☐) or in mixture (■). Depletion rate constants were generated by incubation of the test chemical(s) in 25mg cells/ml for up to 5hrs. Between individual and mixture incubations, significant differences in a chemical's depletion rate constant are indicated by the asterisks (Student's *t*-test). Different letters denote significant differences between compounds in the mixture (ANOVA and Tukey's Honestly Significant Difference, $p<0.05$).

Individual	Depletion rate constant (k_r; 10^{-3} min^{-1})	CL_{int} (mL/h/10^6cells)	CL_H (mL/h/kg)	k_{met} (day^{-1})
9-MA	1.59 (0.83 – 2.36)	0.011 (0.001 – 0.016)	3.30 (1.71 – 4.87)	0.014 (0.0074 – 0.021)
B[a]P	6.51 (3.55– 9.46)	0.044 (0.024 – 0.064)	13.9 (7.66 – 19.9)	0.060 (0.033 – 0.086)
Chrysene	2.06 (0.61 – 3.51)	0.014 (0.004 – 0.024)	4.54 (1.36 – 7.68)	0.020 (0.033 – 0.058)
PCB-153	0.41* (0.005 – 0.76)	0.003* (0.000 – 0.005)	1.12* (0.15 – 2.08)	0.0048* (0.001 – 0.009)
Mixture				
9-MA	2.07 (0.9 – 3.22)	0.139 (0.006 – 0.022)	4.27 (1.91 – 6.62)	0.018 (0.0082 – 0.028)
B[a]P	1.44 (0.3 – 2.58)	0.010 (0.002 – 0.017)	3.13 (0.65 -5.58)	0.013 (0.0028 – 0.024)
Chrysene	0.96 (0.86 – 1.06)	0.006 (0.006 – 0.007)	2.12 (1.89 – 2.35)	0.0091 (0.0081 – 0.0101)
PCB-153	0.03* (0 – 0.03)	0.000* (0 – 0.002)	0.08* (0 – 0.88)	0.000* (0 – 0.004)

Table 2-1: Depletion rate constant (k_r), intrinsic clearance, hepatic clearance and metabolic rate constants for all compounds incubated individually and in mixture. Results presented are mean (95% confidence intervals). The * indicates that the elimination rate was not significantly different from the control.

The calculated values for depletion rate constant, CL_{int}, CL_H and k_{MET} are shown in Table 2-1.

2.4.3 Bioconcentration Factor

Bioconcentration factors (BCFs) were determined using the fish bioaccumulation model of Arnot and Gobas (2004), using the k_{MET} determined as noted above. BCF values were calculated using $k_{MET} = 0$ (assuming no metabolism), k_{MET} values from the present study, as well as experimentally-derived whole organism BCF values from the literature, are shown in Table 2-2. Selection of appropriate BCF values from the literature were based on published criteria for assessing the experimental design and conduct of in vivo bioaccumulation tests (Parkerton et al. 2008). For B[a]P and PCB-153, there were sufficient data available to document a range of BCF values for freshwater fish only. Data for 9-MA was limited to one study on *D. pulex* (Southworth 1978). Chrysene data was also limited, comprising of only one study on fish (juvenile turbot, *Scophthalmus maximus*, Baussant et al. 2001) consequently, two aquatic invertebrate studies were added to the range (*Rhepoxinius abronius*, Boese et al. 1999; *Daphnia magna*, Newsted & Giesy 1987).

When compared to BCF values where $k_{MET} = 0$, all chemical BCF values that incorporated biotransformation data from the present study were significantly reduced (PCB-153 had no measurable metabolism, hence $k_{MET} = 0$ was used in the BCF calculation). BCF values for individually-incubated 9-MA, chrysene, and B[a]P were reduced by 47, 78 and 93%, respectively. The BCF values calculated using k_{MET} values were consistent with empirically-derived BCF values reported in the literature (Table 2-

Chemical	Calculated $BCF_{kmet=0}$	Calculated BCF_{kmet} (individual)	Calculated BCF_{kmet} (mixture)	Measured BCF Values (range)
9-MA	9500	4100 – 6500 (5000)	3400 – 6200 (4400)	4583
B[a]P	26860	1400 – 3400 (2000)	4400 - 17000 (7000)	224 – 3208
Chrysene	24281	3500 – 12000 (5300)	8600 - 9800 (9100)	650 – 6088
PCB-153	13493	13493*	13493*	8913 – 740,000

Table 2-2: Bioconcentration factors for all compounds incubated individually and in mixture, compared to BCF (k_{met}=0) and empirical BCF range. Results presented as the range between the upper and lower 95% confidence intervals, with the mean in brackets.
The * indicates that because k_r was not significantly different from the control, k_{met}=0 was used in the BCF calculation.

2). When incubated in a mixture, significant decline in k_{MET} values and resulted in increased BCF values for B[a]P and chrysene. Comparing between mixture and individual chemical incubations, no difference in calculated BCF values were obtained from 9-MA data. Compared to BCFs determined using k_{MET} = 0, data obtained from mixture incubations of chrysene and B[a]P resulted in BCF values reduced by 63 and 74% respectively.

2.5 Discussion

The BCF of an environmental contaminant may be used to estimate the potential internal dose that may be achieved in an organism, which in turn can indicate the possibility of a toxic response. Therefore, programs such as CEPA, TSCA and REACh use bioaccumulation factors (BAF/BCF) as key indices for evaluating potential harm. The daunting, labour-intensive and expensive task of assessing tens of thousands of new and existing chemicals (US EPA 1976; Brodar et al. 2002; Rogers 2003) has been the impetus for developing rapid *in vitro* and *in silico* screening methods to determine a chemical's bioaccumulative potential.

As biotransformation can reduce the concentration of chemical within an organism, the extent of metabolic breakdown of commercial chemicals can have a significant impact on their bioaccumulation. Most current methods of assessing bioaccumulation (save *in vivo* laboratory and field studies) do not include biotransformation in their determinations, information that is necessary in order to accurately predict bioaccumulation. The range of biotransformation capabilities for environmental contaminants in fish is large, yet for most compounds, biotransformation rates are lacking

(van der Linde et al. 2001), highlighting the crucial need for accurate biotransformation data for more accurate BCF calculations.

Isolated fish hepatocytes have been a valuable tool used in studying hepatic cell biology and biochemistry, including toxicological research, particularly in the study of the biotransformation of xenobiotics (Andersson & Koivusaari 1986; Gill & Walsh 1990; Han et al. 2007, 2008; Kennedy & Tierney 2008; Johnston et al. 1999; Mingoia et al. 2010; Nishimoto et al. 1992; Zaleski et al. 1991). Isolated hepatocytes represent a useful model system for determining the depletion rate constants of a variety of contaminants of concern. This model system has advantages over *in vivo* systems as it reduces the number of animals used, allows for simultaneous testing of several chemicals, results in increased numbers of replicates, reduces inter-individual variability caused by extra-hepatic factors, and ultimately reduces costs and time. It also has advantages over other *in vitro* methods such as sub-cellular fractions and microsomes that lack enzyme cofactors, cellular structure and intracellular transport systems, and show greater non-specific binding which can reduce the concentration of freely available substrate (Jones and Houston, 2004). In the present study, isolated hepatocytes had an intrinsic clearance rate for B[a]P consistent with reported values (Han et al. 2007, 2008).

The substrate depletion method measures the loss of the parent compound rather than measuring metabolite accumulation. Despite the use of a simplified method, the measurement of depletion rate constants has yielded accurate measurements of biotransformation rates, where comparison data is available. Xenobiotic biotransformation can utilize a multitude of metabolic pathways, resulting in the production of numerous Phase I and II metabolites. In order to determine accurate

biotransformation rates using metabolite accumulation data, all pathways and resulting metabolites must be identified and measured. For many compounds, these pathways are not elucidated and the metabolites often require synthesis in order to be quantified (Sjogren et al. 2009); all of this increases the complexity and the potential for uncertainty in the final estimates. However, by using the substrate depletion method, the only assumption is that the initial test chemical concentration is well below the Michaelis-Menten constant (K_m), and that the loss of parent compound is accurately quantified. Substrate depletion has been utilized in prior toxicological research for inclusion in PBPK and mass-balance models (Mazur et al. 2007; Han et al. 2007; 2008; 2009).

The depletion rate constants determined for each of the four chemicals used singly and in a mixture were used to calculate BCFs. The 'well-stirred' liver model (Rowland et al. 1973; Jones & Houston, 2004) was used to scale depletion rate constants from cells to the whole liver. This model has been shown to predict elimination with the same bias and precision as other liver clearance models although it has been described as the least physiologically relevant (Ito & Houston 2004). Along with several other apparent disadvantages (e.g. binding parameters within this model [$f_{u,h}$ and $f_{u,b}$] lose sensitivity at log K_{ow} > 4) it has been shown to provide a reasonable, if conservative, estimates of biotransformation in fish (Han et al. 2007; 2008). Liver clearance (CL_H) calculated by the 'well-stirred' liver model is used to calculate k_{MET} in a bioaccumulation model (Arnot & Gobas 2003; 2004) to calculate BCF values. Extrapolating from depletion rate constant to BCF using the methodology described herein appears to be an excellent starting point for assessing the bioaccumulative abilities of those compounds requiring screening. The

BCF values modelled in this study are in good agreement with values obtained from *in vivo* BCF values in primary literature.

The addition of biotransformation rate constants (k_{MET}) in the BCF calculation resulted in model predictions that more closely match results from *in vivo* BCF values in the literature. In individual treatment groups, BCFs for 9-MA, chrysene, and B[a]P were reduced significantly by the incorporation of biotransformation into the model. The values for hepatic clearance are consistent with what is known for both B[a]P (which is metabolized by fish rapidly (Kennedy et al. 1989; Kennedy 1990; Kennedy & Walsh 1994)), and PCB-153 (which is known to be metabolized slowly, of at all, by fish (Buckman et al. 2004; 2006). All four chemicals have modeled BCF values above 5000 when $k_{MET} = 0$, however, the inclusion of *in vitro* biotransformation data reduced the BCF values to equal to or below 5000 for 9-MA and B[a]P (CEPA [1999] defines a bioaccumulative chemical with a BCF value > 5000). However, if the 95% confidence interval range is taken into account, only B[a]P would be considered to be non-bioaccumulative.

Using BCF values derived from k_{MET} of the mixtures, only 9-MA fell below the CEPA threshold for bioaccumulation. This clearly illustrates the need for a standardized methodology for assessing BCFs that is representative of realistic environmental exposures or the need for more information on specific metabolic pathways and potential interactions between chemicals in terms of their biotransformation potential.

Toxicological assessment, including that for bioaccumulative potential, typically involves individual testing of chemicals, whether they are new drugs or environmental contaminants. Real world scenarios almost invariably involve chemical mixtures, which

can significantly alter the toxicokinetics and toxicodynamics within organisms, depending on the contents of the mixture. No information on potential interactions can be derived from single-compound screening. The present results show interaction between the compounds in terms of biotransformation, resulting in significantly altered depletion rate constants and BCF values. Significant increases in BCF values were seen for chrysene and in particular, B[a]P when they were incubated in a mixture whereas, the metabolism of 9-MA was virtually unchanged. In the mixture, all of the test chemicals are likely biotransformed with the participation of cytochrome P450 isoforms. For example, B[a]P and chrysene have been shown to be substrates for CYP 1A1 (Stegeman & Hahn 1994; Xue & Warshawsky 2005; Bauer et al. 1996). Competitive inhibition of metabolism by B[a]P and chrysene may explain decreases in hepatic clearance of these chemicals when incubated together. Interestingly 9-MA, whose metabolism was not affected, is reported to be metabolized by CYP2B1 (Anzenbacher et al. 1996). However, the concentrations of test chemicals used were low (2.0μM total), well below published K_m values for fish hepatocytes (B[a]P: 40μM, Nishimoto et al. 1992) In both mixture and individual incubations, PCB-153 was shown to be resistant to biotransformation, consistent with findings in other studies (Buckman et al. 2004; 2006).

The effect of competition between the different compounds calls into question whether screening should be done with mixtures instead of individual compounds. PCB-153 is almost always present in the environment in mixtures of PCB congeners known as Aroclors (McFarland & Clarke 1989). PAHs such as B[a]P, chrysene and 9-MA also enter the environment in a mixture, from processes such as incomplete combustion and fossil fuels (Douben 2003). Depending on the constituents of the mixture and their

concentrations present in the environment, an organism's ability to biotransform contaminants might be inhibited due to competition for biotransformation enzymes, or enhanced due to the presence of inducers. Biotransformation rates generated by mixtures may offer more accurate prediction of BCF values and consequently be more protective of aquatic species.

In the present study, an isolated hepatocyte model system was used to determine *in vitro* biotransformation rates using a substrate depletion method in order to determine BCFs. Utilizing this methodology to rapidly assess thousands of chemicals shows promise, and will aid chemical management bodies in meeting their regulatory goals. However, this methodology is not without concerns. For example, assumptions made within calculations and models used may not adequately represent all the factors present in the natural environment. As a surrogate for empirical BCF data and as a tool in CEPA's Phase I assessment, the BCFs calculated using hepatocyte generated biotransformation data can be utilized, but should never supersede empirically-derived BCF and BAF values. New models and tools such as these will allow more accurate assessments of new and current-use chemicals, and improve our ability to protect the environmental and human health.

2.6 References

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3: Effect of temperature on bioconcentration factors incorporating biotransformation rates obtained from an isolated rainbow trout hepatocyte model system

3.1 Abstract

Freshwater fish in temperate climates can be exposed to a wide range of water temperatures on a daily or seasonal basis. Concurrently, in contaminated environments, these fish can be exposed to lipophilic anthropogenic chemicals which may bioaccumulate. It is well documented that temperature plays a dominant role in xenobiotic metabolism rates in ectothermic organisms including teleosts. Determinations of accurate rates of metabolism (by measuring substrate depletion rate constants) under varying temperature regimes are needed to model accurate bioaccumulation or bioconcentration factors (BAF/BCF) for screening-level risk assessments. To determine the extent to which bioaccumulation is environmentally temperature-dependent, *in vitro* rainbow trout (*Onchorynchus* mykiss) hepatocytes isolated from fish acclimated to either 8°C or 18°C, were used to determine biotransformation rate constants of a polychlorinated biphenyl (PCB-153) or one of three polycyclic aromatic hydrocarbons: benzo(a)pyrene (B[a]P), chrysene or 9-methylanthracene (9-MA), incubated individually or in mixture under various temperature treatments. Acute temperature increases increased biotransformation rates of B[a]P and chrysene, but acute temperature decreases had no effect. At acclimation temperatures, chrysene showed thermal compensation, having similar depletion rate constants at different acclimation temperature, however, B[a]P and 9-MA showed significantly increased depletion rate constants in cells from

18°C acclimated fish. Biotransformation rate constants were determined at various incubation temperatures and were used in a bioaccumulation model (Arnot & Gobas bioaccumulation model [2003, 2004]) to determine BCF values. The modelled BCF values generally showed similar trends to depletion rate constants with regard to temperature change, with increases in temperature decreasing predicted BCF values due to a corresponding increase in depletion rate constant. These results emphasize the importance of considering all environmental factors when evaluating chemicals for the potential to bioaccumulate in biota.

3.2 Introduction

Many environmental contaminants of concern possess physical-chemical properties that result in accumulation of chemicals within organisms in higher concentrations relative to the organism's surrounding environment, resulting in increased potential for toxic effects. Understanding bioaccumulation potential can contribute to the quantification and mitigation of risks posed by such compounds (Arnot & Gobas, 2004), and aid in the determination of environmental quality guidelines, the categorization of potentially hazardous chemicals, and in calculations of potential body burdens (Government of Canada 1999; US EPA 2000; OECD 2001).

Nations that have ratified the Stockholm Convention on Persistent Organic Pollutants (UNEP, 2001) are currently undergoing assessments of anthropogenic chemicals to determine the hazards that these chemicals pose to both human health and the environment, and how they can be best regulated. In Canada, attention is focused on the 23,000 chemicals on the domestic substances list (DSL; Government of Canada, 1999). These chemicals are being assessed for their persistence (P), inherent toxicity (T)

and bioaccumulation (B) potential. Little data is available on the bioaccumulative potential of most chemicals on the DSL; less than 0.2 % and 4% have accurate bioaccumulation factor (BAF) or bioconcentration (BCF) values, respectively (Arnot & Gobas, 2006).

In some jurisdictions, chemicals can be considered bioaccumulative if they possess a log octanol-water partition coefficient (K_{ow}) of 5 or greater (Government of Canada, 1999). Determining a chemical's bioaccumulative potential can also be conducted through field or laboratory testing to determine either bioaccumulation factors (BAFs) or bioconcentration factors (BCFs). Sampling both organisms and water in the field to determine the concentration of chemical in each allows for the determination of BAF values (Morrison & Gobas, 2000). While these values are the most environmentally-relevant (dietary, dermal and respiratory uptake are incorporated) and preferred in B assessments, analytical limitations and the lack of guidelines for field assessments make BAF values difficult to generate and less reliable (Burkhard 2003). The comparison between chemical concentrations in fish and the amount of chemical that is freely available in the water can be determined in the laboratory, resulting in a BCF value (Arnot & Gobas, 2006). Laboratory-generated BCF values have more rigorous guidelines (e.g. OECD TG 305 BCF flow-through bioconcentration test [OECD, 1996]), but difficulties such as the requirement of achieving steady state requires prior knowledge of the chemical's kinetics, there are sometimes lengthy depuration times for high log K_{ow} chemicals, and difficulties maintaining constant chemical concentrations in the water and binding of log K_{ow} chemicals to test apparatus increase variation of the derived BCF values (Gobas & Morison 2000; Arnot & Gobas, 2006).

Bioaccumulation models offer the possibility of more rapid B assessments of large numbers of chemicals. These models range from simplistic equilibrium partitioning regression models (Veith et al. 1979; Neely et al 1974) to fugacity-based food-web bioaccumulation models (Camphens &Mackay 1997; Arnot & Gobas 2004; Gobas & Arnot 2010). Current model predictions on chemical bioaccumulation using log K_{ow} values are relatively accurate if a chemical is generally non-metabolizable, such as most polychlorinated biphenyls (PCBs) (Gobas, 1993).

The bioaccumulation potential of a chemical is highly affected by biotransformation; many chemicals such as polycyclic aromatic hydrocarbons (PAHs) can be rapidly metabolized, and without the incorporation of biotransformation information into models, assessments can be highly inaccurate (Baussant et al. 2001). For example, in a comparison between 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT) and one of its analogs with a similar log K_{ow} value (3.98 and 3.96 for DDT and methyl-ethoxychlor, respectively), Kapoor and colleagues (1973) showed that that the two chemicals had very different BCF values (84,500 and 400 for DDT and methyl-ethoxychlor, respectively) in mosquitofish (*Gambusa affinis*) due to the fishes' ability to extensively biotransform and eliminate the analog. The metabolic breakdown and elimination of a chemical can also be modelled, with limited success (van der Linde et al. 2001; Nichols et al. 2006, 2007; Arnot et al. 2008, 2009), but the dearth of experimentally derived *in vitro* and *in vivo* metabolism data for most compounds can result in inaccurate model predictions.

Abiotic factors are known to modify the ability of organisms to biotransform xenobiotics and have the potential to greatly impact bioaccumulation behaviour; these

include salinity (Bawardi et al, 2007), water hardness (Tolls et al, 2000) and pH (Laitinen et al, 1982, 1984) and prior exposure to pollutants (Behrens et al. 2001; Buckman et al. 2007; Sijm et al. 1993; Miranda et al. 1998). Among the stresses facing aquatic organisms today, ubiquitous environmental contaminants, and natural and anthropogenically-induced temperature change may prove to be among the most challenging. Environmental temperature plays a dominant role not only in the general metabolic processes in ectothermic organisms including teleosts, but there is a considerable amount of literature highlighting the effects of both acute and acclimation temperature change on biotransformation rates in fish.

There is, however, limited information on the effects of temperature on bioaccumulation in whole organisms or the mechanistic underpinnings of these changes. For example, increases in BCF values for di(2-ethylhexyl) phthalate (DEHP) from 45 to 6510 were attributed to increases in gill uptake at higher temperatures in sheepshead minnow (*Cyrinodon varegatus*) (Karara & Hayton, 1989). Increased temperature resulted in significantly increased BCF values for DDT and PCBs (Edgren et al. 1979; Veith et al. 1979), bisphenol A (Honkanen & Kukkonen 2006; Heinonen et al. 2002; Honkanen et al. 2001), benzo(a)pyrene (Jimenez et al. 1987), chlorobenzene (Koelmans & Sanchez Jimenz, 1994) and dichlorodiphenyldichloroethene (DDE, Nawaz & Kirk, 1996). Other studies have found that temperature had no effect on BCF values (Gerould 1983), or linked decreasing BCF values with increased temperature (Muijs & Jonker, 2009).

Temperature may act on several toxicokinetic processes to achieve altered chemical accumulation. On a whole organism level, changes in temperature can result in

alterations in processes involved in chemical uptake, biotransformation and elimination. Acute increases in temperature result in increased metabolism, oxygen uptake and gill ventilation, in addition to higher feeding rates (Black et al. 1991; Reid et al. 1996; Winslade, 1974). Temperature increases decrease the concentration of dissolved oxygen, but uptake efficiencies (diffusion of chemical across the membrane of the gill cell) and not uptake rate constants linked to oxygen concentration have been shown to determine the uptake of chemical across the gills (Opperhuizen & Schrap 1988; Sijm et al. 1993). The increase in physiological intake rates can result in higher xenobiotic uptake rates, an affect that is exacerbated by the increase in blood flow to the gills with increased temperature (Karara & Hayton, 1989). Generally, biotransformation rates increase with acute temperature increases, however, given time to acclimate to new temperatures, compensation returns values to baseline levels (Curtis et al. 1990; Kennedy & Walsh, 1991). Results of studies on the effects of temperature change on chemical elimination are equivocal. Increased temperatures can result in increased elimination (Patterson et al, 2007); however, others have found that elimination is affected by the amount of chemical initially available through uptake, and that changes in temperature directly affect uptake, but affect biotransformation and elimination both directly (increasing the rates at which these processes occur) and indirectly (affecting the amount of chemical present initially (Kennedy et al, 1989b).

The overall objective of the present study was to use an established isolated hepatocyte model (Moon et al. 1985; Kennedy et al. 1991; Bains & Kennedy, 2004) to assess the effects of acute and acclimatory temperature change on chemical biotransformation rates and resulting model predictions for BCF values in whole fish.

Using a substrate depletion method, depletion rate constant for several chemicals (benzo(a)pyrene [B[a]P], chrysene, 9-methylanthracene [9-MA] and 2,2',4,4',5,5'-hexachloro-1,1'-biphenyl [PCB-153]) dosed both singly and in a mixture, were determined. Empirically-derived depletion rate constants were extrapolated to whole organism clearance rates and further to BCF values using a fish bioaccumulation model (Arnot & Gobas, 2003, 2004).

3.3 Materials and Methods

3.3.1 Chemicals

Benzo(a)pyrene (log K_{ow}=5.97), chrysene (log K_{ow}=5.81), 9-methylanthracene (log K_{ow}=5.07) and deuterated chrysene were obtained from Sigma-Aldrich (St Louis, MO, USA). PCB-153 (log K_{ow}=7.75) and PCB-209 were obtained from AccuStandard (New Haven, CT, USA). Chemical purities were always >98%. Acetonitrile and hexane were obtained from Caledon Laboratories (Georgetown, ON, CAN).

3.3.2 Animals

Adult male rainbow trout (*Oncorhynchus mykiss*) were purchased from Miracle Springs Trout Farm (Misson, BC). Fish were housed in 500-L flow-through tanks supplied with dechlorinated municipal water at a temperature of 18 ± 1°C or 8± 1°C, under a light:dark cycle of 12:12. The average fish weight was 561 ± 34g (mean ± SEM). Fish were fed commercial salmon pellets (Ewos Pacifica Ltd., Surrey, BC, CAN) and were acclimated to test temperatures for at least two weeks prior to an experiment.

3.3.3 Hepatocyte isolations

Hepatocytes were isolated according to Moon et al (1985) with modifications (Gourley & Kennedy, 2009)Following anesthetization with buffered MS222 (0.05g/L), the liver was cleared of blood by cannulating the hepatic portal vein and perfusing with a solution containing Hanks Balanced Salts Solution (Ca^{2+} and Mg^{2+}-free, Sigma), 10mM HEPES (BioShop, Burlington, ON), 0.81 mM $MgSO_4$, and 5.95mM $NaHCO_3$ (pH=7.7), at a rate of 2.0mL/min/g. The liver was then perfused with the same solution containing collagenase (0.6 g/L Type IV, Sigma), until the hepatocytes began to disaggregate. The liver was then removed, minced and filtered successively through 250 and 75 µm nylon mesh, respectively. Cells were washed with the initial solution containing 20 g/L fatty acid-free bovine serum albumin (Sigma), 0.99 mM $CaCl_2$ (Fisher Scientific, Fairlawn, NJ), and 3 mM D-glucose (Sigma) with low speed centrifugation (46 x *g* for 4 min at 4°C) in a Beckman Coulter GS-15R centrifuge. The cell suspension was concentrated to 25 mg cells/ml (Bains & Kennedy 2004), and incubated for 30 min at the respective acclimation temperature of each fish (8°C or 18°C). Prior to chemical exposure and at the end of each experiment, cell viability was determined by trypan blue exclusion (Gill and Walsh, 1990).

3.3.4 Chemical exposure and incubation

Hepatocytes were incubated with 0.5 µM of the test chemicals (B[a]P, chrysene, 9-MA, PCB-153) either individually or in a mixture of the test chemicals, at the same concentration (0.5 µM each). Briefly, 5µl of a compound dissolved in acetonitrile was added to 500µL of the hepatocyte suspension (0.5µM test chemical and acetonitrile concentration [1%v/v]) in 2mL amber vials (Aglient Technologies, Santa Clara, CA).

Hepatocytes were incubated with chemicals at both 8°C and 18°C. Incubations were terminated at 0, 20, 40, 80, 100 or 120 min by the addition of 1 mL ice-cold hexane and brief vortexing. Controls consisted of heat-treated cells (100°C for 10 min) which were incubated with chemicals under identical conditions.

3.3.5 Chemical analysis

The amount of the parent compound in each vial was determined as follows: the internal standard (final concentration: 0.5µM of deuterated chrysene dissolved in acetonitrile, 1% v/v), was added to the vials which were vortexed for 1 min, then centrifuged at 800 x *g* for 10 min. Each hexane supernatant was transferred to clean 2mL amber vial and analysed using an Agilent 6890 gas chromatograph (GC) in conjunction with an Agilent 5973 mass spectrometer (MS) (Agilent, Mississauga, ON). The column used was an HP-5M5 5% phenyl methyl siloxane-coated capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) protected by a fused-silica deactivated guard column (5 m x 0.530 mm i.d.) (Agilent, Mississauga, ON). The injection volume was 1 µL, and the flow rate of the helium carrier gas was 1 mL/min. The gas chromatograph was programmed with an injection temperature of 63°C, followed by a temperature ramp of 20°C/min to a temperature of 200°C. The temperature was held at 200°C for 1 min, after which a 15°C/min temperature ramp was initiated to a final temperature of 285°C. The mass spectrometer quantified the target compounds at selected ions (*m/z* 192 for 9-MA, *m/z* 228 for chrysene, *m/z* 252 for B(a)P, *m/z* 360 for PCB-153, and *m/z* 240 for deuterated chrysene) using an ion energy of 70eV and an ion source temperature of 230°C.

Extraction efficiency assays were conducted in order to determine the loss of parent compound due to non-specific binding. Hepatocytes were incubated for 1 hr with 30 mM sodium azide to inhibit oxidative metabolism (Zuckerbraun et al. 2007). The extraction efficiency of all compounds was assessed simultaneously, at all time points, and compared to the extraction efficiency of PCB-209, as this compound is known to be non-metabolizable (Buckman et al. 2006). Incubations were performed for the same duration as the test incubations, and were terminated by the addition of 1 mL hexane. For sample analysis, 0.6 mL of hexane supernatant was removed and transferred into a clean 2 mL vial. An internal standard of 0.5 µM of deuterated chrysene was added. Analysis of the extract by GC-MS was as described previously (*m/z* 498 for PCB-209).

3.3.6 Data Analysis

3.3.6.1 Intrinsic and Hepatic Clearance

In order to avoid enzyme saturation, the concentration of test chemicals was selected such that the substrate depletion data followed first-order elimination kinetics, as evidenced by the linearity of the ln concentration decline for the duration of the assay (0.5 µM << *Km*). The rate of substrate depletion was used to determine the depletion rate constant when fitted to the linear equation:

$$\mathrm{Ln}C_t = \mathrm{Ln}C_0 - kt \qquad \text{(Bisswanger, 2008)} \qquad (1)$$

Where C_0 and C_t are the concentrations of parent compound (µM) per mg cells at time 0 and time t (min) respectively, and k is the first order depletion rate constant (per min). This calculated rate constant was then normalized to number of cells (Bains & Kennedy, 2004) in order to determine the rate of substrate elimination within the vial (intrinsic clearance [Cl_{int}]) using the following equations (Han *et al*, 2007):

Cl_{int}=depletion rate constant (k_r; hour^{-1})/concentration of cells (10^6cells/mL) (2)

This rate of intrinsic clearance can then be used to extrapolate from the substrate elimination rate *in vitro* to a rate that modelled *in vivo* clearance from the liver. Using the "well-stirred" liver model as described in Jones and Houston, (2004):

$$CL_H = \frac{Q_H \times (CL_{int} H_T W_L) \times (f_{u,b}/f_{u,h})}{Q_H + (CL_{int} H_T W_L) \times (f_{u,b}/f_{u,h})} \quad (3)$$

hepatic clearance (CL_H) can be determined. Parameters in the model include hepatic blood flow (Q_H; 536.1 mL/h/lg body weight (Nichols et al. 1990)), liver weight (W_L; 12.7g/kg body weight (Nichols et al. 1990)), and hepatocellularity (H_T; 510 x 10^6 cells/g liver weight (Hampton et al 1989)), as well as the fraction of chemical unbound in hepatocyte suspension ($f_{u,h}$) and blood plasma ($f_{u,b}$) which were determined as in Han et al. (2007).

3.3.6.2 Temperature Coefficient (Q_{10})

The temperature coefficient (Q_{10}) was determined using the following equation (Somero & Hochachka 1977; Koivusaari & Andersson 1984; Hochachka & Somero 2002):

$$Q_{10} = (k_1/k_2)^{10/(t1-t2)} \quad (4)$$

with t_1 and t_2 being 18°C and 8°C respectively, and k_1 and k_2 representing the rate constants derived at its respective incubation temperature. These values give a measure of the fold-change of substrate depletion within the incubations due to acute temperature change. Q_{10} values were calculated for individual and mixture incubations at both acclimation temperatures (8°C and 18°C).

3.3.6.3 BCF_{kmet} calculation

The inclusion of metabolism data into the bioconcentration model utilized hepatic clearance (CL_H) as the total clearance (CL_T) in the calculation of the metabolic rate constant k_{MET}.

$$k_{MET} = 0.024 CL_T / V_{ss} \quad (5)$$

where total clearance is divided by the volume of distribution at steady state (V_{ss}) as described in Arnot and Gobas (2004).

Utilizing K_{MET} as an output in the bioaccumulation model proposed by Arnot and Gobas (2003, 2004):

$$BCF = k_1 \Phi / (k_2 + k_e + k_G + k_{MET}) \quad (6)$$

bioconcentration factors (BCF) for the test chemicals were determined for both mixture and individual incubations, and for the different temperature regimes. This equation calculates the ratio of inputs (such as gill uptake, k_1) mitigated by the bioavailable fraction (Φ, the ratio of freely dissolved to total chemical concentration in the water), and effluxes (gill elimination (k_2), excretion (k_e), growth (k_G), and metabolism (k_{MET})). The procedure for these calculations is fully described in Arnot and Gobas (2003, 2004).

3.3.6.4 Statistical Analysis

All statistical analyses were conducted using JMP 7.0.2 (2007). A split-plot with incomplete blocks analysis design was used to compare between the depletion rate constants for all temperature regimes and incubation combinations. Tukey's Honestly Significant Difference test was utilized to determine where significant differences between treatments were present ($p<0.05$). Dunnett's test was employed to compare all chemical depletion rate constants to their respective controls ($p<0.05$) If no significant

difference was found between control and a chemical's depletion rate constant, the k_{MET} value for that chemical was set to zero.

3.4 Results

3.4.1 Depletion rate constant

Figures 3-1 and 3-2 show the depletion of each compound with time under all temperature regimes (acclimation: test incubation temperature; 8:8, 8:18, 18:8, 18:18) for individual and mixture incubations, respectively. Cell viability was >80% for all test incubations. As the initial concentrations were well below published Michaelis-Menten constants (K_m =40µM B[a]P (Nishimoto et al. 1992)), the metabolism of the compounds followed first-order kinetics (equation 1). The depletion rate constants were determined from the slope of these plots and are presented in Table 3-1. Heat-treated control slopes were not significantly different from zero. The extraction efficiency of 9-MA, B[a]P, chrysene and PCB-153 were not significantly different from 100%, so no adjustments to the depletion rate constant were required (Appendix B).

Acclimation temperature had varying effects on chemical biotransformation in individual incubations. Cells incubated at their acclimation temperatures showed a significant difference between 8°C:8°C- and 18°C:18°C-acclimated cells for both B[a]P ($p<0.001$) and 9-MA ($p=0.0328$), with cooler temperatures resulting in lower depletion rate constants. For chrysene, there were no significant differences between depletion rate constants between cells incubated at their respective acclimation temperatures. PCB-153 was not metabolized in any of the temperature regimes (Figure 3-3).

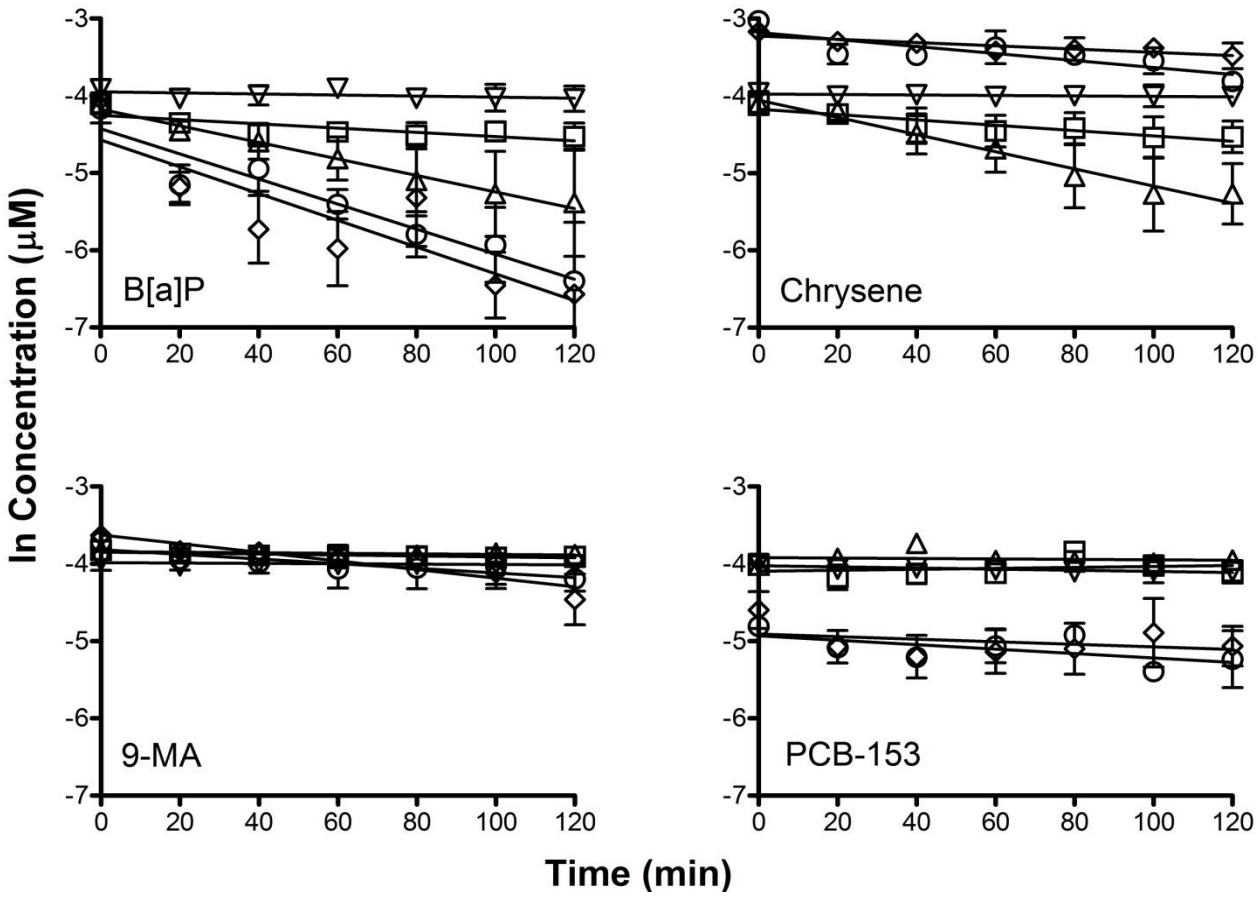

Figure 4: Substrate depletion graphs of 9-MA, B[a]P, PCB-153 and chrysene incubated individually with 25mg hepatocytes/ml at four temperature regimes: two incubations at acclimation temperatures (8:8 □ and 18:18 ◊), an acute temperature increase (8:18 △) and an acute temperature decrease (18:8 ○) compared to the heat-treated control (▽). For each chemical, n=6 (3 fish/acclimation temperature (8°C and 18°C)). All control slopes were not significantly different from zero.

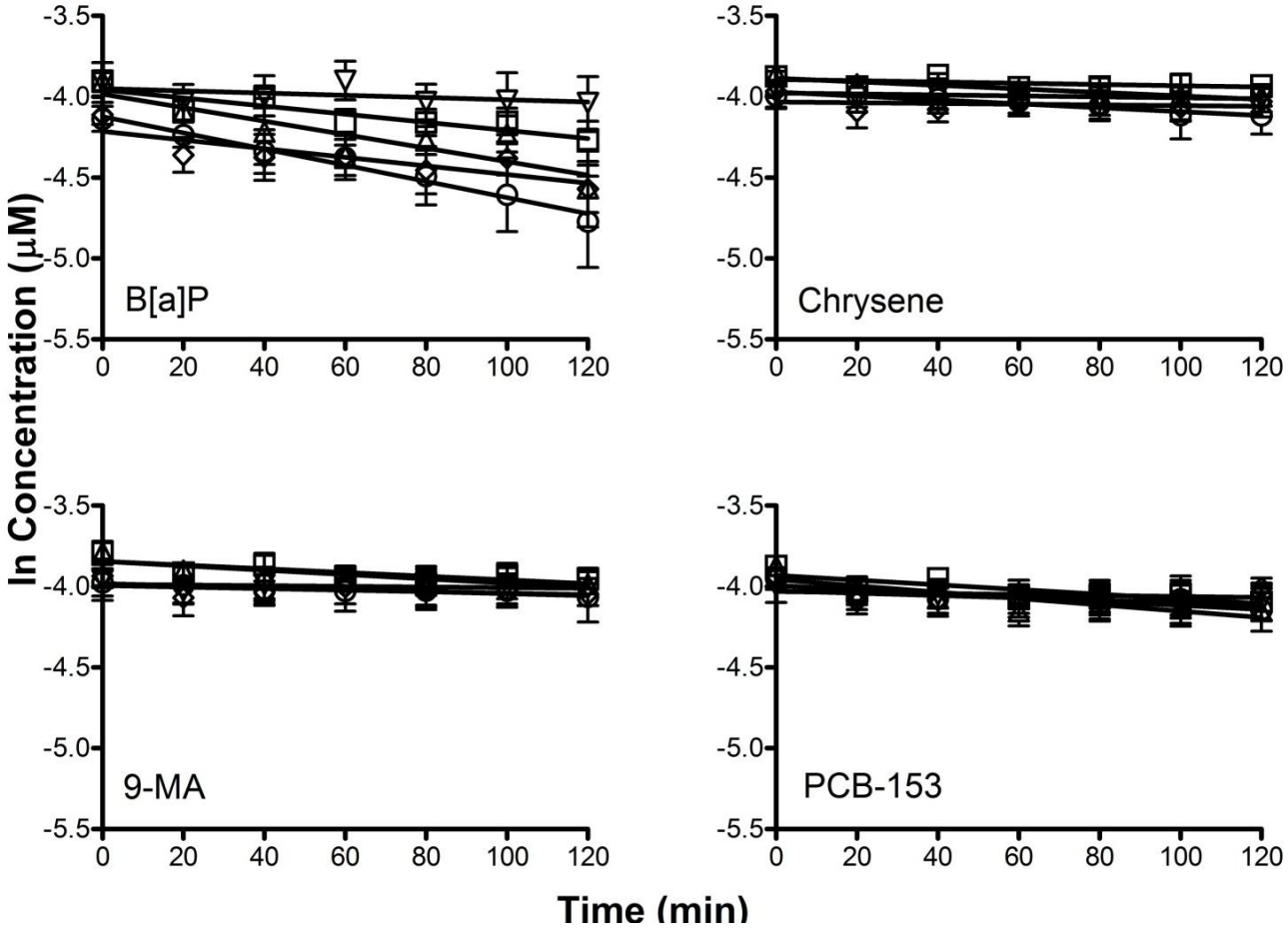

Figure 5: Substrate depletion graphs of 9-MA, B[a]P, PCB-153 and chrysene incubated concurrently in 25mg hepatocytes/ml at four temperature regimes: two incubations at acclimation temperatures (8:8 □ and 18:18 ♢), an acute temperature increase (8:18 △), and an acute temperature decrease (18:8 ○) compared to the heat-treated control (▽). For both acclimation temperatures (8°C and 18°C), n=6. All control slopes were not significantly different from zero.

Individual	Depletion rate constant (k_r; 10^{-3} min^{-1})			
	8-8	8-18	18-8	18-18
9-MA	0.61* (0.18 – 1.04)	0.36 (0 – 0.80)	3.04* (1.17 – 4.90)	5.61* (2.92 – 8.31)
B[a]P	2.64* (0.17 – 5.30)	10.7* (9.15 – 12.27)	16.2* (10.65 – 21.83)	17.3* (5.76 – 28.87)
Chrysene	3.47* (1.92 – 5.02)	11.1* (8.83 – 13.4)	4.52* (1.10 – 7.93)	2.11* (0.89 – 3.32)
PCB-153	0.63 (0 – 2.29)	0.29 (0 – 2.76)	2.86 (0 – 6.96)	1.67 (0 – 6.76)
Mixture				
9-MA	1.80* (0.36 – 3.23)	1.35* (0.48 – 2.22)	0.61* (0.30 – 0.92)	0.47 (0 – 1.49)
B[a]P	2.51* (1.10 – 3.93)	4.16* (1.35 – 6.98)	5.00* (4.11 – 5.89)	2.67* (0.66 – 4.68)
Chrysene	0.75 (0 – 0.12)	1.10* (0.63 – 1.56)	1.20* (0.79 – 1.61)	0.22 (0 – 1.33)
PCB-153	1.46 (0.22 – 2.70)	1.95 (0.52 – 3.38)	1.23 (0.43 – 2.02)	0.30 (0 – 1.76)

Table 3-1: Depletion rate constants (k_r) for 9-methylanthracene, benzo[a]pyrene, chrysene and PCB-153, incubated individually and in mixture, for all temperature regimes. The results presented are mean with 95% confidence intervals presented in brackets.
The * indicates significantly different from control.

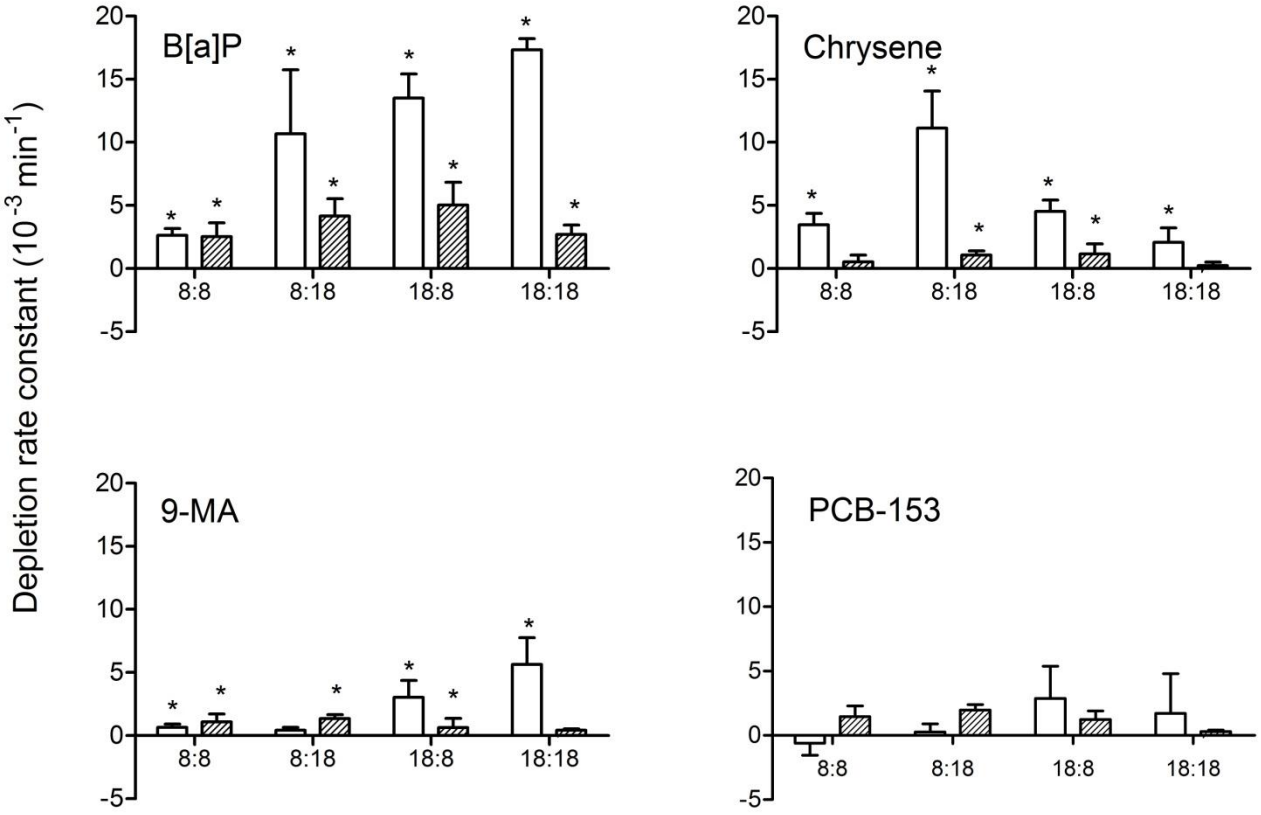

Figure 6: Depletion rate constants (k_r) of 9-MA, PCB-153, chrysene, and B[a]P in individual (open bars) and mixture incubations(hatched bars). The * indicates significant difference from the contol; ie the PCB-153 depletion rate constant at all temperature regimes was not significantly different from the control and hence showed no significant metabolism. There was no significant difference between the depletion rate constants of the chemicals in a mixture when exposed to any temperature regime.

Acute temperature increases (8:8°C *v*. 8:18°C treatment group) resulted in significantly increased depletion rate constants for B[a]P ($p<0.0001$; $Q_{10}=3.71\pm1.51$). Chrysene showed a similar pattern ($p=0.030$; $Q_{10}=3.14\pm0.16$). Acute temperature decreases (18:18°C *v*. 18:8°C treatment group) did not result in significantly changes in depletion rate constants for chrysene or B[a]P. For 9-MA and PCB-153, there was no statistical difference between depletion rate constants measured for any acute temperature change in individual incubations.

When comparing between depletion rate constants for chemicals incubated individually and in a mixture at acclimation temperature, statistically significant differences in depletion rate constants were only present in B[a]P. At 18°C acclimation (18:18°C), the depletion rate constant of B[a]P in a mixture incubation was significantly less than its depletion rate constant in mixture incubation at 8°C (8:8°C) ($p<0.001$). When comparing B[a]P metabolism when incubated in individual and mixture incubations, at 18°C acclimation the individual incubation depletion rate constant was 6.5-fold greater that of the mixture, whereas for the 8°C, the depletion rate constants for B[a]P were almost equal between individual and mixture incubations. Within the mixture, there was no statistically significant difference between depletion rate constant for chemicals when incubated at acclimation temperature.

Comparing individual and mixture incubations, the direction of the acute temperature change affected the magnitude of the difference between depletion rate constants. With an acute temperature increase from 8°C to 18°C, there was a significant difference ($p=0.0081$; $Q_{10}=3.14\pm0.16$ for individual incubations, $Q_{10}=0.91\pm1.10$ for mixture incubations) between the depletion rate constants of chrysene incubated

individually and in mixture, but there was no significant difference between mixture and individual depletion rate constants with an acute temperature decrease. The reverse is true for B[a]P; significant differences ($p=0.0128$; $Q_{10}=1.23\pm0.31$ for individual incubations, $Q_{10}=0.88\pm0.31$ for mixture incubations) are present between depletion rate constants for mixture and individual chemical incubations with an acute decrease in temperature, but with an acute increase in temperature, no significant difference is found. No difference was found between depletion rate constants when comparing within temperature treatments for 9-MA incubated individually or in a mixture. When comparing between depletion rate constants generated in a mixture, there was no statistically significant difference between depletion rate constants for the four test chemicals derived from cells exposed to an acute temperature change.

3.4.2 Bioconcentration Factor

Using the well-stirred liver model (Jones & Houston, 1994) and a fish bioaccumulation model (Arnot & Gobas, 2003), *in vitro* depletion rate constants were extrapolated to estimate whole organism BCF values. The 95% confidence intervals of the depletion rate constants were incorporated into the models to generate a range of BCF values. Tables 3-2, 3-3 and 3- 4 give both calculate biotransformation rate constants (k_{MET}) and BCF values for both individual and mixture incubations for all temperature conditions.

Values for intrinsic clearance (CL_{int}), hepatic clearance (CL_H) and the metabolism rate constant (k_{MET}) all followed the same directional trends as depletion rate constants with regards to acclimation and acute temperature changes. The magnitude of effect that is present in depletion rate constants is also present in BCF values, with increases in

depletion rate constant resulting in decreases in modelled BCF values. However, temperature is also incorporated into the faecal elimination rate constant (k_E) in the bioaccumulation model, so increases or decreases in BCF values are not directly proportional to changes in the depletion rate constant.

The incorporation of biotransformation data into calculations of BCF resulted in improved, more accurate values for B[a]P and chrysene, regardless of temperature (Tables 3-3 and 3-4). With generally lower derived depletion rate constants, the BCF values for 9-MA were reduced to a much lesser extent than either B[a]P or chrysene values (Table 3-2). The depletion rate constant for PCB-153 was not significantly different from the control; hence metabolism information was not incorporated into the BCF equation (modelled BCF values were 22,292 and 17,103 for 8°C and 18°C-acclimated fish, respectively).

When comparing between BCF values modelled using incubations at acclimation temperature, higher acclimation temperatures reduced the BCF values for 9-MA and B[a]P (relative to k_{MET}=0) to a greater extent than colder acclimation temperature. Both B[a]P and 9-MA showed significant differences in the modelled BCF values between acclimation temperatures. BCF values modelled from depletion rate constants derived from 18°C-acclimated and 18°C incubated hepatocytes were reduced 67% and 96% for 9-MA and B[a]P respectively (compared to BCF values were k_{MET} =0). However, using depletion rate constants generated at 8°C, modelled BCF values were reduced only 20% and 82% for 9-MA and B[a]P respectively. In contrast to individual incubations, when incubated in mixture, there was no significant difference between BCF values generated at acclimation temperatures for 9-MA or B[a]P. Chrysene did not show significant

	8:8	8:18	18:8	18:18
$BCF_{Kmet=0}$	10687		9749	
Individual				
k_{MET} (x 10^{-2} day^{-1})	0.55 (0.16 – 20.6)	NSD	2.68 (1.04 – 4.30)	4.91 (2.58 – 7.22)
BCF	8600 (7600 – 10000)	10687	4600 (3500 – 6800)	3200 (2400 – 4700)
% reduction	20%	0%	53%	67%
Mixture				
k_{MET} (x 10^{-2} day^{-1})	1.59 (0.32 – 2.86)	1.20 (0.43 – 1.97)	0.55 (0.27 – 0.82)	NSD
BCF	9400 (4800 – 9400)	7000 (5800 – 9000)	7900 (7300 – 8800)	9749
% reduction	12%	34%	19%	0%

Table 3-2: Metabolism rate constant (k_{MET}) modelled bioconcentration factors, and the percent reduction in modelled BCF values (compared to $BCF_{kmet=0}$) for 9-methylanthracene generated using depletion rate constants from both individual and mixture incubations for all temperature regimes. NSD = k_{MET} was not significantly different from zero, as the elimination rate constant not significantly different from control.

	8:8	8:18	18:8	18:18
$BCF_{Kmet=0}$	40202		29167	
Individual				
k_{MET} (x 10^{-2} day^{-1})	2.46 (0.16 – 4.85)	9.64 (8.28 – 10.9)	14.3 (9.58 – 18.8)	15.2 (5.28 – 24.3)
BCF	7100 (4000 – 16000)	2100 (1800 – 2400)	1300 (1000 – 1900)	1200 (800 – 3300)
% reduction	82%	95%	96%	96%
Mixture				
k_{MET} (x 10^{-2} day^{-1})	2.34 (1.03 – 3.64)	3.85 (1.26 – 6.40)	4.61 (3.80 – 5.41)	2.48 (0.61 – 4.29)
BCF	7300 (5100 – 14000)	4800 (3000 – 12000)	3800 (330 – 4400)	6300 (4000 – 15000)
% reduction	82%	88%	87%	79%

Table 3-3: Metabolism rate constant (k_{MET}) modelled bioconcentration factors, and the percent reduction in modelled BCF values (compared to $BCF_{kmet=0}$) for B[a]P generated using depletion rate constants from both individual and mixture incubations for all temperature regimes.

	8:8	8:18	18:8	18:18
$BCF_{Kmet=0}$	34420		26092	
Individual				
k_{MET} (x 10^{-2} day^{-1})	3.27 (1.82 – 4.68)	10.1 (8.12 – 12.1)	4.24 (1.05 - 7.34)	2.00 (0.85 – 3.13)
BCF	5700 (4200 – 9000)	2100 (1700 – 2500)	4200 (2600 – 11000)	7500 (5300 – 13000)
% reduction	83%	94%	84%	71%
Mixture				
k_{MET} (x 10^{-2} day^{-1})	NSD	1.04 (0.60 – 1.48)	1.14 (0.75 – 1.53)	NSD
BCF	34420	13100 (10000 – 18000)	10800 (9000 – 13000)	26092
% reduction	0%	62%	58%	0%

Table 3-4: Metabolism rate constant (k_{MET}) modelled bioconcentration factors, and the percent reduction in modelled BCF values (compared to $BCF_{kmet=0}$) for chrysene generated using depletion rate constants from both individual and mixture incubations for all temperature regimes. NSD = k_{MET} was not significantly different from zero, as the depletion rate constant not significantly different from control.

differences between BCF values derived at acclimation temperatures in either individual or mixture incubations.

Acute temperature increases tended to decrease BCF values for B[a]P and chrysene in both individual and mixture incubations. Acute temperature increases for B[a]P (from 8°C to 18°C) resulted in reduction in BCF values of 95% for individual incubations, compared to BCF calculated with k_{MET} =0. When B[a]P was incubated in mixture, inclusion of metabolism data generated with acute temperature increase reduced the BCF values by 88% compared to BCF calculated with k_{MET} =0. However, acute temperature decreases did not reduce the modelled BCF values for B[a]P relative to BCF values modelled at acclimation temperature. For chrysene, an increase in the percent reduction of BCF values was the result of acute temperature increase in individual incubations, but acute temperature decreases did not result in a significantly difference between 18:8 and 18:18 BCF values. In a mixture, the depletion rate constant for chrysene was not significantly different from the control (k_{MET} =0) when incubated at acclimation temperature. Acute temperature changes resulted in significant depletion rate constants for chrysene in a mixture; compared to BCF values modelled with (k_{MET} =0), an acute temperature increase reduced the modelled BCF value by 62%, and an acute temperature decrease reduced the modelled BCF by 58%. Acute temperature change did not have a significant effect on BCF values for 9-MA.

Differences are present when comparing between BCF values modelled from individual and mixture incubations exposed to the same temperature regime. Acute temperature changes generally result in significantly different BCF values, similar to the depletion rate constant results. BCF values modelled from individual and mixture

incubations of chrysene at 8°C acclimation, 18°C incubation temperature were significantly different ($p<0.05$). This difference between BCF values results in an 84% reduction in BCF value modelled from individual incubations (compared to BCF values were k_{MET} =0), compared to 58% reduction in BCF modelled from mixture incubations. B[a]P also showed significant differences between BCF values modelled from individual and mixture incubations when exposed to an acute temperature decrease (from 8°C to 18°C). The only instance where there was a significant difference between BCF values modelled from mixture and individual incubations at acclimation temperature was for 18°C acclimated B[a]P. Using mixture incubation depletion rate constants, the modelled BCF value for 18:18 incubated B[a]P was reduced 77% from BCF modelled when k_{MET} =0, compared to 96% for BCF values modelled from individual incubations. Generally, reduced depletion rate constants resulting from mixture incubations resulted in increases in modelled BCF values, compared to BCF values modelled from individual incubations.

3.5 Discussion

A substrate depletion method in an isolated hepatocyte model system was successfully used to assess the biotransformation of xenobiotics. This methodology appears to be an efficient and accurate means of empirically determining depletion rate constants, intrinsic clearance rates, and BCF values of various environmental contaminants. Isolated hepatocytes have been used extensively in the examination of xenobiotic biotransformation (Andersson & Koivusaari 1986; Gill & Walsh 1990; Han et al. 2007, 2008; Kennedy & Tierney 2008; Johnston et al. 1999; Mingoia et al. 2010; Nishimoto et al. 1992; Zaleski et al. 1991; Steward et al, 1990). However, the majority of these studies have assessed biotransformation by the production of metabolites. In this

study, a simpler substrate depletion approach generated useful results, and biotransformation rates for B[a]P calculated in this study are in good agreement with previously published results (Han et al. 2007, 2008).

For both mixture and individual incubations, PCB-153 showed no metabolism in the trout hepatocyte suspension. This was expected, as PCBs as a class are considered generally non-metabolizable. Buckman and colleagues (2004) exposed juvenile rainbow trout to different Aroclor mixtures, and determined that no significant biotransformation occurred for any of the PCB congeners. By using PCB-153 in our hepatocyte incubations, we obtained a useful negative control; a benchmark against which an assessment of whether or not a compound was significantly metabolized.

Incorporating biotransformation rates into modelled bioconcentration factors (BCF) can result in more physiologically relevant bioaccumulation assessments. Modelling the BCF values of chemicals that are readily metabolizable without the inclusion of biotransformational elimination can result in an overestimation of bioaccumulation (Baussant et al, 2001). Including metabolism in BCF models incorporates all routes of elimination (e.g. diffusion across the gills, faecal egestion, and growth), offsets the modelled uptake calculated by physical-chemical parameters (Arnot & Gobas 2003, 2004; Veith et al. 1979), and resulted in lower, more accurate BCF values for metabolizable, high log K_{ow} compounds. Such a method may lead to a more accurate and efficient evaluation of the more than 23,000 chemicals on CEPA's Domestic Substances List (Arnot & Gobas, 2006). While not a replacement for empirically derived BCF and BAF values, modelled BCF values that include metabolism can be compared to guidelines to determine if a chemical can bioaccumulate. Through the use of depletion

rate constants determined *in vitro*, extrapolated BCF values can allow for rapid screening of anthropogenic chemicals.

As shown previously (Han et al. 2007; 2008), depletion rate constant data derived *in vitro* can be extrapolated to whole organism biotransformation rates, and generate bioconcentration factors (BCF) which are comparable to empirically-derived, *in vivo* literature values. Currently, bioaccumulation models allow for the incorporation of metabolism, but with the dearth of available data (van der Linde et al. 2001), metabolism rate constants (k_{MET}) are usually set to zero. Incorporation of metabolism data resulted in reductions of modelled BCF values ranging from 12% to 96% from the calculated BCF with no metabolism data. The premise behind the incorporation of depletion rate constant data into the BCF calculation is to improve the accuracy with which the modelled BCF fits with experimental data (Bassaunt et al. 2001).

Environmental temperature is known to affect xenobiotic biotransformation rates in ectothermic organisms through a variety of mechanisms from changes in lipid membrane viscosity (Hazel & Prosser, 1974), to increases or decreases in the amount of free energy required for enzymatic reactions to proceed (Somero & Hochachka, 2002). Typically, biotransformation rates are constant when incubation temperature matches acclimation temperature (through thermal compensation) by adaptations in the composition of the lipid membrane, and alterations in activity and concentration of enzymes (Kennedy & Walsh, 1996). Acute temperature decreases can result in lower biotransformation rates due to decreases in membrane fluidity and the corresponding effects on membrane-bound enzymes (Hazel & Prosser, 1974) as well as the slowing of enzyme-mediated reaction rates, with the reverse trend with acute temperature increases.

When comparing between acclimation temperatures, thermal compensation played a role in the biotransformation of the chemicals. Chrysene biotransformation showed thermal compensation, having similar depletion rate constants in cells incubated at their acclimation temperatures. Thermal compensation in PAH metabolism has been shown previously (Wills 1983; Karr et al. 1985), as has compensation in many xenobiotic-metabolizing enzymes (Blanck et al. 1989; Kennedy et al. 1991). As well, the production of phase II B[a]P metabolites in *Opsanus beta* hepatocytes showed thermal compensation (Gill & Walsh, 1990). In contrast to chrysene, B[a]P and 9-MA showed increased depletion rate constants with increased acclimation temperature. This differs from previous studies showing that microsomes from cold-acclimated fish had higher enzymatic activities than warm-acclimated fish (Ankley et al. 1985; Carpenter et al. 1990). Increased depletion rate constants with acute temperature increases can be the result of incomplete compensation in the enzymes to the new acclimation temperature (Prosser 1991). While both chrysene and B[a]P are phase I metabolised by CYP 1A, possible incomplete compensation in phase II and other phase I enzymes resulted in the differential compensation in depletion rate constants between the two CYP 1A substrates. Both glutathione-S-transferase (GST) and epoxide hydrolase (EH) have been shown to display low sensitivity to temperature change (Kennedy et al. 1991), and both function in the biotransformation of B[a]P (Varanasi et al. 1989). Little is known about the biotransformation of 9-MA, only that it can be phase I metabolised by CYP 2B (Anzenbacher et al. 1996) and that phase II metabolism does occur (Turcotte 2008).

Acute temperature increases tended to increase depletion rate constants; however, acute decreases in temperature showed no significant effects on the metabolism of these

chemicals from hepatocyte suspensions when compared to their respective acclimation controls. Both B[a]P and chrysene showed a significant increase in depletion rate constant with the acute temperature change from 8°C to 18°C. These results are in accordance with the findings of Gill and Walsh (1990), Ankley et al. (1985), Karr et al. (1985) and others. As PAHs have been found to passively diffuse through the lipid membrane (Kennedy & Walsh, 1996), and are metabolized by CYP450 1A (Stegman & Hahn, 1994), it is not surprising that these xenobiotics would have similar trends in depletion rate constants with regard to thermal changes. While increased temperature increased the depletion rate constant, the expected decline in depletion rate constant with a decrease in temperature was not seen with any chemical. Gill and Walsh (1990) found similar results, showing a significant increase in the rate of Phase II B[a]P metabolites formed by the cold-acclimated fish incubated at warmer temperatures, as compared to the warm-acclimated fish, which showed no change in metabolite formation across incubation temperatures.

In modelling the effect temperature has on BCF values, limitations within model parameters can result in less accurate results. Aside from the measured effect temperature has on depletion rate constant and the extrapolated k_{MET} value, temperature also plays a role in the calculation of the dietary uptake rate constant (k_D), and subsequent faecal elimination rate constant (k_e) calculations. As a result, BCF values calculated using different acclimation temperatures produced modelled BCF that differ even without k_{MET} data incorporated. While the modelled effects of temperature on dietary uptake and faecal egestion are included in the fish bioaccumulation model (Arnot & Gobas, 2003), the effect temperature has on uptake and elimination of xenobiotics across the gills is not

modelled. Studies have shown that uptake of chemical across the gills is significantly affected by temperature (Kennedy et al. 1989a; Jimenez et al. 1987). As a result, the effects of acclimation and acute temperature changes on uptake and elimination of xenobiotics cannot be accounted for totally in the BCF model employed.

When comparing the depletion rate constants of chemicals incubated individually and in the mixture, the mixture rates were generally reduced. In the mixture incubations, hepatocytes are exposed to a total of 2.0 µM test chemicals, a four-fold increase in concentration compared to the individual incubations, and may lead chemical competition for biotransformation pathways. This is clearly evident with the PAHs, particularly B[a]P, as shown in Table 3-1. Both B[a]P and chrysene have been shown to be substrates for CYP1A (Stegeman et al. 1998; Stegeman & Hahn, 1994) and both show reduced depletion rate constants in the mixture compared to individual incubations. However, this does not explain the reduction in depletion rate constant for 9-MA (which has been shown to be metabolized by CYP 2B1 [Azenbacher et al. 1996]), and the total concentration of chemicals are well below the published value for the K_m of B[a]P (40 µM [Nishimoto et al. 1992]). Another likely possibility is the cosolvent effect, resulting in the test chemicals acting as solvents for each other, decreasing the dissolution of the test compounds into the aqueous medium. PAHs and PCBs have both been shown to act as cosolvents (Zemanek et al. 1997; Janikowski et al. 2002; Bardi et al. 2000; Li et al. 1992; 1995). The result of cosolvent effect is a decrease in the amount of chemical available to the enzymes, resulting in decreased depletion rate constants for hepatocyte incubations with chemical mixtures. The reduction in biotransformation in the mixture is important for two reasons; B[a]P and chrysene exist in the environment in complex

mixtures with other classes of chemicals as well as other PAHs (Schwarzenbach et al, 2006), and secondly, understanding the reduction in metabolism and the subsequently reduced elimination that results from concurrent exposure to a mix of chemicals would allow for better regulation of these ubiquitous pollutants.

A general trend of decreased depletion rate constants when chemicals were incubated in mixture, as opposed to individual incubations, resulted in increased modelled BCF values for the chemicals in mixtures. Mixture incubations may be more environmentally relevant, as many pollutants, such as PCBs and PAHs, are present in the environment in complex mixtures (McFarland & Clark, 1989; Douban 2003). The decreased depletion rate constants in mixture incubations are a more conservative measure of biotransformation rate of chemicals (compared to individual incubations) in the hepatocyte suspension, and the increased BCF values that are derived using these lower depletion rate constants result in more protective BCF values. Using the CEPA bioaccumulation endpoint of BCF > 5000 as bioaccumulative, B[a]P, 9-MA, PCB-153 and chrysene all appear to be bioaccumulative when in mixture. BCF values derived using individual incubations tend to be less than those derived using mixture data, but no chemical had a BCF value consistently > 5000. Depending on the temperature regime and the chemical, some chemicals would not be considered bioaccumulative at warmer temperatures (B[a]P at 8°C:18°C, 18°C:8°C and 18°C:18°C; 9-MA at 18°C:18°C; chrysene at 8°C:18°C), but are bioaccumulative at cooler temperatures. Furthermore, consideration of the 95% confidence interval for the modelled BCF values means that one could say that a chemical could be bioaccmulative if their range of BCF values includes 5000.

Generally, BCF values modelled using depletion rate constants from chemicals incubated individually fit well with previously published experimental data, but when chemicals were incubated in mixture, the incorporation of the lower depletion rate constants into the BCF calculation resulted in BCF values that were outside the range of empirical values. In order to generate a range of BCF values from quality data, guidelines for evaluating BCF data were used (Parkerton et al. 2008) to garner BCF values from primary literature (Appendix D). Compared to an empirical BCF range of 224 to 3208, the modelled BCFs for B[a]P that utilize the biotransformation data from mixture incubations all overestimated B[a]P bioaccumulation. For chrysene, the modelled BCFs that included mixture incubation data were 44% to 53% greater than the top end of the empirical BCF range (650 – 6088). The trend is the same for 9-MA; however, as the comparison is against only one empirical BCF value (4538; Southworth et al. 1987), this comparison should be viewed with caution. The disparity between BCF values modelled using mixture depletion rate constants and empirical BCF is not surprising, as BCFs are determined *in vivo* using single chemical exposures.

In comparing the modelled BCF values with the bioaccumulation endpoints set up in the PBT assessments (Government of Canada 1999), it becomes clear that best professional judgement must be used in determining if a chemical is bioaccumulative. B[a]P has modelled BCF values below the endpoint of 5000 for some temperature treatments, but lower temperatures and mixture incubations generally results in BCF values that exceed 5000. Clearly, standardized testing methods need to be put forth in order to produce highly comparable BCF values (validated by *in vivo* studies) with which to assess bioaccumulation.

Through the generation of *in vitro* depletion rate constant data, assessment of the thousands of anthropogenic chemicals previously and currently used for the potential to bioaccumulate may be realistic. The need for this data is critical, as many countries, such as Canada (Government of Canada 1999), the United States (US EPA 1976), and those in the European Union (Bodar et al. 2002), aim to assess presently-used and new chemicals for their persistence in the environment, bioaccumulative potential and inherent toxicity (Phase I, P B T assessments). Incorporating metabolism into the BCF model more accurately reflects the potential body burdens experienced by aquatic biota, and by improving our knowledge of these processes, we can better protect both the environment and human health. As a result of the versatility of the Arnot and Gobas (2003, 2004) fish bioaccumulation model, the inclusion of metabolic rates at different temperatures provides for more environmental relevance in the modelled BCF, further improving the prediction of bioaccumulative potential for pollutants in aquatic systems. These modelled BCFs, while not a replacement for empirically derived, *in vivo* values, are a useful first screening step, ensuring that those that are bioaccumulative are regulated appropriately and prevented from reaching toxic levels in humans and the environment.

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4: Conclusion

The primary objectives of this research were to measure the depletion rate constants of hydrophobic organic chemicals *in vitro* using rainbow trout hepatocytes, and to extrapolate this data to the whole organism level to estimate bioconcentration factors (BCFs). Isolated hepatocytes provided a simplified system and controlled environment where substrate depletion could be measured (instead of traditional metabolite formation) to determine biotransformation rates. The hepatocyte model system eliminates the confounding factors involved with working with whole animals (i.e., inter-individual differences in physiology, distribution of compound), as well as the problems involved in working with subcellular fractions (i.e. greater non-specific binding, required addition of cofactors). Hepatocytes retain cellular structure and function, resulting in greater physiological relevance. Using the substrate depletion method to determine depletion rate constants is advantageous as it allows for easy and accurate quantification of the rate of biotransformation, without prior knowledge of the enzymes involved in the reaction. Compared to measuring product formation, measuring the rate of substrate depletion does not require knowledge or synthesis of the metabolites generated by the reactions, and the measured apparent rate is the sum of all the metabolic pathways.

The measured loss of the parent compound in the cell suspension was used to determine depletion rate constants which were then extrapolated to calculate estimates of the *in vitro* clearance rate (intrinsic clearance, CL_{int}). CL_{int} values were extrapolated to model hepatic clearance (CL_H) using the "well-stirred" liver model (Jones & Houston

2004), which modelled the rate at which a chemical is cleared from the liver of trout. As the liver is the primary site of metabolism for hydrophobic compounds, hepatic clearance is used to represent the rate at which a chemical is cleared from the whole organism through biotransformation, hence CL_H was used in the metabolic rate constant equation to determine k_{MET}. By incorporating a metabolic rate constant into a fish bioaccumulation model, more accurate predictions of BCF were modelled. Two factors that affect biotransformation within an *in vitro* system, exposure to chemicals in a mixture and temperature change, were also evaluated for the magnitude of their effect on the depletion rate constant, and how modelled BCF values were subsequently affected.

While using isolated hepatocytes is an excellent means of assessing the xenobiotic biotransformation and their potential for bioaccumulation, it is not without its flaws. Artefacts of *in vitro* testing, such as non-specific binding of the hydrophobic chemicals to the cells and the vials, could result in less compound bioavailable to the enzymes, and an underestimate of the intrinsic clearance of the compound. As well, modelling BCF values from depletion rate constants is subject to the quality of data and models currently available. The 'well-stirred' liver model includes assumptions that rely heavily on hepatic blood flow and less so on biotransformation; these assumptions have been criticized (Yang et al. 2007). As well, the BCF values modelled in this research are limited to incorporating only hepatic clearance rates, as corresponding values for extrahepatic biotransformation rates (gill, intestinal and others) for the test chemicals were not available. As a means of deriving screening-level values for bioaccumulation assessments, the methodology utilized in this thesis has merit as a means of assessing currently-used and new anthropogenic chemicals.

4.1 Comparison of Intrinsic Clearance and Bioconcentration Factors

Using hepatocytes to determine the depletion rate constant of xenobiotics is a highly physiologically relevant, effective means with which to determine their rate of biotransformation; extrapolating from the depletion rate constant to intrinsic clearance allows for comparison between the results presented in this thesis, and results presented by other researchers. Since most researchers do not determine depletion rate constants and instead measure the enzyme kinetics of xenobiotic metabolism, comparison between results was conducted using intrinsic clearance (CL_{int}). CL_{int} was determined from published results by dividing the theoretical maximum of the metabolic rate (V_{max}) with the concentration of compound required to produce half the V_{max} (K_m) (Rane et al. 1977). Using B[a]P as a model compound, as it has been well researched, intrinsic clearance can be calculated and compared to results to those published in literature. The results of the present study (CL_{int} = 0.044 mL/h/10^6 cells at 13°C) are in close agreement with those of previously published results. Nishimoto and colleagues (1992) reported an apparent V_{max} of 1300 pmol B[a]P metabolized/h/10^6 cells, and a K_m of 29 µM; their resulting intrinsic clearance (CL_{int}) was 0.045mL/h/10^6 cells. Using the results of B[a]P incubated in isolated hepatocytes from mirror carp, a CL_{int} 0.053 mL/h/10^6 cells can be calculated (Zaleski et al. 1991) An intrinsic clearance of 0.029 mL/h/10^6 cells can be determined from the results of Stewart et al (1990). The CL_{int} values reported by Han and colleagues (0.044 mL/h/10^6 cells [2007]; 0.026 to 0.068 mL/h/10^6cells [2008]) were determined using substrate depletion methodology as in the present study, as opposed to measuring the formation of metabolites. One caveat to consider when comparing results generated by measuring metabolite formation to results from generated using the substrate depletion

method is that metabolite formation commonly measures the rate of production of only one or a few metabolites. The resulting calculation could be an under estimation of the rate of intrinsic clearance. However, generally comparable results can be seen in the data presented here, generated by different researchers using different methodologies.

Comparison of the BCF modelled values generated in the present research show good correlation with empirical data and other extrapolated data. By extrapolating the depletion rate constant data presented by Hsiao Lee (2009), and using the methodology in Han et al. (2009), calculation of modelled BCFs for 9-MA, B[a]P and chrysene from depletion rate constants generated using S9 liver fractions was possible. In those experiments, S9 fractions from trout were incubated with all three chemicals, therefore comparison with generated BCF values modelled using depletion rate constants from hepatocyte mixture incubations is most appropriate. Comparing the BCFs values for B[a]P modelled using both S9 and hepatocyte data, the resulting BCF values are not statistically significantly different, as the S9 generated BCF is within the 95% confidence interval range of the modelled BCF values derived in this thesis. In comparison, the BCF values for 9-MA and chrysene were significantly greater for S9-derived than for hepatocyte-derived values, reflecting a greater depletion rate constant in hepatocytes. This also holds true for the comparison between the BCF values modelled using S9 and microsome data in Han et al (2009) compared to the BCF values modelled using hepatocyte derived depletion rate constants (Han et al. 2007, 2008). Han and colleagues (2009) found that BCF values extrapolated from S9 and microsome data overestimated bioaccumulation, likely due to the lack of phase II enzyme activity and increased non-specific binding in the subcellular fractions. Comparing between BCFs extrapolated

from hepatocyte individual chemical incubations, the BCF value (1528) generated by Han et al (2007) are within the 95% confidence interval range of the present results (1400 – 3400), whereas the BCF value of 1206 (Han et al. 2008) is somewhat lower. It is likely that this small difference is not significant, but as there are no statistical error margins given on the BCF values modelled in Han et al (2008), comparisons are difficult. General agreement in the extrapolated values between researchers is evidence of the validity of the methodology proposed in Han et al (2007, 2008, 2009) and further explored in this thesis.

4.2 Effects of Chemical Mixtures

While cellular incubations typically are conducted using individual chemicals, in the environment, complicated mixtures of chemicals are more environmentally relevant as aquatic systems often are contaminated with thousands of different compounds (Rosen 2007; Schwarzenbach et al. 2006). These compounds may have unknown synergistic, antagonistic or additive effects when present in a mixture, affecting biotransformation and subsequently bioaccumulation. Inhibitory effects of some chemicals are well known; piperonylbutoxide (PBO) (Sijm & Opperhuizen 1988; Sijm et al. 1993) and imidazole antifungals (clotrimazole, ketoconazole, miconazole) (Miranda et al. 1998; Levine & Oris 1999) function as CYP450 inhibitors, decreasing biotransformation and increasing bioaccumulation of xenobiotics in fish (Sijm et al. 1993). Inhibition can also occur when two compounds share a similar biotransformation pathway and are present at high concentrations; the result is decreased clearance due to competition (Timchalk et al. 2005). Other ubiquitous compounds present in mixtures can induce enzymes, resulting in increased biotransformation rates. For example, polycyclic aromatic hydrocarbons

(Gerhart & Carlson 1978; James & Bend 1980; Kleinow et al. 1987; Kennedy & Tierney 2008), polychlorinated biphenyls (Sleiderink et al. 1996; Buckman et al. 2007) and organochlorines such as DDT (Lemaire et al. 2010) have been shown to induce CYP450, increasing rates of biotransformation and decreasing bioaccumulation. In the presence of wide spread, ubiquitous pollutants, the effects of these mixtures on biotransformation and bioaccumulation are largely unknown, but by investigating these effects at a cellular level *in vitro*, one can begin to quantify how depletion rate constants are changed due to exposure to a mixture of pollutants, instead of individual incubations.

In the studies presented in this thesis, an important result was the general trend of decreasing xenobiotic intrinsic clearance when compounds were tested in a mixture. Competition is one explanation for the reduced depletion rate constants, but the low concentrations used (2.0µM total), direct competition seems unlikely. The total concentration of the mixture of chemicals used is well below the reported K_m for B[a]P (40 µM, Nishimoto et al. [1992]), and as the majority of PAHs are likely biotransformed by CYP450, these enzymes should be well below saturation, and competition for binding sites should not be present.

One other hypothesis that addresses the reduced biotransformation is the cosolvent effect. This hypothesis suggests that the solution of acetonitrile, B[a]P, chrysene, 9-MA and PCB-153, was more stable, with a higher fugacity capacity, than solutions that contained acetonitrile and only one of the test compounds (Munz & Roberts 1986). The increase in the solution's stability increases its fugacity capacity, resulting in a greater proportion of the test compounds remaining contained in the acetonitrile solution instead of dissolving into the aqueous medium. The effect of cosolvents in PAH phase

partitioning is well documented (Zemanek et al. 1997; Janikowski et al. 2002; Bardi et al. 2000) as is the effect on degradation in the soil in efforts to clean-up PAH contaminated soils and sediments. PCBs have also been shown to be cosolvents within a mixture (Li et al. 1992; 1995). The result of cosolvent effect is a decrease in the amount of chemical available to the enzymes, resulting in decreased depletion rate constants for hepatocyte incubations with chemical mixtures.

4.3 Effects of Temperature Change

Temperature change has also been shown to affect the biotransformation and bioaccumulation of xenobiotics. At a cellular level, acute temperature changes result in direct effects to enzymes, inducing conformation changes in the enzyme and affecting enzymatic interactions with xenobiotics (Hazel & Prosser 1974). In addition, indirect effects, such as changes in the lipid membrane with changes in temperature, affect diffusion and lipid-bound enzymes, changing the rate of biotransformation (Williams & Hazel 1994). These acute temperature changes tend to increase biotransformation rate with increased temperature, whereas temperature decreases tend to decrease biotransformation rates. Generally, the results of the studies contained within this thesis follow the trend of biotransformation rate change with acute temperature change. Significant increases in depletion rate constants were found for individual incubations of B[a]P and chrysene with an acute temperature increase. However, decreases in hepatocyte depletion rate constants with the acute temperature decrease were not seen for any chemical. Similar results were found using toadfish (*Opsanus beta*) hepatocytes incubated with B[a]P (Gill & Walsh, 1990). After a 1.5 h incubation of hepatocytes with B[a]P, there was a significant increase in the rate of Phase II metabolites formed by the

cells of cold-acclimated fish incubated at warmer temperatures, compared to the formation rate at acclimation temperature. However, the warm-acclimated fish showed no change in Phase II metabolite formation with an acute temperature decrease.

When comparing the depletion rate constants generated by cells incubated at their respective acclimation temperatures, thermal compensation of biotransformation was observed in individual and mixture incubations of chemicals. Thermal compensation in PAH metabolism has been presented previously (Wills 1983; Karr et al. 1985), and compensation has been shown in xenobiotic-metabolising enzymes as well (Blanck et al. 1989; Kennedy et al. 1991). This compensation can be the result of changes in enzyme concentrations and shifts in the ratio of enzymes in favour of isoenzymes that are optimally suited for the change in temperature (Hazel & Prosser, 1974). In this research, the exceptions to thermal compensation were B[a]P and 9-MA incubated individually, as the depletion rate constant for acclimation and incubation at 8°C was significantly lower than the depletion rate constant generated with hepatocytes at 18°C. Further testing to determine if the decrease in depletion rate constant at 8°C for B[a]P and 9-MA was due to decreased enzymatic activity or decreased diffusion due to increased lipid membrane rigidity would tease out the reasons for this departure from the general trend of thermal compensation.

Just as temperature has been shown to affect biotransformation, increasing bioaccumulation of xenobiotics with increasing temperature has been shown in the literature. Because the uptake of chemicals generally increases with increasing temperature, even though metabolism and elimination rates also increase, these mechanisms may not compensate for the increased accumulation of xenobiotics. Hence,

BCF values have been shown to increase with increasing temperature (Karara & Hayton 1989; Edgren et al. 1979; Veith et al. 1979; Honkanen & Kukkonen 2006; Heinonen et al. 2002; Honkanen et al. 2001; Jimenez et al. 1987; Koelmans & Sanchez-Jimenez 1994; Nawaz & Kirk 1995). In the present study, the BCF values modelled from depletion rate constants actually declined with increasing temperature because increased uptake of chemicals with increased temperature is not accounted for in the BCF model (Arnot & Gobas, 2003; 2004). Currently, the model calculates uptake of the chemical across the gills (k_1) as a function of gill ventilation rate and uptake efficiency. Recommended values are based on experiments conducted at 12°C (Nichols et al. 1990), and since gill ventilation rates have been shown to increase or decrease with corresponding changes in temperature (Black et al. 1991; Reid et al. 1996) the fish bioaccumulation model does not account for temperature change within its respiratory uptake calculations. Uptake of highly hydrophobic organic contaminants is predominantly through dietary uptake, which is taken into account with respect to temperature through temperature-dependent feeding rates. However, modelled BCF values do not include dietary uptake, as the only routes of chemical uptake included in BCF experiments are respiratory and dermal uptake.

4.4 Modelling Bioconcentration Factors

Within the BCF model, equations and parameters allow for ease of use, yet are customizable for modelling bioconcentration of xenobiotics in specific fish species and aquatic ecosystems. Two variables that are easily measured are water temperature and fish weight. In the studies contained in this thesis, water temperature was intentionally varied as an experimental treatment, and fish weights varied with the availability of fish stocks. However, in changing these parameters, the resulting BCF values are not perfectly

comparable with BCF values modelled using different fish weights or temperatures. Fish weight (W_b) factors into the calculation of all rate constants except k_{MET} (k_1, k_2, k_e, k_D, *and* k_G), hence the BCF model in sensitive to variance in fish weight. Temperature affects the calculated feeding rate, which is reflected in k_e, the egestion rate constant as it is calculated from k_D, the dietary uptake rate constant. Hence, even without metabolic data ($BCF_{kmet=0}$) the confounding effect of temperature on bioconcentration is evident. In particular, the evidence of temperature effect is seen with PCB-153 bioconcentration where large differences are seen in values between 8°C and 18°C ($BCF_{kmet=0,8°C}$ =22292, $BCF_{kmet=0,18°C}$ =17103). Care must be taken when comparing modelled BCF values, and parameters included within the model should be used consistently.

In predicting bioaccumulation of hydrophobic organic compounds, currently available bioaccumulation models cannot account for biotransformation (Mackay & Fraser 2000; Camphens & Mackay 1997), which can result in an overestimation of the concentration of chemical within an organism (Mathew et al. 2008, Baussant et al. 2001). Models have been developed to predict biotransformation rates of xenobiotics (van der Linde et al. 2001, Arnot et al. 2008; 2009) but with limited *in vivo* data to parameterize these biotransformation models, inaccurate predictions can occur. By incorporating rates of biotransformation into models, more accurate bioaccumulation assessments can be predicted. The result of these more accurate estimates of modelled BCFs are values that can be utilized by regulators and managers in bioaccumulative (B) phase I assessments of anthropogenic chemicals.

Under the Canadian Environmental Protection Act (CEPA 1999), the preferred criterion for assessing the bioaccumulative ability of a chemical is its bioaccumulation

factor (BAF); those chemicals with BAFs >5000 are considered bioaccumulative. Modelling BAF considers dietary input, as well as the influx of chemical across the gill surface, which makes it more reflective of real-world scenarios, especially for chemicals with a high K_{ow} values, such as PCB-153 (Gobas 1993). Modelling dietary input requires knowledge of biomagnification of the chemical within the food web (trophic biomagnification), and the rates at which the chemical is broken down by biota within the food web (trophic dilution) (Arnot & Gobas 2004). As readily available trophic biomagnification and dilution data for the selected pollutants are not available, extrapolations of depletion rate constants to BCF values instead of BAF values were conducted. If data on the trophic effects of the selected chemicals were available, more accurate predictions would be possible.

In this research, parameters used in both the "well-stirred" liver model (hepatic clearance [CL_H]) and BCF model were matched with those in Han et al (2007). However, in order to determine the range of variability within these models, values from published literature, derived via experimentation could be used as well. Model parameters that can be experimentally-derived include: hepatocellularity, hepatic blood flow, gill ventilation rate, and total lipid content. Appendix A contains Table A-1, which shows all the parameters within the models used in this thesis, and Tables A-2 to A-5 gives the range of values for the parameters that are experimentally derived. Hepatocyte counts have been determined by a number of researchers (Pesonen & Andersson 1991; Braunbeck & Storch 1992; Hyllner et al. 1989; Mingoia et al. 2010), and results ranged from 100 – 600 million cells/liver. However, the cell count method of determining numbers of cells/liver is prone to error. By determining the protein content of the liver,

and then extrapolating to the number of cells, Hampton and colleagues (1989) arrived at a more accurate hepatocellularity (H_T) value for rainbow trout of 540 $x10^6$ cells/g; this value was used in the models contained in this thesis. Han et al. (2008) determined hepatocellularity in a manner similar to Hampton (1989), and calculated a value of 510 $x10^6$ cells/g. Using values found in literature, hepatic blood flow (arterial and portal) was determined by taking a percentage (25.9%) of the cardiac output. Hepatic blood flow values ranged from 307.7 to 1117.3 ml/h/kg (Barron et al. 1987; Neumann & Holeton 1983). For trout gill ventilation rate the value of 254.4 L/d/kg (Nichols et al. 1990) was used in this model; however, the range of values in the literature extended from 170 L/d (Gobas & Mackay 1987) to 514 L/d (Lin & Randall 1990). Whole body, total lipid content values vary between different species of fish (Lu et al. 2008), so species specific values were used. Rainbow trout total lipid content values ranged from 0.0488 kg/kg (4.48%, Dyer et al. 2000) to 0.152 kg/kg (15.2%, Hoffman et al. 1999); the value used in this model was 0.1 kg/kg (Nichols et al. 1990). Overall, use of these values could expand the range of modelled BCFs possible using the "well-stirred" liver model (Rowland et al. 1973; Jones & Houston, 2004) and the fish bioaccumulation model (Arnot & Gobas 2003, 2004).

Fish parameters were not the only experimental data that had a range of values, the reported log K_{ow} values for the four chosen test chemicals vary as well. For B[a]P, these values ranged across two orders of magnitude, from a log K_{ow} of 5.78 to 7.99 (MacKay et al. 2006). For 9-MA, fewer values were available; the log K_{ow} range was from 5.07 to 5.61 (Mackay et al. 2006) and the log K_{ow} values for chrysene were from

5.61 to 5.91 (MacKay et al. 2006). PCB-153 had the highest average log K_{ow}, but the range spanned from 6.34 to 8.35 (MacKay et al. 2006).

4.5 Future Directions

Hepatocytes are a physiologically-relevant model system for the derivation of biotransformation data, however, one drawback is the limitation that hepatocytes must be isolated and used promptly in order to best represent the *in vivo* processes. Cryopreservation of fish hepatocytes is one solution to overcoming these limitations (Mingoia et al. 2010; Ferraris et al. 1998). Methods to preserve fish hepatocytes have been published (Mingoia et al. 2010; Ferraris et al. 1998), and these have resulted in high yields and high viabilities. This would allow fish hepatocytes to be shipped to laboratories for use in xenobiotic screening, rather than the current method of using subcellular fractions (Mingoia et al. 2010). The benefit of being able to rapidly assess biotransformation and extrapolate to bioaccumulative potential, and to do so using fewer animals than traditional *in vivo* methods and current *in vitro* cellular methods, make the possibility of cryopreserved hepatocytes an attractive option and one that deserves future study.

Another area requiring future study is the quantification of the fraction of chemical that is bound to hepatocytes, and hence unavailable for biotransformation. Highly hydrophobic organic compounds preferentially partition into the lipid phase, non-specifically binding to cell membranes, resulting in a lesser concentration available to be metabolised. This non-specific binding can result in an underestimation of depletion rate constant. Measurements of this parameter can be obtained through equilibrium dialysis,

ultra-filtration or tube absorption methods (Grime & Riley 2006), but a novel method, measuring the partition coefficient of the compound into water and thin-film ethylene-vinyl acetate, can be used to model the fraction of chemical that is unbound (f_u) and available for biotransformation (Frank Gobas, pers. comm. Nov 2010). Understanding the f_u-corrected CL_{int} will allow for better extrapolation of *in vitro* biotransformation data to whole organism metabolism rate constants (k_{MET}).

While improving *in vitro* determination of biotransformation and its incorporation into models to estimate bioaccumulation is important, without environmentally relevant BCF and BAF values to compare the results to, the accuracy of the models comes into question. The importance of comparing bioaccumulation data within teleosts has been stressed (Parkerton et al. 2008), however, in this work, comparison to BCF values derived exclusively from fish was impossible. Both 9-MA and chrysene had very few published BCF values, as the BCF range for 9-MA included only one value, 4538 in *Daphnia pulex* (Southworth 1978) and the BCF range for chrysene was from 650 to 6088 for chrysene (US EPA 1980; Newsted & Giesy 1987), and test organisms included both fish (juvenile turbot) and invertabrates (*Rhepoximus abronius* and *Daphnia magna*). The relevance of BCF values to environmental conditions is also questionable, but without good quality BAF values to compare to, (as of 2006, only 0.2% of currently-used chemicals had BAF measurements (Arnot & Gobas, 2006) these questions cannot be answered. Guidelines are required so that consistent and comparable BAF values can be measured (Burkhard 2003). With a database of values available, modelled results can be compared to empirical *in vivo* values, in order to develop bioaccumulation models.

4.6 Summary

To summarize, the use of hepatocytes as a model metabolic system for determining depletion rate constants of xenobiotics was shown to be a highly reliable and viable system. The derived depletion rate constants were successfully used to model BCF values using the "well-stirred" liver model (Jones & Houston, 2004) and the fish bioaccumulation model (Arnot & Gobas 2003; 2004). These modelled values fit well with experimentally-derived BCF values. Incubating hepatocytes with a mixture of chemicals decreased the depletion rate constant relative to individually incubated chemicals. The result of these decreased depletion rate constants was that BCF values extrapolated from mixture incubations were generally greater than BCF values modelled from individual incubation depletion rate constants. Investigating the effect of temperature on biotransformation provided a range of environmentally relevant hepatocyte depletion rate constants. Through extrapolation of depletion rate constants to BCF values, the effect of temperature on modelled BCF was explored. Understanding the effect temperature has on biotransformation and bioaccumulation is important, as countries that experience large seasonal fluctuations in temperature need to account for this variation in bioaccumulation due to temperature when creating legislation and regulations for the control and management of anthropogenic chemicals. Moreover, the concurrent effects of global climate change and disposal of anthropogenic chemicals need to be understood in order to prevent accumulations of toxic chemicals in biota. By increasing our knowledge of biotransformation and bioaccumulation of xenobiotics, we can better protect our environment and human health.

Appendices

Appendix A – Model Parameters

Table A-1: Parameters contained within clearance equations, "well-stirred" liver model (Jones and Houston 2004) and fish bioaccumulation model (Arnot and Gobas 2003, 2004)

Parameter		**Value**	**Reference**
Depletion rate constant (k_r)	$\mathrm{Ln}C_t = \mathrm{Ln}C_0 - kt$	Derived from the slope of Ln concentration over time	Bisswanger 2008
Intrinsic clearance	$Cl_{int} = k($ hour$^{-1})$ /[cells] (10^6cells/mL)	Varies by chemical	Han et al. 2007
Hepatic clearance - CL_H			
Hepatic blood flow	Q_H	536.1 mL/h/kg body wt	Nichols et al. 1990
Hepatocellularity	H_t	510 x 10^6 cells/g liver weight	Hampton et al. 1989
Trout liver weight	W_L	12.7 g/kg body weight	Nichols et al. 1990
Fraction unbound in hepatocyte suspension	$f_{u,h}$	Log (1 - $f_{u,h}$ /$f_{u,h}$)= 0.067logK_{ow} - 2.215	Han et al. 2007
Fraction unbound in blood plasma	$f_{u,b}$	Log (1 - $f_{u,b}$/$f_{u,b}$)= 0.613logK_{ow}-0.569	Han et al. 2007
BCF= $k_1\ \Phi/(\ k_2 + k_e + k_G + k_{MET})$			
Φ (fraction of chemical that is bioavailable to fish in H_2O) =1/(1+ $X_{POC}\ D_{POC}\ \alpha_{POC} K_{ow} + X_{DOC}\ D_{DOC}\ \alpha_{DOC}\ K_{ow}$)	X_{POC} (concentration particulate organic carbon (POC))	5 x 10^{-7} kg/L	Gobas & Morrison 2000
	X_{DOC} (concentration dissolved organic carbon (DOC))	5 x 10^{-7} kg/L	
	D_{POC} (disequilibrium POC)	1	Arnot & Gobas 2004
	D_{DOC} (disequilibrium DOC)	1	

	α_{POC} (proportionality constants describing similarity in phase partitioning of POC)	0.35	Seth et al 1999
	α_{DOC} (as above for DOC)	0.08	Burkhard, 2000
$k_1 = E_w \times G_v$ (gill uptake rate constant)	E_w (gill uptake efficiency)	$(1.85 + (155/K_{ow})^{-1}$	Arnot & Gobas, 2004
	G_v (trout ventilation rate)	254.4 L/d/kg	Nichols et al. 1990
$k_2 = k_1 / K_{tw}$ (gill elimination rate constant)			
$K_{tw} = \nu_{LT} K_{ow} + \nu_{NT} \beta K_{ow} + \nu_{WT}$	ν_{LT} (lipid content trout)	0.1	Nichols et al. 1990
	ν_{NT} (non- lipid content trout)	0.2	Arnot & Gobas, 2004
	ν_{WT} (water content)	0.7	
	β (proportionality constant reflecting sorption capacity of non-lipid organic material relative to that of octanol)	0.035	Gobas et al. 1999
k_e = 1/8 dietary uptake (fecal elimination rate constant)	$K_e = 0.125 \times K_D$ $= 0.125 E_D G_D / W_B$		Arnot & Gobas, 2003; Arnot & Gobas, 2004
$k_D = E_D \times G_D / W_B$ (dietary uptake rate constant)	E_D (dietary chemical transfer efficency)	$= (3 \times 10^{-7} \times K_{ow} + 2)^{-1}$	Arnot &Gobas, 2004
	G_D (feeding rate)	$=0.022 \times W_B^{0.85} \times e^{(0.06T)}$ (T = water temp)	Arnot &Gobas, 2004
	W_B (trout body wt)	Average weight of fish (kg)	Experimentally determined
k_G (growth rate constant)		$0.0005 \times W_B^{-0.2}$	Arnot & Gobas, 2003; Arnot & Gobas, 2004
k_{MET} (biotransformation rate constant) $= 0.024 CL_T / V_{ss}$	CL_T (total clearance by body metabolism)	$= CL_H + CL_{others}$ (here $CL_{others} = 0$)	
	V_{ss} (volume distribution at steady state)		
$V_{ss} = \nu_{LT} K_{ow}$ +	ν_{LT} (lipid content in	0.1	Nichols et al. 1990

$\nu_{NT}\beta K_{ow} + \nu_{WT}$ / $\nu_{LB}K_{ow} + \nu_{NB}\beta K_{ow} + \nu_{WB}$	trout)		
	ν_{NT} (non- lipid content in trout)	0.2	Arnot &Gobas, 2004
	ν_{WT} (water content in trout)	0.7	
	ν_{LB} (lipid content in blood)	0.014	Nichols et al. 2006, Bertelsen et al. 1998
	ν_{NB} (non- lipid content in blood)	0.147	
	ν_{WB} (water content in blood)	0.839	
	β (proportionality constant reflecting sorption capacity of non-lipid organic material relative to that of octanol)	0.035	Gobas et al. 1999

Table A-2: Hepatocellularity values

Value ($x10^6$ cells/g)	Reference
540 ±12	Han et al. 2008
510	Hampton et al. 1989
860 (female)	Nichols et al. 2006
255	Cowan-Ellsberry et al. 2008
100-200	Pesonen & Andersson 1991
500-600	Braunbeck & Storch 1992
100-200	Hyllner et al. 1989
300	Mingoia et al. 2010

Table A-3: Hepatic blood flow values

Value (mL/h/kg)	Reference
536.1	Nichols et al. 1990
307.7 – 971.3 (temp dependant)	Barron et al. 1987
480.2 – 663.6	Brodeur et al. 2001
713.3 – 1117.3	Neumann et al. 1983
536.1	Abbas & Hayton 1997
518	Schultz & Hayton 1993
728.7 - 977	Keen & Farrell 1994

Table A-4: Trout gill ventilation rates

Value (L/d)	Reference
254.4 (L/d/kg)	Nichols et al. 1990
170	Gobas & Mackay 1987
503.9	Black & McCarthy 1988
387.3 - 514	Lin & Randall 1990
223.2	Smith & Jones 1982

Table A-5: Trout lipid contents

Value (kg/kg)	Reference
0.1	Nichols et al. 1990
0.08	Morrison et al. 1999
0.0488	Dyer et al. 2000
0.152	Hoffman et al. 1999
0.064 – 0.166	Hamilton et al. 2005
0.07 – 0.1	Schultz & Hayton 1999
0.0708 – 0.1163	Cravedi & Tulliez 1982

Appendix B – Extraction Efficiency

In order to properly attribute the loss of parent compound within our test system to metabolism, we first needed to determine the amount non-specifically bound to the cellular matrix, and hence is non-recoverable by our methodology. To accomplish this, metabolism was inhibited using sodium azide (NaN_3). Sodium azide has been shown to inhibit cellular respiration and metabolic activity on foetal cells (Ishikawa et al. 2006), and competitively inhibit ethanol oxidation by CYP2E1, CYP1A2 and CYP3A4 (Salmela et al. 2001).

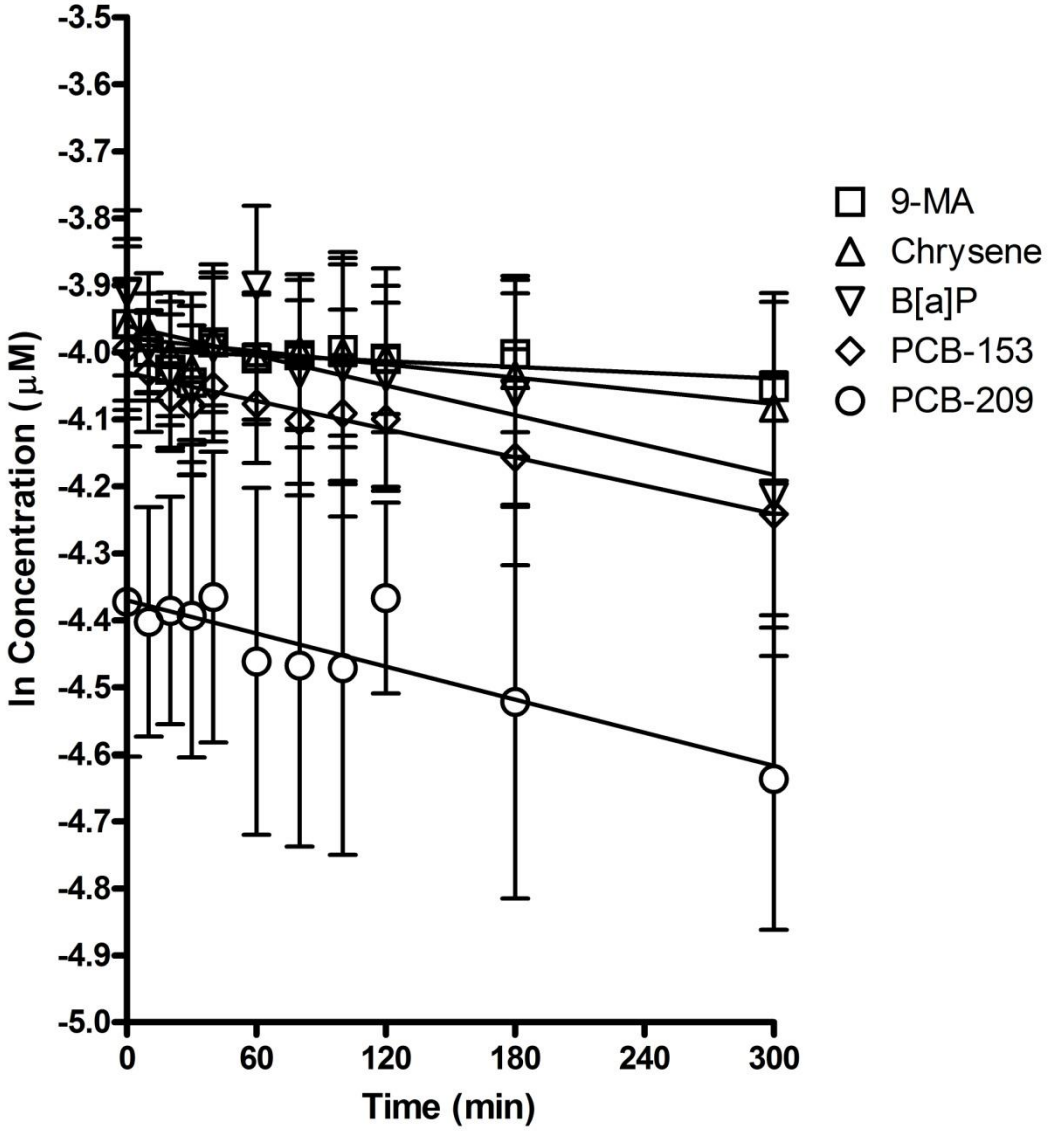
As a positive control for metabolism inhibition, PCB-209 was used in the extraction efficiency experiments as it has shown to be non-metabolizable (Alonso et al. 2008; Buckman et al. 2006). If the decline in concentration recovered over time in the test compound(s) was significantly greater than the disappearance of PCB-209, then we could conclude that metabolism was not inhibited.

The results from our extraction efficiency assays are presented in Figure B-1 and Table B-1. All of our test chemicals (9-methylanthracene, benzo(a)pyrene, chrysene and PCB-153) had extraction efficiencies that were not significantly different from 100% extracted ($p<0.05$), hence no correction was required to adjust the depletion slopes for loss due to non-metabolism.

Table B-1: Recovery of test chemicals from cell matrix

Test Chemical	Depletion rate constant (x10-3) (95% confidence interval)	Significantly different from zero? (p value)	Percent recovered (compared to external standard)
PCB-209	0.82 (2.10 to 0.45)	0.194	68 - 73
PCB-153	0.70 (1.45 to 0.47)	0.065	75 – 84
B[a]P	0.74 (1.61 to -0.12)	0.090	77 – 96
Chrysene	0.33 (1.02 to -0.360	0.339	78 – 89
9-MA	0.14 (0.8 to -5.23)	0.663	82 - 90

Figure B-1: Rate of non-specific binding to hepatocytes within the test system, incubated for 5h at 13°C.



Appendix C – Gas Chromatography – Mass Spectrometry

igure C-1: Abundance (intensity of signal) of the four test compounds in a sample fter incubation with hepatocytes (5hrs at 13°C).

Each peak is labelled with run time, name and chemical structure (identified using mass spectroscopy).

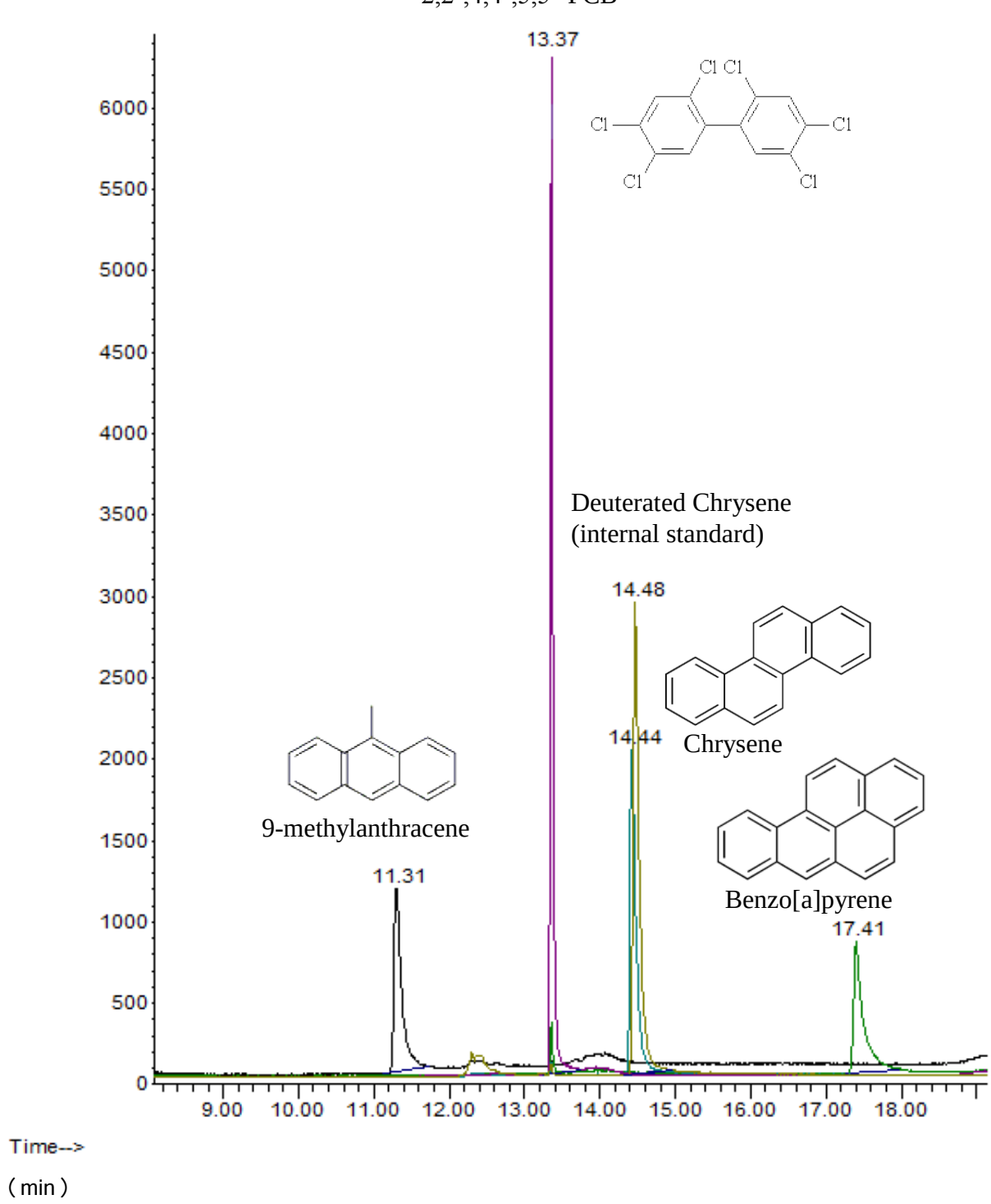
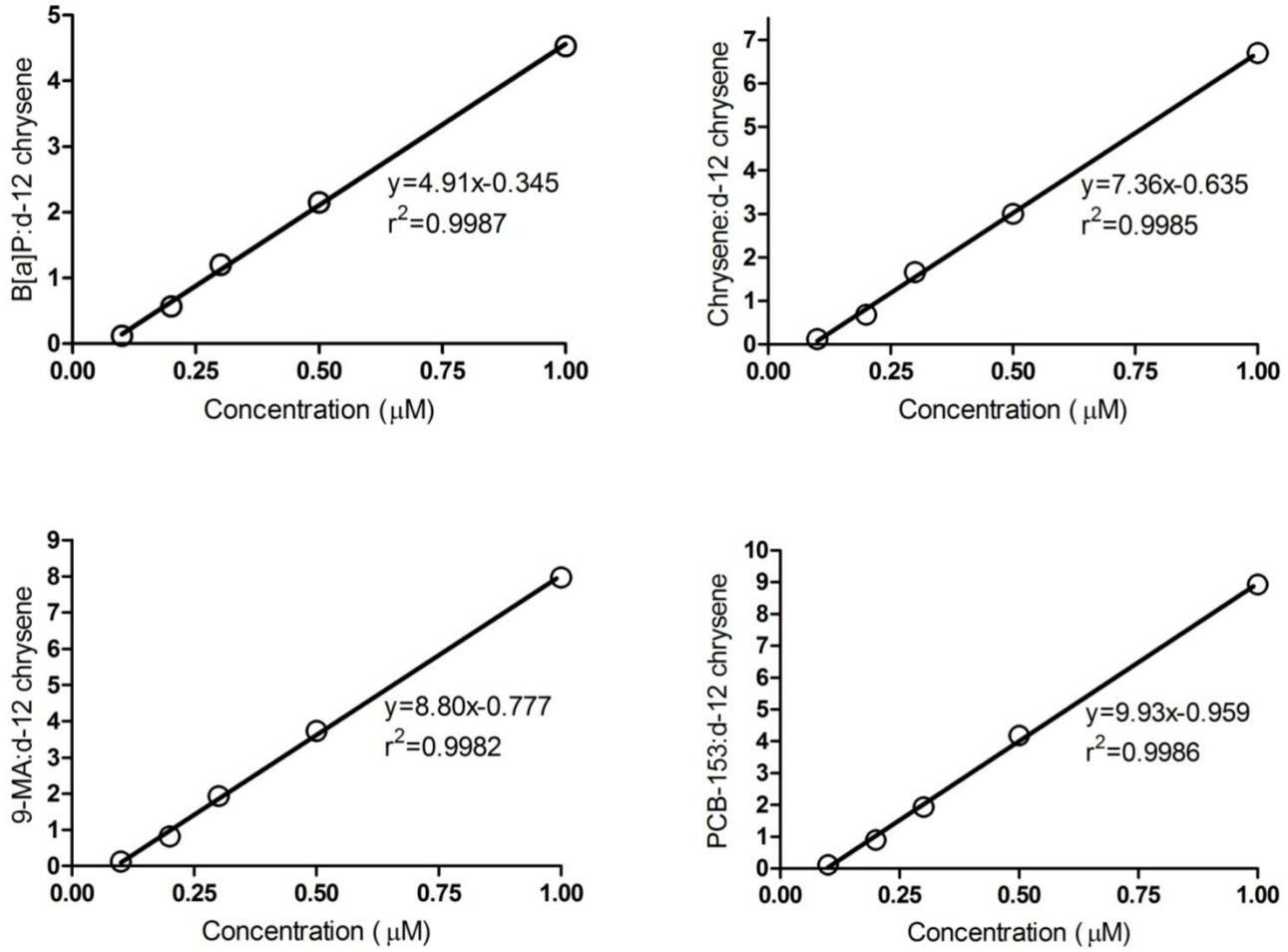


Figure C-2: Standard curves showing response, measure in terms of peak area, of the four test chemicals, relative to the internal standard (d12-chrysene) as a function of the concentration of the test chemical.



Appendix D – Empirical BCF values

Table D-1: Table of BCF values derived *in vivo,* compiled from primary literature.

Chemical	BCF Value	Reference
9-MA	4583	Southworth 1978
B[a]P	224, 282, 367, 377, 3208	McCarthy & Jimenez 1985
	480	Freitag et al. 1985
	490	Gobas & Mackay 1987
	367, 377, 608, 3208	Jimenez et al. 1987
	930	Lu et al. 1977
	2310	Johnsen et al. 1989
	770, 1600	Spacie et al. 1983
	1100	Cohen et al. 1994
	920	Gerhart & Carlson 1978
Chrysene	650	US EPA 1980
	1560	Boese et al. 1999
	1865	Baussant et al. 2001
	6088	Newsted & Giesy 1987
PCB-153	8913	Wei et al. 2001
	42658	Lynch et al. 1982
	45709	Kenaga 1980
	68968, 69183	Muir et al. 1985
	208930	Gobas & Mackay 1987
	446684	Fox et al. 1994
	448000, 450000	Geyer et al. 2000
	741310	Oliver & Niimi 1985

Appendix E – Comparison between fish bioaccumulation models

The primary fish bioaccumulation model utilized in this thesis (Arnot & Gobas 2003) was designed for screening-level bioaccumulation assessments, and does not incorporate environmental factors such as temperature into all its uptake and elimination rate constants. By using a refined version of this fish bioaccumulation model (Arnot et al. 2008) the effect of temperature on gill uptake (and elimination) rate constants and growth rate constant can be modelled for more representative BCF values.

In the fish bioaccumulation model by Arnot et al. (2008), gill ventilation (G_v) is calculated with the following equation:

$$G_v=M/(C_{ox}E_{ox}) \quad \text{(E1)}$$

Where M = metabolic oxygen requirements of the fish; C_{ox} = oxygen concentration in the water; mg/L, and E_{ox}= efficiency of oxygen transfer across the gills.

To calculate C_{ox} (oxygen concentration in the water; mg/L), temperature (T) and oxygen saturation (SAT) were incorporated into the equation:

$$C_{ox}=(14.45 -0.413T + 0.00556T^2) \times SAT \quad \text{(E2)}$$

The efficiency of oxygen transfer across the gills (E_{ox}) was set to the default value of 0.65, and the metabolic oxygen requirements of the fish (M), was determined as follows:

$$Log_{10}M= 2.8 + 0.786(log_{10}W_b)+0.017 \times T \quad \text{(E3)}$$

Where W_b is the empirically derived fish weight. Gill elimination rate constant was calculated as before (Arnot & Gobas 2003) by dividing k_1 by the trout-water partition coefficient.

The growth of a fish was also affected by temperature, and this effect was modelled using the following equation:

$$k_G = G_G(1.113)^{T-20}(1000W_b)^{-0.02} \quad \text{(E4)}$$

where the growth rate coefficient (G_G) is set to the default value of 5.86×10^{-3}.

Calculated values for G_v, k_1 and k_G are given in Table E-1, and a comparison of the BCF values calculated using the different models (k_{MET} values were calculated from the data contained herein) is in Table E-2.

Table E-1: Gill ventilation, gill uptake and growth rate constants; comparison between two fish bioaccumulation models (Arnot and Gobas, 2003 and Arnot et al 2008).

Model	**G_v (L/d)**	**k_1 (L/d/kg)**	**k_G (d^{-1}; 10^{-3})**
Arnot and Gobas, 2003 (13°C)	254.4 L/d/kg	151	0.51
Arnot et al 2008 (8°C)	74.7	74	0.46
Arnot et al 2008 (18°C)	201.7	188	1.33

Table E-2: Modelled BCF values for both individual and mixture incubations at all temperature treatments, using two fish bioaccumulation models

Chemical	Temperature Treatment	Individual		Mixture	
		Arnot & Gobas, 2003	**Arnot et al. 2008**	**Arnot & Gobas, 2003**	**Arnot et al. 2008**
9-MA	8:8	8600* (7600 – 10000)	5100* (4000 – 7000)	9400* (4800 – 9400)	2900* (1900 – 6000)
	8:18	10687	7560	7000* (5800 – 9000)	2900* (2100 – 4800)
	18:8	4600* (3500 – 6800)	3900* (2900 – 5900)	7900* (7300 – 8800)	7100* (6400 – 7900)
	18:18	3200* (2400 – 4700)	2600* (2000 – 4000)	9749	8910
B[a]P	8:8	7100* (4000 - 16000)	2200* (1200– 12000)	7300* (5100 – 14000)	2300* (1600 – 4500)
	8:18	2100* (1800 – 2400)	480* (420 – 560)	4800* (3035 – 12000)	1200* (720 – 3000)
	18:8	1300* (1000 – 1900)	1040* (810 – 1500)	3800* (330 – 4400)	2960* (2600 – 3500)
	18:18	1200* (800 – 3300)	990* (630 – 2600)	6300* (4000 – 15000)	4900* (3100 – 12000)

Table E-2 continued.

Chemical	Temperature Treatment	Individual		Mixture	
		Arnot & Gobas, 2003	**Arnot et al. 2008**	**Arnot & Gobas, 2003**	**Arnot et al. 2008**
Chrysene	8:8	5700* (4159 – 8987)	1800* (1300 – 2700)	34420	17333
	8:18	2100* (1700 – 2500)	480* (400 – 600)	13100* (10000 – 18000)	3600* (2800 – 5300)
	18:8	4200* (2600 – 11000)	3300* (2000 – 8900)	10800* (9000 -13000)	8500* (7100 – 11000)
	18:18	7500* (5300 – 13000)	5900* (4200– 10000)	26092	20548
PCB-153	8	22292	7747	22292	7747
	18	17103	7909	17103	7909

The * indicates the depletion rate constants (k_r) were significantly different from the control, for PCB-153 and others that did not have metabolism in the test system, BCF values with k_{MET} equal to zero were modelled.

All BCF values modelled using Arnot et al. (2008) were lower than their respective BCF value modelled using Arnot and Gobas (2003). This reduction varied between chemicals, with 8% to 58% reductions in 9-MA, 17% to 77% in B[a]P, and 21% to 77% in chrysene.

When comparing BCF values modelled for PCB-153 (k_{MET}=0), the effect of including temperature in k_1, k_2, and k_G becomes apparent. In the 2003 model, BCF values modelled at 18°C were lower than 8°C due to the temperature affects on k_e. However, in the 2008 model, where both uptake and elimination rate constants include temperature parameters, BCF values modelled at 18°C were higher than 8°C, which fits with literature (Karara & Hayton 1989; Veith et al. 1979; Edgren et al. 1979; Jimenez et al. 1987).

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