Improving Bioaccumulation Assessment: Relationship Between In Vitro and In Vivo Biotransformation Rates of Hydrophobic Organic Chemicals in Mammals

by

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Abstract

The effective management of industrial and commercial chemicals in the environment requires good public policy based on sound science. The overall objective of this research is to improve national and international regulatory programs for the environmental management of industrial and commercial chemicals by developing and testing methods for the assessment of bioaccumulation of chemicals in biota. Bioaccumulation is a key consideration in the assessment of the environmental impacts of chemicals on environmental and human health. A review of regulatory approaches to the assessment and management of chemicals shows that current methods for assessing chemical bioaccumulation lack a priori consideration of the ability of organisms to biotransform chemicals and methods to assess bioaccumulation in species other than fish. The specific objective of my research is to develop and test a scientifically sound and cost-effective method for assessing bioaccumulation of chemicals in a mammalian species that incorporates the ability of mammals to biotransform chemicals. A thin-film sorbent-phase dosing method was developed and tested to measure the in vitro biotransformation rates of hydrophobic chemicals in rat and fish liver S9 fractions. The results showed that the biotransformation rates measured using the sorbent-phase dosing system were significantly higher than those measured using conventional solvent-delivery dosing methods. The sorbent-phase dosing system demonstrated several advantages over traditional solvent-dosing methods for hydrophobic chemicals by (i) eliminating incomplete dissolution of very hydrophobic substances in largely aqueous liver homogenates; (ii) providing a method for measuring the unbound fraction of substrate in solution; and (iii) simplifying chemical analysis. Also, an in vitro-to-in vivo extrapolation (IVIVE) method was developed to estimate whole body biotransformation rate constants and biomagnification factors (BMFs) of hydrophobic chemicals in rats from in vitro biotransformation rates. The IVIVE methodology was evaluated and found to be consistent with IVIVE models for pharmaceuticals and produced estimates of rat whole body biotransformation rate constants and BMFs for benzo[a]pyrene which were within the range of empirical values. The proposed IVIVE model for bioaccumulation assessment requires fewer physiological and physiochemical parameters than those used for pharmaceutical drug research; does not involve interconversions between clearance and rate constants in the extrapolation; and may be a useful method for conducting regulatory bioaccumulation assessments in a mammalian species. Finally, recommendations for improving regulatory assessment and control of potentially hazardous commercial chemicals in Canada are presented.

Keywords: Bioaccumulation; biotransformation; hydrophobic chemical; sorbent-phase dosing; in vitro-to-in vivo extrapolation; bioaccumulation modeling

Dedication

I dedicate this thesis to the memory of my father,
Fu-Hai Lee, who always encouraged me to
pursue my dreams.

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Glossary

B	bioaccumulation
BAF	bioaccumulation factor
BCF	bioconcentration factor
BMF	biomagnification factor
CEPA	Canadian Environmental Protection Act
chrysene-d^{12}	deuterated chrysene
CMP	Chemicals Management Plan
CYP	cytochrome P450
DSL	Domestic Substances List
EC_{50}	median effective concentration
EVA	ethylene vinyl acetate
GC/MS	gas chromatography/mass spectrometry
GPE	greatest potential for human exposure
HPLC	high-performance liquid chromatography
iT	inherent toxicity
IVIVE	in vitro-to-in vivo extrapolation
K_{OA}	octanol−air partition coefficient
K_{OW}	octanol−water partition coefficient
LC_{50}	median lethal concentration
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NOEC	no-observed-effect concentration
OECD	Organisation for Economic Co-operation and Development
P	persistence
PAH	polycyclic aromatic hydrocarbon
PBT	persistent, bioaccumulative, and toxic
PCB	polychlorinated biphenyl
PDMS	poly(dimethylsiloxane)
POPs	persistent organic pollutants
PTFE	polytetrafluoroethylene
QSAR	quantitative structure−activity relationship

REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
S9	Supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 × *g* for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes.
SAICM	The Strategic Approach to International Chemicals Management
TSCA	Toxic Substances Control Act
UNEP	United Nations Environment Programme
UVCB	Unknown or Variable composition, Complex reaction products, or Biological materials
WSSD	World Summit on Sustainable Development

Chapter 1.

Introduction

1.1. Background

Chemicals play an important role in modern human life. They are used in a wide variety of products and applications, and can improve living standards and contribute to national and global economy. The global chemical industry has grown rapidly in recent decades. The global chemical output increased from US$ 171 billion in 1970 to US$ 4.12 trillion in 2010 (UNEP, 2013). Although the exact number of chemicals on the global market is unknown, it is estimated that there are more than 140,000 chemicals on the market of the European Union (E.U.), and about 700 new chemicals are introduced into commerce each year in the United States (U.S.) (UNEP, 2013). The total production volume of chemicals increased by 54% from 2000 to 2010 (UNEP, 2013). With the increasing production and consumption of chemicals, chemical emissions and waste generation are of concern. It is reported that 4.9 million metric tonnes of chemicals were released to the environment or disposed of in North America (Canada, Mexico and United States) in 2009 (UNEP, 2013). The production, use and disposal of chemicals may pose adverse effects on the ecosystems (including the atmosphere, water, soil and wildlife) and human health.

It is challenging to manage the large number of chemicals in commerce properly to gain the benefits of chemical uses and avoid adverse impacts on human health and the environment. At the United Nations Earth Summit held in Rio in 1992, Agenda 21 was established to provide a comprehensive plan of action to deal with global environmental problems and to accelerate sustainable development. Chapter 19 of Agenda 21 promotes "environmentally sound management of toxic chemicals, within the principles of sustainable development and improved quality of life for humankind" and states that

chemicals “can be used widely in a cost-effective manner and with a high degree of safety” (UNDSD, 1992). At the United Nations World Summit on Sustainable Development (WSSD) held in Johannesburg in 2002, governments agreed on the commitment of sound management of chemicals and established a goal “to achieve, by 2020, the use and production of chemicals in ways that lead to the minimization of significant adverse effects on human health and the environment” (United Nations, 2002). This led to the establishment of the Strategic Approach to International Chemicals Management (SAICM) in 2006, which is an international policy framework adopted by governments and stakeholders to support the achievement of the WSSD 2020 goal. The SAICM addresses the sound management of chemicals throughout their life-cycle and has a broad scope covering environmental, economic, social, health, and labour aspects of chemical safety (UNEP, 2006). In addition to international non-binding commitments, there are regulatory approaches addressing the assessment and management of chemicals.

1.2. Regulatory Approaches to Chemicals Assessment and Management

To protect human health and the environment, national and international regulatory programs have been developed to identify, evaluate and manage chemical substances that pose the greatest threats to humans and the environment. Of particular concerns are chemicals that have the abilities to persist in the environment, to bioaccumulate in the organisms and food chains, and to cause toxic effects on the organisms, known as persistent, bioaccumulative, and toxic substances (PBTs), and a subset of organic substances that are subject to long-range transport, known as persistent organic pollutants (POPs). The regulatory approaches often involves the scientific identification of PBTs or POPs, risk assessment and evaluation of the identified chemicals, development of management strategies and implementation of management actions to protect human health and the environment. The regulatory framework of major regulatory programs addressing PBTs and POPs at global, regional and national scale, such as the Stockholm Convention on Persistent Organic Pollutants, E.U. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Canadian Environmental Protection

Act (CEPA), U.S. Toxic Substances Control Act (TSCA), and Japanese Chemical Substances Control Law (CSCL), are discussed below.

1.2.1. Stockholm Convention on Persistent Organic Pollutants

The Stockholm Convention on Persistent Organic Pollutants is a global treaty administered by the United Nations Environment Programme (UNEP). It was adapted in 2001 and entered into force in 2004, aiming to protect human health and the environment from POPs (UNEP, 2009). POPs are typically hydrophobic ('water-hating') and lipophilic ('fat-loving') chemicals that have the propensity to enter the gas phase under environmental temperatures and can partition into lipids and accumulate in the fatty tissue of living organisms (Jones & Voogt, 1999). Because of their resistance to breakdown reactions (e.g., photolysis and metabolism), they are prone to long-range atmospheric transport and food-web bioaccumulation, and may cause health effects (e.g., carcinogenicity and endocrine disruption) in humans and wildlife even in remote regions of the earth (Jones & Voogt, 1999; Li, et al., 2006). The Stockholm Convention recognized the need for global action on POPs and established measures to reduce and/or eliminate emissions and discharges of POPs. Any party to the Convention may submit a proposal to list a chemical in Annex A (elimination), Annex B (restriction), and/or Annex C (unintentional production) of the Convention. The proposed chemicals are examined by the Persistent Organic Pollutants Review Committee (POPRC) by applying the screening criteria specified in Annex D, including criteria for persistence, bioaccumulation, potential for long-range environmental transport, and adverse effects (Table 1.1). The POPRC takes into account all information in an integrative and balanced manner to decide whether the screening criteria are fulfilled. If the POPRC is satisfied that the proposed chemical fulfills the screening criteria, then the chemical is examined through a risk profile requiring information specified in Appendix E, including source data, hazard assessment for the endpoints of concern, environmental fate, monitoring and exposure data. If the POPRC decides that the proposal shall proceed, information related to socio-economic considerations specified in Annex F is required for developing a risk management evaluation. Based on the risk profile and risk management evaluation, the POPRC makes a recommendation on whether the chemical should be listed in Annex A, B, or C of the Convention (UNEP, 2009). Starting with twelve initial POPs, colloquially known as the

"dirty dozen", there are currently 26 POPs listed in the annexes of the Convention (UNEP, 2016).

1.2.2. European Union's Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is an E.U. regulation (Regulation (EC) No 1907/2006) for controlling chemicals that replaced older legislation that governed industrial chemicals, such as Regulation (EEC) No 793/93 (evaluation and control of existing chemical substances) and Directive 76/769/EEC (restrictions of marketing and use of chemical substances) (Williams et al., 2009). REACH entered into force in 2007 and was adopted to protect human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness and innovation of the E.U. industry (European Commission, 2006). REACH places the burden of proof on companies, that is, companies must demonstrate how the substance can be safely used, and they must communicate the risk management measures to the users. The registration procedure requires companies (registrants) to register all new and existing chemicals manufactured and available in the E.U. market above 1 tonne per year by submitting a registration dossier to the European Chemicals Agency (ECHA), including a technical dossier (required for all substances subject to the registration obligations) and a chemical safety report (required for chemicals manufactured or imported at more than 10 tonnes a year). The technical dossier contains information on the physicochemical properties, environmental fate and toxicological data of a substance, as well as information on its manufacture, use and exposure. The chemical safety report is a documentation of a chemical safety assessment, containing a detailed summary of the information on environmental and human health hazard assessments, PBT (persistent, bioaccumulative and toxic) and vPvB (very persistent and very toxic) assessment, exposure assessment and risk characterisation. The PBT and vPvB criteria are given in Annex XIII of REACH, as summarized in Table 1.1.

The evaluation procedure in the REACH Regulation includes dossier evaluation and substance evaluation. The dossier evaluation involves a complex procedure in which the ECHA evaluates the testing proposals, conducts compliance checks, drafts a decision,

and evaluates submitted information and comments; third parties may provide information on testing proposals; the registrants can provide comments on the draft decision; and the E.U. Member States can propose amendments to the draft decision to be assessed by the Member State Committee. The substance evaluation is undertaken by the E.U. Member States to evaluate chemicals selected by the ECHA and listed in the community rolling action plan and propose follow-up actions, such as the identification of substances of very high concern (SVHC) and the restriction of substances. The SVHC include those that are carcinogenic, mutagenic or toxic to reproduction (CMR substances), PBT and vPvB chemicals, and chemicals of equivalent concern on a case-by-case basis. The REACH Regulation also promotes alternative methods for the assessment of substances in order to reduce the number of tests on animals.

In the authorisation process, chemicals identified as SVHC by the E.U. Member States or ECHA are added to the Candidate List, and then ECHA prioritizes these substances to determine the ones included in the Authorisation List (Annex XIV of REACH). Manufacturers, importers or downstream users can apply for authorisation for the use of the substance on the Authorisation List. Authorisation for specified uses is granted if the applicant can demonstrate that the risk from the use of the substance is adequately controlled, or if it can be proved that the socio-economic benefits of using the substance outweigh the risks and there are no suitable alternative substances or technologies available. Currently, there are 31 substances contained in the Authorisation List (ECHA, 2016a).

The restriction procedure can be initiated by an E.U. Member State or the ECHA and involves limiting or banning the manufacture, marketing or use of a substance when there is an unacceptable risk to human health or the environment posed by the substance on a community-wide basis. A restriction proposal needs to be prepared according to Annex XV and made available for public consultation. ECHA's Risk Assessment Committee and Committee for Socio-economic Analysis both need to provide opinions. The European Commission makes the ultimate decision by taking a balanced view of the identified risks, benefits and costs of the proposed restriction. Substances that are restricted under REACH are listed in Annex XVII. Currently, there are 61 substances contained in Annex XVII of REACH (ECHA, 2016b).

1.2.3. Canadian Environmental Protection Act

The Canadian Environmental Protection Act, proclaimed in 1988 (CEPA 1988) and amended in 1999 (CEPA 1999), is an important part of Canada's federal environmental legislation aiming to prevent pollution, protect the environment and human health, and contribute to sustainable development (Government of Canada, 1999). The CEPA 1999 requires that the Minister of the Environment and the Minister of Health (the Ministers) take action with respect to the control of toxic substances (Part 5 of CEPA 1999). The Ministers are required to "categorize" and then, if necessary, "screen" substances listed on the Domestic Substances List (DSL) to determine whether they are "toxic" or capable of becoming "toxic" as defined under the Act (referred to as "CEPA-toxic"). The DSL contains substances in commerce in Canada between January 1984 and December 1986 (referred to as existing substances), and there are approximately 23,000 substances on the DSL. As required by the Act, Environment Canada and Health Canada completed the categorization of all substances on the DSL by September 14, 2006 to identify substances that have the "greatest potential for human exposure" (GPE) and that are persistent (P) or bioaccumulative (B) and inherently toxic (iT) to human or non-human organisms (Section 73 of CEPA 1999). Environment Canada is responsible for identifying substances that are P or B and iT to non-human organisms (i.e., ecological categorization) based on the criteria summarized in Table 1.1; Health Canada is responsible for categorizing substances that are GPE and iT to humans (i.e., human health-related categorization). Substances that are not listed on the DSL are considered "new substances" and they are subject to the New Substances Program.

Existing substances that meet the categorization criteria are subject to screening-level risk assessments. Ecological screening assessments are conducted by Environment Canada; screening health assessments are conducted by Health Canada. Based on the results of the screening assessment, substances that require in-depth assessments are added to the Priority Substances List, and substances that are determined to be toxic or capable of becoming toxic under the Act (Section 64 of CEPA 1999) are added to the List of Toxic Substances (Schedule 1 of the Act) subject to risk management measures. Toxic substances released into the environment as a result of human activity are subject to virtual elimination, and they are listed on the Virtual Elimination List to reach ultimate

reduction of the quantity or concentrations in the release below the level of quantification (Section 65 of CEPA). Currently, there are 133 substances contained in the List of Toxic Substances (Environment Canada, 2016a) and 2 substances (hexachlorobutadiene and perfluorooctane sulfonate (PFOS) and its salts) contained in the Virtual Elimination List (Environment Canada, 2016b).

The categorization of all existing substances on the DSL was completed in 2006, and approximately 4,300 substances were identified to meet the categorization criteria. Through the Chemicals Management Plan (CMP), launched in 2006 as a jointly-managed initiative between Health Canada and Environment Canada, the federal government aims to assess these approximately 4,300 priority existing substances by 2020, in support of the Strategic Approach to International Chemicals Management (SAICM) and the 2020 goals set by the World Summit on Sustainable Development (WSSD) for sound management of chemicals. Key activities under the CMP include risk assessment, risk management, research, monitoring and surveillance, compliance promotion and enforcement, and stakeholder engagement and risk communication.

1.2.4. United States Toxic Substances Control Act

The Toxic Substances Control Act (TSCA) is the primary chemicals management law of the U.S. administered by the U.S. Environmental Protection Agency (EPA). It was originally passed in 1976 (US Congress, 1976) and recently amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act (US Congress, 2016), which was signed by the President and went into effect in June 2016. The new law includes a number of significant changes, such as mandatory duties of the EPA to evaluate existing chemicals with clear and enforceable deadlines, to evaluate new and existing chemicals against a new risk-based safety standard, to make an affirmative determination on new chemicals before entry into the marketplace, to seek more information and require companies to conduct additional studies to ensure chemical safety, to provide greater public access to critical chemical information by employing new requirements for confidential business information, and to promote development and implementation of alternative (non-animal) testing methodologies and protocols (Tollefson, 2016; USEPA, 2016).

Under the new Act, EPA is required to prioritize existing chemicals and identify them as high- or low-priority substances, and the high-priority substances are then subject to risk evaluations. High-priority substances refer to those that may present an "unreasonable risk" of injury to health or the environment due to potential hazard and route of exposure; low-priority substances are those that do not meet the standard for high priority. EPA is required to establish a new risk-based safety standard to determine whether a chemical use poses an "unreasonable risk", excluding consideration of costs or other non-risk factors. EPA must take risk management actions to address unreasonable risks by taking into account a range of considerations, including sensitive subpopulations, economic costs, social benefits, and equity concerns. It is required to establish procedure rules of EPA's process and criteria for the prioritization and risk evaluation of chemicals by June 2017. In addition, EPA must have 20 ongoing chemical risk evaluations within 3.5 years of the enactment of the Frank R. Lautenberg Chemical Safety for the 21st Century Act.

1.2.5. Japanese Chemical Substances Control Law

The Japanese Chemical Substances Control Law (CSCL, also known as Kashinho) is one of the primary laws that regulate industrial chemicals in Japan. The CSCL was first enacted in 1973, and most recently amended in 2009 and went into effect in 2010. The CSCL regulates both new and existing chemicals. There are approximately 28,000 existing chemicals that were already manufactured or imported at the time of the enactment of the CSCL in 1973. The CSCL employs a mix of hazard-based and risk-based approaches to classify chemicals into five groups, including (i) class I specified chemicals: persistent, bioaccumulative, and toxic chemicals (with long-term toxicity to humans or predator animals at higher trophic levels); (ii) monitoring chemicals: persistent and bioaccumulative chemicals with unknown toxicity properties; (iii) class II specified chemicals: chemicals that pose a risk of causing damage to human health or the environment based on step-wise risk assessments; (iv) priority assessment chemicals: candidates for the class II specified chemical that may pose a risk on human health or the environment with considerable amount of the chemicals remains in the environment; and (v) general chemicals: chemicals that are not included in any other group. Class I specified chemicals are restricted on license of manufacture, import and usage and they are subject

to virtual prohibition. The assessment and evaluation involves a series of processes that are available on the website of the Ministry of Economy, Trade and Industry (METI) of Japan (METI, 2016). There are 30 class I specified chemicals and 23 class II specified chemicals as of December, 2015 (METI, 2015).

Table 1.1 summarizes the criteria for persistence, bioaccumulation, toxicity, and potential for long-range transport set out in the Stockholm Convention, E.U. Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Canadian Environmental Protection Act (CEPA), U.S. Toxic Substances Control Act (TSCA), and Japanese Chemical Substances Control Law (CSCL). These criteria include similar evaluation metrics, such as half-life in individual environmental medium for persistence evaluation, and bioconcentration factor (BCF), bioaccumulation factor (BAF), and the logarithm of octanol−water partition coefficient (log K_{OW}) for bioaccumulation evaluation. However, the numerical criteria are not harmonized under the various regulations, depending on the desired degree of precaution and on the number of substances to be managed (Webster et al., 2004; van Wijk et al., 2009). For example, the Stockholm Convention aims to ban or restrict the release of chemicals that have global impacts, and the numerical criteria are selected to capture a small number of chemicals with PBT properties similar to the original “dirty dozen”. The objective of the Toxics Release Inventory Program under U.S. Toxic Substances Control Act is to provide information on the release of chemical to local communities, and the numerical criteria are selected to include more chemicals compared to the Stockholm Convention (USEPA, 1999a). The criteria for persistence and bioaccumulation under the Canadian Environmental Protection Act are intended to determine highly persistent and highly bioaccumulative substances for virtual elimination, and these criteria have higher thresholds than those set out in other national and regional regulatory programs (e.g., E.U. REACH, U.S. TSCA and Japanese CSCL). Despite the differences, the various national and international regulatory programs share the ultimate goals of reducing risks and protecting human health and the environment. van Wijk et al. (2009) has written a comprehensive review of the regulatory programs concerning PBTs and POPs. The criteria and limitations of current bioaccumulation assessment are discussed below.

1.3. Limitations of Current Regulatory Bioaccumulation Assessment

Bioaccumulation assessment is an important aspect in chemical categorization and risk assessment under current regulatory approaches. If they are not biotransformed, chemicals that have the ability to bioaccumulate in biota and humans may reach elevated internal concentrations and possibly cause toxic effects (Gobas et al., 2009; Ehrlich et al., 2011). The criteria for bioaccumulation assessment in various regulatory programs are shown in Table 1.1, as part of the PBT assessment. The bioaccumulation criteria rely on the bioconcentration factor (BCF), the bioaccumulation factor (BAF), or the octanol–water partition coefficient (K_{OW}). BAF refers to the ratio of the concentration of a substance in an organism to the concentration in water, based on uptake from the surrounding medium and food; BCF refers to the ratio of the concentration of a substance in an organism to the concentration in water, based only on uptake from the surrounding medium; and K_{OW} refers to the ratio of the concentration of a substance in an octanol phase to the concentration of the substance in the water phase of an octanol–water system that has reached equilibrium. Both the BCF and BAF are organism−water chemical concentration ratios while measured under different conditions. The BCF is based on chemical uptake from the surrounding medium only (i.e., dietary exposure is not included); and the BAF is based on chemical uptake from all possible routes of exposure (i.e., including chemical exposure from the diet and surrounding medium). BCFs can only be measured under controlled laboratory conditions, and BAFs are typically measured under field conditions (Arnot & Gobas, 2006).

Table 1.1. Overview of regulatory criteria for persistence, bioaccumulation, toxicity, and potential for long-range transport in selected regulations

Regulation	Persistence (P)	Bioaccumulation (B) [b]	Toxicity (T) [c]	Potential for Long-range Transport	Reference
UNEP Stockholm Convention on Persistent Organic Pollutants [a]	Half-life in water > 2 months; or half-life in soils > 6 months; or half-life in sediment > 6 months	BCF or BAF > 5000; or log K_{OW} > 5	Evidence of adverse effects to human health or to the environment; or toxicity or ecotoxicity data that indicate the potential for damage to human health or to the environment	Measured levels far from source; or monitoring data in remote area; or multimedia modeling evidence and half-life in air > 2 days	UNEP, 2009 (Appendix D)
E.U. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)	*PBT substances* Half-life in marine water > 60 days; or half-life in fresh or estuarine water > 40 days; or half-life in marine sediment > 180 days; or half-life in fresh or estuarine water sediment > 120 days; or half-life in soil > 120 days	BCF > 2000	NOEC or EC10 < 0.01 mg/L; or substances that are carcinogenic, germ cell mutagenic, or toxic for reproduction; or evidence of specific target organ toxicity after repeated exposure	Not applicable	European Commission, 2006 (Annex XIII)
	vPvB substances Half-life in marine, fresh or estuarine water > 60 days; or half-life in marine, fresh or estuarine water sediment > 180 days; or half-life in soil > 180 days	BCF > 5000	Not applicable		

Regulation	Persistence (P)	Bioaccumulation (B) [b]	Toxicity (T) [c]	Potential for Long-range Transport	Reference
Canadian Environmental Protection Act (CEPA)	Half-life in air ≥ 2 days; or half-life in water ≥ 182 days; or half-life in sediments ≥ 365 days; or half-life in soil ≥ 182 days	BAF ≥ 5000; or BCF ≥ 5000; or log K_{OW} ≥ 5	*CEPA-toxic* Substances that have or may have an immediate or long-term harmful effect on the environment; or substances that constitute or may constitute a danger to the environment; or substances that constitute or may constitute a danger in Canada to human life or health *Inherently toxic to non-human organisms* LC50 or EC50 ≤ 1 mg/L; or NOEC ≤ 0.1 mg/L	Not applicable	Government of Canada, 2000; Government of Canada, 1999
U.S. Toxic Substances Control Act (TSCA): Toxics Release Inventory Program	Half-life ≥ 2 months in water, sediment, or soil; or half-life ≥ 2 days in air	BCF or BAF ≥ 1000	Chemicals known to cause or may cause significant adverse acute human health effects, adverse chronic human health effects (e.g., cancer or teratogenic effects, reproductive dysfunctions, neurological disorders, and heritable genetic mutations), or adverse effect on the environment.	Not applicable	USEPA, 1999a;
U.S. Toxic Substances Control Act (TSCA): New Chemicals Program	*Order pending testing/significant new use rule* Transformation half-life > 2 months	BCF or BAF ≥ 1000		Not applicable	USEPA, 1999b

Regulation	Persistence (P)	Bioaccumulation (B) [b]	Toxicity (T) [c]	Potential for Long-range Transport	Reference
	Ban pending testing Transformation half-life > 6 months	BCF or BAF ≥ 5000	Develop toxicity data where necessary, based upon various factors, including concerns for persistence, bioaccumulation, other physical/chemical factors, and toxicity based on existing data.		
Japanese Chemical Substances Control Law (CSCL)	*Not readily biodegradable* BOD [d] ≥ 60% *Readily biodegradable* BOD [d] < 60%	*Highly bioaccumulative* BCF ≥ 5000 *Not highly bioaccumulative* BCF < 1000; or log K_{OW} < 3.5 *Judgment considering other test data* 1000 ≤ BCF < 5000	Screening toxicity based on results of toxicity tests, including bacterial reverse mutation test, in vitro mammalian chromosome aberration test, repeated dose 28-day oral toxicity study in rodents, and reproduction/developmental toxicity screening test. Screening ecotoxicity based on results of toxicity tests, including algal growth inhibition test, daphnids acute immobilization test, and fish acute toxicity test.	Not applicable	MHLW et al., 2011

[a] UNEP = United Nations Environment Programme

[b] BCF = bioconcentration factor; BAF = bioaccumulation factor; log K_{OW} = the logarithm of octanol−water partition coefficient.

[c] NOEC = no-observed-effect concentration; EC10 = 10% effect concentration; LC50 = median lethal concentration; EC50 = median effect concentration.

[d] BOD = biological oxygen demand (determined following OECD 301C test guideline)

The current bioaccumulation criteria listed in Table 1.1 for assessing the large number of chemicals in commerce are subject to limitations, which may lead to mis-categorization of chemicals with bioaccumulation potential. The first major limitation is that the empirical BCF and BAF data are not available for the great majority of commercial chemicals. For example, the empirical BCF and BAF data are only available for 3.7% of the approximately 11,300 organic chemicals on the Canadian Domestic Substances List (Arnot & Gobas, 2006). As a result, bioaccumulation assessment often relies on the K_{OW} criterion or on bioaccumulation models. K_{OW} is a physical-chemical property used as a surrogate parameter for chemical partitioning between water and lipids in biological organisms (Arnot & Gobas, 2006) and does not account for biological factors such as biotransformation in the bioaccumulation assessment. Biotransformation is an internal biological process that plays an important role in reducing tissue concentration of chemical substances. If chemicals are metabolised rapidly, they may not bioaccumulate in the organism. The reliance on the K_{OW} criterion for bioaccumulation assessment is of particular concern for very hydrophobic chemicals (log K_{OW} > 5). If these chemicals are metabolized at sufficiently high rates, a “false-positive” result may be obtained, i.e., chemicals are considered to be bioaccumulative while in reality they are not. In addition, even if bioaccumulation models incorporate various chemical uptake and elimination processes (including biotransformation) for the calculation of bioaccumulation potential (e.g., BCF and BAF), biotransformation rates are not available for the majority of commercial chemicals. In the absence of such data, the precautionary principle is applied in bioaccumulation modelling: the biotransformation rates of chemicals are assumed to be zero (Arnot and Gobas, 2004). As noted above, this may lead to overestimation of bioaccumulation potential for chemicals that undergo biotransformation.

Second, current bioaccumulation metrics are specific and limited to aquatic organisms; bioaccumulation in non-aquatic organisms is often not considered in bioaccumulation assessment. For example, BAF and BCF are measures for bioaccumulation potential specific to aquatic organisms. The K_{OW} criterion was derived based on the studies investigating bioaccumulation of chemicals in aquatic organisms that depend on exchange with water for respiration. As a result, bioaccumulation in non-aquatic organisms is assessed based on bioaccumulation criteria for aquatic organisms. However, there is evidence that some chemicals that are assessed as non-

bioaccumulative in aquatic organisms are bioaccumulative in non-aquatic organisms. For example, Kitano (2007) showed that five of 21 persistent organic pollutants recognized in the Stockholm Convention at that time do not meet BCF and K_{OW} based bioaccumulation criteria but are highly bioaccumulative in non-aquatic organisms. The failure of the BCF and K_{OW} to identify chemicals that have bioaccumulation potential in non-aquatic organisms is due to the fact that the bioconcentration factor in fish has limited relevance to bioaccumulation in air-breathing organisms (Kelly et al., 2007). Therefore, there is a need to develop methods for assessing bioaccumulation of chemicals in non-aquatic organisms, particularly mammalian wildlife and humans.

Third, there are limitations associated with the experimental determination of BCFs or BAFs for highly hydrophobic chemicals (log K_{OW} > 6). The BCFs are commonly measured following a standardized guideline developed by the Organisation for Economic Co-operation and Development (OECD), that is, the OECD 305–I guideline titled "Aqueous Exposure Bioconcentration Fish Test" (OECD, 2012), which is part of the revision of the 2006 guideline (OECD, 2006). It has been shown that the BCF test is applicable to chemicals with moderate hydrophobicity (log K_{OW} = 1.5 to 6), but the test results for chemicals with log K_{OW} greater than 6 are questionable (Ehrlich et al., 2011). These very hydrophobic chemicals tend to have low water solubility and strong binding affinity to sediment and dissolved organic carbon, resulting in oversaturation of the chemical in test solutions and very low levels of freely dissolved fraction that is bioavailable to fish. This may cause underestimation of the measured BCFs. The problem with BCF measurements for chemicals with low water solubility has been recognized by the OECD and addressed by including a dietary bioaccumulation test, the OECD 305–III guideline titled "Dietary Exposure Bioaccumulation Fish Test", in the OECD 305 guideline (OECD, 2012). However, the endpoint from this test is a dietary biomagnification factor (BMF, defined as the ratio of the concentration of a substance in an organism to that in the organism's food at steady-state) rather than a BAF, and regulatory criteria for BMFs are not available currently.

1.4. Methods for Determining Biotransformation Rates of Xenobiotics for Bioaccumulation Assessment

Biotransformation (or metabolic transformation) is defined as the structural modification of chemical molecules mediated by enzymes within an organism, leading to the formation of relatively polar (water soluble) substances that are easily excreted from the organism (Gibson & Skett, 2001; Asha & Vidyavathi, 2009). Biotransformation is an important internal biological process that plays a vital role in the elimination of xenobiotics (i.e., foreign compounds) from the organism. Biotransformation involves chemical reactions catalyzed by metabolizing enzymes to convert the xenobiotics into metabolites that are more readily eliminated than the parent compound. Biotransformation reactions normally occur in two phases: Phase I (or functionalization) and Phase II (or conjugation) reactions. The Phase I reactions include oxidation, reduction, and hydrolysis and result in the introduction of a chemically reactive functional group (such as —OH, —NH_2, —SH, and —COOH) into the xenobiotic molecule to change it into a more polar chemical. The isoforms of cytochrome P450 (CYP) are the most important Phase I enzymes in the metabolism of xenobiotics (Hodgson & Rose, 2007). The product of Phase I metabolism may act as a substrate for Phase II conjugation reactions to yield products that are usually highly water-soluble and recognized by specific transporters that excrete them. Phase II reactions include glucuronidation, sulfation, methylation and acetylation. Many high log K_{OW} chemicals undergo both Phase I and Phase II metabolism whereas many low log K_{OW} chemicals are present in the form that can be directly conjugated by Phase II pathways (Nichols et al., 2009a).

The CYP enzymes play a critical role in the metabolism of xenobiotics (Martignoni et al., 2006). The CYP enzymes are present throughout the human body, most abundantly in the liver (e.g., CYP3A4, CYP2D6, CYP2C9/10/19, CYP1A2, CYP2E1, CYP1A1, CYP2A6 and CYP2B6) and in a minor fraction in other tissues such as lung (e.g., CYP3A4 and CYP1A1), heart (e.g., CYP2D6 and CYP2E1) and gastrointestinal tract (e.g., CYP3A4, CYP1A1 and CYP1B1) (Anzenbacher & Anzenbacherova, 2001). There are differences in the composition, expression and catalytic activity of CYP enzymes between species (Guengerich, 1997; Martignoni et al., 2006; Bogaards et al., 2000). For example, CYP3A and CYP2C are the most abundant CYP subfamily of human and rat liver, respectively

(Anzenbacher & Anzenbacherova, 2001; Zuber et al., 2002). Han et al. (2009) found that the activity of CYP 1A and CYP 3A (measured using the substrate of 7-ethoxyresorufin-O-dealkylase and testosterone 6β-hydroxylasein, respectively) in trout liver microsomes and S9 fractions were significantly lower than those in rat liver microsomes and S9.

As described above, the lack of consideration of biotransformation capacity is one of the major limitations in current bioaccumulation assessments. To date, no standardized experimental protocol has been universally accepted to estimate the biotransformation rates of commercial chemicals. The biotransformation rates of chemicals can be measured from in vivo experiments, but in vivo experiments are expensive, time consuming, and require a substantial number of animals raising ethical concerns. For example, the OECD 305-I guideline (OECD, 2012) for aqueous exposure bioconcentration fish test requires two groups of fish: an exposure group exposed to the test substance at one or more chosen concentrations and a control group held under identical conditions except for the absence of the test substance. A minimum of 4 fish are sampled on at least 5 occasions during the uptake phase (usually 28 days) and on at least 4 occasions during the depuration phase (usually 14 days). To determine the BCF of the test substance, a minimum of 72 fish per test are used and an approximate cost of US$ 125,000 per chemical is needed (Weisbrod et al., 2009).

Alternative predictive methods to determine the biotransformation rates of commercial chemicals are based on in silico and/or in vitro approaches. These are more rapid, ethical, and cost-effective manner; however they also have limitations. In silico modeling approaches involve the calculation of whole body biotransformation rate constants for organic chemicals using both modeled and empirical data (van der Linde et al., 2001; Arnot et al., 2008a; Arnot et al., 2008b; Arnot et al., 2009). As biotransformation is one of the elimination processes in the organism, the whole body biotransformation rate constant of the chemical can be obtained using a mass balance model that takes the difference between measured total elimination rate constant and the sum of modeled elimination rate constant in the absence of biotransformation (i.e., the sum of rate constants for respiratory elimination, fecal elimination, and growth dilution) (van der Linde et al., 2001; Arnot et al., 2008a). The total elimination rate constants can also be obtained from measured BCF data for fish (Arnot et al., 2008a). Based on this approach, Arnot et

al. (2008b) have compiled a database containing more than 1500 estimates of whole body biotransformation rate constants for approximately 700 organic chemicals in fish. Arnot et al., (2009) also developed a quantitative structure–activity relationship (QSAR) model to predict screening-level whole body biotransformation rate constants and half-lives for organic chemicals in fish. Kuo and Di Toro (2013) have used internal chemical partitioning and solute-solvent interactions as the basis of an in silico biotransformation model to calculate the whole body biotransformation half-lives of neutral and weakly polar organic chemicals in fish. Note that these in silico calculations of biotransformation rate constants or half-lives of organic chemicals have been developed for fish but not for mammalian species. There are limitations to in silico approaches. For example, robust estimates of whole body biotransformation half-lives require high quality empirical data. In some in vivo studies, experimental conditions such as specific feeding rates and growth rates were not reported, and simultaneous exposures to multiple chemicals resulting in substrate competition may have occurred (Arnot et al, 2008a). These shortcomings may affect the accuracy and uncertainty of model predictions. In addition, in silico models have restricted applicability to new chemical classes which may not be adequately represented in the training set of QSARs; the training set should contain large numbers of data including adequate diversity in chemical structures or functionalities to ensure a wide range of applicability (Kuo & Di Toro, 2013).

In in vitro approaches, the whole body biotransformation rate constant is obtained by extrapolating from measured in vitro biotransformation kinetics. In vitro assays followed by in vitro-to-in vivo extrapolation (IVIVE) have been developed and used widely in the pharmaceutical field to predict hepatic and total body clearance of drugs for clinical applications (Rane et al., 1977; Houston, 1994; Obach, 1999; Jones & Houston, 2004). More recently, IVIVE has been applied to bioaccumulation assessments in fish, first proposed by Nichols et al. (2006) and refined recently (Nichols et al. 2013). The first step of this approach involves experimental measurements of in vitro intrinsic clearance of the chemical in a subcellular or cellular hepatic system using a substrate depletion approach (Jones & Houston, 2004). The second step involves an IVIVE approach to obtain the hepatic clearance of the chemical extrapolated from the in vitro measurements using a well-stirred liver model (Wilkinson & Shand, 1975). The third step uses a fish bioaccumulation model to calculate BCFs (Arnot & Gobas, 2004) in which the whole body

biotransformation rate constant derived from the predicted hepatic clearance is used as an input parameter. Biotransformation rates have been determined using various in vitro fish liver preparations such as S9 subcellular fractions (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Han et al., 2009; Escher et al., 2011; Laue et al., 2014), microsomes (Dyer et al., 2008; Han et al., 2009), freshly isolated hepatocytes (Han et al., 2007; Cowan-Ellsberry et al., 2008; Dyer et al., 2008), and cryopreserved hepatocytes (Fay et al., 2014), as well as ex vivo assays using isolated perfused fish livers (Nichols et al., 2009b; Nichols et al., 2013). For most of the chemicals tested, BCFs that were calculated using biotransformation data were closer to BCFs measured in vivo than those for which biotransformation was ignored (Han et al., 2007; Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Fay et al., 2014; Laue et al., 2014). These data suggested that bioaccumulation assessment improved with the incorporation of biotransformation data using an IVIVE-BCF modeling approach. However, this approach has been developed for fish but not yet for mammalian species. Moreover, the in vitro test originally designed for relatively water-soluble drugs may not be readily applicable to highly hydrophobic commercial chemicals (log K_{OW} > 5). As noted above, the conventional in vitro dosing method in which the test chemical is dissolved in a spiking solvent may result in incomplete dissolution of highly hydrophobic chemicals (with low water solubility) (Kwon et al., 2009). Furthermore, the spiking solvent may inhibit enzyme activity (Li et al., 2010) resulting in underestimation of the in vitro biotransformation rate. A solvent-free dosing approach has the potential to overcome some of these problems. Previous studies have shown that hydrophobic test chemicals loaded into solid sorbent phases such as poly(dimethylsiloxane) (PDMS) or ethylene vinyl acetate (EVA) can deliver a steady concentration of dissolved chemical in aqueous media (Mayer et al., 1999; Brown et al., 2001; Kiparissis et al., 2003; Mayer et al., 2008; Kwon et al., 2009; Smith et al., 2010; Kramer et al., 2010; Golding et al., 2008; Meloche et al., 2009). A solvent-free sorbent-phase dosing technique may be useful for measuring in vitro biotransformation rates of very hydrophobic chemicals with bioaccumulation potential.

1.5. Research Objectives

The overall objective of my research was to improve national and international regulatory programs for the environmental management of industrial and commercial chemicals by developing and testing methods for the assessment of bioaccumulation of chemicals in biota. The following specific objectives were established:

1. To develop an in vitro dosing assay using a solvent-free, thin-film sorbent-phase dosing technique to measure the in vitro biotransformation rate constants of hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5) by rat and fish liver S9 subcellular fractions
2. To compare the measured in vitro biotransformation rates of hydrophobic chemicals from the sorbent-phase dosing approach to that from the conventional solvent-delivery doing method for highly hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5)
3. To develop and evaluate an in vitro-to-in vivo extrapolation (IVIVE) approach for calculating the whole body biotransformation rate constants of hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5) in rats
4. To develop a mechanistic bioaccumulation model for rats that includes in vitro biotransformation rates as a key variable to calculate the biomagnification factors (BMFs) of hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5)
5. To provide recommendations for improving regulatory assessment and control of potentially hazardous commercial chemicals in Canada

To accomplish this work, the development of a solvent-free, thin-film sorbent-phase dosing system for measuring the in vitro biotransformation rate constants of hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5) by rat liver S9 subcellular fractions is presented in **Chapter 2** (published as a peer reviewed paper in *Environmental Science & Technology*), including an investigation on the theory of the sorbent-phase dosing approach and a comparison between sorbent-phase dosing and conventional solvent-delivery dosing. The application of thin-film sorbent-phase dosing to measure the in vitro biotransformation rate constants of hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5) by fish liver S9 subcellular fractions is presented in **Chapter 3** (published as a peer reviewed paper in *Environmental Toxicology and Chemistry*), that includes comparisons between sorbent-phase dosing and conventional

solvent-delivery dosing and between single-chemical dosing and multi-chemical dosing. **Chapter 4** (submitted for publication to *Environmental Toxicology and Chemistry*) presents the development and evaluation of an in vitro-to-in vivo extrapolation (IVIVE) approach to determine the whole body biotransformation rate constants for hydrophobic chemicals with bioaccumulation potential (log K_{OW} > 5), the development of a rat bioaccumulation model for calculating the biomagnification factors, and the application of IVIVE in combination with bioaccumulation modeling as a regulatory tool. **Chapter 5** presents the conclusion and associated recommendations for improving regulatory management of potentially hazardous commercial chemicals in Canada from this study and from other related studies in the literature.

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Chapter 2.

Measuring In Vitro Biotransformation Rates of Super Hydrophobic Chemicals in Rat Liver S9 Fractions Using Thin-Film Sorbent-Phase Dosing*

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Yung-Shan Lee prepared the rat liver S9 homogenate, set up the sorbent-phase dosing system, designed the experiments, performed preliminary and primary experiments, performed instrumental analysis, performed data analysis using MATLAB codes written by Dave Campbell, and wrote the paper.

2.1. Summary

Methods for rapid and cost-effective assessment of the biotransformation potential of very hydrophobic and potentially bioaccumulative chemicals in mammals are urgently needed for the ongoing global evaluation of the environmental behaviour of commercial chemicals. We developed and tested a novel solvent-free, thin-film sorbent-phase in vitro dosing system to measure the in vitro biotransformation rates of very hydrophobic chemicals in male Sprague–Dawley rat liver S9 homogenates and compared the rates to those measured by conventional solvent-delivery dosing. The thin-film sorbent-phase dosing system using ethylene vinyl acetate coated vials was developed to eliminate the incomplete dissolution of very hydrophobic substances in largely aqueous liver homogenates, to determine biotransformation rates at low substrate concentrations, to measure the unbound fraction of substrate in solution, and to simplify chemical analysis by avoiding the difficult extraction of test chemicals from complex biological matrices. Biotransformation rates using sorbent-phase dosing were 2-fold greater than those measured using solvent-delivery dosing. Unbound concentrations of very hydrophobic

test chemicals were found to decline with increasing S9 and protein concentrations, causing measured biotransformation rates to be independent of S9 or protein concentrations. The results emphasize the importance of specifying both protein content and unbound substrate fraction in the measurement and reporting of in vitro biotransformation rates of very hydrophobic substances, which can be achieved in a thin-film sorbent-phase dosing system.

2.2. Introduction

National and international regulatory programs, including the Canadian Environmental Protection Act (CEPA), the U.S. Toxic Substances Control Act (TSCA), the E.U. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), and the UNEP Stockholm Convention on Persistent Organic Pollutants use the bioconcentration factor (BCF), bioaccumulation factor (BAF), or the logarithm of octanol–water partition coefficient (log K_{OW}) to assess the bioaccumulative behaviour of commercial chemicals in food-webs (Arnot & Gobas, 2006). However, since the BCF and BAF are not available for the great majority of commercial chemicals, the assessment of bioaccumulation often relies on the application of BCF and BAF bioaccumulation models, Quantitative Structure–Activity Relationships (QSARs) or the K_{OW} criterion (log K_{OW} > 5) (Arnot & Gobas, 2006; Robinson et al., 2004). A key limitation of these assessment techniques is that they are poorly equipped or unable to estimate the rate of biotransformation of the chemical (Weisbrod et al., 2009). Underestimation of biotransformation rates may cause many chemicals to be mis-classified as bioaccumulative when they are not. To date, standardized protocols for determining biotransformation rates of chemicals do not exist. However, there is a growing need to develop methods for determining the rates of biotransformation of chemicals especially for very hydrophobic (log K_{OW} > 5) and poorly volatile (log K_{OA} > 6) chemicals that have a high bioaccumulation potential. Chemicals with a relatively low K_{OW} and/or K_{OA} are quickly eliminated in most organisms and typically do not biomagnify even if they are not subject to biotransformation (Kelly et al., 2007). However, chemicals of high K_{OW} and high K_{OA} are very slowly eliminated and even low rates of biotransformation can dominate the overall depuration rate of the chemical and determine whether or not the substance will

biomagnify. For that reason, several authors have advocated for the development of methods for determining biotransformation rates that minimize animal testing, reduce costs, speed up the chemical evaluation process and use animal models other than fish (Weisbrod et al., 2009; Nichols et al., 2009a)

The application of in vitro assays and in vitro-to-in vivo extrapolation (IVIVE) of biotransformation rates has been proposed as a potential solution (Weisbrod et al., 2009; Nichols et al., 2009a; Nichols et al., 2006; Han et al., 2007; Dyer et al., 2008; Cowan-Ellsberry et al., 2008; Han et al., 2009; Escher et al., 2011; Nichols et al., 2009b). This method has been used extensively in the pharmaceutical field to assess metabolic clearance rates of drugs (Rane et al., 1977; Houston, 1994; Obach, 1999; Jones & Houston, 2004; Mohutsky et al., 2006). In vitro studies involving freshly isolated hepatocytes from fish (Han et al., 2007; Dyer et al., 2008; Cowan-Ellsberry et al., 2008) and rats (Han et al., 2007), fish liver microsomes (Dyer et al., 2008; Han et al., 2009) and fish liver S9 fractions (Cowan-Ellsberry et al., 2008; Han et al., 2009; Escher et al., 2011), perfused fish liver preparations (Nichols et al., 2009b) and models by Cowan-Ellsberry et al. (2008) demonstrate that these methods may also be useful for determining the biotransformation rate of potentially bioaccumulative chemicals. However, the exceptionally high hydrophobicity of potentially bioaccumulative substances may limit the applicability of tests designed for relatively water-soluble pharmaceutical drugs. One limitation concerns the dissolution of extremely hydrophobic substances in a largely aqueous medium (e.g., liver cell suspensions or tissue homogenates). The “solvent-delivery” or “spiking” procedure used for this purpose can lead to incomplete dissolution of the test chemicals in the assay medium (Hansen & Fouts, 1972; Robie et al., 1976; Luisi et al., 1975; Kwon et al., 2009) causing underestimation of the biotransformation rate constant. A second limitation is the introduction of a spiking solvent in the assay which can inactivate metabolic enzymes or may cause competitive inhibition between the spiking solvent (at high concentration) and the test chemical (at low concentrations) (Easterbrook et al., 2001; Hickman et al., 1998; Li et al., 2010). A third limitation concerns the applicability of the dosing regime of current in vitro bioassays to environmental exposures. Solvent-delivery methods for pharmaceutical drugs mimic typical oral drug administration conditions where organisms are exposed to high initial concentrations after oral administration. In contrast, exposures to environmental contaminants typically involve low

concentrations over a prolonged period. Exposure conditions can affect reaction order and rate. For example, high initial concentrations in in vitro bioassays using solvent-delivery procedures can lead to enzyme saturation (if the substrate concentration exceeds the Michaelis–Menten constant), thereby yielding lower substrate biotransformation rate constants than would be achieved at much lower substrate concentrations. A fourth limitation is that the extrapolation of in vitro to in vivo biotransformation rates requires knowledge of the unbound chemical fraction in the incubation medium, which is not determined in the solvent delivery method and therefore requires additional experimentation or computational modeling (Cowan-Ellsberry et al., 2008; Han et al., 2009; Escher et al., 2011). Finally, the application of solvent-delivery methods for the measurement of biotransformation rates of large numbers of chemicals can pose analytical challenges associated with the extraction, separation and analysis of test chemicals in complex biological media such as liver S9, microsomes or hepatocytes.

A solvent-free dosing technique has the potential to overcome these problems. Previous studies have shown that hydrophobic test chemicals loaded into solid sorbent phases such as poly(dimethylsiloxane) (PDMS) or ethylene vinyl acetate (EVA) can deliver a steady concentration of dissolved chemical in aqueous media (Kwon et al., 2009; Mayer et al., 1999; Brown et al., 2001; Kiparissis et al., 2003; Mayer et al., 2008; Smith et al., 2010; Kramer et al., 2010). Sorbent-phase dosing may be useful for measuring biotransformation rates of very hydrophobic chemicals with bioaccumulation potential because (i) it eliminates the problem of incomplete dissolution of very hydrophobic substances in aqueous tissue homogenates, (ii) it avoids the introduction of solvents into the incubation medium, (iii) it determines biotransformation rates at very low substrate concentrations, (iv) it measures the unbound fraction of substrate in solution, and (iv) it avoids chemical extraction from complex biological matrices if the chemical concentration in the sorbent phase is used for the determination of the biotransformation rate.

The objective of this study was to develop a thin-film sorbent-phase dosing system for measuring in vitro biotransformation rates of very hydrophobic chemicals by a rat liver S9 fraction, to compare the performance of this method to that of a conventional solvent dosing system and to investigate the role of the unbound substrate fraction and enzyme concentration on the in vitro biotransformation rate.

2.3. Theory

2.3.1. Sorbent-Phase Dosing

Thin-film sorbent-phase dosing involves the diffusive delivery of the test chemical from a thin film of solid sorbent material such as ethylene vinyl acetate (EVA) into a largely aqueous medium containing metabolic enzymes such as an S9 liver homogenate, liver microsomes or a suspension of hepatocytes. The exchange of the test chemical between the sorbent phase and the medium can be described by a two-compartment mass-transfer model (Figure 2.1):

$$\frac{dC_e}{dt} = k_2 \frac{V_m}{V_e} C_m - k_1 C_e \tag{2.1}$$

$$\frac{\mathrm{d}C_m}{dt} = k_1 \frac{V_e}{V_m} C_e - (k_2 + k_r) C_m \tag{2.2}$$

where C_e and C_m are the concentrations (mol/m^3) of the chemical in the EVA thin film and the incubation medium containing liver homogenate, respectively; V_e and V_m are the volumes of the EVA thin film and the incubation medium (m^3), respectively; k_1 and k_2 are the mass-transfer rate constants (min^{-1}) describing the transfer of the chemical from the thin film to the incubation medium (k_1) and from the medium back to the thin film (k_2), respectively; and k_r is the in vitro biotransformation rate constant (min^{-1}). Equation 2.1 describes the chemical exchange between the sorbent phase and the incubation medium. Equation 2.2 describes the role of biotransformation in the incubation medium.

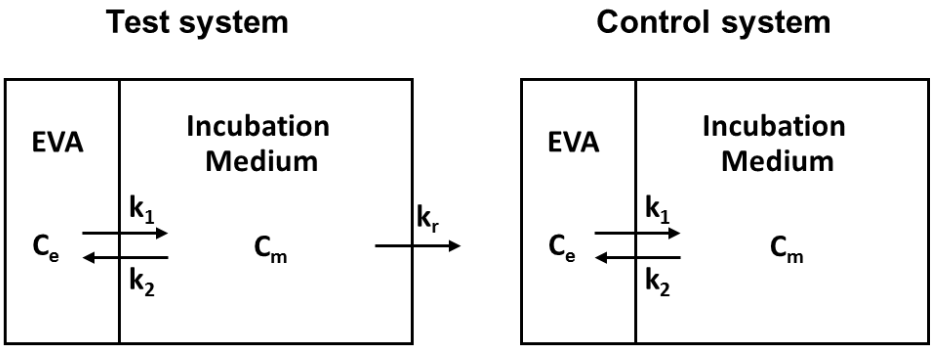

Figure 2.1. **Two-compartment model of the thin-film sorbent-phase dosing system illustrating the chemical substrate concentration in the thin film sorbent (C_e) and in the incubation medium (C_m), the film to medium transfer rate constant k_1, the medium to film transfer rate constant k_2 and the biotransformation rate constant k_r.**

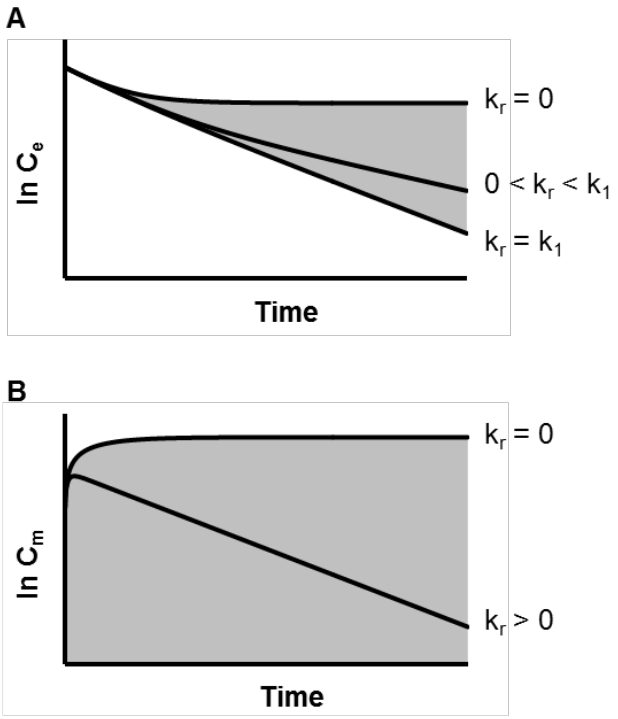

Figure 2.2. **Diagrams illustrating the theoretical time course of the chemical concentration in the thin film sorbent phase (A) and the incubation medium (B) if the biotransformation rate constant $k_r = 0$ (control) and $k_r > 0$ (test). The shading illustrates the approximate range of k_r values that can be determined when measuring concentrations in the sorbent phase (A) and in the incubation medium (B).**

Figure 2.2 illustrates that the biotransformation rate constant k_r can be determined by measuring the time course of the parent (i.e., unmetabolized) test chemical concentration in the sorbent phase (A) and the incubation medium (B) during a test (using a metabolically active medium) and a negative control (using an inactive medium, that is, k_r is zero). Inactive incubation medium can be prepared by heat denaturation of the enzymes, the exclusion of cofactors for the enzymatic reaction from the incubation medium or the addition of inhibitors. The rate constants k_1 and k_2 are determined in the control experiments by measuring the time course of the test chemical concentration in either the sorbent phase or the incubation medium or both media. The biotransformation rate constant k_r, is determined by comparing the concentration time course in either the sorbent or incubation medium of the active system (i.e., test) to the corresponding concentration time course in the inactive system (i.e., control).

Figure 2.2 also illustrates the theoretical working range of thin-film sorbent-phase dosing. When measuring concentration time course in the sorbent phase (A), k_r values ranging from the detection limit (i.e., no statistically significant differences between the sorbent concentration time courses in the test (active medium) and control (inactive medium)) to approximately k_1 (i.e., the film-to-medium chemical delivery rate constant), which is determined in the control experiment. Very high biotransformation rates (i.e., $k_r > k_1$) may therefore not be measurable as temporal concentration changes in the sorbent phase because the sorbent-to-medium delivery rate is the rate controlling step in the biotransformation rate measurement. Such very high k_r values should be reported as values greater than k_1. The value of k_1 is determined in the control experiment and may be increased by adding stirring techniques to the method. For the classification of chemicals for bioaccumulation capacity, it is likely that a minimum in vitro biotransformation rate constant for nonbioaccumulative substances can be defined. If this in vitro biotransformation rate constant is less than k_1, observations indicating that $k_r > k_1$ may be sufficient for chemical classification. When measuring the concentration time course in the incubation medium (B), all k_r values exceeding the lower detection limit (i.e., no statistically significant differences between the incubation medium concentration time courses in the test (active medium) and control (inactive medium)) can theoretically be measured. Very fast biotransformation reactions, including those for which $k_r > k_1$, can also be measured but may produce low concentrations in the incubation medium that do

not meet the analytical detection limit. However, the measurement of the reaction rate in the incubation medium is not limited by the sorbent-to-medium delivery rate.

2.3.2. In Vitro-to-In Vivo Extrapolation (IVIVE)

Several studies have shown that nonspecific binding can affect the extrapolation of in vitro biotransformation rates to in vivo rates (Austin et al., 2002; Riley et al., 2005; McLure et al., 2000; Gertz et al., 2008). This phenomenon is of particular importance to very hydrophobic chemicals with a high bioaccumulation potential because of the extremely high affinities of these chemicals for lipids, proteins and other biomolecules in biota. Therefore, the rate of biotransformation in a biological medium (dC_m/d_t) is often expressed in terms of the unbound or freely dissolved chemical concentration C_{fd}, which is related to the chemical concentrations in the reacting medium C_m by the fraction of unbound or freely dissolved chemical in the incubation medium f_{fd}:

$$-\frac{dC_m}{dt} = \frac{V_{max}}{K_M + C_{fd}} C_{fd} = k_r^* C_{fd} = k_r^* f_{fd} C_m = k_r C_m \quad (2.3)$$

where V_{max} is the maximum velocity of the biotransformation reaction ($mol \cdot m^{-3} \cdot min^{-1}$), K_M is the Michaelis–Menten constant (mol/m^3), k_r^* is the intrinsic in vitro biotransformation rate constant of the unbound test chemical (min^{-1}) and k_r is the apparent in vitro biotransformation rate constant of the test chemical (min^{-1}). At low substrate concentrations (i.e., $C_{fd} << K_M$), the apparent biotransformation rate constant k_r follows first order kinetics and is the product of the intrinsic in vitro biotransformation rate constant k_r^* and the fraction of freely dissolved (or unbound) test chemical in the incubation medium, that is,

$$k_r = \frac{V_{max}}{K_M} f_{fd} = k_r^* f_{fd} \quad (2.4)$$

The unbound fraction f_{fd} is often difficult to determine experimentally for very hydrophobic chemicals, but in a sorbent-phase dosing experiment it can be derived from the chemical concentrations in the film (C_e) and the medium (C_m) in the control experiment (using an enzymatically inactive medium) at steady-state as $C_e/(K_{ew} \cdot C_m)$ where K_{ew} is the thin film to water partition coefficient of the chemical, which can be determined

experimentally or through correlations with the octanol–water partition coefficient (Golding et al., 2008). Theoretically, f_{fd} can be expressed as:

$$f_{fd} = 1/\left[1 + \sum_{i=1}^{n}\left(\frac{V_i}{V_w}K_{iW}\right)\right] \quad (2.5)$$

where V_i/V_w is the relative volume (m^3) of nonaqueous medium constituent i (e.g., protein or lipid) to water, n is all relevant non-aqueous medium constituents that can bind the enzyme substrate other than water and K_{iw} is the medium constituent i to water partition coefficient (unitless). This expression is similar to the equation used for calculating unbound fraction reported by Jones and Houston (2004). Substituting Equation 2.5 in Equation 2.4 shows that for relatively water soluble substances which have a low K_{iw}, f_{fd} approaches 1 and the intrinsic biotransformation rate constant k_r* approaches k_r. An increase in protein content in the incubation (e.g., a higher concentration of S9) can therefore be expected to increase V_{max}, k_r* and k_r. This makes it necessary to normalize k_r to the protein content in the incubation medium when comparing in vitro clearance rates among different experiments and when extrapolating an in vitro biotransformation rate to an in vivo rate. For very hydrophobic substances for which $\Sigma(V_i \cdot K_{iw})/V_w >> 1$ (i.e., the great majority of the chemical in the reaction medium is bound), an increase in protein content (e.g., a more concentrated S9 medium) will increase V_{max} while reducing f_{fd}. For these substances, protein normalization of k_r can produce widely varying determinations of the biotransformation rate which are difficult to extrapolate to in vivo rates unless the fraction of unbound chemical in the test is taken into account. When extrapolating the experimentally observed apparent biotransformation rate constant to an in vivo biotransformation rate constant in a liver organ, it is important to account for differences in f_{fd} between the test system and the actual liver as well as the differences in protein concentrations in the test system and in the actual liver.

2.4. Materials and Methods

Descriptions of the chemical substances used; the preparation of liver S9 homogenates and conditions for analysis by gas chromatography–mass spectrometry (GC/MS) in this study are included in Appendix A.

2.4.1. Thin Film Preparation

A 0.64 g L^{-1} EVA solution was prepared by dissolving EVA beads in dichloromethane. This solution was spiked with one of three test chemicals, that is, chrysene (log K_{OW} = 5.60), benzo[a]pyrene (log K_{OW} = 6.04) and PCB 153 (log K_{OW} = 7.50), to final concentrations of 2.74, 5.05, and 8.66 µg/mL, respectively. Thin films of EVA containing the test chemicals were formed on the interior surface of 2 mL silanized amber glass vials (Agilent, Mississauga, ON) by adding 25 µL of the EVA solution and rolling the vials slowly to evaporate the solvent. Each vial contained 0.016 µL (16 µg) of EVA, producing a film with an average thickness of 0.02 µm (Meloche et al., 2009).

2.4.2. Incubation Conditions of Sorbent-Phase Dosing System

The reactions were started by adding 0.50 mL incubation mixture (preincubated at 37 °C for 5 min) to the EVA-coated vials. The maximum possible concentrations of chrysene, benzo[a]pyrene, and PCB 153 in the incubation medium were 0.6, 1.0, and 1.0 µM, respectively, assuming that 100% of the chemicals were delivered to the incubation medium. The incubation mixture consisted of 0.20 mL phosphate buffer (0.2 M, pH 7.4), 0.10 mL KCl (1.15% w/v), 0.10 mL NADPH–generating system (including 1.6 µmol NADP, 16 µmol glucose-6-phosphate, 1.6 units glucose-6-phosphate dehydrogenase following Van et al. (2007) and 4 µmol $MgCl_2$ prepared in phosphate buffer), and 0.10 mL male Sprague–Dawley rat liver S9 (containing approximately 6 mg S9 protein). Incubations were conducted at 37 ºC in a water bath (Grant OLS200, Cambridge, UK) that rolled the vials horizontally at 80 rpm to optimize contact between the incubation medium and the EVA thin film. Vials were capped with polytetrafluoroethylene (PTFE) lined screw caps during the incubation to prevent evaporative losses of the test chemicals. At 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min, the reactions were stopped by quickly transferring 0.40 mL of incubation medium into 1.0 mL ice-cold hexane (for later extraction and analysis) and by removing the remaining medium from the EVA coated vials. The EVA thin films were then rinsed twice with 0.40 mL of deionized water and 1.0 mL hexane was added to the vials to extract chemicals from the films.

Three independent experiments were conducted to determine the average in vitro biotransformation rates of the test chemicals. In each experiment, a test system using the

incubation conditions described above were run in parallel with two controls: a "no-cofactors control" in which the NADPH–generating system was omitted from the incubation mixture, and a "heat-treated control" which used heat treatment (60 °C for 10 min) for enzyme inactivation. In each experiment, two vials without incubation medium were also incubated to determine the initial concentration of the test chemicals in the EVA thin films (C_e at t = 0).

2.4.3. Incubation Conditions of Solvent-Delivery Dosing System

The in vitro biotransformation rates obtained from the solvent-free sorbent-phase dosing experiments were compared with rates measured using a conventional solvent-delivery dosing system, in which test chemicals dissolved in solvent (acetonitrile) were added directly to the incubation medium. The same S9 preparations were used in both sorbent-phase dosing and solvent-delivery dosing experiments. Identical incubation conditions were used. Briefly, 0.50 mL incubation mixture in 2-mL vials was preincubated at 37 °C for 5 min, and 2.4 μL of test chemicals dissolved in acetonitrile was added to initiate the reactions. Final concentrations of chrysene, benzo[a]pyrene, and PCB 153 were 0.6, 1.0, and 1.0 μM, respectively. The final acetonitrile concentration was < 0.5% (v/v), a concentration demonstrated to have no effect on five major cytochrome P450 activities in rat liver microsomes (Li et al., 2010). Incubations were carried out in triplicate experiments at 37 °C, and reactions were terminated at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min by adding 1.0 mL ice-cold hexane to the incubation medium to stop the reactions. The vials were stored on ice until chemical extraction.

2.4.4. Incubation Conditions for Protein Content Studies

To test the effect of altering protein content on the fraction of unbound substrate and the in vitro biotransformation rate, the sorbent-phase dosing experiments were conducted as described above using 1.2−12 mg S9 protein per incubation by diluting S9 homogenates. Incubations were carried out in triplicate experiments at 37 °C, and reactions were terminated at 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min as described earlier.

2.4.5. Chemical Extraction

Prior to chemical extraction of the films, internal standards (0.21 nmol chrysene-d^{12} for benzo[a]pyrene and chrysene; 0.14 nmol PCB 155 for PCB 153) were added to the hexane extraction solvent. Test chemicals remaining in the film after the termination of the incubation were extracted into the hexane by vortexing the vials vigorously for 30 seconds. Extracts were transferred to a 2-mL autosampler vial for GC/MS analysis. The extraction efficiencies of the three test chemicals from EVA were 102.4 ± 1.4% (mean ± SD).

For extraction of test chemicals from the incubation medium, after the addition of internal standards (0.21 nmol chrysene-d^{12} for benzo[a]pyrene and chrysene; 0.14 nmol PCB 155 for PCB 153), vials containing S9 plus hexane were vortexed for 90 seconds. The vials were then centrifuged at 800 × *g* for 10 min (Thermo IEC Centra-CL2). The upper organic layer was transferred to a 2-mL autosampler vial for GC/MS analysis. The extraction efficiencies were 87.8 ± 1.5%, 77.5 ± 4.4%, and 63.7 ± 13.1% (mean ± SD) for chrysene, benzo[a]pyrene, and PCB 153, respectively.

2.4.6. Data Analysis

To determine the mass-transfer rate constants (k_1, k_2) and in vitro biotransformation rates (k_r) and their 95% confidence intervals from the results of sorbent-phase dosing experiments, a procedure for fitting the experimental data using a nonlinear regression (Bates & Watts, 1988) and a Runge–Kutta numerical differential equation solver (Cheney & Kimcaid, 1994) using MATLAB R2009a (Mathworks, Natick, MA) was developed as described in Appendix A. To derive the biotransformation rate constants in solvent-delivery dosing experiments, the declining concentrations in the incubation medium over time were fitted by a first-order kinetic model:

$$\frac{dC_m}{dt} = -k_r C_m \tag{2.6}$$

where C_m is the control-corrected chemical concentration in the incubation medium (μM); and k_r is the apparent first-order biotransformation rate constant (min^{-1}). k_r was estimated using a linear regression from the slope of ln ($C_m/C_{m,t=0}$) vs time (i.e., ln ($C_m/C_{m,t=0}$) = $-k_r \cdot t$),

where $C_{m,t=0}$ is the initial dosing concentration of the chemical in the incubation medium (μM).

2.5. Results and Discussion

2.5.1. Sorbent-Phase Dosing

Figure 2.3 illustrates that the concentrations of chrysene, benzo[a]pyrene, and PCB153 in thin films exposed to inactive no-cofactor control S9 liver homogenates declined over time to reach plateau concentrations for chrysene and benzo[a]pyrene, but not for PCB153, as the test chemicals transferred from the thin film into the liver homogenates. Corresponding concentrations in the inactive liver homogenates increased over time reaching apparent steady-state concentrations. Concentrations in the thin films exposed to active liver homogenates showed a continuous decline in concentration over time. Corresponding concentrations in the active liver homogenates increased over time, as the test chemical transferred from the thin film to the liver homogenate, then reached a maximum when the net mass transfer rate from film to medium equaled the biotransformation rate and finally declined (except for PCB153) when the biotransformation rate exceeded the net film to medium mass transfer rate. Concentrations of benzo[a]pyrene in the homogenate were well below the reported Michaelis–Menten constant K_M of 14.1 and 14.6 μM in rat liver S9 (Alvares et al., 1968; Zampaglione & Mannering, 1973).

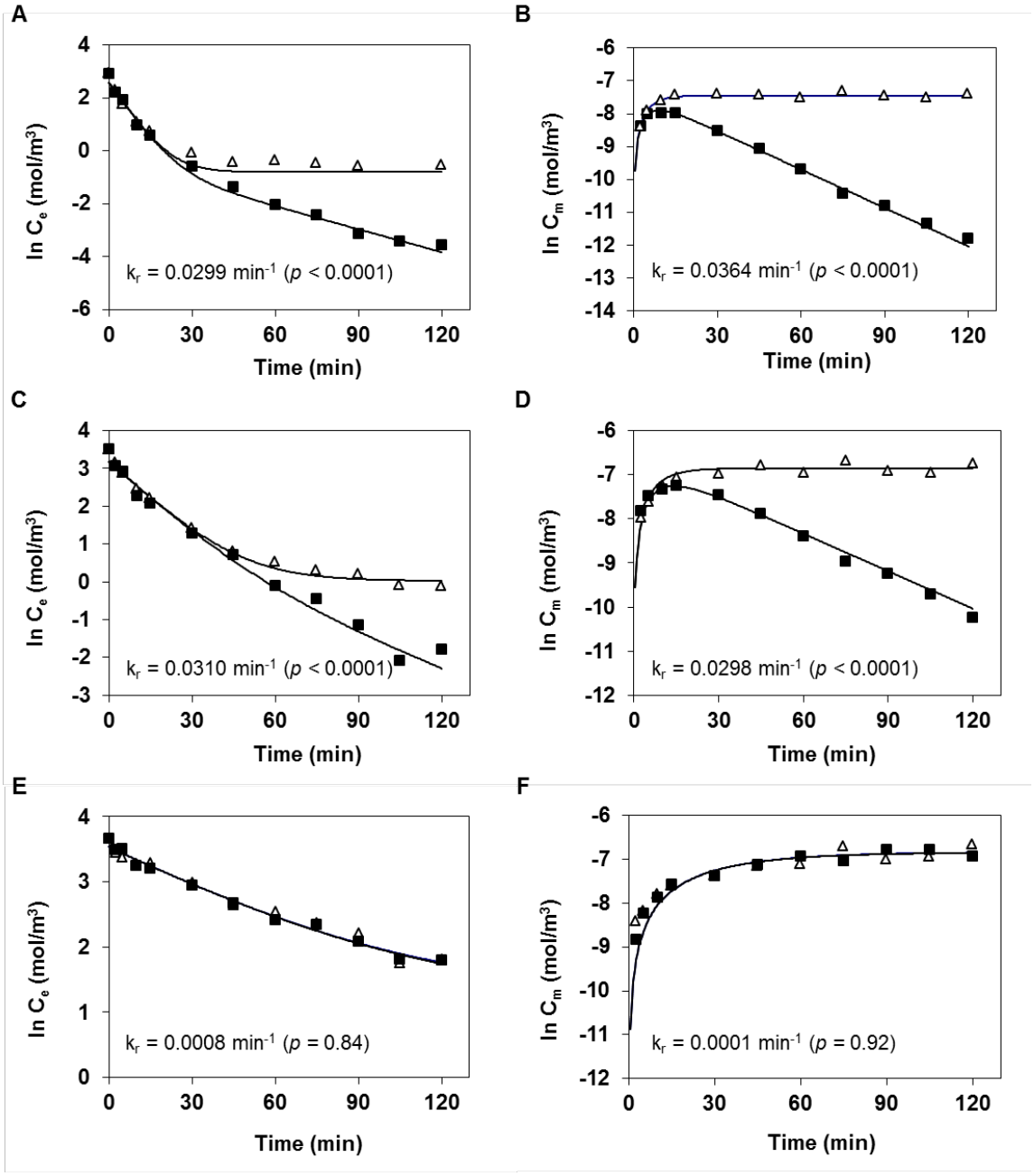

Figure 2.3. Concentration-time profiles in the EVA thin film sorbent phase (left) and in the incubation medium (right) containing active (solid squares) or inactive (open triangles) male Sprague–Dawley rat liver S9 homogenate (no-cofactor control) using the sorbent-phase dosing approach for chrysene (A, B), benzo[a]pyrene (C, D), and PCB 153 (E, F). Solid lines represent nonlinear regressions. Data from one of three experiments are shown.

Chrysene and benzo[a]pyrene concentrations in the film and the liver homogenate showed highly significant differences between the test (active liver homogenate) and control (inactive liver homogenate) as demonstrated by the *p*-values (< 0.0001) of the k_r nonlinear regression determinations (Figure 2.3). Statistically significant differences ($p < 0.05$) in concentrations between the control and test were not observed for PCB153 concentrations in the films or liver homogenates. Hence, PCB153 does not appear to be biotransformed at a significant rate, which is consistent with results from other studies (Muhlebach et al., 1991).

Figure 2.3 shows that the initial concentrations of benzo[a]pyrene, chrysene and PCB 153 in test and no-cofactor control experiments were the same, indicating a similarity in the chemical exchange kinetics in the control and test systems. Such agreement was not observed when using controls prepared by heat treatment (Figure A1 in Appendix A). This indicates that heat treatment affects the diffusion characteristics of the liver homogenate resulting in a change in the film-to-liver homogenate exchange kinetics between control and test which interferes with the measurement of k_r. For this reason, only data from the no-cofactor controls were used for further analysis.

Figure 2.4 illustrates that for all test chemicals, k_1 was greater than k_r. This confirms that the delivery of the test chemicals from the sorbent phase to the liver homogenate was not the rate-limiting step, making it possible to determine k_r from concentration measurements in the thin films. Figure 2.4 also shows that the results from the three independent experiments were highly reproducible and that similar determinations of k_1, k_2, and k_r were obtained for each of the test chemicals by analyzing the chemical concentrations in the film only, the liver homogenate only, or the combined data set of concentrations in film and liver homogenate. This indicates that k_r can be determined by measuring either the concentrations in the film or the liver homogenate or both.

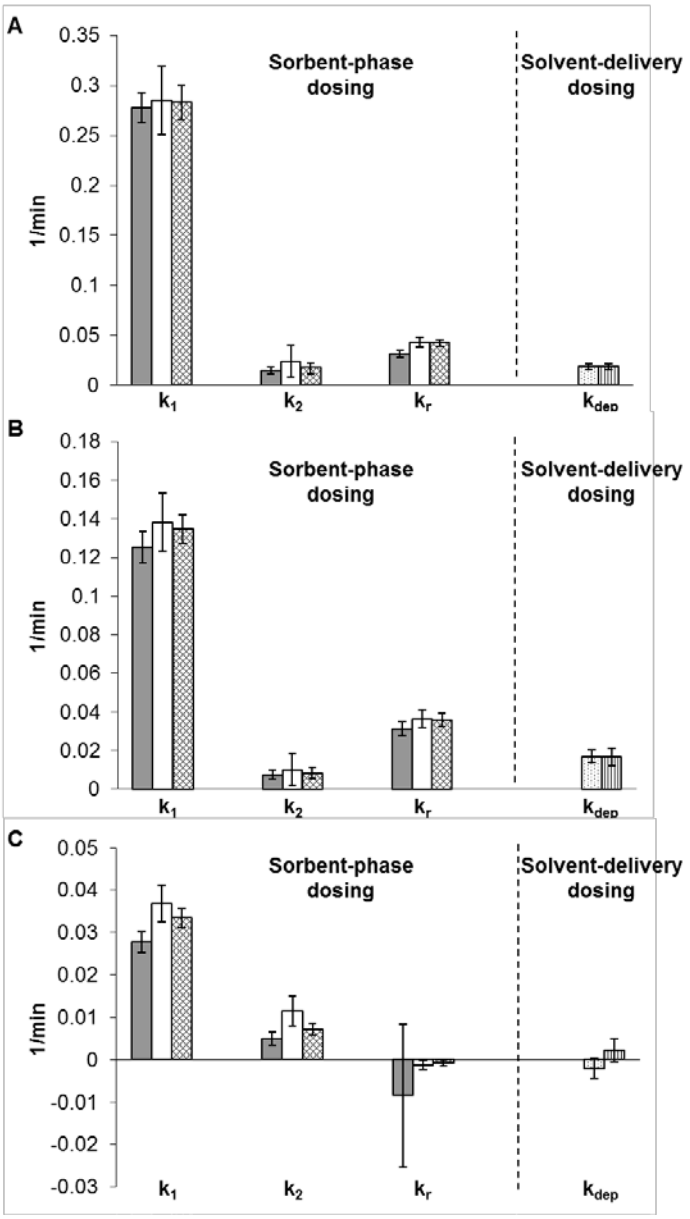

Figure 2.4. **Measured mass-transfer rate constants (k_1 and k_2) and in vitro biotransformation rates (k_r) determined in sorbent-phase dosing experiments (n = 3) and solvent-delivery dosing experiments (n = 3) for chrysene (A), benzo[a]pyrene (B), and PCB 153 (C). Results from sorbent-phase dosing experiments were obtained from concentrations in thin films only (filled bars), the incubation medium only (empty bars), or the combined data set of thin film and incubation medium concentration data (cross-hatched bars) using the no-cofactors control. k_r values obtained from the solvent-delivery dosing experiments were analyzed using the no-cofactor control (dotted bars) or the heat-treated control (striped bars). Results were obtained from three independent experiments and error bars denote 95% confidence intervals.**

Figure A2 in Appendix A illustrates that the rate of chemical delivery from the thin film to the liver homogenate (k_1) decreased with increasing hydrophobicity (log K_{OW}) of the test chemical. A similar trend was observed for k_2. This means that with increasing hydrophobicity, it becomes more difficult to measure high biotransformation rates by analyzing the chemical concentrations in the EVA thin film. Since the measurement of biotransformation rate from concentrations in the liver homogenate medium is not directly affected by the rate of chemical delivery from film to medium, high biotransformation rates for very hydrophobic chemicals can still be measured by analyzing chemical concentrations in the incubation medium.

2.5.2. Solvent-Delivery Dosing

Figure 2.5 illustrates that in solvent-delivery dosing experiments, concentrations of chrysene and benzo[a]pyrene in rat liver S9 declined in a log–linear fashion over the course of the incubation period, while the concentrations of PCB153 did not. Linear regressions showed highly significant declines ($p < 0.05$ for the slopes) for chrysene and benzo[a]pyrene but not for PCB153. The apparent biotransformation rate constants (k_r) determined in triplicate experiments using no-cofactor and heat-treated controls are shown in Figure 2.4. Differences in the determination of k_r between using the two controls were not statistically significant ($p > 0.05$), indicating that in solvent based substrate delivery, the determination of in vitro biotransformation rates was not affected by the heat treatment of the homogenate.

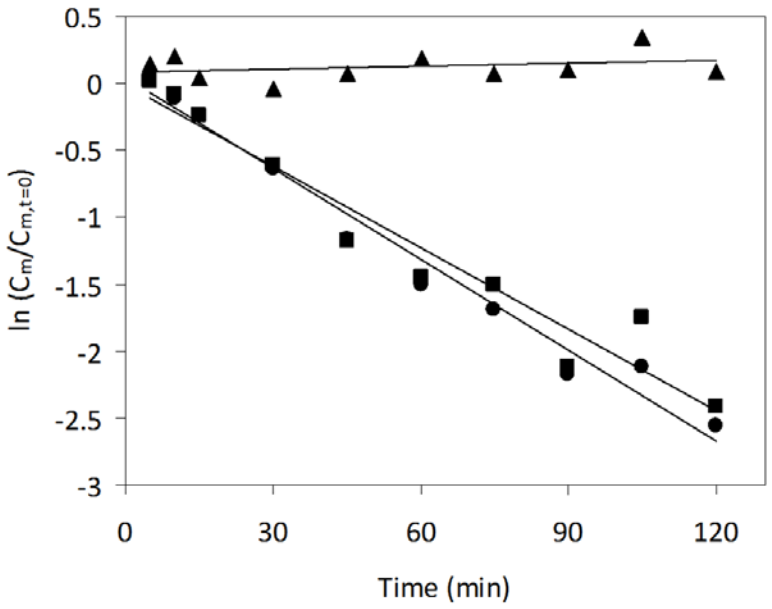

Figure 2.5. Concentration-time profiles expressed as the ratio of chemical concentrations in the incubation medium of the test and control for chrysene (●), benzo[a]pyrene (■), and PCB 153 (▲) in rat liver S9 homogenate using solvent-delivery dosing and no-cofactor controls. Results represent one of three independent experiments.

Figure 2.4 shows that the in vitro biotransformation rates for both chrysene and benzo[a]pyrene using sorbent-phase dosing were two times greater ($p < 0.05$) than those derived from the solvent-delivery dosing experiments using the same liver preparation. The higher in vitro biotransformation rates obtained with the sorbent-phase dosing system may be attributable to (i) lack of enzyme inhibition by a spiking solvent, (ii) low initial substrate concentrations in the liver homogenate thereby reducing the potential for enzyme saturation and preventing precipitation (e.g., microcrystals) of the hydrophobic substrates (Kwon et al., 2009) which can limit access of the substrate to enzymes, and (iii) reduction of enzyme inhibition by metabolic products because of their diffusion into the thin film. Studies have shown that metabolism of benzo[a]pyrene in rats can be inhibited by several of its metabolic products (Shen et al., 1979; Fahl, 1982; Keller et al., 1982). In addition, two-phase bioreactors enhance microbial biotransformation by in situ removal of inhibitory products by the organic phase (Malinowski, 2001; Daugulis, 1997).

The measured apparent in vitro half-life ($\ln 2/k_r$) for benzo[a]pyrene was 22 min using sorbent-phase dosing compared to 41 min using solvent-delivery dosing. These

values are consistent with published in vitro half-lives of benzo[a]pyrene in rat liver microsomes (30 min) (Fahl, 1982; Keller et al., 1982) and rat hepatocytes (60 min) (Han et al., 2007). However, biotransformation rates expressed as clearance rates in units of $mL \cdot h^{-1} \cdot mg\ protein^{-1}$ encompass a wide range of values from 0.63 to 19.4 $mL \cdot h^{-1} \cdot mg\ S9\ protein^{-1}$ (Table A1 in Appendix A). Using the sorbent-phase dosing system, we obtained 0.2 $mL \cdot h^{-1} \cdot mg\ S9\ protein^{-1}$. The differences in measured biotransformation rates likely arise due to interlaboratory differences in experimental approach such as monitoring the rate of metabolite formation versus substrate depletion, and differences in protein concentrations and consequently, in the fraction of unbound chemical substrate used in the in vitro system. The relationship between in vitro biotransformation rate and concentrations of protein and unbound chemical fractions is discussed in the next section.

2.5.3. Unbound Chemical Substrate Fraction

The unbound fractions of chrysene, benzo[a]pyrene and PCB153 in the incubation medium were determined from the concentrations in the film and the incubation medium in the control experiment at steady-state as $C_e/(K_{ew} \cdot C_m)$ where K_{ew} was $10^{6.40}$ for chrysene, $10^{6.52}$ for benzo[a]pyrene and $10^{8.10}$ for PCB153 (Golding et al., 2008). Figure 2.6 illustrates that increasing the amount of liver homogenate protein 10-fold per incubation (from 1.2 to 12 mg) resulted in a statistically significant decline in the fraction of unbound chrysene and benzo[a]pyrene in the liver homogenate from approximately 0.2% to 0.025% (for chrysene) and from 0.12% to 0.018% (for benzo[a]pyrene). However, Figure 2.6 also shows that while the fraction of unbound chemical decreased, there was no statistically significant change in the value of k_r. The significance of the slope of the linear regression of k_r versus protein content was characterized by probability values of p = 0.93 for chrysene and p = 0.09 for benzo[a]pyrene. It appears that for the very hydrophobic chemicals in this study, a 10-fold increase in enzyme activity in incubations (which elevates biotransformation rates) was associated with a similar reduction in the unbound substrate fraction (which reduces biotransformation rates), causing no statistically significant changes in the apparent in vitro biotransformation rates with increasing protein content. A similar observation was observed in a previous study with very hydrophobic substrates (Kalvass et al., 2001). These observations are consistent with the theory expressed by Equation 2.3. These findings imply that protein normalization of measured

biotransformation rate constants of very hydrophobic substances can produce substantial error in the measurement of k_r and that the large variation in observed in vitro intrinsic clearance rates of benzo[a]pyrene among different studies documented in Table A1 of Appendix A may be partly explained by protein normalization of measured biotransformation rates. These findings emphasize the importance of specifying both protein content and unbound substrate fraction in the measurement and reporting of in vitro biotransformation rates for very hydrophobic substances. The study indicates that the intrinsic in vitro biotransformation clearance rate of the unbound chemical is remarkably fast at rates of 10.9 ± 1.5 $mL \cdot min^{-1} \cdot mg$ S9 $protein^{-1}$ (mean ± SD) for chrysene and 15.3 ± 4.1 $mL \cdot min^{-1} \cdot mg$ S9 $protein^{-1}$ (mean ± SD) for benzo[a]pyrene and that the binding of the very hydrophobic chemicals to liver homogenate constituents other than the active sites of biotransforming enzymes exerts a large influence on the apparent biotransformation rate.

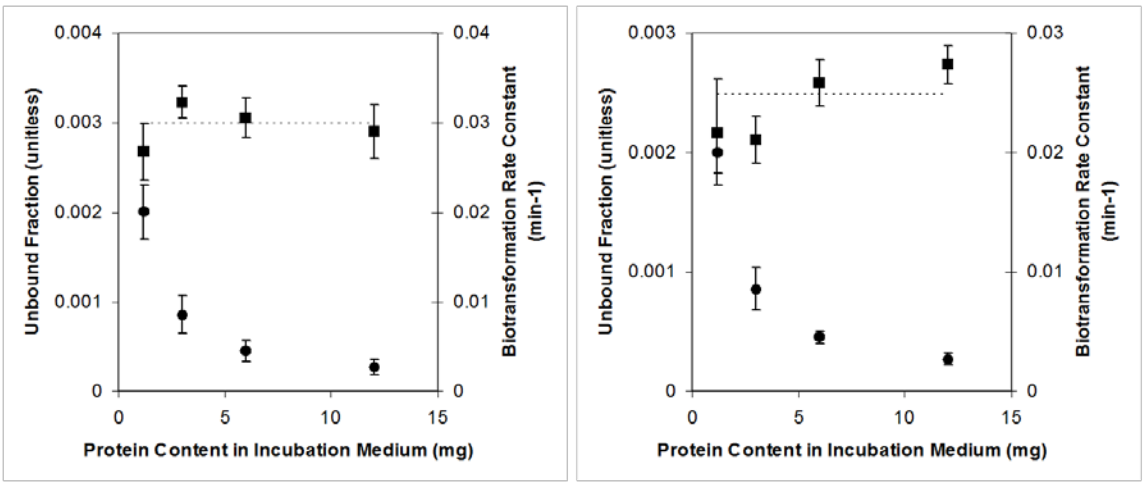

Figure 2.6. Relationship between the rat liver S9 protein content (mg) in the incubation medium and the unbound fraction (unitless) in the incubation medium (●, left axis) and the apparent biotransformation rate constant k_r (min^{-1}) (■, right axis) for chrysene (left) and benzo[a]pyrene (right). Error bars denote 95% confidence intervals. Dotted lines represent the average apparent biotransformation rate constant.

2.5.4. Method Application

The implementation of effective bioaccumulation screening under the UN Convention on Persistent Organic Chemicals, REACH in the European Union, CEPA in Canada and TSCA in the U.S. requires access to methods that can, with relative ease and low cost, determine biotransformation rates of many commercial chemicals (Weisbrod et al., 2009). Of particular importance are methods for chemicals with very high octanol–water and octanol–air partition coefficients as these chemicals have an intrinsic potential for food-web biomagnification. In addition, methods tailored for mammalian species are important because mammals occupy high trophic positions in food-webs and for many chemicals, bioaccumulation measurements in fish do not provide accurate estimates of bioaccumulation in mammals (Kelly et al., 2007; Gobas et al., 2003). The extreme hydrophobicity and very low aqueous solubility of chemicals with a high bioaccumulation potential can pose methodological challenges especially if the method involves chemical dissolution in an aqueous medium. The sorbent-phase dosing technique explored in this study may reduce some of these challenges by eliminating the need for the addition of chemical carrier solvents and by helping to maintain first order kinetics of

biotransformation by the low initial substrate concentrations in the incubation medium. The experimental analysis of chemical concentrations in the thin film does in many cases not require the type of analytical clean up procedures often associated with the extraction and analysis of organic chemicals in complex biological matrices like liver homogenates. Measurement of chemical concentrations in the thin films also provides a relatively simple method for measuring the unbound chemical fraction in the incubation medium, which is important for in vitro to in vivo extrapolations. The findings of this study suggest that the thin-film sorbent-phase dosing approach may be a simple and fast screening tool for measuring the in vitro biotransformation rates of commercial substances with a high bioaccumulation potential in mammalian species.

One of the key limitations of sorbent-phase dosing technique is the use of an appropriate control. The no-cofactor control may be appropriate for chemicals whose biotransformation is primarily mediated by cytochrome P450 enzymes, such as the oxidations of many polycyclic aromatic hydrocarbons (PAHs). However, the heat or chemically treated controls may be necessary for biotransformation reactions mediated by enzymes that do not require NADPH or other cofactors (e.g., soluble enzymes in the liver cytosol). This study showed that heat treatment of the incubation medium affects the film-to-medium exchange kinetics, causing differences in the concentration time course between test and control unrelated to biotransformation. Future studies are needed to develop strategies for using heat-treated controls or for finding alternative chemical controls (e.g., mercury or sodium azide treatment) for assessing the biotransformation ability of chemicals that are not biotransformed by cytochrome P450.

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Chapter 3.

In Vitro Biotransformation Rates in Fish Liver S9: Effect of Dosing Techniques*

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Yung-Shan Lee prepared the fish liver S9 homogenate with Danny Lee, supervised Danny Lee to perform experiments and instrumental analysis, performed data analysis, interpreted the results, and wrote the paper.

3.1. Summary

In vitro biotransformation assays are currently being explored to improve estimates of bioconcentration factors of potentially bioaccumulative organic chemicals in fish. The present study compares thin-film and solvent-delivery dosing techniques as well as single- versus multi-chemical dosing for measuring biotransformation rates of selected polycyclic aromatic hydrocarbons in rainbow trout (*Oncorhynchus mykiss*) liver S9. The findings show that biotransformation rates of very hydrophobic substances can be accurately measured in thin-film sorbent-dosing assays from concentration–time profiles in the incubation medium but not from those in the sorbent phase because of low chemical film-to-incubation-medium mass-transfer rates at the incubation temperature of 13.5 °C required for trout liver assays. Biotransformation rates determined by thin-film dosing were greater than those determined by solvent-delivery dosing for chrysene (octanol–water partition coefficient [K_{OW}] = $10^{5.60}$) and benzo[a]pyrene (K_{OW} = $10^{6.04}$), whereas there were no statistical differences in pyrene (K_{OW} = $10^{5.18}$) biotransformation rates between the two methods. In sorbent delivery–based assays, simultaneous multi-chemical dosing produced biotransformation rates that were not statistically different from those measured in single-chemical dosing experiments for pyrene and benzo[a]pyrene but not for chrysene.

In solvent-delivery experiments, multi-chemical dosing produced biotransformation rates that were much smaller than those in single-chemical dosing experiments for all test chemicals. While thin-film sorbent-phase and solvent delivery–based dosing methods are both suitable methods for measuring biotransformation rates of substances of intermediate hydrophobicity, thin-film sorbent-phase dosing may be more suitable for superhydrophobic chemicals.

3.2. Introduction

Biotransformation may play an important role in the elimination and bioaccumulation of xenobiotic chemicals. However, the lack of a priori consideration of biotransformation rates is a major limitation in current bioaccumulation assessments. The bioconcentration factor (BCF), bioaccumulation factor (BAF), and the octanol–water partition coefficient (K_{OW}) are routinely used to assess the bioaccumulative behavior of chemicals according to national and international regulations such as the Canadian Environmental Protection Act; the US Toxic Substances Control Act; the European Union's Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH); the Japanese Chemical Substances Control Law; and the Stockholm Convention on Persistent Organic Pollutants (Arnot & Gobas, 2006). Empirical BCFs and BAFs do not exist for the vast majority of commercial chemicals. Therefore, to date, bioaccumulation assessments have often relied on the K_{OW} or on bioaccumulation models, which in most cases did not consider biotransformation because of a lack of information on biotransformation rates. This approach can lead to misidentification of the bioaccumulation potential of chemicals in screening and risk assessments. Recently, two approaches have emerged to include biotransformation in bioaccumulation assessments. One approach is the development of quantitative structure–activity relationships for calculating the BCF and BAF that take into account biotransformation (Arnot et al., 2009). The other approach is the application of experimental in vitro biotransformation tests (Nichols et al., 2006; Cowan-Ellsberry et al., 2008; Nichols et al., 2009a; Nichols et al., 2013; Dyer et al., 2008; Han et al., 2009).

In vitro analysis of chemical biotransformation rates in liver preparations has been advocated for predicting biotransformation rates in whole organisms in a manner that is

cost-effective and reduces animal use (Weisbrod et al., 2009). Developing protocols for in vitro biotransformation tests using fish liver is of particular interest because existing bioaccumulation models for fish can use the bioassay data to estimate BCF and BAF values (Nichols et al., 2006; Cowan-Ellsberry et al., 2008; Nichols et al., 2009a; Nichols et al., 2013; Arnot & Gobas, 2004). Various fish liver preparations include 9000-*g* supernatants of liver homogenate (S9) (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Han et al., 2009; Escher et al., 2011), microsomes (Dyer et al., 2008; Han et al., 2009), freshly isolated hepatocytes (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Han et al., 2007; Uchea et al., 2013), and cultured hepatocytes (Uchea et al., 2013). Ex vivo assays using isolated perfused fish livers have also been proposed to measure biotransformation rates that can be extrapolated to the whole body (Nichols et al., 2009b). However, in vitro assays for measuring biotransformation rates of highly hydrophobic chemicals (log K_{OW} > 5) with high bioaccumulation potential can be challenging when using conventional solvent-delivery dosing methods where chemicals are introduced into the incubation medium dissolved in a small volume of an organic solvent (Lee et al., 2012). These challenges include incomplete dissolution of the hydrophobic chemical in the largely aqueous assay medium and inhibition of enzyme activity by the spiking solvent. A solvent-free dosing approach has the potential to overcome the above-mentioned problems and may reduce error in measurements of the in vitro biotransformation rates of very hydrophobic chemicals (i.e., those with high bioaccumulation potential) (Lee et al., 2012). In addition, sorbent-phase dosing allows for direct measurement of the unbound chemical fraction in the incubation and reduces substrate saturation of enzyme by using incubations with an initial substrate concentration of zero.

Solvent-free passive dosing techniques have been developed and applied to improve toxicity testing of highly hydrophobic chemicals by loading the test chemical into a sorbent phase such as poly(dimethylsiloxane), silicone O-rings, or ethylene vinyl acetate (EVA), and then delivering the chemical into the assay medium by passive diffusion (Escher et al., 2011; Mayer et al., 1999; Brown et al., 2001; Kiparissis et al., 2003; Mayer & Holmstrup, 2008; Kwon et al., 2009; Smith et al., 2010; Kramer et al., 2010). Passive dosing using silicone O-rings as the dosing polymer has also been used to measure biodegradation kinetics of phenanthrene and fluoranthene in a bacterial strain at defined dissolved chemical concentrations ranging over 4 orders of magnitude (Smith et al., 2012).

An EVA thin-film sorbent-phase dosing approach has been developed and applied to measure the in vitro biotransformation rates of superhydrophobic chemicals by rat liver S9 fractions, and it was found that the in vitro biotransformation rates measured using the sorbent-phase dosing method were greater than those measured using a solvent-delivery dosing method under the same conditions (Lee et al., 2012).

The objective of the present study was to develop and evaluate the sorbent-phase dosing technique to measure in vitro biotransformation rates of hydrophobic chemicals by liver S9 fractions from rainbow trout (*Oncorhynchus mykiss*). In vitro biotransformation rates obtained from sorbent-phase dosing were compared with biotransformation rates measured using conventional solvent-delivery dosing to evaluate method performance. To date, solvent-free passive dosing techniques have not been applied to fish liver preparations, yet fish remain the predominant species used in bioaccumulation assessment. In addition, we investigated multi-chemical dosing using both sorbent-phase and solvent-delivery dosing approaches; measuring the biotransformation rates of multiple chemicals in the same incubation would reduce time, costs, and animal use. The ultimate goal of the present studies was to improve current bioaccumulation assessments.

3.3. Theory

In thin-film sorbent-phase dosing systems, the test chemical is transferred by passive diffusion from the sorbent phase (EVA thin films) to the incubation medium containing liver S9 with active metabolic capacity (test) or inactivated enzymes (control). The theory and inherent assumptions of the thin-film sorbent-phase dosing approach are presented in Lee et al. (2012). A two-compartment mass-transfer model is used to describe the exchange of the test chemical between the sorbent phase and the incubation medium considering simultaneous diffusive transfer and biotransformation, as described by the Equations 3.1 and 3.2

$$\frac{dC_e}{dt} = k_2 \frac{V_m}{V_e} C_m - k_1 C_e \tag{3.1}$$

$$\frac{\mathrm{d}C_m}{dt} = k_1 \frac{V_e}{V_m} C_e - (k_2 + k_r) C_m \tag{3.2}$$

where C_e and C_m are concentrations (mol/m^3) of the chemical in the EVA sorbent phase and the incubation medium, respectively; V_e and V_m are volumes (m^3) of the EVA thin film and the incubation medium, respectively; k_1 and k_2 are mass-transfer rate constants (min^{-1}) describing the transfer of the chemical from the sorbent phase to the incubation medium (k_1) and from the medium back to the sorbent phase (k_2); and k_r is the in vitro biotransformation rate constant (min^{-1}). It is required that the mass-transfer rate constants (k_1 and k_2) of the chemical in the test are the same in the test and control and that k_r is 0 in the control.

One of the features of the thin-film sorbent-phase dosing technique is that biotransformation rates can be determined by measuring chemical concentrations in the EVA films over time, which is analytically less difficult than measuring concentrations in highly complex liver preparations. However, this requires the diffusive mass-transfer rate of the chemical from the sorbent to the incubation mixture to be greater than the rate of biotransformation (i.e., $k_1 > k_r$) to ensure that the mass transfer is not the rate-limiting step in the biotransformation process (Lee et al., 2012). If this requirement is not met and $k_1 \leq k_r$, then the biotransformation rate can be determined from the chemical concentration in the incubation medium over time but not from the concentrations in the films.

3.4. Materials and Methods

3.4.1. Chemicals

Pyrene, chrysene, benzo[a]pyrene, and chrysene-d^{12} were purchased from Sigma-Aldrich with purities of 98% or higher. Ethylene vinyl acetate (EVA), Elvax 40W, was obtained from DuPont (Willington, DE). Potassium dihydrogen phosphate and high-performance liquid chromatography (HPLC)–grade acetonitrile were obtained from Caledon Laboratories. Potassium phosphate dibasic was obtained from Anachemia Canada (Lachine, QC). Potassium chloride and HPLC–grade hexane were obtained from EMD Millipore (Gibbstown, NJ). All other chemicals, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO).

3.4.2. Animals

Nine male rainbow trout (*Oncorhynchus mykiss*, approximately 1000 g body weight) were obtained from Miracle Springs (Mission, BC). The fish were held in tanks equipped with a dechlorinated tap water flow-through system (13.5 ± 2 °C) under a 16:8-h light:dark cycle for at least two weeks and fed EWOS Pacific 3.0-mm pellets (Surrey, BC) once daily.

3.4.3. Preparation of Trout Liver S9 Fraction

The trout were euthanized by overdose exposure to 0.3 $g \cdot L^{-1}$ tricaine methanesulfonate (MS222, buffered with 0.3 $g \cdot L^{-1}$ sodium bicarbonate). Exposure to this concentration of MS222 for 5 min has no effect on microsomal P450 activities (Kolanczyk et al., 2003). Livers were immediately excised and rinsed in ice-cold 1.15% (w/v) KCl. Each liver was weighed, minced on ice with a razor blade, and homogenized on ice using a Potter-Elvehjem glass tissue grinder with a Teflon pestle (Kontes, Vineland, NJ) in 1 volume ($g \cdot mL^{-1}$) of ice-cold 0.20 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The liver homogenates were pooled into three groups (each group was comprised of three trout livers) and centrifuged at 9000 × *g* for 20 min at 4 °C (Hermle Z360K centrifuge). The 9000-*g* supernatant fraction (S9) was collected and stored at −80 °C until use (held for < 3 months). The protein concentration of the S9 fraction was determined by the method of Bradford (1976) using bovine serum albumin (Fraction V; Sigma-Aldrich) as the standard.

3.4.4. Thin Film Preparation

The EVA thin film was prepared according to Lee et al. (2012). Briefly, a 0.135-$g \cdot L^{-1}$ EVA solution was prepared by dissolving EVA beads in dichloromethane. The test chemicals were pyrene, chrysene, and benzo[a]pyrene, which have log K_{OW} values of 5.18, 5.60, and 6.04, respectively (Mackay et al., 2006). These chemicals were added to the EVA solution individually or as a mixture producing nominal concentrations of 4.05 $\mu g \cdot mL^{-1}$, 4.57 $\mu g \cdot mL^{-1}$, and 12.62 $\mu g \cdot mL^{-1}$, respectively. The maximum possible concentration in the incubation medium (assuming all chemical in the sorbent is instantaneously released into the incubation medium) was 1.0 µM for pyrene and chrysene and 2.5 µM for benzo[a]pyrene. These concentrations are less than reported Michaelis–

Menten constants (K_M) of 15.1 µM for pyrene hydroxylation by isolated hepatocytes from rainbow trout (Law et al., 1991) and 33 µM to 125 µM for benzo[a]pyrene hydroxylation by liver microsomes of rainbow trout (Carpenter et al., 1990). Thin films of EVA containing the test chemicals were formed on the interior surface of 2-mL silanized amber glass vials (Agilent, Mississauga, ON) by adding 25 µL of the spiked EVA solution and rolling the vials slowly to evaporate the solvent. The thin films were 4 nm thick and contained 0.0035 µL (3.4 µg) EVA. Thicker films (20 nm containing 17 µg EVA) were also studied. Film thickness was estimated by dividing the volume of EVA film by the interior surface area of the vial.

3.4.5. Incubation Conditions of Sorbent-Phase Dosing System

The reactions were started by adding 0.50 mL of the incubation mixture containing trout liver S9 (preincubated at 13.5 °C for 5 min) to the EVA–coated vials. The incubation mixture consisted of 0.38 mL phosphate buffer (0.20 M, pH 7.4) containing 1.15% (w/v) KCl, 0.10 mL reduced nicotinamide adenine dinucleotide phosphate (NADPH)–generating system (0.8 µmol nicotinamide adenine dinucleotide phosphate, 8 µmol glucose-6-phosphate, 1.6 units glucose-6-phosphate dehydrogenase, and 4 µmol $MgCl_2$ prepared in phosphate buffer), and 0.020 mL trout liver S9 (containing approximately 3 mg S9 protein in the incubation mixture). Incubations were conducted at 13.5 ± 1 °C in a water bath equipped with an immersion cooler (Grant CS 200G, Cambridge, UK). The vials were capped with polytetrafluoroethylene (PTFE) lined screw caps and rolled horizontally at 80 rpm in the water bath with a roller designed in-house to optimize contact between the incubation medium and the EVA thin film. The reactions were stopped at various times (10 min, 20 min, 30 min, 40 min, 60 min, 80 min, 100 min, and 140 min in single-chemical dosing experiments for pyrene and 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, and 180 min in all other experiments) by quickly transferring 0.40 mL of incubation medium to 1.0 mL ice-cold hexane and removing the remaining medium from the EVA–coated vials. The EVA thin films were then rinsed twice with 0.20 mL of deionized water, and 1.0 mL hexane was added to the vials to extract chemicals from the films. Chemical concentrations were measured in both the EVA and medium phases.

A no-cofactor control system using inactive liver S9 (incubated at 13.5 °C overnight and no NADPH–generating system included in the incubation mixture) was run in parallel with each test system using the incubation conditions described above. Two vials without incubation medium were included in the incubation bath and their films extracted to determine the initial concentration of the test chemicals in the EVA thin films (C_e at t = 0). Test chemicals were dosed individually or in a mixture under the same experimental conditions to examine mixture effects. Triplicate incubations using different pools of liver S9 (3 fish per pool, 9 fish used in total) were conducted to determine the mean in vitro biotransformation rates of the test chemicals.

3.4.6. Incubation Conditions of the Solvent-Delivery Dosing System

The same trout liver S9 preparations and incubation conditions were used in both sorbent-phase dosing and solvent-delivery dosing experiments. Briefly, pyrene, chrysene, and benzo[a]pyrene were dissolved individually or in a mixture in acetonitrile. To initiate the reactions, 2.4 µL of the spiked solvent was added to 0.50 mL incubation mixture in 2-mL vials preincubated at 13.5 °C for 5 min. Final concentrations in the incubation medium were 0.50 µM for pyrene and chrysene and 1.0 µM for benzo[a]pyrene. The final acetonitrile concentration was < 0.5% (v/v). Incubations were conducted at 13.5 ± 1 °C, and reactions were terminated at various times (0 min, 10 min, 20 min, 30 min, 40 min, 60 min, 80 min, 100 min, and 140 min in single-chemical dosing experiments for pyrene and 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, and 180 min in other experiments) by adding 1.0 mL ice-cold hexane to the incubation medium. A no-cofactor control system in which the trout liver S9 had been incubated at 13.5 °C overnight for enzyme inactivation and the NADPH–generating system was omitted from the incubation mixture was run in parallel with a test system using the incubation conditions described above. Test chemicals were dosed individually or in a mixture under the same experimental conditions to examine mixture effects. Triplicate experiments using the same S9 preparations used in the sorbent-phase dosing (3 fish per pool, 9 fish used in total) were conducted to determine the mean in vitro biotransformation rates of the test chemicals.

3.4.7. Chemical Extraction

Chemical extraction procedures were according to Lee et al. (2012). Briefly, prior to chemical extraction from the films, an internal standard (0.21 nmol chrysene-d^{12}) was added to the hexane extraction solvent. Test chemicals remaining in the film after the termination of incubation were extracted into the hexane by shaking the vials on a vortex mixer for 1 min. Extracts were transferred to 2-mL autosampler vials for gas chromatography/mass spectrometry (GC/MS) analysis. Internal standard (0.21 nmol chrysene-d^{12}) was added to the vials containing S9 plus hexane, and the vials were shaken on a vortex mixer for 2 min. The vials were then centrifuged at 800 $\times$ *g* for 10 min (IEC Centra-CL2; Thermo Scientific). The upper organic layer was transferred to a 2-mL autosampler vial for GC/MS analysis.

3.4.8. GC/MS Analysis

Test chemicals were analyzed using an Agilent 6890 GC coupled to an Agilent 5973 MS and an Agilent 7683 autosampler (Agilent, Mississauga, ON). The GC was fitted with a cool-on-column capillary inlet, and the injection volume was 1 µL. Chemicals were separated on an HP-5MS 5% phenyl methylpolysiloxane–coated column (30 m x 0.25 mm inner diameter, 0.25 µm film thickness) connected to a fused-silica deactivated guard column (5 m x 0.53 mm inner diameter). The oven was held at an initial temperature of 60 °C for 0.5 min, then increased at 25 ºC min^{-1} to 200 °C (held for 0.5 min), followed by an increase at 20 °C min^{-1} to a final temperature of 300 °C (held for 4 min). Helium was used as the carrier gas at a constant flow rate of 1.0 mL·min^{-1}. Conditions for MS measurements were as follows: electron impact ionization at 70 eV; ion source temperature at 230 °C; and selected ions at mass-to-charge ratios of 202 (pyrene), 228 (chrysene), 240 (chrysene-d^{12}), and 252 (benzo[a]pyrene). Agilent MSD ChemStation software (G1701CA) was used for instrument control and data processing. The dynamic range and relative response factor (obtained by dividing the ratio of peak area by the concentration of the test chemical to that of the internal standard) for each test chemical were determined using an 8-point calibration curve (concentration range, 1–500 ng·mL^{-1}). Strong linearity (r^2 > 0.99) was shown in the calibration curves, and constant relative response factor values were obtained over the concentration range.

3.4.9. Data Analysis

In the sorbent-phase dosing experiments, the mass-transfer rate constants (k_1, k_2) and in vitro biotransformation rate constants (k_r) and their 95% confidence intervals were estimated as described by Lee et al. (2012) by fitting the measured chemical concentrations in either the sorbent phase or the incubation medium using a nonlinear regression and a Runge–Kutta numerical differential equation solver using MATLAB R2009a (Mathworks, Natick, MA).

To derive the biotransformation rate constants in solvent-delivery dosing experiments, the declining concentrations in the incubation medium over time were fitted by a first-order kinetic model

$$\frac{dC_m}{dt} = -k_r C_m \tag{3.3}$$

where C_m is the control-corrected chemical concentration in the incubation medium (μM) and k_r is the apparent first-order biotransformation rate constant (min^{-1}). The k_r value was estimated using a linear regression from the slope of ln ($C_m/C_{m,t=0}$) versus time (i.e., ln $[C_m/C_{m,t=0}] = -k_r \cdot t$), where $C_{m,t=0}$ is the initial dosing concentration of the chemical in the incubation medium (μM). Only data showing apparent first-order depletion were used for data analysis.

3.4.10. Determination of Unbound Fraction

The unbound fractions of the test chemicals in the incubation medium were determined as C_e/(EVA–water partition coefficient $[K_{ew}] \cdot C_m$) as described by Lee et al. (2012). Briefly, $C_e/(K_{ew} \cdot C_m)$ was obtained from the control data in the sorbent-phase dosing experiments, where C_e/C_m is the ratio of chemical concentration in the sorbent phase to chemical concentration in the incubation medium at steady state in the control, calculated as $(k_2 \cdot V_m)/(k_1 \cdot V_e)$ obtained from Equation 3.1 when $dC_e/dt = 0$ or from Equation 3.2 when $dC_m/dt = 0$ and $k_r = 0$; K_{ew} is $10^{5.84}$, $10^{6.40}$, and $10^{6.52}$ for pyrene, chrysene and benzo[a]pyrene, respectively (Golding et al., 2008).

3.5. Results and Discussion

3.5.1. Thin-Film Sorbent-Phase Dosing of Trout Liver S9

Extraction efficiencies of all test chemicals from the sorbent phase were high at 102 ± 1%, and sorbent concentrations were therefore not corrected for extraction efficiency. Extraction efficiencies from the trout liver incubation mixture were 79 ± 2%, 70 ± 4%, and 61 ± 4% (mean ± standard deviation [SD]) for pyrene, chrysene, and benzo[a]pyrene, respectively. Concentrations of the test chemicals in the incubation medium were corrected for extraction efficiencies to ensure that mass balance was obtained. The lower extraction efficiencies from the incubation medium compared with the sorbent phase illustrate the greater ease and lower error of sorbent-phase extractions.

When test chemicals were dosed individually via the thin-film sorbent phase, concentrations of pyrene, chrysene, and benzo[a]pyrene in the sorbent phase declined over time as the test chemical was transferred from the sorbent phase to the incubation medium (Figure 3.1). The magnitude of concentration decline over time was greatest for pyrene, whereas chrysene and benzo[a]pyrene exhibited similar concentration declines over time (Figure 3.1). For all test chemicals, the rates of concentration decline over time in the test (thin films exposed to active trout liver S9) and control (thin films exposed to inactive trout liver S9) were similar and did not show statistically significant ($p < 0.05$) differences (Figure 3.1). Corresponding concentrations in incubation media containing inactive S9 increased over time and reached a plateau (Figure 3.1). Concentrations in the active liver incubations increased over time, reached a maximum, and then decreased over time for all test chemicals (Figure 3.1). Concentration profiles in the incubation mixture showed highly statistically significant differences between test and control ($p < 0.05$). The findings show that the biotransformation rate constant, k_r, of the chemicals in the present study can be derived from the concentration profiles in the incubation phase but not from those in the sorbent phase. Figure 3.2 illustrates the reasons for these findings. It shows that the similarity between chemical concentration profiles in the sorbent phase of the test and control incubations is a result of the slow chemical delivery rate of the test chemicals from the sorbent phase to the incubation medium (k_1) relative to the biotransformation rate in the incubation medium (k_r). The measured in vitro

biotransformation rate constants for chrysene and benzo[a]pyrene obtained from concentrations measured in the incubation medium were 0.17 ± 0.03 min^{-1} and 0.037 ± 0.006 min^{-1} (mean ± SD), respectively. These rate constants are significantly higher (p < 0.05) than the corresponding k_1 values (Figure 3.2B and 3.2C). For pyrene, k_r was 0.012 ± 0.005 min^{-1} (mean ± SD) and significantly (p = 0.04) smaller than k_1, but k_1 did not exceed k_r by a large enough margin to measure k_r using the sorbent-phase concentration time course.

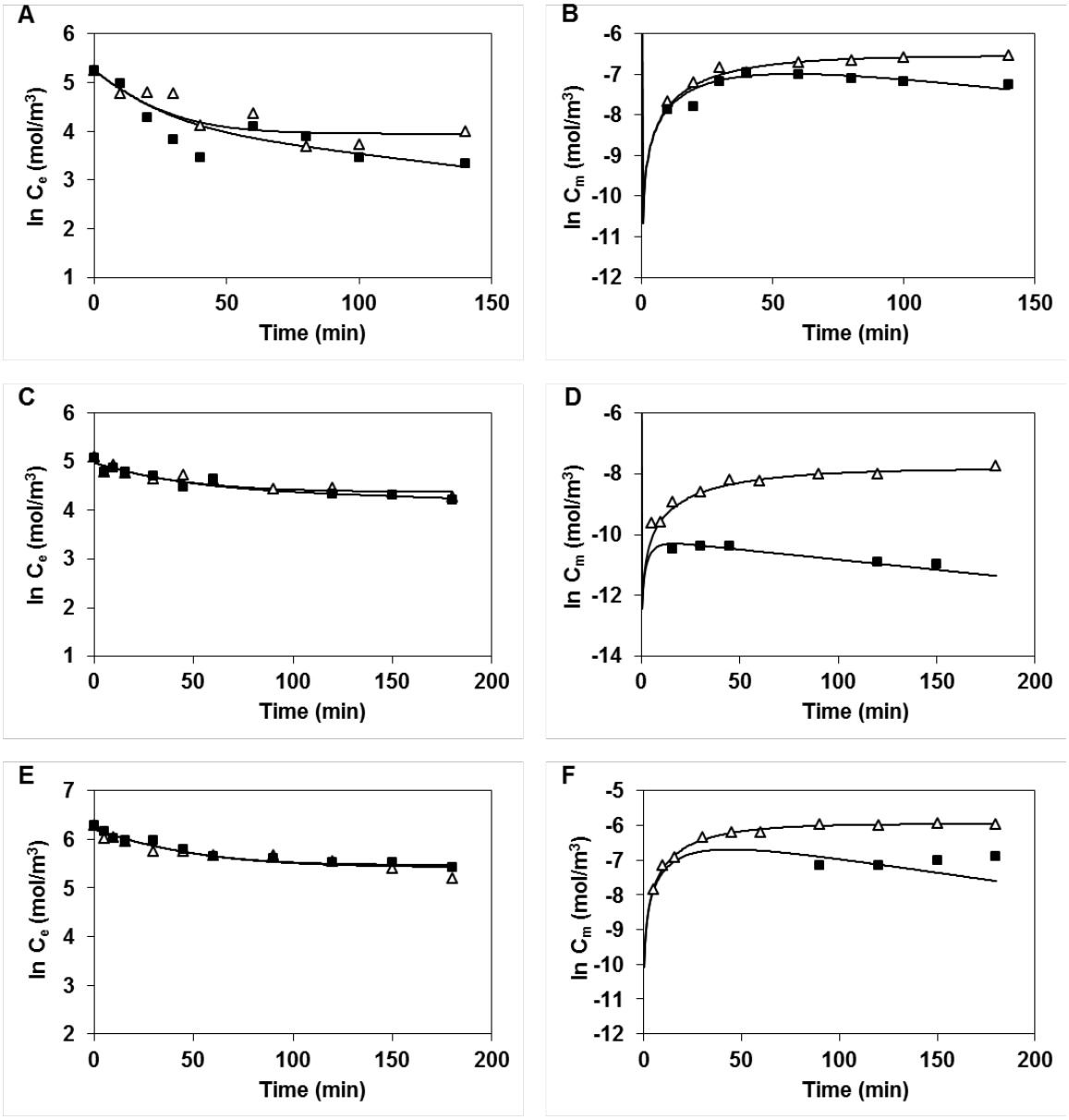

Figure 3.1. **Natural logarithm of concentration-time profiles in the EVA thin film sorbent phase (ln C_e; A, C, E) and in the incubation medium (ln C_m; B, D, F) containing active (solid squares) or inactive (open triangles) rainbow trout liver S9 (control) using the sorbent-phase single-chemical dosing approach for pyrene (A, B), chrysene (C, D), and benzo[a]pyrene (E, F). Solid lines represent nonlinear regressions. Data from one of three experiments are shown.**

In similar experiments of the same test chemicals in rat liver S9 (Lee et al., 2012), delivery rates of chrysene (0.28 min^{-1}) and benzo[a]pyrene (0.13 min^{-1}) from the sorbent phase to the liver incubation medium were much greater than those measured in the present experiment using fish liver S9 (i.e., 0.012 ± 0.005 min^{-1} and 0.012 ± 0.002 min^{-1}, mean ± SD), despite the thinner EVA films (4 nm) used in the fish study compared with the rat study (20 nm). In trout liver incubation experiments at 13.5 °C using thin films of 20-nm thickness, equal to that used in the rat liver incubation studies at 37 °C, k_1 values were 0.0038 ± 0.006 min^{-1} and 0.0013 ± 0.002 min^{-1} for chrysene and benzo[a]pyrene, respectively, 73 and 96 times, respectively, lower than those measured in the rat study (Figure B1 in Appendix B). While the rat and trout liver S9 incubation mixture did vary in composition (12 mg S9 protein·mL^{-1} and 3 mg S9 protein·mL^{-1} in rat and fish, respectively), we expect that the lower incubation temperature in the trout study (13.5 °C) compared with the rat study (37 °C) is the main factor causing the lower sorbent delivery rates in the trout liver S9 incubations. Diffusion coefficients are recognized to decline with decreasing temperature (Niesner & Heintz, 2000). The lower temperature required in bioassays with rainbow trout liver (13.5 °C in the present study) compared with 37 °C in mammalian liver bioassays can therefore limit the ability of measuring biotransformation rates from concentration measurements in the thin films. Sorbent-delivery rates can be increased by employing thinner films. However, there are practical limits to the use of very thin films because of test chemical evaporation from the sorbent phase during film preparation and handling processes, which contributes error. The decline in diffusion rates with decreasing temperature does not affect the ability to measure biotransformation rates from concentration measurements in the incubation medium.

Figure 3.2 shows that for all test chemicals there were no statistically significant differences ($p > 0.05$) between sorbent-to-liver medium mass-transfer rate constants (k_1) obtained from concentrations in the sorbent phase and those obtained from concentrations in the incubation medium. Similarly, medium-to-sorbent mass-transfer rate constants (k_2) determined from concentrations measured in the sorbent phase were not significantly different from those determined using the concentrations in the incubation medium ($p > 0.05$). This illustrates that mass-transfer rate constants can be determined using concentrations in either the sorbent or the medium phase. However, there were differences in the magnitude of error in the determination of the mass-transfer rate

constants. For pyrene, the error in the k_1 and k_2 measurements obtained from concentrations in the sorbent phase was greater than that obtained from concentrations in the medium, but this was not observed for chrysene and benzo[a]pyrene. This may be a result of the greater volatility of pyrene, which introduces variability among replicates because of loss of chemical from the sorbent phase during preparation and handling of the thin films. Pyrene has a lower octanol–air partition coefficient (K_{OA}; log K_{OA} = 8.60) compared with chrysene (log K_{OA} = 10.40) and benzo[a]pyrene (log K_{OA} = 10.80) (Mackay et al., 2006). For benzo[a]pyrene, the error in the k_1 and k_2 measurements obtained from concentrations in the incubation medium was greater than that obtained from concentrations in the sorbent phase. The lower extraction efficiency and fewer detectable measured concentrations for benzo[a]pyrene in the incubation medium (Figure 3.1) compared with those from the sorbent phase are likely the main causes of the difference in error. Therefore, to obtain accurate sorbent-to-medium and medium-to-sorbent mass-transfer rate constants, concentration measurements in the liver medium may be preferred over measurements in the sorbent phase for relatively low-K_{OA} chemicals, whereas concentrations measured in the sorbent phase may be more suitable for high-K_{OW} and high-K_{OA} chemicals.

Concentrations of pyrene and benzo[a]pyrene measured in the incubation mixture were well below (by 1–3 orders of magnitude) reported Michaelis–Menten constants of 15 µM for pyrene measured using isolated hepatocytes from rainbow trout (Law et al., 1991) and 33 µM to 125 µM for benzo[a]pyrene measured using liver microsomes from rainbow trout (Carpenter et al., 1990). This suggests that enzyme saturation likely did not occur and that first-order enzyme kinetics were maintained. Substrate concentrations below the Michaelis–Menten constant are necessary in substrate depletion experiments to avoid enzyme saturation and subsequent underestimation of the depletion rate.

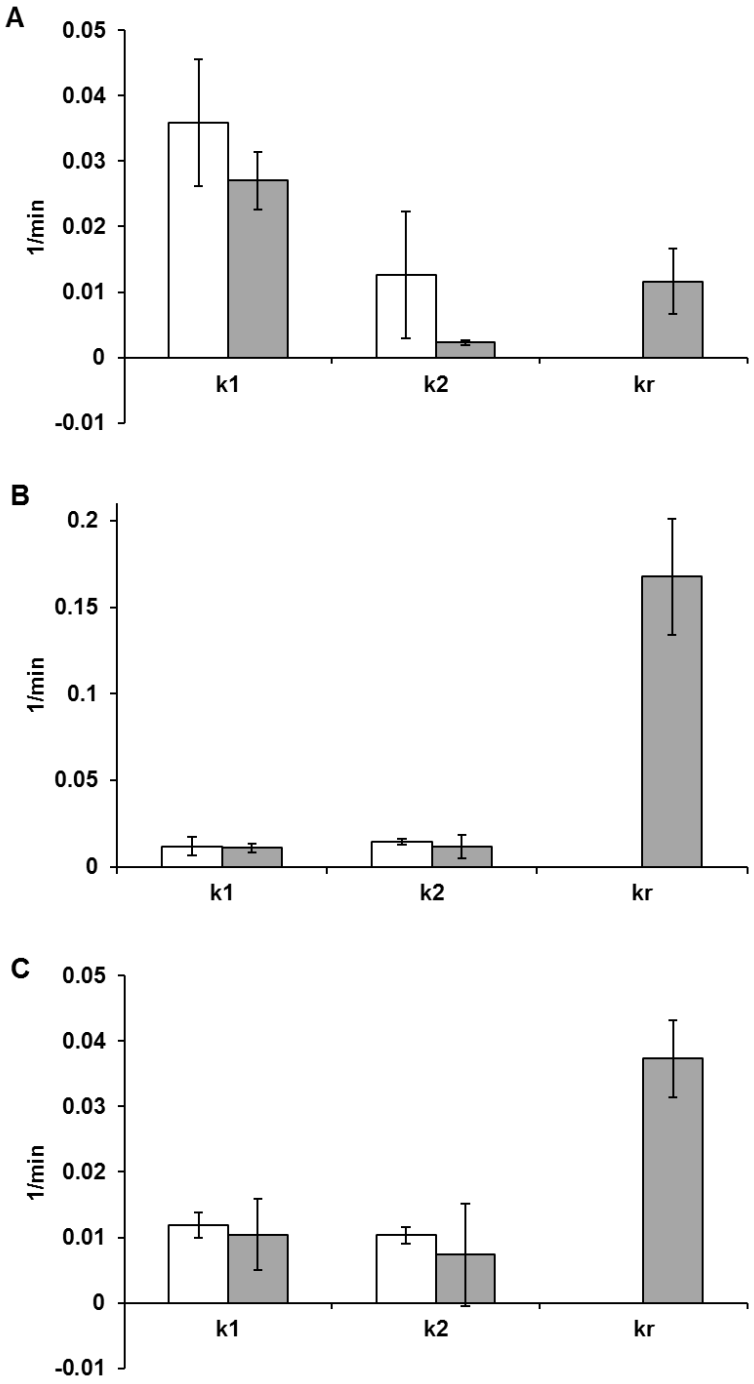

Figure 3.2. **Measured rate constants for mass-transfer (k_1 and k_2) and in vitro biotransformation rate (k_r) in sorbent-phase single-chemical dosing experiments (n = 3) for pyrene (A), chrysene (B), and benzo[a]pyrene (C). Results obtained from three independent experiments using concentrations in the sorbent phase (empty bars) or the liver incubation mixture (filled bars). Error bars represent the standard deviation.**

The extrapolation of in vitro biotransformation rates to in vivo rates requires information about the fraction of unbound substrate in incubation mixtures (Obach, 1997; Obach, 1999; Poulin et al., 2012). In a sorbent-dosing approach, the unbound substrate can be measured from the concentration profiles in the control (inactive S9) incubations (Lee et al., 2012). In the present study, the unbound fractions of pyrene, chrysene, and benzo[a]pyrene in the incubation medium were 0.033 ± 0.001, 0.087 ± 0.053, and 0.04 ± 0.01 (mean ± SD), respectively. The mean measured unbound fractions of pyrene, chrysene, and benzo[a]pyrene were approximately 3-fold, 14-fold, and 13-fold higher than those calculated using a K_{OW}-dependent empirical relationship used by others (Escher et al., 2011; Nichols et al., 2013) for fish liver S9. One of the factors contributing to the observed differences between measured and calculated unbound fractions may be that the empirical relationship was obtained using heat-denatured liver S9 or microsomes. The S9 preparations used in the present experiments were not heat-treated. Using the measured unbound fractions and the measured S9 protein content of 2.83 ± 0.42 mg S9 protein·mL^{-1} (mean ± SD), unbound intrinsic clearance rates of 0.13 ± 0.06 mL·min^{-1}·mg S9 protein^{-1}, 0.69 ± 0.45 mL·min^{-1}·mg S9 protein^{-1}, and 0.33 ± 0.11 mL·min^{-1}·mg S9 protein^{-1} (mean ± SD) for, respectively, pyrene, chrysene, and benzo[a]pyrene can be derived. The unbound intrinsic clearance rates for chrysene and benzo[a]pyrene in rainbow trout liver are much lower than those measured using the same technique in rat liver (Lee et al., 2012): 10.9 ± 1.5 mL·min^{-1}·mg S9 protein^{-1} (mean ± SD) for chrysene and 15.3 ± 4.1 mL·min^{-1}·mg S9 protein^{-1} (mean ± SD) for benzo[a]pyrene. This supports the general assumption that biotransformation rates in fish are lower than those in mammals.

3.5.2. Solvent-Delivery Dosing Using Trout Liver S9

Figure 3.3 illustrates that when dosed individually using the solvent delivery method, the concentrations of pyrene, chrysene, and benzo[a]pyrene in the active liver S9 showed a statistically significant log–linear decline with incubation time ($p < 0.05$ for the slopes), indicating apparent first-order kinetics of substrate depletion within the first hour of incubation (later time points flattening the depletion curves were omitted from data analysis). The estimated in vitro biotransformation rate constants (k_r) for pyrene, chrysene, and benzo[a]pyrene were 0.021 ± 0.005 min^{-1}, 0.008 ± 0.002 min^{-1}, and 0.019 ± 0.006

min^{-1} (mean ± SD), respectively. The k_r values obtained for pyrene and benzo[a]pyrene were similar ($p > 0.05$), and both were significantly higher than the measured k_r value for chrysene ($p < 0.05$). Benzo[a]pyrene was also biotransformed faster than chrysene by liver microsomes from brown bullheads (Pangrekar et al., 1995). The k_r value measured in the present study for pyrene was in good agreement with the value of 0.015 ± 0.002 min^{-1} (mean ± SD) found earlier in solvent-delivery experiments in this laboratory (Johanning et al., 2012). For benzo[a]pyrene, the measured in vitro biotransformation rate normalized to protein concentration in the incubation medium was 0.37 ± 0.12 $mL \cdot h^{-1} \cdot mg$ S9 $protein^{-1}$, which is approximately 5 times higher than that reported by Han et al. (2009) in rainbow trout liver S9. The apparent difference in biotransformation rate may be a result of the higher substrate concentration of 2 µM and the lower protein concentration of 2 mg $protein \cdot mL^{-1}$ in Han et al. (2009) compared with those of the present study, 1.0 µM and 2.8 mg $protein \cdot mL^{-1}$. Both factors can contribute to lower biotransformation rates according to Michaelis–Menten theory.

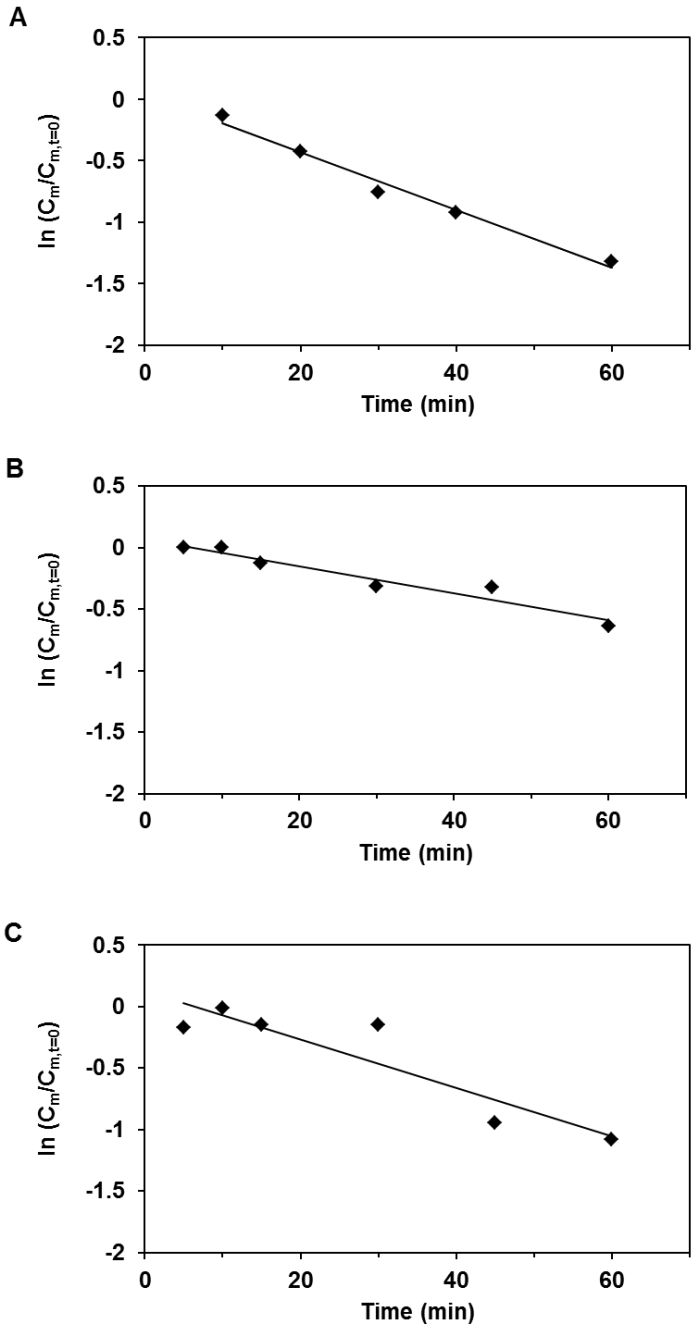

Figure 3.3. **Natural logarithm of concentration-time profiles in the trout liver S9 in the solvent-delivery single-chemical dosing experiments for pyrene (A), chrysene (B) and benzo[a]pyrene (C). Concentrations expressed as the ratio of chemical concentration in the incubation medium in the test (C_m, control adjusted) to initial chemical concentrations in the incubation medium ($C_{m,t=0}$). Data from one of three experiments are shown.**

The in vitro biotransformation rate of pyrene obtained from the solvent-delivery dosing experiments was not significantly different ($p > 0.05$) from that obtained in the sorbent-phase dosing experiments using the same trout liver S9 preparation (Figure 3.4A and Figure 3.4B). For chrysene and benzo[a]pyrene, however, the in vitro biotransformation rates measured using the thin-film sorbent-phase dosing system were significantly higher (20 times and 2 times, respectively) than those measured using the solvent-delivery dosing system ($p < 0.05$). This agrees with experiments using rat liver S9 in which k_r values were also greater than those obtained from solvent-delivery dosing experiments for both chemicals (Lee et al., 2012). The higher biotransformation rates obtained in thin-film sorbent-phase dosing experiments may result from lower initial incubation substrate concentrations in the sorbent delivery–based experiments, a lack of solvent inhibition of enzyme activities, better chemical dissolution in the incubation medium, and reduced biotransformation product inhibition by product diffusion into the sorbent phase. The greatest difference between biotransformation rates measured in sorbent and solvent delivery–based systems was for chrysene. Chrysene has the lowest aqueous solubility of the chemicals tested—2 µg/L at 25 °C compared with 4 µg/L and 130 µg/L for benzo[a]pyrene and pyrene, respectively (Mackay et al., 2006). It has been suggested that when superhydrophobic chemicals are spiked using a carrier solvent and delivered into a largely aqueous liver preparation, their concentrations may locally exceed their aqueous solubility, causing the formation of microcrystals (Kwon et al., 2009) that may limit substrate access to enzymes during the relatively short incubation phase, causing underestimations of in vitro biotransformation rates in the solvent-delivery dosing experiments. For chemicals with greater water solubility, such as pyrene, incomplete dissolution in the incubation medium may not be an important issue, and in vitro biotransformation rates in sorbent-phase dosing and solvent-delivery dosing systems are similar. The results suggest that the solvent-delivery dosing approach is appropriate for measuring in vitro biotransformation rates of less hydrophobic chemicals (e.g., log $K_{OW} < 5$) with relatively high water solubility. The sorbent-phase dosing approach may be more useful for measuring in vitro biotransformation rates for very hydrophobic chemicals (e.g., log $K_{OW} > 5$) with very low water solubility.

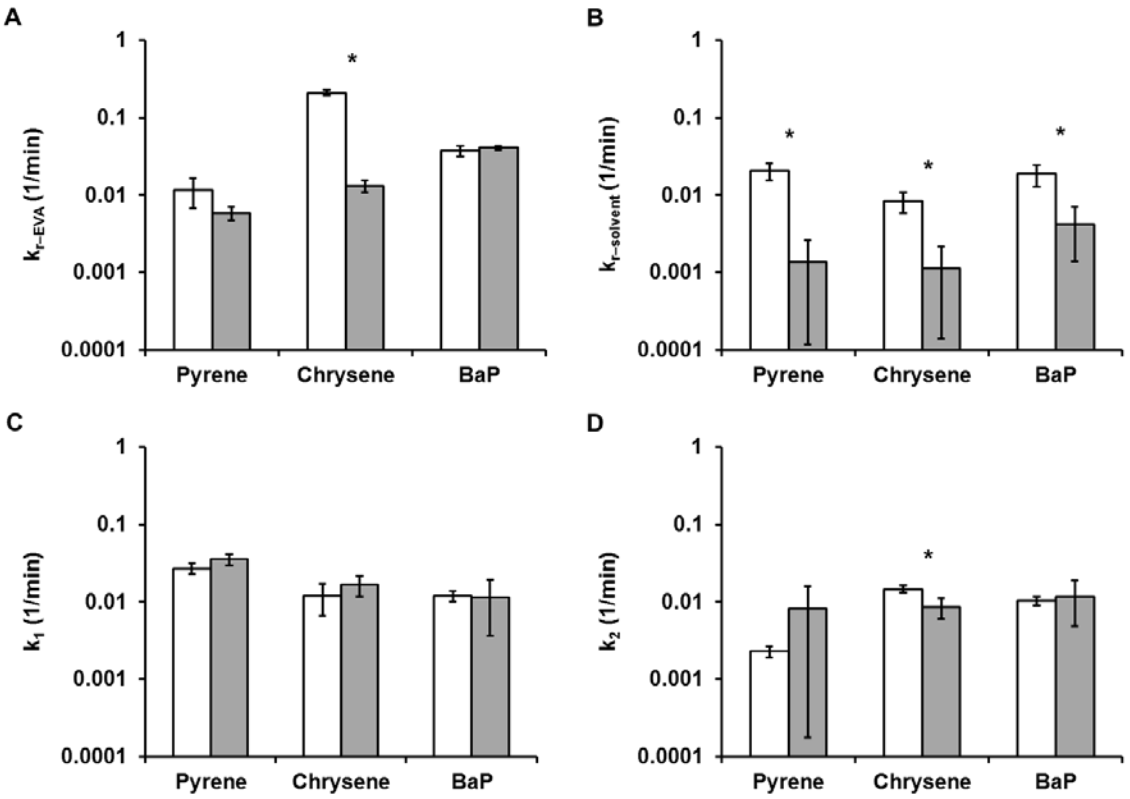

Figure 3.4. Measured in vitro biotransformation rates determined in the sorbent-phase dosing experiments (k_{r-EVA}; A) or in the solvent-delivery dosing experiments ($k_{r-solvent}$; B), sorbent-to-medium mass transfer rate constants (k_1; C), and medium-to-sorbent mass transfer rate constants (k_2; D) obtained from single-chemical dosing experiments (empty bars) or multi-chemical dosing experiments (filled bars). Mass transfer rate constants for pyrene were obtained from concentrations in the liver incubation medium; mass transfer rate constants for chrysene and benzo[a]pyrene (BaP) were obtained from concentrations in the sorbent phase. In vitro biotransformation rates were obtained from concentrations in the liver medium for all test chemicals. Error bars represent the standard deviation (n = 3). Asterisks indicate a significant difference ($p < 0.05$). *Significant difference ($p < 0.05$). EVA = ethylene vinyl acetate.

3.5.3. Dosing with Chemical Mixtures

Thin-Film Sorbent-Phase Dosing

Figure B2 in Appendix B illustrates the time course of chemical concentrations in the EVA film and in the incubation medium in the multi-chemical dosing experiment. Sorbent-to-medium mass-transfer rate constants (k_1) obtained from multi-chemical dosing

experiments were not significantly different from those obtained from single-chemical sorbent-phase dosing experiments for all 3 test chemicals ($p > 0.05$; Figure 3.4C). Also, the medium-to-sorbent mass-transfer rate constants (k_2) were similar in single-chemical and mixture incubations (Figure 3.4D). For chrysene, the difference in k_2 values between single-chemical and chemical mixture incubations was very small but statistically significant ($p = 0.022$) because of very low variability among replicates. The observation that film-incubation medium mass-transfer rate constants were not affected by the presence of other chemicals spiked simultaneously in the sorbent phase is consistent with diffusive mass-transfer of chemicals being controlled by the molecular diffusion coefficients and thickness of the diffusion layers (Kwon et al., 2007), neither of which are affected by multi-chemical dosing conditions. There was also no significant mixture effect on in vitro biotransformation rates (k_r) measured for pyrene and benzo[a]pyrene in the sorbent-phase dosing experiments (Figure 3.4A). This suggests that codelivery of approximately equimolar concentrations of the 3 test chemicals to the incubation medium does not affect the dissolution in the incubation medium or enzyme–substrate interactions of pyrene and benzo[a]pyrene. However, the k_r measured for chrysene in the single-chemical sorbent-phase dosing experiments was significantly higher ($p < 0.05$) than that in the multi-chemical dosing experiments. This may be a result of competitive inhibition of chrysene oxidation by benzo[a]pyrene as both chemicals are catalyzed by CYP1A (Stegeman et al., 1998). Constitutive levels of CYP1A in fish are low (Pangrekar et al., 1995; Schlenk et al., 2008; Uno et al., 2012), and benzo[a]pyrene concentrations in the incubation medium are greater than those of chrysene (Figure B2 in Appendix B), making competitive inhibition more likely.

Solvent-Delivery Dosing

When test chemicals were dosed together as a mixture, no statistically significant declines ($p > 0.05$) in the pyrene and chrysene concentrations with incubation time were observed (Figure B3 in Appendix B). For benzo[a]pyrene there was a statistically significant decline ($p < 0.05$) in concentrations in the incubation medium over time (Figure B3 in Appendix B), but the in vitro biotransformation rate in the mixture incubation was much lower than that in single-chemical incubations. In general, in vitro biotransformation rate constants (k_r) obtained from single-chemical dosing experiments were significantly greater than those obtained from multi-chemical dosing experiments (Figure 3.4B).

Similar results were reported for chrysene and benzo[a]pyrene biotransformation in a solvent delivery dosing experiment with rainbow trout hepatocytes (Trowell, 2010). The results suggest that the biotransformation of the test chemicals is inhibited by the presence of other substrates in the incubation mixture. In solvent-delivery experiments, therefore, chemicals should be dosed individually to measure the full metabolic capacity of the liver preparation. However, the observation that the mixture effect was much smaller in the sorbent-phase dosing experiments than that in the solvent delivery–based dosing experiments (and apparently absent for pyrene and benzo[a]pyrene) suggests that the dosing method also has an effect on the apparent biotransformation rates of chemicals when dosed together. The lower initial substrate concentrations in the incubation medium in the thin-film dosing experiments compared with those in the solvent-delivery dosing experiments may reduce competitive inhibition and be partly responsible for this observation. A sorbent-delivery system may therefore have greater potential for measuring biotransformation rates of multiple chemicals, although further work on this issue appears necessary.

The results of the present study suggest that thin-film sorbent-phase dosing is a particularly useful method for measuring in vitro biotransformation rates of substances that are highly hydrophobic (e.g., log K_{OW} > 5), and hence very difficult to fully dissolve in aqueous media, and that have a high log K_{OA} (e.g., log K_{OA} > 5), which reduces measurement error among replicate thin-film preparations concentration because of reduction of evaporative losses of the test chemical from the films. An important advantage of thin-film sorbent-phase dosing over solvent delivery–based dosing is the direct measurement of the fraction of unbound test chemical in the incubation. The unbound fraction of very hydrophobic chemicals can be very low and play an important role in the in vitro-to-in vivo extrapolation of biotransformation rates and may be difficult to estimate by other means. A disadvantage of the thin-film sorbent-phase dosing method is that the release rate of the chemical from the film to the incubation medium falls with decreasing temperature and with increasing K_{OW}. Although slow thin-film release rates do not pose a fundamental problem to the application of the thin-film sorbent technique to measure biotransformation rates, they do require the adoption of more sensitive techniques for chemical detection and lower detection limits. The sorbent-phase dosing technique has shown potential for simultaneous measurement of biotransformation rates

of multiple chemicals, whereas the solvent delivery−based experiments have indicated that biotransformation rates are best measured when dosing chemicals individually. Further investigations are needed to refine the sorbent-phase dosing system, to explore its application to compounds other than polycyclic aromatic hydrocarbons, and to investigate its use for simultaneous measurement of biotransformation rates of multiple chemicals, with the ultimate goal of improving bioaccumulation assessments.

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Chapter 4.

In Vitro-to-In Vivo Extrapolation of Biotransformation Rates for Assessing Bioaccumulation of Hydrophobic Organic Chemicals in Mammals

4.1. Summary

Incorporating biotransformation in bioaccumulation assessments of hydrophobic chemicals in both aquatic and terrestrial organisms in a simple, rapid and cost-effective manner is urgently needed to improve current bioaccumulation assessments of potentially bioaccumulative substances. One method to estimate whole animal biotransformation rate constants is to combine in vitro measurements of hepatic biotransformation kinetics with in vitro-to-in vivo extrapolation (IVIVE) and bioaccumulation modeling. An established IVIVE modeling approach exists for pharmaceuticals and this has recently been applied to aquatic bioaccumulation assessments in fish (referred to here as IVIVE-Ph). The present study proposes and tests an alternative IVIVE approach for hydrophobic chemicals (log K_{OW} > 4) in rats to support terrestrial bioaccumulation assessment. The IVIVE-B approach requires fewer physiological and physiochemical parameters than the IVIVE-Ph approach and does not involve interconversions between clearance and rate constants in the extrapolation. Using in vitro depletion rates, the results show that the IVIVE-B and IVIVE-Ph models yield similar estimates (within 1.2-fold) of rat whole organism biotransformation rate constants for hypothetical chemicals with log K_{OW} ≥ 4. The IVIVE-B approach generated in vivo biotransformation rate constants and biomagnification factors (BMFs) for benzo[a]pyrene that are within the range of empirical observations. The proposed IVIVE-B approach may be a useful tool for assessing biomagnification factors of hydrophobic organic chemicals in mammals.

4.2. Introduction

International and national regulations controlling hazardous chemicals, including the UNEP Stockholm Convention on Persistent Organic Pollutants, the E.U. Registration,

Evaluation, Authorisation and Restriction of Chemicals (REACH), the Canadian Environmental Protection Act (CEPA) and the U.S. Toxic Substances Control Act (TSCA), specify criteria for categorizing the bioaccumulative behaviour of commercial chemicals based on their bioconcentration factor (BCF), bioaccumulation factor (BAF), and logarithm of octanol-water partition coefficient (log K_{OW}) (Arnot & Gobas, 2006). However, the effectiveness of current regulations for identifying potentially bioaccumulative substances has recently been challenged. First, it was demonstrated that methods and criteria for assessing bioaccumulation in aquatic (i.e., water-breathing) organisms are not always applicable to air-breathing organisms including humans (Kelly et al., 2007; Czub & McLachlan, 2004). Kitano (2007) showed that five of 21 persistent organic pollutants recognized in the Stockholm Convention are bioaccumulative in non-aqueous organisms despite having BCF values in fish below the criterion value. The discrepancy arises because BCF as a measure of bioaccumulation in water-breathing organisms such as fish has limited relevance to air-breathing organisms (Kelly et al., 2007). Therefore, there is a need to develop methods to assess the bioaccumulative potential of chemicals in air-breathing (i.e., terrestrial) organisms, particularly mammalian wildlife and humans. Second, empirical BCF and BAF values do not exist for the great majority of commercial chemicals (Arnot & Gobas, 2006); resulting in a reliance on the application of BCF and BAF bioaccumulation models or the K_{OW} criterion (log K_{OW} > 5) for bioaccumulation assessment. However, K_{OW} is an inherent property of the chemical and provides no information on the potential for metabolism of that chemical, i.e., its biotransformation. This limitation is of particular importance for very hydrophobic chemicals (log K_{OW} > 5, log K_{OA} > 6) as they are often very slowly eliminated from the organism and even low rates of biotransformation can dominate the overall depuration rate of the chemical. Not accounting for biotransformation can overestimate bioaccumulation potential in chemical evaluations performed in silico. Quantitative structure–activity relationships have been developed to predict whole body biotransformation rates of organic chemicals in fish (Arnot et al., 2009; Papa et al., 2014), but this approach has not yet been developed for mammalian species. Third, it has been proposed that the biomagnification factor (BMF) may be a useful metric to identify chemicals of concern for bioaccumulation and that BMFs can be extended beyond fish to other consumer organisms (Gobas et al., 2009).

Terrestrial bioaccumulation models have been developed for assessing bioaccumulation of substances in terrestrial food-webs (Gobas et al., 2003; Kelly & Gobas, 2003; Armitage & Gobas, 2007). However, these models do not provide an algorithm to estimate chemical biotransformation rates a priori. At present, there are no standardized experimental methods to measure biotransformation rates of commercial chemicals in wildlife or humans. However, in vitro measurements of biotransformation rates in liver preparations provide an alternative to in vivo testing that reduce costs, increase throughput, and minimize animal use (Weisbrod et al., 2009). The application of in vitro assays followed by in vitro-to-in vivo extrapolation (IVIVE) has been developed and used widely in the pharmaceutical field to predict hepatic and total body clearance of drugs for clinical applications (Rane et al., 1977; Houston, 1994; Obach, 1999; Jones & Houston, 2004). As first described (Nichols et al., 2006) and refined (Nichols et al., 2013a) by Nichols et al., this approach, in combination with a fish BCF bioaccumulation model, can be applied to bioaccumulation assessments of chemicals in fish. In our study, we refer to this approach as IVIVE-Ph. In vitro depletion rates of test chemicals are measured using isolated perfused fish livers (Nichols et al., 2009; Nichols et al., 2013b) or fish liver preparations including S9 (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Han et al., 2009; Escher et al., 2011; Laue et al., 2014), microsomes (Dyer et al., 2008; Han et al., 2009), freshly isolated hepatocytes (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Han et al., 2007), and cryopreserved hepatocytes (Fay et al., 2014). The measured in vitro biotransformation rate constant for a test chemical is converted into in vitro intrinsic clearance and then extrapolated to hepatic intrinsic clearance. Subsequently, the well-stirred liver model is used to calculate the chemical's hepatic clearance by accounting for three biological factors: hepatic blood flow, enzyme activity and chemical binding in the blood (Wilkinson & Shand, 1975). Assuming no extrahepatic or biliary elimination, the calculated hepatic clearance (representing total body clearance) is then divided by an estimation of the volume of distribution of the chemical (calculated as the ratio of a partitioning-based estimate of the BCF in the absence of biotransformation or organism growth and the blood–water partition coefficient) to obtain the whole organism biotransformation rate constant to be used as an input parameter in the fish BCF model. For most test chemicals, the model-calculated BCFs that incorporate biotransformation information are closer to BCFs measured in whole fish than those that do not consider

biotransformation (Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Laue et al., 2014; Han et al., 2007; Fay et al., 2014).

IVIVE-Ph uses the well-stirred liver model (Wilkinson & Shand, 1975), involves interconversion between clearance and rate constants in the extrapolation process, and requires an estimate of the volume of distribution of chemicals to obtain the whole organism biotransformation rate constants for bioaccumulation modeling. The clearance concept is useful for clinical applications to relate dose to therapeutic concentrations of a drug in plasma, and the well-stirred liver model is useful for predicting the effects of alterations in hepatic blood flow and enzyme activity on the drug concentration-time profile due to disease or drug interactions (Wilkinson & Shand, 1975), but such information is not required for bioaccumulation assessments. In addition, IVIVE-Ph requires information about hepatic blood flow, fraction of unbound chemical in blood, and blood–water partition coefficients. These data are difficult to obtain for many organisms and may not be needed to assess the bioaccumulative behaviour of chemicals with bioaccumulation potential.

Therefore, we propose an alternative IVIVE approach that is based on the extrapolation of in vitro biotransformation rate constants for hydrophobic organic chemicals (log K_{OW} > 5) for the purpose of mammalian bioaccumulation assessment (referred to here as the IVIVE-B approach). This approach does not use the well-stirred liver model for reasons that are described below and there is no interconversion between rate constant measures to clearance measures and back to rate constants during extrapolations. Hepatic blood flow, blood composition, fraction of unbound chemicals in blood, and blood–water partition coefficients are not required in the proposed IVIVE-B approach. This may allow for application of the model to many species, including aquatic organisms, for which such information is not readily available. Furthermore, reducing data requirements removes impediments for including non-aquatic bioaccumulation data in regulatory bioaccumulation assessments.

The objectives of this study were (i) to develop an IVIVE-B approach for assessing rat whole organism biotransformation rate constants for hydrophobic chemicals that have bioaccumulation potential; (ii) to evaluate the proposed IVIVE-B model by comparing the predictions to those obtained from the IVIVE approach for pharmaceuticals (IVIVE-Ph); (iii)

to develop a mechanistic bioaccumulation model for rats that accounts for biotransformation by using in vitro bioassay data to estimate biomagnification factors (BMFs); and (iv) to demonstrate the application of the proposed IVIVE-B modeling approach as a tool for bioaccumulation assessments of hydrophobic chemicals. To date, a bioaccumulation modeling approach that incorporates biotransformation rates extrapolated from measured in vitro biotransformation rates has not been developed for mammals. Rats were chosen to be the model species for mammals because they are widely used in laboratory experiments and their physiological parameters are well established; these were necessary for the IVIVE-Ph inputs. The IVIVE-B approach is developed for extrapolating in vitro biotransformation rate data derived using liver S9 subcellular fractions. The liver S9 fraction was chosen because its preparation is simple and relatively quick compared to other commonly used in vitro systems such as liver microsomes or hepatocytes. Also, the liver S9 fraction contains both microsomal and cytosolic enzymes, providing a more complete enzymatic profile than liver microsomes, and is therefore useful for preliminary bioaccumulation assessments for a large number of chemicals whose biotransformation mechanisms are unknown. Benzo[a]pyrene and chrysene were chosen as model chemicals because both are hydrophobic and have bioaccumulation potential (log K_{OW} > 5) and their in vitro biotransformation rate constants and unbound fractions in the rat liver S9 incubation mixture have been measured previously using a thin-film sorbent-phase dosing method (Lee et al., 2012). The ultimate goal of this study is to improve bioaccumulation assessments for hydrophobic chemicals in terrestrial animals using an alternative and simplified IVIVE-B modeling approach that incorporates empirical biotransformation data.

4.3. Theory

4.3.1. The IVIVE-B Approach for Bioaccumulative Substances in Mammals

The framework of the proposed IVIVE-B approach for potentially bioaccumulative substances in mammals is illustrated in Figure 4.1. The major steps of the IVIVE-B approach are:

Step 1: Experimental measurement of the apparent in vitro biotransformation rate constant k_r using the substrate depletion method. Rates of substrate depletion are measured instead of metabolite formation rates because the metabolites of most commercial chemicals are unknown. The in vitro biotransformation rate constant can be measured in S9 liver preparations using a thin-film sorbent-phase dosing approach that delivers the test chemical from a sorbent phase to the incubation medium (Lee et al., 2012; Lee et al., 2014; Lo et al., 2015a) or by introducing the test chemical to the incubation in a spiking solvent. In the latter method, in vitro biotransformation rate constants, k_r (h^{-1}), can exhibit strong dependence on the initial substrate concentrations (Lo et al., 2015a; Obach & Reed-Hagen, 2002), i.e.,

$$k_{r,C\to 0} = k_r / \left(1 - \frac{C_I}{C_I + K_M}\right) \tag{4.1}$$

where $k_{r,C\to 0}$ (h^{-1}) is the maximum in vitro biotransformation rate constant at infinitesimally low substrate concentration; C_I is the initial concentration of the test chemical (substrate) in the incubation medium (μM); and K_M is the apparent Michaelis–Menten constant (μM) (Obach & Reed-Hagen, 2002; Nath & Atkins, 2006). If C_I is well below a known K_M or if it is acceptable to assume that C_I is well below K_M, then $k_{r,C\to 0}$ can be approximated by k_r. If K_M is unknown, it can be measured by conducting solvent delivery–based depletion experiments using a range of initial substrate concentrations (Lo et al., 2015a; Obach & Reed-Hagen, 2002). If k_r is measured using the thin-film sorbent-phase dosing approach, it may be possible to approximate $k_{r,C\to 0}$ from a single substrate concentration experiment because the substrate concentration in the incubation medium is initially zero and may remain below K_M throughout the incubation period due to slow release of the hydrophobic chemical from the sorbent phase to the incubation medium (Lo et al., 2015a). However, K_M cannot be verified within the constraints of a single substrate concentration experiment.

The in vitro biotransformation rate constant $k_{r,C\to 0}$ can then be normalized to the fraction of substrate that is freely dissolved in the incubation medium ($f_{u,inc}$; unitless), as shown in Equation 4.2:

$$k_r^* = \frac{k_{r,C\to 0}}{f_{u,inc}} \tag{4.2}$$

where k_r^* (h^{-1}) is the in vitro biotransformation rate constant of the unbound chemical in the incubation medium. The value of $f_{u,inc}$ can be measured in the depletion experiment (Escher et al., 2011; Lee et al., 2012), estimated from empirical relationships (Nichols et al., 2013a; Han et al., 2009; Austin et al., 2002), or estimated by assuming that the incubation medium consists of three phases (i.e., lipids, proteins and water) and that the chemical partitions as follows:

$$f_{u,\mathrm{inc}} = \frac{f_{W,\mathrm{inc}}}{f_{L,\mathrm{inc}} \cdot K_{OW} + f_{P,\mathrm{inc}} \cdot K_{PW} + f_{W,\mathrm{inc}}} \tag{4.3}$$

where $f_{L,inc}$, $f_{P,inc}$, and $f_{W,inc}$ are the fractions of lipid, protein, and water of the incubation medium (v/v; unitless), respectively; K_{OW} represents the lipid–water partition coefficient; and K_{PW} is the protein–water partition coefficient, which for some non-ionic hydrophobic organic compound can be estimated as $0.05 \cdot K_{OW}$ (deBruyn & Gobas, 2007).

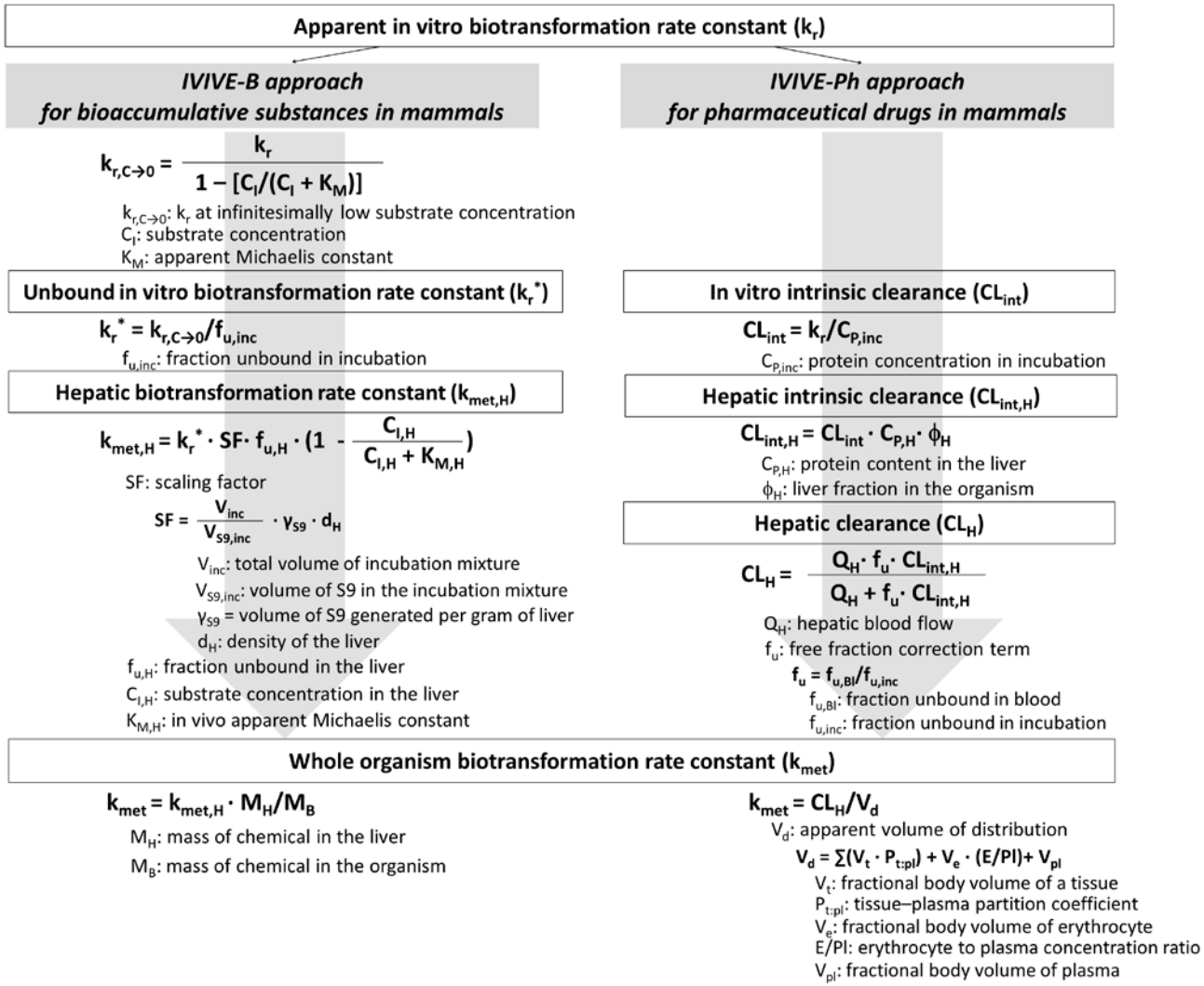

Figure 4.1. Framework of the IVIVE-Ph and IVIVE-B models for predicting the biotransformation rate constants of chemicals in mammals.

Step 2: Calculation of the hepatic biotransformation rate constant ($k_{met,H}$) from the unbound in vitro biotransformation rate constant (k_r^*). This calculation is based on the simplifying assumptions that (i) $k_{met,H}$ for bioaccumulative substances in mammals is determined by liver enzyme function and the fraction of unbound chemical in the liver, and is not importantly influenced by the hepatic blood flow, and that (ii) $k_{met,H}$ exhibits a substrate concentration-dependent relationship that can be described by Michaelis–Menten kinetics. The rationale for not considering hepatic blood flow is twofold. First, hydrophobic chemicals are often slowly metabolized and their hepatic biotransformation rates are not limited by delivery of chemical to the liver via the blood. To use pharmaceutical terminology, such chemicals have low hepatic extraction ratios and their removal by the liver is dependent on enzyme activity and is not affected significantly by liver blood flow (Wilkinson & Shand, 1975). Second, in mammals, dietary uptake is the major route of exposure to hydrophobic organic chemicals of limited volatility. After oral exposure, chemicals first enter the liver from the gastrointestinal tract via the hepatic portal vein, and the extraction of unbound chemical in the liver depends on enzyme activity (Wilkinson & Shand, 1975; Lo et al., 2016), not on blood flow to the liver. The hepatic biotransformation rate constant $k_{met,H}$ (h^{-1}) is calculated as

$$k_{met,H} = k_r^* \cdot SF \cdot f_{u,H} \cdot \left(1 - \frac{C_{I,H}}{C_{I,H}+K_{M,H}}\right) \quad (4.4)$$

where SF is a scaling factor (unitless) that accounts for the dilution of enzymes that occurs during the preparation of the liver fraction; $f_{u,H}$ is the unbound fraction (unitless) of the chemical in the liver; $C_{I,H}$ is the substrate concentration in the liver (µM); and $K_{M,H}$ is the in vivo hepatic Michaelis constant (µM).

For liver S9 preparations, SF can be obtained through a series of volume ratios associated with the stepwise process of the preparations of liver S9 and expressed as

$$SF = \frac{V_{inc}}{V_{S9,inc}} \cdot \frac{V_{S9}}{V_{hom}} \cdot \frac{V_{hom}}{V_H} = \frac{V_{inc}}{V_{S9,inc}} \cdot \frac{V_{S9}}{W_H} \cdot d_H = \frac{V_{inc}}{V_{S9,inc}} \cdot \gamma_{S9} \cdot d_H \quad (4.5)$$

where V_{inc} and $V_{S9,inc}$ are the total volume of incubation mixture (mL) and the volume of S9 (mL) used in the in vitro experiments, respectively; V_{S9} is the volume of liver S9 fraction

(mL) collected after spinning the liver homogenate at 9000 × g; V_{hom} is the volume of liver homogenate (mL) prior to centrifugation in the liver S9 preparation process; V_H is the volume of liver (mL) used for preparing the liver S9 fraction, and it can be obtained from the wet weight of the liver (W_H; g) and the density of the liver (d_H; g/mL); and γ_{S9} is the yield of S9 fraction generated per gram of liver (i.e., V_{S9}/W_H; mL/g liver). Note that V_{hom} cancels out in Equation 4.5, which simplifies the calculation of SF such that the measurement of V_{hom} is not required.

The unbound fraction of the chemical in the liver ($f_{u,H}$) in Equation 4.4 can be estimated by assuming that the liver consists of three phases (i.e., lipids, proteins and water) and that the chemical partitions as follows:

$$f_{u,H} = \frac{f_{W,H}}{f_{L,H} \cdot K_{OW} + f_{P,H} \cdot K_{PW} + f_{W,H}} \quad (4.6)$$

where $f_{L,H}$, $f_{P,H}$, and $f_{W,H}$ are the fractions of lipid, protein, and water of the liver (v/v; unitless), respectively; K_{OW} represents the lipid–water partition coefficient; and K_{PW} is the protein–water partition coefficient, which for some non-ionic hydrophobic organic compound can be estimated as $0.05 \cdot K_{OW}$ (deBruyn & Gobas, 2007).

The concentration dependence of the in vivo hepatic biotransformation rate constant in Equation 4.4 is expressed by $K_{M,H}$. $K_{M,H}$ may be difficult to measure. However, assuming that the in vitro assay includes the majority of the enzymes involved in the biotransformation of the test chemical, it can be assumed that, for the purpose of in vitro-to-in vivo extrapolation, $K_{M,H}$ is approximately equal to K_M. Differences in the substrate concentrations between the in vitro assay and real world in vivo application can therefore be taken into account.

Step 3: Derivation of the whole organism biotransformation rate constant k_{met} from the hepatic biotransformation rate constant ($k_{met,H}$). This calculation is based on the assumptions that (i) the liver is the major organ of xenobiotic biotransformation; and (ii) chemical partitioning within the organism is fast and maintains a near-equilibrium between the chemical in the liver and in the rest of the organism. The whole organism biotransformation rate constant k_{met} (d^{-1}) can be calculated as

$$k_{met} = 24 \cdot k_{met,H} \cdot \frac{M_H}{M_B} \quad (4.7)$$

where M_H and M_B are the mass (g) of the chemical in the liver and in the whole organism (including the liver), respectively. A factor of 24 converts the unit of k_{met} from h^{-1} to d^{-1}. The ratio of M_H/M_B represents the fraction of the total chemical mass in the organism (M_B) that is in the liver (M_H). For many non-ionic hydrophobic substances, it can be estimated by assuming that the liver and the organism consist of three phases, lipids, proteins and water, and that the chemical partitions according to Equation 4.8:

$$\frac{M_H}{M_B} = \phi_H \cdot \frac{f_{L,H} \cdot K_{OW} + f_{P,H} \cdot K_{PW} + f_{W,H}}{f_{L,B} \cdot K_{OW} + f_{P,B} \cdot K_{PW} + f_{W,B}} \quad (4.8)$$

where ϕ_H is the volumetric fraction of the liver in the organism (v/v; unitless), that is, V_H/V_B, where V_B is the volume of the organism (mL); and $f_{L,B}$, $f_{P,B}$, and $f_{W,B}$ are the fractions of lipid, protein, and water of the organism (v/v; unitless), respectively. It is interesting to note that in the extrapolation to k_{met} from in vitro data (Equations 4.1 to 4.8), the term $f_{L,H} \cdot K_{OW} + f_{P,H} \cdot K_{PW} + f_{W,H}$ which appears in Equations 4.6 and 4.8 tend to cancel out. This is because in the model, an increase in the lipid or protein content both reduces the fraction of unbound chemical available for biotransformation in the liver and increases the chemical mass present in the liver. This implies that for the estimation of the whole organism biotransformation rate, the model is insensitive to the actual liver lipid and protein composition. The derivation of Equations 4.3, 4.6 and 4.8 is given in Appendix C.

4.3.2. The IVIVE-Ph Approach for Pharmaceutical Drugs in Mammals

An IVIVE approach to estimate in vivo hepatic clearance of pharmaceuticals in mammals was recently applied to fish and estimates of whole fish biotransformation rate constants were incorporated into aquatic bioaccumulation assessments (Nichols et al., 2006; Nichols et al., 2013a; Cowan-Ellsberry et al., 2008; Han et al., 2009; Escher et al., 2011; Laue et al., 2014; Han et al., 2007; Fay et al., 2014). In the present study, we refer to this approach as the IVIVE-Ph approach and discuss its application to mammals with the purpose of comparing it to the proposed IVIVE-B approach.

The major steps in the IVIVE-Ph method in mammals are illustrated in Figure 4.1 and summarized as follows:

Step 1: The in vitro biotransformation rate constant (k_r; h^{-1}) measured by substrate depletion in liver S9 is normalized to total S9 protein concentration in the incubation medium ($C_{P,inc}$; mg S9 protein/mL) to obtain the in vitro intrinsic clearance (CL_{int}; $mL \cdot h^{-1} \cdot$mg liver S9 protein^{-1}) according to Equation 4.9:

$$CL_{int} = \frac{k_r}{C_{P,inc}} \tag{4.9}$$

Step 2: The in vitro intrinsic clearance is scaled up to the whole liver to obtain the hepatic intrinsic clearance ($CL_{int,H}$; $mL \cdot h^{-1} \cdot g$ organism^{-1}):

$$CL_{int,H} = CL_{int} \cdot C_{P,H} \cdot \phi_H \tag{4.10}$$

where $C_{P,H}$ is the protein content of the liver (mg S9 protein/g liver); and ϕ_H is the wet weight fraction of the liver in the organism (g liver/g organism).

The well-stirred liver model (Wilkinson & Shand, 1975) is then used to calculate hepatic clearance (CL_H; $mL \cdot h^{-1} \cdot g$ organism^{-1}) considering hepatic intrinsic clearance, hepatic blood flow and chemical binding, according to Equation 4.11:

$$CL_H = \frac{Q_H \cdot f_u \cdot CL_{int,H}}{Q_H + f_u \cdot CL_{int,H}} \tag{4.11}$$

where Q_H is the hepatic blood flow (mL blood$\cdot h^{-1} \cdot g$ organism^{-1}) obtained from the product of cardiac output (mL blood$\cdot h^{-1} \cdot g$ organism^{-1}) and the fraction of blood flow that goes through the liver (unitless); and f_u is the free fraction correction term defined as

$$f_u = \frac{f_{u,Bl}}{f_{u,inc}} \tag{4.12}$$

where $f_{u,Bl}$ and $f_{u,inc}$ are the unbound fractions (unitless) of the chemical in blood and in the incubation medium, respectively. Empirical equations for calculating $f_{u,Bl}$ have been reported previously using blood binding data from mammals and fish (for neutral

chemicals with log K_{OW} ranging from –0.78 to 6.19) (Cowan-Ellsberry et al., 2008; Han et al., 2007), and specifically from rainbow trout (for neutral chemicals with log K_{OW} ranging from 1.5 to 8.2) (Nichols et al., 2013a). This approach may be applicable to the IVIVE of potentially bioaccumulative substances in mammals. An alternative approach for estimating $f_{u,Bl}$ is to consider the blood as consisting of three phases, lipids, proteins and water, and assuming that the chemical partitions as follows:

$$f_{u,Bl} = \frac{f_{W,Bl}}{f_{L,Bl} \cdot K_{OW} + f_{P,Bl} \cdot K_{PW} + f_{W,Bl}} \tag{4.13}$$

where $f_{L,Bl}$, $f_{P,Bl}$, and $f_{W,Bl}$ are the fractions of lipid, protein, and water of blood (v/v; unitless), respectively; K_{OW} represents the lipid–water partition coefficient; and K_{PW} is the protein–water partition coefficient, which for some non-ionic hydrophobic organic compound can be estimated as $0.05 \cdot K_{OW}$ (deBruyn & Gobas, 2007). The unbound fraction of the chemical in the incubation medium ($f_{u,inc}$) can be measured experimentally (Escher et al., 2011; Lee et al., 2012), or estimated from empirical relationships (Nichols et al., 2013a; Han et al., 2009) or from Equation 4.3.

Step 3: The whole organism biotransformation rate constant (k_{met}; d^{-1}) is calculated by dividing the hepatic clearance (CL_H; $mL \cdot h^{-1} \cdot g$ organism^{-1}) by the apparent volume of distribution of the chemical (V_d; mL/g organism), that is,

$$k_{met} = \frac{24 \cdot CL_H}{V_d} \tag{4.14}$$

where a factor of 24 is obtained by converting the unit of k_{met} from h^{-1} to d^{-1}. In pharmacology, V_d is defined as the theoretical volume that the administered drug dose would have to occupy (if it were uniformly distributed) to provide the same concentration as that in blood plasma, or alternatively, as the ratio of the total amount of drug in the organism and the drug plasma concentration (Rowland & Tozer, 1989). In previous studies where this approach was applied to fish (Nichols et al., 2006; Nichols et al., 2013a; Cowan-Ellsberry et al., 2008; Han et al., 2009; Escher et al., 2011; Laue et al., 2014; Han et al., 2007; Fay et al., 2014), V_d was viewed as the sorptive capacity of the fish relative to that of blood and calculated as the ratio of a partitioning–based estimate of the BCF (in the absence of biotransformation or organism growth) and the blood–water partition

coefficient (Nichols et al., 2006). The partitioning–based estimate of the BCF may not be meaningful and practical for mammals. Alternatively, the volume of distribution of chemicals in mammals can be calculated using a mechanism–based approach that considers drug lipophilicity and plasma protein binding as two main determinants of V_d as proposed by Poulin and Theil (2002). The volume of distribution in mammals at steady-state is calculated as

$$V_d = \Sigma V_t \cdot P_{t:pl} + V_e \cdot (E/P) + V_{pl} \quad (4.15)$$

where V_t, V_e, and V_{pl} are the fractional body volume (mL/g organism) of a tissue, erythrocyte, and plasma, respectively; E/Pl is the erythrocyte to plasma concentration ratio (unitless), which is set equal to 1 for chemicals that distribute homogeneously into tissues; and $P_{t:pl}$ is the tissue–plasma partition coefficient (unitless), including the non-adipose tissue–plasma partition coefficient ($P_{t:pl\_non\text{-}adipose}$) and the adipose tissue–plasma partition coefficient ($P_{t:pl\_adipose}$). The non-adipose tissues include the bone (plus marrow), brain, gut, heart, kidney, liver, lung, skeletal muscle, skin, and spleen; and the adipose tissue refers to subcutaneous white fat. The tissue–plasma partition coefficient for a non-adipose tissue ($P_{t:pl\_non\text{-}adipose}$; unitless) is calculated as

$$P_{t:pl\_non-adipose} = \frac{K_{OW}\cdot(f_{NL,t}+0.3\cdot f_{PhL,t})+1\cdot(f_{W,t}+0.7\cdot f_{PhL,t})}{K_{OW}\cdot(f_{NL,pl}+0.3\cdot f_{PhL,pl})+1\cdot(f_{W,pl}+0.7\cdot f_{PhL,pl})} \cdot \frac{f_{u,pl}}{f_{u,t}} \quad (4.16)$$

where $f_{NL,t}$, $f_{PhL,t}$, and $f_{W,t}$ are the fractions of neutral lipids, phospholipids, and water of the tissue (v/v; unitless), respectively; $f_{NL,pl}$, $f_{PhL,pl}$, and $f_{W,pl}$ are the fractions of neutral lipids, phospholipids, and water of the plasma (v/v; unitless), respectively; and $f_{u,pl}$ and $f_{u,t}$ are the unbound fractions (unitless) in the plasma and tissue, respectively. The value of $f_{u,pl}$ can be estimated by assuming that the plasma consists of three phases, lipids, proteins and water, and that the chemical partitions as follows:

$$f_{u,pl} = \frac{f_{W,pl}}{f_{L,pl}\cdot K_{OW} + f_{P,pl}\cdot K_{PW} + f_{W,pl}} \quad (4.17)$$

where $f_{L,pl}$, $f_{P,pl}$, and $f_{W,pl}$ are the fractions of lipids (including neutral lipids and phospholipids), proteins, and water of the plasma (v/v; unitless), respectively. $f_{u,t}$ can be estimated from $f_{u,pl}$ based on an empirical equation for mammals (Poulin et al., 2001):

$$f_{u,t} = \frac{1}{1 + [(1 - f_{u,pl})/f_{u,pl}] \cdot 0.5} \quad (4.18)$$

The tissue–plasma partition coefficient for the adipose tissue ($P_{t:pl\_adipose}$; unitless) is calculated as

$$P_{t:p\_adipose} = \frac{K_{VO:W} \cdot (f_{NL,t} + 0.3 \cdot f_{PhL,t}) + 1 \cdot (f_{W,t} + 0.7 \cdot f_{PhL,t})}{K_{VO:W} \cdot (f_{NL,pl} + 0.3 \cdot f_{PhL,pl}) + 1 \cdot (f_{W,pl} + 0.7 \cdot f_{PhL,pl})} \cdot \frac{f_{u,pl}}{1} \quad (4.19)$$

where $K_{VO:W}$ is the olive oil–water partition coefficient (unitless). It was reported that olive oil is a better surrogate of the adipose tissue lipids than octanol (Poulin et al., 2001). The logarithm of $K_{VO:W}$ can be calculated based on an empirical equation for neutral compounds (Poulin & Theil, 2002):

$$log K_{VO:W} = 1.15 \cdot \log K_{OW} - 1.35 \quad (4.20)$$

The derivation of Equations 4.13 and 4.17 is given in Appendix C.

4.3.3. Bioaccumulation Model

For the purpose of bioaccumulation assessment, a whole organism toxicokinetic bioaccumulation model that describes the major uptake and elimination pathways of chemicals in the organism can be used to obtain bioaccumulation metrics BCF and BMF. The major uptake processes of chemicals in mammals include respiratory and dietary uptake, and the major elimination processes include respiratory elimination, fecal elimination, urinary elimination, biliary elimination, biotransformation, lactation, and growth. The organism is described as a single compartment in which the chemical is distributed homogeneously based on the assumption of rapid internal partitioning of the chemical. The change of chemical concentrations in the organism over time is described as

$$\frac{dC_B}{dt} = k_{AU} \cdot C_A + k_D \cdot C_D - (k_{AE} + k_F + k_U + k_{Bi} + k_{met} + k_L + k_G) \cdot C_B \quad (4.21)$$

where C_B, C_A and C_D are the concentrations (mol/m^3) of the chemical in the organism, air and diet, respectively; k_{AU} and k_D are the rate constants (d^{-1}) describing the first-order kinetics of chemical uptake from air and diet, respectively; and k_{AE}, k_F, k_U, k_{Bi}, k_{met}, k_L ,and

k_G are the rate constants (d^{-1}) describing the first-order kinetics of chemical elimination via respiratory elimination, fecal elimination, urinary elimination, biliary elimination, biotransformation, lactation, and growth dilution, respectively. The k_{met} can be obtained from the IVIVE-B or IVIVE-Ph approach. The equations for deriving k_{AU}, k_D, k_{AE}, k_F, k_U, k_{Bi}, and k_L are described in Appendix C. This model can be used to derive an expression for the steady-state biomagnification factor (BMF) if dietary uptake is the major route of exposure and chemical uptake from air is negligible:

$$BMF = \frac{C_B}{C_D} = \frac{k_D}{(k_{AE}+k_F+k_U+k_{Bi}+k_{met}+k_L+k_G)} \quad (4.22)$$

This model can often be further simplified. For example, when applied to adult male mammals, lactation does not need to be considered and growth dilution may be negligible (i.e., $k_L = 0$ and $k_G = 0$). The lipid-normalized BMF (kg lipid/kg lipid) can be obtained by multiplying the unitless steady-state BMF (Equation 4.22) by a factor of $(d_D \cdot f_{L,D})/(d_B \cdot f_{L,B})$, where d_D and d_B are the densities of diet and organism, respectively; and $f_{L,D}$ and $f_{L,B}$ are the lipid fractions (w/w; unitless) of diet and organism, respectively.

4.4. Methods

4.4.1. Model Evaluation

The proposed IVIVE-B model for potentially bioaccumulative substances was evaluated using three approaches. First, we compared the model-calculated biotransformation rate constants in rats (k_{met}) for hypothetical chemicals (log K_{OW} ranging from 0 to 10) with those calculated by the IVIVE-Ph model. The input in vitro biotransformation rate constants were set at 0.1, 0.2, 0.5, 1, 2, 5 and 10 h^{-1} (corresponding to in vitro half-lives of 6.9 h, 3.5 h, 1.4 h, 42 min, 21 min, 8.3 min and 4.2 min, respectively), considering practical experimental conditions of in vitro experiments. The unbound fraction of the hypothetical chemicals in the incubation mixture ($f_{u,inc}$) was calculated according to Equation 4.3.

To examine the assumption of perfusion-independent hepatic biotransformation rate in the proposed IVIVE-B model for potentially bioaccumulative substances in

mammals, the hepatic biotransformation rate constant in the IVIVE-B model was considered as analogous to the hepatic clearance in the IVIVE-Ph model, and the well-stirred liver model (Equation 4.11) that accounts for hepatic blood flow and unbound hepatic intrinsic clearance was used to independently assess the relative contribution of hepatic blood flow and unbound hepatic intrinsic clearance to hepatic clearance for hypothetical chemicals (log K_{OW} ranging from 0 to 10) with in vitro biotransformation rate constants set at 0.1, 0.2, 0.5, 1, 2, 5 and 10 h^{-1}. The well-stirred liver model (Equation 4.11) was rearranged in an additive format as

$$\frac{1}{CL_H} = \frac{1}{Q_H} + \frac{1}{f_u \cdot CL_{int,H}} \tag{4.23}$$

The percentage contribution of hepatic blood flow to hepatic clearance ($CL_{H-Q\%}$; %) can be calculated as

$$CL_{H-Q\%} = \left(\frac{1}{Q_H}\right) / \left(\frac{1}{CL_H}\right) \cdot 100\% \tag{4.24}$$

Similarly, the percentage contribution of unbound hepatic intrinsic clearance to hepatic clearance ($CL_{H-int\%}$; %) can be calculated as

$$CL_{H-int\%} = \left(\frac{1}{f_u \cdot CL_{int,H}}\right) \Big/ \left(\frac{1}{CL_H}\right) \cdot 100\% \tag{4.25}$$

Second, we evaluated the proposed IVIVE-B model by comparing the IVIVE-B model calculated whole body biotransformation rate constants (k_{met}) for benzo[a]pyrene and chrysene in rats to those calculated using the IVIVE-Ph model. Actual in vitro measurements of the depletion rate constant and unbound fraction in incubation mixture ($f_{u,inc}$) (Lee et al., 2012) were used in both models. We also compared the BMF values for benzo[a]pyrene and chrysene in rats calculated by the rat bioaccumulation model using input k_{met} values obtained from the IVIVE-B model to those calculated using k_{met} from the IVIVE-Ph model.

Third, the hepatic and whole body biotransformation rate constant for benzo[a]pyrene in rats calculated from the proposed IVIVE-B model was compared to the measured depuration rate constants for benzo[a]pyrene in rats from in vivo and ex vivo

(e.g., isolated perfused liver) studies reported in the literature. The whole body biotransformation rate constant for benzo[a]pyrene in rats calculated from the IVIVE-B model was used as an input parameter in the rat bioaccumulation model to obtain the BMF. The calculated BMF for benzo[a]pyrene in rats was then compared to empirical BMF data obtained from the literature.

4.4.2. Model Parameterization

The IVIVE-B and IVIVE-Ph models were parameterized for rats. The input parameters for hypothetical chemicals and the two model chemicals (benzo[a]pyrene and chrysene) in both IVIVE models are summarized in Table C1 in Appendix C. For hypothetical chemicals, the log K_{OW} value was set ranging from 0 to 10, and the unbound fraction in incubation mixture ($f_{u,inc}$) was calculated according to Equation 4.3. For benzo[a]pyrene and chrysene, the log K_{OW} values were obtained from Mackay et al. (2006) and adjusted to 37 ºC according to Beyer et al. (2002), and the in vitro biotransformation rate constants and unbound fractions in the incubation mixture ($f_{u,inc}$) were obtained from previous measurements that used a thin-film sorbent-phase dosing approach with liver S9 from male Sprague–Dawley rats (Lee et al., 2012).

In the IVIVE-B model, the volume of liver S9 in incubation mixture ($V_{S9,inc}$) and the total volume of incubation mixture (V_{inc}) were obtained from the experimental conditions of the sorbent-phase dosing experiments using rat liver S9 fractions (Lee et al., 2012). The yield of liver S9 fraction (γ_{S9}) was measured during the preparation of rat liver S9 fractions. The density of the liver (d_H) was measured previously for male Sprague–Dawley rats (Sohlenius-Sternbeck, 2006). The lipid and water fractions in the liver ($f_{L,H}$ and $f_{W,H}$) were reported previously for the rat (Poulin & Krishnan, 1996). The protein fraction in the liver ($f_{P,H}$) was calculated by assuming that the sum of all fractions equalled unity. The lipid and protein fractions in the rat ($f_{L,B}$ and $f_{P,B}$) were reported previously (deBruyn & Gobas, 2006). The water fraction in the rat ($f_{W,B}$) was calculated by assuming that the sum of all fractions equalled unity.

In the IVIVE-Ph model, the S9 protein concentrations in the incubation mixture and in the liver were obtained from the sorbent-phase dosing experiments using rat liver S9

fractions (Lee et al., 2012). The volumetric fraction of the liver in the organism (ϕ_H) was estimated as the measured wet weight fraction (i.e., g liver/g animal) because a mass-to-volume conversion can be ignored for tissues with densities approximating 1 g/mL (Brown et al., 1997). The cardiac output and fraction of blood flow through the liver in the rat were obtained from reported values (Brown et al., 1997). The lipid and water fractions in the blood ($f_{L,Bl}$ and $f_{W,Bl}$) were reported previously for the rat (Poulin & Krishnan, 1996). The protein fraction in the blood ($f_{P,Bl}$) was calculated by assuming that the sum of all fractions equalled unity. The fraction of unbound chemicals in the blood was calculated using Equation 4.13. The volume of distribution (V_d) of chemicals was calculated using a mechanism–based approach developed by Poulin and Theil (2002) using Equations 4.15–4.20. The fractional body volume of tissues (V_t), erythrocyte (V_e) and plasma (V_{pl}), fractions of neutral lipids of tissues ($f_{NL,t}$) and plasma ($f_{NL,pl}$), fractions of phospholipids of tissues ($f_{PhL,t}$) and plasma ($f_{PhL,pl}$), fractions of water of tissues ($f_{W,t}$) and plasma ($f_{W,pl}$), and erythrocyte to plasma concentration ratio (E/Pl) were obtained from Poulin and Theil (2002). Because the calculation of V_d was developed and tested for drugs with relatively low hydrophobicity (log K_{OW} < 3 for the majority of neutral test drugs) compared to hydrophobic chemicals, the calculation of the tissue–plasma partition coefficient for the adipose tissue ($P_{t:pl\_adipose}$) for chemicals with log K_{OW} greater than 3 in the present study was set to be the same as that calculated for chemical with log K_{OW} = 3 to avoid extrapolation of the empirical equation (Equation 4.20).

The input parameters for the rat bioaccumulation model are summarized in Table C2 in Appendix C. The log K_{OA} values for benzo[a]pyrene and chrysene were obtained from Mackay et al. (2006) and adjusted to 37 ºC according to Beyer et al. (2002). Dietary absorption efficiency (E_D) for the chemical was estimated based on measured fecal excretion data for seven polycyclic aromatic hydrocarbons (PAHs) dosed in rat diet (Change, 1943) using linear regression:

$$\frac{1}{E_D} = 1 \times 10^{-6} K_{OW} + 1.303 \quad (n = 7, R^2 = 0.77) \tag{4.26}$$

The $f_{u,inc}$ for benzo[a]pyrene (i.e., 0.00044) was obtained from sorbent-phase dosing experiments (Lee et al., 2012) and was almost identical to the value calculated from Equation 4.3 (0.00046). The $f_{u,inc}$ for chrysene (i.e., 0.00046) was also obtained from

sorbent-phase dosing experiments but was 2.7-fold lower than that calculated from Equation 4.3.

4.4.3. Model Application

The relationship between in vitro biotransformation rate constants and calculated BMFs in rats using the proposed IVIVE-B model in combination with the rat BMF model was established for adult male rats for a set of hypothetical chemicals with log K_{OW} values ranging from 2 to 10 and log K_{OA} values ranging from 4 to 10, at input in vitro biotransformation rate constant of 0, 0.1or 0.5 h^{-1}. The fraction of unbound chemical in the incubation mixture ($f_{u,inc}$) was calculated according to Equation 4.3. The dietary absorption efficiency (E_D) was assumed to be K_{OW}-dependent and calculated according to Equation 4.26 and did not consider any intestinal biotransformation. The values of other input parameters are listed in Tables C1 and C2 in Appendix C for the IVIVE-B and rat BMF models, respectively.

4.5. Results and Discussion

4.5.1. Model Evaluation

First, we compared the whole body biotransformation rate constants (k_{met}) in rats calculated by the proposed IVIVE-B model to those determined by the IVIVE-Ph model for hypothetical chemicals with a wide range of log K_{OW} values. Figure 4.2 illustrates that the k_{met} values calculated by the IVIVE-B and IVIVE-Ph models both decreased sigmoidally with increasing log K_{OW}, and that at log $K_{OW} \geq 4$, k_{met} values remained approximately constant. The change in k_{met} as a function of log K_{OW} can be explained by the change in the fractions of unbound chemical in the incubation medium, blood and liver with log K_{OW}. In the IVIVE-B model, $f_{u,H}$ falls relative to $f_{u,inc}$ with increasing log K_{OW} at low log K_{OW} due to differences in composition of the liver and the incubation medium. At higher log K_{OW}, $f_{u,H}/f_{u,inc}$ reaches a constant value as the chemical is predominantly bound in both the liver and the incubation medium, and the unbound fractions in liver and incubation medium fall with increasing log K_{OW} at essentially the same rate. In the IVIVE-Ph model, the change of k_{met} with log K_{OW} is due to $f_{u,Bl}$ falling relative to $f_{u,inc}$ with increasing log K_{OW} at low log

K_{OW} due to differences in composition of the blood and the incubation medium as well as an increase in V_d with increasing log K_{OW}. At high K_{OW}, the ratio $f_{u,Bl}/(f_{u,inc} \cdot V_d)$ reaches a constant value because the chemical is predominantly bound in both the blood and the incubation medium, and the unbound fractions in blood and incubation medium fall with increasing log K_{OW} at essentially the same rate and V_d is approximately constant with increasing log K_{OW}.

For chemicals with log $K_{OW} < 4$, the k_{met} values calculated by the IVIVE-B model were on average 1.77-, 1.71-, and 1.27-fold lower than those calculated by the IVIVE-Ph model when the in vitro biotransformation rate constant was set to 0.1, 1, and 10 h^{-1}, respectively (Figures 4.2A–4.2C). For chemicals with log $K_{OW} \geq 4$, differences between the two models were less: the IVIVE-B model yielded values of k_{met} on average 1.19-, 1.18-, and 1.06-fold lower than those of the IVIVE-Ph model when the in vitro biotransformation rate constant was set to 0.1, 1, and 10 h^{-1}, respectively. This indicates that the k_{met} values produced by the IVIVE-B and IVIVE-Ph models are in good agreement for chemicals with log $K_{OW} \geq 4$ for in vitro biotransformation rate constants ranging from 0.1 to 10 h^{-1}, corresponding to in vitro half-lives ranging from 6.9 h to 4.2 min.

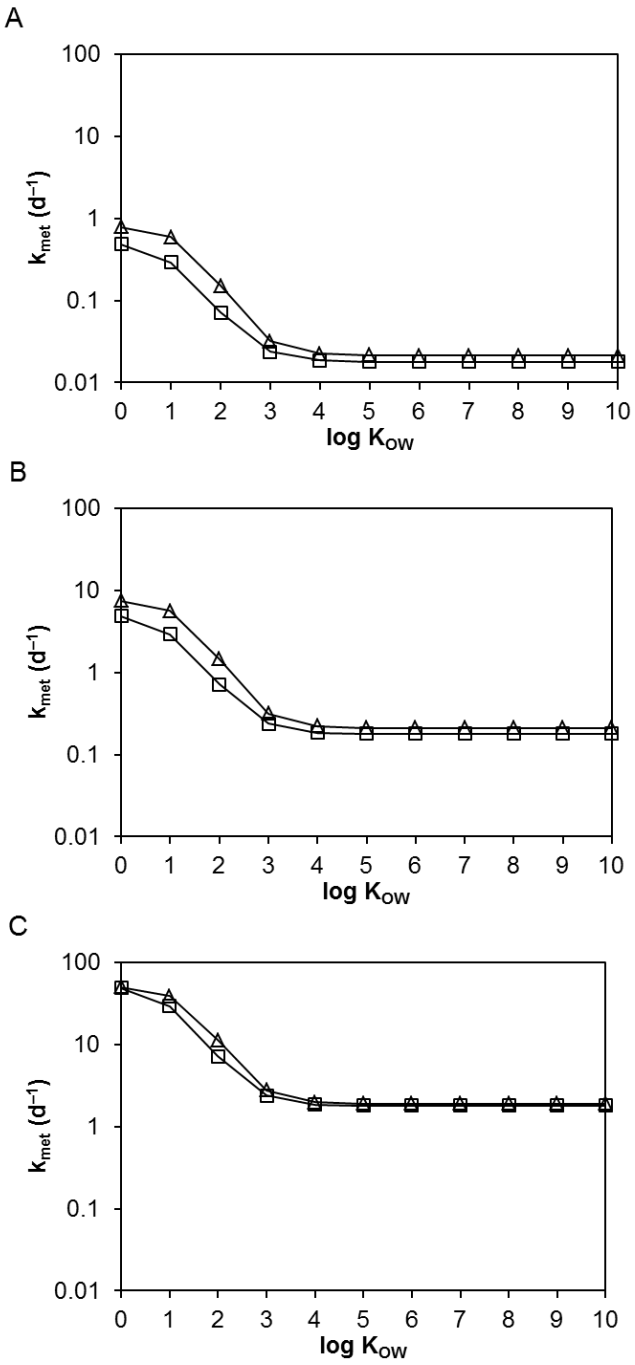

Figure 4.2. Relationship between log K_{OW} and calculated whole body biotransformation rate constants (k_{met}, d^{-1}) for hypothetical chemicals in rats using the IVIVE-B model (open squares) or the IVIVE-Ph model (open triangles) at input in vitro biotransformation rate constants of 0.1 h^{-1} (A), 1 h^{-1} (B) and 10 h^{-1} (C).

One of the major assumptions in the proposed IVIVE-B model is that the biotransformation rate of hydrophobic chemicals in the liver is controlled by the metabolic activity of the liver rather than the hepatic blood perfusion rate. To test this assumption, we compared the calculated hepatic clearance to two determining components, hepatic blood flow and unbound hepatic intrinsic clearance, in the well-stirred liver model for a set of hypothetical chemicals, considering that the hepatic biotransformation rate in the IVIVE-B model is analogous to the hepatic clearance in the IVIVE-Ph model. Figure 4.3 illustrates the relative contribution of hepatic blood flow and unbound hepatic intrinsic clearance to hepatic clearance in the well-stirred liver model for a set of hypothetical chemicals. Figure 4.3A shows that the relative contribution of hepatic blood flow to hepatic clearance ($CL_{H-Q\%}$) decreased with increasing log K_{OW} (when log $K_{OW} < 4$) and achieved a constant value when log $K_{OW} \geq 4$. The $CL_{H-Q\%}$ values increased with increasing in vitro biotransformation rates. Figure 4.3B shows an opposite trend: the relative contribution of unbound hepatic intrinsic clearance to hepatic clearance ($CL_{H-int\%}$) increased with increasing log K_{OW} (when log $K_{OW} < 4$) and remained approximately constant (when log $K_{OW} \geq 4$). The $CL_{H-int\%}$ values increased with decreasing in vitro biotransformation rates. Figure 3B shows that more than 85% of the hepatic clearance is due to the unbound hepatic intrinsic clearance for hydrophobic chemicals (log $K_{OW} \geq 4$) if in vitro depletion half-lives exceed 4.2 min. This supports the assumption in the IVIVE-B modeling approach that for hydrophobic chemicals with bioaccumulative potential (log $K_{OW} > 5$), the hepatic biotransformation rate is controlled by nonspecific chemical binding and inherent metabolic activity in the liver rather than the liver perfusion rate.

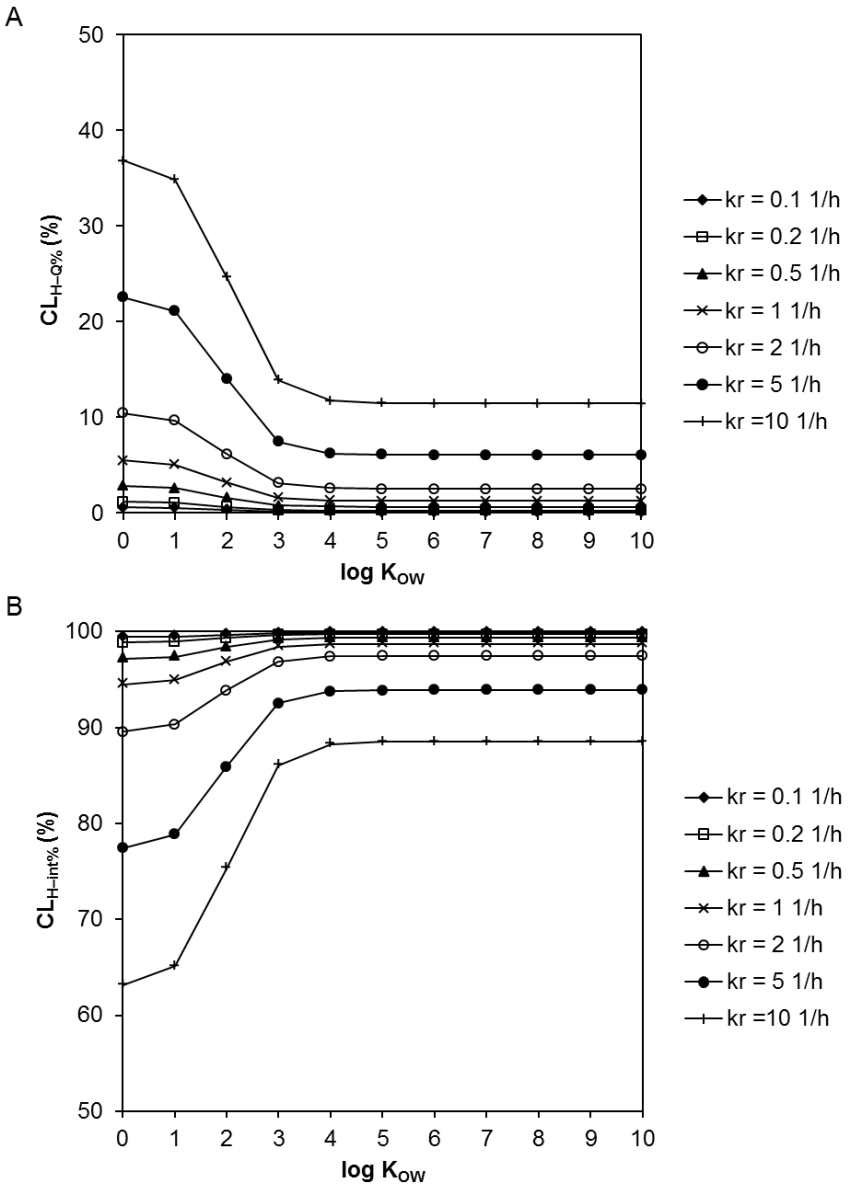

Figure 4.3. The percentage contribution of hepatic blood flow to rat hepatic clearance ($CL_{H-Q\%}$; calculated using Equation 4.24) as a function of log K_{OW} (A), and the percentage contribution of unbound hepatic intrinsic clearance to rat hepatic clearance ($CL_{H-int\%}$; calculated using Equation 4.25) as a function of log K_{OW} (B) derived from the well-stirred liver model (Equations 4.11 and 4.23) at input in vitro biotransformation rate constant (k_r) ranging from 0.1 to 10 h^{-1}.

Figures C1A and C1B in Appendix C further illustrate that at in vitro biotransformation rate constants of 0.1 and 1 h^{-1}, the calculated hepatic clearance was identical to the unbound hepatic intrinsic clearance over the entire range of the log K_{OW}

values, suggesting that chemical binding and inherent metabolic activity in the liver are the major determinants of hepatic clearance for slowly metabolized chemicals, and that hepatic blood flow does not affect the hepatic clearance under such conditions. When the in vitro biotransformation rate constant was set to a high value of 10 h^{-1}, hepatic clearance was controlled by unbound hepatic intrinsic clearance and hepatic blood flow for chemicals with log K_{OW} < 4, but essentially independent of hepatic blood flow and fully controlled by unbound hepatic intrinsic clearance for the more hydrophobic chemicals with a log K_{OW} > 4 (Figure C1C in Appendix C). The results in Figures 4.3 and C1 in Appendix C support the use of the IVIVE-B model for evaluating hydrophobic chemicals with a log K_{OW} greater than approximately 4. Because hepatic clearance data are not required, the IVIVE-B approach simplifies the assessment of bioaccumulation potential in rats and possibly other mammalian species.

Second, the IVIVE-B model was evaluated by comparing the whole body biotransformation rate constants (k_{met}) and BMF values in rats for two hydrophobic model chemicals, i.e., benzo[a]pyrene and chrysene (log K_{OW} of 6.04 and 5.60 at 25 ºC, respectively), to those obtained using the IVIVE-Ph model. The in vitro biotransformation rate constants and unbound fractions in the incubation mixture for benzo[a]pyrene and chrysene were taken from a previous study using rat liver S9 fractions (Lee et al., 2012). For benzo[a]pyrene, the calculated k_{met} in rats was 0.40 ± 0.11 and 0.46 ± 0.12 d^{-1} (mean ± SD, n = 3) for the IVIVE-B and IVIVE-Ph models, respectively. The calculated lipid-normalized BMFs in adult male rats were 0.073 ± 0.017 and 0.063 ± 0.014 kg lipid/kg lipid (mean ± SD, n = 3) using k_{met} calculated from the IVIVE-B and IVIVE-Ph models, respectively. For chrysene, the calculated k_{met} in rats was 1.22 ± 0.14 and 1.33 ± 0.15 d^{-1} (mean ± SD, n = 3) for the IVIVE-B and IVIVE-Ph models, respectively. The calculated lipid-normalized BMFs in adult male rats were 0.033 ± 0.004 and 0.030 ± 0.004 kg lipid/kg lipid (mean ± SD, n = 3) using k_{met} calculated from the IVIVE-B and IVIVE-Ph models, respectively. For both chemicals, the calculated whole body biotransformation rate constants and BMF values using the proposed IVIVE-B model were not statistically different from those calculated using the IVIVE-Ph model (Figure 4.4). The agreement between the models further supports our contention that the IVIVE-B model is a good alternative for estimating whole body biotransformation rate constants and BMFs for hydrophobic chemicals in rats.

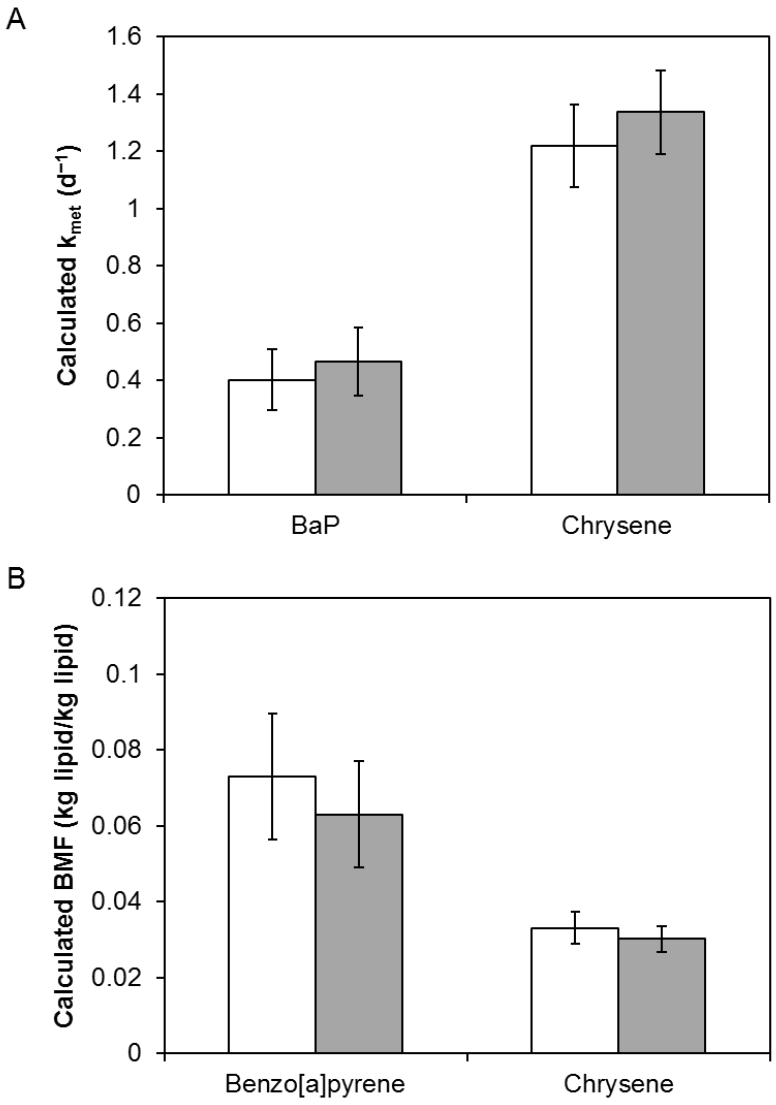

Figure 4.4. The whole body biotransformation rate constants (k_{met}; A) and BMF (B) for benzo[a]pyrene and chrysene in rats calculated from the IVIVE-B (empty bars) and IVIVE-Ph (filled bars) models. Error bars represent the standard deviation (n = 3).

Third, the IVIVE-B model was evaluated by comparing the calculated hepatic biotransformation rate constant ($k_{met,H}$) and whole body biotransformation rate constant (k_{met}) for benzo[a]pyrene in rats to those obtained from empirical observations reported in the literature. Table 4.1 shows that the calculated $k_{met,H}$ for benzo[a]pyrene in rats from the IVIVE-B model (0.38 ± 0.10 h^{-1}; mean ± SD, n = 3) is within the range of the previously reported hepatic elimination rate constants for benzo[a]pyrene (0.05−5 h^{-1}) measured from in vivo rat studies or an ex vivo study using isolated perfused rat liver. Table 4.1 also

shows that the calculated k_{met} for benzo[a]pyrene in rats from the IVIVE-B model (0.40 ± 0.11 d^{-1}; mean ± SD, n = 3) was very close to measured whole body elimination rate constants for benzo[a]pyrene administered via oral gavage and intravenous injection (0.41 and 0.53 d^{-1}, respectively) from a recent in vivo rat study (Moreau & Bouchard, 2015) and with the median value (1.06 d^{-1}) of previously-measured whole body elimination rate constants that varied over almost two orders of magnitude (Table 4.1). The wide range of observed elimination rate constants for benzo[a]pyrene in rats reported in the literature may be associated with different experimental designs and conditions such as different dose levels and routes of administration (e.g., oral gavage and intravenous injection) used in the in vivo experiments. The dose dependence of in vivo biotransformation rate constants as proposed in Equation 4.4 may also partly explain the empirical observations. In addition, greater extrahepatic biotransformation (e.g., intestinal biotransformation), which is not considered in the IVIVE-B or IVIVE-Ph approach may also affect the bioaccumulation behaviour. Furthermore, technical difficulties in conducting in vivo and ex vivo experiments for very hydrophobic chemicals (e.g., incomplete dissolution in aqueous solution) may contribute uncertainty in observed values.

Finally, the proposed IVIVE-B approach was evaluated by comparing the calculated lipid-normalized BMF value for benzo[a]pyrene in adult male rats (0.073 ± 0.017 kg lipid/kg lipid; mean ± SD, n = 3) to the observed BMF value for benzo[a]pyrene in rats. The IVIVE-B BMF value is in good agreement with a previous in vivo determination of the BMF in rats from Kang et al. (2007), i.e., BMF = 0.023 kg lipid/kg lipid, derived from the ratio of the highest concentration of benzo[a]pyrene observed in rat muscle (34.5 ng/g, assuming 2% lipid) to the concentration of benzo[a]pyrene in corn oil (75 μg/mL) administered daily by oral gavage for 30 days. Hence, for benzo[a]pyrene in rats, the proposed IVIVE-B combined with a BMF modeling approach yielded depuration rate constants and BMFs that are in good agreement with those obtained in vivo. Unfortunately, data required for a more thorough evaluation of the IVIVE-B approach are, as far as we know, not available at this point. Additional investigations are needed to further evaluate the proposed IVIVE-B modeling approach.

Table 4.1. Values of hepatic and whole body biotransformation rate constants reported in the literature for benzo[a]pyrene

Animal	In vivo Route of Administration or ex vivo Assay or IVIVE [a] Model	Dose (µmol/kg)	Hepatic Elimination Rate Constant (h^{-1})	Whole Body Elimination Rate Constant (d^{-1})	Reference
Male Sprague–Dawley rats (330–370 g)	IVIVE-B model	—	0.38 ± 0.10	0.40 ± 0.11	The present study
	IVIVE-Ph model	—	—	0.47 ± 0.12	
Male Sprague–Dawley rats (200–250 g)	Oral via gavage	40	—	0.41	Moreau & Bouchard, 2015
	Intravenous	40	—	0.53	
	Intratracheal	40	—	0.91	
	Cutaneous	40	—	1.06	
Male Sprague–Dawley rats (300–460 g)	Intravenous	0.0045	—	1.00 (blood)	Foth et al., 1988
Male Sprague–Dawley rats (260–290 g)	Intravenous	40	0.047	1.33 (blood)	Marie et al., 2010
Male Fisher–344 rats (about 200 g)	Oral via gavage	400	—	2.88 ± 0.10 (plasma)	Ramesh et al., 2002
Male Sprague–Dawley rats (200–300 g)	Intratracheal	0.117	—	9.07 ± 1.01 (blood)	Wiersma & Roth, 1983a
Male Fisher–344 rats (about 200 g)	Oral via gavage	400	0.06 ± 0.007	—	Ramesh et al., 2001
Male Wistar rats (average 344 g)	Intravenous	24	0.13 ± 0.11	—	Moir et al., 1998
		8	1.48 ± 0.22	27.5 ± 5.47 (blood)	
Male Sprague–Dawley rats (190–275 g)	Isolated perfused liver	20 (nmol)	2.34 ± 0.12 [b]	—	Wiersma & Roth, 1983b
			3.76 ± 0.22 [c]	—	
			5.03 ± 0.34 [d]	—	

[a] IVIVE = in vitro-to-in vivo extrapolation
[b] measured at perfusion flow = 7 mL/min
[c] measured at perfusion flow = 10 mL/min
[d] measured at perfusion flow = 20 mL/min

4.5.2. Model Application

The IVIVE-B modeling approach for relating measured in vitro biotransformation rates to the BMF may be useful for bioaccumulation screening. Figure 4.5A illustrates that when no biotransformation is measured in an in vitro assay, chemicals with a log K_{OW} greater than 2 and a log K_{OA} greater than 5 have a biomagnification potential in rats (BMF > 1). These results are in line with previous studies showing that non-metabolized chemicals with a log K_{OW} greater than 2 and a log K_{OA} greater than 5 have the potential to biomagnify in terrestrial food chains (Kelly et al., 2007; Gobas et al., 2003; Kelly & Gobas, 2003). In contrast, Figure 4.5B shows that when chemicals are metabolized slowly (at an in vitro rate constant of 0.1 h^{-1}), only chemicals with a log K_{OW} between 2.5 and 6.5 and a log K_{OA} greater than 5.5 have the potential to biomagnify in rats. Figure 4.5C shows that at the higher in vitro biotransformation rate constant of 0.5 h^{-1}, the estimated BMFs of hydrophobic chemicals in rats are all less than 1. This indicates that even a slow rate of biotransformation can be sufficient to negate biomagnification of the chemical in the food-chain. Figure 4.5C also suggests that the in vitro biotransformation rate constant of 0.5 h^{-1} could be used as a preliminary guideline (cut-off value) for assessing bioaccumulative potential of chemicals that undergo biotransformation in rats. For example, chemicals may be considered to be non-bioaccumulative in rats if the in vitro biotransformation rate constants measured from the in vitro assays are greater than 0.5 h^{-1}. This allows quick chemical screening because full execution of the IVIVE-B model is not required. However, such a guideline should be applied with great care because of the lack of testing of IVIVE-B model and the lack of standardized protocols for measuring in vitro biotransformation rate constants in rats for hydrophobic chemicals for the purpose of bioaccumulation screening.

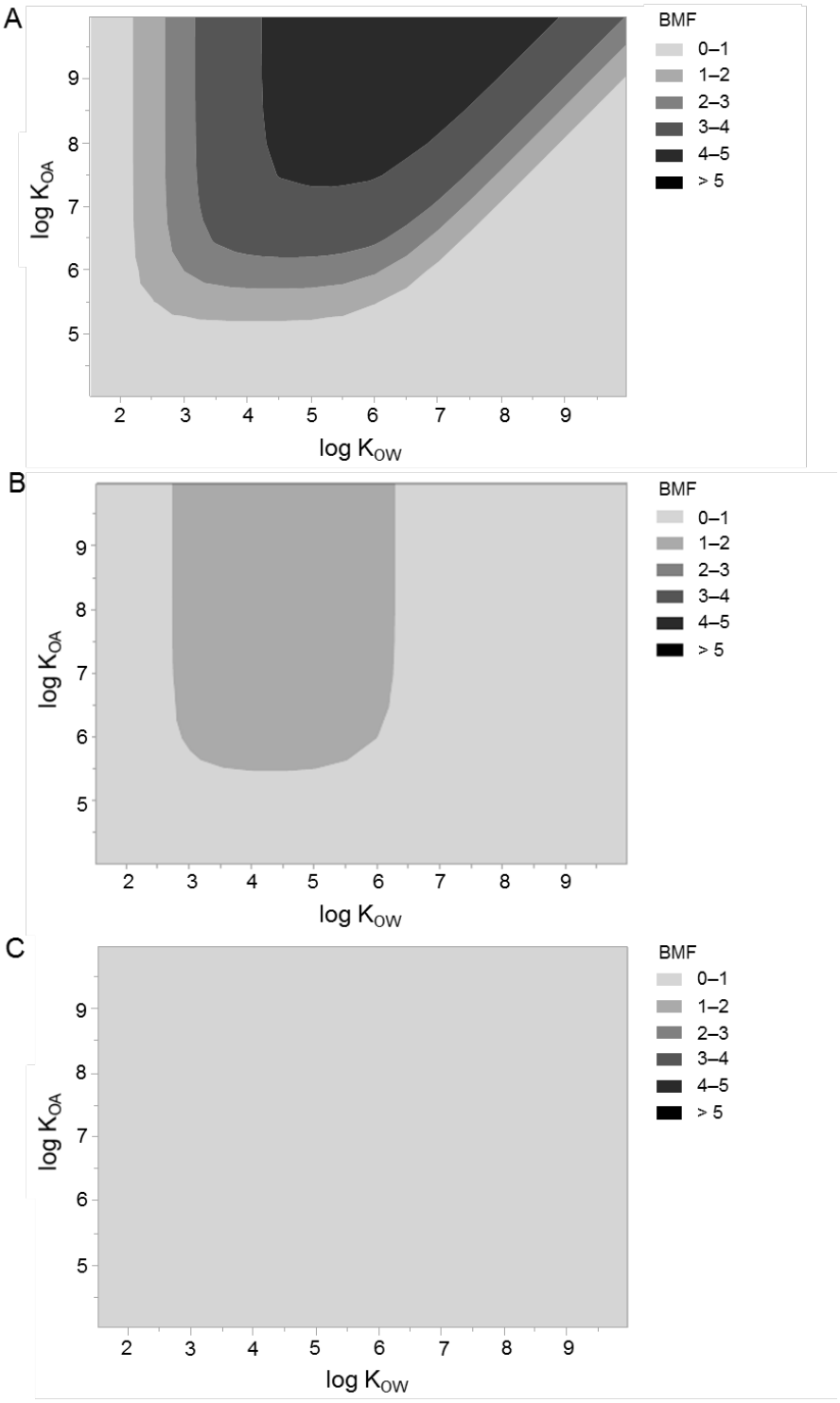

Figure 4.5. Calculated BMF values in adult male rats for hypothetical chemicals as a function of log K_{OW} and log K_{OA} using the IVIVE-B model in combination with a rat BMF model at input in vitro biotransformation rate constant of 0 h^{-1} (A), 0.1 h^{-1} (B) and 0.5 h^{-1} (C).

4.5.3. Advantages and Limitations of the Proposed IVIVE-B Approach

The proposed IVIVE-B approach can be used for estimating in vivo biotransformation rate constants and BMF values for hydrophobic chemicals with bioaccumulative potential (log K_{OW} > 5) in mammals and possibly other terrestrial animals. This approach has several advantages. First, the extrapolation from in vitro to in vivo is relatively straightforward as it involves the extrapolation of rate constants. Clearance and volume of distribution values are useful properties for describing the therapeutic dosages of pharmaceuticals, but are in most cases not required for bioaccumulation assessment of high log K_{OW} chemicals. Second, the well-stirred liver model is not incorporated into the proposed IVIVE-B because it is not needed for bioaccumulation assessment. Consequently, information to parameterize the well-stirred liver model such as cardiac output, fraction of blood flow through the liver, and fraction unbound in blood and estimates of the volume of distribution are not required in the IVIVE-B approach. This is advantageous as several of these parameters may not be available or hard to measure for most species of wildlife and may introduce uncertainty in predictions. Third, the scaling factor involved in the IVIVE-B approach is comprised of several volume ratios which are easy to measure in the preparation of liver S9 fractions and in the in vitro substrate depletion experiments.

The proposed IVIVE-B approach is also subject to limitations. First, the prediction of whole body biotransformation rate constants from the proposed IVIVE-B model is sensitive to the unbound fractions of chemicals in the incubation mixture ($f_{u,inc}$) and in the liver ($f_{u,H}$). The unbound fractions can be estimated (e.g., using Equations 4.3 and 4.6); calculated from empirical relationships (e.g., Han et al. (2009) and Nichols et al. (2013) used binding data for drugs with log K_{OW} ranging from 1.54 to 6.35 (Austin et al., 2002)); or measured (Escher et al., 2011; Lee et al., 2012). The sorbent-phase dosing approach is a useful method for hydrophobic chemicals in largely aqueous incubation mixture because it is solvent-free; the concentrations of very hydrophobic chemicals in the incubation medium often remain very low (hence avoiding saturation effects) due to slow release of the chemical from the sorbent phase (Lee et al., 2012; Lee et al., 2014); and because the in vitro biotransformation rate constant and the unbound fraction in the incubation mixture can be determined in the same experiment. The fraction unbound in

the liver ($f_{u,H}$) is another critical parameter in the IVIVE-B model. In the present study, it was estimated using Equation 4.6 but more investigation is needed to test equation 4.6.

Second, the BMF estimations by the IVIVE-B approach are sensitive to the value used for dietary absorption efficiency (E_D), which is not derived in the in vitro bioassay. Dietary absorption efficiency of chemicals can be affected by the composition of diet (Ramesh et al., 2004) and intestinal biotransformation (Lo et al., 2015b). Values chosen for the assimilation efficiencies for lipid (α_L), protein (α_P) and carbohydrate (α_C) can therefore have a large effect on the BMF estimations. The BMF model is insensitive to assimilation efficiency for water (α_W) as well as the increase in solubility of chemical in bile compared to water (β) as reported previously (Armitage & Gobas, 2007).

Other potential limitations of the IVIVE-B approach result from key assumptions of the model, e.g., the assumption that no extrahepatic metabolism occurs. The small intestines may contribute to the first-pass metabolism of ingested and absorbed chemicals (Ramesh et al., 2004) and intestinal biotransformation has been reported to contribute substantially to biotransformation of hydrophobic organic chemicals in fish. (Lo et al., 2015b). This is a limitation of both the IVIVE-B and IVIVE-Ph approaches. While there is a clear need for further testing of the IVIVE-B approach for bioaccumulation assessment, we submit that the proposed IVIVE-B modeling approach presented and evaluated in this study can be a useful tool for assessing the bioaccumulative potential of hydrophobic chemicals that undergo biotransformation in mammals and possibly other terrestrial and non-aquatic animals.

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Chapter 5.

Improving Management of Hazardous Substances in Canada

5.1. Introduction

Millions of chemical substances have been produced and thousands are now used commercially every day. The wide use of chemicals is of concern because it can pose potential adverse effects on the ecosystem and human health. Regulating hazardous chemicals remains a challenge because it requires a trade-off between the benefits of the use of chemicals and the environmental, human health and social costs to society associated with their use. This dichotomy is best exemplified by DDT. DDT is notorious for its biomagnification through food chains and adverse reproductive effects in wildlife; however, DDT is one of only a few cheap and effective tools for controlling the mosquitoes that carry malaria parasites. Malaria kills over one million people, mainly children, in tropical areas each year (Attaran et al., 2000).

To achieve the benefit of chemical use and protect human and environmental health from potential adverse impacts, various national, regional and international agencies have developed regulatory approaches to assess and control hazardous chemicals. Major programs include the Stockholm Convention on Persistent Organic Pollutants at global scale, the European Union's Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) at regional scale, and the Canadian Environmental Protection Act (CEPA), the United States Toxic Substances Control Act (TSCA), and the Japanese Chemical Substances Control Law (CSCL) at national scale. These programs usually involve the identification of hazardous properties (e.g., persistence, bioaccumulation, toxicity, and potential for long-range transport), the assessment of risks to human health and the environment, and the implementation of management actions. The effectiveness and efficiency of regulatory assessment and control of hazardous chemicals rely on sound science and social involvement (Eden, 1996; Power & McCarty, 2006).

The objectives of this chapter were (i) to review the regulatory framework and process of the assessment and control of hazardous substances under the Canadian Environmental Protection Act, 1999 (CEPA 1999), and (ii) to provide recommendations for improving regulatory management of hazardous substances under CEPA 1999 based on the results of this and other related studies. The recommendations provided in the present study may also be applicable to other regulatory programs concerning the assessment and control of hazardous substances.

5.2. Overview of Assessment and Control of Hazardous Substances under the Canadian Environmental Protection Act, 1999

The Canadian Environmental Protection Act and Toxic Substances Management Policy are the primary elements of Canada's federal legislative framework for the assessment and control of hazardous substances to protect the environment and human health based on principles of sustainable development. The Canadian Environmental Protection Act was proclaimed in 1988 (CEPA 1988) and amended in 1999 (CEPA 1999) (Government of Canada, 1999). Among its many provisions, CEPA 1999 requires that the Minister of the Environment and the Minister of Health (the Ministers) take actions with respect to the control of toxic substances (Part 5 of CEPA 1999).

Under CEPA 1999, the Ministers are required to categorize and screen the substances listed on the Domestic Substances List (DSL), which are substances in commerce in Canada between 1984 and 1986, to determine whether they are toxic or capable of becoming toxic under the Act. A substance is considered toxic "if it is entering or may enter the environment in a quantity or concentration or under conditions that (i) have or may have an immediate or long-term harmful effect on the environment or its biological diversity; (ii) constitute or may constitute a danger to the environment on which life depends; or (iii) constitute or may constitute a danger in Canada to human life or health" (Section 64 of CEPA 1999). These substances are often referred to as "CEPA-toxic" substances. There are approximately 23,000 substances on the DSL, including organic chemicals (50%), unknown or variable composition, complex reaction products, or biological materials (UVCBs) (20%), polymers (18%), inorganic substances (10%), and

other types of substances (2%) (Environment Canada, 2003). These substances are also referred to as “existing substances”. Substances that are not on the DSL are considered to be “new substances” and are subject to the New Substances Program.

The process for assessing and controlling existing substances under CEPA 1999 involves three major phases: categorization, risk assessment, and risk management (Figure 5.1). The purpose of categorization is to identify substances on the DSL that have the greatest potential for exposure of the general population in Canada (GPE), or that are persistent (P) or bioaccumulative (B) and inherently toxic (iT) to human beings or non-human organisms (Section 73 of CEPA 1999). Substances that meet the categorization criteria are subject to risk assessments, starting with screening assessments followed by in-depth assessments if further assessments are required. The primary objective of screening and in-depth assessments is to determine whether a substance is “CEPA-toxic” or capable of becoming “CEPA-toxic”. If a substance is assessed to be “CEPA-toxic”, the Ministers can recommend to add it to the List of Toxic Substances in Schedule 1 of the Act. Substances that are assessed to be “CEPA-toxic” are subject to risk management. Risk management measures are preventive or control actions to reduce or eliminate the release of toxic chemicals into the environment. Substances that meet the criteria for “CEPA-toxic” and are also persistent, bioaccumulative, and produced primarily as a result of human activity are considered to be most dangerous and thereby subject to virtual elimination. The regulatory approaches to categorization, risk assessment, and risk management of existing chemicals under CEPA 1999 are described as follows.

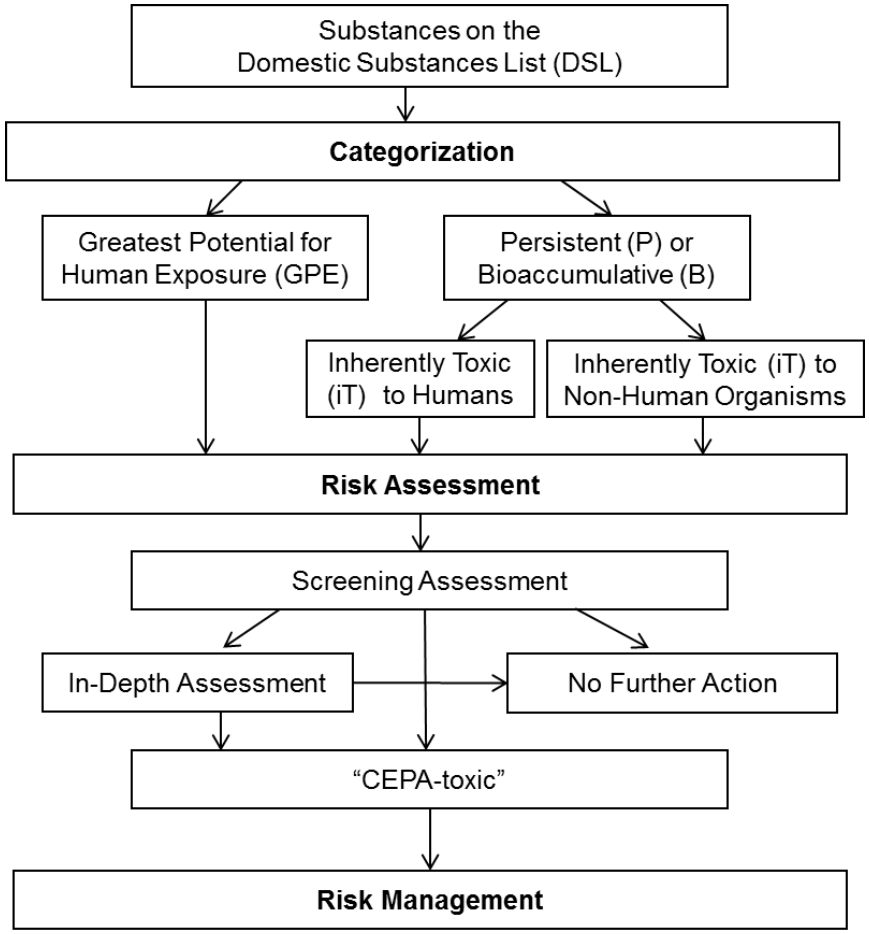

Figure 5.1. Process for assessing and controlling existing substances under the Canadian Environmental Protection Act, 1999

5.2.1. Categorization

Environmental Canada and Health Canada are responsible for conducting ecological categorization and human health categorization, respectively. The purpose of ecological categorization is to identify substances on the DSL that are persistent (P) or bioaccumulative (B) and inherently toxic (iT) to non-human organisms; and the purpose of human health categorization is to identify substances on the DSL that present the greatest potential for human exposure (GPE) and that are inherently toxic (iT) to humans.

The ecological categorization of P, B, and iT to non-human organisms is based on numerical criteria listed in Table 5.1. The cut-off values for assessing P and B are set out in the Persistence and Bioaccumulation Regulations (Government of Canada, 2000). The

cut-off values for assessing iT to non-human organisms are provided in the guidance manual for the categorization of substances on the DSL (Environment Canada, 2003), and these values are in accordance with those used in various E.U. and U.S. Environmental Protection Agency initiatives (Environment Canada, 2003).

Table 5.1. Categorization criteria for persistence, bioaccumulation, and inherent toxicity to non-human organisms under CEPA 1999

Persistence (P)		Bioaccumulation (B)	Inherent Toxicity (iT) to Non-Human Organisms
Half-life in		BAF [a] ≥ 5000	LC50 [d] (EC50 [e]) ≤ 1 mg/L
Air	≥ 2 days	BCF [b] ≥ 5000	NOEC [f] ≤ 0.1 mg/L
Water	≥ 182 days	log K_{OW} [c] ≥ 5	
Sediment	≥ 365 days		
Soil	≥ 182 days		

[a] BAF = bioaccumulation factor;
[b] BCF = bioconcentration factor;
[c] log K_{OW} = the logarithm of octanol−water partition coefficient;
[d] LC50 = median lethal concentration;
[e] EC50 = median effective concentration;
[f] NOEC = no-observed-effect concentration.

Persistence (P) describes the length of time a substance resides in the environment. The P criteria are defined based on the half-lives of chemicals in individual environmental medium of air, water, sediment, or soil (Table 5.1). According to the Persistence and Bioaccumulation Regulations (Government of Canada, 2000), half-life means the period it takes the concentration of a substance to be reduced by half, by transformation, in a medium. Only degradation through chemical, biochemical, and photochemical processes is considered (Robinson et al., 2004).

Bioaccumulation (B) describes the increase in chemical concentrations in organisms relative to those in the diet or surrounding medium. The B criteria are defined based on the bioaccumulation factor (BAF), bioconcentration factor (BCF), or the logarithm of octanol–water partition coefficient (log K_{OW}) (Table 5.1). According to the Persistence and Bioaccumulation Regulations (Government of Canada, 2000), BAF means the ratio of the concentration of a substance in an organism and the concentration in water, based on uptake from the surrounding medium and food; BCF means the ratio of the concentration of a substance in an organism and the concentration in water, based

only on uptake from the surrounding medium; and K_{OW} means the ratio of the concentration of a substance in an octanol phase and the concentration of the substance in the water phase of an octanol–water mixture. The Persistence and Bioaccumulation Regulations (Government of Canada, 2000) state a preference for BAF over BCF; in the absence of BAF or BCF data, log K_{OW} data can be used.

Inherent toxicity (iT) refers to the hazard a substance presents to the environment or human health, which can be represented by the toxic effect caused by the substance, i.e., the toxicity found in a study or predicted due solely to the test substance, or the effect that has not been masked or mitigated by some factor or parameter, as defined by Environment Canada (2003). The numerical criteria for iT to non-human organisms are based on aquatic toxicity data using acute endpoint of LC50 (median lethal concentration) or EC50 (median effective concentration), or using chronic endpoint of NOEC (no-observed-effect concentration) (Table 5.1). In aquatic toxicity testing, LC50 refers to the concentration of a substance in water causing death of 50% of the experimental organisms in the water; and EC50 refers to the concentration of a substance in water inducing toxic effects on 50% of the experimental organisms. NOEC refers to the highest concentration of a substance at which there is no adverse effect observed in a toxicological study. Environment Canada has developed guidance documents for ecological categorization of organic and inorganic substances (Environment Canada, 2003), UVCBs (unknown or variable composition, complex reaction products, or biological materials) (Environment Canada, 2005a), and polymers (Environment Canada, 2005b) on the DSL.

The human health categorization of health-related components (i.e., GPE and iT to humans) is based on an integrated framework developed by Health Canada (2009) for priority setting (i.e., categorization) and risk assessments. The integrated framework is a tools-based approach based on iterative application of increasingly discriminating (i.e., simple and complex) tools for consideration of exposure and hazard (Health Canada, 2009). Health Canada has developed two simple tools, the simple exposure tool (SimET) and simple hazard tool (SimHaz), and two complex tools, the complex exposure tool (ComET) and complex hazard tool (ComHaz). The categorization of health-related components is based on the application of simple tools to all substances on the DSL followed by the partial application of the complex tools to prioritized subsets of substances,

resulting in a draft “maximal list” of substances considered as health priorities for screening assessments (Health Canada, 2009).

The simple exposure tool (SimET) is developed for providing relative ranking of exposure potential for all substances on the DSL based on three lines of evidence: quantity in commerce in Canada, number of companies involved in commercial activities in Canada, and weighting by experts of the potential for human exposure based on consideration of various use codes (Meek & Armstrong, 2007). The SimET categorizes chemicals into three groups: greatest potential for exposure (GPE), intermediate potential for exposure (IPE), and lowest potential for exposure (LPE), based on criteria outlined in the integrated framework document (Health Canada, 2009). The complex exposure tool (ComET) provides more refined, quantitative estimates of exposure than the SimET. The ComET considers the physical/chemical properties and production quantity of the substances, as well as environmental (far-field) and consumer (near-field) exposure with age classes and daily intakes of the general population, resulting in route-, duration- and age group-specific estimates of total exposure (Health Canada, 2009).

The simple hazard tool (SimHaz) is developed for rapid classification of all substances on the DSL into two groups, high-hazard and low-hazard substances, based on information about chemical classification developed by national or international agencies which have reviewed large numbers of substances. The toxicity endpoints considered for identifying high-hazard substances are carcinogenicity, genotoxicity, developmental toxicity, and reproductive toxicity; the endpoints and criteria for identifying high- and low-hazard substances are outlined by Hughes et al. (2009). The ComHaz is a more complex hierarchical tool than SimHaz. The ComHaz considers a range of toxicological endpoints in a step-wise manner and includes a number of endpoint-specific criteria developed for this tool as outlined by Hughes et al. (2009).

The draft “maximal” list of health priorities was released in 2004, containing 1896 substances for further assessments (Health Canada, 2009). The categorization of all existing substances on the DSL was completed in September 2006. Approximately 4,300 substances were identified for further attention (Government of Canada, 2014a), and

approximately 3,250 substances were identified to meet the criteria as either P or B and iT to aquatic organisms (i.e., PiT or BiT) (van Wijk et al., 2009).

5.2.2. Risk Assessment and Management

Substances on the DSL that meet the categorization criteria are subject to risk assessment followed by risk management (if needed) under CEPA 1999 (Figure 5.1). Risk assessment and risk management are included in the Chemicals Management Plan (CMP) launched by the Government of Canada in 2006. The goal of the CMP is to address all 4,300 substances identified in categorization by 2020 (Government of Canada, 2014a). The CMP is a comprehensive initiative aimed at reducing the risks posted by chemical substances to Canadians and their environment. It includes measures to assess and manage the risks posted by chemical substances and integrates government activities involving different laws governing chemicals, including CEPA 1999, the Canada Consumer Product Safety Act, the Food and Drugs Act, the Pest Control Products Act, and others (Government of Canada, 2014a). Under the CMP, risks are addressed following a management cycle (Figure 5.2): (i) CMP risk assessors conduct scientific evaluations to assess potential environmental and health risks posed by chemicals to determine whether management actions are required; (ii) the government develops measures to prevent or manage risks; (iii) the government ensures the compliance of risk management obligations; and (iv) the government invests in research and monitoring to provide essential information about chemical exposures and their effects on human health and the environment (Government of Canada, 2014a).

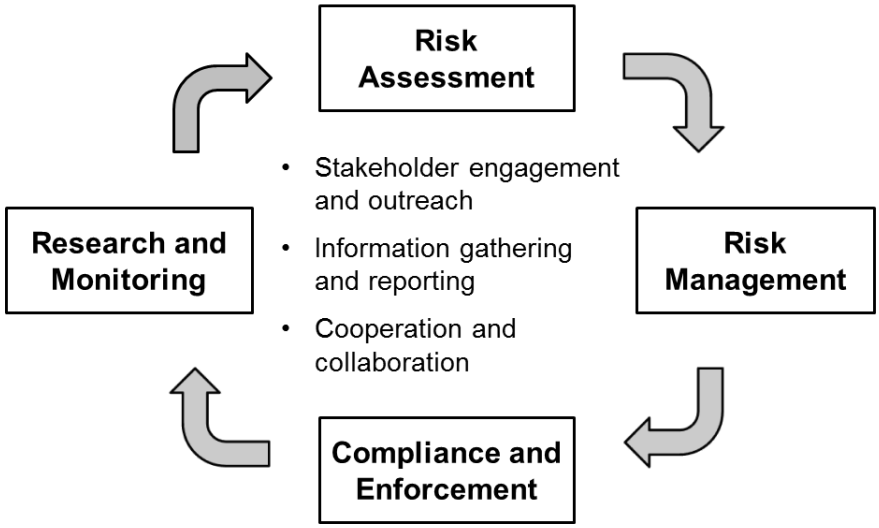

Figure 5.2. The chemicals management cycle under the Chemicals Management Plan in Canada

Note. Adapted from Government of Canada (2014a).

Under CEPA 1999, the objective of risk assessment is to determine whether a substance is "CEPA-toxic", which is then subject to risk management measures. Risk assessments are based on sound science and consider multiple lines of evidence and uncertainties. Environment Canada is responsible for conducting ecological (environmental) risk assessments. The major steps of ecological risk assessment include problem formulation, exposure assessment, effect assessment, and risk characterization; tools and approaches used for conducting ecological screening assessments are developed and described in a guidance manual (Environment Canada, 2006). A weight of evidence approach provides a basis for risk assessment as required under Section 76.1 of CEPA 1999; a guidance document for the use of weight of evidence has been established by Environment Canada (2005c). In addition, a technical approach for "rapid screening" of substances of lower ecological concern has been developed by Environment Canada (2007) for rapid and efficient screening assessment of existing substances that are anticipated to be of low ecological concern, in order to focus resources on the assessment of substances that have a higher probability of causing harm. Health Canada

is responsible for conducting human health risk assessments, which involve hazard identification, hazard characterization, exposure assessment and risk characterization (Health Canada, 2000). The complex tools (ComHaz and ComET) described above take into account exposure and hazard for specific subpopulations or age groups considering a range of toxicological endpoints used in human health risk assessments (Health Canada, 2009). In addition, a "margin of exposure" approach that compares critical effect levels with estimates of exposure taking into account the confidence/uncertainties in the available data provides a basis for decision making in human health risk assessments (Meek & Armstrong, 2007; Health Canada, 2004). The results of risk assessments are stated in screening assessment reports and state of the science reports. A summary of the scientific considerations and proposed measures from the risk assessments must be published in the Canada Gazette for a 60-day public comment period; following the public comment period, the final decision of a risk assessment must be published in the Canada Gazette (Sections 74–76 of CEPA 1999).

The objective of risk management is to develop and implement risk management measures to prevent or manage the risks. The major components of risk management include: (i) the establishment of risk management objectives; (ii) the selection of risk management instruments; (iii) compliance with risk management instruments; and (iv) stakeholder engagement. A variety of risk management instruments can be used, including regulatory measures (e.g., environmental emergency regulations), economic instruments (e.g., financial incentives and subsidies) and voluntary approaches (e.g., performance agreements) (Government of Canada, 2016a). Consulting with interested stakeholders is an important element for the development of risk management instruments. Stakeholders can provide feedback and information on risk management documents. In addition, the CMP Stakeholder Advisory Council, a multi-stakeholder group that contributes to the implementation of the CMP, can offer advice and input to foster dialogue on issues pertaining to the CMP between stakeholders and government and among different stakeholder groups; issues may include chemical policy, risk assessment, risk management, and risk communications (Government of Canada 2014b).

With the commitment to address the approximately 4,300 substances identified as high priorities for assessment by 2020 under the CMP, approximately 2,740 substances

have been assessed and 363 substances have been concluded to be “CEPA-toxic” as of December 2015 (Government of Canada, 2015). For these “CEPA-toxic” substances, 76 final risk management instruments covering 325 substances have been developed, and additional risk management instruments are being developed (Government of Canada, 2015).

5.3. Recommendations for Improving Chemicals Assessment and Management

5.3.1. Recommendations Based on the Results of This Study

Scientific evaluation of bioaccumulative potential for the large number of commercial and industrial chemicals plays an important role in categorization and risk assessment in current regulatory management of hazardous substances under CEPA 1999. Improving bioaccumulation assessment can improve hazard identification and reduce uncertainty in risk assessment (Arnot & Gobas, 2006). Currently, regulatory bioaccumulation criteria rely on the bioaccumulation factor (BAF), the bioconcentration factor (BCF), and the octanol–water partition coefficient (K_{OW}) (Table 5.1). These criteria are based on scientific principles and evidence but they are subject to limitations, as reviewed by Gobas et al. (2009) and discussed in Chapter 1. First, the empirical BAF and BCF data are not available for the great majority of commercial chemicals; thus, the bioaccumulation assessment often relies on the K_{OW} criterion or on bioaccumulation models. This may lead to “false-positive” results (i.e., chemicals are considered to be bioaccumulative while in reality they are not) as biotransformation is not accounted for in the K_{OW} criterion and generally not considered in bioaccumulation models due to the lack of information on biotransformation rates. The neglect of biotransformation in bioaccumulation assessment is of particular concern for highly hydrophobic chemicals (log K_{OW} > 5), because even slow rates of biotransformation can dominate the overall depuration rate of these chemicals and determine whether the substance will bioaccumulate. Second, the current bioaccumulation criteria apply to aquatic organisms that depend on exchange with water for their respiration (e.g., fish), but they may not be sufficient to assess bioaccumulation potential for air-breathing animals (e.g., humans and terrestrial organisms) whose respiring medium is air rather than water. This may lead to

"false-negative" results (i.e., chemicals are not considered to be bioaccumulative while in reality they are) if the assessment is based on the K_{OW} criterion (log $K_{OW} \geq 5$), as it has been demonstrated that substances with the log K_{OW} value between 2 and 5 and the log K_{OA} (the logarithm of octanol–air partition coefficient) value greater than 5 can biomagnify in air-breathing organisms (Armitage & Gobas, 2007; Kelly et al., 2007). Third, for highly hydrophobic chemicals with log K_{OW} greater than 6, conventional bioaccumulation tests may underestimate BCFs or BAFs; these chemicals tend to have low water solubility and strong binding affinity to sediment and dissolved organic carbon, resulting in decrease in freely dissolved fractions that are bioavailable to fish and the possibility of oversaturation of the test solutions (Ehrlich et al., 2011). In addition, in vivo bioaccumulation tests are time-consuming and require substantial resources (for labour and animal costs); it may not be practical or ethically acceptable to conduct in vivo bioaccumulation tests on the large number of commercial chemicals for bioaccumulation screening.

To overcome these problems and improve current bioaccumulation assessment, it is important to develop scientifically sound and cost-effective methods (e.g., in vitro and in silico approaches) that incorporate biotransformation in bioaccumulation assessment, especially for highly hydrophobic organic chemicals with bioaccumulation potential (log $K_{OW} > 5$), in both aquatic and non-aquatic organisms. As the current bioaccumulation assessment relies on fish as the only animal model, it is important to develop methods to include another animal model (e.g., mammals) in regulatory bioaccumulation assessment to better protect terrestrial organisms including humans. In addition, it is important to develop methods that overcome the technical difficulties with dissolution and binding properties of highly hydrophobic organic chemicals to improve in vitro and in vivo experimental tests for bioaccumulation assessment.

In this thesis research, the thin-film sorbent-phase dosing assays for measuring in vitro biotransformation rate constants for hydrophobic chemicals (presented in Chapters 2 and 3) and in vitro-to-in vivo extrapolation approach for bioaccumulative substances (IVIVE-B model) combined with a rat bioaccumulation model for estimating in vivo biotransformation rate constants and biomagnification factors (BMFs) (presented in Chapter 4) can be used to assess the bioaccumulative potential for hydrophobic organic chemicals that undergo biotransformation in rats. The established in vitro sorbent-phase

dosing method and the IVIVE-B bioaccumulation modeling approach can greatly reduce time and animal use compared to in vivo bioaccumulation testing. The thin-film sorbent-phase dosing method has shown to be useful for measuring the in vitro biotransformation rates for chemicals with high hydrophobicity and limited volatility. Compared to the conventional solvent-delivery dosing method, the sorbent-phase dosing method is solvent-free and has a potential to overcome the incomplete dissociation of hydrophobic chemicals in the largely aqueous incubation solution and maintain first order kinetics of biotransformation by the low substrate concentrations in the incubation medium. The IVIVE-B model has demonstrated to be useful for mammalian bioaccumulation assessment for hydrophobic chemicals (log $K_{OW} \geq 4$). Compared to the traditional in vitro-to-in vivo extrapolation approach for pharmaceuticals (IVIVE-Ph), the IVIVE-B model is straightforward without the need to convert estimates between clearance and rate constants and requires less biological and physiochemical parameters that are not available or hard to obtain for many mammalian species. Nevertheless, the conventional solvent-delivery dosing and IVIVE-Ph approaches are useful for less hydrophobic chemicals (e.g., log $K_{OW} < 4$).

Based on the results of this study, a methodological framework for assessing the bioaccumulative potential for non-ionic organic chemicals in mammals is proposed to improve current bioaccumulation assessment by accounting for biotransformation using cost-effective approaches (Figure 5.3). In the proposed framework (Figure 5.3), non-ionic organic chemicals are first divided into three groups based on their hydrophobicity, that is, chemicals with low (log $K_{OW} \leq 2$), low to moderate ($2 < \log K_{OW} < 4$), and moderate to high (log $K_{OW} \geq 4$) hydrophobicity. Based on scientific findings that chemicals with a log K_{OW} greater than 2 and a log K_{OA} greater than 5 have the potential to biomagnify in terrestrial food webs (Armitage & Gobas, 2007; Kelly et al., 2007), chemicals in the low hydrophobicity group do not appear to be of concern for bioaccumulation potential in mammals based on current knowledge. Chemicals in the low to moderate and moderate to high hydrophobicity groups are then considered for their lipid-air partitioning property (log K_{OA}). Chemicals with a low lipid-air partition tendency (log $K_{OA} < 5$) are of limited concern for bioaccumulation potential in mammals because of their high rate of pulmonary elimination. Chemicals with a high lipid-air partition tendency (log $K_{OA} \geq 5$) are then considered for their metabolic capability in the organism. It is suggested that metabolized

chemicals with a moderate to high hydrophobicity (log K_{OW} ≥ 4) can be assessed for mammalian bioaccumulation potential using the developed sorbent-phase dosing and IVIVE-B approaches in combination with a mammalian bioaccumulation model; metabolized chemicals with a low to moderate hydrophobicity (2 < log K_{OW} < 4) can be assessed for mammalian bioaccumulation potential using the conventional solvent-delivery dosing and IVIVE-Ph approaches in combination with a mammalian bioaccumulation model; and non-metabolized chemicals can be assessed for mammalian bioaccumulation potential using a mammalian bioaccumulation model with input biotransformation rate constant set to zero. The proposed methodological framework using in vitro and modeling approaches can increase the throughput of screening the bioaccumulative potential for the large number of non-ionic organic chemicals in mammals.

The proposed framework may also be applicable to aquatic species to improve current bioaccumulation assessment by accounting for biotransformation. However, the results obtained from the sorbent-phase dosing experiments using trout liver S9 fractions (Chapter 3) indicated that the low mass-transfer rate of hydrophobic chemicals from the sorbent phase to the incubation medium due to low incubation temperature required for trout may limit the application of the sorbent-phase dosing method to measure the in vitro biotransformation rates for hydrophobic chemicals in fish. Future investigations are needed to improve the sorbent-phase dosing system at low temperature conditions and to test and evaluate the application of the IVIVE-B approach to aquatic species.

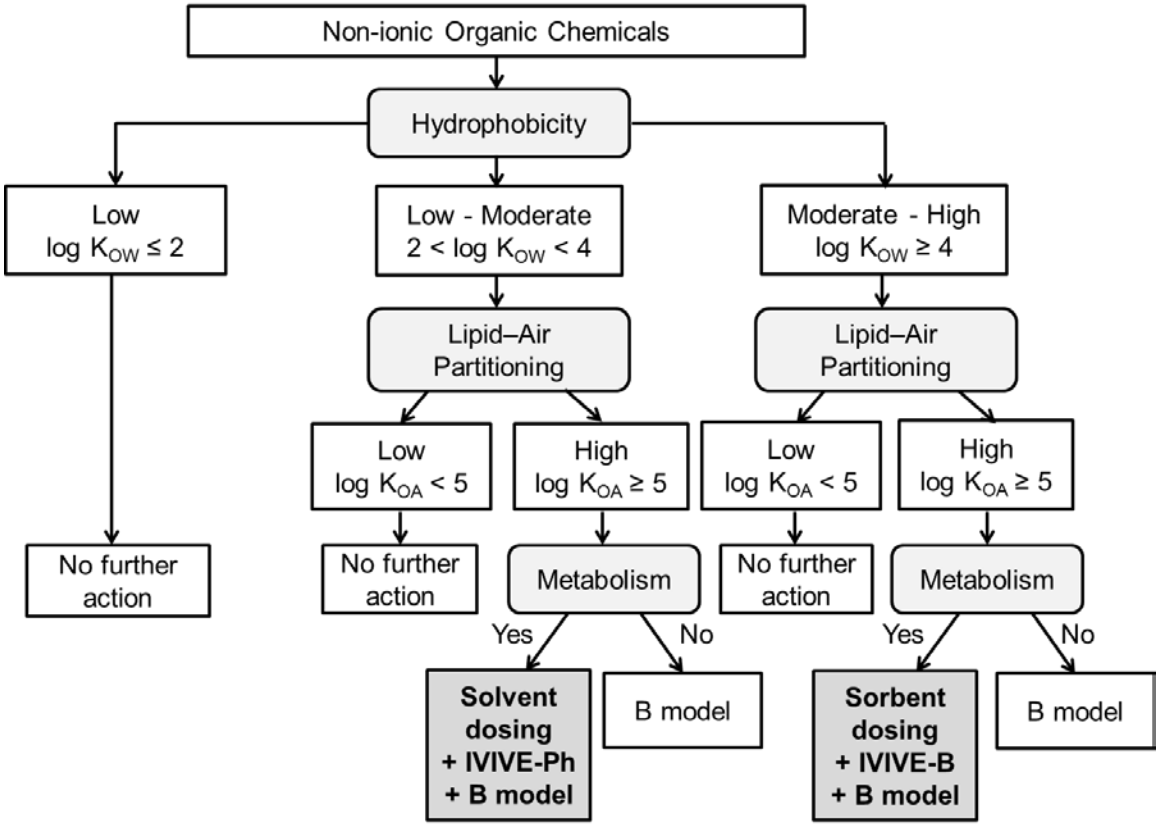

Figure 5.3. Proposed methodological framework for mammalian bioaccumulation assessment using in vitro and modeling approaches

Note IVIVE-B = in vitro-to-in vivo extrapolation approach for bioaccumulative chemicals
IVIVE-Ph = in vitro-to-in vivo extrapolation approach for pharmaceuticals
B model = bioaccumulation model

5.3.2. Additional Recommendations

In addition to bioaccumulation assessment, scientific evaluations of persistence and inherent toxicity of chemicals also play an important role in categorization and risk assessments under CEPA 1999. The criteria for persistence (P) and inherent toxicity (iT) to non-human organisms are summarized in Table 5.1. Notably, there are no specific criteria for identifying substances that are iT to humans; iT to humans is considered integratively with the greatest potential for human exposure (GPE) as health-related components in human health categorization using various exposure and effect tools (e.g., SimET, SimHaz, ComET and ComHaz) developed by Health Canada (2009). Although

the criteria for P and iT to non-human organisms have clear cut-off values based on scientific findings, they are subject to limitations and may need improvement to be more effective in terms of achieving the goals of the legislation.

The P criteria are developed in terms of single-media degradation half-lives (Table 5.1); however, using single-media criteria to assess persistence may be problematic. First, the use of a half-life implies that the degradation reactions follow first-order kinetics, but this is not always the case (Mackay et al., 2003). Second, the degradation half-life of a chemical in a medium may vary depending on environmental conditions (e.g., temperature, pH, the presence of oxidizing or reducing species, and the nature of the microbial community) (Mackay et al., 2003; Boethling et al., 2009). Third, the single-media criteria do not consider mode of entry and the effects of partitioning to other media, thus they may "penalize" the chemical that has only a small fraction present in a specific medium in which it has a long half-life (Webster et al., 1998; Mackay et al., 2003). For example, if a chemical is assessed as persistent because it exceeds the half-life criterion in only one phase, it may become controversial if this chemical does not have a "realistic presence" in that phase due to little exposure or low partition tendency to that phase.

To overcome these problems, Webster et al. (1998) suggested that it is essential to have a set of standard environmental conditions to allow degradation half-lives viewed as chemical properties independent of variation in environmental conditions. In addition, it has been suggested by a number of studies that an "overall persistence" or "overall residence time" obtained from a multimedia mass-balance model under steady-state conditions is regarded as the preferred metric for evaluating environmental persistence (Webster et al., 1998; Mackay et al., 2003; MacLeod & McKone, 2004; Scheringer et al., 2009; Mackay et al., 2014a). The concept of overall persistence treats the environment as a single, unified set of connected media in which irreversible degradation is the only loss considered. An overall residence time expresses the average time that a chemical is likely to reside in a multimedia environment before it degrades, and it can be calculated as the overall mass in the system divided by total loss rate (or total input rate) regardless of degradation kinetics (i.e., first order or not) (Webster et al., 1998; Mackay et al., 2003; Mackay et al., 2014a). The multimedia mass-balance model normally has 4 compartments (i.e., air, water, soil, and sediment) with predefined compartmental

dimensions, and requires input parameters such as physical-chemical property data (e.g., solubility in water, vapour pressure, the octanol–water partition coefficient (K_{OW}) and octanol–air partition coefficient (K_{OA})), degradation half-life in each medium and the fraction of emission to each medium (Boethling et al., 2009). To improve the evaluation of persistence, chemicals may be assessed with respect to their overall persistence or overall residence time in an evaluative multimedia environment.

The criteria for iT to non-human organisms rely on acute (LC50 or EC50) and chronic (NOEC) endpoints under CEPA 1999 (Table 5.1). Major limitations of the iT criteria include: (i) the iT criteria are based solely on aquatic toxicity; (ii) most of the iT evaluations are based on acute aquatic toxicity data due to data availability (Robinson et al., 2004); and (iii) the criteria are based on "external" water concentrations in the aquatic environment. To improve iT assessments, it is important to develop iT criteria for non-aquatic organisms (e.g., mammals and other terrestrial organisms) and better investigate chronic toxicity. It has been suggested that adverse effects associated with long-term exposures are more relevant to assessing the potential toxicity of persistent, bioaccumulative, and toxic chemicals (PBTs) and persistent organic pollutants (POPs) (van Wijk et al., 2009). In addition, a number of studies have suggested that the "internal" effect concentrations at the active site in the organism where the toxic effect takes place should be considered instead of the "external" concentrations in the exposure media (McCarty & Mackay, 1993; Mackay et al., 2001; Gobas et al., 2001; Mackay et al., 2014b). The internal measure of toxicity (internal dose) is called the critical body burden (CBB) or critical body residue (CBR) when referring to the whole body or called the critical tissue burden (CTB) when referring to a specific tissue or organ (McCarty & Mackay, 1993; Mackay et al., 2001). The internal dose metrics (e.g., CBB, CBR and CTB) that employ delivered dose in the organism are more appropriate to compare toxicity between chemicals than external dose metrics (e.g., LC50 and EC50); the external dose metrics are strongly influenced by modifying factors, such as uptake kinetics, metabolism, test duration, size, and lipid content of the test organism (Mackay et al., 2014b). The use of a criterion based on an internal concentration (≤ 1 mmol/kg) has been suggested (Gobas et al., 2001).

Furthermore, it has been indicated that scientific and social inputs are both important in environmental decision-making (Power & McCarty, 2006). Stakeholder participation in environmental decision-making is of increasing concern and has been embedded into national and international policy (Reed, 2008). For example, stakeholder participation in environmental decision-making has been increased at all levels of government in the U.S. (Beierle, 2002). Enhancing stakeholder involvement may improve government decision-making by achieving consensus, improving acceptance of and compliance with decisions, increasing public support for agencies and their programs, and educating the public on the sources of environmental risk and the trade-offs of policy options (Fiorino, 1990).

A stakeholder can be defined as an individual or a representative of a group affected by or affecting the issues in question (Glicken, 2000). Concerning the control of toxic substances under CEPA 1999, stakeholders may include government agencies, academic and technical experts, industries, environmental non-governmental organizations, and the public. Under CEPA 1999, the Minister of the Environment and the Minister of Health (the Ministers) are the ultimate decision makers for the categorization and assessment of hazardous substances; stakeholders are informed of final decisions and can participate in the decision-making process through data submission, consultation, and public comments. For example, interested parties were encouraged to submit any information that may assist Environment Canada in categorization (Environment Canada, 2003). Scientific and technical experts from government, academia, industries, environmental organizations and consultant groups have been invited to participate in a special advisory committee (e.g., the Technical Advisory Group formed in 1998) and attend specific meetings (e.g., peer review meetings) and workshops. During the implementation of the Chemicals Management Plan (CMP), external bodies can participate in the process through the CMP Science Committee, Stakeholder Advisory Council, and Challenge Advisory Panel (Government of Canada, 2016a). CEPA 1999 requires a public comment period within 60 days after publication of proposals and decisions.

In addition, an appeal mechanism exists within the regulatory framework of CEPA 1999. Notice of objection (Sections 77(8) and 332(2) of CEPA 1999) provides a

mechanism for stakeholders to challenge a decision. The notice of objection should be filed within 60 days after publication of the decision in the Canada Gazette, the Minister of Environment Canada then determines if further discussion or a Board of Review (Section 333 of CEPA 1999) are warranted in response to the notice of objection. If a Board of Review is formed, it is required that a conclusion be made as soon as possible and that a report be submitted to the Minister who established the board (Section 340(1) of CEPA 1999). For example, the first Board of Review under CEPA 1999 was established to determine the nature and extent of the danger posted by decamethylcyclopentasiloxane (Siloxane D5). Based on the conclusion of the screening assessment of Siloxane D5 conducted by Environment Canada and Health Canada, it was recommended that Siloxane D5 be added to the Toxic Substances List in Schedule 1 of CEPA 1999. The affected industry stakeholders filed a notice of objection in 2009 claiming that the screening assessment of Siloxane D5 was not consistent with the best available science and requested to establish a Board of Review. The Siloxane D5 Board of Review was established by the Minister of the Environment in 2010, and a report was submitted to the Minister in 2011 concluding that Siloxane D5 does not pose a danger to the environment based on new scientific information (Siloxane D5 Board of Review, 2011; Giesy et al., 2016).

Although stakeholders can file a notice of objection and request for Board of Review to challenge a decision under CEPA 1999, whether a board will be established depends on the decision made by the Minister of the Environment, who will judge on whether there are sufficient new scientific data or information required for establishing the board. To date, there have been several requests for Board of Review concerning various chemicals and issues, but only one request has been accepted so far and a Board of Review on Siloxane D5 was established and completed (Government of Canada, 2016b; Siloxane D5 Board of Review, 2011; Giesy et al., 2016). It is important to note that the decision-making structure under CEPA 1999 is not based on collaborative or shared decision-making (i.e., decisions are made by a consensus of affected parties) (Selin & Chavez, 1995). Under CEPA 1999, the Ministers are the ultimate decision makers and the stakeholders are informed of final decisions. Therefore, the structure of ultimate decision-making cannot be changed under the Act; however, the decision-making process can be improved by using appropriate strategies and approaches for enhancing

stakeholder participation at various stages (i.e., categorization, risk assessment and risk management) of the control of hazardous substances.

The categorization and risk assessment of hazardous chemicals rely significantly upon scientific evidence and judgement. The non-expert stakeholders may not involve actively during the categorization and risk assessment processes. It has been suggested that early involvement of the public in the decision-making process may compromise the objective of efficiency, and stakeholders tend to make inadequate and selective use of scientific information and analysis (Beierle, 2002; Renn, 2006). However, the "expert stakeholders" may play an important role in assisting decision-making of categorization and risk assessment. Under CEPA 1999, expert peer reviews have been conducted for human health risk assessments (Health Canada, 2009). The expert review is a formal, external, and independent review of an intended final work product of risk assessment (Patterson et al., 2007). Patterson et al. (2007) have suggested that the practice of peer review can be extended to other type of peer involvement activities (i.e., peer input and peer consultation). They defined the term "peer" as experts who collectively are of equal standing (i.e., those who have at least the same level of training and experience) as the authors of the risk assessment report, and suggested that peer input can be conducted at an early stage emphasising on appropriate focus, data acquisition, and identification of issues, followed by peer consultation to gather independent expert peer opinion and advice on a work product during its development (Patterson et al., 2007). They concluded that the additional peer input and peer consultation activities that can be performed formally or informally can improve efficiency and quality of risk assessment and further enhance the credibility and public confidence of the results (Patterson et al., 2007).

Compared to the science-based process of categorization and risk assessment, stakeholders (including experts and non-experts) may have more influence on the implementation of risk management. It has been suggested that local and scientific knowledges can be integrated to provide a more comprehensive understanding of complex issues in environmental management (Reed, 2008). One prominent example is given by the U.S. National Academy of Science, which recommends the adoption of an "analytical-deliberation process" by which technical expertise and public value input are integrated (Stern & Fineberg, 1996). A model called "cooperative discourse" has been

developed for conducting such an analytical-deliberation process (Renn, 1999). The process of deliberation proposed in this model involves a panel of "randomly selected citizens" as jurors and representation of interest groups as witnesses; the witnesses provide arguments and evidence to the panels who ultimately make a decision. In the random sampling process, all potentially affected people have an equal chance to be drawn into the sample, and normally only a fraction of those (ranging from 5 to 40%) selected by random sampling agree to participate. The idea of randomly selected citizens rather than actively involved stakeholders can provide an opportunity for those who are normally under represented (e.g., unemployed workers, retired persons, and women with small children) to participate in the collective decision-making process. This model may avoid polarization of options and facilitate a mutual learning process. Under CEPA 1999, a strategy leader and one or more risk managers are responsible for developing a risk management strategy and tools (Government of Canada, 2013). Although the "cooperative discourse model" mentioned above may not be readily applicable to the risk management processes in Canada, it is important to develop strategies to encourage all affected people to participate actively in the process of risk management.

The issue of effective communication between experts and non-experts in risk management is of concern and has been widely addressed in the literature (Pavlou et al., 1998; Yosie & Herbst, 1998; Glicken, 2000; Bier, 2001; Renn, 2001; Power & McCarty, 2006; Beck et al., 2016). The information about risks involves scientific and technical evaluations and assumptions. It is required to translate such risk-based information into understandable language and concepts to facilitate the dialogue between technical experts and non-expects. A number of approaches have been suggested to improve the presentation and communication of risks and uncertainty. For example, visual presentation of risk information is more readily understandable than the same information presented numerically; quantitative scoring can be used to illustrate both the direction and magnitude of confidence (e.g., a quantitative ranking system of low, medium, or high confidence) to enhance understanding of uncertainty; and presenting information in clear and concise manner to avoid the problem with long documents that tend to lose transparency and clarity (Beck et al., 2016). These approaches may be useful for strategy leaders and risk managers to communicate risks and uncertainty with non-technical stakeholders during the implementation of risk management under CEPA 1999.

Furthermore, the legally required Parliamentary review provides a good opportunity to improve the control and management of hazardous substances under CEPA 1999. As required under the Act (Section 343 of CEPA 1999), a committee of one or both Houses of Parliament must review the Act every five years. The first Parliamentary review of CEPA 1999 was completed in 2007 by the designated committees in the House of Commons and the Senate (Environment Canada, 2016). The second Parliamentary review of CEPA 1999 is being undertaking currently by the Standing Committee on Environment and Sustainable Development designated by the House of Commons, and the Committee is expected to complete the review in 2017 (Environment Canada, 2016).

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Chapter 6.

Conclusions

The effective management of industrial and commercial chemicals in the environment requires good public policy based on sound science. Several national and international regulatory programs have been developed to address the control and management of industrial and commercial chemicals, such as the Canadian Environmental Protection Act (CEPA), the U.S. Toxic Substances Control Act (TASC), the Japanese Toxic Substances Control Law (TSCL), the E.U. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), and the Stockholm Convention on Persistent Organic Pollutants. The overall objective of this research is to improve national and international regulatory programs for the environmental management of industrial and commercial chemicals by developing and testing methods for the assessment of bioaccumulation of chemicals in biota. The overall conclusions of the present study are summarized as follows:

First, a thin-film sorbent-phase dosing in vitro S9 liver homogenate bioassay was developed and tested and found to be a useful method for measuring biotransformation rate constants of chemicals required for bioaccumulation assessment in aquatic and non-aquatic organisms. The strengths of the thin-film sorbent-phase dosing are that: (i) it eliminates incomplete dissolution of highly hydrophobic organic chemicals in largely aqueous solution; (ii) it maintains low initial substrate concentrations in the incubation medium; (iii) it simplifies chemical analysis; (iv) it may be able to measure biotransformation rates of multiple chemicals; (v) it measures unbound fraction of chemical in solution; and (vi) it may be useful for screening large number of chemicals. The major limitations of the thin-film sorbent-phase dosing method are that: (i) chemical mass-transfer rates from the sorbent phase to the incubation medium are slower at lower incubation temperature; and (ii) this approach may not be applicable to chemicals with high volatility due to chemical loss in the film preparation process.

Second, a method for in vitro-to-in vivo extrapolation (IVIVE-B) of biotransformation rates was developed and tested for estimating in vivo biotransformation

rate constants and biomagnification factors (BMF) of hydrophobic organic chemicals in rats. The strengths of the IVIVE-B approach are that: (i) it requires less biological parameters than the current in vitro-to-in vivo extrapolation approach for pharmaceuticals (IVIVE-Ph); and (ii) it is possibly applicable to other species than rats. The major limitations of the IVIVE-B approach include that (i) extrahepatic biotransformation (e.g., intestinal biotransformation) is not included; and (ii) this method is limited to non-ionic hydrophobic organic chemicals and may not be applicable to ionizable chemicals or chemicals with high polarity.

Third, a combination of in vitro liver S9 bioassays and IVIVE-B modeling can improve bioaccumulation assessments by including methods for bioaccumulation assessment in non-aquatic organisms and identifying cut-off values for in vitro depletion rates for non-biomagnifying chemicals. The major strength of this approach is that it is potentially useful for bioaccumulation screening. The limitations of this approach include that: (i) this method is limited to non-ionic hydrophobic organic chemicals; and (ii) extrahepatic biotransformation is not included.

Recommendations for future work include: (i) the development and testing of methods for including intestinal biotransformation in bioaccumulation assessments, potentially by developing in vitro methods for measuring intestinal biotransformation rates and by modifying the IVIVE-B approach to include intestinal biotransformation; and (ii) further testing of the IVIVE-B approach to other species and substances.

Appendix A.

Supplemental Information for Materials and Methods

Chemicals

Benzo[a]pyrene, chrysene, and chrysene-d^{12} were purchased from Sigma-Aldrich (St. Louis, MO). 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) and 2,2',4,4',6,6'-hexachlorobiphenyl (PCB 155) were purchased from AccuStandard (New Haven, CT). Ethylene vinyl acetate (Elvax 40W®) was obtained from Dupont (Willington, DE). Potassium dihydrogen phosphate was obtained from Caledon Laboratories (Georgetown, ON). Potassium phosphate dibasic was obtained from Anachemia Canada (Lachine, QC). Potassium chloride and HPLC–grade hexane were obtained from EMD (Gibbstown, NJ). All other chemicals, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Rat Liver S9 Homogenate

Male Sprague–Dawley rats (330–370 g body weight) were obtained from Charles River Laboratories (Wilmington, MA) and housed in quarantine for at least three days upon arrival at the Animal Resource Centre at Simon Fraser University. Animal rooms were maintained at a constant temperature (19–23 °C) and humidity (45–55%) under a 12 h dark/light cycle. The rats were fed 5001 Laboratory Rodent Diet (PMI Nutrition International, Brentwood, MO) and allowed food and water ad libitum. The animals were anesthetized using 5% isoflurane and sacrificed by cervical dislocation. The liver was immediately excised and immersed in ice-cold 1.15% (w/v) KCl. Each liver was weighed, minced on ice with a razor blade, and homogenized on ice using a Potter-Elvehjem glass tissue grinder with a Teflon pestle (Kontes, Vineland, NJ) in one volume (g/ml) of ice-cold 0.2 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The liver homogenates from three livers were pooled and then centrifuged at 9000 $\times$ *g* for 20 min at 4 °C (Hermle Z360K centrifuge). The S9 fraction was collected and stored at –80 °C until use (< 3 months). The protein concentration of the S9 fraction was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

GC/MS Analysis

Analysis of test chemicals was performed using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer and an Agilent 7683 auto sampler (Agilent, Mississauga, ON). The GC was fitted with a cool-on-column capillary inlet and the injection volume was 1 µL. Chemicals were separated on an HP-5MS 5% phenyl methylpolysiloxane–coated column (30 m $\times$ 0.25 mm i.d., 0.25 µm film thickness) connected to a fused-silica deactivated guard column (5 m $\times$ 0.53 mm i.d.). The oven was held at an initial temperature of 60 °C for 0.5 min, then increased at 20 °C/min to 200 °C (1 min), followed by an increase at 15 °C/min to a final temperature of 285 °C (5 min). Helium was used as carrier gas at constant flow rate of 1.0 mL/min. Conditions for MS measurements were: electron impact ionization at 70 eV; ion source temperature at 230 ºC; selected ions at m/z 252 (benzo[a]pyrene), 228 (chrysene), 240 (chrysene-d^{12}), and 360 (PCB 153 and PCB 155). Agilent MSD ChemStation software (G1701CA) was used

for instrument control and data processing. The dynamic range and relative response factor (obtained by dividing the ratio of peak area/concentration of the test chemical to that of the internal standard) for each test chemical were determined using an eight-point calibration curve (concentration range: 1–500 ng/mL). Strong linearity ($r^2 > 0.99$) was shown in the calibration curves and constant relative response factor values were obtained over the concentration range.

Data Analysis

To determine the mass-transfer rate constants (k_1, k_2) and in vitro biotransformation rates (k_r) from the results of sorbent-phase dosing experiment, a methodology was developed by applying a numerical simulation of Equations 3.1 and 3.2 and a statistical model:

$$Ce_i(t) = N(S_{ce}(k_{i1}, k_{i2}, k_{ir}, C_{ei}(t), C_{mi}(t)), \sigma^2_{iCe}) \quad \text{(A1)}$$

$$Cm_i(t) = N(S_{cm}(k_{i1}, k_{i2}, k_{ir}, C_{ei}(t), C_{mi}(t)), \sigma^2_{iCm}) \quad \text{(A2)}$$

Where $C_{ei}(t)$ and $C_{mi}(t)$ are the observations of concentrations of chemical i (where i is chrysene, benzo[a]pyrene or PCB 153 in this study) in the EVA thin film and the incubation medium, respectively, at time t. $S_{ce}(k_{i1}, k_{i2}, k_{ir}, C_{ei(t)}, C_{mi(t)})$ and $S_{cm}(k_{i1}, k_{i2}, k_{ir}, C_{ei(t)}, Cm_{i(t)})$ are the solutions to the differential equations Equation 2.1 and Equation 2.2, respectively, for chemical i. σ^2_{iCe} and σ^2_{iCm} are the residual variance of observations of C_e and C_m for chemical i. The statistical model is based on the assumptions that the observations are normally distributed around the differential equation solution and that the residual variance of observations of C_e and C_m are unique to each chemical. Initial chemical concentrations, $C_{ei}(t=0)$ and $C_{mi}(t=0)$, are required to numerically solve systems of differential equations. The initial conditions for C_m were fixed at zero, as the test chemicals were dosed in the thin film. $C_{ei}(t=0)$ was treated as an additional parameter in the model. For each chemical i in sorbent-phase dosing experiments, the parameters k_{i1}, k_{i2}, k_{ir}, $C_{ei}(t=0)$, σ^2_{iCe} and σ^2_{iCm} were simultaneously determined by solving the differential equations 2.1 and 2.2 using a Runge–Kutta 1–5 (stiff) solver to numerically compute the model trajectories and then minimizing the squared discrepancy between observations $C_{ei}(t)$ and $C_{mi}(t)$, and the differential equation solutions $S_{ce}(k_{i1}, k_{i2}, k_{ir}, C_{ei(t)}, C_{mi(t)})$ and $S_{cm}(k_{i1}, k_{i2}, k_{ir}, C_{ei(t)}, Cm_{i(t)})$ using nonlinear least squares (Bates & Watts, 1988; Cheney & Kimcaid, 1994). The resulting parameter estimates are the maximum likelihood estimates from the statistical model in Equation A1 and Equation A2.

Experimental data measured under test (using active liver S9 homogenate) and control (using inactive enzymes) conditions were used simultaneously to obtain k_1, k_2, and k_r values for each of the test chemicals based on the following assumptions: (i) k_r is zero in the control system; (ii) k_1, k_2, and initial conditions (C_e and C_m at time = 0) are the same for both test and control systems; the initial condition for C_m is fixed at 0; (iii) k_1, k_2, and k_r are constants. Parameters were obtained by analyzing the chemical concentrations in the EVA thin films, in the incubation medium containing liver S9 homogenate, or the combined data set of concentrations in film and homogenates. When only the incubation medium concentration data were used, the measured initial concentration in the EVA thin film was also used to obtain better fitting results. When the EVA film concentrations data were used, the nonlinear least squares fitting was modified to use the 'relative' discrepancy

between fitted and measured concentrations of the chemical (e.g. difference between fitted and measured concentrations divided by measured concentration), to improve the k_r parameter estimation by putting more emphasis on later time points. The computations were performed using MATLAB R2009a (Mathworks, Natick, MA) by applying an ode15s solver and a nonlinear regression function.

Supplemental Data

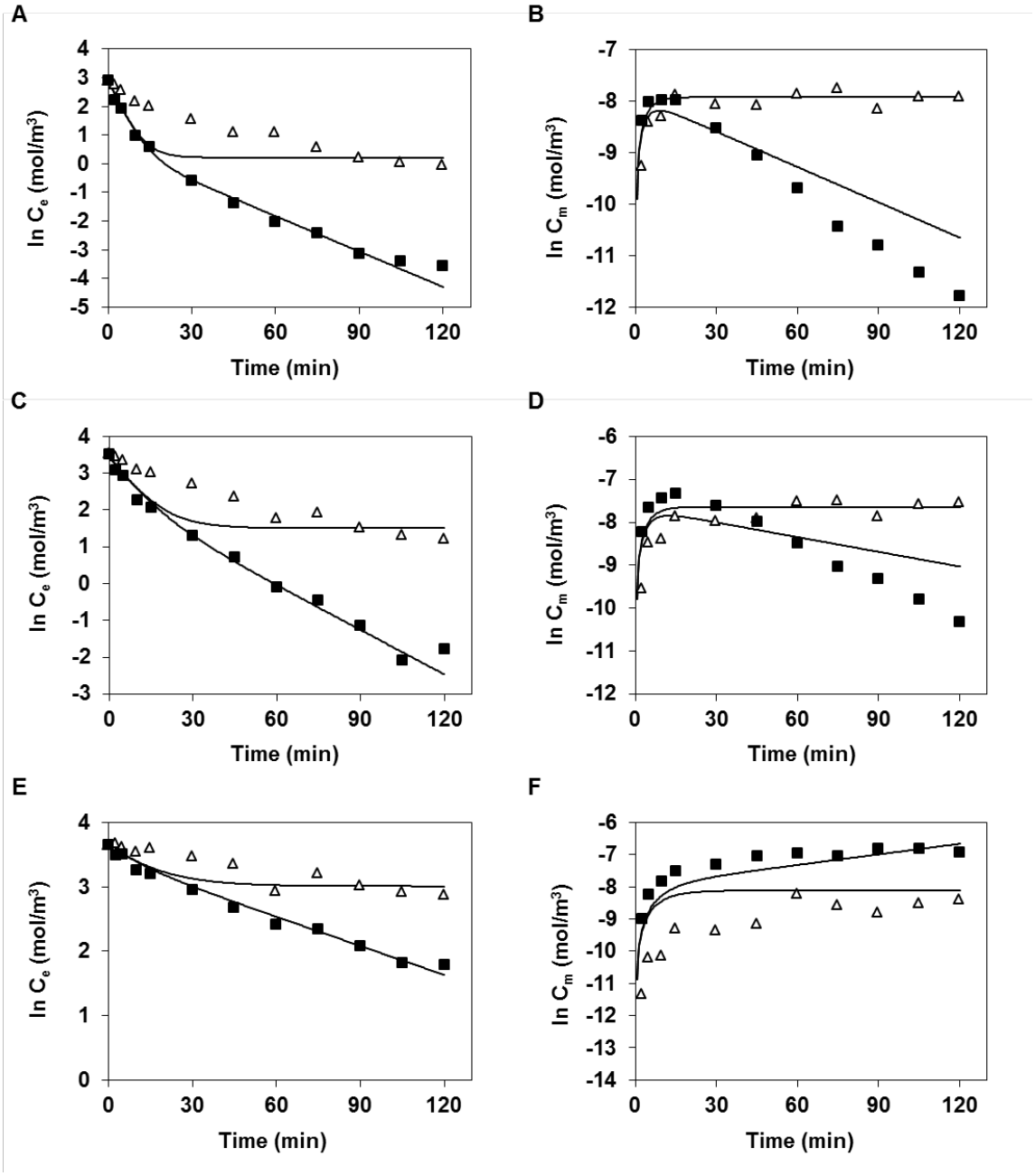

Figure A1. **Concentration−time profiles in the EVA thin films (left) and in the incubation medium (right) containing active (solid squares) and inactive (open triangles) male Sprague–Dawley rat liver S9 homogenate (heat-treated) using the sorbent-phase dosing approach for chrysene (A, B), benzo[a]pyrene (C, D), and PCB 153 (E, F). Solid lines represent nonlinear regressions. Data from one of three experiments are shown.**

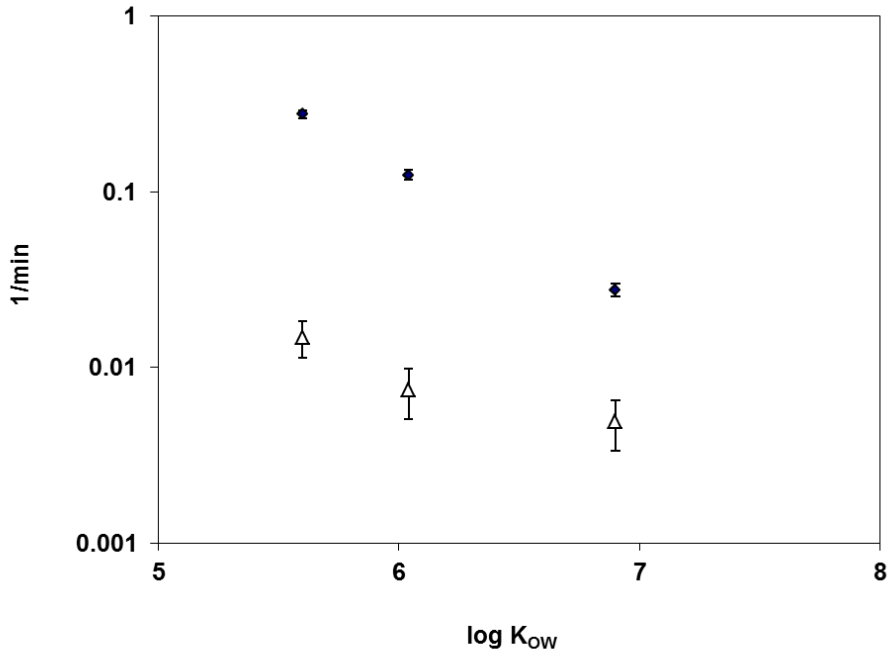

Figure A2. **Relationship between log K_{OW} and mass-transfer rate constants k_1 (solid circles) and k_2 (open triangles) in the thin-film sorbent-phase dosing system. k_1 and k_2 values were estimated by analyzing chemical concentrations in the thin film. Results were obtained from three independent experiments and error bars denote 95% confidence intervals.**

Table A1. Compilation of in vitro intrinsic clearance rates of benzo[a]pyrene in S9 rat liver homogenates and microsomes

In vitro System	Specie	Intrinsic Clearance [a] ($mL\ h^{-1}\ mg\ S9\ protein^{-1}$)	Source
Liver S9 homogenate	Male Sprague–Dawley rats	0.16	The present study
Liver S9 homogenate	Male Sprague–Dawley rats	0.93 [b]	Alvares et al., 1968
Liver microsomes	Male Holtzman rats	0.65 [b]	Zampaglioze & Mannering, 1973
Liver microsomes	Male Sprague–Dawley rats	0.63 [c]	DePierre et al., 1975
Liver microsomes	Male Wistar rats	19.4 [c]	Razzouk et al., 1978
Liver microsomes	Male Sprague–Dawley rats	4.54 [c]	Wiersma & Roth, 1983

[a] Intrinsic clearances were calculated based on V_{max}/K_M for metabolic pathway of benzo[a]pyrene hydroxylation (Alvares et al., 1968; Zampaglioze & Mannering, 1973; DePierre et al., 1975; Razzouk et al., 1978) or sum of metabolic pathways for benzo[a]pyrene (Wiersma & Roth, 1983).
[b] Unit conversion was conducted by assuming that 1 g of liver tissues contains 143 mg S9 proteins (Punt et al., 2008).
[c] Unit conversion was conducted by assuming that 1 mg microsomal protein is equivalent to 4.1 mg S9 protein (Punt et al., 2008).

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Appendix B.

Supplemental Data

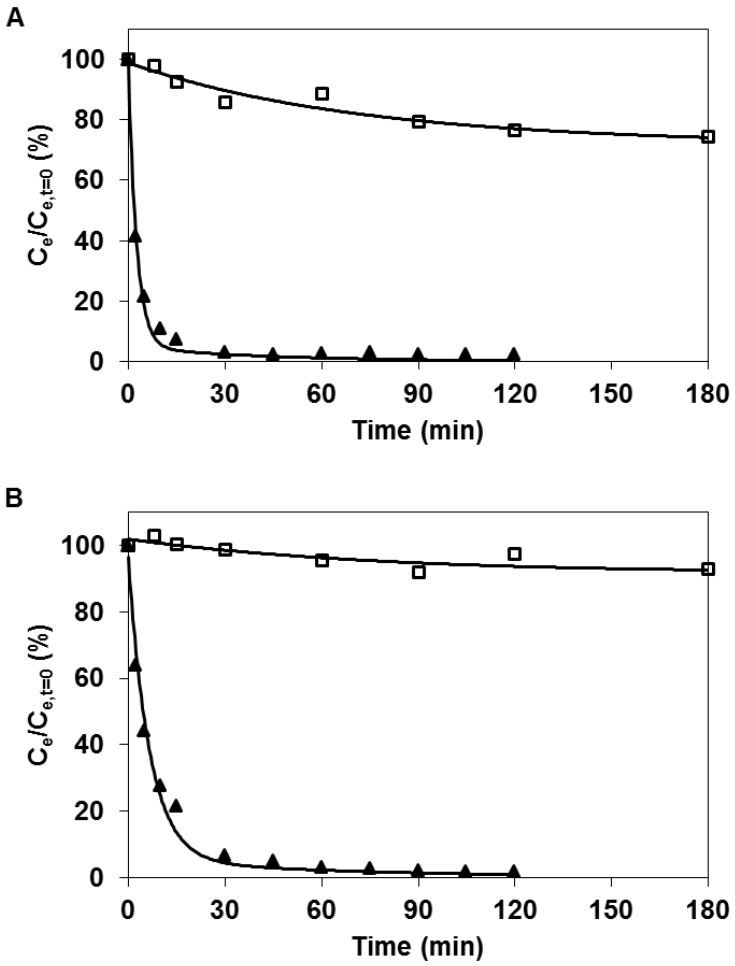

Figure B1. **Concentration-time profiles in the EVA thin film sorbent phase containing inactive liver S9 (control) from rats (solid triangles; data obtained from Lee et al. (2012)) or rainbow trout (open squares) using the sorbent-phase dosing approach with film thickness of 20 nm for chrysene (A) and benzo[a]pyrene (B). Solid lines represent nonlinear regressions.**

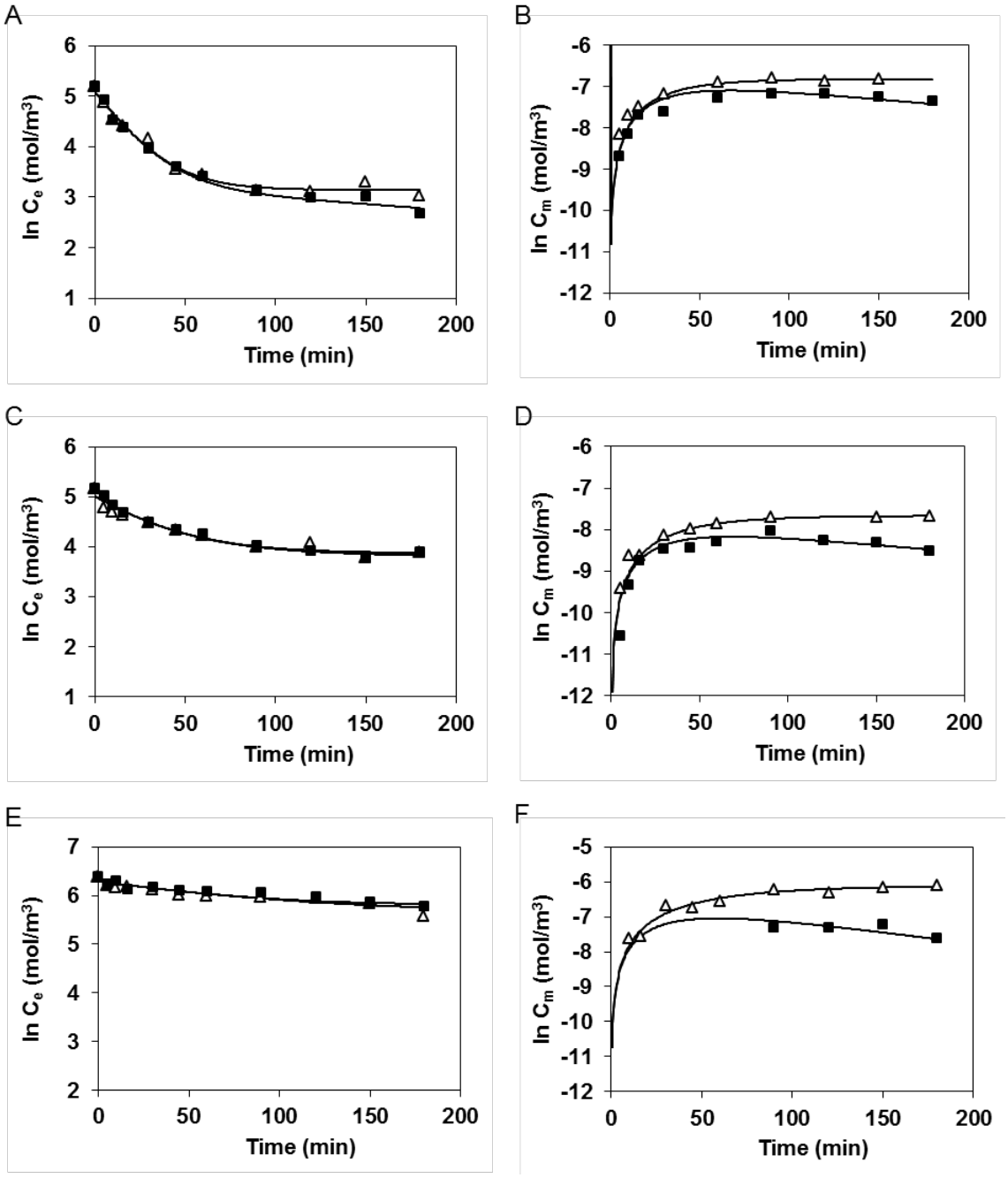

Figure B2. **Concentration-time profiles in the EVA thin film sorbent phase (left) and in the incubation medium (right) containing active (solid squares) or inactive (open triangles) rainbow trout liver S9 (control) using the sorbent-phase multi-chemical dosing approach for pyrene (A, B), chrysene (C, D) and benzo[a]pyrene (E, F). Solid lines represent nonlinear regressions. Data from one of three experiments are shown.**

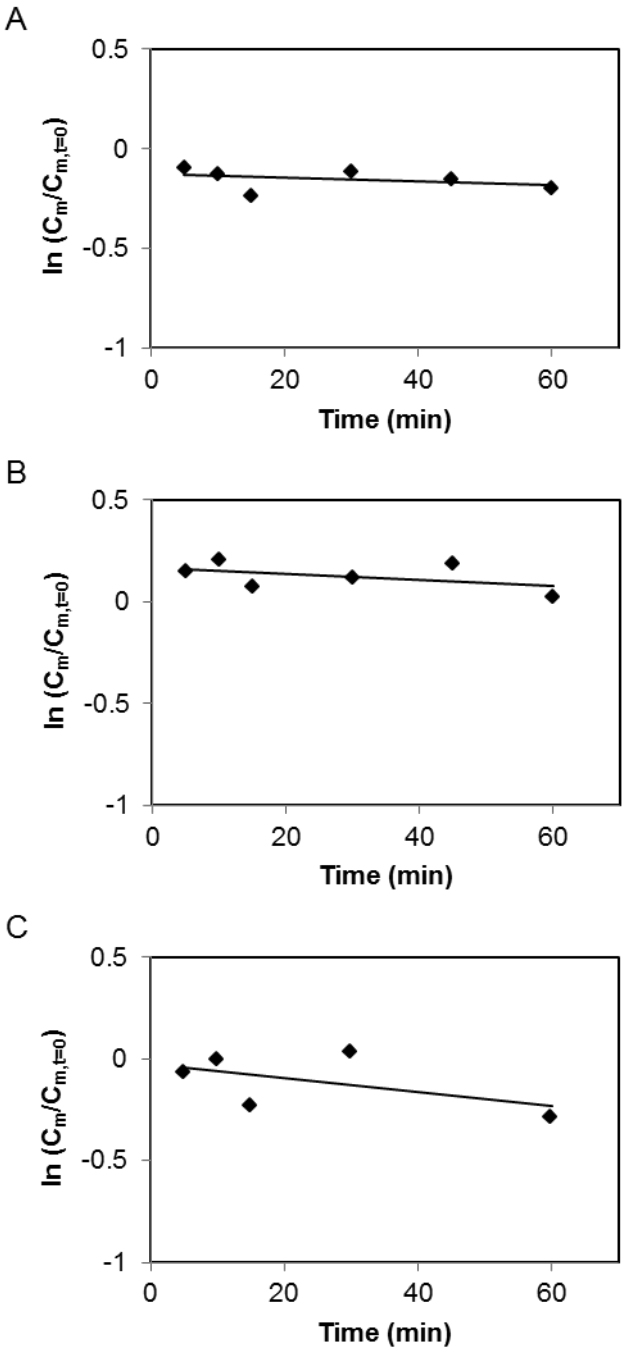

Figure B3. **Concentration-time profiles in the trout liver S9 in the solvent-delivery multi-chemical dosing experiments for pyrene (A), chrysene (B) and benzo[a]pyrene (C). Concentrations expressed as the ratio of chemical concentrations in the incubation medium in the test (control adjusted) to initial chemical concentrations in the incubation medium ($C_{m,t=0}$). Data from one of three experiments are shown.**

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Appendix C.

Derivation of Equations

Derivation of Equations 5.3, 5.6, 5.13 and 5.17

The unbound fraction of the chemical in the incubation medium, liver, blood and plasma calculated using Equations 5.3, 5.6, 5.13 and 5.17, respectively, is derived by assuming that each medium (i.e., incubation medium, liver, blood or plasma) consists of three phases, lipids, proteins and water and that the chemical partitions as follows:

$$f_{u,\mathrm{X}} = \frac{n_{W,\mathrm{X}}}{n_{L,\mathrm{X}}+n_{P,\mathrm{X}}+n_{W,\mathrm{X}}} = \frac{C_{W,\mathrm{X}}\cdot V_{W,\mathrm{X}}}{C_{L,\mathrm{X}}\cdot V_{L,\mathrm{X}}+C_{P,\mathrm{X}}\cdot V_{P,\mathrm{X}}+C_{W,\mathrm{X}}\cdot V_{W,\mathrm{X}}} =$$
$$\frac{C_{W,\mathrm{X}}\cdot(V_{W,\mathrm{X}}/V_{T,\mathrm{X}})}{C_{L,\mathrm{X}}\cdot(V_{L,\mathrm{X}}/V_{T,\mathrm{X}})+C_{P,\mathrm{X}}\cdot(V_{P,\mathrm{X}}/V_{T,\mathrm{X}})+C_{W,X}\cdot(V_{W,\mathrm{X}}/V_{T,\mathrm{X}})} = \frac{C_{W,\mathrm{X}}\cdot f_{W,\mathrm{X}}}{C_{L,\mathrm{X}}\cdot f_{L,\mathrm{X}}+C_{P,\mathrm{X}}\cdot f_{P,\mathrm{X}}+C_{W,\mathrm{X}}\cdot f_{W,\mathrm{X}}} =$$
$$\frac{f_{W,\mathrm{X}}}{(C_{L,\mathrm{X}}/C_{W,\mathrm{X}})\cdot f_{L,\mathrm{X}}+(C_{P,\mathrm{X}}/C_{W,\mathrm{X}})\cdot f_{P,\mathrm{X}}+f_{W,\mathrm{X}}} = \frac{f_{W,\mathrm{X}}}{f_{L,\mathrm{X}}\cdot K_{OW}+f_{P,\mathrm{X}}\cdot K_{PW}+f_{W,\mathrm{X}}} \quad \text{(C1)}$$

where X represents the medium, i.e., the incubation medium, liver, blood or plasma; $n_{L,X}$, $n_{P,X}$, and $n_{W,X}$ are the amount (mol) of the chemical in lipid, protein, and water phase of the medium, respectively; $C_{L,X}$, $C_{P,X}$, and $C_{W,X}$ are the concentrations (mol/mL) of the chemical in lipid, protein, and water phase of the medium, respectively; $V_{L,X}$, $V_{P,X}$, and $V_{W,X}$ are the volume (mL) of lipid, protein, and water phase of the medium, respectively; $V_{T,X}$ is the total volume of the medium (mL); $f_{L,X}$, $f_{P,X}$, and $f_{W,X}$ are the fractions of lipid, protein, and water of the medium, respectively (v/v; unitless); and K_{OW} and K_{PW} are the octanol–water partition coefficient and protein–water partition coefficient, respectively.

Derivation of Equation 5.8

The ratio of the mass of chemical in the liver (M_H; g) relative to that in the organism (M_B; g) can be derived by viewing both the liver and the organism as consisting of three phases, including lipids, proteins and water. M_H/M_B can be derived as

$$\frac{M_H}{M_B} = \frac{V_H\cdot C_H}{V_B\cdot C_B} = \frac{V_H}{V_B}\cdot\frac{f_{L,H}\cdot C_{L,H}+f_{P,H}\cdot C_{P,H}+f_{W,H}\cdot C_{W,H}}{f_{L,B}\cdot C_{L,B}+f_{P,B}\cdot C_{P,B}+f_{W,B}\cdot C_{W,B}} = \phi_H\cdot\frac{f_{L,H}\cdot K_{LW}+f_{P,H}\cdot K_{PW}+f_{W,H}}{f_{L,B}\cdot K_{LW}+f_{P,B}\cdot K_{PW}+f_{W,B}} \quad \text{(C2)}$$

where V_H and V_B are the volumes (m^3) of the liver and the organism, respectively; □ H denotes the ratio of V_H/V_B; C_H and C_B are the concentrations (mol/mL) of the chemical in the liver and the organism, respectively; $C_{L,B}$, $C_{P,B}$, and $C_{W,B}$ are the concentrations (mol/mL) of the chemical in lipid, protein, and water of the organism, respectively; $f_{L,B}$, $f_{P,B}$, and $f_{W,B}$ are the fractions of lipid, protein, and water of the organism, respectively (v/v; unitless).

Equations for Describing the Uptake and Elimination Kinetics in the Bioaccumulation Model for Terrestrial Mammals

Uptake kinetics

The rate constant for respiratory uptake of the chemical (k_{AU}; d^{-1}) is defined as

$$k_{AU} = \frac{E_A \cdot G_A}{V_B} \quad \text{(C3)}$$

where E_A is the inhalation efficiency of the chemical (unitless); G_A is the inhalation rate of the organism (i.e., volume of air respired by the organism per day; m^3/d); and V_B is the volume of the organism (m^3).

The rate constant for dietary uptake of the chemical (k_D; d^{-1}) is defined as

$$k_D = \frac{E_D \cdot G_D}{V_B} \quad \text{(C4)}$$

where E_D is the dietary absorption efficiency of the chemical (unitless); G_D is the ingestion rate of the organism (i.e., volume of food ingested by the organism per day; m^3/d).

Elimination Kinetics

The rate constant for respiratory elimination of the chemical (k_{AE}; d^{-1}) is defined as

$$k_{AE} = \frac{E_A \cdot G_A}{K_{BA} \cdot V_B} \quad \text{(C5)}$$

where K_{BA} is the biota–air partition coefficient describing the distribution of chemical between the organism and air. K_{BA} can be calculated as

$$K_{BA} = \frac{C_B}{C_A} = \frac{f_{L,B} \cdot C_{L,B} + f_{P,B} \cdot C_{P,B} + f_{W,B} \cdot C_{W,B}}{C_A} = f_{L,B} \cdot K_{OA} + f_{P,B} \cdot \chi_P \cdot K_{OA} + \frac{f_{W,B}}{K_{AW}} = \left(f_{L,B} + f_{P,B} \cdot \chi_P\right) \cdot K_{OA} + \frac{f_{W,B}}{K_{AW}} \quad \text{(C6)}$$

where C_A is the concentration of the chemical in air (mol/m^3); K_{OA} is the octanol–air partition coefficient; and K_{AW} is the air–water partition coefficient, which can be calculated as K_{OW}/K_{OA}.

The rate constant for fecal elimination of the chemical (k_F; d^{-1}) is defined as

$$k_F = \frac{E_D \cdot G_F}{K_{BF} \cdot V_B} \quad \text{(C7)}$$

where G_F is the fecal excretion rate of the organism (i.e., volume of fecal matter excreted by the organism per day; m^3/d); and K_{BF} is the biota–feces partition coefficient describing the distribution of chemical between the organism and its fecal matter.

G_F can be calculated from the ingestion rate of the organism considering the digestibility of the ingested diet and expressed as

$$G_F = G_D \cdot \left(1 - f_{L,D} \cdot \alpha_L - f_{P,D} \cdot \alpha_P - f_{C,D} \cdot \alpha_C - f_{W,D} \cdot \alpha_W\right) \quad \text{(C8)}$$

where $f_{L,D}$, $f_{P,D}$, $f_{C,D}$, and $f_{W,D}$ are the fractions of lipid, protein, carbohydrate, and water of the ingested diet, respectively (v/v; unitless); and α_L, α_P, α_C, and α_W are the assimilation efficiencies for lipid, protein, carbohydrate and water, respectively (v/v; unitless).

K_{BF} can be calculated as

$$K_{BF} = \frac{C_B}{C_F} = \frac{f_{L,B}\cdot C_{L,B} + f_{P,B}\cdot C_{P,B} + f_{W,B}\cdot C_{W,B}}{f_{L,F}\cdot C_{L,F} + f_{P,F}\cdot C_{P,F} + f_{C,F}\cdot C_{C,F} + f_{W,F}\cdot C_{W,F}} =$$

$$\frac{f_{L,B}\cdot K_{OW} + f_{P,B}\cdot \chi_P \cdot K_{OW} + f_{W,B}}{f_{L,F}\cdot K_{OW} + f_{P,F}\cdot \chi_P \cdot K_{OW} + f_{C,F}\cdot \chi_C \cdot K_{OW} + f_{W,F}} = \frac{\left(f_{L,B} + f_{P,B}\cdot \chi_P\right)\cdot K_{OW} + f_{W,B}}{\left(f_{L,F} + f_{P,F}\cdot \chi_P + f_{C,F}\cdot \chi_C\right)\cdot K_{OW} + f_{W,F}} \quad \text{(C9)}$$

where C_F is the concentration of the chemical in the fecal matter (mol/m^3); and $f_{L,F}$, $f_{P,F}$, $f_{C,F}$, and $f_{W,F}$ are the fractions of lipid, protein, carbohydrate, and water of the fecal matter, respectively (v/v; unitless).

$f_{L,F}$, $f_{P,F}$, $f_{C,F}$, and $f_{W,F}$ can be calculated from ingested diet and expressed as

$$f_{L,F} = \frac{(1-\alpha_L)\cdot f_{L,D}}{(1-\alpha_L)\cdot f_{L,D} + (1-\alpha_P)\cdot f_{P,D} + (1-\alpha_C)\cdot f_{C,D} + (1-\alpha_W)\cdot f_{W,D}} = \frac{(1-\alpha_L)\cdot f_{L,D}\cdot G_D}{G_F} \quad \text{(C10)}$$

$$f_{P,F} = \frac{(1-\alpha_P)\cdot f_{P,D}}{(1-\alpha_L)\cdot f_{L,D} + (1-\alpha_P)\cdot f_{P,D} + (1-\alpha_C)\cdot f_{C,D} + (1-\alpha_W)\cdot f_{W,D}} = \frac{(1-\alpha_P)\cdot f_{P,D}\cdot G_D}{G_F} \quad \text{(C11)}$$

$$f_{C,F} = \frac{(1-\alpha_C)\cdot f_{C,D}}{(1-\alpha_L)\cdot f_{L,D} + (1-\alpha_P)\cdot f_{P,D} + (1-\alpha_C)\cdot f_{C,D} + (1-\alpha_W)\cdot f_{W,D}} = \frac{(1-\alpha_C)\cdot f_{C,D}\cdot G_D}{G_F} \quad \text{(C12)}$$

$$f_{W,F} = \frac{(1-\alpha_W)\cdot f_{W,D}}{(1-\alpha_L)\cdot f_{L,D} + (1-\alpha_P)\cdot f_{P,D} + (1-\alpha_C)\cdot f_{C,D} + (1-\alpha_W)\cdot f_{W,D}} = \frac{(1-\alpha_W)\cdot f_{W,D}\cdot G_D}{G_F} \quad \text{(C13)}$$

The rate constant for urinary elimination of the chemical (k_U; d^{-1}) is defined as

$$k_U = \frac{G_U}{K_{BU}\cdot V_B} \quad \text{(C14)}$$

where G_U is the urinary excretion rate of the organism (i.e., volume of urine excreted by the organism per day; m^3/d); and K_{BU} is the biota–urine partition coefficient describing the distribution of chemical between the organism and its urine. K_{BU} can be calculated as

$$K_{BU} = \frac{C_B}{C_U} = \frac{f_{L,B}\cdot C_{L,B} + f_{P,B}\cdot C_{P,B} + f_{W,B}\cdot C_{W,B}}{f_{L,U}\cdot C_{L,U} + f_{P,U}\cdot C_{P,U} + f_{W,U}\cdot C_{W,U}} = \frac{f_{L,B}\cdot C_{L,B} + f_{P,B}\cdot C_{P,B} + f_{W,B}\cdot C_{W,B}}{C_{W,U}} = f_{L,B} \cdot K_{OW} +$$

$$f_{P,B} \cdot \chi_P \cdot K_{OW} + f_{W,B} = \left(\mathrm{f_{L,B}} + \mathrm{f_{P,B}} \cdot \chi_\mathrm{P}\right) \cdot \mathrm{K_{OW}} + \mathrm{f_{W,B}} \quad \text{(C15)}$$

where C_U is the concentration of the chemical in urine (mol/m^3); and $f_{L,U}$, $f_{P,U}$, and $f_{W,U}$ are the fractions of lipid, protein, and water of urine, respectively (v/v; unitless). The above expression is simplified by assuming that water is the dominant phase in the composition of urine (i.e., $f_{W,U}$ =1 and $f_{L,U} = f_{P,U} = 0$).

The rate constant for biliary elimination of the chemical (k_{Bi}; d^{-1}) is defined as

$$k_{Bi} = \frac{G_{Bi}}{K_{BBi} \cdot V_B} \tag{C16}$$

where G_{Bi} is the billiary excretion rate of the organism (i.e., volume of bile excreted by the organism per day; m^3/d); and K_{BBi} is the biota–bile partition coefficient describing the distribution of chemical between the organism and its bile. K_{BBi} can be calculated as

$$K_{BBi} = \frac{C_B}{C_{Bi}} = \frac{f_{L,B} \cdot C_{L,B} + f_{P,B} \cdot C_{P,B} + f_{W,B} \cdot C_{W,B}}{\beta \cdot C_{W,B}} = \frac{f_{L,B} \cdot K_{OW} + f_{P,B} \cdot \chi_P \cdot K_{OW} + f_{W,B}}{\beta} = \frac{(f_{L,B} + f_{P,B} \cdot \chi_P) \cdot K_{OW} + f_{W,B}}{\beta} \tag{C17}$$

where C_{Bi} is the concentration of the chemical in bile (mol/m^3), which is defined as $\beta \square C_{W,B}$, where β represents the increase in solubility of chemicals in bile fluids compare to water (unitless).

The elimination rate constant of the chemical for lactation (k_L; d^{-1}) is defined as

$$k_L = \frac{G_M}{K_{BM} \cdot V_B} \tag{C18}$$

where G_M is the lactation rate of the organism (i.e., volume of milk excreted by the organism per day; m^3/d); and K_{BM} is the biota–milk partition coefficient describing the distribution of chemical between the organism and its milk. K_{BM} can be calculated as

$$K_{BM} = \frac{C_B}{C_M} = \frac{f_{L,B} \cdot C_{L,B} + f_{P,B} \cdot C_{P,B} + f_{W,B} \cdot C_{W,B}}{f_{L,M} \cdot C_{L,M} + f_{P,M} \cdot C_{P,M} + f_{W,M} \cdot C_{W,M}} = \frac{f_{L,B} \cdot K_{OW} + f_{P,B} \cdot \chi_P \cdot K_{OW} + f_{W,B}}{f_{L,M} \cdot K_{OW} + f_{P,M} \cdot \chi_P \cdot K_{OW} + f_{W,M}} = \frac{(f_{L,B} + f_{P,B} \cdot \chi_P) \cdot K_{OW} + f_{W,B}}{(f_{L,M} + f_{P,M} \cdot \chi_P) \cdot K_{OW} + f_{W,M}} \tag{C19}$$

where C_M is the concentration of the chemical in milk (mol/m^3); and $f_{L,M}$, $f_{P,M}$, and $f_{W,M}$ are the fractions of lipid, protein, and water of milk, respectively (v/v; unitless). $k_L = 0$ for male animals.

Supplemental Data

Table C1. Input parameters for the IVIVE-B and IVIVE-Ph models in rats

IVIVE-B model				IVIVE-Ph model			
Parameter (symbol)	Value	Unit	Source	Parameter (symbol)	Value	Unit	Source
Log octanol–water partition coefficient at 37 ºC (log K_{OW})	0–10 for hypothetical chemicals 5.87 for benzo[a]pyrene 5.45 for chrysene	Unitless	Mackay et al., 2006; Beyer et al., 2002	Log octanol–water partition coefficient at 37 ºC (log K_{OW})	0–10 for hypothetical chemicals 5.87 for benzo[a]pyrene 5.45 for chrysene	Unitless	Mackay et al., 2006; Beyer et al., 2002
In vitro biotransformation rate constant ($k_{r,C\rightarrow 0}$)	2.15 ± 0.57 for benzo[a]pyrene 2.54 ± 0.30 for chrysene	h^{-1}	Lee et al., 2012	In vitro biotransformation rate constant ($k_{r,C\rightarrow 0}$)	2.15 ± 0.57 for benzo[a]pyrene 2.54 ± 0.30 for chrysene	h^{-1}	Lee et al., 2012
Unbound fraction in incubation ($f_{u,inc}$)	4.44×10^{-4} for benzo[a]pyrene 4.45×10^{-4} for chrysene	Unitless	Lee et al., 2012	Unbound fraction in incubation ($f_{u,inc}$)	4.44×10^{-4} for benzo[a]pyrene 4.45×10^{-4} for chrysene	Unitless	Lee et al., 2012
Fraction of lipids of incubation medium ($f_{L,inc}$)	0.0023	Unitless	Lee et al., 2012	Fraction of lipids of incubation medium ($f_{L,inc}$)	0.0023	Unitless	Lee et al., 2012
Fraction of proteins of incubation medium ($f_{P,inc}$)	0.0115	Unitless	Lee et al., 2012	Fraction of proteins of incubation medium ($f_{P,inc}$)	0.0115	Unitless	Lee et al., 2012
Fraction of water of incubation medium ($f_{W,inc}$)	0.9863	Unitless	Lee et al., 2012	Fraction of water of incubation medium ($f_{W,inc}$)	0.9863	Unitless	Lee et al., 2012

IVIVE-B model				IVIVE-Ph model			
Parameter (symbol)	Value	Unit	Source	Parameter (symbol)	Value	Unit	Source
Liver fraction in organism (ϕ_H)	0.038	Unitless	Lee et al., 2012	Liver fraction in organism (ϕ_H)	0.038	Unitless	Lee et al., 2012
Volume of incubation mixture (V_{inc})	0.5	mL	Lee et al., 2012	S9 protein concentration in incubation ($C_{P,inc}$)	12.18	mg/mL	Lee et al., 2012
Volume of S9 in incubation mixture ($V_{S9,inc}$)	0.1	mL	Lee et al., 2012	S9 protein concentration in liver ($C_{P,H}$)	68.84	mg/g liver	Lee et al., 2012
Yield of S9 per gram of liver (γ_{S9})	1.13	mL/g	Lee et al., 2012	Cardiac output (CO)	19.92	$mL \cdot min^{-1} \cdot g\ organism^{-1}$	Brown et al., 1997
Liver density (d_H)	1.05	g/mL	Sohlenius-Sternbeck, 2006	Fraction of blood flow through liver (LF)	0.183	Unitless	Brown et al., 1997
Fraction of lipids of liver ($f_{L,H}$)	0.06	Unitless	Poulin & Krishnan, 1996	Fraction of lipids of blood ($f_{L,Bl}$)	0.0033	Unitless	Poulin & Krishnan, 1996
Fraction of proteins of liver ($f_{P,H}$)	0.24	Unitless	$1-f_{L,H}-f_{W,H}$	Fraction of proteins of blood ($f_{P,Bl}$)	0.1567	Unitless	$1-f_{L,Bl}-f_{W,Bl}$
Fraction of water of liver ($f_{W,H}$)	0.70	Unitless	Poulin & Krishnan, 1996	Fraction of water of blood ($f_{W,Bl}$)	0.84	Unitless	Poulin & Krishnan, 1996
Fraction of lipids of organism ($f_{L,B}$)	0.05	Unitless	deBruyn & Gobas, 2006	Fractional body volume of erythrocyte (V_e)	0.0367	$mL \cdot g\ organism^{-1}$	Poulin & Theil, 2002
Fraction of proteins of organism ($f_{P,B}$)	0.23	Unitless	deBruyn & Gobas, 2006	Fractional body volume of plasma (V_{pl})	0.0449	$mL \cdot g\ organism^{-1}$	Poulin & Theil, 2002

IVIVE-B model				IVIVE-Ph model			
Parameter (symbol)	Value	Unit	Source	Parameter (symbol)	Value	Unit	Source
Fraction of water of organism ($f_{W,B}$)	0.72	Unitless	$1-f_{L,B}-f_{P,B}$	Fractional body volume of tissue (V_t)	0.041476 for bone 0.0057 for brain 0.027 for gut 0.0033 for heart 0.0073 for kidney 0.0366 for liver 0.005 for lung 0.404 for muscle 0.19 for skin 0.002 for spleen 0.0761 for adipose	mL·g organism^{-1}	Poulin & Theil, 2002
				Fraction of neutral lipids of plasma ($f_{NL,pl}$)	0.00147	Unitless	Poulin & Theil, 2002
				Fraction of neutral lipids of tissue ($f_{NL,t}$)	0.0273 for bone 0.0392 for brain 0.0292 for gut 0.014 for heart 0.0123 for kidney 0.0138 for liver 0.0219 for lung 0.01 for muscle 0.0239 for skin 0.0077 for spleen 0.853 for adipose	Unitless	Poulin & Theil, 2002

IVIVE-B model				IVIVE-Ph model			
Parameter (symbol)	Value	Unit	Source	Parameter (symbol)	Value	Unit	Source
				Fraction of phospholipids of plasma ($f_{PhL,pl}$)	0.00083	Unitless	Poulin & Theil, 2002
				Fraction of phospholipids of tissue ($f_{PhL,t}$)	0.0027 for bone 0.0533 for brain 0.0138 for gut 0.0118 for heart 0.0284 for kidney 0.0303 for liver 0.014 for lung 0.009 for muscle 0.018 for skin 0.0136 for spleen 0.002 for adipose	Unitless	Poulin & Theil, 2002
				Fraction of water of plasma ($f_{W,pl}$)	0.96	Unitless	Poulin & Theil, 2002
				Fraction of water of tissue ($f_{W,t}$)	0.446 for bone 0.788 for brain 0.749 for gut 0.779 for heart 0.771 for kidney 0.705 for liver 0.79 for lung 0.756 for muscle 0.651 for skin 0.771 for spleen	Unitless	Poulin & Theil, 2002

IVIVE-B model				IVIVE-Ph model			
Parameter (symbol)	Value	Unit	Source	Parameter (symbol)	Value	Unit	Source
					0.12 for adipose		
				Fraction of lipids of plasma ($f_{L,pl}$)	0.0023	Unitless	
				Fraction of proteins of plasma ($f_{L,pl}$)	0.0377	Unitless	
				Erythrocyte to plasma concentration ratio (E/Pl)	1	Unitless	

Table C2. Input parameters for the bioaccumulation model for rats using benzo[a]pyrene and chrysene as model chemicals

Parameter	Symbol	Value	Unit	Source
Log octanol–water partition coefficient at 37 °C	log K_{OW}	5.87 [a] 5.45 [b]	Unitless	Mackay et al., 2006; Beyer et al., 2002
Log octanol–air partition coefficient at 37 °C	log K_{OA}	10.38 [a] 9.56 [b]	Unitless	Mackay et al., 2006; Beyer et al., 2002
Weight of organism	W_B	0.364	kg	Lee et al., 2012
Density of organism	d_B	1000	kg/m^3	Assumed
Volume of organism	V_B	3.64×10^{-4}	m^3	W_B/d_B
Inhalation efficiency	E_A	0.7	Unitless	Kelly & Gobas, 2003
Dietary absorption efficiency	E_D	0.42 [a] 0.59 [b]	Unitless	Equation 4.26
Inhalation rate	G_A	0.24	m^3/d	U.S. EPA, 1993
Ingestion rate	G_D	2.76×10^{-5}	m^3/d	Roth et al., 1993
Urinary excretion rate	G_U	1.93×10^{-5}	m^3/d	Roth et al., 1993
Biliary excretion rate	G_{Bi}	1.50×10^{-5}	m^3/d	Kuipers et al., 1985
Fecal excretion rate	G_F	7.77×10^{-6}	m^3/d	Equation S8
Lipid fraction in organism	$f_{L,B}$	0.05	Unitless	deBruyn & Gobas, 2006
Protein fraction in organism	$f_{P,B}$	0.23	Unitless	deBruyn & Gobas, 2006
Water fraction in organism	$f_{W,B}$	0.72	Unitless	$1–f_{L,B}–f_{P,B}$
Lipid fraction in diet	$f_{L,D}$	0.045	Unitless	Goodman et al., 1984
Protein fraction in diet	$f_{P,D}$	0.234	Unitless	Goodman et al., 1984
Carbohydrate fraction in diet	$f_{C,D}$	0.650	Unitless	Goodman et al., 1984
Water fraction in diet	$f_{W,D}$	0.071	Unitless	$1–f_{L,D}–f_{P,D}–f_{C,D}$
Density of diet	d_D	1000	kg/m^3	Assumed
Assimilation efficiency for lipid	α_L	0.97	Unitless	Nishiyama et al., 2009
Assimilation efficiency for protein	α_P	0.96	Unitless	Nishiyama et al., 2009
Assimilation efficiency for carbohydrate	α_C	0.6	Unitless	Assumed
Assimilation efficiency for water	α_W	0.85	Unitless	Armitage & Gobas, 2007
Proportionality constant relating the sorptive capacity of protein to that of octanol	χ_P	0.05	Unitless	deBruyn & Gobas, 2007

Increase in solubility of chemicals in bile fluids compared to water	β	10	Unitless	Gobas et al., 2003

[a] values for benzo[a]pyrene.
[b] values for chrysene.

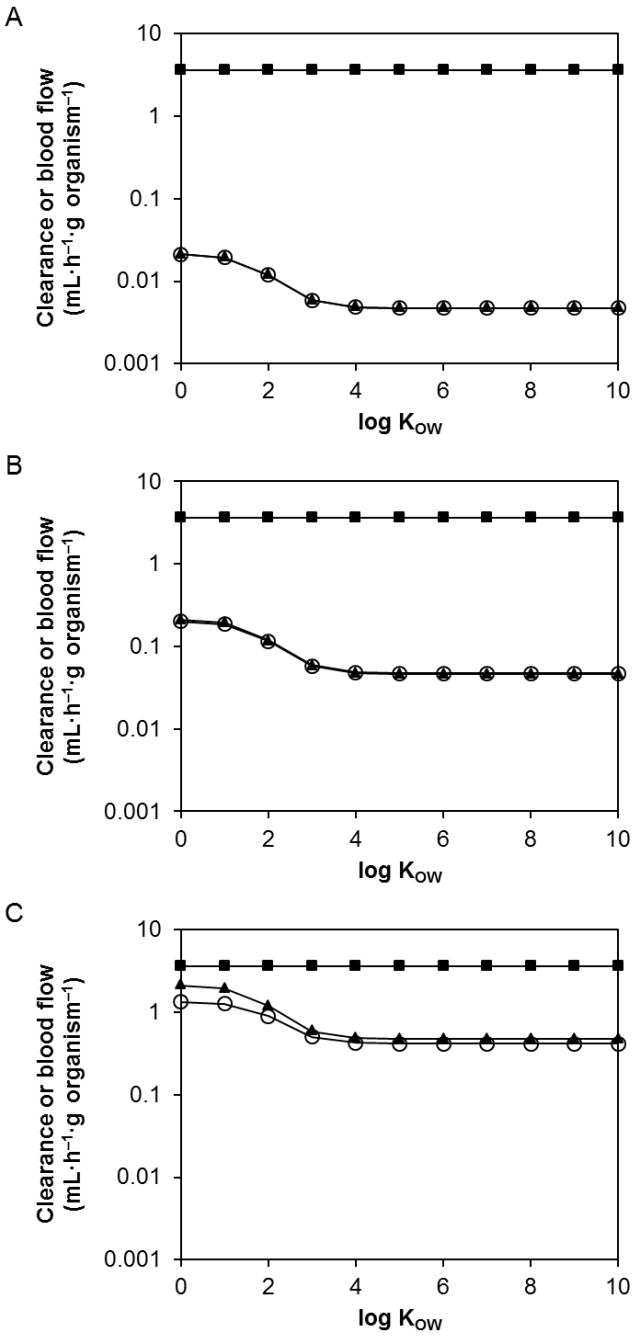

Figure C1. **The relationship between log K_{OW} of hypothetical chemicals and calculated rat hepatic clearance (i.e., CL_H; open circles), hepatic blood flow (i.e., Q_H; filled squares), and unbound hepatic intrinsic clearance (i.e., $f_u \cdot CL_{int,H}$; filled triangles) obtained from the well-stirred liver model (Equation 4.11) at input in vitro biotransformation rate constant of 0.1 h^{-1} (A), 1 h^{-1} (B) and 10 h^{-1}(C).**

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