TOWARDS HIGH THROUGHPUT DETERMINATION OF BIOTRANSFORMATION RATES OF CHEMICAL MIXTURES USING ISOLATED PERFUSED TROUT LIVERS

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ABSTRACT

In the field of environmental risk assessment, aquatic contaminants are typically characterized by their persistence, bioaccumulation potential, and toxicity (PBT). Bioconcentration factor (BCF) is the most common metric for evaluating bioaccumulation potential and is frequently estimated using Quantitative-Structure-Activity-Relationships (QSARs) and other *in silico* tools. These methods can estimate BCF based upon the physicochemical characteristics of a chemical and the toxicokinetic parameters of a model organism for chemicals which do not undergo biotransformation. For chemicals which are actively biotransformed, whole animal *in vivo* exposures have classically been required to determine BCF for regulatory acceptance. Recently, *in vitro* substrate depletion assays have been adopted as an alternative to *in vivo* testing due to concerns of cost and ethics. The results of these assays have been met with doubt due to uncertainties involved with *in vitro-in vivo extrapolation* (IVIVE) methods required to bridge the gap between target organs of biotransformation such as the liver, and whole-organism outcomes. The overall objective of this thesis was to validate an IVIVE approach for estimating biotransformation in rainbow trout (*Oncorhynchus mykiss*) by using the isolated perfused liver method, which represents an intermediate between *in vitro* and *in vivo.*

The first study (Chapter 2) involved comparing direct measurements of hepatic clearance in the isolated perfused liver with *in vitro* determinations of clearance which were previously published as part of a collaborative international trial for the regulatory adoption of *in vitro* substrate depletion assays. This comparison was performed for the model biotransformation substrate chemicals pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor. The hepatic clearance rate of these chemicals was determined in isolated perfused livers by

measuring the difference between chemical concentration as it entered and exited the liver, giving a measure of chemical elimination due to biotransformation. Experiments were performed over a period of several hours to validate physiological performance, and measurements of glucose efflux and pH were used to confirm aerobic respiration and thus metabolic activity. In addition, the influence of protein binding on hepatic clearance was investigated by varying the concentration of bovine serum albumin (BSA) in perfusates spiked with chemical. Measured clearances were in good agreement with *in vitro* substrate depletion models coupled with an IVIVE approach which takes protein binding into account. Overall, this study indicated that uncertainty associated with current IVIVE models is likely due to extrahepatic biotransformation, variability in BCF test designs, and inaccuracies in partitioning estimates or other kinetic processes such as uptake across the gills, rather than the reliability of *in vitro* test methodologies.

The second study (Chapter 3) focused on expanding the domain of applicability of the isolated perfused liver method following validation in Chapter 2 as well as concurrent research investigating mixture experiments in ionizable organic compounds (IOCs). One of the primary concerns about bioaccumulation assessment methods regardless of *in silico, in vitro,* or *in vivo* approaches is the limited number of chemicals which have been successfully tested. To achieve the throughput needed by modern chemical risk assessment frameworks, a mixture experiment was performed using the isolated perfused liver model. This study used a chemical mixture obtained from the United States Environmental Protection Agency (EPA) as part of the EPA's non-targeted analysis collaborative trial (ENTACT). This mixture contained over 500 chemicals, and the study utilized advanced high-resolution-mass-spectrometry (HRMS) methods to detect individual compounds in mixture at the very low concentrations required for mixture

experiments. Hepatic clearance was determined for 20 substances in this mixture simultaneously, representing diverse classes of chemicals including pharmaceuticals, pesticides, and industrial chemicals. This study demonstrated that the isolated perfused liver method can be a valuable tool for bioaccumulation screening, and validated the performance of this model for diverse groups of chemicals.

Chapter 4 discusses the present state of bioaccumulation assessment with regard to biotransformed chemicals, focusing on the role of the isolated perfused liver method and the goals of validation and demonstration set out by this thesis. Many studies have focused on which factors involved in IVIVE drive the discrepancies between BCFs determined by *in vivo* exposures with those predicted by IVIVE approaches. The difference between chemical freely available for biotransformation in the systemic circulation *in vivo* versus *in vitro* has been identified as a source of uncertainty. This ratio has typically been assumed to be identical, as this assumption whilst mechanistically impossible leads to a more reliable prediction of BCF. In Chapter 2, it was demonstrated that the influence of protein binding on hepatic clearance can be incorporated into an IVIVE model to increase reliability, indicating that prior assumptions were inappropriate. Concerns over limited domain of applicability were addressed in Chapter 3 as well as in concurrent work involving IOCs, focusing on the high-throughput nature of these experiments in which six fish were used to screen 20 chemicals.

In summary, the isolated perfused liver model advanced in this thesis work has contributed to the field of bioaccumulation assessment by validating presently adopted *in vitro* assays. Furthermore, this work has expanded the chemical scope of methods developed to achieve high-throughput predictions of biotransformation, serving as the basis for incorporation into reliable IVIVE models.

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LIST OF ABBREVIATIONS

ADME	adsorption, distribution, metabolism, excretion
AGC	automatic gain control
BCF	bioconcentration factor
BSA	bovine serum albumin
C_{AFF}	concentration afferent
C_{EFF}	concentration efferent
CEPA	Canadian Environmental Protection Act
$C_{FISH,SS}$	concentration in fish, steady state
CL_H	hepatic clearance
$CL_{INT,\ IN\ VITRO}$	*in vitro* intrinsic clearance
$CL_{INT,\ IN\ VIVO}$	*in vivo* intrinsic clearance
$C_{W,SS}$	steady-state aqueous concentration
$C_{W,TOT}$	total aqueous concentration
CYP	Cytochrome P450
CSA	Cyclosporin A
$DDMS^2$	data-dependent tandem mass spectrometry
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
E_H	hepatic extraction fraction
ENTACT	EPA's non-targeted analysis collaborative trial
EPA	United States Environmental Protection Agency
EU	European Union
FD	freely dissolved chemical fraction in water
FLD	fluorescence detection
F_U	fraction unbound
$F_{U,ASSAY}$	fraction unbound in assay media
$F_{U,BLOOD}$	fraction unbound in blood
$F_{U,HEP}$	fraction unbound in isolated hepatocytes

$F_{U,P}$	fraction unbound in plasma
$F_{U,S9}$	fraction unbound in the S9 system
HBS	Hank's Balanced Salts
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IOC	ionizable organic compounds
IVIVE	*in vitro-in vivo extrapolation*
K_1	gill uptake rate constant
K_2	gill elimination rate constant
K_E	fecal egestion rate constant
K_{MET}	whole-body metabolism rate constant
K_{OW}	octanol/water partition coefficient
LC	liquid chromatography
LC50	lethal concentration 50
MS 222	ethyl 3-amino-benzoate methane sulfonate
NaOH	sodium hydroxide
OECD	Organisation for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
PBT	Persistence, Bioaccumulation, Toxicity
PBTK	physiologically based toxicokinetic
pKa	acid dissociation constant
pp-LFER	poly parameter linear free energy relationships
Q_H	hepatic blood flow
QSAR	quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RMSE	root mean standard error
RT-HEP	rainbow trout isolated hepatocytes
RT-S9	rainbow trout subcellular fraction
TG	testing guideline

PREFACE

The focus of this thesis work was to use the isolated perfused rainbow trout (*Oncorhynchus mykiss*) liver model to validate a set of prior *in vitro* substrate depletion IVIVE results, explore the mechanistic assumptions and parameterizations of IVIVE models, and finally to demonstrate an expanded applicability domain of alternative testing approaches.

Chapter 1 is a literature review and general introduction to the challenges involved in modern bioaccumulation assessment. This includes considerations of regulatory importance, current and past assessment tools and frameworks, as well as the fish physiology of interest to bioaccumulation science.

Chapter 2 consists of work related to validating an existing IVIVE model using the isolated perfused liver, as well as the mechanistic investigation of dissolved chemical fraction and what influence it has on hepatic clearance. Chapter 2 was prepared in the manuscript style and was published in the peer-reviewed scientific journal *Environmental Science and Technology* (Schultz et al., 2022). Chapter 3 relates to mixture experiments that naturally followed after validation in Chapter 2, and has been prepared for submission to *Environmental Science and Technology*. Due to the similarities in methodology between these two chapters, there is some repetition in the materials and methods.

Chapter 4 is a general discussion, including a brief summary of concurrent work published within the Brinkmann lab group (Bourgeois et al., 2022) that utilized the same method and in which the author of this thesis participated significantly. This chapter evaluates the state-of-the-art in bioaccumulation assessment tools and what role this thesis work fulfills, as well as discussing data gaps and future research recommendations.

CHAPTER 1 GENERAL INTRODUCTION

The production and usage of chemicals of environmental concern continue to be the primary challenge of chemical regulatory bodies within the developed world. To date, over 350,000 chemicals and mixtures have been registered within the global market,[1] many of which present risks to aquatic and terrestrial ecosystems, as well as human populations. During chemical risk assessments, these xenobiotics are required to be characterized by their potential for persistence, bioaccumulation, and toxicity (PBT).[1,2] The evaluation of these PBT criteria using standardized laboratory test methods is standard practice and widely accepted by regulators such as Environment and Climate Change Canada (ECCC), the United States Environmental Protection Agency (USEPA), and the European Chemicals Agency (ECHA).[3] However, the ever-increasing number of chemicals and substances produced and used today puts pressure on the ability of regulators to assess these criteria appropriately. In particular, the comparably low throughput of conventional test methods becomes a limiting factor, especially with regard to the arduous process of obtaining bioaccumulation data of sufficient quality.[4] One such endpoint that is often derived from bioaccumulation studies is the bioconcentration factor (BCF), a measure of a chemical's propensity to accumulate in aquatic organisms, most commonly fish, from its surrounding environment through non-dietary routes of exposure.[5]

Producing empirical BCF data is often cost- and time-prohibitive on a per-chemical basis[6] and as such, simple models that predict bioaccumulation based on physiochemical characteristics of chemicals are commonly used to satisfy these data needs. For chemicals that undergo biotransformation, these estimates are often inaccurate compared to empirical data, leading to, at times, drastic overestimations of a chemical's bioaccumulation potential.[7] Hence,

empirically derived BCF data has remained the gold standard of many bioaccumulation assessments to this day.[6]

Newer methods in bioaccumulation risk assessment focus on increasing throughput of experimental methods whilst avoiding the use of whole animals on both ethical grounds and economic feasibility, which includes *in vitro* biotransformation tests with fish hepatocytes and subcellular fractions.[8] To accomplish this, extrapolation methods must be used to translate findings from the biological level of organization being studied (e.g., tissues, cells, molecules) within alternative *in vitro* testing methods to the *in vivo* level, i.e., the whole organism being assessed. The goal of these methods is often to produce bioaccumulation data of sufficient quality for use by regulators within PBT assessments. Thus, it is a requisite that any new method is capable of generating quantitative estimates of bioaccumulation that strikes a balance between being conservative but also practically feasible. A methodology may be satisfactory for scientists and regulators who are focused on mechanistic accuracy and reproducibility, but if such a method is considered overly conservative it may encounter setbacks in the adoption process by industry.[5,9] Refined alternative methods seek to satisfy this balance and are predicated on reconciling the limitations of classically adopted BCF experiments with the development of state-of-the-art chemical analysis and modeling practices.

Whilst the BCF approach remains the standard for regulatory use globally, there is a growing body of research reflecting a different approach, focused on more rapidly generated empirical measurements of biotransformation which influence BCF,[9] as part of a weight-of-evidence approach.[10] These approaches are rooted in the physiology of model organisms, and constitute both *in vitro* assays focused on a target organ of biotransformation such as the liver or gill, as well as *in silico* approaches based on Quantitative Structure Activity Relationships (QSARs)

which are increasingly informed by physiologically relevant assay results. The inclusion of direct physiological measurements of biotransformation can reduce variability within predictive models.[11,12] The work performed within the scope of this thesis focused on refined physiological measurements of biotransformation and the application of such measurements to newer predictive models of biotransformation for the purposes of BCF assessment.

1.1 Toxicokinetics

The term toxicokinetics refers to the time-dependent, quantitative change in a chemical's concentration and disposition within an organism.[13] An in-depth understanding of toxicokinetic processes is key to predictive modeling of chemical perturbation of many biological processes, and sufficiently accurate toxicokinetic models can be useful tools for assessing both overall chemical fate in an organism, as well as target organ toxicity.[14] Toxicokinetics can be characterized as the sum of the processes of absorption, distribution, metabolism, and excretion (ADME), which will be explained in more depth in the following paragraphs.

Absorption can be defined as the introduction of xenobiotics into the systemic circulation of an organism. This process broadly requires compounds to pass cellular membranes or other barriers *via* passive diffusion or active transport mechanisms to enter the blood stream.[15] Due to physiological diversity among species, the relative contributions of different absorption pathways (i.e., branchial, dermal, dietary) can be variable, and contribute to inter- species differences in toxicity of a given xenobiotic.

Within the ADME processes, distribution refers to the movement of xenobiotics from the systemic circulation into the individual tissues or organs of an organism. The primary factor influencing initial distribution following absorption into the systemic circulation is systemic

blood flow (i.e., cardiac output), whilst the final disposition into exact organs is related to the physiology of the organisms (i.e., blood flow distribution, lipid content of individual tissues and organs) and physicochemical characteristics (e.g., lipophilicity, H-bond donor/acceptor potential) of a xenobiotic and thus its affinity for various biological tissues.[16] Many xenobiotics have an affinity for plasma proteins such as serum albumin or globulins within the systemic circulation. Thus, plasma protein binding is of particular significance in determining the bioavailability of xenobiotics, as it is assumed that only the free or unbound fraction of a compound is biologically active.[16] Physiochemical properties such as lipophilicity, molecular size, and pKa can dictate the target tissue of a xenobiotic.[13]

Metabolism describes the biotransformation of a xenobiotic into a compound that is more readily removed from the body. These processes are catalyzed by enzymes grouped into phase I or phase II reactions, depending on their activity.[16] Phase I enzymes often take part in hydrolytic or redox reactions, creating hydrophilic metabolites that are preferred substrates for phase II enzymes.[15] Phase II enzymes then conjugate these products with endogenous molecules *via* glucuronidation, methylation, acetylation, sulfonation, or glutathione conjugation to create highly water-soluble metabolites, thereby facilitating elimination.[15]

The removal of a xenobiotic or its metabolites is defined as excretion. Many xenobiotics in mammals are eliminated by the kidney, with fecal, biliary, and respiratory excretion also contributing. Renal elimination is the sum of two mechanisms, glomerular filtration and tubular secretion. The rate of elimination by glomerular filtration is a function of renal blood flow, and physicochemical characteristics of size and protein binding, which determine if a compound may pass the glomerular pores. Tubular secretion is the process by which compounds not filtered at the glomerulus cross the tubular membrane, facilitated by active transporters located on the

basolateral and apical sides of the membrane. Some lipophilic or polar compounds may undergo tubular reabsorption by these transporters, thus renal clearance is fully described by these concepts. Biotransformation within the liver creates metabolites that are then secreted into the bile, and may eventually be excreted within the feces, contributing to biliary excretion. This process is dictated by biotransformation enzymes within the liver, and is responsible for the elimination of large conjugates more so than smaller metabolites. An additional consideration is the phenomenon of enterohepatic recirculation, in which metabolites are secreted from the liver via the bile duct and thus re-enter the gastrointestinal tract where they are subsequently absorbed within the intestine.[13]

ADME processes vary depending on both chemical and species factors, such as the specific physiology and suite of biotransformation enzymes available within an organism as well as class of chemical. The effect of these on A, D, and E processes is understood within the literature, with existing *in silico* models sufficient for describing these differences.[17] The same cannot be claimed for M (biotransformative processes), the variation of which across diverse chemical classes has been identified as one source of discrepancy within existing predictive modeling approaches.[5] As such, biotransformation represents one of the most significant obstacles to the creation of accurate predictive models of bioaccumulation. However, the recent development of reliable *in vitro* assays of biotransformation has allowed for the investigation of diverse groups of chemicals not previously described in classical biotransformation studies. It has also been suggested that in combination with novel modeling, results of these assays could be extrapolated to *in vivo*.[10,18] The prospective benefits and current limitations of these models are reviewed in Section 1.4.

1.2 Bioconcentration Factor

Historically, quantifying the bioconcentration factor (BCF) of environmental contaminants in live fish has presented several challenges, many of which are inherent to the use of whole animals in testing. Generally, the BCF is calculated as a ratio between the concentration of a chemical within a fish and its environment at steady state; however, multiple assumptions are inherent to this stipulation.[19] The steady state model of BCF assumes that steady-state absorption and elimination kinetics are present and takes bioavailability from water during aqueous exposure into account.[4] The time required to reach steady-state conditions can vary significantly depending on chemical characteristics, water quality, and intraspecies variability. Furthermore, accurate measurements of bioavailable or unbound chemical within the exposure water can be challenging to obtain within both a laboratory setting, as well as in relation to field studies of regulatory significance.[4]

In an effort to minimize these issues, guidelines have been developed to standardize *in vivo* biotransformation studies.[20] Previous studies have sought to understand the contributing factors of variability in BCF, with some proposing benchmarks that help judge the quality of BCF data. The primary criteria identified for review of whole-animal studies are: (1) water analysis, (2) use of radio-labelled chemicals, (3) aqueous solubility, (4) exposure duration, (5) tissue analysis, and (6) several more minor factors related to study design.[4]

Water analysis refers to the practice of quantifying the concentration of chemical available within water during whole-fish exposures, with guidelines suggesting that multiple samples be taken not only during the establishment of exposure conditions, but also at the same time as samples are collected from test organisms.[4] A study which fails to account for the differences in

initial exposure concentration and actual exposure concentrations introduces significant variability in subsequent BCF calculations.[21,22]

The use of radio-labelled chemicals to quantify exposure concentrations within water and fish can lead to increased variability in BCF calculations.[4] The calculation of BCFs must be specific to the parent compound, and failure to differentiate radio-labelled metabolites from parent compound can result in both over- and underestimations of BCF. The presence of radio-labelled metabolites within an organism during sampling can lead to an increase in apparent concentration when mistaken for parent compound, thus leading to an overestimation of BCF. At the same time, mistaking clearance of the radio-labelled metabolite for the parent compound may cause an increase in the apparent clearance of a compound, resulting in an underestimation of BCF.[4]

The aqueous solubility criterion investigates the difference in the concentration of a chemical used in whole-fish exposures compared to its aqueous solubility during exposure. An experiment where the nominal chemical exposure concentration exceeds its solubility will lead to an inaccurate estimation of BCF, as the stated exposure concentration and actual exposure concentration may vary significantly. Furthermore, the aqueous solubility of many industrial contaminants is uncertain within the literature, introducing variability into testing conditions and exposures within standardized BCF approaches. Exposure duration addresses the assumption of steady-state kinetics within whole-fish BCF testing. Whilst up to a 20% fluctuation from the steady-state plateau is acceptable within testing guidelines, many studies are conducted in a shorter timeframe due to logistical overhead, complexity of whole-animal handling, as well as the variability in half-life across chemicals.[4]

The tissue analysis criterion refers to the selection of specific organs, tissues, or whole fish to determine the concentration of a chemical within an organism. Depending on physiochemical characteristics, chemicals will partition to different tissues within a fish following whole-body exposure, and thus the selection of tissue for final BCF determination is critical.[23] Whole-body analysis is considered ideal; however, many studies utilize different methods based on target organs associated with the analyzed chemical. For example, lipophilic compounds such as polycyclic aromatic hydrocarbons partition rapidly into fatty tissues, and as such, total body lipid content must be considered when quantifying the accumulation of these compounds to create lipid-normalized measures of BCF.[24]

The sixth criterion is made up of toxicity, water quality, and the *n*-octanol-water partition coefficient (K_{OW}) of test chemicals. Toxic effects of test chemicals used in whole-fish exposures may perturb biotransformation or elimination pathways, and stress can increase the respiratory rate of fish during exposure. Concentrations well below the half-maximal effect concentration (LC50) must be utilized in order to eliminate variability introduced by toxicity; however, this stipulation can in some cases conflict with the detection threshold of chemical analyses. Variability in water quality conditions can influence the bioavailability of test chemicals during exposures, especially for ionizable organic chemicals, and failing to conform to standardized guidelines can lead to uncertainty within predicted BCFs. Finally, the BCF method generally requires knowledge of the K_{OW} or hydrophobicity of a compound, as it constitutes a simple measure of bioconcentration tendency in support of experimental design.

These criteria are inherent to all whole-fish aqueous exposure tests with the goal of determining BCFs. Whilst Arnot and Gobas et al.[4] observed a relatively high level of confidence across the BCF studies of thousands of chemicals using these criteria, other studies have pointed

out the sometimes-extreme variation in calculated BCFs for the same chemical across multiple studies. Wassenaar et al.[19] reported substantial variation in calculated BCFs when converting the standard deviation of compiled BCF studies to a 95% confidence range, suggesting that a chemical assessed to have a given BCF may in fact fall on either side of a regulatory cut-off value. This range is problematic considering that multiple regulatory frameworks use common threshold values of calculated BCF in their evaluation of PBT status. For example, under the Canadian Environmental Protection Act (CEPA), a substance is considered bioaccumulative when it has a BCF exceeding 5,000.[25] Under the European Union's Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation, thresholds of 2,000 and 5,000 represent bioaccumulative and very bioaccumulative substances respectively.[26] Thus, a chemical assessed as having a BCF of 2,000 and thus categorized as bioaccumulative under REACH criterion, in fact has a 95% confidence range that crosses into both non-bioaccumulative (below 2,000) and very bioaccumulative (above 5,000).[19] Furthermore, these fixed thresholds are applied broadly to all chemicals used within a jurisdiction, rather than only the groups of chemicals which have been historically well described by reproducible *in vivo* BCF studies. The variation in current BCF methods leads to a range of values for individual chemicals that would otherwise result in recategorization according to PBT criteria depending on jurisdiction, for example the 95% confidence range on an *in vivo* BCF assessment of 5,000 may be as high as 13,122 or as low as 1,905.[19] Based on this, two studies for a given chemical performed according to the same guideline (OECD 305) could very well result in two entirely different classifications of said chemical (e.g., non-bioaccumulative and very bioaccumulative). This calls into question the reproducibility and thus validity of the BCF whole-fish exposure method. Thus, improved methods for determining the accumulation risk of chemicals are urgently required.

1.3 Biotransformation

Metabolism within the ADME framework, now more commonly referred to as chemical biotransformation to distinguish the term from the metabolism of endogenous molecules, has been identified as a central and yet currently least understood determinant of bioconcentration of chemicals in aquatic species, specifically fish.[27] The complement of biotransformation enzymes found in fish species is similar to that of mammals,[13] and as such, concepts developed by pharmacologists are often used as the basis for toxicokinetic discussions in aquatic species. As one example, the cytochrome P450 (CYP) superfamily of phase I biotransformation enzymes is diverse, well- studied, and responsible for the biotransformation of many environmental contaminants ranging from industrial chemicals to pharmaceuticals. CYP enzymes are often most concentrated within the liver, making it the primary site of biotransformation for xenobiotics in fish.[13] Liver biotransformation can be quantified as the hepatic clearance of a compound, which is defined as the volume of blood that is quantitatively cleared of the xenobiotic from systemic circulation as a result of the metabolic action of the liver. Multiple approaches to determine or estimate hepatic clearance of chemicals have been developed over the years, which are discussed in more detail in the subsequent sections.

The acceptance of new approaches in predictive toxicology requires those methods to be not only more economical and ethical in animal use, but to also be more physiologically representative of the organism. Most studies attempting to directly compare the outputs of *in vivo*-*in vitro* extrapolation (IVIVE) models with experiments having focused on polycyclic aromatic hydrocarbons (PAHs), a group representing relatively few of the chemicals of environmental concern today.[5] Furthermore, few studies have been conducted focusing on the

biotransformation of pharmaceuticals within an environmental context.[28] Thus, a knowledge gap exists for this diverse class of potential environmental contaminants.

1.3.1 Subcellular fractions, RT-S9

Among the most recent *in vitro* assessment methods are substrate depletion assays using rainbow trout sub-cellular fractions, specifically post-mitochondrial supernatant (RT-S9). Even though similar methods have been used in mammalian systems for decades, this procedure has only recently been standardized within the Organisation for Economic Co-operation and Development (OECD) Guideline for the Testing of Chemicals 319B.[29] This assay has been performed across a group of chemicals ranging from polycyclic aromatic hydrocarbons (PAHs)[30] to diverse pharmaceuticals[28] to fragrance chemicals.[9,31,32] Although the RT-S9 assay has been standardized and validated for use, *in vitro-in vivo* extrapolation (IVIVE) models need to be used to extrapolate RT-S9 biotransformation data to the *in vivo* level. Particularly, the ability of these models to account for extrahepatic biotransformation and observed protein binding across different chemical families has hampered efforts to develop accurate IVIVE models.[5]

1.3.2 Isolated hepatocytes, RT-HEP

Instead of using S9 subcellular fractions, a similar metabolic system based on isolated rainbow trout hepatocytes (RT-HEP) can be used to study chemical biotransformation *in vitro*. These methods, like those based on RT-S9, have recently been standardized within OECD Guideline 319A.[33] While both systems have some intrinsic advantages and disadvantages, both systems are often used interchangeably. The RT-HEP system uses cryopreserved rainbow trout hepatocytes to obtain *in vitro* measurements of the intrinsic clearance of chemicals.

Consequently, there are analytical differences and considerations involved in its specific use cases. The hepatocyte assay does not require added metabolic cofactors, contains intact cell membranes, and in certain conditions can approximate the *in vivo* situation more closely. Conversely, the hepatocyte assay is more challenging to prepare and has a somewhat higher logistical overhead through the requirement for infrastructure to culture, store, incubate, and handle cells. Similar to RT-S9, RT-HEP have been used to characterize the biotransformation of fragrance chemicals.[31] Comparison studies between the two methods have shown low intra- and inter-laboratory variation, as well as similar results when used as the basis for IVIVE modeling.[18] Although only recently described using standardized assays,[18] these findings reinforce the choice of these assay systems for quantifying biotransformation in context of bioconcentration assessments of environmental contaminants compared to the high degree of variability associated with conventional *in vivo* whole-body exposures.

1.3.3 Isolated perfused livers

An even newer approach in predictive biotransformation modeling is that of the isolated perfused fish liver, a procedure which has been used in mammalian studies for decades but has only recently been considered for use in environmental risk assessment and accelerated regulatory screening within the context of industrial chemicals.[30,34] This method focuses on eliminating several sources of uncertainty within *in vitro-in vivo* extrapolation approaches, such as a lack of tissue structural congruity between the *in vitro* and *in vivo* contexts. The isolated perfused liver method allows for the observation of biotransformation within an intact organ under physiological conditions, eliminating the confounding factors of *in vivo* assays such as extrahepatic biotransformation. The isolated perfused liver model also allows for evaluations of

other physiological processes, e.g., changes in perfusion rates of the tissue by varying hepatic blood flow, altering the fraction unbound through addition of graded levels of albumin, and systematic studies of hepatic extraction efficiency using chemicals of different intrinsic biotransformation capacity. Evaluating the hepatic metabolism of chemicals in a system as close to the *in vivo* organism as possible creates greater certainty in biotransformation studies. In addition, the isolated perfused liver method presents the opportunity to screen mixtures of chemicals over the entirety of their relevant biological action or metabolism within the organism, provided the liver model can be maintained at similar physiological condition and performance as the *in vivo* organism.[34]

Prior to investigations by Nichols et al.[30,34] most isolated perfused liver studies were patterned after mammalian studies commonly using rats in pharmacology, and thus failed to account for the specific differences in fish physiology.[34] Specifically, the utilization of a purpose-made clearing solution to purge blood from fish liver prior to perfusion may represent one of the most critical steps to permit the organ longevity observed in subsequent studies. The stability and physiological performance required to generate meaningful biotransformation data from isolated perfused rainbow trout livers was first validated by Nichols et al.[34] who maintained physiological performance over 10 hours of perfusion by incorporating specific optimizations of mammalian liver perfusion protocols for fish physiological condition. This extended time course allows for the investigation of a more diverse group of chemicals, specifically more lipophilic chemicals, compared to shorter-term *in vitro* assays. This robustness has the added benefit of allowing for step-wise experiments that vary perfusion rate or unbound fraction within the same liver conditions and replicate, increasing throughput and physiological validity. Repeated interval sampling as conducted in Nichols et al.[30,34] allows for pseudo-real time analysis of

afferent and efferent samples for physiological signs of metabolism, such as changes in glycogen mobilization or release of major ions. These studies further validate that – although complex – the liver isolation procedure can become consistent and simple with experience, leading to a high rate of successful perfusion and a reduction in animal use. Although successful from a practical standpoint, these studies reported the need for more focused research on the key model assumptions regarding fraction unbound[35] and potential for extrahepatic biotransformation.[5]

1.4 *In vitro-in vivo* extrapolation (IVIVE*)* using the well stirred model

IVIVE modeling approaches are required to bridge the gap between relatively abstract *in vitro* measurements such as intrinsic hepatic clearance, to whole-organism outcomes such as BCF which have regulatory significance. These approaches generally rely upon well described physiologically based toxicokinetic (PBTK) models, into which high quality *in vitro* data is integrated to result in an *in vivo* predictive outcome. One source of uncertainty within these approaches is the parameterization strategy applied, whilst some models require direct physiological characterization of a parameter such as metabolic protein content available (e.g., using a protein assay kit on metabolic material), others may use an empirically derived or QSAR derived value. IVIVE models are typically structured into uptake and elimination rate constants between physiological compartments, which are in turn informed by estimates of activity within each compartment. For example, a simple model of aquatic bioconcentration (Equation 1.1) may define bioconcentration as the ratio of the concentration of a substance at steady state conditions within fish ($C_{FISH,SS}$; mg kg^{-1} fish) and the environment ($C_{W,SS}$; mg L^{-1}).[35]

$$BCF = \frac{C_{FISH,SS}}{C_{W,SS}} \quad (1.1)$$

Thus, the challenge is to estimate substance concentration within the fish at steady state. A more complex model (Equation 1.2) is required, in which the primary contributors are rate constants describing uptake and loss in gills (k_1, L h^{-1} kg fish; and k_2, h^{-1}, respectively), the concentration of chemical within water ($C_{W,TOT}$; mg L^{-1}) and the fraction of this total which is bioavailable (FD; unitless). Finally, the fecal elimination and metabolism are accounted for (k_E, h^{-1};and k_{MET}; h^{-1}).[35]

$$C_{FISH,SS} = k_1 \times FD \times \frac{C_{W,TOT}}{k_2+k_E+k_{MET}} \quad (1.2)$$

Conceptually, each parameter within the above example model may be broken down further into increasingly detailed characterization of uptake and elimination, however in practice the chief contributors to variability within IVIVE approaches have been estimations of systemic bioavailability (FD; unitless), as well as biotransformation rate in organs such as the liver (k_{MET}; h^{-1}).[35]

The well-stirred model of hepatic clearance (Equation 1.3) is a single-compartment simplification to describe the kinetics of hepatic biotransformation.[36] Hepatic clearance (CL_H; L h^{-1} kg liver) is calculated using measures of hepatic blood flow (Q_H; L h^{-1}), fraction unbound or systemic bioavailability (f_U; unitless) and intrinsic clearance (CL_{INT}; mL h^{-1} mg protein or mL h^{-1} 10^6 cells).[35]

$$CL_H = \frac{Q_H \times f_u \times CL_{int}}{Q_H + f_u \times CL_{int}} \quad (1.3)$$

It is predicated on assumptions that negligible extrahepatic biotransformation occurs, and that the target chemical is in instantaneous equilibrium within the liver. Within the well-stirred model, chemicals can be classified by their hepatic extraction efficiency or ratio (E_H; unitless), defined

as the fraction of chemical entering the liver that is removed by biotransformation during its transit through the liver (Equation 1.4). This ratio requires only the concentration of chemical within the afferent blood flow to the liver (C_{AFF}; μM), and efferent blood flow out of the liver (C_{EFF}; μM).

$$E_H = \frac{(C_{AFF} - C_{EFF})}{C_{AFF}} \quad (1.4)$$

In toxicokinetic modeling, chemicals can be classified as high, low, or intermediate extraction ratio compounds depending on the efficiency with which the liver can eliminate them.[30] The extraction ratio is affected by the rate of blood flow to the liver (Q_H; L h^{-1}) enzymatic biotransformation of free chemical (CL_{INT}; mL h^{-1} mg protein or mL h^{-1} 10^6 cells), binding of chemical to plasma and tissue proteins (f_U; unitless), as well as chemical partitioning into blood cells and excretion into bile. The parameters that have received most attention within predictive toxicokinetic models are the variable enzymatic biotransformation of chemicals and the variation in plasma protein binding and thus freely available chemical.[5]

1.4.1 Fraction unbound

In toxicokinetics, the fraction of freely bio-accessible chemical within the systemic circulation is referred to as the fraction-unbound (f_U; unitless). Chemical that is bound to plasma proteins such as albumin is considered unable to act physiologically within the systemic circulation, and thus only free, unbound chemical is considered for the purposes of target organ toxicity, biotransformation, and dispositional processes outside of plasma protein binding.[13] When integrated into IVIVE modeling, fraction-unbound generally represents a correction factor between the systemic bioavailability of an *in vitro* system such as RT-S9, and that of the *in vivo* system.[35] For example, the IVIVE model created by Nichols et al.[35] parameterizes fraction-

unbound as both the ratio of free chemical fraction within blood plasma ($f_{U,P}$; unitless) as well as the *in vitro* S9 or hepatocyte system ($f_{U,S9}$ or $f_{U,HEP}$; unitless). This parameter has created uncertainty within IVIVE models, and can be difficult to predict experimentally using methods based on the hydrophobicity of a chemical. Furthermore, it has been noted that some models approximate *in vivo* conditions better by simply ignoring differences in systemic bioavailability between *in vivo* and *in vitro* assays, by assuming the fraction-unbound of a chemical is essentially equal between both systems calling into question the validity of this parameterization and correction.[5]

1.4.2 Enyzme kinetics

Within the well-stirred model, the action of the liver's biotransformation enzymes and transporters is referred to as intrinsic clearance (CL_{INT}; mL h^{-1} mg protein or mL h^{-1} 10^6 cells). For high extraction ratio compounds such as polycyclic aromatic hydrocarbons (PAHs), hepatic clearance is not limited by the rate of enzymatic clearance, or intrinsic clearance, but instead limited by the blood flow to the liver. Conversely, for low extraction efficiency compounds, hepatic clearance is insensitive to changes in hepatic blood flow, but highly sensitive to changes in intrinsic clearance, e.g., through enzyme inhibition, toxicity, etc. Moreover, some common biotransformation enzymes are saturable and thus calculating hepatic clearance based upon substrate concentration can be complicated by these capacity-limited systems.[13] These established relationships within the well stirred model can be directly investigated in metabolic experiments by using chemicals of varying extraction efficiency and altering hepatic blood flow.

1.4.3 Problems with existing IVIVE approaches

Determining and validating a measure of bioaccumulation within fish species is confounded by both the variability of existing *in vivo* whole fish approaches, as well as potential uncertainties in alternative *in vitro* approaches. Whilst promising new *in vitro* methods have effectively addressed issues of animal use ethics and cost, there is a lack of consensus on how best to use these methods to parameterize the models required to largely replace *in vivo* approaches in chemical risk assessment.[5] As most IVIVE approaches are predicated on existing BCF models and thus seek to predict BCF as their main result, they require the evaluation of all uptake and elimination rate constants, or similar toxicokinetic descriptors. For the purposes of accelerated screening and IVIVE modeling, biotransformation has presented a significant hurdle due to the vast chemical diversity of environmental contaminants.[27] The significant difference between BCFs calculated by *in vitro* approaches and those of conventional whole-fish exposures is further complicated by the reproducibility of *in vivo* methods versus *in vitro* methods, with previous studies suggesting that *in vitro* approaches have greater reproducibility and accuracy than *in vivo*.[18] This calls into question the current modeling paradigm that seeks to further refine *in vitro* methods into producing coherent BCF data that may itself be flawed in concept.

Laue et al.[5] analyzed a number of different parameterization methods and BCF datasets to study the parameters responsible for uncertainty within IVIVE models for BCF calculations. Several of these factors were identical to issues previously described in Sections 1.2-1.4, such as *in vivo* BCF calculations, namely differences in chemical concentrations and species used for *in vitro* assays. These parameters are largely related to study design and experimental methods and are not exclusive to *in vitro* approaches. The crucial factors of uncertainty inherent to *in vitro* approaches were identified as the quantification of unbound fraction, the inability to account for

extrahepatic biotransformation, as well as the observation of saturation kinetics caused by high concentrations of test chemicals (Section 1.4.2).

1.5 Chemicals analyzed in prior IVIVE work

Although a relatively diverse set of chemicals has been investigated to date in IVIVE experiments, varying quality of data presents challenges towards the goal of a unified dataset for evaluation and model development. For this thesis research, we have selected a varied, yet feasible set of chemicals that have been previously studied *in vitro* with a high degree of reproducibility during an international ring trial experiment which took place as part of the adoption of the OECD TG 319A/B guidance (OECD 2017, henceforth referred to as Ring Trial). These chemicals are introduced in the following sections.

1.5.1 Pyrene and other reference chemicals

Polycyclic aromatic hydrocarbons (PAHs) such as pyrene (1) and phenanthrene (2), or alkylphenols such as 4-*n*-nonylphenol (3) are commonly used in both general toxicological studies as well as biotransformation assays specifically.[37]

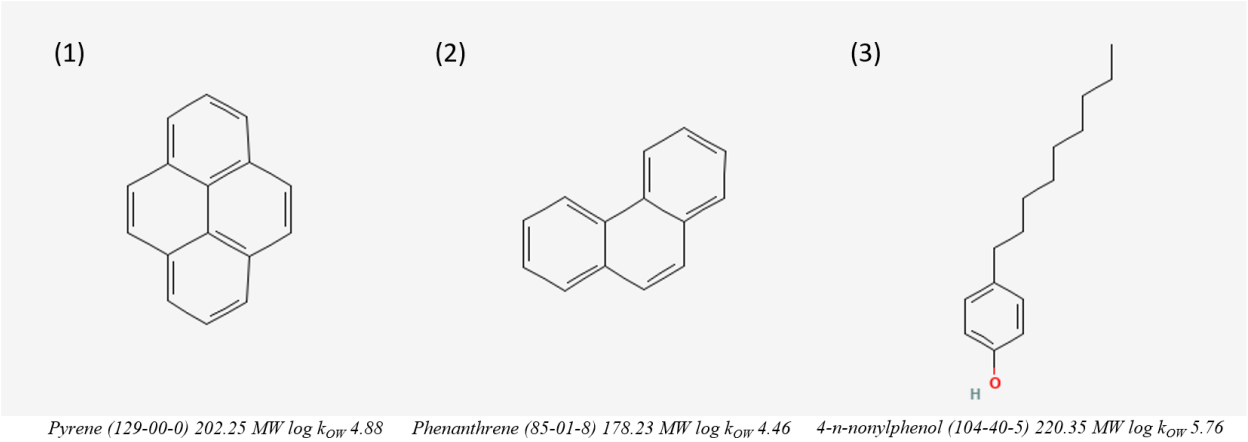
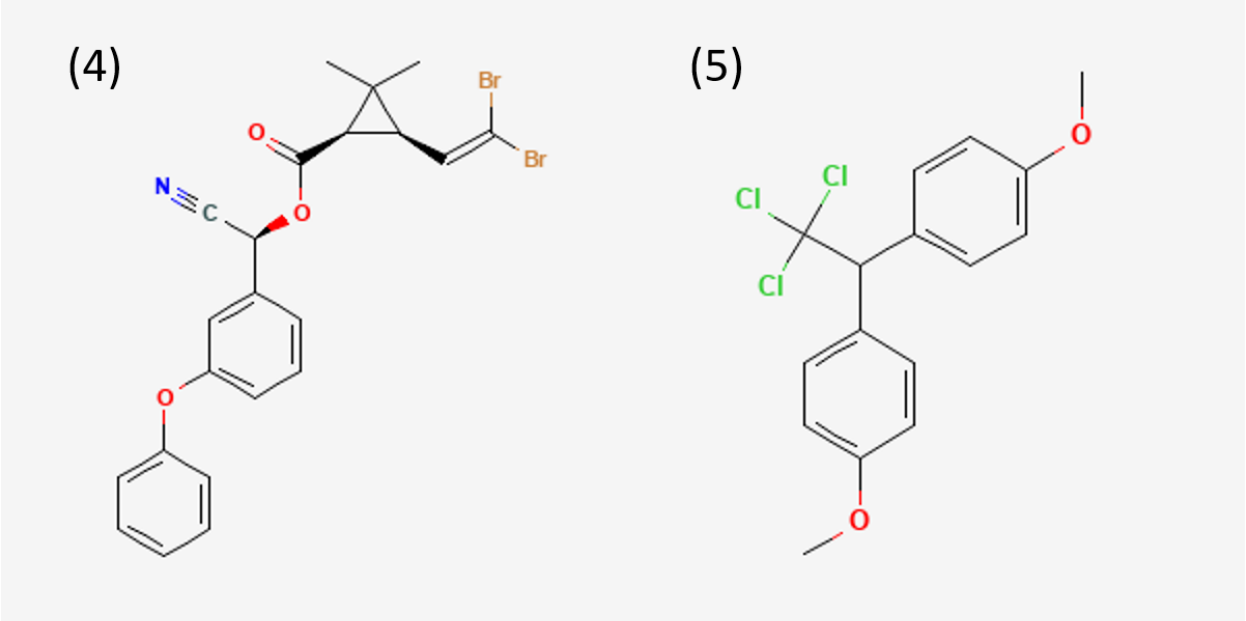


Figure 1.1 Chemical structure and physicochemical characteristics of pyrene, phenanthrene, and 4-n-nonylphenol

This is due to their hydrophobic nature and thus propensity to bioaccumulate, as well as the simplicity of chemical analysis and quantity of existing literature. Furthermore, these chemicals represent common industrial contaminants. PAHs originate from petrogenic or pyrogenic sources, i.e., from spills of fossil fuels or incomplete combustion of organics.[38,39] To date, only one prior study has investigated the hepatic clearance of pyrene or phenanthrene by isolated perfused livers.[30] The proposed study is thus capable of validating and expanding upon this specific chemical. 4-*n*-nonylphenol is representative of the group of alkylphenols. This class of chemicals is considered of environmental relevance due to their extensive use in the production of household detergents, emulsifiers, and other epoxy products.[40] No prior investigation of 4-*n*-nonylphenol exists within an isolated perfused liver system. Deltamethrin (4) and methoxychlor (5) are pesticides representing common hydrophobic environmental contaminants and are included due to prior investigation in the Ring Trial.



Deltamethrin (52918-63-5) 505.2 MW log k_{OW} 6.20 *Methoxychlor (72-43-5) 345.6 MW log k_{OW} 5.08*

Figure 1.2 Chemical structure and physicochemical properties of deltamethrin and methoxychlor

Deltamethrin is a pyrethroid frequently sprayed on ponds to control mosquitos, and is considered highly toxic to aquatic organisms.[41] Methoxychlor is an organochlorine insecticide, banned in various jurisdictions based on PBT criteria in the 2000s. These chemicals serve to increase the chemical diversity of environmental contaminants tested in the proposed study, whilst maintaining comparability with prior *in vitro* testing.

1.5.2 Chemical mixtures

Concurrent work within the Brinkmann lab[42] has demonstrated the ability to detect and quantify the biotransformation of individual pharmaceutical compounds within a mixture using the isolated perfused trout liver model, with good agreement to individual compound substrate depletion methods.[42] Building on this work, the practical detection of multiple chemicals within a perfusion mixture has implications for both accelerated screening and environmental assessment. The feasibility of such an analysis has been questioned in the past, however the recent availability of more sensitive state-of-the-art analytical instrumentation allows for the usage of low concentrations that are less likely to encounter saturation kinetics or other interactions during hepatic biotransformation. Chemical diversity is inherent to relevant mixture compositions, and thus, many possible chemical combinations are considered for mixture analysis and feasibility.

The EPA Non-Targeted Analysis Collaborative Trial (ENTACT) mixture is a diverse mixture of approximately 1,200 chemical substances from the ToxCast library, contained within 10 mixtures each containing between 100 and 400 compounds.[43,44] Developed as part of a multi-phase, cross-laboratory project to enhance the consistent, rapid non-targeted analysis of environmental contaminants. The ENTACT mixture is directly applicable to the study of

biotransformation using isolated perfused livers, representing an opportunity to cross validate the analysis and quantification of chemical mixtures within such a system, as well as generate biotransformation data on a large number of previously unscreened chemicals. Mixtures provide an opportunity to study any group of chemicals that are deemed to be analytically compatible, and the ENTACT mixture is biologically amenable to *in vitro* experiments as well as the isolated perfused liver approach.

1.6 Rationale, objectives, and hypotheses

There is a clear need for alternatives to whole-animal testing for the purposes of environmental risk assessment. Existing methods have significant costs, both ethically and economically, creating barriers to the proper testing and identification of chemicals released into the environment. The development of toxicological methods focused on *in vitro* and *in silico* approaches that accurately represent bioaccumulation within model species while allowing for high throughput screening is key to the future of environmental risk assessment. Using an isolated perfused liver model, the goal of the proposed research was to create and validate a method for determining hepatic biotransformation of diverse chemical mixtures regarding their bioaccumulation potential for use in risk assessment.

1.6.1 Specific Objectives

Objective 1: To compare the biotransformation of pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor within isolated perfused livers and substrate depletion methods. Satisfying this objective provides a starting point for comparison with previous *in vitro* clearance assays.

Objective 2: To validate the detection and quantification of biotransformation of individual chemicals within diverse chemical mixtures in the isolated perfused liver. This objective seeks to demonstrate the high-throughput aspects of the isolated perfused liver model.

Objective 3: To generate a reference dataset of biotransformation data from diverse chemical mixtures using the isolated perfused liver model. Achieving this would contribute immensely to the existing body of biotransformation data for modeling use.

1.6.2 Research hypotheses

H_0-1 a): There is no statistically significant difference in the hepatic clearance of pyrene, phenanthrene 4-n-nonylphenol, deltamethrin, and methoxychlor predicted from substrate depletion assays conducted in the OECD Ring Trial and isolated perfused trout livers performed within the present study.

H_0-1 b): There is no statistically significant difference in the modeled BCF of pyrene, 4-n-nonylphenol, deltamethrin, and methoxychlor as calculated by IVIVE model when using substrate depletion assays versus isolated perfused liver biotransformation data.

H_0-2 a): There is no statistically significant difference in the biotransformation of select mixture chemicals between individual substrate depletion assay experiments and diverse chemical mixture assays of isolated perfused trout livers.

H_0-2 b): There is no statistically significant difference in the modeled BCF of select mixture chemicals between individual substrate depletion assay experiments and diverse chemical mixture assays using isolated perfused trout livers.

CHAPTER 2 VALIDATION OF METHODS FOR *IN VITRO-IN VIVO* EXTRAPOLATION USING HEPATIC CLEARANCE MEASUREMENTS IN ISOLATED PERFUSED FISH LIVERS

PREFACE

The goal of Chapter 2 was to incorporate physiologically relevant, direct measurements of hepatic clearance from the isolated perfused liver model into an existing IVIVE model which could account for difference in protein binding to validate and compare against existing OECD 319 ring trial data for the same set of chemicals. Chapter 2 was prepared in manuscript style and was published in the peer-reviewed scientific journal *Environmental Science & Technology*.

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Author contributions:

Matthew Schultz (University of Saskatchewan) conducted lab work associated with fish dissections, liver perfusions, sample generation, processing, analysis, data generation, figure and table preparation, and drafted the manuscript.

Sophia Krause (Helmholtz Centre for Environmental Research) performed IVIVE data analysis for hepatic clearance data generated in the isolated perfused liver system, and provided comments and edits on the manuscript.

Markus Brinkmann (University of Saskatchewan) provided scientific guidance for the liver perfusion experimental setup, participated in liver perfusion pilot experiments, assisted in apparatus setup, analyzed samples, prepared figures and tables for publication, assisted in

drafting the manuscript, provided comments and edits on the manuscript, and obtained and contributed research funding.

2.1 Abstract

In vitro biotransformation assays using hepatocytes or liver subcellular fractions, combined with *in vitro*−*in vivo* extrapolation (IVIVE) models, have been proposed as an alternative to live fish bioconcentration studies. The uncertainty associated with IVIVE approaches to date has been attributed to assay protocols, model assumptions, or variability of *in vivo* data. An isolated perfused trout liver model that measures hepatic clearance has been proposed for validating IVIVE predictions in the absence of other confounding factors. Here, we investigated the hepatic clearances of five chemicals (pyrene, phenanthrene, 4-*n*-nonlyphenol, deltamethrin, and methoxychlor) in this model and compared measured rates to values predicted from published in vitro intrinsic clearances for validation of IVIVE models. Additionally, we varied protein concentrations in perfusates to test binding assumptions of these models. We found that measured and predicted hepatic clearances were in very good agreement (root mean squared error 16.8 mL h^{-1} g^{-1}) across three levels of protein binding and across a more diverse chemical space than previously studied within this system. Our results show that current IVIVE methods can reliably predict in vivo clearance rates and indicate that discrepancies from measured bioconcentration factors might be driven by other processes, such as extrahepatic biotransformation, etc., and help streamline optimization efforts to the processes that truly matter.

2.2 Introduction

Chemical risk assessment seeks to characterize environmental contaminants based on standardized methods that can be applied by regulators, stakeholders, and researchers. Contaminants in the aquatic environment are often characterized by three information requirements related to their persistence, bioaccumulation potential, and toxicity (PBT).[2] Of

these, studying bioaccumulation potential presents significant challenges due to the costs associated with generating empirical data from whole animal testing, e.g., according to the test guidelines 305 of the Organisation for Economic Co-operation and Development.[20] This guideline outlines methods for determination of the bioconcentration factor (BCF), which is defined as the ratio between a chemical's accumulation within an organism through nondietary routes of exposure compared to its ambient environment. These studies are experimentally challenging and require large numbers of animals, resulting in low throughput.[4] Despite these issues, BCF is still recognized as a standard end point for bioaccumulation studies within risk assessment.[45]

In consequence, BCF studies are not always conducted, and simple models and quantitative structure− activity relationships that estimate BCF based on physiochemical characteristics of chemicals, e.g., the n-octanol−water partitioning coefficient (*log* KOW), are frequently used in chemical risk assessment.[5,26] These models often agree well with empirical models for chemicals that do not undergo biotransformation. For chemicals that undergo biotransformation, however, these estimates are often inaccurate, leading to sometimes dramatic overestimations of a chemical's bioaccumulation potential.[35,46]

Newer modeling approaches and prediction methods seek to eliminate the use of whole fish on both ethical and economic grounds while enabling the incorporation of experimental data from alternative test methods, including those from recently standardized *in vitro* assays using isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes (RT-HEP) or liver subcellular fractions (RT-S9).[18,29,33] Such alternative approaches require extrapolation from a low level of biological organization (*in vitro*) to that of the whole organism (*in vivo*); this process has been termed *in vitro*−*in vivo* extrapolation (IVIVE).[47,48] The goal of these methods is to create data of

sufficient quality for use in environmental risk assessment. However, previous studies have focused on a relatively narrow and well-characterized chemical space (specifically polycyclic aromatic hydrocarbons, PAHs), which is not entirely representative of current risk assessment needs.[30,37,49] Recent studies have sought to expand this understanding using in vitro assays to investigate pesticides, fragrance chemicals, and other emerging contaminants.[31,50,51] Thus, there is a need to expand the validation of IVIVE approaches to a broader chemical space, potentially by using assays of intermediate biological organization to reduce uncertainty.

Incorporating *in vitro* biotransformation data into predictive models through comprehensive IVIVE methods has been demonstrated to enhance predictive performance markedly but is still limited by several factors. One such factor is the influence of chemical binding to lipids and plasma proteins on freely dissolved chemical concentrations and consequently on *in vitro* biotransformation rates. Many researchers in this space found that incorporating the assumption that no difference in binding exists between *in vitro* assays and blood plasma *in vivo*, which is neither supported by theory nor empirical evidence, improved the apparent accuracy of models.[11,52] More recently, however, a consensus has been reached that this practice was misleading and should be avoided, while instead more research is needed to identify the potential reasons for these discrepancies.[5,53] One such reason has been hypothesized to be the potential contribution of extrahepatic biotransformation in organs such as gill and gastrointestinal tract.[13,54] Additionally, the substantial variability of BCF measurements questions the robustness of its use as a comparator for IVIVE predictions.[4,19]

To overcome the current limitations pertaining to the ability to validate current IVIVE approaches, an isolated perfused rainbow trout liver model has been developed that can be used to determine hepatic clearance of chemicals in the absence of these confounding factors.[34] Based

on studies with six PAHs, Nichols et al. showed that hepatic clearances measured in isolated perfused trout livers and those predicted using IVIVE were generally in good agreement.[30]

In the present study, we used the isolated perfused liver model to expand the covered chemical space beyond PAHs by measuring the hepatic clearances of pyrene and phenanthrene (both PAHs, to demonstrate comparability with previous studies), as well as 4-n-nonylphenol, deltamethrin, and methoxychlor. These chemicals were chosen based on both their relative chemical diversity and their previous analysis using standardized in vitro biotransformation assays.[18] Furthermore, we conducted these experiments at three different concentrations of bovine serum albumin (BSA) to investigate the impact of protein binding on free chemical concentrations and thus hepatic clearance.[30]

2.3 Materials and Methods

2.3.1 Organisms

Rainbow trout were acquired as eyed embryos (Troutlodge, Bonney Lake, WA) and raised at the Aquatic Toxicology Research Facility (University of Saskatchewan, Saskatoon, Canada) to suitable size. Fish were fed commercially available size #3 Floating Salmonid Feed (Corey Aquafeeds, Fredericton, Canada) once daily at 1% body weight and maintained at a photoperiod of 16 h light/8 h dark at 12 ± 1 °C. Water chemistry was consistent at pH 7.8, dissolved oxygen >80%, with ammonium, chlorine, nitrate, and nitrite all measuring <0.1 mg L^{-1}. The masses of fish used in this study were between 210 and 440 g, with livers ranging from 1.4 to 4.0 g in mass. Approval from the University Animal Care Committee was obtained (Animal Use Protocol number 20070049), and all animal use was performed in accordance with Canadian Council on Animal Care (CCAC) regulations.

2.3.2 Chemicals

The preparation of perfusion and clearing buffers was adapted from that described by Nichols et al.[30,34] This procedure had been specifically optimized to prolong the perfusion time available for data collection. All chemicals were purchased from Sigma-Alrich, unless noted otherwise. A clearing buffer consisted of a solution of 9.5 g L^{-1} Hank's balanced salts, modified without phenol red, magnesium sulfate, or calcium chloride (HBSS), 2.38 g L^{-1} 4—2(-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.55 g L^{-1} sodium pyruvate, 0.73 g L^{-1} ethylenediaminetetraacetic acid (EDTA), and 17.5 mL of 1 M aqueous sodium hydroxide solution (NaOH) in 1 L of ultrapure water. A perfusion buffer consisted of 90 g of Hank's balanced salts, modified without phenol red, with magnesium sulfate and calcium chloride (HBS), 23.83 g of HEPES, 3.5 g of sodium bicarbonate, 10.0 g of glucose, and 147.0 mL of 1 M sodium hydroxide solution in 10 L of ultrapure water. Control perfusate was gassed for 24 h with carbogen gas (0.5% CO_2/99.5% O_2 mixture), followed by the introduction of bovine serum albumin (BSA) at desired concentrations (1, 2.5, or 10 g L^{-1}) until thoroughly dissolved. Sodium hydroxide and hydrochloric acid were used to adjust and maintain a pH of 7.8 for both clearing and perfusion buffers throughout preparation prior to perfusions.

Pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor all had purities ≥98%. Stock solutions of test chemicals were prepared in acetone, spiked into perfusion buffers at nominal concentrations of 0.1, 0.1, 2.0, 1.0, and 0.32 μM, respectively, and given 24 h to equilibrate prior to perfusions. Acetone concentrations never exceeded 0.05%. Buffers were maintained at 12 °C in a temperature-controlled water bath throughout the experiments.

2.3.3 Liver Isolation and Perfusion

Trout livers were isolated and perfused following methods adapted from Nichols et al.[30,34] Fish were fasted for approximately 24 h and then euthanized with an overdose of buffered ethyl 3-aminobenzoate methanesulfonate (MS 222, 250 mg L^{-1}). Fish were weighed prior to initiating the liver isolation procedure. An initial incision along the ventral midline from the anus to the gill isthmus was made, followed by an incision along the lateral line to the operculum and removal of the muscle flap to expose the body cavity. The intestine was severed at the anus, and the gastrointestinal tract was manipulated to fully expose the liver and esophagus, which was severed to permit the careful removal of the intact organ and viscera for transfer to the perfusion equipment. Any damage to the liver, gallbladder, or hepatic portal vein during this process resulted in rejection from the experiment but was generally rare.

Isolated livers and viscera were transferred in their entirety to a specifically built stainless steel apparatus. The method of full removal and transfer of both liver and viscera and letting perfusate buffer drain freely from the liver was adopted as a change compared to the procedure outlined by Nichols et al.[30,34], who also cannulated efferent blood vessels and collected efferent perfusates from the cannula. This change was employed to reduce exposure of the liver to non-physiological conditions and thus prevent deterioration, as well as to increase the success rate of the procedure as compared to excising the liver and hepatic vasculature alone. The liver and viscera were manipulated to expose the hepatic portal vein within the apparatus, and 5-0 silk suture was placed around the vein and surrounding vasculature as required to secure the vein in place for cannulation. The portal vein was cannulated directly using appropriately sized intravenous catheters secured by the sutures connected to a syringe pump using Tygon tubing,

and the apparatus was placed within a humidified refrigerator maintained at an average internal temperature of 12 °C as monitored by a Type K thermocouple throughout the experiment.

A UP-100 Universal Perfusion System (Harvard Apparatus, Holliston, MA) equipped with a water-jacketed bubble trap was maintained at 12 °C using a recirculating temperature-controlled water bath and was fed by a syringe infusion pump (KD Scientific, KDS 200 Series) using 60 mL BD Luer-Lok syringes. Initially, clearing buffer solution was pumped through at a flow rate of 8.9 mL kg^{-1} body weight min^{-1} to clear the liver of blood. If after 10 min the liver was less than ~95% cleared of blood by visual determination, it was rejected from the experiment and discarded. Once cleared of blood, the perfusate was switched to perfusion buffer spiked with the test chemicals. Afferent and efferent samples were collected in 15 min intervals for the duration of the experiment. Afferent samples were collected by a T-junction sampling port immediately prior to the apparatus chamber. Efferent samples were collected from below the liver in a glass beaker, as the perfusate drained from the liver. Samples of perfusate were analyzed concurrently for pH and glucose over the duration of each experiment using a benchtop pH meter and a hand-held glucose test meter (Contour Next meter, Ascensia, Basel, Switzerland). Glucose efflux and decrease in pH were calculated as indicators of physiological performance of each liver.

Experiments were designed with $n = 3—4$ individual livers per treatment, differing in test chemical, BSA concentration, and duration of experiment. Perfusions followed one of three conditions: (1) perfusion for 2−5 h at 1 g L^{-1} BSA, (2) perfusion for 3 h at 2.5 g L^{-1} BSA, and (3) perfusion for 3 h at 10 g L^{-1} BSA. Concentrations of test chemicals and BSA remained constant throughout a single perfusion experiment.

2.3.4 Sample Extraction and Analysis

For perfusates containing pyrene, phenanthrene, and 4-*n*-nonylphenol, a 333 μL aliquot was taken from both afferent and efferent samples, mixed with 1 mL of chilled acetonitrile (mass-spectrometry grade, Fisher Scientific) in 1.5 mL centrifuge tubes, and stored on ice for the duration of the experiment. Samples were vortex mixed for 30 s and then centrifuged for 10 min at 10 000 ×g. A 1 mL aliquot of the supernatant was removed and transferred to a 2 mL LC vial and stored at 4 °C until analysis using liquid chromatography.

For deltamethrin and methoxychlor perfusates, 500 μL aliquots were taken and mixed with 500 μL of chilled acetonitrile, followed by identical storage and centrifugation compared to prior samples. A 5 μL spike of internal standard (isotopically labeled deltamethrin-(phenoxy-d_5) and hexachlorobenzene-^{13}C6, respectively) was added to each sample. A 500 μL aliquot of supernatant was removed and transferred to a 2 mL LC vial and liquid−liquid extracted using 500 μL of hexanes (ACS grade, Fisher Scientific) by vortex-mixing for 30 s and allowing for phase separation. Finally, a 200 μL aliquot of the supernatant was removed and transferred to a 2 mL LC vial containing a 300 μL glass micro insert for analysis. Blank samples were taken after the addition of BSA, prior to spike chemicals.

Pyrene, phenanthrene, and 4-*n*-nonylphenol were analyzed using an HP Agilent 1100 Series high performance liquid chromatography (HPLC) system with an HP 1046A Programmable Fluorescence Detector. A gradient program ranging from 90% A/10%B to 0%A/100%B at a fixed 0.500 mL minute $^{-1}$ over a period of 35 min was used. Solvent A consisted of HPLC grade water; solvent B consisted of HPLC purity acetonitrile. The following fluorescence excitation/emission wavelengths (nm) were used: pyrene −237/385, phenanthrene −250/390, 4-*n*-nonylphenol −225/315. Data acquisition and peak integration were performed using Agilent

Chemstation software. The quantification of pyrene, phenanthrene, and 4-*n*-nonylphenol was performed based on a seven-point matrix-matched external standard calibration, that is, calibration curves were prepared in perfusate buffer containing the same BSA concentrations. Measured concentrations were generally within ±20% of nominal concentrations.

Deltamethrin and methoxychlor were analyzed using a Thermo Scientific Trace 1300 gas chromatograph equipped with an ISQ 7000 quadrupole mass detector and programmable-temperature vaporizing injector operating in split-less mode. Samples were separated on an Agilent DB-5MS (30 m length, 0.25 mm diameter, 0.25 μm film thickness) capillary column with high purity helium at a flow rate of 1.0 mL min^{-1} as a carrier gas. Data acquisition and processing was performed using Thermo Scientific Chromeleon software in single ion monitoring (SIM) mode.

For the analysis of deltamethrin, the injector temperature, transfer line, and ion source temperatures were 280, 280, and 230 °C, respectively. The gas chromatograph followed a temperature gradient program of 70 °C held for 1 min, ramped at 25 °C min^{-1} to 250 °C, ramped at 5 °C min^{-1} to 280 °C, and held for 8 min. Ions with *m/z* 181 and 186 (retention time, RT, 16.9 min for both analytes) were used for the quantification of native and mass-labeled deltamethrin, respectively. For the analysis of methoxychlor, the injector temperature, transfer line, and ion source temperatures were 270, 300, and 250 °C, respectively. The gas chromatograph followed a temperature gradient program of 90 °C held for 1 min, ramped at 12 °C min^{-1} to 150 °C, ramped at 2 °C min^{-1} to 230 °C, then ramped at 20 °C min^{-1} to 275 °C, and held for 45 s. Ions with *m/z* 227 (RT 43.1 min) and 290 (RT 14.1 min) were used for the quantification of methoxychlor and hexachlorobenzene $^{13}C6$ as internal standards, respectively.

Quantification was performed based on a seven-point calibration standard and recovery-corrected based on internal standard recoveries. Across all analyses for both deltamethrin and methoxychlor, the internal standard recovery ranged from 80 to 120% and measured concentrations were generally within ±30% of nominal concentrations.

2.3.5 Data Analysis

The quantification of hepatic clearance was derived from the total concentration of target chemicals within afferent (C_{AFF}; μM) and efferent (C_{EFF}; μM) samples, yielding hepatic extraction efficiency (E_H; dimensionless; eq 2.1). Calculation of the hepatic clearance was performed according to Nichols et al.,[30] using a body weight normalized perfusion rate (perfusion rate, mL h^{-1} g $liver^{-1}$; eq 2.2) to calculate hepatic clearance of each chemical per sample.

$$E_H = \frac{(C_{AFF} - C_{EFF})}{C_{AFF}} \quad (2.1)$$

$$CL_H = E_H \times perfusion\ rate \quad (2.2)$$

The average hepatic extraction fraction and thus clearance was calculated for each liver, ignoring the initial 60 min of sampling, during which the observed rate of clearance is driven by chemical partitioning rather than biotransformation and therefore not indicative of steady-state clearance. Consequently, the average hepatic clearance values reported were determined based on varying numbers of samples (between 4 and 16) depending on the experimental design. Measurements of glucose efflux as well as pH were used on a qualitative basis to ensure the observed hepatic clearance was the result of physiological activity within each liver, as together these represent an observation of cellular respiration.[30,34]

2.3.6 *In Vitro-In Vivo* Extrapolation

To compare the hepatic clearance values obtained using the isolated perfused trout liver model with values predicted from in vitro data, we applied an IVIVE approach previously published by Krause and Goss.[12] To this end, a well-stirred liver model that explicitly accounts for the blood flow limitation of the perfused livers was implemented in a Microsoft Excel spreadsheet (eq 2.3; attached to this publication in the Supporting Information),

$$CL_H = \frac{Q_H \times \frac{f_{unbound\ blood}}{f_{unbound\ assay}} \times CL_{in\ vitro,int} \times \frac{W_{assay}}{W_{blood}}}{Q_H + \frac{f_{unbound\ blood}}{f_{unbound\ assay}} \times CL_{in\ vitro,int} \times \frac{W_{assay}}{W_{blood}}} \quad (2.3)$$

where CL_H is the body weight-normalized hepatic clearance (mL h^{-1} g^{-1} liver), Q_H is the hepatic blood flow or perfusion rate (mL h^{-1} g^{-1} liver), $f_{unbound\ blood}$ and $f_{unbound\ assay}$ are the unbound chemical fractions in blood and assay media, respectively, $CL_{IN\ VITRO,\ INT}$ is the *in vitro* intrinsic clearance determined using *in vitro* test procedures, and W_{assay} and W_{blood} are the water fractions (mLwater/mLassay/blood) in both assay media and blood, respectively. The term $\frac{f_{unbound\ blood}}{f_{unbound\ assay}}$ or fraction unbound is often referred to as f_U in the literature.[35] $f_{unbound\ blood}$ and $f_{unbound\ assay}$ were calculated based on partition coefficients between assay media and water, as well as liver and water, that were predicted based on combining the contributing sorption to proteins, lipids, and water of the phase of interest as estimated by poly-parameter linear free energy relationships (pp-LFER). This approach allowed us to explicitly account for the differing freely dissolved fractions of the various chemicals between the three different BSA levels that were applied. Using this model, we extrapolated previously published *in vitro* intrinsic clearance values for the chemicals of interest[5,18,55] that were generated using either rainbow trout hepatocytes or RT-S9. For pyrene, an *in vitro* intrinsic clearance value of 1.03 ± 0.12 mL h^{-1} 10^6 $cells^{-1}$ was generated according to

OECD 319A using hepatocytes from the same source of fish as the isolated perfused trout livers (Figure C.1).

For each chemical−BSA combination, we plotted the arithmetic mean and standard deviation of measured hepatic clearance over time (Figures B.1−B.5) and against those predicted using the IVIVE.[12] The root mean squared error (RMSE) was calculated as a measure of the goodness-of-fit. Additionally, we created Bland−Altman plots using Prism 9 software (GraphPad, LaJolla, FL) to estimate potential systematic biases of one method compared to the other.

2.4 Results and Discussion

2.4.1 Physiological Performance of Isolated Perfused Livers

To obtain reliable and robust clearance measurements in isolated perfused trout livers, it is imperative to ensure proper physiological functioning of the organ during the entire experiment.[30,34] A net glucose efflux was measured across all experiments and chemical treatment (Figure 2.1). Values ranged from 2.4 to 79 μmol h^{-1} g^{-1} liver, indicating continuous glycogen mobilization.[13,56] These values are comparable to previously published values by Nichols et al.,[30] which ranged from 8.2 to 72 μmol h^{-1} g^{-1} liver. Furthermore, a continuous decrease in pH was detected between afferent and efferent perfusate samples (Figure 2.1), indicating active respiration of the organ.[56] The drop in pH ranged from −0.20 to −0.01 and was generally more pronounced than previously reported by Nichols et al.,[30] who reported pH decreases ranging from −0.034 to −0.014. These differences might be due to our use of a simple benchtop pH meter, while Nichols et al.[30] used a more sophisticated total blood gas analyzer.

Regardless of the absolute magnitude, the determined decrease in pH and concurrent net glucose efflux together are sufficient to demonstrate physiological performance.

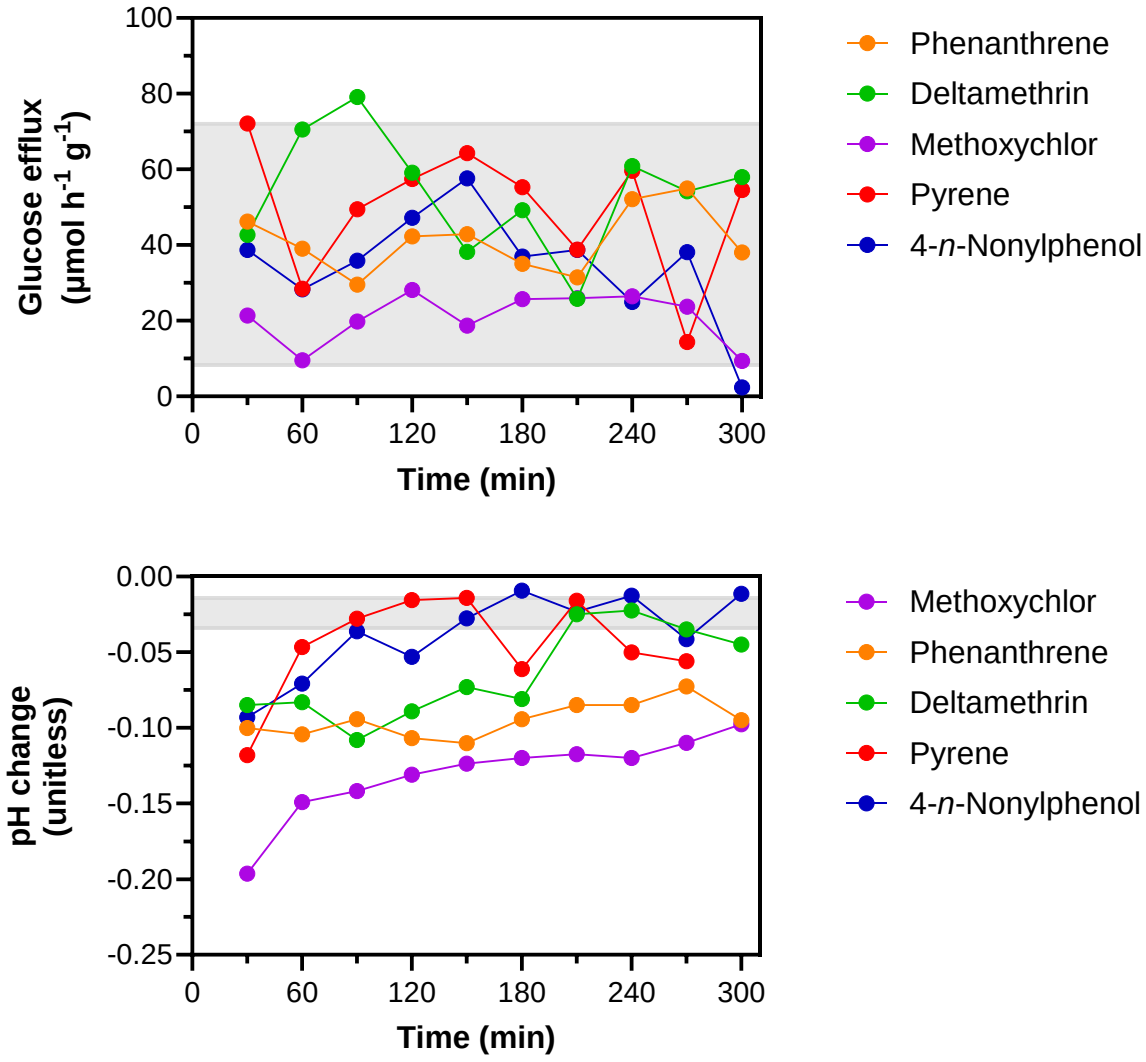

Figure 2.1 Physiological performance of isolated perfused livers was evaluated through measurement of glucose efflux (top) and pH change (bottom) between afferent and efferent samples. Data shown summarizes all perfusions performed across BSA concentrations. Symbols indicate the arithmetic means of all livers across all conditions per chemical. Gray shaded areas indicate the range of reference values from previous studies by Nichols et. al.[26]

2.4.2 Hepatic Clearance in Isolated Perfused Livers

Two sets of chemicals were analyzed in our study: (1) the PAHs pyrene and phenanthrene were studied here to demonstrate proficiency and compare the performance of our experimental setup to the only other published data set by Nichols et al.,[30] while (2) our measurements for 4-*n*-nonylphenol, deltamethrin, and methoxychlor represent the first measurements of hepatic clearance within an isolated perfused trout liver model for these chemicals (Table 2.1, Figures B.1−B.5).

The concentration of BSA changes the amount of free chemical fraction available, which had a significant impact on measured hepatic extraction fractions and clearances of all five chemicals, with the 10 g L^{-1} treatment consistently showing the lowest and the 1.0 g L^{-1} treatment consistently showing the greatest values (Table 2.1). Except for deltamethrin, the 2.5 g L^{-1} treatment consistently fell in between the values measured at 1.0 and 10 g L^{-1}, respectively.

Table 2.1 Experimentally Measured Hepatic Clearance (CL_H, mL h^{-1} g^{-1}) and Hepatic Extraction Fractions (E_H,dimensionless) of the Five Tested Chemicals in Isolated Perfused Trout Livers in the Presence of 1.0, 2.5, and 10 g L^{-1} BSA[a]
[a]All values are expressed as arithmetic means ± standard deviation of n = 3-4 replicate livers per condition.

	CL_H (mL h^{-1} g^{-1})			E_H (%)		
BSA (g L^{-1}	1.0	2.5	10	1.0	2.5	10
Pyrene	46.4±7.44	37.5 ± 3.52	30.2 ± 18.7	80.9 ± 12.5	49.1 ± 8.81	50.1 ± 14.8
Phenanthrene	24.1 ± 5.64	17.1 ± 2.40	9.81 ± 3.63	39.5 ± 13.8	29.6 ± 7.69	18.6 ± 5.00
4-*n*-nonylphenol	76.0 ± 12.7	55.4 ± 17.0	47.1 ± 7.01	99.3 ± 1.47	73.8 ± 18.9	67.5 ± 14.4
Deltamethrin	19.0 ± 8.87	7.54 ± 2.46	12.9 ± 8.87	27.2 ± 10.3	12.4 ± 1.44	21.8 ± 16.2
Methoxychlor	39.4 ± 18.7	33.5 ± 16.9	14.9 ± 6.34	64.1 ± 30.5	60.8 ± 30.6	27.0 ± 11.5

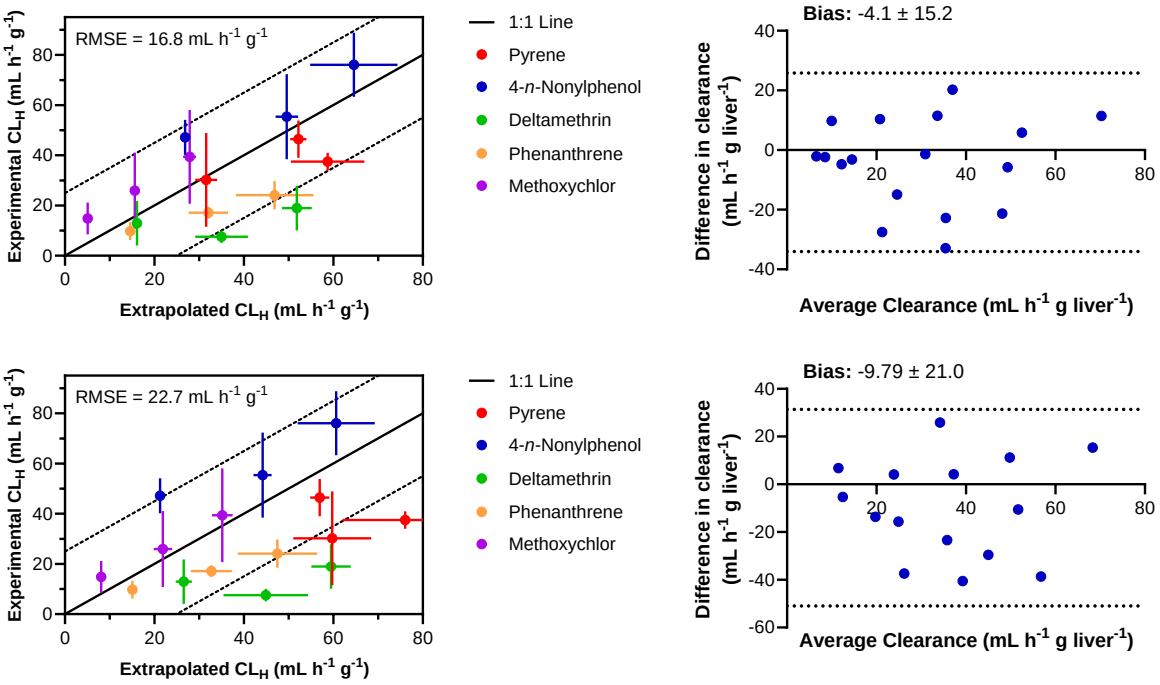

Figure 2.2 Experimental clearance of assayed chemicals in isolated perfused livers compared with extrapolated clearance in isolated hepatocytes or S9. Each chemical is represented by a unique color (see legend) in the presence of 1.0, 2.5, and 10 g L^{-1} BSA through triangles, circles, and squares, respectively. The solid line indicates the 1:1 line; dotted lines correspond to ± the root means squared error (RMSE). Symbols indicate the arithmetic means, error bars the standard deviations of n=3—4 replicate livers per condition. Horizontal error bars represent the variability in extrapolated hepatic clearance values from RT-HEP and RT-S9; vertical error bars indicate variability of clearance measurements across replicate perfused livers. Bland-Altman plots for clearances predicted from isolated hepatocytes or S9 versus measured hepatic clearances were generated to test for systematic differences (biases).

Nichols et al.[30] reported hepatic extraction fractions of 85% and 85% for pyrene and 79% and 54% for phenanthrene at 1.0 and 10 g L^{-1} BSA, respectively, while in our study, we measured hepatic extraction fractions of 80.9 and 50.1 for pyrene and 39.5 and 18.6 for phenanthrene at 1.0 and 10 g L^{-1} BSA, respectively (Table 2.1). While the overall trends agree well between both studies, Nichols et al. measured systematically greater values for both pyrene and phenanthrene. In addition, the authors did not observe the same clear trend in binding dependent clearance, which they speculate could be attributed to saturation under all measured conditions. Indeed, measured concentrations of pyrene and phenanthrene in perfusates were

approximately 0.25 and 1.1 μM, respectively, while those targeted in the present study were somewhat smaller, with nominal concentrations of 0.1 μM for both PAHs. In a different study, Nichols et al.[57] measured the saturable hepatic biotransformation of pyrene and phenanthrene by fitting initial rates of substrate depletion to the Michaelis−Menten model. The Michaelis−Menten constants (K_M; μM) determined in this way were 0.075 and 0.84 μM, respectively, suggesting that slight concentration increases beyond these levels could have marked impacts on the measured clearance rates, thereby explaining the systematic discrepancies between both studies. Indeed, the in vitro intrinsic clearance (1.03 ± 0.12 mL h^{-1} 10^6 $cells^{-1}$) of pyrene (Figure C.1) generated using hepatocytes from the same source of fish as the isolated perfused trout livers reported here is 3-fold lower compared to the average clearance reported by Nichols et al. (3.48 mL h^{-1} 10^6 $cells^{-1}$).[11] Values reported by Nichols et al.[30,34] for S9 in their liver perfusion studies were more in line with those reported in the ring trial.[18] This indicates that the lower overall clearance may be attributed to metabolic variability in the strain of trout used. This might be the result of interstrain variability, as has been previously reported.[58]

In addition to the two PAHs, one goal of this study was to expand upon and validate the isolated perfused fish liver model based on a broader chemical space compared to prior studies. Here, we generated additional hepatic clearance measurements for 4-*n*-nonylphenol, deltamethrin, and methoxychlor, respectively (Table 2.1). Measured hepatic extractions fractions for these chemicals ranged from 27.2 to 99.3% at 1.0 g L^{-1} BSA and 21.8 to 67.5% at 10 g L^{-1} BSA, respectively, generally following the trend 4-*n*-nonylphenol > methoxychlor > deltamethrin. Interestingly, the same trend was not observed when evaluating *in vitro* intrinsic clearance values generated using S9 and hepatocytes, which followed the trend deltamethrin > 4-*n*-nonylphenol > methoxychlor and 4-*n*-nonylphenol > deltamethrin > methoxychlor,

respectively. These apparent discrepancies are the result of differences in protein binding and partitioning, and flow limitations in case of the perfused livers, between the three experimental systems, thereby further underlining the necessity to extrapolate results of *in vitro* assays to the *in vivo* level (or *ex situ*, in the case of isolated perfused livers) using adequate IVIVE models that explicitly represent these aforementioned differences.

2.4.3 Comparison of Measured and Predicted Hepatic Clearance

Previously published *in vitro* intrinsic clearance measurements from the ring trial reports associated with the standardization of OECD guidelines 319A and B were used as inputs for an IVIVE model that was refined from prior studies[35] and described in detail in Krause and Goss.[12] Hepatic clearance values extrapolated from extrapolated from published *in vitro* RT-S9 or RT-HEP intrinsic clearance values were compared with those directly measured in the isolated perfused liver model for both S9 and hepatocyte *in vitro* data separately (Figure 2.2).

As was observed for the impacts of protein binding on directly measured hepatic clearances in isolated perfused livers, hepatic clearance was predicted based on the *in vitro* data and partitioning taking into account the predicted binding in the presence of the different BSA concentrations and followed the same trend. The obvious impacts of protein binding on clearances of all five chemicals, with the 10 g L^{-1} treatment consistently showing the lowest and the 1.0 g L^{-1} treatment consistently showing the greatest values measured values, are another clear indication that setting the term $f = f_U$ unbound assay unbound to 1 is mechanistically inappropriate as it fails to correct for systemic bioavailability. Generally, the agreement between directly measured and extrapolated hepatic clearances was good for both in vitro input data based on hepatocytes and S9 (Figure 2.2), with RMSEs of 16.8 and 22.7 mL h^{-1} g^{-1} liver, respectively.

Deviations of predicted from measured BCFs based on current IVIVE exercises may span several orders of magnitude, especially if the assumption of $f_U = 1.0$ is not entertained.[18] Our data set, on the contrary, indicates that current IVIVE models can be used to extrapolate confidently and quantitatively from in vitro measurements to the organ level, with a coefficient of variation of approximately 20% that falls within the observed variability of standardized in vitro assays.[18,37] Additionally, Bland−Altman analyses indicate that there was very little, if any, systematic bias of 4.1 and 9.8 mL h^{-1} g^{-1} liver between extrapolated and measured hepatic clearances for hepatocytes and S9, respectively (Figure 2.2).

In this light, our data set provides important insights into the potential reasons for the previously observed difference between measured and modeled BCFs. Our study has added data for the three non-PAH chemicals to the breadth of data available for validation of IVIVE models. It has been discussed previously whether uncertainties arose from *in vitro* assays, IVIVE models, or *in vivo* BCFs used for validation, or all of these combined.[5,19] We believe that the present data set provides additional confidence that IVIVE based on current models and *in vitro* assay protocols can yield reliable extrapolations from subcellular preparations or hepatocytes to the organ level, with a level of variation that does not exceed levels of interindividual variability observed in standardized biological test systems. Mismatches between predicted and measured BCFs can therefore be assumed to be rooted in factors beyond the organ level and that have been discussed before,[5,7,18] including neglect of extrahepatic biotransformation, variability in BCFs test designs,[19] enzyme induction during live fish BCF studies, and inaccuracies in partitioning estimates and other kinetic processes (e.g., gill uptake rate constant k_1).

2.4.4 Future Applications and Research Needs

This study has demonstrated that the isolated perfused fish liver model can be reliably used as a tool to quantify hepatic clearance of chemicals. Therefore, this model can be considered valuable for generating high-quality biotransformation data. Without further extrapolation, the obtained hepatic clearance measurements may be used directly as input parameters to physiologically based toxicokinetic models, such as the ones developed in Brinkmann et al.[59]

Furthermore, we show that this quantitative information is useful for validating current approaches for IVIVE. In this way, we demonstrate that current IVIVE models, such as that of Krause and Goss[12] applied here, yield quantitatively accurate predictions of hepatic clearance. That is, if differences in binding between in vitro assays and perfused livers are accurately accounted for by means of pp-LFERs predictors and if flow limitations are explicitly incorporated. In this way, we believe that the data set presented here has the potential to demonstrate whether uncertainties in in vitro assay protocols, IVIVE models, BCFs used for validation, or all these factors combined were the source of commonly observed incongruities in predicted and measured BCFs.

Isolated perfused livers could also be used to further refine IVIVE models, e.g., by systematically studying the dependence of hepatic clearance on perfusion rates. An influence seems plausible based on the assumption that limitations occur through slow desorption of chemicals from plasma proteins, here, albumin, or rate-limiting permeation of chemicals through cell membranes. These factors are not currently considered in most IVIVE models, including the one applied here. Krause and Goss[60] show in their recent publication that both factors could impact the results of IVIVE predictions under some circumstances, and it would therefore be useful to study their impact in the isolated perfused trout liver model in greater detail.

Last, our study has also shown that this method can be expanded to a more diverse chemical space than previously studied. It should thus be the goal of subsequent studies to expand the covered chemical space even further. However, the throughput of the isolated perfused liver model is still limited, and our group has thus begun concurrent work investigating mixtures of chemicals in the isolated perfused liver model, representing a move toward substantially higher throughput screening of environmental contaminants.

2.5 Acknowledgements

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CHAPTER 3 HIGH THROUGHPUT PREDICTION OF HEPATIC CLEARANCE USING ISOLATED PERFUSED FISH LIVERS WITH A NON-TARGET SCREENING MIXTURE

PREFACE

The main goal of Chapter 3 was to demonstrate an expanded domain of applicability for the isolated perfused liver method, principally by successfully analyzing a complex non targeted analysis screening mixture including diverse chemical classes. The materials and methods utilized in this chapter, as well as much of the background information mirror that of Chapter 2 closely due to the overlap in methodologies. The novel aspects of Chapter 3 however are related to the analytical challenges of such a complex mixture as it relates to a biological system such as the isolated perfused liver. The main goal was met in a limited capacity as 20 of the theoretically present 545 chemicals were detected, however this ratio is explained and discussed further in both Chapter 3 and 4. Chapter 3 is intended to be submitted to the peer-reviewed scientific journal *Environmental Science & Technology*, however this submission may require additional data analysis that is considered beyond the scope of the requirements for the MSc program and as such will not be submitted in the state present within this thesis document.

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Author Contributions:

Matthew Schultz (University of Saskatchewan) performed all liver isolation procedures and perfusion experiments, as well as subsequent data generation, data analysis, prepared figures and tables, and drafted the manuscript.

Jonathan K. Challis (University of Saskatchewan) provided analytical feedback and data analysis, as well as provided access to the chemical mixtures utilized in this work.

Jenna Cantin (University of Saskatchewan) assisted with analytical instrument preparation and sample analysis, as well as data analysis.

Markus Brinkmann (University of Saskatchewan) provided guidance on study design, assisted with chemical analysis, data analysis, figures and tables, provided edits and comments on the manuscript, orchestrated the submission of manuscript for publication, and obtained research funding.

3.1 Abstract

Chemical risk assessment often focuses on screening substances for criteria of persistence, bioaccumulation potential, and toxicity (PBT). Of these criteria, bioaccumulation potential presents unique challenges due to the difficulty associated with testing compounds that undergo biotransformation, compounded by the diversity of chemical classes of interest. Current assessment frameworks rely largely upon *in vivo* assays. The adoption of new approach methodologies (NAMs) such as *in vitro* biotransformation assays in combination with *in vitro*-*in vivo* extrapolation (IVIVE) models has been limited due to concerns of overprediction, uncertainty, and limited domain of applicability. These concerns can be addressed using the isolated perfused liver model, representing an intermediate level of biological organization. This model delivers a physiologically relevant measure of hepatic clearance and has been previously validated to demonstrate reductions in uncertainty and overprediction. The present study uses this model to expand the domain of applicability of such methods using a diverse mixture of chemicals to measure hepatic clearance in rainbow trout. Samples were analyzed using liquid-chromatography-high-resolution-mass-spectrometry (LC-HRMS) to semi-quantitatively measure individual chemicals within the mixtures and calculate hepatic extraction fraction. Our results serve to both validate prior *in vitro* methods, as well as demonstrate the potential use of the isolated perfused liver model as a tool for high-throughput bioaccumulation screening within a tiered risk assessment approach.

3.2 Introduction

Bioconcentration is the process through which xenobiotics can accumulate preferentially within biota from their surrounding environment through non-dietary routes of exposure.[61] Typically, this process is either measured empirically, or estimated from quantitative-structure-activity relationships (QSARs) in the earliest stages of chemical risk assessment.[4,20,62] These *in silico* models rely upon estimates of lipophilicity such as the *n*-octanol-water partition coefficient (referred to commonly as *log* K_{OW}), as the fatty tissues of organisms such as fish often sequester neutral hydrophobic chemicals to a greater extent compared to other biological compartments.[63] For chemicals which do not undergo biotransformation, predictive models based upon the linear relationship between *log* K_{OW} and whole-body or tissue lipid content have been used to determine bioconcentration factor (BCF) with great success.[63]

Whilst this approach works well for legacy hydrophobic contaminants of concern, many emerging environmental contaminants fit a different profile such as ionizable organic compounds (IOCs) including many pharmaceuticals, volatile or hard to test chemistries such as UV-stabilizers, or other novel entities which undergo biotransformation.[31,35] The OECD test guideline TG 305[20] provides a means of quantifying BCF within fish using an *in vivo* whole-animal approach and has been adopted as a standardized assay internationally for this purpose, despite concerns regarding animal use, accuracy, and cost. As part of an international effort to reduce, refine, and replace animal testing protocols such as the TG 305 test, the *in vitro* alternative TG 319 test was adopted recently.[29,33] This test provides a means to estimate *in vitro* intrinsic clearance of a chemical using isolated hepatocytes (RT-HEP) or post-mitochondrial supernatant, commonly referred to as S9 subcellular fractions (RT-S9). When coupled with *in*

vitro-in vivo extrapolation (IVIVE) models, this test guideline allows for a relatively robust prediction of BCF.[18]

Several limitations exist within this approach, such as the availability of comprehensive physiological datasets for non-model species upon which IVIVE models rely, as well as uncertainty regarding bioavailable chemical fraction, the contribution of extrahepatic biotransformation or elimination, and concerns over a limited chemical domain of applicability. Recent studies have suggested that differences in freely available chemical fraction can be considered within IVIVE models and accounted for at the organ-level using isolated perfused liver experiments to measure the contribution of systemic bioavailability (commonly referred to as unbound fraction, f_U) towards *in vivo* intrinsic clearance.[53,64] Other studies have sought to address the limited domain of applicability of these approaches, primarily by expanding the number and diversity of chemicals assayed, including mixture conditions.[9,42] As the scale and complexity of environmental contamination increases, and the ability for regulators, researchers, and industry stakeholders to generate high quality bioaccumulation data becomes more pressing, novel approaches such as TG 319A/B require demonstration of higher throughput.

The present study seeks to expand upon the applicability domain of the isolated perfused rainbow trout liver model, which has recently demonstrated validity in both IVIVE outcomes as well as reliability in assaying a mixture of IOCs for hepatic clearance.[42,64] To accomplish this, livers from six fish were perfused with a non-target screening mixture in experiments adopted from previously published methodologies,[30,34,64] and hepatic extraction fraction was determined for a subset of positively identified substances.

3.3 Materials and Methods

3.3.1 Organisms

Rainbow trout (RBT) were obtained from Lyndon Hatcheries (ON, Canada) and kept at the Aquatic Toxicology Research Facility (University of Saskatchewan, Saskatoon, Canada) until suitable size. Water temperature was 12 ± 1 °C with a 16 h light: 8 h dark photoperiod. Fish were fed commercial size #3 Floating Salmonid Feed (Corey Aquafeeds, Fredericton, Canada) once per day at a rate of 1% body weight. Water chemistry was maintained at pH 7.8, dissolved oxygen at >80% saturation, with ammonium, chlorine, and nitrate all below 0.1 mg L^{-1}. Fish used in this study weighed between 220 and 280 g, with wet liver masses ranging from 1.7 to 2.3 g. All experiments conducted in this study were done in accordance with established University Animal Care Committee approval and protocols (Animal Use Protocol 20070049) and Canadian Council on Animal Care (CCAC) regulations.

3.3.2 Mixture Selection and Composition

Reagents required for liver perfusions were obtained from Sigma-Aldrich unless otherwise noted. Preparation of buffers and buffer composition were identical to previously published studies[42,64] using the isolated perfused liver model and are described in detail there. The chemical mixture used within the present study was obtained from the US EPA's Non-Targeted Analysis Collaborative Trial (ENTACT).[43,44] In brief, the ENTACT mixture consists of ten ToxCast mixtures containing between 95 and 365 chemicals each, ranging from amenable mixtures to challenging chemistries with the intention of refining non-target-analysis methods. As these mixtures were formulated in dimethyl sulfoxide (DMSO), they are amenable to biological assays such as the isolated perfused liver method. In the present study, mixtures "503" and "505" were

combined due to concerns of limited volume of chemical stock, as the isolated perfused liver method requires constant perfusion with spiked buffers. Both mixtures were amenable to combination and contained a common selection of 95 chemicals at the same nominal concentration of 50 μM. Spiked buffers were prepared by adding 200 μL of each ENTACT mixture to 1.0 L of perfusion buffer, creating a combined spike concentration of 20 nM of the 95 common chemicals. Due to the complexity of this mixture and lack of comprehensive internal standards for such a large number of chemicals, a set of 25 internal standards covering a similar level of chemical diversity were utilized as follows: Atenolol-D7, Atrazine-D5, Carbamazepione-D10, Chlorpyrifos-D10, Clofibric acid-D4, Clothiandin-D3, Diazinon-D10, Enrofloaxacin-D5, 17β Estradiol-D4, Estrone-D4, 17α Ethynylestradiol-D4, Gemfibrozil-D6, Ibuprofen-D3, Imidacloprid-D4, Josamycin, Ketoprofen-D4, Metaprolol-D7, Naproxen-D3, Propranolol-D7, Sulfamethoxine-D6, Sulfamethazine-13,C6, Sulfapyridine-D4, Sulfamethoxazole-D4, Thiamethoxam-D3 . A final concentration of 1 g L^{-1} of bovine serum albumin (BSA, protease and fatty acid free) was added prior to chemical spiking with mixtures.

3.3.3 Liver Isolation and Perfusion

Liver perfusion followed methods detailed in previous studies,[64] adapted from Nichols et al.[30,34] Fish were fasted for approximately 24 h prior to euthanasia using an overdose of buffered ethyl 3-amino-benzoate methanesulfonate (MS 222, 250 mg L^{-1}). Fish weight was measured prior to liver isolation. As this procedure was identical to prior studies,[42,42] a brief description of this procedure is given.

Fish were dissected from an initial incision near the anus, along the ventral midline to the gill isthmus, followed by an incision to the lateral line and removal of the muscle flap to expose

the internal organs within the body cavity. The intestine was severed proximal to the anus, and the gastrointestinal tract removed to permit the isolation and surgical remove of the liver and associated viscera for transfer to the perfusion apparatus. Within the apparatus, the viscera are manipulated to expose the hepatic portal vein and surrounding vasculature, which is then cannulated using an appropriately sized intravenous catheter secured by sutures within the vein. The apparatus was placed within a climate-controlled refrigeration chamber maintained at an average internal temperature of 12 °C as monitored by a Type K thermocouple throughout the experiment.

A UP-100 Universal Perfusion System (Harvard Apparatus, Holliston, MA) with water-jacketed bubble trap was kept at 12 °C using a recirculating temperature-controlled water bath and was fed by a syringe infusion pump (KD Scientific, KDS 200 Series) using 60 mL BD Luer-Lok syringes maintained at identical conditions.

A total of 6 fish livers were perfused for 3 hours each in the present study at identical chemical spike and BSA concentration. Whilst this duration is shorter than prior validation experiments, it still allows the observation of both partitioning driven clearance within the first hour of perfusion, followed by two hours of largely steady state clearance. Typically, fish are perfused at a rate equal to 8.9 mL kg^{-1} body weight min^{-1};[64] however, due to concerns regarding total volume of chemically spiked stock, this rate was halved in these experiments. Previous unpublished trials using a selection of chemicals from prior IVIVE validation experiments at varying flow rates demonstrated that hepatic clearance within the isolated perfused liver system varies based upon hepatic blood flow and thus flow rate in a predictable manner. Due to the limited chemical stock, a compromise between suitably robust experimental replicates and lowered flow rates was used. As a result of this change, it can be assumed that some chemicals

assayed within the mixture experiments may have exhibited flow-limited clearance. Samples were taken from both afferent and efferent lines at an interval of 15 minutes in parallel with measurements of glucose and pH change to verify physiological performance of the isolated perfused livers.

3.3.4 Sample Extraction and Chemical Analysis

Sample extraction and analysis was informed by and adapted from Bourgeois et al.[42] and follows similar procedures. A total volume of 450 μL of both afferent and efferent samples were taken, with 50 μL of internal standard mixture added, followed by 500 μL of ice-cold acetonitrile in a centrifuge tube. Blank samples were taken of each perfusate mixture after the addition of BSA and prior to the introduction of chemical spikes, as well as prior to the addition of internal standard mixture. Centrifuge tubes were kept chilled on ice, before being vigorously vortexed, followed by centrifugation for 10 min at 10 000 ×g to remove protein precipitates. Then, 200 μL aliquots of the supernatant were transferred into a 2 mL liquid-chromatography vial containing a 300 μL glass micro-insert and stored for analysis at -20 °C.

Chemical analysis was performed using a Vanquish UHPLC Liquid Chromatography system coupled to a QExactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher, Mississauga, ON). LC gradient elution and parameters is given in Appendix C.3. A Suspect-Target-Screening (STS) method was used with the following positive electrospray ionization (ESI) source parameters: sheath gas flow = 35; aux gas flow = 10; sweep gas flow =1; aux gas heater = 400 °C; spray voltage = 3.8 kV; S-lens RF = 60; capillary temperature = 350 °C. Data-dependent MS2 (ddMS2) was performed with the following Full MS/ddMS2 scan settings: 60,000/15,000 resolution, automatic gain control (AGC) target = $5x10^5/1x10^5$, max injection

time = 100 ms/100 ms, full scan range of 70-1,000 *m/z*, MS2 isolation window = 2.0 *m/z*, loop count = 5, and a stepped NCE (normalized collision energy) = 15, 30, 45.

3.3.5 Data Analysis

Data analysis for all samples was conducted using Compound Discoverer 3.3 (Thermo Fisher Scientific), using a data processing workflow adapted from Cardenas et al., (MSc thesis, available online). This method relies upon mass spectra deposited in the Thermo database mzCloud for identification. Chromatographic peaks were manually inspected after feature identification, and were removed on the basis of normalization compared to blank samples. Criteria for peak selection and further data analysis was the presence of an identified feature from within the ENTACT mixture, as well as consistent detection and peak area within afferent samples. This provision validates that all analysis was performed on chemicals that were both present within the ENTACT mixture, as well as present in all afferent samples and thus actually flowing into the isolated perfused liver.

Of the 6 perfusion experiments, a single replicate failed to meet this criterion and was identified as a mechanical failure to perfuse completely. Extraction fractions were based upon the difference in apparent peak area between afferent and efferent samples (Equation 3.1) for n = 5 liver treatments.

$$E_H = \frac{(C_{AFF} - C_{EFF})}{C_{AFF}} \quad (3.1)$$

3.4 Results and Discussion

3.4.1 Physiological Performance of Isolated Perfused Livers

All liver perfusions within the present study exhibited a trend of decreased pH and an increased glucose between afferent and efferent samples, (Figure E.1) indicating aerobic respiration and thus physiological performance.

3.4.2 Hepatic Clearance in Isolated Perfused Livers

The chemicals consistently detected in both afferent and efferent samples came primarily from mixture 503 and included several chemicals present in spiked perfusate at the highest nominal concentration of 20 nM. Whilst the ENTACT mixture was designed as a challenging non-target analysis screening test, the chemicals present in each mixture are inherently amenable to screening together in a biological test system[44], and thus the expected number of chemicals screened for hepatic extraction fraction within the present study is limited largely by the refinement of the data analysis and feature detection suite used. Of the 545 compounds present (5 control samples, 90 common compounds between mixtures 503 and 505, 90 compounds unique to 503, 360 compounds unique to 505), only 20 were detected consistently enough to calculate steady-state extraction fraction, as this calculation requires identification of chemical within both afferent and efferent samples at all timepoints within the experiment. This ratio between expected and detected compounds is not unexplained, however. Within the original EPA ENTACT study, 1,074 out of a total of 1,269 spiked substances were correctly identified within the ten ToxCast mixtures used within ENTACT.[44] Of these 1,074 substances, positive ESI methods (as used in the present study) were responsible for identifying approximately 450 compounds.[44] Combined with the matrix effects of both a biological test method and relatively

unrefined method of feature analysis, a substantial decrease in the ability to consistently detect and semi quantify compounds is expected. Detected compounds showed a wide range of chemical classes, including pharmaceuticals, fungicides, herbicides, and pesticides. Measured hepatic extraction fractions from this subset of chemicals showed quantitative or near-quantitative clearance (an arbitrary ceiling of 85% mean hepatic extraction fraction representing a rapidly metabolized compound) for all detected chemicals within the ENTACT mixtures except pioglitazone and PD-0333941(anti-diabetic pharmaceuticals), laurolactam (polyamide monomer), secbumeton (triazine herbicide), and pirimicarb (carbamate insecticide) (Figure 3.1). Despite this, our results show, for the first time, the application of a non-target screening method to a biological test system designed to assay the hepatic clearance in fish. Whilst prior studies within the isolated perfused liver system had demonstrated the ability to assay mixtures as effectively as individual compounds, this was limited to nine psychotropic pharmaceuticals of relatively similar chemical class.[42]

3.4.3 Limitations of Mixture Approaches

A common shortfall of mixture experiments in biological systems is the need to validate that chemicals in mixture are behaving similarly to individual applications.[65,66] The primary means of avoiding confounding additive, synergistic, or antagonistic effects is to utilize concentrations of chemicals within mixture below the limit of enzyme or transporter saturation. Whilst prior mixture studies in the isolated perfused trout liver validated that hepatic clearance of individual compounds was identical to the same compounds in mixture, the present study did not.[42] Experiments with large non-target screening mixtures such as ENTACT necessarily preclude such validation experiments – if it were feasible to assay such chemicals individually, mixture

experiments would have less merit regarding high-throughput. Furthermore, the environmental reality of aqueous exposure is indeed a complex mixture of co-occurring chemical stressors that include diverse chemicals of varying mechanism of action and apparent clearance mechanism.

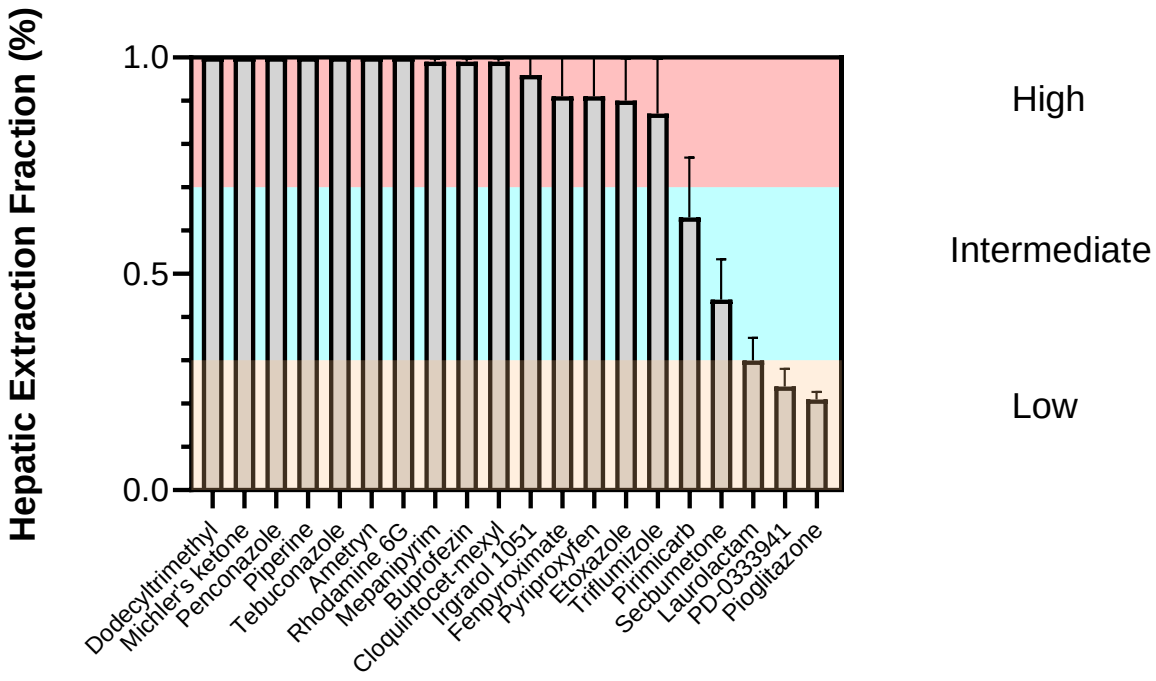

Figure 3.1 Hepatic extraction fraction of detected chemicals within ENTACT mixtures 503+505. Ranges are given for High (70-100%) Intermediate (30-70%) and Low (0-30%) hepatic extraction fractions. Data are mean ± SEM of *n*=5 replicate livers. Error bars are clipped at 1.0, representing the maximum possible hepatic extraction fraction.

3.4.4 Future Applications and Research Needs

Our results demonstrate both higher throughput, and expanded domain of applicability for the isolated perfused liver method of assaying biotransformation capacity in rainbow trout. Whilst these experiments do not produce a BCF or similar B-metric quantification of bioaccumulation potential for regulatory purposes, it has been suggested recently that bioaccumulation assessment may increasingly rely upon direct physiological measurements or *in*

vitro measurements of intrinsic clearance.[9] As such, a key future research need would be the determination of *in vitro* intrinsic clearance utilizing methods such as the OECD TG 319 to compare with the isolated perfused liver results of this study. This comparison would serve to further validate both approaches, and highlight the strengths and weaknesses of each within the context of bioaccumulation assessment. Approaches such as the isolated perfused liver model have now been demonstrated to address many of the concerns regarding uncertainty of IVIVE predictions and limited domain of applicability that have prevented the widespread adoption of methods rooted in the reduction, refinement, and replacement of vertebrate animal testing.[42,64] Several knowledge gaps exist in both the IVIVE prediction models, as well as the knowledge of relevant chemical space.

3.5 Acknowledgements

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CHAPTER 4 GENERAL DISCUSSION

4.1 Thesis objectives and overview

The objectives of this thesis focused largely on a demonstration of proficiency with a novel approach for bioaccumulation screening – the isolated perfused rainbow trout liver model – as well as generation of new data in support of chemical risk assessment. These research goals have been satisfied by the results of the studies structured within this thesis document, and are detailed below.

In terms of specific objectives, Objective 1, the comparison of clearance characteristics of pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor within the isolated perfused liver model has been satisfied by the data presented and discussed in Chapter 2. Specifically, the strong agreement between hepatic clearances measured within the isolated perfused liver system with those extrapolated from *in vitro* assays demonstrated that the latter adequately predicts *in vivo* outcomes, implying that the uncertainty associated with IVIVE modeling is due to extrahepatic contribution or modeling assumptions. Conversely, a lack of agreement in these values would indicate The results of that the *in vitro* methods themselves may introduce variability or fail to represent the biotransformation processes of the trout liver. Chapter 2 also indicated that a critical modeling assumption – the correction factor between protein binding of the *in vitro* assay compared to the *in vivo* system, can be investigated and accounted for in a more mechanistically correct fashion than prior models had assumed. This objective related strongly to an ongoing debate within the field of aquatic bioaccumulation, and bioaccumulation science in general regarding the adoption and use of novel-approach-methods (NAMs). Within the field of bioaccumulation assessment, the NAM in question is the OECD Test Guideline 319A/B, an *in vitro* assessment of intrinsic hepatic clearance from which a BCF

can be estimated using IVIVE models. Despite adoption by the OECD in 2018, this test guideline is still not widely accepted by chemical regulators, the reasons for this are debated heavily. Some experts regard this test as being too abstract or not adequately representing the physiology involved in a process as complex as biotransformation, and caution that the results cannot be interpreted identically as the standard *in vivo* OECD Test Guideline 305. Within the context of this thesis document, this project has created invaluable data for the purposes of back-validating this specific NAM and sought to contribute information in support of the reliability and mechanistic basis of such an approach, with the overall goal of furthering adoption of tests that fit criteria of reduction, refinement, and replacement in vertebrate animal testing.

Objective 2 of this project was in principle, to detect individual chemicals within a diverse chemical mixture in a complex biological system, the isolated perfused rainbow trout liver. Building upon concurrent work by undergraduate research projects that investigated mixture experiments within the same approach, this objective has been satisfied by the data discussed within Chapter 3. Specifically, the diversity of the chemicals evaluated simultaneously from within the EPA ENTACT mixture shows that the isolated perfused liver model combined with modern HRMS methods represents a powerful screening tool approach.

Finally, Objective 3 was to generate a reference dataset of biotransformation data from diverse chemical mixtures, a task which is heavily related to Objective 2 in principle, and thus will be discussed jointly.

4.2 Validation of *in vitro-in vivo* extrapolation

Many sources of uncertainty have been identified to explain the discrepancies between *in vitro* or *in silico* predictive models and BCFs measured *in vivo*, such as inconsistency in gill

uptake rate (k_1), the assumption of systemic bioavailability (fraction unbound; f_U), enzyme saturation, cross-species differences in biotransformation capacity, as well as the contribution of extrahepatic biotransformation. These discrepancies often manifest as a trend of overprediction, in which IVIVE models based upon reliable *in vitro* assay data tend to result in a greater predicted BCFs compared to those obtained from conventional *in vivo* standardized testing. As a result of this trend, recent studies have focused on elucidating which uncertainty factors contribute to this apparent overprediction. The isolated perfused liver model addresses some but not all of these uncertainty factors.

One of the primary sources of uncertainty within IVIVE modeling to date is the contribution of the fraction unbound (f_U) or systemic bioavailability. Typically, fraction unbound is estimated from *log* K_{OW} using quantitative structure-activity relationships (QSARs); however, many studies have shown that QSAR-estimated measurements of fraction unbound result in a less reliable prediction of BCF than simply assuming identical systemic bioavailability (f_U=1.0) between *in vivo* and *in vitro* test systems. In this context, reliability or predictivity refers to alignment with existing *in vivo* BCF experiments. Whilst prior IVIVE studies have either failed to adequately quantify the systemic bioavailability of chemicals assayed for biotransformation, or simply made the mechanistically implausible assumption of identical *in vivo* and *in vitro* bioavailability (f_U=1.0) to reduce overprediction, the results of Chapter 2 show conclusively that this parameter can be a significant contributor to variation in hepatic clearance within the rainbow trout liver. This gives substantial credence to models which attempt to include systemic bioavailability in predictions of both hepatic clearance and overall bioaccumulation potential, as opposed to simply equalizing both *in vivo* and *in vitro* bioavailability to align IVIVE models with *in vivo* results.

The systematic exclusion of extra-hepatic biotransformation in the isolated perfused liver model has the ability to address its contribution to overall whole-body biotransformation. This factor primarily influences *in vivo* assays of BCF, as standardized *in vitro* assays to date utilize substrate depletion methods using hepatocytes and liver sub-cellular preparations and thus necessarily exclude extra-hepatic biotransformation. For example, chemicals may undergo biotransformation in the gill structures, prior to entering the systemic circulation and thus being available for biotransformation within the liver. Furthermore, the influence of gill biotransformation on predicted BCF is confounded by various uncertainties in model parameterization, an issue which is inherent to compartmental models using uptake and elimination rates. The gill surface represents an initial site of biotransformation for many xenobiotics which then enter the systemic circulation and thus may contribute to a first-pass effect. In practice, this contribution is minor due to gill blood flow outpacing gill biotransformation for all but the most rapidly metabolized xenobiotics. These predictions for the gill epithelium to biotransform xenobiotics vary by chemical class, differences in enzyme capacity between gill and liver, as well as the contribution of water chemistry, the latter of which is a common issue in whole animal *in vivo* testing approaches. A similar situation occurs in the GIT, another site of extra-hepatic biotransformation. The isolated perfused liver model can be thought to account entirely for hepatic biotransformation, thus allowing mass-balance models or similar physiologically based toxicokinetic (PBTK) model approaches to refine predictions of systemic effects by using reliable and validated measures of hepatic biotransformation capacity. Effectively, a reliable measure of hepatic biotransformation suggests that uncertainty in an overall bioaccumulation prediction may come from other biological compartments or IVIVE parameterization.

IVIVE approaches rely upon characterization of the underlying organism model, usually using PBTK modeling to determine factors such as blood flow, or inherent biotransformation capacity of tissues such as the gill or liver which are necessary for predictions. Rainbow trout has been adopted as the standardized test organism for many bioaccumulation assessments; however, the question remains regarding the applicability of these methods to other non-model species. IVIVE models have been validated in rainbow trout alone; however, additional *in vitro* studies in common carp, catfish, and other species have been performed. These studies have been largely conducted using subcellular fractions rather than isolated hepatocytes due to the difficulty associated with primary cell culture across species, and thus have not been incorporated into IVIVE models to the extent that rainbow trout have. These *in vitro* studies cannot be readily extrapolated to BCF or similar measures of bioaccumulation potential due to the lack of species characterization for IVIVE modeling to extrapolate from. In contrast, the isolated perfused liver model requires relatively little characterization. To arrive at a reliable measure of intrinsic hepatic clearance, species-specific estimates of hepatic blood-flow, as well as optimizations of buffer composition are needed. Thus, the isolated perfused liver model may be more directly comparable to existing *in vitro* test methods with regard to cross-species comparisons in terms of outcome. With the growing trend towards tiered assessment strategies and de-prioritization in screening, the ability to reliably and rapidly quantify *in vitro* measures of biotransformation capacity instead of BCF or similar bioaccumulation metrics becomes more important in regulatory use.

Regarding enzyme saturation, advances in modern analytical chemistry allow for the use of chemical substrate concentrations well below the Michaelis-Menten affinity constant (K_M). Prior isolated perfused liver studies using PAHs resulted in possible saturation kinetics for pyrene,

however this trend was avoided in the current study by use of lower chemical concentrations within spiked buffers. The use of pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor (i.e., the same compounds used in the validation ring trial of the OECD TG 319 assay) allowed for a comparison of predicted and measured hepatic clearance, in which the isolated perfused liver results displayed equivalent variability to that of an international collaborative ring trial, further reinforcing the confidence in these results.

4.3 Relative performance and viability of mixture experiments in the isolated perfused liver

The use of mixture experiments in this study was complicated by both logistical factors around the acquisition, use, and limited volume of chemical available. Whilst the ENTACT mixture utilized is amenable to biological assays by virtue of being in solution in DMSO, it presented several challenges in analysis due to the sheer complexity of data analysis and analytical chemistry skills required to reliably semi-quantify compounds within this mixture. Despite this, 20 compounds were successfully identified for the use of just six fish.

Concurrent studies using the isolated perfused liver model with a psychotropic drug mixture of nine compounds demonstrated the validity and applicability of such mixture experiments. Whilst *in vitro* test guidelines such as OECD TG 319 can accommodate mixture experiments, such experiments are uncommon due to these associated difficulties. Chemical substrate concentrations below the Michaelis-Menten constant are necessary, as well as a validated analytical method to detect and quantify individual compounds within a complex mixture. Advances in analytical chemistry and the use of non-targeted screening methods have addressed these issues, allowing for detection and quantification of very low concentrations of chemical substrates, as well as identification of similar compounds (i.e., pharmaceuticals) within a

complex mixture. Effectively, spiked mixtures of perfusate within isolated perfused liver experiments present a relatively "clean" mixture profile to analyze, compared to non-target screening methods which typically are applied to environmental samples or complex effluents, increasing the overall confidence in these mixture experiments.

One noted advantage of the isolated perfused liver method is the intact physiological nature of the liver, as compared to *in vitro* test methods using suspensions or homogenizations of hepatocytes of post-mitochondrial supernatant. This intact physiology not only allows for direct physiological measurements of hepatic clearance as discussed previously, but necessarily leaves the various cellular and extracellular membranes intact. Epithelial exchange surfaces within the gills, skin, or gut may play a role in the biotransformation or disposition of xenobiotics within fish. This role is particularly important for ionizable organic compounds (IOCs) which can be fully or partially ionized at physiologically relevant pH values, with many pharmaceuticals found within municipal wastewater treatment effluent falling under this chemical category. The intact membrane physiology of the isolated perfused liver can thus be used to investigate other forms of biotransformation and elimination and their role in bioaccumulation assessment. For instance, active transport has been identified to play a significant role in the elimination of IOCs and other xenobiotics. By contrast, the RT-HEP and RT-S9 methods may lack substantial contribution of these active transporters. Thus, by modulating the activity of these active transporters, differences in the clearance potential of chemical classes, such as IOCs, can be investigated, adding value to this method in investigating physiologically relevant measures of biotransformation and bioaccumulation. Bourgeois et al.[42] demonstrated this using Cyclosporin A (CsA), a broad inhibitor of active transport processes. Hepatic clearance was measured in the presence and absence of CsA to determine which compounds within the psychotropic drug

mixture were reliant upon active transporters for biotransformation and elimination. This result was verified with *in vitro* experiments using RTL-W1 cells measuring the inhibition of Calcein AM efflux (an ATP-binding cassette transport protein substrate). These experiments suggest that active transport can be investigated as an additional layer within a tiered risk assessment strategy to create a more comprehensive understanding of what processes drive the bioaccumulation potential of a chemical of interest.

4.4 Isolated perfused liver approach as a NAM in chemical risk assessment

The isolated perfused liver model shows much promise regarding its application in support of chemical risk assessment; however, it is unlikely to be considered for adoption as a standardized approach for numerous reasons. This approach inherently requires vertebrate animal testing, and is thus incompatible with the forward-thinking non-vertebrate NAM adoption presently being debated by regulators globally. Furthermore, it requires localized or on-site animal use, as opposed to the TG 319 approaches which allow for cryopreserved material to be sourced from a central lab facility or commercial vendors for standardized testing globally. However, the isolated perfused liver approach shows incredible promise for continued research for the purposes of localized screening or development of bioaccumulation models, and reduces animal use despite reliance on vertebrate testing. The high throughput of this approach lends itself to various applications such as larger scale environmental screening, in which environmental mixtures such as wastewater effluents or spill run-off may be rapidly screened for preliminary bioaccumulation assessment. This method also eliminates substantial sources of variability associated with TG 305 and similar tests, such as extrahepatic biotransformation and

other complexities of *in vivo* experiments. Overall, it has been demonstrated that this approach has a place within bioaccumulation assessment beyond the scope of a standardized assay.

4.5 Limitations of current approaches and recommendations for future research

The isolated perfused liver method has demonstrated potential for reliable investigation of biotransformation capacity across a diverse domain of applicability, with equivalent reliability compared to presently adopted *in vitro* methods. A considerable limitation of this approach compared to these previously adopted methods is the logistical considerations of the method. It is necessary to obtain fresh livers from a healthy population of model fish species. Whilst this limitation is technically common to recently standardized *in vitro* assays (OECD TG 319), these assays result in a stable, cryopreserved suspension that is able to be generated within a central laboratory or facility housing animals, then shipped globally for standardized regulatory tests. The isolated perfused liver method by contrast requires a similar up-front logistical consideration (i.e., animal husbandry facilities and skilled personnel); however, it has been demonstrated as part of TG 319 adoption that frozen liver preparations exhibit reduced biotransformation capacity compared to fresh liver preparations. Thus, it must be stated that the isolated perfused liver model is, when compared to current assessment strategies, unsuited for widespread regulatory adoption on this logistical basis alone. Furthermore, mixture experiments have inherent confounding factors related to analytical challenges of even amenable mixture constituents.

An additional limitation of the isolated perfused liver approach is that without detailed characterization of the species used for IVIVE modeling, it results in a simple measure of intrinsic hepatic clearance. Whilst this measure is important for estimating bioaccumulation, intrinsic clearance itself is only the starting point for IVIVE predictions which seek to estimate

BCF or other bioaccumulation metrics for regulatory use. Whilst there are presently no published studies investigating the isolated perfused liver model in other model or non-model species, the adjustments and refinements required are largely physiological in nature, and thus any species with sufficiently described toxicokinetics may be utilized in a modified protocol.

A significant drawback of the isolated perfused liver approach is that it is inherently a vertebrate testing methodology. As regulatory bodies such as ECHA and US-EPA seek to eliminate all vertebrate testing by varying deadlines, there is a growing debate regarding the future of present vertebrate research in favor of invertebrate methods. It is highly unlikely that an invertebrate adoption of a similar method would be feasible, and thus there is concern that despite falling under reduction, refinement, and replacement (of whole animal testing) considerations, the isolated perfused liver method is at the end of the day, a vertebrate test which some would consider having a limited life span within present regulatory frameworks. Despite this, there is considerable debate regarding the adoption of equivalent vertebrate tests for bioaccumulation assessment, and methods such as the isolated perfused liver serve to directly inform future biological tests for biotransformation by investigating mechanistic assumptions such as fraction unbound or systemic bioavailability.

One strong avenue of future research is towards the exploration of environmental mixtures within the isolated perfused liver model. Indeed, the ENTACT mixture investigated here represents a complex, confounding mixture designed to challenge analytical methods that have been developed to analyze environmental samples. Advanced methods such as passive environmental sampling may be used to create diverse chemical mixtures from diffuse sources such as water bodies, wastewater effluents, road run-off, or other environmental contaminant sinks. These mixtures may then be applied to the isolated perfused liver method, possibly

utilizing fish species of specific concern within these localized environments to determine their bioaccumulation potential and thus conduct rapid and efficient risk assessment.

Because the isolated perfused liver model determines hepatic clearance by measuring the depletion of a chemical of interest whether individually or in mixture, it is also equally suited to measuring the metabolites of these compounds. Although not quantified within any experiments in the present study, the investigation of a "perfused liver xeno-metabolome" is theoretically of great value for both mixture effects, as well as cross-species investigation of metabolic pathways for contaminants of interest. Furthermore, the isolated perfused liver method lends itself to this approach by not requiring additional sampling overhead or logistical considerations to determine metabolites, they can instead be measured within the same set of samples that are used to determine intrinsic hepatic clearance.

4.6 Concluding statement

Although it is arguably presently unsuited to adoption in risk assessment frameworks, the isolated perfused liver approach presents incredible potential as a localized screening tool, allowing for the relatively rapid screening of complex mixtures for hepatic biotransformation. With a growing trend towards the inclusion of measures such as intrinsic hepatic clearance or hepatic extraction fraction in tiered assessment strategies, the isolated perfused liver methodology represents a physiologically valid means of reliably determining biotransformation potential within fish species.

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APPENDICES

Appendix A. Experimental apparatus and workflows

A.1 Liver isolation procedure and perfusion apparatus

The liver isolation procedure was initially adapted from Nichols et al., with several notable modifications to suit the laboratory setup at the University of Saskatchewan Toxicology Centre. Whilst Nichols et al., perfused the liver within the body cavity and cannulated both the hepatic portal vein and hepatic artery to achieve flow throughout the liver, our study instead severed the hepatic artery, allowing drainage from both the hepatic artery as well as diffuse flow through the liver exterior. Furthermore, instead of using surgical adhesives to hold the cannula in place in the hepatic portal vein, our study utilized 5-0 surgical sutures with varying techniques to secure the cannula. These differences are primarily related to dissection skills and speed, and are not believed to significantly impact the perfusion.

A conceptual diagram of the overall experiment including apparatus setup, perfusate flow, and measured endpoints is provided below (Figure A.1) What follows is a detailed explanation of the key elements of this perfusion apparatus.

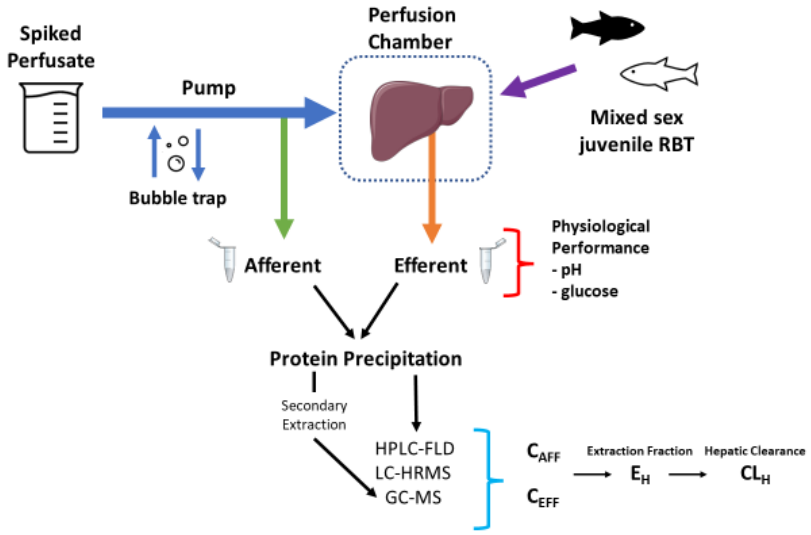

Figure 3

Figure A.1 Conceptual experimental workflow, including transition from physical experimental procedures to downstream data endpoints.

The perfusion chamber consisted of a repurposed miniature refrigerator, with perfusate lines traversing the weather stripping along the door edge to create a 12°C environment. Due to the need to open this door to sample, the temperature inside was set to 10°C to create an average of 12°C. A temperature-controlled water bath was maintained at 12°C using a chiller and fed by a sink tap, this bath also contained 1 L bottles of spiked perfusates. 60 mL BD Luer-lock syringes were filled from these perfusate bottles and placed within a KD Scientific KDS-200 series syringe pump, calibrated for 60mL BD syringes and set at varying flow rates to achieve a physiologically relevant hepatic flow rate. This rate was typically 8.9 mL kg^{-1} body weight min^{-1}, resulting in a range of flow rates between 1 and 4 mL min^{-1} commonly. The perfusate flow then enters an UP-100 Universal Perfusion System (Harvard Apparatus, Holliston, MA) with water-jacketed bubble trap to maintain 12°C as close as possible to the liver. All tubing utilized in these experiments was made of low-leaching Tygon. Prior to entering the perfusion chamber, a T-junction with resealable

screw cap was utilized to take afferent samples. An in-line flow monitor (Sensiron SLF3S-1300F, SF06 based) was utilized to ensure steady flow to the liver. Within the perfusion chamber, the isolated liver and G.I. tract is placed within a stainless-steel mesh on an elevated apparatus to allow for perfusate to drip into collection beakers for sampling. Humidity was maintained within the chamber using a desktop humidifier.

Appendix B. Individual chemical hepatic clearance data

B.1 Hepatic clearance of pyrene, phenanthrene, and 4-*n*-nonylphenol, deltamethrin, and methoxychlor

Time course dependent clearance data for pyrene, phenanthrene, and 4-*n*-nonylphenol, deltamethrin, and methoxychlor are provided below (Figures B 1.1-1.5). These figures demonstrate the region of steady-state clearance (typically 60 minutes after perfusion begins) that follows a rapid distribution phase in which clearance is driven by chemical partitioning into the liver tissue, rather than true metabolic clearance.

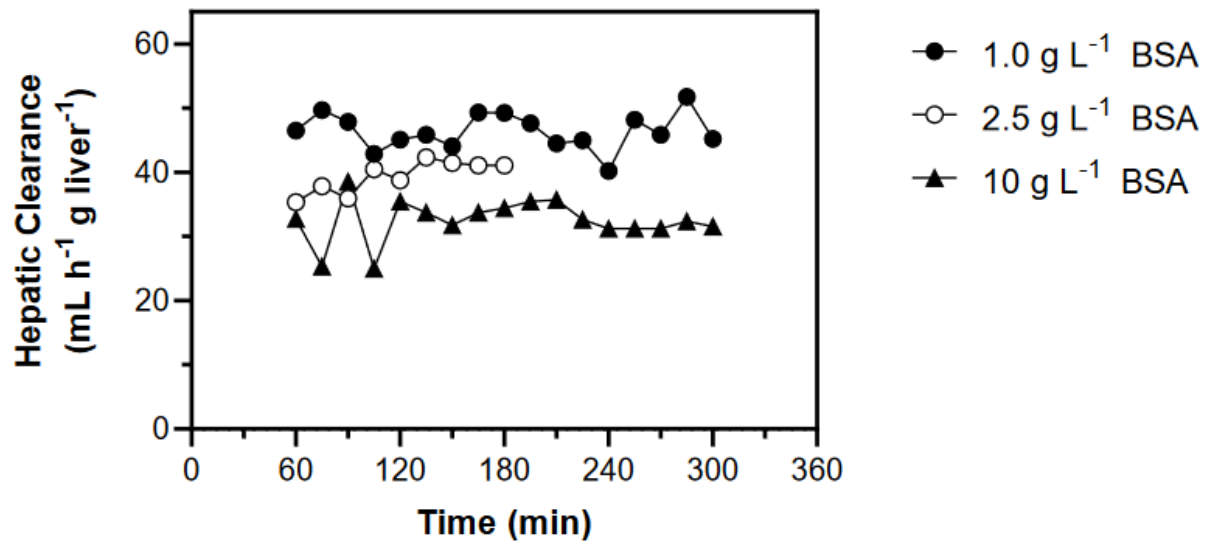

Figure B.1 Experimental hepatic clearance of pyrene in isolated perfused trout livers over time. Clearance was measured in the presence of 1.0, 2.5, and 10 g L^{-1} BSA as indicated through closed circles, open circles, and triangles, respectively. Symbols indicate the arithmetic means of *n*=3—4 replicate livers per condition.

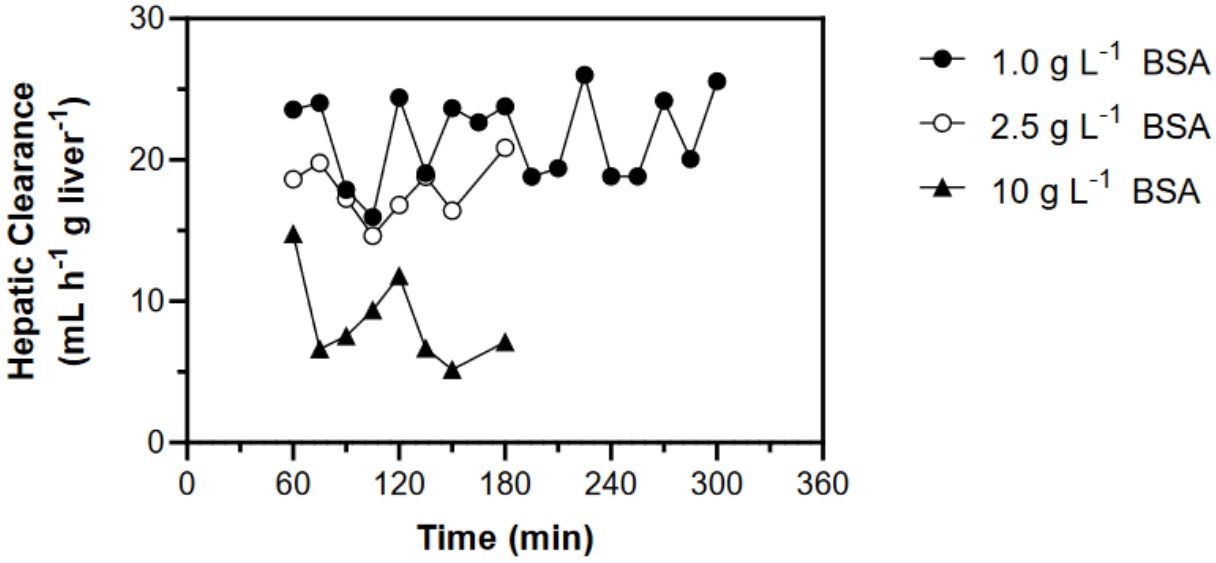

Figure B.2 Experimental hepatic clearance of phenanthrene in isolated perfused trout livers over time. Clearance was measured in the presence of 1.0, 2.5, and 10 g L^{-1} BSA as indicated through closed circles, open circles, and triangles, respectively. Symbols indicate the arithmetic means of *n*=3—4 replicate livers per condition.

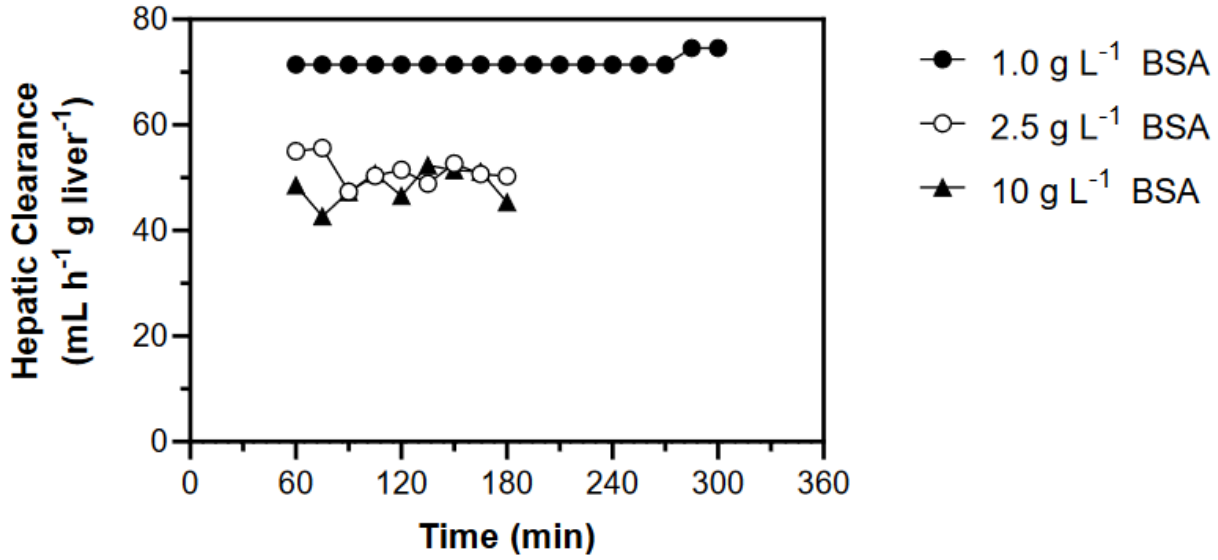

Figure B.3 Experimental hepatic clearance of 4-*n*-nonylphenol in isolated perfused trout livers over time. Clearance was measured in the presence of 1.0, 2.5, and 10 g L^{-1} BSA as indicated through closed circles, open circles, and triangles, respectively. Symbols indicate the arithmetic means of *n*=3—4 replicate livers per condition.

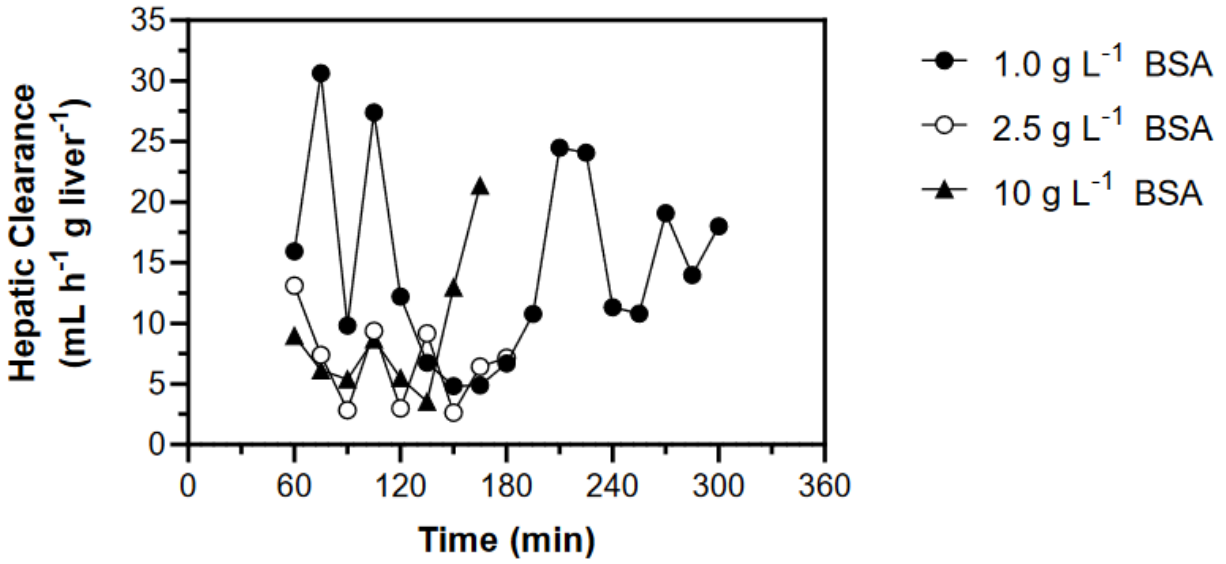

Figure B.4 Experimental hepatic clearance of deltamethrin in isolated perfused trout livers over time. Clearance was measured in the presence of 1.0, 2.5, and 10 g L^{-1} BSA as indicated through closed circles, open circles, and triangles, respectively. Symbols indicate the arithmetic means of *n*=3—4 replicate livers per condition.

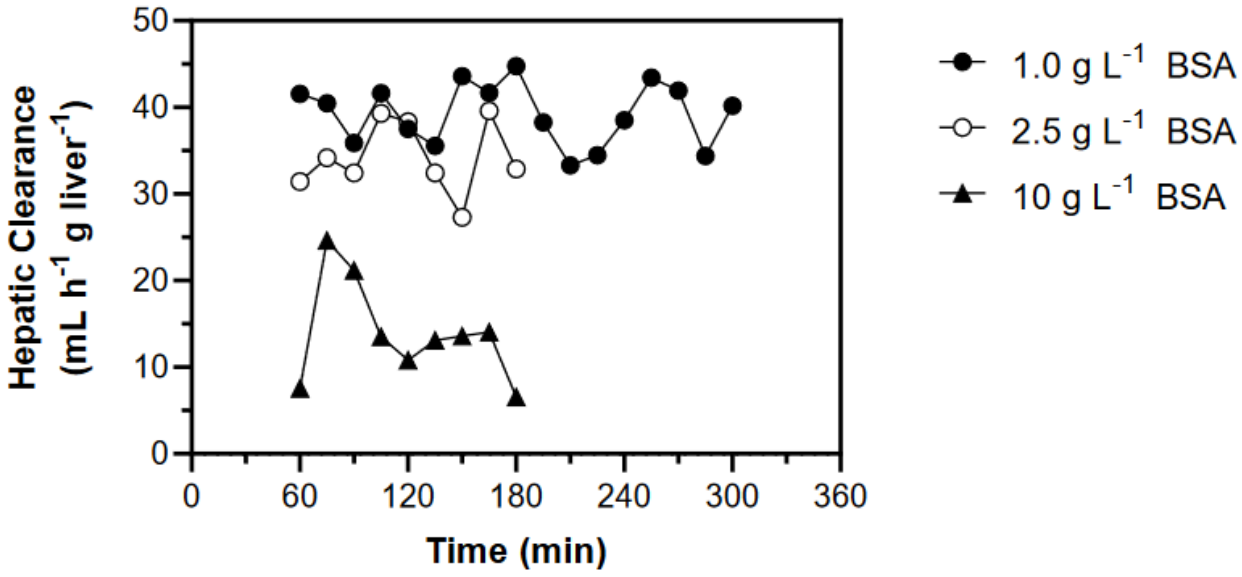

Figure B.5 Experimental hepatic clearance of methoxychlor in isolated perfused trout livers over time. Clearance was measured in the presence of 1.0, 2.5, and 10 g L^{-1} BSA as indicated through closed circles, open circles, and triangles, respectively. Symbols indicate the arithmetic means of *n*=3—4 replicate livers per condition.

Appendix C. *In vitro* substrate depletion data

C.1 In vitro intrinsic clearance data for pyrene

An OECD 319A standardized *in vitro* intrinsic clearance assay was performed using pyrene with the same stock of rainbow trout as used in isolated perfused liver experiments. The purpose of such an assay is to compare the metabolic performance of the population of rainbow trout used, as well as validate comparisons between the isolated perfused liver method and standardized *in vitro* assays for the benchmark PAH pyrene.

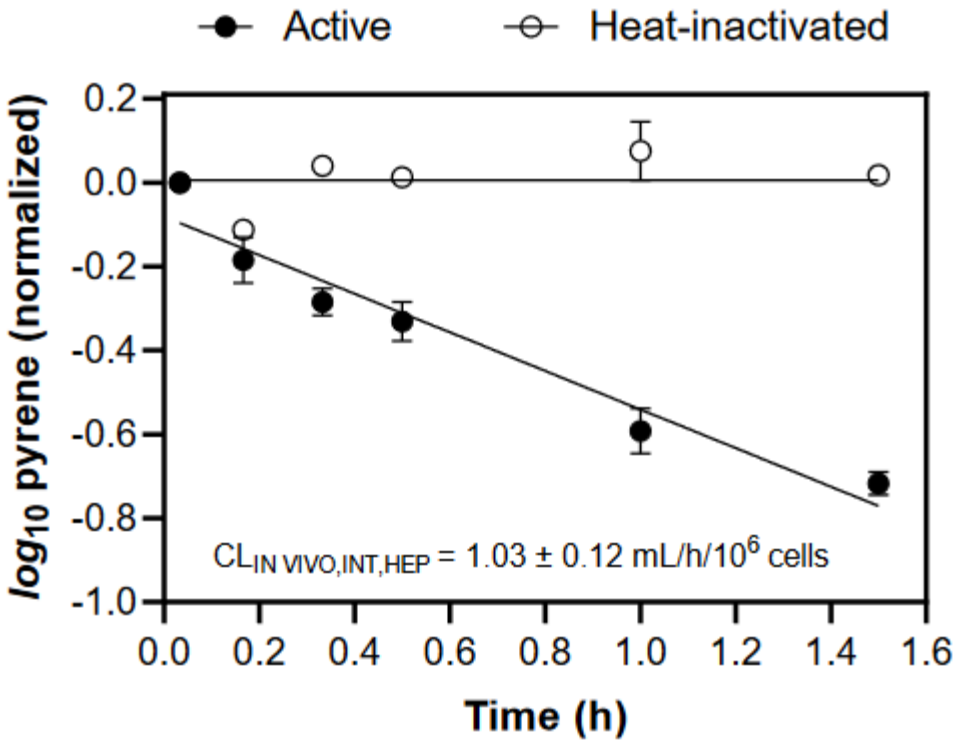

Figure C.1 Log_{10}-transformed substrate depletion curves for pyrene obtained *in vitro* assays with rainbow trout hepatocytes in the presence of live (closed circles) or heat-inactivated (open circles) hepatocytes. Symbols indicate the arithmetic means, error bars the standard deviations of *n*=3 replicate vials per condition. The average *in vitro* intrinsic clearance was determined from these curves and is also reported on the graph.

Appendix D. ENTACT mixture experiment data

D.1 Chemical analysis of US EPA TOXCAST ENTACT Mixtures "503" and "505"

The US EPA TOXCAST ENTACT project distributed ten mixtures of varying constituents, formulated at a nominal concentration of 20mM in dimethyl sulfoxide (DMSO). Exhaustive details of this mixture's formulation, rationale, and uses outside the scope of this paper are available within Ulrich et al.[44] For the purposes of this thesis, mixtures 503 and 505 were utilized for several reasons. Firstly, a sufficient volume of each of these mixtures was available to create the several liters of spiked perfusates required to perfuse a number of fish which would also allow for statistical significance. Secondly, these mixtures contained a common 95 chemicals

consisting of 5 non-target method replicate control samples, and a set of 90 replicate chemicals. Importantly, these chemicals were not part of any “challenge mixtures” utilized by the ENTACT project which would have created isobaric interferences, purity issues, or otherwise be non-amenable. A full list of the chemicals utilized within these mixtures is provided. [link to excel sheet]. Mixture 503 consisted of 5 control replicates and 90 replicate chemicals. Mixture 505 consisted of 5 control replicates, 90 replicate chemicals, and 360 unique chemicals. Thus, the total of 545 chemicals, however in reality the 95 chemicals of the replicate sets were present at double the concentration of the 360 unique chemicals of Mixture 505. This was accounted for and desired, and the results indicated that the consistent detections were primarily from this increased concentration chemical set.

D.2 Hepatic clearance of ENTACT mixture

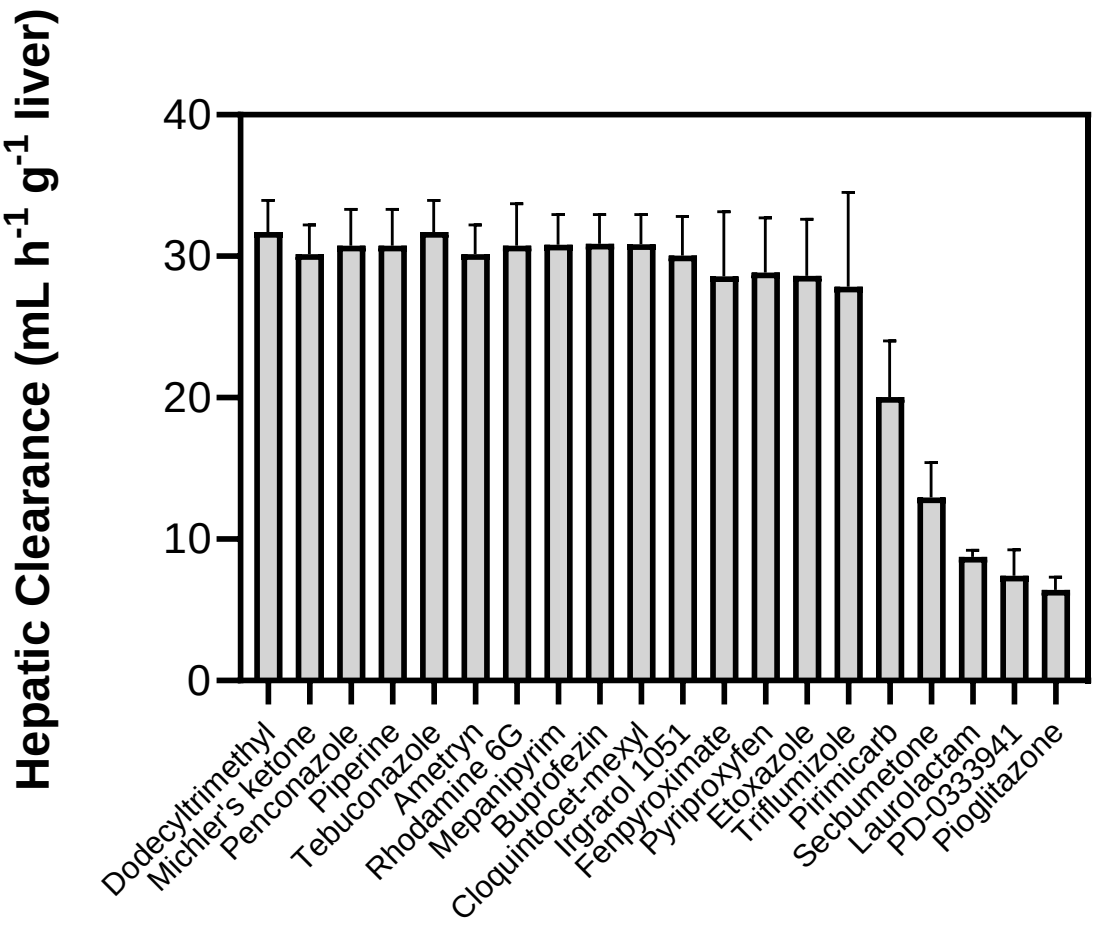

Figure D.1 Hepatic clearance (CL_H) of 20 chemicals consistently detected within afferent and efferent samples. Data are arithmetic mean ± SEM for *n*=5 liver perfusion experiments.

The above figure represents steady state conditions, and were derived following the initial one-hour "distribution phase" of the liver perfusion, during which clearance is driven primarily by chemical partitioning and not by true biotransformation. Time dependent data is provided below for a selected set of chemicals which showed intermediate to low hepatic extraction fraction or clearance. (Figures D 2.2-2.3)

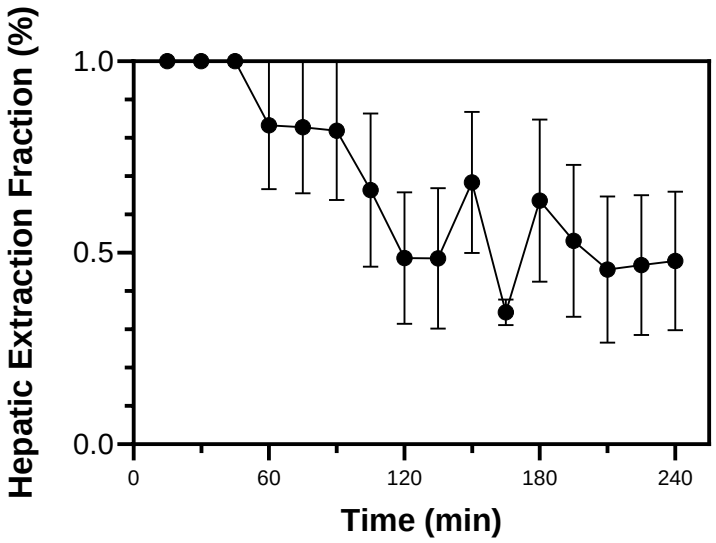

Figure D.2 Hepatic extraction fraction over time of Pirimicarb in *n*=4 liver perfusion experiments. Dots represent mean, error bars represent standard error.

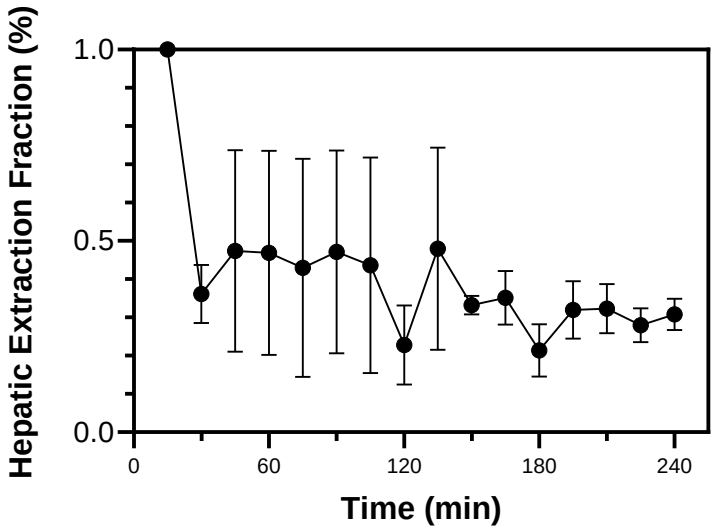

Figure D.3 Hepatic extraction fraction over time of Laurolactam in *n*=3 liver perfusion experiments. Dots represent mean, error bars represent standard error.

Appendix E. Physiological Measurements of Liver Perfusion Experiments

E.1 pH and glucose measurements of ENTACT experiments

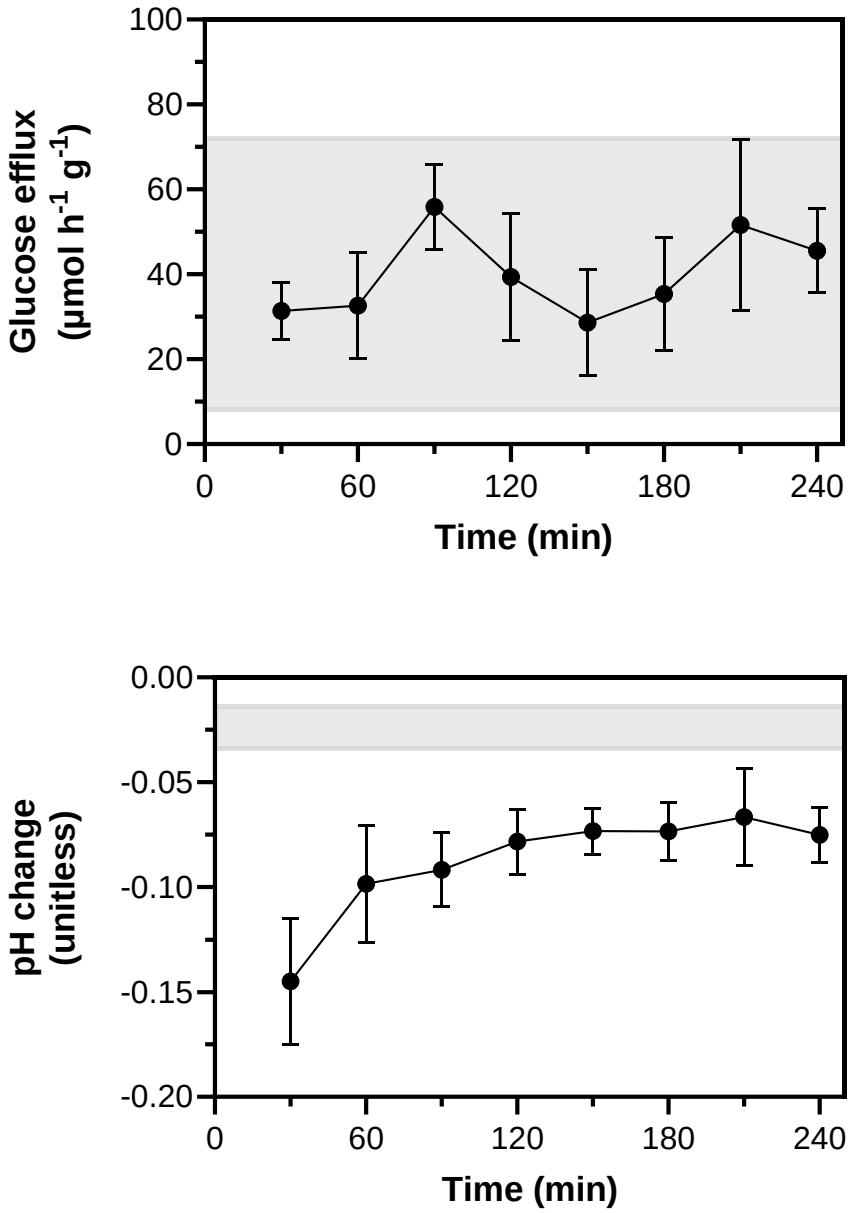

Figure E.1 Physiological performance of isolated perfused livers was evaluated through measurement of glucose efflux and pH change between afferent and efferent samples. Data shown summarizes all perfusions performed with ENTACT mixture (*n=5*). Dots indicate the arithmetic means of all livers. Error bars represent standard error. Gray shaded areas indicate the range of reference values from previous studies conducted by Nichols et al.[30]