THE USE OF ISOLATED RAINBOW TROUT ENTEROCYTES TO ESTIMATE EXTRAHEPATIC METABOLISM OF COMMERCIAL CHEMICALS

by

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Abstract

Thousands of chemicals are awaiting categorization for bioaccumulation potential. An *in vitro* test was developed to measure the biotransformation rates of bioaccumlative commercial chemicals (benzo[a]pyrene, chrysene, 9-methylanthracene, and PCB-209) by freshly isolated rainbow trout (*Oncorhynchus mykiss*) enterocytes. The enterocyte isolation procedure was optimized to yield $4x10^8 \pm 5x10^6$ million cells per fish by systematically investigating pH (7.3-7.4), filtration steps, aeration (air), and DTT (1.0 mM) and collagenase (0.5 mg/ml for 5 min) concentrations. Immunofluorescence microscopy using antibodies to cytokeratin confirmed epithelial origin. Biotransformation rates of test chemicals were not different from control cells in uninduced and β-naphthoflavone-induced fish. In uninduced fish, CYP3A27 and CYP1A1 activity was 5- and 40-fold higher in hepatocytes compared to enterocytes, respectively. CYP1A1 activity was 2-fold higher versus uninduced enterocytes. The findings of this study showed that of enterocyte metabolism of CYP1A1 substrates can be ignored when estimating overall bioaccumulation potential whereas CYP3A27 substrates should be further investigated.

Keywords: biotransformation; metabolism; enterocytes; intestinal epithelial cells; rainbow trout; CYP1A1; CYP3A27;

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Glossary

9-MA	9-Methylanthracene
ATP	Adenosine-5'-triphosphate
BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
B[a]P	B[a]P
BSAF	Biota-Sediment-Accumulation Factor
CEPA	Canadian Environmental Protection Act
CoA	Coenzyme A
CYP	Cytochrome P450
d12-chrysene	Deuterated Chrysene
DSL	Domestic Substances List
DTT	Dithiothreitol
GC-MS	Gas Chromatography-Mass Spectometry
GIT	Gastrointestinal Tract
GST	Gluatathione S-Transferases
HOC	Hydrophobic Organic Carbon
K_{OW}	Octanol-water partition efficient
MEM	Minimum Essential Medium
NAT	*N*-acetyl Transferase

NGS	Normal Goat Serum
OECD	Organisation of Economic Cooperation and Development
O. mykiss	*Oncorhynchus mykiss*
PAH	Polyaromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
PBS	Phosphate-Buffered Saline
PBT	Persistent, Bioaccumulative, and/or Toxic
PBTK	Physiologically-Based Toxicokinetic
QSAR	Quantitative Structure-Activity Relationship
REACh	Registration, Evaluation, Authorisation, and Registration of Chemical Substances
RTGutGC	Rainbow Trout Intestinal Epithelial Cell Line
S9	Supernatant fraction obtained from an organ homogenate by centrifuging at 9000 g for 20 min in a suitable medium
SAM	S-adenosylmethionine
ST	Sulfotransferases
TPBS/BSA	Tween Phosphate Buffered Saline/Bovine Serum Albumin
TSCA	Toxic Substances Control Act
UDP	Uridine Diphosphate
UDPGA	Uridine Diphosphate Glucuronic Acid
UDPGT	Uridine Diphosphate Glucuronsyltransferase
UVCBs	Unknown or Variable Composition, Complex Products, or Biological Materials

1: Introduction

1.1 Accumulation of commercial chemicals in biota

The annual release of several hundred thousand tonnes of anthropogenic chemical compounds from the manufacture of commercial products has become a growing concern. These substances make up over 100,000 commercial chemicals (Voigt et al., 1990) and more substances are added to the list each year. Of particular concern are toxic substances that have the potential to remain in the environment for long periods of time (months to years) or to bioaccumulate in organisms in higher trophic levels which may cause serious harm to human health and wildlife. Such chemicals can be labelled either persistent and/or bioaccumulative, and toxic compounds (PBTs) and are of global concern because many can be transported over long distances from land to air and water. Because Canada is located in the Northern Hemisphere, the colder climate makes our country even more vulnerable due to the global distillation process (Environment Canada, 2011).

1.1.1 Regulation of anthropogenic compounds

In Canada, the Domestic Substances List (DSL) was compiled from a list of chemicals that were manufactured in or imported into Canada in amounts >100kg, or used in Canada for commercial manufacturing purposes between January 1, 1984 and December 31, 1986. The DSL was comprised of 23,000 chemicals when it was first published in the Canadian Environmental Act (CEPA, 1999) (DSL, Government of Canada, 1999), and as of 2009, the Act was amended to add 500 more substances to the inventory (CEPA, 1999, Section 71 Notice).

CEPA (1999) is an important part of Canada's federal environmental legislation aimed at preventing pollution and protecting the environment and human health.

One of the primary goals of CEPA (1999) has been to subject substances on the DSL to a two-phase evaluation (Phase I and Phase II) to be completed by Environment Canada. Phase I assessments categorize commercial chemicals according to regulations, and a chemical is considered hazardous if the substance is persistent and/or bioaccumulative, inherently toxic and predominantly anthropogenic (see Table 1 for criteria). Chemicals that are deemed hazardous by this evaluation proceed to a Phase II Screening level risk assessment where a substance can be further categorized as a Track 1 substance, and added to Schedule 1 of Toxic Substances List, or added to the Priority Substances List for further assessment, or classified as not requiring any further action.

Table 1: CEPA's criteria for assessing persistence, bioaccumulation, and toxicity of commercial chemicals on the DSL (Government of Canada, 1999; Government of Canada, 2000).

Persistence		**Bioaccumulative**	**Toxic**
Medium	**Half-life**		
Air	2 days	[1]BAF $\geq$ 5,000	[4]CEPA-toxic or CEPA-toxic equivalent
Water	$\geq$ 182 days	[2]BCF $\geq$ 5,000	
Sediment	$\geq$ 365 days	Log [3]$K_{OW} \geq 5.0$	
Soil	$\geq$ 182 days		

[1]BAF: Bioaccumulation Factor, [2]BCF: Bioaccumulation Factor, [3]K_{OW}: Octanol-water partition coefficient, [4]CEPA: Canadian Environmental Protection Agency

A chemical is considered a Track 1 substance if it is an anthropogenic chemical that is concentrated in the environment in addition to meeting the PBT criteria listed in Table 1; if so, it will be targeted for virtual elimination from the environment. This would result in the ultimate

reduction of the quantity or concentration of a toxic substance in the release into the environment below concentrations that can be accurately measured or, the "level of quantification." Chemicals on the Toxic Substance List are described as being ‘CEPA toxic’ if they are entering the environment in a quantity or concentration or under conditions that:

a) have, or may have, an immediate or long-term harmful effect on the environment or its biological diversity;

b) constitute, or may constitute, a danger to the environment on which life depends;

c) constitute, or may constitute, a danger in Canada to human life or health

If a chemical is considered ‘CEPA toxic’ then the federal government will work with provinces, territories, industry, non-government organizations and other interested parties to develop a management plan to reduce or eliminate the harmful effects the substance has on the environment and the health of Canadians. Substances that are to be assessed on a priority basis to determine whether they are ‘CEPA’ toxic and pose a significant risk to the health of Canadians or to the environment are added to the Priority Substances List. A substance is “CEPA-toxic equivalent” if it satisfies the definition of “CEPA-toxic” as a result of a systematic, risk-based assessment. Inherent toxicity is a term introduced and used under CEPA 1999 that is distinguished from CEPA-toxic; inherent toxicity refers to the hazard a substance presents to an organism. It is demonstrated by the concentration of the substance that produces a toxic effect in an organism, tested under laboratory conditions, or in other studies. No further action at the time of the assessment is required if a chemical is not considered a hazard to human health or the environment.

Many other countries, including the United States (under the Toxic Substances Control Act (TSCA)) (TSCA, 1976), and the European Union (under the Registration, Evaluation,

Authorisation, and Restriction of Chemical Substances (REACh) program (UNEP, 2006) and several other countries, have also taken an initiative to share the responsibility of removing or lessening the release of PBT compounds into the environment.

Currently, Environment Canada and Health Canada are responsible for completing the regulatory PBT assessment, with no contributions from the industry that registers the chemical, which further increases the cost of fulfilling the goals of Act (CEPA, 1999). Therefore, CEPA has made it a priority to improve the regulation of PBT compounds by focusing on the assessment and classification of these substances.

1.1.2 Current screening assessment criteria

Currently, both *in vivo* and *in vitro* assays are being used by regulatory agencies to measure bioaccumulation. *In vivo* measurements include the bioaccumulation factor (BAF) and bioconcentration (BCF) test, whereas the octanol-water partition coefficient (K_{OW}) is a commonly used approach that does not include any biological factors contributing to bioaccumulation. The criteria for bioaccumulative potential endpoints used by CEPA, TSCA, and the REACh program are summarized in Table 2.

Table 2: An overview of regulatory bioaccumulation endpoints and criteria used by various agencies around the world.

Regulatory agency	Bioaccumulation endpoint	Criteria (log values)	Program
Environment Canada	[1]K_{ow}	≥ 100 000 (5)	4CEPA (1999)
Environment Canada	[2]BCF	≥5 000 (3.7)	CEPA (1999)
Environment Canada	[3]BAF	≥5 000 (3.7)	CEPA (1999)
European Union 'bioaccumulative'	BCF	≥2 000 (3.3)	[5]REACh†
European Union 'very bioaccumulative'	BCF	≥5 000 (3.7)	REACh
United States 'bioaccumulative'	BCF	1 000 (3)-5 000 (3.7)	[6]TSCA, [7]TRI
United States 'very bioaccumulative'	BCF	≥5 000 (3.7)	TSCA, TRI
United Nations Environment Programme	K_{ow}	≥100 000 (5)	Stockholm Convention
United Nations Environment Programme	BCF	≥5 000 (3.7)	Stockholm Convention

[1] K_{OW}: Octanol-water partition coefficient, [2]BCF: Bioaccumulation Factor, [3]BAF: Bioaccumulation Factor, [4]CEPA: Canadian Environmental Protection Agency, [5]Registration, Evaluation, Authorization, and Restriction of Chemical substances, [6]Toxic Substances Control Act, [7]Toxic Release Inventory

When empirical BAF or BCF data is unavailable, quantitative structure-activity relationship (QSAR) models have been used that use the physico-chemical properties of a chemical to make predictions about bioaccumulation potential (Weisbrod et al., 2007). The current methods used to screen chemicals for bioaccumulation are described in Section 1.3, along with the advantages and disadvantages of using each test. First, the *in vivo* test organism commonly used in these studies will be described.

1.2 Rainbow trout as a model organism

International and national regulations often use *Oncorhynchus mykiss* (rainbow trout) for freshwater pollution studies and research in aquatic toxicology, including bioaccumulation, studies because it is a hardy fish that is easy to spawn, fast growing, and can be fed an artificial diet (Cowx, 2005). In Canada, CEPA (1999) selected rainbow trout as a test species for several reasons: the fish is common to all parts of Canada, it is ecologically and economically relevant, and has already been widely used as a test species for assessing the ecological effects of a variety of stressors (Government of Canada, 2010). In addition, the rainbow trout is already included in many bioaccumulation models as a higher trophic level organism (Law, et al., 1991; Gobas and MacKay, 1987; Mackay, 1982; Neely, et al., 1974; Nichols, et al., 1990; Veith, et al., 1979).

1.3 Methods for estimating bioaccumulation

Currently, the CEPA-approved methods to measure bioaccumulation include the bioaccumulation factor (BAF) and bioconcentration factor (BCF) test, and the octanol-water partition coefficient (K_{OW}). The order of preference is BAF over BCF, which in turn is preferred over a log K_{OW} endpoint. Several computational models (equilibrium partitioning, two-compartment kinetic, fugacity, physiologically-based toxicokinetic models (PBTK)) have also been used to predict the bioaccumulation potential of high K_{OW} compounds when empirical BAF and BCF data is not available (Law, et al., 1991; Gobas and MacKay, 1987; Mackay, 1982; Neely, et al., 1974; Nichols, et al., 1990; Veith et al., 1979). Each of these tests is described in more detail in the following sections.

1.3.1.1 Bioconcentration factor test (BCF)

Bioconcentration is the process in which chemical substances accumulate in aquatic organisms exposed via the aquatic medium accumulate in the organism where dietary exposure

is not included. The Organisation of Economic Cooperation and Development Test Guideline Fish Bioconcentration Factor (BCF) test (OECD 305E, 1996) has been used internationally as a standard test to measure the bioconcentration of a chemical in fish. The OECD recommended various freshwater, estuarine and marine fish species and decisions on the test species used should be based on whether the fish is readily available, can be obtained in convenient sizes and can be maintained under disease and parasite-controlled conditions in the laboratory. Other criteria selecting fish species include recreational, commercial, ecological importance as well as comparable sensitivity, past successful use, etc. (OECD, 1996).

The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. The concentration of the test substance in/on the fish (or specified tissue thereof) is followed through both phases of the test. In addition to the test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects in the bioconcentration test to a matching control group and to obtain background concentrations of test substances. The BCF is the ratio of the chemical concentration in the organism to the total chemical concentration in the bulk water phase in steady-state conditions, where the total concentration includes the freely dissolved chemical concentration in the water plus chemical sorbed to particulate and organic matter (Arnot and Gobas, 2006). The disadvantages are that the OECD BCF test may underestimate bioaccumulation, it cannot predict biomagnifications (see below) and its high cost. It has been estimated that using the OECD test, each chemical assessment would require 4 months to perform, use at least 108 fish, and cost approximately $125,000 to conduct (Weisbrod et al., 2007), making this approach unfeasible for the assessment of all 23,000 chemicals on the DSL.

1.3.1.2 Bioaccumulation factor (BAF) and biota-sediment accumulation factor (BSAF) tests

BAFs and BSAFs are considered superior to BCFs because chemical concentrations are determined typically from field measurements which integrate all exposure routes (e.g., gut, gill, skin) in one test and include the complexities of the real ecosystem. For poorly soluble chemicals, the BCF may underestimate exposure because these substances are likely to be 'bound' or 'sorbed' to hydrophobic organic matter as opposed to being suspended in the water. As a result, these chemicals are not expected to pass through biological membranes easily and are not so easily taken up by the organism. In steady-state conditions, BAFs are determined by calculating the ratio of the chemical in the organism to the concentration of the chemical in all routes of exposure, whereas BSAF is defined as the ratio of chemical concentration in an organism to the chemical concentration in the sediment. BSAFs are useful if water concentrations are too low, and the bioaccumulation factor can be expressed relative to the concentration of the chemical in the sediment (Gobas, 2001). Currently, there is a lack of a standard protocol for BAF and BSAF determination in field situations which makes it difficult to compare values determined by different researchers. In general, BAF and BSAF tests require the following ecosystem conditions and chemical properties: spatial and temporal gradients in chemical concentrations, chemical distribution between the sediment and the water column, life history patterns of the organism, the chemical's hydrophobicity, and metabolism of the chemical in all organisms composing the food web. A successful sampling design procedure, based upon the above factors, must define the frequency of sample collection, the spatial distribution of sample collection locations, and total the number of samples to be collected. Unfortunately, adequate field data sets are extremely limited because these tests require coordinated fish, water, and sediment data over time. In most field studies, fish and sediment samples may only be collected once in a field season because of the labour required for their collection. The major

disadvantage of the BAF and BSAF tests are the costs associated with optimizing the sample collection and analysis process, which can outweigh the costs of a BCF test (Burkhard, 2003)

1.3.1.3 Drawbacks of *in vivo* approaches

The *in vivo* approaches (BAF and BCF) are considered to be a more reliable indication of a substance's ability to bioaccumulate in biota because parameters such as metabolism are considered (EPA, 2000) however, the problem is that empirically-derived BAF and BCF information is available for less than 4% of the commercial chemicals on the DSL (Arnot and Gobas, 2006). Furthermore, as stated above, a disadvantage of the BCF and the BAF test is the high cost and relatively long time required to gather data even for one chemical.

1.3.1.4 Octanol-water partition coefficient (K_{OW})

As an alternative to *in vivo* tests, regulatory authorities have had to turn to other methods such as determining the octanol-water coefficient, to estimate the bioaccumulation potential of a broad range of chemicals. The octanol-water partition coefficient (K_{OW}) is the ratio of the concentration of a chemical in octanol to that in water at equilibrium and at a specified temperature (Finizio, 1997), and is often expressed as a logarithm (log K_{OW}). A higher log K_{OW} value is indicative of a lipophillic molecule that is more likely to bioaccumulate in biological tissue. This parameter is frequently used because it is simple, inexpensive, and can provide a rough estimate of bioaccumulation for a wide range of compounds in a short period. However, for highly hydrophobic compounds (log K_{OW} > 5), direct experimental measurement of K_{OW} using traditional shake-flask methods is extremely difficult because the emulsified phases are persistent and difficult to clear even with prolonged centrifugation (Danielsson and Zhang, 1996). Another drawback of this test is that it can be quite variable in its predictions for the

same chemical depending on whether direct partitioning (shake-flask and slow-stirring) or high-performance liquid chromatographic are used (Leo et al., 1971).

In addition, modifying factors such as metabolism or abiotic degradation are not allowed for if only simple physico-chemical descriptors such as log K_{OW} are used. Metabolism of a chemical can render it less likely to biomagnify in the food-chain when hydrophobic parent compounds are enzymatically converted to a more water-soluble product (see Section 1.4 below).

1.3.1.5 Computational models to predict bioaccumulation

Models have become more extensively employed to predict bioaccumulation because empirical BAF or BCF data are often not available either because compounds have recently been introduced, and/or the information is considered by industry to be too difficult or costly to generate. In the past 40 years, numerous computational models have been created to measure the bioaccumulation potential of chemicals in fish. The earliest models were simple equilibrium partitioning models that focused on the relationship between log K_{OW} and log BCF (Neely et al., 1974; Veith, et al., 1979). These were followed by simple two-compartment kinetic models that illustrate the movement of chemicals between fish and water (Neely et al. 1974). Fugacity-based bioconcentration models were also developed which established the theoretical basis for future bioconcentration models allowing them become more diversified (Mackay, 1982). Physiologically-based toxicokinetic (PBTK) models that incorporated knowledge of fish tissue physiology, interactions of chemicals between tissue compartments and fish responses to chemicals, were later developed in the 1990's (Nichols et al., 1990; Law et al., 1991). Fugacity models were continually improved to better reflect processes that affect bioconcentration (Gobas and MacKay, 1987), and as researchers became more aware of the contribution of dietary and

gill uptake of hydrophobic compounds to biomagnifications and bioaccumulation (Bruggemana et al., 1981; Connolly and Pedersen, 1988; Muir and Yarechewski, 1988; Norstrom, et al., 1976), models that included these features were created (Clark et al., 1990; Thomann and Connolly, 1984).

Although the authors of many current models, such as the Arnot and Gobas Fish Bioaccumulation Model (Arnot and Gobas, 2003:2004) acknowledge that biotransformation plays an important role in a chemical's bioaccumulative potential, most models incorporate the assumption that the metabolic transformation rate constant (K_M) is equal to zero. This is because metabolism data is available for only a small number of chemicals. Research has shown that inclusion of biotransformation information in bioaccumulation models would more accurately reflect the true bioaccumulative potential of a chemical (Arnot and Gobas, 2003).

Research on measuring the biotransformation rate of environmental organic contaminants is very limited in comparison to the research of pharmaceutical substances. Drug biotransformation models, such as MetabolExpert (CompuDrug, Sedona, AZ), that can predict the metabolic fate of drugs and drug-like organic compounds in humans, animals, or in plants are commercially available. This provides confidence that a model to predict the biotransformation rate of hydrophobic organic carbons can also be made.

1.4 Overview of xenobiotic metabolism in fish

The aquatic environment plays an important role in the fate of hydrophobic organic carbons (HOCs) because large amounts of these chemicals are released into the environment by urban and industrial discharges, runoff from agricultural soils, and leaching through soil (Mackay and Boethling, 2000). HOCs in the water phase have a tendency to partition out of the water phase and associate with organic-rich phases such as food particulates or biota (CCME,

1992). As a result, fish living in the aquatic media are primarily exposed to hydrophobic chemical contaminants through ingestion rather than via the gill or skin.

Xenobiotics are foreign substances found in living organisms. If they are hydrophobic/lipophillic organic chemicals (HOCs), they may be readily absorbed from the gastrointestinal tract and distributed to other tissues. Metabolic biotransformation converts HOCs into a more water-soluble form by adding hydrophilic functional groups that makes them more likely to partition into the water phase, and in some cases, allows them to be actively excreted by energy-dependent pumps. Once the xenobiotic is metabolized, its biological activity may be changed and its half-life, and consequently its accumulation, are reduced.

1.4.1 Phase I and Phase II reactions

A two-phase process is responsible for the biotransformation of nonpolar chemicals to more water-soluble metabolites that generally results in detoxification and elimination of the parent compounds. The two groups of reactions responsible for this process are called Phase I and Phase II reactions.

1.4.2 Phase I reactions

The three major types of phase I reactions include oxidation, reduction, and hydrolysis reactions. The biotransformation of xenobiotics are dominated by the cytochrome P450 (CYP450) family of drug metabolizing enzymes, where the process of transferring electrons to electron deficient acceptors (usually an essential cofactor), such as $NADP^+$, are catalyzed by dehydrogenases. The incorporation of molecular oxygen (from water) into molecules are catalyzed by oxygenases, whereas peroxidases catalyze the derivation of oxygen from peroxide cofactors (Schlenk, et al., 2008). Figure 1 is an example of a phase I reaction where

benzo[a]pyrene (B[a]P) is metabolized to a more polar product via CYP and hydrolysis reactions.

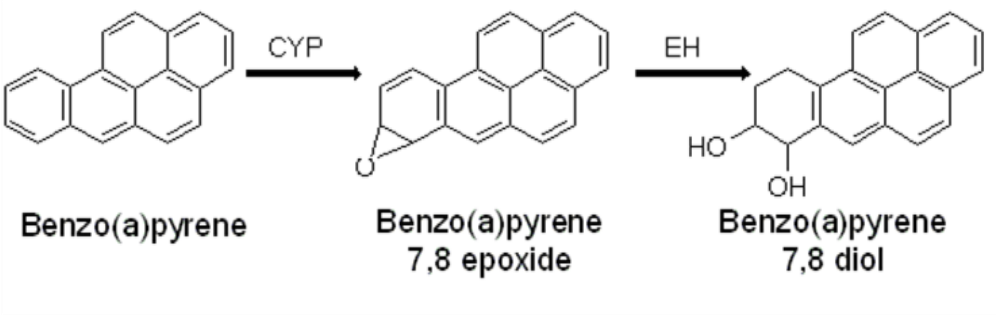


Figure 1: Example of a Phase I reactions catalyzed by cytochrome P450 and hydrolysis biotransformation reactions. B[a]P is metabolized by CYP450 enzymes and epoxide hydrolase (EH) to form a more water-soluble metabolite. CYP: cytochrome P450, H: hydrogen, O: oxygen.

1.4.2.1 Cytochrome P450 enzyme system

The most important enzyme system catalyzing phase I metabolic reactions is the Cytochrome P450 (CYP450) monooxygenase system. These are the most numerous of all xenobiotic-metabolizing enzymes and have the broadest substrate specificities. The CYP450 superfamily of enzymes has existed for over 3.5 billion years (Nelson et al., 1993). Animals, plants, and microorganisms all contain CYP450, and CYP450s have been found in all mammalian tissues examined. They are found in the greatest abundance in the liver, and are typically associated with the endoplasmic reticulum or mitochondria. In fish, CYP450s have been found in almost every tissue examined, although with different patterns of isozymes. Isoforms well known to be part of the biotransformation of xenobiotics include families CYP450 1, CYP450 2, and CYP450 3 (Lewis, 2001). Up until 2009, the number of CYP450 genes discovered in the Japanese puffer fish (*Takifugu rubripes)* was 55, and 81 CYP450 genes for the Zebrafish *(Danio rerio).*

1.4.2.2 Nomenclature

The standard classification system for CYP450 enzymes is to categorize families and subfamilies on the basis of either their specific functions or presence in species where the nomenclature for the gene is designated with CYP (cytochrome P450), Arabic number (family), letter (subfamily, and Arabic number (specific protein). Thus, CYP1A1 refers to the gene for the P450 in family 1, subfamily A, subfamily member 1. CYPs in the same family show more than a 40% sequence similarity, and those within a subfamily are more than 55% similar (Nelson, et al., 1993).

1.4.2.3 Structure

CYP450 is not a single entity but includes a large number of isoforms. There have been over 1000 isoenzymes isolated (Lewis, 2001). The basic structure of each isoenzyme is a heme-binding skeleton, where the heme iron in CYP450 is usually in the ferric (Fe^{3+}) state. Once reduced, the ferrous (Fe^{2+}) form can then bind ligands such as O_2 or carbon monoxide (CO). The amino acid sequence around the cysteine residue that forms the thiolate bond with the heme moiety is highly conserved in all CYP450 enzymes and is converted to an inactive product when this bond is broken **(**Ioannides, 2008).

In eukaryotes, CYP450s are located in the membrane of the smooth endoplasmic reticulum and are bound to the mitochondrial membrane, whereas in bacteria, they are present in the cytosol in a soluble form. The activity is dependent on the presence of a NADPH-cytochrome P450 reductase (Lewis, 2001) as well as phospholipids which have been shown to be important in substrate binding of CYP enzymes (Murtazina et al. 2004).

1.4.2.4 Biochemistry

The P450 system is involved in various xenobiotic metabolic pathways (Nebert, 1989), and the overall reaction can be summarized as follows:

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

Electrons are first transferred from NAD(P)H to NADPH-cytochrome reductase in the microsomal cycle.

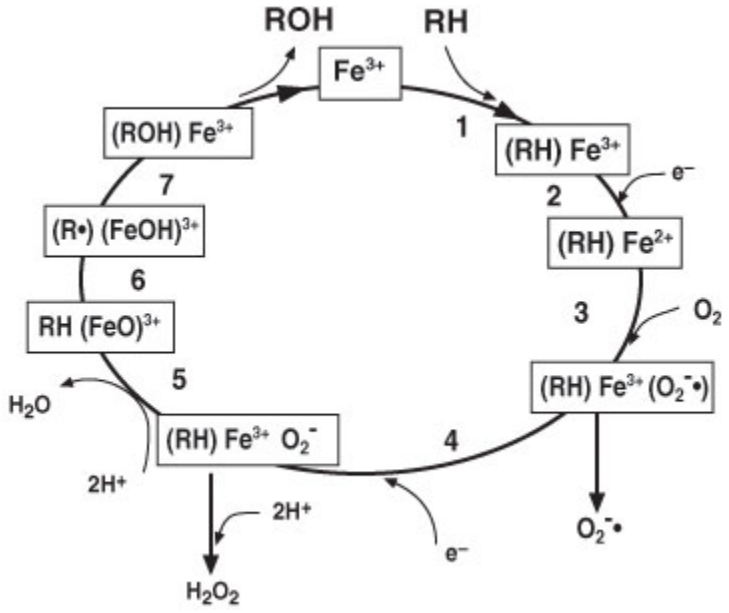


Figure 2: Cytochrome P450 oxidation (catalytic) cycle (Adapted from Orellana B and Guajardo T, 2004). R: carbon side chain, C: carbon, H: hydrogen, O: oxygen, Fe: iron.

The catalytic cycle is started with the binding of the substrate to the oxidized (Fe^{3+}) CYP complex and an electron is transferred from NADPH to the complex (steps 1 and 2, Figure 2). The reduced CYP complex is then bound to oxygen, which is coordinated to iron *trans* to thiolate (step 3, Figure 2), followed by a second electron donated by either NADPH-cytochrome P450 reductase or NADH-cytochrome b_5 reductase (step 4). The bond in molecular oxygen is then cleaved to release a water molecule (step 5) and oxygen is inserted into the substrate (as an

hydroxyl) through generation of hydroxide and carbon free radicals. Lastly, the P450 enzyme is restored to its initial ferric state when ROH dissociates from the complex (Parkinson, 2001).

1.4.2.5 Function

CYP450s have numerous catalytic functions that contribute to biotransformation including monooxygenase, oxidase, reductase, and peroxygenase activity. Monooxygenase activities with xenobiotics include hydroxylations, epoxidations, N-, O-, or S-dealkylations, deaminations, sulfoxidations, desulfurations and oxidative dehalogenations (Figure 3).

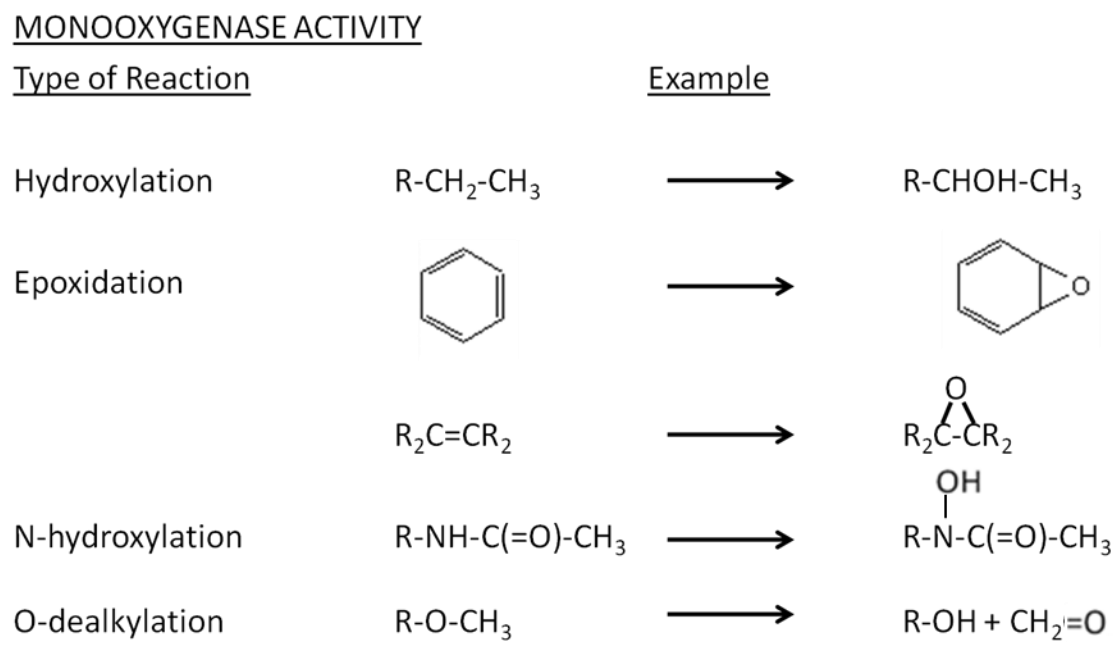


Figure 3: Example of biotransformation reactions resulting from monooxygenase activity (Adapted from Ortiz-Delgado, 2002). R: carbon side chain, C: carbon, H: hydrogen, O: oxygen, N: nitrogen.

Oxidase activities are commonly referred to as uncoupling of the P450 reaction cycle or oxidase activity of P450 (Goeptar et al., 1995), in which molecular oxygen receives an electron from reduced P450 and a superoxide anion radical ($O_2^{-\bullet}$) is formed in the process. Spontaneous

or enzyme-catalyzed dismutation of superoxide radicals result in the production of H_2O_2. The overall reaction is:

$$NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O_2$$

Figure 4 provides examples of cytochrome P450-mediated oxidation of catechols and xenobiotics such as benzene (Ingelman-Sundberg and Hagbjörk 1982).

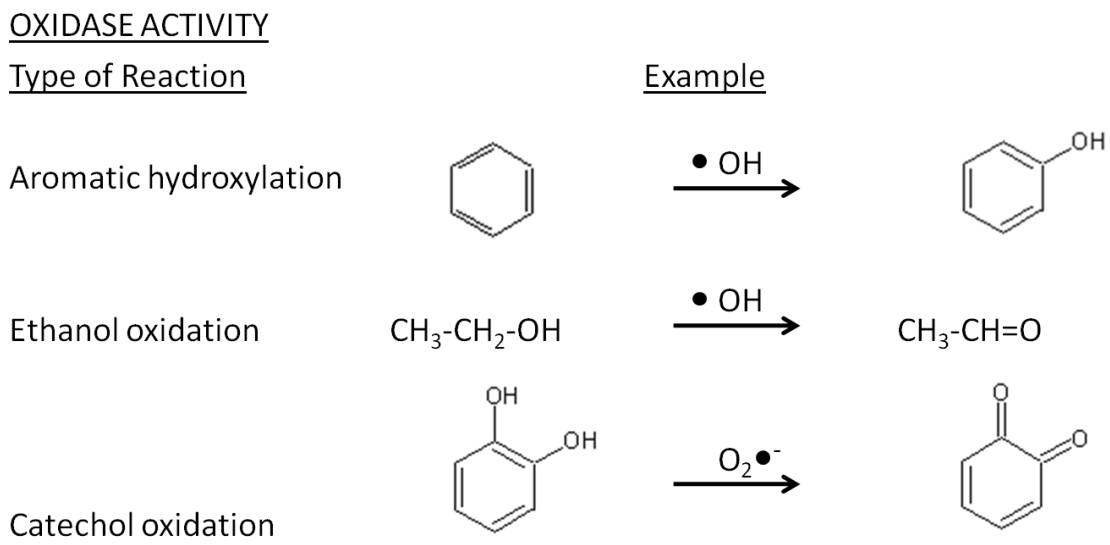


Figure 4: Example of biotransformation reactions resulting from oxidase activity (Adapted from Ortiz-Delgado, 2005). C: carbon, H: hydrogen, O: oxygen, N: nitrogen.

P450 enzymes can also perform peroxygenases functions (Figure 5) in wide range of peroxide-dependent substrate hydroxylation reactions (de Montellano, 1986).

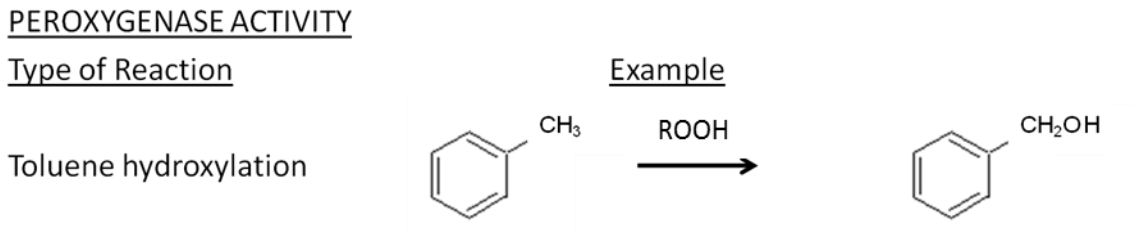


Figure 5: Example of biotransformation reactions resulting from peroxygenase activity (Adapted from Ortiz-Delgado, 2002). R: carbon side chain, C: carbon, H: hydrogen, O: oxygen.

Finally, P450 enzymes can also function as reductases. These reactions occur most optimally under anaerobic conditions when NADPH is present and electrons are directly transferred to reducible substrates to produce free radical intermediates. For example, halogenated alkane and quinine reactions are frequently observed (Goeptar et al., 1995; Ingelman-Sundberg and Hagbjörk, 1982; de Montellano 1971). Examples of azo and nitro reduction, and reductive dehalogenation are shown in Figure 6.

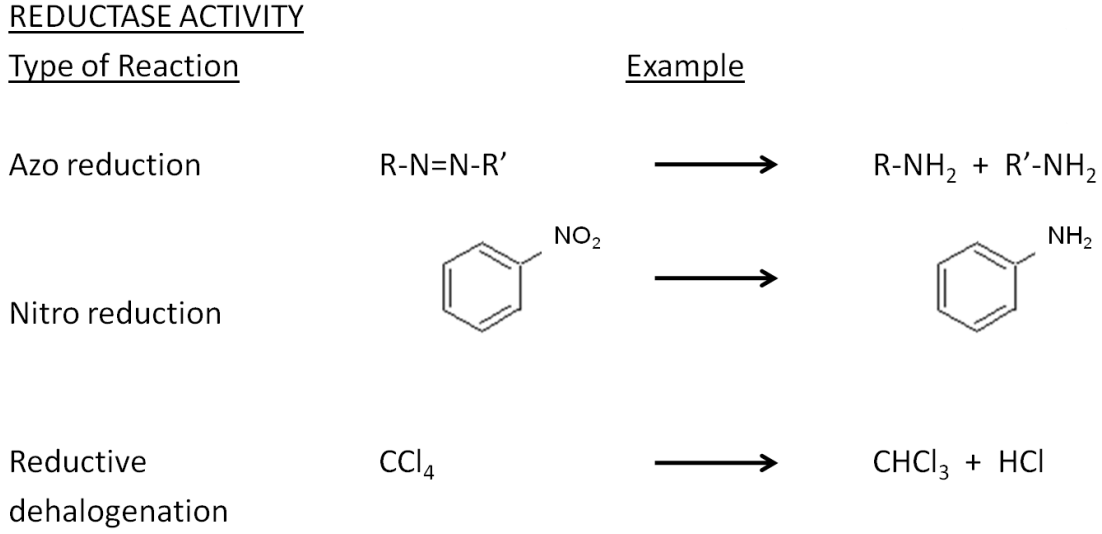


Figure 6: Example of biotransformation reactions resulting from reductase activity (Adapted from Ortiz-Delgado, 2002). R: carbon side chain, R' rest of the carbon molecule, C: carbon, H: hydrogen, O: oxygen, N: nitrogen, Cl: chlorine.

Such reactions are present in anaerobic microorganisms although transient free radicals may be produced by reductase activity in eukaryotes.

1.4.2.6 Distribution of CYP in fish

In fish, CYPs are typically present in high levels in the liver, accounting for 1 to 2% of the mass of hepatocytes (Lester et al. 1993; Lewis 2001), and are also found in the intestine, kidney, brain, and skin (Arukwe et al., 2002; Hegelund et al., 2003; Ortiz-Delgado et al. , 2002).

1.4.2.7 Hydrolytic reactions

Enzyme-mediated hydrolytic reactions include catalysis of reactions with esters, amides, and epoxides. Esterases and amidases are found in several subcellular fractions of cells from all tissues examined with the highest levels found in the liver, kidney and intestine. Xenobiotics containing functional groups such as aldehydes, ketones, and alcohols are susceptible to reduction through enzymatic reactions catalyzed by aldehyde/ketone reductases, alcohol dehydrogenases and aldehyde dehydrogenases, or nonenzymatically by the action of biological reducing agents. These enzymes are typically associated with microsomes (Lech and Vodicnik, 1985), but have been found in the cytoplasm of cells as well (Adamson, 1967).

1.4.3 Phase II reactions

In Phase II reactions, organic molecules are conjugated to endogenous substrates such as carbohydrates, amino acids or peptides, methyl- or acetyl groups or inorganic sulphate, and the processes consists of the transfer of the conjugating agent from its bound coenzyme form to the xenobiotic, catalyzed by specific transferases. Fish have the ability to conjugate a wide variety of xenobiotics or their Phase I metabolites by transferring a polar molecule (such as carbohydrates, amino acids or peptides, methyl- or acetyl groups or inorganic sulphate) from its

bound coenzyme form to the xenobiotic. Specific transferases such as uridine diphosphate-glucuronosyltransferase (UDPGT), sulfotransferases (ST), glutathione S-transferases (GSTs), and several others catalyze this process.

1.4.3.1 Glucuronidation reactions

The UDP-glucuronosyltransferases (UGTs) represent a major group of phase II conjugating enzymes that are located in the endoplasmic reticulum that plays an important role in the biotransformation of natural toxins and anthropogenic toxicants. Glucuronidation involves the conjugation of metabolically-activated UDP-glycoside (UDPGA) such as UDP-glucuronic acid or UDP-glucose to a xenobiotic, forming a D-glucuronide to increase the water solubility of substrates which facilitates transport and excretion (Mackenzie et al., 1997). In fish species, UGT has been found to be high in the intestine and liver, and in measurable activities in the gill, kidney, and muscle tissue (Clarke et al., 1991; George et al., 1998; James et al., 1998; Singh et al., 1996).

1.4.3.2 Sulfation reactions

The transfer of sulphate occurs when conjugation of xenobiotics, steroids, and bile acids to inorganic sulphate is catalyzed by sulfotransferases. From the reaction of adenosine-5'-triphosphate (ATP) and inorganic sulphate, 3'-phosphoadenosine-5'-phophosulfate is generated as the sulphate group donor. The SO^{3-} group of the 3'-phosphoadenosine-5'-phophosulfate is transferred with the subsequent release of adenosine-3', 5'-diphosphate. The products of these reactions are ionized organic sulphates that have an increased water solubility and which are more readily excreted than the parent compound or hydroxylated metabolite. The sulfonation ability in rainbow trout has been found to be relatively low (Andersson et al., 1983; Parker et al., 1981; Statham et al., 1975); however, the proportion of sulfonate conjugates of phenol was

greater than its glucuronide conjugates in seven out of eight freshwater species examined (Layiwola and Linnecar, 1981).

1.4.3.3 Glutathione *S*-Transferases

The glutathione *S*-transferases are a supergene family of phase II enzymes that provide cellular protection against the toxic effects of a variety of environmental chemicals. Glutathione conjugation reactions are catalyzed by glutathione S-transferases (GSTs) to protect the cell and its components by catalyzing the first step in the formation of N-acetylcysteine derivatives of a variety of xenobiotics. The most important reaction

1.4.3.4 Methylation reactions

Methylation is a two-step process where the biosynthesis of S-adenosylmethionine (SAM), and the transfer of methyl groups from this coenzyme to the xenobiotic or metabolite by specific transmethylases. It plays an important role in breaking down endogenous substrates such as histamine, serotonin, and norepinephrine (de Bruin 1976), but methylation has been found to have limited contribution to the biotransformation of HOCs (Sipes, 1986).

1.4.3.5 Amino acid conjugation reactions

Amino acid conjugation is the attachment of amino acids, such as glycine and taurine, to xenobiotics that have a carboxylic acid functional group. The enzymes involved in this process need to be activated by ATP and acetyl-coenzyme A or phenylacetyl CoA (Bend et al., 1980), of the carboxylic acid to a thioester derivative of CoA. The synthesizing enzymes are ATP-dependent acid:CoA ligases and the coenzyme A thioester then transfers its acyl moiety to the amino group of the acceptor amino acid, a reaction catalyzed by N-acyltransferases, forming the conjugate and regenerating CoA. Ligases and N-acyltransferases are soluble enzymes and have

been found in the matrix of hepatic mitochondria in fish (Bend et al., 1980). The most common amino acid conjugation in fish is with taurine, a reaction catalyzed by taurine N-acyltransferase. Other amino acids can be conjugated with xenobiotics in fish, but are much less common (Huang and Collins, 1962).

1.5 Overview of first pass metabolism

With fish, as with mammals, significant metabolism of dietary toxicants is often believed to occur only after these compounds are transported to the liver, the organ which generally contains the largest and highest concentration of xenobiotic metabolizing enzymes. Most studies on HOC metabolism are conducted with the liver (Trowell, 2010; Cowan-Ellsberry et al, 2008; Han et al., 2008; Han et al., 2007; Nishimoto et al. 1992), however, in the past 25 years, the contributions of first-pass metabolism to breaking down a wide range of xenobiotics to a significant extent has become more apparent in the intestine of fish (Kleinow, et al., 1998; Van Veld, et al., 1987; Van Veld, et al., 1988; Van Veld, et al., 1985). First-pass metabolism is described as the significant metabolism of xenobiotics bound to food or other particulates in the small intestine which can affect whether a chemical will enter the systemic circulation or be excreted (Figure 6).

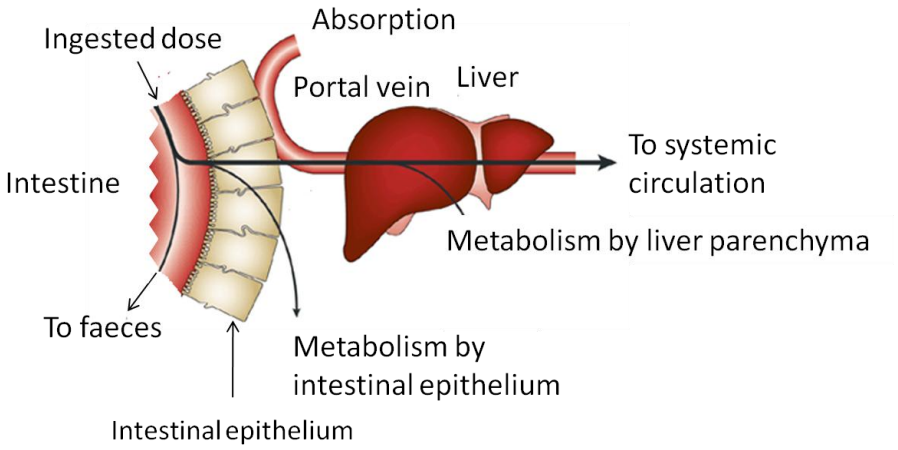


Figure 6: A schematic of first-pass metabolism. HOCs bound to food or other particulates enter the intestinal lumen. The first cells they encounter are the intestinal epithelial cells (enterocytes) that line the lumen. Depending on the chemical structure, the HOCs may be metabolized within the enterocytes or are carried unchanged to the liver via the portal circulation. (Adapted from van de Waterbeemd and Gifford, 2003).

Only a few studies have focused on the metabolism of hydrophobic organic carbons in fish intestine and our knowledge about the extent of biotransformation in this organ is very limited. Currently, B[a]P is the only anthropogenic compound that has been studied in fish intestinal metabolism research (Kleinow, 2001; van Veld et al., 1988: 1987: 1985); and it is therefore important to measure the extent of biotransformation of other anthropogenic chemicals to better understand the behaviour of these chemicals when ingested by fish.

Hydrophobic chemical contaminants (such as benzo[a]pyrene) released into the aquatic environment will bind strongly onto organic solid phases or be present in dissolved forms that are poorly absorbed at gill and skin surfaces. Therefore, fish are exposed to these substances primarily via ingestion of contaminated food (or sediment for some species) and subsequent absorption within the gastrointestinal tract (GIT). The amount of ingested chemicals that reaches the systemic circulation can be reduced by intestinal metabolism prior to entering the systemic

circulation and is therefore fundamental to include in any study examining the biotransformation of xenobiotics sorbed to particulates in the intestine of fish.

1.6 Gastrointestinal tract anatomy

The gastrointestinal system of fish is generally comprised of the esophagus, stomach, pyloric caeca, intestine and the rectum (Figure 7). In general, the digestive tract of fish is simpler than that of its mammalian counterpart where the distinct regions of the small (duodenum, jejunum, ileum) and large intestine of mammals are not apparent in fish (Kapoor et al., 1975). Many fish also lack a well-defined stomach and the length of the intestine can be variable, depending on diet of the fish (Ferraris and Ahearn, 1984). A common modification of the gastrointestinal tract in some fish species is the presence of the pyloric cecae.

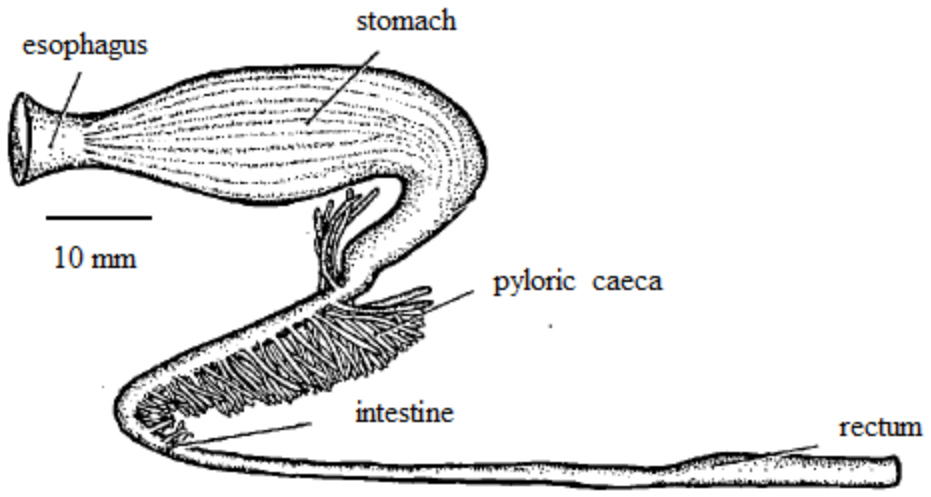


Figure 7: A diagram of the gross anatomy of a *Salmo trutta* (brown trout) gastrointestinal tract showing the various regions (Burnstock, 1959).

Fish intestines have a layer of serosal cells outside the muscle-coats and the circular layer is about twice as thick as the longitudinal layer. The stratum compactum and granulosum are very prominent where the tunica propria contains no glands and is bound by a much-folded

mucosal epithelium containing many mucus-cells (Figure 8). The pyloric ceca in brown trout has been shown to be histologically identical with the intestine (Burnstock, 1959). Epithelial cells lining the intestinal tract, also known as enterocytes, are thought to be responsible for the majority of metabolic activity of xenobiotics (Tocher et al., 2002, 2004; Oxley et al. 2005; Bogevik et al., 2008) because it is the first layer of cells contacted by a xenobiotic when it enters the intestine.

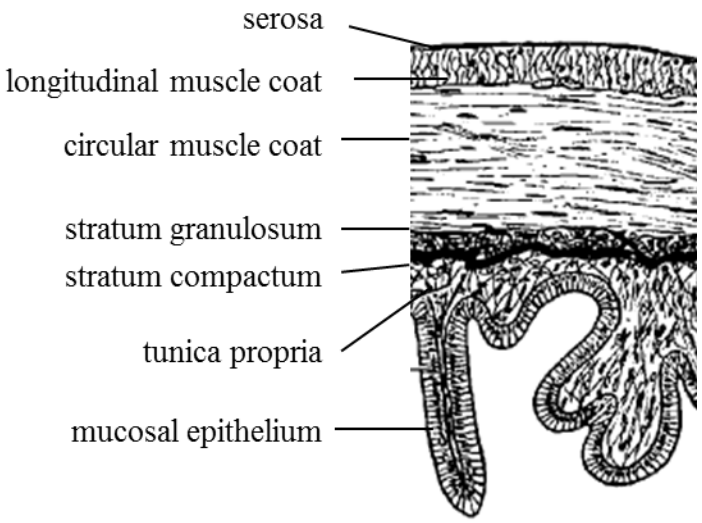


Figure 8: Transverse section through the wall of the intestine of brown trout (Burnstock, 1959).

1.7 Use of freshly isolated enterocytes as a test model of intestinal xenobiotic metabolism

The DSL contains a wide variety of chemicals ranging from simple organic chemicals, pigments, organometallic compounds, surfactants, polymers, metal elements, metal salts and other inorganic substances, products of biotechnology as well as substances that are of "Unknown or Variable Composition, complex reaction products, or Biological materials" (referred to as UVCBs) that need to be screened. Using subcellular fractions such as cytosolic,

S9, or microsomes alone may underestimate biotransformation if the metabolic enzymes are not localized in these membranes. Therefore, an *in vitro* assay that incorporates as many metabolic enzymes as possible with a high throughput of screened chemicals and technical ease compared to whole organism test is desirable.

Isolated enterocytes are a suspension of mucosal epithelial cells isolated from freshly-excised intestine; consequently, these cells possess most of the functional capabilities of the intact epithelium except cell polarity. These cells have been used extensively for fish fatty acid metabolism (Bogevik et al. 2008; Oxley et al. 2005) and uptake (Pérez 1999; Larsson et al. 1998) studies as well as characterizing the cells for respiratory, digestive hydrolase and Mg^{2+} - dependent ATPase activities and parathyroid hormone receptor signalling pathways and ligand binding (Rotllant et al. 2006).

Cell have been held in suspension where they can remain viable for several hours (Pérez 1999; Burke and Handy 2005). In comparison to subcellular fractions, the advantages of enterocytes are that all metabolic enzymes normally present in the epithelial linging of the intestine are present in intact cells, reducing equivalents are generated by cell metabolism, xenobiotic transporters are still present and operational, and any detected metabolism can be attributed to the specific cell type (Brandon et al. 2003). The disadvantages of using enterocytes to measure biotransformation is that the isolation process is time-consuming (3 h), only the preselected cells can be studied, and cell damage can occur during the isolation (Brandon et al., 2003).

Unlike hepatocytes, which have been extensively used to measure xenobiotic metabolism in rainbow trout (Trowell, 2010; Cowan-Ellsberry et al, 2008; Han et al., 2008; Han et al., 2007)

and other fish species (Nishimoto et al. 1992) enterocytes have yet to be used to study the biotransformation of organic chemical contaminants in fish.

Intestinal metabolism of HOC's in fish have been studied using microsomal and perfused *in situ* preparations (Kleinow, et al., 1998; Van Veld, et al., 1987; Van Veld, et al., 1988; Van Veld, et al., 1985). Other intestinal test models that were considered for this project include S9 fractions, cytosolic fractions, and cell cultures. The advantages and disadvantages of each approach are discussed below.

1.7.1 Tested *in vitro* methods to measure xenobiotic metabolism in fish intestine

Microsomes are subcellular fractions enriched in endoplasmic reticulum membranes that are prepared by differential centrifugation after enterocytes are sonicated and homogenized. The major advantage of using microsomal fractions is that it is a well characterized, inexpensive, and simple system to study xenobiotic metabolism where inter-individual variation can be observed (Brandon et al., 2003). However, the major drawbacks of this approach is that microsomal fractions are enriched with CYPs and UGTs and have no competition with other enzymes that may otherwise metabolize the xenobiotic, resulting in higher biotransformation rates in microsomes compared to whole cells (Sidelmann et al., 1996). In addition, subcellular fractions do not contain cytosolic metabolizing enzymes or soluble Phase II enzymes, such as esterases or peroxidases.

Isolated intestinal perfusion have also been used to study biotransformation of xenobiotics in fish intestine (Kleinow et al., 1998). In this study, a section of the intestine was removed/isolated from the fish and radiolabelled B[a]P was introduced into the intestinal lumen or the blood perfusing the isolated segment. The degree of biotransformation was then determined by draining the intestinal segment of the infusate for liquid scintillation counting and

metabolite analysis. This approach is considered to be the best representation of the *in vivo* situation as it keeps the original three-dimensional architecture and contains all cell types in the intestine. The drawback of this approach is that it requires significant technical expertise; it relies on radiolabelled substrates and only one experiment (with one or a mixture of chemicals) can be conducted with each fish, which would increase the cost of the screening test if thousands of chemicals need to be assessed.

1.7.2 Other intestinal models considered for biotransformation *in vitro* test

The cytosolic fraction contains soluble phase II enzymes such as *N*-acetyl transferase (NAT) and sulfonotransferase; this fraction may obtained by differential centrifugation. The addition of exogenous cofactors for NAT, sulfotransferase (ST), and GST is necessary for the catalytic process. The main advantage of the cytosolic fraction system is the presence of a few enzymes at higher concentrations compared to S9 fractions (see below) and the biotransformation of chemicals can be studied for each enzyme or in combination depending on the cofactors added. A disadvantage of this test system is that it includes only soluble phase II enzymes located in the cytosolic fraction and therefore does not include UGTs (major group of phase II conjugating enzymes) located on the endoplasmic reticulum; therefore, the contributions of this enzyme cannot be studied.

The S9 fraction contains both microsomal and cytosolic fractions. As with microsomes, an NADPH-regenerating system or NADPH is required to supply the reducing power for the CYP enzymes. In addition, for the phase II enzymes, addition of exogenous cofactors is necessary for UGT, NAT, ST, and GST. Compared with microsomes or cytosolic fractions alone, S9 fractions offer a more complete representation of the metabolic profile, as they contain both phase I and phase II activity. The major disadvantage of S9 fractions is the overall lower

specific enzyme activity compared to microsomal (higher concentrations of CYP and UGTs) or cytosolic fractions (higher concentrations of NAT, ST, and GST) and some metabolites may not be biotransformed (Brandon et al., 2003).

Cultured cell lines for biotransformation studies are less popular compared to other models described above. This is mainly due to their dedifferentiated cellular characteristics and incomplete expression of all families of metabolic enzymes. The usefulness of immortalized cell lines as an *in vitro* model is due to their continuous propagation which reduces animal use (Brandon et al., 2003). The first intestinal cell line from fish (RTgutGC) was derived from the intestine of rainbow trout by Kawano et al., (2011) and has been cryopreserved successfully though its complement of xenobiotic metabolizing enzymes has not been studies. An established fish hepatocellular carcinoma cell line derived from *Poeciliopsis lucida* is commercially available (http://www.atcc.org). The major advantage of using a cultured cell line is the ease of cell culturing and relatively stable enzyme concentrations and it is the absence or low expression level of many important phase I and phase II metabolizing enzymes which limits the application of cultured cell lines (Brandon et al., 2003).

1.8 Goal of the overall project

A cost- and time-effective screening method is urgently needed that includes biotransformation information of commercial chemicals while reducing the number of animals used. To fulfil this need, our research group at Simon Fraser University is assessing various rapid and inexpensive methods to estimate HOC biotransformation in the intestine and liver of rainbow trout to refine the fish bioaccumulation model developed by Arnot and Gobas (2003; 2004). My contribution to the overall project was focused on measuring the metabolism of

commercial compounds, and to estimate the extent that first-pass metabolism contributes to the overall breakdown of hydrophobic organic compounds in rainbow trout.

1.9 Specific aims

The aim of my study is to develop an improved *in vitro* method to measure intestinal metabolism by rainbow trout. I then tested the method by measuring in-vitro substrate depletion rates for high K_{OW} chemicals that are potentially bioaccumulative in trout enterocytes. My specific aims were:

1) To optimize an enterocyte isolation method to yield cells with consistently high cell viabilities (> 90%) over a 5 h incubation period

2) To confirm the purity of enterocyte preparations using an antibody to an epithelial cell marker and immunofluorescence microscopy

3) To measure the metabolism of four hydrophobic chemicals listed on the DSL in freshly-isolated trout enterocytes in the presence and absence of inducing agents

4) To compare the CYP 3A27 and CYP 1A1 activity levels in trout enterocytes and hepatocytes in the presence and absence of inducing agents

2: Materials and Methods

2.1 Animals

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

2.2 Chemical compounds

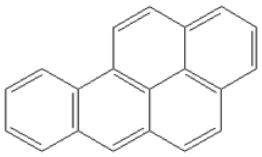
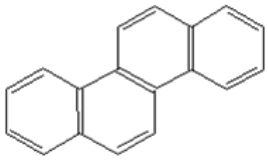
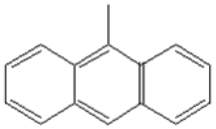
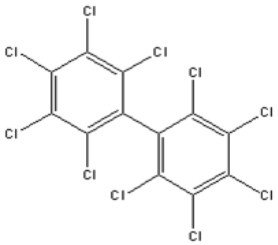
2.2.1 Selected test compounds

The chemical compounds selected for this study are on the Canadian Domestic Substances List, and include B[a]P (B[a]P), chrysene, 9-methylanthracene (9-MA), and polychlorinated biphenyl 209 (PCB 209). Currently, the metabolism of B[a]P has been extensively studied in fish and *in vivo* data is available for validation of the *in vitro* results. B[a]P has been shown to be readily metabolized (Han et al. 2009; Han et al. 2008; Cowan-Ellsberry et al. 2008; Van Veld, R. Vetter, et al. 1987; Kleinow et al. 1998)) and was selected as a positive control whereas chrysene and 9-MA are metabolized by S9 liver homogenates to a lesser extent. PCB 209 was chosen as a non-metabolizable (negative) control because metabolism of PCB 209 is negligible due to a complete substitution by chlorine (Buckman et al., 2006).

2.2.2 Structure and physico-chemical properties of chemicals

The chemical structure, log K_{OW}, and molecular weight of B[a]P, chrysene, 9-MA, and PCB 209 are summarized in Table 3.

Table 3: Molecular structure, log K_{OW}, and molecular weight of benzo[a]pyrene (B[a]P), chrysene, 9-methylanthracene (9-MA), and polychlorinated biphenyl (PCB) 209.

Chemical Name	Chemical Structure	Log K_{OW}	Molecular Weight (g/mol)
B[a]P		5.97	252
Chrysene		5.73	228
9-MA		5.07	192
PCB 209		8.27	498

B[a]P, chrysene, 9-MA, and deuterated chrysene (d12-chrysene) were obtained from Sigma-Aldrich (St Louis, MO) and PCB-209 was obtained from AccuStandard (New Haven, CT).

All chemicals were >98% pure. Acetonitrile and hexane were obtained from Caledon Laboratories (Georgetown, ON, CAN).

2.3 Enterocyte isolation procedure

2.3.1 Preparation of solutions

Two buffer solutions (A and B) were used in the isolation procedure to remove enterocytes from the epithelial lining of the intestine. Buffer A contained 27 mM sodium citrate (Bioshop, Burlington, ON), 96 mM NaCl (Caledon Laboratories, Georgetown, ON, CAN), 1.5 mM KCl (EMD Chemicals Inc., Darmstadt, Germany), 5.6 mM K_2HPO_4/KH_2PO_4 (Anachemia Canada Inc., Montreal, QC, CAN/(Caledon Laboratories, Georgetown, ON, CAN)), and 1.0 mM diothiotreitol (DTT) (Bioshop, Burlington, ON). The second buffer (buffer B) contained 1.5 mM ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific Company, Fairlawn, NJ), 140 mM NaCl, 16 mM anhydrous Na_2HPO_4 (EM Sciences, Darmstadt, Germany), and 1.0 mM DTT. Buffer A and B were prepared the day before an experiment and stored at 4^oC until use. DTT was added on the day of enterocyte isolation to each solution after aeration and pH adjustment and prior to filling the intestinal lumen with each buffer.

Eagle's Minimum Essential Medium with Hanks salts (MEM) (Sigma Aldrich Inc., St. Louis, MO) buffered with 10.6 g/L of $NaHCO_3$ (EMD Chemicals Inc., Darmstadt, Germany) was the source of salts, amino acids, vitamins, sugars, and other nutrients to maintain a healthy enterocyte preparation. MEM plus $NaHCO_3$ was prepared in large volumes (1 to 2L) and filter sterilized using a sterilized and disposable 0.22 μM vacuum driven filtration system (Millipore Corporation, Billerica, MA). The solution was then aliquotted into sterile glass bottles and refrigerated at 4^oC until it was ready for use. HEPES (15 mM) was also added to buffers A and B, as well as to MEM solutions.

On the day of the experiment, all three solutions (buffer A and B, and MEM) were gassed with room air for 20 min and allowed to sit for 20 min before adjusting the pH to 7.3 - 7.4. The solutions were stored at 4^{o}C and transferred to room temperature when ready for use where the temperature of each solution was maintained on ice for the duration of the isolation.

2.3.2 Cell isolation

Trout enterocytes were isolated using a modification of the method as described in Pérez et al., (1999) which was a modified procedure from Hegazy et al., (1983). In each experiment, one rainbow trout was anesthetized by immersion in 0.5 g/L of MS 222 (Sigma Chemical Co., St. Louis, MO) buffered with 0.5 g/L of $NaHCO_3$ for 5 min. Cessation of opercular movement was used as an indication of the proper level of anaesthesia. The mass of the fish was then recorded and the fish was transferred to an ice-cold surface for the removal of the intestine.

A ventral incision was made from the gills to the caudal fin to expose the intestine. The intestine was immediately removed from the fish using scissors by cutting the area after the pyloric caecum and before the rectum. Following removal from the fish, the proximal end of the intestine was attached to a luer lock valve with a haemostat to perfuse the lumen of the intestine (Figure 7). The plastic 10 ml syringe was then filled with 10 ml of ice-cold solution A.

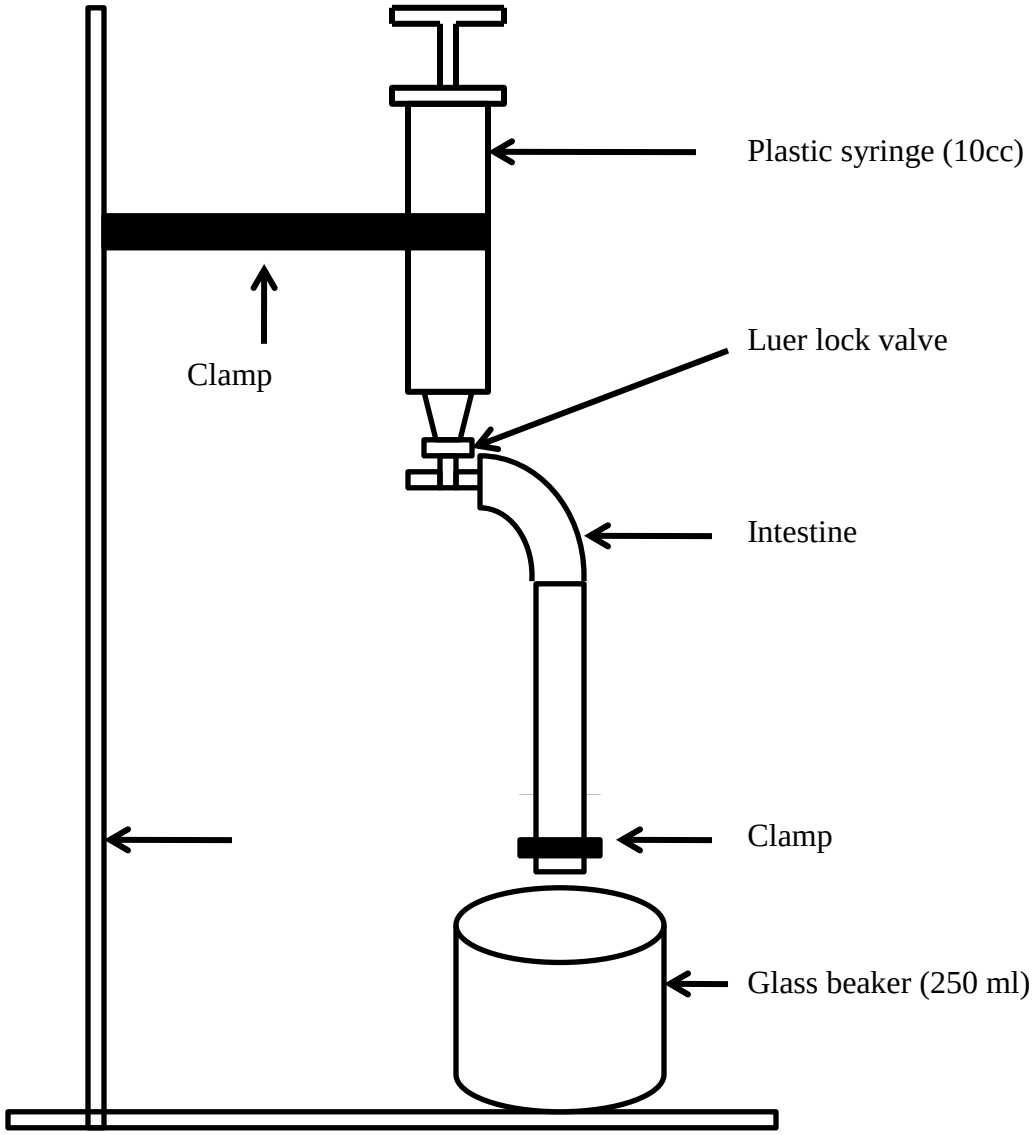


Figure 9: Schematic of the experimental set up used to perfuse the fish intestine with solutions for the enterocyte isolation procedure.

The lumen of the intestine was first flushed with a buffer A (pH 7.4) to remove food residues and mucus by hydrostatic pressure exerted by the syringe, followed by 10-min incubation with the same buffer to equilibrate the cells to the solution and to loosen epithelial cell connections to the intestine. The luminal contents were then emptied and discarded. Once the intestine was rinsed, the second syringe containing 10 ml of ice-cold solution B was

introduced into the lumen and incubated for 3 min and then the intestine containing solution B was gently palpated for 2 min to loosen epithelial cells. The luminal contents were then collected in 100 ml ice-cold MEM solution. This treatment was repeated three times. A clamp was attached to the bottom of the intestine to prevent the emptying of solution A and B during incubation periods.

The cell suspension was centrifuged using the Hermle Z360K centrifuge at 200 x *g* for 8 min and the cell pellet resuspended for 5 min in 20 ml of MEM plus 0.5 mg/ml (final concentration) of collagenase type IV (Sigma-Aldrich St Louis, MO) to produce a single cell suspension. Cells were filtered through a 250-µm nylon mesh to remove larger particles. The enterocytes were then washed twice with 20 ml MEM solution followed by centrifugation at 200 x *g* for 8 min and resuspended in 5 ml MEM solution for determining the yield and viability of the cell preparation.

2.3.3 Cell yield and viability

To a 10 µl aliquot of the cell preparation (in MEM), 10 µl of 0.4% Trypan blue solution in 0.81% NaCl (pH 7.23) was added and mixed by gentle pipetting. The mixture was then transferred to a hemacytometer where cells were viewed under the differential interference contrast setting of the microscope (Zeiss, AxioCam MRm), and photomicrographs were taken using Axio Vision (v 4.7.1.0) software.

Cell yield was determined by counting the live (cells that excluded Trypan blue) and dead (cells took up dye) cells in five randomly selected squares in the 1 mm center square in one chamber of the hemacytometer (Figure 8). Cell density was based on an average from 3 separate counts. As each square of the hemocytometer represents a total volume of 0.1 mm^3, and 1 cm^3 is

equivalent to approximately 1 ml, cell concentration (cells/ml), total yield, and percent viability (%) of the preparation was determined using the following calculations:

Cells per ml = the average count per square X number of squares in the centre square (1)

X dilution factor X 10^4

Total Cells = cells per ml X original volume of fluid of cell sample (2)

Cell viability (%) = total live cells total / total counted cells X 100 (3)

Only enterocyte preparations with a viability of greater than 85% were used in metabolism and enzyme activity experiments.

2.4 Confirmation of enterocyte identity using an epithelial specific marker and immunofluorescence staining

To confirm that the cell suspension obtained from fish intestines were of epithelial origin, the cells were stained with antibodies to cytokeratin. Keratins are a group of water-insoluble proteins that form monofilaments, a class of intermediate filament. These filaments form part of the cytoskeletal complex in epidermis and in most epithelial tissues (Asch and Asch, 1986; Clausen et al. 1986). Other researchers have successfully stained human enterocytes using the cytokeratin monoclonal antibody (AE1/AE3) that was used in the current study (To et al. 2004). The stringent, but broad, specificity of the pooled AE1/AE3 antibody has been shown to be useful as a general stain for cells of epithelial origin (Millipore Corporation, Billerica, MA).

2.4.1 Cell fixation

After centrifugation at 150 x *g* for 8 min, freshly-isolated enterocytes from a single fish were fixed by resuspending the cell pellet in 4% paraformaldehyde solution (in MEM). Cells were incubated with the fixative for 15 min at room temperature.

2.4.2 Adherence of cells to poly-L-lysine coated glass cover slips

Using a grease pencil, a well was made on each glass cover slip poly-L-lysine (0.01%) was then added until the solution completely filled the circled area. Coverslips were allowed to air dry. Fixed enterocytes were adhered to the coverslip by adding an aliquot of the cell suspension to the area for 30 min, followed by three gentle washes with phosphate-buffered saline (PBS).

2.4.3 Cell membrane permeabilization

The cell membranes of enterocytes were permeabilized by adding -20^{o}C acetone to fixed enterocytes and incubating coverslips in the freezer (-20^{o}C) for 5 min. Following incubation, coverslips were transferred to room temperature and allowed to air dry.

2.4.4 Blocking of non-specific binding by secondary antibody

To minimize background staining from non-specific binding by the secondary antibody, samples were treated with 5% normal goat serum (NGS) in Tween 20 phosphate buffered saline/0.1% bovine serum albumin (TPBS/BSA) for 20 min at room temperature, followed by three washes with PBS.

2.4.5 Incubation with primary and secondary antibody

Coverslips were incubated in a solution of 50 μg/ml mouse anti-cytokeratin AE1/AE3 monoclonal antibodies (in 1% NGS) (Millipore Corporation, Billerica, MA) at 4^{o}C overnight in a humidified chamber. Each coverslip was then transferred to a well in a 6-well plate and washed three times with PBS. To each well, a 12 μg/ml solution Alexa Fluor 488-conjugated goat anti-mouse IgG (in TPBS/BSA) (Sigma, Saint Louis, MO) and incubated for 60 min at room temperature. Coverslips were then washed three times with TPBS/BSA.

Control samples were prepared in the same way except that 1% NGS and TPBS/BSA were used instead of the primary and secondary antibodies, respectively. Cells were examined using a WaveFX spinning confocal microscope (Quorum Technologies, Guelph, ON) and images were processed using Volocity software (Perkin Elmer, MA, USA).

2.5 Measurement of cytochrome P450 3A27 and P450 1A1 enzyme activity

2.5.1 Description of luminescence assay

The P450-Glo^{TM} assays (Pro-Mega) employ a luminescence method for measuring Cytochrome P450 (CYP450) activity and were designed to measure the activities of CYP450 enzymes from recombinant and native sources as well as to test the effects of chemical compounds on CYP450 activities. The assays were performed by incubating freshly-isolated cells (enterocytes or hepatocytes) with a luminogenic substrate. The substrates are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6`-hydroxy-2`-benzothiazolyl)-4-thiazolecarboxylic acid] and are CYP450 enzyme substrates but not substrates for lucerifase. The derivatives are converted by CYP450 enzymes to a luciferin product that is detected in a second reaction with the Luciferin Detection Reagent. The amount of light produced in the second reaction is proportional to the activity of the CYP450 (Figure 9) (ProMega, 2009).

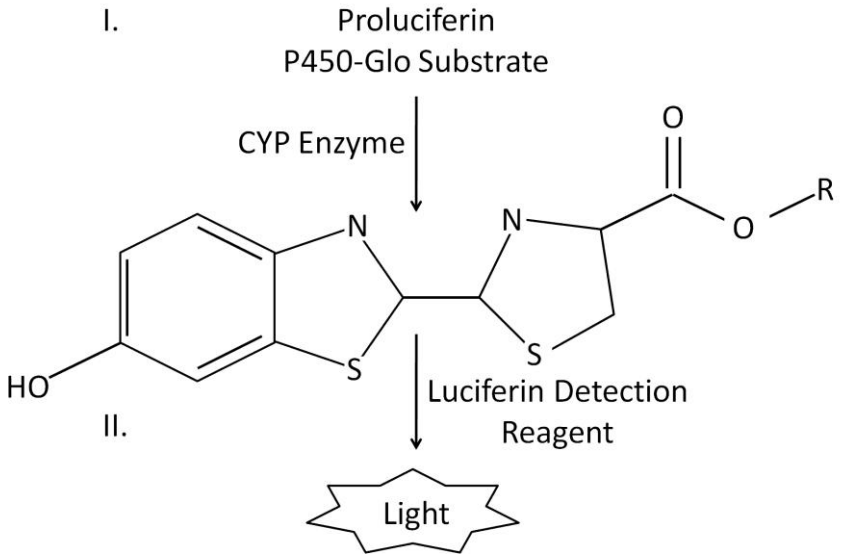


Figure 10: Schematic of the assay for cytochrome P450 activity. CYP enzymes in the sample convert a luminogenic (Reaction I) to a luciferin product. Addition of the luciferin detection reagent (Reaction II) stops the reaction and generates light in proportion to the CYP activity. R = organic substituent.

The substrate provided with the kit was created to probe CYP450 3A4, a mammalian cytochrome P450 isoform responsible for the metabolism of a very wide range of chemicals. This isoform has the most broad substrate range of all known P450s and catalyzes the oxidation of many chemotherapeutic compounds including CYP3A4 has been identified as an important isoform in human enterocytes (Paine et al., 1997; Watkins et al., 1987). In *O. mykiss*, no CYP3A4 isoform exists but a reciprocal best blast search revealed that the homologue with the highest percent similarity is CYP 3A27 (55% identity (277/507), 75% positives (379/507), 1% gaps (4/507)) (Appendix 1). Furthermore, the highest expression of CYP3A27 in *O. mykiss* was found in the intestine (Lee et al., 1999). Therefore, we hypothesized that the commercially-available substrate would provide a measure of CYP3A27 activity in *O. mykiss* enterocytes and hepatocytes.

2.5.2 Overview of biochemical assay

To a 2 ml frosted microcentrifuge tube, 12.5 µl of a 2X CYP450 reaction mixture (ProMega^{TM}) was added to 12.5 µl of the cell suspension and incubated at 13^{o}C for 180 min. To stop the reaction and initiate luminescence, a 1:1 volume ratio of the reconstituted luciferin detection reagent was then added to the substrate plus cell mixture. The luminescence was allowed to stabilize for 20 min at room temperature. Total luminescence was measured with a luminometer (Turner Designs 20/20, CA, USA,) in standard mode with a 2-sec delay and 10-sec integration. The enzyme activity in the negative control samples was terminated by heat inactivation at 95^{o}C for 10 min.

2.5.3 Buffers and solutions

The lyophilized luciferin detection reagent and buffer were transferred from a -20^{o}C freezer and equilibrated to room temperature. One bottle of reconstitution buffer 10 ml was transferred to an amber bottle containing the lyophilized luciferin detection reagent and the suspension was mixed by swirling the bottle several times to obtain a homogenous solution. Luciferin-PFBE (CYP3A4) and Luciferin-CEE (CYP1A1) were provided as aqueous solutions and were kept on ice when assays were being conducted. The provided stock solution of CYP450 substrate was diluted with MEM to make a 2X concentration CYP450 reaction mixture (400 mM).

2.5.4 Cell based enzyme activity assay

Freshly isolated enterocytes and hepatocytes were adjusted to a concentration of 2x10^{6} cells/ml and a sample (~1x10^{6} cells) was removed for the negative control. Hepatocytes were provided by Jennifer Trowell using a collagenase perfusion procedure as described in Moon et al. (1985) with modifications (Gourley and Kennedy, 2009).

Freshly isolated hepatocytes from the same fish, generously provided by Jennifer Trowell, were also used for comparison in both CYP3A27 and CYP1A1 enzyme assays. MEM alone was found to exhibit no luminescence when the detection reagent was added. Each of the above treatments was prepared in triplicate using freshly-prepared enterocytes and hepatocytes.

To each sample, 50 µM (final concentration) of Luciferin-PFBE (in MEM) and a 50 µl aliquot of either live or heat-inactivated enterocytes, or live hepatocytes was added to a 2 ml frosted microcentrifuge tube, and incubated at 13^{o}C for 3 h. Following incubation, the luminescence of each sample was read using a luminometer as described above. Finally, detection reagent (100 µl) was added to each sample, and mixed by finger vortexing, and maintained at room temperature for 20 min before luminescence was read again. The CYP1A1 assay was conducted using the same conditions except that the final concentration of Luciferin-CEE was 100 µM as recommended by the manufacturer.

2.5.5 Calculation of luminescence

The luminescence of each sample was determined by calculating the ratio of luminescence measured before and after adding the detection reagent, followed by normalization of luminescence per 1 million cells. The mean of the ratios from the triplicate samples per treatment type was then calculated.

2.6 Intraperitoneal injection of the CYP450 1A1 inducer, ß-naphthoflavone

Four days prior to the cell isolation, fish were injected intraperitoneally with 50 mg/kg ß-naphthoflavone dissolved in coconut oil-using a 21 gauge syringe. The volume of the inducing preparation injected into the fish was determined by fish weight to attain the desired concentration. The CYP induction period was based on a previous study that showed that the

level of CYP1A1 activity is not significantly different between 4-7 days after induction (Smith and Wilson 2010).

2.7 Exposure of cells to selected chemical compounds

2.7.1 Incubation conditions

Chemical incubations were conducted in 2 ml amber glass vials (Agilent Technologies, Santa Clara, CA) to prevent photodegradation of the test compounds during the experiment. Following isolation, the concentration of the enterocyte preparation was adjusted to $2x10^6$ cells/ml, a 500 µl aliquot (1 million cells) was transferred to each glass vial and 5 µl of the test chemical(s) was added to each sample by conventional solvent phase dosing. Stocks of chemical(s) were dissolved in acetonitrile and the final concentration of B[a]P and each chemical (B[a]P, chrysene, 9-MA, and PCB 209) in the mixture incubations was 0.25 µM. This concentration was chosen for B[a]P because it was environmentally relevant and was well below their published Michaelis-Menten constants (K_m) (Nishimoto et al., 1992), and chrysene and 9-MA have been shown to be metabolizable by S9 fractions in rainbow trout (Lee, 2009). The volume of solvent in each sample never exceeded 1% of the total volume. Incubation times for metabolism experiments were 0, 10, 20, 30, 60, 120, and 240 min with triplicate samples for each time point. At the end of the designated incubation period, an internal standard of deuterated chrysene (d12-chrysene) (0.25 µM) dissolved in acetonitrile, was added to each sample. The purpose of the internal standard was to control for inconsistencies in the volume of the extract removed for analysis from sample to sample, in addition to any changes in GC-MS conditions.

Reactions were terminated by adding 1 ml of ice-cold hexane to each sample vial followed by brief vortexing. Control samples were prepared by either heat-inactivation of the

cells at 90^{o}C for 10 min prior to addition of the test chemical. In some experiments, metabolic activity was inhibited by adding sodium azide (0.3 mM) for 30 min prior to the start of incubation. Otherwise, the control samples were handled in the same manner as live cells.

2.8 Chemical extraction from intestinal cells

After the addition of 1-ml hexane, each sample was vigorously vortexed for 45 seconds to extract chemicals from cells and the incubation media. After separation of the organic and aqueous layers, 0.6 ml of the hexane extract was transferred to a clean 2 ml amber glass vial.

In some experiments, the hexane extract was concentrated. After evaporation of the hexane in the fume hood at room temperature overnight, the residue was redissolved in 100 µl of hexane and transferred to a 400 µl flat bottom glass insert (Agilent Technologies, Santa Clara, CA).

2.9 Gas chromatography-mass spectrometry analysis (GC-MS) of extracted chemicals

The hexane extract was analysed using an Agilent 6890 gas chromatograph (GC) in conjunction with an Agilent 5973 mass spectrometry (MS) detector (Agilent, Mississauga, ON). The column was an HP-5M5 5% phenylmethyl siloxane-coated capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) protected by a fused-silica deactivated guard column (5 m x 0.530 mm i.d.) (Agilent, Mississauga, ON). The injection volume was 1 µL, and the helium carrier gas flow rate was 1 mL/min. The GC was programmed with an injection temperature of 63°C, followed by a temperature ramp of 20°C/min to a temperature of 200°C. The temperature was held at 200°C for 1 min, after which a temperature ramp of 15°C/min was initiated to a maximum temperature of 285°C. The MS quantified the target compounds at select ions (*m/z* 192 for 9-MA, *m/z* 228 for chrysene, *m/z* 252 for B(a)P, *m/z* 498 for PCB-209 and *m/z* 240 for deuterated chrysene) using an ion energy of

70eV and an ion source temperature of 230°C. The parameters on the GC-MS were optimized by Victoria Otton.

Chromatograms for chemicals detected in each sample generated by the mass-spectrometer were collected using Enhanced ChemStation software (G1701CA Version C 00.00 21-Dec-1999, Agilent Technologies, 1989-1999). The data collected by the mass-spectrometer was plotted using three axes: time, abundance, and mass/charge (m/z) and the amount of each test compound and the internal standard was determined by measuring the ratio of peak areas.

2.10 Quantifying metabolites and calculating rates of metabolism

2.10.1 Depletion rate constant

In each experiment, the natural logarithm of each chemical(s) concentration was plotted against time for live and sodium azide control samples. Linear regression was then performed on each treatment and the depletion rate constant for each chemical was calculated from the slope. Depletion rate constants were not adjusted to account for extraction efficiency because it has been shown that the extraction efficiency of 9-MA, B[a]P, chrysene and PCB 209 (PCB 153 was tested) from hepatocytes were not significantly different from 100% (Trowell, 2010).

The concentration of the chemicals used in this study (0.25 μM) were assumed to follow first-order kinetics because preliminary studies measuring the depletion of B[a]P by enterocytes showed that the rate was not constant. This suggests that the concentration of the chemical added to the test system did not saturate the metabolic enzymes of rainbow trout enterocytes, and the maximum rate of depletion has yet to be reached.

2.11 Statistical analysis

A Student‘s *t* test was used to compare depletion rate constants of compounds in live cells in comparison to control samples ($p<0.05$). All statistical analysis was conducted using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com”.

3: Results

3.1 Enterocyte isolation optimization factors and cell yield

Initial experiments typically yielded 2.53 $\pm$ 1.96 x 10^7 cells/intestine with viabilities of 92 $\pm$ 5% after isolation and 84.5 $\pm$ 4.5% over 5 h (n=6). To improve the quality, and yield of enterocytes, several factors related to the isolation conditions and media were systematically investigated. The experimental factors found to be important to a successful cell isolation (healthy cells, single cell suspension, high yield) are summarized in Table 4. The pH of the buffers used in the isolation procedure, and media (MEM) used to collect the enterocytes also played a large role in maintaining a healthy cell preparation. Various factors affecting pH were tested, and the results are summarized in Table 5. The optimized enterocyte isolation procedure yielded ~420 $\pm$ 50 million cells from a single intestine, producing initial cell viabilities of 96 $\pm$ 3% and 90 $\pm$ 4.5% after 4 h of incubation at 13^{o}C (n=9). Figure 10 shows a light microscopic image of a typical enterocyte preparation.

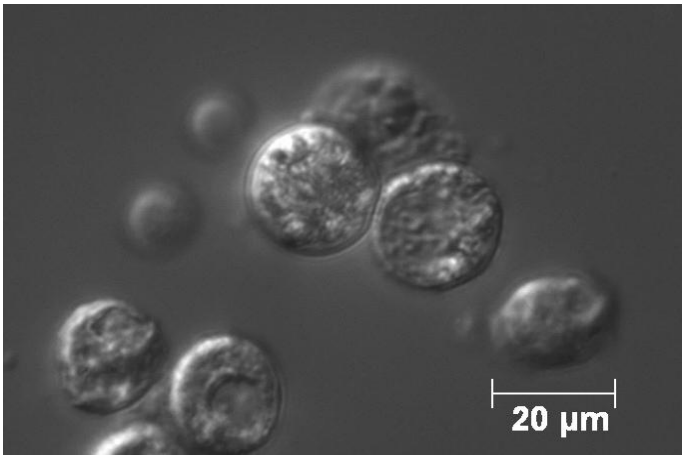


Figure 10: Differential interference contrast image of freshly-isolated enterocytes from *O. mykiss* intestine.

3.2 Factors affecting cell health and yield

3.2.1 Concentration of DTT used to reduce mucus viscosity to aid in removal from the intestine

In the protocol by Pérez (1999), 0.5 mM DTT was added only to buffer B. In my experiments, the concentration of DTT was increased to 1.0 mM and was added to both buffer solutions A and B to reduce the viscosity of the mucus inside the intestine. Following the 10-min incubation period with solution A, a large proportion of mucus in the intestine is removed, thus exposing the epithelial cell layer. Further incubations with buffer solution B plus DTT visibly reduced the viscosity of any remaining mucus.

3.2.2 Media

MEM was used as a medium to collect isolated enterocytes as per Pérez et al., (1999) and as an incubation medium because it contains sufficient amounts of nutrients (inorganic salts, essential and non-essential amino acids, vitamins, and glucose) to maintain a healthy cell suspension over 5 h. Other researchers have been successful using variations of Ringers saline solution as a nutrient media to isolate enterocytes from rainbow trout and gilt-head sea bream intestine (Burke and Handy, 2005; Dópido et al., 2004; Rotllant et al., 2006). MEM was preferred in our experiments because it was nutrient rich and easy to prepare. The benefit of using a commercially prepared media in comparison to self-prepared solutions is that the quality of the ingredients in the media is guaranteed via quality control tests.

3.2.3 Concentration of collagenase used to produce a single cell suspension

Upon isolation, large masses of enterocytes are encapsulated in mucus, and required treatment with collagenase to produce a single cell suspension so that the concentration of the preparation could be determined. Collagenase is protease commonly used to break down the

native collagen in the basal lamina and extracellular matrix. The optimal concentration of collagenase used to treat the isolated enterocytes for 5 min was determined to be 0.5 mg/ml.

3.2.4 Filtering of luminal contents before or after treatment of collagenase

Prior to collecting the cells in MEM, the enterocyte preparation eluted from the intestine was filtered through a 200 µm pore sized nylon mesh to remove digestate and mucus in initial experiments (Pérez 1999). I determined that if cells were filtered following collagenase treatment where debris was removed from a single cell suspension instead of masses of cells a higher yield of cells was achieved (370 – 470 million).

3.2.5 Size of nylon filters used to remove debris and remaining mucus from isolated luminal contents

In my initial experiments, the luminal contents were first passed through nylon mesh with a larger pore size (250 µm) to remove large particles of debris followed by a second filtration step through a medium pore sized nylon mesh (200 µm). Because the two pore sizes of the nylon mesh are similar, this filtration system did not make any difference in removing any debris. This part of the procedure was then modified by using 200 µm nylon mesh to remove digestate and mucus, followed by a second filtration through a smaller pore sized nylon mesh (75 µm) to remove any remaining debris from the eluted luminal contents. For the final optimized cell isolation procedure, only a 200 µm pore sized nylon mesh was used to remove any debris or mucous from the collagenase treated cell preparation. The second filtration step (75 µm) was later removed as it did not decrease number of cells isolated per fish.

Table 4: Factors varied to optimize the yield and viability of *O. mykiss* enterocytes. n/a = not applicable

Factor	Variables Tested	Optimal Variable	Effect on Isolation Procedure	Effect on yield or viability
pH	7.3 – 7.6	7.3 – 7.4	Minimized cell blebbing	Increased cell viability from
Concentration of DTT	0.5, 1.0 mM	1.0 mM	Reduced viscosity of mucous exposes epithelial cell layer in intestine, and increased ease of separation from its cells	Increased yield by 10-fold
Concentration of Collagenase used in a 5 min treatment	0.1, 0.5, 1.0 mg/ml	0.5 mg/ml	Single cell suspension	n/a
Filtration Step	Filtering before or after collagenase treatment	After	Higher cell yield as masses of cells do not get stuck in the filter	Increased yield by 10-fold
Filtration system	250/200 µm vs 200/75 µm vs 200 µm	200 µm only	Removed most digestate from cell suspension	n/a

3.3 Factors affecting the pH of isolation solutions and incubation media

3.3.1 Aeration of solutions

During the isolation procedure, it was important to maintain the pH of all of solutions in the range of 7.3 – 7.4 to yield a high number of cells isolated, and to maintain a healthy suspension of cells that had viabilities of greater than 85% for the entire experiment (5h) when tested using trypan blue exclusion. The composition of the source of aeration was also important to sustaining the pH in the optimal range. Carbogen was initially used to aerate all of the

solutions as per Pérez (1999); however, the amount of carbon dioxide (95%) in carbogen was found to cause the pH to fluctuate outside the optimal range over the duration of the experiment.

3.3.2 Solution rest period prior to pH adjustment

In the original protocol, the pH was adjusted immediately after aeration because the health of the cells and the isolation procedure are pH sensitive. I found that more stable pH was obtained if aerated solutions of MEM and buffers A and B were allowed to sit for 20 min before the pH was adjusted.

3.3.3 Addition of sodium bicarbonate before and after aeration

Sodium bicarbonate ($NaHCO_3$) was originally added to MEM prior to aeration. The protocol was later modified to add $NaHCO_3$ after solutions were aerated to increase the compounds effectiveness at maintaining a solutions pH (Gourley and Kennedy, 2009)

Table 5: Factors varied to maintain the pH in the optimal range (7.3 – 7.4) of buffer solutions A and B, and MEM used in the isolation of *O. mykiss* enterocytes (Carbogen: 95% O_2/5% CO_2, Air: 20.95% O_2/0.039% CO_2).

Factor	Variable Tested	Optimized Variable	Effect on pH or cell health
Aeration	Air vs Carbogen	Air	Less pH fluctuation
Resting period before pH adjustment	0 vs 20 min	20 min	Allow solution to equilibrate with air
Addition of $NaHCO_3$ to solutions	Before vs After Aeration	After	Increase buffering effectiveness

3.3.4 Verification of the epithelial nature of the isolated cells using an epithelial-specific antibody

As described in the Materials and Methods, the antibody used to identify cells as epithelial was the pan-cytokeratin antibody raised in mice. Confocal microscopy was used to examine adherent fixed cells that had been permeabilized and treated with the antibody. Control cells received the same treatment except that samples were treated with either non-specific immuglobulin or 1% NGS instead of the primary antibody. The results are shown in Figure 11. Cells showed positive staining with the cytokeratin antibody compared to the control cells, hence, the isolated cells were of epithelial origin. Exposure time, sensitivity, and laser intensity for control samples were 3.47, 2.21, and 1.19 times higher than samples treated with pan cytokeratin antibodies.

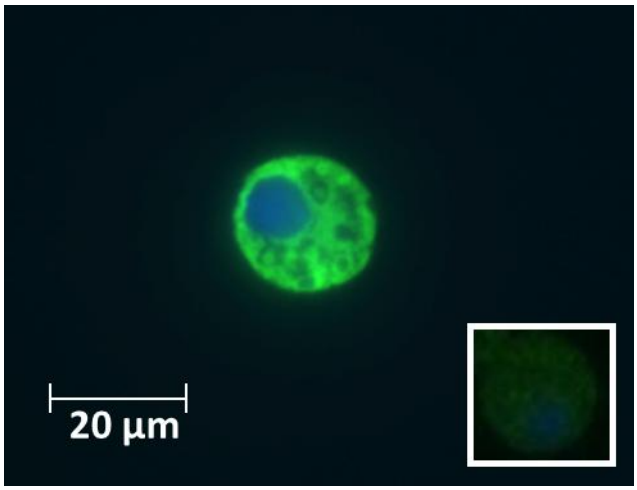


Figure 11: Confocal laser scanning microscopy image of enterocyte stained with anti-cytokeratin monoclonal antibody (green) and nuclear staining with DAPI (blue). Control cells are shown in inset under the same exposure (no primary antibody).

3.4 Biotransformation Rates (k_r)

3.4.1 Chemical mixture (B[a]P, chrysene, 9-MA, and polychlorinated biphenyl 209)

The biotransformation of a mixture of chemicals containing 0.25 μM each of B[a]P, chrysene, 9-MA, and PCB 209 dissolved in acetonitrile over 2 h by live enterocytes compared to sodium azide control cells is shown in Figures 13-16. As expected, PCB 209, fully substituted with chlorine atoms, was not metabolized by enterocytes (Figure 16). B[a]P, chrysene, and 9-MA showed a trend of decreasing concentration over the course of the experiment, however, the rates of metabolism were not statistically different from the controls (Table 6 and Figure 13-16).

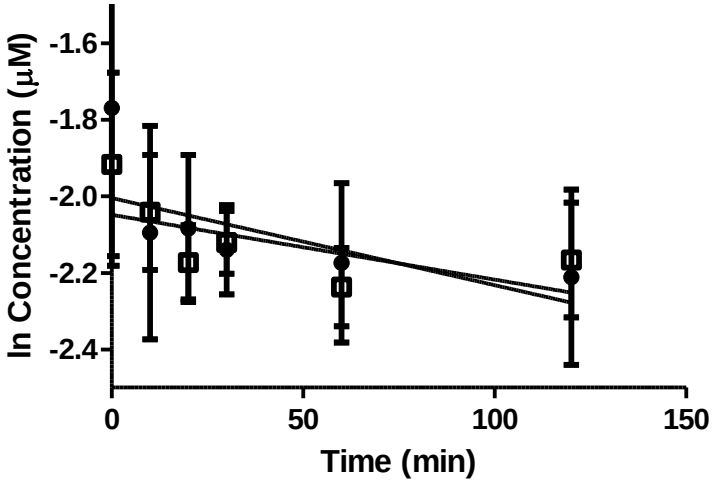


Figure 12: Change in the natural logarithm of the concentration of B[a]P (μM) in a mixture also containing chrysene, 9-MA, and PCB 209 during 120 min of incubation with live (●) or sodium azide-treated (□) enterocytes. The values shown are the mean ± SD of three independent experiments.

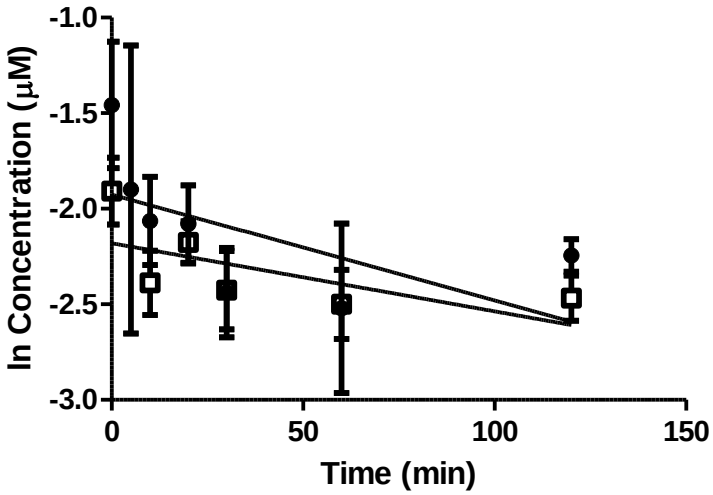


Figure 13: Change in the natural logarithm of the concentration of 9-MA (µM) in a mixture also containing B[a]P, chrysene, and PCB 209 during 120 min of incubation with live (●) or sodium azide-treated (□) enterocytes. Values shown are the mean ± SD of three independent experiments.

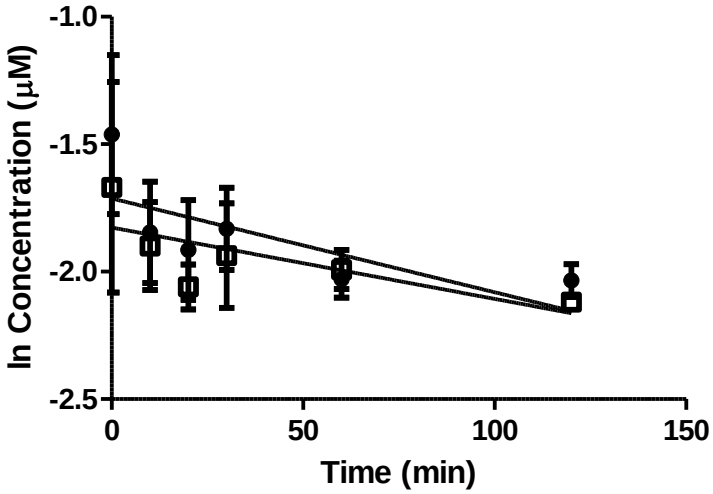


Figure 14: Change in the natural logarithm of the concentration of chrysene (µM) in a mixture also containing chrysene, PCB 209, 9-MA during 120 min of incubation with live (●) or sodium azide-treated (□) enterocytes. Values shown are the mean± SD of three independent experiments.

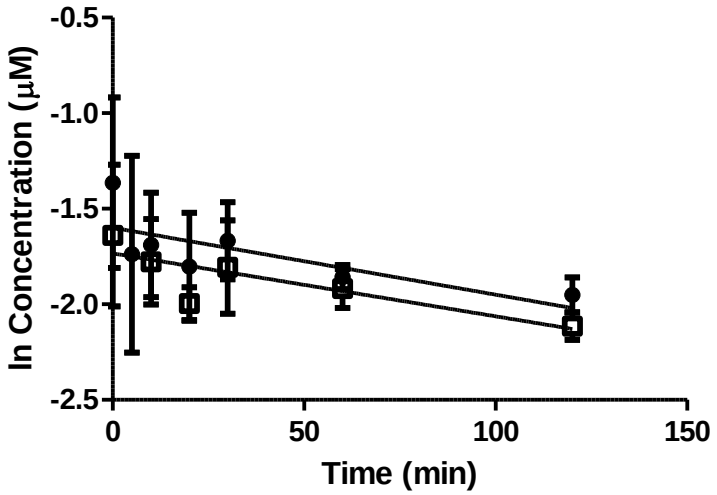


Figure 15: Change in the natural logarithm of the concentration of PCB 209 (µM) in a mixture also containing B[a]P, chrysene, and 9-MA during 120 min of incubation with live (•) or sodium azide-treated (□) enterocytes. Values shown are the mean ± SD of three independent experiments.

Table 6: Depletion rate constants for B[a]P, chrysene, 9-MA, and PCB-209. The results presented are the mean of independent experiments with 95% confidence intervals presented in brackets.

Chemical	**k_r of live cells ($x10^{-3}$/min/10^6 enterocytes)**	**p-value**	**k_r of sodium azide controls ($x\ 10^{-3}$/min/ enterocytes)**	**p-value**	**p-value (live vs. sodium azide)**
B[a]P	2.23 (0 – 7.30)	0.0999	1.70 (0 – 4.65)	0.1978	0.8598
Chrysene	3.68 (0 – 8.26)	0.0862	2.80 (0 – 7.73)	0.1068	0.8105
9-MA	5.54 (0 – 13.2)	0.0296	3.56 (0 – 7.95)	0.1820	0.2464
PCB 209	3.50 (0 – 9.40)	0.2267	3.30 (0 – 8.26)	0.1761	0.966

3.4.2 Metabolism of B[a]P in uninduced and induced fish

To determine whether induction by β-naphthoflavone would increase the rate of B[a]P metabolism by entercoytes, the biotransformation of B[a]P by live enterocytes from uninduced and induced fish was determined. In these experiments, control cells were inactivated using sodium azide. There was a trend of decreasing B[a]P in cells from both uninduced and induced fish when compared to controls. For uninduced fish, the rate of enterocyte B[a]P metabolism was 3.76 x 10^{-3} /min/10^{6} enterocytes (0 – 11.8 x 10^{-3} /min/10^{6} enterocytes) compared to the control of 1.57 x 10^{-4} /min/10^{6} enterocytes (0 – 9.94 x 10^{-3} /min/10^{6} enterocytes). For the cells from induced fish (Figure 18), the rates for the live and control cells were 4.39 x 10^{-3} /min/10^{6} enterocytes (0 – 5.41 x 10^{-3}/min/10^{6} enterocytes) and 5.67 x 10^{-3}/min/10^{6} enterocytes (0 – 6.37 x 10^{-3}), respectively. Statistical analysis revealed that the rate of biotransformation of B[a]P by live enterocytes in both test systems was not statistically different from the controls.

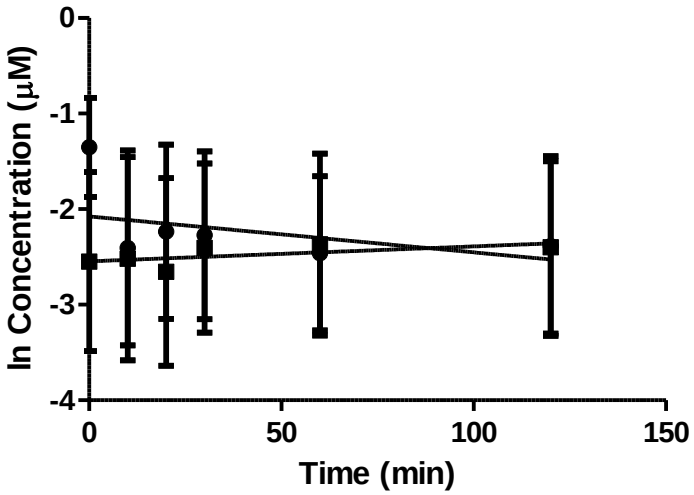


Figure 16: Change in the natural logarithm of the concentration of B[a]P (µM) during 120 min of incubation with live (●) or sodium azide-treated (■) enterocytes isolated from uninduced fish. Values shown are the mean ± SD from three independent experiments.

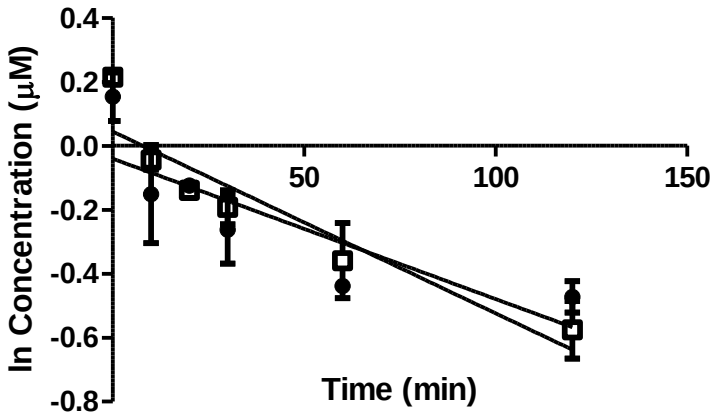


Figure 17: Change in the natural logarithm of the concentration of B[a]P (µM) during 120 min of incubation with live (●) or sodium azide-treated (□) enterocytes isolated from induced fish. Values shown are the mean ± SD of three independent experiments.

3.5 Comparison of control sample treatments

It was believed that part of the problem with the apparent lack of activity may have been due to the non-zero slope of the heat-treated control cells, perhaps due to sequestration of the test chemical resulting in poor extractability over the incubation period. Therefore, I directly compared the B[a]P recovery from heat-treated and sodium azide treated control cells. The results are shown in figure 19. Statistical analysis revealed that the slope of the heat-treated controls was not significantly different from that of the sodium azide treated controls.

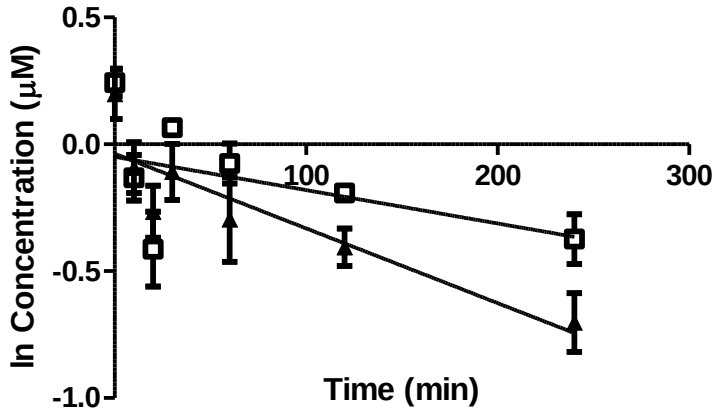


Figure 18: Change in the natural logarithm of the concentration of B[a]P (µM) during 240 min of incubation with heat-inactivated (▲), or sodium azide-treated (□) enterocytes isolated from induced fish. Values shown represent the mean + SD of three independent experiments.

3.5.1 Relative CYP450 3A27 activity of enterocytes vs. hepatocytes in uninduced fish

To determine whether the enterocytes had active CYP450 activity, CYP3A activity was semi-quantitatively measured in freshly-isolated entercytes and hepatocytes from the same fish using the Promega luminescent assay purchased from Fisher. In this assay, the amount of light produced in the reaction between the luciferin product and the detection reagent is proportional to the activity of the CYP450.

The data in Figure 20 show that when normalized to the number of cells present, isolated enterocytes had a higher CYP450 3A27 activity than hepatocytes isolated from the same fish. Enzyme activity was reduced by 70% when enterocytes were heat-treated prior to the assay.

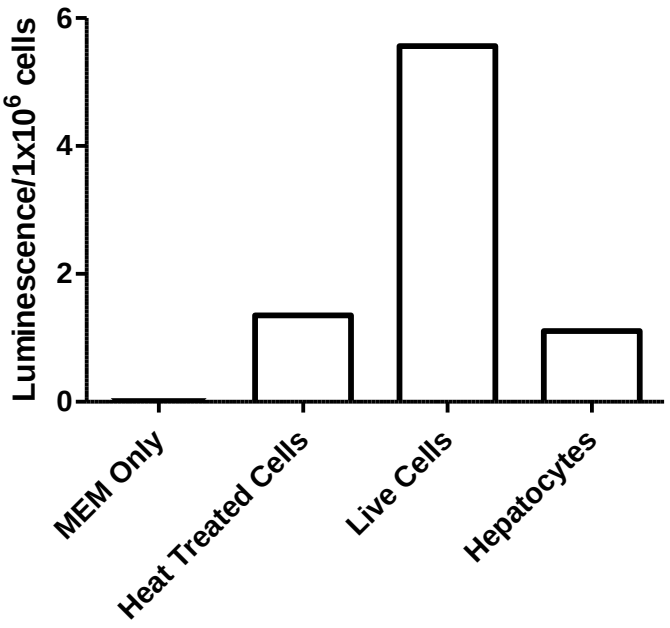


Figure 19: CYP450 3A27 activity in freshly-isolated *O. mykiss* enterocytes and hepatocytes from the same animal using a luminescence assay with a human CYP3A4 substrate. Heat treatment was at 95°C for 10 min. The luminescence in the graph is defined as the ratio of luminescence before and after adding detection reagent. Values shown are the mean of three replicates from a single fish and each replicate deviated less than 10% from the mean.

3.5.2 Effect of CYP1A1 induction on the enzyme activity in enterocytes and hepatocytes

To determine whether enterocytes contained the CYP1A1 isoform, and whether the enzyme was inducible, fish were injected with β-naphthoflavone. Fish were injected with β-naphthoflavone between 4 days prior to organ harvesting. The relative CYP1A1 activity of enterocytes and hepatocytes (live and heat treated) were determined using a 1A1 substrate with the ProMega luminescence assay. For uninduced fish, CYP1A1 activity of live enterocytes was not significantly greater than in heat-treated cells, i.e., 1A1 activity was not detectable using this method. Induction with β-naphthoflavone increased the CYP1A1 activity in enterocytes approximately two-fold over background levels.

With induction, the CYP1A1 activity in hepatocytes increased approximately 10-fold. When compared with enterocytes, hepatocytes from induced fish contained approximately 400 times the activity when normalized to the number of cells present.

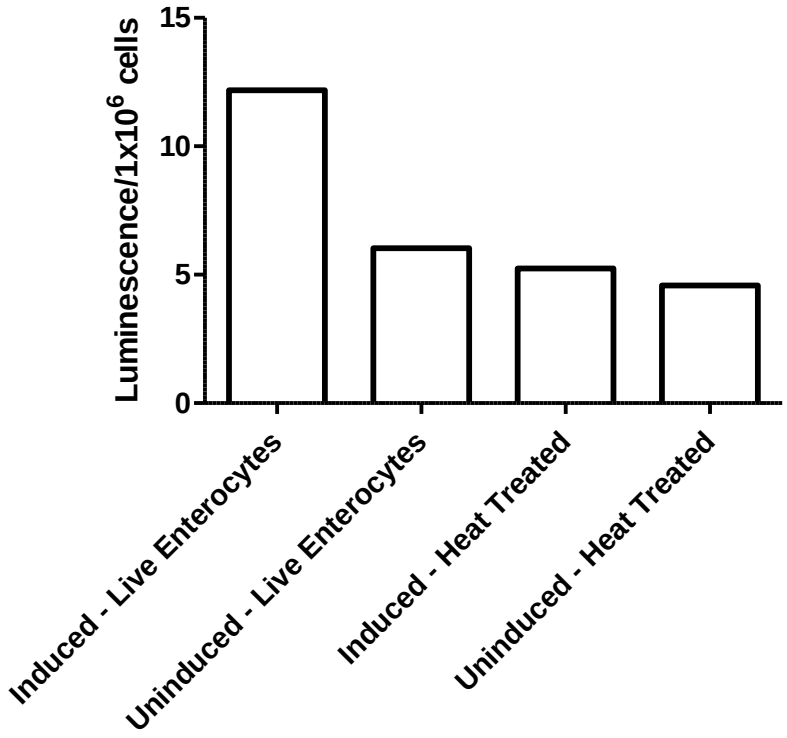


Figure 20: CYP450 1A1 activity in freshly-isolated enterocytes from uninduced fish and fish induced with beta-naphthloflavone measured using a commercial luminescence assay. Heat treatment was at 95^{o}C for 10 min. Induced fish were intraperitoneally injected with 50 mg/kg of ß-naphthoflavone for 3-5 days. Values shown are the mean of three replicates from a single fish and each replicate deviated less than 10% from the mean.

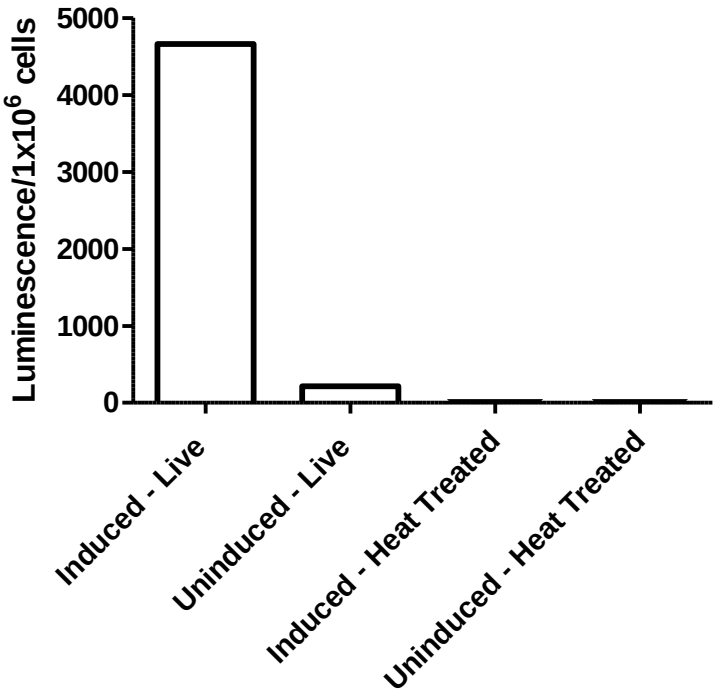


Figure 21: CYP450 1A1 activity in freshly-isolated hepatocytes from uninduced fish and fish induced with beta-naphthloflavone measured using a commercial luminescence assay. Heat treatment was at 95^{o}C for 10 min. Induced fish were intraperitoneally injected with 50 mg/kg of ß-naphthoflavone for 3-5 days. Values shown are the mean of three replicates from a single fish and each replicate deviated less than 10% from the mean. Note the difference in the scale of the y-axis in coamparison to Fig. 20.

4: Discussion

Anthropogenic activities have created large amounts of chemicals that are eventually released into our environment every year, and there are growing concerns over the global distribution of these chemicals. The Canadian government has established a Priority Substance List that identifies substances to be assessed on a priority basis to determine if they pose a risk to the health of Canadians or to the environment (CEPA, 1999). Currently, there are ~23,000 chemicals listed as substances that are awaiting evaluation for their bioaccumulation potential as one of the three criteria including persistence, bioaccumulative potential and toxicity screening criteria. Bioaccumulative chemicals have the potential to bioaccumulate in organisms, which can lead to the biomagnification of the compound to harmful concentrations in upper trophic level organisms and human beings. Currently, the endpoints used to assess bioaccumulative potential include *in vivo* approaches in fish that yield values such as the bioaccumulation factor (BAF) and bioconcentration factor (BCF), and *in vitro* approaches such as octanol-water partition coefficient (K_{OW}). Computational models have also been developed to make predictions based on the physico-chemical characteristics of a chemical (Arnot and Gobas, 2003; 2004; Gobas and MacKay, 1987; Law, et al., 1991; Mackay, 1982; Neely, et al., 1974; Nichols, et al., 1990; Veith et al., 1979). BAF and BCF tests provide valuable information on bioaccumulation as the whole organism is used in tested, but are expensive, time consuming, and would require a huge number of animals to test the 23,000 chemicals on the DSL. K_{OW} is a rapid and inexpensive test that is suitable for testing a broad range of chemicals; however, does not take into consideration any changes in chemical properties if it is metabolized once it enters an

organism. Computational models include dietary uptake and elimination parameters, including biotransformation, to predict bioaccumulative potential of chemicals. Due to the lack of biotransformation data available in literature, these models currently assume that there is no metabolism of chemicals in the organism. Such an approach may overestimate bioaccumulative potential and cause mis-classification of a substance. To address the need of a rapid and inexpensive approach to assess the bioaccumulative potential of a chemical, our research group is interested in developing a model that includes an *in vitro* test to measure biotransformation of a chemical. My contribution to the project was to develop an *in vitro* method to measure the biotransformation of anthropogenic chemicals using freshly isolated enterocytes from rainbow trout intestine.

The biotransformation of commercial chemicals by fish intestinal enterocytes was included in the overall project because many of the compounds on the DSL have highly hydrophobic properties that will likely bind to food or sorb to particulate matter in the aquatic media. Therefore, fish are primarily exposed to HOC's in their diet where first-pass metabolism can occur. First-pass metabolism is fundamental to consider in the overall breakdown of chemicals because it can contribute to the breakdown of chemicals to a significant extent. Biotransformation of the compound can result in metabolites that are more easily excreted and lower the bioaccumulative potential of the parent compound.

4.1 Selection of an intestinal test model

Freshly isolated enterocytes were selected as a test system in favour of other approaches (i.e. microsomal fractions, perfused *in situ* preparations, etc.) to study the biotransformation of HOC's in fish intestine primarily because intact cells contain all metabolic enzymes normally present in the intestinal epithelial layer. This is in addition to the presence of functional

xenobiotic transporters, generation of reducing agents through metabolic processes and attribution of metabolism to a specific cell type. This approach has been used in rat intestinal metabolism studies (Grafström et al, 1997; Stohs et al, 1977) but it is novel to the area of intestinal biotransformation of environmental contaminants in fish.

As noted above several researchers have used rainbow trout enterocytes as a test system to study intestinal activities (e.g., fatty acid metabolism studies, uptake, and characterization (Pérez, 1999; Oxley et al., 2005; Burke and Handy 2005; Kwong et al., 2010). A comparison of these methods to isolate the enterocytes to the current study is shown in Table 7. None of the studies provided the cell yield from one fish.

Table 7: A comparison of the percent cell viabilities and loss of lactate dehydrogenase (where available), pH of solutions, and temperature the procedure of the current study to other studies using rainbow trout enterocytes. Statistics for cell viabilities are shown for studies when available. n/a: data not available

Reference	Viability	Loss of LDH	pH	Temp (^{o}C)
Current study (n=9)	96 $\pm$ 3% at isolation 90 $\pm$ 4.5% after 4h	n/a	7.3 - 7.4	13
Pérez et al., 1999 (n=12)	88.9 $\pm$ 3.2 % at isolation 78% after 4h	0.14%/min after 1h 0.24%/min after 2h	7.3	13
Tocher et al., 2004 (n=120)	>95% at isolation >85% after 2h	n/a	n/a	20
Burke and Handy, 2005 (n=54)	82% at isolation 78% after 4h	n/a	7.4	Room temperature
Kwong et al., 2010 (n=n/a)	93 $\pm$ 3% at isolation >85% after 3h	n/a	7.4	15

On average, the isolation procedure used in the current study yielded higher percent cell viabilities at isolation (96 $\pm$ 2%) and over 4h (90 $\pm$ 4.5%) than all of the other approaches demonstrates that the cells obtained from the present procedure are more stable than reported for other methods. The factor that contributed most to successfully optimizing the enterocyte procedure was the addition of DTT to aid in removing any mucus lining the intestine by reducing the mucus viscosity. DTT is a reducing agent commonly used to decrease the viscosity of mucus that breaks disulphide bonds by dissociating mucin fibers into their monomeric subunits. This chain-breaking process nearly abolishes the viscoelastic gel properties of mucus (Mestecky, 2005). Incubation with DTT in the rinsing buffer may also remove dead enterocytes present in the mucus from the cell preparation as the rinsing buffer is discarded. Live intact cells lining the epithelium are detached by chemical digestion, using EDTA, in the isolation buffer, and by mechanical digestion (gentle palpations). EDTA is a chelating agent that neutralizes calcium and magnesium ions that enhance cell-to-cell adhesion and facilitates the release of individual enterocytes from the intestine. A single cell suspension is produced by gently pipetting the cells while being treated with collagenase. Filtration of enterocyte preparation after the cells were treated with collagenase was also crucial to increasing the cell yield of the procedure because clumped cells would not be included in the sample.

The elution approach used by all researchers is similar in the key steps taken where the luminal contents of the intestine is rinsed first, followed by chemical and mechanical digestion to detach cells from the tissue, separation of clumps to generate a single cell suspension (where necessary), and filtration of the preparation to remove any mucus or cellular debris. Each procedure varies in the key ingredients added to the rinsing, isolation, and incubation solutions, cell detachment and separation approaches, and whether the preparation was filtered before or after clumped cells were separated. A comparison of these factors of each study is shown in

Table 8 and the concentration of the key reagents can be found in Table 9. The incubation medium that had the lowest drop in viability over time (4% over 4h) was the physiological saline solution (Burke and Handy, 2005). The most notable difference between the solution that Burke and Handy (2005) used was the concentration of glucose added to the media, where it was almost 2-fold higher than what is found in MEM, Medium 199, and the modified Cortland's solution. This suggests that enterocytes need to be provided with an ample amount of glucose to remain viable over time.

Table 8: A comparison of the important elements (rinsing and isolation buffers, cell detachment and separation approach, filtration before/after separation of clumped cells, and incubation medium) of studies using freshly isolated trout enterocytes to study intestinal activities.

Reference	Variable in Enterocyte Isolation Procedure					
	Rinsing Buffer	Isolation Buffer	Cell Detachment Approach	Cell Separation	Filtration Before/After Cell Separation Step	Incubation Medium
Current study	Na-Citrate + DTT	EDTA + DTT	Gentle palpation	Collagenase and gentle pipetting	After	MEM
Pérez et al., 1999	Na-Citrate	EDTA + DTT	Gentle palpation	Collagenase	Before	MEM
Tocher et al., 2004	HBSS + EDTA	Collagenase + EDTA	Mincing	Collagenase	After	Medium 199
Burke and Handy, 2005	Physiological saline	DTT	Agitation by Pasteur pipette	n/a	After DTT buffer	Physiological saline
Kwong et al., 2010	Modified Cortland's saline	EDTA + DTT	Cell scraping	Gentle pipetting	After	Modified Cortland's saline

Table 9: Reagents in the incubation media used to incubate rainbow trout enterocytes

Reference	Reagent Concentration (mM)									
	NaCl	KCl	$CaCl_2$	$NaHCO_3$	$MgSO_4$	Na_2HPO_4	NaH_2PO_4	Glucose	L-Glutamine	HEPES
Current study	137	5.4	1.8	4.2	0.81	3.4	-	5.6	2	15
Perez et al., 1999	137	5.4	1.8	4.2	0.81	3.4	-	5.6	2	10
Tocher et al., 2004	116	5.4	1.8	26.2	0.81	-	1	5.6	2	10
Burke and Handy, 2005	125	3	1.8	10	2	1	-	10	2	-
Kwong et al., 2010	133	5	1	1.9	1.9	2.9	-	5.5	-	-

4.2 Biotransformation rates (k_r)

In biotransformation experiments, I measured the metabolism of the selected test compounds by rainbow trout enterocytes isolated from uninduced fish and fish treated with ß-naphthoflavone to induce CYP1A1 activity (induced fish) were measured. In uninduced fish, a mixture of chemicals containing B[a]P, chrysene, 9-MA, and PCB 209 was incubated with freshly isolated epithelial cells, where the metabolism of these chemicals by live enterocytes was not found to be statistically different from the control samples (Figures 12-15). This is consistent with the results from the CYP assays, in which the CYP1A1 activity of enterocytes isolated from uninduced fish was found to be approximately 40-fold lower than what was measured in hepatocytes and not statistically significantly different from heat-killed cells. Both B[a]P and chrysene are substrates of CYP1A1 (Weiden et al., 1994) and because of the low CYP1A1 activity found in uninduced fish, any metabolism of the two compounds that may have occurred was too low to be detected. CYP2B1 has been shown to be responsible for the metabolism of 9-MA (Anzenbacher et al., 1996) and in hepatocytes isolated from uninduced fish, the substrate depletion rate was lower than B[a]P and chrysene (Trowell, 2010). PCB 209, which was selected as a non-metabolizing control, was not metabolized, a result that is consistent with literature (Buckman et al. 2006).

These results are consistent to those of other studies using uninduced fish to measure the biotransformation of environmental contaminants. Several studies have shown that dietary intake of PAH's and starvation have opposing effects on intestinal monooxygenases (van Veld, 1988). In a study by van Veld et al., (1988) *Leiostomus xanthurus* (teleost spot), the microsomal

monooxygenase system of this fish was found to be very sensitive to dietary PAH exposure; fish that were exposed to dietary B[a]P showed a 10-fold increase in aryl hydrocarbon monooxygenase (AHH) activity compared to control fish and a level that approached the activity in the liver. Another group, exposed fish to 3-methylcholanthrene (3-MC), a more potent monooxygenase inducer, in their diet showed a 36-fold increase in intestinal EROD activity and a 17-fold increase in AHH activity. The intestinal AHH and EROD activity 15- and 11-fold higher than in the liver respectively. In contrast, when fish were starved, EROD and AHH activities were zero in intestinal microsomes, which suggests that monooxygenase activities are heavily dependent on inducing compounds that are ingested (van Veld et al., 1988). For the current study, the CYP1A1 activity in enterocytes was 2-fold higher in induced cells in comparison to uninduced cells where an increase in enzyme activity is consistent with other studies (Gravato and Guilhermino, 2009; Pangrekar et al., 2003; van Veld et al., 1987). However, I hypothesize that the degree of induction may be increased in intestinal preparations if fish were induced via the diet as opposed to intraperitoneal injection.

The absence of any measurable metabolism of 9-MA to a significant extent can also be attributed to using epithelial cells isolated from fish fed a standard diet, where enzyme activity was not induced. Only a few studies have focused on the biotransformation of 9-MA in species across all taxa, and it has been suggested that either CYP1A1 or CYP2B1, or both enzymes metabolize the substance (Von Tungeln and Fu, 1986; Stegeman and Hahn, 1994). In a rat study, the metabolism of 9-MA by liver microsomes from animals pre-treated with 3-methylcholanthrene (3-MC). This suggests that 9-MA is a CYP1A1 substrate, as 3-MC is a commonly used as an CYP1A1 inducer (Von Tungeln and Fu, 1986). This may also be the case in fish as this CYP enzyme has a large number of conserved regions when compared to

mammals (Nebert and Gonzalez, 1987). One study has suggested that 9-MA is metabolized by CYP2B1 in rat liver microsomes (Anzenbacher et al., 1996), and an N-terminus analysis has shown that 50% of the CYP2B1 amino acid sequence in rats is similar to what is found in fish. Furthermore, protein analysis has shown cross-reactivity with antibodies against both scup P450B and rat CYP2B1 (Stegeman and Hahn, 1994).

The absence of any measurable degree of metabolism in the chemical mixture experiments may have also been related to the experimental procedure. The concentrations of chemicals (0.25 μM) used in these experiments were just above the detection limit and the signal-to-noise ratio during GC-MS analysis of a 0.6 ml sample of the hexane extract was relatively low, thereby increasing the error of the data collected. In later biotransformation experiments (induced/uninduced), the extracts were reconstituted in a volume of 100 μl to concentrate the sample which successfully increased the signal-to-noise ratio, and potentially reducing the error resulting from GC-MS analysis. Nevertheless, this did not reveal any significant metabolism.

Some experiments were conducted using enterocytes isolated from uninduced fish and compared B[a]P metabolism to cells from induced fish. Both uninduced and induced fish were fed a standard commercial pellet diet, with the exception that induced fish were injected with ß-naphthoflavone to induce CYP1A1 activity. The results of this set of experiments showed that induction did not significantly increase the rate of metabolism of B[a]P by live cells (induced/uninduced). This result was unexpected as several other studies have shown that fish induced for CYP1A1 activity have a higher rate of metabolism in fish intestinal tissue of CYP1A1 substrates than uninduced fish (Gravato and Guilhermino 2009; Van Veld et al., 1987; Pangrekar 2003) (Table 10). A possible explanation is that intraperitoneal injection may not be

suitable for inducing enzyme activity in fish intestine. Prior to entering the intestine, substances in the intraperitoneal space first enter the liver via the hepatic portal vein, where the chemical can be broken down. This can ultimately reduce the amount of the inducing agent that reaches the intestine resulting in a negligible increase in enzyme activity.

Induction by feed could be more effective approach to induce enzyme activity compared to induction by intraperitoneal injection. As stated above, enzyme induction via spiked feed showed the highest percent increase (850%) of metabolism of a CYP1A1 substrate by intestinal tissue from uninduced whereas induced fish and the percent increase for liver microsomes was only 150% when induced with the same dietary dose of B[a]P (van Veld et al., 1987). This may be the reason behind the discrepancy in the percent increase in metabolism of CYP1A1 substrates between my study, and the study conducted by van Veld and his colleagues. As mentioned above, fish in my experiments were induced by ip injection with ß-naphthoflavone and not the diet, where induction in the intraperitoneal cavity may not have sufficient to trigger CYP1A1 production in the intestine.

The lack of any detectable metabolism of B[a]P by live cells in comparison to sodium azide-treated could also be due to the low activity of the number of cells (1 million) used in each sample, where the breakdown of B[a]P may have been too low for any detectable metabolism to occur.

Other studies have used liver preparations from induced and uninduced fish (Table 9), but the effects of enzyme induction on the liver using the same concentrations used appear to be less dramatic than what was found in intestinal microsomes (van Veld et al., 1987).

Table 10: Summary of the results from studies using uninduced and induced fish to measure the biotransformation of B[a]P and chrysene.

Fish Species	Sample	Exposure to chemical via	Inducer/Route	Treatment	Biotranformation rate	% Induced/ Uninduced	Reference
Dicentrarchus labrax	Liver homogenate	Water medium	B[a]P/Water	Uninduced	~18[b*]		Gravato and Guilhermino, 2009
				Induced (8 μg/L)	33 ± 10[b*]	183%	
				Induced (16 μg/L)	36 ± 8[b*]	200%	
Oncorhynchus mykiss	Intestinal microsomes	Diet	B[a]P/diet	Uninduced	0.04 ± 0.02[c]		Van Veld et al., 1987
				Induced (16 mg/kg)	0.34 ± 0.10[c]	850%	
	Liver microsomes	Diet	B[a]P/diet	Uninduced	0.31 ± 0.24[c]	158%	
				Induced (16 mg/kg)	0.49 ± 0.19[c]		
Ameriurus nebulous	Liver microsomes	To incubation test system[2]	3-MC/ ip injection	Uninduced	33.57[d]		Pangrekar et al., 2003
				Induced	45.48[d]	135%	

[1]: 0.25 μM of B[a]P was added to each sample. [2]: 15 and 5 mM of chrysene was added to uninduced and induced samples respectively. [*]: Estimated values from data summarized in bar graphs. Exact numbers were not provided. [a]: units: /min/10^6 enterocytes. [b]: units: ng BaP equivalents/mg protein. [c]: units: nmol BaP metabolites/min/mg microsomal protein. [d]: units: chrysene metabolites formed (pmol/min/mg microsomal protein)

In the natural environment aquatic, animals live in a range of habitats that can range from pristine environments where chemicals on the DSL are not present, to habitats where contaminants are released into the ecosystem on a regular basis. Fish living in pristine environments are much less likely to be exposed to xenobiotics and bioaccumulation of HOCs becomes less of a concern compared to animals living in contaminated sites are exposed to xenobiotics regularly and enzyme activity is induced by a wide variety of compounds. Situations where bioaccumulation of chemicals can reach levels that can cause harm to biota are of greater concern; therefore, using induced specimens in bioaccumulation tests would be more reflective of the metabolic potential of fish living in areas chemicals on the DSL are present. This approach would allow measurement of metabolic potential of rainbow trout enterocytes to break down selected test chemicals that considers the route of exposure to HOCs, without the complexities of the real-world scenario where other compounds can affect biotransformation via inhibitory, potentiating, synergistic, or competitive processes. Biotic (predators, plants) and abiotic (water, sunlight, temperature) factors in the natural environment can also be controlled in a laboratory setting.

4.3 CYP assays

In this study, the CYP3A27 and CYP1A1 enzyme activity in rainbow trout enterocytes and hepatocytes was investigated. The CYP3A enzymes are the major constituent of the enzymes present in the intestine and liver of most mammals and other species, including fish (Celander et al., 1996, 1989; Hegelund and Celander, 2003: Hegelund et al., 2004; Husoy et al., 1994), however, the expression of CYP3A enzymes is higher (~7-fold) in the intestine compared to the liver (Lee and Buhler, 2003). CYP3A27/CYP3A45 are involved in the metabolic clearance of numerous chemically diverse compounds including toxins, carcinogens, therapeutic drugs, and hormones (Aoyama et al., 1990; Gillam et al., 1993; Li et al., 1995; Smith et al., 1996; Waxman et al., 1998).

These enzymes are believed to act as the first line of defense to prevent bioaccumulation of lipophillic substances that enters the body through the digestive tract (Guengerich and Shimada, 1991; 1998). They are functionally the most versatile form of CYP, having a broad substrate specificity for xenobiotics such as pharmaceuticals (rifampicin), as well as procarcinogens (aflatoxin B1), and endogenous and exogenous substrates such as steroids (dexamethasone and pregnenolone-16alpha-carbonitrile), bile acids, eicosanoids, and retinoids (Aoyama et al., 1990; Gillam et al., 1993; Li et al., 1995; Smith et al., 1996; Waxman et al., 1998). Functional properties of the recombinant protein of CYP3A27 and its paralog CYP3A45 (94% sequence similarity) expressed by a baculovirus in insect cells confirm the catalytic activities of the two CYP450s when testosterone was used as a substrate (Lee and Buhler, 2002; 2003). The abovementioned factors provides evidence that CYP3A enzymes play an important role in first-pass metabolism.

The current study is unique in that the enzyme activity of CYP3A27 in fish intestinal and hepatic cells were measured from the same animal; we found that enterocyte enzyme activity was 5-fold higher in hepatocytes when normalized per million cells. The only other known study comparing CYP3A enzymes in fish intestine and liver measured the mRNA levels of CYP3A30/56 using RT-PCR. This study found a ratio of intestinal to hepatic mRNA expression in *Fundulus heteroclitus* (killifish) of 0.61. These CYP paralogs appear to be specific to killifish. The use of non-homologous antibodies raised against trout to detect the presence of CYP3A in killifish suggests that CYP3A30/56 has a high sequence similarity to CYP3A27/45. These data indicate that although expression levels of these isozymes are likely to be higher in the liver, it does not indicate how active the enzymes are in the presence of a substrate (Hegelund and Celander, 2003). Our data show that at least in *O. mykiss*, CYP3A27 intestine/liver activity was 5. The biotransformation *in vitro*

method using enterocytes could also be used to test other test compounds that are substrates of other CYP isozymes, or hydrolases such as esterases and pancreatic lipases.

Research on CYP3A enzymes in fish is very limited, where further investigation is necessary to determine the importance of CYP3A enzymes in intestinal metabolism of HOCs. Research on the biotransformation of pharmaceutical compounds has been extensively studied in mammalian intestinal models, and much is known about the CYP3A4 isoform responsible for metabolizing these compounds. Unlike the hydrophobic properties of the chemicals on the DSL (log K_{OW} >5), the polar and hydrophilic nature of pharmaceuticals (log K_{OW} < 4.5) does not make these compounds a concern for bioaccumulation (Stamm et al., 2008) and making it difficult to apply this knowledge in fish intestinal studies on organic commercial chemicals.

4.4 Advantages and limitations of *in vitro* approach used in the current study

Several factors were considered during the selection of an appropriate intestinal model to study the biotransformation of xenobiotics in the current study. These include the complexity, cost, and ethical acceptance of the procedure to study the biotransformation of xenobiotics, as well as the number of chemicals that can be tested per fish, resemblance of the true *in vivo* situation, and the stability of enzymes over time. The number of chemicals that could be tested per fish and the complexity of a model relate to the overall cost of measuring the biotransformation of xenobiotics. The complexity of each model was determined from a number of factors: the length of the isolation method, the level of expertise required to perform the procedure and the number of chemicals that could be tested per fish. The latter is an important factor to consider because of the thousands of chemicals that await screening. Ethical considerations for each model were also considered with the goal of minimizing the number of fish used to test each chemical. The resemblance to the *in vivo* situation as well as the stability of metabolic enzymes and ability to freeze-thaw the preparation

were also evaluated for each intestinal model. A comparison of developed intestinal models to *in vivo* animal models including microsomes, S9 fractions, enterocytes, and isolated perfused intestines were selected based on these criteria and the results are summarized in Table 10. The advantages and disadvantages of each model can be found in Table 11.

The enterocyte intestinal model has never been used to measure the biotransformation of HOC's in fish. It was selected for the current study because cytosolic (NATs, STs, and GSTs), phase I (oxidation, reduction, hydrolysis), and phase II (UGTs, GSTs) metabolic enzymes are present in whole cells making the model suitable for testing the broad range of substrates listed on the DSL. As with microsomal and S9 preparations, degradative enzymes such as proteases are not present in the preparation, and thus will not interfere with the biotransformation of compounds. Of note, the enterocyte isolation procedure takes a similar amount of time to prepare microsomal fractions from the time that fish are sacrificed (1.5-2h). A large number of chemicals can also be tested for biotransformation per fish at a lower cost thereby reducing the number of animals used in comparison to *in vivo* tests without completely compromising the structural resemblance to the whole organism. The major drawback is that for CYP1A1 substrates at least, induction via the diet will likely be required so that measurable substrate depletion rates are attained. Lastly, enterocytes, unlike cytosolic, S9, and microsomal fractions cannot be freeze thawed for later use, must be used the same day as the isolation.

Table 11: A comparison of the various in vitro methods of intestinal preparations compared to in vivo experiments. Factors considered included complexity (determined by length of isolation and level of expertise required), number of compounds per fish, cost, applicability, resemblance to *in vivo* situation, and stability of enzyme over time, for the selection of an appropriate intestinal model to test the biotransformation of HOC's. A score of 1 indicates the factor is the most favourable for the test model, and a score of 5 indicates that the factor is the least favourable for that model. This table was modified from Brandon et al. (2003).

In vitro Method	Complexity	Compounds per fish	Cost	Ethically acceptable	Resemblance to *in vivo*	Enzyme stability over time	Ability to freeze-thaw
Microsomal and cytosolic fractions	1	Several	1	2	6	2	Y
S9 fractions	1	Several	1	2	5	2	Y
Cultured cell lines[a]	2	n/a	2	1	4	1	Y
Enterocytes	3	Several	3	3	3	3	N
Perfused intestine	4	One	4	4	2	3	N
In vivo animal model	5	One	5	5	1	1	N

a: model is in the process of being developed. Y = preparations can be freeze-thawed and N = preparations cannot be freeze-thawed for later use

Table 12: An overview of different *in vitro* models and their advantages and disadvantages (modified from Brandon et al., 2003)

In vitro technique	Advantages	Disadvantages
Microsomes	Affordable	Only CYP and UGT enzymes
Cytosolic fractions	NAT, ST, and GST activity depends on cofactors present	Only NAT,ST, and GST
	High enzyme activities	
S9 fractions	Both phase I and 2	Lower specific enzyme activity than microsomes and cytosol fraction
Cell lines	Easy to culture	
	Relatively stable enzyme expression levels	Low expression levels of many enzymes
Transgenic cell lines	Easy to culture	Only the introduced recombinant few isozymes are expressed at high levels
	Higher expression levels	
	Study of one isozyme or a combination of CYPs	
Enterocytes	Study of enzyme inducers possible	Other metabolizing components are not included
	Drug transporters still present and operational	
Perfused intestine	All cell types	Delicate model, requires high level of technical training
	Three-dimensional architecture	Limited viable period

Other models that have been used to study the intestinal metabolism of xenobiotics include microsomal fractions and perfused *in situ* intestinal preparations. van Veld and his colleagues have had success using microsomal fractions to study the metabolism of B[a]P in killifish intestine following the intestinal absorption of dietary B[a]P (van Veld et al., 1987). Microsomal fractions are often used because the procedure is simple, cost and time effective, and preparations can be freeze-thawed for later use. The number of animals used for testing bioaccumulation of a single test compound is also reduced as several chemicals can be assessed using the same microsomal fraction prepared from the same fish. This test model was not selected for the current study because only CYP and UGT are present and therefore microsomes are not be suitable for testing a broad range of substrates. In addition, it is more difficult to extrapolate rates to the whole organism.

Another method that has been used to study the biotransformation of xenobiotics in fish intestine is isolated intestinal perfusions (Kleinow et al., 1998). This approach is considered the best representation of the *in vivo* situation as it keeps the original three-dimensional architecture of the organ and contains all cell types and enzymes present in the intestine. One of the disadvantages of the isolated perfusion model include the poor reproducibility of results because of high interindividual variation because only one chemical can be tested per fish for a single time point. Experiments conducted using enterocytes, cytosolic, S9, and microsomal fractions allow for replication of samples at

several time points over the course of the study. Lastly, the number of animals used to test each chemical would the same number required for a BAF or BCF test.

Biotransformation studies have never been conducted on fish intestinal epithelial cell lines because a well-characterized model has only recently been developed for the investigation of xenobiotic metabolism Kawano et al., (2011). Hepatic cell lines prepared from trout (RTG-2) have been shown to metabolize B[a]P (Smolarek et al., 1987) but are not commonly used for metabolism studies mainly due to the dedifferentiated cellular characteristics and incomplete expression of all families of metabolic enzymes (Crommelin and Sindelar, 1997). Cell lines have been prepared from other tissues and organs of fish including the gills, spleen, and kidney (Bols and Lee, 1991), and only recently has an epithelial cell line been developed for fish intestinal tissue (RTgutGC) by Kawano and colleagues (2011). The cell line was derived from primary cultures prepared from the gut of a rainbow trout and divided into new culture vessels followed by propagation and has been shown to express CYP1A1 activity when induced by B[a]P and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Phase I and Phase II enzymes have not been characterized in the RTgutGC, and only until the enzymes present in the cell line is well understood can this approach be considered for use in biotransformation studies. Compared to freshly-isolated enterocytes, cell lines are generally easier to culture prepare and have relatively stable concentrations. Once established, cell lines are easily available, have consistent characteristics from batch to batch, reduce the number of animals used to assess chemicals, and would cost much less than a BAF or BCF test. However, an important disadvantage is the absence or low expression level of most important phase I and phase II metabolizing enzymes, which limits its use in

biotransformation studies (Brandon et al., 2003). It is also difficult to investigate individual CYPs or other enzymes due to their low expression levels (Table 11). Finally, the approval of regulatory agencies to use of *in vitro* cell cultures in human toxicology provides confidence that substitution of animal experiments, including fish tests, can be done.

4.5 Should intestinal metabolism of chemicals be included in fish bioaccumulation models?

The test compounds selected for the current study, (B[a]P, chrysene, 9-MA), were not biotransformed at a detectable rate in uninduced enterocytes. Nevertheless, I believe that intestinal biotransformation of commercial compounds should still be included in fish bioaccumulation models because two earlier studies have provided evidence that intestinal first-pass metabolism can contribute significantly to the biotransformation of PAH's in fish, such as B[a]P (Kleinow et al., 1998; van Veld et al., 1987). In addition, the results of the CYP3A27 activity assays also suggests that the intestine may play a larger role than the liver as a major site of biotransformation of these substrates and is therefore important to consider in future biotransformation studies. The current study used enterocytes as an *in vitro* test model to measure biotransformation of organic commercial compounds mainly because whole cells contain a more complete set of enzymes than cytosolic, S9, and microsomal fractions. Enterocytes are a suitable intestinal model for studying the biotransformation of large numbers of compounds with a wide range of chemical properties because the system contains all the enzymes (phase I, phase II, and cytolsolic enzymes) normally present in the intestine capable of biotransformation. The current study confirmed that the metabolizable test compounds

(B[a]P, chrysene, 9-MA) are not biotransformed at a detectable rate in uninduced cells, and that CYP1A1 induction by ß-napthoflavone resulted in a significantly higher rate of metabolism. However, induction of enzyme activities may generate higher enzyme activity that reflects a more natural induction environment, i.e., via the diet rather than by intraperitoneal injection. Because of time constraints, inducing metabolic enzymes such as via their diet was not performed, but I anticipate that this will result in a detectable amount of metabolism. Because a fish enterocyte test model is novel to the study of HOC metabolism, it was necessary to spend more time and effort on optimizing the isolation procedure and confirming epithelial identity and relative CYP3A27 and CYP1A1 activity prior to confidently using the cells for biotransformation experiments.

4.6 Future directions

To date, research in this area is limited and further investigations are required to better understand intestinal metabolism of hydrophobic environmental contaminants in fish. Investigation on the effect of dietary induction of metabolic enzymes in fish on the rate of HOC metabolism should be pursued. In addition, other substrates should also be tested with enterocytes such as CYP3A and epoxide hydrolase substrates. Cell lines should also be considered as an intestinal test model for biotransformation for rapid testing once development of a rainbow trout intestinal epithelial cell line inducible by CYP1A1 substrates becomes more readily available. As data is collected on the recent RTgutTC cell line, it will become clear whether this cell line will be useful for measuring in vitro biotransformation rates. Regulatory agencies in Europe are moving towards using cell lines for toxicological work to allow for quick and inexpensive screening to minimize the use of animals.

Appendices

Appendix 1: Pairwise amino acid sequence alignment of *O. Mykiss* CYP3A27 protein and Human CYP3A4 using Blastp

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