DEVELOPMENT OF ALTERNATIVE METHODS TO ASSESS THE TOXICITY AND BIOACCUMULATION POTENTIAL OF CHEMICALS IN THE AQUATIC ENVIRONMENT

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List of Abbreviations

- **AAT**: Aminoacyl transferase

- **ADH**: Alcohol dehydrogenase

- **ADME:** Absorption, Distribution, Metabolism and Excretion

- **ALDH**: Aldehyde dehydrogenase

- **AO**: Aldehyde oxidase

- **AOP**: Adverse Outcome Pathway

- **AzoRT**: Azoreductase

- **BCF:** Bioconcentration Factor

- **BCF_p**: Organism-water partition coefficient

- **BSA**: Bovine Serum Albumin

- **C_{HEP}**: Concentration of hepatocytes

- **CL_T**: Metabolic clearance of all organs

- **CL_H**: Metabolic hepatic clearance

- **CL_{INT}**: *In vitro* intrinsic clearance

- **$CL_{INT\ LIVER}$**: Intrinsic clearance in liver

- **CoA**: Coenzyme A

- **CO**: Cardiac output

- **C_{DOC}**: Dissolved organic carbon content

- **C_{POC}**: Particulate organic carbon content

- **CYP**: Cytochrome P450

- **DMEM:** Dulbecco's Modified Eagle Medium

- **DMSO**: Dimethyl sulfoxide

- **DSL:** Domestic Substance List

- **DTD**: Diaphorase

- **D_{DOC}**: Disequilibrium factor for dissolved organic carbon

- **D_{POC}**: Disequilibrium factor for particulate organic carbon

- **E_D**: Dietary chemical transfer efficiency

- **E_W:** Gill chemical uptake efficiency

- **EH**: Epoxide hydrolase

- **FBS**: Fetal Bovine Serum

- **FET:** Fish Embryo Test

- **FD; ϕ:** Fraction of a chemical that is bioavailable to the fish in water

- **FMO**: Flavin-containing monooxygenase

- **f_U**: Hepatic clearance binding term

- **$f_{U,P}$; $f_{u,b}$**: Unbound fraction of test compound in blood plasma

- **$f_{U,HEP}$; $f_{u,h}$**: Unbound fraction of test compound in the clearance assay

- **GC-MS**: Gas Chromatography-Mass Spectrometry

- **GSH:** Glutathione

- **GST**: Glutathione S-transferase

- **G_V**: Ventilation rate

- **G_D**: Feeding rate

- **HLC:** Henry's Law Constant

- **HR-MS**: High Resolution-Mass Spectrometry

- **k**: Terminal rate constant

- **K_{MET}:** Whole body biotransformation rate

- **K_{OW}:** Octanol-water partition coefficient

- **K_1:** Gill uptake rate constant

- **K_2**: Elimination rate constant

- **K_E**: Faecal egestion rate constant

- **K_G**: Growth dilution rate constant

- **L_B**: Lipid content of the organism

- **LC-MS**: Liquid Chromatography-Mass Spectrometry

- **L_{HEP}**: Total number of hepatocytes in the fish liver

- **LF**: Fraction of blood flow through liver

- **LPO**: Lipoxygenase

- **LW**: Liver weight

- **L-15**: Leibovitz medium

- **MA:** Michael Acceptor

- **MAO**: Monoamine oxidase

- **MIE:** Molecular Initiating Event

- **MW:** Molecular weight

- **NIVA:** Norwegian Institute for Water Research

- **NTR**: Nitroreductase

- **OECD**: Organization for Economic Cooperation and Development

- $\mathbf{P_{b:w}}$: Blood: water partition coefficient

- $\mathbf{Q_H}$: Hepatic blood flow

- $\mathbf{Q_{HFRAC}}$: Liver blood flow as fraction of cardiac output

- $\mathbf{Q_C}$: Cardiac output

- **QSAR**: Quantitative Structure-Activity Relationship

- **REACH:** Registration, Evaluation, Authorisation and restriction of Chemical substances

- **SETAC**: Society for Environmental Toxicology and Chemistry

- **SULT**: Sulfotransferase

- **S9:** Subcellular liver fractions

- **UGT**: UDP-glucuronosyl transferase

- $\mathbf{v_{LT}}$: Lipid content in trout

- $\mathbf{v_{NT}}$: Non-lipid content in trout

- $\mathbf{v_{WT:}}$ Water content in trout

- $\mathbf{V_{D,BL}}$**;** $\mathbf{V_d}$: Apparent volume of distribution

- $\mathbf{v_{LWB}}$: Fractional whole-body lipid content

- $\mathbf{W_B}$: Wet weight of the organism

- α_{DOC}: Dissolved organic carbon content binding constant

- α_{POC}: Particulate organic carbon content binding constant

- β: Non-lipid organic matter-octanol proportionality constant

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Abstract

The toxicity and bioaccumulation potential of chemicals are properties that need to be assessed in risk assessment. In the context of the aquatic environment, both properties were traditionally evaluated in the whole fish. However, due to the reluctance to use a large number of animals for experimentation and high cost of *in vivo* testing, alternative techniques have been developed to assess these properties. This thesis describes three distinct investigations towards the development of alternative methods for predicting the toxicity and bioaccumulation potential of chemicals.

The first study of this thesis is centred on the development of a list of reference compounds to evaluate non-animal methods to *in vivo* bioaccumulation studies in fish. The selection of representative chemicals was developed following a novel strategy built from previous criteria proposed for the validation of experimental tests and considering relevant aspects for the bioaccumulation of organic chemicals. A revision and a comparison of the most used alternative approaches to *in vivo* bioaccumulation studies were undertaken in this thesis. In particular, a variety of *in vitro* and *in silico* methods were explored and compared in terms of their reliability to predict the whole body biotransformation rate and bioconcentration factor of chemicals in fish. As a consequence of this investigation, an insight into the main challenges and future perspectives for each of the methods evaluated was conducted to provide a foundation for future research. The last research study is focused on the verification of the prediction of protein binding for cyclic compounds and the development of a decision tree strategy to prioritise chemicals for *in vivo* toxicity testing. The last two objectives were developed based on the integration of different alternative methods to assess the toxicity of chemicals. This thesis concludes with a summary and a discussion of the work undertaken and suggestions for future work.

Contents

Appendix IV (provided as electronic supplementary data)

Chapter 1. Introduction

1.1. Chemicals in the Aquatic Environment and Their Risk Assessment

Man-made and naturally occurring chemical substances can ultimately enter into the aquatic environment. Aquatic ecosystems act as a major recipient of chemical substances and their transformation products, resulting in a significant pollutant burden. In particular, there are four possible origins of chemical substances in the environment [1]:

1. *Chemicals from Anthropogenic Production and Use*. Man-made chemicals are produced from different types of industries (e.g. agricultural, chemical, pharmaceutical and electronic industries) and have a wide range of uses (e.g. cosmetics, fertilisers, food additives, pharmaceuticals and biocides). These chemicals are also called xenobiotics and represent a risk to aquatic organisms that are exposed to them.
2. *Inert Materials*. Inorganic compounds are the essence of geological systems such as metals from rocks. The heavy metals represent substances of concern due to their potential adverse effects.
3. *Fossil Fuels.* Fossil fuels contain mainly organic compounds such as hydrocarbons. Hydrocarbons are used for several processes such as electricity generation and production of a broad range of synthetic substances (e.g. pesticides, dyes and plastics).
4. *By-products of Organisms.* These are produced from biochemical reactions in the organism itself. Generally, substances produced by organisms are readily biodegradable in the environment.

Of these sources, the most significant in terms of the potential harm to aquatic ecosystems are organic chemicals arising from anthropogenic use and these will be the focus of this

thesis. Modern society has a conundrum: man-made chemicals are needed to maintain health and a high quality of life, however the adverse effects of these on the environment are a potential problem. Therefore, chemicals undergo a process of risk assessment in an attempt to ensure that they are used and disposed of safely [1]. A fundamental component of risk assessment procedures is the measurement of the potential hazardous effects of chemicals on aquatic species through toxicity tests [2]. Aquatic toxicity tests should be conducted according to good laboratory practice (GLP). When conducting a toxicity test, the following aspects should be taken into account [2]:

1. *Dose Selection.* The selection of the dose level depends on the study. There should be at least three dose levels (low, middle and high), in addition to control groups. The high dose should produce evidence of toxicity, the middle dose should be moderately toxic and no toxicity is expected for the low dose.
2. *Test Species*. The selection of appropriate species is based on several considerations such as how well they represent the environment and knowledge and experience of their maintenance. Although there is a diversity of aquatic model organisms, fish are the most commonly tested species in ecotoxicological studies due to their high trophic position and similarities with mammals. *Danio rerio* (zebrafish) and *Oryzias latipes* (medaka) are commonly used in ecotoxicology and biomedical research due to their following advantages: 1) ease of maintenance and manipulation in the laboratory; 2) high fecundity and rapid development; and 3) transparency of the chorion (eggshell) that enables ready identification of abnormalities induced by chemicals [3, 4].
3. *Endpoint and Test Duration.* The endpoint measured is the biological response of the test organism to a concentration of a chemical over a defined period of time. Examples of toxicological endpoints include the median lethal concentration (LC_{50}) and the median effective concentration (EC_{50}). In terms of test duration, toxicity

studies can be divided into acute and chronic tests. In acute toxicity tests, the adverse effect is measured over a relatively short timeframe (between 2 and 7 days), whereas chronic studies are carried out over a prolonged period and usually consider sub-lethal effects.

4. *Test Substance Characteristics.* This refers to the physico-chemical properties of the substance of concern. Before conducting a toxicity test, all available information related to the test substance should be gathered. Water solubility and volatility in solution are the most relevant chemical properties considered in waterborne exposures [5].

In order to ensure high quality toxicity data, experiments should be conscientiously planned and carried out according to standardised test protocols issued by international bodies such as the Organisation for Economic Cooperation and Development (OECD). *In vivo* testing methods, which imply the use of the whole fish, have been widely used to assess the potential toxicity and bioaccumulation of chemicals. Table 1.1 lists the toxicity and bioaccumulation tests in fish that are conducted in compliance with the OECD guidelines. Due to their reliance on animal use, however, *in vivo* methods have been continually criticised by animal welfare organisations. In addition, other factors such as the high cost of maintaining animals in laboratory conditions have led to the development other methods which apply the 3Rs (reduction, refinement and replacement of animals in experiments) concepts [12]. Alternatives are explained in the next section.

Table 1.1 Toxicity and bioaccumulation tests in fish

Test	No. OECD guideline
Fish, Acute Toxicity Test	203 [6]
Fish, Prolonged Toxicity Test 14-day Study	204 [7]
Fish, Early-life Stage Toxicity Test	210 [8]
Fish, Juvenile Growth Test	215 [9]
Bioaccumulation in Fish Aqueous and Dietary Exposure	305 [10]
Fish Sexual Development Test (FSDT)	234 [11]

1.2. The REACH Legislation: An Opportunity for Change

The Registration, Evaluation, Authorisation and restriction of Chemical substances (REACH) regulation is the European Union (EU)'s largest piece of legislation for the assessment of chemicals [13]. REACH entered into force on 1st June 2007 and eliminates the distinction between existing and new substances, subjecting all substances to the same standards. One of the main goals of REACH is the protection of the environment and human health from chemicals produced, used or imported in quantities of 1 tonne or more a year [14]. Since there are complete sets of toxicity and bioaccumulation data for fewer than 5% of chemicals on the market, an increased use of experimental animal testing has been reported to be likely in the coming years. Consequently, REACH advocates the use of alternative methods to *in vivo* fish testing to assess the toxicity and bioaccumulation of chemicals.

There are several alternatives proposed to *in vivo* testing using fish. While some approaches imply the use of toxicity tests in aquatic invertebrates to assess the toxicity of chemicals (see examples in Table 1.2), others rely on the use of *in vitro* and *in silico* (computer-based) techniques to prevent unnecessary testing with fish. *In vitro* and *in silico* methods represent the focus of this thesis.

Table 1.2 Toxicity tests in aquatic invertebrates

Test	No. OECD guideline
Daphnia magna, Acute Immobilisation Test	202 [15]
Daphnia magna, Reproduction Test	211 [16]
Freshwater Alga and Cyanobacteria, Growth Inhibition Test	201 [17]

In vitro methods are becoming widely used in aquatic ecotoxicology to reduce the number of fish required in scientific research and risk assessment [18]. *In vitro* approaches involve the use of test systems based on lower levels of biological organisation such as fish embryos, tissues, cells, subcellular fractions and molecules. Of these, toxicity tests with embryos provide a feasible alternative to experimentation with adult fish as they possess similarities in physiology to the adult [18].

In silico methods are considered to be an efficient alternative due to their low cost and speed with respect to testing methods [19]. *In silico* approaches encompass Quantitative Structure-Activity Relationship (QSAR) models, structural alerts, grouping chemicals and read-across. Of these, QSAR models are one of the most powerful tools in aquatic toxicology. The principle of QSAR is the development of relationships between the physico-chemical properties of chemicals with their given biological activity by using a mathematical model. Developing a QSAR model involves the following steps [20]:

1. *Selection of the Endpoint of Interest.*
2. *Gathering Data from Available Resources.*
3. *Assessment of the Quality of the Gathered Data*. Not only should data relating to chemical structure be checked (correct identification of a compound by its nomenclature, CAS number or chemical structure), but also toxicological data gathered should be assessed for quality. This is because high quality data are needed to generate accurate models. There are formal scoring methods for data quality assessment, for instance the Klimisch criteria can be used to allocate data to one of

four classes for quality [21]. Similarly, the ToxRTool scheme, based on the use of Klimisch criteria, can be used to assess the quality of toxicological data [22].

4. *Obtaining Descriptors of Chemical Structure and Properties.* Software such as EPI Suite, from the United States Environmental Protection Agency (US EPA) (www.epa.gov), is able to calculate a set of physico-chemical parameters relevant to the modelling and prediction of toxicity. Amongst them, the octanol-water partition coefficient (K_{OW}) is the main descriptor used in aquatic toxicity prediction due to its good correlation with biological and environmental processes [20]. This is because log K_{OW} acts as a surrogate for the hydrophobicity of a substance. Other valuable descriptors are Henry's Law Constant (HLC) which gives an indication of the volatility of a chemical compound in solution and descriptors for water solubility (S).
5. *Generating the Model.* Although there are many different ways to create a model using various statistical analysis techniques, linear regression analysis is the most common method used to build QSAR models.

Another *in silico* approach to predict toxicity and especially specific interactions is based on the use of structural alerts. Structural alerts are chemical sub-structures or molecular fragments that are associated with toxicological activity [23]. Initially, structural alerts were developed and introduced for human toxicology, in particular for endpoints such as mutagenicity and carcinogenicity, and subsequently they were applied to aquatic toxicology [24]. Although the structural alert approach does not provide a quantitative estimate of toxicity (e.g. LC_{50} and EC_{50}), it can be used to identify chemicals with a potential for binding to biological molecules such as protein and DNA, and hence may be related to specific modes of toxic action [25,26]. Structural alerts have been compiled within programs such as the OECD QSAR Toolbox (www.qsartoolbox.org) to allow for their use within the REACH framework for various purposes, such as prioritising chemicals before conducting an *in vivo* toxicity test and category formation. Grouping for category formation, is another *in silico*

approach by which chemicals with similar structural physico-chemical for toxicological properties, particularly those that follow a regular (quantitative) pattern, are grouped in the same category [27]. Once a chemical category has been formed, data gaps for properties (e.g. those related to toxicology) can be filled by other techniques, such as QSAR models and read-across. Read-across is a method by which toxicological data available for a reference set of chemicals are used to interpolate the toxic activity of a query compound [28]. In the context of non-animal approaches, read-across represents an effective approach as it allows for extrapolation of data from chemicals with test data to those with no data.

1.3. Persistence, Bioaccumulation and Toxicity Potential of Chemicals

Compounds are characterised by physico-chemical and molecular shape properties which determine their persistence, bioaccumulation and toxicity potential [5]. There is an interest in identifying and regulating persistent, bioaccumulative and toxic chemicals due to their adverse impacts on human health and the environment. The persistence of a substance can be defined as its presence in the environment before its degradation by physical, chemical and biological processes [29]. This section describes in detail the assessment of the toxicity and bioaccumulation of chemicals in aquatic ecotoxicology.

1.3.1 Bioaccumulation of Organic Chemicals

Information on chemical accumulation in aquatic organisms is important for understanding the environmental behaviour of a compound and its possible biomagnification throughout higher trophic levels [29]. Two different terms are used with regard to chemical accumulation in fish: bioaccumulation and bioconcentration. Bioaccumulation is the accumulation of a substance from all routes of exposure (from the solution and diet), whereas bioconcentration refers only to its accumulation from the environment that occurs in a waterborne exposure i.e. across the gills and skin [30].

The potential of a compound to accumulate is usually expressed by the bioconcentration factor (BCF). Traditionally, BCFs were determined in the whole body of fish by a flow-through test according to OECD Test Guideline (TG) 305 [31], which required the use of at least two test concentrations per substance. To comply with the 3Rs principles, this guideline was modified, providing a list of recommendations such as the use of only one test concentration per substance to reduce the number of fish required for experimentation [10]. *Cyprinus carpio* (common carp) and *Oncorhynchus mykiss* (rainbow trout) are the most common fish species used for *in vivo* testing [32]. In particular, common carp has been used in Japan to test chemicals to meet obligations under the Chemicals Substances Control Law [33]. Unfortunately, although approximately 300 chemicals are tested every year in Japan, only data for about 800 "existing" chemicals are available online at the Chemical Risk Information Platform of the National Institute of Technology and Evaluation (NITE)'s website (www.nite.go.jp).

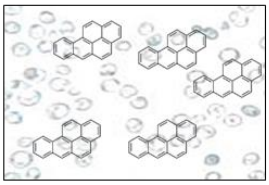
In vitro methods for bioaccumulation mainly involve the use of cells or subcellular fractions to study the processes governing chemical bioaccumulation, i.e. Absorption, Distribution, Metabolism and Excretion (ADME) [30]. Whilst *in vitro* methods for absorption are based predominantly on mammalian species, a variety of cell-based assays has been developed to study xenobiotic biotransformation in fish. *In vitro* tests for xenobiotic metabolism include the use of microsomes [34], subcellular fractions such as S9 [35,36], freshly isolated hepatocytes [37,38], cryopreserved hepatocytes [39,40] and aggregates cultures [41]. Primary hepatocytes can form tree-dimensional spheroidal cultures under specific laboratory conditions (e.g. constant rotation speed over exposure of one day) [41].The majority of such assays are derived from rainbow trout liver due to the fact that such species possess a standardised procedure for isolation of hepatocytes. In an *in vitro* scenario, the potential biotransformation of a compound is usually expressed by the intrinsic clearance (CL_{INT}), which is calculated from a substrate-depletion approach. CL_{INT} data can be

incorporated into established Physiologically-Based ToxicoKinetics (PBTK) models [42,43] to estimate the whole body biotransformation rate (K_{MET}). Figure 1.1 shows the key steps and calculations required for the calculation of K_{MET} from a clearance assay using freshly isolated hepatocytes from rainbow trout. It should be stressed that although information on xenobiotic biotransformation can be determined using *in vitro* assays, their applicability is currently limited due to lack of assay validation and technical limitations [44].

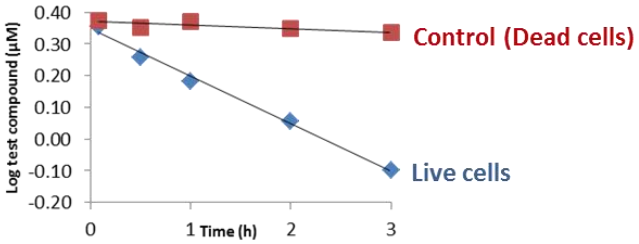
Only a couple of studies have investigated the feasibility of fish embryos to assess chemical bioaccumulation [45,46]. This could be explained by the difficulty in determining the internal chemical concentration in such small test organisms. To overcome this, a simplistic approach has been proposed by which the chemical concentration in embryos was determined indirectly by quantifying the depletion of chemical concentration in the exposure solution [46]. The applicability of such an approach, however, was limited for compounds with significant biotransformation potential whose BCF values were overestimated.

Step 1. Isolation of hepatocytes

Step 2. Incubation assay
(Hepatocytes in suspension with test compound)



Step 3. Calculation of depletion rate constant (k) (h^{-1})
(Log C_t = Log C_O – k/2.3 t)



Step 4. Calculation of intrinsic clearance rate (CL_{INT}) (mL/h/10^6 cells)
($CL_{INT} = k/C_{HEP}$)

Step 5. In vitro-in vivo data extrapolation
(Incorporation of CL_{INT} into PBTK models)

Determination of whole body biotransformation rate **(K_{MET}) (d^{-1})**

Figure 1.1 Steps required for the calculation of whole body biotransformation rate (K_{MET}) (d^{-1}) (days). C_{HEP} = Concentration of hepatocytes used in the clearance assay; Log C_o = Initial concentration of test chemical (µM); Log C_t = Final concentration of test chemical; PBTK = Physiologically-Based ToxicoKinetics Model.

Due to the limited applicability of *in vitro* methods, some governmental agencies rely on predictive models to estimate BCF. Computer-based models for bioaccumulation have been developed over more than 30 years [47]. Traditionally, QSAR models for bioaccumulation were focused on regression analysis between *in vivo* log BCF values and hydrophobicity expressed by log K_{OW}, although with poor correlations being found for ionic substances, those prone to metabolism and hydrophobic substances (Log K_{OW} > 6). Dimitrov et al. [48] established a non-linear relationship model between log K_{OW} and *in vivo* log BCF data to

estimate the maximum bioconcentration potential (log BCF_{max}) of organic chemicals, which is shown in Figure 1.2. This non-linear model was later modified to correct for the effects of metabolism, molecular size, ionisation and water solubility on the maximum bioconcentration potential [49]. However, despite the novelty of such approach, Dimitrov's models were not available to the public. As a consequence, governmental agencies and industry usually rely on the predictions made by the log K_{OW}-based model developed by Meylan et al. [50], which is available in the EPI Suite Software (www.epa.gov). In contrast to the numerous models built for BCF, there have been only few attempts to model the bioaccumulation factor (BAF) [51,52].

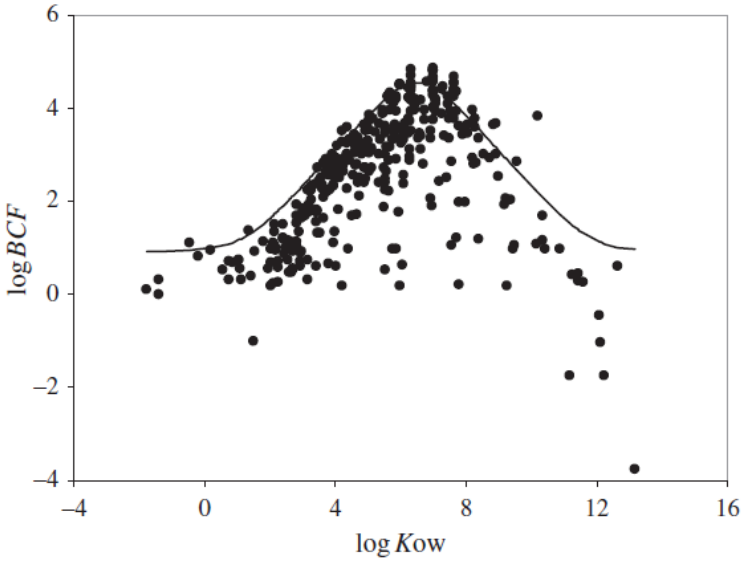


Figure 1.2 The relationship between log BCF and log K_{OW} illustrating the maximal bioconcentration (log BCF_{max}) model developed by Dimitrov et al. [48] shown as a solid line.

Other *in silico* approaches have been focused on building kinetic mass balance models to take into account the ADME processes to predict chemical bioaccumulation [53,54]. By using this approach, BCF is calculated as follows:

$$\text{Bioconcentration factor (BCF)} = K_1 \phi / (K_2 + K_E + K_G + K_{MET}) \text{ (L/Kg)} \qquad (1.1)$$

Where ϕ is the fraction that is bioavailable to the fish in water (unitless); K_1 is defined as the gill uptake rate constant (L/Kg x day (d)); K_E corresponds to the faecal egestion rate constant

(d^{-1}); K_2 is the elimination rate constant (d^{-1}); K_G is the growth dilution rate constant (d^{-1}); and finally K_{MET} corresponds to the metabolic rate (d^{-1}). All these parameters are usually estimated for a fish of 1 Kg. Compared to conventional log K_{OW}-based models, the kinetic approach offers the advantage of incorporating K_{MET} data estimated from *in vitro* clearance assays to refine BCF. The refinement of BCF using *in vitro* data has been conducted using S9 fractions [36,55,56], freshly prepared hepatocytes [37,38] and cryopreserved cells [40]. However, the chemicals that have been tested in these clearance assays were not supported by high quality *in vivo* BCF data, and consequently it was not possible to explore the effect of metabolism on the total bioconcentration.

It should be added that the prediction of metabolic susceptibility of chemicals in fish is usually based on predictions for mammalian metabolic data [49]. Nonetheless, the QSAR model of Arnot et al. [57] can be used to predict K_{MET} of a compound in fish. This model was created from metabolic biotransformation data estimated from *in vivo* data for bioaccumulation. In particular, data for BCF, elimination rate constant and whole body biological half-lives (HL) were used to estimate K_{MET} by re-arranging Equation 1.1. The predicted K_{MET} data were modelled in a multiple linear regression using similar descriptors to those employed in biodegradation models [57]. The QSAR model of Arnot et al. [57] is available in the EPI Suite Software (www.epa.gov) and predicts K_{MET} for a range of fish weights (10, 100 and 1000 g).

1.3.2 Toxicity of Organic Chemicals

As many toxic effects are dose-dependent, the potential hazard of chemicals has been assessed though a dose-response evaluation to determine, for acute effects, the concentration causing 50% lethality (LC_{50}). Traditionally, the potential hazardous effects of chemicals were assessed in the whole fish according to official guidelines (see Table 1.1.). Approximately half of the *in vivo* LC_{50} data in fish have been reported for rainbow trout,

Pimephales promelas (fathead minnow) and *Lepomis macrochirus* (bluegill), contrasting with the low percentage of data (< 1%) for model species such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*)[58]. At present, the effect of biological factors (e.g. fish species and life stage) and test conditions (e.g. temperature and pH) on *in vivo* data are not well-understood due to the fact that such variables are not reported in toxicity tests.

A variety of *in vitro* methods have been developed to assess the toxicity of chemicals. To date, the Fish Embryo Acute Toxicity Test (FET) [59] provides a viable alternative to experimentation on adults. Moreover, good correlations have been reported between acute toxicological data to embryos and adults [14]. Cell cultures are another promising alternative to acute fish toxicity tests [24]. In order for cell-line-based toxicity assays to reflect *in vivo* conditions, they should be derived from the tissues where chemicals exhibit toxicity and account for the chemical fraction available to cells [60,61]. It should be noted that alternative approaches have mainly been proposed for acute fish toxicity testing. This contrasts with the lack of alternatives developed for chronic toxicity tests due to their lack of *in vivo* data, and hence the difficulty in their replacement [24]. Nonetheless, molecular assays could play an important role in predicting the chronic effects of chemicals. This could be assisted, for instance, by using molecular biomarkers to predict endocrine disruption, genotoxicity and immune modulation [4]. In particular, toxicogenomic techniques such as microarrays can identify genes that are associated with long-term toxic response [62].

1.4. Mechanism of Toxic Action

A key aspect for understanding the toxicity of chemicals is unravelling their mechanism of toxic action. This process involves the identification of the toxicant-biological target interaction, also called the molecular initiating event (MIE), and knowledge of the toxicological responses at higher levels of biological organisation. All this information, from the MIE processes to downstream physiological responses, is framed into the concept

recently termed the “Adverse Outcome Pathway” (AOP), which is represented schematically in Figure 1.3 [63].

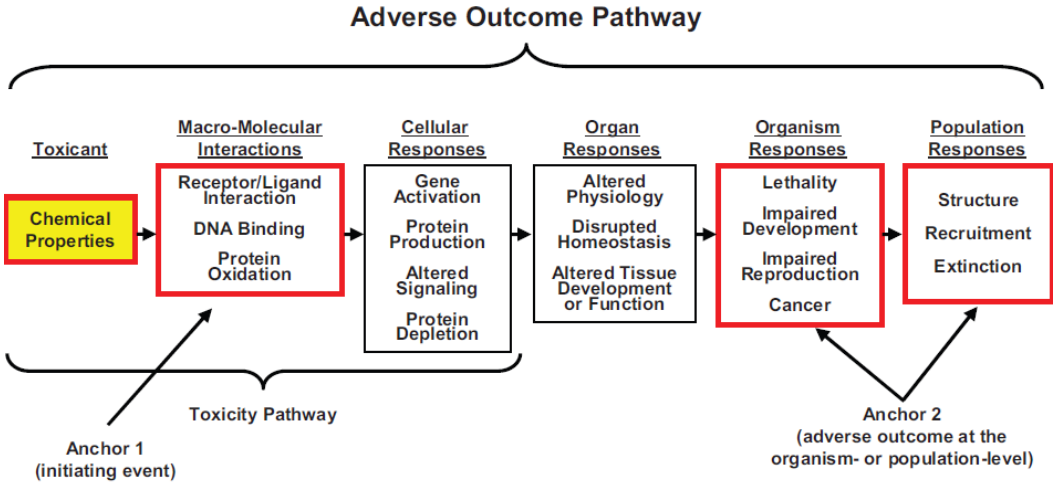


Figure 1.3 Conceptual diagram of the Adverse Outcome Pathway adapted from Ankley et al. [63].

The most common mechanism of acute aquatic toxicity is narcosis; approximately 60% of all industrial organic chemicals act via this mechanism. Toxicity due to narcotic mechanisms results from a reversible hydrophobic interaction between the toxicant and cellular membranes [64]. Non-polar narcosis corresponds to an unspecific minimum level of toxicity exerted by any chemical (also referred to as baseline toxicity). Log K_{OW} has been used in QSAR models to estimate the acute toxicity of narcotic chemicals, which include a diversity of compounds such as aromatic and aliphatic hydrocarbons, alcohols, ketones, aromatic nitro and amino compounds [65]. Over the past three decades, numerous QSAR models have been developed for acute aquatic toxicity for three trophic levels [66]. Other chemicals can initiate the toxicological response by electrophilic mechanisms. In such mechanisms, the toxicant forms covalent bonds with proteins and DNA resulting in a specific and irreversible toxic response. One example of an electrophilic reaction is Michael type addition that involves the addition of a SH group from a protein (for instance on the fish gill, causing membrane irritation) at an electron-deficient β-carbon of an electrophile, known as a Michael acceptor (MA). This reaction ultimately forms a chemical-protein adduct. The net result of this

covalent interaction is the permanent disruption of proteins, which can be observed as an increased toxicity above that elicited by narcosis (often termed excess toxicity).

Structural alerts for Michael acceptors from the literature, as well as other reaction mechanistic domains, were reviewed and compiled by Enoch et al. [25,26]. In particular, the structural alerts within the Michael addition domain were classified into four classes: polarised alkenes, polarised alkynes, quinones and related chemicals and acid imides [25]. Table 1.3 shows examples of substructures of structural alerts for Michael acceptors encoded into SMILES strings [67]. It should be stressed that structural alerts can be developed based on mechanistic knowledge related to protein binding, which is gained from the analysis of the chemical structure, and does not necessarily involve empirical evidence [25,26]. Therefore, the verification of structural alerts based on experimental data is required before reactivity predictions become applied for regulatory purposes [68]. Here, *in chemico* reactivity data could play an important role for the verification of the prediction of chemical reactivity. For instance, Schultz et al. [67] employed a set of Michael acceptors for the verification of the structural alerts. *In chemico* assays involve the use of proteins such as glutathione (GSH) or peptides to determine the reactivity of chemicals based on a depletion of substrate approach [69].

Table 1.3 Examples of structural alerts for Michael acceptors, adapted from Schultz et al. [67]

SMILES strings	Message
C≡CC=O	ethynylene or acetylenic with a carbonyl
[CH2]=C(C)C=O	α-C atom alkyl-substituted with a carbonyl
C=CN(=O)=O	olefinic nitro
C=CS=O	vinyl or vinylene with a S=O group
C≡Cc1ncccc1	*ortho*-ethynylene azaarene
O=C1C=C[CH]=CC1=O	*ortho*-quinone
[CH3]=[CH][CH]=O	acrolein (2-propenal)

1.5. Integrated Testing Strategies (ITS) in Aquatic Toxicology

Integrated Testing Strategies (ITS), also referred to as Intelligent Testing Strategies, represent another promising approach to reduce the number of fish required for experimentation [70,71]. These are built from the combination of a set of alternative methods to provide a Weight of Evidence (WoE) with regard to the toxicity and/or bioaccumulation potential of a query compound. The components of ITS can be divided into two categories: non-testing approaches (e.g. read-across, QSARs and waiving decision schemes built from cut-off values for key chemical descriptors [72,73]) and testing approaches (e.g. tests using fish surrogates and *in vitro* methods). When results from alternative methods are in concordance for a given examined compound and ecotoxicological endpoint of interest, conducting *in vivo* tests in fish are thus less likely to be required for its risk assessment. It should be noted that risk assessment requires very high confidence concerning the toxicity and bioaccumulation potential of an examined compound. As a consequence, although ITS have been proposed to evaluate the toxicity [74] and bioaccumulation potential [30,75] of chemicals, there is a need to formalise ITS to provide potential hazard information with high reliability [24].

1.6. Validation: The Final Step for the Implementation of Alternative Methods

Validation is the key to obtain regulatory status of alternative methods [76,77]. Validation has been defined as the process by which the reliability and relevance of a procedure are established for a particular purpose. Depending on the type of non-animal method developed, specific validation requirements should be applied. For instance for *in silico* models, a validated QSAR should incorporate [78]: 1) a defined endpoint; 2) an unambiguous algorithm; 3) a defined domain of applicability; 4) appropriate measures of goodness-of-fit, robustness and predictivity; and 5) a mechanistic interpretation, if possible. The validation of *in vitro* assays are more difficult than for *in silico* models, since they need to be designed, managed and conducted to very high standards [24]. At present, the Fish Embryo Acute

Toxicity Test (FET) [59] is the only alternative that has been validated as an accepted *in vitro* method. The validation principles for *in vitro* testing include, amongst others: 1) an assessment of a quality of overall design; 2) demonstration of the intra- and inter-laboratory reproducibility of the test method; and 3) to demonstrate the assay performance in relation to existing toxicity data [76,77]. To accomplish the latter, test compounds that are used in the validation of *in vitro* methods should be supported by *in vivo* high quality toxicity data to facilitate a better understanding of the relationship between *in vivo* and *in vitro* data. Therefore, lists of reference compounds selected based on their high quality *in vivo* toxicity data should be developed to facilitate future inter-method correlations and data comparison. Whilst such a reference list has been provided for developing alternatives to acute fish toxicity [79], there are no reference lists proposed for chronic toxicity and bioaccumulation studies.

1.7. Objectives of this Thesis

This research has been undertaken within the AlterREACH project. This project is funded by the Norwegian Research Council (NRC) and is coordinated by the Norwegian Institute for Water Research (NIVA) in Oslo, Norway. Framed into the REACH legislation, the AlterREACH project aims to develop and evaluate non-animal methods to assess the adverse effects of chemicals and their bioaccumulative potential to aquatic species, particularly in fish. In particular, the following objectives were achieved in the present thesis:

1. The establishment of a list of reference compounds to develop and evaluate alternative methods to *in vivo* bioaccumulation studies in fish (*Chapter 2*).
2. The development of log BCF_{max} models for rainbow trout and common carp that assisted in the creation of the reference list for bioaccumulation studies (*Chapter 2*).
3. The development of an *in vitro* clearance assay using freshly prepared trout hepatocytes to study the metabolic biotransformation for a set of reference

chemicals supported by high quality *in vivo* BCF data (*Chapter 3*).

4. The evaluation and comparison of available alternative methods in terms of their reliability to estimate K_{MET} and BCF for a set of reference chemicals. Alternative K_{MET} methods include the clearance assay (objective 3) together with different PBTK calculation models derived from the literature and the QSAR model of Arnot et al. [57] to predict K_{MET}. Alternative BCF methods include log BCF-based and kinetic mass balance models (*Chapter 3*).
5. The verification of structural alerts for Michael acceptors using the growth inhibition assay in *Tetrahymena pyriformis* (protozoan) and *in chemico* GSH reactivity data (*Chapter 4*).
6. The development of an ITS for prioritising chemicals for *in vivo* testing. This ITS was built from the combination of the non-animal methods used for the verification of structural alerts for Michael acceptors *(Chapter 4).*

Chapter 2. Development of a List of Reference Compounds to Evaluate Alternative Methods *to In Vivo* Fish Bioaccumulation Tests[1]

2. 1. Introduction

The potential of a compound to bioaccumulate is one of several properties that need to be evaluated in risk assessment procedures. Although bioaccumulation refers to the accumulation of a substance in an organism from all routes of exposure (from the solution and diet), the potential bioaccumulation of a compound is usually expressed by the bioconcentration factor (BCF) that refers only to its accumulation through waterborne exposure. In aquatic risk assessments, BCFs have been measured in fish according to the Organisation for Economic Cooperation and Development (OECD) Test Guideline (TG) 305 "Aqueous and dietary exposure" [10,31]. This test is demanding in terms of resources and the use of a large number of animals per test substance. Coupled with this, compliance with legislation such as the European Union Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) regulation [13] has the potential to increase the demand for animal testing to assess BCFs for a large number of chemicals unless further action is taken to limit animal testing. In order to reduce cost and limit animal use, other methods such as *in silico* (computer-based) and *in vitro* techniques have been proposed as alternatives to *in vivo* testing in fish to comply better with the principles of the 3Rs (reduction, refinement and replacement) for animal testing [12].

In silico models for bioaccumulation have been developed over more than 30 years, mostly in the form of Quantitative Structure-Activity Relationships (QSARs) [47]. As chemical uptake is mainly a steady-state phenomenon controlled predominantly by passive diffusion processes and lipid partitioning, early QSAR models were built from the relationships

[1] This Chapter is based on a publication whose link to the source is provided in the Appendix III.

between the observed log BCF and hydrophobicity, often represented by the logarithm of octanol/water partition coefficient (log K_{OW}). Although there is a strong relationship with hydrophobicity, the maximal bioconcentration potential of a compound may be reduced by poor chemical bioavailability, ionisation and other factors that are associated with the Absorption, Distribution, Metabolism and Excretion (ADME) of chemicals [48]. Consequently, more recent approaches have developed mass balance models for a better interpretation of the ADME processes governing the bioaccumulation of neutral compounds [53,54] and ionisable compounds [80].

Of the ADME processes, absorption and metabolism have been implicated as the most important factors introducing uncertainty into predictive models for bioaccumulation [30]. To deal with factors affecting chemical absorption, *in silico* approaches have considered molecular properties to screen for chemicals that may have limited bioaccumulation as a result of molecular constraints. In particular, molecular weight (MW) and the maximum inter-atomic distance between two atoms in the chemical structure (D_{max}) have been demonstrated to be useful descriptors [81,82]; however, there has been little consensus in their use [83]. This can be explained partly by the fact that other features such as low bioavailability and metabolic biotransformation may also contribute to reduce the bioaccumulation of large molecules [83]. To deal with uncertainties associated with metabolism, modelling studies have incorporated chemical biotransformation data into log K_{OW}-based prediction models to correct for the effect of metabolism in aquatic bioaccumulation [49]. Although partly successful, these predictions of metabolic susceptibility have been based on models for mammalian systems due to the lack of *in vivo* metabolic data for fish. Models for predicting the metabolic susceptibility in fish include: 1) a QSAR model developed by Arnot et al. [57] for the whole body biotransformation half-lives K_{MET}(HL) built from predicted *in vivo* K_{MET} rate data, which were estimated from measured BCF and total elimination rate constant in fish [84,85]; and 2) a more sophisticated

biotransformation half-lives model that was developed using the Iterative Fragment Selection method to identify fragments associated with chemical degradation [86].

A variety of *in vitro* methods have been developed to study the absorption and metabolism of chemicals [30]. Whilst *in vitro* methods for absorption are based predominantly on mammalian systems, test systems for metabolism have also been developed for fish. Current biotransformation assays are based on a substrate-depletion approach for the calculation of the hepatic clearance rate which can be incorporated into established physiologically-based models for the estimation of K_{MET} [42,43]. Although standardised protocols for *in vitro* methods such as subcellular fractions (S9) and primary hepatocyte cell assays have been proposed [35,41], standardised protocols for other biotransformation assays are still to be developed. It should be stressed that, despite the fact that information on absorption and metabolism properties can be obtained through *in vitro* methods, their applicability for assessing chemical bioaccumulation is currently limited by the lack of assay validation [44]. There is a need, therefore, to enable the development, standardisation and validation of *in vitro* methods for the prediction of *in vivo* bioaccumulation within a regulatory context.

In order to ensure that non-animal methods can be used as surrogates for, or as a complement to, *in vivo* fish testing, the establishment of a high quality and well-parameterised relationship between *in vivo* and *in vitro* data is required. A small number of such comparisons have been reported for BCFs [36-40], but they have been applied to a limited selection of chemicals. A representative list of chemicals, chosen on the basis of defined criteria for chemical diversity and data quality, is thus required to facilitate targeted comparison of the different approaches used in bioaccumulation testing.

The aim of the present study, therefore, was to develop a list of reference compounds for future development and evaluation of the applicability of alternative test methods to assess bioaccumulation in fish. The selection of reference compounds was conducted according to

a number of criteria that were integrated into a three tiers strategy. The selection strategy was applied to identify a diverse set of chemical classes supported by high quality *in vivo* data for BCF, K_{MET} and analysed metabolites for rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). In addition, broad coverage of bioconcentration potential, molecular properties (MW, D_{max}), and metabolic properties (metabolic pathways, K_{MET} (HL) were also required for the selected compounds. This Chapter describes the chemical selection process and the use of *in silico* techniques that were employed to assist in the creation of the reference list of chemicals.

2.2. Materials and Methods

2.2.1 Chemical Selection Strategy

The selection of reference chemicals was conducted according to a set of criteria organised into three Tiers as shown in Figure 2.1. The criteria were established using expert judgement based on previous criteria of the validity of the test procedures [77], and relevant considerations for the assessment of chemical accumulation in aquatic organisms. In essence, the purpose of Tiers I and II was to obtain high quality *in vivo* data for key bioaccumulation endpoints, whereas the purpose of Tier III was to ensure chemical diversity. The three tiers of chemical selection are described in detail below.

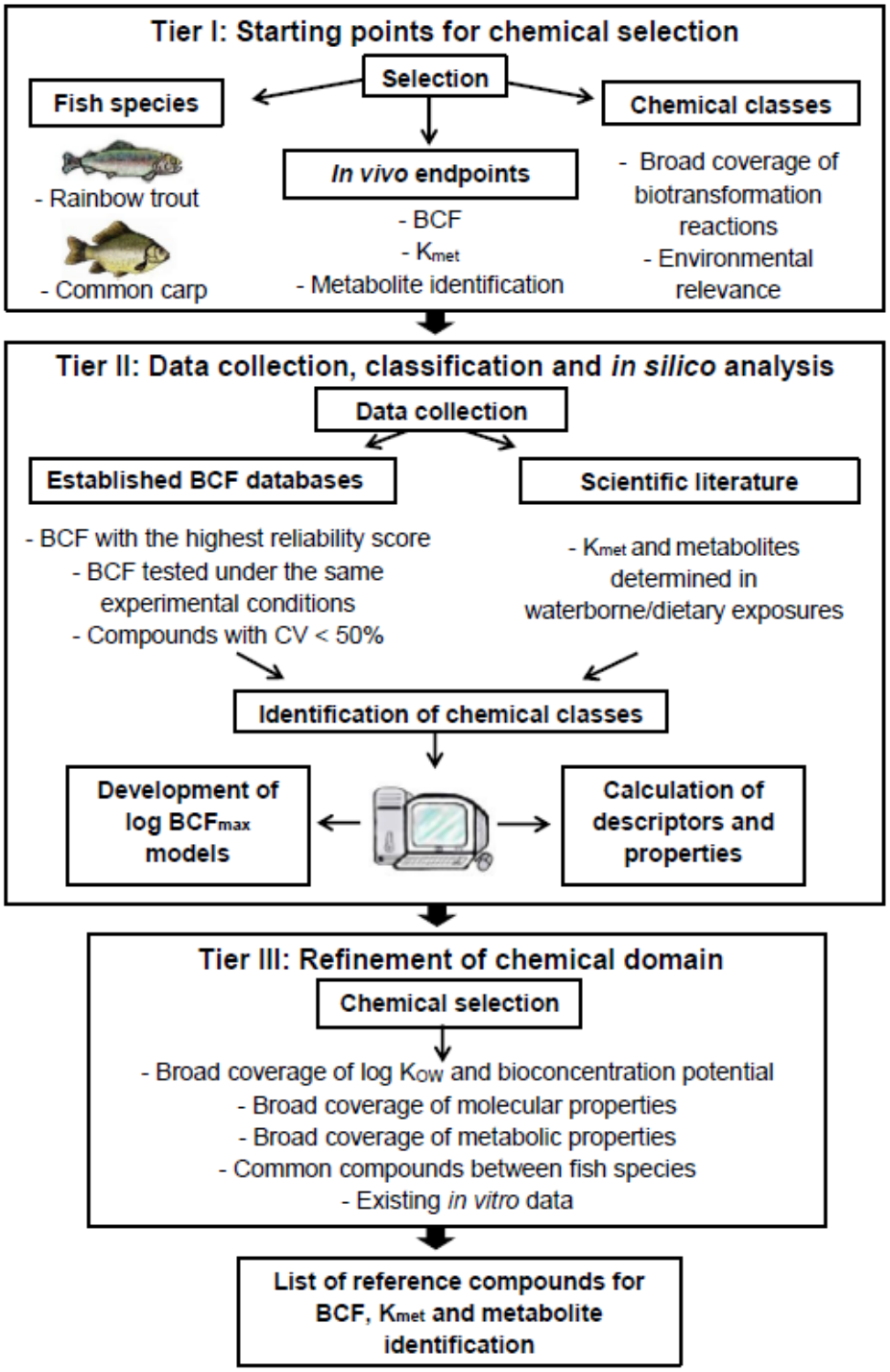


Figure 2.1 The three tier selection strategy used for the development of a list of reference compounds for evaluating alternative methods to fish bioaccumulation tests.

2.2.2 Starting Points for Chemical Selection

Before data compilation, the first step in the strategy was the selection of relevant *in vivo* bioaccumulation endpoints, fish species and chemical classes for the development of a reference list of chemicals (Figure 2.1).

Data for *in vivo* BCF, K_{MET} and characterisation of the metabolic pathway of the chemicals (expressed by metabolite identification) were selected from *in vivo* measurements related to bioaccumulation for two reasons. Firstly, the many BCF data that were available for organic chemicals and which were determined according to official guidelines [10,31]; and secondly, the possibility of measuring such properties through *in vitro* metabolic test systems such as S9 and isolated hepatocytes [35-40]. *In vitro* test systems can also provide specific information on the metabolic pathway of test chemicals by identifying and quantifying their resulting metabolites [87]. With regard to fish species, rainbow trout and common carp were chosen in the present study as being OECD recommended test species for *in vivo* bioaccumulation studies [10,31] and for which different alternative testing approaches have been proposed [30]. With regard to chemical classes, only organic chemicals were considered in the present study since mechanisms other than hydrophobicity driven by passive diffusion could be involved in the bioaccumulation of organometallic compounds and organic salts [49]. As the use of *in vitro* metabolism assays may become more common in bioaccumulation studies in the future, the selection of relevant chemical classes was based on the inclusion of structurally diverse chemicals that span known biotransformation reactions in fish [29,88]. Table 2.1 lists the 19 chemical classes that were considered for the development of the reference list with their main biotransformation pathways and enzymes involved. The metabolism of xenobiotics is often classified into Phase I and Phase II biotransformation reactions [29,88]. In Phase I, reactive and polar groups are added to compounds that are being metabolised. There are three types of Phase I reactions: oxidation,

reduction and hydrolysis. Phase II reactions increase the polarity of chemicals by conjugation with a polar molecule (e.g. uridine diphosphate (UDP) glucuronic acid). Another crucial aspect for the creation of a reference list was the inclusion of chemicals of environmental concern. These included persistent organic compounds such as Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs), and others with known industrial applications such as aliphatic halogenated hydrocarbons that have been used as industrial solvents, organophosphates and amides (pesticides), azo compounds (dyes) and heterocyclic compounds.

Table 2.1. Chemicals classes considered for the development of a reference list of chemicals for alternatives to *in vivo* BCF tests [29,88]

No.	Chemical classes	Major Phase I Metabolic Reactions (Enzymes)	Major Phase II Metabolic Reactions (Enzymes)
1	Aliphatic hydrocarbons	Oxidative dehalogenation (CYP), Epoxidation of alkenes (CYP), Hydroxylation (CYP)	Glutathione conjugation (GST)
2	Benzenes	Hydroxylation (CYP)	Glucuronidation (UGT)
3	Biphenyls	Hydroxylation (CYP)	Glucuronidation (UGT)
4	Polycyclic aromatic hydrocarbons	Hydroxylation (CYP), Epoxidation (CYP), Hydrolysis (EH)	Glutathione conjugation (GST), Glucuronidation (UGT)
5	Ethers	Dealkylation (CYP)	
6	Carboxylic acids and esters	Hydrolysis of esters	Amino acid conjugation (Acetyl-CoA, AAT), Glucuronidation (UGT)
7	Alcohols	Oxidation (ADH)	Sulfonation (SULT)
8	Phenols		Glucuronidation (UGT), Sulfonation (SULT)
9	Aldehydes	Oxidation (ALDH), Oxidation of aromatic aldehydes (AO)	
10	Quinones	Reduction (DTD)	
11	Nitroaromatic compounds	Reduction (CYP NRT)	
12	Azo compounds	Reduction (CYP AzoRT)	
13	Amines	Oxidation of secondary amines (MAO), Oxidation of tertiary amines (FMO), Reduction (CYP)	Glucuronidation (UGT), Sulfonation (SULT), Acetylation (Acetyl-CoA)
14	Amides and carbamates	Oxidation (FMO)	Glucuronidation (UGT)
15	Organosphosphates	Oxidation (CYP)	
16	Organosulfur compounds	Oxidation desulfuration (FMO)	
17	Epoxides	Hydrolysis (EH)	
18	Polyunsaturated fatty acids	Oxidation (LPO)	
19	Heterocyclic compounds	Oxidation (CYP), Reduction (CYP), Epoxidation (CYP)	Glucuronidation (UGT)

AAT: Aminoacyl transferase, Acetyl-CoA: Acetyl-coenzyme A, ADH: Alcohol dehydrogenase, ALDH: Aldehyde dehydrogenase, AO: Aldehyde oxidase, CYP: Cytochrome P450, CYP AzoRT: Cytochrome P450 azoreductase, CYP NRT: Cytochrome P450 Nitroreductase, DTD: DT Diaphorase, EH: Epoxide hydrolase, FMO: Flavin-containing monooxygenase, GST: Glutathione S-transferase, LPO: Lipoxygenase, MAO: Monoamine oxidase, SULT: Sulfotransferase, UGT: Uridine diphosphate glucuronosyl transferase.

2.2.3 Data Collection

The next step in the strategy was the compilation of BCF, K_{MET} and metabolite data for rainbow trout and carp from different information sources. A thorough scientific literature search was conducted to compile chemicals supported by K_{MET} data and information on identified metabolites. BCF values were obtained from established databases including the Environment Canada's Domestic Substance List (DSL) and non-DSL databases, both reviewed by Arnot and Gobas [32], and the EURAS-CEFIC database [89]. These databases were selected for two main reasons. Firstly, they assessed *in vivo* BCF data based on the quality principles reported in OECD TG 305 [10,31]; and secondly, they compiled data from other important sources including the ECOTOX (http://cfpub.epa.gov/ecotox) and the Japanese Ministry of Economy, Trade and Industry-National Institute of Technology and Evaluation (METI-NITE) database (http://www.nite.go.jp/index-e.html).

Table 2.2 lists the general features of the different databases in terms of their availability and format, BCF data contained therein and the assessment score. It should be noted that although the databases differ in the number of criteria and scoring system to assess the quality of the *in vivo* BCF, they all agree with the recommendations for identification of high quality BCF data proposed by Parkerton et al. [90]: the correct analysis of test substance in fish and exposure medium, lack of significant toxic effects on exposed fish and achievement of steady state with unambiguous units.

Table 2.2 A summary and comparison of the features of the established BCF databases

	Environment Canada BCF databases	**EURAS-CEFIC database**
Source	On request from http://www.hc-sc.gc.ca/	Freely available from http://ambit.sourceforge.net/euras/
Format	Microsoft excel spreadsheet	Microsoft excel spreadsheet
No. BCF values	5317	1130
No. chemicals	822	549
Species	Fish (82%), Invertebrates (15%), Autotrophs (4%)	Only fish (90% for common carp)
Score system	1 (high quality) 2 (moderate quality) 3 (low quality)	Klimisch score: 1 (reliable without restrictions) 2 (reliable with restrictions) 3 (not reliable) 4 (not assignable)

Only BCF data for rainbow trout and common carp supported by the highest quality score and measured under the same experimental conditions were selected for inclusion in the reference list. The experimental considerations were: 1) analytical determination of the test compounds in the whole fish (wet weight); 2) experimental tests conducted in a flow-through system and; 3) using the steady state method for the calculation of BCF. For chemicals with multiple BCF values, only those presenting coefficient of variance (100 x standard deviation/average value; CV %) lower than 50% were considered (Figure 2.1). Single BCF values for each chemical were obtained by averaging the multiple data points after the removal of statistically significant outliers. Outliers were identified using the boxplot graph representation in the SPSS software version (v.) 18 (http://www.spss.co.in). In this simple analysis, outliers were identified outside the T-bars (95% confidence intervals of the data). With regard to chemicals supported by *in vivo* K_{MET} and metabolite identification data, both waterborne and dietary exposures were considered for the compilation of chemicals from the literature due to the lack of sufficient *in vivo* fish studies covering metabolism of chemicals.

2.2.4 Identification of Chemical Classes

Compounds processed in Tier II were assigned to chemical classes according to the categories established in Tier I (Table 1.1) and the presence of other functional groups that were not listed in Table 1.1 (e.g. nitrile, peroxide, ketone). A further sub-classification according to structural properties (e.g. aliphatic vs aromatic fragments) was conducted for those chemical classes that contained a large number of chemicals. Industrial names were used for chemicals with complex chemical structures (e.g. dyes).

2.2.5 Calculation of Descriptors and Properties

Chemical structures of the compiled compounds were obtained from the EPI Suite version 4.1 (http://www.epa.gov). The International Chemical Identifier (InChI) was obtained from the OpenBabel v. 2.3.1 software (http://openbabel.org/) and used to identify replicate compounds among databases and fish species. Chemical structures were recorded as SMILES strings for descriptor calculation and entered into different EPI Suite models to calculate: 1) log K_{OW} and MW from KOWWIN v. 1.68; 2) Henry's Law Constant (HLC) from HenryWin v. 3.20 using the bond contribution method; and 3) K_{MET} (HL) normalised to a 10 g fish from BCFBAF v. 3.01, which was based on the QSAR model developed by Arnot et al. [57]. Common names extracted from ChemSpider (http://www.chemspider.com/) were used for those chemicals presenting complex International Union of Pure and Applied Chemistry (IUPAC) names.

D_{max} values were calculated from the geometry optimised 3-D structures (in xyz format). The 3-D structures were obtained from SMILES strings using a Python v 2.7.3 script. The 3D geometries were generated using OpenBabel v. 2.3.2 (http://openbabel.org/); accessed using Python via the Pybel module v. 1.8 (http://openbabel.org/wiki/Python) and locally optimised using the MMFF94 force-field [91]. The MOPAC input files were extracted, and

MOPAC v. 2012 (http://openmopac.net/) was run to optimise the chemical structures using the AM1 Hamiltonian. The following keywords were employed: charge=0 and PRT INT (setting no charge and exporting the interatomic distances, respectively). D_{max} values were obtained from the MOPAC.out file, where D_{max} was defined as the maximum interatomic distance between non-hydrogen atoms. The D_{max} values were extracted automatically from the MOPAC.out file using an in-house Perl script.

The prediction of metabolic pathway and resulting metabolites was made using the Meteor Nexus v.1.5.1 software (Lhasa Limited, Leeds, England (www.lhasalimited.org/meteor/). Meteor predicts biotransformation reactions from the structure of the parent compound by applying rules that were created based on knowledge on mammalian metabolism [92]. Such knowledge was extracted from the literature and/or confidential information from pharmaceutical industries. The Meteor Nexus software uses absolute and relative reasoning to make biotransformation predictions. Absolute reasoning describes the probability of biotransformation taking place (probable, plausible, equivocal, doubted and improbable), whereas relative reasoning allows further ordering of all possible metabolic outcomes. Levels of probable, plausible and equivocal, were selected for the analysis of the total number of resulting metabolites of the examined compounds, whereas predictions identified as probable and/or plausible were used to characterise the metabolic pathways of compounds identified in Tier II.

2.2.6 Development of Log BCF_{max} Models

The objective of the development of log BCF_{max} models was to identify chemicals whose BCF values were poorly correlated (over-predicted) with log K_{OW}. Compounds supported by *in vivo* BCF data for rainbow trout and common carp were classified into six categories, noted below, depending on the difference between their reported predicted maximal BCF and experimental values. To provide an estimate of the maximal bioconcentration potential of

the examined chemicals, a model for the maximal log BCF (log BCF_{max}) for both fish species was thus required. To accomplish this, Equation 2.1 developed by Bintein et al. [93] was modified to accommodate a subset of *in vivo* BCF data, using the Minitab v. 16 statistical software (http://www.minitab.com).

$$\text{Log BCF} = 0.91 \log \text{Kow} - 1.97 \log (6.8 \times 10^{-7} \text{Kow} + 1) - 0.79 \qquad (2.1)$$

$$n = 154,\ r^2 = 0.950,\ s = 0.347,\ F = 464$$

Where n is the number of observations; r^2 is the square of the correlation coefficient; s is the standard error, and F is Fisher's statistic

As a difference of 0.5 log BCF is assumed reasonable to account for the variability resulting from experimental procedures [94], compounds whose residuals were between 0 and 0.5 log units from this log BCF_{max} were considered well-predicted (W). In a similar manner, compounds whose residuals were less than 0 were considered under-predicted (U) and compounds whose residuals were greater than 0.5 log units were considered over-predicted (O) by the log BCF_{max} model. Under-predicted and over-predicted compounds were further classified into: 1) highly under-predicted compounds (U2) (residuals < -1); 2) slightly under-predicted (U1) (residuals = -0.5 to 0); 3) slightly over-predicted (O1) (residuals > 0.5-1); 4) moderately over-predicted (O2) (residuals = 1 to 1.5); and 5) highly over-predicted (O3) (residuals > 1.5).

2.2.7 Refinement of Chemical Domain

The objective of Tier III was to pursue chemical diversity within the reference list in terms of physico-chemical, molecular and metabolic properties for each of the chemical classes identified in Tier II. Hydrophobicity (Log K_{OW}) was selected amongst other physico-chemical properties due to its strong influence on BCF [47]; MW and D_{max} were selected as they have been widely used to investigate the effect of molecular mass and size on chemical

bioaccumulation [81,82]; and finally, K_{MET} (HL), metabolic pathway and number of metabolites of chemicals were selected amongst other metabolic properties. All these properties were calculated using the software as described above. When compounds in the same chemical class were identified as having the same bioconcentration potential for a similar range of log K_{OW}, compound selection was then based on ensuring a broad range of molecular and metabolic properties. It should be noted that chemicals that were in common between rainbow trout and common carp and other chemicals that were supported by existing *in vitro* data were included in the reference list regardless of their physico-chemical, molecular and metabolic properties.

2.3. Results and Discussion

The present study aimed to establish a list of reference compounds for the development, assessment and validation of alternative methods to *in vivo* bioaccumulation studies in fish. As no official guidance is provided for conducting such a selection process, the present study presents a novel approach to select and evaluate such reference compounds.

Different strategies have been developed to select representative compounds from existing databases depending on the number of compounds in the databases and purpose of the study. Particularly in drug discovery, computational techniques, such as cluster analysis and dissimilarity-based compound selection, have been used to ensure the selection of structurally diverse sets of compounds for testing [95]. In contrast, the chemical selection procedure is rationalised in (eco)toxicological studies by considering aspects important for the evaluation of alternative methods through the establishment of a list of selection criteria, and therefore, not necessarily employing high-throughput screening approaches. Similar to other chemical selection strategies in (eco)toxicology [79,96-98], the strategy followed in the present study was based on the establishment of a list of criteria, evaluation of available experimental *in vivo* data, and the use of *in silico* techniques to complement the selection

process. It should be noted, however, that whilst for toxicity studies there is a need to consider the mechanism of toxic action to ensure either consistency or diversity, chemical bioaccumulation is governed to a large degree by ADME processes that are more clearly linked to physico-chemical and molecular properties.

The selection process performed in the present study involved three Tiers: Tier I selected relevant *in vivo* bioaccumulation endpoints, fish species and chemical classes for the development of a list of reference compounds; Tier II compiled and analysed chemicals supported by high quality *in vivo* data for the established endpoints and chemical classes in Tier I; and Tier III applied further criteria to ensure a broad chemical domain. Tier I has been explained in detail in Materials and Methods, and therefore, it will not be discussed again in this section.

2.3.1 Compounds Supported by In Vivo BCF Data

Data Extraction

A total of 361 BCF values for rainbow trout and another 840 BCF values for common carp were obtained from the Canadian DSL and non-DSL and EURAS-CEFIC BCF databases based on the highest reliability score and application of the same test conditions. Initially, all BCF values that were extracted for common carp were assessed as having the highest reliability score since they were measured in compliance with Good Laboratory Practise in Japan to meet the Chemical Substance Control Law [33]. In contrast, for rainbow trout 331 BCF values failed to meet one or more of the established quality criteria of the databases [32,89], for example the toxic effects reported for two dioxin-like compounds, uncertain correction of the radiolabel analysis for some organophosphates and insufficient exposure duration to achieve 80% of steady state for the majority of polychlorinated compounds.

Multiple BCF values were obtained for the majority of compounds for rainbow trout; all chemicals assessed in common carp had two BCF values. Compounds containing multiple BCFs with a coefficient of variance (CV) that was lower than 50% were considered for the development of list of reference compounds. A total of 51 out of 59 compounds were selected for rainbow trout; a total of 224 compounds out of 420 were selected for common carp. Generally, rejected compounds (CV > 50%) showed a significant difference between their BCFs reported at different test concentration as is represented in Figure 2.2 for a rejected compound, pentachloronitrobenzene (CAS no. 62-68-8). This could be an indication of toxic effects and/or enzyme saturation in test organisms produced at high concentrations [32].

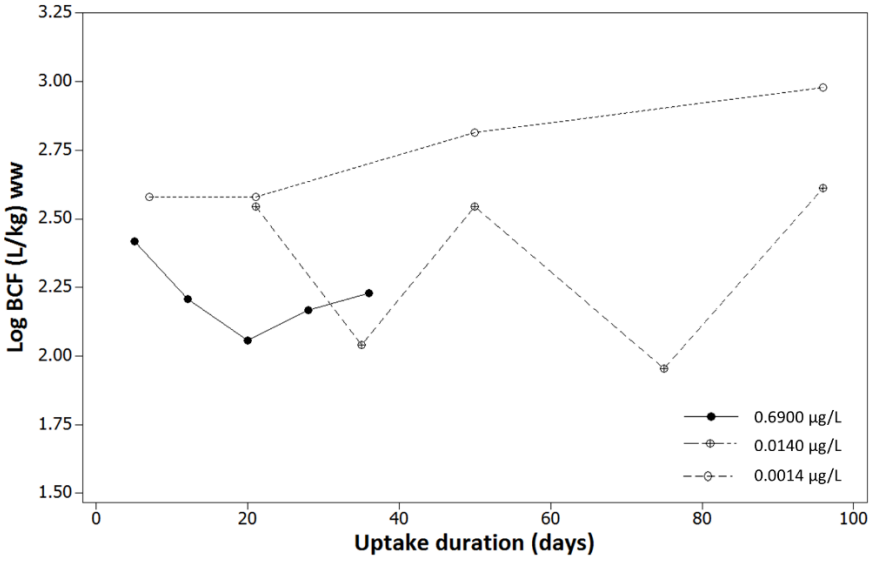


Figure 2.2 Relationship between log BCF and uptake duration phase depending on test concentration for pentachloronitrobenzene.

Single BCF values for selected compounds (CV < 50%) were obtained by averaging the multiple data points after the removal of two outliers that were identified for two compounds for rainbow trout, and thus were excluded in the calculation of the average values for these compounds.

Identification of Chemical Classes

The selected chemicals supported by high quality *in vivo* BCF data for both rainbow trout and common carp were classified into 18 chemical classes and additional subclasses on the basis of their functional groups and structural properties. Table 2.3 shows the chemical classes identified and number of compounds in each according to fish species. As can be seen, the majority of compounds were found to be aliphatic and aromatic hydrocarbons (classes 1-5), phenols, nitrobenzenes and amines, with a relatively small number of organosulfur compounds, carboxylic acids, alcohols, amides and organophosphates.

Table 2.3. Chemical classes and subclasses for compounds with *in vivo* BCF data

No.	Chemical classes	Subclasses	Trout	Carp	Total
1	Aliphatic linear hydrocarbons	Alkenes, Alkanes	0	24	24
2	Aliphatic cyclic hydrocarbons	Cycloalkanes, Cycloalkenes	1	13	14
3	Benzenes	Alkylbenzenes, Benzenes	17	28	45
4	Biphenyls	Chlorobiphenyls, Bromobiphenyls	5	5	10
5	Polycyclic aromatic hydrocarbons (PAHs)	Naphthalenes, Other PAHs	3	7	10
6	Ethers	Aliphatic ethers, Aromatic ethers	2	9	11
7	Carboxylic acids and esters	Any subclassification was conducted	6	1	7
8	Alcohols	Alcohol derivatives, Alcohols	0	7	7
9	Phenols	Alkylphenols, Nitrophenols, Phenol derivatives	3	35	38
10	Ketones	No subclassification	0	4	4
11	Quinones	No subclassification	0	7	7
12	Nitrobenzenes	Alkylnitrobenzenes	12	13	25
13	Amines	Alipathic amines, Naphthalenamines, Benzoamines, Other amines	0	25	25
14	Amides and carbamates	Aliphatic amides, Aromatic amides, Carbamates	0	7	7
15	Organophosphates	Aliphatic organophosphates, Aromatic organophosphates, Thiophosphates	1	8	9
16	Heterocyclic compounds	Saturated, Unsaturated	1	16	17
17	Dyes (azo compounds)	No subclassification	0	5	5
18	Minority groups	Nitriles, Organosulfur compounds, Phenyls, Hydrazobenzenes, Peroxides	0	10	10

Table 2.3 covered the majority of the chemical classes that were proposed for inclusion in the reference list based on their known biotransformation reactions (Table 2.1) with the exception of aldehydes, epoxides and polyunsaturated fatty acids. Of these, only two epoxides were found initially within the common carp data, however they were not considered further due to a CV > 50%. The lack of diversity for some classes of chemicals in the original BCF databases, such as reactive compounds (e.g. epoxides), can be explained by the fact that such chemicals are likely to cause toxicity higher than the 10 % mortality validity limit set for OECD tests even at low exposure concentrations [10,31], and hence may not be good candidates for *in vivo* bioaccumulation assessments. This observation is supported by the toxic effects reported for dioxin-type compounds described above for rainbow trout. Nonetheless, a broad range of chemical structures and other chemical groups that were not initially considered in Tier I, such as ketones, carbamates, esters, nitriles and peroxides, were considered for the development of the list of reference compounds.

Table S1 lists the 265 chemicals that entered into Tier III according to the chemical classes noted in Table 2.3. Table S1 is provided as electronic supplementary data (Appendix IV). Physico-chemical properties (log Kow, log HLC), molecular properties (MW, D_{max}), metabolic properties (metabolic reactions, number of metabolites, K_{MET} (HL)) and data related to the measurement of the *in vivo* BCF (test temperature, final wet weight and lipid content of test organisms, reference database) are shown in Table S1.

Compounds in Common Between Fish Species

Of the 265 chemicals, ten chemicals were in common between rainbow trout and common carp including five substituted benzenes (**44, 45, 53, 64, 71)** (see Table S1), 2,2',5-trichlorobiphenyl (**83**), pentachlorophenol (**151**) and three nitrobenzenes (**177, 178, 182**). For all chemicals in common, lower values of log BCF were obtained for common carp than for rainbow trout. This could be caused by differences in the temperature of the test system,

since the temperature used to measure BCFs in common carp was 22 °C and a temperature of 15 °C was reported for rainbow trout (see Table S1). Temperature is considered to be one of the factors affecting bioconcentration kinetics since it may influence the biological enzymatic rates and other temperature-dependent biological processes [29]. In fact, a comparative study has shown that *in vitro* metabolite formation was 10 to 100 times faster in carp than trout [99], which could explain the consistently lower BCF values observed for common carp in the present comparison.

2.3.2 Development of Log BCF_{max} Models

The first step for the identification of compounds with lower log BCF values than expected from the relationship with log K_{OW} correlation was the development of a model for maximal log BCF for rainbow trout and common carp (log BCF_{max}). Equation 2.1 was modified to accommodate a subset of chemicals with high BCF values for each of the fish species. The bilinear models built for rainbow trout and common carp are shown as a solid line in Figure 2.3 and are described by Equations 2.2 and 2.3 respectively. These models represent the worse-case scenario for BCF where accumulation is driven purely by passive diffusion processes.

$$\text{Log } BCF_{max,\,Rt} = 0.88 \log K_{OW} - 1.73 \log (2.25 \times 10^{-6} K_{OW} + 1) - 0.08 \quad (2.2)$$

$$\text{Log } BCF_{max,\,Cc} = 0.89 \log K_{OW} - 1.51 \log (1.41 \times 10^{-6} K_{OW} + 1) - 0.88 \quad (2.3)$$

It should be noted that the data used in Equations 2.2 and 2.3 were based on selecting chemicals with high BCF values (shown as open circles in Figure 2.3) to obtain the maximal BCF value and, therefore, were not subjected to any statistical treatment.

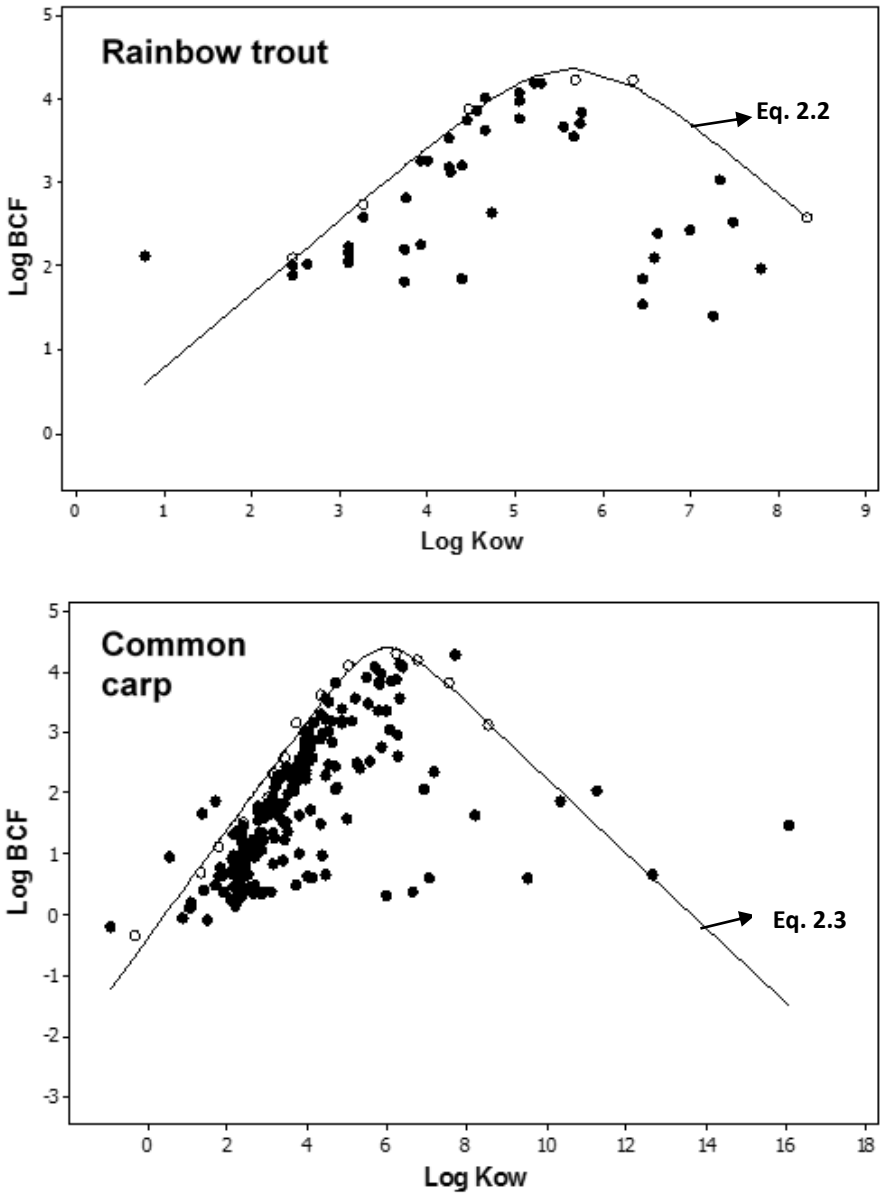


Figure 2.3 Relationship between log BCF and log K_{OW} for rainbow trout and common carp compounds. Solid line: log BCF_{max} model developed from a set of chemicals with representative maximal values (represented as open circles).

Compounds supported by *in vivo* BCF data were then split into six different categories depending on their residual values from Equations 2.2 and 2.3. Following the rationale explained above, 6% of compounds were classified as being highly and slightly under-predicted (U2,U1), 31% well-predicted (W), another 35% slightly over-predicted (O1) and finally 28% of substances were identified as being moderately and highly over-predicted (O2,O3). Over-predicted compounds represent groups of interest to allow for the

investigation of factors reducing BCF of chemicals such as high rates of metabolic reactions, poor bioavailability due to a high degree of ionisation and lack of undergoing uptake due to molecular constraints.

Compounds that were under-predicted (U2,U1) included a set of hydrophilic heterocyclic compounds (**234,235,237,238**), aliphatic amides (**218,224**) and highly hydrophobic hydrocarbons (log K_{OW} > 7) such as dechloran A (**38**), 1,3,5-tris (1,1-dimethylethyl)benzene (**78**), decabromobiphenyl (**87**) and tris (1-methylethyl)naphthalene (**96**) (see Table S1). The high bioconcentration potential of highly hydrophobic hydrocarbons (**38,78,87**) could be attributable to a lack of metabolic biotransformation, since halogen groups and/or alkyl groups at the target site of metabolic reaction, or at surrounding positions, may have the potential to inhibit partly or completely the metabolic biotransformation of these compounds [29].

Well-predicted and slightly over-predicted compounds (W, O1) included the majority of neutral compounds such as cyclic aliphatic, benzenes, biphenyls, PAHs and all compounds classified as ethers. This observation is supported by their relatively high values of K_{MET} (HL) and lack of polar groups in the chemical structure that may make them less susceptible to a metabolic attack [47]. However, compounds with log K_{OW} < 3 with polar groups and relatively low values of K_{MET} (HL) such as alcohols (**119**), phenols (**123-130**), quinones (**164**), nitrobenzenes (**171-180**) and organophosphates (**225**) were also classified as W and O1 (Table S1). This is in agreement with previous *in silico* predictions suggesting that high biotransformation rates have only a minimal impact on the BCF for compounds with log K_{OW} < 3 [43,84]. High rates of chemical flux across the gills could be more important for the overall BCF than the biotransformation rates for such compounds. Conversely, more hydrophobic compounds (log K_{OW} > 3) with polar groups such as ketones (**160,161,163**), quinones (**169,170**), nitrobenzenes (**187,188,191**), amines (**212,216**), amides (**219-221,223**) were

identified as being over-predicted (O2,O3) (Table S1). The lower than expected log BCF values may be a result of metabolism as previous modelling studies have shown that relatively low biotransformation rate may have a large influence on bioaccumulation for hydrophobic compounds (log K_{OW} > 3) [43,84].

As expected, the observed log BCF of the majority of ionisable compounds in the present study showed low bioconcentration potential, as chemical properties other than hydrophobicity account for the bioaccumulation of ionisable compounds. Ionisable compounds included organic acids such as phenols, carboxylic acids and phosphates and organic bases such as amines and heterocyclic compounds containing a nitrogen atom in the ring. Descriptors other than log K_{OW} have been considered in recent *in silico* studies to improve the prediction of bioaccumulation for ionisable compounds [80]. Alternative descriptors include the logarithm of the distribution coefficient (log D), which is the ratio of concentration of unionised forms of a compound in octanol and the total concentration of unionised and ionised forms in water. It should be noted that the log K_{OW} of ionisable compounds provided in the present study refers to their neutral form. The absorption of ionisable compounds is dependent on several factors such as the log K_{OW} of the neutral form and the degree of ionisation, i.e. the negative logarithm of the equilibrium constant for dissociation (pKa). No significant reduction in the uptake rate is predicted for weak organic acids and weak bases at a pH of 7 [80]; however, a moderate reduction in the uptake is expected at higher pH (pH > 9) and, therefore, further analysis of the degree of ionisation for ionisable compounds will be required to address this in detail.

It is worth noting that as the bioaccumulation of a compound is a complex function comprising diverse chemical properties and biological processes, the low bioconcentration of some of the examined compounds could be associated with more than one factor [83]. For instance, the significant low bioconcentration potential of dyes (**251-255**) (Table S1) could

be a result of several processes such as a potential fast metabolic biotransformation, absorption limitation due to their large molecular size (D_{max} = 1.44 to 2.64) and poor bioavailability due to ionisation and binding to proteins.

2.3.3 Compounds Supported by In Vivo K_{MET} Data

Data for 14 chemicals with measured *in vivo* K_{MET} values were found for rainbow trout, which contrasted with the lack of information for K_{MET} for common carp. These chemicals are listed in Table S2, provided as electronic supplementary data in the Appendix IV, and encompassed five phenyls (**1-5**) [100,101] and nine heterocyclic pesticides (**6-14**) [101,102]. Of these, tetrachlorobiphenyl (**1**) and DDT (**2**) were also supported by *in vivo* BCF data (see compounds **85** and **261** in Table S1). Experimental details used for the assessment of K_{MET}, such as type of exposure, tissue analysed and test concentration are provided in Table S2. K_{MET} values were calculated by comparing the HL of test compounds with known recalcitrant PCBs in a non-linear relationship between log K_{OW} and HL [103]. Based on this approach, chemicals whose HL fall on, or near, this non-linear relationship were assumed to be recalcitrant to biotransformation, whereas those chemicals with data that fall below this relationship were suggested to be more readily biotransformed. This method allows for the quantification of the biotransformation rates of a number of organic chemicals that were tested under the same experimental conditions [103].

2.3.4 Compounds Supported by In Vivo Analysed Metabolites

Data for eight chemicals whose resulting metabolites were analysed in an *in vivo* system using rainbow trout were compiled from the literature. These chemicals are provided in Table S3 (electronic supplementary data, Appendix IV) and included four perfluoroalkylated compounds (**1-4**)[104,105], decabromodiphenyl ether (BDE) (**5**)[106] and three carboxylic acid pharmaceuticals (**6-8**) [107,108]. Although few metabolites were monitored for each

compound, the biotransformation pathway was proposed for fluorotelomer acrylate (**4)** and carboxylic acids (**6-8**). Experimental details used for the analysis of metabolites and references are provided in Table S3. Depending on the study, different routes of exposure (dietary, waterbone, intraperitoneal injection) as well as fish tissues for analysis (muscle, blood, liver bile, and kidney) were used to investigate the biotransformation pathways of these compounds (see Table S3). Worthy of mention is that both aspects may influence the formation and accumulation of resulting metabolites from the parent compound. For instance, a different metabolic pattern was found for BDE (**5**), where debromominated diphenyl ether metabolites (De-BDEs) were the main metabolites in liver, whereas methoxylated diphenyl ethers (MeO-DBEs) were found in higher concentration in blood [106]. With regard to common carp, the biotransformation of three PAHs [109] and four polybrominated diphenyl ether congeners [110] have been reported, but these data were not included in the present study as neither study detected any metabolites of the parent compounds in the tissue analysed.

2.3.5 Refinement of Chemical Domain

All the chemicals that were compiled in Tier II were reduced in number according to the Tier III criteria (Figure 2.1). A total of 144 compounds out of the 265 compounds passing Tier II were selected to provide *in vivo* BCF data (see reference compound (RC) in Table S1). Selected chemicals are listed in Table 2.4 with some of the properties considered most relevant for the selection process. Generally, fewer chemicals were selected (< 50%) for the chemical classes that contained a large number of chemicals and low structural diversity (e.g. aliphatic linear and cyclic hydrocarbons, benzenes, ethers, phenols and nitrobenzenes) than for more diverse chemical groups with fewer chemicals (e.g. organophosphates, amides and heterocyclic compounds) (Table 2.3). The selection covers chemicals used in a range of industrial applications such as: 1) intermediates in the production of pharmaceuticals

(**91,123**), cosmetic and fragrances (**192)**, plastics (**46,225**), detergents (**36**); 2) pesticides including insecticides (**35, 38, 221,227, 248,250, 261**), nematocides (**13,98**), fungicides (**262**) and herbicides (**222**); 3) dyes (**251-255**); 4) and industrial solvents (**14,21,229,234**). Of the selected compounds, naphthalene (**88**), pentachlorophenol (**151**) and nonyl-phenol (**154**) should be considered of high environmental relevance due to their multiple industrial applications.

For compounds supported by K_{MET} and metabolite identification, 13 compounds were selected on the basis of the existing *in vitro* data and broad coverage of chemical properties considered for selection (see RC in Tables S2 and S3 respectively). In terms of existing *in vitro* data, four fungicides (**6**,**7**,**10**,**11**) with *in vivo* K_{MET} data were previously tested in rat and rainbow trout hepatic microsomes with the aim to investigate their cross-species comparison [111]. A high degree of metabolic conservation was found between both species. The biotransformation of four compounds supported by *in vivo* analysed metabolites was also investigated from an *in vitro* scenario (see Table S3 for details). For instance, the metabolism of decabromodiphenyl ether (**5**) was investigated using liver microsomal fractions from common carp and rainbow trout [112], and 8:2 Fluorotelomer acrylate (**4**) was tested in S9 fractions isolated from rainbow trout stomach and liver [113].Table 2.5 and Table 2.6 list the selected chemicals for *in vivo* data for K_{MET} and metabolites respectively.

The ensemble of reference compounds for *in vivo* BCF, K_{MET} and metabolite data encompasses a broad range of bioconcentration potential, log K_{OW} (-0.93 to 12.11), molecular properties (MW: 68.1 to 959 g/mol and D_{max}: 0.29 to 2.64 nm), K_{MET} (HL) (0-33100 d) and diverse metabolic biotransformation pathways.

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
Aliphatic linear hydrocarbons									
3	Dichlorodifluoromethane	1.82	1.24	0.78	W	Cc	120.91	0.29	0.619
11	2-Methyl-1,3-butadiene	2.58	1.92	1.04	O1	Cc	68.12	0.56	0.591
13	1,2-Dibromo-3-chloropropane	2.68	2.01	1.04	O1	Cc	236.33	0.48	0.340
14	Tetrachloroethene	2.97	2.26	1.71	O1	Cc	165.83	0.56	3.35
19	2,4,4-Trimethyl-1-pentene	4.08	3.25	2.76	W	Cc	112.22	0.65	3.00
20	1-Nonene	4.62	3.70	3.18	O1	Cc	126.24	0.99	2.77
21	1,1,2,3,4,4-Hexachloro-1,3-butadiene	4.72	3.78	3.82	U1	Cc	260.76	1.19	26.8
23	2,5,8-Trimethyl-1-nonene	6.01	4.39	3.35	O2	Cc	168.33	1.24	5.89
24	Hexadecane	8.20	3.38	1.65	O3	Cc	226.45	2.18	19.9
Aliphatic cyclic hydrocarbons									
26	Cyclohexane	3.18	2.45	1.87	O1	Cc	84.16	0.54	0.762
30	4-Ethenyl-cyclohexene	3.73	2.94	2.18	O1	Cc	108.18	0.61	1.55
33	1,2,3,4,5-Pentabromo-6-Chlorocyclohexane	4.71	3.77	2.45	O2	Cc	513.09	0.66	2.52
35	Heptachlor	5.86	4.38	3.97	W	Cc	373.32	0.68	50.1
36	Cyclododecane	6.12	4.39	3.85	O1	Cc	168.33	0.89	11.7
38	Dechloran A	11.27	1.48	2.03	U2	Cc	653.73	1.13	33100
Benzenes									
39	Chlorobenzene	2.64	1.97	1.24	O1	Cc	112.56	0.47	0.532
44	1,2-Dichlorobenzene	3.28	2.54	2.26	W	Cc	147.00	0.56	3.42
			2.80	2.58	W	Rt			
45	1,3-Dichlorobenzene	3.28	2.54	2.24	W	Cc	147.00	0.56	3.67
			2.80	2.74	W	Rt			
46	1-Methylethenylbenzene	3.44	2.68	1.84	O1	Cc	118.18	0.66	0.940

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
48	1,2,4-Trimethylbenzene	3.63	2.85	2.13	O1	Cc	120.20	0.63	0.821
53	1,2,3-Trichlorobenzene	3.93	3.36	3.26	W	Rt	181.45	0.59	5.59
			3.12	2.82	W	Cc			
56	Ethenylethylbenzene	3.93	3.12	2.66	W	Cc	132.21	0.86	2.71
62	1,2-Dichloro-4-(trifluoromethyl)benzene	4.24	3.62	3.18	W	Rt	215.00	0.69	9.61
63	1,2,4-Trichloro-5-methylbenzene	4.47	3.81	3.88	W	Rt	195.48	0.59	4.93
64	1,2,3,4-Tetrachlorobenzene	4.57	3.66	3.03	O1	Cc	215.89	0.59	8.70
			3.88	3.85	W	Rt			
68	1,3-Bis(1-methylethyl)benzene	4.90	3.92	3.16	O1	Cc	162.28	0.89	4.01
71	Pentachlorobenzene	5.22	4.14	3.55	O1	Cc	250.34	0.64	13.7
			4.28	4.19	W	Rt			
73	1,2,4,5-Tetrachloro-3,6-dimethylbenzene	5.67	4.37	3.55	O1	Rt	243.95	0.70	6.98
76	Hexabromobenzene	7.33	3.44	3.02	W	Rt	551.49	0.67	7.32
78	1,3,5-Tris(1,1-dimethylethyl)benzene	7.72	3.67	4.30	U2	Cc	246.44	0.94	77.0
Biphenyls									
81	3,5-Dichlorobiphenyl	5.05	4.19	3.77	W	Rt	223.10	0.92	20.2
83	2,2',5-Trichlorobiphenyl	5.69	4.35	4.07	W	Cc	257.55	0.93	94.3
			4.37	4.23	W	Rt			
84	3,3'-Diethylbiphenyl	5.83	4.38	3.80	O1	Cc	210.32	1.11	30.8
85	2,2',3,3'-Tetrachlorobiphenyl	6.34	4.16	4.23	W	Rt	291.99	0.93	155
PAHs									
88	Naphthalene	3.17	2.44	1.97	W	Cc	128.18	0.59	4.53
91	9H-Fluorene	4.02	3.19	2.75	W	Cc	166.22	0.84	1.37
93	Anthracene	4.35	3.48	3.30	W	Cc	178.24	0.90	2.54
95	1,2,3,4-Tetrachloronaphthalene	5.75	4.37	3.70	O1	Rt	265.95	0.70	25.2
96	Tris(1-methylethyl)naphthalene	7.54	3.78	3.82	U1	Cc	254.42	1.05	22.4

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
97	Octachloronaphthalene	8.33	2.61	2.58	W	Rt	403.73	0.78	218
Ethers									
98	2,2'-Oxybis (1-chloropropane)	2.39	1.75	0.91	O1	Cc	171.07	0.56	0.437
101	Dibenzyl ether	3.48	2.72	2.45	W	Cc	198.27	0.84	0.075
103	1,2,3-Trichloro-4-methoxybenzene	4.01	3.43	3.25	W	Rt	211.48	0.74	18.5
106	2-(2-Methylpropoxy)naphthalene	4.65	3.73	2.85	O1	Cc	200.28	1.06	5.93
107	Pentachloroanisole	5.30	4.31	4.19	W	Rt	280.37	0.83	69.0
108	Hexabromodiphenyl ether	8.55	3.16	3.15	W	Cc	643.59	1.10	40.3
Carboxylic acids and esters									
109	Chlorobenzilate	3.99	3.17	2.65	O1	Cc	325.19	1.03	2.13
112	Abietic acid	6.46	4.09	1.84	O3	Rt	302.46	1.16	49.4
114	Palustric acid	7.27	3.49	1.40	O3	Rt	302.46	1.20	84.8
115	12,14-Dichlorodehydroabietic acid	7.81	3.05	1.97	O2	Rt	369.33	1.17	53.3
Alcohols									
116	1,1,1,3,3,3-Hexafluoro-2-propanol	1.11	0.60	0.20	W	Cc	168.04	0.46	0.370
118	1,1,1-Trichloro-2-methyl-2-propanol	2.09	1.48	0.26	O2	Cc	177.46	0.49	0.405
120	2,6-Dichloro-benzenemethanol	2.36	1.72	0.32	O2	Cc	177.03	0.63	0.228
121	4-Chlorobenzhydrol	3.35	2.60	1.77	O3	Cc	218.68	0.98	0.636
Phenols									
123	4-Nitrophenol	1.91	1.32	0.65	O1	Cc	139.11	0.63	0.073
129	3-Nitro-p-cresol	2.46	1.81	0.95	O1	Cc	153.14	0.68	0.051
134	2-Chloro-5-methylphenol	2.70	2.02	0.50	O3	Cc	142.59	0.59	0.227
135	4-Chloro-2-methylphenol	2.70	2.02	1.14	O1	Cc	142.59	0.61	0.209
136	2,6-Dichlorophenol	2.80	2.11	1.06	O2	Cc	163.00	0.56	0.350
137	3,4-Dichlorophenol	2.80	2.11	1.70	W	Cc	163.00	0.56	0.528
140	2,5-Dichlorophenol	2.80	2.11	1.15	O1	Cc	163.00	0.56	0.436

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
143	2,4-Dichloro-6-nitrophenol	3.20	2.47	1.28	O2	Cc	208.00	0.62	0.190
144	1,1'-Biphenyl-4-ol	3.28	2.54	1.64	O1	Cc	170.21	0.93	0.630
145	2,4,5-Trichlorophenol	3.45	2.69	2.60	W	Cc	197.45	0.62	0.734
147	4-(Phenylmethyl)phenol	3.54	2.77	1.39	O2	Cc	184.24	0.92	0.231
148	2-Methoxytetrachlorophenol	3.92	3.36	2.26	O2	Rt	261.92	0.78	5.91
151	Pentachlorophenol	4.74	3.80	2.10	O3	Cc	266.34	0.62	2.20
			4.00	2.65	O2	Rt			
153	2,6-Bis(1,1-dimethylethyl)-4-ethyl-phenol	5.52	4.29	3.49	O1	Cc	234.38	0.93	1.42
154	Nonyl-phenol	5.99	4.39	0.33	O3	Cc	220.36	1.57	1.33
156	2,6-Dicyclohexylphenol	6.30	4.36	2.95	O2	Cc	258.41	1.01	1.06
158	Hexachlorophene	6.92	4.12	2.07	O3	Cc	406.91	1.01	3.78
Ketones									
160	Diphenylmethanone	3.15	2.43	0.84	O3	Cc	182.22	0.97	0.248
161	Michler's ketone	3.50	2.74	1.52	O2	Cc	268.36	1.44	0.298
162	Oxybenzone	3.52	2.75	1.98	O1	Cc	228.25	1.21	0.173
163	2-(1-Cyclohexenyl)cyclohexanone	3.73	2.94	0.49	O3	Cc	178.28	0.85	0.480
Quinones									
165	1-Amino-9,10-anthracenedione	3.53	2.76	1.99	O1	Cc	223.23	0.96	0.126
166	1-Hydroxy-9,10-anthracenedione	3.64	2.86	2.26	O1	Cc	224.22	0.96	0.070
169	2-Ethyl-9,10-anthracenedione	4.38	3.50	0.99	O3	Cc	236.27	1.15	0.432
170	7-Oxobenz[de]anthracene	4.73	3.79	2.07	O3	Cc	230.27	0.96	0.387
Nitrobenzenes									
171	Nitrobenzene	1.81	1.23	0.63	O1	Cc	123.11	0.61	0.099
175	1-Methyl-3-nitrobenzene	2.36	1.72	0.76	O1	Cc	137.14	0.71	0.335
177	1-Chloro-4-nitrobenzene	2.46	2.08	2.00	W	Rt	157.56	0.61	0.644
			1.81	1.12	O1	Cc			

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
178	1-Chloro-2-nitrobenzene	2.46	2.08	2.09	U1	Rt	157.56	0.61	0.644
			1.81	1.16	O1	Cc			
181	2-Ethylnitrobenzene	2.85	2.16	1.06	O2	Cc	151.17	0.77	0.372
182	1,2-Dichloro-4-nitrobenzene	3.10	2.65	2.07	O1	Rt	192.00	0.60	1.03
			2.38	1.67	O1	Cc			
188	1,2,3-Trichloro-4-nitrobenzene	3.74	3.20	2.19	O2	Rt	226.45	0.61	1.62
189	1,3,5-Trichloro-2-nitrobenzene	3.74	2.95	2.44	O1	Cc	226.45	0.69	1.71
191	1,2,3,4-Tetrachloro-5-nitrobenzene	4.39	3.74	1.85	O3	Rt	260.89	0.68	2.14
192	Musk xylol	4.45	3.56	3.56	W	Cc	297.27	0.92	0.070
Amines									
193	1,8-Naphthalenediamine	1.34	0.81	0.70	W	Cc	158.20	0.63	0.042
194	N-Ethyl-benzenamine	2.11	1.50	0.91	O1	Cc	121.18	0.90	0.125
195	2,5-Dimethylbenzenamine	2.17	1.55	0.48	O2	Cc	121.18	0.69	0.095
197	N,2-Dimethylbenzenamine	2.17	1.55	0.72	O1	Cc	121.18	0.76	0.317
204	Triallylamine	2.58	1.92	0.47	O2	Cc	137.23	0.99	0.122
206	1,1'-Biphenyl-2-amine	2.84	2.15	1.36	O1	Cc	169.23	0.93	0.750
207	2,4,6-Trichlorobenzenamine	3.01	2.30	1.92	W	Cc	196.46	0.67	0.979
208	N,N-Diethyl-benzenamine	3.15	2.43	1.93	W	Cc	149.24	0.81	0.143
212	N-Nitroso-N-phenylbenzenamine	3.16	2.43	1.36	O2	Cc	198.23	0.91	0.123
213	4,4'-Methylenebis (2-chlorobenzenamine)	3.47	2.71	2.33	W	Cc	267.16	1.11	0.552
215	N-Phenyl-1-naphthalenamine	4.47	3.58	3.22	W	Cc	219.29	1.08	2.21
216	N-Phenyl-2-naphthalenamine	4.47	3.58	2.32	O2	Cc	219.29	1.26	2.51
217	N,N-Dioctyl-1-octanamine	10.35	2.05	1.86	W	Cc	353.68	2.30	20.0
Amides and carbamates									
218	N,N-Dimethylformamide	-0.93	-1.22	-0.19	U2	Cc	73.10	0.42	0.007

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
219	N,N-Diethyl-3-methylbenzamide	2.26	1.63	0.29	O2	Cc	191.28	1.01	0.206
220	3,5-Dimethylphenyl methylcarbamate	2.27	1.64	0.25	O2	Cc	179.22	1.06	0.051
221	Fenocarb	2.86	2.17	0.36	O3	Cc	207.27	0.81	0.132
222	Linuron	2.91	2.21	1.25	O1	Cc	249.10	0.92	0.148
223	3-Hydroxy-N-phenyl-2-naphthalenecarboxamide	4.47	3.58	0.66	O3	Cc	263.30	1.36	0.186
Organophosphates									
225	Triethyl phosphate	0.87	0.39	-0.04	W	Cc	182.16	0.88	0.028
226	Tris(1-chloro-2-propanyl) phosphate	2.89	2.19	0.39	O3	Cc	327.57	0.87	0.136
227	Fenitrothion	3.30	2.56	1.60	O1	Cc	277.23	1.11	0.785
228	Gardcide	3.81	3.01	1.65	O2	Cc	365.97	1.08	1.57
229	Tributyl phosphate	3.82	3.02	1.01	O3	Cc	266.32	1.32	0.289
230	Foxim	4.39	3.51	3.00	O1	Cc	298.30	1.25	1.10
231	2-Methylphenyl diphenyl phosphate	5.25	4.16	2.51	O3	Cc	340.32	1.12	0.506
232	2-Ethylhexyldiphenyl phosphate	6.30	4.36	2.63	O3	Cc	362.41	1.38	0.559
233	Triphenyl phosphite	6.62	3.99	2.39	O3	Rt	310.29	1.12	4.04
Heterocyclic compounds									
234	1,4-Dioxane	-0.32	-0.67	-0.35	U1	Cc	88.11	0.41	0.031
237	N,N-Dimethyl-1,2-dithiolan-4-amine	1.38	0.84	1.68	U2	Cc	149.27	0.66	0.026
238	4-Ethenyl-pyridine	1.71	1.14	1.87	U2	Cc	105.14	0.68	0.151
241	Benzothiazole	2.17	1.55	0.66	O1	Cc	135.18	0.66	0.112
243	3-Methyl-thiophene	2.36	1.72	0.61	O2	Cc	98.16	0.51	0.291
244	2-(3-Oxo-1H-indol-2-ylidene)-1H-indol-3-one	3.11	2.39	0.39	O3	Cc	262.27	1.27	1.85
245	9H-Carbazole	3.23	2.50	2.13	W	Cc	167.21	0.93	1.42
246	Dibenzofuran	3.71	2.92	3.17	U1	Cc	168.20	0.85	0.824

Table 2.4 Reference compounds with *in vivo* BCF data and other significant properties (cont.)

ID	Chemical name	Log K_{OW}	Pred. Log BCF_{max}	*In vivo* log BCF	BP	Sp	MW	D_{max}	K_{MET} (HL)
247	10H-Phenothiazine	3.82	3.02	2.57	W	Cc	199.27	0.99	0.820
248	Dibenzothiophene	4.17	3.32	3.16	W	Cc	184.26	0.85	0.901
249	N,N'-dicyclohexyl-thiourea	4.16	3.32	0.60	O3	Cc	240.41	1.05	0.236
250	Synepirin 500	6.07	4.39	3.06	O2	Cc	345.53	1.39	7.46
Dyes									
251	Disperse Blue 143	2.20	1.58	0.50	O2	Cc	408.46	1.84	0.001
252	Disperse Yellow 42	4.33	3.46	1.49	O3	Cc	369.40	1.44	0.391
253	Disperse Yellow 163	5.00	3.99	1.58	O3	Cc	417.26	1.65	1.07
254	Pigment Yellow 12	7.05	4.05	0.62	O3	Cc	629.51	2.34	0.139
255	Pigment Orange 13	9.55	2.54	0.60	O3	Cc	623.51	2.64	78.7
Others									
256	1,4-Benzenedicarbonitrile	1.09	0.59	0.12	W	Cc	128.13	0.68	0.232
257	4-Cyano-4'-hexylbiphenyl	6.31	4.36	3.55	O1	Cc	263.39	1.65	49.7
258	Diphenylmethane	4.02	3.19	2.92	W	Cc	168.24	0.95	0.559
259	Phenyl xylylmethane	5.11	4.07	3.20	O1	Cc	196.29	1.08	2.46
260	Triphenylchloromethane	5.58	4.32	2.55	O3	Cc	278.78	0.94	2.12
261	DDT	6.79	4.18	4.19	W	Cc	354.49	1.04	161
262	Thiram	1.70	1.13	0.48	O1	Cc	240.42	1.17	0.228
263	2-Naphthyl disulfide	6.66	4.24	0.39	O3	Cc	318.45	1.34	94.3
264	2,2'-Dichlorohydrazobenzene	4.34	3.47	3.64	U1	Cc	253.13	1.17	15.2
265	Dicumyl peroxide	5.88	4.39	2.77	O3	Cc	270.37	1.06	2.79

BP = bioconcentration prediction (O1 = slightly over-predicted; O2 = moderately over-predicted; O3 = significantly over-predicted; U1 = slightly under-predicted; U2 = highly under-predicted; W = well predicted); D_{max} = maximum interatomic distance between two atoms in the chemical structure (nanometers); ID = identification number in Table S1; K_{MET}(HL) = Whole body biotransformation half-lives (days); Log BCF = logarithm of bioconcentration factor (Litres per kilogram wet weight fish); Log K_{OW} = logarithm of octanol–water partition coefficient calculated from KOWWIN v. 1.68; MW= molecular weight (grams per mole) calculated from KOWWIN v. 1.68; PAH = polycyclic aromatic hydrocarbon; Sp = species (Cc = common carp; Rt = rainbow trout); Pred. log BCF_{max}= predicted maximal log BCF using equations 2.2 and 3.2 according to fish species.

Table 2.5 Reference compounds with *in vivo* whole body biotransformation rate (K_{MET}) data

ID	Chemical name	Log K_{OW}	K_{MET}	Sp	D_{max}	MW	K_{MET} (HL)
Phenyls							
1	2,2',3,3'-Tetrachlorobiphenyl	6.34	0.00	Rt	0.93	292	155
2	DDT	6.79	0.01	Rt	1.04	354	161
4	2,2',3,3',5,6'-Hexachlorobiphenyl	7.62	0.01	Rt	0.93	361	343
Heterocyclic compounds							
6	Myclobutanil	3.5	0.20	Rt	1.1	289	5.35
7	Propiconazole	4.13	0.57	Rt	1.18	342	6.20
10	Metconazole	4.19	0.58	Rt	1.05	320	4.58
11	Triadimefon	2.94	0.54	Rt	1.14	294	2.68
14	Fipronil	6.64	1.01	Rt	1.21	437	3.77

D_{max}: Maximum inter-atomic distance between two atoms in the chemical structure (nm); ID: Identification number in Table S2; K_{MET}: *In vivo* whole body biotransformation rate (1/d); K_{MET} (HL): Whole body biotransformation half-lives (days); Log K_{OW}: Logarithm of octanol-water partition coefficient calculated from KOWWIN v. 1.68; MW: Molecular weight (g/mol) calculated from KOWWIN v. 1.68; Sp: Species (Rt: Rainbow trout).

Table 2.6. Reference compounds with *in vivo* metabolite data

ID	Chemical name	Log K_{OW}	Metabolites	Sp	D_{max}	MW	K_{MET} (HL)
Perflouroalkylated hydrocarbons							
3	8:2 Fluorotelomer alcohol	5.75	8:2 FTCA; 8:2 FTUCA	Rt	1.34	464	24.4
4	8:2 Fluorotelomer acrylate	7.11	8:2 FTOH; 8:2 FTUCA; 7:3 FTCA; 8:2 FTCA; PFOA; 8:2 FTOH-Glu	Rt	1.7	518	20.7
Phenyls							
5	Decabromodiphenyl ether	12.11	De-DBEs; MeO-BDEs	Rt	1.08	959	581
Carboxylic acids							
6	Diclofenac	4.02	4'-OH-DCF; 5-OH-DCF; DCF- A.Glu; 4'-OH-DCF-Sul; 5-OH-DCF-Sul; 4'-OH-DCF-A.Glu; 5-OH-DCF- A.Glu; 3'-OH-DCF-A.Glu; 4'-OH-DCF-E.Glu	Rt	0.96	296	6.13
8	Ibuprofen	3.79	Carboxyl-IBF; 2-OH-IBF; IBF-A.Glu; OH-IBF-A.Glu; 3-OH-IBF; IBF-Tau	Rt	1.03	206	1.87

D_{max}: Maximum inter-atomic distance between two atoms in the chemical structure (nm); ID: Identification number in Table S3; K_{MET}: Whole body biotransformation half-lives (days); Log K_{OW}: Logarithm of octanol-water partition coefficient calculated from KOWWIN v. 1.68; MW: Molecular weight (g/mol) calculated from KOWWIN v. 1.68; Sp: Species (Rt: Rainbow trout); Metabolites: FTCA: Fluorotelomer saturated acid; FTUCA: Fluorotelomer unsaturated acid; FTOH: Fluorotelomer alcohol; FTOH-Glu: Fluorotelomer glucuronide conjugate; PFOA: perfluorooctanoate; De-BDEs: Debrominated diphenyl ethers; MeO-BDEs: Methoxylated brominates diphenyl

ethers; OH-DCF: Hydroxylated diclofenac; OH-DCF-Sul: Sulfate conjugate of hydroxylated diclofenac; DCF-A.Glu: Acyl glucuronide of diclofenac; DCF-E.Glu: Ether glucuronide of hydroxylated diclofenac; Carboxyl-IBF: Carboxyl ibuprofen; OH-IBF: Hydroxylated ibuprofen; IBF-A.Glu: Acyl glucuronide of ibuprofen; OH-IBF-A.Glu: Acyl glucuronide of hydroxylated ibuprofen; IBF-Tau: Taurine conjugate of ibuprofen.

2.3.6 Lists of Reference Compounds: Further Considerations and Implications

The successful development and validation of non-animal methods to *in vivo* bioaccumulation studies in fish are dependent on the rational selection of key chemical classes supported by a broad chemical domain and high quality *in vivo* data. However, further refinement of the reference list to accommodate project-specific requirements and specific purposes of the studies to be conducted is likely to be warranted. For instance, chemical purity, commercial availability, and availability of reliable quantitative analytical methods (e.g. analytical sensitivity, accuracy and reproducibility) are likely to represent practical selection criteria for such refinements. Chemical properties crucial for the performance of experimental approaches such as high volatility in solution (log HLC > 3 [114]), a high degree of adsorption to the test vessels (log K_{OW} > 6 [47]) and rapid hydrolysis in water (HL < 12 hours [115]) should also be considered to ensure the stability of the chemical in any assay performed. It should be noted that these additional criteria are likely to depend on the assay format, and no measures were taken to exclude those from the reference list. Moreover, additional compounds could be added to the reference list to expand the chemical domain for those chemical classes that were not represented in the original databases (aldehydes, epoxides and polyunsaturated fatty acids).

It is anticipated that the present list of reference chemicals may benefit the development and validation of alternative methods. Various *in vitro* test systems such as microsomes, subcellular fractions and isolated hepatocytes are currently deployed in different studies worldwide, and harmonisation of experimental efforts aided by the use of common reference compounds may facilitate a more rapid standardisation of alternative methods for future regulatory use. The present reference list also aims to provide a better foundation for

future *in vitro* to *in vivo* BCF extrapolations, as well as identifying compounds that may be particularly challenging for *in silico* predictions. For instance, reference compounds identified as over-predicted by log K_{OW} and susceptible to metabolic biotransformation may be particularly relevant for such studies. Additionally, *in vitro* derived data could enhance the knowledge of *in vivo* absorption and metabolism processes, allowing a better understanding of how both processes can influence *in vivo* assessment of chemical bioaccumulation in fish. Moreover, *in vitro* biotransformation data could be incorporated into the log BCF_{max} models developed for rainbow trout and common carp to correct for the effect of metabolism on BCF and to refine the knowledge of metabolic pathways in fish. Finally, greater acceptance of alternative methods in regulatory frameworks may accommodate implementation of both testing (e.g. *in vitro* assays) and non-testing (e.g. *in silico* methods) in Integrated Testing strategies (ITS) to prioritise chemicals for *in vivo* testing and aid hazard assessment in general [30,75].

2.4 Conclusion

In order to reduce the number of fish required for experimentation, there is a need to develop and validate non-animal methods to assess bioaccumulation of chemicals. Successful development of alternative test systems to *in vivo* testing could provide not only accurate information on ADME processes for a given compound, but also be used in regulatory processes and thus reducing animal use.

The present work provides a transparent description of the selection of reference chemicals for future development and evaluation of alternative testing approaches using a three-tiered approach. This approach was based on: Tier I) selection of relevant *in vivo* bioaccumulation properties, fish species and chemical classes; Tier II) data collection, evaluation and analysis of the chemicals supported by high quality *in vivo* BCF data ; and Tier III) a refinement process to ensure a representation of a large chemical domain in terms of physico-chemical,

molecular and metabolic properties. As a consequence of this work, a reference list of 144 chemicals with high quality BCF data, eight chemicals with K_{MET} data and five compounds with metabolite data have been proposed. It is envisioned that this list may facilitate the development of alternative approaches to regulatory *in vivo* testing, enhance the understanding of the relationship between *in vivo* and *in vitro* data, refine *in silico* prediction of BCF and metabolic properties of chemicals and facilitate larger implementation of alternative testing and non-testing approaches within regulatory frameworks.

Chapter 2 has focused on the establishment of a list of reference compounds to enable the development and evaluation of non-animal methods to assess chemical bioaccumulation. Once such a list was established, a set of compounds supported by high quality *in vivo* BCF data was selected to investigate the reliability of the most common alternative methods used for the estimation of the whole body biotransformation rate and BCF. This work is explained in more detail in Chapter 3.

Chapter 3. A Review and Comparison of Alternative Methods to *In Vivo* Bioaccumulation Studies in Fish

3.1 Introduction

The bioaccumulation of a compound is the result of Absorption, Metabolism, Distribution and Excretion (ADME) processes [30]. The potential bioaccumulation of chemicals directly from water is usually expressed by the bioconcentration factor (BCF), which may be measured in the whole fish, for instance according to the Organisation for Economic Cooperation and Development (OECD) Guideline 305 [10]. In order to reduce the cost and the number of fish used in *in vivo* testing studies, non-animal methods including *in silico* and *in vitro* methods have been proposed to assess the BCF of chemicals [116].

Early *in silico* models were built on the relationship between the experimental log BCF and hydrophobicity, expressed by the logarithm of octanol-water partition coefficient (log K_{OW}), as a surrogate for biological lipids [47]. However, compounds which are ionised and others prone to metabolism and highly hydrophobic chemicals (log $K_{OW} > 6$) show a poor correlation with log K_{OW} [48]. To deal with some of these uncertainties, Meylan al [50] used an innovative approach based on the development of linear regression models for non-ionisable (neutral) and ionisable compounds applying different log K_{OW} ranges (e.g. 1 to 7 and > 7). To account for the ADME processes of chemical bioaccumulation, other *in silico* studies have been focused on building kinetic mass balance models, such as the model developed by Arnot and Gobas [53,54] normalised for fish of a weight of 1 Kg. Such mass balance models were improved recently by Nichols et al. [117] through the incorporation of new experimental data for the calculation of extrapolating parameters such as the fraction that is bioavailable to the fish in water (ϕ). In addition, Nichols et al. [117] proposed designing the mass balance model for a 10 g fish based on the rationale that the majority of *in vivo* BCF data have been measured in small fish. Although some of these models are available within the EPI Suite

software (http://www.epa.gov) to enable their use, a comparison between log K_{OW}-based and kinetic mass balance models in terms of their ability to predict BCF of chemicals is still to be conducted.

Of the ADME processes of chemicals, metabolism has been identified as the main uncertainty in predictive studies due to the lack of *in vivo* metabolic data for fish to be used for modelling [30]. Nonetheless, Arnot et al. [84] predicted the whole body biotransformation rate (K_{MET}) in fish based on *in vivo* data available for bioaccumulation by re-arranging the mass balance BCF model equation (see Equation 1.1). In doing this, a database of predicted K_{MET} in fish was created [85] and used for the development of a Quantitative Structure-Activity Relationship (QSAR) [57] model to predict whole body biotransformation rates in fish based on half-lives (HL). K_{MET} data for chemicals can also be predicted by the incorporation of their *in vitro* intrinsic clearance rate (CL_{INT}) into established Physiologically-Based ToxicoKinetic (PBTK) models designed for a 1 Kg rainbow trout [42,43] (see Figure 1.1 for more details). CL_{INT} data were calculated using *in vitro* test systems including S9 subcellular fractions and isolated hepatocytes, both derived from the livers of fish donors [37,38]. Hans et al. [37] and Cowas-Ellsberry et al. [38] were the first authors to perform *in vitro-in vivo* data extrapolations to calculate K_{MET} from *in vitro* intrinsic clearance; however, they used different values for key extrapolation factors such as the weight of fish liver. Consequently, Nichols et al. [117] have recently proposed a standardised PBTK calculation model defined for rainbow trout, to enable future *in vitro-in vivo* extrapolations.

The applicability of *in vitro* methods for metabolism is currently limited due to data variability and technical challenges [44]. In particular, the question remains whether the biological features of the fish donors could be partly responsible for a high data variability reported for *in vitro* clearance using rainbow trout hepatocytes [37,39,40]. As a result of better analytical technologies not yet being developed, only a limited number of chemicals have been tested

in such bioassays [37,39,40]. Therefore, there is a need to develop and improve *in vitro* metabolic assays to enable their validation and utility for risk assessment procedures. The benefits of the validation of *in vitro* test systems using fish cells can be enormous. For example, accurate *in vitro* metabolic data not only could unravel the impact of biotransformation processes on the maximal BCF of chemicals expected by their passive diffusion processes, but also they could refine BCF estimates by incorporating *in vitro* K_{MET} data into mass balance BCF models [37,38].

The aim of this study was to review and compare the non-animal methods described above which have been proposed as alternatives to *in vivo* bioaccumulation studies in fish. The specific aims included: 1) to investigate the metabolic biotransformation fora set of compounds supported by *in vivo* BCF data in a clearance assay using freshly isolated trout hepatocytes, along with providing an explanation of the potential factors affecting data variability; 2) to compare the QSAR model of Arnot [57] with different PBTK calculation models in terms to their ability to estimate K_{MET}. The intrinsic clearance rates of test compounds were integrated into the PBTK models provided by Hans et al. [37], Cowas-Ellsberry et al. [38] and Nichols et al. [117] for K_{MET} estimation; and 3) to compare EPI-log K_{OW}-based and mass balance BCF models using the goodness of fit between *in vivo* and predicted BCF data for the chemicals examined. Fish weights of 10 g and 1 Kg using the *in vitro* K_{MET} calculated by Nichols et al. [117] model were employed to investigate their impact on the predicted BCF. This study also provided an insight into the challenges and future perspectives for each of the alternatives examined.

3.2 Materials and Methods

It should be noted that this work was undertaken as part of the PhD programme at the Norwegian Institute for Water Research (NIVA) in Oslo, Norway, from January to June in 2013.

3.2.1 Test Compounds

A total of ten compounds including diverse halogenated benzenes, two polycyclic aromatic hydrocarbons (PAHs), one heterocyclic compound (dibenzofuran) and one polychlorinated biphenyl (PCB14) were tested in the trout clearance assay (see Table 3.1). All test chemicals were obtained from Sigma-Aldrich Norway (www.sigmaaldrich.com/norway) with purities between 94 and 99% and, therefore, they were not purified prior to testing. The majority of these chemicals were selected from the reference list developed for the evaluation of alternatives to bioaccumulation in fish (Chapter 2) [118]. Chemicals that were not included in the reference list, such as benzo(a)pyrene (BaP) and pentachloronitrobenzene (PCNB), were also considered for *in vitro* testing. BaP was considered as a benchmark compound in this study based on the widely reported *in vitro* data for this compound [35,37,40]; PCNB was selected to enable metabolic data comparison with the other two hydrophilic chloronitrobenzenes (see Table 3.1). Whilst any *in vivo* BCF data were found for BaP from the established databases (see Table 2.2), data measured at three different concentrations were found for PCNB (see Figure 2.2), from which only values obtained at the lowest concentration (0.0014 µg/L) were considered for the average of the BCF. A set of key properties such as the predicted metabolic pathway, the bioconcentration prediction (BP) and molecular properties of the test compounds were considered prior to testing (Table 3.1).

Table 3.1 Test compounds considered for *in vitro* testing with relevant properties and chemical analysis parameters

CAS NR	Chemical name and abbreviation	Log Kow[a]	*In vivo* BCF (Fs)	Predicted metabolic pathway (Enzyme) [b]	BP	m/z quant.	m/z Qualifier ion	Internal standard
88-73-3	1-Chloro-2-nitrobenzene (CNB)	2.46	123 (Rt) 14 (Cc)	1) 4-Hydroxylation (CYP) > O-Sulphation (SULT), Glucuronidation (UGT) ; 2) Reduction of Nitro Group (CYP450 reductase/XO/AO)	U1 (Rt) O1 (Cc)	157	159	d10- biphenyl
618-62-2	1,3-Dichloro-5-nitrobenzene (DCNB)	3.10	170 (Rt)	Reduction of Nitro Group (CYP450 reductase/XO/AO)	W	145	191	d8-acenaphthylene
132-64-9	Dibenzofuran (DBF)	3.71	1490 (Cc)	1) Hydroxylation of Fused Benzenes (CYP) > O-Sulphation (SULT), Glucuronidation (UGT); 2) Hydroxylation of Fused Benzenes (CYP)	U1	168	Not used	d8-acenaphthylene
54135-80-7	2,3,4-Trichloroanisole (TCA)	4.01	1778 (Rt)	1) Oxidative O-Demethylation (CYP); 2) 5-Hydroxylation (CYP) > Glucuronidation (UGT), O-Sulphation (SULT), Oxidative O-Demethylation (CYP)	W	212	210	d8-acenaphthylene
120-12-7	Anthracene (AT)	4.35	2012 (Cc)	1) Hydroxylation of Fused Benzenes (CYP) > Glucuronidation (UGT), O-Sulphation (SULT); 2) Dihydrodiols (CYP, EH)	W	178	Not used	d8-dibenzothiophene
87-86-5	Pentachlorophenol (PCP)	4.74	447 (Rt) 126 (Cc)	Nd	O2 (Rt) O3 (Cc)	266	264	d8-dibenzothiophene
82-68-8	Pentachloronitrobenzene (PCNB)	5.03	590 (Rt)	Reduction of Nitro Group (NTR)> Glucuronidation (UGT), O-Sulphation (SULT)	O2	295	212	d8-dibenzothiophene
34883-41-5	3,5-Dichlorobiphenyl (PCB14)	5.05	5888 (Rt)	4-Hydroxylation (CYP) > Glucuronidation (UGT), O-Sulphation (SULT)	W	222	224	d8-dibenzothiophene
636-28-2	1,2,4,5-Tetrabromobenzene (TBB)[c]	5.55	4677 (Rt)	Nd	O1	394	393	d8-dibenzothiophene
50-32-8	Benzo(a)pyrene (BaP)	6.11	Nd	1) Hydroxylation (CYP); 2) Dihydrodiols formation (CYP,EH)	Nd	252	132	d12-perylene

BP: Bioconcentration predictions based on log BCF_{max} model predictions using Equations 2.2 and 2.3 (U1: Under-predicted (residuals = -0.5 to 0), W: Well-predicted (residuals = 0 to 0.5), O1: Over-predicted (residuals = 0.5 to 1); O2: Over-predicted (residuals = 1 to 1.5); O3: Over-predicted(residuals > 1.5); CAS NR: Chemical Abstracts Service Registry Number; Enzymes (AO: Aldehyde oxidase, CYP: Cytochrome P450, EH: Epoxide hydrolase, GST: Glutathione S-transferase, SULT: Sulfotransferase, UGT: UDP-glucuronosyl transferase, XO: Xanthine oxidase); Fs: Fish species (Rt: Rainbow trout, Cc: Common carp); Log K_{OW}: Predicted logarithm of octanol-water coefficient, m/z quant: m/z used for quantification; Nd: No data predicted for metabolic pathways. [a] KOWWIN v.1.68 (EpiSuite); [b] Meteor NEXUS v.1.5.1 software; [c] Compound tested only in preliminary experiments.

3.2.2 Fish Culture

Juvenile rainbow trout of approximately 200 to 500 g obtained from the Valdres Rakfisk BA hatchery (Valdres, Norway) were kept at the Department of Biology, University of Oslo (Norway), in the following culture conditions: 6 ± 2 °C, pH 6.6, 100% oxygen saturation and under a 12h light/12h dark photo cycle. The fish were fed daily with commercial pellets (Skretting, Stavanger, Norway) that corresponded to approximately 0.5% of total body weight.

3.2.3 Hepatocyte Isolation

The procedure for the isolation of hepatocytes from rainbow trout is described by Tollefsen et al. [119]. In brief, juvenile rainbow trout were killed by a blow to the head, then fish liver and intestines were exposed by dissection and finally liver cells were isolated by a two-step liver perfusion procedure. The resulting single cell suspension was checked for cell viability (criteria > 85% viable) using the trypan blue method. The cell concentration was determined using Coulter counter. Cells were diluted to 2 x 10^6 cells/mL in Leibovitz media (L-15).

3.2.4 Hepatocyte Incubation

A detailed protocol for this technique is provided as Supplementary Data in the Appendix I. The hepatocyte incubation was performed in 1 mL of a 2 x 10^6 cells/mL suspension in 5 mL sample vials with orbital shaking (100 rpm) at 11 °C. A volume of 5 µL of test chemical dissolved in dimethyl sulfoxide (DMSO) were added to each of the incubation glass vials that were swirled gently to ensure sample homogeneity. The incubation was terminated at 0, 1, 2, 3, 4, 5 h into Eppendorf tubes containing 400 µL of dichloromethane with internal standard added to correct for the loss of analyte during sample tubes. Sample tubes were mixed briefly by vortexing and centrifuged at 20800 centrifugal force (xg) for 10 minutes at 4 °C. The supernatant was transferred to Gas Chromatography Mass Spectrometry (GC-MS) vials and

stored until chemical analysis at 4 °C. The control (dead) cells were obtained by heating at 100 °C for 15 minutes to inactivate cells and then were treated in the same way as fresh hepatocytes.

3.2.5 Analytical Methods

The target analytes were determined on an Agilent 7890A gas chromatograph (GC Agilent JW Scientific, Santa Clara, CA) linked to an Agilent 5975c inert XL EI/CI mass spectrometer, MS (Agilent JW Scientific) operated in single-ion monitoring mode (SIM) with electron impact ionisation (70 keV). Separation was on a DB-5MS column (30 m, -0.25 mm i.d. and 0.25 µm film thickness, Agilent JW Scientific) following a pulsed splitless injection (1 µL injection, pulse pressure 20 psi for 1.2 min, injector temperature of 300 ^{o}C). The helium carrier gas flow was set to 1.0 mL min^{-1}. The GC oven temperature programme started with a step at 60 ^{o}C (held for 2 min) before an increase to 200 ^{o}C (at the rate 20 ^{o}C min^{-1}), followed by an increase to 225 ^{o}C (at the rate of 4 ^{o}C min^{-1}) and then again increased to 315 ^{o}C (at the rate of 30 ^{o}C min^{-1}) with this temperature held constant for a further 2 min. The temperatures for the ion source, quadrupole and transfer line were set to 230, 150 and 280 ^{o}C, respectively. The relative response of surrogate internal standards and 8-point calibration curves were used for the quantification. The retention time and the m/z compared to the external standard was used for identification. The internal standards, m/z qualifier ion and m/z used for the quantification of the chemicals examined are shown in Table 3.1. The chemical analysis of the compounds tested in this study was conducted at NIVA by Andreas S Hogfeldt.

3.2.6 Intra and Inter Assay Variability

Variability within and between assays were reported as a percent Coefficient of Variation (100 x standard deviation/average value; % CV), which were calculated using Microsoft Excel v.2010.

3.2.7 Predicting K_{MET} and BCF from the Intrinsic Clearance

Figure 3.1 shows the key equations required for the calculation of K_{MET} from the *in vitro* data generated in the clearance assay. Definitions of all parameters and their units are listed in Table 3.2. To calculate K_{MET} and BCF, the first step was the calculation of the first-order elimination rate constant (k) (h-1) using the following equation:

$$\text{Log } C_t = \text{Log } C_O - k/2.3\ t \qquad (3.1)$$

Where C_O and C_t are the concentration of test chemicals (μM) at time 0 and t, respectively. The intrinsic clearance rate (CL_{INT}) was normalised to the hepatocytes concentration (C_{HEP}) (2 x 10^6 cells/mL) and loss of the chemical in control samples according to Equation 3.2:

$$CL_{INT} = CL_{INT,\ ACTIVE\ HEPATOCYTES} - CL_{INT,\ CONTROL} \qquad (3.2)$$

As can be seen in Figure 3.1, the intrinsic clearance in liver ($CL_{INT\ LIVER}$), hepatic clearance (CL_H) and finally K_{MET} were calculated from the input of other physiological parameters. These were the liver weight (LW), the total number of hepatocytes in the fish liver (L_{HEP}), the hepatic blood flow (Q_H), the hepatic clearance binding term (f_U) and the apparent volume of distribution ($V_{D,BL}$). The term f_U is the ratio of the unbound (free) fraction of test compound in blood ($f_{U,b}$) between the unbound fraction in the *in vitro* clearance assay ($f_{U,h}$). V_d relates the amount of test compound in the body to the concentration in the blood at steady state. Data for LW, L_{HEP}, Q_H, f_U, and $V_{D,BL}$ were calculated from the parameters used by Has et al. [37], Cowas-Ellsberry et al. [38] and Nichols et al. [117]. These are shown in Table 3.3 to allow comparison. It should be noted that same variable could differ in nomenclature depending on the calculation model (e.g. $V_{D,BL}$ also termed V_d).

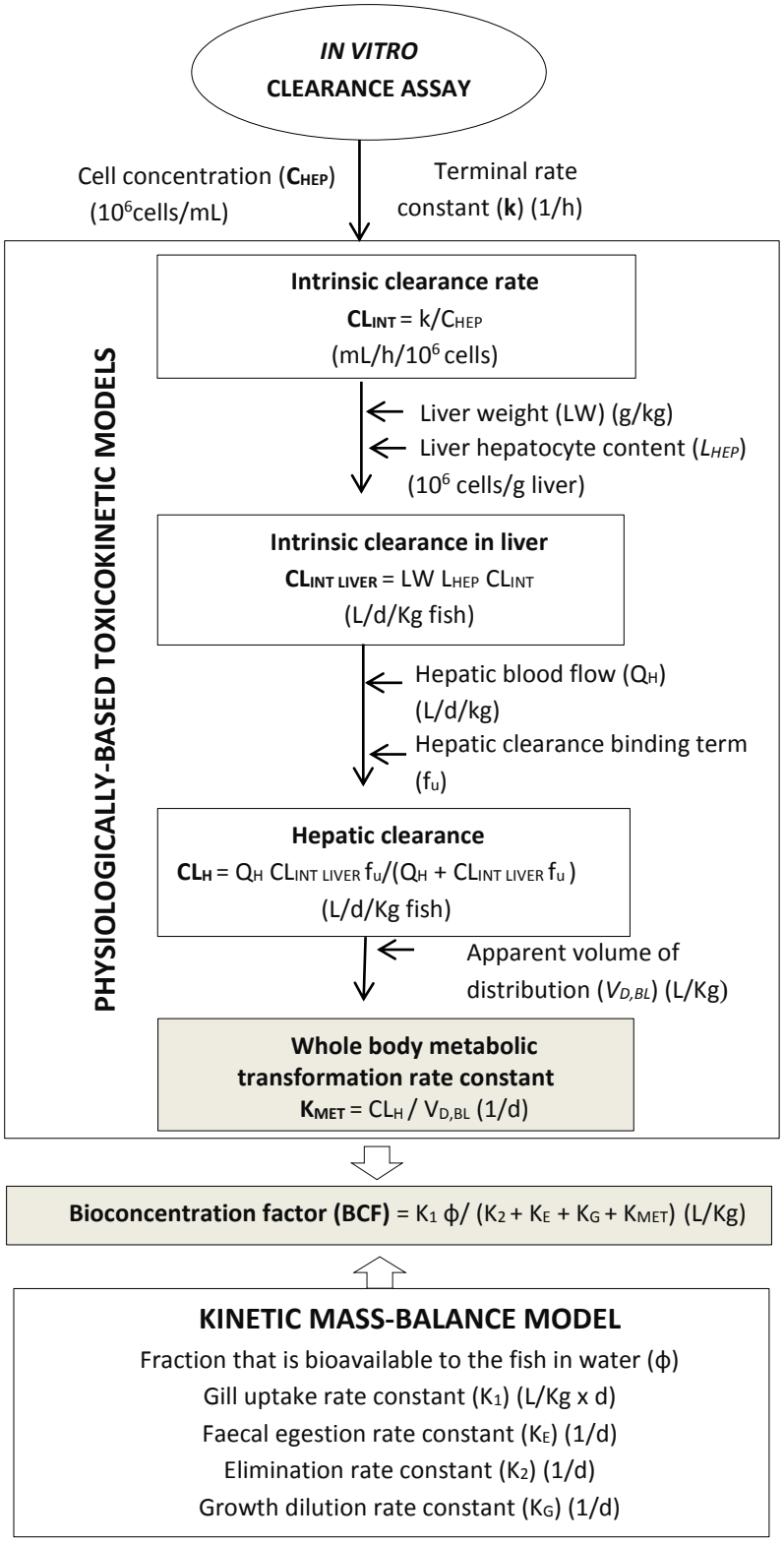


Figure 3.1 *In vitro-in vivo* data extrapolation process to calculate K_{met} and BCF.

Table 3.2 Parameters used in the *in vitro-in vivo* data extrapolation

Definition	Symbol	Units
Fraction of a chemical that is bioavailable to the fish in water	ϕ; FD	Unitless
Gill uptake rate constant	K_1	L/Kg x d
Gill chemical uptake efficiency	E_W	Unitless
Ventilation rate	G_V	L/d
DOC content	C_{DOC}	Kg/L
POC content	C_{POC}	Kg/L
Disequilibrium factor for DOC	D_{DOC}	Unitless
Disequilibrium factor for POC	D_{POC}	Unitless
DOC content binding constant	α_{DOC}	Unitless
POC content binding constant	α_{POC}	Unitless
Elimination rate constant	K_2	1/d
Lipid content in trout	v_{LT}	Kg/Kg
Non-lipid content in trout	v_{NT}	Kg/Kg
Water content in trout	v_{WT}	Kg/Kg
Non-lipid organic matter-octanol proportionality constant	β	Unitless
Lipid content of the organism	L_B	Unitless
Faecal egestion rate constant	K_E	1/d
Dietary chemical transfer efficiency	E_D	Unitless
Feeding rate	G_D	Kg/d
Temperature	T	°C
Growth dilution rate constant	K_G	1/d
Octanol-water partition coefficient	K_{ow}	Unitless
Wet weight of the organism	W_B	Kg
Whole body transformation rate	K_{MET}	1/d
Metabolic clearance of all organs	CL_T	L/d/Kg
Metabolic hepatic clearance	CL_H	L/d/Kg
Apparent volume of distribution	$V_{D,BL}$; V_d	L/kg
Total number of hepatocytes in the fish liver	L_{HEP}	10^6 cells/g liver
Organism-water partition coefficient	BCF_p	Unitless
Blood: water partition coefficient	$P_{b:w}$	Unitless
Fractional whole-body lipid content	v_{LWB}	Unitless
Depletion rate constant	k	1/h
Concentration of hepatocytes	C_{HEP}	10^6 cells/mL
In vitro intrinsic clearance	CL_{INT}	mL/h/10^6 cells
Intrinsic clearance in liver	$CL_{INT\ LIVER}$	L/d/Kg fish
Liver weight	LW	g/kg
Hepatic blood flow	Q_H	L/d/Kg
Liver blood flow as fraction of cardiac output	Q_{HFRAC}	Unitless
Cardiac output (with temperature adjusted)	Q_C	L/d/Kg
Fraction of blood flow through liver	LF	Unitless
Cardiac output	CO	mL/min/Kg
Hepatic clearance binding term	f_U	Unitless
Unbound fraction of test compound in blood plasma	$f_{U,P}$; $f_{u,b}$	Unitless
Unbound fraction of test compound in the clearance assay	$f_{U,HEP}$;$f_{u,h}$	Unitless

DOC: dissolved organic carbon; POC: Particulate organic carbon

Table 3.3 Parameters used by each of the PBTK calculation models considered

Parameter (Units)	Calculation models		
	Hans [37]	**Cowas-Ellsberry [38]**	**Nichols [117]**
LW (g/Kg)	12.7	8.5	15
L_{HEP} (10^6 cells/mL)	510	255	500
Q_H (L/d/Kg)	Q_H = LF CO CO = 34.5 ml/min/kg LF_{Hans} = 0.259 $LF_{Cowas-Ellsberry}$ = 0.20		$Q_H = Q_{HFRAC} Q_C$ Q_{HFRAC} = 0.259 Q_C = ((0.23T)-0.78) (W_B /500)^-0.1)24 T= 15 °C
f_U	$f_U = f_{u,b} / f_{u,h}$ $f_{u,b\text{-}Hans} = 1/ (10^{-0.613logkow-0.569} + 1)$ $f_{u,h\text{-}Hans} = 1/ (10^{-0.676logkow-2.215} + 1)$ $f_{u,b\text{-}Cowas\text{-}Ellsberry} = 1/ (10^{-0.605logkow-0.558} + 1)$ $f_{u,h\text{-}Cowas\text{-}Ellsberry} = 1/ (10^{-0.685logkow-2.237} + 1)$		$f_u = f_{U,P} / f_{U,HEP}$ $f_{u,p} = \nu_{WBL} / P_{BW}$ $f_{u,HEP} = 1/((C_{HEP} / 2) \times 10^{0.676logKow-2.215}+1)$ $P_{BW} = (10^{0.73logKow} \times 0.16) + \nu_{WBL}$ ν_{WBL} = 0.839
V_d (L/Kg)	$V_d = BCF_p / P_{b:w}$ $BCF_p = \nu_{LT} K_{ow} + \nu_{NT} \beta K_{ow} + \nu_{WT}$ β = 0.035; ν_{LT} = 0.1; ν_{NT} = 0.2; ν_{WT} = 0.7 $P_{b:w} = \nu_{LB} K_{ow} + \nu_{NB} \beta K_{ow} + \nu_{WB}$ β = 0.035; ν_{LB} = 0.014; ν_{NB} = 0.147; ν_{WB} = 0.839		$V_{D,BL} = BCF_p / P_{BW}$ $BCF_p = \nu_{LWB} K_{ow}$ ν_{LWB} = 0.05 $P_{BW} = (10^{0.73logKow} 0.16) + \nu_{WBL}$ ν_{WBL}= 0.839

As Figure 3.1 shows, K_{MET} calculated from the *in vitro* clearance rate was then incorporated into the mass balance BCF model provided by Nichols et al. [117], which was compared with the model of Arnot and Gobas [53,54] that is referred to as EPI-mass balance model in this study. Parameters for each of the mass balance models are shown in Table 3.4 to allow comparison. The BCF values of test compounds were estimated as a result of other physiological processes including the chemical uptake from the water via gills (K_1), elimination processes through gills (K_2), faecal egestion (K_E) and growth dilution (K_G). The mass balance BCF equation also takes into account the fraction of chemicals that are bioavailable to the fish in water (ϕ or FD).

Table 3.4 Parameters used by each of the mass balance models considered

Parameter (Units)	EPI-mass balance model [53,54]	Nichols mass balance model [117]
Φ; FD	$\phi = 1/(1+\alpha_{POC} D_{POC} C_{POC} K_{ow} + \alpha_{DOC} D_{DOC} C_{DOC} K_{ow})$ $\alpha_{POC} = 0.35; \alpha_{DOC} = 0.08;$ $D_{POC} = 1; D_{DOC} = 1$ $C_{POC} = 5 \times 10^{-7}$; $C_{DOC} = 5 \times 10^{-7}$	$FD = 1/(1+\alpha_{POC} C_{POC} K_{ow} + \alpha_{DOC} C_{DOC} K_{ow})$ $\alpha_{POC} = 0.35; \alpha_{DOC} = 0.08$ $C_{POC} = 4.6 \times 10^{-6}; C_{DOC} = 1 \times 10^{-6}$
K_1 (L/Kg d)	$K_1 = E_W G_V / W_B$ $E_W = (1.85 + (155/K_{ow}))^{-1}$ $G_V = 254.4$	$K_1 = 1/((0.01+1/K_{ow})W_B^{0.4})$
K_2 (1/d)	$K_2 = K_1 / BCF_p^b$ $BCF_p = \nu_{LT} K_{ow} + \nu_{NT} \beta K_{ow} + \nu_{WT}$ $\beta = 0.035; \nu_{LT} = 0.1; \nu_{NT} = 0.2; \nu_{WT} = 0.7$	$K_2 = K_1 / L_B K_{OW}$ $L_B = 0.05$
K_E (1/d)	$K_E = 0.125 G_D / E_D W_B$ $E_D = 3 \times 10^{-7} K_{OW} + 2$ $G_D = 0.022 W_B^{0.85} e^{(0.06T)}$ T=10 °C	$K_E = 0.125 G_D / E_D W_B$ $E_D = 5.1 \times 10^{-8} K_{OW} + 2$ $G_D = 0.022 W_B^{-0.15} e^{(0.06T)}$ T=15°C
K_G (1/d)	$K_G = 0.0005 W_B^{-0.2}$	$K_G = 0.0005 W_B^{-0.2}$

3.2.8 Predicting K_{MET} and BCF from EPI Suite v.4.1

Chemical structures of test compounds were recorded as SMILES strings and entered into the BCFBAF software v.3.01 (http://www.epa.gov) to calculate: 1) K_{MET} normalised to 1 Kg fish, which was based on the QSAR model developed by Arnot et al. [57]; and 2) BCF predicted from the mass balance model created by Arnot and Gobas [53,54] assuming no biotransformation and incorporating biotransformation rates calculated from biological HL; and 3) BCF predicted form the EPI-log K_{OW}-based model built by Meylan et al. [50].

3.3. Results and Discussion

There is an urgent need to develop, improve and validate non-animal methods to assess the bioaccumulation potential of chemicals in fish. Validated alternatives to *in vivo* studies are likely to be implemented into tiered strategies to reduce the number of fish required to assess chemical bioconcentration [30,75]. The integration of *in vivo*, *in vitro* and *in silico* methods is likely to allow for the successful development and standardisation of alternatives to *in vivo* bioaccumulation tests. Based on the integration of *in vivo-in vitro-in silico* data, this Chapter reviewed and compared the current alternatives to *in vivo* bioaccumulation in fish.

3.3.1 In Vitro Clearance Assay: Development of an Experimental Protocol

The first aim of this Chapter was to investigate the ability of the *in vitro* clearance assays using freshly isolated rainbow trout hepatocytes to generate clearance data for a set of reference compounds. As a standardised protocol is still to be developed, the experimental procedure taken in this study was based on the methodology carried out by Fay and colleagues [40]. A similar protocol was also learnt in a course undertaken in the 6th Society for Environmental Toxicology and Chemistry (SETAC) World congress held in 2012 in Berlin.

Before performing definitive assays, preliminary experiments were undertaken to develop an experimental protocol. One of the objectives of the preliminary experiments was the selection of appropriate test concentrations based on their first-order kinetics [35]. It should be stressed that there is a lack of consensus in the literature for the selection of test concentration. For example, the benchmark compound, BaP, has been tested at different concentrations including 4 µM [39], 2 µM [37], and 0.5 µM [40] using a cell concentration of 2 x 10^6 cells/mL. Therefore, the first experiment consisted of testing BaP at 4, 2, 1 and 0.5 µM in order to investigate its first order kinetics. Figure 3.2 shows the depletion curves for each of these test concentrations.

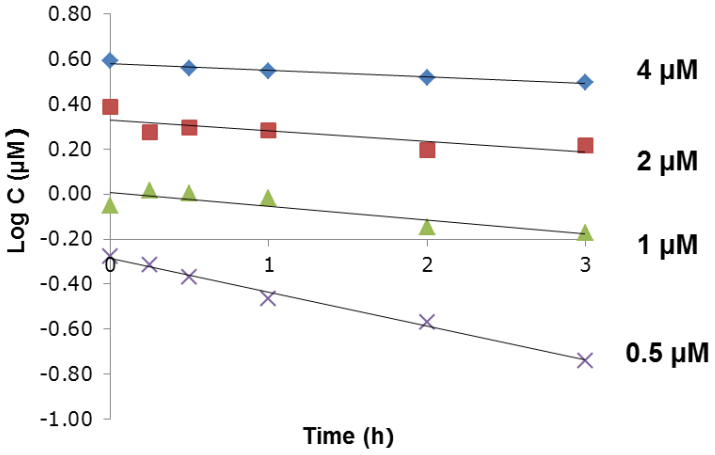


Figure 3.2. Depletion of BaP at 4, 2, 1 and 0.5 µM in freshly isolated hepatocytes. Log C: Logarithm of test concentration; BaP: benzo(a)pyrene.

Apparent higher depletion rates were obtained at lower concentrations, suggesting poor bioavailability of BaP and/or enzyme saturation at higher concentrations. Based on the same rationale, the other test chemicals listed in Table 3.1 were tested at 2, 1 and 0.5 µM to investigate their first order kinetics. A concentration of 4 µM was not considered further to reduce the final number of samples per test substance and to avoid possible toxic effects at this concentration. When testing chemicals with unknown metabolic biotransformation, a sample of BaP at 0.5 µM was considered as an additional control to ensure that the metabolic clearance occurred. Table 3.5 shows the CL_{INT} data at each of these test concentrations for the compounds examined. Attempts at measuring DBF, AT, PCN, PCB14 at some concentrations failed. As higher CL_{INT} values were obtained at the lowest concentration for the majority of chemicals, a concentration of 0.5 µM was selected. Additionally, by testing chemicals at 0.5 µM, concentrations above the aqueous solubility for hydrophobic chemicals were avoided.

Table 3.5 CL_{INT} data for test chemicals at 2, 1 and 0.5 µM

C	CL_{INT} (mL/h/10^6 cells)									
	CNB	**DCNB**	**DBF**	**TCA**	**AT**	**PCP**	**PCNB**	**PCB14**	**BaP**	**TBB**
2	0	0.017	Nd	0.049	0.096	0.063	Nd	0.037	0.064	0
1	0	0.000	0.086	0.068	0.131	0.046	Nd	0.012	0.048	0
0.5	0	0.015	0.040	0.093	Nd	0.053	Nd	Nd	0.182	0

Chemicals (AT: anthracene; BaP: benzo(a)pyrene; CNB: 1-chloro-2-nitrobenzene; DBF: dibenzofuran; DCNB: 1,3-dichloro-5-nitrobenzene; PCN: pentachloronitrobenzene; PCB14: 3,5-dichlorobiphenyl; PCP: pentachlorophenol; TBB: 1,2,4,5-tetrabromobenzene; TCA: 2,3,4-trichloroanisole); C: Nominal test concentration (µM); CL_{INT} = *In vitro* clearance rate; Nd: No data available.

Another crucial question to be determined for experimental testing was whether the depletion rates should be normalised to the loss of the examined chemicals in control samples or not. *In vitro* clearance rates are usually determined with no control normalisation, although some chemical losses over time have been reported in control samples, being attributed to the method used to boil hepatocytes [40]. According to the preliminary results, the depletion of the parent compound in denatured samples could be related to the intrinsic properties of test chemicals rather than the method used to boil hepatocytes [40]. For instance, Figure 3.3 shows the depletion curves in control (open circles) and treated (solid circles) samples for BaP and TBB determined at 0.5 µM. As can be seen, whilst the concentration of BaP in control samples was constant over time, a steady loss was observed for TBB. Such loss of TBB in denatured samples was also found at 1 and 2 µM, and therefore this compound was rejected for further experimentation. Consequently, CL_{INT} values were normalised with respect to controls based on Equation 3.2 to avoid the over-estimation of metabolic biotransformation for certain types of chemicals and also to take into account chemical losses due to abiotic degradation processes (e.g. volatilisation) and the adsorption of the test chemicals to plastic materials.

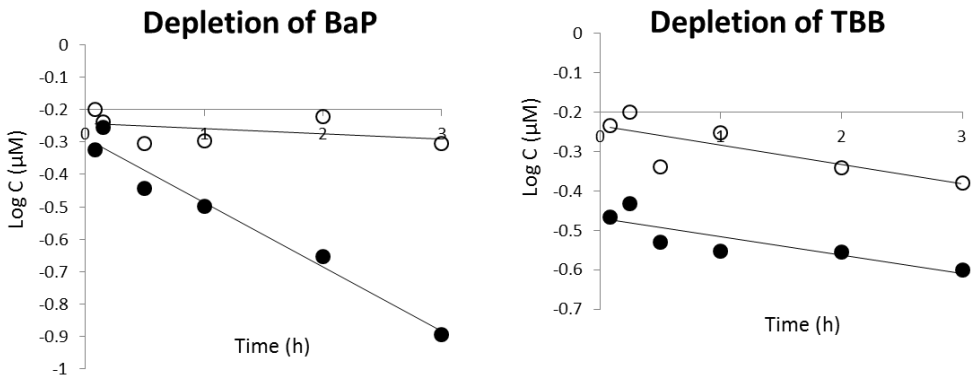


Figure 3.3 Depletion of BaP and TBB at 0.5 µM. Log C: Logarithm of test concentration (0.5 µM); BaP: benzo(a)pyrene; TBB: tetrabromobenzene; Solid circles: treated samples; Open circles: control samples.

Finally, to avoid the high rate of CV (50-70%) reported in the literature [37,39,40], the intrinsic clearance rates of test chemicals were calculated using cells from the same fish donor. This involved, however, the use of two replicates in order to test all chemicals at the same time.

3.3.2 In Vitro Clearance Assay: Results and Data Variability

Table 3.6 shows the average of the metabolic clearance (CL_{INT}) values for test compounds from two individual experiments. These results were in a good agreement with the CL_{INT} data determined at 0.5 µM in the preliminary experiments, with the exception of TCA (Table 3.5).

Table 3.6 CL_{INT} data measured from two experiments using freshly isolated hepatocytes

	CL_{INT} (mL/h/10^6 cells) mean, ±SD, CV								
Exp	**CNB**	**DCNB**	**DBF**	**TCA**	**AT**	**PCP**	**PCNB**	**PCB14**	**BaP**
1	0.001 ±0.001 141%	0.021 ±0.030 141%	0.015 ±0.001 2%	0.018 ±0.002 12%	0.061 ±0.009 14%	0.046 ±0.003 6%	0.848 ±0.086 10%	0.015 ±0.005 40%	0.104 ±0.00 76%
2	0.004 Nd	0.000 ±0.000	0.064 ±0.014 21%	0.041 ±0.013 31%	0.268 ±0.003 1%	0.038 ±0.021 55%	1.008 ±0.001 0.1%	0.011 ±0.015 141%	0.197 ±0.07 4%
Mean	**0.002**	**0.011**	**0.039**	**0.029**	**0.164**	**0.042**	**0.928**	**0.013**	**0.151**
SD	±0.002	±0.015	±0.035	±0.016	± 0.146	± 0.006	±0.113	± 0.003	±0.06
CV	110%	141%	88%	56%	89%	14%	12%	22%	6 44%

Chemicals (AT: anthracene; BaP: benzo(a)pyrene; CNB: 1-chloro-2-nitrobenzene; DBF: dibenzofuran; DCNB: 1,3-dichloro-5-nitrobenzene; PCN: pentachloronitrobenzene; PCB14: 3,5-dichlorobiphenyl; PCP: pentachlorophenol; TCA: 2,3,4-trichloroanisole); CL_{INT} = *In vitro* clearance rate; Exp: Experiment; Nd: No data available; CV: Coefficient of variation; SD: Standard deviation. Duplicates were used in each assay.

Only anthracene (AT), pentachloronitrobenzene (PCNB) and benzo(a)pyrene (BaP) showed significant metabolism (CL_{INT} > 0.15) contrasting with the low and negligible clearance rates obtained for the majority of the compounds. The low intrinsic clearance for 3,5,-dichlorobiphenyl (PCB14) supports the low *in vivo* values of K_{MET} reported for PCBs [100] as well as its good correlation with log K_{OW} (Table 3.1). In contrast, higher clearance rates were expected for pentachlorophenol (PCP) based on its reported *in vivo* metabolic data [120] and poor correlation with log K_{OW} (over-prediction) (Table 3.1). Whilst similar CL_{INT} data were determined for polycyclic aromatic hydrocarbons (see AT and BaP, Table 3.6), different intrinsic clearance rates were obtained for nitrochlorobenzenes, of which only PCNB was cleared at a high rate (Table 3.6). This may be attributable to a lack of passive diffusion for the hydrophilic nitrochlorobenzenes through the cell membrane [121]. The highest rate of clearance detected for PCNB supports the significant role of nitroreductases in the biotransformation of nitroaromatic compounds [88].

Coefficient of variation (CV) values for intra and inter assays are shown in Table 3.6. The CV values for non- and or poorly metabolised compounds such as CNB, DCNB and DBF should be treated with caution, as their mean and standard deviation (SD) values were similar to each other resulting in CVs > 100%. The CVs of inter-assays in this study ranged from 12 to 89%, slightly higher than those reported in recent studies [40]. Various factors could influence the experimental variability obtained such as differences in testing procedures, chemical analysis and enzymatic activities of the fish donors. The latter could be influenced by gender, age and diet [29]. The gender of fish donor was unlikely to contribute to the data variability since non-sexually mature fish donors were used. In addition, no metabolic gender differences from *in vitro* conditions have been reported for rainbow trout [122].

The age of the animal (expressed by differences in body weight) is likely to be a potential factor affecting CL_{INT} variability, as a good correlation ($r^2 = 0.72$) was found between the CL_{INT} values for BaP determined at 0.5 µM and the weight of the fish donors (see Figure 3.4). Remarkably, such linear regression could be improved ($r^2 = 0.99$) when an apparent outlier was removed. It should be noted that CL_{INT} data for BaP were taken from both preliminary and definitive experiments. This finding agrees with the higher metabolic rates reported in fat cells isolated from young rats compared to those determined in old donors [123]. Although the relationship between whole body metabolic rates and body mass has been investigated widely in the literature [124], little is known concerning such relationships at the cellular level, thus representing a topic of future research.

It should be added that season could also influence the metabolic capabilities of the fish donors [125,126]. The potential influence of season on CL_{INT} data variability was not explored in this study due to the fact experiments were conducted within the same period (April to June in 2013).

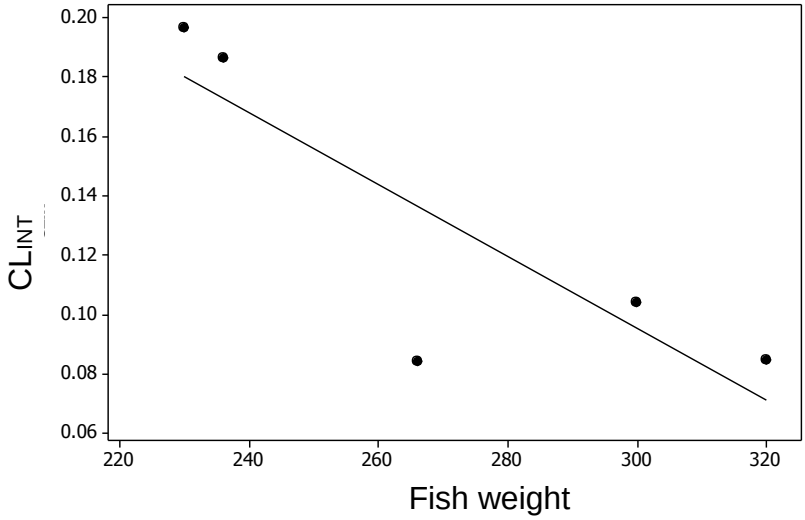


Figure 3.4 CL_{INT} data versus the fish donor weight for BaP at 0.5 µM

3.3.3 In Vitro Clearance Assay: Challenges and Future Perspectives

Table 3.7 lists the two main challenges found for the *in vitro* clearance assay using freshly isolated rainbow trout hepatocytes.

Table 3.7 Challenges of the clearance assay using rainbow trout hepatocytes

Challenges and proposed solutions	Drawbacks
1. Low sensitivity to poorly metabolised chemicals	
- Use young donor animals (< 200 g)	- Difficulties in cell isolation procedure - More fish will be needed
- Increase cell concentration (> 2 x 10^6 cells/mL)	- More fish will be needed
- Increase temperature (> 11 °C)	- Decrease of cell viability - No representative for *in vivo* conditions
2. Time consuming procedure and low reproducibility	
- Use multi-channel pipettes, well-plates, LCMS analysis	- Establishment of a new protocol

The difficulty in measuring low biotransformation rates could be explained partly due to the fact that *in vitro* biotransformation rates in fish have been determined to be much slower than those reported for rat hepatocytes (between 8.3 to 315 fold of difference) [37]. It is likely that for this reason, low or non-clearance rates were obtained for the majority of the test chemicals in this study supporting similar results obtained with liver S9 fractions [127].

Nonetheless, future clearance assays based on isolated hepatocytes using young fish donor (< 200 g) and/or using cell concentrations higher than 2 x 10^6 cells/mL may represent alternatives to increase the sensitivity of measuring the depletion rates of poorly metabolised compounds.

Another challenge for the clearance *in vitro* assay was the use of a time consuming experimental procedure due to the several steps involved. For example, the incubation was conducted in borosilicate glass tubes, then the reaction was terminated in Eppendorf tubes and eventually subsamples were transferred to glass vials for chemical analysis. It is expected that future technological improvements such as the integration of the incubation with the chemical analysis in the same support will allow for testing a greater number of compounds.

Despite these drawbacks, *in vitro* clearance assays using fish hepatocytes may represent a promising alternative for fish ecotoxicology. For instance, the CL_{INT} value for BaP in this investigation (CL_{INT} = 0.151, Table 3.6) was comparable to those reported using cryopreserved trout hepatocytes (CL_{INT} = 0.214) determined at the same concentration [40], suggesting the reliability of such assays to study xenobiotic biotransformation. Moreover, compared with subcellular assays (e.g. S9 and microsomes), isolated hepatocytes have showed higher values for CL_{INT} [36] and metabolic activity [128], making them better candidates for routine screening in risk assessment. In addition, a promising benefit of the standardisation and validation of the clearance assay with hepatocytes would be the investigation of the differences between metabolic activities reported within and among fish species in the literature [129].

A step-wise selection of test chemicals is the key to study the biotransformation of chemicals. In order to investigate the impact of metabolic biotransformation on chemical bioconcentration, the selection of test chemicals should be based on the relationship between their predicted maximal log BCF and experimental values. It was expected in this

study that compounds with high bioconcentration potential (W,U1), i.e. similar values to their predicted maximal log BCF values, would show low values for CL_{INT} values and vice versa. This was observed for the majority of chemicals with the exception of pentachlorophenol (PCP) and 1,2,4,5-tetrabromobenzene (TBB), whose other chemicals properties could play a role in their limited BCF.

Amongst all chemical properties that could affect the accumulation of a compound, the effect of molecular properties on BCF has been extensity investigated [81,82,130,131]. As a consequence, a list of cut-off values for molecular weight (MW) and maximum molecular diameter (D_{max}) have been proposed as indicators of low bioaccumulation. In particular, values of 700 g/mol and 1.7 nm have been proposed as cut-off values for MW and D_{max} respectively in bioaccumulation assessment [132]. These molecular properties are unlikely to contribute to the low *in vivo* bioaccumulation reported for PCP and TBB based on these cut-off values. A high volatility in solution, expressed by a logarithm of Henry's Law Constant (log HLC), is also unlikely to contribute to the limited *in vivo* bioconcentration for PCP and TBB, as their log HLC are lower than the cut-off value proposed as indicator of high volatility in solution, log HLC > 3 (see Table S1)[114]. An exposure pH higher than 8.5 used for *in vivo* testing could explain the low *in vivo* BCF value for PCP [133,134]. It should be emphasised here that molecular properties, log HLC, and the prediction of metabolic pathways should be taken into account when selecting potential metabolised compounds whose *in vivo* BCF values were over-predicted by log K_{OW}. For example, no probable biotransformation pathways were predicted for PCP and TBB (see Table 3.1), suggesting that this piece of information should be considered to provide a high confidence to select well-metabolised compounds.

3.3.4 Comparison of Alternatives to Predict K_{MET}

The second aim of this study was to investigate current alternative methods in terms of their ability to predict whole body biotransformation rate (K_{MET}) in 1 Kg fish. The K_{MET} values for test compounds were calculated using the following approaches: 1) the QSAR model built by Arnot et al [57], which is referred to as EPI-QSAR in this study; and 2) Physiologically-Based ToxicoKinetic (PBTK) models that involved the extrapolation of the generated *in vitro* clearance data (Table 3.6) to an estimated whole body metabolism in liver. In such models, K_{MET} is defined by the ratio between the hepatic clearance (CL_H) and the apparent volume of distribution ($V_{D,BL}$) (Figure 3.1). A comparison of different PBTK calculation models that have been used for fish [37,38,117] was also conducted in this study to investigate the physiological parameters affecting K_{MET}. Table 3.8 shows the K_{MET} data predicted according to each of these non-animal methods. Figure 3.5 shows the plot of predicted K_{MET} data versus test compound according to the model employed.

Table 3.8 Comparison of predicted K_{MET} values for test chemicals

Chemical name	Log Kow	K_{MET} (d^{-1}) (1 Kg)			
		PBTK Nichols [117]	PBTK Hans [37]	PBTK Cowan-Ellsberry [38]	EPI-QSAR [57]
CNB	2.46	0.027	0.008	0.002	0.398
DCNB	3.10	0.047	0.024	0.008	0.206
DBF	3.71	0.068	0.060	0.028	0.266
TCA	4.01	0.035	0.041	0.021	0.012
AT	4.35	0.136	0.208	0.115	0.086
PCP	4.74	0.025	0.056	0.030	0.099
PCNB	5.03	0.380	0.825	0.523	0.059
PCB14	5.05	0.006	0.018	0.009	0.010
BaP	6.11	0.030	0.211	0.106	0.243

AT: Anthracene; BaP: Benzo(a)pyrene; CNB: 1-Chloro-2-nitrobenzene; DBF: Dibenzofuran; DCNB: 1,3-Dichloro-5-nitrobenzene; PCN: Pentachloronitrobenzene; PCB14: 3,5-Dichlorobiphenyl; PCP: Pentachlorophenol; TCA: 2,3,4-Trichloroanisole; K_{MET} : Whole body biotransformation rate (d^{-1}).

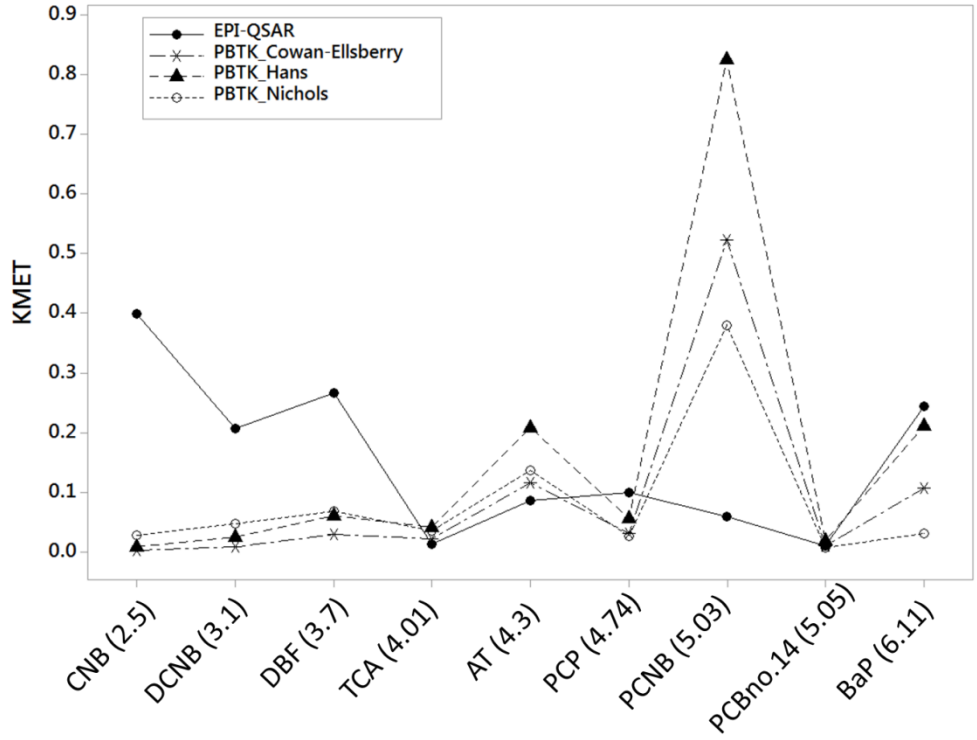


Figure 3.5 Predicted K_{MET} data vs test chemical. AT: Anthracene; BaP: Benzo(a)pyrene; CNB: 1-Chloro-2-nitrobenzene; DBF: Dibenzofuran; DCNB: 1,3-Dichloro-5-nitrobenzene; PCN: Pentachloronitrobenzene; PCB14: 3,5-Dichlorobiphenyl; PCP: Pentachlorophenol; TCA: 2,3,4-Trichloroanisole, K_{MET}: Whole body biotransformation rate (d^{-1}).

From the comparison of K_{MET} data, a number of observations are apparent. The first is that for relatively hydrophilic compounds (log K_{OW} < 3), significantly higher K_{MET} values were predicted using the EPI-QSAR model than those predicted from PBTK models that showed good similarities in predictions (see Figure 3.5). This could be attributable to the data that were used for the development of the EPI-QSAR model. In particular, due to the lack of reported measured metabolic rates in fish, the EPI-QSAR model was built from a database of fish biotransformation rates that were estimated from available bioaccumulation data by re-arranging Equation 3.3 [84].

$$\text{Bioconcentration factor (BCF)} = K_1\,\phi / (K_2 + K_E + K_G + K_{MET})\ \text{(L/Kg)} \qquad (3.3)$$

In particular, when whole body BCF values were available, the K_{MET} was calculated as:

$$K_{MET} = (K_1 \phi/BCF) – (K_2 + K_E + K_G) \qquad (3.4)$$

And if the total elimination rate constant (K_T) was reported, K_{MET} was calculated as follows:

$$K_{MET} = K_T - (K_2 + K_E + K_G) \qquad (3.5)$$

Whole body biological HL(d) was also converted to a total elimination rate constant as follows:

$$K_T = \ln(2)/HL \qquad (3.6)$$

In Equations 3.4 and 3.5, data for K_1, ϕ, K_2 , K_E , K_G were based on predictions [84]. However, this approach did not correct for the effect of hydrophobicity on measured BCF and K_T, both being log K_{OW}-dependent. For example, it is well-known that the most important route of elimination for hydrophilic chemicals is across the gill [121]. As a consequence, greater rates of elimination have been reported for such chemicals than for more hydrophobic chemicals (log K_{OW} > 3), as illustrated in Figure 3.6 for various neutral (non-ionisable) aromatic compounds [135]. Therefore, when using Equations 3.4 and 3.5, high K_{MET} values are estimated for chemicals with log K_{OW} < 3 by the EPI-QSAR model as a result of their high measured elimination rates and low BCF values, and not as a consequence of significant metabolism.

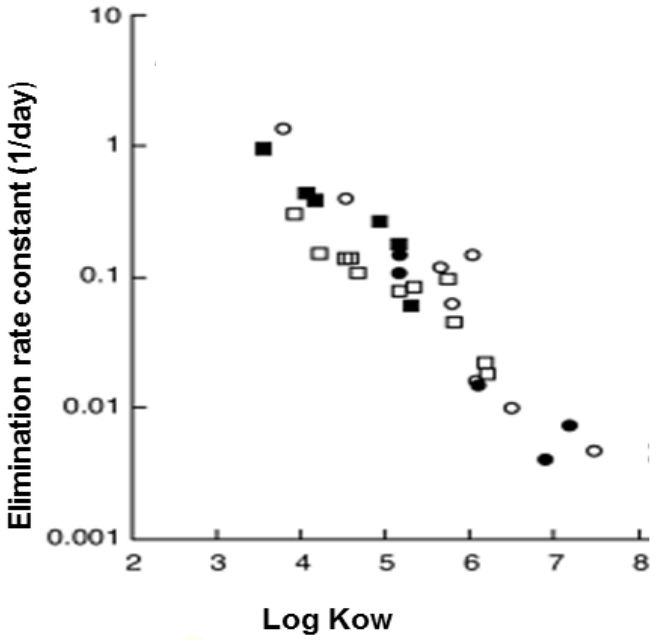


Figure 3.6 Dependence of elimination and branchial uptake rate constants on log K_{OW} for neutral compounds adapted from Erickson and McKim study [135]. Solid squares: chlorinated benzenes; solid circles: chlorinated benzenes/biphenyls; open squares: chlorinated benzenes/napthalenes; open circles: brominated benzenes/biphenyls and chlorinated biphenyls.

The second observation from the comparison of K_{MET} data is the low metabolic rates predicted for hydrophobic chemicals by the Nichols PBTK approach with respect to the other calculation approaches (Table 3.8). Conversely, higher metabolic rates are predicted by the Nichols model for chemicals with log K_{OW} < 3 than by the other models. It is noted that benzo(a)pyene (BaP) and 1-chloro-2-nitrobenzene (CNB), whose reported CL_{INT} data were 0.151 and 0.002 respectively, showed similar K_{MET} values (Table 3.8). In order to investigate this more thoroughly, physiological parameters calculated using each of the PBTK models were compared for BaP and CNB (Table 3.9).

Table 3.9 Parameter comparison for 1-chloro-2-nitrobenzene (CNB) and benzo(a)pyrene (BaP) for each of the calculation models

Parameter	Chemical	Nichols model [117]	Hans model [37]	Cowan-Ellsberry model [38]
fu	**CNB**	0.099	0.132	0.132
	BaP	0.015	0.055	0.055
$CL_{INT\ LIVER}$ (L/d/Kg fish)	**CNB**	0.360	0.311	0.104
	BaP	27	23.473	7.85
Q_H (L/d/Kg)	**CNB**	24.542	12.680	9.792
	BaP	24.542	12.680	9.792
CL_H (L/d/Kg)	**CNB**	0.035	0.041	0.0082
	BaP	0.402	1.182	0.592
V_d (L/Kg)	**CNB**	1.33	4.9617	4.9617
	BaP	13.95	5.588	5.588

As can be seen, different values for the hepatic clearance binding term (f_u), intrinsic clearance in liver ($CL_{INT\ LIVER}$), hepatic clearance (CL_H) and apparent volume of distribution ($V_{D,BL}$) were calculated for CNB and BaP. This is due to the dependence of these variables on log K_{OW} (see equations listed in Table 3.3). Of these, PBTK models are highly sensitive to changes in $V_{D,BL}$, as it appears in the denominator of K_{MET}. The value of $V_{D,BL}$ is calculated as the ratio of BCF_p/P_{BW}. P_{BW} is termed as the fractional water content of blood; BCF_p is the organism-water partition coefficient. It should be noted that whilst Hans et al. [37] and Cowan-Ellsberry et al. [38] used the same equations to calculate BCF_p and P_{BW} (see Table 3.3), Nichols et al. [117] employed the equation given by Fitzsimmons et al. [136] to calculate P_{BW}, and a simplified equation for BCF_p. The analysis of the data revealed that by using the Nichols model, much lower and higher $V_{D,BL}$ values were obtained for BaP and CNB respectively than the other approaches (see Table 3.9), which resulted in similar K_{MET} values for both compounds (Table 3.8). The parameter f_u also has a strong influence on K_{MET} [117,37]. f_u is the ratio between the unbound fraction of test compound in blood plasma ($f_{u,h}$ o $f_{U,P}$) and the unbound fraction of test compound in the *in vitro* clearance assay ($f_{U,P}$ o $f_{U,HEP}$) (Table 3.3). To correct for the cell

concentration in the *in vitro* clearance assay, Nichols et al. [117] incorporated the cell concentration (C_{HEP}) into the $f_{u,h}$ equation given by Hans et al. [37] (see Table 3.3.). Nichols also proposed the use of the equation for P_{BW} given by Fitzsimmons et al. [136] to calculate $f_{U,P}$. By using such equations, however, a lower value of f_u was determined for BaP than the values predicted by Hans and Cowan-Ellsberry models (see Table 3.9), contributing to its low K_{MET}.

The final observation is related to the good concordance of predictions for 2,3,4-trichloroanisole (TCA) and 3,5-dichlorobiphenyl (PCB14) (Figure 3.5) made by all alternative models. This finding suggests a high level of confidence to identify low and non-metabolised chemicals with log K_{OW} > 4 by using either the EPI-QSAR or any of the PBTK models.

3.3.5 Comparison of Alternatives to Predict BCF

The final aim of this Chapter was the comparison of the most common non-animal models used in risk assessment to predict BCF of chemicals. Values of BCF for test chemicals were calculated according to the following methods: 1) the log K_{OW}-based model developed by Meylan et al. [50] that is referred to as the EPI-log K_{OW}-based model in this study; 2) the kinetic mass balance of Arnot and Gobas [53,54] (Table 3.4), assuming no biotransformation and incorporating biotransformation rates calculated from biological half-lives and referred to as the EPI-mass balance with K_{MET} = 0 and K_{MET} (HL) respectively; and 3) the recent mass balance provided by Nichols et al. [117] (Table 3.4), referred to as the Nichols mass balance model, using two different fish masses: 10 g and 1 Kg (Table 3.4). Nichols et al. [117] accounted for the effect of metabolism by incorporating the K_{MET} data estimated from the *in vitro* clearance assay. Table 3.10 shows the predicted BCF values for test compounds according to each of these predictive BCF models.

Table 3.10 Comparison of the predicted BCF values for the examined chemicals using different methods

Chemical name (log K_{OW})	*In vivo* BCF (L/Kg)	Predicted BCF (L/kg)				
		Nichols mass balance model		EpiSuite (BCFBAF v.3.01)		
		10 g (*in vitro* K_{MET})	1 kg (*in vitro* K_{MET})	EPI-mass balance with K_{MET} (HL)	EPI-mass balance K_{MET} (HL) =0	EPI-Kow- based model
CNB (2.46)	68	14 (0.027)	14 (0.027)	15 (2.629)	19	14
DCNB (3.10)	170	62 (0.047)	61 (0.047)	121 (0.186)	132	51
DBF (3.71)	1490	248 (0.068)	216 (0.068)	1179 (0.031)	1348	243
TCA (4.01)	1778	491 (0.035)	424 (0.035)	547 (0.028)	578	136
AT (4.35)	2012	879 (0.136)	433 (0.136)	1121 (0.155)	2743	401
PCP (4.74)	286	2330 (0.025)	1500 (0.025)	254 (1.109)	9569	1110
PCNB (5.03)	590	1161 (0.380)	245 (0.380)	270 (1.018)	4046	535
PCB14 (5.05)	5888	4691 (0.006)	3457 (0.006)	3790 (0.054)	14140	6645
BaP (6.11)	Nd	7321 (0.030)	1560 (0.030)	364 (0.622)	21010	5147

AT: anthracene; BaP: benzo(a)pyrene; BCF: Bioconcentration Factor (Litres/ Kg fish); CNB: 1-chloro-2-nitrobenzene; DBF: dibenzofuran; DCNB: 1,3-dichloro-5-nitrobenzene; HL: Half-lives; K_{MET}: Whole body metabolic biotransformation rate (d^{-1}) in parenthesis; PCN: pentachloronitrobenzene; PCB14: 3,5-dichlorobiphenyl; PCP: pentachlorophenol; TCA: 2,3,4-trichloroanisole.

It should be noted that the first kinetic mass balance model for aquatic chemical bioaccumulation was developed by Arnot and Gobas [52,53]. This was normalised for a fish of 1 Kg and integrated into the EPI Suite software (http://www.epa.gov). Recently, Nichols et al. [117] defined such a mass balance BCF model for 10 g fish based on the rationale that the majority of the *in vivo* BCF data have been determined in small fish or juveniles of large species. However, such a model assumed that the biotransformation rates do not change

with fish mass, which means that the *in vitro* K_{MET} data predicted for 1 Kg fish are incorporated into a BCF model normalised for 10 g fish. To investigate the effect of K_{MET} on chemical bioconcentration according to the fish weight, both 10 g and 1 Kg were used as inputs for modelling using the same K_{MET} value determined by the Nichols PBTK model (Table 3.8). Results show that for chemicals with log K_{OW} < 4, similar BCF values were predicted regardless of the fish mass used (Table 3.10). This finding is in good agreement with previous predictions that showed high metabolic rates have little impact on the bioconcentration for hydrophilic compounds [30,84]. In contrast, for more hydrophobic compounds, higher BCF values were predicted for a 10 g fish than 1 Kg (Table 3.10), supporting the concept of strong influence of metabolic rates on the BCF for hydrophobic chemicals [30,84]. As there is empirical evidence that the metabolic rates scale with the body mass of the organism [57,124], such differences in predicted BCF values depending on the fish weight were a result of the lack of K_{MET} transformation used for a 10 g BCF. Therefore, *in vitro* K_{MET} data should be normalised to the same fish mass used in the Nichols mass balance model to correct for the effect of metabolism on BCF. For instance, this can be performed by using the following equation provided by Arnot et al. [57]:

$$K_{MET,0.10} = K_{MET,1} (W_X/W_N) - 0.25 \quad (3.7)$$

Where W_N and W_X refers to 1 and 0.01 Kg respectively according to this example. K_{MET} is the whole body metabolic rate (1/d). The term $K_{MET,1}$ is the K_{MET} predicted for a 1 Kg fish calculated from the Nichols PBTK calculation model. The term $K_{MET,0.10}$ is the K_{MET} predicted for a fish of 0.01 Kg weight.

Nichols and colleagues [117] also employed different factors to calculate the following parameters: 1) the fraction that is bioavailable to the fish in water (ϕ), which was termed FD; 2) the gill uptake rate constant (K_1); and 3) elimination rate constant (K_2) (Table 3.4). Based on experimental measurements, new values for the dissolved organic carbon content (C_{DOC})

and the particulate organic carbon content (C_{POC}) are recommended for the calculation of ϕ/FD. Based on the comparison performed for PBTK models (Table 3.9), values of ϕ, K_1 and K_2 for CNB and BaP were compared according to the mass balance model used, assuming no metabolism (K_{MET} = 0) (Table 3.11). The analysis of the data showed that lower values of ϕ and K_1 for CNB and BaP were obtained by the Nichols mass balance model with respect to the EPI-mass balance model, resulting in a strong impact on the outcome of BCF for hydrophobic compounds such as BaP (Table 3.11). It should be noted that a value of BCF of 11469 for BaP is more "realistic" than a value of 21010 based on the evidence of the lack of *in vivo* BCF values for the chemical compiled in Chapter 2 higher than 21000. This finding indicates that the new parameters used for ϕ calculation by the Nichols mass balance model improve the BCF predictions for non-metabolised hydrophobic compounds.

Table 3.11 Parameters comparison for 1-chloro-2-nitrobenzene (CNB) and benzo(a)pyrene (BaP) for each of the mass balance models assuming no metabolism (K_{MET} = 0)

Parameter	Chemical	Nichols mass balance model	EPI-mass balance model
ϕ/FD	**CNB**	0.99	0.99
	BaP	0.52	0.78
K_1 (L/Kg/d)	**CNB**	74.25	106.55
	BaP	99.99	137.50
K_2 (1/d)	**CNB**	5.15	3.37
	BaP	< 0.01	< 0.01
Predicted BCF (L/kg fish)	**CNB**	14	19
	BaP	11469	21010

An interesting observation is apparent from the comparison of predicted BCF data (Table 3.10). Generally, BCF values predicted by the EPI-log K_{OW}-based model were lower than those made by EPI- and Nichols mass balance models (Table 3.10). This may occur because the EPI-log K_{OW}-based model was built from BCF values that were measured in warm water fish species [50], mainly for fathead minnow, and the majority of the reported data in this study

were determined in rainbow trout, a cold species. From a kinetic processes scenario, warm water fish species are likely to achieve the steady state earlier than cold water species, which may result in lower BCFs than those reported for cold water fish [121]. A similar finding was found when comparing the common chemicals with data for common carp and rainbow trout in the creation of the reference list (Chapter 2).

Finally, the analysis of the relationship between *in vivo* and predicted log BCF data, according to each model, revealed that the EPI-mass balance model incorporating K_{MET} made better predictions ($r^2 = 0.92$) than the other predictive approaches ($r^2 < 0.60$) (as is shown in Figure 3.7). Interestingly, the reported K_{MET} data from such a model were determined, and not based on predictions as was expected initially. K_{MET} data calculated based on experimental HLs are shown in parenthesis in Table 3.10, and differed from the K_{MET} predicted by EPI-QSAR model developed by Arnot et al. [57] (see Table 3.8). It should be stressed that no information was found in the EPI Suite Software (http://www.epa.gov) related to the original source of experimental HL data and the method followed to transform the experimental HL into rate constants. It is hypothesised, however, that these experimental HL were measured in the whole body fish and then transformed into metabolic rate constants following Equations 3.5 and 3.6.

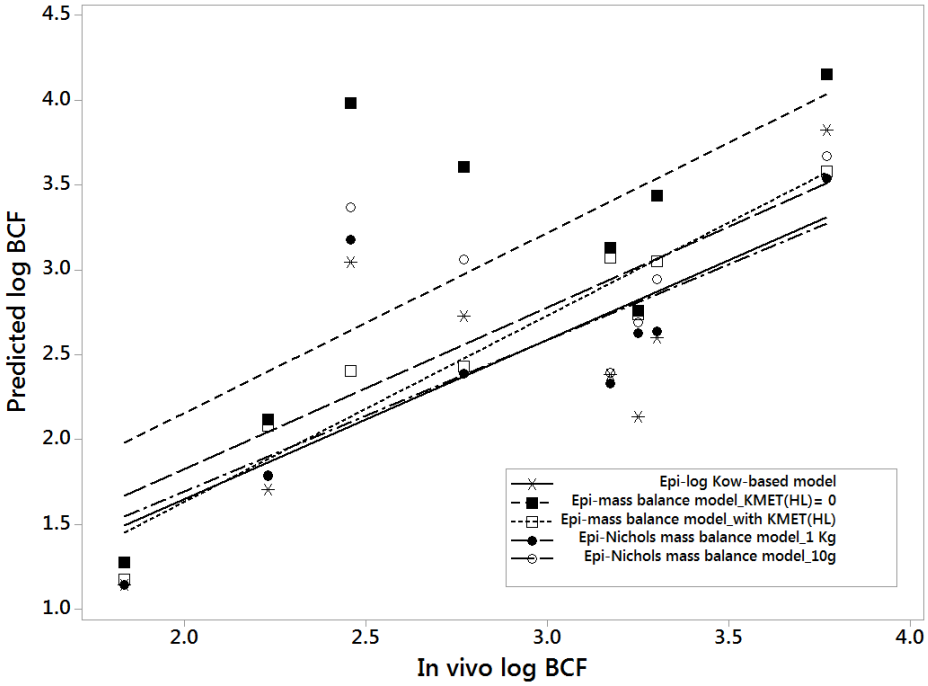


Figure 3.7 Predicted log BCF vs *in vivo* log BCF for each predictive BCF models investigated. The lines plotted represent the QSAR as stated in the figure legend.

3.3.6 Alternatives for K_{MET} and BCF: Challenges and Future Perspectives

The challenge of the *in silico* K_{MET} models reviewed in this study was to obtain reliable data for metabolic rates of test chemicals. This could be explained as predictive models for K_{MET} were not developed from *in vivo* metabolic data, since only a few studies have been conducted so far (see section 2.3.3 for more details). In order to build reliable K_{MET} models, more *in vivo* studies will be required to measure the whole body metabolic rates in fish for chemicals covering a broad range of log K_{OW}. Nonetheless, the whole body metabolic rates may follow a similar trend to those reported between branchial absorption rates and log K_{OW} for guppy [137], as shown in Figure 3.8. This is because a compound needs to be absorbed and distributed to the target tissue before being metabolised. A question remains, however, concerning the metabolic clearances of hydrophilic chemicals measured in *in vivo* conditions. Based on the *in vitro* results obtained (Table 3.6), very low metabolic rates are expected for these compounds. It should be noted that obtaining reliable K_{MET} data will be also relevant

for mass balance BCF models, since the analysis showed that even low variations of metabolic activity had a strong impact on BCF for hydrophobic test chemicals.

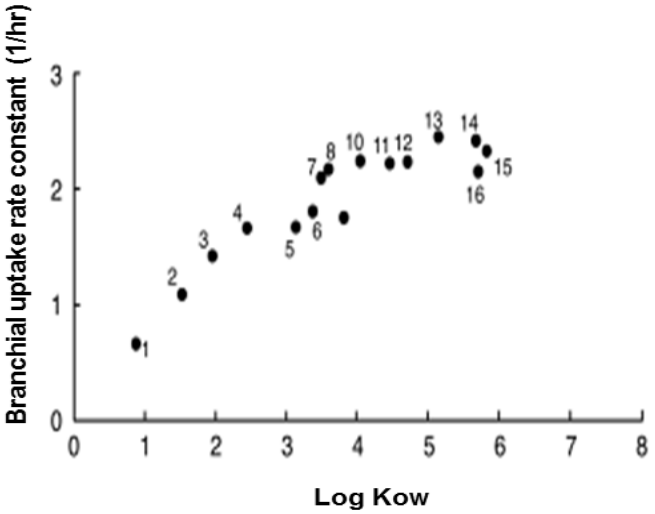


Figure 3.8 Relationship between branchial uptake rate constant in guppies and log K_{OW} adapted from Saarikoski et al. [137]. 1: Butyric acid; 2: Phenol; 3: Benzoic aicid; 4: 4-Phenylbutyric acid; 5:2,4-Dichlorophenol; 6: 2-sec Butyl-4,6-dinitrophenol; 7: 3,4-Dichlorobenzoic acid; 8: 2,6-Dibromo-4-nitrophenol; 9: 2,4,5-Trichlorophenol; 10: 2,4,6-Trichlorophenol; 11: 2,3,4,6-Tetrachlorophenol; 12: Tetrachloroverathrol; 13: Pentachlorophenol; 14: Pentachloroanisol; 15: 2,4,6-Trichloro-5-phenylphenol; 16: DDT.

Different challenges were found depending on the *in silico* BCF models evaluated. For example, the main challenge of log K_{OW}-based models was the comparison of BCF data for different fish species. It is expected that the development of log K_{OW}-based models for single fish species, such as those developed for rainbow trout and common carp in Chapter 2, will allow for an accurate BCF data comparison. The main challenge of mass balance models was associated with the use of different fish weights. For instance in this study, clearance data determined using fish donors with weights ranging from 220 to 320 g were incorporated into a PBTK model design for a fish of 1 Kg to predict K_{MET}, and such predicted K_{MET} data were then integrated into a mass balance model normalised to a 10 g fish for BCF prediction. The extrapolation factors of both PBTK and mass balance models should be normalised to a 1 Kg to avoid the uncertainty associated with the fish mass. This would also involve the data

normalisation of the *in vitro* CL_{INT} values determined for a specific weight of the fish donor to 1 Kg. In order to do that, more effort from experimental approaches will be required with a two-fold purpose: 1) to corroborate the correlation between CL_{INT} and the weight of the fish donors observed for BaP in this study (Figure 3.4); and 2) to develop a QSAR model to estimate the CL_{INT} value in a 1 Kg fish for a specific compound.

3.4 Conclusion

The potential of a compound to accumulate in aquatic organisms is usually expressed by bioconcentration factor (BCF). Traditionally, data for BCF for organic chemicals were determined in the whole fish according to the OECD TG 305 [31]. Due to metabolism reducing chemical bioaccumulation significantly, the potential of a compound to undergo metabolism has been identified as another key measurement in risk assessment. Over the last decade, there has been an increase in the number of alternatives proposed to *in vivo* testing in fish, necessitating a comprehensive analysis of their potential use in chemical bioaccumulation assessment. This Chapter has accordingly reviewed and compared the most common alternatives including an *in vitro* clearance assay using fresh hepatocytes and *in silico* models to predict K_{MET} and BCF of chemicals. Results showed that the *in vitro* clearance assay using freshly isolated trout hepatocytes represents a powerful tool to identify readily metabolic chemicals as well as to investigate the impact of significant metabolism processes on their maximal BCF. A high variability in K_{MET} predictions was obtained when different models were compared, suggesting that further developments and improvements are needed. With regards to BCF models, a kinetic mass balance model incorporating K_{MET} predicted based on experimental HL represents a potential surrogate to *in vivo* bioaccumulation studies with respect to traditional log K_{OW}-based and a mass balance model defined for a 10 g fish. As a consequence of this work, an in-depth analysis of the current challenges and future perspectives of the examined alternative methods to *in vivo* testing was also provided.

Chapter 3 has reviewed and compared the most common alternative methods used for the estimation of BCF and K_{MET} for a set of reference compounds selected from the reference list established in Chapter 2. Compared to toxicity tests, the assessment of chemical bioaccumulation is more complex as diverse processes are involved, i.e. Absorption, Distribution, Metabolism and Excretion (ADME). Therefore, considerable work was undertaken in this thesis towards the development of alternative methods for chemical bioaccumulation. Non-animal methods for aquatic toxicity will be covered in Chapter 4, by conducting a mechanistic analysis of the toxicity of cyclic compounds. Chapter 4 also highlights the importance of integrating different alternative methods to provide a high level of confidence regarding the toxic mechanism of cyclic compounds.

Chapter 4. *In silico* Mechanistic Analysis of the Toxicity of Cyclic Compounds[2]

4.1. Introduction

Under the Registration, Evaluation, Authorisation and restriction of Chemical substances (REACH) legislation, animal testing might be required for the registration of about 35,000 chemicals produced, used or imported in the European Union for which sufficient toxicological information is lacking [13]. REACH has accordingly advocated the use of alternative methods to *in vivo* toxicity tests to assess the toxicity of chemicals. Some of the alternatives to *in vivo* tests in fish are based on the use of aquatic invertebrates to assess the toxicity of chemicals. Amongst all, the growth inhibition assay with the ciliated protozoan *Tetrahymena pyriformis* represents a potential fish surrogate due to the good correlation between *T. pyriformis* and fish acute toxicities [138]. In addition, toxicity tests in aquatic surrogates are frequently used as part of Integrated Testing Strategies (ITS) to prioritise chemicals for further fish toxicity testing [74].

Non-testing strategies rely on the use of Quantitative Structure-Activity Relationship (QSAR) models to predict the adverse effect of chemicals. The majority of QSAR models for ecotoxicology have been built on descriptors for hydrophobicity (expressed by the logarithm of the octanol-water partition coefficient, log K_{OW}), for instance the non-polar narcosis QSAR model developed by Ellison et al. [139] to predict the 50% inhibition of growth concentration (IGC_{50}) to *T. pyriformis*. However, chemicals interacting by electrophilic mechanisms with biological macromolecules can exhibit excess toxicity above the baseline of narcosis, and therefore their toxicity cannot be predicted easily from QSARs based on log K_{OW} alone [140]. Common approaches for the prediction of chemical reactivity involve the use of quantum

[2] This Chapter is based on a published article whose link to the source is provided in the Appendix III.

mechanical descriptors for modelling chemical reactivity [141, 142] and the identification of chemicals for potential protein binding based on structural alerts [68]. Structural alerts that are associated with toxicological activity [25,26] have been encoded computationally into *in silico* profilers within the OECD QSAR Toolbox (www.qsartoolbox.org). There are two sets of protein profilers: the Organisation for Economic Cooperation and Development (OECD) set and the Optimised Approach based on Structural Indices Set (OASIS), which are used for various purposes such as forming categories. The most common approach to build a category is based on the principle that similar chemicals should have a common reactive centre [27,143]. Recent studies have shown that one of the better ways to define a chemical category for toxicological assessment is setting the structural boundaries of the category by applying mechanistic chemistry and grouping chemicals by their ability to undergo a common Molecular Initiating Event (MIE) [68]. MIE refers to the toxicant-biological target interaction that leads to toxicological responses at higher levels of biological organisation (see Figure 1.3 for more details). Therefore, it is important to verify the applicability domain of chemical reactions to correctly assign a chemical to a category, and thus allowing for filling data gaps for untested chemicals when applying read-across [144].

The Michael addition domain is one of the most important mechanistic applicability domains relating to reactive toxicity [68,144]. Michael acceptors are soft electrophiles containing a polarised α,β-unsaturated carbonyl or carbonyl fragment. The common characteristic of the structural alert for Michael acceptors is a C=C or C≡C group with a neighbouring electron-withdrawing moiety. Figure 4.1 shows an example of the reaction between a Michael acceptor (acrolein) with a soft nucleophile resulting in a chemical-protein adduct.

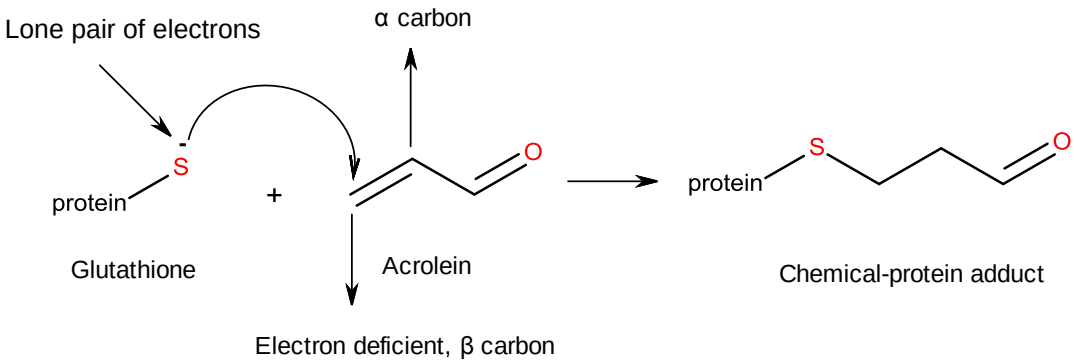


Figure 4.1 Michael addition reaction between glutathione (nucleophile) and acrolein (Michael acceptor) (electrophile). The curly arrow indicates the direction of electron movement from the nucleophile to the β-carbon (positive charge) of the electrophile. Representation based on Figure 1 published by Enoch et al. [25].

The reactivity of Michael acceptors can be measured under standardised experimental conditions using techniques known as *in chemico* methods [145]. *In chemico* techniques employ the use of biological nucleophiles (e.g. peptides or proteins) to quantify the reactivity of a compound [69]. In *in chemico* assays, the measured endpoint is the effect concentration of electrophile that depletes 50% of glutathione (GSH) (RC_{50}) after 120 min incubation time at 25 °C and pH 7.4 [134,146]. This protocol has been employed to establish an extensive database quantifying the reactivity of Michael acceptors and other electrophiles (www.qsartoolbox.org).

In chemico reactivity data play an important role in the verification of the prediction of chemical reactivity. For example, Schultz et al. [67] verified the structural alerts for a list of Michael acceptors using *in chemico* reactivity data. However, the Michael addition domain should cover a larger range of chemicals than considered so far since Michael acceptor compounds are very important industrial chemicals including polymers, textiles and auxiliary materials in medicine [147]. Recent investigations have also used *T. pyriformis* toxicity data to refine the applicability domain of selected structural alerts [148-150]. This multiple data integration not only offers the possibility of the verification of *in silico* predictions of chemical

reactivity, but also could allow for the development of an ITS to prioritise chemicals for aquatic toxicity testing.

Therefore, the aims of this study were: 1) to verify the extension of the applicability domain for Michael acceptors within the *in silico* profilers of the OECD QSAR Toolbox using experimental data. The applicability domain of the structural alerts for cyclic compounds that are potential Michael acceptors was probed using the *in chemico* GSH reactivity and *T. pyriformis* growth inhibition assays; and 2) to develop an ITS by combining data from the above *in silico, in vitro* and *in chemico* methods to prioritise chemicals for *in vivo* toxicity testing.

4.2. Materials and Methods

This work was undertaken in collaboration with Professor Terry W Schultz from the University of Tennessee (United States). Professor Schultz and colleagues conducted the experimental work, i.e. the *in chemico* testing and *T. pyriformis* growth inhibition assays, whereas the *in silico* analysis and the development of an ITS were conducted at LJMU as part of this research.

4.2.1 Test Compounds

Thirty cyclic chemicals containing a range of six-membered cyclic chemicals with different substituents were selected for evaluation; the structures of these compounds are summarised in Figure 4.2. All chemicals were purchased from commercial sources (Sigma-Aldrich.com or Alfa.com) in the highest purity available (95% minimum) and were not further purified prior to testing.

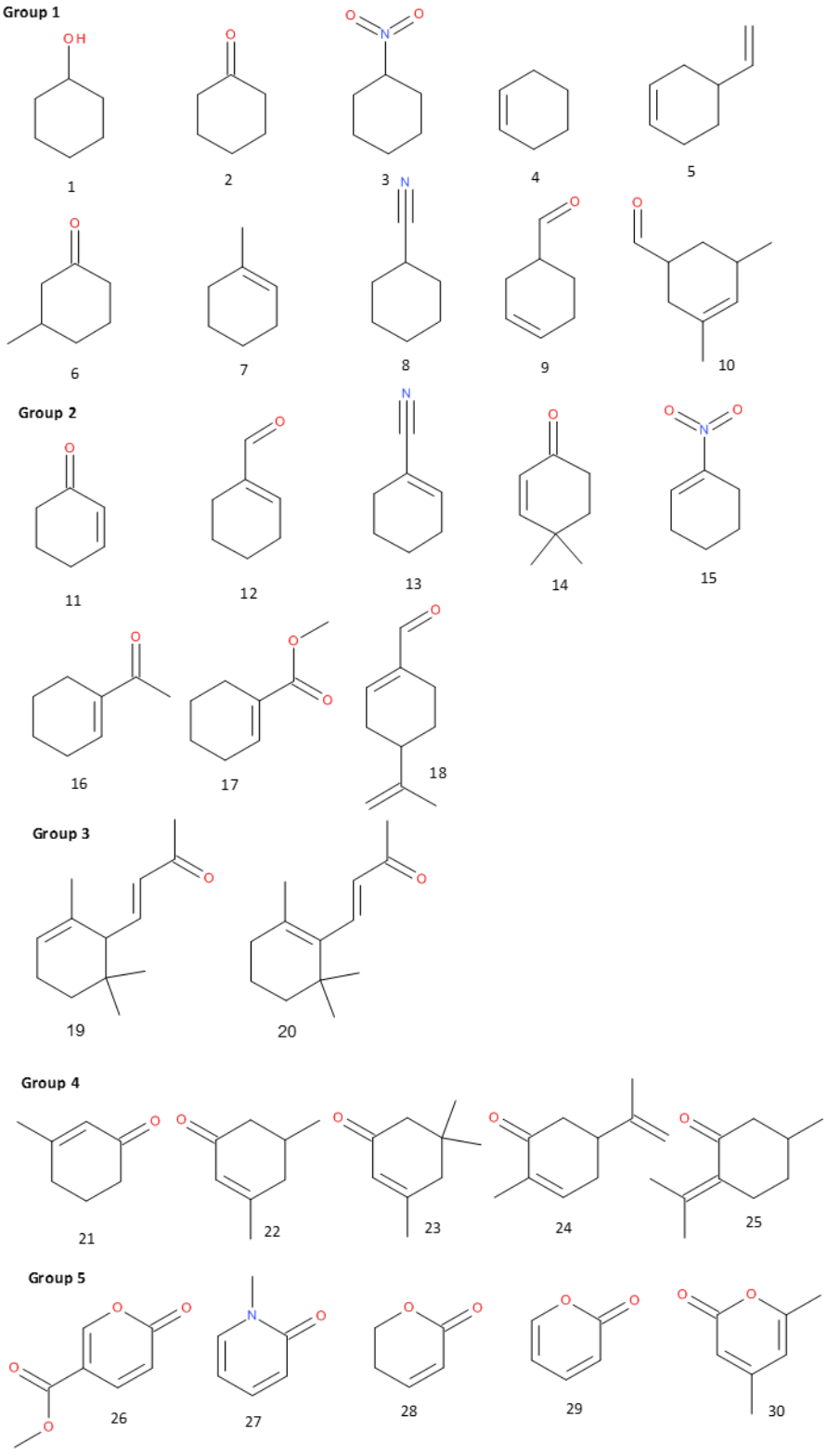
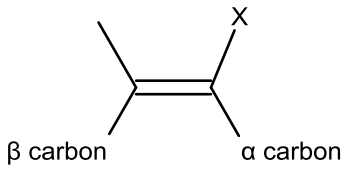


Figure 4.2 Chemicals considered in this study. Identification number related to Table 4.1. Group 1: polarised alkanes or non-polarised-alkenes; Group 2: polarised α,β-unsaturated alkenes; Group 3: polarised ketones substituted at the β-carbon; Group 4: α,β-unsaturated alkenes with substituents at the α- and/or β-carbon atoms; Group 5: α,β-unsaturated heterocyclics.

The chemicals tested were classified into five groups depending on the presence or absence of the polarised α,β-unsaturated fragment, the general structural alert for Michael acceptors (Figure 4.3), and other structural features in the molecule that may affect the reactivity with GSH.



X= CO, CN, NO_2 groups

Figure 4.3 Polarised α,β-unsaturated fragment.

- *Group 1.* The first group were polarised alkanes or non-polarised alkenes. It was expected that these chemicals would not be reactive with GSH as they do not contain the general structural alert for a Michael acceptor in their structure.
- *Group 2.* This group contains polarised α,β-unsaturated alkenes with no substituents at the α- or β-carbon atoms (Figure 4.3). It was hypothesised that these chemicals would be reactive with GSH as they do not have any mitigating factors which may affect reactivity.
- *Group 3.* The third group of chemicals were acyclic polarised ketones substituted at the β-carbon by a sterically hindered cyclohexene ring. These chemicals were of interest due to the potential for a displaced steric effect as a result of the substituted cyclohexene ring.
- *Group 4.* Chemicals in this group were α,β-unsaturated alkenes with substituents at the α- and/or β-carbon atoms. Although these chemicals may react with GSH as they meet the general structural alert for a Michael acceptor, it was hypothesised that electron-donating substituents at the α- and/or β-carbon would affect reactivity.
- *Group 5***.** The fifth group of chemicals is the polarised α,β-unsaturated heterocyclics, either O-containing pyranones or N-containing chemicals. It was hypothesised that although these chemicals may be reactive with GSH due to the general structural

alert for a Michael acceptor, heterocyclic features may be a mitigating factor affecting reactivity.

4.2.2 Protein Binding Prediction

The cyclic test chemicals were profiled for protein binding using the OASIS and OECD profilers contained within version 3.1 of the OECD QSAR Toolbox (www.qsartoolbox.org). Chemical structures were entered into the OECD QSAR Toolbox as SMILES string notations. The SMILES strings of each compound were previously obtained and verified in the KOWWIN v 1.68 software (www.epa.gov). If a structural alert was triggered for a particular chemical within one, or both, of the profilers this information was recorded. Chemical transformation due to metabolism and/or autoxidation was not considered in this investigation.

4.2.3 In Chemico GSH Reactivity

Reactivity with the thiol group of GSH was measured using a simple and rapid spectrophotometric-based assay [147]. Briefly, free thiol was quantified after its reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) with the absorption of the product measured at 412 nm. Experiments were performed with freshly prepared GSH (1.375 mM; 0.042 g of reduced GSH into 100 ml of phosphate buffer at pH 7.4) and freshly prepared stock solutions in dimethyl sulfoxide (DMSO). By combining the correct amounts of GSH solution, stock solution, and buffer the final concentration of thiol was brought to 0.1375 mM, in a manner that the concentration of DMSO in the final solution was always < 10%. Following range-finding experiments, definitive experiments were performed with concentrations adjusted to 90, 80, 60, 40, 20 and 10% of the stock solutions. Associated with each assay was a control containing GSH and a blank without GSH.

The RC_{50} values (the concentration giving 50% reaction in a fixed time of 2 h) were determined from nominal chemical concentrations (dependent variable) and absorbance

normalised to the control (independent variable) using Probit Analysis in the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Chemicals with a RC_{50} value of greater than 135 mM were considered to be non-reactive due to the fact that a contaminant at the level of 1% could be the cause of such reactivity. Similarly, RC_{50} values of greater than 70 mM were treated as "suspect" as a contaminant at the 2% level could be the cause of such reactivity.

4.2.4 In Vitro Toxicity Data

The protocol described by Schultz [138] was used to measure the 50% inhibition of growth concentration (IGC_{50}) to *T. pyriformis* of the cyclic compounds after a 40 h exposure period. The population density of *T. pyriformis* was quantified spectrophotometrically at 540 nm. Following range-finding experiments, definitive experiments were performed with concentrations adjusted to cover the highest concentration eliciting no effect on population growth to the lowest concentration inhibiting population growth completely.

Coupled to each assay, a control containing no test material and *T. pyriformis* and a blank containing neither test material nor *T. pyriformis* [139] were used in order to indicate the suitability of the medium and also to help interpret the results produced under test conditions. The IGC_{50} value was calculated (in millimolar units) by absorbance normalised to controls (independent variable) and the nominal concentration of the toxicant (dependent variable) using the Probit Analysis in SAS software (SAS Institute, Cary, NC).

4.2.5 In Silico Analysis to Identify Excess and Baseline Toxicities

The QSAR model for non-polar narcosis published by Ellison et al. [139], shown as Equation 4.1, was used to calculate the baseline toxicity of the cyclic chemicals. The log K_{OW} for all chemicals was calculated using the KOWWIN v1.68 software.

$$\text{Log } 1/IGC_{50} = 0.78 \log K_{OW} - 2.01 \qquad (4.1)$$

$$n = 87,\ r^2 = 0.96,\ s = 0.20,\ F = 2131$$

Where, n is the number of observations, r^2 is the square of the correlation coefficient adjusted for degrees of freedom, s is the standard error on the estimate and F is Fisher's statistic.

The residual value for each compound was obtained from the difference between the experimental values and the predicted toxicity calculated from Equation 4.1. Since the standard error of the estimate for the baseline model for the IGC_{50} (Eq. 1) is 0.2 log units, experimental toxicity values with residuals < 0.4 log units (twice the s value for Eq. 4.1) were considered not different from narcosis-level compounds. In a similar manner, chemicals more toxic than this limit (residuals > 0.4 log units) were classified as exhibiting excess toxicity.

4.2.6 Development of an ITS to Prioritise Cyclic Compounds for In Vivo Testing

The development of an ITS was conducted to classify cyclic compounds into different categories to prioritise chemicals for further *in vivo* aquatic toxicity testing. This strategy was built based on: 1) previous ITS proposed for environmental toxicity testing [69,74]; and 2) a classification approach that combined a QSAR analysis and the presence of structural alerts to identify chemicals with different priorities for experimental testing [140]. The *in silico*, *in vitro* and *in chemico* results were combined in a decision tree strategy to prioritise cyclic compounds for further *in vivo* testing.

4.3. Results and Discussion

4.3.1 Verification of Structural Alerts

The integration of *in vitro* and *in chemico* data is becoming more important to verify predictions of chemical reactivity [148-150]. Based on the combination of non-animal assays, the first aim of this Chapter was to verify the structural alerts for Michael acceptors in existing protein binding profilers.

A total of 30 chemicals were profiled using the *in silico* OASIS and OECD profilers in the OECD QSAR Toolbox. The results are summarised in Table 4.1. The results showed 22 chemicals contained a structural alert (in a least one of the profilers), of which 19 were identified as being experimentally reactive with GSH. A further eight chemicals failed to trigger an alert in either profiler, these chemicals were all experimentally non-reactive. The analysis of the relationship between log $1/IGC_{50}$ and log K_{OW} (Figure 4.4) revealed that 12 chemicals exhibit excess toxicity. A detailed discussion of the results for each group of chemicals is provided below.

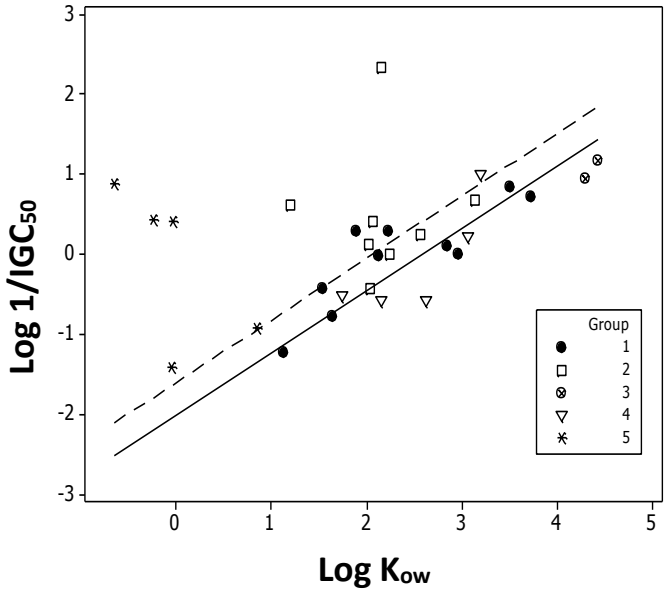


Figure 4.4 Relationship between log K_{OW} and log $1/IGC_{50}$ for the cyclic compounds. The solid line: QSAR model for non-polar narcosis published by Ellison et al. [139]. Dashed-line: QSAR model + 0.4 log $1/IGC_{50}$ units.

Table 4.1 Summary of experimental and *in silico* data

ID(G)	Chemical name	Log K_{OW}	*In silico* profiler		Log $1/RC_{50}$	*In chemico*	Log $1/IGC_{50}$	Toxicity to *T. pyriformis*	TC
			OASIS	OECD					
1(1)	Cyclohexanol	1.64	None	None	NR at 100mM	NR	-0.77	NSDB	3
2(1)	Cyclohexanone	1.13	None	None	NR at 100mM	NR	-1.23	NSDB	3
3(1)	Nitrocyclohexane	2.23	None	None	NR at 15mM	NR	-0.28	NSDB	3
4(1)	Cyclohexene	2.96	None	None	NR at 2.5mM	NR	-0.01	NSDB	3
5(1)	4-Vinyl-1-cyclohexene	3.73	None	None	NR at 0.5mM	NR	0.71	NSDB	3
6(1)	3-Methy-1-cyclohexanone	1.54	None	None	NRAS	NR	-0.43	NSDB	3
7(1)	1-Methyl-1-cyclohexene	3.51	None	None	NRAS	NR	0.84	NSDB	3
8(1)	Cyclohexanecarbonitrile	2.12	None	None	NR at 100mM	NR	-0.03	NSDB	3
9(1)	3-Cyclohexene-1-carboxyaldehyde	1.89	SB	SB	NR at 22mM	NR	0.29	XS	1
10(1)	3,5-Dimethyl-3-cyclohexene-1-carboxaldehyde	2.85	SB	SB	NR at 2mM	NR	0.11	XS	1
11(2)	2-Cyclohexen-1-one	1.2	MA	MA	-0.49	Reactive	0.61	XS	1

AC: acylation; ID: Identification number; G: chemical group; MA: Michael addition; NR: no reactive; NSDB: not significantly different from baseline; TC: Testing category (1: priority for acute toxicity tests; 2: priority for chronic toxicity tests; 3: no priority for experimental testing); SB: Schiff base formation; XS: excess toxicity.

Table 4.1 Summary of experimental and *in silico* data (cont.)

ID(G)	Chemical name	Log K_{OW}	*In silico* profiler		Log $1/RC_{50}$	*In chemico*	Log $1/IGC_{50}$	Toxicity to *T. pyriformis*	TC
			OASIS	OECD					
12(2)	1-Cyclohexene-1-carboxaldehyde	2.02	SB	SB/MA	0.98	Reactive	0.12	XS	1
13(2)	1-Cyanocyclohexene	2.04	MA	MA	0.68	Reactive	-0.43	NSDB	2
14(2)	4,4-Dimethyl-2-cyclohexen-1-one	2.07	MA	MA	0	Reactive	0.4	XS	1
15(2)	1-Nitro-1-cyclohexene	2.15	MA	MA	-1.6	Reactive	2.33	XS	1
16(2)	1-Acetyl-1-cyclohexene	2.24	MA	MA	0.32	Reactive	0	NSDB	2
17(2)	Methyl-1-cyclohexene-1-carboxylate	2.56	MA	MA	0.23	Reactive	0.24	NSDB	2
18(2)	4-Isopropenyl-cyclohexene-1-carboxaldehyde	3.14	SB	SB/MA	0.75	Reactive	0.67	NSDB	2
19(3)	4-(2,6,6-Trimethyl-2-cyclohexen-1-yl-)3-buten-2-one	4.29	MA	MA	NR at 0.2mM	NR	0.95	NSDB	2
20(3)	4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	4.42	None	MA	NR at 0.2mM	NR	1.17	NSDB	2
21(4)	3-Methyl-2-cyclohexen-1-one	1.75	MA	None	1.1	Reactive	-0.52	NSDB	2

AC: acylation; ID: Identification number; G: chemical group; MA: Michael addition; NR: no reactive; NSDB: not significantly different from baseline; TC: Testing category (1: priority for acute toxicity tests; 2: priority for chronic toxicity tests; 3: no priority for experimental testing); SB: Schiff base formation; XS: excess toxicity.

Table 4.1 Summary of experimental and *in silico* data (cont.)

ID(G)	Chemical name	Log K_{OW}	*In silico* profiler		Log $1/RC_{50}$	*In chemico*	Log $1/IGC_{50}$	Toxicity to *T.pyriformis*	TC
			OASIS	OECD					
22(4)	3,5-Dimethyl-2-cyclohexen-1-one	2.16	MA	None	1	Reactive	-0.57	NSDB	2
23(4)	3,5,5-Trimethyl-2-cyclohexen-1-one	2.62	MA	None	1.4	Reactive	-0.57	NSDB	2
24(4)	5-Isopropenyl-2-methyl-2-cyclohexen-1-one	3.07	MA	MA	NR At 8mM	NR	0.23	NSDB	2
25(4)	2-Isopropylidene-5-methylcyclohexan-1-one	3.2	MA	None	NR at 10mM	NR	1.01	XS	1
26(5)	Methylcoumalate	-0.65	MA	AC/MA	-0.04	Reactive	0.89	XS	1
27(5)	1-Methyl-2-pyridone	-0.05	MA	AC/MA	2	Reactive (suspect)	-1.41	XS	1
28(5)	5,6-Dihydro-2H-pyran-2-one	-0.03	MA	AC/MA	NR at 100mM	NR	0.42	XS	1
29(5)	2H-Pyran-2-one	-0.24	MA	AC/MA	NR at 200mM	NR	0.43	XS	1
30(5)	4,6-Dimethyl-a-pyrone	0.85	MA	AC/MA	1.9	Reactive (Suspect)	-0.93	XS	1

AC: acylation; ID: Identification number; G: chemical group; MA: Michael addition; NR: no reactive; NSDB: not significantly different from baseline; TC: Testing category (1: priority for acute toxicity tests, 2: priority for chronic toxicity tests, 3: no priority for experimental testing); SB: Schiff base formation; XS: excess toxicity.

Cyclic Polarised Alkanes and Cyclic Non-polarised Alkenes (Group 1)

Ten cyclic chemicals contained either a polarised alkane moiety or non-polarised alkene unit (see Table 4.1). No structural alerts for protein binding were identified in Group 1 compounds with the exception of 3-cyclohexene-1-carboxyaldehyde (**9**) and 3,5-dimethyl-3-cyclohexene-1-carboxaldehyde (**10**). These two cyclic aldehydes triggered a structural alert for Schiff base formation in both protein binding profilers due to the presence of the carbonyl moiety (see compounds **9** and **10** in Table 4.1). The lack of GSH reactivity for the two Schiff base formers (aldehydes) is expected as this mechanism requires a nucleophilic nitrogen atom, rather than the sulphur, attacking the carbonyl carbon as shown in Figure 4.5 for compound **9** [151]. In addition, these Schiff base formers showed excess toxicity towards *T. pyriformis* in the QSAR analysis (Figure 4.4), which could be a result of the depletion of proteins containing nitrogen reacting with them. The absence of structural alerts triggered for Michael acceptors for this group of chemicals was verified by the lack of reactivity towards GSH, as expected initially based on their lack of an α, β-unsaturated fragment (see Figure 4.2).

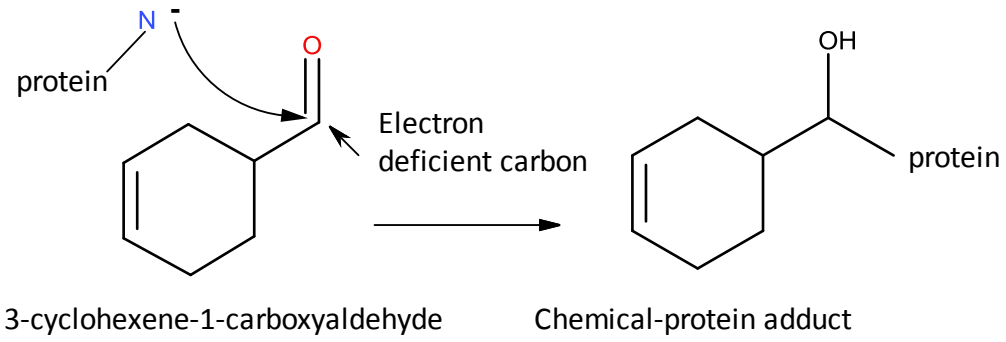


Figure 4.5 Schiff base reaction between 3-cyclohexene-1-carboxyaldehyde (ID 9) and a nitrogen containing nucleophile.

Unsubstituted Cyclic Polarised Alkenes (Group 2)

There are eight cyclic chemicals in the dataset that contain an un-substituted polarised alkene moiety (where the alkene is part of the ring system polarised by either an aldehyde, ketone, ester, cyano or nitro group; see Figure 4.1 for more details). All of these chemicals triggered a structural alert related to Michael addition for covalent protein binding in at least one of the profilers (Table 4.1). In addition, compounds **12** and **18** in which the alkene is polarised by an aldehyde moiety also trigger an alert for Schiff base formation (Table 4.1). As expected these *in silico* predictions were verified by the results of the *in chemico* data that showed all of the chemicals to be reactive towards GSH. In addition, four of the Group 2 compounds (**11,12,14,15**) exhibited excess toxicity towards *T. pyriformis* (Figure 4.4).

Acyclic α,β-unsaturated Polarised Ketones (Group 3)

There were two acyclic α,β-unsaturated polarised ketones in the dataset that feature a cyclohexene ring attached to the β-carbon atom (compounds **19, 20**). Although these chemicals were both profiled as being capable of reacting via Michael addition, neither exhibited either reactivity towards glutathione nor showed excess toxicity (Table 4.1). Their lack of reactivity could be explained by a steric hindrance at the β-carbon atom caused by the methyl substituents present on the cyclohexene ring [142,151]. These compounds are also relatively hydrophobic (log K_{OW} > 4) which leads to low water solubility potentially limiting the usefulness of the reactivity assays. Aqueous methanol reaction conditions would be needed to assess the reactivity of chemicals with poor solubility.

Substituted Cyclic Alkenes Polarised by a Ketone (Group 4)

All substituted cyclic alkenes polarised by a ketone had a methyl substituent at the β-carbon, with the exception of compound **24** whose methyl group was at the α-carbon (see Figure 1). All these chemicals were identified as being reactive by the OASIS protein binding profiler,

whereas only compound **24** was predicted to be reactive by the OECD profiler via Michael addition (Table 4.1). This is due to the restricted applicability domain in the OECD profiler that excludes chemicals that are di-substituted at the β-carbon atom. The evaluation of the experimental reactivity data showed that only three of these chemicals were reactive towards GSH (compounds **21**, **22**, **23**) and none of them showed excess toxicity. Interestingly, the reactive chemicals with a methyl group at the β-carbon were those in which the alkene double bond was within the ring system (for example, compare the ring systems of compound **21** and **25** in Figure 4.1). The difference in reactivity can be explained by the release of the ring strain energy in chemicals of this type that has the effect of lowering the energy of activation [142]. Finally, the experimental data showed that the presence of a methyl group at the α-carbon atom results in the loss of Michael addition reactivity, which is in agreement with a number of studies [68,142,153].

Polarised α,β-unsaturated Heterocyclics (Group 5)

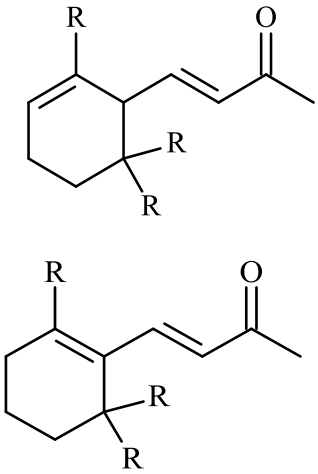
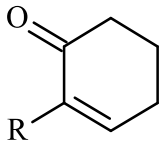
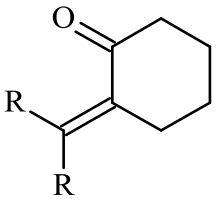
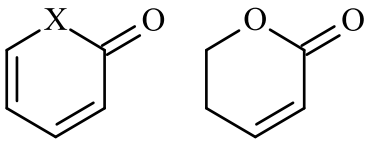
The final five chemicals in the dataset were six-membered ring systems containing either a nitrogen or oxygen atom. Whilst all these chemicals were profiled by the protein binding by OASIS profiler as being reactive via Michael addition, the OECD profiler identified potential reactivity via either a Michael addition mechanism or an acylation mechanism (Table 4.1). With the exception of compound **26,** none of these chemicals were positively identified as being reactive in the *in chemico* glutathione-based assay. The ester group attached to the heterocyclic ring could be responsible for the GSH reactivity shown for compound **26** [151]. All five heterocyclic compounds exhibited excess toxicity towards *T. pyriformis* (Figure 4.4). This finding suggests that these chemicals prefer to react via the acylation mechanism. The acylation mechanism involves the attack of a carbonyl moiety by proteins containing nitrogen atom as a nucleophilic centre [25] and, therefore, such reactivity would not be expected to be observed in a thiol-based GSH assay.

Structural Alert Refinement

The analysis above suggests that several refinements are required to the structural alerts in the protein binding profilers of the OECD QSAR Toolbox for some chemicals of groups 3, 4 and 5. The suggested refinements are summarised in Table 4.2 and discussed in more detail below.

In the analysis of the third group of chemicals, there is strong evidence that the presence of a substituted cyclohexene ring system attached to the β-carbon inhibits the reactivity via the Michael addition mechanism. Thus, chemicals containing such a moiety should be excluded from the Michael addition alert for acyclic polarised alkenes. A further restriction in the domain of the structural alerts present in both profilers was suggested from the analysis of the Group 4 chemicals. This is required to reflect the lack of Michael addition reactivity when the α-carbon is substituted by an alkyl group. For this group of chemicals, this study also showed the need for an expansion of the domain of the structural alert present in the OECD profiler to reflect the reactivity of cyclohexanones substituted by an alkyl group at the β-carbon. The experimental data suggest that Group 5 of chemicals react via acylation instead of Michael addition. The applicability domain of both profilers needs a restriction to exclude these chemicals from the Michael acceptor domain.

Table 4.2 Suggested refinements to the protein binding profilers in the OECD QSAR Toolbox

Group	Profiler	Structural alert	Applicability domain
3	Both		Restrict the Michael acceptor domain to exclude chemicals matching the alert. R = alkyl N.B. any substituted cyclohexene is included
4	Both		Restrict the Michael acceptor domain to exclude chemicals matching the alert. R = alkyl
4	OECD		Expand the Michael acceptor domain to include chemicals matching the alert. R = alkyl
5	Both		Restrict the Michael acceptor domain to exclude chemicals matching the alert. X = O, NH, NR (R = alkyl)

4.3.2 Proposed ITS to Prioritise Cyclic Compounds for In Vivo Testing

The applicability and acceptance of ITS for regulatory purposes are becoming essential with the introduction of REACH legislation [154]. The second objective of this Chapter was, therefore, to provide an example of an ITS for aquatic ecotoxicology studies to prioritise chemicals for experimental testing. Such a strategy was developed based on previous ITS that included the following steps: 1) collection of available toxicity data on the query compound; 2) the use of *in silico* methods to make predictions on its toxicity; 3) performing *in vitro* assays (e.g. fish surrogates tests, fish cells and fish embryos); 4) WoE evaluation on all data; and 5) finally determination as to whether it is required to perform fish toxicity OECD tests [74,69].

The strategy developed for the classification of cyclic compounds into three categories to prioritise chemicals for further aquatic toxicity testing is represented as a decision-tree diagram in Figure 4.6. In brief, the first step of the strategy proposed in this study was the identification of compounds exhibiting excess toxicity and narcotic (baseline) toxicity based on their absolute residuals values from Equation 4.1 (see above). Once compounds with excess and narcotic toxicity were identified, *in silico* data (i.e. the presence of a structural alert triggered by either profiler) and *in chemico* data (reactivity with GSH) were used. Whilst all compounds with excess toxicity were considered as a priority for experimental testing, only those that showed baseline toxicity and GSH reactivity and/or triggered an alert by either profiler were considered as the highest priority for chronic experimental testing (Figure 4.6). The identification of compounds relevant for chronic studies was conducted on the basis of the assumption that slow reactions between xenobiotics and proteins could have a potential hazardous effect to aquatic organisms when such interaction is persistent in the long term. In comparison to acute toxicity studies, chronic toxicity tests are not frequently conducted due to the high cost and long period involved [24]. The prediction of the chronic toxicity of a compound is usually based on the Acute-Chronic Toxicity (ACT) ratio [155]. The

ACT ratio refers to the median lethal or effective acute concentration divided by chronic value. This study has accordingly integrated acute and chronic toxicity tests in the same classification scheme to identify both potential short and long term hazards, thus representing the first ITS where both types of tests are integrated in the same scheme.

As has been explained previously in the verification of structural alerts for some cyclic compounds (e.g. group 3), the presence of a structural alert in a chemical structure does not guarantee that the reaction will take place [25]. Steric hindrance and electronic effects caused by substituents at, or near, the reaction site can affect partially, or even totally, the reaction between GSH (or another nucleophile) and the toxicant. In particular, it has been reported that steric hindrance effects can influence at least 25% of the overall reaction rate of the Michael addition reaction [152]. As a consequence, reactivity data with GSH provided crucial information on the MIE of the Michael type reaction (e.g. the existence of mitigating factors) which was used to refine the prediction of chemical reactivity. In the ITS proposed, such information is framed by a dashed line and was crucial to identify compounds that do not require further experimental testing (non-reactive baseline toxicants without alert triggered) (see Figure 4.6).

The proposed strategy could be taken as a foundation to develop future ITSs based on other fish surrogates (e.g. Daphnia, fish embryo) and toxicological endpoints (e.g. lethal concentration), both frequently used in risk assessments. According to the classification scheme illustrated in Figure 4.6, 12 chemicals were considered as having the highest priority for acute toxicity tests, 10 other chemicals as being relevant for chronic toxicity tests and eight chemicals were identified as compounds without a need to undergo experimental testing. Categories for each of the examined compounds are provided in Table 4.1 and explained in more detail below.

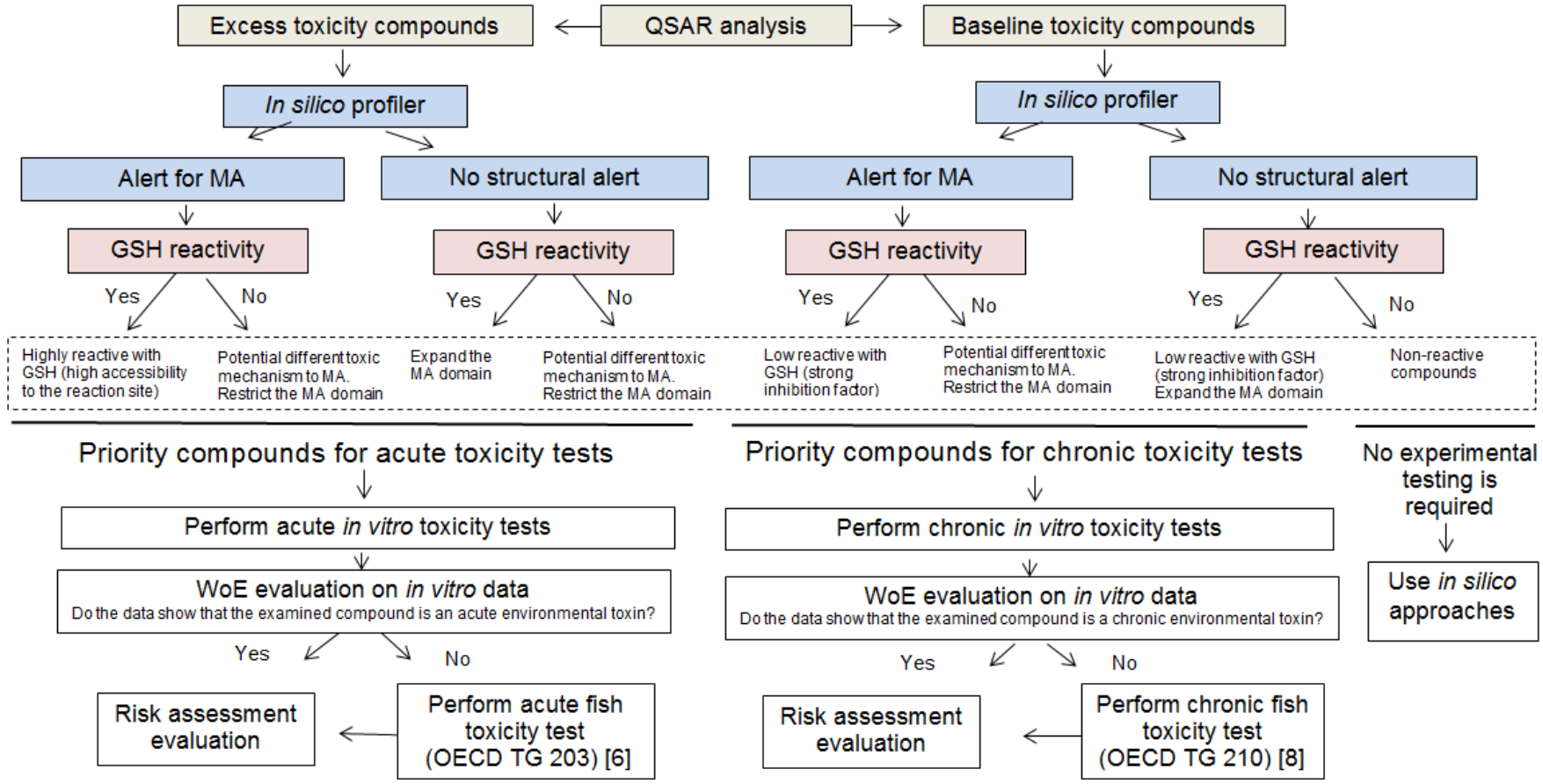


Figure 4.6 Proposed ITS for Michael acceptors to prioritise cyclic compounds for further testing. GSH: Glutathione; QSAR: Quantitative Structure-Activity Relationship; MA: Michael Acceptor; WoE: Weight of Evidence.

Compounds with the Highest Priority for Acute Toxicity Tests

Compounds with the highest priority for acute toxicity tests were all chemicals that exhibited excess toxicity towards *T. pyriformis.* Interestingly, all compounds with excess toxicity triggered a structural alert by either profiler (Table 4.1), supporting the reliability of *in silico* profilers to identify reactive compounds. Of these chemicals, compounds **9**, **11**, **12**, **14** and **15** were highly reactive with GSH. In the analysis of their chemical structure, no methyl substituents were found at the α,β -C atoms suggesting a greater accessibility to the reaction site. In contrast, compounds **27-30** did not react with GSH. Such compounds were associated with the acylation mechanism by the OECD protein binding profiler indicating that the electrophilic toxic mechanism involved a nitrogen atom as the nucleophilic centre rather than sulphur as explained before. A lysine-based depletion assay would be required to clarify the type of electrophilic reaction of these chemicals.

Chemicals showing excess toxicity should be considered as compounds of environmental concern since their reaction with biological macromolecules is irreversible [25]. Testing such compounds through a variety of *in vitro* toxicity assays such as the Fish Embryo Test (FET) [14] and fish cells test systems (both primary and cell lines cultures) would be necessary to generate more data to support their excess toxicity observed to *T. pyriformis*. Finally, if the *in vitro* data generated are not conclusive enough on the potential acute toxicity of the examined compound, the acute fish toxicity test [6] should be conducted.

Compounds with the Highest Priority for Chronic Toxicity Tests

Compounds with the highest priority for chronic toxicity tests were baseline compounds that showed either *in chemico* GSH reactivity or a structural alert triggered. Generally, these compounds included all the chemicals containing mitigating factors affecting reactivity such as a steric hindrance to the site of reaction (Group 3) and methyl group acting as electron-

donating substituents at the α and β C -atoms (Group 4). This finding shows how combining different non-animal test methods can provide an insight into the mechanism of the reaction. It is expected that even slow reaction rate with GSH of these chemicals could be toxic towards aquatic organisms in a long term exposure. Conducting additional *in vitro* and *in vivo* chronic toxicity tests would be of interest to expand the knowledge of the long term toxic effect of such chemicals.

Compounds with No Need for Experimental Testing

Compounds with no need for experimental testing were narcotic compounds that neither triggered a structural alert by the *in silico* profilers nor were reactive with GSH. All these chemicals belong to the Group 1 (polarised alkanes and non-polarised alkenes). For these chemicals, there is enough WoE of the lack of their reactivity to biological targets, and therefore further *in vitro* and *in vivo* testing should be not required. Alternatively, QSARs or read-across can be used to predict the toxicity of these non-reactive chemicals.

4.4. Conclusion

The integration of alternative methods is becoming more important in regulatory toxicology. This study has showed two outcomes of integrating *in vitro*, *in chemico* and *in silico* methods to provide relevant information on the Michael addition electrophilic mechanism of action. Firstly, this study has verified the structural alerts present in the *in silico* profilers within the OECD QSAR Toolbox using experimental data. The results showed that the applicability domain of some of the structural alerts required refinement and improvement, and therefore suggestion are given. Secondly, an ITS was developed based on the combination of multiple pieces of information to identify: 1) chemicals with a high priority for acute toxicity tests; 2) chemicals with a high priority for chronic toxicity tests; and 3) chemicals with no need for experimental testing. The results showed a good concordance between *in silico, in chemico*

and *in vitro* data, which could be integrated into ITS to make a decision whether a query compound will require *in vivo* fish toxicity testing.

Chapter 5. Discussion and Suggestions for Future Work

To conclude this thesis, a summary and discussion of the research undertaken will be provided in the first section of this Chapter. A description of three proposed plans for future research will be described in the second section. The suggestions for future work were derived from the identification of knowledge gaps from the research conducted in this thesis, and they are expected to provide a basis for ongoing research. Finally, a thesis overview and final thoughts on alternative methods are provided in the last section.

5.1. Summary and Discussion of the Work Undertaken

Chapter 1: Introduction

The development of non-animal methods to assess the toxicity and bioaccumulation potential of organic chemicals in fish is the central theme of this thesis. In Chapter 1, a description is given of different *in vitro* and *in silico* techniques used in aquatic ecotoxicology with a particular emphasis of why these methods have become important in risk assessment. To provide a better understanding of the research topic, Chapter 1 also introduced the origin of the pollutants that enter into the aquatic environment, the aspects that should be taken into account for the design of toxicity tests and the process of validation for the implementation of alternative methods.

Chapter 2: Development of a List of Reference Compounds to Evaluate Alternative Methods to In Vivo Fish Bioaccumulation Tests

The validation of alternative methods is the key for their implementation in risk assessment to reduce the number of animals required to assess the bioaccumulation potential and toxicity of chemicals [76,77]. To enable the validation of alternatives, lists of reference

compounds supported by high quality *in vivo* data are required to enable data comparison. Whilst lists of reference compounds have been provided to develop different alternatives to animal (eco)toxicity studies [79,98], no reference list has been proposed for *in vivo* bioaccumulation in fish. This knowledge gap led to the development of a reference list of chemicals to develop and validate alternative methods for predicting bioaccumulation with a particular focus on metabolic assays, as part of this thesis.

It should be stressed that a considerable effort was made to establish a step-wise approach to select reference compounds for chemical bioaccumulation, as there is no official guidance of chemical selection procedures in general. Initially, it was planned to perform a similar approach to the compound selection strategy provided by Olah et al. [156] for drug discovery, which would have implied the use of *in silico* tools employed in industrial research. Generally, chemical selection procedures for drug discovery rely on a random selection of a structurally diverse set of chemicals based on a broad chemical space distribution resulting from the structure-biological activity relationship [95]. Examples of *in silico* techniques used for compound selection include clustering, cell-based and dissimilarity-based compounds selection methods [157,158]. Such methodology was rejected, however, due to two main reasons: 1) the heterogeneity of the data, since one of the selection criteria was that compounds supported by three *in vivo* measurements related to bioaccumulation should be included in the reference list; and 2) the intrinsic difficulty in selecting different chemical classes identified by employing a random selection. It was expected that the chemical selection could be biased for those classes containing a minority of chemicals, since different chemical classes can have a similar coverage for a given descriptor. Consequently, a list of selection criteria was established to minimise random compound selection as much as possible. In doing this, the criterion of the inclusion of different chemical classes was prioritised to ensure a broad range of key chemical properties (see Figure 2.1). Such selection criteria were integrated into a three-tier strategy. Tier I consisted of the selection of: 1)

relevant *in vivo* bioaccumulation endpoints (bioconcentration factor (BCF), whole body biotransformation rates (K_{MET}) and measurements related to metabolite identification); 2) fish species (common carp and rainbow trout); and 3) and chemical classes that are shown in Table 2.2. Tier II involved data collection, evaluation and analysis of the chemicals supported by *in vivo* data for the selected *in vivo* endpoints. Tier III was based on a refinement selection process to guarantee a broad range of chemical domain in terms of physico-chemical, molecular and metabolic properties. From the use of the above selection strategy, 144 chemicals were selected for BCF, another eight for K_{MET} and five compounds were supported by *in vivo* data for metabolism.

The availability and the quality of *in vivo* data were key issues for compound selection. For example, to provide a representative list, the selection was focused on the fish species that possessed more measurements for bioaccumulation, i.e. rainbow trout and common carp. Another relevant aspect resulting from the creation of a relevance list was the identification of compounds whose BCF values showed poor correlation with the logarithm of octanol-water coefficient (K_{OW}). Such compounds were considered to be of interest for *in vitro* metabolic assays based on the rationale that significant metabolic biotransformation can reduce the maximal bioconcentration of chemicals. To identify compounds that may potentially be metabolised, maximal log BCF models (log BCF_{max}) were built from the relationship between *in vivo* log BCF and log K_{OW} for rainbow trout and common carp, representing the first models that have been developed for these fish species.

It should be added that although the list of reference compounds mainly addresses the development and validation of non-animal approaches for bioaccumulation studies in fish, other benefits can be derived from its common use, as for instance: 1) the investigation of the Absorption, Metabolism, Distribution and Excretion (ADME) properties of reference chemicals based on the integration of study results derived from various *in vitro* assays; and

2) the development of other chemical selection strategies for other fish species and/or toxicity endpoints based on the chemical selection strategy proposed. In particular, a list of reference compounds could be developed for chronic toxicity studies based on the data compiled by Raimondo et al. [155].

Chapter 3: A Review and Comparison of Alternative Methods to In Vivo Bioaccumulation Studies in Fish

Over the last decade, various non-animal methods have been developed to assess the bioaccumulation of chemicals. Whilst some of these methods involved the use of fish hepatocytes or subcellular fractions (e.g. microsomes, S9) to assess the xenobiotic biotransformation based on a depletion approach, others use *in silico* models to estimate K_{MET} and BCF of chemicals [30,116]. Predictive K_{MET} approaches include Physiologically-Based ToxicoKinetic (PBTK) and Quantitative Structure-Activity Relationship (QSAR) models. Predictive BCF approaches include log K_{OW}-based and kinetic mass balance models. Before alternatives become implemented in risk assessment to meet the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation [13], an in-depth analysis of their potential use as a surrogate and/or complement to *in vivo* bioaccumulation testing is likely to be required.

As a consequence, Chapter 3 reviewed and compared the current alternatives for bioaccumulation for a set of compounds selected from the reference list developed in Chapter 2. The non-animal methods examined were: 1) the *in vitro* clearance assay using freshly isolated hepatocytes from rainbow trout; 2) three PBTK calculation models and a QSAR model to predict K_{MET}; and 3) log K_{OW}-based and mass balance models for estimating BCF. Results showed that the rainbow trout clearance assay can be used to identify readily metabolised chemicals and investigate the effect of metabolic processes on the maximum bioconcentration of chemicals. High variability in predicted K_{MET} data was obtained from the

comparison between three PBTK models with an established QSAR model, suggesting further development and improvement needs. With regards to predictive BCF models, a kinetic mass balance model incorporating K_{MET} predicted based on experimental half-lives (HL) represents a potential surrogate to *in vivo* bioaccumulation studies. Overall, although a significant effort is being conducted to develop non-animal methods to enable their use in risk assessment, further experimental work is still needed to: 1) set up a feasible protocol for the *in vitro* clearance assay to increase its sensitivity; 2) explore the biological factors of the fish donors (e.g. age) that can affect the variability of *in vitro* data; and 3) investigate the *in vivo* whole body metabolic rates of compounds covering a broad range of hydrophobicity (log K_{OW}). Chapter 3 also highlighted the importance of the fish body mass in mass balance models for predicting BCF of chemicals, suggesting a need for consensus in selecting a single fish mass for future *in vitro-in vivo* data extrapolations.

Chapter 4: In Silico Mechanistic Analysis of the Toxicity of Cyclic Compounds

There is an increasing desire in (eco)toxicology to integrate various tests to elucidate the toxic mechanism of chemicals. In a regulatory toxicology context, a set of alternative methods are usually integrated into decision trees that are termed as Integrated Testing Strategies (ITS), which are used to make a conclusion as to whether or not a compound represents a toxic hazard before conducting animal tests [69,74]. The combination of different experimental results has also been used for the verification of *in silico* predictions to define their applicability domain. This has been shown for the experimental verification of the structural alerts within protein profilers of the Organisation for Economic Co-operation and Development (OECD) QSAR Toolbox (www.qsartoolbox.org) by combining *in chemico* (reaction with Glutathione (GSH)) and *Tetrahymena pyriformis* toxicity data [148-150].

Chapter 4 described two different outcomes derived from the integration of multiple methods when investigating the toxic mechanism of 30 cyclic compounds acting by the

Michael addition reaction. The first section involved the verification of structural alerts encoded into the two *in silico* protein profilers of the OECD QSAR Toolbox by using in *chemico* GSH and *T. pyriformis* toxicity data. The results showed that the applicability domain of some of the structural alerts required refinement and improvement; and hence, more work is required in this area. In particular, the following cyclic compounds should be excluded from Michael addition domain: 1) compounds with a ring system attached to the β-carbon; 2) cyclic compounds containing an alkyl group at the α-carbon; and 3) heterocyclic compounds containing either a nitrogen or oxygen atom in the ring system.

The second section consisted of the development of an ITS for aquatic toxicity testing to classify cyclic compounds into: 1) chemicals with a high priority for acute toxicity tests; 2) chemicals with a high priority for chronic toxicity tests; and 3) chemicals with no need for experimental testing. To classify examined compounds into these categories, a decision tree was built from: 1) the QSAR analysis of the toxicity data to *T. pyriformis;* 2) the presence/absence of a structural alert for Michael addition domain; and 3) *in chemico* GSH data. Overall, a good concordance between *in silico*, *in chemico* and *in vitro* data was found when applying the ITS proposed, which can be used as a foundation to develop other prioritisation strategies for different aquatic species and/or endpoints.

5.2. Suggestions for Future Work

Suggestions for future work following this thesis are discussed below.

5.2.1 Investigation of the Factors Affecting In Vivo BCF Data Variability

It is expected that a large number of chemicals will be tested according to the recent OECD 305 TG guideline [10] to meet the new REACH legislation. It should be noted that although a review of BCF for organic chemicals has been provided [32], the effect of experimental variables on chemical bioconcentration is not well understood. Moreover, ambiguous results

have been reported in the literature concerning the influence of test concentration on BCF. For example, while no significant differences between BCF data measured at the lowest and highest test concentrations were reported by Creton and colleagues [159], BCF data variability according to test concentration was found for chemicals whose coefficient of variance was higher than 50% when developing a reference list (see Figure 2.2 for more details). Similar to *in vivo* toxicity studies [58], there is also a knowledge gap in the potential effects of other abiotic factors such as the temperature and the pH of the test system on the bioconcentration of a compound. The latter has been identified as a key variable for the bioaccumulation of ionisable compounds [80,134,135].

The biological features of test organisms also have an impact on chemical bioconcentration. The body weight of test animals is one of the most important variables in bioaccumulation studies due to its influence on Absorption, Distribution, Metabolism and Excretion (ADME) processes. In particular, previous work has reported that small fish have higher rates of absorption, metabolism and excretion of chemicals than larger fish [29]. It is believed that such physiological differences, depending on body mass, may affect the time required to attain the steady state (equilibrium) of chemical bioconcentration. For example, compounds tested in small fish may reach steady state earlier as a result of their higher rates of absorption, metabolism and excretion than those test chemicals measured in larger fish. This aspect should be considered as crucial for the bioconcentration of hydrophobic chemicals, due to the fact these chemicals require a significant amount of time to attain effective equilibrium [160].

Therefore, an in-depth analysis of *in vivo* BCF data is required to investigate the effect of both experimental conditions and the body mass of test fish on chemical bioconcentration. It is expected that a better understanding of the sources of BCF data variability will not only allow

for the development and improvement of alternatives to whole body fish testing, but also will help to select appropriate conditions for future *in vivo* BCF assessment.

5.2.2 Future Directions of In Vitro Methods to Study Xenobiotic Metabolism

The following aspects are considered as being relevant for the development and improvement of *in vitro* clearance assays using freshly isolated rainbow trout hepatocytes.

Expand the Work Undertaken in Chapter 3

As explained in Chapter 3, a total of nine chemicals were tested using cells from the same fish donor to minimise the influence of the biological variables on experimental data, which implied, however, the use of two replicates per test substance in order to test all chemicals at one time. Therefore, future experiments could consist of testing fewer chemicals with three replicates per compound to obtain lower intra-assay data variability. Of the compounds examined, 1,3-dichloro-5-nitrobenzene, dibenzofuran and pentachlorophenol should be rejected for future experiments as they were not cleared by the hepatocytes (see results in Tables 3.6). It should be noted that test concentration higher than 0.5 µM are required to test pentachloronitrobenzene to avoid its low detection limit for analysis, and exposure periods shorter than 5 hours should be used to reduce the number of samples per chemical.

Another important question that remains unanswered is whether hepatocytes isolated from younger fish donors have higher metabolic rates than those isolated from older fish, as described in section 3.3.2. In order to investigate this more thoroughly, the measurement of metabolic activity of enzymes from hepatocytes isolated from different aged fish donors should be conducted. Key metabolic enzymes to be measured are 7-ethoxyresorufin-O-dealkylation (EROD), testosterone 6β-hydroxylation, 1-chloro-2,4-dinitrobenzene (CDNB)-glutathione conjugation and p-nitrophenol-glucuronidation [39].

Development of a Protocol for Daily Routine

As observed in Chapter 3, the *in vitro* clearance protocol used to study the biotransformation of chemicals involved several steps and materials: 1) the metabolic incubation in borosilicate glass tubes; 2) the aliquot of subsamples to stop the reaction into 1.5 mL Eppendorf tubes; and 3) finally the transfer of supernatant to vials for chemical analysis through Gas Chromatography Mass Spectrometry (GC-MS). The ideal experiments to study the metabolism of xenobiotics would be those in which the biological test system and chemical analysis are integrated in the same material, similar to the methodology conducted with an effect-directed analysis (EDA). EDA combines bioassays with analytical chemistry in 96-well plates to analyse environmental pollutants in risk assessment [161]. Another advantage of such analysis is that chemical analysis is performed using Liquid Chromatography Mass Spectrometry (LC-MS) that can be coupled to High Resolution Mass Spectrometry (HR-MS). LC-MS offer several advantages in comparison with GC-MS, such as a rapid chemical analysis with a very high sensitivity [161]. However, some types of chemicals such as essential oils, fragrances and non-polar compounds are particularly well-suited to GC-MS as they do not ionise well by LC-MS.

Figure 5.1 shows an example of a 96 well-plate test system that could be used to study xenobiotic biotransformation, being suitable for both GC-MS and LC-MS analysis. For the analysis of certain types of chemicals (e.g. non-polar and volatile chemicals), an additional step will be required to extract some supernatant for the analysis of parent compound by GC-MS (see Figure 5.1). The use of such a test system could offer several advantages with respect to the current experimental procedure including: 1) higher reproducibility and reliability, as a result of the use of a greater number of replicates and multi-channel pipettes to stop the reaction, thus reducing the time gaps amongst sub-samples; and 2) elucidating

the metabolic pathways of test chemicals by analysing the resulting metabolites of test compound by LC-MS.

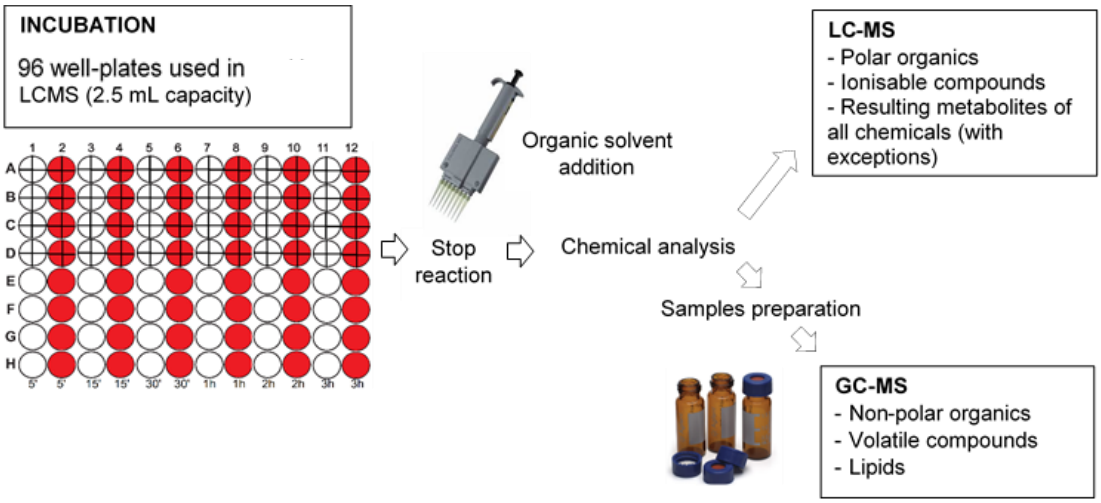


Figure 5.1 Experimental system proposed to study xenobiotic biotransformation. Open circles: Test compound A, Solid circles: Test compound B; Crossed circles: control samples

The use of the above, or a similar, test system is expected to expand the knowledge of the metabolism of structurally diverse chemicals. We believe that data on CL_{INT} and metabolites identified for a broader chemical domain will allow for the construction of *in silico* models to predict CL_{INT} and metabolic pathways of chemicals. Such metabolic data generated could also be incorporated into the log BCF_{max} models developed for rainbow trout and common carp to correct for the effect of metabolism on maximal bioconcentration.

It should be noted that the clearance assays performed in this thesis consisted of an incubation of test compounds with freshly prepared hepatocytes in suspension. Research is also needed to develop clearance assays in other cell-based systems such as monolayers and aggregates cultures [41,128]. It is expected that clearance data generated in other test systems may differ from those determined in a suspension system. For instance, a comparative study showed that clearance rates of well-metabolised compounds were lower in monolayers than in suspension due to the relatively small surface area for chemical diffusion in monolayer test systems [162].

The Use of Cryopreserved Hepatocytes in the Clearance Assays

The use of cryopreserved hepatocytes in clearance assays is important to comply with the 3Rs for animal use. Previous work has shown that cryopreserved hepatocytes are suitable for bioaccumulation risk assessment, as they maintain metabolic activities comparable to freshly isolated hepatocytes [39]. Initially, it was aimed to use cryopreserved cells together with freshly isolated hepatocytes in the clearance assays to reduce the number of fish for experimentation. As a consequence, preliminary experiments were undertaken to investigate the feasibility of cryopreserved hepatocytes for use in clearance assays. Freshly isolated hepatocytes with 95% viability from two isolations were cryopreserved following the protocol included as Appendix I, and then thawed according to protocol provided as Appendix II in the following days. For all thawed cells batches, low percentages of cell viability (~ 50%) and yield recovery (~ 25%) were obtained. The low percentage of cell viability could be a result of an inappropriate use of cryobox, as the foam was not removed, and/or a toxic effect caused by the DMSO (from the cryopreservation buffer). The low percentage of cell viability obtained in this study was similar to the yield recovery of 37% reported in previous studies [39]. It was concluded that further experimental work will be required to obtain a higher percentage of both cell viability and recovery in order to use cryopreserved hepatocytes for *in vitro* clearance assays.

5.2.3 Establishment of a List of Reference Compounds for Developing AOPs

A list of reference compounds can be established for aquatic ecotoxicology to provide a better understanding of toxic mechanisms of chemicals through the development of Adverse Outcome Pathways (AOPs). Investigating the AOP of chemicals is becoming a crucial topic of research in (eco)toxicology [26, 163], and recommendations for their appropriate development and assessment have been provided in the recent OECD guideline [164].

Different applications for developing non-animal methods can be derived from established AOPs including: 1) the establishment of (Q)SAR, facilitating predictive and mechanism-based toxicology; 2) the elaboration of prioritising strategies to reduce animal use; and 3) the development of novel *in vitro* toxicity screening tests [165]. As a consequence, a list of reference compounds selected from high quality toxicity data might be needed for future *in vitro* testing aimed at establishing AOPs.

A variety of molecular test systems can be used to study the toxic mechanism of chemicals at cellular and molecular levels. Amongst them, the microarray test represents a powerful tool in research to study the gene expression of the toxic effects in organisms. Of relevance is that changes in gene expression are toxicant specific and can be linked to their mechanism of toxic action [62,166]. Furthermore, microarray assessment could be a potential alternative to median effective concentration (EC_{50}) and median lethal concentration (LC_{50}) standardised tests [62]. Therefore, not only can a proposed list of reference chemicals contribute to the development of AOPs, but it can also be used for the evaluation and validation of the microarray test as an alternative to animal toxicity testing.

The following steps describe the methodology that could be used for the development of such a reference list.

Step 1: Starting Points for Chemical Selection

Similar to the strategy taken in Chapter 2, the selection of relevant fish species, toxicity endpoints and chemical classes for aquatic ecotoxicology should be conducted before data compilation. *Danio rerio* (zebrafish) and *Oryzias latipes* (medaka) could be fish species candidates for two reasons: 1) their embryos are frequently used to assess the toxicity of chemicals as they present several advantages with respect to the whole fish [3,4]; 2) their genomes have been sequenced [167-169]. The selection of appropriate toxicity endpoints

should be established using expert judgment and considering relevant aspects such as data availability. It should be noted here that *in vitro* toxicity endpoints may be chosen instead of *in vivo* measurements due to the larger data availability [14]. Additionally, a good correlation between whole fish and embryo acute toxicities has been shown for zebrafish [14]. The EC_{50} and LC_{50}, both determined from 24 to 96 hours, represent endpoints of interest due to their wide use in ecotoxicology. It is expected that relevant chemical classes for ecotoxicology may cover those proposed for bioaccumulation (Chapter 2, Table 2.1) as well as compounds exhibiting: 1) non-polar narcosis; 2) polar narcosis; and 3) all electrophilic reactions involved in protein and DNA binding [25,26].

Step 2: Data Compilation, Evaluation and Chemical Classification

Data for EC_{50} and LC_{50} determined in zebrafish and medaka embryos could be compiled from different publicly available sources such as the ECOTOX database (cfpub.epa.gov/ecotox), ECETOC (www.ecetoc.org) and ECHA (echa.europa.eu). Recent scientific publications, such as the list of chemicals supported by zebrafish toxicity data compiled by Lammer et al. [14] could also be taken into account. Once all experimental data are collated and organised, assessment of the quality of the data should be performed according to the Klimisch criteria or the scheme for data quality provided by Przybylak et al. [22]. It should be stressed that only compounds assessed with the highest reliability score should be considered for the reference list. After this, the classification of compiled chemicals according to the chemical classes established in Step 1 should be conducted. A further sub-classification would be expected for those classes containing a large number of chemicals. Due to the difficulty in classifying chemicals with multiple functional groups in the molecule, special considerations should be given for such chemicals.

Step 3: In Silico Analysis

A set of physico-chemical, molecular and toxicological properties should be calculated for the chemicals compiled in Step 2. Log K_{OW}, logarithm of Henry's Law Constant (log HLC) and water solubility may account for key chemical properties; molecular weight (MW) and maximum molecular diameter (D_{max}) should be designated for molecular properties; the toxic mechanism of action and the presence/absence of structural alerts for protein and DNA binding should account for toxicological properties. The mechanism of action of chemicals should be predicted according to the following Verhaar classes [170,171]: 1) non-polar narcotics; 2) polar narcotics; 3) reactive chemicals; 4) specifically acting chemicals; and 5) unknown. Examples of some specific mechanisms of toxic action that should be considered include Acetylcholinesterase (Ach E) inhibition and oxidative phosphorylation [172]. Another aspect of interest should be predicting metabolism for the examined compounds, since some resulting metabolites can be more toxic than the parent compound [173]. Some of the above properties may be calculated by using the OECD QSAR Toolbox (www.qsartoolbox.org), EPI Suite software (www.epa.gov) and commercial software such as Meteor Nexus (www.lhasalimited.org)

For compounds with sufficient data, QSARs could be developed from the linear regression between the inverse of the logarithm of the selected endpoint and log K_{OW}. Such analysis aims to discriminate narcosis-level compounds and chemicals exhibiting excess toxicity. In addition, baseline narcotics and compounds with excess toxicity could be split into different groups depending on their predicted toxic mechanism of action and structural alert triggered, based on a similar approach to the Integrated Testing Strategy (ITS) proposed for Michael acceptors shown in Figure 4.6.

Step 4: Refinement of Chemical Domain

A final selection process would be conducted by using a similar methodology to that employed in Chapter 2. For each of the chemical classes identified in Step 2, a selection should be performed to achieve a broad chemical domain for log K_{OW}, molecular properties (MW, D_{max}) and toxic mechanism. Additionally, the final list should include the following: 1) common compounds for established endpoints and fish species, as they represent chemicals of interest for exploring the link between EC_{50} and LC_{50} as well as interspecies differences; and 2) compounds supported by a high weight of evidence on the toxic mechanism. For instance, compounds predicted to be reactive with an alert triggered by the protein/DNA profilers, and conversely predicted narcotic compounds with no structural alert triggered by the OECD profilers.

It is expected that *in vitro* data generated for such reference chemicals by using a battery of *in vitro* test systems, and subsequently incorporated into a common database, will make a significant contribution for aquatic ecotoxicology.

5.3. Thesis Overview and Future Directions of Alternative Methods for Aquatic Toxicology

This thesis has been centred on the development and improvement of non-animal methods to assess the toxicity and bioaccumulation potential of organic chemicals. This involved the use of multiple disciplines in the area of aquatic toxicology including: 1) the analysis and evaluation of *in vivo* data for chemical bioaccumulation; 2) the development of *in silico* models for bioaccumulation and the refinement of structural alerts for protein binding; and 3) the establishment of an *in vitro* clearance assay to study xenobiotic biotransformation.

In order to reduce the number of fish required for experimentation, further work is still needed for the final implementation of alternative methods. The use of a holistic approach might be the key to develop and improve non-animal methods. Similar to musicians of the

orchestra playing together, the integration of multiple results obtained from different assays could play an important role in providing accurate information concerning the mechanism of toxic action of chemicals.

More experimental efforts are expected in the coming years to develop and validate *in vitro* assays for aquatic bioaccumulation. This is because *in vitro* methods to study xenobiotic metabolism for fish are being currently developed and no assays have been proposed to determine the absorption of chemicals. The validation of these methods will be crucial for their appropriate implementation in risk assessment. It is expected that the list of reference compounds established in Chapter 2 will assist in this process.

Finally, more *in vivo* testing using the whole fish is also likely to be required for the suitable development of alternative methods. This is because the effect of biological and experimental factors on *in vivo* data are not well-understood in both toxicity and bioaccumulation studies. A better understanding of the influence of these factors on *in vivo* data variability could not only allow for a rational development of *in vitro* assays, but also it could explain the data variability reported for *in vivo* studies.

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7. Appendices

Appendix I. Protocol of Cryopreservation of Trout Hepatocytes

Appendix II. Protocol of Thawing of Cryopreserved Trout Hepatocytes

Appendix III. References of the Published Articles

Appendix IV. Tables S1, S2 and S3 Included as Electronic Supplementary Data

Appendix I: Cryopreservation of Trout Hepatocytes

This protocol is exemplified for a final concentration of 15 million cells per vial and a total of 10 vials.

1. Determine the concentration and viability of freshly isolated hepatocytes.
2. Transfer 150 million of hepatocytes into a 50 mL centrifuge tube.
3. Centrifuge cells at 50 g for 3 min at 4 °C to sediment cells. Aspirate supernatant to just above cell pellet and add cryopreservation buffer up to 5 mL.
4. Suspend cells in solution and then slowly add 2.5 mL of cryopreservation buffer containing 12% Dimethyl Sulfoxide (DMSO) while gently shaking cells.
5. After 5 minutes on ice, slowly add 7.5 mL of cryopreservation buffer containing 16% DMSO while gently shaking cells. Final volume is 15 mL at 1 million cells/mL.
6. After 5 minutes on ice, suspend cells by gently shaking and transfer 1.5 mL of hepatocytes suspension to cryovials.
7. Place vials into the freezing container (previously filled up with isopropanol) and keep it at – 80 °C overnight (not recommendable for more than 2 days).
8. After the controlled rate freezing is completed by the freezing container, place the cryovials into a liquid nitrogen storage tank. Removing cells from freezer to storage tank should take less than 1 minute.

Additional notes

- Keep cells and medium on ice throughout entire procedure and always shaking when adding a new medium.
- DMSO is required to protect cells and is added in a two-step gradient to adapt cells to DMSO. The final concentration of DMSO should be less than 10%.

Cryopreservation Buffer (DMEM with 20% FBS and 0.25% BSA)

- 40 mL Dulbecco's Modified Eagle Medium (DMEM)
- 10 mL Fetal Bovine Serum (FBS)
- 0.125 g Bovine Serum Albumin (BSA)

Mix and adjust to 7.8

Cryopreservation Buffer w/12% DMSO

- 4.4 mL cryopreservation buffer
- 0.6 mL DMSO

Cryopreservation Buffer w/16% DMSO

- 10.5 mL cryopreservation buffer
- 2 mL DMSO

Appendix II: Thawing of Cryopreserved Trout Hepatocytes

This protocol is exemplified for thawing a total of 45 million frozen cells contained in 3 cryovials (15 million cells/vial).

1. Prepare recovery medium at a room temperature using sterile conditions. The recovery medium contains Dulbecco's Modified Eagle Medium (DMEM), 10% of Fetal Bovine Serum (FBS) and 0.25 % of Bovine Serum Albumin (BSA). For a total volume of 150 mL, the amounts for each reagent are:

Reagent	
DMEM	135 mL
FBS	15 mL
Low Fatty Acid (BSA)	0.375 g

2. Mix the recovery medium for approximately 5 min in the incubator refrigerated at 28 °C, adjust its pH to 7.8 and then filter the recovery medium with a sterile filter.
3. Adjust the pH of Leibovitz medium (L-15) (without phenol) to 7.8.
4. Aliquot 42 mL of recovery medium into a 50 mL centrifuge tube for thawing 2 vials containing approximately 30 million frozen cells, and aliquot 21 mL in another centrifuge tube for 1 vial (15 million frozen cells).
5. Set up an area for thawing cryovials using a room-temperature bath.
6. Once the cryovials are taken from the liquid nitrogen storage tank, place them immediately into a water bath with gentle shaking for 2 minutes or until their contents freely move and/or a small ice crystal remains.
7. Pour the contents of cryovials into the recovery medium in centrifuge tubes. Once you poured the content of cryovials, pipette 1 mL of recovery medium to each cryovial to re-suspend any remaining cells, invert once to mix and then pour the re-suspended 1 mL into the centrifuge tubes.
8. Invert tubes once and centrifuge them at 500 rpm for 5 minutes at 4 °C.
9. Aspirate supernatant being careful not to disturb cell pellet.
10. Quantify sufficient to make a volume of 5 mL with L-15 medium and re-suspend cell pellet by tapping side of centrifuge tube against finger/hand. Note: If there are more than 2 centrifuge tubes (based on the number of vials thawed), combined cells into 2 centrifuge tubes and quantify sufficient to make a volume of around 45 mL with L-15 medium for each.
11. Invert tubes once and centrifuge at 500 rpm for 5 minutes at 4 °C.
12. Aspirate supernatant being careful not to disturb cell pellet.
13. Quantify sufficient to make a volume of 5 ml with L-15 and re-suspend cell pellet by tapping side of centrifuge tube against finger/hand. Combine cells into one centrifuge tube and quantify sufficient to make a volume of 45 mL with L-15 medium.
14. Invert tube once and centrifuge at 500 rpm for 3 minutes at 4 °C.
15. Aspirate supernatant and suspend cells in approximately 0.75 mL of L-15 medium per cryovial thawed.
16. Count cell density and cell viability using Trypan blue. Note: the cell viability should be higher than 80%.
17. Calculate the percentage of recovery: total number of cells/total number of cells cryopreserved) x 100. Note: the recovery percentage should be around 20%.

Appendix III. References of the Published Articles

Article 1 (cited in Chapter 2). Rodriguez-Sanchez N, Cronin MTD, Lillicrap A, Madden JC, Piechota P, Tollefsen KE. 2014. Development of a list of reference chemicals for evaluating alternative methods to in vivo fish bioaccumulation tests. *Environ Toxicol Chem* 33:2740-2752. http://onlinelibrary.wiley.com/doi/10.1002/etc.2734/full

Article 2 (cited in Chapter 4). Rodriguez-Sanchez N, Schultz TW, Cronin MTD, Enoch SJ. 2013. Experimental verification of structural alerts for the protein binding of cyclic compounds acting as Michael acceptors. *SAR QSAR Environ Res* 24:963-977. http://www.tandfonline.com/doi/abs/10.1080/1062936X.2013.820793#.VTDemU10zcs