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Title:

Development of a list of reference chemicals for evaluating alternatives methods to *in vivo* fish bioaccumulation tests

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Abstract

The desire to reduce the number of animals used in experiments has highlighted the need to standardise and validate *in vitro* methods as alternatives to bioaccumulation studies using fish. The present work details a process based on five criteria to develop a list of reference compounds to evaluate alternative test methods to standard assays using rainbow trout (*Oncorhynchus mykiss*). The approach was based on: 1) inclusion of relevant chemical classes for bioaccumulation and supported by data on bioconcentration factor (BCF), whole body biotransformation rate (K_{met}) and metabolic pathways (criteria 1-2); 2) cover a broad range of bioconcentration potencies, logarithmof octanol-water coefficient (Log Kow), metabolic susceptibility, molecular weight and maximum molecular diameter (criteria 3-4); and 3) identification of chemicals that are unsuitable for *in vitro* testing according to cut-off values for hydrolysis, volatility in solution and lipophilicity (criterion 5). *In silico* techniques were employed to predict maximal log BCF, K_{met} and the metabolic pathway for those chemicals for which *in vivo* data for some of these properties were not available. Of the 139 compounds considered as reference compounds, 51 were supported by high quality *in vivo* BCF, 22 compounds were supported by either *in vivo* K_{met} or metabolic biotransformation data and ten chemicals did not pass volatility and lipophilicity cut-off values. The list of reference compounds is anticipated to provide a transparent basis for future experimental assessment of the applicability of alternative methods for bioaccumulation assessment within the larger scientific community.

Keywords: Bioaccumulation, reference list, Bioconcentration factor, Alternative testing, *In vitro*

Introduction

The potential of a compound to bioaccumulate is one of many hazardous properties that needs 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 environment and diet), the bioaccumulation of chemicals is usually expressed by the bioconcentration factor (BCF) that refers only to its accumulation from the environment in a waterborne exposure. In aquatic risk assessments, BCFs have been measured in fish according to the Organisation for Economic Cooperation and Development (OECD) Test Guideline305 [1-2].

In vivo test systems for bioaccumulation are demanding in terms of resources and the use of large number of animals per test substance. Coupled with this, compliance with legislation such as the European Union REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) regulation [3] has the potential to increase the demand for animal testing to assess bioaccumulation for a large number of chemicals. Other methods such as *in silico* (computer-based) and *in vitro* techniques have been proposed as alternatives to *in vivo* testing since they comply better with the principles of the 3Rs (reduction, refinement and replacement) for animal testing [4].

In silico models for bioaccumulation have been developed for more than 30 years, mostly in the form of Quantitative Structure-Activity Relationships (QSARs) [5]. As chemical bioaccumulation is a steady-state phenomenon controlled predominantly by passive diffusion processes and lipid partitioning, the majority of these mathematical models have been based on relationships between the observed log BCF and hydrophobicity, often represented by the logarithm of n-octanol/water partition coefficient (log Kow). Whilst there is a strong relationship with hydrophobicity, the maximum bioconcentration of a chemical may be reduced by ionisation, poor chemical bioavailability in the water column and others factors that are

associated with the Absorption, Distribution, Metabolism and Excretion (ADME) properties of the chemicals [6-7].

Of the ADME properties, absorption and metabolism have been implicated as factors introducing uncertainty into models for bioaccumulation [8]. To deal with factors that affect chemical absorption, *in silico* approaches have considered molecular properties to screen chemicals with limited bioaccumulation as a result of molecular constraints. In particular, molecular weight (MW) and maximum inter-atomic distance between two atoms in the chemical structure (D_{max}) have been demonstrated to be useful descriptors [9-10]. Molecular descriptors have resulted in a variety of molecular cut-off values; however, there has been little consensus in the use. This can be explained partly by the fact that other features such as low bioavailability and extensive biotransformation of chemicals may also contribute to reduce bioaccumulation of large molecules [11]. To deal with uncertainties associated with metabolism, modelling studies have been incorporated chemical biotransformation data into the log Kow-based models to correct for the effect of metabolism in aquatic bioaccumulation [12]; however, the prediction of metabolic susceptibility employed have been based on mammalian predictions due to the lack of metabolic *in vivo* data for fish.

A variety of fish cell-based methods have been developed to study the biotransformation of chemicals, mainly based on a depletion approach to calculate the hepatic clearance rate [8]. *In vitro* hepatic clearance data can be incorporated into physiologically-based models that allow for the extrapolation to whole animal biotransformation rates (K_{met}) and the prediction of BCF [13-14]. *In vitro* test systems can also provide specific information on the metabolic pathway of a compound by identifying its resulting metabolites [15]. Although standardised protocols for subcellular fractions (S9) and primary hepatocytes spheroids in rainbow trout (*Oncorhynchus mykiss*) have recently been proposed [16-17], the applicability of *in vitro* assays for assessing chemical bioaccumulation is currently limited by methodological and technical

shortcomings as well as assay variability [18]. 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 [19].

In order to ensure that non-animal methods can be used as surrogates for whole fish testing, the establishment of a high quality and well-parameterised relationship between *in vivo* and estimated data is required. A small number of such comparisons have been reported for bioaccumulation assessments [20-21], but they have been applied to a limited selection of chemicals. Therefore, a representative list of chemicals for bioaccumulation, chosen on the basis of defined criteria, is required in order to allow a scientifically transparent process for future data comparisons.

The aim of this study was, therefore, to develop a list of reference compounds for rainbow trout for the evaluation of alternative methods as a potential surrogate, or compliment, to *in vivo* studies to assess chemical bioaccumulation. The development of a reference list was conducted according to a set of criteria that were applied to include a variety of chemical classes supported by data on BCF, K_{met} and their potential biotransformation pathways. A broad coverage of log Kow, range of bioconcentration potential and molecular properties (MW and D_{max}), and the identification of benchmark (control) chemicals and others with potential *in vitro* difficulties based on key physico-chemical properties (hydrolysis, volatility in solution and lipophilicity) were also pursued. This study shows the importance of *in silico* techniques to assist in the creation of the reference list of chemicals by the use of established *in silico* models and software for the prediction of chemical properties considered.

Materials and methods

Strategy for developing a reference list of compounds

The development of a list of reference compounds was conducted according to the following criteria:

1. To include different chemical classes that were established to cover a broad range of metabolic reactions studied in fish and chemicals of environmental concern.
2. To identify chemicals supported by *in vivo/in silico* data on BCF, K_{met} and metabolic pathway for rainbow trout.
3. To cover a broad range of lipophilicity and bioconcentration potential.
4. To cover a broad range of molecular properties and metabolic susceptibility.
5. To identify chemicals with *in vitro* testing difficulties according to cut-off values for hydrolysis, volatility in solution and lipophilicity.

These criteria were established using expert judgement based on previous criteria of the validity of the test procedures [22] and specific considerations for chemical bioaccumulation.

Criterion 1

Due to *in vitro* metabolism assays becoming more frequent in bioaccumulation studies, the first step in the strategy was the selection of the chemical classes that should be included in the reference list to cover all biotransformation routes characterised in fish [23]. Table 1 shows the 18 chemical groups that were considered in this study with their main metabolic reaction and enzymes. Of these, Polycyclic Aromatic Hydrocarbons (PAHs) represent one of the most studied groups of chemicals using *in vitro* methods. For instance, benzo(a)pyrene is usually taken as a benchmark compound for the development of clearance assays such as S9 [16] and primary hepatocytes [20]. It should be noted that although little is known on fish metabolism for some chemical classes such as heterocyclic compounds, they represent a group of interest for research because of their wide agrochemical and pharmacological applications [24]. Other chemicals such as polychlorinated biphenyls (PCBs) and organophosphates (OP) were also included in this study since they have been considered chemicals of environmental concern.

[Table 1 here]

Criterion 2

The next step in the strategy was the selection of the appropriate fish species from which to obtain *in vivo* data, bearing in mind that *in vivo* data are available for many fishes including freshwater and marine species. Rainbow trout was chosen as being one of the eight OECD recommended test species for conducting flow-through *in vivo* bioconcentration studies [1-2] and for which different alternative approaches have been proposed [8].

Once the fish species was chosen, chemicals supported by *in vivo* data on BCF, K_{met} and knowledge of the metabolic pathway for rainbow trout were compiled from different sources of information such as the scientific literature and BCF databases. In selecting chemicals based on available *in vivo* BCF data, those BCF values with the highest quality/reliability score assigned by the parent databases (refer to Table 2) and measured under the same experimental conditions were preferred. The experimental considerations were: 1) analytical determination of tissue concentrations of the test compounds in whole fish (wet weight) and; 2) experimental tests being conducted in a flow-through system and using the steady-state method for the calculation of the BCF. In addition, experimental data from organometallic compounds and organic salts were removed from the chemical selection due to the possibility that mechanisms other than hydrophobicity could strongly affect bioaccumulation of a compound [12]. Single BCF values for each chemical were obtained by averaging the multiple data points after the removal of statistically significant outliers and single BCF values for a test concentration. It should be noted that compounds with coefficient of variation (CV) of the reported BCF data higher than 0.5 and those presenting inconsistencies with their analogue chemicals were not considered further for the development of the reference list.

When there were no *in vivo* BCF, K_{met} and metabolic pathways data for a compound on the reference list, *in silico* techniques were used to predict these properties. These involved: 1) the bilinear model developed by Bintein et al [25] (stated as Equation 1) to build a maximal log BCF model (log BCF_{max} model) for rainbow trout; 2) the Arnot et al [26] QSAR model

developed from fish *in vitro* metabolism data to predict K_{met}; and 3) Meteor, a commercial software for the prediction of the metabolic pathway of chemicals.

Reference chemicals were thus classified into four types of compounds according to the presence of *in vivo* or *in vitro* data for BCF, K_{met} and metabolic pathways as is shown in Figure 1.

[Figure 1 here]

Criteria 3 and 4

These criteria refer to achieving a broad range of lipophilicity (expressed by log Kow), bioconcentration potencies, molecular properties and metabolic susceptibility. Log Kow was selected amongst other physico-chemical descriptors due to its strong influence on BCF [5]. To establish a range of bioconcentration potencies, Gold-Standard BCF compounds were classified into three ranges depending on the difference between their reported *in vivo* BCF data and the predicted maximal BCF values. As a difference of 0.5 log BCF is assumed reasonable to account for the variability resulting from experimental procedures [27], compounds whose residuals were lower than 0.5 log units for this maximal log BCF were considered well-predicted by log Kow. In this manner, compounds whose residuals were between 0.5 and 1 log units were considered moderately over-predicted, and compounds whose residuals were greater than 1 log unit were classified as highly over-predicted by the model. Among molecular descriptors, MW and D_{max} were selected as they have been used widely to investigate the effect of molecular mass and size on chemical bioaccumulation [9-10]. Finally, predicted K_{met} data were used as a measure of metabolic susceptibility.

Criterion 5

The last criterion was established to ensure chemical stability during the experimentation due to as this is considered one of the essential criteria for the validity of the test procedures [22]. Therefore, the identification of compounds that may be subject to abiotic degradation and/or

potentially significant adsorption to the test vessels was required to ensure their stability in *in vitro* test systems. The following chemical properties were considered relevant for the bioavailability and stability of compounds in the water phase: 1) volatility in solution (expressed by Henry's Law constant (HLC)); 2) hydrolysis (expressed by half-life (HL) in water); and 3) lipophilicity (log Kow). The cut-off values for these properties were applied to identify compounds that were highly volatile in solution (log HLC <-11 atm$(molL^{-1})^{-1}$), readily hydrolysed (HL <12 hours), and highly lipophilic (log Kow>8). The cut-off value for log HLC was taken from the physico-chemical constraints or indicators of low bioaccumulation proposed by Nendza et al [28]. The guidance on bioconcentration and bioaccumulation for the implementation of the REACH legislation [18] provided the cut-off value for HL on the basis of the assumption that the rate of hydrolysis of chemicals should be greater than 12 hours for it to be sufficiently absorbed by the organisms being exposed. The cut-off value of log Kow was taken from the analysis of the relationship between log Kow and *in vivo* log BCF of rainbow trout compounds conducted in this study, representing a potential threshold where reliable log BCF predictions could be obtained.

Data extraction

Reference chemicals were compiled from different sources of information. A thorough literature search was conducted to compile chemicals with *in vivo* data on K_{met} and metabolic pathways for rainbow trout. However, a wider coverage of literature, involving other species and bioaccumulation endpoints, was needed with the aim to include all relevant chemicals established for the development of the reference list of chemicals (Table 1) and cover a broad range of chemical properties considered (criteria 3 and 4).

Chemicals selected based on high quality *in vivo* BCF values for rainbow trout were obtained from the Environment Canada Domestic Substance List (DSL) and non-DSL Environment Canada databases, both reviewed by Arnot et al. [29] and the EURAS-CEFIC database [30].

Table 2 lists the general features of the different databases in terms of their availability and format, BCF data contained therein and the score used to assess the quality of the data. It should be noted that although the databases differ in the number of criteria and scoring system, they all covered the crucial aspects reported in the guidance proposed by Parkerton et al [31] for evaluating *in vivo* fish BCF data. Such aspects include the correct analysis of test substance in both fish tissue and exposure medium, no significant adverse effects on exposed fish and achievement of steady-state with unambiguous units.

[Table 2 here]

In silico tools

Calculation of physico-chemical descriptors

Chemical structures of the compounds considered were obtained from the EPISuite v. 4.1 and were recorded as SMILES strings. SMILES strings were entered into different EPISuite models to calculate: 1) Log Kow from KOWWIN v.1. 68; 2) HLC from HenryWin v. 3.20; and 3) HL from the Fugacity model V.

Calculation of molecular descriptors

KOWWIN v.1. 68 was used to calculate the MW of chemicals. D_{max} data were calculated from the geometry optimised 3-D structures (in xyz format). The 3-D structures were obtained using a Python v 2.7.3 script. The 3D geometries were generated using OpenBabel v. 2.3.2 (http://:www.openbabel.org), accessed using Python via the Pybel module v. 1.8, and locally optimised using the MMFF94 force-field [32]. 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; D_{max} being 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.

Identification of outliers

Outliers for multiple BCF data were identified using the boxplot graph representation in the SPSS software v.18 (http://www.spss.co.in). In this simple analysis, outliers were identified as non-normally distributed when identified outside the T-bars (95% confidence intervals of the data).

Development of a max log BCF model

In order to calculate the potential of maximal bioconcentration (log BCF_{max})for those chemicals that did not have *in vivo* BCF data for rainbow trout (see Figure 1), the development of a log BCF_{max} model was required. Equation (1), developed by Bintein et al [25], was re-built for a subset of chemicals that were supported by the highest *in vivo* BCF values using the Minitab v. 16 statistical software (http://www.minitab.com). In addition, the log BCF_{max} model developed allowed for the identification of benchmarks or positive controls on the basis of their good correlation with log Kow (a difference of 0.5 log units between their predicted log BCF values log and observed log BCF).

$$\log BCF = 0.91 \log Kow - 1.97 \log (6.8\ 10^{-7}\ Kow + 1) - 0.79 \quad (1)$$

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

Where:

n is the number of observations

r^2 is the square of the correlation coefficient

s is the standard error

F is Fisher's statistic

The model described by Bintein et al [25] was selected in preference to others due to the fact it was obtained using BCF values for freshwater fish (1/3 for rainbow trout), measured in whole fish (wet weight) and under flow-through conditions.

Prediction of metabolic-related properties

K_{met} data estimated for a one kg fish were obtained from BCFBAF v.3.01 model of EPISuite and which is based on the QSAR model developed by Arnot et al [26].

The prediction of metabolic pathway and resulting metabolites was made using the Meteor software (Lhasa Limited, Leeds, England (www.lhasalimited.org/meteor/). Three levels were selected for the analysis: probable, plausible and equivocal. The structure of parent compounds were entered into .sdf format and the resulting metabolic pathway and metabolites were stored being available in the Supplementary Information.

Results and discussion

This study aimed to develop a list of reference compounds for the development, assessment and validation of the performance of alternatives methods to *in vivo* bioaccumulation studies for rainbow trout. As no official guidance is provided for conducting such a selection process, the current study presents a novel approach to identify, select and evaluate reference compounds.

Similar to other chemical selection strategies in toxicity studies [33-35], the strategy followed in this study was based on a list of criteria established and the use of *in silico* techniques to assist in the selection process. It should be noted, however, that whilst for toxicity studies there is a need to consider the toxic mechanism and/or mode of action to ensure either consistency or diversity, bioaccumulation is governed by ADME processes that are more clearly linked to physico-chemical and molecular properties.

According to the five criteria detailed above, a total of 139 chemicals were considered as being the best candidates for the development and assessment of non-animal methods for bioaccumulation (Table 3). Reference chemicals included the 18 chemicals classes listed in

Table 1 and a broad range of lipophilicity (log Kow: -2.25 to 12.11), bioconcentration potential (W, O1, O2), MW (30 to 959 g/mol), D_{max} (0.18-2.65 nm) and metabolic susceptibility (K_{met}: 0 to 37.6). Details of the metabolic pathway and resulting metabolites for each compound are provided in Supplementary Information. Approximately half of the reference compounds were supported by *in vivo* data for some of these properties, and therefore they were considered Gold Standard compounds due to their role in the evaluation of the applicability of alternative methods. A set of 10 compounds were identified as a challenge for *in vitro* testing due to they did not pass the cut-off values for log HLC and log Kow.

[Table 3 here]

Gold Standard-BCF compounds

Initially, investigation of the databases identified 354 *in vivo* BCF values for a total of 59 chemicals that were obtained under the same experimental conditions and assessed with the highest reliability score. Table 4 lists the number of BCF values for individual chemicals, CV, the database from which they were retrieved and experimental features such as test concentration. As can be seen, the Environment Canada DSL and Non-DSL Databases contributed in approximately equal terms to the total number of experimental data, whereas a low percentage of compounds were in common between the EURAS-CEFIC database and either of the Environment Canada databases. Moreover, the majority of these compounds were found to be halogenated benzenes (40 %) and chloronitrobenzenes (20%) with a small number of compounds of environmental concern such OPs (**53**).

[Table 4]

BCFs values for 79 chemicals failed to meet one or more of the established quality/reliability criteria of the databases [29-30] and thus they were not considered for the creation of a reference list of chemicals. Some examples of these unreliable compounds include the toxic effects reported for two dioxin-like compounds (e.g. tetradioxin), uncertain correction of the radiolabel

337 analysis for the parent compound for some organophosphates (e.g. tricresyl phosphate) and
338 insufficient exposure duration to achieve 80% of steady-state for the majority of
339 polychlorinated compounds (e.g. mirex).
340 Only experimental data for rainbow trout were considered in order to avoid the variability in
341 BCF that may be caused by data obtained from different species. Such variability of fish species
342 may be a result of differences in biological factors and uptake kinetics [36]. However,
343 differences in organism size and lipid content of the same fish species may explain the BCF
344 variability obtained for the same compounds. Other factors such as strain, culturing conditions
345 and different metabolic capacities due to different feeding regimens or/and seasonal variation
346 could potentially explain some of the variability found in *in vivo* BCF data; however, they were
347 not assessed due to the lack of such data in the original databases.
348 Multiple BCF values were obtained for the majority of chemicals (Table 4). Compounds with
349 CV > 0.5 were not included in the list of reference chemicals. Of these 59 chemicals, six
350 compounds (**20, 21, 23, 35, 31, 37)** had CV higher values than the established and thus were
351 rejected. Additionally, 1,4-dichlobenzene (**12)** and 1,3,5-trichloro-2-ntirobenzene (**16)** were
352 rejected as some discrepancies were found in comparison with their analogues.
353 Single BCF values for each chemical were obtained by averaging the multiple data points after
354 removal of statistically significant outliers. Furthermore, single data for a test concentration
355 were also rejected for the average of the multiple BCF data points. Figure 2 shows the box plot
356 representation of the range of BCF values for the compounds considered. Three statistical
357 outliers were identified (values for compounds **14, 24** and **33)**, which were excluded from use
358 in the calculation of the average values for these compounds.
359 [Figure 2 here]
360 *Development of a log* BCF_{max} *model for rainbow trout compounds and assignment of*
361 *bioconcentration potencies*

The 51 Gold Standard-BCF compounds obtained above were used to build a max log BCF model for rainbow trout compounds. Equation 1 (stated above) was modified to accommodate a subset of *in vivo* BCF data (represented as open circles in Figure 3). The bilinear log BCF_{max} model built is shown as a solid line in Figure 3 and was calculated using Equation 2. This model represents the worst-case scenario of bioconcentration driven by passive diffusion processes and which should be considered specific for rainbow trout.

$$\text{Log } BCF_{max} = 0.88 \log Kow - 1.73 \log (2.25\ 10^{-6}\ Kow + 1) - 0.08 \quad (2)$$

It should be noted that the data used in Equation 2 (six compounds in total) were selected a priori to obtain the maximal BCF value and, therefore, there is no statistical significance to this relationship.

[Figure 3 here]

Compounds were classified into three bioconcentration potency ranges depending on the difference between their reported *in vivo* BCF data and predicted maximal BCF values (well-predicted (residuals<0.5); moderately over-predicted (residuals=0.5-1) and highly over-predicted (residuals>1)); Following this rationale, 29 compounds were classified as being well-predicted compounds, 9 to be marginally over-predicted and another 13 substances were identified as being significantly over-predicted.

The majority of well-predicted compounds were neutral compounds such as biphenyls (**38-40, 45, 48**), halogenated benzenes (**11, 13, 17, 19, 32-34, 41, 56)** and alkylbenzenes (**24, 25, 30**). This observation is supported by the lack of polar groups in the chemical structure that may make them less susceptible to a metabolic attack [5]. Following the same rationale, most compounds that were moderately over-predicted by Equation 2 were polar compounds such as nitrochlorobenzenes (for example compounds **6-8)**. However, hydrophobic compounds (log Kow <3) with polar groups in their structure (**2-5**) were also well predicted by Equation 2. This finding indicates that the high biotransformation potential of hydrophilic compounds is unlikely

387 to affect their bioaccumulation significantly. This is in agreement with previous *in silico*
388 predictions that observed that high rates of chemical flux across the gills could be more
389 significant than the biotransformation rates for bioaccumulation of hydrophilic compounds [15,
390 37].
391 Highly over-predicted compounds included other nitrobenzenes (**14, 15, 28**), triphenyl
392 phosphite (**53**), pentabromomethylbenzene (**54**) and ionic compounds such as phenolics **(18,**
393 **36**) and hydrophobic organic acids (**49-52, 55, 58**). The low observed log BCF of the OP
394 compound may be a result of metabolism, since modelling studies that have shown that
395 relatively low biotransformation rates may have a large influence on bioaccumulation for
396 hydrophobic compounds [15,37]. As expected, the observed log BCF of ionisable compounds
397 in this study was low, as the bioaccumulation of ionisable compounds is not primarily driven
398 by hydrophobicity [6]. Rather, a mechanistic model for the uptake and elimination of ionisable
399 compounds via fish gills [38] showed that although ionisable compounds are less bioavailable
400 than neutral species, in terms of crossing biological membranes, they can maintain a high
401 diffusion across epithelial cell membrane which is comparable to neutral molecules.
402 Consequently, descriptors other than log Kow have been considered in recent *in silico* studies
403 to improve the predictions of bioaccumulation for ionisable compounds. Alternative descriptors
404 include the logarithm of the distribution coefficient (log D), which is the ratio of concentration
405 of unionised forms of a compound in octanol and the total concentration of unionised and
406 ionised forms in water [39-40].
407 It is worth noting that due to the fact that the bioaccumulation of a compound is a complex
408 function comprising diverse physiological and biological processes, the reduced
409 bioconcentration of some of these highly over predicted substances could be associated with
410 more than one factor [11]. For example, the relatively high molecular size (D_{max}= 1.16 to 1.20),

411 such as that of carboxylic acid compounds (**49-52, 55),** may also have contributed to reduction
412 in BCFs.
413 Gold Standard- metabolic compounds
414 Of the 139 reference compounds listed in Table 3, 22 were classified as Gold Standard
415 metabolic compounds including Gold Standard -K_{met} and MP compounds. Table 5 lists the *in*
416 *vivo* data and other experimental details, amongst others, type of exposure, uptake phase and
417 test concentration. In particular, eight pesticides (triazoles) (**1-8**) [41], two insecticides (**9-10**)
418 [42] and four PCBs (**11-14**) [43] were found with *in vivo* K_{met} data determined through a dietary
419 exposure using juvenile rainbow trout. The K_{met} data were calculated by comparing their HL
420 with known recalcitrant PCBs in non-linear relationship between log Kow and HL developed
421 by Fisk et al [44]. Based on this approach, chemicals whose HL fall on, or near, this non-linear
422 relationship are assumed to not undergo high metabolism processes (recalcitrant), whereas
423 those chemicals that fall below this relationship are suggested to be biotransformed. This
424 method allows for the quantification of the biotransformation rates of organic chemicals that
425 are tested using the same experimental conditions.
426 A total of eight chemicals were compiled from the literature whose resulting metabolites were
427 analysed in an *in vivo* system, and which are referred to Gold Standard-MP compounds in Table
428 3. As Table 5 shows, these chemicals included four perfluoroalkylated compounds (**15-18**) [45-
429 46], decabromodiphenyl ether (**19**) [47] and three carboxylic acid pharmaceuticals (**20-22**) [48-
430 49]. Although few metabolites were monitored for each compound, the whole
431 biotransformation pathway was proposed for compounds **18**,**20,21,22.** Depending on the study,
432 different routes of exposure (dietary, waterbone, intraperitoneal injection) as well as fish tissues
433 for analysis (muscle, blood, liver bile, kidney) were used to investigate the biotransformation
434 pathways of Gold Standard-MP compounds. Worthy of mention is that both aspects may
435 influence the formation and accumulation of resulting metabolites from the parent compound.

For instance, a different metabolic pattern was found for decabromodiphenyl ether (**19)**, where debrominated diphenyl ethers metabolites (De-BDEs) were the main metabolites in liver, whereas methoxylated diphenyl ethers (MeO-BDEs) were found in higher concentration in blood [47]. It should be noted that different metabolites of ibuprofen (IBF) were found by comparing two types of exposure: a waterborne exposure with four additional pharmaceuticals [48] and on its own [49]. Whilst the hydroxylated and acyl glucuronide metabolites of IBF were reported in both studies, taurine conjugates of IBF were only reported in organisms that were exposed to a single waterborne exposure of IBF [49].

[Table 5 here]

Supplementary compounds

Although this study prioritised the selection of chemicals for *in vivo* data for rainbow trout, not all chemical classes listed in Table 1 were covered. As it was observed above, *in vivo* BCF compounds were mostly halogenated aromatic chemicals. This lack of diversity for some types of chemicals such as reactive compounds, could be explained by the fact such chemicals are likely to cause higher mortalities and adverse effects than the 10% of the limit established for the validity of OECD protocols [1-2] and hence will not be good candidates for *in vivo* bioaccumulation assessments. This observation is supported by the toxic effect reported for dioxin-type compounds described above. Nonetheless, the identification of the lack of *in vivo* data for certain chemical classes could provide a basis for the selection of chemicals for future *in vivo* BCF testing in rainbow trout [2].

To facilitate the correct development and validation of alternative methods for bioaccumulation, 67 compounds were added to the list to cover all relevant chemical classes presented in Table 1. Generally, the complementary chemicals were extracted from the review of biotransformation in fishes [23], metabolism studies on different species such as mammals and others using rainbow trout aimed to provide additional information for future *in vitro* assays.

Supplementary chemicals encompassed a set of halogenated compounds (**6-9**) [50],six PAHs (**42-47**) [51], five heterocyclic compounds (**58-62**) [23,24,52] the majority of OPs (**66-69**) [53], and the complete set of organosulfur compounds **(71-77)** [23,54-56], amines and amides (**99-114**) [23,52,57], aldehydes (**115-118)** [58-59], alcohols (**119-122**) [23,60-61], quinones (**127-131**) [23,62-64], epoxides (**132-136**) [65] and polyunsaturated fatty acids (**137-139)** [23]**.**

Compounds with testing difficulties

All 139 compounds compiled in Table 3 were screened according to the cut-off values defined in the last criterion of our strategy. Of these, six compounds (**57,61,62,84,106,114**) had log HLC lower values than the established cut-off, and four chemicals (**29,41, 137,139**) did not pass the criteria for lipophilicity. Since these compounds may be highly volatilite in solution and there may be potentially significant adsorption to the test vessels respectively, special considerations should be taken into account to ensure their chemical stability in the *in vitro* assays. It should be noted that although all compounds passed the criteria for hydrolysis, chemicals with HL of 208 hours (**100,101,119**), and even those with values of 360 (as indicated in Table 3) could require further attention in long-term assays to avoid the loss of the parent compound.

Other reported properties that may limit the bioaccumulation of chemicals, such as ready biodegradability and phototransformation were not taken into account in this study. This is due to the fact that readily biodegradable molecules can bioaccumulate if their uptake rate is greater than the rate of degradation [19], and for phototransformation processes are expected to be less significant under laboratory lighting conditions than under field conditions [31].

List of reference compounds: Further considerations and implications

The present list of 139 chemicals (Table 3) could undergo a refinement of the chemical selection process under project-specific requirements. For instance, other essential criteria for the selection of test chemicals [22] such as known and high consistent purity and commercial

availability should be applied to the present list of chemicals to select a set of compounds for *in vitro* testing. Moreover, the possibility of a compound to be quantifiable by an analytical method and its existing *in vitro* data for rainbow trout (as indicated in Table 3) could be also taken into account in the making-decision process.

When selecting chemicals within the same chemical group, chemicals with broader values for log Kow, molecular properties and K_{met} should be selected with the aim to ensure a wider domain for these properties according to criteria 3 and 4 of this study. Furthermore, additional compounds can be added to the list expanding the chemical domain as appropriate.

Previous work has assigned positive controls in reference lists proposed for the development of alternatives methods to *in vivo* testing [33, 35]. Of the 139 reference chemicals presented in this study, chemicals with a neutral (non-ionised) structure that were well-predicted by Equation 2 (identified in Table 3) could be considered as positive controls or benchmark compounds since their bioaccumulation is expected to be driven mainly by passive diffusion processes. However, and no less important, is the consideration of the over-predicted chemicals (O1 and O2 compounds in Table 3)due to the fact they might be susceptible to moderate metabolism (e.g. nitrocholobenzenes) and/or poor bioavailability (e.g. ionisable compounds); and thus *in vitro* test systems for metabolism may assist in elucidating where significant biotransformation processes impact on BCF, helping to clarify uncertain *in vivo* OECD measurements. Similarly, future development and improvement of aquatic non-animal tests for absorption could provide more information on the uptake processes of low bioavailable chemicals and a better understanding of molecular constraints on chemical absorption at a cellular level.

We believe that a successful development and validation of fish *in vitro* assays is the key for the correct use of non-animal methods in bioaccumulation tests. This is due to fact the various benefits can be obtained from the validation of such assays.. For instance, accurate *in vitro* data could enhance the knowledge of *in vivo* absorption and metabolism processes, allowing a better

511 understanding of how both processes can influence *in vivo* assessment of chemical
512 bioaccumulation in fish. In addition, *in vitro* metabolic data could be incorporated into the log
513 BCF_{max} model developed for rainbow trout compounds to correct for the effect of metabolism
514 on bioaccumulation and refine the estimates of k_{met} and metabolic pathways. And from
515 regulatory perspective, *in vitro* assays potentially could be used together with *in silico* methods
516 in a tiered approach to prioritise chemicals for future *in vivo* testing in order to reduce animal
517 use.

518 **Conclusions**

519 There is an urgent need to develop and validate non-animal methods to assess bioaccumulation
520 of chemicals in fish. A successful development of alternative test systems to *in vivo* testing
521 could provide not only accurate information on ADME processes for a given compound, but
522 also they could be used in risk assessment procedures to reduce the number of fish for
523 experimentation.

524 The present work has introduced a fully transparent description of an approach applied to
525 develop a list of reference chemicals for the development of non-animal methods to assess
526 chemical bioaccumulation. The rationale employed in this study was based on five established
527 criteria. An *in silico* approach was required to develop a log BCF_{max} model for rainbow trout,
528 explore the bioconcentration potential of examined chemicals and assist in the development of
529 the list of reference compounds. As a consequence of this work, a reference list of 139
530 chemicals including 18 different chemical classes is proposed to facilitate the evaluation of
531 alternative methods to *in vivo* testing for rainbow trout. It is envisioned that using this list of
532 reference compounds may enhance our understanding of the relationship between *in vivo* and
533 *in vitro* data by providing a common basis for experimental effort, and through such effort
534 facilitate the refinement of *in silico* prediction of BCF, K_{met} and metabolic pathways of
535 chemicals for one of the most common fish species used in regulatory testing.

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Figure legends

[Figure 1] Classification of reference compounds into four groups based on the presence of *in vivo* or *in vitro* data on BCF, K_{met} and metabolic pathways.

[Figure 2] Boxplot representation of the range of BCF values for the 59 chemicals listed in Table 4. Outliers represented as open circles.

[Figure 3] Relationship between and log BCF (L/Kg) ww log Kow for the Gold Standard-BCF compounds. Solid line: log BCF_{max} model (Equation 2) developed from a set of chemicals with high values (represented as open circles). Long-dashed line: Log BCF_{max} model-0.5. Short-dashed line: Log BCF_{max} model -1. W: well-predicted compounds (residuals < 0.5 log units), O1: marginally over-predicted compounds (residuals > 0.5), O2: Highly over-predicted compounds (residuals > 1) according to log BCF_{max} predictions.

768 **Figure 1**

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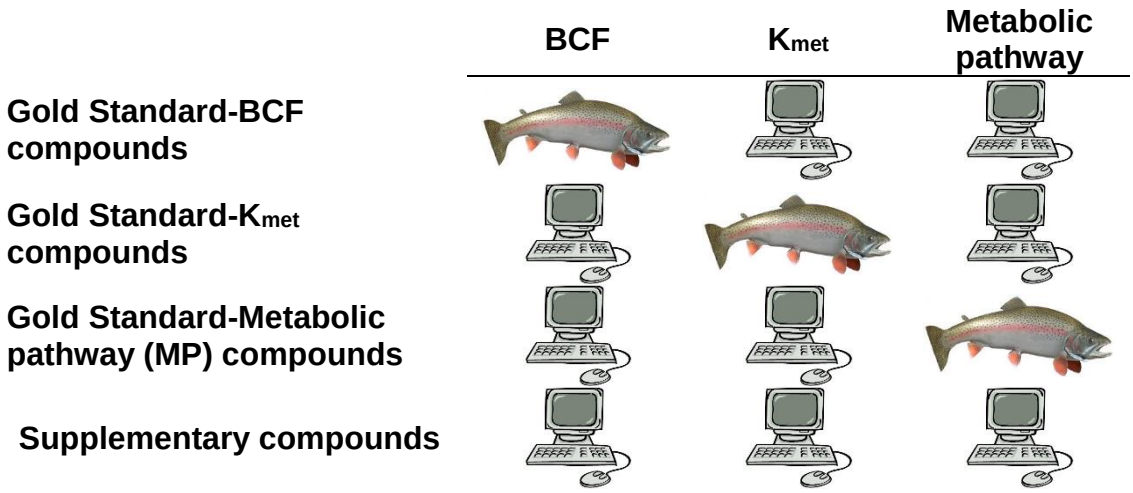

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779 **Figure 2**

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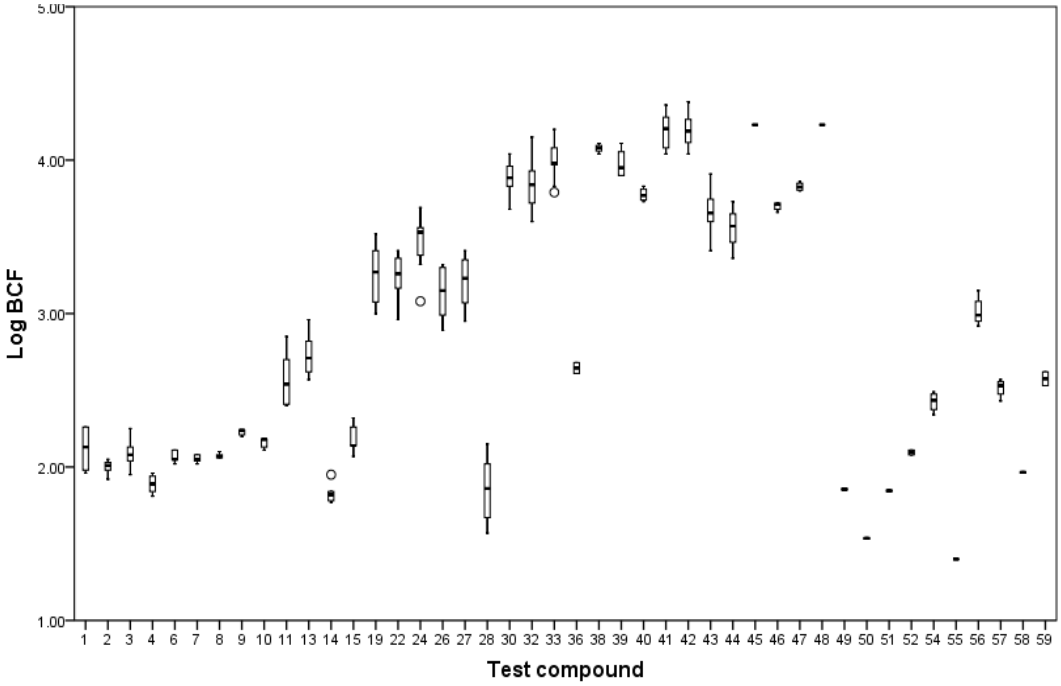

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786 **Figure 3**

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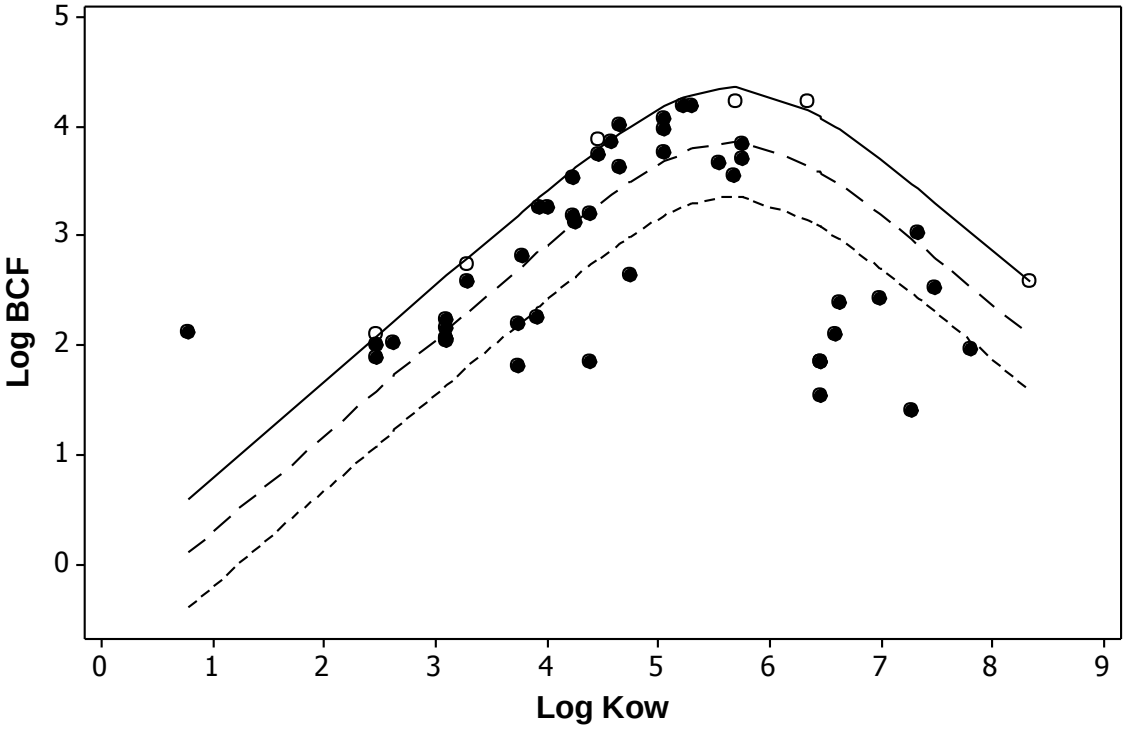

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Table 1. Chemicals classes considered for the development of a reference list of chemicals and their main known biotransformation reactions in fish.

No.	**Chemical class**	**Studied biotransformation reaction (enzyme)** [23]
1	Aliphatic halogenated hydrocarbons	Phase I: Oxidative dehalogenation(CYPs) Phase II: GSH conjugation (GSTs)
2	Aromatic halogenated hydrocarbons	
3	Heterocyclic compounds	Phase I: Oxidation, reduction (CYPs) Phase II: Glucuronidation (UGTs)
4	Polycyclic aromatic hydrocarbons	Phase I: Hydroxylation, (CYPs) Phase II: GSH conjugation (GSTs)
5	Polychlorinated biphenyls	Phase I: Hydroxylation (CYPs) Phase II: Glucuronidation (UGTs)
6	Organosphosphorus	Phase I: Oxidative desulfuration (CYPs) Hydrolysis (CES)
7	Organosulfur compounds	Phase I: Oxidation (FMOs)
8	Carboxylic acids	Phase II: Amino acid conjugation (AAT)
9	Nitroaromatic compounds	Phase I: Reduction (NTR)
10	Aliphatic amines	Phase I: Oxidation (CYPs, MAO,FMOs) Reduction (CYPs) Phase II: Glucuronidation (UGTs) Sulfonation (SULT) Acetylation (Acetyl-CoA)
11	Aromatic amines	
12	Amides	Phase II: Glucuronidation (UGTs)
13	Aldehydes	Phase I: Oxidation (AO, ALDH)
14	Alcohols	Phase I: Oxidation (ADH) Phase II: Sulfonation (SULT)
15	Phenols	Phase II: Glucuronidation (UGTs) Sulfonation (SULT)
16	Quinones	Phase I: Reduction (DTD)
17	Epoxides	Phase I: Hydrolysis (EH)
18	Polyunsaturated fatty acids	Phase I: Oxidation (LPO)

AAT: Aminoacyl transferase, Acetyl-CoA: Acetyl-coenzyme A, ADH: Alcohol dehydrogenase, ALDH: Aldehyde dehydrogenase, AO: Aldehyde oxidase, CES:Carboxylesterase, CYPs: Cytochrome P450, DTD: DT Diaphorase, EH: Epoxide hydrolase, FMOs: Flavin-containing monooxygenase, GSTs:Glutathione S-transferase, LPO: Lipoxygenase, MAO: monoamine oxidase, NTR: Nitroreductasa, SULT: Sulfotransferasa, UGTs: UDP-glucuronosyl transferase.

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808 **Table 2.** A summary and comparison of the data and features of the 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%) autotroph (4%)	Only fish (90% for Common carp)
Score system	1 (high), 2 (moderate), 3 (low)	Klimisch score: 1 (reliable without restrictions), 2 (reliable with restrictions), 3 (not reliable), 4 (not assignable)

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818 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
819 studies.

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
1	1	γ-Lindane	58-89-9	GS-BCF	4.26	290.83	0.68	3.13	0.01	1-2	W, control	1	Nd
2	1,12	Perfluorooctanesulfonamide	754-91-6	GS-MP	5.80	499.14	1.40	4.34	0.01	3,T5		[45]	Nd
3	1,14	10:2 Fluorotelomer alcohol	865-86-1	GS-MP	7.08	564.13	1.60	3.63	0.00	4-5,T5		[45]	Nd
4	1,14	8:2 Fluorotelomer alcohol	678-39-7	GS-MP	5.75	464.12	1.34	4.35	0.01	6-7,T5		[45]	[13]
5	1	8:2 Fluorotelomer acrylate	27905-45-9	GS-MP	7.11	518.17	1.70	3.60	0.01	8-9,T5		[47]	[66]
6	1	Tetrachloromethane	56-23-5	SP	2.44	153.82	0.50	2.06	0.17	10		[50]	Nd
7	1	Trichloroethane	79-00-5	SP	2.01	133.40	0.37	1.68	0.50	11-12		[50]	Nd
8	1	Vinyl chloride	75-01-4	SP	1.62	62.50	0.37	1.34	0.72	13-14	HL=360 h	[50]	Nd
9	1	Dichloromethane	75-09-2	SP	1.34	84.93	0.24	1.09	0.88	15		[50]	Nd
10	2	1,2-Dichlorobenzene	95-50-1	GS-BCF	3.28	147.00	0.56	2.58	0.06	16-17	W, control	1	Nd
11	2	1,3-Dichlorobenzene	541-73-1	GS-BCF	3.28	147.00	0.56	2.74	0.06	18-19	W, control	1	Nd
12	2	1,2,3-Trichlorobenzene	87-61-6	GS-BCF	3.93	181.45	0.59	3.26	0.04	20-21	W, control	1	Nd
13	2	1,2,3,4-Tetrachlorobenzene	634-66-2	GS-BCF	4.57	215.89	0.59	3.85	0.02	22-23	W, control	1	Nd
14	2	Pentachlorobenzene	608-93-5	GS-BCF	5.22	250.34	0.64	4.19	0.02	24	W, control	1	Nd
15	2	1,3-Dibromobenzene	108-36-1	GS-BCF	3.77	235.90	0.60	2.82	0.08	25-26	W, control	3	Nd
16	2	1,3,5-Tribromobenzene	626-39-1	GS-BCF	4.66	314.80	0.60	4.02	0.05	27	W, control	3	Nd

820 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
821 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
17	2	1,2,4-Tribromobenzene	615-54-3	GS-BCF	4.66	314.80	0.60	3.63	0.05	28	W, control	3	Nd
18	2	1,2,4,5-Tetrabromobenzene	636-28-2	GS-BCF	5.55	393.70	0.67	3.67	0.04	29	O1	3	Nd
19	2	Hexabromobenzene	87-82-1	GS-BCF	7.33	551.49	0.67	3.02	0.03	30	W, control	2	Nd
20	2	1,2,4-Trichloro-5-methylbenzene	6639-30-1	GS-BCF	4.47	195.47	0.59	3.88	0.04	31-32	W, control	3	Nd
21	2	1,2,4,5-Tetrachloro-3,6-dimethylbenzene	877-10-1	GS-BCF	5.67	243.95	0.70	3.55	0.03	33	O2	2	Nd
22	2	Pentachlorotoluene	877-11-2	GS-BCF	5.76	264.36	0.75	3.83	0.02	34	O2	2	Nd
23	2	Pentabromomethylbenzene	87-83-2	GS-BCF	6.99	486.62	0.70	2.43	0.02	35	O2	2	Nd
24	2	Pentabromoethylbenzene	85-22-3	GS-BCF	7.48	500.65	0.72	2.52	0.02	36	O1	2	Nd
25	2	2,4-Dichloro-1-(trifluoromethyl)benzene	320-60-5	GS-BCF	4.24	215.00	0.69	3.52	0.02	37	W, control	2	Nd
26	2	1,2-Dichloro-4-(trifluoromethyl)benzene	328-84-7	GS-BCF	4.24	215.00	0.69	3.18	0.02	38-39	W, control	3	Nd
27	2	1,2,3-Trichloro-4-methoxybenzene	54135-80-7	GS-BCF	4.01	211.47	0.75	3.25	0.01	40-42	W, control	3	Nd
28	2	Pentachloroanisole	1825-21-4	GS-BCF	5.30	280.36	0.80	4.19	0.00	43	W, control	2	Nd
29	2	Decabromodiphenyl ether	1163-19-5	GS-MP	12.11	959.17	1.08	-0.62	0.00	44,T5	Highly lipophilic	[47]	[67]
30	3	2,3-Dichloro-1,1'-biphenyl	16605-91-7	GS-BCF	5.05	223.10	0.93	4.08	0.01	45-46	W, control	2	Nd

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824 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
825 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
31	3	2,5-Dichloro-1,1'-biphenyl	34883-39-1	GS-BCF	5.05	223.10	0.93	3.98	0.01	47-49	W, control	2	Nd
32	3	3,5-Dichloro-1,1'-biphenyl	34883-41-5	GS-BCF	5.05	223.10	0.92	3.77	0.01	50	W, control	2	Nd
33	3	2,2',5-Trichloro-1,1'-biphenyl	37680-65-2	GS-BCF	5.69	257.54	0.93	4.23	0.00	51-52	W, control	2	Nd
34	3	2,2',5-Trichloro-1,1'-biphenyl	20020-02-4	GS-BCF	5.75	265.95	0.70	5.23	0.01	53	W, control	2	Nd
35	3	2,2',3,3'-Tetrachloro-1,1'-biphenyl	38444-93-8	GS-BCF and K_{met}	6.34	291.99	0.93	4.23	0.00	54-55	W, control	2, [43]	Nd
36	3	p,p'-Dichlorodiphenyltrichloroethane	50-29-3	GS-K_{met}	6.79	354.49	1.04	3.85	0.01	56-57		[43]	Nd
37	3	2,2',3,3',4,6'-Hexachloro-1,1'-biphenyl	38380-05-1	GS-K_{met}	7.62	360.88	0.89	3.19	0.00	58-60		[43]	Nd
38	3	2,2',3,3',5,6'-Hexachloro-1,1'-biphenyl	52744-13-5	GS-K_{met}	7.62	360.88	0.93	3.19	0.01	61-62		[43]	Nd
39	3	2,2',3,3',6,6'-Hexachlorobiphenyl-1,1'-biphenyl	38411-22-2	GS-K_{met}	7.62	360.88	0.93	3.19	0.00	63-65		[43]	Nd
40	4	1,4-Dichloronaphthalene	1825-31-6	GS-BCF	4.46	197.06	0.77	3.75	0.02	66-67	W, control	3	Nd
41	4	Octachloronaphthalene	2234-13-1	GS-BCF	8.33	403.73	0.78	2.58	0.00	68	W, control. Highly lipophilic	2	Nd

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827 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
828 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Refed
42	4	9H-Fluorene	86-73-7	SP	4.02	166.22	0.84	3.43	0.15	69-70	HL=360 h	[51]	Nd
43	4	Phenanthrene	85-01-8	SP	4.35	178.23	0.87	3.70	0.08	71-72		[51]	[68]
44	4	Anthracene	120-12-7	SP	4.35	178.23	0.90	3.70	0.08	73-74		[51]	Nd
45	4	Fluoranthene	206-44-0	SP	4.93	202.25	0.91	4.11	0.08	75		[51]	Nd
46	4	Benzo(a)pyrene	50-32-8	SP	6.11	252.31	1.04	4.26	0.23	76-77		[51]	[16,20,58]
47	4	Benzo(a)anthracene	56-55-3	SP	5.52	228.29	1.15	4.34	0.07	78-79		[51]	Nd
48	5	Dehydroacetic acid	520-45-6	GS-BCF	0.78	168.15	0.82	2.12	36.91	80	U, HL=360 h	1	Nd
49	5	Myclobutanil	88671-89-0	GS-K_{met}	3.50	288.78	1.10	2.98	0.20	81-82		[41]	[69]
50	5	Propiconazole	60207-90-1	GS-K_{met}	4.13	342.22	1.18	3.52	0.57	83-84		[41]	[69]
51	5	Cyproconazole	94361-06-5	GS-K_{met}	3.25	291.78	1.07	2.77	0.37	85-86		[41]	Nd
52	5	Penconazole	66246-88-6	GS-K_{met}	4.67	284.18	1.01	3.94	0.21	87-88		[41]	Nd
53	5	Metconazole	125116-23-6	GS-K_{met}	4.19	319.83	1.05	3.57	0.58	89-90		[41]	[69]
54	5	Triadimefon	43121-43-3	GS-K_{met}	2.94	293.75	1.14	2.50	0.54	91-93		[41]	[69]
55	5	Tetraconazole	112281-77-3	GS-K_{met}	4.25	372.15	1.14	3.62	0.24	94-96		[41]	Nd

829 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
830 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
56	5	Tebuconazole	107534-96-3	GS-K_{met}	3.89	307.82	1.31	3.32	0.55	97-98		[41]	Nd
57	5	Fipronil	120068-37-3	GS-K_{met}	6.64	437.15	1.21	3.96	1.01	99-100	Highly volatile in solution	[41]	Nd
58	5	1,6-Hexalactam	105-60-2	SP	0.66	113.16	0.51	0.50	11.38	101-102	HL=360 h	[56]	Nd
59	5	Levamisole	14769-73-4	SP	2.87	204.29	1.06	2.43	1.10	103-104	HL=360 h	[24]	Nd
60	5	Paramethadione	115-67-3	SP	1.08	157.17	0.74	0.86	2.92	105-106		[24]	Nd
61	5,9	4-Nitroquinoline oxide	56-57-5	SP	0.82	190.16	0.72	0.64	3.87	107	Highly volatile in solution	[23]	Nd
62	5,9	Nitrofurantoin	67-20-9	SP	-0.17	238.16	1.09	-0.23	37.60	108	Highly volatile in solution	[23]	Nd
63	6	Triphenyl phosphite	101-02-0	GS-BCF	6.62	310.28	1.10	2.39	0.05	109	O2	[23]	Nd
64	6	Diazinon	333-41-5	SP	3.86	304.35	1.11	3.29	0.15	110-112		[23]	[58]
65	6	Chlorpyrifos	2921-88-2	SP	5.11	350.59	1.15	4.21	0.10	113-114		[23]	[21]
66	6	Cyanophos	2636-26-2	SP	2.76	243.22	1.08	2.34	0.15	115-116		[52]	[52]
67	6,7	Fenthion	55-38-9	SP	4.08	278.33	1.03	3.48	0.08	117-118		[52]	[52]
68	6,9	Methyl parathion	298-00-0	SP	2.75	263.21	1.06	2.33	0.22	119-120		[52]	[52]
69	6,9	Chlorothion	500-28-7	SP	3.39	297.65	0.93	2.89	0.14	121-122		[52]	[52]

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833 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
834 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
70	6,9	Parathion	56-38-2	SP	3.73	291.26	1.16	3.18	0.12	123-124		[23]	[58]
71	7	Diphenyl disulfide	882-33-7	SP	4.31	218.34	0.92	3.67	0.29	125-126	HL=360 h	[53]	Nd
72	7	Sulfanilamide	63-74-1	SP	-0.55	172.20	0.76	-0.57	13.79	127		[54]	Nd
73	7	Dibenzothiophene	132-65-0	SP	4.17	184.26	0.85	3.55	0.23	128-129	HL=360 h	[55]	Nd
74	7,10	Thiourea	62-56-6	SP	-1.31	76.12	0.50	-1.23	37.60	130	HL=360 h	[23]	Nd
75	7,10	Aldicarb	116-06-3	SP	1.36	190.26	0.88	1.11	3.21	131-132		[23]	[58]
76	7,12	Thiobencarb	28249-77-6	SP	3.90	257.78	1.36	3.33	1.07	133-134		[23]	Nd
77	7,5	Methimazole	60-56-0	SP	-0.49	114.17	0.53	-0.51	25.46	135	HL=360 h	[23]	Nd
78	8	Pimaric acid	127-27-5	GS-BCF	6.45	302.45	1.16	1.85	0.00	136-138	O2	1	Nd
79	8	Isopimaric acid	5835-26-7	GS-BCF	6.45	302.45	1.15	1.54	0.00	139-141	O2	1	Nd
80	8	Abietic acid	514-10-3	GS-BCF	6.46	302.45	1.16	1.84	0.00	142-144	O2	1	Nd
81	8	Neoabietic acid	471-77-2	GS-BCF	6.59	302.45	1.16	2.10	0.00	145-147	O2	1	Nd
82	8	Palustric acid	1945-53-5	GS-BCF	7.27	302.45	1.20	1.40	0.00	148-150	O2	1	Nd
83	8	12,14-Dichlorodehydroabietic acid	57055-39-7	GS-BCF	7.81	369.33	1.20	1.97	0.00	151-152	O2	1	Nd
84	8	Diclofenac	15307-86-5	GS-MP	4.02	296.15	0.96	3.43	0.03	153-155, T5	Highly volatile in solution	[49]	Nd
85	8	Naproxen	22204-53-1	GS-MP	3.10	230.26	1.21	2.64	0.12	156-158,T5	HL=360 h	[49]	Nd

835 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
836 studies (cont.)

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ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
86	8	Ibuprofen	15687-27-1	GS-MP	3.79	206.28	1.03	3.23	0.11	159-160,T5	HL=360 h	[49-50]	[70]
87	9	1-Chloro-4-nitrobenzene	100-00-5	GS-BCF	2.46	157.55	0.66	2.00	0.34	161-162	W	1	Nd
88	9	1-Chloro-2-nitrobenzene	88-73-3	GS-BCF	2.46	157.55	0.60	2.09	0.38	163-164	W	2	Nd
89	9	1-Chloro-3-nitrobenzene	121-73-3	GS-BCF	2.46	157.55	0.61	1.89	0.32	165-166	W	2	Nd
90	9	1,2-Dichloro-4-nitrobenzene	99-54-7	GS-BCF	3.10	192.00	0.71	2.07	0.19	167-168	O1	1	Nd
91	9	1,4-Dichloro-2-nitrobenzene	89-61-2	GS-BCF	3.10	192.00	0.62	2.05	0.20	169-171	O1	2	Nd
92	9	2,4-Dichloro-1-nitrobenzene	611-06-3	GS-BCF	3.10	192.00	0.62	2.07	0.20	172	O1	2	Nd
93	9	1,3-Dichloro-5-nitrobenzene	618-62-2	GS-BCF	3.10	192.00	0.62	2.23	0.20	173	W	2	Nd
94	9	1,2-Dichloro-3-nitrobenzene	3209-22-1	GS-BCF	3.10	192.00	0.62	2.16	0.20	174-175	W	2	Nd
95	9	1,2,4,-Trichloro-5-nitrobenzene	89-69-0	GS-BCF	3.74	226.44	0.67	1.80	0.14	176-177	O2	2	Nd
96	9	1,2,3-Trichloro-4-nitrobenzene	17700-09-3	GS-BCF	3.74	226.44	0.60	2.19	0.13	178-179	O2	2	Nd
97	9	1,2,4,5-Tetrachloro-3-nitrobenzene	117-18-0	GS-BCF	4.39	260.89	0.62	3.20	0.07	180-181	O1	2	Nd
98	9	1,2,3,4-Tetrachloro-5-nitrobenzene	879-39-0	GS-BCF	4.39	260.89	0.67	1.85	0.10	182-183	O2	2	Nd

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ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
99	10	Ethylamine	75-04-7	SP	-0.15	45.08	0.44	-0.22	2.17	184-185	HL=360 h	[55]	Nd
100	10	Butylamine	109-73-9	SP	0.83	73.14	0.70	0.64	0.97	186-188	HL=208 h	[55]	Nd
101	10	Hexylamine	111-26-2	SP	1.82	101.19	0.95	1.51	0.47	189-191	HL=208 h	[55]	Nd
102	10	2-Amino-2-propanol	78-96-6	SP	-1.19	75.11	0.54	-1.13	8.33	192-193	HL=360 h	[55]	Nd
103	10	Trimethylamine	75-50-3	SP	0.04	59.11	0.62	-0.05	10.14	194	HL=360 h	[23]	Nd
104	11	N,N-Dimethylaniline	121-69-7	SP	2.17	121.18	0.94	1.82	2.81	195-196		[23]	[58]
105	11	2-Aminofluorene	153-78-6	SP	3.10	181.23	1.02	2.64	1.24	197-199		[23]	Nd
106	11	Kynurenine	343-65-7	SP	-2.25	208.21	0.97	-2.06	19.68	200-201	HL=360 h. Highly volatile in solution	[56]	Nd
107	11	Tryptamine	61-54-1	SP	1.27	160.22	0.93	1.03	0.58	202-204	HL=360 h	[56]	Nd
108	11	Benzenamine	62-53-3	SP	1.08	93.13	0.61	0.86	6.40	205-206	HL=360 h	[23]	Nd
109	12	Butyramide	541-35-5	SP	-0.18	87.12	0.60	-0.24	27.92	207	HL=360 h	[55]	Nd
110	12	E,E-N-Isobutyl-2,4-decadienamide	18836-52-7	SP	4.20	223.35	1.85	3.58	0.43	208-210	HL=360 h	[55]	Nd
111	12	Acetyl-1-pyrroline	99583-29-6	SP	1.66	111.14	0.64	1.37	1.89	211-212	HL=360 h	[55]	Nd
112	12	2-Isopropyl-N,2,3-trimethylbutyramide	51115-67-4	SP	2.48	171.28	0.78	2.09	2.18	213-214		[55]	Nd

840 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
841 studies (cont.)

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843 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
844 studies (cont.)

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ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Ref ed
113	12	N-Ethyl(E)-2,(Z)-6-nonadienamide	608514-56-3	SP	2.80	181.27	1.39	2.37	1.09	215-216	HL=360 h	[55]	Nd
114	12	Piperine	94-62-2	SP	3.69	285.34	1.53	3.15	0.22	217-219	Highly volatile in solution	[55]	Nd
115	13	Acetaldehyde	75-07-0	SP	-0.17	44.05	0.31	-0.23	3.82	220-221	HL=360 h	[57]	Nd
116	13	Acrolein	107-02-8	SP	0.19	56.06	0.40	0.08	2.88	222-224	HL=360 h	[58]	Nd
117	13	Endrin aldehyde	7421-93-4	SP	4.80	380.91	0.72	4.03	0.01	225-227		[58]	Nd
118	13	Formaldehyde	50-00-0	SP	0.35	30.03	0.18	0.22	3.79	228	HL=360 h	[58]	Nd
119	14	Ethanol	64-17-5	SP	-0.14	46.07	0.31	-0.21	7.28	229	HL=208 h	[23]	Nd
120	14	Allyl alcohol	107-18-6	SP	0.21	58.08	0.43	0.10	4.94	230-231	HL=360 h	[23]	Nd
121	14	1-Propanol	71-23-8	SP	0.35	60.10	0.43	0.22	5.03	232-233	HL=360 h	[59]	Nd
122	14	Cyclohexanol	108-93-0	SP	1.64	100.16	0.50	1.36	2.01	234-235	HL=360 h	[60]	Nd
123	15	Pentachlorophenol	87-86-5	GS-BCF	4.74	266.34	0.62	2.65	0.09	236	O2	1	[71]
124	15	4,5-Dichloro-2-methoxyphenol	2460-49-3	GS-BCF	2.63	193.03	0.77	2.03	0.61	237-238	W	1	Nd
125	15	2-Methoxytetrachlorophenol	2539-17-5	GS-BCF	3.92	261.92	0.81	2.26	0.04	239	O2	1	Nd
126	15	Phenol	108-95-2	SP	1.51	94.11	0.50	1.24	6.61	240-241	HL=360 h	[57]	[58]
127	16	Phenanthrenequinone	84-11-7	SP	3.56	208.21	0.93	3.04	0.44	242-243		[23]	Nd

846 **Table 3.** List of reference compounds for the development and evaluation of alternative methods to *in vivo* fish bioaccumulation
847 studies (cont.)

ID	Cc (T1)	Chemical name	CAS RN	Type (F1)	Log Kow	MW (g/mol)	D_{max} (nm)	Log BCF	K_{met} (1/d)	MP and Ms (pp)	Notes	Ref	Refed
128	16	9,10-Anthraquinone	84-65-1	SP	3.34	208.21	0.96	2.84	0.54	244		[61]	Nd
129	16	1,4-Benzoquinone	106-51-4	SP	0.25	108.09	0.49	0.14	10.88	245	HL=360 h	[62]	Nd
130	16	1,4-Naphthoquinone	130-15-4	SP	1.66	158.15	0.72	1.37	0.58	246-247	HL=360 h	[63]	Nd
131	16	2-Hydroxy-1,4-naphthoquinone	83-72-7	SP	0.78	174.15	0.81	0.60	1.16	248-249	HL=360 h	[64]	Nd
132	17	2-Ethenyloxirane	930-22-3	SP	0.73	70.09	0.56	0.56	2.69	250-251	HL=360 h	[65]	Nd
133	17	1,2-Epoxyoctane	2984-50-1	SP	2.83	128.21	1.05	2.40	0.68	252-253	HL=360 h	[65]	Nd
134	17	9,10-Phenanthrene oxide	585-08-0	SP	3.22	194.23	0.85	2.74	1.59	254-255	HL=360 h	[65]	Nd
135	17	1-Phenyloxirane	96-09-3	SP	1.59	120.15	0.73	1.31	5.73	256-257	HL=360 h	[65]	Nd
136	17	(2R,3S)-2,3-diphenyloxirane	1439-07-2	SP	3.22	196.24	1.16	2.74	5.20	258-259	HL=360 h	[65]	Nd
137	18	Arachidonic acid	506-32-1	SP	8.07	304.47	2.65	2.81	0.01	260-262	HL=360 h. Highly lipophilic	[23]	Nd
138	18	Eicosapentaenoic acid	25378-27-2	SP	7.85	302.45	2.52	3.00	0.00	263-266	HL=360 h	[23]	Nd
139	18	Docosahexaenoic acid	6217-54-5	SP	8.62	328.49	1.23	2.34	0.00	267-270	HL=360 h. Highly lipophilic	[23]	Nd

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851 CAS RN: Chemical Abstracts Service Registry Number, Cc: Chemical class according to Table 1, D_{max}: Maximum inter-atomic distance
852 between two atoms in the chemical structure (nm) calculated using MOPAC v.2012, ID: Identification number, K_{met}: Whole body
853 biotransformation rate (1/day (d)). *In vivo* values for GS-K_{met} compounds and *in silico* values for GS-BCF, GS-MP and SP obtained from
854 BCFBAF v.3.01 for one Kg fish, Log BCF: Logarithm of the average of bioconcentration factor (BCF) values (L/Kg ww (wet weight)). *In vivo*
855 values for GS-BCF compounds and *in silico* values for GS-K_{met}, GS-MP and SP compounds obtained from Equation 2, Log Kow: Logarithm of
856 octanol-water partition coefficient calculated from KOWWIN v.1.68, MP: Metabolic pathway. *In vivo* biotransformation routes for GS-MP
857 compounds are showed in Table 5 (T5). Predicted metabolic pathways for all reference compounds were calculated using Meteor software and
858 are provided in Supplementary Information (pages (pp), Ms: Resulting metabolites from the parent compound. Metabolites analysed in a *in vivo*
859 systems for GS-MP compounds are showed in Table 5 (T5). Structures of potential metabolites were calculated using Meteor software and are
860 provided in Supplementary Information (pages (pp), Notes: HL: Half-Life (h: hours) calculated from Fugacity model from the EpiSuite v.4.1, W:
861 well-predicted compounds (residuals < 0.5 log units), O1: marginally over-predicted compounds (residuals > 0.5 log units), O2: highly over-
862 predicted compounds (residuals > 1 log units) and U: under-predicted compounds according to Equation 2, Ref: Reference (1: DSL
863 Environment Canada BCF database, 2: non-DSL Environment Canada BCF database, 3: Common between EURAS-CEFIC database and
864 DSL/non-DSL Environment Canada BCF database), Ref ed: Reference of exiting *in vitro* data in rainbow trout, Type: Type of compound
865 according to Figure 1. GS-BCF: Gold-Standard BCF compounds, GS-K_{met}: Gold-Standard- K_{met} compounds, GS-MP: Gold-Standard metabolic
866 pathway compounds, SP: Supplementary compounds.
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869 **Table 4.** Experimental details of Gold-Standard BCF compounds

ID	CAS RN	Chemical name	n BCF	Log BCF	CV	Test concentration (µg/L)	Uptake phase (days)	T (°C)	Rng Ww_f (g)	Rng lipid content (%)	DB
1 (48)	520-45-6	Dehydroacetic acid	4	2.12	0.36	3.6	5 to 20	15	140	Nd	1
2 (87)	100-00-5	1-Chloro-4-nitrobenzene	5	2	0.11	0.78	5 to 36	15	165	8.40	1
3 (88)	88-73-3	1-Chloro-2-nitrobenzene	5	2.09	0.26	0.72	5 to 36	15	165	8.40	2
4 (89)	121-73-3	1-Chloro-3-nitrobenzene	5	1.89	0.15	0.8	5 to 36	15	165	8.40	2
5 (124)	2460-49-3	4,5-Dichloro-2-methoxyphenol	1	2.03	0	7	20	15	140	8.00	1
6 (90)	99-54-7	1,2-Dichloro-4-nitrobenzene	5	2.07	0.1	0.73	5 to 36	15	165	8.40	1
7 (91)	89-61-2	1,4-Dichloro-2-nitrobenzene	5	2.05	0.06	0.77	5 to 36	15	165	8.40	2
8 (92)	611-06-3	2,4-Dichloro-1-nitrobenzene	5	2.07	0.04	0.75	5 to 36	15	165	8.40	2
9 (93)	618-62-2	1,3-Dichloro-5-nitrobenzene	5	2.23	0.05	0.76	5 to 36	15	165	8.40	2
10 (94)	3209-22-1	1,2-Dichloro-3-nitrobenzene	5	2.16	0.07	0.77	5 to 36	15	165	8.40	2
11 (10)	95-50-1	1,2-Dichlorobenzene	10	2.58	0.43	0.05,0.94	7 to 119	15	242-400	7.2-10.7	1
12	106-46-7	1,4-Dichlorobenzene	21	2.76	0.47	0.03,0.07,0.08,0.67	7 to 119	15	175-400	5.0-10.7	1
13 (11)	541-73-1	1,3-Dichlorobenzene	10	2.74	0.36	0.03,0.69	7 to 119	15	242-400	7.2-10.7	1
14 (95)	89-69-0	1,2,4-Trichloro-5-nitrobenzene	5	1.8	0.18	0.68	5 to 36	15	165	8.40	2
15 (96)	17700-09-3	1,2,3-Trichloro-4-nitrobenzene	5	2.19	0.23	0.66	5 to 36	15	165	8.40	2

870 **Table 4.** Experimental details of Gold-Standard BCF compounds (cont.)

ID	CAS RN	Chemical name	n BCF	Log BCF	CV	Test concentration (µg/L)	Uptake phase (days)	T (°C)	Rng Ww_f (g)	Rng lipid content (%)	DB
16	18708-70-8	1,3,5-Trichloro-2-nitrobenzene	5	2.92	0.24	0.54	5 to 36	15	165	8.40	2
17 (15)	108-36-1	1,3-Dibromobenzene	1	2.82	0	<0.01	90	15	280	Nd	3
18 (125)	2539-17-5	2-Methoxytetrachlorophenol	1	2.26	0	1	20	15	140	8	1
19 (12)	87-61-6	1,2,3-Trichlorobenzene	8	3.26	0.43	<0.01,0.07	22 to 119	15	277-400	8-10.7	1
20	108-70-3	1,3,5-Trichlorobenzene	8	3.49	0.64	<0.01,0.04	22 to 119	15	277-400	8-10.7	1
21	120-82-1	1,2,4-Trichlorobenzene	20	3.38	0.51	<0.01,0.05	7 to 119	15	183-400	6.3-10.7	1
22 (27)	54135-80-7	1,2,3-Trichloro-4-methoxybenzene	13	3.25	0.32	<0.01*,0.01,0.07	7 to 96	15	183-400	6.3-10.7	3
23	67-72-1	Hexachloroethane	10	2.84	0.57	<0.01,<0.01	7 to 119	15	242-400	7.2-10.7	1
24 (25)	320-60-5	2,4-Dichloro-1-(trifluoromethyl)benzene	10	3.52	0.34	0.03,0.21	21 to 96	15	183-312	5.0-8.0	2
25 (26)	328-84-7	1,2-Dichloro-4-(trifluoromethyl)benzene	1	3.18	0	<0.01	90	15	280	Nd	3
26 (1)	58-89-9	γ-Lindane	12	3.13	0.45	<0.01,0.03,2.01*	5 to 96	15	175-312	5.3-8.0	1
27 (97)	117-18-0	1,2,4,5-Tetrachloro-3-nitrobenzene	12	3.2	0.36	<0.01,0.01,0.64	20 to 96	15	165-312	6.9-8.0	2

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873 **Table 4.** Experimental details of Gold-Standard BCF compounds (cont.)

ID	CAS RN	Chemical name	n BCF	Log BCF	CV	Test concentration (µg/L)	Uptake phase (days)	T (°C)	Rng Ww_f (g)	Rng lipid content (%)	DB
28 (98)	879-39-0	1,2,3,4-Tetrachloro-5-nitrobenzene	8	1.85	0.47	<0.01,0.61	12 to 96	15	165-288	6.7-8.4	2
29 (40)	1825-31-6	1,4-Dichloronaphthalene	1	3.75	0	<0.01	90	15	280	Nd	3
30 (19)	6639-30-1	1,2,4-Trichloro-5-methylbenzene	10	3.88	0.24	<0.01,<0.01,0.05	21 to 96	15	202-312	5.0-8.0	3
31	95-94-3	1,2,4,5-Tetrachlorobenzene	7	3.9	0.53	<0.01,0.02	39 to 119	15	202-312	5.0-8.0	1
32 (13)	634-66-2	1,2,3,4-Tetrachlorobenzene	17	3.85	0.38	<0.01,<0.01,0.03	39 to 119	15	258-312	7.0-10.70	1
33 (16)	626-39-1	1,3,5-Tribromobenzene	11	4.02	0.33	<0.01,<0.01*,0.02	4 to 96	15	183-312	6.90-8.0	3
34 (17)	615-54-3	1,2,4-Tribromobenzene	1	3.63	0	<0.01	90	15	280	Nd	3
35	87-68-3	Hexachlorobutadiene	10	3.94	0.62	<0.01,<0.01	7 to 119	15	242-400	7.20-10.70	1
36 (123)	87-86-5	Pentachlorophenol	2	2.65	0.11	91.2	6	12.5	19- 39	6.30-8.95	1
37	82-68-8	Pentachloronitrobenzene	14	2.41	0.74	<0.01,0.01,0.69	5 to 96	15	165-312	6.30-8.45	2
38 (30)	16605-91-7	2,3-Dichloro-1,1'-biphenyl	3	4.08	0.08	<0.01	75 to 96	15	223-278	6.60-8.20	2

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878 **Table 4.** Experimental details of Gold-Standard BCF compounds (cont.)

ID	CAS RN	Chemical name	n BCF	Log BCF	CV	Test concentration (µg/L)	Uptake phase (days)	T (°C)	Rng Ww_f (g)	Rng lipid content (%)	DB
39 (31)	34883-39-1	2,5-Dichloro-1,1'-biphenyl	4	3.98	0.24	0.01	35 to 96	15	191-341	6.60-8.20	2
40 (32)	34883-41-5	3,5-Dichloro-1,1'-biphenyl	4	3.77	0.1	0.02	35 to 96	15	191-341	6.60-8.20	2
41 (14)	608-93-5	Pentachlorobenzene	6	4.19	0.29	<0.01,<0.01	43 to 119	15	277-400	8.20-10.70	1
42 (28)	1825-21-4	Pentachloroanisole	8	4.19	0.26	<0.01,0.01	35 to 96	15	202-312	6.70-8.0	2
43 (18)	636-28-2	1,2,4,5-Tetrabromobenzene	8	3.67	0.35	<0.01,0.02	35 to 96	15	202-312	6.70-8.0	3
44 (21)	877-10-1	1,2,4,5-Tetrachloro-3,6-dimethylbenzene	3	3.55	0.41	0.01	50 to 96	15	262-288	6.70-8.0	2
45 (33)	37680-65-2	2,2',5-Trichloro-1,1'-biphenyl	2	4.23	0	0.02	75 to 96	15	278-341	6.60-8.20	2
46 (34)	20020-02-4	1,2,3,4-Tetrachloronaphthalene	4	3.7	0.07	<0.01	35 to 96	15	191-341	6.60-8.20	2
47 (22)	877-11-2	Pentachloromethyl benzene	4	3.83	0.07	<0.01	35 to 96	15	191-341	6.60-8.20	2
48 (35)	38444-93-8	2,2',3,3'-Tetrachloro-1,1'-biphenyl	2	4.23	0.03	2.8	14 to 20	15	140	Nd	2
49 (78)	127-27-5	Pimaric acid	2	1.85	0.02	2.7	14 to 20	15	140	Nd	1

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882 **Table 4.** Experimental details of Gold-Standard BCF compounds (cont.)

ID	CAS RN	Chemical name	n BCF	Log BCF	CV	Test concentration (µg/L)	Uptake phase (days)	T (°C)	Rng Ww_f (g)	Rng lipid content (%)	DB
50 (79)	5835-26-7	Isopimaric acid	2	1.54	0.02	2.7	14 to 20	15	140	Nd	1
51 (80)	514-10-3	Abietic acid	2	1.84	0.01	2.1	14 to 20	15	140	Nd	1
52 (81)	471-77-2	Neoabietic acid	2	2.1	0.05	0.7	14 to 20	15	140	Nd	1
53 (63)	101-02-0	Triphenyl phosphite	1	2.39	0	0.81	96	12	Nd	Nd	1
54 (23)	87-83-2	Pentabromomethylbenzene	4	2.43	0.15	<0.01	35 to 96	15	191-341	6.60-8.20	2
55 (82)	1945-53-5	Palustric acid	2	1.4	0	1.1	14 to 20	15	140	Nd	1
56 (18)	87-82-1	Hexabromobenzene	5	3.02	0.22	<0.01	21 to 96	15	191-341	6.60-8.20	2
57 (24)	85-22-3	Pentabromoethylbenzene	4	2.52	0.13	<0.01	35 to 96	15	191-341	6.60-8.20	2
58 (83)	57055-39-7	12,14-Dichlorodehydroabietic acid	2	1.97	0.02	3.2	14 to 20	15	140	Nd	1
59 (41)	2234-13-1	Octachloronaphthalene	2	2.58	0.2	0.01	75-96	15	278-341	6.60-8.20	2

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884 CAS RN: Chemical Abstracts Service Registry Number, CV: Coefficient of variance, DB: Database reference (1: DSL Environment Canada BCF
885 database, 2: non-DSL Environment Canada BCF database, 3: Common between EURAS-CEFIC database and DSL/non-DSL Environment
886 Canada BCF database), ID: Identification number (identification number in Table 3), Log BCF: Logarithm of the average of bioconcentration
887 factor (BCF) values (L/Kg ww (wet weight)), Nd: No data reported, T: Temperature (°C), Rng ww: Range of final wet weight (mg) of test species,
888 Rng lipid content: range of final lipid content (%) of test species.

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890 **Table 5.** Experimental details of Gold-Standard metabolic compounds

ID	CAS NR	Chemical name	K_{met} (1/d)	Metabolites analysed	Metabolic pathway proposed	TE	FTA	E (days)	TC (μg/g ww or μg/L)	T (°C)	Ww_i (g)	Ref
1 (50)	88671-89-0	Myclobutanil	0.200	Nd	Nd	DM	C	8	30.15	12	18	[41]
2 (51)	60207-90-1	Propiconazole	0.572	Nd	Nd	DM	C	8	24.96	12	18	[41]
3 (52)	94361-06-5	Cyproconazole	0.369	Nd	Nd	DM	C	8	23.83	12	18	[41]
4 (53)	66246-88-6	Penconazole	0.211	Nd	Nd	DM	C	8	31.46	12	18	[41]
5 (54)	125116-23-6	Metconazole	0.580	Nd	Nd	DM	C	8	28.14	12	18	[41]
6 (55)	43121-43-3	Triadimefon	0.541	Nd	Nd	DM	C	8	29.02	12	18	[41]
7 (56)	112281-77-3	Tetraconazole	0.237	Nd	Nd	DM	C	8	30.95	12	18	[41]
8 (57)	107534-96-3	Tebuconazole	0.552	Nd	Nd	DM	C	8	26.43	12	18	[41]
9 (58)	120068-37-3	Fipronil	1.006	Nd	Nd	DM	C	32	7.68	12	10	[42]
10 (36)	50-29-3	p,p'-Dichlorodiphenyltrichloroethane	0.011	Nd	Nd	DM	C	32	0.42	12	10	[42]
11 (35)	38444-93-8	2,2',3,3'-Tetrachlorobiphenyl	0.001	OH-PCBs	Hydroxylation	DM	C	30	<0.01	12	80	[43]
12 (37)	38380-05-1	2,2',3,3',4,6'-Hexachlorobiphenyl	0.004	OH-PCBs	Hydroxylation	DM	C	30	0.04	12	80	[43]
13 (38)	52744-13-5	2,2',3,3',5,6'-Hexachlorobiphenyl	<0.001	OH-PCBs	Hydroxylation	DM	C	30	0.03	12	80	[43]
14 (39)	38411-22-2	2,2',3,3',6,6'-Hexachlorobiphenyl	0.002	OH-PCBs	Hydroxylation	DM	C	30	0.02	12	80	[43]
15 (2)	754-91-6	Perfluorooctanesulfonamide	Nd	PFOS	Nd	DM	M	30	10.9	12	Nd	[45]

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892 **Table 5.** Experimental details of Gold-Standard metabolic compounds (cont.)

ID	CAS NR	Chemical name	K_{met} (1/d)	Metabolites analysed	Metabolic pathway proposed	TE	FTA	E (days)	TC (μg/g ww, μg/L)	T (°C)	Ww_i (g)	Ref
16 (3)	865-86-1	10:2 Fluorotelomer alcohol	Nd	10:2 FTCA 10:2 FTUCA	Nd	DM	M	30	5.00	12	Nd	[45]
17 (4)	678-39-7	8:2 Fluorotelomer alcohol	Nd	8:2 FTCA 8:2 FTUCA	Nd	DM	M	30	6.70	12	Nd	[46]
18 (5)	27905-45-9	8:2 Fluorotelomer acrylate	Nd	8:2 FTOH 8:2 FTUCA 7:3 FTCA 8:2 FTCA PFOA 8:2FTOH-Glu	β-like oxidation mechanism: 8:2FTUCA>7:3 β-keto acid>7:2 ketone>PFOA	DM	L,Bl, k,Bi	5	93.00	18	45	[47]
19 (29)	1163-19-5	Decabromodiphenyl ether	Nd	De-BDEs MeO-BDEs	Nd	II	M,L, Bl	28	0.1;0.5	15	100	[47]
20 (84)	15307-86-5	Diclofenac	Nd	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	Hydroxylation> Glucuronidation Sulfatation	WM	Bi	10	1.8;43	14	33	[48]
21 (85)	22204-53-1	Naproxen	Nd	DNPX NPX-A.Glu DNPX-A.Glu	Demethylation> Glucuronidation	WM	Bi	10	1.6;40	14	33	[48]

893 **Table 5.** *In vivo* experimental data for Gold-Standard metabolic compounds (cont.)

ID	CAS NR	Chemical name	K_{met} (1/d)	Metabolites analysed	Metabolic pathway proposed	TE	FTA	E (days)	TC (μg/g ww, μg/L)	T (°C)	Ww_i (g)	Ref
			Nd	Carboxyl-IBF 2-OH-IBF IBF-A.Glu OH-IBFs--A.Glu	Hydroxylation> Glucuronidation Sulfatation	WM	Bi	10	1;25	14	33	[48]
22 (86)	15687-27-1	Ibuprofen	Nd	2-OH-IBF 3-OH-IBF IBF-A.Glu OH-IBFs-A.Glu IBF-Tau	Nd	WS	Bi	4	0.17;1.9;13;145	14	58	[49]

CAS RN: Chemical Abstracts Service Registry Number, DNPX: 6-*O*-desmethylnaproxen, E: Exposure duration (days) (Including uptake phase and intraperitoneal injection),FTA: Fish target analysed (Bi: Bile, Bl: Blood, C: Carcas, L: Liver, K: Kidney, M: Muscle), IBF: Ibuprofen, ID: Identification number (identification number in Table 3), K_{met}: Whole body biotransformation rate (1/days), Nd: No data reported, NPX: Naproxen, Ref: Reference, T: Temperature (°C), TC: Test concentration (Dietary and Intraperitoneal injection exposure: μg/g ww, waterborne exposure: μg/L), TE: Type of exposure (D: Dietary, W: Waterborne, S: Single, M: Mixture of chemicals, II: Intraperitoneal injection), Ww_i: Initial wet weight of fish (mg),Metabolites (PFOS: Perfluorooctanesulfonate, 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, DCF-A.Glu: Acyl glucuronide of E.Glu: Ether glucuronide of hydroxylated diclofenac,NPX-A.Glu: Acyl glucuronide of naproxen,DNPX-A.Glu: Acyl glucuronide of 6-*O*-desmethylnaproxen, 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).

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