In vitro metabolism using rainbow trout liver S9
Summary report of the HESI Bioaccumulation Committee

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Abstract

Standard methods to assess bioaccumulation, such as QSARs, computer modeling, and the in vivo OECD 305 Test Guideline do not incorporate estimates of metabolism and are not valid for many chemical classes. In order to improve the in vitro assessment of bioaccumulation/bioconcentration assessment and enable concerted evaluations of thousands of commercial substances in a scientifically correct, timely and cost effective manner, this study was undertaken to examine and pre-validate the utility of rainbow trout S9 fractions to predict in vivo fish metabolism. The project aimed to standardize both the S9 isolation and incubation methodology and resulted in the development of a standardized method for both protocols that was recently published in Current Protocols. Funding was provided from the European Commission, Joint Research Center, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods (JRC/IHCP/ECVAM) and the European Chemical Industry Council (CEFIC), and five laboratories from North America, Europe, and Australia worked to validate this methodology, testing the approach with 15 different chemicals. The purpose of this report is to summarize and describe an overview of the project findings, limitations, lessons learned, and future recommendations for rainbow trout liver S9 metabolism assays. Raw data and additional details are available upon request by contacting Michelle Embry (membry@ilsi.org).

1. Introduction

1.1 Background

Bioaccumulation is defined as the biological sequestering of xenobiotics and/or their metabolites by uptake via food, water, air, or sediment, such that the concentration in the organism is greater than that in its surroundings or food and is the result of absorption, distribution, metabolism, and excretion (ADME) processes. Since the United Nations Stockholm Convention on persistent organic pollutants (POPs) was adopted in 2001, there has been significant activity concerning the assessment of persistent, bioaccumulative, toxic (PBT) substances worldwide. For example, the European Commission REACh (Registration, Evaluation, Authorisation, and Restriction of Chemicals) regulation requires chemical substances produced above 100 tons/year to be evaluated for their potential to bioaccumulate in the environment. This requirement is contingent upon the chemical's octanol-water partition coefficient (log K_{ow}) being ≥3. In general, a bioconcentration factor (BCF) is established and chemical substances with BCF > 2000 (or log K_{ow} >4.5) are considered as bioaccumulative. A chemical is classified as very bioaccumulative if the BCF > 5000 (or log K_{ow} >5). In order to avoid unnecessary testing on animals, REACh requests the use of existing information

from standard and non-standard methods, *in vitro* methods, *in silico* methods, read-across, weight-of-evidence, etc. in an intelligent testing strategy for assessing the bioaccumulative potential of a substance.

In order to address the scientific challenges associated with conducting bioaccumulation assessments for chemicals regulated under various PBT programs, there is a clear need to develop alternative methods for evaluating the thousands of chemicals that will need to be assessed over the next few years, taking into account integrated testing strategies and a tiered, weight of evidence approach. Because *in vivo* (e.g., OECD 305) bioaccumulation data are relatively scarce, the majority of preliminary bioaccumulation assessments currently rely on QSAR (Quantitative Structure Activity Relationships) and log K_{ow}-based model estimates for fish, though for many chemicals, these models are not valid and outside of the domain for certain chemical classes and do not account for the impact of metabolism in the organism.

Incorporating metabolism in fish is critical to improving bioaccumulation estimates especially those produced utilizing many of the existing computational models. The existing *in vivo* OECD 305 fish bioconcentration factor (BCF) guideline does account for metabolism but requires large numbers of fish, is costly and is labor and resource intensive. There is a need to close this gap between *in silico* (QSAR) and *in vivo* (OECD 305 guideline) approaches with an efficient *in vitro* testing strategy that includes not only an assessment of passive uptake but also biotransformation along with proper scaling from test tube to whole animal. Metabolism data can be assessed by using an *in vitro* system that can be reliably extrapolated to *in vivo* rates biotransformation and utilized in a prediction model for BCF determination.

In vitro methods (e.g., isolated hepatocytes, subcellular fractions, and cell lines) to measure the metabolism of chemicals are available, and have been used for decades in drug development and pre-clinical testing, though to a lesser extent in environmental hazard assessment. Fish liver S9 fractions have both Phase I and Phase II xenobiotic metabolizing enzymes and initial studies have demonstrated that they are an appropriate *in vitro* test system for evaluating xenobiotic metabolism (Johanning et al., 2007; Sahi et al., 2007; Johanning et al., 2008).

In order to improve the *in vitro* assessment of bioaccumulation/bioconcentration assessment and enable concerted evaluations of thousands of commercial substances in a scientifically correct, timely and cost effective manner, this study was undertaken to examine and pre-validate the utility of rainbow trout S9 fractions to predict *in vivo* fish metabolism (Cowan-Ellsberry et al, 2008; Nichols et al., 2006; Weisbrod et al, 2009).

1.2 Objectives

This project evaluated the sensitivity, specificity, and reproducibility of a rainbow trout liver S9 assay and its ability to predict *in vivo* rates of fish metabolism. The objectives were to:

- Assess the reproducibility (intra- and inter-laboratory variability) and transferability of the *in vitro* rainbow trout liver S9 fraction isolation assay
- Assess the reproducibility (intra- and inter-laboratory variability) and transferability of the *in vitro* rainbow trout liver S9 fraction incubation assay
- Evaluate its relevance to predict *in vivo* k_{MET} to be used in bioconcentration/bioaccumulation models.

1.3 Project partners and funding

Five laboratories (Eawag/University of Queensland, The Dow Chemical Company CellzDirect/Life Technologies, CanTest/Maxxam Analytics, and The Procter & Gamble Company) participated in the study, with statistical support provided by AstraZeneca and project management support provided by ILSI-HESI. In addition, rainbow trout liver S9 was provided by the USEPA (Duluth, MN) and the project was actively monitored by a multi-sector team of scientific advisors.

This project was funded by the European Commission via ECVAM (Contract CCR.IHCP.C434207.X0) and CEFIC (LRI-ECO6.2-ILSIHESI-0804).

1.4 Summary report

The purpose of this report is to summarize and describe an overview of the project findings, limitations, lessons learned, and future recommendations for rainbow trout liver S9 metabolism assays. Raw data and additional details are available upon request by contacting Michelle Embry (membry@ilsi.org). Additional detailed information is also available in the final contract report submitted to the European Commission, namely ECVAM (contract CCR.IHCP.C434207.X0).

2. Materials and Methods

2.1 S9 preparation

The rainbow trout liver S9 preparation protocol will be published in the August 2012 online issue of *Current Protocols in Toxicology – In vitro 53:14.10.1-14.10.28* (Johanning *et al.*, 2012). Please refer to this publication for a detailed description of the method. The method will also be made available in the ECVAM Database on Alternative Methods:
http://ecvam-dbalm.jrc.ec.europa.eu

2.2 S9 incubation

The rainbow trout liver S9 incubation protocol was published in the August 2012 online issue of *Current Protocols in Toxicology – In vitro 53:14.10.1-14.10.28* (Johanning *et al.*, 2012). Please refer to this publication for a detailed description of the method. The method will also be made available in the ECVAM Database on Alternative Methods:
http://ecvam-dbalm.jrc.ec.europa.eu

2.3 Test chemical selection

In order to facilitate the development of the *in vitro* assay for assessing the metabolic stability of chemicals, a common list of test chemicals was developed. An initial list of 1156 records was provided by Dr. Jon Arnot (University of Toronto, personal communication) containing measured BCF values from Arnot and Gobas (2006). These records were compared to predicted BCF values using the mass-balance BCF model described in Arnot and Gobas (2004) assuming no *in vivo* biotransformation. The difference in the predicted BCF values and the measured values provided a potential measure of the impact of *in vivo* biotransformation on the BCF. Furthermore, initially it was assumed that the greater the difference between predicted and measured BCF values at any log K_{ow} value, the greater the likelihood that the difference was due to *in vivo* biotransformation, although clearly other non-metabolic processes could have contributed to these observed differences. Using this general approach and further data collection, a list of approximately 100 candidate chemicals with moderate to high expected levels of impact of *in vivo* biotransformation on the BCF were identified. These same data were used by Arnot et al. (2008a, 2008b, 2009) to develop a method for estimating the *in vivo* metabolic biotransformation rate (k_{MET}) and eventually to develop a quantitative structure-activity relationship (QSAR) for predicting screening level primary biotransformation rate constants from chemical structure.

The winnowing of these 100 chemicals to a small core set required evaluation of the physical, chemical, biotransformation potential, and analytical aspects of each of the chemicals as well as an evaluation of the quality of the empirical BCF values. The criteria for selecting the final chemical list included:

- log K_{ow} in the range of 4-6 where BCF potential is greatest
- *in vivo* fish BCF data available and of sufficient quality
- indication (*in vivo*) of the degree and type of biotransformation
- properties that are suitable for evaluation using non-radiolabeled (cold) analytical techniques
- non-ionic at physiological pH
- diverse chemical classes represented
- diverse group of potential biotransformation pathways

As part of the selection process, preference was given to test chemicals with measured *in vivo* rates of biotransformation or bioaccumulation in fish. This was to allow for comparison with extrapolated *in vivo* rates of metabolism from measured *in vitro* data. A key element of the criteria was the ability to trace data back to the primary source of the biotransformation or BCF data. In many instances the information was not available and quickly reduced the list of potential chemicals.

An additional key aspect considered in the selection of some of the chemicals was the availability of analytical methods and instrumentation suitable for measuring the analytes in *in vitro* matrices. Chemicals predicted to have a high bioaccumulation (`B') potential (e.g., log K_{ow} >4) tend to be difficult to test, particularly under *in vitro* conditions due to their poor aqueous solubility producing reduced or uncertain bioavailability. Both fish liver S9 and hepatocyte tests are conducted in small volumes (0.2 – 5.0 mL) and have a much greater surface/sorptive area to test volume ratio and/or evaporation potential as compared to *in vivo* assays, potentially compromising analytical detection due to limited bioavailability. Furthermore, some chemicals may be cytotoxic at 1-10 μM; hence, testing and analytical detection limits below these concentrations are required because these types of *in vitro* metabolic tests require sub-toxic exposures. In determining if an analytical method was suitable, we considered measurement needs (e.g., total parent chemical vs. bioavailable chemical vs. metabolites). Due to the potentially unknown nature of biotransformation pathways *in vitro*, we determined that quantification of the loss of parent over time would

provide the most expedient measure of biotransformation rates. Importantly, limits of quantitation (LOQ) per analytical method needed to be no greater than 5% of the initial exposure solution, typically around 0.5 - 0.005 μM.

Chemical form at environmental pH was also evaluated, with a bias toward selecting organic chemicals that are nonionic at ecological or physiological pH. Ionizable moieties or polar groups may provide unique analytical challenges beyond those associated with non-polar organics. Charged species have limited membrane permeability, which could influence the apparent biotransformation potential observed in fish hepatocytes, but would not influence tests with liver S9 subcellular fractions, confounding the comparison of biotransformation rates between the two systems (Dimitrov et al. 2003).

Finally, all test compounds needed to be readily available, of high purity, and reasonably priced or donated by manufacturers so that all laboratories involved in the project could share test chemicals from the same lot/batch.

Considering all the above criteria the management team agreed on an initial list of 6 compounds as well as additional 9 chemicals which met most of the criteria. Log K_{ow} values in these tables are from EPI Suite v. 3.12 (http://www.epa.gov/oppt/exposure/index.htm) and the default log BCF was estimated using Arnot and Gobas (2003) model for "lower" trophic level fish. Test chemicals are listed in **Table 1**.

2.4 Analytical methods

A set of analytical methods were developed for all fifteen (15) chemicals utilizing a similar approach based on parent compound disappearance. The parent test chemicals were separated utilizing specific columns and detected by select ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS). An internal standard (deuterated form of the compound or a known standard) was added to each sample to facilitate quantification. In some cases, and external standard was also used. A summary of the methods used is provided in **Table 2** and the Final report for the ECVAM Study Contract (Johanning and Embry, 2010) and detailed methods are available upon request.

2.5 Statistical analysis

Statistical analysis was performed by Dr. Alan Sharpe (AstraZeneca). A description of the statistical methods utilized is given below.

2.5.1 Calculation of transformation rate (k)

For each chemical, the transformation rate (k) was determined by plotting the ln μmoles/mg protein (measured loss of test chemical rate) vs. time. The transformation rate k was utilized and subjected to the different statistical analysis and used to determine the intra- and inter-laboratory variability. The R^2 goodness of fit values from the linear regression used to determine k and the p-value ($p < 0.01$) indicating the statistical significance of the regression was calculated.

2.5.2 Intra-laboratory variability

Intra-laboratory variability is an indication of how much variability exists in results obtained for the same assay undertaken repeatedly at the same laboratory and can give an indication of how reproducible or reliable an assay is.

2.5.3 Inter-laboratory variability

Inter-laboratory variability is an indication of how much variability exists in results obtained from different laboratories for the same assay and can give an indication of how transferable an assay is between laboratories.

In order to obtain information on the intra- and inter-laboratory variability of the *in vitro* rainbow trout liver S9 assay, the following statistical methodologies were applied to the data.

2.5.4 Analysis of variance

For each of the compounds, a 1-way Analysis of Variance has been undertaken to determine whether there were significant differences between laboratories.

2.5.5 Mandel's h statistic

Mandel's h statistic is a measure of how much the mean value for each laboratory differs from the overall mean of all laboratories. Comparison against a critical value for Mandels h statistic can give an indication of whether a laboratory is producing outlying values compared to other laboratories.

2.5.6 Grubbs test

Grubb's test provides an additional statistical test to indicate whether the mean value from an individual laboratory may be considered an outlier when compared the mean of the remaining laboratories. Laboratories producing mean values which could be considered as outlying when compared to other laboratories produce a Grubb's statistic which is significant at the p=0.01 level.

2.5.7 Homogeneity of variances

For each compound, Bartlett's test for homogeneity of variances has been undertaken to assess whether there

is a significant difference between the variances obtained by each of the laboratories.

2.5.8 Cochran's test

Cochran's test provides an additional statistical test to assess whether the variability in results obtained from an individual laboratory is significantly greater than that found in other laboratories. Laboratories producing variability which could be considered as outlying when compared to other laboratories produce a Cochran's statistic which is significant at the p=0.01 level.

3. Results – Single S9 Preparation

3.1 Chemicals evaluated using rainbow trout liver S9 Batch A (prepared by CellzDirect / Life Technologies)

3.1.1 Initial results

This pool of rainbow trout liver S9 (rainbow trout strain: Shasta and Kamloops strain hybrids) was prepared from livers flushed and isolated at the Battelle PNNL (Dr. Irv Schultz' laboratory). These livers were quickly frozen and shipped on dry ice to the leading laboratory (CellzDirect/Life Technologies) where they were processed into the liver S9 fraction.

Initial incubations with the first 6 chemicals (see **Table 1**) demonstrated low or undetectable metabolic turnover rates using Batch A S9 for chlorpyrifos, DBE, methoxychlor, and 4-nonlyphehol. The first-order rate constant (k) equals the slope of the linear fit of ln [parent chemical] vs. time (hours). First-order metabolic turnover is therefore indicated a negative slope and an R^2 value close to 1 (generally >0.7). Very few runs with these four chemicals showed first-order metabolic turnover in any of the five laboratories. Pyrene and FMHE demonstrated slight metabolism, though overall activity was quite low.

3.1.2 S9 analysis

Due to this lower observed activity in the rainbow trout liver S9 Batch A metabolic rates, additional studies were conducted to assess the rainbow trout liver S9 isolation protocol and measure its activity using various methods and to characterize factors that may affect activity.

3.1.2.1 Fresh / Frozen tissue

Frozen tissue liver S9 was compared to freshly prepared (livers not frozen prior to isolation) liver S9 and results demonstrated a significant decrease in activity when isolated liver was frozen prior to preparation of the S9 fraction). (**Figure 1**).

3.1.2.2 Fish strain

Various rainbow trout strains (Emerson, Shasta-Kamloops, Erwin, Steelhead-Kamloops and Eagle Lake-Oden State Hatchery) from various regions demonstrate significant differences in metabolism. (**Figure 2**).

3.1.2.3 Temperature

Enzyme activities were also tested with rainbow trout liver S9 fraction samples at different temperatures (12 and 18ºC). The results indicated that they were no significant differences in the enzyme activity (testosterone and lauric acid hydroxylation, 7-hydroxycoumarin sulfation and glucuronidation and estradiol glucuronidation). (**Figure 3**).

3.1.2.4 Sex and lifecycle

To-date, data from different laboratories indicate that immature rainbow trout (~1 year) do not exhibit significant differences when male and female metabolic enzyme activities are compared. These enzymes most likely will change during a fish life cycle but they are no comprehensive studies that provide this information during the entire life cycle of the rainbow trout or other fish. (**Figure 4**).

3.2 Chemicals evaluated using rainbow trout liver S9 Batch B (prepared by USEPA)

3.2.1 S9 Batch B isolation and characterization

Due to significant metabolic differences between S9 isolations prepared from different rainbow trout strains and preparation procedures (frozen vs fresh – see above) and the low turnover seen in several of the first 6 chemicals using the original preparation, a separate S9 pool (Batch B) was isolated. The same general protocol as described in the *Current Protocols in Toxicology* publication was followed, with the following exceptions:

- The rainbow trout were Erwin strain, obtained from the USGS fish hatchery in LaCrosse, Wisconsin, USA
- Livers were not frozen prior to preparation of the S9 fraction, therefore fresh tissue was utilized as recommended
- Several livers were perfused concurrently, and pools of 3 – 5 fish were used to make the S9 fractions.

Enzyme activity of S9 produced from the same rainbow trout strain used for S9 Batch B were followed for almost two years and tested approximately every six months for testosterone hydroxylation. These samples contained 250 mM sucrose and enzyme activity did not changed significantly throughout the experimentation. In addition, rainbow trout liver S9 samples lacking 250 mM

sucrose were tested after two years and enzyme activity showed a significant decrease at that point *(Johanning et al., 2010).*

3.2.2 Chemicals evaluated

Nine chemicals were evaluated in three laboratories (CellzDirect / Life Technologies, Dow, and University of Queensland) using S9 Batch B: Decanol, BaP, C16EO8, fenthion, zoxamide, deltamethrin, 17α-ethinylestradiol, diclofenac, and DBP (see **Table 1** for more information). Measurable first-order loss rates were measured in all chemicals in all three laboratories, though there was a high degree of variability observed (**Figure 5**). See below for additional discussion.

3.2.3 Data analysis

A complete statistical analysis was conducted on all of the data generated from each of the laboratories and a full report is available upon request. An additional analysis was also performed with acceptance criteria of $R^2>0.7$ and $p<0.05$ (i.e. only significant regression slope from the assay), though this analysis did not greatly affect the overall conclusions.

In examining all of the generated data, CellzDirect / Life Technologies showed the lowest metabolic rates overall. We think that a possibility may be that different laboratories were treating time zero differently. CellzDirect/Life Technologies recommended adding all reagents to the stopping solution and test chemical as the last step to avoid any probable start of reaction. University of Queensland, which had outsourced the chemical analysis of some of the additional 9 chemicals to an external analytical laboratory (Queensland Health Forensic and Scientific Services, Coopers Plains, Australia), showed the highest intra-laboratory variability for several compounds, though all of the laboratories showed high intra- and inter-laboratory variability when all compounds were compared **(Tables 3 – 4).**

In addition, an analysis was performed comparing only Dow and CellzDirect / Life Technologies data because of the high intra-laboratory variability seen with University of Queensland as well as because analytical methods were similar in these two laboratories. Data with $R^2<0.7$ and $p>0.05$ were investigated further to assess whether there were any specific patterns or reasons why these data were unable to fulfill the acceptance criteria.

While the mean rate generally differed between the two laboratories, intra-laboratory variability was broadly similar with only one significant difference in variances highlighted. Dow and CellzDirect / Life Technologies were the closest in terms of which materials and protocol were followed. This may suggest that while there is some work to do in understanding why the inter-laboratory differences exist, the intra-laboratory variability may be governed by the some of the details in the protocol and some differences in the analytical methods used **(Tables 5 – 6).**

Looking at differences in intra-laboratory variability does not tell the whole story of course. Even comparing just two laboratories there is a large range of coefficient of variation (standard deviation (SD)/mean) values over all compounds, hence while the labs may be consistently variable between themselves for most of the compounds, they may also be consistently highly variable in some cases.

3.2.3.1 General conclusions

Based on the statistical analysis described above, there was, in general, a high degree of inter- and intra-laboratory variability. Though there is not a single factor that can be identified as contributing to this high degree of variability, several conclusions can be made by examining data from the chemicals individually. It is hypothesized that much of the variability seen with these chemicals was due to inconsistencies with the analytical method. Due to time constraints, preliminary incubations were not performed with these nine test chemicals to determine appropriate incubation times and substrate concentrations. This may help to explain some of the observed variability. Data summaries are presented in **Tables 7 – 15.**

3.2.3.2 Diclofenac

This chemical showed measurable loss rates in all three laboratories with the overall lowest intra- and inter-laboratory variability. This pharmaceutical compound is water soluble, can be analyzed by LC-MS/MS, and analytical methods were readily available. Diclofenac is a substrate for CYP2C9 in mammalian *in vitro* metabolism studies and has been well-studied in the pharmaceutical sector (**Table 7**).

3.2.3.3 Benzo(a)pyrene

Analytical methods for BaP are readily available, as this chemical has been very well-studied in fish and other non-mammalian species. In examining the data, laboratories familiar with the analytical method required for lipophilic chemicals like BaP demonstrated the lowest variability, highlighting that experience in handling and analyzing particular chemicals contributes to the success of the assay **(Table 8).**

3.2.3.4 Zoxamide

As with fenthion, there was inconsistency in the time courses with triplicate runs. Zoxamide is very rapidly metabolized and CellzDirect/Life Technologies and Dow had the same time courses and lower coefficient of variation (CoV) as opposed to the University of Queensland **(Table 9).**

3.2.3.5 Fenthion

Runs with fenthion demonstrated inconsistency in the time courses with triplicate runs. Fenthion was analyzed with the same instrumentation and analytical method in all three laboratories, though results indicate similar conclusions as with zoxamide, with CellzDirect and Dow having the same time courses and lower CoV as opposed to University of Queensland, deviating from these two laboratories **(Table 10).**

3.2.3.6 Deltamethrin

This chemical illustrates the need for robust analytical method development for "sticky" compounds. The three laboratories used the same analytical method, though CellzDirect/Life Technologies and University of Queensland showed low R^2 values (<<0.7). Dow deltamethrin runs has a low CoV and high R^2 value and was the laboratory with the most experience in analyzing this compound **(Table 11).**

3.2.3.7 17α-Ethinylestradiol

This chemical's analytical method requires derivatization for both GC/MS and HPLC. It is hypothesized that the large number of vial transfers required for the derivatization may have affected recovery, as evidenced by heat-treated and T=0 recovery values. All three laboratories showed low R^2 values and high CoV **(Table 12).**

3.2.3.8 Decanol

The analytical method available for decanol was very difficult, with the chemical peak appearing very close to the time of sample injection. Only one laboratory (CellzDirect/Life Technologies) demonstrated fast metabolism; Dow and University of Queensland were unable to attain lower limits of quantitation and unable to detect the compound at 0.5 μM. Therefore, their experiments were run at 1 μM. In addition, Dow was unable to obtain three replicate experiments resulting in only one experiment for analyses. University of Queensland and Dow also demonstrated problems recoveries at T=0 **(Table 13).**

3.2.3.9 C16E08

This chemical illustrates the need for robust analytical method development for "sticky" compounds. Both CellzDirect/Life Technologies and Dow showed similar metabolic rates and shared the same analytical method. However, low CoVs for CellzDirect and the Dow were observed as well as unusually high recoveries (>500%), high variability and low R^2 for University of Queensland **(Table 14).**

3.2.3.10 DBP

This chemical showed very fast metabolism but also very different metabolic rates in all three laboratories. This compound had a very difficult chemistry to work with and various analytical methods and test concentrations were used **(Table 15).**

4. Results – Multiple S9 Preparations

4.1 Data summary

Metabolism of three chemicals (pyrene, FMHE, and 4-nonylphenol) was examined using four separate S9 pools prepared by different laboratories: Batch A (CellzDirect – see above), Batch B (USEPA – see above), Batch C (CanTest/Maxxam Analytics), and Batch D (Dow). As discussed above, Batch A S9 had lower activity and little to no turnover was seen the chemicals listed. Rainbow trout strains and other parameters for each S9 batch are included in **Table 16.**

4.1.1 Pyrene

Data obtained from the four S9 pools indicate that activity in Batch B > Batch C >Batch D (which is nearly equal to the low activity seen in Batch A) **(Table 17).**

4.1.2 4-NP

Data obtained from the four S9 pools indicate that activity in Batch B > Batch C >Batch D (which is nearly equal to the low activity seen in Batch A) **(Table 18).**

4.1.3 FMHE (Fluoroxypyr-1-methylheptyl-ester)

Data obtained from the four S9 pools indicate that activity in Batch B >Batch C&D > Batch A **(Table 19).**

4.1.4 Methoxychlor

Data obtained from the four S9 pools indicate that activity in Batch C >Batch B>>Batch A. **(Table 20).** Results from batch D were not obtained.

4.2 Discussion

The large observed differences in metabolic rate between trout liver S9 preparations, particularly between Batch B/C and Batch D can be explained by differences in S9 preparation. Batch B and batch C did not freeze the livers prior to isolation of S9, whereas the batch D livers were frozen. As was seen with the preparation of

the Batch A rainbow trout liver S9 above, freezing the livers results in a decrease in metabolic activity by half the activity. The rainbow trout liver S9 (Batch A) showed lower activity in subsequent enzyme activity assays (testosterone hydroxylation) (**Figure 1**) when compared to other batches (e.g. Batch B). It is important to note that sampling handling such as freezing the tissue prior to S9 processing affects overall enzyme activity. We strongly recommend the use of fresh over frozen tissue. We were unable to process Batch A on site and frozen tissue had to be used compromising enzyme activity. For this reason, the laboratories and management team recommended to use another trout liver S9 preparation for the remaining 9 chemicals. This liver S9 (Batch B) batch was prepared on site from fresh liver tissue.

In addition, trout used for the various preparations were different strains and from different regions of the country. However, it is generally agreed that the freezing of the liver prior to S9 preparation was most likely the largest contributor to the observed variability. Differences in metabolic enzyme activities in strains, stages during the life cycle and water parameters used during growth and maintenance of the fish should be further investigated to confirm the observations up to date.

5. Bioavailability

In vitro metabolic measurements using fish liver S9 fractions have the potential to provide rapid and cost-effective measurements of biotransformation potential by measuring intrinsic clearance rate of the parent chemical. The first step in the extrapolation process is the estimation of liver *in vivo* intrinsic clearance by incorporating scaling factors relating the experimental test conditions from S9 metabolic stability evaluations to the whole liver. As a second step, this intrinsic clearance in the liver is combined with information on liver blood flow and the scaling factor f_u. The term f_u is the ratio of the free fraction of chemical in the blood to the free fraction of the chemical in the *in vitro* test system. There is currently no empirical equation for estimating the free fraction in S9 *in vitro* tests. Therefore, we measured S9 and blood binding for five of the test chemicals used in the S9 metabolism study (pyrene, methoxychlor, fluoroxypyr, chlorpyrifos, and nonylphenol).

A detailed summary of the results of this work can be found in Escher et al (2011).

6. Extrapolation Model

Results from the 9 chemicals assayed using trout liver S9 Batch B (Section 3.2) were used to estimate BCF using the extrapolation model described in Cowan-Ellsberry et al. (2008). Preliminary analysis of this data showed that the model is relatively insensitive to differences in in vitro S9 rates. Despite relatively large differences in measured S9 loss rates (as evidenced by the standard deviation), there is little change in the estimated BCF values.

As a result of these findings, a team has been working to evaluate the various model parameters, with a focus on the following:

- Performing a critical analysis of the model assumptions. For example, the current model assumes a 1 kg fish with 10% lipid content.
- Performing a sensitivity analysis of the model to demonstrate the key parameters that drive the calculations.
- Re-analyzing the critical parameters affecting BCF output identified in the Cowan-Ellsberry et al (2008) paper:
  - Blood flow through the liver
  - Fish blood characteristics – such as binding
  - Free fraction of the chemical in the S9 assay (assumptions related to the binding correction term, f_u)

New mathematical algorithms will provide improved predictions of critical model input parameters and a formal sensitivity analysis will provide additional guidance on model inputs that contribute the greatest amount of variability and uncertainty in predicted levels of accumulation.

7. Key learnings and next steps

Overall, the results of this study resulted in standardization of the trout liver S9 fraction isolation and incubation protocols over the two-year duration of the project. As previously noted, these protocols were published in the August 2012 issue *of Current Protocols in Toxicology, In vitro* (Johanning et al., 2012).

In addition, this study resulted in the identification of several critical factors that should be considered in future research projects.

- Collect all data related to the fish used for S9 isolation (strain, aquaculture conditions (water chemistry (e.g. dissolved oxygen, hardness), temperature, fish density, and photoperiod), age, life cycle, and gender).
- Prepare liver S9 fraction using fresh tissue – do not freeze livers prior to S9 isolation if at all possible. Process (perfuse and excise the liver after fish has

been euthanized, homogenize and centrifuge) as many fish as possible within one hour from the start of the first fish to be pooled. It is recommended to process no more than 5 – 6 fish in one hour.

- Add 250 mM sucrose in homogenization buffer and prepare a stock of pooled trout liver S9 fraction at an approximate total protein 25 mg/mL.

- Incubate the test chemicals at the fish holding or acclimation temperature (typically somewhere12.5 ± 2.5 °C) or and 100 mM phosphate buffer at pH = 7.8.

- It is imperative and crucial to develop the analytical method (GC-MS; LC-MS/MS; HPLC) for the particular test chemical before any trout liver S9 fraction incubation. Consider LOQ (sensitivity) before choosing test chemical concentrations. Make sure the test chemical recoveries are > 80%.

- Establish in preliminary incubations: A- Incubation Time (up to 120 min) and B- Protein Concentration (somewhere 0.2- 2 mg/mL) and determine that the reaction rate follows first-order reaction kinetics and linearity within the established concentration range used in the calibration curve.

- Collect at least 6 time points and use the following time courses:
  - for non-esters: e.g. 0, 20, 40, 60, 90 and 120 min
  - for esters or "ester-like": e.g. 0, 2.5, 5, 7.5, 10 and 20 min

- All chemicals should demonstrate first-order enzyme kinetics.

- The test chemical should be added last. This addition designates the initiation of the reaction.

- Glass vessels (test tubes) should be used for the incubations instead of plastic to avoid test chemical sticking to the plastic surfaces.

- The assays require appropriate controls to address:
  - Volatility
  - Adsorption
  - Non-enzymatic degradation

These controls (negative) include heat-treated trout liver S9 (inactive or heat-treated) to determine whether there is non-enzymatic degradation of the test chemical. Although positive controls were not included in the incubation assays, assessment of phase I (e.g. testosterone or lauric acid hydroxylation) and II (e.g. 7-hydroxycoumarin sulfation and glucuronidation or estradiol glucuronidation) enzymatic activity should be performed to test the stability and activity of the trout liver S9 fraction.

Additional controls such as no-cofactors may be included. These controls will assess whether other non- Phase I (CYP450) and II (e.g. UGTs, SULTs) enzyme systems such as carboxyl esterases (very active in fish) play a role in the metabolism of the test chemicals.

- The results thus far indicate that the assay works best for the data to be used in the BCF determination when metabolism rates are rapid. However, the assay can detect slow, moderate to fast metabolism of test chemicals.

Once the critical parameters mentioned above have been identified, the five main steps for the trout liver S9 *in vitro* metabolism assay are:

- **Step 1: Robust analytical method development.** The first step before embarking in the trout liver S9 *in vitro* metabolic assay is to develop a robust analytical method for the test chemical under investigation. We find that this is the biggest limiting step. It is **extremely important** that the laboratory performing the analysis has experience in test chemicals with high Kow > 3, i.e. lipophilic and handling biological "matrixes" such as the rainbow trout liver S9 fraction.
- **Step 2: Preliminary incubations.** These incubations are necessary to determine the optimal active rainbow trout liver S9 protein concentration, test chemical concentration (recommendation start at around 0.5-1 μM) and incubation time.
- **Step 3: Final incubations.** Once the assay conditions have been identified, proceed with the final incubations
- **Step 4: Analysis of parent chemical and calculation of transformation rate (k)** based on loss of parent compound.
- **Step 5: Prediction of *in vivo* BCF based on in vitro K_{met}** using the available models.

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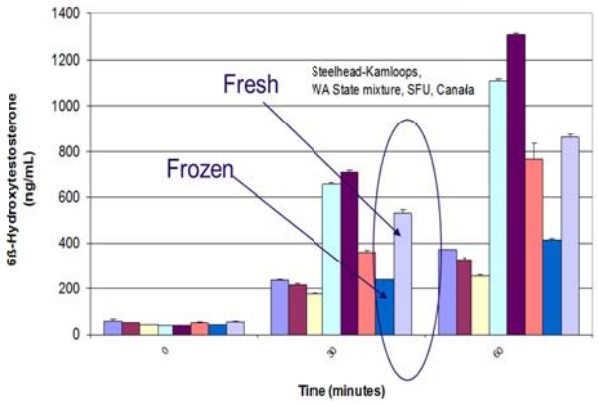
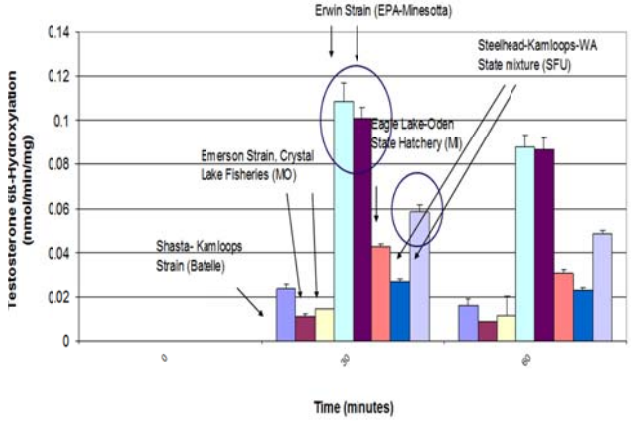
Figure 1. Fresh vs frozen tissue preparation effect. Formation of 6β-hydroxytestosterone from testosterone (by the CYP3A enzyme system) in rainbow trout liver S9 from various trout strains overtime (at T= 0, 30 and 60 min)



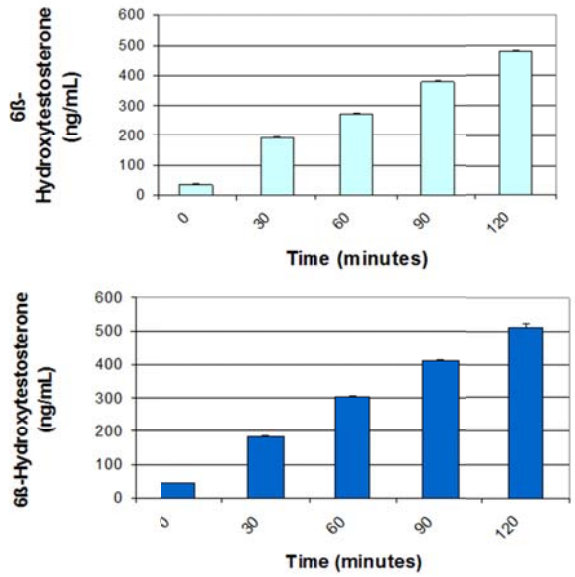
This graphic representation is to illustrate and highlight the circled area showing the difference in enzyme activity (by 6β-hydroxytestosterone metabolite formation from testosterone) in tissue from the same strain and batch of fish (male trout Steelhead-Kamloops WA State strain) that were processed from frozen (blue bar) and fresh tissue (lilac bar). The frozen samples (blue bar) are about half the activity when compared to the fresh tissue samples (lilac bar).

Figure 2. Trout strain effect. Testosterone 6β-hydroxytestosterone activity (CYP3A) in rainbow trout liver S9 fractions from various strains.



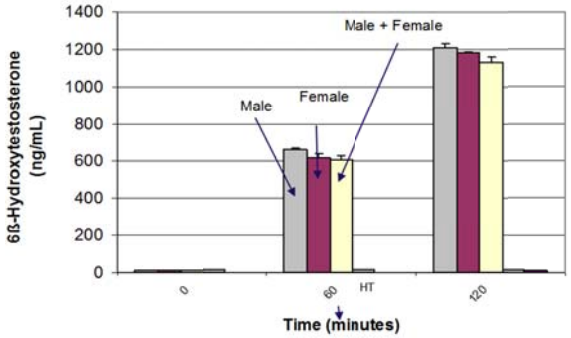
This graph shows testosterone hydroxylation activity in rainbow trout liver S9 fractions from different strains and sex. These samples include enzyme activities in rainbow trout liver S9 fractions from: Shasta-Kamloops strain (Battelle PNNL lab) (purple bar, Batch **A**, frozen tissue from male trout), Emerson strain from Crystal Lake Fisheries (red bar, male trout; yellow bar, female trout; both from frozen tissue), Erwin strain (EPA-MN) (light blue bar, male trout, Batch **B**; purple bar, female trout; both from fresh tissue), Eagle-Lake Oden State Hatchery (coral bar, fresh tissue from male trout, Batch **D**), and Steelhead-Kamloops mixture WA state mixture (blue bar, male trout from frozen tissue; lilac bar, fresh tissue from male trout, Batch **C**).

Figure 3. Rainbow trout liver S9 incubation temperature effect. Formation of 6β-hydroxytestosterone (from 500uM testosterone) in rainbow trout S9 liver fractions (Emerson Strain, Crystal Lake Fisheries, MO, USA) incubations held at 12ºC (top) and 18ºC (bottom)



This graphic representation shows metabolite formation (6β-hydroxytestosterone) in rainbow trout liver S9 fractions incubated at two different temperatures, T= 12 ºC (top) and 18 ºC (bottom).Samples were incubated overtime up to 120 min.

Figure 4. Sex effect, male vs female rainbow trout liver S9 fraction. Formation of 6β-hydroxytestosterone (CYP3A) in male, female, and pooled rainbow trout liver S9 fractions prepared from fresh tissue (Emerson Strain, Crystal Lake Fisheries, MO, USA) (different batch from the one depicted in Figure 2).



This graph shows of 6β-hydroxytestosterone formation in rainbow trout liver S9 fractions from male, female and pooled (male + female). Incubations were performed overtime up to 120 min. Arrows indicate the different samples from male rainbow trout (grey bar), female rainbow trout (red bar) and pooled male + female rainbow trout (yellow bar). HT denotes heat-treated liver S9 (male trout).

Figure 5. Measured loss rates for 9 test chemicals in all 3 laboratories. Error bars represent standard error; each laboratory had 3 runs to assess intra-laboratory variability.

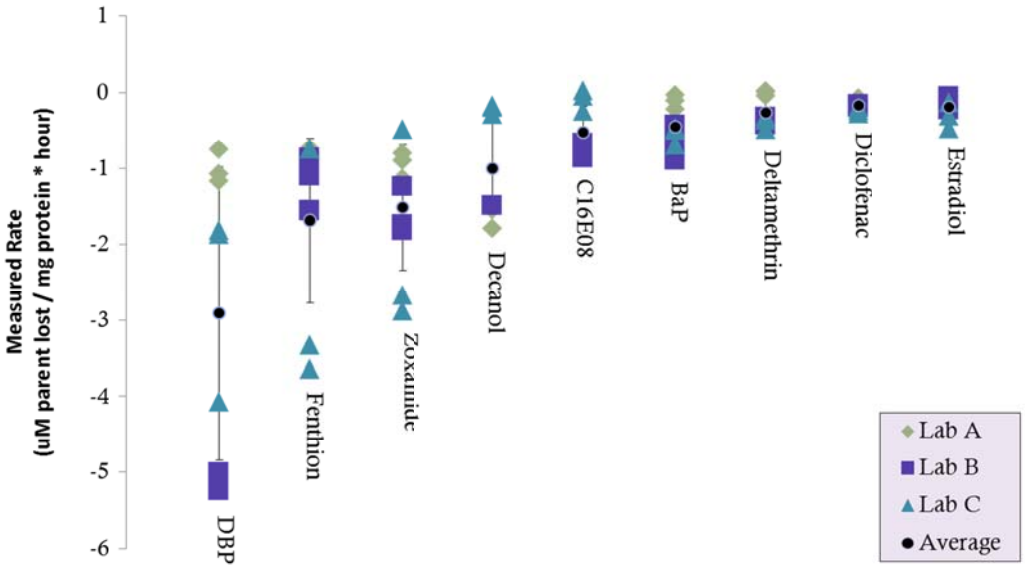


Table 1: Test chemicals

The first 6 chemicals in the table (shaded) were used for the initial proof of concept studies

Category	Chemical	CAS No.	Log K_{ow}	Measured Log BCF	BCF Species	REF
Alcohol (phenol)	4-nonylphenol	104405	5.99	2.31-2.64	Fathead Minnow	1,2
PAH	Pyrene	129000	4.93	1.7-3.4	Sheepshead minnow & others	3,4,5
Ester (Aromatic)	fluroxypyr 1-methylheptyl ester	81406373	4.82	1.64	Rainbow Trout	6
Ether	Dibenzyl Ether	103504	3.48	1.6-1.9	Common Carp	7
Organo-phosphate	Chlorpyrifos	2921882	4.66	3.12	Rainbow Trout	8
Organo-chlorine	Methoxychlor	72435	5.67	2.0-3.18	Sheepshead Minnow Mosquitofish	9,10
Aliphatic Alcohol	1-Decanol[a]	112-30-1	4.77-5.13*	1.78 (Exxal)*	Rainbow Trout*	11
PAH	Benzo (a) Pyrene (BaP)	50328	6.11	2.4-2.9	Bluegill	12, 13
Aliphatic Ether	C16EO8	5698-39-5	4.54	2.6	Fathead Minnow	14
Organo-phosphate	Fenthion	55389	4.09	2.1-2.9	guppy goldfish killifish mtn cloud fish	15, 16
Benzamide Fungicide	Zoxamide	156052685	3.76	2.6	Bluegill	17
Pyrethroid	Deltamethrin	52918635	6.18	1.6-2.5	Rainbow Trout	18, 19
Hormone	17α-Ethinylestradiol	57636	4.2	<2.7	Fathead Minnow	20
NSAID	Diclofenac	15317796	4.02-4.51	Wide ranging with tissue and conc.	Rainbow Trout	21
Phthalate	Dibutyl phthalate[b]	84-74-2	4.89**	4.89**		

1. *Snyder et al. (2001)*
2. *Giesy et al. (2000)*
3. *Carlson et al. (1979)*
4. *Ogata et al. (1984)*
5. *Jonsson et al. (2004)*
6. *Rick et al. (1996)*
7. *ExxonMobil Chemical Company. (2006)*
8. *Baron and Woodburn (1995)*
9. *Kapoor, I. P. et al. (1970)*
10. *Hansen and Parrish (1977)*
11. *de Wolf and Parkerton, 1999*
12. *Jimenez et al. 1987*
13. *Spacie et al. 1983*
14. *Tolls et al. 2000*
15. *de Bruin and Hermens 1991*
16. *Tsuda et al. 1997*
17. *Robinson. 1998*
18. *Muir et al. 1994*
19. *http://www.inchem.org/documents/ehc/ehc/ehc97.htm#SectionNumber:4.4*
20. *Länge et al. 2001*
21. *Schwaiger et al. 2004*

*Corresponds to Dodecanol, none available for decanol

**Corresponds to values for Diethyl Hexyl Phthalate, none available for Dibutyl Phthalate

[a]dodecanol was substituted for 1-Decanol due to analytical method availability of the later

[b]Diethyl Hexyl Phthalate was substituted for Dibutyl phthalate due to analytical method availability of the later

Table 2. Analytical methods used for each test chemicals in the various laboratories

Test Chemical	Analytical Instrumentation				
	CellzDirect	Dow	UQ	Maxxam	P&G
4-NP	GC-MS	N/A	HPLC	N/A	GC-MS
Pyrene	GC-MS	GC-MS	HPLC fluorescence detection	GC-MS	GC-MS
Beate had chechFMHE	GC-MS	GC-MS	GC-MS	LC-MS	GC-MS
DBE	GC-MS	N/A	GC-MS	N/A	GC-MS
Chlorpyrifos	GC-MS	GC-MS	HPLC	LC-MS	GC-MS
Methoxychlor	GC-MS	N/A	GC-ECD	N/A	GC-MS
BaP	GC-MS	GC-MS	HPLC	N/A	N/A
C1608	LC-MS-MS	LC-MS-MS	HPLC, GC-MS	N/A	N/A
DBP	GC-MS	GC-MS	HPLC	N/A	N/A
Decanol	GC-MS	GC-MS	GC-MS	N/A	N/A
Deltamethrin	GC-MS	GC-MS	GC-MS	N/A	N/A
Diclofenac	LC-MS/MS	LC-MS/MS	HPLC	N/A	N/A
EE2	LC-MS/MS	LC-MS/MS	HPLC, GC-MS	N/A	N/A
Fenthion	GC-MS	GC-MS	GC-MS	N/A	N/A
Zoxamide	LC-MS/MS	LC-MS/MS	HPLC	N/A	N/A

N/A = Not Applicable

Table 3: Summary statistics of k for each compound (all laboratories, all data)

Compound	Avg k	SD	CoV	Min	Max	n
BaP	0.448	0.289	64.490	0.035	0.901	9
C1608	0.526	0.342	65.103	-0.031	0.864	9
DBP	2.910	1.929	66.295	0.750	5.248	9
Decanol	1.001	0.744	74.358	0.169	1.790	7
Deltamethrin	0.260	0.197	75.735	-0.018	0.482	9
Diclofenac	0.168	0.065	38.464	0.073	0.274	9
EE2	0.189	0.134	70.884	0.042	0.469	9
Fenthion	1.657	1.104	66.640	0.740	3.639	9
Zoxamide	1.521	0.827	54.366	0.485	2.879	9

Table 4. Summary statistics of k for each compound\laboratory

Cmpd	Lab	Avg k	SD	CoV	Min	Max	n
BaP	CD	0.121	0.095	79.095	0.035	0.223	3
	DOW	0.675	0.237	35.145	0.428	0.901	3
	UQ	0.548	0.123	22.530	0.465	0.690	3
C16E08	CD	0.716	0.012	1.671	0.703	0.727	3
	DOW	0.776	0.100	12.850	0.668	0.864	3
	UQ	0.086	0.144	167.399	-0.031	0.247	3
DBP	CD	1.002	0.222	22.208	0.750	1.172	3
	DOW	5.151	0.125	2.429	5.010	5.248	3
	UQ	2.578	1.290	50.017	1.807	4.067	3
Decanol	CD	1.626	0.142	8.720	1.540	1.790	3
	DOW	1.491	-	-	1.491	1.491	1
	UQ	0.213	0.060	28.185	0.169	0.281	3
Delta-methrin	CD	0.008	0.034	429.875	-0.018	0.047	3
	DOW	0.388	0.058	14.918	0.322	0.429	3
	UQ	0.384	0.088	22.788	0.313	0.482	3
Diclofenac	CD	0.095	0.024	24.746	0.073	0.120	3
	DOW	0.178	0.021	11.955	0.159	0.201	3
	UQ	0.231	0.042	18.043	0.191	0.274	3
EE2	CD	0.114	0.064	56.465	0.042	0.166	3
	DOW	0.154	0.095	61.495	0.050	0.235	3
	UQ	0.299	0.174	58.006	0.122	0.469	3
Fenthion	CD	1.322	0.505	38.156	0.742	1.657	3
	DOW	1.080	0.418	38.678	0.814	1.561	3
	UQ	2.568	1.592	61.966	0.740	3.639	3
Zoxamide	CD	0.950	0.173	18.165	0.806	1.141	3
	DOW	1.605	0.309	19.265	1.250	1.817	3
	UQ	2.009	1.324	65.914	0.485	2.879	3

CD = CellzDirect / Life Technologies
UQ = University of Queensland

Table 5: Summary statistics of k for each compound (Dow and CD only)

Compound	Average k	St Dev	CoV	Min	Max	n
BaP	0.398	0.344	86.470	0.035	0.901	6
C1608	0.746	0.071	9.569	0.668	0.864	6
DBP	3.077	2.279	74.064	0.750	5.248	6
Decanol	1.593	0.134	8.421	1.491	1.790	4
Deltamethrin	0.198	0.213	107.297	-0.018	0.429	6
Diclofenac	0.137	0.050	36.248	0.073	0.201	6
EE2	0.134	0.076	56.422	0.042	0.235	6
Fenthion	1.201	0.435	36.222	0.742	1.657	6
Zoxamide	1.277	0.423	33.111	0.806	1.817	6

Table 6: Summary statistics of k for each compound\laboratory (Dow and CD only)

Compound	Lab	Avg k	SD	CoV	Min	Max	n
BaP	CD	0.121	0.095	79.095	0.035	0.223	3
	DOW	0.675	0.237	35.145	0.428	0.901	3
C1608	CD	0.716	0.012	1.671	0.703	0.727	3
	DOW	0.776	0.1	12.85	0.668	0.864	3
DBP	CD	1.002	0.222	22.208	0.75	1.172	3
	DOW	5.151	0.125	2.429	5.01	5.248	3
Decanol	CD	1.626	0.142	8.72	1.54	1.79	3
	DOW	1.491	-	-	1.491	1.491	1
Delta-methrin	CD	0.008	0.034	429.875	-0.018	0.047	3
	DOW	0.388	0.058	14.918	0.322	0.429	3
Diclofenac	CD	0.095	0.024	24.746	0.073	0.12	3
	DOW	0.178	0.021	11.955	0.159	0.201	3
EE2	CD	0.114	0.064	56.465	0.042	0.166	3
	DOW	0.154	0.095	61.495	0.05	0.235	3
Fenthion	CD	1.322	0.505	38.156	0.742	1.657	3
	DOW	1.08	0.418	38.678	0.814	1.561	3
Zoxamide	CD	0.95	0.173	18.165	0.806	1.141	3
	DOW	1.605	0.309	19.265	1.25	1.817	3

Table 7. Diclofenac Summary Data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	0.274	0.0310	0.828	0.000
	2	0.227	0.0280	0.803	0.000
	3	0.191	0.0310	0.704	0.000
DOW	1	0.174	0.0270	0.717	0.000
	2	0.159	0.0180	0.833	0.000
	3	0.201	0.0200	0.859	0.000
CD	1	0.093	0.0110	0.816	0.000
	2	0.073	0.0250	0.344	0.010
	3	0.120	0.0120	0.869	0.000

Table 8. BaP Summary Data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	0.465	0.0761	0.756	0.000
	2	0.489	0.0408	0.900	0.000
	3	0.690	0.0387	0.952	0.000
DOW	1	0.428	0.0310	0.927	0.000
	2	0.901	0.0696	0.918	0.000
	3	0.696	0.0448	0.941	0.000
CD	1	0.035	0.0214	0.141	0.125
	2	0.104	0.0171	0.698	0.000
	3	0.223	0.0417	0.642	0.000

Table 9. Zoxamide summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	2.879	0.3300	0.854	0.000
	2	0.485	0.3190	0.126	0.148
	3	2.662	0.1940	0.926	0.000
DOW	1	1.250	0.1160	0.879	0.000
	2	1.817	0.0690	0.977	0.000
	3	1.747	0.0670	0.977	0.000
CD	1	1.141	0.0690	0.945	0.000
	2	0.806	0.0440	0.955	0.000
	3	0.902	0.0380	0.972	0.000

Table 10. Fenthion summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	3.327	0.3082	0.943	0.000
	2	3.639	0.3908	0.925	0.000
	3	0.739	0.116	0.717	0.000
DOW	1	0.864	0.1430	0.695	0.000
	2	1.561	0.2080	0.790	0.000
	3	0.814	0.4510	0.178	0.091
CD	1	0.742	0.1000	0.775	0.000
	2	1.568	0.1180	0.917	0.000
	3	1.657	0.1340	0.906	0.000

Table 11. Deltamethrin summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	0.482	0.0008	0.115	0.257
	2	0.358	0.0793	0.560	0.000
	3	0.313	0.0947	0.421	0.005
DOW	1	0.429	0.0460	0.846	0.000
	2	0.414	0.0310	0.920	0.000
	3	0.322	0.0330	0.854	0.000
CD	1	-0.005	0.0350	0.001	0.899
	2	0.047	0.0150	0.393	0.005
	3	-0.018	0.0510	0.008	0.731

Table 12. 17α-Ethinylestradiol summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	0.122	0.0850	0.114	0.172
	2	0.307	0.0980	0.397	0.007
	3	0.469	0.1190	0.494	0.001
DOW	1	0.235	0.0260	0.831	0.000
	2	0.050	0.0320	0.132	0.139
	3	0.176	0.0470	0.467	0.002
CD	1	0.134	0.0210	0.714	0.000
	2	0.042	0.0410	0.062	0.320
	3	0.166	0.0370	0.564	0.000

Table 13. Decanol summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	0.281	0.0780	0.461	0.003
	2	0.169	0.0450	0.516	0.003
	3	0.188	0.0440	0.531	0.001
DOW	1	1.491	0.1450	0.883	0.000
	2				
	3				
CD	1	1.540	0.0600	0.977	0.000
	2	1.790	0.0620	0.981	0.000
	3	1.549	0.0700	0.969	0.000

Table 14. C16EO8 summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	-0.031	0.2026	0.002	0.880
	2	0.043	0.0662	0.025	0.528
	3	0.247	0.0674	0.456	0.002
DOW	1	0.668	0.0193	0.844	0.000
	2	0.864	0.0290	0.982	0.000
	3	0.795	0.0856	0.987	0.000
CD	1	0.719	0.0187	0.989	0.000
	2	0.703	0.0187	0.989	0.000
	3	0.727	0.0301	0.973	0.000

Table 15. DBP summary data

Lab	Rep	Rate - k	SE(k)	R2	p-value
UQ	1	1.861	0.6740	0.337	0.015
	2	4.067	0.6789	0.692	0.000
	3	1.807	0.1331	0.920	0.000
DOW	1	5.248	0.1550	0.986	0.000
	2	5.010	0.2820	0.952	0.000
	3	5.196	0.2760	0.957	0.000
CD	1	0.750	0.0680	0.883	0.000
	2	1.172	0.0910	0.912	0.000
	3	1.083	0.0650	0.946	0.000

Table 16. Parameters for various S9 preparations

S9 pool	Rainbow Trout Strain	Sex	Fresh / Frozen
Batch A (CellzDirect/Life Technologies)	Shasta-Kamloops	Male	Frozen
Batch B (USEPA)	Erwin	Both	Fresh
Batch C (CanTest/Maxxam)	Steelhead-Kamloops	Male	Fresh
Batch D (Dow)	Eagle Lake Hatchery	Male	Frozen

Table 17. Pyrene data obtained from 4 different S9 pools

S9 pool	Rep	Rate - k	SE(k)	R2	p-value
Batch A (CellzDirect / Life Technologies)	1	0.297	0.014	0.963	0.00
	2	0.150	0.013	0.908	0.00
	3	0.198	0.011	0.951	0.00
Batch B (USEPA)	1	0.114	0.035	0.622	0.00
	2	3.406	0.083	0.982	0.00
	3	4.932	0.050	0.998	0.00
Batch C (CanTest / Maxxam)	1	0.822	0.068	0.900	0.00
	2	1.008	0.100	0.863	0.00
	3	0.876	0.062	0.930	0.00
Batch D (Dow)	1	0.306	0.022	0.834	0.00
	2	0.002	0.147	0.000	0.982
	3	0.140	0.050	0.328	0.013

Table 18. 4-NP data obtained from 4 different S9 pools

S9 pool	Rep	Rate - k	SE(k)	R2	p-value
Batch A (CellzDirect / Life Technologies)	1	-0.61	0.040	0.127	0.146
	2	-0.088	0.033	0.311	0.016
	3	0.479	0.074	0.722	0.000
Batch B (USEPA)	1	0.310	0.081	0.511	0.002
	2	0.545	0.095	0.671	0.00
	3	0.332	0.029	0.889	0.00
Batch C (CanTest / Maxxam)	1	0.172	0.014	0.905	0.00
	2	0.325	0.014	0.973	0.00
	3	0.384	0.014	0.979	0.00
Batch D (Dow)	1	0.204	0.021	0.123	0.168
	2	0.007	0.081	0.006	0.773
	3	0.069	0.095	0.412	0.005

Table 19. FMHE data obtained from 4 different S9 pools

S9 pool	Rep	Rate - k	SE(k)	R2	p-value
Batch A (CellzDirect / Life Technologies)	1	2.840	0.302	0.847	0.00
	2	6.187	0.425	0.930	0.00
	3	2.327	0.833	0.328	0.013
Batch B (USEPA)	1	24.130	0.490	0.998	0.00
	2	10.905	1.174	0.935	0.00
	3	22.046	1.144	0.981	0.00
Batch C (CanTest / Maxxam)	1	6.515	1.140	0.671	0.00
	2	9.169	1.075	0.820	0.00
	3	7.478	0.931	0.801	0.00
Batch D (Dow)	1	8.010	0.613	0.914	0.00
	2	8.695	0.914	0.850	0.00
	3	7.667	0.918	0.813	0.00

Table 20. Methoxychlor data obtained from 3 different S9 pools

S9 pool	Rep	Rate - k	SE(k)	R2	p-value
Batch A (CellzDirect / Life Technologies)	1	0.066	0.026	0.299	0.023
	2	0.050	0.030	0.157	0.115
	3	0.004	0.010	0.010	0.699
Batch B (USEPA)	1	0.253	0.063	0.506	0.001
	2	0.179	0.017	0.872	0.00
	3	0.212	0.024	0.830	0.00
Batch C (CanTest / Maxxam)	1	0.654	0.039	0.945	0.00
	2	0.319	0.041	0.793	0.00
	3	0.161	0.034	0.763	0.002
Batch D (Dow)	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-

Table 21. Estimated BCF values using the Cowan-Ellsberry et al (2008) extrapolation model

Chemical	log K_{ow}	est. BCF [k_{met} = 0]	*in vivo* BCF (TG305)	S9 data		extrapolation model	
				measured loss rate (average)	stdev	estimated BCF	BCF stdev
BaP	6.11	26,826	251 - 794	-0.45	0.29	383	0.149
C16E08	4.54	8,934	398	-0.53	0.34	372	0.120
DBP	4.5	2,882	n/a	-2.91	1.93	341	0.005
Decanol	3.76	565	60*(n-dodecanol)*	-1.00	0.74	227	0.015
Deltamethrin	6.18	27,752	39 - 316	-0.26	0.20	383	0.114
Diclofenac	4.51	2,493	40 - 316	-0.17	0.06	342	0.043
17α-Estradiol	4.2	461	501	-0.19	0.13	207	0.038
Fenthion	4.09	1,185	126 - 794	-1.69	1.08	290	0.003
Zoxamide	3.76	565	398	-1.52	0.83	227	0.003