Organisation for Economic Co-operation and Development

ENV/JM/MONO(2018)13

Unclassified

English - Or. English

27 August 2018

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Validation Report of the two new Test Guidelines on Determination of In Vitro Intrinsic Clearance Using Cryopreserved Rainbow Trout Hepatocytes or Liver S9 Sub-Cellular Fractions

SERIES ON TESTING AND ASSESSMENT
NO. 281

JT03435123

This document, as well as any data and map included herein, are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 281

Validation Report of the two new Test Guidelines on Determination of In Vitro Intrinsic Clearance Using Cryopreserved Rainbow Trout Hepatocytes or Liver S9 Sub-Cellular Fractions

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2018

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 35 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in twelve different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides**; **Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; Safety of Manufactured Nanomaterials;** and **Adverse Outcome Pathways.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

For this and many other Environment, Health and Safety publications, consult the OECD's World Wide Web site (www.oecd.org/ehs)

or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France

Fax : (33-1) 44 30 61 80

E-mail : ehscont@oecd.org

© OECD 2018
Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

Foreword

This document contains the report of the international validation studies conducted in 2014 and 2015 on the determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes or liver S9 sub-cellular fractions (adopted as OECD Test Guideline 319A and 319B).

The project to develop this Guidance Document was co-led by the European Commission (EC-JRC) and the United States.

The Working Group of the National Coordinators of the Test Guidelines Programme endorsed the ring-test report at its 30th meeting in April 2018. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the report on 30 June 2018.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

Table of contents

INTRODUCTION

1. Hydrophobic organic chemicals released to the environment may accumulate in fish and other aquatic animals. In general, this behavior reflects the tendency of such compounds to partition out of water and into tissue lipids. Other chemicals accumulate in fish because of their affinity for specific proteins in blood and tissues. In either case, this accumulation increases the risk of toxicity to exposed animals and the animals that consume them, including humans. For this reason, the potential for chemical bioaccumulation in fish is commonly evaluated when performing chemical hazard assessments, and limits on acceptable levels of accumulation have been prescribed under various legislative frameworks (Gobas et al., 2009).

2. The potential for a chemical to accumulate in fish may be determined directly using standardized testing methods (e.g., OECD Test Guideline 305; OECD, 2012), but these methods are expensive, time-consuming, and require a substantial number of animals. More commonly, bioaccumulation assessments are performed using predictive computational models. One-compartment mass-balance models are preferred for many screening-level assessments, in part because of their relative simplicity. These models can account for differences in the nature of an exposure (e.g., route and environmental conditions such as temperature) as well as attributes of the exposed organism (e.g., size). Examples include the K_{OW} (based) Aquatic Bioaccumulation Model (KABAM; USEPA, 2016) used by USEPA for pesticide risk assessment and the BCFBAF module provided as part of USEPA's Estimation Program Interface Suite (EPI Suite; USEPA, 2012), both of which are based on well-known descriptions given by Arnot and Gobas (2003, 2004). Models of greater complexity, including physiologically based toxicokinetic (PBTK) models for fish, have also been promoted as tools for bioaccumulation assessment (Stadnicka et al., 2014; Brinkmann et al., 2015). Because they describe chemical accumulation in specific tissues and organs, PBTK models provide a direct link between chemical accumulation at a site of action and observed effects. In addition, these models are well suited to relate *in vitro* effects information to exposures that would be required to elicit these effects *in vivo* ("reverse toxicokinetics").

3. A critical input to both model types is the rate of hepatic biotransformation. Researchers have long known that biotransformation may substantially reduce the extent of chemical bioaccumulation in fish (Southworth et al., 1980; Oliver and Niimi, 1985; de Wolf et al., 1992). However, unlike many other inputs to the models (e.g., rates of chemical flux across the gills and gut) the rate of metabolism for a particular compound cannot be predicted with any confidence from its relatively hydrophobicity (e.g., log K_{OW}). For this reason, biotransformation represents the principal source of uncertainty in many bioaccumulation assessments for fish (Nichols et al., 2009).

4. Thus, a need exists for methods to estimate metabolic activity in fish and incorporate this information into established computational models. One promising approach involves the measurement of metabolic activity using *in vitro* systems derived from liver tissue (Nichols et al., 2006). This approach borrows from methods pioneered by the pharmaceutical industry for preclinical screening of drug candidates (Rodrigues, 1997), and yields an estimate of intrinsic clearance, which is the rate of hepatic metabolism under non-saturating conditions. The estimated intrinsic clearance rate may be used directly as an input to PBTK models for fish (in the mass-balance equation for liver tissue). Alternatively, this value may be extrapolated to the whole animal to calculate the metabolism rate constant (k_M) commonly represented in one-compartment bioaccumulation

models. To date, several research groups shown that by incorporating *in vitro* metabolism data into one-compartment models for fish, modeled (*in silico*) bioaccumulation predictions are improved; that is modeled predictions are substantially closer to measured values than predictions obtained assuming no metabolism (Han et al., 2007, 2009; Cowan Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010; Laue et al., 2014). Additional work has been performed to improve methods for cryopreservation of rainbow trout hepatocytes (Mingoia et al, 2010; Fay et al., 2014a) and refine *in vitro-in vivo* extrapolation (IVIVE) factors (Fay et al., 2014a; Nichols et al., 2013).

5. The routine use of *in vitro* assays to support bioaccumulation assessment requires, however, that the methods be reliable (repeatable/reproducible) and transferable. Additional questions relate to utility of different *in vitro* test systems and the need to normalize for differences in activity of starting biological material. In this report we describe the results of an international ring trial involving six testing laboratories. The ring trial was conducted with the primary goal of assessing intra- and inter-laboratory variability in assays to measure *in vitro* intrinsic clearance ($CL_{\text{IN VITRO, INT}}$) using rainbow trout liver S9 sub-cellular fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP). By performing these assays in parallel, we sought information which could be used to select a preferred method, or alternatively, assess the method domain for applicability. Issues related to variation in biological material as well as laboratory-specific biases in study findings were addressed by repeated use of pyrene as a potential reference chemical.

6. It is important to note that this ring trial was conducted to inform the development of two OECD Test Guidelines (OECD Project 3.13), and that these proposed Test Guidelines describe the use of RT-S9 and RT-HEP from rainbow trout (*Oncorhynchus mykiss*) to determine the $CL_{\text{IN VITRO, INT}}$ of a test chemical using a substrate depletion approach. An associated OECD Guidance Document describes how to best perform these methods and how $CL_{\text{IN VITRO, INT}}$ can be used to inform *in silico* prediction models of bioaccumulation in fish; however, these extrapolation methods are explicitly not part of the Test Guidelines.

MATERIALS AND METHODS

Test chemical selection

7. The five test chemicals 4-*n*-nonylphenol (4NP), fenthion (FEN), methoxychlor (MC), deltamethrin (DM), cyclohexyl salicylate (CS), and pyrene (PYR) as reference chemical, were selected on the basis of their relative hydrophobicity, ease of analysis, existence of measured bioaccumulation data for fish, and desire to select test chemicals representing diverse chemical classes. Table 1 shows the structure, measured or predicted log K_{OW} value, and measured and modeled bioconcentration factor (BCF) for each test chemical. The BCF is defined as the steady-state chemical concentration in a fish divided by that in water, assuming a water-only exposure. Modeled BCFs were obtained assuming no metabolism, and reflect the extent of accumulation expected from simple partitioning considerations. Absent biotransformation, each test chemical would be expected to accumulate in fish to a relatively high level (predicted BCFs range from about 600 to 23,000). In each case, however, measured BCFs are considerably lower than modeled values, indicating substantial metabolism. All test chemicals except for DM have been

tested previously using *in vitro* assays derived from rainbow trout liver, thereby providing an opportunity to compare measured rates of activity to published values.

Chemicals and Supplies

8. PYR, 4NP, FEN, MC, and DM were obtained from Sigma-Aldrich (St. Louis, MO). CS was provided by Givaudan Schweiz AG (Dübendorf, Switzerland). The internal standards anthracene (ANT), 4-*n*-nonylphenol-d4 (4NP-d4), methyl laurate (ML), and permethrin (PM) were supplied by Sigma Aldrich. Fenthion-d6 (FEN-d6) and methoxylchor-d6 (MC-d6) were obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Catalog and lot numbers for test chemicals and internal standards are provided in ANNEX 2, Table 1A2. Dulbecco's modified Eagle's medium (low glucose, DMEM) and Leibovitz-15 (L-15) medium were purchased from Life Technologies (Carlsbad, CA). β-nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Oriental Yeast Co. (Osaka, Japan) or Enzo Life Sciences (Exeter, UK). Adenosine 3'-phophate 5'-phosphosulfate lithium salt (PAPS) was purchased from Sigma Aldrich or EMD Millipore (Temecula, CA). Glutathione (GSH) was obtained from Sigma Aldrich or Thermo Fisher Scientific (ACROS Organics; Geel, Belgium). All other reagents, solvents, and cofactors were obtained from Sigma Aldrich.

Animals

9. Rainbow trout (*Oncorhynchus mykiss*), Erwin strain, were obtained from the USGS Upper Midwest Environmental Sciences Center in La Crosse, WI and acclimatized for >6 months. The fish were fed a commercial trout chow (Classic trout; Skretting USA) and maintained at 11 ± 1 °C under a 16:8-h light:dark cycle. Water used for fish holding was obtained directly from Lake Superior (single pass, sand filtered and UV treated) and had the following characteristics: alkalinity 43-47 mg/L as $CaCO_3$, pH 7.2-7.8; and dissolved O_2 85-100% of saturation. All rainbow trout holding conditions and experimental procedures were approved by an Institutional Animal Care and Use Committee in accordance with principles established by the Interagency Research Animal Committee.

10. The mean weight of fish sampled to obtain RT-S9 was 322.9 ± 42.0 g, while that of fish used to obtain RT-HEP was 395.5 ± 83.4 g. The sexual maturity of each animal was evaluated by determining its gonadosomatic index (GSI; equal to gonad weight divided by the weight of the animal). Measured GSI values suggested that all fish were very early stages of sexual maturation (Gomez et al., 1999; Le Gac et al., 2001).

Preparation and Characterization of RT-S9 and RT-HEP

11. RT-S9 and RT-HEP were generated in one location (US EPA, MED, Duluth) and shipped to the other participating laboratories (ANNEX 3). RT-S9 were prepared as described by Johanning et al. (2012a). Each lot of tested biological material contained RT-S9 from 3 (CS, 4NP, FEN, DM, PYR) or 6 (MC) fish of mixed sex. Individual lots were aliquoted into 1.8 mL cryogenic sample vials, frozen by immersion in liquid N_2 and stored at -80 ºC. RT-HEP were obtained from animals of mixed sex according to Fay et al. (2015). Each sample lot contained cells from 7 animals. The fresh cells were suspended in buffer containing dimethyl sulfoxide (DMSO), aliquoted into 1.8 mL cryogenic sample vials, and transferred to the vapor phase of liquid N_2 for freezing and storage. RT-HEP and RT-S9 were shipped together in the vapor phase of liquid N_2 (CXR500 cryogenic shipper;

LABRepCo, Horsham, PA). Acceptance of each shipment was contingent upon the presence of liquid N_2 at the time of receipt.

12. Cryopreserved RT-HEP and RT-S9 from each sample lot were characterized to determine 7-ethoxyresorufin-*O*-deethylase activity (EROD; a surrogate for CYP1A1 activity), uridine 5'-diphosphate-glucuronosyltransferase (UGT) activity, and glutathione-*S*-transferase (GST) activity (Table 2). The RT-S9 were characterized as described by Nichols et al. (2013), while cell lysates were evaluated using methods given by Fay et al. (2014b). The protein content of crude RT-S9 and cell lysates was determined using Peterson's modification of the Lowry method (Sigma technical bulletin TP0300; Sigma Aldrich).

Recovery yield and viability of thawed hepatocytes

13. RT-HEP used in substrate depletion assays were evaluated to determine viability and cell recovery yield after thawing using 0.04% trypan blue (Fay et al., 2015). These suspensions were then diluted to the desired concentration of viable cells (1 or 2 x 10^6 cells/mL) in L-15 and recounted for accuracy.

Preparation of enzymatically inactive material

14. RT-HEP suspensions (2 x 10^6 cells/mL in L-15 medium) and RT-S9 (25 mg/mL protein in 100 mM potassium phosphate buffer) were inactivated by boiling for 15 min in a 100 ºC water bath. The final volume was adjusted by addition of L-15 or phosphate buffer to maintain the concentration of enzymatically inactive material. These samples were prepared in advance and stored at –80 ºC. Enzymatically inactive S9 protein was homogenized prior to use with a mortar and pestle so that it could be pipetted into the assay system. All heat-inactivated samples were prepared in advance in one location (U.S. EPA, MED, Duluth) and stored at –80 ºC before shipping to the other participating laboratories.

Experimental Design

15. The study design for the current ring trial was informed by the results of two previous multi-laboratory studies performed using RT-HEP (Fay et al, 2014a) and RT-S9 (Johanning et al., 2012b). An analysis of these findings was conducted using a linear-mixed effects (LME) model (McCulloch et al., 2008) to determine which study factors contributed significantly to variability in measured rates of chemical depletion. The LME model was fit with restricted maximum likelihood to model intrinsic clearance as a population parameter, chemical and sampling time point as fixed effects, and laboratory, run (number of independent assays) and replicate vial (number of vials per time point) as random effects. Likelihood-ratio tests (LRT; Graybill et al., 1976) were performed to determine whether the contribution of a variance component to overall variability in the data was statistically significant ($\alpha = 0.05$). The LME model results were then used to perform a set of Monte Carlo simulations, from which an optimal study design was selected. The results of this modeling exercise are provided in ANNEX 4.

16. The LME model results showed that laboratory effects contributed the most to variability in measured rates of intrinsic clearance. To minimize this factor in the current ring trial, all samples generated for each test chemical were shipped to one laboratory for analysis. The results of the LRT analysis showed that the contribution of vial replicate effects to overall variability was not statistically significant. The contribution of run effects

was not statistically significant for active hepatocyte samples, but was statistically significant for heat-inactivated hepatocytes, and for both active and heat-inactivated S9 fractions.

17. The final study design involved 6 laboratories (A-F), each of which evaluated 6 test chemicals (CS, 4NP, FEN, DM, MC, and PYR) using both RT-HEP and RT-S9. Assays performed using CS, 4NP, FEN, DM, and MC were conducted with chemical-specific lots of biological material, while PYR was run in parallel as a reference chemical with each of the other test chemicals to obtain a dataset covering all lots of biological material. Three assays ("independent runs"), conducted on different days, were performed for each chemical and biological matrix. Each assay was run using a single reaction vial (single vial approach), with one subsample withdrawn at each of 7 sampling time points.

Substrate depletion assays

18. *In vitro* intrinsic clearance rates were measured using a substrate depletion approach (Johanning et al., 2012a; Fay et al., 2015). Standard Operating Procedures (SOPs) were developed for each test method and are included in ANNEXES 5 and 6. Preliminary assays were run with each test chemical to evaluate the concentration-dependence of activity, assess the kinetics of depletion, and optimize the sampling schedule. This information was then used to identify the lowest starting concentration that would yield high quality measurements across most or all of the sampling times, taking into consideration the rate of activity and the analytical method limit of detection. Additional preliminary studies were conducted to determine the stopping conditions (solvent type and ratio of sample to solvent), optimize extraction procedures, and ensure that there were no analytical interferences. Reaction conditions employed for each test chemical are summarized in Table 3.

Chemical Analyses

19. Samples containing CS and MC were analyzed by GC/MS. Deltamethrin was analyzed by GC/MS/MS, FEN and 4NP were analyzed by LC/MS/MS, and PYR was analyzed by HPLC with fluorescence detection. All samples generated for each test chemical were shipped to one laboratory for analysis. Details pertaining to methods, instrumentation, and the laboratory responsible for analysis of each test chemical are provided in ANNEX 7.

Determination of In Vitro and In Vivo Intrinsic Clearance

20. Measured chemical concentrations were $\log_{10}$-transformed and plotted against time to determine a first-order elimination rate constant (k; equal to -2.3 x slope) with units of inverse time (1/h). Rate constants determined in the RT-S9 assay were divided by protein content (mg/mL) to calculate an *in vitro* intrinsic clearance rate ($CL_{\text{IN VITRO, INT}}$; mL/h/mg protein). For the RT-HEP assay, $CL_{\text{IN VITRO,INT}}$ (mL/h/10^6 cells) was determined by dividing k by the measured concentration of viable cells. Measured rates of $CL_{\text{IN VITRO,INT}}$ determined using RT-HEP and RT-S9 were extrapolated to common units of $CL_{\text{IN VIVO,INT}}$ (L/d/kg fish) to permit direct comparisons between the two *in vitro* test systems. Scaling factors used to perform these extrapolations (163 mg S9 protein/g liver and 510×10^6 hepatocytes/g liver) were developed in earlier work with sexually immature trout of the same age, source, and strain (Nichols et al., 2013b; Fay et al., 2014a). Liver size as a fraction of total body weight

was set equal to the value (0.015) determined by Schultz et al. (1999) for small trout typical of those used in bioconcentration testing efforts.

Intra- and inter-laboratory variability

21. Intra- and inter-laboratory variability was characterized as the percent coefficient of variation among repeated measurements (% CV; standard deviation/ average value × 100;). Intra-laboratory variability in RT-HEP yield and viability were calculated from a laboratory's daily averages for each RT-HEP lot. Inter-laboratory variability in these measurements was then calculated for each RT-HEP lot from overall averages determined by each laboratory.

22. Intra-laboratory variability in $CL_{\text{IN VITRO, INT}}$ was calculated for each test chemical, the two *in vitro* systems and the 6 laboratories. Inter-laboratory variability in $CL_{\text{IN VITRO, INT}}$ was determined for each test chemical and *in vitro* system using average clearance values from three runs.

23. Although explicitly not part of the Test Guidelines, additional analysis was performed to facilitate comparison between the two *in vitro* test systems. Each $CL_{\text{IN VITRO, INT}}$ value determined from a singlet assay performed during one experimental day was converted to an estimate of $CL_{\text{IN VIVO,INT}}$. Intra-laboratory variability in $CL_{\text{IN VIVO,INT}}$ was calculated from the mean and standard deviation of $CL_{\text{IN VIVO,INT}}$ values determined for three different days. Inter-laboratory variability in $CL_{\text{IN VIVO,INT}}$ was calculated from the overall averages determined by each laboratory for each test chemical.

RESULTS

Characterization of RT-S9 and RT-HEP

24. The results of characterization assays performed using RT-S9 and RT-HEP are shown in Table 2. Each assay was conducted under saturating substrate conditions. As such, the data can be used to assess variability in V_{max} values for each reaction pathway among the pooled lots of biological material. EROD, UGT, and GST activities, measured using RT-HEP, averaged 5.43 ± 1.20 pmol/min/mg protein, 225 ± 19 pmol/min/mg protein, and 425 ± 32 nmol/min/mg protein, respectively. A similar analysis of RT-S9 data yielded means of 5.49 ± 0.44 pmol/min/mg protein, 1178 ± 109 pmol/min/mg protein, and 889 ± 159 nmol/min/mg protein, respectively. All measured activities were similar to those reported previously for RT-S9 and RT-HEP obtained using rainbow trout from the same source (Fay et al., 2014a; Fay et al., 2017, Nichols et al., 2013), suggesting that this material was typical of that used earlier.

Recovery Yield and Viability of Thawed RT-HEP

25. The average yield and viability of thawed RT-HEP, determined by each laboratory for each of the 5 RT-HEP lots are reported as ANNEX 8. Averaged across all 6 laboratories, the yield for each RT-HEP lot was consistently near 30% (28.1% to 35.3%), while RT-HEP viability was greater than 85% (85.5% to 87.2%). The intra-laboratory variability in replicated yield determinations ranged from 2.1% to 37.7%. The intra-laboratory variability in replicated viability measurements was quite low, resulting in %

CVs that ranged from 0.2% to 5.8%. The inter-laboratory variability in RT-HEP cell yield determinations was higher, with % CVs ranging from 14.4% to 40.7%. The inter-laboratory variability in viability for each RT-HEP lot was low, with % CVs ranging from 4.6% to 6.5%.

In Vitro Intrinsic Clearance

26. Figures 1 - 5 show the complete set of depletion data for all five test chemicals, generated using both RT-S9 and RT-HEP. Figures 6 – 10 show the depletion data for pyrene (reference chemical) using both RT-S9 and RT-HEP that was run in parallel with all of the test chemicals. In nearly all cases, the depletion data exhibited the expected log-linear decrease in chemical concentration. Lines shown in each panel represent linear regression equations fitted to the data from individual incubations. Tables 4 & 5 show the *in vitro* intrinsic clearance rates for RT-HEP and RT-S9, respectively.

27. There was a slow rate of chemical depletion from enzymatically inactive (heat-inactivated) RT-S9 controls for CS, DM and FEN (Fig. 1- 3). In contrast, there was little or no indication of chemical depletion from enzymatically inactive RT-HEP for any of the test chemicals.

28. Negative (enzymatically inactive) controls were incorporated into the assays to account for possible abiotic loss processes such as hydrolysis or volatilization. In principal, it would be possible to use data from these controls to correct measured rates of depletion in active samples, provided that the abiotic loss processes exhibited first-order kinetics. In practice, however, this is challenging, particularly if the abiotic loss term approaches or exceeds the rate of biotransformation. Follow-up studies were conducted with DM, CS, FEN, MC, and 4NP to determine whether this apparent loss from heat-inactivated RT-S9 samples was real or an artifact. For this effort, RT-S9 samples from the same experimental lots were inactivated by withholding all co-factors and allowing the samples to stand at room temperature overnight. In all but one case (4NP), depletion rates for samples treated in this manner were indistinguishable from 0. For 4NP, optimal results (i.e., no detectable depletion) were obtained using heat-inactivated samples; nevertheless, the loss from samples inactivated by time and absence of cofactors was negligible. It is unlikely, therefore, that the apparent loss of chemical from heat-inactivated RT-S9 samples reflected a true loss of chemical from solution. Instead, deposition of denatured protein onto the wall of the reaction vial with repeated vortexing may have had a progressive impact on the chemical concentration remaining in solution; however, this remains to be determined. Based on these findings, $CL_{\text{IN VIVO,INT}}$ values for all chemicals and both test systems were calculated using measured rates of depletion from active samples without additional correction.

29. Measured rates of $CL_{\text{IN VITRO,INT}}$ for the 5 test chemicals are shown in Figures 11 and 12. The rank order of clearance determined using RT-HEP was: CS > FEN > 4NP > DM > MC, while that determined using RT-S9 was: CS > FEN > DM > 4NP > MC. For RT-HEP, clearance rates averaged for each chemical across all 6 laboratories ranged from 0.08 to 10.80 mL/h/10^6 cells, a 135-fold difference. The range in activity was somewhat lower for RT-S9 (0.32–21.50 mL/h/mg protein, a 67-fold difference). Measured $CL_{\text{IN VITRO,INT}}$ values and coefficients of variation (CV) that describe the variability in measured clearance rates are shown in Tables 4 & 5. Intra-laboratory CVs, determined from measured clearance rates for three independent runs, averaged 16.4% ± 12.8% across all 5 test chemicals, both *in vitro* test systems, and all 6 testing laboratories (overall range of 0.4% to 51.8%). The mean of all intra-laboratory CVs calculated using data from RT-HEP

(18.6%) was greater than that calculated using data from RT-S9 (14.1%), but this difference was not significant. Inter-laboratory CVs calculated for each test chemical and *in vitro* system using average clearance values from 3 runs averaged 25.1% ± 6.1% (range of 9.4% to 37.2%). In most cases, the mean of intra-laboratory CVs calculated for a given chemical and test system were substantially smaller than the corresponding inter-laboratory CV. Inter-laboratory CVs developed using data from RT-HEP (32.4% ± 4.1%) were significantly larger than those generated using data from RT-S9 (17.7% ± 6.8%). The lowest calculated inter-laboratory CV (9.4%) was associated with metabolism of MC of RT-S9. As noted above, MC was the most slowly metabolized of all test chemicals in both systems. Overall, however, there were no clear trends for either test system regarding inter-laboratory CVs.

Use of Pyrene as a Reference Chemical

30. Substrate depletions assays with PYR were conducted in parallel with those conducted for the other 5 test chemicals. As indicated previously, these other 5 test chemicals were evaluated using different lots of biological material. The results for PYR therefore provide an opportunity to evaluate lot-to-lot differences in metabolic activity, as well was differences between the in vitro assays. Depletion curves are provided in Figures 6 – 10. Measured $CL_{\text{IN VITRO,INT}}$ and calculated $CL_{\text{IN VIVO,INT}}$ are shown in Tables 7 and 8. $CL_{\text{IN VITRO,INT}}$ values for the 5 RT-HEP lots varied by a factor of 1.61 (highest/lowest based on averages calculated across all laboratories), by a factor of 1.66 for the 5 lots of RT-S9. These lot-to-lot differences in activity were significant for RT-HEP (one-way ANOVA; $p = 0.046$), but not for RT-S9 ($p = 0.062$). Intra-laboratory CVs averaged 16.7% ± 13.3% across both in vitro test systems and all 6 testing laboratories (range of 1.6% to 58.7%). The mean of all intra-laboratory CVs calculated using data from RT-HEP (19.1%) was greater than that calculated using data from RT-S9 (14.2%), but this difference was not significant ($p = 0.061$). Averaged across all 5 RT-HEP lots, the mean inter-laboratory CV for PYR was 27.9% ± 11.0% (range 14.9% to 41.6%), while that determined for the 5 lots of RT-S9 was 27.% ± 14.0% (range from 13.5% to 44.3%).

Clearance Comparisons Among In Vitro Test Systems

31. Although explicitly not part of the Test Guidelines, additional analysis was performed to facilitate comparison between the two *in vitro* test systems (Figure 13) for potential use and application for bioaccumulation assessment. Using appropriate scaling factors, measured rates of $CL_{\text{IN VITRO,INT}}$ for RT-HEP and RT-S9 were converted to common units of *in vivo* intrinsic clearance ($CL_{\text{IN VIVO,INT}}$; L/d/kg fish). The scaling factors used to perform these extrapolations (163 mg S9 protein/g liver and 510×10^6 hepatocytes/g liver) were developed in earlier work with sexually immature rainbow trout of the same age, source, and strain (Nichols et al., 2013; Fay et al., 2014a). The resulting $CL_{\text{IN VIVO,INT}}$ values differed by no more than a factor of 3, and were generally much closer.

32. A second comparison was then performed by using these intrinsic clearance values as inputs to a well-stirred liver model, commonly employed for *in vitro-in vivo* extrapolation of hepatic biotransformation data (Rowland et al., 1973; Wilkinson and Shand, 1975).

$$CL_H = \frac{Q_H f_U CL_{IN\ VIVO,INT}}{Q_H + f_U CL_{IN\ VIVO,INT}}$$

33. In this model, CL_H is hepatic clearance (L/d/kg fish), Q_H (L/d/kg fish) is the liver blood flow rate, and f_U (unitless) is a binding term used to correct for binding effects *in vitro* and in plasma. For this evaluation, Q_H was calculated as 0.259 times the estimated cardiac output in small (10 g) trout commonly employed for standardized bioaccumulation testing (Nichols et al., 2013). The binding term f_U was calculated as the ratio of unbound chemical fractions in blood plasma ($f_{U,P}$) and in the *in vitro* test system ($f_{U,S9}$ or $f_{U,HEP}$). Empirically-based algorithms used to estimate these binding terms are described by Nichols et al. (2013). Hepatic clearance rates predicted by this model for each compound differed by less than a factor of 2.6 (Figure 14). This improved agreement, relative to that for calculated $CL_{IN\ VIVO,INT}$ values, can be attributed to the fact that $CL_{IN\ VIVO,INT}$ values for several compounds were approaching the estimated rate of liver blood flow (i.e., the theoretical maximum value).

Statistical analysis to inform Test Guideline study design

34. As described in detail in ANNEX 4, the design for this multi-laboratory ring-trial was developed based on a detailed analysis of data from previous studies. However, the results of this ring-trial were statistically analyzed to support and inform the test design for the two OECD Test Guidelines (*Determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes[RT-HEP]* and *Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction [RT-S9]*). Full details of the statistical analyses performed are included in ANNEX 9. Briefly, analyses were performed to determine the minimum number of time points necessary to determine the $CL_{IN\ VITRO,\ INT}$ rate, i.e. to calculate the regression and derive the slope, as well we the appropriate R^2 value. In addition, an analysis was performed to determine the necessary number of independent runs to calculate the $CL_{IN\ VITRO,\ INT}$ rate.

35. Conclusions from this analysis support the need for a minimum of six time points to determine the $CL_{IN\ VITRO,\ INT}$ rate, i.e., to calculate the regression and derive the slope, with an R^2 value >0.85. In the case of chemicals that are more slowly metabolized (e.g., a very shallow slope), the R^2 may not be ≥0.85. In this instance, careful consideration should be given to whether the slope is significantly different than zero before including or excluding the run. In addition, each test should consist of at least two independent runs to determine $CL_{IN\ VITRO,\ INT}$. Each independent run should be performed on a different day or on the same day provided that for each run: a) independent fresh stock solutions and working solutions of the test chemical are prepared and b) independently prepared (i.e., thawed and diluted) biological material is used. If the calculated regression from the two runs with active material are significantly different (e.g., t-test of the slopes with $p<0.05$), then a third run should be performed.

BCF predictions

36. As above, although explicitly not part of the Test Guidelines, additional analysis was performed to demonstrate the use of these *in vitro* data for bioaccumulation assessment. In a recent report, Nichols et al. (2013) described a pair of models that employ *in vitro* intrinsic clearance rate to predict BCF values in rainbow trout. One model (HEP-BCF) was provided for data derived from isolated hepatocytes, while a second (S9-BCF) was developed for data derived from liver S9 fractions. These models predict the BCF for a "standardized" fish (10 g rainbow trout containing 5% whole-body lipid), which is typical of fish commonly tested *in vivo* under OECD TG305 (OECD, 2012).

37. Following the approach outlined previously, $CL_{IN\ VITRO,INT}$; mL/h/10^6 cells or mL/h/mg protein from the *in vitro* tests was multiplied by either the S9 content of liver tissue (L_{S9}; 163 mg mg S9 protein /g liver) or hepatocellularity (L_{HEP}; 510 × 10^6 hepatocytes/g liver),and by the liver weight as a fraction of body weight (L_{FBW}) to yield the $CL_{IN\ VIVO,INT}$; L/d/kg fish. The $CL_{IN\ VIVO,INT}$ is converted to an estimate of CL_H; L/d kg fish using a well-stirred liver model (Nichols et al., 2013). For this assessment, the binding term used in the liver model was set to f_u=modelled (i.e., assuming that biotransformation enzymes operate against the free or unbound chemical fraction *in vivo* [plasma] and *in vitro*) or f_u=1 (i.e., assuming the same availability of the chemical to metabolic enzymes *in vitro* and *in vivo)*. A whole-body biotransformation rate constant (k_{MET}; 1/d) was calculated by dividing CL_H by the chemical's apparent volume of distribution, referenced to the chemical concentration in blood ($V_{D,BL}$; L/kg) (Nichols et al., 2006). The V_D is estimated as the ratio of fish/water and blood/water partition coefficients, each of which is calculated using log K_{ow}-based algorithms. The total chemical concentration in fish at steady state ($C_{FISH,SS}$; mg/kg) is predicted using the 1-compartment model given by Arnot and Gobas (Arnot and Gobas, 2003), which includes rate constants that describe chemical uptake and loss across the gills, and fecal egestion. Finally, $C_{FISH,SS}$; mg/kg is divided by the chemical concentration in water ($C_{W,TOT}$) resulting in a BCF value expressed on a total chemical basis (BCF_{TOT}; L/kg).

38. Table 9 shows a comparison of estimated BCF values using the Nichols et al., (2013b) BCF model predictions assuming no biotransformation and predicted BCFs using $CL_{IN\ VITRO,INT}$ determined in both test systems with two binding assumptions (f_u=modelled and f_u=1.0) (Nichols et al., 2013) and ranges of available measured *in vivo* BCF values (e.g., OECD TG305; OECD, 2012). The Nichols model was parameterized for a 10g fish, 5% lipid, at 12°C.

39. BCF predictions using the RT-S9 and RT-HEP *in vitro* assays were within ~2-fold agreement between the two assays for the modelled binding assumption, and within <1.2-fold for the f_u=1 assumption. For all chemicals tested, *in vitro* biotransformation decreased predicted BCF values (versus model predictions assuming no biotransformation), depending on the rate of intrinsic clearance as well as the binding (f_u) assumptions. Overall, the data generated in this study yield predicted BCF values which are generally closer to measured values than predictions obtained assuming no metabolism. As such, the results of this study are consistent with findings provided in several earlier reports (Han et al., 2007, 2009; Cowan Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010; Laue et al., 2014).

CONCLUSIONS & DISCUSSION

- The present ring trial builds on previous efforts (Johanning et al., 2012b; Fay et al., 2014a) to evaluate the reliability of *in vitro* methods used to measure intrinsic hepatic clearance in rainbow trout.
- The present ring trial differs from earlier efforts with respect to its scope (a larger number of participating laboratories) and the use to two *in vitro* metabolizing systems (RT-S9; RT-HEP). Following recommendation given by Fay et al. (2014a), this study also employed pyrene as a positive control compound to aid in the interpretation of the ring trial findings.

- As in the study conducted by Fay et al. (2014a), all of the analyses for each test chemical concentration were performed in one laboratory, focusing the reliability assessment on user-associated sources of variability in the *in vitro* test systems.
- Measurable rates of activity (negative depletion slope statistically different from 0) were observed for all test chemicals in both test systems. Measured $CL_{\text{IN VITRO, INT}}$ for individual test chemicals differed by ~ 2 orders of magnitude. All of these measurements were well within the capabilities of the assay (not too slow or too fast).
- The reliability of each *in vitro* method was assessed by quantifying intra- and inter-laboratory variability (% CV) in repeated *in vitro* intrinsic clearance determinations ($CL_{\text{IN VITRO, INT}}$). Generally, calculated levels on intra- and inter-laboratory variability associated with either test system were comparable to values reported earlier by Fay et al. (2014a; for cryopreserved hepatocytes).
- When averaged across all laboratories for each of the 5 test chemicals, the intra-laboratory variability in measured $CL_{\text{IN VITRO, INT}}$ for both test systems was consistently smaller than the corresponding level of inter-laboratory variability. Overall, the intra-laboratory CVs ranged from 0.4% to 51.8% (with 56 CVs <40% and 4 CVs in range of 46-53%), averaging 16.4%, while inter-laboratory CVs ranged from 9.4% to 37.2%, averaging 25.1%. Intra-laboratory CVs for the reference chemical, pyrene, ranged from 1.6% to 58.7%, averaging 16.7%. Inter-laboratory CVs for pyrene ranged from 13.5% to 44.3%, averaging 27.4%.
- In several instances, the intra-laboratory variability calculated for an individual chemical was quite small (< 10%). Generally, the intra-laboratory variability in $CL_{\text{IN VITRO, INT}}$ determined using RT-S9 was smaller than the variability in $CL_{\text{IN VITRO, INT}}$ measured using RT-HEP. This difference may have been associated with increased variability inherent to the handling (i.e., thawing, counting, viability determination) of cryopreserved RT-HEP.
- Calculated levels of intra- and inter-laboratory variability in $CL_{\text{IN VITRO, INT}}$ were similar across all test chemicals and did not suggest any pattern with respect to the absolute rate of activity. Although there are clear limits on rates of activity that can be measured using these *in vitro* systems, the present dataset suggests that the reliability of the method is relatively constant across the range of measurable activity levels.
- Mean $CL_{\text{IN VITRO,INT}}$ values for PYR, determined using five pooled lots of RT-S9 varied by a factor of 1.7, while mean values determined for five pooled lots of RT-HEP differed by a factor of 1.6. These findings evidence a high level of consistency for biological material isolated from a defined strain of rainbow trout, and are similar to results reported by Fay et al. (2014a).
- The utility of using PYR as a positive control compound to determine correct test conditions, etc. was demonstrated in several isolated cases (e.g., no activity which may be due to a lack of enzyme cofactors).
- The potential for a laboratory-specific bias in study findings (e.g., one laboratory generating consistently higher or lower rates of activity) was evaluated by comparing measured rates of PYR metabolism to metabolism rates determined for the other test chemicals, across all of the testing laboratories (rank order assessment). These comparisons did not provide any evidence of a laboratory-specific bias; however, such comparisons may be useful in future, particularly for laboratories that have relatively little experience with the assays.
- Although explicitly not part of the Test Guidelines, the additional analysis performed to facilitate comparison between the two *in vitro* test systems showed:
    - When expressed in common units ($CL_{\text{IN VIVO,INT}}$; mL/h/g fish), intrinsic clearance rates determined for each test chemical using both *in vitro* systems were very

similar (1.2 to 2.3-fold difference). Importantly, there were no obvious trends in the data which would suggest that one or the other test system consistently yields a higher or lower rate of activity. Similar findings were published recently by Fay et al. (2017).

- When used as inputs to an established bioconcentration model for rainbow trout (Nichols et al., 2013), the data generated in this study yield predicted BCF values which are generally closer to measured values than predictions obtained assuming no metabolism. As such, the results of this study are consistent with findings provided in several earlier reports (Han et al., 2007, 2009; Cowan Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010; Laue et al., 2014).

- Overall, these findings suggest that both in vitro assays are highly reliable, and that either assay may be used with confidence to generate data which may be used to refine modeled bioaccumulation predictions. Presently, we cannot conclude that one or the other assay system is preferred for this application. Additional work with chemical substrates representing a wider range of structures and inherent metabolic stability is needed to determine whether the domain for applicability of these two test systems differs.

ACKNOWLEDGEMENTS

40. This ring-trial was coordinated and sponsored by the Health and Environmental Sciences Institute (HESI) Bioaccumulation Technical Committee. Foundational work to support the development of these methods was provided by the European Commission (Study Contract CCR.IHCP.C434207.X0) and CEFIC (European Chemical Industry Council; Contract LRI-ECO6.2-ILSIHESI-0804). The contributions from all of the laboratories and experts involved in this ring trial are highly appreciated, as is the feedback provided from the OECD Project 3.13 Expert Group.

REFERENCES

Arnot, J. A. and Gobas, F. A. P. C. 2003. A generic QSAR for assessing the bioaccumulation potential of organic chemicals in aquatic food webs. QSAR and Combinatorial Science 22:337−345.

Arnot, J. A. and Gobas, F. A. P. C. 2004. A food web bioaccumulation model for organic chemicals in aquatic ecosystems. Environmental Toxicology and Chemistry 23:2343–2355.

Arnot, J.A., Meylan, W., Tunkel, .J, Howard, P.H., Mackay, D., Bonnell, M., and Boethling, R.S. 2009. A quantitative structure-activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. Environmental Toxicology and Chemistry 28: 1168-1177.

Brinkmann, M., Freese, M., Pohlmann, J.D., Kammann, U., Preuss, T.G., Buchinger, S.,Reifferscheid, G., Beiermeister, A., Hanel, R., and Hollert, H. 2015. A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*). Science of the Total Environment 536:279-87

Cowan-Ellsberry, C.E., Dyer, S.D., Erhardt, S., Bernhard, M.J., Roe, A.L., Dowty, M.E., and Weisbrod, A.V. 2008. Approach for extrapolating in vitro metabolism data to refine bioconcentration factor estimates. Chemosphere 70:1804-1817.

de Wolf, W., de Bruijn, J.H.M., Seinen, W., and Hermens, J.L.M. 1992. Influence of biotransformation on the relationship between bioconcentration factors and octanol–water partition coefficients. Environmental Science & Technology 26:1197–1201.

Dyer, S.D., Bernhard, M.J., Cowan-Ellsberry, C., Perdu-Durand, E., Demmerle, S., and Cravedi, J.-P. 2008. In vitro biotransformation of surfactants in fish. Part I: Linear alkylbenzene sulfonate (C12-LAS) and alcohol ethoxylate (C13EO8). Chemosphere 72:850-862.

Fay, K.A., Fitzsimmons, P.N., Hoffman, A.D., and Nichols, J.W. 2014a. Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish. Xenobiotica 44:345-351.

Fay, K.A., Mingoia, R.T., Goeritz, I., Nabb, D.L., Hoffman, A.D., Ferrell, B.D., Peterson, H.M., Nichols, J.W., Segner, H., and Han, X. 2014b. Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential. Environmental Science & Technology 48:8170-8178.

Fay, K.A., Nabb, D.L., Mingoia R.T., Bischof, I., Nichols, J.W., Segner, H., Johanning, K., and Han, X. 2015. Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (*Oncorhynchus mykiss*). Current Protocols in Toxicology 65:4.42.1-29.

Fay, K.A., Fitzsimmons, P.N., Hoffman, A.D., and Nichols, J.W. 2017. Comparison of trout hepatocytes and liver S9 fractions as in vitro models for predicting hepatic clearance in fish. Environmental Toxicology and Chemistry 36:463-471.

Graybill, F.A. 1976. Theory and application of the linear model. Duxberry Press, New Scituate.

Gobas, F.A., de Wolf, W., Burkhard, L.P., Verbruggen, E., and Plotzke K. 2009. Revisiting bioaccumulation criteria for POPs and PBT assessments. Integrated Environmental Assessment and Management 5:624-637.

Gomez, C.F., Constantine, L., and Huggett, D.B. 2010. The influence of gill and liver metabolism on the predicted bioaconcentration of three pharmaceuticals in fish. Chemosphere 81:1189-1195.

Gomez, J.M., Mourot,B., Fostier, A., and Le Gac, F. 1999. Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (*Oncorhynchus mykiss*). Journal of Reproduction and Fertility 115:275-285.

Han, X., Nabb, D., Mingoia, R., and Yang, C.-H. 2007. Determination of xenobiotic intrinsic clearance in freshly isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) and rat and its application in bioaccumulation assessment. Environmental Science & Technology 41:3269-3276.

Han, X., Mingoia, R.T., Nabb, D.L., Yang, C.H., Snajdr, S.L., and Hoke, R.A. 2008. Xenobiotic intrinsic clearance in freshly isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*): Determination of trout hepatocellularity, optimization of cell concentrations and comparison of serum and serum-free incubations. Aquatic Toxicology 89:11-17.

Han, X., Nabb, D. L., Yang, C.-H., Snajdr, S. I., Mingoia, R. T. 2009. Liver microsomes and S9 from rainbow trout (*Oncorhynchus mykiss*): Comparison of basal-level enzyme activities with rat and determination of xenobiotic intrinsic clearance in support of bioaccumulation assessment. Environmental Toxicology and Chemistry 28:481−488.

Johanning, K., Hancock, G., Escher, B., Adekola, A., Bernhard, M.J., Cowan-Ellsberry, C., Domoradzki, J., Dyer, S., Eickhoff, C., Embry, M., Erhardt, S., Fitzsimmons, P., Halder, M., Hill, J., Holden, D., Johnson, R., Rutishauser, S., Segner, H., Schultz, I., and Nichols, J. 2012a. Assessment of metabolic stability using the rainbow trout (*Oncorhynchus mykiss*) liver S9 fraction. Current Protocols in Toxicology 53:14.10.1-28.

Johanning, K., Hancock, G., Escher, B., Adekola, A., Bernhardt, M., Cowan-Ellsberry, C., Domoradski, J., Dyer, S., Eickhoff, C., Erhardt, S., Fitzsimmons, P., Halder, M., Nichols, J., Rutishauser, S., Sharpe, A., Segner, H., Schultz, I., and Embry, M. 2012b. In vitro metabolism using rainbow trout liver S9. Summary report of the HESI Bioaccumulation Committee. Available at: http://www.hesiglobal.org/files/public/Committees/Bioaccumulation/Presentations%20and%20Data%20Resources/S9_report_FINAL_20Nov2012.pdf.

Laue, H., Gfeller, H., Jenner, K.J., Nichols, J.W., Kern, S., and Natsch, A. 2014. Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of in vitro intrinsic clearance. Environmental Science and Technology 48:9486-9495.

Le Gac, F., Thomas, J.L., Mourot, B., and Loir, M. 2001. In vivo and in vitro effects of prochloraz and nonylphenol ethoxylates on trout spermatogenesis. Aquatic Toxicology 53:187-200.

McCulloch, C.E. Searle, S.R. and Neuhaus, J.M. 2008. Generalized, Linear, and Mixed Models, 2nd Edition, 424 pages, Wiley.

Mingoia, R.T., Glover, K.P., Nabb, D.L., Yang, C.-H., Snajdr, S.I., and Han, X. 2010. cryopreserved hepatocytes from rainbow trout (*Oncorhynchus mykiss*): A validation study to support their application in bioaccumulation assessment. Environmental Science and Technology 44:3052-3058.

Nichols, J.W., Schultz, I.R., and Fitzsimmons, P.N. 2006. In vitro-in vivo extrapolation of quantitative hepatic biotransformation data for fish: I. A review of methods, and strategies

for incorporating intrinsic clearance estimates into chemical kinetic models. Aquatic Toxicology 78:74-90.

Nichols, J. W., Bonnell, M., Dimitrov, S., Escher, B., Han, X., and Kramer, N. 2009. Bioaccumulation assessment using predictive approaches. Integrated Environmental Assessment and Management 5:577-597.

Nichols, J.W., Hoffman, A.D., ter Laak, T.L., and Fitzsimmons P.N. 2013. Hepatic clearance of six polycyclic aromatic hydrocarbons by isolated perfused trout livers: Prediction from in vitro clearance by liver S9 fractions. Toxicological Sciences 136: 359-372.

Nichols, J.W., Huggett, D.B., Arnot, J.A., Fitzsimmons, P.N., and Cowan-Ellsberry C.E. 2013. Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in vitro intrinsic clearance. Environmental Toxicology and Chemistry 32:1611-1622.

OECD 2012. Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing. doi: 10.1787/9789264185296-en.

Oliver, B.G. and Niimi, A.J. 1985. Bioconcentration factors of some halogenated organics for rainbow trout: limitations in their use for prediction of environmental residues. Environmental Science & Technology 19:842-849.

Rodrigues, A.D. 1997. Preclinical drug metabolism in the age of high-throughput screening: an industrial perspective Pharmaceutical Research, 14:1504–1510.

Rowland, M., Benet, L.Z., and Graham G.G. 1973. Clearance concepts in pharmacokinetics. Journal of Pharmacokinetics and Biopharmaceutics 1:123–136.

Stadnicka-Michalak, J., Tanneberger, K., Schirmer, K., and Ashauer, R. 2014. Measured and modeled toxicokinetics in cultured fish cells and application to in vitro-in vivo toxicity extrapolation. PLoS One 9, e92303.

Southworth, G.R., Keffer, C.C., and Beauchamp, J.J. 1980. Potential and realized bioconcentration. A comparison of observed and predicted bioconcentration of azaarenes in the fathead minnow (*Pimephales promelas*). Environmental Science and Technology 14:1529-1531.

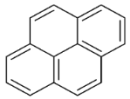
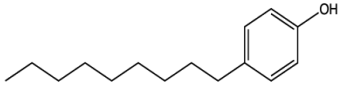
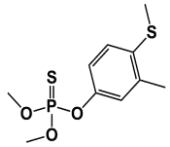
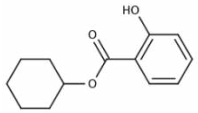
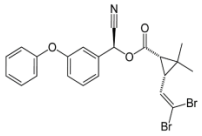
USEPA. 2012. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11. United States Environmental Protection Agency, Washington, DC, USA.

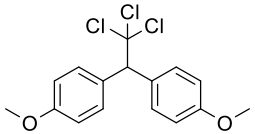
USEPA. 2016. KABAM (KOW (based) Aquatic BioAccumulation Model). United States Environmental Protection Agency, Washington, DC, USA.

Wilkinson, G.R. and Shand, D.G. 1975. Commentary: a physiological approach to hepatic drug clearance. Clinical Pharmacology & Therapeutics 18:377-390.

TABLES

Table 1. Test chemicals used to evaluate the reliability of in vitro substrate depletion assays (RT-S9; RT-HEP)

Chemical	Structure	Log Kow[a]	Predicted bioconcentration factor (BCF; L/kg)[b]	Range of empirical BCF (L/kg)	Previous use in *in vitro* studies with rainbow trout HEP, S9
Pyrene		4.88	3490	78[c] – 1578[d]	*Mingoia et al., 2010* *Nichols et al., 2013*
4-n-Nonylphenol		5.76	16,549	240[e] - 344[f]	*Mingoia et al., 2010* *Han et al., 2007, 2008, 2009* *Fay et al., 2014a*
Fenthion		4.09	607	185[g] – 16,600[h]	*Fay et al., 2014a*
Cyclohexyl salicylate		4.70	2371	400[i]	*Laue et al., 2014*
Deltamethrin		6.20	22,900	115[j] – 1400[k]	

Methoxychlor		5.08	522	174[l]	*Fay et al., 2014a*

[*]Additional details on available empirical BCF studies are included in ANNEX 10; [a]CLogP estimated values; [b]Modeled estimates obtained using Nichols et al., (2013) assuming no biotransformation; [c]Average of 4 measurements from Jonsson et al., 2004 (sheepshead minnow); [d]Average of 5 measurements from Carlson et al., 1979 (fathead minnow); [e]Average of 3 measurements from Giesy et al., 2000 (fathead minnow); [f]Average of 3 measurements from Snyder et al., 2001 (fathead minnow); [g]Average of 36 studies / measurements from Tsuda et al., 1993, 1995, 1996, 1997 (medaka, guppy, goldfish, carp, minnow); [h]Value from DeBruijn and Hermens, 1991 (guppy); [i]Average of 2 measured steady state values for zebrafish (RIFM study); cited in Laue et al, 2014; [j]Modeled value based on measured parent chemical concentration at the lowest DOC from Muir et al., 1994 (rainbow trout); [k]Value from Fackler, 1990 (bluegill); [l]Measured 140d value from Hansen and Parrish, 1977 (sheepshead minnow)

Table 2. Characterization of RT-HEP and RT-S9a

Lot	Test compound	Yield (%)[b]	Viability (%)[b]	Protein content (mg/mL)[c]	EROD activity (pmol/min/mg)[d]	UGT activity (pmol/min/mg)[d]	GST activity (nmol/min/mg)[d]
RT-HEP							
1	Cyclohexyl salicylate	46.2 ± 5.5	81.0 ± 0.8	2.38 ± 0.04	5.10 ± 0.45	213 ± 14	386 ± 36
2	Fenthion	57.5 ± 6.3	87.3 ± 5.0	2.81 ± 0.03	6.12 ± 0.32	201 ± 13	462 ± 53
3	4-*n*-Nonylphenol	66.9 ± 9.2	83.0 ± 2.0	3.28 ± 0.06	7.02 ± 0.59	238 ± 13	436 ± 30
4	Deltamethrin	65.7 ± 10.2	88.3 ± 2.5	2.86 ± 0.02	3.85 ± 0.24	247 ± 7	398 ± 35
5	Methoxychlor	53.8 ± 6.7	82.7 ± 5.1	2.93 ± 0.09	5.05 ± 0.46	228 ± 46	441 ± 27
	All lots	58.0 ± 8.6	84.5 ± 3.2	2.85 ± 0.34	5.43 ± 1.20	225 ± 19	425 ± 32
RT-S9							
1	Cyclohexyl salicylate	---	---	23.1 ± 0.7	6.02 ± 0.30	1208 ± 51	906 ± 14
2	Fenthion	---	---	22.6 ± 0.4	5.52 ± 0.36	1226 ± 35	1088 ± 149
3	4-*n*-Nonylphenol	---	---	23.8 ± 0.3	4.83 ± 0.14	1197 ± 46	706[e]
4	Deltamethrin	---	---	21.0 ± 0.3	5.36 ± 0.23	990 ±13	756 ± 66
5	Methoxychlor	---	---	24.4 ± 1.6	5.71 ± 0.13	1271 ± 21	989 ± 31
	All lots			23.0 ± 1.3	5.49 ± 0.44	1178 ± 109	889 ± 159

Abbreviations: EROD = ethoxyresorufin-O-deethylase activity, UGT = uridine diphosphate glucuronosyltransferase activity, GST = glutathione-S-transferase activity.
[a]Data for each lot are reported as the mean ± SD (n = 3). Summary data (RT-HEP or RT-S9) are given as the mean ± SD for all lots.
[b]Determined for thawed hepatocytes.
[c]For RT-HEP, this refers to the protein content of lysates created by sonication of 1 x 10^6 viable hepatocytes/mL. For RT-S9, this refers to the protein content of crude RT-S9 prior to freezing.
[d]Determined for cryopreserved RT-HEP and RT-S9.
[e]Based on one replicate

Table 3. Study designs employed by participating laboratories

Test chemical	RT-S9 or RT-HEP lot number	RT-S9 or RT-HEP conc. (mg/mL or 10^6 cells/mL	Starting chemical conc. (µM)	Sampling times (min)	Stopping solvent	Internal standard[a]
RT-HEP						
Cyclohexyl salicylate	1	1.0	1.0	2,5,8,11,14,17,20	400 µL MTBE	0.1 µM ML
Fenthion	2	2.0	0.2	2,5,10,20,30,40,50	400 µL ACN	0.025 µM FEN-d6
4-*n*-Nonylphenol	3	2.0	2.0	2,5,10,20,30,40,50	500 µL ACN	0.4 µM 4NP-d4
Deltamethrin	4	2.0	1.0	2,10,20,40,60,90,120	400 µL DCM	0.5 µM PM
Methoxylchlor	5	2.0	0.32	2,7,20,60,120,180,240	400 µL DCM	0.1 µM MC-d6
Pyrene[c]	1-5	2.0	0.025	2,5,10,20,30,40,50	400 µL ACN	0.002 µM ANT
RT-S9						
Cyclohexyl salicylate	1	0.25	1.0	2,4,6,8,10,12,14	400 µL MTBE	0.1 µM ML
Fenthion	2	1.0	0.2	2,4,6,8,10,12,14	400 µL ACN	0.025 µM FEN-d6
4-*n*-Nonylphenol	3	1.0	2.0	2,5,10,15,20,30,40	500 µL ACN	0.4 µM 4NP-d4
Deltamethrin	4	1.0	1.0	2,10,20,30,40,50,60	400 µL DCM	0.5 µM PM
Methoxychlor	5	1.0	0.32	2,7,20,60,120,180,240	400 µL MeOH	0.1 µM MC-d6
Pyrene[c]	1-5	1.0	0.025	2,4,6,8,10,12,14	400 µL ACN	0.002 µM ANT

Abbreviations: MTBE = methyl *tert*-butyl ether, DCM = dichloromethane, ACN = acetonitrile, MeOH – methanol, ML = methyl laurate, FEN-d6 = fenthion-d6, 4NP-d4 = 4-*n*-nonylphenol-d4, PM = permethrin, MC-d6 = methoxychlor-d6, ANT = anthracene.
aConcentrations are those in the stopping solvent prior to the addition of sample.
[b]Pyrene was run in parallel with all other test compounds (see text for details).

Table 4. In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$; mL/h/mg protein) of 5 test chemicals, measured using cryopreserved rainbow trout hepatocytes (RT-HEP)

Test chemical	Laboratory	Intra-laboratory variability				Inter-laboratory variability		
		$CL_{IN\ VITRO,INT}$	SD	CV (%)	Mean CV (%)[b]	$CL_{IN\ VITRO,INT}$[b]	SD	CV (%)
Cyclohexyl salicylate	A	5.87	0.77	13.0	22.7	10.80	3.95	36.6
	B	15.87	3.77	23.8				
	C	15.23	0.66	4.3				
	D	9.83	3.66	37.3				
	E	9.64	2.20	22.9				
	F	8.36	2.91	34.8				
Fenthion	A	2.25	0.28	12.4	16.3	1.54	0.57	37.2
	B	2.09	0.74	35.6				
	C	0.95	0.17	17.7				
	D	1.34	0.07	5.2				
	E	1.72	0.32	18.4				
	F	0.89	0.08	8.5				
4-*n*-Nonylphenol	A	0.36	0.052	14.3	16.8	0.57	0.17	29.3
	B	0.86	0.048	5.6				
	C	0.49	0.124	25.3				
	D	0.62	0.151	24.6				
	E	0.56	0.064	11.5				
	F	0.54	0.104	19.4				
Deltamethrin	A	0.38	0.018	4.8	16.5	0.34	0.10	30.2
	B	0.44	0.126	28.6				
	C	0.18	0.021	12.0				
	D	0.36	0.044	12.1				
	E	0.41	0.030	7.2				
	F	0.25	0.085	34.1				
Methoxychlor	A	0.05	0.007	13.9	20.9	0.08	0.02	28.7
	B	0.10	0.007	6.8				
	C	0.06	0.004	6.5				
	D	0.10	0.016	15.1				
	E	0.09	0.042	49.3				
	F	0.10	0.034	33.9				

Table 5. In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$; mL/h/mg protein) of 5 test chemicals, measured using rainbow trout liver S9 fractions (RT-S9)[a]

Test chemical	Laboratory	Intra-laboratory variability				Inter-laboratory variability		
		$CL_{IN\ VITRO,INT}$	SD	CV (%)	Mean CV (%)[b]	$CL_{IN\ VITRO,INT}$[b]	SD	CV (%)
Cyclohexyl salicylate	A	28.32	5.13	18.1	12.8	21.50	4.27	19.9
	B	21.29	4.05	19.0				
	C	22.05	1.70	7.7				
	D	19.92	2.28	11.4				
	E[c]	22.30	0.20	0.9				
	F	15.09	2.97	19.7				
Fenthion	A[c]	9.59	0.49	5.1	10.3	9.71	2.61	26.8
	B	13.47	0.71	5.3				
	C	10.02	2.72	27.2				
	D	7.70	0.96	12.4				
	E	11.40	1.10	9.7				
	F	6.12	0.15	2.4				
4-*n*-Nonylphenol	A	0.87	0.15	17.0	14.0	0.78	0.15	19.9
	B	0.84	0.07	7.9				
	C	1.00	0.08	7.6				
	D	0.73	0.15	20.8				
	E	0.61	0.09	13.9				
	F[c]	0.61	0.10	16.7				
Deltamethrin	A	1.44	0.06	4.2	28.7	1.49	0.19	12.7
	B	1.83	0.94	51.0				
	C	1.57	0.18	11.4				
	D	1.33	0.12	9.2				
	E	1.33	0.59	44.6				
	F	1.45	0.75	51.8				
Methoxychlor	A	0.35	0.007	2.0	4.8	0.32	0.03	9.4
	B	0.35	0.035	9.8				
	C	0.30	0.001	0.4				
	D	0.30	0.009	2.9				
	E	0.35	0.012	3.3				
	F	0.29	0.031	10.7				

[a]Intra-laboratory viability was quantified as the percent coefficient of variation (CV) of mean *in vitro* intrinsic clearance rates ($CL_{IN\ VITRO,INT}$; ml/h/mg protein) determined for each test chemical by each laboratory (n = 3 except where noted). Inter-laboratory variability was quantified as the CV of mean $CL_{IN\ VITRO,\ INT}$ values determined for each chemical by all participating laboratories (n = 6).
[b]Mean across all laboratories; [c]n = 2

Table 6. In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$) and in vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$) of 5 test chemicals, measured using rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP).

Test chemical		*RT-HEP*[a]		*RT-S9*[b]	
	Lab	$CL_{IN\ VITRO,INT}$	$CL_{IN\ VIVO,INT}$	$CL_{IN\ VITRO,INT}$	$CL_{IN\ VIVO,INT}$
Cyclohexyl salicylate	A	5.87	1077.73	28.32	1661.82
	B	15.87	2913.73	21.29	1249.30
	C	15.23	2796.23	22.05	1293.89
	D	9.83	1804.79	19.92	1168.91
	E	9.64	1769.90	22.30	1308.56
	F	8.36	1534.90	15.09	885.48
Fenthion	A	2.25	413.10	9.59	562.74
	B	2.09	383.72	13.47	790.42
	C	0.95	174.42	10.02	587.97
	D	1.34	246.02	7.70	451.84
	E	1.72	315.79	11.40	668.95
	F	0.89	163.40	6.12	359.12
4-*n*-Nonylphenol	A	0.36	66.10	0.87	51.05
	B	0.86	157.90	0.84	49.29
	C	0.49	89.96	1.00	58.68
	D	0.62	113.83	0.73	42.84
	E	0.56	102.82	0.61	35.79
	F	0.54	99.14	0.61	35.79
Deltamethrin	A	0.38	69.77	1.44	84.50
	B	0.44	80.78	1.83	107.38
	C	0.18	33.05	1.57	92.13
	D	0.36	66.10	1.33	78.04
	E	0.41	75.28	1.33	78.04
	F	0.25	45.90	1.45	85.09
Methoxychlor	A	0.05	9.18	0.35	20.54
	B	0.10	18.36	0.35	20.54
	C	0.06	11.02	0.30	17.60
	D	0.10	18.36	0.30	17.60
	E	0.09	16.52	0.35	20.54
	F	0.10	18.36	0.29	17.02

[a] $CL_{IN\ VIVO,INT} = CL_{IN\ VITRO,INT} \times L_{HEP} \times L_{FBW} \times 24$; $L_{HEP} = 510 \times 10^6$ cells / g liver; $L_{FBW} = 0.015$ g liver / g fish; 10g fish;

[b] $CL_{IN\ VIVO,INT} = CL_{IN\ VITRO,INT} \times L_{S9} \times L_{FBW} \times 24$; $L_{S9} = 163$ mg / g liver; $L_{FBW} = 0.015$ g liver / g fish; 10g fish

Table 7. In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$) of pyrene, measured in conjunction with in vitro studies for 5 test chemicals

Test chemical	*In vitro* system – Lot number[b]	Lab	Intra-laboratory variability				Inter-laboratory variability[a]		
			$CL_{IN\ VITRO,INT}$[c]	SD	CV (%)	Mean CV (%)[d]	$CL_{IN\ VITRO,INT}$[e]	SD	CV (%)
CS	RT-HEP – 1	A	0.84	0.11	13.1	19.1	1.86	0.77	41.6
		B	2.45	0.20	8.1				
		C	1.49	0.36	24.1				
		D	3.01	0.22	7.4				
		E	1.86	0.61	33.1				
		F	1.49	0.43	28.7				
	RT-S9 – 1	A	16.33	1.41	8.7	14.9	16.38	2.21	13.5
		B[f]	20.07	6.87	34.2				
		C	17.01	0.63	3.7				
		D	14.21	1.91	13.5				
		E[f]	16.66	2.74	16.5				
		F	14.02	1.79	12.8				
FEN	RT-HEP – 2	A	1.37	0.22	16.1	16.8	2.36	0.72	30.6
		B	3.51	1.25	35.7				
		C	1.98	0.17	8.8				
		D	2.55	0.33	12.9				
		E	2.62	0.59	22.7				
		F	2.12	0.10	4.5				
	RT-S9 – 2	A[f]	25.15	0.41	1.6	11.5	21.72	4.99	23.0
		B	19.37	0.34	1.8				
		C	17.58	2.35	13.4				
		D	15.13	0.88	5.8				
		E	26.58	7.10	26.7				
		F	26.52	5.17	19.5				

[a]Summary statistics represent the inter-laboratory variability in measured $CL_{IN\ VITRO,INT}$ values, and were developed using mean $CL_{IN\ VITRO,INT}$ values determined for each test chemical and *in vitro* test system (n = 6 except for MC where n =5).
[b]RT-HEP = cryopreserved rainbow trout hepatocytes, RT-S9 = rainbow trout liver S9 fractions
[c]Mean of $CL_{IN\ VITRO,INT}$ values determined in independent runs performed in parallel with substrate depletion experiments for each test chemical (n = 3, except where noted). Units: RT-HEP – mL/h/10^6 cells; RT-S9 – mL/h/mg protein.
[d]Mean across all laboratories
[e]Mean across all laboratories
[f]n = 2

Table 8. In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$) and in vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$) of pyrene, measured in conjunction with in vitro studies for 5 test chemicals in rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP).

Test Chemical	*In vitro* system – Lot number	Lab	$CL_{IN\ VITRO,INT}$*	$CL_{IN\ VIVO,INT}$ (L/d/kg fish)
CS	RT-HEP-1	A	0.84	154.22
		B	2.45	449.82
		C	1.49	273.56
		D	3.01	552.64
		E	1.86	341.50
		F	1.49	273.56
		AVG	**1.86**	**340.88**
	RT-S9-1	A	16.33	958.24
		B	20.07	1177.71
		C	17.01	998.15
		D	14.21	833.84
		E	16.66	977.61
		F	14.02	822.69
		AVG	**16.38**	**961.37**
FEN	RT-HEP-2	A	1.37	251.53
		B	3.51	644.44
		C	1.98	363.53
		D	2.55	468.18
		E	2.62	481.03
		F	2.12	389.23
		AVG	**2.36**	**432.99**
	RT-S9-2	A	25.15	1475.80
		B	19.37	1136.63
		C	17.58	1031.59
		D	15.13	887.83
		E	26.58	1559.71
		F	26.52	1556.19
		AVG	**21.72**	**1274.63**
4NP	RT-HEP-3	A	0.98	179.93
		B	1.60	293.76
		C	1.52	279.07
		D	1.66	304.78
		E	1.42	260.71
		F	1.77	324.97
		AVG	**1.49**	**273.87**
	RT-S9-3	A	26.71	1567.34
		B	27.85	1634.24
		C	28.8	1689.98
		D	20.27	1189.44
		E	32.89	1929.99
		F	26.35	1546.22
		AVG	**27.15**	**1592.87**
DM	RT-HEP-4	A	1.36	249.70
		B	1.88	345.17
		C	1.33	244.19
		D	1.59	291.92
		E	1.29	236.84
		F	1.59	291.92

		AVG	**1.51**	**276.62**
	RT-S9-4	A	32.42	1902.41
		B	31.53	1850.18
		C	15.05	883.13
		D	16.84	988.17
		E	12.56	737.02
		F	14.17	831.50
		AVG	**20.43**	**1198.73**
MC	RT-HEP-5	A	0.73	134.03
		B	---	---
		C	1.68	308.45
		D	2.10	385.56
		E	1.46	268.06
		F	1.40	257.04
		AVG	**1.47**	**270.63**
	RT-S9-5	A	29.03	1703.48
		B	---	
		C	24.18	1418.88
		D	18.31	1074.43
		E	14.26	836.78
		F	10.29	603.82
		AVG	**19.21**	**1127.48**
OVERALL AVG RT-HEP			**1.74**	**319.00**
OVERALL AVG RT-S9			**20.98**	**1231.02**

Units: RT-HEP – mL/h/10^6 cells; RT-S9 – mL/h/mg protein.

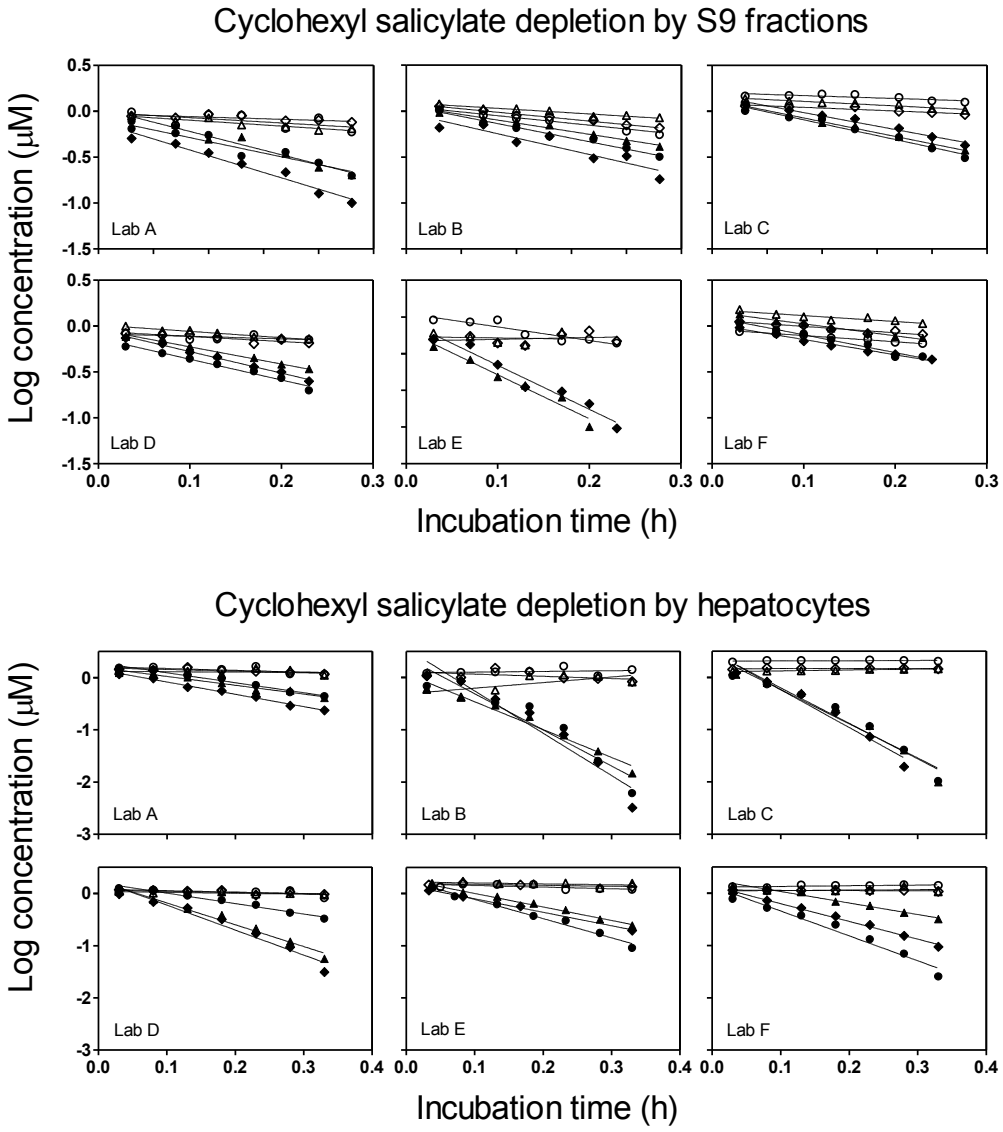
Table 9. Comparison of measured and modeled chemical bioconcentration factors (BCFs; L/kg)

Chemical	Empirical BCF[a]	Nichols et al. (2013b) BCF model predictions assuming no biotransformation[b]	Nichols et al. (2013b) BCF model predictions obtained using *in vitro* rates of biotransformation (RT-HEP and RT-S9)[b,c]			
			RT-HEP $f_U = f_{U,P}/f_{U,HEP}$	RT-HEP $f_U = 1.0$	RT-S9 $f_U = f_{U,P}/f_{U,S9}$	RT-S9 $f_U = 1.0$
Cyclohexyl salicylate	400	2371	217 ± 11	181 ± 1	448 ± 50	181 ± 1
Fenthion	185 – 16,600	607	277 ± 43	117 ± 2	192 ± 19	113 ±1
4-*n*-Nonylphenol	240 - 344	16,549	2909 ± 676	321 ± 14	3891 ± 590	381 ± 22
Deltamethrin	115 -1400	22,900	4214 ± 1216	315 ± 31	2472 ± 306	292 ± 9
Methoxychlor	174	5229	3835 ± 398	446 ± 84	3359 ± 113	423 ± 18
Pyrene[h]	78 - 1578	3490	709 ± 78	213 ± 2	316 ± 21	204 ± 1

[a]See table 1 and Annex 10 for additional information on in vivo BCF studies
[b]The models were run assuming a 10 g fish containing 5% lipid that is exposed at 12 °C.
[c]For all chemicals except pyrene, reported BCFs represent the inter-laboratory mean ± SD (n = 6). Inter-laboratory means were based on intra-laboratory means for each laboratory. Intra-laboratory means were based in turn on *in vitro* datasets for 3 independent runs. For pyrene, BCFs represent the mean ± SD of all inter-laboratory means (n = 5), where PYR was run in parallel with a given test chemical.

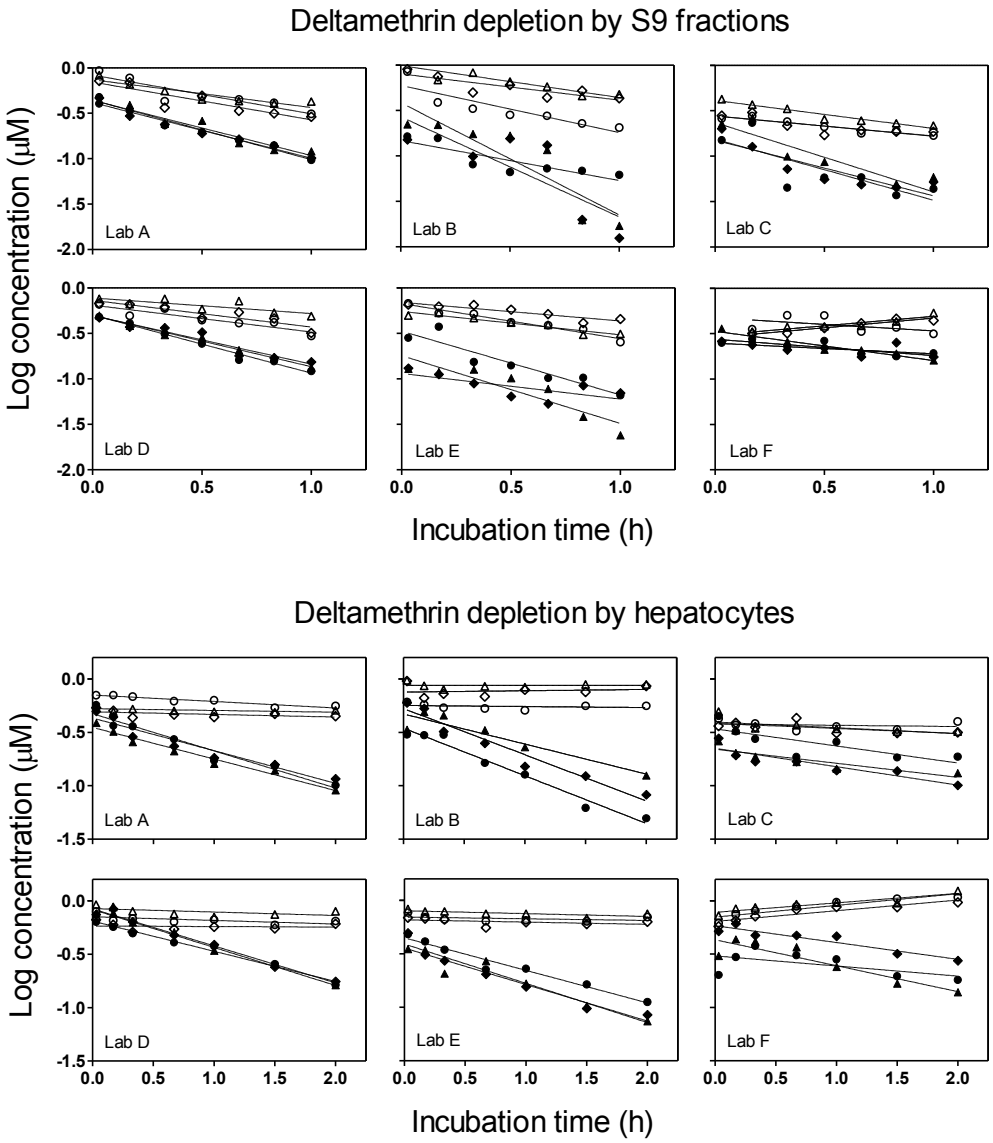
FIGURES

Figure 1. RT-HEP and RT-S9 substrate depletion curves from individual laboratories for cyclohexyl salicylate (CS).



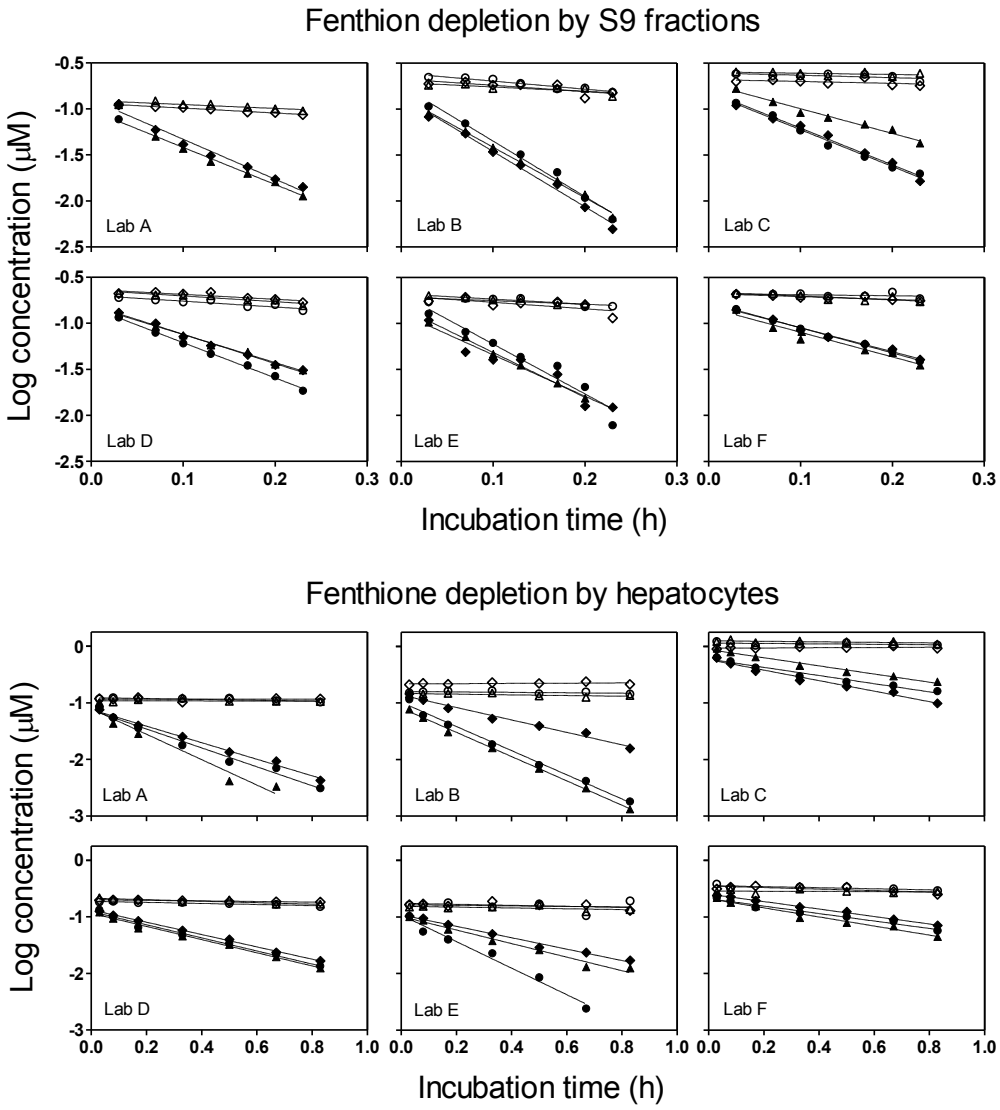
Each graph represents the data from one laboratory for one test chemical. The filled symbols represent data derived from live cells while the open symbols represent data from enzymatically inactive controls. Replicate depletions do not take into account differences in measured cell concentrations between the runs for the RT-HEP experiments. RT-S9 experiments are normalized for protein concentration (0.25 mg/mL). Different symbol shapes represent the average values from triplicate runs for each experiment.

Figure 2. RT-HEP and RT-S9 substrate depletion curves from individual laboratories for deltamethrin (DM).



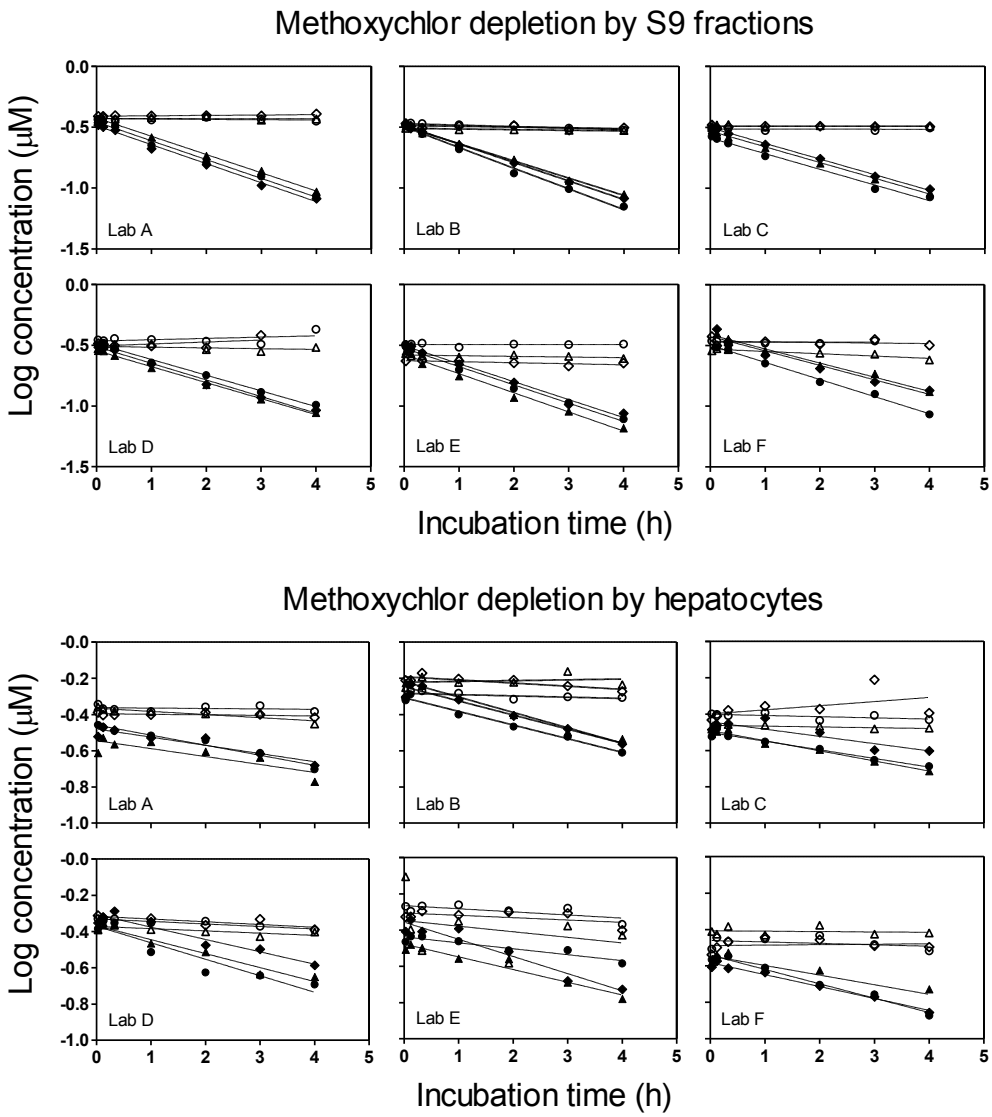
Each graph represents the data from one laboratory for one test chemical. The filled symbols represent data derived from active biological material while the open symbols represent data from enzymatically inactive controls. Replicate depletions do not take into account differences in measured hepatocyte concentrations between the runs for the RT-HEP experiments. RT-S9 experiments are normalized for protein concentration (1 mg/mL). Different symbol shapes represent the average values from triplicate runs for each experiment.

Figure 3. RT-HEP and RT-S9 substrate depletion curves from individual laboratories for fenthion (FEN).



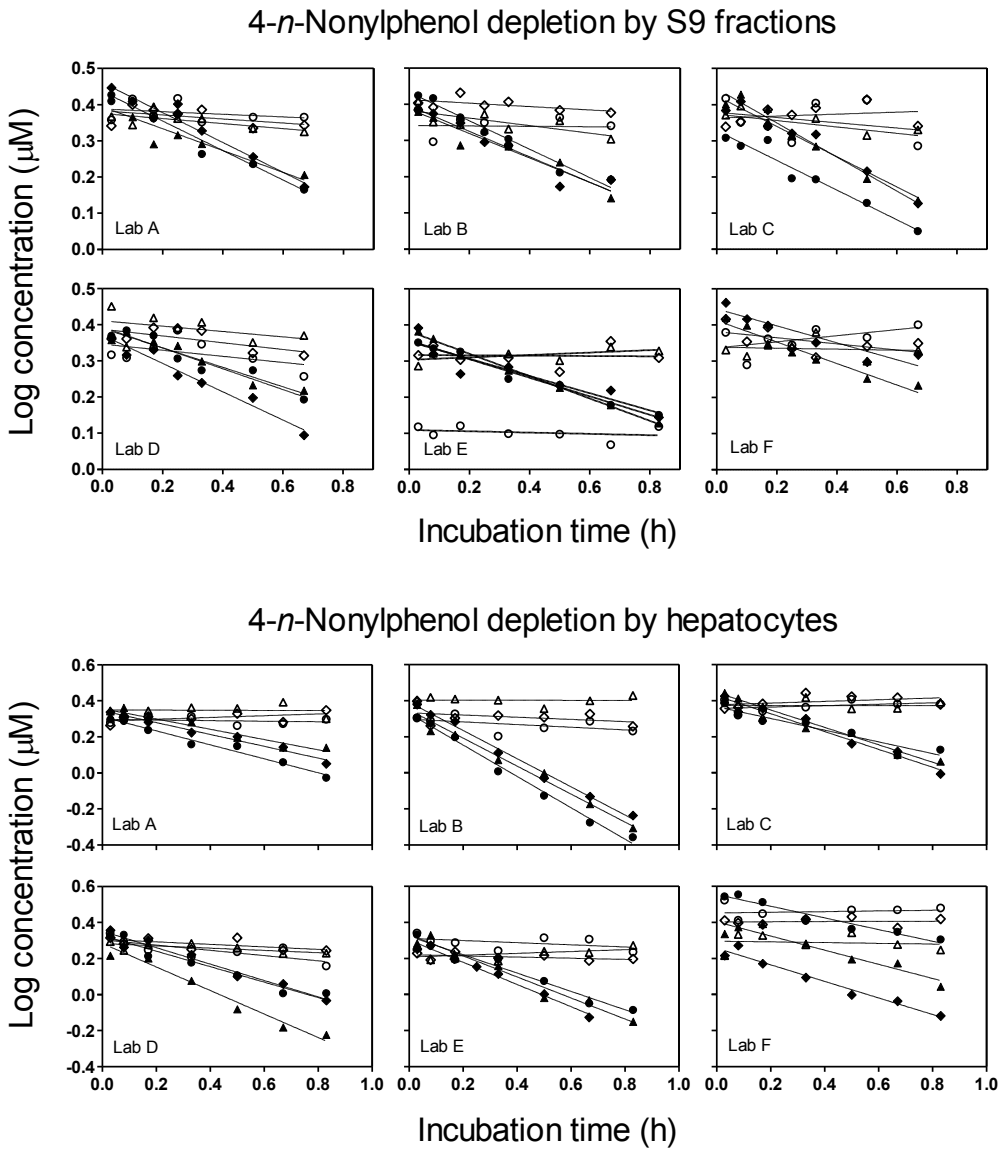
Each graph represents the data from one laboratory for one test chemical. The filled symbols represent data derived from active biological material while the open symbols represent data from enzymatically inactive controls. Replicate depletions do not take into account differences in measured hepatocyte concentrations between the runs for the RT-HEP experiments. RT-S9 experiments are normalized for protein concentration (1 mg/mL). Different symbol shapes represent the average values from triplicate runs for each experiment.

Figure 4. RT-HEP and RT-S9 substrate depletion curves from individual laboratories for methoxychlor (MC).



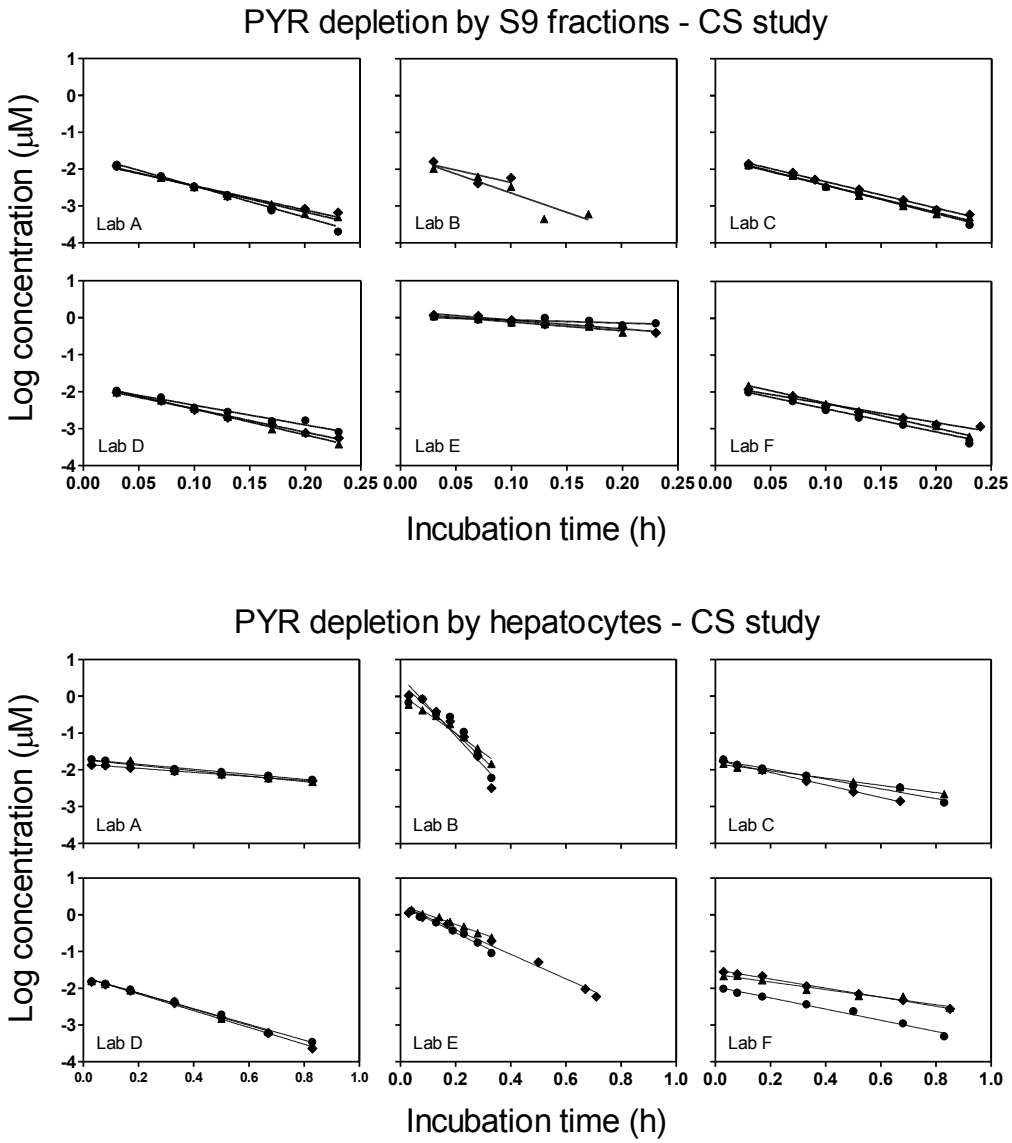
Each graph represents the data from one laboratory for one test chemical. The filled symbols represent data derived from active biological material while the open symbols represent data from enzymatically inactive controls. Replicate depletions do not take into account differences in measured hepatocyte concentrations between the runs for the RT-HEP experiments. RT-S9 experiments are normalized for protein concentration (1 mg/mL). Different symbol shapes represent the average values from triplicate runs for each experiment.

Figure 5. RT-HEP and RT-S9 substrate depletion curves from individual laboratories for 4-n-nonylphenol (4NP).



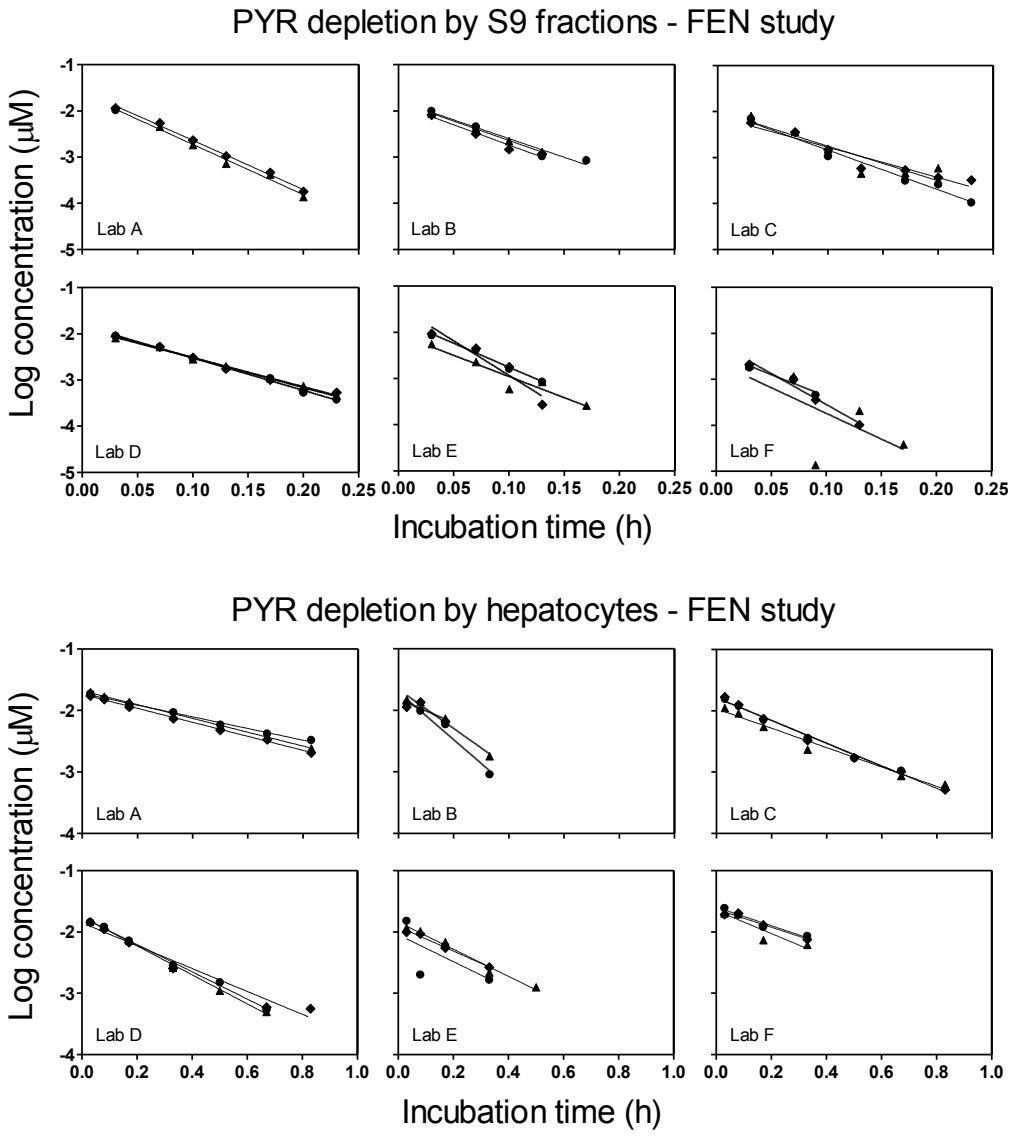
Each graph represents the data from one laboratory for one test chemical. The filled symbols represent data derived from active biological material while the open symbols represent data from enzymatically inactive controls. Replicate depletions do not take into account differences in measured hepatocyte concentrations between the runs for the RT-HEP experiments. RT-S9 experiments are normalized for protein concentration (1 mg/mL). Different symbol shapes represent the average values from triplicate runs for each experiment.

Figure 6. In vitro biotransformation of pyrene (PYR) by rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP) – lot 1 (cyclohexyl salicylate [CS] studies).



In this figure, each panel shows data generated using one lot of biological material. Different symbol shapes represent measured concentrations from 3 independent experiments performed on different days. Previous studies with PYR have shown that there is no loss of chemical from inactive controls (Nichols et al., 2013; Fay et al., 2017). These experiments were therefore performed without inactive controls to reduce the sample burden. Replicate depletion curves shown for the hepatocyte assays do not take into account small differences in measured hepatocyte concentration between runs (typically ± 20% of nominal).

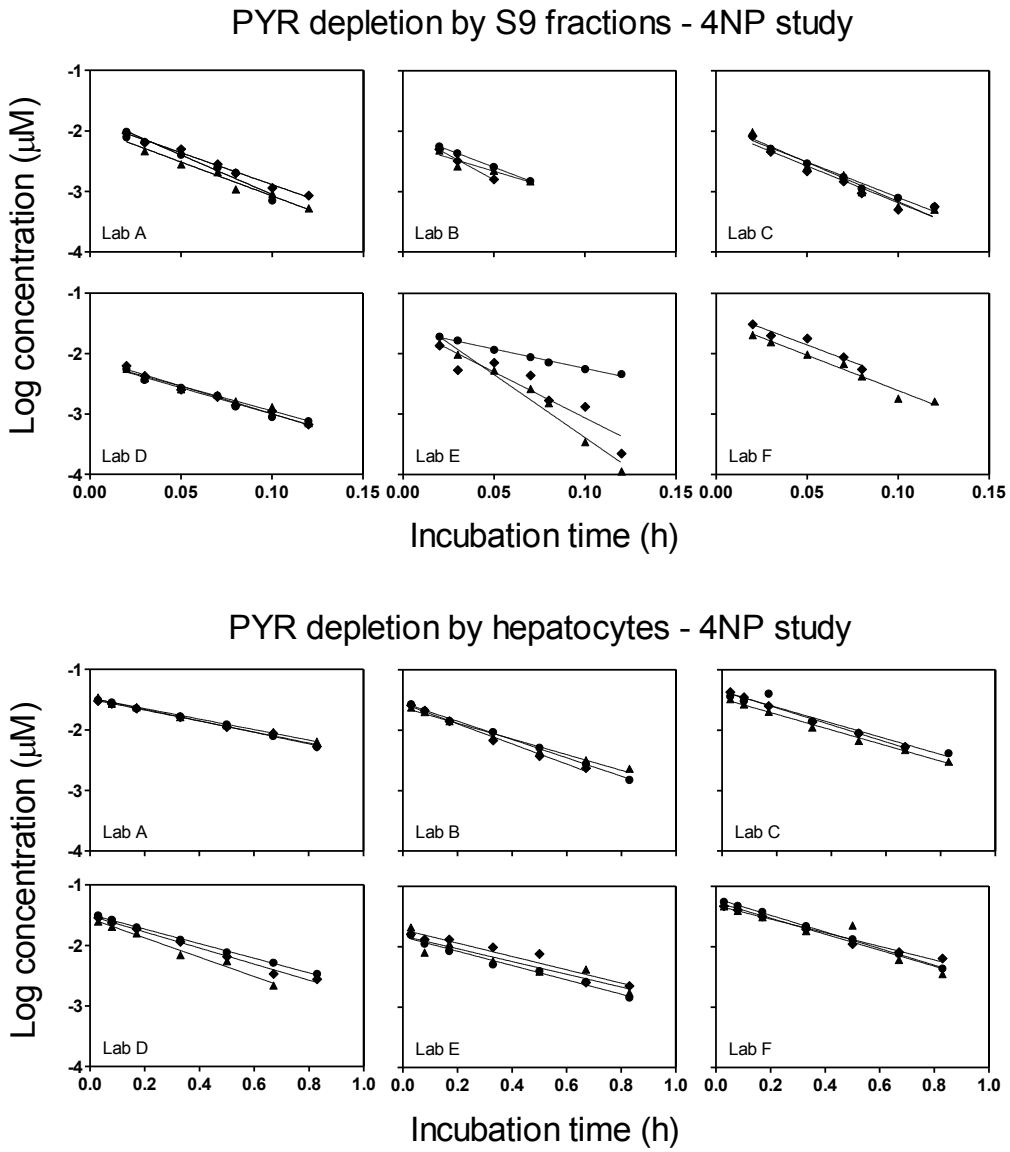
Figure 7. In vitro biotransformation of pyrene (PYR) by rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP) – lot 2 (fenthion [FEN] studies).



In this figure, each panel shows data generated using one lot of biological material. Different symbol shapes represent measured concentrations from 3 independent experiments performed on different days. Previous studies with PYR have shown that there is no loss of chemical from inactive controls (Nichols et al., 2013; Fay et al., 2017). These experiments were therefore performed without inactive controls to reduce the sample burden. Replicate depletion curves shown for the hepatocyte assays do not take into account small differences in measured hepatocyte concentration between runs (typically ± 20% of nominal)

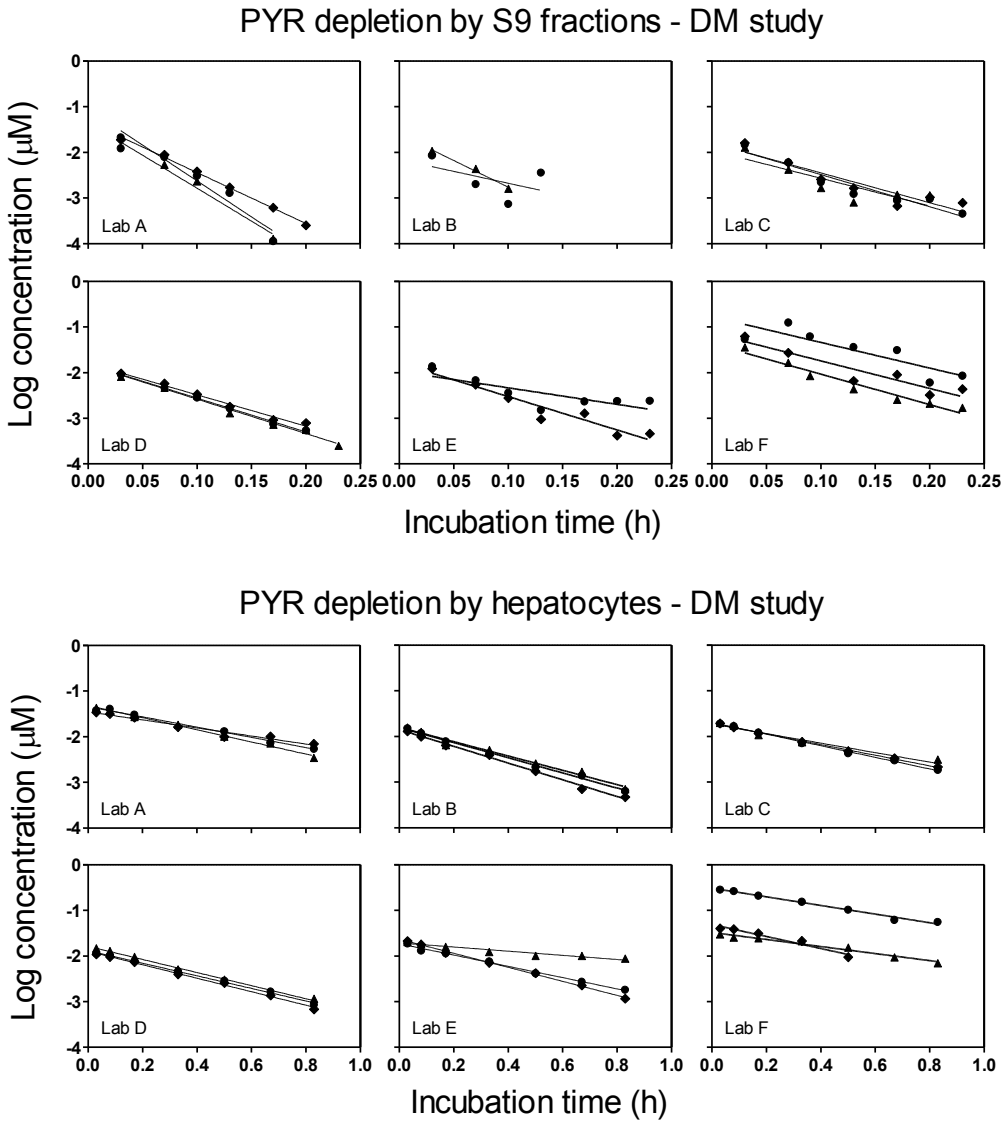
.

Figure 8. In vitro biotransformation of pyrene (PYR) by rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP) – lot 3 (4-n-nonylphenol [4NP] studies).



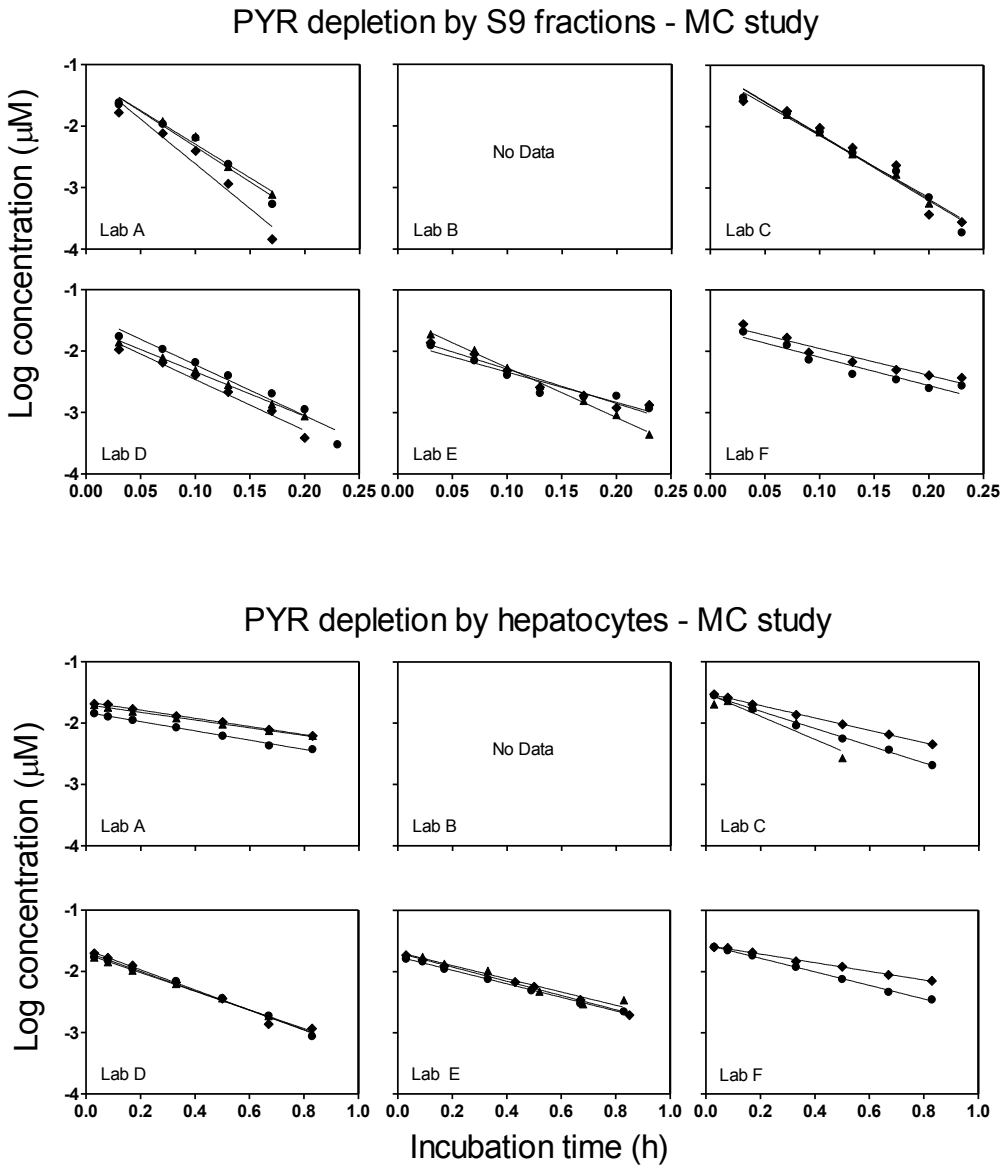
In this figure, each panel shows data generated using one lot of biological material. Different symbol shapes represent measured concentrations from 3 independent experiments performed on different days. Previous studies with PYR have shown that there is no loss of chemical from inactive controls (Nichols et al., 2013; Fay et al., 2017). These experiments were therefore performed without inactive controls to reduce the sample burden. Replicate depletion curves shown for the hepatocyte assays do not take into account small differences in measured hepatocyte concentration between runs (typically ± 20% of nominal).

Figure 9. In vitro biotransformation of pyrene (PYR) by rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP) – lot 4 (deltamethrin [DM] studies).



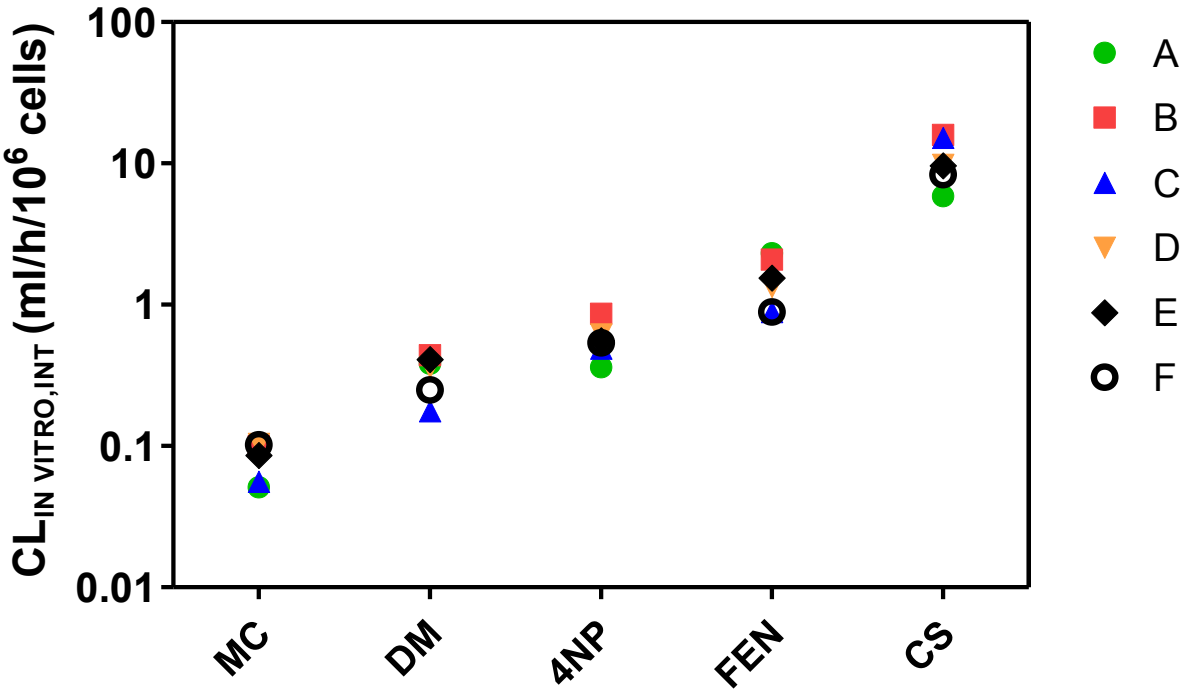
In this figure, each panel shows data generated using one lot of biological material. Different symbol shapes represent measured concentrations from 3 independent experiments performed on different days. Previous studies with PYR have shown that there is no loss of chemical from inactive controls (Nichols et al., 2013; Fay et al., 2017). These experiments were therefore performed without inactive controls to reduce the sample burden. Replicate depletion curves shown for the hepatocyte assays do not take into account small differences in measured hepatocyte concentration between runs (typically ± 20% of nominal).

Figure 10. In vitro biotransformation of pyrene (PYR) by rainbow trout liver S9 fractions (RT-S9) and cryopreserved rainbow trout hepatocytes (RT-HEP) – lot 5 (methoxychlor [MC] studies).



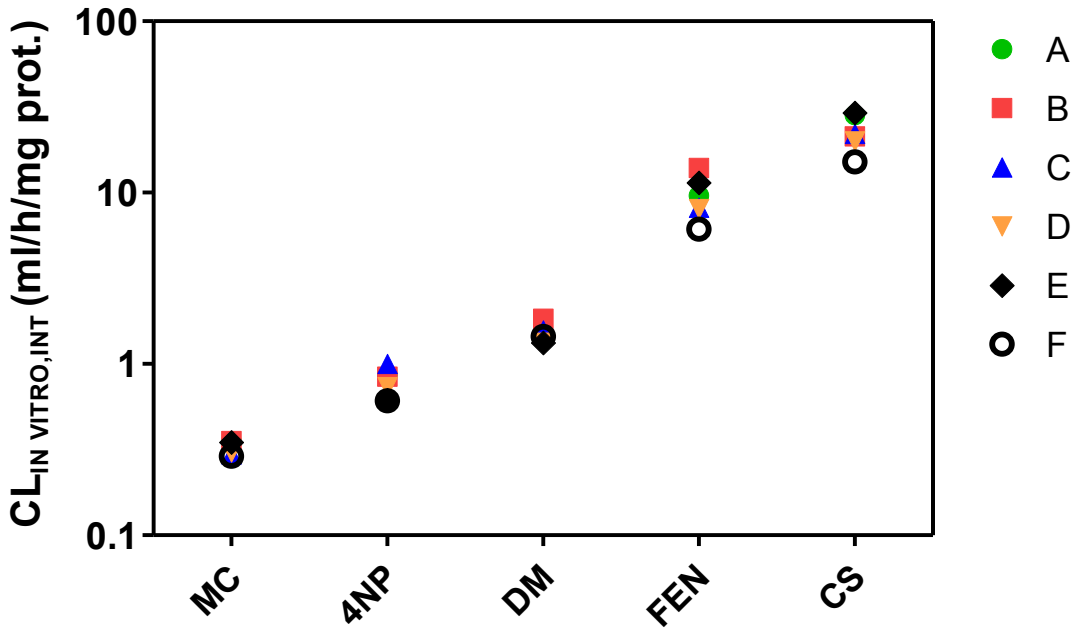
In this figure, each panel shows data generated using one lot of biological material. Different symbol shapes represent measured concentrations from 3 independent experiments performed on different days. Previous studies with PYR have shown that there is no loss of chemical from inactive controls (Nichols et al., 2013; Fay et al., 2017). These experiments were therefore performed without inactive controls to reduce the sample burden. Replicate depletion curves shown for the hepatocyte assays do not take into account small differences in measured hepatocyte concentration between runs (typically ± 20% of nominal).

Figure 11. In vitro intrinsic clearance rates (CLIN VITRO,INT) for methoxychlor (MC), deltamethrin (DM), 4-nonylphenol (4NP), fenthion (FEN), and cyclohexyl salicylate (CS), determined using cryopreserved rainbow trout hepatocytes (RT-HEP).



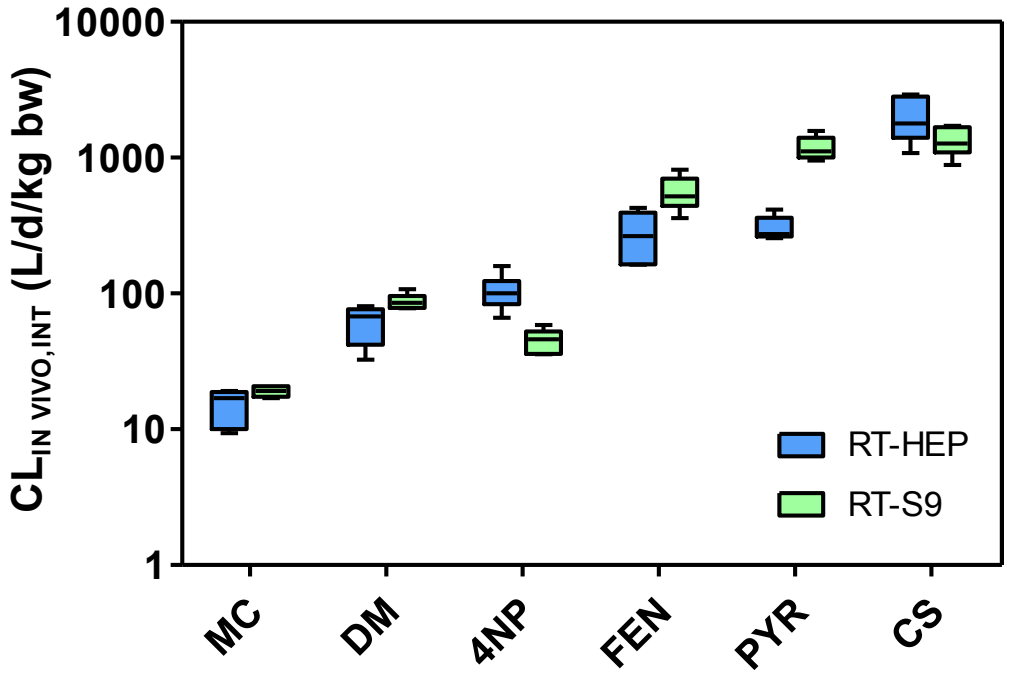
The symbols represent intrinsic clearance rates measured by 6 different laboratories (A-F). Each symbol represents the mean of 3 independently determined values.

Figure 12. In vitro intrinsic clearance rates (CLIN VITRO,INT) for methoxychlor (MC), 4-nonylphenol (4NP), deltamethrin (DM), fenthion (FEN), and cyclohexyl salicylate (CS), determined using rainbow trout liver S9 fractions (RT-S9).



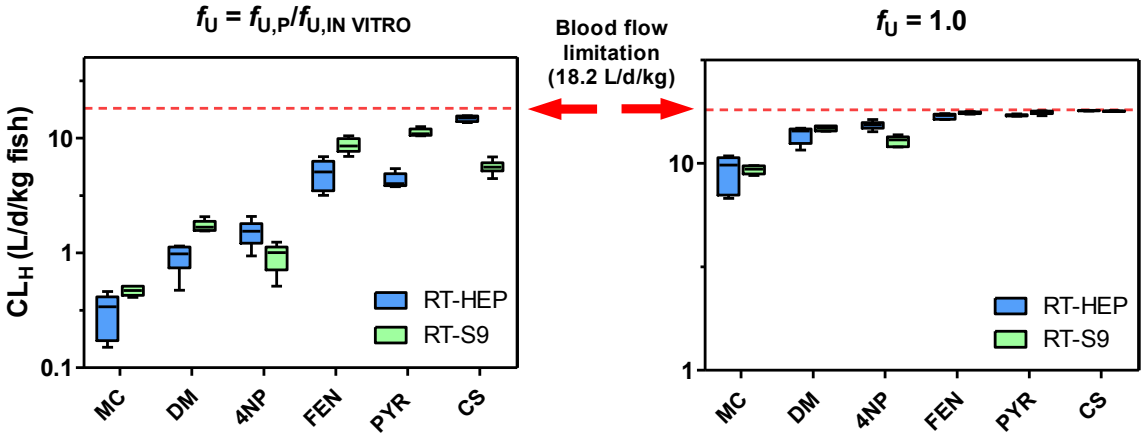
The symbols represent intrinsic clearance rates measured by 6 different laboratories (A-F). Each symbol represents the mean of 3 independently determined values.

Figure 13. Estimated in vivo intrinsic clearance rates (CLIN VIVO,INT) for methoxychlor (MC), deltamethrin (DM), 4-nonylphenol (4NP), fenthion (FEN), pyrene (PYR), and cyclohexyl salicylate (CS).



CL*IN VIVO,INT* values were calculated from measured rates of in vitro intrinsic clearance obtained using cryopreserved rainbow trout hepatocytes (RT-HEP) or trout liver S9 fractions (RT-S9). Means calculated for all laboratories are shown as horizontal lines. Values shown for MC, DM, 4NP, FEN, and CS represent data generated using chemical-specific lots of biological material, while those given for PYR represent studies performed using all 5 lots of tested material. Boxes denote the 25th and 75th percentiles while top and bottom whiskers extend up to 1.5 times the interquartile range.

Figure 14. Estimated hepatic clearance values (CLH) for methoxychlor (MC), deltamethrin (DM), 4-nonylphenol (4NP), fenthion (FEN), pyrene (PYR), and cyclohexyl salicylate (CS).



CLH values were calculated using a well-stirred liver model under two different binding assumptions (fU = fU,P/fU,HEP or S9 or fU = 1.0; see text for details). In vitro intrinsic clearance rates used as inputs to these calculations were generated using cryopreserved rainbow trout hepatocytes (RT-HEP) or rainbow trout liver S9 fractions (RT-S9). Means calculated for all laboratories are shown as horizontal lines. Values shown for MC, DM, 4NP, FEN, and CS represent data generated using chemical-specific lots of biological material (RT-HEP or RT-S9), while those given for PYR represent studies performed using all 5 lots of tested material. Boxes denote the 25th and 75th percentiles while top and bottom whiskers extend up to 1.5 times the interquartile range.

ANNEX 1 - Abbreviations

ANT	Anthracene
BCF	Bioconcentration factor (L/kg)
CL_H	*in vivo* hepatic clearance (L/d kg fish)
$CL_{, IN VITRO, INT}$	*in vitro* intrinsic clearance, (mL/h/10^6 cells or mL/h/mg protein)
$CL_{, IN VIVO, INT}$	*in vivo* intrinsic clearance (L/d/kg fish)
CS	Cyclohexyl salicylate
CV	Coefficient of variation
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DM	Deltamethrin
EROD	Ethoxyresorufin-O-deethylase
FEN	Fenthion
FEN-d6	Fenthion-d6
f_U	binding term used to correct for binding effects *in vitro* and in plasma (unitless)
GC	Gas Chromatography
GSH	L-Glutathione
GSI	Gonadosomatic index
GST	Glutathione transferase
HPLC	High Performance Liquid Chromatography
IVIVE model	*In vitro* to *in vivo* extrapolation model
k_e	Elimination rate constant
K_M	Michaelis-Menten constant
k_{MET}	Whole-body biotransformation rate constant (1/d)
LME model	Linear-mixed effects model
log K_{ow}	n-Octanol-water partition coefficient
L-15	Leibovitz-15
LOQ	Limit of quantification
MC	Methoxychlor
MC-d6	Methoxychlor-6
MS	Mass spectrometry

MTBE	methyl tert-butyl ether
NADPH	Nicotinamide adenine dinucleotide 2'-phosphate4NP 4-*n*-nonylphenol
4NP-d4	4-*n*-nonylphenol-d4
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate
PBTK	physiologically based toxicokinetic
pKa	Acid dissociation constant
PM	Permethrin
Q_H	Liver blood flow rate (mL/h/g liver)
RT-HEP	Rainbow trout hepatocytes
RT-S9	Rainbow trout liver S9 sub-cellular fraction
PYR	Pyrene
SOP	Standard Operating Procedure
SULT	Sulfotransferase
TG	Test Guideline
UDPGA	Uridine 5'-diphosphoglucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
V_{max} concentration	Maximum enzymatic rate at saturating test chemical

ANNEX 2: Details pertaining to test chemicals and internal standards

TABLE 1 A2. Details pertaining to test chemicals and internal standards

Chemical name	CAS no.	Supplier	Catalog no.	Lot no.	% Purity	MW (g/mol)
Test chemicals						
Pyrene	129-00-0	Sigma-Aldrich	185515	bcbk2867v	98.7	202.25
4-*n*-nonylphenol	104-40-5	Sigma-Aldrich	442873	lc07805v	99.9	220.35
Cyclohexyl salicylate	25485-88-5	Givaudan	8819601	ve003164	99.8	220.26
Fenthion	55-38-9	Sigma-Aldrich	36552	szbc178xv	97.9	278.33
Methoxychlor	72-43-5	Sigma-Aldrich	49054	lc09014	99.9	345.65
Deltamethrin	52918-63-5	Sigma-Aldrich	45423	szbc059xv	99.6	505.20
Internal standards						
Anthracene	120-12-7	Sigma-Aldrich	48567	Lcl0254v	99.0	178.23
4-*n*-nonylphenol-d4	1173019-62-9	Sigma-Aldrich	614343	mbbb2035v	98.0	224.27
Methyl laurate	111-82-0	Sigma-Aldrich	61689	bcbn8014v	99.5	214.34
Fenthion-d6	1189662-83-6	C/D/N Isotopes	D-6462	c126	97.0	284.36
Methoxychlor-d6	106031-79-2	C/D/N Isotopes	D7030	e244	98.6	351.69
Permethrin	52645-53-1	Sigma-Aldrich	45614	sbd142xv	98.1	391.29

ANNEX 3: Participants and tasks

Table 1A3: Participants and tasks

Participants	Tasks
US-EPA	• Isolation, characterization of RT-S9 and RT-HEP and shipment to laboratories • Analytical measurements: pyrene, fenthion
DuPont (USA)	• Incubations with RT-S9 and RT-HEP • Analytical measurements: 4-n-nonylphenol
Dow (USA)	• Incubations with RT-S9 and RT-HEP • Analytical measurements: Deltamethrin
Givaudan (CH)	• Incubations with RT-S9 and RT-HEP • Analytical measurements: Cyclohexyl salicylate
Fraunhofer (D)	• Incubations with RT-S9 and RT-HEP • Analytical measurements: Methoxychlor
Procter & Gamble (USA)	• Incubations with RT-S9 and RT-HEP
KJ Scientific / SCJ (USA)	• Incubations with RT-S9 and RT-HEP
HESI (USA)	• Coordination and reporting

ANNEX 4: Optimization of Study Design

A statistical analysis of two earlier ring trials was conducted using a linear-mixed effects (LME) model (McCulloch et al., 2008). The LME model findings were then used to inform a subsequent Monte Carlo simulation modeling effort. The goal of this analysis was to optimize the study design for the current ring trial.

A previous multi-laboratory study, conducted using cryopreserved trout hepatocytes (Fay et al., 2014a), was performed by three laboratories and involved six test chemicals. Substrate depletion assays were conducted using active and heat-inactivated hepatocytes. Three assays were run for each chemical on three separate days (runs), and each assay included seven separate time points with three replicates (vials) per time point for both active and heat-inactivated controls. A second multi-laboratory study, performed using trout liver S9 fractions (Johanning et al., 2012), involved three laboratories and three test chemicals. The assays were conducted using active and heat-inactivated S9 fractions, and were run three times for each chemical on three different days (runs). Substrate concentrations were determined at six time points for active S9 samples and three time points for heat-inactivated controls (beginning, middle and end of assay), with three replicates (vials) per time point.

The LME model was used to determine which study factors contributed substantially to variability in measured rates of depletion. This model may be stated as

$$y_{ijklm} = \mu_i + a_{ij} + a_k + b_{l(k)} + c_{m(kl)} + e_{ijklm}$$

where,

y_{ijklm} = log concentration from m^{th} vial and l^{th} run from k^{th} lab with samples exposed to the i^{th} chemical for the j^{th} time point

μ_i = mean baseline (fixed) effect specific to the i^{th} chemical

a_{ij} = j^{th} time point (fixed) effect specific to the i^{th} chemical

a_k = k^{th} lab effect (random), assumed to be normally distributed with a mean of 0 and variance of σ_a^2

$b_{l(k)}$ = l^{th} run effect nested within k^{th} lab, assumed to be normally distributed with a mean of 0 and variance of $\sigma_{b(a)}^2$

$c_{m(kl)}$ = m^{th} vial effect nested within l^{th} run from k^{th} lab, assumed to be normally distributed with a mean of 0 and variance of $\sigma_{c(ab)}^2$

e_{ijklm} = residual error, assumed to be normally distributed with mean of 0 and variance of σ_e^2

The LME model was fit with restricted maximum likelihood to model chemical depletion rate as a population parameter, chemical and time point as fixed effects, and laboratory, run and replicate vial as random effects. Likelihood-ratio tests (LRT; Graybill et al., 1976) were then performed to determine whether the contribution of a variance component to the overall variability in the data was statistically significant.

The LME model results showed that each of the variance components arising from laboratory effects, run effects, and vial replicate effects contributed to total variability observed in depletion data from previous laboratory studies (Table 1A4). However,

laboratory effects contributed the most variability while there was very little variability associated with replicate vial effects. The highest variance associated with laboratory effects was determined for the study involving liver S9 fractions (Johanning et al., 2012). In this effort, participating laboratories provided their own analytical support for all three test chemicals. In contrast, all samples for each chemical tested in the hepatocyte ring trial (Fay et al. 2014) were sent to one laboratory for analysis. To minimize this factor (laboratory variability) in the current ring trial it was recommend that a single laboratory conduct all analyses for each chemical as part of the final round of testing.

The results of the LRT analysis (Table 2A4) showed that the contribution of vial replicate effects to variability was not statistically significant. Based on this information, we reduced the overall sample number in the current ring trial by >50% by reducing the number of replicate vials from 3 to 1 for both active and heat-inactivated, hepatocyte and S9 samples. The contribution of run effects was not statistically significant for active hepatocyte samples, but was statistically significant for heat-inactivated hepatocytes and for both active and heat-inactivated S9 fractions.

Finally, estimated parameters from the LME model were used to perform a set of Monte Carlo simulations. To identify the optimal experimental design we employed a grid search by varying the number of chemicals, laboratories, and test runs. For each combination of grid parameters we generated 1000 simulated datasets and computed estimates for the depletion rate. A 95% confidence interval was then calculated covering the true value of the depletion rate, as well as the mean confidence width. Several screening criteria were applied to these simulations to develop a study design that provided robust statistical power while reflecting practical considerations such as time, cost, the availability of biological material: 1) coverage probability of a 95% confidence interval should be no less than 0.95; 2) total number of samples per chemical per biological material (hepatocytes or S9) should be no more than 250; 3) number of available laboratories participating in the current ring trial would be between 5-9; 4) number of test chemicals would be between 3-7; 5) number of runs per chemical per biological material would be between 2-4; 6) number of time points per chemical per biological material would be between 3-8. Based on the results of this effort, we developed the final study design involving 6 laboratories, 5 test chemicals, 3 independent runs per chemical, a single vial per run, and 7 sampling time points.

TABLE 1A4. Variance components from the linear mixed effects (LME) model[a]

Variable	Active samples		Heat-inactivated samples	
	Hepatocytes	S9 fractions	Hepatocytes	S9 fractions
Lab	0.209	0.404	0.109	0.253
Run (Lab)[b]	0.055	0.209	0.093	0.166
Replicate vial (Run, Lab)[b]	1.14×10^{-5}	1.23×10^{-5}	7.13×10^{-6}	7.26×10^{-5}
Residual error	0.412	0.399	0.219	0.430

[a]All variances are reported as the standard deviation of the mean
[b]Nested parameters

TABLE 2A4. Likelihood-ratio test (LRT) for significance of random effects[a]

Isolated hepatocytes

Random effect	Active samples		Heat-inactivated samples	
	-2 log likelihood ratio	P-value	-2 log likelihood ratio	P-value
Vial ($\sigma_{c(ab)}^2$)	<0.001	0.9995	<0.001	0.9996
Run ($\sigma_{b(a)}^2$)	2.45	0.118	48.46	<0.0001*

Liver S9 fractions

Random effect	Active samples		Heat-inactivated samples	
	-2 log likelihood ratio	P-value	-2 log likelihood ratio	P-value
Vial ($\sigma_{c(ab)}^2$)	<0.001	0.9996	<0.001	0.9998
Run ($\sigma_{b(a)}^2$)	64.62	<0.0001*	28.45	<0.0001*

*denotes values that differ significantly from 0

REFERENCES

Fay, K.A., Mingoia, R.T., Goeritz, I., Nabb, D.L., Hoffman, A.D., Ferrell, B.D., Peterson, H.M., Nichols, J.W., Segner, H., and Han, X. 2014. Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential. Environmental Science & Technology 48:8170-8178.

Graybill, F.A. 1976. Theory and application of the linear model. Duxberry Press, New Scituate.

Johanning, K., Hancock, G., Escher, B., Adekola, A., Bernhardt, M., Cowan-Ellsberry, C., Domoradski, J., Dyer, S., Eickhoff, C., Erhardt, S., Fitzsimmons, P., Halder, M., Nichols, J., Rutishauser, S., Sharpe, A., Segner, H., Schultz, I., and Embry, M. 2012. In vitro metabolism using rainbow trout liver S9. Summary report of the HESI Bioaccumulation Committee. Available at: http://www.hesiglobal.org/files/public/Committees/Bioaccumulation/Presentations%20and%20Data%20Resources/S9_report_FINAL_20Nov2012.pdf.

McCulloch, C.E. Searle, S.R. and Neuhaus, J.M. 2008. Generalized, Linear, and Mixed Models, 2nd Edition, 424 pages, Wiley.

ANNEX 5: RT-HEP SOP

Trout cryopreserved hepatocyte substrate depletion assay Standard Operation Procedure

Version 1.1

for the OECD in vitro test guideline project

1. PURPOSE

This procedure describes the experimental conditions and steps for incubation of a test chemical (TC) with cryopreserved hepatocytes isolated from rainbow trout to estimate the *in vitro* intrinsic clearance of the parent compound.

2. PRINCIPLE

In vitro determination of metabolic stability

Metabolic stability experiments are conducted using a substrate depletion approach wherein the biotransformation rate is determined by measuring the disappearance of parent chemical from the reaction mixture. The incubation system consists of thawed, live cryopreserved hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) in culture medium. Simultaneous incubations conducted using heat-inactivated hepatocytes are used to distinguish between enzymatic biotransformation and other potential loss processes including abiotic degradation, volatilization, and adsorption to the reaction vessel. Each reaction is performed at a constant temperature corresponding to the acclimatization temperature of the source fish. The substrate depletion reaction is initiated by the addition of test compound to the incubation system. A sufficient number of sampling time points are obtained to develop a high-quality regression of log-transformed chemical concentration data. The slope of this log-linear depletion is normalized to the suspension cell concentration to provide an *in vitro* intrinsic clearance.

Preliminary incubations were performed to establish reaction conditions and sampling time points appropriate to a given compound. Important variables include the starting concentration of test compound, the cell concentration and viability, and the total reaction run time. Additional experiments were conducted to characterize the activity of cryopreserved hepatocytes using standard substrates for one or more Phase I and II metabolic reactions. Analytical methods for the test substance also were developed and validated before conducting any substrate depletion studies. The analytical methods were demonstrated to have sufficient sensitivity to detect the decreased concentration of analyte due to biotransformation.

3. HEALTH PRECAUTIONS

Tissue should be handled with caution, and treated as if there is a potential presence of infectious agents. Wear appropriate laboratory coat, gloves and eye protection during all laboratory operations. Use caution when working with organic solvents and test chemicals. Read the appropriate Material Safety Data Sheet (MSDS) for each test chemical and solvent, and handle solvents in the fume hood when possible. Follow additional in-house safety guidelines.

4. LIST OF TERMS

4.1 DEFINITIONS

Reference Chemical	A chemical with a known *in vitro* biotransformation rate by cryopreserved trout hepatocytes.
Heat-inactivated hepatocytes	Hepatocytes that have been heat-inactivated by boiling.
% Coefficient of Variation	100 × the standard deviation divided by the average
% viability	100 × the number of cells excluding trypan blue divided by the total number of cells
% yield	100 × the number of live cells recovered after thawing divided by the number of live cells initially cryopreserved

4.2 ABBREVIATIONS

ACN	Acetonitrile
AE	Acetone
BSA	Bovine serum albumin
CS	Cyclohexyl salicylate
CV	Coefficient of variation
DCM	Dichloromethane (methylene chloride)
DM	Deltamethrin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FEN	Fenthion
HEP	Thawed, live cryopreserved trout hepatocytes
HI	Heat-inactivated
HIHEP	Heat-inactivated trout hepatocytes
L-15	Leibovitz -15 medium
IMS	Intermediate stock
I STD	Internal standard
MC	Methoxychlor
ML	Methyl laurate
MSDS	Material Safety Data Sheet
MTBE	Methyl tert-butyl ether
MTX	Matrix blank
PYR	Pyrene
SOP	Standard operation procedure
SS	Superstock
TBD	To Be Determined
TC	Test Chemical
4NP	4-n-nonylphenol

5. STUDY DESIGN

HEP and HIHEP will be sampled as singlet assays over three different days for each test chemical. Using separate vials of the same prepared suspension of thawed hepatocytes, depletions of the reference chemical (PYR) will be run in parallel with (or just prior to) the test chemical for each experimental day. Depletions of PYR using heat-inactivated cells will only be conducted in the pilot experiment. A generalized study design is provided in Table 1. Specific details regarding the order in which test chemicals will be sampled and experimental specifics are provided in ANNEX 1.

Table 1. Study design for the assessment of one test chemical (X)

Experimental day	Substrate depletion experiment with live hepatocytes		Substrate depletion experiment with HI hepatocytes
1	test chemical X	reference chemical (PYR)	test chemical X
2	test chemical X	reference chemical (PYR)	test chemical X
3	test chemical X	reference chemical (PYR)	test chemical X

6. MATERIALS AND EQUIPMENT

*Note: We recommend specific brands of equipment and reagents in some cases; however**, equivalent equipment or reagents may be substituted if not stated otherwise**. It is the researcher's responsibility to determine the substitute suitable for a particular application. Items that are mandatory for use are designated as such. Equipment, specific lot and expiration data for reagents may be recorded on the Record of Reagents, Chemicals and Instruments (ANNEX 3). Deviations during the experiment may be recorded in the "Comments" sections of other worksheets.*

6.1 EQUIPMENT AND LABWARE

6.1.1 Fixed Equipment

- Analytical balance for mg quantities
- Vortex mixer, Thermo Scientific* MaxiMix/Vortex Mixer (cat. no. 12-815-50)
- Sample incubation equipment. Common options include:
  - Shaking water bath with chiller, VWR water bath shaker 18 L (cat. no. 89032-226) with VWR AD 7 L rfg/htg circulator, SS, 120V (cat. no. 89202-970)
  - Shaking incubator with heating and cooling functions, Denville Scientific (cat. no. S2085-HC)
  - Thermomixer block with shaking capabilities, Ditabis Model MKR 23 230 V for Europe (cat. no. 980523001); Jade Scientific 115 V for USA (cat. no. 98021150)
- Refrigerated centrifuge, Thermo Scientific IEC Centra GP8R (cat. no. 29530)
- Small benchtop refrigerated centrifuge for microfuge tubes, USA Scientific Eppendorf (cat. no. 22620601)
- pH meter, Fisher Scientific Accumet AB150 pH meter (cat no.13-636-AB150A)
- Compound microscope capable of 20 × magnification
- Vacuum pump – optional
- 4ºC refrigerator, LABRepCo (cat.no. LABL-23-SD)
- -20 ºC freezer, LABRepCo (cat. no. LABH-14-FA)
- Cryogenic container or dewar containing liquid nitrogen, Coleparmer, Taylor-Wharton (cat. no. EW-03779-60)

6.1.2 Labware

- Glassware for making up and storing chemical solutions,
- Pipette tips, 0.1-10, 0.5-200, 100 -1000 µL, Daigger (cat. nos. EF2033B, EF2036B, EF2037B, respectively)

- Pipetman 10, 100, 200 and 1000 µL, Daigger (cat. nos. EF9930B, EF9930D, EF9930E, EF9930F, respectively)
- Serological pipets, 10, 25 mL, Sigma-Aldrich (cat. nos. CLS4100, CLS4250, respectively)
- Portable Pipet-aid, Daigger, Drummond Scientific (cat. no. EF20391E)
- Disposable bottle-top vacuum filters, 0.22µm, 250mL, Sigma-Aldrich, Corning (cat. no. CLS430767)
- 7 mL scintillation vials, Perkin Elmer (cat. no. 6000167) OR Cole Parmer, Kimble solvent saver (cat. no. EW-08918-14) OR VWR Wheaton (Cat. no. 986492 Europe; note that these are described as 6 mL scintillation vials but they have the exact same dimensions as the above 7 mL vials in the USA) – ***mandatory***
- Holder for scintillation vials, Thomas Scientific (cat. no. 9720D10) - optional
- 1.5 mL microcentrifuge tubes, Sigma-Aldrich, Eppendorf Safe-Lock (cat. no. T9661)
- Microcentrifuge tube rack, Sigma-Aldrich (cat. no. R5651)
- Sample vials with fused inserts and caps (300 µL), Chromocol (cat. no 03-FISV(A)) or Waters (186001126c) – ***mandatory***
- Sample vials (1.5 mL), Agilent (cat. no. 5182-0715)
- Sample vial screw caps (for 1.5 mL standard sample vials, above), Agilent (cat. no. 5182-0717)
- Eppendorf Repeater® Plus pipette, Eppendorf, Fisher (cat. no. 022260201) -optional
- Combitips for Repeater® Plus pipette) for 0.2 mL volume, Eppendorf (cat no. 022266004) (Optional)
- Timer, Sigma-Aldrich (cat. no. 22754-U)
- Disposable hemacytometers (Neubauer improved), Incyto Co. C-Chip, DHC-N01-5, VWR (cat. no. 82030-468 USA, cat. no. 631-1098 Europe) - ***mandatory***
- Tally counter, Sigma-Aldrich (cat. no. Z169021)
- Cryogloves to remove cells from liquid nitrogen, Fischer Scientific (cat. no. 11-394-200)
- 50 mL tube rack, Sigma-Aldrich (cat. no R5651)
- 50 mL conical centrifuge tubes, Fisher Scientific, Falcon (cat. no. 14-959-49A)
- Pasteur pipettes (9 in or 228 mm) (Fisher Scientific cat. no. 13-678-8D)- optional
- Spatulas for weighing chemicals

6.2 CHEMICALS AND CELL CULTURE REAGENTS

6.2.1 Reagents, Media, Sera

- Sodium hydroxide (NaOH) for pH adjustment, Sigma-Aldrich (cat. no. S2770)
- Hydrochloric acid (HCl) for pH adjustment, Sigma-Aldrich (cat. no. H9892)
- Dulbecco's Modified Eagle Medium (DMEM), low glucose with phenol red, Gibco, Life Technologies (cat. no.12320-032 USA or 22320-022 Europe) - ***mandatory***
- Fetal Bovine Serum (FBS), qualified, not heat-inactivated, Gibco, Life Technologies (cat. no. 26140-079 USA, 10270-106 Europe)
- Bovine Serum Albumin, Sigma-Aldrich (cat. no. A7030)
- Leibovitz-15 (L-15) with glutamine, without phenol red, Gibco, Life Technologies (cat. no. 21083) - ***mandatory***
- Trypan blue, 0.4%, Sigma-Aldrich (cat. no. T8154) – ***mandatory***

6.2.2 Chemicals (test chemicals, internal standards, extraction solvents)

- Test Chemicals and reference chemical (***mandatory***; details in ANNEX 1, Table A1.1)
- Internal standards (***mandatory***; details in ANNEX 1, Table A1.1)

- Stopping and extraction solvents, HPLC grade (e.g. acetonitrile, methylene chloride; ANNEX 1, Table A1.1)
- Solvents to dissolve test chemicals, HPLC grade (e.g. acetone, acetonitrile; ANNEX 1, Table A1.4)

6.3 BIOLOGICAL MATERIAL

Cryopreserved hepatocytes (HEP), EPA lots 1,3,4,5,7,8. Each lot was pooled from 5 fish (*Oncorhynchus mykiss*, Erwin strain, 392 ± 84 g, mixed gender, sexually immature), frozen as 1.5 mL, 10 × 10^6 cells/mL. Fish were fed a commercial trout chow (Silver Cup; Nelson and Sons Inc, Murray, UT) and held on a 16:8 light: dark photo period at 11 ± 1 ºC. All animals were fasted 24 h prior to use. Specific lots of cryopreserved hepatocytes designated for use with each test chemical are provided in ANNEX 1.

- Heat-inactivated (HI) trout hepatocytes (HIHEP), EPA. A suspension of primary trout hepatocytes (2× 10^6 cells/mL) in L-15 medium was boiled for 15 min in a 100ºC water bath. The final volume of the suspension was adjusted by addition of L-15 medium to maintain the initial concentration of biological material.

7. SOLUTIONS PREPARATION

Refer to the Certificate of Analysis and MSDS for each test chemical and reagent to determine appropriate storage and handling conditions. The purity of the test compound is critically important and should be taken into account when calculating the mass needed to create stock and spiking solutions if the purity is < 95%. For this study, all test chemicals and internal standards are of sufficient purity (ANNEX 1, Tables A1.2 and A1.3), so that no adjustments are required.

7.1 MEDIA

Media should be pH adjusted on the day of the experiment to 7.8 ± 0.1 at 11ºC. The pH of a solution is dependent upon its temperature. Please consult your pH equipment specifics on how to adjust the pH of your solution when it is at a temperature other than 11ºC.

7.1.1 Recovery medium

About 55 mL of recovery medium (DMEM supplemented with 10% FBS and 0.25% BSA) are needed per set of clearance reactions (thawing 2 cryovials provides for two 1 mL suspensions of 2 ×10^6 cells/mL). If three reactions will be performed within one week, it is recommended to prepare 170 mL of recovery medium, sterile filter and aliquot 55 mL each into three 50 mL centrifuge tubes (50 mL vials actually can hold 55 mL). All prepared media should be used within one week.

Table 2. Recovery medium

Reagent	Per 170 mL
DMEM	153 mL
FBS	17 mL
BSA	0.425 g

Preparation prior to the experimental day (use sterile technique):

- Combine the reagents listed in Table 2. Record actual volumes and mass on the Media Preparation Worksheet (ANNEX 3).
- Invert to mix.
- If needed, adjust pH with 1 N NaOH as needed to facilitate dissolution of the BSA. The target pH is 7.8 at 11ºC.
- Filter sterilize using sterile filters and vacuum pump or equivalent system.
- In a biohood, aliquot 55 mL into three sterile 50 mL centrifuge tubes.

- If a lot of foam is present, allow the media to sit overnight at 4°C.
- Use within 1 week of preparation. Store at 4°C.

Preparation on the experimental day (sterile technique not required):

- In a 100 mL flask, adjust the pH of approximately 60 mL of the Recovery medium to 7.8 at 11°C using 1N HCl or NaOH. Record the actual pH and temperature on the Media Preparation worksheet (ANNEX 3). Sterile technique is not required.
- Bring medium to room temperature (~25 °C).
- Transfer 42 mL of Recovery medium to a 50 mL centrifuge tube and 13 mL of recovery medium to a separate 50 mL centrifuge tube and maintain both at room temperature.

7.1.2 L-15 medium

Preparation on the experimental day:

- Adjust the pH of approximately 150 mL L-15 media to 7.8 at 11°C using 1N HCl or NaOH. Record actual pH and temperature on the Media Preparation worksheet (ANNEX 3).
- Maintain on ice or in 4°C refrigerator.

7.2 STOCK SOLUTIONS

7.2.1 Test Chemical stock solutions

All chemical stock solutions must be prepared in glassware. Test chemical (TC) Superstock solutions will be made fresh when beginning incubations with a new test chemical. All test chemical incubations will be completed within 2 weeks. If incubation experiments with a given TC extend beyond two weeks, a fresh Superstock may be required. Spiking stock solutions (dilutions from the Superstock) shall be made up fresh the day of the experiment. Depending upon the desired incubation concentration of the TC and the molecular weight of the TC, an Intermediate stock may be necessary. Specific preparation guidance for each test chemical is provided in ANNEX 1, Table A1.4.

- Record stock preparations on the Superstock and Intermediate Stock Preparations worksheet and the Spiking Stock Preparations worksheet (ANNEX 3)
- Remove a 1 mL aliquot of the final Spiking stock, preserve in a standard 1.5 mL HPLC/GC vial with screw top for shipping with the test chemical incubation samples. Samples of Spiking stock will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (Section A2.1) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.2.2 Reference chemical (PYR) stock solutions

Reference chemical (PYR) stocks for depletion experiments shall be made up as a concentrated Superstock, an Intermediate stock and the Spiking stock. The Superstock and Intermediate stock are made up fresh every two weeks, while the spiking stock is made up daily by diluting the Intermediate stock. All PYR stock solutions are prepared in acetone. The Superstock is prepared at a concentration of 5 mM. The Superstock is diluted 25 fold to a 200 µM concentration (Intermediate stock). This Intermediate stock is diluted 40 fold to a 5 µM concentration (Spiking stock). An example of this preparation is provided below.

Table 3. Example preparation of PYR stocks

Prior to the day of the experiment (2 weeks stability) Day of the experiment

	Intermediate stock (200 µM):
Superstock (5 mM)	25 x dilution of Superstock

PYR (mg)	Volume Acetone (mL)	Volume of Superstock (mL)	Total volume with Acetone (mL)
10.1	10.0	0.400	10.0

Spiking Stock (5 µM): 40 × dilution of Intermediate stock	
Volume of Intermediate stock (mL)	Total volume with acetone (mL)
0.25	10.0

Preparation prior to the experimental day:

- Prepare a 5 mM PYR Superstock in acetone. For example, weigh out 10.1 mg of PYR and dissolve in 10 mL of acetone to create the Superstock. Mix well.
- Record stock preparations on Superstock and Intermediate Stock Preparations worksheet (ANNEX 3).
- Remove 400 µL of the Superstock and bring up to 10 mL with acetone to create the 200 µM Intermediate Stock. Mix well. Store at 4°C.

Preparation on the experimental day:

- Remove 250 µL of the Intermediate stock and bring up to 10 mL with acetone to create the 5 µM Spiking Stock. Mix well. Record on the Spiking Stock Preparations worksheet (ANNEX 3).
- Remove a 1 mL aliquot of the final PYR Spiking stock, preserve in a standard 1.5 mL HPLC/GC vial with screw top for shipping with the PYR incubation samples. Samples of Spiking stock will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (A2.1) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.3 STOPPING SOLUTION WITH INTERNAL STANDARD

7.3.1 Test chemical stopping solution with internal standard

One bulk solution of stopping solution may be used for all depletion experiments for a given TC if all reactions occur within **2 weeks** of the initial preparation. Visible contamination, or data suggesting contamination, degradation of the internal standard, or incorrect preparation will require fresh preparation of the stopping solution. For each TC, the type of solvent, internal standard and the concentration of the internal standard are detailed in ANNEX 1 (Table A1.5).

- Dissolve test chemical into the appropriate solvent to achieve the Superstock concentration.
- Record details of preparation on the Stopping solution with Internal Standard Preparation worksheet (ANNEX 3).
- Remove a 1 mL aliquot of the stopping solution for shipment to the laboratory analyzing the incubation samples containing the corresponding test chemical. If one bulk stopping solution is used for experiments of a given test chemical, then only one sample needs to be provided to the analytical laboratory. These samples will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (A2.2) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.3.2 Pyrene stopping solution containing anthracene

One bulk solution of stopping solution for PYR depletions may be used for all sets of experiments performed in concert with a TC. All reactions should occur within **2 weeks** of the stopping solution

preparation. Visible contamination, or data suggesting contamination, degradation of the internal standard, or incorrect preparation will require fresh preparation of the stopping solution. Stopping solution for PYR reactions contains anthracene (ANT) as an internal standard; Table 3 and ANNEX 1 (A1.5) detail the procedure for preparing PYR stopping solution.

Table 4. Example preparation of acetonitrile (ACN) Stopping solution containing anthracene (ANT).

Superstock (SS; 1 mM)		Intermediate stock 1 (IMS 1; 20 µM): 50 × dilution of SS		Intermediate stock 2 (IMS 2; 0.4 µM): 50 × dilution of IMS 1		Stopping solution (0.002 µM): 100 × dilution of IMS 2	
ANT (mg)	Volume ACN (mL)	Volume of SS (mL)	Total volume with ACN (mL)	Volume of IMS 1 (mL)	Total volume with ACN (mL)	Volume of IMS 2 (mL)	Total volume of Stopping solution (mL)
8.91	50	1.0	50	1.0	50	0.500	100

- Prepare a 1.0 mM ANT Superstock of stopping solution in acetonitrile. For example, weigh out 8.91 mg of ANT (178.23 g/mol) and dissolve in 50 mL of acetonitrile. Mix well.
- Record details of preparation on the Stopping solution with Internal Standard Preparation worksheet (ANNEX 3).
- Remove 1.0 mL of the Superstock and bring up to 50 mL with acetonitrile to create the 20 µM Intermediate Stock 1. Mix well. Store at 4°C.
- Remove 1.0 mL of the Intermediate stock 1 and bring up to 50 mL with acetonitrile to create the 0.4 µM Intermediate stock 2. Mix well. Store at 4°C.
- Remove 500 µL of the Intermediate stock 2 and bring up to 100 mL with acetonitrile to create the 0.002 µM Stopping solution. Mix well. Store at 4°C.
- For each preparation (e.g. for each test chemical), aliquot a 1.0 mL sample of the stopping solution for shipment to the PYR analytical laboratory. These samples will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (A2.2) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

8. PROCEDURES

All deviations from the following procedures must be detailed on the appropriate worksheets in the "Comments" sections (ANNEX 3).

8.1 PRIOR TO THE EXPERIMENT

1. Set up a room temperature water bath.
2. Prepare Recovery medium (Section 7.1.1).

3. Prepare Superstocks and Intermediate stocks of the test chemical and reference chemical (Section 7.2). Record details on the Superstock and Intermediate Stock Preparation worksheet (ANNEX 3)
4. Prepare Stopping solutions with internal standards (Section 7.3). Record details on the Stopping Solution with Internal Standard Preparation worksheet (ANNEX 3).
5. Label tubes and vials for the substrate depletion experiment. See ANNEX 2 for the labeling scheme. Labels for the microfuge tubes and HPLC/GC analytical vials will be provided for incubation samples, matrix blanks, spiking solvent and stopping solution. Note that for specific chemicals, glass Hirschmann tubes may be used in place of the plastic microfuge tubes. In these cases, specific guidance will be provided (TBD; ANNEX 1).
    a. 50 mL centrifuge tube (1)
    b. cell counting microfuge tubes (5: 2 pre-dilution, 3 post-dilution)
    c. 7 mL sample scintillation vials (3: test chemical HEP, test chemical HIHEP, PYR HEP)
    d. microcentrifuge tubes for sample time points (23: 7 for each of the 3 samples above, 2 matrix blanks (HEP/HIHEP))
    e. HPLC/GC sample vials, 300 µL with fused inserts (23: 7 for each of the 3 incubations, 2 matrix blanks (HEP, HIHEP))
    f. HPLC/GC sample vials, standard 1.5 mL (8:, 2 spiking solutions (TC/PYR), and 6 stopping solutions (3 each TC/ PYR)*)

    **stopping solutions only need to be sampled on the first day of the experiment if the same preparation is used for all experiments in the set.*

8.2 DAY OF THE EXPERIMENT

8.2.1 Preliminary steps

1. Turn on the centrifuges (for the 50 mL tube as well as the 1.5 mL microfuge tubes) and adjust the temperature to 4°C.
2. Turn on the water bath or incubation equipment for running reactions and bring to a constant temperature (for these experiments, T= 11 °C).
3. Dispense the appropriate stopping solution (with internal standard) used to terminate reactions into the pre-labeled microfuge tubes. For tubes receiving aliquots from the PYR incubation, fill microfuge tubes with the 400 µL of acetonitrile containing 0.002 µM ANT. Likewise, prepare the receiving tubes for the TC with the appropriate stopping solution. Refer to ANNEX 1 (Table A1.2) for stopping solutions and volumes specific to each TC. Keep the tubes containing acetonitrile on ice or in a 4°C refrigerator.

   Acetonitrile must be kept cold to fully precipitate protein from the reactions.
4. Prepare the Spiking stock solutions of the TC and PYR (Section 7.2). Aliquot 1.0 mL of the TC Spiking stock into 1.5 mL standard HPLC/GC vials for inclusion in the incubation sample shipments to the TC analytical laboratory.
5. Remove HIHEP (2 x 10^6 cells/ mL) from the freezer and thaw; 1 mL is needed to run the experiment. Excess thawed sample may be refrozen for later use.

8.2.2 Thawing of cryopreserved cells.

1. Adjust the pH of the L-15 and the Recovery media (prepared previously) to 7.8 ± 0.1 at 11°C using 1N HCl or NaOH (Section 7.1.1). Maintain the L-15 on ice or at 4ºC.
2. Allow approximately 55 mL of Recovery medium to come to room temperature. Transfer 13 mL of Recovery medium to a second 50 mL centrifuge tube, and maintain both tubes at room temperature (~ 25ºC) (Section 7.1.1).
3. Remove 2 vials of HEP from liquid nitrogen vapor (transport vials on dry ice if located in a different lab) and immediately thaw the vials in a room temperature water bath. Record time on Cell Recovery and Suspension Dilution Worksheet (ANNEX 3). Thawing should be conducted so that the frozen suspension is completely submerged below the waterline. Hold the vials by their caps so that the caps are above the water level. Gently move vials side to side or in a cross pattern until the contents freely move and a small ice crystal remains. For most labs with a room temperature near 25 ºC, this thawing process will take approximately 2 min 15 sec.
4. Pour the contents of 2 vials into the tube containing 42 mL of room temperature Recovery medium. Note that 2 vials should be added to one tube. Recovery will be diminished if only one vial is added at this step.
5. Transfer 1 mL of recovery medium from the extra tube (containing ~13 mL) to each cryovial, rinse and resuspend any remaining cells left in the cryovial. Recap the vial and invert once to mix. Add contents from the rinse to the 50 mL tube containing the hepatocyte/recovery medium suspension. The final volume expected is ~47 mL.
6. Gently invert the tube containing cells and Recovery medium once, and centrifuge at 50 x g for 5 min, 4°C. Check that the 'brake' function on the centrifuge is turned off.
7. Remove tubes from the centrifuge and aspirate the supernatant to the point where the centrifuge tube begins to taper (~4 mL mark), being careful not to disturb the cell pellet. The supernatant can be aspirated either manually by using a pipette, or by using a vacuum pump. Do not discard the supernatant by pouring.

   Importantly, in an inter-laboratory comparison, it was observed that the level to which the supernatant was aspirated above the pellet introduced variability for cell recovery/yield between the labs. Aspirating too close to the pellet will decrease yield. To obtain consistent results, aspirate the supernatant to the point at which the tube begins to taper for all wash steps.
8. Add ~5 mL L-15 (pH adjusted, 4°C or ice cold) to each tube and resuspend the cell pellet by gently tapping the side of centrifuge tube against the back of the opposite hand.
9. Upon resuspension of the pellet, bring the tube to a final volume of 45 mL with the L-15. Invert once and centrifuge again at 50 x g for 5 minutes at 4°C.
10. Aspirate the supernatant to the top of the conical portion of centrifuge tube, being careful not to disturb the cell pellet.
11. Wash cells a second time by adding ~ 5 mL L-15 (pH adjusted, 4°C or ice cold) and resuspend the cell pellet by lightly tapping the side of centrifuge tube against the back of the opposite hand.
12. Upon resuspension of the pellet, add L-15 to a final volume of ~45 mL. Invert the tube once and centrifuge at 50 x g for 3 minutes at 4°C.
13. Aspirate the supernatant to **just below** (~ 2 mm) the conical taper portion of centrifuge tube and resuspend cells in ~1.5 mL L-15 by gently tapping side of centrifuge tube against the opposite hand. The final volume should be approximately 3 mL at this point.

14. Place the tube with resuspended cells on ice and prepare to count the cells.

8.2.3 Cell counting and suspension dilution

1. Prepare a 6× dilution by transferring 440 µL of L-15 to a 1.5 mL microcentrifuge tube, add 60 µL of 0.4% trypan blue solution and vortex.

   Note that a 6 × dilution is not required, but is offered as initial guidance. The researcher may choose to alter the dilution factor depending upon the cell yield from two cryovials. Alternative dilutions should be prepared so that the final concentration of the trypan blue is 0.04%. Dilutions should result in 50-150 total cells present in a given counting quadrant (200-600 total cells/side of hemacytometer).

2. Gently invert/swirl the tube containing the hepatocytes to resuspend the cells. Quickly transfer 100 µL of the cell suspension to the tube containing the L-15 and trypan blue. Gently invert the microcentrifuge tube to mix the cells with the dye.

   Prepare each dilution of cells in L-15 and trypan blue immediately before counting since trypan blue is cytotoxic.

3. Carefully suspend cells in the microfuge tube containing trypan blue, quickly transfer 10 µL of the suspension into one of the V-shaped wells, and gently expel the sample. The area under the coverslip will fill by capillary action. Load each side of the hemocytometer.

4. Place the loaded hemacytometer on the microscope stage and bring the counting grid on one side into focus at low power (20 × magnification).

5. Count the unstained (live) cells in the 4 corners of the grid (Fig. 2, squares C, D, E, and F). When counting cells within a corner grid (e.g. square F, Fig. 2), count all cells wholly contained within outside corner grid lines, and count the cell if it is touching a top or right edge, as described in Fig. 2. Do not count the cell if it is touching the bottom or left edge.

6. Record the total number of unstained (live) cells from all 4 corners on the Pre-Dilution Cell Count Worksheet (ANNEX 3).

7. From the same 4 corners, count the stained (dead) cells and add this number to the live cell count to determine the total number of cells (stained + unstained). Record the total number of cells on the Pre-Dilution Cell Count Worksheet.

8. Count both sides of the hemacytometer.

9. Determine % viability for each side and record. The average viability must be 80% to satisfy the acceptance criteria (section 9.1).

   % viable = (total live cells /total cell count) x 100

   If the viability is below 80%, continue to perform the assay and save the samples. Perform a rerun (4th experiment). If the viability is poor only for one run, exclude those samples from analysis. If poor viability is consistent, determine if other labs have similar results for the same lot of cells. Evaluate if any steps in the preparation deviated from the SOP, including excessive time of cells in trypan blue.

10. Determine the viable cell concentration for each side and record.

    Viable cell concentration (cells/mL) = (# of live cells/number of fields counted) x dilution (6) x 10,000

    Note: the total number of live cells counted in the 4 corners from one side of the hemacytometer would be divided by 4 (the number of fields counted).

11. Perform 2 cell counts using both sides of the hemacytometer (4 counts total). Prepare a new trypan blue dilution from the cell suspension just prior to each count so that the cells do not remain in tyrpan blue more than 2 min prior to counting. Average the viability and viable cell concentration from all 4 counts and record on the Pre-Dilution Cell Count Worksheet.
12. Once the viable cell concentration is determined, dilute cells to 2 x 10^6 cells/mL. To determine the volume needed for a 2 x 10^6 cells/mL suspension, carefully measure the volume of the remaining suspension using a serological pipet, multiply by the calculated average cell concentration, and divide by the desired cell concentration (2 x 10^6 cells/mL) to obtain the final desired volume. The amount of L-15 to add to the suspension is the difference between the current volume and the desired volume. Record on the Cell Recovery and Suspension Dilution worksheet (ANNEX 3).

    Example:
    volume of suspension = 3.3 mL
    concentration determined from cell counting = 2.8 × 10^6 cells/mL

    (2.8 × 10^6 cells/mL x 3.3 mL)/ 2.0 × 10^6 cells/mL = 4.6 mL
    4.6 mL – 3.3 mL = 1.3 mL
    Add 1.3 mL of L-15 to the suspension.

13. Perform 3 cell counts (both sides of the hemacytometer; 6 total) on the diluted suspension to confirm cell density using the Post-dilution Cell Count Worksheet (ANNEX 3). This second set of counts may be made during or after the incubations as time allows. The dilution of the suspension in L-15 and trypan blue will be at the discretion of the researcher, but will likely be less than the dilution performed above (e.g., 6 ×).

    Note: Two vials containing 1.5 mL of 10 ×10^6 cells/ml suspension each can be expected to provide ~ 7.5 – 10 million hepatocyte (~25-33% yield), which corresponds to ~4-5 mL of suspension at a concentration of 2× 10^6 cells/mL. After aliquoting 2 mL for incubations, excess suspension should be available for cell counting.

    Determine the agreement among the three average live cell counts as % CV = 100* (stdev/average). If the % CV is greater than 20, an additional dilution in L-15 and trypan blue and cell count is needed to obtain an accurate estimation of the live cell concentration. Repeat until the % CV for all counts is ≤ 20.

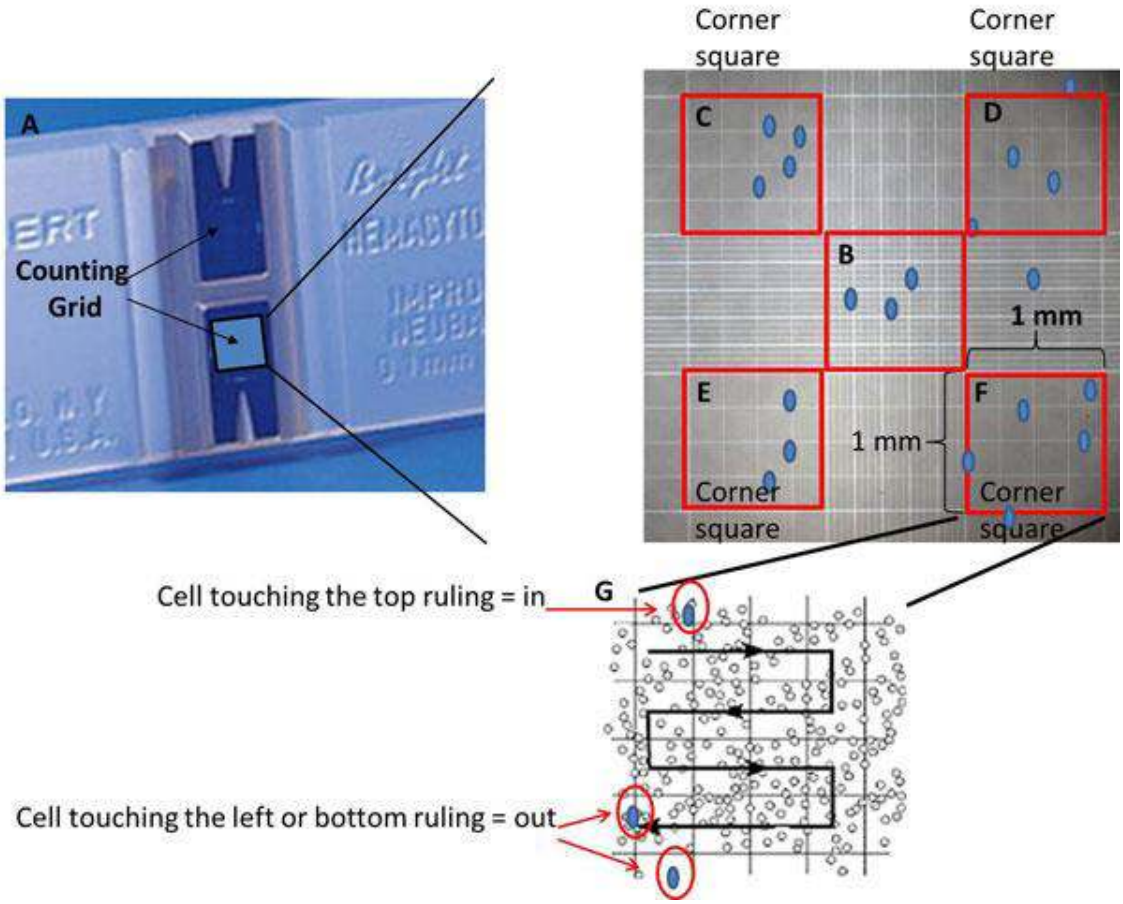


Figure 2.Graphic representation of the cell count. Counting grid on a hemacytometer (Neubauer improved). Note that this study will use plastic, disposable hemacytometers unlike the glass hemacytometer pictured here (A). Visual representation of counting grid magnified to 20 x, count 4 corners (C,D,E, and F). Follow the general rule for counting each corner grid by following path (G).

8.2.4 Substrate Depletion Assay

1. Resuspend the live cells by gentle inversion, and transfer 1 mL of live cells diluted to 2 x 10^6 cells/mL into each of two 7 mL scintillation vials.
2. Resuspend the HI cells by gentle inversion and transfer 1 mL of this suspension to a third 7 mL scintillation vial.
3. Place all three vials into a shaking water bath or incubator, and pre-incubate samples to constant temperature (11 ± 1°C) with gentle shaking for 10 minutes.
4. Start the reaction by adding 5 µL of the prepared Spiking stock containing the test chemical or reference chemical (PYR).

   The time points for HEP and HIHEP samples incubated with the test chemical will be identical. The spiking and sampling of the vials may be staggered so that the aliquots are sampled at precise times. For example, dosing of the HIHEP may be staggered 30 seconds after dosing the Hep vial. Each aliquot taken as a time point for the HIHEP would be similarly staggered 30 seconds after the HEP samples were taken. An example sampling scheme is provided in ANNEX 1, Table A1.6.

Depending upon the specific time points predetermined for each test chemical, the live hepatocyte suspension spiked with the reference chemical (PYR) may be sampled prior to the test chemical or during the test chemical incubations.

5. At each designated time point (7 total; ANNEX 1), gently swirl the cells to ensure a uniform suspension, remove an aliquot of 100 µL, and transfer this aliquot to the labeled microfuge tube containing the appropriate solvent containing internal standard (ANNEX 1). Dispense the aliquot and rinse the pipet tip in the solvent by aspirating and dispensing 3 times. Record all information on the Clearance Assay Worksheet (ANNEX 3).
6. Manually swirl/mix each reaction vial every 15 min for the duration of the experiment if time points are longer than 15 min apart.
7. After the experiment is complete, vortex the microfuge tubes containing the incubation samples (hepatocyte suspension plus solvent) on a vortex mixer. See ANNEX 1 for chemical-specific instructions, including extraction with the addition of a second solvent where appropriate, solvent temperature considerations, centrifugation, etc.
8. Transfer 300 µL of the supernatant or the organic phase to analytical HPLC/GC sample vials.
9. Secure samples by tightly screwing on the vial tops.
10. Store at -20°C until analysis.

8.2.5 Preparation of matrix blanks

These samples may be prepared during or after the substrate depletion assays. They will be included in the shipments to the analytical labs for the test chemical as well as the reference chemical. See ANNEX 2 for labeling convention.

1. Prepare one matrix blank using the live hepatocyte suspension and one matrix blank using the heat-inactivated hepatocyte suspension for the test chemical on each experimental day (6 total/ test chemical). Using the excess hepatocyte suspension (post-dilution), pipet 100 µL into the appropriate solvent (and volume) for the test chemical (ANNEX 1). **The solvent for the matrix blanks should not include the internal standard**. Prepare these matrix blanks as described for the corresponding TC (steps 7-10, Section 8.2.4).

9. DATA ANALYSIS AND REPORTING

The following acceptance criteria and requirements represent conditions of satisfaction which should be met in order for a test to pass. If these are not met, the test may need to be repeated. All deviations from the SOP must be recorded in the "Comments" section on the appropriate worksheet.

9.1 ACCEPTANCE CRITERIA AND REQUIREMENTS

9.1.1 Experimental Acceptance Criteria

1. **Media pH.** The pH of the Recovery Medium and L-15 should be adjusted to 7.8 ± 0.1 at 11 ± 1 ^{o}C the day of the experiment.
2. **Cell counting and viability.**
   - Cell suspensions diluted to the reaction concentration (e.g. 2 x 10^6 cells/mL) should be diluted and stained with 0.04% trypan blue three separate times. Each dilution should be counted on both sides of a hemacytometer. Live and total cells will be recorded. The average live cell concentration from each of the three replicates should vary less than 20 % CV. If the variability is greater than 20 % CV, a fourth dilution and cell count is required (or more).

- Cell viability in the hepatocyte suspension should be ≥ 80%.
- The diluted cell suspension (incubation suspension) concentration should be within 25% of its target concentration (e.g. 2.0 ± 0.5 x 10^6 cells/mL). If the concentration falls below this range, proceed with the experiment but conduct a rerun. If the concentration falls above this range, dilute cells again. Conduct the substrate depletion assay on the newly diluted suspension.

9.1.2 Analytical Acceptance Criteria

- Test chemical analytical runs will contain a standard curve determined using 7 calibration standards. Five standards spanning the concentration range of the incubation samples, with a correlation coefficient of 0.95 will be required for calculations.
- A mid-range standard will be analyzed after every 14 sample injection. The % RSD throughout the run should be 5% or less for non-matrix standards.
- All matrix spike samples should be within 20% of expected value. The analytical performing laboratory should make three levels of their own matrix spikes at each analytical run.

9.1.3 Requirements

1. **Dissolution of test chemical**. The stock solution of the compound under study should be dissolved as recommended by the specific test methods determined by the analytical labs performing the chemical analyses.
2. **Superstock and Intermediate stock test chemical expiration**. Analytical labs performing the chemical analyses should perform stability test on the TC in spiking solvent. TC Superstock and Intermediate stock solutions should be made fresh and used within two weeks or within the stability time frame, whichever comes first.
3. **Spiking stock solutions**. These solutions should be made fresh the day of the experiment by diluting the corresponding Superstock as appropriate.
4. **Stopping solution containing internal standard**. Analytical labs performing the chemical analyses should perform stability test on the internal standard in stopping solution. Stopping solutions should be made fresh and used within two weeks or within the stability time frame, whichever comes first.
5. **Media Expiration.** Recovery Media should be prepared using sterile technique, stored at 4°C and assigned an expiration date of one week.
6. **Preliminary experiments.** Preliminary studies data should be available and final conditions established by the analytical lab performing the TC analyses. All participating laboratories in the Ring Trial should utilize these the established experimental conditions.
7. **Incubation reaction vials.** It is critical that the vials utilized by all participating laboratories will be glass 7 mL scintillation vials. Note that the same vials are described as 6 mL scintillation vials in Europe. Recommendations are given in the SOPs. Plastic should be avoided at all times for the incubation step.
8. **Total percentage of organic solvent in incubation mixture.** The total percentage of organic solvent in the reaction mixture should be < 1 to avoid potential inhibition of metabolic enzyme activity.
9. **Incubation temperature.** The incubation temperature should be constantly maintained at 11 ±1 °C for the duration of the experiment. This temperature reflects the maintenance temperature of the source fish.
10. **Experimental design.** All experiments with the TC will be repeated as singlet assays on three separate days. Time points, chemical concentration, cell concentration and other experimental conditions will be consistent for the three experiments.

11. **Reference chemical.** Pyrene will be used as the reference chemical to determine functionality of the system. The analytical lab performing the analysis, i.e. US EPA, will establish the reference chemical conditions.
12. **Negative Controls.** Heat-inactivated cryopreserved hepatocytes are incorporated into each substrate depletion experiment to account for possible chemical losses due to abiotic degradation, volatilization, and adsorption to the reaction vessel. Heat-inactivated samples will be sampled under the same conditions as the live.
13. **Matrix blank samples.** Matrix blank samples containing the biological material (HEP and HIHEP) and solvent(s) without addition of test chemical or internal standard will be prepared each experimental day for the test chemical analytical laboratory. These samples will be analyzed only if contamination is suspected in the incubation samples.

9.2 DATA REPORTING

Data templates to report the results will be provided to the analytical laboratories. Each laboratory will use these templates to report valid and failed experiments. These files containing the data should be sent to ILSI HESI for data analyses by the SAS Statisticians. All printed and signed originals should be sent to ILSI HESI as well for archiving. Results of the intrinsic clearance will be reported for each test chemical used in the *in vitro* metabolism experiments performed by the participating laboratories. Copies of all Worksheets from each laboratory will be sent to ILSI HESI for data archiving.

9.3 STATISTICAL ANALYSES

Concentrations of each test chemical utilized in the OECD Ring Trial will be log transformed and plotted against the reaction time. Depletion rate constants (k; hr-1) will be calculated from the slope using linear regression. One-way analysis of variance (ANOVA) of the loss of parent will be determined. Significance will be determined at $\alpha = 0.05$. SAS will be utilized for statistical analysis of data.

10. REFERENCES

Further information on substrate depletion experiments using fish hepatocytes may be found in the following publications (this list is not exhaustive):

Dyer, S. D., et al. (2008). "In vitro biotransformation of surfactants in fish. Part I: Linear alkylbenzene sulfonate (C12-LAS) and alcohol ethoxylate (C13EO8)." Chemosphere 72(5): 850-862.

Fay, K. A., et al. (2014). "Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish." Xenobiotica 44(4): 345-351.

Fay, K. A., et al. (2014). "Intra- and Interlaboratory Reliability of a Cryopreserved Trout Hepatocyte Assay for the Prediction of Chemical Bioaccumulation Potential." Environmental Science & Technology 48(14): 8170-8178.

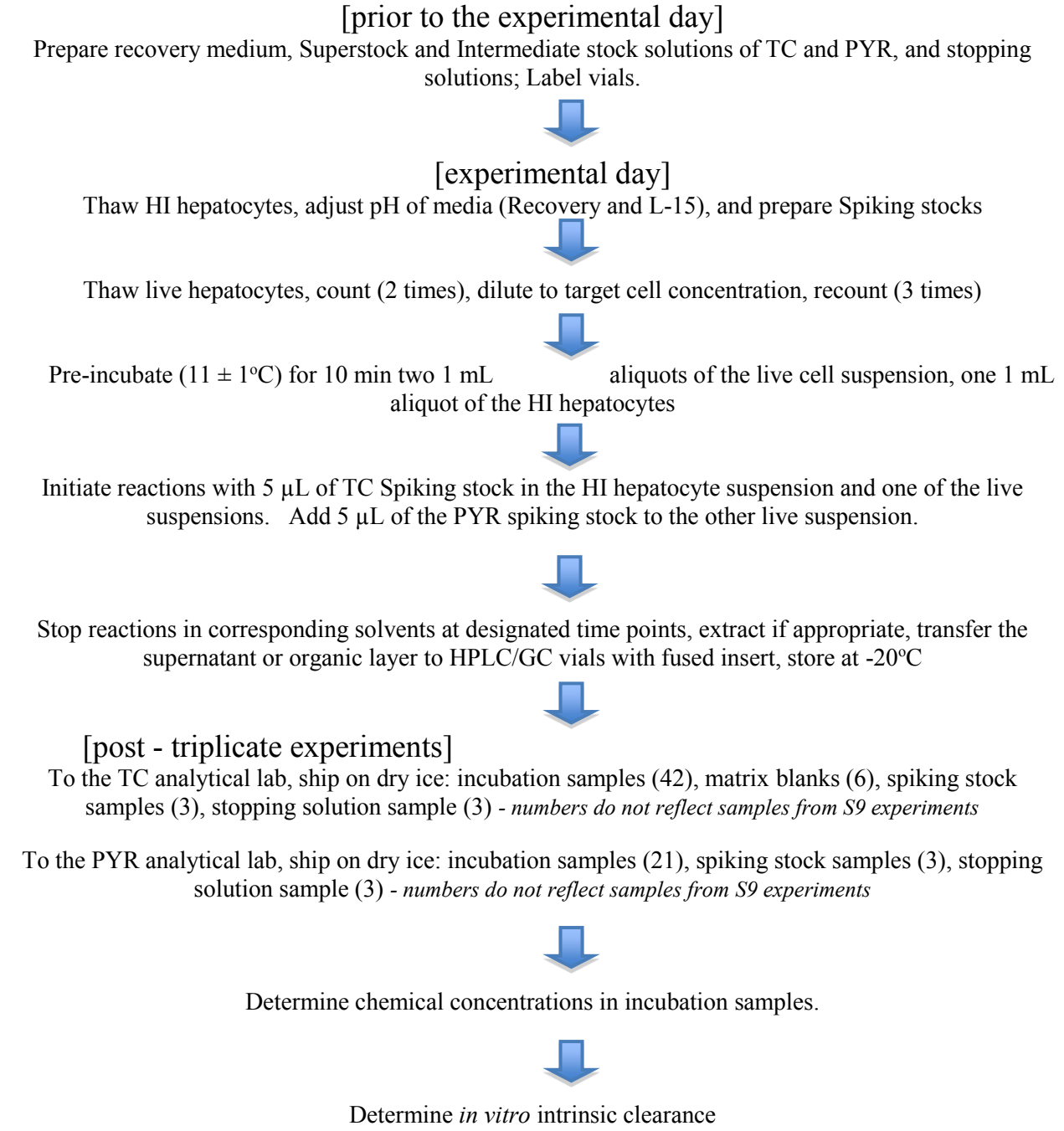
Gomez CF, 2011. Metabolism of three pharmaceuticals in rainbow trout and channel catfish cell suspensions and the effect on predicted bioconcentration. Chapter 4. Dissertation, University of North Texas.

Han, X., et al. (2007). "Determination of Xenobiotic Intrinsic Clearance in Freshly Isolated Hepatocytes from Rainbow Trout (Oncorhynchus mykiss) and Rat and Its Application in Bioaccumulation Assessment." Environmental Science and Technology 41: 3269-3276.

Mingoia, R. T., et al. (2010). "Cryopreserved Hepatocytes from Rainbow Trout (Oncorhynchus mykiss): A Validation Study to Support Their Application in Bioaccumulation Assessment." Environmental Science & Technology 44(8): 3052-3058.
Nabb, D. L., et al. (2006). "Comparison of basal level metabolic enzyme activities of freshly isolated hepatocytes from rainbow trout (Oncorhynchus mykiss) and rat." Aquatic Toxicology 80(1): 52-59.

ANNEX 1: EXPERIMENTAL SUMMARY

Fig. A1.1 Diagrammatic representation of the incubation procedure for the substrate depletion experiment using suspensions of trout cryopreserved hepatocytes.



.

Table A1.1 Study design and experimental details

Expt.	Chemical [1,2]	Cell Lot	Incubation conc (µM)	Cell conc (10^6 cells/mL)	Time points (min)	Stopping solution[3], volume (µL)	Internal standard[4]	Internal standard conc. (µM)	Extraction solvent, volume (µL)	Extraction procedure	Analytical laboratory[5]
pilot	PYR	1, HI	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA
1	4NP	5, HI		2	2,5,10,20,30,40,50	cold ACN, 500	4NP-d4	0.400		B	DUP
	PYR (+)	5	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA
2	FEN	4, HI				DCM, 400	FEN-d6			C	EPA
	PYR (+)	4	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA
3	CS	3, HI				cold MTBE, 400	ML		-	D	GIV
	PYR (+)	3	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA
4	MC	8, HI				DCM	MC-d6			E	FB
	PYR (+)	8	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA
5	DM	7, HI				cold ACN, 400	PM		DCM	F	DOW
	PYR (+)	7	0.025	2	2,5,10,20,30,40,50	cold ACN, 400	ANT	0.002	-	A	EPA

[1] + = reference chemical

[2]Test chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[3]Stopping solutions: ACN = acetonitrile, DCM = Dichloromethane (methylene chloride)

[4]Internal standards: ANT = anthracene, 4NP-d4 = 4-n-nonylphenol-d4, FEN-d6 = fenthion- d6, ML= methyl laurate, MC-d6 = methoxychlor–d6, PM= permethrin; details in Table A1.3

[5]Analytical laboratory: EPA = Environmental Protection Agency, DUP = DuPont, GIV = Givaudan, FB = Fraunhofer IME/ University of Bern, DOW = Dow Chemical; details in Table A1.4

Extraction procedures:

A – PYR: 100 µL will transferred from the reaction vial at each timepoint into Eppendorf tubes containing 400 µL 0.002 µM ANT in ACN. Vortex 10 min at 2300 rpm, and refrigerate overnight. Centrifuge all samples for 10 min at 20,000 × g, and transfer 300 µL of supernatant to a HPLC vial for shipment to EPA.

B- 4NP: 100 µL will transferred from the reaction vial at each timepoint to Eppendorf tubes containing 500 µL 0.4µM 4NP-d4 in ACN. Vortex for 10 min at 2300 rpm, and refrigerator overnight. Centrifuge all

samples for 10 min at 20,000 × g, and then transfer 180 µL of supernatant HPLC vial for shipment to DuPont.

For each sample, please save the remaining supernatant in separate set of vials, to be held at your lab.

Table A1.2 Test Chemical (TC) Details

TC	Chemical abbrev.	CAS No.	Supplier	Cat. No.	Lot No.	% Purity	MW (g/mol)
Pyrene	PYR	129-00-0	Sigma-Aldrich	185515	bcbk2867v	98.7	202.25
4-n-nonylphenol	4NP	104-40-5	Sigma-Aldrich	442873	lc07805v	99.9	220.35
Fenthion	FEN	55-38-9	Sigma-Aldrich	36552	szbc178xv	97.9	278.33
Cyclohexyl Salicylate	CS	25485-88-5	Givaudan	8819601	ve003164	99.8	220.26
Methoxychlor	MC	72-43-5	Sigma-Aldrich	49054	lc09014v	99.9	345.65
Deltamethrin	DM	52918-63-5	Sigma-Aldrich	45423	szbc059xv	99.6	505.20

Table A1.3 Internal Standard (I STD) Details

I STD	Chemical abbrev.	CAS No.	Supplier	Cat. No.	Lot No.	% Purity	MW (g/mol)
Anthracene	AN	120-12-7	Sigma-Aldrich	48567	lc10254v	99	178.23
4-n-nonylphenol-d4	4NP-d4	1173019-62-9	Sigma-Aldrich	614343	mbbb2035v	98	224.27
Fenthion- d6	FEN-d6	1189662-83-6	C/D/N Isotopes	D-6462	c126	97	284.36
Methyl laurate	ML						
Methoxychlor-d6	MC-d6	106031-79-2	C/D/N Isotopes	D-7030	e244	98.6	351.69
Permethrin	PM	52645-53-1	Sigma-Aldrich	45614	szbd142xv	98.1	391.29

Table A1.4 Test Chemical (TC) Stock preparations

TCl[1]	MW (g/mol)	Stock solvent[2]	Example TC mass (mg)	Example Superstock volume (mL)	Superstock conc (mM)	Dilution of Superstock[3]	Intermediate stock conc. (µM)	Dilution of Intermediate stock to Spiking Stock	Spiking stock conc. (µM)
PYR	202.25	AE	10.1	10.0	5.0	25 ×	200	40 ×	5.0
4NP	220.35	ACN	11.02	10.0	5.0	12.5 ×	--	--	400
FEN	278.33								
CS	220.26								
MC	345.65								
DM	505.20								

[1] Test (and Reference) chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[2] Solvents: AE = acetone, ACN = acetonitrile, DMSO = dimethyl sulfoxide, DCM = Dichloromethane (methylene chloride), MTBE = methyl-tert-butyl ether

[3] Some test chemical spiking stocks may require the preparation of an intermediate stock, others may be prepared by directly diluting a Superstock.

Table A1.5 Stopping solution with Internal Standard (I STD) preparation

TC[1]	I STD[2]	I STD MW (g/mol)	Stop. soln[3]	Example I STD mass (mg)	Example Superstock volume (mL)	Superstock I STD conc. (mM)	Dilution of Superstock	Interm. stock 1[4] conc (µM)	Dilution of Interm. Stock 1	Interm. Stock 2[4] conc. (µM)	Dilution of Interm. Stock 2	Stopping solution conc (µM)
PYR	ANT	178.23	ACN	8.91	50.0	1.0	50 ×	20	50 ×	0.4 µM	200 ×	0.002
4NP	4NP-d4	224.27	ACN	5.6	10.0	2.5	25 ×	100	250 ×	--	--	0.400
FEN	FEN-d6	284.36										
CS	ML											
MC	MC-d6	351.69										
DM	PM	391.29										

[1] Test (and Reference) chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[2] Internal standards: ANT = anthracene, 4NP-d4 = 4-n-nonylphenol-d4, FEN-d6 = fenthion- d6, ML= methyl laurate, MC-d6 = methoxychlor–d6, PM= permethrin. Details in Table A1.3

[3] Solvents: AE = acetone, ACN = acetonitrile, DMSO = dimethyl sulfoxide, DCM = Dichloromethane (methylene chloride), MTBE = methyl-tert-butyl ether

[4] Some test chemical spiking stocks may require the preparation of intermediate stocks, others may be prepared by directly diluting a Superstock.

Table A1.6. Example of time staggering.
In this example, the live hepatocyte suspension (HEP) was spiked with 4-n-nonylphenol (4NP) at time 0'0". The heat-inactivated hepatocyte suspension (HIHEP) was spiked with 4NP 30 seconds later (0'30"). The heat-inactivated suspension was sampled at each time point 30 seconds after the live suspension to maintain a consistent elapsed time.

Sample ID	Targeted Time (min' sec")	Clock Time (min'sec")	Sample ID	Targeted Time (min'sec")	Clock Time (min'sec")
EPA-4NP-01-HEP -1	2	2'00"	EPA-4NP-01-HIHEP -1	2	2'30"
EPA-4NP-01-HEP -2	5	5'00"	EPA-4NP-01-HIHEP -2	5	5'30"
EPA-4NP-01-HEP -3	10	10'00"	EPA-4NP-01-HIHEP -3	10	10'30"
EPA-4NP-01-HEP -4	20	20'00"	EPA-4NP-01-HIHEP -4	20	20'30"
EPA-4NP-01-HEP -5	30	30'00"	EPA-4NP-01-HIHEP -5	30	30'30"
EPA-4NP-01-HEP -6	40	40'00"	EPA-4NP-01-HIHEP -6	40	40'30"
EPA-4NP-01-HEP-7	50	50'00"	EPA-4NP-01-HIHEP-7	50	50'30"

Table A1.7 Analytical laboratory shipment information.

Analytical Laboratory	**Analyte**	**Address**	**Contact information**
EPA	PYR, FEN	**U.S. EPA** Mid-Continent Ecology Division 6201 Congdon Blvd, Duluth, MN 55804 USA	
DUP	4NP	**DuPont Haskell Global Centers for Health and Environmental Sciences** Building S315/lab room 1132 1090 Elkton Rd Newark, DE 19711 USA	
GIV	CS	**Givaudan Schweiz AG** Ueberlandstrasse 138 CH-8600 Dubendorf Switzerland	
DOW	DM	**Dow Chemical Company** 1803 Building, Door E, Lab 485 Midland, MI 48674 USA	
FB	MC	**Fraunhofer IME** Auf dem Aberg 1 57392 Schmallenberg Germany	

ANNEX 2: LABELING CONVENTIONS

Pre-printed labels (Direct Thermo Cryo-Tags; solvent-resistant printing, cyro-stable) will be provided to each laboratory. Labels will use the following codes:

Laboratory abbreviations (Field 1):
DOW - Dow Chemical Company
DUP - DuPont-Haskell Global Centers for Health and Environmental Sciences
EPA - U.S. Environmental Protection Agency ***** *the EPA is not anticipated to produce samples for the final dataset, but may be involved in some preliminary studies, troubleshooting, etc.*
FB - Fraunhofer IME/ University of Bern
GIV - Givaudan Schweiz AG
PG - Proctor & Gamble
SCK - SC Johnson and Son/ KJ Scientific

Chemical abbreviations (Field 2):
PYR - Pyrene[1]
4NP - 4-*n*- nonylphenol
CS - Cyclohexyl Salicylate
DM - Deltamethrin
FEN - Fenthion
MC - Methoxychlor

Biological material abbreviations (final Field)
HEP –hepatocyte in vitro experiment/ live cell experiment
HIHEP – Heat-inactivated cell experiment
S9 –S9 in vitro experiment/ active S9 experiment, *see S9 SOP*
HIS9- heat-inactivated S9 experiment, *see S9 SOP*

A2.1 SPIKING STOCK

(Aliquots to be sent to the corresponding analytical lab with incubation samples.)

For each experiment, researchers performing depletion experiments will provide to the analytical laboratory a sample of the spiking solution used to dose the reaction samples containing either live hepatocytes or heat inactivated hepatocytes. Spiking solutions for the test chemical as well as the reference chemical (PYR) will be prepared fresh the day of the experiment. Please label each spiking solution with the following convention, using the abbreviations provided below:

Field 1	Field 2	Field 3	Field 4	Field 5
originating lab	test chemical abbreviation[1]	experimental day (01, 02, or 03)	SPK	biological material (HEP, S9)

[1]Spiking stocks prepared for reactions with the PYR during a specific test chemical experiment will be labelled as above, but the field for the test chemical will be denoted as PYR, test chemical abbreviation.

Examples:

Note: The following are examples. Please modify according to your laboratory abbreviation and chemical:

EPA - 4NP - 02 - SPK- HEP denotes the sample originated at the EPA laboratory, contains 4NP, and was used in the second experimental day to spike suspensions of hepatocytes (live and HI).

EPA – PYR, 4NP - 02 - SPK- HEP denotes the sample originated at the EPA laboratory, contains pyrene, and was used as a spiking stock for the reference chemical during the second 4NP experimental day with hepatocytes (live and HI).

A2.2 STOPPING SOLUTION CONTAINING INTERNAL STANDARD

Reactions (see ANNEX 1) will be stopped using solvent containing an internal standard. For these experiments, samples of the stopping solution will be included in the shipment to the analytical laboratory for the corresponding test chemical. Solvent with internal standard may be made up for both S9 and HEP experiments or may be prepared separately for experiments with each biological material. The stopping solution will be labeled as follows:

Field 1	Field 2	Field 3	Field 4
originating lab	Corresponding test chemical abbreviation[1]	I STD	biological material (HEP, S9)[2]

[1]Stopping solution prepared for reactions with the reference chemical (PYR) during a specific test chemical experiment will be labelled as above, but the field for the TC will be denoted as PYR, TC abbreviation.

[2] If the same preparation of stopping solution is used for experiments with both Hepatocytes and S9 fraction, include both abbreviations in Field 4.

Example:

EPA- FEN- I STD – HEP, S9 denotes the sample originated at the EPA, contains stopping solution with the internal standard for fenthion experiments (fenthion- d6), and was used in both the Hepatocyte and S9 depletion experiments.

EPA-PYR, FEN-I STD – HEP denotes the sample originated at the EPA, contains stopping solution for the pyrene experiment run in parallel with fenthion depletions using hepatocytes (i.e, acetonitrile with 0.002 µM anthracene).

A2.3 INCUBATION SAMPLES

(stopped reactions from each time point)

Researchers performing depletion experiments will remove 7 aliquots from the reaction sample at pre-determined time points, generating 7 subsamples for chemical analysis per reaction. These depletion experiments will be performed for each test chemical as singlet assays over three separate experimental days for both live and heat-inactivated hepatocyte in vitro systems. Each live hepatocyte suspension prepared for an experimental day will also be tested with PYR as a reference chemical/ potential benchmark chemical in a separate vial. Incubation samples for each test chemical will be labeled with the following fields:

Field 1	Field 2	Field 3	Field 4	Field 5
originating lab	test chemical abbreviation[1]	experimental day (01, 02, or 03)	biological material (HEP or HIHEP)	time point (1-7)

Samples obtained from reactions with the reference chemical (PYR) during a specific test chemical experiment will be labelled as above, but the field for the test chemical will be denoted as PYR, test chemical abbreviation.

Note: The following is an example. Please modify according to your laboratory abbreviation and chemical:

Experiment 1 (01)

Labels for active hepatocytes, test chemical 4NP	Labels for heat-inactivated hepatocytes, test chemical 4NP	Labels for the reference chemical (PYR) samples, conducted during 4NP depletions
EPA-4NP-01-HEP-1	EPA-4NP-01-HIHEP-1	EPA-PYR, 4NP-01-HEP-1
EPA-4NP-01-HEP-2	EPA-4NP-01-HIHEP-2	EPA-PYR, 4NP-01-HEP-2
EPA-4NP-01-HEP-3	EPA-4NP-01-HIHEP-3	EPA-PYR, 4NP-01-HEP-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Experiment 2 (02)

Labels for active hepatocytes, test chemical 4NP	Labels for heat-inactivated hepatocytes, test chemical 4NP	Labels for the reference chemical (PYR) samples, conducted during 4NP depletions
EPA-4NP-02-HEP-1	EPA-4NP-02-HIHEP-1	EPA-PYR, 4NP-02-HEP-1
EPA-4NP-02-HEP-2	EPA-4NP-02-HIHEP-2	EPA-PYR, 4NP-02-HEP-2
EPA-4NP-02-HEP-3	EPA-4NP-02-HIHEP-3	EPA-PYR, 4NP-02-HEP-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Experiment 3 (03)

Labels for active hepatocytes, test chemical 4NP	Labels for heat-inactivated hepatocytes, test chemical 4NP	Labels for the reference chemical (PYR) samples, conducted during 4NP depletions
EPA-4NP-03-HEP-1	EPA-4NP-03-HIHEP-1	EPA- PYR, 4NP-03-HEP-1
EPA-4NP-03-HEP-2	EPA-4NP-03-HIHEP-2	EPA- PYR, 4NP-03-HEP-2
EPA-4NP-03-HEP-3	EPA-4NP-03-HIHEP-3	EPA- PYR, 4NP-03-HEP-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Laboratories may choose to prepare and assay S9 preparations along with hepatocyte preparations on the same day for a given test chemical. Similar labels would contain the term S9 or HIS9 in place of HEP/ HIHEP. See the SOP for S9 depletions.

A2.4 MATRIX BLANKS

Researchers performing depletion experiments will prepare 2 matrix blanks (designated MTX) each experimental day for both HEP and HIHEP. Matrix blanks will be prepared as an incubation sample without TC or internal standards in the stopping solution. Samples will be shipped to the TC analytical lab. Matrix blanks for the PYR samples will not be prepared except in the pilot experiment.

Matrix blank samples for each test chemical will be labeled with the following fields:

Field 1	Field 2	Field 3	Field 4	Field 5
originating lab	test chemical abbreviation	experimental day (01, 02, or 03)	MTX	biological material (HEP, HIPEP)

Examples:

Note: The following are examples. Please modify according to your laboratory abbreviation and chemical:

EPA - 4NP - 02 – MTX - HEP denotes the sample is a matrix blank originated at the EPA laboratory, corresponding to the second 4NP experimental day, prepared with the post-dilution suspension of live hepatocytes. Field 2 (4NP) does NOT denote the presence of 4NP, but identifies the experimental day.

EPA –4NP - 02 – MTX- HIHEP denotes the sample is a matrix blank originated at the EPA laboratory, used in the second 4NP experimental day using heat-inactivated hepatocytes. Field 2 (4NP) does NOT denote the presence of 4NP, but identifies the experimental day.

ANNEX 3: WORKSHEETS

A3.1 GENERAL WORKSHEETS

RECORD OF REAGENTS, CHEMICALS AND INSTRUMENTS

RECOVERY MEDIUM PREPARATION

REFERENCE CHEMICAL STOCK PREPARATION

A3.2 4-*N*- NONYL PHENOL (4NP)

TEST CHEMICAL STOCK PREPARATION

STOPPING SOLUTION WITH INTERNAL STANDARD PREPARATION

PRE-DILUTION CELL COUNTING

POST-DILUTION CELL COUNTING

CELL RECOVERY

CLEARANCE ASSAY

A3.1 GENERAL WORKSHEETS

RECORD OF REAGENTS, CHEMICALS AND INSTRUMENTS
RECOVERY MEDIUM PREPARATION
REFERENCE CHEMICAL STOCK PREPARATION

RECORD OF REAGENTS, CHEMICALS AND INSTRUMENTS

Instruments, reagents and chemicals used from _____ to _____

Reagent/Chemical Name	Supplier	Catalog number	Lot number	Expiration Date
DMEM	Gibco	12320-032 (US) or 22320-022 (Europe)		
FBS				
BSA				
L-15	Gibco	21083		
Sodium Hydroxide				
Hydrochloric Acid				
Trypan Blue	Sigma	T8154		
Acetonitrile				
Methylene Chloride				
Methyl tert-butyl ether				

Equipment	Model	ID number	Notes
Balance			
Centrifuge 1			
Centrifuge 2			
pH meter			
Freezer			
Refrigerator			
Incubation equipment			
Compound microscope			
Vortex mixer			
Cryogenic storage			

MEDIA PREPARATION

Lab: _____ Test Chemical: _____ Date: _____ Initials: _____ HEP

Preparation of Recovery Medium

Refer to Sections 7.1 in the Standard Operation Procedure.

Reagent	Per 170 mL prep:	actual
DMEM	153 mL	
FBS	17 mL	
BSA	0.425 g	

Experimental Day pH adjustment Media Preparation

Refer to Section 7.2 in the Standard Operation Procedure.

Experimental Date	Researcher initials	Recovery Medium (55 mL)		L-15 (150 mL)	
		pH	Temperature (°C)	pH	Temperature (°C)

Comments:

A3.2 4NP WORKSHEETS

TEST CHEMICAL STOCK PREPARATION

STOPPING SOLUTION WITH INTERNAL STANDARD PREPARATION

PRE-DILUTION CELL COUNTING

POST-DILUTION CELL COUNTING

CELL RECOVERY

CLEARANCE ASSAY

SUPERSTOCK AND INTERMEDIATE STOCK PREPARATIONS

Lab: _____Test Chemical: __4NP___Date:_____ Initials: _____ S9 / HEP (circle)

Preparation of 4-n-nonylphenol (4NP) stocks

Refer to Section 7.3 and ANNEX 1, Table A1.4.

Stock, abrev.	Mass 4NP (mg)	ACN Volume (mL)	Stock Conc. (mM), *Target = ___5mM___* = 1000 * mg TC/ 220.35[1]/ mL solvent
Example	11.02	10	5 mM
Actual Superstock, SS			

[1] MW = molecular weight of test chemical

Comments:

Preparation of PYRENE (PYR) stocks

Refer to section 7.1 and ANNEX 1, Table A1.4.

Stock, abrev.	Mass PYR (mg)	Acetone Volume (mL)	Stock Conc. (mM), *Target = 5.0 mM* = 1000* mg PYR/ 202.25 / mL acetone
Example	10.1	10.0	5 mM
Superstock, SS			

Stock, abbrev.	Volume SS	Total volume acetone (mL)	Stock Conc. (uM), *Target = 200 µM* = µM SS * mL SS / total mL
Example	400 µL	10.0	200 µM
Intermediate, IMS			

Comments:

SPIKING STOCK PREPARATIONS

Lab: _____Test Chemical: ___4NP___ Initials: _____ S9 / HEP (circle)

Remove 1 mL aliquots of the final Spiking stocks for both the test chemical and PYR. Preserve in the standard 1.5 mL HPLC/GC sample vial for shipping with the incubation samples.

Preparation of test chemical (4NP) Spiking Stocks (SPK)

Refer to Section 7.2 and ANNEX 1, Table A1.5.

Date Superstock (SS)/Intermediate stock (IMS) prepared: _____Diluting solvent:____ACN____

Date	Experimental day	Volume 4NP SS	Total volume solvent (mL)	Spiking stock Conc. (µM) = µM IMS* mL SS or IMS/ total mL
	Example	0.40	5	400 µM
	01			
	02			
	03			

Comments:

Preparation of Reference chemical (PYR) Spiking Stocks (SPK)

Refer to section 7.2 and ANNEX 1, Table A1.5.

Date Superstock (SS)/Intermediate stock (IMS) prepared: _____

Date	Experimental day	Volume IMS (mL)	Total volume acetone (mL)	Spiking stock Conc. (µM) = µM IMS* mL IMS/ total mL
	Example	0.25	10.0	5 µM
	01			
	02			
	03			

Comments:

STOPPING SOLUTION WITH INTERNAL STANDARD PREPARATION

Lab: _____Test Chemical: ___4NP_____ Initials: _____ S9 / HEP (circle)

Remove three 1 mL aliquots of both the test chemical stopping solution and the PYR stopping solution to1.5 mL standard HPLC/GC vials for shipping with the incubation samples.

Internal standard (4NP): ____**4NP-d4**_____ MW (g/mol):_____224.27___ Solvent: _____ACN___

Internal standard (PYR): ____**ANT**_____ MW (g/mol):_____178.23___ Solvent: _____ACN___

Preparation of 4-*n*-nonylphenol (4NP) stopping solution containing 0.4 µM 4NP-d4 in ACN

Refer to Section 7.3.1 and ANNEX 1, Table A1.5.

	Amount Internal standard (mg)	Final ACN volume for Superstock (SS) (mL)	Added vol. SS to IMS (mL)	Final ACN vol. of IMS (mL)	Added volume IMS (mL)	Final ACN volume for Stopping solution (mL)	Final conc Stopping solution
Example	5.6	10.0	0.40	10.0	1.0	250	0.400 µM
Actual							

Preparation of Reference chemical (PYR) stopping solution containing 0.002 µM ANT in ACN

Refer to Section 7.3.2 and ANNEX 1, Table A1.5.

	Amount Internal standard (mg)	Final ACN vol. for Superstock (SS) (mL)	Added vol. of SS to IMS 1 (mL)	Final vol. IMS 1 (mL)	Added vol. of IMS 1 to IMS 2 (mL)	Final vol. IMS 2 (mL)	Added vol. of IMS 2 to Stopping Soln. (mL)	Final vol. Stopping soln (mL)	Final conc Stopping solution
Example	8.91	50.0	1.0	50.0	1.0	50.0	1.0	200	0.002 µM
Actual									

Comments:

PRE-DILUTION CELL COUNT WORKSHEET

Lab: _____Test Chemical: _____Experimental Day: ____ Date: _____ Initials: _____

Hepatocyte Batch ID:	
Number of vials thawed:	
Time of removal from liq N_2	

Refer to Section 8.2.3

Calculations:
(total live count/total cell count) x 100 = % Viable
(total live count/# of fields counted (4)) x dilution factor (6) x 10,000 = Concentration, cells/mL

Cell counts

Replicate 1

Dilution factor:	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration, cells/mL			

Replicate 2

Dilution factor:	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration, cells/mL			

Final Average

	Rep 1	Rep 2	Ave
% viable			
Concentration, cells/mL			

Comments:

POST-DILUTION CELL COUNT WORKSHEET

Lab: _____Test Chemical: _____Experimental Day: ____ Date: _____ Initials: _____

Hepatocyte Batch ID:	
Number of vials thawed:	

Refer to Section 8.2.3

Calculations:
(total live count/total cell count) x 100 = % Viable
(total live count/# of fields counted (4)) x dilution factor x 10,000 = Concentration, cells/mL

Cell counts

Replicate 1

Dilution factor:	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration, cells/mL			

Replicate 2

Dilution factor:	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration, cells/mL			

Replicate 3

Dilution factor:	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration, cells/mL			

Final Average

	Rep 1	Rep 2	Rep 3	Ave
% viable				
Concentration, cells/mL				
			% CV	

If the variability of the 3 average cell counts > 20 % CV, perform additional dilutions/counts as needed.

Comments:

CELL RECOVERY AND SUSPENSION DILUTION WORKSHEET

A	B	C	D	E	F	G
Date	Experimental Day	No. vials thawed	Volume of suspension pre - cell counts (mL)	Average live cell concentration (10^6 cells/mL)	Total No. Recovered (live) cells (10^6 cells)	% Cell recovery
	01					
	02					
	03					
				Pre-dilution cell count sheet, Final average	D*E	100* F/(C*15)

Lab: _____Test Chemical: __4NP__Cell Lot: _____ Initials: _____ Thaw time: _____

Refer to Section 8.2.3

Table 1: Cell recovery

Column F: **Total number of recovered (live) hepatocytes** = (average viable cell concentration * suspension volume **prior** to cell counting)

Column G: **% Cell Recovery** = 100 × (number of recovered hepatocytes/ number of viable cells initially frozen)

Table 2: Suspension dilution

A	B	C	D	E	F	G
Date	Experimental Day	Volume of suspension post- cell counts (mL)	Average live cell concentration (10^6 cells/mL)	Desired live cell concentration (10^6 cells/mL)	Final volume of suspension (mL)	Volume to add to suspension (mL)
	01			2.0×10^6		
	02			2.0×10^6		
	03			2.0×10^6		
			Table 1, column E	2.0×10^6	(D/E)*C	F-C

Column F: **Final volume** = (average live cell concentration / desired live cell concentration)* suspension volume **after** cell counts

Column G: **Total volume to add to the suspension** = Final volume – Post-cell count volume

Comments:

HEPATOCYTE CLEARANCE ASSAY WORKSHEET
Lab: _____Test Chemical: __4NP___Experimental Day: ____ Date: _____ Initials: _____

Test Chemical Information:

Chemical Name	4-*n*- nonylphenol (4NP)	PYR (reference chemical)
Chemical MW (g/mol)	220.35	202.25
Chemical supplier, cat. #	Sigma Aldrich	Sigma-Aldrich, 185515
Lot #	lc07805v	bcbk2867v
Spiking stock solvent	acetonitrile	acetone
Spiking Stock Concentration	400 µM	5 µM
Incubation Concentration	2 µM	0.025 µM
Stop Solvent	acetonitrile	acetonitrile
Internal Standard (I STD)	4NP-d4	ANT
I STD Supplier, cat. #	Sigma Aldrich, 614343	Sigma-Aldrich, 48567
I STD Lot #	mbbb2035v	lc10254v
I STD Stock Concentration	0.4 µM	0.0031 µM

Experimental Conditions:

	4NP, HEP	4NP, HIHEP	PYR, HEP
Live cell lot #	5	Not applicable	5
Cell Concentration	2×10^6 cells/mL	2×10^6 cells/mL	2×10^6 cells/mL
Reaction Vessel	7-mL scintillation vial, loosely capped	7-mL scintillation vial, loosely capped	7-mL scintillation vial, loosely capped
Reaction Temperature	11°C	11°C	11°C
Replicates	One reaction	One reaction	One reaction
Time Points	2,5,10,20,30, 40,50	2,5,10,20,30, 40,50	2,5,10,20,30, 40,50
Reaction Buffer	L-15 medium	L-15 Medium	L-15 medium
Reaction Volume	1000 µL	1000 µL	1000 µL
Dose Vehicle	acetonitrile	acetonitrile	acetone
Dose Volume	5 µL	5 µL	5 µL
Reaction Stop Volume	100 µL	100 µL	100 µL
Stop Solution Volume	500 µL	500 µL	400 µL
Extraction Solvent	N/A	N/A	Not applicable

Extraction Solvent Volume	N/A	N/A	Note applicable
Final Transfer volume to ship	180 µL	180 µL	300 µL

Lab: _____Test Chemical: ____4NP__Experimental Day: ____ Date: _____ Initials: _____

Sample Pre-incubation time _____min Start time _____

Elapsed time from Thawing to Start (see Cell Recovery Worksheet) _____

Refer to Section 8.2.4.

	Sample ID	Targeted time (min)	Actual Time, if different	Notes
PYR, HEP		2		
		5		
		10		
		20		
		30		
		40		
		50		
4NP, HEP		2		
		5		
		10		
		20		
		30		
		40		
		50		
4NP, HIHEP		2		
		5		
		10		
		20		
		30		
		40		
		50		

PYR: 100 µL will transferred from the reaction vial at each timepoint into Eppendorf tubes containing 400 µL 0.002 µM ANT in ACN. Vortex 10 min at 2300 rpm, and refrigerate overnight. Centrifuge all samples for 10 min at 20,000 × g, and transfer 300 µL of supernatant to a HPLC vial for shipment to EPA.

4NP: 100 µL will transferred from the reaction vial at each timepoint to Eppendorf tubes containing 500 µL 0.4µM 4NP-d4 in ACN. Vortex for 10 min at 2300 rpm, and refrigerator overnight. Centrifuge all samples for 10 min at 20,000 × g, and then transfer 180 µL of supernatant HPLC vial for shipment to DuPont. **For each sample, please save the remaining supernatant in separate set of vials, to be held at your lab.**

Comments:

ANNEX 6: RT-S9 SOP

Trout S9 substrate depletion assay Standard Operation Procedure

for the OECD Project 3.13 "New TG *In Vitro* Fish Hepatic Metabolism"

Version 1.1

1. PURPOSE

This procedure describes the experimental conditions and steps for incubation of a test chemical with enzymatically-active liver S9 fraction isolated from rainbow trout to estimate the *in vitro* intrinsic clearance of the parent compound.

2. PRINCIPLE

In vitro determination of metabolic stability

Metabolic stability experiments are conducted using a substrate depletion approach wherein the biotransformation rate is determined by measuring the disappearance of parent chemical from the reaction mixture. The incubation system consists of thawed, active S9 fraction prepared from rainbow trout (*Oncorhynchus mykiss*) liver in buffer supplemented with enzymatic cofactors and alamethicin. Simultaneous incubations conducted using heat-inactivated S9 fraction are used to distinguish between enzymatic biotransformation and other potential loss processes including abiotic degradation, volatilization, and adsorption to the reaction vessel. Each reaction is performed at a constant temperature corresponding to the acclimatization temperature of the source fish. The substrate depletion reaction is initiated by the addition of test compound to the incubation system. A sufficient number of sampling time points are obtained to develop a high-quality regression of log-transformed chemical concentration data. The slope of this log-linear depletion is normalized to the protein content to provide an *in vitro* intrinsic clearance.

Preliminary incubations were performed to establish reaction conditions and sampling time points appropriate to a given compound. Important variables include the starting concentration of test compound, the protein concentration, and the total reaction run time. The trout liver S9 fraction was characterized using standard substrates for one or more Phase I and II metabolic reactions. Analytical methods for the test substance also were developed and validated before conducting any substrate depletion studies. The analytical methods were demonstrated to have sufficient sensitivity to detect the decreased concentration of analyte due to biotransformation.

3. HEALTH PRECAUTIONS

S9 fraction should be handled with caution, and treated as if there is a potential presence of infectious agents. Wear appropriate laboratory coat, gloves and eye protection during all laboratory operations. Use caution when working with organic solvents and test chemicals. Read the appropriate Material Safety Data Sheet (MSDS) for each test chemical and solvent, and handle solvents in the fume hood when possible. Follow additional in-house safety guidelines.

4. LIST OF TERMS

4.1 DEFINITIONS

Reference Chemical	A chemical known to be biotransformed in vitro by trout liver S9 fraction.
Heat-inactivated S9 fraction	Liver S9 fraction isolated from homogenized trout livers and enzymatically inactivated by boiling.

4.2 ABBREVIATIONS

ACN	Acetonitrile
AE	Acetone
ANT	Anthracene
CS	Cyclohexyl salicylate
DCM	Dichloromethane (methylene chloride)
DM	Deltamethrin
FEN	Fenthion
HI	Heat-inactivated
HIS9	Heat-inactivated trout liver S9 fraction
IMS	Intermediate stock
I STD	Internal standard
K-PO4	100 mM potassium phosphate
MC	Methoxychlor
ML	Methyl laurate
MSDS	Material Safety Data Sheet
MTBE	Methyl tert-butyl ether
MTX	Matrix blank
PM	Permethrin
PYR	Pyrene
S9	Trout liver S9 fraction
SOP	Standard Operation Procedure
SS	Superstock
TBD	To Be Determined
TC	Test Chemical
4NP	4-n-nonylphenol

5. STUDY DESIGN

Active and heat-inactivated S9 will be sampled from individual runs over three different days for each test chemical. Using separate vials of the same prepared solution of S9 in 100 mM phosphate buffer with cofactor supplementation, depletions of a reference chemical (pyrene; PYR) will be run in parallel with (or just prior to) the test chemical. Depletions of PYR using heat-inactivated S9 will only be conducted in the pilot experiment. A generalized study design is provided in Table 1. Specific details regarding the order in which test chemicals will be sampled and experimental specifics are provided in ANNEX 1.

Table 1. Study design for the assessment of one test chemical (X)

Experimental day	Substrate depletion experiment with active S9 fraction		Substrate depletion experiment with HIS9 fraction
1	test chemical X	reference chemical (PYR)	test chemical X
2	test chemical X	reference chemical (PYR)	test chemical X
3	test chemical X	reference chemical (PYR)	test chemical X

6. MATERIALS AND EQUIPMENT

*Note: We recommend specific brands of equipment and reagents in some cases; however**, equivalent equipment or reagents may be substituted if not stated otherwise**. It is the researcher's responsibility to determine the substitute suitable for a particular application. Items that are mandatory for use are designated as such. Equipment and specific lot and expiration data for reagents may be recorded on the Record of Reagents, Chemicals and Instruments (ANNEX 3). Deviations during the experiment may be recorded in the "Comments" sections of other worksheets.*

6.1 EQUIPMENT AND LABWARE

6.1.1 Fixed Equipment

- Analytical balance for mg quantities
- Vortex mixer, Thermo Scientific* MaxiMix/Vortex Mixer (cat. no. 12-815-50)
- Sample incubation equipment. Common options include:
  - Shaking water bath with chiller, VWR water bath shaker 18 L (cat. no. 89032-226) with VWR AD 7 L rfg/htg circulator, SS, 120V (cat. no. 89202-970)
  - Shaking incubator with heating and cooling functions, Denville Scientific (cat. no. S2085-HC)
  - Thermomixer block with shaking capabilities, Ditabis Model MKR 23 230 V for Europe (cat. no. 980523001); Jade Scientific 115 V for USA (cat. no. 98021150)
- Refrigerated centrifuge, Thermo Scientific IEC Centra GP8R (cat. no. 29530)
- Small benchtop refrigerated centrifuge for microfuge tubes, USA Scientific Eppendorf (cat. no. 22620601)

- pH meter, Fisher Scientific Accumet AB150 pH meter (cat no.13-636-AB150A)
- 4°C refrigerator, LABRepCo (cat. no. LABL-23-SD)
- -20°C freezer, LABRepCo (cat. no. LABH-14-FA)
- -80°C freezer, LABRepCo (cat. no. ULT390-10-A)

6.1.2 Labware

- Glassware for making up and storing chemical solutions,
- Pipetman 10, 100, 200 and 1000 µL, Daigger (cat. nos. EF9930B, EF9930D, EF9930E, EF9930F, respectively)
- Pipette tips, 0.1-10, 0.5-200, 100 -1000 µL, Daigger (cat. nos. EF2033B, EF2036B, EF2037B, respectively)
- Serological pipets, 10, 25 mL, Sigma-Aldrich (cat. nos. CLS4100, CLS4250, respectively) - optional
- Portable Pipet-aid, Daigger, Drummond Scientific (cat. no. EF20391E) - optional
- 7 mL scintillation vials, Perkin Elmer (cat. no. 6000167) OR Cole Parmer, Kimble solvent saver (cat. no. EW-08918-14) OR VWR Wheaton (Cat. no. 986492 Europe; note that these are described as 6 mL scintillation vials but they have the exact dimensions as the above 7 mL vials in the USA) – ***mandatory***
- Holder for scintillation vials, Thomas Scientific (cat. no. 9720D10) - optional
- 1.5 mL microcentrifuge tubes, Sigma-Aldrich, Eppendorf Safe-Lock (cat. no. T9661)
- Sample vials with fused inserts (300 µL), Chromocol (cat. no 03-FISV(A)) or Waters (186001126c) – ***mandatory***
- Sample vials (1.5 mL), Agilent (cat. no. 5182-0715)
- Sample vial screw caps (for 1.5 mL standard sample vials, above), Agilent (cat. no. 5182-0717)
- Eppendorf Repeater® Plus pipette, Eppendorf, Fisher (cat. no. 022260201) -optional
- Combitips for Repeater® Plus pipette) for 0.2 mL volume, Eppendorf (cat no. 022266004) - optional
- Timer, Sigma-Aldrich (cat. no. 22754-U)
- Parafilm, Sigma-Aldrich (cat. no. P7793)
- Microcentrifuge tube rack, Sigma-Aldrich (cat. no. R5651)
- Spatulas for weighing chemicals

6.2 CHEMICALS

6.2.1 Cofactors, alamethicin, and salts

- Nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH), Oriental Yeast Co. (cat. no. 44332900); OR Enzo Life Sciences (cat. no. 480-004-G001)
- Uridine 5'-diphosphoglucuronic acid, trisodium salt (UDPGA), Sigma–Aldrich (cat. no. U6751)
- L-Glutathione reduced (GSH), Sigma (cat. no. G6529)

- Adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS), Sigma (cat. no. A1651) or EMD Millepore (cat.no. 118410)
- Alamethicin from Trichoderma viride, Sigma-Aldrich (cat. no. A4665)
- Potassium phosphate dibasic (K_2HPO_4), Sigma-Aldrich (cat. no. 60353)
- Potassium phosphate monobasic (KH_2PO_4), Sigma-Aldrich (cat. no. P5655)

6.2.2 Test chemicals, internal standards and solvents

- Test Chemicals and reference chemical (***mandatory***; details in ANNEX 1, Table A1.1)
- Internal standards (***mandatory***; details in ANNEX 1, Table A1.1)
- Stopping and extraction solvents (e.g. acetonitrile, methylene chloride; ANNEX 1 Table A1.1)
- Solvents to dissolve test chemicals and alamethicin (e.g. methanol, acetone, acetonitrile; ANNEX 1, Table A1.4)

6.3 BIOLOGICAL MATERIAL

- Frozen trout liver S9 fraction, EPA lots 1, 2, 6, 8, 9, 10, 11. Each lot was pooled from 3 fish (*Oncorhynchus mykiss*, Erwin strain, 330 ± 46 g, mixed gender, sexually immature), frozen as 150 µL, 21.0 - 24.4 mg S9/mL. Fish were fed a commercial trout chow (Silver Cup; Nelson and Sons Inc, Murray, UT) and held on a 16:8 light: dark photo period at 11 ± 1 °C. All animals were fasted 24 h prior to use. Specific lots of S9 designated for use with each test chemical are provided in ANNEX 1, Table A1.1.
- Heat-inactivated (HI) trout liver S9 fraction (HIS9), EPA. Trout liver S9 fraction was diluted 1:1 (2×) with 100 mM phosphate buffer and boiled for 15 min in a 100°C water bath. The final volume of the HIS9 was adjusted by addition of 100 mM phosphate buffer to maintain the initial concentration of biological material. The final protein concentration was determined to be 12.3 mg S9 protein/mL.

7. SOLUTIONS PREPARATION

Refer to the Certificate of Analysis and MSDS for each test chemical and reagent to determine appropriate storage and handling conditions. The purity of the cofactors and test compounds are critically important and should be taken into account when calculating the mass needed to create solutions of a specific concentration. In general, adjustments are not recommended if the purity is > 95%. All cofactors should be of sufficient purity so that adjustments are not required, with the exception of PAPS. For this study, all test chemicals and internal standards are of sufficient purity (ANNEX 1, Tables A1.2 and A1.3) so that adjustments are not required. Some of the stock solutions may be prepared in advance, or reagents weighed out prior to the experimental day.

7.1 PHOSPHATE BUFFERS

Note: Use Milli-Q-purified, ultrapure water, or equivalent in all potassium phosphate recipes.

7.1.1 Potassium phosphate dibasic buffer, 100 mM

- Transfer 1.742 g potassium phosphate dibasic (K_2HPO_4) to a 100-ml volumetric flask and bring up to volume with ultrapure water. Store at 4° C.
- Prepare fresh monthly and discard if visibly contaminated.

7.1.2 Potassium phosphate monobasic buffer, 100 mM

- Transfer 0.681 g potassium phosphate monobasic (KH_2PO_4) to a 50 mL volumetric flask and bring up to volume with ultrapure water. Store at 4 °C.
- Prepare fresh monthly, and discard if visibly contaminated.

7.1.3 Potassium phosphate buffer, pH 7.8, 100 mM (K-PO4 buffer)

- Mix together 100 mM potassium phosphate dibasic (see recipe, above) and 100 mM potassium phosphate monobasic (see recipe, above) to achieve a pH of 7.8 at 11°C.

  For example, to create 100 mL of buffer, combine 88 mL of 100 mM potassium phosphate dibasic with 12 mL of 100 mM potassium phosphate monobasic.
- Add additional potassium phosphate dibasic (base) or potassium phosphate monobasic (acid) to adjust the pH. Store at 4° C.
- Prepare fresh monthly. Discard if visibly contaminated.

7.2 ALAMETHICIN

7.2.1 Alamethicin stock solution in methanol, 10 mg/mL

- Add 0.1 mL of methanol per milligram of alamethicin creating a 10 mg/mL solution.
- Recap the vial, vortex, and store as 25 µL aliquots of stock solution in microcentrifuge tubes (e.g., 1.5 mL Eppendorf) at -20°C until use.

7.2.2 Alamethicin working solution in K-PO4 buffer, 250 µg/mL.

- The day of the experiment, dilute one 25 µL aliquot of 10 mg/mL stock solution with 975 µL K-PO_4 buffer (7.1.3) for a final concentration of 250 µg/mL.

7.3 COFACTORS

All reaction co-factors are provided in substantial excess of amounts needed to support Phase I and II metabolic pathways. Stated molar concentration values are approximate.

7.3.1 NADPH, ~20 mM

Note: NADPH may be pre-weighed prior to the experiment and stored at −20 °C. Record weight on vials.

- Dissolve 16.67 mg of β-nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH) in 1 mL of ice-cold K-PO_4 buffer (7.1.3) and vortex until completely dissolved.
- This solution should be prepared fresh on the day of the experiment and stored on ice.

7.3.2 UDPGA, ~20 mM

Note: UDPGA may be pre-weighed prior to the experiment and stored at −20 °C. Record weight on vials.

- Dissolve 12.93 mg of uridine 5' -diphosphoglucuronic acid, trisodium salt (UDPGA) in in 1 mL of ice-cold K-PO_4 buffer (7.1.3) and vortex until completely dissolved.
- This solution should be prepared fresh on the day of the experiment and stored on ice.

7.3.3 GSH, ~ 50 mM

Note: GSH may be pre-weighed prior to the experiment and stored at −20 °C. Record weight on vials.

- Dissolve 15.37 mg of L-glutathione (GSH) in 1 mL of ice-cold K-PO_4 (7.1.3) buffer and vortex until completely dissolved.
- This solution should be prepared fresh on the day of the experiment and stored on ice.

7.3.4 PAPS, pH 8.0, 10 mM

The purity of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) will likely be < 95%, requiring adjustment for solution preparation. Because of the considerable expense of this cofactor, we recommend making up one concentrated solution (10 mM) prior to the experimental day and freezing it as 50 µL aliquots. On the day of the experiment, one aliquot may be thawed and diluted to the working concentration (1 mM).

- Calculate the amount of chemical required to prepare a 10 mM PAPS solution. An accurate adjustment for chemical purity would take into account the lot-specific information of the purity of the anhydrous acid, % water, % lithium and % solvent. However, for the purposes here, a simple adjustment for purity of anhydrous acid and water content will be sufficient.

  Chemical mass × anhydrous acid content × (1- water content)/ 507.26 g/mol = mol anhydrous acid

  Volume of K-PO_4 buffer (mL) to add = (mmol of anhydrous acid/ 10 mM) × 1000 mL/L

  Example:

  10 mg chemical
  65 % pure anhydrous acid
  14.3 % water
  Molecular Weight of anhydrous acid = 507.26 g/mol

  10 mg × 0.65 × (1-0.143)/ 507.26 mg/mmol = 0.01098 mmol anhydrous acid
  (0.01098 mmol anhydrous acid/ 10 mmol/L) × 1000 mL/L = 1.098 mL

- Adjust a small volume (e.g., 25 mL) of ice-cold K-PO_4 buffer to pH 8.0 by addition of dibasic potassium phosphate solution (7.1.1)
- Pre-chill microfuge tubes (e.g., -20°C for 10 min).
- Weigh PAPS in a pre-chilled microfuge tube and dissolve in ice-cold **pH 8** K- PO_4 buffer (PAPS is most stable when frozen at pH 8).
- Aliquot 50 µL each into pre-chilled microfuge tubes and immediately freeze at -80°C.

7.4 MASTER MIX

- Just prior to the experiment (8.2.1, step 12), dissolve each of the pre-weighed cofactors (NADPH, UDPGA, GSH; Section 7.3) in 1.0 mL of K- PO_4 buffer (7.1.3). The resulting concentrations are 20 mM NADPH, 20 mM UDPGA, and 50 mM GSH, respectively. Vortex and store on ice.
- Dilute 50 µL of pre-frozen 10 mM PAPS solution with 450 µL of K- PO_4 buffer (7.1.3) to make a 1 mM PAPS solution. **Prepare immediately before use**.
- Combine the following and vortex to create the Master Mix (total volume = 2.0 mL):
  - 500 µL of 20 mM NADPH

- 500 µL of 20 mM UDPGA
- 500 µL of 50 mM GSH
- 500 µL of 1 mM PAPS

- Re-vortex prior to aliquoting into reaction vials.

7.5 STOCK SOLUTIONS

7.5.1 Test Chemical stock solutions

Test chemical (TC) Superstock and Intermediate stock solutions have an expiration date of 2 weeks, and it is optimal that all incubations for a test chemical be performed with the same Superstock within that 2 week period. Spiking stock solutions (dilutions from the Superstock) shall be made up fresh the day of the experiment. Depending upon the desired incubation concentration of the test chemical and the molecular weight of the test chemical, an Intermediate stock may be necessary. Specific preparation guidance for each test chemical is provided in ANNEX 1, Table A1.4.

- Record stock preparations on the Superstock and Intermediate Stock Preparations worksheet and the Spiking Stock Preparations worksheet (ANNEX 3)
- Remove a 1 mL aliquot of the final Spiking stock, preserve in a standard 1.5 mL HPLC/GC vial with screw top for shipping with the test chemical incubation samples. Samples of Spiking stock will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (Section A2.1) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.5.2 Reference chemical (PYR) stock solutions

Reference chemical (PYR) stocks for depletion experiments shall be made up as a concentrated Superstock, an Intermediate stock and the Spiking stock. The Superstock and Intermediate stock are made up fresh every two weeks, while the spiking stock is made up daily by diluting the Intermediate stock. All pyrene stock solutions are prepared in acetone. The Superstock is prepared at a concentration of 5 mM. The Superstock is diluted 25 fold to a 200 µM concentration (Intermediate stock). This Intermediate stock is diluted 40 fold to a 5 µM concentration (Spiking stock). An example of this preparation is provided below.

Table 2: Example preparation of PYR stocks

Prior to the day of the experiment (2 weeks stability)

Superstock (5 mM)		Intermediate stock (200 µM): 25 x dilution of Superstock	
PYR (mg)	Volume Acetone (mL)	Volume of Superstock (mL)	Total volume with Acetone (mL)
10.1	10.0	0.400	10.0

Day of the experiment

Spiking Stock (5 µM): 40 × dilution of Intermediate stock	
Volume of Intermediate stock (mL)	Total volume with acetone (mL)
0.25	10.0

Preparation prior to the experimental day:

- Prepare a 5 mM PYR Superstock in acetone. For example, weigh out 10.1 mg of pyrene and dissolve in 10 mL of acetone to create the Superstock. Mix well.

- Record stock preparations on Superstock and Intermediate Stock Preparations worksheet (ANNEX 3).
- Remove 400 µL of the Superstock and bring up to 10 mL with acetone to create the 200 µM Intermediate Stock. Mix well. Store at 4°C.

Preparation on the experimental day:

- Remove 250 µL of the Intermediate stock and bring up to 10 mL with acetone to create the 5 µM Spiking Stock. Mix well. Record on the Spiking Stock Preparations worksheet (ANNEX 3).
- Remove a 1 mL aliquot of the final PYR Spiking stock, preserve in a standard 1.5 mL HPLC/GC vial with screw top for shipping with the pyrene incubation samples. Samples of Spiking stock will only be analyzed in the case of an apparent problem with the corresponding incubation samples. See ANNEX 2 (A2.1) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.6 STOPPING SOLUTION WITH INTERNAL STANDARD

7.6.1 Test chemical stopping solution with internal standard

One bulk solution of stopping solution may be used for all depletion experiments for a given test chemical if all reactions occur within **2 weeks** of the initial preparation. Visible contamination, or data suggesting contamination, degradation of the internal standard, or incorrect preparation will require fresh preparation of the stopping solution. For each test chemical, the type of solvent, internal standard and the concentration of the internal standard are detailed in ANNEX 1 (Table A1.5).

- Dissolve test chemical into the appropriate solvent to achieve the Superstock concentration.
- Record details of preparation on the Stopping solution with Internal Standard Preparation worksheet (ANNEX 3).
- Remove three 1 mL aliquot of the stopping solution for shipment to the laboratory analyzing the incubation samples containing the corresponding test chemical. Store each in separate HPLC/GC analytical vials. If more than one stopping solution is prepared for the experiments with a given test chemical, three 1 mL aliquots need to be shipped for each preparation. See ANNEX 2 (A2.2) for the labelling convention. Store at -20°C until shipment to the analytical laboratory.

7.6.2 Pyrene stopping solution containing anthracene

One bulk solution of stopping solution for PYR depletions may be used for each set of experiments performed in concert with a test chemical. All reactions should occur within 2 weeks of the stopping solution preparation. Visible contamination or data suggesting contamination, degradation of the internal standard, or incorrect preparation will require fresh preparation of the stopping solution. Stopping solution for pyrene reactions contains anthracene as an internal standard; Table 3 and ANNEX 1 (Table A1.5) detail the procedure for preparing the PYR stopping solution.

Table 3. Example preparation of acetonitrile (ACN) Stopping Solution containing anthracene (ANT).

Superstock (SS; 1 mM)	Intermediate stock 1 (IMS 1; 20 µM): 50 × dilution of SS	Intermediate stock 2 (IMS 2; 0.4 µM): 50 × dilution of IMS 1	Stopping solution (0.002 µM): 100 × dilution of IMS 2

ANT (mg)	Volume ACN (mL)	Volume of SS (mL)	Total volume with ACN (mL)	Volume of IMS 1 (mL)	Total volume with ACN (mL)	Volume of IMS 2 (mL)	Total volume of Stopping solution (mL)
8.91	50	1.0	50	1.0	50	0.500	100

- Prepare a 1.0 mM ANT Superstock of stopping solution in acetonitrile. For example, weigh out 8.91 mg of ANT (178.23 g/mol) and dissolve in 50 mL of acetonitrile. Mix well.
- Record details of preparation on the Stopping solution with Internal Standard Preparation worksheet (ANNEX 3).
- Remove 1.0 mL of the Superstock and bring up to 50 mL with acetonitrile to create the 20 µM Intermediate Stock 1. Mix well.
- Remove 1.0 mL of the Intermediate stock 1 and bring up to 50 mL with acetonitrile to create the 0.4 µM Intermediate stock 2. Mix well.
- Remove 500 µL of the Intermediate stock 2 and bring up to 100 mL with acetonitrile to create the 0.002 µM Stopping solution. Mix well. Store at 4°C.
- Remove three 1 mL aliquot of the PYR stopping solution containing ANT for shipment to the EPA. Store each aliquot in separate HPLC/GC analytical vials. If more than one stopping solution is prepared for the experiments with a given test chemical, three 1 mL aliquots need to be shipped for each preparation. See ANNEX 2 (A2.2) for the labelling convention. Store at -20°C until shipment to the EPA.

8. PROCEDURES

Note that the procedures outlined below follow, to an extent, the method described in Johanning et al., 2012 with some modifications. **All deviations from the following procedures must be detailed on the appropriate worksheets in the "Comments" sections (ANNEX 3).**

8.1 PRIOR TO THE EXPERIMENT

1. Prepare an adequate volume of K-PO_4 buffer (Section 7.1.3) and adjust the pH to 7.8 at 11°C. Store at 4°C for up to 1 month. Discard if visibly contaminated or data suggests contamination.

   Approximately 100 mL are needed per test chemical.

2. Weigh out the required amounts of NADPH, UDPGA, and GSH to make up 1.0 mL of cofactor solutions. Store, undissolved, in 1.5 mL microfuge tubes at -20°C until the day of the experiment.

   If pre-weighing cofactors for several experiments, write the mass of chemical on the tube for recording later.

3. Prepare the PAPS as frozen 50 µL aliquots of a 10 mM solution in K-PO_4 buffer, pH 8 (Section 7.3.4). Record details on the Alamethicin and PAPS Superstock preparation worksheet (ANNEX 3).
4. Prepare a 10 mg/mL alamethicin in methanol. Store a 25 µL aliquots and store at -20°C (Section 7.2.1). Record details on the Alamethicin and PAPS Superstock preparation worksheet (ANNEX 3).
5. Prepare Superstocks and Intermediate stocks of the test chemical and reference chemical (Section 7.5). Record details on the Superstock and Intermediate Stock Preparation worksheet (ANNEX 3).

6. Prepare Stopping solutions with internal standards (Section 7.6). Record details on the stopping solution with Internal Standard Preparation worksheet (ANNEX 3).
7. Label tubes and vials for the substrate depletion experiment. See ANNEX 2 for the labeling scheme. Labels for the microfuge tubes and HPLC/GC analytical vials will be provided for incubation samples, matrix blanks, spiking solvent and stopping solution. Note that for specific chemicals, glass Hirschmann tubes may be used in place of the plastic microfuge tubes for the collection of incubation samples at each time point. In these cases, specific guidance will be provided (TBD; ANNEX 1).
    a. Tube for Master Mix (> 2.0 mL volume) (1)
    b. 7 mL sample scintillation vials (3: test chemical S9, test chemical HIS9, PYR S9)
    c. microcentrifuge tubes for sample time points (23: 7 for each of the 3 samples above, 2 matrix blanks (S9/HIS9)).
    d. HPLC/GC sample vials, 300 µL with fused inserts (23: 7 for each of the 3 incubations, 2 matrix blanks (S9/HIS9))
    e. HPLC/GC sample vials, standard 1.5 mL (8:, 2 spiking solutions (TC/PYR), and 6 stopping solutions (3 each TC/ PYR)*)

    **Stopping solutions only need to be sampled on the first day of the experiment if the same preparation is used for all experiments in the set. If the same stopping solution was used for HEP experiments and a sample has already been prepared, no further samples are necessary.*

8.2 DAY OF THE EXPERIMENT

8.2.1 Preliminary steps

1. Turn on the water bath or incubation equipment for running reactions and bring to a constant temperature (for these experiments, T = 11 ± 1°C)
2. Dispense the appropriate stopping solution (with internal standard) used to terminate reactions into the pre-labeled microfuge tubes. For tubes receiving aliquots from the PYR incubation, fill microfuge tubes with the 400 µL of acetonitrile containing 0.002 µM ANT. Likewise, prepare the receiving tubes for the TC with the appropriate stopping solution. Refer to ANNEX 1 (Table A1.2) for stopping solutions and volumes specific to each TC. Keep the tubes containing acetonitrile on ice or in a 4°C refrigerator.

Acetonitrile must be kept cold to fully precipitate protein from the reactions.

3. Prepare the Spiking stock solutions of the TC and PYR (Section 7.5). Aliquot 1.0 mL of the TC Spiking stock into 1.5 mL standard HPLC/GC vials for inclusion in the incubation sample shipments to the TC analytical laboratory.
4. Dissolve one tube of pre-weighed NADPH, UDPGA and GSH each in 1.0 mL K-PO_4 buffer (Section 7.1.3). Vortex each and store on ice. Record the mass of each on the Reaction Mixture Preparation Worksheet.
5. Prepare the 250 µg/mL working solution of alamethicin in K-PO_4 buffer by diluting 25 µL of the premade 10 mM alamethicin (Section 7.2.2) with 975 µL K-PO_4 buffer (Section 7.1.3). Vortex and store on ice.
6. Thaw one tube of active S9 and one tube of HIS9. The active S9 should be thawed in an ice-water bath (e.g., beaker containing ice and water). See ANNEX 1, Table A1.1 for lot-specific information for each test chemical. Record on the Reaction Mixture Preparation Worksheet.

Excess active S9 should not be re-frozen for later use in metabolic stability assays. Excess HIS9 may be re-frozen for later use.

7. Dilute the active S9 (150 µL) with K-PO_4 buffer (Section 7.1.3; lot-specific volumes detailed in ANNEX 1, Table A1.1), so that the appropriate concentration of protein is delivered to the reaction system in 100 µL (100 µL of S9 is diluted 10 × with Master Mix in the final reaction solution). Mix well and store on ice.

The dilution values were determined as follows:

The total amount of protein in the S9 tube (mg/mL x 150 µL = µg protein) was divided by 10 x the target incubation protein concentration (mg/mL) to get the desired total volume (µL). The difference between the desired total volume and the 150 µL is the amount of K-PO_4 to add to the S9 tube.

Example:

Lot 1 protein concentration: 23.6 mg/mL
Target incubation protein concentration in the pilot pyrene experiment: 1 mg/mL
10 x concentration: 10 mg/mL

23.6 mg/mL x 150 µL = 3540 µg protein
3540 µg protein/10 mg/mL = 354 µL
354 µL-150 µL = 204 µL K-PO_4 to add

8. Aliquot 200 µL HIS9 and dilute as detailed in ANNEX 1, Table A1.1, so that the appropriate concentration of protein is delivered to the reaction system in 100 µL. All HIS9 in this study is from a batch measured to contain 12.3 mg protein/mL. For experiments with a target incubation protein concentration of 1 mg/mL, add 46 µL of K-PO_4 buffer (Section 7.1.3) to 200 µL HIS9. Mix well and store on ice. Excess diluted HIS9 will be used to prepare a matrix blank.

12.3 mg/mL x 200 µL = 2460 µg protein
2460 µg/protein/ 10 = 246 µL
246 µL – 200 µL = 46 µL K-PO_4 to add

9. Prepare the reaction vials (test chemical S9, test chemical HIS9, reference chemical S9) with biological material and K-PO_4 buffer (Section 7.1.3).
    a. Add 400 µL of K-PO_4 buffer (Section 7.1.3) to each of the three 7 mL scintillation vials.
    b. Add 100 µL of the diluted S9 to each of two 7 mL scintillation vials (test chemical S9, reference chemical S9)
    c. Add 100 µL of the diluted HIS9 to the third 7 mL scintillation vial (test chemical HIS9)
10. To each of the above reaction vials, add 100 µL of the 250 µg/mL alamethicin solution. Pre-incubate reaction vials on ice for 15 min.
11. Thaw one tube of frozen PAPS (Section 7.3.4) on ice and dilute with 450 µL ice-cold K-PO_4 buffer (Section 7.1.3) to make a ~1 mM solution.

Note that PAPS will lose its effectiveness quickly once thawed and diluted. Prepare just before combining into the Master Mix.

12. Combine 500 µL each of the NADPH, UDPGA, GSH solutions, and the diluted PAPS to make up a Master Mix (Section). Note that diluted PAPS will be added last (below). Rinse the PAPS tube with the Master Mix solution to capture the total 500 µL volume. Vortex and store on ice.
13. Add 400 µL of the above Master Mix to each of the pre-incubated reaction vials. Gently swirl each vial until thoroughly mixed. Figure 1, below, details the contents of the reaction vials at this step, just prior to dosing.

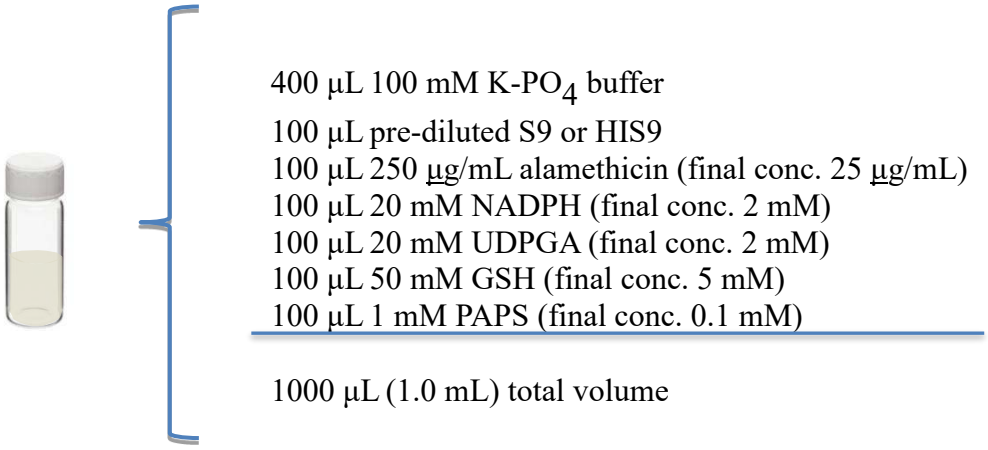


Figure 1. Description of reaction mixture contents in a 7 mL scintillation vial prior to dosing, including volumes and final concentrations of the components.

15. Place all three vials (Test chemical S9, Test chemical HIS9, and PYR S9) into a shaking water bath or incubator and pre-incubate samples at constant temperature (11 ± 1°C) with gentle shaking for 10 minutes.

8.2.2 Substrate Depletion Assay

1. Start the reaction by adding 5 µL of the prepared Spiking stock containing the test chemical or reference chemical (PYR). Swirl reaction vials upon addition of the chemical to ensure thorough mixing. Record start time on the S9 Clearance Assay worksheet (ANNEX 3).

   The time points for S9 and HIS9 samples incubated with the test chemical will be identical. The spiking and sampling of the vials may be staggered so that the aliquots are sampled at precise times. For example, dosing of the HIS9 may be staggered 30 seconds after dosing the S9 vial. Each aliquot taken as a time point for the HIS9 would be similarly staggered 30 seconds after the S9 samples were taken. An example sampling scheme is provided in ANNEX 1, Table A1.6.

   Depending upon the specific time points predetermined for each test chemical, the S9 reaction vial spiked with the reference chemical (PYR) may be sampled prior to, after, or during the test chemical incubations. If the incubations for the TC and PYR are not started at the same time, the vial(s) used in the incubation which is started later should be kept on ice and transferred to the incubator 10 min prior to the reaction initiation.

2. At each designated time point (7 total; ANNEX 1, Table A1.1), gently swirl the reaction mixture, remove an aliquot of 100 µL, and transfer this aliquot to the labeled microfuge tube containing the corresponding stopping solution with internal standard (ANNEX 1). Dispense the aliquot and rinse

the pipet tip in the solvent by aspirating and dispensing 3 times. Record all information on the Clearance Assay Worksheet (ANNEX 3).

3. After the experiment is complete, vortex the microfuge tubes containing the incubation samples on a vortex mixer. See ANNEX 1 for chemical-specific instructions, including extraction with the addition of a second solvent where appropriate, solvent temperature considerations, centrifugation, etc.
4. Transfer the appropriate volume of the supernatant or the organic phase to analytical HPLC/GC sample vials (ANNEX 1, Table A1.1 extraction procedures)
5. Secure samples by tightly screwing on the vial tops.
6. Store at < -20°C until analysis.

8.2.3. Preparation of blanks

These samples may be prepared during or after the substrate depletion assays. They will be included in the shipments to the analytical labs for the test chemical as well as the reference chemical. See ANNEX 2 for labeling convention.

1. Pepare one matrix blank using the active S9 and one matrix blank using the heat-inactivated S9 on each experimental day (6 total/ test chemical). Using the excess Master Mix and diluted S9/ HIS9 solutions, prepare mock incubation samples (Figure 1) without test chemical PYR. The mock incubation samples may be scaled down (< 1.0 mL) as necessary. Pipet 100 µL of mock incubation sample (S9 and HIS9 each) into the appropriate solvent (and volume) for the test chemical (ANNEX 1). **The solvent for the matrix blanks should not include the internal standard**. Prepare these matrix blanks as described for the corresponding test chemical (steps 4-7, Section 8.2.2).

9. DATA ANALYSIS AND REPORTING

The following acceptance criteria and requirements represent conditions of satisfaction which should be met in order for a test to pass. If these are not met, the test may need to be repeated. All deviations from the SOP must be recorded in the "Comments" section on the appropriate worksheet.

9.1 ACCEPTANCE CRITERIA AND REQUIREMENTS

9.1.1 Experimental Acceptance Criteria

1. **K-PO_4 pH.** The pH of the 100 mM potassium phosphate buffer should be adjusted to 7.8 ± 0.1 at 11 ± 1 ºC weekly.

9.1.2 Analytical Acceptance Criteria

- Test chemical analytical runs will contain a standard curve determined using 7 calibration standards. Five standards spanning the concentration range of the incubation samples, with a correlation coefficient of 0.95 will be required for calculations.
- A mid-range standard will be analyzed after every 14 sample injection. The % RSD throughout the run should be 5% or less for non-matrix standards.
- All matrix spike samples should be within 20% of expected value. The analytical performing laboratory should make three levels of their own matrix spikes at each analytical run.

9.1.3 Requirements

1. **Dissolution of test chemical**. The stock solution of the compound under study should be dissolved as recommended by the specific test methods determined by the analytical labs performing the chemical analyses.
2. **Superstock and Intermediate stock test chemical expiration**. Analytical labs performing the chemical analyses should perform stability test on the test chemical in spiking solvent. Test chemical Superstock and Intermediate stock solutions should be made fresh and used within two weeks or within the stability time frame, whichever comes first.
3. **Spiking stock solutions**. These solutions should be made fresh the day of the experiment by diluting the corresponding Superstock as appropriate.
4. **Stopping solution containing internal standard**. Analytical labs performing the chemical analyses should perform stability test on the internal standard in stopping solution. Stopping solutions should be made fresh and used within two weeks or within the stability time frame, whichever comes first.
5. **K-PO_4 expiration.** K-PO_4 buffer should be prepared fresh monthly and stored at 4ºC. Any visual contamination or data suggesting contamination warrants disposal of the buffer and fresh preparation.
6. **Preliminary experiments.** Data from preliminary studies should be available and final conditions established by the analytical lab performing the test chemical analyses. All participating laboratories in the Ring Trial should utilize these established experimental conditions.
7. **Incubation reaction vials.** It is critical that the vials utilized by all participating laboratories will be glass 7 mL scintillation vials. Note that the same vials are described as 6 mL scintillation vials in Europe. Recommendations are given in the SOPs. Plastic should be avoided at all times for the incubation step.
8. **Total percentage of organic solvent in incubation mixture.** The total percentage of organic solvent in the reaction mixture should be < 1% to avoid potential inhibition of metabolic enzyme activity.
9. **Incubation temperature.** The incubation temperature should be constantly maintained at 11 ±1 ºC for the duration of the experiment. This temperature reflects the maintenance temperature of the source fish.
10. **Experimental design.** All experiments with the test compound will be conducted as single replicate assays on three separate days. Time points, chemical concentration, protein concentration and other experimental conditions will be consistent for the three experiments.
11. **Reference chemical.** Pyrene will be used as the reference chemical to determine functionality of the system. The analytical lab performing the analysis, i.e. US EPA, will establish the reference chemical conditions.
12. **Negative Controls.** Samples with heat-inactivated S9 fraction are incorporated into each substrate depletion experiment to account for possible chemical losses due to abiotic degradation, volatilization, and adsorption to the reaction vessel. Heat-inactivated samples will be sampled under the same conditions as the active S9.
13. **Matrix blank samples.** Matrix blank samples containing the biological material (S9 and HIS9) and solvent(s) without addition of test chemical or internal standard will be prepared each experimental day for the test chemical analytical laboratory. These samples will be analyzed only if contamination is suspected in the incubation samples.

9.2 DATA REPORTING

Data templates to report the results will be provided to the analytical laboratories. Each laboratory will use these templates to report valid and failed experiments. These files containing the data should be sent to ILSI HESI for data analyses by the SAS Statisticians. All printed and signed originals should be sent to ILSI

HESI as well for archiving. Results of the intrinsic clearance will be reported for each test chemical used in the *in vitro* metabolism experiments performed by the participating laboratories. Copies of all Worksheets from each laboratory will be sent to ILSI HESI for data archiving.

9.3 STATISTICAL ANALYSES

Concentrations of each test chemical utilized in the OECD Ring Trial will be log transformed and plotted against the reaction time. Depletion rate constants (k; hr-1) will be calculated from the regression slope using linear regression. One-way analysis of variance (ANOVA) of the loss of parent will be determined. Significance will be determined at $\alpha = 0.05$. SAS will be utilized for statistical analysis of data.

10. REFERENCES

Further information on substrate depletion experiments using fish liver S9 fraction may be found in the following publications (this list is not exhaustive):

Connors, K. A., et al. (2013). "Comparative pharmaceutical metabolism by rainbow trout (*Oncorhynchus mykiss*) liver S9 fractions." Environmental Toxicology and Chemistry 32(8): 1810-1818.

Han, X., et al. (2009). "Liver microsomes and S9 from rainbow trout (*Oncorhynchus mykiss*): Comparison of basal-level enzyme activities with rat and determination of xenobiotic intrinsic clearance in support of bioaccumulation assessment." Environmental Toxicology and Chemistry 28(3): 481-488.

Johanning, K., et al. (2012). "Assessment of Metabolic Stability Using the Rainbow Trout (*Oncorhynchus mykiss*) Liver S9 Fraction." Current Protocols in Toxicology, John Wiley & Sons, Inc.: 14.10.11-14.10.28.

Laue, H, et. al. (2014). "Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of *in vitro* intrinsic clearance." Environmental Science and Technology 48(16):9486-95.

Nichols, J. W., et al. (2013). "Hepatic Clearance of 6 Polycyclic Aromatic Hydrocarbons by Isolated Perfused Trout Livers: Prediction From *In Vitro* Clearance by Liver S9 Fractions." Toxicological Sciences 136(2): 359-372.

ANNEX 1: Experimental Summary

Figure A1.1 Diagrammatic representation of the incubation procedure using S9 fraction

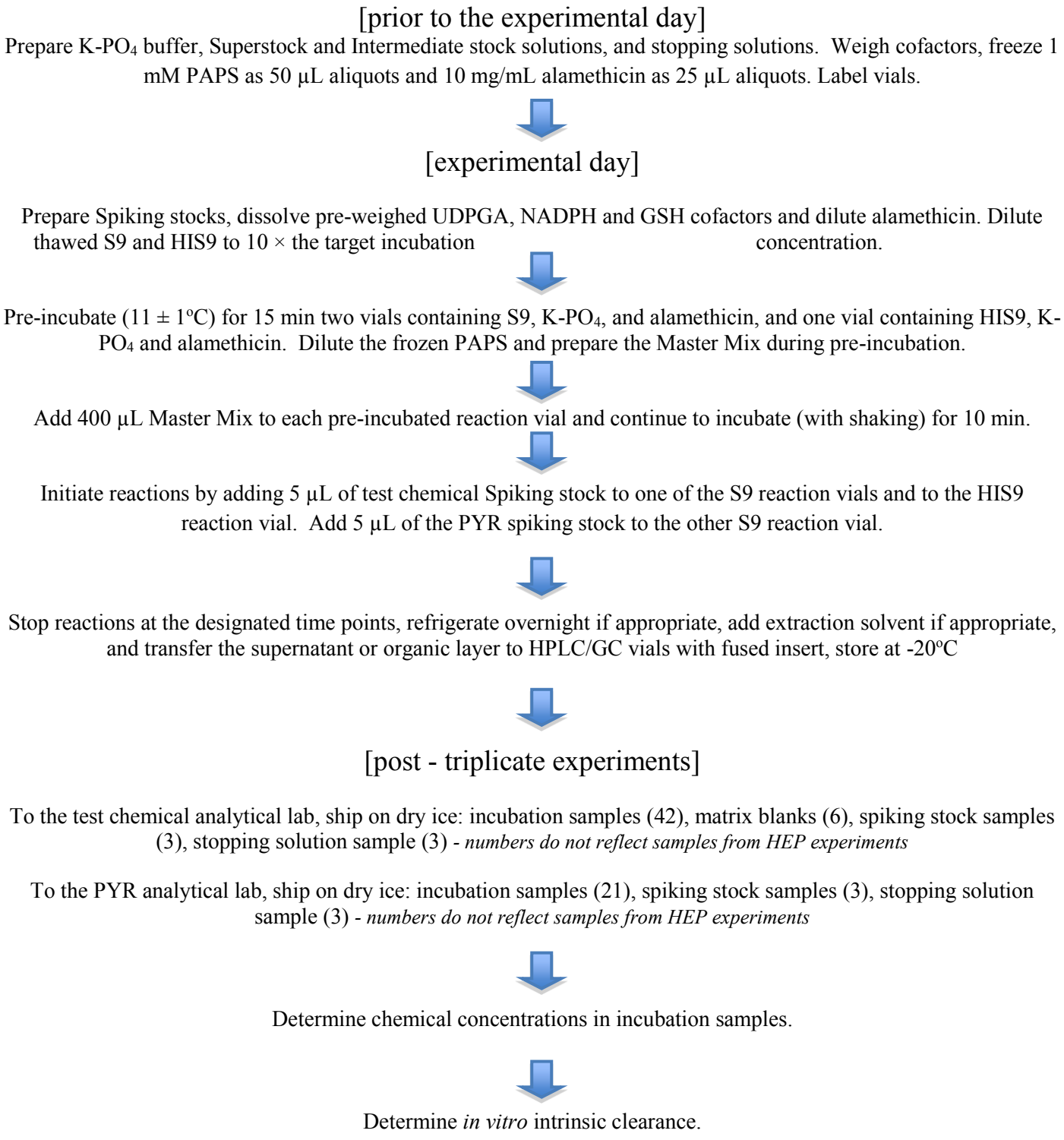


Table A1.1 Study design and experimental details

Expt	Chem[1,2]	S9 Lot	Chem Incub. conc (µM)	S9 incub. conc (mg/mL)	Volume (µL) K-PO4 to add to 150 µL S9 [3]	Volume (µL) K-PO4 to add to 200 µL HIS9	Time points (min)	Stopping solution[4], volume (µL)	I STD[5]	I STD conc. (µM)	Extract. solvent, volume (µL)	Extract. Method	Analyt. Lab[6]
pilot	PYR	1,HI	0.025	1.0	204	46	2,4,6,8,10,12,14	cold ACN, 400	ANT	0.002	-	A	EPA
1	4NP	2, HI	2.0	1.0	207	46	2,5,10,15,20,30,40	cold ACN, 400	4NP-d4	0.400		B	DUP
	PYR (+)	2	0.025	1.0	204	46	2,4,6,8,10,12,14	cold ACN, 400	ANT	0.002	-	A	EPA
2	FEN	6, HI						DCM	FEN-d6			C	EPA
	PYR (+)	6	0.025				2,4,6,8,10,12,14	cold ACN, 400	ANT		-	A	EPA
3	CS	8, HI						cold MTBE, 400	ML			D	GIV
	PYR (+)	8	0.025				2,4,6,8,10,12,14	cold ACN, 400	ANT		-	A	EPA
4	MC	11, HI						DCM	MC-d6			E	FB
	PYR (+)	11	0.025				2,4,6,8,10,12,14	cold ACN, 400	ANT		-	A	EPA
5	DM	10, HI						cold ACN, 400	PM		DCM,	F	DOW
	PYR (+)	10	0.025				2,4,6,8,10,12,14	cold ACN, 400	ANT		-	A	EPA

[1] + = reference chemical

2Test chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[3]protein concentrations (mg/mL) of the frozen S9 lots: lot 1, 23.6; lot 2, 23.8; lot 6, 22.6; lot 8, 23.1; lot 11, 24.4; lot 10, 21.0.

[4]Stopping solutions: ACN = acetonitrile, DCM = Dichloromethane (methylene chloride)

[5]Internal standards: ANT = anthracene, 4NP-d4 = 4-n-nonylphenol-d4, FEN-d6 = fenthion- d6, ML = methyl laurateMC-d6 = methoxychlor–d6, PM= permethrin; details in Table A1.3

[6]Analytical laboratory: EPA = Environmental Protection Agency, DUP = DuPont, GIV = Givaudan, FB = Fraunhofer IME/ University of Bern, DOW = Dow Chemical; details in Table A1.4

Extraction procedures:

A – PYR: 100 µL will transferred from the reaction vial at each timepoint into Eppendorf tubes containing 400 µL 0.002 µM ANT in ACN. Vortex 10 min at 2300 rpm, and refrigerate overnight. Centrifuge all samples for 10 min at 20,000 × g, and transfer 300 µL of supernatant to a HPLC vial for shipment to EPA.

B- 4NP: 100 µL will transferred from the reaction vial at each timepoint to Eppendorf tubes containing 500 µL 0.4µM 4NP-d4 in ACN. Vortex for 10 min at 2300 rpm, and refrigerator overnight. Centrifuge all samples for 10 min at 20,000 × g, and then transfer 180 µL of supernatant HPLC vial for shipment to DuPont. **For each sample, please save the remaining supernatant in separate set of vials, to be held at your lab.**

Table A1.2 Test Chemical (TC) Details

TC	Chemical abbrev.	CAS No.	Supplier	Cat. No.	Lot No.	% Purity	MW (g/mol)
Pyrene	PYR	129-00-0	Sigma-Aldrich	185515	bcbk2867v	98.7	202.25
4-n-nonylphenol	4NP	104-40-5	Sigma-Aldrich	442873	lc07805v	99.9	220.35
Fenthion	FEN	55-38-9	Sigma-Aldrich	36552	szbc178xv	97.9	278.33
Cyclohexyl Salicylate	CS	25485-88-5	Givaudan	8819601	ve003164	99.8	220.26
Methoxychlor	MC	72-43-5	Sigma-Aldrich	49054	lc09014v	99.9	345.65
Deltamethrin	DM	52918-63-5	Sigma-Aldrich	45423	szbc059xv	99.6	505.20

Table A1.3 Internal Standard (I STD) Details

I STD	Chemical abbrev.	CAS No.	Supplier	Cat. No.	Lot No.	% Purity	MW (g/mol)
Anthracene	ANT	120-12-7	Sigma-Aldrich	48567	lc10254v	99	178.23
4-n-nonylphenol-d4	4NP-d4	1173019-62-9	Sigma-Aldrich	614343	mbbb2035v	98	224.27
Fenthion- d6	FEN-d6	1189662-83-6	C/D/N Isotopes	D-6462	c126	97	284.36
Methyl Laurate	ML						
Methoxychlor-d6	MC-d6	106031-79-2	C/D/N Isotopes	D-7030	e244	98.6	351.69
Permethrin	PM	52645-53-1	Sigma-Aldrich	45614	szbd142xv	98.1	391.29

Table A1.4 Test Chemical (TC) Stock preparations

TCl[1]	MW (g/mol)	Stock solvent[2]	Example TC mass (mg)	Example Superstock volume (mL)	Superstock conc (mM)	Dilution of Superstock[3]	Intermediate stock conc. (µM)	Dilution of Interm. stock to Spiking Stock	Spiking stock conc. (µM)
PYR	202.25	AE	10.1	10.0	5.0	25×	200	40 ×	5.0
4NP	220.35	ACN	11.02	10.0	5.0	12.5×	--	--	400
FEN	278.33								
CS	220.26								
MC	345.65								
DM	505.20								

[1] Test (and Reference) chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[2] Solvents: AE = acetone, ACN = acetonitrile, DMSO = dimethyl sulfoxide, DCM = Dichloromethane (methylene chloride), MTBE = methy-tert-butyl ether

[3] Some test chemical spiking stocks may require the preparation of an intermediate stock, others may be prepared by directly diluting a Superstock.

Table A1.5 Stopping solution with Internal Standard (I STD) preparation

TC[1]	I STD[2]	I STD MW (g/mol)	Stop. soln[3]	Example I STD mass (mg)	Example Superstock volume (mL)	Superstock I STD conc. (mM)	Dilution of Superstock	Interm. stock 1[4] conc (µM)	Dilution of Interm. Stock 1	Interm. Stock 2[4] conc. (µM)	Dilution of Interm. Stock 2	Stopping solution conc (µM)
PYR	ANT	178.23	ACN	8.91	50.0	1.0	50 ×	20	50 ×	0.4 µM	200 ×	0.002
4NP	4NP-d4	224.27	ACN	5.6	10.0	2.5	25 ×	100	250 ×	--	--	0.400
FEN	FEN-d6	284.36										
CS	ML											
MC	MC-d6	351.69										
DM	PM	391.29										

[1] Test (and Reference) chemicals: PYR = pyrene, 4NP = 4 n-nonylphenol, FEN = fenthion, CS = cyclohexyl salicylate, MC = methoxychlor, DM = deltamethrin; details in Table A1.2

[2] Internal standards: ANT = anthracene, 4NP-d4 = 4-n-nonylphenol-d4, FEN-d6 = fenthion- d6, ML = methyl laurate, MC-d6 = methoxychlor–d6, PM= permethrin. Details in Table A1.3

[3] Solvents: AE = acetone, ACN = acetonitrile, DMSO = dimethyl sulfoxide, DCM = Dichloromethane (methylene chloride), MTBE = methyl-tert-butyl ether

4 Some test chemical spiking stocks may require the preparation of intermediate stocks, others may be prepared by directly diluting a Superstock.

Table A1.6. Example of time staggering.
In this example, the active S9 reaction mixture (S9) was spiked with 4-n-nonylphenol (4NP) at time 0'0". The heat-inactivated S9 reaction mixture (HIS9) was spiked with 4NP 30 seconds later (0'30"). The heat-inactivated suspension was sampled at each time point 30 seconds after the active S9 to maintain a consistent elapsed time.

Sample ID	Targeted Time (min' sec")	Clock Time (min'sec")	Sample ID	Targeted Time (min'sec")	Clock Time (min'sec")
EPA-4NP-01-S9 -1	2	2'00"	EPA-4NP-01-HIS9 -1	2	2'30"
EPA-4NP-01-S9 -2	5	5'00"	EPA-4NP-01-HIS9 -2	5	5'30"
EPA-4NP-01-S9 -3	10	10'00"	EPA-4NP-01-HIS9 -3	10	10'30"
EPA-4NP-01-S9 -4	15	15'00"	EPA-4NP-01-HIS9 -4	15	15'30"
EPA-4NP-01-S9 -5	20	20'00"	EPA-4NP-01-HIS9 -5	20	20'30"
EPA-4NP-01-S9 -6	30	30'00"	EPA-4NP-01-HIS9 -6	30	30'30"
EPA-4NP-01-S9-7	40	40'00"	EPA-4NP-01-HIS9-7	40	40'30"

Table A1.7 Analytical laboratory shipment information.

Analytical Laboratory	Analyte	Address	Contact information
EPA	PYR, FEN	**U.S. EPA** **Mid-Continent Ecology Division** 6201 Congdon Blvd, Duluth, MN 55804 USA	
DUP	4NP	**DuPont Haskell Global Centers for Health and Environmental Sciences** Building S315/lab room 1132 1090 Elkton Rd Newark, DE 19711 USA	
GIV	CS	**Givaudan Schweiz AG** Ueberlandstrasse 138 CH-8600 Dubendorf Switzerland	
DOW	DM	**Dow Chemical Company** 1803 Building, Door E, Lab 485 Midland, MI 48674 USA	
FB	MC	**Fraunhofer IME** Auf dem Aberg 1 57392 Schmallenberg Germany	

ANNEX 2: LABELING CONVENTIONS

Pre-printed labels (Direct Thermo Cryo-Tags; solvent-resistant printing, cyro-stable) will be provided to each laboratory. Labels will use the following codes:

Laboratory abbreviations (Field 1):

DOW - Dow Chemical Company

DUP - DuPont-Haskell Global Centers for Health and Environmental Sciences

EPA - U.S. Environmental Protection Agency * *the EPA is not anticipated to produce samples for the final dataset, but may be involved in some preliminary studies, troubleshooting, etc.*

FB - Fraunhofer IME/ University of Bern

GIV - Givaudan Schweiz AG

PG - Proctor & Gamble

SCK - SC Johnson and Son/ KJ Scientific

Chemical abbreviations (Field 2):

PYR - Pyrene[1]

4NP - 4-*n*- nonylphenol

CS - Cyclohexyl Salicylate

DM - Deltamethrin

FEN - Fenthion

MC – Methoxychlor

Biological material abbreviations (final Field)

S9 – S9 in vitro experiment/ active S9 experiment

HIS9- heat-inactivated S9 experiment

HEP – hepatocyte in vitro experiment/ live cell experiment, *see Hepatocyte SOP*

HIHEP – Heat-inactivated cell experiment, *see Hepatocyte SOP*

A2.1 SPIKING STOCK

(Aliquots to be sent to the corresponding analytical lab with incubation samples.)

For each experiment, researchers performing depletion experiments will provide to the analytical laboratory a sample of the spiking solution used to dose the reaction samples containing either live hepatocytes or heat inactivated hepatocytes. Spiking solutions for the test chemical as well as the reference chemical (PYR) will be prepared fresh the day of the experiment. Please label each spiking solution with the following convention, using the abbreviations provided below:

Field 1	**Field 2**	**Field 3**	**Field 4**	**Field 5**
originating lab	test chemical abbreviation[1]	experimental day (01, 02, or 03)	SPK	biological material (HEP, S9)

[1]Spiking stocks prepared for reactions with the reference chemical (PYR) during a specific test chemical experiment will be labelled as above, but the field for the test chemical will be denoted as PYR, test chemical abbreviation.

Examples:

Note: The following are examples. Please modify according to your laboratory abbreviation and chemical:

EPA - 4NP - 02 - SPK- S9 denotes the sample originated at the EPA laboratory, contains 4NP, and was used in the second experimental day to spike S9 reaction mixtures (active and HI).

EPA – PYR, 4NP - 02 - SPK- S9 denotes the sample originated at the EPA laboratory, contains pyrene, and was used as a spiking stock for the reference chemical during the second 4NP experimental day with S9 (active and HI).

A2.2 STOPPING SOLUTION CONTAINING INTERNAL STANDARD

Reactions (see ANNEX 1) will be stopped using solvent containing an internal standard. For these experiments, samples of the stopping solution will be included in the shipment to the analytical laboratory for the corresponding test chemical. Solvent with internal standard may be made up for both S9 and HEP experiments or may be prepared separately for experiments with each biological material. The stopping solution will be labeled as follows:

Field 1	**Field 2**	**Field 3**	**Field 4**
originating lab	Corresponding test chemical abbreviation[1]	I STD	biological material (HEP, S9)[2]

[1]Stopping solution prepared for reactions with the reference chemical (PYR) during a specific test chemical experiment will be labelled as above, but the field for the test chemical will be denoted as PYR, test chemical abbreviation.
[2] If the same preparation of stopping solution is used for experiments with both Hepatocytes and S9 fraction, include both abbreviations in Field 4.

Example:
EPA- FEN- I STD – HEP, S9 denotes the sample originated at the EPA, contains stopping solution with the internal standard for fenthion experiments (fenthion- d6), and was used in both the Hepatocyte and S9 depletion experiments.
EPA-PYR, FEN-I STD – S9 denotes the sample originated at the EPA, contains stopping solution for the pyrene experiment run in parallel with fenthion depletions using S9 (i.e, acetonitrile with 0.002 μM anthracene).

A2.3 INCUBATION SAMPLES

(stopped reactions from each time point)

Researchers performing depletion experiments will remove 7 aliquots from the reaction sample at pre-determined time points, generating 7 subsamples for chemical analysis per reaction. These depletion experiments will be performed for each test chemical as singlet assays over three separate experimental days for both S9 and HIS9 in vitro systems. Each S9 reaction mixture prepared for an experimental day will also be tested with PYR as a reference chemical/ potential benchmark chemical in a separate vial. Incubation samples for each test chemical will be labeled with the following fields:

Field 1	**Field 2**	**Field 3**	**Field 4**	**Field 5**
originating lab	test chemical abbreviation[1]	experimental day (01, 02, or 03)	biological material (S9 or HIS9)	time point (1-7)

Samples obtained from reactions with the reference chemical (PYR) during a specific test chemical experiment will be labelled as above, but the field for the test chemical will be denoted as PYR, test chemical abbreviation.

Note: The following is an example. Please modify according to your laboratory abbreviation and chemical:

Experiment 1 (01)

Labels for active S9 fraction, test chemical 4NP	**Labels for heat-inactivated S9 fraction, test chemical 4NP**	**Labels for the reference chemical (PYR) samples, conducted during 4NP depletions**

EPA-4NP-01-S9-1	EPA-4NP-01-HIS9-1	EPA-PYR, 4NP-01-S9-1
EPA-4NP-01-S9-2	EPA-4NP-01-HIS9-2	EPA-PYR, 4NP-01-S9-2
EPA-4NP-01-S9-3	EPA-4NP-01-HIS9-3	EPA-PYR, 4NP-01-S9-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Experiment 2 (02)

Labels for active S9 fraction, test chemical 4NP	**Labels for heat-inactivated S9 fraction, test chemical 4NP**	**Labels for the reference chemical (PYR) samples, conducted during 4NP depletions**
EPA-4NP-02-S9-1	EPA-4NP-02-HIS9-1	EPA-PYR, 4NP-02-S9-1
EPA-4NP-02-S9-2	EPA-4NP-02-HIS9-2	EPA-PYR, 4NP-02-S9-2
EPA-4NP-02-S9-3	EPA-4NP-02-HIS9-3	EPA-PYR, 4NP-02-S9-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Experiment 3 (03)

Labels for active S9 fraction, test chemical 4NP	**Labels for heat-inactivated S9 fraction, test chemical 4NP**	**Labels for the reference chemical (PYR) samples, conducted during 4NP depletions**
EPA-4NP-03-S9-1	EPA-4NP-03-HIS9-1	EPA- PYR, 4NP-03-S9-1
EPA-4NP-03-S9-2	EPA-4NP-03-HIS9-2	EPA- PYR, 4NP-03-S9-2
EPA-4NP-03-S9-3	EPA-4NP-03-HIS9-3	EPA- PYR, 4NP-03-S9-3
And so on up to - 7	And so on up to - 7	And so on up to - 7

Laboratories may choose to prepare and assay S9 preparations along with hepatocyte preparations on the same day for a given test chemical. Similar labels would contain the term HEP/ HIHEP in place of S9 or HIS9. See the SOP for hepatocyte depletions.

A2.4 MATRIX BLANKS

Researchers performing depletion experiments will prepare 2 matrix blanks (designated MTX) each experimental day for both S9 and HIS9. Matrix blanks will be prepared as an incubation sample without test chemical or internal standards in the stopping solution.

Matrix blank samples for each test chemical will be labeled with the following fields:

Field 1	**Field 2**	**Field 3**	**Field 4**	**Field 5**
originating lab	test chemical abbreviation	experimental day (01, 02, or 03)	MTX	biological material (S9, HIS9)

Examples:

Note: The following are examples. Please modify according to your laboratory abbreviation and chemical:

EPA - 4NP - 02 – MTX - S9 denotes the sample is a matrix blank originated at the EPA laboratory, corresponding to the second 4NP experimental day, prepared with the master mix and diluted S9 used to generate the reaction mixture. Field 2 (4NP) does NOT denote the presence of 4NP, but identifies the experimental day.

EPA –4NP - 02 – MTX- HIS9 denotes the sample is a matrix blank originated at the EPA laboratory, corresponding to the second 4NP experimental day, prepared with the master mix and diluted HIS9 used to generate the negative control reaction mixture. Field 2 (4NP) does NOT denote the presence of 4NP, but identifies the experimental day.

ANNEX 3: WORKSHEETS

A3.1 GENERAL WORKSHEETS

RECORD OF REAGENTS, CHEMICALS AND INSTRUMENTS

PHOSPHATE BUFFER PREPARATION

ALAMETHICIN AND PAPS SUPERSTOCK PREPARATIONS

REFERENCE CHEMICAL STOCK PREPARATION

RECORD OF REAGENTS, CHEMICALS AND INSTRUMENTS

Instruments, reagents and chemicals used from _____ to _____

Reagent/Chemical Name	Supplier	Catalog number	Lot number	Expiration Date
NADPH				
UDPGA				
GSH				
PAPS				
Alamethicin				
Monobasic potassium phosphate				
Dibasic potassium phosphate				
Acetone				
Acetonitrile				
Methylene Chloride				
Methyl *tert*-butyl ether				

Equipment	Model	ID number	Notes
Balance			
Centrifuge 1			
pH meter			
Freezer			
Refrigerator			
Incubation equipment			
Vortex mixer			

Comments:

PHOSPHATE BUFFER PREPARATION

Lab: _____ Test Chemical: _____ Date: _____ Initials: _____ S9

Buffers used from _____ to _____.

Preparation of 100 mM potassium phosphate stocks (Section 7.1)

Reagent	Stock Conc.	Mass (mg)	Volume ultrapure water (mL)
Dibasic (K_2HPO_4) potassium phosphate buffer	100 mM		
Monobasic (KH_2PO_4) potassium phosphate buffer	100 mM		

Reagent	Stock Conc.	Volume Dibasic (K_2HPO_4) buffer (mL)	Volume Monobasic (KH_2PO_4) buffer (mL)	Total volume (mL)	pH at 11°C
Potassium phosphate buffer (K-PO_4), pH 7.8 +/- 0.1 at 11°C	100 mM				

Comments:

ALAMETHICIN AND PAPS SUPERSTOCK PREPARATION

Lab: _____ Test Chemical: _____ Date: _____ Initials: _____ S9

Reagent	Stock concentration (mg/mL)	Mass alamethicin (mg)	Volume Methanol (mL)
Alamethicin Superstock	10		

Preparation of 10 mg/mL alamethicin in methanol (Section 7.2.1)

Preparation of PAPS in K-PO4 buffer (pH 8) (Section 7.3.4)
Anhydrous purity _____ Water content _____ Mol anhydrous acid _____

Calculation of volume K-PO_4 to add to bottle*:*

Chemical mass × anhydrous acid content × (1- water content)/ 507.26 g/mol = mol anhydrous acid*

Volume of K-PO_4 buffer (mL) to add = (mmol of anhydrous acid/ 10 mM) × 1000 mL/L

Reagent	Stock concentration (mM)	Mass PAPS (mg)*	Volume K-PO_4 buffer (mL)
PAPS Superstock	10		

**mass of impure PAPs as provided by vendor*

Comments:

A3.2 4NP WORKSHEETS

SUPERSTOCK AND INTERMEDIATE STOCK PREPARATION

STOPPING SOLUTION WITH INTERNAL STANDARD PREPARATION

COFACTOR AND MASTER MIX PREPARATION

CLEARANCE ASSAY

SUPERSTOCK AND INTERMEDIATE STOCK PREPARATIONS

Lab: _____Test Chemical: 4NP Date:_____ Initials: _____ S9 / HEP (circle)

Preparation of 4-*n*-nonylphenol (4NP) stocks

Refer to Section 7.6 in the S9 SOP and ANNEX 1, Table A1.4.

Stock, abrev.	Mass 4NP (mg)	ACN Volume (mL)	Stock Conc. (mM), Target = ___5mM___ = 1000 * mg TC/ 220.35[1]/ mL solvent
Example	11.02	10	5 mM
Actual Superstock, SS			

[1] MW = molecular weight of test chemical

Comments:

Preparation of PYRENE (PYR) stocks

Refer to section 7.6 and ANNEX 1, Table A1.4.

Stock, abrev.	Mass PYR (mg)	Acetone Volume (mL)	Stock Conc. (mM), Target = 5.0 mM = 1000* mg PYR/ 202.25 / mL acetone
Example	10.1	10.0	5 mM
Superstock, SS			

Stock, abbrev.	Volume SS	Total volume acetone (mL)	Stock Conc. (uM), Target = 200 µM = µM SS * mL SS / total mL
Example	400 µL	10.0	200 µM
Intermediate, IMS			

Comments:

STOPPING SOLUTION WITH INTERNAL STANDARD PREPARATION

Lab: _____Test Chemical: _____ Initials: _____ S9 / HEP (circle)

Remove three 1 mL aliquots of both the test chemical stopping solution the PYR stopping solution. Preserve in the 1.5 mL standard HPLC/GC vial for shipping with the incubation samples.

Internal standard (4NP): **deuterated 4-n-nonylphenol (4NP-d4)** MW (g/mol): 224.27 Solvent: ACN

Internal standard (PYR): **anthracene (ANT)** MW (g/mol): 178.23 Solvent: ACN

Preparation of 4-*n*-nonylphenol (4NP) stopping solution containing 0.4 µM 4NP-d4 in ACN

Refer to Section 7.6.1 and ANNEX 1, Table A1.4.

	Amount Internal standard (mg)	Final ACN volume for Superstock (SS) (mL)	Added vol. SS to IMS (mL)	Final ACN vol. of IMS (mL)	Added volume IMS (mL)	Final ACN volume for Stopping solution (mL)	Final conc Stopping solution
Example	5.6	10.0	0.40	10.0	1.0	250	0.400 µM
Actual							

Preparation of Reference chemical (PYR) stopping solution containing 0.002 µM ANT in ACN

Refer to Section 7.6.2 and ANNEX 1, Table A1.4.

	Amount Internal standard (mg)	Final ACN vol. for Superstock (SS) (mL)	Added vol. of SS to IMS 1 (mL)	Final vol. IMS 1 (mL)	Added vol. of IMS 1 to IMS 2 (mL)	Final vol. IMS 2 (mL)	Added vol. of IMS 2 to Stopping Soln. (mL)	Final vol. Stopping soln (mL)	Final conc Stopping solution
Example	8.91	50.0	1.0	50.0	1.0	50.0	1.0	200	0.002 µM
Actual									

Comments:

SPIKING STOCK PREPARATIONS

Lab: _____Test Chemical: _____ Initials: _____ S9 / HEP (circle)

Remove 1 mL aliquots of the final Spiking stocks for both the test chemical and PYR. Preserve in the standard 1.5 mL HPLC/GC sample vial for shipping with the incubation samples.

Preparation of test chemical (4NP) Spiking Stocks (SPK)

Refer to Section 7.5.1 and ANNEX 1, Table A1.4.

Date Superstock (SS)/Intermediate stock (IMS) prepared: _____Diluting solvent:____ACN____

Date	Experimental day	Volume 4NP SS	Total volume solvent (mL)	Spiking stock Conc. (µM) = µM IMS* mL SS or IMS/ total mL
	Example	0.40	5	400 µM
	01			
	02			
	03			

Comments:

Preparation of Reference chemical (PYR) Spiking Stocks (SPK)

Refer to section 7.5.2 and ANNEX 1, Table A1.4.

Date Superstock (SS)/Intermediate stock (IMS) prepared: _____

Date	Experimental day	Volume IMS (mL)	Total volume acetone (mL)	Spiking stock Conc. (µM) = µM IMS* mL IMS/ total mL
	Example	0.25	10.0	5 µM
	01			
	02			
	03			

Comments:

REACTION MIXTURE PREPARATION

Lab: _____ Test Chemical: _____ Date: _____ Initials: _____ S9

Dilution of Alamethicin *(Refer to Section 7.2.2)*

Reagent	Volume 10 mg/mL alamethicin in methanol	Volume K-PO_4 (pH 7.8 at 11°C) *Target = 975 µL*
250 µg/mL alamethicin	25 µL	

Cofactor Preparation *(Refer to Section 7.3)*

Cofactor	Stock Conc. approximate	Mass (mg)	Volume 100 mM K-PO_4 buffer (mL)
NADPH	20 mM		
UDPGA	20 mM		
GSH	50 mM		

Dilution of 10 mM PAPS to 1 mM PAPS *(Refer to Section 7.3.4)*

Reagent	Volume 10 mM PAPS	Volume K-PO_4 (pH 7.8 at 11°C) *Target = 450 µL*
1 mM PAPS	50 µL	

Preparation of Master Mix *(Refer to Section 7.4)*

Reagent	Target volumes (per 2.0 mL prep):	Actual volumes (µL)
20 mM NADPH	500 µL	
20 mM UDPGA	500 µL	
50 mM GSH	500 µL	
1 mM PAPS	500 µL	

Preparation of Diluted Biological Material *(Refer to Section 8.2.1)*

Biological Material	Target incubation concentration (mg/mL)	Volume biological material (un-diluted)	Volume K-PO_4 (pH 7.8 at 11°C) added
S9, Lot #		150 µL	
HIS9		200 µL	

S9 CLEARANCE ASSAY WORKSHEET
Lab: _____Test Chemical: _____Experimental Day: ____ Date: _____ Initials: _____

Test Chemical Information:

Chemical Name	4-n- nonylphenol (4NP)	PYR (reference chemical)
Chemical MW (g/mol)	220.35	202.25
Chemical supplier, cat. #	Sigma Aldrich	Sigma-Aldrich, 185515
Lot #	lc07805v	bcbk2867v
Spiking stock solvent	acetonitrile	acetone
Spiking Stock Concentration	400 µM	5 µM
Stop Solvent	acetonitrile	acetonitrile
Internal Standard (I STD)	4NP-d4	AN
I STD Supplier, cat. #	Sigma Aldrich, 614343	Sigma-Aldrich, 48567
I STD Lot #	mbbb2035v	lc10254v
I STD Stock Concentration	0.4 µM	0.002 µM

Experimental Conditions:

	4NP, S9	4NP, HIS9	PYR, S9
S9 lot #	2	Not applicable	2
S9 incubation concentration (mg/mL)	1.0	1.0	1.0
Reaction Vessel	7-mL scintillation vial, loosely capped	7-mL scintillation vial, loosely capped	7-mL scintillation vial, loosely capped
Reaction Temperature	11°C	11°C	11°C
Replicates	One reaction	One reaction	One reaction
Time Points	2,5,10,15,20,30,40	2,5,10,15,20,30,40	2,4,6,8,10,12,14
Reaction Buffer	100 mM K-PO_4	100 mM K-PO_4	100 mM K-PO_4
Reaction Volume	1000 µL	1000 µL	1000 µL
Dose Vehicle	acetonitrile	acetonitrile	acetone
Dose Volume	5 µL	5 µL	5 µL
Chemical Incubation Concentration	2µM	2µM	0.025 µM
Reaction Stop Volume	100 µL	100 µL	100 µL
Stop Solution Volume	500 µL	500 µL	400 µL
Extraction Solvent	Not applicable	Not applicable	Not applicable
Extraction Solvent Volume	Not applicable	Not applicable	Not applicable
Final Transfer Volume to Ship	180 µL	180 µL	300 µL

Lab: _____Test Chemical: _____Experimental Day: ____ Date: _____ Initials: _____

Sample Pre-incubation time _____min Start time _____

Refer to Section 8.2.2.

	Sample ID	Targeted time (min)	Actual Time, if different	Notes
PYR, S9		2		
		4		
		6		
		8		
		10		
		12		
		14		
4NP, S9		2		
		5		
		10		
		15		
		20		
		30		
		40		
4NP HIS9		2		
		5		
		10		
		15		
		20		
		30		
		40		

PYR: 100 µL will transferred from the reaction vial at each timepoint into Eppendorf tubes containing 400 µL 0.002 µM ANT in ACN. Vortex 10 min at 2300 rpm, and refrigerate overnight. Centrifuge all samples for 10 min at 20,000 × g, and transfer 300 µL of supernatant to a HPLC vial for shipment to EPA.

4NP: 100 µL will transferred from the reaction vial at each timepoint to Eppendorf tubes containing 500 µL 0.4µM 4NP-d4 in ACN. Vortex for 10 min at 2300 rpm, and refrigerator overnight. Centrifuge all samples for 10 min at 20,000 × g, and then transfer 180 µL of supernatant HPLC vial for shipment to DuPont. **For each sample, please save the remaining supernatant in separate set of vials, to be held at your lab.**

Comments:

ANNEX 7: Chemical analyses

Samples containing Cyclohexyl Salicylate (CS) were analyzed at the Givaudan research facility (Givaudan Schweiz AG) in Dübendorf, Switzerland. The analyses were performed by GC/MS on a Thermo Scientific TSQ 8000 triple quadrupole mass spectrometer connected to a Thermo Scientific Trace 1310 gas chromatograph. Chromatography was carried out on a 5% phenyl-methylpolysiloxane capillary column (HP-5MS, 15 m × 0.25 mm, 0.25 µm; Agilent Technologies). Helium was used as the carrier gas, and the flow rate was 1.0 mL /min. One µL samples were injected with pulsed splitless injection. The pulse pressure was set to 1.5 bar for 1 min. The temperature program started at 40°C for 2 min, increased by 40°C/min, and ended at 280°C for 1 min. The mass spectrometer was operated in selected reaction monitoring (SRM) mode for CS analysis, detecting the SRM transitions from 120 to 92 (collision energy 15 eV) and 138 to 92 (*m/z*) (collision energy 25 eV). Methyl laurate was used as internal standard. For methyl laurate analysis, the mass spectrometer was operated in selected ion monitoring (SIM) mode, detecting ions with *m/z* values 74.10 and 87.10. Calibration standards were prepared in MTBE in the presence of the corresponding matrix (heat-inactivated RT-HEP or heat-inactivated RT-S9 plus all cofactors except PAPS).

Deltamethrin (DM) was analyzed by GC/MS/MS at the The DOW Chemical Company laboratories in Midland, Michigan, USA. Samples were analyzed using an Agilent 7890B GC coupled to an Agilent 7000C triple quadrupole MS. Permethrin was used as the internal standard. The analyses were performed using an Agilent HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm), pulsed splitless injection at 250°C, and a GC temperature program of 100°C for 1.0 min, ramped to 200°C at 45°C/min with 0.5 min hold, ramped to 290°C at 13°C/min with 0.5 min hold, and ramped to 310°C at 45°C/min with 2.5 min hold. Permethrin was analyzed using Q1 and Q3 masses of 183 and 128, respectively. DM was analyzed using Q1 and Q3 masses of 181 and 152, respectively. Preliminary studies showed that matrix did not have an effect on the analysis. As a result, calibration standards were prepared in dichloromethane in the absence of matrix.

Samples containing methoxychlor (MC) were analyzed by GC/MS at the Fraunhofer Institute for Molecular Biology and Applied Ecology in Achen, Germany. The analyses were performed on a GC/MS system consisting of a HP5890 series II GC coupled to a HP5972 mass sensitive detector (Hewlett-Packard). Separations were performed on 5% phenyl polysilphenylene-siloxane capillary columns (BPX-5, 30 m length x 0.25 mm i.d., 0.25 µm df, SGE). Deuterated MC (MC-d6) was used as internal standard. Calibration standards were prepared in dichloromethane (RT-HEP) or methanol (RT-S9) in the presence of matrix.

Fenthion (FEN) was analyzed at a U.S. EPA laboratory (ORD/NHEERL/Mid-Continent Ecology Division) in Duluth, Minnesota, USA. The analyses were performed on an Agilent HPLC/Triple Quadrupole 6410 tandem mass spectrometer (LC/MS/MS; Agilent Technologies, Santa Clara, CA). Chromatography was carried out using an Agilent Zorbax Extend-C18 column, (3.5 micron, 2.1 x 100mm). Mobile phase A was 2% ethanol, 1% methanol, 2% 250 mM ammonium acetate in methanol, 96% water, and 0.01% of 25% ammonium hydroxide, pH 8.2. Mobile phase B was 2% ethanol, 2% water, 1% 250mM ammonium acetate in methanol, 95% methanol, and 0.01% of 25% ammonium hydroxide. The isocratic flow rate was 0.45 mL/min at 80% mobile phase B, and the injection volume was 25 µL. FEN and its internal standard FEN-d6 eluted at

4.1 min and were detected as transitions from 279 to 169 (m/z; FEN) and 285 to 169 (m/z; FEN-d6). Transitions from 279 to 247 (FEN) and 285 to 250 (FEN-d6) were used as qualifier transitions.

Samples containing 4-NP with 4NP-d4 as internal standard were analyzed by LC/MS/MS at the DuPont Haskell Global Centers for Health and Environmental Sciences, Newark, Delaware. The analyses were performed using an API 4000 triple quadrupole mass spectrometer equipped with atmospheric-pressure chemical ionization source (Applied Biosystems/MDS Sciex) USA. Chromatographic separation was achieved using a Waters Acquity UPLC system with a Kinetex C18 column (1.7µm, 2.1x100 mm; Phenomenex). Mobile phase A and B were 95%water/5%MeoH and 100% MeOH, respectively. Gradient elution was performed starting at 30% B and ramping to 99% B over 5 min, with a total run time of 5.80 min. 4NP and 4NP-d4 were detected in negative multiple reaction monitoring mode using Q1/Q3 ion transitions at m/z 119.0/105.8 and 223.0/109.8, respectively. Analyst 1.4.2 software (Applied Biosystems/MDS Sciex) was used for data acquisition and quantification.. 4NP was quantified using calibration standards with 1/x weighting and quadratic regression, and was normalized to the internal standard response. All calibration standards were prepared in acetonitrile in the presence of matrix.

Pyrene (PYR) was also analyzed at the U.S. EPA laboratory in Duluth, Minnesota, USA. The analyses were performed using an Agilent 1260 HPLC system equipped with a multiwavelength fluorescence detector. Chromatography was performed on a Hypersil Green PAH column (2.1 x 100 mm, 5 µm particle size; Thermo Scientific). The aqueous phase contained 10% acetonitrile and 90% deionized water, while the organic phase consisted of 95% acetonitrile and 5% deionized water. Samples were eluted by isocratic flow (0.5mL/min) using 55% organic phase. PYR and its internal standard Anthracene (ANTH) were detected using the following excitation/emission wavelengths: PYR - 260/390; ANTH - 260/420.

ANNEX 8: Yield and viability of thawed RT-HEP

RT-HEP lot no.	Lab	Intra-laboratory				Inter-laboratory			
		Avg. %[a] yield	CV (%)	Avg. %[a] viability	CV (%)	Avg. % yield	CV (%)	Avg. % viability	CV (%)
1	A	26.1	4.8	81.0	1.1	28.6	14.4	85.5	6.1
	B	25.0	9.0	92.9	0.9				
	C	30.3	2.1	81.7	1.3				
	D	24.8	9.0	91.2	2.3				
	E	35.4	13.4	82.4	5.8				
	F	29.8	36.8	83.6	3.6				
2	A	24.5	29.2	82.1	1.8	30.4	34.4	86.7	5.8
	B	26.1	13.1	94.5	0.7				
	C	33.0	7.6	86.4	2.2				
	D	31.3	10.1	90.5	2.9				
	E	49.1	17.9	81.4	3.9				
	F	18.6	9.8	85.5	2.7				
3	A	27.1	25.5	82.1	1.4	35.3	19.1	87.2	4.6
	B	33.1	6.9	93.1	0.5				

	C	43.4	4.0	83.8	1.6				
	D	34.8	7.4	89.9	1.5				
	E	43.3	19.1	86.1	1.6				
	F	30.2	5.4	88.1	4.3				
4	A	22.1	8.3	80.8	0.2	28.1	40.7	86.3	6.5
	B	20.5	6.9	95.1	0.7				
	C	33.5	9.1	83.8	1.2				
	D	24.4	15.2	90.7	2.8				
	E	49.0	29.0	85.9	2.4				
	F	19.1	37.7	81.5	4.5				
5	A	30.8	6.7	80.4	0.5	30.3	23.2	87.0	5.1
	B	29.5	4.0	92.8	0.7				
	C	30.5	10.4	85.6	3.1				
	D	29.2	21.5	90.8	1.7				
	E	42.0	9.4	87.2	5.0				
	F	19.9	5.5	85.0	2.8				

[a]All reported averages are based on 3 independent determinations

REFERENCES

Johanning, K., Hancock, G., Escher, B., Adekola, A., Bernhardt, M., Cowan-Ellsberry, C., Domoradski, J., Dyer, S., Eickhoff, C., Erhardt, S., Fitzsimmons, P., Halder, M., Nichols, J., Rutishauser, S., Sharpe, A., Segner, H., Schultz, I., and Embry, M. 2012. In vitro metabolism using rainbow trout liver S9. Summary report of the HESI Bioaccumulation Committee. Available at: http://www.hesiglobal.org/files/public/Committees/Bioaccumulation/Presentations%20and%20Data%20Resources/S9_report_FINAL20Nov2012.pdf

ANNEX 9: Statistical Analysis to Inform Test Guideline Study Design

Prepared by:
John W. Green, Ph.D., Ph.D.
Principal Consultant: Biostatistics, DuPont

Background

The United States and European Commission are developing of two test guidelines (TGs) focusing on fish in vitro metabolism using S9 and cryopreserved hepatocytes respectively, as well as a guidance document on how information derived from these TGs can be used for bioaccumulation assessment.

Reference chemical (pyrene) depletions are conducted with each experiment using HEP or S9. This means from each fish used, the liver S9 fraction or hepatocytes are isolated, incubated, and the parent compound is extracted at regular intervals. From these depletions, the in vitro intrinsic clearance is calculated. This is done by determining the concentrations at each time point, plotting log10 (concentration) against time, and fitting a straight line to the data. From the slope of the fitted regression line, the elimination rate constant, ke, is calculated as 2.30 x slope. The ratio elimination rate/ biological concentration is then calculated). From there, further calculations permit the prediction of the bioconcentration factor. This report is concerned only with the regression lines referenced above.

The current study design has labs performing 3 independent experiments, deriving a slope (first order log-linear depletion kinetics), and then reporting the mean value as the output of the TG. That value is then used in an extrapolation model to calculate an adjusted BCF value. The objective of this statistical analysis was to determine whether criteria could be developed to allow stopping after two experiments and to identify situations where three experiments were needed. As part of this, criteria were needed to (1) assess the quality of fit of each regression line, (2) determine whether the slopes of the regressions lines were sufficiently similar that a third experiment and regression were not needed, (3) decide when three experiments were done, whether to use all three or select two similar regressions and disregard the third. As part of objective (1), criteria were needed to identify unusual observations (outliers) that negatively impacted the quality of fit and, where found, to repeat the regression with those observations removed. Also a minimum number of points required for a regression to be considered valid were needed. The latter is needed in the event that the outlier identification process left so few observations as the render the subsequent data unrepresentative.

Methodology

The residuals about the regression line for the model logconc=time were examined for each compound, lab and day. The variance of each set of residuals was calculated. From that the distribution of such variances for each compound was developed. The 10th, 50th, and 90th percentiles (P10V, P50V, and P90V) of each variance distribution were calculated. The distribution of slope estimates was likewise characterized for each compound, yielding 10th, 50th, and 90th percentiles (P10S, P50S, and P90S). These values are given in Table 1A9 along with the median intercept value, Int, for each compound. The specific times at which LogConc was measured in the database varied somewhat among labs using the same compound and varied greatly among compounds. The observed times were approximately evenly spaced between the minimum and maximum times. In the simulations, for each compound, the seven times simulated were min+k*(max-min)/6, k=0, 1, 2, …, 6. Since only the slope is used in applications of these regressions, there was no need to vary the intercepts within the same compound, but they were varied among compounds.

Table 1A9. Observed slopes and variances

	Slopes			Intercept	Variances		
CHEM	P10	Median	P90	Median	p10	Median	p90

CSHEP	-8.11	-3.92	-1.91	0.26	0.00067	0.00776	0.05585
CSS9	-3.65	-2.36	-1.29	0.05	0.00022	0.00061	0.00613
DMHEP	-0.43	-0.30	-0.13	-0.37	0.00075	0.00316	0.00781
DMS9	-0.82	-0.65	-0.28	-0.69	0.00102	0.00805	0.03504
FENHEP	-2.26	-1.33	-0.66	-0.97	0.00032	0.00224	0.00760
FENS9	-6.02	-3.92	-1.22	-0.86	0.00023	0.00112	0.00860
MCHEP	-0.09	-0.07	-0.04	-0.45	0.00020	0.00067	0.00186
MCS9	-0.16	-0.14	-0.12	-0.50	0.00009	0.00040	0.00082
NPHEP	-0.78	-0.47	-0.32	0.35	0.00034	0.00080	0.00137
NPS9	-11.42	-0.35	-0.24	0.39	0.00011	0.00047	0.00170
PYRHEP	-1.90	-1.58	-0.66	-1.69	0.00031	0.00164	0.00606
PYRS9	-10.63	-8.68	-5.63	-1.61	0.00082	0.00334	0.01180

Table 2A9. Observed times of measurement

Compound	time1	time2	time3	time4	time5	time6	time7
CSHEP	0.03	0.15	0.26	0.37	0.48	0.60	0.71
CSS9	0.03	0.09	0.14	0.20	0.26	0.31	0.37
DMHEP	0.03	0.36	0.69	1.02	1.34	1.67	2.00
DMS9	0.03	0.19	0.36	0.52	0.68	0.84	1.00
FENHEP	0.03	0.17	0.30	0.43	0.57	0.70	0.83
FENS9	0.03	0.07	0.10	0.13	0.17	0.20	0.23
MCHEP	0.03	0.71	1.39	2.07	2.74	3.42	4.10
MCS9	0.03	0.69	1.36	2.02	2.68	3.34	4.00
NPHEP	0.03	0.17	0.30	0.44	0.57	0.71	0.84
NPS9	0.02	0.15	0.29	0.43	0.56	0.70	0.83
PYRHEP	0.03	0.16	0.30	0.43	0.56	0.70	0.83
PYRS9	0.03	0.14	0.24	0.35	0.46	0.56	0.67

Table 3A9 provides a summary of one measure of the quality of the regression lines, namely the R-square or adjusted R-square value. The value of this table is to serve as a guideline for future experiments. Should the R-square value for a future regression line be low compared to the values established in the development of this guideline, e.g., fall below the 10th percentile of the distribution or below 85%, then consideration should be given to omitting that line from further use.

Table 3A9. Quality of regression lines

	R-Square					RMSE				
chem	p10r	p25r	p50r	p75r	p90r	p10s	p25s	p50s	p75s	p90s
CSHEP	0.93	0.95	0.97	0.98	0.99	0.028	0.035	0.095	0.182	0.259
CSS9	0.86	0.96	0.97	0.98	0.99	0.016	0.020	0.027	0.052	0.086
DMHEP	0.63	0.84	0.95	0.97	0.98	0.030	0.044	0.062	0.080	0.097
DMS9	0.38	0.75	0.83	0.94	0.98	0.035	0.061	0.098	0.140	0.239
FENHEP	0.97	0.98	0.98	0.99	1.00	0.020	0.038	0.052	0.069	0.097
FENS9	0.06	0.95	0.99	0.99	1.00	0.017	0.024	0.037	0.067	0.102
MCHEP	0.76	0.89	0.95	0.97	0.98	0.016	0.019	0.028	0.037	0.047
MCS9	0.98	0.99	0.99	1.00	1.00	0.010	0.012	0.022	0.025	0.031
NPHEP	0.91	0.95	0.96	0.98	0.99	0.020	0.023	0.031	0.036	0.041
NPS9	0.78	0.91	0.95	0.97	0.99	0.012	0.019	0.024	0.033	0.045
PYRHEP	0.85	0.92	0.98	1.00	1.00	0.019	0.030	0.050	0.079	0.106
PYRS9	0.98	0.99	0.99	1.00	1.00	0.038	0.041	0.071	0.099	0.130

pXr=xth percentile of R-square distribution, x=10, 25, 50, 75, 90

Table 3A9 indicates that for most experiments, the R-square values were generally greater than 85%, though those for DMS9 and FENS9 included a small proportion of notably low values. The use of adjusted R-square does not alter this assessment in an appreciable manner. The variability in the data is displayed as the root-mean-squared error, RMSE, given on the right of Table 3. This is an indication of the standard deviation of the data about the regression line. It will be clear from the RMSE values that the low R-square values for FENS9 was not attributable to high variance. It should be observed, for example, that the highest RMSE value was for PYRS9, which did not have low R-square values. Thus, the utility of the distribution of RMSE values is limited.

Simulation Study

Data were simulated for each combination of PxV and PyS for each compound using the median intercept for that compound, where each of x and y ranged over 10, 50, 90 independently of each other for days 1 and 2, treating days 1 and 2 as independent realizations of the same set of conditions. Seven measurement times are simulated for each day. Those times varied by compound and are given in Table 2. The data were generated first as random normal variates with mean zero and variance PxV, then that value was added to the response predicted by the equation LogConc= Intercept+PxS*Time. This process simulated random variation about the regression line. The estimated slope can be compared to the slope used as a basis for the simulation, PyS. This process simulated repeated studies under nominally the same conditions and captures the variability observed in the available data.

The slope estimates for the day 1 and day 2 lines were compared. (Test for this comparison is provided below.) If they were not significantly different, then the mean slope of these two was reported and sampling stopped. Otherwise, a day 3 dataset was generated and the three slopes were compared and the mean slope of the three was calculated or one slope was discarded and the slope of the two that agree was reported. An assessment was made whether all three slopes agreed or whether one of them was sufficiently different from the other two as to be ignored. The mean of the two or three retained slopes was calculated and reported.

What is reported here is the proportion of datasets that stop after two days, the distribution of slope estimates when sampling stops after 2 days, the distribution of slope estimates when sampling continues for three days, the proportion of datasets that are sampled for three days but one of the three slope estimates is discarded due to deviation from the other two.

In Tables 4A9-9A9, by comparing the various percentiles and mean to the slope simulated (tslope), it is possible to get a good idea of the ability to estimate the true slope under the conditions present during the development of the guideline. The spread from p10 to p90 indicates the tightness (or lack thereof) of the estimated slopes, given the variability of the data. It is evident that the true slope had little impact on the ability to estimate the slope. It will be observed also that increased variance (vp=90 vs vp=10) was associated with an increased spread (e.g., p90 increased and p10 decreased) in slope estimates (difference between p10 and p90 in slope distribution), though it was not dramatic.

Table 10A9 does the same for those data where experiments were run on all three days using CS. Little difference was found between Tables 9 and 4. Similar results were found for the other compounds and are not provided in this summary.

The simulations indicate that in 85-90% of situations, it should be possible to stop sampling after two days. There is no appreciable loss of information or quality of estimation from doing so.

Following Table 10A9 are rules used for deciding (1) whether to stop after two days, (2) whether all three days are used when sampling continues or selecting the best two, (3) what constitutes an outlier to be omitted and justifies fitting a new regression line with outliers omitted. As to the minimum number of observations required to fit a reliable regression line, further simulations are under way to address that in depth. For the present, it seems unwise to use fewer than four observations and the rules provided rarely found more than two outliers and often found none in the data provided.

Table 4A9. Summary of regressions after two days: Compound=CS, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
Cshep	895	-8.108	-8.109	-8.068	-8.088	-8.108	-8.128	-8.148	10	10
Cshep	861	-8.110	-8.109	-7.969	-8.035	-8.115	-8.181	-8.249	10	50
Cshep	875	-8.114	-8.109	-7.761	-7.904	-8.122	-8.308	-8.482	10	90
Cshep	888	-3.916	-3.916	-3.877	-3.895	-3.915	-3.937	-3.954	50	10
Cshep	889	-3.916	-3.916	-3.786	-3.849	-3.916	-3.984	-4.047	50	50
Cshep	884	-3.917	-3.916	-3.528	-3.729	-3.914	-4.123	-4.287	50	90
Cshep	873	-1.913	-1.911	-1.875	-1.894	-1.915	-1.932	-1.949	90	10
Cshep	867	-1.915	-1.911	-1.778	-1.847	-1.913	-1.985	-2.051	90	50
Cshep	869	-1.912	-1.911	-1.569	-1.715	-1.905	-2.103	-2.249	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
Css9	875	-3.650	-3.649	-3.605	-3.627	-3.650	-3.674	-3.696	10	10
Css9	882	-3.650	-3.649	-3.577	-3.613	-3.650	-3.691	-3.720	10	50
Css9	872	-3.655	-3.649	-3.429	-3.532	-3.660	-3.777	-3.872	10	90
Css9	879	-2.355	-2.357	-2.313	-2.334	-2.354	-2.377	-2.399	50	10
Css9	872	-2.359	-2.357	-2.280	-2.320	-2.360	-2.399	-2.433	50	50
Css9	872	-2.367	-2.357	-2.124	-2.248	-2.370	-2.489	-2.598	50	90
Css9	870	-1.294	-1.294	-1.248	-1.269	-1.295	-1.319	-1.339	90	10
Css9	869	-1.294	-1.294	-1.222	-1.255	-1.290	-1.334	-1.368	90	50
Css9	852	-1.294	-1.294	-1.060	-1.176	-1.291	-1.422	-1.532	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 5A9. Summary of regressions after two days: Compound=MC, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
MChep	873	-0.091	-0.091	-0.087	-0.089	-0.091	-0.093	-0.095	10	10
MChep	882	-0.091	-0.091	-0.084	-0.087	-0.091	-0.094	-0.097	10	50
MChep	880	-0.090	-0.091	-0.080	-0.085	-0.090	-0.096	-0.101	10	90
MChep	857	-0.067	-0.067	-0.063	-0.065	-0.067	-0.069	-0.071	50	10
MChep	872	-0.067	-0.067	-0.061	-0.064	-0.067	-0.070	-0.073	50	50
MChep	874	-0.067	-0.067	-0.055	-0.061	-0.067	-0.073	-0.078	50	90
MChep	880	-0.040	-0.040	-0.036	-0.038	-0.040	-0.042	-0.044	90	10
MChep	881	-0.040	-0.040	-0.033	-0.036	-0.040	-0.043	-0.046	90	50
MChep	878	-0.040	-0.040	-0.029	-0.034	-0.040	-0.046	-0.051	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
MCs9	875	-0.157	-0.157	-0.155	-0.156	-0.158	-0.159	-0.160	10	10
MCs9	876	-0.158	-0.157	-0.152	-0.155	-0.158	-0.160	-0.163	10	50
MCs9	856	-0.158	-0.157	-0.150	-0.154	-0.158	-0.162	-0.165	10	90
MCs9	873	-0.141	-0.141	-0.138	-0.139	-0.141	-0.142	-0.143	50	10
MCs9	872	-0.141	-0.141	-0.136	-0.138	-0.141	-0.144	-0.146	50	50
MCs9	868	-0.141	-0.141	-0.134	-0.137	-0.141	-0.145	-0.148	50	90
MCs9	880	-0.119	-0.119	-0.117	-0.118	-0.119	-0.121	-0.122	90	10
MCs9	870	-0.120	-0.119	-0.114	-0.117	-0.120	-0.123	-0.124	90	50
MCs9	897	-0.120	-0.119	-0.112	-0.116	-0.119	-0.123	-0.127	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 6A9. Summary of regressions after two days: Compound=DM, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
DMhep	885	-0.432	-0.433	-0.418	-0.425	-0.433	-0.440	-0.447	10	10
DMhep	867	-0.434	-0.433	-0.405	-0.418	-0.434	-0.449	-0.463	10	50
DMhep	881	-0.434	-0.433	-0.388	-0.411	-0.433	-0.456	-0.479	10	90
DMhep	849	-0.300	-0.300	-0.286	-0.293	-0.300	-0.308	-0.314	50	10
DMhep	882	-0.300	-0.300	-0.269	-0.286	-0.301	-0.316	-0.329	50	50
DMhep	859	-0.302	-0.300	-0.255	-0.279	-0.301	-0.326	-0.348	50	90
DMhep	857	-0.131	-0.131	-0.117	-0.124	-0.132	-0.139	-0.146	90	10
DMhep	865	-0.132	-0.131	-0.102	-0.117	-0.133	-0.148	-0.161	90	50
DMhep	867	-0.132	-0.131	-0.085	-0.108	-0.132	-0.156	-0.177	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
DMs9	873	-0.823	-0.823	-0.788	-0.804	-0.823	-0.842	-0.859	10	10
DMs9	876	-0.820	-0.823	-0.728	-0.767	-0.817	-0.873	-0.919	10	50
DMs9	863	-0.827	-0.823	-0.628	-0.725	-0.822	-0.926	-1.030	10	90
DMs9	876	-0.645	-0.647	-0.612	-0.628	-0.647	-0.662	-0.679	50	10
DMs9	868	-0.652	-0.647	-0.561	-0.603	-0.650	-0.699	-0.745	50	50
DMs9	861	-0.652	-0.647	-0.442	-0.549	-0.648	-0.763	-0.860	50	90
DMs9	871	-0.282	-0.281	-0.247	-0.264	-0.283	-0.300	-0.314	90	10
DMs9	874	-0.279	-0.281	-0.189	-0.232	-0.275	-0.330	-0.370	90	50
DMs9	863	-0.286	-0.281	-0.094	-0.182	-0.271	-0.397	-0.494	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 7A9. Summary of regressions after two days: Compound=FEN, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
FENhep	870	-2.260	-2.260	-2.237	-2.248	-2.260	-2.273	-2.282	10	10
FENhep	867	-2.260	-2.260	-2.200	-2.229	-2.260	-2.290	-2.322	10	50
FENhep	863	-2.260	-2.260	-2.143	-2.203	-2.260	-2.318	-2.375	10	90
FENhep	885	-1.332	-1.332	-1.310	-1.319	-1.332	-1.345	-1.356	50	10
FENhep	860	-1.332	-1.332	-1.272	-1.298	-1.330	-1.362	-1.391	50	50
FENhep	874	-1.324	-1.332	-1.210	-1.265	-1.321	-1.384	-1.436	50	90
FENhep	871	-0.663	-0.664	-0.640	-0.651	-0.663	-0.676	-0.686	90	10
FENhep	868	-0.660	-0.664	-0.596	-0.628	-0.662	-0.692	-0.720	90	50
FENhep	883	-0.660	-0.664	-0.550	-0.598	-0.659	-0.722	-0.774	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
FENs9	873	-6.016	-6.017	-5.936	-5.972	-6.016	-6.057	-6.094	10	10
FENs9	872	-6.015	-6.017	-5.841	-5.922	-6.016	-6.103	-6.193	10	50
FENs9	874	-6.004	-6.017	-5.509	-5.752	-6.007	-6.257	-6.474	10	90
FENs9	874	-3.922	-3.920	-3.843	-3.884	-3.923	-3.959	-3.999	50	10
FENs9	875	-3.913	-3.920	-3.732	-3.824	-3.923	-4.006	-4.084	50	50
FENs9	869	-3.911	-3.920	-3.443	-3.658	-3.897	-4.152	-4.399	50	90
FENs9	872	-1.220	-1.220	-1.147	-1.181	-1.219	-1.257	-1.300	90	10
FENs9	876	-1.222	-1.220	-1.051	-1.135	-1.226	-1.311	-1.383	90	50
FENs9	879	-1.224	-1.220	-0.757	-0.969	-1.223	-1.463	-1.695	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 8A9. Summary of regressions after two days: Compound=NP, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
NPhep	860	-0.784	-0.783	-0.761	-0.772	-0.784	-0.796	-0.807	10	10
NPhep	871	-0.782	-0.783	-0.746	-0.763	-0.782	-0.801	-0.819	10	50
NPhep	872	-0.784	-0.783	-0.735	-0.758	-0.785	-0.808	-0.833	10	90
NPhep	868	-0.472	-0.471	-0.447	-0.459	-0.473	-0.485	-0.495	50	10
NPhep	877	-0.471	-0.471	-0.437	-0.453	-0.470	-0.489	-0.506	50	50
NPhep	855	-0.473	-0.471	-0.424	-0.446	-0.473	-0.499	-0.519	50	90
NPhep	866	-0.322	-0.322	-0.299	-0.310	-0.322	-0.335	-0.346	90	10
NPhep	889	-0.321	-0.322	-0.284	-0.304	-0.323	-0.340	-0.356	90	50
NPhep	853	-0.320	-0.322	-0.274	-0.295	-0.321	-0.345	-0.368	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
NPs9	871	-11.419	-11.419	-11.404	-11.411	-11.418	-11.426	-11.433	10	10
NPs9	882	-11.418	-11.419	-11.391	-11.403	-11.418	-11.433	-11.445	10	50
NPs9	855	-11.419	-11.419	-11.365	-11.393	-11.420	-11.446	-11.468	10	90
NPs9	888	-0.351	-0.350	-0.339	-0.344	-0.351	-0.358	-0.365	50	10
NPs9	874	-0.350	-0.350	-0.321	-0.335	-0.351	-0.365	-0.376	50	50
NPs9	882	-0.349	-0.350	-0.299	-0.322	-0.350	-0.375	-0.401	50	90
NPs9	880	-0.237	-0.237	-0.224	-0.230	-0.236	-0.244	-0.251	90	10
NPs9	882	-0.235	-0.237	-0.207	-0.221	-0.236	-0.251	-0.262	90	50
NPs9	872	-0.236	-0.237	-0.185	-0.207	-0.235	-0.264	-0.288	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 9A9. Summary of regressions after two days: Compound=PYR, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
PYRhep	864	-1.896	-1.895	-1.874	-1.884	-1.896	-1.907	-1.919	10	10
PYRhep	872	-1.895	-1.895	-1.843	-1.866	-1.894	-1.921	-1.948	10	50
PYRhep	881	-1.894	-1.895	-1.794	-1.844	-1.892	-1.946	-1.994	10	90
PYRhep	867	-1.582	-1.582	-1.559	-1.571	-1.582	-1.592	-1.604	50	10
PYRhep	861	-1.581	-1.582	-1.531	-1.552	-1.580	-1.608	-1.631	50	50
PYRhep	856	-1.580	-1.582	-1.473	-1.528	-1.581	-1.632	-1.681	50	90
PYRhep	881	-0.664	-0.665	-0.642	-0.652	-0.664	-0.675	-0.688	90	10
PYRhep	885	-0.666	-0.665	-0.612	-0.638	-0.666	-0.695	-0.720	90	50
PYRhep	852	-0.665	-0.665	-0.562	-0.611	-0.664	-0.720	-0.773	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
PYRs9	866	-10.626	-10.626	-10.578	-10.600	-10.627	-10.651	-10.672	10	10
PYRs9	888	-10.626	-10.626	-10.536	-10.576	-10.626	-10.675	-10.719	10	50
PYRs9	853	-10.628	-10.626	-10.461	-10.540	-10.629	-10.710	-10.791	10	90
PYRs9	860	-8.684	-8.684	-8.640	-8.661	-8.684	-8.706	-8.730	50	10
PYRs9	848	-8.683	-8.684	-8.586	-8.633	-8.685	-8.733	-8.779	50	50
PYRs9	866	-8.686	-8.684	-8.518	-8.604	-8.686	-8.770	-8.853	50	90
PYRs9	865	-5.632	-5.632	-5.588	-5.608	-5.633	-5.655	-5.679	90	10
PYRs9	882	-5.632	-5.632	-5.538	-5.584	-5.633	-5.680	-5.722	90	50
PYRs9	887	-5.627	-5.632	-5.446	-5.531	-5.627	-5.725	-5.813	90	90

Stop#=number (out of 1000 simulated datasets) when only two days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Table 10A9. Summary of regressions over three days: Compound=CS, Times=7

Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
Cshep	105	-8.109	-8.109	-8.075	-8.090	-8.109	-8.123	-8.147	10	10
Cshep	139	-8.096	-8.109	-7.988	-8.042	-8.100	-8.162	-8.193	10	50
Cshep	125	-8.099	-8.109	-7.770	-7.918	-8.076	-8.280	-8.400	10	90
Cshep	112	-3.911	-3.916	-3.876	-3.892	-3.908	-3.929	-3.947	50	10
Cshep	111	-3.921	-3.916	-3.809	-3.848	-3.929	-3.989	-4.072	50	50
Cshep	116	-3.890	-3.916	-3.539	-3.716	-3.875	-4.061	-4.271	50	90
Cshep	127	-1.911	-1.911	-1.868	-1.892	-1.909	-1.930	-1.956	90	10
Cshep	133	-1.925	-1.911	-1.818	-1.861	-1.916	-1.982	-2.049	90	50
Cshep	131	-1.905	-1.911	-1.587	-1.752	-1.907	-2.046	-2.195	90	90
Chem	Stop#	mean	tslope	p90	p75	p50	p25	p10	sp	vp
Css9	125	-3.652	-3.649	-3.611	-3.629	-3.652	-3.673	-3.700	10	10
Css9	118	-3.649	-3.649	-3.574	-3.614	-3.649	-3.683	-3.721	10	50
Css9	128	-3.640	-3.649	-3.414	-3.517	-3.649	-3.754	-3.880	10	90
Css9	121	-2.357	-2.357	-2.316	-2.336	-2.353	-2.380	-2.403	50	10
Css9	128	-2.359	-2.357	-2.295	-2.327	-2.357	-2.395	-2.432	50	50
Css9	128	-2.354	-2.357	-2.133	-2.240	-2.365	-2.456	-2.573	50	90
Css9	130	-1.293	-1.294	-1.257	-1.269	-1.292	-1.316	-1.333	90	10
Css9	131	-1.287	-1.294	-1.225	-1.257	-1.288	-1.316	-1.356	90	50
Css9	148	-1.280	-1.294	-1.074	-1.155	-1.259	-1.405	-1.512	90	90

Stop#=number (out of 1000 simulated datasets) when all three days were needed
Sp=10, 50, or 90 is the percentile of the slope distribution (Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Table 1) used for simulation
pX, x=10, 25, 50, 75, 90 is the indicated percentile of slopes from simulated data
tslope is the slope that was simulated
mean=mean of slopes from simulated data

Rule for stopping after 2 days

Compare the slopes using a t-test. If they are not different, stop. If they are different, continue with the third day. The formula for this t-test is

$$T = \frac{Slope_1 - Slope_2}{\sqrt{SE_1^2 + SE_2^2}}, \quad (1)$$

where SE_1 is the standard error of the estimated slope, $Slope_1$, from the first regression line. If the absolute value of T is less than 2, then stop: There is no need to repeat the study on day 3.

Rule for deciding which 2 or 3 slope values to use if the study is done on three days.

Compute the T-test in (1) for all pairs of slopes, 1 and 2, 1 and 3, 2 and 3. This step was reached because 1 and 2 were different by Rule ii). If 1 and 3 are not different, use just those two slopes. If 2 and 3 are not different, then use just those two slopes. Otherwise, use all three slopes.

NOTE: It is possible in some software, such as the REG procedure in SAS, to develop a single model that includes all three days and automate the comparison of slopes.

Rule used for determining outliers to omit from a regression.

There are simple ways to identify outliers. Most of them require calculating the residuals from regression. The i^{th} residual, e_i, is the difference between the observed LogConc, Y_i, and that predicted by the regression line, $\hat{Y}_i$, so

$$e_i = Y_i - \hat{Y}_i. \quad (2)$$

Then calculate the lower and upper quartiles, Q1 and Q3, of the set of these residuals from single fitted regression model. From that, calculate the inter-quartile range, IQR=Q3-Q1. Any residual greater than Q3+1.5*IQR or less than Q1-1.5*IQR is an outlier. No software is needed to do this. This is a good general rule for outlier detection called Tukey's rule. For small datasets, it does not find many outliers.

For such small datasets as in this application (seven observations), the simple method is of limited value. With small datasets: Exclude any observation where DFFITS>1 or abs(rstudent)>2 or abs(dfbetas_time)> $2/\sqrt{n}$, where rstudent=studentized residual and is a measure of the deviation of an observation from the fitted regression line; dffits and dfbetas_time are functions describing the impact on the overall goodness of fit and the slope estimate, respectively, of omitting the given observation. Definitions of these terms are provided below. All of these are automatically calculated (if requested) by the REG procedure in SAS and in some other software packages. Minitab has DFFITS and standardized residuals (similar to studentized). These functions are available in R, also. Unfortunately, Excel does not include these functions, so the definitions are provided below to allow programming.

a) Hat matrix. The entire matrix is not needed, just the diagonal term corresponding to the ith observation. This given by

$$h_i = \frac{1}{n} + \frac{(x_i - \bar{x})^2}{SS_x}, \quad (3)$$

where $SS_x = \sum_{i=1}^{n}(x_i - \bar{x})^2$, (4)

and $x_1, x_2, \ldots, x_n$ are the measurement times, $\bar{x}$ is the mean measurement time, and n is the number of measurement times (n=7 in the data provided).

b) DFFITS

$$DFFITS = \frac{h_i e_i}{1-h_i}, \quad (5)$$

where $e_i = Y_i - \hat{Y}_i$ is the ith residual, that is, the difference between the observed LogConc, Y_i, and that predicted by the regression line, $\hat{Y}_i$.

A value of DFFITS greater than 1 in absolute value is an outlier.

c) Studentized residual

$$RSTUD_i = \frac{e_i}{s(i)\sqrt{1-h_i}}, \quad (6)$$

where s(i) is obtained by solving the following for $s^2(i)$ and taking the square-root.

$$(n-2)s^2(i) = (n-1)MSE - \frac{e_i^2}{1-h_i}. \quad (7)$$

An absolute value of $RSTUD_i > 2$ is an outlier.

d) DFBETAS

This is another diagnostic that is focused specifically on whether an outlier has a notable effect on the slope of the regression line.

$$DFBETAS_{Slope} = \frac{x_i - \bar{x}}{SS_x}\frac{e_i}{1-h_i}. \quad (8)$$

A value of DFBETAS_Slope greater than $2/\sqrt{n}$ in absolute value is an outlier.

Minimum number of points required for a regression

This analysis considers the minimum number of points required for a regression to be considered valid. This is needed in the event that the outlier identification process left so few observations as the render the subsequent data unrepresentative. There are seven sampling times in the current design, which will be referred to as Plan A. If outliers are removed to provide a good fit of the regression line, the number of points used will be less than seven. Clearly, if only one point remains, no line can be fit at all. How many points can be removed without degrading the quality of the regression beyond acceptability? To explore this issue, three additional simulations were done. In Plan T, experiments were simulated to include only 5 sampling times but spaced so as to cover the same range as the current seven sampling time design covers. In Plan X17, seven sampling times were simulated and the first and last times were excluded. In Plan X12, seven sampling times were simulated and times 1 and 2 were excluded. In every case, only five sampling

times were included in the regression. A similar procedure could be followed with a total of four sampling times used in the regression, but that has not yet been done and is probably not needed.
Tables 11A9-16A9 summarize the findings. Rather than present tables analogous to Tables 4-9 of Part 1, it seems more informative to compare the variability in the estimated slopes in a different way. The spread between the 10th and 90th percentiles, P10 to P90, of the distribution of slope estimates is a measure of the uncertainty in the slope estimates. To compare two different sampling schemes, then, one can compare the width of the intervals (P10, P90) under the different schemes. This is presented in separate tables for each compound.
It is interesting that the success rate of stopping after the first two runs is essentially unchanged under sampling Plans T, X12, and X17 as under Plan A. The difference is in the uncertainty of the slope estimates.

Table 11A9. Comparison of sampling schemes for compound CS

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
CSHEP	10	10	1.14	1.73	1.79
CSHEP	10	50	1.13	1.65	1.68
CSHEP	10	90	1.12	1.82	1.67
CSHEP	50	10	1.21	1.82	1.85
CSHEP	50	50	1.25	1.80	1.81
CSHEP	50	90	1.11	1.70	1.79
CSHEP	90	10	1.30	1.89	1.77
CSHEP	90	50	1.26	1.83	1.67
CSHEP	90	90	1.31	1.87	1.81
CSS9	10	10	1.20	1.72	1.75
CSS9	10	50	1.31	2.10	1.88
CSS9	10	90	1.32	1.93	2.00
CSS9	50	10	1.28	1.94	1.72
CSS9	50	50	1.10	1.78	1.74
CSS9	50	90	1.24	1.92	1.79
CSS9	90	10	1.19	1.72	1.67
CSS9	90	50	1.24	1.95	1.87
CSS9	90	90	1.25	1.86	1.90

Sp=10, 50, or 90 is the percentile of the slope distribution (Part 1, Table 1) used for simulation
vp=10, 50, or 90 is the percentile of the variance distribution (Part 1, Table 1) used for simulation
RATIOTA=(P90T-P10T)/(P90A-P10A), where
i) P90T=90th percentile of slope distribution under Plan T
ii) P90A=90th percentile of slope distribution under Plan A

RATIOX17A=(P90X17-P10X17)/(P90A-P10A), where
i) P90X17=90th percentile of slope distribution under Plan X17, so the times used reduce the range covered compared to Plan A

RATIOX17A==(P90X17-P10X17)/(P90A-P10A), where
i) P90X12=90th percentile of slope distribution under Plan X12, so again, the times used reduce the range covered compared to Plan A

So, the variability in slope estimates arising from plan T is 11-32% greater than the slope estimates in the full seven point sampling scheme. The variability in Plan X17 is 65-110% greater and the variability in Plan X17 is 67 to 100% more variable than the original seven point plan. It is doubtless true that a plan that excludes three sampling

points from the extreme range of sampling times (e.g., times 1-3, times 1,2, and 7, times 1, 6, and 7, or times 5, 6, and 7) will produce more variable slope estimates. If interior times are removed, there should be less impact on the slope estimate, similar to that under Plan T. These results are consistent with well understood properties of regression modeling, where better results generally come from fitting a line (or curve) to a wide range of explanatory variables compared to a narrow range.

Table 12A9. Comparison of sampling schemes for compound DM

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
DMHEP	10	10	1.14	1.70	1.80
DMHEP	10	50	1.25	1.80	1.79
DMHEP	10	90	1.20	1.98	1.78
DMHEP	50	10	1.22	1.87	1.82
DMHEP	50	50	1.12	1.78	1.78
DMHEP	50	90	1.17	1.69	1.79
DMHEP	90	10	1.15	1.75	1.77
DMHEP	90	50	1.16	1.69	1.77
DMHEP	90	90	1.18	1.80	1.90
DMS9	10	10	1.13	1.58	1.67
DMS9	10	50	1.21	1.79	1.91
DMS9	10	90	1.13	1.82	1.78
DMS9	50	10	1.17	1.78	1.64
DMS9	50	50	1.25	1.77	1.97
DMS9	50	90	1.12	1.74	1.67
DMS9	90	10	1.24	1.78	1.83
DMS9	90	50	1.25	1.90	1.85
DMS9	90	90	1.27	1.76	1.82

Table 13A9. Comparison of sampling schemes for compound FEN

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
FENHEP	10	10	1.24	1.84	1.78
FENHEP	10	50	1.15	1.83	1.82
FENHEP	10	90	1.16	1.69	1.68
FENHEP	50	10	1.22	1.76	1.84
FENHEP	50	50	1.26	1.87	1.77
FENHEP	50	90	1.17	1.67	1.77
FENHEP	90	10	1.14	1.80	1.75
FENHEP	90	50	1.21	1.76	1.75
FENHEP	90	90	1.17	1.90	1.72
FENS9	10	10	1.18	1.75	1.75
FENS9	10	50	1.15	1.83	1.77
FENS9	10	90	1.12	1.85	1.71
FENS9	50	10	1.20	1.77	1.81
FENS9	50	50	1.15	1.80	1.79
FENS9	50	90	1.17	1.82	1.77

FENS9	90	10	1.22	1.85	1.86
FENS9	90	50	1.20	1.83	1.78
FENS9	90	90	1.20	1.86	1.84

Table 14A9. Comparison of sampling schemes for compound MC

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
MCHEP	10	10	1.13	1.72	1.73
MCHEP	10	50	1.17	1.78	1.79
MCHEP	10	90	1.21	1.71	1.83
MCHEP	50	10	1.12	1.75	1.74
MCHEP	50	50	1.26	1.93	1.99
MCHEP	50	90	1.21	1.82	1.72
MCHEP	90	10	1.11	1.69	1.65
MCHEP	90	50	1.22	1.84	1.61
MCHEP	90	90	1.21	1.83	1.82
MCS9	10	10	1.24	1.72	1.76
MCS9	10	50	1.11	1.74	1.72
MCS9	10	90	1.19	1.78	1.76
MCS9	50	10	1.03	1.76	1.67
MCS9	50	50	1.20	1.70	1.64
MCS9	50	90	1.21	1.86	1.91
MCS9	90	10	1.25	1.87	1.88
MCS9	90	50	1.24	1.80	1.79
MCS9	90	90	1.20	1.81	1.83

Table 15A9. Comparison of sampling schemes for compound NP

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
NPHEP	10	10	1.14	1.82	1.72
NPHEP	10	50	1.16	1.73	1.75
NPHEP	10	90	1.16	1.72	1.69
NPHEP	50	10	1.13	1.66	1.68
NPHEP	50	50	1.22	1.80	1.78
NPHEP	50	90	1.26	1.71	1.70
NPHEP	90	10	1.19	1.78	1.78
NPHEP	90	50	1.21	1.79	1.82
NPHEP	90	90	1.18	1.83	1.83
NPS9	10	10	1.07	1.66	1.66
NPS9	10	50	1.17	1.75	1.76
NPS9	10	90	1.25	1.80	1.84
NPS9	50	10	1.24	1.92	1.81
NPS9	50	50	1.21	1.76	1.76
NPS9	50	90	1.24	1.85	1.81
NPS9	90	10	1.19	1.76	1.71
NPS9	90	50	1.17	1.82	1.81
NPS9	90	90	1.21	1.83	1.81

Table 16A9. Comparison of sampling schemes for compound PRY

COMPOUND	sp	vp	RATIOTA	RATIOX17A	RATIOX12A
PYRHEP	10	10	1.19	1.87	1.75
PYRHEP	10	50	1.20	1.78	1.77
PYRHEP	10	90	1.22	1.87	1.78
PYRHEP	50	10	1.20	1.86	1.63
PYRHEP	50	50	1.27	1.96	1.75
PYRHEP	50	90	1.14	1.79	1.78
PYRHEP	90	10	1.24	1.78	1.73
PYRHEP	90	50	1.15	1.63	1.77
PYRHEP	90	90	1.10	1.63	1.59
PYRS9	10	10	1.18	1.69	1.82
PYRS9	10	50	1.20	1.79	1.80
PYRS9	10	90	1.23	1.87	1.86
PYRS9	50	10	1.25	1.86	1.80
PYRS9	50	50	1.17	1.68	1.76
PYRS9	50	90	1.23	1.91	1.85
PYRS9	90	10	1.18	1.79	1.79
PYRS9	90	50	1.19	1.81	1.76
PYRS9	90	90	1.18	1.78	1.56

ANNEX 10: Empirical BCF values for 6 test chemicals

Table 7 of the Study Report shows a comparison of calculated BCF values using the BCFBAF model (BCF regression model as well as the Arnot and Gobas BCF model with and without biotransformation rate predictions), predicted BCFs using $CL_{IN\ VITRO,\ INT}$ in both test systems with two binding assumptions, and measured *in vivo* BCF values. It is important to note that there are known and well-documented *in vivo* BCF data quality issues, and that methods for evaluating BCF data confidence are critical for identifying sources of uncertainty and error in the measured values (e.g., Arnot and Gobas, 2006; Parkerton et al., 2008). The table below summarizes some critical data quality considerations for *in vivo* BCF measurements that should be taken into account when comparing with the *in vitro* to *in vivo* extrapolated (modelled) BCFs for the ring test chemicals in Table 7 of the Study Report. Additional evaluation criteria for many of the studies can be found in Arnot and Gobas, 2006.

Chemical	Study	Species	BCF (L/kg)	Comments
Pyrene	Jonsson et al., 2004	Sheepshead minnow	78 (Average from 4 measurements / studies)	Mixtures study; Arnot & Gobas database quality score = 1; two exposure concentrations
	Carlson et al., 1979	Fathead minnow	1578 (Average from 5 measurements / studies)	Mixture toxicity study (6 PAHs); concentrations (total PAH) near chronic baseline toxicity range; Issues with maintaining consistent water concentrations during the study.
	Ogata et al., 1984	Goldfish	N/A	Test concentration above water solubility Uncertain exposure duration; reported at "steady state"
	deVoogt et al	Guppy	N/A	Chemical not measured in fish – "Banerjee method" Water concentration only measured at the end of exposure duration
4-NP	Snyder et al., 2001	Fathead minnow	344 (Average from 3 measurements / studies)	Arnot & Gobas database quality score = 1
	Giesy et al., 2000	Fathead minnow	240 (Average from 3 measurements / studies)	Arnot & Gobas database quality score = 1
	Ekelund et al., 1990	Stickleback	N/A	Used total radiolabel with no correction for parent; insufficient exposure duration

Chemical	Study	Species	BCF (L/kg)	Comments
	McLeese et al., 1981	Atlantic salmon	N/A	Used total radiolabel with no correction for parent; insufficient exposure duration
Fenthion	Tsuda et al., 1993, 1996, 1995, 1997	Medaka (high-eyes) Guppy Goldfish Killifish Carp	185 (Average from 36 studies / measurements)	Some studies performed on pesticide mixtures; excluded those with low data quality scores in Arnot & Gobas
	DeBruijn and Hermens, 1991	Guppy	16,600	Mixture study with 15 pesticides; log BCF (lipid) > log Kow; high uncertainty
Cyclohexyl salicylate	Laue et al., 2014 (RIFM study)	Zebrafish	400 (Average from 2 measurements)	OECD 305 TG study; range includes all BCF values (i.e., BCF_{ss} and BCF_k) normalized to a 5% lipid content fish
Deltamethrin	Muir et al., 1994	Rainbow Trout	115	Measured for rainbow trout based on measured parent chemical; concentration; Arnot & Gobas database quality score = 1
	USEPA RED for Delta (Nov 2016) – MRID Schocken, 1993	Bluegill	698 BCFss (reported)	Used total radiolabel with no correction for parent
	Fackler, 1990	Bluegill	1400 Whole body tissue	Used total radiolabel with no correction for parent Not growth or lipid normalized
	Schettgen, 2000 [thesis]	Rainbow trout	860	Exposure concentrations within a factor of 2 of the acute LC50 values; well above OECD TG recommendations; Relative STDEV of the BCF measurements high (~43%)
Methoxychlor	Hansen & Parrish, 1977	Sheepshead minnow	174	Arnot & Gobas database quality score = 1

REFERENCES

Arnot, J.A., and Gobas, F.A.P.C. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessment for organic chemicals in aquatic organisms. Environmental Reviews 14: 257-297.

Carlson, R., Oyler, A., Gerhart, E., Caple, R., and Welch, K. 1979. Implications to the aquatic environment of polynuclear aromatic hydrocarbons liberated from northern great plains coal. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/3-79/093.

de Bruijn, J. and Hermens, J. 1991, Uptake and elimination kinetics of organophosphorous pesticides in the guppy *(Poecilia reticulata)*: Correlations with the octanol/water partition coefficient. Environmental Toxicology and Chemistry 10:791-804.

de Voogt, P., Van Hattum, B., Leonards, P., Klamer, J.C., Govers, H. 1991. Bioconcentration of polycyclic heteroaromatic hydrocarbons in the guppy (*Poecilia reticulata*). Aquatic Toxicology 20:169-194.

Ekelund, R., Bergman, A., Granmo, A., and Berggren M. 1990. Bioaccumulation of 4-nonylphenol in marine animals- A re-evaluation. Environmental Pollution 64:107-120.

Fackler, P. 1990. Deltamethrin: Bioconcentration and elimination of carbon 14-residues by bluegill (*Lepomis macrochirus*): Lab Project Number: SLI 89-11-3143:1719 0789 6115 140. Unpublished study prepared by Springborn Laboratories, Inc. 55p. USEPA MRID# 41651040.

Giesy, J.P., Pierens, S.L., Snyder, E.M., Miles-Richardson, S., Kramer, V.J., Snyder, S.A., Nichols, K.M., and Villeneuve D.A. 2000. Effects of 4-nonylphenol on fecundity and biomarers of estrogenicity in fathead minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry 19:1368-1377.

Hansen, D.J., and Parrish, P.R. 1977. Suitability of sheepshead ninnows (*Cyprinodon variegatus*) for life-cycle toxicity tests. In: F.L.Mayer and J.L.Hamelink (Eds.), Aquatic Toxicology and Hazard Evaluation, 1st Symposium, ASTM STP 634, Philadelphia, PA:117-126.

Jonsson, G., Bechmann, R. K., Bamber, S. D. and Baussant, T. 2004. Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (*Cyprinodon variegatus*) exposed to contaminated seawater. Environmental Toxicology and Chemistry 23:1538–1548.

Laue, H., Gfeller, H., Jenner, K.J., Nichols, J.W., Kern, S., and Natsch, A. 2014. Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of in vitro intrinsic clearance. Environmental Science & Technology 48:9486-9495.

McLeese, D.W., Zitko, V., Sergeant, D.B., Burridge, L., and Metcalfe, C.D. 1981. Lethality and accumulation of alkylphenols in aquatic Fauna. Chemosphere 10:723-730.

Muir, D.C.G., Hobden, B.R., and Servos, M.R. 1994. Bioconcentration of pyrethroid insecticides and DDT by rainbow trout: Uptake, depuration, and effect of dissolved organic carbon. Aquatic Toxicology 29:223-240.

Ogata, M., Fujisawa, K., Ogino, Y., and Mano, E. 1984. Partition coefficients as a measure of bioconcentration potential of crude oil compounds in fish and shellfish. The Bulletin of Environmental Contamination and Toxicology 33:561-567.

Parkerton, T. F., Arnot, J. A., Weisbrod, A. V., Russom, C., Hoke, R. A., Woodburn, K., Traas, T., Bonnell, M., Burkhard, L. P., and Lampi, M. A. 2008. Guidance for evaluating in vivo fish bioaccumulation data. Integrated Environmental Assessment and Management 4:139-155.

Schettgen, C. 2000. Bioaccumulation of triclosan at different water pH values and of the pyrethroids cyfluthrin, cypermethrin, deltamethrin and permethrin. Dissertation. University of Oldenburg.

Schocken, M. and Grandy, K. 1993. Issues related to maintaining consistent, nominal concentrations of deltamethrin in Aquatic Toxicology and Bioconcentration Studies: Lab Project Number: 90-4-3307: 90-12-3597. Unpublished study prepared by Springborn Laboratories, Inc. 16 p. USEPA MRID# 43072702.

Snyder, S.A., Keith, T.L., Pierens, S.L., Snyder, EM, and Giesy, J.P. 2001. Bioconcentration of nonylphenol in fathead minnows (Pimephales promelas). Chemosphere 44:1697-1702.

Tsuda, T., S. Aoki, M. Kojima, and T. Fujita. 1993. Accumulation and Excretion of Organophosphorous Pesticides by Carp Cyprinus carpio. Comp.Biochem.Physiol.C 104(2):275-278.

Tsuda, T., S. Aoki, T. Inoue, and M. Kojima. 1995. Accumulation and Excretion of Diazinon, Fenthion and Fenitrothion by Killifish: Comparison of Individual and Mixed Pesticides. Water Res. 29(2):455-458

Tsuda, T., M. Kojima, H. Harada, A. Nakajima, and S. Aoki. 1997. Relationships of Bioconcentration Factors of Organophosphate Pesticides Among Species of Fish. Comp.Biochem.Physiol.C 116(3):213-218.

Tsuda, T., M. Kojima, H. Harada, A. Nakajima, and S. Aoki. 1996. Accumulation and Excretion of Fenthion, Fenthion Sulfoxide and Fenthion Sulfone by Killifish (Oryzias latipes). Comp.Biochem.Physiol.C 113(1):45-49.