OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

WASHINGTON, D.C. 20460

MEMORANDUM

DATE: December 12, 2023

SUBJECT: Use of *In Vitro* Data to Determine Data Derived Extrapolation Factors (DDEFs) for Human Health Risk Assessment for Select Organophosphates (OPs)

PC Codes: See Table 1
Decision No.: 579172
Risk Assessment Type: NA
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DP Barcode: D468397
Regulatory Action: NA
Case No.: NA
CAS No.: See Table 1
40 CFR: See Table 1

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Table 1. List of Select OP Chemicals Used for *In Vitro* Testing			
Chemical	**PC Code**	**CAS No.**	**40 CFR**
Bensulide	009801	741-58-2	§180.241
Chlorethoxyfos	129006	54593-83-8	§180.486
Dichlorvos (DDVP)*	084001	62-73-7	§180.235
Dicrotophos	035201	141-66-2	§180.299
Dimethoate	035001	60-51-5	§180.204
Ethoprop	041101	13194-48-4	§180.262
Fenamiphos	100601	22224-92-6	§180.349
Malathion	057701	121-75-5	§180.111
Methamidophos**	101201	10265-92-6	NA
Naled	034401	300-76-5	§180.215
Parathion	057501	56-38-2	NA
Phorate	057201	298-02-2	§180.206
Phosmet	059201	732-11-6	§ 180.261
Phostebupirim (Tebupirimiphos)	129086	96182-53-5	NA
Terbufos	105001	13071-79-9	§ 180.352
Tribufos	074801	78-48-8	§180.272

* DDVP is a registered pesticide and is also a metabolite/degradate of naled (also tested) and trichlorfon (PC code 057901). Trichlorfon inhibits AChE without oxon activation but was not tested.

** Methamidophos is a more potent metabolite/degradate of acephate (PC Code 103301) and is not a currently registered pesticide. Acephate was not tested even though it inhibits AChE without oxon activation.

The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

1.0 Introduction

In 2007, the National Research Council (NRC) published a report titled "Toxicity Testing in the 21st Century: A Vision and a Strategy" advocating a new testing paradigm that sought to reduce the reliance on use of laboratory animals and move towards the adoption of more efficient and human-relevant methods, such as *in vitro* and *in silico* predictive approaches. EPA's Office of Pesticide Programs (OPP) has developed a strategic vision for implementing the principles outlined in the 2007 NRC report. This strategic vision focuses on advancing and evaluating new approach methods (NAMs), including computational and predictive modeling approaches, *in vitro* techniques, and targeted *in vivo* testing, as potential supplements or replacements for conventional chemical testing methods involving whole animals. OPP's strategy is consistent with the EPA's objective to reduce animal testing while safeguarding human health and the environment. The publication of the EPA NAMs Work Plan in 2020[1], with subsequent updates in 2021, further underscores the Agency's commitment to this goal. OPP has taken proactive measures to spearhead a wide range of initiatives aimed at developing and implementing NAMs in pesticide risk assessment to reduce its reliance on animal testing, while also providing opportunities to incorporate more human relevant information. One of these initiatives involves the use of NAMs to minimize the reliance on default assumptions for risk assessment, including the application of 10X default uncertainty factors (UFs) applied for interspecies and intraspecies extrapolations, respectively.

In line with this objective, OPP received and reviewed *in vitro* acetylcholinesterase (AChE) inhibition data generated for organophosphate (OP) compounds to better inform the uncertainty in human health risk assessments. Common to all OPs is the ability to inhibit the enzyme AChE, thereby impeding the breakdown of the neurotransmitter acetylcholine and leading to neurotoxicity. AChE inhibition is the basis of the current human health risk assessments for OPs. OPP considered the use of these *in vitro* AChE inhibition data to develop the interspecies and/or intraspecies data-derived extrapolation factors (DDEFs) in accordance with EPA's 2014 *Guidance for Applying Quantitative Data to Develop DDEFs for Interspecies and Intraspecies Extrapolation*[2] (referred to hereafter as EPA's DDEF Guidance).

In 2020, this approach and the derived results were evaluated by a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP)[3]. The SAP supported the overall study design and methodological approaches used to generate the proposed DDEFs[4]. However, the panel also highlighted certain areas for improvement which were acknowledged in the Agency's response to the SAP's final report[5]. OPP has since conducted additional analyses and incorporated further characterization based on the SAP recommendations. This memo aims to offer a brief overview of the DDEF concept, a summary of the *in vitro* methods and analyses, a description of the revised analyses performed since the SAP, and the Agency's conclusions regarding interspecies DDEFs for select OPs that are currently registered and undergoing Registration Review.

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[1] https://www.epa.gov/chemical-research/new-approach-methods-work-plan

[2] https://www.epa.gov/risk/guidance-applying-quantitative-data-develop-data-derived-extrapolation-factors-interspecies. https://www.epa.gov/sites/default/files/2015-01/documents/ddef-final.pdf.

[3] FIFRA SAP. September 2020. https://www.regulations.gov/docket/EPA-HQ-OPP-2020-0263

[4] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0054

[5] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0057

2.0 Development of DDEFs for Interspecies and Intraspecies Extrapolation

2.1 Background

For human health risk assessment, the application of default UFs is a common practice used to extrapolate toxicity data derived from animal models to human populations and to account for differences across the human population. Interspecies UFs (UF_A) account for inherent uncertainties and variability between animal models and humans, while intraspecies UFs (UF_H) account for inherent uncertainties and variability within the human population. In a typical risk assessment, a default UF of 10X is applied to both interspecies and intraspecies extrapolation, respectively. Both default UFs consist of a factor of 3X for pharmacokinetics (PK) and 3X for pharmacodynamics (PD). PK is defined as the determination and quantification of the time course and dose dependency of absorption, distribution, metabolism, and excretion (ADME) of the chemical agent, whereas PD is defined as the determination and quantification of the sequence of events at the cellular or molecular levels leading to a toxic response. Generated from chemical or class-specific information, DDEFs more accurately reflect the variations between species and/or within humans, thus they may be used to replace the default uncertainty values to reduce uncertainty in risk assessment.

As described in EPA's DDEF Guidance, DDEFs can also be developed separately for PK and PD, potentially resulting in the ability to calculate a total of four DDEFs given sufficient information. The interspecies extrapolation from animal data to humans entails two DDEFs: 1) the extrapolation factor covering interspecies PK variability (EF_{AK}); and 2) the extrapolation factor covering interspecies PD variability (EF_{AD}). Similarly, within the context of intraspecies extrapolation to account for human variability, two DDEFs are included: 1) the extrapolation factor covering intraspecies PK variability (EF_{HK}); and 2) the extrapolation covering intraspecies PD variability (EF_{HD}). Subsequently, the composite factor (CF) can be calculated using the following equation (Equation 1).

$$CF = EF_{AK} \times EF_{AD} \times EF_{HK} \times EF_{HD} \qquad \text{Equation 1}$$

The calculation of the CF is analogous to calculating total UFs when using the default 10X values for interspecies extrapolation and intraspecies variation. If data are only available to develop DDEFs for certain extrapolation components, the remaining extrapolation components are determined using the default value of 3X.

As described in the EPA's DDEF guidance, information on mode of action (MOA) is important in DDEF derivation, even when a comprehensive understanding of the mechanism is not available. DDEFs should be determined in the context of toxicity endpoints most relevant for risk assessment purposes. In the case of OPs, the common mechanism of toxicity is their shared ability to inhibit AChE.

2.2 Mode of Action/Adverse Outcome Pathway for Ops

OP pesticides are a group of closely related insecticides that affect the functioning of the nervous system. In 1999, EPA determined that the OPs form a common mechanism group based on their shared ability to bind to and phosphorylate AChE in both the central (brain) and peripheral nervous

systems[6]. Some OPs must be metabolized (activated) to an oxon metabolite, which is the active AChE inhibiting moiety. The initiating event in the MOA/adverse outcome pathway (AOP) for OPs involves inhibition of AChE via phosphorylation of the serine residue at the active site of the enzyme, thereby leading to accumulation of acetylcholine and ultimately to neurotoxicity (see Figure 1). AChE inhibition is consistently observed in the OP toxicology databases in multiple species, durations, lifestages, and routes.

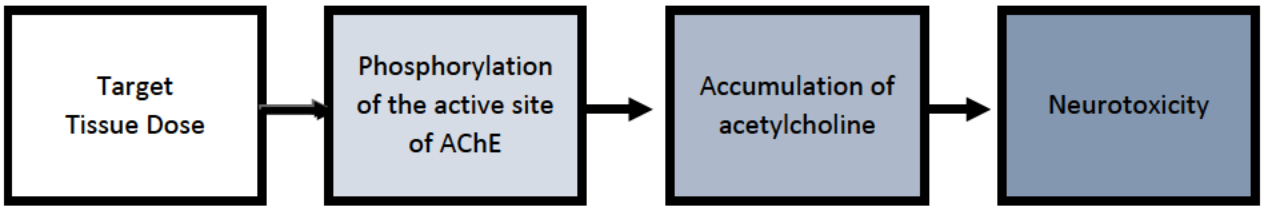

Figure 1. Adverse outcome pathway for Ops

The Agency has used and continues to use AChE inhibition as the endpoint to derive points of departure (PODs) for OP human health risk assessments. This science policy is grounded in extensive research spanning decades, showing that AChE inhibition is the initial key event in the adverse outcome pathway that leads to cholinergic neurotoxicity. Using AChE inhibition data to derive PODs was also supported by the FIFRA SAP in 2008[7] and 2012[8] for the OP pesticide, chlorpyrifos. This approach was deemed to provide the most robust response data for estimating human health risk.

The extensive dataset available for OPs, coupled with the widespread acceptance of the neurotoxic MOA for OPs attributed to AChE inhibition, presents an opportunity to utilize *in vitro* AChE inhibition data, a pharmacodynamic process, in determining the PD DDEFs for OPs. This strategy holds the potential to reduce the Agency's reliance on default UFs for OP risk assessments. In addition, this approach is consistent with the Agency's efforts to reduce laboratory animal use and utilize human relevant data to inform human health risk assessments.

2.3 *In vitro* AChE inhibition Data

Since 2016, researchers from Mississippi State University have been engaged in data generation, acting on behalf of OP pesticide registrants (AMVAC Chemical Corporation, FMC Corporation, and Gowan Company) and their consultant (Exponent). More specifically, *in vitro* AChE inhibition data were collected for several OP compounds to inform interspecies and intraspecies PD DDEFs. Paraoxon (parent: parathion) was used as the positive control given its well characterized kinetics. These experiments generated *in vitro* AChE inhibition data in rats and humans, as well as AChE inhibition kinetic constants (bimolecular rate constant k_i, dissociation constant K_I, and phosphorylation constant k_p) for the OP compounds listed in Table 2. In general, the compounds tested were either the parent OP and/or metabolites, depending on whether the parent or the metabolite is an active AChE inhibitor (Table 2). Detailed findings can be found in the following study reports:

[6] US EPA OPP, 31-JUL-2006, Docket ID EPA-HQ-OPP-2006-0618-0002; Organophosphorus Cumulative Risk Assessment, 2006 Update. https://www.regulations.gov/document/EPA-HQ-OPP-2006-0618-0002.

[7] 2008 SAP: https://www.epa.gov/sap/fifra-scientific-advisory-panel-historical-meetings

[8] 2012 SAP: https://www.regulations.gov/docket?D=EPA-HQ-OPP-2012-0040

- MRID 50773501 (12/10/2018): malaoxon (the active metabolite of malathion) and omethoate (the active metabolite of dimethoate)
- MRID 50773502 (12/18/2018): DDVP, naled, dicrotophos, tribufos, phorate oxon sulfone (phorate metabolite), phorate oxon sulfoxide (phorate metabolite), ethoprop, methamidophos (acephate metabolite), fenamiphos, terbufos oxon sulfone (terbufos metabolite), terbufos oxon sulfoxide (terbufos metabolite), chlorethoxyfos oxon (chlorethoxyfos metabolite), and tebupirimphos oxon (tebupirimphos metabolite)
- MRID 50773503 (12/10/2018): bensulide oxon (the active metabolite of bensulide) and phosmet oxon (the active metabolite of phosmet)

The *in vitro* AChE inhibition data utilized in the present analysis can be found in Appendix A.

Table 2. OP Compounds Tested

Parent OP	Registrant	Oxon activation required	Compound(s) tested
Acephate[1]	AMVAC	No	Methamidophos
Chlorethoxyfos	AMVAC	Yes	Chlorethoxyfos oxon
DDVP (Dichlorovos)	AMVAC	No	DDVP
Dicrotophos	AMVAC	No	Dicrotophos
Ethoprop	AMVAC	No	Ethoprop
Fenamiphos	AMVAC	No	Fenamiphos
Naled	AMVAC	No	Naled
Parathion	N/A	Yes	Paraoxon (positive control)
Phorate	AMVAC	Yes	Phorate oxon sulfoxide Phorate oxon sulfone
Terbufos	AMVAC	Yes	Terbufos oxon sulfoxide Terbufos oxon sulfone
Tebupirimphos	AMVAC	Yes	Tebupirimphos oxon
Tribufos	AMVAC	No	Tribufos
Dimethoate	FMC	Yes	Omethoate
Malathion	FMC	Yes	Malaoxon

Table 2. OP Compounds Tested			
Parent OP	**Registrant**	**Oxon activation required**	**Compound(s) tested**
Bensulide	Gowan	Yes	Bensulide oxon
Phosmet	Gowan	Yes	Phosmet oxon

[1] Acephate metabolizes/degrades to methamidophos. Methamidophos is no longer a registered pesticide in the United States. The parent OP acephate, does not require oxon activation to inhibit AChE, however it was not tested.

Briefly, a continuous spectrophotometric assay was used to determine dose-dependent AChE activities for several OP compounds as listed in Table 2, with paraoxon serving as the positive control. The assays were performed using "erythrocyte ghost" preparations (i.e., red blood cell (RBC) membranes separated from hemoglobin and other cytoplasmic constituents) that were obtained from either human or rat RBCs as the source of AChE.

Human AChE samples were acquired from 18 healthy individuals, comprising both sexes, including 14 subjects ranging in age from 10 – 60 years old, as well as 4 cord blood samples. These samples were sourced from multiple racial and ethnic groups (i.e., from Caucasian, African American, and Hispanic subjects). For rats, AChE inhibition data were obtained from three individually pooled samples per sex from adult rats (Sprague Dawley [Crl:CD(SD)BR]). The male samples (n=3) were prepared from the pooled blood of five male rats. Similarly, the female samples (n=3) were prepared from the pooled blood of five female rats.

3.0 EPA's Evaluation of DDEF Guidance *In Vitro* Criteria

In vitro systems provide several advantages in defining PD DDEFs. These systems circumvent resource limitations associated with *in vivo* studies and offer distinct opportunities to directly study response development in humans. In *in vitro* systems, the influence of PK can be well controlled and response data can be well characterized, mostly due to the avoidance of experimental constraints (i.e., number of doses/concentrations used for testing), which allows an opportunity to separate the PK and PD components of uncertainty. To be used for quantitative purposes*, in vitro* data should be interpreted in the context of the intact system to avoid taking isolated findings out of context. The EPA's DDEF guidance recommends a series of questions to be considered when applying *in vitro* data to determine DDEFs. OPP has concluded that all criteria have been appropriately considered and met for use of these *in vitro* data for estimating interspecies PD DDEFs for OP compounds. OPP previously noted concerns with sample size and the ability of the data to reflect diversity in the human population for generating intraspecies PD DDEF values, and these concerns were shared by the 2020 SAP. As a result, the remainder of this document will focus on the use of the available *in vitro* data to support interspecies DDEF values only. The following presents OPP's evaluation of the EPA's DDEF guidance questions as they relate to using the rat and human *in vitro* AChE inhibition data for generating *interspecies* PD DDEFs for the tested OP compounds.

- **Was the toxicologically active form of the agent studied?**

Table 2 above lists the tested OP compounds, along with the associated registrant and information regarding their requirement for activation to an oxon form to inhibit AChE. Generally, those containing the P=S moiety require activation, while those containing the P=O group do not. From the OPs subjected to testing, DDVP, dicrotophos, ethoprop, fenamiphos, naled, and tribufos do not require oxon activation, so testing was conducted on the parent compounds. DDVP is a metabolite/degradate of naled as well as another currently registered OP pesticide, trichlorfon, which was not tested. The DDVP data cannot be used to inform potential DDEF values for trichlorfon since it inhibits AChE without oxon activation. Similarly, methamidophos was tested since it is a metabolite/degradate of acephate. However, no explanation was provided by the registrant as to why *in vitro* testing was not performed for the parent, acephate, since it also inhibits AChE without oxon activation. Data for methamidophos can therefore only be used to inform potential DDEF values for methamidophos (which is no longer registered as an active ingredient in the U.S., but exposures are evaluated as part of the acephate risk assessment). Tribufos was tested but did not inhibit AChE in the *in vitro* assay; therefore, data were not available for analysis for tribufos. In this case, it is likely that chemical or assay-specific factors contributed to the divergence between *in vitro* and *in vivo* AChE activity for tribufos, however, for the majority of tested chemicals, there was concordance in the results from the *in vitro* and *in vivo* data.

Chlorethoxyfos, terbufos, phorate, tebupirimphos, dimethoate, malathion, bensulide and phosmet do require oxon activation to inhibit AChE, so the testing was conducted on their respective oxon metabolites. For phorate, Bowman and Cassida (1958)[9] determined that phorate oxon sulfoxide and phorate oxon sulfone were the most potent AChE inhibitors among phorate metabolites. For terbufos, U.S. EPA (2006)[10] reported that the key active metabolites were terbufos oxon sulfoxide and terbufos oxon sulfone. Thus, two metabolites were tested for both phorate and terbufos. Even though some of the compounds have chiral centers giving rise to optical isomers, the pesticides are employed as racemic mixtures, therefore, testing isolated enantiomers is not considered necessary.

- **How directly was the measured response linked to the adverse effect?**

The Agency has a long-standing history and policy of using AChE inhibition as the endpoint to derive PODs for human health risk assessment of OPs. As discussed in more detail in Section 2.2, OPs form a common mechanism group based on their shared ability to bind to and phosphorylate AChE. A MOA/AOP for OPs has been established for decades, which involves inhibition of the enzyme AChE via phosphorylation of the serine residue at the active site of the enzyme as the initiating event, leading to accumulation of acetylcholine and resulting in neurotoxicity in the central and/or peripheral nervous system. In this experiment, the measurement pertains to AChE inhibition, which is a direct link to the response being evaluated by OPP. The degree of neurotoxicity is determined by the rate and extent of AChE inhibition, which is readily quantifiable in *in vitro* systems.

- **Are the biological samples used in the assays derived from equivalent organs, tissues, cell types, age, stage of development, and sex of the animals/humans**

[9] Bowman, J.S. and Casida, J.E., 1958. Further studies on the metabolism of thimet by plants, insects, and mammals. Journal of Economic Entomology, 51(6), pp.838-843.

[10] US EPA (2006). Interim Reregistration Eligibility Decision for Terbufos. http://archive.epa.gov/pesticides/reregistration/web/pdf/terbufos_red.pdf

in which the target organ toxicity was identified?

Blood samples were collected from healthy animal and human subjects. The experiment used "erythrocyte ghost" preparations, which refer to RBC membranes isolated from hemoglobin and other cytoplasmic constituents. These cell membranes were obtained from human or rat blood, which contains AChE, from both sexes. Rat samples were taken from adult animals, while human samples were collected from donors ranging in age from 10 – 60 years old, as well as 4 cord blood samples. Per the EPA's DDEF guidance, an animal typical of the responding species/strain/sex/lifestage should be used for generating data for interspecies PD DDEFs, rather than an animal model that may be unusually sensitive. Age differences in AChE activity have been observed in rats; however, it has been demonstrated that the increased sensitivity observed in juvenile rats can be largely attributed to PK differences, rather than PD differences. Additionally, in a study conducted by Poet et al. (2017)[11], a physiologically based pharmacokinetic-pharmacodynamic (PBPK-PD) model was accompanied by Monte Carlo analysis to estimate DDEF for intraspecies extrapolation, considering different life stages. Their findings suggested that potential variations in RBC AChE inhibition across different life stages were primarily linked to the metabolic clearance of chlorpyrifos and its oxon. While the study focused on intraspecies DDEF, the conducted sensitivity analysis indicated that the variation in AChE inhibition constants, which can be estimated in human and rat cells, do not significantly contribute to AChE inhibition levels. This is further supported by comparable brain AChE IC_{50} values obtained by Meek et al. (2021)[12] across post-natal day (PND) 1, PND 12, and PND 70 rats. Therefore, for the purposes of generating PD interspecies DDEFs, the adult rat samples are considered appropriate. Furthermore, as discussed in the Agency's response to the SAP's final report, additional rat samples would have a negligible impact on the interspecies DDEF results.

- **What is the range of variability (e.g., diverse human populations and life stages) that the biological materials cover?**

Human AChE used in the experiments was derived from blood samples obtained from healthy individuals of both sexes (8 male and 10 female), multiple racial and ethnic groups (13 Caucasian and 5 non-Caucasian), and across lifestages (adults 16 to 60 years old, juveniles 10 to 13 years old, and cord blood samples). For the purposes of generating interspecies PD DDEFs, the range of variability in these samples is considered sufficient.

- **If the effect occurs or can be measured in several tissues, is the studied tissue or tissue preparation an appropriate surrogate? Or, in situations where the effect is not localized, is the effect consistent across tissues?**
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AChE is found throughout the body in the peripheral and central nervous system, including in cholinergic neurons, in the vicinity of synapses and in other non-neural tissues. This enzyme is responsible for the breakdown of acetylcholine which terminates the action in the synapse between

[11] Poet, T.S., et al., 2017. Use of a probabilistic PBPK/PD model to calculate Data Derived Extrapolation Factors for chlorpyrifos. Regulatory Toxicology and Pharmacology, 86, June 2017, pp 59-73. https://www.sciencedirect.com/science/article/abs/pii/S0273230017300351

[12] Meek, E.C., Carr, R.L. and Chambers, J.E., 2021. In vitro age-related differences in rats to organophosphates. Toxicology in Vitro, 72, p.105102.

neurons and muscle fibers or glands. As discussed earlier, the initiating event in the MOA/AOP for OPs involves inhibition of AChE, thereby leading to accumulation of acetylcholine and ultimately to neurotoxicity.

Per OPP's *Science Policy on the Use of Data on Cholinesterase Inhibition for Risk Assessments*[13], AChE inhibition measured in the blood can serve as a surrogate measure for the peripheral nervous system in animals, and for both the peripheral and central nervous systems in humans. In fact, for most single chemical OP assessments, RBC AChE inhibition was found to be similar or more sensitive than the brain AChE across the OPs in Registration Review and was used as the basis of risk assessment endpoints for human health risk assessment. As OPP has previously acknowledged, empirical data pertaining to the comparison of PD parameters (like the inhibition constants measured in the *in vitro* experiments) across RBC AChE and brain AChE are sparse, thereby limiting the available information to characterize the similarity of RBC and brain AChE sensitivity to inhibition by OPs. Nevertheless, there are existing studies that have demonstrated similar AChE inhibition constants for RBC and brain within species (e.g., Basova and Rozengart, 2009[14]; Herkert et al., 2012[15]; Singh, 1985[16]). Another study (Coban et al., 2016[17]) also found similar inhibition constants across different species and tissues, such as rat or mouse brain AChE vs. human RBC AChE.

The representativeness of RBC AChE inhibition for brain AChE inhibition is also supported by the similar structures of the different forms of AChE within mammalian species. AChE is a relatively well-conserved enzyme that is found across vertebrate and invertebrate species. As described in MRID 50773505, amino acid sequences of the catalytic domains of brain AChE and RBC AChE are identical within a given mammalian species. The term catalytic domain here refers not only to the catalytic active site, but also the peripheral anionic site and gorge that connects the catalytic active site and peripheral anionic site. Therefore, the catalytic domain constitutes >90% of the sequence in mature enzyme. The remaining is the anchor region, which is involved in attaching the enzyme to the cell membrane and is not involved in catalysis.

Taking all of this into account, the use of RBC AChE in the *in vitro* experiments is considered appropriate for the purposes of generating interspecies PD DDEFs.

- **Does the design of the study allow for statistically valid comparisons based on such factors as replicate and sample size?**

Inhibition kinetics were measured for the OPs listed in Table 2, which includes the positive control OP paraoxon. RBC AChE data were derived from 6 pooled Sprague Dawley rat sources and 18 human

[13] Science Policy on the Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphorous and Carbamate Pesticides. 2000. https://www.epa.gov/sites/default/files/2015-07/documents/cholin.pdf

[14] Basova, N.E. and Rozengart, E.V., 2009. Identical reactivity of brain and erythrocyte cholinesterases of some mammals. Journal of evolutionary biochemistry and physiology, 45, pp.211-220.

[15] Herkert, N.M., Freude, G., Kunz, U., Thiermann, H. and Worek, F., 2012. Comparative kinetics of organophosphates and oximes with erythrocyte, muscle and brain acetylcholinesterase. Toxicology letters, 209(2), pp.173-178.

[16] Singh, A.K., 1985. Kinetic analysis of inhibition of brain and red blood cell acetylcholinesterase and plasma cholinesterase by acephate or methamidophos. Toxicology and applied pharmacology, 81(2), pp.302-309.

[17] Coban, A., Carr, R.L., Chambers, H.W., Willeford, K.O. and Chambers, J.E., 2016. Comparison of inhibition kinetics of several organophosphates, including some nerve agent surrogates, using human erythrocyte and rat and mouse brain acetylcholinesterase. Toxicology letters, 248, pp.39-45.

sources, including subjects of various age, sex, and race/ethnicity. For each subject, the inhibition reactions were conducted in duplicate. The 95% confidence intervals around the interspecies ratios in the present IC_{50} analysis as well as in previous analyses presented to the 2020 SAP using the bimolecular rate constants, indicate a relatively high degree of precision in the estimated interspecies PD DDEFs. It is possible that increasing the number of sampled rat and/or human individuals could further reduce interspecies uncertainty, but the existing data and analyses indicate sufficiency of the present sample size and suggest that adding additional individuals would likely have minimal impact on the point estimates.

- **Was chemical uptake considered when the chemical was applied to the samples so as to give comparable intracellular concentrations across tissues?**

Care was taken to avoid non-specific binding. No additional proteins were added to the assays for enzyme stabilization to avoid introducing any additional potential binding sites for the test compounds.

- **Were similar tissues or samples evaluated across species?**

AChE was derived from blood samples in both rats and humans using the same laboratory methods.

- **Do the concentrations in the *in vitro* studies allow for comparison with *in vivo* conditions?**

EPA's DDEF guidance states that "Optimally, the concentrations used in studies of the critical effect(s) include the concentration at the POD." Since the dose response data were generated using nearly the full range of potential inhibition in the *in vitro* system (approximately 10-90% inhibition of AChE activity), the *in vitro* concentrations tested for each OP compound encompass the tissue concentrations expected at the PODs.

4.0 Derivation of Interspecies PD DDEFs

OPP concluded that all DDEF criteria have been appropriately considered and met for using the rat and human *in vitro* AChE inhibition data to estimate the interspecies PD DDEFs for several OP compounds. The original analysis submitted by the registrants and presented to the 2020 SAP focused on assessing inhibition kinetics using the bimolecular rate constant (*ki*). However, since the DDEF guidance specifies using a "concentration" as the unit of measure, and the bimolecular rate constant is a point estimate derived from data from multiple times and concentrations, the SAP suggested reanalyzing the data using the *in vitro* concentration data directly. More specifically, the SAP recommended focusing on the dose-response relationship between the administered OP concentration and the resulting reduction in AChE activity.

In response to the SAP's recommendations, OPP initiated discussions with the registrants and Exponent to explore a revised approach aimed to facilitate a more direct comparison of interspecies variability using the *in vitro* dose-response data, in line with the EPA's DDEF guidance. Subsequently in 2021, Exponent submitted new analyses in which proposed interspecies PD DDEF values were calculated using half-maximal inhibitory concentration (IC_{50} values; MRID 51739001). The IC_{50} represents the concentration of an OP that results in a 50% inhibition of baseline AChE activity.

The new analysis, which focused on the derivation of interspecies PD DDEFs using IC_{50} data, (MRID 51739001) underwent a thorough review by OPP to verify its appropriateness and data integrity. During this review, OPP identified several transcriptional and typographical errors within the underlying data. These issues were resolved by Exponent and the contracted academic lab at Mississippi State University, and further subjected to a data quality audit sponsored by the registrants. The rectified data were subsequently used by OPP to recalculate interspecies PD DDEFs and to further evaluate the stability of these values. The corrected, *in vitro* AChE inhibition data utilized in the present analysis can be found in Appendix A. A detailed discussion regarding OPP's analyses and results are summarized below.

4.1 Statistical Approach

IC_{50} values were determined by fitting the statistical models to the dose-response relationship between OP concentration (i.e., dose) and AChE activity (i.e., response). EPA scientists used the same statistical methods used in Exponent's report (MRID 51739001) to reanalyze the corrected data, select a statistical model, and estimate the interspecies ratios of inhibitory concentration associated with 10%, 25%, 50%, and 75% reduction of baseline AChE activity (i.e., IC_{10}, IC_{25}, IC_{50}, and IC_{75}) for each chemical. Consistent with EPA's DDEF guidance, the rat:human IC_{50} ratio (which is the ratio that represents the central tendency estimate of the PD species difference) is being used to estimate the interspecies PD DDEF. The IC_{10}, IC_{25}, and IC_{75} were estimated to determine if the values obtained for the IC_{50} were stable and conserved across the full range of potential inhibition tested in the *in vitro* system. Note that the derivation of IC_{10}, IC_{25}, IC_{50}, and IC_{75} was conditional on random effects being equal to zero. The point estimates were derived following the method of Ritz et al. 2015[18].

Nonlinear Fixed- and Mixed- Effects Models

In the reanalysis of the *in vitro* dataset, a three-parameter log-logistic nonlinear dose-response model was used to determine the interspecies pharmacodynamic DDEFs for each of the tested OPs. The model was fit to the dose response relationship between the reduction in AChE activity at the last time point (5 minutes) and the administered OP concentration. The longest time point was selected because the IC value can only be estimated at a single time point, and the dose-response relationship was most developed at the last time point. The utilized nonlinear model includes the IC_{50} as a parameter, thus allowing for easy estimation of the interspecies ratio from the fitted models. The remaining inhibitory concentration values (i.e., IC_{10}, IC_{25}, and IC_{75}) can be readily derived from the fitted models. The three-parameter log-logistic function assumes that the lower limit of the inhibition curve is known (i.e., AChE activity of 0) and has the form:

$$y = \frac{d}{1 + \exp^{(b(\log(x) - \log(e)))}}$$

where:

b = Slope
d = Upper Limit
e = IC_{50}

[18] Ritz, C., Baty, F., Streibig, J.C. and Gerhard, D., 2015. Dose-response analysis using R. PloS one, 10(12), p.e0146021.

Five different nonlinear models of this form were used for consideration of the interspecies DDEF; the equations for these models are presented and discussed below. The first of these was a nonlinear model with fixed effects for each species, while the remaining four models included both fixed effects and random effects that accounted for variability between individual sources[19]. Inclusion of random effects may help to account for between- and within-source variation in AChE activity as well as species-specific sources of variation. Models were fit to the data and analyzed in R using the '*drc*' package for the model with only fixed effects and the '*medrc*' package for the models that include both fixed and random effects (Ritz et al., 2015[20]; Gerhard & Ritz, 2017[21]).

Model 1 - No random effects

For the *i* th source $[i = 1, \dots, m]$ and the *j* th observation $[i = j, \dots, n]$, the 3-parameter log-logistic function with only fixed effects takes the form:

$$\mathrm{AChE}_{ij} = \mu_{ij} + \epsilon_{ij}$$

$$\mu_{ij} = \frac{d_i}{1 + \exp^{\left(b_i(\log(OP_{ij}) - \log(e_i))\right)}}$$

$$p_i = \begin{cases} p_H \text{ if source i is human} \\ P_R \text{ if source i is rat} \end{cases}$$

Where the residual error (ϵ_{ij}) is mean-zero normally distributed ($\epsilon_{ij} \sim N(0, \sigma^2)$) and the parameter *p* may refer to any of the three log-logistic parameters *b*, *d*, or *e* (i.e., slope, upper limit, and IC_{50}, respectively).

Model 2 - Source random effects, full variance-covariance structure

Model 2 extends model 1 by including random effects for each log-logistic function parameter, which are contained within vector ψ_i with variance-covariance matrix (*G*).

$$\psi_i = [\psi_{bi}, \psi_{di}, \psi_{ei}]$$

$$\mu_{ij} = \frac{d_i + \psi_{di}}{1 + \exp^{\left((b_i + \psi_{bi})(\log(OP_{ij}) - \log(e_i + \psi_{ei}))\right)}}$$

$$\psi_i \sim N(0, G)$$

$$G = \begin{bmatrix} g_b & g_{bd} & g_{be} \\ g_{bd} & g_d & g_{de} \\ g_{be} & g_{de} & g_e \end{bmatrix}$$

Again, $\epsilon_{ij} \sim N(0, \sigma^2)$ and it now estimates residual within-source variability (i.e., the variability in observations from the duplicate measurements for each source).

[19] The term 'source' refers to blood samples either collected from an individual human subject or pooled together from a group of rats.

[20] Ritz, C., Baty, F., Streibig, J.C. and Gerhard, D., 2015. Dose-response analysis using R. PloS one, 10(12), p.e0146021.

[21] Gerhard, D. and Ritz, C., 2017. Marginalization in nonlinear mixed-effects models with an application to dose-response analysis. arXiv preprint arXiv:1707.02502.

Model 3 - Source random effects, diagonal variance-covariance structure

Model 3 is analogous to model 2 except that no off-diagonal covariance elements are estimated for ψ_i, and is thus more parsimonious:

$$G = \begin{bmatrix} g_b & 0 & 0 \\ 0 & g_d & 0 \\ 0 & 0 & g_e \end{bmatrix}$$

Model 4 - Species-specific source random effects, diagonal variance-covariance structure

Model 4 is an extension of model 3 that allows separate random effect variances to be estimated for each species-specific parameter:

$$\psi_i \sim N(0, G_s)$$

where *s* is an indicator of the source species for a given observation. The structure of G_s is therefore either:

$$G_H = \begin{bmatrix} g_{Hb} & 0 & 0 \\ 0 & g_{Hd} & 0 \\ 0 & 0 & g_{He} \end{bmatrix} \quad \text{or} \quad G_R = \begin{bmatrix} g_{Rb} & 0 & 0 \\ 0 & g_{Rd} & 0 \\ 0 & 0 & g_{Re} \end{bmatrix}$$

for humans or rat sources, respectively.

Model 5. Species-specific source random effects, diagonal variance-covariance structure, species-specific residual error

Model 5 extends model 4 so that residual error (ϵ_{ij}) is also species specific:

$$\epsilon_{ij} \sim N(0, \sigma^2)$$

where σ^2 will be either σ_H^2 or σ_R^2 depending on whether an observation is from a human or rat source, respectively.

Model Selection and Regression Diagnostics

For each OP, the Bayesian information criterion (BIC) to assess model quality, and the preferred model was selected based on the lowest BIC value of the fit models. All levels of the interspecies inhibitory concentration ratios (i.e., IC_{10}, IC_{25}, IC_{50}, and IC_{75}) were determined from the selected model. The model assumptions and outliers were assessed using residual plots generated for the preferred model. During fitting, warnings and errors were generated for some mixed effects models, and these were evaluated and, where possible, resolved. In many cases, these could be resolved by providing starting values for fixed effect parameters based on estimates from the fit excluding random effects (i.e., that from model 1). Resolution of other errors required transformation of the chemical concentration values; this transformation does not impact the interspecies IC_{50} ratio estimate.

4.2 Results

The sections below provide the summary results for the interspecies data (rat to human) analyses.

Tribufos did not produce any AChE inhibition even at a final concentration of 1 mM[22]; therefore, values are not reported for tribufos. There were persistent, unresolvable convergence errors when fitting the data for omethoate, which precluded reporting values for this chemical as well.

4.3 Interspecies Data Analyses

The table below provides the summary results for the interspecies ratios of the IC_{50} values obtained from the Agency reanalysis of the AChE inhibition data obtained for the OP compounds listed; neither tribufos nor omethoate are shown in Table 3 or used in this analysis due to the absence of *in vitro* AChE inhibition and issues fitting the statistical models to the data, respectively (fitting issues for omethoate are discussed further below). Per the SAP's recommendation, the interspecies ratios were also calculated for different inhibitory concentrations (IC_{10}, IC_{25}, and IC_{75}) and are presented in Figure 2 and Appendix B Table 1.

Persistent convergence issues existed when fitting the models to data for omethoate. The fixed-effects model (Model 1) converged, but none of the models that included random effects (Model 2 – Model 5) converged. The lack of convergence for Model 2 – Model 5 could not be resolved. The fitted result from Model 1 indicated a very gradual dose response curve for humans and residual analysis showed a strong positive skew. These factors called into question the validity of the model fitted to the omethoate data and, therefore, these results have been omitted from the present analysis.

In the residual analysis for malaoxon, a unique outlying dose-response curve was identified for one of the human sources (Hispanic Male, 23y); the dose response curve for this source was unusually gradual and, therefore, produced an anomalously high IC_{50} value. Thus, a separate set of models was run for this chemical excluding the outlying values. The resulting interspecies ratios are shown below for fits conducted with and without this outlier (Table 3; malaoxon and malaoxon with one source removed). However, there were persistent convergence issues for the malaoxon dataset when the outlier data was removed. Following outlier removal, models 2, 4, and 5 failed to converge; model 3 did converge and resulted in a lower BIC than model 1. The difference between the results with and without the outlier are substantial, with the removal of the outlier producing a more conservative result (Table 3).

Fenamiphos exhibited the lowest IC_{50} interspecies ratio of the tested compounds (Table 3, Figure 2). Further, unlike the other OP compounds tested, the interspecies ratios calculated at the other inhibitory concentrations (i.e., IC_{10}, IC_{25}, and IC_{75}) differed substantially from one another and from that calculated at the IC_{50} (Table 3, Figure 2, and in Figure 1 in Appendix B). This is likely being driven by the data from two rat sources which exhibited much steeper dose response curves than the other tested rat sources (Appendix B, Figure 2). These two sources are not anomalous in the overall dataset for this chemical as there are analogous curves observed in the dose response data from human sources (Appendix B, Figure 3). By contrast to those found in the human dataset, the steep curves found in the rat dataset exert a strong force shaping the overall model to the data for this species due to the relatively low number of sampled individuals. The impact on the interspecies ratios is most pronounced at lower concentrations of fenamiphos in the incubation where the dose response curve is steepest (i.e., lower levels of AChE inhibition), which contributes to the appearance of the rat being

[22] An *in vivo* dose level orders of magnitude above the limit dose of 1,000 mg/kg/day would be needed to achieve a plasma concentration of 1 mM

considerably more sensitive than the human.

Table 3: Estimated IC_{50} interspecies ratio and 95% confidence interval			
Chemical	**Rat/Human IC_{50} Estimate**	**95% Confidence Interval**	
		Lower	**Upper**
Bensulide Oxon	0.66	0.55	0.79
Chlorethoxyfos	0.54	0.46	0.62
DDVP	1.47	0.98	1.99
Dicrotophos	0.80	0.72	0.89
Ethoprop	1.52	1.27	1.79
Fenamiphos	0.42	0.37	0.49
Malaoxon	0.60	0.35	1.81
Malaoxon (one source removed)	1.19	0.93	1.52
Methamidophos	0.89	0.76	1.02
Naled	0.66	0.50	0.95
Phorate oxon sulfone	0.81	0.62	1.03
Phorate oxon sulfoxide	0.71	0.65	0.78
Phosmet oxon	1.03	0.86	1.22
Tebupirimphos oxon	0.61	0.49	0.74
Terbufos oxon sulfone	1.60	1.21	2.12
Terbufos oxon sulfoxide	1.40	1.29	1.50

The interspecies PD DDEFs at the IC_{50} ranged from 0.42 to 1.60 (Table 3). The majority of the DDEF values were approximately 1 or less; DDVP, ethoprop, malaoxon (without the outlier), terbufos oxon sulfone, terbufos oxon sulfoxide, and phosmet oxon were slightly greater than one. Convergence issues (omethoate), skew (phorate oxon sulfone and phorate oxon sulfoxide), and outlier data (malaoxon) impacted a minority of the analyzed chemicals. The observations at the IC_{50} were stable and broadly conserved at other inhibitory concentrations (i.e., IC_{10}, IC_{25}, and IC_{75}; Figure 2 and Appendix B, Table 1). The confidence intervals were narrow around the point estimates with increasing inhibitory concentrations (i.e., from IC_{10} to IC_{75}), which reflects the lower interindividual variability at high treatment response levels (Figure 2 and Appendix B, Table 1). There are some chemicals for which this trend appears reversed (i.e., higher variability at higher inhibitory concentrations, e.g., fenamiphos in Figure 2); however, this is an artifact of the scale of the error bars relative to that of the point estimate and this phenomenon resolves when the results are plotted on a log scale (Appendix B, Figure 1). For some of the tested chemicals, the width of the 95% confidence intervals suggests some uncertainty around the calculated interspecies ratios at each of the examined inhibitory concentrations (Table 3, and Appendix B, Table 1). There are numerous plausible sources for this uncertainty including inherent variability in *in vitro* assays and aspects of the study design. Most notable of the latter would likely be sample size, as larger studies provide more precise estimates and therefore narrower confidence intervals; however, aspects like spacing between tested concentrations and incubation duration could also play a role in uncertainty in these values. Nevertheless, the central tendency of the interspecies ratios is approximately 1 (Table 3 and Appendix B, Table 1), and this trend is broadly conserved across the examined inhibitory concentrations for the tested chemicals (Figure 2).

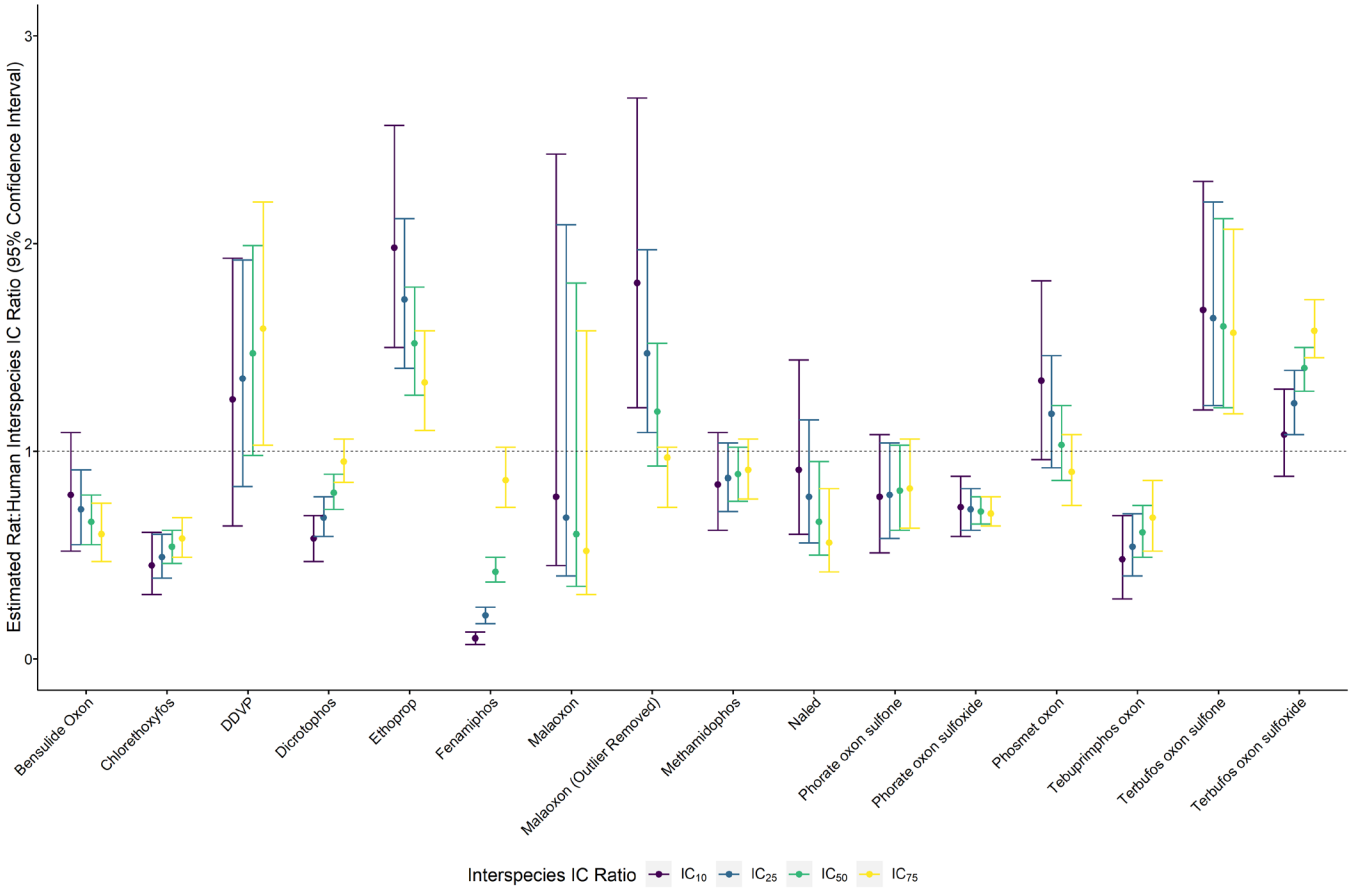


Figure 2: **Comparison of estimated IC_{10}, IC_{25}, IC_{50}, and IC_{75} interspecies ratios.** Error bars indicate the 95% confidence interval around the estimate.

5.0 Conclusions

In vitro data were generated by academia on behalf of pesticide registrants to calculate PD DDEF values for several OP compounds in accordance with EPA's DDEF guidance. OPP reviewed and independently reanalyzed the data to estimate inhibitory concentrations for each of the OPs tested. The interspecies ratios for the tested chemicals were approximately 1 across the range of examined inhibitory concentrations (i.e., IC_{10} to IC_{75}). Although EPA's DDEF guidance allows for interspecies PD values other than 1, OPP has concluded that the totality of the data supports using an interspecies PD DDEF of 1X for the tested OPs (excluding omethoate and tribufos), which is considered to be a health protective approach. This takes into consideration the minor variability seen across the data and inhibitory concentrations evaluated, the high degree of interspecies homology (both in the protein structure and structure of rat and human AChE), as well as data that demonstrate differences in AChE activity between rats and humans are largely driven by differences in PK (particularly metabolic clearance) rather than PD.

The data are considered reliable for supporting an interspecies PD DDEF of 1X for human health risk assessment for the following OPs:

- Bensulide (Bensulide oxon)
- Chlorethoxyfos
- DDVP[23]
- Dicrotophos
- Ethoprop
- Fenamiphos
- Malathion (Malaoxon)
- Methamidophos[24]
- Naled
- Phorate (Phorate oxon sulfone and sulfoxide)
- Phosmet (Phosmet oxon)
- Tebupirimphos (Tebupirimphos oxon)
- Terbufos (Terbufos oxon sulfone and sulfoxide)

[23] DDVP is a registered pesticide, as well as a metabolite/degradate of naled (also tested) and trichlorfon. DDVP data will not be used for either naled or trichlorfon, since these OPs do not require oxon activation to inhibit AChE.

[24] Methamidophos is not currently registered and is also a metabolite/degradate of acephate. Data for methamidophos will not be applied to the parent compound, acephate, since acephate does not require oxon activation to inhibit AChE.

Appendix A.

The following file contains *in vitro* data, the R code used to analyze it, and a brief readme file that describes the contents of the portfolio and how to use it.

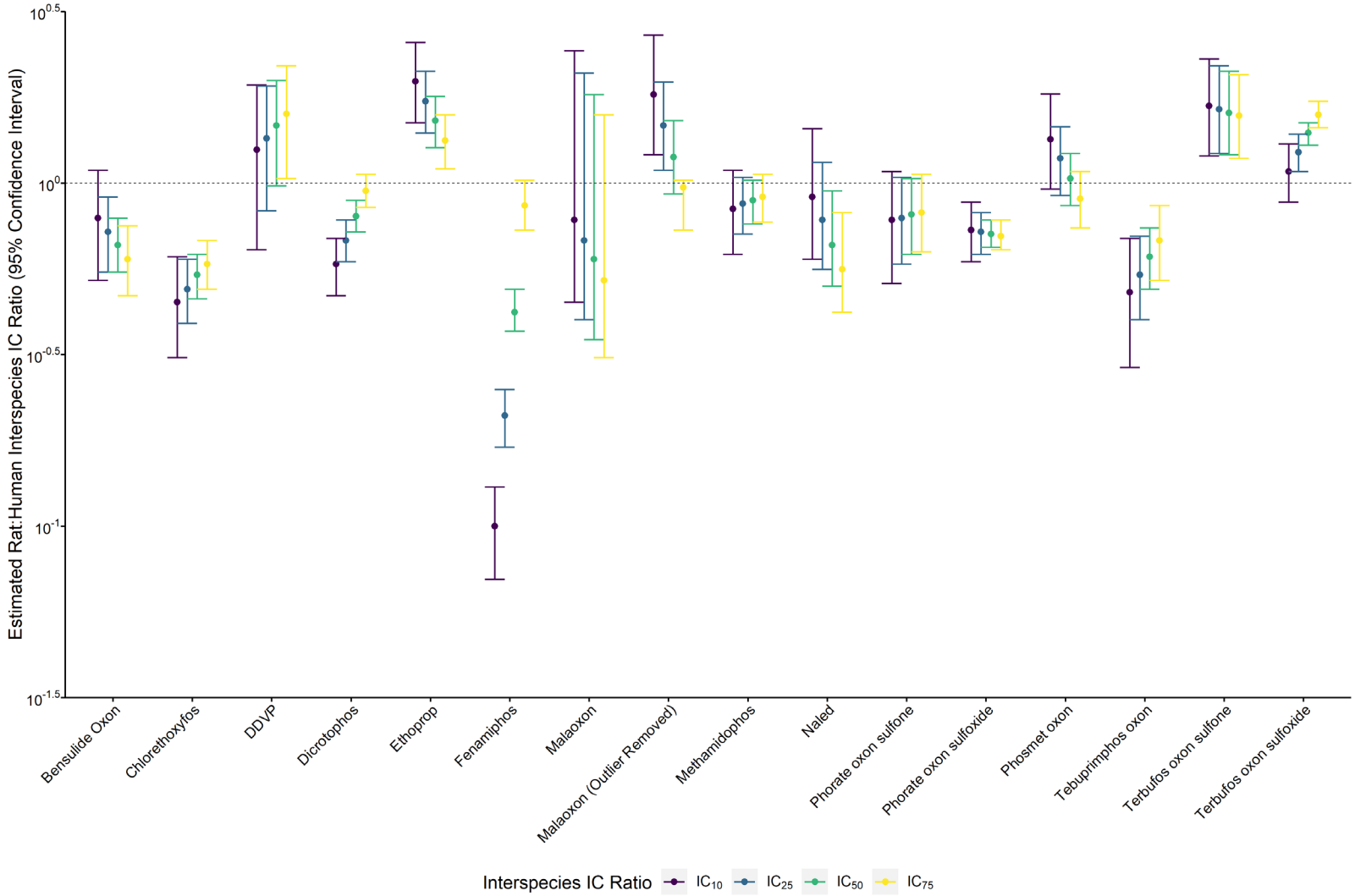
ddef_invitro_data_r
code_readme.pdf

“ddef_invitro_data_rcode_readme.pdf”

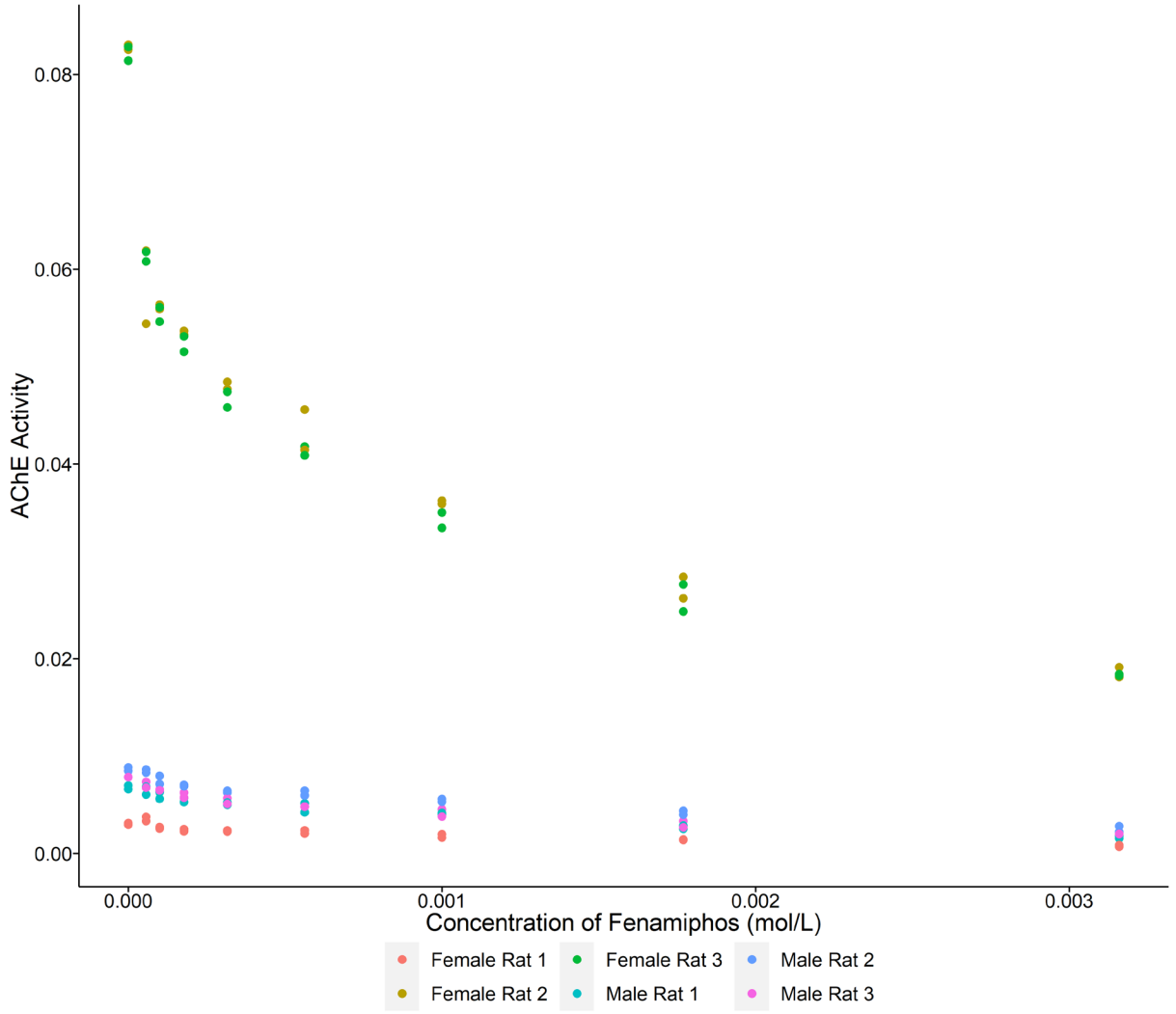
Appendix B.

Appendix B, Table 1: Estimated IC_{10}, IC_{25}, IC_{50}, and IC_{75} interspecies ratios with their associated 95% confidence intervals

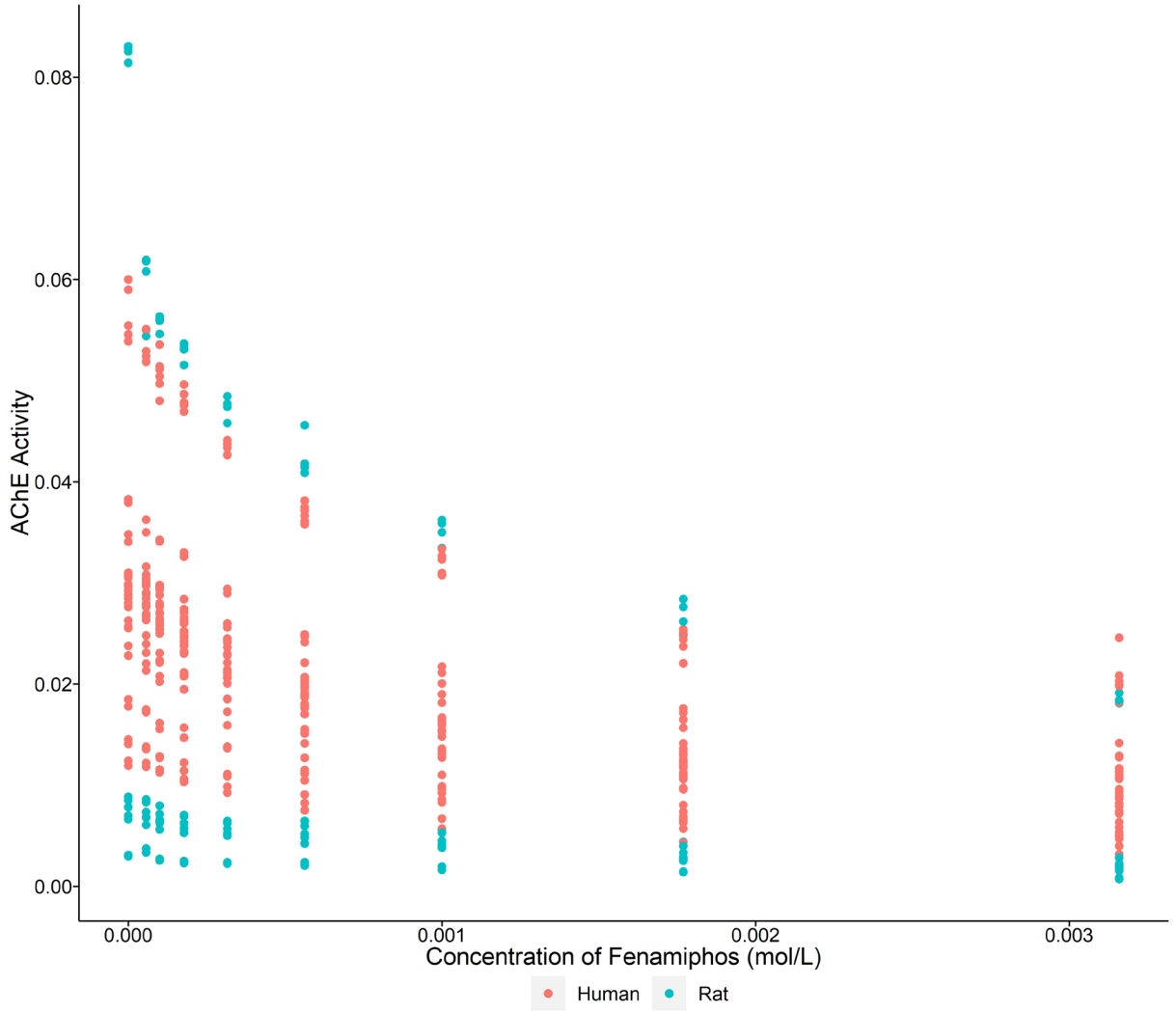
Chemical	Rat/Human IC_{10} Estimate	95% Confidence Interval		Rat/Human IC_{25} Estimate	95% Confidence Interval		Rat/Human IC_{50} Estimate	95% Confidence Interval		Rat/Human IC_{75} Estimate	95% Confidence Interval	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
Bensulide Oxon	***0.79***	0.52	1.09	***0.72***	0.55	0.91	***0.66***	0.55	0.79	***0.60***	0.47	0.75
Chlorethoxyfos	***0.45***	0.31	0.61	***0.49***	0.39	0.60	***0.54***	0.46	0.62	***0.58***	0.49	0.68
DDVP	***1.25***	0.64	1.93	***1.35***	0.83	1.92	***1.47***	0.98	1.99	***1.59***	1.03	2.20
Dicrotophos	***0.58***	0.47	0.69	***0.68***	0.59	0.78	***0.80***	0.72	0.89	***0.95***	0.85	1.06
Ethoprop	***1.98***	1.50	2.57	***1.73***	1.40	2.12	***1.52***	1.27	1.79	***1.33***	1.10	1.58
Fenamiphos	***0.10***	0.07	0.13	***0.21***	0.17	0.25	***0.42***	0.37	0.49	***0.86***	0.73	1.02
Malaoxon	***0.78***	0.45	2.43	***0.68***	0.40	2.09	***0.60***	0.35	1.81	***0.52***	0.31	1.58
Malaoxon (one source removed)	***1.81***	1.21	2.7	***1.47***	1.09	1.97	***1.19***	0.93	1.52	***0.97***	0.73	1.02
Methamidophos	***0.84***	0.62	1.09	***0.87***	0.70	1.04	***0.89***	0.76	1.02	***0.91***	0.77	1.06
Naled	***0.91***	0.60	1.44	***0.78***	0.56	1.15	***0.66***	0.50	0.95	***0.56***	0.42	0.82
Phorate oxon sulfone	***0.78***	0.51	1.08	***0.79***	0.58	1.04	***0.81***	0.62	1.03	***0.82***	0.63	1.06
Phorate oxon sulfoxide	***0.73***	0.59	0.88	***0.72***	0.62	0.82	***0.71***	0.65	0.78	***0.70***	0.64	0.78
Phosmet oxon	***1.34***	0.96	1.82	***1.18***	0.92	1.46	***1.03***	0.86	1.22	***0.90***	0.74	1.08
Tebupirimphos oxon	***0.48***	0.29	0.69	***0.54***	0.40	0.70	***0.61***	0.49	0.74	***0.68***	0.52	0.86
Terbufos oxon sulfone	***1.68***	1.20	2.30	***1.64***	1.22	2.20	***1.60***	1.21	2.12	***1.57***	1.18	2.07
Terbufos oxon sulfoxide	***1.08***	0.88	1.30	***1.23***	1.08	1.39	***1.40***	1.29	1.5	***1.58***	1.45	1.73



Appendix B, Figure 1: **Comparison of estimated IC_{10}, IC_{25}, IC_{50}, and IC_{75} interspecies ratios.** Results are plotted on a logarithmic scale. Error bars indicate the 95% confidence interval around the estimate.



Appendix B, Figure 2: **Comparison of the dose response curves from rat sources for fenamiphos**. The 'dose' is the *in vitro* chemical concentration during the experiment and the 'response' is the measured AChE activity.



Appendix B, Figure 3: **Comparison of the dose response curves from rat and human sources for fenamiphos**. The 'dose' is the *in vitro* chemical concentration during the experiment and the 'response' is the measured AChE activity.