UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

DATE: 24-AUG-2023

SUBJECT: Evaluation of the Developmental Neurotoxicity Potential of Acephate/Methamidophos to Inform the FQPA Safety Factor

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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/201402/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.

Introduction

The organophosphates (OPs) are a class of pesticides that have an established mode of action/adverse outcome pathway (MOA/AOP) involving inhibition of the enzyme acetylcholinesterase (AChE) via phosphorylation of the serine residue at the active site of the enzyme. This inhibition leads to accumulation of acetylcholine (a neurotransmitter) and ultimately to neurotoxicity in the central and/or peripheral nervous system. The need for additional developmental neurotoxicity (DNT) data to assess potential effects in the developing young, including the DNT guideline study for any pesticide, is considered in the context of the entire database and in accordance with the 40 CFR Part 158 toxicology data requirements for pesticides[1]. Based on the available data for OPs, EPA previously issued data call-ins for *in vivo* DNT studies; however, none of the 18 submitted DNT studies for OPs identified endpoints that were more sensitive than AChE inhibition. Consequently, EPA has continued to use AChE inhibition as the critical endpoint for OP human health risk assessments. However, in recent years, scientific evidence has raised uncertainty about whether AChE inhibition will be protective of potential neurodevelopmental effects for OPs, especially given the lack of a MOA/AOP for the potential neurodevelopmental effects.

In 2015, EPA released a literature review on neurodevelopmental effects and Food Quality Protection Act Safety Factor (FQPA SF) determination for the organophosphate (OP) pesticides (A. Lowit, D331251[2],15-SEP-2015). The review was then updated in 2016 to incorporate additional studies and address public comments (A. Aldridge et al., D437043[3], 29-DEC-2016). In the 2015/2016 review, data from three primary lines of evidence – epidemiological studies, studies in laboratory animals, and *in vitro* assays – were evaluated in a weight of evidence (WOE) approach to assess the DNT potential of OPs. Although the MOA/AOP is not established for any potential developmental neurotoxic outcomes, OPP took a conservative approach by performing the 2015/2016 review for the OPs as a group based on the assumption that, like AChE inhibition and subsequent neurotoxicity, DNT outcomes would share a common MOA/AOP.

At the time of the 2015/2016 review, uncertainties regarding potential neurodevelopmental effects and their relative sensitivity to AChE inhibition for OPs was most notably raised by epidemiological studies; however, limitations were identified in these studies that make it

[1] https://www.ecfr.gov/current/title-40/chapter-I/subchapter-E/part-158?toc=1
[2] https://www regulations.gov/document/EPA-HQ-OPP-2009-0059-0030
[3] https://www regulations.gov/document/EPA-HQ-OPP-2008-0351-0107

difficult to causally or quantitatively link exposure of individual OPs to the investigated outcomes. Ultimately, out of an abundance of caution, the 10X FQPA SF for the OPs was retained at that time due to the overarching uncertainties in the human dose-response relationship for potential neurodevelopmental outcomes and its quantitative relationship to AChE inhibition. As a result, in the last risk assessment for acephate and its metabolite/degradate methamidophos (D. Drew; 28-MAR-2018; D446177), the FQPA 10X SF was retained or a database uncertainty factor was applied for the population subgroups that include infants, children, youths, and women of childbearing age for all exposure scenarios.

Recognizing the uncertainty in the human dose-response relationship for neurodevelopmental outcomes, EPA has pursued the development of approaches to facilitate quantitative or semi-quantitative comparisons between doses which elicit AChE inhibition and those which are associated with potential neurodevelopmental outcomes. Since the 2015/2016 review, high quality data on underlying biological processes of neurodevelopment have become available as a result of an international effort to develop new approach methodologies (NAMs) for evaluating DNT hazard. This international effort recognized the strengths and limitations of the available DNT studies through a series of meetings with scientists, regulators, and stakeholders (Lein et al., 2007; Coecke et al., 2007; Crofton et al., 2011; Bal-Price et al., 2012; Aschner et al., 2017; Fritsche et al., 2017; Fritsche et al., 2018a; Fritsche et al., 2018b; Bal-Price et al., 2018; Sachana et al., 2019)[4], leading to the development of a battery of *in vitro* assays that assess processes critical to development of the nervous system (referred to hereafter as DNT NAM battery), and provide chemical-specific evaluation of DNT hazard potential. By focusing on critical biological processes underlying neurodevelopment, the DNT NAM battery can provide relevant information regarding DNT hazard potential of individual chemicals and evaluate early perturbations that are difficult to obtain or evaluate *in vivo*. Therefore, assessment of whether a chemical may impact these upstream critical processes provides an evaluation of its ability to yield a myriad of potential downstream DNT impacts, including complex neurological deficits seen in the human population ranging from subtle learning disabilities to severe neural tube defects (*e.g.,* spina bifida).

The *in vitro* data from the DNT NAM battery can provide a scientifically robust, data-driven basis for evaluating potential DNT hazard and its quantitative relationship to AChE inhibition for

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[4] Lein et al. (2007) Meeting report: alternatives for developmental neurotoxicity testing. Environ Health Perspect. 115(5):764-8; Coecke et al. (2007) Workgroup report: incorporating in vitro alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. Environ. Health Perspect. 115(6):924-931; Crofton et al. (2011) Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. ALTEX. 28(1):9-15; Bal-Price et al. (2012) Advancing the science of developmental neurotoxicity (DNT): testing for better safety evaluation. ALTEX 29(2):202-15; Aschner et al. (2017) Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: example lists and criteria for their selection and use. ALTEX. 34(1):49-74; Fritsche et al. (2017) OECD/EFSA workshop on developmental neurotoxicity (DNT): The use of non-animal test methods for regulatory purposes. ALTEX. 34(2):311-315; Fritsche et al. (2018a) Consensus statement on the need for innovation, transition and implementation of developmental neurotoxicity (DNT) testing for regulatory purposes. 354:3-6.; Fritsche et al. (2018b) Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation. Toxicol Sci. 165(1):14-20. https://doi.org/10.1093/toxsci/kfy175; Bal-Price et al. (2018) Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods. Toxicol Appl Pharmacol. 354:7-18. https://doi.org/10.1016/j.taap.2018.02.008; Sachana et al. (2019) International Regulatory and Scientific Effort for Improved Developmental Neurotoxicity Testing. Toxicol. Sci. 167(1):45-57.

individual OPs. In 2020, EPA convened a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) to review the DNT NAM battery with the OPs as a case study[5]. Overall, the SAP agreed that the current DNT NAM battery reflects, if not directly models, critical processes for neurodevelopment and that data from the battery can be used as part of a WOE evaluation. Activity observed in the DNT NAM battery can also be used in kinetic models, such as high-throughput toxicokinetic (HTTK) or refined physiologically-based pharmacokinetic (PBPK) models, where the *in vitro* concentrations that are associated with bioactivity observed in the DNT NAM battery can be directly compared with an internal dose metric (e.g., average blood concentration) associated with chemical-specific points of departure (PODs). For OPs, such comparison evaluates the relative sensitivity of activity in the DNT NAM battery to AChE inhibition given the PODs for OP human health risk assessments are based on 10% AChE inhibition. AChE endpoints can be derived from *in vivo* animal studies or predicted using PBPK models when available. Therefore, *in vitro* data from the DNT NAM battery can be used as part of a WOE approach, to characterize the uncertainty in the dose-response relationship for neurodevelopmental outcomes and AChE inhibition by comparing the average blood concentration associated with a 10% inhibition of AChE (from *in vivo* animal studies) with the *in vitro* bioactive concentrations from the DNT NAM battery assays.

Notably, the data from the DNT NAM battery for numerous OP compounds has demonstrated that differences exist across OP chemicals with respect to their potential to elicit neurodevelopmental effects. No consistent pattern (e.g., differences in degree of activity/inactivity; activity in different assays that represent different critical processes) has emerged to suggest that all OPs share a common pathway for potential DNT or to support the assumption that all OP compounds have similar concerns related to DNT. The differences observed in the DNT NAM battery data also emphasize differences in DNT potential across OPs that were previously identified in epidemiological and laboratory animal studies. Accordingly, EPA has reevaluated its approach to assessing the DNT potential of the OPs.

Based on the best available science, OPP has determined that DNT potential of OPs should be evaluated on a chemical-by-chemical basis, not as a group (M. Perron, TXR 0058584, D467385, 10-APR-2023). Moving forward, as with all pesticides subject to FQPA, EPA will continue to assume a default 10X safety factor to protect for infants and children unless reliable data exists to support application of a different safety factor. An appropriate FQPA safety factor for each OP will be assessed on an individual chemical basis taking into consideration the strengths and limitations of all scientifically sound data and information. To inform the FQPA SF determination for OPs, DNT potential will be evaluated using chemical-specific data for each OP in a WOE evaluation. This WOE evaluation considers whether the OP compound has the potential to elicit DNT outcomes, as well as the relative sensitivity of potential DNT to AChE inhibition (i.e., comparison of relevant dose(s) where potential DNT outcomes may occur in relation to AChE inhibition).

Unlike several other OPs, acephate does not require bioactivation prior to inhibiting AChE; however, its metabolite/degradate, methamidophos, is a more potent AChE inhibitor. Therefore, this document provides a WOE evaluation of chemical-specific data for both acephate and methamidophos. The following sections will provide:

[5] https://www regulations.gov/document/EPA-HQ-OPP-2020-0263-0006

- an overview of the DNT NAM battery and methods used to analyze the data from the battery;
- a summary of the results from the DNT NAM battery for acephate and methamidophos;
- a summary of available *in vivo* DNT studies for acephate and methamidophos in laboratory animals;
- a summary of epidemiological studies that investigated associations between acephate and/or methamidophos and DNT outcomes;
- discussion of the results from all lines of evidence, including the strengths and limitations of each; and
- EPA's conclusion on the DNT potential for acephate/methamidophos, including the quantitative relationship of potential DNT outcomes to AChE inhibition, to inform the FQPA SF.

Overview of the DNT NAM battery

The current DNT NAM battery consists of multiple *in vitro* assays that utilize either human or rat neural cell models. Assays in the DNT NAM battery were developed by the US EPA Office of Research and Development (ORD) and international collaborators, with a goal of facilitating faster, less expensive, and more human relevant DNT screening and evaluation. The overarching goal was to develop a battery of assays that measure critical neurodevelopmental processes *in vitro* including proliferation of neuroprogenitor cells, differentiation of neuroprogenitors into glial and neuronal subtypes, apoptosis, migration of neurons and oligodendrocytes, neurite outgrowth (NOG), synaptogenesis, and neural network formation (Figure 1). When development of the DNT NAM battery was initiated, it was recognized that brain development is complex, comprising of distinct neurodevelopmental processes occurring at different ages, across specific brain regions, involving many different cell types. The concept of evaluating 'key' neurodevelopmental processes was designed to address this issue, given that these processes must occur for proper nervous system development and function, and the mechanisms underlying these processes are well conserved. By focusing on critical biological processes that are the underpinnings of the apical endpoints, the DNT NAM battery can provide relevant information regarding DNT potential of individual chemicals related to critical processes of neurodevelopment and evaluate early perturbations that are difficult to obtain or evaluate *in vivo*.

Chemical-induced alterations in these processes would indicate a potential hazard for neurodevelopment as these processes are highly conserved in mammals. Activity in these assays should not be construed as evidence of DNT *in vivo*. Although activity may be observed in the battery, it may not necessarily represent an adverse change that is typically linked to tissue-level or apical effects in a MOA/AOP. As described in the "*Toxicity Testing in the 21st Century*" report[6], to develop an AOP, not only is it necessary to establish plausible relationships among the key events, but quantitative relationships also need to be established. In other words, how much of a change in one key event is needed to result in an adverse effect at the next level of biological organization? Thus, certain exposures to a chemical may impact normal physiological responses in a way that may not necessarily be adverse. Consequently, the AOP concept requires an understanding of adaptive/homeostatic capacity of biological systems and their limits, relative to concentration and duration of exposure. OPP is taking a health-protective

[6] http://www.nap.edu/catalog.php?record_id=11970

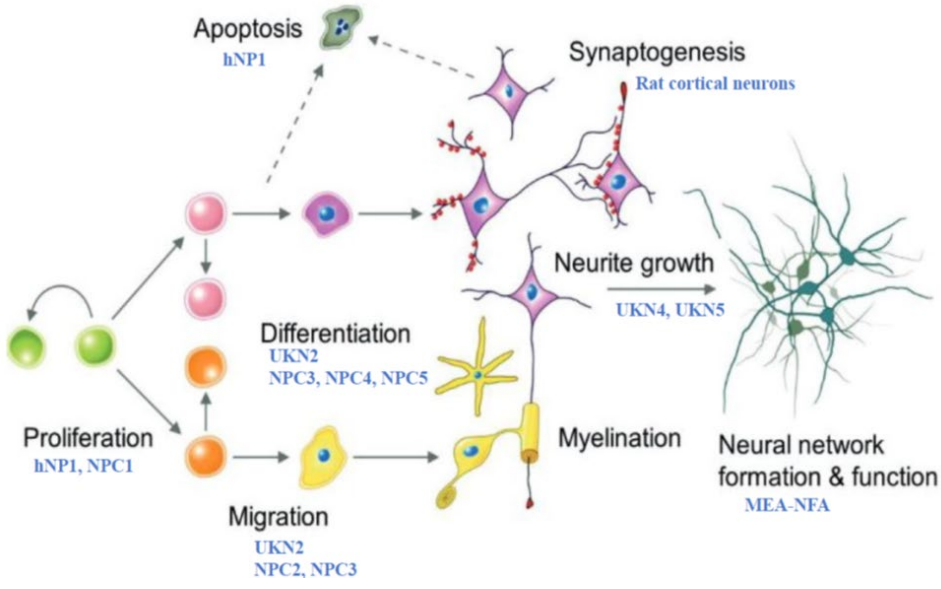
approach at this time by assuming that observed activity (in the form of true positive results) in the battery is associated with adversity.

Rather than trying to investigate every potential element that may be involved in a cascade of events that influence neurodevelopment (e.g., neurotransmitters, hormones, gene expression, neurodevelopmental stage, etc.), the DNT NAM battery focuses on key neurodevelopmental processes and therefore takes an integrative approach. This allows for the evaluation of the interplay of the hundreds - if not thousands - of upstream molecular steps involved in neurodevelopment and the potential consequences of their perturbation in critical processes that are associated with a wide spectrum of downstream DNT outcomes. This strategy obviates the need to elucidate every molecular step in a MOA/AOP for every potential DNT outcome, which would be a resource-intensive process in terms of time, animals, and cost. This, in turn, results in faster, more biologically and human-relevant evaluations, and ultimately health-protective decision-making.

The microelectrode array-based network formation assay (MEA-NFA; rat cortical neurons) and high-content imaging (HCI) assays for proliferation (human neural progenitor cell line (hNP1)), apoptosis (hNP1 cells), NOG (human embryonic stem (hN2) or induced pluripotent stem cell derived (CDI) neurons and rat cortical neurons), and synaptogenesis (rat cortical neurons) were developed by ORD. The assays developed by ORD have been described in detail in an Agency Issue Paper provided to the 2020 SAP[2]. International collaborators (University of Konstanz (UKN); Leibniz Institute for Environmental Medicine (IUF)) developed assays that are both overlapping and complementary to the ORD assays (Masjosthusmann et al., 2020)[7]. Using human primary neuroprogenitor cells (Lonza) in neurosphere cultures, IUF has developed assays for proliferation (NPC1), radial glial migration (NPC2), neuronal differentiation (NPC3), and oligodendrocyte differentiation (NPC5). Assays developed at the UKN include migration of human neural crest from h9 embryonic stem cells (UKN2), NOG in Lund human mesencephalic embryonic neuronal precursor (LUHMES) cells (UKN4), and NOG in human peripheral nervous system cells (immature dorsal root ganglion) cells from h9 embryonic cells (UKN5). A complete summary of the assays and strategies can be found in Sachana *et al*., 2021[8]. Each assay has a corresponding measurement of cytotoxicity which can be used to evaluate bioactivity occurring below the threshold of cytotoxicity. Changes in assay endpoints in the presence of cytotoxicity indicate that they are likely to be the result of non-specific cytotoxicity.

[7] https://doi.org/10.2903/sp.efsa.2020.EN-1938
[8] https://www ncbi.nlm nih.gov/pmc/articles/PMC7912397/

Figure 1. Critical neurodevelopmental processes and battery of *in vitro* assays (blue text)



The assays in the DNT NAM battery have been extensively characterized and reviewed. The methods, data from positive control and reference chemical testing, and the readiness of these assays have been evaluated and published in the peer-reviewed literature (Bal-Price *et al.*, 2018; Sachana *et al.*, 2021). For example, during development of the MEA-NFA, data from positive and negative control chemicals were first published for the assay in 2016 (Brown *et al.*, 2016[9]), and this was followed by publication of results with reference chemicals (Frank *et al.*, 2017[10]) that demonstrated or lacked putative evidence of DNT *in vivo*[11], and then by screening of larger sets of chemicals (Shafer *et al.*, 2019[12]). A similar approach was followed for all the other assays in the current DNT NAM battery and the primary literature for each battery is summarized in Sachana *et al.*, 2021.

In 2020, EPA convened a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) to review the DNT NAM battery with the OPs as a case study[13]. The Agency's Issue Paper supporting the SAP review provides additional characterization of the assays developed by EPA, including information on coefficients of variation, metrics of assay performance, and intralaboratory reproducibility. Overall, the SAP agreed that the current DNT NAM battery reflects, if not directly models, critical processes for neurodevelopment and that data from the battery can be used as part of a WOE evaluation, but also noted several processes

[9] Brown et al. (2016) Editor's Highlight: Evaluation of a Microelectrode Array-Based Assay for Neural Network Ontogeny Using Training Set Chemicals. Toxicol. Sci. 154(1):126-139. https://doi.org/10.1093/toxsci/kfw147

[10] Frank et al. (2017) From the Cover: Developmental Neurotoxicants Disrupt Activity in Cortical Networks on Microelectrode Arrays: Results of Screening 86 Compounds During Neural Network Formation. Toxicol. Sci. 160(1):121-135. https://doi.org/10.1093/toxsci/kfx169

[11] Note: Some chemicals were tested using doses and/or exposure routes that are not relevant for human health risk assessment.

[12] Shafer TJ, Brown JP, Lynch B, Davila-Montero S, Wallace K, Friedman KP. 2019. Evaluation of chemical effects on network formation in cortical neurons grown on microelectrode arrays. Toxicol Sci. 169(2):436-455

[13] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0006

and cell types that the panel believed to be missing in the battery. As discussed in the Agency's response to the SAP[14], the current battery is not entirely lacking these processes and cell types and/or these perceived limitations could be addressed by utilizing information from other available studies. The panel recommended the DNT battery "be a living and evolving process that can be revised and improved with new technology, assays, information on validity and reliability and *in vivo* translation"; however, the panel also noted that "this is not meant to preclude the ability of the Agency to utilize all valid and relevant data in their efforts to determine risks for human health".

International review and acceptance of the battery has also progressed since the 2020 SAP. Some organizations, such as European Food Safety Authority (EFSA) and European Chemicals Agency (ECHA), currently consider data from the DNT NAM battery as part of their evaluations. Further, an Expert Group on DNT was convened by the Organization of Economic Cooperation and Development (OECD) to develop a guidance document that describes the use of the battery as part of an Integrated Approach for Testing and Assessment (IATA) for DNT[15]. This guidance went through two rounds of review by OECD member states and partners (e.g., non-governmental organizations (NGOs) and industry), and was approved by the OECD Working Group of the National Coordinators for the Test Guidelines Programme (WNT) at its meeting in April 2023. This guidance includes several case-studies for application of the battery to DNT decision-making. In addition, the guidance includes additional technical characterization of the assays, as it contains appendices that contain a "ToxTemp" form for each assay (Krebs *et al.*, 2019). These "ToxTemp" forms contain information regarding the biological/human relevance, technical performance, appropriate assay positive controls, and domains of applicability for each assay.

DNT NAM Battery Analysis Methods

Assay data from ORD and international collaborators were compiled and analyzed using the publicly available ToxCast Analysis Pipeline (tcpl) R package (version 2.1.0). The ToxCast database contains high throughput data for chemicals of interest to US EPA, and tcpl is used for storage, normalization, and visualization of high throughput data. In brief, the chemical concentration-response data were normalized on a plate-by-plate basis to the median of the control wells and curve-fit using tcpl. Curve-fitting was performed in the increased bioactivity (up) or decreased bioactivity (down) direction for each assay endpoint, with a few exceptions where the biology precluded a bi-directional response (e.g., viability was only considered in the down direction). The 2020 Agency Issue Paper included 34 MEA-NFA endpoints (17 endpoints fit in the up and down direction), while the present analysis excluded the 17 'up' endpoints based on recent findings that these endpoints are predominantly inactive and have yet to be validated with positive controls (Carstens *et al*., 2022[16]). The efficacy cutoff was defined as three times the baseline median absolute deviation (BMAD), where BMAD approximates baseline noise using the vehicle control wells and the two lowest concentrations of test chemical wells on each plate, with a few exceptions (please see Table A.1 for additional details). The potency value was

[14] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0057
[15] https://www.oecd.org/env/ehs/testing/guidance-evaluation-of-data-developmental-neurotoxicity-in-vitro-testing.pdf
[16] https://pubmed ncbi nlm nih.gov/35172012/

indicated by the concentration at 50% maximal activity values (AC_{50}) for any positive assay endpoints in the suite of assays.

In some cases, more than one sample (with three technical replicates) of a compound may have been tested in an assay. Two samples were tested in the majority of the ORD assays for acephate and methamidophos. If positive results were obtained from both samples (tests of the sample run from different cultures or days) for the same endpoint, the mean of the individual sample values was used as the AC_{50} for that endpoint. In cases where the results from two samples of the same compound were not concordant, a number of factors were evaluated to decide how to treat the discordant data. This included consideration of any information regarding sample stability, evaluation of the robustness of positive responses, and consideration of ToxCast flags (discussed below in more detail).

True positive responses were determined by several criteria: 1) For all concentration response curves, the goodness of model curve fitting was evaluated using 9 cautionary flags generated in tcpl (Table A.2). Curves with 3 or more flags were considered not reliable (and hence not considered true positive results). The tcpl flags were designed to capture general indicators of excessive noise, borderline activity, or overfitting and may not fully capture the goodness of fit; therefore, expert review was used when necessary. 2) A true positive was also defined by 'selective' activity, or bioactivity occurring below cytotoxicity. Selective activity was computationally defined as a response with a selectivity score of > 0.3 (Stiegler *et al*., 2011[17]; Krug *et al*.[18], 2013; Frank *et al*., 2018[19]). The selectivity score was calculated as the cytotoxicity potency (log10-AC_{50} of the cytotoxicity endpoint for each assay) minus the potency of a positive response (log10-AC_{50}). 3) When a chemical did not demonstrate selective bioactivity, but demonstrated cytotoxicity in the DNT NAMs, a generalized value of cell stress and cytotoxicity-mediated effects of each chemical was considered (i.e., the ToxCast cytotoxicity-associated "burst" value). The "burst" is defined as a lower bound estimate for a concentration that might cause cell stress or cytotoxicity based on a battery of more than 800 *in vitro* assay endpoints (predominantly non-neuronal from high-throughput assays) (Judson *et al.,* 2016[20]). Previous work indicated that chemicals can be cytotoxic at concentrations occurring below the "burst" value (Carstens *et al*., 2022), suggesting that neuronal cell types and/or neurodevelopmental processes can be more sensitive to cytotoxicity compared to generalized *in vitro* cell stress models and should be considered in the interpretation of DNT potential. Therefore, cytotoxic activity in any DNT NAMs occurring below the "burst" was considered when determining a true positive. Additional information on ToxCast data generation and different levels of data analysis can be found in Table A.2 and the Agency website [21]. 4) Lastly, in order to determine true positive activity across different types of assay endpoints, the data were examined holistically, and an expert review was conducted to assess the robustness of the positive endpoints and determine if they represented genuine bioactivity or spurious responses. For example, Carstens *et al*. (2022) reported that MEA-NFA endpoints are highly correlated and sensitive to chemicals that exhibit *in vivo* DNT. Therefore, a low hit rate in the MEA-NFA assay may be indicative of

[17] https://pubmed ncbi nlm nih.gov/21342877/
[18] https://pubmed ncbi nlm nih.gov/23179753/
[19] https://pubmed ncbi nlm nih.gov/28973552/
[20] https://pubmed ncbi nlm nih.gov/27208079/
[21] https://www.epa.gov/chemical-research/toxcast-data-generation-toxcast-pipeline-tcpl

spurious activity. For more information on DNT NAM technologies and data analysis methods, refer to the 2020 SAP Agency Issue Paper and Carstens *et al*., 2022.

Estimation of average blood concentrations using kinetic modeling

The DNT NAM battery data provides an opportunity to examine the relative sensitivity of potential DNT activity to doses that inhibit AChE. Specifically, average blood concentrations estimated at PODs based on 10% AChE inhibition can be compared to AC_{50} values from the DNT NAM battery to examine whether and to what degree PODs based on AChE are protective of the NAM-based concentrations. This comparison is possible because both the average blood concentration and DNT NAM battery-based AC_{50} are internal concentrations, and each reflect a different biological endpoint. A kinetic model is required to estimate the appropriate internal concentrations at the AChE-based POD. Similar to the other aspects of risk assessment, a tiered approach should be utilized when selecting an appropriate kinetic model to maximize efficiency, minimize the use of animals, and fit the specific purpose. On the continuum of kinetic models that may be applied for estimating the internal concentration, at one extreme are models that require a minimum amount of chemical-specific data (such as the HTTK model[22]). At the other extreme are highly refined PBPK models that require a significant amount of time and resources for development and evaluation. The amount of data and the type of model necessary for estimating the internal concentrations should depend on the purpose of the application and the degree of uncertainty permissible given that purpose.

For acephate and methamidophos, the DNT NAM battery did not show any true positive hits for acephate or methamidophos (discussed in detail below). Therefore, use of a kinetic model was not necessary for acephate or methamidophos because there were no AC_{50} values to compare to AChE-based PODs.

Results

DNT NAMs – In Vitro Activity

Acephate: Acephate did not show any activity in assays measuring proliferation, apoptosis, cytotoxicity, neuronal migration, neuronal differentiation, NOG, network formation and function, synaptogenesis, or oligodendrocyte differentiation using both human and rat cell lines.

Methamidophos: Methamidophos demonstrated activity in one assay endpoint in human cells measuring a decrease in NOG initiation. Following consultation with ORD scientists, the activity was not considered a true positive given the activity was borderline (the biological response was not robust compared to the variability in controls) and occurring only at the highest concentration tested (100 µM) (Appendix C).

[22] Details of the HTTK model can be found in the 2020 SAP Agency Issue Paper and peer reviewed literature: Pearce RG, Setzer RW, Strope CL, Wambaugh JF, Sipes NS. httk: R Package for High-Throughput Toxicokinetics. J Stat Softw. 2017 Jul 17;79(4):1-26. https://doi.org/10.18637/jss.v079.i04. PMID: 30220889 Linakis MW, Sayre RR, Pearce RG, Sfeir MA, Sipes NS, Pangburn HA, Gearhart JM, Wambaugh JF. Development and evaluation of a high throughput inhalation model for organic chemicals. J Expo Sci Environ Epidemiol. 2020 Sep;30(5):866-877. https://doi.org/10.1038/s41370-020-0238-y. Epub 2020 Jun 16. Erratum in: J Expo Sci Environ Epidemiol. 2020 Jul 9; PMID: 32546826

In rat assays, methamidophos demonstrated activity in five assay endpoints, including four assay endpoints related to neural network formation in the MEA-NFA and one endpoint measuring cytotoxicity in the same assay. Two of the active MEA-NFA endpoints demonstrated selective activity (activity occuring below a threshold of cytotoxicity). Although two samples of methamidophos were tested in the MEA NFA, activity was only observed with one sample. Upon expert review of the concentration response curves for the five assay endpoints, the results demonstrated low confidence curves related to noisy data and borderline activity at the highest concentration (Appendix B). Based on previous findings, the MEA-NFA, which includes 17 endpoints, typically demonstrates activity in a majority of endpoints in response to chemicals with evidence of *in vivo* DNT (Carstens et al 2022). Given that methamidophos was active in only a few MEA-NFA endpoints and demonstrated low confidence curves, as well as the lack of activity in the other sample, it was concluded that there were no true positives for methamidophos in the MEA-NFA.

Consequently, the positive results in the rat DNT NAM endpoints were not considered true positives due to borderline or noisy concentration response curves, as well as the lack of activity in the second methamidophos sample. As a result, methamidophos is considered to lack *in vitro* DNT activity, similar to its parent compound.

Appendix A contains additional details on the DNT NAM battery, including efficacy cutoff methods for each DNT NAM endpoint in the ToxCast Data analysis pipeline (Table A.1), ToxCast Data Pipeline for MEA-NFA and HCI assays (Table A.2), a summary of assay endpoints measured for acephate and methamidophos in human and rat neuronal cell lines (Table A.3), and assay activity and endpoints measured for methamidophos in human and rat neuronal cell lines (Table A.4)[23]. Concentration response curves for methamidophos in rat and human cell lines are included in Appendix B and C, respectively.

Comparing NAM and AChE-based internal concentrations: Since there were no true positive hits for either acephate or methamidophos, the HTTK approach to calculate internal concentrations for comparison to AChE-based PODs was not conducted.

In vivo DNT studies (guideline)

For acephate and methamidophos, guideline *in vivo* DNT studies are available and were incorporated in the previous risk assessment. In both studies, the only effect observed was AChE inhibition.

In the guideline *in vivo* DNT study in rats for acephate (MRID 46151802; N. McCarroll, TXR 0052318, D298039, 24-JAN-2006), pregnant rats were dosed via gavage from gestation day (GD) 6 through lactation day (LD) 6 at dose levels of 0, 0.5, 1, and 10 mg/kg/day. There were no adverse effects observed in maternal animals (no LOAEL established). Pups were similarly dosed from postnatal days (PNDs) 7-21. There were no adverse effects on offspring survival, body weight, clinical signs, functional observational battery (FOB), motor activity, developmental landmarks, auditory startle reflex, learning and memory, brain weights, brain

[23] Since there was no assay activity observed for acephate, there is not a table comparable to Table A.4, nor concentration response curves included in the appendix for acephate.

morphology or neuropathology. On PND 21, substantial brain AChE inhibition (↓29% in males and ↓25% in females) was observed in pups at 0.5 mg/kg/day (offspring LOAEL; no offspring NOAEL established), with similar or greater inhibition (up to approximately 60%) at the higher doses tested. Similarly, red blood cell (RBC) AChE inhibition was observed at all doses on PND 21 (16-50% in males and 14-63% in females). This results in apparent increased quantitative susceptibility in pups; however, AChE inhibition was not measured in maternal animals in this study. However, there are numerous studies available in the acephate database that demonstrate considerable AChE inhibition would have been observed in maternal animals at a similar dose level (0.5 mg/kg/day) if AChE measurements had been included in the DNT study. For example, in the gestational CCA study in rats (MRID 46151805; P. Chin, TXR 0054967, D360436, 25-AUG-2009), dams dosed from GD 6-21 exhibited 17% brain AChE inhibition at 0.5 mg/kg/day and up to 62% at 10 mg/kg/day.

In the methamidophos guideline *in vivo* rat DNT study (MRID 45666401; TXR 0058601), pregnant rats were dosed continuously via the diet at dose levels of 0, 1, 10 or 30 ppm (0, 0.1, 0.9, 2.5 mg/kg/day during gestation, and 0, 0.2, 2.4, and 7.9 mg/kg/day during lactation) from GD 0 – LD 21. There were no significant changes in maternal mortality, clinical signs, body weights, body weight gains, food consumption, reproductive performance, or abbreviated FOB. However, brain and RBC AChE inhibition was observed at all doses tested (8-83% and 12-84%, respectively). The maternal LOAEL is established at 1 ppm (0.1 mg/kg/day) based on brain and RBC cholinesterase inhibition in dams, with no maternal NOAEL identified. In offspring, there were no adverse effects observed on survival, food consumption, abbreviated FOB, brain weights, brain morphology, or neuropathology. Similar to the maternal animals, the only effect observed in offspring was AChE inhibition. RBC inhibition was observed on PND4 and PND21 at doses ≥0.9 mg/kg/day (20-41% on PND4 and 12-53% on PND21). Brain inhibition was observed at 2.5 mg/kg/day on PND4 (41%) and ≥0.9 mg/kg/day on PND21 (13-43%). The offspring LOAEL is established at 0.9 mg/kg/day, with an offspring NOAEL of 0.1 mg/kg/day.

In vivo DNT studies (from the literature)

In the updated 2016 literature review on neurodevelopmental effects for the organophosphates (OPs) (A. Aldridge *et al*. D437043, 29-DEC-2016), two additional methamidophos studies (Castro *et al*., 2000[24], Lima *et al.* 2013[25]) that evaluated neurobehavioral effects were identified. There were no additional *in vivo* studies identified in the recent toxicological literature search conducted in 2023 (included in the second revised human health risk assessment for acephate and methamidophos in support of registration review [(R. Louden et al., D463758, 24-AUG-2023)]), that were considered relevant to DNT. Results from the studies identified in the updated 2016 literature review should be considered with caution, as they both only tested one dose level (1 mg/kg/day) and the purity of the test substance was not reported. Additionally, the Lima *et al.* (2013) study dosed mice via subcutaneous injection (not a relevant route of exposure

[24] de Castro VL, Chiorato SH, Pinto NF. Relevance of developmental testing of exposure to methamidophos during gestation to its toxicology evaluation. Toxicology Letters. (2000); 118(1-2):93-102. https://doi.org/10.1016/s0378-4274(00)00271-x

[25] Lima CS, Dutra-Tavares AC, Nunes F, Nunes-Freitas AL, Ribeiro-Carvalho A, Filgueiras CC, Manhães AC, Meyer A, Abreu-Villaça Y. Methamidophos exposure during the early postnatal period of mice: immediate and late-emergent effects on the cholinergic and serotonergic systems and behavior. Toxicol Sci. 2013 Jul;134(1):125-39. https://doi.org/10.1093/toxsci/kft095. Epub 2013 Apr 17. PMID: 23596261; PMCID: PMC3840737.

for human health risk assessment). However, these studies provide additional support that there is low concern for DNT-related effects following exposure to methamidophos and AChE inhibition is a protective endpoint.

In the de Castro *et al*. (2000) study with rats, maternal animals were dosed by gavage with 1 mg/kg/day of methamidophos (purity not reported) from GD 6-15. There were no adverse effects observed in maternal animals. Parameters examined in maternal animals included: body weight, clear signs of toxicity, pregnancy rate and pup viability. There was also no adverse effect on neuromotor measures of activity (including swimming performance and open-field measurements) reported in rat pups following treatment of maternal animals with methamidophos, although high variability of the measures was discussed. AChE inhibition was not measured in this study; however, a pilot study evaluating nonpregnant female rats dosed with 1 mg/kg/day methamidophos for 10 days showed 16% plasma AChE inhibition, but brain AChE was apparently not measured (no citation provided; however, the results from this preliminary study were discussed in the methods section of the main study as justification for dose selection and appear to have been conducted in the same laboratory). This suggests that dams treated at the same dose over the same exposure duration in the deCastro *et al.* (2000) study most likely experienced AChE inhibition as well. Furthermore, based on the extensive database for methamidophos that includes numerous studies that measure AChE inhibition, significant AChE inhibition would be expected at 1 mg/kg/day.

In Lima *et al.* (2013), mice were exposed (via subcutaneous injection) to 1 mg/kg/day methamidophos (purity not reported) during the early postnatal period (PND 3-9). There were no adverse DNT-related observations in mice treated with 1 mg/kg/day from PND 3-9. However, the mice experienced considerable brain AChE inhibition throughout dosing, with ~36% and 46% inhibition occurring at 1 and 4 hr after the first dose, 53% and 61% inhibition occurring at 1 and 4 hr after the last dose, and ~19% brain inhibition occurring the day after the last dose.

Epidemiology studies

An extensive search of the literature was performed in 2015 to identify epidemiological investigations of the association between OP exposures and potential DNT outcomes (A. Lowit, D331251[26],15-SEP-2015). The review was then updated in 2016 to incorporate additional studies and address public comments (A. Aldridge et al., D437043[27], 29-DEC-2016). Exposure in most of these studies was assessed using biomarkers. In limited instances, exposure was assessed using a direct measure of an OP pesticide (e.g., chlorpyrifos measures in blood) or a metabolite specific to a particular OP (e.g., malondialdehyde (MDA) as a specific metabolite of malathion). There were no studies with specific biomarkers for acephate or methamidophos included in the 2015/2016 review.

The majority of the epidemiology studies in the 2015/2016 review used non-specific biomarkers of OP exposure, with urinary dialkyl phosphates (DAPs) being the most commonly measured biomarkers. DAPs are considered non-toxic metabolites[28], and each DAP is a breakdown

[26] https://www.regulations.gov/document/EPA-HQ-OPP-2009-0059-0030
[27] https://www.regulations.gov/document/EPA-HQ-OPP-2008-0351-0107
[28] https://www.cdc.gov/biomonitoring/OP-DPM_FactSheet.html#:~:text=Once%20they%20enter%20the%20body,an%20exposure%20to%20organophosphate%20insecticides

product from multiple OPs, making it impossible to separate exposure and associated effects for individual, specific OPs. Another limitation associated with using urinary DAPs as biomarkers as an exposure measure includes temporal variability, with levels often varying substantially over short time scales (i.e., day-to-day). As such, quantification of DAPs in a single urine sample may not represent an individual's typical exposure to OP pesticides since it only represents a "snapshot" in time that can underestimate or overestimate typical exposures. Additionally, urinary DAP levels may also reflect direct exposure to DAPs, rather than exposure to an OP, because DAPs can form through degradation processes in/on food and in the environment. For example, DAPs can be present in commodities prior to consumption (e.g., Chen et al., 2012; Zhang et al., 2008[29]). Therefore, measured DAP levels can be a reflection of OP exposure, direct DAP exposure (e.g., through consumption of commodities containing DAPs), or a combination thereof. Furthermore, multiple FIFRA SAPs have identified uncertainties in the epidemiological data[30], including but not limited to relatively modest sample sizes, concerns with the representativeness of a single point exposure, potential for exposure misclassification, and questions about "biologic plausibility due to lack of clarity on mechanism of action". As a result, although the measures in the epidemiological studies involving DAPs may provide qualitative evidence that exposure to one or more OPs occurred, the actual level of such exposure during critical window(s) of susceptibility is not known. Consequently, these studies are unable to provide a robust quantitative evaluation of the sensitivity of potential DNT effects relative to AChE inhibition for any specific OP. Moreover, studies using DAPs are not relevant for acephate and methamidophos since neither degrade into DAPs.

In support of the risk assessment for acephate, HED conducted a systematic review of the epidemiology with the aim of identifying epidemiological studies that reported effect measures specific to associations between acephate and methamidophos exposure and health outcomes, including potential DNT outcomes (E. Jones et al., D467800, 28-JUL-2023). This included studies identified in a previous epidemiology literature review that was completed for acephate/methamidophos (S. Recore et al., D423142, 30-SEP-2014). The 2023 review used the methods described in OPP's "Framework for Incorporating Human Epidemiologic & Incident Data in Risk Assessments for Pesticides[31]", the methods described in Sections 3.0-3.5 of the *Acephate: Tier II Review of Human Incidents and Epidemiology for the Draft Risk Assessment*, and generally followed the guidance provided by the National Toxicology Program/Office of Health Assessment and Translation (NTP/OHAT)[32]. The 2023 systematic literature review considered publications available in peer-reviewed literature databases (PubMed, PubMed

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[29] Chen et al. (2012) Preformed biomarkers including dialkylphosphates (DAPs) in produce may confound biomonitoring in pesticide exposure and risk assessment. J Agric Food Chem. 60(36):9342-51; Zhang et al. (2008) Dialkylphosphates (DAPs) in fruits and vegetables may confound biomonitoring in organophosphorus insecticide exposure and risk assessment. J Agric Food Chem. 56(22):10638-45.

[30] Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting held April 10-12, 2012 on "Chlorpyrifos Health Effects" - https://www regulations.gov/document/EPA-HQ-OPP-2012-0040-0029 Transmittal of Meeting Minutes of the April 19-21, 2016 FIFRA SAP Meeting Held to Consider and Review Scientific Issues Associated with "Chlorpyrifos: Analysis of Biomonitoring Data" - https://www.regulations.gov/document/EPA-HQ-OPP-2016-0062-0140

[31] US EPA. December 28, 2016. Office of Pesticide Programs' Framework for Incorporating Human Epidemiologic & Incident Data in Risk Assessments for Pesticides. https://www3.epa.gov/pesticides/EPA-HQ-OPP-2008-0316-DRAFT-0075.pdf

[32] Handbook for Conducting a Literature-Based Health Assessment Using OHAT Approach for Systematic Review and Evidence Integration, January 9, 2015.

Central, Scopus, and Science Direct) and a HED-maintained electronic library of published articles from the Agricultural Health Study (AHS)[33], a prospective cohort study of farmer pesticide applicators and their families in Iowa and North Carolina of the United States.

HED identified nine publications that reported on the potential association between acephate/methamidophos exposure and potential DNT health effects in children in five study populations including 1) the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) longitudinal birth cohort of pregnant women and their children in a farmworker community in the Salinas Valley of California (Gunier et al., 2017[34]; Hyland et al., 2021[35]; Hyland et al., 2022[36]; Gunier et al., 2022[37]); 2) a population-based study of children living in an agricultural area in San Joaquin Valley of California (von Ehrenstein et al., 2019[38]); 3) a pooled analysis of two records-based case-control studies of mother-infant pairs in California (Rull et al., 2006[39]); 4) a birth cohort of infants in China (Silver et al., 2017[40]; Silver et al., 2018[41]); and, 5) a case-control study of children 0-5 years old in Thailand (Juntarawijit et al., 2020[42]).

Investigated outcomes of the epidemiology studies were grouped into the following six general domains of neurodevelopment and neurobehavior: motor/sensory function of development; learning, memory, intelligence, and cognitive development; attention, hyperactivity, and externalizing and internalizing behaviors; risk-taking behaviors and delinquent acts; autism spectrum disorder; and neural tube defects (see Appendix D). The majority of these outcomes

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[33] https://aghealth nih.gov/

[34] Gunier, R. B., Bradman, A., Harley, K. G., Kogut, K., & Eskenazi, B. (2017). Prenatal Residential Proximity to Agricultural Pesticide Use and IQ in 7-Year-Old Children. *Environ Health Perspect, 125*(5), 057002. d https://doi.org/10.1289/ehp504

[35] Hyland, C., Bradshaw, P. T., Gunier, R. B., Mora, A. M., Kogut, K., Deardorff, J., . . . Eskenazi, B. (2021). Associations between pesticide mixtures applied near home during pregnancy and early childhood with adolescent behavioral and emotional problems in the CHAMACOS study. *Environ Epidemiol, 5*(3). https://doi.org/10.1097/ee9.0000000000000150

[36] Hyland, C., Bradshaw, P., Deardorff, J., Gunier, R. B., Mora, A. M., Kogut, K., . . . Eskenazi, B. (2022). Interactions of agricultural pesticide use near home during pregnancy and adverse childhood experiences on adolescent neurobehavioral development in the CHAMACOS study. *Environ Res, 204*(Pt A), 111908. https://doi.org/10.1016/j.envres.2021.111908

[37] Gunier, R. B., Deardorff, J., Rauch, S., Bradshaw, P. T., Kogut, K., Sagiv, S., Eskenazi, B. (2022). Residential proximity to agricultural pesticide use and risk-taking behaviors in young adults from the CHAMACOS study. *Environ Res, 215*(Pt 2), 114356. https://doi.org/10.1016/j.envres.2022.114356

[38] von Ehrenstein, O. S., Ling, C., Cui, X., Cockburn, M., Park, A. S., Yu, F., . . . Ritz, B. (2019). Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: population based case-control study. *BMJ, 364*. https://doi.org/10.1136/bmj.l962

[39] Rull, R. P., Ritz, B., & Shaw, G. M. (2006). Neural tube defects and maternal residential proximity to agricultural pesticide applications. *Am J Epidemiol, 163*(8), 743-753. https://doi.org/10.1093/aje/kwj101

[40] Silver, M. K., Shao, J., Zhu, B., Chen, M., Xia, Y., Kaciroti, N., . . . Meeker, J. D. (2017). Prenatal naled and chlorpyrifos exposure is associated with deficits in infant motor function in a cohort of Chinese infants. *Environ Int, 106*, 248-256. https://doi.org/10.1016/j.envint.2017.05.015

[41] Silver, M. K., Shao, J., Ji, C., Zhu, B., Xu, L., Li, M., . . . Meeker, J. D. (2018). Prenatal organophosphate insecticide exposure and infant sensory function. *Int J Hyg Environ Health, 221*(3), 469-478. https://doi.org/10.1016/j.ijheh.2018.01.010

[42] Juntarawijit, Y., Chaichanawirote, U., Rakmeesri, P., Chairattanasakda, P., Pumyim, V., & Juntarawijit, C. (2020). Chlorpyrifos and other pesticide exposure and suspected developmental delay in children aged under 5 years: a case-control study in Phitsanulok, Thailand. *F1000Res, 9*, 1501. https://doi.org/10.12688/f1000research.27874.5

were investigated in a single study population, with one to three publications typically available per outcome. Outcomes were measured by questionnaire and clinical exam using validated tests of neurodevelopment; however, no two studies used the same outcome measurement.

Regarding the exposure assessment of the relevant epidemiology studies, authors of the cohort of mother-infant pairs in China (Silver et al., 2017; Silver et al., 2018) directly measured acephate and methamidophos exposure through umbilical cord blood (both studies utilized the same blood samples for analyzing pesticide exposure. However, most studies relied on a GIS-based exposure assessment method that estimated exposure based on geographic proximity of the pregnant mother's residence to treated agricultural fields. Briefly, studies used pesticide use reporting data from the California Department of Pesticide Regulation (DPR)[43] and geographic proximity of residences to treated agricultural fields at a distance (usually 1-2 km) that was shown to be most strongly correlated with concentrations of agricultural pesticides in dust samples collected from homes in that region (Harnly et al., 2009;[44] Gunier et al., 2011)[45] to estimate potential pesticide exposure during specific timepoints (i.e., before, during and after pregnancy).

Certain analyses included the residence reported on the birth certificate and other analyses determined the exact maternal address during prenatal and postnatal periods reducing the uncertainty in the exposure assessment. Using this method and the CA DPR pesticide use reports, the amount of pesticides applied in nearby agricultural fields within a certain radius of the pregnant woman's residence for the duration of the exposure time period (i.e., preconception, pregnancy, first year of life) based on a known address for each trimester or on the address reported on the birth certificate was used to estimate exposure. The address on the birth certificate is less specific than addresses by trimester as it does not account for possible residential mobility during pregnancy[46]. One publication (Gunier et al., 2017) used the GIS-based method to estimate exposure to acephate and other pesticides and then used maternal urinary DAP concentrations as a covariate in the statistical model to adjust for other pesticide exposures. The GIS-based assessments may have limited ability to investigate the relationship with acephate and methamidophos specifically if there is a strong degree of correlation across different pesticides. As such, these investigations may be unable to distinguish between factors associated with geographic proximity to agricultural land and living, pesticide use in general, and specific pesticides.

Statistically significant associations were reported between acephate exposure and full-scale IQ (Gunier et al., 2017) and hyperactivity (Hyland et al., 2021) in the CHAMACOS prospective cohort study population, and autism spectrum disorder (von Ehrenstein et al., 2019) in the Salinas Valley, CA case-control study population. All three studies relied upon a GIS-based assessment approach to estimate pesticide exposure at the maternal residence. Overall, the magnitude of the effect estimates that reported positive associations were relatively small (i.e., close to the null – 1.0 for ORs and RRs), were not statistically significant or were borderline

[43] https://www.cdpr.ca.gov/docs/pur/purmain htm

[44] Harnly, M.E., Bradman, A., Nishioka, M., et al., 2009. Pesticides in dust from homes in an agricultural area. Environ. Sci. Technol. 43 (23), 8767–8774.

[45] Gunier, R.B., Ward, M.H., Airola, M., et al., 2011. Determinants of agricultural pesticide concentrations in carpet dust. Environ. Health Perspect. 119 (7), 970–976.

[46] Past studies have indicated that around 11 – 32 % of pregnant women move their residence at least one time throughout pregnancy, and the median move distances were between 4.2 – 10 km (Lupo et al., 2010; Strickland et al., 2017; Pereira et al., 2016).

statistically significant, were not consistently observed across analyses within the studies (e.g., with or without adjustment for exposure to other pesticides, maternal-reported vs. youth-reported), and/or were only observed following stratified analyses. Below are short summaries of the statistically significant associations observed.

- Gunier et al. (2017) reported on the association between acephate exposure and learning and cognition in the CHAMACOS cohort study. Authors observed a statistically significant association between a standard deviation (SD) increase in acephate use within 1 km of maternal residence during pregnancy and decreased Full-Scale IQ; however, an association was not observed when exposure to other pesticides was considered (i.e., adjusted for in the statistical analysis). A statistically significant association between a SD increase in acephate use within 1 km of maternal residence during pregnancy and decreased Verbal Comprehension was also observed when exposure to other pesticides was not considered. Analyses considering exposure to other pesticides were not reported for Verbal Comprehension. There were no associations observed with working memory, processing speed, or perceptual reasoning.

- Hyland et al. (2021) reported borderline statistically significant associations between a two-fold increase in acephate applications during *childhood* (0-5 yrs) and *maternal*-reported hyperactivity among all children, hyperactivity among girls only, and externalizing problems among girls only in the CHAMACOS cohort. Similar analyses using *youth*-reported hyperactivity did not observe a significant association. There were no other significant associations observed between acephate applications during *childhood* and internalizing problems, depression, anxiety, or attention problems (using maternal- or youth-reported data). There were no significant associations observed between acephate application during *pregnancy* and any investigated outcomes.

- von Ehrenstein et al. (2019) observed a statistically significant association between acephate exposure at 3-months pre-pregnancy and autism spectrum disorder with intellectual disability comorbidity, among the San Joaquin Valley case-control participants and that significant association remained after adjustment for exposure to other pesticides. A significant association was not observed for exposure at 3-months pre-pregnancy and all autism spectrum disorders. There was also no evidence of a significant association between acephate exposure and autism spectrum disorder (with or without intellectual disability) for acephate exposure during any other exposure time period investigated.

No evidence of a significant association was reported for three outcomes: motor/sensory function and developmental delay in the cohort in China and the case-control study in Thailand; risk taking behavior in the CHAMACOS cohort; and neural tube defects in the registry-based case-control study in California (six of the nine identified publications).

The 2023 epidemiological review concluded that there was *insufficient epidemiological evidence* of a clear associative or causal relationship between acephate or methamidophos exposure and the six DNT outcomes in children at this time including: motor/sensory function and development; cognitive development; attention, hyperactivity, and externalizing and internalizing behavior; risk-taking behavior; autism spectrum disorder; and neural tube defects. This conclusion was based on small body of studies (i.e., typically only one or two studies per health outcome) that often had substantive limitations with respect to their exposure assessment

approach and/or outcome assessment, a lack of consistent evidence of a positive association, and the potential for bias in the available studies. Additional details including more in-depth discussion of the strengths and limitations of the studies reviewed here can be found in the *Acephate: Tier II Review of Human Incidents and Epidemiology for the Draft Risk Assessment* (E. Jones et al. D467800, 28-JUL-2023).

Discussion

As previously mentioned, OPP took a conservative approach by performing the 2015/2016 review for the OPs as a group based on the assumption that, like AChE inhibition and subsequent neurotoxicity, DNT outcomes would share a common MOA/AOP and therefore similar potential DNT concerns would exist across OPs. At that time, the uncertainties in the human dose-response relationship for potential neurodevelopmental outcomes and its quantitative relationship to AChE inhibition prevented reduction of the 10X FQPA SF for the OPs.

Based on the best available science that indicates differences in DNT potential exist across OPs, OPP has determined that DNT potential of OPs should be evaluated on a chemical-by-chemical basis. Therefore, the purpose of this evaluation was to assess the DNT potential of acephate and its metabolite/degradate, methamidophos, using chemical-specific data to evaluate the sensitivity of potential DNT effects relative to AChE inhibition. For this evaluation, the chemical-specific studies available included *in vitro* and *in vivo* assays, as well as epidemiological studies. In the case of DNT, exposure to xenobiotics during critical stages of development may result in altered neural development leading to potential lifelong ramifications. Assessing the potential DNT hazard and/or risk from a chemical exposure is a complex process that involves multiple evidence streams that converge in a WOE driven evaluation. Historically, most of the DNT information has been collected in toxicology studies using animal models. Incorporation of additional information from epidemiological studies and *in vitro* assays provides for a more robust evaluation. However, it is well recognized that any given assay or study, including the *in vivo* DNT guideline study, will not fully evaluate all elements of the nervous system. As such, the strengths and limitations associated with each line of evidence need to be taken into consideration and balanced with those associated with other lines of evidence, including the quality and human relevance of the data obtained from other studies and assays.

The basic purpose of DNT guideline testing in animals is to assess the potential of chemicals to cause adverse neurodevelopmental outcomes. This is achieved through a series of evaluations that measure the functional and/or structural integrity of the developing nervous system. The strengths of the *in vivo* DNT guideline study reside in its ability to evaluate multiple functional domains using a whole organism. Testing with laboratory animals captures intact biological and physiological conditions, including absorption, distribution, metabolism, and interactive biology, in a living system. This includes evaluation of numerous endpoints (functional, behavioral, and anatomical) in the nervous system at multiple time points across different lifestages. However, it does not completely evaluate all aspects of nervous system structure and function/behavior. Because the DNT guideline study infers DNT effects on the basis of apical endpoints, with little or no information on the underlying biological processes responsible for the observed phenotype, it is unknown if the observed effects truly represent developmental toxicity derived from nervous system disruption. Reliable detection, measurement, and interpretation of treatment-related DNT effects in the guideline study depends on appropriate study design and conduct that adequately controls for confounding factors such as variability (*e.g.,* due to dosing regimen, age at treatment

and assessment, or inherent measure variability), impact of systemic maternal and/or offspring toxicity, experimental procedures, environmental conditions, etc.[47] Despite these uncertainties, the guideline studies, as well as the available literature studies that evaluated neurobehavioral effects in laboratory animals, clearly demonstrated that AChE was the most sensitive (and only) adverse effect observed.

Epidemiological studies are aimed at investigating associations between a risk factor (e.g., chemical exposure) and particular health outcomes in humans. An obvious strength of an epidemiological study is its evaluation of the relevant species (humans) and groups of interest (e.g., elderly, children, etc.) at relevant exposure levels thereby obviating the need to extrapolate across species or from high exposure levels. Another strength is the ability of epidemiological studies to research a wide range of health outcomes, some of which may be difficult to evaluate in experimental animals or an appropriate animal model may not exist for evaluation. However, major limitations in epidemiological studies are associated with the inability to control the populations and exposures being investigated. Errors in epidemiological studies typically arise from chance, bias, and confounding. Researchers attempt to minimize the impact of these errors through study design and execution (e.g., sufficient sample size, proper selection of study subjects, measurement of potential confounders). Multiple FIFRA SAPs have identified uncertainties in the epidemiological data in the 2015/2016 review[48], including but not limited to relatively modest sample sizes, concerns with the representativeness of a single point exposure, potential for exposure misclassification, and questions about "biologic plausibility due to lack of clarity on mechanism of action".

Direct measurement of all exposures of interest, including the chemical under evaluation and potential confounders, can be challenging and resource intensive in environmental epidemiological studies. As a result, information from questionnaires, interviews, or other proxies are frequently used to determine exposure in lieu of direct measurements. Limitations in exposure measurements often make it difficult to utilize the results of epidemiological studies to perform a robust evaluation of dose-response and preclude the use of these data for deriving PODs for risk assessment. As discussed earlier, the 2015/2016 review primarily utilized studies using biomarkers, known as DAPs, that were not specific to any particular OP, including acephate or methamidophos, and may not even reflect exposure to any OPs (e.g., direct consumption of DAPs on commodities). Along with the additional limitations associated with DAPs discussed earlier (e.g., temporal variability, sampling at a single time point), EPA has also noted that studies utilizing DAPs as biomarkers of OPs have not consistently observed associations with potential DNT outcomes. As a result, although the use of DAPs may provide qualitative evidence that exposure to one or more OPs occurred, the actual level of such exposure during critical window(s) of susceptibility is not known and the use of DAPs or other non-specific biomarkers as an exposure measure restricts the ability of the study to inform chemical-specific DNT potential. Further, DAPs are not relevant for acephate and methamidophos, given

[47] Limitations associated with the DNT guideline have been described in more detail in the 2020 Agency Issue Paper https://www regulations.gov/document/EPA-HQ-OPP-2020-0263-0006

[48] Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting held April 10-12, 2012 on "Chlorpyrifos Health Effects" - https://www.regulations.gov/document/EPA-HQ-OPP-2012-0040-0029
Transmittal of Meeting Minutes of the April 19-21, 2016 FIFRA SAP Meeting Held to Consider and Review Scientific Issues Associated with "Chlorpyrifos: Analysis of Biomonitoring Data" - https://www regulations.gov/document/EPA-HQ-OPP-2016-0062-0140

neither degrade into DAPs. Consequently, this WOE has focused on epidemiological studies specific to acephate and/or methamidophos.

For this WOE analysis, OPP considered nine epidemiologic publications that reported on the association between acephate/methamidophos exposure and potential DNT outcomes among infants and children. The nine publications reported mixed results across five cohort and case-control study populations and six different neurologic outcomes. Evidence of statistically significant associations were reported between acephate exposure and Full-scale IQ (Gunier et al., 2017) and hyperactivity (Hyland et al., 2021) in the CHAMACOS prospective cohort study population, and autism spectrum disorder (von Ehrenstein et al., 2019) in the Salinas Valley, CA case-control study population. No evidence of a significant association was reported for the other three outcomes: motor/sensory function and developmental delay; risk taking behavior; and neural tube defects (six of the nine identified publications).

Several challenges were identified that introduced uncertainty in the evaluation of the relationship between acephate/methamidophos exposure and DNT outcomes. Six of the nine studies reported positive associations that were not significant (e.g., odds ratio (OR) > 1.00, but not statistically significant) and in most cases were not observed consistently. Additionally, for many of the studies, including the three studies that reported a significant association, pesticide exposure was not directly measured, and exposure estimates instead relied on geographic proximity of residence to treated agricultural fields. The GIS-based assessments may have limited ability to investigate the relationship with acephate and methamidophos specifically if there is a strong correlation across different pesticides applied in that study area. As such, these investigations may be unable to distinguish between factors associated with geographic proximity to agricultural land and living areas, pesticide use in general, and exposure to specific pesticides. Further, the majority of these health outcomes were investigated in a single study population making it challenging to evaluate patterns or trends in the evidence. As a result, the overall epidemiological evidence is considered insufficient to conclude that there is a clear associative or causal relationship between exposure to acephate or methamidophos and subsequent DNT outcomes. Consequently, there was not compelling epidemiological evidence to link acephate/methamidophos exposure to DNT outcomes.

In contrast to the guideline and epidemiological studies, the DNT NAM battery is not performed using an intact organism and may lack potentially impactful aspects of a living organism exposure, such as blood-brain and blood-placental barriers, and may have limited metabolic capacity compared to *in vivo* models. The concept of evaluating key neurodevelopmental processes was designed to address the complexity of brain development, given processes must take place across all brain regions and neurotransmitter types for proper nervous system development, and the mechanisms underlying these processes are well conserved. Even though *in vitro* assays do not entirely recapitulate an intact organism, estimates of sensitivity (61-87%) and specificity (71-93%) for different groups of DNT assays indicate that they are capable of detecting effects and discerning between potential type I and type II errors (Harrill et al. 2018[49];

[49] Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR. 2018. Testing for developmental neurotoxicity using a battery of *in vitro* assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 354:24-39.

Shafer et al. 2019; Masjosthusmann et al. 2020[50]) and are expected to improve as more chemicals are tested and the battery continues to evolve. By focusing on these critical biological processes, the DNT NAM battery allows OPP to evaluate potential upstream disruptions that are associated with a spectrum of neurodevelopmental health outcomes. As a result, OPP can protect for a wide range of neurodevelopmental effects by using the DNT battery in a WOE evaluation.

In the 2020 SAP Report[51], the panel agreed that the DNT NAM battery reflects, if not directly models, critical processes of neurodevelopment, but also noted several processes and cell types that were perceived to be missing in the battery. As discussed in the Agency's response to the SAP[52], the current battery is not entirely lacking in these processes and cell types. For instance, although the panel encouraged development and inclusion of glial-based targeted NAMs in the battery, there are several assays in the battery that include glia and allow for potential detection of effects through a glial mediated mechanism despite not specifically measuring glial endpoints. The 2020 SAP also acknowledged that the battery will continue to evolve as the science advances, but this does not preclude the use of data from the battery in a WOE evaluation. Taken together with the recent adoption by the OECD WNT of a guidance document on the use of the battery as part of an IATA for DNT, there is consensus that data from the DNT NAM battery can and should be utilized as part of a WOE evaluation.

It is within the challenges associated with the *in vivo* DNT guideline and epidemiology studies that the value of NAMs become evident. Integrating *in vitro* and computational information with available *in vivo* and epidemiology data as part of an overall WOE evaluation can address some of the limitations encountered in the standard testing paradigm (*e.g.,* high variability, low throughput, high cost, or confounding factors). Moreover, this integrative approach also helps to address data interpretation challenges such as human relevance, biological processes leading to apical endpoints, and the role/contribution of confounding factors (e.g., maternal systemic toxicity) in eliciting effects. In the case of acephate/methamidophos, limited bioactivity was observed as previously described, and it was concluded that there were no true positive responses in the human or rat assays.

Results are consistent across multiple lines of evidence (*in vivo* and *in vitro*), demonstrating no evidence of DNT and supporting that AChE inhibition is protective of potential DNT effects. In fact, the DNT NAM battery which assesses hundreds of different endpoints showed no evidence of true DNT responses in either human or rat assays for both acephate and methamidophos. Additionally, the *in vivo* guideline DNT studies, as well as the available literature studies that evaluated neurobehavioral effects in laboratory animals clearly showed that AChE inhibition was the most sensitive (and only) adverse effect observed. For its part, the epidemiology evidence related to DNT outcomes across nine studies was insufficient to establish a clear associative or causal relationship between acephate/methamidophos exposure and DNT outcomes. Therefore, there was not compelling epidemiology evidence to link acephate/methamidophos exposure to DNT outcomes. Based on the WOE analysis presented, AChE inhibition continues to be

[50] Masjosthusmann S, Blum J, Bartmann K, Dolde X, Holzer AK, Stürzl LC, Keßel EH, Förster N, Dönmez A, Klose J. 2020. Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity. EFSA Supporting Publications. 17(10):1938E

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

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

considered the most sensitive endpoint and selecting a POD based on this effect would result in a health-protective risk assessment.

Conclusions

The DNT potential of acephate and its metabolite/degradate, methamidophos, was evaluated using chemical-specific data across multiple lines of evidence (toxicology studies, epidemiological studies, and *in vitro* DNT NAMs battery). Overall, the preponderance of the animal and *in vitro* evidence shows little support for adverse neurodevelopmental outcomes particularly at doses eliciting significant AChE inhibition. Some associations were observed in the small body of epidemiology evidence; however, confidence in the epidemiological database is undermined by several deficiencies identified in the available studies, including uncertainty due to inconsistency of findings within and across studies, limitations in their design, potential for bias, health outcomes being investigated in a single study population, and the exposure assessment approach (*e.g*., lack of direct measurements of acephate and/or methamidophos exposure). The totality of the data, therefore, indicates that there is little to no evidence of adverse neurodevelopmental outcomes due to acephate/methamidophos exposure, particularly at doses that elicit AChE inhibition and are the basis for the current PODs in the risk assessment. Therefore, AChE inhibition continues to be the most sensitive and health-protective endpoint for human health risk assessment, as supported by: (1) the lack of any true positive results from the DNT NAM battery using human and rat cells, (2) lack of DNT effects observed in the *in vivo* guideline DNT studies for acephate and methamidophos, and (3) lack of adverse DNT effects in the literature studies available for methamidophos (including a study with mice dosed via subcutaneous injection). Epidemiology evidence related to DNT outcomes demonstrated there was insufficient evidence of a clear associative or causal relationship between acephate/methamidophos exposure and DNT outcomes. Therefore, there was no compelling epidemiology evidence to link acephate/methamidophos exposure to DNT outcomes.

Appendix A

Table A.1. Efficacy cutoff methods for each DNT NAM endpoint in the ToxCast Data Analysis Pipeline (tcpl).

The tcpl level 5 methods ('mc5.mthds') for the efficacy cutoff are indicated for each multi-concentration (mc) screening assay endpoint. The efficacy cutoff methods include 3X the baseline median absolute deviation (bmad3), 20%, (pc20), bmad5, pc25, bmad2, and pc10. When multiple mc5 methods are indicated, the efficacy cutoff is defined as the maximum of all values given by the assigned level 5 methods.

Assay endpoint name	mc5 mthds
CCTE_Shafer_MEA_dev_firing_rate_mean_dn	bmad3
CCTE_Shafer_MEA_dev_burst_rate_dn	bmad3
CCTE_Shafer_MEA_dev_active_electrodes_number_dn	bmad3
CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn	bmad3
CCTE_Shafer_MEA_dev_per_burst_interspike_interval_dn	bmad3
CCTE_Shafer_MEA_dev_per_burst_spike_percent_dn	bmad3
CCTE_Shafer_MEA_dev_burst_duration_mean_dn	bmad3
CCTE_Shafer_MEA_dev_interburst_interval_mean_dn	bmad3
CCTE_Shafer_MEA_dev_network_spike_number_dn	bmad3
CCTE_Shafer_MEA_dev_network_spike_peak_dn	bmad3
CCTE_Shafer_MEA_dev_spike_duration_mean_dn	bmad3
CCTE_Shafer_MEA_dev_network_spike_duration_std_dn	bmad3
CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean_dn	bmad3
CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_dn	bmad3
CCTE_Shafer_MEA_dev_per_network_spike_spike_percent_dn	bmad3
CCTE_Shafer_MEA_dev_correlation_coefficient_mean_dn	bmad3
CCTE_Shafer_MEA_dev_mutual_information_norm_dn	bmad3
CCTE_Shafer_MEA_dev_LDH_dn	bmad3
CCTE_Shafer_MEA_dev_AB_dn	bmad3
UKN5_HCS_SBAD2_neurite_outgrowth_dn	bmad3, pc20, bmad5, pc25
UKN5_HCS_SBAD2_cell_viability_dn	bmad3, pc20, bmad5, pc25
UKN2_HCS_IMR90_neural_migration_dn	bmad3, pc20, bmad5, pc25
UKN2_HCS_IMR90_cell_viability_dn	bmad3, pc20, bmad5, pc25
UKN4_HCS_LUHMES_neurite_outgrowth_dn	pc25
UKN4_HCS_LUHMES_cell_viability_dn	pc25
IUF_NPC1a_proliferation_BrdU_72hr_dn	pc30, bmad2
IUF_NPC1a_proliferation_area_72hr_dn	pc30, bmad2
IUF_NPC1_viability_72hr_dn	pc30, bmad2
CCTE_Mundy_HCI_Cortical_NOG_BPCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_NOG_NeuriteCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_NOG_NeuriteLength_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_NOG_NeuronCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_BPCount_loss	bmad3, pc30

Assay endpoint name	mc5 mthds
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteLength_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuronCount_loss	bmad3, pc30
CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_SynapseCount_loss	bmad3, pc30
CCTE_Mundy_HCI_hN2_NOG_BPCount_loss	bmad3, pc30
CCTE_Mundy_HCI_hN2_NOG_NeuriteCount_loss	bmad3, pc30
CCTE_Mundy_HCI_hN2_NOG_NeuriteLength_loss	bmad3, pc30
CCTE_Mundy_HCI_hN2_NOG_NeuronCount_loss	bmad3, pc30
CCTE_Mundy_HCI_iCellGABA_NOG_NeuriteCount_loss	pc30
CCTE_Mundy_HCI_iCellGABA_NOG_NeuriteLength_loss	pc30
CCTE_Mundy_HCI_iCellGABA_NOG_NeuronCount_loss	pc30
CCTE_Mundy_HCI_iCellGABA_NOG_BPCount_loss	bmad3, pc30
CCTE_Mundy_HCI_hNP1_Casp3_7_gain	bmad3, pc30
CCTE_Mundy_HCI_hNP1_CellTiter_loss	bmad3, pc30
CCTE_Mundy_HCI_hNP1_Pro_MeanAvgInten_loss	bmad3, pc30
CCTE_Mundy_HCI_hNP1_Pro_ObjectCount_loss	bmad3, pc30
CCTE_Mundy_HCI_hNP1_Pro_ResponderAvgInten_loss	bmad3, pc30
IUF_NPC2a_radial_glia_migration_72hr_dn	bmad2, pc10
IUF_NPC2a_radial_glia_migration_120hr_dn	bmad2, pc10
IUF_NPC2b_neuronal_migration_120hr_dn	pc30, bmad2
IUF_NPC2c_oligodendrocyte_migration_120hr_dn	pc30, bmad2
IUF_NPC3_neuronal_differentiation_120hr_dn	pc30, bmad2
IUF_NPC4_neurite_length_120hr_dn	pc30, bmad2
IUF_NPC4_neurite_area_120hr_dn	pc30, bmad2
IUF_NPC5_oligodendrocyte_differentiation_120hr_dn	bmad2
IUF_NPC2-5_cytotoxicity_72hr	bmad2, pc10
IUF_NPC2-5_cytotoxicity_120hr	bmad2, pc10
IUF_NPC2-5_cell_number_120hr_dn	pc30, bmad2
IUF_NPC2-5_viability_120hr_dn	pc30, bmad2
IUF_NPC1_cytotoxicity_72hr	bmad2, pc10

Table A.2. ToxCast Data Pipeline for MEA-NFA and HCI assays.

ToxCast Data Pipeline Level	MEA-NFA: Methods Applied	HCI assays: Methods Applied
mc0: pre-processed data input	Data are pre-processed to obtain AUC values by assay component	Data are raw input
mc1: mapping to well and column indexes	Auto	
mc2: transformation	No transformation	
mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control. The response was multiplied by -1 for the "up" endpoints such that all endpoints are curve-fit in the positive direction.	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control.
Mc4: BMAD calculation type for curve-fitting	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells and the 2 lowest concentrations of the test wells on each plate.	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells and the 2 lowest concentrations of the test wells on each plate.
Mc5: Hitcall and potency determination	The cutoff for a positive response in each assay endpoint was set as 3*BMAD.	The cutoff for a positive response was the greater of 30% or 3*BMAD.
Mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hitcalls with less than 50% efficacy (17) were assigned to all; additionally cell viability assays	

Table A.3. Summary of assay endpoints measured for acephate and methamidophos in human and rat neuronal cell lines.

Chemical	Activity type	Assay	Species/cell line name	# True Positive/ # Total Endpoints Measured	AC50 (µM)[1]
Acephate	Proliferation	NPC1	Human (hNPC)	0/4	-
		HCI	Human (hNP1)	0/3	-
	Apoptosis	HCI	Human (hNP1)	0/2	-
	Cytotoxicity	NPC2	Human (hNPC)	0/4	-
	Migration	NPC2	Human (hNPC)	0/4	-
	Neuronal Differentiation	NPC3	Human (hNPC)	0/1	-
	Neurite outgrowth	NPC4	Human (differentiated from hNPC)	0/2	-
		HCI	Human (hN2)	0/4	-
		HCI	Human (CDI)*	0/4	-
		HCI	Human (CDI iCell GABA)**	0/4	
	Oligodendrocyte differentiation	NPC5	Human (oligodendrocyte progenitor)	0/1	-
	Network formation and function	MEA	Rat (primary rat cortical)	0/19	-
	Neurite outgrowth	HCI	Rat (primary rat cortical)	0/4	-
	Synaptogenesis	HCI	Rat (primary rat cortical)	0/8	-
Methamidophos	Proliferation	NPC1	Human (hNPC)	0/4	-
		HCI	Human (hNP1)	0/3	-
	Apoptosis	HCI	Human (hNP1)	0/2	-
	Cytotoxicity	NPC2	Human (hNPC)	0/4	-
	Migration	NPC2	Human (hNPC)	0/4	-
	Neuronal Differentiation	NPC3	Human (HCS)	0/1	-
	Neurite outgrowth	NPC4	Human	0/2	-
		HCI	Human (hN2)	0/4	-
		HCI	Human (CDI)*	0/4	-

		HCI	**Human (CDI iCell GABA)**	**0/4**	
	Oligodendrocyte differentiation	**NPC5**	**Human (oligodendrocyte progenitor)**	**0/1**	**-**
	Network formation and function	**MEA**	**Rat (primary rat cortical)**	**0/19 including one cytotoxicity measurement**	**-**
	Neurite outgrowth	**HCI**	**Rat (primary rat cortical)**	**0/4**	**-**
	Synaptogenesis	**HCI**	**Rat (primary rat cortical)**	**0/8**	**-**

MEA = microelectrode array network formation assay; HCI = high-content imaging; hNP1= human neural progenitor cell line; NPC1-4 = human primary neuroprogenitor cells (Lonza) in neurosphere cultures & NPC5 = human glial cells in neurosphere cultures developed at Leibniz Institute for Environmental Medicine (IUF). hN2 = human neuronal cell line; CDI = Cellular Dynamics Inc.; induced pluripotent stem cell derived neurons; (NOG (CDI) and NOG (iCell GABA)

[1] AC50s were not reported due to the lack of true positive results

***** At the time of this memo, the NOG (CDI) assay endpoints were labeled 'CCTE_Mundy_HCI_CDI_NOG' in the CompTox dashboard (invitrodb v3.5). In the next release of the dashboard (invitrodb v4.0+), these assay endpoints will have the label 'CCTE_Mundy_HCI_iCellGluta_NOG'.

******At the time of this memo, data from the NOG (iCell GABA) were not reported in the CompTox dashboard. The data are available at doi: 10.23645/epacomptox.22149200 and will be included in the next CompTox dashboard data update with the endpoint labels 'CCTE_Mundy_HCI_iCellGABA_NOG' (estimated release in summer 2023).

Table A.4. Assay activity and endpoints measured for methamidophos in human and rat neuronal cell lines.

Activity type	Assay short name	Species	Sample ID	AC_{50} (µM)	Cytotoxicity Measure (Yes/No)	Selective Activity (Yes/No)	Notes from Expert Review
Human Assays							
Apoptosis	HCI_hNP1_Casp3_7_gain	Human	Sample 1	-	No	No	
	HCI_hNP1_Casp3_7_gain	Human	Sample 2	-	No	No	
	HCI_hNP1_CellTiter_loss	Human	Sample 1	-	Yes	No	
	HCI_hNP1_CellTiter_loss	Human	Sample 2	-	Yes	No	
Proliferation	HCI_hNP1_Pro_MeanAvgInten_loss	Human	Sample 1	-	No	No	
	HCI_hNP1_Pro_MeanAvgInten_loss	Human	Sample 2	-	No	No	
	HCI_hNP1_Pro_ObjectCount_loss	Human	Sample 1	-	Yes	No	
	HCI_hNP1_Pro_ObjectCount_loss	Human	Sample 2	-	Yes	No	
	HCI_hNP1_Pro_ResponderAvgInten_loss	Human	Sample 1	-	No	No	
	HCI_hNP1_Pro_ResponderAvgInten_loss	Human	Sample 2	-	No	No	
	IUF_NPC1_Cytotoxicity_72hr	Human	Sample 1	-	Yes	No	
	IUF_NPC1_Viability_72hr_dn	Human	Sample 1	-	Yes	No	
	IUF_NPC1a_Proliferation_BrdU_72hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC1a_proliferation_area_72hr_dn	Human	Sample 1	-	No	No	
Cytotoxicity	IUF_NPC2-5_Cell_number_120hr_dn	Human	Sample 1	-	Yes	No	
	IUF_NPC2-5_Cytotoxicity_120hr	Human	Sample 1	-	Yes	No	
	IUF_NPC2-5_Cytotoxicity_72hr	Human	Sample 1	-	Yes	No	
	IUF_NPC2-5_Viability_120hr_dn	Human	Sample 1	-	Yes	No	
Migration	IUF_NPC2a_Radial_glia_migration_120hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC2a_Radial_glia_migration_72hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC2b_Neuronal_migration_120hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC2c_Oligodendrocyte_migration_120hr_dn	Human	Sample 1	-	No	No	
Neuronal differentiation	IUF_NPC3_Neuronal_differentiation_120hr_dn	Human	Sample 1	-	No	No	
Neurite outgrowth	IUF_NPC4_Neurite_area_120hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC4_Neurite_length_120hr_dn	Human	Sample 1	-	No	No	
	IUF_NPC4_Neurite_area_120hr_up	Human	Sample 1	-	No	No	
	IUF_NPC4_Neurite_length_120hr_up	Human	Sample 1	-	No	No	
	CCTE_Mundy_HCI_hN2_NOG_BPCount_loss	Human	Sample 2	-	No	No	
	CCTE_Mundy_HCI_hN2_NOG_NeuriteCount_loss	Human	Sample 2	-	No	No	

Activity type	Assay short name	Species	Sample ID	AC_{50} (µM)	Cytotoxicity Measure (Yes/No)	Selective Activity (Yes/No)	Notes from Expert Review
	CCTE_Mundy_HCI_hN2_NOG_NeuriteLength_loss	Human	Sample 2	44.4	No	Yes	Borderline activity, only at high concentration (100 µM)
	CCTE_Mundy_HCI_hN2_NOG_NeuronCount_loss	Human	Sample 2	-	Yes	No	
	CCTE_Mundy_HCI_CDI_NOG_BPCount_loss	Human	Sample 1	-	No	No	
	CCTE_Mundy_HCI_CDI_NOG_NeuriteCount_loss	Human	Sample 1	-	No	No	
	CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss	Human	Sample 1	-	No	No	
	CCTE_Mundy_HCI_CDI_NOG_NeuronCount_loss	Human	Sample 1	-	Yes	No	
	CCTE_Mundy_HCI_iCellGABA_NOG_BPCount_loss	Human	Sample 2	-	No	No	
	CCTE_Mundy_HCI_iCellGABA_NOG_NeuriteCount_loss	Human	Sample 2	-	No	No	
	CCTE_Mundy_HCI_iCellGABA_NOG_NeuriteLength_loss	Human	Sample 2	-	No	No	
	CCTE_Mundy_HCI_iCellGABA_NOG_NeuronCount_loss	Human	Sample 2	-	Yes	No	
Oligodendrocyte differentiation	IUF_NPC5_Oligodendrocyte differentiation_120hr_dn	Human	Sample 1	-	No	No	
Rat Assays							
Neurite outgrowth	HCI_Cortical_NOG_BPCount loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_NOG_BPCount loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_NOG_NeuriteCount loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_NOG_NeuriteCount loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_NOG_NeuriteLength loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_NOG_NeuriteLength loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_NOG_NeuronCount loss	Rat	Sample 1	-	Yes	No	
	HCI_Cortical_NOG_NeuronCount loss	Rat	Sample 2	-	Yes	No	
Synaptogenesis/ Maturation	HCI_Cortical_Synap&Neur_Matur_BPCount_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_BPCount_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteCount_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteCount_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteLength_loss	Rat	Sample 1	-	No	No	

Activity type	Assay short name	Species	Sample ID	AC_{50} (µM)	Cytotoxicity Measure (Yes/No)	Selective Activity (Yes/No)	Notes from Expert Review
	HCI_Cortical_Synap&Neur_Matur_NeuriteLength_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron_loss	Rat	Sample 2	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_NeuronCount_loss	Rat	Sample 1	-	Yes	No	
	HCI_Cortical_Synap&Neur_Matur_NeuronCount_loss	Rat	Sample 2	-	Yes	No	
	HCI_Cortical_Synap&Neur_Matur_SynapseCount_loss	Rat	Sample 1	-	No	No	
	HCI_Cortical_Synap&Neur_Matur_SynapseCount_loss	Rat	Sample 2	-	No	No	
Network formation and function	MEA_dev_Alamar blue_dn	Rat	Sample 1	-	Yes	No	
	MEA_dev_Alamar blue_dn	Rat	Sample 2	-	Yes	No	
	MEA_dev_LDH_dn	Rat	Sample 1	29.09	Yes	No	Noisy/borderline activity
	MEA_dev_LDH_dn	Rat	Sample 2	-	Yes	No	
	MEA_dev_active_electrodes_number_dn	Rat	Sample 1	0.44	No	Yes	Noisy/borderline activity
	MEA_dev_active_electrodes_number_dn	Rat	Sample 2	-	No	No	
	MEA_dev_burst_duration_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_burst_duration_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_burst_rate_dn	Rat	Sample 1	-	No	No	
	MEA_dev_burst_rate_dn	Rat	Sample 2	-	No	No	
	MEA_dev_bursting_electrodes_number_dn	Rat	Sample 1	0.10	No	Yes	Noisy/borderline activity
	MEA_dev_bursting_electrodes_number_dn	Rat	Sample 2	-	No	No	
	MEA_dev_correlation_coefficient_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_correlation_coefficient_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_firing_rate_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_firing_rate_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_inter_network_spike_interval_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_inter_network_spike_interval_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_interburst_interval_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_interburst_interval_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_mutual_information_norm_dn	Rat	Sample 1	-	No	No	

Activity type	Assay short name	Species	Sample ID	AC_{50} (µM)	Cytotoxicity Measure (Yes/No)	Selective Activity (Yes/No)	Notes from Expert Review
	MEA_dev_mutual_information_norm_dn	Rat	Sample 2	-	No	No	
	MEA_dev_network_spike_duration_std_dn	Rat	Sample 1	-	No	No	
	MEA_dev_network_spike_duration_std_dn	Rat	Sample 2	-	No	No	
	MEA_dev_network_spike_number_dn	Rat	Sample 1	-	No	No	
	MEA_dev_network_spike_number_dn	Rat	Sample 2	-	No	No	
	MEA_dev_network_spike_peak_dn	Rat	Sample 1	25.11	No	No	Noisy/borderline activity
	MEA_dev_network_spike_peak_dn	Rat	Sample 2	-	No	No	
	MEA_dev_per_burst_interspike_interval_dn	Rat	Sample 1	-	No	No	
	MEA_dev_per_burst_interspike_interval_dn	Rat	Sample 2	-	No	No	
	MEA_dev_per_burst_spike_percent_dn	Rat	Sample 1	-	No	No	
	MEA_dev_per_burst_spike_percent_dn	Rat	Sample 2	-	No	No	
	MEA_dev_per_network_spike_spike_number_mean_dn	Rat	Sample 1	65.90	No	No	Noisy/borderline activity
	MEA_dev_per_network_spike_spike_number_mean_dn	Rat	Sample 2	-	No	No	
	MEA_dev_per_network_spike_spike_percent_dn	Rat	Sample 1	-	No	No	
	MEA_dev_per_network_spike_spike_percent_dn	Rat	Sample 2	-	No	No	
	MEA_dev_spike_duration_mean_dn	Rat	Sample 1	-	No	No	
	MEA_dev_spike_duration_mean_dn	Rat	Sample 2	-	No	No	

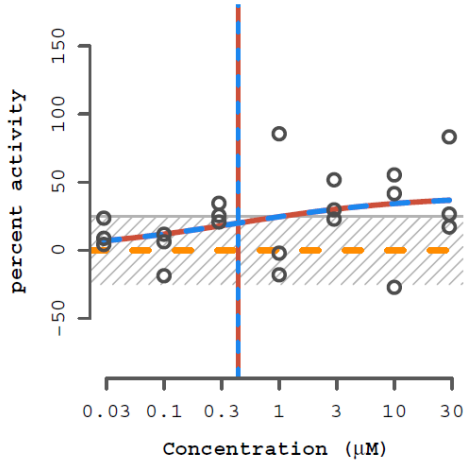
Key:

- The assay name is comprised of the name of the assay, type of cell line used and assay endpoint.
- MEA = microelectrode array network formation assay; HCI = high-content imaging; hNP1= human neural progenitor cell line; NPC1-4 = human primary neuroprogenitor cells (Lonza) in neurosphere cultures & NPC5 = human glial cells in neurosphere cultures developed at Leibniz Institute for Environmental Medicine (IUF).
- Assay with no activity is represented by "-" in the AC_{50} column.
- Average AC_{50} calculated when positive results were obtained from both samples for a single DNT endpoint.
- Assay selectivity: The assay is considered selective (i.e., the lowest concentration-related effects occurred at lower concentrations than cytotoxicity) if the selectivity scores are > 0.3. Assay selectivity is not determined if there is no activity, and it is represented by "-" in the Assay selectivity column.
- Sample 1 = sample identification (SPID) Number EPAPLT0167A08; Sample 2 = SPID Number TT0000177B02

Appendix B.

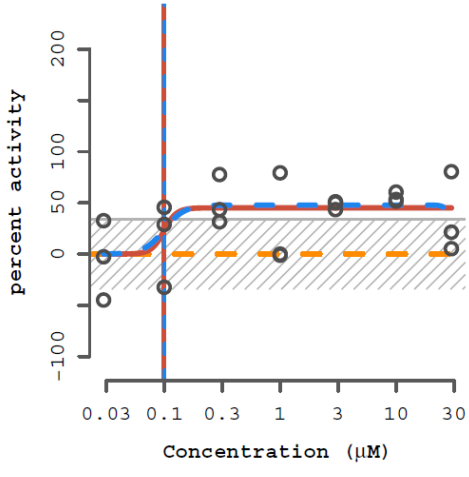
Concentration Response Curves for Methamidophos in Rat Cell Lines.

CCTE_Shafer_MEA_dev_active_electrodes_number_dn



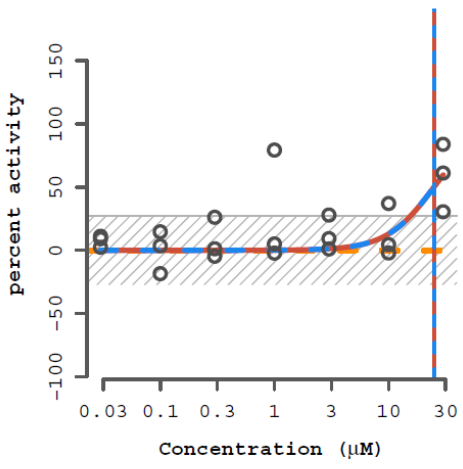

```
ASSAY:    AEID2498 (CCTE_Shafer_MEA_dev_active_electrode

NAME:     Methamidophos
CHID:     24177    CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID:     41354425

HILL MODEL (in red):
       tp        ga        gw
val:   40        -0.357    0.579
sd:    26        1.39      0.767

GAIN-LOSS MODEL (in blue):
       tp        ga        gw        la        lw
val:   40        -0.357    0.579     3.06      7.96
sd:    26        1.39      0.767     149000    960000

       CNST         HILL         GNLS
AIC:   213.64       205.48       209.48
PROB:  0.01         0.87         0.12
RMSE:  36.97        27.01        27.01

MAX_MEAN: 42.4       MAX_MED: 41.7       BMAD: 8.38

COFF: 25.2    HIT-CALL: 1     FITC: 42    ACTP: 0.99

FLAGS: 10; 17
```

CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn



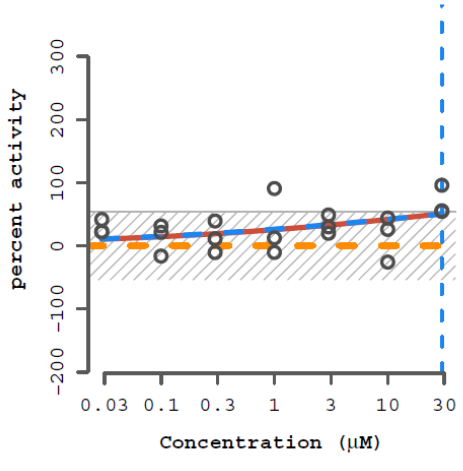

```
ASSAY:    AEID2500 (CCTE_Shafer_MEA_dev_bursting_electro

NAME:     Methamidophos
CHID:     24177    CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID:     41355245

HILL MODEL (in red):
       tp        ga        gw
val:   45.1      -1        8
sd:    7.48      0.094     74.7

GAIN-LOSS MODEL (in blue):
       tp        ga        gw        la        lw
val:   47.8      -0.999    4.99      1.48      17.7
sd:    7.6       0.133     17.7      0.0505    145

       CNST         HILL         GNLS
AIC:   226.05       210.53       213.58
PROB:  0            0.82         0.18
RMSE:  46.49        28.53        28.62

MAX_MEAN: 55.2       MAX_MED: 53.3       BMAD: 11.4

COFF: 34.2    HIT-CALL: 1     FITC: 41    ACTP: 1

FLAGS: 17
```

CCTE_Shafer_MEA_dev_network_spike_peak_dn



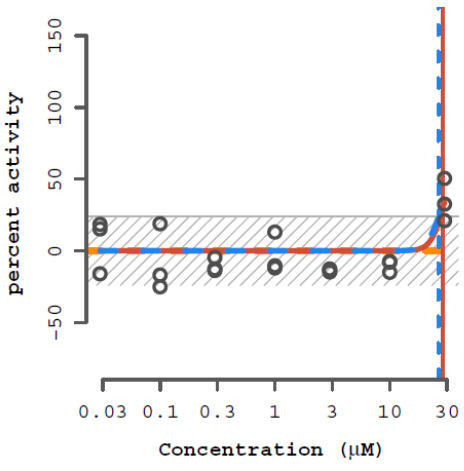

```
ASSAY:   AEID2512 (CCTE_Shafer_MEA_dev_network_spike_pe

NAME:    Methamidophos
CHID:    24177   CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID:    41360217

HILL MODEL (in red):
      tp       ga       gw
val:  101      1.4      2.08
sd:   199      0.906    4.03

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  101      1.4      2.08     1.99     14
sd:   201      0.913    4.05     499      13600

      CNST        HILL        GNLS
AIC:  203.35      193.37      197.37
PROB: 0.01        0.88        0.12
RMSE: 32.19       22.75       22.75

MAX_MEAN: 58.5      MAX_MED: 61.1      BMAD: 9.06

COFF: 27.2   HIT-CALL: 1    FITC: 42   ACTP: 0.99

FLAGS: 6
```

CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_dn




```
ASSAY:   AEID2520 (CCTE_Shafer_MEA_dev_per_network_spik

NAME:    Methamidophos
CHID:    24177   CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID:    41363937

HILL MODEL (in red):
      tp       ga       gw
val:  115      1.82     0.3
sd:   129      2.74     0.193

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  101      1.48     0.306    3.3      7.1
sd:   NaN      NaN      NaN      NaN      NaN

      CNST        HILL        GNLS
AIC:  219.65      207.78      211.9
PROB: 0           0.88        0.11
RMSE: 41.9        28.4        28.45

MAX_MEAN: 68.6      MAX_MED: 55.4      BMAD: 18

COFF: 54.1   HIT-CALL: 1    FITC: 42   ACTP: 1

FLAGS: 6; 11
```

CCTE_Shafer_MEA_dev_LDH_dn



ASSAY: AEID2529 (CCTE_Shafer_MEA_dev_LDH_dn)

NAME: Methamidophos
CHID: 24177 CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID: 41366832

HILL MODEL (in red):

	tp	ga	gw
val:	60.5	1.46	8
sd:	451	0.896	25.6

GAIN-LOSS MODEL (in blue):

	tp	ga	gw	la	lw
val:	46.9	1.42	8	3.29	1.12
sd:	764	2.15	28.2	322	400

	CNST	HILL	GNLS
AIC:	187.49	183.12	187.12
PROB:	0.09	0.8	0.11
RMSE:	19.31	14.19	14.19

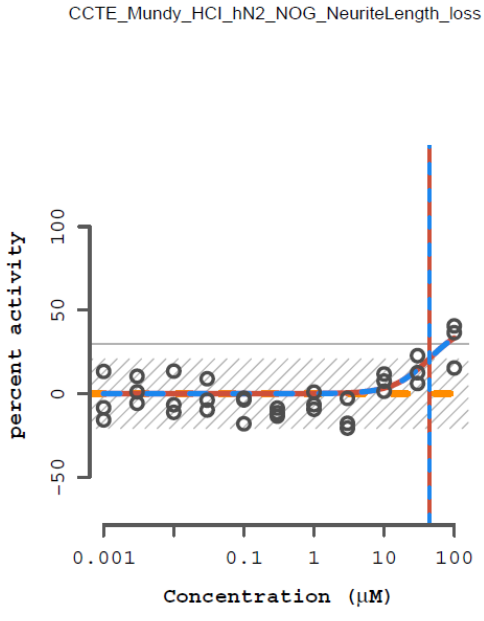
MAX_MEAN: 34.6 MAX_MED: 32.5 BMAD: 8.03

COFF: 24.1 HIT-CALL: 1 FITC: 42 ACTP: 0.91

FLAGS: 6; 17

Appendix C.

Concentration Response Curves for Methamidophos in Human Cell Lines




```
ASSAY:   AEID2791 (CCTE_Mundy_HCI_hN2_NOG_NeuriteLength_

NAME:    Methamidophos
CHID:    24177   CASRN: 10265-92-6
SPID(S): EPAPLT0167A08
M4ID:    36488299

HILL MODEL (in red):
      tp       ga       gw
val:  42       1.65     1.65
sd:   24       0.418    1.07

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  42       1.65     1.65     2.84     7.87
sd:   24       0.418    1.07     824      7740

      CNST       HILL       GNLS
AIC:  272        259.06     263.06
PROB: 0          0.88       0.12
RMSE: 14.46      10.22      10.22

MAX_MEAN: 30.8      MAX_MED: 36.5      BMAD: 6.96

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 1

FLAGS: 6; 17
```

Appendix D. Summary of Available Studies Investigating Association Between Acephate/Methamidophos and Potential Neurodevelopmental/ Neurobehavioral Outcomes in Children

Outcome	Outcome Measurement	Exposure Measurement	Study Design	Result	Study Population	Study Quality	Author
Motor/ Sensory Function or Developmental Delay	Motor function: Peabody Developmental Motor Scales at 6 weeks and 9 months	Umbilical cord blood	Cohort	No evidence of a significant association between methamidophos and motor function outcomes. Acephate was detected in less than 10% of samples and authors did not report results for acephate.	Fuyang Zhejiang, China Mother-child pairs	Low	Silver et al., 2017
	Sensory function: Grating Visual Acuity, Auditory Brainstem Response at 6 weeks and 9 months	Umbilical cord blood	Cohort	No evidence of a significant association between methamidophos and sensory function outcomes. Acephate was detected in less than 10% of samples and authors did not report results for acephate.			Silver et al., 2018
	Modified Denver Development Screening Test between 0-5 years	Self-reported maternal prenatal and postnatal pesticide use.	Case-control	No evidence of a significant association between methamidophos and neurodevelopmental delay.	Thailand Mother-child pairs	Moderate	Juntarawijit et al., 2020
Learning, Memory, Intelligence, Cognitive Development	Wechsler Intelligence Scale for Children at 7 years	GIS-based Assessment toxicity weighted- agricultural pesticide application within 1 km of maternal residence at birth. Spot urine measurement of six DAPs	Cohort	Without adjustment for exposure to other pesticides, evidence of a statistically significant association between a standard deviation (SD) increase in acephate use within 1 km of maternal residence during pregnancy and a 2.3 point decrease in Full-Scale IQ (β = -2.3 points; 95% CI: -3.9, -0.6) and a 2.7 point decrease in Verbal Comprehension (β = -2.7 points; 95% CI: -4 3, -1.2) in children at 7-years old. With adjustment for exposure to other pesticides, no evidence of a statistically significant association between a SD increase in acephate use within 1 km of maternal residence during pregnancy and a 0.6 point decrease in Full-Scale IQ at 7-years of age (n=255) (β = -0.6; 95% CI: -4.8, 3.6). Authors did not report results for any of the other outcomes (Working Memory, Processing Speed, Verbal Comprehension, and Perceptual Reasoning).	CHAMACOS Salinas Valley, CA, US Mother-child pairs	Moderate	Gunier et al., 2017
Attention Hyperactivity Internalizing Behavior	Neurobehavioral development and emotional problems Behavior Assessment System for Children, 2nd edition at 16 and 18 years (n=593)	GIS-based – Assessment agricultural pesticide application within 1-km of maternal residence during and after pregnancy.	Cohort	Evidence of a borderline statistically significant association between a 2-fold increase in acephate applications within 1 km of the residence *during childhood (0-5 yrs)* and increased *maternal-reported* hyperactivity among all children (β = 1.6; 95% CrI: 0.1, 3.0) and among girls only (β = 1.9; 95% CrI: 0.1, 3.8). No evidence of a significant association observed when evaluated using *youth-reported* hyperactivity (β = 0.8; 95% CI: -0.9, 2.4). Evidence of a borderline statistically significant association between a 2-fold increase in acephate applications *during childhood (0-5 yrs)* and *maternal-reported* externalizing problems among girls only (β = 1.6; 95% CrI: 0.0, 3.3). Youth-reported externalizing problems were not tested. No evidence of significant associations observed between two-fold increase in acephate applications *during pregnancy* and any investigated outcome (either maternal- or youth-reported).			Hyland et al., 2021
	Neurobehavioral development and emotional problems with Adverse Childhood Experiences at 16 and 18 years	GIS-based Assessment agricultural pesticide application within 1-km of maternal residence after pregnancy.	Cohort	No evidence of a significant association of interaction between a two-fold increase in acephate exposure and adverse childhood experiences on any maternal- or youth-reported neurobehavioral development and emotional problem outcomes (internalizing problems, hyperactivity, attention problems) (-2.5 < all βs < 1.5; all 95% CIs encompassed the null value 0.0)			Hyland et al., 2022

Outcome	Outcome Measurement	Exposure Measurement	Study Design	Result	Study Population	Study Quality	Author
Risk Taking Behavior	Risk-Taking Behavior and Adverse Childhood Experiences from childhood at 18 years	GIS-based Assessment agricultural pesticide application within 1-km of maternal residence after pregnancy.	Cohort	No evidence of a significant association between acephate exposure and any risk-taking behavior outcomes.			Gunier et al., 2022
Autism Spectrum Disorder	Autism Spectrum Disorder with intellectual disability (Diagnostic and Statistical Manual of Mental Disorders, fourth edition, revised)	GIS-based Assessment agricultural pesticide application within 2- km of maternal residence before, during and after pregnancy	Case-control	Evidence of a statistically significant association between *3-months pre-pregnancy* acephate exposure and autism spectrum disorder with intellectual disability comorbidity without adjustment for other pesticides (OR = 1.54; 95% CI: 1.11, 2.13) and when adjusted for other pesticides (OR = 1.47; 95% CI: 1.04, 2.09). No evidence of a significant positive association between acephate exposure during any other time period and autism spectrum disorder with or without intellectual disability comorbidity (0.93 < all other ORs < 1.26; all 95% CIs encompassed the null value of 1.0).	San Joaquin Valley, CA, US Mother-child pairs	Moderate	von Ehrenstein et al., 2019
Neural Tube Defects	California Birth Defects Monitoring Program Case Definition for Neural Tube Defects	GIS-based Assessment agricultural pesticide application within 1,000-m of maternal residence during pregnancy	Case-control	No evidence of a significant association between acephate or methamidophos exposure and neural tube defects (1.2 < all ORs < 2.0; all 95% CIs encompassed the null value of 1.0; with n = 13-33 exposed cases).	CA, US Birth Defects Registry	Moderate	Rull et al., 2006