Comments from the
California Office of Environmental Health Hazard Assessment and the
California Department of Pesticide Regulation on the
US EPA's Proposed Use of New Approach Methodologies
To Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity
for Human Health Risk Assessment

Docket HQ-OPP-2020-0263

We thank the US EPA for the opportunity to comment on the issue paper "Use of New Approach Methodologies to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment." Overall, we support the development of new approach methodologies (NAMs) and are actively working towards their use in our prioritization and risk assessments. The *in-vitro* test methodologies that have been proposed for skin irritation, eye irritation, and skin sensitivity that were originally proposed in the US EPA Interim Science Policy: Use of Alternative Approaches for Skin Sensitization as a Replacement for Laboratory Animal Testing (April 2018), along with the ensuing meetings and publications (Hoffmann et al 2018; Kleinstreuer et al 2018, etc.) detail the difficulty of implementing *in-vitro* replacements for relatively simply toxicological endpoints. Developmental neurotoxicity (DNT) is noteworthy in its multifactorial nature, and is admittedly much more complex that an irritative reaction. Much thought needs to be given to replacement of whole animal studies for a complex and sometimes multi-generational endpoint such as DNT.

Our review finds that the proposal to replace DNT testing and current risk assessment practices with NAMs (1) is premature, (2) does not demonstrate that the NAMs cover the important aspects of DNT necessary to support appropriately health-protective decisions, (3) is based on an analysis that is too limited in scope due to its narrow focus on a single class of pesticides, and (4) is not adequate to characterize DNT risks in diverse human populations. Accordingly, we respectfully offer the following comments on the proposal.

1. **The proposal is premature in that it would abandon the DNT test without establishing the replacement battery of NAMs as an alternative that would allow agencies to make decisions that are appropriately health-protective (section 2.1, page 7).**

– The National Toxicology Program recently convened a collaborative workshop on screening for DNT [Behl et al (2019) Toxicol Sci 167: 6-14]. Workshop participants discussed key issues and knowledge gaps that "need to be addressed for its use in regulatory decision making." They recommended that more DNT compounds be included to better characterize the sensitivity and specificity of these assays. The current aim is to develop approaches to screen and prioritize chemicals for further *in-vivo* testing, not as a complete replacement for *in-vivo* DNT testing at this time. The US EPA proposal neither acknowledges nor cites this pertinent paper from the NTP.

– The US EPA's Office of Pesticide Programs acknowledges that since 1998 they received approximately 100 DNT tests, comprising 32 on organophosphates, *N*-methyl carbamates, and pyrethroids, and 68 on other pesticides, of which 24 (35%) were used to derive points of departure (page 8). Thus, the DNT test often identifies the most sensitive health outcome, and to abandon it at this time would be to abandon a critical support for health-protective decisions.

– The proposal is to shift to targeted testing based on commonly accepted modes of action (page 8). But this approach will work only for chemicals where there is a single mode of action that is known. It will not work for the majority of chemicals where the mode of action is *not* known or where there may be multiple modes of action resulting in one or more manifestations of DNT. The DNT test accounts for such chemicals by evaluating the potential for multiple adverse outcomes without the requirement that there be a single mode of action that is known in advance.

– The US EPA bases this shift on only three classes of pesticides, and these classes are not representative of the majority of other pesticides. As recounted above, the US EPA did not find the DNT test to be critical for the three classes of pesticides that form the basis of its proposal, while for other pesticides, 35% of DNT tests identified sensitive health outcomes. Therefore, it is imprudent and premature to base a general shift in testing strategy on only the three classes of pesticides.

– If the US EPA were to shift away from the *in-vivo* DNT test, a likely consequence is that fewer of these tests would be conducted. State and other environmental health agencies, which now rely on these studies to evaluate the potential for DNT, would lose a critical tool for making decisions that are appropriately health-protective. For example, DPR is required to evaluate reproductive and developmental toxicity data for the registration of new pesticide active ingredients (Title 3 California Code of Regulations sections 6158-6159).

– Many of these test methods have not been validated and to use NAMs in a regulatory context, a framework is needed. Selecting one or two approaches as proposed defeats the purpose of determining criteria for endpoints as complex

as neurodevelopment. It is concerning that the proposal considers only two assays for DNT, as many complex pathways are involved in neurodevelopment. It is important to integrate various NAMs, as each assay can fill data gaps and make up for what the others lack.[1] Only then will this approach be ready for risk assessment. Focusing on just two methods is neither adequate nor appropriate.

– Neurodevelopment includes key events such as neural induction, precursor cell proliferation, pattern formation, cell migration, neuronal and glial differentiation, formation of axons and dendrites, axonal guidance and target recognition, cell survival and apoptosis, synapse formation and pruning, and neurotransmitter specification. Furthermore, these cellular events are fundamental principles of neurodevelopment that are conserved across species ranging from the nematode (*Caenorhabditis elegans*) to humans and these can be used to fill data gaps.

Minor error:

– The proposal asserts that a limitation of the DNT test is that coefficients of variation are commonly higher than the mean (page 7). The coefficient of variation is the standard deviation divided by the mean, so the assertion should have been written either that the coefficient of variation is commonly greater than 1 or that the standard deviation is commonly greater than the mean.

– On this point, coefficients of variation can be large for motor activity but very small for brain morphometry. The motor activity variation is well recognized, and guidance for analysis and interpretation was developed in a joint effort by the US EPA and Health Canada under the auspices of NAFTA (https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/developmental-neurotoxicity-study-guidance).

[1] For example, MEAs measure electrical activity. It is possible that a compound from the same group of chemicals is negative for a DNT endpoint in the MEA assay; however, a historical or traditional *in-vivo* studies have other findings. HCI or any imaging assay may not be enough to provide information on a DNT endpoint if there is a lack of information from the MEA. There needs to be a screening process (molecular target approach, perhaps concentration response), followed by QSAR analysis (this may be medium through put), followed by other assays such as MEAs (measurement of electrical activity) and FLIPR (measurement of Ca2+ flux which is crucial to neurodevelopment), followed by imaging, that can be run in parallel to MEAs (sholl analysis can quantify what is seen qualitatively in imaging and morphology), and then whole tissue analysis where neural connections are intact and not dissociated can be integrated as part of the evidence. Furthermore, 3D cultures, or organoids (stem cells) are also useful assays for evaluating neurodevelopment. All of these methods provide advantages and disadvantages.

2. The proposal asks the wrong question. It is not sufficient that the replacement battery of NAMs be reviewed for "relevance to DNT screening and evaluation" (section 2.3, page 10). Rather, the question is whether the replacement battery might miss important aspects of DNT, thus being inadequate to guide and support health-protective decisions.

– Unlike other biological endpoints where NAMs have been adopted (such as acute irritation or acute toxicity), DNT is a complex response mediated by multiple different pathways. Single chemical triggers may have disparate results, depending on slight differences in treatment dose or timing. The proposal does not demonstrate that the NAMs battery can account for this complexity in DNT effects.

– The proposal relies on a single case study as if this would provide sufficient evidence for a shift in testing strategy. This is problematic. The case study of organophosphate pesticides, which inhibit acetylcholinesterase, cannot provide this evidence, as organophosphates are not representative of other pesticides, especially those that operate through one or more different modes of action. Moreover, DNT is a complex phenomenon and protection against DNT cannot be achieved by focusing on acetylcholinesterase inhibition.

– Even for chemicals that inhibit acetylcholinesterase, that mechanism might not fully capture other aspects of DNT. For example, the California Department of Pesticide Regulation's 2019 Risk Characterization Document for chlorpyrifos used a published DNT test to establish a lower endpoint than what had been identified by the acetylcholinesterase inhibition studies [https://www.cdpr.ca.gov/docs/whs/pdf/chlorpyrifos_final_tac.pdf]. In other words, the acetylcholinesterase inhibition studies failed to capture the adverse health impacts that were identified by DNT tests, and this would have led to inadequately health-protective decisions.

– Similarly, under Proposition 65, the maximum allowable dose levels for chlorpyrifos were based on the same neurodevelopmental effect [https://oehha.ca.gov/media/downloads/crnr/chlorpyrifosfsor07082020.pdf].

– A long-standing issue in the application of *in-vitro* tests has been that they do not reflect the metabolism of animals or humans *in vivo*. The US EPA acknowledges this limitation, stating, in particular, "The metabolic capacity of these DNT-NAMs is not well understood (section 2.3.7, page 65)." The proposal glosses over this point, rationalizing that important metabolites for several organophosphates were known and tested separately. For many other chemicals, it is not likely that metabolites will be known and tested, thus this is a substantial limitation that argues against a major shift in testing strategy for DNT at this time.

- Building on this point, the proposal does not account for the toxicokinetics of placental transfer to the developing fetus. This is another substantial limitation that should be addressed before considering a major shift in testing strategy.

- A relevant question is to identify the characteristics of chemicals that cause DNT. As in the chlorpyrifos example above, the acetylcholinesterase studies failed to capture adverse health outcomes identified by the DNT studies. The proposal's basis on a single mode of action is inconsistent with a recommendation of the National Academy of Sciences that "encourages the cataloging of pathways, components, and mechanisms that can be linked to particular hazard traits" [NASEM (2017) *Using 21st Century Science to Improve Risk-Related Evaluations*, chapter 7, page 133].

- To support the proposed shift in testing strategy, the proposal should include a demonstration that the battery of NAMs identifies a DNT hazard for those chemicals where the DNT test identified a hazard. As mentioned above, the NAMs are not specific for DNT, and they may not be as sensitive as the DNT test. Therefore, the NAMs would not certain identify neurodevelopmental hazards that, as a practical matter, we already know exist because the DNT test has identified them. This failure to identify currently known neurotoxicity hazards represents a concerning reduction in health protectiveness. Moreover, the battery of NAMs was not tested on non-organophosphate chemicals to show cross-class applicability for pesticides that operate through mechanisms other than acetylcholinesterase inhibition.

- In addition, any NAM test battery would need to account for the full range of durations and timing of exposures, including pre-fertilization, pre-implantation, all *in-utero* developmental benchmarks, parturition, and *ex-utero* exposures. Well known developmental toxicants vary in their effect depending on when the mother or developing organism was exposed.

3. A single case study on organophosphates is too limited in scope to serve as the basis for a general proposal to replace standard uncertainty factors with *in-vitro*-derived extrapolation factors (section 3.1, page 68).

- For seven of the 16 organophosphate pesticides considered in section 3 of the proposal, there were model-fit concerns or issues. Eleven more organophosphate pesticides with test results reported in section 2 were not even considered in section 3. This is indicative of an approach that is neither well supported nor generally applicable.

- The case study is specific for the derivation of interspecies and intraspecies toxicodynamic extrapolation factors for acetylcholinesterase inhibition of red blood cell membranes. This may be a possible approach for organophosphate

pesticides, but there is no evidence for its applicability to other chemicals, as discussed in point #2 above.

– The US EPA states that they cannot predict the impact of the transition to these studies, and that they cannot be quantitatively correlated to whole-animal doses. This will add additional uncertainty in determining regulatory targets.

4. The analysis is not adequate to demonstrate health protection for diverse human populations, susceptible populations and life-stages, pre-existing health conditions, and the cumulative effects of exposure to multiple chemical and nonchemical stressors.

– It is important to consider the full range of human variability (including biologically susceptible and socially vulnerable individuals) in chemical risk assessment in order to protect sensitive populations [Koman et al (2019) PLoS Biol 17]. However, intra-species variability has not been adequately addressed in the current proposal, and evidence suggests the default intra-species uncertainty factor of 10X is under-protective with respect to uniquely susceptible or vulnerable populations [National Research Council (2009) *Science and Decisions: Advancing Risk Assessment*; OEHHA (2008) Air Toxics Hot Spots Program Technical Support Document for the Derivation of Noncancer Reference Exposure Levels, https://oehha.ca.gov/air/air-toxics-hot-spots; Janssen et al (2012), https://www.nrdc.org/sites/default/files/strengthening-toxic-chemical-risk-assessments-report.pdf].

– The sample sizes are too small for confident conclusions. For example, there were only four infant samples and five non-Caucasian samples (section 3.3.2, page 73). The US EPA acknowledged that "there is low confidence in the results of these analyses . . . [which] would not be representative of the subpopulations being evaluated." This severely limits the generalizability of conclusions to diverse population groups and thus would not support appropriately health-protective decisions.

Conclusions

At this time, it is imprudent and premature to abandon the DNT guideline test in favor of a replacement battery of NAM tests. The analysis is not adequate to demonstrate that the proposed approach would support appropriately health-protective decisions on DNT for a wide variety of chemicals.

The analysis to support the derivation of extrapolation factors is inadequate to ensure that toxicity values would be health-protective for variability in response to chemical exposure in different segments of a diverse human population.

We respectfully urge the US EPA not to implement this proposal, to continue to develop NAM tests, to conduct a thorough evaluation of NAM tests for a wide variety of chemicals, and to invite the broader participation of state environmental health agencies and other stakeholders who might have different views in these activities.

Sincerely,

Vincent Cogliano, PhD
Deputy Director for Scientific Programs
California Office of Environmental Health Hazard Assessment

Karen Morrison, PhD
Assistant Director and Chief Science Advisor
California Department of Pesticide Regulation