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

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

APR 04 2019

Memorandum

SUBJECT: Transmittal of Meeting Minutes and Final Report for the Federal Insecticide Fungicide and Rodenticide Act, Scientific Advisory Panel (FIFRA SAP) Meeting held on December 4 and 6, 2018

TO: Richard Keigwin
Director
Office of Pesticide Programs

FROM: Shaunta Hill-Hammond, PhD
Designated Federal Official
Science Advisory Board Staff Office

Steven M. Knott for

THRU: Steven Knott, MS
Executive Secretary, FIFRA SAP
Office of Science Coordination and Policy

Hayley Hughes, DrPH, MPH, CSP
Director
Office of Science Coordination and Policy

Hayley Hughes

Please find attached the meeting minutes and final report for the FIFRA Scientific Advisory Panel open public meeting held in Arlington, Virginia, on December 4 and 6, 2018. This report addresses a set of scientific issues being considered by the U. S. Environmental Protection Agency regarding a new approach methodology for inhalation risk assessments.

Attachment:

cc:
Erik Baptist
Nancy Beck
Charlotte Bertrand
Alexandra Dunn
Cheryl Dunton
Arnold Layne
Anna Lowit
Monique Perron
Linda Strauss
Dana Vogel
OPP Docket

FIFRA Scientific Advisory Panel:
Robert E. Chapin, Ph.D.
George Corcoran, Ph.D.
Sonya K. Sobrian, Ph.D.
Clifford P. Weisel, Ph.D.
Raymond S.H. Yang, Ph.D.

FQPA Science Review Board Members:
Holger P. Behrsing, Ph.D.
James Blando, Ph.D.
Jennifer M. Cavallari, Sc.D.
Marie C. Fortin, Ph.D.
Stephen G. Grant, Ph.D.
Jon A. Hotchkiss, Ph.D.
Allison Jenkins, M.P.H
Kathryn Page, Ph.D.
Robert J. Mitkus, PhD.
Emily N. Reinke, Ph.D.
Nikaeta Sadekar, Ph.D.
Kristie Sullivan, M.P.H.
Lisa M. Sweeney, Ph.D.

FIFRA Scientific Advisory Panel
Meeting Minutes and Final Report
No. 2019-01

Peer Review on Evaluation of a Proposed Approach to Refine the Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM)

December 4 and 6, 2018
FIFRA Scientific Advisory Panel Meeting

Held at

U.S. Environmental Protection Agency
Conference Center
Lobby Level One Potomac Yard (South Bldg.)
2777 S. Crystal Drive, Arlington, VA 22202

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NOTICE

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) is a federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides advice, information, and recommendations to the U.S. Environmental Protection Agency (EPA or Agency) Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The SAP serves as a primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. The FQPA Science Review Board members serve the FIFRA SAP on an *ad hoc* basis to assist in reviews conducted by the FIFRA SAP. The meeting minutes and final report are provided as part of the activities of the FIFRA SAP.

The FIFRA SAP carefully considered all information provided and presented by the Agency, as well as information presented by the public. The minutes represent the views and recommendations of the FIFRA SAP and do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the federal government. The mention of trade names or commercial products does not constitute an endorsement or recommendation for use.

The meeting minutes and final report do not create nor confer legal rights nor impose legally binding requirements on the EPA or any other party. The meeting minutes and final report of the December 4 and 6, 2018, FIFRA SAP meeting represent the SAP's consideration and review of scientific issues associated with the "Peer Review on Evaluation of a Proposed Approach to Refine the Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM)." Steven Knott, M.S., FIFRA SAP Executive Secretary, reviewed the minutes and final report. Robert E. Chapin, Ph.D., FIFRA SAP Chair, and Shaunta Hill-Hammond, Ph.D. Designated Federal Official, certified the minutes and final report that is publicly available on the SAP website at http://www.epa.gov/sap under the heading of "Meetings" and in the public e-docket, Docket No. EPA-HQ-OPP-2018- 0517, accessible through the docket portal: http://www.regulations.gov. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/sap. Interested persons are invited to contact Steven Knott, M.S., FIFRA SAP Executive Secretary, via e-mail at knott.steven@epa.gov.

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December 4 and 6, 2018
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Held at

U.S. Environmental Protection Agency
Conference Center
Lobby Level One Potomac Yard (South Bldg.)
2777 S. Crystal Drive, Arlington, VA 22202

Robert E. Chapin, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel

RE Chapin
Signature

APR 04 2019
Date

Shaunta Hill-Hammond, Ph.D.
Designated Federal Official
Environmental Protection Agency

Steven M. Knott for
Signature

APR 04 2019
Date

Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel Meeting
December 4 and 6, 2018

Peer Review on Evaluation of a Proposed Approach to Refine the Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM)

PARTICIPANTS

FIFRA SAP Chair

Robert E. Chapin, Ph.D., Independent Consultant, Preston, Connecticut

Designated Federal Official

Shaunta Hill-Hammond, Ph.D., Biologist/Designated Federal Official
US. Environmental Protection Agency, Washington, District of Columbia
202-564-3343, hill-hammond.shaunta@epa.gov

FIFRA Scientific Advisory Panel Members

George Corcoran, Ph.D., Chairman and Professor, Department of Pharmaceutical Sciences Wayne State University, Detroit, Michigan

Sonya K. Sobrian, Ph.D., Associate Professor, Howard University College of Medicine, Howard University, Washington, District of Columbia

Clifford P. Weisel, Ph.D., Professor, Rutgers University, Piscataway, New Jersey

Raymond S.H. Yang, Ph.D., Professor (Emeritus), College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado

FQPA Science Review Board Members

Holger P. Behrsing, Ph.D., Principal Scientist and Head, Respiratory Toxicology Program, Institute for *In Vitro* Sciences, Inc., Gaithersburg, Maryland

James Blando, Ph.D., Associate Professor, Old Dominion University, School of Community and Environmental Health, Norfolk, Virginia

Jennifer M. Cavallari, ScD., Associate Professor, Department of Community Medicine and Health Care UConn School of Medicine, Farmington, Connecticut

Marie C. Fortin, Ph.D., Jazz Pharmaceuticals, Ewing, New Jersey

Stephen G. Grant, Ph.D., Dr. Kiran C. Patel College of Osteopathic Medicine, Nova Southeastern University, Fort Lauderdale, Florida

Jon A. Hotchkiss, Ph.D., Senior Inhalation Toxicologist, Toxicology, Environmental Research and Consulting Laboratory, The Dow Chemical Company, Midland, Michigan

Allison Jenkins, M.P.H, Texas Commission on Environmental Quality, Austin, Texas

Kathryn Page, Ph.D., Senior Scientist, Toxicologist, Product Safety, The Clorox Company Pleasanton, California

Robert J. Mitkus, Ph.D., Regulatory Toxicologist, BASF Corporation, Durham, North Carolina

Emily N. Reinke, Ph.D., Biologist, Health Effects Division, Toxicology Directorate, U.S. Army Public Health Center, Gunpowder, Maryland

Nikaeta Sadekar, Ph.D., Human Health Scientist, Inhalation Toxicology, Research Institute for Fragrance Materials, Woodcliff Lake, New Jersey

Kristie Sullivan, M.P.H., Vice President for Research Policy, Physicians Committee for Responsible Medicine, Washington, District of Columbia

Lisa M. Sweeney, Ph.D., CHMM, UES, Inc. Beavercreek, Ohio

LIST OF ACRONYMS AND ABBREVIATIONS

ACRONYMS	DESCRIPTION
AIC	Akaike Information Criterion
AOP	Adverse Outcome Pathway
BMD	Benchmark Dose
BMDL	Benchmark Dose Lower Confidence Limit
BMR	Benchmark Response
CFD	Computational Fluid Dynamic
D50	Particles with diameters corresponding to 50% sampling efficiency
EPA or Agency	U.S. Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GSD	Geometric Standard Deviation
HEC	Human Equivalent Concentrations
IRIS	Integrated Risk Information System
LDH	Lactate Dehydrogenase
LOAEL	Lowest Observed Adverse Effect Level
MOA	Mode-of-Action
MDL	Method Detection Limit
MPPD	Multiple-Path Particle Dosimetry
MMAD	Mass Median Aerodynamic Diameter
NAM	New Approach Methodology
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs
PBPK	Physiologically Based Pharmacokinetic
PNNL	Pacific Northwest National Laboratory
POD	Point of Departure
PSD	Particle Size Distribution
RfD	Reference Dose
RH	Relative Humidity
SAP	Scientific Advisory Panel
TEER	Transepithelial Electrical Resistance
UF	Uncertainty Factors
URT	Upper Respiratory Tract

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) completed its review of the set of scientific issues being considered by the U.S. Environmental Protection Agency (EPA or Agency) regarding a new approach for the assessment of inhalation toxicity. The meeting minutes and final report of the December 4 and 6, 2018, FIFRA SAP meeting represent the SAP's consideration and review of scientific issues associated with the "Peer Review on Evaluation of a Proposed Approach to Refine the Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM)." Advanced notice of the meeting was published in the Federal Register on August 8, 2018.

The review was conducted in an open public meeting held in Arlington, Virginia. The Agency position paper, charge questions, and related documents in support of the SAP meeting are posted in the public e-docket at http://www.regulations.gov (ID: EPA-HQ-OPP-2018- 0517). Dr. Robert E. Chapin chaired the meeting. Dr. Shaunta Hill-Hammond served as the Designated Federal Official.

In preparing these meeting minutes and final report, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. The meeting minutes and final report address the information provided and presented at the meeting, especially the Panel response to the Agency charge questions.

U.S. EPA presentations were provided during the FIFRA SAP meeting by the following (listed in order of presentation):

- Introduction - Evaluation of a Proposed Approach to Refine Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM) - Anna Lowit, Ph.D., Health Effects Division, Office of Pesticide Programs, EPA
- Evaluation of a Proposed Approach to Refine Inhalation Risk Assessment for Point of Contact Toxicity: A Case Study Using a New Approach Methodology (NAM) – Monique Perron, Sc.D., Health Effects Division, Office of Pesticide Programs, EPA

PUBLIC COMMENTERS

Oral statements were presented by:

- Doug Wolf, Ph.D., Senior Technical Leader; Sheila Flack, Ph.D., Technical Expert; Alex Charlton, Ph.D., Technical Expert; and Paul Hinderliter, Ph.D., Technical Expert, Syngenta Crop Protection, LLC, Product Safety
- Song Huang, Ph.D., Chief Scientific Officer, Epithelix Sàrl
- Amy Clippinger, Ph.D., Director, PETA International Science Consortium
- Clive Roper, Ph.D., Head, *In Vitro* Sciences, Charles River Edinburgh, Ltd.

Written statements were provided by:

- Monita Sharma, Ph.D., Nanotoxicology Specialist and Amy J. Clippinger, Ph.D., Director, PETA International Science Consortium

EXECUTIVE SUMMARY

The EPA's Office of Pesticide Programs (OPP) is considering a new approach (New Approach Methodology or NAM) proposed by Syngenta Crop Protection (Syngenta) for the assessment of inhalation toxicology, using a respiratory irritant, the fungicide chlorothalonil, as an example. The approach draws from the vision proposed by the National Research Council for Toxicity Testing in the 21st Century (NRC, 2007). A no observed adverse effect level (NOAEL) was not obtained in previous short-term studies, due to severe local irritation effects. This irritation would also preclude obtaining reliable dose-response data and a credible NOAEL from a 90-day study, so risks from short- and intermediate-term inhalation exposures could not be predicted. Thus, the usual approach for assessing inhalation health risks was considered unworkable. In an attempt to provide the Agency with *some* means of assessing short- and intermediate-term inhalation risks, Syngenta assembled a suite of technologies for addressing specific questions raised by the Agency per their risk assessment mandates. Syngenta proposed that their new methodology approach addresses deficiencies in traditional approaches (i.e., a 90-day rat inhalation toxicity study for extrapolation to humans) by relying on *in vitro* experiments and simulations with greater human relevance.

The FIFRA SAP was charged with providing recommendations to the Agency in considering the derivation of the point of departure (POD) from an *in vitro* assay and the integration of the *in vitro* POD for calculation of human equivalent concentrations (HECs) for the inhalation risk assessment. The pesticide chlorothalonil was presented as a case study to solicit advice on the proposed overall approach for application to other pesticides or industrial chemicals in the future.

The Panel commended Syngenta for the leadership they showed in pushing this approach even absent any mandates from the Agency and trying to realize the aspirations of the National Research Council (NRC, 2007). Similarly, the Panel commended the Agency for all of its efforts to advance the adoption of *in vitro* models, particularly those incorporating human cells, to reduce the use of animals while protecting human health. The Panel expressed appreciation for the opportunity to learn and comment on the approach presented using chlorothalonil as an example.

The Panel addressed five charge questions. The Panel provided the following overall summary of the major conclusions and recommendations detailed in the report.

Charge Question 1: Biological Understanding of Mechanisms and Adverse Outcome Pathways - Major Conclusions and Recommendations

- The Panel was divided about whether sufficient data were presented to fully inform the Panel on the mechanism of chlorothalonil's toxicity. All the data presented were at maximal-effect concentrations, and the many roles of inflammation in the clinical signs and etiology of pathology were not presented. The Panel was divided on whether sufficient evidence was presented to support the contention that cytotoxicity was the basis for the contact irritation and respiratory toxicity and, therefore, allow its immediate translation to an *in vitro* assay. There was also concern that the acute exposure data from the *in vitro* method were of unknown relevance or power to allow the appropriate decisions to

protect from repeated (90-day) exposures, which was one of the initiating impulses for the proposal. Finally, the Panel was divided on the proposal's ability to quantify cell death and some were concerned that visual estimations obtained by pathology were ignoring decades of understanding and accounting for the many steps involved in cell death and removal.

Charge Question 2: The Use of *In Vitro* Endpoints to Report Cell Damage and Response - Major Conclusions and Recommendations

- The Panel agreed that the proposed MucilAir^{TM} model has the potential to evaluate local respiratory irritation, corrosion, or cytotoxicity. The Panel agreed that conceptually using an *in vitro* test system such as MucilAir^{TM} to assess the toxicological profile of irritants or cytotoxicants is appropriate and could eventually be widely implemented once the following shortcomings in this proposal are addressed.
    - The proposal uses data from a 24-hour exposure to predict responses after 90-days of exposure. This extrapolation was clearly not supported by the data, and no compelling justification was offered. This could be addressed by performing the appropriate experiments, as the model is viable *in vitro* for up to a year.
    - Experiments should be performed to show the equivalence between the direct liquid exposure (as presented) and the aerosol or particulate exposures more likely to be found in occupational situations.
    - Another concern was the use of cells from a single region of the respiratory tree. One cell type as surrogates for the many different cell types lining the respiratory tree; active agent vs. commercial formulation in the *in vitro* exposures, and the lack of analytic verification of the amount of active agent present in the model system during or after exposures; the size and diversity of the donor pool, and the complete lack of a direct comparison of the outcomes after similar exposures performed using *in vivo* and *in vitro* models. All of these are solvable issues.

- Endpoints/Selected Measures
    - Some Panelists were concerned that the endpoints selected were effective at reporting significant disorganization or damage, but there were no measures of more subtle changes whose long-term expression might reflect increasing damage.
    - The endpoints were often changed only at the two highest doses, which concerned some Panel members. While it is reassuring that lower exposures are without effect, developing a more finely-divided dose-response curve will likely be necessary for a full POD identification.
    - Some Panelists were concerned about the combining of data from endpoints of differing toxicity and recommended that the Agency develop guidance to focus on the most sensitive endpoint. This should be coupled with a more robust (defensible and usual) development of the benchmark dose. Additionally, reproducibility of response across labs and across replicates were of concern to the Panel.

- Given these, the Panel felt that these measures were generally of value and were fit for this purpose.

Charge Question 3: The Strengths and Limitations of the Computational Fluid Dynamic (CFD) Model to Calculate Deposition - Major Conclusions and Recommendations

- The Panel supported the use of the CFD model as an innovation in handling the complexities of flow in a dynamic and branching system. The Panel also recommended that the EPA and/or Syngenta address many of the embedded assumptions about the model and its use by:
  - Providing greater detail on the proposed particle size distribution (including the degree of concordance with the Respicon data and Oxford Laser-VisiSize data);
  - Demonstrating that the lab spray results are congruent with or similar to field spray data;
  - Considering the lung as a target organ of concern (in concert with exploration of the impact of oronasal and/or mouth breathing);
  - Determining the potential for additional upper respiratory tract (URT) deposition of chlorothalonil during exhalation;
  - Moving beyond an "n of 1" for human upper respiratory tract geometry, addressing CFD model parameter uncertainty and variability, and selecting of parameter values appropriate to the relevant exposure scenarios (e.g., level of effort);
  - Addressing questions about the precision of the current URT CFD model;
  - Addressing the potential for application of different/additional modeling approaches to dosimetry calculation (e.g., Multiple-Path Particle Dosimetry (MPPD) model, physiologically based pharmacokinetic (PBPK) models);
  - Considering alternative dose metrics for the risk assessment point of departure; and
  - Expanding the use of the rat CFD model simulation findings to build confidence in the approach.

Charge Question 4: The Calculation of Human Equivalent Concentrations (HEC) - Major Conclusions and Recommendations

- The Panel agreed with the general approach of calculating the HEC using the *in vitro* POD and data generated from a dosimetry model, and overall, found the three steps taken to calculate the HEC were justified.
  - The Panel recommended basing the mass median aerodynamic diameter (MMAD) and particle size distribution (PSD) on empirical sampling and usage data, and not on assumptions or lab predictions.
  - The Panel agreed that sensitivity analyses for all the breathing parameters would be warranted, and probably insightful.
  - Some Panelists were concerned about using transformed data for the benchmark dose (BMD) analysis, as it appears to be more protective in this instance but also appears to ignore other Agency guidance.

- Panel members made several suggestions to improve the transparency and process for determining the HEC. Among the changes will be a consideration of Uncertainty Factors, and the need for a thoughtful specification of which ones are necessary and their values. These are discussed at length.
- Panel members recommended that a set of guidelines be developed for an HEC based on an *in-vitro* approach for risk assessment. The guidelines could provide decision criteria for evaluating the appropriateness of the model system for the endpoints, toxicants being considered, and the computational model for describing the human physiology to determine the Uncertainty Factors (UF) for the *in vitro* to human *in vivo* extrapolations.

Charge Question 5: The Strengths and Limitations of this Approach for Compounds Causing Portal-of-Entry Toxicity - Major Conclusions and Recommendations

- Compared to the current *in vivo* rodent models, the Panel recognized the need for a more powerful, discriminating, and resilient approach to the inhalation toxicity problem that can accommodate the hundreds of potential exposures. The proposed approach has strength, relevance, and utility. The proposal using chlorothalonil was generally well-reasoned and serves as an instructive example. Both the Agency and Syngenta are to be commended for exploring this approach.

- Some of the numerous strengths of this approach are:
  - It focuses on a relevant human *in vitro* model for local lung toxicity.
  - It tries to address an unmet regulatory need using a novel method that has considerable scientific merit.
  - It shows how CFD modeling can be used to estimate site-specific dosimetry. Many other strengths are listed.

- Unsurprisingly, *in vitro* methods come with their own shortcomings and assumptions, which need to be acknowledged and investigated so as to correctly account for them. Among these are
  - Intraspecies variability;
  - Unknown degrees of variability among 3D models of human airways;
  - Possible shortcomings of the emerging proprietary tissue-specific models, and the variance introduced by the use of different cell types.
  - Endpoints must be carefully chosen for relevance, sensitivity, and durability over a wide exposure range;
  - This NAM requires a relatively good understanding of the relevant adverse outcome pathway (AOP). Absent this knowledge, such a model will be less powerful or useful;
  - Estimates of exposure are a critical piece of these models, which will need additional work, particularly with sensitivity analyses to help identify the most critical components;
  - The Panel found value in the concept of an Agency-issued guidance document on the key components of computing the best HEC;

- Various physico-chemical properties of exposures (mixtures, active agents, formulations) need to be addressed. Some of the Panel recommended starting from relative simplicity (active agents rather than formulations);
- Panel members suggested a decision tree as one approach to help registrants decide which test or test parameters would be most appropriate in their situation;
- The relationship between length of exposure *in vitro* and length of exposure being modeled *in vivo* needs to be systematically addressed; and
- Any *in vitro* test should be grounded in AOP-based information and accommodate the expected mode of action.

- Lest this list of requirements appear impossibly long, the Panel emphasized again the benefit (and difficulty) of reducing these generic values (reduced animal use, improved human relevance, increased speed and resilience of method) to practice, and the Panel reiterated the compelling need to do so. The Panel also recognized that some information on some of these limitations may already exist. A program to leverage existing data on known chemicals and to systematically evaluate these *in vitro* models and compare the predicted protective levels with those levels already derived from *in vivo* methods will be both instructive and necessary to help generate trust in the new methods. The criteria required of such assays, already developed by OPP, are a strong place to start. Negative predictors are just as important as positive predictors. The Panel recognized that the animal data were imperfect, but these at least have the value of having real-world history and at least *some* sense of how protective they have been.

DETAILED PANEL DELIBERATIONS AND RESPONSE TO CHARGE

Charge Question 1.

Please comment on the biological understanding of the irritation caused by exposure to contact irritants, such as chlorothalonil, via the inhalation route and how this understanding informs the applicability of the *in vitro* testing considered in the EPA's issue paper (U.S. EPA, 2018a). As part of its submission (Syngenta Crop Protection, 2018a) and summarized in Section 2.2.4 of the Agency's issue paper (U.S. EPA, 2018a), Syngenta has provided a biological understanding of the irritation resulting from chlorothalonil exposure. This includes an AOP where epithelial cell damage occurs from initial respiratory exposure to chlorothalonil and causes cell death. Following repeated exposure, the repeated cell death results in a metaplastic response and differentiation of respiratory epithelium into stratified squamous epithelium.

Panel Response 1:

The charge to comment on the "biological understanding" of the irritation caused by exposure to contact irritants, such as chlorothalonil, is confounded by different interpretations of the charge. Prior to the December meeting, many Panel members noted that the charge was to understand the respiratory irritant effects of the agent chlorothalonil. During the meeting, it became clear that the intent of Syngenta's proposal was to provide a model for the late "unresolved" metaplastic effects of the agent in a submitted 2-week dosing study. Additionally, during the meeting, the Agency advised the Panel not to consider the new approach in a similar context to those of existing animal test systems, and while consideration of the new approach (an *in vitro* assay - MucilAir™) requires stringent review, to not hold the new approach to standards beyond those imposed or accepted for the existing test systems.

The use of animal data as a "gold standard" for comparison of old and new methods may not be appropriate where the use of human models is prioritized. However, the existing animal data produced in the initial registration of chlorothalonil were essential to the development, assessment, and use of this new approach, and so should not be completely ignored. To some degree, these various charges were interdependent and at odds; the Panel attempted to address them all.

As to an understanding of the clinical respiratory toxic effects of chlorothalonil, described by Syngenta as "labored/rapid breathing, gasping, wheezing and rales," the Panel noted there were not sufficient data in the proposal to provide a reasonable biological understanding of this physiological effect. All *in vivo* rat data provided (U.S. EPA, 2014) demonstrated full respiratory effects, and this endpoint was not provided quantitatively, so there was no variation in effect. Although these data were pointedly not cited in the presentation by Syngenta, cellular damage to the respiratory system, originally described as "degeneration and/or necrosis" and expanded on in their oral presentation as "necrosis and ulceration" was noted in all treated animals, in addition to the respiratory effects. Since no sub-cytotoxic effects were documented, the Panel agreed that the interpretation that airway epithelial cytotoxicity was intrinsic to the contact irritation and/or respiratory toxic effects was unjustified, given that all data were derived from a plateau of

maximal effects on the induction curves of both endpoints. Syngenta provided no rationale to discount the possibility that sub-cytotoxic effects could induce the physiological reaction in the absence of overt cell death.

Moreover, the Panel noted that other factors have been observed in nasal irritation and respiratory toxicity, including, but not limited to, inflammation, olfactory and other sensory nerve effects. Inflammation was observed in the *in vivo* data but was dismissed as resolving with time. It must be noted that the existing animal data were not germane to the level of exposure required to initiate physiological effects. Similarly, it was stated that olfactory effects could be discounted because of the modeled deposition profiles. This assumed that all effects were modulated only by the amount of contact, discounting the possibility that olfactory effects were much more sensitive and could be induced at levels that were still not associated with overt degeneration in other parts of the pathway.

Although unclear in Syngenta's written proposal, during their oral presentation it became clear that the proposed *in vitro* model was, at least partly, meant to satisfy an EPA request for a 90-day chronic exposure study. Thus, instead of concentrating on establishing the threshold of acute effects that the Panel considered to be lacking in the original data, the follow-up was more concerned with accounting for long-term effects. Once again, all exposures in the 2-week study induced both symptoms of respiratory toxicity and airway degeneration. Squamous metaplasia of the larynx was the only effect that did not completely resolve after a further 2-week recovery time, and this observation became the focus of the follow-up studies, including the move to an *in vitro* system that is often used in a repeat exposure (as indicated in public comments by Dr. Huang), multi-week scenario, and includes relevant cell types.

For a number of reasons, including:

- the length of time, 14-days (as tested) compared to 90-days (as required by the EPA),
- the metaplasia seen in the rat larynx might be associated with the specific anatomy of the rat airway, and
- the suspicion that even this lingering effect would resolve, if given a longer recovery.

Many Panel members were confused when this squamous metaplasia effect was given as the outcome of the adverse outcome pathway, instead of contact irritation resulting in respiratory toxicity. In the context of squamous metaplasia, the Panel generally agreed that the process involved cell death, although the pathway could have begun with earlier initiating events such as exposure to reactive oxygen species. In the written proposal, oral presentation, and later as "clarification," Syngenta stated unequivocally that the only biological effect of chlorothalonil was cytotoxicity, yet this is confounded by the transient inflammation noted in rat studies. Some Panel members would have preferred this assertion to be supported with evidence, rather than simply asserted as "common knowledge." Indeed, additional mechanisms of chlorothalonil toxicity have been previously described elsewhere (Parsons, 2010).

There was also some concern among the Panel that despite the ability of the CFD model to distinguish areas of deposition, it appears that cytotoxic and degenerative effects in different areas of the airway were invoked interchangeably in the proposal. Also, there is a general assertion that the CFD model system was concurrently applicable to the whole pathway, rather

than just the region of the respiratory tract from which the human donor cells were obtained. Confirmation that this assertion is true would be important for the future application of the model, since regional variability in cellular response might necessitate the use of different donor models.

The Panel did not reach consensus on the contention that cytotoxicity as the basis of the *in vivo* contact irritation and respiratory toxic effects of chlorothalonil has been established definitively enough to allow for translation to an *in vitro* assay. Some Panel members considered that cytotoxicity was appropriate as a regulatory endpoint representing irritation, despite the fact that it was not well justified in the proposal. Other Panel members considered that the AOP leading to metaplasia was germane to the evaluation of the irritant effect of the chemical. In particular, there was precedent that cytotoxicity in other organotypic test systems representing other human organs have gained acceptance as representative models. Specific instances include test guidelines for eye irritation using reconstructed human cornea-like epithelium (Organisation for Economic Co-operation and Development (OECD):TG492 and U.S. EPA, 2015), skin irritation and skin corrosion using the reconstructed human epidermis model (OECD TG 431 and TG 439). Another reason put forward for accepting cell death as an endpoint was the assertion that it may act as a converging key event for divergent toxic pathways.

Panel members also noted that a greater effort was necessary to justify accepting simple cytotoxicity as representative of *in vivo* respiratory effects. In general, there were two methods for justifying such a translation: 1) as a mechanistic precursor effect, or 2) simply as a consistent and reliable biomarker. Since no data were available to reflect the onset of symptoms in the *in vivo* model, neither of these conditions were fulfilled. This highlighted a fundamental problem with the application: it attempted to both replace existing methodology with new methodology and provide actionable data from that new methodology at the same time. The limited *in vivo* data cannot be invoked as evidence for concentrating on a cell death endpoint without first ensuring that the *in vivo* data support such a translation, and then showing that the *in vitro* data were at least in some way, reiterating the *in vivo* data. This was not a case where new methodologies were being created in a vacuum. Since there are such existing methodologies, it was imperative to understand the relative efficacy of a new system at determining or estimating human toxicity, in addition to factors such as throughput, money saved, and animals spared. The panel noted that more clear presentation and justification of the putative AOP underlying the approach could also have provided underlying support.

Another aspect of balancing the Agency's charge to evaluate the biological understanding of the proposal both in the context of existing *in vivo* data and as freestanding information was the question of duration. In response for further acute data, the submitted *in vitro* data did provide the no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) data that were missing from the acute *in vivo* studies (if the translation in systems was accepted). However, if the proposal is also to be responsive to the request for a 90-day study, many on the Panel expressed reservations that this can be done with a single acute study. The Panel discussed the possibility of repeated dosing in the *in vitro* system, and clearly the system does at least potentially have the ability to provide such a capability, with the need to determine what the *in vitro* equivalent of a subchronic exposure would entail. There was a general concern among panelists about the inadequacy of using the *in vitro* system to replace a 90-day subchronic

study, complete with recovery periods with a single acute study. There was also concerns that the model would not persist for longer periods, and repeated doses, if there was significant cell death as a primary effect. The Panel considered that more useful BMD could be calculated from the cumulative effects of chronic exposures to sub-cytotoxic doses. There was significant interest from the Panel in determining whether the *in vitro* system was capable of demonstrating the same types of changing cell landscape over time as was exhibited after the *in vivo* exposure.

Finally, there was concern among the Panel that generalized "cell death" is no longer an appropriate endpoint. In fact, in Syngenta's presentation, much of the data involved tissue disorganization, presumably secondary to cell death, as the *in vivo* endpoint. One advantage of the proposed *in vitro* model is that it can recapitulate such an effect. However, it was expressed that subjectively ranking histological effects, while visual, was not as quantitative as was possible with current technologies. Many panelists expressed a belief that morphometric measurements would allow for better comparison between the concept of cell death *in vivo* with cell death as represented by the 3D model system. It was also not clear whether decades of progress in defining mechanisms of cell death, complete with easily applied markers, have been incorporated into the assay system, to ensure that the type of cell death observed *in vivo* (associated with human health effects) is successfully reiterated *in vitro*.

Panel Deliberations – Charge question 2

Please comment on the strengths and limitations of using the *in vitro* test systems to evaluate a variety of membrane and cell damage endpoints (transepithelial electrical resistance, lactate dehydrogenase release, and resazurin metabolism) as markers of cellular response as described in MRID 50317702 (Syngenta Crop Protection, 2017) and summarized in Section 2.2.4 of the EPA's issue paper (U.S. EPA, 2018a). Please include in your comments a consideration of the study design and methods, appropriateness of the selected measures, robustness of the data, and sufficiency of reporting.

Panel Response 2:

MucilAir™, as an *in vitro* system, has several advantages in that it is a three-dimensional model comprised of human airway epithelial cells that allows direct exposure to chemicals at the air-liquid interface, and recapitulates some critical functions of the human respiratory tract including barrier function, mucous production, and cilia function. The comments below summarize the discussion of several aspects of the studies that were reviewed by the Panel and include the study design, method of application to the MucilAir™ system, donor tissue characteristics, *in vitro* endpoints selected and relevance to irritation, validation and reproducibility, and reporting details.

In general, the Panel agreed that this model has the potential to evaluate the type of effect of concern, namely local respiratory irritation, corrosion, or cytotoxicity. The Panel agreed that conceptually, using an *in vitro* test system such as MucilAir™ to assess the toxicological profile of irritants or cytotoxicants was appropriate and could eventually be widely implemented. However, the study, as conducted, has several shortcomings that are outlined below, together with specific strategies to address these issues.

Study Design and Methods

One of the greatest concerns of the Panel regarding the study design was the reliance on a single, 24-hour study as a replacement of a 90-day animal study. The study design, as presented, was not considered sufficient to replace a 90-day animal study, even when an AOP suggested acute irritation/cytotoxicity was the critical adverse effect. If the model as presented is used to replace a subchronic animal study, the Panel insisted that repeated dosing is necessary to assess the potential effects of repeated exposure. The study presented in the Agency's issue paper (U.S. EPA, 2018a) only looked at acute effects with cell death as the endpoint. The Panel considered that this approach could be suitable to derive short-term exposure limits, but not exposure limits for chronic effects.

One of the major, yet overlooked, advantages of MucilAir™ is that this system is viable and remains fully differentiated and functional for over one year in culture (Epithelix, 2019). Therefore, the Panel recommended conducting further studies to assess the effect of repeated exposures in the model to see if results indicate a lower POD and, if this is the case, that this POD be used for the derivation of the HECs. Different study durations (e.g., 14-days, 28-days, 90-days) should be investigated to identify a POD that is representative of subchronic exposure and that has the best predictive value. If it is proven that repeated exposure over a specific duration does not change the outcome when compared to a shorter duration, then the approach could be optimized to the shorter study duration. For example, if data demonstrate that the same results are obtained following three months of dosing versus one month of dosing, then it could be acceptable to conduct the study for a 1-month duration. Some Panel members also expressed the desire to assess recovery following repeated exposure.

Some members of the Panel expressed their concern that the approach used to dose the cells as performed within the study (i.e., pipetting the chlorothalonil onto the MucilAir™ system), may not adequately represent an *in vivo* inhalation exposure. These members suggested that the study should be conducted using different exposure methodologies (e.g., liquid, aerosol).

Several members of the Panel recommended that the tissue used *in vitro* be appropriately representative of the vulnerable tissue *in vivo*. If the maximal deposition is modeled to occur in the vestibule of the nasal region (given the particle size used for the modeling), then the nasal epithelium also needs to be represented in the *in vitro* testing. Since the Agency's issue paper (U.S. EPA, 2018a) states that this epithelium is more akin to the epidermis, other *in vitro* models (e.g., skin models) could be used for that purpose. The same goes for deep lung tissue, as effects were observed in that region, despite low predicted deposition as per computational modeling. This could be particularly important when evaluating chronic exposure.

In addition, Panel members also insisted that additional studies are needed to confirm that the results obtained in MucilAir™ derived from nasal tissue are representative of the finding obtained using the same model derived from tissue from other regions (i.e., tracheal and bronchial tissue models). As stated in the study information, only the nasal tissue model was available when the chlorothalonil study was conducted. Panel members lacked confidence in the discussion that the additional models would respond the same way, without data supporting that assertion. Another concern arose when it was explained by Syngenta, that the nasal tissue model

cells were (or usually were) obtained from nasal polyps. Panel members raised questions regarding whether those cells would respond differently than cells derived from normal nasal epithelium.

Panel members also noted that it appeared that chlorothalonil was not measured in media or tissue extracts at any point during the incubation period and questions were raised about its relative stability in cell culture media vs biological matrices. It is best to measure the media concentration analytically to verify the delivered dose compared to the nominal concentrations used. In addition, the Panel members recommended evaluating the system without cells and determining the analytical concentrations of chlorothalonil throughout the system to determine whether the test substance sorbs to parts of the system. In addition, Syngenta has shown that inactive ingredients can modulate the outcomes (i.e., the toxicity of the formula) in this model. Since the whole formulation was used in the case study, it is unclear whether the active ingredient chlorothalonil, would have been more toxic at the same concentration without the inactive ingredients. Moving forward, Panel members recommended that the active ingredient should be tested alone (or with a simple vehicle) to avoid confounding effects of the formulation.

Justification of the number of donors was not included in the study information and there was extensive discussion by the Panel on the MucilAir^{TM} donor tissues and reasons for the five donors per group. Some Panel members commented on the lack of data on differences in donors and cell models that could impact responses or introduce additional uncertainty. Members of the Panel had questions about the variability between the replicates per donor per dose since these data were not shown as error bars on the graphs, or standard deviation in the tables during Syngenta's oral presentation. It was also discussed that since tissues grow/mature independently in their respective inserts, one can observe differences in biomass. For Transepithelial Electrical Resistance (TEER), it could help explain donor-donor differences as cellular proliferation rates may impact tissue biomass as well.

Panel members noted the inclusion of cultures from multiple individuals was an interesting aspect of this study but that the range of baseline (control) responses across individuals should be presented. Some Panel members acknowledged that while information on intraspecies variability can be gathered *in vitro* by using cells derived from single donors, the number of donors needed to obtain biologically significant information regarding interindividual variability would be much larger than the sample size used in this study and this is in fact out of scope for this NAM. The use of pools of donors should be investigated as an approach to augment throughput and decrease variability.

Overall, at a minimum, the Panel recommended that studies be conducted over longer durations and that the study duration be optimized to substantiate the use of this NAM as a replacement for subchronic inhalation studies for irritants. Further, comparative toxicity studies with several irritants should be conducted (using nasal- tracheal- and bronchial- derived cells) to prove the assertion that the nasal epithelium is representative of the airways in general and this comparative investigation should be included in the model design or evaluation process. The Panel also agreed that a database of historical controls should be established and used in defining a study acceptability criterion.

Appropriateness of Selected Measures

TEER, lactate dehydrogenase (LDH) release, and resazurin metabolism are standard markers of overt toxicity. The pivotal hypothesis in the Agency's issue paper (U.S. EPA, 2018a) is that by protecting for the initial cell damage caused by chlorothalonil exposure, effects that would be caused from repeated exposure would also be prevented. However, since the chosen markers are markers of overt toxicity, the current study design does not allow for an assessment of the potential sublethal (sub-cytotoxic) effects that upon repeated exposure would lead to the same phenotype over time. Nevertheless, the Panel noted that the chosen markers of toxicity would be acceptable in a context where repeated daily exposure over a longer period of time (as discussed above) would be employed.

Syngenta presented information on TEER correlating well with other markers of cell injury/death. The Panel recommended the addition of this information, and any other information showing the other endpoints (e.g., LDH and resazurin) and their correlation in other studies, be included in the documents provided for the Agency's review. Some Panel members commented on the need to include morphometric assessment of exposure response/injury/adaptation. Furthermore, although the MucilAir^{TM} system could be used to assess early critical key endpoint(s), the Panel did not believe that a single endpoint analysis would be sufficient to capture the different types of mechanisms of irritation/corrosion/cell damage.

The Panel commented that the dose-response curves, as presented in the study, were mainly "flat" for most doses administered and because a significant change only occurred in the highest two doses administered, it may not produce a model that can accurately reflect the POD. This fact was acknowledged on Page 7 of the EPA's issue paper (U.S. EPA, 2018a) "…occurred, uncertainty remained due to the broad dose intervals of the existing data that commonly resulted in binary "all or nothing" dose-response curves and could limit accuracy of POD determination." Panel members commented that it is important to have a full view of the response behavior by observing data across a range of responses, not just the last two data points, as produced in this study.

In addition, the Panel agreed that while all three measurements should always be run in parallel, the most sensitive measurement (in this study, resazurin metabolism) should be considered as the critical effect and used for defining the POD, especially considering that LDH has been previously shown to not be a great marker of toxicity in this system (Balogh Sivars et al., 2018). By taking the geometric mean (as proposed in the issue paper (U.S. EPA, 2018a)), the results are skewed in favor of a less protective value. Some Panel members were concerned with the fact that Syngenta stated that for resazurin, results from lower doses needed to be combined with the control to produce a significant difference and requested that these data be included in future submittals to the EPA.

Overall, the Panel agreed that although TEER, LDH, and resazurin metabolism are markers of overt cytotoxicity, they could be suitable in a context where the cells are exposed repeatedly to the test substance over a subchronic period of time. Cytotoxicity is regarded as a point of convergence (e.g., node) of different AOPs leading to the health outcome of concern, and is therefore, considered acceptable in a context where the intent is to assess chemicals for their potential to lead to that outcome. The most sensitive of the three endpoints should be used for the

determination of the POD as different toxicants have different mechanisms of actions and some endpoints might not have a good predictive value for all types of irritants/corrosive/cytotoxicants.

Data Analysis, Results and Study Reporting

Some members of the Panel explained that the proposed calculations to derive the POD are not acceptable. What constitutes "a response," which for risk assessment purposes we would consider an "adverse effect" is based on the variability of the assay (i.e. on standard deviation). Since their pool of donors is very small, and the tissue replicates measurements and controls showed large variability from sample to sample, the threshold of what constitute an effect is not anchored in physiology, but rather is a representation of the variability of the assay with these donors in that lab and at that time. The greater the variability, the higher the POD will be and in this case, the benchmark response is only a reflection of the variability. For this approach to be acceptable, it will need to be anchored in physiology and the *in vitro* markers of toxicity will need to be correlated to more sophisticated approaches looking at "true" viability (e.g., live-dead assay, high content imaging, etc.). Only then would we have an actual sense of what this assay represents and based on that we will be able to define the level of toxicity (i.e., benchmark response) that is toxicologically and biologically relevant and consequently what the appropriate extrapolation or adjustment values should be. Furthermore, the method detection limit (MDL) should be reported and used to interpret the effects in the context of the assay limitations and the physiological relevance.

Some members also expressed that the BMD modeling performed is not consistent with the Agency's benchmark dose technical guidance document (U.S. EPA, 2012). The BMD is based on experimental replicates when it should be based on the data from all donors across dose groups. Tissue replicates should be averaged, and then donor data averaged. Then the donor average and measure of variance should be used for the modeling across doses (the same way it would be done with animal data). It is highly recommended that the BMD modeling be done according to the process outlined below as the per current guideline.

Robustness, Confidence in the Approach, and Validation

Many, but not all, Panel members agreed that the technical reproducibility should be documented during the method validation stages and culture conditions should be prescribed in a guidance document. Outlier treatment and accept/reject criteria should be preestablished during the validation phase. The endpoints chosen and the intrinsic variability of the system command that such criteria be established before this NAM can be used and accepted.

The Panel was concerned about the lack of study validation presented in the study materials. It is important to note that "Study validation" in this document refers to 1) how reliable the test is when performed over time (i.e., repeatability over time under similar or the same circumstances), and 2) to what degree it correlates with, and thus predicts, an *in vivo* response.

There was no effort presented to repeat the study in different labs or even in the same lab or to use known controls from Syngenta's portfolio. Panel members would like to see evidence that this method is applicable to other irritants where thresholds (e.g., NOAELs and LOAELs) have been established in the literature, ideally with human data. The robustness cannot be assessed

until data substantiate the relationship between effects in the *in vitro* model and *in vivo* response in humans. A pivotal component of the validation will be to establish the relationship between an effect seen in this model and *in vivo*, human, effects. This might inform the development of an *in vitro-in vivo* extrapolation factor.

The specificity and sensitivity of the method were not described, nor evaluated. This too will be required before this approach can be used to establish safe values with confidence. Per the discussion above, the confidence in the approach as it stands is low, but the Panel made several recommendations to substantially improve and confirm the value of this NAM.

Summary

Overall, the Panel considered that the model has the potential to provide information regarding the irritant, corrosive, or cytotoxic potential of a toxicant via inhalation. However, before this NAM can be used, several questions remain, and further experiments are recommended. These may include, the effect of study duration (with a recommendation to conduct subchronic repeated exposures), the effect of the region of origin of the cells, and the effect of individual donors vs. pools of donors. The current endpoints (TEER, LDH, and resazurin) should be adequate in the context of a repeated dose study. Changes in data analysis, specifically with regards to benchmark dose modeling, are recommended to be in alignment with current practices, and the most sensitive adverse effect should be used and considered the "critical effect" and used as the point of departure.

Overall, with validation, the model will have the potential to improve the testing of inhalation toxicants for local effects and will provide a more humane assay. With optimization and a few adjustments, the model will likely be more predictive and relevant to humans than traditional models.

Panel Deliberations – Charge question 3

Please comment on the strengths and limitations of using the CFD model results to calculate cumulative deposition, including the assumptions and calculations made to account for polydisperse particle sizes as discussed in the EPA's issue paper (U.S. EPA, 2018a). A CFD model for the upper airway of a human was used in the proposed approach to determine surface deposition of discrete particle sizes (monodisperse) in regions of the respiratory tract and adjusted for amount of active ingredient as described in MRID 50610403 (Syngenta Crop Protection, 2018b) and summarized in Section 2.2.3 of the Agency's issue paper (U.S. EPA, 2018a). Since operators are exposed to distributions of particle sizes (polydisperse), percent contributions of each discrete particle size were calculated based on a particle size distribution derived for operators applying liquid formulations and used to determine cumulative deposition in each region of the respiratory tract as described in MRID 50610402 (Syngenta Crop Protection, 2018a) and summarized in Section 2.2.5 of the Agency's issue paper (U.S. EPA, 2018a).

Panel Response 3:

The Panel was asked to "comment on the strengths and limitations of using the CFD model results to calculate cumulative deposition, including the assumptions and calculations made to account for polydisperse particle sizes."

The Panelists deemed that use of the CFD model is an innovative approach to determining human airway exposure to chlorothalonil, and that the calculations performed to account for polydisperse particles are supported by the information provided. For the most part, the proposed process improves upon the current process the EPA would use for the interpretation of *in vivo* data with consideration of the deposition of chlorothalonil particles in the human respiratory system used to determine actual deposited doses to the tissue.

Going forward, the Panel would like to see better justification for the chosen inputs and assumptions for the model provided upfront. That additional justification and documentation could have provided answers to many of the questions that arose while reviewing the documents. The Panel also recommended that the EPA and/or Syngenta (1) provide greater detail on the proposed particle size distribution (including the degree of concordance with Respicon and Oxford Laser-VisiSize data); (2) consider the lung as a target organ of concern (in concert with exploration of the impact of oronasal and/or mouth breathing); (3) determine the potential for additional upper respiratory tract (URT) deposition of chlorothalonil during exhalation; (4) move beyond an "n of 1" for human upper respiratory tract geometry, addressing CFD model parameter uncertainty and variability, and selecting of parameter values appropriate to the relevant exposure scenarios (e.g., level of effort); (5) address questions about the precision of the current URT CFD model; (6) address the potential for application of different/additional modeling approaches to dosimetry calculation (e.g., Multiple-Path Particle Dosimetry (MPPD) model, PBPK models); (7) consider alternative dose metrics for the risk assessment point of departure; and (8) expand the use of the rat CFD model simulation findings to build confidence in the approach. Each of these concerns is discussed in greater detail below.

<u>Particle size distribution</u>
The assumed inhalable fraction of particles (<100 μm) used in the CFD model was based on literature information and authoritative sampling conventions. A series of laboratory experiments were conducted at 20˚C and ambient relative humidity (RH) with a fixed distance of approximately 2.5 feet between the Occupational Safety and Health Administration versatile tube sampler, which collects inhalable particles (<100 μm), and three different nozzles/sprays operated at a pressure of 40 pounds per square inch (psi). The spray experiments were conducted with water and 5% chlorothalonil as an emulsion within water. A particle size distribution with a mass median aerodynamic diameter (MMAD) of 35 μm with a geometric standard deviation (GSD) of 1.5 was calculated from literature data for a spray applicator. During the laboratory spray test, particle distribution was also measured using a four-stage impactor sampler (Respicon Air Sampler with size cutoffs of Respirable 4 μm, Thoracic 10 μm, and Inhalable 100 μm) and with an Oxford Lasers N60V probe with VisiSize particle size software. Details on the exact VisiSize system used were not provided. The raw results from the Respicon Air Sampler and Oxford Laser-VisiSize are given in Appendices of a report from Syngenta (Syngenta Crop Protection, 2018c). The Oxford Lasers – VisiSize system collects particle counts for particles

starting at 10 µm. The reports states: “Mathematical descriptions for each fraction have been defined and used to generate particle size distributions (PSDs) for risk assessment (TSI Incorporated, 1997). According to these criteria, the inhalable fraction refers to particles with diameters corresponding to 50% sampling efficiency (D50) of 100 µm, and the thoracic and respirable corresponding to D50 of 10 µm and 4 µm, respectively (Figure 5).” The Panel noted that this description does not provide sufficient detail to determine how the calculations of the particle size distribution were done nor why the GSD of 1.5 obtained from TSI 1977 was appropriate for the spray systems being modeled (TSI Incorporated, 2013).

The Panel further recommended that the calculated particle size distribution be compared to the measured values collected using the Respicon Air Sampler and the Oxford Lasers N60V probe with VisiSize software. An examination of the data in the Appendices to the Syngenta Report cited above shows particle counts reported by the VisiSize software are within a factor of two of each other across bin sizes from 10 µm to 100 µm. The concentration in the raw data reported for Respicon Respirable and Thoracic impactor stages appear to be as much as 10% and 30%, respectively, of the inhalation concentration. The Panel recommended that the calculated particle size distribution, MMAD and GSD be compared to the measured particle size distribution from the laboratory experiments of the three spray nozzles.

A second concern that the Panel has with the use of the laboratory spray data and the calculated PSD as inputs for the CFD model is whether they are representative of the exposures to applicators. The PSD of aqueous droplets and aerosols sprayed into the air can shift depending upon meteorological conditions, e.g., ambient temperature, RH, and wind speed (Ho et al.,1974). Low RH, high temperature and high wind speed can cause evaporation of water vapor from aerosols resulting in a shift to a smaller PSD (and smaller MMAD) compared to that emitted from the spray nozzles as determined in the reported laboratory experiments. Decreases in particle size distribution will potentially change the deposition pattern within the lung, with more particle deposition deeper into the respiratory tract. Loss of water from the aerosols will not change the chlorothalonil air concentration but will result in higher concentrations in the aerosols, potentially changing the dose delivered to individual cells.

The laboratory setup had a distance of approximately 2.5 feet between the spray and sampler which might represent the distance between an applicator’s breathing zone and the nozzle from a wand sprayer. However, the application modeled was for a drum spraying system mounted on a tractor. Since the breathing zone of an applicator operating a tractor-mounted spraying system is further than 2.5 feet from the nozzles, this would result in lower concentrations of aerosols from any individual nozzle. However, tractor spraying likely has multiple nozzles that would result in higher concentrations than from a single nozzle. This was not included in the simulation. The longer residence time in the air could further shift the particle size distribution to a smaller MMAD as larger particles settle faster and more evaporation of water from the aerosols could occur. The Panel suggested exploring both mathematical models for calculating particle size distribution changes based on the meteorological conditions and distance between the nozzle and the breathing zone of the applicator and to make field measurements of particle size distribution during chlorothalonil spraying. The Panel also suggested that a sensitivity analysis be done across a range of meteorological conditions and distance between the spray nozzle and breathing zone as part of the mathematical exposure calculation. The field measurements can be done

using personal impactor samplers and/or real-time particle counters, such as Optical Particle Counters whose particle counts could be converted to mass assuming spherical aqueous aerosols with a density of 1, since the spray formulation is predominantly water.

Another issue the Panel raised with the representativeness of the laboratory spraying simulation was the use of a single pressure of 40 psi to generate the spray for the three nozzles evaluated. The pressure applied to a nozzle can alter the amount of spray and its particle size distribution. While the 40 psi pressure used in the laboratory test is consistent with what is applied in a drum application system, a range of pressures likely occurs during actual field applications. For example, hand carrier applicators are pressurized by a hand pump. Thus, the pressure starts higher and declines between the hand pumping and pesticide application. There could be a range of pressures from a drum system that has multiple nozzles. The Panel recommended that a range of pressures should be tested for both size distribution and amount of spray released.

It is recognized that the distribution of droplet sizes from applicators is skewed to droplets of hundreds of microns with its tail in the inhalation particle size range. This could be consistent with an MMAD of 35 μm for just the inhalable fraction (<100 μm). However, the current presentation is insufficient to evaluate the accuracy of that value and the proposed GSD of 1.5. It is critical to confirm that particles <10 μm were insignificant, since that is a key assumption for the CFD model input and can alter whether particles from spraying enter the alveolar region, which was not modeled in the current approach.

A nose-breathing only, monodispersed CFD model was used. That model has an inherent assumption that there are no changes in particle size within the respiratory tract. However, due to the humidity within the respiratory tract, hydrophilic particles are known to increase in size after being inspired (Broday and Georgopoulos, 2001). While this is not likely to alter the results for the particle size deposition for the assumed conditions, if there are conditions where there are smaller particles (<10 μm) being inhaled, growth of particle size in the respiratory tract may become important. This should be considered if it is determined that the particle size distribution includes fine particles and should be evaluated if the proposed CFD approach is applied to other pesticides and industrial chemicals and/or exposure scenarios.

While the CFD model was presented for a single scenario, spraying of chlorothalonil, this was presented as a conceptual approach that could be used for other pesticides and industrial chemicals. The Panel, therefore, recommended that full sensitivity analyses be done on the input parameters described above and others that may have a range of values so that confidence in the CFD model is assured and the assumptions about the most sensitive inputs can be examined when applying it for different conditions.

Consideration of the Lung as a Potential Human Toxicity Concern and Oronasal Breathing

A significant concern of the Panel about the CFD approach, as implemented in the current case study, was that it neglected to address the lung as a potential target organ. The lung was identified as a target organ even in an obligate nose-breather, the rat (U.S. EPA, 2014). Predictions (presented in Table 5 and MPPD simulations) indicate that smaller particles in the inhalable range pass through the trachea deeper into the lung (Syngenta Crop Protection, 2018b).

While in humans, fractional lung deposition (which is highly dependent on particle size) may be much lower than that delivered to the upper respiratory tract and larynx, it is not zero.

A CFD model with proper assumptions provides a valid approach for calculating cumulative deposition. For the specific application described, there are several assumptions for which the Panel recommended better documentation.

The CFD model used a breathing rate for a sedentary adult male who was a nose breather. Individuals spraying chlorothalonil are likely to breathe at a higher rate for at least part of the time than the assumed sedentary breathing rate, since applicators exert themselves and carry equipment. A higher breathing rate (discussed in greater detail below under "CFD Model Parameter Assumptions") would increase the mass of aerosols inhaled, increase the linear velocity of the air through the respiratory tract, and cause more air to penetrate deeper into the lungs. Higher breathing rates are also associated with a shift in an individual from being a nose breather to mouth breather. These conditions could change the deposition pattern. Inclusion of oronasal breathing in the model to ascertain its effect on compound deposition should be considered. The Panel suggested using a CFD model that can examine the deposition for both mouth and nose breathers and recommends that a sensitivity analysis for breathing rate be conducted. The Panel would like to see the Source to Outcome approach extended to computational modeling of lung deposition in humans during mouth breathing (as a "worst case" scenario) and possibly to human scenarios with 100% nasal breathing and with mouth breathing augmenting nasal breathing. Habitual oronasal breathing is not unusual (4/30 subjects) and switching from nasal to oronasal breathing at higher ventilation rates is the norm (20/30 subjects) (Niinimaa et al., 1981). It may be that these elements do not add greater understanding to the approach and may not be needed in future cases, but this first application of the approach merits consideration of this concern.

Consideration of further URT deposition during exhalation

The CFD modeling of the URT assumes no deposition during exhalation of the compound, but no evidence was provided in support of this assumption (Syngenta Crop Protection, 2018b). The Panel recommended that inclusion of exhalation and oronasal breathing in the model to ascertain their effect on compound deposition should be considered. Particles deposited during inhalation can be assumed to be "stuck," but the regional deposition of entrained particles in the exhaled breath may lead to a different deposition pattern – or just increase the tissue dose. The modeling of lung deposition (recommended above) could support or challenge the validity of the assumption that no (significant) deposition of chlorothalonil occurs in the upper airway during exhalation. As with consideration of oronasal breathing, it may be that this element does not add greater understanding to the approach and may not be needed in future cases, but this first application of the approach merits consideration of this concern.

CFD Model Parameter Assumptions: Justification, Uncertainty, and Variability

The general ideas of "variability" and "uncertainty" are unavoidable when we deal with populations, as is the case in risk assessment. Furthermore, transparency on the sources of parameter values and the scenarios they are intended to represent would also be desirable.

Inclusion of sensitivity analyses of the upper airway CFD model (Syngenta Crop Protection, 2018b) would have greatly enhanced the understanding of the uncertainty and potential variability of CFD modeling outcomes for use in risk assessment. The model geometry is based on an "n of 1" individual (Kabilan et al., 2016). The current submission does not place this geometry in any context to indicate whether this individual is likely to be representative of the population. Syngenta provided no evidence in this submission to support their assertion that the CFD modeling "is applicable across individuals" (Syngenta Crop Protection, 2018a). EPA provided limited support for the representativeness of the CFD model ("within the range" of other simulations) (U.S. EPA, 2018a). Sensitivity analyses would identify key model parameters that could focus an assessment of the representativeness of the CFD model, and the Panel recommended that such an analysis be undertaken.

In the present report by Syngenta Crop Protection (2018b), the human nasal-breathing model was based on a 35 year-old healthy male, weighing 68 kg and 67 inches tall (Kabilan et al. 2016). In Corley et al. (2012), the human CFD model was based on multi-slice CT imaging of the head and torso of a female 84 years of age. In the Corley et al. (2015) paper on cigarette smoke aldehyde constituents, a new 18-year old male volunteer was used to build the oral breathing human model. The question then is whether or not the CFD model simulations for aerosol dosimetry in human on chlorothalonil would have been different if each of these three different human CFD models was used for three separate simulation runs. This question seems to be of particular relevance since the "limitation" of using a sole volunteer was specifically raised in the Discussion of the Corley et al. (2012) paper. The Panel recommended that simulations with these additional human URT geometries be conducted as a first step toward understanding interindividual pharmacokinetic variability of chlorothalonil. As with sensitivity analyses mentioned above, the application of the model may not require CFD modeling using multiple volunteers each time it is applied. Also, there may be existing data to address this question.

The Panel encouraged EPA and/or Syngenta to consider using a Bayesian approach to continuously update and strengthen the CFD modeling as new information becomes available. The integration and application of Markov Chain Monte Carlo, or similar statistical/mathematical methodologies to address the issues of variability and uncertainty in the CFD modeling could also be considered.

The selected breathing frequency and inhalation rate for the CFD model (20 breaths/minute [min], 7.4 L/min) differs from the rate for the HEC calculation (12.7 breaths/min, 8.3 L/min, (U.S. EPA, 2018a); a sensitivity analysis of the region-specific doses calculated by the model could provide insight as to whether this difference had an impact on conclusions drawn in the overall assessment. No rationale for the selected rate was provided, though it seems intended to represent a "high end" resting rate for activity such as sitting on a tractor. The Panel recommended that, in general, the choice of representative vs. conservative parameter values should be addressed.

Furthermore, it might be more appropriate to label "driving a tractor" as a light activity rather than a sedentary activity. The rate of 7.4 L/min is less than the recommended adult "light intensity" rate of ~12 L/min (mean) or 16 L/min (95^{th} percentile) (U.S. EPA, 2011) and would be more representative of someone actively driving a tractor. The differential is even greater when

compared to the mixing/loading scenario (16.7 L/min) to be addressed in the future and the mixing/loading/application scenario (26.7 L/min) (Syngenta Crop Protection, 2018c). Dr. Paul Hinderliter (Syngenta) relayed findings that increased breathing frequency results in higher deposition rates but no change in distribution. Syngenta/ Pacific Northwest National Laboratory's (PNNL) findings should be documented and submitted to the docket or incorporated into revised risk assessment documentation. At these higher rates, however, oral breathing is expected and could alter deposition patterns.

Another question raised by the Panel is to what extent are the CFD model parameters that drive the deposition predictions dependent on age or sex of the applicator.

The CFD model assumed 20°C, and ambient humidity. However, the effect of temperature and humidity on deposition was not discussed, either qualitatively or quantitatively. Dr. Paul Hinderliter indicated that the nose is pretty good at regulating temperature and humidity. While concerns about ambient application conditions are somewhat mitigated by modulating processes *in vivo*, the Panel noted that supporting references would nonetheless be appreciated.

Model precision

It is not clear that the CFD model mesh is sufficiently fine to accurately estimate the dose to specific "hot spots." Regional doses are presented as distributions (that is, percentiles). The authors state that the 75^{th} percentiles are stable, but higher percentiles would not be. The Panel questioned whether the stability would vary with the number of mesh segments for a given region. If that is not the case, why not? What is the number of facets for each region? A Panel member found the 75^{th} percentile doses to the rat larynx to be approximately linear with respect to airborne concentration ($r^2 = 0.991$), but the deviation between the dose at the lowest concentration and the trendline was 19%. Lack of similar simulations for the human makes it hard to assess the true stability and precision of the human dosimetry computations. The Panel recommended that additional information on stability and mesh dimensions be provided for both rat and human simulations.

Alternative deposition modeling options and Potential Expansions of Modeling Approaches

While the EPA and/or Syngenta appear to have determined that CFD modeling of upper airways best suits their purposes, other modeling options were suggested by one or more members of the Panel.

The CFD model provides the potential to derive a better site-specific dose (mass/unit area) compared to the MPPD model, however, MPPD is freely available, widely used, its simulations are reproducible, and there are well established estimates of the surface area throughout the human and rodent respiratory tracts. Panel members recommended that a comparison between the regional doses predicted by these two methodologies should be done as further confirmation of the model. This idea may not be fruitful because the granularity of the CFD model is much greater than MPPD plus regional surface areas derived from morphometric or stereological assessments.

The CFD model used did not include clearance mechanisms, nor was it run for repeated exposures that might simulate a 90-day sub-chronic exposure. Panel members recommended that

the EPA and/or Syngenta should consider whether pharmacokinetic alterations are expected (in rats or humans) that would alter deposition or clearance with repeated exposure to chlorothalonil.

A Panelist noted that earlier studies on acrolein and aldehyde components of cigarette smoke employed CFD modeling integrated with PBPK modeling to advance human risk assessment (Corley et al., 2012; 2015). The rationale for the choice of level of detail (i.e., the decision of not including PBPK modeling studies in the present proposal) in the model chosen by Syngenta should be made clear in the supporting documents.

Selection of Dose Measure

The CFD simulations suggest the existence of localized regions with higher deposition. These hot spots differ from the MucilAir^{TM} 3D air-liquid system that was used for the testing. Panel members suggested that the implications of this difference should be considered. In addition, the selection of a 75th percentile dose as the POD should be more rigorously justified.

Making Use of the Rat Data

While the NAM approach emphasizes human-relevant simulation, *in silico* methods, and *in vitro* testing, the "parallelogram approach" still has merit, if applied using existing rat *in vivo* data in a weight of evidence approach. The predicted 75th percentile dose in rat transitional epithelium is not that much lower than the larynx. Panel members asked why no significant toxicity was observed there, or has this information not been teased out of the *in vivo* studies? The greater concordance observed in rat dosimetry-*in vivo* severity correlation, the greater confidence one can have in applying the same strategies to predict human *in vivo* effects. It is recognized that the converse is not necessarily true; that lack of concordance between rat *in vitro* and *in vivo* approaches may not indicate a lack of concordance between human *in vivo* and *in vitro* approaches. This is because of potential methodological differences between rat *in vitro* and *in vivo* studies. Panel members recommended that the EPA and/or Syngenta should maximize the insights that can be gained from past rat studies even as they move toward reduced animal testing in the future.

Panel Deliberations – Charge question 4

Please comment on the calculation of the human equivalent concentrations. Human equivalent concentrations were calculated for operators applying liquid formulations in the proposed approach using the benchmark dose level from the *in vitro* measurements and the cumulative deposition as described in MRID 50610402 (Syngenta Crop Protection, 2018a) and summarized in Section 2.2.5 of the Agency's issue paper (U.S. EPA, 2018a).

Panel Response 4:

The Panel agreed with the general approach of calculating the HEC using the *in vitro* POD and data generated from a dosimetry model. However, the Panel has suggested a number of refinements to the calculations that follow.

Overall, the three steps taken to calculate the HEC were justified. However, Panel members had suggestions on how to address some of the uncertainty in the calculations. First and foremost, the

Panel confirmed the importance of incorporating the suggestions given in response to the other charge questions. In addition, members of the Panel suggested that additional refinements of data be considered.

- All members suggested that the determination of the MMAD for the particles and the PSD be based on empirical sampling results and usage data. The Panel supported the continued work of the EPA and Syngenta as they seek to refine the human-relevant particle size distribution experienced during spraying. Also, should the Agency accept the mathematically derived human relevant PSD, comparisons should be made against the sampling data, to ascertain the concordance. Furthermore, sensitivity analyses should explore alternate MMADs as well as GSDs that describe particle exposures.

- Panel members believed that sensitivity analyses around the breathing parameters were warranted. The CFD modeling of deposited particle mass per breath was performed under the breathing parameters of 7.4 L/min and 20 breaths/min. However, in the HEC calculation, the number of breaths per minute is decreased to 12.7/ minute, so the adjustment is a factor of 12.7/20 = 0.635. However, the scenario is supposed to represent a minute volume of 8.3 L/min, which would be an adjustment of 8.3/7.4 = 1.12, therefore it is critical to know what is "rate limiting" in the CFD model - the number of breaths or the amount of air taken in. The breathing rate should better reflect the exposure scenario where exertion is required during tractor or backpack application of the product. An active breathing rate would be more appropriate.

- Panel members thought that the Agency is justified in the use of the transformed data in the BMD analysis. However, one Panel member believed that a different rationale should be considered within the Benchmark Dose Lower Confidence Limit (BMDL) analysis. The Agency pointed out that in their independent BMD analysis, the BMDL calculation using the transformed data resulted in lower Akaike Information Criterion (AIC) values as compared to the untransformed data. Yet, the Agency opted to use the BMDL from the transformed data simply because it was 'lower'. One Panel member thought that the rationale is unconvincing: among adequately fitting models, AIC should be used to choose the best model (which in this case was provided by the models using untransformed data), as is consistent with Agency BMDs guidance.

- Syngenta's HEC calculation is based on a comparison of an adjusted BMDL (mg/cm^2) to site-specific deposition (mg/cm^2) of a 1 mg/L aerosol in various lung tissues. The HEC is calculated by solving a simple proportion and makes sense: as expected, the sites with the highest total deposition, e.g., larynx, have the lowest HECs. However, the BMDL is based on a 24-hour exposure, while the site-specific deposition to which the BMDL is being compared is an 8-hour occupational exposure duration (the spray applicator exposure scenario). One Panel member thought that the BMDL should be adjusted up (by a factor of 3 for example assuming a linear relationship, as commonly performed by Agency ORE assessors) to make a valid comparison with the shorter applicator exposure duration. The Panel disagreed with adjusting up the BMDL based on exposure duration as both the toxicodynamics and time-course of the progression of toxicity remains unknown.

- One Panel member had concerns regarding the method used to derive the BMD due to its being chosen individually for the endpoints assessed and appeared to be based on the results from each endpoint, which seems inappropriate. A protocol outlining what would be required to generate a positive result should be determined prior to data generation and would provide confidence that the method to derive significance is not being chosen to show evidence of change when it is not present, but to show when a positive result is occurring. All three methods were standard EPA analyses, but differed for specific endpoints. TEER used relative deviation from mean response of the control group, LDH used the point at which the response reaches a specific value (no explanation was provided to support this designation of value), and the Resazurin results from lower doses were combined with the control, and then results from the two highest doses were used to compare relative deviation from the combined group (this is inappropriate – either the wrong doses were selected for this endpoint, or the endpoint was not an appropriate choice to represent the effects of the chemical, or both).

- One Panel member suggested an alternate approach to BMD modeling. The BMD is based on experimental replicates when it should be based on the data from all donors across dose groups. Tissue replicates should be averaged, and then donor data averaged. The donor average and measure of variance should be used for the modeling across doses (the same way it would be done with animal data). In addition, while all measurements should be run, Panel members agreed that the most sensitive measurement (in this study, Resazurin metabolism) should be considered as the critical effect and used for defining a POD. Especially considering that LDH has been previously shown to not be a great marker of toxicity in this system (Balogh Sivars et al. 2018), by taking the mean of the three analyses the results are skewed in favor of a less protective value.

A number of Panel members expressed confusion over the equation used to calculate the HEC and a lack of transparency. Panel members suggest that instead of using the actual deposited dose, the fraction deposited, and surface area be included, as this parallels better and with approaches used for local effects (e.g., dermal irritation and sensitization).

- There was some uncertainty as to the relevance of the aerosol concentration of 1 mg/L in the final calculation. It is believed to be from the assumption of a 1 mg/L aerosol used in the CFD results and presented in Table 2.2.3.1 in the Agency report. Additional clarity should be provided in justifying this step in the calculation, especially as others look to use this case study as an example for applying NAM in risk assessment.

Panel members had several suggestions for the Agency with respect to the HEC calculation, both in general and with respect to the use of the HEC in similar studies utilizing *in vitro* results.

- Panel members suggested that the Agency prepare a guidance document that delineates the use and calculation of the HEC for studies utilizing *in vitro* results when possible, or after more experience is gained with these approaches. In addition, guidance on how the use of *in vitro* results from NAM affect the HEC methodology would be useful.

- The Panel's suggestions were mixed regarding whether the HEC should be renamed or differentiated when used in the context of NAM and *in vitro* data. Some Panel members suggested that the EPA develop a terminology that clarifies what data are being used to calculate the HEC. While others suggested that since the resulting value from a NAM or *in vivo* approach is intended to represent the concentration relevant for human exposure, the same traditional naming convention of HEC should be used regardless of the data used to generate it.

The Panel suggestions were also mixed regarding the approach to determine the UF and whether this NAM requires additional consideration and/or adaptation of UF. However, members of the Panel agreed that a paradigm shift was needed to define the underlying assumptions allowing for extrapolating *in vitro* data to human *in vivo* data and that some of these assumptions will differ from the considerations employed for deriving an HEC from animal data.

Some Panel members reiterated the use of the parallelogram approach. This would consist of using *in vivo/in vitro* and human/animal experiments to represent four sides of a parallelogram to generate an adequate extrapolation model from *in vitro* human cell studies to humans and strengthen our ability to quantify the potential risk to humans. Guidelines have been developed for selection of animals for use in *in vivo* studies to be somewhat representative (species, gender, age, number, genetic make-up, etc.) of humans for the endpoint to be considered, though an exact match is rarely, if ever achieved. Hence, the HEC derived was adjusted using UFs for the extrapolation from animal to humans. It is noted that even so, under-prediction and over-prediction of risk can arise because of differences that had not been understood in sensitivities of different species, differences in metabolism, differences in uptake and distribution within the body, etc. It was stated that as the scientific community (industry, governmental [including the EPA], non-profit and academic contributors) develop *in vitro* models based on human cell lines and mathematical models that consider human physiology. The differences between the *in vitro* models and a living human organism, which has feedback mechanism, repair cells, and metabolism that respond to exposure to toxic agents but may not be present or respond in an identical fashion in the *in vitro* model need to be considered. One Panel member suggested that this could be accomplished through the use of *in vitro* to *in vivo* UF in an analogous fashion to the animal to human extrapolations adjustments. For some model systems and chemicals the adjustment could be minimal, such as for a potent contact toxicant well represented by the *in vitro* model, resulting in an UF of 1, i.e., no adjustment to the calculated HEC, while in other cases it might be large when the mode of action is not adequately represented in the *in vitro* system. Such a UF value could also be used when there is incomplete understanding of toxicity of an agent, such as an incomplete knowledge of an AOP. Here the UF would be used to account for the uncertainty in the HEC estimate, which could then be revised once the additional information about the AOP is determined. The proposed *in vitro* to *in vivo* UF is separate from intraspecies variability across people for which a 10x UF is often used.

- One Panel member suggested, and other Panel members agreed, that since this study presents acute toxicity findings for respiratory irritation, and not the repeat dose effects requested by the EPA, the exposure duration UF should remain at 10. However, the

intraspecies UF used seems over-restrictive for a direct acting irritant since the European Union National Academy of Sciences and the EPA both align on an UF of 3 for direct acting irritants.

- Some Panel members were confident that the reduction of the toxicokinetic and toxicodynamic interspecies UF to one was justified due to the use of human tissues.

- The Panel agreed that the model should include repeated exposures. It is argued in the issue paper (U.S. EPA, 2018a) that study duration does not need to be accounted for with an UF. However, given that a 24-hour study is acute, if the intent is to protect from chronic effects, then an UF should be included for extrapolating from acute to chronic, even if the putative AOP presented suggests that by protecting from (acute) cell death we concurrently protect from long-term effects, because there isn't a sufficient understanding of the potential effects of repeated sub-cytotoxic exposures and this isn't addressed by the model.

- Overall, with optimization and a few adjustments this approach will likely be more predictive and relevant to humans than traditional animal models. However, it is reported in the issue paper (U.S. EPA, 2018a) that the most health protective HEC based on the *in vitro* assay is 0.037 mg/L. However, the HEC based on *in vivo* inhalation LOAEL in rats where clinical signs consisting of hypoactivity, gasping, lacrimation, nasal discharge, piloerection, ptosis, and respiratory gurgle were observed is 0.001 mg/L; a 37-fold difference. Further, when this HEC was compared on a daily dose basis to the reference dose (RfD), by multiplying the HEC by the daily breathing volume and applying a 10X UF and the RfD (which also includes the safety factor) by the standard 70-kg body weight, the HEC would lead to an acceptable exposure that is 37 times higher than the RfD. This means that the dose received via the inhalation port of entry on a daily basis could be 37 times the dose that was established to protect from chronic systemic toxicity. Based on these comparisons, there is a concern that the factors chosen to derive the HEC are insufficient and not health protective. One Panel member thought that to refine the approach the benchmark dose-response must be anchored into physiologically relevant changes by correlating the *in vitro* markers with human *in vivo* response, using the available human data. Workers and exposed populations deserve that the Agency document and describe the quantitative relationship between the HEC as calculated in the issue paper (U.S. EPA, 2018a) and the irritation threshold in humans. Only after this qualitative relationship between the *in vitro* model and the target species (human) is established, will we be able to confirm that the HEC derivation is acceptable and that no additional safety factors are needed.

- The Panel did not reach consensus on the utility and necessity of comparing the HEC derived from *in vitro* methodology to human inhalation studies. Some Panel members believed that as this new approach to calculate the HEC is being implemented, its appropriateness can be confirmed by comparing the HEC with a documented "no observed effect concentration" in human inhalation studies where available. Some members on the Panel, therefore, suggested that the HEC be calculated using this approach for pesticides or industrial compounds whose HEC have been well established

based on human inhalation studies for comparison. One Panel member was concerned that such a comparison would result in holding *in vitro* studies to a higher standard than current approaches.

Based on the above arguments, Panel members recommended that a set of guidelines be developed for a HEC based on an *in vitro* approach for risk assessment. The guidelines could provide decision criteria for evaluating the appropriateness of the model system for the endpoints and toxicants being considered and the computational model for describing the human physiology to determine the UF for the *in vitro* to human *in vivo* extrapolations.

Panel Deliberations – Charge question 5

The proposed approach to refine inhalation risk assessments for contact irritants has been presented with chlorothalonil as a proof of concept. Please comment on the strengths and limitations of using this proposed approach for chlorothalonil and other contact irritants, as well as its potential to be used for other chemicals that cause portal of entry effects in the respiratory tract.

Panel Response 5:

In vitro testing using human cells has great promise and offers many potential benefits, such as reduced reliance on *in vivo* animal testing and a reduced burden on animal welfare, potentially avoiding the pitfalls of animal to human extrapolation, and faster screening throughput for chemical safety evaluations.

Panel members well recognized the need for quicker evaluations for the large number of chemicals needing assessment and the innumerable combinations forming mixtures, with one member estimating more than seven hundred thousand chemicals in the EPA's Pesticide Chemical Search Database (U.S. EPA, 2018b) and millions of potential mixtures and formulations. The proposed approach is a step forward in the use of human modeling and tissues for assessment of the inhalation toxicity of certain chemicals.

The case-study of chlorothalonil discussed by the Panel is well-reasoned. The Panel noted that an *in vitro* assay can be appropriate to evaluate direct acting toxicants. The MucilAir™ system has been used in over 100 publications starting in 2008. Although not all of these are relevant to the current question, some may provide additional supporting information to increase the comfort of applying this approach to other chemicals. The overall approach, to utilize a human *in vitro* model of local lung toxicity to refine the human health risk assessment for chlorothalonil serves as an instructive example. It is an example of *in vitro* to *in vivo* extrapolation and the Agency should be commended for entertaining this approach.

One strength of the approach is that it seeks to identify and utilize a relevant human *in vitro* model for the endpoint of concern: local lung toxicity. It is important to note that this case-study did not consider or evaluate systemic toxicity. Another strength of the overall approach is that it proposes a modern, novel toxicological approach to the current risk assessment for chlorothalonil, for which a NOAEC has not been attained for the inhalation route using animal

studies. A third strength is the demonstration of how CFD modeling can be used to estimate site-specific deposition in the relevant target organ. Additional strengths include:

- Use of human-derived cells and a CFD model that considers human respiratory anatomy
- The ability to use many doses and replicates
- The tissue model is well-established in the literature and widely used
- The CFD deposition modeling and 10-dose experimental design allows for a quantitative risk assessment using an *in vitro* approach
- Ability to discern upstream toxic endpoints and provide mechanistic understanding
- Retention of intraspecies uncertainty factor
- Potential for toxicity investigation using tissues from sensitive subpopulations
- Cytotoxicity as a measure allows the capturing of several possible mechanisms leading to cell death

EPA should continue to explore and carefully consider the utilization of human *in vitro* methods. *In vitro* methods should be evaluated to assure they protect the health and welfare of the public and the environment. The Panel found the approach of using chemical case-studies as examples of *in vitro* test application to be very beneficial in their discussion and greatly assisted in framing the issue under consideration by the Panel.

In vitro testing methods have their own set of limitations and will not necessarily resolve all the uncertainties that exist with currently accepted *in vivo* studies. While likely to be potentially very helpful, it is not likely a "magic bullet" that will fully resolve the common uncertainties in risk assessment. It is also important to recognize at the outset that some of the deficiencies of this specific *in vitro* approach that the Panel identified are also deficiencies of the current *in vivo* approach.

- Intraspecies variability still exists with *in vitro* studies and in fact may actually be higher when using donors who are not inbred (as is done with many animal tests).
    - It was noted in this proof-of-concept case-study model evaluated for chlorothalonil that only 5 donors were used, all of whom were Caucasian, with the female donors being relatively close in age. Despite this relative similarity among donors, there was still variability in the results and this variability could be much higher should a wider and more representative donor population be used. This is particularly important because this can result in much less precise estimates of the benchmark response (BMR), since what qualifies as a BMR is currently determined by the variability in the assay, and hence can impact the BMD and POD of the risk assessment. A "look across" analysis to evaluate the magnitude of the variability would be helpful, especially when different cell lines or products are being considered for future *in vitro* tests.
        - *In vitro* testing should attempt to utilize donors that are representative of the appropriate target population, but it is crucially important that health protective and scientifically defensible methods are utilized when estimating the BMR and BMD.

        - The standard measures of variability (e.g., standard deviation) of the data were used and deemed adequate for assessing variability.
        - An assessment of the impact of this variability on the interpretation is necessary because in the current approach, the greater the variability, the higher the POD will be.
        - Sensitivity analyses were suggested as an important step in understanding the impact of variability on the final result.
        - Some Panel members believed that the BMR should be defined by correlation with a quantitative measure of cell death or histopathology.

- It would also be helpful in the future to know if other three-dimensional airway models—either marketed by other companies or produced within a laboratory—can be used interchangeably with this or other similar approaches.
    - In order to allow for the use of other similar models, the EPA should stipulate or refer to acceptable performance standards that are established for *in vitro* tissue models and these performance standards should be publicly available.
    - Manufacturers of *in vitro* tissue models will likely have their own proprietary products, which may vary from manufacturer to manufacturer and this may result in variability. There may even be important variability from batch to batch. Therefore, the EPA should require *in vitro* tissue model providers to benchmark their product and, if necessary, evaluate batch performance on the performance standards. They must also use appropriate and standardized experimental controls. This performance information should be publicly available.
        - The OECD skin irritation guideline cited below is a precedent for the use of performance standards and can serve as a model for this activity.
            - Reference: OECD (2015), Test No. 439: *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264242845-en.

- Tissues can also change their behavior and express phenotype based on the culture medium and other preexisting conditions of the donors (host factors) and even prior exposures (smoker, etc.). This may be a particularly important asset of this *in vitro* system as it could be used to target unique populations, (e.g., sensitive sub-populations such as asthmatics).
- The specific choice of cells used in culture for *in vitro* methods must be carefully considered and should be representative of the target organs for toxic chemical exposures. Critical parameters such as sensitivity and cellular response should be similar, and representative of the populations exposed.
    - In this particular case-study with chlorothalonil, the study exclusively utilized cells that were harvested from the nasal passages, and it was unclear whether this harvest location produced *in vitro* cultures that would respond in a similar way and with similar sensitivity to other locations, such as in the lung that could be

exposed to a test chemical. Syngenta did state that they believed the cell type (epithelial) would be anticipated to respond similarly to the endpoint being assessed (cytotoxicity representing irritation) as other epithelial cells in the lung, although they presented no data or rationale for this assertion.

- It is very important that the cells used in *in vitro* cultures are representative of the cells that will receive a dose in the population under consideration for the risk assessment. Some Panel members expressed that the vestibule should also have been accounted for, since it received the largest dose. It may be possible that some *in vitro* models, using cells which functionally behave in a similar manner as other target cells of interest, could be applied more broadly to other exposure routes after careful consideration by the EPA scientists.

- *In vitro* testing protocols are still subject to the challenge of choosing appropriate adverse endpoints for consideration.
  - Although it is generally accepted that the endpoints of TEER, LDH, and resazurin are effective markers of cell damage and tissue integrity, some Panel members noted that these endpoints might not be sensitive indicators of important precursor steps in the process of cytotoxicity.
  - Variability in the measured response for an adverse endpoint should also be considered and maximum variability thresholds should be part of the method acceptance criteria. The impact variability will have on the interpretation should also be considered.
  - While it is important that the endpoint be sensitive, measurable, and represent an underlying pathological response, it should also be physiologically relevant.
  - Some Panel members noted that a better understanding of the specific correlation and quantitative relationship between these endpoints and health effects in the target species (e.g., human) would facilitate a more accurate interpretation of the meaning of the study results.

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Estimates of exposures for relevant scenarios and the corresponding target cellular dose are critically important when using *in vitro* assays for safety evaluations of chemicals.

- The results of the *in vitro* assay may not be applicable or even result in errors when characterizing the risk, if the exposure and hence the cellular dose are not estimated accurately. It is crucial that the HEC be computed accurately and that the results be reproducible.
  - For the case-study of chlorothalonil, there was a lot of discussion about the models used to estimate exposures and the resulting cellular dose. For example, Panel members discussed at length that the particle size distributions assumed in the study materials submitted were highly subject to operational parameters of the nozzles used and concern was expressed about the actual particle size distributions experienced in the field versus that assumed by the CFD model used to estimate cellular doses.

- Panel members were concerned that the particle size used for modeling may not have been representative of actual field conditions and may have been too large with a standard deviation that was too narrow.
- The EPA should publish a concise up-to-date guidance document that is widely accessible that provides details on the specific procedures and variables used to compute HEC, perhaps with a corresponding example. This document should also include common variables used and their corresponding default values or distributions of values that can be used. The formulas and specific values used for modeling should be explicitly outlined in a guidance document (e.g., surface areas for each region, breathing rates, etc.).

  Panel members found it difficult to evaluate the HEC in the case-study discussed and did not find documentation on the EPA's website or the EPA documents helpful as many were very dated. As a result, Panel members struggled to understand and evaluate the HEC values used in the chlorothalonil case-study. This difficulty can manifest itself in highly variable non-standardized approaches and non-harmonization of approaches leading to significant imprecision in the estimation of the HEC values for future risk assessments. It was noted that if the Panel found it confusing and difficult, users of future risk assessments, such as public health professionals or industrial hygienists, are likely to find the exposure estimates even less clear and hence less helpful.
- Chemicals with different physicochemical properties should be carefully considered. Important parameters such as volatility and the form of the chemical as present in the environment must be carefully considered.

    - It must also be recognized that many active ingredients are formulated in many different products resulting in a wide range of mixtures that are placed on the market for consumer use. The EPA should carefully consider mixtures and their impact on the exposures received.
        - Tests should be interpreted with care and knowledge that it is possible that the combination of active ingredients with in-active ingredients may alter the toxicity significantly.
        - Mixtures with different levels of active ingredients may significantly alter toxicity.
        - Importantly, the active ingredient tested in a standard vehicle should inform the development of the HEC for that ingredient since inactive ingredients can influence the toxicity of the active ingredient and this could result in a HEC that is not protective for a different formula.
        - One panel member felt there was no a priori reason why mixtures cannot be assessed in the presented approach and suggested, barring any physicochemical incompatibilities, that approaches be built around regulatory needs for information on single chemicals or mixtures.

- This chlorothalonil case study demonstrated that understanding the chemical's physicochemical parameters is important, there was considerable discussion about the chemical's volatility and how it was applied and in what form, whether it was dissolved, emulsified, volatile, etc. This type of information can greatly impact the appropriate method in which the chemical is applied to the *in vitro* culture, because the mode of application of the chemical to the *in vitro* culture may significantly impact the results and responses seen. For example, chemicals that are more volatile may behave very differently or even be lost from the culture due to volatilization.
    - Several Panel members suggested that for some scenarios, such as an evaluation of risk due to aerosol exposure or pipetting techniques such as those used in the case study for chlorothalonil might not be an appropriate dosing scheme.
    - However, other Panel members recognized that the pipetting application technique gives a more accurate measure of mass per unit area of cells exposed, is more practical, and should provide acceptable results.

- Data generated from *in vitro* tests, as with any data in a risk assessment, should be fit-for-purpose and have their limitations and applicability discussed in the narrative of the test results to prevent the misapplication of these data to other chemicals if significant differences in their properties will significantly impact the interpretation of the results and application to other exposure scenarios.

- Sensitivity analyses may be helpful in future risk assessments to determine which variables utilized in models for computing the HEC have the greatest impact on uncertainty in the model results. Understanding the uncertainty will greatly impact the interpretation of the results.
- The development of a decision tree was suggested as a potential way to standardize and assure consistency when making decisions about which toxicity testing protocols should be used to evaluate specific exposure scenarios. An example decision tree may include the following elements:

    - Level 1 – What levels of information on toxicity and exposure are available to guide the appropriate approach
        - *In vitro* model, *in vivo* model, *in silico*, chemical structural-activity relationship, epidemiologic or human clinical testing, or other
        - Availability of toxicity data on chemical(s) (some criteria considerations: endpoints (single or multiple), contact or systemic, parent compound or metabolite, typically part of a mixture with similar endpoints, health outcome …)

- Available exposure data for chemical (some criteria considerations: routes of exposure, population demographics, activity that leads to exposure including exertion level, etc.)

- Level 2 – Assuming a human cell-based *in vitro* approach is selected
  - Selection of human cell model (some criteria considerations: endpoint(s) relevance to health outcome, contact toxicant and cell/tissue response, single acute, sub-chronic or chronic exposure, appropriateness of model as representative of target organ/tissue, representativeness of model for target population [e.g., age, gender, ethnicity, health status], need for repair mechanisms-role of homeostasis in response to agent, etc.)
  - Selection of exposure dose (delivery method to cells, physicochemical properties of toxicant, exposure intensity, duration, frequency, form of agent and concentration in vehicle, …)
  - Modeling exposure pattern in target organ – need for sensitivity analyses for each input
  - Type of mathematic model describing organ (Computational Fluid Dynamic Model, respiratory tract dosimetry model, stochastic model, etc.)
  - Exposure characteristics (exposure intensity, duration, frequency; delivery mechanism based on exposure evaluation, representative of exposed population, incorporate repair mechanism, single acute, sub-chronic or chronic exposure, form/concentration of toxicant and type of carrier, etc.)

- Level 3 – Human Equivalent Concentration
  - Select equation (target organ/tissue – surface area, repair mechanism, physicochemical properties, concentration adjustments, need for sensitivity analyses of inputs, form/concentration of toxicant, etc.)
  - Need for uncertainty factors to adjust *in vitro* to *in vivo* (delivery processes not accounted for, repair processes, missing information in mode of action or adverse outcome pathway leading to uncertainty, etc.)
  - Need for intraspecies uncertainty factor (demographic differences – age, gender, ethnicity, variation in polymorphism/genetic differences, effect of health status, range of exertion, etc.)
  - Need for study duration uncertainty factor (e.g., a study that was acute and the intent is to protect from chronic exposures)

- Level 4 – Compare results to existing data from other approaches

It was not clear that the format of the *in vitro* 24-hour assay was representative of sub-chronic exposures with repeated doses and potential recovery and re-exposure of the cells *in vitro*.

- The modeling approach pursued by Syngenta posited that a single 24-hour *in vitro* exposure to the target tissue would provide data that would stand in for results obtained from a 90-day rat study. They presented an AOP that they felt supported this prediction. The Panel responded that the underlying AOP for the chlorothalonil case study was not sufficiently developed and, therefore, would not support the suggestion that multiple days of dosing would not have changed the outcome of the risk assessment. In addition, some Panel members felt that the justification of an *in vitro* test based on AOP may be limited at times because the knowledge of the full pathological process may not be known in sufficient detail. Justification of an *in vitro* test based on an AOP must be carefully considered when conducting risk assessments.
- The Panel noted that Syngenta should take advantage of the ability of the MucilAir™ system to remain viable in culture for many months and conduct repeated-dose experiments.
- In regard to future applicability, some Panel members thought it may be possible that data from one or more acute-exposure *in vitro* assays could predict effects seen in repeated-dose studies, but more information was needed before this could be concluded with any certainty. Other Panel members considered that only shorter-term (e.g., one month or 14-days) *in vitro* studies could be used to predict sub-chronic studies.
- The information submitted and reviewed by the SAP for the chlorothalonil case study suggested that the length of exposure time, length of recovery between exposures, and re-exposure were important considerations when evaluating cellular responses such as metaplasia.
- This case study consisting of a 24-hour test is not considered long enough to ensure there is any evaluation of these longer term or repeat-dose exposures. Some Panel members indicated that this was a data gap in the case study.
    - While the Syngenta data in this case study may have unclear applicability to longer term sub-chronic studies, the data did show that a progression from 2 hour exposure to 6 hour exposure did not show a substantial difference in the pathology endpoints in acute inhalation studies.

    - With regard to the applicability of these *in vitro* tests to longer term exposure interpretation, several Panel members agreed that it would be important for these tests to be capable of measuring endpoints at sub-cytotoxic levels and that the impact of a recovery period and re-exposure may be an extremely important consideration in the applicability of these tests to longer term exposure scenarios (e.g., sub-chronic and chronic). Other Panel members, noted that assessment of subcellular changes was not necessary to provide PODs for human health risk assessment, which are based on adverse, functional or structural changes at the organ or organism level. Demonstration showing the applicability of shorter term acute *in vitro* tests to longer term risk evaluations may be possible in some unique

scenarios, such as evaluations of sensitizers, but this should be very carefully and cautiously considered by the EPA's scientists. Generally speaking, acute tests should not be extrapolated to sub-chronic exposures unless there is information to support such extrapolation. In addition, several Panel members highlighted that when selecting a POD, the most sensitive endpoint between the three measurements should be selected as the "critical effect."

Any *in vitro* test should reflect key events in the AOP and be representative of the expected Modes-of-Action (MOA) of the chemical being evaluated for safety.

- A wide range of endpoints, beyond frank toxicity, may be applicable and necessary when conducting *in vitro* testing. For example, in this case study several Panel members suggested that markers of sensory irritation might have been appropriate and that for example, over 50% of the occupational exposure limits are based on sensory irritation rather than cell necrosis. Clear specification of the purpose and goal of any test, with specification as to why the endpoint chosen is applicable to the risk under evaluation, should be clearly delineated. The *in vitro* test must be "fit-for-purpose" and limitations in the general applicability of any test for other purposes should be specified.
    - Some Panel members noted that the case study with chlorothalonil skipped the hazard identification step by focusing only on cell necrosis. However, other Panel members noted that the hazard identification was conducted previously and was based on a full and complete animal toxicity database. They also discussed that the purpose of this SAP was not hazard identification.
- Among many parameters that can be considered when deciding on the applicability of an *in vitro* test system, the expected MOA and AOP should be key considerations.
- Panel members noted that assessment of the reliability of the model/approach for future uses need not include prospective trials comparing *in vitro* results to *in vivo* results with dozens of chemicals and acknowledged that comparisons to current nonclinical *in vivo* models or model results may or may not be useful.
    - Relevance could be supported with an AOP and other information, such as an assessment of the reliability of the test system.
        - Some comparative data, such as in this case, using the system to assess some inhaled pharmaceuticals and other chemicals may be useful.
    - Reliance on an AOP can support the use of upstream effects – such as cell death, as in this case – to make decisions that do not require *in vivo* testing. Once the AOP has provided biological relevance for the upstream effect and the test system addressing that endpoint is considered reliable, use with other chemicals to assess this specific endpoint might be possible. Detailed information and explanation about how the AOP was constructed, and how the endpoints were selected to fit into the AOP would be useful in order to support the careful consideration to other chemicals with similar modes of action.
- Other Panel members highlighted that several gaps must be addressed in the proposed approach before it can be widely implemented, primarily in consideration of the existing knowledge. Specifically, it is highly concerning that the HEC, derived using this

approach, is higher than the air concentration that resulted in adverse effects in animals and this suggests that there might be an issue with the proposed approach that is not accounted for properly. Of primary importance, it was argued that the HEC and BMDL must be anchored into a physiologically human-relevant threshold (i.e., a concentration that is known NOT to elicit effects). Considering that irritation is an "immediate" effect, this could be done by considering human studies such as field studies (with personal samplers), clinical studies, or epidemiological studies. The Panel did not suggest that human studies are required, but simply that if they are available the Panel wanted to note that they can be very helpful. Some Panel members suggested the *in vitro* to *in vivo* extrapolation could be done using the parallelogram approach and comparing human *in vitro* with animal *in vitro* and animal *in vitro* to animal *in vivo* extrapolate from human *in vitro* to human *in vivo*, but this approach bears greater uncertainty.

- It will likely be difficult to select the proper *in vitro* test, if there is significant and clinically relevant uncertainty about the behavior, toxicity, adverse outcome pathways, or organ systems that may be impacted by exposure to a chemical. In this scenario, a hierarchical "Integrated Approach to Testing and Assessment, where one or more *in vitro* tests are part of a battery of several evaluations, may be necessary. Some Panel members suggested a common approach to the utilization of *in vitro* tests and suggested that it include initial cheminformatics assessment of the test material to identify structural alerts, potential MOA, estimate of the toxic category, regional dosimetry, etc., that would help in selecting the appropriate *in vitro* test system(s).

- It is also important to recognize that such an *in vitro* test does not allow for assessment of the effects of the chemical after it is absorbed and distributed throughout the body, or effects of systemic metabolism, and that chemicals may pose significant systemic toxicity to multiple organ systems that are not evaluated using this approach.

Building confidence in the accuracy and applicability of test results, to ensure an effective risk assessment, should be underpinned by a transparent evaluation of any new method or approach according to predetermined performance criteria. This reliability and confidence building process should seek input from a wide range of scientists, including scientists who perform risk assessments (e.g., toxicologists), as well as those who use risk assessments (e.g., public health, industrial hygienists).

There was a lot of discussion about what information would be needed for the *in vitro* model to be applied in the proposed context. A majority of the Panel members agreed that building confidence in these *in vitro* test systems does not require comparison with animal studies, although existing animal data should not be excluded from the weight of evidence and certainly has value in the evaluation process.

However, in order to accept this paradigm shift and start identifying acceptable limits of exposure based on multiple pieces of evidence, including *in vitro* approaches, we need to have a high confidence that it will be human health protective. For this, we need some confidence that

the data and evidence are relevant to humans. Therefore, to bridge this type of assay to the human population of interest, is it critical to anchor the results to relevant health outcomes. A systematic comparison, as part of an evaluation program, with existing chemical irritants for which we have human (e.g., occupational) data would potentially prove useful with regards to informing *in vitro* to human extrapolation.

In general, any new method or approach should be evaluated for its reliability and relevance for the purpose for which its being proposed through a transparent process with previously determined criteria. The use of the criteria developed by OPP for the evaluation of NAMs is extremely helpful in this regard and is outlined in Appendix B of the Agency's issue paper (U.S. EPA, 2018a). These are based on internationally-harmonized criteria and include: decision context, biological relevance, reference chemical set justification, reliability within context of use, transparency, description of uncertainty, access by third parties, and independent scientific review. EPA's discussion of whether the approach meets the criteria for its intended use is for the most part persuasive; additional information would help to increase confidence.

Starting out with a proof-of-concept evaluation for *in vitro* studies, it would be helpful to leverage existing data on a set of reference chemicals based on their expected mode of action with initial consideration for chemicals that have extensive and well understood toxicity to validate the assay. Information supporting the reproducibility of the MucilAir^{TM} system and other similar systems are also needed and should be considered when proposing use of these systems. This will help further improve confidence in the performance of these *in vitro* studies, while also helping the risk assessor understand the limitations of any *in vitro* study used. It is also important to emphasize that this information may already be available in the literature or within testing facilities.

- Standardization and harmonization of testing protocols will be necessary to end users, especially those with a global footprint.
- For some uses, negative predictive value is just as important as positive predictive value; Panel members suggested a broader understanding is needed of the ability of the test to correctly predict a positive response and the ability of the test to correctly predict a negative response.
- It is clear that animal studies have limitations, and many argue that in-fact they may not be the best predictor of toxicity. Indeed, the current animal-based paradigm may be missing crucial effects, either because the animal model isn't giving us this information, or because the tests aren't efficient enough to allow testing of the many potential chemicals, mixtures, and sensitive populations. However, there has to be a method to evaluate the performance and predictive ability of any new test method under consideration. Careful thought should be given as to how this can be done. For example, it was suggested that if a comparison were made of the results for an *in vitro* test method to previously obtained results from chemicals with already existing animal and human data on well known hazards, that this could possibly serve as some assurance that the *in vitro* test predicts risks accurately.
    - Several Panel members suggested that we should be cautious about using the animal data as a standard by which we judge a new methodology, and that it

should be used in a weight-of-evidence approach rather than as a strict comparator.
    - A minority of the Panel suggested that the presently proposed entire suite of methodologies (i.e., the *in vitro* work, CFD modeling, etc.) be applied to well-studied chemicals with established Integrated Risk Information System (IRIS) Risk Assessments, such as formaldehyde and acrolein. A comparison of the risk assessment derived from a NAM against the existing IRIS risk assessment may provide insights into the performance of the NAM. This could be done for a pre-defined set of test chemicals with related modes of action or AOPs.
    - Another Panel member suggested a more general use for weight-of-evidence concepts such as study quality, systematic review, and the IRIS methodology for assigning confidence.
    - Another Panel member suggested that case studies—including the one outlined here and others, are a way to build confidence in NAMs via a long-term and iterative process, always keeping in mind the context of the method.

- Performance of *in vitro* test methods should be periodically reassessed as new information becomes available to determine whether they continue to provide accurate risk estimates.
- The implications of the results for any *in vitro* test should be framed within an overall "weight of evidence" approach to assess the risk of any chemical exposure.
- For chlorothalonil and other chemicals for which histopathology results are available from *in vivo* exposures, such as metaplasia, it would be desirable for the key lesion from the AOP or MOA be shown to be replicated by the *in vitro* model. Such demonstration would be of limited scope, solely for the intent of adding confidence to the weight of evidence evaluation of fitness-for-use.

Overall, the Panel generally agreed that the proposed strategy is a step in the right direction and that after consideration of the Panel's recommendations, using next generation, appropriate and relevant models, such as this one, will contribute to better hazard and risk assessment, ultimately improving the way we protect workers and the general public.

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