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Case Study on the use of an Integrated Approach for Testing and Assessment (IATA) for New Approach Methodology (NAM) for Refining Inhalation Risk Assessment from Point of Contact Toxicity of the Pesticide, Chlorothalonil.

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT

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Foreword

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Roper CS[1)], Hargrove MM[2)], Sullivan K[3)] and Wolf D[2)] for illustrating practical use of IATA and submitted to the 2021 review cycle of the IATA Case Studies Project.

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The case study was reviewed by the project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022.

The case study is illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, the cases study should not be interpreted as official regulatory decisions made by the authoring member countries.

This document is published under the responsibility of the Chemicals and Biotechnology Committee of the OECD.

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Abbreviations and Acronyms

AOP	Adverse Outcome Pathway
AIC	Akaike Information Criterion
BMD	Benchmark Dose
BMD_{sd}	BMD for One Standard Deviation Change
$BMDL_{sd}$	Lower Bound of the 95% Confidence Interval on the BMD_{sd}
BMI	Body Mass Index
BMR	Benchmark Response
CFD	Computational Fluid Dynamic
CBF	Ciliary beating frequency
DF	Dry flowable
EF_{AD}	Extrapolation Factor for Interspecies Toxicodynamics
EF_{AK}	Extrapolation Factor for Interspecies Toxicokinetics
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA SF	Food Quality Protection Act Safety Factor
GSD	Geometric Standard Deviation
HEC	Human Equivalent Concentration
HED	Human Equivalent Dose
IVIVC	*In Vitro – In Vivo* Correlations
LC_{50}	Median Lethal Concentration
LDH	Lactate Dehydrogenase
LOAEC	Lowest Observed Adverse Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
LOC	Level of Concern
MMAD	Mass Median Aerodynamic Diameter
MOE	Margin of Exposure
MPPD	Multiple Path Particle Deposition
MCC	Mucociliary clearance
NAM	New Approach Methodology
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NoEL	No Effect Level
OECD	Organisation for Economic Co-operation and Development
POD	Point of Departure
PSD	Particle Size Distribution
RfC	Reference Concentration
ROS	Reactive oxygen species
SAP	Scientific Advisory Panel
SC	Suspension Concentrate

SDG	Suspension Dispersion Granule
SDS	Sodium Dodecyl Sulphate
TD	Toxicodynamic
TEER	Trans-epithelial Electrical Resistance
TK	Toxicokinetic
UF_A	Interspecies Factor for Animal-to-Human Extrapolation
UF_H	Intraspecies Factor for Differences in Sensitivity Among Humans
UVCB	Unknown or Variable Composition, Complex Reaction Products or Biological Materials
WDG	Water Dispersible Granule

Executive Summary

Crop protection products require assessment by regulatory authorities for inhalation toxicity based on pattern of use. Determining inhalation hazard and risk has traditionally been based on the use of an *in vivo* study. Chlorothalonil (CAS No. 1897-45-6) containing formulation (Bravo 720 SC) was assessed for respiratory toxicity in a repeated dose inhalation toxicity study in the rat resulting in a dose-dependent- increase in squamous metaplasia of the larynx at all doses. Further animal tests would not provide relevant human toxicity data due to the known higher sensitivity of the rat than the human (primarily due to the complexity of the rat nasal turbinates).

A proof of concept, preliminary experiment using individual donor human MucilAir™, an *in vitro* 3D model for the upper airway (Vinall, 2017). MucilAir™ tissues were exposed to serial dilutions of chlorothalonil in Bravo 720 SC for 24 hours. Toxicity endpoints of trans-epithelial electrical resistance (TEER), lactate dehydrogenase (LDH) release and resazurin reduction were measured from the MucilAir™ tissues. Computational fluid dynamics (CFD; Flack *et al.*, 2018), particle size distribution (PSD; Flack and Hedson, 2018) and operator breathing measurements were performed. From these experiments, a preliminary risk assessment was calculated, and issue paper published (EPA, 2018a) which resulted in a US EPA Science Advisory Panel (SAP) meeting (EPA, 2018b) and subsequent SAP report with recommendations for future work to further refine the model (EPA, 2019).

MucilAir™ tissues were exposed to the same serial dilutions of chlorothalonil in Bravo 720 SC for 8- and 24-hour single exposures and repeat daily exposures for 5 days (Paulo, 2020). The same end points were measured with the same calculations performed as the original study. Similar CFD calculations were performed to calculate human equivalent concentrations (HECs) and these were used to derive human equivalent concentrations (HEC) for operators; applicators or mixers/ loaders. The draft risk assessment for inhalation exposure was then formally performed (EPA, 2021).

The short- and intermediate -term inhalation MOEs ranged from 5 to 660,000, assuming baseline clothing (*i.e.*, no respirator) and were not of concern. The crop with the highest application rate in each crop category (*i.e.*, orchards, high acreage field crops, and typical acreage field crops), was assessed and was representative of the remaining crops at lower application rate. Cranberries represents typical acreage field crops, soybeans represented high acreage field crops, and pistachios represented orchard crops.

These results compellingly verify the applicability of this testing strategy as an IATA for identification of safety respiratory toxicants in operator exposure risk assessment.

1 Introduction

This Case Study was developed to demonstrate how an *in vitro* 3D human respiratory model, particle size distribution (PSD), *in silico* computational fluid dynamics, human respiratory tract structure and *in vivo* human operator exposure measurements can be utilised to replace an *in vivo* repeated dose respiratory toxicology study such as OPPTS 870.3465, 40 CFR Part 798, Organisation for Economic Co-operation and Development (OECD) TG 412 (OECD, 2018a) and OECD TG 413 (OECD, 2018b) to determine respiratory operator exposure risk for a US EPA submission.

The human and rat respiratory tracts are complex with many similarities, but also fundamentally important differences. The rat has a complex system of nasal turbinates through which inspired air, and any gaseous, particulate, or liquid particles, must first pass before reaching the conducting airways and finally the lower airway. The human airway is arguably simpler with inspired air more directly reaching the conducting airway. The rat nasal tissue is considered to be more sensitive than the human to respiratory toxicants due to the relative number of cells and complexity of the turbinates through which the inspired air must first travel.

Chlorothalonil is a broad-spectrum fungicide that acts as a respiratory irritant.

As part of the pesticide re-registration process, EPA historically required registrants and manufacturers to conduct sub-chronic inhalation studies in laboratory animals to evaluate the potential health effects of pesticides in residential or occupational settings. In advance of conducting sub-chronic inhalation studies for re-registration, a two-week aerosol inhalation range-finding toxicity study was conducted in male Sprague Dawley rats with the commercial formulation, Bravo Weather Stik® 720 SC (*aka* Bravo 720 SC), at targeted concentrations ranging from 0.001 to 0.015 mg chlorothalonil/L of air (Bain, 2013). During the two-week study, toxicologically significant observations related to respiration (wheezing, sneezing, irregular respiration, and gasping) were initially observed in two out of 25 animals in the high exposure group but resolved over the second week of treatment. A concentration dependent reduction in feed consumption and body weight gains was also observed, with net weight loss occurring in the highest exposure group. As anticipated from prior acute inhalation studies and longer-term studies by other routes of exposure, the primary pathological findings included concentration-dependent epithelial cell degeneration and necrosis with associated inflammation and inflammatory cell infiltration, hyperplasia and squamous metaplasia in respiratory tissues lining the nasal cavity, larynx, trachea, and lung of male Sprague Dawley rats. All microscopic findings in respiratory tissues were indicative of a contact irritant/ cytotoxicant that showed partial (nose and larynx) or full (trachea and lungs) recovery at the end of a 14-day post-exposure recovery period depending upon the exposure concentration. A No Observed Adverse Effect Level (NOAEL) was not established under these exposure conditions with nasal respiratory epithelium and larynx being particularly susceptible tissues. The value of conducting longer-term inhalation rodent bioassays become questionable for chemicals like chlorothalonil, where the local portal of entry contact cytotoxicity is the most sensitive endpoint that limits exposure concentrations and durations. This is especially applicable for a species that, unlike humans, are obligate nose-breathers with significantly different nasal and laryngeal airway anatomy leading to potentially important differences from humans in target tissue doses.

Additional animal studies to determine a No Effect Level (NoEL) would be unnecessary if a scientifically valid *in vitro* approach could be identified. As part of the Integrated Approach to Testing and Assessment

(IATA) project, this document presents a Case Study to evaluate a New Approach methodology (NAM) for assessment of human operator exposure to the pesticide, chlorothalonil. Such an alternative approach was created based upon an understanding of the mode of action that leads to squamous metaplasia of the larynx. A 3D in vitro model of respiratory epithelium (MucilAir™) was used to define the dose -response including a NoEL of the initial key event. To integrate these new studies for inhalation risk assessments, computational aerosol dosimetry models are needed to relate realistic human exposure scenarios to the dose-response relationships determined *in vitro* and from *in vivo* animal studies. Accurate and relevant risk evaluation based on actual inhalation exposure scenarios and target site-specific respiratory surface concentrations is one such strategy to describe human health risks.

The NAM consisting of separate studies and calculations was, therefore, designed resulting in this Case Study. The exposure data that includes measured aerosol characteristics of non-volatile pesticide formulations provide an improved input to exposure models. Coupling a CFD airflow model with the 3D respiratory test system (MucilAir™) data was used to determine a human equivalent concentration (HEC). The *in vitro* assay was used to define the dose-response and a NoEL of the initial key event. CFD models can be used to describe target site-specific dosimetry for the human and can calculate surface concentrations of deposited aerosol formulations in discrete regions of the respiratory tract. This does need to be coupled to breathing rate, as determined by operators performing different procedures, such as mixing and loading. This approach can provide a more accurate reflection of the deposition necessary to initiate the cascade of events that result in an irritant mediated response in the upper respiratory tract.

1.1 History of the NAM Approach for Chlorothalonil

A NAM was proposed to refine inhalation risk assessment that could be used for direct contact irritants, such as chlorothalonil, using a point of departure (POD) from an *in vitro* test system derived from human airway primary cells. To calculate a human equivalent concentration (HEC) for the purpose of human health risk assessment, the *in vitro* POD is used in conjunction with aerosol deposition results from a CFD model of the upper human respiratory tract, which allows for the integration of relevant PSDs for expected human exposures.

The proposal was initially presented to the US EPA in 2014, who recognized the value of this proposal for chlorothalonil, as well as other contact irritants, and supported the movement to a NAM in lieu of *in vivo* laboratory animal testing. Early in the process, the US EPA reached out to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to collaborate on the review of the proposed approach. The EPA encouraged further development and determined that external peer review and public dialogue would be needed prior to applying the proposed approach to human health risk assessments for contact irritants, such as chlorothalonil. A preliminary risk assessment and "issue" paper was then published by EPA (EPA, 2018a). In December 2018, the proposed approach was presented to a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) using chlorothalonil as a proof of concept (EPA, 2018b). Advice was solicited from the SAP on the methods used to derive the POD from the *in vitro* assay and integration of the *in vitro* POD for calculation of HECs for inhalation risk assessment. Additionally, the SAP was asked to review how the CFD model was applied for the approach. The SAP final report (EPA, 2019) supported use of the approach for contact irritants and provided recommendations to improve and/ or further support the approach. In particular, the SAP recommended collecting additional information on the impact of repeat dosing on the *in vitro* measurements, consideration of clearance, and differences in nasal and oral breathing deposition. Further data was then generated and used for a formal draft risk assessment (EPA, 2021).

1.2 Traditional Approach to Generating Data for Inhalation Risk Assessment

Determining inhalation hazard and risk has traditionally been based on the use of an *in vivo* study to determine a safe HEC of the chemical in the air. For evaluating effects *via* the inhalation route, registrants and manufacturers conduct sub-chronic inhalation toxicity studies according to test guideline requirements (OPPTS 870.3465, 40 CFR Part 798, OECD TG 412, and OECD TG 413). In these studies, several groups of experimental animals (rat is the preferred species) are exposed daily for a defined period to graduated concentrations of test substance (one concentration per group) as a gas, volatile substance, or aerosol/ particulate. During the period of administration, the animals are observed daily to detect clinical signs of toxicity. At the end of the study, animals are euthanized, necropsied, and appropriate histological examinations carried out. These studies are used to determine the lowest concentration where adverse effects are observed following repeated inhalation exposure, which is referred to as the lowest observed adverse effect concentration (LOAEC). The highest concentration tested at which no adverse effects were observed would be used to establish a no observed adverse effect concentration (NOAEC) for the study.

When selecting endpoints for human health risk assessment, toxicological data is reviewed to identify toxicity endpoints (effects observed in toxicity studies that are considered treatment related/ adverse), as well as the dose levels needed to elicit these effects following chemical exposure. These dose levels are then used to identify a POD. The POD is typically a dose where no adverse effects have been observed and is used as a quantitative starting point for risk assessment for the route (in this Case Study, it is inhalation) and duration (single day to chronic) of exposure under evaluation.

If a route-specific inhalation study has been selected to evaluate inhalation exposures from a chemical, exposure concentrations in the animal study are converted to HECs. This conversion allows for exposure duration adjustments (daily and weekly) to account for differences between the animal toxicity study and expected human exposures. The conversion also allows for application of a dosimetry adjustment factor that accounts for the physical nature of the inhaled material (*i.e.*, gas, volatile substance, or aerosol/ particulate), and species differences in ventilation rate and respiratory tract architecture that contribute to the pharmacokinetic differences between the test species and humans.

To provide appropriate safety margins for assessing human health risks, uncertainty factors (UF) are applied. Typically, this includes a 10X Interspecies Factor for Animal-to-Human Extrapolation (UF_A) and a 10X Intraspecies Factor for Differences in Sensitivity Among Humans (UF_H). If the reference concentration (RfC) methodology has been applied, the interspecies extrapolation factor may be reduced from 10X to 3X due to the calculation of HECs that account for pharmacokinetic (not pharmacodynamic) interspecies differences. Additional factors may also be applied to account for deficiencies or uncertainties in the toxicology database (*e.g.*, extrapolation from a LOAEL to a NOAEL, uncertainty from a data gap or extrapolation to longer durations).

2 Purpose

2.1 Purpose of Use

The purpose of use is to generate safety data for a human risk assessment for the pesticide, chlorothalonil, knowing that the traditional *in vivo* approach was not appropriate.

This Case Study was developed to demonstrate how an *in vitro* 3D human respiratory model, *in silico* computational fluid dynamics, and *in vivo* human operator exposure measurements can be utilised to replace an *in vivo* repeated dose respiratory toxicology study, such as OPPTS 870.3465, 40 CFR Part 798, Organisation for Economic Co-operation and Development (OECD) TG 412 (OECD, 2018a) and OECD TG 413 (OECD, 2018b) to determine respiratory operator exposure risk. The data was submitted to the US EPA who then performed a risk assessment (EPA, 2021) utilizing the *in vitro* and *in silico* data and the draft risk assessment, is summarised also in this Case Study.

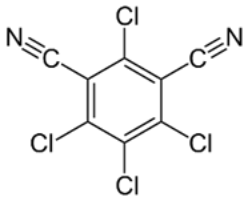
This Case Study utilises procedures and processes that were discussed with the US EPA in order to fulfil their needs for a NAM for use in a risk assessment. Therefore, there may be differences in the requirements and interpretations from other regulatory agencies. It is recommended that discussions with agencies should take place to ensure that their requirements are fulfilled. Examples include, but are not limited to, BMD analysis calculations, GLP compliance, bioanalysis and choice of endpoints. To help agencies, registrants and researchers, the Considerations for Using Respiratory Toxicology NAMs Considerations for Using Respiratory Toxicology NAMs has been created and is included at the end of this Case Study.

This Case Study uses a combination of a human *in vitro* 3D model for the upper airway tract, MucilAir™, a CFD model for the human upper airway tract, standard US EPA calculations for benchmark dose levels (BMDL) and human operator breathing measurements to identify a safe operator exposure level to the pesticide, chlorothalonil, following field application. This NAM replaces the traditional approach of using animals to calculate the HEC, but more importantly, provides a human relevant HEC which is used in the risk assessment for operators safely using chlorothalonil containing formulations and sprays.

2.2 Target Chemical

The pesticide, chlorothalonil (2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile, CAS No. 1897-45-6) is a broad-spectrum, non-systemic protectant pesticide mainly used as a fungicide to control fungal foliar diseases of vegetable, field, and ornamental crops. It is also used as a wood protectant, anti-mould and anti-mildew agent, bactericide, microbiocide, algaecide, insecticide, and acaricide. Residential/ non-agricultural uses include use on golf courses, on home gardens, as a wood preservative, and in paint formulations. The chemical structure of chlorothalonil is presented in Figure 1.

Figure 1. Chemical Structure of Chlorothalonil.



A chlorothalonil containing formulation (Bravo 720 SC) was assessed for respiratory toxicity in a repeated dose inhalation toxicity study in rats. This study showed a dose dependent increase in squamous metaplasia of the larynx at all doses tested. Additional animal studies to determine a NoEL would be unnecessary if a scientifically valid *in vitro* approach could be identified. Such an alternative approach was created based upon an understanding of the mode of action that leads to squamous metaplasia of the larynx. Chlorothalonil is a contact irritant that has been found to be toxic *via* the inhalation route. It is classified as Toxicity Category I for acute inhalation (median lethal concentration (LC_{50}) ≤0.05 mg/L). Non-lethal effects observed in acute inhalation studies included clinical signs indicative of respiratory tract effects, such as nasal discharge, difficulty breathing, decreased activity/ lethargy, respiratory rales, ptosis, and piloerection. Consistent with its effects as a respiratory irritant, chlorothalonil also causes severe eye irritation (Toxicity Category I) in acute studies. A 90-day inhalation toxicity study was not feasible due to the irritant nature of chlorothalonil and animal welfare concerns.

2.3 Endpoints

The MucilAir™ assay measures a variety of membrane and cell damage endpoints. Trans-epithelial electrical resistance (TEER) measures the integrity of tight junctions between cells in the membrane. Lactate dehydrogenase (LDH) release serves as an indicator of membrane damage as a marker of cytotoxicity. Resazurin reduction is a measure of metabolic activity of the MucilAir™ tissues. Together, these endpoints can be used to calculate a BMDL which is then aligned with measurements of human operator exposure and CFD representation of the human airway tract to calculate the final endpoint of a POD for risk assessment.

2.4 Exposure Information

When preparing to spray and spraying pesticides, the primary routes of exposure are dermal and inhalation. The dermal route is calculated from skin absorption data (OECD 428, 2004) and from skin irritation (OECD 404 (2015) and OECD 439 (2020a), skin corrosion (OECD 431, 2019a) and skin sensitization (OECD 406 (1992), OECD 429 (2010), OECD 442C (2020b), OECD 442D (2018c) and OECD 442E (2018d)) testing. This Case Study identifies the NAM to consider operators exposed to the pesticide *via* the inhalation route. This Case Study has a direct *in vitro – in vivo* correlation since human cell toxicity data is converted to exposure to the conducting airway utilizing CFD and breathing information measured directly from operators performing the spraying. Since the test is performed in human tissues, there is no interspecies extrapolation required. The 3D tissue, Epithelix MucilAir™, model test is similar to the 3D regulatory tests for skin irritation (OECD 439) and skin corrosion (OECD 431) using MatTek EpiDerm™ or SkinEthic™ RHE and ocular irritation (OECD 492, 2019b) using MatTek EpiOcular™ or SkinEthic™ HCE or similar models.

3 Hypothesis for Performing IATA

The anatomy and physiology of human and rodent respiratory tracts differ in several ways that can impact changes in airflow and deposition of inhaled substances and, therefore, influence the animal to human dose response extrapolation. For example, airway size (length and diameter), cell types and distribution, and composition of secretory products vary across species (Clippinger *et al*., 2018a, Lippman and Schlesinger, 1984, EPA, 1994). Additionally, branching patterns differ across species. Human airways have a more symmetrical dichotomous pattern than rodents. The more symmetrical dichotomous pattern is prone to deposition at branching points leading to higher concentrations at these points compared to rodents (Clippinger *et al*., 2018a; Lippman and Schlesinger, 1984).

The structures that provide an initial barrier to inhaled air and particles are the nasal cavity and larynx, which have notable differences between rats and humans. The nasal cavity consists of nasal turbinates, where particles deposit primarily through inertial impaction. Humans have three turbinates that are relatively simple in shape, while the architecture of the nasal turbinate systems in rats is more convoluted than humans with complex folding and branching patterns (Harkema *et al.*, 2006). In conjunction with the obligate nasal breathing of rodents, this results in greater deposition in rats as compared to humans.

There is also significant interspecies variability in overall surface area and cellular composition or distribution of the nasal surface epithelium. On average, the surface area of the human extra-thoracic, tracheobronchial, and pulmonary regions are 200 cm^2, 3200 cm^2, and 54 m^2, respectively. In contrast, the average surface area in the rat in those regions are 15 cm^2, 22.5 cm^2, and 0.34 m^2, respectively (EPA, 1994). In most animal species, there are four types of nasal epithelium:

(i) squamous epithelium
(ii) non-ciliated cuboid or columnar transitional epithelium
(iii) ciliated pseudostratified cuboid or columnar respiratory epithelium and
(iv) olfactory epithelium.

However, the distribution of these epithelial populations and nasal cell types within these populations differ across species (Harkema *et al.*, 2006). Furthermore, rats have a higher percentage coverage of the nasal cavity in olfactory epithelium that leads to a more heightened sense of smell as compared to humans.

In addition, there is an anatomical difference between rats and humans in the larynx. The larynx is involved in sound production and protects against food aspiration. In rats, cartilage associated with the ventral pouch is U-shaped and the larynx and trachea in rats form a relatively straight line from the nasal turbinates, which enhances the deposition of aerosols in the rat larynx (Kaufmann *et al.*, 2009). As a result, the larynx can be a common site of injury in laboratory inhalation toxicity studies with rats. In contrast, in humans the U-shaped pouch is absent, and the larynx is more sharply angled to the oro-nasal cavity (Kaufmann *et al.*, 2009). As a result, when considering risk assessment for humans, determining the relevance of laryngeal lesions seen in rat *in vivo* studies is complicated by these anatomical differences. Due to critical differences between rat and human respiratory tracts, the ability of *in vivo* testing to correctly predict effects in humans can be affected. As a result, NAMs that take into consideration the differences will serve as a refinement for human health risk assessment.

There are several *in vitro* tools available to evaluate inhalation toxicity that have been summarized in detail by Clippinger *et al.* (2018a) and Singh *et al.* (2021). These include lung-on-a-chip models, *ex vivo* lung slices, *in vitro* cell cultures and 3-dimensional (3D) models. 3D models cultured from airway epithelial cells at the air-liquid interface can mimic the different regions of the human respiratory tract, including barrier function, mucous production, and cilia function. 3D models have been used successfully to study infection and toxicity in the respiratory system (Mathis *et al.,* 2013; Neilson *et al.,* 2015; Essaidi-Laziosi *et al.,* 2017 and Welch *et al.*, 2021) and are the focus of this current Case Study. As stated earlier, 3D models are also extensively used in hazard and risk assessment of chemicals using OECD test guideline model tests. Singh *et al.* (2021) reviewed the current available technologies and included the engineering and computational models alongside the biological available models as well as identifying applications for these methods.

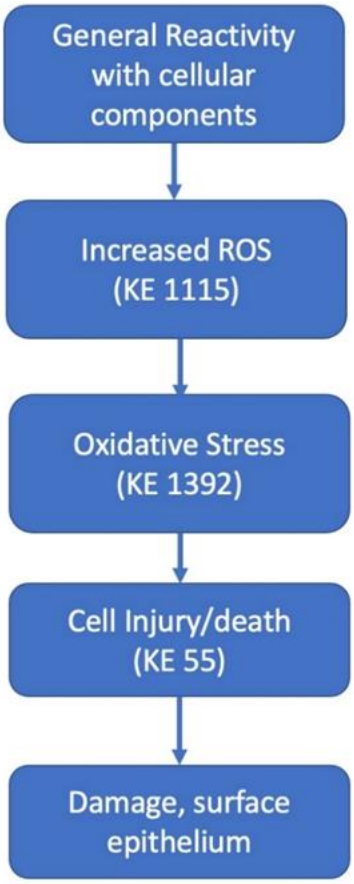
An understanding of *in vitro* and *in vivo* dosimetry is essential when using any of the *in vitro* systems. Although NAMs are often validated by comparing to *in vivo* tests, due to the inherent differences between animals and humans, this comparison is challenging. Therefore, human relevant exposure information was integrated into the evaluation of the *in vitro* results for this Case Study.

4 Approaches Used

4.1 Biology/ Adverse Outcome Pathway

A biological understanding of the irritation resulting from chlorothalonil exposure has been developed. This includes an Adverse Outcome Pathway (AOP) where epithelial cell damage occurs from initial inhalation exposure to chlorothalonil and causes cell death (Figure 2). Following repeated exposure, the cell death results in a metaplastic response and transformation of respiratory epithelium into stratified squamous epithelium. Based on the AOP, all subsequent response is due to repeated acute toxicity. This AOP is defined in https://aopwiki.org/events/1115.

Figure 2. Partial Adverse Outcome Pathway Illustrating Exposure to Chlorothalonil Leading to Cell Death and Damage to the Surface Epithelium (adapted from Hargrove et al., 2021)



Due to its four electrophilic chlorine atoms (Figure 1), chlorothalonil is highly reactive towards intracellular thiols, such as glutathione, that are important antioxidant components. With exposure to high enough amounts of chlorothalonil, the antioxidant system is overwhelmed leading to cell damage and subsequent cell death. Therefore, available i*n vitro* models were considered for assessing damage to respiratory epithelial cells and identified MucilAir™ as the optimal model for the proposed approach as these are nasal derived human 3D models. Chlorothalonil is needed at the cell surface in this pathway. Therefore, the *in vitro* system is mimicking the *in vivo* exposure of the initial interaction of chlorothalonil with respiratory cells. Furthermore, by protecting for the initial cell damage caused by chlorothalonil exposure, effects that would be caused from repeated exposure would also be prevented.

This Case Study identifies a NAM for using a POD derived from an *in vitro* assay (MucilAir™). To calculate HECs for the purposes of human health risk assessment, the *in vitro* POD was used in conjunction with surface concentrations of deposited chlorothalonil particles derived from a CFD model. As a proof of concept, the calculated HECs were used to provide potential risk estimates for chlorothalonil.

A source to outcome approach has been utilised as a framework for integrating human exposure and hazard characterization for a refined inhalation risk assessment. This approach is comprised of 4 components: source, exposure, dosimetry, and outcome. These are used to refine the inhalation risk assessment for chlorothalonil.

4.2 Test System: MucilAir™

The Epithelix MucilAir™ test system is similar in structure and function to the tracheobronchial epithelium, exhibiting a pseudostratified, ciliated epithelium which secretes mucus. Therefore, this accurately reflects the structure of the ciliated pseudostratified respiratory epithelium found in the respiratory tract of all mammals. MucilAir™ has been shown to replicate the physiological and barrier functions of the airway epithelial cells including mucociliary clearance, making it an appropriate model for *in vitro* assessment of human respiratory irritation represented by direct cytotoxicity. The ability for the MucilAir™ assay to metabolise chemicals is described by Cevena *et al.* (2019) and further confirmed by toxicogenomic analysis by Baxter *et al.* (2015), Haswell *et al.* (2018) and Cervena *et al.* (2019). Cytokine release has also been observed by Metz *et al.* (2018) and Welch *et al.* (2021).

MucilAir™ is a 3D *in vitro* test system derived from nasal, tracheal, or bronchial tissues of healthy donors cultured at the air-liquid interface. Human nasal-derived MucilAir™ was supplied by Epithelix Sàrl, 14 Chemin des Aulx, CH-1228 Plan-Les-Ouates, Geneva, Switzerland. The human nasal tissue model was the only model available from Epithelix at the time of the planning and conduct of the initial study; serendipitously, this was the model that would be chosen now as it is nasal derived tissue which contact toxicity primarily occurs. The MucilAir™ assay was chosen as the test system for this study as this is an *in vitro* model for the respiratory airways. Certificates of analysis including donor information (*e.g.*, age, sex, smoker), cell information (*e.g.*, cell type, date of seeding), and quality control results (*e.g.*, sterility, tissue integrity, *etc.*) were provided for each donor.

4.3 Biomarkers of Toxicity/ Irritation

Cell viability can be determined using numerous parameters, but it is typically defined by the integrity of the outer cell membrane. If the cell membrane is damaged, substances that are typically prohibited from traversing the cell membrane can cross it. As a result, measurements may evaluate membrane integrity directly or by using dyes that indicate substances have moved across the membrane due to cell damage. Therefore, cell damage is evaluated using measurements of TEER, resazurin metabolism, and LDH release. As such, these measurements are being used to determine if cell damage and/ or death has

occurred from the initial respiratory exposure to chlorothalonil described in the AOP above. TEER measures the integrity of tight junctions between cells in the membrane, LDH release serves as an indicator of membrane damage as a marker of cytotoxicity, and resazurin reduction as a measure of metabolic activity of MucilAir™ tissues.

Since TEER is used to measure the integrity of tight junctions between cells in the membrane, decreases in this measurement would indicate loss of barrier integrity, but does not necessarily mean that cell death has occurred. Resazurin is a non-fluorescent dye that can be reduced by viable cells with active metabolism resulting in a fluorescent chemical, resorufin. As a result, the measured fluorescence is proportional to the number of viable cells and reduced fluorescence indicates low cell viability. LDH is an enzyme released when cells suffer sufficient membrane damage indicative of cytotoxicity that leads to cell death. The released LDH can convert resazurin into its fluorescent metabolite, resorufin. Therefore, similarly to resazurin metabolism, the measured fluorescence is proportional to the number of viable cells; however, in this case, conversely, an increase in fluorescence indicates low cell viability. Evaluation of these *in vitro* endpoints using MucilAir™ has been shown to predict *in vivo* respiratory toxicity (Sivars *et al.*, 2018), where TEER and resazurin measurements resulted in 88% sensitivity and 100% specificity.

5 Preliminary Test

5.1 Preliminary Test: MucilAir™ 24 hour Exposure (Vinall, 2017)

An initial, proof of concept study was performed (Vinall, 2017). MucilAir™ tissues were exposed to dilutions of chlorothalonil for 24 hours. TEER and LDH release were measured at 0 hour (predose) and 24 hours. Resazurin metabolism was measured at 24 hours only. Six MucilAir™ replicates (tissue units) were exposed to each dose level or control. The Bravo 720 SC formulation was diluted in saline giving ten chlorothalonil concentrations ranging from 1.995 to 199.5 mg/L (6 replicates/ dose/ donor). Controls (6 replicates per control) were included to demonstrate appropriate performance of the assay. Sodium dodecyl sulphate (SDS; 4 mM) was used as the positive control (Welch *et al*, 2021). Physiological saline (sodium chloride; 0.9%, w/v) was used as the negative control (Welch *et al*, 2021). Controls for measurement of the maximum LDH release (LDH_{max}) from MucilAir™ cells were also included. These comprised cells treated with Promega Lysis Solution for *ca* 2 hour (one set of controls prepared each day of testing).

MucilAir™ media was pre-warmed prior to use. Receipt, cell husbandry, and experimental use of MucilAir™ tissues was in accordance with the manufacturer's supplied protocols and previous uses of this model at the performing Test Facility, Charles River, Edinburgh, UK. On delivery, MucilAir™ units were transferred to 24-well plates containing prewarmed MucilAir™ medium (700 µL). The condition of MucilAir™ tissues was then determined by viewing representative tissues (n = 4 per plate) by microscopy to verify cellular form and correct cilia function. Tissues were maintained in a humidified incubator set to maintain a temperature of 37°C with a 5% CO_2 environment for *ca* 1 week prior to use. Media was replaced at 3 day intervals during this preincubation.

TEER across MucilAir™ tissues was measured in accordance with the manufacturer's supplied protocols. MucilAir™ units were transferred to 24-well plates containing physiological saline (700 µL/well). A further aliquot of physiological saline (220 µL/well) was added to each apical chamber. TEER was then measured using a Millicell ERS-2 meter prior to dosing (0 hour) and at 24 hour post-dose. Background resistance for the support membranes was accounted for by subtracting a nominal value (100 Ω) from each reading, as recommended by the manufacturer.

These measurements are being used to determine if cell damage and/ or death has occurred from the initial respiratory exposure to chlorothalonil described in the AOP above. Tissues were exposed for 24 hours. Cell viability (irritation) was assessed by TEER, LDH release and resazurin metabolism.

Culture media was collected from the basal chamber for analysis in the LDH release assay prior to dosing (0 hour) and at 24 hour post dose. The media was replaced *ca* 24 hour prior to the pre-dose (0 hour) collection to ensure that the measurement made before and after dosing corresponded to equivalent release time periods. Following transfer of MucilAir™ units to physiological saline, duplicate aliquots of the media remaining in wells (100 µL) were transferred to 96-well plates and analysed for LDH content.

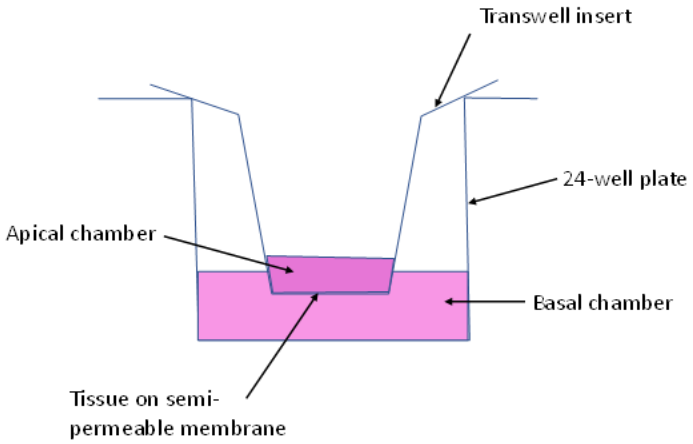
Using a pipette, the dosing solutions (30 µL) were applied directly onto the centre of the apical surface of MucilAir™ tissues, taking care not to scratch the surface with the pipette tip. The units were gently tapped to ensure that the dosing solutions or controls were dispersed across the apical surface. Six replicates

were treated with each dose level or positive (SDS; 4 M) or negative (Physiological saline) control. MucilAir™ units were exposed to dosing solutions for 24 hours in a humidified incubator set to maintain a temperature of 37°C in a 5% CO_2 atmosphere. At the end of the exposure period, the apical surface of tissues was rinsed three times with saline (*ca* 1.5 mL in total) to remove residual dosing solution. After incubation, the media samples were collected for LDH analysis.

The release of LDH from cells was measured using the CytoTox ONE Homogeneous Membrane Integrity Assay (Promega), following the manufacturer's protocol. Cytotoxicity was assessed at 0 hour (predose) and following the 24 hour exposure to the dosing solutions. Substrate Mix was reconstituted according to the manufacturer's instructions. The collected culture supernatants were cooled to ambient temperature (*ca* 20 min) before adding Substrate Mix (100 µL) to each well. The reaction proceeded at ambient temperature for 10 min and was then ended by addition of Stop Solution (50 µL). Plates were covered with tin foil to protect from light during incubation. Fluorescence of wells was measured at an emission wavelength of 590 nm with excitation at 544 nm ($544_{ex}/590_{em}$) within 2 hours of stopping the reaction. An additional set of control wells for background fluorescence, containing only culture media, was also prepared. The LDH_{max} release value for healthy cells was assayed in MucilAir™ tissues disrupted with Lysis Solution (2 hour treatment), as per manufacturer's instructions (this assumed that sufficient Lysis Solution was available in the wells to kill all cells). These values were used to calculate the relative release in other tissues.

The metabolic competence of cells was assessed by measuring the ability of MucilAir™ to reduce resazurin to resorufin. This was assessed following the manufacturer's supplied protocol. Following TEER measurement at the 24-hour timepoint, apical physiological saline was removed and MucilAir™ units were transferred to 24-well plates containing resazurin solution (6 µM in saline; 500 µL/well). A further aliquot of resazurin solution was applied to the apical surface of each unit (220 µL/well). Plates were then incubated for 1 hour ± 5 min in a humidified incubator set to maintain a temperature of 37°C in a 5% CO_2 atmosphere. After incubation, duplicate samples (100 µL) were collected from the apical chamber for analysis. Collected samples were transferred to 96-well plates and resorufin measured by fluorescence emission at an emission wavelength of 590 nm with excitation at 544 nm ($544_{ex}/590_{em}$). Additional control wells for the background absorbance of the assay plates were included with each experimental batch. These wells contained aliquots of unreacted resazurin solution (100 µL; 6 µM in saline). All samples were analysed fresh and discarded following analysis. A schematic representation of the experimental procedure for the resazurin reduction assay is provided in Figure 3.

Figure 3. Schematic Representation of the Experimental Procedure for the Resazurin Reduction Assay (Hargrove et al., 2021).



TEER measurements were performed using a Millicell® ERS-2 meter. Fluorescence data from the LDH release assay and resazurin reduction assay were collected using a Thermo Scientific Fluoroskan Ascent microplate fluorimeter, with assay setup and data capture by Thermo Scientific Ascent Software. Assay data was transferred to Microsoft Excel 2007 for further analysis. The background resistance of the Transwell® support membranes was accounted for by subtracting a nominal value (100 Ω) from each reading, as recommended by the manufacturer.

Generation of reaction products from the LDH assay was measured by fluorimetry with excitation at 544 nm and emission at 590 nm. Measured fluorescence values were corrected for the fluorescence of cell culture media by subtracting the mean emission of background (*i.e.*, MucilAir™ assay medium only) controls included in each assay. Release of LDH from treated tissues was expressed as a percentage of the maximum LDH content of healthy cells, measured in undosed cells disrupted with Lysis Solution.

Reduction of resazurin to resorufin by viable cells was measured by fluorimetry with excitation at 544 nm and emission at 590 nm. Readings were corrected by subtracting the mean value of background controls (unreacted resazurin solution). MucilAir™ metabolic activity in treated cells was expressed relative to vehicle control (saline) treated tissues after correcting for background.

Cell morphology and health was verified on arrival and periodically during the tissue maintenance period by light microscopy. Cell monolayers appeared to be intact with the effects of cilia beating being clearly visible.

Tissues from all donors responded in a similar manner to negative control treatments. In all negative control tissues, a reduction in TEER was observed following 24 hour incubation. This was attributed to the removal of mucus from the surface of the cells during rinsing steps and stress to tissues due to experimental manipulations. Pre-dose (0 hour) and post-dose (24 hour) LDH release was similar for all donors and are summarised in Table 1.

Table 1. Negative Control Treatments for TEER and LDH Release

	Mean TEER (Ω)		Mean TEER (Ω x cm^2)		Mean LDH Release (%)	
Donor	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour
1	1919	1538	637	510	0.86	0
2	1387	645	460	214	2.05	3.52
3	2371	1300	787	431	0.95	1.05
4	2187	1391	726	462	0.45	1.11
5	1957	1219	649	404	0.89	0.45

Tissues from all donors responded in a similar manner to positive control treatments. In all positive control tissues, a considerable reduction (*ca* 95%) in TEER was observed following 24 hour incubation. Pre-dose (0 hour) LDH release was low (<4%) for all donors and increased considerably (*ca* 200%) for all donors after 24 hour treatment. After the positive control treatment (24 hour) resazurin metabolism was reduced to 0-14% relative to negative control tissues. TEER readings, LDH percentage, from pre-dose (0 hour) and 24 hour post-dose, and resazurin metabolism release at 24 hour post-dose are summarised in Table 2.

Table 2. Positive Control Treatments for TEER, LDH Release and Resazurin

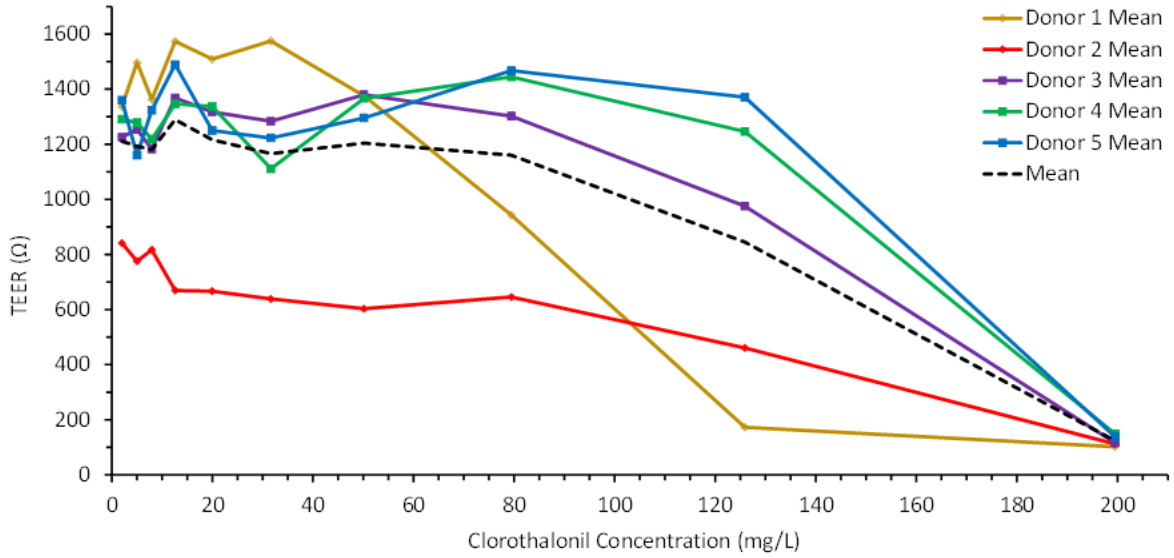
	Mean TEER (Ω)		Mean TEER (Ω x cm^2)		Mean LDH Release (%)		Resazurin (%)
Donor	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour	24 hour
1	1866	109	619	36	2.66	180	0.05
2	1982	98	658	33	2.09	269	0.19
3	2449	117	813	39	1.53	227	14.2
4	2125	98	705	33	1.53	187	7.08
5	2251	84	747	28	3.41	210	4.47

Prior to dosing with Bravo 720 SC, the mean TEER reading for the individual donors varied from the pooled donor mean by *ca* 250 Ω. The TEER response to Bravo 720 SC (chlorothalonil) dilutions varied by donor. Mean TEER readings, from pre-dose (0 hour) and 24 hour post-dose, for all donors are summarised in Table 3 and Figure 4.

Table 3. Mean TEER Reading Pre Dose and at 24 hour Post Dose for Donors over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	Mean TEER Reading (Ω)					TEER (Ω x cm^2)	
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Mean	SD
Predose	1951	1769	2270	1999	2116	671	62
1.995	1337	842	1226	1291	1361	402	71
5.012	1497	776	1254	1279	1160	396	88
7.913	1363	817	1182	1218	1324	392	72
12.59	1574	670	1369	134	1490	348	207
19.95	1510	667	1317	1338	1250	404	107
31.62	1575	639	1284	1111	1223	387	113
50.12	1376	603	1380	1367	1296	400	112
79.43	943	646	1303	1445	1467	385	118
125.9	173	461	976	1246	1371	281	170
199.5	102	112	116	149	138	41	6

Figure 4. Effect of Bravo 720 SC Treatment (24 hour) on TEER Across MucilAir™

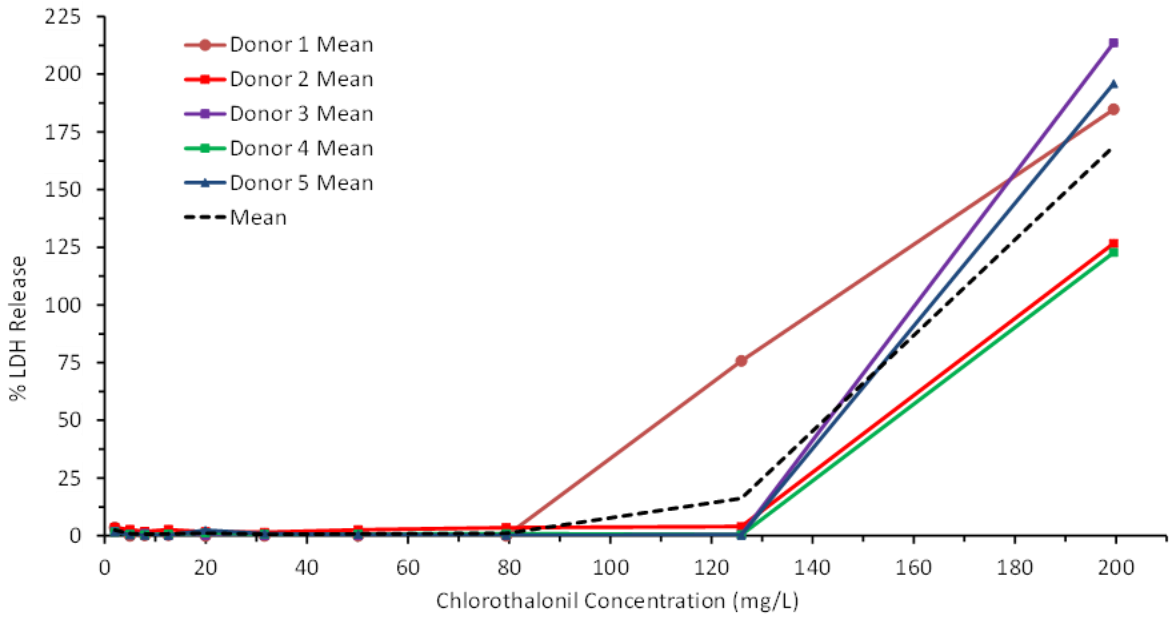


Prior to dosing, the mean percentage LDH release for the donors varied from 0.71% to 2.40% of the appropriate LDH_{Max} controls. The percentage LDH release response to 24 hour treatment with Bravo 720 SC dilution varied by donor, with Donor 3 showing the greatest overall percentage LDH release. The mean percentage LDH release results, from pre dose (0 hour) and 24 hour post-dose, for all donors are summarised in Table 4 and Figure 5.

Table 4. Mean LDH Release Pre-Dose and at 24 hour Post-Dose for Donors over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	LDH Release (%)				
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
Predose	0.71	1.26	1.38	0.82	2.40
1.995	3.57	3.56	1.90	1.63	1.74
5.012	0.00	2.67	0.81	0.62	0.38
7.913	0.23	1.90	1.04	0.43	0.22
12.59	0.31	2.67	0.37	0.67	0.26
19.95	0.00	1.74	0.32	1.19	2.42
31.62	0.00	1.52	0.15	0.74	0.90
50.12	0.00	2.62	0.33	0.71	0.62
79.43	0.00	3.53	0.47	0.97	0.22
125.9	75.76	4.01	0.56	0.44	0.31
199.5	184.78	126.73	213.58	122.73	195.96

Figure 5. Effect of Bravo 720 SC Treatment (24 hour) on LDH Release from MucilAir™

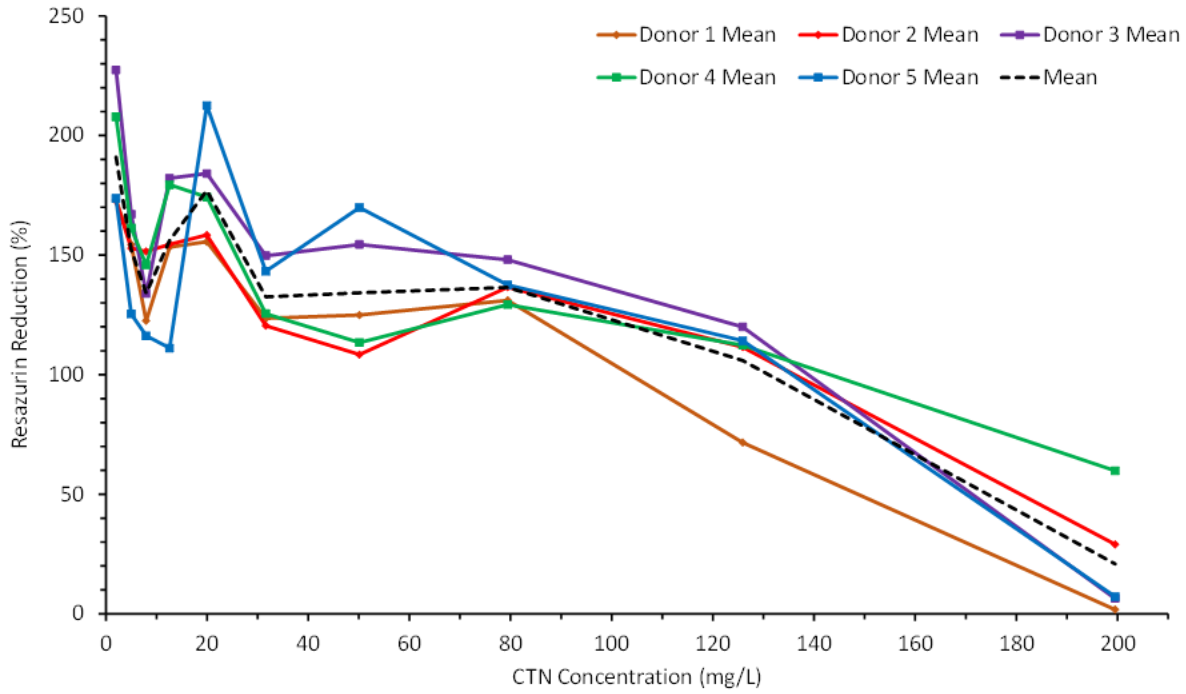


The resazurin metabolism response to 24 hour treatment with Bravo 720 SC dilutions, as a percentage of the negative control treatment, followed a similar pattern for all donors. The percentage of resazurin metabolism decreased in a concentration-specific manner, with the most pronounced change in percentage resazurin metabolism observed in the two highest concentrations (125 and 199 mg/L). The mean percentage resazurin metabolism release results at 24 hour post-dose, for all donors, are summarised in Table 5 and Figure 6.

Table 5. Mean Resazurin Release at 24 hour Post-Dose for Donors over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	% Resazurin Metabolism				
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
1.995	173	173	227	208	174
5.012	154	153	167	161	125
7.913	123	152	134	146	116
12.59	154	155	182	179	111
19.95	156	158	184	174	213
31.62	124	121	150	126	143
50.12	125	108	154	113	170
79.43	131	137	148	129	138
125.9	71.6	112	120	112	114
199.5	1.77	29.0	6.43	59.8	7.19

Figure 6. Effect of Bravo 720 SC Treatment (24 hour) on Rezazurin Metabolism by MucilAir™



All donors showed a dose related decrease in TEER readings and resazurin metabolism following challenge with Bravo 720 SC dilutions, with more pronounced responses observed at higher chlorothalonil concentrations. All donors showed a dose related increase in LDH release following challenge with Bravo 720 SC dilutions, with more pronounced responses observed at higher chlorothalonil concentrations. Observations of decreased TEER and resazurin metabolism in conjunction with increased LDH release are consistent with tissue level irritation. The response of MucilAir™ tissues to 24-hour treatment with positive control, negative control and chlorothalonil treatments differed slightly between donors, although the same general patterns were followed by all groups. Differences observed between donors were attributed to inter-individual variability in response to chlorothalonil challenge.

5.2 Preliminary Test: Benchmark Dose (BMD) Modelling of Vinall (2017) MucilAir™ Data (Lei *et al.*, 2018)

For each endpoint, BMD modelling was used to determine a BMD corresponding to a change in the mean response equal to one standard deviation (SD) change from the control mean (BMD_{sd}) and the lower bound of the 95% confidence interval on the BMD_{sd} ($BMDL_{sd}$). Use of the BMD_{sd} is consistent with the EPA's Benchmark Dose Technical Guidance (EPA, 2012). Benchmark response (BMR) selections are made on a case-by-case basis and take into account statistical and biological information. In the absence of information to determine the level of response to consider adverse, a change equal to one standard deviation from the control mean is used. BMD values were converted from formulation concentration to a tissue concentration using the internal diameter of the MucilAir™ well inserts (33.18 mm^2), as described in Equation 1.

$$BMDL_{sd}\ (mg/cm^2) = BMDL_{sd}\ (mg/L) \times \frac{30\ \mu L \times 1 \times 10^{-6}\ L/\mu L}{31.88\ mm^2 \times 0.01\ cm^2/mm^2} \qquad \text{Equation 1}$$

Doses were log transformed and fit with a modified Hill model. The EPA performed its own BMD analyses on the untransformed data for comparison and found the Hill model to best fit the data. Both models (Hill for untransformed and modified Hill for transformed) were found to fit the data well visually. Akaike information criterion (AIC) values indicate the relative fit of a model for a dataset (*i.e.*, a lower AIC value indicates that a model fits the data better than a model with a higher AIC). Overall, the untransformed data provided similar or lower AIC values than the transformed data; however, the BMD and BMDL values obtained using the transformed data were lower and, therefore, considered protective. For this study, similar BMD results were obtained for all three endpoints (Table 6).

Table 6. Chlorothalonil BMDL Values Calculated from Vinall (2017) MucilAir™ Data

	BMDL			
Donor	Dilute Formulation (mg/L)			Tissue Concentration (µg/cm^2)
	TEER	LDH Release	Resazurin	Geometric Mean
1	51.2	67.6	66.7	5.55
2	53.1	92.8	80.5	6.64
3	88.2	90.2	91.9	8.14
4	110	91.0	42.5	6.80
5	124	102	113	10.2
Geometric Mean	80.1	87.9	75.0	7.30

5.3 Preliminary Test: Computational Fluid Dynamics-based Aerosol Dosimetry Modelling (Corley *et al.*, 2018)

A quantitative assessment of site-specific aerosol deposition patterns and local surface doses of chlorothalonil within regions of the conducting airways of the human using anatomically and physiologically correct, 3D CFD airflow and Lagrangian aerosol transport models (also known as computational fluid-particle dynamics or CFPD models) was performed. Simulations were conducted under previously reported inhalation bioassay conditions across a broad range of aerosol sizes that encompass the sizes expected for occupational and residential exposure for humans. The resulting computational models provide the necessary common denominator of localized tissue doses critical to integrating data and defining appropriate comparative dose-metrics within a source-to-outcome risk assessment approach.

Two human models, one for nasal and one for oral breathing, were derived from multislice CT imaging of a 35-year-old healthy male volunteer, weighing 68 kg and 67 inches tall (Corley *et al.*, 2021).

CFD airflow simulations were performed using STAR-CCM+. The standard Lagrangian particle tracking algorithm in STAR-CCM+ was utilized in the CFPD model with the following assumptions:

1. One-way coupling of airflow with aerosol transport (*e.g.*, aerosol droplets do not affect airflows.
2. No aerosol agglomeration, hygroscopic growth, or electrostatic interactions.
3. Aerosol droplet diameter was assumed to be constant for each simulation.
4. Aerosol density was based upon water (1 g/cm^3), the diluent used in inhalation studies and application methods.
5. All aerosols were introduced in specified X-Y coordinates within the nasal or oral inlets based upon localized airflow and aerosol exposure concentrations at each time-step and thus, considered fully inhalable at all sizes.
6. No-slip boundary condition was used for aerosols at the airway wall.
7. Once the aerosol collides with the wall it was considered ‘stuck’ at the point of impact and does not slide along the wall or re-enter the airflow.
8. The only forces assumed to act on the aerosols were drag and gravity with the gravity force directed for a prone rat and upright human.

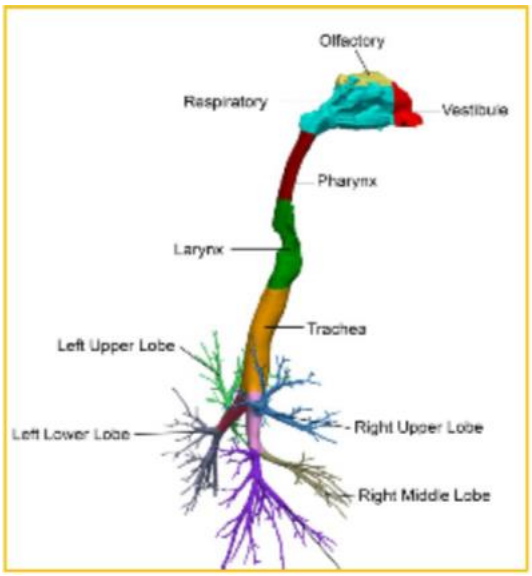
9. Mechanisms for aerosol deposition appropriate for the physical characteristics of the simulated aerosols included sedimentation, inertial impaction, and diffusion although the latter mechanisms are more important for smaller (<1 µm) aerosols.
10. Airways were assumed smooth and rigid as is the current standard for CFD simulations of the upper respiratory tract and
11. Each simulation assumed a 1 mg/L aerosol concentration with resting nasal breathing or resting oral breathing.

Details are presented in Corley *et al*. (2018) as an internal report and then formally published in Corley *et al.* (2021). Table 7 shows the conditions used in these simulations. The CFD model is shown in Figure 7.

Table 7. Computational Mesh Characteristics and Particles Tracked for Human CFD Models (from Corley et al., 2021)

Characteristic	Human (Nasal)	Human (Oral)
General surface Mesh Statistics		
Surface Facets	132,265	345,781
Prism Boundary Layers	20	15
Cells in Boundary Layer	2,645,300 (est)	4,250,764
Boundary Layer Thickness (µm)	500	500
Total Polyhedral and Prismatic Cells in Mesh	2,845,876	4,880,020
Nodes	6,433,388	12,022,936
Maximum Y+ Value (dimensionless)	0.122 (vestibule)	0.09 (bronchi)
Meshing/ Simulation Software (Star-CCM+) Version	8.02	14.04.011
Boundary Inlet		
Surface Facets	3,690 (3151 quadrilateral, 539 polygonal)	9,620 (4650 quadrilateral, 4970 polygonal)
Surface Area (m^2)	1.51431×10^{-4}	1.42×10^{-4}
Representative no. of parcels tracked/ simulation	4.0×10^5	9.91×10^6

Figure 7. CFD Model Diagram of Human Respiratory Tract (Corley et al., 2018)



The nasal breathing simulations (Yugulis and Corley, 2020) were performed for monodisperse, spherical particles sizes of 1, 3, 5, 10, 15, 20, and 30 µm. This range of aerosol sizes was chosen to cover expected

sizes in residential or occupational exposures and to facilitate calculations of polydisperse aerosol deposition in future risk assessments (Flack *et al.,* 2019). Since total particle deposition was approximately 99% at 30 µm, simulations for particles >30 µm were not included in this approach since negligible penetration of the larger particles was predicted. The particle size was extended to 50 µm for the oral breathing simulations.

Since the CFD model is essentially generating results for a generic water droplet (*i.e.*, non-chemical specific), the results were adjusted for chlorothalonil in the diluted product. The deposition of chlorothalonil is proportional to the amount of active ingredient being applied; therefore, the CFD results were multiplied by the maximum percent of chlorothalonil in a diluted product (4.9%, w/w). The total aerosol dose per surface area for each region of the nasal breathing model is provided in Table 8.

Table 8. Aerosol Dose per Surface Area for each Region of the Nasal Breathing Model

Aerosol Diameter (µm)	Regional Aerosol Deposition (Total mg/cm^2 Deposited Surface Area)					
	Vestibule	Respiratory	Olfactory	Pharynx	Larynx	Trachea
1	7.73×10^{-4}	6.33×10^{-4}	9.12×10^{-4}	3.27×10^{-4}	4.01×10^{-4}	1.38×10^{-4}
3	7.08×10^{-4}	3.73×10^{-4}	7.72×10^{-4}	2.52×10^{-4}	4.65×10^{-4}	1.45×10^{-4}
5	5.35×10^{-3}	4.78×10^{-4}	1.76×10^{-3}	2.37×10^{-4}	5.66×10^{-4}	1.27×10^{-4}
10	4.83×10^{-2}	3.18×10^{-3}	2.82×10^{-4}	1.57×10^{-3}	3.47×10^{-3}	4.49×10^{-4}
15	6.44×10^{-2}	2.61×10^{-3}	1.23×10^{-4}	2.51×10^{-4}	2.20×10^{-3}	1.31×10^{-3}
20	6.49×10^{-2}	2.21×10^{-3}	1.18×10^{-4}	3.61×10^{-4}	6.92×10^{-4}	3.74×10^{-4}
30	6.33×10^{-2}	6.57×10^{-4}	0.00	1.94×10^{-4}	2.68×10^{-4}	$5.18x\ 10^{-5}$

Deposition at the 75th percentile was selected because it is the highest concentration area that is not affected by stochastic variations in the modelling. These results are summarised in Table 9.

Table 9. Human CFD Simulation Results for 1 mg/L Aerosol, for Aerosol Sizes Ranging from 1 to 30 µm MMAD.

Aerosol Diameter (µm)	Deposition at 75th Percentile (mg Chlorothalonil/cm2/breath) Adjusted for 4.9% (w/w) Chlorothalonil					
	Vestibule	Respiratory	Olfactory	Pharynx	Larynx	Trachea
1	1.05×10^{-3}	7.46×10^{-4}	1.28×10^{-3}	4.19×10^{-3}	5.29×10^{-4}	1.80×10^{-4}
3	8.30×10^{-4}	5.95×10^{-4}	1.11×10^{-3}	3.09×10^{-4}	6.08×10^{-4}	1.89×10^{-4}
5	1.40×10^{-3}	7.01×10^{-4}	3.90×10^{-3}	3.63×10^{-4}	7.55×10^{-4}	1.56×10^{-4}
10	3.98×10^{-2}	1.10×10^{-3}	4.33×10^{-4}	1.32×10^{-3}	3.43×10^{-3}	3.19×10^{-4}
15	7.12×10^{-2}	7.10×10^{-4}	2.38×10^{-4}	8.56×10^{-4}	2.07×10^{-3}	3.42×10^{-4}
20	6.76×10^{-2}	5.57×10^{-4}	1.59×10^{-4}	4.53×10^{-4}	6.55×10^{-4}	1.37×10^{-4}
30	3.69×10^{-2}	4.63×10^{-4}	0.00	1.38×10^{-4}	2.52×10^{-4}	5.22×10^{-5}

5.4 Preliminary Test: Calculation of HEC

The CFD model provided results for discrete particles sizes (*i.e.*, monodisperse) ranging from 1 to 30 µm in a single breath; however, spray applicators will be exposed to distributions of these particle sizes (*i.e.*, polydisperse). The percent contribution of each discrete particle size was determined mathematically using the PSD for the "adjusted" inhalable fraction for applicators (*i.e.*, MMAD = 35 µm, GSD = 1.5) and are presented in Table 10. For details, see Flack and Ledson (2018).

Table 10. Percent Contribution of Discrete Particles to the Relevant Particle Size Distributions (MMAD = 35 µm, GSD = 1.5)

Aerosol Diameter (m)	Percent Contribution (%)	Decimal Contribution
1	3.43×10^{-14}	3.43×10^{-16}
3	6.06×10^{-6}	6.06×10^{-8}
5	0.0034	3.4×10^{-5}
10	1.44	0.0144
15	12.8	0.128
20	32.9	0.329
30	52.9	0.529

For each region of the upper respiratory tract, the deposition of each particle size was calculated by multiplying the percent contribution of a particle size by the predicted deposition from the CFD model (assuming the maximum percent of chlorothalonil in a diluted product of 4.9% (w/w)). For example, the deposition in the larynx of a 10 µm particle would be calculated by multiplying 3.43×10^{-3} mg/cm^2/breath (Table 9) by 0.0144 (Table 10). After calculating the deposition of each particle size for a given region in this manner (Table 11), the cumulative (Spray Applicator) site-specific deposition per breath was then calculated as the sum of depositions across particle sizes (Table 11).

Table 11. Cumulative Particle Deposition for 1 mg/L Aerosol in Site-specific Regions of the Respiratory Tract for each Exposure Scenario

Aerosol Diameter (m)	Cumulative Deposition Amount (mg Aerosol/cm^2/breath)					
	Vestibule	Respiratory	Olfactory	Pharynx	Larynx	Trachea
1	2.56×10^{-19}	4.39×10^{-19}	1.44×10^{-19}	1.81×10^{-19}	6.17×10^{-20}	2.56×10^{-19}
3	3.61×10^{-11}	6.73×10^{-11}	1.87×10^{-11}	3.68×10^{-11}	1.15×10^{-11}	3.61×10^{-11}
5	2.38×10^{-08}	1.05×10^{-07}	1.23×10^{-08}	2.57×10^{-08}	5.30×10^{-09}	2.38×10^{-08}
10	1.58×10^{-05}	6.24×10^{-06}	1.90×10^{-05}	4.94×10^{-05}	4.59×10^{-06}	1.58×10^{-05}
15	9.09×10^{-05}	3.05×10^{-05}	1.10×10^{-04}	2.65×10^{-04}	4.38×10^{-05}	9.09×10^{-05}
20	1.83×10^{-04}	5.23×10^{-05}	1.49×10^{-04}	2.15×10^{-04}	4.51×10^{-05}	1.83×10^{-04}
30	2.45×10^{-04}	0.00	7.30×10^{-05}	1.33×10^{-04}	2.76×10^{-05}	2.45×10^{-04}
Cumulative (Spray Applicator)	5.35×10^{-04}	8.91×10^{-05}	3.51×10^{-04}	6.63×10^{-04}	1.21×10^{-04}	5.35×10^{-04}

Before calculating HECs, relevant breathing rates and exposure duration must be incorporated to determine the total daily deposition of aerosol for each region of the upper respiratory tract since the site-specific deposition estimates in Table 9 were calculated per breath. The total deposition for each region of the respiratory tract was calculated by multiplying the cumulative site-specific deposition (from Table 11) by a breathing rate of 12.7 breaths/min, an exposure duration of 8 hours, and a conversion factor of 60 min/hour. The breathing rate was derived from the minute ventilation of 8.3 L/min and the exposure duration is the default used by EPA to evaluate occupational handler activities. The ventilation rate of 8.3 L/min was an assumption made by the EPA as a policy decision. Different breathing rates may be assumed. To account for different activity levels, lower or higher breathing rates are assumed on the exposure side when calculating unit exposures. Site-specific total deposition values are presented in Table 12.

Table 12. Total Deposition of Aerosol in Site-specific Regions of the Respiratory Tract for each Exposure Scenario

Exposure Scenario	Total Deposition Amount (mg Aerosol/cm^2)				
	Respiratory	Olfactory	Pharynx	Larynx	Trachea
Spray Applicator	3.26	0.543	2.137	4.042	0.738

Since each CFD simulation assumed a 1 mg/L aerosol concentration, site-specific HECs were calculated by simply dividing the geometric mean $BMDL_{sd}$ of 0.0073 mg chlorothalonil/cm^2 by the total deposition calculated for each region of the upper respiratory tract in Table 12. Site-specific HECs are presented in Table 13.

Table 13. HEC Values

Exposure Scenario	HEC (mg Chlorothalonil/L)				
	Respiratory	Olfactory	Pharynx	Larynx	Trachea
Spray Applicator	0.002	0.013	0.003	0.002	0.010

These initial results were submitted to the EPA. A preliminary risk assessment (EPA, 2018a) was generated. The data was evaluated by the EPA SAP (EPA, 2018b) who published a report with recommendations (EPA, 2019). Based on recommendations from this SAP meeting and report, a second study was performed and some changes to the utilisation of the particle size and CFD modelling data to further refine this preliminary risk assessment. This new work, which was used in the EPA risk assessment is described in Section 7.

6 Main Test

6.1 Main Test: MucilAir™ 8 hour, 24 hour and Repeat Dosing Exposures (Paulo, 2020)

The previous study (Vinall, 2017) was repeated with different exposure scenarios. The aim of this study was to examine the response of MucilAir™ pooled donor tissues, when challenged with a formulation (Bravo 720 SC) containing chlorothalonil. Ten dilutions of the formulation were prepared and six MucilAir™ tissues were exposed to each dilution. Three exposure scenarios were tested, as listed below, and all concentrations were tested concurrently, to minimise variability in experimental conduct.

- Scenario 1: A single 8 hour treatment (to replicate a typical 'working day' exposure)
- Scenario 2: A single 24 hour treatment (to replicate Vinall, 2017)
- Scenario 3: Repeated treatment comprising consecutive daily treatment for 5 days (with dose removal before each re-dose) to replicate a 'working week'. This scenario was representative of a 'worst case' exposure as operator exposure would not be constant for 5 days.

Vehicle controls (physiological saline), positive controls (SDS; 4 mM) and air-liquid interface (ALI) controls were included in each scenario (Welch *et al.*, 2021). In addition to these controls, a Bravo 720 SC blank (chlorothalonil absent) formulation was included in Scenario 3 only. These tests were performed as described above (Vinall, 2017) with the following exceptions: different exposure scenarios and the replacement of individual donor with pooled donor MucilAir™ tissues. MucilAir™ pooled donor tissues and culture medium were obtained from Epithelix Sàrl. The 'pooled donor' variant is prepared from 14 donors.

Cell morphology and health was verified visually on arrival at the Test Facility, Charles River, Edinburgh, UK, and periodically during the tissue maintenance period by light microscopy. Cell layers appeared to be intact with clearly visible cilia beating.

In all vehicle control tissues, for all three scenarios, there was a reduction in the TEER reading following treatment with physiological saline. This was potentially attributed to the removal of mucus from the surface of the cells during rinsing steps and stress caused to tissues due to experimental manipulations. There was little or no release of LDH at pre-dose (0 hour) and post-dose (or following 3 day exposure, scenario 3 only) for all testing scenarios. The mean TEER and LDH results for pre-dose and at 8 hour, 24 hour and 5 days (3 days for LDH) post dose are summarised in Table 14.

Table 14. Negative Control Treatments for TEER and LDH Release

Scenario	Mean TEER Reading (Ω x cm^2)		Mean % LDH	
	Pre-dose	Post-dose	Pre-dose	Post-dose
1	716	376	*0.00*	*0.00*
2	701	591	*0.00*	*0.00*
3	617	542	*0.00*	1.01 *

Negative values corrected to 0.00
** Media analysed after 3 day exposure*

In positive control tissues, a considerable reduction in TEER was observed following treatment in all three dosing scenarios. This corresponded to the resazurin data where a reduction in resazurin metabolism was observed (*ca* 40% in the 8 hour group, *ca* 8% in the 24 hour group and <0.01% in the 5 day group) relative to vehicle control tissues. LDH release was low for all pre-dose measurements and increased considerably (197% and 137% in the 8 and 24 hour groups, respectively). For Scenario 3, the intermediate LDH release was considerably lower (*ca* 50%). It was hypothesised that this was due to LDH being released rapidly after direct contact with the toxicant and then degrading over the 3 day period as the half-life of LDH in culture medium is *ca* 9 h (Promega, 2009). At 5 days, negligible levels of LDH were detected. The mean TEER, LDH release from pre-dose and post-dose, and resazurin metabolism post-dose for all exposure scenarios are summarised in Table 15.

Table 15. Positive Control Treatments for TEER, LDH Release and Resazurin Metabolism

Scenario	Mean TEER Reading (Ω x cm^2)		Mean % LDH		Mean % Resazurin
	Pre-dose	Post-dose	Pre-dose	Post-dose	Post-dose
1	604	60	*0.00*	197	39.3
2	622	14	*0.00*	137	8.23
3	682	57	*0.00*	49.9 *	*0.00*

Negative values corrected to 0.00
** Media analysed after 3 day exposure*

Untreated ALI controls showed a slight decrease in TEER in all exposure scenarios as well as a slight increase in LDH release. Resazurin metabolism decreased with increasing exposure time which was attributed to stress under experimental conditions. Overall, the data from the ALI tissues were similar to the vehicle control treated tissues and are summarised in Table 16. Evaluation of ALI, vehicle and positive control data indicates that the assay performed within the normal parameters for a study of this type.

Table 16. ALI Control Treatments for TEER, LDH Release and Resazurin Metabolism

Scenario	Mean TEER Reading (Ω x cm^2)		Mean % LDH		Mean % Resazurin
	Pre-dose	Post-dose	Pre-dose	Post-dose	Post-dose
1	674	445	*0.00*	22.3	88.7
2	728	603	*0.00*	*0.00*	55.1
3	678	699	*0.00*	25.6 *	35.8

Negative values corrected to 0.00
** Media analysed after 3 day exposure*

Bravo 720 SC Blank formulation was only tested in Scenario 3. It showed a slight increase in LDH release following the 3 days of treatment and was similar to that observed after 5 days exposure. A slight decrease in TEER was observed after 5 days exposure, which could be attributed to the removal of mucus from the surface of the cells during redosing and rinsing steps, and stress to tissues due to experimental manipulations. Resazurin, as a percentage of the vehicle control, was 101% for the blank formulation, indicating that cells were healthy following the 5 days of treatment with the formulation excipients, demonstrating that any observed toxicity was due to chlorothalonil and none of the other formulation components. For all endpoint assessments, the results from this treatment group were similar to the results of the corresponding ALI and vehicle control groups indicating that exposure to the blank formulation resulted in no adverse effects on cell viability. Mean TEER, percentage LDH release, and resazurin metabolism for cells treated with the blank formulation are summarised in Table 17.

Table 17. Bravo 720 SC Blank Formulation Control Treatment for TEER, LDH Release and Resazurin Metabolism

Scenario	Mean TEER Reading (Ω x cm^2)		Mean % LDH		Mean % Resazurin
	Pre-dose	Post-dose	Pre-dose	Post-dose	Post-dose
3	674	445	*0.00*	22.3	88.7

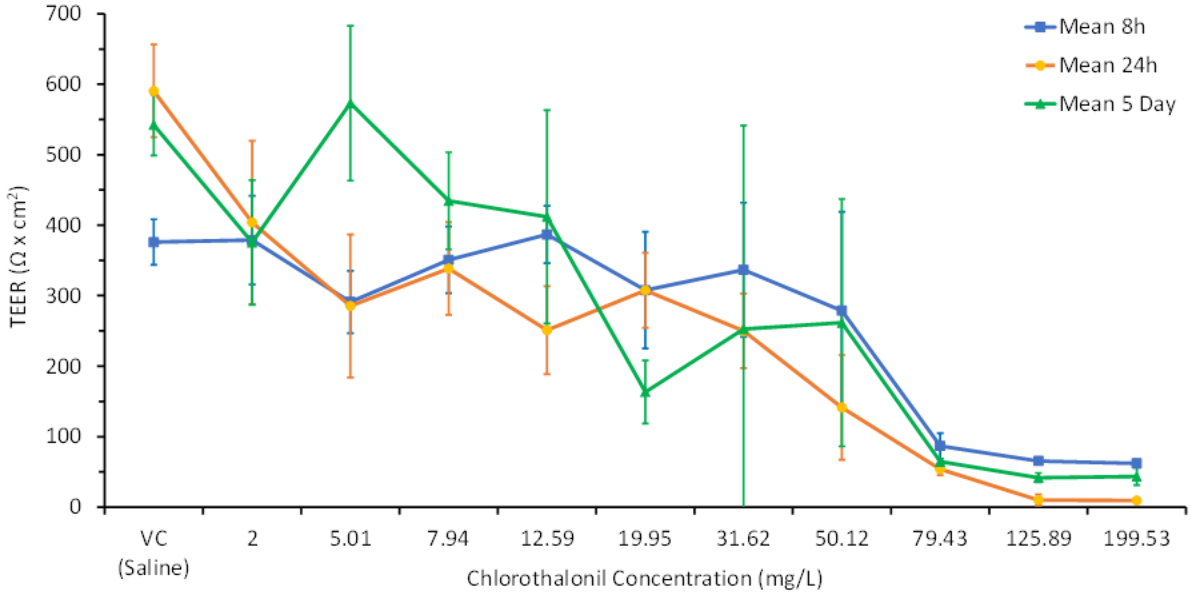
Negative values corrected to 0.00
** Media analysed after 3 day exposure*

TEER readings, in response to treatment with Bravo 720 SC dilutions, were similar across the testing scenarios. At all timepoints post-dose, a concentration dependent decrease in TEER was observed, with the highest three chlorothalonil concentrations (79.43, 125.89 and 199.53 mg/L) causing the most pronounced changes in TEER. Mean TEER readings from pre-dose and post-dose for all exposure scenarios are summarised in Table 18 and Figure 8. The pre-dose values were included as an assay quality control check of membrane viability prior to dosing.

Table 18. Mean TEER Reading Pre-Dose and Post-Dose for Scenarios 1, 2 and 3 over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	Mean TEER Reading (Ω x cm^2)					
	Scenario 1		Scenario 2		Scenario 3	
	Pre-dose	8 hour	Pre-dose	24 hour	Pre-dose	5 days
2.00	540	379	670	404	576	376
5.01	641	291	693	285	588	573
7.94	504	351	716	339	617	435
12.59	571	387	695	251	602	412
19.95	541	308	686	308	653	163
31.62	679	337	652	250	704	253
50.12	678	279	688	141	623	262
79.43	677	87	665	54	544	65
125.89	659	66	641	10	602	42
199.53	631	62	650	9	604	44

Figure 8. Effect of Bravo 720 SC Treatment (8 hour, 24 hour and 5 Day) on TEER Across MucilAir™



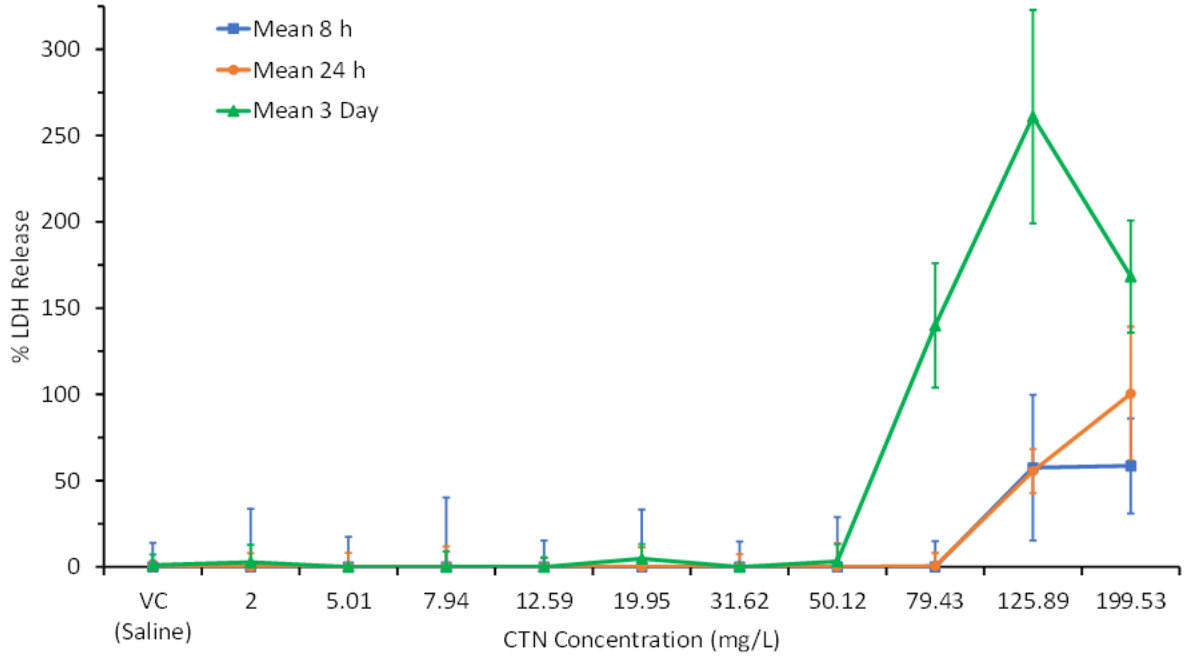
Prior to dosing, the mean percentage of LDH release for all tissues was <0.01% of the appropriate LDH benchmark controls. This indicates that the tissues were viable before treatment with chlorothalonil. The percentage LDH release response to treatment with Bravo 720 SC was similar across the 8 and 24 hour treatment groups where LDH release was only seen at the two highest concentrations (125.89 and 199.53 mg/L). The intermediate samples (collected after 3 days of exposure) from the 5 day treatment showed high LDH release at the three highest concentrations of chlorothalonil tested (79.43, 125.89 and 199.53 mg/L) indicating high toxicity at these doses. Due to this toxicity, it was hypothesised that LDH was released early in the treatment and was depleted by Day 5, where the data showed irregular LDH release response with no concentration specific pattern (the half-life of LDH in culture medium is *ca* 9 h). The mean percentage LDH release results from pre-dose and post dose for Scenario 1, Scenario 2 and the intermediate samples from Scenario 3 are summarised in the Table 19 and Figure 9. LDH release results from the intermediate media samples for Scenario 3 are presented below as these are more reflective of the extent of toxicity due to the *ca* 9 h half-life of LDH in culture medium.

Table 19. Mean LDH Release Pre Dose and Post Dose for Scenarios 1, 2 and 3 over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	Mean % LDH Release					
	Scenario 1		Scenario 2		Scenario 3	
	Pre-dose	8 hour	Pre-dose	24 hour	Pre-dose	3 days
2.00	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	2.70
5.01	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
7.94	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
12.59	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
19.95	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	4.85
31.62	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
50.12	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	3.15
79.43	*0.00*	*0.00*	*0.00*	0.46	*0.00*	140
125.89	*0.00*	57.5	*0.00*	55.5	*0.00*	261
199.53	*0.00*	58.6	*0.00*	100	*0.00*	168

Negative values corrected to 0.00
** Media analysed after 3 day exposure*

Figure 9. Effect of Bravo 720 SC Treatment (8 hour, 24 hour and 5 Day) on LDH Release from MucilAir™



** Media analysed after 3 day exposure*

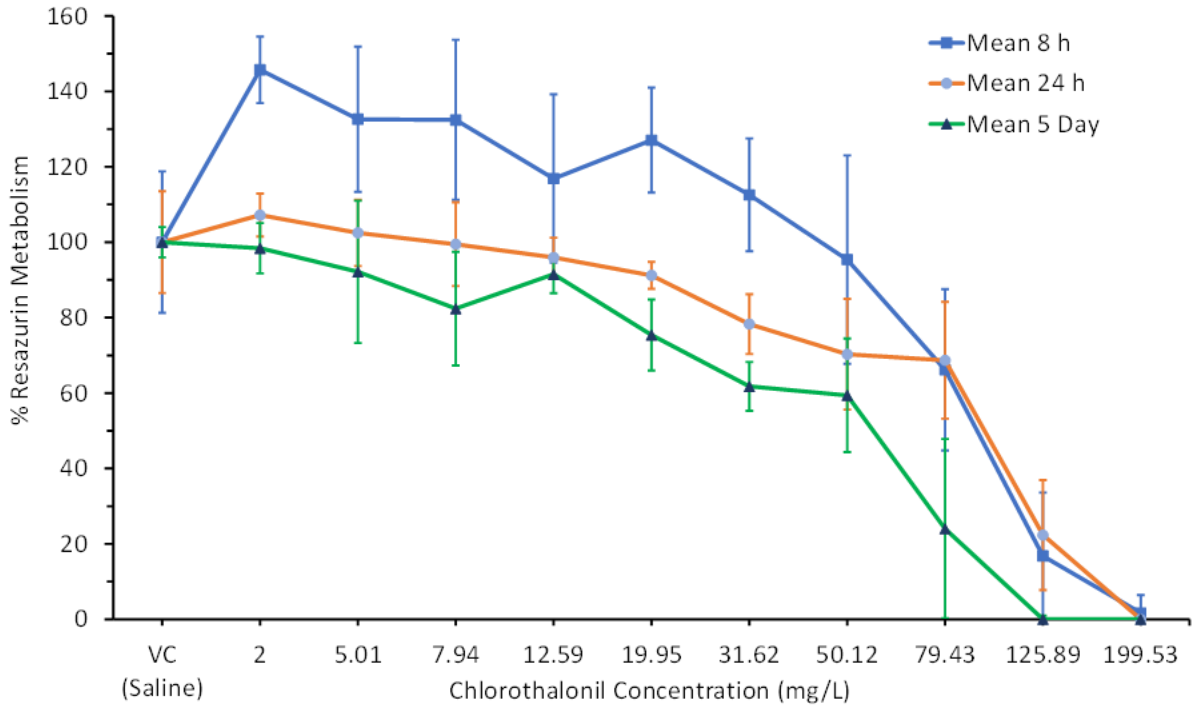
Resazurin metabolism, following treatment with Bravo 720 SC dilutions, followed a similar pattern for all testing scenarios. The percentage of resazurin metabolism decreased in a concentration-specific manner, with the most pronounced decrease observed at the two highest concentrations of chlorothalonil tested (125.89 and 199.53 mg/mL). Percentage of resazurin metabolism also decreased across the testing scenarios, concurrent with time of exposure. The mean percentage resazurin metabolism release results for all exposure scenarios are summarised in Table 20 and Figure 10.

Table 20. Mean Resazurin Metabolism Post Dose for Scenarios 1, 2 and 3 over the Range of Chlorothalonil Concentrations Tested

Chlorothalonil Concentration (mg/L)	Mean % Resazurin Metabolism		
	Scenario 1 (8 hour)	Scenario 2 (24 hour)	Scenario 3 (5 d)
2.00	146	107	98.4
5.01	133	102	92.2
7.94	132	99.5	82.4
12.59	117	96.0	91.5
19.95	127	91.2	75.4
31.62	113	78.3	61.8
50.12	95.4	70.3	59.4
79.43	66.2	68.7	24.0
125.89	16.8	22.4	*0.00*
199.53	1.68	*0.00*	*0.00*

Negative values corrected to 0.00

Figure 10. Effect of Bravo 720 SC Treatment (8 hour, 24 hour and 5 Day) on Resazurin Metabolism by MucilAir™



In all scenarios, there was a dose related decrease in TEER readings and resazurin metabolism following challenge with Bravo 720 SC dilutions, with a more pronounced response observed at higher chlorothalonil concentrations. These responses are consistent with tissue irritation. At the 8- and 24-hour timepoints, there was a dose-related increase in LDH release following challenge with Bravo 720 SC dilutions. Pronounced responses were seen only at the highest two chlorothalonil concentrations (125.89 and 199.53 mg/L) tested. Five day treatment with chlorothalonil gave inconsistent LDH release results with no clear dose related pattern. As such, intermediate media samples taken following the 3 day exposure, revealed high LDH release at the three highest concentrations tested (79.43, 125.89 and 199.53 mg/L). It was hypothesised that the toxicity of the high doses observed in Scenario 3 caused LDH to be released early, *i.e.*, on contact, and for it to be depleted over time, thus giving low and irregular results observed at

Day 5. Observations of decreased TEER and resazurin metabolism, in conjunction with increased LDH release confirmed contact toxicity. These findings were seen at concentrations across all three exposure scenarios tested in this MucilAir™ study.

6.2 Main Test: BMD Modelling of MucilAir™ Data from Paulo (2020)

A BMD analyses using constant variance (Stevens, 2020) was performed on this data (Table 21). As opposed to the Vinall (2017) study, where BMD values were approximately the same for all three endpoints, TEER and resazurin measurements were found to be more sensitive than LDH in this study. Little variation in toxicity was observed following a single 24 hour exposure compared to an exposure comprising of consecutive daily treatments. The results also demonstrated that a single 24 hour exposure is a conservative estimate compared to a single 8 hour treatment designed to replicate a typical 'working day' exposure.

Table 21. Results of BMD Modelling of Chlorothalonil MucilAir Datasets With or Without Outliers Removed

Scenario	LDH*		TEER		Resazurin	
	$BMDL_{1SD}$ (mg/L)	$BMDL_{1SD}$ (mg/L) With Outliers Removed	$BMDL_{1SD}$ (mg/L)	$BMDL_{1SD}$ (mg/L) With Outliers Removed	$BMDL_{1SD}$ (mg/L)	$BMDL_{1SD}$ (mg/L) With Outliers Removed
1 (8 hour)	50.0	47.0	50.0	36.1	30.8	30.8
2 (24 hour)	73.8	72.4	2.03	7.53	13.4	13.9
3 (5 day)	67.6	57.5	4.42	13.4	8.62	8.38

For all three exposure scenarios, LDH release was only increased at the highest doses tested and the BMD values were similar to those obtained in the first study (Vinall, 2017). For resazurin metabolism and TEER, the BMD values were lower than those obtained in the first study and may be attributed to the use of pooled donors in this study rather than single donors used in the first study. In this study, the single 24 hour exposure provided lower BMD values than the single 8 hour exposure for resazurin metabolism and TEER indicating the single 24 hour exposure provides a conservative estimate for a typical workday. It was concluded that the single 24 hour and repeated 24 hour, 5-day exposure scenarios provided similar results; therefore, similar HECs would be calculated for each exposure scenario. This is consistent with the biological understanding (Figure 2), such that protection for the initial cell damage caused by a contact irritant like chlorothalonil will prevent effects that would be caused from repeated exposure.

Better model fits were observed for the resazurin metabolism measurements as compared to the TEER measurements due to greater variability in the TEER measurements. As a result, the BMD values from the resazurin metabolism measurements were selected for use in this Case Study to calculate HECs and BMDL values for resazurin metabolism were converted to tissue concentrations using Equation 1 and are presented in Table 22. Since the repeated exposure scenario was not significantly different, the single dose values were utilized for subsequent HEC calculations.

Table 22. Chlorothalonil BMDL Values Calculated from Resazurin Data from Paulo (2020) MucilAir™ Data

	BMDL		
Exposure Time	8 hour	24 hour	24 hour Repeated (5 days)
Dilute Formulation (mg/L)	30.8	13.4	8.62
Tissue Concentration (µg/cm^2)	2.8	1.2	0.8

6.3 Main Test: Calculation of Retained Doses Using CFD Modelling

In order to utilize the *in vitro* data for human health risk assessment, a dosimetry model was required to calculate external concentrations that would produce the surface concentrations of deposited chlorothalonil in each region of the upper respiratory tract. Dosimetry models are used to determine internal doses of a chemical and provide information that aids in the understanding of the relationship between an external exposure and a biological response. Deposition of chlorothalonil was predicted in site-specific regions of the human upper respiratory tract (*i.e.*, vestibule, respiratory, olfactory, pharynx, larynx, and trachea) using a three-dimensional CFD model (Corley *et al.*, 2021) similar to previously published models (Corley *et al.,* 2012, Corley *et al*., 2015, Kabilan *et al*., 2016). CFD has been used in many scientific fields to analyse fluid flows and there is a multitude of literature available on CFD theory and application. CFD models for the upper respiratory tract have been developed for several species, including rats (*e.g.*, Kimbell *et al*., 1993; Kimbell *et al.*, 1997), monkeys (*e.g.*, Kepler *et al*., 1998), and humans (*e.g.*, Subramaniam *et al*., 1998). For these models, a computational mesh based on species specific anatomical data are used to develop airflow patterns that are used in conjunction with boundary conditions, diffusivity, and mass transfer coefficients to predict localized deposition of inhaled aerosol in units of mass per unit area (*e.g.*, mg/cm^2/breath).

Each simulation of the CFD model was based on a 68 kg male subject assuming 1 mg/L aerosol concentration, minute volume of 7.4 L/min, and breathing frequency of 20 breaths/min. Since the CFD model is essentially generating results for a generic water droplet, the nasal and oral CFD deposition results are considered chemical agnostic (*i.e.*, non-chemical specific). Simulations for nasal breathing were performed for monodisperse, spherical particles sizes of 1, 3, 5, 10, 15, 20, and 30 µm. An example of for the 10 µm PSD is provided in Table 23. For full details and results, see Corley *et al.* (2018).

Table 23. Predicted Aerosol Deposition for 10 µm PSD in Regions of the Human Upper Respiratory Tract CFD Model for Nasal Breathing

Airway Region	Surface Area (cm^2)	Total Deposition (% Inhaled)	Surface Area Deposited (cm^2)	Fraction Surface Area Deposited	Total Deposited Aerosol (mg)	Average of Deposited SA (mg/cm^2)
Vestibule	33.0	27.1	2.06	0.063	9.97×10^{-2}	5.91×10^{-2}
Respiratory	181.4	12.7	14.7	0.081	4.67×10^{-2}	6.61×10^{-3}
Olfactory	21.3	0.024	0.315	0.015	8.89×10^{-5}	3.60×10^{-4}
Pharynx	29.1	1.92	4.50	0.155	7.05×10^{-3}	1.87×10^{-3}
Larynx	33.0	5.99	6.35	0.192	2.20×10^{-2}	3.72×10^{-3}
Trachea	55.3	0.993	8.12	0.147	3.65×10^{-3}	4.48×10^{-4}
Total	353.0	48.8	36.03	0.102	1.79×10^{-1}	

Deposition was predicted for site specific regions of the human upper respiratory tract (*i.e.*, vestibule, respiratory, olfactory, pharynx, larynx, and trachea; Figure 7). Since total particle deposition was *ca* 99% at 30 µm, simulations for particles greater than 30 µm are not shown since negligible penetration of the larger particles was predicted. Based on comments received from the SAP, the impact of oral breathing

on aerosol deposition for the same particle sizes evaluated in the nasal breathing model were evaluated, as well as an additional particle size of 50 µm.

For smaller particle sizes (1-5 µm), minimal deposition was predicted for regions of the upper respiratory tract. These smaller particles pass the tracheobronchial region where they are suspended in the airways or deposited deeper in the respiratory tract to the lungs, which is not described by the current CFD model. For larger particles (10-20 µm), considerable deposition occurs in the upper respiratory tract with the nasal model filtering out a higher percentage of aerosol than the oral model. At particle sizes ≥30 µm, both the nasal and oral models account for nearly complete deposition in the upper respiratory tract. Deposition in the nasal model predicted more than 90% of the 30 µm particles will be filtered out by the vestibule. Similarly, for the oral model, more than 90% of the 50 µm were predicted to deposit in the oral cavity.

In addition to investigating deposition from oral breathing, a clearance model was applied to the deposition results based on the SAP recommendations (EPA, 2019). The total deposition from a single breath predicted by the CFD model will be reduced due to physical clearance mechanisms, such as mucociliary clearance, resulting in retained doses (*i.e.*, total deposition – total cleared = retained dose) for each particle size and site-specific region as summarised in Table 24(Szarka *et al.*, 2020).

Table 24. Retained Aerosol Doses of Monodisperse Particles in the Nasal Breathing Model

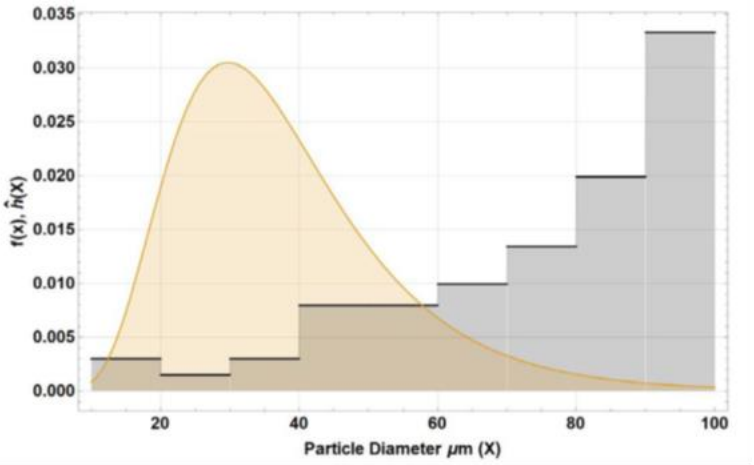
Diameter of Aerosol (µm)	Retained Aerosol Doses (µg/cm^2)					
	Respiratory	Olfactory	Pharynx	Larynx	Trachea	Vestibule
1	43.4	62.5	22.4	27.5	94.6	2,522
3	25.6	52.9	17.3	31.9	99.4	2,310
5	32.8	121	16.3	38.8	87.1	17,458
10	218	19.3	108	238	308	157,615
15	179	8.43	172	151	898	210,153
20	152	8.09	248	47.5	256	211,785
30	45.1	0.00	13.3	18.4	35.5	206,563

For both the nasal and oral models, the trachea was found to have the highest retained doses for all particle sizes modelled in the upper respiratory tract, with exception of the vestibule in the nasal model.

Though simulations were conducted using monodisperse particles (*i.e.*, all particles assumed to be the same size in each simulation), humans will be exposed to a distribution of the modelled particle sizes (*i.e.*, polydisperse); therefore, PSDs of inhaled aerosols were incorporated to calculate retained doses from polydisperse particle exposures. Empirical and theoretical PSDs covering the inhalable range for humans (<100 µm) were utilized to calculate the polydisperse particle exposures.

Data are available from the American Society of Agricultural and Biological Engineers (ASABE) standardized reference agricultural nozzles, which are also part of the AgDRIFT® (ver. 2.1.1) nozzle data library, to construct an empirical PSD for droplets generated from application of nozzle technology. Data for a reference nozzle producing fine to medium droplet sizes was used for this Case Study; however similar empirical PSDs would be expected for other nozzles for particle sizes up to 100 µm. Since the overall PSD for the nozzle data includes particles sizes greater than the inhalable range for humans, only data ≤100 µm was used to create a histogram density (Figure 11). The mass median aerodynamic diameter (MMAD) for this distribution would be *ca* 80 µm.

Figure 11. Probability Density Function, f(x), of Theoretical Distribution (MMAD = 35 µm, GSD = 1.5) and Histogram Density, h(x) of the Empirical Distribution for 0 ≤ x ≤100 µm



To cover the inhalable range and scenarios where particle sizes are not expected to be as large as those generated using nozzle technology, two theoretical distributions with MMADs of 10 µm and 35 µm were also used to create probability density functions for particles sizes up to 100 µm. A geometric standard deviation (GSD) of 1.5 was assumed for both theoretical distributions.

Deposition in the trachea (other than the vestibule) showed the highest polydisperse retained dose in the upper respiratory tract for both oral and nasal breathing. The polydisperse retained doses using the modified cubic spline curve for the nasal breathing model are summarized in Table 25.

Table 25. Site-Specific Polydisperse Retained Doses (mg aerosol/cm^2) for Nasal Breathing Model Using the Modified Cubic Spline Curve

Airway Region	Total Aerosol Deposited per Surface Area (mg Aerosol/cm^2)		
	Empirical	Theoretical MMAD = 35 m, GSD =1.5	Theoretical MMAD = 10 m, GSD =1.5
Vestibule	38.9	103	104
Respiratory	1.21 x 10^{-2}	3.88 x 10^{-2}	1.22 x 10^{-1}
Olfactory	1.69 x 10^{-4}	9.09 x 10^{-4}	3.85 x 10^{-2}
Pharynx	7.31 x 10^{-3}	4.07 x 10^{-2}	7.92 x 10^{-2}
Larynx	6.41 x 10^{-3}	1.48 x 10^{-2}	1.21 x 10^{-1}
Trachea	1.95 x 10^{-2}	4.93 x 10^{-2}	2.70 x 10^{-1}

MMAD = mass median aerodynamic diameter; GSD = geometric standard deviation.

6.4 Main Test: Calculation of Human Equivalent Concentrations and Human Equivalent Doses for Inhalation Risk Assessment

Site-specific HECs were calculated for each PSD and duration using Equation 2:

$$HEC = \frac{BMDL \times DF}{D_R \times DF} \times AC = \frac{BMDL}{D_R} \times AC$$ Equation 2

Where

- HEC = human equivalent concentration (mg chlorothalonil/L air).
- BMDL = lower bound of the 95% confidence interval on the BMD (mg chlorothalonil/cm^2).
- D_R = polydisperse retained dose (mg aerosol/cm^2) from Table 23.
- D_F = dilution factor (mg chlorothalonil/mg aerosol).
- AC = aerosol concentration (mg aerosol/L air) = 1 (based on the CFD model assumptions).

While the CFD model can predict deposition in the nasal vestibule, retained doses calculated for the vestibule were not used to calculate HECs for chlorothalonil. The vestibule is the most anterior part of the nasal cavity. While the rest of the nasal cavity is lined with respiratory epithelium, the vestibule is lined with the same epithelium as human skin. It also has small hairs to help filter and prevent materials from entering the respiratory tract. As a result, the vestibule functions similarly to the skin and the cell types are different from those used in the MucilAir™ assay for evaluating upper respiratory tract irritation.

Site specific human equivalent doses (HEDs) were then calculated using Equation 3 for each PSD and duration. Since the CFD model predicted deposition for a 68 kg male subject with a minute volume of 7.4 L/min, these assumptions were used to calculate the HEDs for anticipated human exposures.

Human Equivalent Dose (mg/kg/day) = HEC x CF x D Equation 3

Where

- HEC = human equivalent concentration (mg chlorothalonil/L air).
- CF = human-specific conversion factor = 7.4 L/min * 60 min/hour ÷ 68 kg.
- D = anticipated daily duration.

Site-specific HEDs were calculated for 8 hour and 24 hour for each PSD, which corresponds with the exposures used in the *in vitro* assay (Paulo, 2020), as well as the anticipated daily durations expected for occupational and non-occupational bystander scenarios, respectively. Additionally, due to residential uses of a chlorothalonil trigger spray product, the 8 hour BMDL was used to calculate a 2 hour HEC and HED for the theoretical PSD with a MMAD of 35 µm and GSD of 1.5. A summary of the HECs and HEDs for each airway region and exposure duration using empirical and theoretical PSDs are provided in Table 26.

Table 26. Site Specific Human Equivalent Concentrations and Human Equivalent Doses for Nasal Breathing Model

Airway Region	Total Aerosol Deposited per Surface Area (mg Aerosol/cm^2)			
	8 hour		24 hour	
	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)
Respiratory	0.232	12.1	0.099	15.6
Olfactory	16.5	863	7.08	1110
Pharynx	0.383	20.0	0.164	25.7
Larynx	0.437	22.8	0.187	29.3
Trachea	0.144	7.50	0.062	9.64

HEC = human equivalent concentration
HED = human equivalent dose

HECs and HEDs for each PSD and duration were calculated. These are presented for a PSD of MMAD = 35 µm with a GSD of 1.5(in Table 27) and MMAD = 10 µm with a GSD of 1.5 (Table 28).

Table 27. Human Equivalent Concentration and Human Equivalent Dose for Theoretical PSD with MMAD = 35 µm and GSD = 1.5

Airway Region	2 hour		8 hour		24 hour	
	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)
Respiratory	0.072	0.941	0.072	3.77	0.031	4.84
Olfactory	3.08	40.2	3.08	161	1.32	207
Pharynx	0.069	0.899	0.069	3.60	0.030	4.63
Larynx	0.190	2.48	0.190	9.90	0.082	12.73
Trachea	0.057	0.742	0.057	2.97	0.024	3.82

HEC = human equivalent concentration
HED = human equivalent dose

Table 28. Human Equivalent Concentration and Human Equivalent Dose for Theoretical PSD with MMAD = 10 µm and GSD = 1.5

Airway Region	8 hour		24 hour	
	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)
Respiratory	0.023	1.20	0.010	1.54
Olfactory	0.073	3.80	0.031	4.88
Pharynx	0.035	1.85	0.015	2.37
Larynx	0.023	1.21	0.010	1.55
Trachea	0.010	0.54	0.004	0.70

HEC = human equivalent concentration
HED = human equivalent dose

The trachea provided the most health protective values for human health risk assessment for all PSDs. As expected, HECs and HEDs decreased with decreasing MMAD given the CFD model predicted most of the larger particles are filtered by the nasal vestibule and the greatest deposition in the upper respiratory tract was observed for the theoretical PSD with a MMAD of 10 µm (GSD = 1.5). Consequently, the theoretical PSD with a MMAD of 10 µm (GSD = 1.5) provides the lowest HEC and HED and would, therefore, provide a worst-case scenario.

PSDs and durations considered appropriate for each exposure scenario were selected and a summary of HECs and HEDs used for evaluating inhalation exposures from conventional uses of chlorothalonil are presented in Table 29.

Table 29. Human Equivalent Concentrations and Human Equivalent Doses Calculated for the Trachea and Used for Inhalation Risk Assessment of Conventional Uses of Chlorothalonil.

Empirical		Theoretical (MMAD =' 35 m,' GSD = 1.5)				Theoretical (MMAD =' 10 m,' GSD = 1.5)	
8 hour		2 hour		8 hour		24 hour	
HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)	HEC (mg/L)	HED (mg/kg/day)
0.144	7.50	0.057	0.742	0.057	2.97	0.004	0.70

HEC = human equivalent concentration
HED = human equivalent dose

7 Uncertainty Factors (UFs)

Default 10X interspecies UF_A and 10X intraspecies UF_H are divided into two components representing toxicokinetic (TK) variability (3X) and toxicodynamic (TD) variability (3X). Since the CFD model directly predicts the deposition of aerosols in the human respiratory tract (TK) and the *in vitro* studies directly measured endpoints in a system derived from human cells (TD), the default 10X Extrapolation Factor for Interspecies (EF_{AD}) can be reduced to 1X. Furthermore, chlorothalonil is a direct acting irritant with toxicity occurring at the point of contact in the respiratory tract such that absorption, distribution, metabolism, and excretion characteristics are not likely to have a significant effect on the response among the human population. As such, the Extrapolation Factor for Interspecies (EF_{AK}) TK component of UF_A can be reduced to 1X. Therefore, the default 10X intraspecies variation UF_H can be reduced to 3X. The FQPA SF is 1X, when applicable. As a result, the level of concern (LOC) for inhalation exposures is 3 (1X interspecies, 3X intraspecies, and 1X FQPA SF when applicable). This was a policy decision for the US EPA. Different UFs may be relevant for different agencies.

8 Risk Assessments

8.1 Residential Handler Exposure/ Risk Estimates

Chlorothalonil is currently registered for use on residential garden, ornamental shrubs, plants, and trees. It should be noted that there is one registered chlorothalonil product label (EPA Reg. No. 67572-2) for use by homeowners that requires specific clothing/ PPE (*e.g.*, long-sleeve shirt/ long pants and gloves) which would normally preclude it from consideration in the residential handler assessment. However, it was considered in the residential handler assessment since the product is specifically labelled for residential use and is packaged in a 24 oz, 32 oz, or 1-gallon trigger spray bottle. For residential handler exposures from the trigger spray product, the 2 hour value (Table 27) calculated using the theoretical PSD with a MMAD of 35 µm was selected. The short term inhalation residential handler margin of exposure (MOE) is greater than the LOC of 3 and are not of concern as summarized in Table 30.

Table 30. Residential Handler Non-Cancer Exposure and Risk Estimates for Chlorothalonil for Mixer/ Loader/ Applicator

Exposure Scenario	LOC	Inhalation Unit Exposure (mg/lb ai)	Maximum Application Rate	Amount Handled Daily	Inhalation	
					Dose (mg/kg/day)	MOE
Garden/ ornamental hose-end sprayer	3	0.034	0.0073 lb ai/gal	11 gal	0.000034	22,000
Garden/ ornamental trigger spray		0.061	0.0014 lb ai/bottle	2 bottles	0.0000021	350,000

8.2 Non-occupational Ambient Exposure Assessment from Non-Point Sources

Bystanders who live or work near treated fields are potentially exposed to emissions that travel off-site. There is the potential for inhalation exposure to chlorothalonil *via* ambient air resulting from multiple agricultural applications across large regions which are also referred to as non-point sources. A bystander volatilization inhalation exposure assessment for chlorothalonil utilizing the currently available inhalation toxicity and publicly available air monitoring data was developed. Chlorothalonil was detected in multiple ambient air studies.

The chlorothalonil bystander volatilization inhalation exposure assessment compared the maximum 24 hour air concentration detected in each of the monitoring studies to the acute HEC for residential bystanders. The acute scenarios were protective of longer-term durations since the inhalation PODs are the same. The 24 hour HEC of 0.004 mg/L (Table 29) was used based on the theoretical distribution with a MMAD of 10 µm and GSD of 1.5. This comparison was performed to represent a potential resident who lives next to a treated field and may be exposed to the peak concentration of chlorothalonil volatilizing off the field over a 24 hour period. For the purposes of the post-application bystander inhalation quantitative

assessment, only acute 24 hour post application ambient air concentrations were incorporated into the ambient air study site data, which provided chlorothalonil volatilization MOE calculations for each site. None of the air concentrations resulted in risks of concern (MOE ≥3).

8.3 Occupational Handler Exposure and Risk Estimates

Based on the anticipated use patterns and current labelling, types of equipment and techniques that can potentially be used, occupational handler exposure is expected from the registered uses. The quantitative inhalation exposure/ risk assessment developed for occupational handlers was based on the typical use scenarios.

The PSDs used for the mixer/ loader and mixer/ loader/ applicator exposure to liquid formulations/ solutions were based on both theoretical data (MMAD of 35 µm and GSD of 1.5) and empirical nozzle data from agricultural spray equipment (MMAD of *ca* 80 µm) to provide lower and upper bounding estimates. A range of risk estimates were provided since there is potential for the particles sizes from mixing and loading activities to be smaller than those from applying liquids using spray nozzle technology.

The PSD used for the applicator and flagger exposure to liquid solutions was based on empirical nozzle data from agricultural spray equipment (ASAE Reference Droplet Spectra Distributions, as provided in AGDISPTM and AgDRIFT® models).

It was considered that the CFD modelling used to determine the inhalation endpoints was applicable to solid particles as well as the liquid particles. Although PSDs are not available for the specific chlorothalonil solid formulations, product information for solid dispersible granules (SDG) indicate 99% of the particles are less than 44 µm. This approach is consistent with the requirements of the EPA's Worker Protection Standard which, when included on all labels, precludes direct exposure pathways. Furthermore, an individual SDG granule/ particle is large enough to see with the naked eye, where the visibility threshold for a micron is in the range of 30-40 µm. Therefore, the PSD used for the mixer/ loader, applicator, and loader/ applicator exposure to granule or DF/ WDG/ SDG formulations was based on theoretical data with MMAD of 35 µm and GSD of 1.5.

For occupational exposures, the 8 hour values calculated using the empirical PSD and the theoretical PSD with a MMAD of 35 µm were selected (Table 29).

The PSDs used for the mixer/ loader/ applicator exposure to solid formulations applied as liquid solutions were based on both theoretical data (MMAD of 35 µm and GSD of 1.5) and empirical nozzle data from agricultural spray equipment to provide lower and upper bounding estimates. Since the handlers can be exposed to smaller particles while mixing/ loading the solid formulations and to larger particles while applying the liquid solutions with spray equipment, a range of risk estimates is provided.

The short- and intermediate-term inhalation MOEs ranged from 5 to 660,000, assuming baseline clothing (*i.e.*, no respirator) and were not of concern. The crop with the highest application rate in each crop category (*i.e.*, orchards, high acreage field crops, and typical acreage field crops), was assessed and was representative of the remaining crops at lower application rate. Cranberries represents typical acreage field crops, soybeans represented high acreage field crops, and pistachios represented orchard crops.

9 Application of IATA

9.1 Summary of Data

The purpose of this IATA is to clarify the use of human *in vitro* data derived from an upper airway model for the derivation of a POD for the purposes of risk assessment. This Case Study utilises the EpiThelix MucilAir™ test system with the pesticide, chlorothalonil. Knowing the AOP for toxicity and PSD is critical in decision making for which test system to use. For example, small particle sizes, e.g., <5 µm, could reach the alveolar tissues and as such, both upper and lower airway (e.g., MatTek EpiAlveolar™ and ImmuONE™ ImmuLUNG™ *etc*) as well as for oral breathing, the buccal and gingival models (*e.g.*, MatTek EpiOral™ and EpiGingival™, respectively) may need to be used as stand alone or in addition. If there is data from animal studies that shows only region-specific toxicity, as demonstrated in this Case Study, then it can be used to justify the choice of upper or lower airway model to use.

Human equivalent concentration and human equivalent doses were successfully calculated using the POD derived from the MucilAir™ studies, as summarised in Table 29. These values were used to generate the risk assessments resulting in an MOE for the garden/ ornamental hose-end sprayer of 22,000 and for the garden/ ornamental trigger sprayer of 350,000.

The PODs were used to calculate the short- and intermediate-term inhalation MOEs for chlorothalonil under a wide variety of uses and these MOEs ranged from 5 to 660,000, assuming baseline clothing (*i.e.*, no respirator) and were not of concern.

Importantly, the resultant risk assessments did not require the generation of animal data from a standard 90-day subchronic inhalation toxicity, such as that described in OECD Test Guideline No. 413 (OECD, 2018). Therefore, the IATA contributes not only to generating a human relevant risk assessment, but also provides a route for inhalation toxicity testing with animal replacement.

This IATA utilises human derived tissue (MucilAir™), so is human relevant. The test system is obtained from the tissues that are impacted by the contact toxicity produced by chlorothalonil. Both experiments utilised human nasal derived MucilAir™. The only difference being that Vinall (2017) was performed using individual donor derived tissues and Paulo (2020) was performed with pooled donor derived tissues. The positive (SDS; 4 mM) and negative control (physiological saline) performed correctly for both experiments in all scenarios. Welch *et al.*, (2021) used a range of SDS concentrations and the results for the 5 mM SDS group are similar to the results with SDS (4 mM) in these studies. The positive control data at 24 h is compared in Table 31. The LDH data was calculated differently in Welch *et al.*, (2021) and, therefore, is not included.

Table 31. A Comparison of Effect of SDS (4 mM) for MucilAir™ from Vinall (2017) and Paulo (2020) and SDS 5 mM from Welch et al., (2021).

Experiment	TEER (Ω x cm^2)				% LDH				% Resazurin	
	Predose		24 hour		Predose		24 hour		24 hour	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD

Vinall (2017)	708	34	34	4	2	1	215	36	5.20	5.85
Paulo (2020)	622	14	14	4	2	6	137	32	8.23	1.40
Welch (2021)	489	139	23	3	-	-	-	-	13.0	12.9

Cervena *et al.* (2019) demonstrated consistent TEER values (*ca* 600-700 Ω x cm^2) and LDH release (*ca* 1-3%) over a period of 28 days in control tissues. These control values were similar to the control and pre-dose TEER values recorded in the Case Study experiments.

The initial study and POD were also peer reviewed at an independent US EPA SAP meeting (EPA, 2018b) which published recommendations in the subsequent SAP report (EPA, 2019). The recommendations were followed resulting in further testing and model refinement. The SAP report (EPA, 2019) was broadly supportive of the NAM and the testing strategy with no concerns about the reliability of the test system (MucilAir™).

Therefore, these data were considered to be reliable.

9.2 Application of IATA

This IATA can be applied to any contact respiratory toxicants to replace *in vivo* acute (OECD TG 412) and chronic (OECD TG 413) respiratory toxicology studies. The test system (MucilAir™) tissues are derived from the human nasal tissue which are the tissues which are most sensitive to these respiratory toxicants. Since the nasal tissue is comprised of the same cells in the conducting airway tract, this test system is also considered to be protective of the entire conducting respiratory tract. This is also confirmed by the PSD used to perform the CFD modelling.

Toxicants that require metabolism can also be evaluated as these cells are human nasal derived and retain metabolic functions of the same cells *in vivo* (*i.e.*, in the operator). The ability for MucilAir™ to metabolise chemicals is described by Cevena *et al.* (2019) and further confirmed by toxicogenomic analysis by Baxter *et al.* (2015) and Haswell *et al.* (2018). Therefore, it is postulated that this test systems and series of experiments would be suitable for these chemicals as well.

CFD is routinely used in many scientific disciplines to observe and predict liquid and gaseous airflow. This is used in engineering from sewerage and rainwater system simulations to aircraft and car design. This is well documented and understood and, therefore, fit for purpose.

For the *in vivo* (usually rat) tests, particles are ideally created to be 1-5 µm as this is the particle size that most effectively enters the rat respiratory tract. This particle size range is relevant for the rats as the physiology of the rat respiratory (conducting) tract is very much more complex than the human, which results in far more of the larger particles being removed. This Case Study uses PSD that reflects those to which the operator may be exposed, and which are deposited in different sites in the respiratory tract. Therefore, use of PSD identification is fit for purpose.

Using this approach, the short- and intermediate-term inhalation MOEs for chlorothalonil ranged from 5 to 660,000, assuming baseline clothing (*i.e.*, no respirator) and were not of concern. These results compellingly verify the applicability of this testing strategy as an IATA for identification of safety respiratory toxicants in operator exposure risk assessment.

9.3 Uncertainty

This approach can reduce the uncertainty of the inhalation risk assessment for point of contact toxicity by directly measuring the response in human tissues using the *in vitro* assay and predicting deposition in the human upper respiratory system with CFD models. The MucilAir™ model is derived from cells of human donors and simulates the structure and function of the human upper respiratory system with

pseudostratified, ciliated epithelium which secrete mucus. As such, the uncertainty that arises from extrapolation from an animal model, particularly given the anatomical and physical differences between animal and human respiratory tracts, can be avoided using this *in vitro* assay.

However, some uncertainty may arise from delivering the test item to the tissues by pipetting the liquid rather than aerosolization, which is the expected inhalation exposure for humans. Aerosolization of the formulation or dilutions could be applied to this by using equipment that creates aerosols such as a nebuliser. However, this would also require an exposure device too, such as a cloud chamber, and the mass of deposited formulation would need to be measured either using a quartz microbalance or collecting fractions for analysis *via* a suitable analytical methodology so bringing in new areas of uncertainty. This latter method is the standard approach within the *in vivo* rat assay and so this uncertainty cancels out by utilising the same techniques as those already used *in vivo*. The current approach negates the need for these complexities as the entire dose applied to the cells is known from the mass of test item weighed out, the serial dilutions performed, and the volume applied to the tissues. If more refined information is needed, the exposure chamber/ nebuliser methodology could be utilised.

In the *in vivo* rat test, the particle sizes are fixed to a much smaller level, typically 1-5 µm, to deposit as much of the test item/ formulation into the deep lung. This does not necessarily replicate the particle sizes to which the operator is exposed. There is often test item collected onto the face, eyes and in the mouth of the rats, even when suitable dosing systems are used. Therefore, the uncertainty in the current NAM is similar to, or improved on, the uncertainty in the *in vivo* rat test. Choosing and justifying the administration of test item to the test system reduces the uncertainty of the assay.

The MucilAir™ model using human nasal tissue was used. The cellular composition of the nasal, tracheal, and bronchial epithelia is the same and consists of basal, ciliated, and goblet cells. Therefore, similar responses are expected across tissue types for the evaluation of cell damage from irritation. At the time of this initial work (Vinall, 2017), only this nasal tissue model was available. However, in hindsight, the same model would have been chosen as the observed rat toxicity was in the nasal turbinates. Other versions were available during the planning and conduct for the second *in vitro* study (Paulo, 2020), but nasal MucilAir™ was chosen as this was the most suitable (nasal derived) model available. For other chemicals, different tissue models may be more suitable based on the toxic effects known about the chemical of interest. Example models include but are not limited to MatTek EpiOral™, EpiGingival™, EpiAirway® and EpiAlveolar®, EpiThelix MucilAir™ and SmallAir™ or ImmuONE™ ImmuLUNG™ and ImmuPHAGE™, amongst others available and in development. Choosing and justifying the test system reduces the uncertainty of the assay.

For chlorothalonil, MucilAir™ was the optimal *in vitro* model for this Case Study. This included consideration of ease of use and maintenance, ability to model cell-cell interactions in response to toxicants, representation of *in vivo* tissue organization, ability to simulate mechanical action of the respiratory tract, suitability for the potential for long term tests, and applicability of results to *in vivo* inhalation toxicity. The 3D *in vitro* assays, such as MucilAir™, are the best available tool to evaluate human respiratory tract toxicity given the current state of the science. Evaluation of the *in vitro* endpoints (TEER, LDH, and resazurin) using MucilAir™ has been shown to predict *in vivo* respiratory toxicity (Sivars *et al.,* 2018). TEER and resazurin measurements have been shown to provide 88% sensitivity and 100% specificity. Unlike other assays that have been shown to have transferability issues, MucilAir™ does not appear to have this limitation since it can remain in a homeostatic state for a long period of time. The good transferability and high reproducibility of MucilAir™ within and across laboratories has been documented in a recent study (Hoffmann *et al.,* 2018). Choosing and justifying the endpoints measured reduces the uncertainty of the assay.

Dosing of Bravo 720 SC formulation diluted with water directly onto the surface of the MucilAir™ tissue as a “liquid” application was different to the exposure of the liquid droplets containing dilutions of Bravo 720 SC by an operator or bystander inhaling the spray where an aerosol exposure is observed.

However, the actual concentration of the inhaled liquid or aerosol would be the same however the test would be performed. The only difference is that we are comparing a liquid and an aerosol exposure. The mass of chemical per area may be lower *in vivo* but the total surface area exposed *in vivo* may be higher. This will depend on the physical properties of the applied solution e.g., viscosity of the liquid, the surface tension, and the area of the exposed apical chamber area of MucilAir tissues. This question was discussed many times. Together, the CFD and *in vitro* test replicates what is happening in humans during an occupational exposure. The CFD model identifies where the inhaled particle will land in the respiratory tract. Since there is no inhalation and exhalation in MucilAir™, it does not make a great deal of difference if the exposure is *via* a "cloud" or as a bolus "liquid" dose. If the "cloud" exposure is from, for example, a nebuliser, there is a steady build-up of the particles on the mucus surface. These particles coalesce and mix with the MucilAir™ mucus. This is readily observed when using a cloud chamber such as the Vitrocell Cloud chamber (www.vitrocell.com) which contains a quartz microbalance. As the nebulised fraction lands on the balance, a real time increase in mass can be seen until all the formulation has settled. Therefore, similar levels of liquid are exposed to same area of MucilAir™ irrespective of the choice of using the liquid or cloud route with both mixing with the mucus and then reaching the tissue itself. The liquid dosing is also more quantitative and simpler to perform. If refinements are required to the model, then further testing can be planned with the more complex exposure systems. A useful review of the systems is given by Primavessy *et al.* (2021)

The rat *in vivo* is not representative of a realistic exposure either. The exposure of 6 h daily may not reflect the occupational, patient or consumer exposure. The particles are generated to be small enough to reach the alveolar sac, which may not relate to the actual size of the particles. For the *in vitro* approach, much higher levels of chemicals can be exposed to the *in vitro* models than can be administered to animals *e.g.*, where the chemical (e.g., UVCB) is not volatile. In those cases, there is often no observable effect at any dose level since it is not possible to volatilise the chemical to high enough levels to result in a toxic effect. With the *in vitro* liquid dosing approach, even neat material can be applied to identify a true hazard rating. These models generate data that show the actual NoEL for hazard identification and then this can be related back to exposures which are low (low volatility) using the CFD and BMD modelling to quantify risk. This also causes validation problems as the *in vivo* test often gives a hazard classification which is identified as being higher than the achieved dose level which did not result in any observed effect. This then becomes the hazard classification, when the actual hazard may be this or a much lower level. Using these *in vitro* models and similar NAM approaches, the true NoEL values can be identified to provide more accurate hazard classifications.

There is some uncertainty that arises due to duration differences between the MucilAir™ and expected handler exposures in the Vinall (2017) study; however, the MucilAir™ exposures are considered protective since the MucilAir™ tissues were exposed for 24 hours to the chlorothalonil dilutions. This is three times longer than the expected occupational exposures (*i.e.*, 8 hour) and could have resulted in additional cell damage in the assay that may not occur during typical human exposure durations. This was confirmed to be protective in the Paulo (2020) study. The *in vivo* rat test uses exposures of 6 h followed by recovery of 18 hour. However, the rat *in vivo* model does allow for repeat chronic dosing usually 6 h daily for 5 days, 2 days recovery and then repeat to achieve the standard 28- and 90-day chronic tests. Therefore, the *in vivo* rat test has further inherent uncertainty. Choosing and justifying the exposure duration reduces the uncertainty of the assay.

Intraspecies variability was uncertain in the Vinall (2017) study. The MucilAir™ tissues only represented 5 individual healthy donors. Variability across these donors was relatively low; however, the low number of donors with a similar age range may not be considered representative of the human population. As such, the default intraspecies uncertainty factor remained at 10X. This was, again, resolved in the Paulo (2020) study with the use of the pooled donor (14 donors) MucilAir™. Choosing and justifying the number of donors or the use of pooled donors reduces the uncertainty of the assay. The *in vivo* rat test uses juvenile rats. Operators should not be juvenile workers and, as such, the human healthy donors used would be

more representative of the operator population than the juvenile rats. Furthermore, new *in vitro* test systems are coming on to the market from donors with diseases such as COPD or asthma which may represent the operator population further. Where these operators need to be considered, additional satellite test groups may be investigated. These vulnerable populations are not included in the animal tests either. Therefore, this uncertainty is lower than for the *in vivo* rat test that this replaces.

Where the objective is to protect the rat from the effects of an unknown toxicant, then the rat 3D test system should be considered, *e.g.*, MatTek rat EpiAirway™ (Hayden *et al.* 2018), alongside or instead of the human test system. By performing these tests in rat *in vitro* models, toxicity in the animal can be screened in advance prior to conducting the *in vivo* test. This can be used in improving the dose range finding resulting in less animals being used. However, this Case Study demonstrates that the human *in vitro* respiratory toxicity NAM is now the gold standard.

There are limited experimental data available to evaluate the model performance of the CFD model. Comparisons can be made with alternative modelling approaches to supplement the limited data available, *e.g.*, other CFD model simulations, multiple path particle deposition (MPPD) model (MPPD: Multiple-Path Particle Dosimetry Model - ARA) or Aerosolved (https://www.intervals.science/resources/aerosolved). However, there are several differences between the current CFD approach and experimental/ alternative modelling approaches that make direct comparisons difficult. The data indicate the current CFD model simulations for a single male were within the range observed for other CFD simulations (Keeler *et al.,* 2016), results using the MPPD model (Anjilvel and Asgharian, 1995; Asgharian *et al.,* 2001), and data using nasal moulds (Kelly *et al.*, 2005; Shanley *et al.*, 2008). Critically, CFD is used in mainstream engineering to identify fluid and gas flow as a matter of general practice which lowers the uncertainty of this model due to its widespread use in other industries.

Since the AOP relates to tissue cell damage and death, an important additional endpoint of pathology should be incorporated. The EPA SAP (EPA, 2019) identified this as an important additional endpoint. The 3D models, such as MucilAir™ and EpiAirway®, lend themselves well to pathology assessment. SDS is the positive control in this Case Study. SDS was used as a model irritant following application to MucilAir™ (Welch *et al.*, 2021). Additional endpoints included pathology, morphology, and cytokine release (IL-6 and IL-8) alongside TEER, LDH release and resazurin metabolism. By applying pathology analysis, a further area of uncertainty would be additionally reduced. Where the toxicity is different, other biomarkers may be more relevant including the use of OMICs technologies. Importantly, the primary end point in the animal tests (OECD TG 412 and OECD TG 413) is pathology. Therefore, inclusion of a veterinary pathology assessment of the human *in vitro* tissues would better reflect the animal tests and the AOP. Cervena *et al.* (2019) used reactive oxygen species (ROS) production and cell morphology changes alongside LDH and TEER. Sivars *et al.* (2018) used cytotoxicity, cell barrier integrity, viability, morphology, ciliary beating frequency (CBF), mucociliary clearance (MCC), and cytokine release for predictive accuracy for respiratory toxicity. The difference with their methodology was that % change from baseline was the critical criterion for identifying effects of the inhaled drug substances.

9.4 Uncertainty Factors Table

The following factors were considered to contribute to uncertainty to this Case Study. When applying this process to other occupational, consumer or clinical scenarios, different considerations are identified in Section 11.

Target	Uncertainty (Low, Medium, High)	Impact of uncertainty on hypothesis
Dose application; liquid *versus* aerosol	Low	None, as this is an overexposure compared to any aerosolised exposures. The AOP is contact toxicity.

Exposure duration e.g. 6, 8, 24 hour or repeated dose	Low	A decision as to whether the experiment should be chronic or acute is always needed. The exposure ties should relate to occupational exposure. In this case, it is a single overexposure with no recovery and as such, is likely to have resulted in a worst-case scenario for the overexposed cells.
Choice of model; MucilAir™, EpiAirway®, SmallAir™ *etc*	Low	This was dependant on the AOP, which was contact toxicity in the upper airway. MucilAir™ or EpiAirway® are both good choices for upper airway toxicity.
Age and sex of donors *versus* population	Low	This is poorly covered in the animal *in vivo* test which uses in-bred juvenile animals (5 per sex per group), which is less reflective of a human population. The adult healthy human derived tissues are a better reflection of the operator population, especially when the pooled donor version of MucilAir™ is used. There were 5 donors and 6 replicates per donor in the preliminary study (Vinall, 2017). For the main test (Paulo, 2020), we chose to use 5 replicates per group of the pooled donor MucilAir™. For this current Case Study, since the AOP and observed toxicity was due to contact toxicity, this variability is also not a factor.
Health status of operators (*e.g.* asthma)	Low	This is already covered in the safety margins applied and is not a normal testing approach in the standard battery of tests or in animals.
Choice of CFD model simulation	Low	This is widely used in the engineering field. This is now reported in Corley *et al.* (2021). An alternative model is MPPD (https://www.ara.com/mppd/)
Different or multiple breathing patterns could be modelled.	Low	For the current scenario, these measurements represented the operator's exertion levels for these activities. Different activities may require different breathing rates.
For future extrapolations to significantly less than 1 mg/L aerosol exposure concentrations, CFPD simulations could be repeated to confirm local dose predictions since deposited surface areas may decrease.	Low	All simulations were conducted for a single, inhaled breath associated with resting or light activities with aerosol droplets that leave the trachea not available for the return during exhalation

9.5 Strategy and Integrated Conclusion

The strategy used to develop the integrated conclusion is quite simply given in Equation 4.

CFD + *in vitro* MucilAir™ = BMD Equation 4

The benchmark dose level was determined from the CFD determinations to demonstrate where the inhaled particles would be deposited and then the *in vitro* MucilAir™ was used to determine the toxicity from those deposited particles.

Using this approach, the short- and intermediate-term inhalation MOEs for chlorothalonil ranged from 5 to 660,000, assuming baseline clothing (*i.e.*, no respirator) and were not of concern. These results compellingly verify the applicability of this testing strategy as an IATA for identification of safety respiratory toxicants in operator exposure risk assessment.

It is recommended that an understanding of the PSD and potential AOP are critical in utilising this type of testing strategy. The PSD will determine the choice of test system and the AOP will confirm the endpoints for analysis and BMD modelling. All test systems and endpoints should be thoroughly justified before initiating testing.

This Case Study provides results that compellingly verify the applicability of this testing strategy as an IATA for identification of safety respiratory toxicants in operator exposure risk assessment. This resulted in a

direct replacement of animals whilst providing a more realistic, human relevant risk assessment for chlorothalonil. It is, therefore, considered to be a good testing strategy for inhalation toxicity testing and risk assessment evaluation, provided that the PSD and AOP are understood to justify the test system and end points to be measured.

10 Considerations for Using Respiratory Toxicology NAMs

This section is intended to provide registrants, researchers, and regulatory bodies consideration for how to create a respiratory toxicology testing programme following the principles and ideas used for the creation of this current Case Study. This does not guarantee regulatory acceptance and registrants and researchers should discuss their plans with the agency or regulatory body to whom they intend to submit the data to. The work performed in this Case Study was designed for submission to the US EPA and followed discussion with the US EPA at each step. The AOP, contact toxicity, was identified and this drove the decisions taken throughout. The term "agency" refers to any government, intergovernmental agency or regulatory authority or body that regulates the safety of chemicals, crop protection products, pharmaceuticals, household products and cosmetics etc. It is recommended to locate the latest versions of all documents prior to making decisions and discussing with agencies. This is not meant to be an exhaustive list of considerations. Test article and test item is the chemical under investigation and, for simplicity, the term, test article, will be used throughout.

10.1 Regulatory Agency

Each agency has its own criteria for advising, reviewing, and accepting or rejecting any test or tests including NAMs. Therefore, early discussions with the agency will have benefits for the successful outcome of any testing programme and calculations. Differences between agencies interpretations and views include, but are not limited to, the following.

10.1.1 GLP Compliance

In this Case Study, the US EPA did not require any component of this NAM to be performed to GLP standards. Agencies are likely to require compliance to GLP standards for safety tests.

10.1.2 Dose Analysis

In this Case Study, the US EPA did not require the concentration of chlorothalonil in the dosing solutions (dilutions of Bravo 720 SC in water) to be confirmed using a suitable fit-for-purpose bioanalytical technique (e.g., LC-MS/MS, GC-MS, ICP-MS, UPLC, HPLC etc.). Other agencies may expect this to be included for either dosing solutions or nebulised chemicals or vapours, gases, or other particulates, which is standard practice for the *in vivo* OECD test guideline tests.

Note: GLP tests invariably have bioanalytical analysis of exposed doses. Therefore, this should be the same for *in vitro*, GLP-compliant, safety assessment tests.

10.1.3 BMD Modelling

The US EPA has published guidance on BMD modelling in a technical guidance document (EPA, 2012) and a manual (EPA; 2015). EFSA has published guidance for BMD modelling (EFSA; 2017a) and software for BMD modelling (EFSA, 2017b).

10.1.4 Uncertainty Factors (UFs)

The US EPA created a policy to cover the use of NAMs and chose the UFs based on decisions within that policy. Other agencies will apply different UFs based on either published or unpublished policy or following review of the data submitted. Typical UFs include but are not limited to the following examples.

- UF_A to cover the interspecies uncertainty for animal-to-human extrapolation.
- UF_H to cover the intraspecies uncertainty for differences in sensitivity among humans.
- UF_{TK} to cover the uncertainty in toxicokinetics, although in this Case Study, this referred to the deposition of the chemical into the human respiratory tract which was identified by the CFD calculations.
- UF_{TD} to cover the uncertainty in toxicodynamics across the population.

It is outside the scope of this section to identify all uncertainty factors or to identify what those factors may be for each agency, although these are typically, but not limited to 1X, 3X and 10X.

10.1.5 Level of Concern (LOC)

The US EPA has identified a LOC of 3 above which is not considered to be of concern. Other agencies may apply different LOCs based on either published or unpublished policy or following review of the data submitted. It is outside the scope of this section to identify these LOC values.

10.1.6 Margin of Safety (MOS)

Each agency or regulatory body has its own published or unpublished values for MOS. It is outside the scope of this section to identify these MOS values.

10.2 Choice of CFD Model

There are different CFD models to choose from including creation of a bespoke model for the NAM in question. The CFD model used in this NAM is published (Corley *et al.* 2021). The Multiple-Path Particle Dosimetry Model (MPPD) is a freely available software which is downloadable at https://ara.com/mppd/. Aerosolved is also a free software available for download at https://www.intervals.science/resources/aerosolved.

10.3 Choice of PSD Model

The PSD model chosen is published as Flack *et al.* (2019).

10.4 AOP

Where possible, the AOP should be identified. Existing AOPs are identified in https://aopwiki.org/aops. A new AOP or AOPs may need to be identified.

10.5 Study Design

There are many components to creating a suitable study design. The most important are to identify the purpose of the work *i.e.*, generation of data for a risk assessment or for hazard identification and classification. Critical factors to consider and justify are exposure times, occupational/ consumer/ patient exposure scenarios, washout periods and acute *versus* chronic exposures. Clippinger *et al.* (2018b) identified acute as up to 24 h and chronic as longer than 24 h and this definition is used here. Although most uses of MucilAir™ have been short, Cervena et al. (2019) performed experiments for up to 28 days. The additional considerations and complexities required for these longer duration experiments are not discussed here. It is assumed that the laboratory performing these experiments follows good aseptic techniques alongside good cell husbandry procedures. Cell husbandry, choice of media and other basic information is usually available from the model manufacturers or may be created during the development of the NAM.

For hazard identification, a short-term exposure with high concentrations, or even neat (undiluted), of test article will be exposed to the tissue. These concentrations may exceed the levels to which an operator or consumer may be exposed to due to the chemical volatility. This is a problem with hazard identification with animals as the classification is often given as the maximum dose achieved. Using this NAM, it will be possible to identify the true hazard for each chemical.

For risk assessment, the standard animal test approach (OECD 412, 2018a and OECD 413, 2018b) is to expose the animal to very small particles (usually smaller than those created in an occupational, consumer or patient scenario) for 6 h every day. These small particle sizes are created to ensure the inhaled material reaches the alveolar tissue. The chronic testing is typically 6 h per day for 5 days with 2 days recovery for 28 or 90 days or daily for 28 or 90 days. These testing scenarios do not take in use exposure scenarios into consideration.

The next generation risk assessment approach is to identify the exposure and then replicate it. This will be different for a commercial operator spraying a crop protection product, a consumer spraying a pesticide on the lawn or a consumer using a deodorant spray or a perfume or a patient inhaling a drug. For example, the consumer may only be exposed to the deodorant spray for a few seconds, the consumer wears a perfume all day (24 h), the commercial operator may spray the crop for 8 h in a day and the consumer may only be spraying the lawn for an hour. It would be possible to generate experiments to identify these exposures. The patient may inhale a single short, metered dose from an inhaler once or twice a day or undergo a continuous infusion through a facemask.

The chronic exposures are difficult to perform in the human 3D tissue models as the cells grow very slow and there are high risks to the cells being contaminated, not necessarily by the analysts, but more likely by the test article formulations which may not be possible to prepare aseptically or may not be able to be treated to be microbiologically clean. Currently, there is good evidence to show that these models can be used for 2 weeks by most competent labs. The 2 week experimental period may be enough for many chronic scenarios. Additional control tissues may be needed such as blank (formulation without test article), double blank (*i.e.*, physiological saline, but undergoes the same processing) and triple blank (*i.e.*, no exposure and no processing) as well as any necessary tissue controls required for the assays. Cervena et al (2019) successfully performed their experiments over a 28-day duration.

It is also important to note that these chronic exposures will be different to those performed in the animal tests (*e.g.*, OECD 412 & OECD 413). Additionally, the data from the animal studies may also not have been using the correct particle size (as small particles are generated to reach the lower ling of the rat) or high enough concentrations cannot be formed in nebulisers to observe toxicity. There are also the species differences as well as the differences between whole animal (*in vivo*) and cells (*in vitro*). Therefore, any comparisons or *in vitro - in vivo* correlations (IVIVC) between these tests will be almost impossible to make. Where improvements in IVIVC are required, animal versions of the human *in vitro* models have been

developed, such as the MatTek rat EpiAlveolar® model (Hayden *et al.*, 2018). There are currently other models under development.

10.6 Test Article/ Formulation

This Case Study used the crop protection pesticide test article, chlorothalonil, tested in the commercial formulation, Bravo 720 SC (diluted with water to replicate spray dilutions of Bravo 720 SC). As one of the justifications for creating this NAM was the big differences in biology and physiology between the human and the rat, this NAM can be applicable to any chemicals including crop protection products, pharmaceuticals, chemicals, cosmetic chemicals, fragrances, and household chemicals. Ideally, the formulation should be the same as that which the human (operator, patient, or consumer) may be exposed, *i.e.*, the commercially available formulation. This is important when performing a risk assessment. When using these models for hazard classification, the pure chemical (or UVCB) should be used, *i.e.*, the chemical entity for which a hazard is to be identified.

10.7 Choice of Test System

The test system choice will depend on the particle size distribution which is the main factor in identifying where a particle may be deposited within the human respiratory tract. The particle type, e.g., water, liquid, solid, shape, size and size distribution are all factors that affect this deposition. In general, very large particles will reach the nasal region only whereas very small particles or vapours and gases will reach the alveolar region. The CFD modelling of the measured PSD identifies the regions where deposition may occur in the human (and/or animal, depending on the CFD model used and the test requirements). In the Case Study, the deposition was demonstrated to be in the nasal and upper airways only from PSD and CFD analysis. When this work was initiated, in 2014, the only model available was MucilAir™, which is the human nasal derived model. It is the nasal tissue in the rat, which is most sensitive to contact toxicity, therefore, serendipitously, this was the correct choice of test system.

The following tissue models are available and as innovation continues in this area, there will be additional models to consider as well. Additional information is also summarised in Clippinger *et al.* (2018b). The suppliers and their models are identified as follows.

- Upper airway:
  - Epithelix MucilAir™, MatTek EpiAirway®
- Conducting/ bronchiole:
  - Epithelix SmallAir™
- Alveolar:
  - MatTek EpiAlveolar®
  - ImmuONE ImmuLUNG™
  - Invitrolize ALIsens

There are many human derived cellular models. They do not have the complex structure or biological complexity of the 3D models. However, they do have relevance for certain uses, such as respiratory sensitization, immune responses, or mechanistic questions. Examples are included below.

- Macrophage:
  - ImmuPHAGE™
  - Alveolar models with macrophages as co-culture
- Sensitization:
  - SenzaGen GARD®air
  - ImmuONE ImmuPHAGE™
  - Invitrolize ALIsens

Human primary cells (*e.g.*, human bronchial epithelial (HBE) cells or small airway epithelial cells (SAEC)) or human-derived cell lines (*e.g.*, BEAS-2B, Calu-3, A549, or NCI-H292) may also be appropriate, especially where there is AOP to support a simpler testing approach. This is further discussed in Clippinger *et al.* (2018b). Human precision cut lung slices may also be a suitable test system. This is described by Lui *et al.* (2019).

10.8 Number of Replicates or Donors

OECD 412 (2018), Summary states "At a minimum, the main study comprises groups of 5 male and 5 female rodents…". Rats are "young adults 7 to 9 weeks of age" (OECD 412, 20118; p4) juvenile and inbred. Currently, the manufacturers create models using adult donors only, which makes these models more relevant to occupational and consumer use scenarios where the users are adult. Epithelix offer limited numbers of individual donors and pooled donor pool options.

In the current Case Study, both experiments used 6 replicates, but there were differences. Vinall (2017) used 5 individual donors with 6 replicates per donor (30 MucilAir™ tissues per dose level). There was discussion about this at the EPA SAP (2018) and clarified in the report (EPA, 2019). It was agreed that the pooled donor MucilAir™ was advantageous as it utilised a larger population and a similar pooled donor approach is used in *in vitro* metabolism studies. This reduced the number of MucilAir™ dose level down to 6 in Paulo (2020).

Therefore, it would be reasonable to use at least 5 replicates. Since there are limited donor options available from some suppliers, then the following should be considered appropriate:

- If only 1 donor is available, then use 10 replicates per dose level.
- If only 2 donors are available, then use at least 3 replicates per donor, *i.e.*, at least 6 replicates per dose level.
- If pooled donor models are available, use at least 5 replicates per dose level.

Where the AOP, mode of action, or envisioned purpose of the approach does not require consideration of intra- or inter-individual variability, such as the current case study with contact toxicity, then the number of replicates could be, justifiably, reduced.

10.9 Liquid *versus* Vapour, Gaseous and Nebulised Exposure

In the current Case Study and other examples (e.g., Welch *et al.*, 2021), the formulations were applied as liquids whereas the occupational exposure would be to the fine spray or water droplets. This was extensively discussed between the registrant, Syngenta, and the US EPA. The liquid dosing results in a known, controlled, application and is used in other OECD tests (such as ocular irritation, and skin irritation and skin corrosion). The aerosol/ nebuliser exposure is difficult to control to provide a precise quantitative exposure (as we also know this is the case *in vivo* with a lot of material not being inhaled by animals which may be ingested, become exposed to the skin, or lost completely). As there is no exhalation in this *in vitro* model, there is only build of spray or liquid whichever way the tissue is exposed. In addition, if the water spray particles were generated in the in vitro system, for example by use of a spray device or nebuliser, they will condense onto the mucous, coalesce and mix with the mucous before eventually reaching the tissue. Therefore, this route of dosing was identified to be worst-case exposure scenario and protective.

There will be good examples for when the exposure should be following nebulisation of the formulation or test article, such as powders or nanoparticles, which also cause physical damage and nanoparticles which cannot be dissolved into a dosing solution (e.g., water, physiological saline, mineral oil, olive oil etc). These

particles could be applied directly as the powder in a spatula where precise weighing is possible, similarly to the exposures for the *in vitro* skin irritation (OECD, 2020a), and eye irritation (OECD, 2019b) tests.

When performing the exposures, similar procedures will be employed to generate the particles as are used in the *in vivo* tests. Therefore, it is not considered to be a great technological leap for experienced technical teams. There are different exposure systems such as the Vitrocell cloud chamber, the Vitrocell smoking robot, twin stage impinger or a device lab created by the lab specifically for the purpose of producing an exposure system relevant to the risk assessment. For a useful review, see Primavessy *et al.* (2021).

10.10 Choice of Endpoints

The AOP is critical in identifying the endpoints to consider and choose. Although there may be insufficient information on the toxicity of the chemical in which case some default endpoints are recommended. For the *in vivo* tests (such as OECD 412 & OECD 413), the primary endpoint is pathology. Therefore, pathology should be considered initially and where there is no AOP, this endpoint is essential. The current Case Study did not need to use pathology as the LDH release and resazurin biomarkers confirmed cell death which is related to respiratory irritation. Pathology is less sensitive than the biomarkers as cell death occurs before this can be observed by a veterinary pathologist. Each endpoint should relate to the AOP or any known toxicities that have been observed elsewhere. There are continuous innovations and developments in pathology slide stains and techniques. Similarly, new biomarker tests are being developed which are used in add on tests within both animal and non-animal model testing. The following is not an exhaustive list of endpoints, but instead a reasonable list of known endpoints available today.

<u>Pathology</u>

- H&E stain
- Vimentin; fibrosis and cancer
- Mucociliary clearance, cilia beating frequency and cilia loss
- Cell morphology

<u>Biomarkers</u>

- Barrier Integrity or Epithelial Functionality
    - TEER
    - Paracellular permeability (Papp)
    - (Pathology)
- Cell Death
    - LDH Release
- Cell Stress (may be identified to relate to cell viability)
    - Mitochondrial activity
        - MTT, MTS, WST-1, resazurin
        - Alamar blue/ Presto blue
    - Oxidative stress
    - Response to DNA damage
- Immunology
    - Cytokine production
        - Interleukins (Welch *et al*. 2021)
    - Immune cell function
        - Phagocytosis
- Fibrosis
    - Fibronectin
    - Pro-Collagen I α1
- Genetox
    - Comet
- Omics

10.11 Drug Delivery or Chemical Absorption

This can be used to make predictions of systemic delivery and/ or absorption which may be then used to identify toxicities in other tissues such as the liver and kidney or efficacy in drug evaluations. The media is easily removed at different time points and the test article can be determined in the media using suitably validated analytical methods. This approach is well established in other areas, such as dermal absorption (OECD, 2004).

10.12 At Risk Populations: Children and Diseased Adults

The current animal test approach accounts for these populations with the use of the UFs. However, the NAM approach allows us to identify ways to prioritise these populations with or without the use of UFs. These are example approaches and further NAMs may be developed based on these and other ideas. The CFD model can be reparametrized to the exposure in children and has been described (Tsega, 2018 and Su *et al.* 2020). Human disease derived respiratory models have been created by Epithelix and MatTek. These are models based on the standard models; MucilAir™ and EpiAirway®, except that the tissues are obtained from patients with well characterised diseases such as COPD (Chronic Obstructive Pulmonary Disease), asthma, cystic fibrosis (several mutations), allergic rhinitis and smokers. *In vitro* PDX models have been developed on the MucilAir™ platform (OncoTheis). Other disease models may be available and are continually being developed.

10.13 Animal Test Methods

The choice of using the rat or animals should be the last resort due to the weaknesses identified in the sensitivity of the rat, differences in cell populations, physiology, and the greater complexity of the nasal turbinates of the rat compared to the human and the greater complexity of the lower lung in the human as already described in this Case Study.

In line with the 3Rs (Russell and Burch; 1959), the animal should be the final choice and not the default first choice. However, there will be cases where insufficient data is generated using NAM approaches. Where this is chosen, reduction and refinement should be further considered based on information generated from the NAM testing programme. Therefore, a reduced OECD 412 or OECD 413 test may be appropriate to answer the questions which could not be answered using the NAMs developed.

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Annex

The standard regulatory test that has been replaced by this Case Study is the Subchronic Inhalation Toxicity: 90-day Study (OECD TG 413, 2018b). A summary of this test is given below to illustrate the no of animals not used and the designs for this test.

References	OECD (2018). Test No. 413: Subchronic inhalation toxicity: 90-day study
Species/strain	Rat (preferred)
Sex	Male and female
Age range	7-9 weeks on randomization to test groups
Doses	At least 4 concentrations (1 producing no effect)
No of animals	At least 10 male and 10 female animals per dose group
Satellite groups	A satellite group of 5 males per concentration (*i.e.*, at least 20 animals)
Control animals	Filtered air exposed animals
Administration route	Inhalation
Exposure period	6 hours per day, 5 days per week, 90 days (others may be justified)
MMAD	=2 µm with GSD of 1-3 for rats. Note, this may not relate to the human exposure.