Defined Approach for Detection of Eye Irritants and Corrosives for Pesticide Formulations

N Choksi[1], AJ Clippinger[2], S Gehen[3], M Corvaro[4], S Ng[5], SN Kolle[6], A van Cott[7], A Hofstra[8], M Inforzato[9], N Ryan[10], E Webb[11], W Casey[12], D Allen[1]

[1]ILS, RTP, NC, USA; [2]PISC, Philadelphia, PA, USA; [3]Corteva Agriscience, Indianapolis, IN, USA; [4]Corteva Agriscience, Rome, Italy; [5]The Chemours Company, Wilmington, DE, USA; [6]BASF SE, Ludwigshafen, Germany; [7]BASF Corp, RTP, NC, USA; [8]Syngenta Canada Inc, Guelph, ON, Canada; [9]Syngenta Crop Protection LLC, Greensboro, NC, USA; [10]Bayer Crop Science, RTP, NC, USA; [11]Bayer Crop Science, St. Louis, MO, USA; [12]NIH/NIEHS/DNTP/NICEATM, RTP, NC, USA.

Introduction

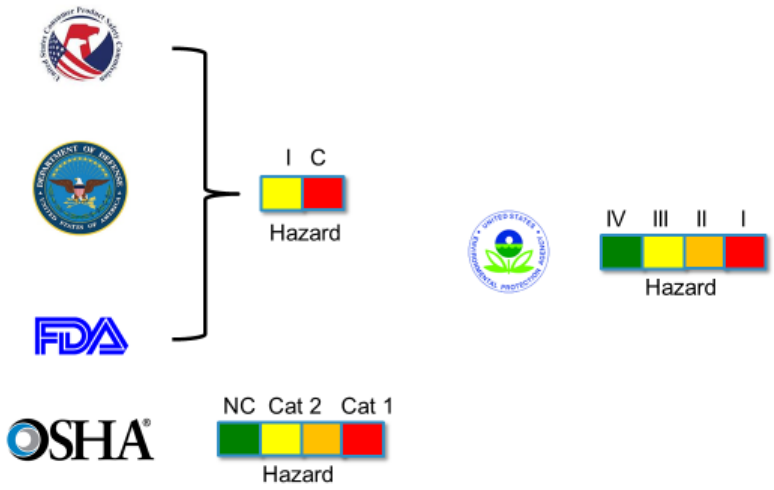
- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) developed "A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States" that describes three strategic goals:
    - Connect end users with developers of new approach methodologies
    - Foster the use of efficient, flexible, and robust practices to establish confidence in new methods
    - Encourage the adoption and use of new methods and approaches by federal agencies and regulated industries

- One approach to establishing confidence in new methods is through public-private partnerships. These allow cross-sector communication and cooperation among federal agencies and the private sector, to facilitate sharing knowledge, experience, and data.
- In conjunction with PETA International Science Consortium Ltd. (PISC), the U.S. Environmental Protection Agency (EPA), and CropLife America companies, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is coordinating a three-phase study to:
    - Assess the applicability of seven *in vitro* eye irritation/corrosion methods to pesticide formulations

- Develop a defined testing approach for prediction of U.S. and international irritancy classifications

U.S. and International Irritancy Classifications

- Eye irritation data are used by U.S. and international agencies to assess human ocular health hazard.
- Data may be used to develop precautionary labels related to protective clothing requirements for applicators.
- The figure below provides a general overview of classifications systems used at individual U.S. agencies.
  - Color coding scheme indicates relative level of human hazard (i.e., red category is ocular corrosive; green category is ocular non-corrosive/minimal irritant).
  - Different classification schemes at agencies are based on different regulatory needs.



Study Design and Logistics

- Test formulations were selected to
  - Include a range of hazard classifications according to the EPA and UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS) classification systems
  - Include suspension concentrate, emulsifiable concentrate, and soluble liquid formulation types

    - Support comparison to high-quality *in vivo* data

- Coded formulations, donated by companies listed below, were distributed by the National Toxicology Program to the testing laboratories.
    - BASF
    - FMC
    - Monsanto (now Bayer Crop Science)
    - Dow-DuPont (Corteva Agriscience)
    - Syngenta
- Phase goals (**Table 1**):
    - **Phase 1:** Initial testing with EPA Category I/GHS Category 1 and EPA Category IV/GHS Not Classified formulations to identify test methods for inclusion in later phases
    - **Phase 2:** Expand testing to include formulations classified as EPA Category II/III and GHS Category 2 to refine test methods for potential use in a defined approach
    - **Phase 3:** Greater expansion of formulation categories in test methods identified for incorporation in a potential defined approach for ocular irritation classification
- **Table 2** lists the methods utilized, the applicable Organisation for Economic Co-operation and Development (OECD) test guidelines (TG), and the laboratories conducting each test.

Table 1 Study Phases

Phase	Activities	Completion Dates
Pre-Study Phase	• Formation of stakeholder study group o Scientists representing ICCVAM agencies, industry, and international regulatory and non-governmental organizations o Assist with formulation procurement, study evaluation, and data review • Selection of *in vitro* test methods	March 2018
Phase 1	• Testing of six formulations (three EPA Category I/GHS Category 1 and three EPA Category IV/GHS Not Classified formulations) in all *in vitro* test methods	September 2018
Phase 2	• Testing of 10 formulations in all *in vitro* test methods	March 2019
Phase 3	• Testing of approximately 30 formulations in selected *in vitro* test methods	September 2019

Table 2. *In Vitro* Methods Used in Prospective Testing

Test Method	OECD TG	Testing Laboratory
Bovine Corneal Opacity and Permeability	OECD TG 437	Institute for In Vitro Sciences
Neutral Red Release	-	Institute for In Vitro Sciences
Isolated Chicken Eye	OECD TG 438	Citoxlab
EpiOcular (EO) (EIT method)	OECD TG 492	MatTek
EO (Time-to-toxicity method; ET50-neat protocol)	-	MatTek
EO (Time-to-toxicity method; ET50-dilution protocol)	-	MatTek
Porcine Cornea Reversibility Assay	-	MB Research Labs

In Vitro Methods Background

Bovine Corneal Opacity and Permeability

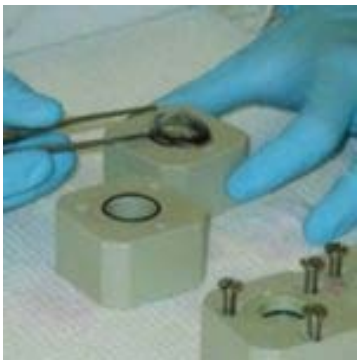


Image from Institute for In Vitro Sciences

- Bovine corneal tissue, obtained as a byproduct from a slaughterhouse, is mounted in chamber.
- Formulations are applied to the epithelial surface of the cornea.
- After designated exposure period, two endpoints are assessed.
    - Opacity – determined by light transmission through cornea
    - Permeability – determined by amount of fluorescein dye that penetrates through cornea
- Irritancy classification
    - *In vitro* irritancy score (IVIS) is calculated as mean opacity + (15 × mean permeability).
    - Histopathology is used to analyze the degree and depth of corneal damage.
    - If conflicting classifications are obtained from IVIS and histopathology evaluation, the more severe classification is used for irritancy classification.

Neutral Red Release


Image from Institute for In Vitro Sciences (https://iivs.org/testing-services/assays/cytotoxicity/neutral-red-uptake/)

- Cultured normal human epidermal keratinocytes are pre-exposed to neutral red medium.
- After pre-exposure, dilution series of test formulation is applied for 1 minute to culture surface and then removed.
- Neutral red release by cells is measured spectrophotometrically.
- Irritancy classification

- Cytotoxicity is measured at each concentration.
- Concentration that causes 50% neutral red release (NRR50) is determined for classification.

Isolated Chicken Eye

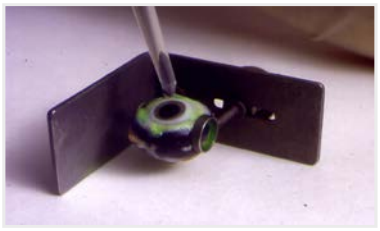
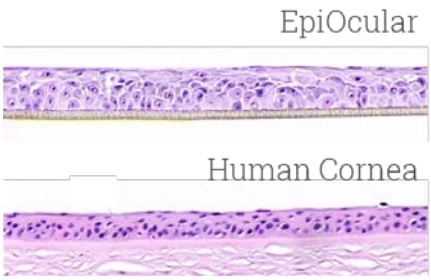


Image from Menk Prinsen, TNO

- Freshly isolated chicken corneas, obtained as a byproduct from a slaughterhouse, are mounted.
- Formulation is applied for 10 seconds to the corneal surface and then rinsed off.
- Four endpoints are assessed at pre-defined time points up to 240 minutes after exposure.
  - Thickness – determined by amount of swelling using an optical pachymeter on a slit-lamp microscope
  - Opacity – determined by light transmission through cornea
  - Integrity – determined by fluorescein retention
  - Morphology – determined by visual inspection of the eye
- Classification for each endpoint is determined.
- Irritancy classification
  - A combination of endpoints is used to determine hazard classification according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS).
  - Histopathology is used to analyze the degree and depth of corneal damage.
  - If conflicting classifications are obtained from GHS hazard classification and histopathology evaluation, the more severe classification is used for irritancy classification.

EpiOcular: EIT Method



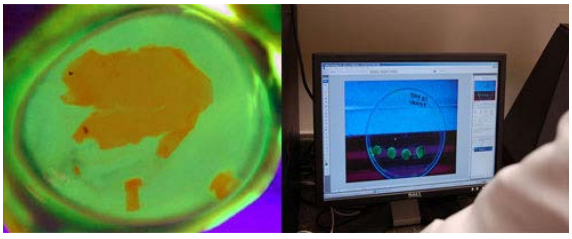
Images from MatTek Corporation (https://www.mattek.com/products/epiocular/)

- Nonkeratinized epithelium is prepared from normal human keratinocytes.
- Cells are seeded in an insert that contains a porous membrane to allow nutrients to reach the cells.
- Formulation is applied for a pre-defined exposure period and then rinsed off.
- Irritancy classification
    - Cell viability is measured after exposure and a post-exposure incubation period using a vital dye (e.g., MTT).

EpiOcular: Time-to-toxicity Method

- The same cell construct and application procedure as EIT method is used.
- Two different protocols are used to assess toxicity:
    - **Neat Protocol:** Formulations tested undiluted and tissue viability measured at pre-defined time points up to 60 minutes after application
    - **Dilution Protocol:** Formulations tested at 20% concentration and tissue viability measured at pre-defined time points up to 256 minutes after application
- Irritancy classification
    - Cell viability is measured at different time points for each protocol.
    - Data are used in a decision tree to determine hazard labeling.

Porcine Cornea Reversibility Assay


Images from MB Research Labs (http://www.mbresearch.com/porcora.htm)

- Excised porcine corneal tissues, obtained as a byproduct from a slaughterhouse, are cultured in plates.
- Tissues are exposed to formulation for 5 minutes.

- Fluorescein stain is used to visualize tissue damage.
- Irritancy classification
    - Area of damage is assessed over three weeks
    - Data used to determine potential reversibility of formulation-induced damage

Phase 1 Results

- Phase 1 formulations were categorized as EPA Category I/GHS Category 1 or EPA Category IV/GHS Not Classified based on historical animal data.
- **Table 3** lists the classification criteria for each *in vitro* test method.
- No single test method assigned a correct classification for all six pesticide formulations, but none misclassified all tested formulations (**Table 4**).
- All methods are included in Phase 2, where 10 formulations that represent a range of eye irritancy classifications will be evaluated.

Table 3. Phase 1 Results Classification Key*

	EPA Category IV/GHS Category NC	EPA Category I/GHS Category 1
BCOP-OECD	IVIS ≤3 and histopathology classifies as EPA Category III or IV/GHS Not Classified	IVIS >55 or histopathology classifies as EPA Category I/GHS Category 1
NRR	NRR50 >250 mg/mL	NRR50 <50 mg/mL
ICE-OECD	GHS Not Classified and histopathology classifies as No Prediction	GHS Category 1 or histopathology classifies as GHS Category 1
PorCORA	NA	Irreversible
EO-OECD	Tissue viability >60%	NA
EO-neat ET50	ET50 ≥60 min	NA
EO-dil. ET50	ET50 ≥256 min	NA
EO-CON4EI	GHS Not Classified	GHS Category 1

Abbreviations: BCOP = bovine corneal opacity and permeability; CON4EI = Consortium for *In Vitro* Eye Irritation Testing Strategy Project; dil. = dilution protocol; EO = EpiOcular; ET50 = exposure time required to reduce tissue viability to 50%; ICE = isolated chicken eye; IVIS = *in vitro* irritation score; NA = not applicable; NC = not classified; NRR = neutral red release; PorCORA = procine cornea reversibility assay.

*BCOP-OECD, ICE-OECD, and EO-OECD classifications based on decision criteria present in OECD test guidelines for individual test methods. Histopathology classification criteria for BCOP and ICE, and classification criteria for NRR, EO-neat ET50, and EO-dil. ET50 were based on criteria utilized by each testing laboratory. EO-CON4EI classification based on decision tree shown below:

EO-CON4EI Classification Decision Tree

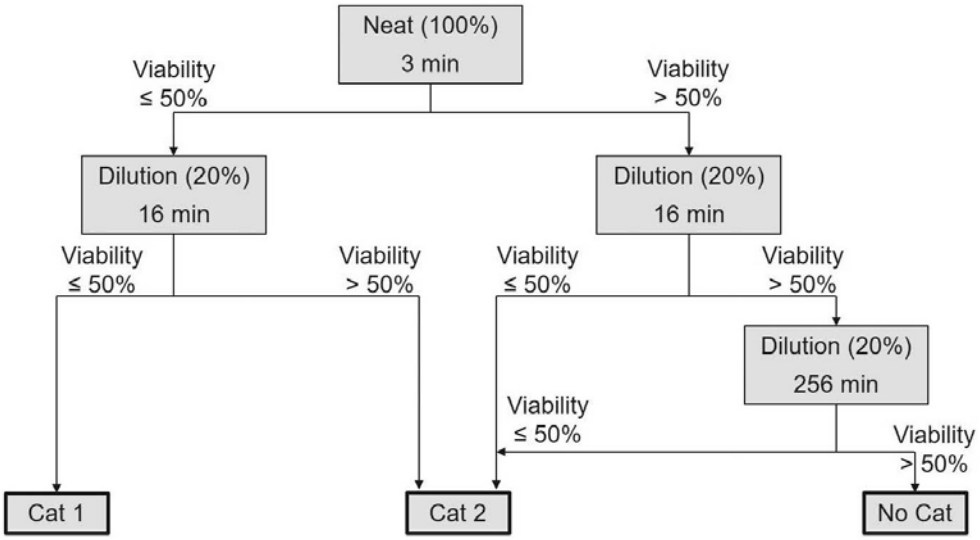


Image from Kandarova et al. 2018. Toxicol In Vitro, 49:34-52.

Table 4. Phase 1 *In Vitro* Classification Results Relative to *In Vivo* Classification Results

Table 4a. EPA Category IV/GHS Category NC

	Formulation A	Formulation B	Formulation C
BCOP-OECD[1]	Agree	Agree	Agree
NRR[2]	Disagree	Agree	Agree
ICE-OECD[3]	Disagree	Agree	Disagree
PorCORA[4]	No Prediction	No Prediction	No Prediction
EO-OECD[2]	Agree	Agree	Agree
EO-neat ET50[5]	Agree	Agree	Agree
EO-dil. ET50[5]	Agree	Agree	Agree
EO-CON4EI[6]	Agree	Agree	Agree

Table 4b. EPA Category I/GHS Category 1

	Formulation D	Formulation E	Formulation F
BCOP-OECD[1]	Agree	Disagree	Agree
NRR[2]	Agree	Agree	Agree
ICE-OECD[3]	Disagree	Disagree	Agree
PorCORA[4]	Agree	Agree	Disagree
EO-OECD[2]	No Prediction	No Prediction	No Prediction
EO-neat ET50[5]	No Prediction	No Prediction	No Prediction
EO-dil. ET50[5]	No Prediction	No Prediction	No Prediction
EO-CON4EI[6]	Disagree	Disagree	Agree

Abbreviations: BCOP = bovine corneal opacity and permeability; CON4EI = Consortium for *In Vitro* Eye Irritation Testing Strategy Project;
dil. = dilution protocol; EO = EpiOcular; ET50 = exposure time required to reduce tissue viability to 50%; ICE = isolated chicken eye;
NRR = neutral red release; PorCORA = procine cornea reversibility assay.

Color key: Green = *in vitro* method correctly classified the test formulation; Red = *in vitro* method incorrectly classified the test formulation; Orange = *in vitro* classification does not allow for definitive classification of formulation in either category (e.g., EO-OECD classification system indicates no classification prediction can be made when tissue viability ≤60%; therefore, formulations that produce this response cannot be classified using the EO-OECD classification system).

[1]Classification based on most severe response obtained from IVIS or histopathology results. IVIS and histology classifications consistent for Formulations A-C. Histology classification showed greater level of irritation than IVIS for Formulations D and F.
[2]Classification based on most severe response obtained in two runs.
[3]Classification based on most severe response obtained from ICE score or histopathology results.
[4]Classification based on reversibility.
[5]Classification based on most severe response obtained in 2-3 runs.
[6]Classification based on decision tree presented in Kandarova et al. 2018. (Toxicol In Vitro 49:34-52). Mean of all runs used for decision tree calculations.

Conclusions and Future Directions

- Phase 1 results showed that no single test method could be used to assign a correct classification for all six pesticide formulations relative to their *in vivo* classifications. Results suggest that combining results of multiple tests in an integrated approach may be useful in classification of these formulations (e.g., within the confines of the current decision criteria the EpiOcular method correctly classified all the Category IV/NC formulations and the Neutral Red Release method correctly classified all the Category I/1 formulations).
- Phase 2 testing is currently ongoing; pesticide formulations with a broader range of eye irritancy classifications than Phase 1 are being tested using all *in vitro* methods.
- Based on Phase 1 and 2 results, one or more of the test methods may be used in Phase 3 to test an expanded set of pesticide formulations. The outcomes of this analysis will suggest endpoints that can form the basis of a defined approach for pesticide formulations testing for eye irritation/corrosion potential.

Acknowledgements

This project was funded in whole or in part with federal funds from the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN273201500010C.

The views expressed above do not necessarily represent the official positions of any federal agency. Since the poster was written as part of the official duties of the authors, it can be freely copied.

Subscribe to the NICEATM News Email List

To get announcements of NICEATM activities, visit the NIH mailing list page for NICEATM News at https://list.nih.gov/cgi-bin/wa.exe?SUBED1=niceatm-l&A=1 and click “Subscribe”