

Use of *In vitro* and *Ex vivo* Eye Irritation/ Corrosion Test Methods for the Toxicity Assessment of Pesticides

4th Webinar in the Series on the Use of New Approach Methodologies for the Risk Assessment of Pesticides

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2 June 2023

Eye Exposure to Agrochemicals

- Occurs by accidental splashing or exposure to chemical particles, vapors or gases.
- Substance contacting the surface of the human eye, naturally gets expelled in less than 2 min (more than 80%). So short term exposures assays were developed for extrapolation of toxicological effects.
- The traditional regulatory test method for Ocular Toxicity Evaluation is *in vivo* rabbit Draize test (OECD TG 405).

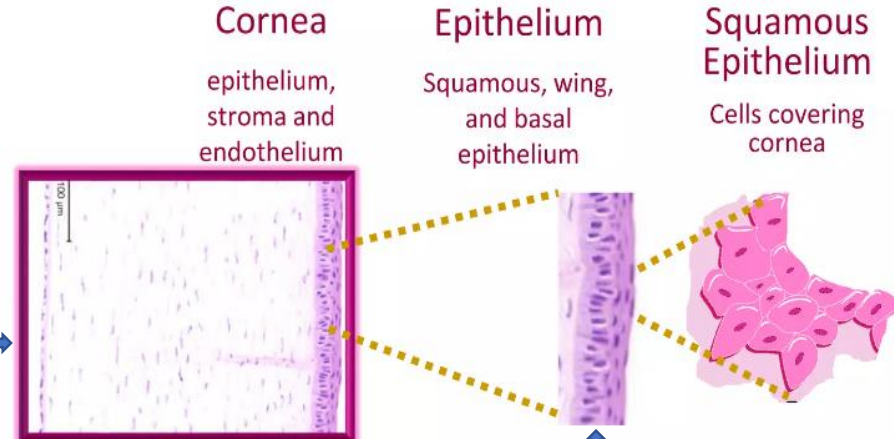
Eye Damage Classification

UN GHS Category	Classification Criteria	US EPA Category
Cat 1	Serious eye damage /irreversible effects on the Eye irritation / reversible effects on the eye (fully reverses the effects within observation period of 21 days)	I
Cat 2A	Eye irritation / reversible effects on the eye (fully reverses the effects within observation period of 21 days)	II
Cat 2B	Mild Eye irritation / reversible effects on the eye (fully reverses the effects within observation period of 7 days)	III / IV
No Cat	Non-Corrosive/ Nonirritant/ not classified	

Eye Damage and Irritation Predicting Methods

Organotypic Ex vivo Assays:

1. BCOP LLBO test
2. Isolated Chicken Eye (ICE) test
3. Isolated Rabbit Eye (IRE) test
4. Porcine cornea opacity/reversibility assay (PorCORA)
5. Ex Vivo Eye Irritation (EVEIT) test



Cytotoxic and Cell Function Based In vitro Assays:

1. Short Time Exposure (STE) in vitro test
2. Fluorescein Leakage (FL) test
3. Neutral Red Release (NRR) test
4. Cytosensor Microphysiometer (CM) test

Reconstructed 3D Human Tissue Assays:

1. Reconstructed Human Cornea-like Epithelium (RhCE) test
2. EpiOcular Time-to-Toxicity (ET50) protocols using RhCE models and EYEIRR-ISVR
3. LabCyte CORNEA MODEL24 (EIT)
4. Vitrigel-Eye Irritancy test (EIT)

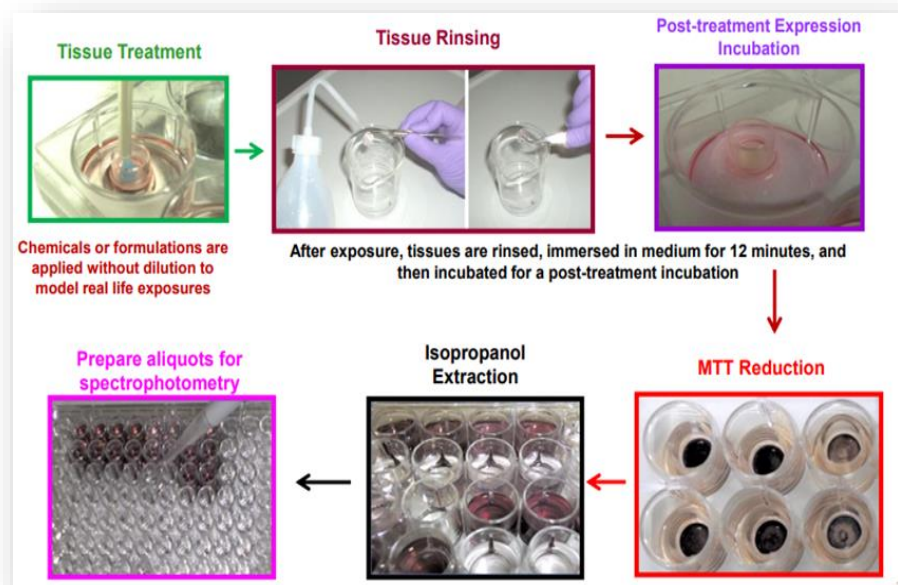
Macromolecular Matrix Assays:

1. Ocular Irritation (In Chemico)
2. Optisafe test

Reconstructed 3D Human Tissue assays

Assay	Reconstructed Human Cornea-like Epithelium (RhCE) test	EpiOcular Time-to-Toxicity (ET50) protocols using RhCE models and EYEIRR-ISVR
Validity	Validated, OECD 492	Validated, OECD 492 B
Test System	Reconstructed non-keratinized multi-layered human corneal epithelium	Reconstructed non-keratinized multi-layered human corneal epithelium
Exposure	1-30 min for liquids (neat) •3-24 hours for solids (neat)	liquids: 5 min neat, 16 min 20% w/v, 120 min 20% w/v •solids: 30 min neat, 120 min neat
End point	Cytotoxicity (MTT)	Cytotoxicity (MTT)
Applicability	UN GHS NC- all types of chemicals, including agrochemical formulations	UN GHS Cat 1, 2 and NC

Assay	LabCyte CORNEA MODEL24 EIT
Validity	Optimised
Test System	Me-too assay to Rh CE
Exposure	1 min for liquids & 24h for solids
End point	Cytotoxicity (MTT, WST-8 or WST-1 assay)
Applicability	UN GHS Cat 1, 2 and NC

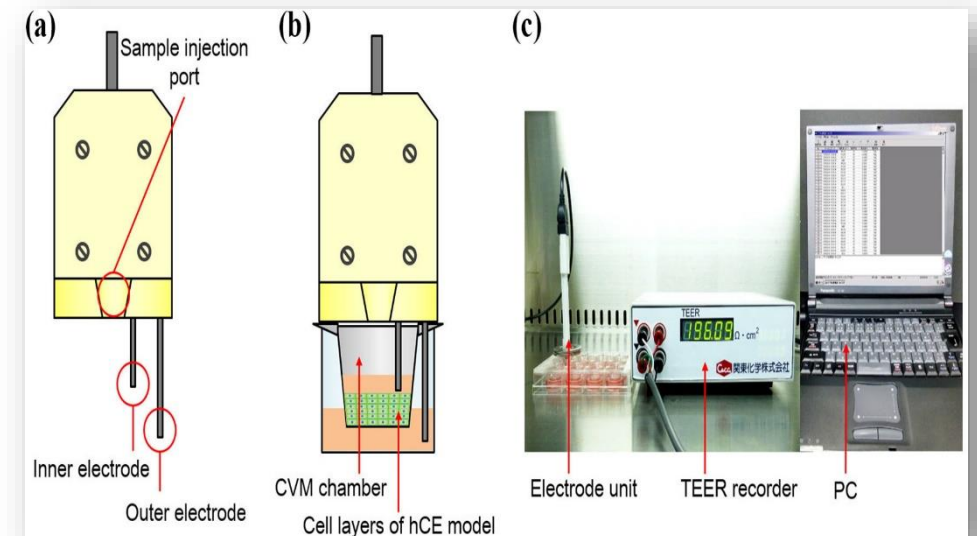


Reconstructed 3D Human Tissue assays

Test Method	No Category	No prediction can be made
EpiOcular TM EIT (for both protocols)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
SkinEthic TM HCE EIT (for the liquids' protocol)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
SkinEthic TM HCE EIT (for the solids' protocol)	Mean tissue viability > 50%	Mean tissue viability ≤ 50%
LabCyteCORNEA-MODEL24EIT (for both protocols)	Mean tissue viability > 40%	Mean tissue viability ≤ 40%
MCTT HCE TM EIT (for the liquids' protocol)	Mean tissue viability > 35%	Mean tissue viability ≤ 35%
MCTT HCE TM EIT (for the solids' protocol)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%

	No Category	Category 2	Category 1
SkinEthic HCE TTL (for the liquids protocol)	Mean tissue viability > 50% within all-time treatments	Any other combination of values ¹	Mean tissue viability ≤ 50% within all-time treatments
SkinEthic HCE TTS (for the solids protocol)	Mean tissue viability > 40% after 30 minutes and > 60% after 120 minutes	Any other combination of values ¹	Mean tissue viability ≤ 40% after 30 minutes and ≤ 60% after 120 minutes

Assay	Vitrigel-Eye Irritancy test (EIT)
Validity	Validated, OECD 494
Test System	Reconstructed non-keratinized multi-layered human corneal epithelium
Exposure	3 min at 2.5% (w/v)
End point	Damage to epithelial barrier function measured by time-dependent changes in Transepithelial Electrical Resistance (TEER)
Applicability	UN GHS NC

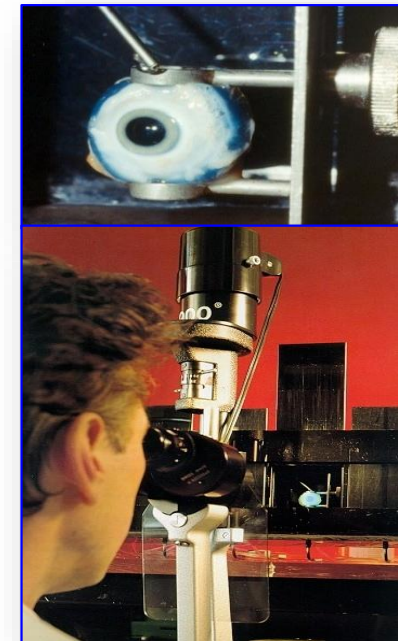


Organotypic Ex vivo Assays

Assay	Bovine Corneal Opacity and Permeability (BCOP LLBO) test method with the optional addition of histopathology analysis	Isolated Chicken Eye (ICE) test
Validity	Validated , OECD TG 437AC	Validated , OECD TG 438
Test System	Corneas isolated from bovine eyes obtained from animals at slaughterhouses	Chicken eyes isolated from abattoir animals
Exposure	10 min for liquids (neat) and surfactants (10% w/v) •4 hours for non surfactant solids	All chemicals (neat) for 10 min
End point	Corneal opacity •Loss of barrier function (permeability)	Corneal opacity, fluorescein retention, corneal swelling morphological damage and histology
Applicability	UN GHS Cat 1 - all types of chemicals	UN GHS Cat 1 - all types of chemicals

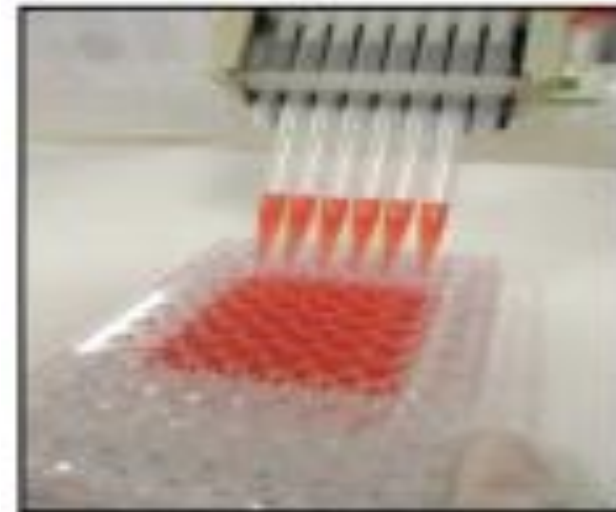
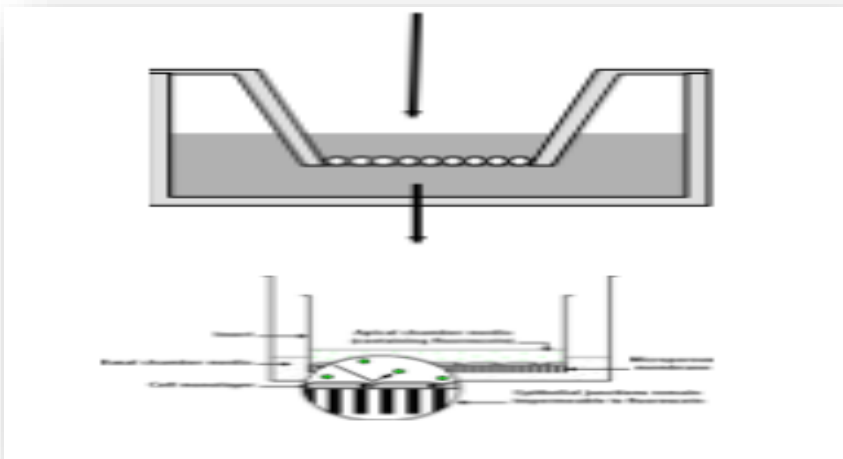


Opacitometer 1	Opacitometer 2	
OP-KIT* and Duratec	LLBO **	UN GHS
IVIS ≤ 3	LIS ≤ 30	No Category
3 < IVIS ≤ 55	LIS > 30 and lux/7 ≤ 145 and OD ₄₉₀ ≤ 2.5	No stand-alone prediction can be made
IVIS > 55	<ul style="list-style-type: none"> LIS > 30 and lux/7 ≤ 145 and OD₄₉₀ > 2.5 or LIS > 30 and lux/7 > 145 	Category 1



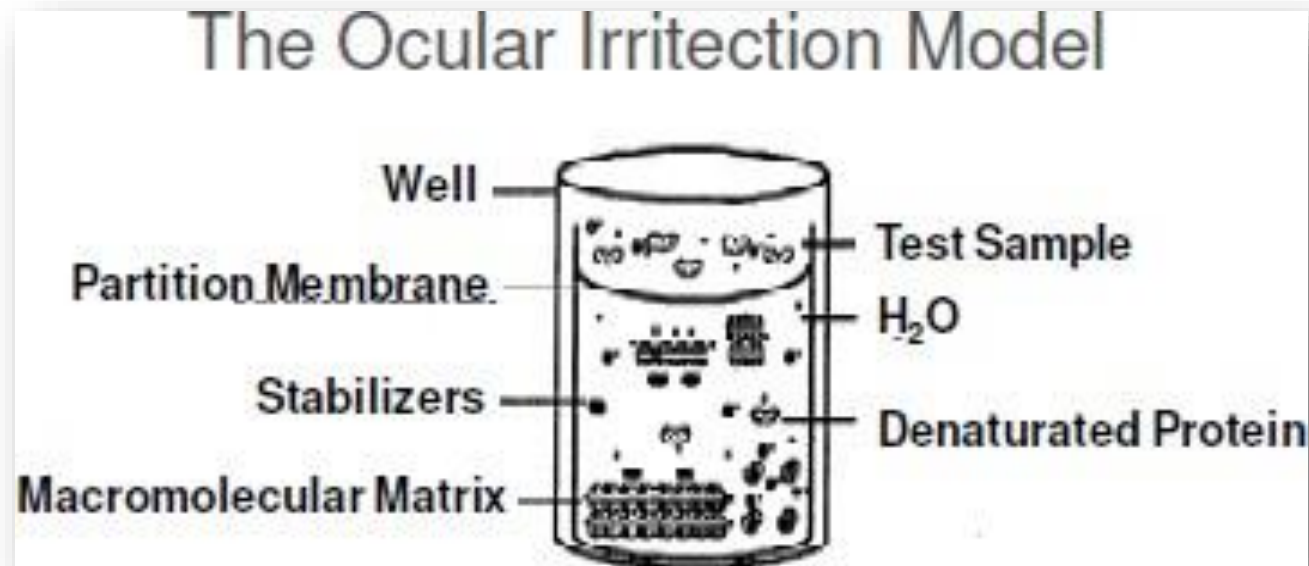
Cytotoxicity and cell function based in vitro assays

Assay	Fluorescein Leakage (FL) test	Short Time Exposure (STE) in vitro test
Validity	Validated, OECD TG 460	Validated, OECD TG 491
Test System	Confluent monolayer of MDCK CB997 tubular epithelial cells	Confluent monolayer of SIRC cell line
Exposure	Series of 5 concentrations exposed each for 1 min	5 min at 5% and 0.05%
End point	Trans-epithelial permeability to fluorescein	Cytotoxicity (MTT assay)
Applicability	UN GHS Cat 1 - only to soluble chemicals and those that form a stable suspension during testing	UN GHS Cat 1 - only to soluble chemicals and those that form a stable suspension during testing



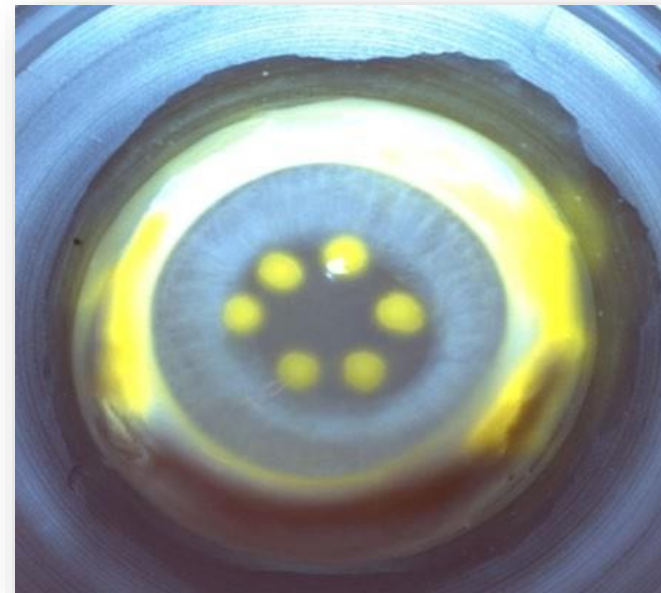
Macromolecular matrix assays

Assay	In vitro Macromolecular Test Method Ocular Irritation (In Chemo)	Optisafe test
Validity	Validated, OECD TG 496	ICCVAM validated
Test System	Transparent macromolecular matrix (composed of a mixture of proteins, glycoproteins, carbohydrates, lipids and low MW components that mimics the highly ordered structure of the cornea)	Transparent macromolecular matrix
Exposure	5 different concentrations exposed each for 24 hours	5 different doses concentrations each for 18 hours
End point	Turbidity at 405 nm ("opacity")	Turbidity at 405 nm ("opacity")
Applicability	UN GHS Cat 1 and NC Fast, simple, inexpensive and readily available	UN GHS No Cat & US EPA IV Fast, simple, inexpensive and readily available



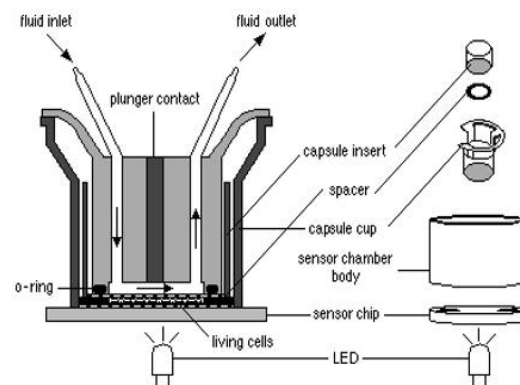
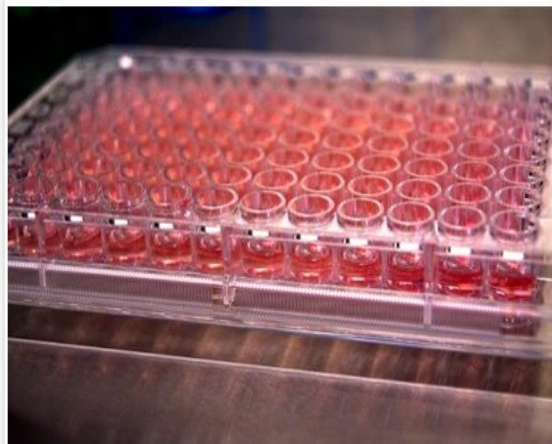
Other Organotypic Ex vivo Assays

Assay	Isolated Rabbit Eye (IRE) test	Porcine cornea opacity/reversibility assay (PorCORA)
Validity	Inter lab validation & peer review	Optimised
Test System	Ex vivo rabbit eyes mounted on specialized whole globe eye or corneal holders	Excised porcine corneas
Exposure	10 sec to identify a severe irritant (corrosive) •1 min (or longer) for less severely irritating substances	5 min exposure
End point	•Corneal swelling, and subjectively corneal opacity •injury to the epithelium (measure of fluorescein retention)	Corneal epithelial recovery over 21 days by fluorescein stain retention. Corneal injury reversibility using Sodium Fluorescein stain to detect compromised epithelial barrier function
Applicability	UN GHS Cat 1 using a top-down approach)	UN GHS Cat 1 - all types of chemicals



Other Cytotoxicity and cell function based in vitro assays

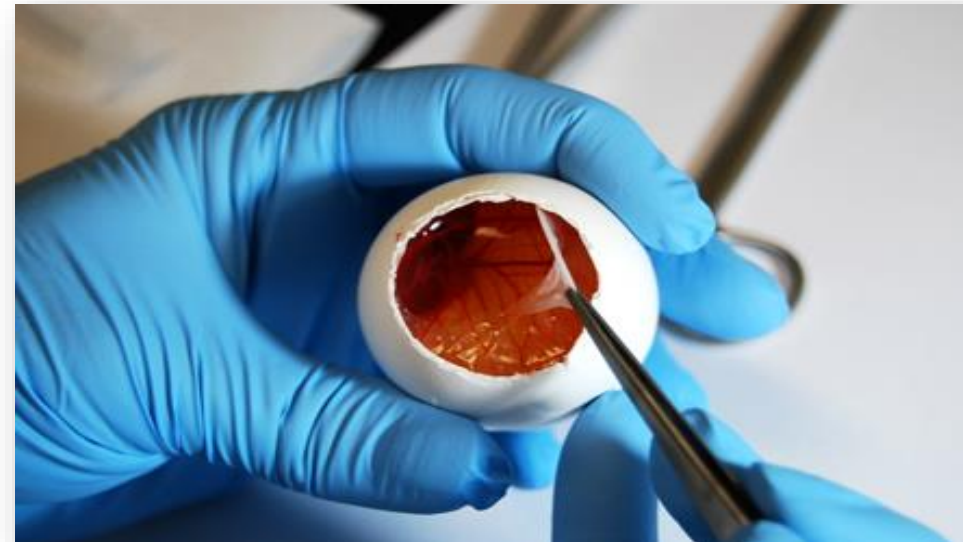
Assay	Neutral Red Release (NRR) test	Cytosensor Microphysiometer (CM) test
Validity	Validation and peer review	Validated
Test System	Confluent monolayer of Fibroblast cell line	Sub-confluent monolayer of mouse L929 fibroblasts
Exposure	1- 5 min	sequential exposure to seven increasing concentrations each for 13.5 min followed by washing and measurement (20 min cycle duration)
End point	Cytotoxicity (Neutral Red)	Cellular metabolic rate (acidification, pH change) using CM apparatus
Applicability	UN GHS NC - for water - soluble test chemicals UN GHS Cat 1 - agrochemical formulations with Proof-of-concept study	UN GHS Cat. 1 - only to soluble chemicals and those that form a stable suspension during testing UN GHS NC - only to soluble surfactants and those that form a stable suspension during testing US EPA for Cat. III and IV



Other Ex vivo assays

Assay	Slug mucosal irritation (SMI) assay	Hen's Egg Test on the Chorio-Allantoic Membrane (HET CAM)
Validity	Optimised	Validation and peer review
Test System	Mucosal surface of slugs (<i>Arion lusitanicus</i>)	Chorioallantoic membrane (CAM) of Fertilized chicken eggs
Exposure	Mucus produced from the mucosal surface of slugs, LDH activity, Protein content	300 seconds
End point	Turbidity at 405 nm ("opacity")	UN GHS Cat. 1 - based on coagulation) UN GHS NC - based on coagulation, haemorrhage and lysis
Applicability	UN GHS Cat 1 , Cat. 2A/B and NC	UN GHS No Cat & US EPA IV Fast, simple, inexpensive and readily available

Assay	Chorioallantoic Membrane Vascular Assay (CAMVA)
Validity	Validation
Test System	Blood vessels of the chorioallantoic membrane (CAM) of Fertilized chicken eggs
Exposure	30 min
End point	Vascular change to the CAM such as haemorrhaging or hyperaemia (capillary injection) & occurrence of vessels devoid of blood flow (ghost vessels)
Applicability	UN GHS Cat 2A/2B



Integrated Approach on Testing and Assessment (IATA) – Eye Damage

M1 : Existing human data

M2 : Existing *in vivo* data (OECD 405)

M3 : Existing *In Vitro* data

M4 : Other existing Non –OECD *in vivo* data

M5 : Other non – OECD *In vitro* data

M6 : Existing skin corrosion data (collective)

M7 : Physiochemical properties

M8: Non testing data – Substance : (Q)SAR, read across, grouping , expert systems. Mixtures : bridging principles & Theory of additivity

Part-I : Existing Data

M9: WoE approach elements

Part – 2 : WoE

M4: OECD adopted *In vitro* test methods

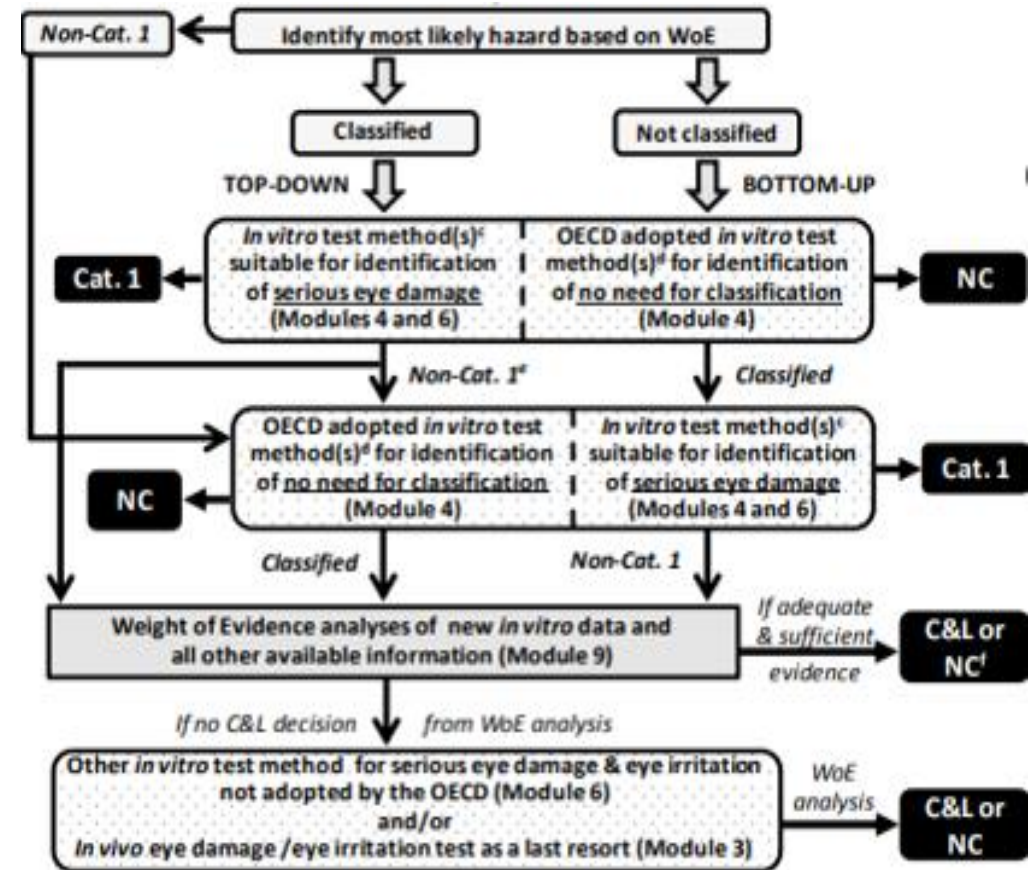
M6: Other non-OECD adopted test methods

M3: Last resort - *In vivo* animal test OECD 405

Top-Down approach – UN GHS Cat 1

Bottom-Up approach – UN GHS NC

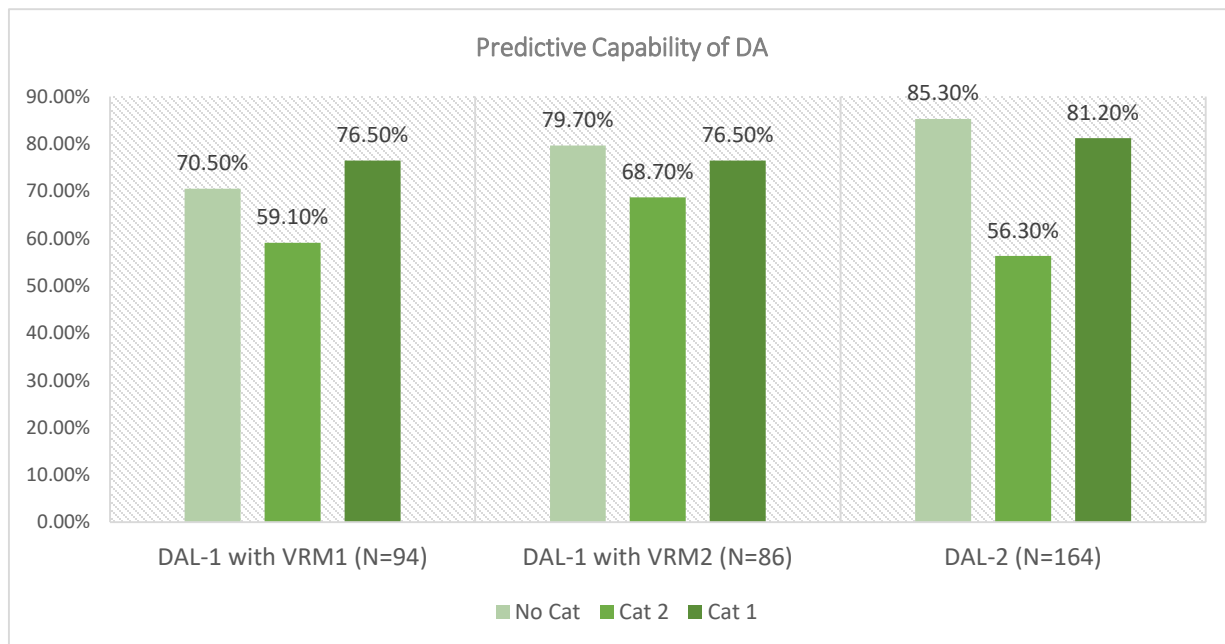
Part – 3: Testing Data



Defined Approach Methodologies (DA)

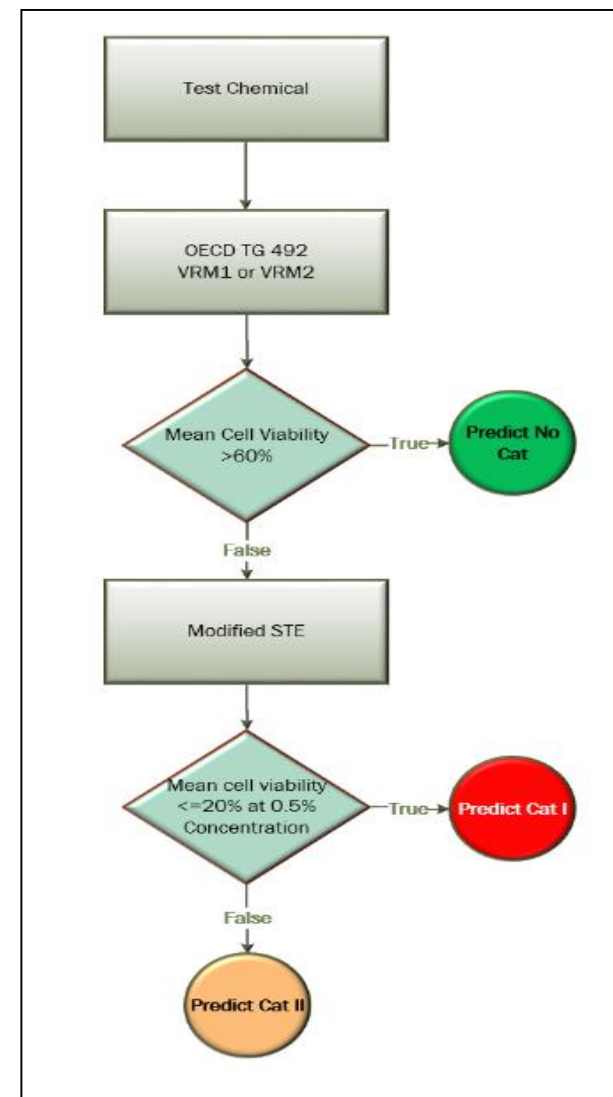
OECD 467

1. **Defined Approaches 1 (DAL-1)**, based on physicochemical properties and *in vitro* data (RhCE and BCOP LLBO), for neat non-surfactant liquids : **UN GHS Cat 1, 2 and NC**
2. **Defined Approaches 2 (DAL-2)**, based on *in vitro* data (BCOP LLBO & STE), for non-surfactant neat liquids, liquids and solids dissolved in water : **UN GHS Cat 1, 2 and NC**

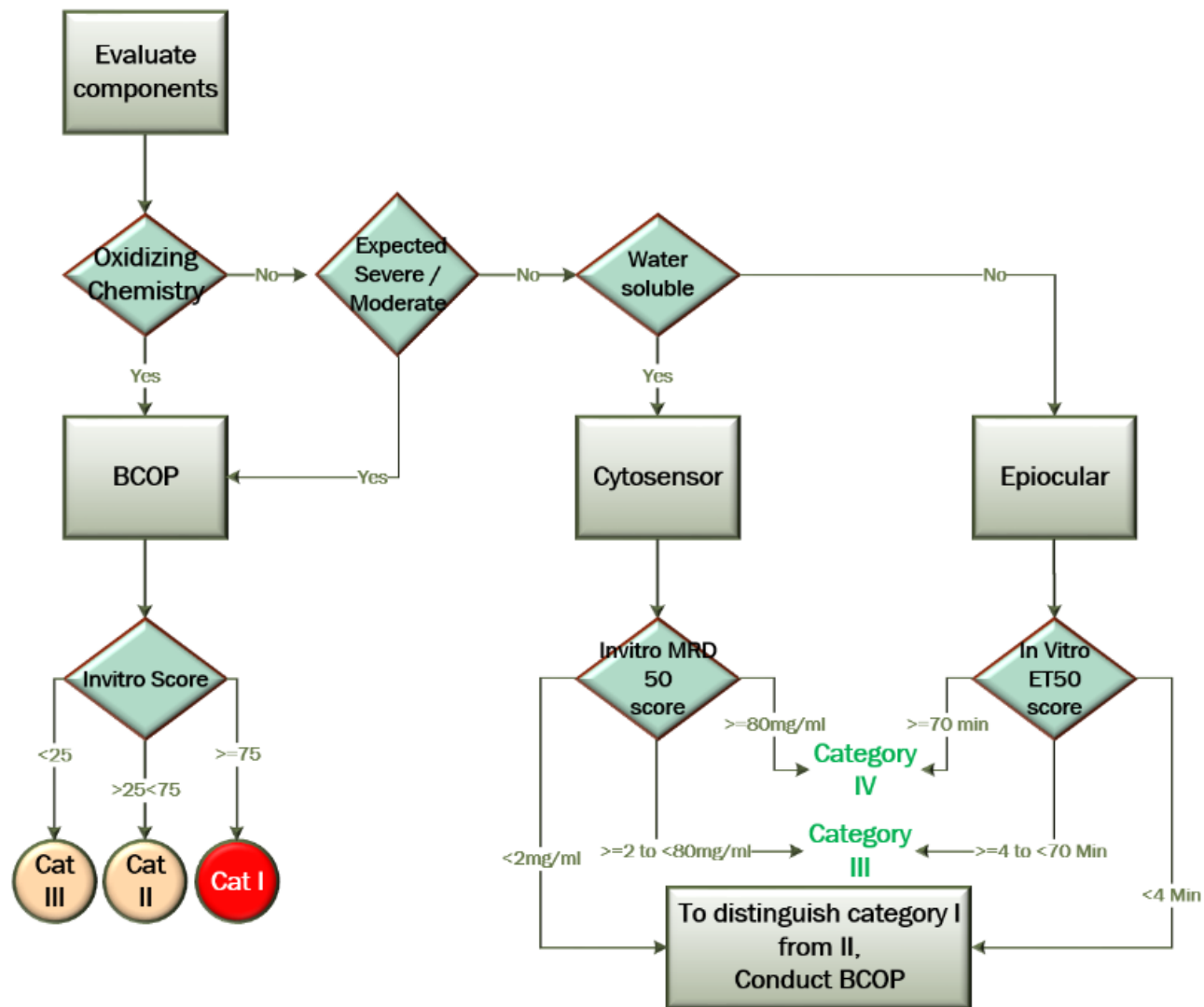


Defined Approach for Surfactants

(N. Al'ep'ee et al. 2023)



Decision Tree Approach - Eye Hazard Labelling



Label Elements for serious Eye damage/irritation

	UN GHS Cat 1	UN GHS Cat 2A	UN GHS Cat 2B
Symbol			No symbol
Signal Word	Danger	Warning	Warning
Hazard statement	Causes serious eye damage	Causes serious eye irritation	Causes eye irritation

References:

1. OECD Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Serious Eye Damage and Eye Irritation Series on Testing & Assessment No. 263; ENV/JM/MONO(2017)15; (2017)
2. Use Of An Alternate Testing Framework For Classification Of Eye Irritation Potential Of EPA Pesticide Products, US EPA (2015)
3. Chapter 3.3; Serious Eye Damage/Eye Irritation. Part 3; Health Hazards, UN GHS (2015)
4. Test Guideline No. 467 Defined Approaches for Serious Eye Damage and Eye Irritation; OECD Guidelines for the Testing of Chemicals (2022)
5. Alternative approaches for the assessment of serious eye damage/eye irritation; Webinar Series on the Use of New Approach Methodologies (NAMs) in Risk Assessment, by Joao (2021)
6. Amy J. Clippinger, Hans A. Raabe, David G. Allen, Neepa Y. Choksi, Anna J. van der Zalm, Nicole C. Kleinstreuer, João Barroso & Anna B. Lowit (2021) Human-relevant approaches to assess eye corrosion/irritation potential of agrochemical formulations; Cutaneous and Ocular Toxicology, 40:2, 145-167, DOI: 10.1080/15569527.2021.1910291.
7. N. Al'ep'ee et al. Development of a Defined Approach for Eye hazard identification of chemicals having surfactant properties according to the three UN GHS categories. Toxicology In Vitro 89 (2023) 105576.
8. APSL Clearance number : APSL_P36_01/06/2023



Thank you

