National Institute of Environmental Health Sciences
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Prospective and Retrospective Evaluation of the Eye Irritation Potential of Agrochemical Formulations

National Institutes of Health
U.S. Department of Health and Human Services

Prospective and Retrospective Evaluation of the Eye Irritation Potential of Agrochemical Formulations

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
Department of Health and Human Services

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FOREWORD

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is an NTP office focused on the development and evaluation of alternatives to animal use for chemical safety testing. NICEATM was established by the ICCVAM Authorization Act of 2000 (42 U.S.C. 285 *l*-3) to provide support to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). NICEATM and ICCVAM work collaboratively to evaluate new and improved testing approaches applicable to the needs of U.S. federal agencies.

NICEATM publishes reports of its test method development and evaluation activities in the scientific literature. Through NTP, NICEATM also issues reports of ICCVAM test method evaluations and other communications and makes these available on the NTP website, where they are available free of charge. Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please contact NICEATM.

TABLE OF CONTENTS

Foreword ii
Table of Contents iii
Tables v
Figures v
About This Report vi
Authors vi
Contributors vi
Review ix
Publication Details x
Abstract xi
Preface xii
1. Introduction 1
2. Retrospective Analysis 4
2.1 Materials and Methods 4
2.1.1 Agrochemical Formulation Data Submissions 4
2.1.2 Test Methods 4
2.1.3 Data Analyses 6
2.2 Results 6
2.2.1 Submitted Data from CLA Companies 6
2.2.2 Concordance Analysis 7
2.2.3 Range of Responses of Methods Evaluated in Retrospective Evaluation 9
2.3 Summary of Retrospective Data Evaluation 11
3. Prospective Testing 11
3.1 Materials and Methods 12
3.1.1 Phases and Goals 12
3.1.2 Formulation Selection 12
3.1.3 Participating Laboratories 14
3.1.4 Study Management 14
3.1.5 Test Methods 14
3.1.6 Classification Criteria and Data Analysis 16
3.2 Results 19
3.2.1 Phase 1 19
3.2.2 Phase 2 21
3.2.3 Range of Responses of Methods Evaluated in Phases 1 and 2 24
3.2.4 Range of Responses of Methods Evaluated in Phase 1 and 2 Results, and Retrospective Analysis 26
4. Results/Discussion 28

5. References.....30

TABLES

Table 1. EPA and GHS Ocular Irritation Classification Systems....3
Table 2. Submitted In Vitro Test Data....7
Table 3. Concordance Analysis Between Submitted In Vitro and Rabbit Data....8
Table 4. Tested Formulations....13
Table 5. Evaluated In Vitro Methods and Testing Laboratories....14
Table 6. Phase 1 and 2 In Vitro Results Classification Key and Criteria for Concordance with In Vivo Results....17
Table 7. Concordance of Phase 1 In Vitro Results with In Vivo Classifications....20
Table 8. Concordance of Phase 2 In Vitro Results with In Vivo Classifications....22

FIGURES

Figure 1. Distribution of In Vitro Phase 1 Data vs. EPA Hazard Classification Based on Historical Rabbit Data....9
Figure 2. Distribution of In Vitro Phase 1 and 2 Data vs. EPA Hazard Classification Categories Based on Historical Rabbit Data....24
Figure 3. Distribution of In Vitro Phase 1 and 2 and Available Retrospective Data vs. EPA Hazard Classification Categories Based on Historical Rabbit Data....27

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REVIEW

The analyses and results described in this document have been reviewed by, and reflects comments received from, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Ocular and Dermal Irritation Expert Group (ODIEG). The ICCVAM ODIEG is comprised of experts from five different ICCVAM member agencies who are listed below.

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ABSTRACT

Agrochemical manufacturers must meet regulatory requirements to provide information about the acute, subchronic, and chronic effects potentially caused by their products and active ingredients. Studies run to obtain these results can require up to 100 animals per product and 7000 animals per active ingredient. In the United States alone, this results in the use of approximately 600 rabbits per year for eye irritation testing of agrochemical formulations and highlights the value of implementing non-animal approaches. While several in vitro[1] methods have been found to be appropriate for specific applications, no single method has been identified as a complete replacement for the rabbit eye test for classification and labeling of agrochemical formulations. Development of a defined approach would leverage the strengths of different non-animal eye irritation test methods to predict the complete range of ocular irritation potential of agrochemical formulations. However, development of such approaches requires data from representative formulations that have been tested in multiple in vitro methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods worked to develop a defined approach for eye irritation testing. To support this effort, CropLife America member companies provided data on formulations tested in the in vivo rabbit test method and in one or more in vitro test methods. Results compiled for 232 agrochemical formulations were reviewed to determine if a combination of in vitro methods could accurately assign U.S. Environmental Protection Agency (EPA) eye irritation hazard classifications. However, there was insufficient overlap of formulations tested in multiple methods to conduct comparative analyses with which to justify a proposed testing strategy.

Accordingly, new data for a short list of agrochemical formulations were generated in a variety of in vitro test methods, including the bovine corneal opacity and permeability assay (BCOP), neutral red release assay, isolated chicken eye assay, porcine cornea reversibility assay, and EpiOcular (EO) test method. In addition to the standard BCOP and EO testing protocols, protocols that adjusted incubation time, testing concentration, and/or analysis method were evaluated. Altogether, 16 donated formulations were evaluated in eight different test method protocols. In vitro test results were compared to regulatory hazard classifications (United Nations Globally Harmonized System and EPA) that were assigned based on retrospective rabbit test method data.

Our analyses showed that no single method produced results that completely aligned with the rabbit test. While a combination of test methods may provide better information, development of such integrated strategies is still confounded by the variability and questionable human relevance of the reference animal data. We discuss issues associated with a reliance on animal data as a reference for the evaluation of new testing approaches and the potential advantages of a practical, more human-relevant strategy for hazard classification.

[1] In this report, the phrase “in vitro test method” encompasses test methods where living tissues are taken directly from a living organism and tested outside the natural conditions (i.e., ex vivo test method) and where replicate biological matter (e.g., cell lines) outside of a living organism is tested (i.e., in vitro test method).

PREFACE

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is an office within the Division of the National Toxicology Program, National Institute of Environmental Health Sciences. NICEATM focuses on the development and evaluation of alternatives to animal use for chemical safety testing. It provides technical and scientific support for the Interagency Coordinating Committee on the Validation of Alternative Toxicological Methods (ICCVAM) and ICCVAM workgroup activities, peer review panels, expert panels, workshops, and validation efforts.

In addition to providing support for ICCVAM, NICEATM:

- Supports NTP activities, especially those contributing to the U.S. government's interagency Tox21 initiative.
- Conducts analyses and evaluations, and coordinates independent validation studies on novel and high-priority alternative testing approaches.
- Provides information to test method developers, regulators, and regulated industry through its website and workshops on topics of interest.

NICEATM's activities are guided in part by the "Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States" issued by ICCVAM in 2018. One objective articulated in the Strategic Roadmap was that ICCVAM agencies would utilize public-private partnerships to promote cross-sector communication and cooperation. An implementation plan developed for the Strategic Roadmap stated that NICEATM, ICCVAM, and collaborators would advance the use of integrated approaches to testing and assessment and defined approaches to enable prediction of skin and eye irritation hazard. The project described in this report was undertaken to address both of these objectives.

1. INTRODUCTION

The in vivo Draize rabbit eye test has been used for more than 75 years to assess the irritation and corrosion potential of chemicals and substances that may come into contact with the eye (Draize et al. 1944). However, the rabbit test relies upon subjective assessments of reversibility and damage. Therefore, results are potentially confounded both by interobserver variation and animal variation. Consequentially, the likelihood of repeat testing yielding the same classification has been demonstrated to be <50% for substances which fall into the mild to moderate irritation range (Luechtefeld et al. 2016). Studies have also suggested that the responses observed in animal tests are not always relevant to the responses observed in humans (Verstraelen et al. 2013). These considerations, in conjunction with animal welfare concerns and international regulations banning or restricting animal testing of chemicals, have led to the development and evaluation of methods that may reduce or replace animal testing (Oliveira et al. 2015).

Several in vitro test methods[2] have been validated for the identification of severe eye irritants and corrosives, and for identifying chemicals as "not classified" (NC) or not requiring signal words based on decision criteria for the United Nations Globally Harmonized System for Classification and Labelling of Chemicals (GHS) and U.S. Environmental Protection Agency (EPA) hazard classification and labeling system (**Table 1**). Some of these methods (OECD 2017a, b, c) have been adopted as test guidelines issued by the Organisation for Economic Co-operation and Development (OECD), which assist in acceptance of data across countries and reduce repeated testing.

In recent years, there has been movement towards identification of a combination of in vitro methods that could identify the full range of eye irritancy. Since each in vitro method models specific eye irritation endpoints, combinations of test methods are proposed to more fully assess the eye irritation potential of a substance. This is consistent with an OECD guidance document (OECD 2019) emphasizing the use of integrated testing strategies that employ multiple assays with potentially different domains of applicability and coverage of key biological events. Development of an integrated testing strategy for assessing eye irritation would leverage the strengths of different non-animal test methods to predict the complete range of ocular irritation potential of agrochemical formulations.

Agrochemical formulations are typically mixtures composed of one or more active ingredients combined with one or more "inert" constituents to optimize activity and enhance delivery of the active ingredient(s) (Kolle et al. 2017). Due to the complex nature of mixtures and formulations, these substances are not typically included as reference chemicals in test method validation efforts. Results from prospective in vitro eye irritation testing of agrochemical formulations have, to date, reported discordant results with classifications based on in vivo rabbit studies, but these studies have been limited in scope based on the number of formulations evaluated and methods evaluated (Kolle et al. 2017; Settivari et al. 2016). However, utilization of a representative set of such chemicals to evaluate a set of test methods that collectively assess the

[2] The phrase "in vitro test method" encompasses test methods where living tissues are taken directly from a living organism and tested outside the natural conditions (i.e., ex vivo test method) and where replicate biological matter (e.g., cell lines) outside of a living organism is tested (i.e., in vitro test method).

key biological events that produce eye irritation represents an opportunity to more reliably predict human effects of these substances and reduce the number of animals tested

Table 1. EPA and GHS Ocular Irritation Classification Systems

EPA Classification			GHS Classification		
Category	Classification[a]	PPE	Category	Classification[b]	PPE
I	Corrosive (irreversible destruction of ocular tissue), or corneal involvement or irritation lasting for more than 21 days after administration of substance	Eye protection	1	Effects on the cornea, iris, or conjunctiva that are not expected to reverse or do not fully reverse within 21 days	Eye protection
II	Corneal involvement or irritation clearing in 8 to 21 days after administration of substance	Eye protection	2A	Effects on the cornea, iris, or conjunctiva that fully reverse within 21 days	Eye protection
III	Corneal involvement or irritation clearing in ≤7 days after administration of substance	No minimum	2B	Effects on the cornea, iris, or conjunctiva that fully reverse within 7 days	Eye protection
IV	Irritation clearing in <24 hours after administration of substance	No minimum	NC	No effects are produced, or minimal effects observed that do not lead to classification	None noted

Abbreviations: NC = not classified; PPE = personal protective equipment

[a]A positive response for the EPA classification system is defined as a corneal opacity or iritis score ≥1, or conjunctival redness or chemosis score ≥2 in a single animal at any observed time point up to 21 days after substance administration.

[b]A Category 1 GHS classification is applied when a substance produces either (a) mean corneal opacity score ≥3 or iritis score ≥1.5 (over Days 1, 2, and 3) in at least two animals or (b) a score >0 on Day 21. A Category 2A and 2B classification is applied when a substance produces either (a) mean corneal opacity or iritis score ≥1 or (b) conjunctival redness score ≥1 (over Days 1, 2, and 3) in at least 2 animals.

This report describes retrospective and prospective studies conducted to evaluate the usefulness and limitations of a group of in vitro test methods that could potentially be combined into a defined approach to assign hazard classification and labeling for eye irritation potential. While the focus of the evaluation was on EPA hazard classification, study results also were evaluated for GHS classification and labeling.

2. RETROSPECTIVE ANALYSIS

Previous experience has shown that public-private partnerships are key to the successful development, acceptance, and implementation of alternative test methods. To leverage results from previously conducted studies, the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) partnered with companies belonging to the industry consortium Crop Life America (CLA) to develop a high-quality database of paired in vitro and in vivo eye irritation studies.

2.1 Materials and Methods

2.1.1 Agrochemical Formulation Data Submissions

Data were submitted by CLA companies (BASF, Bayer/Monsanto, FMC, Corteva Agriscience, and Syngenta). Availability of (1) associated historical rabbit data or EPA and GHS ocular irritancy classification (**Table 1**) and (2) results from an in vitro eye irritation method were required for inclusion in the analyses. Additional information submitted included, but were not limited to, formulation type and active ingredient.

2.1.2 Test Methods

In vitro results were available from at least one of five test methods: bovine corneal opacity and permeability (BCOP); isolated chicken eye (ICE); EpiOcular™ (EO); neutral red release (NRR); and chorioallantoic membrane vascular assay (CAMVA). General information regarding conduct of each test method protocol was provided by the submitting companies.

2.1.2.1 Bovine Corneal Opacity and Permeability

The BCOP method was conducted according to OECD test guideline (TG) 437 (OECD 2017a). Briefly, bovine eyes for testing (collected after slaughter for human consumption) were prepared and mounted into a corneal holder. The eyes were preincubated in complete Eagle's modified essential medium (complete EMEM) without phenol red. The medium was then replaced and an initial opacity measurement was conducted. The medium was replaced with medium containing test article, negative control, or positive control. Corneas were incubated for up to 4 hours, removed, and then washed. The anterior chamber of the corneal holder was refilled with complete EMEM without phenol red, and an opacity measurement was performed immediately and after incubation. After the second opacity measurement, sodium fluorescein solution was added to the chambers and corneas were incubated for approximately 90 mins to assess permeability. The medium was

removed and transferred to a 96-well plate. Complete EMEM without phenol red was added to the wells and optical density at 490 nm (OD_{490}) measured. Opacity and mean permeability values were used to calculate the in vitro irritancy score (IVIS) for each treatment group using the equation noted in OECD TG 437 (OECD 2017a).

2.1.2.2 Isolated Chicken Eye

The ICE method was conducted according to OECD TG 438 (OECD 2017b). Briefly, chicken heads were collected after slaughter for human consumption. The whole eye and nictitating membrane were removed and maintained at appropriate humidity. The prepared eye was placed in a steel clamp and acclimatized. After reference measurements were taken, the test material was applied to the entire corneal surface. After exposure, the corneal surface was rinsed with physiological saline at ambient temperature. Corneal thickness and corneal opacity, and fluorescein retention were measured at predetermined time intervals. Eyes were then processed for histopathology. Corneal swelling, corneal opacity, and fluorescein retention were calculated as described in OECD TG 438 and used for regulatory classification (OECD 2017b).

2.1.2.3 EpiOcular

Two different EO protocols were used to generate the submitted data. In one case, EO was conducted according to OECD TG 492, which is based on a threshold of cell viability to delineate potential eye irritants. In another case, EO was conducted using the time-to-toxicity protocol, which is based on the time required to cross the 60% viability threshold (i.e., estimated time to reduce cell viability by 40%, or ET40).

The method described in OECD TG 492 (EO-OECD) is based on a reconstructed human cornea-like epithelium tissue model. Briefly, test articles or controls were applied to tissues and incubated. Inserts containing the tissues were removed from the wells and rinsed. The inserts were then incubated with assay medium. The inserts were incubated with 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide dye, rinsed with Dulbecco's phosphate-buffered saline, and incubated with isopropanol overnight. The next day, the plates were placed on an orbital shaker for 2-3 hours at room temperature. Solution was then placed on a 96-well plate and absorbance measured at 570 nm.

For the second protocol, the methodology described for OECD TG 492 was followed with one difference (OECD 2017c). After test articles or negative or positive controls were applied, the tissues were incubated for varying time periods until the 60% viability threshold was obtained.

2.1.2.4 Neutral Red Release

Normal human epidermal keratinocytes were grown to approximately 75 to 90% confluency in standard culture conditions. The routine culture medium was decanted, and neutral red medium was added. The neutral red medium was then decanted, and routine culture medium was added. Test article dilutions were applied to plated cells and then each well was rinsed with Dulbecco's phosphate-buffered saline. After rinsing, neutral red solvent was added to each treatment well and plates were shaken. Absorbance at 550 nm (OD_{550}) was measured and recorded.

2.1.2.5 Chorioallantoic Membrane Vascular Assay

One-day-old fertilized eggs were placed in a humidified incubator for 3 days. On Day 3, eggs were checked for contamination, cleaned with ethanol if necessary, and rotated. The next day a rectangular window was cut into the eggshell. A Teflon ring was placed on a vascularized area of 10-day old chorioallantoic membrane, inside of which test articles and controls applied. The treated eggs were placed in an incubator for approximately 30 mins. At the end of the incubation period, the chorioallantoic membrane of each treated egg was examined for vascular effects (hemorrhaging, capillary injection, and ghost vessels).

2.1.3 Data Analyses

Submitted in vitro and rabbit data were compiled into a single spreadsheet. Classifications based on in vitro test method results, using previously developed decision criteria, were compared to historical rabbit classifications to evaluate concordance. A result was judged to be concordant when classification based on in vitro results agreed with classification based on rabbit data. A result was judged to be discordant when classification based on in vitro results did not agree with classification based on rabbit data.

For each in vitro test method, classifications were compared to in vivo classifications using a bottom-up and top-down approach. Specifically, for the top-down approach, the ability of an in vitro test method to identify severe eye irritants and corrosives was evaluated. Likewise, for the bottom-up approach, the ability of an in vitro test method to identify formulations that do not require eye irritation labels was evaluated.

Formulations evaluated in at least two test methods were identified and evaluated to determine whether they could be used in the development of an integrated testing strategy.

2.2 Results

2.2.1 Submitted Data from CLA Companies

Data on 232 formulations were received from five different agrochemical companies (**Table 2**). BCOP, EO, and ICE results were submitted by at least one

company. No overlap in tested formulations was noted between companies, based on the information provided.

Table 2. Submitted In Vitro Test Data

Company	BCOP	EO	ICE	NRR	CAMVA
1	-	52[a]	-	68	-
2	97	97[b]	10	-	-
3	14	-	-	-	4
4	14	5	56	-	-
5	-	-	25	-	-

[a]All results were obtained from studies conducted using a time-to-toxicity EO protocol.
[b]All results were obtained from studies conducted using the EO-OECD protocol.

2.2.2 Concordance Analysis

Concordance analysis results are provided in **Table 3**. The top-down approach concordance rates between in vitro and rabbit results ranged from 77% to 86% for the GHS classification system and 79% to 86% for the EPA classification system. For the bottom-up approach, the concordance rates ranged from 18% to 79% for the GHS classification system and 21% to 80% for the EPA classification system.

In the data provided by the agrochemical companies, 97 formulations were tested in both the BCOP and EO (OECD protocol) methods. Additionally, a different set of 66 formulations were tested in both EO (time-to-toxicity protocol) and NRR methods.

Table 3. Concordance Analysis Between Submitted In Vitro and Rabbit Data

Test Method	Classification System	n	Concordance		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
			%	No.	%	No.	%	No.	%	No.	%	No.
BCOP (Top-down)	GHS	97	77	75/97	14	3/21	95	72/76	5	4/76	86	18/21
	EPA	97	80	78/97	14	3/21	99	75/76	1	1/76	86	18/21
BCOP (Bottom-up)	GHS	97	79	77/97	85	46/54	72	31/43	28	12/43	15	8/54
	EPA	97	75	73/97	38	13/34	95	60/63	5	3/63	62	21/34
BCOP-Expanded (Top-down)	GHS	104	78	81/104	21	5/24	95	76/80	5	4/80	79	19/24
	EPA	101	81	82/101	14	3/21	99	79/80	1	1/80	86	18/21
BCOP-Expanded (Bottom-up)	GHS	104	79	82/104	86	51/59	69	31/45	31	14/45	14	8/59
	EPA	101	76	77/101	43	15/35	94	62/66	6	4/66	57	20/35
ICE (Top-down)	GHS	65	86	56/65	0	0/9	100	56/56	0	0/56	100	9/9
	EPA	65	86	56/65	0	0/9	100	56/56	0	0/56	100	9/9
ICE (Bottom-up)	GHS	65	60	39/65	63	12/19	59	27/46	41	19/46	37	7/19
	EPA	65	66	43/65	64	21/33	69	22/32	31	10/32	36	12/33
EO (ET50; Bottom-up) [a]	GHS	51	65	33/51	58	18/31	75	15/20	25	5/20	42	13/31
	EPA	51	53	27/51	49	21/43	75	6/8	25	2/8	51	22/43
EO (Time-to-Toxicity; Bottom-up) [a]	GHS	97	18	17/97	9	5/54	28	12/43	72	31/43	91	49/54
	EPA	97	21	20/97	23	18/77	10	2/20	90	18/20	77	59/77
NRR (Top-down)	GHS	66	79	52/66	85	11/13	77	41/53	23	12/53	15	2/13
	EPA	66	79	52/66	85	11/13	77	41/53	23	12/53	15	2/13
NRR (Bottom-up)	GHS	66	71	47/66	85	28/33	58	19/33	42	14/33	15	5/33
	EPA	66	80	53/66	82	37/45	76	16/21	24	5/21	18	8/45

[a]EO assay decision criteria do not distinguish between corrosive/severe eye irritants (Category 1/Category I) and moderate eye irritations (Category 2/Category II). Therefore, a top-down evaluation could not be conducted.

2.2.3 Range of Responses of Methods Evaluated in Retrospective Evaluation

BCOP, NRR, and EO quantitative results were graphed to determine if novel decision criteria could be developed to increase alignment between in vitro and rabbit eye irritation classification categories (**Figure 1**). As shown in the following graphs, the large range of values observed for all the rabbit EPA classifications limits the ability to modify current test method decision criteria for application to agrochemical formulations.

Figure 1. Distribution of In Vitro Phase 1 Data vs. EPA Hazard Classification Based on Historical Rabbit Data

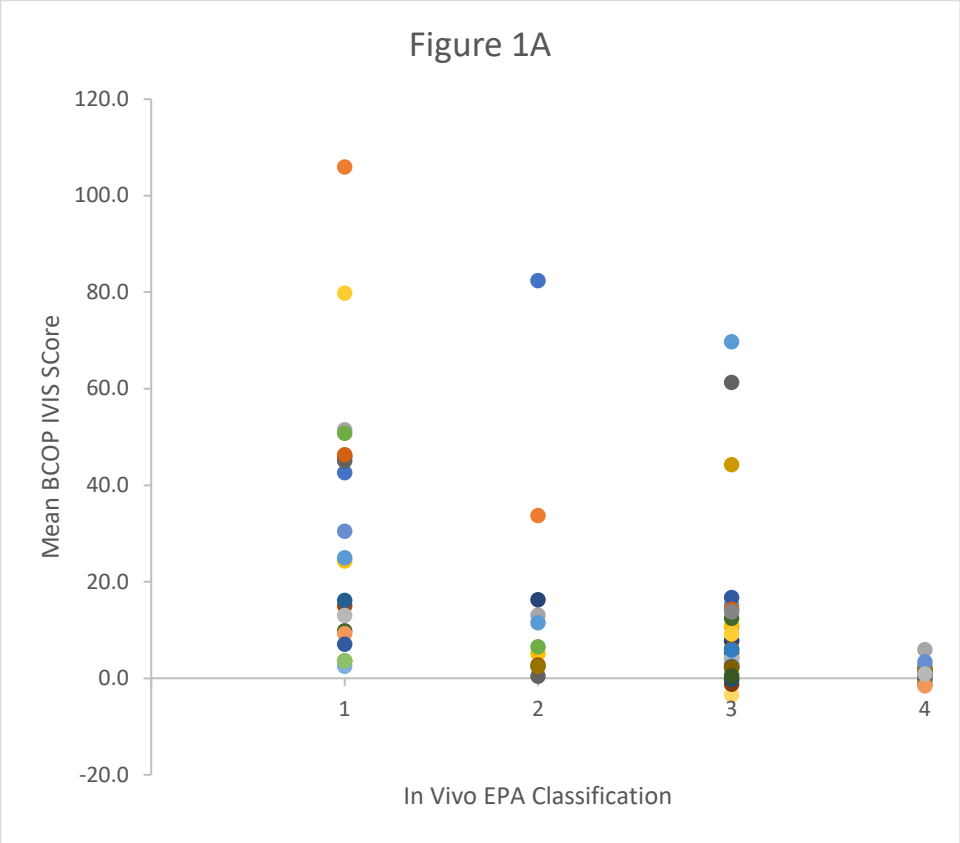
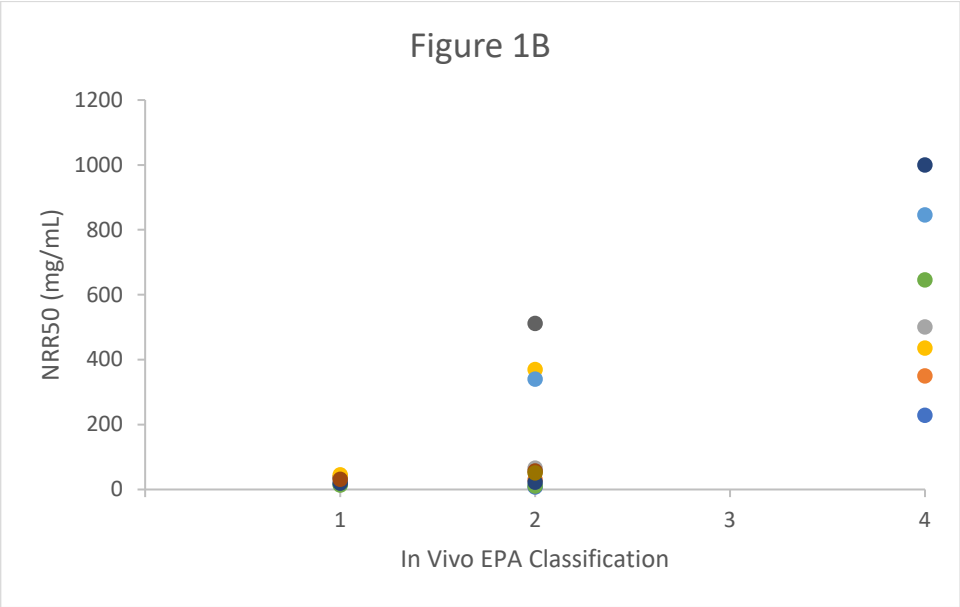


Figure 1A: Mean BCOP IVIS vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.



Abbreviation: NRR50: 50% reduction in neutral red release

Figure 1B. NRR50 vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

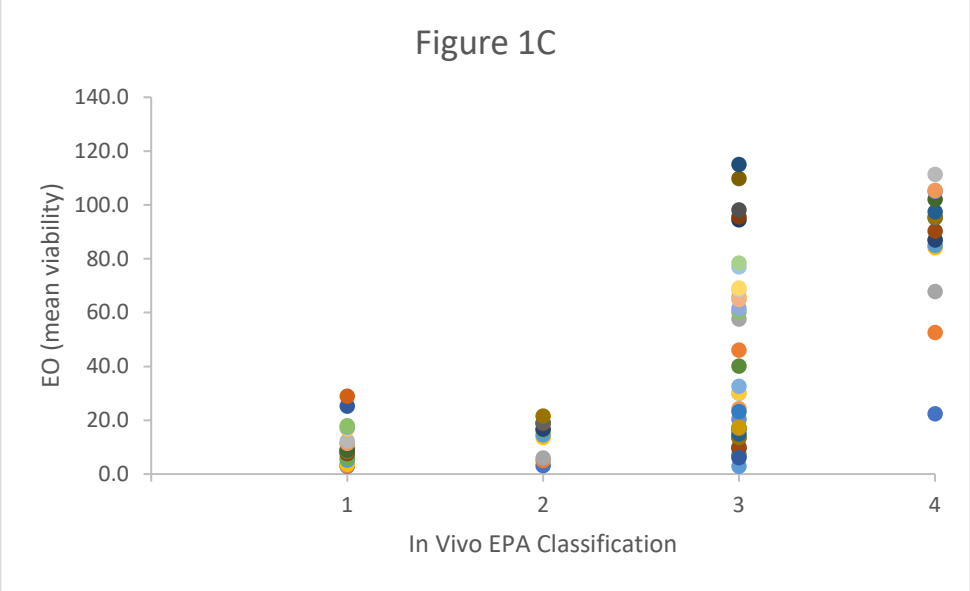


Figure 1C. EO (mean viability) vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

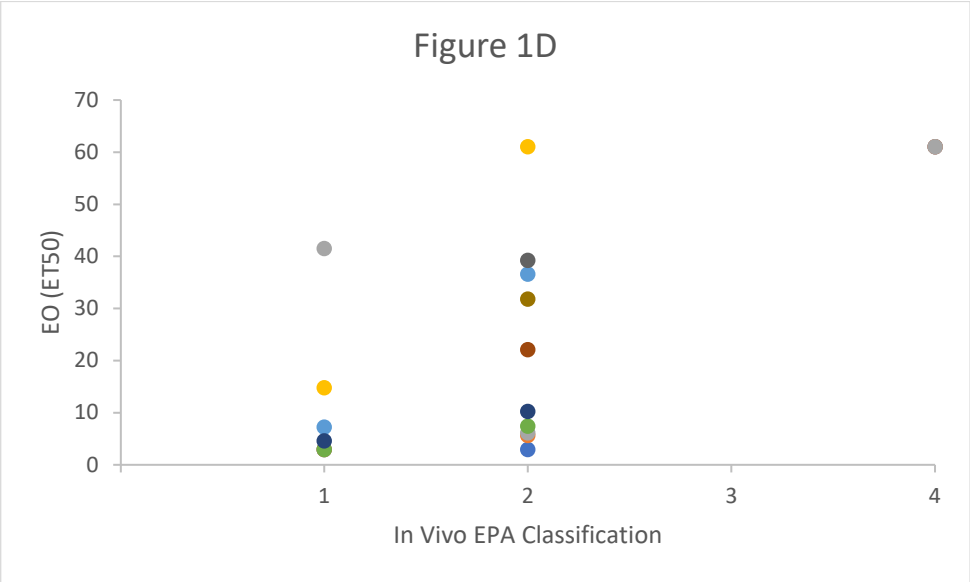


Figure 1D. EO (ET50) vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

2.3 Summary of Retrospective Data Evaluation

For formulations for which data from in vitro test methods were available, alignment of classification based on these data with that based on in vivo data was generally at least 60%. Evaluation of test methods for inclusion and use in a defined approach requires the same substances to be tested in multiple test methods. This allows for identification of strengths and weaknesses of each method for classification of formulations as eye irritants or non-irritants. In the data provided by the agrochemical companies, 97 formulations were tested in both the BCOP and EO (OECD protocol) methods. Additionally, a different set of 66 formulations was tested in both EO (time-to-toxicity protocol) and NRR methods. With minimal overlap among multiple test methods, there were limited evaluations that could be conducted to develop a defined approach. Therefore, it was determined that a prospective in vitro testing was needed to develop a data set of formulations tested in the same test methods.

3. PROSPECTIVE TESTING

The PETA Science Consortium International e.V., CLA companies, and NICEATM collaborated to evaluate a set of 16 agrochemical formulations in a common set of seven in vitro eye irritation and corrosion test method protocols: BCOP, ICE, three different EO protocols, NRR, and the porcine cornea reversibility assay (PorCORA). The testing was conducted as a proof-of-principle to determine if these specific methods might be useful in a testing strategy to determine the eye irritation potential of agrochemical formulations. Because no human data are available for these substances, hazard classifications assigned based on data from the in vivo rabbit test was used for comparison, despite known limitations of this method (Clippinger et al, 2021).

3.1 Materials and Methods

3.1.1 Phases and Goals

The study was conducted in two phases. In Phase 1, EPA Category I/GHS Category 1 and EPA Category IV/GHS not classified (NC) formulations (n=6, 3 for each category) were tested to determine which methods should proceed to further testing and which should be excluded, based on Phase 1 performance. Phase 2 (n=10) expanded the number of EPA Category I/GHS Category 1 and EPA Category IV/GHS NC formulations tested and also included one each of EPA Category II/GHS 2A and EPA Category III/GHS NC irritants. This phase focused on determining the suitability of each of the test methods to be included in a potential defined approach.

3.1.2 Formulation Selection

Formulations (**Table 4**) were donated by CLA companies (BASF, Bayer/Monsanto, FMC, Corteva Agriscience, and Syngenta). Tested formulations were selected to (1) include a range of hazard classifications, and (2) focus on three of the most common agrochemical formulation types based on the dataset of 233 formulations provided by CLA: suspension concentrates, emulsifiable concentrates, and soluble liquids. Availability of historical rabbit data or EPA and GHS ocular irritancy classification was required for inclusion of a formulation for in vitro testing. Availability of individual rabbit data enabled the identification of the driver of EPA Category I/GHS Category 1 classification (i.e., persistence of a response until observation Day 21, observation of a severe response in at least one animal) to allow us to interrogate any discordance in corrosive results (Barroso et al. 2017).

The National Institute of Environmental Health Sciences Chemistry and Absorption, Distribution, Metabolism and Excretion Resources Group received, coded, and supplied all formulations to each participating testing laboratory. Coded formulations were packaged and shipped to the testing laboratories (**Table 5**) according to established regulatory procedures. Participating laboratory personnel were instructed to handle all formulations as hazardous and potentially carcinogenic. Health and safety information was provided to each facility in a sealed package, which provided hazard information and emergency instructions.

Table 4. Tested Formulations

Phase	Formulation Code	Active Ingredient (AI)	% AI	% Organic Solvent	% Surfactant	Formulation Type	EPA Class	EPA Class Driver	GHS Class	GHS Class Driver
1	A	Afidopyropen	NP	10	66	EC/ME	IV	NA	NC	NA
1	B	Spirotetramat	43.815	0	6.5	SC	IV	NA	NC	NA
1	C	Fenbuconazole	25	NP	NP	SC	IV	NA	NC	NA
1	D	Pyraclostrobin, Mefentrifluconazole	NP	NP	NP	EC	I	Persistence	1	Persistence
1	E	Afidopyropen	NP	50	44	EC	I	Persistence	1	Persistence
1	F	2,4-D TIPA salt	71	0	0	SL	I	Persistence	1	Persistence
2	G	Chlorpyrifos-methyl + Deltamethrin	21.6/3.7	70	4	EC	I	Persistence	1	Persistence
2	H	2,4-D Choline salt	65.5	0	0	SL	I	Persistence	1	Persistence
2	I	Methomyl	NP	56	NP	SL	I	Persistence	1	Persistence
2	J	Benzovindiflupyr/Solatenol	NP	43	3	EC	I	Persistence	1	Persistence
2	K	Glyphosate	88.67	0	9.2	SL	II	NA	2A	NA
2	L	Propiconazole	NP	5.65	33.9	EC	III	NA	NC	NA
2	M	Propamocarb hydrochloride	92.13	0	0	SL	IV	NA	NC	NA
2	N	Penoxsulam	21.9	0	11.5	SC	IV	NA	NC	NA
2	O	Glyphosate	84.4	0	10	SL	IV	NA	NC	NA
2	P	Mesotrione	NP	0	0.2	SC	IV	NA	NC	NA

Abbreviations: EC = emulsifiable concentrate, ME = microencapsulated; SC = suspension concentrate, SL = soluble liquid; NA = not applicable; NC = not classified; NP = not provided.

3.1.3 Participating Laboratories

Four independent testing laboratories conducted eight different protocols using five different in vitro test methods (**Table 5**). Test methods for which an OECD test guideline was used to develop the protocol are noted. The remaining test methods were conducted using in-house testing protocols. All testing was conducted under "GLP-like" conditions. All methods are described below.

Table 5. Evaluated In Vitro Methods and Testing Laboratories

Test Method	OECD TG	Testing Laboratory
Bovine Corneal Opacity and Permeability (BCOP-OECD)	OECD TG 437	Institute for In Vitro Sciences
BCOP – Extended Incubation Period[a] (BCOP-Extended)	-	Institute for In Vitro Sciences
Neutral Red Release (NRR)	-	Institute for In Vitro Sciences
Isolated Chicken Eye (ICE-OECD)	OECD TG 438	Citoxlab
Porcine Cornea Reversibility Assay (PorCORA)	-	MB Research Labs
EpiOcular (EO-OECD)	OECD TG 492	MatTek Life Sciences
EO (Time-to-toxicity method; EO-neat ET50)	-	MatTek Life Sciences
EO (Time-to-toxicity method; EO-dilution ET50)	-	MatTek Life Sciences

Abbreviation: TG = test guideline.
[a]Protocol evaluated in Phase 2 only.

3.1.4 Study Management

Scientists from NICEATM, PETA Science Consortium International e.V., and EPA comprised the study management team that reviewed and approved the study design, study timeline, and deliverables. During each testing phase, each laboratory provided test result summaries to NICEATM once testing of all formulations was completed. Additionally, a final report was provided by each testing laboratory after completion.

3.1.5 Test Methods

3.1.5.1 BCOP Standard Protocol

The protocol described in OECD TG 437 (OECD 2017a; referred to below as "BCOP-OECD") was followed for this evaluation. Detailed methodological information is provided in Section 2.1.2.1.

3.1.5.2 BCOP Extended Incubation Period Protocol

This testing approach (referred to below as "BCOP-extended") was added in Phase 2 to assess whether a longer incubation time could improve the method's performance for agrochemical formulations. The methodology described in OECD TG 437 (OECD 2017a) was

followed, except for one difference. After test article, negative control, or positive control was applied, the corneas were incubated at 32 ± 1ºC for 20 h.

3.1.5.3 Isolated Chicken Eye

Detailed methodological information is provided in Section 2.1.2.2.

3.1.5.4 Neutral Red Release

Detailed methodological information is provided in Section 2.1.2.4.

3.1.5.5 Porcine Corneal Opacity Reversibility

Porcine eyes (Spear Products, Coopersburg, PA) were received on ice. Corneas were dissected from the surrounding tissues and placed in six-well plates with HBSS. Corneas were then placed into a 24-well plate containing HBSS and then filled with a mixture of agarose and gelatin supplemented with media and antibiotics and allowed to solidify at room temperature. The corneas were transferred to large deep-well dishes and media was added to cover the limbal conjunctival junction and leave the corneal epithelia exposed. The mounted corneas were acclimatized overnight.

After equilibration, the media was removed and corneas were treated topically with 10 µl of the test article, positive control, or negative control. After 5 min, the corneas were rinsed twice with sterile Dulbecco's phosphate-buffered saline containing phenol red. Cultures were subsequently maintained in M-199 media supplemented with fetal bovine serum, sodium bicarbonate, L-glutamine, amphotericin B, gentamicin, penicillin, and streptomycin.

On Days 1, 2, 3, 7, 10, 14, and/or 21, the degree of corneal injury was visualized with sodium fluoride stain on a transilluminator. The area of sodium fluoride retention on each cornea was scored on a scale of 0 to 4. After data collection, a digital image of each cornea was acquired for reference, pre-warmed media was added to the dish, and the dish was returned to the incubator. On Day 21 of the study or the first observation point at which a treated or control cornea was observed to be clear of stain retention, observation of that cornea was halted and the cornea preserved in 10% formalin.

3.1.5.6 EO Standard Protocol

The protocol described in OECD TG 492 (OECD 2017c; referred to below as "EO-OECD") was followed for this evaluation. Detailed methodological information is provided in Section 2.1.2.3.

3.1.5.7 EO Time-to-Toxicity – Neat Method Protocol

The methodology described in OECD TG 492 (OECD 2017c) was followed for the time-to-toxicity – neat method protocol (referred to below as "EO-neat ET50"), except for one difference. After neat test articles or negative or positive controls were applied, the tissues

were observed at 3, 30, or 60 min, and the time recorded at which cell viability was reduced by 50%.

3.1.5.8 EO Time-to-Toxicity – Dilution Method Protocol

The methodology described for the time-to-toxicity – neat method protocol was followed for the time-to-toxicity – dilution method protocol (referred to below as "EC-dilution ET50"), except that test articles were tested at a 20% dilution in water.

3.1.6 Classification Criteria and Data Analysis

Predictions of EPA and GHS eye irritation hazard classifications were based on the individual in vitro test results. Decision criteria described in the relevant OECD test guidelines were used to assign classifications from BCOP-OECD, ICE-OECD, and EO-OECD test results (OECD 2017a, b, c). For the BCOP, in addition to classification based on the OECD test guideline, a separate classification was assigned incorporating histopathology results. Consideration of histopathology for classifications based on BCOP-extended, NRR, EO-neat ET50, and EO-dilution methods used criteria developed by the individual testing laboratories. The Consortium for in vitro Eye Irritation (CON4EI) developed EpiOcular classification criteria (EO-CON4EI) that were also used in this study (Kandarova et al. 2018). The classification key used in this evaluation is provided in **Table 6**.

Tested formulations were classified using both the EPA and GHS classification systems as ocular corrosives or irritants (EPA Category I, II, or III, or GHS Category 1 or 2), or chemicals not requiring classification and labeling (EPA Category IV or GHS NC) based on historical rabbit data. Classifications based on in vitro test method results were compared to historical rabbit classifications to evaluate concordance. A designation of "no prediction can be made" (NPCBM) was assigned when in vitro results and decision criteria did not allow for classification of formulation ocular irritancy potential in a specific hazard classification category. For example, a Category IV classification can be made using the EO-OECD classification system when tissue viability is greater than 60%. On the other hand, no definitive classification can be assigned when tissue viability is less than or equal to 60%. Therefore, if a formulation was classified as Category I/1, Category II/2A, or Category III/NC based on rabbit data and the tissue viability in the in vitro test was less than or equal to 60%, a "NPCBM" result would be noted because it could not be determined whether the result was discordant or concordant.

Table 6. Phase 1 and 2 In Vitro Results Classification Key and Criteria for Concordance with In Vivo Results

	In Vivo Classification Based on Historical Results (EPA/GHS)											
	Category IV/Category NC			**Category III/Category NC**			**Category II/Category 2A**			**Category I/Category 1**		
	Concord.[a]	**NPCBM**[a]	**Discord.**[a]	**Concord.**	**NPCBM**	**Discord.**	**Concord.**	**NPCBM**	**Discord.**	**Concord.**	**NPCBM**	**Discord.**
BCOP-OECD	IVIS ≤3 and histopath as III or IV/NC, or negative	IVIS ≤3 and histopath as negative-slight	IVIS >3	NA	IVIS >3 and ≤55	IVIS <3 or >55	NA	IVIS >3 and ≤55	IVIS <3 or >55	IVIS >55 or histopath as I/1, severe, or moderate-severe	NA	IVIS <55
BCOP-Extended	IVIS <15	NA	IVIS >15	NA	IVIS >15 and ≤55	IVIS <15 or >55	NA	IVIS >15 and ≤55	IVIS <15 or >55	IVIS >55	NA	IVIS <55
NRR	NRR50 >250 mg/mL	NA	NRR50 ≤250 mg/mL	NA	NRR50 >50 mg/mL	NRR50 <50 mg/mL	NA	NRR50 >50 mg/mL	NRR50 <50 mg/mL	NRR50 <50 mg/mL	NA	NRR50 >50 mg/mL
ICE-OECD	NC and histopath as NP	NP and histopath as NP	Any other combo	NA	NP and histopath as NP	Any other combo	NA	NP and histopath as NP	Any other combo	Cat 1 or histopath as Cat 1	NA	NC or NP and histopath as NP
PorCORA	NA	Revers.	Irrevers.	NA	Revers.	Irrevers.	NA	Revers.	Irrevers.	Irrevers.	Revers.	NA
EO-OECD	Viability >60%	NA	Viability ≤60%	NA	Viability ≤60%	Viability >60%	NA	Viability ≤60%	Viability >60%	NA	Viability ≤60%	Viability >60%
EO-neat ET50	ET50 ≥70 min	NA	ET50 <70 min	ET50 ≥4 and <70	NA	ET50 <4 or ≥70	NA	Any ET50	NA	ET50 <4 min	NA	ET50 ≥4 min
EO-dil. ET50	ET50 ≥256 min	ET50 >64 and <256 min	ET50 <64 min	NA	ET50 ≥16 and <256 min	ET50 <16 or >256 min	NA	ET50 ≥4 and <64 min	ET50 <4 or >64 min	ET50 <4 min	ET50 >4 and <16 min	ET50 ≥16 min
EO-CON4EI	NC	NA	Cat 1 or 2	NA	Cat 2 or NC	Cat 1	NA	Cat 2 or NC	Cat 1	Cat 1	NA	Cat 2 or NC

Abbreviations: Cat = Category; CON4E = Consortium for in vitro Eye Irritation Testing Strategy Project; combo = combination; Concord. = concordant result; dil. = dilution protocol; Discord.: discordant result; ET50 = exposure time required to reduce tissue viability to 50%; histopath = histopathology; Irrevers. = irritation did not reverse during 21-day observation period; IVIS = in vitro irritancy score; NA = not applicable; NC = not classified; NP = no prediction; NPCBM = no prediction can be made; NRR50 = concentration of test substance that causes 50% release of incorporated neutral red dye; Revers. = irritation reversed during 21-day observation period.
[a]Criteria for concordance, discordance, and NPCBM designations are described in the 'classification criteria and data analysis' section

3.2 Results

3.2.1 Phase 1

The Supplemental Information file provides results for all formulations in each of the methods tested in Phase 1. While none of the in vitro methods used in Phase 1 produced results that always aligned with the classifications assigned by the rabbit test, none produced discordant results for all the test formulations (**Table 7**). The EO-OECD, ICE-OECD, and PorCORA methods could not assign classifications for all tested formulations because the results were outside of the decision criteria for definitive classification of either corrosive or NC substances. Of the formulations that could be classified by these three methods, two of four formulations tested by ICE-OECD, two of two formulations tested by PorCORA, and three of three formulations tested by EO-OECD showed concordance between rabbit and in vitro data. The only discordant substance in the PorCORA assay was identified as reversing at Day 21, and thus was a borderline corrosive/severe irritant. The EC-dilution ET50 and EO-CON4EI methods each showed concordance for four of the six formulations tested. Since all of the methods used in Phase 1 showed promise for further evaluation, all methods were included in Phase 2.

Table 7. Concordance of Phase 1 In Vitro Results with In Vivo Classifications

	Category IV/Category NC			Category I/Category 1		
Formulation	**A**	**B**	**C**	**D**	**E**	**F**
BCOP-OECD[a]	Concordant	Concordant	Concordant	Concordant	Discordant	Concordant
NRR[b]	Discordant	Concordant	Concordant	Concordant	Concordant	Concordant
ICE-OECD[c]	NPCBM	Concordant	NPCBM	Discordant	Discordant	Concordant
PorCORA[d]	NPCBM	NPCBM	NPCBM	Concordant	Concordant	NPCBM
EO-OECD[b]	Concordant	Concordant	Concordant	NPCBM	NPCBM	NPCBM
EO-neat ET50[e]	Concordant	Concordant	Concordant	Concordant	Discordant	Concordant
EO-dil. ET50 [e]	Concordant	Concordant	Concordant	Discordant	Discordant	Concordant
EO-CON4EI[f]	Concordant	Concordant	Concordant	Discordant	Discordant	Concordant

Abbreviations: CON4EI = Consortium for in vitro Eye Irritation Testing Strategy Project; dil. = dilution protocol; ET50 = exposure time required to reduce tissue viability to 50%.

[a]Classification based on most severe response obtained from in vitro irritancy score or histopathology results.

[b]Classification based on most severe response obtained in two runs.

[c]Classification based on most severe response obtained from ICE score or histopathology results.

[d]Classification based on reversibility.

[e]Classification based on most severe response obtained in two to three runs.

[f]Classification presented in Kandarova et al. (2018). Mean of all runs used for decision tree calculations.

3.2.2 Phase 2

The Supplemental Information file provides results for all formulations in each of the methods tested in Phase 2. Similar to Phase 1 results, none of the results produced by the in vitro methods in Phase 2 aligned with rabbit data for all 10 test formulations, but each method produced results that aligned for some portion of the full list of test formulations (**Table 8**). Due to the limits of the decision criteria for the PorCORA method which are focused on identifying corrosives/severe irritants, only two formulations could be classified.

Table 8. Concordance of Phase 2 In Vitro Results with In Vivo Classifications

	Category IV/Category NC				Cat. III/ Cat. NC	Cat. II/ Cat. 2A	Category I/Category 1				
Formulation	**M**	**N**	**O**	**P**	**L**	**K**	**G**	**H**	**I**	**J**	**Concord. %**
BCOP-OECD[a]	NPCBM	NPCBM	NPCBM	Concord.	Discord.	Discord.	Concord.	Concord.	Concord.	Concord.	71% (5/7)
BCOP-Extended[b]	Concord.	Concord.	Concord.	Concord.	Discord.	Discord.	Concord.	Discord.	Concord.	Discord.	60% (6/10)
NRR[c]	Discord.	Concord.	Discord.	Discord.	Discord.	Discord.	Concord.	Concord.	Discord.	Discord.	30% (3/10)
ICE-OECD[d]	Concord.	Concord.	NPCBM	Concord.	NPCBM	Discord.	Discord.	Concord.	Concord.	Concord.	75% (6/8)
PorCORA[e]	NPCBM	NPCBM	NPCBM	NPCBM	NPCBM	NPCBM	NPCBM	Concord.	NPCBM	Concord.	100% (2/2)
EO-OECD[e]	Concord.	Concord.	Discord.	Concord.	NPCBM	NPCBM	NPCBM	NPCBM	NPCBM	NPCBM	75% (3/4)
EO-neat ET50[f]	Discord.	Concord.	Discord.	Concord.	Concord.	NPCBM	Concord.	Concord.	Concord.	Discord.	67% (6/9)
EO-dil. ET50[f]	NPCBM	Concord.	Discord.	Concord.	NPCBM	NPCBM	NPCBM	Discord.	Discord.	Discord.	33% (2/6)
EO-CON4EI[g]	Discord.	Concord.	Discord.	Concord.	NPCBM	NPCBM	Concord.	Discord.	Discord.	Discord.	38% (3/8)

Abbreviations: Cat. = Category; CON4EI = Consortium for in vitro Eye Irritation Testing Strategy Project; Concord. = concordant results; Concord. % = concordant result percentage; dil. = dilution protocol; Discord. = discordant results; ET50 = exposure time required to reduce tissue viability to 50%; Form. = formulation.

[a]Classification based on most severe response obtained from IVIS or histopathology results.

[b]Classification based on IVIS.

[c]Classification based on most severe response obtained in two runs.

[d]Classification based on most severe response obtained from ICE score or histopathology results.

[e]Classification based on reversibility.
[f]Classification based on most severe response obtained in two to three runs.
[g]Classification presented in Kandarova *et al*. (2018). Mean of all runs used for decision tree calculations.

3.2.3 Range of Responses of Methods Evaluated in Phases 1 and 2

The results from both phases were combined to determine if natural separation of in vitro data points could be identified for the different rabbit classification categories (**Figure 2**). Based on the following graphs, the limited number of tested Category II and III formulations combined with the large range of values observed for the Category I and IV formulations limits the ability to modify current decision criteria for application to agrochemical formulations.

Figure 2. Distribution of In Vitro Phase 1 and 2 Data vs. EPA Hazard Classification Categories Based on Historical Rabbit Data

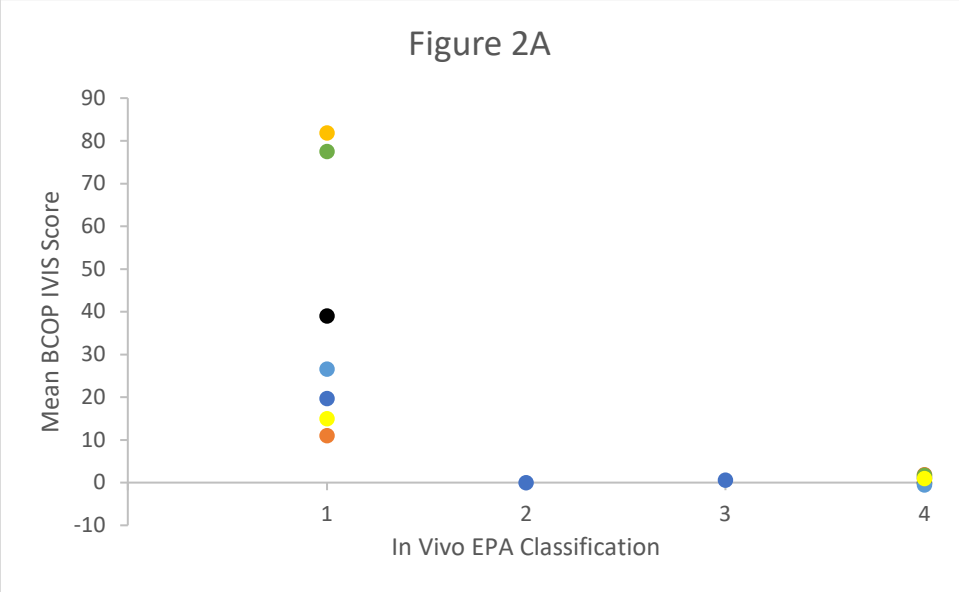


Figure 2A: Mean BCOP IVIS vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations

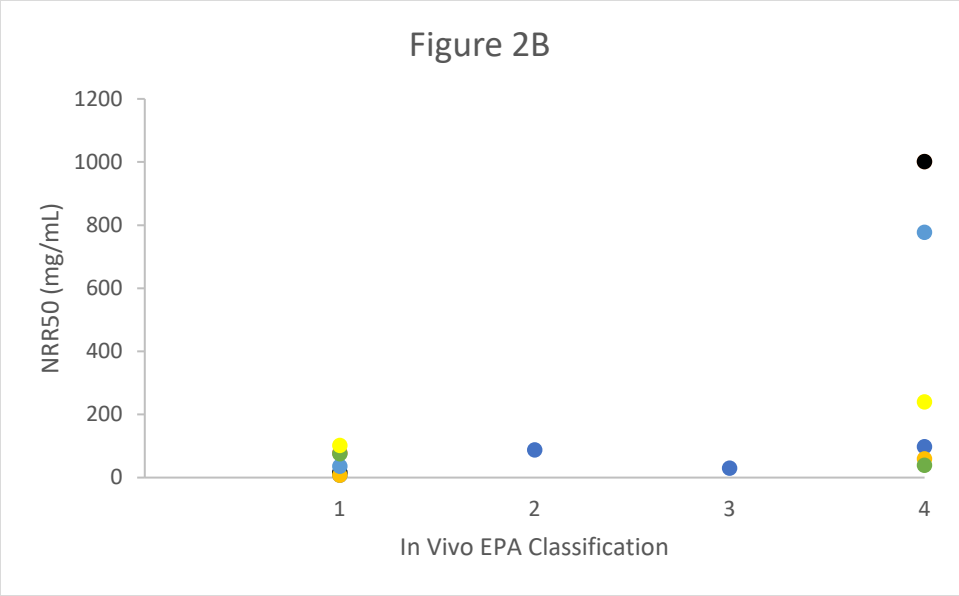


Figure 2B. NRR50 vs. in vivo hazard EPA classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations

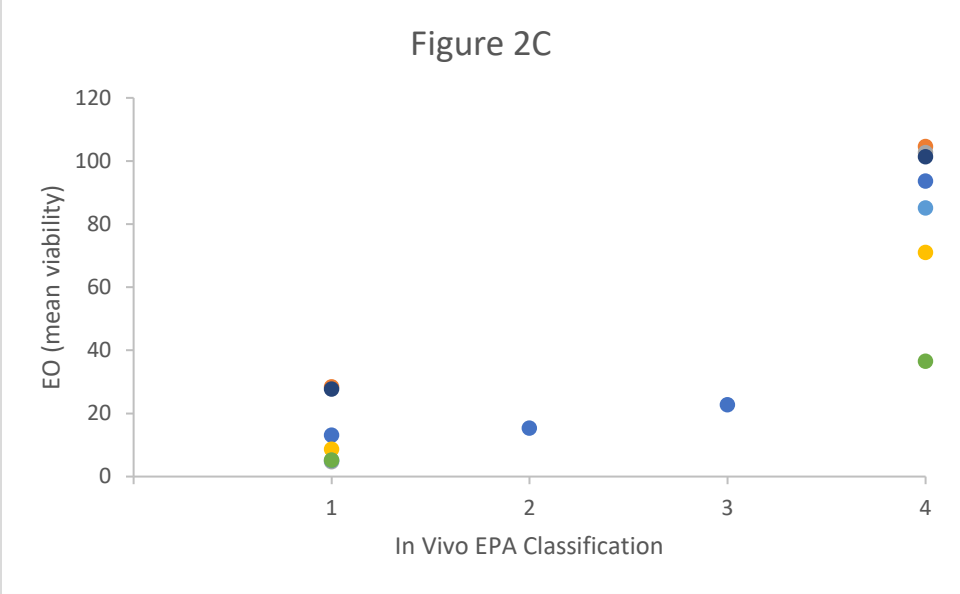


Figure 2C. EO (mean viability) vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

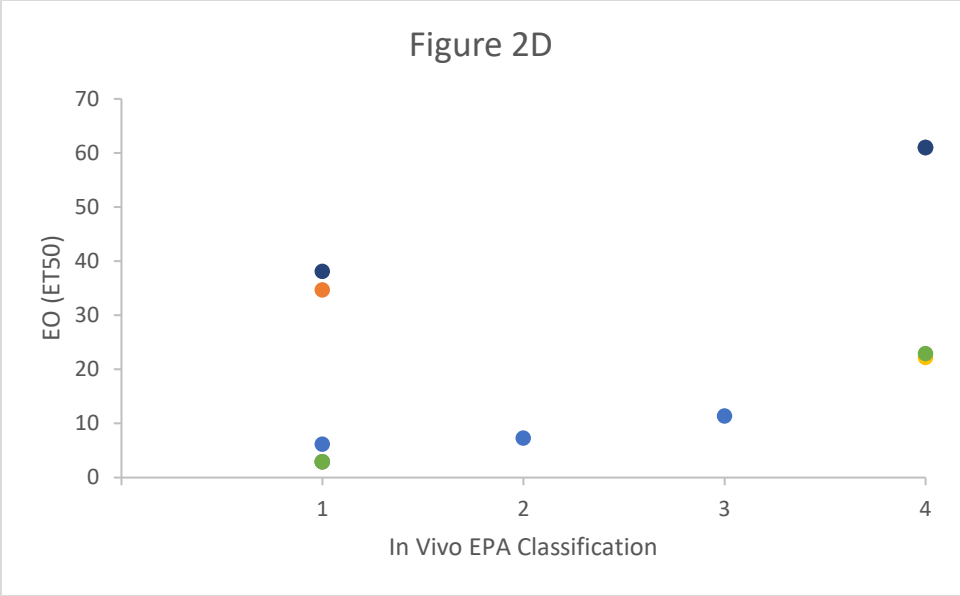


Figure 2D. EO (ET50) vs. in vivo hazard EPA classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

3.2.4 Range of Responses of Methods Evaluated in Phase 1 and 2 Results, and Retrospective Analysis

To increase the number of available data points, results from both phases of the prospective testing were combined with available retrospective data to determine if revised decision criteria could be applied to the in vitro data points for the different in vivo classification categories (**Figure 3**). Based on the following graphs, the large range of values observed for the graphed in vivo EPA classification categories limits the ability to modify current decision criteria for application to agrochemical formulations. Further analyses that do not use the rabbit data as a reference are underway to glean additional insights.

Figure 3. Distribution of In Vitro Phase 1 and 2 and Available Retrospective Data vs. EPA Hazard Classification Categories Based on Historical Rabbit Data

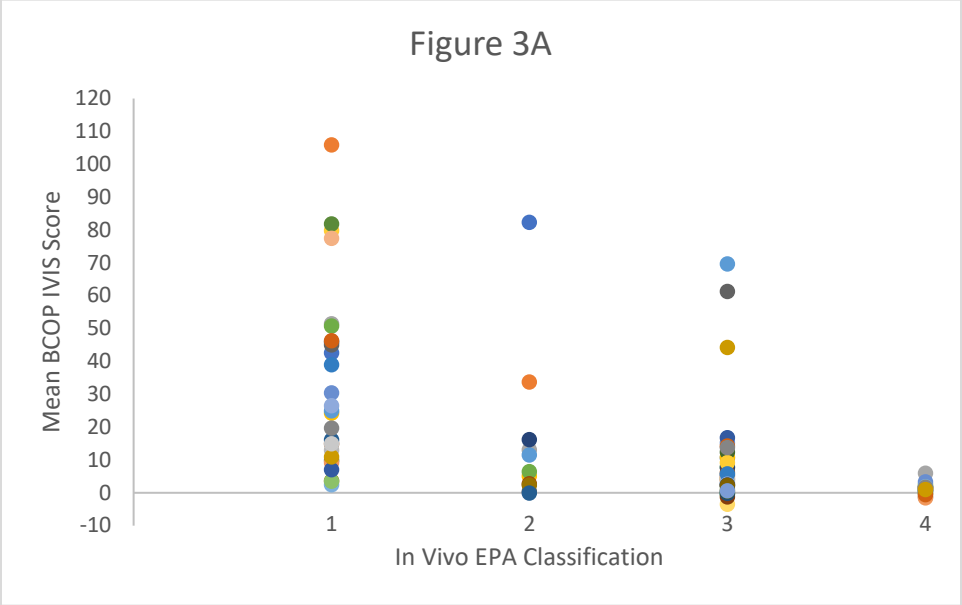


Figure 3A: Mean BCOP IVIS vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

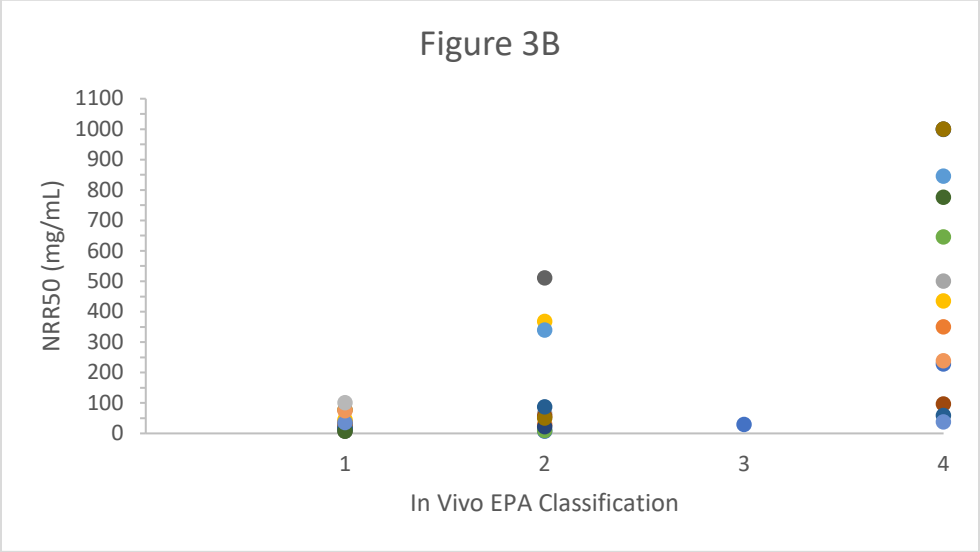


Figure 3B. NRR50 vs. in vivo hazard EPA classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

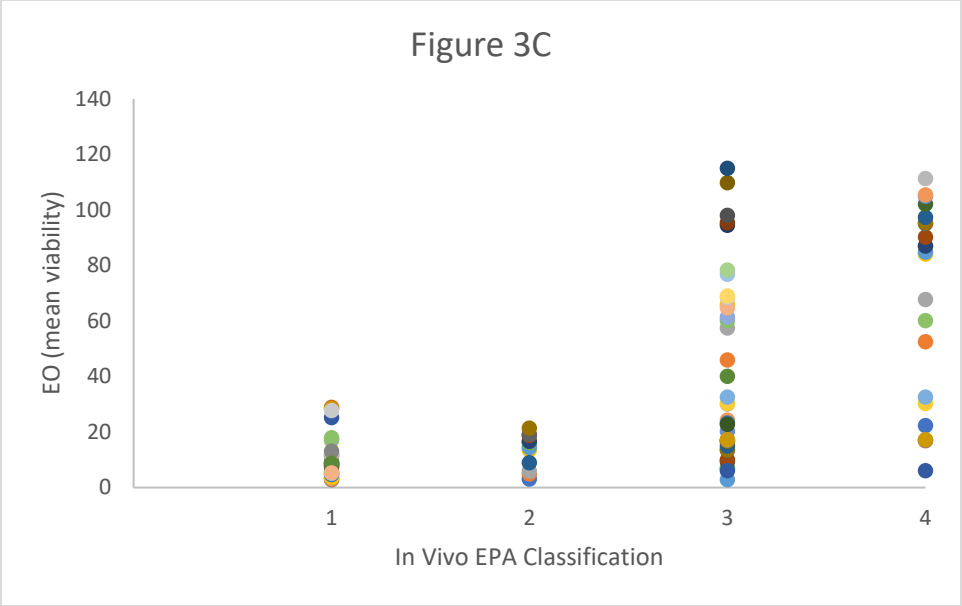


Figure 3C. EO (mean viability) vs. in vivo EPA hazard classification for tested formulations based on historical rabbit results. Different points on graph represent tested formulations.

4. RESULTS/DISCUSSION

The test methods evaluated in this study represent a variety of domains of applicability and coverage of key biological events. For example, the BCOP and ICE methods provide a full-thickness model to assess corneal effects (e.g., damage to corneal epithelium, corneal stroma). Inclusion of histopathology in these models can provide information about depth of injury. PorCORA can assess reversibility of effects, but such effects are limited to those observed in the epithelium. Questions also remain regarding the potential impact of interspecies differences on the utility of the in vitro methods (e.g., BCOP, ICE, PorCORA) when used to predict the human response; however, endpoints measured by these and the associated limitations would seem to be analogous to those measured by the currently used rabbit test. Three-dimensional reconstructed human corneal tissue models are of particular interest because they measure cytotoxicity, a critical event in the irritation pathway, in cells from the species of interest.

A recently submitted publication characterizes the available in vivo and in vitro test methods with respect to their relevance to human ocular anatomy, anticipated exposure scenarios, and the mechanisms of eye irritation/corrosion (Clippinger et al., 2021). The in vitro methods were shown to be at least as relevant to the effects observed in the human eye when compared to the rabbit eye. These observations underscore the importance of not using the rabbit test as a reference to validate new methods.

The current study was conducted as a proof-of-principle to determine if specific methods could be used to develop a testing strategy to determine the eye irritation potential of agrochemical formulations. This is important because agrochemical formulations are not among the reference

substances included in previous validation studies that demonstrated the usefulness and limitations of many of these methods (OECD 2017a, b, c).

Further analyses of the data presented above are underway to see how the in vitro data align with each other. Additionally, data are being evaluated quantitatively, where possible, to provide clarity on the extent to which the results are discordant. For example, we are examining the repeated test results on the same substance narrowly falling on either side of the Category I threshold. While this would be a discordant outcome from the point of view of hazard classification, such results would actually be more quantitatively concordant than two results at the extreme ends of Category I. These analyses will also take into consideration the reproducibility of the rabbit test to provide proper context to any direct comparisons between the in vitro and rabbit test.

It should also be noted that several evaluated test methods do not have empirically derived decision criteria for specific regulatory classification groups. For example, the OECD EO classification system provides classification criteria for chemicals that do not require classification (i.e., Category IV/Category NC); however, there are no criteria available for severe/corrosive eye irritants (i.e., Category I/Category 1). Therefore, it is unknown whether these in vitro methods correctly identified the tested formulations.

Ideally, future evaluations and acceptance decisions will be based on the extent to which methods align with the mechanisms associated with eye irritation in humans, and not simply based on the extent of concordance with the rabbit test method.

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