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

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Addendum to the 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
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TABLE OF CONTENTS

TABLES

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REVIEW

The analyses and results described in this document have been reviewed by, and reflect 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 six different ICCVAM member agencies who are listed below.

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PREFACE

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is an office within the Division of Translational Toxicology, 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 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

This report serves as an addendum to the NIEHS report, "Prospective and Retrospective Evaluation of the Eye Irritation Potential of Agrochemical Formulations" (Choksi et al., 2021) describing the two-phased evaluation of agrochemical formulations conducted previously. Herein we describe a third phase of prospective studies conducted to evaluate the usefulness and limitations of a group of in vitro test methods[1] that could potentially be combined into a defined approach to assign hazard classification and personal protective equipment (PPE) labeling for eye irritation potential based on the United Nations Globally Harmonized System for Classification and Labeling of Chemicals (GHS) and U.S. Environmental Protection Agency (EPA) hazard classification systems (EPA, 2004; United Nations, 2019; **Table 1**). While the scope of this report is focused on the methods and results associated with Phase 3 prospective testing, we have also included results from Phases 1 and 2 to provide a comprehensive view of the full study results in one place. By doing so, we can provide the results used for analyses and interpretation of the full data set generated throughout all phases of this study. Given the recognized limitations of the in vivo test method (Clippinger et al., 2021; Luechtefeld et al., 2016), particularly with respect to mild and moderate irritants, we have not included concordance analyses herein but instead provided results that can be assessed across all assays included in all three study phases.

[1]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).

Table 1. EPA and GHS Ocular Irritation Classification Systems

EPA Category	EPA Classification[a]	EPA PPE Requirement	GHS Category	GHS Classification[b]	GHS PPE Requirement
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[c]	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[c]	NC	No effects are produced, or minimal effects observed that do not lead to classification	None noted

[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 of three tested 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 chemosis or conjunctival redness score ≥2 (over Days 1, 2, and 3) in at least two of three tested animals.
[c]EPA may recommend inclusion of eye protection labelling for Category III substances, if deemed appropriate.
Abbreviations: NC = not classified; PPE = personal protective equipment.

As detailed previously (Choksi et al., 2021), PETA Science Consortium International e.V., CropLife America (CLA) companies, and NICEATM collaborated in Phases 1 and 2 of this study to evaluate a set of 16 agrochemical formulations in a common set of in vitro eye irritation and corrosion test method protocols. Based on an assessment of those results, and considering other factors (e.g., the relevance of each method to humans, applicability to agrochemical formulations, inclusion in an Organisation for Economic Co-operation and Development [OECD] Test Guideline [TG]), the EpiOcular™ (EO) standard protocol and the bovine corneal opacity and permeability (BCOP) standard protocol (with histopathology) were selected to proceed with Phase 3, which expanded the number of mild and moderate irritant formulations tested (i.e., formulations classified based on the in vivo rabbit test as GHS Category 2A or 2B, or EPA Category II or III). The common set of test methods included in the evaluation

was also expanded in Phase 3. In addition to the Phase 3 formulations, the 16 formulations from Phases 1 and 2 were tested in the SkinEthic Time-to-Toxicity approach for liquids (TTL) and the EyeIRR-IS method. Additionally, 12 middle irritancy formulations were tested in the in vitro depth of injury (IVDoI) method.

A total of 29 formulations with available historical in vivo test have been tested in as many as five in vitro eye irritation and corrosion test methods (**Table 2**). Hazard classifications based on each test method and associated decision criteria were used to assign the categories that were then used for determining the extent of agreement across all test methods.

Table 2. Index of Prospective Testing Phase of Agrochemical Formulations in Non-Animal Methods

Formulation Code	Historical In Vivo Rabbit EPA Class.	Historical In Vivo Rabbit GHS Class.	BCOP	EO	TTL	IVDoI	EyeIRR-IS
A	IV	NC	Phase 1	Phase 1	Phase 3	IS	Phase 3
B	IV	NC	Phase 1	Phase 1	Phase 3	IS	Phase 3
C	IV	NC	Phase 1	Phase 1	Phase 3	IS	Phase 3
D	I	1	Phase 1	Phase 1	Phase 3	IS	Phase 3
E	I	1	Phase 1	Phase 1	Phase 3	IS	Phase 3
F	I	1	Phase 1	Phase 1	Phase 3	IS	Phase 3
G	I	1	Phase 2	Phase 2	Phase 3	IS	Phase 3
H	I	1	Phase 2	Phase 2	Phase 3	IS	Phase 3
I	I	1	Phase 2	Phase 2	Phase 3	IS	Phase 3
J	I	1	Phase 2	Phase 2	Phase 3	IS	Phase 3
K	II	2A	Phase 2	Phase 2	Phase 3	IS	Phase 3
L	III	NC	Phase 2	Phase 2	Phase 3	IS	Phase 3
M	IV	NC	Phase 2	Phase 2	Phase 3	IS	Phase 3
N	IV	NC	Phase 2	Phase 2	Phase 3	IS	Phase 3
O	IV	NC	Phase 2	Phase 2	Phase 3	IS	Phase 3
P	IV	NC	Phase 2	Phase 2	Phase 3	IS	Phase 3
Q	II	NC	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
R	II	2A	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
S	III	2B	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
T	III	NC	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
U	II	2A	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
V	III	2B	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
W	III	NC	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
X	II	2A	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
Y	II	2A	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
Z	III	NC	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
AA	II	2A	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
AB	III	2B	Phase 3	Phase 3	Not tested	Not tested	Not tested
AC	III	NC	Phase 3	Phase 3	Phase 3	Phase 3	Phase 3
Total			29	29	28	28	28

Abbreviations: BCOP = bovine corneal opacity and permeability; Class. = classification; EO = EpiOcular; IS = independent study; IVDoI = in vitro depth of injury; NC = not classified; TTL = SkinEthic Time-to-Toxicity approach for liquids.

2. MATERIALS AND METHODS

2.1 Phase 3 Goals

Prospective testing in Phase 3 was focused on compiling a comprehensive dataset of results from multiple in vitro eye irritation test methods for a reference list of agrochemicals. These results will be used as a proof-of-principle to determine whether these method(s) might be useful in defined approaches to determine the eye irritation potential of agrochemical formulations.

Accordingly, in Phase 3, 13 formulations classified as moderately (EPA Category II; GHS Category 2A) or mildly irritating (EPA Category III; GHS Category 2B), based on existing in vivo rabbit data and according to at least one of the two classification systems, were tested in EO and BCOP (n=5 EPA II/GHS 2A; n=1 EPA II/GHS NC; n=3 EPA III/GHS 2B; n=4 EPA III/GHS NC).

All formulations from all phases were also tested in TTL and EyeIRR-IS, except formulation AB for which the donated volume was insufficient to test in these methods. The formulations tested in TTL and EyeIRR-IS represented the full range of classifications (n=7 EPA I/GHS 1; n=6 EPA II/GHS 2A; n=1 EPA II/GHS NC; n=2 EPA III/GHS 2B; n=5 EPA III/GHS NC; n=7 EPA IV/GHS NC).

A subset of 12 formulations classified as moderately or mildly irritating based on existing in vivo rabbit data according to at least one of the two classification systems were tested in IVDoI (n=5 EPA II/GHS 2A; n=1 EPA II/GHS NC; n=2 EPA III/GHS 2B; n=4 EPA III/GHS NC).[2]

2.2 Formulation Selection

Formulations (**Table 3**) were donated by CLA companies (BASF, Bayer/Monsanto, Corteva Agriscience, Corteva Agriscience/Dow AgroSciences, Dow AgroSciences/DuPont, 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 information 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). Additionally, this information enabled interrogation of the reliability of the in

[2] Phase 1 and 2 agrochemical formulations (n=16) were tested in IVDoI in an independent study, and results are reported herein. All testing was performed by the same laboratory using identical methodologies. However, a single run was conducted for the independent study, whereas Phase 3 formulations were tested in three runs.

vivo test (i.e., number of animals tested vs. number of animals driving the classification).

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 4**) 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 3. Tested Formulations and Classifications Based on Historical In Vivo Data

Phase	Formulation Code	Active Ingredient	Formulation Type	EPA Class.[a]	EPA Category I Driver	GHS Class. [a]	GHS Category 1 Driver	No. Animals Tested [No. Driving Class.]
1, 3	A	Afidopyropen	EC/ME	IV	NA	NC	NA	3 [3]
1, 3	B	Spirotetramat	SC	IV	NA	NC	NA	3 [3]
1, 3	C	Fenbuconazole	SC	IV	NA	NC	NA	9 [9]
1, 3	D	Pyraclostrobin, Mefentrifluconazole	EC	I	Persistence	1	Persistence	1 [1]
1, 3	E	Afidopyropen	EC	I	Persistence	1	Persistence	1 [1]
1, 3	F	2,4-D TIPA salt	SL	I	Persistence	1	Persistence	6 [NR]
2, 3	G	Chlorpyrifos-methyl + Deltamethrin	EC	I	Persistence	1	Persistence	3 [1]
2, 3	H	2,4-D Choline salt	SL	I	Persistence	1	Persistence	3 [1]
2, 3	I	Methomyl	SL	I	Persistence	1	Persistence	6 [1]
2, 3	J	Benzovindiflupyr/Solatenol	EC	I	Persistence	1	Persistence	1 [1]
2, 3	K	Glyphosate	SL	II	NA	2A	NA	3 [1]
2, 3	L	Propiconazole	EC	III	NA	NC	NA	3 [NR]
2, 3	M	Propamocarb hydrochloride	SL	IV	NA	NC	NA	3 [3]
2, 3	N	Penoxsulam	SC	IV	NA	NC	NA	3 [3]
2, 3	O	Glyphosate	SL	IV	NA	NC	NA	3 [3]
2, 3	P	Mesotrione	SC	IV	NA	NC	NA	3 [3]
3	Q	Glyphosate	SL	II	NA	NC	NA	3 [1]
3	R	Glyphosate; Triclopyr	SL	II	NA	2A	NA	3 [1]
3	S	Glyphosate	SL	III	NA	2B	NA	3 [1]
3	T	Folpet; Oxathiapiprolin	SC	III	NA	NC	NA	3 [1]
3	U	Triclopyr-2-butoxyethyl ester	EC	II	NA	2A	NA	3 [1]
3	V	2,4-Dichlorophenoxyacetic acid; Triisopropanolamine salt and Picloram triisopropanolamine salt	SL	III	NA	2B	NA	3 [2]
3	W	Glyphosate	SL	III	NA	NC	NA	3 [1]
3	X	Pyraclostrobin; Mefentrifluconazole	EC	II	NA	2A	NA	3 [1]
3	Y	Afidopyropen	EC	II	NA	2A	NA	3 [1]
3	Z	Lambda-Cyhalothrin	EC	III	NA	NC	NA	3 [1]
3	AA	Difenoconozale	EC	II	NA	2A	NA	3 [1]
3	AB	Fluroxypyr-meptyl; Cyhalofop-butyl	EC	III	NA	2B	NA	3 [1]
3	AC	Picoxystrobin; Cyproconazole	EC	III	NA	NC	NA	3 [NR]

Abbreviations: Class. = classification; EC = emulsifiable concentrate, ME = microencapsulated; SC = suspension concentrate, SL = soluble liquid; NA = not applicable; NC = not classified; NR = not reported.

[a] EPA and GHS classifications based on historical in vivo data.

2.3 Participating Laboratories

Five independent testing laboratories conducted prospective testing of formulations in the five in vitro test methods (**Table 4**). Test methods for which an OECD test guideline was followed are noted. The remaining test methods were conducted using in-house testing protocols. While testing did not always include review by a quality assurance unit and thus was not technically GLP-compliant, all testing was conducted according to the principles of GLP. All methods are described below.

Table 4. Evaluated In Vitro Methods and Testing Laboratories

Test Method	Test Method Details	Testing Laboratory
BCOP with histo	Standard protocol as described in OECD TG 437 (OECD, 2023a), with predictions based on incorporation of IVIS and histo findings	Institute for In Vitro Sciences
EO	Standard protocol as described in OECD TG 492 (OECD, 2023b)	MatTek Life Sciences
IVDoI-10%	10% protocol (surfactants tested at 10%; non-surfactants tested at 100%)	Lebrun Labs
IVDoI-Neat	Neat protocol (all test formulations tested at 100%)	Lebrun Labs
TTL	Standard protocol as described in OECD TG 492B (OECD, 2022a)	EpiSkin
EyeIRR-IS	Standard protocol as described in Cottrez et al. (2021)	ImmunoSearch

Abbreviations: BCOP = bovine corneal opacity and permeability; EO = EpiOcular; histo = histopathology; IVDoI = in vitro depth of injury; IVIS = in vitro irritancy score; OECD TG = Organisation for Economic Co-operation and Development test guideline; TTL = SkinEthic Time-to-Toxicity approach for liquids.

2.4 Study Management

Scientists from Inotiv-NICEATM and PETA Science Consortium International e.V. comprised the study management team that reviewed and approved the study design, study timeline, and deliverables. 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.

2.5 Test Methods

2.5.1 BCOP

The protocol for BCOP OP-KIT described in OECD TG 437 (OECD, 2023a) was followed for this evaluation. 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 formulation, 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 minutes 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 (OD490) measured. Opacity (measured with OP-KIT opacitometer) and mean permeability values were used to calculate the in vitro irritancy score (IVIS) for each treatment group using the following equation noted in OECD TG 437 (OECD, 2023a).

IVIS with OP-KIT = mean opacity (read-out OP-KIT) + (15 x mean permeability OD490)

2.5.2 EO

The protocol described in OECD TG 492 (OECD, 2023b) for the EpiOcular™ Eye Irritation Test was followed for this evaluation. Briefly, test formulations 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 (PBS), 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.

2.5.3 IVDoI

IVDoI testing was conducted according to two protocols, differentiated in this report as "IVDoI-10%" and "IVDoI-Neat."

2.5.3.1 IVDoI-10%

The protocol described by Lebrun et al. (unpublished) was followed for this evaluation. Briefly, test substances were pre-screened to differentiate surfactants and non-surfactants by diluting to 10% in blanking buffer, vortexing, and measuring froth above the meniscus. Substances with froth extending 0.2 cm above the meniscus were deemed surfactants and were tested at 10% dilution in water. All other substances were tested neat.

Rabbit eyes (collected after slaughter for human consumption) in Hank's balanced salt solution were obtained, iced, from Pel-Freez Biologicals (Rogers, AR). After removing the eyelids and ocular muscles, a 0.25% solution of Lissamine™ Green B was applied. The eyes were then rinsed with PBS, and a dissecting microscope was used to examine the integrity of the corneal surface. Eyes with observed green staining were excluded from testing, while intact eyes were placed in a 12-well plate with sterile cell culture media and incubated for 2 hours. Eyes were then placed on holders, and test materials were pipetted into an 8-mm dosing ring placed on the central cornea. After 1 minute, the material was removed, and the eyes rinsed with sterile wash buffer. Eyes were then returned to 12-well plates and incubated for 24 hours. Corneas (with iris attached to aid preservation of corneal shape) were then separated from the

rest of the tissue, placed in fixative, and processed for fluorescent labeling.

The direct stromal depth of injury (DoI) was measured using imaging software and calculated as (thickness of nonviable stroma) / (total stromal thickness) x 100.

When the result was negative (i.e., DoI = 0%), the lab repeated the test on a different eye keeping all conditions the same, with one exception: eyes were exposed to the test formulations for 6 min. This extended exposure, referred to as a "metabolic test," is intended to confirm negative results by providing additional time for the metabolic conversion of substrates into active (i.e., irritant) products.

2.5.3.2 IVDoI-Neat

The procedures described above for the IVDoI-10% protocol were followed for this evaluation, except that all test formulations were tested neat, regardless of whether they were identified as surfactants during pre-screening.

2.5.4 TTL

The protocol described in OECD TG 492B (OECD, 2022a) for SkinEthic™ human corneal epithelium Time-to-Toxicity for approach for liquids was followed for this evaluation. Briefly, formulations were applied to reconstructed human cornea-like epithelium (RhCE, maturation day 5) for exposure durations of 5, 16, and 120 minutes, and then rinsed. Formulations were tested neat for 5-minute exposures and diluted 20% (w/v) in water for 16- and 120-minute exposures. Percent viability of the tissues exposed to the test formulation was determined, relative to that of tissue treated with the negative control, for each of the three exposure times.

2.5.5 EyeIRR-IS

The protocol described in Cottrez et al. (2021) were followed for this evaluation. Briefly, the tissue surface was moistened with PBS and incubated for 10 minutes at 37°C. An epithelium was then topically treated with 50±2 µL of the test chemical preparation (corresponding to 100 $\mu L/cm^2$) and incubated for 10 minutes at room temperature. The test formulation was then gently rinsed from the tissue by spraying sterile PBS against the cell culture insert wall (not directly on the tissue). The tissues still resting on their insert support were then "soaked" in 5 mL of culture medium for 30 minutes to ensure complete removal of any remaining test formulation. The rinsing medium was removed, and fresh culture medium was added. Tissues were then incubated for 6 hours. Total RNA was extracted, and quantitative reverse transcription-polymerase chain reaction was performed for gene expression analysis of 10 genes. An algorithm based on gene expression modulation was used to calculate a liquid irritation index (LII).

2.6 Classification Criteria

The classification criteria used in this evaluation are described below and summarized in **Tables 5 - 7**.

2.6.1 BCOP

Predictions of eye irritation hazard classifications were based on a combination of the individual in vitro test results (IVIS scores) and histopathological findings.

Histopathology results and depth and degree of corneal injury were analyzed by experts at the testing laboratory, and the following general guidance (adapted from decision criteria presented in Redden et al. (2009)) was used to categorize eye irritation potential:

- Minimal: damage or loss limited to the surface squamous cell layer in the epithelium.
- Mild: damage or loss extends to the wing cell layers in the epithelium; basal cell layer and basal lamina remain intact.
- Moderate: damage typically involves all layers of the epithelium and may cause stromal keratocyte damage no deeper than the upper third to half of the stroma.
- Severe: keratocyte damage extends into the lower half of the stroma and may include damage to the endothelium.

Decision criteria described in OECD TG 437 (OECD, 2023a) were used to predict GHS classifications based on IVIS score calculations. Decision criteria described in EPA's alternate testing framework (EPA, 2015) were used to predict EPA classifications based on IVIS score calculations.

Additional criteria which incorporate histopathology findings (**Table 5**) were developed to assign final classifications, as presented in the results of this study. The final classifications were determined by the more severe outcome (i.e., the result of the IVIS score calculation alone vs. the result of the histopathological findings of ocular injury, where "minimal" correlates with EPA IV/GHS NC, "mild" with EPA III/GHS 2B, "moderate" with EPA II/GHS 2A, and "severe" with EPA I/GHS 1).

Final predictions of GHS and EPA classifications are distinguished henceforth as "BCOP-OECD+histo" and "BCOP-EPA+histo", respectively.

2.6.2 EO

Predictions of eye irritation hazard classifications were based on the individual in vitro test results. Decision criteria described in OECD TG 492 (OECD, 2023b) were used to predict GHS classifications ("EO-OECD"). EO results were not used to predict EPA classifications.

2.6.3 IVDoI

Each formulation was tested in triplicate, and predictions of eye irritation hazard classifications were based on agreement of at least two of the three individual test runs.[3] Lebrun Labs developed decision criteria (unpublished) that were used to assign both EPA and GHS classifications in this study. The same classification criteria were used for both protocols ("IVDoI-10%" and "IVDoI-Neat"). Briefly:

- DoI > 20% was classified as EPA I/GHS 1.
- 15% ≤ DoI ≤ 20% was classified as EPA II/GHS 2A.
- DoI < 15% was classified as EPA III/GHS 2B.
- Where the direct stromal DoI = 0%, an additional metabolic test was performed to distinguish between non-irritants. The procedure for the metabolic test is the same as described above, except a tear solution (saline with ascorbic acid) is first added to the dosing ring, followed by the test material, for an exposure duration of 6 minutes. If any degree of stromal damage is observed after washing, the material is classified as EPA II/GHS 2A. If no stromal damage is observed, the substance is classified as EPA IV/GHS NC.

2.6.4 TTL

Each formulation was evaluated in duplicate runs using a single batch of RhCE tissues. Predictions of eye irritation hazard classifications were based on the individual in vitro test results. Decision criteria described in OECD TG 492B (OECD, 2022a) were used to predict GHS classifications ("TTL-OECD"). TTL data were not used to predict EPA classifications.

2.6.5 EyeIRR-IS

Each formulation was tested neat and diluted at 30% in PBS, on the same tissue batch. Predictions of eye irritation hazard classifications were based on agreement of at least two independent test runs performed on different tissue batches. Decision criteria described by Cottrez et al. (2021) were used to assign GHS classifications. EyeIRR-IS data were not used to assign EPA classifications.

2.7 Alignment Analysis

Given the limitations and low reliability of the in vivo rabbit eye test, it was not appropriate to assess performance of the other methods based solely on concordance of predictions with that of the in vivo data. Therefore, we evaluated alignment of predictions across the individual in vitro test methods and the historical in vivo rabbit eye test data.

[3] Excludes Phase 1 and 2 agrochemical formulations (n=16), which were tested in an independent study. For these formulations, the result of the single run was used as the GHS prediction.

2.7.1 Alignment of EPA Predictions Across Methods

We evaluated alignment of predictions across methods with EPA classification criteria (i.e., BCOP-EPA+histo, IVDoI-10% and IVDoI-Neat, and historical in vivo rabbit eye test data). Classifications based on in vitro test method results and historical rabbit classifications were compared to enable an evaluation of alignment. Since histopathology was conducted for all eyes regardless of the IVIS score as part of the BCOP-EPA+histo method, we incorporated the histopathological findings with IVIS results to determine an overall BCOP-EPA+histo prediction for use in the alignment analysis (**Table 5**). We considered histopathological classifications of "severe," "moderate," "mild," and "minimal" equivalent to predictions of EPA I, II, III, and IV, respectively. The overall BCOP-EPA+histo prediction used in the alignment analysis was the more severe of the predictions based on IVIS alone or histopathology alone. For example, the overall BCOP-EPA+histo prediction for a formulation with IVIS 24 (corresponding to EPA III) and histopathology findings of "moderate" (corresponding to EPA II) would be EPA II.

For each formulation, we used these guidelines to evaluate alignment of EPA predictions across methods and determine whether a majority of methods (i.e., at least 2 of 3 methods) achieved the same prediction (henceforth referred to as "majority EPA prediction"). For formulations where a majority alignment was not achieved, we listed the majority EPA prediction as "inconclusive". We also evaluated PPE labeling.

2.7.2 Alignment of GHS Predictions Across Methods

We evaluated alignment of predictions across methods with GHS classification criteria (i.e., BCOP-OECD+histo, EO-OECD, IVDoI-Neat and IVDoI-10%, TTL-OECD, EyeIRR-IS, and historical in vivo rabbit eye test data). Classifications based on in vitro test method results and historical rabbit classifications were compared to enable an evaluation of alignment. The classification critieria for the IVDoI protocols and the historical in vivo rabbit eye test data allow for classification of the full spectrum of eye irritation/corrosion potential (i.e., GHS 1, 2A, 2B, or NC). However, based upon the OECD TGs, the classification criteria of the BCOP and EO methods only allow for classification of corrosives and non-irritants (i.e., GHS 1 and NC) and non-irritants (i.e., GHS NC), respectively. Furthermore, while the classification criteria of the TTL and EyeIRR-IS methods allow for classification of non-corrosive irritants (i.e., GHS 2), they do not allow for subclassification to distinguish moderate and mild eye irritants (i.e., GHS 2A and 2B, respectively). Thus, we used the following guidelines to address differences in classification criteria:

- BCOP-OECD+histo: Since histopathology was conducted for all eyes regardless of the IVIS score as part of the BCOP-OECD+histo method, we incorporated the histopathological findings with IVIS results to determine an overall BCOP-OECD+histo prediction for use in the alignment analysis (**Table 5**). This concept is in line with recommendations of OECD Guidance Document 160, which encourages using histopathological evaluations to develop decision

criteria that may further improve the accuracy of BCOP predictions (OECD, 2017). We considered histopathological classifications of "severe," "moderate," "mild," and "minimal" equivalent to predictions of GHS 1, 2A, 2B, and NC, respectively. The overall BCOP-OECD+histo prediction used in the alignment analysis was the more severe of the predictions based on IVIS alone or histopathology alone. For example, the overall BCOP-OECD+histo prediction for a formulation with IVIS 2.5 (corresponding to GHS NC) and histopathology findings of "mild" (corresponding to GHS 2B) would be GHS 2B. For formulations where the prediction based on IVIS resulted in "no standalone prediction" (i.e., $3 < \text{IVIS} \leq 55$, indicating some level of irritation), and histopathology findings of "minimal" (corresponding to GHS NC), the prediction would be GHS 2B.

- EO-OECD: For formulations where the EO-OECD method resulted in "no prediction can be made", we excluded the EO-OECD result from the alignment analysis. We based alignment on the other evaluated test methods that achieved definitive predictions.

- TTL-OECD: For formulations where the TTL-OECD method resulted in a prediction of GHS 2, the result was considered in alignment with predictions of other evaluated test methods that achieved classification predictions of GHS 2A and 2B.

- EyeIRR-IS: For formulations where the EyeIRR-IS method resulted in a prediction of GHS 2, we considered the result in alignment with other evaluated test methods that achieved classification predictions of GHS 2A and 2B.

For each formulation, we used these guidelines to evaluate alignment of GHS predictions across methods and determine whether a majority of methods (i.e., at least 3 of 5 methods, or at least 4 of 6 methods) achieved the same prediction (henceforth referred to as "majority GHS prediction"). For formulations where a majority alignment was not achieved, we listed the majority GHS prediction as "inconclusive". We also evaluated PPE labeling.

Table 5. Classification of BCOP Results Incorporating Histopathology

Classification system	Classification based on IVIS only[a]	Histopathological findings of ocular injury	Final classification based on IVIS + histopathology
EPA	I	Minimal	I
EPA	I	Mild	I
EPA	I	Moderate	I
EPA	I	Severe	I
EPA	II	Minimal	II
EPA	II	Mild	II
EPA	II	Moderate	II
EPA	II	Severe	I
EPA	III	Minimal	III
EPA	III	Mild	III
EPA	III	Moderate	II
EPA	III	Severe	I
GHS	1	Minimal	1
GHS	1	Mild	1
GHS	1	Moderate	1
GHS	1	Severe	1
GHS	NC	Minimal	NC
GHS	NC	Mild	2B
GHS	NC	Moderate	2A
GHS	NC	Severe	1
GHS	NPCBM	Minimal	2B
GHS	NPCBM	Mild	2B
GHS	NPCBM	Moderate	2A
GHS	NPCBM	Severe	1

[a]IVIS thresholds for EPA classification derived from EPA's alternate testing framework (EPA, 2015); IVIS thresholds for GHS classification derived from OECD TG 437 (OECD, 2023a).
Abbreviations: IVIS = in vitro irritancy score; NC = not classified; NPCBM = no (stand-alone) prediction can be made.

Table 6. Phase 3 In Vitro Classification Criteria for EPA Ocular Irritancy Categories

	IV	III	II	I
BCOP-EPA+histo[a]	NA	IVIS < 25 and histo = minimal or mild	IVIS < 75 and histo = minimal, mild, or moderate	IVIS ≥ 75; or histo = severe
IVDoI-10% and IVDoI-Neat	Stromal DoI = 0% and meta test = negative	0% < Stromal DoI < 15%	Stromal DoI = 0% and meta test = positive; or 15% ≤ DoI ≤ 20%	Stromal DoI > 20%

[a]IVIS thresholds derived from EPA's alternate testing framework (EPA, 2015). Final BCOP-EPA+histo classifications used for analyses were driven by the most severe response obtained from IVIS or histopathology (see **Table 5**). Note that, using these classification criteria, a prediction of EPA Cat. IV is not possible.
Abbreviations: DoI = depth of injury; histo = histopathology; IVIS = in vitro irritancy score; meta = metabolic; NA = not applicable; neg = negative; pos = positive.

Table 7. Phase 3 In Vitro Classification Criteria for GHS Ocular Irritancy Categories

	NC	2B	2A	1	NPCBM
BCOP-OECD+histo[a]	IVIS ≤ 3 and histo = minimal	3 < IVIS ≤ 55 and histo = minimal or mild; or IVIS ≤ 3 and histo = mild	IVIS ≤ 55 and histo = moderate	IVIS > 55; or histo = severe	NA
EO-OECD	Viability > 60%	NA	NA	NA	Viability ≤ 60%
IVDoI-10% and IVDoI-Neat	Stromal DoI = 0% and meta test = negative	0% < Stromal DoI < 15%	Stromal DoI = 0% and meta test = positive; or 15% ≤ Stromal DoI ≤ 20%	Stromal DoI > 20%	NA
TTL-OECD[b]	Viability > 50% for all three exposure times	Any other combination	Any other combination	Viability ≤ 50% for all three exposure times	NA
EyeIRR-IS[b]	LII < 10 at 30% and LII < 10 at 100%	LII < 10 at 30% and LII ≥ 10 at 100%	LII < 10 at 30% and LII ≥ 10 at 100%	LII ≥ 10 at 30% (independently of the LII value obtained at 100%)	NA

[a]IVIS thresholds derived from OECD TG 437 (OECD, 2023a). Final BCOP-OECD+histo classifications used in analyses were driven by the most severe response obtained from IVIS or histopathology (see **Table 5**).
[b]Classification criteria for test method do not distinguish between GHS 2A/2B subcategories.
Abbreviations: DoI = depth of injury; histo = histopathology; IVIS = in vitro irritancy score; LII = liquid irritation index; meta = metabolic; LIS = laser light-based opacitometer irritancy score; NA = not applicable; NC = not classified; NPCBM = no (stand-alone) prediction can be made.

3. RESULTS

The Supplemental Information file provides results for all formulations in each of the methods tested in Phase 3. Classifications based on in vitro test method results and historical rabbit classifications were compared to enable an evaluation of alignment (**Tables 8 and 9**). For EO-OECD, a designation of “no prediction can be made" (NPCBM) was assigned when in vitro results and decision criteria did not allow for classification of ocular irritancy potential in a specific hazard classification category. In the EO-OECD test method, no definitive classification can be assigned when tissue viability is less than or equal to 60%.

Table 8. EPA Hazard Classifications Based on Phases 1, 2, and 3 Results for In Vitro and In Vivo Test Methods

Formulation Code	BCOP-EPA+histo: IVIS	BCOP-EPA+histo: Histo	BCOP-EPA+histo: Overall[a]	IVDoI-10%	IVDoI-Neat	Historical In Vivo	Majority EPA Prediction
A	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
B	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
C	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
D	III	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
E	III	Mild (III)	III	I[b]	I[b]	I	I (2/3; 67%)
F	II	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
G	I	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
H	II	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
I	I	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
J	III	Severe (I)	I	I[b]	I[b]	I	I (3/3; 100%)
K	III	Minimal (IV)	III	II[b]	IV[b]	II	Inconclusive
L	III	Minimal (IV)	III	IV[b]	IV[b]	III	III (2/3; 67%)
M	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
N	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
O	III	Minimal (IV)	III	II[b]	II[b]	IV	Inconclusive
P	III	Minimal (IV)	III	IV[b]	IV[b]	IV	IV (2/3; 67%)
Q	III	Moderate (II)	II	II	II	II	II (3/3; 100%)
R	II	Moderate (II)	II	I	I	II	II (2/3; 67%)
S	III	Mild (III)	III	IV	II	III	III (2/3; 67%)
T	III	Mild (III)	III	IV	IV	III	III (2/3; 67%)
U	III	Moderate (II)	II	II	II	II	II (3/3; 100%)
V	II	Moderate (II)	II	IV	I	III	Inconclusive
W	III	Mild (III)	III	II	IV	III	III (2/3; 67%)
X	III	Moderate (II)	II	I	I	II	II (2/3; 67%)
Y	III	Mild (III)	III	IV	III	II	III (2/3; 67%)
Z	III	Minimal (IV)	III	IV	IV	III	III (2/3; 67%)
AA	III	Minimal (IV)	III	IV	II	II	II (2/3; 67%)
AB	III	Moderate (II)	II	Not tested	Not tested	III	Inconclusive
AC	III	Mild (III)	III	I	I	III	III (2/3; 67%)
Majority EPA Prediction Rate			**17/25; 68%**	**NA**	**17/25; 68%**	**24/25; 96%**	

[a]Overall in vitro classification driven by most severe response obtained from IVIS or histopathology results.
[b]In vitro classification based on results of a single run conducted in an independent study.
Green: alignment across a majority (i.e., at least 3 of 5, or 4 of 6) of methods.

Yellow: misalignment with a majority of methods; PPE labeling unchanged.
Blue: misalignment with a majority of methods; PPE labeling overprotective relative to that of the majority.
Red: misalignment with a majority of methods; PPE labeling underprotective relative to that of the majority.
Abbreviations: histo = histopathology; IVIS = in vitro irritancy score; NA = not applicable.

Table 9. GHS Hazard Classifications Based on Phases 1, 2, and 3 Results for In Vitro and In Vivo Test Methods

Formulation Code	BCOP-OECD+ histo: IVIS	BCOP-OECD+histo: Histo	BCOP-OECD+ histo: Overall[a]	EO-OECD	IVDoI-10%	IVDoI-Neat	TTL-OECD[c]	EyeIRR-IS[c]	Historical In Vivo	Majority GHS Prediction
A	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
B	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
C	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
D	NPCBM	Severe (1)	1	NPCBM	1[b]	1[b]	2	2	1	1 (3/5; 60%)
E	NPCBM	Mild (2B)	2B	NPCBM	1[b]	1[b]	2	1	1	1 (3/5; 60%)
F	NPCBM	Severe (1)	1	NPCBM	1[b]	1[b]	1	1	1	1 (5/5; 100%)
G	1	Severe (1)	1	NPCBM	1[b]	1[b]	2	1	1	1 (4/5; 80%)
H	NPCBM	Severe (1)	1	NPCBM	1[b]	1[b]	1	1	1	1 (5/5; 100%)
I	1	Severe (1)	1	NPCBM	1[b]	1[b]	2	1	1	1 (4/5; 80%)
J	NPCBM	Severe (1)	1	NPCBM	1[b]	1[b]	2	1	1	1 (4/5; 80%)
K	NC	Minimal (NC)	NC	NPCBM	2A[b]	NC[b]	2	2	2A	2A (3/5; 60%)
L	NC	Minimal (NC)	NC	NPCBM	NC[b]	NC[b]	2	NC	NC	NC (4/5; 80%)
M	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
N	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
O	NC	Minimal (NC)	NC	NPCBM	2A[b]	2A[b]	2	NC	NC	NC (3/5; 60%)
P	NC	Minimal (NC)	NC	NC	NC[b]	NC[b]	NC	NC	NC	NC (6/6; 100%)
Q	NC	Moderate (2A)	2A	NPCBM	2A	2A	2	2	NC	2A (4/5; 80%)
R	NPCBM	Moderate (2A)	2A	NPCBM	1	1	1	1	2A	1 (3/5; 60%)
S	NC	Mild (2B)	2B	NPCBM	NC	2A	2	2	2B	2B (4/5; 80%)
T	NC	Mild (2B)	2B	NC	NC	NC	2	NC	NC	NC (4/6; 67%)
U	NPCBM	Moderate (2A)	2A	NPCBM	2A	2A	2	1	2A	2A (4/5; 80%)
V	1	Moderate (2A)	1	NPCBM	NC	1	1	1	2B	1 (4/5; 80%)
W	NPCBM	Mild (2B)	2B	NPCBM	2A	NC	2	2	NC	2B (3/5; 60%)
X	NPCBM	Moderate (2A)	2A	NPCBM	1	1	2	1	2A	2A (3/5; 60%)
Y	NC	Mild (2B)	2B	NPCBM	NC	2B	2	2	2A	2B (4/5; 80%)
Z	NPCBM	Minimal (NC)	2B	NC	NC	NC	NC	NC	NC	NC (5/6; 83%)
AA	NC	Minimal (NC)	NC	NPCBM	NC	2A	2	2	2A	2A (4/5; 80%)
AB	NPCBM	Moderate (2A)	2A	NPCBM	Not tested	Not tested	Not tested	Not tested	2B	Inconclusive
AC	NPCBM	Mild (2B)	2B	NPCBM	1	1	2	NC	NC	Inconclusive
		Majority GHS Prediction Rate	21/27; 78%	80-100%[d]	NA	22/27; 81%	19/27; 70%	24/27; 89%	22/27; 81%	

[a]Overall in vitro classification driven by most severe response obtained from IVIS or histopathology results.
[b]In vitro classification based on results of a single run conducted in an independent study.

[c]Classification criteria for test method do not distinguish between GHS 2A/2B subcategories.
[d]Majority GHS prediction rate when calculated as number of formulations for which EO-OECD predicted NC out of total number of formulations for which any majority GHS prediction was determined is 100% (8/8). Majority GHS prediction rate when calculated as the number of formulations for which EO-OECD predicted NC out of total number of formulations for which the majority GHS prediction was NC is 80% (8/10).
Green: alignment across a majority (i.e., at least 3 of 5, or 4 of 6) of methods.
Yellow: misalignment with a majority of methods; PPE labeling unchanged.
Blue: misalignment with a majority of methods; PPE labeling overprotective relative to that of the majority.
Red: misalignment with a majority of methods; PPE labelling underprotective relative to that of the majority.
Abbreviations: histo = histopathology; IVIS = in vitro irritancy score; NA = not applicable; NC = not classified; NPCBM = no (stand-alone) prediction can be made.

3.1 Alignment of EPA Predictions Across Methods

Data from BCOP-EPA+histo and IVDoI-Neat were used to assess alignment across in vitro methods and the in vivo rabbit test and to determine majority EPA predictions (**Table 8**). The IVDoI-10% data were excluded from this analysis to prevent majority EPA predictions being weighted toward a method with multiple protocols.

Majority EPA predictions were determined for 86% (25/29) of the tested formulations and considered inconclusive for the remaining four formulations. Of the 25 formulations for which majority EPA predictions were determined, predictions were aligned across two of the three methods for 17/25, and predictions were aligned across all three methods for the remaining 8.

Both BCOP-EPA+histo and IVDoI-Neat aligned with the majority EPA predictions at a lower rate compared with the in vivo rabbit test. While the in vivo rabbit test resulted in a classification that differs from the majority prediction for a single formulation, BCOP-EPA+histo and IVDoI-Neat each produced a classification that differs from the majority classification for 8 formulations. However, for both BCOP-EPA+histo and IVDoI-Neat, only 2 of the classifications would produce different PPE labeling requirements than those associated with the majority EPA prediction (formulations E and AA for BCOP-EPA+histo, and formulations S and AC for IVDoI-Neat). For BCOP-EPA+histo, predictions for formulations E and AA are underprotective of potential eye irritation compared to that of the majority EPA prediction. For IVDoI-Neat, predictions for formulations S and AC are overprotective compared to that of the majority EPA prediction. The PPE requirements associated with the in vivo rabbit test prediction is overprotective of potential eye irritation compared to that of the majority EPA prediction for a single formulation (formulation Y).

3.2 Alignment of GHS Predictions Across Methods

Data from BCOP-OECD+histo, EO-OECD, IVDoI-Neat, TTL-OECD, and EyeIRR-IS were used to assess alignment across non-animal methods and the in vivo rabbit test and to determine majority GHS predictions (**Table 9**). The IVDoI-10% data were excluded from this analysis to prevent majority predictions being weighted toward a method with multiple protocols. It should also be noted that while EO-OECD provides classification criteria for chemicals that do not require classification (i.e., GHS NC), there are no criteria available for identifying eye irritants (i.e., GHS Categories 1, 2A, and 2B). Since it is not feasible to compare alignment with other in vitro methods and the in vivo rabbit test, EO-OECD results of NPCBM were excluded from the alignment analysis. Furthermore, since TTL-OECD and EyeIRR-IS do not provide criteria for differentiating between GHS subcategories 2A and 2B, all TTL-OECD and EyeIRR-IS results of GHS Category 2 were considered in alignment with predictions of other evaluated test methods that achieved classification predictions of GHS Category 2A and 2B.

The GHS alignment analysis resulted in determination of majority predictions for 93% (27/29) of formulations. Furthermore, all 27 predictions were achieved based on alignment of three or more test methods.

The in vivo rabbit test resulted in a classification that differs from the majority GHS prediction for 5 formulations. Of these, PPE labeling requirements associated with the in vivo rabbit test prediction were affected for 2 formulations (formulations Q and W underprotective of potential eye irritation relative to that of the majority GHS prediction).

Of the in vitro test methods, only EyeIRR-IS (89%; 24/27) aligned with majority GHS predictions at a higher rate than the in vivo rabbit test (81%; 22/27). IVDoI-Neat (81%; 22/27) equaled the in vivo test while BCOP-OECD+histo (78%; 21/27) and TTL-OECD (70%; 19/27) were lower. Because EO-OECD can only predict non-irritants (OECD, 2023b), the majority GHS prediction rate is not directly comparable with that of the other methods. To address this difference, we calculated the EO-OECD majority GHS prediction rate as (1) the number of formulations for which it predicted NC out of the total number of formulations for which any majority GHS prediction was determined (i.e., 100%; 8/8); and (2) the number of formulations for which it predicted NC out of the total number of formulations for which the majority GHS prediction was NC (i.e., 80%; 8/10). For the latter calculation, 2 formulations (L and O) were NPCBM. Accordingly, we considered the overall majority GHS prediction rate for EO-OECD to be 80-100%.

EyeIRR-IS predictions misaligned with majority GHS predictions for 3 formulations (formulations D, U, and X), though the PPE requirements associated with the EyeIRR-IS predictions for the 3 formulations are the same as that of the majority GHS predictions.

IVDoI-Neat predictions misaligned with majority GHS predictions for 5 formulations. Of these, PPE labeling requirements were affected for 3 formulations (2 formulations [K and W] were underprotective relative to that of the majority GHS prediction, while 1 formulation [formulation O] was relatively overprotective).

BCOP-OECD+histo predictions differed from the majority GHS predictions for 6 formulations. Of these, PPE labeling requirements were affected for 4 formulations (2 formulations [K and AA] were underprotective, and 2 formulations [T and Z] were overprotective relative to that of the majority GHS prediction).

TTL-OECD predictions misaligned with majority GHS predictions for 8 formulations. Of these, PPE labeling requirements were affected for 3 formulations. For all 3 formulations, PPE labeling requirements were overprotective relative to that of the majority GHS prediction.

4. DISCUSSION

Defined approaches use results from multiple in vitro mechanistic assays in specific combinations and a structured data interpretation procedure to derive an objective, reproducible prediction. OECD issued TG 467 (OECD, 2022b), which describes defined approaches for identifying chemicals with serious eye damage or eye irritation potential. However, the applicability of the defined approaches described in the TG are limited to non-surfactant neat liquids, and liquids and solids dissolved in water. Therefore, defined approaches that are applicable to agrochemical formulations are needed.

This study was conducted as a foundational step in developing defined approaches to fill this need for both GHS and EPA hazard classification systems to evaluate the usefulness and limitations of different in vitro test methods. Therefore, it is important to evaluate the methods within the context of these results so that defined approaches can be strategically developed to maximize strengths and overcome limitations of individual test methods.

Three in vitro test methods/protocols used in this evaluation have classification criteria for EPA categories (i.e., BCOP-EPA+histo, IVDoI-10%, and IVDoI-Neat) (**Table 6**). As stated previously, data from only one IVDoI protocol could be used in the alignment analysis to prevent majority EPA predictions being overly weighted toward this method. The two protocols seem to perform similarly, based on the observation that both produced the same prediction for 22 of 28 formulations. Of the six formulations for which the two protocols produced different predictions, the IVDoI-Neat protocol produced more conservative predictions for 4 formulations (S, V, Y, and AA) while the IVDoI-10% protocol produced more conservative predictions for only remaining 2 formulations (K and W). Therefore, the alignment analysis of EPA predictions across methods included three methods (i.e., BCOP-EPA+histo, IVDoI-Neat, and historical in vivo data; **Table 8**). Of the 25 formulations for which a majority EPA prediction was achieved, 17 were based on alignment of 2 methods, and only 8 were based on alignment of all 3 methods. The lack of complete alignment can be partially attributed to limitations of the BCOP-EPA+histo classification criteria, according to which a prediction of EPA IV is not possible (see **Table 6**). Despite this limitation, only 2 BCOP-EPA+histo PPE labelings (formulations E and AA) were underprotective relative to the majority EPA prediction (**Table 8**). IVDoI-Neat did not produce any underprotective predictions relative to the majority EPA predictions.

The alignment analysis of GHS predictions, based on available criteria, included six methods (i.e., BCOP-OECD+histo, EO-OECD, IVDoI-Neat, TTL-OECD, EyeIRR-IS, and historical in vivo data; **Table 9**). The determination of a majority GHS prediction for 27 of 29 formulations suggest that all five in vitro methods are applicable to agrochemical formulations. Furthermore, majority GHS prediction rates of all five in vitro methods were ≥70%. It should be noted that since EO-OECD does not provide sub-classification criteria for the prediction of eye irritants, its alignment with majority predictions is based on the subset of 8 formulations for which EO-OECD resulted in a classification of GHS NC and not the 27 formulations for which a majority EPA prediction was achieved. Therefore, we calculated the EO-OECD majority GHS prediction rate two different ways to glean a fair assessment. Specifically, EO-OECD majority GHS prediction rate was 100% (8/8) when calculated as the number of formulations for which EO-OECD predicted NC out of total number of formulations for which any majority GHS prediction was determined (as was done for all other methods); and EO-OECD majority GHS prediction

rate was 80% (8/10) when calculated as the number of formulations for which EO-OECD predicted NC out of total number of formulations for which the overall majority GHS prediction was NC. Either way, these data suggest EO-OECD is highly useful for the accurate prediction of agrochemical substances not requiring classification and labeling for eye irritation (i.e., GHS NC).

Four of the five in vitro test methods described in the current report (i.e., BCOP, EO, TTL, and EyeIRR-IS) were among the test methods reviewed by Clippinger and colleagues (2021) to characterize their relevance to human ocular anatomy, anticipated exposure scenarios, and mechanisms of eye irritation and corrosion in humans. The authors demonstrated that these in vitro methods are at least as, or potentially more, reflective of human biology compared with the in vivo test. Further, semi-automated quantitative in vitro measurements result in significantly more reproducible data than observational in vivo endpoints based on subjective criteria. Additionally, some in vitro methods (e.g., BCOP) provide an opportunity for a mechanistic assessment of eye irritation, whereas the in vivo rabbit test evaluates apical outcomes of ocular exposure and provides limited mechanistic information.

The test methods evaluated in this study represent a variety of domains of applicability and coverage of key biological events. For example, the BCOP method provides a full-thickness model to assess corneal effects (e.g., damage to corneal epithelium, corneal stroma). As outlined in OECD Guidance Document No. 160 (OECD, 2018), inclusion of histopathology in this model can provide information about depth of injury. While the BCOP stand-alone method may be used for identifying chemicals inducing serious eye damage (GHS Category 1) and those not requiring classification and labeling (GHS NC), the classification schema used in this study to incorporate histopathology enables prediction of the full range of ocular irritancy potential, including subcategorization of GHS 2A/2B.

The results demonstrate the importance of histopathological evaluation in refining overall BCOP predictions. The schema used to incorporate histopathological findings with the IVIS (described in **Table 5**) resulted in a more severe overall BCOP-EPA+histo classification for 8 formulations (Formulations D, F, H, J, Q, U, X, and AB; **Table 8**). Additionally, the schema resulted in a more severe overall BCOP-OECD+histo classification for 4 formulations (Formulations Q, S, T, and Y), and enabled classification for the 12 formulations for which the IVIS alone would result in NPCBM (Formulations D, E, F, H, J, R, U, W, X, Z, AB, and AC; **Table 9**).

Questions remain regarding how interspecies differences may impact the utility of certain in vitro methods (e.g., BCOP, IVDoI) for predicting the human response; however, the same concerns surround the currently used in vivo rabbit test, and the in vitro methods do not suffer from the observed issues around subjectivity of the in vivo response interpretation/scoring schema. While the IVDoI results reported herein are promising, we acknowledge that neither IVDoI-Neat nor IVDoI-10% protocol have been formally validated for the detection of eye irritation. Further investigations are required to better assess IVDoI as a standalone method.

Overall, the results of this study suggest that BCOP with histopathology, EO, TTL, IVDoI, and EyeIRR-IS are all applicable to agrochemical formulations, and that they all may be useful in the development of defined approaches to predict eye irritation potential of these types of chemical products. As outlined in the ICCVAM Validation Workgroup's report on Validation,

Qualification, and Regulatory Acceptance of New Approach Methodologies (ICCVAM, 2024), future evaluations and acceptance decisions should strongly consider 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 in vivo rabbit test.

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