NTP
National Toxicology Program

Evaluation of Glyphosate, (Aminomethyl)phosphonic Acid, and Glyphosate-Based Formulations for Genotoxicity and Oxidative Stress Using *In Vitro* Approaches

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Toxicity Report No. 16: 13-week study with glyphosate in feed (1992)

- Nominated by California Regional Water Quality Control Board North Coast Region (1981)
  - Glyphosate being found in water runoff in areas of use
- NTP selected glyphosate for toxicity evaluation because of:
  - Expanding use
  - Potential for human exposure
  - The lack of published reports concerning comprehensive toxicity or carcinogenicity evaluations

Toxicity Report No. 16: 13-week study with glyphosate in feed (1992)

Top dose for rats ~3,400 mg/kg/day (males & females)

Top dose for mice ~10,800 and ~12,000 mg/kg/day (males & females, respectively)

- No gross lesions at necropsy (rats or mice)
- Micronucleus assay was negative in male and female mice (also 13-week exposure via feed)
- Bacterial mutagenicity tests were negative
- ADME studies indicated low absorption and rapid elimination
  - Pre-treatment with Roundup did not change elimination of an oral dose of glyphosate

Toxicity Report No. 16: 13-week study with glyphosate in feed (1992)

- Cytoplasmic alterations in salivary glands of rats (all dose levels) and mice (higher doses)
- Increases in serum bile acids, alkaline phosphatase, and alanine aminotransferase activities in rats (but no liver lesions) at higher doses
- Reduced sperm counts in rats at higher doses
- Diarrhea in male and female rats in top dose group for first 50 days of study

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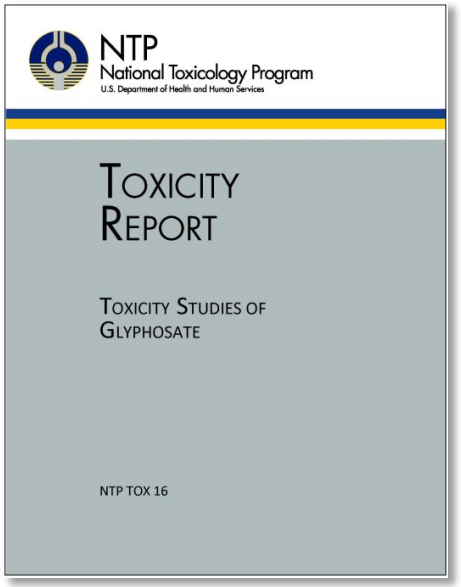

Revisiting Glyphosate Testing at NTP

Rationale

- Different interpretations of the potential health effects of glyphosate exposure
  - Regulatory agencies have concluded that glyphosate is unlikely to be a carcinogenic risk to humans
  - IARC monograph 112 identified glyphosate as “probably carcinogenic to humans (Group 2A)”
    - Limited evidence in humans for the carcinogenicity of glyphosate
    - Sufficient evidence in experimental animals for the carcinogenicity of glyphosate

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    - Limited evidence in humans for the carcinogenicity of glyphosate
    - Sufficient evidence in experimental animals for the carcinogenicity of glyphosate
- Major public concern about exposure risks
- Reported differences in toxicity of glyphosate versus glyphosate-based formulations (GBFs)

Key Characteristics of Carcinogens

- **International Agency for Research on Cancer (IARC) Monograph 112**: Glyphosate is "probably carcinogenic to humans (Group 2A)" (2015)
  - Limited evidence in humans for the carcinogenicity of glyphosate
  - Sufficient evidence in experimental animals for the carcinogenicity of glyphosate
    - Strong evidence for genotoxicity
    - Strong evidence for induction of oxidative stress

Smith et al. (2016) Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *EHP*.124(6): 713-21, PMID 26600562

Approach

- Test glyphosate and glyphosate-based formulations (GBFs) for genotoxicity and induction of oxidative stress (key characteristics of carcinogens)
- Test glyphosate and GBFs side-by-side to assess whether glyphosate is the biologically active component
  - GBFs are mixtures, and the formulations vary in their composition. Few studies have differentiated the effects of glyphosate from GBFs.
  - Animal studies have been conducted with glyphosate, whereas humans are exposed to GBFs.
- Use *in vitro* screening approach due to need for high quality mechanistic data and large number of test articles

Test Articles

Active ingredients, GBFs, and one glyphosate metabolite (19 test articles)

Active ingredients (5)

- Glyphosate (free acid)
  - Technical Grade
- Glyphosate isopropyl amine
  - Salt form used for majority of GBFs
- Metolachlor, mesotrione, and diquat dibromide
  - Herbicides in GBFs tested by NTP

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- Metolachlor, mesotrione, and diquat dibromide
  - Herbicides in GBFs tested by NTP

GBFs (13)

- 9 agricultural-use GBFs
- 4 residential-use GBFs

Glyphosate metabolite (1)

- (Aminomethyl)phosphonic acid (AMPA)
  - Microbial metabolite, also found in human blood in cases of severe glyphosate poisoning

Selection of Top Concentrations for Chemicals

Testing guidelines

- OECD Test Guidelines for *in vitro* mammalian cell genotoxicity assays
  - For *in vitro* genotoxicity testing, if no precipitate or limiting cytotoxicity is observed, the highest test concentration should be limited to **10 mM**
- International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use: Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2(R1)
  - "Second, a limit of **1 mM** maintains the element of hazard identification, being higher than clinical exposures to known pharmaceuticals, including those that concentrate in tissues (Goodman & Gilman, 2001), and is also higher than the levels generally achievable in preclinical studies *in vivo*."
  - "For pharmaceuticals with unusually low molecular weight (*e.g.*, less than 200) higher test concentrations should be considered."

Concentration Range: 0.01 to 10 mM

Glyphosate-Based Formulations (13)

All Genetox Tests: Top dilution of 1:100

Test Article	%Glyphosate
Agricultural A	20.5
Agricultural B	41.0
Agricultural C	41.0
Agricultural D	41.0
Agricultural E	44.9
Agricultural F	48.7
Agricultural G	48.8
Agricultural H	50.2
Agricultural I	53.8

Glyphosate-Based Formulations (13)

All Genetox Tests: Top dilution of 1:100

Test Article	%Glyphosate	Other Actives
Agricultural A	20.5	20.5% S-Metolachlor, 2.05% Mesotrione
Agricultural B	41.0	
Agricultural C	41.0	
Agricultural D	41.0	
Agricultural E	44.9	
Agricultural F	48.7	
Agricultural G	48.8	
Agricultural H	50.2	
Agricultural I	53.8	
Residential J	1.92	
Residential K	18.0	0.73% Diquat Dibromide
Residential L	41.0	
Residential M	50.2	

Mechanisms of genotoxic activity

- Litron MultiFlow® DNA Damage Assay (TK6 cells)
  - Identifies signatures of clastogenic or aneugenic activity
- Litron MicroFlow® Assay (TK6 cells)
  - Based on OECD Guideline 487 for *in vitro* micronucleus assays (MN arise from clastogenic or aneugenic mechanisms)
- Bacterial Mutagenicity Testing
  - NTP studies informed by OECD Guideline 471 and conducted using GLP

Litron MultiFlow® DNA Damage Assay

Identify Genotoxic Mode of Action in TK6 cells

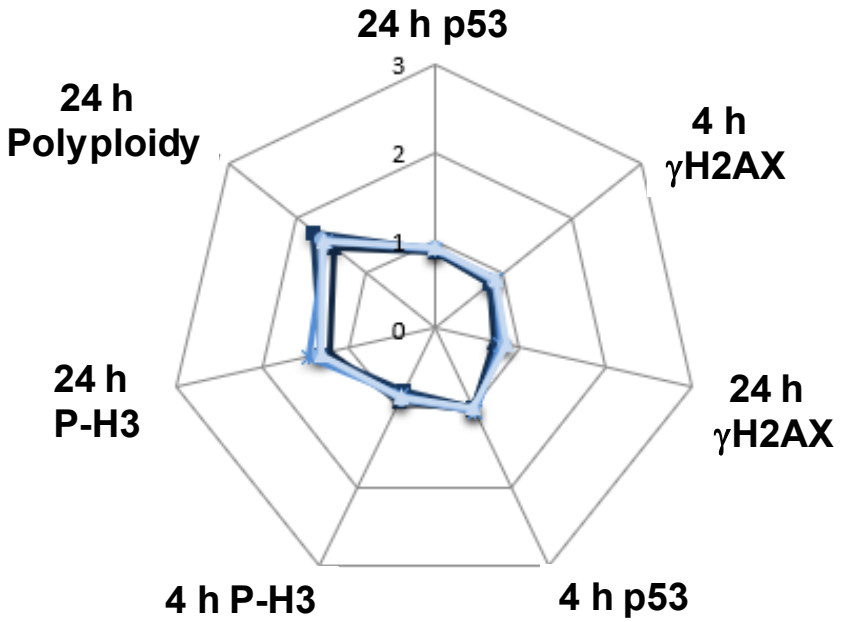
- Discriminate clastogens from aneugens using 3 biomarkers:
  - translocation of p53 to the nucleus
  - phosphorylation of H2AX (γH2AX) and histone H3
- 20-point dose-response curve (sqrt 2 spacing) using 96-well plate format
- 4 h & 24 h continuous exposure timepoints
- Cytotoxicity evaluated at 24 h
- Data are integrated using a probability matrix algorithm trained using data from known clastogens, aneugens, and non-genotoxicants
- Machine learning models and global evaluation factors are used to identify mode of action

Glyphosate, Glyphosate IPA, and AMPA

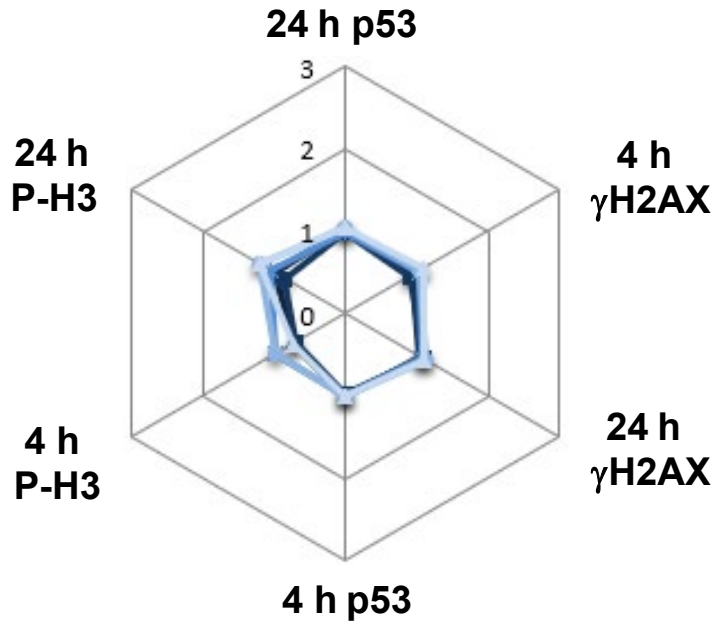
MultiFlow DNA Damage Assay: Genotoxicity (TK6 cells)

- Glyphosate, glyphosate IPA, and AMPA were identified as **non-genotoxicants** in the MultiFlow DNA Damage Assay (+/-S9)

Glyphosate -S9



Glyphosate +S9

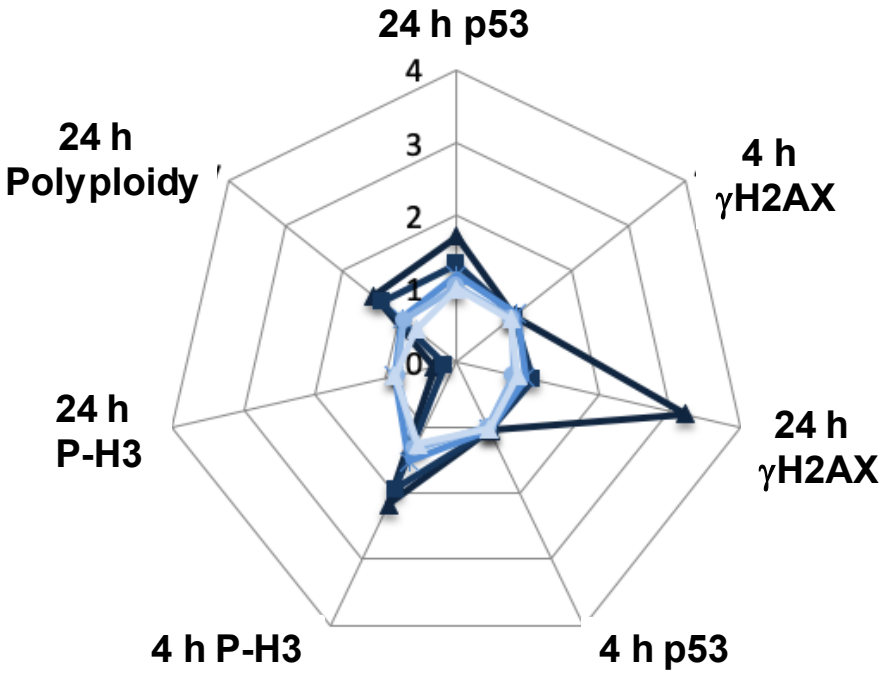


2 µM 4 µM 5 µM 7 µM 10 µM

Glyphosate-Based Formulations

MultiFlow DNA Damage Assay: Genotoxicity (TK6 cells)

Agricultural GBF A
Clastogenic Signature

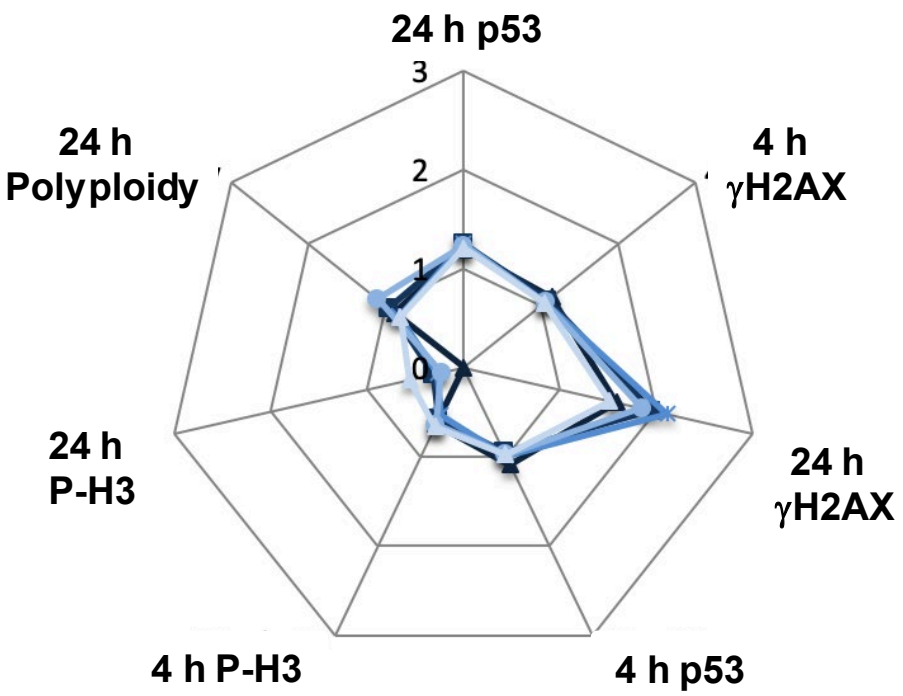


Agricultural GBF A +S9
Non-genotoxic

Glyphosate-Based Formulations

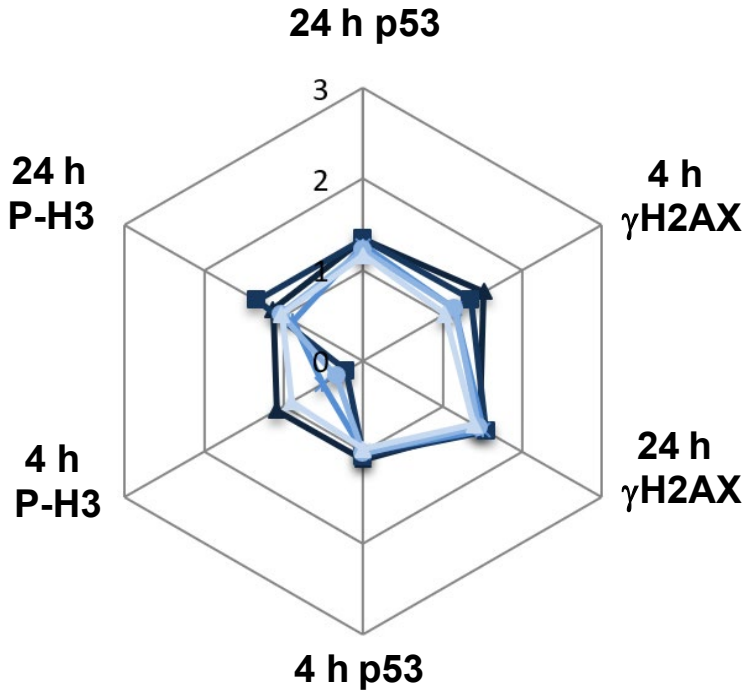
MultiFlow DNA Damage Assay: Genotoxicity (TK6 cells)

Agricultural GBF I Clastogenic Signature



1/800 1/200

Agricultural GBF I +S9 Clastogenic Signature

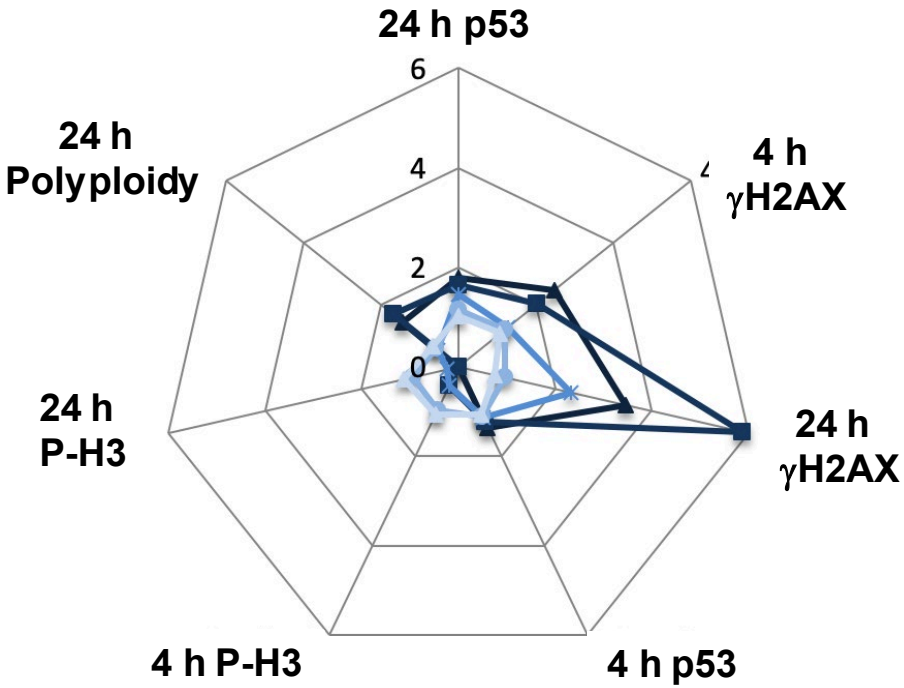


1/400 1/100

Active Ingredients in GBFs

MultiFlow DNA Damage Assay: Genotoxicity (TK6 cells)

Metolachlor
Clastogenic Signature



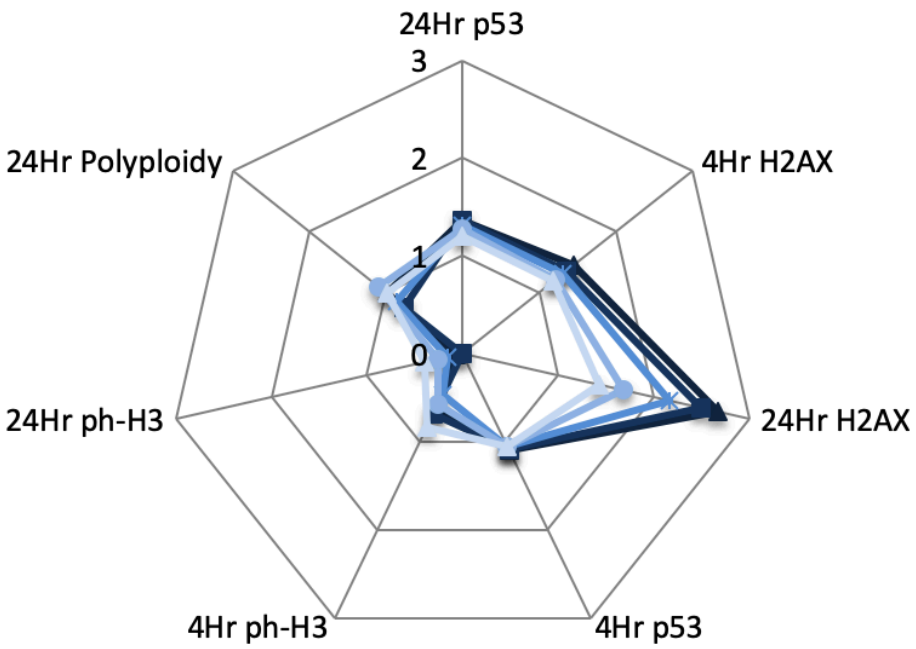
Metolachlor +S9
Non-genotoxic

0.16 mM 0.62 mM

Active Ingredients in GBFs

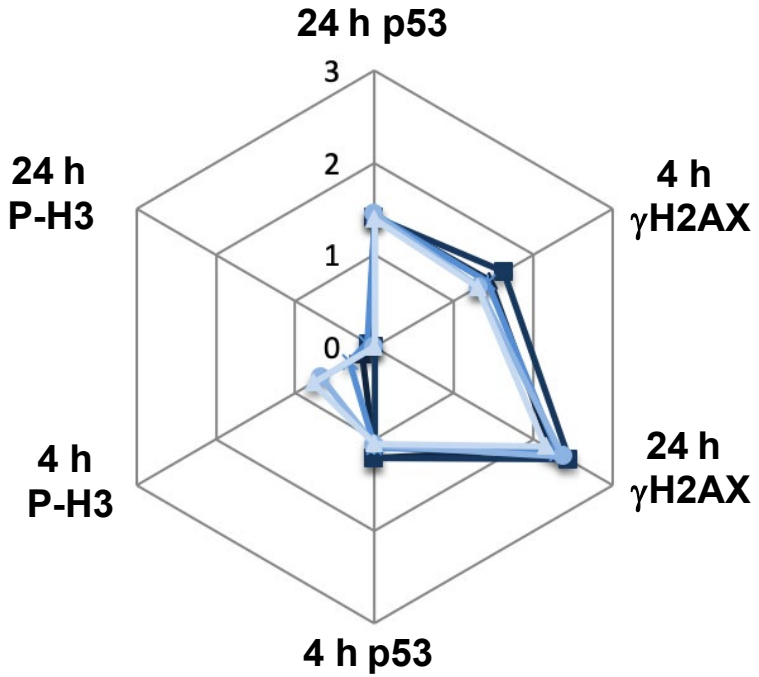
MultiFlow DNA Damage Assay: Genotoxicity (TK6 cells)

Diquat Dibromide Clastogenic Signature



0.04 mM 0.16 mM

Diquat Dibromide +S9 Clastogenic Signature



0.6 mM 2.5 mM

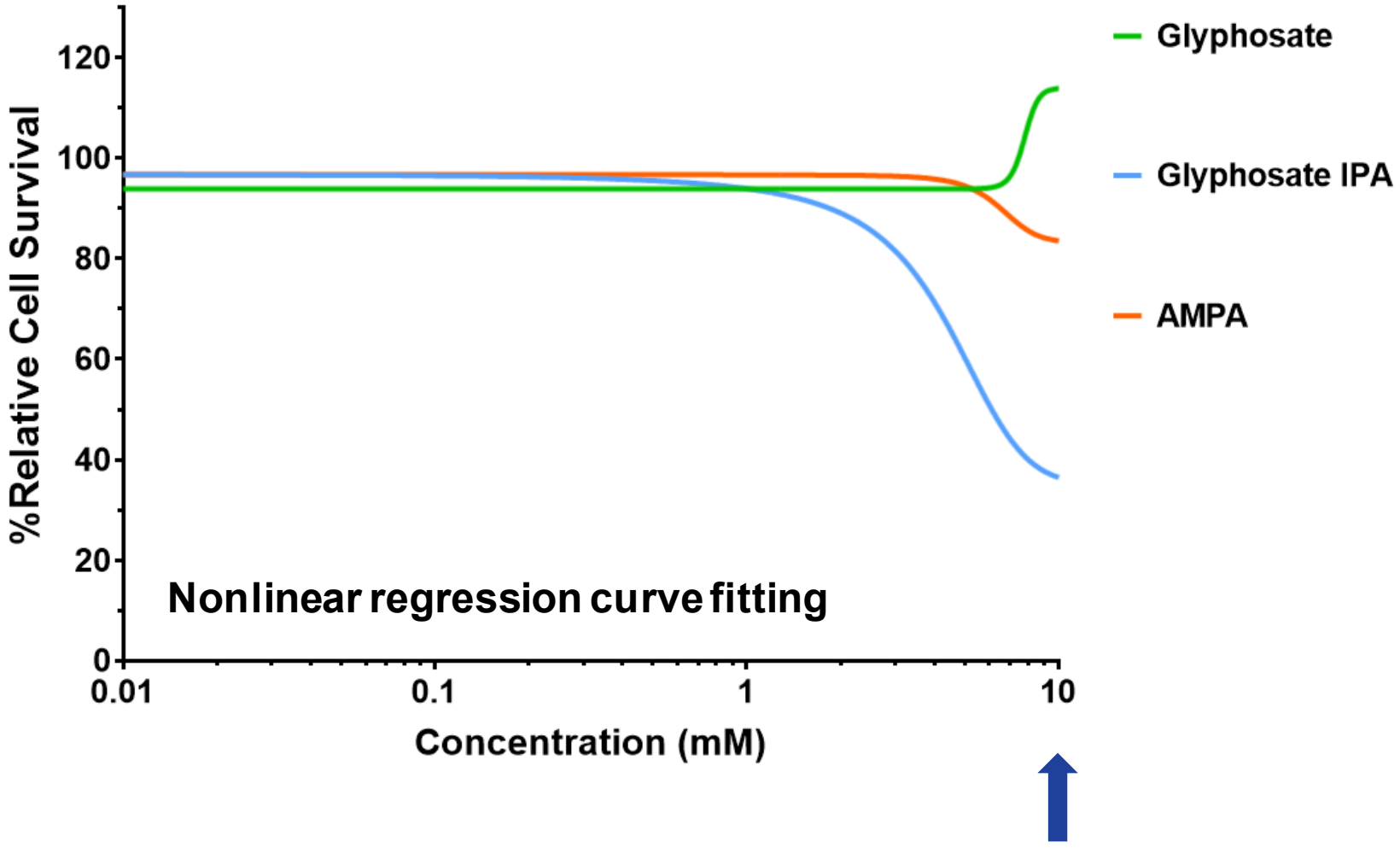
Summary of MultiFlow Testing

Genetic Toxicity Results

	MultiFlow Assay	
Test Article	-S9	+S9
Glyphosate	Negative	Negative
Glyphosate IPA	Negative	Negative
AMPA	Negative	Negative
Diquat Dibromide	Clastogenic	Clastogenic
Metolachlor	Clastogenic	Negative
Mesotrione	Negative	Negative
Agricultural A	Clastogenic	Negative
Agricultural B	Negative	Negative
Agricultural C	Negative	Negative
Agricultural D	Negative	Negative
Agricultural E	Negative	Negative
Agricultural F	Negative	Negative
Agricultural G	Negative	Negative
Agricultural H	Negative	Negative
Agricultural I	Clastogenic	Clastogenic
Residential J	Negative	Negative
Residential K	Negative	Negative
Residential L	Negative	Negative
Residential M	Negative	Negative

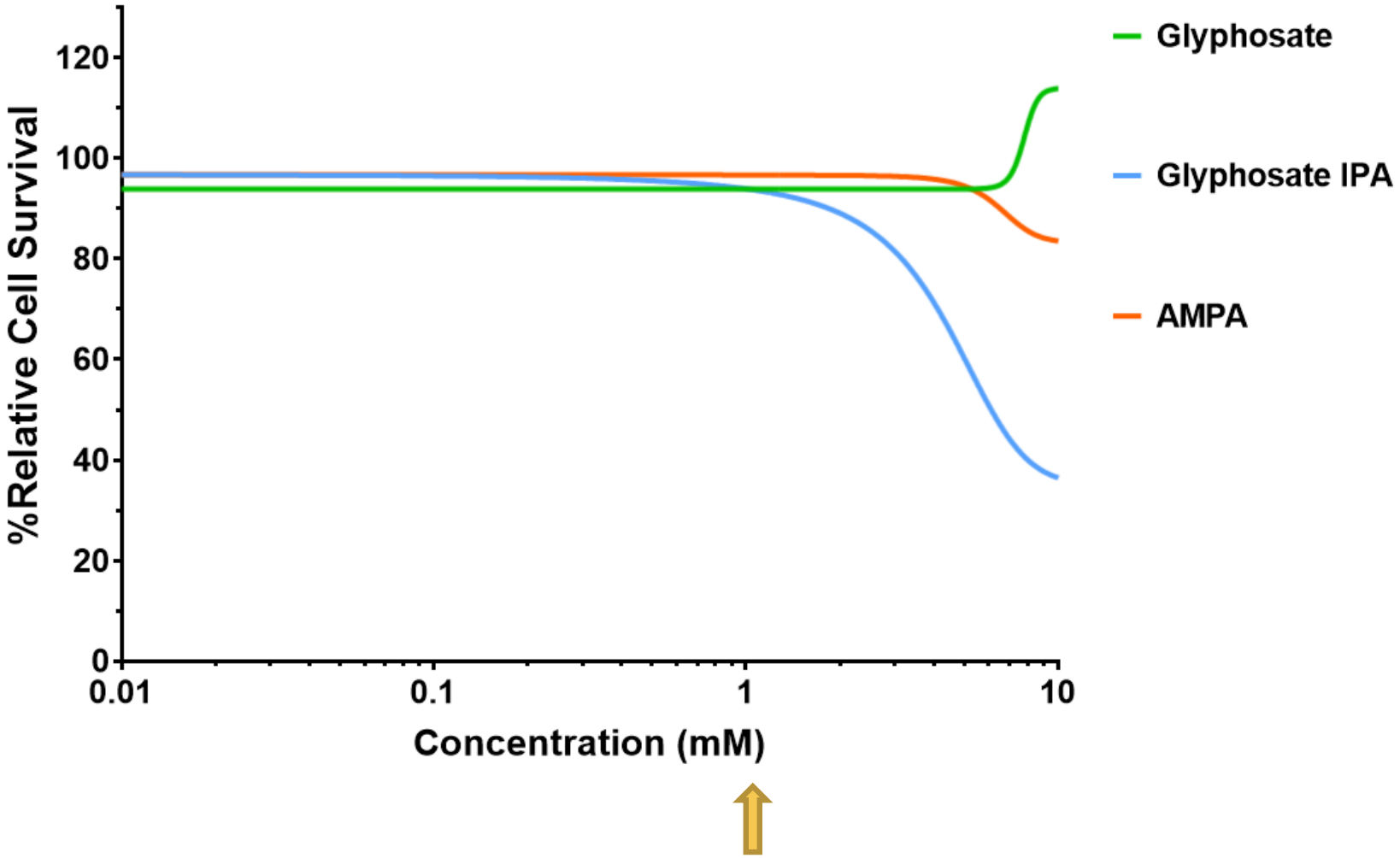
Glyphosate, Glyphosate IPA, and AMPA

MultiFlow DNA Damage Assay: Cytotoxicity (TK6 cells)



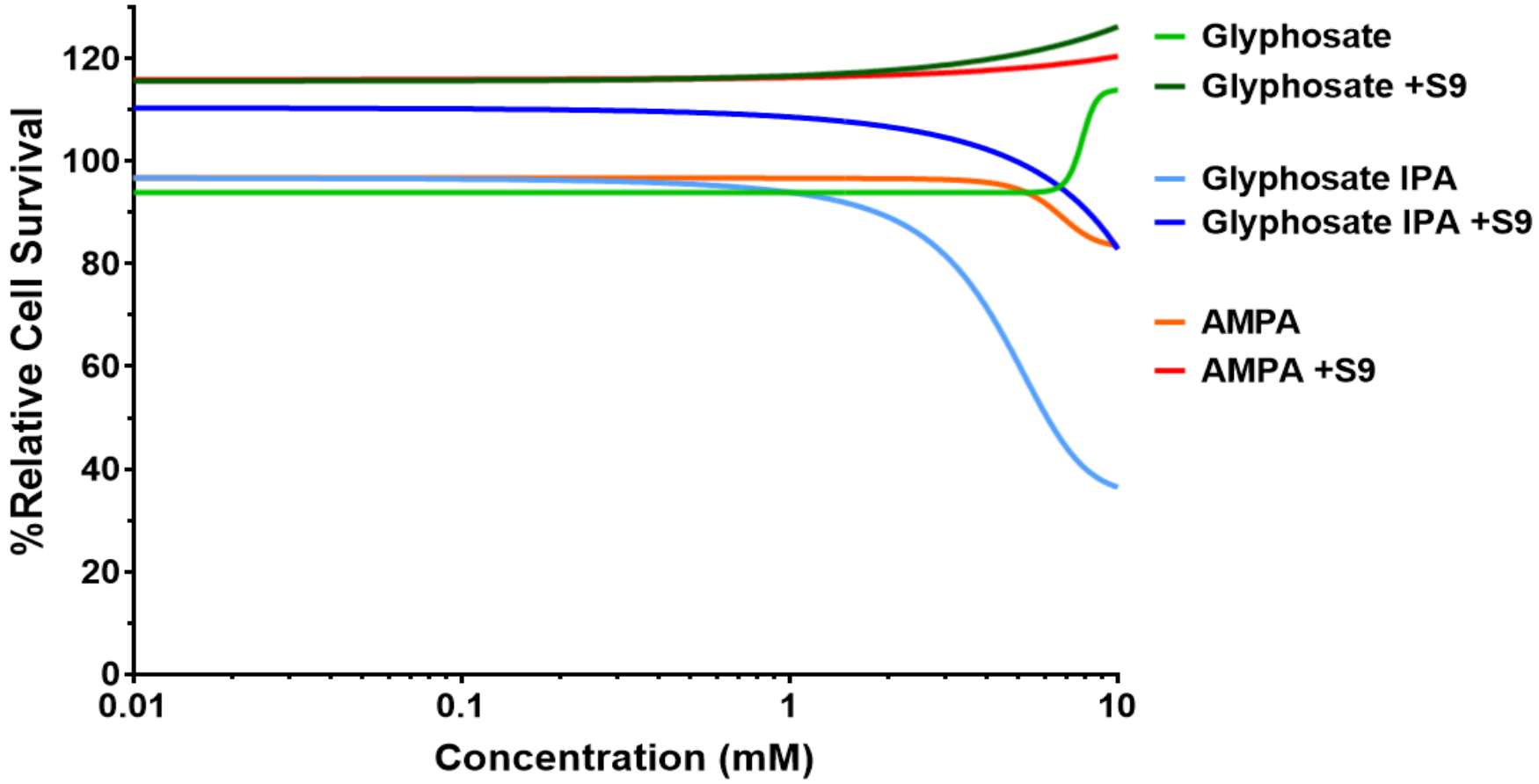
Glyphosate, Glyphosate IPA, and AMPA

MultiFlow DNA Damage Assay: Cytotoxicity (TK6 cells)



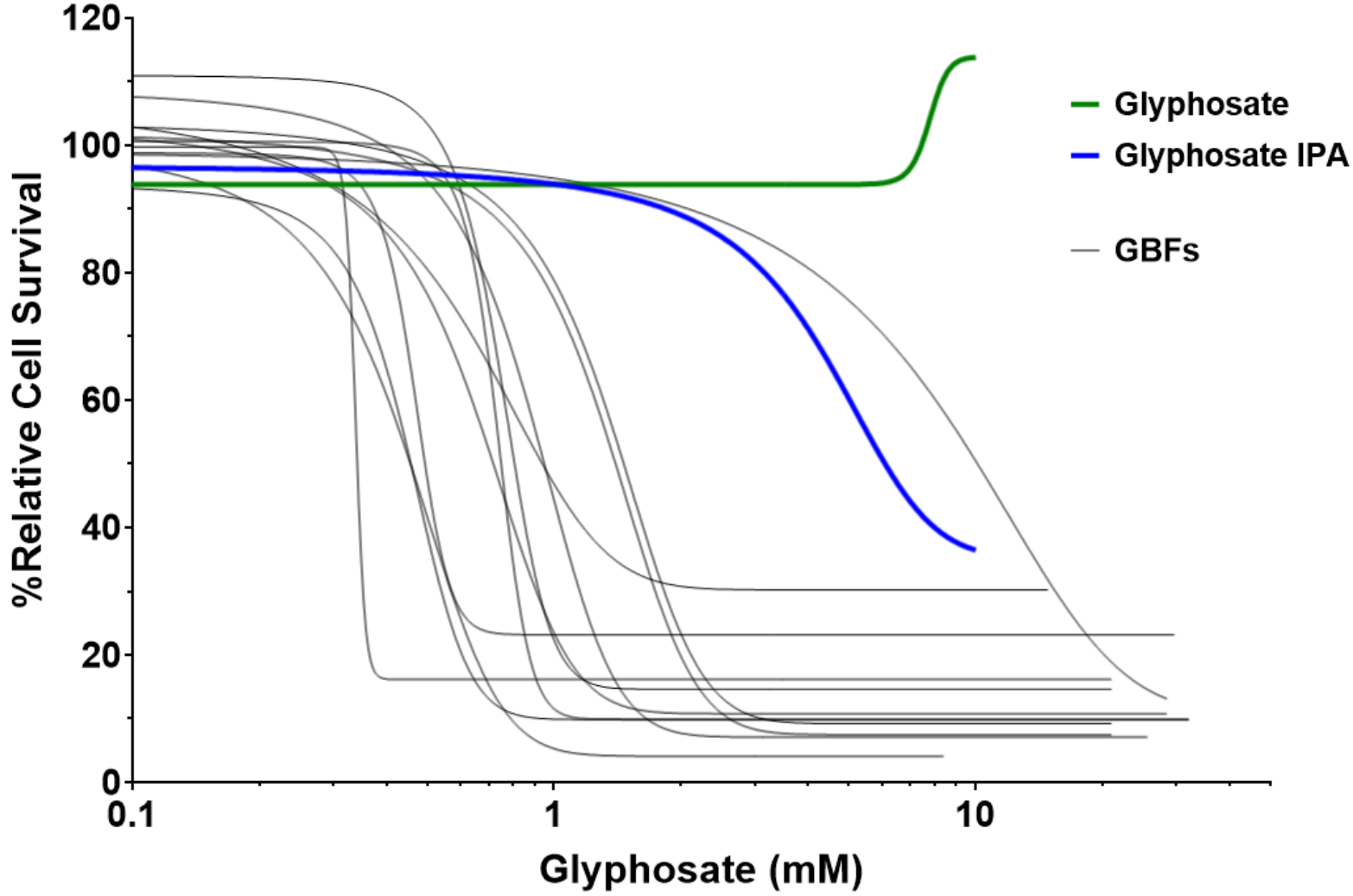
Glyphosate, Glyphosate IPA, and AMPA

MultiFlow DNA Damage Assay: Cytotoxicity (TK6 cells)



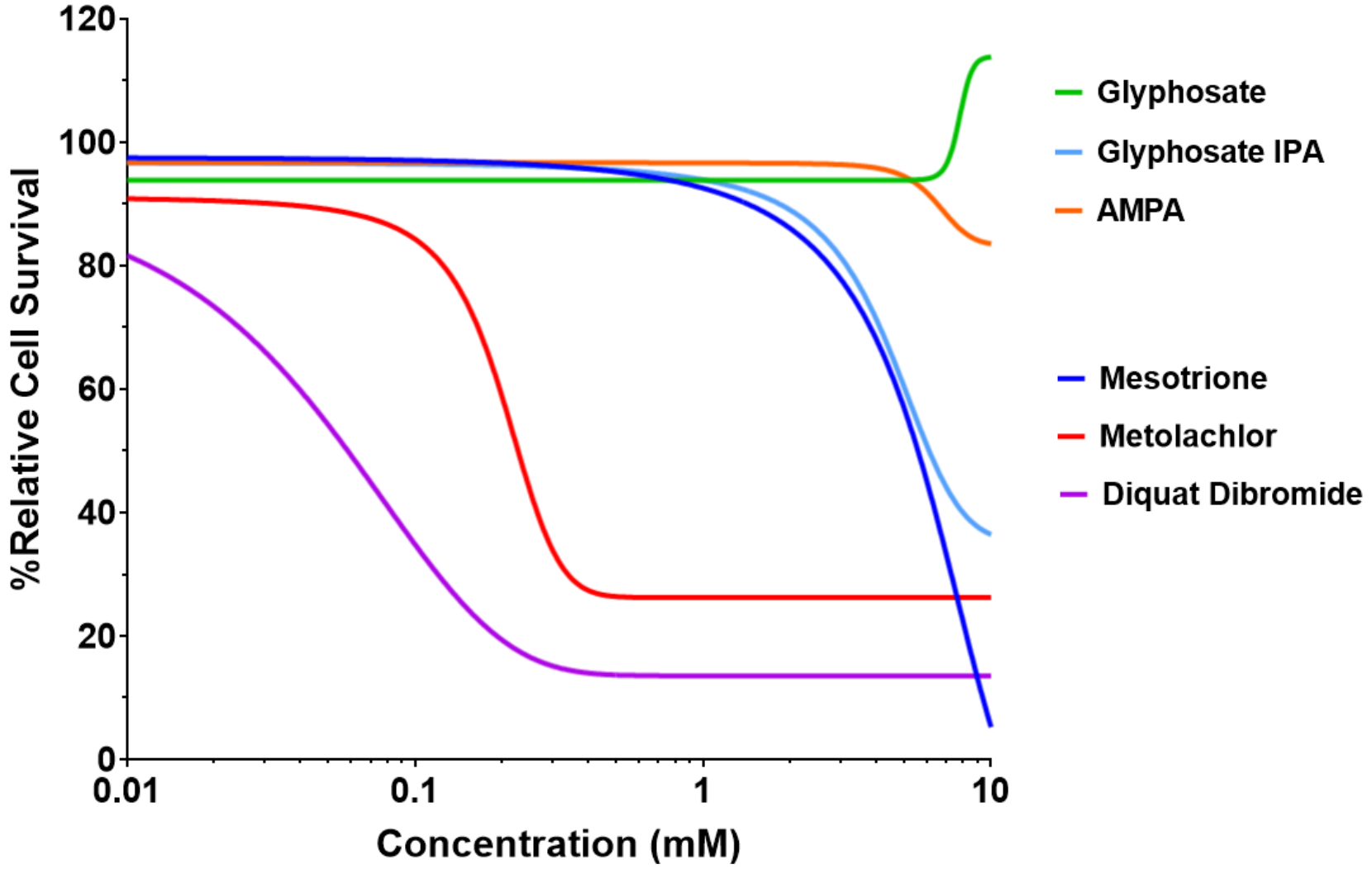
Glyphosate versus GBFs

MultiFlow DNA Damage Assay: Cytotoxicity (TK6 cells)



Glyphosate versus Other Actives

MultiFlow DNA Damage Assay: Cytotoxicity (TK6 cells)



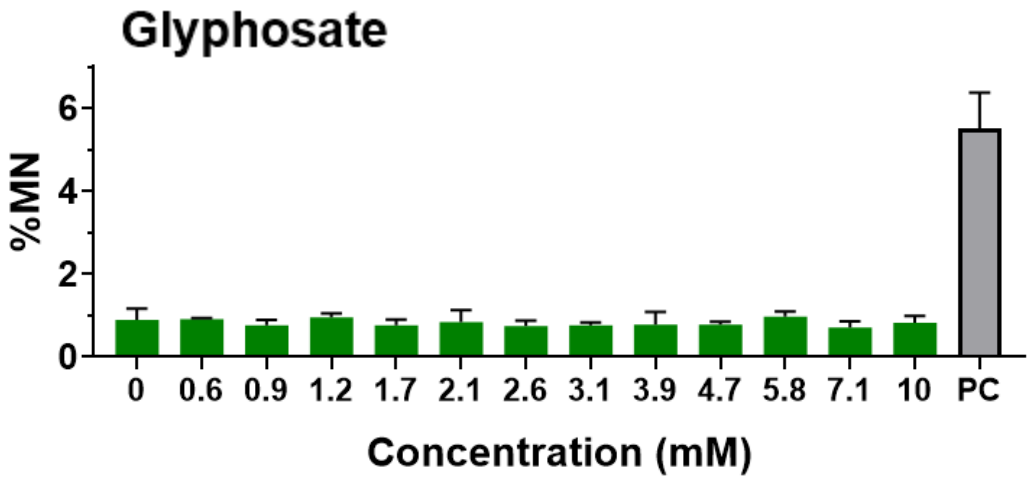
Litron MicroFlow® Assay

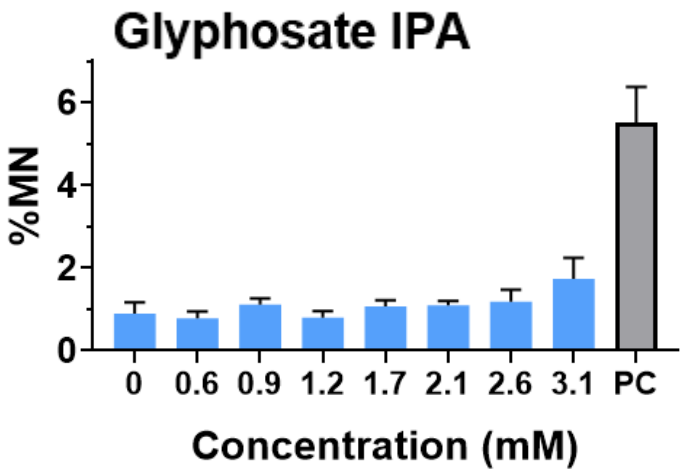
Micronuclei – biomarkers of chromosomal damage or changes in chromosome number

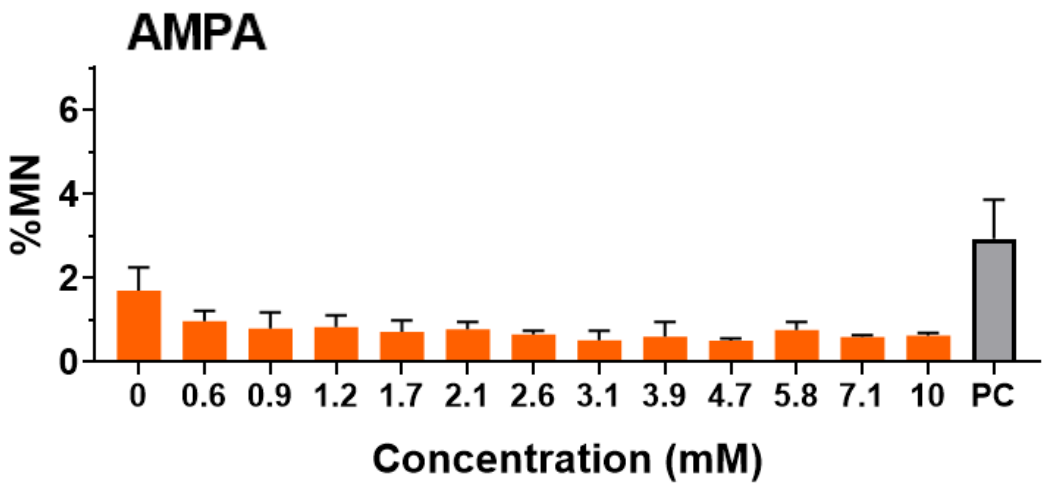
- Flow cytometry assay based on OECD Guideline 487 for *in vitro* micronucleus assays
- Human lymphoblastoid TK6 cells
- 24 h continuous exposure, 4 h exposure +S9, 4 h exposure -S9
  - 3 arms of study x 19 test articles = 57 tests
- 12-point dose-response curves using 96-well plate format
- Limit of 55% ± 5% cytotoxicity

Glyphosate, Glyphosate IPA, and AMPA

MicroFlow MN assay (TK6 cells) 24 h exposure

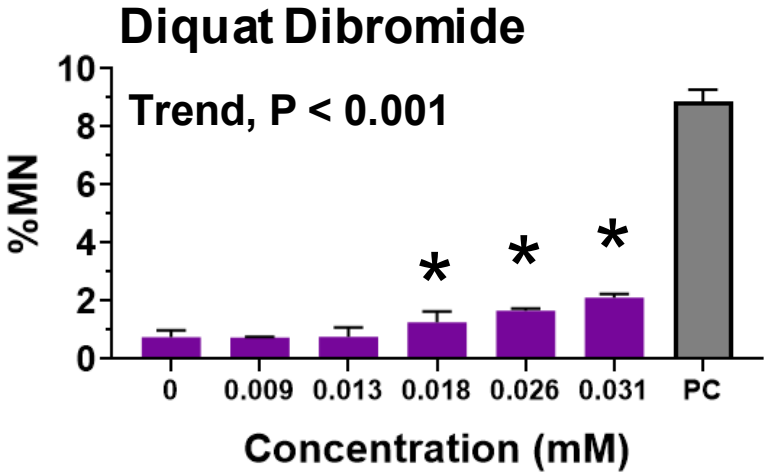


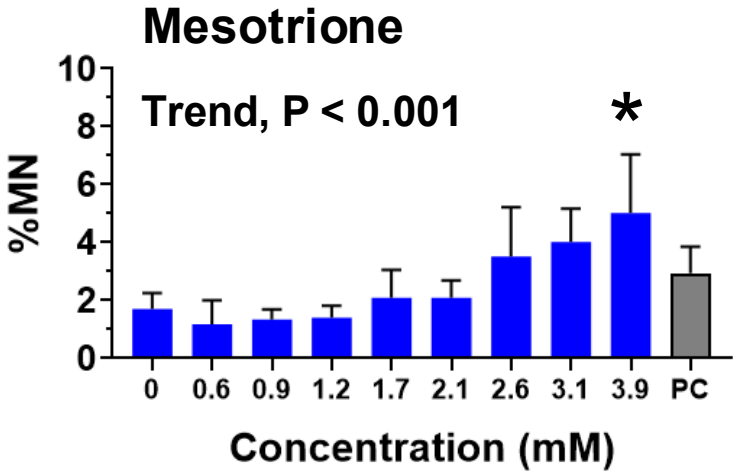


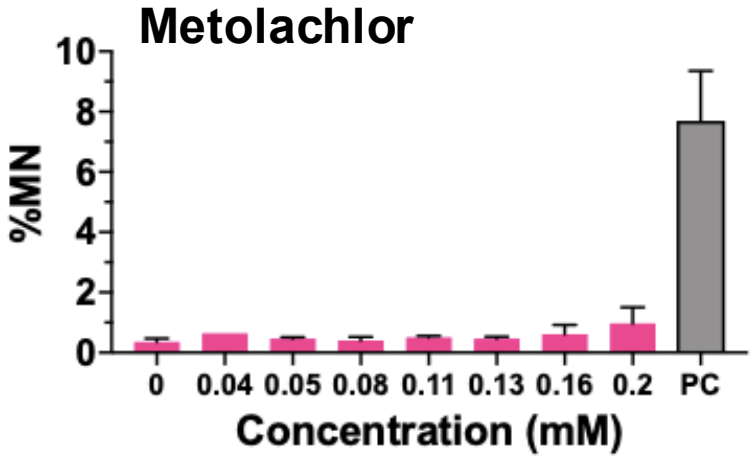


Actives Other than Glyphosate

MicroFlow MN assay (TK6 cells) 24 h exposure







Summary of MultiFlow & MicroFlow Testing

Genetic Toxicity Results

	MultiFlow Assay		MicroFlow Assay		
Test Article	-S9	+S9	24 h	4 h +S9	4 h –S9
Glyphosate	Negative	Negative	Negative	Negative	Negative
Glyphosate IPA	Negative	Negative	Negative	Negative	Negative
AMPA	Negative	Negative	Negative	Negative	Negative
Diquat Dibromide	**Clastogenic**	**Clastogenic**	**Positive**	Negative	**Positive**
Metolachlor	**Clastogenic**	Negative	Negative	Negative	Negative
Mesotrione	Negative	Negative	**Positive**	Negative	Negative
Agricultural A	**Clastogenic**	Negative	**In Progress**		
Agricultural B	Negative	Negative			
Agricultural C	Negative	Negative			
Agricultural D	Negative	Negative			
Agricultural E	Negative	Negative			
Agricultural F	Negative	Negative			
Agricultural G	Negative	Negative			
Agricultural H	Negative	Negative			
Agricultural I	**Clastogenic**	**Clastogenic**			
Residential J	Negative	Negative			
Residential K	Negative	Negative			
Residential L	Negative	Negative			
Residential M	Negative	Negative			

Bacterial Mutagenicity Testing

Based on OECD Guideline 471 (GLP)

Tester Strain	BP at Primary Reversion Site	Reversion Event
S. typhimurium TA97a	$(C)_6$	Frameshift
S. typhimurium TA98	$(GC)_4$	Frameshift
S. typhimurium TA100	GC	Base Substitution
S. typhimurium TA1535	GC	Base Substitution
E. coli uvrA WP2 (pKM101)	AT	Base Substitution

Study Design

Pre-incubation protocol

5 to 7 Doses + vehicle control and appropriate positive controls for each tester strain

+/- Phenobarbital/benzoflavone-induced SD male rat liver S9 mix

Top concentration of 6,000 µg/plate for non-cytotoxic chemicals

Top dilution of 1:100 for non-cytotoxic GBFs

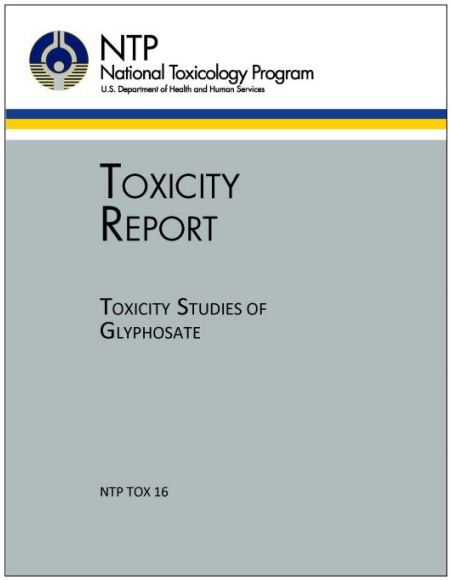
Bacterial Mutagenicity Testing

Glyphosate, Glyphosate IPA, AMPA and other actives

Test Article	-S9	+S9
Glyphosate	Negative	Negative
Glyphosate IPA	Negative	Negative
AMPA	Negative	Negative

NTP Toxicity Report No. 16 (1992):

Glyphosate
TA100, TA1535, TA97, & TA98
Aroclor 1254-induced rat (or hamster) liver S9
Top dose of 3,333 µg/plate
Pre-incubation protocol
Negative in all strains +/-S9



Bacterial Mutagenicity Testing

Glyphosate, Glyphosate IPA, other actives, and AMPA

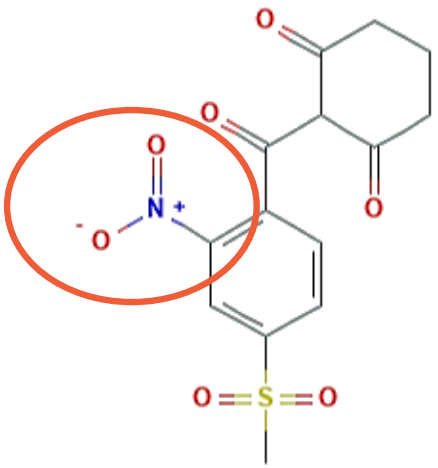
Test Article	-S9	+S9
Glyphosate	Negative	Negative
Glyphosate IPA	Negative	Negative
AMPA	Negative	Negative
Diquat Dibromide	Negative	Negative
Metolachlor	Negative	Negative
Mesotrione	**Positive**	**Positive**

Bacterial Mutagenicity Testing

Mesotrione

Tester Strain	BP at Primary Reversion Site	Reversion Event	-S9	+S9
TA97a	$(C)_6$	Frameshift	500 – 6,000	1,500 – 6,000
TA98	$(GC)_4$	Frameshift	500 – 6,000	3,000 – 6,000
TA100	GC	Base Substitution	1,500 – 6,000	1,500 – 6,000
TA1535	GC	Base Substitution	1,500 – 6,000	Equivocal
E. coli	AT	Base Substitution	Negative	Negative

- Low potency
- Attenuation with rat liver S9



Bacterial Mutagenicity Testing

Glyphosate-Based Formulations

Test Article	%Glyphosate	+/-S9
Agricultural A	20.5	Negative
Agricultural B	41.0	Negative
Agricultural C	41.0	Negative
Agricultural D	41.0	Negative
Agricultural E	44.9	Negative
Agricultural F	48.7	Negative
Agricultural G	48.8	Negative
Agricultural H	50.2	Negative
Agricultural I	53.8	Negative

Agricultural GBF A (top conc. 1:100) contains
2.05% mesotrione (Ames positive)

Bacterial Mutagenicity Testing

Glyphosate-Based Formulations

Test Article	%Glyphosate	+/-S9
Agricultural A	20.5	Negative
Agricultural B	41.0	Negative
Agricultural C	41.0	Negative
Agricultural D	41.0	Negative
Agricultural E	44.9	Negative
Agricultural F	48.7	Negative
Agricultural G	48.8	Negative
Agricultural H	50.2	Negative
Agricultural I	53.8	Negative
Residential J	1.92	Negative
Residential K	18.0	Negative
Residential L	41.0	Negative
Residential M	50.2	Negative

Summary of Genetic Toxicity Testing

Glyphosate, other actives, and AMPA

	MultiFlow Assay		MicroFlow Assay			Ames Assay	
Test Article	-S9	+S9	24 h	4 h +S9	4 h –S9	-S9	+S9
Glyphosate	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Glyphosate IPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
AMPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Diquat Dibromide	**Clastogenic**	**Clastogenic**	**Positive**	Negative	**Positive**	Negative	Negative
Metolachlor	**Clastogenic**	Negative	Negative	Negative	Negative	Negative	Negative
Mesotrione	Negative	Negative	**Positive**	Negative	Negative	**Positive**	**Positive**

- Glyphosate, glyphosate IPA, and AMPA do not show genotoxicity in this battery of *in vitro* tests
- Actives in GBFs other than glyphosate show genotoxic activity in several assays

Summary of Genetic Toxicity Testing

Glyphosate-based formulations

	MultiFlow Assay		MicroFlow Assay			Ames Assay	
Test Article	-S9	+S9	24 h	4 h +S9	4 h –S9	-S9	+S9
Glyphosate	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Glyphosate IPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
AMPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Diquat Dibromide	Clastogenic	Clastogenic	Positive	Negative	Positive	Negative	Negative
Metolachlor	Clastogenic	Negative	Negative	Negative	Negative	Negative	Negative
Mesotrione	Negative	Negative	Positive	Negative	Negative	Positive	Positive
Agricultural A	Clastogenic	Negative	In Progress			Negative	Negative
Agricultural B	Negative	Negative				Negative	Negative
Agricultural C	Negative	Negative				Negative	Negative
Agricultural D	Negative	Negative				Negative	Negative
Agricultural E	Negative	Negative				Negative	Negative
Agricultural F	Negative	Negative				Negative	Negative
Agricultural G	Negative	Negative				Negative	Negative
Agricultural H	Negative	Negative				Negative	Negative
Agricultural I	Clastogenic	Clastogenic				Negative	Negative
Residential J	Negative	Negative				Negative	Negative
Residential K	Negative	Negative				Negative	Negative
Residential L	Negative	Negative				Negative	Negative
Residential M	Negative	Negative				Negative	Negative

Summary of Genetic Toxicity Testing

Glyphosate-based formulations

	MultiFlow Assay		MicroFlow Assay			Ames Assay	
Test Article	-S9	+S9	24 h	4 h +S9	4 h –S9	-S9	+S9
Glyphosate	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Glyphosate IPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
AMPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Diquat Dibromide	Clastogenic	Clastogenic	Positive	Negative	Positive	Negative	Negative
Metolachlor	Clastogenic	Negative	Negative	Negative	Negative	Negative	Negative
Mesotrione	Negative	Negative	Positive	Negative	Negative	Positive	Positive
Agricultural A	Clastogenic	Negative	In Progress			Negative	Negative
Agricultural B	Negative	Negative				Negative	Negative
Agricultural C	Negative	Negative				Negative	Negative
Agricultural D	Negative	Negative				Negative	Negative
Agricultural E	Negative	Negative				Negative	Negative
Agricultural F	Negative	Negative				Negative	Negative
Agricultural G	Negative	Negative				Negative	Negative
Agricultural H	Negative	Negative				Negative	Negative
Agricultural I	Clastogenic	Clastogenic				Negative	Negative
Residential J	Negative	Negative				Negative	Negative
Residential K	Negative	Negative				Negative	Negative
Residential L	Negative	Negative				Negative	Negative
Residential M	Negative	Negative				Negative	Negative

Summary of Genetic Toxicity Testing

Glyphosate-based formulations

	MultiFlow Assay		MicroFlow Assay			Ames Assay	
Test Article	-S9	+S9	24 h	4 h +S9	4 h –S9	-S9	+S9
Glyphosate	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Glyphosate IPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
AMPA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Diquat Dibromide	Clastogenic	Clastogenic	Positive	Negative	Positive	Negative	Negative
Metolachlor	Clastogenic	Negative	Negative	Negative	Negative	Negative	Negative
Mesotrione	Negative	Negative	Positive	Negative	Negative	Positive	Positive
Agricultural A	Clastogenic	Negative	In Progress			Negative	Negative
Agricultural B	Negative	Negative				Negative	Negative
Agricultural C	Negative	Negative				Negative	Negative
Agricultural D	Negative	Negative				Negative	Negative
Agricultural E	Negative	Negative				Negative	Negative
Agricultural F	Negative	Negative				Negative	Negative
Agricultural G	Negative	Negative				Negative	Negative
Agricultural H	Negative	Negative				Negative	Negative
Agricultural I	Clastogenic	Clastogenic				Negative	Negative
Residential J	Negative	Negative				Negative	Negative
Residential K	Negative	Negative				Negative	Negative
Residential L	Negative	Negative				Negative	Negative
Residential M	Negative	Negative				Negative	Negative

Summary of Genetic Toxicity Tests

- Glyphosate, glyphosate IPA, and AMPA did not show genotoxic activity and were not cytotoxic to human lymphoblastoid TK6 cells
  - Based on this data set, it is unlikely that any genotoxic activity of GBFs is due to glyphosate
- Some GBFs showed genotoxic activity that, in some cases, could potentially be attributed to herbicides other than glyphosate

Oxidative Stress and Other Endpoints

- Human cell lines
  - HepaRG liver cells and HaCaT cells (spontaneously immortalized skin keratinocytes)
- Experimental design
  - 384-well plate format, 10-pt dose-response curves, 3 replicates, 4 and 24 h time points

Oxidative Stress and Other Endpoints

- Human cell lines
  - HepaRG liver cells and HaCaT cells (spontaneously immortalized skin keratinocytes)
- Experimental design
  - 384-well plate format, 10-pt dose-response curves, 3 replicates, 4 and 24 h time points
- Assays
  - CellTiter-Glo with bright field imaging of wells (cell viability)
  - ROS-Glo (detects hydrogen peroxide)
  - GSH-Glo (reduced levels of glutathione as an indicator of oxidative stress)
  - Mitochondrial Membrane Potential (functional electron transport)
  - γH2AX (biomarker for DNA double strand breaks)

High Throughput Screening Assays

Preliminary findings

- **Cell viability**
  - Glyphosate and AMPA did not affect cell viability at concentrations up to 10 mM
  - Some GBFs decreased cell viability at concentrations from 0.1 to 1.0 mM glyphosate equivalents
    - Toxicity of GBFs was not correlated with the concentration of glyphosate in the GBFs
- **Oxidative stress**
  - Glyphosate, AMPA, and GBFs did not induce H2O2 in HepaRG or HaCaT cells at the 4 h or 24 h time points
- **DNA damage**
  - Glyphosate, AMPA, and GBFs did not increase γH2AX in HepaRG or HaCaT cells at the 4 h or 24 h time points

Glyphosate v. Glyphosate-Based Formulations

- In NTP *in vitro* studies, there was no evidence that glyphosate, glyphosate IPA, or AMPA were genotoxic or that they induced oxidative stress
  - Glyphosate was also negative in a micronucleus study in which male and female mice ingested glyphosate in feed at very high doses (NTP Toxicity Report No. 16)
- Herbicides in GBFs other than glyphosate showed genotoxic activity

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