QUEEN'S UNIVERSITY BELFAST | IGFS THE INSTITUTE FOR GLOBAL FOOD SECURITY

High Content Analysis

Julie P Meneely

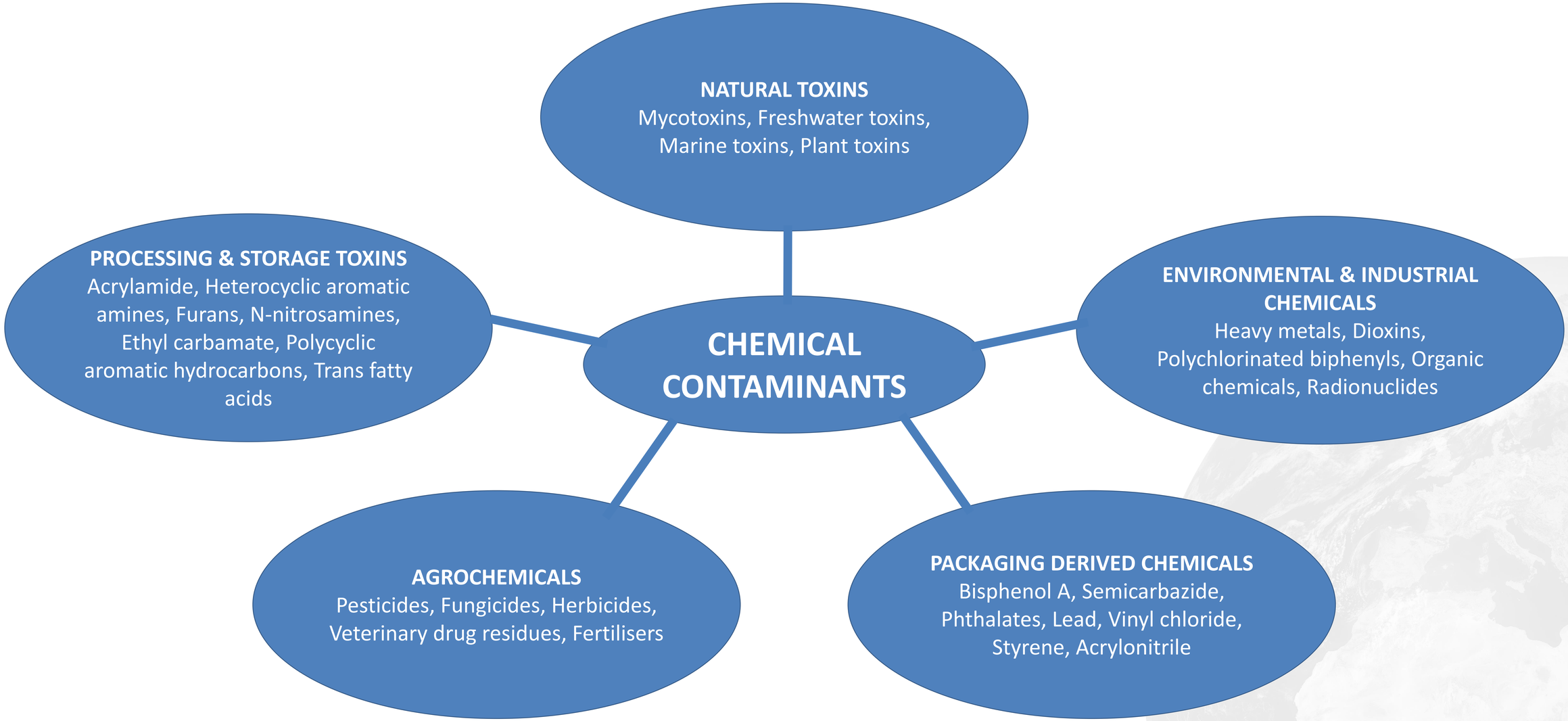
Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast

Challenges in food and feed safety research, 27-29 November 2018
Prague, Czech Republic

MultiCoop

UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

BOKU Universität für Bodenkultur Wien
University of Natural Resources and Applied Life Sciences, Vienna


NATURAL TOXINS
Mycotoxins, Freshwater toxins, Marine toxins, Plant toxins
PROCESSING & STORAGE TOXINS
Acrylamide, Heterocyclic aromatic amines, Furans, N-nitrosamines, Ethyl carbamate, Polycyclic aromatic hydrocarbons, Trans fatty acids
CHEMICAL CONTAMINANTS
ENVIRONMENTAL & INDUSTRIAL CHEMICALS
Heavy metals, Dioxins, Polychlorinated biphenyls, Organic chemicals, Radionuclides
AGROCHEMICALS
Pesticides, Fungicides, Herbicides, Veterinary drug residues, Fertilisers
PACKAGING DERIVED CHEMICALS
Bisphenol A, Semicarbazide, Phthalates, Lead, Vinyl chloride, Styrene, Acrylonitrile

In vivo toxicity testing

Advantages:
Physiological interactions between cells and tissues revealed.
International harmonisation.
Simple to perform.
Animal models exist for human diseases.
Relatively inexpensive.

Disadvantages:
Ethical concerns.
Species differences makes prediction of human responses difficult.
Require large amounts of test substance.
Natural variances not reflected due to use of inbred strains.
Complex data.
Unrealistic exposure scenarios.
Lack of reproducibility.

In vitro toxicity testing

Advantages:
Widely established methodologies.
Low cost.
High numbers of replicates tested.
Novel technologies, miniaturisation and automation available.
Few ethical concerns.
Straightforward interpretation.

Disadvantages:
Cells maintained under non-physiological conditions
Low cell densities result in impaired intracellular signalling.
Culture conditions are not stable.
Lack of biotransformation capabilities.
Lack of differentiation therefore organ functionality in immortalised cell lines.
Cell cross-contamination.
Cell culture contamination.
Not sensitive predictors of human toxicity.

Traditional cytotoxicity endpoints and assays

Cell Number	•Trypan blue, SRB: (Sulforhodamine B), Methylene blue staining, Resazurin, ALP: (Alkaline phosphatase enzyme), Hoechst
Cell Viability	•LDH: (Lactate dehydrogenase) leakage, Crystal violet, Calcein-AM, Fluorescein diacetate
Cellular ATP	•ATP
Cellular Metabolic Function	•MTT: (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), XTT: (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide), WST-1: (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate), TMRE: (tetramethylrhodamine, ethyl ester)
Membrane permeability	•LDH, MTT, Caspase-based, Annexin, Granzyme-based
Nuclear Size	•Hoechst, BrdU: (5-Bromo-2´-Deoxyuridine), DAPI, TUNEL, Ethidium homodimer, Propidium dye, Caspase-based
Lysosomal Activity	•NR: (Neutral red), Granzyme-based, Cathepsin D activity
Intracellular Calcium	•Fluo-3, Fluo-4

In vitro toxicity testing

Rapid, inexpensive, simple, sensitive, homogeneous, automated

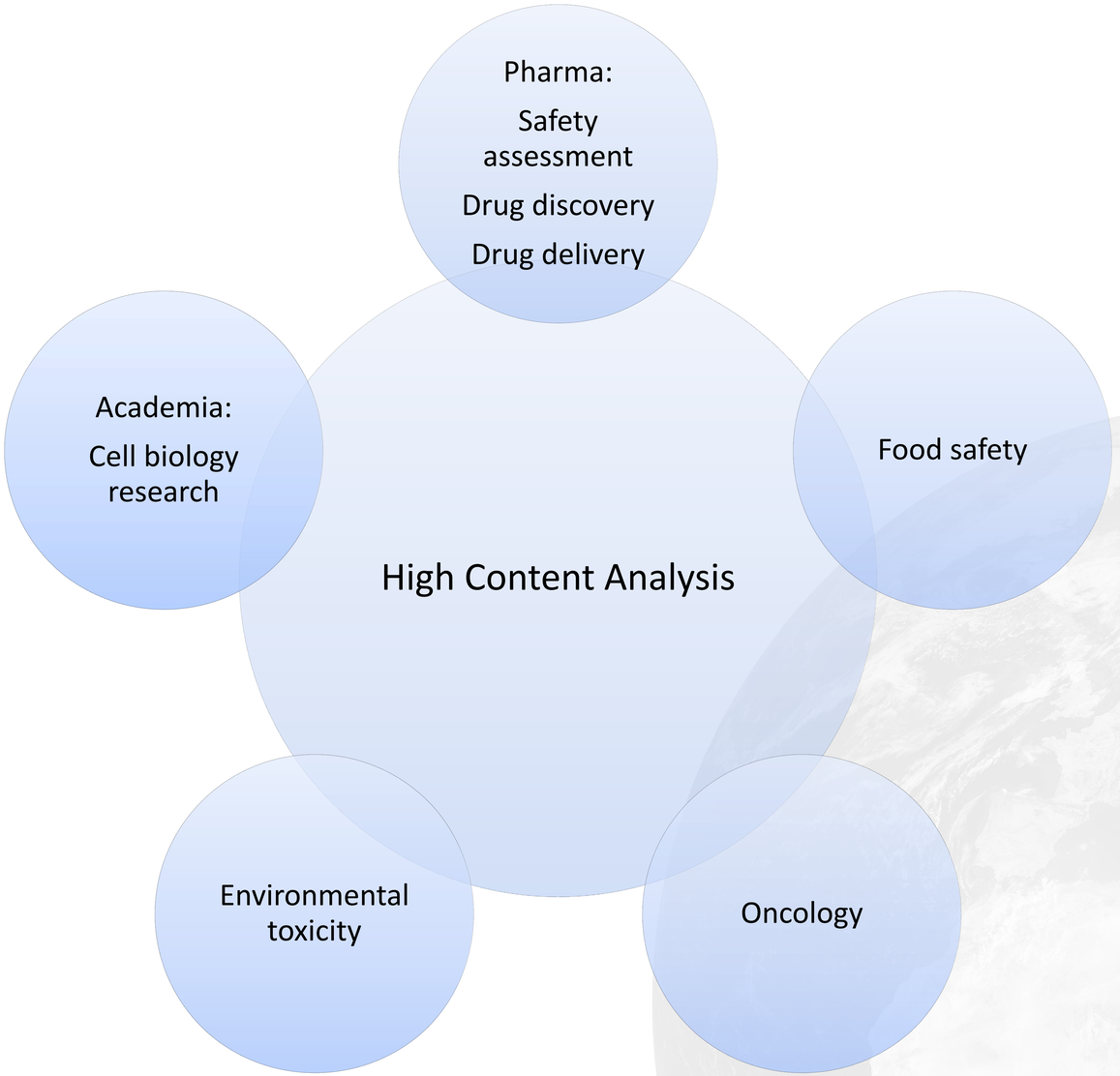
State-of-the-art techniques using luminescence and fluorescence offer sensitivity, simplicity, reproducibility and are suitable for high-throughput screening (HTS) and high-content screening (HCS)

Major advances in automated microscopes and microtitre plate readers, image analysis software and the development of a wide range of subcellular fluorescent probes

High Content Screening

High Content Analysis:

- Automated technology
- Physiological incubation of cells in microtitre plates
- Automated liquid handling and multiprobes
- Epifluorescence or laser-based microscopy
- Fluorescence image acquisition
- Quantitative morphometric analysis of individual cells and their organelles
- Use began in drug discovery

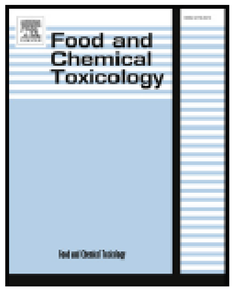


Food and Chemical Toxicology 121 (2018) 527–540

Contents lists available at ScienceDirect

Food and Chemical Toxicology

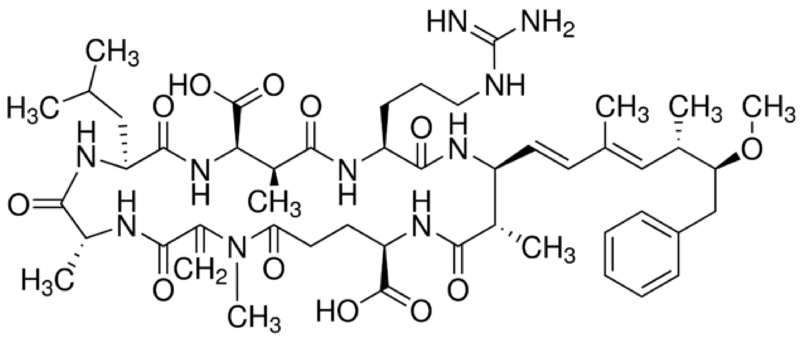
journal homepage: www.elsevier.com/locate/foodchemtox



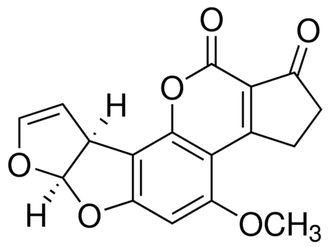
Assessing the combined toxicity of the natural toxins, aflatoxin B_1, fumonisin B_1 and microcystin-LR by high content analysis

Julie P. Meneely[a,*], Jana Hajšlová[b], Rudolf Krska[c], Christopher T. Elliott[a]

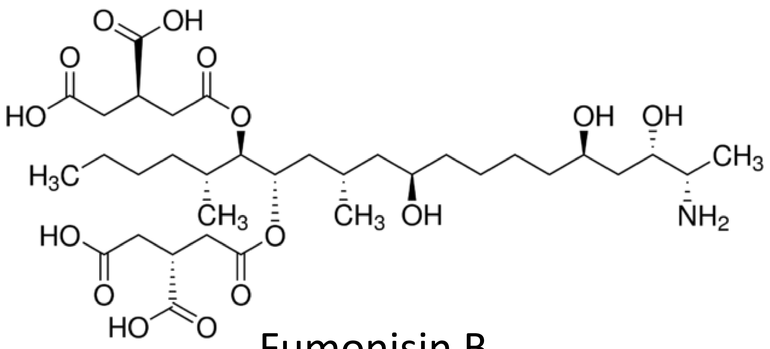
[a] Institute for Global Food Security, School of Biological Sciences, Queen's University, Belfast, BT7 1NN, United Kingdom
[b] Faculty of Food & Biochemical Technology, Department of Food Analysis & Nutrition, University of Chemistry & Technology, Technická 3, 166 28, Prague 6, Czech Republic
[c] Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenz Str. 20, 3430, Tulln, Austria



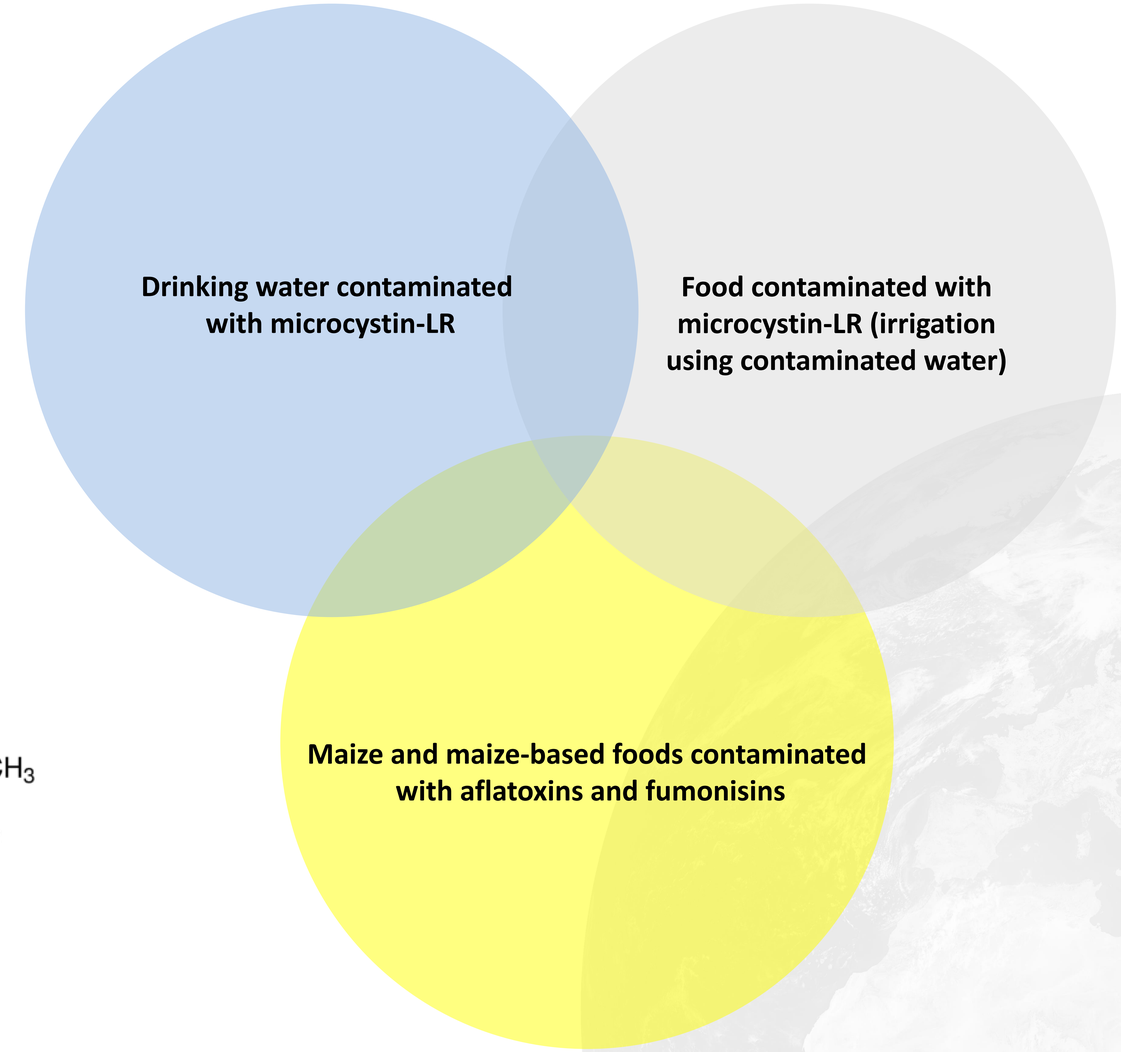
Microcystin-LR



Aflatoxin B_1

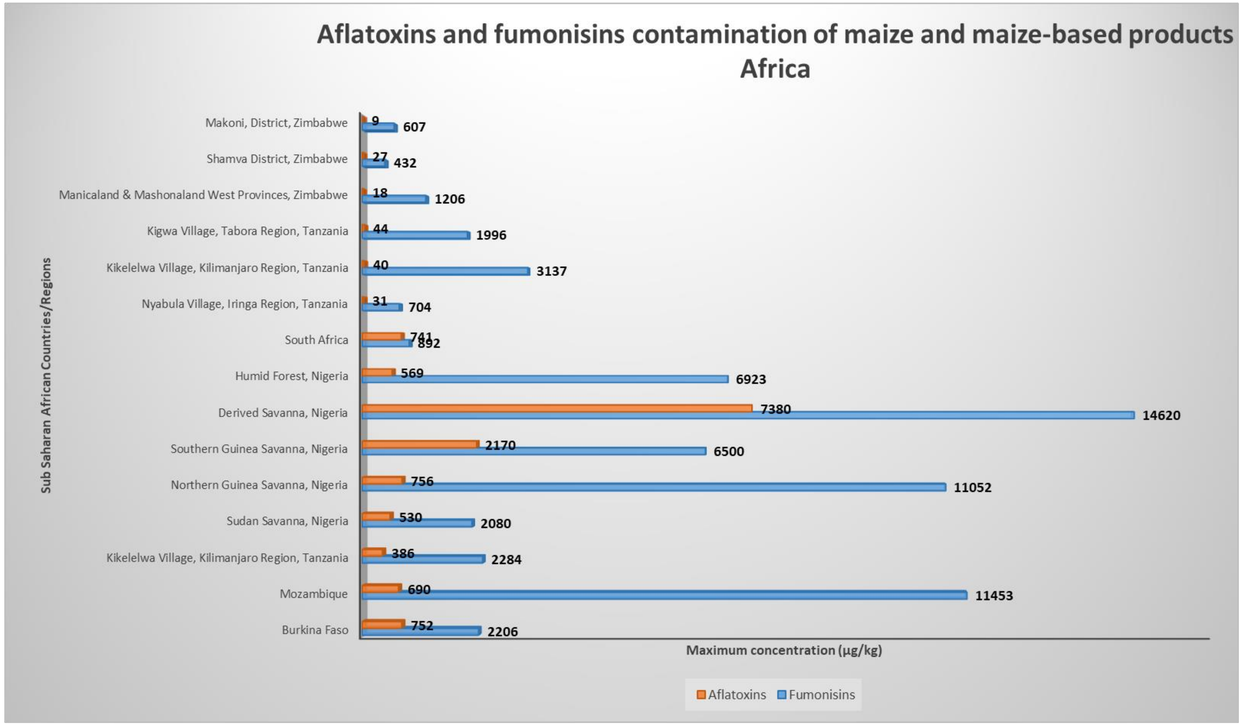


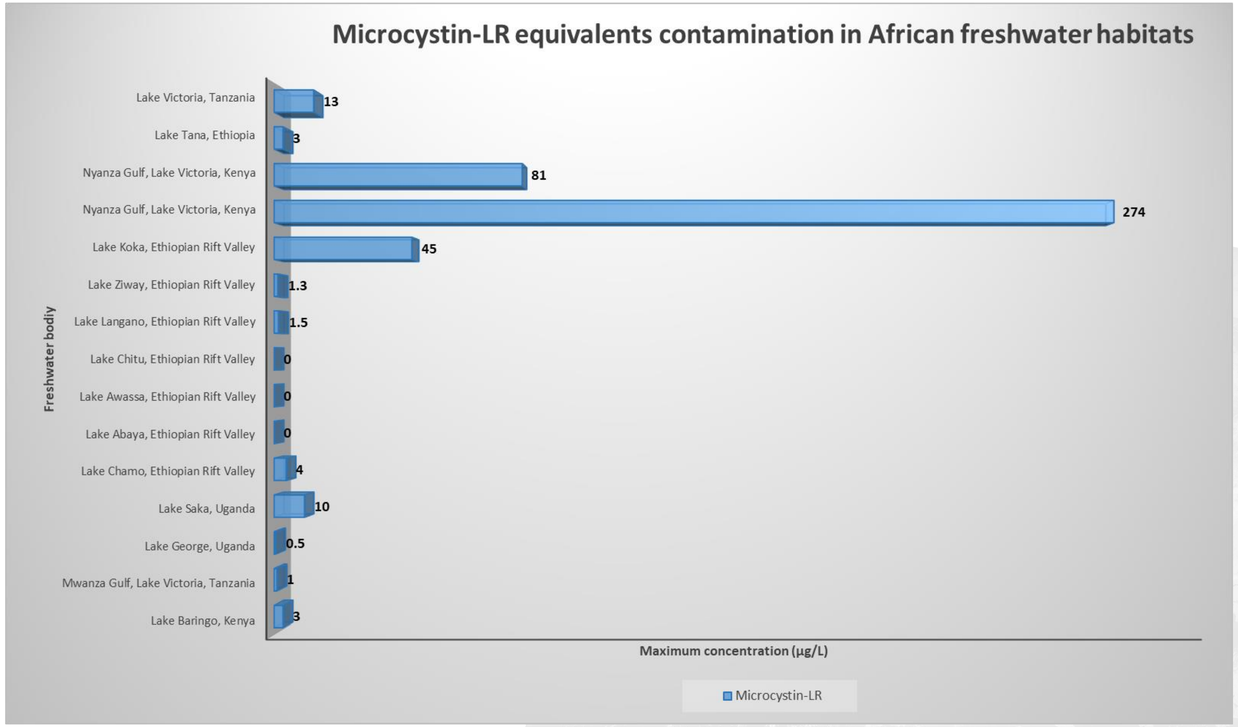
Fumonisin B_1



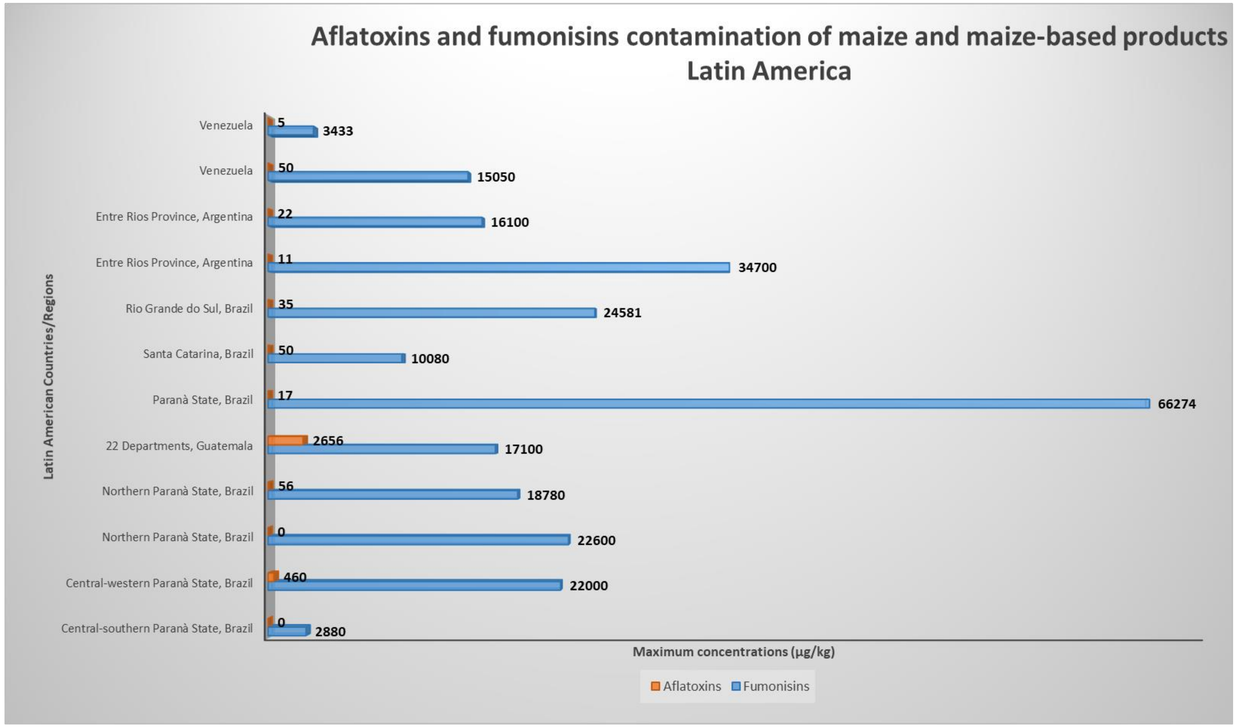
QUEEN'S UNIVERSITY BELFAST | IGFS THE INSTITUTE FOR GLOBAL FOOD SECURITY

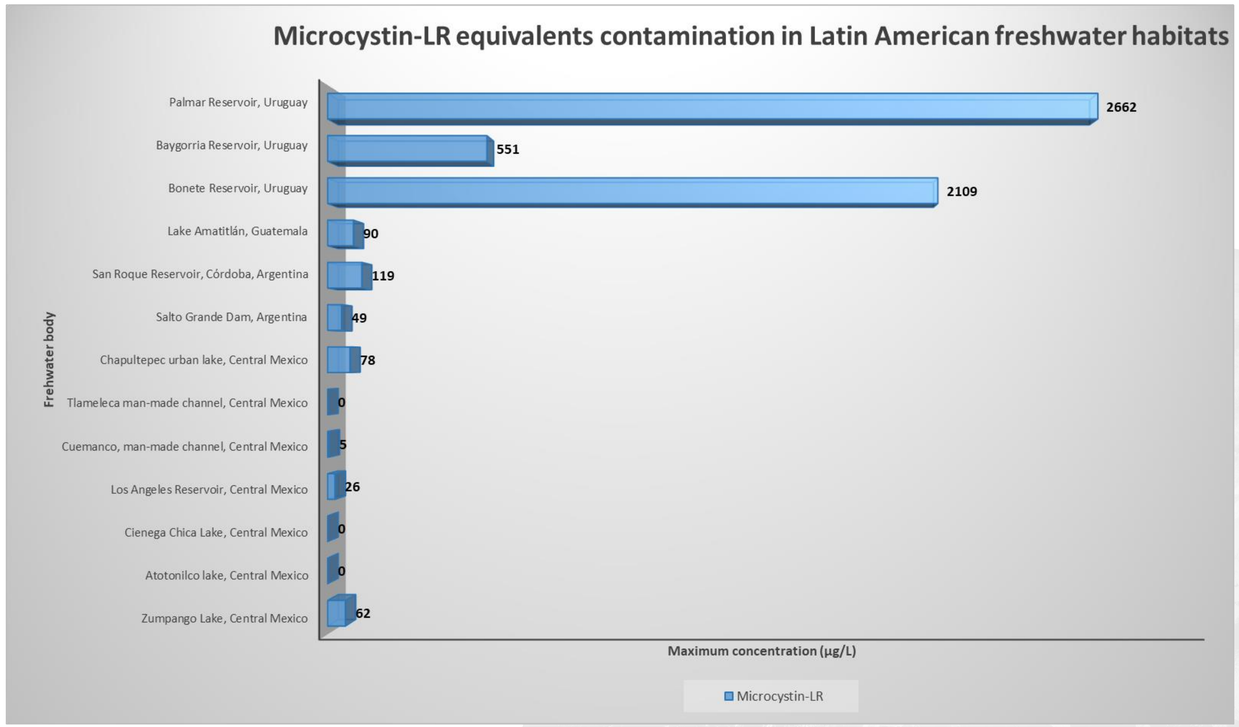
Contamination levels of aflatoxins, fumonisins and microcystins in Africa



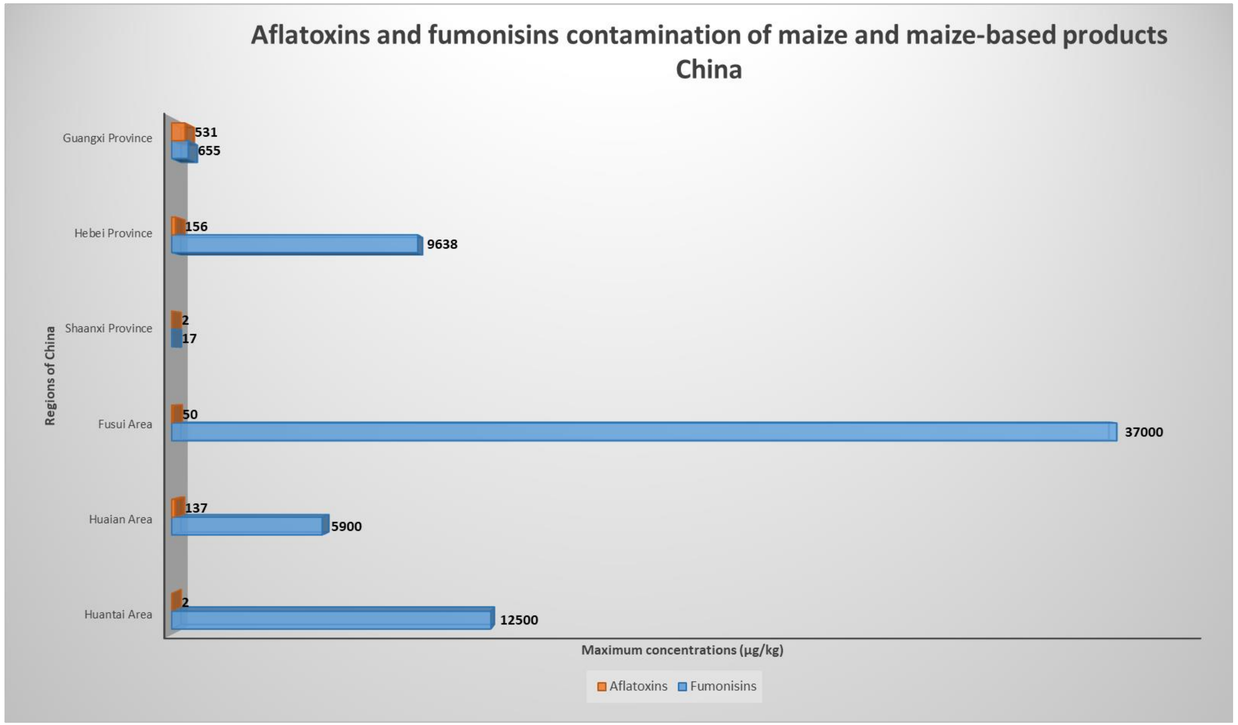


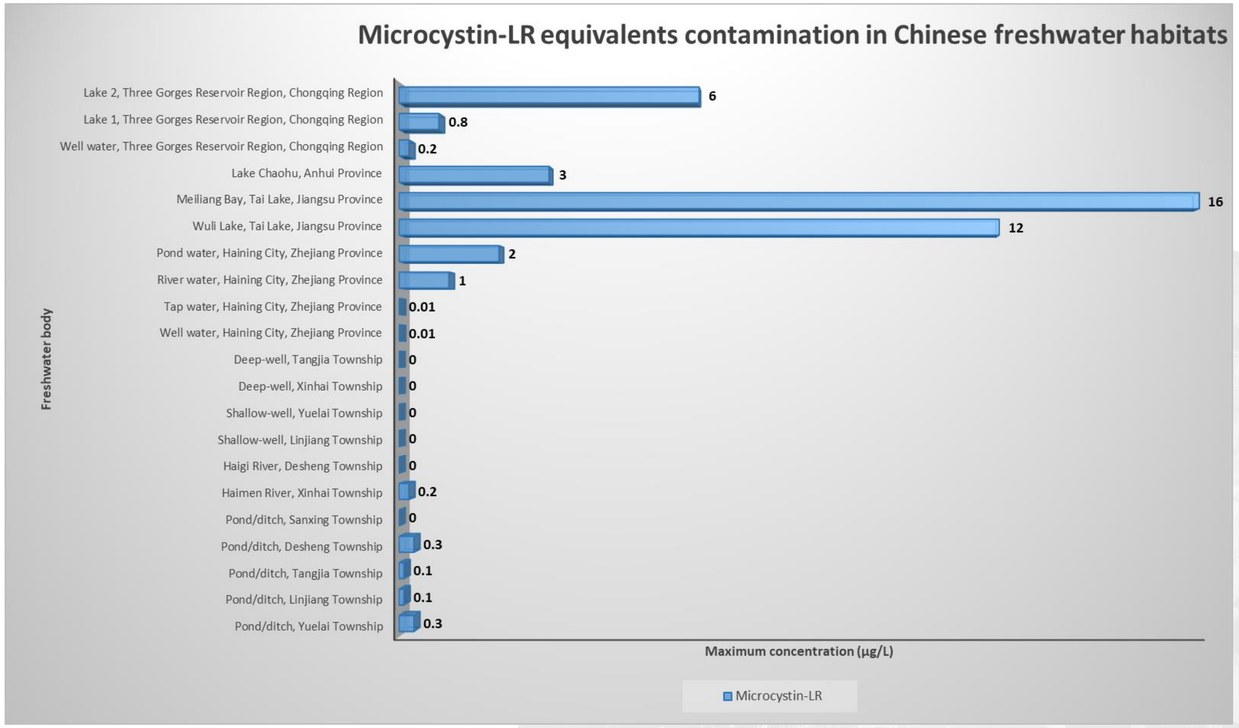
Contamination levels of aflatoxins, fumonisins and microcystins in Latin America





Contamination levels of aflatoxins, fumonisins and microcystins in China





Country	Biomarker	Concentration (µg/L)	Estimated exposure (µg/kg/bw/d)	Reference
Cameroon	Urinary AFM_1	1.38	1.15	Abia et al, 2013
Nigeria	Urinary AFM_1	1.5	2.5	Ezekiel et al, 2014
Brazil	Urinary AFM_1	0.0069	0.0018	Jager et al, 2014
Brazil	Urinary AFM_1	0.0042	0.0014	Jager et al, 2014
Fushui County, China	Urinary AFM_1	3.2	3.68	Zhu et al, 1987
Shanghai, China	Urinary AFM_1	5.2	4.33	Qian et al, 1994
Cameroon	Urinary FB_1	14.8	123.3	Abia et al, 2013
Nigeria	Urinary FB_1	12.8	76	Ezekiel et al, 2014
Huaian County, China	Urinary FB_1	13.63	7.67	Xu et al, 2010
Fusui County	Urinary FB_1	0.72	2.12	Xu et al, 2010
Mexico	Urinary FB_1	0.147	0.368	Gong et al, 2008
South Africa	Urinary FB_1	0.225	8.14	Westhuizen et al, 2011
Anhui Province, China	MC-LR (serum)	0.39	0.065	Chen et al, 2009
Three Gorges Reservoir, China	Estimated from food And water consumption	/	0.203	Li et al, 2011
Caruaru, Brazil	MC-LR (serum)	133	11.1*	Pouria et al, 1998

*Acute poisoning of dialysis patients during haemodialysis

In vitro testing using high content analysis

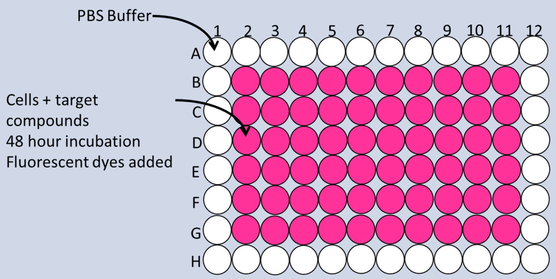
- Cell health profiling / cytotoxicity / single toxins / combined toxins
  - Cell number
  - Nuclear morphology
  - Mitochondrial health
- Interactions of toxins
  - Antagonistic
  - Additive
  - Synergistic
- Appropriate cell lines
  - Human hepatocellular carcinoma (HepG2 cells)
  - Human epithelial colorectal adenocarcinoma (Caco-2) cells
  - Madin-Darby bovine kidney epithelial (MDBK) cells
- Realistic concentrations
  - Reported biomarker/serum concentrations in the literature

Relating the *in vitro* concentrations used to *in vivo* concentrations in humans

Toxin	*In vitro* Concentration (µg/L)	*In vivo* concentration (µg/kg/bw/d)	Range population exposures (µg/kg/bw/d)	Tolerable Daily Intake (TDI) (µg/kg/bw)
Aflatoxin B_1	0.1	0.0003	0.0014 – 4.33	ALARA
	2	0.006		
	10	0.033		
	100	0.33		
	500	1.6		
Fumonisin B_1	200	0.67	0.368 – 123.3	2
	1000	3.33		
	2000	6.67		
	4000	13.3		
	8000	26.6		
Microcystin-LR	0.2	0.0006	0.065 – 0.203	0.04
	1	0.003	11.1 – fatal poisoning	
	5	0.017		
	50	0.17		
	250	0.83		

QUEEN'S UNIVERSITY BELFAST | IGFS THE INSTITUTE FOR GLOBAL FOOD SECURITY

1. Cell/sample preparation

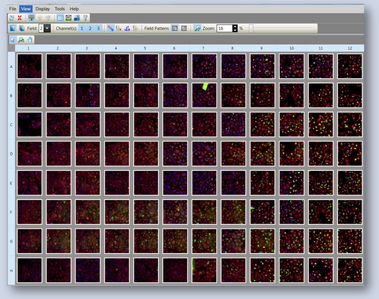


Exposure analyses were performed in triplicate on three independent occasions and the results expressed as the mean percentage of the solvent control ± standard error of the mean (SEM) of the exposures.

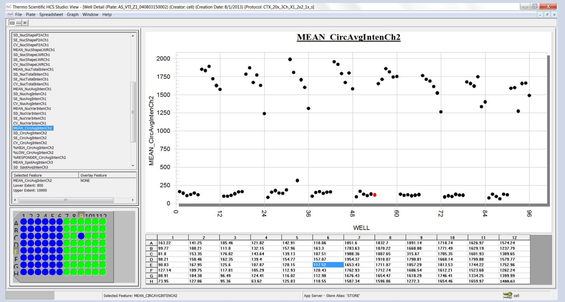
2. Image & data collection

Configure imaging methods
Phenotype selection
Scanning

3. Image & data visualisation



Fluorescent images acquired



Quantitative data collected for individual cells/cell populations

Fluorescent probes:

1. Hoechst nuclear stain, cell-permeant dye that emits blue fluorescence when bound to DNA.
   - Cell number
   - Nuclear area
   - Nuclear intensity
2. MitoTracker® Orange CMTMRos, cell-permeant orange dye that accumulates in mitochondria.
   - Mitochondrial mass
   - Mitochondrial membrane potential

Assay configuration:

- Optimise exposure time
- Identify objects
  - Background
  - Size/Shape
  - Intensity
- Validate objects
- Selection of objects
- Scan plate

Data:

- Selected object count
- Mean object area Ch1
- Average intensity Ch1
- Mean object area Ch2
- Average intensity Ch2

Cellular parameters measured

Parameter	Significance
Cell number (CN)	Damage to cell structure and function are the first consequences of a toxic impact on cells, therefore a live cell count is a very sensitive indicator of cell stress. Decrease = cell death Increase = proliferation
Nuclear Area (NA)	When subjected to acute toxic injury, cells will swell initially and finally rupture or shrink. – necrosis (traumatic cell death). A decrease in NA (cell shrinkage) signifies apoptosis or programmed cell death.
Nuclear Intensity (NI)	When cellular injury results in nuclear condensation and cell shrinkage, an increase is observed for NI. This correlates to NA.
Mitochondrial Mass (MM)	Enhanced biogenesis of mitochondria can increase MM due to increased mitochondrial respiration and this often corresponds with reduced MMP. Activated during times of cellular stress.
Mitochondrial Membrane Potential (MMP)	Apoptotic cells often exhibit a significant decrease in MMP, however, in the very early stages of apoptosis a relative increase in MMP can occur.

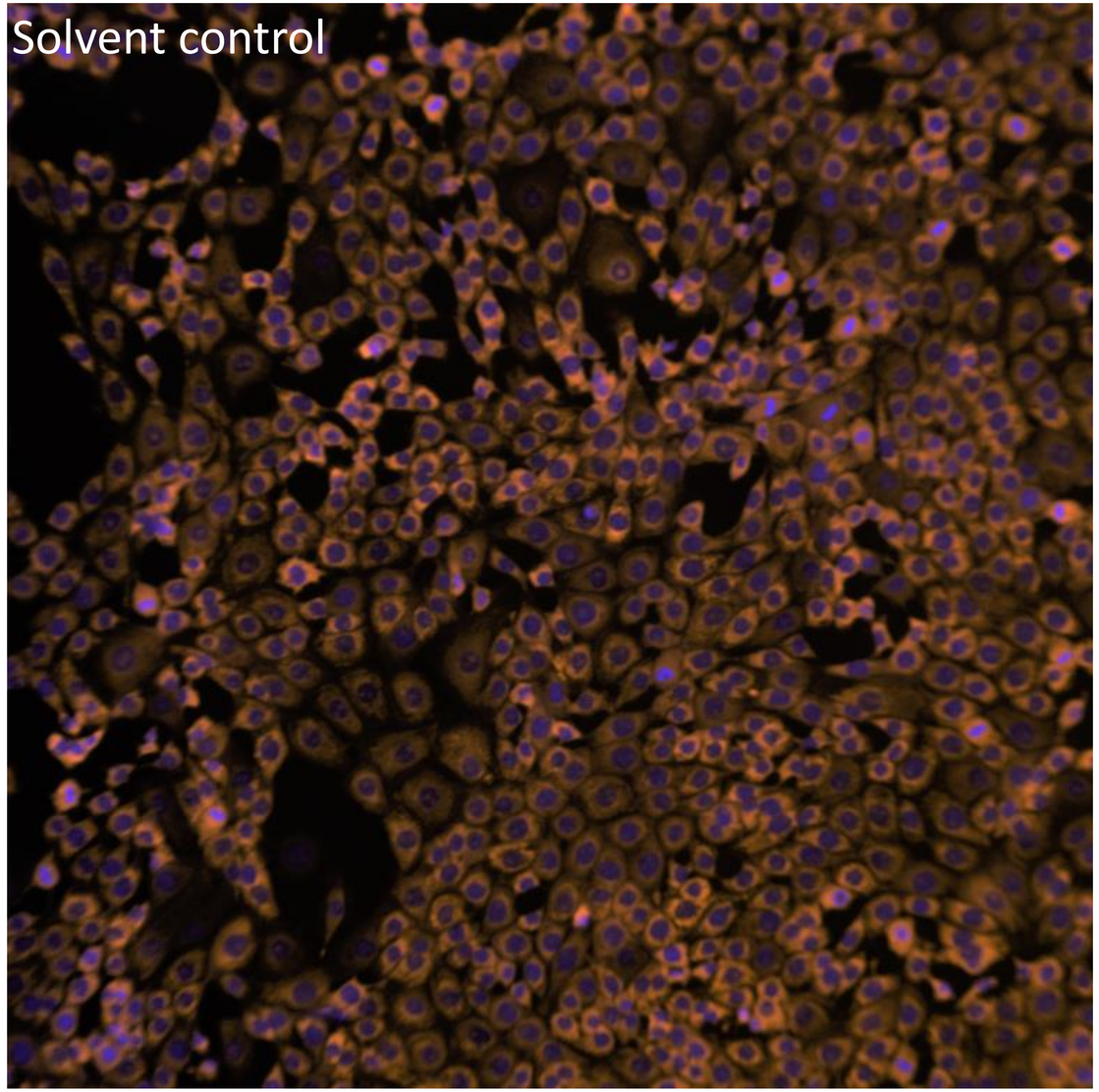
Results: Cytotoxic effects of individual biotoxins

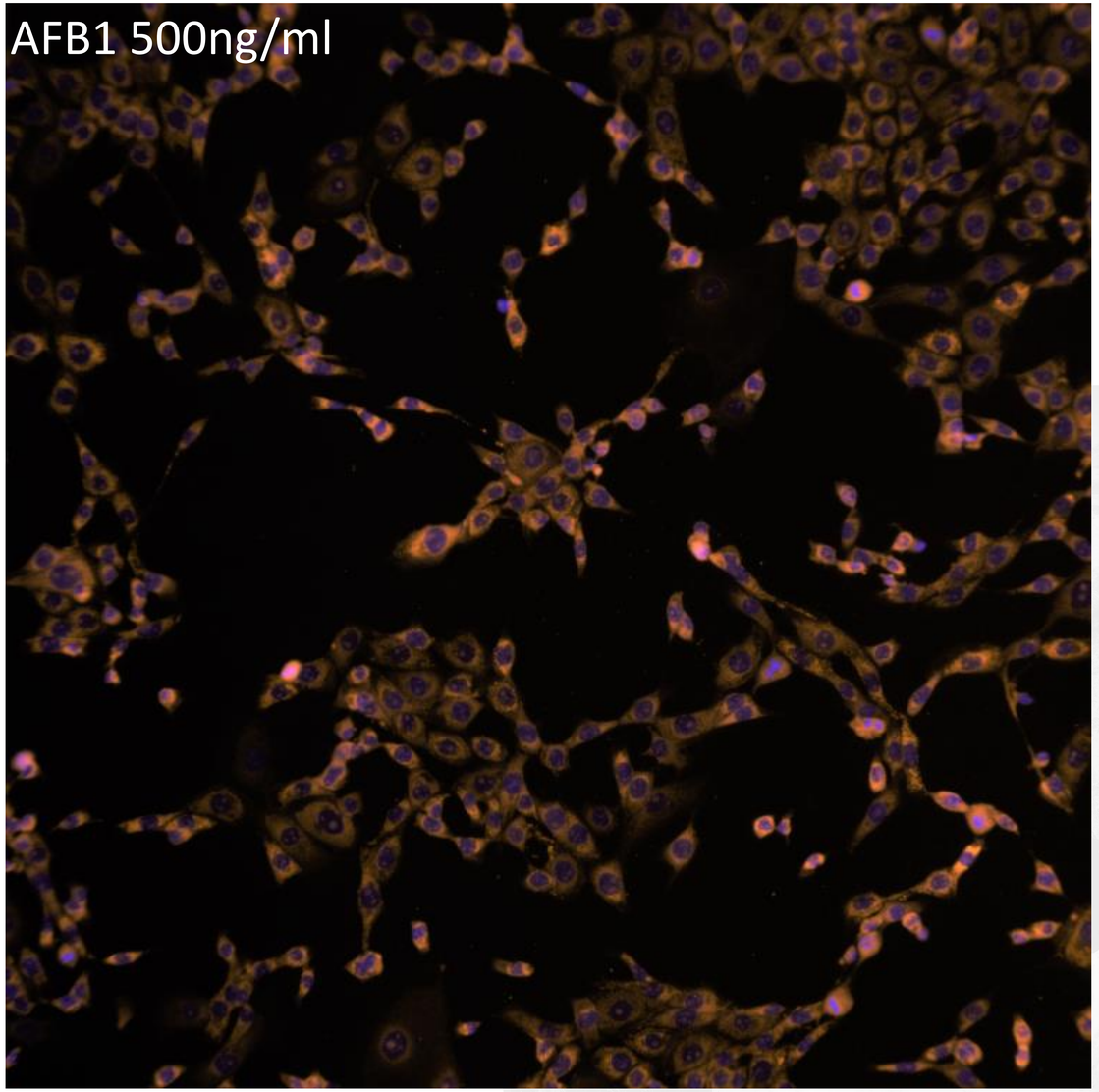
- No cytotoxicity was observed for FB_1 or MC-LR at the concentrations investigated.
- AFB_1 at the highest concentrations triggered cellular injury in all cell lines tested.

Cytotoxic effects of AFB_1 at 500 µg/L ≡ 1.6 µg/kg/bw/d

Endpoint	HepG2 cell line	Caco-2 cell line	MDBK Cell line
Cell number (CN)	↓ 17.3% ($p \leq 0.001$)	No effect	↓ 13.5% ($p \leq 0.05$)
Nuclear Area (NA)	↑ 17.0% ($p \leq 0.001$)	↑ 10.1% ($p \leq 0.001$)	↑ 6.6% ($p \leq 0.05$)
Nuclear Intensity (NI)	No effect	No effect	No effect
Mitochondrial Mass (MM)	↑ 12.3% ($p \leq 0.001$)	↑ 8.3% ($p \leq 0.001$)	No effect
Mitochondrial Membrane Potential (MMP)	No effect	No effect	No effect

Significant differences at the 95% confidence level between the control and treated groups were determined by one-way ANOVA and Dunnett's multiple comparison assessment.


Solvent control


AFB1 500ng/ml

Cytotoxic effects of binary mixture AFB_1/MC-LR exposure

Cytotoxic effects of AFB_1/MC-LR at 500 µg/L & 250 µg/L ≡ 1.6 & 0.83 µg/kg/bw/d

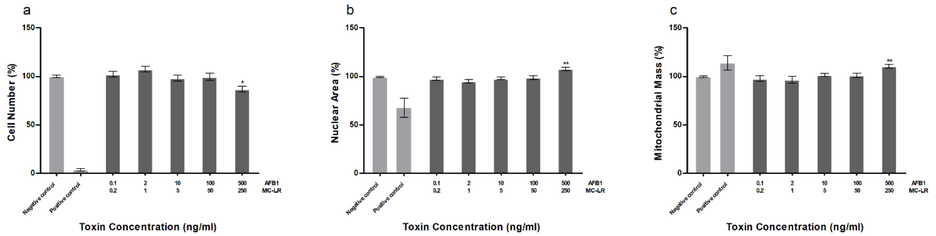
Endpoint	HepG2 cell line	Caco-2 cell line	MDBK Cell line
Cell number (CN)	↓ 11.3% ($p \leq 0.01$)	No effect	No effect
Nuclear Area (NA)	↑ 8.2% ($p \leq 0.05$)	No effect	↑ 3.7% ($p \leq 0.05$)
Nuclear Intensity (NI)	No effect	No effect	No effect
Mitochondrial Mass (MM)	↑ 10.6% ($p \leq 0.05$)	↑ 5.4% ($p \leq 0.01$)	No effect
Mitochondrial Membrane Potential (MMP)	No effect	No effect	No effect

HepG2 cells: Same endpoints affected as for AFB_1 single exposure. Effects are less significant.

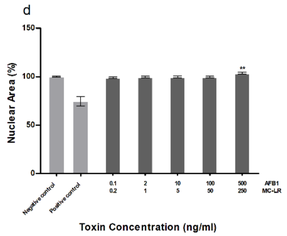
Caco-2 & MDBK cells: Only 1 endpoint affected compared to AFB_1 single exposure. Effects are less significant.

Treatment with binary mixture - Aflatoxin B_1/Microcystin-LR

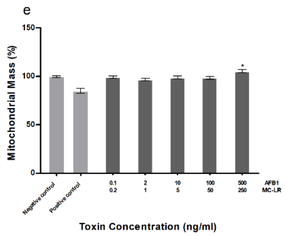
HepG2 Cell Line

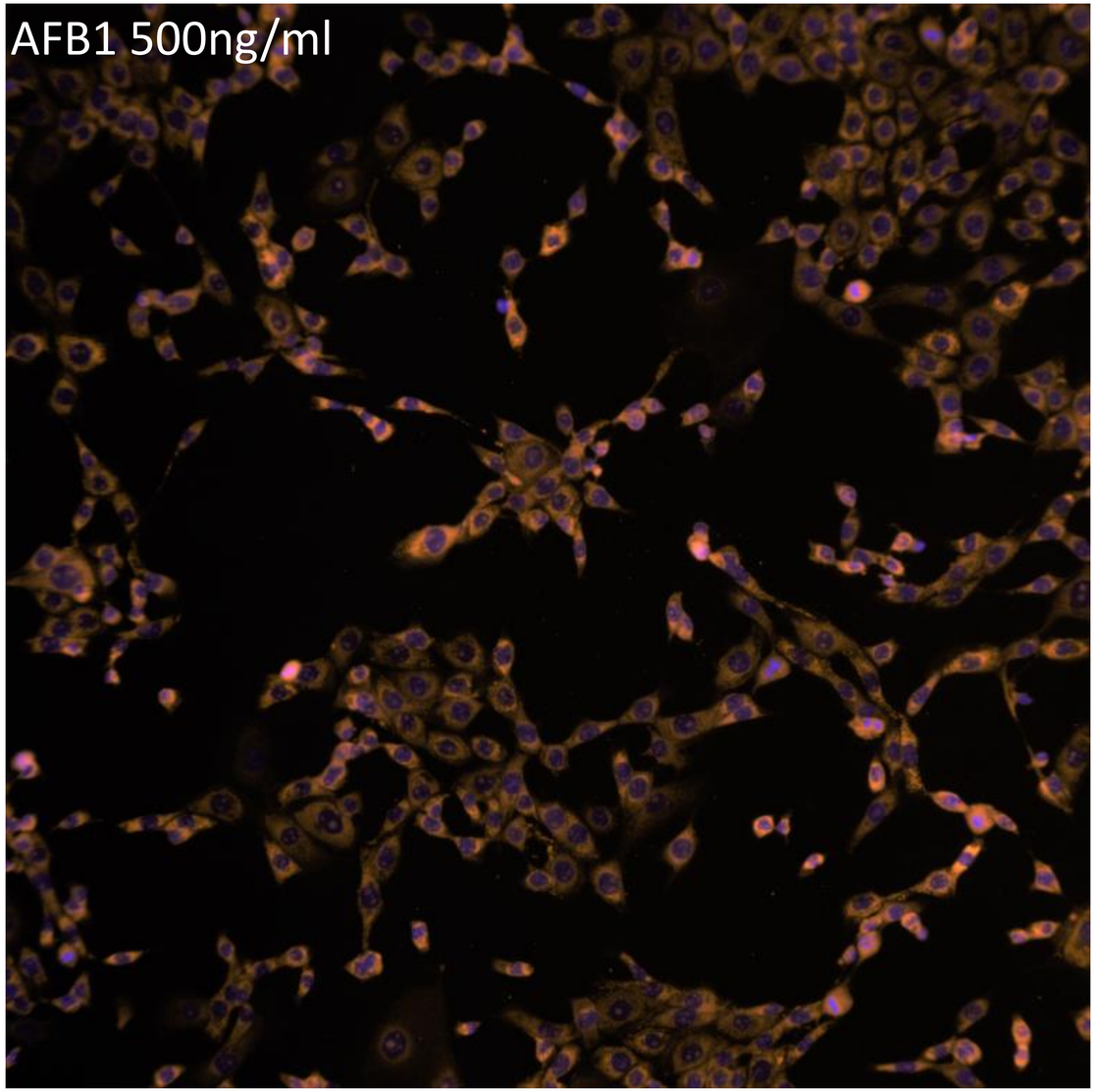


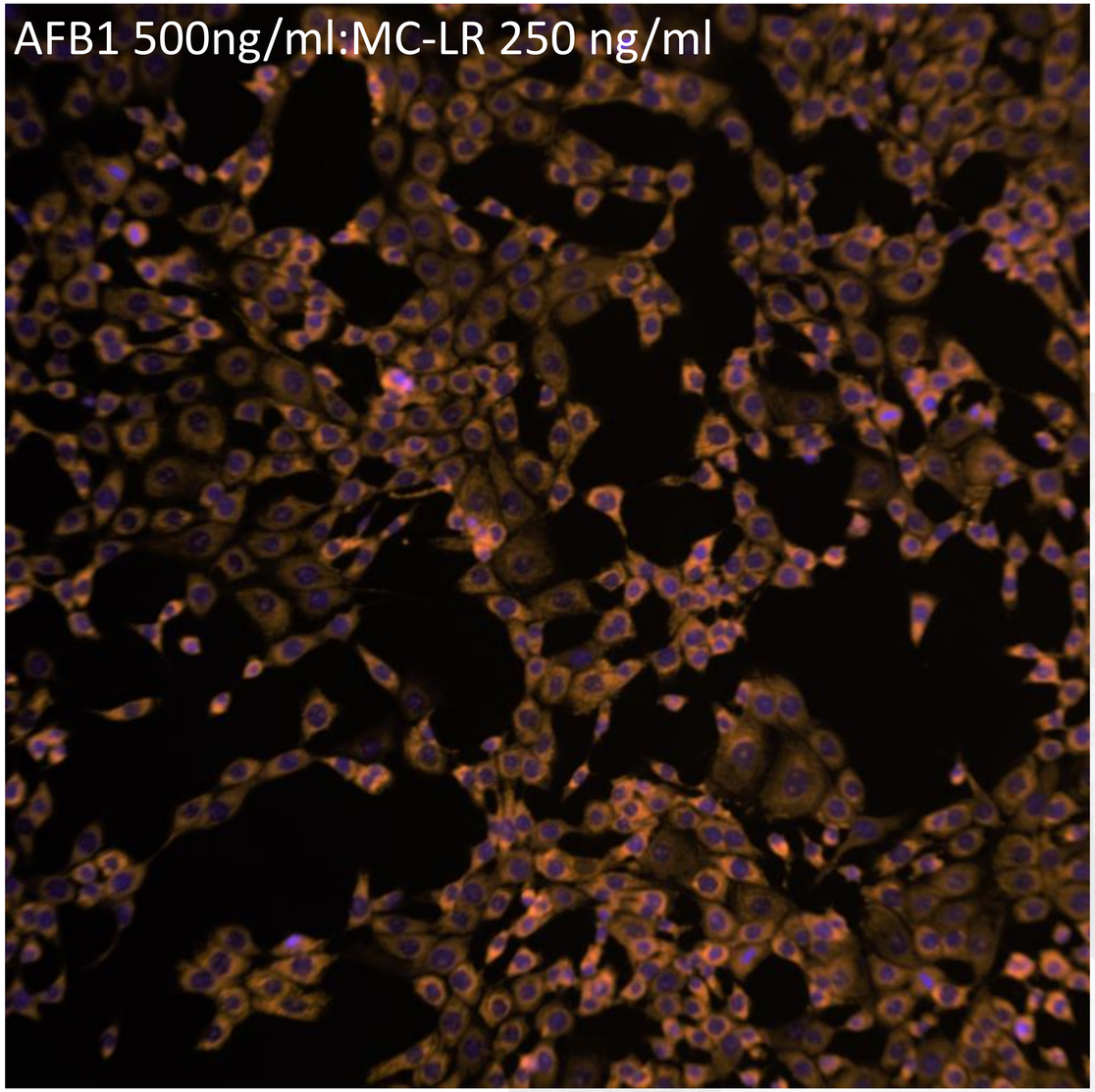
MDBK Cell Line



Caco-2 Cell Line




AFB1 500ng/ml

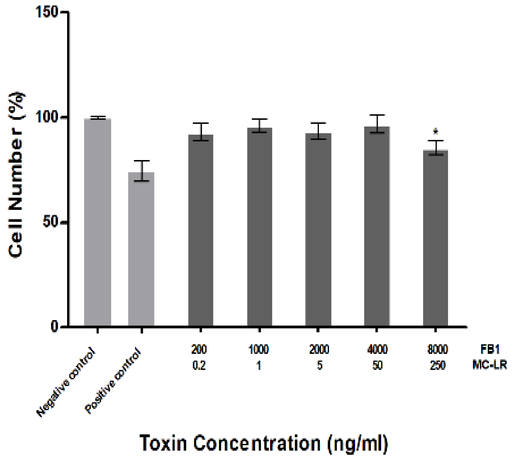

AFB1 500ng/ml:MC-LR 250 ng/ml

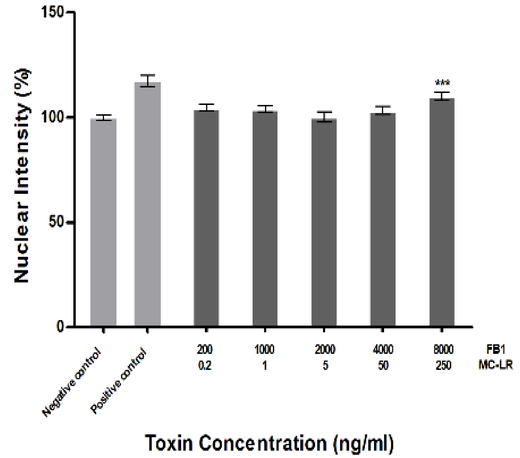
Cytotoxic effects of binary mixture FB_1/MC-LR exposure

Cytotoxic effects of FB_1/MC-LR at 8000 µg/L & 250 µg/L ≡ 26.6 & 0.83 µg/kg/bw/d	
Endpoint	**MDBK Cell line**
Cell number (CN)	↓ 14.1% ($p \leq 0.05$)
Nuclear Area (NA)	No effect
Nuclear Intensity (NI)	↑ 10.2% ($p \leq 0.001$)
Mitochondrial Mass (MM)	No effect
Mitochondrial Membrane Potential (MMP)	No effect

Treatment with binary mixture - Fumonisin B_1/Microcystin-LR

MDBK Cell Line





Cytotoxic effects of ternary mixture AFB_1/FB_1/MC-LR exposure

Cytotoxic effects of AFB_1/FB_1/MC-LR at 500 µg/L & 250 µg/L ≡ 1.6, 26.6 & 0.83 µg/kg/bw/d			
Endpoint	**HepG2 cell line**	**Caco-2 cell line**	**MDBK Cell line**
Cell number (CN)	↓ 13.0% ($p \leq 0.05$)	No effect	↓ 11.5% ($p \leq 0.05$)
Nuclear Area (NA)	↑ 11.3% ($p \leq 0.001$)	No effect	↑ 3.8% ($p \leq 0.05$)
Nuclear Intensity (NI)	No effect	No effect	↑ 6.8% ($p \leq 0.05$)
Mitochondrial Mass (MM)	↑ 11.3% ($p \leq 0.01$)	↑ 6.8% ($p \leq 0.01$)	↑ 5.0% ($p \leq 0.05$)
Mitochondrial Membrane Potential (MMP)	↑ 10.9% ($p \leq 0.01$)	No effect	No effect

Assessment of the interactive effects of the toxin mixtures (additive, antagonistic, synergistic)

Several experimental designs exist for the study of drug/contaminant interactions:

1. Comparison of theoretical expected values derived from single contaminant exposure studies with the observed values derived from co-exposure experiments (Weber et al., 2005)
2. Chou-Talalay (2006) method using isobologram analyses and quantification of the antagonism or synergy by calculation of a combination index (Le et al., 2018).

Expected mean values:
Binary mixture: mean (expected for $AFB_1 + FB_1$) = (mean (AFB_1) + mean (FB_1)) – 100%
Ternary mixture: mean ($AFB_1 + FB_1$ + MC-LR) = (mean ($AFB_1 + FB_1$) + mean (MC-LR)) -100%.

Expected standard error of the mean (SEM) values:
Binary mixture e: SEM (expected for AFB_1 + MC-LR) = $[(\text{SEM for } AFB_1)^2 + (\text{SEM MC-LR})^2]^{1/2}$
Ternary mixture: SEM (expected for $AFB_1 + FB_1$ + MC-LR) = $[(\text{SEM for } AFB_1 + FB_1)^2 + (\text{SEM for MC-LR})^2]^{1/2}$

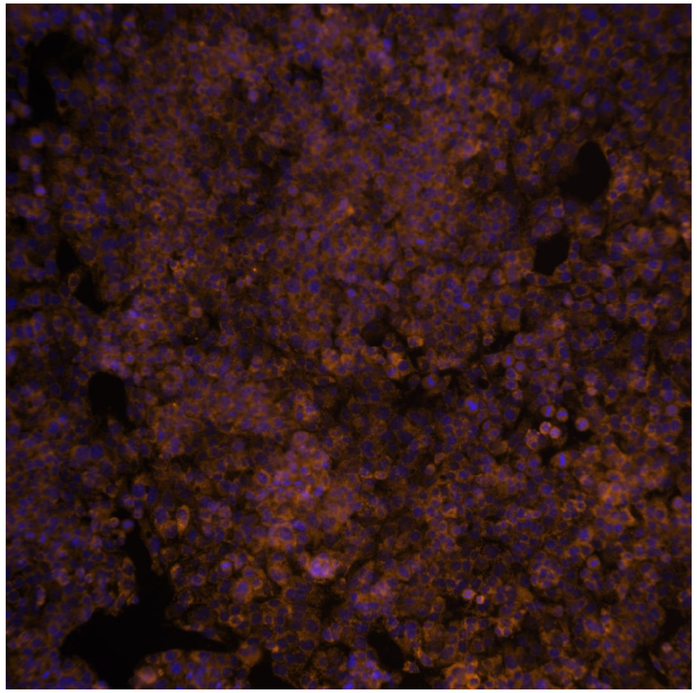
Assessment of the interactive effects of the toxin mixtures (additive, antagonistic, synergistic)

Additive effects:
Measured parameters are not significantly above or below the expected values.

Antagonism:
Measured parameters > expected values for cell number, nuclear intensity and mitochondrial membrane potential and < the expected values for nuclear area and mitochondrial mass.

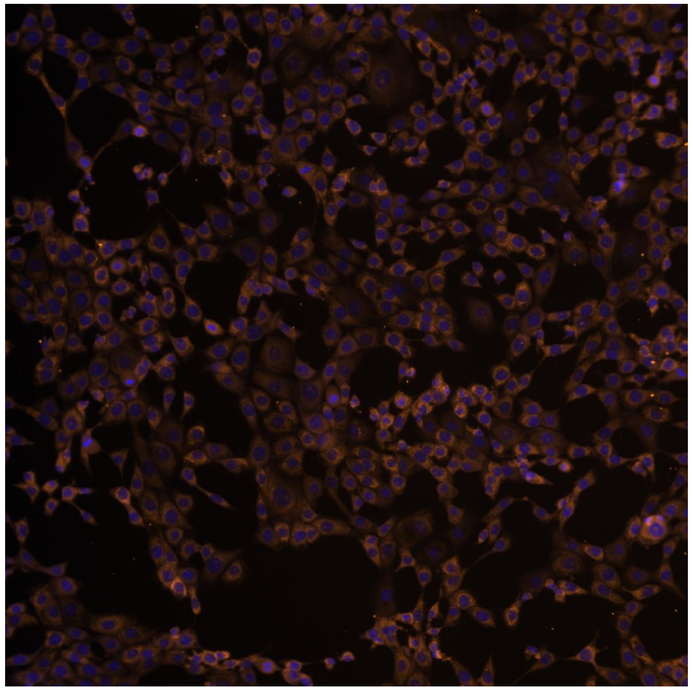
Synergy:
Measured parameters < expected values for cell number, nuclear intensity and mitochondrial membrane potential and > the expected values for nuclear area and mitochondrial mass.

HepG2 Cells



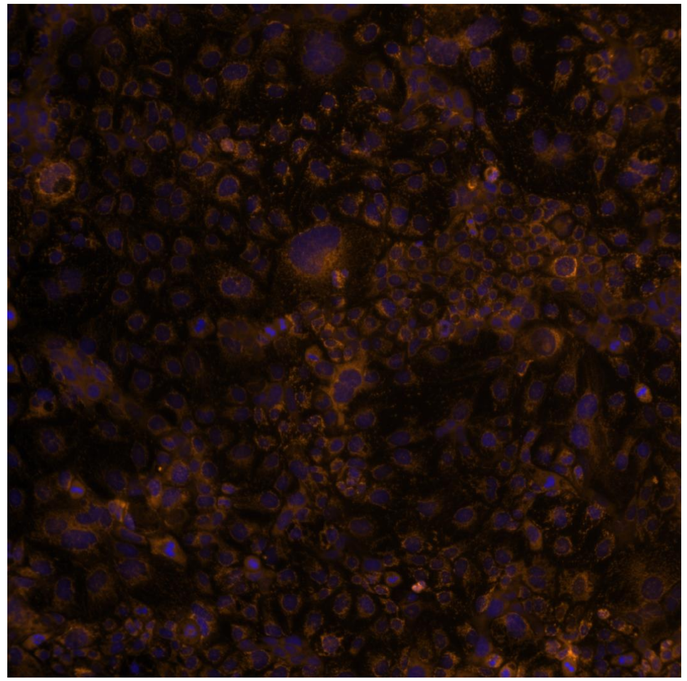
Endpoint	AFB_1/MC-LR (ng/ml)				
	0.1	2	10	100	500
	0.2	1	5	50	250
Cell Number	Antagonism	Antagonism	Antagonism	Antagonism	Antagonism
Nuclear Area					Antagonism
Nuclear Intensity					Synergy
Mitochondrial Mass					
Mitochondrial Membrane Potential					
			FB_1/MC-LR (ng/ml)		
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number	Antagonism				
Nuclear Area	Antagonism				Antagonism
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					
			AFB1/FB1/MC-LR (ng/ml)		
	0.1	2	10	100	500
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number	Antagonism	Antagonism			
Nuclear Area	Antagonism	Antagonism			
Nuclear Intensity		Synergy			
Mitochondrial Mass					
Mitochondrial Membrane Potential					

MDBK Cells



Endpoint	AFB_1/MC-LR (ng/ml)				
	0.1	2	10	100	500
	0.2	1	5	50	250
Cell Number					
Nuclear Area					Antagonism
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					
			FB_1/MC-LR (ng/ml)		
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number					
Nuclear Area					Antagonism
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					Synergy
			AFB1/FB1/MC-LR (ng/ml)		
	0.1	2	10	100	500
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number					
Nuclear Area					Antagonism
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					

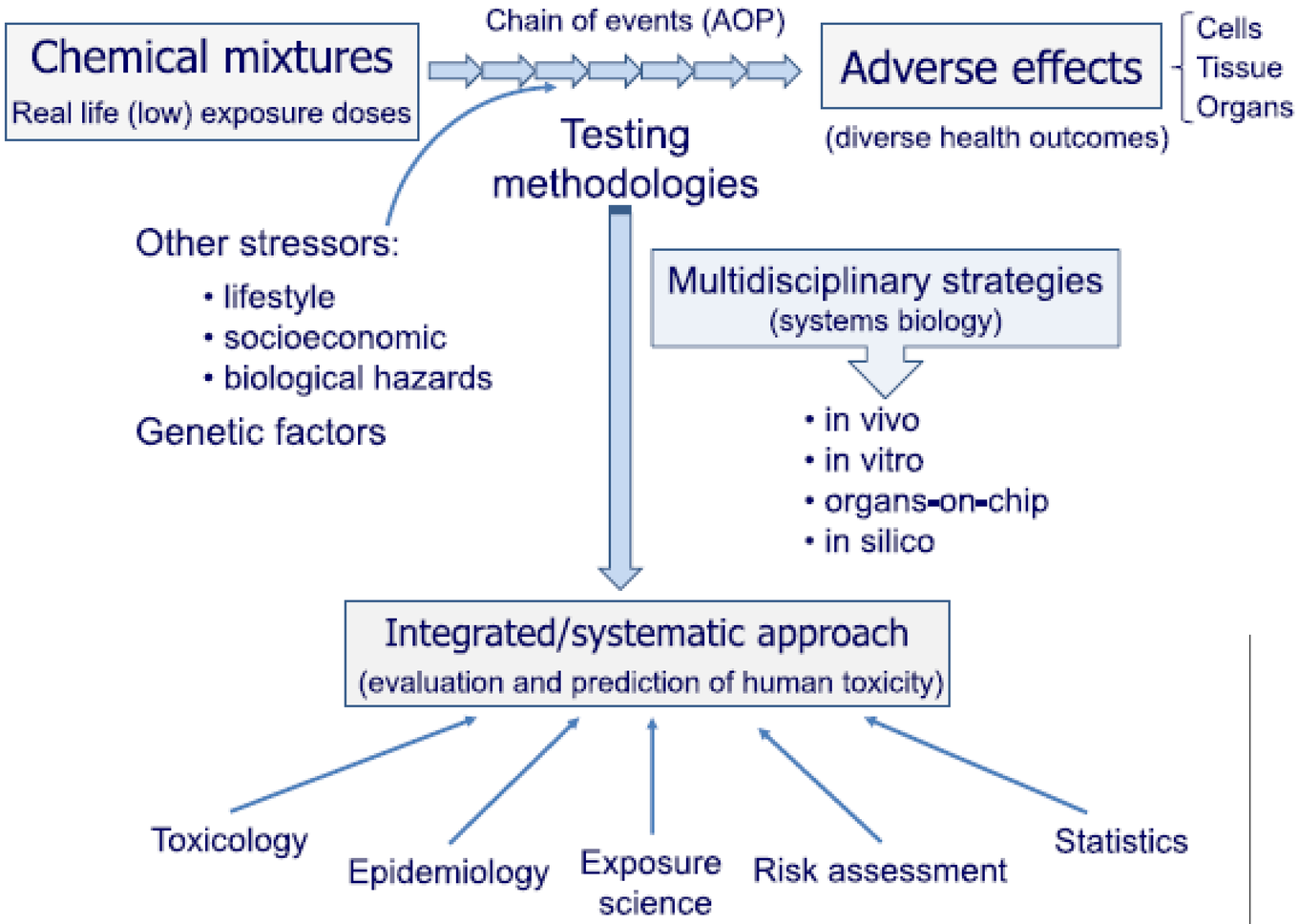
Caco-2 Cells



Endpoint	AFB_1/MC-LR (ng/ml)				
	0.1	2	10	100	500
	0.2	1	5	50	250
Cell Number					
Nuclear Area					
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					
			FB_1/MC-LR (ng/ml)		
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number					
Nuclear Area					
Nuclear Intensity					Synergy
Mitochondrial Mass					
Mitochondrial Membrane Potential					
			AFB1/FB1/MC-LR (ng/ml)		
	0.1	2	10	100	500
	200	1000	2000	4000	8000
	0.2	1	5	50	250
Cell Number					
Nuclear Area					
Nuclear Intensity					
Mitochondrial Mass					
Mitochondrial Membrane Potential					

Summary & conclusions

1. The cytotoxicity revealed for AFB_1, AFB_1/MC-LR, FB_1/MC-LR and AFB_1/ FB_1/MC-LR at the highest concentrations tested indicate there is potentially a real threat to populations if exposed to these concentrations.
2. The antagonism exhibited between AFB_1 and MC-LR at all concentrations tested contradicts the hypothesis that co-exposure to these toxins may exacerbate incidences of liver cancer BUT this may be as a result of the cell line tested in this study.
3. Synergistic effects were observed for some endpoints at the highest concentrations tested in this study (AFB_1/MC-LR and FB_1/MC-LR) – lacking exposure data for MC-LR.
4. At low concentrations synergy was exhibited for the ternary mixture (AFB_1/FB_1/MC-LR) (NI) equivalent to exposures of 0.006, 3.33 and 0.003 µg/kg/bw/d for AFB_1, FB_1 and MC-LR, respectively. Chronic exposure risk?
5. Further investigations are required using alternative cell lines and other additional endpoints such as ROS and caspase-3 activity.



Multiple hazard approach for long-term health outcomes and integrated approach of multiple lines of evidence for toxicity testing and prediction (AOP: adverse outcome pathways).

Food and Chemical Toxicology 103 (2017) 188–193

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

Human exposure to chemical mixtures: Challenges for the integration of toxicology with epidemiology data in risk assessment

Antonio F. Hernández [a, *], Aristidis M. Tsatsakis [b, **]

[a] Department of Legal Medicine and Toxicology, University of Granada School of Medicine, Granada, Spain
[b] Center of Toxicology Science & Research, Medical School, University of Crete, Heraklion, Crete, Greece

www.multicoop.eu

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 692195.

Disclaimer: *The information expressed in this presentation reflects the authors' views; the European Commission is not liable for the information contained therein.*