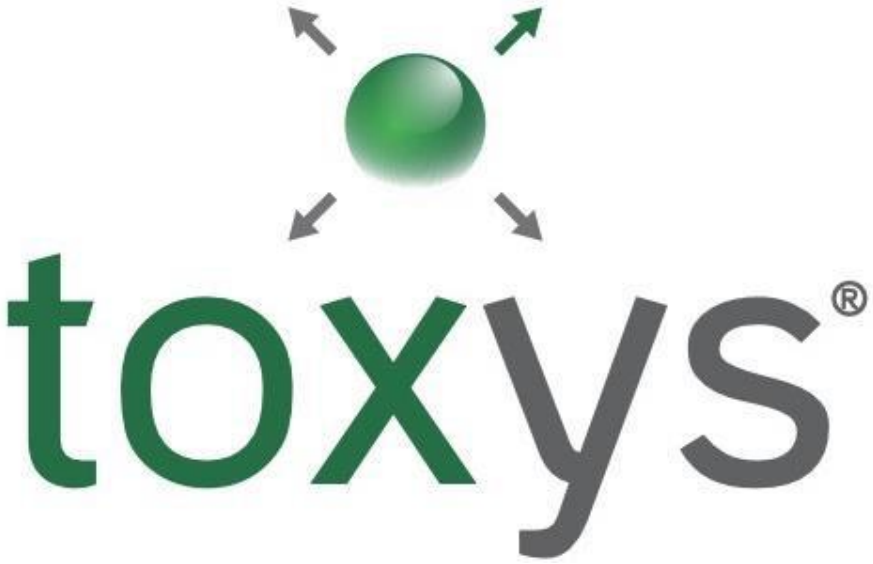

ToxProfiler Report

Final version

Toxys project code:	23063
Sponsor reference:	MA-RR-23-5046
Date:	2023 09 08

Toxys B.V.

Authors: B. ter Braak PhD, L. Loonstra-Wolters MSc., K. Elbertse MSc., R. Derr MSc.

Table of Content

Study administration

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Study director Study coordinator Study operators	B. ter Braak, PhD R. Derr, MSc. L. Loonstra-Wolters, MSc. K. Elbertse, MSc.
Sponsor:	Honeywell International 115 Tabor Rd. Morris Plains New Jersey 07950 United States
Sponsor contact:	Kamlesh Sodani e-mail: [redacted]@honeywell.com
Number of test articles:	8 Oxidative stress: CDDO-me Cell cycle stress: Cisplatin ER stress: Tunicamycin Autophagy: Amiodarone Ion stress: Cadmium Chloride Protein stress: Cadmium Chloride Inflammation: human TNF alpha Cytotoxicity: Cadmium Chloride
Date of testing:	Between August 15th, 2023 and August 25th, 2023
Number and sequence of tests:	- Concentration range finding with parental HepG2 cells. - ToxProfiler with 7 reporter lines with 3 independent biological replicates.
Data storage:	All primary test results are stored at least three years at Toxys and are available upon request. All the quantified data (.txt) per replicate/reporter can be shared upon request.

Executive summary

Objective: The goal of this project is to assess the toxic properties and investigate the toxicological mode-of-action of 8 compounds asked to test by Honeywell in the ToxProfiler assay.

Methods: The ToxProfiler assay consists of seven human reporter cell lines that were applied to study chemically induced activation of seven distinct stress pathways; oxidative stress (SRXN1-GFP), cell cycle stress (p21-GFP), ER stress (CHOP-GFP), autophagy (LC3-GFP), ion stress (MT1X-GFP), protein stress (HSPA1B-GFP) and inflammation (ICAM1-GFP). The differential induction of the GFP reporters as well as cytotoxicity of the tested compounds were determined by live cell confocal microscopy. Using our automated image segmentation and quantification pipelines, the stress biomarker induction was accurately quantified with a single cell resolution. A custom R-package "modelpod" was used to determine the lowest concentratio40n of the test compounds that resulting in a significant induction of the stress biomarker.

Results: The assay control compounds significantly induced their respective stress pathway. Cytotoxicity was observed for 6 out of the 8 test compounds in the tested concentration range. None of the ToxProfiler endpoints was induced by PFPrA and TFA Na in the tested concentration range. In Table 1 an overview of the ToxProfiler reporter results is presented. The POD plotter software was applied to determine if a stress pathway was significantly affected in a concentration dependent fashion. The same PoD approach was applied on the PI (cytotoxicity).

Table 1. Summary ToxProfiler assay results. Using the POD plotter software, significant concentration dependent effects are quantified. A red color indicates a significant induction of the corresponding readout, a green color indicates that there was no significant induction of the corresponding ToxProfiler readout in the tested concentration range.

Compound name	Oxidative stress	Cell cycle stress	ER stress	Auto-phagy	Ion stress	Protein stress	Inflam-mation	Cyto-toxicity
PFDA								
PFNA								
PFOA								
HFPO-DA (GenX)								
PfPeA								
HFBA								
PFPrA								
TFA Na								

Conclusions:

- The validity of the ToxProfiler assay was confirmed by treatment to various reference compounds and assessing the specificity of the different reporter cell lines.
- For all significant responses a point of departure (PoD) was determined.
- Cytotoxicity was observed for 6/8 compounds in the tested concentration range.
- ER stress was the most common (6/8) stress pathway to be induced.
- Autophagy was induced by 5/8 compounds.
- 4/8 compounds significantly induced cell cycle stress and protein stress.
- Oxidative stress was induced by 3/8 compounds.
- 2/8 compounds induced ion stress.
- None of the stress pathways was induced by PFPrA and TFA Na nor was cytotoxicity observed till 10mM.
- A clear correlation was found between (ToxProfiler) stress pathway activation and carbon length of the tested PFAS.

Background

Treatment to chemicals can lead to cell injury. Such cell injury initiates cell stress response signaling pathways. Strong activation of these pathways reflects the onset of toxicity. The ToxProfiler platform consists of a panel of seven reporter cell lines cultured as a 2D monolayer, in which biomarkers of several of such cell stress response pathways have been tagged with a fluorophore (GFP)[1,2]. With this platform we can dynamically observe activation of these stress pathways (upon chemical treatment) using a high throughput confocal imaging system[1,2,3]. The in-house developed automated image acquisition and process pipeline allows for a time efficient and high throughput screening[1,2,3,4]. The data is presented in concentration response plots from which point of departures (PoDs) are derived. The significant stress pathway responses are visualized all together in a hierarchical clustering with a heatmap, the resulting stress signaling fingerprints can then be easily compared, facilitating a chemical read-across based on these features. The analyses provide detailed mechanistic information. In figure 1 an overview of ToxProfiler readouts is presented.

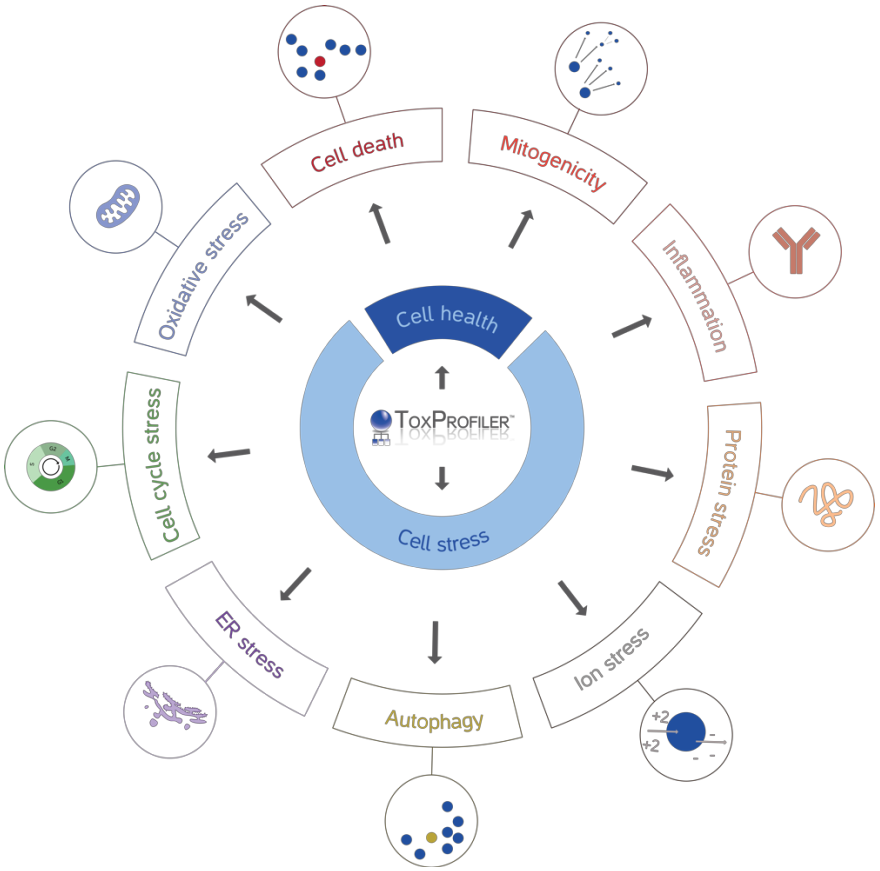


Figure 1. Overview of the stress pathways and biomarkers covered by the ToxProfiler reporter system.

[1] Wink S, et al. High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. Arch Toxicol. 2017 Mar;91(3):1367-1383.

[2] Wink S, et al. Dynamic imaging of adaptive stress response pathway activation for prediction of drug induced liver injury. Arch Toxicol. 2018 May;92(5):1797-1814.

[3] Niemeijer M, et al. Systems Microscopy Approaches in Unravelling and Predicting DILI. 2018 In: Drug-Induced Liver Toxicity. Springer New York, pp 611-625.

[4] Wink S, et al. Quantitative high content imaging of cellular adaptive stress response pathways... Chem Res Toxicol. 2014 Mar 17;27(3):338-55.

Test protocol

The ToxProfiler assay requires a standard cell culture facility, an automated high throughput confocal imager and a dedicated analysis desktop with proprietary software. The ToxProfiler reporter cells were maintained by culturing them in 2D on uncoated cell culture dishes with DMEM medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. During the chemical treatment and reporter analysis the ToxProfiler cells were cultured in 384-well imaging plates.

Cytotoxicity testing/concentration range finding
For chemical testing, first a concentration range finding was performed using parental HepG2 cells (strain HB-8065). Parental HepG2 cells were treated with 14 different concentrations, at a 1/3 log difference between each concentration, of the test substances. The maximum included concentration was 10 mM. However, for most of the compounds, solubility limited the maximal concentration in the test assay. For compounds dissolved in DMSO the maximal concentration was set to 0.4% of the maximal solubility. Cell death was measured as fraction of Propidium Iodide (PI, a stain for late apoptosis/necrosis) positive cells after 24 h treatment. Cadmium Chloride was included as a positive control and used as a validity check for the concentration range finding assay. Based on this data the appropriate concentrations were selected for the ToxProfiler reporter assay. When significant cell death (>15% PI positive cells) was observed, the lowest concentration at which cell death was observed was included as the highest concentration together with the subsequent 6 sub-cytotoxic concentrations. When no cell death was observed the 7 highest concentrations are included for the ToxProfiler assay.

ToxProfiler assay
The seven independent HepG2 BAC-GFP reporter cell lines were seeded with 8,000-9,000 cells per well in uncoated 384-well imaging plates. Two days after seeding, HOECHST (#33342, a DNA stain) was added. After one hour incubation, the HOECHST medium was removed and the compound treatment was performed in fresh cell culture medium containing the PI stain. Cells were imaged using an Operetta CLS imager at 24 h after treatment. Two images per well were taken (technical replicates) using four different channels (GFP; stress response activation, HOECHST: nuclei, Digital Phase Contrast: cytoplasm, PI: cell death). The GFP levels of the ToxProfiler reporters were quantified in their expected subcellular location (see table 2) and determined on a single cell level using automated segmentation pipelines (see figure 2). This resulted in concentration response graphs, point of departures (PoDs) and hierarchical clustering overviews for easy interpretation of the generated data.

Table 2. ToxProfiler reporter lines with thei4r corresponding positive control compounds and timepoints.

Reporter	Corresponding Pathway	+ Control compound	Subcellular localisation	Time point
HepG2-SRXN1-GFP	Oxidative stress	CDDO-me	Cytoplasmatic	24 h
HepG2-p21-GFP	Cell cycle stress	Cisplatin	Nuclear	24 h
HepG2-CHOP-GFP	ER stress	Tunicamycin	Nuclear	24 h
HepG2-LC3-GFP	Autophagy	Amiodarone	Cytoplasmatic foci	24 h
HepG2-MT1X-GFP	Ion stress	Cadmium Chloride	Cytoplasmatic	24 h
HepG2-HSPA1B-GFP	Protein stress	Cadmium Chloride	Cytoplasmatic	24 h
HepG2-ICAM1-GFP	Inflammation	hTNFα	Cytoplasmatic membrane	24 h

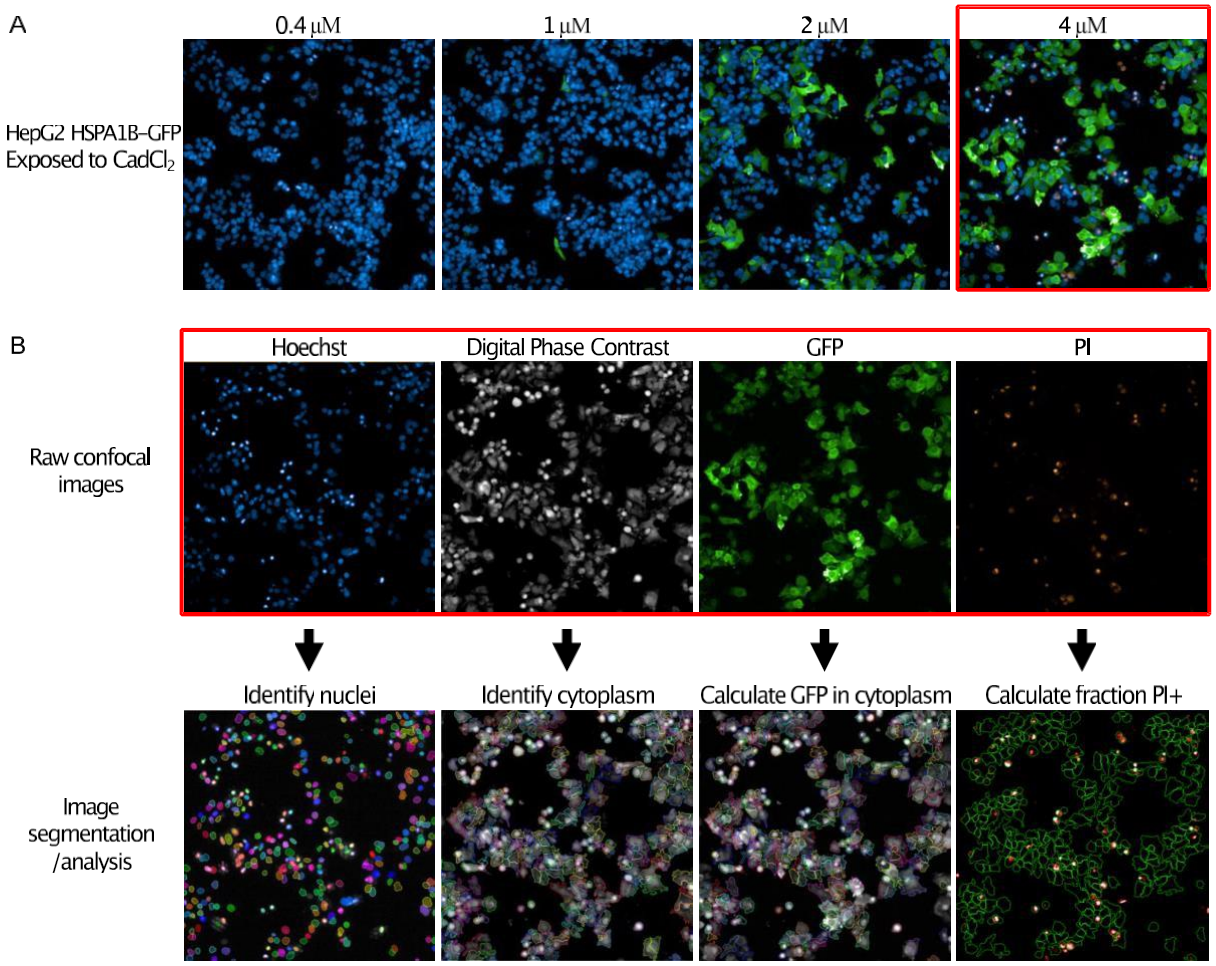


Figure 2. Automated image segmentation and quantification description. A) Example of a concentration response with the raw confocal images of Cadmium Chloride treatment on HepG2-HSPA1B-GFP. B) Example of the automated image segmentation and analysis pipeline. This process is performed on all images.

Positive reference treatments with CDDO-me (oxidative stress), cisplatin (cell cycle stress), tunicamycin (ER stress), amiodarone (autophagy), cadmium chloride (ion stress, protein stress and cytotoxicity), human TNF alpha (inflammation) were included in each plate of the respective ToxProfiler reporter, which allowed for plate-wise min-max normalization. Solvent concentrations were kept constant over the concentration range and never exceeded 0.4% for DMSO.

All data was corrected for potential auto-fluorescence. For which the parental HepG2s were exposed to the same conditions (compounds/concentrations). The mean fluorescence caused by the compound was then subtracted from the ToxProfiler results of the respective compound/concentration. This approach is only feasible when the auto-fluorescence do not disturb the image segmentation process.

This experiment was conducted as a non-GLP study, however general principles to conduct proper scientifically correct *in vitro* experiments were adhered to, and particular care was taken to proper handling of test compound (stock) solutions to prevent/minimize degradation of the test articles, based on instructions/compound information from the suppliers.

Data analysis

For the analysis of the images, a Harmony 5.1® based image segmentation and analysis pipeline was applied that returns the GFP/PI induction in a specific sub-cellular localization and per individual cell (Figure 2). Data quality checks are performed in the harmony software and further processing of the data was performed using a pipeline of custom R-scripts. To be able to compare the GFP reporter activation for different compounds from one screen to another, a min-max plate-wise normalization step was performed using the positive and negative control samples that were included on every plate. Point of departures (PoDs) were calculated using a custom R-package "modelpod". Concentration response curves were fitted with *loess* regression. The intersect of this fitted curve with the mean of the solvent control plus two times the standard deviation of the concentration-response curve regression gives a value corresponding to the lowest concentration at which we observe a significant (positive or negative) effect (the PoD).

Test criteria

The ToxProfiler assay is considered to have a positive response when a PoD was calculated. The PoDs were determined not only for reporter responses, but also for PI (cytotoxicity). For each reporter a positive reference control was included, the respective GFP induction of this compound should have a value of at least 2x the standard deviation above the background GFP response induced by the solvent.

Concentration-range finding results

Test parameters

- A single 24 h continuous exposure to the parental HepG2 cell line.
- Propidium iodide (PI) dye was used to quantify fraction of dead cells.
- Chemical information and included concentrations on all the test and reference compounds can be found in table 3 and 4
- PFDA and PFNA precipitated in assay medium in the tested concentrations.
- PFDA, PFNA and PFOA showed auto-fluorescence in the HOECHST channel in only the highest tested concentrations.
- The observation of compound precipitation and auto-fluorescence did not affect the concentration selection of these compounds. In all cases cytotoxicity determined the selected concentrations
- Concentration response plots for the cytotoxicity can be found in figure 3.
- Assay performed following standard protocols.

Table 3. Chemical information and concentration ranges for the concentration range finding experiments.

Compound name	Full name	CAS#	Carbon length	MW	Provider	Product#
PFDA	Perfluorodecanoic acid	335-76-2	C10	514.08	Sigma-Aldrich	177741-5G
PFNA	Perfluorononanoic acid	375-95-1	C9	464.08	Sigma-Aldrich	394459-5G
PFOA	Perfluorooctanoic acid	335-67-1	C8	414.07	Sigma-Aldrich	171468-5G
HFPO-DA (GenX)	2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid	13252-13-6	C6	330.05	Enamine	EN300-18553200
PfPeA	Perfluoropentanoate	2706-90-3	C5	264.05	Sigma-Aldrich	396575-5ML
HFBA	Heptafluorobutyric acid	375-22-4	C4	214.04	Sigma-Aldrich	164194-5G
PFPrA	Perfluoropropionic acid	422-64-0	C3	164.03	Sigma-Aldrich	245917-10G
TFA Na	Sodium trifluoroacetate	2923-18-4	C2	136.01	Sigma-Aldrich	132101-25G

Table 4. Chemical information and concentration ranges for the concentration range finding experiments.

#	Compound reference (name/number)	Solvent	Stock conc. (in mM)	Lowest conc. (in μM)	Highest conc. (in μM)	# of Conc.	Difference between conc.
1	PFDA	DMSO	2500	0.46	10000	14	1/3 log
2	PFNA	DMSO	2500	0.46	10000	14	1/3 log
3	PFOA	DMSO	2500	0.46	10000	14	1/3 log
4	HFPO-DA (GenX)	DMSO	2500	0.46	10000	14	1/3 log
5	PfPeA	DMSO	2500	0.46	10000	14	1/3 log
6	HFBA	DMSO	2500	0.46	10000	14	1/3 log
7	PFPrA	DMSO	2500	0.46	10000	14	1/3 log
8	TFA Na	DMSO	2500	0.46	10000	14	1/3 log
Internal reference compound*							
A	Cadmium Chloride	DMSO	50	0.01	200	14	1/3 log

***supplied by Toxys. Reference compound was obtained from Sigma-Aldrich.**

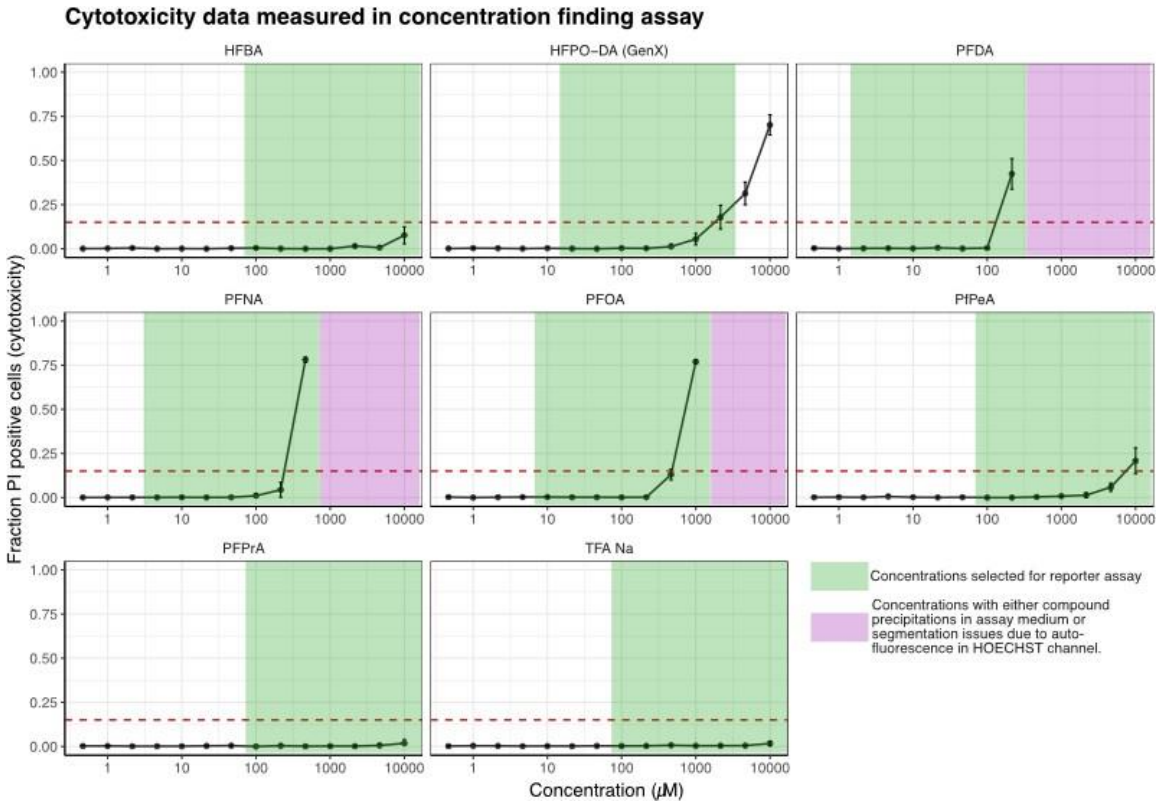


Figure 3. Cytotoxicity measured in 14 concentrations for the concentration range finding experiments. Normalized concentration response plots of cell death data of all compounds included in this project at timepoint 24h as measured with the PI stain in the parental HepG2 (wild type) line. The black dots represent the mean over two technical replicates with error bars representing the standard deviation. The red line indicates the used threshold of cytotoxicity; 15% PI positive cells. The green shaded area represents the 7 selected concentrations for the reporter assay. The pink shaded area shows the concentrations at which either precipitations in assay medium were observed or the compound concentration that causes issues with the HOECHST segmentation.

ToxProfiler assay results

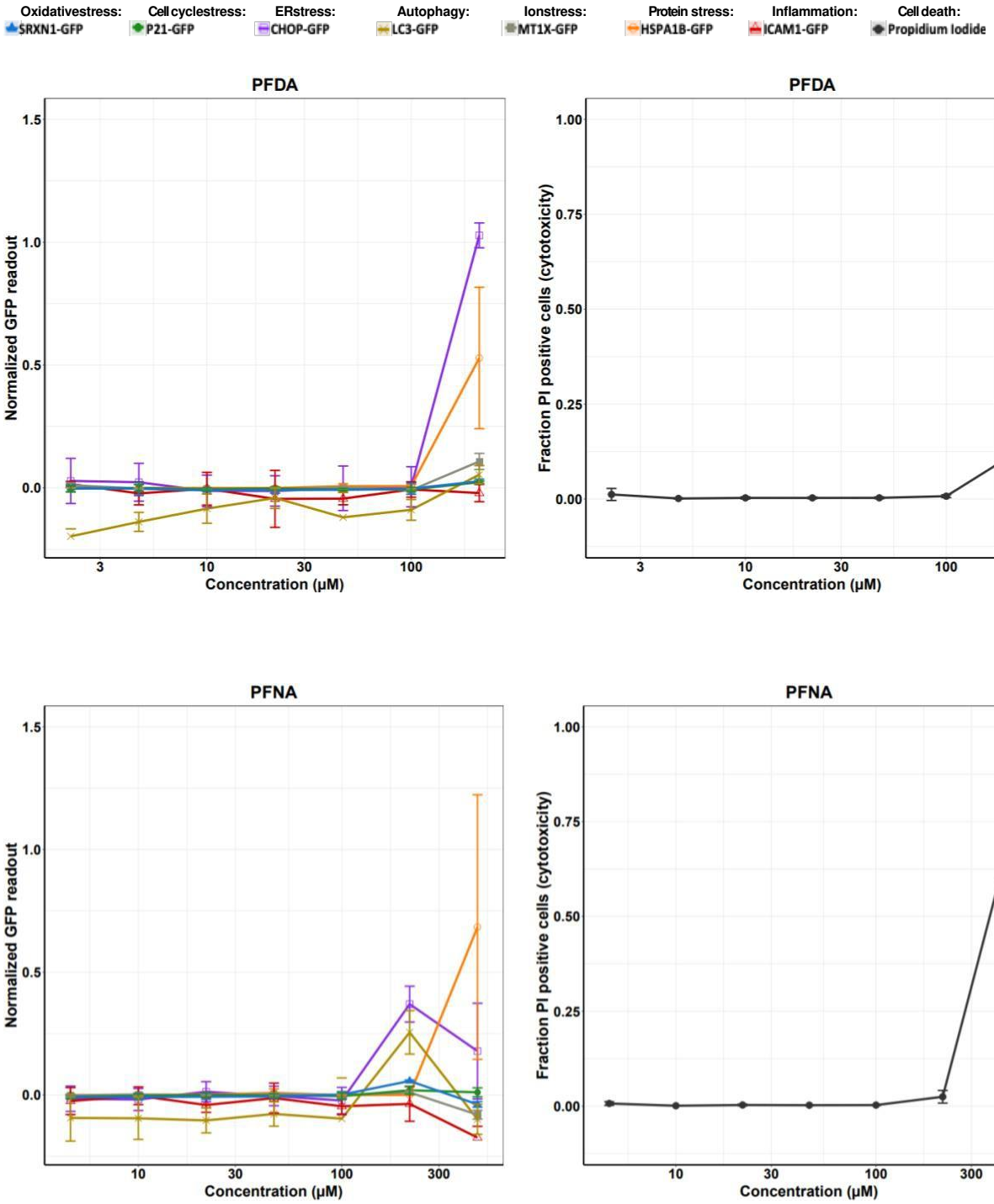
Test parameters

- A single 24 h continuous exposure to the 7 independent ToxProfiler reporter cell lines.
- Information on the tested concentrations can be found in table 5.
- Number of independent biological replicates: 3.
- Number of technical replicates (for each biological replicates): 2.
- Quantified GFP- and cytotoxicity- concentration response curves can be found in figure 4.
- A heatmap with a hierarchical clustering with all the ToxProfiler data can be found in figure 5.
- Points of departures (PoDs) from ToxProfiler endpoints are listed in table 6.
- Assay performed following standard protocols.

Table 5. Concentration ranges for the ToxProfiler reporter assay.

#	Compound name	Stock	Lowest conc.	Highest conc.	# of Conc.	Difference between conc.
1	PFDA	2.5 M	2.15 µM	215 µM	7	1/3 log
2	PFNA	2.5 M	4.64 µM	464 µM	7	1/3 log
3	PFOA	2.5 M	10 µM	1000 µM	7	1/3 log
4	HFPO-DA (GenX)	2.5 M	21.54 µM	2154 µM	7	1/3 log
5	PfPeA	2.5 M	100 µM	10000 µM	7	1/3 log
6	HFBA	2.5 M	100 µM	10000 µM	7	1/3 log
7	PFPrA	2.5 M	100 µM	10000 µM	7	1/3 log
8	TFA Na	2.5 M	100 µM	10000 µM	7	1/3 log
Internal reference compounds*						
1	CDDO-me	0.125 mM	0.03 nM	0.5 µM	7	5-fold
2	Cisplatin	3.33 mM	0.1 µM	10 µM	7	1/3 log
3	Tunicamycin	2.5 mM	0.1 µM	10 µM	7	1/3 log
4	Amiodarone	5 mM	0.2 µM	20 µM	7	1/3 log
5	Cadmium Chloride	1.08 mM	0.04 µM	4.31 µM	7	1/3 log
6	human TNF alpha	10 mg/ml	0.6 pg/ml	10 ng/ml	7	5-fold

***Supplied by Toxys. All reference compounds are obtained from Sigma-Aldrich except for human TNF alpha. Human TNF alpha was purchased at R&D systems.**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P21-GFP
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CHOP-GFP
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LC3-GFP
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Protein stress:
HSPA1B-GFP
Inflammation:
ICAM1-GFP
Cell death:
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PFDA
Normalized GFP readout
Concentration (µM)
PFDA
Fraction PI positive cells (cytotoxicity)
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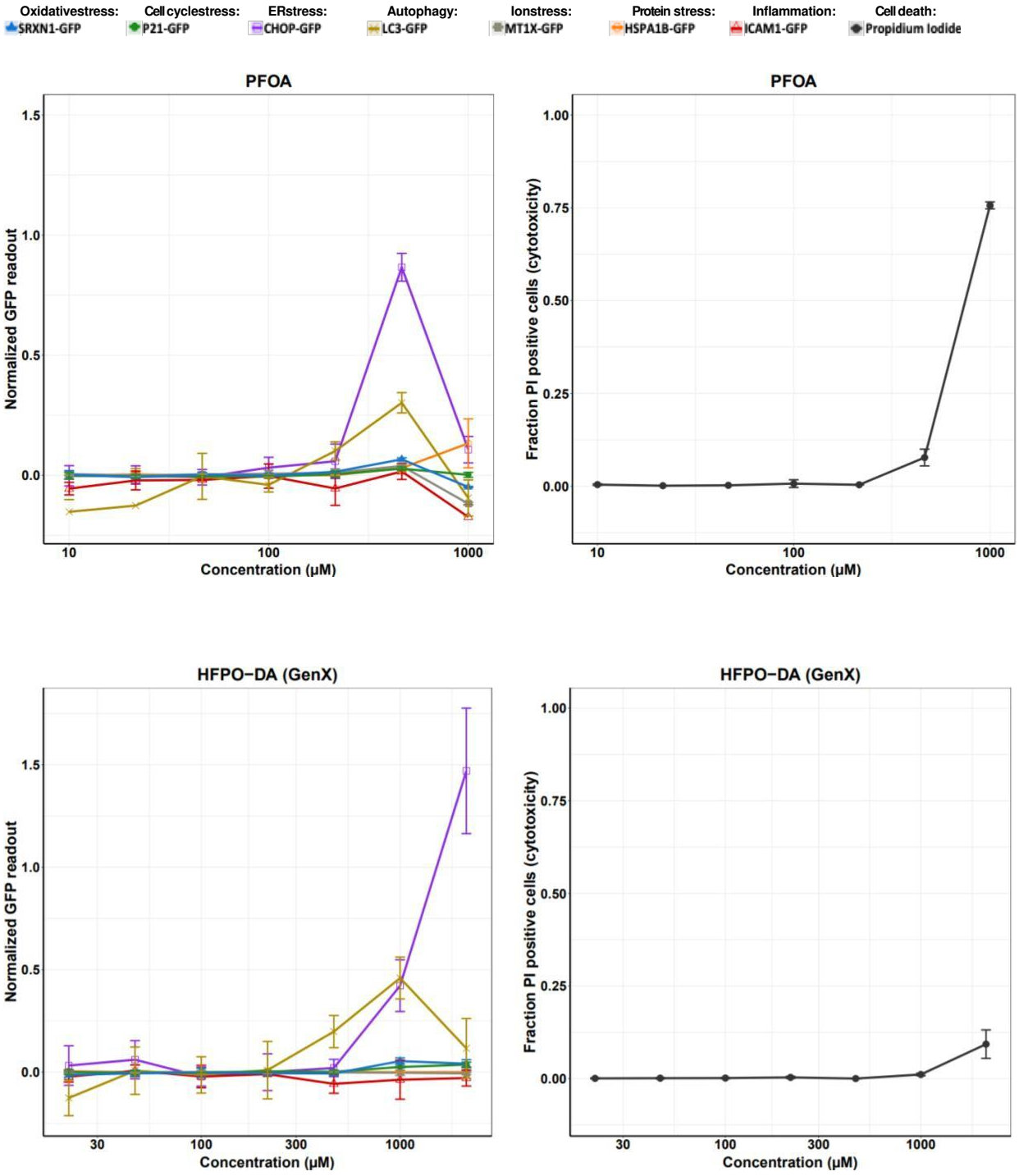
Concentration (µM)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PFOA
Normalized GFP readout
Concentration (µM)
PFOA
Fraction PI positive cells (cytotoxicity)
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HFPO−DA (GenX)
Fraction PI positive cells (cytotoxicity)
Concentration (µM)


Oxidativestress:
SRXN1-GFP
Cell cyclestress:
P21-GFP
ERstress:
CHOP-GFP
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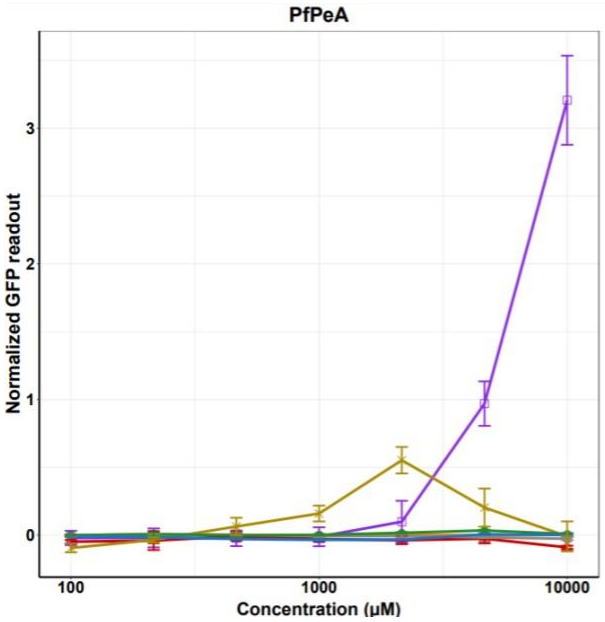
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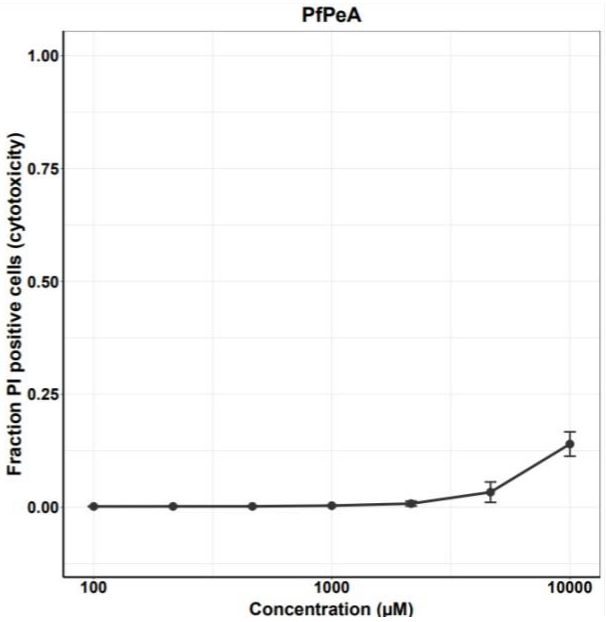
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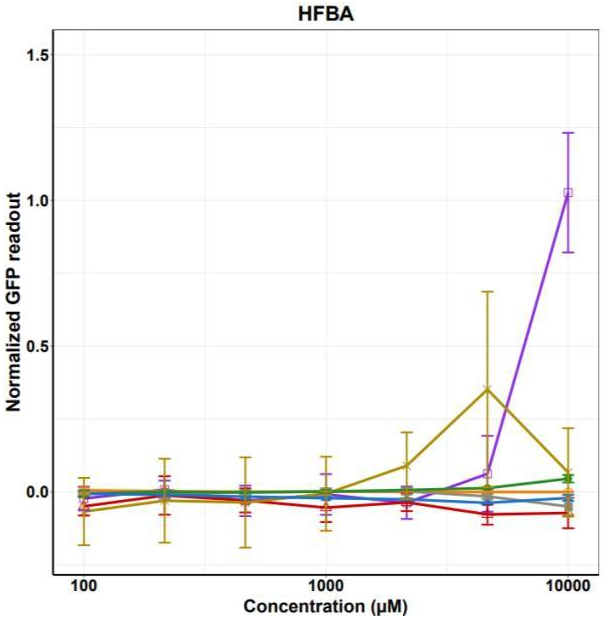
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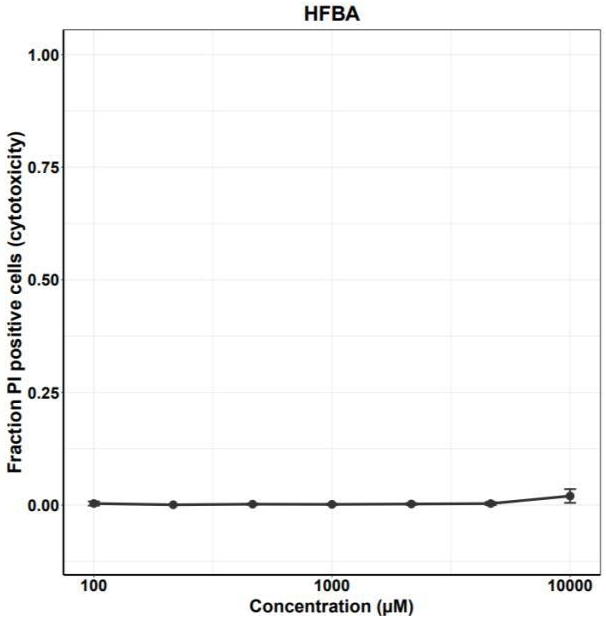
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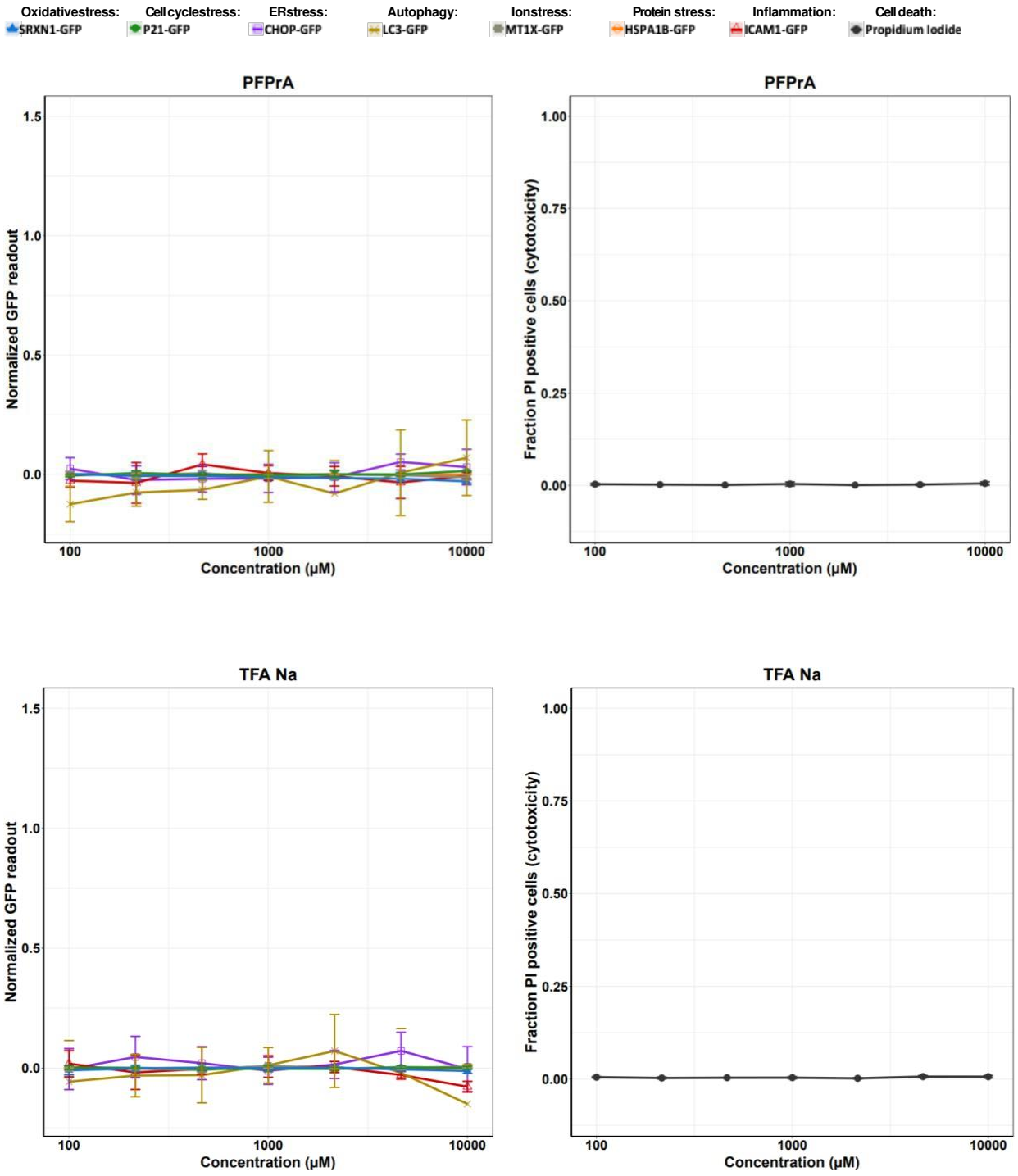


Figure 4. Concentration response plots of the 7 ToxProfiler GFP reporters for each compound. The graph on the left represents the quantified and normalized GFP responses in which each color represents one of the 7 ToxProfiler GFP reporters. The curves on the right represents cell death data, in which the black dots represent the average fraction of cell death (PI positive cells) over three biological replicates. The error bars represent the standard deviation over the three biological replicates.

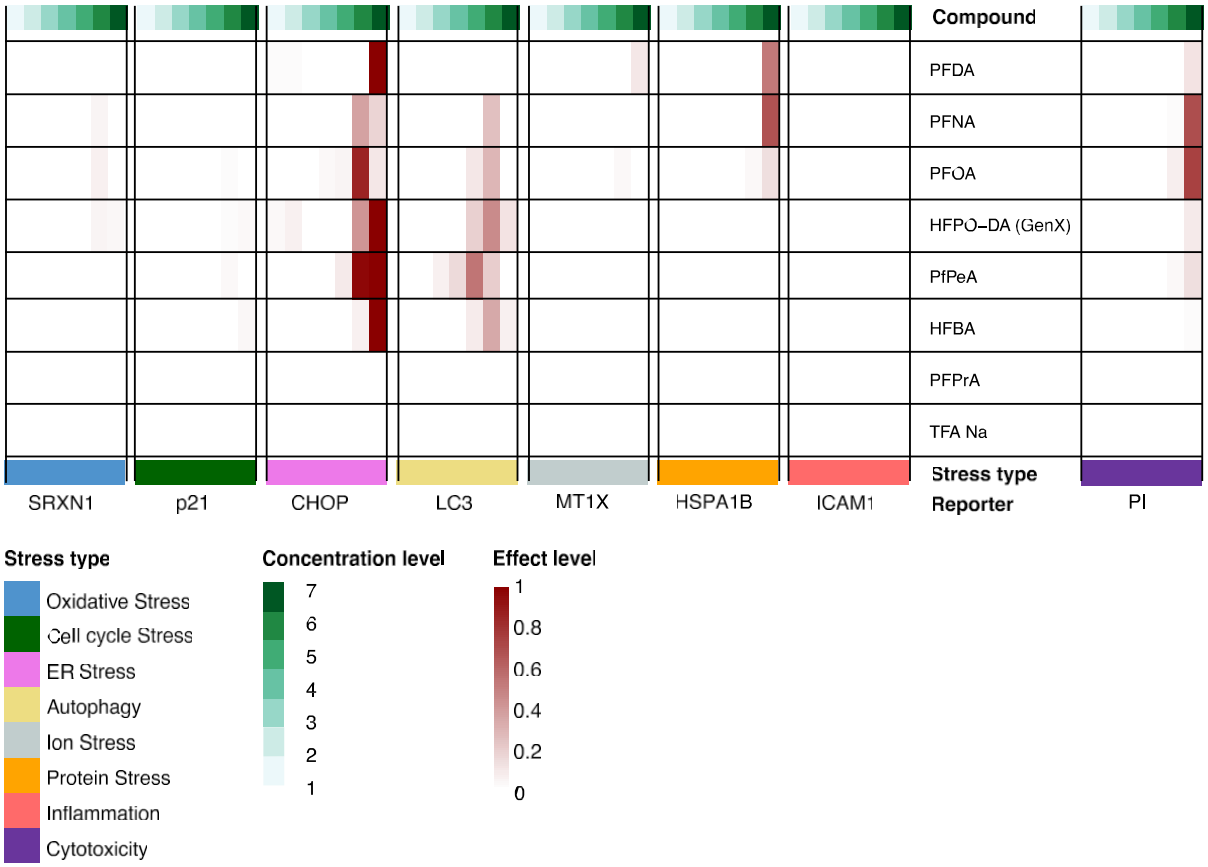


Figure 5. Heatmap of all the quantified and normalized data. Each row represents a compound with 7 concentration levels in which 7 represents the highest concentration. The different columns represent the different readouts: the 7 ToxProfiler reporters and PI. The red gradient indicates an induction of the integrated GFP intensity/PI signal as compared to the solvent control. A white color indicates similar levels as the control. Note that the different chemicals are not screened on an equimolar level but rather on an equitoxic level. The chemicals are ordered based on their carbon length with the C10 on top.

Table 6. Point of departures (PoDs) per reporter with cytotoxicity information for all compounds. For each compound the lowest PoD was marked with a ***bold/italic/underlined*** font to indicate the primary response. If the difference between the lowest POD and the successive POD of that chemical was <2fold also the second POD was highlighted. PoDs are shown in µM. An empty well indicates that the compound at the tested concentration range did not significantly affect that specific readout.

Compound Name	Carbon Length	ToxProfiler (TP) stress pathway							Cytotoxicity	TP Rank
		Oxidative SRXN1	Cell cycle P21	ER CHOP	Autophagy LC3	Ion MT1X	Protein HSPA1B	Inflammation ICAM1	PI	
PFDA	C10			***134***		***151***	***153***		151	1
PFNA	C9	***150***		***150***	***150***		353		251	2
PFOA	C8	***215***	450	***215***	***300***	***300***	450		300	3
HFPO-DA	C6	650	1000	700	***300***				1379	4
PfPeA	C5		4642	3000	***1000***				2200	5
HFBA	C4		7000	6000	***2154***				7700	6
PFPrA	C3									7-8
TFA Na	C2									7-8

Conclusions and discussion

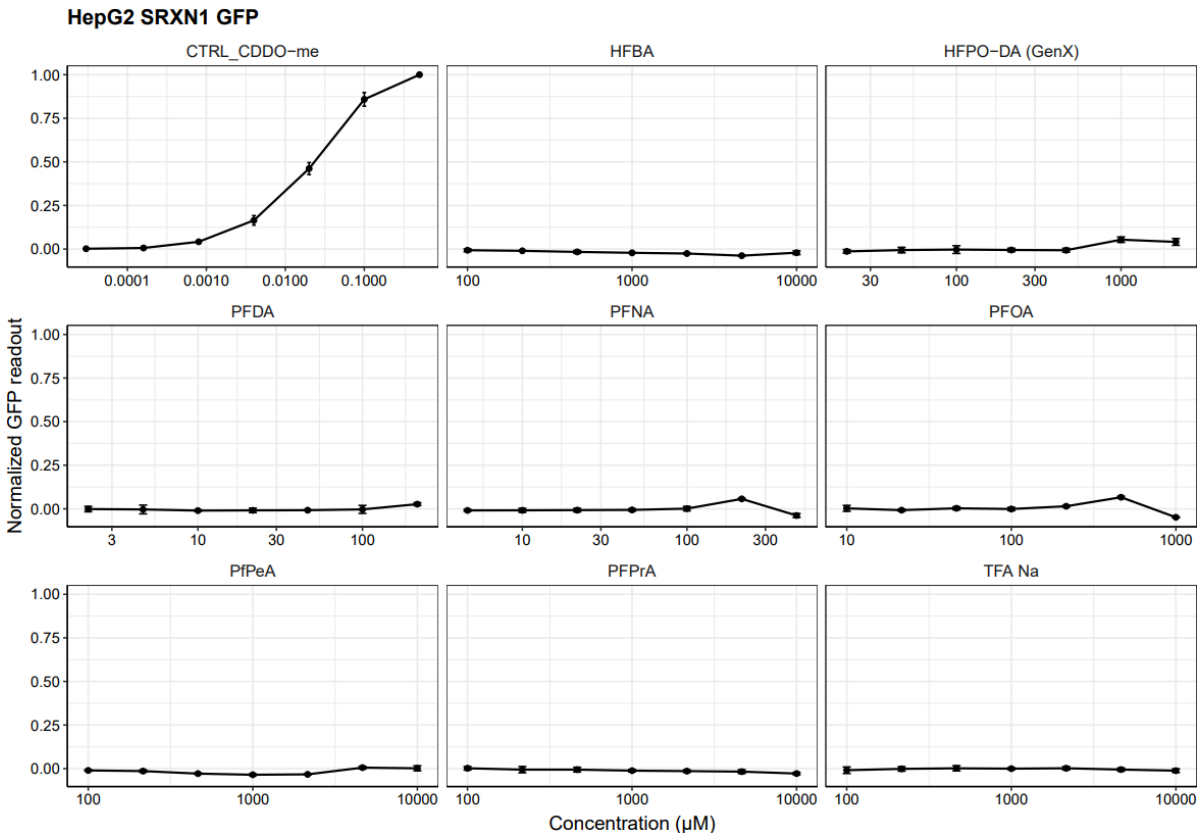
Overall conclusions per compound

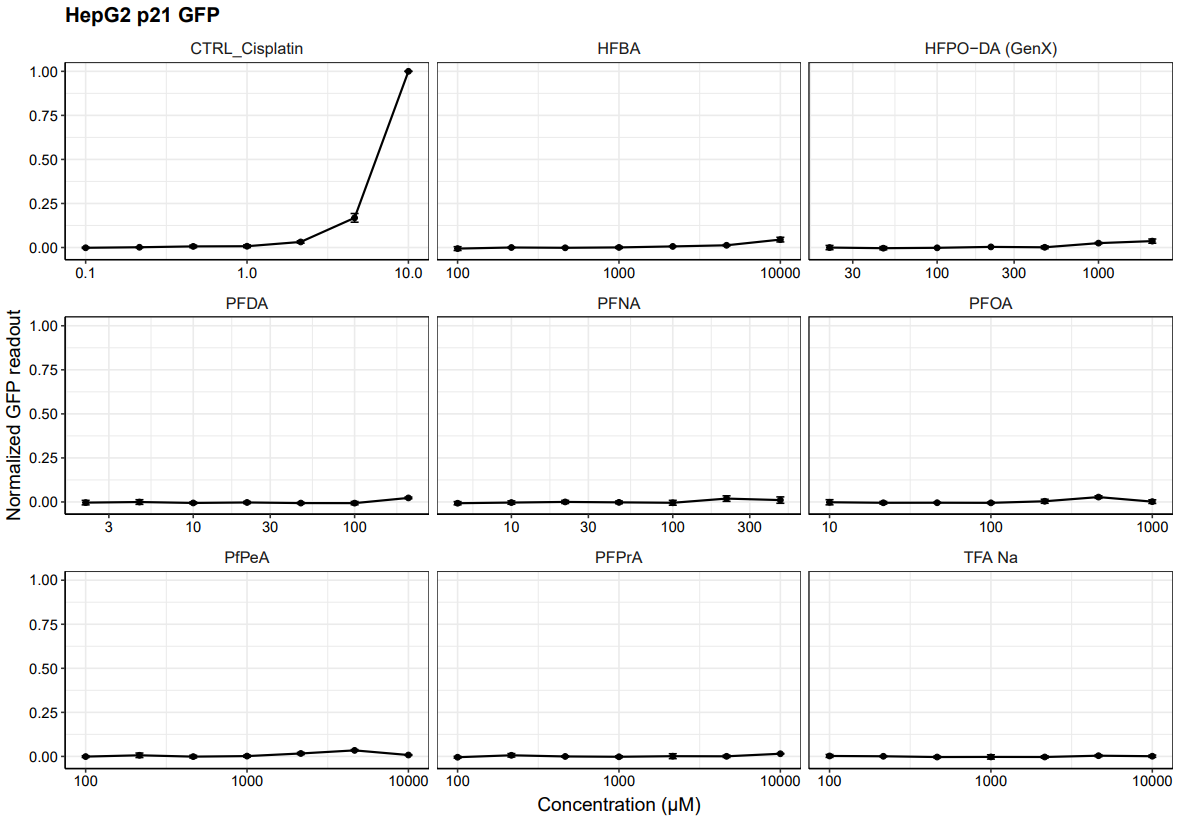
In table 7 an overall conclusion per compound is presented that is based on cell count. PI and GFP reporter data. Only when the effect was significant (and thus a PoD was determined) the effect was listed in the overall conclusion table.

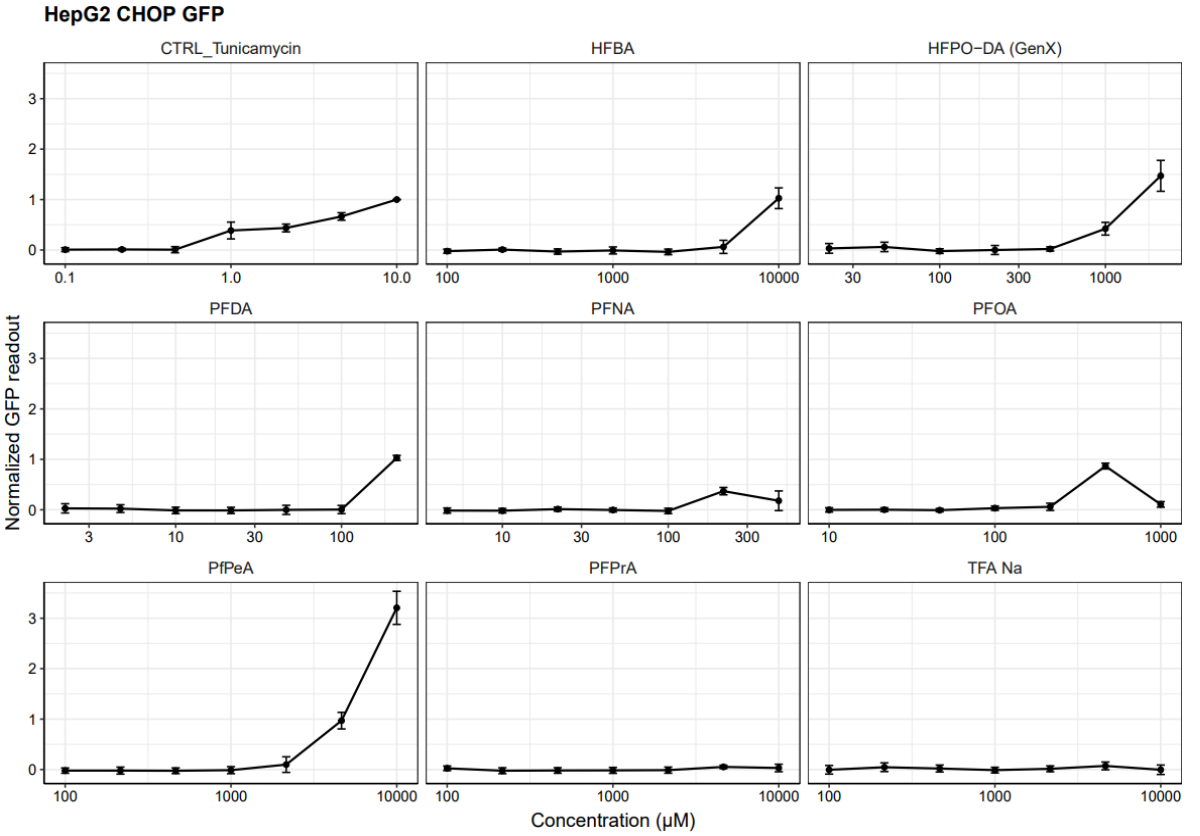
Table 7. Overview of the primary toxic properties of the tested compounds. The conclusions are based only on the ToxProfiler assay results and only on the tested concentration range (table 4).

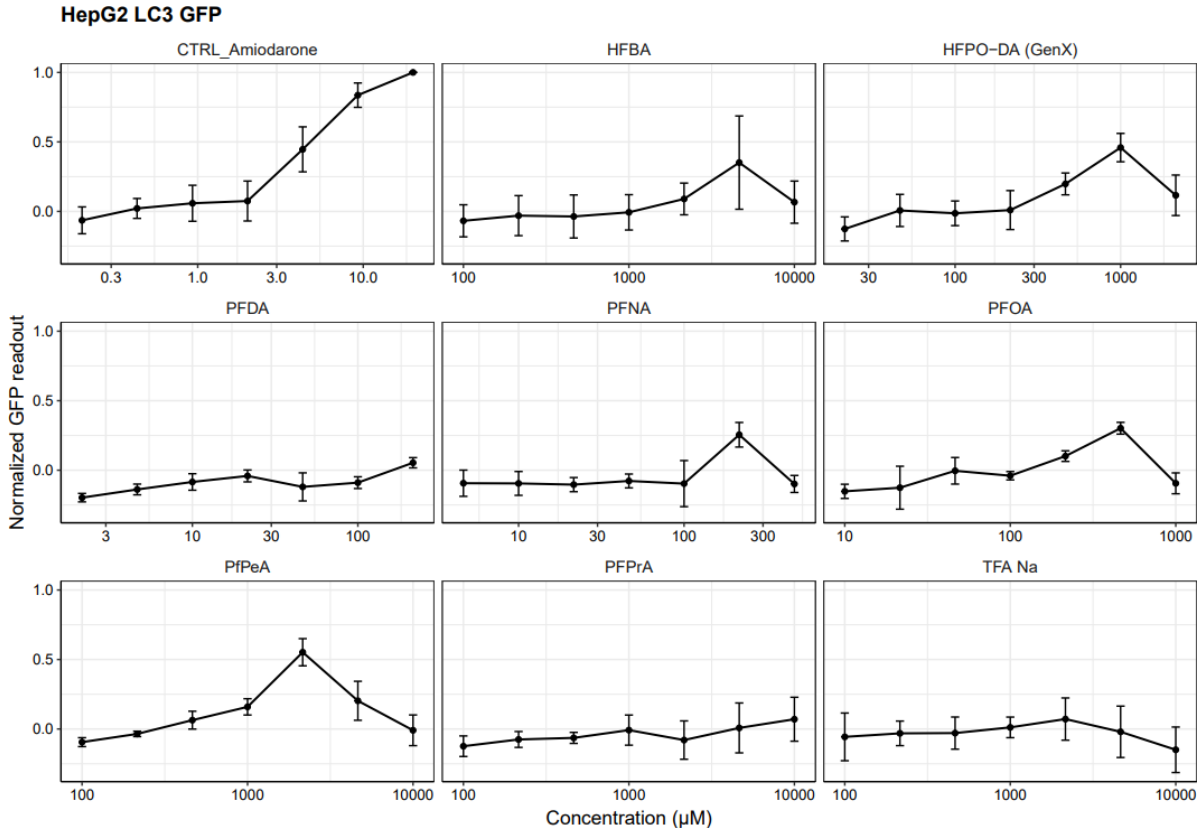
Compound name	Brief overall conclusion
PFDA	Compound induces ER stress in a concentration dependent manner at ≥134 µM. Ion stress, protein stress and cytotoxicity are induced at ≥150µM.
PFNA	Compound induces autophagy, oxidative stress and ER stress in a concentration dependent manner at ≥150µM. Protein stress is induced at ≥353µM. Cytotoxicity is induced at a concentration of ≥251µM.
PFOA	Compound induces oxidative stress and ER stress in a concentration dependent manner at ≥215µM. Ion stress and autophagy are induced at ≥300µM. Both cell cycle stress and protein stress are observed at ≥450µM. Cytotoxicity is induced at a concentration of ≥300µM.
HFPO-DA (GenX)	Compound affects the autophagy response in a concentration dependent manner at ≥300µM. Oxidative stress, ER stress and cell cycle stress are induced at ≥650µM, ≥700µM and ≥1000µM, respectively. Cytotoxicity is induced at a concentration of ≥1379µM.
PfPeA	Compound affects the autophagy response in a concentration dependent manner at ≥1000µM. ER stress and cell cycle stress are induced at ≥3000µM and ≥4642µM, respectively. Cytotoxicity is induced at a concentration of ≥2200µM.
HFBA	Compound affects the autophagy response in a concentration dependent manner at ≥2154µM. ER stress and cell cycle stress are induced at ≥6000µM and ≥7000µM, respectively. Cytotoxicity is induced at a concentration of ≥7700µM.
PFPrA	This compound did not induce any of the ToxProfiler endpoints in the tested concentration range. Also, no cytotoxicity was found in the tested concentrations (up to 10mM).
TFA Na	This compound did not induce any of the ToxProfiler endpoints in the tested concentration range. Also, no cytotoxicity was found in the tested concentrations (up to 10mM).

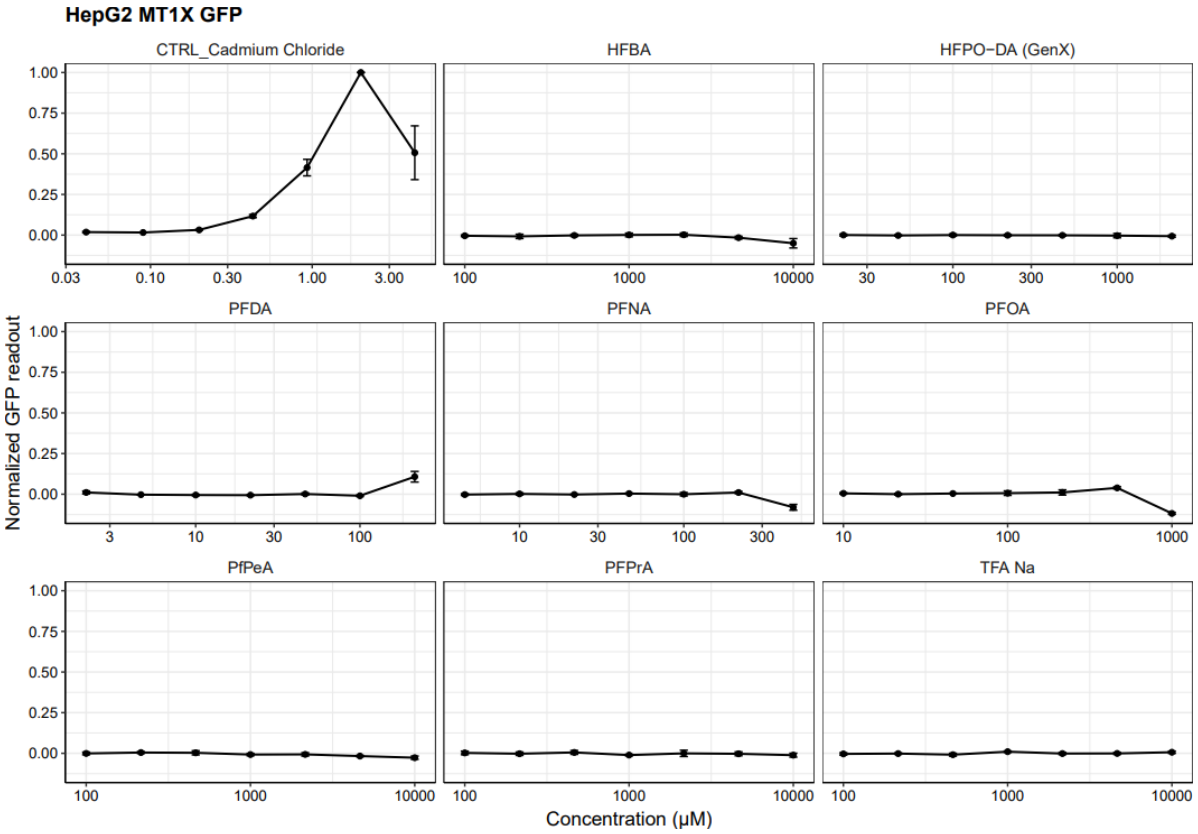
Annex I: Concentration response curves per reporter/chemical

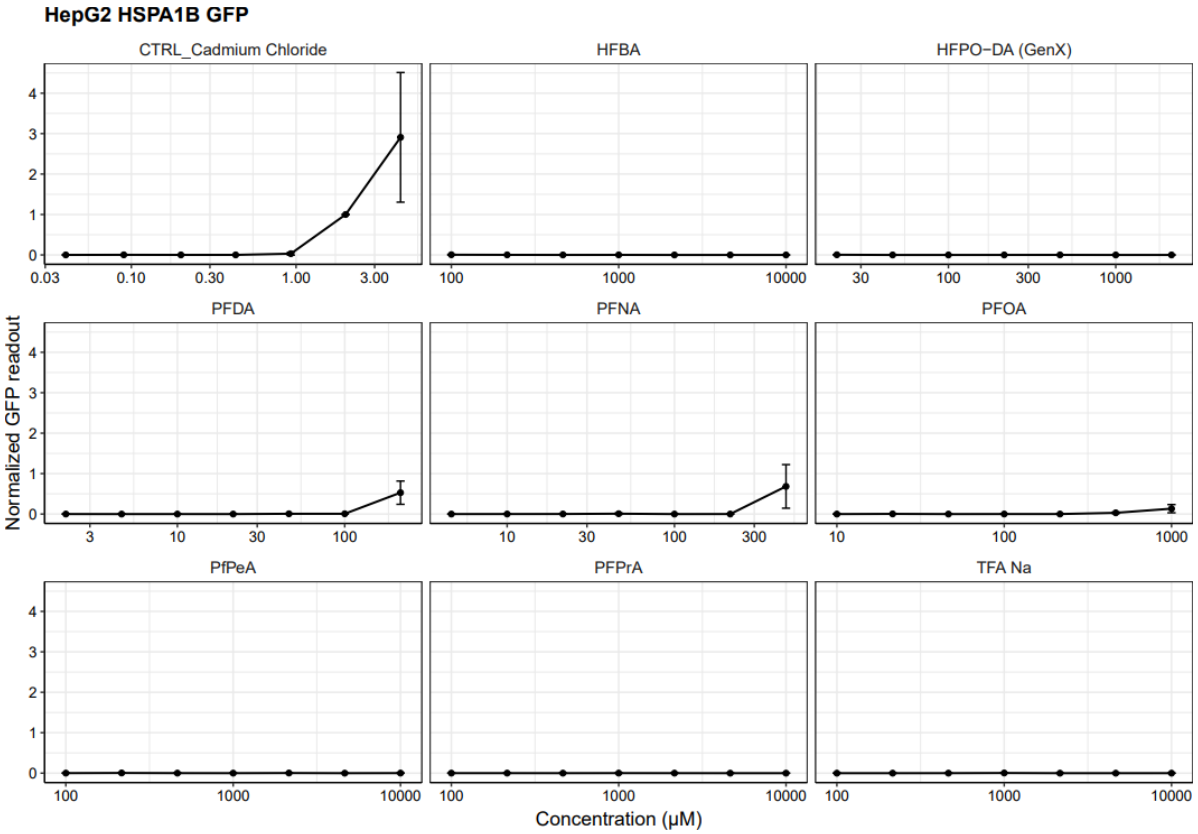


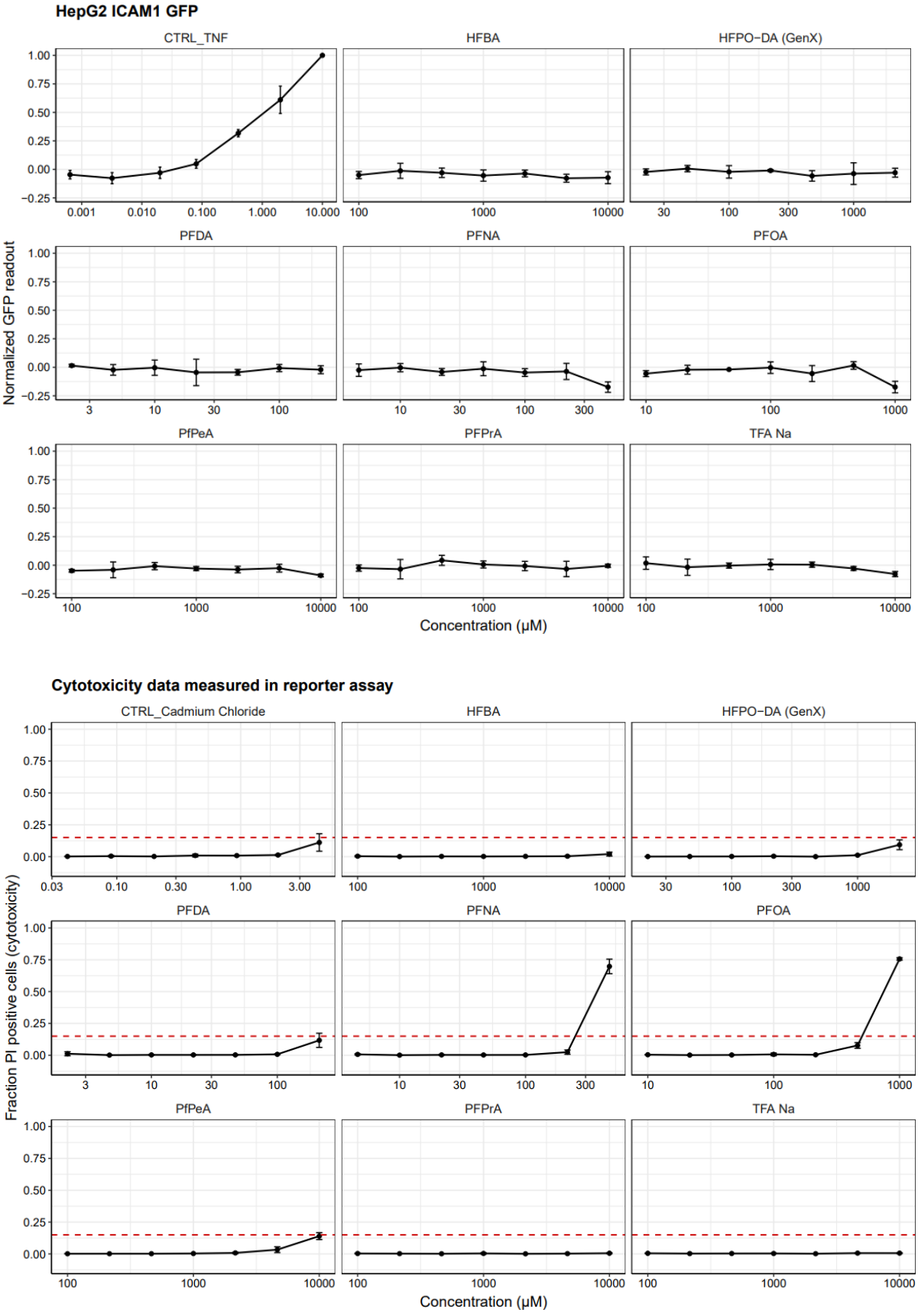



HepG2 CHOP GFP
CTRL_Tunicamycin
HFBA
HFPO−DA (GenX)
PFDA
PFNA
PFOA
PfPeA
PFPrA
TFA Na
Normalized GFP readout
Concentration (μM)


HepG2 LC3 GFP
CTRL_Amiodarone
HFBA
HFPO−DA (GenX)
PFDA
PFNA
PFOA
PfPeA
PFPrA
TFA Na
Normalized GFP readout
Concentration (μM)


HepG2 MT1X GFP
CTRL_Cadmium Chloride
HFBA
HFPO−DA (GenX)
PFDA
PFNA
PFOA
PfPeA
PFPrA
TFA Na
Normalized GFP readout
Concentration (µM)


HepG2 HSPA1B GFP
CTRL_Cadmium Chloride
HFBA
HFPO−DA (GenX)
PFDA
PFNA
PFOA
PfPeA
PFPrA
TFA Na
Normalized GFP readout
Concentration (µM)



Supplemental figure 1. Concentration response plots of the test compounds per ToxProfiler readout. On the X-axis the concentrations are depicted (in µM). On the y-axis the plate-wise normalized biomarker-GFP

measurements or the fraction PI positive (dead) cells are shown. Error bars represent the standard deviation over the three biological replicates.