Schug et al.:

Extending the Concept of Predicting Fish Acute Toxicity *In Vitro* to the Intestinal Cell Line, RTgutGC

Supplementary Data

Tab. S1 Final exposure concentrations used for cell viability testing

Chemical	$logK_{ow}$	Concentrations (mg/L)					
		1	2	3	4	5	6
EugF	1.83	300	150	75	30	10	1
MetA	2.17	800	400	200	100	50	25
Lil	2.94	120	90	60	30	10	2
DaB	3.68	20	10	5	4	2	1
Hel	4.33	40	30	25	20	10	2.5
Pa	4.37	10	7.5	5	2.5	1	0.1
Vel	4.60	12	8	5	2.5	1	0.1
Ver	4.75	15	10	7.5	5	2.5	1
Nir	4.99	6	3	2	1	0.5	0.1
Cet	5.09	5	3	2	1	0.5	0.25
Cax	5.09	6	4	2	1	0.5	0.1
Exa	5.15	2.5	1.5	1	0.5	0.25	0.1
Alp	5.20	4	2	1	0.5	0.1	0.01
MuD	5.52	4	2	1.5	1	0.5	0.1
To	5.70	2	1	0.75	0.5	0.1	0.01
Vul	6.25	0.4	0.3	0.2	0.1	0.05	0.025

doi:10.14573/altex.1905032s

Tab. S2: **Analytical conditions for quantification via LC-MSMS**
The LC-MS/MS instrument included two binary pumps (LC-20AD) operating in gradient mode, an autosampler SIL-30AC (temperature 15°C), a column oven CTO-20AC (column Kinetex C18 2.6 µ 100 A° 50 mm x 2.1 mm column ref: 00B-4462-AN, Phenomenex, USA, temperature 30°C), a diverter valve to limit the injection of interfering compounds into the ESI source of the MS/MS (temperature = 550°C, voltage = 3000 V, declustering potential = 60 V, 4000 Qtrap from ABSciex, Switzerland). MS/MS operated in MRM mode. All LC units came from Shimadzu, Switzerland. Solvent A = H_2O + 0.1% formic acid; Solvent B = Methanol + 0.1% formic acid. All quantifications were done by external calibration with an internal standard (ISTD). After addition of the appropriate amount of ISTD, all samples were filtered through RC 0.2 µm 13 mm filters before injection. Limit of quantification (LOQ) is given based on injection volume.

Chemical	Separation conditions	Injection volume (µl)	MRM transition	ISTD	LOQ (µg/L)
MetA	total flow = 0.5 mL/min, 50% B up to 90% B in 2.5 min, maintained 0.3 min, then decreased to 50% B in 0.3 min, maintained 0.9 min	2	152.2/120.0	DaB	1
Lil	total flow = 0.5 mL/min, 55% B up to 62% B in 3.7 min, then up to 95% B in 0.3 min, maintained 0.5 min, then decreased to 50% B in 1.3 min	2	159.2/129.2	DaB	3
DaB	total flow = 0.5 mL/min, 50% B up to 95% B in 4 min, maintained 0.5 min, then decreased to 50% B in 0.2 min, maintained 1.3 min	2	193.2/137.0	To	2
Hel	total flow = 0.5 mL/min, 70% B up to 85% B in 3 min, maintained 0.5 min, then up to 95% B in 0.5 min, maintained 0.5 min, decreased to 70% B in 0.5 min, maintained 1.5 min	2	229.183/83.1	To	3
Pa	total flow = 0.5 mL/min, 60% B up to 80% B in 3 min, maintained 0.5 min, then up to 95% B in 1.0 min, maintained 1 min, decreased to 60% B in 0.1 min, maintained 1.4 min	10	209.2/177.2	To	10
Nir	total flow = 0.5 mL/min, 50% B up to 95% B in 4 min, maintained 0.5 min, then decreased to 50% B in 0.2 min, maintained 1.3 min	5	205.25/55.1	DaB	2
Cet	total flow = 0.5 mL/min, 60% B up to 95% B in 4 min, maintained 0.5 min, then decreased to 60% B in 0.2 min, maintained 1.3 min	2	237.2/193.1	DaB	3
Alp	total flow = 0.5 mL/min, 60% B up to 78% B in 2 min, then up to 95% B in 3 min, maintained 1.6 min, then decreased to 60% B in 0.1 min, maintained 1.3 min	2	283.2/127.0	DaB	1
MuD	total flow = 0.5 mL/min, 60% B up to 78% B in 2 min, then up to 95% B in 3 min, maintained 1.6 min, then decreased to 60% B in 0.1 min, maintained 1.3 min	2	237.2/55.1	DaB	3
To	total flow = 0.5 mL/min, 50% B up to 95% B in 4 min, maintained 0.5 min, then decreased to 50% B in 0.2 min, maintained 1.3 min	2	259.3/175.1	DaB	1
Vul	total flow = 0.5 mL/min, 50% B up to 95% B in 4 min, maintained 0.5 min, then decreased to 50% B in 0.2 min, maintained 1.3 min	10	259.3/161.1	DaB	1
Cax	total flow = 0.5 mL/min, 60% B up to 74% B in 1.7 min, then up to 95% B in 2.8 min, maintained 1.0 min, then decreased to 60% B in 0.2 min, maintained 1.3 min	2	237.2/193.1	DaB	3
Exa	total flow = 0.5 mL/min, 60% B up to 78% B in 2 min, then up to 95% B in 3 min, maintained 1.6 min, then decreased to 60% B in 0.1 min, maintained 1.3 min	2	223.2/55.1	DaB	5
Vel	total flow = 0.5 mL/min, 70% B up to 87% B in 3.5 min, then up to 95% B in 0.3 min, maintained 0.7 min, then decreased to 70% B in 0.3 min, maintained 1.7 min	2	197/179	To	100

Tab. S3: **Analytical conditions for quantification of Eug and Ver**

Eugenol F and Verdox were quantified using GC since sensitivity in LC-MS/MS was not appropriate for these two compounds.

Chemical	Instrument	Quantification method
EugF	GC-MS	Eugenol was quantified using GC-MS (7890 Agilent chromatograph combined with a 5977 Agilent mass spectrometer, both from Agilent, Switzerland). Samples were extracted using SPME DVB/Carboxen/PDMS fiber, using Tonalide as externalized standard. SPME Equilibration time = 5 min @ 50°C, extraction time 8 = min. GC column was a DB17ms (J&W, USA) 30 m, 0.25 mm ID, 0.25 µm film (total He flow = 40 mL/min, oven program: 80°C 1 min, 18°C/min, 260°C 1 min). Injection was conducted in split/splitless injector in splitless mode with a specific SPME liner (desorption time = 8 min, liner from Supelco, Switzerland). MS was operated in SIM mode (Eugenol: 149 m/z ; To: 164 m/z). LOQ < 500 µg/L.
Ver	GC-FID	Verdox was quantified with GC-FID (6890 Agilent chromatograph, Agilent, Switzerland). Samples were fully extracted with MTBE (3 x 500 µl). 1 µL of extracted sample was directly injected into split/splitless injector (250°C, split 1:20, 133.5 kPa He). GC column was a DBXLB (J&W, USA) 10m, 0.18 mm ID, 0.18 µm film (total He flow = 40 mL/min, oven program: 100°C 0.5 min, 30°C/min, 250°C 0.5 min). FID was operated at 250°C, with H2 flow 40.0 mL/min, air flow 450 mL/min, N2 makeup flow 45.0 mL/min, data acquisition rate 50Hz. LOQ < 100 µg/L.

Tab. S4: **Normalized measured concentration for the test chemicals**

Measured concentrations at the beginning of the experiment t_{0h} were normalized to the concentrations in the dosing mixtures (DM). Concentration after 2-3 h of exposure ($t_{2\text{-}3h}$) and at the end of the experiment (t_{24h}) were normalized to t_{0h}. Test chemicals are ordered according to the $logK_{ow}$.

Chemical	$logK_{ow}$	Concentration at t_{0h} normalized to DM		Concentration at $t_{2\text{-}3h}$ normalized to t_{0h}		Concentration at t_{24h} normalized to t_{0h}	
		Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
EugF	1.83	88	17	102	17	105	15
MetA	2.17	99	4	100	3	100	6
Lil	2.94	102	5	101	6	101	3
DaB	3.68	n.d.	n.d.	92	1	99	20
Hel	4.33	110	19	103	30	32	12
Pa	4.37	152	21	88	7	50	9
Vel	4.60	133	11	92	10	57	11
Ver	4.75	130	10	85	2	74	8
Nir	4.99	107	8	92	16	46	17
Cet	5.09	113	7	85	9	72	3
Cax	5.09	127	n.d.	80	2	68	10
Exa	5.15	110	13	61	2	30	15
Alp	5.20	119	25	64	3	30	1
MuD	5.52	n.d.	n.d.	61	1	33	4
To	5.70	n.d.	n.d.	55	3	43	3
Vul	6.25	110	9	56	13	16	1

n.d., not determined

Tab. S5: *In vitro* EC_{50} based on three cell viability endpoints

EC_{50} values were derived based on metabolic activity (Alamar Blue), cell membrane integrity (CFDA-AM) and lysosomal integrity (Neutral Red) using the nominal and geometric mean of three measured concentrations from three biological replicates. For Vul no full dose-response was recorded and no EC_{50} values were determined.

Chemical	Alamar Blue				CFDA-AM				Neutral Red			
	EC_{50} (mg/L) nominal		EC_{50} (mg/L) geomean		EC_{50} (mg/L) nominal		EC_{50} (mg/L) geomean		EC_{50} (mg/L) nominal		EC_{50} (mg/L) geomean	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
EugF	38.9	7.0	40.3	7.3	97.6	43.6	96.7	41.1	75.5	27.8	77.4	27.4
MetA	155.6	31.6	173.5	24.5	278.6	107.8	310.9	113.9	211.0	56.6	235.1	53.7
Lil	48.7	9.5	51.8	9.9	79.5	10.2	85.0	11.1	70.0	2.3	74.7	2.1
DaB	5.5	1.2	5.8	0.8	5.7	0.8	14.1	2.5	10.4	2.9	10.5	2.3
Hel	24.9	10.7	19.4	10.2	30.0	6.1	23.3	5.6	32.1	7.0	26.2	7.6
Pa	5.4	0.4	4.2	0.2	5.8	0.1	4.4	0.3	6.7	0.8	5.0	0.3
Vel	4.0	0.5	2.1	0.2	7.4	0.2	3.3	0.4	6.5	0.1	3.0	0.4
Ver	7.5	0.6	4.5	1.1	12.6	2.1	8.0	2.0	12.0	0.6	7.0	0.9
Nir	4.4	1.2	2.5	0.7	5.3	0.8	3.0	0.5	3.9	0.7	2.2	0.4
Cet	2.4	0.6	1.7	0.5	3.7	0.5	2.6	0.3	4.7	0.5	3.1	0.2
Cax	4.3	0.0	12.0	2.6	3.9	0.6	11.1	3.6	4.8	0.2	13.0	3.0
Exa	1.6	0.1	0.7	0.1	2.1	0.4	1.0	0.3	1.4	0.5	0.6	0.2
Alp	1.9	0.3	0.7	0.1	2.5	0.2	1.0	0.1	2.2	0.1	0.9	0.0
MuD	2.1	1.3	0.5	0.1	2.4	1.5	0.6	0.5	2.0	1.2	0.5	0.4
To	0.9	0.2	0.4	0.1	0.4	0.1	0.4	0.1	0.9	0.1	0.4	0.1
Vul	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not determined

Tab. S6: Non-toxic concentrations (NtCs)

NtCs were derived based on the three cell viability endpoints expressed as nominal and geometric mean of three measured concentrations at t_{0h}, $t_{2\text{-}3h}$ and t_{24h} from three biological replicates.

Chemical	Alamar Blue		CFDA-AM		Neutral Red	
	NtC (mg/L) nominal	NtC (mg/L) geomean	NtC (mg/L) nominal	NtC (mg/L) geomean	NtC (mg/L) nominal	NtC (mg/L) geomean
EugF	10.5	11.3	10.00	7.90	17.38	19.91
MetA	50.0	56.2	64.42	71.78	113.50	127.35
Lil	22.0	23.7	47.10	49.66	46.45	48.98
DaB	1.0	1.0	n.d.	n.d.	4.49	4.03
Hel	6.9	4.4	2.50	1.60	14.35	9.98
Pa	1.0	0.5	2.50	1.39	4.47	2.28
Vel	1.0	1.0	2.50	2.10	4.13	3.64
Ver	2.2	1.3	2.50	1.50	1.00	0.72
Nir	2.0	1.1	1.57	0.90	0.10	0.10
Cet	2.0	5.8	0.50	2.57	0.50	2.57
Cax	0.5	0.3	0.25	0.17	0.50	0.32
Exa	0.9	0.4	0.25	0.09	0.21	0.07
Alp	0.1	0.02	0.03	0.005	0.50	0.14
MuD	0.8	0.3	0.50	0.14	0.30	0.10
To	0.3	0.2	n.d.	0.0004	0.010	0.011

n.d., not determined

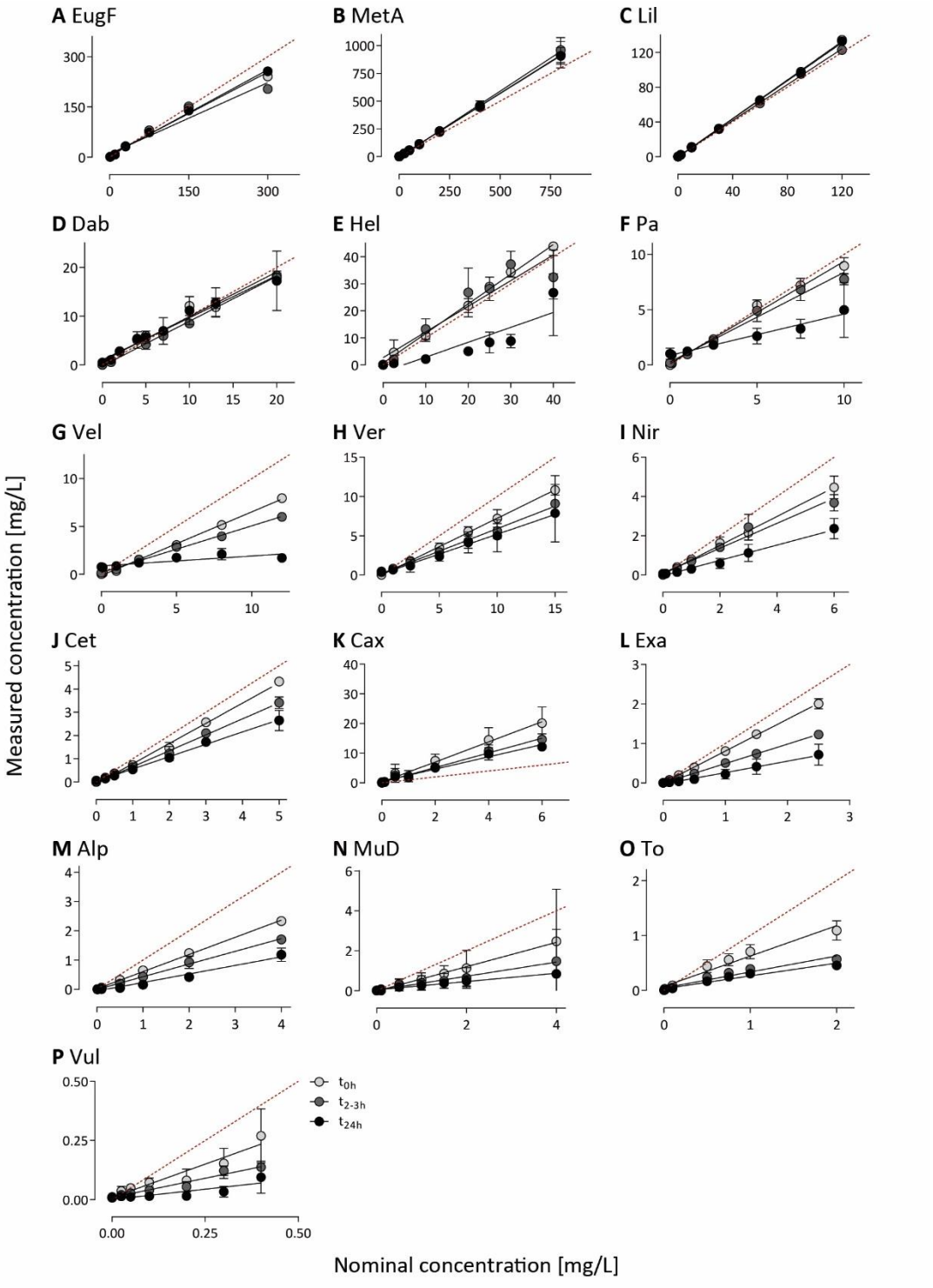

Fig. S1: Measured chemical concentration
Measured chemical concentration at t_{0h}, $t_{2\text{-}3h}$ and t_{24h} is plotted against the nominal concentration. The red dashed line indicates the line of unity. Data are shown as mean ±SD of three biological replicates, and chemicals are ordered with increasing $\log K_{ow}$ from the lowest $\log K_{ow}$ = 1.83 (Panel A) to highest $\log K_{ow}$ = 6.25 (Panel P).

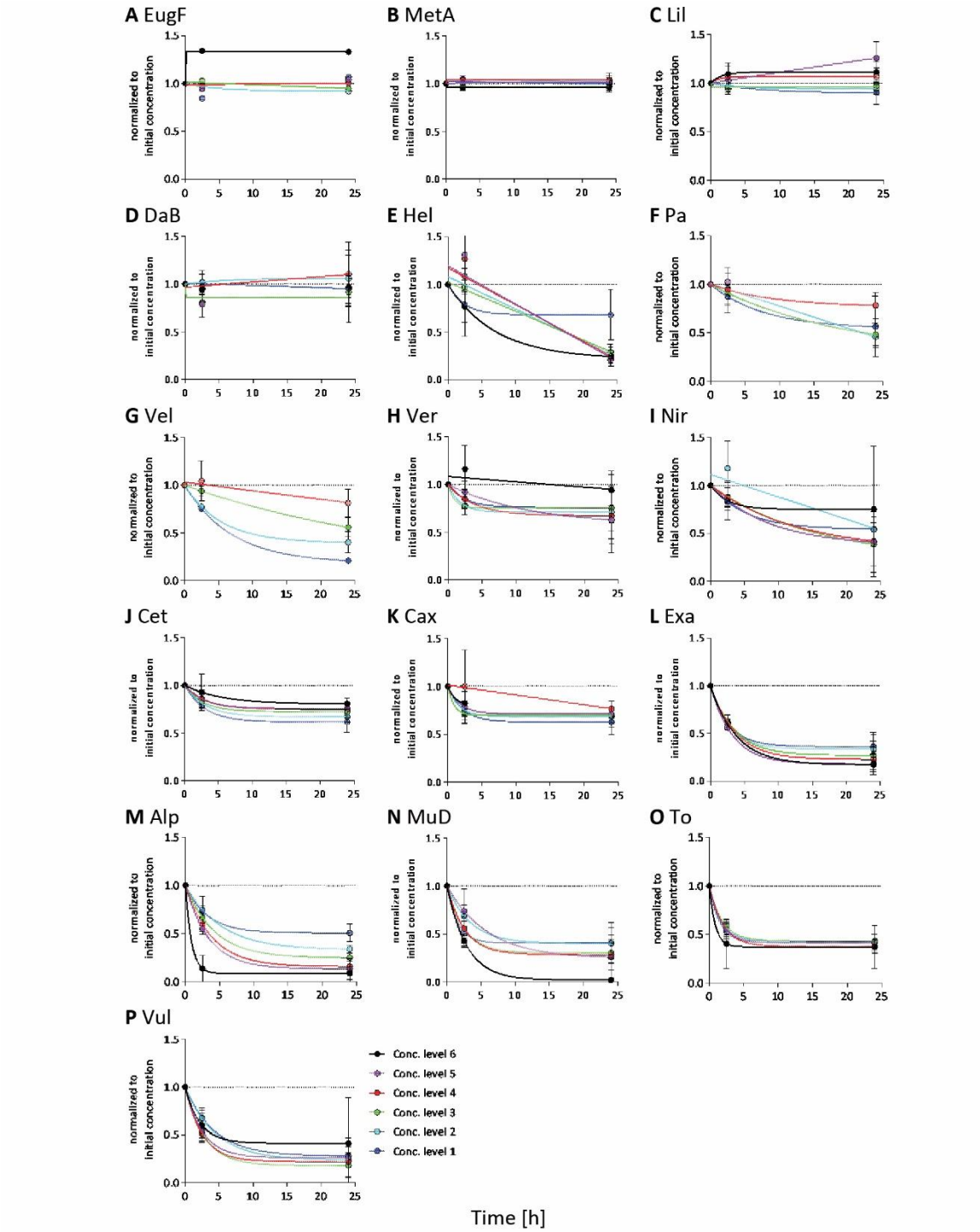

Fig. S2: Concentration loss over time
The measured concentration after 2-3 h and 24 h was normalized to the initial concentration (t_{0h}) and is plotted over time. Different concentration levels per chemical are shown with different colors, with concentration level 1 being the highest and level 6 the lowest concentration (Tab. S2.1). Chemicals are ordered according to $logK_{ow}$ from the lowest $logK_{ow}$ = 1.83 (Panel A) to highest $logK_{ow}$ = 6.25 (Panel P).

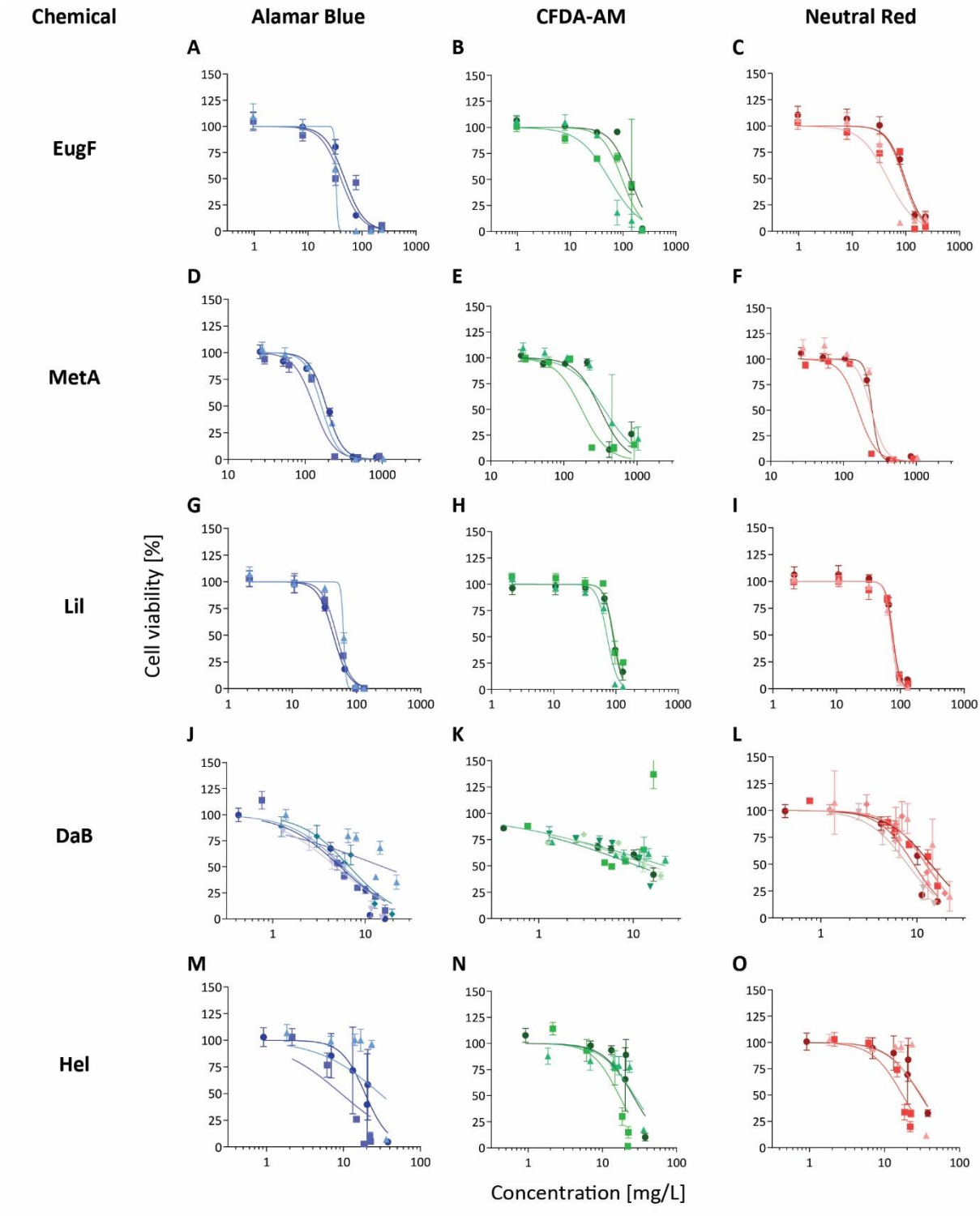

Fig. S3-1: Concentration-response curves for all three endpoints
Cell viability data were based on the geometric mean of the measured concentration. For each endpoint, the three biological replicates with mean ±SD of three technical replicates and the fitted concentration-response curve are shown. The figure is continued below. Chemicals are listed according to their logK_{ow}.

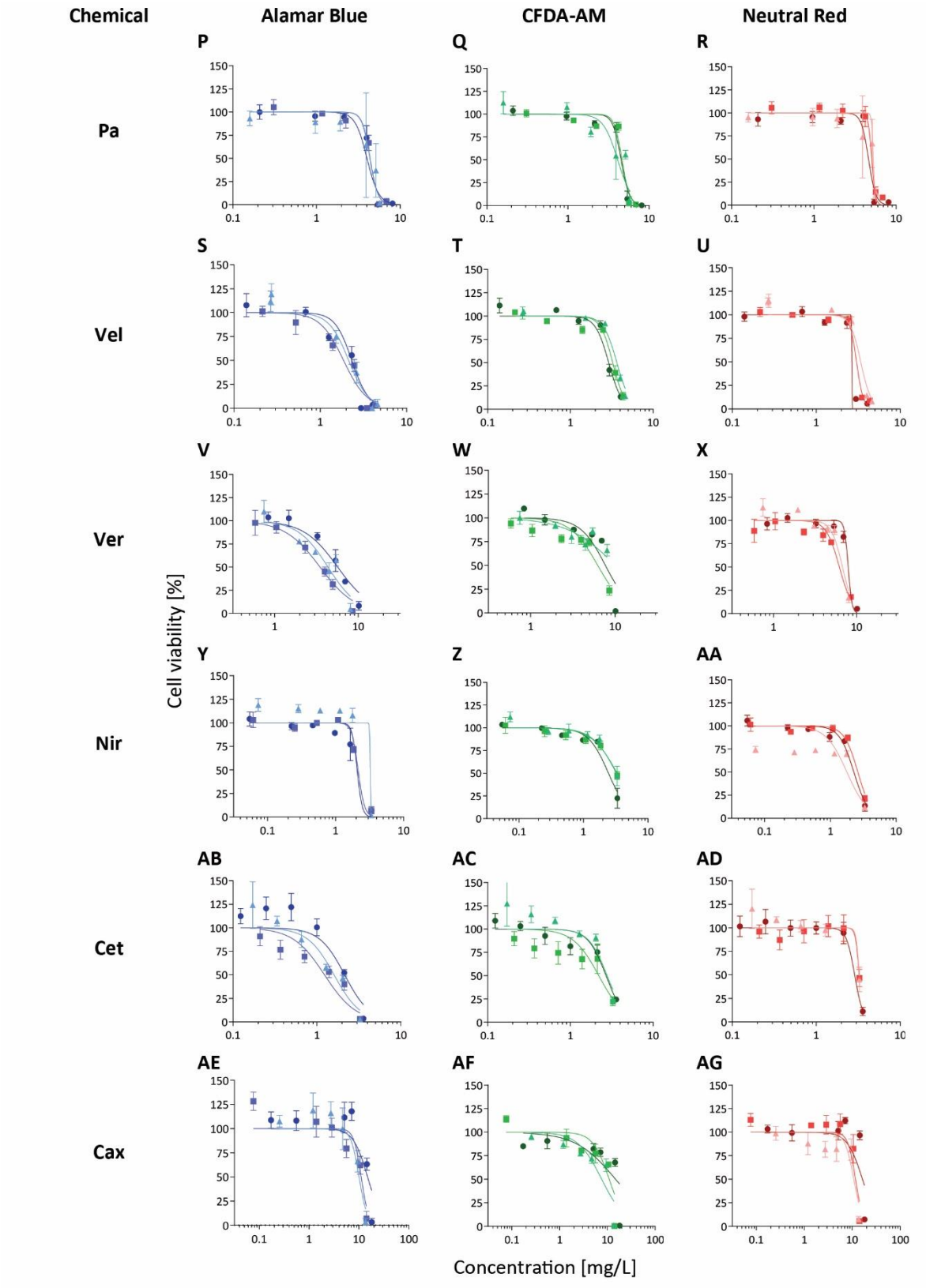

Fig. S3-2: Concentration-response curves for all three endpoints (continuation)

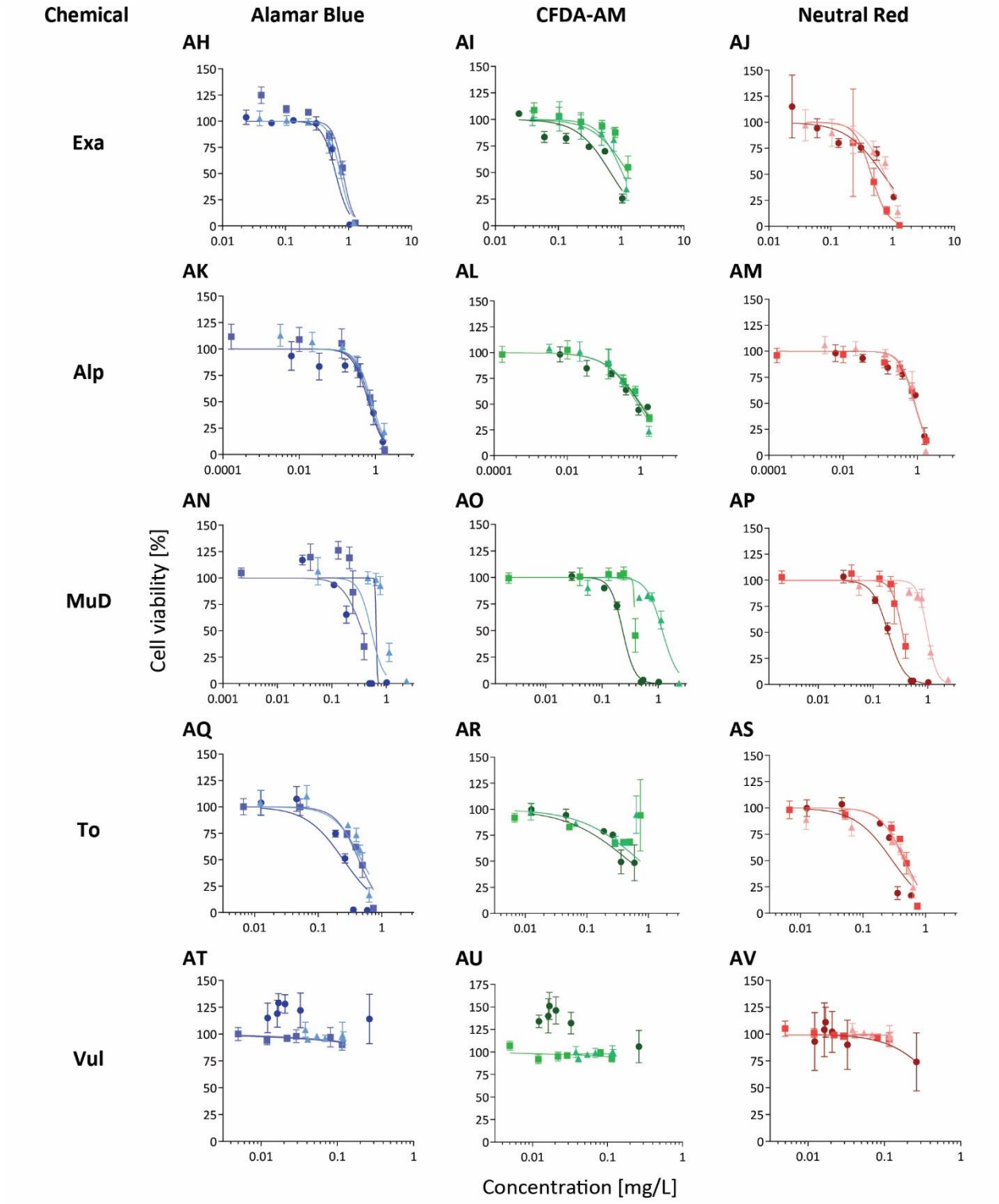

Fig. S3-3: Concentration-response curves for all three endpoints (continuation)

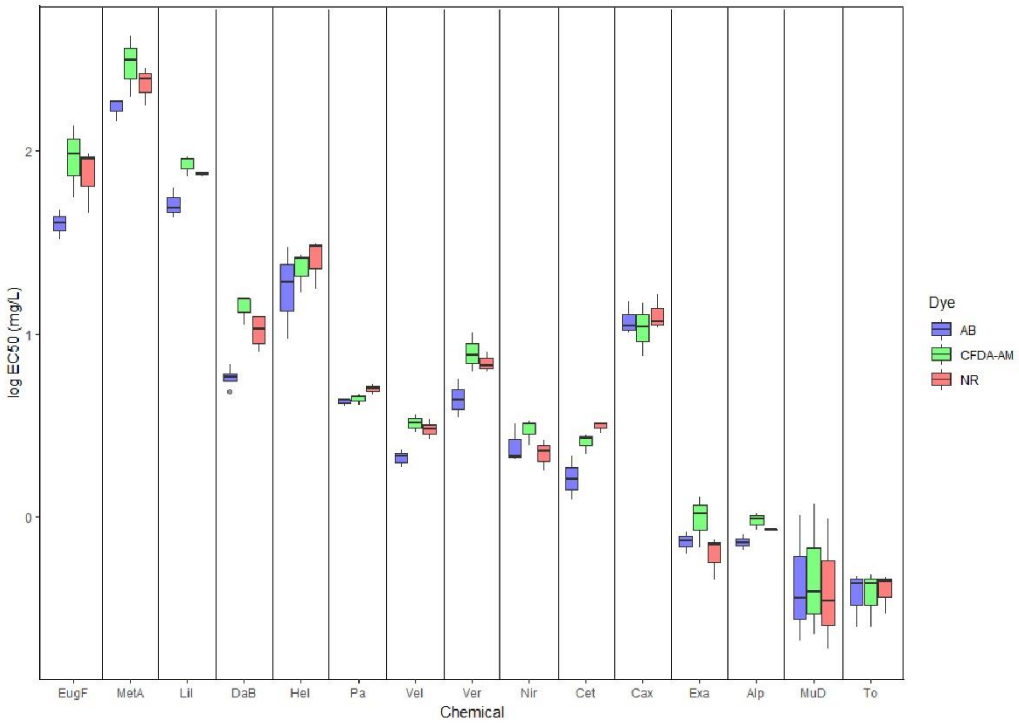

Fig. S4: Boxplot of log EC_{50} values per dye and chemical
Boxplots show the 95%-quantiles of the EC_{50} values per cell viability endpoint and chemical.

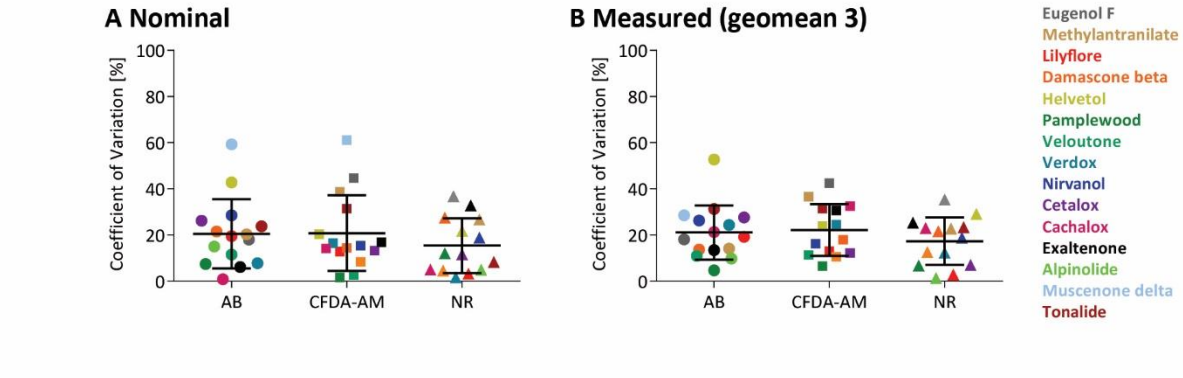

Fig. S5: Coefficients of variation (CoVs)
The coefficients of variation (CoVs) were calculated for EC_{50} values based on nominal (Panel A) or the geometric mean of measured concentrations (Panel B). CoVs are shown color-coded for the individual chemicals based on the three different cell viability measurements: Metabolic activity measured with Alamar Blue, membrane integrity measured with CFDA-AM, and lysosomal integrity measured with Neutral Red. Bars indicate the means ±SD. No significant differences between differently derived EC_{50} values or cell viability dyes could be detected by the non-parametric Kruskal-Wallis test.

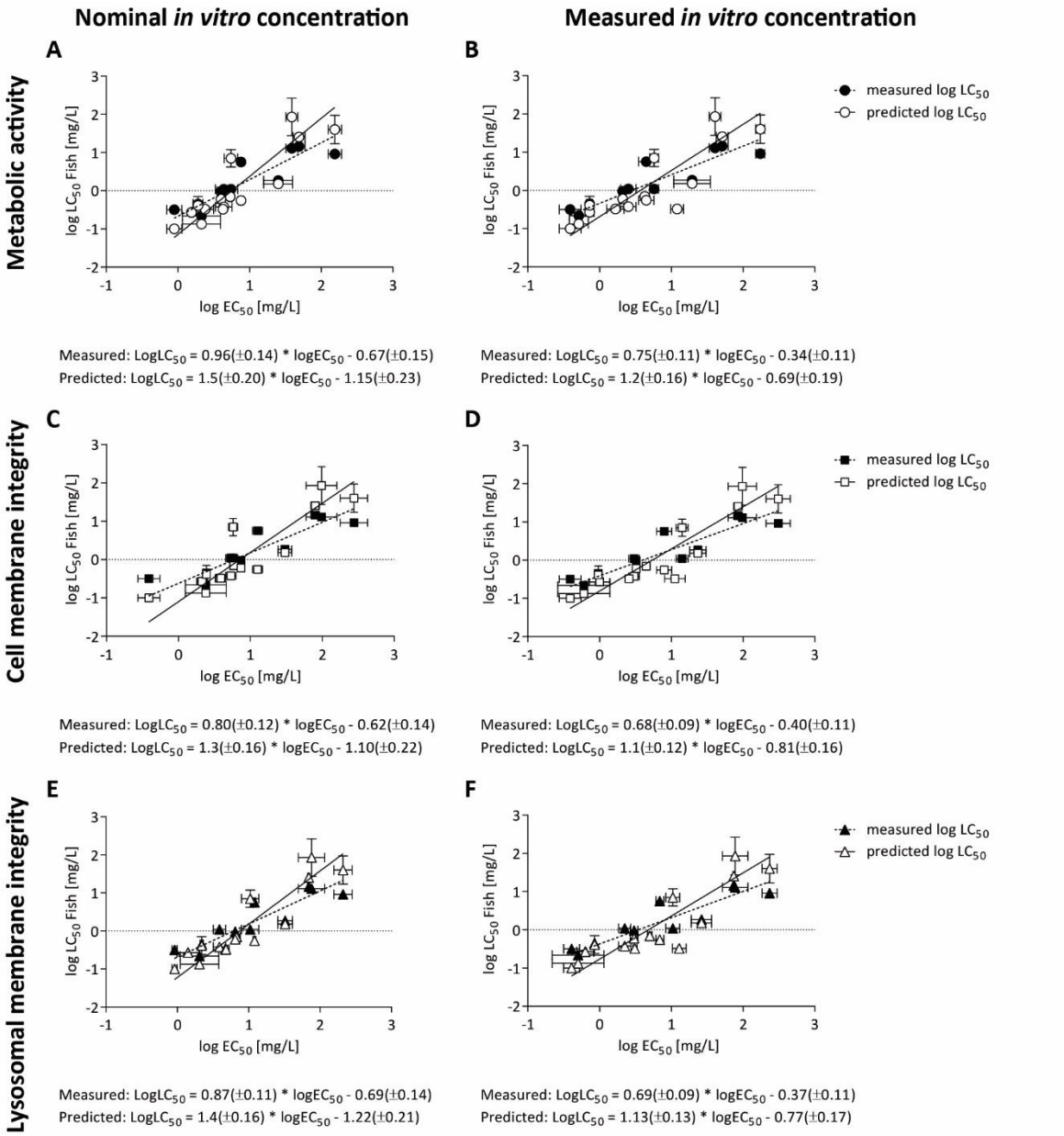

Fig. S6: *In vitro* to *in vivo* extrapolation for fragrances for all three cell viability endpoints
A linear regression between EC_{50} values, based on cell metabolic activity (Panel A,B), cell membrane integrity (Panel C, D) and lysosomal membrane integrity (Panel E, F), compared to fish LC_{50} values was established. RTgutGC EC_{50} values were calculated using the nominal (Panel A, C, E) and geometric mean of three measured concentrations (t_{0h}, $t_{2\text{-}3h}$ and t_{24h}) (Panel B, D, F) and plotted against measured (black symbols) and predicted (white symbols) LC_{50} fish data. Cell line-based data are shown as mean ±SD of three biological replicates, fish data as mean ±SD of 2-3 replicates from ECOSAR predictions or Firmenich internal data (Tab. 1). A Deming (model II) regression, assuming equal uncertainties for x- and y-values was fitted against the measured (dashed line) and predicted (solid line) fish LC_{50} data.

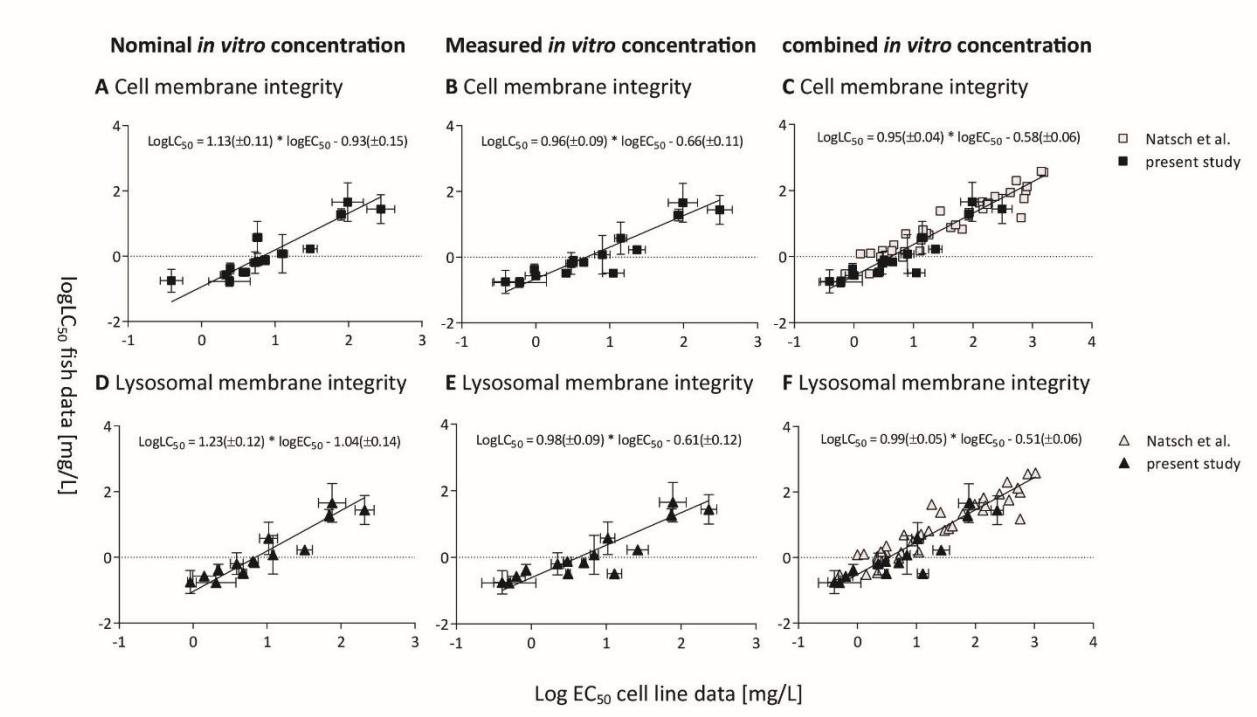

Fig. S7: *In vitro* to *in vivo* extrapolation for the endpoints cell membrane integrity and lysosomal integrity
A linear regression between EC_{50} values, based on the endpoint cell membrane integrity and lysosomal integrity, compared to fish LC_{50} values was established. RTgutGC EC_{50} values were calculated using the nominal (Panel A, D) and geometric mean based on three measured concentrations (t_{0h}, $t_{2\text{-}3h}$ and t_{24h}) (Panel B, E) and plotted against the mean of measured and predicted LC_{50} fish data. Cell line-based data are shown as mean ±SD (RTgutGC, n = 3). Panel C,D shows RTgutGC EC_{50} values based on measured concentration combined with data available from Natsch et al. 2018 (grey symbols, RTgill-W1, n = 1). Solid line and equation represent a Deming (Model II) regression assuming equal uncertainties for x- and y-values.

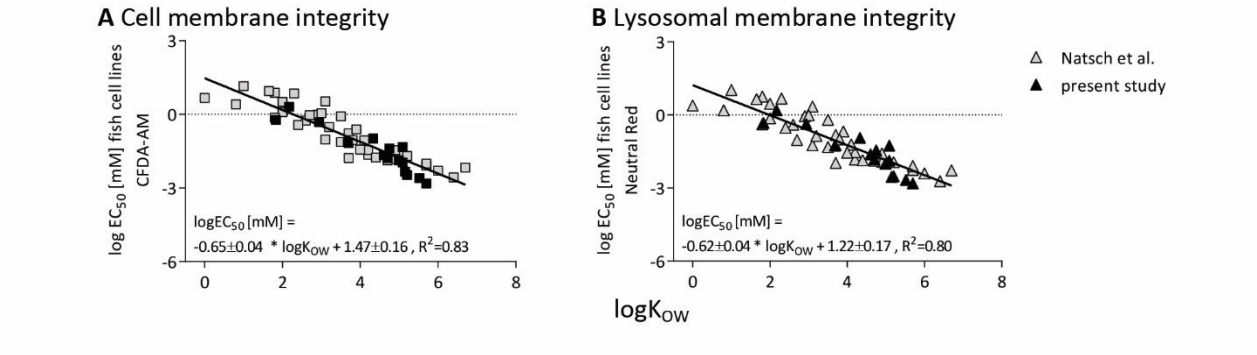

Fig. S8: QSARs for cell membrane and lysosomal integrity
Regression analysis between cell membrane integrity, measured with CFDA-AM (Panel A), and lysosomal integrity, measured with Neutral Red (Panel B), and the measured $logK_{ow}$ of the test chemicals. Solid lines and equation present a linear regression fitted against the mean of all data points.

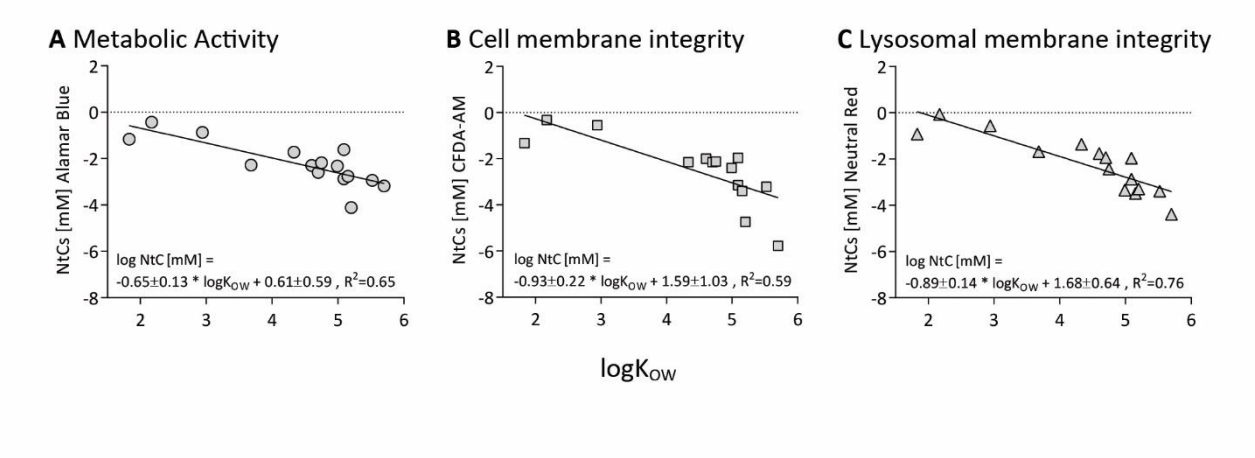

Fig. S9: QSARs for non-toxic concentrations (NtCs)
Regression analysis between measured NtCs and $logK_{ow}$ of the test chemicals based on metabolic activity (Panel A), cell membrane integrity (Panel B) and lysosomal membrane integrity (Panel C). Solid lines and equation present a linear regression fitted against the mean of all data points.

Reference

Natsch, A., Laue, H., Haupt, T. et al. (2018). Accurate prediction of acute fish toxicity of fragrance chemicals with the RTgill-W1 cell assay. *Environ Toxicol Chem 37*, 931-941. doi:10.1002/etc.4027