Supporting Information:

Characterization of the mercapturic acid pathway, an important phase II biotransformation route, in a zebrafish embryo cell line

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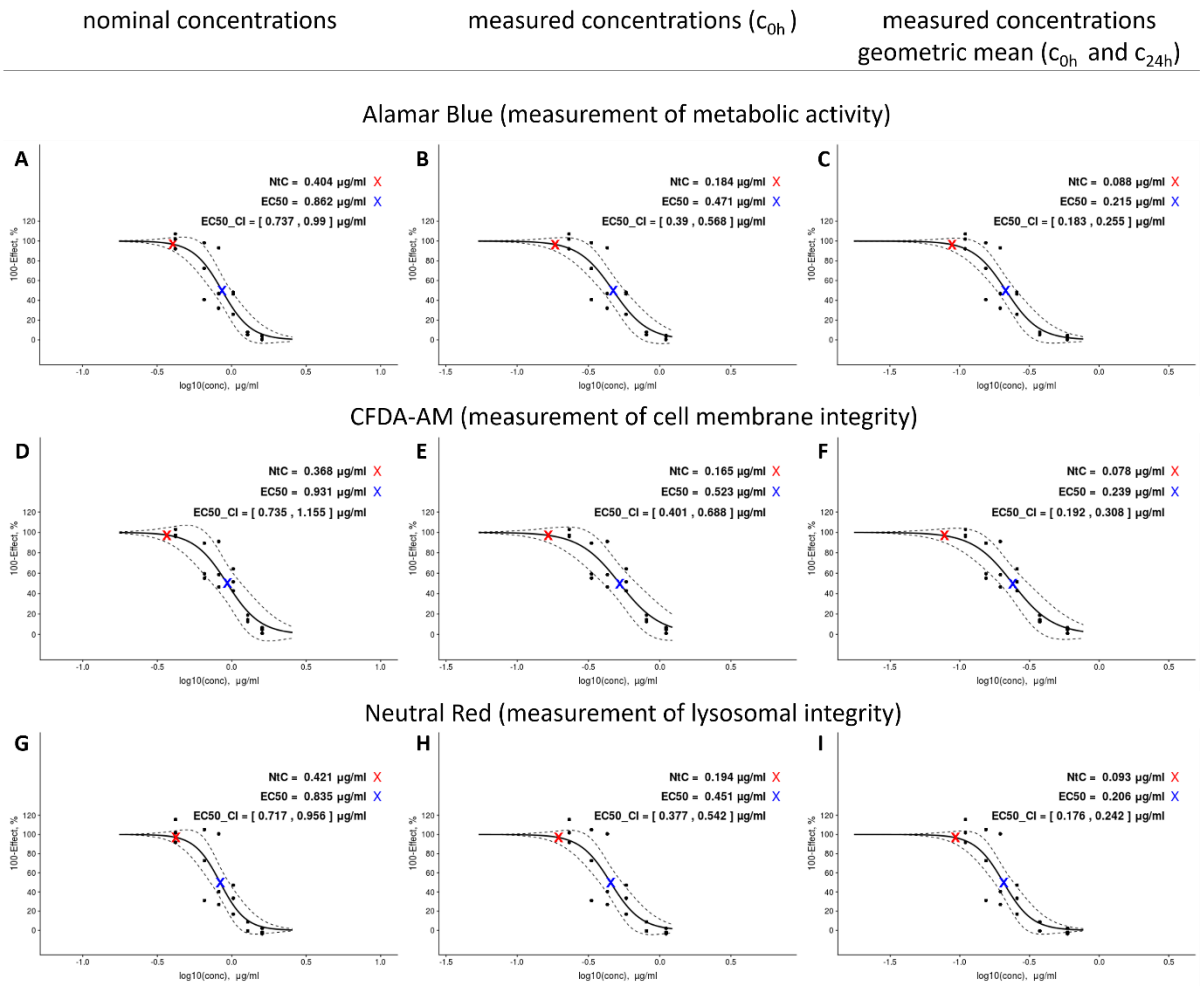
SI Table 1: Nominal and measured 1-chloro-2,4-dinitrobenzene (CDNB) concentrations at the beginning C_{0h} and at the end C_{24h} of the experiment and the resulting geometric mean. C1-6 are the concentrations used, SD is the standard deviation, and Δ% the difference between the nominal and measured concentration in percent.

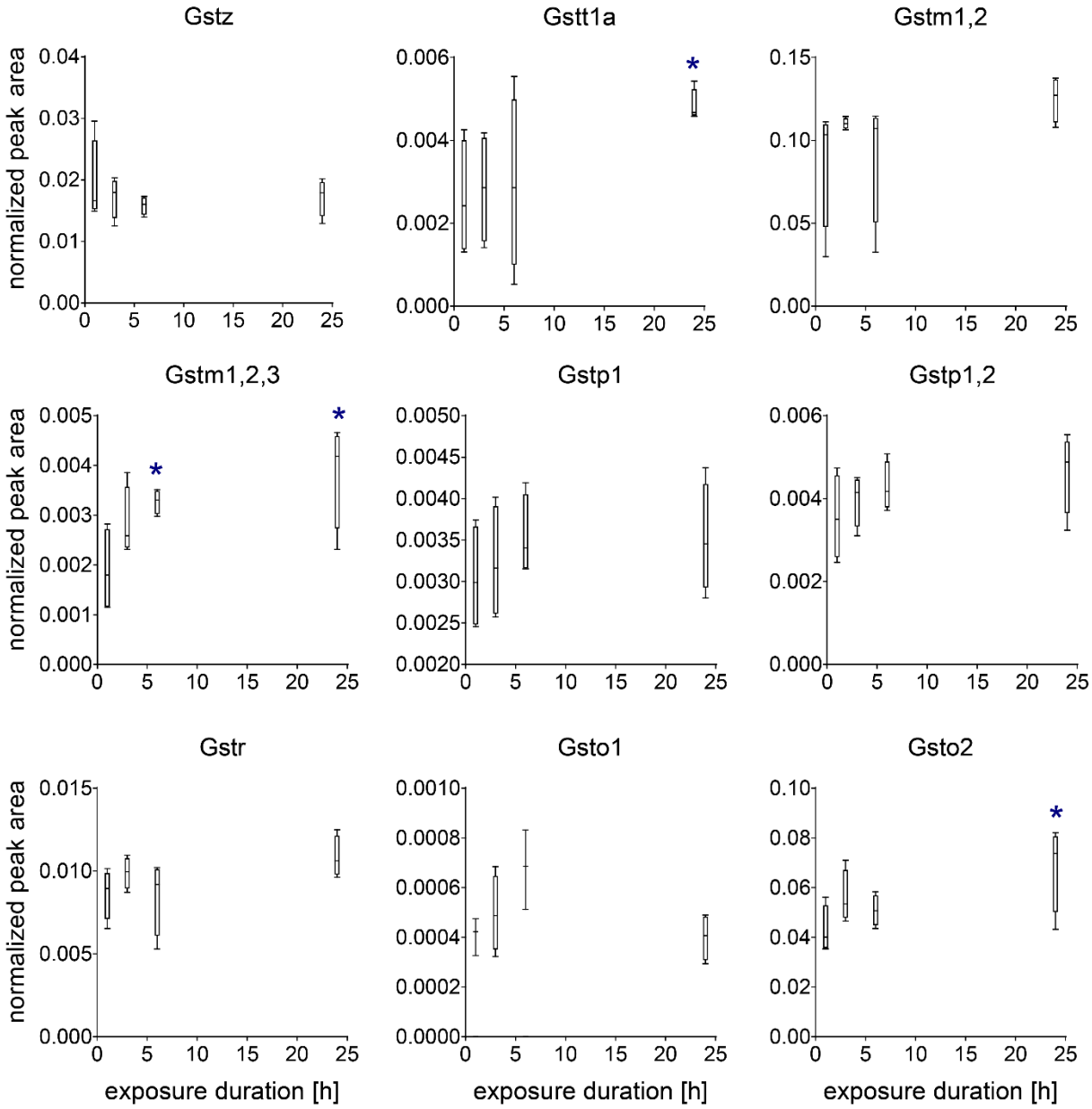
	nominal concentration [µg/ml]	**C_{0h}** measured concentration [µg/ml]		**C_{24h}** measured concentration [µg/ml]		geometric mean (C_{0h} and C_{24h})	difference nominal and **C_{0h}** measured concentration	
		average	SD	average	SD		*Δ%*	*average Δ%*
C1	1.600	1.109	0.151	0.203	0.000	0.592	30.708	42.156
C2	1.280	0.808	0.067	0.046	0.136	0.375	36.901	
C3	1.024	0.582	0.036	0.000	0.042	0.258	43.197	
C4	0.819	0.426	0.046	0.000	0.025	0.195	48.026	
C5	0.655	0.332	0.037	0.000	0.011	0.154	49.313	
C6	0.419	0.231	0.023	0.000	0.070	0.110	44.789	
Control	0.000	0.000	0.000	0.000	0.000	0.000		

SI Table 2: Mean of EC_{50} and non-toxic concentration (NtC) values for 1-chloro-2,4-dinitrobenzene (CDNB) exposure obtained with the cell viability measurements performed with the three fluorescent dyes Alamar Blue, 5-carboxyfluorescein diacetate acetoxy-methyl ester (CFDA-AM) and Neutral Red and calculated based on nominal, measured (C_{0h}) and geometric mean of measured CDNB concentrations at the beginning and the end of the experiment (c_{0h} and c_{24h}).

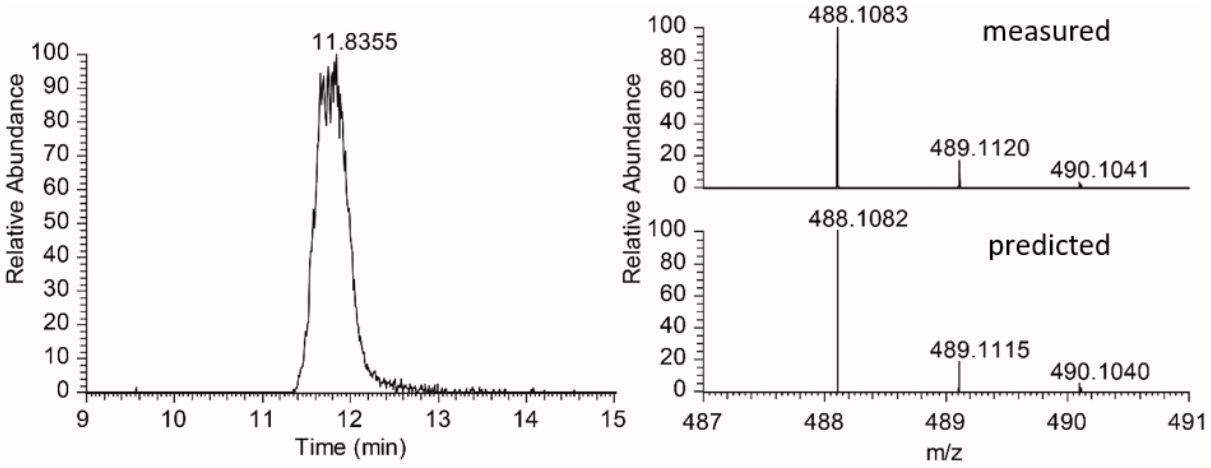
	nominal concentration		measured concentration (C_{0h})		geometric mean (C_{0h} and C_{24h})	
	ng/ml	µM	ng/ml	µM	ng/ml	µM
EC_{50}	886	4.4	428	2.4	220	1.1
NtC	398	2	181	0.9	86	0.4



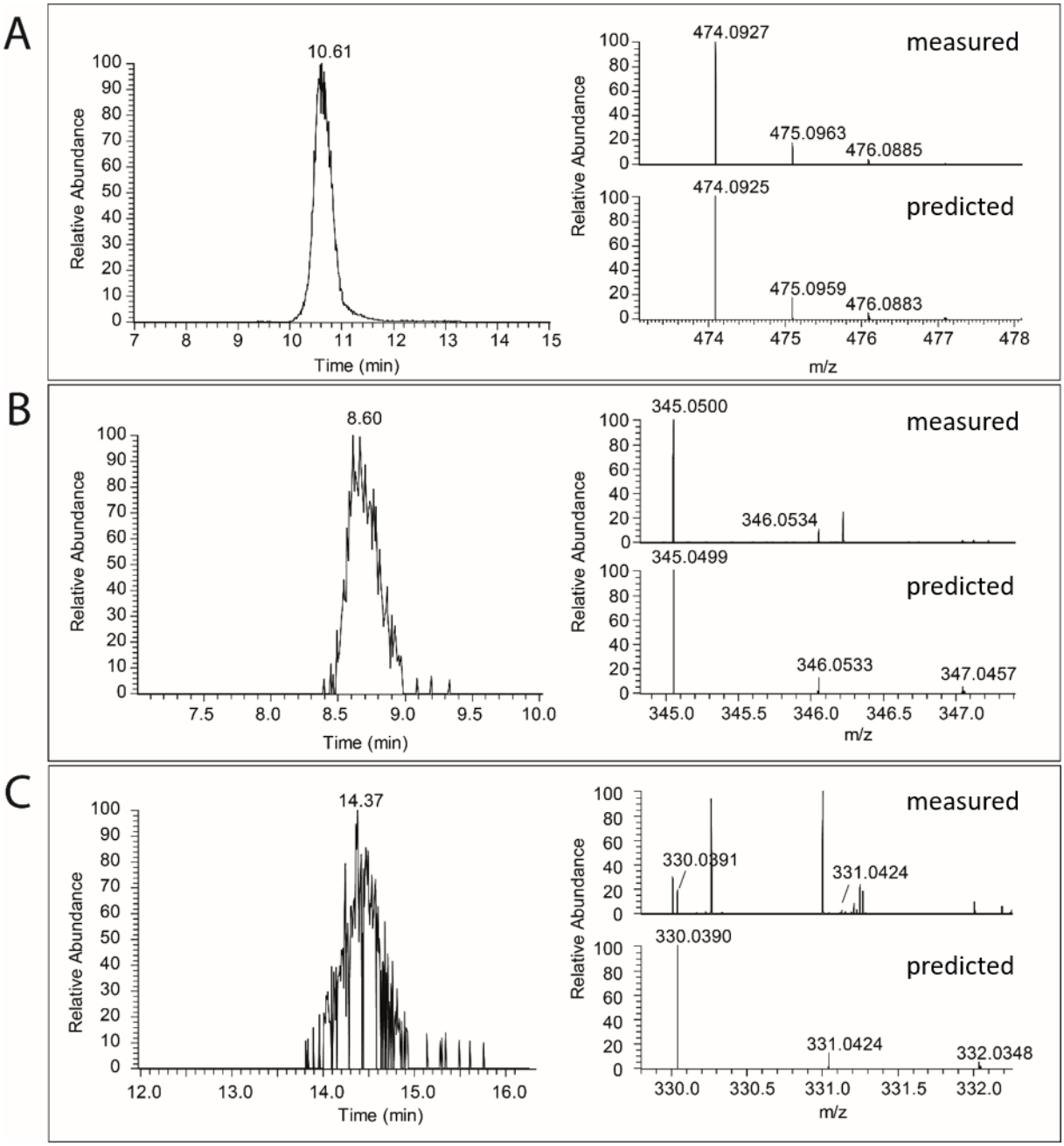
SI Figure 1: Dose-response data for cell viability measured with the use of three fluorescent dyes, Alamar Blue for metabolic activity (A-C), 5-carboxyfluorescein diacetate acetoxy-methyl ester (CFDA-AM) for cell membrane integrity (D-F) and Neutral Red for lysosomal integrity (G-I), as described in [1, 2]. Data is shown as % cell viability normalized to the CDNB-free solvent control as a function of log CDNB nominal concentration (A, D and G), log CDNB concentration measured in samples collected at the beginning of the experiments (c_{0h}) (B, E and H) and log CDNB geometric mean of concentrations measured in samples collected at the beginning (c_{0h}) and at the end of the experiments (c_{24h}) (C, F and I). The curve fit and the calculation of the non-toxic concentration (NtC) and the half-maximal effect concentration (EC_{50}) were performed using an algorithm developed by Stadnicka-Michalak et al.[3]. The figure shows the fitted sigmoidal curve together with 95% confidence intervals and measured data where each dot represents one of three independent biological replicates. The NtC and EC_{50} values are indicated by the red and blue crosses, respectively. EC50_CI represents the EC_{50} confidence interval. The EC_{50} values calculated based on the three fluorescent dyes data did not differ significantly between each other ($p > 0.01$, one-way ANOVA).



*SI Figure 2: Expression of cytosolic GSTs in PAC2 cells cultured in L15 medium supplemented with 5% FBS for 1, 3, 6, and 24 h. The expression is shown as peak area normalized to the housekeeping proteins β-actin and 40S ribosomal protein S18. The values are shown as boxplots (minimum, first quartile, median, third quartile, and maximum) of four independent biological replicates. The normalized peak area of peptides belonging to the same enzyme (in case of proteotypic peptides) or several isoenzymes from the same class (in case of shared peptides) were cumulated. Significantly upregulated GSTs are marked by *, p=0.05.*



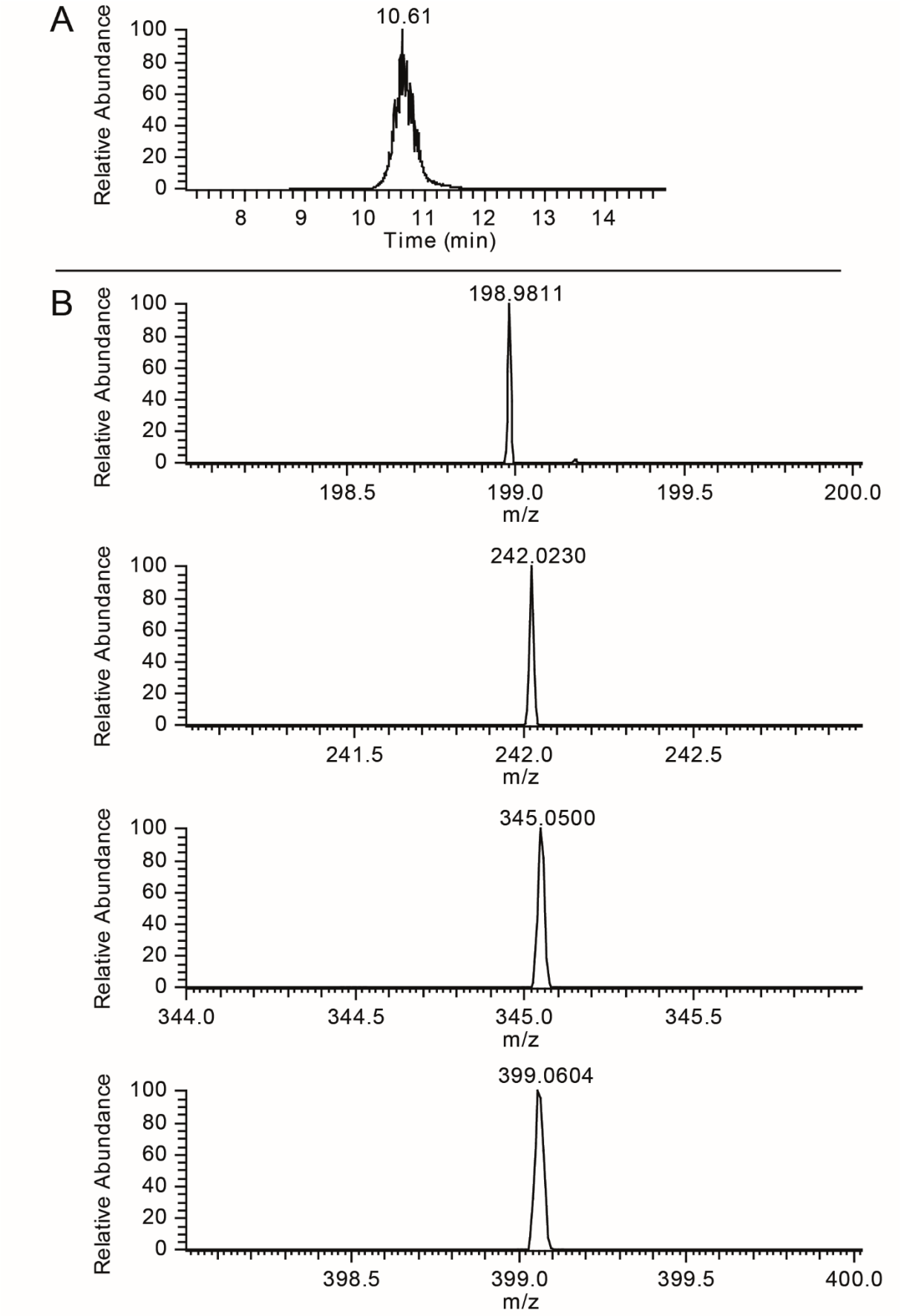
SI Figure 3: Chromatogram in full scan mode and mass spectra of 2,4-dinitrotoluene-S-glutathione (DNT-SG, internal standard).The measured isotope distribution is depicted on top of the predicted isotope distribution. m/z: mass-to-charge ratio.



SI Figure 4: Chromatogram in full scan mode and mass spectra of A: 2,4-dinitrophenyl-S-glutathione (DNP-SG), B: 2,4-dinitrophenyl cysteinglycine (DNP-CG) and C: 2,4-dinitrophenyl N-acetylcysteine (DNP-NAC). The measured isotope distribution is shown on top of the predicted isotope distribution. m/z: mass-to-charge ratio.

SI Table 3: Difference (ppm) between the measured and predicted isotopes of the protonated molecular ions of 2,4-dinitrotoluene-S-glutathione (DNT-SG, internal standard), 2,4-dinitrophenyl-S-glutathione (DNP-SG), 2,4-dinitrophenyl cysteinglycine (DNP-CG) and 2,4-dinitrophenyl N-acetylcysteine (DNP-NAC) in full scan (MS).

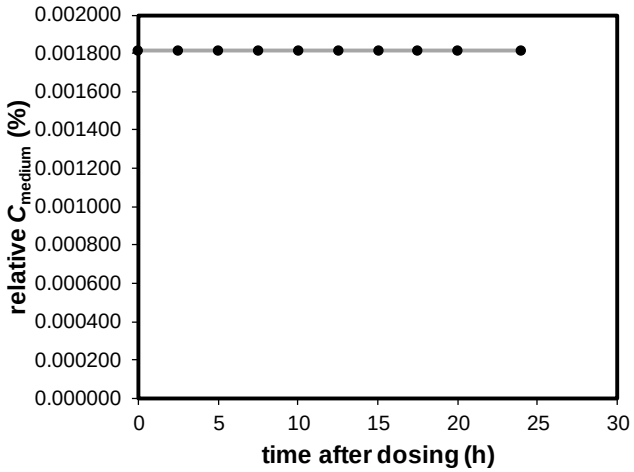
measured m/z	predicted m/z	difference ppm
DNT-SG		
488.1083	488.1082	0.20
489.1120	489.1115	1.02
490.1041	490.1040	0.20
DNP-SG		
474.0927	474.0925	0.42
475.0963	475.0959	0.84
476.0885	476.0883	0.42
DNP-CG		
345.0500	345.0499	0.29
346.0534	346.0533	0.29
DNP-NAC		
330.0391	330.0390	0.30
331.0424	331.0424	0.00



SI Figure 5: Reconstructed ion chromatogram of the m/z=474.09 (DNP-SG) precursor ion (A) and MS2 accurate mass spectra of the monoisotopic fragment ions (B). m/z: mass-to-charge ratio.

Model output

C_{medium} over time		
hours after dosing	C_{PS} (mmol kg^{-1})	C_{medium} (mmol L^{-1})
0	0.000000	0.001817
2.51	0.000068	0.001816
5.01	0.000135	0.001816
7.52	0.000201	0.001816
10.03	0.000265	0.001815
12.53	0.000327	0.001815
15.04	0.000388	0.001814
17.55	0.000448	0.001814
20.00	0.000505	0.001814
24.00	0.000594	0.001813
	% medium depletion (AUC analysis)	**100.00%**
	$C_{medium,eq}$ (mmol L^{-1})	**0.0016571**



Calculation

C_{medium} (mmol L^{-1})	Start	0.001817
C_{medium} (mmol L^{-1})	End	0.001813
$Loss_{medium}$ (mmol L^{-1})		0.000004
$Loss_{medium}$ (%)		0.2

SI Figure 6: Kinetic model of the 1-chloro-2,4-dinitrobenzene (CDNB) concentration in T75 cell culture flasks filled with L15 medium supplemented with 5% FBS in a cell-free system. The concentration changes upon partitioning into plastic [4].

Generation of DNP-CG through gas-phase reactions in the ESI source

In some sample chromatograms we observed a double peak at a retention time of 8.6 and 10.6 min with the mass m/z=345.0500. This mass corresponds to the 1-chloro-2,4-dinitrobenzene (CDNB) biotransformation product 2,4-dinitrophenyl cysteinglycine (DNP-CG). However, since the CDNB biotransformation product 2,4-dinitrophenyl-S-glutathione (DNP-SG) elutes at 10.6 min, with exactly the same retention time (RT) as the second peak, we conclude that the m/z=345.0500 signal detected at RT of 10.6 corresponds to a DNP-SG fragment produced in the ESI source through breaking of the peptide bond.

References

(1) Tanneberger, K., Knobel, M., Busser, F. J., Sinnige, T. L., Hermens, J. L., and Schirmer, K. (2013) Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol 47*, 1110-1119.

(2) Fischer, M., Belanger, S. E., Berckmans, P., Bernhard, M. J., Bláha, L., Coman Schmid, D. E., Dyer, S. D., Haupt, T., Hermens, J. L. M., Hultman, M. T., Laue, H., Lillicrap, A., Mlnaříková, M., Natsch, A., Novák, J., Sinnige, T. L., Tollefsen, K. E., von Niederhäusern, V., Witters, H., Županič, A., and Schirmer, K. (2019) Repeatability and Reproducibility of the RTgill-W1 Cell Line Assay for Predicting Fish Acute Toxicity. *Toxicological Sciences 169*, 353-364.

(3) Stadnicka-Michalak, J., Knobel, M., Zupanic, A., and Schirmer, K. (2018) A validated algorithm for selecting non-toxic chemical concentrations. *ALTEX 35*, 37-50.

(4) Fischer, F. C., Cirpka, O. A., Goss, K. U., Henneberger, L., and Escher, B. I. (2018) Application of Experimental Polystyrene Partition Constants and Diffusion Coefficients to Predict the Sorption of Neutral Organic Chemicals to Multiwell Plates in in Vivo and in Vitro Bioassays. *Environ Sci Technol 52*, 13511-13522.