Supplementary Information

Mixtures of chemical pollutants at European legislation safety concentrations: how safe are they?

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SUMMARY OF SUPPLEMENTARY INFORMATION

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Table S1. Exceedance of AA-EQS in Europe (from Kodeš *et al.*, 2013). In addition the table gives typical chemical monitoring data in surface waters from the literature (average, 90th percentile and maximum concentrations).

Chemical substances	**AA-EQS (µg/L)**	**MAC-EQS (µg/L)**	**Exceedance of AA-EQS (µg/L)**	**Maximum concentrations (µg/L)**
Atrazine	0.6	2.0	Less than 5% of samples from PL and GB(Kodeš *et al.*, 2013) Per90: 0.006(Loos *et al.*, 2009)	0.046(Loos *et al.*, 2009)
BaP	0.00017 (0.05)[1]	0.1	Some samples from BE, PL, BA(Kodeš *et al.*, 2013)	
Cadmium	0.08	0.45	In several countries(Kodeš *et al.*, 2013)	
Chlorfenvinphos	0.1	0.3	Some samples from PL (25%)(Kodeš *et al.*, 2013)	
Chlorpyrifos	0.03	0.1	Only in PL 40% of samples(Kodeš *et al.*, 2013)	
DEHP (Bis(2-ethylhexyl) phthalate)	1.3	n.a.	Most of the samples from SK; FR, GB and PL less than 10%(Kodeš *et al.*, 2013)	
Diclofenac	0.1	n.a.	Per90: 0.043(Loos *et al.*, 2009) Ebro river (Spain); average 0.051(Gros *et al.*, 2009) Switzerland: Average 0.065(Kase *et al.*, 2011)	0.247(Loos *et al.*, 2009) 0.219(Gros *et al.*, 2009)
Diuron	0.2	1.8	PL: 30% of the samples(Kodeš *et al.*, 2013) Per90: 0.115(Loos *et al.*, 2009)	0.864(Loos *et al.*, 2009)
17beta-Estradiol	0.0004	n.a.	Median: 0.00019(Williams *et al.*, 2012)	0.00057(Williams *et al.*, 2012) < 0.000037(Esteban *et al.*, 2014)
Fluoranthene	0.0063	1.0	Some samples from BE and PL(Kodeš *et al.*, 2013)	
Isoproturon	0.3	1.0	About 50% of the samples from PL were close to EQS, while less than 5% of samples form IT, IE, BE, and BA (few samples) were above EQS(Kodeš *et al.*, 2013) Per90: 0.086(Loos *et al.*, 2009)	2.0(Loos *et al.*, 2009)
Ni	4	n.a.	Samples from RS, PL, MK, IT, CY, and BG were reported above EQS(Kodeš *et al.*, 2013)	
4-Nonylphenol	0.3	2.0	All reported concentrations were below EQS(Kodeš *et al.*, 2013) Per90: 0.268(Loos *et al.*, 2009)	4.5(Loos *et al.*, 2009)
Simazine	1.0	4.0	About 25% of the samples from PL and less than 5% from IT were reported above EQS(Kodeš *et al.*, 2013) Per90: 0.034(Loos *et al.*, 2009)	0.169(Loos *et al.*, 2009)
Carbamazepine	0.5		Average: 0.248(Loos *et al.*, 2009) Ebro river (Spain); average 0.019(Gros *et al.*, 2009)	11.6(Loos *et al.*, 2009) Ebro river (Spain): 0.060(Gros *et al.*, 2009)
Sulfamethoxazole	0.6		Average: 0.076(Loos *et al.*, 2009) Ebro river (Spain); average 0.011(Gros *et al.*, 2009)	4.1(Loos *et al.*, 2009) Ebro river (Spain): 0.050(Gros *et al.*, 2009)
Triclosan (Irgasan)	0.02		European surface waters	0.020(Xie *et al.*, 2008)

DEET	41		Germany; rivers of Hessisches Ried: 0.124[8]	1.3(Quednow & Puttmann, 2009)
Bisphenol A	1.5		Per90: 0.064 μg/L(Loos, R. *et al.*, 2009)	0.323(Loos, R. *et al.*, 2009)

BA: Bosnia Herzegovina; BE: Belgium; BG: Bulgaria; CY: Cyprus; FR: France; GB: Great Britain; IE: Ireland; IT: Italy; MK: Macedonia; PL: Poland; RS: Serbia; SK: Slovakia

[1]: AA-EQS value of Directive 2008/105/EC

n.a. – not applicable; Per90: 90th percentile

The objective of the Water Framework Directive (WFD) – to achieve good status by 2015 – will be met only in around half of the European waters. This applies for chemical and biological quality parameters for chemical and ecological status, respectively. Around 50% of European surface water bodies exceed at least one of the priority substances' Annual Average EQS (AA-EQS (or national EQS for the river basin specific pollutants)). However, most of the EQS exceedances (in some countries) are caused by only a small number of "ubiquitous" substances (mercury, cadmium, tributyltin, brominated diphenylethers (BDEs), PAHs (Σ Benzo(b)fluoranthene, Benzo(k)fluoranthene; Σ Benzo(g,h,i)perylene, Indeno(1,2,3-cd)pyrene), nickel, and DEHP). Maximum Allowed Concentration (EQS (MAC-EQS) levels are more often exceeded, but it is difficult to capture short-term pollution events under the normal sampling regime of WFD compliance monitoring (one sample per month)

Table S2. Bioluminescent reporter *E. coli* strains and their model toxicant

E. coli strain	Promoter	Type of stress sensed	Model toxicant/positive control (inducing concentration, mg/L)	Reference
RFM443	*recA*	DNA damage	Nalidixic acid (10)	[9]
RFM443	*katG*	Oxidative - peroxides	Hydrogen peroxide (10)	[10]
MG1655	*micF*	Oxidative - superoxides	Paraquat (500)	[9]
PHL	*zntA*	Excess Cd(II), Pb(II) and Zn(II)	$CdCl_2$ (50)	[11]
MG1655	*arsR*	Excess arsenic	Sodium arsenite (10)	[9]
DE112	*fabA*	Membrane damage	Phenol (1000)	Lab collection
DE112	*grpE*	General/protein damage	Ethanol (4%)	Lab collection
RFM443	*marR*	Antibiotics and phenolics (Oxidative stress)	Chloramphenicol (1)	[12]
RFM443	*cydA*	Respiratory inhibitors.	Na cyanide (10)	Lab collection
MG1655	*sodA*	Oxidative - superoxides	Paraquat (500)	[11]
MG1655	*yqjF*	Specific nitro aromatics	2,4 Dinitrotoluene (156)	[13]
MG1655	*soxS*	Antibiotic and oxidative stress agents	Paraquat (500) or Chloramphenicol (1)	[12]

Table S3. List of Primers used for qRT-PCR and the genes they are directed against.

Cell type	Gene symbol	Gene name	Primer (5'-3')
HeLa	*EF1a*	Elongation factor 1a	Forward: TCTGGTTGGAATGGTGACAA Reverse: ACGAGTTGGTGGTAGGATGC
	AR	Androgen receptor	Forward: GCGCCAGCAGAAATGATTGCACTA Reverse: ACACTGTCAGCTTCTGGGTTGTCT
	ERα	Estrogen receptor alpha	Forward: ACACATTTCTGTCCAGCACCCTGA Reverse: CACCACGTTCTTGCACTTCATGCT
	ERβ	Estrogen receptor beta	Forward: TGGAGTCTGGTCGTGTGAAG Reverse: GTCGGCACTTCTCTGTCTCC
	MT2A	Metallothionein 2A	Forward: AGGGCTGCATCTGCAAAG Reverse: GGTCACGGTCAGGGTTGTA
	CYP1A1	Cytochrome P450, family 1 subfamily A, polypeptide 1	Forward: AGTGGCAGATCAACCATGACCAGA Reverse: TGCATTTGGAAGTGCTCACAGCAG
	GSTK1	Glutathione S-transferase kappa1	Forward: ATCCAGAGATGCTGGAGAAAGCGT Reverse: CTTCACCTTTGGCGTTGCGATCTT
	COX2	Cyclooxygenase-2	Forward: AGGGTTGCTGGTGGTAGGAATGTT Reverse: AAGTGCTTGGCTTCCAGTAGGCAG
	IL-6	Interleukin-6	Forward: GCAGAAAAAGGTGGGTGTGT Reverse: GCAGAAGAGAGCCAACCAAC
	IL-8	Interleukin-8	Forward: CAGGAATTGAATGGGTTTGC Reverse: AAACCAAGGCACAGTGGAAC
	p53	Tumor supressor protein p53	Forward: GTCTTTGAACCCTTGCTTGC Reverse: CCACAACAAAACACCAGTGC
LMH	*EF1a*	Elongation factor 1a	Forward: CTGGATTGCCACACTGCTCACA Reverse: GATTTCAGGAACTTCGGGCCATCC
	AR	Androgen receptor	Forward: ACGAGTACCGGATGCACAAATCCA Reverse: TTCTGGTTCTTCAGGCCATCCACT
	ESR1	Estrogen receptor 1	Forward: TTTCCAGTGCTCACCCTGCATTTG Reverse: AGTCTCCAGCTCAGTGCCTTGAAT
	ESR2	Estrogen receptor 2 (beta)	Forward: ACGCACACCTCTGTCTGTTTCTGA Reverse: TCTTGCAGGACTGTTCTGAGGCTT
	MT	Metallothionein	Forward: GCACGTGTGGAGACAACTGCAAAT Reverse: ACAGCCCTTGGCACAGTTGTT
	CYP1A1	Cytochrome P450, family 1 subfamily A, polypeptide 1	Forward: GAGTTTGACCTTCAGCACCGACAC Reverse: TCGAAGCTCTGCTTCTCCTCCATC
	GST	Glutathione S-transferase	Forward: GCACGTGTGGAGACAACTGCAAAT Reverse: ACAGCCCTTGGCACAGTTGTT
	COX2	Cyclooxygenase-2	Forward: GCCTACTAGAAGTCGACCATCGCA Reverse: ACTCCTGGTCGAGTGGTGATGAAG
	IL-8	Interleukin-8	Forward: GATCCCTTGGAAGCCACTTCAGTC Reverse: GTCGGCATGAGCTGACTCTGACTA
	p53	Tumor supressor protein p53	Forward: CCTGCTTGATGGACGAGAGTTGGT Reverse: TGGTGACGTAGACGGACATGCT
ZFL	*ef1a*	Elongation factor 1a	Forward: GTACTACTCTTCTTGATGCCC Reverse: GTACAGTTCCAATACCTCCA
	ar	Androgen receptor	Forward: ACAACACACCTGGATGGGAGTGAT Reverse: TGACCTGTAGCAGCACAAACTCCT
	esr1	Estrogen receptor 1	Forward: AAGAACTCGTCCACATGATCGCCT Reverse: AGACTCCGAAATCGAGCCACAGTT
	esr2a	Estrogen receptor 2a	Forward: TACGACTTCAGCACTCTGCCCTTT Reverse: CCGCTTTACCAGTGGTTTGCTGTT
	esr2b	Estrogen receptor	Forward: TGTTCGAGTTTGCCACAGACTCCT Reverse: ACAGATGCTCGATGCCTTTGTTGC
	mt2	Metallothionein 2	Forward: CCTGCAAGTGCACTAATTGCCAGT Reverse: ACGCAGACGTGGAGTAGACAAACA

	cyp1A	Cytochrome P450, family 1 subfamily A, polypeptide 1	Forward: AGGCTGGTGATGGAGCATTACGAT Reverse: ATCGGACACTTGCAGGTTGGAGTT
	mgst1	Microsomal Glutathione S-transferase 1	Forward: GCACTTCCGGGTGTTTGTAGTGTC Reverse: GTGAGCACCCTGTAGGCCATAGAT
	cox2	Cyclooxygenase-2	Forward: CACTGTTGCCGGACAACTTTCAGA Reverse: TCCAGCAGTCTGTTTGGTGAAGGA
	il8	Interleukin-8	Forward: CAGGTGATCCGGGCATTCATGGT Reverse: AATGAGCTTGAGAGGTCTGGCTGT
	p53	Tumor supressor protein p53	Forward: AGTTAAGTGATGTGGTGCCTGCCT Reverse: ATCAGCTTCTTTCCCTGTTTGGGC

Figure S1

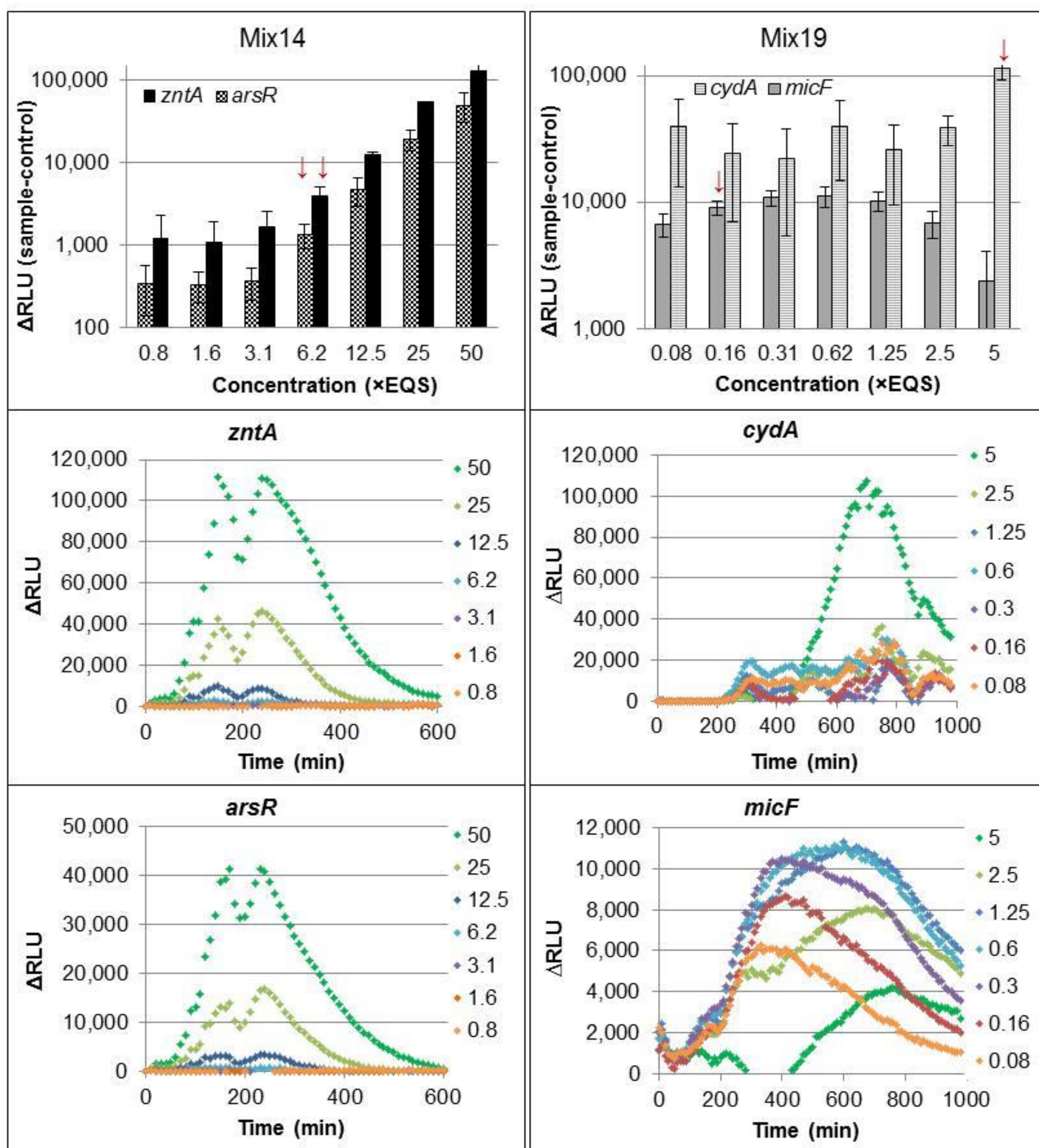

Figure S1. Induction of reporter genes in *E. coli*. Genetically modified *E. coli* strains harbouring different sensing elements fused to bioluminescent reporter genes (*luxCDABE*) were exposed to serial concentrations of Mix14 (left) and Mix19 (right). The top panels show the dose-dependent maximum response in logarithmic scale (over 500 min exposure for *zntA*, *arsR* and *micF* and over 800 min exposure for *cydA*) of the bacterial reporter strains that responded to the mixtures (lowest concentration detected is marked with a red arrow; p-values < 0.05). The response kinetics for these bacterial reporters is shown in the graphs below for the different concentrations tested. Data reflect the difference in luminescence intensity in the presence and absence of the test mix, presented in the microtiter plate reader's (Wallac Victor1) arbitrary luminescence units (ΔRLU). Error bars represent ±SD, *n*=3.

Figure S2

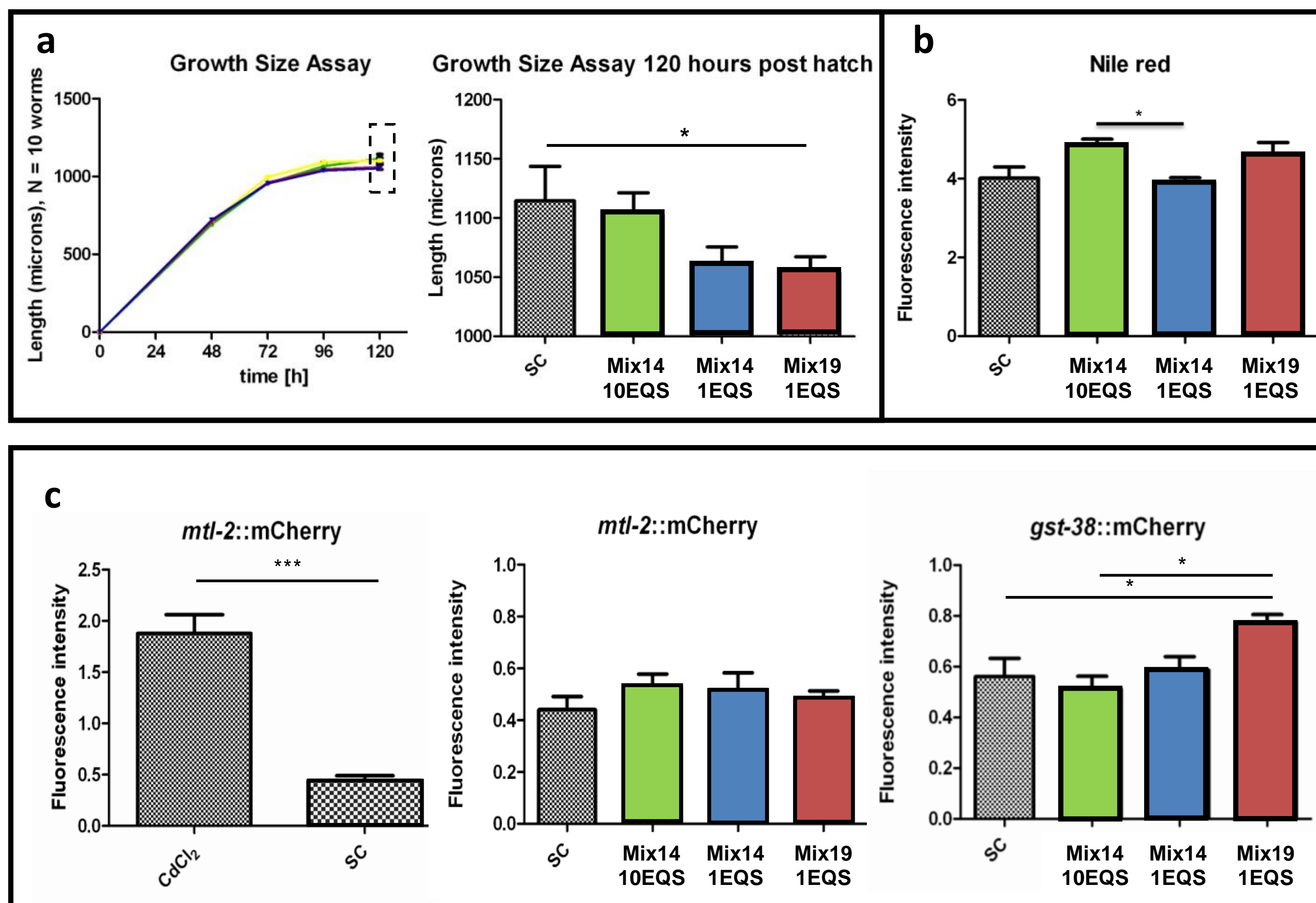

Figure S2. Nematode phenotype analyses. Nematodes were exposed to solvent control (SC) or to Mix14 (at concentration of single chemicals equivalent to 10× or 1×EQS) and Mix19. Changes in development (nematode length) was monitored over time (*n*=10 per condition) (Panel a). Growth, though uniform between exposures during the first 72 h, started to deviate at later time points. At 120 h, exposure to Mix19 induced a statistically significant inhibition ($p<0.05$) in development. (Panel b) Nile Red staining was used to visualize and quantify (using Image-ProExpress, *n*=10 per condition) the accumulation of lipids in storage compartments. (Panel c) A suite of fluorescently tagged transgenes were monitored in age synchronous nematodes exposed for 48h (from L1 to pre-adult L4 stage). Expression of *mtl-2* was strongly induced in worms exposed to 10 µM Cd (left panel) but not by the Mix14 or Mix19 (middle panel). In contrast *gst-38,* a glutathione-S-transferase involved in phase II detoxification was differentially expressed in nematodes exposed to Mix19 (compared to the solvent control). Values are arbitrary units following the baseline normalization with an invariant *unc-47*:GFP, where * = $p<0.05$, *** = $p<0.001$. Error bars represent ±SD. *n*=10.

Figure S3

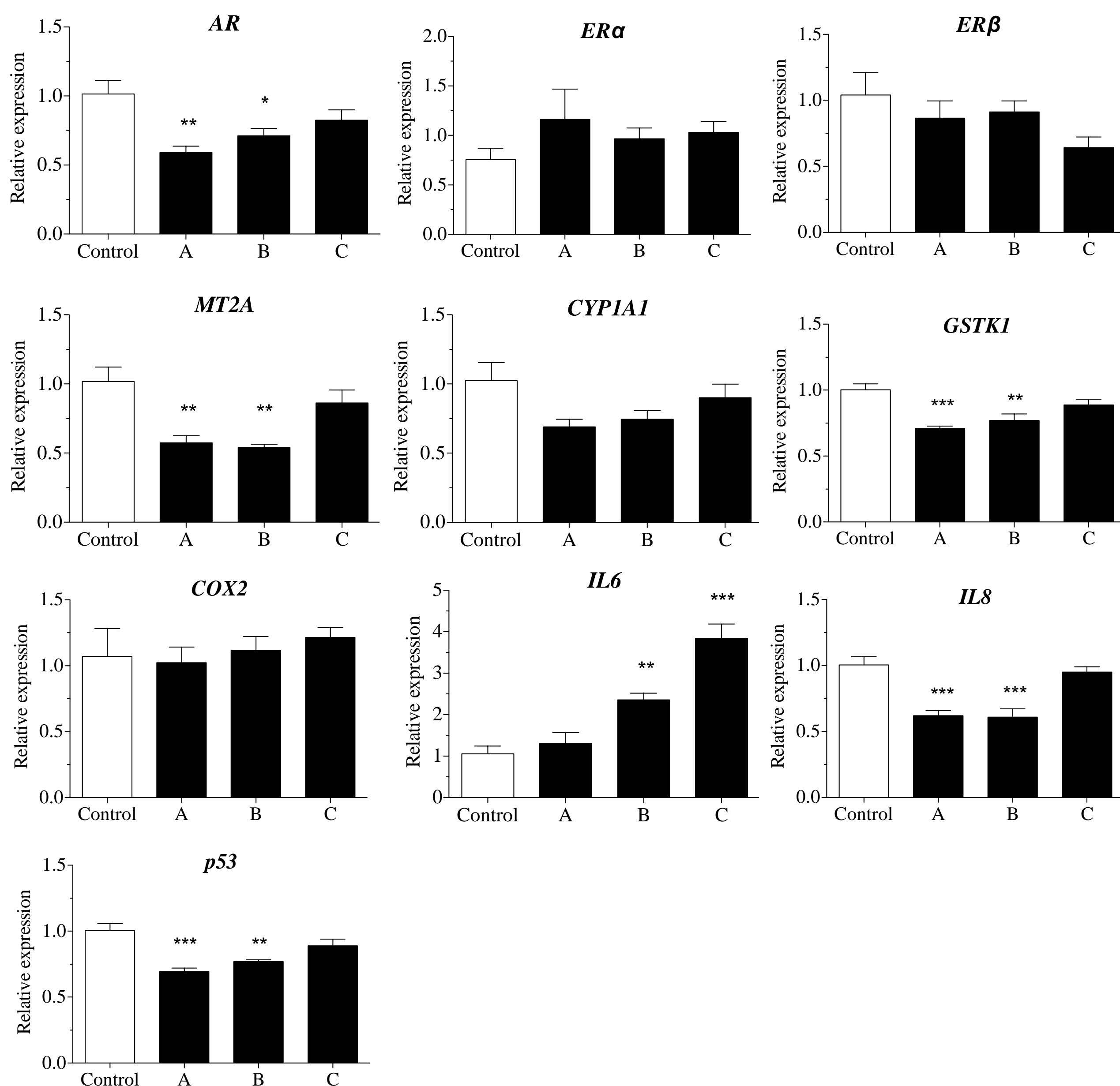

Figure S3. Regulation of gene expression in HeLa cells exposed for chemical mixtures. Cultured HeLa cells were treated for 24 h with mixtures Mix14 at 1×EQS and 10×EQS and Mix19 at 1×EQS. Thereafter was total RNA isolated followed by qRT-PCR analysis. Control cells are treated with the chemical solvent solutions and the expression level in the control are set to 1. The relative expression levels of 10 genes, normalized against *elongation factor 1a*, are shown. The statistical methods are ANOVA followed by Dunnet post test (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$). Error bars represent ±SD. *n*=4.

Supplementary Materials and Methods

Preparation of reference mixtures and stability study

Two chemical mixtures Mix14 and Mix19 were studied, containing 14 and 19 different compounds at concentrations equivalent to the Annual Average Environmental Quality Standard (AA-EQS), as listed in Table 1 of the main manuscript. The chemicals included in the mixtures were the regulated substances atrazine, diuron, isoproturon, simazine, benzo[a]pyrene, triclosan, fluoranthene, cadmium, nickel, DEHP, 17β-estradiol, 4-Nonylphenol, chlorphenvinphos and chlorpyrifos, and the emerging pollutants bisphenol A, carbamazepine, sulfamethoxazole, triclosan and DEET. The chemicals used for the preparation of the reference mixtures were of high purity (at least ≥ 96%) and were supplied by Sigma Aldrich, except for atrazine, which was supplied by Dr Ehrenstorfer.

A total of five reference materials (i.e. three organic and two inorganic reference materials) for Mix14 and Mix19 have been produced as 1000 and 10000-fold concentrated mixtures. The organic reference materials (in methanol) have been prepared separately from the inorganic ones (in 2% nitric acid in water). Additional reference materials were prepared for Mix14 as 10000-fold concentrated solutions, to allow the assessment of effects at a wider range of concentrations.
ISPRA RM040 Reference Organic Mixture (10000×EQS) (in Methanol)
ISPRA RM041 Reference Organic Mixture (1000×EQS) (in Methanol)
ISPRA RM042 Reference Organic Mixture (1000×EQS) (in Methanol)
ISPRA RM043 Reference Inorganic Mixture (10000×EQS) (in 2% Nitric acid)
ISPRA RM044 Reference Inorganic Mixture (1000×EQS) (in 2% Nitric acid)

A short-term stability study on the candidate reference materials was carried out to take into account any improper shipment and storage conditions during the exercise. The temperatures of -20±4 °C and 4±4 °C were chosen as reference temperatures (where degradation and instability was supposed to not occur) for the organic reference materials (i.e. ISPRA RM040, ISPRA RM041, ISPRA RM042) and the inorganic reference materials (i.e. ISPRA RM043 and ISPRA RM044), respectively.
The stability experiment was designed applying an isochronous study. According to this approach, all the samples were stored at the reference temperature and moved to the test temperature (i.e. 24 °C) in different times (i.e. 0, 1, 4 and 8 weeks). Therefore, all the samples

were analysed at the end of the study under repeatability conditions (ISO Guide 35; Lamberty et al., 1998; Linsinger et al., 2001) minimising the variations in analytical responses. The results are expressed as the ratio between the concentrations of samples stored at the test temperature for different time intervals (i.e. 0, 1, 4 and 8 weeks) and the concentrations observed in the samples that remained at the reference temperature for the entire study.
For this purpose, a total of 20 units (i.e. five units for each time interval) for each reference material were analysed once.

LC/MS analyses

For the analyses of the organic micropollutants an *Acquity*® ultra high pressure liquid chromatography (UHPLC) system (*Waters Corporation*, Milford, MA, USA) coupled to a hybrid triple-quadrupole linear ion trap mass spectrometer (5500 *QTRAP*®) with a turbo ion spray source from *AB SCIEX* (Foster City, CA, USA) were used. The *QTRAP*® system was operated for quantification of the target analytes in selected reaction monitoring (SRM) acquisition mode (MS/MS) with both positive and negative electrospray ionization. Unequivocal identification was provided by the acquisition of two SRM transitions per compound in most cases (Table S4). The protonated or deprotonated molecular ion of each compound was chosen as the precursor ion.

Ultra high pressure LC separations were performed with a reversed-phase BEH C18 analytical column (*Waters*; 50 × 2.1 mm, 1.7 µm). For the analyses performed in positive mode, the compounds were separated using Milli-Q water/methanol 95:5% (v/v), with 0.1% acetic acid employed as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B at a flow rate of 0.4 mL/min. The gradient elution started with 90% mobile phase A held for 1 min and then ramped within 8 min to 95% mobile phase B, held for 0.1 min, and then reverted to initial conditions via a 0.1 min ramp, allowing 2 min of stabilization time. The total analysis time was 12 min.

For the analyses performed in negative mode, the compounds were separated using Milli-Q water with 0.03% ammonium hydroxide employed as mobile phase A and methanol as mobile phase B at a flow rate of 0.3 mL/min. The gradient elution started with 65% mobile phase A held for 1 min and then ramped within 5 min to 50% mobile phase B, held for 2 min, then ramped up to 90% mobile phase B within 2 min and then reverted to initial conditions allowing 2 min of stabilization time. The total analysis time was 12 min.

The operating conditions for the analyses performed in both positive and negative ionization modes were as follows: ion spray voltage 4500 V; curtain gas 25 (arbitrary units); ion source

gases GS1 and GS2 were 55 and 45 psi, respectively; probe temperature 550 °C. Nitrogen served as nebulizer and collision gas.

Careful optimization of the compound-dependent MS parameters was performed for each chemical substance. Optimization of MS parameters (declustering potential (DP) and collision energy (CE)) was performed by flow injection analysis for each compound. The entrance potential (EP) for precursor ions and the collision cell exit potential (CXP) for product ions were not changed for any of the compounds because they had very little influence on the optimization process. They were set to default values of 10 (EP) and 11 or 13 (CXP). The declustering potential was optimized for each compound in order to obtain the maximum response for the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ion and to prevent in-source fragmentation or adducts. The table below summarizes the precursor ions and suitable MS/MS transitions selected by the optimization procedure. All data were acquired and processed using the *Analyst®* 1.6 software package.

Table S4. SRM operative parameters. Q1: parent ion (m/z); Q3: product ion (m/z), ID: analyte name; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: cell exit potential.

Q1	Q3	ID	DP	EP	CE	CXP
294	250	Diclofenac	-42	-10	-16	-11
294	214	Diclofenac	-42	-10	-29	-11
300	256	13C6-Diclofenac	-173	-10	-15	-11
227	212	Bisphenol A	-120	-10	-25	-11
227	133	Bisphenol A	-120	-10	-36	-11
239	224	13C12-Bisphenol A	-120	-10	-29	-11
271	143	Estradiol	-75	-10	-66	-11
271	145	Estradiol	-75	-10	-51	-11
273	147	13C2-Estradiol	-215	-10	-54	-11
287	35	Triclosan	-69	-10	-45	-11
287	142	Triclosan	-69	-10	-50	-11
299	35	13C12-Triclosan	-69	-10	-44	-11
237	194	Carbamazepine	250	10	28	13
237	165	Carbamazepine	250	10	60	13
247	204	Carbamazepine-d10	234	10	31	13
192	91	DEET	244	10	41	13
192	119	DEET	244	10	24	13
198	91	DEET-d6	80	10	42	13
233	72	Diuron	169	10	25	13
233	133	Diuron	169	10	53	13
240	78	Diuron-d6	156	10	24	13
254	156	Sulfamethoxazole	150	10	22	13
254	92	Sulfamethoxazole	150	10	38	13
260	98	13C6-Sulfamethoxazole	70	10	36	13

GC/MS analyses

Atrazine, Chlorfenvinphos, Chlorpyriphos, Chlorpyriphos, DHEP, Fluoranthene, Isoproturon, 4-Nonylphenol and Simazine analyses were carried out with a gas-chromatograph coupled with a single quadrupole mass spectrometer, (GC-MS, Agilent model 6890 coupled to an MSD 5975 inert mass-selective detector) operating with EI ionization in the selected ion monitoring (SIM) mode. The column used was a DB 5MS, 30 m × 0.25 mm ID and 0.25 µm film thickness. The carrier gas was helium at a constant flow rate of 1.2 mL/min. The injector was operated in splitless mode at a temperature of 270 °C. The GC oven was programmed as follows: 60 °C hold for 1 min; increase at a rate of 30 °C/min to 130 °C; hold at 130 °C for 1 min; increase at 7 °C/min to 180 °C; hold at 180 °C for 6 min, increase at a rate of 15 °C/min to 280 °C and hold at 280 °C for 0 min, increase at a rate of 20 °C/min to 310 °C and hold at 310 °C for 8 min. The interface temperature was maintained at 280 °C.

ICP-MS analyses

Ni and Cd were measured using a quadrupole ICP-MS Agilent 7500ce, equipped with octopole collision/reaction cell in order to minimize the isobaric and spectral interferences. The matrix effect was corrected using the internal standard technique. The instrumental conditions of the ICP-MS were according to the manufacturer’s instructions. External calibration and quality control were applied using reference solutions certified according to ISO Guide 34 and ISO 17025.

Table S5. Reconstitution of the concentrated reference materials (ISPRA RM). A final volume of 1L for the exposure mixture is given as an example.

	ISPRA RM040	ISPRA RM041	ISPRA RM042	ISPRA RM043	ISPRA RM044	Methanol	2% nitric acid	MQ water or buffer
Mix14 (10×EQS)	1 mL			1 mL				1L
Mix14 (1×EQS)		1 mL			1 mL			1L
Mix19 (1×EQS)			1 mL		1 mL			1L
Solvent Control						1 mL	1 mL	1L

C. elegans: development endpoints

Pharyngeal Pumping Food intake

Pharyngeal pumping was assessed in N2 nematodes (subjected to the above mentioned RMs) by counting pharyngeal bulb contraction over a time period of 2×30 sec. Pumping was assessed by means of high magnification Apochromatic Zoom and FusionOptics™ microscopy (Leica M205C) and quantified from 15 nematodes per condition.

Imaging for the Nile Red Assay

Single worms were picked onto a glass slide with a drop of M9 and immobilized with sodium azide (2%). The filter G-2A (Ex 510nm-560nm) was used to quantify the fluorescence. Images were captured with a Nikon DS-2Mv digital camera and NIS-Elements F 2.20 software linked to a Nikon ECLIPSE TE2000-S inverted microscope. The fluorescence intensities from 10 worms per condition were analysed using ImageJ.

Movement-Assay

The movement of wild-type nematodes (challenged with the respective mixtures) was assessed after 48 h, 72 h and 96 h by determining the distance travelled on agar within a 30 sec timeframe. Movement was quantified from 15 nematodes per condition using the Image-Pro Express software (Media Cybemetrics, Inc.).

Statistical analyses

Data obtained from the Nile Red staining were analysed using the one-way ANOVA followed by the Tukey's Multiple Comparison Test to test for significant differences between the RMs. The phenotypic assays were assessed by means of the two-way ANOVA. All tests were executed with Graphpad Prism.

Saccharomyces cerevisiae cytotoxic and genotoxic assay

Strains and growth condition

Strains and genotypes are described in the Table S6. The parental strain FYAK26/8-10B1 carries a deletion in the multidrug transporters PDR5 (pleiotropic drug response), SNQ2 (disruption confers sensitivity to 4-nitroquinoline-N-oxide) and YOR1 (yeast oligomycin resistance) (Schmitt *et al.*, 2005a).

Yeast cells were grown on minimal YNB medium (per 1 L of final solution): 1.7 g yeast nitrogen base (without amino acids and without (NH4)2SO4), 10.5 g citric acid buffer, and 0.5 g amino acid drop out mix. The pH was adjusted to 6.4. After autoclaving, glucose solution with a final concentration of 0.5% and 10 mL of a sterile solution of L-leucine (4

mg/mL) and histidine (2 mg/mL) were added to the growth medium. YNB was purchased from DIFCO, all amino acids, 4-nitroquinoline-*N*-oxide (4-NQO), and *N*-methyl-*N*-nitrosourea (NMU) were obtained from Sigma-Aldrich. All components were of analytical grade.

For cytotoxicity assessment growth inhibition assays were performed to determine EC_{50} values. For genotoxic test a transcription activation assay, involving the DNA damage inducible RAD54 promoter fused to the yeast enhanced green fluorescent protein (GFP) of *Aequorea victoria* (Cormack *et al.*, 1996), was used as a genetic marker for general toxicant-inducible DNA integrity damage. Thereby the induction of green fluorescence serves as the genotoxic endpoint (Mateus *et al.*, 2000).

Table S6. Yeast strains used in this study.

Name	Plasmid	Relevant Genotype	Origin
FYAK26/8-10B1 (sensitive mutant, parental strain	-	MATa ura3-52 trpΔ63 leu2Δ1 his3Δ200 GAL2+ pdr5Δ1::hisG snq2::hisG yor1::hisG	Kolaczkowski *et al.*, 1998
#261 (indicator strain: genotoxicity)	pY-$P_{RAD54-s}$-yEGFP3	As FYAK26/8-10B1 plus [$P_{RAD54-s}$-yEGFP3 URA3 LEU2]	AG Molecular Bioenergetics
#545 (control strain: genotoxicity)	pY-$P_{RAD54-s}$-yEGFP3Δ	As FYAK26/8-10B1 plus [$P_{RAD54-s}$ URA3 LEU2]	AG Molecular Bioenergetics
#544 (indicator strain: acute toxicity)	pY-P_{PMA1}-yEGFP3/PEST	As FYAK26/8-10B1 plus [P_{PMA1}-yEGFP3/PEST URA3 LEU2]	AG Molecular Bioenergetics
#549 (control strain: acute toxicity)	pY-P_{PMA1}-yEGFP3	As FYAK26/8-10B1 plus [P_{PMA1}-yEGFP3 URA3 LEU2]	AG Molecular Bioenergetics

Assay conditions and fluorescence monitoring

In all tests 96-well microtiter plates were used. For pre-cultures, cells were grown in liquid medium (10 mL) and incubated overnight at 30 °C at 250 rpm. The cell were centrifuged and resuspended in fresh medium at a final number of 1×10^6 cells/mL by measuring optical density (OD) of 0.25 AU at the Eppendorf Biophotometer Plus spectrophotometer.

10^6 cells/mL in a volume of 200 µL media/well, were exposed to 2 different mixtures, Mix14 and Mix19, at different concentrations (ranging from 1× to 250×EQS for Mix14, and from 0.5× to 25×EQS for Mix19). Positive controls were tested and they ranged from 1 to 1000 µg/L for 4-NQO, from 1 to 5000 µg/L for NMU and from 10 to 5000 µg/L for cadmium (Cd). The exposure were done at 8 h incubation (for growth and genotoxicity) and 4 h incubation (for acute toxicity) at 30 °C and continuously agitated at 120 rpm. Additional controls were: i) negative control cultures (YNB medium with yeast cells and 10 µL of solvent carrier) to indicate maximum proliferative capacity and unimpaired fluorescence intensity; ii) blank controls (compounds in appropriate concentrations and YNB medium

without yeast cells) to indicate endogenous compound absorbance and fluorescence; and iii) YNB medium to monitor potential contamination and medium absorbance and fluorescence (Schmitt *et al.*, 2004).

Fluorescence (λ_{ex} 485 nm; λ_{em} 535 nm) and turbidity (OD 600 nm) were obtained (Tecan Spectra Fluoro Plus) at time zero (start of the experiment). At the end of the incubation period fluorescence and absorption values were read out. Chronic and genotoxic toxic effects were calculated by growth inhibition (G_i) and fluorescence induction ratio (IR), respectively according to Schmitt *et al.* (2005b). Data were not reliable when Gi was >20%. For genotoxicity, a chemical was considered genotoxic when IR>1.4 (Schmitt *et al.*, 2005b).

***Vibrio fischeri* acute toxicity**

In the Microtox® test, the toxicity level of the mixtures was determined by a decrease in the bioluminescence emitted naturally by the bacterium *Vibrio fischeri*. The test was based on the ISO 11348-3 guideline. The microplate format of the assay was used. Each sample was adjusted to achieve neutral pH in the range 6.5-7.5. The initial luminescence of bacteria was recorded. Subsequently, samples, negative and positive controls were added into each well and luminescence was recorded after 15 and 30 minutes of exposure. 3,5-Dichlorophenol or potassium dichromate were used as positive controls. The evaluation of the generated data by fitting a dose response curve was carried out with GraphPad Prism 5 Software (La Jolla, California, USA). This fit provided EC_{50} and EC_{10} values.

Dictyostelium discoideum

The amoebic cells of *D. discoideum* (0.75×10^6 cells/mL) were incubated with 3 mL of each samples and 1 mL of AX-2 medium (culture medium). Cell viability and lysosomal membrane stability (LMS) were evaluated after 3 h of exposure while the replication rate was assessed after 24 h of treatment. Cell viability and replication rate were carried out as described in Dondero *et al.* (2006) and LMS as described in Sforzini *et al.* (2011).

Statistical analysis

The test was performed in four replicates and significant differences were tested using the non-parametric Mann-Whitney U-test.

Three spined stickleback *(Gasterosteus aculeatus (L.)* immune activities

Thirty adult sticklebacks (5.6 ± 0.2 cm, 1.9 ± 0.3 g), from one spawn, were obtained in home husbandry (INERIS, Verneuil-en-Halatte, France). Before experiments, the fish were maintained in a tank (50 L, 19 ± 1 °C, 350 µS/cm) with a 14/10 h light/dark cycle for one month. During this period, sticklebacks were fed daily with frozen red mosquito larvae and brine shrimp (3% of body weight/day; Europrix, France).

Leucocyte isolation

Each fish was sacrificed by cervical dislocation, measured and weighed. Spleen tissues were removed under aseptic conditions and gently pressed through sterilized nylon mesh (40 µm, Dutscher) with Leibovitz 15 (L15) medium (Sigma) containing heparin lithium (10 U/mL, Sigma), penicillin (500 U/mL, Biochrom AG) and streptomycin (500 µg/mL, Biochrom AG) to obtain leucocyte suspension. Then, leucocytes were adjusted at 10^6 cells/mL with Malassez haemocytometer to perform analyses.

Ex vivo exposures

For the *ex vivo* exposures, 30 leucocyte suspensions were used for each test. Each concentration was prepared freshly. Immediately after the mixture dilution process, for each leucocyte suspension and each mixture, 500 µL of leucocyte suspension were mixed in Micronics (Dutscher) with 2 µL of mixture for each concentration tested. In the same way, 2 µL of solvent was mixed with 500 µL of leucocyte suspension to obtain a solvent control using to check the quality of the leucocyte suspension. All samples (controls and leucocyte suspension mixed with pollutant) were incubated at 4 °C for 18 h until analyses.

Innate immune biomarkers analysis

Analyses were carried out on whole leucocytes, using a Cyan™ ADP flow cytometer (Beckman Coulter). For each leucocyte sample, 10000 cells were counted.

Leucocyte distribution was obtained using FSC and SSC parameters for size and complexity, respectively. Cellular mortality was detected using a double markedly method without any inhibitory effect on cellular function (Idziorek *et al.*, 1995). The YO-PRO®-1 (1 mM in DMSO, Invitrogen) and Propidium Iodide (PI, 1.5 mM in water, Invitrogen) were used in order to obtain cellular fluorescence parameters indicating the presence of apoptotic (FL1, green fluorescence) and necrotic (FL3, red fluorescence) leucocytes, respectively (Bado-Nilles *et al.*, 2014). These two markers allow ultrasensitive detection of double-stranded nucleic acids. Nevertheless, activation of P2X7 receptor in apoptotic cells enable penetration of YO-PRO®-1 (Baraldi *et al.*, 2004) in contrary to PI, which is excluded from viable cells due to their membrane impermeant characteristics. Cell necrosis and apoptosis were detected

after 10 min of incubation on ice with YO PRO®-1 (5 µM) and PI (7.5 µM) to limit potential dyes interference with cellular activities, membrane permeability and background staining.

Leucocyte respiratory burst, based on the technique described in Chilmonczyk and Monge (1999), was optimized for three-spined stickleback. Determination of reactive oxygen species (ROS) by unstimulated cell depends upon the cell incorporating 2'-7'-dichlorofluorescin diacetate (H_2DCF-DA, Sigma), which is a stable non-fluorescent molecule which is hydrolyzed to DCFH by cytosolic enzymes. When leucocytes are stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma), the more specific inductor of respiratory burst (Ambrozova *et al.*, 2011; Chadzinska *et al.*, 2012), H_2DCF-DA is also hydrolyzed by H_2O_2. Finally, the DCFH obtain is oxidised to the fluorescent dichlorofluorescein (DCF) to permit quantification by flow cytometry of unstimulated and stimulated cell. Stimulation index of respiratory burst was determined, after 30 min of incubation at room temperature, as the ratio of fluorescence of PMA stimulated cells (H_2DCF-DA at 60 µM plus PMA at 15 µM) to that of unstimulated cells (H_2DCF-DA at 60 µM).

The lysosomal membrane integrity (LMI) was detected as previously described in Bado-Nilles *et al.* (2013). Briefly, samples were incubated using AO (10 µM) during 20 min in the dark and at room temperature in order to obtain cellular fluorescence parameters indicating the presence of lysosomes with intact lysosomal membrane (FL3).

The phagocytosis activity was measured using Fluorescent microsphere (2.7×10^{10} particles/mL, Fluorospheres® carboxylate-modified microsphere, diameter 1 µm, Molecular Probes) as previously described in Gagnaire *et al.* (2006).

Statistical analysis

Results were expressed as means ± standard error with n = 30. Verification of normality and of homogeneity of covariance matrices (homoscedasticity) were conducted using respectively the Anderson–Darling test and the Bartlett test on XLStat 2008 (Addinsoft). If values were not normally distributed, the data was log-transformed using $F(x) = \log(x)$, prior to parametric analysis. Finally, a one-way ANOVA was used to assess the effect of each mixture at each concentration in relation to mean solvent control values. The Student Newman-Keuls's test was used for all multiple comparisons. All hypotheses were tested for statistical significance at the level of $p \leq 0.05$.

Atlantic salmon – regulation of molecular biomarkers

Exposure

Immature Atlantic salmon (*Salmo salar*, mean weight and length 10±2.5 g and 9±2 cm, respectively) were obtained from Lundamo hatcheries (Trondheim, Norway) and kept in 50 L tanks at 7±0.5 °C and for a 14/10 h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facilities. The experiment was performed after 24 h acclimation period. Five groups of 10 fish were exposed once for 5-days to Mix14 0.016×EQS, Mix14 0.16×EQS and Mix19 0.016×EQS, one group was exposed to the carrier vehicle methanol (0.01%) and one blank control. The final concentration of methanol was similar in all exposure groups. During the experimental period, fish were starved and duplicate sampling of 5 fish from each exposure group was sacrificed for gene expression and enzyme activity, respectively at days 3 and 5 after exposure. No fish mortalities were observed. Samples were collected from each exposure group after the fish were anaesthetized with benzocaine (5 mg/L) and blood was collected before sacrifice. After sacrifice, the liver was excised and weighed.

ELISA assay

The semi-quantitative Vitellogenin (Vtg) ELISA was performed according to standard protocol (Arukwe *et al.*, 1997). Plasma samples were diluted to 1:500 in coating buffer (0.05 M sodium-bicarbonate buffer, pH 9.5). The diluted samples were adsorbed to microtiter wells (overnight at 4 °C) and incubated with polyclonal rabbit Artic char Vtg antibody PO-1 or rabbit anti salmon zona radiata protein (Zrp) O-146 (diluted 1:2000 – Biosense Laboratories) for 1 h at 37 °C. Goat anti-rabbit (CYP1A) peroxidase-conjugated secondary antibody (GAR-HRP, Bio-Rad) diluted 1:3000 and H_2O_2/o-phenylenediamine dihydrochloride (OPD) were used for ELISA detection at 492 nm using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA).

RNA purification and cDNA synthesis

Total RNA was purified from liver tissues homogenized in Trizol reagent according to manufacturer's protocol. Total cDNA for the real-time PCR reactions were generated from 1 μg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

Quantitative (real-time) polymerase chain reaction (PCR)

Quantitative (real-time) PCR with gene sequence primer pair was used for evaluating Vtg or ERα gene expression profiles. For each treatment, the expression of Vtg, ERα or Zrp was

analyzed as described in Arukwe *et al.* (2005), using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25 µL DNA amplification reaction contained 12.5 µL of iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 1 µL of cDNA and 200 nM of each forward and reverse primers (Vtg: forward-aagccacctccaatgtcatc; reverse-gggagtctgtcccaagacaa: or ERα: forward-tccaggagctgtctctccat; reverse-gatctcagccataccctcca: or Zrp: forward-tgacgaaggtcctcaggg; reverse-agggtttggggttgtggt). The 3 step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 sec), 57- (Vtg) or 55 °C (ERα and Zrp) (30 sec), and 72 °C (30 sec), followed by a melting analysis at 95 °C for 1 min, 55 °C for 30 sec and thereafter decreasing fluorescence detection with increasing temperature between 55-95 °C. Controls lacking cDNA template (minus RT sample) were included to determine the specificity of target cDNA amplification as described previously in Arukwe *et al.* (2005). Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve was based on equal amplification efficiency with unknown samples and this is usually checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated using known amounts of plasmid containing the Vtg, ERα or Zrp amplicon.

Tetrazolium MTT test

The MTT assay is a basal cytotoxicity assay that assesses the ability of cells to reduce the yellow tetrazolium salt (MTT) to formazan. This ability indicates an integrity and functionality of the cellular mitochondria.

MTT test with human cells

Two cell lines, namely RPTEC/TERT1 and HUVEC/TERT7, overexpressing the catalytic subunit of human telomerase (hTERT) have been used in this exercise. hTERT immortalized cells by stabilizing/elongating the lengths of telomeres in the cells (Bodnar *et al.*, 1998; Harley *et al.*, 1990; Olovnikov *et al.*, 1996) keeping the cell specific characteristics of the normal, mortal counterparts (Chang *et al.*, 2005; Wieser *et al.*, 2008). Additionally, two commonly used human cell lines, HepG2 and MCF-7 are included within this study, representing liver and breast cancer epithelium, respectively.

RPTEC/TERT1, human renal proximal tubular epithelial cells (Evercyte GmbH) were cultivated in ProxUp medium (Evercyte GmbH). *HUVEC/TERT7: human umbilical vein endothelial cells* (Evercyte GmbH) were cultivated in EndoUp-2 medium (Evercyte GmbH) in culture dishes pre-coated with gelatine (1% in PBS). *HepG2, hepatocellular* carcinoma *cells* (ATCC) were cultivated in DMEM/Ham´F F12 (Biochrom) supplemented with 4 mM L-Glutamin (Biochrom) and 10% FCS (PAA). *MCF-7 breast cancer cell line* (ATCC) were cultivated in MEM Earls Salt (Biochrom) supplemented with 2 mM L-Glutamin (Biochrom), 1 mM Na-pyruvate (Biochrom), 1x non-essential aminoacids (Biochrom), 0,01 mg/mL Insulin (Sigma) and 10% FCS (PAA).

Exposure

Cells were seeded in the inner wells of a 96-well plate. The outer wells were filled with 200 µL PBS to avoid evaporation of the medium. When the cells had reached about 70-90% confluent, treatment medium was applied to the cells. Incubation time was set to 24 h at 37 °C and 5% CO_2. Thereafter, 10 µL/well MTT-solution (2 µg/µL, Promega) were added and cells were incubated for another 4 h. 100 µL of 10% SDS in 0.01 M HCl were added to the wells. The plates were incubated for another 20 h; then the absorption was measured at 570 and 690 nm with an Infinite® M200 microplate reader (Tecan). The value for the reference wavelength of 690 nm was then subtracted from the absorption at 570 nm.

MTT test with RTG-2 Rainbow trout gonad cell

The method used for the assay was developed on the basis of the Protocol No. 17 INVITTOX ECVAM and scientific literature (Caminada *et al.*, 2006; Davoren *et al.*, 2007; Jin *et al.*, 2010; Twentyman *et al.*, 1987).

The test samples Mix14 and Mix19 and the solvent control were reconstituted in the culture medium of the cells. The pH of the mixtures and that of the solvent was not compatible with cell survival and was therefore adjusted to pH = 7.2 -7.6.

The measurements of absorbance (Abs) were performed with the spectrophotometer TECAN Infinite F200Pro at the wavelength of 570 ± 10 nm (shaking duration: 5, multiple reads per well: circle filled 5x5, number of flashes per well: 3) and correct with respect to white.

The toxicity was expressed as the percentage of cell survival after 72 h of exposure. Cell survival was determined by the ratio: [(Abst / ABSC) x100]. Abst = average value of Abs in the treatments; ABSC = average value of Abs in the controls.

The compliance of the procedure was evaluated by performing a test with a reference toxicant cadmium chloride monohydrate ($CdCl_2$). Table S7 shows the values of internal reference for this toxic and interlaboratory variability (n = 6).

Table S7. Internal reference values for $CdCl_2$ in the MTT assay.

		Mean (n= 6)	ST	CV%
EC_{50} (72 h)	mg/L	15.96	4.40	6.29
lim inf 95%	mg/L	6.23		
lim sup 95%	mg/L	25.64		

Neutral red (NR) test

For analysis of acute cytotoxicity, the H4IIE-luc cells were used as a model. The H4IIE-luc were plated in DMEM-F12 with phenol red (Sigma Aldrich, USA) containing 10% fetal calf serum and the density of cells were 15000 per well. After 24 h cells were exposed to samples. At the end of the test, exposure medium was removed and 100 µL of medium with neutral red (0.05 mg/mL) is added to each well. After 1 h of incubation, medium with neutral red is removed and lysis buffer for neutral red is added. Plates are shaken for 20 minutes and absorbance is measured at 570 nm.

xCELLigence systems

Isolation of primary cultures of hepatocytes from Juvenile Atlantic salmon (Salmo salar L.)
Hepatocytes were isolated from 8 experimentally reared juvenile Atlantic salmon (288-375 g) with a two-step perfusion method previously described in Søfteland *et al.* (2009). Cell viability was determined by the Trypan Blue exclusion method, according to the manufacturer's protocol (Lonzo). The cell suspensions were plated on 2 $\mu g/cm^2$ laminin (Sigma-Aldrich, Oslo, Norway) coated culture plates (TPP, Trasadingen, Switzerland), and the hepatocytes were kept at 10 °C in a sterile incubator without additional O_2/CO_2 (Sanyo, CFC FREE, Etten Leur, Netherland). Cell concentrations of 0.2×10^6 cells per well in xCELLigence 96-well plates (in 0.2 mL complete L-15 medium) were used.
Chemical exposure
The primary cells were cultured for 36-40 h prior to chemical exposure with a change of medium (containing 10% fish serum (FS)) after 18-20 h. The cells were exposed for 24 h to

solvent control, Mix14 (1× and 10×EQS) and Mix19 (1×EQS). Cells from four fish were used per treatment. The exposure medium contained 1% FS and was substituted with new medium after 18-20 h and the chemical exposure was sustained for another 24 h.

Cytotoxicity assessment

Real time impedance data obtained by the xCELLigence systems (Roche Diagnostics, Oslo, Norway) was applied to assess cytotoxicity of the chemical mixtures. The xCELLigence system quantifies electrical impedance across electrodes in 96-well cell culture E-Plates. The impedance measurement gives quantitative information regarding cells' biological status including morphology, cell number and viability. The real time cell monitoring was conducted at 10 °C in an incubator without additional O_2/CO_2 (Sanyo, CFC FREE, Etten Leur, Netherland), using the RTCA single plate xCELLigence platform. The data was collected with intervals of 2 min after contaminant exposure for 12 h, then every 15 min for 120 h. The cell index (CI) is a parameter that is derived from the measured cell-electrode impedance data that quantifies the status of the cells, the CI values presented here were calculated from 4 replicate values.

Data analysis

GraphPad Prism 6.0 software was used for the statistical analyses of the xCELLigence response curves using one-way analysis of variance together with a Dunnett's post hoc test ($p < 0.05$) to detect treatment variation in contaminant-exposed hepatocytes. Mean±SE were calculated for four replicates (n=4).

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