Joint Danube Survey 3

icpdr iksd
International Commission for the Protection of the Danube River
Internationale Kommission zum Schutz der Donau

Chapter (summary report) on: Large volume sampling and effect-based screening

Version: 1.0 rev
Date: 26.08.2014

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Imprint

Published by:
ICPDR – International Commission for the Protection of the Danube River

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1 Introduction

Many organic compounds and their transformation products occur in waters and may pose a risk to human and environmental health. Their chemical structures are often unidentified and they are mainly present in low concentrations with an unknown contribution to mixture toxicity effects (Escher et al. 2014, Escher et al. 2013, Umbuzeiro et al. 2011). Therefore, traditional water monitoring using priority lists or river basin specific compounds is increasingly supplemented by multi-target, non-target and bioanalytical techniques (Hecker and Hollert 2009, Krauss et al. 2010, Richardson and Ternes 2011, 2014). These approaches aim to unravel adverse effects potentials and link them to known, unknown or so far neglected compounds (e.g., transformation products) in a non-deterministic manner (Brack 2003). Effect-based screening is therefore an important prerequisite for a holistic and risk-based river basin management to support the WFD (Brack et al. 2014, Brack et al. 2009, Malaj et al. 2014).

The effect-based screening in highly diluted large rivers such as the River Danube requires significant pre-concentration and the extraction of large water volumes for subsequently applying a large number of different bioassays and multi-target analysis. At the same time the transport to the laboratory and the preparation of extracts of large water volumes are a big challenge. Therefore, a newly developed mobile large-volume extraction device (LVSPE) was used to extract water samples of up to 1000 litres on-site during the JDS3 (Scholz 2013, Schulze et al. 2014).

The extracts were analysed for 264 water phase relevant organic compounds using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) in support of the effect-based screening with a set of different *in vitro* and *in vivo* bioassays. The bioassays cover the following endpoints:

- *Organism based bioassays (in vivo)*
    a. *algal growth inhibition (biological quality element)*
    b. *algal photosynthesis inhibition (biological quality element)*
- *Cell-based bioassays (in vitro)*
    a. *(anti-)estrogen-like activity (female sex hormone system)*
    b. *(anti-)androgen-like activity (male sex hormone system)*
    c. *glucocorticoid-like activity (development, metabolism, immune response)*
    d. *thyroid hormone-like activity (metabolism, development)*
    e. *mutagenic activity (damages of genes and cells)*
    f. *adaptive stress responses (protective response to chemicals)*
    g. *dioxin-like activity (xenobiotic metabolism, chronic)*
    h. *pregnane X receptor mediated activity (xenobiotic metabolism)*
    i. *acetylcholinesterase inhibition (neurotoxicity)*

The algae bioassays refer directly to a biological quality element and thus represent aquatic ecosystem relevant endpoints. The selected cell-based bioassays present important steps in toxicity pathways; for

example induction of xenobiotic metabolism, specific and reactive modes of toxic action, and activation of adaptive stress response pathways (Escher et al. 2014).

The main objective of the LVSPE sampling was to enable combined biological and chemical analysis. However, multi-target enrichment methods have typically recoveries below 100% for some compounds. Thus, the parallel usage for biological and chemical analyses does not allow the compensation of compound losses during *on-site* extraction and sample handling for example by using isotope labelled internal standards. Thus, some uncertainness needs to be accepted for the benefit of parallel chemical and biological analyses. We estimate this uncertainty by comparison with direct analysis of water samples. The JDS3 was an excellent platform to demonstrate the feasibility of an effect-based screening at a river basin scale.

2 Methods

2.1 Large volume solid phase extraction (LVSPE)

2.1.1 Principles of LVSPE

The LVSPE consists of a vacuum sampling system (borosilicate glass vessel connected to a membrane pump), a filtration cartridge to remove residual suspended particulate matter (glass fibre deep filter with a size cut-off of <0.63 µm), and extraction cartridges filled with different solid phases to adsorb dissolved semi-polar to polar organic compounds (Schulze et al. 2014).

A water volume of 500 mL per sampling step was taken using the vacuum system. The filtration unit was mounted in the inflow tubing. The water was released to a stainless steel chamber from the sampling vessel. In the steel chamber, the water was pressurised and pumped through the extraction cartridges, which were mounted in sequence (**Error! Reference source not found.**).

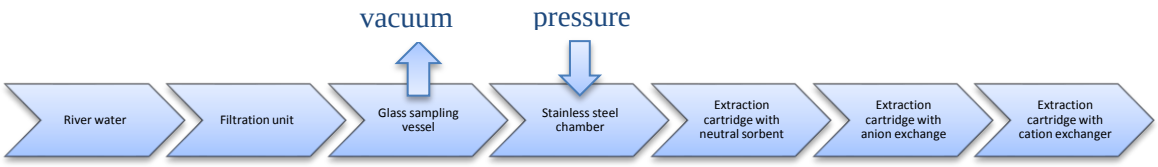


Figure 1 Flowchart of the large –volume extraction device

The first, neutral sorbent was a polystyrene-divinylbenzene co-polymer (PS-DVB; 160 g Chromabond® HR-X, Macherey Nagel, Düren, Germany) for the extraction of neutral polar to semi-polar organic compounds. The second sorbent was a weak anionic exchanger (100 g Chromabond® HR-XAW) based on the PS-DVB sorbent for the extraction of acidic compounds, which are anionic at the typical pH of surface water. The third sorbent was a weak cationic exchanger (100 g Chromabond® HR-XCW) also based on the PS-DVB sorbent for the extraction of basic compounds that are cationic at a water pH of 6-8.

2.1.2 Sampling

The collection of LVSPE samples was performed at 22 sampling sites during the JDS3 transect including 5 tributaries (Table 3). Total volumes of 650 litres of water were collected within 1 hour after reaching the sampling site, centrifuged for suspended particulate matter (SPM) (see chapter 22) removal and then stored in a stainless steel chamber. A total of 500 litres was extracted. At two sites (JDS 33 and JDS 57), samples of 1000 litres were extracted for additional experiments. The samples were stored in isolation boxes for transport at approximately 10 °C. At UFZ, the samples were maintained at 4 °C until further preparation.

2.1.3 Sample processing

Freeze-dried solid phases were extracted with ethyl acetate and methanol in series (neutral sorbent), methanol containing 2% of 7N ammonia in methanol (weak anion exchanger) and methanol with 1% formic acid (weak cation exchanger). The extracts were combined, neutralised, filtered (GF/F, Whatman) to remove remaining precipitates and reduced in volume to a final concentration factor of 1000 for aliquotation. For further analysis, aliquots were reduced until dryness using rotary (40 °C water bath temperature) and nitrogen evaporation. All freeze-dried samples and extracts were stored at -20 °C.

2.2 Chemical analysis with liquid chromatography coupled to high resolution mass spectrometry

Aliquots of the extracts for chemical analysis were reconstituted in methanol to a concentration factor of 1000 of water sample corresponding to 1 mL of final extract. Before analysis, a mixture of 38 isotope-labelled internal standards was added.

The chemical screening was conducted by liquid chromatography-high resolution mass spectrometry (LC-HRMS) using an Agilent 1200 LC coupled to a Thermo LTQ Orbitrap XL. Samples were analysed by positive and negative mode electrospray ionization at a nominal resolving power of 100,000 (Hug et al. 2014). For calibration, 1 L water sample aliquots from a pristine streamlet (Harz mountains, Germany) were spiked with a mixture of the target compounds at seven concentration levels between 1 and 1000 ng/L and extracted using a multilayer SPE cartridge containing 200 mg of Chromabond HR-X, 100 mg of Chromabond HR-XAW, and 100 mg of Chromabond HR-XCW, eluted as described above and extracts adjusted to a final concentration factor of 1000.

With the LC-HRMS target screening, altogether 264 compounds could be analysed, covering a wide range of compounds from different sources and chemical classes including pesticides, biocides, pharmaceuticals, industrial chemicals, artificial sweeteners, UV filters, and surfactants.

2.3 Bioanalysis with *in vitro* and *in vivo* bioassays

2.3.1 Bioassays

Aliquots of the extracts were tested in the bioassays detailed in

Table 1. Most samples were tested with a highest concentration level of relative enrichment factor (REF) of 500 and in dilution series to obtain full dose-response curves. Fabrication and solvent blank samples were tested in parallel using the same relative enrichment factors.

2.3.1.1 Growth inhibition and Photosystem II inhibition of Chlamydomonas reinhardtii

The growth inhibition of green algae represents a standardised acute toxicity endpoint used in chemical risk assessment (OECD 2011) and has been expanded to include Photosystem II (PSII) inhibition (Nestler et al. 2012). Green algae are often among the most sensitive whole organism assays and thus used as a biological quality elements in the WFD. In this study the algae *Chlamydomonas reinhardtii* was used.

2.3.1.2 Mutagenicity

The Ames test is the most widespread test for the detection of the mutagenic potential of chemicals and environmental mixtures (Reifferscheid et al. 2012). The method bases on the chemically-induced reversion of auxotrophic *Salmonella typhimurium* mutants to prototrophic metabolism (Ames et al. 1975). To detect possible bioactivation of substances, rat liver homogenate and cofactors (S9-mix) can be added to the test system to simulate exogenous biotransformation potential (Maron and Ames 1983). We used the Ames fluctuation assay with the tester strain *Salmonella thyphimurium* TA98 with and without S9 accordingly to Reifferscheid et al. (2012).

2.3.1.3 Adaptive Stress Responses

The adaptive stress response pathways are key players in controlling the cell homeostatis and / or for repairing damages by transcriptional activation of cytoprotective genes (Simmons et al. 2010). In order to investigate the p53 mediated apoptosis in response to deoxyribonucleic acid damage, the p53-bla HCT-116 gene-reporter assay was used (Yeh et al. 2014). The NF-κB-bla THP-1 gene-reporter assay was used to analyse the samples for induction of inflammation (Invitrogen 2009). The ARE-bla Hep G2 gene-reporter assay was performed to investigate the samples for the inductions of the Nrf-2 mediated oxidative stress pathway (Invitrogen 2006).

2.3.1.4 Estrogen Receptor

The MELN assay was employed to assess the presence of substances able to interact with and activate the human estrogen receptor (ER), and thus presenting estrogenic activity in the samples (Balaguer et al. 1999)

2.3.1.5 Aryl hydrocarbon Receptor

CAFLUX assay was employed to assess dioxin-like (aryl hydrocarbon receptor - mediated) toxicity. Chronic adverse effects of xenobiotics such as interference with liver functions, immunity, endocrine and nervous system as well as embryo toxicity and carcinogenicity were experimentally related to Aryl hydrocarbon receptor (AhR)-dependent events (Janošek et al. 2006).

2.3.1.6 Pregnane X Receptor

In this study, we explored the potential use of the HG5LN-hPXR assay (Lemaire et al 2006) as a detector of PXR-active substances in JDS samples. The human pregnane X receptor (PXR) is a nuclear receptor that plays a crucial role in detoxification processes by mediating the transcription of genes that code for xenobiotic biotransformation enzymes. As such, PXR is a molecular target for a wide range of xenobiotics including pharmaceuticals, pesticides, steroids, phthalates or alkylphenols, though at relatively high concentrations, i.e. in the µg/L range and upper (Creusot et al. 2010, Lemaire et al. 2006, Mnif et al. 2007).

2.3.1.7 Glucocorticoid Receptor

The GR CALUX assay was performed to assess the glucocorticoid-like (glucocorticoid receptor – mediated) activity of the JDS3 samples (van der Linden et al. 2008). Glucocorticoids are important

steroid hormones controlling metabolism, immune responses and inhibition of inflammation as well as cellular proliferation (Sonneveld et al. 2007, van der Linden et al. 2008).

2.3.1.8 Thyroid Receptor

The potencies of the JDS3 samples to activate the thyroid hormone receptor – which plays a key role in growth, development and energy homeostatis – was investigated by employing the GH3-TRE-luc assay (Freitas et al. 2011).

2.3.1.9 Acetylcholinesterase (AChE) inhibition

The in vitro inhibition of acetylcholinesterase (AChE) is a rapid assay to determine the chemical interference with the enzymatic conversion of AchE to acetyl and choline in the neuronal synaptic cleft. The measurement of AChE inhibition has predominantly been associated with the neurotoxicity of organophosphate and carbamate insecticides, but may also be affected by other organic compounds of unknown structure (Holth and Tollefsen, 2007).

Table 1 Summary of the bioassays, the modes of action and the principles of the assays

Bioassay	Mode of action of active chemicals	Principle of the assay	Reference
Growth inhibiton test (GI)	Algal growth inhibition	Quantification of growth and growth inhibition of *Chlamydomonas reinhardtii* algal biomass as a function of time; 96-well microplate, 24, 48 and 72 h incubation	(OECD 2011)
Photosystem II (PSII) efficiency	Photosystem II (PSII) inhibition	Measurement of maximal PSII photochemical efficiency in *Chlamydomonas reinhardtii*; 96-well microplate, 24, 48 and 72h incubation	(Nestler et al. 2012)
Ames fluctuation assay (TA98)	Mutagenic activity (frame shift mutation)	Quantification of the mutagenic activity in TA98 cell lines of *Salmonella thyphimurium with and without S9* ; 384-well microplate, 24 h exposure	(Reifferscheid et al. 2012)
p53-bla HCT-116 (p53)	p53 mediated apoptosis in response to DNA damage (adaptive stress response)	Beta-lactamase reporter gene activation upon activation of the p53 receptor in HCT-116 cells; cytotoxicity measured in parallel using resazurin; 384- well microplate, 40 h incubation	(Yeh et al. 2014)
ARE-bla Hep G2 (ARE-BLA)	Induction of the Nrf-2 mediated oxidative stress pathway (adaptive stress response) in a metabolically active cell line	Beta-lactamase reporter gene activation upon activation of the Nrf-2 mediated oxidative stress pathway in Hep G2 cells; cytotoxicity measured in parallel using resazurin; 384- well microplate, 15 h incubation	(Invitrogen 2006)
NF-κB-bla THP-1	Induction of inflammation (adaptive stress response)	Beta-lactamase reporter gene assay to induce inflammation in THP-1 cells; cytotoxicity measured in parallel using resazurin; 384- well microplate, 24 h incubation	(Invitrogen 2009)
MELN (ER, anti-ER)	Binding to and activation of the human estrogen receptor (ER)	Luciferase reporter gene activation upon activation of the ER receptor in MCF-7 cells; 96-well microplate, 24h incubation	(Balaguer et al. 1999)
GR Calux®	Binding to and activation of the human glucocorticoid receptor (GR; development, metabolism, immune response)	Chemical activated Luciferase gene expression (Calux®) activation upon activation of the GR receptor in U2OS cells; 96-well microplate, 24 h incubation	(van der Linden et al. 2008)
GH3-TRE-luc	Induction of thyroid hormone receptor (metabolism, development)	Beta-lactamase reporter gene activation upon activation of the GH3 receptor in TRE- luc cells; cytotoxicity measured in parallel using resazurin; 96- well microplate , 24 h incubation	(Freitas et al. 2011)
CAFLUX (AhR)	Activation of aryl hydrocarbon receptor (AhR; xenobiotic metabolism, dioxin-like activity)	GFP reporter gene activation upon activation of AhR in H1G1.1c3 cells; 96-well microplate , 24h incubation	(Aarts et al. 1998)
HG5LN-hPXR	Binding to and activation of the human pregnane X receptor (PXR; xenobiotic metabolism)	Luciferase reporter gene activation upon activation of the PXR receptor in HeLa cells (GAL4RE-Luc/GAL4-hPXR); 96-well microplate, 24 h incubation	(Lemaire et al. 2006)
***In vitro* acetylcholine esterase (AChE) activity**	Acetylcholinesterase (AChE) inhibition	Characterisation of AChE activity (enzyme kinetics and enzyme saturation studies) following the production of 5-thio-2-nitrobenzoic acid, 96 well microplate, 3h incubation	(Holth and Tollefsen 2012)

2.3.2 Classification of bioanalytical results

The preliminary results of biotesting were classified in two ways: A qualitative classification of samples where no quantitative data were available yet and a semi-quantitative classification for bioassay where preliminary results were available.

- *Qualitative classification (A)*

E	*effect*
P-	*weak effect*
N	*no effect (effect similar to blank or no effect at maximum tested REF)*
C	*negative effect (cytotoxicity masking induction)*
C-	*weak cytotoxic effect*

A semi-quantitative classification was applied to the bioassays indicative of adaptive stress responses. This classification is preliminary and is based on the effect concentration causing an induction ratio (IR) of 1.5 ($EC_{IR1.5}$), in units of relative enrichment factor (REF). The REF is the dimensionless quotient of all concentration and dilutions steps during the preparation and biotesting of the samples and is used as the dose-metric for concentration-effect curves (Escher et al. 2013). The REF, at which the effect threshold of an IR of 1.5 is exceeded, is dependent on, both, the assay and the sample potency (Escher et al. 2014). Therefore the effect in relation to the effect of a blank sample (ultrapure water treated in the same way as the samples) was used to classify the strength of effect. In the case that the blank sample did not induce an effect in the assay, the effect of the sample was compared to the maximum REF used in the assay.

- *Semi-quantitative classification (B):*

E+	*strong effect ($EC_{IR1.5}$ of sample more than 100 times lower than $EC_{IR1.5}$ of the blank, i.e., sample 100 times more potent than the blank)*
E	*effect ($EC_{IR1.5}$ of sample more than 10 times lower than $EC_{IR1.5}$ of the blank, i.e., sample 10 times more potent than the blank)*
E-	*weak effect ($EC_{IR1.5}$ of sample up to 10 times lower than $EC_{IR1.5}$ of the blank, i.e., sample less than 10 times more potent than the blank)*
N	*no effect (effect similar to blank or no effect at maximum tested REF)*
C	*negative effect (cytotoxicity masking induction)*

3 Results

3.1 Results of chemical screening

Of the 264 compounds which were analysed, 91 could be detected in at least one sample. Method detection limits determined based on the replicate analysis of calibration standards were below 2 ng/L for 156 compounds, between 2 and 5 ng/L for 67 compounds, between 5 and 20 ng/L for 30 compounds, and between 20 and 150 ng/L for 11 compounds.

Compared to the direct analysis of water samples (see chapter: 26) the concentrations determined were in general up to a factor of 2 to 3 lower, which is exemplified for a set of compounds in Figure 2. The reasons for these differences are losses of compounds occurring during sampling (breakthrough through LVSPE cartridge) as well as sample and extract handling (sample transfer, evaporation). These compound losses could not be compensated for by internal standard addition, as an on-site extraction was conducted and the extracts were used for biological testing.

Furthermore, enriched LVSPE extracts showed about 2 times higher matrix effects as compared to water samples, which could only partly be compensated for by internal standard addition. Thus, it has to be stressed here that the concentrations determined by chemical screening of LVSPE extracts have to be considered as an underestimation of the real water concentrations. However, in contrast to direct water analysis of water samples, LVSPE provides the opportunity to directly compare chemical and ecotoxicological analyses. This needs to be considered when interpreting the chemical analytical and bioassay data.

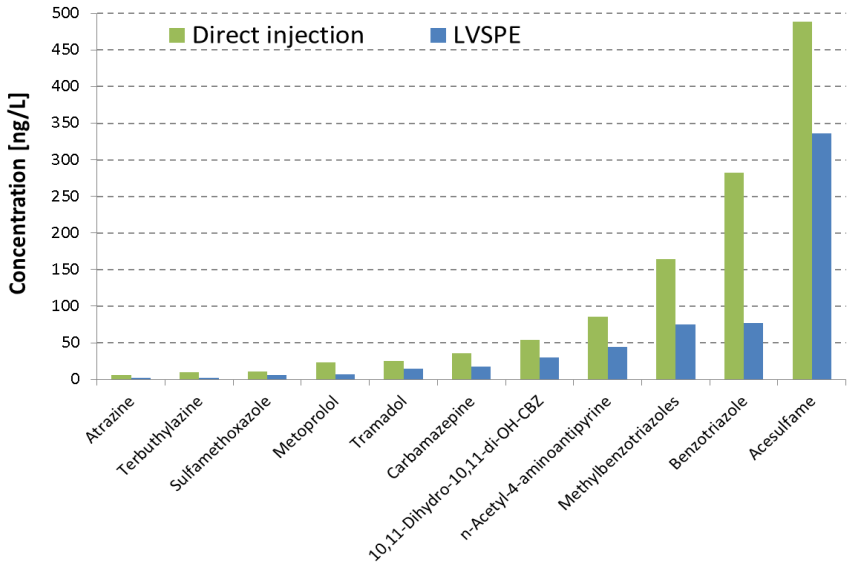
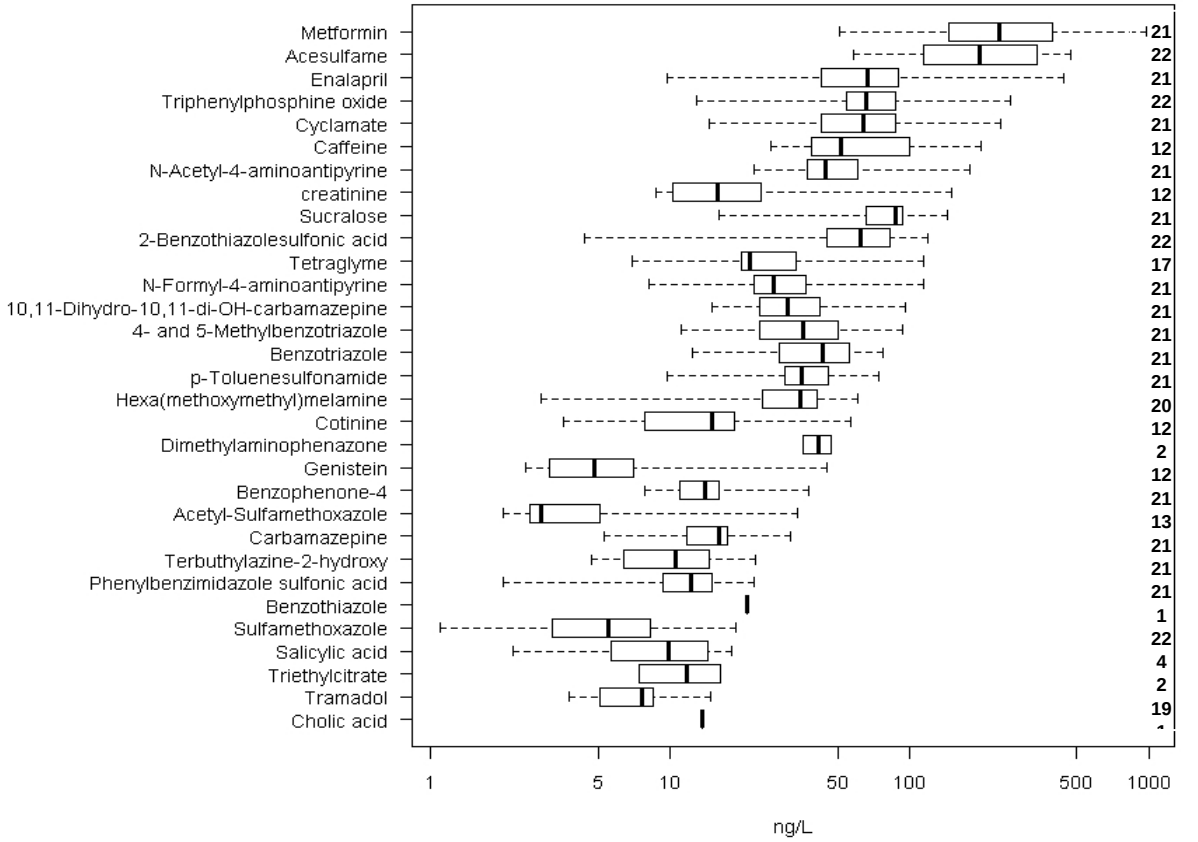
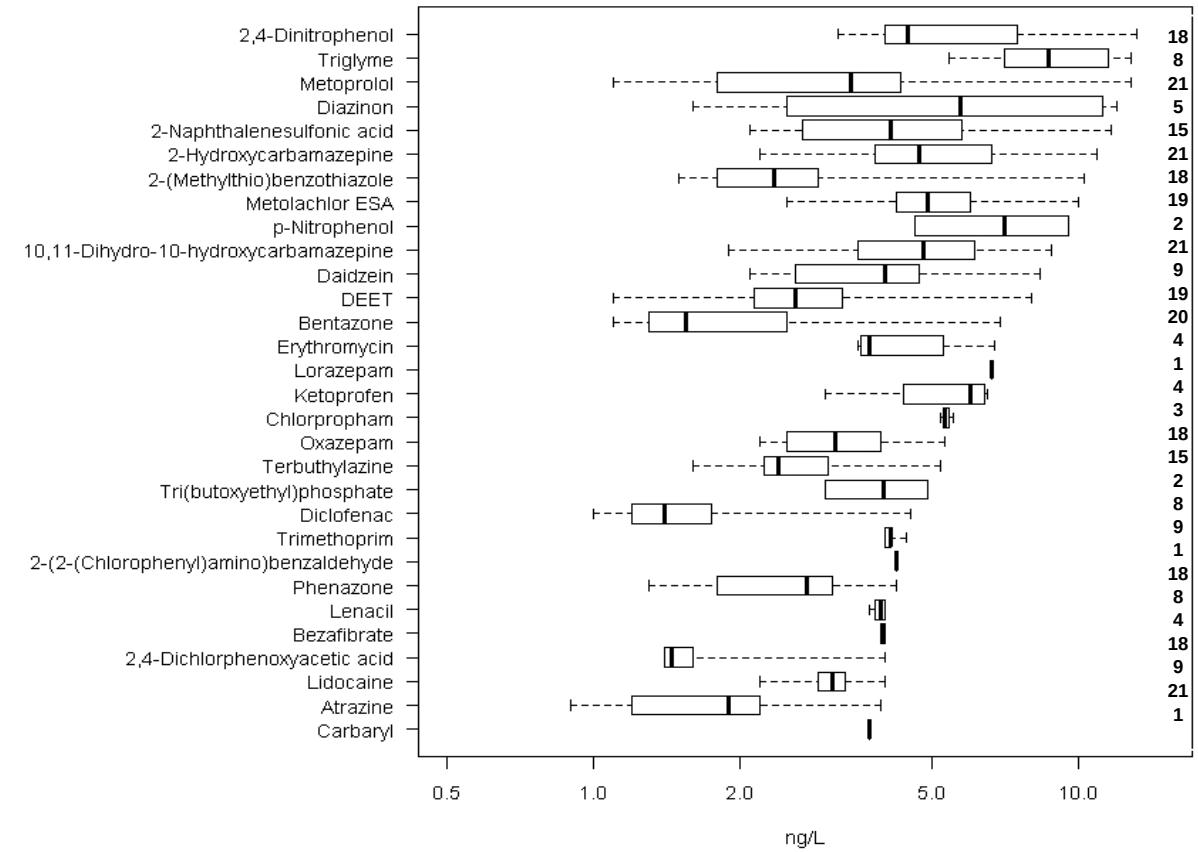


Figure 2 Concentration of selected compounds in sample JDS27 as determined by direct water injection (see chapter 26) and after LVSPE

An overview of the compounds detected and the concentration ranges is given in Figure 3 while those compounds which could not be detected above their MDL are listed in

Table 2. Among the compounds most frequently detected at relatively high concentrations were pharmaceuticals (metformin, enalalpril, carbamazepine), their transformation products (TPs; N-acetyl and N-formyl-4-aminoantipyrine, both derived from metamizole, TPs of carbamazepine), artificial sweeteners (acesulfame, cyclamate, sucralose), benzotriazoles and methylbenzotriazole corrosion inhibitors, and industrial chemicals such as benzothiazole sulfonic acid, triphenylphosphine oxide, p-toluenesulfonamide and hexa(methoxymethyl)melamine.
Widely used and legacy herbicides and their TPs (bentazone, atrazine, terbuthylazine, metolachlor, metolachlor ESA, isoproturon, mecoprop) were frequently detected at concentrations below 10 ng/L. Only in a small number of samples the insecticides diazinon (n=5), acetamiprid (n=2) and the fungicides carbendazim (n=9) and tebuconazole (n=1) concentrations were detected below 10 ng/L.


Metformin 21
Acesulfame 22
Enalapril 21
Triphenylphosphine oxide 22
Cyclamate 21
Caffeine 12
N-Acetyl-4-aminoantipyrine 21
creatinine 12
Sucralose 21
2-Benzothiazolesulfonic acid 22
Tetraglyme 17
N-Formyl-4-aminoantipyrine 21
10,11-Dihydro-10,11-di-OH-carbamazepine 21
4- and 5-Methylbenzotriazole 21
Benzotriazole 21
p-Toluenesulfonamide 21
Hexa(methoxymethyl)melamine 20
Cotinine 12
Dimethylaminophenazone 2
Genistein 12
Benzophenone-4 21
Acetyl-Sulfamethoxazole 13
Carbamazepine 21
Terbuthylazine-2-hydroxy 21
Phenylbenzimidazole sulfonic acid 21
Benzothiazole 1
Sulfamethoxazole 22
Salicylic acid 4
Triethylcitrate 2
Tramadol 19
Cholic acid
1 5 10 50 100 500 1000
ng/L


2,4-Dinitrophenol 18
Triglyme 8
Metoprolol 21
Diazinon 5
2-Naphthalenesulfonic acid 15
2-Hydroxycarbamazepine 21
2-(Methylthio)benzothiazole 18
Metolachlor ESA 19
p-Nitrophenol 2
10,11-Dihydro-10-hydroxycarbamazepine 21
Daidzein 9
DEET 19
Bentazone 20
Erythromycin 4
Lorazepam 1
Ketoprofen 4
Chlorpropham 3
Oxazepam 18
Terbuthylazine 15
Tri(butoxyethyl)phosphate 2
Diclofenac 8
Trimethoprim 9
2-(2-(Chlorophenyl)amino)benzaldehyde 1
Phenazone 18
Lenacil 8
Bezafibrate 4
2,4-Dichlorphenoxyacetic acid 18
Lidocaine 9
Atrazine 21
Carbaryl 1
0.5 1.0 2.0 5.0 10.0
ng/L

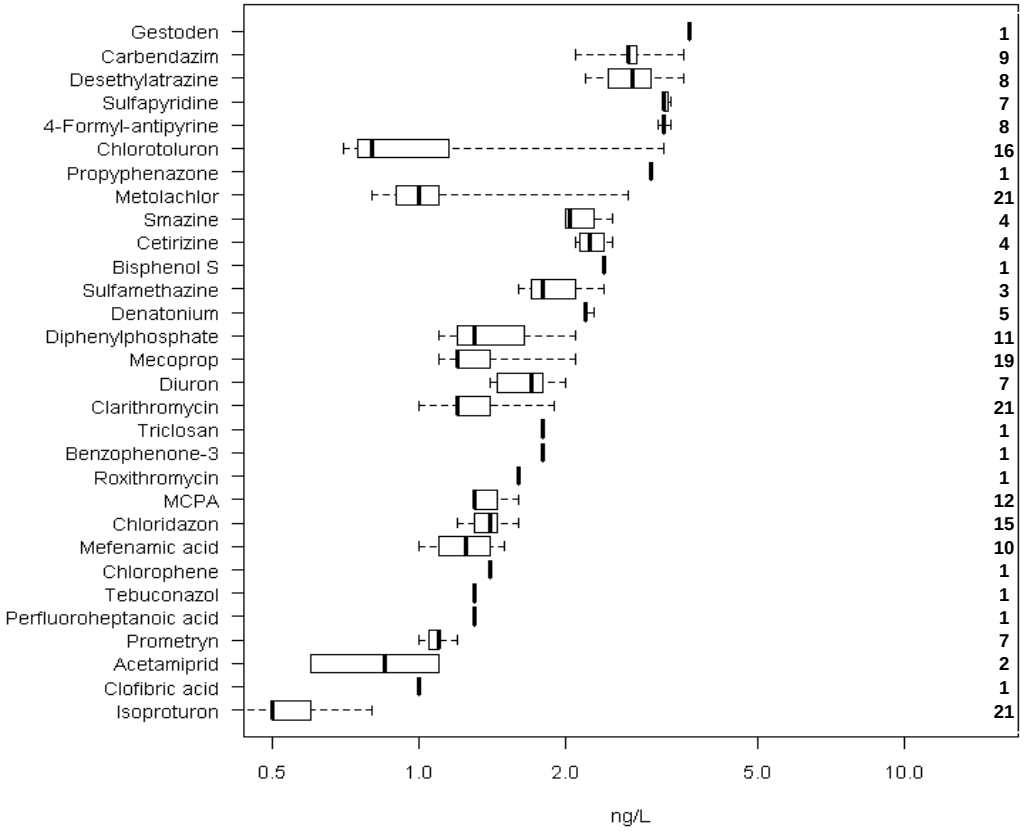


Figure 3 Overview of concentration of all 91 compounds detected in the 22 LVSPE samples; data are shown as median values, 25/75-percentiles (boxes) and maximum/minimum (whiskers); the values on the right denote the number of detections; note the logarithmic scales

Table 2 List of compounds not detected in the LVSPE samples; MDLs are given in brackets

1,2-Benzisothiazolinone (1)	Chloroxuron (0.9)	Imazalil (2)	Pirimicarb (1)
2,6-Dichlorobenzamide (4)	Citalopram (2)	Indomethacin (3)	Pirimiphos-methyl (5)
2-Acetonaphthone (4)	Clomazone (0.8)	Irgarol (1)	Pravastatin (5)
2-aminobenzimidazole (4)	Clonidine (0.8)	ISO E Super (21)	Prednisolone (4)
2-Isopropyl-6-methyl-pyrimidin-4-ol (20)	Clopidogrel (2)	Ketoconazole (3)	Prednisone (4)
2-Isopropylthioxanthone (4)	Clotrimazole (1)	Lauric isopropanolamide (1)	Primidone (20)
2-Methylbenzothiazole (0.7)	Clozapine (1)	Lauryl diethanolamide (9)	Prochloraz (0.8)
2-Naphthoic acid (20)	Cortisone (6)	Lincomycin (5)	Progesterone (3)
2-Octyl-4-isothiazolinone (0.8)	Crotamiton (1)	Linuron (0.9)	Propamocarb (2)
3,4-dichlorophenylurea (1)	Cyclophosphamide (10)	Mebendazol (2)	Propanil (3)
3,5,6-Trichloro-2-pyridinol (1)	Cypermethrin (68)	Medroxyprogesterone (2)	Propiconazole (1)
3-Cl-2-hydroxypropyltrimethylammonium (25)	Cyproterone (3)	Metamitron (4)	Propoxycarbazone (10)
3-Iodopropynyl butylcarbamate (10)	Desethylterbuthylazine (2)	Metazachlor (0.9)	Propranolol (2)
4-Amino-N,N-dimethylbenzenesulfonamide (2)	Desisopropylatrazine (2)	Methiocarb (0.7)	Propylparaben (1)
4-Androstene-3,17-dione (3)	Dexamethasone (1)	Monensin (0.5)	Prosulfocarb (0.5)
4-Bromophenol (50)	Diazepam (2)	Monobenzylphthalate (1)	Prothioconazole-desthio (1)
4-Hydroxybenzotriazole (5)	Dichlorvos (10)	Monoethylphthalate (10)	Pyrantel (0.8)
4-Hydroxytamoxifen peak1 (2)	Didecyldimethylammonium (2)	Mono-isobutylphthalate (10)	Pyrazophos (2)
4-Hydroxytamoxifen peak2 (2)	Diflufenican (1)	N,N-Dimethyldodecylamine-N-oxide (2)	Quinoxyfen (2)
4-methylbenzylidene camphor (3)	Dimethoate (1)	Naproxen (7)	Raloxifene (1)
4-Methylcoumarin (1)	Diphenhydramine (2)	n-Butylbenzene sulfonamide (20)	Simetryn (0.9)
7-Hydroxy-4-methylcoumarin (2)	Drospirenone (1)	Nonivamide (3)	Sotalol (20)
Acid orange 7 (free acid) (5)	Ethion (4)	Norethindrone (1)	Spiroxamine (2)
Albendazole (2)	Ethofumesate (1)	Norgestimat (4)	Tamoxifen (0.6)
Ametryn (1)	Ethyl tosylamide (2)	Norgestrel (3)	Terbinafine (1)
Anastrozole (1)	Ethyl-azinphos (3)	Octocrylene (45)	Testosterone (2)
Azinphos-methyl (1)	Ethylparaben (2)	Pantoprazole (6)	Tetrabromobisphenol A (20)
Azoxystrobin (1)	Exemestane (1)	Paroxetine (6)	Tetrachlorosalicylanilide (1)
Benalaxyl (1)	Fenpropimorph (2)	p-Chlorocresol (10)	Thiabendazole (2)
Bendiocarb (0.9)	Fenuron (1)	Pendimethalin (3)	Thiacloprid (1)
Benzenesulfonic acid (150)	Finasteride (2)	Penicillin G (1)	TMDD (10)
Benzethonium (2)	Fipronil (1)	Pentobarbital (15)	Trenbolone (2)
Benzyldimethyldodecylammonium (2)	Flumequine (20)	Pentoxifylline (3)	Triamcinolone (5)
Benzyldimethylhexadecylammonium (2)	Flurtamone (2)	Perfluorobutanoic acid (1)	Triclocarban (1)
Betamethasone (2)	Flusilazole (0.9)	Perfluorodecanoic acid (3)	Trifloxystrobin (0.2)
Bifenox (4)	Flutamide (1)	Perfluorohexanoic acid (2)	Tri-isobutylphosphate (100)
Bisphenol A-(2,3-dihydroxypropylether) (3)	Furosemide (5)	Perfluorooctanesulfonic acid (2)	Trimethyloctylammonium (3)
Boscalid (5)	Gemfibrozil (10)	Perfluorooctanoic acid (2)	Triphenyl phosphate (2)
Bromoxynil (1)	Hexadecylpyridinium (2)	Perfluorotetradecanoic acid (5)	Tris(1,3-dichloro-2-propyl)phosphate (5)
Butylparaben (2)	Hexadecyltrimethylammonium (0.9)	Pethoxamid (1)	Tris(1-chloro-2-propyl)phosphate (50)
Canrenone (0.9)	Hydrocortisone (4)	Picolinafen (2)	Tris(2-chloroethyl)phosphate (3)
Carfentrazone-ethyl (4)	Ibuprofen (50)	Pindolol (3)	Verapamil (2)
Chloramphenicol (2)	Icaridin (1)	Piperonylbutoxide (2)	Warfarin (2)

3.2 Results of biological screening

The assessment of the LVSPE samples on a number of bioassays (

Table 1) is currently in progress. Available preliminary results of the bioassays are summarized in Table 3. The conclusions are to be seen as preliminary and can only reflect the state of knowledge based on the currently available data.

Table 3 Summary of preliminary bioassay results as (A) qualitative and (B) semi-quantitative classification; tributaries are highlighted in red[a]

Station code	Name	Algae GI	Algae PSII	Ames -S9	Ames +S9	p53	ARE	NF-κB	ER	PXR	AhR	GR Calux®	GH3-TRE	AChE inhibition
	Classification	**(A)**	**(A)**	**(A)**	**(A)**	**(B)**	**(B)**	**(B)**	**(A)**	**(A)**	**(A)**	**(A)**	(A)	**(A)**
JDS8	Oberloiben	E-	E-	E	E	E-	E-	E	E-	NA	E	N	C	N
JDS22	Budapest downstream M0 bridge	N	E	E-	E	E-	E-	E-	E	E-	E	N	C	N
JDS27	Hercegszanto	E-	N	N	E	E-	E-	E	E	E	E	N	C	N
JDS29	/Drava (rkm 1.4)	P-	E	N	E	E-	E-	E-	E	E	E-	N	C	E
JDS30	Downstream Drava (Erdut/Bogojevo)	N	E	N	E	E-	E-	E	E	E-	E-	E-	C	N
JDS32	Upstream Novi-Sad	NA	NA	N	E	E-	E	E-	E	NA	E	N	C	N
JDS33	Downstream Novi-Sad	N	N	E-	E	E-	E-	E-	E	E	E	E-	C	N
JDS35	/Tisa (rkm 1.0)	E-	E	N	E	E-	E	E	C	E	E	E	C	N
JDS36	Downstream Tisa/Upstream Sava (Belegis)	E	E-	E-	E	E-	E-	C	N	NA	E	E	C	N
JDS37	/Sava (rkm 7.0)	E	E	N	N	E-	E-	E	E-	E	E-	E-	C	E
JDS39	Downstream Pancevo	E-	E	NA	NA	E-	E-	E-	E-	E	E	E-	C	N
JDS41	/Velika Morava	E-	E-	N	E-	C	E	C	E	E	E	N	C	N
JDS44	Irongate reservoir (Golubac/Koronin)	E-	E	N	N	E-	E	E	E	E	E	E-	C	E
JDS53	Downstream Zimnicea/Svishtov	E	E-	N	E	N	E-	E-	N	NA	E-	N	C	N
JDS55	Downstream Jantra	E-	E-	N	E	C	C	E-	N	NA	E-	N	C	N
JDS57	Downstream Ruse	E	E-	N	E-	E-	E-	E-	N	E	E	N	C	N
JDS59	Downstream Arges	E	E-	N	E	E-	E	E-	E-	E	E	N	C	N
JDS60	Chiciu/Silistra	E	E-	N	E	E-	E	E-	N	NA	NA	N	C	N
JDS63	/Siret (rkm 1.0)	E-	E-	E	E	E-	E	E-	C	E	E	N	C	N
JDS64	/Prut (rkm 1.0)	P	N	N	E-	N	N	N	N	NA	NA	N	N	E-
JDS65	Reni	E	E-	E-	E	E-	E	E-	C	NA	NA	N	C	N
JDS67	Sulina - Sulina arm	E-	E-	N	N	E-	C	E-	C	E-	E	N	C	N

[a] See section 0 for explanation of the codes, E: effect, E-: weak effect; E+: strong effect, N: no effect; C: cytotoxic effect, C-: weak cytotoxic effect, NA: not yet analysed

3.2.1 Growth inhibition of *Chlamydomonas reinhardtii*

The growth inhibition of green algae represents a standardised acute toxicity endpoint used in chemical risk assessment (OECD 2011) and has been expanded to include Photosystem II (PSII) inhibition (Nestler et al. 2012). Green algae are often among the most sensitive whole organism assays and thus used as a biological quality elements in the WFD.

Screening the extracts at REF100 in the assay identified that all extracts caused some level of growth inhibition, with 3 extracts being non-toxic, 9 were weakly toxic and 10 were toxic to the algae. Similar screening using PSII inhibition as an endpoint revealed that 2 were non-toxic, 11 were weakly toxic and 9 were toxic to the algae. The corresponding negative controls also showed some degree of toxicity in the bioassays tested at a REF100, thus indicating that introduction of toxic compounds in the extraction process may have occurred and thus potentially overestimating the toxicity of certain extracts.

3.2.2 Mutagenicity

The Ames test is the most widespread test for the detection of the mutagenic potential of chemicals and environmental mixtures (Reifferscheid et al. 2012). The method bases on the chemically-induced reversion of auxotrophic *Salmonella typhimurium* mutants to prototrophic metabolism (Ames et al.

1975). To detect possible bioactivation of substances, rat liver homogenate and cofactors (S9-mix) can be added to the test system to simulate exogenous biotransformation potential (Maron and Ames 1983).

All samples were tested in 3 replicates at a REF of 1000, since no cytotoxicity occurred. Such effects would cause a decrease in revertant numbers and thus lead to false positive results. The blanks were not mutagenic. In the combination of TA98 without S9, mutagenicity was found for the sites JDS8 and JDS63. Weak effects were observed for samples of the sites JDS22, JDS33, JDS36 and JDS65.For the combination of TA98 with S9 an increased number of active samples were proven. All samples - except the sites JDS37, JDS67 and JDS39 (not analysed) - showed a mutagenic potential by the use of S9. However, the samples JDS41, JDS57 and JDS64 showed only weak mutagenicity. This indicates that at a large number of sites substances entering the River Danube and not being mutagenic as parent compound might get bioactivate and enhance mutagenic potential upon metabolism by organisms in the water column.

3.2.3 Adaptive Stress Responses

The adaptive stress response assays targeted oxidative stress (ARE-BLA), inflammation (NF-κB-BLA) and p53 mediated apoptosis in response to DNA damage (p53-BLA). In the ARE-BLA assay, which responds to chemicals that produce reactive oxygen species and those that are direct electrophiles, 8 samples were positive (JDS 32, 35, 41, 44, 53, 59, 60, 65, 67), 11 were weakly positive (JDS8, 22, 27, 29, 30, 33, 36, 37, 39) and one had no effect up to a REF of 500 (JDS64). JSD64 also had no effect in the NF-κB-BLA and p53-BLA assays. Two samples were cytotoxic (JDS55, 67) and thus oxidative stress response could not be ruled out but was masked by cytotoxicity.

The samples tended to have less effect in the p53-BLA assay, which responds to genotoxic chemicals, with no samples having a positive response in the p53-BLA assay. Instead, the majority of the samples were only weakly positive, 4 were cytotoxic (JDS 41, 55, 57, 63) and 2 had no effect (JDS 53, 64). In the NF-κB-BLA assay 6 samples were positive (JDS 8, 27, 30, 35, 37, 44), 13 were weakly positive (JDS 22, 29, 32, 33, 39, 53, 55, 57, 59, 60, 63, 65, 67) and one had a similar effect as the control samples (JDS 64). Cytotoxicity masked induction for JDS 36 and 41.

3.2.4 Estrogen Receptor

The MELN assay was employed to assess the presence of substances able to interact with and activate the human estrogen receptor (ER), and thus presenting estrogenic activity in the samples.

Several samples were found to be cytotoxic in the MELN assay at a REF of 300 and above. Also, a slight effect was seen in one of the two blanks samples at a REF of 1000. Therefore only effects observed at a REF of 100 or below were considered as positive in the assessment of estrogenic activity.

Estrogenic activity was found in several of the JDS samples, at non cytotoxic concentrations. The most active samples were JDS41 (positive response at a REF<3) and JDS22, 27, 29, 30, 32, 44 (positive response at a REF comprised between 3 and 30). In these samples, concentrations of estradiol-equivalents (E2-EQ) are in the 0.01-0.1 ng E2-EQ / L range. However, these values have to be refined by establishing complementary assays and thus are preliminary.

3.2.5 Aryl hydrocarbon Receptor

CAFLUX assay was employed to assess dioxin-like (aryl hydrocarbon receptor - mediated) toxicity. Chronic adverse effects of xenobiotics such as interference with liver functions, immunity, endocrine and nervous system as well as embryo toxicity and carcinogenicity were experimentally related to Aryl hydrocarbon receptor (AhR)-dependent events (Janošek et al. 2006). Most of the samples were cytotoxic in the CAFLUX assay at high concentrations and the cytotoxicity prevented the assessment the dioxin-like potential at relative enrichment factor (REF) of 500 and in case of some samples even

at REF of 167. Dioxin-like activity was detectable in most of the JDS samples at non cytotoxic concentrations. Available preliminary data indicate that it may be in some samples below limit of quantification of toxic equivalent (TEQ) relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In case where sufficient induction was reached, the EC_{20} values of dioxin-like response (concentrations causing 20% response relative to maximum induction caused by TCDD in the bioassay) were for most samples in the range of REF from 10 to 100. When quantifiable, the dioxin-like equivalent occurred in the LVSPE samples in the pg TEQ/L range and it did not differ greatly among sites. According to preliminary results, samples JDS22 and JDS59 belong among those with relatively greater dioxin-like activity. Similar levels of TEQ (6-10 pg/L) were also determined from passive sampling with silicone rubber samplers (see chapter 29). Presence of AhR-active substances at comparable concentrations have been previously detected by bioassays in water from other European rivers (Jálová et al. 2013)

3.2.6 Pregnane X Receptor

In this study, we explored the potential use of the HG5LN-hPXR assay (Lemaire et al 2006) as a detector of PXR-active substances in JDS samples. The human pregnane X receptor (PXR) is a nuclear receptor that plays a crucial role in detoxification processes by mediating the transcription of genes that code for xenobiotic biotransformation enzymes. As such, PXR is a molecular target for a wide range of xenobiotics including pharmaceuticals, pesticides, steroids, phthalates or alkylphenols, though at relatively high concentrations, i.e. in the µg/L range and upper (Creusot et al. 2010, Lemaire et al. 2006, Mnif et al. 2007). All the 12 samples tested so far were able to activate the PXR in this assay. These effects were observed at relatively high concentrations, i.e. at REF ranging from 30 to 300. No cytotoxic effect was observed at these concentrations and the blanks were negative, thus suggesting that PXR active substances are indeed present in the JDS samples. Similarly to these findings, previous studies have shown the widespread occurrence of PXR activity in different environmental matrices such as wastewater effluents, river surface water or sediments (Creusot et al. 2013, Creusot et al. 2010). Although identification of environmental PXR ligands is still a matter of research, phthalates and alkylphenols were identified as contributors to PXR activity in French river sediments (Creusot et al. 2013).

3.2.7 Glucocorticoid Receptor

The GR CALUX assay was performed to assess the glucocorticoid-like (glucocorticoid receptor – mediated) activity of the JDS3 samples (van der Linden et al. 2008). Glucocorticoids are important steroid hormones controlling metabolism, immune responses and inhibition of inflammation as well as cellular proliferation (Sonneveld et al. 2007, van der Linden et al. 2008).

All of the 22 samples were tested in 3 replicates at a REF 100, since acute cytotoxicity in the neutral red assay with U2-OS (GR-CALUX) cells were observed for all of the 22 samples at REF >100-250. In the GR-CALUX, cytotoxicity would reduce luminescent cells and thus signal strength, which would deliver false negative findings. Receptor-mediated endocrine activity in the GR-CALUX could be demonstrated for the samples JDS30, JDS33, JDS35, JDS 37, JDS39 and JDS44. Blanks showed no activity in the GR-CALUX.

3.2.8 Thyroid Receptor

None of JDS samples caused induction in the GH3-TRE assay, but were cytotoxic at high REFs. The lack of induction of the thyroid receptor was not unexpected because most environmental chemicals that interfere with the thyroid function are not binding to the thyroid receptor but are rather goitrogens, which suppress the function of the thyroid gland by interfering with iodine uptake, such as inorganic oxyanions, such as perchlorate and nitrate (Pickford 2010), which would not be extracted by LVSPE samples and which would not be active in the GH3-TRE assay. Relatively few organic chemicals that could be present in are active in the T-Screen assay (Freitas et al. 2011, Schriks et al. 2006). The results of absence of thyroid receptor agonist were consistent with previous studies on water samples (Escher et al. 2014, Inoue et al. 2009, Jugan et al. 2009).

3.2.9 Acetylcholinesterase (AChE) inhibition

The in vitro inhibition of acetylcholinesterase (AChE) is a rapid assay to determine the chemical interference with the enzymatic conversion of AchE to acetyl and choline in the neuronal synaptic cleft. The measurement of AChE inhibition has predominantly been associated with the neurotoxicity of organophosphate and carbamate insecticides, but may also be affected by other organic compounds of unknown structure (Holth and Tolelfsen, 2007).

All extracts from the JDS3 were screened for AChE inhibition using a maximum REF of 100. The results showed that 18 were non-toxic, 1 was weakly toxic and 3 were toxic to the algae. Interestingly, 2 out of 3 solvent blanks caused AChE inhibition at the REF used in the assay. Potential contamination by toxic compounds introduced by the extraction and use of solvents should therefore be determined to assess to which degree this affects the toxicity of the extracts.

4 Conclusions

Large volume solid phase extraction was successfully applied at 22 sampling sites of the JDS 3 to realise effect-based screening in a river basin scale for the first time. The samples were analysed with liquid chromatography – high resolution mass spectrometry for semi-polar to polar organic compounds as well as a set of 9 *in vitro* and 2 *in vivo* bioassays to assess the mode of action of organic compounds present in the samples.

The chemical screening resulted in the detection of 91 compounds in at least one sample. Among mostly identified in relatively high concentrations were pharmaceuticals, their transformation products, artificial sweeteners, corrosion inhibitors, and industrial chemicals. Widely used and legacy herbicides and their TPs were frequently detected. It must be stressed that the concentrations determined were in general up to a factor of 2 to 5 lower than corresponding analyses by direct water injection and thus an underestimation of real water concentrations is obviously.

Despite the overall low concentrations of organic compounds compared to other rivers in Europe (Loos et al. 2010, ter Laak et al. 2010), all extracts were effective in one or more bioassays with the endpoints mutagenicity, dioxin-like and PXR mediated activity, oxidative stress responses, and estrogenicity as well as green and Photosystem II inhibition of green algae. The sample with lowest toxicity was JDS 64 (Prut) that only showed weak mutagenicity (after S9 activation) and neurotoxicity. Samples JDS33 (downstream Novi Sad) and JDS63 (tributary Siret) were among the most toxic samples, which were effective in almost all bioassays.

The bioassays are not fully evaluated and thus the toxicological potentials of the samples might be over- or underestimated. Presented conclusions are therefore to be seen preliminary as well and can only reflect the state of knowledge based on the available data.

Finally, this study demonstrated the feasibility of an effect-based screening in a river basin wide scale using on-site LVSPE even under conditions of high dilution such as in Danube River.

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6 Acknowledgements

We are gratefully to the technical and logistical help of Margit Petre, Anett Kloß, Jörg Ahlheim, Riccardo Massei, Melis Muz, Tomislav Andjiv and the co-workers of Environmental Institute. This study was supported by the ICPDR, the NORMAN Association and the SOLUTIONS project (funded by the European Union grant agreement no. 603437). The development of LVSPE was supported by the German Federal Ministry of Education and Research (funding number 02WRS1282I).