Toxicological and chemical investigation of untreated municipal wastewater: fraction- and species-specific toxicity

Jelena Hrubik^a, Branka Glisic^a, Aleksandra Tubic^b, Ivana Ivancev-Tumbas^b, Radmila Kovacevic^a,
Dragana Samardzija^a, Nebojsa Andric^a, Sonja Kaisarevic^{a*}

^a University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Laboratory
for Ecotoxicology, Novi Sad, Serbia

^b University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and
Environmental Protection, Novi Sad, Serbia

*Corresponding author:

Dr. Sonja Kaisarevic

University of Novi Sad Faculty of Sciences

Department of Biology and Ecology

Trg D. Obradovica 2

21000 Novi Sad

Serbia

Tel. ++381-21-485-2675

Fax. ++381-21-450-620

E-mail. sonja.kaisarevic@dbe.uns.ac.rs

ABSTRACT

Absence of a municipal wastewater (WW) treatment plant results in the untreated WW discharge into the recipient. The present study investigated toxic effects and chemical composition of water extracts and fractions from untreated WW and recipient Danube River (DR). Samples were prepared by solid-phase extraction and silica gel fractionation and screened for EROD activity and cytotoxicity using aquatic models, comprising of fish liver cells (PLHC-1) and a model of the early development of zebrafish embryos, while rat (H4IIE) and human (HepG2) hepatoma cells served as mammalian models. Polar fraction caused cytotoxicity and increased the EROD activity in PLHC-1 cells, and increased mortality and developmental abnormalities in developing zebrafish embryos. In H4IIE, polar fraction induced inhibition of cell growth and increased EROD activity, whereas HepG2 exerted low or no response to the exposure. Non-polar and medium-polar fractions were ineffective. Tentative identification by GC/MS showed that WW is characterized by the hydrocarbons, alkylphenols, plasticizers, and a certain number of benzene derivatives and organic acids. In DR, smaller number of organic compounds was identified and toxicity was less pronounced than in WW treatments. The present study revealed the potent toxic effect of polar fraction of untreated WW, with biological responses varying in sensitivity across organisms. Obtained results confirmed that fraction- and species-specific toxicity should be considered when assessing health risk of environmental pollution.

Keywords: Cytotoxicity, EROD, Polar fraction, Species-specific toxicity, Wastewater

1. INTRODUCTION

Mixtures found in the environment contain a variety of compounds with different physico-chemical properties and limited information about their toxic properties or mode of action. Although the “whole mixture” was found to disturb different physiological processes in vitro (Traven et al., 2008; Tubic et al., 2013), a growing body of research is aimed at increasing the understanding of the contribution of the chemical fractions to the toxicity of mixtures. Among the mixtures of anthropogenic origin, municipal wastewaters (WW) are known for their complexity and high organic load discharged into the receiving waters (Terzic et al., 2008). The need for tools capable of yielding quantitative and qualitative information on municipal WW characterization has already been highlighted (Mihajlovic et al., 2014). However, providing information on their toxicological effects remains a challenge.

Biological methods used to characterize toxicity of the environmental matrices, including WW, rely on one or more of the well-established non-human in vitro systems. Among them, fish and rat cell lines have been widely exploited as adequate target subjects due to either their ecological importance or their ability to act as surrogates for human cells or tissues. Some of the frequently used models in environmental toxicology research are fish liver cells, such as PLHC-1 (Traven et al., 2008; Tubic et al., 2013) or RTL-W1 (Grung et al., 2011), rat liver cell lines, such as H4IIE (Kaisarevic et al., 2009; Tubic et al., 2013), or modified H4IIE cells (Otte et al., 2013). The response of classical targets for chemical action in the cells, such as arylhydrocarbon receptor (AhR)-mediated activity (Jalova et al., 2013), cytotoxicity or genotoxicity (Smital et al., 2011), is taken as a measure of potential toxic properties of the municipal WW. In addition to cell cultures, adult zebrafish and/or their embryos are a widely accepted vertebrate model for toxicity

assessment (Hill et al., 2005) and have been used in the estimation of toxic effects of municipal WW (Galus et al., 2013).

Although the spectrum of experimental models is even broader, the information about the responses of human cells to complex environmental mixtures, especially the AhR-mediated responses, is presently lacking, thus limiting our understanding of adverse impact of chemicals on human health. In general, studies exploiting human cells in order to examine the effect of environmental mixtures of toxicants are scarce, despite their potential to significantly contribute to an effective line-of-evidence in risk assessment procedures (Hara-Yamamura et al., 2013). Human hepatocellular carcinoma cell line HepG2 have a good potential for employment in toxicological research, as they retain the enzymatic apparatus typically involved in hepatic detoxification processes, such as, for instance, cytochrome P450 (CYP) monooxygenases. In addition, they exhibit the majority of cellular features of the normal human hepatocytes (Bouma et al., 1989). Therefore, HepG2 represents a suitable model for assessing the response to chemical exposure.

In the city of Novi Sad, Serbia, in the absence of a municipal WW treatment plant, the untreated WW is discharged directly into the Danube River (DR), and thus represents a potentially serious pollution source. We conducted the present study in order to investigate toxic effects of non-treated municipal WW complex mixture and recipient DR water following WW discharge, estimate fraction- and species-specific toxicity, and link those effects with the chemical composition of samples. The samples were prepared using solid phase extraction (SPE) with hydrophilic-lipophilic Oasis HLB sorbent, following silica gel fractionation into non-polar, medium-polar and polar fractions. For the total extracts of water samples and their fractions, a battery of toxicity assays was conducted using two aquatic models (fish liver PLHC-1 cells and zebrafish embryos) and two mammalian models (rat H4IIE and human HepG2 hepatoma cells).

Obtained results revealed the potent toxic effect of polar fraction of WW, with biological responses varying in sensitivity across organisms. The fractions were further subjected to non-target GC/MS analysis for tentative identification of compounds, to better understand fractions' effects on different biological models.

2. MATERIAL AND METHODS

2.1 Sampling

Samples were collected from two sampling sites, as indicated in Figure 1. Municipal WW of the city of Novi Sad, Serbia, was taken from the collector station GC-2 (N 45°15'438" E 19°51'233"). This is a sewage discharge servicing the northern half of the city (covering an area of approximately 900 ha) with approximately 100,000 inhabitants. It is also a collector for the greatest amount of pre-treated industrial WW (Mihajlovic et al., 2014). Since there is no municipal WW treatment plant in Novi Sad, the untreated WW is discharged directly into the Danube River. Wastewater was collected twice as a 24 h composite sample, while DR was also collected twice, as a spot sample. Wastewater samples (400 mL) proportional to time were taken by collecting the same volume at 30 min intervals during the 24 h period pertaining to 16th/17th November 2011 (henceforth referred to as the November sample) and two weeks later on 30th November/1st December 2011 (December sample). Samples collected in a polypropylene bottles during 24 h were mixed into the final 20 L sample, and were kept in glass bottles until the extraction (no longer than 48 h). Spot samples of the DR surface water (20 L) were taken 800 m downstream from the WW discharge point (N 45°16'005" E 19°51'571") on the 17th and 19th November (mixed into the November sample), as well as on the 1st and 3rd December 2011 (mixed into the December sample).

124

125 *2.2 Sample preparation*

126 Two composite wastewater and two spot river water samples were prepared by solid-
127 phase extraction (SPE) based on modified protocols (Grung et al., 2007; Smital et al., 2011) and
128 as previously described (Tubic et al., 2013). Each of the four collected samples (WW from both
129 November and December and DR water from November and December samples) was used to
130 prepare the total extract for bioassay and fractionated non-polar, medium-polar and polar extracts
131 for both chemical analysis and bioassays. Procedural blanks were prepared for both chemical and
132 biological analysis. Such preparation of samples allowed us to test fraction-specific toxicity in
133 different bioassays, i.e. to investigate which fraction/extract distinguishes for its toxic potency
134 and is the main carrier of toxicity of the samples.

135

136 *2.2.1 Total extracts*

137 Prior to extraction, 2 L of each sample was filtered through a glass fiber filter (ROTH MN 85/70,
138 0.6 μm) in order to remove suspended matter. Filtered samples were then divided in two portions
139 of 1 L and each portion was passed through preconditioned (7.5 mL dichloromethane/7.5 mL
140 methanol/7.5 mL of distilled water) Oasis HLB cartridges (500 mg; 0.6 μm ; Waters Corporation).
141 Thus, the enrichment was performed by percolating two identical aliquots of the wastewater
142 sample (1 L) through each Oasis cartridge (the total volume extracted was 2 L). After washing
143 with distilled water, columns were dried in air and eluted with methanol (2×10 mL; J.T. Baker).
144 Next, the elutes from two Oasis cartridges were combined into one, before being evaporated
145 under the stream of N_2 to approximately 2 mL, and divided into two identical aliquots of 1 mL.
146 The aliquots were transferred into 4 mL vials with Teflon-lined screw caps and stored at 4°C
147 until further analysis. One aliquot (1 mL) intended for biological analysis was used to study the

activity of both DR and WW samples. Prior to bioassay testing, it was dried under N₂ stream and finally dissolved in 0.15 mL of dimethyl sulfoxide (DMSO; Sigma).

2.2.2 Fractionated extracts

The other aliquot (1 mL) of total extract of each sample underwent an additional separation step using silica gel (Ahel and Giger, 1985). For the non-polar fraction, 1 mL of the total concentrated extract was carefully evaporated to dryness using N₂ stream and the residue was re-dissolved in 1-2 mL of n-hexane (J.T. Baker). It was applied to the top of the silica gel column (5 cm × 1.0 cm i.d.) filled in n-hexane. The non-polar compounds were subsequently eluted with 25 mL of n-hexane. The remaining residue in a 4 mL vial was re-dissolved in 1–2 mL dichloromethane (J.T. Baker) and transferred to the same silica gel column. Medium-polar compounds were eluted with 30 mL of dichloromethane. The polar fraction was obtained by subjecting methanol (J.T. Baker) to the same procedure as that applied to residue solvent and eluent (30 mL). Each of the three fractions obtained were evaporated and transferred to 2 mL vials fitted with Teflon-lined screw caps. The final volume of non-polar and medium-polar fractions was 0.5 mL, while it was 2 mL for polar fraction. After the analysis, non-polar and medium-polar fractions were evaporated under N₂ stream, dissolved in 0.15 mL of DMSO and subjected to bioanalyses. The polar fraction was divided into two portions of 1 mL, one of which was used in chemical analysis by methylation in acidic conditions (Santos-Delgado et al., 2000) and the other was intended for bioassays as non-methylated sample. Non-methylated polar fraction was evaporated under N₂ stream, dissolved in 0.075 mL of DMSO and subjected to bioanalyses.

Procedural blanks were obtained by processing the eluate from the SPE columns after the percolation of 100 mL distilled water through the cartridge. The results obtained were used to

eliminate the false positive findings originating from the materials used in the laboratory for both chemical analysis and bioassays.

2.2.3 Methylation procedure for chemical analysis of polar extracts

In accordance with (Santos-Delgado et al., 2000), 1 mL of the methanol extract was transferred to the glass vial of 12 mL, and 250 μ L of cc H₂SO₄ was slowly added, after which the extract was placed in an ultrasonic bath for 1 min. Next, the extract was submerged for 12 min in a water bath heated to 60°C. After the extract was cooled, 6 mL of 2% solution of KCl was added. Finally, formed methyl esters were extracted with 1 mL of n-hexane, after which 0.5 mL of the extract was used for analysis.

2.3 Cell culture-based bioassays: sulforhodamine B (SRB) cytotoxicity assay and CYP1A induction

Fraction- and species-specific toxicity of WW and DR samples was tested by two in vitro bioassays on three cell lines, comprising of fish *Poeciliopsis lucida* desert topminnow hepatocellular carcinoma PLHC-1, rat hepatoma H4IIE and human hepatocellular carcinoma HepG2. Cytotoxicity of samples was tested by sulforhodamine B (SRB) assay, based on the measurement of the amount of protein dye SRB incorporated into viable cells. This assay is a linear relationship with the cell number (Skehan et al., 1990). Induction of CYP1A, a parameter used in the estimation of AhR-mediated activity, was measured by determining its 7-ethoxyresorufin-*o*-deethylase (EROD) activity in microEROD analysis.

H4IIE and PLHC-1 were grown and seeded for the experiments, as described previously (Tubic et al., 2013). HepG2 cells, kindly provided by Dr. Tvrtko Smital (IRB, Zagreb, Croatia), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with

10% fetal bovine serum (FBS; PAA Laboratories), sodium bicarbonate and penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂. For all experiments, HepG2 cells at 80-90% confluence in 75 cm² flasks were used. The cells were first resuspended in 10 mL of culture medium/75 cm² flask, after which the suspension was further diluted in the 1:4 ratio for the SRB, and 1:3 for the microEROD assay. Using these final suspensions, 0.1 mL was added per well in 96-well plates.

In both SRB and EROD assay, 24 h after plating, the cells were exposed to serial dilutions of total extracts, non-polar, medium-polar, or polar non-methylated fractions from WW and DR for 48 h. In each experiment, the cells were treated in triplicate wells of a 96-well plate. Experiments were repeated three times for both assays. The tested concentrations were expressed as amounts of extracted compounds corresponding to the specific amount of water (water equivalents; WEQ) per mL of the culture medium (mL WEQ/mL). In microEROD analysis, the cells were also exposed to 5 nM TCDD as a reference compound for 48 h. Each plate contained control (growth medium and 0.15% DMSO) and cell-free blank wells. No effect of procedural blanks on the survival of PLHC-1 and H4IIE was noted, as measured by SRB assay (the cytotoxicity index values ranged from 1.8% to 15%).

Following incubation, SRB and microEROD assays were performed, as described previously (Tubic et al., 2013). To account for differences in the magnitude of response, and thus be able to compare values among the cell lines pertaining to different species, we have expressed the SRB and EROD data in relative units.

The results of the SRB assay are presented as cytotoxicity index (CI), which was calculated using the equation $CI (\%) = (1 - A_{\text{(sample)}}/A_{\text{(control)}}) \times 100$, where the A values represent the measured absorbances. Hence, the control values are presented as 0%, while the treatment

effects are expressed as a positive (cytotoxic effect) or a negative value (stimulation of cell proliferation).

In the EROD assay, the maximum activity induced by TCDD was set to 100% (TCDDmax) and the effect of treatments was expressed as % of the TCDDmax activity. TCDD EC50 and EC25 values (concentrations that induce 50% and 25% of the maximum TCDD response) were calculated by fitting the data to a classical sigmoidal four-parameter dose-response model. Where complete dose-response curves were obtained, bioassay-derived TCDD equivalent concentrations (TCDD-EQ), expressed as pg TCDD-EQ/mL, were calculated as a ratio of the TCDD EC25 value and the sample concentration that induced 25% of the TCDDmax. All calculations were performed using the GraphPad Prism 5 (GraphPad Software, Inc.). SRB and EROD data are expressed as means +/- standard error of three replicate assays.

2.4 Zebrafish embryo developmental toxicity

Toxicity of municipal WW was also tested using *Danio rerio* (eng. zebrafish) embryos obtained from the wild type (AB strain). We tested the polar fraction of the November sample only, since it showed the most consistent response among the cell lines and was also active in the EROD assay on PLHC-1 cells. Shoal of adult fish was a generous gift from Dr. Zsolt Csenki, Szent Istvan University, Godollo, Hungary. The husbandry, spawning and collecting of zebrafish embryos were performed as described previously (Glisic et al., 2016). Since the embryos used in the present study were no more than 5 days old, the license was not required by the Council of Europe (1986), Directive 86/609/EEC or the University of Novi Sad Ethics Committee.

Zebrafish embryos were cultured at controlled temperature ($26\pm1^{\circ}\text{C}$) and illumination (12:12 h light-dark cycle). Prior to commencing the exposure experiments, the embryos were examined, followed by removal of the coagulated and the non-fertilized embryos. Only fertilized

and healthy embryos were randomly placed in a 96-well plate (one embryo per well; a total of 10 to 20 treated or untreated embryos per plate), representing one experiment within the test. Experiment was repeated two to three times. Two hours post fertilization (hpf), the embryos were treated with the polar fraction of the November WW sample in the final concentration of 4 and 8 mL WEQ/mL or the vehicle control (reconstituted water and 0.15% DMSO) for 96 h. The treatments were carried out in 100 μ L volume/1 embryo/well in a 96-well plate. Mortality and developmental malformation percentages were analyzed at different time points throughout the 24 hour period. The developmental phenotypes of the embryos were observed and imaged using Leica MZ16 magnifier (30 $\times$ magnification).

2.5 Chemical analysis

Chemical analysis of all prepared extracts was performed with the aid of Gas chromatograph Agilent 7890A with 5975C MSD. The internal standard phenantrene-d₁₀ was used (0.4 μ g/mL). A chromatographic column Agilent J & W Scientific DB-5MS 30 m $\times$ 0.25 mm ID $\times$ 0.25 μ m was used. Helium was used as the carrier gas. While the initial oven temperature was 60°C, after 3 minutes, it was incremented at a rate of 3°C/min until 300°C was reached. This temperature (300°C) was maintained for 10 min. Injection was performed in splitless mode with the injector temperature of 270°C. The detector temperature was 150°C and mass of 35-700 amu was scanned at the EI voltage of 70 eV. Tentative identification, obtained via recognition using the GC/EI-MS with a good library database match (Richardson, 2002) of compounds, was performed by *Deconvolution Reporting Software* and *Automated Mass Spectral Deconvolution and Identification System* (AMDIS). All spectra obtained by AMDIS were compared with the NIST reference spectra database, with search matches for all tentatively identified compounds

exceeding 70%.

2.6 Statistical analysis

The results of cell culture-based bioassays were analyzed by one-way ANOVA, Tukey's multiple comparison test, to check for the differences in the responses in different treatments i.e. fraction-specific toxicity. The results of zebrafish embryo developmental toxicity assay were analyzed by two-way ANOVA to check for the time-dependent effects of treatments, and also by one-way ANOVA, Tukey's multiple comparison test to check for the dose-dependent effects of treatments. All calculations were performed using the GraphPad Prism 5 software. The results of the statistical analysis are presented in Supplementary Data, and were fully considered for the discussion of the assays' results.

3. RESULTS

3.1 Cytotoxicity assay

The effects of DR and WW samples on cell survival, determined using the SRB assay, are presented in Figure 2, while the results of the statistical analysis are given as Supplementary Data, Table S1. In DR exposure, the observed responses considerably varied depending on the cell line. More specifically, in the PLHC-1 cells, a mild cytotoxic effect was observed in all DR samples. The most pronounced cytotoxic effect was noticed in the polar fraction from November, reaching 37% of inhibition vs. control. However, all observed effects varied between experiments, which resulted in the lack of statistically significant difference in comparison to control and other treatments. In the H4IIE, the December sample induced a weak stimulation of cell proliferation (mostly pronounced in non-polar fraction), while in HepG2, all DR fractions

were ineffective. Again, statistical analysis did not confirm any significant difference among these treatments and in comparison to control. The cell response to WW samples was consistent, since only cytotoxic effects were observed, with small variations between the experiments. As confirmed by the statistical analysis, non-polar and medium-polar fractions showed no effect or only a weak effect on the cell survival in all cell lines, while strong and consistent cytotoxic effect was observed in the PLHC-1 and H4IIE cells following exposure to the highest doses of the total extract (up to 78% and 85%, respectively) and polar fraction (up to 64% and 70%, respectively). Moreover, the same samples exhibited a mild but statistically significant cytotoxic effect on the HepG2 cell survival (up to 37% of inhibition vs. control). In general, the effects of November and December samples were very similar, with total extracts and polar fractions of all samples being the most effective.

3.2 CYP1A induction potency

We have tested fish PLHC-1, rat H4IIE and human HepG2 cells in terms of EROD induction with the standard reagent TCDD (results not shown). In all cell lines, dose-dependent increase in EROD activity with typical sigmoidal curves was recorded; however, sensitivity of the response to TCDD varied. The EC₅₀ value obtained for TCDD was 76 pM for PLHC-1, 36 pM for H4IIE and 1,300 pM for HepG2. These values are comparable to those obtained in other studies, where EC₅₀ of 30 pM (Traven et al., 2008) or 83 pM (Villeneuve et al., 2001) for PLHC-1; 50 pM (Zeiger et al., 2001) or 20 pM (Villeneuve et al., 2001) for H4IIE; and 680 pM for HepG2 (Zeiger et al., 2001) were reported.

The effects of DR and WW samples on CYP1a induction potency, determined using the EROD assay, are presented in Figure 3, while the results of the statistical analysis are given as

Supplementary data, Table S2. None of the DR samples induced EROD activity in PLHC-1, H4IIE and HepG2 cell lines. No or low potency to induce EROD activity was also noted for the non-polar and medium-polar fractions obtained from the municipal WW. Potency to provoke EROD activity was found in the total extract and polar fraction of the WW samples. In PLHC-1 cells, only the polar fraction of the November sample induced a mild but significant increase in the EROD activity (up to 30% of the TCDDmax; TCDD-EQ = 1.6 pg TCDD-EQ/mL). In rat H4IIE cells, both November and December polar fractions induced EROD activity (up to 70 % and 35% of TCDDmax; TCDD-EQ = 0.9 and 0.6 pg TCDD-EQ/mL). The total extract from the November sample showed slightly lower EROD-inducing potency than the polar fraction (TCDD-EQ = 0.7 pg TCDD-EQ/mL). On the other hand, the total extract from the December sample exhibited higher response than the corresponding polar fraction, reaching 70% of the TCDDmax and 1 pg TCDD-EQ/mL. Interestingly, the highest dose of the polar extract from the November sample was able to elicit a mild but statistically significant increase in EROD response in human HepG2 cell line (up to 13% of TCDDmax). Similar differences in the EROD response among fish, rat and human cell lines were obtained when expressed as a percentage of control (data not shown).

3.3 Zebrafish embryo developmental toxicity

Since polar fraction showed the highest toxicity in the cell lines, we have further evaluated the effect of November polar fraction using the zebrafish embryo model (Figure 4). The results of zebrafish embryo developmental toxicity assay were statistically analyzed for the time- and dose-dependent effects of treatments (Supplementary Data, Table S3). In the control group, cumulative embryo mortality was 30%, which is at the upper range of the validity threshold for undeveloped embryos at 24 hpf (Frayse et al., 2006). The highest concentration of

polar fraction (8 mL WEQ/mL) caused cumulative mortality of 87.5 % in zebrafish embryos, whereas mortality in the 4 mL WEQ/mL group was similar to the control value. The highest mortality rate was observed in the first 24 h of exposure (Figure 4A). Overall gross deformities and severe malformation in development were observed in 27 of the 30 zebrafish embryos exposed to the highest dose of polar fraction (Figure 4B). Moreover, 10% (3 out of 30) of exposed embryos that had survived the initial polar fraction-induced mortality exerted developmental abnormalities, such as tail deformation (Figure 4C).

3.4 Chemical characterization

The list of tentatively identified compounds in DR and municipal WW is given in Table 1, where these are arranged by structural properties or by technical/commercial application. Greater variety of organic compounds was found in the WW samples (35-46 compounds) than in the DR (9-17 compounds). Although the sampling period was only two weeks long, some differences in November and December samples were found at both sampling spots. In the WW samples, differences were especially prominent for aliphatic hydrocarbons and their derivatives, since the majority of compounds from this group were tentatively identified only in the non-polar fraction from the November sample. Similarly, most of the alcohols and ketones were tentatively identified in the November sample. Presence of most of the compounds belonging to other groups was consistent throughout the sampling period. Among medium-polar and polar fraction compounds, alkylphenols (compounds with estrogenic activity) were prominent, as well as antioxidant buthylated hydroxytoluene, indol-related compounds (fragrances) and 2-(methylthio)-benzothiazole. In the DR samples, presence of compounds from most of the groups strongly depended on the sampling period. For example, ketones, phthalates, alkylated benzenes, 2-methyl benzothiazole, and vanillin were found in one sample only. In both the WW and DR samples, a

small number of compounds was identified, such as alcohols dodecanol and hexadecanol and aldehyde propanal.

These results show that most of the contaminants are eluted in the medium-polar fraction; however, in the polar fraction, we were able to tentatively identify only a few compounds. In general, the ability to tentatively identify compounds was hindered by the technical limitations of the GC/MS procedure used. Overall, a significantly lower number of compounds was tentatively identified in the DR when compared to the WW samples (out of 23 compounds in the DR, only 7 were also present in the WW samples).

4. DISCUSSION

In the city of Novi Sad, Republic of Serbia, municipal wastewater is being released into the Danube River without any treatment. In the untreated wastewaters, the expected organic load is very high and is characterized by a complex chemical composition (Terzic et al., 2008). Evaluation of contribution of the component(s) to the overall mixture toxicity based on applied toxicological tests shows that the polar fraction of the WW extract has a dominant effect on the cell survival and induction of CYP1A enzyme. Cytotoxicity and CYP1A induction are usually associated with the presence of non-polar compounds, such as PAHs, polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs). These compounds significantly contribute to the AhR-mediated (Brack et al., 2008; Van den Berg et al., 1998) or cytotoxic effects (Kaisarevic et al., 2009) in the cells. However, if present in samples, they are mostly eluted in non-polar to medium-polar fractions, while only derivatives such as methylated or alcyated PAHs can be expected in polar fraction (Kaisarevic et al., 2009). In addition, absence of CYP1A-inducing potency of non- and medium-polar fraction indicates that typical AhR

agonists, such as PAHs, PCBs and PCDD/Fs, are not the key pollutants in the WW and DR samples investigated in the present study. A particularly interesting observation is that the polar fraction of the WW November sample induced EROD activity in PLHC-1, while the total extract was ineffective. We suggest that this effect can be caused by antagonistic reactions among compounds in the total extract, and/or inhibition of enzyme activity, which can occur at high concentrations of compounds. In the present study, this effect was most likely observed in PLHC-1 only, due to the cell specificity and structural differences between fish and mammalian AhR (Abnet et al., 1999).

Our findings show that polar fraction is the main carrier of the toxic effect of WW. In general, polar fraction contains multitude of compounds comprising diverse functional groups and is considerably less defined and investigated than the non-polar fraction (Lubcke-von Varel et al., 2011). It contains numerous chemicals for which the toxicological data and the knowledge on their potential to cause adverse effects are scarce, including potential to act as an AhR agonist. Chemical analysis performed on the polar fraction in the present study revealed presence of phenols, substituted benzenes and benzene derivatives, as well as organic acids. Thus far, no evidence has been provided to indicate that these compounds are capable of acting as AhR agonists or inducing the CYP1A expression in liver cells. One of the limitations of our work stems from the omission to perform more advanced analyses by liquid chromatography and tandem spectroscopy detection. Such investigation would allow us to ascertain the presence of other chemicals in the polar fraction, particularly pharmaceutical products and surfactants that, according to Smital et al. (2011), could be expected in such samples. In addition, others have shown that pharmaceuticals and personal care products (PCPs) are common WW contaminants (Li, 2014). Ability of these compounds, alone or in a mixture, to provoke EROD activity in gonadal fish cell line (Fernandez et al., 2013) and hepatoma PLHC-1 cells (Wassmur et al., 2013)

has been demonstrated. For example, exposure of PLHC-1 cells to bezafibrate, antidepressants, clofibrate, ibuprofen, and naproxen led to induction of EROD activity (Thibaut and Porte, 2008). Due to the nature of the collection area, which represents an urban discharge, it is quite possible that the polar fraction of the WW used in the present study contained a significant amount of pharmaceuticals or PCPs that may have contributed to the observed EROD activity. Several recent studies have highlighted the polar fraction as the main carrier of adverse effects on several important physiological processes. Potency of the polar fraction from sediment to exert the AhR-mediated (Grung et al., 2011; Lubcke-von Varel et al., 2011), mutagenic, estrogenic and thyroid effects (Lubcke-von Varel et al., 2011) has been documented. Moreover, the polar fraction obtained from the raw WW showed potency to induce EROD activity in PLHC-1 cells (Smital et al., 2011). Our results are similar to the findings of (Smital et al., 2011), who reported TEQ values of total extracts and polar fractions of raw WW and secondary effluent in the range of approximately 0.1-2.2 ng/L. Still, these results should be interpreted with caution, since high cytotoxicity of the samples can mask cellular responses with respect to EROD induction (Smital et al., 2011).

In practice, toxic responses of fish or rat cells to the environmental mixture/fractions are taken as a measure of the level of threat a polluted environment poses to the health of the organism. This approach is well suited for understanding the ecological risk; however, the risk to human health may not be sufficiently addressed. Presently, extrapolation of toxicological data across species is one of the main obstacles encountered in the toxicological research (Habert et al., 2014). The illustration of the activity pattern of the fractions of WW samples (Table 2) clearly shows that the toxic responses obtained in the present study were largely dependent on the origin of the cell lines. Our findings indicate that polar fraction of municipal WW exerted toxic effect in both fish and rat liver cells. In addition, in a more physiological setting, polar fraction caused

434 high degree of mortality and developmental abnormalities in zebrafish embryos. However, these
435 effects were observed in aquatic and rat models only. Response of human HepG2 cell line in
436 terms of cell growth and induction of enzymes involved in the metabolism of chemicals was
437 considerably lower. The difference in the toxic effects was probably due to variation in the
438 biological properties of models, such as membrane permeability or differences in metabolic
439 capacity among human, rat and fish/embryos. Estimated binding affinity of TCDD to the human
440 AhR is more than 10-fold lower than the observed affinity for the rodent species (Ramadoss and
441 Perdew, 2004; Zeiger et al., 2001). In addition, lower expression level of AhR in human
442 hepatocytes (Safe, 1984) and species differences in the hepatic expression of CYP1A1 and
443 CYP1A2 have been demonstrated (Dere et al., 2011; Forgacs et al., 2013). Moreover, based on
444 our EROD activity results, it seems that the rat H4IIE AhR transactivation and CYP1A
445 expression exert greater sensitivity to polar fraction of WW than the fish PLHC-1. This result
446 could be attributed to the significant structural differences between fish and mammalian AhR. It
447 has been demonstrated that fish contain at least two different types of AhR, designated as AhR1
448 and AhR2 (Hahn et al., 1997). Moreover, significant differences in the transactivation activity
449 have been observed between the fish AhR1 and AhR2, as well as the fish and the mammalian
450 AhR (Abnet et al., 1999). Combined, these differences result in a species-specific response to
451 dioxin-like chemicals, as shown elsewhere (Olsman et al., 2007; Villeneuve et al., 2001). In the
452 present study, we further demonstrated that the magnitude of response to the environmental
453 samples, such as WW extracts, the hydrophilic polar fraction in particular, is also considerably
454 different among human, rat and fish cell lines. It appears that potency of the non-dioxin-like AhR
455 agonist (polar fraction) for CYP1A induction and cell growth reduction largely depends on the
456 species, similar to the observed effect of dioxin-like AhR agonist. Based on our results, we can
457 conclude that, compared to rat and fish cells, human hepatoma cell line is less sensitive to

exposure to the environmental pollutants, thus creating an inconsistent toxic response across species. Consequently, the strategy presently employed in toxicity assessment of environmental samples using non-human models is inadequate for inter-species extrapolation and assessment of the effect of environmental chemical mixtures on the human cells and tissues.

Chemical composition of WW in general is very complex. The most prominent contaminant classes in municipal WW of the Western Balkan countries are those derived from aromatic surfactants, while the most abundant individual compounds belong to several classes of pharmaceuticals and PCPs (Terzic et al., 2008). Milic et al. (2014) showed that phthalates, PAHs, glycols and derivatives, caffeine, bisphenol A, terpene, and fatty acids belong to the compounds occurring most frequently in the WW and the Danube surface water in the vicinity of the Novi Sad locality. Although in the present study we were not able to determine presence of all these compound groups, we can conclude that the results reported here reveal a rather typical contamination profile. The smaller number of compounds detected in DR in comparison to WW might be the consequence of high dilution in river water and complex nature of the simultaneous degradation, sorption and/or biotransformation that occurs in river water. For the same locality, Milic et al. (2014) also reported that some of the compounds identified in DR were not detected in WW samples, suggesting that these compounds likely originate from another sources. Chemical analysis did not yield information regarding the specific compound(s) that might be responsible for the particular toxic response; rather, it provided a quality assessment of chemicals present and basic information regarding polarity, allowing us to better understand fractions' effects on different biological models.

In conclusion, the present study highlights and supports that the polar fraction should receive more attention in water analysis and assessment in order to obtain a more precise

probability of the mixture adverse effects on organisms. Our results demonstrate some striking differences among fish, rat and human cells regarding both cytotoxicity index and CYP1A induction. Together with other similar studies (Dere et al., 2011; Zeiger et al., 2001), these findings confirm the necessity of widening the base of experimental data for human risk assessment of environmental matrices by screening their toxicity in immortalized cells of human origin that have a high degree of fidelity to the normal human cells. These changes should provide a more realistic picture of the risk to human health arising from environmental contamination.

Acknowledgment

This work was supported by the grants from the Ministry of Education, Science and Technological Development, Republic of Serbia [grants number 173037 and 172028], and the grant from Swiss National Science Foundation [grant number SCOPES-IZ73Z0_128025]. The authors wish to thank T. Smital for providing us with PLHC-1 and HepG2 cells and Z. Csenki for providing us with adult zebrafish.

498

499 **REFERENCE**

- 500 Abnet, C. C., Tanguay, R. L., Heideman, W., Peterson, R. E., 1999. Transactivation activity of human,
501 zebrafish, and rainbow trout aryl hydrocarbon receptors expressed in COS-7 cells: greater insight into
502 species differences in toxic potency of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl
503 congeners. *Toxicol. Appl. Pharmacol.* 159, 41-51.
- 504 Ahel, M., Giger, W., 1985. Identification of specific organic wapter pollutants on the Sava River by high-
505 resolution chromatographic techniques-assisted mass spectrometry. *Kem. Ind.* 34, 295-309.
- 506 Bouma, M. E., Rogier, E., Verthier, N., Labarre, C., Feldmann, G., 1989. Further cellular investigation of
507 the human hepatoblastoma-derived cell line HepG2: morphology and immunocytochemical studies of
508 hepatic-secreted proteins. *In Vitro Cell Dev. Biol.* 25, 267-275.
- 509 Brack, W., Blaha, L., Giesy, J. P., Grote, M., Moeder, M., Schrader, S., Hecker, M., 2008. Polychlorinated
510 naphthalenes and other dioxin-like compounds in Elbe River sediments. *Environ. Toxicol. Chem.* 27, 519-
511 528.
- 512 Dere, E., Lee, A. W., Burgoon, L. D., Zacharewski, T. R., 2011. Differences in TCDD-elicited gene
513 expression profiles in human HepG2, mouse Hepa1c1c7 and rat H4IIE hepatoma cells. *BMC Genomics.*
514 12, 193.
- 515 EEC, 1986. Council Directive 86/609/EEC of 24 November 1986 on the Approximation of Laws,
516 Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals
517 Used for Experimental and Other Scientific Purposes. European Economic Community, Brussels,
518 Belgium.
- 519 Fernandez, C., Carbonell, G., Babin, M., 2013. Effects of individual and a mixture of pharmaceuticals and
520 personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal
521 cell line (RTG-2). *J. Appl. Toxicol.* 33, 1203-1212.

522 Forgacs, A. L., Dere, E., Angrish, M. M., Zacharewski, T. R., 2013. Comparative analysis of temporal and
523 dose-dependent TCDD-elicited gene expression in human, mouse, and rat primary hepatocytes. *Toxicol.*
524 *Sci.* 133, 54-66.

525 Fraysse, B., Mons, R., Garric, J., 2006. Development of a zebrafish 4-day embryo-larval bioassay to
526 assess toxicity of chemicals. *Ecotox. Environ. Safe.* 63, 253-267.

527 Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J. Y., 2013. Chronic effects of exposure
528 to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquat. Toxicol.* 132-133, 212-222.

529 Glisic, B., Hrubik, J., Fa, S., Dopudj, N., Kovacevic, R., Andric, N., 2016. Transcriptional profiles of
530 glutathione-S-Transferase isoforms, Cyp, and AOE genes in atrazine-exposed zebrafish embryos. *Environ.*
531 *Toxicol.* 31, 233-244.

532 Grung, M., Lichtenthaler, R., Ahel, M., Tollefsen, K. E., Langford, K., Thomas, K. V., 2007. Effects-
533 directed analysis of organic toxicants in wastewater effluent from Zagreb, Croatia. *Chemosphere.* 67, 108-
534 120.

535 Grung, M., Naes, K., Fogelberg, O., Nilsen, A. J., Brack, W., Lubcke-von Varel, U., Thomas, K. V., 2011.
536 Effects-directed analysis of sediments from polluted marine sites in Norway. *J. Toxicol. Environ. Health.*
537 *A.* 74, 439-454.

538 Habert, R., Muczynski, V., Grisin, T., Moison, D., Messiaen, S., Frydman, R., Benachi, A., Delbes, G.,
539 Lambrot, R., Lehraiki, A., N'Tumba-Byn, T., Guerquin, M. J., Levacher, C., Rouiller-Fabre, V., Livera,
540 G., 2014. Concerns about the widespread use of rodent models for human risk assessments of endocrine
541 disruptors. *Reproduction.* 147, R119-129.

542 Hahn, M. E., Karchner, S. I., Shapiro, M. A., Perera, S. A., 1997. Molecular evolution of two vertebrate
543 aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. *Proc. Natl. Acad. Sci. U S A.*
544 94, 13743-13748.

545 Hara-Yamamura, H., Nakashima, K., Hoque, A., Miyoshi, T., Kimura, K., Watanabe, Y., Okabe, S., 2013.
546 Evaluation of whole wastewater effluent impacts on HepG2 using DNA microarray-based transcriptome
547 analysis. *Environ. Sci. Technol.* 47, 5425-5432.

548 Hill, A. J., Teraoka, H., Heideman, W., Peterson, R. E., 2005. Zebrafish as a model vertebrate for
549 investigating chemical toxicity. *Toxicol. Sci.* 86, 6-19.

550 Jalova, V., Jarosova, B., Blaha, L., Giesy, J. P., Ocelka, T., Grabic, R., Jurcikova, J., Vrana, B.,
551 Hilscherova, K., 2013. Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive
552 and composite samples from municipal waste and surface waters. *Environ. Int.* 59, 372-383.

553 Kaisarevic, S., Varel, U. L., Orcic, D., Streck, G., Schulze, T., Pogrmic, K., Teodorovic, I., Brack, W.,
554 Kovacevic, R., 2009. Effect-directed analysis of contaminated sediment from the wastewater canal in
555 Pancevo industrial area, Serbia. *Chemosphere.* 77, 907-913.

556 Li, W. C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil.
557 *Environ. Pollut.* 187C, 193-201.

558 Lubcke-von Varel, U., Machala, M., Ciganeck, M., Neca, J., Pencikova, K., Palkova, L., Vondracek, J.,
559 Loffler, I., Streck, G., Reifferscheid, G., Fluckiger-Isler, S., Weiss, J. M., Lamoree, M., Brack, W., 2011.
560 Polar compounds dominate in vitro effects of sediment extracts. *Environ. Sci. Technol.* 45, 2384-2390.

561 Mihajlovic, I., Pap, S., Sremacki, M., Brboric, M., Babunski, D., Dogo, M., 2014. Comparison of
562 spectrolyser device measurements with standard analysis of wastewater samples in Novi Sad, Serbia. *Bull.*
563 *Environ. Contam. Toxicol.* 93, 354-359.

564 Milic, N., Spanik, I., Radonic, J., Turk Sekulic, M., Grujic, N., Vyviurska, O., Milanovic, M., Sremacki,
565 M., Vojinovic-Miloradov, M., 2014. Screening Analyses of Wastewater and Danube Surface Water in
566 Novi Sad Locality, Serbia. *Fresen. Environ. Bull.* 23, 372-377.

567 Olsman, H., Engwall, M., Kammann, U., Klempt, M., Otte, J., Bavel, B., Hollert, H., 2007. Relative
568 differences in aryl hydrocarbon receptor-mediated response for 18 polybrominated and mixed halogenated
569 dibenzo-p-dioxins and -furans in cell lines from four different species. *Environ. Toxicol. Chem.* 26, 2448-
570 2454.

571 Otte, J. C., Keiter, S., Fassbender, C., Higley, E. B., Rocha, P. S., Brinkmann, M., Wahrendorf, D. S.,
572 Manz, W., Wetzel, M. A., Braunbeck, T., Giesy, J. P., Hecker, M., Hollert, H., 2013. Contribution of

573 Priority PAHs and POPs to Ah Receptor-Mediated Activities in Sediment Samples from the River Elbe
574 Estuary, Germany. PLoS One. 8, e75596.

575 Ramadoss, P., Perdew, G. H., 2004. Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe
576 to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured
577 cells. Mol. Pharmacol. 66, 129-136.

578 Richardson, S. D., 2002. The role of GC-MS and LC-MS in the discovery of drinking water disinfection
579 by-products. J. Environ. Monit. 4, 1-9.

580 Safe, S., 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry,
581 toxicology, and mechanism of action. Crit. Rev. Toxicol. 13, 319-395.

582 Santos-Delgado, M. J., Crespo-Corral, E., Polo-Diez, L. M., 2000. Determination of herbicides in soil
583 samples by gas chromatography: optimization by the simplex method. Talanta. 53, 367-377.

584 Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H.,
585 Kenney, S., Boyd, M. R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl.
586 Cancer Inst. 82, 1107-1112.

587 Smital, T., Terzic, S., Zaja, R., Senta, I., Pivcevic, B., Popovic, M., Mikac, I., Tollefsen, K. E., Thomas,
588 K. V., Ahel, M., 2011. Assessment of toxicological profiles of the municipal wastewater effluents using
589 chemical analyses and bioassays. Ecotox. Environ. Safe. 74, 844-851.

590 Terzic, S., Senta, I., Ahel, M., Gros, M., Petrovic, M., Barcelo, D., Muller, J., Knepper, T., Marti, I.,
591 Ventura, F., Jovancic, P., Jabucar, D., 2008. Occurrence and fate of emerging wastewater contaminants in
592 Western Balkan Region. Sci. Total Environ. 399, 66-77.

593 Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish
594 hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A. Toxicol. In Vitro. 22,
595 1128-1135.

596 Traven, L., Zaja, R., Loncar, J., Smital, T., Micovic, V., 2008. CYP1A induction potential and the
597 concentration of priority pollutants in marine sediment samples-in vitro evaluation using the PLHC-1 fish
598 hepatoma cell line. Toxicol. In Vitro. 22, 1648-1656.

599 Tubic, A., Leovac, A., Hrubik, J., Glisic, B., Kaisarevic, S., Ivancev-Tumbas, I., Kovacevic, R., 2013.
600 Toxicological profiles assessment of the water and sediments from the Krivaja and Jegricka Rivers,
601 Serbia. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 48, 1201-1215.

602 Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P.,
603 Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K.,
604 Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M.,
605 Waern, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for
606 humans and wildlife. Environ. Health Perspect. 106, 775-792.

607 Villeneuve, D. L., Khim, J. S., Kannan, K., Giesy, J. P., 2001. In vitro response of fish and mammalian
608 cells to complex mixtures of polychlorinated naphthalenes, polychlorinated biphenyls, and polycyclic
609 aromatic hydrocarbons. Aquat. Toxicol. 54, 125-141.

610 Wassmur, B., Grans, J., Norstrom, E., Wallin, M., Celander, M. C., 2013. Interactions of pharmaceuticals
611 and other xenobiotics on key detoxification mechanisms and cytoskeleton in *Poeciliopsis lucida*
612 hepatocellular carcinoma, PLHC-1 cell line. Toxicol. In Vitro. 27, 111-120.

613 Zeiger, M., Haag, R., Hockel, J., Schrenk, D., Schmitz, H. J., 2001. Inducing effects of dioxin-like
614 polychlorinated biphenyls on CYP1A in the human hepatoblastoma cell line HepG2, the rat hepatoma cell
615 line H4IIE, and rat primary hepatocytes: comparison of relative potencies. Toxicol. Sci. 63, 65-73.

616

616

617 Table 1. Organic compounds tentatively identified by the GC/MS in the Danube River water and
 618 municipal wastewater of the city of Novi Sad, Serbia (Library search match $\geq 70\%$)

Compounds:	Samples:		Wastewater		Fraction
	Nov.	Dec.	Nov.	Dec.	
Aliphatic hydrocarbons and derivatives					
Heptadecane			+		n
Hexacosane			+		n
Heptacosane			+		n
Octacosane			+		n
Docosane			+		n
Tricosane			+		n
Tetracosane			+		n
Heneicosane			+		n
triacontane			+		n
1-Hexadecene	+		+	+	n
6,6-dimethyl-2-methylene-(1S)-bicyclo[3.1.1]heptanes				+	m
1-methyl-4-(1-methylethyl)-1,3-cyclohexadiene			+	+	m,p
Alcohols					
2-(2-butoxyethoxy)-ethanol		+			n
1-Dodecanol	+	+			n,m
1-Hexadecanol	+	+			n,m
α,α 4-trimethyl-3-cyclohexene-1-methanol		+			m
α -methyl benzenmethanol			+	+	m
2-butoxy-ethanol	+		+		m
Benzyl alcohol	+				m
1-Octadecanol			+		m
Phenylethyl alcohol			+	+	m
Propofol			+	+	p
Aldehydes					
Propanal	+	+			m
Ketones					
1-(3-aminophenyl)-ethanone	+		+	+	m

1-(4-hydroxy-3-methoxyphenyl)-ethanone		+	m
1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone		+	m
2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	+	+	m
Heptanone	+		m
1-(2,4,6-trimethylphenyl)-ethanone	+		m
5-hexyldihydro-2(3H)-furanone		+	m, p
Phthalates			
Di-n-butylphthalate	+		n
Phenols			
2-methyl phenol		+	m
4-methyl phenol		+	m,p
2-methoxy phenol		+	m
4-methoxy phenol (Mequinol)		+	m
2-ethyl phenol		+	m
4-ethyl phenol		+	m
3,4-dimethoxy-phenol		+	m
4-(1-methylethyl)-phenol		+	m
2,4-dimethyl phenol		+	m
2,5-dimethyl phenol		+	m
3,5-dimethyl phenol		+	m,p
2,4,6-trimethyl-phenol		+	m
3-methyl-5(1-methylethyl)-methyl carbamate phenol		+	m
Eugenol		+	m
Substituted benzenes and benzene derivates			
Indene	+		n
1,3,5-trichlorobenzene	+		n
1,3-bis(1-methylethyl)-benzene	+		n
Butylated hydroxytoluen		+	n, m, p
1,4-dimethoxybenzene		+	m
1,2,3-trimethoxybenzene		+	m
1-ethyl-4-methyl-benzene	+	+	n
2,3-dihydro-5-methyl-1H-Indene	+	+	m, p
Indole		+	m
3-methyl-1H-Indole		+	m
Organic acids and esters after methylation			

Phenylmethyl ester of acetic acid	+		m
Tetradecanoic acid	+		m
9-octadecenoic acid, (Z)-Methyl ester	+	+	m, p
9,12-octadecadienoic acid, (Z,Z)-methyl ester		+	p
Amines			
3,4-dichloro-benzenamine		+	m
Food additives			
Vanillin	+	+	m, p
Plasticizers			
Triphenyl phosphate		+	m
PAHs			
3,4-dihydro-1(2H)-naphthalenone		+	m
Benzothiazoles			
2-methyl benzothiazole	+		m
2-(methylthio)-benzothiazole		+	m
Biphenyl			
o-hydroxybiphenyl		+	m
Steroles			
Cholesterol		+	m
Nitriles			
Benzyl nitrile		+	m

n - non-polar fraction, m - medium-polar fraction, p - polar fraction, *Nov.* – November, *Dec.* - December

Table 2. Cytotoxicity and EROD activity pattern of non-polar, medium-polar, polar fractions, and total extract of the wastewater samples collected in November and December in PLHC-1, H4IIE, and HepG2 cell lines (strong effect = significant response above 50%; mild effect = significant response below 50% of maximal (100%) cytotoxicity (SRB) or TCDD (EROD)) .

Wastewater samples																
November									December							
Extract/fraction	Non-polar		Medium-polar		Polar		Total		Non-polar		Medium-polar		Polar		Total	
Fish PLHC-1																
Rat H4IIE																
Human HepG2																
Assays	SRB	EROD	SRB	EROD	SRB	EROD	SRB	EROD	SRB	EROD	SRB	EROD	SRB	EROD	SRB	EROD
		strong effect				mild effect				low/no effect						

FIGURE CAPTIONS

Figure 1. Map of the sampling area. The municipal wastewater (WW) discharge location and the Danube River (DR) sampling site are indicated.

Figure 2. Cytotoxicity of water extracts. PLHC-1, H4IIE and HepG2 cells were exposed to solvent (–) or non-polar (Δ), medium-polar ($\square$), polar fractions ($\blacksquare$), and total extract ($\bullet$) of the Danube River water and wastewater samples collected in November and December. Cytotoxicity was determined by the SRB test after 48 h of incubation. Responses are expressed as the level of cytotoxicity (%) relative to the solvent-treated control cells (0%). Each point denotes the mean $\pm$ SEM of three replicate assays.

Figure 3. EROD activity of water extracts. PLHC-1, H4IIE and HepG2 cell lines were exposed to solvent ($\circ$), non-polar (Δ), medium-polar ($\square$), polar fractions ($\blacksquare$), and total extract ($\bullet$) of the Danube River water and wastewater samples collected in November and December. CYP1A induction was determined by the microEROD assay after 48 h of incubation. Responses are expressed as percentages of the maximum response observed in the TCDD treatment for each cell line. Each point denotes the mean $\pm$ SEM of three replicate assays.

Figure 4. Mortality and developmental abnormalities in zebrafish embryos exposed to polar fraction of wastewater. (A) Embryos were exposed to polar fraction at 2 hpf and cumulative and percentage mortalities were calculated. Values denote mean $\pm$ SEM of 2-3 independent

672 experiments with a total of 10-20 embryos per experiment. Image of a typical toxic effect of polar
673 fraction (B) and developmental abnormalities (C) on zebrafish embryos observed at 48 hpf.

Figure 2

Figure 3

Figure 4

A

B

C