ARTICLE IN PRESS

Environmental Pollution xxx (2016) 1–11

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

# Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis☆

Maria König [a], Beate I. Escher [b, c, d, e, *], Peta A. Neale [b, e], Martin Krauss [a], Klára Hilscherová [f], Jiří Novák [f], Ivana Teodorović [g], Tobias Schulze [a], Sven Seidensticker [a, h], Muhammad Arslan Kamal Hashmi [a], Jörg Ahlheim [a], Werner Brack [a, h]

[a] UFZ – Helmholtz Centre for Environmental Research, Effect-Directed Analysis, 04318 Leipzig, Germany
[b] Australian Rivers Institute, School of Environment, Griffith University, Southport, QLD 4222, Australia
[c] UFZ – Helmholtz Centre for Environmental Research, Cell Toxicology, 04318 Leipzig, Germany
[d] Eberhard Karls University Tübingen, Environmental Toxicology, Center for Applied Geosciences, 72074 Tübingen, Germany
[e] The University of Queensland, National Research Centre for Environmental Toxicology (Entox), Brisbane, QLD 4108, Australia
[f] Masaryk University, Research Centre for Toxic Compounds in the Environment (RECETOX), Kamenice 753/5, 62500 Brno, Czechia
[g] University of Novi Sad, Faculty of Sciences, Trg Dositeja Obradovica 2, 21000 Novi Sad, Serbia
[h] RWTH Aachen University, Institute for Environmental Research (Biology V), Aachen, Germany

ARTICLE INFO

*Article history:*
Received 10 August 2016
Received in revised form 19 October 2016
Accepted 5 November 2016
Available online xxx

*Keywords:*
Bioassay
Xenobiotic metabolism
Specific modes of action
Adaptive stress response
Bioanalytical equivalent concentration

ABSTRACT

Complex mixtures of micropollutants, including pesticides, pharmaceuticals and industrial chemicals emitted by wastewater effluents to European rivers may compromise the quality of these water resources and may pose a risk to ecosystem health and abstraction of drinking water. In the present study, an integrated analytical and bioanalytical approach was applied to investigate the impact of untreated wastewater effluents from the city of Novi Sad, Serbia, into the River Danube. The study was based on three on-site large volume solid phase extracted water samples collected upstream and downstream of the untreated wastewater discharge. Chemical screening with liquid chromatography high resolution mass spectrometry (LC-HRMS) was applied together with a battery of *in vitro* cell-based bioassays covering important steps of the cellular toxicity pathway to evaluate effects on the activation of metabolism (arylhydrocarbon receptor AhR, peroxisome proliferator activated receptor gamma PPARγ), specific modes of action (estrogen receptor ERα, androgen receptor AR) and adaptive stress responses (oxidative stress, inflammation). Increased effects, significantly changed contamination patterns and higher chemical concentrations were observed downstream of the wastewater discharge. A mass balance approach showed that enhanced endocrine disruption was in good agreement with concentrations of detected hormones, while only a smaller fraction of the effects on xenobiotic metabolism (<1%) and adaptive stress responses (0–12%) could be explained by the detected chemicals. The chemical and effects patterns observed upstream of the discharge point were fairly re-established at about 7 km downstream, demonstrating the enormous dilution capacity of this large river.



## 1. Introduction

Surface waters typically contain complex mixtures of micropollutants including pharmaceuticals, pesticides, industrial chemicals and their transformation products (Loos et al., 2009). These compounds are emitted to surface water by point sources including treated or untreated wastewater from municipal and industrial sources or livestock enterprises and by diffuse sources such as run-off from urban and agricultural areas (Heeb et al., 2012). These chemical mixtures may cause adverse effects to aquatic organisms and may pose a risk to human health. Many investigations have focused on the occurrence and effects of estrogens in wastewater affected water bodies (Sumpter, 1998;

☆ This paper has been recommended for acceptance by Dr. Chen Da.
* Corresponding author. UFZ – Helmholtz Centre for Environmental Research, Cell Toxicology, 04318 Leipzig, Germany.
*E-mail address:* beate.escher@ufz.de (B.I. Escher).

http://dx.doi.org/10.1016/j.envpol.2016.11.011
0269-7491/

Please cite this article in press as: König, M., et al., Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis, Environmental Pollution (2016), http://dx.doi.org/10.1016/j.envpol.2016.11.011

Vethaak et al., 2005). Awareness and research on compounds affecting other important hormonal physiological processes has increased in recent years. This includes progestagenic and glucocorticogenic activity, and adaptive stress responses, such as oxidative stress and inflammation (Escher et al., 2012, 2014; Schriks et al., 2010). These toxicological endpoints can be addressed with bioanalytical tools complementing targeted chemical analysis, which may fail to cover the high diversity of possible toxicants occurring in water (Escher and Leusch, 2012). A combination of both approaches may help to assess the overall potency of the complex mixtures and to identify major drivers of their effects.

Untreated wastewaters, which are emitted to the River Danube by big cities including Novi Sad, are believed to compromise water quality and the function of the river as a drinking water resource, for bathing and fishing, and as a habitat for aquatic organisms. For the last 15 years water quality throughout the whole Danube basin has been intensively investigated in three Joint Danube Surveys (JDS, Liška et al., 2015; Liška et al., 2008). The first of these sampling surveys took place in 2001 with follow up campaigns in 2007 and 2013. A recent investigation (Neale et al., 2015) of 22 water samples taken along a stretch from Austria to the Black Sea during the 3rd Joint Danube Survey (JDS3) detected up to 94 different organic chemicals at relatively low concentrations compared to other European rivers (Loos et al., 2010; ter Laak et al., 2010).

The relatively low contamination level could be confirmed as measurable effects after enrichment by a factor of ten to 1000 with bioanalytical tools (Neale et al., 2015). The most frequently detected biological responses were activation of receptors for activation of xenobiotic metabolism (aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR)) and estrogenicity, adaptive stress responses to oxidative stress and genotoxicity, and fish embryo acute toxicity. Furthermore, other studies of the JDS3 classified the chemical and ecological status of the Danube River upstream and downstream of Novi Sad as moderate to good (Liška et al., 2015; Mischke and Opitz, 2005). However, the stretch between Novi Sad and Belgrade was identified as one of the hot spots for fecal pollution (Liška et al., 2015). Fecal pollution is an ongoing problem in the Danube due to the incompletely treated or untreated wastewater disposed in many sections in the river and its tributaries (Kirschner et al., 2009). While the high dilution of the untreated sewage may account for low micropollutant loads found in the JDS3, the fecal pollution markers are an indication that on a local scale much higher contamination of micropollutants and risks to ecosystem and quality of drinking water abstracted downstream may occur.

This study, which is part of the EU project SOLUTIONS (Brack et al., 2015), demonstrates an integrated toxicological-chemical approach to assess and visualize the impact of point sources on river water quality using the River Danube impacted by untreated wastewater from Novi Sad (Serbia) as an example. Samples were taken at three sites, one upstream (2 km) and two downstream (236 m and 7 km) of the wastewater discharge using on-site large-volume solid phase extraction (LVSPE). The upstream and downstream sites are from the same river stretch as the previous sampled sites 32 and 33 of JDS3 (Neale et al., 2015), but closer to the shoreline with potentially greater anthropogenic impact. In addition we investigated an additional site at the wastewater discharge. These sites were not only selected because of the missing wastewater treatment plants, but also due to human health concerns triggered by legal commercial and recreational fishing in the investigated section as well as the fact the main underground aquifers and abstraction points are located between two of the sampling sites. The samples were analyzed for 276 organic chemicals and 21 toxicological endpoints in 15 cellular reporter gene bioassays. The applied bioassays cover important steps of the cellular toxicity pathway, including activation of metabolism, endocrine disruption, adaptive stress responses and non-specific toxicity (Escher et al., 2014; Escher and Leusch, 2012; Macova et al., 2010). A mass balance approach was further applied to identify the major chemical drivers of the measured effects and assess quantitatively their relative contribution to the overall biological effect.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The chemicals used as reference compounds in the *in vitro* assays (Table 1) were purchased from the following sources: 17ß-estradiol, metribolone, cyproterone acetate, dexamethasone, rosiglitazone and 2-chloro-5-nitro-N-4-pyridinylbenzamide from Sigma Aldrich (Steinheim, Germany), dihydrotestosterone and flutamide from Sigma Aldrich (Czech Republic), 2,3,7,8-tetrachlorodibenzo-p-dioxin from Dr. Ehrenstorfer Standards (Germany), tBHQ from Sigma Aldrich (Castle Hill, Australia), mifepristone and promegestone from Steraloids (New Port, RI, USA), mitomycin from Merck Millipore (Baywater, Australia) and TNFα from Life Technologies (Mulgrave, Australia). The selection of analytes for targeted screening was based on Hug et al. (2014) who included a large number of contaminants from different classes known to occur in surface water and wastewater. The list of target analytes was further extended for typical endocrine disruptors and hormones. All solvents used had a purity of at least 99%.

### 2.2. Sampling sites

The three sampling sites were chosen to examine the influence of a direct untreated wastewater discharge on the Danube Riverside at Novi Sad, Serbia. The first sampling site was two kilometers upstream of the wastewater discharge in a rural and suburban area (Site 1, NS1, 45°14′15.9″ N, 19°51′06.5″ E), the second was 236 m downstream (Site 2, NS2, 45°15′12.5″ N, 19°51′21.0″ E) within the city collecting only municipal sewage and rain water runoff from the city of Novi Sad and the third (Site 3, NS3, 45°14′44.1″ N, 19°55′18.5″ E) was seven kilometers downstream of the wastewater discharge again in a rural area close to a small fishing village already outside the city (Supporting Information (SI) Fig. S1). It should be noted that between NS2 and NS3 a second wastewater discharge of predominantly industrial wastewater enters the River, because of an industrial area upstream of NS3. Further details are described in the SI, Section S1.

### 2.3. Large volume solid phase extraction (LVSPE)

A solid phase extraction device for large volumes was used for on-site processing of river water. The LVSPE consists of a vacuum sampling system, a filtration device (Sartopure GF + MidiCap, 0.65 μm separation size, Sartorius) connected to the inlet tube, a stainless steel pressure chamber and a stainless steel extraction cartridge filled with 160 g of a neutral sorbent polystyrene divinylbenzene co-polymer (Chromabond® HR-X, Macherey Nagel, Düren, Germany) (Schulze et al., 2015). In principle, a 500 mL water sample was taken, released to pressure and finally pumped through the extraction cartridge. The cartridge was preconditioned with 800 mL methanol/ethyl acetate (1:1 *v/v*; Merck, LC-MS grade), methanol and LC-MS grade water (Supelco).

Total volumes of 1000, 850 and 1000 L of water were extracted at NS1, NS2 and NS3 by repeated collection of 500 mL sub-samples over 48 h (NS1), 60 h (NS2) and 24 h (NS3), respectively, and compared to a procedural blank with LC-MS grade water and a

Please cite this article in press as: König, M., et al., Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis, Environmental Pollution (2016), http://dx.doi.org/10.1016/j.envpol.2016.11.011