Water **2015**, *7*, 1959-1968; doi:10.3390/w7051959

OPEN ACCESS

water

ISSN 2073-4441
www.mdpi.com/journal/water

Technical Note

Preliminary Toxicological Evaluation of the River Danube Using *in Vitro* Bioassays

Clemens Kittinger [1,*], Rita Baumert [1], Bettina Folli [1], Michaela Lipp [1], Astrid Liebmann [1], Alexander Kirschner [2], Andreas H. Farnleitner [3], Andrea J. Grisold [1] and Gernot E. Zarfel [1]

[1] Institute of Hygiene, Microbiology and Environmental Medicine, Medical University Graz, Graz 8010, Austria; E-Mails: rita.baumert@medunigraz.at (R.B.); bettina.folli@medunigraz.at (B.F.); michaela.lipp@medunigraz.at (M.L.); astrid.liebmann@gmx.at (A.L.); andrea.grisold@medunigraz.at (A.J.G.); gernot.zarfel@medunigraz.at (G.E.Z.)

[2] Division Water Hygiene, Institute for Hygiene and Applied Immunology, Medical University of Vienna, Vienna 1090, Austria; E-Mail: alexander.kirschner@meduniwien.ac.at

[3] Institute of Chemical Engineering, Research Group Environmental Microbiology and Molecular Ecology, Vienna University of Technology, Vienna 1090, Austria; E-Mail: andreas.farnleitner@wavenet.at

* Author to whom correspondence should be addressed; E-Mail: clemens.kittinger@medunigraz.at; Tel.: +43-316-3857-3600; Fax: +43-316-3857-9637.

Academic Editor: Say-Leong Ong

Received: 13 February 2015 / Accepted: 23 April 2015 / Published: 30 April 2015

Abstract: The Joint Danube Survey 3, carried out in 2013 was the world's biggest river research expedition of its kind. The course of the second largest river of Europe passes large cities like Vienna, Budapest and Belgrade and is fed from many tributaries like Inn, Thisza, Drava, Prut, Siret and Argeș. During the 6 weeks of shipping the 2375 km downstream the River Danube from Germany to the Black Sea an enormous number of water samples were analyzed and collected. A wide spectrum of scientific disciplines cooperated in analyzing the River Danube waters. For toxicological analysis, water samples were collected on the left, in the middle, and on the right side of the river at 68 JDS3 sampling points and frozen until the end of the Danube survey. All samples were analyzed with two *in vitro* bioassays tests (umuC and MTS). Testing umuC without S9 activation and MTS test did not show positive signals. But umuC investigations of the water samples came up with toxic signals on two stretches, when activated with S9

enzymes. The override of the limiting value of the umuC investigation with prior S9 activation started downstream Vienna (Austria) and was prolonged until Dunaföldvar (Hungary). This stretch of the River Danube passes a region that is highly industrialized, intensively used for agricultural purposes and also highly populated (Vienna, Bratislava and Budapest). The elevated values may indicate these influences.

Keywords: Joint Danube Survey; Joint Danube Survey 3 (JDS3); UV mutagenesis gene C (umuC); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS); toxicity; river; surface water

1. Introduction

The 2872 km long River Danube, the second longest river in Europa, passes ten countries until it flows into the Black Sea forming a large river delta. The drainage basin is around 817,000 km^2 large, including the waste waters of this mostly densely populated area. The course of the river passes large cities like Vienna, Budapest and Belgrade and is fed from many tributaries like Inn, Tisza, Drava, Sava, Pruth, Siret and Argeș.

The Joint Danube Survey 3 (JDS3) 2013 was the world's biggest river research expedition of its kind [1]. Until now the JDS has been carried out three times every six years. Between 13 August and 26 September, samples were taken along a 2563 km stretch of the River Danube starting in Böfinger Halde (Germany, river 2581 km) to the Danube Delta (river 18 km). Besides collecting water samples and directly surveying the microbiological status, many other river relevant parameters from water, sediment and suspended solids were evaluated by laboratories all across Europe: e.g., hydromorphology, basic chemistry, biological key elements like fish, macrozoobenthos, phytobentos, phytoplankton, macrophytes, *etc.*

One aspect of the investigation was the primary evaluation of the toxicological burden over the whole river course. In order to provide a first toxicological investigation and status assessment of the River Danube, two widely used, easily applicable toxicological tests were applied for all JDS3 samples (umuC and MTS). These tests have been used for the investigation of surface waters by other groups [2–6] and have been additionally established and used for the investigation of the River Mur in Styria, Austria [7]. The investigation of the water samples with these protocols is very reliable in terms of unspecific screening for toxic signals in surface or waste water samples [4–6]. These tests need only a small amount of the test liquid and can react on high numbers of mutagenic and cytotoxic substances and are therefore suitable for looking for unknown hazardous substances originating from all sources [3,8,9]. Compared with other investigations carried out during the JDS3, the results of the toxicity survey may lead to a new discussion on the methodology in the search for toxic substances and to new insights into the toxicological burden of the Danube.

2. Materials and Methods

2.1. Water Samples

Samples were taken all over the River Danube course at 68 positions (Figure 1). At a sampling point (SP) samples were always taken from the left side (L), in the middle (M) and from the right side (R) (resulting in 171 samples) of the River Danube, with the exception of the tributary samples that were mostly collected only once in the middle (11) (Table 1).

Figure 1. Overview of the Joint Danube Survey 3 (JDS3) sampling points along the river Danube. The map was taken with kind permission of the ICPDR.

Subsamples of 50 mL from the sample bottle taken for the microbiological investigations (surface water collected 0.3 m under the river surface) were filled into sterile non-toxic 50-mL plastic vials and immediately stored at −20 °C until analysis in the home laboratory. Before being used in the experiments, the samples, were thawed on ice, vortexed and filtrated to eliminate bacteria via 0.45 µm syringe filter (TPP, Techno Plastic Products, Switzerland). Freezing of the samples might alter the composition and amount of toxic compounds in the sample. Although studies of Armishaw *et al.* showed for pesticide spiked material no alteration over 168 days of freezer storage, this cannot be predicted for hundreds of toxic substances in surface water [10]. The stability of the JDS3 water samples stored at 4 °C was also investigated on three exemplary samples during the study and showed

that most substances were relatively stable over a period of 173 days [1]. The small sample volume, the storage at −20 °C and the possibility to test a large sample number was a requirement for the screening investigation.

Table 1. List of the JDS3 sampling points (SP), the orange highlighted sampling points were only collected midstream.

SP	Name of SP	River km	SP	Name of SP	River km
JDS1	Böfinger Halde	2581	JDS35	Tisa	1215
JDS2	Kelheim, gauging station	2415	JDS36	DS Tisa/US Sava (Belegis)	1200
JDS3	Geisling power plant	2354	JDS37	Sava	1170
JDS4	Deggendorf	2285	JDS38	Upstream Pancevo	1159
JDS5	Mühlau	2258	JDS39	DownstreamPancevo	1151
JDS6	Jochenstein	2204	JDS40	Upstream Vel. Morava	1107
JDS7	US dam Abwinden-Asten	2120	JDS41	Velika Morava	1103
JDS8	Oberloiben	2008	JDS42	DS Velika Morava	1097
JDS9	Klosterneuburg	1942	JDS43	Banatska Palanka	1071
JDS10	Wildungsmauer	1895	JDS44	IGR Golubac/Koronin	1040
JDS11	US Morava (Hainburg)	1881	JDS45	IGR Tekija/Orsova	954
JDS12	Morava	1880	JDS46	Vrbica/Simijan	926
JDS13	Bratislava	1869	JDS47	Upstream Timok	849
JDS14	Gabcikovo reservoir	1852	JDS48	Timok	845
JDS15	Medvedov/Medve	1806	JDS49	Pristol/Novo Salo	834
JDS16	Moson Danube	1794	JDS50	Downstream Kozloduy	685
JDS17	Klizska Nema	1790	JDS51	Iskar	637
JDS18	Vah	1766	JDS52	Downstream Olt	602
JDS19	Iza/Szony	1761	JDS53	Downstream Zimnicea/Svistov	550
JDS20	Szob	1707	JDS54	Jantra	537
JDS21	US Budapest - Megyeri Bridge	1660	JDS55	Downstream Jantra	532
JDS22	DS Budapest—M0	1632	JDS56	Russenski Lom	498
JDS23	Rackeve-Soroksar arm-end	1586	JDS57	Downstream Ruse	488
JDS24	Dunaföldvar	1560	JDS58	Arges	432
JDS25	Paks	1533	JDS59	Downstream Arges	429
JDS26	Baja	1481	JDS60	Chiciu/Silistra	378
JDS27	Hercegszanto	1434	JDS61	Giurgeni	235
JDS28	US Drava	1384	JDS62	Braila	167
JDS29	Drava	1379	JDS63	Siret	154
JDS30	DS Drava (Erdut/Bogojevo)	1367	JDS64	Prut	135
JDS31	Ilok/Backa Palanka	1300	JDS65	Reni	130
JDS32	US Novi Sad	1262	JDS66	Vilova/Kilia Arm	18
JDS33	DS Novi Sad	1252	JDS67	Sulina Arm	26
JDS34	US Tisa (Stari Slankamen)	1216	JDS68	St.Gheorge Arm	104

2.2. Toxicity Assay: umuC

An SOS/umuC assay was carried out to search for mutagenicity. The assay was carried out according to Reifferschied *et al.*, following the modifications of the ISO 13829 standard [11]. The umuC assay was conducted with or without S9 enzymatic activation (Trinova Biochem, Gießen, Germany). Filtrated water samples as described above were applied to the test without pH correction

as the pH values were between 8.0 and 8.5 over the whole stretch of the Danube River [1]. Tests were carried out in 96 well plates (TPP, Techno Plastic Products, Trasadingen, Switzerland). The absorbance at 600 nm and 420 nm was measured with a Zenyth 3100 Multimode Detector (Beckman Coulter, Austria). All experiments were carried out in triplicates and mean and standard error of the mean (SEM) were calculated. According to the ISO 13829 the growth rate (G) was calculated with Equation (1).

$$G = \frac{OD600sample - OD600blank}{OD600control - OD600blank} \quad (1)$$

A growth reduction of 25% compared to the growth control was considered to be a cytotoxic water sample. The induction rate (I_R) was calculated with Equation (2):

$$IR = \frac{1}{G} \times \frac{A420sample - A420blank}{A420control - A420blank} \quad (2)$$

According to ISO 13829 an induction rate of ≥1.5 was taken as a signal for mutagenic potency in the water samples.

2.3. Cytotoxicity Assay: MTS

For determination of cytotoxic potential of the water samples a MTS test (Promega, Mannheim, Germany) was carried out. The test is based on the yellow salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is converted into the blue/violet water insoluble salt formazan. The conversion into formazan is mediated by dehydrogenases of intact mitochondria and therefore provides insight into cell viability. HepG2 (DSMZ ACC 180) cells were used for cytotoxicity assays. HepG2 cells are capable of phase one liver enzymatic reaction and are highly sensitive against polycyclic aromatic hydrocarbons and genotoxic effects can be seen after challenging with carcinogenic mycotoxins. These cells also react positively to Arsenic and carcinogenic metals like Cadmium [12]. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Promega, Vienna, Austria) with 10% fetal bovine sera (FBS, Promega, Austria) and 100 U/mL penicillin/streptomycin (Sigma Aldrich, Vienna, Austria) at 37 °C and 5% CO_2. Passages 3 to 6 were taken for the experiments. Cell number was titrated to find out the best ratio between cell number and maximum signal response. A cell number of 1×10^4 cells/well was found to be ideal. For the cytotoxic analysis, cells were freshly seeded into 96 well plates (Thermo Scientific, Vienna, Austria) and allowed to attach for 4 h. After that, 40% of the medium was replaced by filtrated water samples and incubated for 20 h at 37 °C and 5% CO_2. After the incubation, 20 µL of the Dye Solution was added. The plates were incubated for up to 4 h at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 492 nm was measured with a Zenyth 3100 Multimode Detector (Beckman Coulter, Vienna, Austria). Deionized water served as control. Experiments were carried out in triplicates. Viability (V_C) of the cells incubated with deionized water was taken 100% and the viability of the river samples was put into relation to them and calculated with Equation (3).

$$V_C = \frac{100 \times Abs492\ Water\ Sample}{Abs492\ Control\ Sample} \quad (3)$$

A reduction of the viability to 70% compared to the test sample was taken as a cytotoxic response [3].

3. Results and Discussion

All samples of the JDS3 sampling points were investigated for a toxic signal with the umuC test and the MTS. Experiments were carried out in triplicates and means and standard deviations are given as line and error bars in the figures.

3.1. UmuC Results without Enzymatic S9 Activation

The umuC investigation of the River Danube Samples without S9 activation did not show any raised values (Figure 2). The only exception was one value of the triplicates at sample position JDS31 M that was elevated to 1.79. But because the two other midstream values were 0.95 and 0.92, this high single value of 1.79 has to be interpreted as an outlier. In addition, the mean value was below the limit value of 1.5. The results go also well with previous river studies, were the samples without S9 activation did not came up with a toxic signal [7]. Evaluation of growth of the umuC *Salmonella* as requested in ISO 13829 did also not show any inhibition.

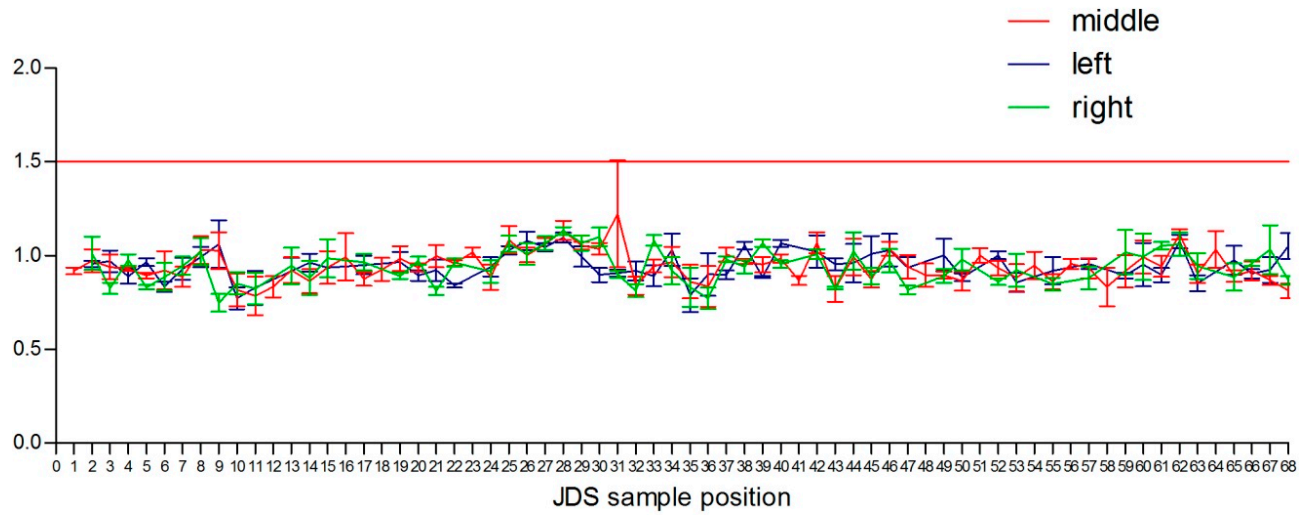

Figure 2. Results from umuC testing of the River Danube without enzymatic activation. The red line at 1.5 represents the limit value according to ISO 13829.

3.2. UmuC Results with Enzymatic S9 Activation

Investigation of the River Danube samples with enzymatic S9 activation showed exceedance of the limit value of 1.5 and elevated values before and after a few JDS sampling points (Figure 3). The values of all investigated sampling points had little standard deviations and were thus considered reliable. Values started to rise from JDS13 (Bratislava, SVK, river 1869 km) on until JDS28 (upstream Drava, HR, river 1632 km). The limiting value was exceeded at JDS15 (Medvedov, SVK, river 1806 km), JDS20 (Szob, HU, river 1707 km), JDS22 (downstream Budapest, HU, river 1632 km), JDS23 (Rackeve-Soroksar branch, HU, river 1586 km), JDS24 (Dunarföldvar, HU, river 1560 km) and JDS25 (Paks, HU, river 1533 km). Elevated values were also observed at JDS55 L (downstream Jantra, RO, river 532 km) but stayed below the limit of 1.5.

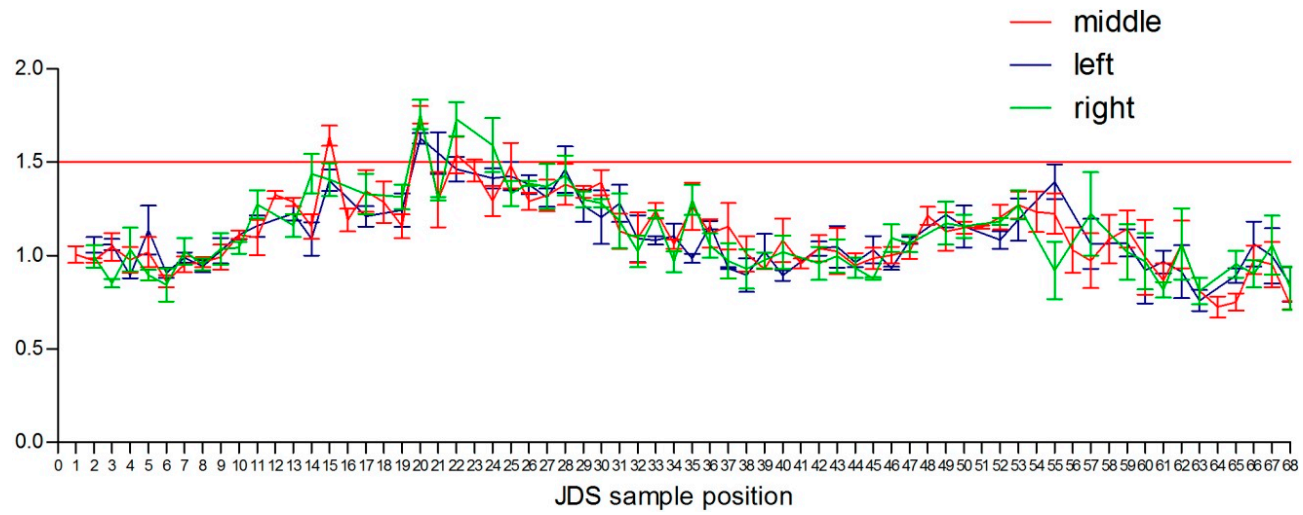

Figure 3. Results from umuC testing of the river Danube with enzymatic activation. The red line at 1.5 represents the limit value. Values at JDS15 M, JDS20 L,M,R, JDS21 L, and JDS22 R, clearly override the limit value. Values are also increased before and after JDS55 but do not exceed the limiting value. Growth of *Salmonella* is impaired beginning at JDS60 but does not fall below 75% compared to the growth control.

When elevated values were observed, they were mostly elevated at all three horizontal sampling point (e.g., JDS 20 left, middle and right). This leads to the conclusion, that the toxic signal has come from a point upstream as it has to be spread all over the whole width of the river. The definite source of the toxic signals is difficult to find, as the umuC is sensitive for at least 400 chemicals tested by Reifferscheid *et al.* [13]. One group of toxicants that need prior S9 activation and are known to be pollutant in surface waters are polychlorinated biphenyls (PCBs) [14,15] although they were found at very low levels in the River Danube [1].The possible sources are the large municipal waste water treatment plants, the outfall of large factories in these areas, and the agricultural land use of the watershed area for these sites.

The reduced growth rate from JDS60 to JDS68 triggered the values to around 0.80 to 0.85 which is close to the cytotoxic limit value according to ISO 13829 (Materials and Methods 2.2). The growth rate dropped by around 15%–20% which might be a reference for cytotoxicity in this stretch of the River Danube, but there was no parallel growth reduction found in the MTS test with eukaryotic cells (see below).

3.3. MTS Testing

For all investigated samples the MTS test did not show any toxic signals (Figure 4) and there were no differences all over the River Danube stretch. Although HepG2 liver cells are capable of phase one enzymatic liver modification and suitable for primary investigation [16] there was no detectable reduction of the cell viability. The values of the River Danube samples tend to be even a little bit elevated (10%–20%) compared to the control (deionized water), as they were only filtrated and contain still their natural salt concentration. The filtrated Danube water was osmotically better for the cells than the control and this must be the reason for the slightly elevated values. The MTS test did not lead

to positive results with the applied cell line. Extending the tests to other cell lines (e.g., epithelial cell lines like IEC-18, fibroblastic cell lines like BALB/c 3T3 [17–19]) could bring further insights.

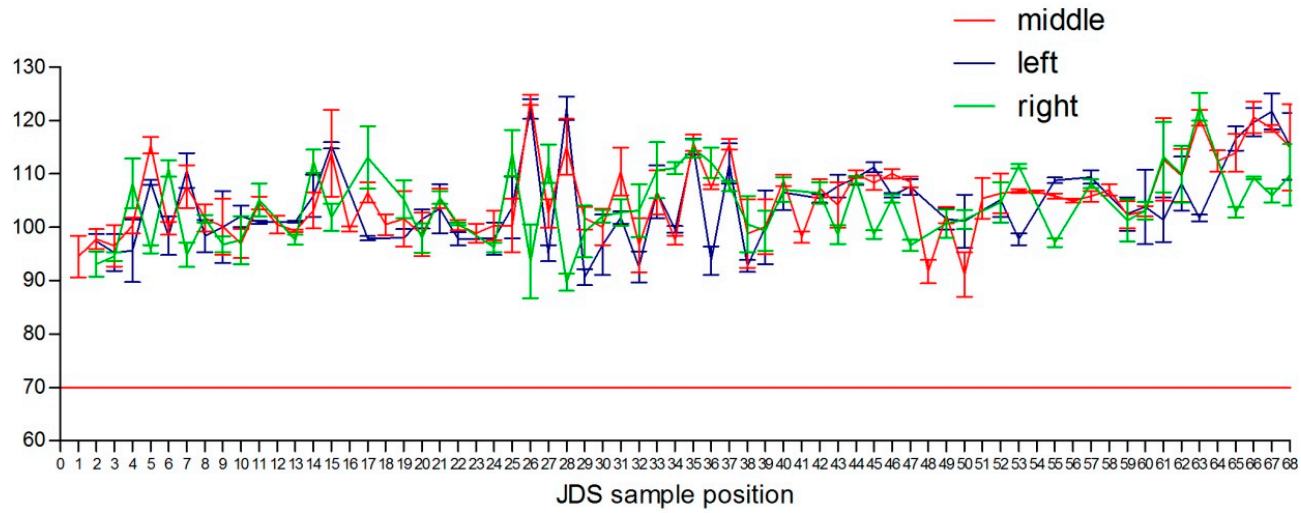

Figure 4. Values of the MTS results of the River Danube sampling points (x-axis). The y-axis represents percentage of viability compared to the control (deionized water, was set as 100%). The red line at 70% represents the limit value for an inhibition of growth caused by a toxic compound or a combination of compounds.

4. Conclusions

The examination of the JDS 3 River Danube samples provided a primary toxicological evaluation of the Danube and its major tributaries. The dense mesh of samples offered a unique chance for an assessment of this large transnational river system. Our data suggest that the Danube water in the river stretch between JDS13 and JDS 28 with elevated umuC values after S9 activation may carry a mutagenic burden. A direct comparison to the prior Danube surveys is not possible because toxicology was not investigated during JDS1 and only for sediment samples during JDS2. Further analysis at a high temporal resolution is needed to proof that our findings are consistent over time.

Acknowledgments

The Joint Danube Survey was organized by the International Commission for the Protection of the Danube River (ICPDR). The study was supported by the Austrian Science Fund (FWF), project nr P25817-B22. We further thank Georg Reischer, Stefan Jakwerth and Stoimir Kolarevic for their help in sampling.

Author Contributions

Clemens Kittinger and Gernot Zarfel had the original idea for the study and with Andreas H. Farnleitner and Andrea J. Grisold carried out the design. Rita Baumert, Bettina Folli, Michaela Lipp and Astrid Liebmann carried out the laboratory work. Clemens Kittinger was responsible for data cleaning. Clemens Kittinger and Alexander Kirschner drafted the manuscript, which was revised by all authors. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare to have no conflicts of interests and no financial relationships that might lead to a conflict of interests.

References

1. JDS 3. *Joint Danube Survey 3. A Comprehensive Analysis of Danube Water Quality*; ICPDR—International Commission for the Protection of the Danube River: Vienna, Austria, 2015.
2. Leusch, F.D.; Khan, S.J.; Gagnon, M.M.; Quayle, P.; Trinh, T.; Coleman, H.; Rawson, C.; Chapman, H.F.; Blair, P.; Nice, H.; *et al.* Assessment of Wastewater and Recycled Water Quality: A Comparison of Lines of Evidence from *in Vitro*, *in Vivo* and Chemical Analyses. *Water Res.* **2014**, *50*, 420–431.
3. Zegura, B.; Heath, E.; Cernosa, A.; Filipic, M. Combination of *in Vitro* Bioassays for the Determination of Cytotoxic and Genotoxic Potential of Wastewater, Surface Water and Drinking Water Samples. *Chemosphere* **2009**, *75*, 1453–1460.
4. Giuliani, F.; Koller, T.; Wurgler, F.E.; Widmer, R.M. Detection of Genotoxic Activity in Native Hospital Waste Water by the umuC Test. *Mutat. Res.* **1996**, *368*, 49–57.
5. Hamer, B.; Bihari, N.; Reifferscheid, G.; Zahn, R.K.; Muller, W.E.; Batel, R. Evaluation of the SOS/umu-Test Post-Treatment Assay for the Detection of Genotoxic Activities of Pure Compounds and Complex Environmental Mixtures. *Mutat. Res.* **2000**, *466*, 161–171.
6. Dizer, H.; Wittekindt, E.; Fischer, B.; Hansen, P.D. The Cytotoxic and Genotoxic Potential of Surface Water and Wastewater Effluents as Determined by Bioluminescence, Umu-Assays and Selected Biomarkers. *Chemosphere* **2002**, *46*, 225–233.
7. Kittinger, C.; Marth, E.; Reinthaler, F.F.; Zarfel, G.; Pichler-Semmelrock, F.; Mascher, W.; Mascher, G.; Mascher, F. Water Quality Assessment of a Central European River—Does the Directive 2000/60/EC Cover all the Needs for a Comprehensive Classification? *Sci. Total Environ.* **2013**, *447*, 424–429.
8. Hernando, M.D.; Heath, E.; Petrovic, M.; Barcelo, D. Trace-Level Determination of Pharmaceutical Residues by LC-MS/MS in Natural and Treated Waters. A Pilot-Survey Study. *Anal. Bioanal Chem.* **2006**, *385*, 985–991.
9. Macova, M.; Toze, S.; Hodgers, L.; Mueller, J.F.; Bartkow, M.; Escher, B.I. Bioanalytical Tools for the Evaluation of Organic Micropollutants during Sewage Treatment, Water Recycling and Drinking Water Generation. *Water Res.* **2011**, *45*, 4238–4247.
10. Armishaw, P.; Millar, R. A Natural Matrix (Pureed Tomato) Candidate Reference Material Containing Residue Concentrations of Pesticide Chemicals. *Fresenius J. Anal. Chem.* **2001**, *370*, 291–296.
11. Reifferscheid, G.; Heil, J.; Oda, Y.; Zahn, R.K. A Microplate Version of the SOS/umu-Test for Rapid Detection of Genotoxins and Genotoxic Potentials of Environmental Samples. *Mutat. Res.* **1991**, *253*, 215–222.

12. Knasmuller, S.; Mersch-Sundermann, V.; Kevekordes, S.; Darroudi, F.; Huber, W.W.; Hoelzl, C.; Bichler, J.; Majer, B.J. Use of Human-Derived Liver Cell Lines for the Detection of Environmental and Dietary Genotoxicants; Current State of Knowledge. *Toxicology* **2004**, *198*, 315–328.
13. Reifferscheid, G.; Heil, J. Validation of the SOS/umu Test using Test Results of 486 Chemicals and Comparison with the Ames Test and Carcinogenicity Data. *Mutat. Res.* **1996**, *369*, 129–145.
14. Flint, S.; Markle, T.; Thompson, S.; Wallace, E. Bisphenol A Exposure, Effects, and Policy: A Wildlife Perspective. *J. Environ. Manag.* **2012**, *104*, 19–34.
15. Haarstad, K.; Bavor, H.J.; Maehlum, T. Organic and Metallic Pollutants in Water Treatment and Natural Wetlands: A Review. *Water Sci. Technol.* **2012**, *65*, 76–99.
16. Baderna, D.; Colombo, A.; Romeo, M.; Cambria, F.; Teoldi, F.; Lodi, M.; Diomede, L.; Benfenati, E. Soil Quality in the Lomellina Area using in Vitro Models and Ecotoxicological Assays. *Environ. Res.* **2014**, *133*, 220–231.
17. Walum, E.; Hedander, J.; Garberg, P. Research Perspectives for Pre-Screening Alternatives to Animal Experimentation: On the Relevance of Cytotoxicity Measurements, Barrier Passage Determinations and High Throughput Screening *in Vitro* to Select Potentially Hazardous Compounds in Large Sets of Chemicals. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 393–397.
18. Baderna, D.; Colombo, A.; Amodei, G.; Cantu, S.; Teoldi, F.; Cambria, F.; Rotella, G.; Natolino, F.; Lodi, M.; Benfenati, E. Chemical-Based Risk Assessment and *in Vitro* Models of Human Health Effects Induced by Organic Pollutants in Soils from the Olona Valley. *Sci. Total Environ.* **2013**, *463–464*, 790–801.
19. Kallweit, A.R.; Baird, C.H.; Stutzman, D.K.; Wischmeyer, P.E. Glutamine Prevents Apoptosis in Intestinal Epithelial Cells and Induces Differential Protective Pathways in Heat and Oxidant Injury Models. *JPEN J. Parenter. Enteral Nutr.* **2012**, *36*, 551–555.