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Integrating bioassays, chemical analysis and *in silico* techniques to identify genotoxicants in surface water

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Abstract

Identification of hazardous compounds, as the first step of water protection and regulation, is still challenged by the difficulty to establish a linkage between toxic effects and suspected contaminants. Genotoxic compounds are one type of highly relevant toxicants in surface water, which may attack the DNA and lead to cancer in individual organism, or even damaged germ cells to be passed on to future generations. Thus, the establishment of a linkage between genotoxic effects and genotoxicant is important for environmental toxicologists and chemists. For this purpose, in the present study *in silico* methods were integrated with bioassays, chemical analysis and literature information to identify genotoxicants in surface water. Large volume water samples from 22 sampling sites of the Danube were collected and subjected to biological and chemical analysis. Samples from the most toxic sites (JDS32, JDS44 and JDS63) induced significant genotoxic effects in the micronucleus assay, and two of them caused mutagenicity in the Ames fluctuation assay. Chemical analysis showed that 68 chemicals were detected in these most toxic samples. Literature findings and *in silico* techniques using the OECD QSAR Toolbox and the ChemProp software package revealed genotoxic potentials for 29 compounds out of 68 targeted chemicals. To confirm the integrative technical data, the micronucleus assay and the Ames fluctuation assay were applied with artificial mixtures of those compounds and the raw water sample extracts. The results showed that 18 chemicals explained 48.5% of the genotoxicity in the micronucleus assay. This study highlights the capability of *in silico* techniques in linking adverse biological effect to suspicious hazardous compounds for the identification of toxicity drivers, and demonstrates the genotoxic potential of pollutants in the Danube.

Key words: genotoxicity; *in silico* techniques; Ames fluctuation assay; micronucleus assay; genotoxicants identification

Highlights:

- Bioassays of Ames fluctuation and micronucleus cover essential genotoxic endpoints
- *In silico* techniques reduced complexity of aquatic mixture with low workload
- An artificial mixture explained 48.5% of genotoxicity in the micronucleus assay

1. Introduction

As required in the European Water Framework Directive (WFD), all water bodies (rivers, lakes, transitional waters, and coastal waters) must achieve 'good water status'(EC 2000). To identify the hazardous compounds which are increasing the threat of pollution, environmental toxicologists and chemists developed panels of techniques for priority chemical analysis and monitoring (Rubirola et al. 2017, Zheng et al. 2015). However, due to the complexity of aquatic systems, identification chemicals that are causative of adverse effects is still a great challenge in ecological status evaluation programs. (Brack et al. 2016, Neale et al. 2015). Thus, the linkages between toxic effects and suspected hazardous compounds are still a major challenge for capturing main threats to aquatic environments (Neale et al. 2015).

Genotoxic compounds, as one type of highly relevant toxicants in environments, can directly or indirectly affect the DNA by inducing gene mutations and/or mutagenic potential, and by changing chromosome structures and numbers (Fenech 1993). Moreover, numerous anthropogenic chemicals in aquatic systems had already been proven to cause genetic damages in aquatic organisms (Brinkmann et al. 2014b, Kosmehl et al. 2007, Kosmehl et al. 2004), which have similar effects on humans (Fenech 1993, Poser et al. 2004). Therefore, linking genotoxic effects and key genotoxic drivers is important for water quality monitoring programs. The reiterative fractionation combined with effect assessment and target/non-target analyses in effect-directed analysis (EDA) could aid characterization and identification of the potential key genotoxic drivers in surface water (Muz et al. 2017). EDA has to rely on advanced analytic instruments, complex analysis process, high sample consumption and high-resolution chemical analysis methods (Brack et al. 2016), which lead to a costly and laborious effort for identification of chemicals of concern. Consequently, the aim of the current study was to link genotoxic effects and genotoxicants by integrating bioassays, chemical analysis and *in silic*o techniques with low workload.

In this study, a large volume solid phase extraction (LVSPE) was conducted to collect and extract the Danube surface water samples from 22 sites (Schulze et al. 2017). To evaluate the comprehensive and realistic toxicity of water pollution, zebrafish that have been used as sentinels for the quality of waters (Hill et al. 2005, ISO 2013, Rocha et al. 2011) were employed to screen the toxic hotspots of the Danube. Next, the Ames fluctuation assay using the strain TA98 and the micronucleus assay were carried out to investigate the genotoxic effects of the identified hotspot samples (Bekaert et al. 1999, Le Curieux et al. 1995, Li et al. 2012, Reifferscheid et al. 2012). These two measurements characterize the chromosomal damage in mitogen-stimulated cells, and gene mutations that lead to a frameshift, respectively (Kirkland et al. 2011, Reifferscheid et al. 2008). Chemical analysis in our previous study reported the occurrence and concertation of 264 chemicals in the Danube (Neale et al. 2015). In consideration of the high workflow required to comprehensively analyze genotoxicity of all detected substances, *in silico* toxicology prediction approaches and previous literature data were used to link genotoxic effects with the identified chemicals. The OECD quantitative structure activity relationships (QSAR) Toolbox is commissioned to identify relevant structural characteristics and potential mechanisms or modes of actions (Weichenthal et al. 2010) of the target chemical (OECD, 2015), which is mainly developed from mechanistic knowledge to model the genotoxicity of frameshift in *Salmonella typhimurium*. However, this software can only be developed from chemicals known to be excess-toxic or from theoretically known mechanisms. The ChemProp software package is developed based on environmentally relevant physical-chemical compound properties/partitioning properties, pure compound descriptors and properties, and even degradation (UFZ Department of Ecological Chemistry, 2016). It offers fully automated read-across based on atom-centered fragments (ACF), and it is suggested recently for the predictive identification of excess-toxic compounds and the absence of excess toxicity (Kühne et al. 2013, Schramm et al. 2011). These two software tools are widely used to predict the toxicity of environmental chemicals (Brinkmann

et al. 2014c, Svetnik et al. 2003), and also as toxicological screening considering mechanistic targets which are active at cellular pathways (Xiao et al. 2016). Thus, the QSAR toolbox and ChemProp were employed to establish linkages between genotoxic effects and relevant chemicals in the Danube. Finally, to confirm the contribution of suspected genotoxic compounds, artificial mixtures of those compounds were tested in the micronucleus assay and the Ames fluctuation assay. By comparing the effects of the artificial mixtures and the corresponding raw extracts, it was possible to determine contribution of the genotoxicants to the genotoxic effects in surface water samples.

The *in silico* techniques used in the current study provided the possibility to establish a target analysis for aquatic samples, which reduced the complexity of aquatic mixture with low workload. The combined approach presented here can serve as part of an integrated strategy to identify legacy and emerging pollutants, and thus can be useful for water quality monitoring and assessment within implementation of the EU Water Framework Directive (WFD) (Di Paolo et al. 2016).

2. **Materials and method**

2.1 Sampling

Sampling was performed across the Danube and selected tributaries in 2013 (Liška et al. 2015). Twenty-two surface water samples were collected using LVSPE (Neale et al. 2015, Schulze et al. 2017). Briefly, up to 1000 L of water were passed through a stainless steel chamber containing neutral sorbent Chromabond® HR-X, anionic exchanger Chromabond® HR-XAW and cationic exchanger Chromabond® HR-XCW (Macherey-Nagel, Düren, Germany) after removing suspended particulate matter with a GF+ Sartopure deep filter (pore size: 0.63 µm; Sartorius). Afterwards, freeze-dried sorbents were extracted using mixtures of ethyl acetate, methanol (neutral sorbent), methanol with 2% of 7N ammonia in methanol (Supelco; weak anionic exchanger) and methanol with 1% formic acid (Merck; weak cationic

exchanger). The extracts were combined, filtered and concentrated to dryness via rotary and nitrogen evaporation prior to shipping, then re-suspended in DMSO or methanol (Neale et al. 2015).

2.2 Mixture preparation

Mixtures of predicted genotoxic compounds were prepared for confirmation of the effects in the micronucleus assay and Ames fluctuation assay. The genotoxic candidates 1H-benzotriazole (≥ 99.0 %)，2-(methylthio)benzothiazole (97%), 2,4-dinitrophenol (≥ 98.0%), 4- and 5-methyl-1h-benzotriazol (98%), acesulfame (≥ 99.0%), atrazine (≥ 99.0%), carbamazepine (≥ 99.0%), chloridazon, cyclamate, daidzein (≥ 98.0%), N,N-Diethyl-m-toluamide (DEET, 97%), isoproturon, metolachlor, n-acetyl-4-aminoantipyrine (≥ 98.5%), N-formyl-4-aminoantipyrine, sulfamethoxazole, terbuthylazine (≥ 98.0%), 4-formyl-antipyrine (97%), trimethoprim (≥ 98.0%), diazinon and diclofenac (≥ 98.5%) were purchased from Sigma-Aldrich (Germany). All agents were used as supplied, and dissolved in HPLC-grade dimethylsulfoxide (DMSO) as 20 g/L stock solutions. The final three mixtures for spiking contained the 400- and 1000-fold enrichment level based on the analytically determined values and ratios of the single compounds (Neale et al. 2015).

2.3 Fish embryo toxicity test with the zebrafish (*Danio rerio*) (zFET)

In the present study, zFET was carried out according to the OECD Test Guideline 236: Fish Embryo Acute Toxicity Test (OECD, 2013), with slight modifications. To decrease sample consumption, 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were used with respective test solutions in volume of 200 µl per well. Fertilization of freshly collected eggs was evaluated using a stereomicroscope, and tests were only conducted if the fertilization rate for a batch of eggs was at least 90%. In order to achieve the earliest possible exposure, fertilized eggs were immediately transferred to dishes containing Danube water extracts at the

range of a relative enrichment factor (REF, as detailed in (Escher and Leusch 2011)) from 62.5 to 1000 (1:2). Zebrafish eggs were placed in the test solutions within 2 h postfertilization (hpf, corresponding to 16–64 cell stage). For each concentration, 10 zebrafish embryos were transferred individually from dishes to the 96-well plate. Test plates were covered with transparent self-adhesive sealing film (Greiner Bio-One, USA) and were incubated at 26 ± 1 °C for 48 h. Thereafter, mortality according to the criteria defined in the OECD guideline was recorded using an inverse microscope. 3,4-dichloroaniline at 3.7 mg/L was used as the reference. The data was expressed in units of REF causing 50% mortality (LC50).

2.4 Genotoxicity assays

Micronucleus assay and Ames fluctuation tests were used as mechanism-specific assays to analyze the genotoxic response to JDS3 samples.

Micronucleus assay: The micronucleus assay protocols for cell culture and the micronucleus assay with rainbow-trout liver (RTL-W1) cells were based on the method described by Rocha et al. (2009). Cells were cultured at 20 °C in Leibovitz's L15 medium with L-glutamine (Sigma–Aldrich) containing 9% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1% (v/v) penicillin/streptomycin solution (Biochrom) (Klee et al. 2004). A volume of 2 mL of the cell suspension at a density of $5–6\times10^4$ cells/ml was cultured for 24 h before treatment. After 24 h, the cells were exposed to Danube water sample (at the REF of 400, 200, 100, 50, 25) for 20 h and followed by replacement with fresh media (incubation: 72 h). Finally, cells were stained using acridine orange to detect the presence of micronuclei. A total number of 2000 RTL-W1 cells of each concentration were randomly selected and analyzed for micronucleus formation, as an indicator of aneugenic and clastogenic genotoxicity. The frequency of micronuclei in RTL-W1 cells was assessed for the identified hotspots. The micronucleus frequency is given as induction factors (IF) relative to the negative controls (Brinkmann et al. 2014a).

Ames fluctuation assay: The Ames fluctuation assay was based on the method described by Maron and Ames, and Reifferscheid et al. (Maron and Ames 1983, Reifferscheid et al. 2005). The mutagenic activity was determined using the His-deficient strain TA 98 of the bacterium *Salmonella typhimurium*. To evaluate toxicity under metabolic activity, a liver homogenate S9-fraction from phenobarbital/β-naphthoflavon treated rats (protein concentration 30.5 mg/ml S9 mix) was added to a buffered co-factor mixture to a parallel setup of each experiment. Bacteria were exposed to the extracted samples at REF 62.5 to 1000 and 1000 fold environmental concentration mixture (1:2) in 24-well microtiter plates. After shaking (150 rpm) for 100 min at 37 °C, the content of the 24-well plates were transferred into 384-well plates with 48 wells per replicate (controls and sample dilutions), and an indicator medium for bacterial growth was added. Plates were then incubated for 48 h at 37°C. Since only reverted bacteria can survive in a histidine deficiency solution, the acidification is an indicator of the reverse mutation of bacteria. Such an acidification is indicated by a change in color of the pH indicator bromocresol purple. The mutagenicity of the tested sample was determined by manual counting of the number of wells that shifted from purple to yellow.

2.5 Prediction of genotoxic compounds

To link the biological genotoxic responses to the relevant genotoxic compounds, software modeling was carried out for the chemicals detected at the hotspots. The detected chemicals were inquired from previous studies (Neale et al. 2015, Schulze et al. 2017). To probe the highest priority compounds with genotoxic potential in the Danube, the *in vitro* mutagenicity (Ames test) alerts with the of OECD QSAR Toolbox software package (OECD, 2015) were deployed. This QSAR Toolbox (*In vitro* mutagenicity (Ames test) by ISS) is based on the mutagenicity/carcinogenicity module of the Toxtree software. It works as a decision tree for estimating *in vitro* (Ames test) mutagenicity, based on a list of 30 structural alerts. The structural alerts for mutagenicity are molecular functional groups or substructures known to

199 be linked to the mutagenic activity of chemicals. As one or more structural alerts embedded in
200 a molecular structure are recognized, the system flags the potential mutagenicity of the
201 chemical. The ACF-based read-across in ChemProp estimated the mutagenicity with regard to
202 the Ames test and the chemical domain in ChemProp were employed to predict toxic
203 compounds due to chemical similarity in the context of structure-activity relationships (Kühne
204 et al. 2007, Schwöbel et al. 2009). The ChemProp is based on 2D structures including an
205 automated substructure search, and is accomplished by tools to characterize the applicability
206 domain in terms of property and chemical space, and to provide uncertainty estimations.
207 Structural input of each chemical was achieved by SMILES strings. There are modules to edit
208 and visualize molecule compilation, for automated classification according to compound
209 classes, and for structure related database searching. The results of prediction were presented
210 as genotoxic chemical groups in QSAR toolbox and numbers in ChemProp. The ChemProp
211 read-across indicated if a compound is active (1) or inactive (0), and the ChemProp chemical
212 domain gave advice if the result of read-across is reliable (3 or 2), to be used with causion (1)
213 or simply the QSAR does not predict correctly (0 = out of domain) (Kühne et al. 2009).

214 2.6 Statistical analysis

215 All spreadsheet calculations were performed using Microsoft Excel™ 2007, Sigma Plot 12.0
216 (Systat Software Inc., San Jose, CA), Origin Pro 8.5.1(Origin Lab Corporation) and the
217 software Prism 6.0 (GraphPad Software Inc., San Diego, USA). All datasets of different
218 treatments were tested for statistically significant differences using one-way analysis of
219 variance (Prášková et al. 2011). Dunnett's test was used to identify significant differences
220 between treatments and controls ($p < 0.05$).

221 **3. Results and Discussion**

222 3.1 Screening toxic hotspots with zFET

To screen the toxic hotspots in the sampling area, zebrafish embryos were exposed to 22 Danube surface water extracts for 48 h to evaluate the comprehensive and realistic toxic effects. Results (Fig. 1) showed that all 22 extracts were capable of causing mortality with LC50 values from REF 110.5 ± 23.4 to 460.8 ± 83.4. Two extracts (JDS32 and JDS63) showed strong embryo-toxic effects, with LC50 values at REF 110.5 ± 23.4 and 173.4 ± 44.8, respectively. The major lethal effects were lacking heartbeat and tail not detached. Moreover, embryo coagulation was found to be the most frequent effect being recorded for higher concentrations of the Danube samples in the wider range-finding tests in the current study. A previous zebrafish embryo study also speculated that coagulation was a sensitive parameter in the zFET test (Hagenaars et al. 2011). Normally, the whole yolk of coagulated embryos is completely denatured (dark under the microscope). However, for JDS44, coagulation only appeared at the end of rapidly dividing cells of the yolk (Fig. 2), indicating that embryos coagulated at the blastula stage, which occurred as soon as exposure to JDS44 was initiated. These phenomena was also noticed in a water quality assessment study where zebrafish embryo coagulated within only 12h of exposure (Hallare et al. 2005). Based on these findings and the evaluation of the results, JDS32, JDS44 and JDS63 were considered as most toxic sites in the current study.

3.2 Genotoxicity of Danube surface water samples

Samples from three identified toxic hotspots were subjected to the micronucleus assay with 4-nitroquinoline-N-oxide (NQO) as a reference for genotoxicity in three independent replicates (Fig. 3). The induction of micronuclei in RTL-W1 cells increased with increasing of NQO concentrations, finally reaching a maximum induction factor (IF) of 8.0. All three toxic hotspots (JDS32, JDS44 and JDS63) caused significant induction of micronuclei, and showed a strong increase of the IF with increasing concentrations, nearly reaching that of the reference. The order of IFs was JDS32 (IF = 7.67) > JDS44 (IF = 7.55) > JDS63 (IF = 7.11).

Boettcher et al. reported the micronuclei frequency in RTL-W1 cells for Danube upstream extracts, where the IF was 5.17 for Rottenacker, 4.5 for Ehingen and 4.0 for Riedlingen, respectively (Boettcher et al. 2010). This indicates that the genotoxic effects appeared not only at several single points, but also occurred at both upstream and downstream sites. When comparing to a previous study of *in situ* Danube samples, the highest IF for fish blood samples was around 5.0 at downstream of JDS60 (Deutschmann et al. 2016), which is slightly lower than that in the current study. In a study of the Serbian part of the Danube, a high percentage of comet tails was found for haemocytes of freshwater fish mussels (Kolarević et al. 2013). These phenomena demonstrate that genotoxic compounds really affect fish, and the predictive ability of the current *in vitro* assay is necessary. In addition, the LVSPE used in the current study was capable of sampling genotoxic compounds.

The results from the Ames fluctuation assay on mutagenicity of the three most toxic sites are shown in Table 2. Two samples (JDS32 and JDS63) presented mutagenic activity with the strain TA98 with supplementation using the S9 mix, *i.e.* potential metabolic activation of genotoxic compounds. An increase in the number of revertants was observed for JDS 32 at REF 125, 250, 500 and 1000 by means of the S9 mix necessary for expressing the potential DNA damage, which indicates the presence of pro-mutagens (Fig. 4). With JDS63, the mutagenicity occurred even without the S9 mix, which shows that JDS63 contains readily genotoxic compounds. A steady increase in revertant numbers appeared from 7.9 to 27.7 at REFs from 31.25 to 1000 for JDS63 in the Ames fluctuation assay with TA98 and S9 supplementation (Fig. 4). These results are in agreement with a survey along the causeway of the Danube Canal in Vienna, where mutagenicity in *Salmonella typhimurium* TA98 and YG1024 with metabolic activation were observed (Kataoka et al. 2000). A maximum number of revertants in the assay with S9 was found at REF 1000 (23.3 for JDS63), which is even higher than that for upper Danube sediment and suspended particulate matter (SPM) extracts

(Keiter et al. 2006). However, no mutagenic activity was detected for the sample JDS44 (Table 2).

In summary, the genotoxic results indicate that samples from all three most toxic sites can cause genotoxicity by chromosomal damage in mitogen-stimulated cells. Additionally, samples from JDS63 and JDS32 induced frameshift mutations. The latter was only after metabolic activation through S9 supplementation, indicating the presence of pro-mutagens in JDS32.

3.3 Linking genotoxic effects and suspected genotoxic compounds

According to our previous reports, 68 out of 270 chemicals were detected at the most toxic sites JDS32, JDS44 and JDS63 (Neale et al. 2015, Schulze et al. 2017). To analyze the genotoxic potential of all these 68 detected substances, readouts from the OECD QSAR Toolbox and the ChemProp software package were integrated with literature data in the current study. As shown in Table S1, 21 chemicals were predicted to be active in the Ames test with alerts based on the Bursi dataset from Kazius et al. (2005). However, four of these (atrazine, desethylatrazine, phenazone, p-nitrophenol) were predicted inactive by ChemProp with high reliablity (at 3 in the chemical domain). Further, two (p-nitrophenol, sucralose) of them gave negative results in previous research (Eichenbaum et al. 2009, Shastry et al. 2012). Five chemicals (2,4-dinitrophenol (2,4-DNP), carbendazim, diuron, metolachlor, trimethoprim) were predicted active according to ChemProp, and only three (2,4-DNP, metolachlor and trimethoprim) were predicted active by both models. Altogether, 19 chemicals (17 by OECD QSAR Toolbox and 5 by ChemProp, three of which predicted active by both, Fig.5) were predicted as mutagens in the Danube. The two *in silico* tools predicted suspected genotoxic compounds differently, since these two software packages have different focuses. In consideration of the rationality and the logicality of these two methods (Johann et al. 2016), all these 19 predicted chemicals were included into artificial mixtures the

subsequent occurrence analysis and bioassay. While not only point mutations as revealed by the Ames fluctuation assay and thus predicted by the respective *in silico* models, genotoxicity can be also in the form of single- and double-strand breaks, loss of excision repair, cross-linking, alkali-labile sites, and structural and numerical chromosomal aberrations. In previous studies, different mutagenic and genotoxic effects were reported for these chemicals (Table 3). *e.g.*, metolachlor was found to induce DNA strand breakage and micronuclei in fish (Polard et al. 2011), acesulfame induced DNA damage in bone marrow cells of mice (Bandyopadhyay et al. 2008), and a significant increase in DNA strand breakage was measured in oyster spermatozoa after exposure to diuron (Akcha et al. 2012). However, ten chemicals (carbamazepine, chlorotoluron, cyclamate, daidzein, DEET, diazinon, diclofenac, genistein, metformin, isoproturon) were shown to exhibit different genotoxic effects in previous literature, but were predicted inactive in the Ames fluctuation assay by the OECD QSAR toolbox or ChemProp. This demonstrated the limitations of the integration of such *in silico* techniques, as long as they cover only specific genotoxic effects, which should be improved in future.

Therefore, all 29 chemicals (1H-benzotriazole, 2-(methylthio)benzothiazole, 2,4-DNP, 4-and 5-methyl-1H-benzotriazol, 4-formyl-antipyrine, acesulfame, acetyl-Sulfamethoxazole, atrazine, carbamazepine, carbendazim, chloridazon, chlorotoluron, cyclamate, daidzein, DEET, desethylatrazine, diazinon, diclofenac, diuron, genistein, gestoden, isoproturon, metformin, metolachlor, N-acetyl-4-aminoantipyrine, N-formyl-4-aminoantipyrine, sulfamethoxazole, terbuthylazine and trimethoprim; 19 by software and 10 by previous studies, Fig. 5) were selected to design artificial genotoxic mixtures to elucidate above predictions.

3.4 Occurrence-related analysis

As shown in Table 3, the predicted 29 suspected genotoxic chemicals were detected at sites JDS32, JDS44 and JDS63 with concentrations ranging from 4.08 to 2046 pM. The most frequently detected chemicals which were presented at relative high concentrations are pharmaceuticals and their transformation products (TPs), artificial sweeteners (acesulfame, cyclamate), and industrial chemicals (1H-benzotriazole), which consequently, are suspected to be the main cause for the genotoxicity in the Danube. Previous genotoxicity investigations have already proven that pharmaceuticals, artificial sweeteners and industrial chemicals can induce various genotoxic effects (Bandyopadhyay et al. 2008, Bolt 2003, Snyder and Green 2001). Eighteen genotoxic candidates were detected at all three hotspots (JDS32, JDS44 and JDS63) that induced micronuclei in RTL-W1 cells (Fig. 3). This leads to the presumption that among these chemicals could be main drivers for micronuclei formation in the Danube. Literature data show that carbamazepine, chloridazon and daidzein can increase micronuclei formation (Table S1). Moreover, concentration-response studies reported that some of the detected chemicals can induce genotoxic effects at very low concentrations, such as metolachlor at 0.01 µg/L (Mai et al. 2012), and 2,4-DNP at 0.05 µg/L (Lee et al. 2003), which consistently matched the current presumption. In addition, 4-formyl-antipyrine and trimethoprim may have a capacity to induce mutagenicity with the supplementation of S9, as they only emerged in JDS32 and JDS63, but not in JDS44. A *Salmonella/microsomal* screening study has proven that Trimethoprim produced significant mutagenicity in the TA98 strain with supplementation using an S9 mix (Rasool et al. 1987). Diazinon and diclofenac may be culprits for the effects in the TA98-S9 setup, because they merely appeared in JDS63 which showed positive results in the Ames fluctuation assay even without S9 mix. A *Daphnia magna* study reported that DNA damage was observed after 48 and 96 h exposure of diclofenac at concentration of 9.8 µM (Gómez-Oliván et al. 2014). However, the concentration of diclofenac was only 3.6 pM in the current study, which may not be that relevant to the mutagenic effects of JDS 63.

3.5 Mixture genotoxicity of the predicted and analyzed suspect contaminants

In order to verify these hypotheses, three corresponding mixtures (Table 4) for different experimental setups were subjected to the micronucleus assay and Ames fluctuation assay to evaluate their genotoxicity. As shown in Fig. 6, a significant increase in micronuclei frequency was observed for the micronuclei-mix, demonstrating the genotoxicty of those chemicals. A comparison of the micronuclei frequency for the micronuclei-mix with that for the raw JDS32 extract (which induced the highest micronuclei frequency, Fig. 3) revealed that the micronuclei frequency of the artificial mixture can explain 48.5% of the effect of raw JDS32 extract. This explanation percentage is much higher than that in an EDA study, where the identified compounds only explained less than 1% of the effect of the raw sample (Muz et al. 2017). Hence, the combination of bioassays, literature data, and chemical analysis in the current study seems to have been effective to reveal micronuclei inducers in realistic environments. However, chemicals outside of the current selection could still have genotoxic potential, since the frequency of micronuclei in RTL-W1 detected for the micronulei-mix was lower than that for JDS32, JDS44 and JDS63 at REF 400. Chemical analysis may not be able to present concentration of all relevant pollutions. Surprisingly, no mutagenic activity was detected in the +S9-mix and –S9-Mix, while the in silico methods identified 19 potential mutagens by predicting their activity in the Ames assay, they seem to be less relevant for the mutagenicity of the raw extracts. This can be understood as the synergism of mutagenic effects of typical water contaminants even without components at a significant individual effect.

4 Conclusions

In the current study, *in silico* approaches were integrated with bioassays, literature data and chemical analysis to link genotoxic effects and suspected hazards compounds in surface water.

Eighteen chemicals (1h-benzotriazole, 2-(methylthio)benzothiazole, 2,4-dinitrophenol, 4- and 5-methyl-1h-benzotriazol, acesulfame, atrazine, carbamazepine, chloridazon, cyclamate, daidzein, DEET, isoproturon, metolachlor, n-acetyl-4-aminoantipyrine, n-formyl-4-aminoantipyrine, sulfamethoxazole and terbuthylazine) were identified from three most toxic sites as potential be genotoxicants in the Danube. These findings can help to establish an overview of the pressures, measures and expectations for pollution by hazardous substances in the Danube.

In particular, the bioassay results for the artificial mixture representing micronuclei-inducing potential according literature data showed that predicted chemicals can explain 48.5% of the effect caused by the raw JDS extract. This demonstrates that the presented integration of results could effectively link toxic effects and toxicants, and might aid in the identification of drivers of toxicity. The approach reduced the complexity of environmental samples with non-experimental techniques. *In silico* techniques in this study decreased workload and provided toxic targets for environmental adverse effect investigation. The integrative approach presented here can serve as part of a standard strategy to identify legacy and emerging pollutants for water quality assessment.

However, other chemicals may also have contributed to the genotoxic effect of the surface water samples, and should be subject to further analysis. The work with modelling and literature data demonstrated that *in silico* approaches cannot cover all suspected hazardous compounds. Our study indicates that especially a more complete set of predictive models is needed for *in silico* methods to be useful for the assessment of genotoxicity. While other effects, such as ER/AR or AhR agonism, rely on a single mode of action, genotoxicity can have a multitude of mechanisms. Consequently, each requires a valid prediction model of its own. The reliability and versatility of these non-experimental techniques needs to be improved through future research.

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