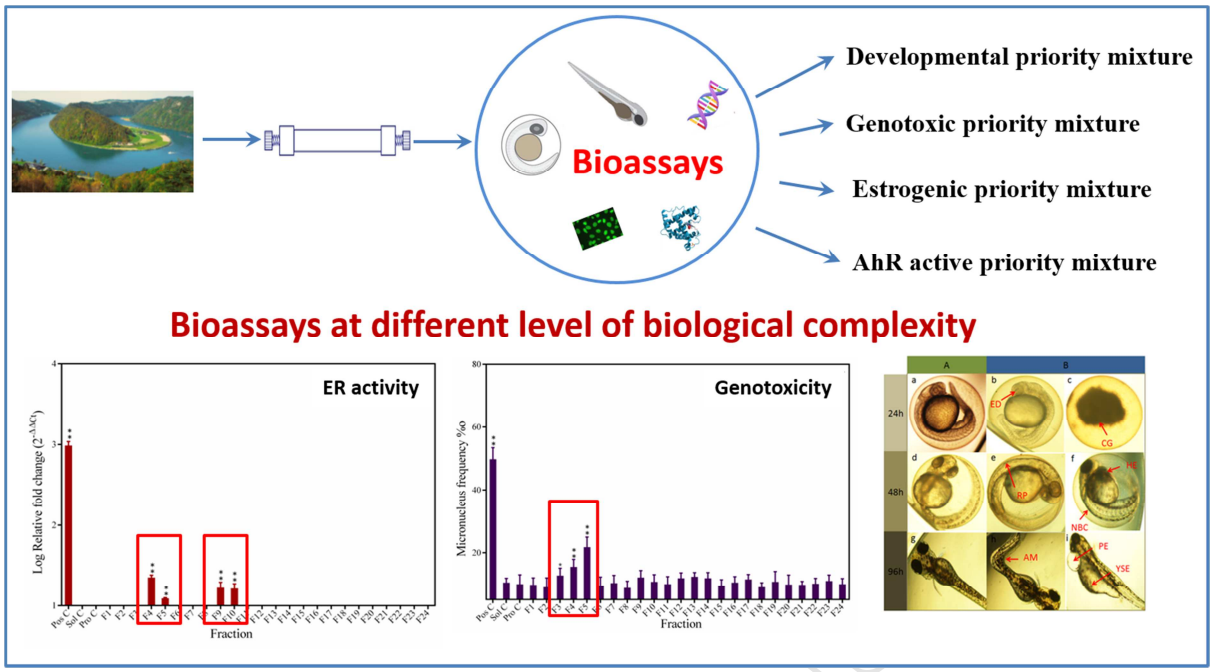

Integrated zebrafish-based tests as a novel strategy investigation for water quality assessment

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ABSTRACT

Water pollution risks to human health and the environment are emerging as serious concerns in the European Union and worldwide. With the aim to achieve good ecological and chemical status of all European water bodies, the "European Water Framework Directive" (WFD) was enacted. With the framework, bioanalytical techniques have been recognized as an important aspect. However, there are limitations to the application of bioassays directly for water quality assessment. Such approaches often fail to identify pollutants of concern, since the defined priority and monitored pollutants often fail to explain the observed toxicity. In this study, we integrated an effect-based risk assessment with a zebrafish-based investigation strategy to evaluate water sample extracts and fractions collected from the Danube. Four tiered bioassays were implemented, namely RNA-level gene expression assay, protein-level ethoxyresorufin-*O*-deethylase (EROD) assay, cell-level micronucleus assay and organism-level fish embryo test (FET). The results show that teratogenicity and lethality during embryonic development might be induced by molecular or cellular damages mediated by the aryl hydrocarbon receptor (AhR) -mediated activity, estrogenic activity and genotoxic activity. With the combination of high-throughput fractionation, this effect-based strategy elucidated the major responsible mixtures of each specific toxic response. In particularly, the most toxic mixture in faction F4, covering a log *Kow* range from 2.83 to 3.42, was composed by 12 chemicals, which were then evaluated as a designed mixture. Our study applied tiered bioassays with zebrafish to avoid interspecies differences and highlights effect-based approaches to address toxic mixtures in water samples. This strategy can be applied for large throughput screenings to support the main toxic compounds identification in water quality assessment.

Keywords: tiered bioassays, zebrafish, large throughput screening, toxicity-based mixture elucidation

1. Introduction

Water pollution poses risks to human health and environments, which is emerging as a serious concern in the European Union and worldwide. To ensure a high level of protection against risks to or *via* the aquatic environment, the WFD launched a strategy for dealing with aquatic pollutions of water bodies (EC 2000). Environmental quality standards were established for a defined set of priority substances, and member states are required to address these priority substances and other specific pollutants in their monitoring programs (EC 2000). However, numerous environmental risk studies indicate that substance-by-substance water quality monitoring could not explain the real environmental effects and target chemical analysis alone may lead to risk underestimation. That is in great deal due to the fact that aquatic ecosystems are contaminated with complex mixtures of a vast number of chemicals (Hecker and Hollert 2009). These mixtures would likely to induce various combined effects (Tamura et al. 2017) that are typically stronger than the effects caused by the individual compounds (additive, sometimes synergistic) (Bliss 1939, Otitoloju 2002). Thus, effect-based bioassays strategies which account for the toxic effects of mixtures and non-target chemical are necessary for water quality assessment (EC 2012).

To investigate toxic effects of water pollution through biomonitoring programs, arrays of *in vitro* bioassays were used to develop high-throughput screening (HTS) of water pollutions, covering different toxicity endpoints in diverse experimental models (Di Paolo et al. 2016, Judson et al. 2010). However, *in vitro* assays used for providing chemical toxic data may lead to misinterpretations in high organism level water quality assessment due to differences in sensitivity between test systems uncertainty factor when transferring measured effects between diverse biological levels (Judson et al. 2014). Even though the adverse outcome pathway concept

provides a logical sequence of causally-linked events at different levels of biological organization (Ankley et al. 2010), the methodology focuses mainly on single chemical or similarly-acting toxicants (Andersen et al. 1997, Cosme et al. 2015, Escher et al. 2017, Russom et al. 2014) which ignored the mixture combined effects . Thus, there are still deficits regarding the investigation of different level biological responses of complex aquatic mixtures for real impacts evaluation in water quality assessment (Brack et al. 2016, Di Paolo et al. 2016).

Zebrafish (*Danio rerio*) is a widely used model organism in water quality assessment by reasons such as small size, easy cultivation, high fecundity, rapid development, external fertilization, and transparent embryos (Braunbeck et al. 2005, Di Paolo et al. 2015b). Lethal and sub-lethal assays with embryos and early larval stages are available for comprehensive toxic effect evaluation, making use of unobstructed observations of main morphological changes by simply using only low magnification light microscope (Hill et al. 2005). Moreover, its embryogenesis and respestive genetic basis making use of histochemical markers, have been largely studied (Long et al. 1997, Puelles et al. 2000, Spitsbergen and Kent 2003). Furthermore, different methods with zebrafish are available to investigate mechanism-specific effects being particularly suited to complement cell-based microscale testing for comprehensive and realistic biological effect evaluations (Hill et al. 2005). For instance, genotoxicity can be investigated by applying the micronucleus assay to detect DNA chromosomal damage in a proliferating cell population regardless of the karyotype (Bolognesi et al. 2006), being *in vitro* technique to investigate the genotoxicity of chemicals and environmental mixtures (Di Paolo 2016). AhR-mediated activity analyzed by means of the expression of xenobiotic-metabolizing enzymes cytochrome P450 activity, allows to detect effects of dioxins and dioxin-like compounds in the aquatic environment (Schiwy et al. 2015) . The estrogenic activity investigated by the induction of vitellogenin gene (*vtg1*) transcripts enables to analyze endocrine disrupting effects of chemicals and mixtures on

the RNA level (Driever et al. 1996, Reinardy et al. 2013). Bioassays with zebrafish embryos allow comparably small sample volumes to be simultaneously screened in a single multi-well plate or a series of petri dishes on organism-level. They thus allow large throughput screenings for different toxicity investigation with minimized sample consumption and reduced workload. Zebrafish up to 120 hours post-fertilization (hpf) can be used (2010/63/EU) for test under European legislation, which greatly simplifies experimental planning, and thus speeds up performance of respective studies (Strähle et al. 2012). All these properties place bioassays with zebrafish in an attractive position for aquatic mixture biological effects investigation and aquatic risk assessment (Di Paolo et al. 2015b).

Consequently, the goal of the current study was to establish an integrative zebrafish testing strategy to investigate molecular, cellular and whole organism toxic effects by tiered bioassays for each specific toxic mixture elucidation in water quality assessment. The assessment strategy was developed using Danube large-volume solid phase extraction (LVSPE) samples (Schulze et al. 2017). Firstly, the FET was applied to identify toxicological hotspots regarding their relevance for environmental toxicity and human health (Maurici et al. 2005, OECD 2012). Secondly, to reduce the complexity of the environmental mixtures and to elucidate the toxic effect of each water mixtures, the most effective hotspot sample was subjected to high performance liquid chromatography (HPLC) fractionation. Afterwards, in order to identify the most potent fraction for each level biological effect, four tiered bioassays were conducted, namely the RNA-level gene expression assay, the protein-level EROD assay, the cell-level micronucleus assay, and the organism-level FET. The most bioactive fractions were then identified on the basis of observed biological effects. The compounds composition were identified with log *Kow* range from HPLC analysis, and further confirmation was done by the toxicity comparison between bioactive fraction and a corresponding artificial mixture.

The panel of zebrafish-based bioassays assembled here is one possible example of a testing strategy towards the implementation of bioanalytical investigation for water quality assessment. There have been only a few studies undertaken on interrelations of effects on tiered biological levels for toxicity evaluation and toxicants identification on the assessment of toxic mixtures by fractionation. To our knowledge, this is the first time such a concept is applied to LVSPE samples using zebrafish early live stages.

2. Materials and Methods

2.1. Sampling and extraction

Sampling was performed across the Danube and selected tributaries during the 3rd Joint Danube Survey (JDS3) between August and September 2013 (Liška et al. 2015). The sampling locations are shown in Table S1 and Figure S1. A total of 22 water samples were collected and extracted using a LVSPE device (Schulze et al. 2017) as detailed in (Neale et al. 2015a). Aliquots of extracts of each sample were concentrated to dryness via rotary and nitrogen evaporation prior to shipping and then re-suspended in acetonitrile or dimethylsulfoxide (DMSO). At last, the concentration factors were fixed to 50,000 and 200,000 for the fractionation and acute toxicity test respectively.

2.2. Reversed-phase high-performance liquid chromatography (RP-HPLC) fractionation

Fractionation and analysis were performed using a HPLC system equipped with the Agilent 1200 series (Agilent, Waldbronn, Germany). RP-HPLC was applied to separate standard chemicals and a Danube hotspot extract at the concentration factor of 50,000 at 30°C by a C18 column (250 × 4 mm^2, 5 µm particle size; Macherey-Nagel, Düren, Germany). The fractionation method was carried out according to the method developed by Suzuki et al. (2004), with slight

modifications (Xiao et al. 2017). Briefly, 40 µl of each extract was fractionated using the following gradient with a flow rate of 0.2 mL/min: 0 to 4.0 min, 10% acetonitrile in methanol/water (80/20, *v/v*); 4.0 to 7.0 min, a linear gradient of 10% to 100% acetonitrile in methanol/water (80/20, *v/v*); 7.0 to 50.0 min, 100% acetonitrile. Fractions were collected every 2 min (the last one after 4 min) in 6-well plates using an automatic fraction collector (Agilent). Subsequently, fractions were evaporated overnight in a sterile working bench, and then directly frozen at -20°C for bioassays. In order to determine the log K_{OW} range of each fraction, a total of 28 chemicals (Table S2) with a log K_{OW} range from 2.03 to 6.76 was analyzed using a photodiode array detector (DAD; Agilent). DAD signals from 210 to 360 nm were recorded and used to model the relationship between retention time and log K_{OW} (Grung et al. 2007).

2.3 Bioanalysis

2.3.1Exposure and acute toxicity assay

The FET was conducted to assess developmental toxicity at 24 hpf, abnormal phenomena at 48 hpf, hatching rate at 96 hpf based on Hollert et al. (2003) and the OECD 236 guideline (OECD 2006) with slight modifications. Briefly, fertilized zebrafish (*Danio rerio*) embryos were transferred into 6 well-plates and static exposure to samples were performed using 0.5% DMSO as carrier. Each exposure test was repeated in three independent experiments. In addition to the tested samples, each experiment contained a positive control condition (3,4-dichloroaniline at the concentration of 3.7 mg/L for developmental toxicity test, 2,3,7,8-tetrachlordibenzo-*p*-dioxin at the concentration of 15 pg/L for EROD activity assay, 17α-ethinylestradiol at the concentration of 1 µg/L for estrogenicity assay, nitroquinoline oxide at the concentration of 19 µg/L for micronucleus assay), a process control (acetonitrile after 50 min HPLC), a solvent control (0.5% DMSO). In the FET, each exposure condition contained 40 embryos. The raw JDS extracts and

the LVSPE process control were tested in five 1:2 dilution steps at the range of a relative enrichment factor (REF) from 62.5 to 1000. The REF takes all enrichment and dilution steps into account (Neale et al. 2015a, Schulze et al. 2017). Artificial water (ISO7346-3 1996)was used as exposure medium. Embryos were inspected using an inverted microscope (Eclipse TS 100, Nikon GmbH, Germany) at the magnification of 40× at 24 hpf, 48 hpf, and 96 hpf. Lethal and sub-lethal endpoints were scored according to the OECD guideline and DIN 38415-6 (DIN38415-6 2001), respectively. The highest concentration that did not induce any embryo abnormal effect was used for the EROD activity and estrogenicity assays.

2.3.2 In vivo AhR-mediated activity assay with zebrafish larvae.

The *in vivo* AhR-mediated activity assay was performed according to Schiwy et al. (2015), with slight modifications. Briefly, 20 hatched larvae were anesthetized with benzocaine (40 g/L in ethanol) for circa 20 minutes after 96 h of exposure to the samples or controls. Then these larvae were transferred to 2 mL reaction tubes and flash frozen in liquid nitrogen. All following steps were carried out on ice. A volume of 400 µL Na_2HPO_4 buffer was added to each reaction tube. Then the larvae were homogenized by a dispersing device (VDI 12, S12N5S, VWR international, Germany) for 10 s at 1,450 rpm and centrifuged at 10,000 *g* under 4 °C for 15 min (ROTINA 420R, Andreas Hettich GmbH & Co, KG, Germany). Subsequently, the supernatant of the homogenate, a protein standard, and a resorufin standard were transferred into a 96-well plate according to a distinct plate layout. 7-Ethoxyresorufin and NADPH were added to measure resorufin production and thus the AhR-mediated activity by means of a microplate reader (Infinite® M 200, Tecan Group Ltd., Switzerland) at 540 nm excitation and 590 nm emission. The protein concentration was determined fluorometrically using the fluorescamin method at an excitation of 380 nm and an emission of 460 nm (Kennedy and Jones 1994). *In vivo* AhR-

184 mediated activities were expressed as a product of resorufin in pM per milligram protein per
185 minute.

186 2.3.3 *In vivo* estrogenicity assessment.

187 The *in vivo* estrogenicity assessment was performed according to Reinardy et al. (2013), with
188 slight modifications. Samples were exposed in 6 well-plates with 40 larvae in each well for 96 h.
189 Afterwards the larvae were transferred into 2 mL reaction tubes and placed on ice for circa 10
190 min for rapid euthanasia. Excess water was removed, the larvae were transferred to 2 mL tubes
191 and samples were stored at -80 °C for further analysis. Isolation, purification, and quantification
192 of total RNA, first-strand cDNA synthesis, and quantitative real-time PCR were performed
193 according to a previous study (Reinardy et al. 2013). Primers were selected by Primer Blast
194 (NCBI). The β-actin gene (ref seq. NM_131031.1) was used as the reference housekeeping gene
195 with the use of the following forward and reverse primers: 5'-
196 ACACAGCCATGGATGAGGAAATCG and 5'-TCACTCCCTGATGTCTGGGTCGT. The *vtg1*
197 (ref seq. NM_0010044897.2) cDNA was amplified by use of the forward primer 5'-
198 ATCAGTGATGCACCTGCCCAGATTG and the reverse primer 3'-
199 ACGCAAGAGCTGGACAAGCTGAA. The estrogenicity of samples was evaluated by
200 calculating the fold-changes in the gene of interest *vtg1* compared with *β-actin* through
201 efficiency-adjusted $2^{-\Delta\Delta Ct}$ (Gosselin et al. 2010). DMSO-exposed larvae (0.5% DMSO) were used
202 as the negative control condition for relative quantification.

203 2.3.4 *In vitro* genotoxicity in zebrafish liver cell line.

204 The zebrafish liver (ZF-L) cell line was cultured according to the protocols published by Ghosh
205 et al. (1994). Cells were cultured at 26 °C in Leibovitz's L15 medium with L-glutamine (Sigma–

Aldrich) containing 10% fetal bovine serum (FBS, Biochrom, Germany) and 1% (v/v) penicillin/streptomycin solution. The micronucleus assay was performed according to a recently developed method (Di Paolo 2016). Briefly, cells were rinsed twice with phosphate buffered saline (PBS, Sigma-Aldrich) and suspended with trypsin. To avoid cytotoxicity in the assay, the highest exposure concentration of samples was defined based on a pretest using the MTT assay. A volume of 2 ml of the cell suspension at a density of 5 – 6×10^4 cells/ml was seeded onto ethanol pre-cleaned microscopic glass cover slips in 6-well plates (TPP, Trassadingen, Switzerland) and incubated for 20 h at 26°C. Subsequently, the medium was completely exchanged with the exposure medium (containing 0.5% of the investigated fractions) and then incubated for 48 h at 26°C. Subsequently, cells were fixed for 10 min in a PBS-diluted (1:1 *v/v*) mixture of methanol and glacial acetic acid (4:1 *v/v*). Fixation was repeated for 10 min in the undiluted mixture. After air-drying, the cover slips were mounted onto glass slides using DePeX (Serva, Germany). Acridine orange was used for staining of the cells after fixation (Brinkmann et al. 2014). A total number of 2000 cells per slide were analyzed under an epifluorescence microscope (Nikon Instruments, Germany) at 100× magnification. The scoring criteria are according to ISO 21427: (a) score only cells with intact cellular structure, micronuclei shall have (b) the same staining intensity and (c) a maximum size of about 30% of the main nucleus. Furthermore, cells had to be (d) clearly separated from the nucleus. Results were recorded as per-mille (‰) of micronucleated cells compared with the total number of counted cells.

2.4. Statistical analysis

The lethal and sub lethal effect rate of FET from three independent experiments was plotted using the software Prism 6.0 (GraphPad Software Inc., USA). The half-maximal lethality concentrations (LC_{50}) were obtained from the two-parameter logistic regressions using log

concentration (agonist) vs. response with variable slope, where the top and bottom of the curve was set to 0% and 100% respectively. Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, USA). One-way ANOVA followed by Dunnett's test (*$p \leq 0.05$ and **$p \leq 0.01$) was used to determine significant differences between samples and respective negative controls.

3. Results and discussion

3.1 Toxicity to zebrafish embryonic-larval stages

All 22 extracts of surface water samples from the Danube that were analyzed with the FET. The results indicated that all 22 extracts were capable of causing mortality to some extent (Figure 1). While the extract of site JDS32 located upstream Novi-sad in Serbia was clearly the most toxic sample, with an LC_{50} value at the REF 110.5, and thus was selected as the hotspot of pollution for further investigation.

For the tiered investigation of toxic effects of complex environmental mixtures, the hotspot extracts was separated using RP-HPLC into 24 fractions, which were then evaluated with the FET. Six fractions (F1, F2, F3, F4, F5 and F7) caused significant increase ($p < 0.05$) in the occurrence of abnormal development of zebrafish embryos at 48 hpf (between 13.3 and 50.0 %) in comparison with the solvent control condition (5.0 %, Figure 2B). Sub-lethal effects of eye deficit, rare pigments, no blood circulation, hemagglutination, axial malformation, pericardial edema and yolk sac edema, as well as coagulation in terms of lethality were frequently detected in these six active fractions during embryonic development (Figure 3). F4, F6 and F7 inhibited the embryonic hatching at 96 hpf, with hatching rates of 53.3 ± 12.6%, 75.0 ± 5.0% and 80.0 ± 10.0%, respectively, in comparison to that of solvent controls of 93.3% ± 5.8 ($p<0.05$) (Figure 2C). All these observed biological effects on embryos or early larval stages of zebrafish indicated

252 that chemicals present as components in the environmental mixture of JDS32 can pose a risk to
253 fish (Embry et al. 2010). Furthermore, the FET results can provide insights into the toxic
254 potential to older life stages of fish (Braunbeck et al. 2015). Though the analysis of lethal and
255 sublethal morphological endpoints in live and whole organisms in a simple and fast testing
256 system (Van den Bulck et al. 2011), the FET provided information of toxic effects on different
257 organs and individual. This assay gives a powerful insight into organism-level as well as
258 population-level risk assessment, and can be used as input for planning of further investigations
259 of involved mechanisms of toxicity.

260 3.2. AhR-mediated activity

261 The *in vivo* molecular AhR-mediated activity assay showed that five fractions (F3, F4, F5, F6
262 and F8) significantly increased CYP1A enzyme activity in zebrafish larvae, with EROD activity
263 ranging from 0.059 ± 0.009 to 0.095 ± 0.008 pmol/(min×mg protein) (Figure 2D). When
264 compared to the AhR-mediated activity of raw JDS32 extract (Neale et al. 2015b), the effect
265 analyzed with H4G1.1c2 cells was slightly higher than that of the most active fraction F6 in the
266 current study, but less than the calculated sum effects of all bioactive fractions. A possible reason
267 could be greater sensitivity of zebrafish larva compared to H4G1.1c2 cells. This assumption is
268 consistent with AhR pathway studies where early life stages of fish have proven to be
269 significantly sensitive to AhR-mediated activity (Elonen et al. 1998, Zodrow et al. 2004). Thus,
270 the current *in vivo* analysis could be a useful tool for detection of mixture AhR-mediated activity.

271 Several studies on zebrafish development reported that the activation of AhR-mediated activity
272 can induce pericardial edema, hemorrhages, craniofacial deformities (Billiard et al. 2006), heart
273 malformations, defective cardiovascular system, growth inhibition and mortality (Di Paolo et al.
274 2015a, Xiao et al. 2016). Similar results also presented in the current study, as among six EROD

active fractions, four (F3, F4, F5 and F7) increased the occurrence of eye deficit, hemagglutination, pericardial edema and no blood circulation (Figure 3). In fact, a study of heart malformation with embryonic zebrafish demonstrated that the cardiovascular system is the key and primary target for AhR-mediated developmental toxicity (Antkiewicz et al. 2005). Thus, we suppose that the activation of AhR is a factor that contributes to induction of cardiovascular abnormal phenomena. A further effect of an increase in AhR activation may ultimately affect larval development (Andreasen et al. 2002) and growth of adult fish (Carney et al. 2006, Xiao et al. 2016), which should be evaluated more carefully in future research, as it could impair reproduction success (Pocar et al. 2005). Therefore, it is considered that the protein-level AhR investigation with zebrafish larva can improve the translation of molecular mechanism-specific toxicity into organism-level toxicity towards the development of novel risk assessment technique with fewer animals.

3.3. Estrogenicity

The estrogenic activity detection by means of the *vtg1* gene expression assay showed that fraction F4, F5, F9 and F10 produced positive fold-changes up to 23 (Figure 2E). An early stage development study has reported that treatment of zebrafish embryos with 17-α-ethinylestradiol (0.1–10 μM) produced dose–response effects on mortality, non-hatching and a phenotype characteristic of ‘curved tail down’ (Kishida et al. 2001). In the current study, developmental toxicity phenomena such as coagulation, reduced pigmentation, axial malformation, yolk sac edema (Figure 3) and not hatching at 96 hpf (Figure 2C) appeared in estrogenicity active fractions (F4 and F5). Even though the knowledge of mechanistic linkages among the *vtg1* gene expression and production of fertile eggs are not completely explained, VTG protein has already been anchored to initiate ER banding event (Ankley et al. 2010). A study on vitellogenesis

298 confirmed that the expression of *vtg1* can be changed by aquatic estrogenic contamination, and
299 then affect embryonic development during embryogenesis (Sumpter and Jobling 1995). An *in*
300 *vitro* study of ER target genes reported that estrogens play an essential role in the growth,
301 differentiation, and homeostasis of a number of target tissues, including reproductive tracts (both
302 male and female), mammary glands, bone, brain, and liver (Li et al. 2013). Thus, we demonstrate
303 that the applied zebrafish gene-level estrogenicity assessment method can contribute to
304 investigate organism-level toxicity endpoints with relevance for population-level consequences,
305 which is of high importance for water quality assessment.

306 3.4. Genotoxicity

307 Genotoxic activity, as another important water quality assessment parameter, was investigated
308 by micronucleus assay with ZF-L cell line in the current study. Fractions F3, F4 and F5 showed
309 significant micronucleus inductions, with the induction values (‰) of 12.83 ± 2.37, 15.50 ± 2.38
310 and 21.75 ± 3.20, respectively (Figure 2F). For these three bioactive fractions, corresponding
311 toxic effects were also observed in the respective FET (Figure 2B). Positive correlation between
312 embryo toxicity and genotoxicity has been observed in the study of Wessel et al. (2007).
313 Furthermore, Anderson and Wild concluded that genotoxicity may have consequences on
314 reproduction through gamete loss due to cell death, embryonic mortality, and mutations
315 (Anderson and Wild 1994). Thus, the applied cell-level genotoxicity assessment test presents
316 toxic potential of aquatic mixture with zebrafish-based *in vitro* assay, assuring that this important
317 mechanism of toxicity is investigated in the same model species as the other applied methods.

318 3.5. Fraction-related toxicity

319 As can be seen from figure 4, F3 exhibited positive effects in EROD assay and micronucleus
320 assay, indicating likely contains AhR-active and genotoxic chemical mixture. F6 gave the highest

AhR-mediated activity, but did not cause any embryonic toxic effect at 24 hpf and 48hpf, and only presented reduce hatching effect at 96 hpf. These observations suggested that the AhR-mediated activity could mainly contribute to the effect of non-hatching, and F6 may contains rather specifical-acting substances for AhR-mediated activity. Similar findings were observed for F9 and F10, which caused fold changes in *vtg1* gene expression assay, but did not present organism level toxic response in the FET; and for F8, which was active in the molecular level assays of AhR-mediated activity, but inactive regarding developmental toxicity. These findings demonstrated the specific contaminations of F9, F10 and F8 regarding to estrogenicity and AhR-mediated activity respectively. Besides, environmental biomarker studies demonstrated that molecular bioassays could be used as sensitive biomarkers to indicate the toxic potential of chemicals or environmental mixtures (Shigenaga and Ames 1991). In particular, EROD has been proposed as sensitive 'early warning' tool to predict the anticipated changes at higher levels of biological organization, i.e. population, community or ecosystem (Cajaraville et al. 2000, Whyte et al. 2008). However, the most active fractions F6 and F8 in the EROD assay did not induce any estrogenic activity in *vtg1* gene expression assay. A similar finding was reported in an anti-estrogenicity study where AhR agonists caused inhibition of estrogen induced responses (Smeets et al. 1999). Moreover, the most polar fractions F1 and F2, which induced yolk sac edema and reduced pigmentations, did not induce any of the evaluated mechanism-specific toxic effect. These finding indicated that F1 and F2 did not contain dioxin-like chemicals (DLCs) and endocrine-disrupting chemicals (EDCs) at concentrations sufficiently highly to induce the corresponding effects. As shown in Table S3, 32 polar chemicals with a log K_{OW} less than 2.23 were detected in raw JDS32 sample with concentrations up to 2.046 nM (Table S3), but none of them is known as DLCs or EDCs. An *in vitro* study reported that the polar fraction also promoted the generation of reactive oxygen species, decreased membrane potential (ΔΨm), loss of

345 mitochondrial membrane mass and induction of apoptosis (Xia et al. 2004).Thus, there are
346 possibly other molecular events involved that we not investigated in the current study.

347 3.6. Identification of the most potent mixtures

348 Figure 4 depicts that F4 presented positive effects in all mechanism-specific endpoints bioassays
349 and embryo developmental bioassay. Such toxicological profile of fractions demonstrated that the
350 complex environmental sample is capable of causing various biological effects at different levels
351 of biological organization, supporting the need for an integrated testing strategy. Moreover, F4
352 showed activity for the apical endpoints in most of current bioassays (Figure 2), and was
353 therefore identified as the most potent mixture for the Danube.

354 In an attempt towards identification of compounds composition of the most potent mixture, a
355 total of 28 chemicals with a wide log K_{OW} range (2.03-6.76) (Table S2) were applied in RP-
356 HPLC (Grung et al. 2007). By plotting the log K_{OW} of the identified compounds versus fraction
357 number, a linear response was obtained (Figure S2) (Thomas et al. 1999). According to a
358 previous chemical-analytical study of JDS32 (Neale et al. 2015b) (Table S3), the herbicide
359 atrazine and the pharmaceutical carbamazepine which were found within the F3 log K_{OW} range,
360 can increase the frequencies of micronuclei (Çelik 2006). Furthermore, the AhR-dependent
361 inhibition of CYP1 can be significantly altered by the isoflavone daidzein (Choi and Kim 2008).
362 Similarly, the F4 log K_{OW} range chemicals triphenylphosphine oxide might influence activation
363 of the AhR (Mikstacka et al. 2008); genistein was found to induce ERα expression in maturing
364 mouse ovaries (Jefferson et al. 2002); metolachlor was proven to induce larval abnormalities and
365 DNA damage in a population of exposed oysters at environmentally relevant concentrations (Mai
366 et al. 2012). However, no chemical with a log K_{OW} more than 5.12 was detected (Table S3). The
367 previous chemical analysis might not have covered DLCs and EDCs that induced positive effects

368 in fractions F6, F8, F9 and F10. This supports the need of bioassays to direct chemical analysis in
369 water quality monitoring.

370 3.7. Artificial mixture toxicity confirmation

371 To further confirm that the identified chemicals are the most toxic potent mixture, F4 log K_{OW}
372 chemicals were prepared and analyzed with the current investigation strategy. On the basis of the
373 chemical linear relationship of log K_{OW} and retention time (Table S2, Figure S2), the log K_{OW} of
374 F4 should range from 2.83 to 3.42 (Grung et al. 2007, Xiao et al. 2017). Our previous chemical
375 analysis study reported that only 12 chemicals within F4 log K_{OW} range were detected in raw
376 JDS32 extract, *i.e.* triphenylphosphine oxide, genistein, isoproturon, diphenyl phosphate, lenacil,
377 mecoprop, metolachlor, 2-(methylthio)benzothiazole, clarithromycin, terbuthylazine, gestoden
378 and oxazepam (Neale et al. 2015b) (Table S3). Hence, an artificial mixture composed of these 12
379 chemicals with 250 times concentrated Danube surface water was prepared to investigate various
380 biological effects (Table S3). In Figure 4, tiered biological effects were found for the mixture and
381 F4. In the estrogenicity assay, the *vtg*1 gene expression induced by exposure to mixture was
382 slightly higher than that found for F4. The micronuclei frequency and AhR-mediated activity of
383 mixture showed no significant differences (p>0.05) to that of F4. It indicates that EROD activity
384 can be caused by a multitude of substances, some of which at rather low concentrations in a given
385 sample (Louiz et al. 2008, Petrulis et al. 2001), and mixture design may missed some of them.
386 The FET showed that the mixture induced mortality, abnormal development and non-hatching of
387 the zebrafish embryos, slightly weaker than that of F4. Thus, the detected chemicals could
388 successfully explain most of developmental toxic effects. This gives strong evidence that the
389 integrative zebrafish investigation strategy developed herein is capable to point towards main
390 components of mixture for toxic effects of water samples confirming.

3.7. Future needs in integrated bioassay investigation strategies

In consideration of the requirement that a minimum of sample amount provides a maximum of output in biological studies, only one concentration was tested for each fraction to retrieve meaningful results in each bioassay, and three independent replicates were implemented to ensure the accuracy of the findings. Fraction dilution into different concentrations is required as input parameters to develop concentration-response-curves for the experimental data qualification in future research. In addition, further target and non-target chemical analysis will be implemented to identify each specific toxic driver in the active fractions.

4. Conclusions and outlooks

The successful application of an integrative zebrafish investigation strategy for aquatic mixture characterization was demonstrated by means of complementary evaluation of mechanism-specific toxicity and apical effects in a complex water sample. The four-tiered zebrafish-based bioassays clarified the occurrence of toxic effects at molecular, cellular and whole organism of the water sample and respective fractions. The characterization of the toxic effects caused by each fraction supported fraction-related toxicity. The fact that all bioanalyses were implemented in zebrafish as the model organism avoided interspecies differences such as transformation rates in different organisms. Finally, the artificial mixture evaluation confirmed that our investigation strategy is a useful tool to characterize aquatic mixtures and the respective involved toxic mechanisms in surface water.

The present study can serve as a step towards the implementation of bioanalytical techniques in water quality monitoring. The outcomes of the present study contribute to demonstrate the performance and usefulness of integrative biomonitoring strategies for water quality assessment.

These efforts provide the scientific underpinnings and regulatory reference for derivation of environmental quality standards in the context of the EU WFD.

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Supporting Information

Detailed information on the sampling information, a list of single compounds used for establishing the relationship between *log* K_{ow} and retention time (RT), the linear regression between *log* K_{ow} and RT, chemical data of JDS32 and artificial mixture, as well as biological effects of artificial mixture is available in the Supplementary Information.

Notes: The authors declare no competing financial interest.

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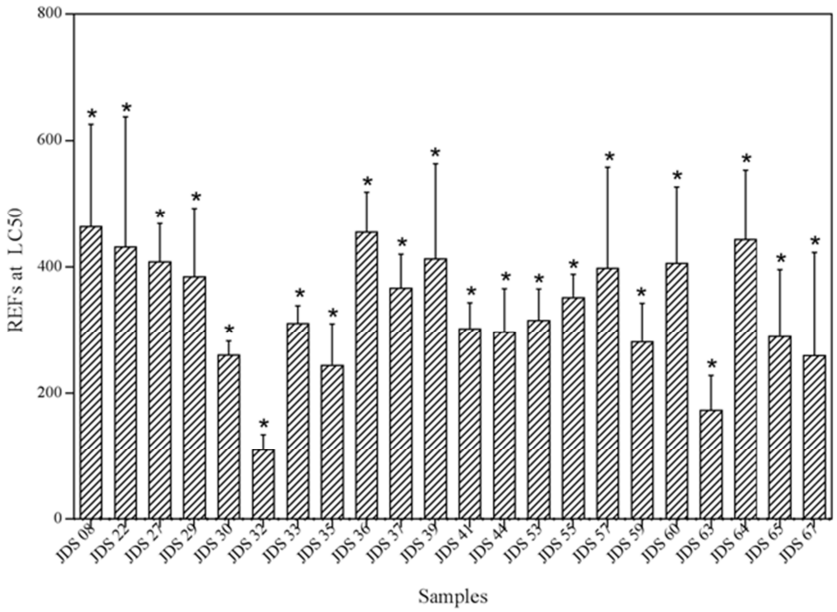

Figure 1: Lethal effect concentrations (LC50) at 48 hpf expressed as relative enrichment factors (REF) of Joint Danube Survey 3 (JDS 3) samples. Data in columns (mean ± SD, n = 3, ** $p<0.01$, *$p<0.05$) depict developmental toxicity with zebrafish embryo that were exposed to JDS3 water extracts at REF 62.5 to 1000.

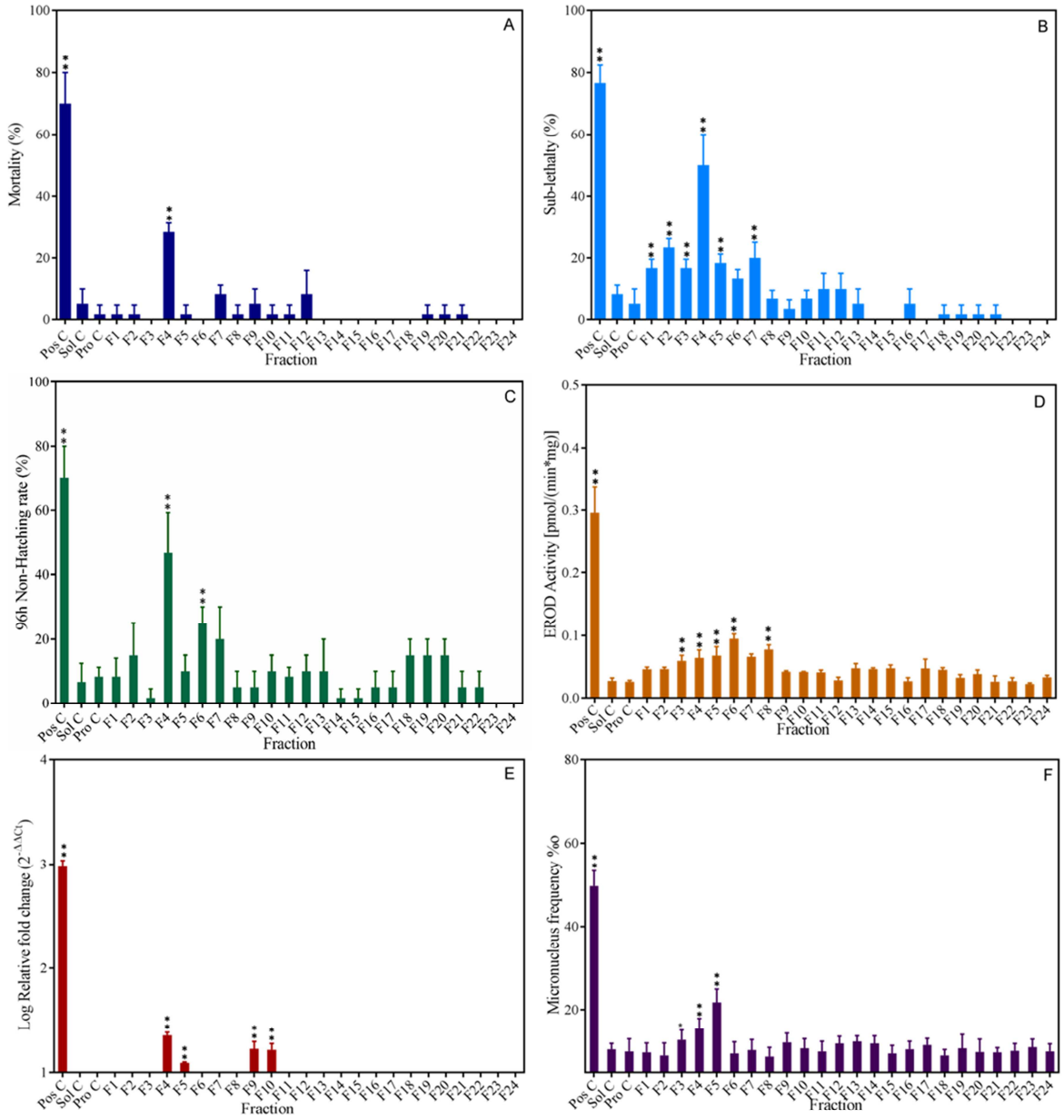

Figure 2. Lethal, sub-lethal and mechanism-specific toxicity endpoints in Danio rerio embryonic-larval stages (A to E) or liver cell line (F) exposed to a fractionated Danube surface water extract. Lethality at 24 hpf (A), sub-lethality at 48 hpf (B), non-hatching rate at 96 hpf (C), AhR-mediated activity as 7-Ethoxyresorufin-O-deethylase (EROD) in at 96 hpf zebrafish larvae (D), estrogenicity as relative fold changes (efficiency-adjusted 2−ΔΔCt) of vtg1 gene expression in 96 hfp zebrafish larvae (E), and genotoxicity as the micronucleus frequency (‰) in a zebrafish liver cell line (F). Data in columns (mean ± SD, n = 3, **: p<0.01, *: p<0.05) depict in zebrafish of control conditions (Pos C: positive controls, Sol C: solvent controls, , Pro C: process control; and 24 RP-HPLC fractions (F1 to F24) tested at the REF of 200 for the FET test (A, B and C) and the micronucleus assay (F), at REF of 50 for the EROD assay (D) and vtg1 assay (E).

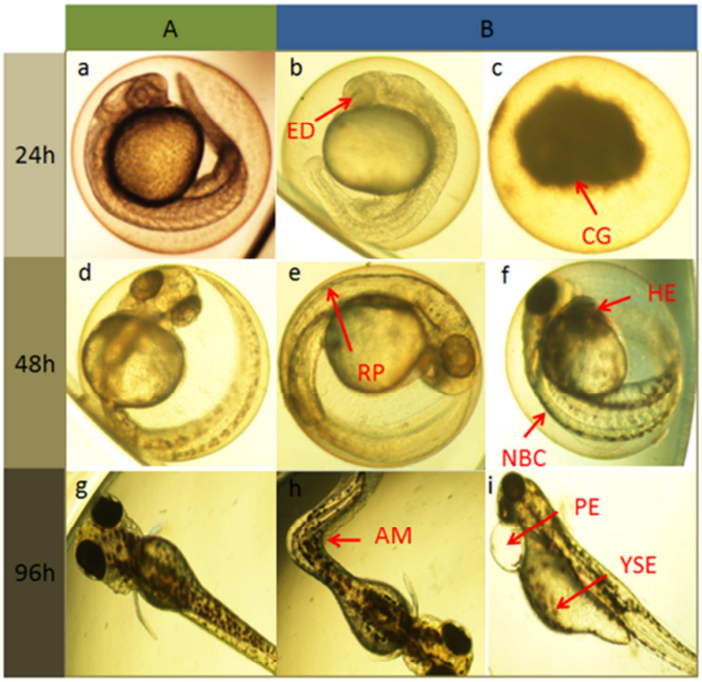

Figure 3. RP-HPLC fraction induced various developmental effects in zebrafish embryos and larvae. The solvent control group of 0.5% DMSO in artificial water (A) demonstrated normal development at 24 (a), 48 (b) and 96 h (c) The groups treated with fractions F1-F8 (B) presented lethal effects such as CG, coagulation (F4); as well as sublethal effects such as: ED, eye deficit (F3); RP, reduced pigmentation (F5); NBC, no blood circulation (F3); HE, hemagglutination (F7); AM, axial malformation (F4); PE, pericardial edema (F5); YSE, yolk sac edema (F4).

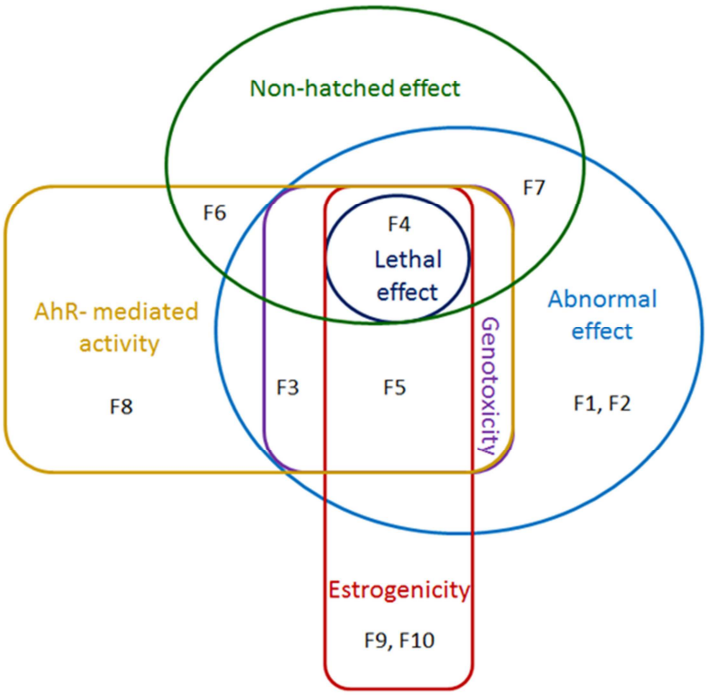

Figure 4. Venn diagram of active fractions in the FET tests (ellipses) and mechanism-specific toxicity bioassays (rectangles). Observed developmental toxicity endpoints were lethality (dark blue), abnormal effect (light blue), and non-hatching (green). Mechanism-specific bioassays investigated AhR-mediated activity(yellow), estrogenicity (red) and genotoxicity (purple).

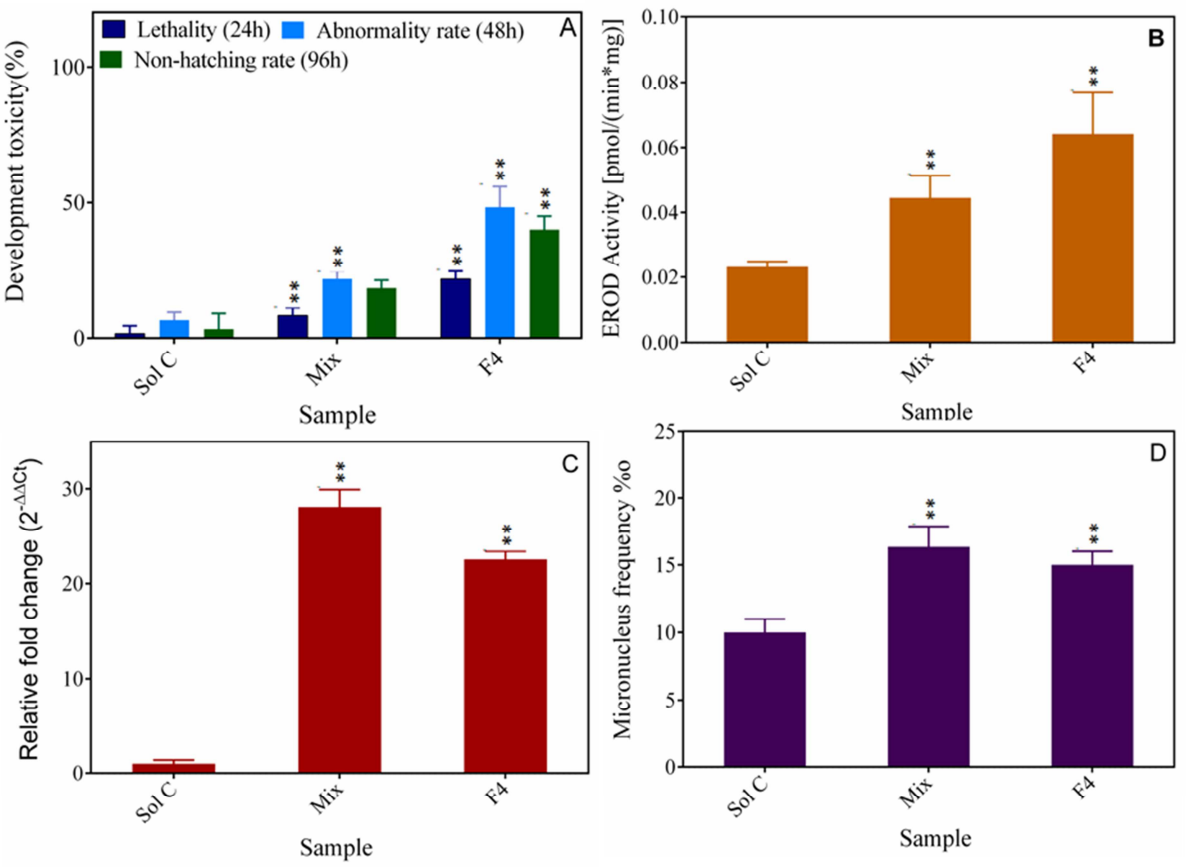

Figure 5. Integrative zebrafish investigation strategy identified the occurrence of different toxic effects in the solvent control condition (Sol C), the mixture (Mix) and the F4 fraction (F4). Data in columns (mean ± SD, n = 3, **: p<0.01) depict developmental toxicity between 24 and 96 h of exposure (A), EROD activity as 7-Ethoxyresorufin-O-deethylase (EROD) in 96 hpf zebrafish larvae (B), estrogenic activity as relative fold changes (efficiency-adjusted 2−ΔΔCt) of vtg1 gene expression at 96 hfp larval zebrafish (C) and genotoxicity as the micronucleus frequency in zebrafish liver cell line (D).

List of Figures

Highlights

- bioassays at different level of biological complexity
- Minimum sample consumption for large throughput screenings of water samples
- Zebrafish-based bioassays were implemented avoiding interspecies differences
- Toxicity-based assessment of mixtures in surface water