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Toxicity of 10 organic micropollutants and their mixture: Implications for aquatic risk assessment

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Abstract

Micropollutants, as a serious water pollution issue, raise considerable toxicological concerns, particularly when present as components of complex mixtures. Due to the interactions of environmental pollution components (contaminant), the micropollutant problem is increasingly complex, thus, water quality of organic chemical contamination assessed substance-by-substance might lead to underestimation in aquatic environmental risk assessment. To assess the aquatic environmental risk of micropollutants mixture, a total of 10 organic micropollutants were selected and analysed by an approach of integration of literature data, laboratory experiments and prediction techniques. The experiment results showed that all 10 micropollutants were capable of causing toxicity in zebrafish embryos, aquatic invertebrates and algae with the LC50 (50% lethal concentration) values from 1.14 mg/L to 14.37 mg/L. Triclosan, carbamazepine, diazinon and diuron were the most hazardous compounds in the Danube River and the Rhine River. The artificial mixture presented a strong antagonistic relationship, which demonstrated an independent action (IA) model of the mixture. Based on the observed toxicity data, the risk quotients (RQs) of environmental mixtures of the Danube River and the Rhine River were extrapolated. It can be concluded that the micropollutant mixture may pose a potential risk for aquatic ecosystems with the present environmentally measured concentrations in the Danube River and Rhine River. Mixture risk assessment results suggested that the toxicity of studied chemicals might be induced by dissimilar actions, which is in agreement with the mixture toxicity prediction of the IA model. The observed findings could be useful to establish an overview of the pressures, vision, measures and expectations for hazardous substances pollution, which can help in making to informed decisions to reduce the concentration and bioactive fraction of pollutants.

Keywords: Micropollutants; zebrafish embryo; mixture toxicity; independent action model; necessity of mixture risk assessment

1. Introduction

Currently, one of the key environmental problems facing humanity is the increasing worldwide contamination of fresh water systems with thousands of industrial and natural chemical compounds (Schwarzenbach et al. 2006). It has been shown that between the years 1930 and 2000, global production of anthropogenic chemicals increased from 1 million to 400 million tons per year (Gavrilescu et al. 2015). Statistics published by EUROSTAT in 2016 reveal that between 2004 and 2013, nearly 50% of the total production of chemicals is represented by environmentally harmful compounds and approximately 20% of them had significant acute impacts on the environment (EUROSTAT 2016). The macro-chemicals among them, such as acids, salts, nutrients and natural organic matter, occurring at mg/L to g/L concentrations, are already relatively well investigated (Schwarzenbach et al. 2006). However, thousands of synthetic and natural micropollutants, which are present at low or very low concentrations (pg/L to ng/L) in the aquatic environment, are still far more difficult to assess regarding their source, behaviour and in particular effects on the aquatic environment (Gerbersdorf et al. 2015). Moreover, the continuous input of micropollutants in receiving waters is a growing environmental issue, since many of them are known to be non-biodegradable, persistent and bioaccumulative (Schwarzenbach et al. 2006). Thus, the aquatic micropollutants have become a worldwide affair of increasing environmental concern.

Even though environmental concentrations of these micropollutants are low, many of them raise considerable toxicological concerns (Schwarzenbach et al. 2006), which is a serious water pollution issue and may cause an increase in poisoning of fish and other aquatic species and thus

an environmental imbalance. For instance, the widely used biocide triclosan has been frequently detected in wastewater (0.07-14 ,000 µg/L), streams (50-2,300 ng/L) and seawater (50-150 ng/L) (Bureau 2003), and was found to be highly toxic to green algae, which may thus compromise the balance of the ecosystem (Tatarazako et al. 2003). Moreover, some chemicals are not degraded at all or only very slowly, such as the most frequently detected pharmaceutical residues carbamazepine and diclofenac (Scheytt et al. 2005). They are produced and administered with the aim of causing a biological effect. Once entering the aquatic environment, these chemicals act as persistent or pseudo-persistent compounds that sustain a multigenerational exposure for the resident organisms (Daughton and Ternes 1999). Thus, regulation programmes, such as the European chemical legislation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), which focus on single chemical assessment, were established to manage the safe use of chemicals (Agency 2007).

However, the contaminants in natural systems rarely occur as individual chemicals, but are usually accompanied by mixture of thousands of compounds (Neale et al. 2015b). Interactions between each mixture component may occur at various endpoints in the toxicodynamic as well as the toxicokinetic phase (Backhaus and Faust 2012, Groten et al. 1997). Mixture toxicity studies show that considerable combination effects could occur when all components are applied at concentrations below their no observed effect concentration (NOEC), or even with mixtures of compounds that cause no effects as single substance at their water solubility limit (Smith et al. 2013). Therefore, water quality investigated substance-by-substance may lead to underestimation in aquatic environmental risk assessment, and the evaluation of the toxic effects of micropollutant mixtures is necessary.

The goal of the current study was to assess the environmental risk of micropollutant mixtures by an approach which assembled literature data, laboratory experiments and predicted techniques for 3 aquatic organisms. Firstly, organic micropollutants were selected from the list of 214 chemicals identified as relevant river pollutants by Busch et al. (2016)), due to their occurrence in European river systems and hazard quotients > 10^{-4}. The selection process was based on 1) the prioritization score (Neale et al. 2017); 2) diversity of chemical use groups, and 3) a specific mode of action expected to lead to enhanced developmental toxicity on the zebrafish embryo. The selected micropollutants covered biocides, herbicides, fungicides, pharmaceutical industrial compounds and natural chemicals. As priority water contaminants, five pesticides and three pharmaceuticals were selected in the current research, since an industrial development and the trade-off to environment study reported that the toxic pollution load released from agriculture is approximately 1.5 times higher than that from pharmaceuticals in water (Salman 2011). Because industrial chemicals and natural chemicals are very important causes of water pollution, the most frequently detected industrial chemical, bisphenol A, and the most abundant soy-derived phytoestrogen genistein were included in the current study (Haynes-Johnson et al. 1999, Makarevich et al. 1997, Nynca et al. 2013, Whitehead et al. 2002). Therefore a total of 10 frequently detected organic micropollutants from European surface waters were selected to analyse their toxic effects in the current study (**Table 1**). Then, the acute toxicity assay with zebrafish embryos, which can provide comprehensive and realistic insights into the bioavailable toxicity potential of contaminated water (Rocha et al. 2011), was applied for each micropollutant individually and in environmental mixtures. On the strength of concentration-response information of individual micropollutants and mixtures in the zebrafish embryos assay, the interactions of the micropollutant mixture were analysed by combination of toxic mechanism

data available from the literature and the prediction model of concentration addition (CA and independent action (IA). Afterwards, the Danube River and the Rhine River, as two of the largest rivers in Central and Western Europe, which are sources of drinking water for more than 20 million people, were investigated to collect the data of occurrence and concentration levels of the 10 studied organic micropollutants in surface water and effluent water. Finally, based on the observed mixture interactions and chemical information in the Danube River and Rhine River, the environment risks and complications of micropollutant mixtures were assessed with three classic aquatic taxa (algae, invertebrate and fish) by risk quotient (RQ) assessment. The current mixture results could help for the development of interactions-based risk assessment to facilitate a more realistic evaluation of environmental mixture hazards.

2. Materials and methods

2.1 Chemicals

Stock solutions of 10 organic chemicals were prepared in dimethyl sulfoxide (DMSO) at 20g/L. Bisphenol A (99%), diclofenac (analytical standard), diuron (98%), carbamazepine (99%), diazinon (analytical standard), triclosan (analytical standard), flusilazole (analytical standard), cyprodinil (analytical standards), penconazole (analytical standards) and genistein (98%) were supplied by Sigma-Aldrich (Deisenhofen, Germany).

2.2 Zebrafish embryo toxicity test

To analyse the comprehensive aquatic toxicity of micropollutants and their mixtures, zebrafish (*Danio rerio*) were maintained according to Braunbeck and Lammer (Braunbeck and Lammer 2005) with the modifications given by Schiwy et al. (2015). The fish were kept in glass aquaria at a water temperature of 26 ± 1 °C, a pH value of 7.8, and a hardness of approximately 1.96

mmol/L. A constant day to night rhythm (14/10 h) was maintained. The fish were fed with commercially available dry flake food (e.g., TetraMin™ flakes; Tetra, Melle, Germany) and live nauplius larvae of *Artemia sp.* (Silver Star Artemia, Inter Ryba GmbH, Zeven, Germany) once daily ad libitum. Groups of 3-month-old zebrafish with a ratio of 3:2 (males/females) were used for egg production. For the following bioassays, fertilised fish eggs were visually selected using a binocular microscope (SMZ 1500, Nikon GmbH, Düsseldorf, Germany).

Only normally developed fish eggs in at least in the 8-cell stage were used to assess mortality after 48 hours post fertilisation (hpf). In general, the test was conducted based on the Organisation for Economic Co-operation and Development (OECD) guideline for fish embryo toxicity tests (FET) (OECD 2006). Briefly, fertilized eggs were selected for the test and placed in 96-well microplates (Figure S1). In each well, 1 egg was exposed to 200 µL of test solution resulting in 0.5% DMSO in aerated artificial water (ISO 7346-3 1996). Each substance and mixture was tested in at least 5 different concentrations, and 10 eggs were employed for each concentration. All experiments were independently repeated 3 times. Eggs were incubated at 26 °C. After 48 hpf, eggs were examined using an inverse microscope. All testing plates were covered with oxygen-permeable adhesive sealing to allow oxygen influx during this 48 h exposure (Strecker et al. 2011). A negative control (artificial water), a solvent control (artificial water containing 0.5% DMSO) and a positive control (3,4-dichloroaniline, 3.7 mg/L in artificial water) were included in every exposure group. Only batches with effect levels above 10% in the positive control were included in evaluation. Mean spontaneous lethal effects were ≤10%. Fertilisation rates were >80%. Lethal endpoints were scored according to the criteria defined in the OECD guideline (ISO 2013) and the study from Shao et al. (2018): coagulation of the embryo, no heartbeat and non-detachment of the tail.

2.3 Exposure

To give a more realistic evaluation of the toxicity of environmental micropollutants, solutions of organic micropollutants were used individually and in mixtures (**Table 2**). Zebrafish embryos were treated with serial dilutions of each chemical individually and in combination. The mixture was prepared with a fixed constant ratio (1:1) based on the individual LC50 values (50% lethal concentration) (mg/L). Five dilutions (serial dilution factor = 2) of each chemical and combination plus 1 negative control, 1 DMSO control and 1 positive control were tested in 3 independent experiments. According to European legislation (EuropeanUnion 2010) and the respective regulation TierSchG (TierSchG 2006) experiments with zebrafish embryo and larvae up to 120 hpf are not considered animal experiments.

2.4 Statistical concentration-response analysis

Statistical concentration-response regression analyses for the single emerging micropollutants and their mixtures were conducted using a best-fit approach (Scholze et al. 2001) by GraphPad Prism 6.0. Two non-linear regression models (logit, Weibull) which both describe the monotonic sigmoid concentration-response relationship, were used independently to fit each test data. The top and bottom of the curve were set to 0 (no lethal embryo after exposure) and 1 (all exposure embryo present lethal effects), respectively. Both were fitted to the data by the method of maximum likelihood. A potential over-dispersion in the data was evalated using the Williams' method, with Fisher's scoring as the optimizsation technique. The best fitting model was selected on the basis of a statistical goodness-of-fit criterion. Excel was used for calculation of the EC*x* (the median effective concentrations that cause x% effect with respect to a negative control).

Statistical uncertainties of the estimated effect concentrations were expressed as 95% confidence intervals and approximated by applying the bootstrap method (Efron and Tibshirani 1994).

2.5 Mixture toxicity predictions based on CA and IA

To analyse the possible interactions of micropollutants in the mixture, toxicities of the mixture were computed based on the predictive equations of concentration addition (CA) (Eq. (1)) and independent action (IA) (Eq. (2)), two of the most widely used definitions of additivity (Altenburger et al. 2004, Faust et al. 2001). CA is based on the assumption that mixture components have the same sites and similar modes of action (Weichenthal et al.), and is computed by equation (Altenburger et al. 2004):

$$ECx_{mix} = \left(\sum_i^n \frac{p_i}{EC_{xi}}\right)^{-1} \quad (1)$$

where ECx_{mix} is the effect concentration of the mixture provoking x% effect, EC_{xi} is the concentration of the component i provoking the same effect (x%) as the mixture when applied individually and p_i is the molar ratio of the *i*th component in the mixture.

IA is based on the assumption that mixture components have dissimilar MOA (mode of action). The following equation applies for IA (Altenburger et al. 2004).

$$\mathrm{E}(C_{mix}) = 1 - \prod_{i=1}^{n}\left(1 - E(C_i)\right) \quad (2)$$

where C_{mix} and $E(c_{mix})$ are the total concentration and total effect of the mixture, respectively, and $E(c_i)$ is the effect of the *i*th component with the concentration c_i in the mixture. The C_i in Eq. (2) can be replaced by ($p \times c_{mix}$). The $E(C_i)$ can be calculated from the function that described the concentration–response curve of the *i*th component (Altenburger et al. 2004, Qin et al. 2011).

2.6 Risk quotient assessment of single micropollutants

The aquatic risks by the micropollutants were assessed on the basis of the risk quotient (RQ) which tries to estimate the actual potential risk (probability of an expected effect, i.e., potential danger, caused by an environmental concentration) of a pollutant. This quotient is calculated as the ratio between measured environmental concentrations (MECs) or predicted environmental concentrations (PECs) and predicted no effect concentrations (PNECs) (Sanderson et al. 2003). Risk quotients (RQs) were then estimated as follows (von der Ohe et al. 2011):

$$\mathrm{RQ} = \frac{PEC\ or MEC}{PNEC} \tag{3}$$

No observed effect concentration (NOEC) values were used for calculation of the emerging micropollutant PNEC [PNEC = (NOEC or LC50 or EC50)/AF (assessment factor)]. The NOEC for zebrafish were represented by the LC10 in the zFET. By lack of NOEC values, LC50 or EC50 values were used. An AF of 1000 was utilised for the given micropollutant, because in the current study, all data came from short-term assays (Bureau 2003).

2.7 Risk quotients assessment of mixture

The risk quotients of mixtures were extrapolated according to the mixture interaction analysis. According to the study of Backhaus and Faust (2012), the risk quotients of mixtures could be calculated by summing up the single micropollutant PEC/PNEC ratios, if the mixture showed a similar mode of action . The final risk quotient for the mixture, $RQ_{PEC/PNEC}$:

$$\mathrm{RQ}_{PEC/PNEC} = \sum_{i=1}^{n} \frac{PEC_i\ or\ MEC_i}{PNEC_i} = \sum_{i=1}^{n} \frac{PEC_i\ or\ MEC_i}{\min(EC50_{algae}, EC50_{daphnids}, EC50_{fish})_i \times (1/AF_i)} \tag{4}$$

where PEC_i and MEC_i are predicted environmental concentration and measured environmental concentration of the *i*th component in the mixture, respectively, $\min(EC50_{algae}, EC50_{daphnids}, EC50_{fish})_i$ is the minimum EC50 value of the *i*th component and AF*i* is the assessment factor for the *i*th component.

If $RQ_{PEC/PNEC}$ is above 1, the RQ of the mixture can be calculated by the sum of the toxic units (STU) in a next step, RQ_{STU}

$$RQ_{STU} = \max(STU_{algae}, STU_{daphnids}, STU_{fish}) \times AF$$

$$= \max\left(\sum_{i=1}^{n} \frac{PEC_i \text{ or } MEC_i}{EC50_{i,algae}}, \sum_{i=1}^{n} \frac{PEC_i \text{ or } MEC_i}{EC50_{i,daphnids}}, \sum_{i=1}^{n} \frac{PEC_i \text{ or } MEC_i}{EC50_{i,fish}}\right) \times AF \quad (5)$$

where $max(STU_{algae}, STU_{daphnids}, STU_{fish})$ is the maximum STU, and the AF equals 1,000 for the limnic aquatic environment according to the council on registration, evaluation and authorisation of chemicals (REACH) (Agency 2007). If the RQ_{STU} is above the threshold of 1, IA should be considered (Backhaus and Faust 2012), and the risk quotients of mixtures may be extrapolated by the maximal risk quotient of a single component, since the independent mode of action for IA.

3 Results and discussion

3.1 Embryo toxicity of individual emerging micropollutants

Based on the fingerprinting identified micropollutants study (Peta A. Neale 2017), a total of 10 frequently detected organic micropollutants (**Table 1**) from European surface waters were selected to analyse their toxic effects in the current study. The concentration-response curves for each individual micropollutant in the zebrafish embryo toxicity test (48 hpf examination) are

shown in **Figure 1**, with concentration effect parameters (location and slope, **Table 2**). All 10 micropollutants were capable of causing mortality in zebrafish embryos, where clear concentration-response relationships were obtained. Based on the two non-linear regression models probit and Weibull, LC50 (the concentration that induced lethal effects on 50% tested embryos) values were calculated ranging from 1.14 (triclosan) to 53.05 (carbamazepine) mg/L. Among these micropollutants, the chemical carbamazepine displayed a flat and incomplete concentration-response curve. It presents a plateau with respect to zebrafish embryo mortality, the induction of which did not progress for drug dosage above approximately 6.25 mg/L. The reason for this behaviour is probably due to the solubility of carbamazepine in 0.5% DMSO medium. With regard to the other 9 chemicals, complete concentration-response relationships could be recorded for zebrafish embryo after an exposure of 48 hpf (**Figure 1**). The LC50 values ranged between 1.14 mg/L and 14.37 mg/L with an order as follows: triclosan < cyprodinil < genistein < penconazole < flusilazole < diazinon< diuron < diclofenac < bisphenol A < carbamazepine. However, according to the LC10 and LC20 values, cyprodinil was the most toxic emerging micropollutant for zebrafish embryos, followed by triclosan, with an exposure concentration as low as 0.313 to 5 mg/L.

3.2 Toxic mechanism of individual emerging micropollutants

In fact, organism mortality can be induced by various different toxicity pathways as a result of combined actions, even though the complete mechanisms of lethal effect on embryos are rarely clear. A previous study proved that disturbance of the oxidant-antioxidant balance can cause defective embryonic development (Ornoy 2007), while embryo developmental toxicity and genotoxicity presented a positive correlation, which may have consequences for embryonic mortality and mutations (Anderson and Wild 1994, Wessel et al. 2007). As shown in **Figure 2**,

several possible pathways of toxic actions have been described for the 10 micropollutants by previous studies. Seven out of 10 micropollutants (bisphenol A, diclofenac, diuron, diazinon, triclosan, flusilazole and carbamazepine) were found to exhibit genotoxicity or mutagenicity. Five out of 10 micropollutants (bisphenol A, diclofenac, diazinon, cyprodinil and genistein) were found to have estrogenic effects or potential estrogenic effects. For the same toxic endpoint, there were maybe different toxicity mechanisms, such as triclosan leading to genotoxicity by increasing micronucleus frequency (Binelli et al. 2009), diazinon inducing genotoxicity by activation of sister-chromatid exchange and DNA damage (Nishio and Uyeki 1981) and bisphenol A presenting genotoxicity by introduction of chromosomal aberrations (Allard and Colaiácovo 2010) and mutagenicity in the Ames test. In addition, some chemicals induce toxic effects by attacking specific tissues or organs. The main acting site for diclofenac is the liver (Bort et al. 1999, Laine et al. 2007), and for penconazole, it is testicular tissues (El-Sharkawy and El-Nisr 2013). Besides, the biological effect of narcosis, which was induced by a wide range of chemicals at high concentrations (Veith and Broderius 1990), was not considered in the current study, since that micropollutants are present at low or very low concentrations in environments.

3.3 Experimental and predicted toxicity of the micropollutant mixture

To identify the interaction effects within the micropollutant mixture in zebrafish embryos, a mixture composed of these 10 micropollutants was prepared (**Table 3**). In the same way as that for the single micropollutants, the concentration–response (mortality) data of the 10-chemical mixture based on the LC50 values of each single chemical was determined using the zebrafish embryo toxicity test.

The observed 95% effect of the 10-chemical mixture, which was better described by the IA model than the CA model, demonstrated a strong antagonism (**Figure 3**). These findings are in accordance with the findings of Belden et al. (2007), where the IA model was proven more accurate than the CA model. According to the postulation of the IA model, the investigated micropollutants may primarily attack different molecular target sites, and after distinct chains of reactions, led to a common toxicological endpoint of lethal embryo in the current study (Faust et al. 2003). In other words, even if all investigated micropollutants caused developmental toxicity in zebrafish embryos, they may have dissimilar MOA for the zebrafish embryo mortality in the current study. It is consistent with the above toxicity mechanism analysis (**Figure 2**) that the zebrafish embryo mortality due to these 10 micropollutants may be induced by different mechanisms.

Therefore, in view of the toxicity mechanisms and the close agreement of the observed experiment effects and predictions, the toxicity of the 10 studied micropollutant-mixture may be induced by dissimilar actions by attacking different subsystems or molecular targets of the affected organism (Kortenkamp 2014).

3.4 Risk quotient assessment of micropollutants and their mixtures

To assess the environmental implications of the micropollutants, aquatic risks were determined with RQs for surface water and wastewater effluent with regard to representative species of the food chain that are typically used in acute toxicity tests. The MECs used in this study were maximum actual measured environmental concentrations that were mainly reported for Danube River surface water, Rhine River surface water and wastewater effluents (Neale et al. 2015, Peta A. Neale 2017) (**Table 4**). All 10 micropollutant MEC values are in the range of ng/L, and most

of the Danube River-related MEC values are higher than that of the Rhine River and higher than that of Rhine effluent wastewater, except for bisphenol A and flusilazole. Thus, it is assumed that the water quality in the Danube River is worse than that in the Rhine River. However, studies reporting actual environmental measured concentrations for penconazole and cyprodinil in the Danube surface water, genistein in the Rhine surface water and genistein, triclosan and flusilazole in Rhine waste water are lacking. These chemicals and the necessity to produce sufficient data on them should be given more attention in future research. The PNEC (predicted no effect concentration) values were obtained by dividing the NOEC or LC50, EC50 value of each emerging micropollutant by a safety factor of 1,000. As shown in **Table 4**, all data are in the range of μg/L, and most of the zebrafish data > invertebrates data > algae data, which could be because of aquatic food chain effects (Hela et al. 2005). Algae appeared to be the most sensitive taxa for most emerging micropollutants, which is in agreement with previous studies (Radix et al. 2000, Schmitt‐Jansen and Altenburger 2005).

RQs for all micropollutants detected are presented in **Figure 4** ranged from 5.65E-06 (carbamazepine in Rhine surface water for zebrafish) to 28.57 (triclosan in Rhine waste water for algae) for individual chemicals and from 2.72 (RQ_{STU} of Danube surface water) to 29.68 ($RQ_{PEC/PNEC}$ of Rhine waste water for algae) for their mixtures. Lines denote the levels of concern (RQ=1). Cyprodinil is reported in the literature as not posing any unacceptable risks to algae (Ardal 2014), and no actual environmental measured concentrations for the Danube River are available. RQs of genistein in Rhine effluent water and surface water were not calculated also because of lack of environmental concentrations data.

For Danube surface water, three out of 10 micropollutants, namely carbamazepine, diazinon and triclosan, reached the concern risk level (RQ=1) to invertebrates and algae (**Figure 4**). These

findings were confirmed by the studies of Hernando et al. and Hela et al., in which carbamazepine and diazinon presented high risk in both effluent water and surface water (Hernando et al. 2006) for zooplankton and fish (Hela et al. 2005). Thus, these three chemicals could be the most hazardous micropollutants in the Danube River, and it should be given more attention in the future chemical regulation program. In particular, RQs of carbamazepine and diazinon in Danube surface water even higher than that in Rhine effluent water, which indicates that the Danube has a heavy load of these two micropollutants. For the Rhine surface water and effluent water, only triclosan was found to exhibit a relatively high risk to algae, which is comparable with that in the U.S., Sweden and Switzerland, where the triclosan RQ ranged within 0.2-6 in surface water and effluent (Samsøe-Petersen et al. 2003). Besides, a retrospective monitoring study reported that triclosan (period 1994-2003 and 2008) and its potential transformation product methyl-triclosan (MTCS; period 1994-2008) were detected at 0.2-3.4 ng/g and 1.0-33.0 ng/g respectively in fish muscle tissue, at 1.0-4.0 ng/g in suspended particulate matter of the Rhine river, which increased until about 2003-2005. This indicates that triclosan is the most priority micropollutant in the Rhine River, which may lead to hazard to the organisms in this river. Diuron was another chemical of note, which was found to pose a medium aquatic risk ($0.1 < RQ < 1$) to algae in all three water resources. These results are in accordance with the findings of Schuler et al., who found that diuron also presented medium aquatic risk to algae in south Florida (Schuler and Rand 2008). Thereby, diuron was found to be a frequently used herbicide both in European and in the U.S, and it could be a common hazard micropollutant (Deng et al. 2012). While no exceedance of the critical RQ of 1 was found for zebrafish exposure, it must be noted that in such complex organisms, molecular and cellular effects can also lead to adverse outcomes that were not investigated using the fish embryo test. Diazinon and diuron

were reported to induce oxidative stress, gene expression changes and behavioural alterations at concentrations below the NOEC (Velki et al. 2017a, Velki et al. 2017b). In addition, bisphenol A showed low risk for all three taxa in the Rhine River, which were still higher than those in the Danube River. Therefore, the industrial chemicals may exhibit lower damage for the aquatic organisms than pesticides and pharmaceuticals. Nevertheless, RQs of diuron, carbamazepine, diazinon, triclosan, cyprodinil and flusilazole in Rhine effluent water were higher than those for surface water but still lower than those for the Danube River, with the exception of diuron. The possible reason may be the biotransformation with the micro-organisms in the effluent (Stasinakis et al. 2009), which may be helpful for the further wastewater treatment plants (Ruel et al. 2012).

As **Figure 4** shows, $RQ_{PEC/PNEC}$s values of the micropollutant mixture were above the threshold value of 1 in Danube surface water, Rhine surface water and Rhine effluent water (Figure 3). A similar phenomenon has previously been reported in the antibiotics mixture study, where an RQ higher than 1 was found for the mixture in wastewater effluents where mixture components showed low effect levels in photosynthetic aquatic organisms (González-Pleiter et al. 2013). According to the calculation principle of mixture RQ, the RQ of the mixture in the current study should be calculated by the sum of the toxic units RQ_{STU}. As shown in **Figure 4**, the mixture RQ_{STU} values ranged between 2.72 and 29.40, with an order as follows: $RQ_{Danube} < RQ_{Rhine\ surface\ water} < RQ_{Rhine\ waste\ water.}$ In other words, the investigated micropollutants in the current study induced the highest risk in the Rhine waste water, which is contrary to the conclusion of single micropollutant assessments. Based on the study of Backhaus and Faust, consideration of IA should be made if the RQ_{STU} is above the threshold of 1, which is consistent with the above interaction effects analysis that toxicity of the 10 studied micropollutant mixture might be

induced by dissimilar actions **(Figure 3)**. This indicates that the mixture risk assessment calculated with $RQ_{PEC/PNEC}$s or RQ_{STU} cannot present the real risk of all different environmental chemical mixtures.

4. Conclusions

Enormous research effort has been directed towards the assessment of implications of micropollutants in the aquatic environment. The results from the zebrafish embryo toxicity test demonstrated that all 10 emerging micropollutants were capable of causing mortality on zebrafish embryos. A combination of zebrafish embryo assay, the prediction model of independent action (IA) and risk assessment of RQ indicates that the toxic effects of the micropollutants mixture might be induced by dissimilar actions. This finding could be helpful for the development of mixture toxicity models and modifying guidelines of environmental risk assessment. RQs of the micropollutant mixtures were above the threshold value of 1 in Danube surface water, Rhine surface water and Rhine effluent water, indicating a potential ecological risk for the aquatic organisms. Single chemicals being exposed to several aquatic organisms for risk assessment is difficulty to reflect the overall implications of micropollutants. The mixture analysis allows effects of interactions to be included, which could facilitate to predict their potential hazards. While in consideration of the complexity of environment mixtures and their interactions, a concerted effort is required to develop more intelligent and systematic approaches for mixture-based risk assessment.

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Figure list

Figure 1. Individual concentration-response curves for the zebrafish embryo mortality of 10 emerging chemicals. The experimental data depicts lethal toxicity endpoint at 48hpf zebrafish embryo (1st replicate: red triangle; 2nd replicate: orange triangle; 3rd: purple rhombus). The regression curves (black lines) are shown with their 95 % confidence intervals (dashed lines), in which the top and bottom of the curve was set to 0% and 100%, respectively.

Figure 2. Known toxic actions of each micropollutant. a. (Hilliard et al. 1998), (Takahashi et al. 2001), (Chen et al. 2002); b. (Nassef et al. 2010) (Gottfried et al. 2013) (Bort et al. 1999, Laine et al. 2007) (Gröner et al. 2015) (Krishnamoorthy et al. 1998); c. (Hayes 1982) (Bank 1995, Chauhan et al. 1998, Saxena et al. 2004);d. (Hamm et al. 2001) (Vigfusson et al. 1983) (Teimouri et al. 2006) (Manabe et al. 2006); e (Binelli et al. 2009, Ciniglia et al. 2005, Foran et al. 2000, Schuur et al. 1998); f. (Beketov and Liess 2008, Estève et al. 2009, Fang et al. 2013, Go et al. 2015); g. (Farag and Ibrahim 2007, Ozakca and Silah 2013); h. (Bekaert et al.) (Egan et al. 2009), i. (Fritz et al. 1998, Zhang et al. 1999) (Spinozzi et al. 1994)

Figure 3. Predicted and observed mortality of the micropollutant mixture in zebrafish embryo test. Mixture composed of triclosan, cyprodinil, genistein, penconazole, flusilazole, diazinon, diuron, diclofenac, bisphenol A and carbamazepine. Prediction curves were derived from CA and IA. Experiment data are presented as mean± standard deviation of three independent fish embryo assay. Dashed lines denote the 95 % confidence intervals of the experimental data.

Figure 4 Ecological risk of each micropollutants by risk quotients and their mixtures by summing up the single micropollutant PEC/PNEC ratios ($RQ_{PEC/PNEC}$) and the sum of the toxic units ($RQ_{STU)}$ in Danube and Rhine

Table 1 Summary of the selected test chemicals, their proposed annual average concentration environmental quality standard (AA-EQS) and maximum hazard quotient in Busch et al. (Busch et al. 2016) given in this study.

Chemicals	CAS No.	Molecular Weight (g/mol)	Chemical use group	Annual Average Environmental Quality Standards (µg/L)	Maximum hazard quotient(Busch et al. 2016)
Bisphenol A	80-05-7	228,28	Industrial Chemical	0.24[a]	2.10×10^{-2}
Diclofenac	15307-86-5	296,148	Pharmaceutical	0.05[a]	6.77×10^{-2}
Diuron	330-54-1	233.1	Herbicide	0.07[a]; 0.2[b]	7.06×10^{-1}
Carbamazepine	298-46-4	236.28	Pharmaceutical	2.0[a]	1.48×10^{-3}
Penconazole	66246-88-6	283.06	Pharmaceutical	8.6[a]	3.51×10^{-4}
Diazinon	333-41-5	304.35	Insecticide	0.012[a]	4.67×10^{0}
Triclosan	3380-34-5	289.55	Biocide	0.02[a]	4.22×10^{-1}
Cyprodinil	121552-61-2	225.29	Fungicide	0.33[a]	5.34×10^{-3}
Flusilazole	85509-19-9	315,39	Fungicide		1.29×10^{-2}
Genistein	446-72-0	270.24	Phytoestrogen		6.13×10^{-4}

[a](Commission 2011); [b](Oekotoxzentrum 2017)

Table 2. The LC_{10}, LC_{20} and LC_{50} values of individual emerging micropollutants in the zebrafish embryo mortality test. RM: regression model; Tet1 and Tet2 are two concentration effect parameters: location and slope; LC10, LC20 and LC50 are the concentrations that induced lethal effects on 10% tested embryos, 20% tested embryos and 50% tested embryos, respectively.

Compound	Regression model			Dose effect parameters (mg/L)		
	RM	Tet1 (location)	Tet2 (slope)	LC_{10}	LC_{20}	LC_{50}
Bisphenol A	Logit	34.59	8.23	7.77	9.75	14.37
Diclofenac	Logit	24.23	5.50	4.64	6.52	11.64
Diuron	Logit	26.17	5.96	4.02	5.51	9.41
Carbamazepine	Weibull	3.65	1.10	1.03	4.94	53.05
Penconazole	Logit	21.53	4.69	2.46	3.66	7.24
Diazinon	Weibull	22.38	4.97	3.33	4.71	7.97
Triclosan	Logit	30.99	5.73	0.47	0.65	1.14
Cyprodinil	Logit	21.96	4.16	0.35	0.55	1.19
Flusilazole	Logit	25.64	5.58	3.20	4.47	7.92
Genistein	Weibull	25.46	5.17	1.17	1.64	2.71

RM: regression model; Tet1 and Tet2 are two concentration effect parameters location and slope; LC_{10}, LC_{20} and LC_{50} are the concentrations that induced lethal effects on 10% tested embryos, 20% tested embryos and 50% tested embryos, respectively.

Table 3. Composition of mixtures in the test (concentrations and mixture ratio)

Compound	Concentrations(in the test mixture)		Mixture ratio (fraction)
	mg/L	mol/L	10-compounds Mixture
Bisphenol A	14.37	6.30E-05	0.24
Diclofenac	11.64	3.93E-05	0.15
Diuron	9.41	4.04E-05	0.15
Carbamazepine	6.25	2.65E-05	0.10
Penconazole	7.24	2.55E-05	0.10
Diazinon	7.97	2.62E-05	0.10
Triclosan	1.14	3.93E-06	0.01
Cyprodinil	1.19	5.26E-06	0.02
Flusilazole	7.92	2.51E-05	0.09
Genistein	2.71	1.00E-05	0.04

Table 4. Measured environmental concentration (MEC) and predicted no effect concentration (PNEC) values of 10 micropollutants

	MEC (ng/L)					
	Concentration in Danube	Concentration in Rhine		PNEC (ng/L)		
Chemicals	Surface water	Surface water	Waste water	Zebrafish	Algae	Invertebrate
Bisphenol A	68.00[a]	410.00[b]	1.60[c]	14.37	3100.00[d]	15400.00[e]
Diclofenac	4.44[f]	1.00[c]	5.00[c]	11.64	49200.00[g]	15200.00[g]
Diuron	2.10[f]	1.00[c]	5.00[c]	9.41	8.00[h]	6330.00[i]
Carbamazepine	31.90[f]	0.30[c]	6.00[c]	53.05	17500.00[j]	25.00[j]
Penconazole	-[k]	1.00[c]	4.00[c]	1.56	3620.00[h]	674000.00[i]
Diazinon	11.87[f]	0.50[c]	2.00[c]	7.97	10.00[k]	920.00[g]
Triclosan	1.74[f]	4.00[c]	40.00[*]	1.14	1.40[l]	180.00[l]
Cyprodinil	n.d	0.50[c]	3.30[c]	1.19	-[m]	690.00[n]
Flusilazole	9.30[o]	1.00[c]	10.00[*]	7.92	15000.00[p]	270.00[q]
Genistein	31.35[f]	-[r]	-[*]	2.71[r]	24439000.00[i]	23767000.00[i]

a:(Loos et al. 2010).b: (Fromme et al. 2002). c: (Peta A. Neale). d: (Dorn et al. 1987).e: (Dorn et al. 1987). f:(Neale et al. 2015).g: (Cleuvers 2004).h: (Ricart et al. 2009).i: (Tixier et al. 2000).j: (Ferrari et al. 2004).k: (Chitescu et al. 2015, Purdešová et al. 2013). h: (Durjava et al. 2013).i: (Agency).g: (González-Pleiter et al. 2013).k: (Butler et al. 1975).l: (Capdevielle et al. 2008).m: (Ardal 2014). n: (Beketov and Liess 2008). O: (Chitescu et al. 2015). p: (Bedil et al. 2015).q: (Suárez-Serrano et al. 2010). r : (Hoerger et al. 2009). n.d: no available data; -: not detected; [*]: Predicted environmental concentrations (PECs) were estimated from the surface water concentrations (assuming 10-fold enrichment) (Polesel et al. 2014).

Toxicity of 10 organic micropollutants and their mixture: Implications for aquatic risk assessment

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Highlights (85 characters) :

- All 10 micropollutants were capable of causing toxicity on zebrafish embryos (78characters)
- An independent action model of the mixture was found by an integrative approach (81 characters)
- The micropollutants pose a potential risk in the Danube River and Rhine River (79 characters)

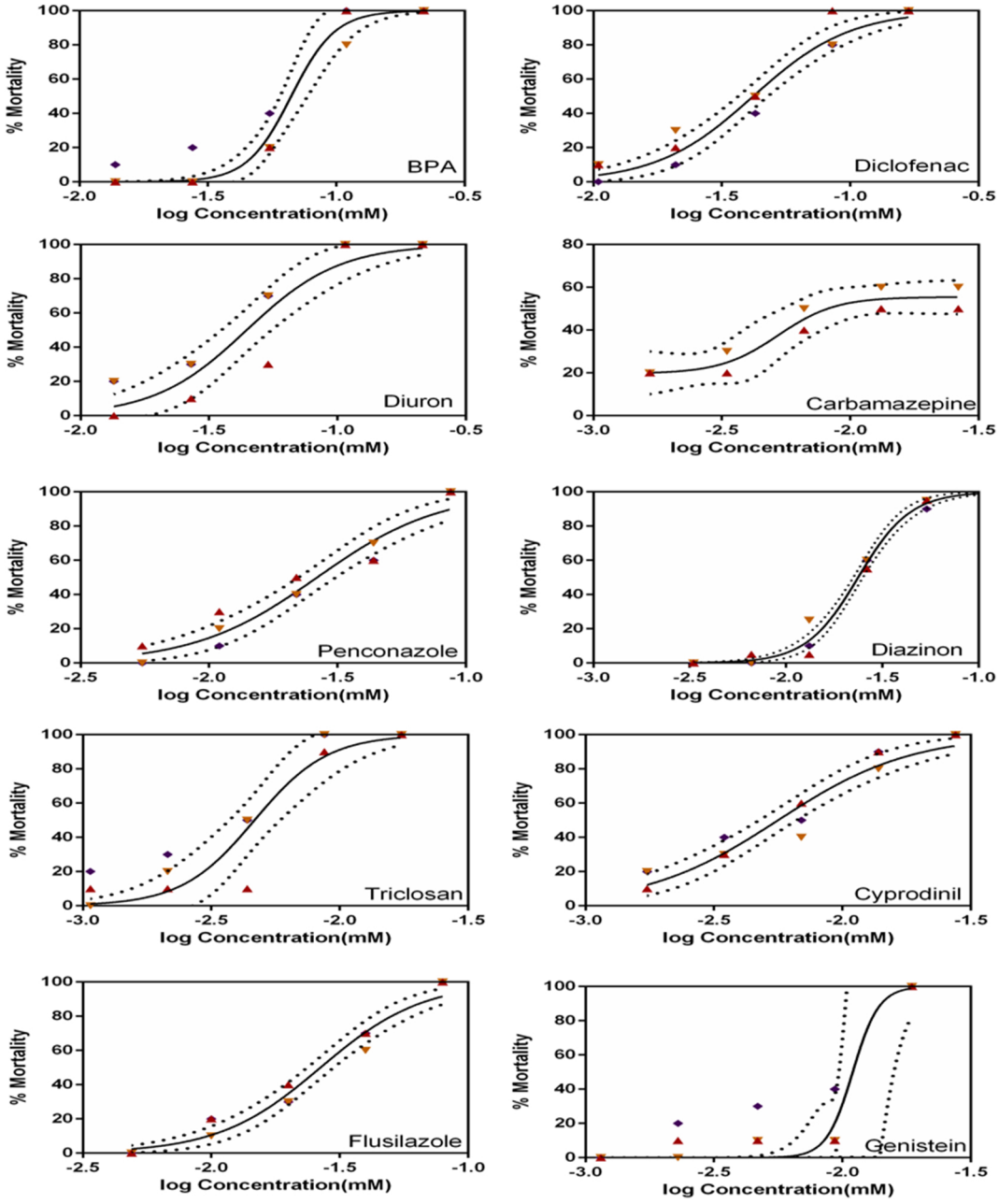
% Mortality
log Concentration(mM)
BPA
Diclofenac
Diuron
Carbamazepine
Penconazole
Diazinon
Triclosan
Cyprodinil
Flusilazole
Genistein

Figure 1

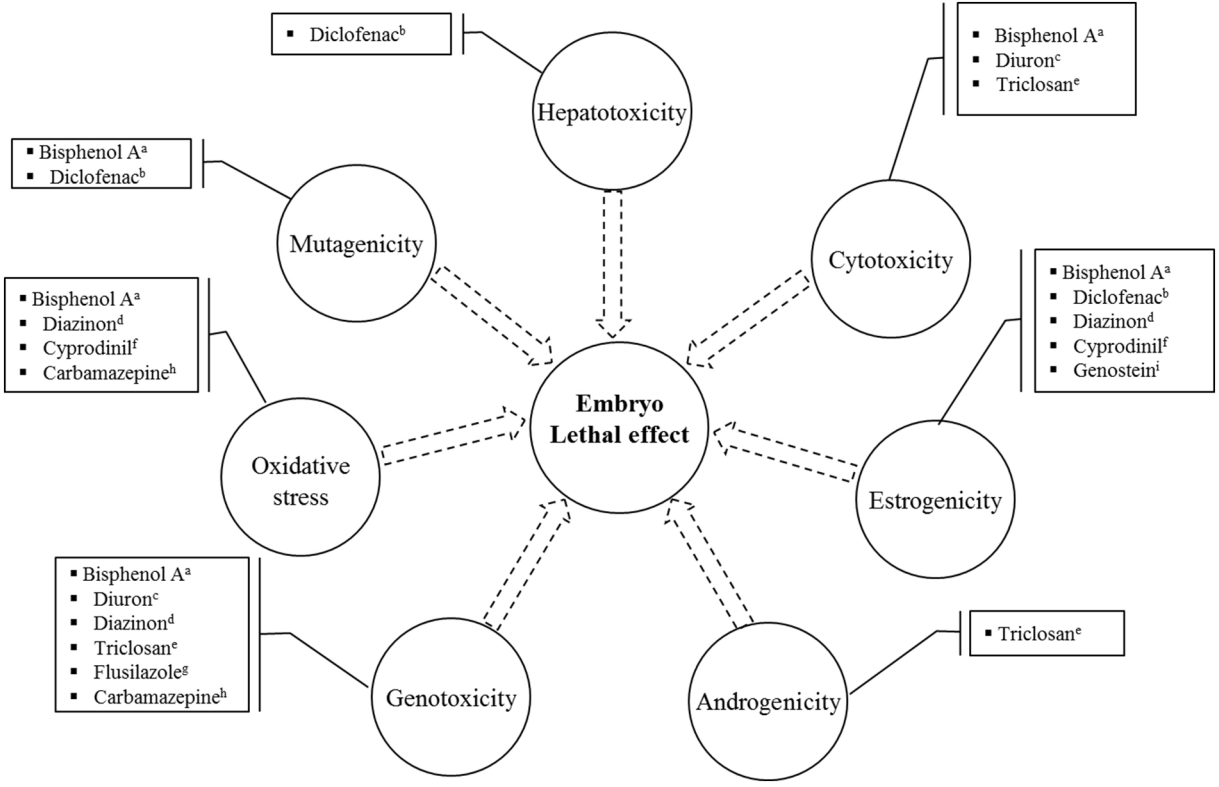
Diclofenac[b]
Hepatotoxicity
Bisphenol A[a]
Diuron[c]
Triclosan[e]
Bisphenol A[a]
Diclofenac[b]
Mutagenicity
Cytotoxicity
Bisphenol A[a]
Diazinon[d]
Cyprodinil[f]
Carbamazepine[h]
Bisphenol A[a]
Diclofenac[b]
Diazinon[d]
Cyprodinil[f]
Genostein[i]
Embryo
Lethal effect
Oxidative
stress
Estrogenicity
Bisphenol A[a]
Diuron[c]
Diazinon[d]
Triclosan[e]
Flusilazole[g]
Carbamazepine[h]
Triclosan[e]
Genotoxicity
Androgenicity

Figure 2

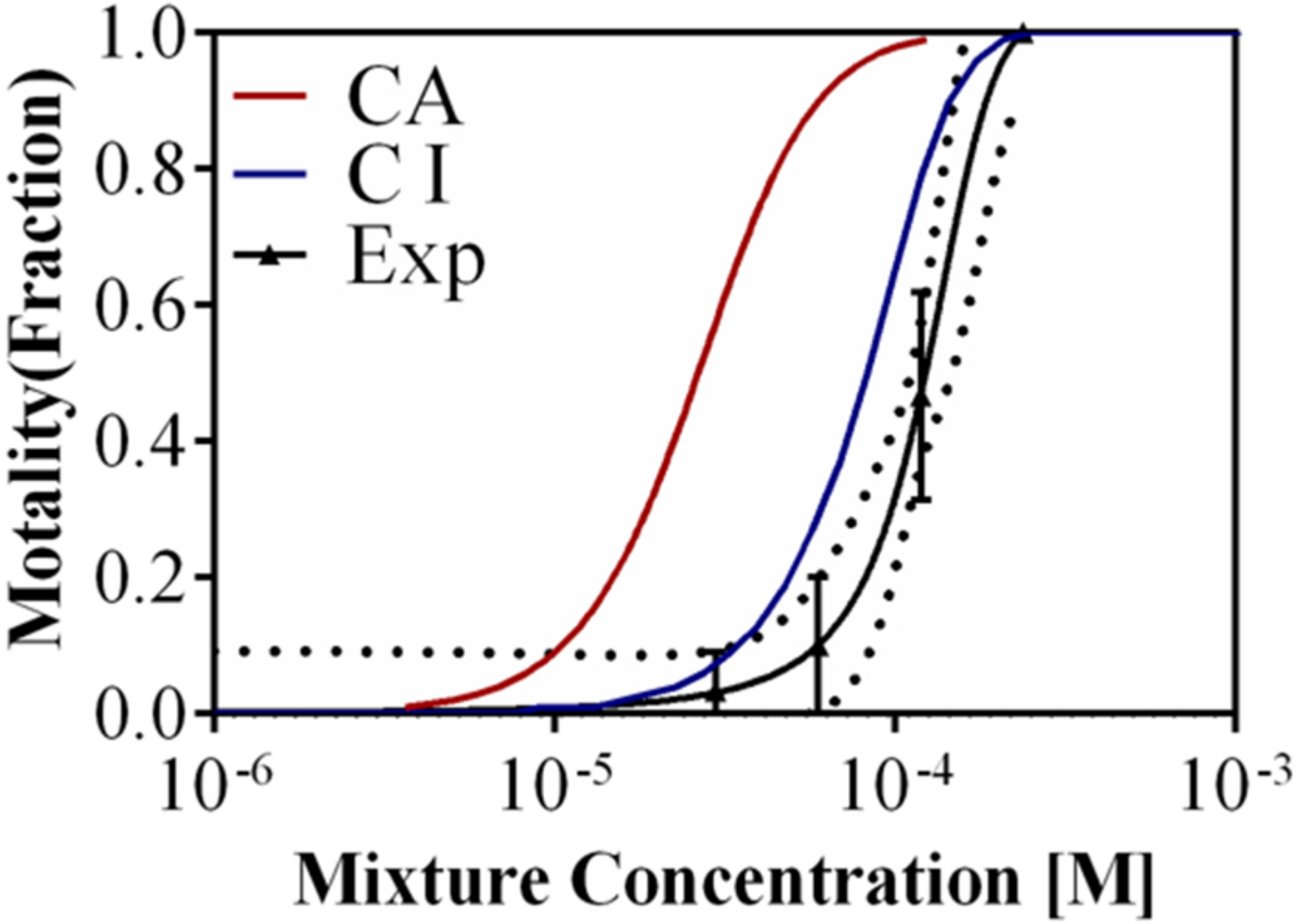
1.0
0.8
0.6
0.4
0.2
0.0
Motality(Fraction)
CA
C I
Exp
10-6
10-5
10-4
10-3
Mixture Concentration [M]

Figure 3

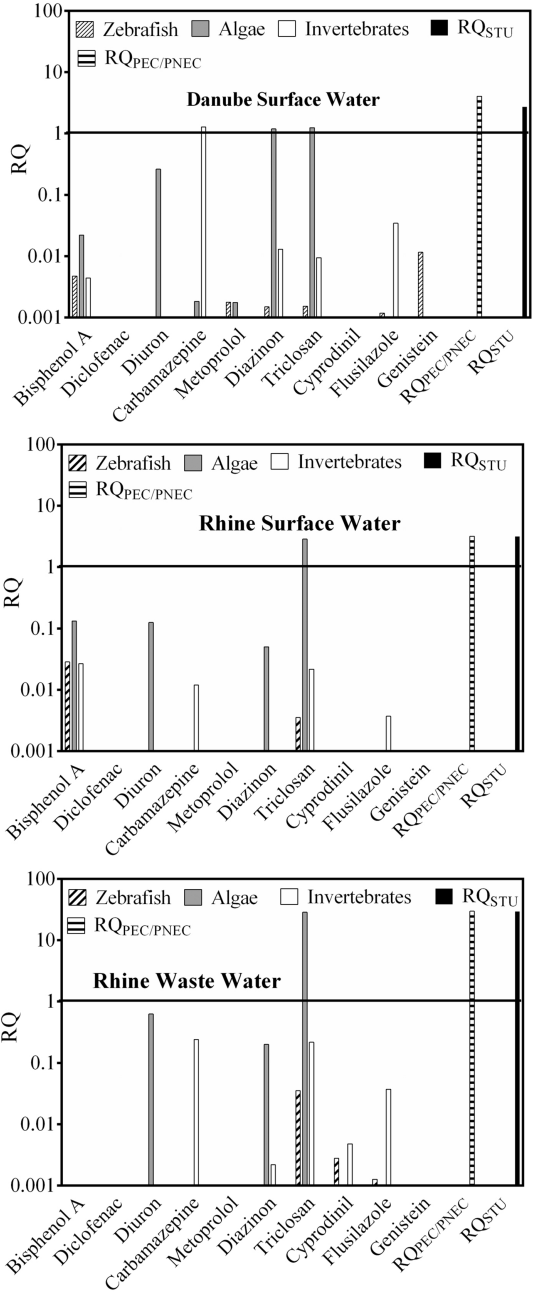
Zebrafish
Algae
Invertebrates
RQSTU
RQPEC/PNEC
Danube Surface Water
RQ
100
10
1
0.1
0.01
0.001
Bisphenol A
Diclofenac
Diuron
Carbamazepine
Metoprolol
Diazinon
Triclosan
Cyprodinil
Flusilazole
Genistein
RQPEC/PNEC
RQSTU
Zebrafish
Algae
Invertebrates
RQSTU
RQPEC/PNEC
Rhine Surface Water
RQ
100
10
1
0.1
0.01
0.001
Bisphenol A
Diclofenac
Diuron
Carbamazepine
Metoprolol
Diazinon
Triclosan
Cyprodinil
Flusilazole
Genistein
RQPEC/PNEC
RQSTU
Zebrafish
Algae
Invertebrates
RQSTU
RQPEC/PNEC
Rhine Waste Water
RQ
100
10
1
0.1
0.01
0.001
Bisphenol A
Diclofenac
Diuron
Carbamazepine
Metoprolol
Diazinon
Triclosan
Cyprodinil
Flusilazole
Genistein
RQPEC/PNEC
RQSTU

Figure 4