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Behavioral profile alterations in zebrafish larvae exposed to environmentally relevant concentrations of eight priority pharmaceuticals

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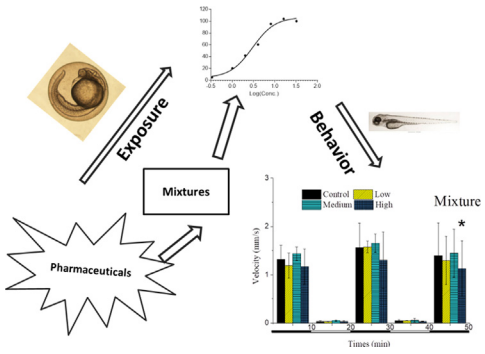
HIGHLIGHTS

- Behavioral alterations are suitable to show the effects of pharmaceuticals.
- 0.1 μg/l triclosan caused accelerated erratic movement in larvae.
- Mixture at the environmental-related concentrations caused decreased swimming speed.

GRAPHICAL ABSTRACT



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ABSTRACT

Although the effects of pharmaceuticals on aquatic organisms have been widely investigated during the last decades, toxic effects, especially delayed toxicity, during the developmental stage at environmental relevant concentrations were rarely known. In this study, a sensitive assay based on behavioral alterations was used for studying the delayed toxicity during the developmental stage on zebrafish embryos. Eight pharmaceuticals that were frequently detected with concentrations ranging from ng/l to μg/l were screened for this study. Behavioral alterations of zebrafish at 118 hpf (hours post fertilization) after exposing to eight single pharmaceuticals with concentrations in the ranges of environmental detected and their mixtures during embryonic development (2–50 h post fertilization, hpf) were observed. Multiple endpoints, including mortality, hatching rate, swimming speed and angular velocity were evaluated. Results showed that behavioral profile alterations in zebrafish larvae are promising for predicting delayed sublethal effects of chemicals. Delayed hatch was observed at 72 hpf following embryonic exposure to triclosan (1 μg/l) and carbamazepine (100 μg/l) up to 50 hpf. The zebrafish larval locomotor behavior following embryonic exposure to 0.1 μg/l triclosan and 1 μg/l caffeine in the early stages of

Abbreviations: DMSO, dimethyl sulfoxide; FET, Fish Embryo Test; LOEC, lowest observed effect concentration.

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development (2–50 hpf) was altered. Furthermore, the effects of the mixture of 8 pharmaceuticals each with the highest environmental concentration on larval behavior were observed during embryonic development. Generally, this study showed that the effects of pharmaceuticals singly or their mixtures in surface waters cannot be ignored.

1. Introduction

Globally, pharmaceuticals are produced and used each year. A proportion of pharmaceuticals and their metabolites finally are released into the environment through a variety of pathways (Jaffrézic et al., 2017; Desbiolles et al., 2018). Consequently, pharmaceuticals are given priority in regulatory water quality monitoring (Di Paolo et al., 2016) and have been observed in surface waters at concentrations ranging from ng/l to μg/l (Hughes et al., 2013; aus der Beek et al., 2015; Hossain et al., 2018; Fekadu et al., 2019).

Although the concentrations of pharmaceuticals in surface waters are unlikely to result in lethal toxicity, the sublethal effects of emerging pollutants on locomotor activities, feeding and reproduction of non-target species are an increasing concern (Brodin et al., 2013; Di Paolo et al., 2015; Tousova et al., 2017). Investigating changes in behavior gradually becomes a widely accepted method for studying the sublethal effects of compounds on non-target aquatic organisms (Tiedeken and Ramsdell, 2009; Ali et al., 2012; Legradi et al., 2015). Zebrafish (*Danio rerio*) is employed as a vertebrate model for investigating toxicological effects on behavior and neurodevelopment (Rihel et al., 2010; Padilla et al., 2011; Chen et al., 2017; Velki et al., 2017; Gauthier and Vijayan, 2018; Michelotti et al., 2018). Zebrafish embryos are small and transparent. The larvae hatch from chorion at 2 or 3 days post fertilization (dpf), and all major organs are well developed at 5 dpf (De Esch et al., 2012). These characteristics of zebrafish determine that behavioral assessment can be carried out during a short period with a small volume of test medium.

Visual motor response has already been used for assessing effects of chemical compounds on zebrafish behavior (Nüßer et al., 2016; Chen et al., 2017; Velki et al., 2017). Zebrafish are phototactic, and thus an alternating dark-and-light period enables the detection of behaviors under different stress scenarios (Legradi et al., 2015). During recent years, multi-endpoints, including swimming speed (Xia et al., 2017), moved distance (Chen et al., 2017; Velki et al., 2017; Michelotti et al., 2018), angular velocity (Michelotti et al., 2018), thigmotaxis preference (Schnörr et al., 2012) and feeding performance (Nassef et al., 2010) have been used in previous studies for fish behavioral assessment, and changes in these endpoints are correlated with external stress.

Based on measured environmental concentrations of pharmaceuticals and the frequencies of their concentrations over predicted no effects levels in European surface waters, eight pharmaceuticals from five therapeutic categories with potential environmental risks were screened for zebrafish behavioral study. These pharmaceuticals included the three antimicrobials clarithromycin, triclosan and sulfamethoxazole, the two anti-inflammatories ibuprofen and diclofenac, the anticonvulsant carbamazepine, the lipid-lowering agent bezafibrate and the stimulant caffeine.

Delayed toxicity as a special case of chronic toxicity was observed with marine flatfish sole (Foekema et al., 2008, 2014) and freshwater zebrafish (Di Paolo et al., 2015; Lovato et al., 2016) during early-stage embryonic development. But, to our knowledge, the delayed effect of pharmaceuticals on fish species is still unknown. Furthermore, compounds often occur in the natural environment as complex mixtures rather than single alone, thus the knowledge of mixture cannot be excluded (Altenburger et al., 2015, 2018; Brack et al., 2017, 2018).

The first aim of this study was to characterize the occurrence of delayed effects on zebrafish larvae up to 118 hpf after exposure to the pharmaceuticals during the embryonic development (2 to 50 hpf) followed by transference to clean artificial freshwater. The second aim of this study was to investigate the combined effects of the selected pharmaceuticals as mixtures under three different scenarios (worst, medium and best-case). Considering that the effects of the compounds could not be completely evaluated by one single endpoint assessment, we analyzed several endpoints (e.g., mortality, hatching rate, swimming speed and angular velocity).

2. Materials and methods

2.1. Test pharmaceuticals

The 8 pharmaceuticals (ibuprofen, diclofenac, caffeine, carbamazepine, clarithromycin, sulfamethoxazole, bezafibrate and triclosan) were supplied by Sigma-Aldrich (Deisenhofen, Germany). Stock solutions of tested pharmaceuticals were prepared in dimethyl sulfoxide (DMSO). The relevant physicochemical characteristics of the test pharmaceuticals are presented in Table 1. Diclofenac and clarithromycin have low solubility in water. In order to observe the potential effects of pharmaceuticals, diclofenac and clarithromycin were tested using a maximum concentration without precipitation observed in DMSO-water miscible systems. The other pharmaceuticals were tested at concentrations below the solubility in water at 20 °C.

2.2. Mixtures

Mixtures of 8 pharmaceuticals at three concentration levels simulating worst, medium and best-case scenarios were designed. Concentrations were based on the highest measured concentrations in European surface waters (Table 2). In the synthetic mixture M1, the concentration of each compound was similar to the highest reported concentration in European surface waters, which was regarded as worst-case scenario in this study. In the mixture M2, the concentration of the each component was 10 times lower than the highest measured concentrations, and close to or little higher than the mean concentrations, representing the medium-case scenario. In the mixture M3 (best-case), the concentrations of the compounds were 100 times lower than the highest measured concentrations and similar or lower than the mean concentrations, representing the best-case scenario.

2.3. Fish embryo acute toxicity test up to 48 hpf

The zebrafish embryo acute toxicity test up to 48 hpf was applied to assess the lethal toxicity of the test compounds and their mixtures. Zebrafish eggs utilized in this study were produced at our own facilities by a wild-type zebrafish strain from West Aquarium GmbH (Bad Lauterburg, Germany). The fish rearing and acute toxicity test protocols were followed as described in the FET (Fish Embryo Test) OECD guideline (OECD, 2013) with slight modifications. Briefly, viable fertilized eggs at 2 hpf (64-cell stage) were exposed individually in 200 μl/well to freshly prepared test solutions in artificial freshwater in 96-well plates. Five nominal concentrations of each compound (dilution ratio 1:2) were used for LC_{50} determination. The maximum concentrations applied for the individual tests are shown in Table 2. For quality assurance an artificial freshwater control, a solvent control (0.5% DMSO), and a positive control (4 mg/l 3,4-dichloroaniline) were also performed

Table 1
Physicochemical characteristics of the 8 priority pharmaceuticals for European surface waters selected for behavioral tests in this study.

Compound	CAS	Mode of action	Log Kow	Solubility[a] (mg/l)	Highest test concentration (mg/l)
Ibuprofen	15687-27-1	Antiinflammatory	3.97	21	12.5
Diclofenac	15307-86-5	Antiinflammatory	4.51	2.37	50
Caffeine	58-08-2	Neuroactive	−0.07	21,600	100
Carbamazepine	298-46-4	Anticonvulsant	2.45	18	6.25
Clarithromycin	81103-11-9	Antibiotic	3.16	1.693	12.5
Sulfamethoxazole	723-46-6	Antibiotic	0.89	610	100
Triclosan	3380-34-5	Antibiotic	4.76	6.05	5
Bezafibrate	41859-67-0	Lipid-lowering agent	4.25	–	6.25

[a] LogKow and water solubility values were acquired from PubChem database. Water solubility of the bezafibrate was unknown.

in each experiment, and ten embryos were exposed per each test concentration or control condition. After 48 h of exposure at 26 ± 0.5 °C in the incubator under a 14 h light: 10 h dark cycle, lethal endpoints were recorded according to the guideline. Acute toxicity values obtained at 48 h were the fundamental for behavioral test that was observed without lethal toxicity. The mortalities of positive controls were higher than 40%, and all experiments were repeated independently three times.

2.4. Delayed effects followed by transference to clean water up to 118 hpf

For the observation of delayed effects, namely hatching rate and behavioral tests, the exposure to the single compounds and mixtures was done up to 50 hpf, followed by transference of embryos to clean water up to 118 hpf. Hatching rate was observed daily and measurement of behavioral endpoints were carried out at 118 hpf. For each single pharmaceutical, five nominal concentrations (0.01, 0.1, 1, 10 and 100 μg/l) and one artificial freshwater control were tested. For the pharmaceutical mixtures, three concentration levels as described above and the artificial freshwater control were tested. There was no significant difference ($p > 0.05$) between behavioral profiles of larvae which were maintained up to 50 hpf in the solvent control condition (DMSO 0.1%) and the profiles of those larvae kept in artificial freshwater. For each test condition, 20 normally developed embryos at 2 hpf were exposed in glass beakers containing 40 ml of the respective exposure solution with final 0.1% DMSO, except for the negative control which contained artificial freshwater only. Chemical exposure solutions were prepared by diluting stock solutions containing 0.01, 0.1, 1, 10 and 100 mg/l of each compound in DMSO to 0.01, 0.1, 1, 10 and 100 μg/l in the exposure medium, respectively. The pH of all solutions was measured and then the glass beakers were sealed with parafilm to minimize evaporation. After 48 h of exposure, the solutions were removed, and the embryos were gently washed three times with 20 ml artificial freshwater. Finally, 40 ml of artificial freshwater were refilled into the glass beakers. During the whole exposure period, exposure vessels were observed every 24 h. The hatching rate was recorded, and dead embryos or larvae were noted and removed immediately. Larvae exhibiting severe malformations such as curved body axis were excluded from the behavioral analysis to avoid the interference of morphological effects. The total numbers of dead or malformed larvae accounted for <10% in all conditions. There was no significance between the treated groups and the controls in embryo mortalities or morphology defects. At 117 hpf, for each condition 16 larvae were transferred into 96-well microtiter plates as one larva in 300 μl of artificial freshwater per well. At 118 hpf, after 5 min of acclimation in the dark in the observation chamber DanioVision (Noldus, Netherlands), video recording of the behavior began in the dark, followed by 10-minute alternated light and dark periods for a total of 50 min at 26 °C. The test of behavior was carried out in a quiet environment to exclude external disturbance. In total, 48 larvae from three replicates at each concentration level for each pharmaceutical or their mixture were analyzed. The imaging of larval locomotion was analyzed with the EthoVision XT 10 software package (Noldus Information Technology, USA) with evaluation of the swimming speed and the angular velocity. Swimming speed is an indicative of mobile activity and absolute angular velocity based on the heading of the center-point reflects rotation activity of larvae, thus the changes in any of two endpoints are correlated with external stress. Zebrafish larvae below 120 hpf are not protected animal stages according to EU Directive 2010/63/EU (European Union, 2010) and TierSchG (Tierschutzgesetz) and the respective regulation TierSchVersV (Tierschutz-Versuchstierverordnung). No animal test authorization was required for our experiments, since the final measurements were terminated before 120 hpf. At the end of the experiments the larvae were euthanized by a prolonged immersion in a benzocaine solution of 4 g/l in ethanol. Since larvae showed greatly individual variations in locomotion response, extra 3 replicates for high concentrations (1, 10 and 100 μg/l) of each pharmaceutical (i.e., in total 6 replicates) were tested to certify the potential effects, data were not shown in this study.

2.5. Statistical analysis

For the acute toxicity tests, 48 h LC_{50} values (expressed in mg/l) were determined by four parameter logistic regression based on the grouped results for mortality endpoint assessment obtained from

Table 2
Composition of mixtures and concentrations of 8 priority selected pharmaceuticals in European surface waters.

Compound	Mean concentration (μg/l)[a]	Highest concentration (μg/l)[a]	Concentrations in the mixture (μg/l)			Mixture ratio (%)
			High (M1)	Medium (M2)	Low (M3)	
Diclofenac	0.31	18.74	19	1.9	0.19	14.82
Triclosan	0.04	0.22	0.2	0.02	0.002	0.16
Carbamazepine	0.22	11.56	11	1.1	0.11	8.58
Bezafibrate	0.18	15.06	15	1.5	0.15	11.7
Sulfamethoxazole	0.3	11.92	12	1.2	0.12	9.36
Ibuprofen	0.38	31.32	30	3	0.3	23.4
Caffeine	0.75	39.81	40	4	0.4	31.2
Clarithromycin	0.17	1.31	1	0.1	0.01	0.78

[a] The highest and mean concentrations in European surface waters were acquired from our unpublished manuscript Zhou et al. (under revision).

three independent experiments using GraphPad Prism 5 (GraphPad Software Inc., USA). For the behavioral endpoints, the significance of differences was analyzed using SPSS Statistics 17.0 (SPSS Inc., USA). Data were first analyzed using one way ANOVA, followed by a post hoc test (LSD test, two-tailed) to compare between groups. Data were presented as mean ± SEM (standard error of the mean), and a p-level of 0.05 was used as the minimal criterion of significance. Graphs were plotted using Origin 9.0 software (OriginLab Corp., USA).

3. Results

3.1. Acute toxicity of pharmaceuticals

No significant mortality for 48 h of exposure individually to ibuprofen, caffeine, clarithromycin, sulfamethoxazole, and bezafibrate were observed compared with the controls. The most toxic compound was triclosan, with a 48 h LC_{50} value of 1.50 ± 0.48 mg/l for zebrafish embryos, followed by diclofenac with a 48 h LC_{50} of 14.15 ± 0.99 mg/l. The acute toxicity of carbamazepine showed an incomplete concentration-response curve in the range of tested concentrations, with circa 50% of mortality in the highest tested concentration (6.25 mg/l). The three mixtures of the 8 pharmaceuticals did not produce any visible acute toxic effects in embryos up to 48 h.

3.2. Hatching rates at 72 h

Significant reduction of hatching rates was observed at 72 h after exposure for 48 h (up to 50 hpf) to 1 μg/l triclosan ($p < 0.05$). This exposure condition showed only circa 25% of hatched embryos, while circa 60% and between 40 and 65% of embryos were hatched in the water control and in the higher triclosan exposure concentrations (10 and 100 μg/l), respectively (Fig. 1). Also, the embryos exposed to the highest concentration of ibuprofen (100 μg/l) caused significant hatching reduction at 72 hpf ($p < 0.05$, Fig. 1). Hatching rates at 72 hpf after exposure to the other pharmaceuticals or to the mixtures showed no significant differences compared with these of the water controls (data not shown). At 96 hpf, the percentages of hatched larvae in all controls and exposed groups were above 90% and no further statistically significant differences were found between each other.

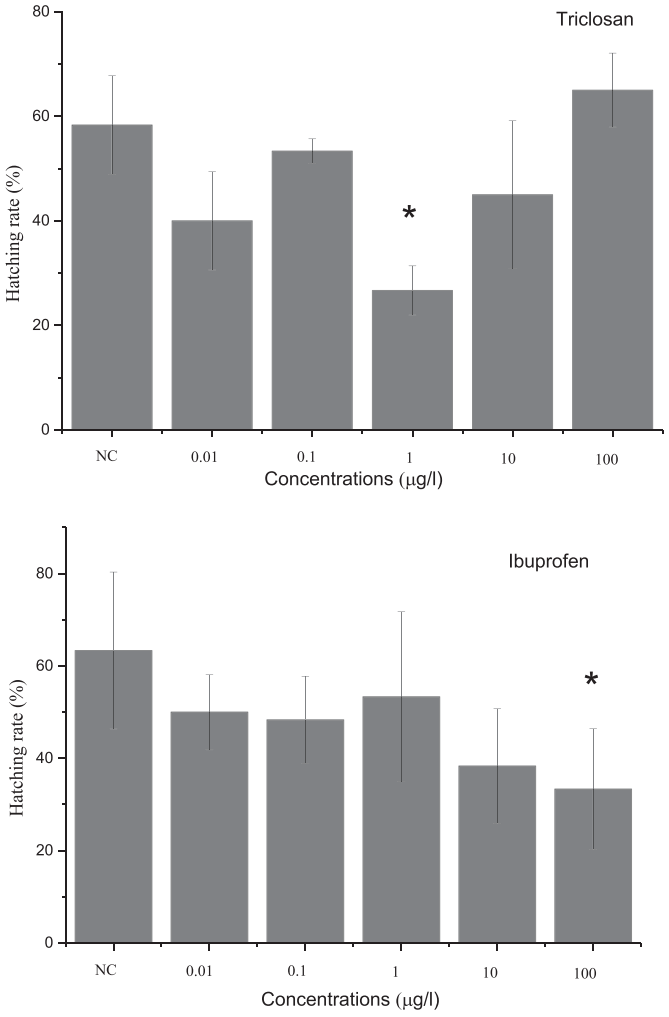

Fig. 1. Percentage of hatched larvae at 72 hpf after 48 h exposure to the triclosan, ibuprofen and negative controls (i.e., water controls). Bars represent the mean values for 3 replicates and error bars stand for SEM. 20 embryos per replicate of each concentration were used for percentage calculation. Values that are significantly different from the control are indicated by asterisks ($p < 0.05$).

3.3. Locomotion patterns after exposure to single compounds and their mixtures

Locomotor activity endpoints, namely swimming speed and angular velocity, were analyzed for 118 hpf zebrafish larvae to detect behavioral alterations and potential neurological impairments caused by the 48 h embryonic exposure to the single pharmaceuticals and their mixtures. The interference of morphological effects (e.g. malformations, edema) on behavioral assessment was avoided by selecting a concentration range which caused no observable lethal or sub-lethal toxicity. For all conditions, the swimming speeds in dark periods were significantly higher ($p < 0.05$) than respective values in light periods (Fig. 2). In contrast, the angular velocity in dark periods was significantly lower ($p < 0.05$) than respective values in light periods (Fig. 3), which means that the larvae under visual conditions prefer to rotate in situ rather than directly move to response to external pressure. In general, high individual variability regarding the swimming speed and angular velocity was observed either in negative controls or in any other case of exposures.

3.3.1. Swimming speed

Considering swimming speed, no significant effects compared to the control were observed for diclofenac (Fig. 2A), bezafibrate (Fig. 2D), sulfamethoxazole (Fig. 2E), ibuprofen (Fig. 2F), and caffeine (Fig. 2G). Triclosan (Fig. 2B), carbamazepine (Fig. 2C), clarithromycin (Fig. 2H) and the mixture under worst-case (Fig. 2M) affected swimming speed during the dark periods, but no significant differences were found during the light periods.

Larvae up to 50 hpf exposed to the highest carbamazepine concentration (100 μg/l) tended to swim faster (1.81 mm/s) than those from water control (1.41 mm/s) during dark periods, with significant differences ($p < 0.05$) in the first dark period. Exposure to the dilution series of clarithromycin (Fig. 2H) produced an inhibitory dose–response pattern, in which increased concentrations caused decreased swimming speed. The swimming speed for 100 μg/l clarithromycin-exposed larvae (0.89 mm/s) was significantly lower ($p < 0.05$) than those presented by larvae from the control condition (1.31 mm/s). Swimming speeds at lower concentrations (0.01, 0.1, 1 and 10 μg/l) were slightly lower (1.29 to 1.07 mm/s) than the control, but no significant differences were found ($p > 0.05$). Although the decreased dose–response pattern was not particularly obvious for triclosan (Fig. 2B), a reduced swimming speed (1.12 mm/s) was presented by the larvae exposed to the highest concentration of 100 μg/l triclosan when compared to the control (1.49 mm/s) during the third dark period ($p < 0.05$). For the mixture-exposed groups, there was no significant difference in swimming speed exposure to the low (M3) and medium (M2) concentration ranges relatively to the control, but a significant decrease ($p < 0.05$) in swimming speed for the larvae treated with the high-level concentration (M1) was found (Fig. 2M).

3.3.2. Absolute angular velocity

Diclofenac, carbamazepine, bezafibrate, sulfamethoxazole, ibuprofen and clarithromycin and mixtures did not exert any significant effects on the absolute angular velocity of the larvae. For zebrafish larvae following embryonic exposure to triclosan (Fig. 3B) a pattern of increasing absolute angular velocities at increasing concentrations occurred at the second and third dark periods. During the second dark period, larvae exposed to 1 and 100 μg/l triclosan performed turning angles higher than respective control ($p < 0.05$). During the third dark period, an increased absolute angular velocity was presented by larvae exposed to 0.1, 1, 10, and 100 μg/l triclosan when compared with control conditions ($p < 0.05$). For caffeine (Fig. 3G), an increased absolute angular velocity, which were significantly higher than the control, was observed at 1, 10, and 100 μg/l during the second and third dark periods ($p < 0.05$). A slight but not significant reduction in absolute angular velocity was observed for larvae exposed to the mixture with the highest concentration range (M1) when compared with control condition.

4. Discussion

4.1. Immediate toxicity to embryos

Among these 8 pharmaceuticals, triclosan, diclofenac and carbamazepine were capable of causing acute toxicity at early embryonic stages. The 48 h LC_{50} values obtained for diclofenac (14.15 mg/l) and triclosan (1.50 mg/l) were in similar range as previously determined values for adult medaka (*Oryzias latipes*) after 96 h exposure (Nassef et al., 2009). It is of relevance to mention that diclofenac and triclosan, having relatively high logKow values (4.5–4.8, Table 1), are known to possibly cause delayed toxicity and have a tendency for bioaccumulation (Daley et al., 2009; Di Paolo et al., 2015). These aspects should be taken in consideration in future studies. Although the LC_{50} value of carbamazepine failed to be detected, acute lethal effects for zebrafish were still observed at the highest tested concentration (6.25 mg/l). The fact that the mixtures did not cause lethality in 48 h embryos suggests that mortality might not be a concern for environmentally representative exposures to pharmaceuticals. Nevertheless delayed or chronic toxicity was still investigated to properly assess the hazard potential of these chemical mixtures.

4.2. Hatching rates and behavioral profiles after transference to clean water

Among four out of eight tested pharmaceuticals, a tendency to increased or decreased locomotion was revealed and a significant difference was observed. However, the differences were not stably occurred during the whole dark periods (Chen et al., 2017; Velki et al., 2017), which was partly attributed to the high individual variations of zebrafish larvae in locomotion response. The variations in behavioral profiles in larval stages are related to modes of action of tested pharmaceuticals, similar results were also found in previous studies (e.g., Drummond and Russom, 1990; Rihel et al., 2010). The antimicrobials triclosan and clarithromycin depressed the locomotor activity as measured by the swimming speed in zebrafish larvae. In contrary the stimulant caffeine accelerated erratic movement as indicated by increased angular velocity, and an increased swimming speed was observed for the anticonvulsant carbamazepine exposure. It is clear that not all of compounds were detected with stress-induced locomotion response, since compounds with a similar mode of action can also have differences in toxicity potency, time to effect onset, and duration of

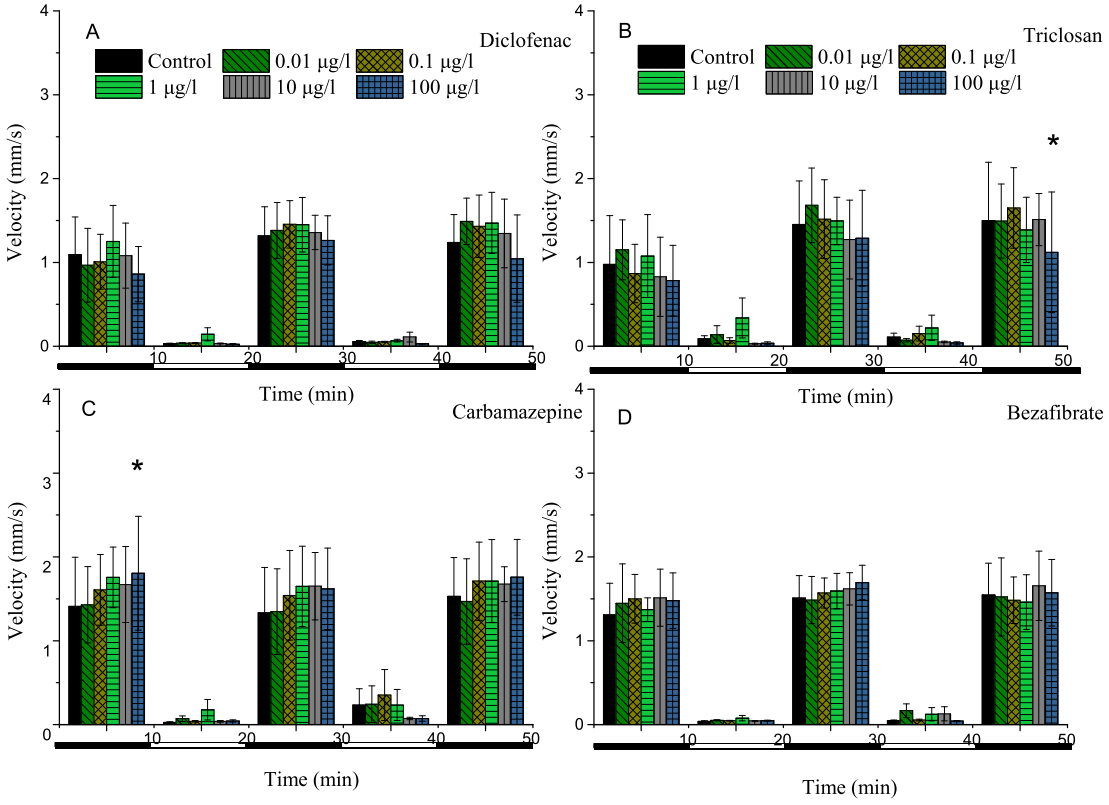

Fig. 2. Swimming speed (mm/s) of 5 dpf larvae in each ten-minute light and dark periods after 48 h embryonic exposure to the single compounds diclofenac (panel A), triclosan (panel B), carbamazepine, (panel C), bezafibrate (panel D), sulfamethoxazole (panel E), ibuprofen (panel F), caffeine (panel G), and clarithromycin (panel H) at 6 concentrations (0, 0.01, 0.1, 1, 10 and 100 μg/l) or to low-, medium- and high-level mixtures (M). The white and black bars at the bottom represent the 10-minute light and dark periods, respectively. Bars represent the mean swimming speed values for 3 experimental replicates ($n = 42–48$) ±SEM (standard error of mean). Asterisks indicate a significant difference from the control in the respective light or dark period ($p < 0.05$).

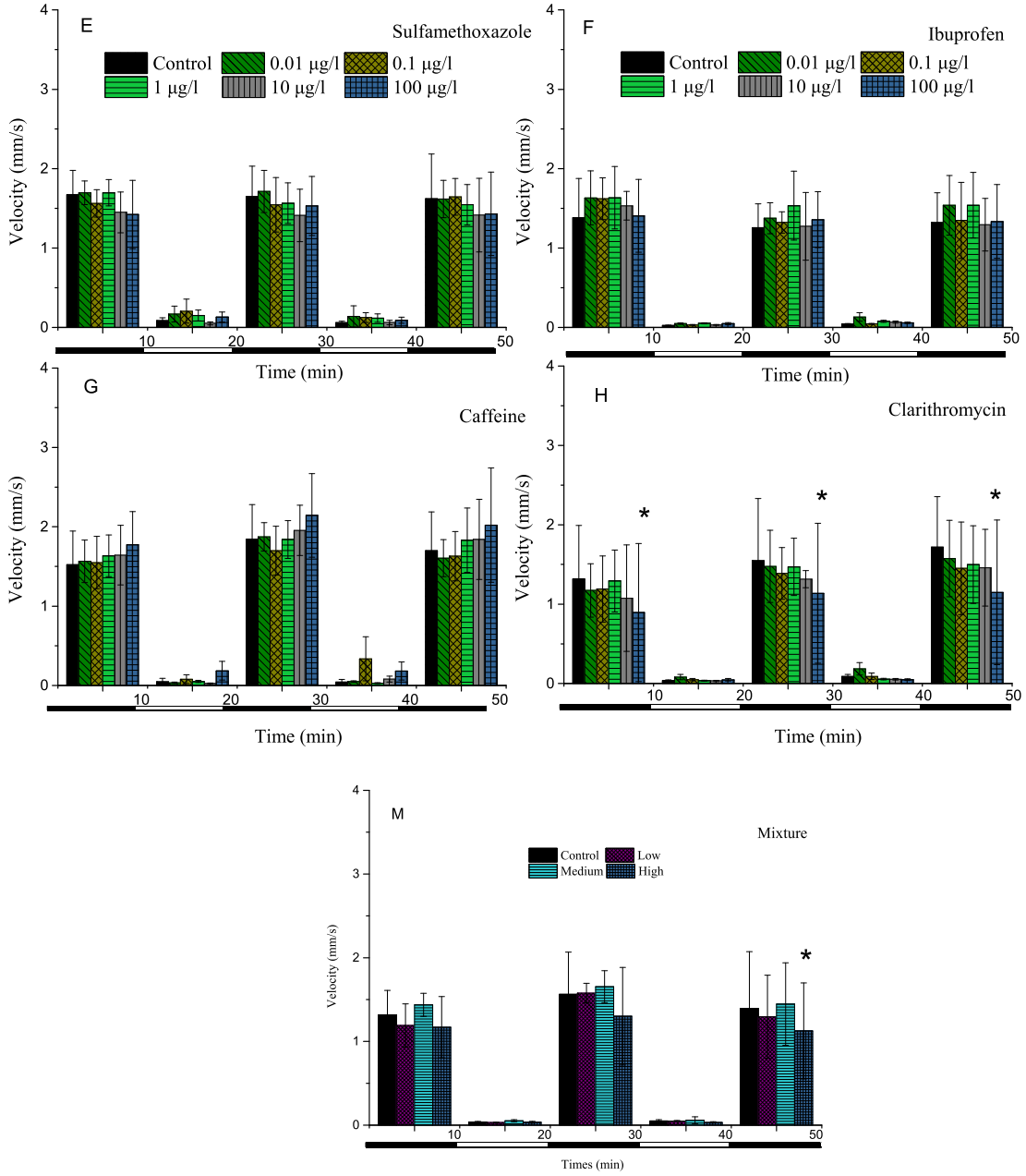

Fig. 2 (*continued*).

effects, inducing even different behavioral effect profiles (Chevalier et al., 2015).

A significant hatching delay occurred at 72 hpf after exposure to ibuprofen (100 μg/l), similar results were reported previously (David and Pancharatna, 2009; Xia et al., 2017). Possible involved mechanisms are related to the fact that the nonsteroidal anti-inflammatory ibuprofen can inhibit cyclooxygenases, which catalyze the biosynthesis of prostaglandin (Cleuvers, 2004) and are necessary for the development of zebrafish embryos (Grosser et al., 2002; Cha et al., 2005). Although no delayed effects of ibuprofen in zebrafish locomotion were observed in this study, immediate effects were previously found for an invertebrate species. For example, acute exposure lasting for 1.5 h to ibuprofen 0.01 μg/l, a concentration lower than the observed mean concentration in European surface waters (0.38 μg/l), resulted in a significant decrease in the activity of amphipoda (*Gammarus pulex*) (De Lange et al., 2006). Therefore, there is indication that ibuprofen can cause delayed hatching in fish and also have immediate effects on other aquatic organisms in the environment.

Both diclofenac and ibuprofen are nonsteroidal anti-inflammatory drugs and have the same mode of action, but no obvious effects of diclofenac on zebrafish were detected in the tested concentration ranges. Similarly, Nassef et al. (2010) found that exposure to 1 mg/l diclofenac for 9 days had no significant effect on the swimming speed of adult medaka fish. Lee et al. (2011) did not observe any effect of 100 μg/l diclofenac on medaka after 3 months exposure from egg phase to adult phase, Moreover, 100 μg/l is greater than the highest detected diclofenac concentration in European surface waters near sewage treatment plants (18.74 μg/l). It is hence unlikely that this drug would pose potential environmental risks for fish species.

The lowest carbamazepine concentration at which significant effects were detected for larval locomotion (100 μg/l) was one order of magnitude higher than the highest concentration detected in European

effluent-influenced surface waters (11.56 μg/l). A decrease in feeding behavior and swimming speed was observed in adult Japanese medaka fish when exposed to 6.15 mg/l carbamazepine (Nassef et al., 2010), which was three orders of magnitude higher than the concentrations detected in surface waters. Previous studies did not show any adverse effect of carbamazepine at environmentally relevant concentrations (De Lange et al., 2006; Guler and Ford, 2010). Thus a preliminary conclusion should be that current carbamazepine alone in surface waters does not represent an urgent risk in terms of behavioral alterations.

For caffeine, a stimulant of the central nervous system, concentration-dependent stimulation of angular velocity and hatching rate were detected. The LOEC (lowest observed effect concentration) for behavior (1 μg/l) was 40 times lower than the maximum concentration detected in European surface waters near wastewater treatment plants (39.81 μg/l), which indicates the potential environmental effects of caffeine on non-target species in aquatic ecosystem. Caffeine is highly water soluble (Log Kow = −0.07) and can be easily eliminated by the liver in adult fish. However, prolonged half-life of caffeine may exist in immature liver of vertebrates (Parsons and Neims, 1981; Hering-Hanit and Gadoth, 2003), and thus absorbed caffeine during zebrafish embryonic developmental stages may not be completely eliminated at 118 hpf due to the immature liver of larvae, and may still cause stimulation of the central nervous system.

Exposure to the antimicrobial triclosan in general caused a tendency for reduced swimming speed with increased exposure concentrations. The effects of triclosan on locomotion behavior were also found in other fish. For example, the swimming speed of adult Japanese medaka was decreased by exposure to 0.17 mg/l triclosan for 9 days (Nassef et al., 2010) and erratic swimming in rainbow trout (*Oncorhynchus mykiss*) was observed upon exposure to 71 μg/l triclosan for 61 days (Orvos et al., 2002). Interestingly, for triclosan, a significant hatching delay was observed at low concentrations (1 μg/l), but no significant effects at higher concentrations (10 and 100 μg/l). This complex non-monotonic concentration-related effect of triclosan on zebrafish early-life stages was also observed in previous studies. For example, Falisse et al. (2017) showed delayed hatching after 72 h exposure to 50 μg/l of triclosan, while no significant hatching delay appeared after exposure to 100 μg/l. Parenti et al. (2019) observed significantly increased activities of catalase (CAT), glutathione peroxidas (GPx), and glutathione S transferase (GST) at 0.1 μg/l triclosan during the 24 h exposure, but no significant increase was shown at higher concentration.

The other two antimicrobials showed different dose–response curves, larvae exposed to clarithromycin showed a reduction in swimming speed and no obvious trend were found for sulfamethoxazole exposure. In fact, despite the similar use the mechanisms of actions are different for these three antimicrobial compounds. Clarithromycin prevents bacteria by inhibiting the translation of peptides, sulfamethoxazole prevents bacteria by interfering with the synthesis of folate, and triclosan prevents bacteria primarily by inhibiting fatty acid synthesis (Finberg et al., 2004; Adzitey, 2015). Therefore changes in behavior pattern probably occur through different mechanisms of action (e.g., avoidance, repellency, neurotoxicity) (Nüßer et al., 2016).

Behavioral effects following exposure of zebrafish to the mixture under the worst scenario (M1) were detected. Generally, the toxicity prediction of a mixture is based on concentration addition for its components with similar modes of action and independent action for its components with dissimilar modes of action (Faust et al., 2001; Altenburger et al., 2004). Although the concentrations of each component in the mixture M1 are lower than the lowest observed effect levels for swimming speed, the mixture M1 still caused decreased swimming activity, which could be explained by combined effects of mixture. Interestingly, for angular velocity the concentrations of two components (i.e. carbamazepine and caffeine) in mixture M1 were higher than the respective lowest observed effect levels for angular velocity, but no

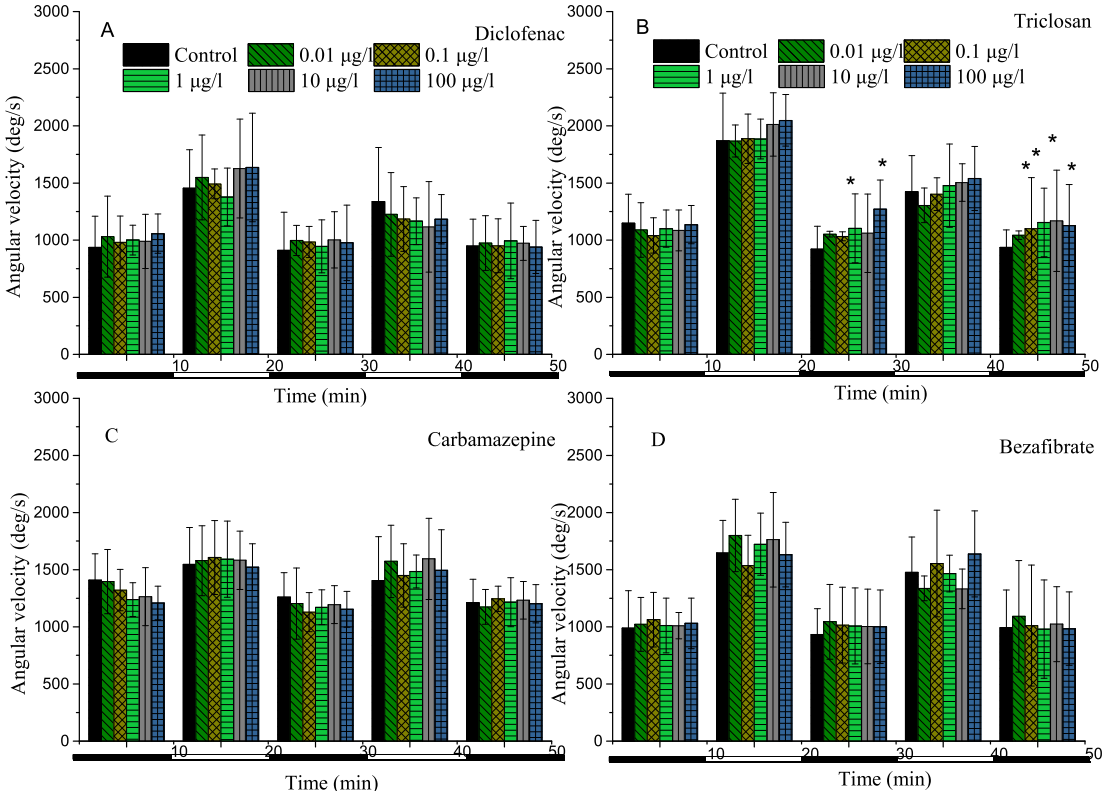

Fig. 3. Angular velocity (deg/s) of 5 dpf larvae in each ten-minute light and dark exposure to 8 single pharmaceuticals at 6 concentrations gradients (0, 0.01, 0.1, 1, 10 and 100 μg/l) or to low-, medium- and high-level mixtures. Velocity was analyzed for 50 min in alternating dark and light. The white and black bars at the bottom represent 10-minute light and dark periods, respectively. Bars represent 3 replicates (n = 42–48 larvae) ±SEM. Asterisks indicate a significant difference from control ($p < 0.05$).

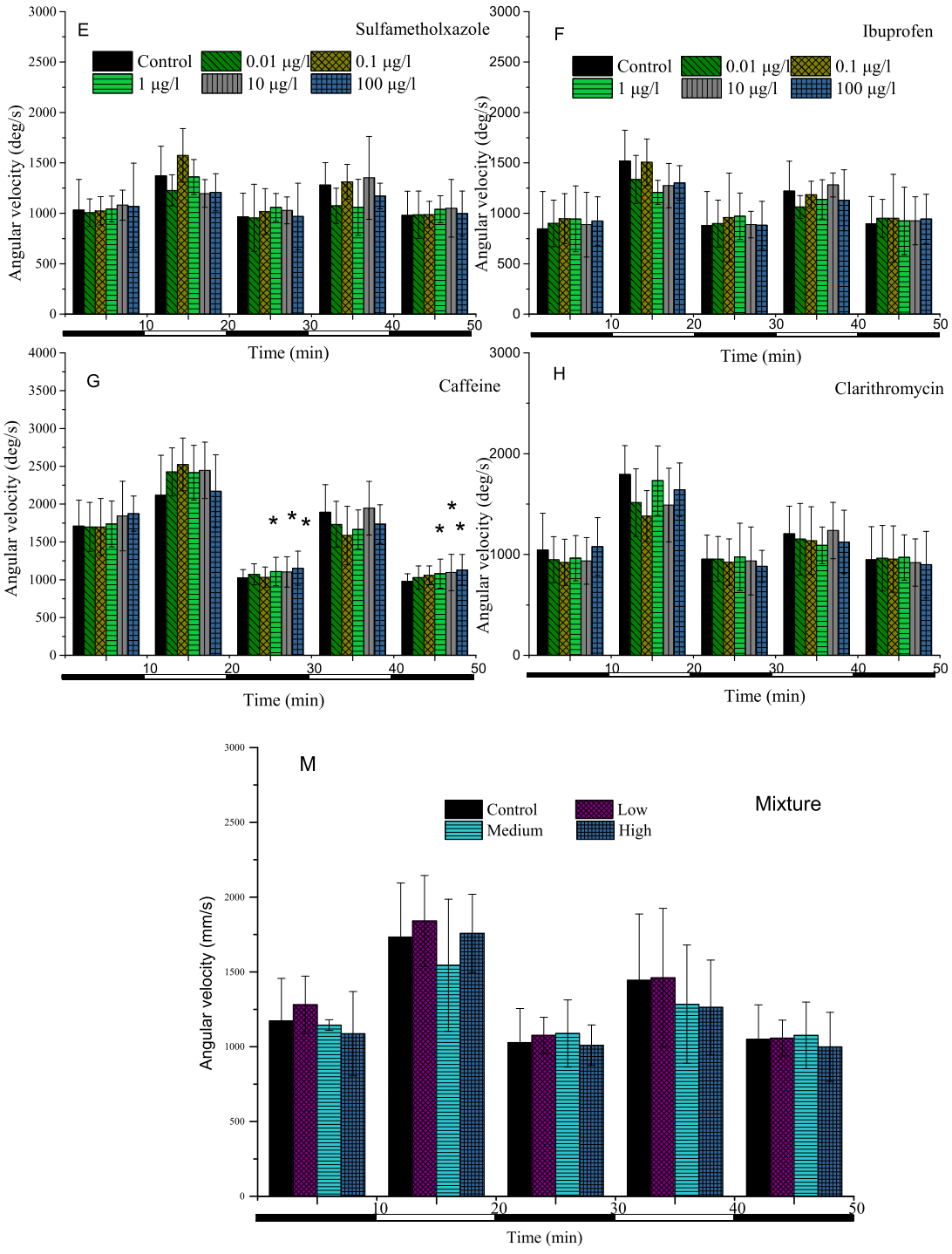

Fig. 3 (*continued*).

significant variation was detected relatively to control. One reason might be that the behavioral effects of carbamazepine and caffeine were counteracted by the combined effects of other components. Meanwhile, the existence of drug interactions could also alter behavior effects of the mixture, but we did not observe the interactions between two of them, since we give priority to the effects of pharmaceuticals on fish species at the development stage with low concentrations. These interactions should be given further attention. For example, erythromycin could interfere with the metabolism of carbamazepine, causing decreased carbamazepine clearance and elevated carbamazepine level (Stafstrom et al., 1995).

4.3. Relevance of delayed larval behavioral effects for toxicity assessment

The effects of compounds on the larval locomotion could be evaluated not only by swimming speed but also by angular velocity (e.g., Liu and Fetcho, 1999; Lorent et al., 2001). Absolute angular velocity characterizes the changes in direction of zebrafish movement and is a sensitive locomotor variable in motor function, especially the erratic swim pattern (Cachat et al., 2011; Rosemberg et al., 2012; Tran and Gerlai, 2013). In our results it is clear that angular velocity was sensitive to dark and light alterations, exhibiting significant difference between the light and dark period. Increased angular velocity indicates the

increased escape behaviors, which often is correlation with increased erratic movement (Cachat et al., 2011). Angular velocity and swimming speed are complementary endpoints for selecting potentially toxic compounds, being of relevance for instance for chemicals that modulate anxiogenesis and escape response (Budick and O'Malley, 2000).

Delayed effects in swimming speed and angular velocity could be detected in the larval stage after exposure to compounds during early stages of embryonic development (2–50 hpf). The occurrence of developmental toxicity in embryos may be related to disruption of the development of the nervous system. During the exposure period, neurons begin to be generated from neural stem cells (6 hpf) and are firstly connected by axons (48 hpf) (De Esch et al., 2012), while brain ventricles also are formed at 48 hpf (De Esch et al., 2012; Legradi et al., 2015). Pollutants could cross placenta and deposit in yolk affecting embryo developmental processes. Our further studies detected increased antioxidative capacity in larvae exposure to 0.1 μg/l triclosan from 2 hpf to 50 hpf (Fig. S1), which probably explains the increased locomotion at 118 hpf to some extent. Similarly, Parenti et al. (2019) reported an increase of activities of antioxidant enzymes in zebrafish embryos exposed for 24 h to triclosan at 0.1 μg/l. Although no significant increase in larval locomotion were detected for 1 μg/l carbamazepine, but increased antioxidative capacity in larvae was still observed.

It is clear that early developmental stages of zebrafish are sensitive to toxicants, and hence the zebrafish behavior test can act as a sensitive biological system for screening the pharmaceuticals that may exert effects on the nervous system and for early detecting the potentially high-risk substance (Legradi et al., 2018). Analysis of the effects of drug treatments on multi-endpoints revealed that swimming speed, angular velocity, and hatching rate are complementary behavioral endpoints. When assessed together they can help to clarify involved mechanisms of behavioral alteration. Furthermore, zebrafish displays a series of behavioral patterns which are comparable to rodents and other species in terms of their genome, brain patterning and neurological system (Ali et al., 2012; Roberts et al., 2013; Legradi et al., 2015). Thus compounds that affect zebrafish in the early stage may also have effects on other species.

The LOECs of triclosan (0.1 μg/l) and caffeine (1 μg/l) in this study were in the range of environmentally relevant concentrations. Of particular interest for future investigations and risk assessment strategies is that the obtained LOEC of triclosan for locomotion is just slightly higher than the observed mean concentration in European surface waters (0.04 μg/l). Since environmental risk is often expressed as a ratio of measured environmental concentration and toxicologically effective concentration, studying the developmental effects of pharmaceuticals to zebrafish not only favors the understanding of chemical toxicity, but also provides reliable data for environmental risk assessment.

5. Conclusion

The present study demonstrated that delayed effects could occur in zebrafish larvae due to embryonic exposure to pharmaceuticals in the range of concentrations detected in effluent-influenced surface waters. Early developmental stages of zebrafish are sensitive to toxicants, thus the zebrafish behavior test can act as a sensitive biological system for screening for pharmaceuticals that may exert effects on the nervous system, and for early detecting potentially high-risk substances. The assessment of multiple endpoints can favor the understanding of how the exposure to pharmaceuticals can lead to effects on zebrafish behavior.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.300.

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