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Microparticle formulations alter the toxicity of fenofibrate to the zebrafish *Danio rerio* embryo

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ABSTRACT

A wide variety of active pharmaceutical ingredients are released into the environment and pose a threat to aquatic organisms. Drug products using micro- and nanoparticle technology can lower these emissions into the environment by their increased bioavailability to the human patients. However, due to this enhanced efficacy, micro- and nanoscale drug delivery systems can potentially display an even higher toxicity, and thus also pose a risk to non-target organisms. Fenofibrate is a lipid-regulating agent and exhibits species-related hazards in fish. The ecotoxic effects of a fenofibrate formulation embedded into a hydroxypropyl methylcellulose microparticle matrix, as well as those of the excipients used in the formulation process, were evaluated. To compare the effects of fenofibrate without a formulation, fenofibrate was dispersed in diluted ISO water alone or dissolved in the solvent DMF and then added to diluted ISO water. The effects of these various treatments were assessed using the fish embryo toxicity test, acridine orange staining and gene expression analysis assessed by quantitative RT polymerase chain reaction. Exposure concentrations were assessed by chemical analysis. The effect threshold concentrations of fenofibrate microparticle precipitates were higher compared to the formulation. Fenofibrate dispersed in 20%-ISO-water displayed the lowest toxicity. For the fenofibrate formulation as well as for fenofibrate added as a DMF solution, greater ecotoxic effects were observed in the zebrafish embryos. The chemical analysis of the solutions revealed that more fenofibrate was present in the samples with the fenofibrate formulation as well as fenofibrate added as a DMF solution compared to fenofibrate dispersed in diluted ISO water. This could explain the higher ecotoxicity. The toxic effects on the zebrafish embryo thus suggested that the formulation as well as the solvent increased the bioavailability of fenofibrate.

1. Introduction

The globally rising consumption of pharmaceutical products is reflected by the occurrence of pharmaceuticals in many water bodies. The largest proportion of pharmaceuticals is released into the environment due to human excretion or veterinary applications (Halling-Sørensen et al., 1998; Aus der Beek et al., 2016). Wastewater treatment plants cannot guarantee complete removal of these compounds and active pharmaceutical ingredients (APIs) can enter the environment. Concentrations of pharmaceuticals in surface waters range from nanogram to microgram per liter (Ternes, 1998; Heberer, 2002; Andreozzi et al., 2005; Petrie et al., 2015).

Fenofibrate is a pro-drug and is hydrolyzed *in vivo* to its active form, fenofibric acid (Keating, 2011; Shipman et al., 2016). The

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pharmaceutical activates the peroxisome-proliferator-activated receptor α (PPARα), and induces the expression of genes essential for fatty acid activation, fatty acid transport and β-oxidation, thus regulating lipoprotein and the metabolism of fatty acids (Mandard et al., 2004; Farnier, 2008; Alagona, 2010; Keating, 2011; Ghonem et al., 2015,). The administration of fenofibrate reduces plasma triglyceride levels and increases the amount of high density lipoprotein (HDL) cholesterol (Kersten et al., 2000; Gilde et al., 2006; Farnier, 2008), being effective in the treatment of hypertriglyceridemia or atherogenic dyslipidemia (Attridge et al., 2013).

Fenofibrate was detected at concentrations of 0.08ng·L^{-1} (samples collected in 2012) and 0.16 μg/L (samples collected in 2001) in Chinese source water samples and the effluents of European sewage water treatment plants, respectively (Andreozzi et al., 2005; Ido et al., 2017). However, studies revealed EC_{50}-values above these environmental concentrations. In this context, chronic exposure to fenofibrate resulted in decreased reproduction in *Brachionus calyciflorus* and *Ceriodaphnia dubia* with EC_{50}-values of 1.44mg·L^{-1} and 0.76mg·L^{-1}, respectively, as well as mutagenic effects in *Salmonella typhimurium* (Isidoriet al., 2007). Moreover, fenofibric acid was found to be toxic towards *Vibrio fischeri* and *Daphnia magna* with EC_{50}-values of 1.72mg·L^{-1} and 4.90mg·L^{-1}, respectively (Rosal et al., 2010).

The use of particles in the nano-and microparticle range increases the bioavailability and solubility of a drug and leads to a sustained release of APIs and improved biocompatibility in treated patients (Kumari et al., 2010; Chen et al., 2019; Han et al., 2019). This can potentially lead to a dose reduction and a reduction in the amount of pharmaceuticals prescribed, which eventually reaches the environment. However, the nanomaterials themselves can cause negative effects on non-target organisms (Rizzo et al., 2013; Piechulek and von Mikecz, 2018). Metal and metal-oxide nanoparticles such as silver-NPs, which are used in medicine for their anti-microbial activity, were observed to cause neurotoxicity, developmental effects and lethality in zebrafish as well as impact other aquatic organisms (Griffitt et al., 2008; Farre et al., 2009; Muth-Kohne et al., 2013). Metal-based microparticles can negatively impact the development of zebrafish as well as fertility and broodsize in crustaceans (Tomilina et al., 2011, 2014). Furthermore, polymeric nanomaterials such as poly lactic-co-glycolid acid (PLGA) or the phthalat of hydroxypropyl methylcellulose (HPMC) were observed to cause negative effects in the zebrafish embryo (Hering et al., 2020).

In this study, we investigated the ecotoxicity of fenofibrate either dispersed in 20%-ISO-water or added to 20%-ISO-water as a di-methyl-formamid (DMF) solution (in the following described as "fenofibrate in DMF") as well as a fenofibrate microparticle formulation stabilized by HPMC (fenofibrate-HPMC) and its respective excipients at morphological, cellular and gene transcription levels in the zerbafish (*Danio rerio*) embryo. Zebrafish embryos have been shown to represent suitable models for the ecotoxicological assessment of small-scale materials such as metal and fluorescent silica nanoparticles and polymeric nanomaterials (Griffitt et al., 2008; Fako and Furgeson, 2009; Fent et al., 2010; Muth-Kohne et al., 2013; Ozel et al., 2013). Specifically, this study assessed apoptosis and changes in expression of selected genes of the neuro-, immune- and endocrine system by acridine orange staining and quantitative polymerase chain reaction (qPCR), respectively. For gene expression analysis, we chose genes involved in different biological pathways which have been shown to be affected by fenofibrate, such as glia cell development, endocrine regulation and immune function. To determine any effects on glia cell development, the transcripts of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) and myelin basic protein (*mbp*) were analysed, which were demonstrated to respond to fenofibrate treatment in zebrafish (Ashikawa et al., 2016). Additionally, the transcription of genes involved in the immune and reproductive endocrine system was studied and thus, genes encoding for vitellogenin (*vtg*)−1 and *vtg*-3 and for cytokine interleukin-8 (*il-8*) were analysed. When gene expression analysis was conducted, *vtg-3* is already expressed when estrogen was present, and *vtg-1* was expressed in zebrafish embryos even at 24hpf (Muncke and Eggen, 2006; Hao et al., 2013). The regulation of cholesterol, a precursor of 17β-estradiol, by lipid regulators like fenofibrate can lead to a regulation of 17β-estradiol and subsequently modulate vitellogenin (Cui et al., 2013). PPARα agonists may also be able to directly impair estrogen metabolism (Mandard et al., 2004). As it was seen that fenofibrate has an anti-inflammatory effect by decreasing the amount of cytokines (Gilde et al., 2006; Farnier, 2008), interleukin-8 (*il-8)* was selected to investigate possible effects of fenofibrate on the immune system.

2. Materials and methods

2.1. Fish husbandry and egg collection

Wildtype zebrafish (West Aquarium GmbH, Bad Lauterberg, Germany) were kept in UV-sterilized and activated carbon filtered water at 27 °C ± 1 °C under a 10 h/14 h dark-light cycle. Fraunhofer IME holds a permit to culture zebrafish according to the German Animal Welfare Act (§ 11 Absatz 1 Ziffer 1 TierSchG). The fish were fed with Tetramin flakes (Tetra GmbH, Melle, Germany) and with live nauplia of *Artemia salina* in the afternoon. In the morning, spawning trays were placed in the fish tanks (50–350 fish per aquarium) and left for approximately 60–90 min. After removal of the spawning trays, eggs were collected and rinsed with fresh aquarium water. The eggs were transferred to a vessel filled with aquarium water.

2.2. Test compounds

Fenofibrate, the fenofibrate-HPMC formulation as well as the excipients to generate the formulation were provided by MyBiotech GmbH (Ueberherrn, Germany). Fenofibrate was dispersed in either ISO water (DIN EN ISO 7346-3, 1996) 5-fold diluted with distilled water (20%-ISO-water) or 100% di-methyl-formamid (DMF). These stock solutions of either 20%-ISO-water or DMF were further diluted with 20%-ISO-water to obtain the test concentrations. The final DMF-concentration in each test solution was 0.1%. A DMF-control of 0.1% was tested in parallel to exclude any toxic effects caused by the solvent. For fenofibrate-HPMC, the test concentrations were based on the fenofibrate content within the fenofibrate-HPMC formulation. A stock solution of 90mg·L^{-1} fenofibrate-HPMC was prepared and diluted to the respective test concentrations of 1.00, 1.97, 3.90, 7.70, 15.20 and 30mg·L^{-1} of fenofibrate (nominal concentrations). The test concentrations were predetermined by the drug content of fenofibrate-HPMC. The API was tested at the same nominal concentrations. The content of the excipients in 90mg·L^{-1} of fenofibrate-HPMC was calculated and a stock solution with the respective content of excipients was prepared in 20%-ISO-water. This stock solution was diluted in 20%-ISO-water to reach the test concentrations of fenofibrate-HPMC. Fenofibrate has a logK_{OW} of 5.2 (National Center for Biotechnology Information 2020a), https://pubchem.ncbi.nlm.nih.gov/compound/Fenofibrate) and is barely soluble in water. Despite the low solubility, we tested fenofibrate in aqueous solution (20%-ISO-water20%-ISO-water) to study whether exposure via the water phase still induces effects. The exposure to fenofibrate dispersed in 20%-ISO-water represents the condition of exposure expected in the environment. The exposure in 20%-ISO-water is a direct comparison to the exposure conditions to the fenofibrate-HPMC formulation, since the formulation was also prepared in 20%-ISO-water.

2.3. Characterization of particle size

The determination of particle size (Feret's diameter) of fenofibrate particles after addition of the crystalline drug or a drug solution in DMF to ISO water was conducted by light microscopy using a Leica DM750 M Microscope system. A representative number of particles (at least 5000) was counted using the ImageJ software (National Institute of Health,

Bethesda, Maryland, USA). There was no difference in the size of the crystals observed between the different concentration ranges.

2.4. Fish embryo toxicity test (FET)

The FETs were conducted under standardized conditions and in accordance to the test guideline OECD 236 (OECD, 2013). The FET is not considered an animal testing method according to the German *Tierschutz-Versuchstierverordnung* (§ § 1-48 TierSchVersV 1.08.2013) and the European guideline 2010/63/EU (European Union, 2010). Thus, a declaration of animal welfare compliance is not necessary. The FET allows for investigating possibly teratogenic substances and their effects on the morphological development of the zebrafish embryo over time as described before and modified by our laboratory (Schulte and Nagel, 1994; Nagel and Insberner, 1998; Bachmann, 2002; Nagel, 2002; Braunbeck et al., 2005; Braunbeck and Lammer, 2006; Bodewein et al., 2016). The effects which were assessed included malformation of somites, detachment of tail, edema, yolk sac deformations, malformation of tail, head as well as of the *chorda dorsalis* (Nagel, 2002; Braunbeck et al., 2005). Effects were considered lethal when coagulation, no formation of somites, no detachment of tail, teratoma and the lack of a heartbeat at 48hpf occurred (Braunbeck et al., 2005; Nagel, 2002; DIN 38415-6, 2001). An atlas of the assessed effects is attached in the supplementary information (Supp. Tab.1). In this study, we focused on edema, mortality and slow blood flow, as these were the major effects observed in the embryos.

We conducted the FETs as previously described (Muth-Kohne et al., 2013; Sonnack et al., 2015). After harvesting the eggs, embryos were transferred into 96-well U-bottom-shaped polystyrene microtiter test plates (Greiner Bio-one, Germany) with 200 µl of the test substance or 20%-ISO-water as control for each well.

The exposure time for each test was 96 hours. After 48hpf, 75% of the solutions were exchanged.

At 24hpf, 48hpf, 72hpf and 96hpf, the embryos were evaluated for morphological defects via bright field microscopy. The pH of the test solutions was determined at the start of testing (0hpf), after the exchange of solutions (48hpf) and at the end of the FET (96hpf) and ranged between 6.5 and 8.0. At the end of testing, the embryos of each treatment were euthanized on ice, and either discarded or used for gene expression analysis (see *2.7*). The tests were run in parallel with two replicates for each treatment with 24 embryos per replicate and the 20%-ISO-water control. Additionally, a solvent control with 12 embryos per replicate was run in parallel. As the assessment of effects was based in the ISO-control, the solvent was only uused in order to show that any effect is not based on solvent treatment. For analysis, the data of the replicates were pooled. This test setup was replicated three times.

To determine the fenofibrate concentration for every exposure scenario, we conducted a bridging study. The exposure experiments for fenofibrate dispersed in 20%-ISO-water, fenofibrate in DMF and the fenofibrate-HPMC formulation were repeated. The tests were performed as described above, however, only one replicate per test concentrations was prepared. Samples were taken for chemical analysis at test start (fresh solution) and after 48hpf (aged solution). As a medium exchange was performed after 48 h, no chemical verification was done after 96hpf. Per concentration, one sample was analysed. At test start, 3 ml of the freshly prepared solution with which the well plates were filled were used for the analysis. The samples after 48hpf were pooled from the wells of one concentration to collect a sufficient amount of the solution for the analysis.

2.5. Chemical water analysis

Deuterated fenofibrate-d6 was used as internal standard. Samples of 3 ml volume were diluted with 0.6 ml methanol and were then analyzed by direct high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) using positive electrospray ionization (ESI+). Data were collected on a Waters 2695 LC system coupled to a Waters Quattro-Micro tandem mass spectrometer. The liquid chromatography was done in a Phenomenex Gemini® C18 HPLC column (150 mm × 3 mm, 5 µm particle size) at a flow rate of 0.5ml•min^{-1} and a column temperature of 30 °C. The mobile phase consisted of solvent mixtures of water, methanol, formic acid and ammonium acetate. The mass transition used for the quantification for fenofibrate was *m/z* 361.11 ⇨ *m/z* 138.96 and for fenofibrate-d6 *m/z* 367.24 ⇨ *m/z* 138.89; the confirmation of the substance identity for both compounds were carried out via the mass transitions 361.11 ⇨ *m/z* 233.06 and *m/z* 367.24 ⇨ *m/z* 234.07.

A matrix calibration with 20%-ISO-water was performed using 8 calibration samples in a concentration range from 0.8µg•L^{-1} to 100 µg fenofibrate/L, the coefficient of determination (r^2) of the linear calibration function was determined to 0.9999. The analytical method was successfully validated for the matrix 20%-ISO-water medium on three fortification levels according to the EU guideline SANCO/3029/99 (European Commission, 11/07/2000) at a limit of quantification (LOQ) of 1.00 µg/L. The accuracy (overall mean recovery) was 96.9% and the precision was 4.6% (mean RSD of the recovery values). The matrix independence of the measurements for the other matrices was proven in further experiments.

Two quality control (QC) samples with fenofibrate concentrations of 2.0 and 75µg·L^{-1} were used for the ongoing verification of the matrix calibration, the mean recovery of the measured QC samples was 98.5 ± 2.6%. Matrix-charged procedural blanks and controls were run to exclude possible cross-contaminations during laboratory work.

2.6. Acridine orange staining

To detect cell death, acridine orange staining was performed. The embryos were exposed to the test substances at nominal concentration of 1.00mg·L^{-1}, 1.97mg·L^{-1}, and 3.90mg·L^{-1} until 48hpf, dechorionated and stained in 2µg·ml^{-1}acridineorange dissolved in 20%-ISO-water for 30 min. The embryos were washed in 20%-ISO-water three times for ten minutes. They were transferred to fresh 20%-ISO-water, anaesthetized with Tricaine and placed on microscope slides with 3% methyl-cellulose. For the fluorescence analysis, an excitation wavelength of 480 nm (bandpass filter 480/40) was selected. Z-Stacks images were taken at fluorescence and brightfield illumination. ImageJ (NIH, Bethesda, USA) was applied to generate full-focus images (*extended depth of field*) and to count the number of stained cells within the region of interest (ROI, here the region from the caudal end of yolk sac extension to the caudal peduncle of the tail). For the analysis of fenofibrate in DMF, the data on the number of particles from the 20%-ISO-water control was taken from the data set of fenofibrate dispersed in 20%-ISO-water, as both experiments were performed in parallel. All data, including fenofibrate in DMF, were normalized to the 20%-ISO-water control, which represents the natural background of apoptosis in zebrafish embryos. As we normalized the fenofibrate-HPMC treatment to the 20%-ISO-water control as well, we wanted to treat every sample identically to avoid any bias. For each exposure scenario, experiments were conducted in three biological, independent replicates. For each concentration of a treatment, 3 to 5 individuals were used for analysis. For a few concentrations, less than 3 animals could be analyzed due to the premature death of the animals during the imaging treatment.

2.7. qPCR analysis

To analyze any effects of fenofibrate at the gene transcription level, qPCR was performed. The embryos were exposed to the test substances until 96hpf. Embryos of the control and the three lowest concentrations (1.00mg·L^{-1}, 1.97mg·L^{-1}, 3.90mg·L^{-1}) were used for qPCR. Ten embryos per treatment group were homogenized using the FastPrep®-24 (MP Biomedical, Eschwege, Germany) and total mRNA was extracted according to the manufacturer's protocol using the RNeasy®

PowerLyzer® Tissue&Cells Kit (QIAGEN, Hilden, Germany).

Subsequently, cDNA was generated using the SensiFast™ cDNA kit (Bioline, Luckenwalde, Germany). QPCR was conducted using the SensiFast™ SyBR No RoxKit (Bioline, Luckenwalde, Germany) and the LightCycler® 96 (Roche Molecular Systems, Mannheim, Germany). The cycling conditions were: 95 °C for 120 s for initial denaturation, followed by 35 cycles of 95 °C for 5 s, 65 °C for 10 s and 72 °C for 10 s, followed by the melting phase of95 °C for 10 s, cooling to 65 °C for 60 s and slowly heating the samples to 97 °C in which fluorescence was measured at each 0.1 °C rise for 1 s. The data were analyzed according to the ΔΔCt-method (Livak and Schmittgen, 2001). The expression of *hmgcr, mbp, vtg-1, vtg-3* and *il-8* was normalized to the expression of the housekeeping genes *rpl-8* and *β-actin* (Table 1) (Sahoo and Oikari, 2013; Vandesompele et al., 2002). For the primer design of *vtg-1* and *vtg-3*, the program Primer3 was used (Koressaar and Remm, 2007; Untergasser et al., 2012). The reference sources of the other primer pair sequences are listed in Table 1. For fenofibrate in DMF, fold-changes were determined in comparison to the DMF-control; for all other tested substances, the 20%-ISO-water control was used. For fenofibrate in DMF and fenofibrate dispersed in 20%-ISO-water, RNA extraction and qPCR analysis were performed on the same embryos which were exposed in the FET. For the qPCR analysis of embryos exposed to the excipients and the formulation, embryos were exposed to the three test concentrations independently from the FET. Four independent, experimental replicates were analyzed for each treatment.

2.8. Analysis and statistics

FET data were initially analyzed with Microsoft Excel to obtain an overview of the effects. In the following, ToxRat® (Version 2.10; ToxRat Solutions GmbH, Alsdorf, Deutschland) was used to obtain concentration-response curves and derive EC_{50}- and LC_{50}-values by Probit analysis including 95% confidence intervals (CI). The EC-levels were based on the number of embryos showing any effect (independent of the type of effect or the number of effects per embryo) compared to the number of the embryos showing no effect (see *2.4* and Supp. Tab.1). Further statistics for the FETs included testing for normal distribution with the Shapiro-Wilk-Test ($p = 0.01$) and variance homogeneity by the Levene's Test ($p = 0.01$). After this, a non-parametric trend analysis by contrast was performed ($p = 0.05$).If variance homogeneity was given, the Williams multiple sequential *t*-test was performed to determine significant differences between exposure conditions ($p = 0.05$). If no variance homogeneity was given and depending on whether data was parametric or non-parametric, the multiple sequentially-rejective Welsh-*t*-test after Bonferroni-Holm or the multiple sequentially-rejective Median (2 × 2-Table) Test after Bonferroni-Holm were conducted, respectively (ToxRatPro 3.1.0). The results of the acridine orange staining and qPCR analysis were tested for normal distribution using the Shapiro-Wilk-Test ($p = 0.01$). Variance homogeneity was tested with the Levene`s test ($p = 0.01$). Parametric data were compared using One-Way-ANOVA ($p = 0.05$) with the Tukey-Test ($p = 0.05$) for post-hoc-testing. Non-parametric data or data with variance heterogeneity were analyzed using the Kruskal-Wallis-ANOVA ($p = 0.05$) and the Dunn's Test as post-hoc-test ($p = 0.05$). Statistics were conducted using Origin 2019b (Origin 2019b, OriginLab Corporation, Northampton, USA).

Table 1
Primer sequences used for qPCR.

gene		primer sequence	AccessionNumber	Reference
rpl-8	F:	TTG TTG GTG TTG TTG CTG GT	NM_200713	Zhang et al. 2017
	R:	GGA TGC TCA ACA GGG TTC AT		
β-actin	F:	AAG AGC TAT GAG CTG CCT GA	AF057040.1	Sahoo et al. 2013
	R:	ACC GCA AGA TTC CAT ACC CA		
mbp	F:	CCG TCG TGG AGA CGT CAA	AY860977	Ashikawa et al., 2016
	R:	CGA GGA GAG GAC ACA AAG CT		
hmgcr	F:	AGC TGT CAC TCA TGT CTG CC	NM_001079977.2	Ashikawa et al., 2016
	R:	CAG CAG GCG ACA TCT CAA GA		
il-8	F:	AAG CCG ACG CAT TGG AAA AC	XM_001342570	Mottaz et al., 2017
	R:	GTT GTC ATC AAG GTG GCA ATG A		
vtg-1	F:	CAA GCT CCT CTA TGT CCA GC	NM_001044897.3	in this paper
	R:	GTG TGC CAA GTA CCG ATC TT		
vtg-3	F:	AGC ACC TGG AGT CTC AAA CA	NM_131265.1	in this paper
	R:	ACT GCT CTG ACA CAA ACC ATG		

3. Results

3.1. Characterization of particle size

Fenofibrate added from DMF solution formed needle-shaped precipitates with a particle size of 10.66± 7.60 μm, while fenofibrate particles dispersed in 20%-ISO-water had a particle size of 13.907 ± 7.609 μm (Fig. 1). Due to the poor aqueous solubility of the API there was no significant difference observed between the particle sizes at different fenofibrate concentrations. For the fenofibrate-HPMC formulation, a particle size of 2.04 μm ± 0.12 μm has been reported (Jung et al., 2021). There was no difference observed in the size of the crystals between the different concentration ranges.

3.2. LC-MS measurement of fenofibrate in samples of the bridging study at test start and after 48 hpf

The measured fenofibrate concentrations differed among fenofibrate application forms. For the different test concentrations of fenofibrate dispersed in 20% ISO, the measured concentrations ranged from 2.94 to 9.93% of the nominal concentrations (Suppl. Table 2). In the group of fenofibrate in DMF, the measured concentrations ranged from 8.32% to 38.38% of the nominal concentrations. The highest measured fenofibrate concentration was 3.90mg·L^{-1}, which was equivalent to 13.00% of the nominal concentration (Suppl. Table 2). Also, for fenofibrate in DMF, the% of nominal values decreased with increasing fenofibrate concentrations. For fenofibrate-HPMC, measured concentrations were between 8.32% and 17.30% of the nominal concentrations (Supp. Tab. 2).

All samples displayed a reduced fenofibrate concentration after 48hpf in comparison to the samples taken at test start (Supp. Tab. 2). The loss of fenofibrate was over 10-fold for the highest concentration of fenofibrate in DMF and the HPMC-formulation after 48hpf.

Taken together, the fenofibrate concentrations applied during the bridging study and measured in the samples of the microformulation and fenofibrate in DMF at test start and 48hpf were higher than the concentrations of fenofibrate dispersed in 20%-ISO-water samples. We were able to determine the actual EC_{50}-and LC_{50}-values of the bridging study (see *3.4.3*), based on the concentrations measured at test start. Based on the biological effects seen in the bridging study, a specific correction factor for each of the different fenofibrate applications was calculated by dividing the EC_{50}-and LC_{50}-values of the measured concentration by the EC_{50}- and LC_{50}-values based on the nominal concentrations. This factor was then applied to the nominal test concentrations as well as to the EC_{50}- and LC_{50}-values of the previous FET exposure in order to determine the corrected EC_{50}- and LC_{50}-values as well as the test

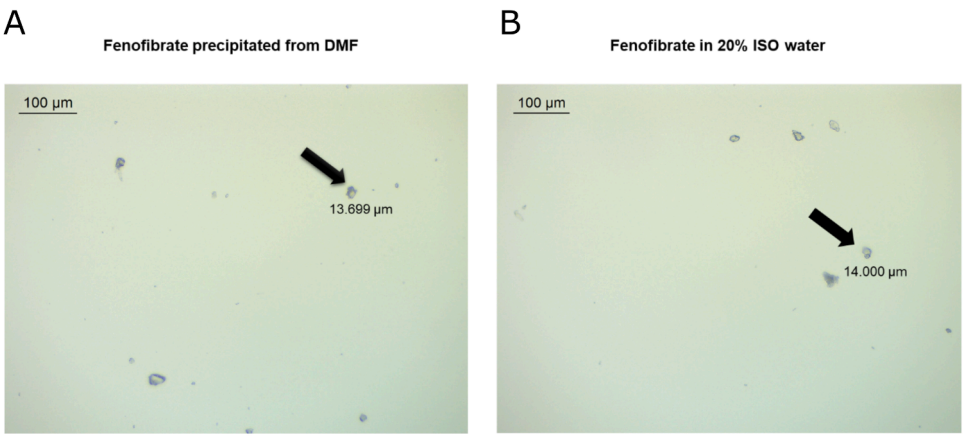

Fig. 1. Representative images of the particles measured for determination of particle size (Feret's diameter) by light microscopy (10-fold magnification).

concentrations of the prior conducted experiments (Table 2, Table 3, Suppl. Table 3 and Suppl. Tab. 4). As the measured fenofibrate concentrations originate from the bridging study and not from the experiments used to generate the data on lethal and sub-lethal endpoints, the reported EC_{50}- and LC_{50}-values and the test concentrations adjusted via the correction factor are, however, estimated and not based on measured values.

3.3. Biological results of the bridging study to assess fenofibrate EC/LC-values

The morphological effects seen in embryos in the bridging study were comparable to the tests performed prior to the bridging study. Based on the nominal concentrations, we determined EC_{50}-values of 5.97mg·L^{-1} (nominal) for the fenofibrate-HPMC formulation, 3.14mg·L^{-1} (nominal) for fenofibrate in DMF and 21.89mg·L^{-1} (nominal) for fenofibrate dispersed in 20%-ISO-water (Fig. 2). The EC_{50}-values determined by the measured concentrations were 0.730mg·L^{-1}, 0.610mg·L^{-1} and 0.930mg·L^{-1} at 96hpf, respectively (Table 2). Thus, the calculated EC_{50}-values based on the measured concentration were only 4.25–19.43% of the EC_{50}-values determined by the nominal concentrations.

Based on nominal concentrations, LC_{50}-values of 12.11mg·L^{-1} and 7.74mg·L^{-1} at 96hpf were determined (Table 2) for the fenofibrate-HPMC formulation and fenofibrate in DMF, respectively. The LC_{50}-values for fenofibrate dispersed in 20%-ISO-water could not be determined at 96hpf due to a lack of mortality. Based on the measured concentrations, the LC_{50}-values decreased to 1.26mg·L^{-1} and 1.19mg·L^{-1}, for fenofibrate-HPMC formulation and fenofibrate in DMF, respectively, with percentages of 10.41% and 15.38% of those determined on the basis of nominal concentration (Fig. 2).

The EC_{50}- and LC_{50}-curves for the results of the bridging study based on the measured concentrations are presented in Supplementary Figure 2. In the following, we will focus on the estimated EC_{50}- and LC_{50}-values as well as the estimated concentrations based on the correction factor (Table 3, Sup. Table 4).

3.4. Fenofibrate and the fenofibrate-HPMC formulation cause similar sub-lethal and lethal effects in the developing zebrafish embryo

The predominantly occurring effects in the embryo were reduced or ceased bloodflow, edema and coagulation (Fig. 3). Visual data as examples for the various effects are presented in Supplementary Fig. 1.

3.4.1. Effects of fenofibrate-HPMC and excipients on zebrafish embryo

Embryos exposed to the excipients did not display any sub-lethal or lethal effects above the control level (Fig. 3A). Thus, neither an EC_{50}- nor a LC_{50}-value could be determined (Fig. 2A, B). For fenofibrate-HPMC, a concentration-dependent increase in sub-lethal and lethal effects was observed. The lowest observed effect concentration was 0.476mg·L^{-1} (estimated), with an average total effect of 59.91% ($p<0.05$). Embryos exposed to the two highest test concentrations of 1.85mg·$L^{-1}$and 3.66mg·L^{-1} (estimated) displayed effects amounting to 95.18% ($p<0.05$) and 98.63% ($p<0.05$) of the embryos, respectively, at 96hpf. The mortality increased from 53.12% at 1.85mg·L^{-1} to 81.26%at 3.66mg·L^{-1} (Fig. 3B). Applying the correction factor determined by the measured concentrations during the bridging study, the estimated EC_{50}- and LC_{50}-values of fenofibrate-HPMC were reduced to 0.420mg·L^{-1} and

Table 2
EC_{50}- and LC_{50}-values of the bridging study with fenofibrate dispersed in 20%-ISO-water, fenofibrate in DMF and fenofibrate-HPMC based on nominal as well as measured concentrations at 48hpf and 96hpf. From these nominal and measured values, a factor was determined for the EC_{50}- and LC_{50}-values for fenofibrate dispersed in 20%-ISO-water, fenofibrate in DMF and fenofibrate-HPMC at 96hpf.

		EC 50 [mg/L]		LC50 [mg/L]	
		48hpf	96hpf	48hpf	96hpf
nominal concentration	Fenofibrate-HPMC	n.d.	5.97	n.d.	12.11
	Fenofibrate in DMF	51.72 (27.37–262.16)	3.14 (2.56–3.83)	n.d.	7.74 (2.43–51.17)
measured concentration	Fenofibrate in ISO 20%	n.d.	21.89	n.d.	n.d.
	Fenofibrate-HPMC	n.d.	0.73	n.d.	1.26
	Fenofibrate in DMF	5.40 (3.15–19.23)	0.61	n.d.	1.19
	Fenofibrate in ISO 20%	n.d.	0.93	n.d.	n.d.
factor ms[c]/nom[c]	Fenofibrate-HPMC		0.122		0.104
	Fenofibrate in DMF		0.194		0.154
	Fenofibrate in ISO 20%		0.0425		n.d.

Table 3
EC50- and LC_{50}-values in $mg \cdot L^{-1}$ of embryos exposed to fenofibrate dispersed in 20%-ISO-water, fenofibrate in DMF and fenofibrate-HPMC as well as its excipients at 24hpf, 48hpf, 72hpf and 96hpf. EC_{50}- and LC_{50}-values are based on nominal concentrations as well as the values determined by the correction factor.

		EC 50 [mg/L]				LC 50 [mg/L]			
		24hpf	48hpf	72hpf	96hpf	24hpf	48hpf	72hpf	96hpf
nominal concentration	Excipients	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)	n.d. (nd.)	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)
	Fenofibrate-HPMC	n.d. (n.d.)	n.d. (n.d.)	10.24 (6,90/ 16,95)	3.44 (2.44/ 4.69)	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)	14.24 (10.41/ 21.56)
	Fenofibrate in DMF	n.d. (n.d.)	11.03 (6.80–22.59)	2.66 (1.92/ 3.50)	1.24 (1.04/ 1.44)	n.d. (n.d.)	n.d. (n.d.)	29.58 (23.58/ 39.66)	7.26 (6.41/8.27)
	Fenofibrate in ISO 20%	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)	16.1 (8.23/ 58.80)	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)	n.d. (n.d.)
values determined by the factor derived from the chemical analysis	Fenofibrate-HPMC				0.420				1.48
	Fenofibrate in DMF				0.241				1.12
	Fenofibrate in ISO 20%				0.684				n.d.
factor ms[c]/nom[c] derived from the bridging study	Fenofibrate-HPMC				0.122				0.104
	Fenofibrate in DMF				0.194				0.154
	Fenofibrate in ISO 20%				0.0425				n.d.

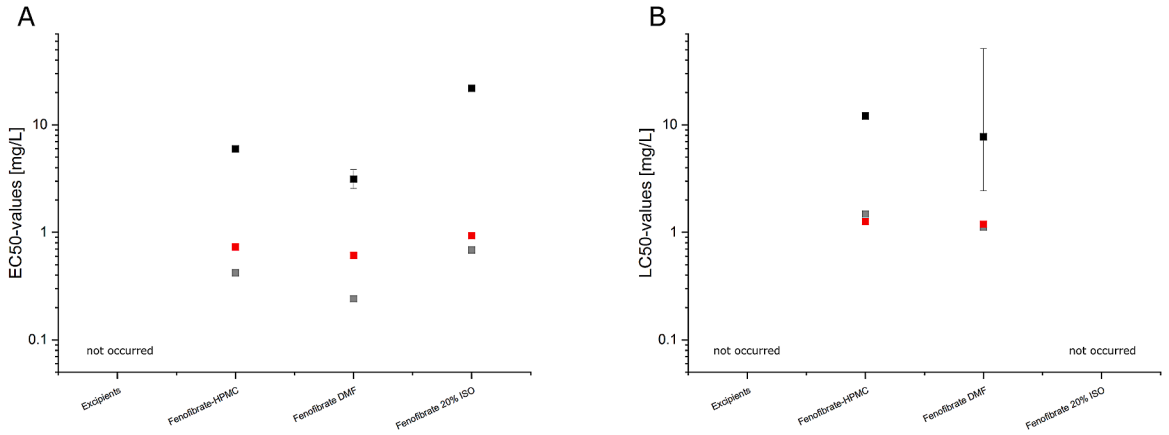

Fig. 2. $EC5_0$-value (A) and LC_{50}-value (B) of embryos exposed to the excipients, the fenofibrate-HPMC, fenofibrate in DMF or fenofibrate dispersed in 20%-ISO-water at 96hpf. No EC_{50}- or LC_{50}-value for the excipients could be determined (not occurred). The black squares display the EC_{50}- or LC_{50}-values determined by the nominal concentration and the red squares display the EC_{50}- or LC_{50}-values based on the measured concentrations of the bridging study. The grey squares display the estimated EC_{50}- and LC_{50}-values of the original study based on the factor derived from the bridging study. The EC_{50}- and LC_{50}-values were determined via Probit analysis. The error bars display the upper and lower 95% confidence intervals.

$1.48 mg \cdot L^{-1}$, respectively (Table 3).

3.4.2. *Effects of fenofibrate in DMF on zebrafish embryos*

Effects seen in embryos exposed to the DMF-control were below 10% and thus not different from the 20%-ISO-water control, meeting the validity criterion of control embryo survival (Fig. 3C). Embryos exposed to $0.194 mg \cdot L^{-1}$ fenofibrate in DMF, displayed 41.92% of sub-lethal effects, which consisted mostly of edema (35.64%) and slow blood flow (24.48%) at 96hpf (Fig. 3C). At $1.49 mg \cdot L^{-1}$, 97.97% of the embryos displayed effects ($p<0.001$), with a mortality of 52.17% (Fig. 2C). Embryos exposed to $5.82 mg \cdot L^{-1}$ showed 100% total effects ($p<0.001$) with a mortality of 84.82% at 96hpf (Fig. 3C). Based on measured concentrations determined by the chemical analysis performed during the bridging study, an EC_{50}-value of $0.241 mg \cdot L^{-1}$ and an LC_{50}-value of $1.12 mg \cdot L^{-1}$ were estimated (Table 3).

3.4.3. *Effects of fenofibrate dispersed in 20%-ISO-water on zebrafish embryos*

Embryos exposed up to an estimated concentration of $0.327 mg \cdot L^{-1}$ did not show any effects above 20% (Fig. 3D). At $0.646 mg \cdot L^{-1}$ and $1.28 mg \cdot L^{-1}$, effects of 53.49% and 71.84% ($p<0.001$ for $1.20 mg \cdot L^{-1}$), respectively, were observed at 96hpf. The effects mostly encompassed sub-lethal effects like edema (40.49% and 56.22%) and slow blood flow (36.33% to 44.12%) and no lethal effects occurred. Including the respective correction factor based on the measured concentrations during the bridging study, the EC_{50}-value is estimated to be $0.684 mg \cdot L^{-1}$ at 96hpf. An LC_{50}-value could not be determined (Table 3).

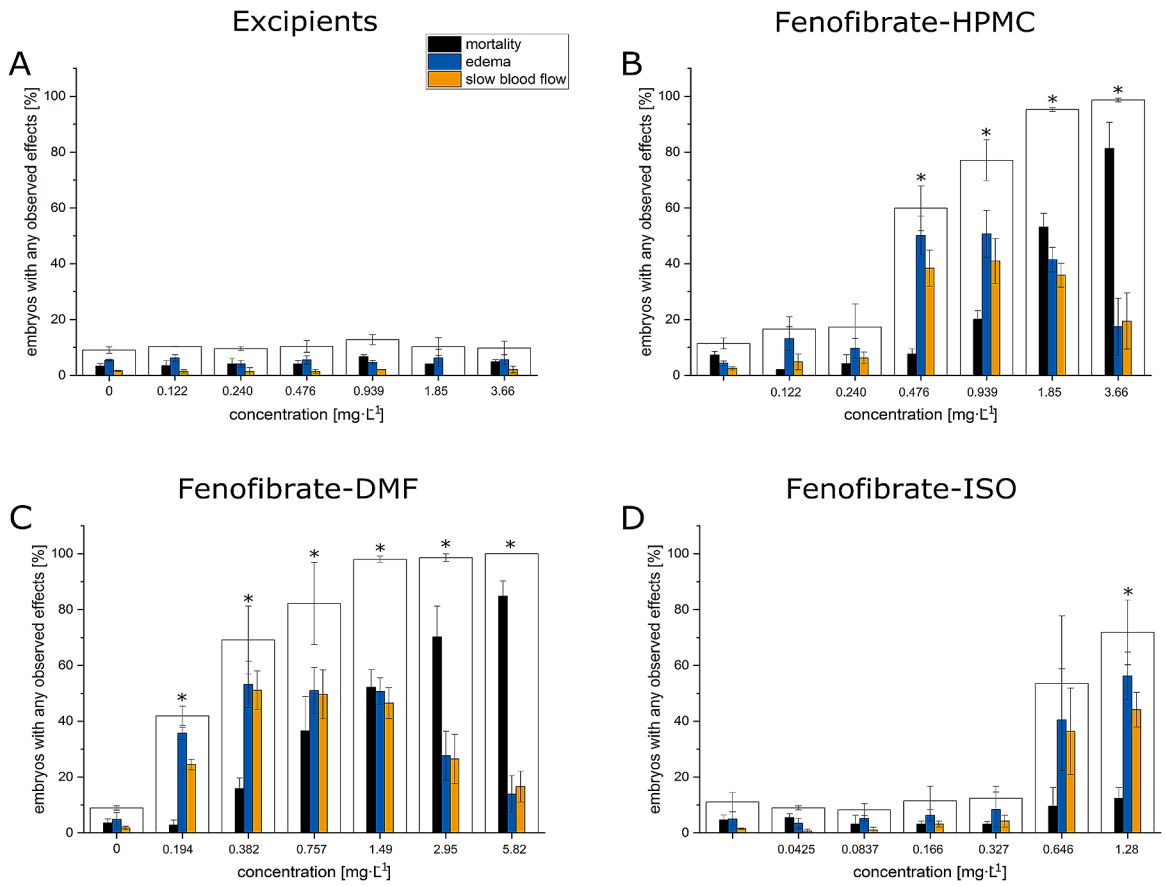

Fig. 3. Sub-lethal and lethal effects caused by fenofibrate dispersed in 20%-ISO-water (A), dispersion of fenofibrate in DMF (B), the excipients (C) and fenofibrate-HPMC (D) in zebrafish embryos at 96hpf are depicted as well as the respective standard error. Embryos exposed to fenofibrate in DMF and the formulation displayed the most effects, whereas embryos exposed to the excipients were not affected. The concentrations display the estimated values.

3.5. *Apoptosis staining of zebrafish embryos after fenofibrate treatment*

Apoptotic cell counts in the 20% ISO control and the DMF-control group,the results were similar and did not show a statistically significant difference. In order to base all values on the same control, also embryos exposed to fenofibrate in DMF were compared to the 20% ISO control. The amount of apoptotic cells in zebrafish embryos exposed to DMF as a control was only 80.39% of the number of apoptotic cell counts in the 20%-ISO-water control. This difference was, however, not statistically different.

Embryos exposed to fenofibrate in DMF showed an increase in apoptosis at 0.194mg·L^{-1} (estimated), which was caused by an outlier at this concentration rather than an effect of fenofibrate in DMF (Fig. 4B). Embryos exposed to fenofibrate-HPMC did not show any significant differences in the proportion of apoptotic cells when compared to the 20%-ISO-water control (Fig. 4C). A decreased amount of apoptotic particles was found for embryos exposed to 0.240mg·L^{-1} fenofibrate-HPMC (estimated) compared to the lowest concentration of excipients ($p = 0.015$). The excipients did not change apoptosis when compared to the 20%-ISO-water control (Fig. 4C).

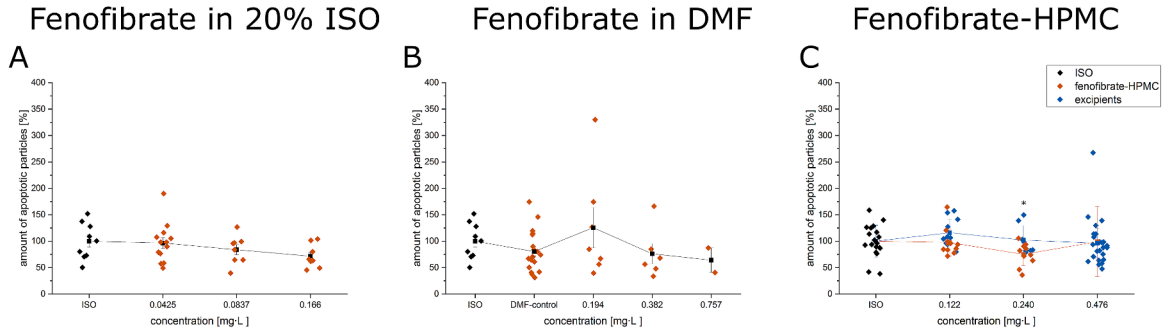

Fig. 4. Results of apoptotic staining of the zebrafish embryos exposed to fenofibrate dispersed in 20%-ISO-water (A), fenofibrate in DMF (B) and fenofibrate-HPMC with its respective excipients (C) at 48hpf ($n = 3$). The dots show the proportional amount of apoptotic cells compared to the control level for each individual embryo (black=control animals, red=treated animals). The average number of apoptotic cells in control embryos was set to 100%. The line connected black squares displays the mean values of each treatment with the respective standard error. The blue dots and line (D) show the proportional amount of apoptotic cells in embryos exposed to the excipients. The concentrations display the estimated values. For analysis, One-Way-ANOVA was applied for parametric and Kruskal-Wallis-ANOVA for non-parametric data. ($p = 0.015$; *).

3.6. Gene transcription analysis after fenofibrate treatment

In embryos exposed to fenofibrate dispersed in 20%-ISO-water, no statistically significant change in gene expression at any concentration was observed for *hmgcr, vtg-1, mbp* and *vtg-3*. Embryos exposed to 0.0425mg•L^{-1} (estimated) fenofibrate dispersed in 20%-ISO-water displayed reduced gene expression of *il-8* when compared to the control and the other tested concentrations ($p \leq 0.02$), but recovered to control expression levels at the higher concentrations (Fig. 5A). Embryos exposed to fenofibrate in DMF did not display significant changes in gene expression for *hmgcr, vtg-1, il-8* and *vtg-3*. A significant reduction in the expression of *mbp* ($p = 0.011$) was observed at 0.757mg•L^{-1} (estimated) and a trend was already noted at the lower concentrations (Fig. 5B). Embryos exposed to fenofibrate-HPMC did not show a significant change in gene expression. However, a trend towards lower *mbp* expressions at all three tested concentrations was seen. Additionally, an upwards tendency in *il-8* expression with increasing concentration was indicated (Fig. 5C). The excipients caused an almost five-fold increase in *il-8* transcription at a concentration of 0.122mg•L^{-1} (estimated; based on the factor of the HPMC-formulation), which decreased with increasing concentrations (Fig. 5D). However, due to data variation this was not significant and an actual increase is presumptive. Furthermore, the excipients caused a trend towards decreased expression of *hmgcr* at a concentration of 0.476mg•L^{-1} (estimated).

4. Discussion

The exposure of fenofibrate dispersed in 20%-ISO-water and the exposure to fenofibrate-HPMC prepared in 20%-ISO-water reflected the conditions present in the aquatic environment. Fenofibrate in DMF was used as a comparison to the formulation, as both were supposed to increase the availability to the embryo. Crystalline fenofibrate is practically insoluble in water (water solubility <1 mg•L^{-1}), with an aqueous solubility of 0.42 mg•L^{-1} having been reported (National Center for Biotechnology Information 2020b), https://pubchem.ncbi.nlm.nih.gov/source/hsdb/7736). We expected that only a very small proportion of the nominal fenofibrate concentrations were dissolved in water and available to the fish embryo. To ensure that fenofibrate would have some level of biological impact on the zebrafish, we chose high nominal concentrations, which led to effective fenofibrate concentrations in the test solutions.

Little is known about possible adverse effects of fenofibrate in fish. In one study fenofibrate was tested up to a concentration of 169µg•L^{-1} (DMF used as solvent) in fathead minnow, *Pimephales promelas*, in an

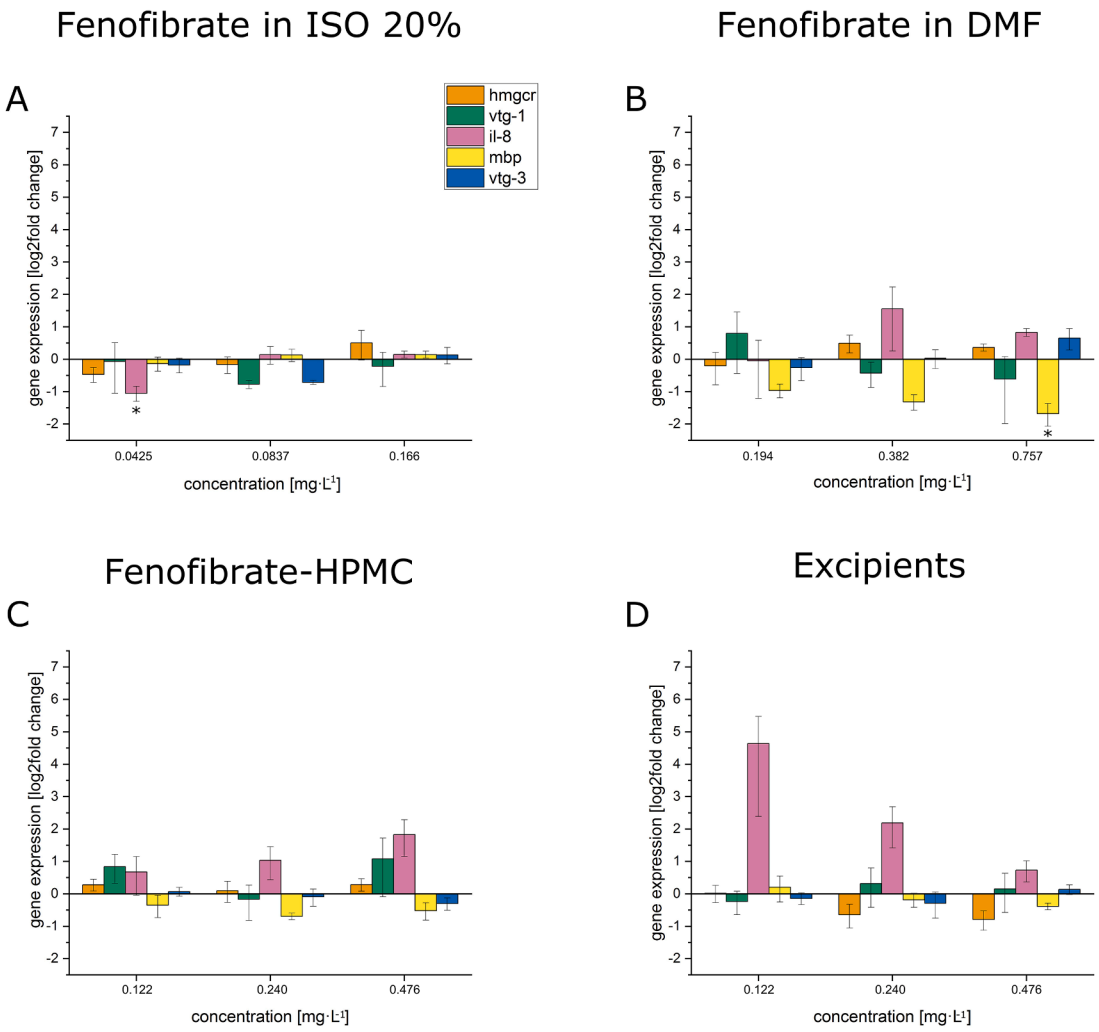

Fig. 5. Results of qPCR performed after 96hpf exposure of zebrafish embryos to fenofibrate dispersed in 20%-ISO-water(A), fenofibrate in DMF (B), fenofibrate-HPMC (C) and its excipients (D). Analysed genes were *hmgcr, vtg-1, il-8, mbp* and *vtg-3*. Data analysis was conducted according to the ΔΔCt-method of Livak and Schmittgen (2001) (Livak and Schmittgen, 2001). The bars show the mean ($n = 3$–5) relative expression of each transcript normalized to *rpl-8* and *β-actin* transcript abundance in the same sample, depicted as log2FC. Error bars show the standard error. The concentrations display the estimated values. One-Way-ANOVA was applied for parametric and Kruskal-Wallis-ANOVA for non-parametric data and asterisks depict statistical differences (A *$p = 0.02$; B*$p = 0.011$).

early life-stage test until 28 days after hatch, and no significant changes in growth and survival were determined (Overturf et al., 2012). Thus, the aim of the present study was to further investigate the potential effects of fenofibrate on aquatic vertebrates at greater concentrations, using the zebrafish embryo as a model organism.

The analysis of the test solutions showed that only a small fraction of the applied fenofibrate was dissolved in the samples of fenofibrate dispersed in 20%-ISO-water (see Supp. Tab. 2). This reduced fenofibrate concentration also explains the smaller number of embryos with any observed effects in the 20%-ISO-water treatment groups. Fenofibrate applied within an HPMC-matrix or DMF, however, resulted in increased measured concentrations of fenofibrate at test start.

Fenofibrate in the formulation is likely to dissociate from HPMC due to sample preparation during chemical analysis. Yet, only the amount of fenofibrate that was initially dissolved was determined by chemical analysis. Although we cannot differentiate between dissolved fenofibrate and fenofibrate still bound to HPMC, we can state that HPMC increased the amount of fenofibrate available to the zebrafish embryos (Supp. Tab. 2). Although we did not include a filter step in the preparation of the fenofibrate samples for chemical analysis, and there might be a possibility for fenofibrate crystals to be present, the difference in dissolved fenofibrate in the various treatments (ISO, DMF, formulation) confirms our hypothesis that solvents and the formulation increase the solubility of fenofibrate.

Despite an overall low solubility of fenofibrate in all treatment groups, the measured concentrations of fenofibrate were sufficient to cause concentration-dependent effects in the embryos and enabled us to determine EC_{50}- and LC_{50}-values for fenofibrate in DMF ($0.241 mg \bullet L^{-1}$/$1.12 mg \bullet L^{-1}$, estimated), fenofibrate dispersed in 20%-ISO-water ($0.684 mg \bullet L^{-1}$/n.d., estimated) and the HPMC-formulation ($0.420 mg \bullet L^{-1}$/$1.48 mg \bullet L^{-1}$, estimated) at 96hpf (Table 3). Applying the correction factor determined by the bridging study, the estimated EC_{50}-value (96hpf) of fenofibrate-HPMC was 1.72-times and of fenofibrate dispersed in 20%-ISO-water 2.84-times higher than the estimated EC_{50}-value of fenofibrate in DMF (Table 3). Thus, the formulation as well as the solvent DMF appeared to enhance the solubility of fenofibrate, resulting in an increase in (bio-) availability of fenofibrate (Savjani et al., 2012), and – to an extent- morphological effects. The bioavailability of fenofibrate was potentially increased due to an effect of DMF on the permeability of the chorion as observed with DMSO (Kais et al., 2013). DMSO and DMF were shown to affect the membrane structure of tumor cells and interfere with the glycoproteins on the cell surface (Borenfreund et al., 1975). Both solvents increase the permeability of cells (Borenfreund et al., 1975; Furmanski and Lubin, 1972; Gries, 1971). Thus, it can be assumed that DMF increases the uptake of fenofibrate. Concerning the formulation, HPMC is a water soluble, hydrophilic coating agent which is applied in the coating of pharmaceuticals for controlled drug release and increase of drug solubility (reviewed in Li et al., 2005; Al-Tabakha, 2010; Ghadermazi et al., 2019). Upon exposure to an aqueous medium, HPMC starts swelling and a gel-layer is formed, leading to drug release via erosion and/or diffusion through the gel-layer (Li et al., 2005).

A decline in all test concentrations of the three treatments was found in samples taken at 48hpf, presumably due to absorption of fenofibrate to the test plate or the chorion due to the estimated partition coefficient of fenofibrate (5.2) (National Center for Biotechnology Information 2020a), https://pubchem.ncbi.nlm.nih.gov/compound/Fenofibrate). This could also impact the availability of fenofibrate to the embryo. The usage of DMF or HPMC as a carrier could reduce the absorption, increase the fenofibrate content in the samples, and thereby the bioavailability to the embryos. Additionally, small particles ranging in the nanoparticle size were shown to increase the bioavailability of pharmaceuticals (Kumari et al., 2010). The difference in particle size between fenofibrate in DMF and fenofibrate dispersed in 20%-ISO-water was small. The particle size of the fenofibrate-HPMC formulation with 2 μm in diameter was smaller than the particles formed by fenofibrate in DMF. However, 21% of the HPMC-formulation particles were shown to assume a diameter below 1 μm (Jung et al., 2021). The pore size of the zebrafish embryo chorion ranges between 0.5 μm to 1.5 μm in diameter (Laale, 1977; Lee et al., 2007; Schreiber et al., 2009; Duan et al., 2013; Kristofco et al., 2018). Therefore, particles could enter the chorion and cause effects. Nevertheless, both treatments caused effects in a similar concentration range and instead of particle size, the ecotoxicity was probably dependent on the increased solubility by the formulation and DMF.

Fenofibrate concentrations of $0.16 \mu g \cdot L^{-1}$ and $<0.02 \mu g \cdot L^{-1}$ were measured in the effluents of sewage treatment plants, and even in drinking water, respectively (Ternes, 2001; Andreozzi et al., 2005; Monteiro and Boxall, 2010). Moreover, a fenofibrate concentration of $0.55 \mu g \cdot L^{-1}$ (maximal measured environmental concentration) was detected in German surface waters (Ternes, 1998; Andreozzi et al., 2005; Bergmann et al., 2011; Ido et al., 2017). Translating our observations to the environment, a safety factor of 1000 should be considered (applicable if acute toxicity data of only one environmentally relevant species is available). The estimated LC_{50}-values (96hpf) of $1.48 mg \cdot L^{-1}$ and $1.12 mg \cdot L^{-1}$ for the formulation and fenofibrate in DMF determined by the estimation factor (Table 3), respectively, would lead to a predicted no effect concentration (PNEC) of $1.42 \mu g \cdot L^{-1}$ and $1.09 \mu g \cdot L^{-1}$. However, this PNEC is above the environmental concentrations of fenofibrate. Thus, it can be assumed that the risk of fenofibrate towards fish is low. It needs to be kept in mind that the EC/LC values concerning the FET studies are estimations based on the bridging study which comprised only one experiment.

Although a higher ecotoxic potential of the formulation compared to fenofibrate dispersed in 20%-ISO-water was indicated, the risk for the aquatic environment does not necessarily increase. Unless vast amounts of fenofibrate-HPMC are accidentally released, the discharge of the formulation from wastewater treatment plants into the environment is very unlikely. The medical application of the formulation would lead to a reduced pharmacologically required dosage of fenofibrate due to the increased bioavailability in patients, while achieving the same therapeutic effect of the API. However, further work is required for an accurate risk assessment for fenofibrate and its formulation.

To assess environmentally relevant side effects, gene expression analyses and determination of apoptosis were performed. Fibrates such as fenofibrate act as an agonist at the receptor PPARα (Desvergne and Wahli, 1999; Kersten et al., 2000; Mandard et al., 2004). PPARα can bind to and regulate the expression of the gene encoding for acyl-CoA-oxidase which catalyzes the first reaction of β-oxidation in the peroxisome and leads to the production of hydrogen peroxide (Dreyer et al., 1992; Kliewer et al., 1992; Tugwood et al., 1992; Yoo et al., 1999; Reddy and Hashimoto, 2001; Mandard et al., 2004). Hydrogen peroxide belongs to the group of reactive oxygen species (ROS), which can cause oxidative stress and apoptosis (Lennon et al., 1991; Dypbukt et al., 1994; Gardner et al., 1997; Hampton and Orrenius, 1997; Davies, 1999; Saito et al., 2006; Halliwell, 2011; Redza-Dutordoir and Averill-Bates, 2016). Zebrafish also expresses PPARα (Ibabe et al., 2002). The morphological effects and mortality observed in the embryos exposed to higher concentrations of fenofibrate in DMF and fenofibrate-HPMC may have been related to changes in apoptosis.

However, the majority of embryos of all exposure groups did not display any changes in apoptosis. In rats, it was found that PPARα influences the expression of superoxide dismutase and is involved in the protection against oxidative stress (Yoo et al., 1999; Mandard et al., 2004). It was also seen that PPARγ is required for catalase expression (Girnun et al., 2002; Mandard et al., 2004; Lushchak, 2011) and an increase in catalase expression in cells treated with fenofibrate was observed (Zuo et al., 2015). Therefore, the absence of altered apoptosis seen in the embryos might be caused by a rather protective function of PPARα. This suggests that apoptosis is not a suitable endpoint for assessing the toxicity of fenofibrate. This can be different for other substances (Schiller et al., 2013; Bodewein et al., 2016) and thus, needs to be assessed for each substance accordingly.

We investigated the potential effects of fenofibrate and its formulation on glial cell development, the estrogen response and the immune response by the analysis of gene transcription of *hmgcr* and *mbp,* of *vtg-1* and *vtg-3*, and of *il-8*. The genes *hmgcr* and *mbp* were chosen, since Ashikawa et al. (2016) detected an increase in the expression of these genes in 5d old zebrafish embryos after exposure to 700 nM fenofibrate (0.253 $mg \cdot L^{-1}$). In zebrafish, *mbp* mRNA is detected from 72hpf (Buckley et al., 2010a). However, in our study, expression of *mbp* was reduced in embryos exposed to an estimated concentration of 0.757$mg \cdot L^{-1}$ fenofibrate in DMF and a trend towards decreased *mbp* expression was indicated in embryos exposed to fenofibrate-HPMC. Though gemfibrozil – a PPARα agonist like fenofibrate (Ashikawa et al., 2016; Friedland et al., 2012) – was also found to increase *mbp* expression in human cells via PPARβ (Jana et al., 2012), PPAR activity was shown not to affect *mbp* expression in zebrafish larvae ((Buckley et al., 2010b); Preston and Macklin, 2015). Moreover, we tested at 96hpf as compared to 120hpf in the study of Ashikawa et al. (2016). These reasons might explain the observed differences in gene expression. Contrary to the data of Ashikawa et al. (2016), we did not detect an increase in the expression of *hmgcr*. In zebrafish embryo mutants without a receptor for low-density lipoprotein (LDL)-cholesterol, an increased level of LDL-cholesterol as well as an increased *hmgcr* expression was found (O'Hare et al., 2014). This finding suggests a connection between the physiological concentration of LDL and *hmgcr* expression. Thus, it can be assumed that a lower level of LDL-cholesterol induced by the application of fenofibrate would cause a decrease in the expression of *hmgcr*.

Rats fed with PPARα agonists displayed an impaired estrogen metabolism (Mandard et al., 2004). In another study, the fibrate class of lipid regulators was reported to have endocrine disrupting potential (Delfosse et al., 2015; Maradonna and Carnevali, 2018). Moreover, fenofibrate caused an estrogenic response in a yeast estrogen screen and the E-screen assay (Isidori et al., 2009). Chronic exposure to benzafibrate - another fibrate- over 21 days affects gonadal steroidogenesis and spermatogenesis of adult male zebrafish and reproduction of crustaceans was impaired by fibrates (Isidori et al., 2007; Velasco-Santamaría et al., 2011). *Vtg-1* and *vtg-3* are expressed in the zebrafish embryo and are responsive to 17β-estradiol, which induces the production of vitellogenin - the female specific yolk protein, which is already present in the zebrafish embryo - via an estrogen receptor (ER)-responsive element (ERE) in the promoter region of *vtg* genes (Arukwe A, 2003; Gruber et al., 2004; Versonnen and J.C.R., 2004; Levi et al., 2009; Lam et al., 2011; Hao et al., 2013; Hara et al., 2016). Upon exposure to estrogen or endocrine disruptors, the production of vitellogenin can be induced (Örn et al., 2006; Zhong et al., 2014; Caballero-Gallardo K., 2016). Thus, there is a possibility that fenofibrate affects the endocrine system in the zebrafish. However, fenofibrate does not appear to affect estrogen signaling in the early life stages of the zebrafish. To further assess a potential estrogenic effect of fenofibrate, expression of other genes such as the aromatase gene *cyp19a1b* or the ER gene *esr2a* could be investigated, which are essential for endocrine signaling (Hawkins et al., 2000; Bardet et al., 2002; Lassiter et al., 2002; Menuet et al., 2002; Levi et al., 2009; Hao et al., 2013).

Finally, activation of PPARα by fibrates was observed to impact cytokine expression levels (Lee et al., 2007; Ghonem et al., 2015; Zuo et al., 2015). The gene *il-8* is expressed in the zebrafish larvae and is needed for the mediation of the neutrophilic response (de Oliveira et al., 2013). We therefore chose *il-8* to investigate possible effects of fenofibrate on the immune response of the zebrafish embryos. Fenofibrate in DMF or fenofibrate-HPMC induced an upward trend and the excipient a downward trend of *il-8* transcripts. Since none of these changes were significant, further investigations could either prove or disprove a possible effect on the immune system.

A significant change in gene expression in embryos was observed for fenofibrate in DMF, only. Thus, fenofibrate does not affect the expression of the genes investigated in this study in a statistically significant way at the early life stages of the zebrafish. As these two treatments showed similar patterns of expression of the genes encoding for *mbp* and *il-8*, a specific effect of fenofibrate on these genes may be indicated.

5. Conclusion

Embryos exposed to fenofibrate in DMF or the HPMC-formulation showed increased effects on survival and morphological defects. This suggests that the uptake of fenofibrate was improved by the fenofibrate-HPMC formulation and the solvent DMF. Fenofibrate-HPMC increased the ecotoxic potential of fenofibrate compared to the API dispersed in 20%-ISO-water. The finding that the formulation improved the bioavailability of fenofibrate for fish embryos was in line with our hypothesis, that formulations designed to increase the bioavailability of a drug in humans also improve bioavailability in fish. Although this would entail an increase in the ecotoxic potential of the drug, the requirement for lower prescription doses in such particulate formulations would effectively lead to a reduced release into the environment. Due to the predicted fate in the environment where fenofibrate is expected to adsorb to suspended solids and sediment, the availability of the drug and thus the exposure to fish can be considered low, unless no direct ingestion occurs.

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Michael J. ParnhamResources, Writing – Review & Editing, Funding acquisition

Walter BöhmerFormal analysis and Investigation (chemical analysis), Writing

Marc WeilerResources

Nazende Günday-TüreliConceptualization, Resources, Writing – Review & Editing, Methodology

Akif-EmreTüreliResources

HarshvardhanModhFormal analysis and Investigation (particle analysis) and visualization (particle size Figure 1)

Paul W.S. HengFormal analysis and Investigation (particle analysis) and visualization (particle size Figure 1)

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We hereby state that all authors have approved of the final article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Supplementary materials

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11 Absatz 1 Ziffer 1 TierSchG Tierschutzgesetz in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl. I S. 1206, 1313), das zuletzt durch Artikel 280 der Verordnung vom 19. Juni 2020 (BGBl. I S. 1328) geändert worden ist.
1-48 TierSchVersV Tierschutz-Versuchstierverordnung vom 1. August 2013 (BGBl. I S. 3125, 3126), die zuletzt durch Artikel 394 der Verordnung vom 31. August 2015 (BGBl. I S. 1474) geändert worden ist.
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