



## Zebrafish early life stages as alternative model to study ‘designer drugs’: Concordance with mammals in response to opioids

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### ARTICLE INFO

#### Keywords:

New Psychoactive Substances  
Zebrafish  
Opioids  
Toxicity  
Behaviour  
Biotransformation

### ABSTRACT

The number of new psychoactive substances (NPS) on the illicit drug market increases fast, posing a need to urgently understand their toxicity and behavioural effects. However, with currently available rodent models, NPS assessment is limited to a few substances per year. Therefore, zebrafish (*Danio rerio*) embryos and larvae have been suggested as an alternative model that would require less time and resources to perform an initial assessment and could help to prioritize substances for subsequent evaluation in rodents. To validate this model, more information on the concordance of zebrafish larvae and mammalian responses to specific classes of NPS is needed. Here, we studied toxicity and behavioural effects of opioids in zebrafish early life stages. Synthetic opioids are a class of NPS that are often used in pain medication but also frequently abused, having caused multiple intoxications and fatalities recently. Our data shows that fentanyl derivatives were the most toxic among the tested opioids, with toxicity in the zebrafish embryo toxicity test decreasing in the following order: butyrfentanyl > 3-methylfentanyl > fentanyl > tramadol > O-desmethyltramadol > morphine. Similar to rodents, tramadol as well as fentanyl and its derivatives led to hypoactive behaviour in zebrafish larvae, with 3-methylfentanyl being the most potent. Physico-chemical properties-based predictions of chemicals' uptake into zebrafish embryos and larvae correlated well with the effects observed. Further, the biotransformation pattern of butyrfentanyl in zebrafish larvae was reminiscent of that in humans. Comparison of toxicity and behavioural responses to opioids in zebrafish and rodents supports zebrafish as a suitable alternative model for rapidly testing synthetic opioids.

### 1. Introduction

New psychoactive substances (NPS), also called “designer drugs”, are emerging in the illicit drug market at a fast pace. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has reported monitoring of 790 NPS by the end of 2019 (EMCDDA, 2020). Synthetic cannabinoids (28%), cathinones (36%) and opioids (3%) are the main

groups of NPS that have been recorded and monitored. Although synthetic opioids represent only a small proportion of all NPS known to EMCDDA, they are of special concern for three main reasons: (a) opiates and opioids are often prescribed for pain relief (Vardanyan and Hruby, 2014), leading to their high use both licitly and illicitly; (b) they are highly potent, thus only a small error in dosage can result in a lethal overdose (Gergov et al., 2009); (c) clandestine laboratories often

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<https://doi.org/10.1016/j.taap.2021.115483>

Received 23 September 2020; Received in revised form 2 March 2021; Accepted 5 March 2021

Available online 13 March 2021

0041-008X/© 2021 The Author(s).

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produce synthetic analogues which have no medical use and are frequently sold under the guise of, for example, heroin (Turock et al., 2009; Breindahl et al., 2016). In the late 1990's, opioid prescriptions as pain medications increased significantly, resulting in a steady rise in opioid consumption in tandem with overdose and addiction problems (Dowell et al., 2016; Jones et al., 2018).

Opioids act primarily by binding to the mu ( $\mu$ ), kappa ( $\kappa$ ) or delta ( $\delta$ ) opioid receptors and can produce analgesic effects, anaesthetic effects, sedation and drowsiness (Benyamin et al., 2008; Byas-Smith et al., 2005; Hug Jr., 1992; Mahesh Trivedi and Gwinnut, 2007). Even for opioids mainly acting by binding to the mu receptors only, there are differences. Opioids display varying overall potencies, for example, with regard to the achieved analgesic profiles. This can be explained by, e.g., differences in the affinity and efficacy at the receptor. Thus, opioids have been categorized into low, medium and high potency opioids (Drewes et al., 2013). The major concerns in the opioid abuse are associated with the high potency synthetic opioids. In the United States and Canada, the current opioid epidemic is being driven by the use of synthetic opioids, particularly fentanyl derivatives. The fast growing market of synthetic opioids, their easy availability and lack of information on their effects pose a range of challenges for public health and drug policy as well as for the drug monitoring systems for their inclusion in routine drug testing (EMCDDA, 2020). Data on toxicity and mechanisms of action are lacking for many NPS, where often only a few representatives of a particular group may have some information available from animal toxicity tests or human user experience, which could allow judging on their potency (Pantano et al., 2019). Due to limited funds and the time and resource-intensive nature of rodent-based toxicity tests, only a few additional substances per year can be subjected to toxicological characterization. This is clearly insufficient, given the fast pace of NPS introduction on the market and their growing availability to global users (Dignam and Bigham, 2017; Elliott et al., 2017). While in vitro tests could provide additional means for rapid testing of some endpoints, they are at present not yet able to replace testing for complex effects which may occur in drug users, such as behavioural responses, chronic toxicity, and developmental toxicity. Therefore, it would be beneficial if there were an alternative animal model representative of whole-animal responses, which would allow testing the NPS toxicity more quickly and efficiently than is currently possible with rodents.

Such alternative approach could involve testing with zebrafish embryos and early larvae, which have been gaining popularity as a test organism complementing rodents in understanding the pharmacology of drugs, especially for toxicity and behavioural effects. Early life stages of zebrafish, spanning the period from fertilization to the start of independent feeding, are considered to be an alternative, non-animal test system. This model has a variety of applications in human hazard assessment, including acute systemic toxicity (Ali et al., 2011; Lammer et al., 2009; Padilla et al., 2012), chronic toxicity (Volz et al., 2011), teratogenicity (Gustafson et al., 2012; Selderslaghs et al., 2009), neurobehaviour (Kokel et al., 2010; Selderslaghs et al., 2010) and specific organ toxicities (Berghmans et al., 2008; Parnig et al., 2002). Zebrafish and mammals have highly conserved pharmacological targets (Rinkwitz et al., 2011; Howe et al., 2013). Studies have shown high similarity of fish and mammalian responses to small molecules that cause reproductive toxicity, behavioural effects, teratogenicity, carcinogenicity, cardiotoxicity, ototoxicity, or liver toxicity (Caballero and Candiracci, 2018; Eimon and Rubinstein, 2009; Kithcart and MacRae, 2017; Levin et al., 2003; Milan et al., 2003; Parnig et al., 2002).

The expression and function of opioid receptors in zebrafish have been shown to be both biologically and pharmacologically comparable to mammals, including rodents and humans. Moreover, zebrafish opioid receptor transcripts could be detected early in the development (before 3 h post fertilization) (Sanchez-Simon and Rodriguez, 2008; Gonzalez-Nunez and Rodriguez, 2009). Pain perception has been well studied in fish, including zebrafish, and behavioural studies are often used as indicators to study pain (Mettam et al., 2011; Reilly, 2008; Sneddon,

2009).

As part of the research focused on the potential use of the zebrafish model for toxicological characterization of NPS, the aim of the present study was to assess the toxicity and behavioural responses to opioids in the early life stages of zebrafish. By testing several opioids belonging to different potency categories, we addressed the following research questions: (a) whether opioids elicit similar or differing toxicity and behavioural effects in the early stages of zebrafish compared to mammals; (b) whether the opioids with varying potencies in mammals follow the same pattern in zebrafish and (c) whether there are similarities in the biotransformation of opioids between zebrafish larvae and mammals.

## 2. Materials and methods

### 2.1. Selection and acquisition of test compounds

We chose six opioids representing low, medium and high potency categories to assess their toxicity and behavioural effects in zebrafish larvae. These substances included morphine, a naturally derived opioid, chosen as a classical medium potency opioid with a long history of both licit and illicit use. Further, tramadol and its metabolite, *O*-desmethyltramadol, were chosen as low potency opioids and also due to tramadol's mixed mode of action. Tramadol's actions on the opioid receptors are mediated by its metabolite *O*-desmethyltramadol, while tramadol itself acts on the monoamine neurotransmission (Meyer et al., 2015). Therefore, we were interested in assessing the differences in the effects of tramadol and *O*-desmethyltramadol in zebrafish larvae. Finally, three fentanyl derivatives were chosen as high potency opioids. Three out of the five most harmful synthetic opioids listed by EMCDDA belong to the fentanyl group. For the three fentanyl drugs that we have selected, several intoxications have been reported in the recent years. These substances included fentanyl itself, which is a synthetic licit and illicit opioid, 3-methylfentanyl, which has the strongest analgesic potency of all fentanyls, and butyrfentanyl, which has caused several deaths, including a case documented at our institute (Staheli et al., 2016). Based on their high octanol-water partition coefficient and the inherent high potency, we assumed that fentanyl and derivatives (i.e., butyrfentanyl and 3-methylfentanyl) should demonstrate the highest toxicity in zebrafish. Based on the effects of opioids in mammals, we assumed that, like rodents, zebrafish larvae should respond with hypoactive behaviour to treatment with opioids.

The list of chemicals including their physico-chemical properties is provided in Table 1 and their chemical structures are given in Fig. 1. All six chemicals had analytical purity >98.5%; they were obtained from Lipomed (Arlesheim, Switzerland) with the permission from the Swiss Federal Office for Public Health to use controlled substances. For drug exposures, stock solutions were prepared in an appropriate volume of reconstituted water to achieve the desired final concentrations.

### 2.2. Zebrafish husbandry

Zebrafish (*Danio rerio*) of OBI wild-type strain (Leipzig, Germany) and wild mix background were maintained at 28 °C under a 14-h light/10-h dark cycle. Fish were reared in recirculating flow-through systems filled with a mix of desalted and tap water, treated with active carbon filter and UV light. Zebrafish were fed with a combination of live artemia and dry flakes twice daily. Eggs for exposure studies were collected from group crosses and were either exposed immediately for early exposures or maintained in reconstituted water (294.0 mg/l CaCl<sub>2</sub>·2H<sub>2</sub>O, 123.2 mg/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 64.74 mg/l NaHCO<sub>3</sub> and 5.75 mg/l KCl) until 5 days post fertilization (dpf) in a Petri dish (ca. 50 embryos per dish), placed in an incubator with the same conditions as mentioned above. All procedures were in accordance with the animal protection guidelines and were approved by the Swiss Cantonal Veterinary Office.

**Table 1**

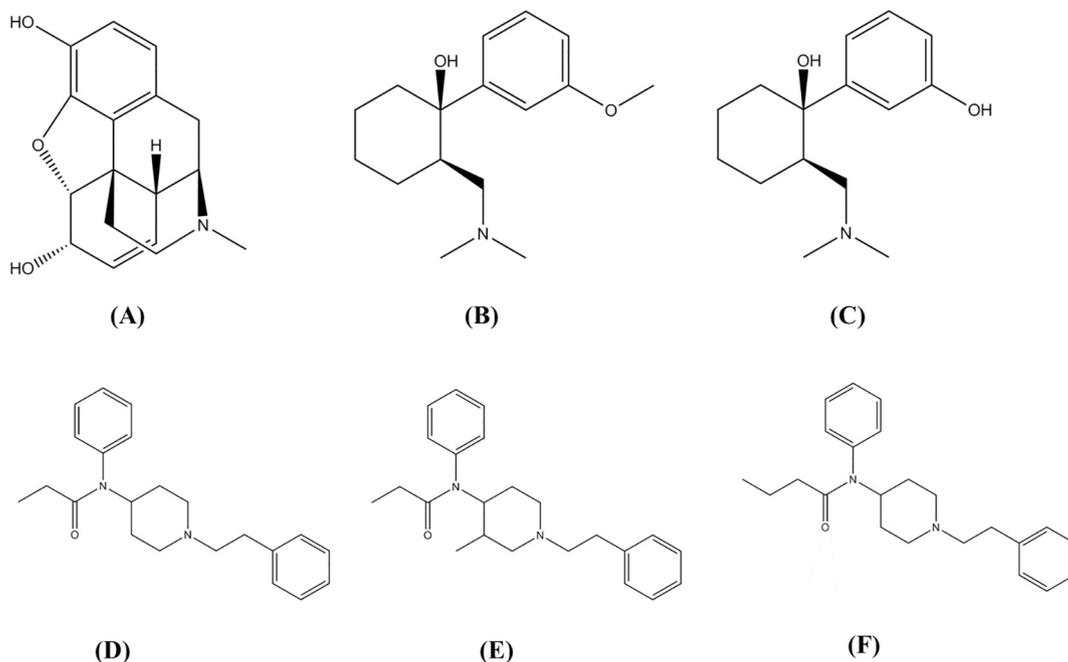
Physico-chemical properties of opioids used in this study. The logarithm of the octanol/water partition coefficient (logP) is a measure of lipophilicity. LogD describes lipophilicity at pH 7.4. The estimates for the dissociation constant (pKa) are given for the strongest basic site.

Compound	CAS number	Referred to in this work as	Molecular weight (g/mol)	log P <sup>a</sup>	log D <sup>b</sup>	pKa <sup>c</sup>
Morphine HCl.monohydrate	52-26-6	Morphine	339.8	0.72	-0.13	9.12
Tramadol HCl	36282-47-0	Tramadol	299.84	3.01	0.52	9.23
O-desmethyl-cis tramadol HCl	1018989-94-0	O-desmethyltramadol	285.81	2.45	0.07	8.97
Fentanyl citrate	990-73-8	Fentanyl	528.6	3.89	3.01	8.77
d, l-cis 3-methylfentanyl HCl	42045-86-3	3-methylfentanyl	386.96	4.31	2.88	9.08
Butyrfentanyl HCl	1443-52-3	Butyrfentanyl	350.5	4.38	3.31	8.77

<sup>a</sup> Prediction from EPI Suite™ software (Version 4.1, U.S. Environmental Protection Agency).

<sup>b</sup> Prediction from Advanced Chemistry Development, Inc. (ACD/Labs), Toronto, Canada.

<sup>c</sup> Prediction from ChemAxon, DrugBank (Version 5.0).



**Fig. 1.** Chemical structures of the tested opioids. (A) morphine; (B) tramadol; (C) O-desmethyltramadol; (D) fentanyl; (E) 3-methylfentanyl; (F) butyrfentanyl. Morphine (A) is a naturally occurring alkaloid containing a 4, 5-epoxymorphinan ring. Tramadol (B) and its metabolite O-desmethyltramadol (C) belong to the 3-amino-1-phenylpropan-1-ol series. Fentanyl (D) and its derivatives belong to the anilidopiperidine series; methyl substitution on the piperidine ring at 3rd position gives 3-methylfentanyl (E) and butyryl group instead of propionyl group gives butyrfentanyl (F).

### 2.3. Zebrafish embryo toxicity (zFET) test

zFET test was performed in triplicate according to the Organization for Economic Co-operation and Development (OECD) Test Guideline 236 (OECD, 2013) with a few modifications. Concentrations for the zFET test were chosen from an initial range-finding test (RFT) which was performed as a single replicate. The concentrations assessed in both tests for each chemical are shown in the supplementary table S1. For the RFT, 10 eggs per chemical concentration, and 20 eggs for the negative (blank) control, were exposed in a 96-well plate (one egg per well in 0.5 mL solution) from approximately two-cell stage to 120 h post fertilization (hpf) without renewing the drug solutions. For the zFET tests, 10 eggs per concentration, including a negative (blank) control, were exposed starting at approximately two- to sixteen-cell stage in a 24-well plate (one egg per well in 1 mL solution). Each day, exposure solutions were replaced with freshly prepared solutions. The embryos were monitored from 24 hpf to 120 hpf, which corresponds to 1 dpf and 5 dpf, respectively. Mortality and developmental abnormalities were recorded to calculate the lethal (LC<sub>50</sub>) and sublethal (EC<sub>50</sub>) metrics, respectively.

### 2.4. Short-term toxicity test

A maximum non-toxic concentration (MNTC) was calculated for each tested compound based on the short-term exposure test where larvae of 4 dpf were exposed for 24 h. The tests were performed in duplicates. At 5 dpf, larvae were monitored for any abnormalities and MNTC was defined as the concentration at which no significant effects were observed. The MNTC was then used to determine the maximum safe concentration that could be tested in the locomotor behaviour assessment, where test concentrations were chosen such that the MNTC was not surpassed.

### 2.5. Locomotor behaviour assessment

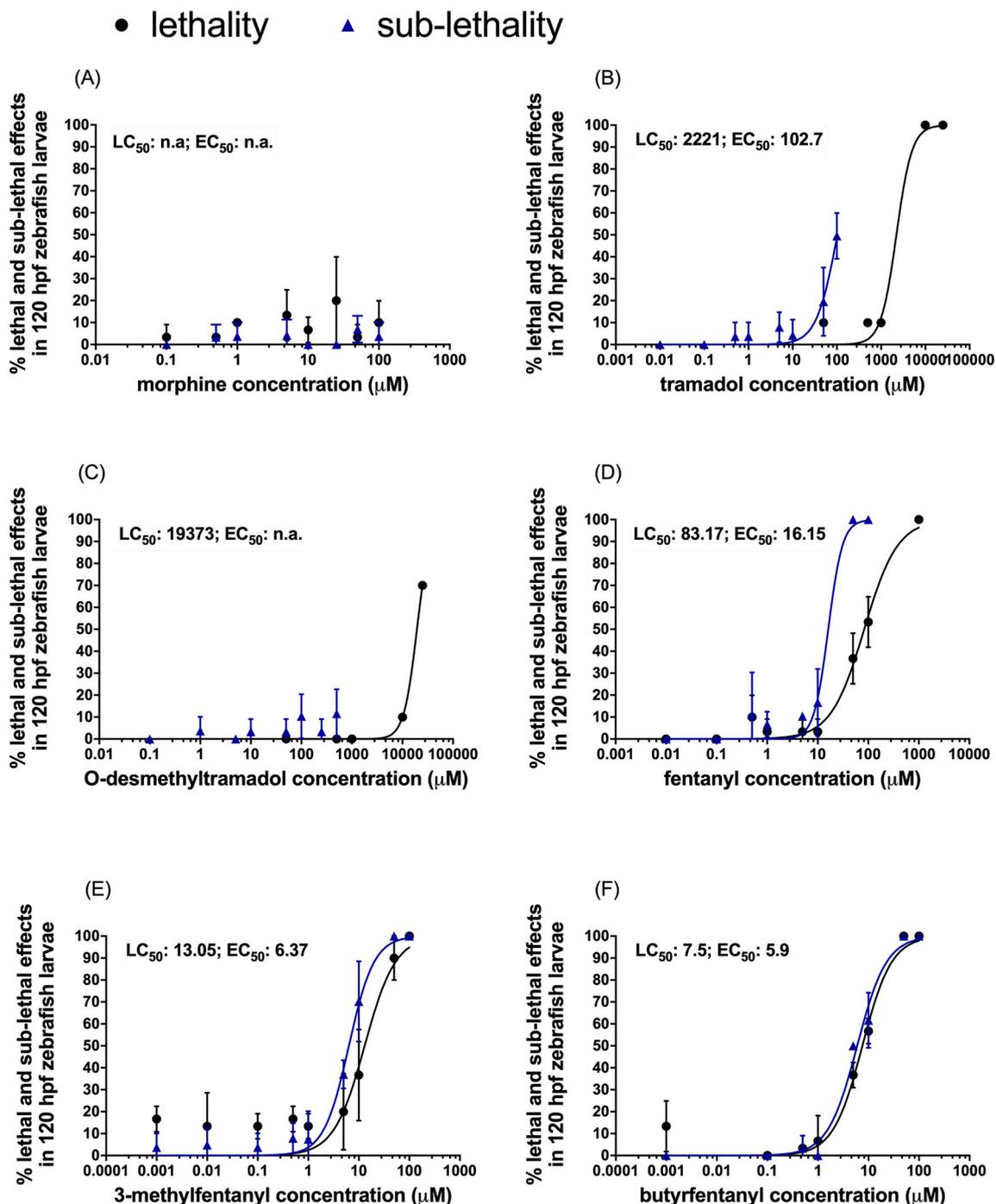
In the morning, unexposed larvae aged 5 dpf were distributed one larva per well in a 48-well plate with well volume of 500 µl and then acclimatized for 3 h in the housing incubator. After acclimatization, the test chemical was added and larvae were immediately placed on the recording platform for tracking the locomotor activity. Locomotor activity was assessed using the ZebraLab™ behaviour tracking system (Version 3, View Point, Lyon, France) consisting of a video camera of 25 frames per second. Detection threshold was set to 20 for tracking the

animal excluding any background. The protocol consisted of 70 min tracking with light and dark phases alternating every 10 min starting with light. Data were exported and analysed for every two min integration period.

## 2.6. Prediction of internal opioid concentrations

The bioconcentration factor (BCF) and time to reach steady-state ( $t_{ss}$ ) in the larvae were predicted for all test chemicals using previously described models (Hendriks et al., 2001; Stadnicka et al., 2012; Kirla

et al., 2016). To investigate if zebrafish larvae can biotransform tramadol to *O*-desmethyltramadol at the conditions used for behaviour experiments, larvae were frozen after 70 min of drug exposure and analysed for tramadol and *O*-desmethyltramadol by LC-MS/MS using the method described in (Staheli et al., 2016). To study the uptake kinetics and biotransformation patterns for butyrfentanyl, exposed larvae were pooled and frozen at different time points (16 larvae per time point). On the day of analysis, frozen larvae samples were thawed, homogenized, extracted with acetonitrile and analysed by LC-MS/MS method developed previously (Staheli et al., 2016). A Phenomenex



**Fig. 2.** Developmental toxicity of opioids. Zebrafish embryo toxicity (zFET) test was performed to determine the lethal ( $\text{LC}_{50}$ ) and sublethal ( $\text{EC}_{50}$ ) concentrations by exposing embryos to different opioids (A-F) from 0 to 5 days post fertilization (dpf) and monitoring the mortality and abnormalities. Data were obtained from three independent replicates with 10 embryos per treatment per replicate, except for tramadol and *O*-desmethyltramadol, where the black curves are based on the range-finding test from a single replicate. Values are reported as mean  $\pm$  SD.

Synergy Polar RP column (Aschaffenburg, Germany) was used to separate the analytes.

## 2.7. Statistical analysis

All data were exported and analysed using Microsoft Excel 2010. All graphs were plotted in GraphPad Prism® (Version 6 for Windows, GraphPad software, San Diego, California, USA). For the zFET test, lethal and sublethal concentrations were calculated using sigmoidal four parameter dose-response curves in GraphPad Prism® 6. For the locomotor activity assessment, statistical analysis was performed using RStudio (Version 0.98.486, USA) following commonly agreed methods for this type of data (MacPhail et al., 2009). In more detail, data were segregated into light and dark subsets and Repeated Measures Analysis of Variance was performed for each subset at each lighting condition. Each concentration was compared to control and among each other. Statistical significance was set at  $\alpha = 0.003$ .

## 3. Results

### 3.1. Lethal and sublethal effects in the zFET test

LC<sub>50</sub> and EC<sub>50</sub> were determined from the zFET tests (Fig. 2, Table 2). Morphine at the tested concentrations did not cause any dose-dependent mortality in the zFET test (Fig. 2A) or in the RFT even at the highest concentration of 25 mM (Supplementary table S1). There was also no developmental toxicity observed in the zFET test (Fig. 2A). Therefore, no LC<sub>50</sub> or EC<sub>50</sub> could be determined for morphine.

Tramadol did not cause mortality in the zFET test. Therefore, LC<sub>50</sub> was determined from the RFT (Fig. 2B), where lethal effects such as egg coagulation and lack of heartbeat were observed. Sublethal effects, such as deformed chorda and protruded mouth abnormalities, were observed in the zFET test at 50  $\mu$ M and higher concentrations; these effects became apparent mostly post-hatching. At lower concentrations, an occasional delayed hatching was noticed. However, by 5 dpf all larvae were hatched.

Similarly to tramadol, *O*-desmethyltramadol did not cause lethality in the zFET test and LC<sub>50</sub> was calculated from the RFT (Fig. 2C). Sublethal effects such as deformed chorda and abnormalities in protruded mouth were occasionally observed in the zFET test post-hatching, but no concentration-dependent drug effects were noticed. Therefore, EC<sub>50</sub> could not be determined for *O*-desmethyltramadol.

Exposure to fentanyl caused dose-dependent mortality in the zFET test (Fig. 2D). Absence of heartbeat was the main lethal endpoint. Cumulative mortality increased with time, especially post-hatching. Sublethal effects were observed as early as 48 hpf and steadily increased in number and severity over the duration of treatment. The main sublethal effects noted prior to hatching were pericardial oedema, yolk sac

oedema and slower heart rate. Post-hatching, deformed chorda and abnormalities in protruding mouth were observed additionally.

Similarly to fentanyl but with higher potency, 3-methylfentanyl caused dose-dependent mortality in the zFET test (Fig. 2E). Egg coagulation and lack of heartbeat were the main lethal effects. Sublethal effects were seen already at the lowest concentration tested (0.001  $\mu$ M) and increased with dose, affecting 100% larvae at the highest concentration of 50  $\mu$ M. Arrested growth, pericardial oedema and decreased heart rate were observed before hatching. After hatching, deformed chorda and yolk sac oedema were also detected.

Butyrfentanyl was the most potent among the compounds tested, with the lowest LC<sub>50</sub> value and 100% mortality observed at the two highest concentrations tested (Fig. 2F). Lack of heartbeat was the main lethal effect observed. In contrast to 3-methylfentanyl, sublethal effects started occurring only at 5  $\mu$ M and above and included pericardial oedema, malformed chorda, arrested growth and slowed heartbeat.

### 3.2. Short-term toxicity test to determine maximum non-toxic concentration

To determine the maximum non-toxic concentration (MNTC) to be used in locomotor behaviour experiments, a short-term 24 h exposure was carried out starting at 4 dpf (Fig. S1). For morphine, this test was not performed as there were no morphological effects observed from zFET test and therefore a 50  $\mu$ M concentration was used as highest for behavioural studies. For tramadol, no effects were observed at all concentrations tested except for one embryo at the highest concentration (Supplementary fig. S1A). Similarly, *O*-desmethyltramadol exposed larvae showed no effects at any tested concentrations during the 24 h exposure (Supplementary fig. S1B). Larvae treated with the highest concentration of fentanyl showed pericardial oedema and yolk sac oedema, while no effects were observed at lower concentrations (Supplementary fig. S1C). A short-term exposure to 3-methylfentanyl caused mortality at 15 and 25  $\mu$ M treatments and resulted in abnormalities at 10  $\mu$ M and above, which included pericardial oedema, irregular heartbeat, yolk sac oedema and deformed head and chorda (Supplementary fig. S1D). Butyrfentanyl at 10  $\mu$ M and higher caused sublethal effects such as posture imbalance and heart oedema (Supplementary fig. S1E).

### 3.3. Locomotor activity

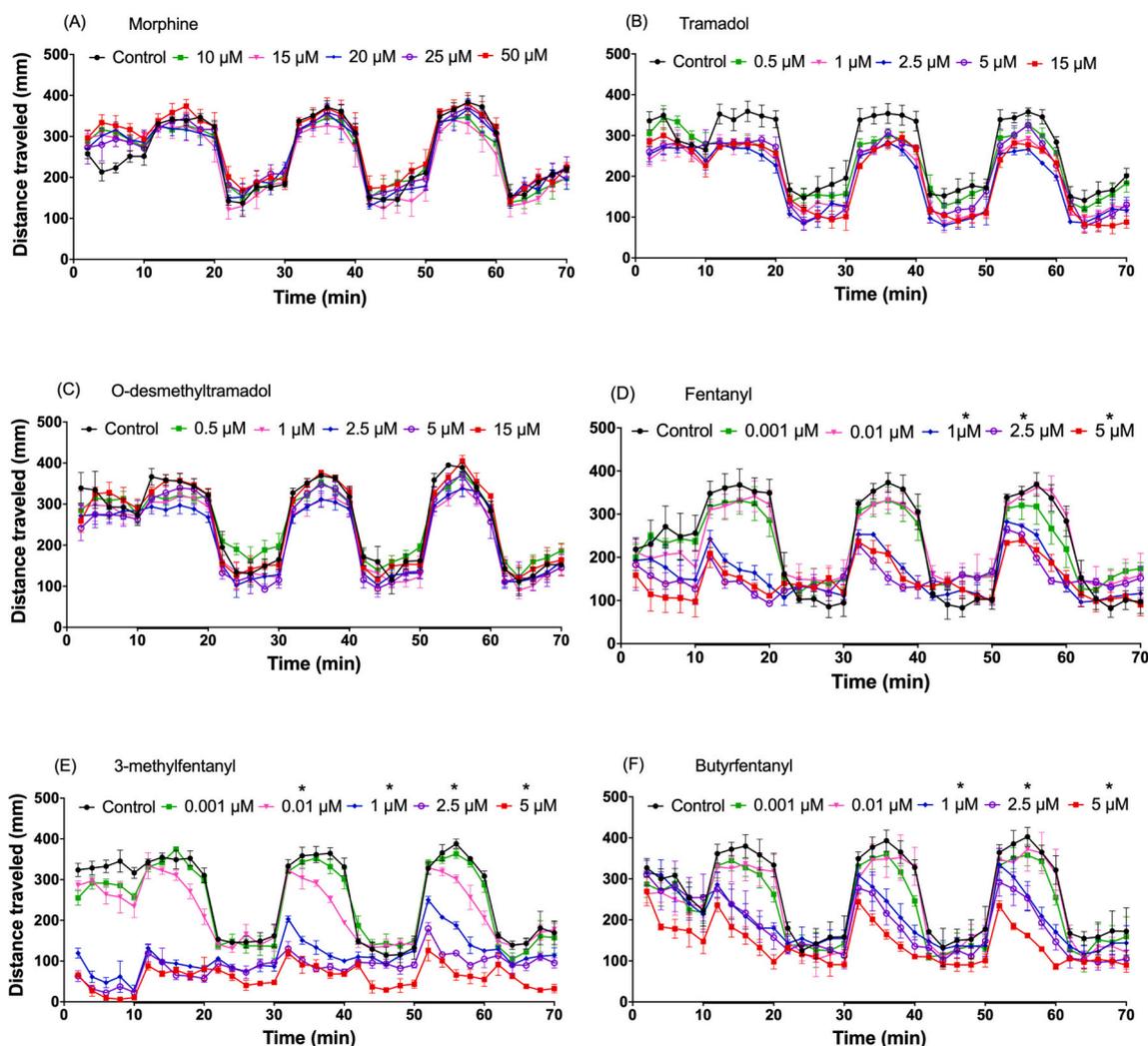
Effects on behaviour were assessed by monitoring the locomotor activity of the larvae (Fig. 3). The concentration that starts to produce a significant behaviour effect was termed the lowest significant behaviour effect concentration (LSBEC) (Table 2). To determine the impact of morphine and tramadol on the behaviour of zebrafish larvae, locomotor activity was assessed for two different concentration ranges, low and high. Morphine did not significantly alter the locomotion at any tested

**Table 2**

Overview of data collected in the course of this study. The shown values include the experimentally measured lethal (LC<sub>50</sub>) and sublethal (EC<sub>50</sub>) toxicity and the lowest significant behaviour effect concentration (LSBEC) values, as well as bioconcentration factor (BCF) and time to steady-state (*t*<sub>ss</sub>) predicted by modeling. LC<sub>50</sub> and EC<sub>50</sub> were determined from the zebrafish embryo toxicity (zFET) test and behaviour was assessed by tracking the locomotor activity of the larvae, both tests performed in triplicates. LC<sub>50</sub> and EC<sub>50</sub> values are presented as mean with confidence intervals (CI<sub>95</sub>). Note that LC<sub>50</sub> and EC<sub>50</sub> for morphine and EC<sub>50</sub> for *O*-desmethyltramadol could not be determined as no respective effects were observed neither in the zFET test nor in the range-finding test. For tramadol and its metabolite *O*-desmethyltramadol, LC<sub>50</sub> values were calculated from a range-finding test conducted as a single replicate, because no mortality was observed in the zFET test. BCF and *t*<sub>ss</sub> were predicted based on physico-chemical properties and using a one-compartment toxicokinetic model.

Opioid	LC <sub>50</sub> ( $\mu$ M)	CI <sub>95</sub>	EC <sub>50</sub> ( $\mu$ M)	CI <sub>95</sub>	LSBEC ( $\mu$ M)	Predicted BCF (l/kg)	Predicted <i>t</i> <sub>ss</sub> (days)
Morphine	n.a.		n.a.		n.a.	0.9	35.4
Tramadol	2221 <sup>a</sup>	483.8-10,196.0 <sup>a</sup>	102.7	83.2-126.8	1	1.1	9.3
<i>O</i> -desmethyltramadol	19,373 <sup>a</sup>	19,370.0-19,375.0 <sup>a</sup>	n.a.		n.a.	0.98	23.3
Fentanyl	83.1	67.8-102.0	16.1	10.8-24.2	1	51.9	1.6
3-methylfentanyl	13	8.7-19.6	6.3	5.3-7.6	0.01	38.7	1.5
Butyrfentanyl	7.5	6.2-9.1	5.9	5.1-6.9	1	102.8	1.7

<sup>a</sup> As lethality could not be determined from the zFET test, LC<sub>50</sub> values for these two chemicals were obtained from a range-finding test conducted as a single replicate.



**Fig. 3.** Locomotor behaviour effects of opioids in zebrafish larvae. Locomotor activity was tracked in 5 days post fertilization (dpf) larvae after immediate exposure to different concentrations of opioids (A-F). Data were obtained from three independent replicates with 8 larvae per treatment per replicate. Values are reported as mean  $\pm$  SEM. \* above a particular concentration indicates the statistically significant difference compared to control.

concentration (Supplementary table S2A), neither in the low (Supplementary fig. S2) nor high (Fig. 3A) range. Therefore, LSBEC could not be determined for morphine. The locomotor activity of tramadol exposed larvae decreased at the high concentration range with the impact being significant at concentrations  $\geq 1$   $\mu$ M (Fig. 3B) (Supplementary table S2B). In the low concentration range (i.e., 1  $\mu$ M), no significant changes in the activity were induced (Supplementary fig. S3). *O*-desmethyltramadol did not significantly alter the locomotion of the larvae at any tested concentration (Fig. 3C, supplementary table S2C). Therefore, the LSBEC of *O*-desmethyltramadol could not be determined. Fentanyl significantly reduced the locomotor activity of the larvae at 1  $\mu$ M and above, mainly under the stimulus condition, i.e. dark (Fig. 3D, Supplementary table S2D). 3-methylfentanyl produced significant reduction in the locomotor activity in a dose-dependent manner, starting at 0.01  $\mu$ M (Fig. 3E) both in the light and dark conditions (Supplementary table S2E). Butyrfentanyl significantly reduced the locomotor activity in a dose-dependent manner starting at 1  $\mu$ M in the dark (Fig. 3F, Supplementary table S2F).

### 3.4. Bioconcentration factor and steady state predictions

The BCF and time to reach steady-state ( $t_{ss}$ ) that were predicted for the tested opioids based on their physico-chemical properties and using a one-compartment toxicokinetic model are shown in Table 2. A

relationship between the logD values of the chemicals and the predictions made could be observed, that is, in general, with the decreasing logD values (Table 1), their predicted BCF decreases while time to steady-state ( $t_{ss}$ ) increases (Table 2).

### 3.5. Uptake and biotransformation

In mammals, analgesic activity of tramadol is mainly driven by its metabolite *O*-desmethyltramadol (Meyer et al., 2015). In our study, when we observed the toxic and behavioural effects of these compounds separately, it was not clear whether the effects seen from tramadol were caused by itself or its biotransformation product. Therefore, we analysed whether zebrafish larvae can biotransform tramadol. To analyse biotransformation of tramadol to *O*-desmethyltramadol, zebrafish larvae were exposed for 70 min to the high range of tramadol concentrations similar to those used for locomotor activity assay. With increasing exposure concentration, internal concentration of tramadol increased. However, *O*-desmethyltramadol was not detected in any of the analysed samples (Supplementary table S3). The limit of quantification (LOQ) was about 0.001  $\mu$ M for *O*-desmethyltramadol. The lowest concentration of tramadol that could be measured was 10 times higher than the LOQ of *O*-desmethyltramadol.

Butyrfentanyl biotransformation was studied in zebrafish larvae in order to compare the pattern to that in humans as analysed in blood

samples and in a documented post-mortem case at our institute (Stacheli et al., 2016). For this, we studied the uptake of butyrfentanyl by exposing the zebrafish larvae to 5  $\mu\text{M}$  and quantifying the internal concentrations over time. Butyrfentanyl was quantifiable in the whole-body homogenates from the first measured time point (30 min). The uptake increased gradually, reaching steady-state around 6 h, with an internal concentration of  $165 \pm 5 \text{ mg/kg}$  (Fig. 4). The experimental data on the uptake fits well with the predicted uptake based on the pH corrected octanol-water partition coefficient, that assumes passive uptake of butyrfentanyl. Zebrafish larvae biotransformed butyrfentanyl by oxidation to hydroxy-butyrfentanyl followed by conjugation to the corresponding glucuronide (Fig. 4). Over the studied time interval, about 8–26% of the parent compound taken up underwent oxidation and 1–9% underwent glucuronidation while the rest remained unchanged (Supplementary table S4).

## 4. Discussion

### 4.1. Correlation of physico-chemical properties to the toxic effects observed

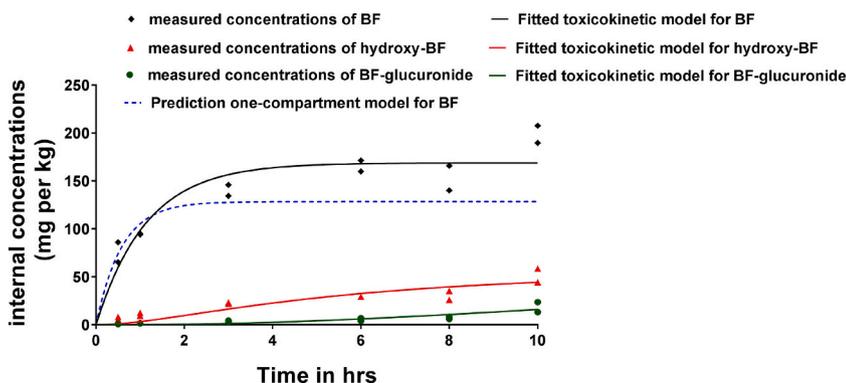
No significant lethal and sublethal effects upon morphine treatment in zebrafish embryos/larvae was observed, likely due to the low uptake rate during aqueous exposure, suggested by the low pH-corrected octanol-water partition coefficient of this compound. The uptake could also be influenced by the speciation of morphine, which depends on the pH and temperature. At physiological pH and the exposure temperature (28 °C), charged species ( $\text{HOB}^{\text{NH}^+}$ ) of morphine would dominate, affecting morphine permeation through the membranes (Stevens and Balahura, 2007). Low uptake of morphine administered in exposure water has been reported in goldfish with uptake being less than 1% of the amount present in water (Newby et al., 2009). Compound uptake in the zebrafish embryo increases with increasing lipophilicity of the respective chemical leading to high internal concentrations (de Koning et al., 2015). Sulfamethoxazole, which has a logP (0.79) close to that of morphine and, like morphine, predominantly exists in ionic form under the exposure pH (7.4), similarly resulted in very low uptake with a relative internal concentration of 0.18 compared to the external medium concentration (Brox et al., 2016). The predicted BCF and  $t_{\text{ss}}$  of morphine (Table 2) also agrees with its low accumulation in zebrafish embryos. These findings support the notion that the lower the logP, the lower the substance's likelihood to be toxic to the developing zebrafish (Padilla, 2013). Another aspect important for the bioavailability of the drug is its solubility. It was suggested that morphine hydrochloride's solubility in exposure medium can be decreased by the presence of chloride ions in the medium due to common-ion effects (Stevens and Balahura, 2007).

In a large scale toxicity assessment of compounds in zebrafish embryos and larvae exposed for 1–5 dpf, an  $\text{LC}_{50}$  of 123.8 mM from 1 to 3 dpf and 23.39 mM at 4 and 5 dpf was obtained for morphine hydrochloride (Ali et al., 2011). In our study, the highest concentration tested

in the range-finding test (25 mM) (Supplementary table S1) gave only 10% mortality at 4 and 5 dpf. While the pH of the test medium was the same in both studies, there are two main differences: First, and the most important difference is that, while the embryos have been exposed by 2 hpf in our study, in Ali's study exposure started only at 24 hpf. Second, while we refreshed the test solutions each day to ensure the same exposure concentration in the medium, Ali and co-workers have used a static non-replacement regime throughout the exposure duration, meaning there was no refreshment of test solutions. They observed buffer loss over the exposure period, which could increase the concentration of the drug. While assessing the effects of morphine on the immune system in zebrafish embryos, Mottaz and coworkers (Mottaz et al., 2017) measured the internal concentration of morphine sulphate. Fish exposed to 1 mg/l (1.5  $\mu\text{M}$ ) of morphine from  $\approx 1\text{hpf}$  to 4 dpf accumulated 11.7 ng/g (0.0117 mg/kg) wet weight. Assuming the similar uptake of morphine hydrochloride (1 mg/l is 3.1  $\mu\text{M}$ ), 4 days of exposure in our study would result in a maximum internal concentration of 0.3 mg/kg at 100  $\mu\text{M}$  exposure or 94 mg/kg at 25 mM exposure, the maximum concentration tested in this study. With an oral bioavailability of 20–40%, morphine with an  $\text{LD}_{50}$  of 470–745 mg/kg in rodents (Higashikawa and Suzuki, 2008) would need a minimum of 94 mg/kg–298 mg/kg to be lethal. In another study, only about 5% of exposed morphine was detected in the embryo after an exposure to 10 nM morphine from 5 hpf to 24 hpf and 48 hpf (Sanchez-Simon et al., 2010). Comparing this 5% uptake to the present study would result in 0.015 mg/kg internal concentration at 100  $\mu\text{M}$  exposure. Thus, the internal concentrations achieved in zebrafish embryos in our study could have been too low to produce effects.

In the zFET test, tramadol is more toxic in zebrafish embryos than its metabolite *O*-desmethyltramadol. This is consistent with the higher logD value of tramadol than *O*-desmethyltramadol, predicting lower uptake of *O*-desmethyltramadol into zebrafish embryo. Tramadol toxicity could also be exerted by a mechanism other than activation of mu opioid receptors by its active metabolite *O*-desmethyltramadol. For example, tramadol is also known to act as inhibitor of monoamine reuptake and as inhibitor of ligand-gated ion channels (Doostmohammadi and Rahimi, 2020), and it can also induce oxidative stress (Plhalova et al., 2020). The absence of tramadol toxicity observed in our experiments at low concentrations (< 10  $\mu\text{M}$ ) is consistent with other studies where tramadol exposure up to 16.75  $\mu\text{M}$  produced no lethal or sublethal effects except for delayed hatching reported by Sehonova et al. (Sehonova et al., 2016; Bachour et al., 2020; Plhalova et al., 2020).

Fentanyl derivatives with the higher logP values showed higher potency. Based on the  $\text{LC}_{50}$  values, butyrfentanyl was almost 11 times more toxic than fentanyl and 3-methylfentanyl about 6 times more toxic than fentanyl. The physico-chemical properties-based modeling also predicted the highest accumulation of butyrfentanyl, followed by fentanyl and 3-methylfentanyl. While the logP values of fentanyl, 3-methylfentanyl and butyrfentanyl do not differ much, the additional difference in their toxicities could be due to differential receptor affinity



**Fig. 4.** Uptake kinetics and biotransformation of butyrfentanyl (BF) in zebrafish larvae. Larvae at 5 dpf were exposed to 5  $\mu\text{M}$  BF up to 10 h. At different time points during the uptake, butyrfentanyl and metabolites in the whole-body homogenates were measured by LC-MS/MS. Data were obtained from two independent replicates with each replicate containing 16 pooled fish per time point. Symbols represent experimental data; solid lines show the fitted toxicokinetic model; dashed line shows the prediction of uptake based on one-compartment model.

and thereby different potency. Compared to all tested chemicals, butyrfentanyl and 3-methylfentanyl showed most severe toxicity from 24 to 48 hpf (data not shown), reflecting their likely effects on the development of heart, brain and bilateral organization, which are the major events happening during embryo development at these stages (Kimmel et al., 1995). The pericardial oedema of the larvae, which was observed for all fentanyls, might have resulted from the homeostasis imbalance due to the failure of water permeability barrier. This toxic response has also been observed upon exposure of embryos to other chemicals, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hill et al., 2004).

#### 4.2. Behavioural effects of opioids in zebrafish larvae

Morphine had no effects on the locomotor activity in zebrafish larvae at 5 dpf. This result is consistent with previous findings at comparable concentrations tested (Ali et al., 2012). However, these authors observed a biphasic response in the locomotor activity, visible only at concentrations  $\geq 3$  mM, i.e. initial excitation at 3–12 mM followed by sedation at much higher concentrations of  $\geq 12$  mM. However, it has to be emphasized that the exposure setups used in the two studies are very different, with their chronic (96 h) exposure and 70 min exposure in our study. In another study with a more comparable exposure period of 1 h, which analysed the choice-behaviour in 2 week old larvae, morphine at 0.8  $\mu$ M (within our low concentration test range of 0.5–5  $\mu$ M) did not alter the swimming activity (Breteau et al., 2007). Therefore, the morphine concentration reached at target sites during the 70 min exposure duration might have been too low to cause any changes in locomotor activity.

The measured outcome of locomotor activity shows tramadol to be more potent in zebrafish larvae compared to its metabolite *O*-desmethyltramadol. In mammals, *O*-desmethyltramadol produced as a metabolite of tramadol is known to be 200 times more potent than the parent compound because of its higher affinity to opioid receptors (Meyer et al., 2015). However, when we administered *O*-desmethyltramadol as an individual substance, we did not observe any changes in the locomotor activity. There could be at least two explanations. The first possibility is that, in mammals, *O*-desmethyltramadol is produced internally as a metabolite of tramadol *in vivo*. However, in our work, *O*-desmethyltramadol itself was administered as a drug. Therefore, as discussed above, a too low uptake of this compound could have precluded the observation of any effects. The other possibility concerns the mixed mechanism of action of tramadol. Both tramadol and *O*-desmethyltramadol are centrally acting opioid analgesics. In addition, tramadol can interfere with serotonin neurotransmission in a way similar to drugs such as meta-chlorophenylpiperazine (mCPP) (Kirla et al., 2018) and para-methylthioamphetamine (Gobbi et al., 2002). In our previous study (Kirla et al., 2018), we observed a decreased locomotor activity in zebrafish larvae exposed to mCPP, which is a serotonin receptor agonist and serotonin reuptake inhibitor. Therefore, tramadol induced hypoactive behaviour in zebrafish larvae could be driven via the serotonin neurotransmission system rather than via the opioid system. This is also supported by tramadol's weaker affinity to mu ( $\mu$ ) opioid receptor, which is the predominantly expressed receptor in the larval stage (Sanchez-Simon and Rodriguez, 2008; Gonzalez-Nunez and Rodriguez, 2009).

Of all the opioids tested, 3-methylfentanyl had the highest potency for reducing the locomotor activity of the larvae. The fentanyl-based series of compounds are among the most potent analgesics because of their highly selective affinity to mu ( $\mu$ ) opioid receptors (Bagley et al., 1991; Vardanyan and Hruby, 2014). Moreover, changes in the scaffold structure of fentanyl results in different biological and pharmacological activities. A 3-methyl substitution at the third position of the piperidine ring causes a sharp increase of  $\mu$ -affinity and selectivity. This increases the potency of 3-methylfentanyl to cause hypoactive behaviour in zebrafish larvae. To summarize, the behavioural effects of different

opioids tested in zebrafish shows that fentanyl derivatives, 3-methylfentanyl followed by butyrfentanyl are the most potent opioids with regard to behavioural effects.

#### 4.3. Evaluation of concordance between zebrafish larvae and mammals

The potencies of all tested chemicals were compared to that of fentanyl, as is frequently performed in rodents. To compare the toxicity between zebrafish larvae and mammals, LC<sub>50</sub> values were used (Table 3). Centrally acting analgesics are known to decrease the locomotor behaviour activity in rodents (Fidecka et al., 1978). The analgesic effect of opioids is known to also result in sedation and thereby have less activity in rodents (Craft et al., 2006; Taylor et al., 2016). Therefore, we compared the behavioural effects measured in zebrafish larvae to the analgesic potency in mammals (Table 4). For this, the lowest concentration that altered the locomotor activity in the larvae, i.e., the LSBEC, was compared to the effective analgesic dose (ED<sub>50</sub>) in mammals.

Morphine effects on the locomotion in rodents are varied. One study reported a biphasic response, with 1–10 mg/kg showing excitation and a higher concentration of 32 mg/kg showing decreased activity upon intraperitoneal injection (Li et al., 2013). In contrast, in another study that also reported a biphasic response with 1–10 mg/kg morphine administered subcutaneously, an initially suppressed and later increased locomotor activity was observed (Craft et al., 2006). Other studies showed no significant alterations in locomotion at 4–7 mg/kg upon intraperitoneal injection (Rezayof et al., 2009; Rezayof et al., 2013). Nevertheless, as discussed above, results from the study of continuous morphine exposure in zebrafish embryos at much higher concentrations suggest that morphine can induce behavioural effects, but only at the concentrations higher than tested here (Ali et al., 2012).

In zebrafish larvae, lethality with tramadol was not observed in the zFET test; however, mild sub-lethal effects were noted. Tramadol use during early pregnancy in humans has been reported to be non-lethal to the developing foetus. However, 0.05% of infants showed congenital malformations such as cardiovascular defects and defective foot (Bloor et al., 2012; Källén and Reis, 2015). The LD<sub>50</sub> potency ratio of tramadol compared to fentanyl in zebrafish larvae is close to the range found in rodents (Table 4). However, the ratio of the concentration producing hypoactivity to fentanyl is 500 times higher in zebrafish larvae compared to the analgesic dose in rodents (Table 4). For tramadol, the hypoactive behaviour and analgesic activity could be different because the analgesic activity is known to be mediated by tramadol's active metabolite *O*-desmethyltramadol (Meyer et al., 2015). However, when we analysed for *O*-desmethyltramadol in the tramadol exposed larvae during 70 min, no *O*-desmethyltramadol could be found; even the LOQ of *O*-desmethyltramadol being 10 times lower (0.001  $\mu$ M) than tramadol. This suggests that the hypoactive behaviour observed could be mediated by tramadol itself.

3-methylfentanyl LD<sub>50</sub> in rodents is two times lower (Mefentanyl) than the LC<sub>50</sub> observed in zebrafish larvae, what can be considered comparable, especially given the different route of administration in rodents and zebrafish. Butyrfentanyl toxicity in zebrafish and mammals could not be compared to LC<sub>50</sub> of fentanyl because of the lack of data on their LD<sub>50</sub> in rodents. In mammals, butyryl substitution is supposed to have lower opioid potential than fentanyl itself. However, we observed that during development, butyrfentanyl resulted in higher lethality than fentanyl. This suggests that the developmental stages of an organism could be more sensitive to the toxic effects of these chemicals compared to the fully developed organism. Fentanyl is used for pain treatment during pregnancy in humans and animals. Developmental effects and neonatal effects of fentanyl have been reported from clinical studies as well as case studies. The adverse events and toxicities that were reported have occurred due to respiratory depression, cardiovascular effects and neuronal-related events such as seizures, effects on eye and brain coordination (Ancora et al., 2017; Hardwick Jr. et al., 1997; Muller and Vogtmann, 2000; Ostwal et al., 2015).

**Table 3**

Comparison of the lethal concentrations of different opioids to fentanyl in rodents and zebrafish larvae. LD<sub>50</sub> or LC<sub>50</sub> ratio to fentanyl is obtained by dividing the LD<sub>50</sub> or LC<sub>50</sub> of fentanyl to the LD<sub>50</sub> of each chemical in rodents and LC<sub>50</sub> in zebrafish larvae. IV: *intravenous administration*; IP: *intraperitoneal administration*.

Opioid	LD <sub>50</sub> in rodents, mg/kg	LC <sub>50</sub> in zebrafish, μM	LD <sub>50</sub> or LC <sub>50</sub> ratio to fentanyl	
			Rodents	Zebrafish
Morphine	Oral: 470 <sup>a</sup> , 745 <sup>b</sup>	n.a	0.13	n.a
Tramadol	Oral: 300-350 <sup>c</sup> , IV-50-100 <sup>c</sup>	2221	Oral: 0.17–0.2, IV: 0.03–0.06	0.037
O-desmethylnaloxone	n.a	19,373	n.a	0.004
Fentanyl	Oral: 62 <sup>a</sup> , IV: 3.1 <sup>d</sup> IP: 76 <sup>e</sup>	83.1	1	1
3-methylfentanyl	IP: 24.8 <sup>f</sup>	13	3.1	6.35
Butyrfentanyl	n.a	7.5	n.a	14.34

<sup>a</sup> Higashikawa and Suzuki, 2008.

<sup>b</sup> Ali et al., 2011.

<sup>c</sup> Matthiesen et al., 1998.

<sup>d</sup> DrugBank, 2021.

<sup>e</sup> Fentanyl, N.C.F.B.I., n.d..

<sup>f</sup> Mefentanyl, N.C.F.B.I., 2021.

**Table 4**

Comparison of the analgesic potency of opioids to fentanyl in rodents and lowest significant behaviour effective concentration (LSBEC) to fentanyl in zebrafish larvae. Potency ratio to fentanyl is obtained by dividing the ED<sub>50</sub> or LSBEC of fentanyl to that of each chemical both in rodents and zebrafish larvae.

Opioid	ED <sub>50</sub> mg/kg (analgesia) rodents	LSBEC (μM) in zebrafish	analgesic/locomotor activity potency ratio to fentanyl	
			Rodents	Zebrafish
Morphine	0.33 <sup>a</sup>	n.a.	0.018	n.a.
Tramadol	4.2 <sup>b</sup> , 8.9 <sup>c</sup>	1	0.001, 0.0006	1
O-desmethylnaloxone	2.94 <sup>c</sup>	n.a	0.002	n.a.
Fentanyl	0.006 <sup>a</sup>	1	1	1
3-methylfentanyl	0.00058–0.0068 <sup>a</sup>	0.01	0.9–10.5	100
Butyrfentanyl	0.047 <sup>a</sup>	1	0.13	1

<sup>a</sup> Higashikawa and Suzuki, 2008.

<sup>b</sup> Hennies et al., 1988.

<sup>c</sup> Dursteler et al., 2006.

The span between lethality and sublethality causing concentrations (i.e., LD<sub>50</sub> and ED<sub>50</sub> values) of butyrfentanyl and 3-methylfentanyl is very narrow, suggesting a high risk when consumed (Higashikawa and Suzuki, 2008). In humans, several intoxication cases are known to have resulted from the consumption of butyrfentanyl (Bäckberg et al., 2015; Staeheli et al., 2016) and 3-methylfentanyl (Martin et al., 1991; Ojanpera et al., 2006; Ojanpera et al., 2008). The narrow span between lethal and sublethal concentrations of fentanyl derivatives is perfectly reflected in the zebrafish model as well, with LC<sub>50</sub> and EC<sub>50</sub> differing by less than 30% in the case of butyrfentanyl and by only 2 times for 3-methylfentanyl (Fig. 2). Another reason for the deaths associated to fentanyl and its derivatives is the adulteration of the illicit traditional drugs with these high potent ones (Algren et al., 2013). This is also one of the main contributors to the ongoing opioid epidemic. Hypoactive behaviour induced by fentanyl in zebrafish larvae is concordant to the suppression of the locomotor activity in rodents in an open-field exploration test after intraperitoneal injection (Fidecka et al., 1978). Opioid receptor expression has been studied at early developmental stages in zebrafish and homology to the mammalian opioid system was found to a large extent (Gonzalez-Nunez and Rodriguez, 2009). Fentanyl, 3-methylfentanyl and butyrfentanyl effects on the locomotor activity leading to hypoactive behaviour can be explained by their analgesic and sedative actions upon binding to the opioid receptors. When compared to fentanyl, the analgesic potency in rodents and potency to cause hypoactivity in zebrafish larvae is the highest for 3-methylfentanyl (Table 4). The potency ratio to fentanyl, however, is about 10 times higher for 3-methylfentanyl and 7 times higher for butyrfentanyl in zebrafish larvae than in rodents.

The uptake of butyrfentanyl in zebrafish larvae is driven by passive diffusion processes as supported by the similarity of the predicted and measured internal concentration values. The biotransformation of butyrfentanyl majorly by phase-I to OH-butyrfentanyl and phase-II to

glucuronide in zebrafish larvae is similar to OH-butyrfentanyl observed in authentic human samples and OH-butyrfentanyl and butyrfentanyl-glucuronide observed in a post-mortem case. CYP2D6 and CYP3A4 are suggested to be the main enzymes involved in the biotransformation of butyrfentanyl. In the post-mortem case, carboxy-butyrfentanyl was also found as a major metabolite (Staeheli et al., 2016; Steuer et al., 2016). However, it has been suggested that the carboxy-butyrfentanyl producing pathway is activated only as an alternative if CYP3A4 is inhibited. Thus, under natural conditions of CYP3A4 function, this metabolite would not have been produced. We have looked for carboxy-butyrfentanyl in zebrafish larvae as well, but could not detect it, suggesting that in live zebrafish larvae, CYP3A4 function was normal.

In conclusion, zebrafish embryos and larvae are sensitive to synthetic opioids with toxicity and behavioural effects comparable to rodents. Moreover, the low, medium and high potency opioids show similar response patterns in zebrafish and mammals, and biotransformation of butyrfentanyl was also comparable. Thus, zebrafish model can be used to gain information on the developmental effects of opioids, occurring when pregnant women use them for pain medication or abuse them. Further, this test organism may be used to assess opioid-adulterated drug suspects, frequently contributing to the recently observed increase in drug-related deaths. When the potencies were compared with fentanyl taken as a reference, differences were seen especially for the analgesic versus locomotor activity effects with zebrafish larvae being more sensitive than rodents. Because of the different routes of exposure in zebrafish larvae and mammals, the internal concentration measurements and distribution studies could aid in explaining the differences. Here, characterization of the internal concentrations and distribution kinetics might provide a clue for the observed differences between the organisms. Overall, zebrafish early stages appear to represent a promising model for early screening of NPS suspects allowing to rapidly gain information on the drug's toxicity and behavioural effects.

## Declaration of Competing Interest

There are no competing financial interests by any of the authors.

## Acknowledgements

We thank the following members of the Department of Environmental Toxicology (Eawag, Switzerland): M. Zimmermann and P. Reichlin for the assistance in maintaining the zebrafish facility, R. Schönenberger, P. Longree (Department of Environmental Chemistry, Eawag) for the assistance in acquiring permission to use controlled substances from the Swiss Federal Office for Public Health. We thank S. Staeheli and M. Poetzsch for their help with the analytical measurements. We express gratitude to Emma Louise Kessler, MD for her generous legacy she donated to the Institute of Forensic Medicine at the University of Zurich, Switzerland for research purposes.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2021.115483>.

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