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Extensive review of fish embryo acute toxicities for the prediction of GHS acute systemic toxicity categories

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ABSTRACT

Distribution and marketing of chemicals require appropriate labelling of health, physical and environmental hazards according to the United Nations global harmonisation system (GHS). Labelling for (human) acute toxicity categories is based on experimental findings usually obtained by oral, dermal or inhalative exposure of rodents. There is a strong societal demand for replacing animal experiments conducted for safety assessment of chemicals. Fish embryos are considered as alternative to animal testing and are proposed as predictive model both for environmental and human health effects. Therefore, we tested whether LC_{50}s of the fish embryo acute toxicity test would allow effectively predicting of acute mammalian toxicity categories. A database of published fish embryo LC_{50} containing 641 compounds was established. For these compounds corresponding rat oral LD_{50} were identified resulting in 364 compounds for which both fish embryo LC_{50} and rat LD_{50} was available. Only a weak correlation of fish embryo LC_{50} and rat oral LD_{50} was obtained. Fish embryos were also not able to effectively predict GHS oral acute toxicity categories. We concluded that due to fundamental exposure protocol differences (single oral dose versus water-borne exposure) a reverse dosimetry approach is needed to explore the predictive capacity of fish embryos.

1. Introduction

The estimation of mammalian acute systemic toxicity is important for the international distribution and marketing of chemicals. Acute toxicity studies are mainly conducted to respond to the regulatory request for hazard classification and labelling by various sector-specific regulations for industrial chemicals, agrochemicals, biocides and pharmaceuticals (reviewed in Seidle et al., 2010). In order to increase international regulatory consistency and efficiency, the United Nations global harmonisation system (GHS) has implemented rules for classification of (human) acute systemic toxicity categories (UN, 2011; Seidle et al., 2011). This classification is based on experimental findings obtained by oral, dermal or inhalative single dose exposures of rodents and calculation of effect concentrations for lethality (Seidle et al., 2010). According to certain thresholds for the LD_{50} five acute toxicity categories are assigned: category I–III (labelled as "danger"), category IV (labelled as "warning") and category V (no label). Category V comprises compounds with low acute systemic toxicity. The thresholds for the different categories are specific for the exposure route (oral, dermal or inhalative) and label warnings normally reflect the most severe hazard category (UN, 2011).

Acute toxicity classification has been critically discussed based on scientific and ethical grounds (e.g., Seidle et al., 2010, 2011; Robinson et al., 2008; Creton et al., 2010; Kinsner-Ovaskainen et al., 2009). Therefore, various approaches have been developed to refine, reduce or replace the use of animal experiments to derive LD_{50}s. For instance, the OECD has approved modifications of the initial protocols for the oral and inhalation routes (OECD 401 and OECD 403), such as the *Up and Down*, *Fixed Dose* and *Acute Toxic class methods* that reduce the number of animals to derive an LD_{50} or acute toxicity category by about 70% (OECD 420, 423,425, 436; Seidle et al., 2010). Also the need to consider exposure via multiple routes has been questioned since the acute toxicity classification would usually have been achieved by sole conduction of oral exposure experiments (Moore et al., 2012; Seidle et al., 2011). In the pharmaceutical sector the conduction of acute toxicity tests has been reduced since the information obtained from acute toxicity studies is of little or no value in the drug development process (Robinson et al., 2008). Hence, they were no longer considered required prior to human clinical trials for pharmaceuticals (Seidle

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et al., 2010). Given the observed correlation of *in vitro* cytotoxicity with human blood plasma LC_{50} (Ekwall et al., 1998; Sjöström et al., 2008) it was suggested to reduce the number of animal tests at least by using *in vitro* toxicity data to derive starting concentrations for acute toxicity assessment (Seidle et al., 2010).

Despite the evolutionary distance also fish embryos have been suggested as predictive model for human or mammalian acute systemic toxicity (Parng et al., 2002; Ali et al., 2011). In compliance with international animal welfare regulations, the fish embryo model provides an ethically acceptable small scale analysis system with the complexity of a complete organism (Halder et al., 2010; Embry et al., 2010; Strähle et al., 2012). Fish embryos are characterised by a high versatility of applications in toxicology ranging from prediction of acute fish toxicity (Lammer et al., 2009; Belanger et al., 2013; Knöbel et al., 2012), fish early life stage toxicity (Volz et al., 2011; Villeneuve et al., 2014), to endocrine disruption (Brion et al., 2012; Thienpont et al., 2011), organ toxicity (Scholz, 2013), teratogenicity (Brannen et al., 2010; Gustafson et al., 2012; Selderslaghs et al., 2012) and the aforementioned mammalian acute systemic toxicity.

The studies that investigated the potential capacity of fish embryos to predict mammalian (oral) acute systemic toxicity have obtained only a weak or partial correlation (Parng et al., 2002; Ali et al., 2011). These investigations were focused on the comparison of fish embryo LC_{50} and mammalian LD_{50} obtained from oral or intravenous application of a limited number of – mostly pharmaceutical – compounds (18 and 60, respectively). This weak correlation is probably related to fundamental differences in exposure and pharmacokinetics in rodents and fish embryos (single oral dose versus water-borne exposure; plasma peak concentrations versus partition equilibrium). However, to meet classification and labelling, precise LD_{50} prediction would not be necessary (Seidle et al., 2010) and hence, the weak or partial correlation of fish embryo and mammalian acute toxicity might be sufficient to predict appropriate acute toxicity categories. At least fish embryo data may allow to reliably identify compounds with high acute toxicity. Therefore, in order to critically evaluate the predictive capacity of fish embryos, LC_{50}s were collected from the scientific literature and compared to rat LD_{50} and corresponding acute toxicity categories. The analysis was limited to oral toxicities in rats since the largest set of data was available for this exposure route and species, and since oral toxicity is – if compared to dermal and inhalation routes – for most of the compounds more sensitive than other exposure routes and the main driver of classification.

2. Material and methods

2.1. Literature survey for fish embryo LC_{50}

Fish embryo acute toxicity LC_{50} were obtained from the articles of Lammer et al. (2009), Belanger et al. (2013) and Knöbel et al. (2012), by a search of the open literature for studies not included in these reviews (using a combination of the key words "fish embryo" and "toxicity" and manual selection) and from internal unpublished data. Publications available until April 2013 have been included. According to the EU directive on the protection of animals used for scientific purposes we considered only LC_{50} data that were obtained with embryonic stages until the stage of independent feeding (EU, 2010). This stage in the zebrafish embryo refers to about 5 days post fertilisation (dpf) (Strähle et al., 2012). However, we also considered data from a study of Padilla et al. (2012) in which zebrafish embryos were exposed until 5 dpf but analysed at 6 dpf after a recovery rate of 24 h. The recovery period in this study aimed at protecting the staff in charge of the analysis of the embryos but may lead to higher lethality rates if compared to 5 dpf. For other fish species (*Oryzias latipes*, 240 hpf; *Psetta maxima*, 144 hpf) longer exposure periods were allowed if they were considered as embryonic stages.

Turbot (*P. maxima*) was the only marine species with fish embryo data and most of the 10 compounds tested by turbot exhibited an about 1000-fold lower LC_{50} if compared to other species (Mhadhbi et al., 2012; Mhadhbi and Beiras, 2012; Mhadhbi and Boumaiza, 2011). Since it is not clear whether the higher sensitivity is associated with a specific higher sensitivity of turbot or deviations in the exposure and evaluation protocols, the turbot data have not been considered for the analysis.

For the comparative analysis with rat oral LD_{50} only fish embryo studies were considered that were able to derive an LC_{50} within the tested range of concentrations.

2.2. Origin of rat LD_{50}

Rat LD_{50} corresponding to compounds for which fish embryo LC_{50} were available were obtained from the ChemIDPlus Advanced database of the United States National Library of Medicines (available at http://chem.sis.nlm.nih.gov/chemidplus/) or via the eChemPortal of the OECD (available at http://www.echemportal.org). The latter refers to different databases and the exact origin is available in the Supplement excel file. The data are listed in Supplement excel Table 2.

2.3. Identification of physico-chemical characteristics

Physicochemical characteristics ($\log K_{ow}$, Henry's law coefficient, water solubility and pk_a where appropriate) were mainly obtained from the ChemIDPlus Advanced database. If the data were not available in the ChemIDPlus Advanced database they were predicted with the ECOSAR software (Clements and Nabholz, 1994) by entering the chemical structure.

2.4. Modelling of fish embryo LC_{50}

A large number of data (>300 compounds) have been analysed in a screening study of the US EPA (Padilla et al., 2012). Since this study was reporting cumulative AC_{50} values that integrated lethal and sublethal effects (hatching, morphology), the raw data were reanalysed in order to obtain LC_{50}. For most of the compounds Padilla et al. (2012) tested only 2 embryos per concentration. In this case the LC_{50} was calculated as the geometric mean of the highest concentration with no mortality and the lowest concentration with 100% mortality. For a few compounds, up to 14 embryos per concentration were tested. For these compounds the LC_{50}s were calculated with the Hill slope equation using the software JMP 10.0 (SAS, Cary, NC):

$$Y = \mathrm{Min} + \frac{\mathrm{Max} - \mathrm{Min}}{1 + \left(\frac{x}{EC_{50}}\right)^{-p}} \quad (1)$$

Min and Max values were set to 0 and 100%. x and y represented the nominal exposure concentration and the survival rate (percentage), respectively. p refers to the slope of the sigmoidal concentration–response curve.

3. Regression analysis

Regression analysis was conducted using a Deming (type II) regression in order to consider variability for both the independent and dependent variable. The regression analysis was performed using the Software Sigma Plot 12.0. (Systat Software GmbH, Erkrath, Germany). The regression analysis of $\log K_{ow}$ versus fish embryo LC_{50} was conducted in two steps. Initially all values for

compounds with a logK_{ow} between −1 and 5 with a predicted narcosis mode-of-action were considered for the regression analysis. The narcosis mode of action was predicted based on QSAR models of Verhaar (Verhaar et al., 1992) and Russom (Russom et al., 1997) using the software ChemProp 5.2.7 (ChemProp, 2012). Then, the residues for the fish embryo LC_{50} were calculated and outliers were identified with a box plot analysis using the software IBM SPSS (IBM, Ehningen, Germany). Outliers represented values more than 1.5-fold of the interquartile distance below or above the 25% or 75% percentile value and were removed since they may not represent compounds with a narcosis mode of action. Subsequently the regression analysis was repeated excluding the outliers.

4. Results and discussion

4.1. Compilation of a data base for fish embryo LC_{50}

A total of 71 studies – including those reviewed by Lammer et al. (2009) and Belanger et al. (2013) – that reported fish embryo mortality and were published until April 2013 were identified (see Fig. 1 and Supplement excel Table 1). These studies and internal unpublished data of the authors covered 641 compounds, of which for 471 compounds at least one study has revealed an LC_{50} in either the catfish (*Clarius gariepinus,* one compound) fathead minnow (*Pimephales pomelas*, 5 compounds), medaka (*O. latipes*, 3 compounds) and zebrafish (*Danio rerio*, all other compounds). The entire dataset of fish embryo LC_{50}, corresponding physico-chemical properties and source of data is available as a Supplement excel file.

For 70 compounds the LC_{50}s of individual compounds were analysed for different exposure durations (24–120 h starting from fertilisation) within the same study (Supplement Table S1). The data showed a relatively strong cumulative toxicity for 7 compounds indicated by lower LC_{50} (<5-fold) in exposures for 72–120 h in comparison to the LC_{50} obtained after 48 h of exposure (0–48 hpf). For 8 compounds a relative LC_{50} of 2–3 was observed. The 0–48 hpf LC_{50} was used as a reference value, since this time window was analysed in the majority of the studies that considered different exposure durations. For two compounds – 2,4-dichlorophenoxy acetic acid (2,4-D) and malathion – deviating results from two different studies were observed. For 2,4-D, Ton et al. (2006) already observed mortality in the 0–48 hpf exposure window, while no toxicity was observed for this exposure window in the range of solubility by an internal study (UFZ, unpublished results). However, Ton et al. (2006) did not reported whether a pH adjustment was conducted. Hence, the observed deviation may result from a reduced pH in the testing solution. 2,4-D is a weak acid and due to its pk_a of 2.73 dissolution results in a pH of around 3. For our internal analysis pH was adjusted to 7 (UFZ, unpublished results). For malathion both available studies indicated a cumulative toxicity but the relative LC_{50}s (i.e. LC_{50} of exposure periods ⩽48 h vs. >48 h) varied from 3 to 34.

In order to consider the partial higher sensitivity of fish embryos in prolonged exposures and to avoid false negatives in the subsequent comparison with rat LD_{50} the fish embryo mortality data were aggregated by using the lowest available LC_{50} for each compound. The identified compounds covered a wide range of logK_{ow}s (−8 to 8) and fish embryo LC_{50}s (10^{-6} to 10^{3} mM). A majority of compound were exhibiting a logK_{ow} of −1 to 5 and LC_{50}s of 10^{-4} to 10^{1} mM (Fig. 1). On average organic compounds with lower logK_{ow} showed a higher toxicity within a logK_{ow} between −1 and 5. A slope of −0.92 (confidence interval −1.10

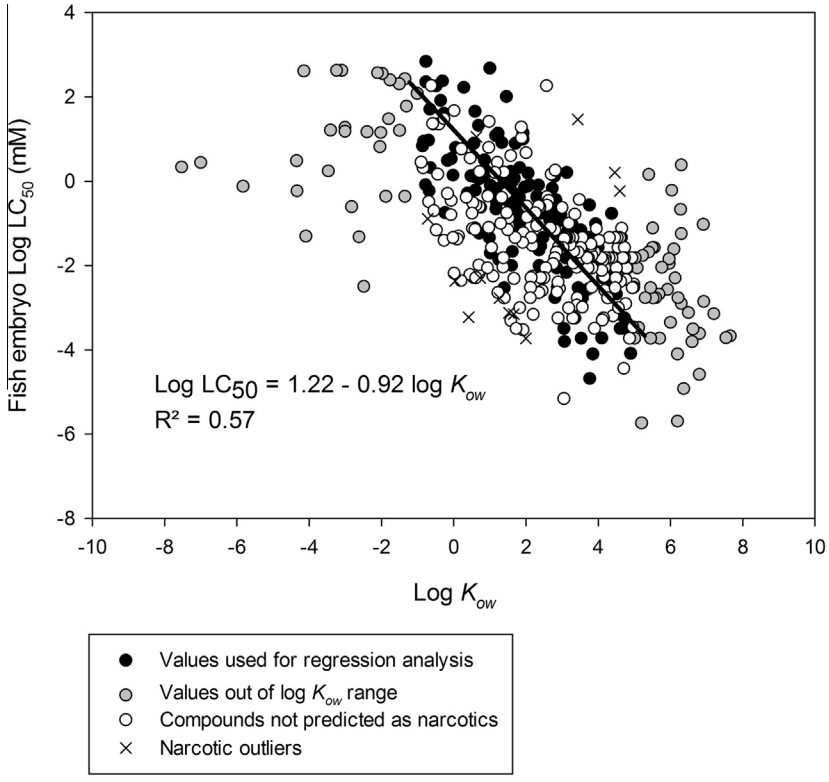

Fig. 1. Distribution of available fish embryo LC_{50} in relation to the logk_{ow}. The figure summarises data obtained from about 471 compounds with exposure durations from 24–120 h. In case that different exposure windows and durations were analysed in one study, the most sensitive data point was displayed. Similarly, if LC_{50}s were available from different studies, the lowest LC_{50} was displayed. Compounds that did not provoke any mortality in the tested range of concentration or where the mortality was <50% in the tested range of concentrations are included in a table in the Supplement (Table S2). The regression analysis was conducted for compounds with a logk_{ow} range from −1 to 5, predicted as narcotics and after removal of outliers. For details on effect levels, species, exposure period, data origin and outlier analysis see section 2 and Supplements.

to −0.75) and an intercept of 1.22 was observed for a regression analysis of the logK_{ow} and compounds with a predicted narcosis mode of action. The slope close to −1 indicates that hydrophobicity of the compounds is a major driver of their toxicity in fish embryos. A very similar slope (−0.94) was also observed for acute adult acute fish toxicity data (Russom et al., 1997).

For 168 compounds (Supplement Table S2) all studies indicated no mortality or the mortality was below 50%. It was difficult to relate a lack of toxicity to physico-chemical properties of the compounds or a weak sensitivity of the fish embryo, since in many cases only a limited range of concentrations was tested. Furthermore, many studies analysed a maximum exposure duration of 48 h and in some cases (e.g., Luviquat and 2,4-D) longer exposure periods were necessary to provoke toxicity. However, compounds with no LC_{50} were included in the comparison with acute oral systemic toxicity categories (see below).

4.2. Correlation of fish embryo LC_{50} and rat oral LD_{50}

The acute systemic toxicity classification according to the GHS system is based on determination of LD_{50} via oral, dermal and/or inhalation exposure of mammalian models, mainly rat our mouse. In order to explore the potential predictive capacity of fish embryo LC_{50} for mammalian acute systemic toxicity, we limited our comparative analysis to rat oral LD_{50}, since for this species and exposure route the greatest number of LD_{50} corresponding to fish embryo LC_{50} were available and since classification is usually based on the oral route (see above). For 364 compounds with available fish embryo LC_{50}s corresponding rat oral toxicity data were identified. The rat LD_{50} ranged from 0.02 to 50100 mg/kg. For 37 compounds tested up to maximum concentrations of 2000–10,000 mg/kg, no acute oral rat toxicity was observed. In order to conduct a correlation analysis, compounds for which no acute toxicity was reported within the tested range of concentrations were not considered. A linear regression analysis of fish embryo LC_{50} and rat oral LD_{50} did only reveal a weak correlation between fish and rat (Log Rat LD_{50} (mmol/kg) = 0.84 + 0.35 ∗ Log fish embryo LC_{50} (mmol/L); Fig. 2). Partially similar and partially deviating observations were made by previous studies using zebrafish embryos. Parng et al. (2002) compared the fish embryo LC_{50} and rodent LD_{50} of 20 compounds. No clear correlation was obtained for these compounds but fish embryo LC_{50} were generally close or below the rodent LD_{50}. Ali et al. (2011) compared 60 compounds and were able to obtain a correlation. The obtained slope of the regression curve of 0.84 for the dependency of rodent LD_{50} on fish embryo LC_{50} was much higher as the slope observed in this study (0.34). This deviation could result from the limited number of compounds tested by Ali et al. (2011), potential similarities of pharmacokinetics of the compounds in their dataset or a different exposure setup. Ali et al. (2011) did expose fish embryos from 24 to 120 hpf while the LC_{50}s used in the present study (which includes data of Parng et al., 2002 and Ali et al., 2011) were based on different exposures scenarios mainly starting from fertilisation and conducted for 48 h.

4.3. Potential impact of protocol variability on the fish embryo LC_{50} – rat oral LD_{50} correlation

The correlation analysis was based on effect concentrations that were derived from different protocols with various deviations in assay conditions. Therefore, the impact of species sensitivity, protocol differences and the chorion as potential source of variability/error is discussed below.

(1) *Species sensitivity:* The dominant species used for determination of fish embryo acute toxicity is zebrafish (>98% of all studies). This excludes a major variability introduced by species difference. Furthermore, a species-specific analysis has been made in previous studies for the comparison of acute (adult) fish versus fish embryo toxicity. These previous correlation analyses have shown that the influence of the species is relatively low. I.e. regardless whether the correlation analysis was restricted to certain species or not, or if different species were compared, the overall correlation was quite high (Belanger et al., 2013; Lammer et al., 2009). For mammalian toxicity overall species peculiarities may also be excluded as a reason for the weak fish embryo LC_{50} – rat LD_{50} correlation. A comparison of rat and mouse LC_{50} for compounds included in this study indicated a high correlation (R^2 = 0.85) with a slope (0.91) not significant different from 1 and a small intercept (0.08) (Supplementary Fig. S2).

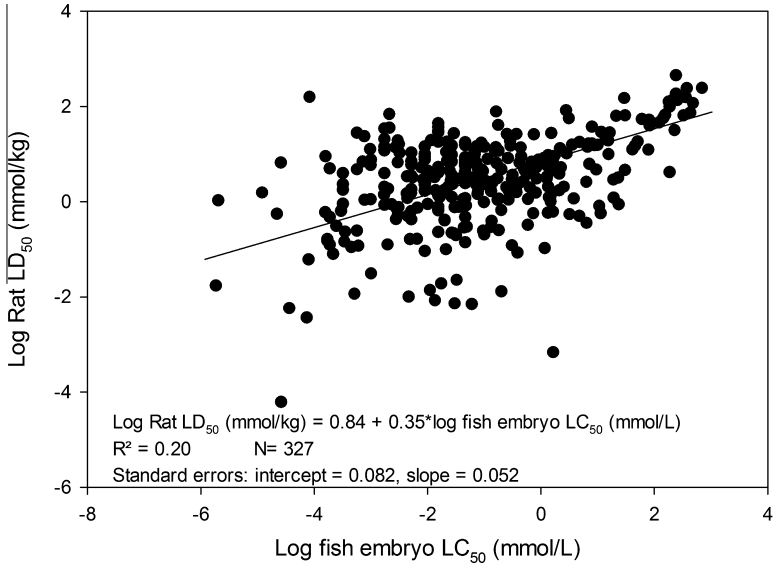

Fig. 2. Correlation of fish embryo LC_{50} and rat LD_{50}. The analysis was based on 326 compounds for which a pair of rat LD_{50} corresponding to available fish embryo LC_{50} could be identified. For fish embryos the lowest LC_{50} was used in case that a compound was analysed in different studies and/or different time windows. The slope of the regression was significantly different to 0 and 1 ($p < 0.0001$).

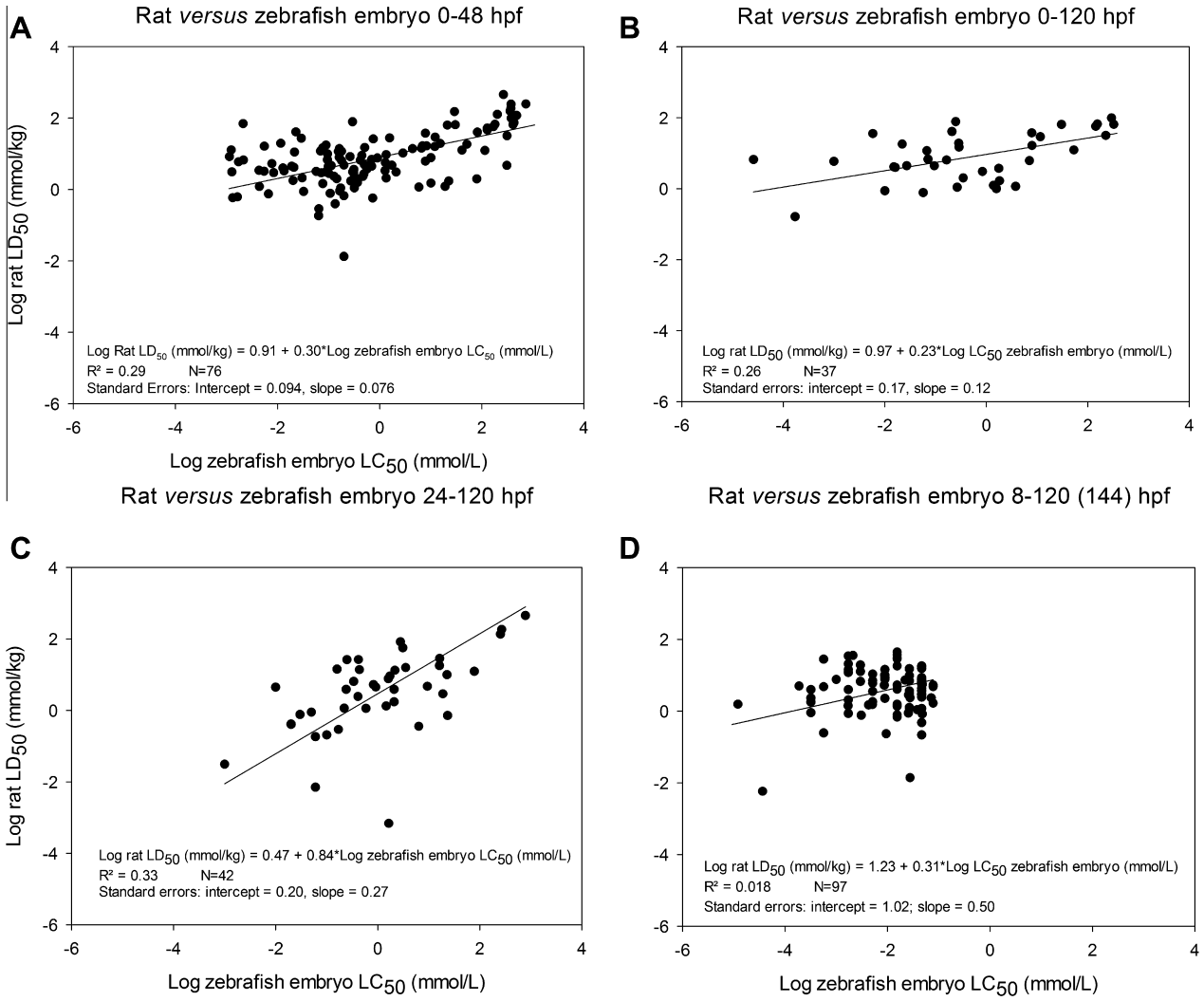

Fig. 3. Correlation of fish embryo LC_{50} and rat LD_{50} for selected exposure protocols. The analysis was based on four major exposure durations used in the fish embryo acute toxicity test (A – 0–48, B – 0–120, C – 24–120 and D – 8–120 h post fertilisation (hpf) with an additional recovery period of 24 h). The data represent subsets of those used in Fig. 2. The slope of the regression was significantly different to 0 and 1 in all analyses ($p < 0.0001$). An exposure start of 0 hpf indicates that exposure was initiated within at least 2 h after fertilisation. Note that most of the LC_{50}s in D are based on geometric means and hence, equal LC_{50} concentrations are obtained for many compounds since the same series of molar concentrations were tested.

(2) *Protocol differences:* A variety of different conditions is used for conduction of the fish embryo acute toxicity test. Exposure duration, exposure medium, exposure volume, the type of exposure vessels, illumination, use of solvents, number of embryos per replicate and whether pH and oxygen concentrations are measured represent the major differences (Table 1 and Supplement excel file 1). In order to analyse the impact of exposure protocols we compared results from experiments with different exposure duration as a potential major experimental conditions that could affect the LC_{50} (Fig. 3A–D). Restriction of the correlation to specific exposure durations (Fig. 3) did not improve the correlation. However, for one exposure protocol (8–120 hpf with a recovery period of 24 h) used in the study of Padilla et al. (2012) a significantly weaker correlation and higher standard error of correlation coefficients have been observed. It is likely that this is primarily caused by the different range of LC_{50} concentrations that could be used for this correlation since the maximum test concentration was 80 μM. Whether the weaker correlation is also caused by the low number of embryos used per replicate or the recovery period requires further experimental verification.

The relatively weak effect of exposure duration on the correlation is also indicated by the comparison of fish embryo acute to adult 96 h acute fish toxicity. This comparison has shown that the correlation is not severely altered if fish embryo test data are restricted to e.g., studies with 96 h of exposure (Belanger et al., 2013). Hence, whether fish embryo tests with different exposure periods are included or not does not significantly affect the overall correlations – albeit it may lead to differences of a few individual compounds.

(3) *The chorion as a potential uptake barrier:* Fish embryo tests include a period of development inside the chorion. In zebrafish embryos hatching occurs around 48–72 hpf and hence the chorion could represent a potential barrier for compound uptake until this stage. However, electron microscopy has shown that the chorion has pores of 0.17 μm^2 (Cheng et al., 2007). This suggests that the chorion may only represent a barrier for e.g., particles or high molecular weight compounds, as indicated by experiments with Luviquat, carbon nanotubes or fluorescein dextran >3000 Da (Cheng et al., 2007; Creton, 2004; Henn and Braunbeck, 2011). Nearly all compounds in the database established for this study have molecular weights in the range of 100–400 Da with a few

Table 1
Major protocols used for the fish embryo tests. The publications of Lammer et al. (2009) and Belanger et al. (2013) represent reviews that refer to a greater number of primary publications. Other included examples refer to studies which have tested at least three compounds. The experimental conditions refer to the selected example references. An exposure start at 0 hpf (hours post fertilisation) indicates that fish embryo incubation was initiated within at least 2 h after fertilisation.

Exposure period (hpf)	Solvents used	Exposure medium	Exposure regime	Exposure vessels	Peculiarities	Example references
0–48	No solvent, 1% DMSO	ISO15088, ISO15088 5x diluted	Static, sealed plates and/or daily renewal	24 well plates, petri dish, crystallisation dish or glass vials		(Lammer et al. (2009), Belanger et al. (2013), Knöbel et al. (2012))
0–72	No solvent, DMSO (0.05, 0.1 or 1%), sea salt (1 g/L), tap water	ISO15088, Tris-buffer	Daily renewal, static/sealed	24 well plates		(Foerster (2008), Baumann (2008), Weigt et al. (2011), Ton et al. (2006), Vaughan and van Egmond (2010))
0–96	No solvent	ISO15088	Daily renewal, static	24/96 well plates		(Foerster (2008), Belanger et al. (2013), Groth et al. (1993), Strecker et al. (2011))
0–120	No solvent, 0.01% DMSO	ISO15088, ISO15088 5x diluted, Sea salt (60 mg/L)	Static, sealed plates and/or daily renewal	24/96 well plates, glass vials		(Knöbel et al. (2012), Lammer et al. (2009), Carlsson et al. (2013))
24–120	No solvent	10% Hanks	Static	96 well plates		(Ali et al. (2011))
8–120 (144)	0.4% DMSO	10% Hanks	Sealed plates, daily renewal	96 well plates	Many compounds tested with only 2 embryos per concentration. Exposure cessation at 120 hpf, lethality analysed at 144 hpf.	(Padilla et al. (2012))

exceptions above 1000 (e.g., 1758 for enamectin and 400,000 for luviquat). Despite some conflicting reports on uptake of low molecular weight fluorescence dyes into the perivitelline space (Kais et al., 2013), the presence or absence of the chorion is unlikely to represent a major limiting factor. This is also supported by the high correlation of fish embryos LC_{50} obtained with prehatched stages up to 48 h to adult acute fish toxicity (Lammer et al., 2009).

Hence, it is unlikely that the observed weak correlation of fish embryo LC_{50} to rat LD_{50} is caused mainly by variability and limitations of protocols. In contrast, the weak correlation might be associated to fundamental differences in exposure and pharmacokinetics of both test systems. In fish embryos, exposure is water borne and a partition equilibrium between the exposure medium and the fish embryo is established. In oral dosing of rat a single dose is administered resulting in a compound-specific time course of plasma concentrations. The initial uptake and distribution, metabolic conversion and renal clearance affect this time course and the effective tissue concentrations. This is also supported by analysis of the dependency of rat LD_{50} used in this study on the hydrophobicity ($\log K_{ow}$) of the test compounds. In contrast to fish embryos only a very weak correlation with a slope close to zero was observed (Supplement Fig. S1).

4.4. Prediction of acute toxicity categories with fish embryos

Despite the weak correlation between rat LD_{50} and fish embryo LC_{50} we hypothesised that fish embryo data might be useful to predict acute toxicity classes – at least to identify highly toxic compounds with an acceptable low false positive or negative rate. This would allow identifying substances for which further mammalian animal studies could be waived. In order to determine whether fish embryo data could be used in a categorisation approach for acute toxicity classes we grouped the 364 compounds for which corresponding oral rat LD_{50}s were available according to the thresholds for oral acute toxicity of the GHS. Compounds for which no LD_{50} was reported but that were tested up to at least 2000 mg/kg were included and considered as compounds with low toxicity that would not be labelled (category V). For each GHS acute toxicity category the corresponding fish embryo toxicity range was identified (Table 2, Fig. 4A) and analysed. The comparison of the geometric means of fish embryos for each acute toxicity categories indicated a trend for categories II to V, i.e. higher fish embryo toxicities were observed for compounds with higher oral rat toxicity (Table 2). However, if the range of fish embryo LC_{50}s and the distribution of individual effect concentration was compared, only a very weak association with the rat acute toxicity was observed. Even identification of highly toxic compounds would be difficult using fish embryo data. For compounds for which no LC_{50} could be obtained in fish embryos, we compared the corresponding acute oral systemic toxicity categories. This comparison may be partially confounded given that some compounds have only been tested in a limited range of concentrations. However, a partial association with the low acute systemic toxicity categories IV and V was observed (79% of 150 compounds for which corresponding LD_{50} were available) indicating that many of the

Table 2
Associations of fish embryo toxicities with acute systemic (oral) toxicity categories. Grouping was conducted according to rat LD_{50} data and the thresholds of the global harmonisation systems. For each category the corresponding fish embryo toxicities were identified. The analysis was restricted to 364 compounds for which both rat LD_{50} and fish embryo LC_{50} were available. Compounds that did not provoke fish embryo toxicity in the tested range for concentrations were not considered.

Toxicity category (GHS)	No. of compounds	Range of fish embryo toxicity (mg/L)	Geometric mean fish embryo toxicities (mg/L)
Category 1 (label: danger): $\leqslant$5 mg/kg	9	0.0084–241	2.7
Category 2 (label: danger): >5 $\leqslant$ 50 mg/kg	24	0.00070–115	1.1
Category 3 (label: danger): >50 $\leqslant$ 300 mg/kg	75	0.0071–3152	9.6
Category 4 (label: warning): >300 $\leqslant$ 2000 mg/kg	133	0.00085–39796	14
Category 5 (no label): >2000	125	0.0024–77,768	26

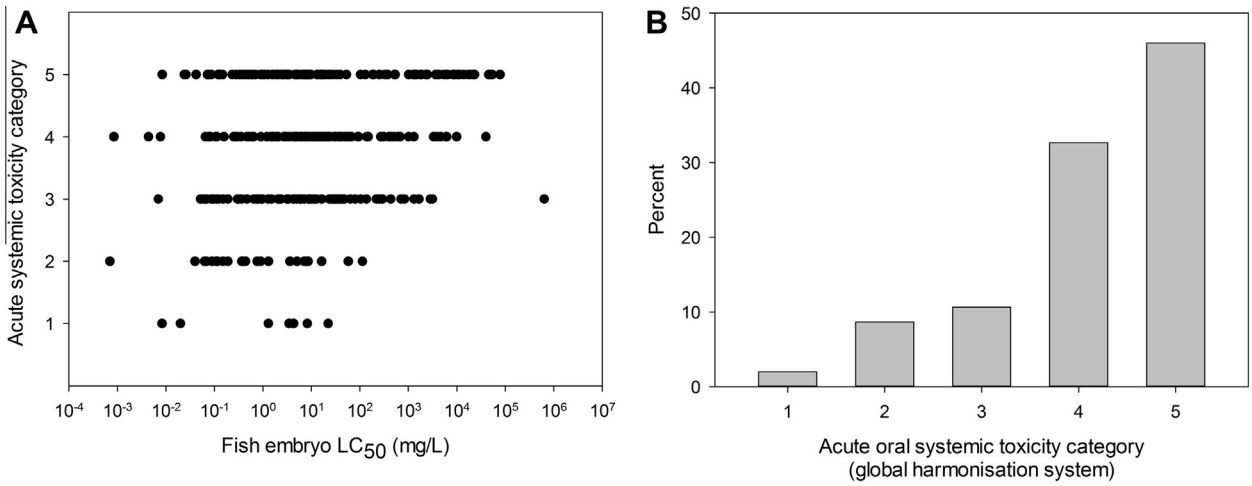

Fig. 4. Association of fish embryo toxicities with acute systemic (oral) toxicity categories. Grouping was conducted according to rat LD_{50} data and the thresholds of the global harmonisation systems (see Table 1). (A) – For each category the corresponding fish embryo toxicities were identified. The analysis was restricted to 387 compounds for which both rat LD_{50} and fish embryo LC_{50} were available. Compounds that have not shown any acute systemic oral toxicity in rats up to a test concentration of 2000 mg/L were assigned to category 5. (B) – For compounds that did not provoke any mortality in the fish embryo test the percentage of compounds that would be grouped into one of the five acute oral systemic toxicity categories was calculated. The analysis is based on 150 compounds for which corresponding rat oral systemic LD_{50} could be identified.

compounds with no toxicity in fish embryos also exhibit a low toxicity in mammals (See Fig. 4).

The fundamental differences in exposure and pharmacokinetics have been also identified as limiting factor to predict acute systemic toxicity for alternative approaches based on *in vitro* cellular models (Ekwall et al., 1998; Sjöström et al., 2008; Noorlander et al., 2008). Therefore, comparison of *in vitro* effect concentrations to effective plasma concentrations of humans or rodents was considered as a key to improve the correlation. Approaches to provide pharmacokinetic data that allow to identify plasma concentrations corresponding to LD_{50} have been initiated (Noorlander et al., 2008). However, the number of compounds for which such data are available is currently very limited and no systematic survey or approach to generate these data has been initiated. In order to investigate the principal predictive capacity of fish embryos it would be necessary to identify plasma concentrations corresponding to LD_{50} concentrations. If a correlation with fish embryo data could be established subsequent approaches would be needed to predict oral, dermal or inhalative effect concentrations using e.g., physiologically based kinetic modelling (Louisse et al., 2010).

5. Declaration of interest

S. Scholz, J. Ortmann and N. Klüver are employees of the Helmholtz Centre for Environmental Research, a public research institute of the Helmholtz association. M. Léonard is employed by L'Oréal and L'Oréal was financially supporting the research presented. The study did not include any products or compounds manufactured by L'Oréal.

6. Disclaimer

The views, conclusions and recommendation expressed in this article are those of the authors and do not necessarily represent views or policies of organisations to which the authors are affiliated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2014.06.004.

References

Ali, S., Mil, H.G.J.v., Richardson, M.K., 2011. Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. PLoS One 6, e21076.
Baumann, L., 2008. Effects of endocrine disruptors in zebrafish (*Danio rerio*) as revealed with the fish sexual development test (MSc Thesis), Faculty of for Biosciences, Univ. Heidelberg, pp. 91.
Belanger, S.E., Rawlings, J.M., Carr, G.J., 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. Environ. Toxicol. Chem. 32, 1768–1783.
Brannen, K.C., Panzica-Kelly, J.M., Danberry, T.L., Augustine-Rauch, K.A., 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res. B Dev. Reprod. Toxicol. 89, 66–77.
Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.-K., Chung, B.-C., Kah, O., 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. PLOS One 7, e36069.
Carlsson, G., Patring, J., Kreuger, J., Norrgren, L., Oskarsson, A., 2013. Toxicity of 15 veterinary pharmaceuticals in zebrafish (*Danio rerio*) embryos. Aquat. Toxicol. 126, 30–41.
ChemProp, 2012. Chemical Properties Estimation Software System 5.2.7, 2012. UFZ Department of Ecological Chemistry. Available for download at <www.ufz.de/index.php?en=6738>.
Cheng, J., Flahaut, E., Cheng, S.H., 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. Environ. Toxicol. Chem. 26, 708–716.
Clements, R.G., Nabholz, J.V., 1994. ECOSAR: a computer program and user's guide for estimating the ecotoxicity of industrial chemicals based on structure activity relationships. EPA-748-R-93-002. U.S. Environmental Protection Agency, Office of Pollution Prevention and Toxics 7403, Washington, DC.
Creton, S., Dewhurst, I.C., Earl, L.K., Gehen, S.C., Guest, R.L., Hotchkiss, J.A., Indans, I., Woolhiser, M.R., Billington, R., 2010. Acute toxicity testing of chemicals-Opportunities to avoid redundant testing and use alternative approaches. Crit. Rev. Toxicol. 40, 50–83.
Creton, R., 2004. The calcium pump of the endoplasmic reticulum plays a role in midline signaling during early zebrafish development. Dev. Brain Res. 151, 33–41.

Ekwall, B., Barile, F.A., Castano, A., Clemedson, C., Clothier, R.H., Dierickx, P., Ferro, M., Fiskesjo, G., Garza-Ocanas, L., Gomez-Lechon, M.J., Gulden, M., Hall, T., Isomaa, B., Kahru, A., Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lewan, L., Loukianov, A., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Stammati, A., Tanaka, N., Valentino, M., Walum, E., Zucco, F., 1998. MEIC evaluation of acute systemic toxicity - Part VI. The prediction of human toxicity by rodent LC_{50} values and results from 61 in vitro methods. ATLA – Altern. Lab. Anim. 26, 617–658.

Embry, M.R., Belanger, S.E., Braunbeck, T.A., Galay-Burgos, M., Halder, M., Hinton, D.E., Léonard, M.A., Lillicrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat. Toxicol. 97, 79–87.

EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Off. J. Eur. Union L276, 34–79.

Foerster, F., 2008. Effects of the exposure duration of various chemicals on the fish embryo test with the zebrafish *Danio rerio* (MSc Thesis), Faculty of for Biosciences, Univ. Heidelberg, pp. 75.

Groth, G., Schreeb, K., Herdt, V., Freundt, K.J., 1993. Toxicity studies in fertilized zebrafish eggs treated with N-methylamine, N, N-dimethylamine, 2-aminoethanol, isopropylamine, aniline, N-methylaniline, N, N-dimethylaniline, quinone, chloroacetaldehyde, or cyclohexanol. Bull. Environ. Contam. Toxicol. 50, 878–882.

Gustafson, A.L., Stedman, D.B., Ball, J., Hillegass, J.M., Flood, A., Zhang, C.X., Panzica-Kelly, J., Cao, J., Coburn, A., Enright, B.P., Tornesi, M.B., Hetheridge, M., Augustine-Rauch, K.A., 2012. Inter-laboratory assessment of a harmonized zebrafish developmental toxicology assay – progress report on phase I. Reprod. Toxicol. 33, 155–164.

Halder, M., Léonard, M., Iguchi, T., Oris, J.T., Ryder, K., Belanger, S.E., Braunbeck, T.A., Embry, M.R., Whale, G., Norberg-King, T., Lillicrap, A., 2010. Regulatory aspects on the use of fish embryos in environmental toxicology. Integr. Environ. Assess. Manag. 6, 484–491.

Henn, K., Braunbeck, T., 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*). Comp. Biochem. Phys. C. 153, 91–98.

Kais, B., Schneider, K.E., Keiter, S., Henn, K., Ackermann, C., Braunbeck, T., 2013. DMSO modifies the permeability of the zebrafish (*Danio rerio*) chorion-implications for the fish embryo test (FET). Aquat. Toxicol. 140–141, 229–238.

Kinsner-Ovaskainen, A., Bulgheroni, A., Hartung, T., Prieto, P., 2009. ECVAM's ongoing activities in the area of acute oral toxicity. Toxicol. In Vitro 23, 1535–1540.

Knöbel, M., Busser, F., Rico Rico, A., Kramer, N.I., Hermens, J.L.M., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S., 2012. Predicting adult fish acute lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties and exposure concentration analysis. Environ. Sci. Technol. 46, 9690–9700.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Phys. C. 149, 196–209.

Louisse, J., de Jong, E., van de Sandt, J.J.M., Blaauboer, B.J., Woutersen, R.A., Piersma, A.H., Rietjens, I.M.C.M., Verwei, M., 2010. The use of in vitro toxicity data and physiologically based kinetic modeling to predict dose-response curves for in vivo developmental toxicity of glycol ethers in rat and man. Toxicol. Sci. 118, 470–484.

Mhadhbi, L., Beiras, R., 2012. Acute toxicity of seven selected pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the marine fish (Turbot, *Psetta maxima*). Water Air Soil Pollut. 223, 5917–5930.

Mhadhbi, L., Boumaiza, M., 2011. Toxic effects of acute exposure of diazonin in turbot (*Psetta maxima*) early life stage (ELS). Int. J. Environ. Res. 6 (6), 139–144.

Mhadhbi, L., Fumega, J., Boumaiza, M., Beiras, R., 2012. Acute toxicity of polybrominated diphenyl ethers (PBDEs) for turbot (*Psetta maxima*) early life stages (ELS). Environ. Sci. Pollut. Res. 19, 708–717.

Moore, N.P., Andrew, D.J., Bjerke, D.L., Creton, S., Dreher, D., Holmes, T., Prieto, P., Seidle, T., Rowan, T.G., 2012. Can acute dermal systemic toxicity tests be replaced with oral tests? A comparison of route-specific systemic toxicity and hazard classifications under the globally harmonized system of classification and labelling of chemicals (GHS). Regul. Toxicol. Pharmacol. 66, 30–37.

Noorlander, C., Zeilmaker, M., Eijkeren, J.v., Bourgeois, F., Beffers, R., Brandon, E., Bessems, J., 2008. Data collection on kinetic parameters of substances. Pilot phase - A methodological report – December 2008 CCR.IHCP.C432921.XO. Available at: <http://ecvam.jrc.it>.

Padilla, S., Corum, D., Padnos, B., Hunter, D.L., Beam, A., Houck, K.A., Sipes, N., Kleinstreuer, N., Knudsen, T., Dix, D.J., David, R.M., 2012. Zebrafish developmental screening of the ToxCast(TM) Phase 1 chemical library. Reprod. Toxicol. 33, 174–187.

Parng, C., Seng, W.L., Semino, C., McGrath, P., 2002. Zebrafish: a preclinical model for drug screening. Assay Drug Dev. Technol. 1, 41–48.

Robinson, S., Delongeas, J., Donald, E., Dreher, D., Festag, M., Kervyn, S., Lampo, A., Nahas, K., Nogues, V., Ockert, D., 2008. A European pharmaceutical company initiative challenging the regulatory requirement for acute toxicity studies in pharmaceutical drug development. Regul. Toxicol. Pharmacol. 50, 345–352.

Russom, C.L., Bradbury, S.P., Broderius, S.J., Hammermeister, D.E., Drummond, R.A., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 16, 948–967.

Scholz, S., 2013. Zebrafish embryos as an alternative model for screening of drug-induced organ toxicity. Arch. Toxicol. 87, 767–769.

Seidle, T., Robinson, S., Holmes, T., Creton, S., Prieto, P., Scheel, J., Chlebus, M., 2010. Cross-sector review of drivers and available 3Rs approaches for acute systemic toxicity testing. Toxicol. Sci. 116, 382–396.

Seidle, T., Prieto, P., Bulgheroni, A., 2011. Examining the regulatory value of multi-route mammalian acute systemic toxicity studies. ALTEX – Altern. Anim. Exp. 28, 95.

Selderslaghs, I.W.T., Blust, R., Witters, H.E., 2012. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod. Toxicol. 33, 142–154.

Sjöström, M., Kolman, A., Clemedson, C., Clothier, R., 2008. Estimation of human blood LC50 values for use in modeling of in vitro-in vivo data of the ACuteTox project. Toxicol. In Vitro 22, 1405–1411.

Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., Schumacher, A., Selderslaghs, I., Weiss, C., Witters, H., Braunbeck, T., 2012. Zebrafish embryos as an alternative to animal experiments – a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod. Toxicol. 33, 128–132.

Strecker, R., Seiler, T.-B., Hollert, H., Braunbeck, T., 2011. Oxygen requirements of zebrafish (*Danio rerio*) embryos in embryo toxicity tests with environmental samples. Comp. Biochem. Phys. C 153, 318–327.

Thienpont, B., Tingaud-Sequeira, A., Prats, E., Barata, C., Babin, P.J., Raldua, D., 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environ. Sci. Technol. 45, 7525–7532.

Ton, C., Lin, Y., Willett, C., 2006. Zebrafish as a model for developmental neurotoxicity testing. Birth Defects Res. A 76, 553–567.

UN, 2011. United Nations, New York and Geneva: Globally harmonized system of classification and labelling of chemicals (GSH). Fourth revised edition. Available at: <www.unece.org>.

Vaughan, M., van Egmond, R., 2010. The use of the zebrafish (*Danio rerio*) embryo for the acute toxicity testing of surfactants, as a possible alternative to the acute fish test. ATLA 38, 231–238.

Verhaar, H.J.M., Van Leeuwen, C.J., Hermens, J.L.M., 1992. Classifying environmental pollutants. 1: Structure-activity relationships for prediction of aquatic toxicity. Chemosphere 25, 471–491.

Villeneuve, D., Volz, D.C., Embry, M.R., Ankley, G.T., Belanger, S.E., Léonard, M., Schirmer, K., Tanguay, R., Truong, L., Wehmas, L., 2014. Investigating alternatives to the fish early life-stage test: a strategy for discovering and annotating adverse outcome pathways for early fish development. Environ. Toxicol. Chem. 33, 158–169.

Volz, D.C., Belanger, S., Embry, M., Padilla, S., Sanderson, H., Schirmer, K., Scholz, S., Villeneuve, D., 2011. Adverse outcome pathways during early fish development: a conceptual framework for identification of chemical screening and prioritization strategies. Toxicol. Sci. 123, 349–358.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens. Toxicology 281, 25–36.