Short Communication

Zebrafish Embryo and Acute Fish Toxicity Test Show Similar Sensitivity for Narcotic Compounds

Anita Birke¹ and Stefan Scholz²

¹BioChem agrar, Machern, Germany, work partially conducted as a visiting scientist at the UFZ; ²Helmholtz Centre for Environmental Research – UFZ, Department of Bioanalytical Ecotoxicology, Leipzig, Germany

Abstract

The zebrafish embryo test has been discussed as an alternative test system to provide data on acute fish toxicity required by diverse regulations. A meta-analysis of zebrafish embryo acute toxicity (ZFET) data has revealed conflicting evidence that narcotic compounds (i.e., compounds with baseline toxicity) may exhibit weaker sensitivity in the ZFET compared to the acute (adult) fish toxicity test (AFT). Therefore, six compounds with presumably narcotic or unknown mode of action, and for which a previous meta-analysis had indicated weaker sensitivity, were experimentally analyzed for their fish embryo acute toxicity and exposure concentrations were monitored. The data indicated that ZFET and AFT for the selected compounds had similar sensitivity and differences were in the range of species differences of the AFT.

1 Introduction

The estimation of acute fish toxicity is required worldwide by diverse regulations for the hazard and risk assessment of chemicals, pesticides, biocides, (veterinary) drugs, and feed additives (reviewed in Scholz et al., 2013). Typically, this information is provided by experimental data based on the acute exposure of fish for 96 h to the test chemicals according to an internationally agreed testing guideline (OECD, 1992). The fish embryo test has been suggested as one potential alternative approach to reduce the number of tests using juvenile or adult fish (reviewed in Scholz et al., 2013), since the fish embryo is considered a non-protected life stage by some animal welfare regulations (Halder et al., 2010; Embry et al., 2010). In the case of zebrafish, which is one of the most commonly used model organisms for fish embryo tests, stages up to 5 dpf (days post fertilization) are commonly used in fish embryo toxicity tests (Strähle et al., 2012). Various meta- and experimental analyses have revealed a high correlation of zebrafish acute embryo toxicity (ZFET) with acute fish toxicity (AFT) with on average similar sensitivity (Lammer et al., 2009a; Busquet et al., 2014; Belanger et al., 2013). The on average high correlation and sensitivity as well as the high reproducibility has led to establishment of the OECD testing guideline 236: “Fish Embryo Acute Toxicity (FET) Test” (OECD, 2013; Busquet et al., 2014).

However, concern has been raised about the applicability domain of the ZFET, given that, for example, neurotoxic compounds appear to exhibit no or weak acute toxicity in fish embryos (Klüver et al., 2015; Sobanska et al., 2018; Glaberman et al., 2016). The weak toxicity of neurotoxic compounds in fish embryos was explained by the lack of respiratory failure syndrome in fish embryos due to uptake of oxygen by diffusion and it being independent of the function of the cardiovascular system. Given that neurotoxic compounds are likely to impact on behavior, it was suggested to assess embryonic movement to identify neurotoxic compounds in order to predict their acute toxicity in later life stages (Klüver et al., 2015). Conflicting evidence was provided for the applicability of the ZFET for presumably narcotic (baseline toxic) compounds. A QSAR established for ZFET baseline toxicity was very similar to the available AFT QSARs (Klüver et al., 2016). However, the meta-analysis of Sobanska et al. (2018) identified 9 out of 47 narcotic compounds that exhibited an LC₅₀ that was more than 10-fold higher than the AFT LC₅₀ in one of four species used for comparison (zebrafish, rainbow trout, fathead minnow, bluegill). For this meta-analysis, a database of 153 study entries representing 123 compounds with corresponding AFTs was established. Given the lack of studies that had been conducted strictly according to the OECD TG 236, the database also considered studies performed similarly to the TG 236. To avoid bias by studies deviating from TG 236, certain

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Correspondence: Stefan Scholz, PhD, Helmholtz Centre for Environmental Research – UFZ, Dpt. of Bioanalytical Ecotoxicology, Permoserstr. 15, 04318 Leipzig, Germany (stefan.scholz@ufz.de)

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quality criteria were considered, i.e., only studies were accepted that had the LC50 inside the water solubility range, exposure duration from 96 to 120 h, a predicted, buffered or measured pH range in exposure solutions between 5–9, a concentration range including baseline toxicity for non-toxic compounds, and a log $K_{ow} < 4$ if exposure concentrations were not measured (Sobanska et al. 2018). We hypothesized that part of the discrepancy may be due to experimental limitations, since ZFET data included in the meta-analysis were often derived from studies of small scale high-throughput testing that lacked verification of exposure concentrations. Therefore, we selected six compounds for which a weak toxicity in the ZFET had been previously reported for experimental verification by conducting a ZFET according to OECD TG 236 (OECD, 2013) including chemical analysis of exposure solutions. The compounds were selected from the previous meta-analysis (Sobanska et al., 2018) according to two criteria: availability of analytical methods and differences in hydrophobicity that span a wide range. Five of the chosen compounds exhibit a narcotic mode of action (MoA, 4-chloroaniline, aniline, acetochlor, folpet, pyraclostrobin); for one compound (3-iodo-2-propynyl-N-butylcarbamate) the MoA is unknown.

2 Animals, materials and methods

The zebrafish embryo acute toxicity test was conducted according to the TG 236 with minor modifications. Details on all materials and methods including methods used for the analysis of exposure media (Tab. S1), and physico-chemical properties of exposure chemicals and measured concentration of exposure solutions (Tab. S2) can be found in supplementary file 1¹. Exposure concentrations were measured for all test chemicals using HPLC-MSMS. Origin of acute fish toxicity data is given in supplementary file 2².

3 Results and discussion

3.1 Analysis of exposure concentrations

It has been recognized that exposure concentrations can drop during incubation in plastic microtiter plates owing to adsorption of hydrophobic compounds or compound volatilization. This can affect the estimation of effect concentrations when test organisms are incubated in plastic microtiter plates as is the case in the ZFET (Schreiber et al., 2008; Riedl and Altenburger, 2007). In line with this, the meta-analysis of FET data by Sobanska et al. (2018) indicated that for substances with a log $K_{ow} > 4$ and a log $K_{aw} > -4$ often no mortality was observed (Sobanska et al., 2018). Loss of test compound would result in an overestimation of effect concentration, particularly if it was determined based on nominal concentration. To avoid this, pre-saturation of test plates 24 h prior to the start of the exposure and/or determination of the

effect concentration based on the measured concentration was recommended by the OECD TG 236 (OECD, 2013). Measured concentrations are not available for most of the fish embryo and also many acute fish tests conducted to date, which may account for some of the observed discrepancies between AFT and ZFET data (Sobanska et al., 2018).

However, in the present study we did not observe any drop of exposure concentrations that could be associated with adsorption of the test compound to microtiter plates. Chemical analysis indicated stable exposure concentrations (i.e., deviation of less than 20% from the concentration at the start of the exposure) for all substances except folpet. Folpet is known to be rapidly hydrolyzed with a reported half-life of 0.7 h (pH 7, 25°C; EFSA, 2009). Therefore, the stability of folpet was monitored initially in exposure medium for 7 h. As expected, the concentration dropped below the detection limit of 0.012 µM within 5 h and a half-life of 29 minutes was calculated (Fig. S1¹). Accordingly, optimal testing of folpet would require a flow-through system, such as proposed by Lammer et al. (2009b). However, in order to compensate at least partially for the hydrolytic loss, a semi-static exposure with 12-h renewal intervals was used. For pyraclostrobin, chemical analysis revealed exposure concentrations below 80% of the nominal concentrations already at the start of the exposure (Tab. S3¹). However, the concentration remained stable over the exposure duration, excluding adsorption to the microwell plates. Instead, incomplete dissolution may account for the lower measured concentration. Therefore, as recommended by the OECD, the geometric mean of measured concentrations detected at the beginning and end of the exposure interval was used to derive effect concentrations (OECD, 2013). Due to the rapid drop below the detection limit within the 12-h renewal interval no geometric mean could be calculated for folpet. Hence, the nominal concentrations were used to derive the LC50 concentrations and it is likely that effect concentrations are thus overestimated for this compound.

3.2 Determination of effect concentrations

For all compounds a concentration-dependent increase in mortality approaching a 100% mortality level was observed and could be used to model concentration-response curves and calculate LC50 values (Tab. 1, Fig. S2¹). The effect concentrations decreased with increasing exposure time, particularly within the first 48 h of exposure (Tab. 1, Fig. S2¹). Given that for the selected compounds death was generally caused by coagulation, this indicates that the internal equilibrium concentration may have only been approached at the end of the exposure. Observations that for many compounds no equilibrium internal concentrations had been established after 24 h of exposure have been reported previously (e.g., El-Amrani et al., 2012; Brox et al., 2014, 2016). The observed toxic ratio (TR, ratio of predicted baseline versus observed LC50) confirmed the classification of aniline, acetochlor, and pyraclostrobin as narcotic compounds (i.e., compounds with toxicity in the range of the calculated baseline tox-

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Tab. 1: LC50 with confidence intervals, modelling parameters and comparative baseline and acute toxicity data

Previously published LC50 for zebrafish embryos (ZFET) and acute fish toxicity (AFT) were obtained from the meta-analysis of Sobanska et al. (2018); individual references and test conditions are given in Tab. S4¹) and refer to exposure durations of 96 or 120 h. DR, *Danio rerio* (zebrafish); LM, *Lepomis macrochirus* (bluegill); OM, *Oncorhynchus mykiss* (rainbow trout); PP, *Pimephales promelas* (fathead minnow).

Substance (MoA)	LC ₅₀ 24 hpf (μmol/l)	LC ₅₀ 48 hpf (μmol/l)	LC ₅₀ 72 hpf (μmol/l)	LC ₅₀ 96 hpf (μmol/l)	FET-LC ₅₀ -values (μmol/l) from meta-analysis	ZFET baseline LC50 (μmol/l)	TR (ZFET)	ZFET/AFT ratio
4-Chloroaniline (Narcosis) Slope	291 (267-314) 8.43	194 (182-206) 6.14	164 (158-169) 6.53	151 (140-162) 5.21	345 (Burkhardt-Holm et al., 1999)	2076	14	0.55 (DR) 8.0 (LM) 1.4 (OM) 0.84 (PP)
Acetochlor (Narcosis) Slope	126 (107-145) 7.34	72.8 (40.3-105) 67	53.2 (47.4-58.9) 13.2	34.6 (32.2-37.1) 6.19	57.7 (Truong et al., 2014) 26.6 (Padilla et al., 2012)	48.3	1.4	6.5 (LM) 16 (OM)
Aniline (Narcosis) Slope	1910 (615-3210) 24	1790 (1590-1990) 4.73	1720 (1450-1990) 3.24	1660 (1430-1880) 3.32	9300 (Groth et al., 1993)	8929	5.4	2.6 (DR) 3.2 (LM) 4.7 (OM) 1.8 (PP)
3-Iodo-2-propynyl-N-butylcarbamate (Unknown) Slope	1.44 (1.38-1.5) 8.56	1.35 (1.25-1.45) 9.62	1.34 (1.25-1.42) 11.5	1.34 (1.25-1.42) 11.5	2.55 (Padilla et al., 2012) 20.2 (Truong et al., 2014)	393	293	1.7 (LM) 4.8 (OM) 1.9 (PP)
Pyraclostrobin* (Narcosis) Slope	0.264 (0.239-0.288) 8.94	0.184 (0.152-0.216) 70.3	0.178 (0.176-0.181) 36.2	0.138 (0.122-0.154) 3.69	0.187 (Padilla et al., 2012) 0.498 (Truong et al., 2014)	0.43	3.1	4.7 (LM) 8.6 (OM)
Folpet (Narcosis) Slope	3.78 (3.39-4.17) 8.97	2.7 (2.32-3.08) 4.92	2.47 (2.18-2.77) 9.35	2.09 (1.31-2.86) 20.3	8.88 (Padilla et al., 2012)	162	77	8.5 (LM) 9.9 (OM) 3.4 (PP)

*based on measured exposure concentrations

icity). A slightly enhanced TR was observed for 4-chloroaniline, which may indicate that this compound does not act through baseline toxicity but also exhibits a specific mode of action. Furthermore, the TR of folpet (77) supports a potential specific or reactive mode of action for this compound.

When the 96-h LC50s were compared to previous zebrafish embryo studies (Tab. 1), lower effect concentrations were obtained for nearly all compounds. The maximum differences ranged from 1.4-fold (3-iodo-2-propynyl-N-butylcarbamate) to 5.6-fold (aniline). Only for acetochlor slightly higher effect concentrations were obtained in our study compared to the study of Padilla et al. (2012). Due to this increased sensitivity, all derived effect concentrations were closer to the range observed for AFT in rainbow trout, fathead minnow, bluegill, or zebrafish (Tab. 1). The relative effect concentration (LC50_{ZFET}/LC50_{AFT}) of the ZFET ranged from 0.55 (4-chloroaniline) to 16 (acetochlor) and was also dependent on the species that was used for the com-

parative AFT value. For instance, relative effect concentrations of ZFET and exposure to acetochlor were lower compared to the LC50_{AFT} of bluegill (6.4). The on average slightly higher effect concentrations for ZFET may relate to species sensitivity rather than stage-specific sensitivities. This is indicated by the two compounds (4-chloroaniline, aniline), for which a zebrafish LC50_{AFT} was available and was closer to the LC50_{ZFET} than the LC50s of other species. A factor of 10 – based on mean species differences of the AFT – has been previously selected as a threshold to indicate whether ZFET data is exhibiting a weaker sensitivity (Sobanska et al., 2018). If this factor is applied to the data obtained in the present study, none of the compounds is classified as a compound with weaker sensitivity in the ZFET. Only for aniline, a weaker sensitivity may be assigned if data are compared to the LC50_{AFT} of bluegill.

It is difficult to conclude why a higher sensitivity was obtained for nearly all compounds in our study since for previous studies

no data on measured concentrations were available. In case of pyraclostrobin, the deviation of measured from nominal concentrations and the use of measured concentrations for calculation of the LC50 may have contributed to the difference in sensitivity (Tab. S3¹). For other compounds, the low exposure volumes (100 compared to 250 µl) in previous studies (Padilla et al., 2012; Truong et al., 2014) could have led to a drop of exposure concentrations due to accumulation in the fish embryo, particularly for compounds with higher hydrophobicity. Plastic polystyrene microtiter plates were used in the previous as well as in our study and the measured concentration did not indicate that adsorption to microplates accounted for the sensitivity differences. Finally, a rapid loss of compound due to, e.g. hydrolysis, may be partially compensated by the frequent renewal of exposure solutions. This was indicated by similar effect concentrations obtained for folpet compared to AFT data. However, the relevance of such compounds for acute exposure scenarios should be questioned or appropriate exposure protocols to maintain stable exposure concentrations should be developed.

4 Conclusions

The previously observed weaker sensitivity of ZFET for some presumably narcotic compounds and one compound with an unknown mode of action was not found to the same extent in this study. Effect concentrations within a factor of 10 of the LC50_{AFT} were obtained for all studied compounds. Given the lack of chemical analysis data in the previous study, it was difficult to conclude whether the higher sensitivity observed in this study was due to more stable exposure concentrations, and/or the use of measured concentration (in case of deviation from nominal) for calculation of the LC50. The study confirms that, as previously suggested by quantitative structure activity relationships, a similar sensitivity can be expected for narcotic compounds for the baseline toxicity in fish embryos and juvenile/adult fish.

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Conflict of interest

The authors declare that they have no conflict of interest.

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