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Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals

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Abstract

A 4-day embryo-larval zebrafish test, from blastula stage to hatching included, was developed. The observations of embryo developmental were made at different development stages, for which morphological, physiological, and behavioral endpoints were selected and quantified for unexposed and exposed embryos. The sensitivity and the ability of these endpoints to inform about mode of action (MoA) were established in testing three model toxicants with well-known toxic effects (propranolol, malathion, cadmium). Lethal, sublethal (heart rate/edema, spontaneous movements, and hatching rate/time disturbance), and teratogenic effects were detected for all the studied compounds. This bioassay allows characterization of impairments at different biological levels: neuromuscular, physiological, morphological, and behavioral, and brings useful information about the toxic MoA of the chemicals on nontarget organisms. In this sense to answers the chemical industries and international organization (EMEA) requirements for the environmental risk assessment of new chemicals and pharmaceuticals.

Keywords: Zebrafish; Embryotoxicity; Mode of action; Bioassay; Risk assessment

1. Introduction

Currently, hazard assessment of chemicals for fish is based on international standards (ISO, ASTM) and guidelines (OECD) based on global toxicity endpoints as mortality, growth, and reproduction impairments. For chemical industries and some countries the state of mind is now to reduce the cost of these experiments and the number of used organisms, in concern for animal welfare. A significant example is the awareness of the ecotoxicologist community of the pharmaceutical industry to apply the principles of replacement, reduction, and refinement (the 3Rs), established by Russell and Burch (1959), in the context of regulatory environmental assessments (Hutchinson et al., 2003). One of the alternatives proposed is to use the early life stage (ELS) of fish as an experimental model (Oberemm, 2000; Nagel, 2002; Hutchinson et al., 2003), because it is no longer necessary to demonstrate that the fish embryo and larva are generally the most sensitive stages in the life cycle of the teleost (Laale and Lerner, 1981; McKim, 1985; von Westernhagen, 1988; Lele and Krone, 1996).

Table 1 shows an overview of International Standards and guidelines based on the ELS test using freshwater fish. From the oldest standard (OECD 210, 1992; ASTM E 1241-98, 1992) to the most recent (DIN 38415-T6, 2002), under consideration for ISO standardization, a decrease of the test duration and a reduction of the number of ELSs are taken into account. The OECD guidelines, and corresponding standards, are based on the study of at least two developmental stages: embryo, pro-larva and larva[1] (only OECD 210). The

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[1]The early life stage terminology followed is defined by Baxter (1988). Embryo, stage before hatching, pro-larva; stage between hatching and yolk sac resorption; larva, stage between yolk-stage resorption and juvenile morphology.

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Table 1
Overview of the toxic bioassay guidelines based on the early life stage of freshwater fish

Considered life stages[a]	Toxicological endpoints	Toxicological characteristic	Test duration[b]	Recommended species	References
Embryo Pro-larva Larva	Cumulative mortality Hatching (rate, half time) Pro-larval length and weight Abnormal larvae (morphology, behavior)	Lethal Sublethal	30–32 days	*Danio rerio* *Onchorhynchus mykiss* *Oryzias latipes* *Pimephales promelas*	OECD 210 (1992) ASTM E1241-98 (1992)
Embryo Pro-larva	Cumulative mortality Hatching (rate, half time) Pro-larval length and weight Abnormal larvae (morphology, behavior)	Lethal Sublethal	8–10 days	*D. rerio* *O. mykiss* *O. latipes* *Cyprinus carpio* *P. promelas*	OECD 212 (1998) ISO 12890 (1999)
Embryo	Coagulated eggs Development of somites Tail detachment Heartbeat	Lethal	48 h	*D. rerio*	DIN 38415-T6 (2002)[c]

The OECD guidelines take into account the OECD members' related national standards.
[a]Early life stage terminology (Baxter, 1988): embryo, stage before hatching, pro-larva; stage between hatching and yolk-sac resorption; larva, stage between yolk-sac resorption and juvenile morphology.
[b]Time duration for tests with *D. rerio.*
[c]In proposal at ISO section TC147/SC5.

toxic effect is assessed through the analysis of various criteria, defined as endpoints: mortality, hatching (rate, time), and (pro-)larva morphology (length, weight, abnormality). The DIN standard is proposed to replace the short acute adult test for wastewater assessment. It relies only on the embryo stage and the selected endpoints refer to rough embryogenesis impairments, with somite development, tail detachment, heartbeat, and coagulated egg. The authors state that the embryos presenting such abnormalities, even if they are still alive after 48 h of exposure, are no longer viable. The main interest of the DIN standard endpoint selection is certainly the objectivity of the results analysis, using binary choice (present/absent).

Information about early lethality is necessary for the hazard assessment of chemicals, but the used criteria cannot inform about the ability of the others embryos to survive over the key period of hatching. Let us cite von Westernhagen (1988) writing that "*viable hatch* is a more sensitive indicator of pollutant effects than *hatchability*." Indeed, in the alive embryos not showing major impairments at 48 h postfecundation (hpf), as required by the DIN standard, substantial numbers of nonviable larvae may be included. Determination of the rate of hatching (viable hatch) would be more relevant for assessing the sublethal effect of pollutant.

Moreover, Hutchinson et al. (2003) performed bioassays allowing information about the toxic mode of action (MoA) of the new developed chemicals to be acquired. The proposed standards with embryos do not offer such a possibility. Starting from the DIN standard and including the hatching period, it seems possible to answer this expectation by describing more precisely the embryo development perturbations induced by toxicants. One of the solutions can be the monitoring over time of the appearance and the evolution of endpoints related to various biological levels (anatomy, physiology, behavior). Indeed, during embryogenesis a lot of biochemical and molecular mechanisms occur among cells, receptors, tissues, and organs. These mechanisms could be influenced more or less specifically by a great number of pollutants, with possible impact on the tissue differentiation and organization, observable at the macroscopic level (Laale and Lerner, 1981; Schulte and Nagel, 1994; Ensenbach, 1998).

The aim of this study was to develop a 4-day embryo-larval bioassay using the zebrafish (*Danio rerio*), from early blastula to hatching. Zebrafish is an attractive species for studies focusing on ELSs. Adult zebrafish can produce a large number of translucent embryos every week, by external fertilization, and the embryogenesis is well characterized (Hisaoka and Battle, 1958; Kimmel et al., 1995; Lele and Krone, 1996). The main objectives of this work were (i) to select endpoints and (ii) to test their relevance, in term of sensitivity and ability to inform about MoA, in testing three model toxicants with well-known toxic effects.

To tackle the first objective, we selected endpoints and we monitored their variation over embryo development. A particular effort was done on the selection of quantifiable endpoints. Indeed analysis of embryotoxicological results is commonly based on an estimation of