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REVIEW ARTICLE

Recent advances and future trends in zebrafish bioassays for aquatic ecotoxicology

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Abstract – Zebrafish (*Danio rerio*), a cyprinid teleost, has become an ideal model species for aquatic ecotoxicology due to the broad spectra of methodologies that have been developed since the 1980s. The zebrafish has many advantages as a model organism e.g. small size, ex utero development of the embryo, short reproductive cycle, and transparent embryos. In addition, the zebrafish shares a high degree of homology with the human genome. It has become a powerful model organism for genetics, development, environmental toxicology, several human diseases and pharmacology. Zebrafish bioassays can be used for environmental monitoring including pollutant evaluations, such as toxic heavy metals, endocrine disruptors, and organic pollutants. The large number of transparent embryos gained from zebrafish females, in vitro and rapid embryonic development and ready-to-use methods of biotechnology enables us to use mostly automatized high-throughput screening not only in pharmaceutical drug development protocols but in aquatic ecotoxicology as well.

Keywords – Aquatic ecotoxicology, zebrafish, bioassays, water pollutant, wastewater, PAHs, PCBs, PPCPs, EDCs

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Introduction

Ecotoxicology is still a rather young discipline of environmental sciences that requires the application of knowledge in several domains (e.g. ecology, toxicology, chemistry, microbiology, engineering, mathematics, climatology, public policy, and environmental legislation), in order to fully understand and adequately interpret the hazards derived from the chemical contaminants continually released into aquatic environments worldwide. The discharge of chemicals like e.g. heavy metals, pesticides, PAHs (Polycyclic Aromatic Hydrocarbons), hormones, pharmaceuticals, nanomaterials are of serious concern in terms of their ecotoxicity, as well as their interactions in mixtures emitted by industrial and municipal effluents from different sources of pollution. In receiving waters cocktails of chemicals interact with specific biochemical characteristics of aquatic systems, with pelagic and benthic organisms, as well as with their associated parasites, and microbial communities (Blaise 2013).

Initially, until human populations remained small, anthropogenic influences on aquatic environments remained insignificant and localized. Later on, major aquatic environmental problems appeared by using flush toilets (WCs) and adjacent sewage systems. Consequently, contaminated municipal wastewater including urine and feces were disposed directly to surrounding surface waters. Until the latter part of the twentieth century, wastewater was almost always unprocessed. As soon as the vicinity of effluent pipes became fouled, the length of the effluent pipe was increased. Effluents fed into surrounding waters without cleaning caused many major catastrophes. For example, several people (tens to hundreds) died of mercury intoxication in Japan following the Minamata incident in 1956, as untreated effluents from a chemical factory were discharged in a bay where local inhabitants took their household water and ate the fish. Although the acute catastrophe emerged in a single year, the mercury contamination of the bay occurred and persisted between 1932 and 1968, and up to the present, around 2000 people have died of mercury intoxication, and more than 10,000 people have received some compensation for mercury-intoxication-caused health problems (Tsuda et al., 2009). Unprocessed paper- and pulp-mill effluents used to be a major environmental question in Western Europe and North America. In the 1960s, the paper and pulp-mill industry of Sweden and Finland produced an amount of effluent corresponding to the effluent produced by 100,000,000 people (Nikinmaa 2014). At that time, all the biota in water areas close to the paper and pulp mills was dead. Also, as a result of effluent discharge, the persistent organic pollutant (POP) concentrations (including polychlorinated biphenyls, PCBs) were so high that e.g. the reproduction of seals was significantly affected. Since then, development in paper- and pulp-mill technology have enabled the industry to be much more environmentally friendly e.g. the use of chlorine in bleaching has been modified, and the mills reuse most water. Consequently, the previously extinct fish and gray seal populations in the Baltic Sea have increased and recovered markedly (Elmgren 2001).

Already in the 1960s the initial results of fish bioassays confirmed that industrial effluents were clearly deleterious to aquatic life. Many industrialized countries (within the Organization for Economic Co-operation and Development) found it appropriate to create environmental agencies to cope with rampant aquatic contamination during the 1970s (Blaise and Gagné, 2013).

Moreover, pollutants of household origin should also be considered. The U.S. EPA defines pharmaceuticals and personal care products (PPCPs) as “any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock” (Cizmas et al., 2015). This definition encompasses thousands of chemicals that make up fragrances, cosmetics, over-the-counter drugs, and veterinary medicines, etc. Indeed, pharmaceuticals and personal care products include numerous chemical classes. PPCPs are generally excreted and emitted into the sewerage system following use. The compounds may then be released into surface waters or enter terrestrial systems when sewage effluent is used for irrigation or where sewage sludge is applied as a fertilizer to agricultural land (Boxall et al., 2012).

Zebrafish: the rise of a powerful animal model

Figure 1. Zebrafish (*Danio rerio*) (https://en.wikipedia.org/wiki/Zebrafish)

George Streisinger (born in 1927, in Budapest, Hungary) can be considered as the founding father of zebrafish (*Danio rerio*, Figure 1) research. In 1981 Streisinger et al. reported methods to produce homozygous diploid zebrafish by using hydrostatic pressure or heat shock. This study greatly promoted the development of genetic analyses in zebrafish. Since then zebrafish has become an ideal model species for aquatic ecotoxicology due to the broad spectra of methodologies that have been developed (for details please browse through “The Zebrafish Book”, available online).

In this short review we cannot provide a complete list of the most important milestones of relevant bioassays based on zebrafish, but the generation of mutant and transgenic lines has to be mentioned hereby.

In 1996, Driever et al. and Haffter et al. reported largescale genetic screens for mutation in zebrafish. They obtained numerous mutations and identified the mutation genes and their functions in zebrafish embryogenesis. These results provided a powerful basis for development studies using zebrafish as a model organism. The zebrafish has many advantages as a model organism, such as small size, ex utero development of the embryo, short reproductive cycle, and transparent embryos. In addition, the zebrafish shares a high degree of homology with the human genome. Thus, the zebrafish is becoming a powerful model organism for studying genetics, development, environmental toxicology, pharmacology, DNA damage repair, cancer, and other diseases (Bradford et al., 2017). Zebrafish could be used for studies on ecotoxicological monitoring and numerous pollutant evaluations, such as toxic heavy metals, endocrine disruptors, and organic pollutants (Martínez-Sales et al., 2015). However, new biotechnologies can now improve detection sensitivity. Recently, with the development of transgenic technology, especially the applications of both luciferase (LUC) and green fluorescent protein (GFP) reporters in this field, monitoring efficiency and sensitivity have been dramatically improved (Dai et al, 2014).

Specific strains of zebrafish suitable e.g. for detecting water pollutants are available all year round from the Zebrafish International Resource Center (ZIRC), which is housed on the University of Oregon campus in Eugene, US. Its mission is to provide broodstock maintained in a central live gene bank of wildtype, transgenic, and mutant lines of zebrafish and to distribute characterized, healthy, and breedable lines to the research community (Varga 2016) or even to laboratories of aquatic ecotoxicology. Husbandry standards and procedures for maintaining healthy strains of zebrafish are continually evaluated, optimized, and developed in Europe as well, at the European Zebrafish Resource Center (EZRC) in Germany.

The genomic sequence of zebrafish is publicly available so that it can be used for investigating specific effects allowing the application of this animal model in environmental studies (Howe et al., 2013). Genomic sequence can also provide information on morphology related molecular mechanisms responding to changing environmental conditions (Hollert and Keiter, 2015). Many of the crucial pathways that regulate vertebrate development are mostly conserved between humans and zebrafish, so that about 70 % of all genes associated with human diseases have functional homologs in zebrafish (Langheinrich 2003). Consequently, zebrafish can be considered as highly significant model organism to investigate the health-related risks of pollutants either for humans or for the environment (Dai et al, 2014). The use of embryos as replacement method for animal experiments has been receiving increased attention. Zebrafish embryos are suitable for the analysis of several endpoints, either in acute developmental toxicity or in functional genetical and physiological analyses (Strähle et al., 2012). Zebrafish as model organism has gained increasing relevance for the assessment of ecotoxicological risk concerning chemicals and environmental mixtures in the prediction of acute toxicity (Braunbeck et al., 2015). Both the hazard potential of polluted sediments including molecular and mechanism-specific alterations (Hallare et al., 2011; Vincze et al., 2014) and the fractions of effect-directed analyses (Di Paolo et al., 2015) can be investigated using zebrafish embryos.

Some relevant zebrafish bioassays for aquatic ecotoxicology

Fish as aquatic vertebrate class is a taxonomic group which has traditionally been used as an essential element of complex toxicity testing protocols. Fish differ from other vertebrates and also from most invertebrates e.g. in their metabolic capacities, in biotransformation competence for a few chemical classes (Hutchinson et al., 2014). In addition to this, due to the occasionally significant pollution levels and the onset of chemicals release, fish are exposed to direct chemical pollution (Hamilton et al., 2017). In many cases the mass mortality of fish has been proved to be a suitable tool to indicate the need for restrictions in chemical discharge via direct contamination and by continuous release from wastewater treatment plants (Kibiria 2011). Fish tests have been applied traditionally in procedures for checking the quality of water resources of human drinking water. Given the relevance of fish in water contamination monitoring, fish tests have intensively been implemented in aquatic toxicity testing both at national and international levels (Carvan et al., 2000).

Numerous test guidelines using fish as test organism has been established at OECD for the evaluation of: acute toxicity (OECD 203), early life-stage toxicity (OECD 210), short term toxicity on embryo and sac-fry (OECD 212), and juvenile growth (OECD 215). The need to evaluate acute toxicity of single compounds has been surpassed by the identification of complex toxic effects derived from the mixtures of compounds (at much lower concentration levels) due to the recent progress in the maintenance of appropriate water quality in numerous countries. However, despite all efforts to control chemical pollution – populations of endangered fish have not recovered in many regions (Johnson and Sumpter, 2014). Considering the potentially deleterious effects following long-range exposure to sublethal concentration of chemical mixtures, the development of procedures to identify more specific ways of toxic action (e.g. endocrine disruption) has been privileged. In order to keep up with these tendencies, OECD expert groups are developing updated test guidelines incorporating more sophisticated endpoints continuously (Braunbeck and Lammer, 2006). For example, “Fish, Acute Toxicity Test” has been originally adopted in 1981 by OECD as part of the “OECD Guidelines for Testing of Chemicals” series and it has been updated several times up to the current 11th version updated in July 2018 (see also OECD 230, 324, 236 in reference list below). For example, according to the protocol described in the OECD 203 guideline, the fish are exposed to the test chemical for a period of 96 hours. Clinical signs and mortalities are recorded and the concentrations which kill 50% of the fish (LC50) are determined, where possible. The selection of species depends in part on the chemical to be tested (industrial chemical, pharmaceutical, biocide or plant protection product), and on the environmental exposure that fish may receive (see in Section 2 of OECD 203). Zebrafish is one of the eight freshwater fish species which are recommended for using in this test protocol.

Since 2005, fish embryo toxicity testing has been made mandatory for routine sewage surveillance in Germany; since then, conventional fish tests are no longer accepted for routine whole effluent testing. According to the protocol originally designed for whole effluent testing, modifications have been introduced to make the protocol fit for chemical testing (Embry et al., 2010). Germany’s flagship projects have resulted in several zebrafish bioassays for aquatic ecotoxicology. For example, a “Special Issue of Environmental Science and Pollution Research” (Hollert and Keiter, 2015) contains relevant papers presented at a symposium on “Methods for the assessment of sediment toxicity using zebrafish (*Danio rerio*)” at Aachen University in 2013. Studies on new molecular and mechanism-specific bioassays are published in this special issue highlighting the following topics (selected references):

- Braunbeck et al. (2015) describes the origin, utilization and potential future applications of the fish embryo test (FET)
- Kamstra et al. (2015) presents a review on zebrafish as a model for DNA methylation within environmental toxicology
- Legradi et al. (2015) describes the comparability of behavioral tests using zebrafish larvae in the assessment of neurotoxicity
- Redelstein et al. (2015) reports on sediment-bound metals concerning bio-accumulation and molecular impacts on zebrafish embryos
- Thellmann et al. (2015) characterizes Fish embryo tests in zebrafish as a relevant tool in the evaluation of waste water treatment plants efficiency regarding the reduction of ecotoxic potentials either in surface water or in the sediment

- Garcia-Käufer et al. (2015) describes their experiments on teratogenic and genotoxic effects of sediment in freshwater samples derived from Rhine and Elbe Rivers (Germany), evaluated using zebrafish embryos in a multi-endpoint testing protocol
- Wang et al. (2015) reports on their investigations on the toxicity of sediment cores sampled from Yangtze River mouth, tested on zebrafish (Danio rerio) embryos
- Hafner et al. (2015) presents investigations on sediment toxicity of German rivers applying standardized bioassay battery.

Moreover, existing assays has been developed within a joint research project called "DanTox" (funded by the German Federal Ministry of Education and Research, 2010-2013) in which novel mechanism-specific contact assays were developed for the assessment of the bioavailable toxicity of sediments. New and relevant knowledge either on the application of such bioassays or on the underlying cellular mechanisms have been obtained. In addition to this, microarray analyses were found to be suitable for the identification of gene sets representing different important cellular pathways and responding to environmental contaminants (Hollert and Keiter, 2015).

Those environmental estrogens that can alter hormone signaling in human body are termed as endocrine-disrupting chemicals (EDCs). Exposure to these chemicals can be associated with decreased semen quality and sperm count, malformation of urogenital tract, or even with increased occurrence of breast cancer and testicular cancer (Jana and Sen, 2012; Rachoń 2015). Similarly in fish, environmental estrogens exposure affects sexual development resulting in the feminization of males and altered sexual behavior (Söffker and Tyler, 2012). Over 900 chemical compounds with endocrine-disrupting activity have been identified, of which 200 had proven estrogenic effects (Kennedy et al., 2013). Large-scale international research projects have been established aiming to screen the endocrine-disrupting activity in order to avoid human and environmental health risks which are associated with exposure (e.g. Vandenberg et al., 2016). Using estrogen-responsive transgenic (TG) zebrafish, Lee et al (2012) and others identified target tissues for environmental estrogens even at environmentally relevant concentrations. Estrogenic endocrine-disrupting chemicals (EDCs) induced specific expression of green fluorescent protein (GFP) in different tissues of TG fish e.g. in the liver, skeletal muscle, forebrain, and ganglions, even in tissues which have not been identified previously as targets for estrogens in fish.

Transgenic zebrafish as biosensor can also be used e.g. for sensing aquatic heavy metal pollution (Pawar et al., 2016).

Future directions

The Zebrafish Information Network (ZIFN) has also responded to the increased use of zebrafish for translational research and the need to access human disease providing relevant data by annotating zebrafish models of human disease as well as by providing dedicated user interfaces and search platforms to access human disease information. In order to aid the development of therapeutic strategies several zebrafish models of human diseases have been created using either genetic information or experimental outcomes to understand corresponding gene pathways and phenotypic effects (Bradford et al., 2017).

Zebrafish has become a powerful animal model for numerous human diseases and consequently for pharmaceutical drug discovery and development during the past few decades (MacRae and Peterson, 2015). The large number of transparent embryos gained from zebrafish females, ex utero and rapid embryonic development and ready-to-use methods of biotechnology enables us to use mostly automatized high-throughput screening not only in pharmaceutical drug development protocols but in aquatic ecotoxicology as well.

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