Zebrafish as a possible bioindicator of organic pollutants with effects on reproduction in drinking waters

M. Martínez-Sales*, F. García-Ximénez, F.J. Espinós

Aquaculture and Environmental Research Group (ACUMA), Polytechnic University of Valencia, 46022 Valencia, Spain

ARTICLE INFO

ABSTRACT

Organic contaminants can be detected at low concentrations in drinking water, raising concerns for human health, particularly in reproduction. In this respect, we attempted to use the zebrafish as a bioindicator to detect the possible presence of these substances in drinking water, aiming to define the most relevant parameters to detect these substances, which particularly affect the development and reproduction of zebrafish. To this end, batches of 30 embryos with the chorion intact were cultured in drinking waters from different sources, throughout their full life-cycle up to 5 months, in 20 L tanks. Six replicates were performed in all water groups, with a total of 24 aquariums. Two generations (F0 and F1) were studied and the following parameters were tested: in the F0 generation, survival and abnormality rates evaluated at 5 dpf (days post-fertilization) and at 5 mpf (months post-fertilization), the onset of spawning and the fertility rate from 3 mpf to 5 mpf, and the sex ratio and underdeveloped specimens at 5 mpf. Furthermore, in the F0 offspring (F1), survival and abnormality rates were evaluated at 5 dpf and the hatching rate at 72 hpf. These results revealed that the hatching rate is the most sensitive parameter to distinguish different levels of effects between waters during the early life stages, whereas the rate of underdeveloped specimens is more suitable at later life stages. Regarding adult reproduction, fertility rate was the most sensitive parameter. The possible reversibility or accumulative nature of such effects will be studied in future work.

© 2015 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V.

Introduction

The detection and monitoring of organic pollutants present in drinking water such as medical substances (Heberer, 2002), some active pharmaceutical ingredients (Kallenborn et al., 2008; Galus et al., 2013) and some persistent organic pollutants (POPs) (Ericson et al., 2008; Wilhelm et al., 2010; Ullah et al., 2011; Eschauzier et al., 2012) are relevant due to their possible effects on human reproductive function in metropolitan areas (Braw-Tal, 2010; Vested et al., 2014).

Animals and humans are exposed in nature to combinations of environmental pollutants. So, different environmental chemicals may interact with each other and thereby induce weaker (antagonistic), additive or stronger (synergistic) combined effects than would be expected from single compounds (Monosson, 2005; Kortenkamp, 2007). In this sense, it must be highlighted that the knowledge of the impact of these chemical interactions is still insufficient (Monosson, 2005; Schwarzenbach et al., 2006; Kortenkamp, 2007; Holmstrup et al., 2010). Moreover, the problem is exacerbated in drinking water

* *Corresponding author*. E-mail: mimarsa@alumni.upv.es (M. Martínez-Sales).

from metropolitan areas, as the concentrations of emerging contaminants are low but numerous and variable over time (Westerhoff et al., 2005; Khetan and Collins, 2007; Kim et al., 2007), with municipal wastewater being the main source of most of these compounds (Metcalfe et al., 2003, 2014). So, due to the complexity of their control and detection, bioindicators can be used as an alternative to monitor their presence.

In this context, zebrafish is currently being used as a model to monitor toxic heavy metals, endocrine disruptors and organic pollutants for toxicology studies (Dai et al., 2014), as well as to assess certain contaminants in water quality studies (Ansari and Sharma, 2009; Molinari et al., 2009). Zebrafish biology has been extensively studied and well described (Westerfield, 2007; Nüslein-Volhard et al., 2002), and many morphological endpoints have been established from embryos to adult in environmental toxicity studies (Zhang et al., 2003). Consequently, as our purpose in the long term is to determine if zebrafish (*Danio rerio*) can be used to detect environmental pollutants in drinking waters with effects on reproduction, an essential and preliminary aspect consists of defining and narrowing down those parameters/endpoints that may be useful to detect the effects of these environmental factors, especially on the development and reproduction of zebrafish when they are cultured in drinking waters from different sources throughout their life cycle.

The parameters evaluated in this study were selected in an attempt to cover the most relevant effects from environmental pollutants possibly present in drinking water on development, and especially on reproduction. So, these parameters were studied from survival and development to reproduction, contemplating the full life-cycle, as environmental exposures during critical periods of development may result in permanent alterations in the biological system of adults (Lyche et al., 2013), or even in subsequent generations. In this context, as pointed out by Skinner (2011), a number of environmental factors and toxicants (bisphenol A, dioxins, *etc.*) have been shown to promote epigenetic transgenerational inheritance of disease states or phenotypic variation.

1. Materials and methods

1.1. *Zebrafish maintenance*

A wild zebrafish colony was reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5°C, in a 3:2 ratio (females:males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Francisco-Simão et al., 2010). The light cycle was regulated at 14 hr light/10 hr dark (Matthews et al., 2002; Brand et al., 2002).

1.2. *Experimental design*

Embryos were obtained by siphoning from the original colony. Batches of 30 embryos at the midblastula transition (MBT) stage with the chorion intact were selected under a stereo microscope and separated from those that degenerated and those that initiated aberrant parthenogenetic development.

Embryos were not dechorionated because they were used to detect substances in different drinking waters that cannot be suitable for dechorionated embryos (Martinez-Sales et al., 2014). In the present study, four different drinking waters were evaluated and classified, depending on their source, into: three waters from different tap water distribution networks (A, B and C) and one bottled spring water that was established as a control group due to the quality of the water. Type A was tap water from a city located in a region with intensive farming activity, from the hydrological basin of the Túria River. Type B was from the tap water distribution network of a medium-sized city, supplied from the Túria and Xúquer Rivers. Finally, type C was tap water from a city also located in a region with intensive agricultural activity, but from the hydrological basin of the Xúquer River. Types A and C came from groundwater prospecting.

Previous to the water addition to the aquariums, receptacles (where the water was stored) were maintained during at least a week open, with a large exchange surface to favor chlorine evaporation (Westerfield, 1995). Batches of embryos were left in Petri dishes and cultured until 5 dpf (days post-fertilization) at 28.5°C in the different waters. Abnormality rates at 5 dpf (pericardial edema, curled tails and skeletal deformities: lordosis, scoliosis and abnormal skeletal development) and survival rates at 5 dpf were evaluated. Six replicates were performed in all water groups. Next, from 5 dpf to complete adulthood (5 months post-fertilization) 30 larvae were left in aquariums (20 L), with a total of 24 aquariums, in these four different waters. The aquariums had water recirculation systems but without active carbon filters. According to the Westerfield (2007) recommendations, a quarter of the total water of the aquarium was removed weekly to be replaced by clean water, in order to avoid ammonium concentration. After three months, marbles were placed in each aquarium with the aim of siphoning all aquariums 2 or 3 times a week for two months, to evaluate the onset of spawning and the fertility rate. Sex ratio of the surviving adults and survival and abnormality rates at 5 mpf were also evaluated. Moreover, in the F0 offspring (F1 larvae) the survival and abnormality rates at 5 dpf and the hatching rate at 72 hpf were evaluated. Sterilized media and materials in aseptic conditions were used. All chemicals were from Sigma-Aldrich (Madrid, Spain).

It should be stated that all environmental conditions were identical in all aquariums and that the spatial distribution of the 24 aquariums was randomized. The chemical parameters established for tap water for human consumption in the Royal Decree 140/2003 of 7 February, by which health criteria for the quality of water intended for human consumption are established, are suitable for the breeding and maintenance of zebrafish (Westerfield, 2007). Results were analyzed using the Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved. To analyze the onset of spawning, a simple Analysis of Variance (ANOVA) test was used. Finally, once all the information was collected and adults' sex was identified, specimens were euthanized with clove oil. The experimental procedures and the animal care in the present work fully agree with the standards for use of animals established by the Ethical Committee of the Polytechnic

University of Valencia, which has specifically approved this study.

2. Results

2.1. Survival and abnormality rates at five days

Survival rate at 5 dpf was evaluated to rule out the presence of acute or long-term toxicants (macropollutants), as the aim was to detect micropollutants (especially organic pollutants with effects on reproduction). Embryo survival rates, evaluated at 5 dpf, in the F0 generation were high in all groups, with no significant differences between waters (Table 1). The abnormality rates in the F0 generation, also evaluated at 5 dpf, were low in all groups (varying from 0% to 0.56%), without significant differences between waters (data not presented in tables). The survival rates at 5 dpf in F0 offspring (F1 larvae) were also high in all groups, only reaching significant differences between the control group and all other groups (A, B and C), where the control group achieved the highest survival rate. Significant differences ($p < 0.05$) were observed in survival rates at 5 dpf for each water, between the F0 generation and F0 offspring (F1 larvae), except in the control group, which maintained the same survival rate. Survival rates in F0 offspring (F1 larvae) slightly, but significantly, decreased compared to the F0 generation in the rest of the waters (A, B and C). Regarding survival rate from 5 dpf to 5 mpf in F0, differences between water groups did not reach significant levels, although they came close to doing so ($p = 0.0617$) (Table 1).

In the case of abnormalities at 5 dpf in F0 offspring (F1 larvae), pericardial edema, curled tails and skeletal deformities (lordosis, scoliosis, and abnormal skeletal development) were the main malformations observed, although other types of malformation were also detected. A slight non-significant increase in abnormality rates in F0 offspring (F1 larvae) (A: 1.54%; B: 1.20%; C: 1.35%; control group: 0.66%) compared to F0 generation (A: 0.56%; B: 0%; C: 0.55%; control group: 0%) was observed in all groups, whatever the water type, the lowest rate being found in the control group. At 5 mpf, the only abnormalities detected were skeletal deformities in water B (two specimens developed lordosis) and water C (one specimen developed scoliosis), with no significant differences between waters.

2.2. Hatching rate

In zebrafish, hatching occurs between 48 and 72 hr post-fertilization (hpf), when organogenesis is almost complete (Kimmel et al., 1995), so the hatching rate was evaluated at 72 hpf in our experiment. Thus, in the analysis of the hatching rate at 72 hpf in F0 offspring (F1 larvae), significant differences were observed between waters, where the lowest rate was reached in group B (86.47%: 761/880) and the highest rate in the control group (99.50%: 1215/1221), while in groups A (96.53%: 473/490) and C (97.41%: 828/850) there were no significant differences.

2.3. Onset of spawning

Adult zebrafish reach sexual maturity within three months after hatching (Ma et al., 2000), so the onset of spawning was evaluated from 3 months on in the F0 generation. No statistically significant differences were detected with one-way ANOVA ($p = 0.9757$), the mean number of days from 3 mpf being 16.5 ± 4.82 days in water A, 19.5 ± 4.82 days in water B, 17.3 ± 4.82 days in water C and 18 ± 4.82 days in the control group. Similar observations were made with the onset of presence of fertilized eggs ($p = 0.9183$). This indicates that the beginning of reproductive activity is similar in females and males, the mean number of days being 18.5 ± 4.65 days in water A, 22.1 ± 4.65 days in water B, 18.1 ± 4.65 days in water C and 18.5 ± 4.65 days in the control group.

2.4. Fertility rate

Regarding the fertility rate in F0, significant differences ($p < 0.05$) were observed between waters evaluated during the 4th and 5th mpf. The statistically worst result was obtained in water B (34.31%: 895/2608) and the best result in the control group (74.37%: 1274/1713). Groups A (42.60%: 490/1150) and C (57.36% 857/1494) reached intermediate values.

2.5. Sex ratio and underdeveloped specimens (runts)

Regarding sex ratio, there were no significant differences ($p = 0.4125$) between waters at 5 mpf in F0, the male percentage being high (varying from 64% to 73%) in all waters compared to the female percentage (varying from 27% to 36%).

On the other hand, some runt fishes were observed (clearly smaller than the other fishes and without a morphologically identifiable sex) at 5 mpf in F0, showing differences between waters ($p = 0.0456$) when analyzed. The worst results were in groups B (7%: 9/128) and C (8.5%: 11/129), and the best result in the control group (0%: 0/89). Group A (5%: 7/140) reached an intermediate value.

Table 1 – Survival rate of zebrafish (*Danio rerio*) specimens cultured in different waters according to their source at 5 dpf and 5 mpf in F0 and F0 offspring (F1).					
		Water A	Water B	Water C	Control group
Survival rate (5 dpf)	F0	177/180 (98.33%)	179/180 (99.44%)	180/180 (100%)	178/180 (98.88%)
	F0 offspring (F1)	454/490 (92.65%)[b]	829/880 (94.20%)[b]	814/857 (94.98%)[b]	1200/1221 (98.28%)[a]
Survival rate (from 5 dpf to 5 mpf)	F0	140/177 (79.09%)	128/179 (71.50%)	129/180 (71.66%)	118/178 (66.29%)
Columns with different superscripts are statistically different ($p < 0.05$).					

3. Discussion

In zebrafish, most full life-cycle studies have been carried out in toxicology, where the substance concentration effects to be evaluated have been previously established (Soares et al., 2009; Galus et al., 2013; Dai et al., 2014). In this study, in contrast, there is no intention to detect specific contaminants, but rather the effect of the mixture of a variety of different substances present in drinking water, on the development and reproduction in zebrafish. In fact, the substances that could be present in the different waters are completely unknown, as are their number and concentrations. For this reason, we attempted to evaluate the most relevant parameters/endpoints of zebrafish throughout their life-cycle to detect the possible presence of emerging contaminants at trace levels in drinking water that could affect survival, development and especially reproduction.

Survival rates at 5 dpf and at 5 mpf have been established as endpoints to assess the acute toxicity in many works (Voelker et al., 2007; Zhu et al., 2008; Shi et al., 2008; He et al., 2011; Keiter et al., 2012). In our study, survival and abnormality rates at 5 dpf in the F0 generation and in F0 offspring (F1 larvae) were high (>92%) and low (<7%) respectively, whatever the water source. At 5 mpf, survival rates (from 66.29% to 79.09%) were within normal values for this species (Santos et al., 2006). These results suggest no relevant presence of lethal substances to zebrafish embryos in the waters studied.

Although our final objective is to detect organic environmental pollutants with effects on reproduction, the mortality evaluation at 5 dpf and at 5 mpf allows us to rule out the presence of lethal toxicants in the studied waters, which, if present, could alter the assessment of the effects on reproduction. According to the hatching rate at 72 hpf, there were differences between waters. The control group (99.50%) reached the best result, agreeing with control data from other studies (Bourrachot et al., 2008; He et al., 2011; Liu et al., 2014).

The period around hatching is a critical stage during embryogenesis (Henn, 2011). This process is a combination of biochemical (action of the enzyme chorionase) and physical (movements of the embryo) mechanisms, which may be differently affected by chemicals (Bourrachot et al., 2008). Indeed, some pharmaceutical substances (David and Pacharatna, 2009), endocrine disruptors (Han et al., 2011) and insecticides (Mandrell et al., 2012), among others (Duan et al., 2008), have decreased or inhibited the hatching rate in zebrafish, and most of these substances have been detected in wastewaters (Brossa et al., 2005) or even in drinking waters (Stackelberg et al., 2004). Hence, according to the results obtained in the present study, the hatching rate at 72 hpf would be a suitable endpoint to assess the presence of organic pollutants that may affect reproduction in drinking waters.

It is well known that spawning in domesticated zebrafish is influenced by photoperiod (Breder and Rosen, 1966). In our case, the light cycle was regulated, as stated in the Materials and methods section. No differences were observed between waters in the onset of spawning or in the onset of appearance of fertilized eggs. So, these two parameters do not appear to be relevant endpoints to detect the possible presence of organic pollutants in drinking waters at trace levels, or perhaps the

substances that could have an effect are not present in the studied waters or, at least, are not present at harmful concentrations. In fact, studies have shown that chronic estrogen exposure of zebrafish to 17α-ethinylestradiol, which may be present in wastewaters and surface waters (Canonica et al., 2008), induced delayed onset of spawning and reduced fecundity and fertilization success at ng/L concentrations (Schäfers et al., 2007; Segner, 2009).

The fertility rate is used in many toxicological studies as endpoint/parameter (Ankley and Johnson, 2004; Liu et al., 2014). Significant differences between waters were observed in our study. The control group (74.37%) obtained the best result, which agrees with the control data from other studies (He et al., 2011; Keiter et al., 2012). These results suggest that fertility rate is a rather sensitive parameter to detect the presence of organic pollutants at trace levels. Obviously, the results obtained in the present study do not allow us to elucidate which of these substances are present in water, but do enable us to detect their joint presence or their absence.

It has been demonstrated that many pollutants detected in wastewater effluents and surface waters (Botella et al., 2004; Brossa et al., 2005; Canonica et al., 2008; Galus et al., 2013) have reduced the fertility rate, as for example persistent organic pollutants (Njiwa et al., 2004), endocrine disruptors (Larsen et al., 2009), and pharmaceutical substances (Nash et al., 2004), among others. The effects on fertility rate could be of female origin, male origin or both. Another possible source could be the watery environment where the external fecundation took place. The breakdown of these three possibilities will be studied in the next study undertaken.

Regarding sex ratio, this did not show significant differences between waters, being within normal values (from 64% to 73% males), which is in accordance with the sex ratios reported as normal in other studies on zebrafish (60 males:40 females) (Fenske et al., 1999), (68 males:32 females) (Örn et al., 2003), (56 males:44 females) (Vaughan et al., 2001; Hsioa and Tsai, 2003), while also being within the normal range in zebrafish raised in captivity (Hill and Janz, 2003).

Sex ratio is a relevant endpoint used in numerous toxicological studies (Hill and Janz, 2003; Baumann et al., 2013; Liu et al., 2014), for example in the evaluation of endocrine disruptors (Örn et al., 2006). However, the complexity of sex determination in zebrafish may prevent the sex ratio from being used as a sensitive indicator of chemical effects without rigorous control of both environmental and genetic factors (Lawrence et al., 2007), as it is well known that environmental factors, including pH, stocking density and temperature affect sex differentiation in fish (Baroiller et al., 1999). However, it must be emphasized that in our case environmental parameters were tightly controlled during the experiment.

Concerning underdeveloped specimens, significant differences between waters were observed. The control group showed the best result (0%) and waters B and C obtained the worst results (7% and 8.5% respectively). It has been reported that some endocrine disruptors (Van der Ven et al., 2003) detected in wastewater effluent and surface waters (Brion et al., 2004), as well as some pharmaceutical and personal care products (Galus et al., 2013) detected in the aquatic environment at ng/L and μg/L concentrations (Metcalfe et al., 2003;

Kuster et al., 2008; Benotti et al., 2009) have been shown to affect zebrafish development. Thus, according to the results obtained in the present work, the underdevelopment rate would be a proper endpoint to assess the presence of organic pollutants in drinking water.

In a broader perspective, there are substances that could affect one or several of the parameters studied. Thus, some endocrine disruptors (He et al., 2011) affected both hatching and fertility rate but did not alter growth in zebrafish. However, other endocrine disruptors only affected hatching rate (Carreño et al., 2007). Other substances like pharmaceutical and personal care products affected both fertility rate and growth, but did not alter hatching rate. Nevertheless, other pharmaceutical and personal care products affected hatching rate and growth but did not affect fertility rate (Galus et al., 2013, 2014). Furthermore, some persistent organic pollutants have been shown to affect the fertility rate, hatching rate and growth in zebrafish (Njiwa et al., 2004).

On the other hand, with respect to the waters studied, according to their source and based upon results obtained, our control group presented the best results. The worst results were obtained in water B in those parameters/endpoints that reached significant differences between waters (hatching rate, fertility rate and underdeveloped specimens). A possible reason to justify this would be that the source of water B, as stated in the Materials and methods section, is surface water, while waters A and C were from groundwater prospecting. The discharge of industrial and municipal wastewaters, whether treated or not, can be considered a constant polluting source that modifies surface water hydrochemistry, whereas groundwater contamination is not usually direct, as it must pass through various geological strata, where many physical–chemical processes may intervene to at least partially purify fluid wastes.

In conclusion, according to the results obtained in all studied parameters, it must be considered that high survival rates allow toxicities to be ruled out. From all the reproductive parameters studied, the hatching, fertility and underdevelopment rates seem to be the most sensitive parameters to detect environmental pollutants in drinking water that affect reproduction during the full life-cycle of zebrafish. The possible cumulative effects through time or those transmissible to the next generation *via* epigenetic mechanisms, with effects on reproduction, will be studied in future work.

REFERENCES

Ankley, G.T., Johnson, R.D., 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. Inst. Lab. Anim.Res. 45 (4), 469–483.

Ansari, B.A., Sharma, D.K., 2009. Toxic effect of synthetic pyrethroid deltamethrin and neem based formulation achook on zebrafish, *Danio rerio* (Cyprinidae). Trends Biosci. 2 (2), 18–20.

Baroiller, J.F., Guiguen, Y., Fostier, A., 1999. Endocrine and environmental aspects of sex differentiation in fish. Cell. Mol. Life Sci. 55 (7), 910–931.

Baumann, L., Holbech, H., Keiter, S., Kinnberg, K.L., Knörr, S., Nagel, T., et al., 2013. The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the fish sexual development test. Aquat. Toxicol. 128–129, 34–42.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2009. Pharmaceuticals and endocrine disrupting compounds in US drinking water. Environ. Sci. Technol. 43 (3), 597–603.

Botella, B., Crespo, J., Rivas, A., Cerrillo, I., Olea-Serrano, M.F., Olea, N., 2004. Exposure of women to organochlorine pesticides in Southern Spain. Environ. Res. 96 (1), 34–40.

Bourrachot, S., Simon, O., Gilbin, R., 2008. The effects of waterborne uranium on the hatching success, development, and survival of early life stages of zebrafish (*Danio rerio*). Aquat. Toxicol. 90 (1), 29–36.

Brand, M., Granato, M., Nüslein-Volhard, C., 2002. Keeping and raising zebrafish. In: Nüslein-Volhard, C., Dahm, R. (Eds.), Zebrafish: A Practical Approach. Oxford University Press, Oxford, England, p. 16.

Braw-Tal, R., 2010. Endocrine disruptors and timing of human exposure. Pediatr. Endocrinol. Rev. 8 (1), 41–46.

Breder, C.M., Rosen, D.E., 1966. Modes of Reproduction in Fishes. Natural History Press, New York.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., et al., 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile-and adult-life stages in zebrafish (*Danio rerio*). Aquat. Toxicol. 68 (3), 193–217.

Brossa, L., Marcé, R.M., Borrull, F., Pocurull, E., 2005. Occurrence of twenty-six endocrine-disrupting compounds in environmental water samples from Catalonia, Spain. Environ. Toxicol. Chem. 24 (2), 261–267.

Canonica, S., Meunier, L., Von Gunten, U., 2008. Phototransformation of selected pharmaceuticals during UV treatment of drinking water. Water Res. 42 (1–2), 121–128.

Carreño, J., Rivas, A., Granada, A., Lopez-Espinosa, M.J., Mariscal, M., Olea, N., et al., 2007. Exposure of young men to organochlorine pesticides in Southern Spain. Environ. Res. 103 (1), 55–61.

Dai, Y.J., Jia, Y.F., Chen, N., Bian, W.P., Li, Q.K., Ma, Y.B., et al., 2014. Zebrafish as a model system to study toxicology. Environ. Toxicol. Chem. 33 (1), 11–17.

David, A., Pancharatna, K., 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 27 (3), 390–395.

Duan, Z.H., Zhu, L., Zhu, L.Y., Yao, K., Zhu, X.S., 2008. Individual and joint toxic effects of pentachlorophenol and bisphenol A on the development of zebrafish (*Danio rerio*) embryo. Ecotoxicol. Environ. Saf. 71 (3), 774–780.

Ericson, I., Nadal, M., Van Bavel, B., Lindström, G., Domingo, J.L., 2008. Levels of perfluorochemicals in water samples from Catalonia, Spain: is drinking water a significant contribution to human exposure? Environ. Sci. Pollut. Res. 15 (7), 614–619.

Eschauzier, C., Beerendonk, E., Scholte-Veenendaal, P., De Voogt, P., 2012. Impact of treatment processes on the removal of perfluoroalkyl acids from the drinking water production chain. Environ. Sci. Technol. 46 (3), 1708–1715.

Fenske, M., Maack, G., Ensenbach, U., Segner, H., 1999. Identification of estrogens-sensitive developmental stages of

zebrafish, *Danio rerio*. Proceedings of the 9th Annual Meeting of Society of Environmental Toxicology and Chemistry. 25–29 May, Leipzig, Germany.

Francisco-Simão, M., Cardona-Costa, J., Pérez Camps, M., García-Ximénez, F., 2010. Ultraviolet radiation dose to be applied in recipient zebrafish embryos for germ-line chimaerism is strain dependent. Reprod. Domest. Anim. 45 (6), 1098–1103.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., et al., 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquat. Toxicol. 132–133, 200–211.

Galus, M., Rangarajan, S., Lai, A., Shaya, L., Balshine, S., Wilson, J.Y., 2014. Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring. Aquat. Toxicol. 151, 124–134.

Han, Z.H., Jiao, S.J., Kong, D.Y., Shan, Z.J., Zhang, X.W., 2011. Effects of β-endosulfan on the growth and reproduction of zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 30 (11), 2525–2531.

He, J.H., Yang, D.R., Wang, C.Y., Liu, W., Liao, J.H., Xu, T., et al., 2011. Chronic zebrafish low dose decabrominated diphenyl ether (BDE-209) exposure affected parental gonad development and locomotion in F1 offspring. Ecotoxicology 20 (8), 1813–1822.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131 (1–2), 5–17.

Henn, K., 2011. Limits of the Embryo Toxicity Test with *Danio rerio* as an Alternative to the Acute Fish Toxicity Test. University of Heidelberg, Germany (PhD thesis).

Hill, R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*). I. Effects on sex ratio and breeding success. Aquat. Toxicol. 63 (4), 417–429.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., et al., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408 (18), 3746–3762.

Hsioa, C.D., Tsai, H.J., 2003. Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo. Dev. Biol. 262 (2), 313–323.

Kallenborn, R., Fick, R., Lindberg, R., Moe, M., Nielsen, K.M., Tysklind, K., et al., 2008. Pharmaceutical residues in Northern European environments: consequences and perspectives. Pharmaceuticals in the Environment. Springer, Berlin, Heidelberg.

Keiter, S., Baumann, L., Färber, H., Holbech, H., Skutlarek, D., Engwall, M., et al., 2012. Long-term effects of a binary mixture of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish (*Danio rerio*). Aquat. Toxicol. 118–119, 116–129.

Khetan, S.K., Collins, T.J., 2007. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. Chem. Rev. 107, 2319–2364.

Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J., Snyder, S.A., 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking waste and waters. Water Res. 41 (5), 1013–1021.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203 (3), 253–310.

Kortenkamp, A., 2007. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. Environ. Health Perspect. 115 (S1), 98–105.

Kuster, M., López de Alda, M.J., Hernando, M.D., Petrovic, M., Martín-Alonso, J., Barceló, D., 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). J. Hydrol. 358 (1–2), 112–123.

Larsen, M.G., Bilberg, K., Baatrup, E., 2009. Reversibility of estrogenic sex changes in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 28 (8), 1783–1785.

Lawrence, C., Ebersole, J.P., Kesseli, R.V., 2007. Rapid growth and outcrossing promote female development in zebrafish (*Danio rerio*). Environ. Biol. Fish 81 (2), 239–246.

Liu, S.Y., Jin, Q., Huang, X.H., Zhu, G.N., 2014. Disruption of zebrafish (*Danio rerio*) sexual development after full life-cycle exposure to environmental levels of triadimefon. Environ. Toxicol. Pharmacol. 37 (1), 468–475.

Lyche, J.L., Grzés, I.M., Karlsson, C., Nourizadeh-Lillabadi, R., Berg, V., Kristoffersen, A.B., et al., 2013. Parental exposure to natural mixtures of POPs reduced embryo production and altered gene transcription in zebrafish embryos. Aquat. Toxicol. 126, 424–434.

Ma, C.G., Fan, L.C., Ganassin, R., Bols, N., Collodi, P., 2000. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc. Natl. Acad. Sci. U. S. A. 98 (5), 2461–2466.

Mandrell, D., Truong, L., Jephson, C., Sarker, M., Moore, A., Lang, C., et al., 2012. Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. J. Lab. Autom. 17 (1), 66–74.

Martinez-Sales, M., García-Ximénez, F., Espinós, F.J., 2014. Zebrafish (*Danio rerio*) as a possible bioindicator of epigenetic factors present in drinking water that may affect reproductive function: is chorion an issue? Zygote 5, 1–6.

Matthews, M., Trevarrow, B., Matthews, J., 2002. A virtual tour of the guide for zebrafish users. Lab. Anim. 31 (3), 34–40.

Metcalfe, C.D., Miao, X.S., Koening, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ. Toxicol. Chem. 22 (12), 2881–2889.

Metcalfe, C., Hoque, M.E., Sultana, T., Murray, C., Helm, P., Kleywegt, S., 2014. Monitoring for contaminants of emerging concern in drinking water using POCIS passive samplers. Environ. Sci. Process. Impacts 16 (3), 473–481.

Molinari, A., Troisi, S., Fallico, C., Paparella, A., Straface, S., 2009. Broom fibre PRB for heavy metals groundwater remediation. EGU General Assembly Conference Abstracts. 11-8075 April, Vienna, Austria.

Monosson, E., 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. Environ. Health Perspect. 113 (4), 383–390.

Nash, J.P., Kime, D.E., Van der Ven Leo, T.M., Wester, P.W., Brion, F., Maack, G., et al., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethinyl estradiol causes reproductive failure in fish. Environ. Health Perspect. 112 (17), 1725–1733.

Njiwa, J.R.K., Müller, P., Klein, R., 2004. Life cycle stages and length of zebrafish (*Danio rerio*) exposed to DDT. J. Health Sci. 50 (3), 220–225.

Nüsslein-Volhard, C., Gilmour, D.T., Dahm, R., 2002. Introduction: zebrafish as a system to study development and organogenesis. In: Nüslein-Volhard, C., Dahm, R. (Eds.), Zebrafish: A Practical Approach. Oxford University Press, Oxford, England, pp. 1–5.

Örn, S., Holbech, H., Madsen, T.H., Norrgren, L., Petersen, G.I., 2003. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyl testosterone. Aquat. Toxicol. 65 (4), 397–411.

Örn, S., Svenson, A., Viktor, T., Holbech, H., Norrgren, L., 2006. Male-biased sex ratios and vitellogenin induction in zebrafish exposed to effluent water from a Swedish pulp mill. Arch. Environ. Contam. Toxicol. 51 (3), 445–451.

Santos, M.M., Micael, J., Carvalho, A.P., Morabito, R., Booy, P., Massanisso, P., et al., 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 142 (1–2), 151–155.

Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., Segner, H., 2007. Concentration-and time-dependent effects of the synthetic estrogen, 17alpha-ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. J. Toxicol. Environ. Health 70 (9), 768–779.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstelter, T.B., Johnson, C.A., Von Gunten, U., et al., 2006. The challenge of micropollutants in aquatic systems. Science 313 (5790), 1072–1077.

Segner, H., 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 149 (2), 187–195.

Shi, X.J., Du, Y.B., Lam, P.K.S., Wu Rudolf, S.S., Zhou, B.S., 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicol. Appl. Pharmacol. 230 (1), 23–32.

Skinner, M., 2011. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. Epigenetics 6 (7), 838–842.

Soares, J., Coimbra, A.M., Reis-Henriques, M.A., Monteiro, N.M., Vieira, M.N., Oliveira, J.M.A., et al., 2009. Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol. Aquat. Toxicol. 95 (4), 330–338.

Stackelberg, P.E., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Henderson, A.K., Reissman, D.B., 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Sci. Total Environ. 329 (1–3), 99–113.

Ullah, S., Alsberg, T., Berger, U., 2011. Simultaneous determination of perfluoroalkyl phosphonates, carboxylates and sulfonates in drinking water. J. Chromatogr. A 1218 (37), 6388–6395.

Van der Ven, L.T.M., Wester, P.W., Vos, J.G., 2003. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 22 (4), 908–913.

Vaughan, M., Van Egmond, R., Tyler, C.R., 2001. The effect of temperature on sexual differentiation and development in the zebrafish (*Danio rerio*). Proceedings of the 11th Annual Meeting of the Society of Environmental Toxicology and Chemistry. 6–10 May, Madrid, Spain.

Vested, A., Giwercman, A., Bonde, J.P., Toft, G., 2014. Persistent organic pollutants and male reproductive health. Asian J. Androl. 16 (1), 71.

Voelker, D., Vess, C., Tillmann, M., Nagel, R., Otto, G.W., Geisler, R., et al., 2007. Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. Aquat. Toxicol. 81 (4), 355–364.

Westerfield, M., 1995. The Zebrafish Book. University of Oregon, Eugene, OR.

Westerfield, M., 2007. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 5th Ed. University of Oregon Press, Eugene, US.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39 (17), 6649–6663.

Wilhelm, M., Bergmann, S., Dieter, H.H., 2010. Occurrence of perfluorinated compounds (PFCs) in drinking water of North-Rhine-Westphalia, Germany and new approach to assess drinking water contamination by shorter-chained C4–C7 PFCs. Int. J. Hyg. Environ. Health 213 (3), 224–232.

Zhang, C.J., Willett, C., Fremgen, T., 2003. Zebrafish: an animal model for toxicological studies. Curr. Protoc. Toxicol. 1–7.

Zhu, X.S., Zhu, L., Duan, Z.H., Qi, R.Q., Li, Y., Lang, Y.P., 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. 43 (3), 278–284.