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Additional Information

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

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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.

Key words

organic pollutants

bioindicator

development and reproduction parameters

drinking water

zebrafish

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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) is 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 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 (Metcalf 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 mid blastula 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 Túrria River. Type B was from the tap water distribution network of a medium-sized city, supplied from the Túrria 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. Type 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 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.

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 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 17 α -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 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 (Metcalf 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.

4 Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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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 ($p < 0.05$).