ZEBRAFISH (*Danio rerio*) AS BIOINDICATOR OF EPIGENETIC FACTORS PRESENT IN DRINKING WATER THAT MAY AFFECT DEVELOPMENT AND REPRODUCTIVE FUNCTION

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Valencia, 15th January 2016

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

acuicultura ACUMA® medioambiente

UNIVERSIDAD POLITÉCNICA DE VALENCIA

Departamento de Ciencia Animal

Centro de Investigación Acuicultura y Medio Ambiente

Laboratorio de Reproducción y Biotecnología Animal

Esta tesis ha sido escrita y presentada como uno de los requisitos para optar al grado de Doctor por el departamento de Ciencia Animal de la Universidad Politécnica de Valencia.

Los directores de la presente Tesis certifican que el trabajo se encuentra terminado y reúne todos los requisitos legales y académicos necesarios para su presentación.

En Valencia a 15 de Enero de 2016

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AGRADECIMIENTOS

Dedico esta tesis a todos aquellos que, de alguna manera u otra, me han ayudado e inspirado a lo largo de toda la investigación, pero sobretodo, me han enseñado que más que el destino lo verdaderamente importante es el camino.

En especial a mis tutores Fernando y Paco (Doctores del saber, pero sobretodo de la vida), a mi súper compi y amigo Javi, a Conchi, a mis compañeros y compañeras del departamento (concretamente a Mara, Amparo, Estrella, Carmen, Raquel, Marina, Vero, Vic, Mamen y Morini), a mis amigas y amigos, a mi familia y a mi chico Rodrigo, mi fuente de inspiración. Sin vosotros este momento nunca hubiera llegado.

¡Gracias!

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SUMMARY

Emerging organic pollutants include a wide array of different compounds such as pharmaceutical and personal care products, endocrine disruptors, and persistent organic compounds (POPs), among others. The main characteristic of these numerous substances is that they do not need to be persistent in the environment to cause negative effects, even long term, since their high transformation and removal rates can be offset by their continuous introduction into the environment. One of the main sources of these contaminants is untreated urban wastewaters and wastewater treatment effluents. Most current wastewater treatment plants are not designed to treat these types of substances, for that reason a high portion of these pollutants and their metabolites can escape and enter to the urban water cycle reaching, therefore, the drinking water supply. The problem is exacerbated by the fact that many of these compounds are non-regulated, since are newly introduced. For the same reason, although concentrations are generally low (ng/l- μg/l), there are worries about their potential and unknown risks of exposures. In this sense, it is known that emerging organic pollutants can have long-lasting effects on development and reproduction, sometimes even in subsequent generations, via epigenetic mechanisms or by mutagenic effects.

For all the above reasons and due to the complexity of the chemical detection of these compounds, bioindicators such as fishes can be used as an alternative, at least complementary, to monitor their presence. In this sense, in the present thesis was studied whether zebrafish (*Danio rerio*) could be established as a bioindicator to detect the presence of these substances in drinking waters through the study of the effects on development and, especially, on reproduction parameters.

To accomplish this objective, four studies in drinking waters from different sources were carried out. Firstly, it was compared whether keeping the chorion intact or, on the contrary, permeabilized with pronase with the aim to figure out if the chorion acts as a barrier to emerging pollutants. Results from this study suggest using embryos with the chorion intact from the outset when drinking water from different sources was to be tested.

In the second study were defined and narrowed down the most sensitive biological parameters to detect the effects of emerging organic pollutants on the development and, especially, on the zebrafish reproduction when they were cultured throughout their life-cycle in drinking waters. Results showed that the hatching, fertility and underdeveloped rates were the most sensitive parameters.

Once the most sensitive parameters were established, in the third study was analyzed the possible cumulative effect along two generations and/or the possible reversibility of the effects from emerging organic pollutants in zebrafish specimens reared in drinking water. Results showed a non-reversible effect on fertility rate and an alteration of sex ratio towards females in one of the studied water, although in this last case the alteration was reversible. A transgenerational alteration in the germline via epigenetic mechanism from the previous generation is proposed as the most plausible explanation to this effect.

Finally, in the last step to establish the zebrafish as a bioindicator, it was developed the fourth study with the aim to discriminate the effects from organic pollutants through three different pathways: male, female or water where the fertilization took place. Results showed a decrease in the fertility rate and in the hatching rate, due to an effect of the water where fertilization took place. The most plausible explanation could be the

presence of substances which affect the micropyle and chorion. In addition, it was observed a decrease in the fertility rate due to a female effect, but in this case by an alteration of the oocyte quality.

Taking into account the results obtained, it could be stated that the zebrafish is a suitable bioindicator to detect the effects from organic pollutants at very low concentration, when are reared in drinking water throughout their life-cycle.

RESUMEN

Los contaminantes orgánicos emergentes incluyen una amplia gama de compuestos diferentes, como los productos farmacéuticos y de cuidado personal, los disruptores endocrinos y los compuestos orgánicos persistentes, entre otros. La principal característica de estas numerosas sustancias es que no necesitan ser persistentes en el medio ambiente para causar efectos negativos, incluso a largo plazo, ya que sus altas tasas de transformación y eliminación pueden ser compensadas por su continua introducción en el medio ambiente. Una de las principales fuentes de estos contaminantes son las aguas residuales urbanas no tratadas y los efluentes de tratamiento de aguas residuales. La mayoría de las plantas de aguas residuales actuales no están diseñadas para el tratamiento de este tipo de sustancias, por ello una alta porción de estos contaminantes y sus metabolitos pueden escapar y entrar al ciclo urbano del agua alcanzando, por lo tanto, el suministro de agua potable. El problema se agrava porque muchos de estos compuestos no están regulados, por ser de hecho de nueva implantación. Por la misma razón, aunque las concentraciones son generalmente bajas (ng/l- µg/l), se desconocen los posibles riesgos a la exposición de estos compuestos. Por otro lado, se sabe que los contaminantes orgánicos emergentes pueden tener efectos a largo plazo sobre el desarrollo y la reproducción, a veces incluso en las generaciones posteriores, a través de mecanismos epigenéticos y/o por efectos mutagénicos.

Por todas las razones citadas anteriormente y debido a la complejidad en la detección química de estos compuestos, los bioindicadores como los peces pueden ser usados como una alternativa, al menos complementaria, para controlar su presencia. En este sentido, en la presente tesis se estudió si el

pez cebra (Danio rerio) se podría establecer como bioindicador para detectar la presencia de estas sustancias en aguas potables a través del estudio de los efectos sobre el desarrollo y, en especial, sobre los parámetros de reproducción.

Para lograr este objetivo, se llevaron a cabo cuatro estudios en aguas potables de diferentes orígenes. En primer lugar, se comparó mantener el corion intacto o, por el contrario, permeabilizarlo con pronasa para averiguar si el corion actúa como una barrera a los contaminantes emergentes. Los resultados de este estudio sugieren el uso de embriones con el corion intacto desde el principio, cuando va a ser usada agua potable procedente de diferentes orígenes.

En el segundo estudio se definieron y acotaron los parámetros biológicos más sensibles para detectar los efectos de los contaminantes orgánicos emergentes sobre el desarrollo y, sobre todo, sobre la reproducción del pez cebra cuando se criaron durante todo su ciclo de vida en aguas potables. Los resultados mostraron que los parámetros más sensibles fueron la tasa de eclosión, la tasa de fecundidad y la tasa de especímenes subdesarrollados.

Una vez establecidos los parámetros más sensibles, en el tercer estudio fue analizado el posible efecto acumulativo a lo largo de dos generaciones y / o la posible reversibilidad de los efectos de los contaminantes orgánicos emergentes en especímenes de pez cebra criados en aguas potables. Los resultados mostraron un efecto irreversible en la tasa de fecundidad y una alteración de la proporción sexual hacia hembras en una de las aguas estudiadas, aunque en este último caso la alteración fue reversible. Una alteración transgeneracional en la línea germinal a través de mecanismos

epigenéticos de la generación anterior se propone como la explicación más plausible para este efecto.

Finalmente, en el último paso para establecer el pez cebra como bioindicador, se desarrolló el cuarto estudio con el objetivo de discriminar los efectos de los contaminantes orgánicos a través de tres vías diferentes: macho, hembra o agua donde la fertilización se llevó a cabo. Los resultados mostraron una disminución de la fertilidad y la tasa de eclosión, debido a un efecto del agua donde la fertilización tuvo lugar. La explicación más plausible podría ser la presencia de sustancias que afectan al micropilo y al corion. Además, se observó una disminución en la tasa de fertilidad debido a un efecto hembra, pero en este caso por una alteración de la calidad de los ovocitos.

Teniendo en cuenta los resultados obtenidos, se puede afirmar que el pez cebra es un bioindicador adecuado para detectar los efectos de los contaminantes orgánicos en concentraciones muy bajas, cuando se crían en el agua potable durante todo su ciclo de vida.

RESUM

Els contaminants orgànics emergents inclouen una àmplia gamma de compostos diferents, com els productes farmacèutics i de cura personal, els disruptors endocrins i els compostos orgànics persistents, entre uns altres. La principal característica d'aquestes nombroses substàncies és que no necessiten ser persistents en el medi ambient per causar efectes negatius, fins i tot a llarg termini, ja que les seves altes taxes de transformació i eliminació poden ser compensades per la seva contínua introducció en el medi ambient. Una de les principals fonts d'aquests contaminants són les aigües residuals urbanes no tractades i els efluents de tractament d'aigües residuals. La majoria de les plantes d'aigües residuals actuals no estan dissenyades per al tractament d'aquest tipus de substàncies, per això una alta porció d'aquests contaminants i els seus metabòlits poden escapar i entrar al cicle urbà de l'aigua aconseguint, per tant, el subministrament d'aigua potable. El problema s'agreuja perquè molts d'aquests compostos no estan regulats, per ser de nova implantació. Per la mateixa raó, encara que les concentracions són generalment baixes (ng/l- µg/l), es desconeixen els possibles riscos a l'exposició d'aquests compostos. D'altra banda, se sap que els contaminants orgànics emergents poden tenir efectes a llarg termini sobre el desenvolupament i la reproducció, de vegades fins i tot en les generacions posteriors, a través de mecanismes epigenetics i/o per efectes mutagenètics.

Per totes les raons donades anteriorment i a causa de la complexitat en la detecció química d'aquests compostos, els bioindicadors com els peixos poden ser usats com una alternativa, almenys complementària, per controlar la seva presència. En aquest sentit, en la present tesi es va estudiar si el peix zebra (Danio rerio) es podria establir com a bioindicador

per detectar la presència d'aquestes substàncies en aigües potables a través de l'estudi dels efectes sobre el desenvolupament i, especialment, sobre els paràmetres de reproducció.

Per aconseguir aquest objectiu, es van dur a terme quatre estudis en aigües potables de diferents orígens. En primer lloc, es va comparar mantenir el cori intacte o, per contra, permeabilitzar-lo amb pronasa per esbrinar si el cori actua com una barrera als contaminants emergents. Els resultats d'aquest estudi suggereixen l'ús d'embrions amb el cori intacte des del principi, quan va a ser usada aigua potable procedent de diferents orígens.

En el segon estudi es van definir i van fitar els paràmetres biològics més sensibles per detectar els efectes dels contaminants orgànics emergents sobre el desenvolupament i, sobretot, sobre la reproducció del peix zebra quan es van criar durant tot el seu cicle de vida en aigües potables. Els resultats van mostrar que els paràmetres més sensibles van ser la taxa d'eclosió, la taxa de fecunditat i la taxa d'espècimens subdesenvolupats.

Una vegada establerts els paràmetres més sensibles, en el tercer estudi es va analitzar el possible efecte acumulatiu al llarg de dues generacions i / o la possible reversibilitat dels efectes dels contaminants orgànics emergents en espècimens de peix zebra criats en aigües potables. Els resultats van mostrar un efecte irreversible en la taxa de fecunditat i una alteració de la proporció sexual cap a femelles en una de les aigües estudiades, encara que en aquest últim cas l'alteració va ser reversible. Una alteració transgeneracional en la línia germinal a través de mecanismes epigenètics de la generació anterior es proposa com l'explicació més plausible per a aquest efecte.

Finalment, en l'últim pas per establir el peix zebra com a bioindicador, es va desenvolupar el quart estudi amb l'objectiu de discriminar els efectes dels contaminants orgànics a través de tres vies diferents: mascle, femella o aigua on la fertilització es va dur a terme. Els resultats van mostrar una disminució de la fertilitat i la taxa d'eclosió, a causa d'un efecte de l'aigua on la fertilització va tenir lloc. L'explicació més plausible podria ser la presència de substàncies que afecten al "micropilo" i al cori. A més, es va observar una disminució en la taxa de fertilitat a causa d'un efecte femella, però en aquest cas per una alteració de la qualitat dels ovòcits.

Tenint en compte els resultats obtinguts, es pot afirmar que el peix zebra és un bioindicador adequat per detectar els efectes dels contaminants orgànics en concentracions molt baixes, quan es crien en l'aigua potable durant tot el seu cicle de vida.

GENERAL INTRODUCTION

BACKGROUND

Human fertility rates are decreasing all over the world, especially in some Western countries (Skakkebæk *et al*., 2006). This decline is considerate as a multifactorial issue, related in many cases with our modern lifestyle. Developed countries such as Spain are associated with low fertility rates (Skakkebæk *et al*., 2006).

It is generally assumed that these trends are because of social and cultural changes. However, it must not to rule out the biological and environmental factors as an additional contribution of the low fertility rates.

A possible sign of decreasing reproductive health is the high demand for infertility clinical treatment. In fact, according to the European Institute of Fertility, in Spain about 7% of children were born after the assisted reproduction treatment. Females and males seems to be both involved in this anomaly. However, a high demand for ICSI (intracytoplasmic sperm injection) (Andersen and Erb, 2006) reflects the preponderance of a male factor, due to a testicular failure and poor semen quality.

Others factors that seems to acquire increasing importance in this infertility are related to the presence of certain emerging pollutants in the environment with effects on reproduction such as epigenetic factors (endocrine disruptors, drugs, pharmaceutical substances, persistent organic pollutants, pesticides, etc.). The presence of these substances is increasing over the past few years, as well as, their complexity and variety. Furthermore, there are many substances with epigenetic effects that are currently unknown (Baccarelli and Bollati, 2009). The problem is exacerbated by the fact that these substances have been found at low concentrations (ng/l or µg/l) in the waters supply and, in many cases, are

not regulated and as a consequence are neither detected nor removed (Richardson and Ternes, 2011). For that reason, conventional treatments which have been designed to common regulated pollutants are inefficient.

Additional measures to the obsolete conventional treatments could be the use of fish as bioindicators (De Andrade Brito *et al*., 2012; Young *et al.*, 2014). In this sense, zebrafish is acquiring more relevance to assess substances in toxicological studies (Dai *et al*., 2014), teratogenic studies (Loucks and Ahlgren, 2012) and, as well as, epigenetic studies in waters (Corrales *et al*., 2014).

Based on the above, in the current thesis it is proposed as a hypothesis that it is possible to use the zebrafish (through their life-cycle) as a bioindicator to detect in drinking water the possible presence of epigenetic factors that could affect the human reproduction.

1. – EPIGENETIC FACTORS

1.1. - Epigenetic

The term epigenetics refers to the study of changes in gene activity that is not caused by changes in the DNA sequence (Skinner *et al*., 2010). These changes are effected by several molecular mechanisms as DNA methylation (Kazazian, 2004), histone modifications (Turner, 1998) and non coding RNAs (Wan and Bartolomei, 2008). These three epigenetic mechanisms, work in an interactive cooperation during the establishment and maintenance of epigenetic marks on the DNA, to regulate cellular development, differentiation, and function (Li, 2002; Stearns *et al*., 2007; Namihira *et al*., 2008). The effect of these epigenetic mechanisms can operate at cellular, individual or population level.

An important aspect to mention of epigenetics is that the epigenetic marks are mitotically and meiotically stable which means that all cells that come from that initial cell will have the same epigenome. The epigenetic machinery can be affected by different factors such as chemical environment, social behavior, nutritional deficiencies, etc. In fact, epigenetic changes are a biological response to environmental stress factors which, in some cases may be transmitted to the offspring (Ficociello *et al*., 2010).

1.2. – Environment and epigenesis

Organisms throughout their life cycle are constantly exposed to environmental influences that pose a threat to the stability of their genome and/or epigenome. Several examples have been studied in various organisms, in which environmental influences such as exposure to chemicals (Anway *et al*., 2005; Vandegehuchte *et al*., 2009), nutritional supplements/nutrient availability (Wolff *et al*., 1998; Cooney *et al*., 2002; Dolinoy *et al*., 2006; Kaminen-Ahola *et al*., 2010), maternal behavior (Weaver *et al*., 2004), pathogens (Boyko *et al*., 2007), or temperature (Lang-Mladek *et al*., 2010) cause alterations in gene expression that persist throughout life and sometimes appear to be transmitted to the next generation (see figure 1).

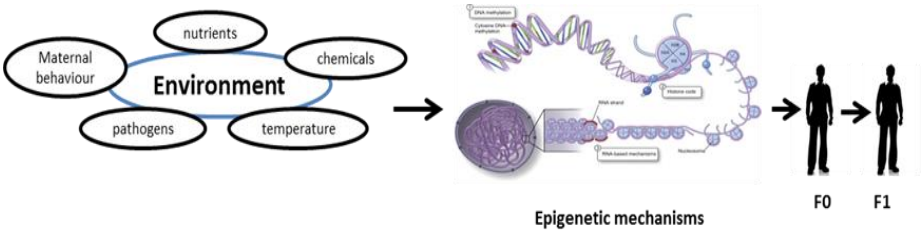

Figure 1: Environmental influences alter gen expressions through epigenetic mechanisms and sometimes, if the germ line is affected, these alterations are transmitted to the next generation.

A number of environmental pollutants such as pharmaceutical substances, endocrine-disrupting chemicals and persistent organic pollutants (POPs), between others (Diethylstilbestrol (DES), Bisphenol A (BPA), etc.), has been widely demonstrated to be able to modify the epigenetic marks and to induce persistent and heritable changes of epigenetic status (Baccarelli and Bollati, 2009).

Whereas mechanisms of action of some of these agents are understood, for others the mode of action remains to be completely elucidated (Marsit *et al*., 2006). Because these epigenetic changes are small, potentially cumulative, and they may develop over time, it may be difficult to establish the cause- effect relationships among environmental factors, epigenetic changes and diseases.

1.3. – Epigenetic effects transmitted trans-generationally

Many environmental factors and toxicants do not have the capability to modify DNA sequence or promote genetic mutations (Jirtle and Skinner, 2007; Szyf, 2007; Skinner *et al*., 2010). This is because of the stability of the genome. However, it has been studied that many environmental factors have promoted abnormal phenotypes or diseases without changes in the

DNA sequence. These alterations have been carried out by molecular processes that influence the genome, such as epigenetics (Jirtle and Skinner, 2007). The majority of the environmental factors acts on somatic tissues and influence the individual exposed. However, it has been demonstrated that when the germ-line is directly affected, these environmental factors can promote a heritable transmission to the next generation. This heritable transmission of modified phenotypes is called as transgenerational inheritance (Jirtle and Skinner, 2007; Whitelaw and Whitelaw, 2008; Skinner *et al*., 2010).

Several chemicals as for example benzopyrene (Csaba and Inczefi-Gonda, 1998), diethylstilbestrol (DES) (Newbold *et al*., 2006), and vinclozolin (Anway *et al*., 2005) have been reported to induce transgenerational phenotypic effects.

Permanent alteration in the epigenetic programming of the germ line appears to be the mechanism involved in the transgenerational phenotype (Skinner *et al*., 2011). The critical period for this epigenetic regulation of the germ line is established during the period of primordial germ cell migration and gonadal sex determination.

It is important to highlight that in some cases exposures to environmental factors as endocrine disruptors to a gestating female allows multiple generations to be exposed (Ryökkynen *et al*., 2005), but in this case does not constitute a transgenerational inheritance, but a multigenerational exposure because a exposition to the pollutant is required.

1.4. – Epigenetic factors that affect the development and reproduction

Several epigenetic factors can alter the epigenome, but in this study we are going to focus only in environmental chemical factors that alter the development and reproduction in vertebrates, especially those present in water.

The most sensitive developmental periods to environmental exposures are both the embryonic and early postnatal periods (Heindel *et al*., 2005; Heindel *et al*., 2006; Anway *et al*., 2006). Some effects of this exposure could be manifested later on reproduction in adults through the alteration of egg and sperm. Furthermore, available evidence from animal models as zebrafish indicates that exposure to xenobiotics during critical periods of development may induce persistent and transmissible changes of epigenetic status (Baccarelli and Bollati, 2009).

The most relevant group of compounds capable to modified the epigenetic marks with effects on development and reproduction can be classified in: endocrine disruptors, pharmacological substances and persistent organic pollutants (POPs). These substances have been selected due to the fact that they are the most abundant and important compounds present in water sources, especially in drinking water supplies at low concentrations (Westerhoff *et al*., 2005; Khetan and Collins, 2007; Kim *et al*., 2007). Most of these substances which are collectively referred as “Emerging Organic Contaminants” (Kuster *et al*., 2008) are still unregulated despite to be biologically active. Furthermore, their environmental significance, as well as, their fate in the environment and their effects remains poorly understood (Pal *et al*., 2010). The presence of these contaminants in water

is not exclusive, what means that these groups of pollutants could be present as single chemicals or as a mixture of them.

- ***Endocrine Disruptors***

Endocrine-disrupting chemicals (EDCs) are a group of exogenous agents that affect the homeostasis, reproduction, development and behaviour in organisms by interfering with the synthesis, release, metabolism and/or function of endogenous hormones. Moreover, such alterations can be manifested even in subsequent generations (Skinner *et al*., 2011).

Diverse endocrine disruptors in the environment include natural hormones, phytoestrogens, synthetic hormones, dioxin, organochlorines, brominated flame retardants, perfluorinated substances, phthalates, bisphenol A, alkylphenols, between others. Some pharmaceutical substances and persistent organic pollutants (POPs) have endocrine effects, but in this point we will refer only to EDCs which display estrogenic activity and interfere with normal estrogen signaling.

EDCs have been detected in air, soil, water, wildlife and human tissues and especially in fish, because the aquatic environment is the ultimate sink for various xenobiotics that enter the environment mainly through sewage discharge (Scholz and Mayer, 2008).

There is substantial evidence indicating that endocrine disruptors contribute to the risk of cancer, developmental problems, diabetes, and possibly also obesity and the metabolic syndrome. Also, endocrine disruptors can contribute to infertility and subfertility in different species even in humans (De Coster *et al*., 2012; Vested *et al*., 2014a). In fish, many studies have demonstrated that EDCs have detrimental effects on reproduction, such as poor-quality gametes, altered sexual behaviour,

decreased spawning events, reduced number of sperm and eggs and delayed hatching and malformation in offsprings (Huang *et al*., 2015).

- ***Pharmacological substances***

Pharmacological substances are any chemical or substance that affects the physiology and the function of the body, of a human or animal. These substances can be artificial or natural. Their presence in the aquatic ecosystems is particularly worrying, as the probability of them being biologically active is rather high.

In the European Union (EU) around 3,000 different pharmaceutical substances are used in human medicine belonging to different medical classes such as contraceptive, painkillers, antibiotics, anticancer drugs, blood-pressure medications, antidepressants, anxiolytics, etc (Khetan and Collins, 2007). However, only a small subset of these compounds has been investigated in environmental studies so far (Richardson and Ternes, 2014). Some of these substances have demonstrated to promote epigenetic alterations which can be classified between: those with effects on reproduction as for example diethylstilbestrol (DES) (Bromer et al., 2009) and tamoxifen (Tryndyak *et al*., 2006) and those with effects on different organ as liver as for example phenobarbital and oxazepam (Pogribny and Rusyn, 2013) between others.

Pharmaceuticals have the potential to interfere detrimentally with the normal development and reproduction of organism (Khetan and Collins, 2007). In fact, long-term exposure to the pharmaceutical ethynylestradiol caused reproductive impairment in zebrafish (Nash *et al*., 2004). Moreover, chronic, aqueous exposure of zebrafish to environmentally relevant

concentrations (µg/l) of acetaminophen, venlafaxine, carbamazepine and gemfibrozil decreased reproductive output (Galus *et al*., 2013).

The main routes to enter into the environment are discharges from wastewater treatment plant (Gros *et al*., 2010), that are usually a consequence of their incomplete removal (Petrovic *et al*., 2005), hospital effluents, land applications (e.g., biosolids and water reuse), concentrated animal feeding operations, and direct disposal/introduction to environment (Daughton and Ternes, 1999).

- ***Persistent Organic Pollutants (POPs)***

Persistent organic pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate through the food chain, and pose a risk of causing adverse effects to human health and the environment (Pozo *et al*., 2006). Furthermore, as it happens in endocrine disruptors and pharmaceutical substances, POPs may induce persistent and heritable changes of epigenetic status (Baccarelli and Bollati, 2009).

Persistent organic pollutants such as polychlorinated biphenyls (PCB), dichlorodiphenyldichloroethanes (DDT), and polybrominated diphenyl ethers (PBDE) are well established pollutants. The release of these chemicals into the environment is mainly through the wastewater discharge, as a consequence of agricultural production, pest control, and modern manufacturing processes and their by-products in the industry.

Exposure to POPs has been associated with adverse effects on the immune system (Sørmo *et al*., 2009), neurobehavioral development (Fonnum and Mariussen, 2009), endocrine functions (Talsness, 2008; Lyche *et al*., 2010; Lyche *et al*., 2011) and mainly on reproduction (Talsness, 2008). Special attention has been on their potential to operate as endocrine disruptors

and with their effects on reproduction in a wide range of species including humans (Lyche *et al*., 2010; Lyche *et al*., 2011; Walker and Gore, 2011).

Traditionally, effects from pollutants were associated and assessed from single chemicals. However, POPs occur as mixtures in nature. So, the current knowledge on the combined toxic effects and modes of actions of exposures to mixtures is very limited (Nourizadeh-Lillabadi *et al*., 2009).

- ***Others***

Other relevant reproductive toxicants to point out due to their adverse effects on female and male reproductive systems are: 1, 3 - Butadiene, tobacco smoke (primary), acetylsalicylic acid, alcohol, and cocaine, between others. These reproductive toxicants may interfere with the sexual functioning or reproductive ability of exposed individuals from puberty throughout adulthood (La Vignera *et al*., 2013; Soeteman-Hernández *et al*., 2013; Guerrero-Bosagna and Skinner, 2014).

2. – DEPURATION AND PURIFICATION OF WATERS

2.1. – Urban water cycle

Traditionally water treatments have been focused on nutrients, microorganism, heavy metals and priority pollutants (compounds with known health effects such as pesticides, industrial chemicals, petroleum hydrocarbons, etc.). However, according to current researches, hundreds of organic pollutants so-called “Emerging Organic Pollutants” have been detected in wastewaters and as a result in surface waters, reaching the urban water cycle. Their toxicological effects are difficult to assess, for that reason, concentrations limits are not yet established in drinking waters and in wastewaters (Pal *et al*., 2014). Furthermore, most of these substances

are unregulated because have been recently introduced or detected in the environment (Houtman, 2010).

The urban water cycle (UWC) (see figure 2) is composed by different sub-cycles that in a direct or indirect way, with more or less exhaustive water treatments, are back into the urban supply. The effluents that normally fed the water supply are the following: the wastewater treatment effluents that pour into the surface water and after treatment pour into the water supply (indirect reuse), the wastewater treatment effluents that is treated by advanced wastewater treatment processes to meet drinking water standards and is directly pour into the water supply system (direct reuse), the groundwater from local precipitations and from surface waters and, finally, the groundwater from the wastewater treatment effluent. In this last case, is necessary a soil-aquifer treatment before recharging the aquifer.

The UWC varies from city to city depending on climate, hydraulics, population, political and other factors as working cost. The UWC described in this work is a generic cycle.

The use of wastewater effluents to augment the water supplies it is known as the process “Indirect Potable Water Reuse” (National Research Council, 1998). This reuse is commonly initiated in areas with growing urban populations and constraints on the availability of new water sources. One of the main concerns in the reuse of water is the possible input of emerging organic pollutants in the drinking water and, thus, the effect on human health through chronic exposures (Stackelberg *et al*., 2004).

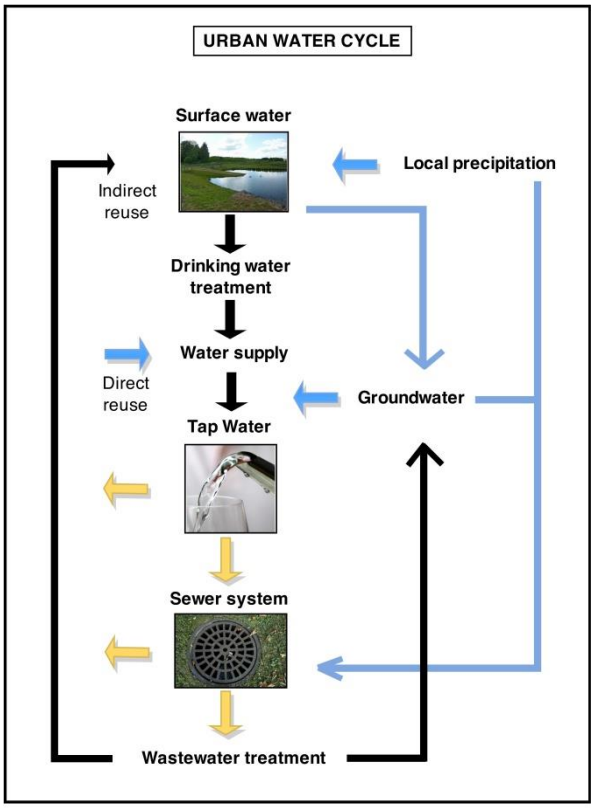

Figure 2: The Urban Water Cycle. Adapted from: Pal et al., 2014.

In Spain, the process of reusing water was proposed for the first time in the National Water Planning through the "AGUA programme" (Muñoz *et al.*, 2010). Nowadays, this programme it has been modified by the new "National Plan for Water Reuse" which was set forth in the Royal Decree 1620/2007 of December 7, where it was laid down the legal framework of reusing treated water. It should be stated that in both cases is not proposed the reuse of treated water, at least directly, for human consumption.

2.2. – Water for human consumption

- ***Legislation***

Drinking water supplies have to follow certain legal requirements to protect the human being from pollution. Thus, at European level it is established the Council Directive 98/83/EC of 3 November, on the quality of water intended for human consumption. The objective of this Directive shall be to protect human health from the adverse effects of any contamination of water intended for human consumption by ensuring that it is wholesome and clean. This Directive has been transposed into Spanish normative by the Royal Decree 140/2003 of 7 February by which health criteria for the quality of water intended for human consumption are established. Finally, the Decree 58/2006 of 5 May develops the Royal Decree at regional level (Valencian Community).

- ***Classification***

Drinking water can be classified according to their mode of supply into: tap water supplied through the distribution network and drinking water packaged for human consumption.

The drinking water that is normally packaged for human consumption is the following: mineral water, spring water and prepared bottled water for public consumption.

Given the clear differences between the mineral water and the spring water and the prepared bottled water for public consumption, regulations are different. The firsts (mineral and spring water) are regulated by the Royal Decree 1798/2010 of 30 December and the second (prepared bottled water) by the Royal Decree 1799/2010 of 30 December.

2.3. – Wastewater and purification treatments

Wastewater effluents are any water that has been adversely affected in quality by anthropogenic influence. Wastewater flows are not uniform, but vary from day to day, from month to month and from year to year. Thus, the design of a treatment plant should be established taking into account the wastewater to be treated. The conventional wastewater treatment generally consists of primary, secondary and sometimes a tertiary stage, with different biological and physicochemical processes available for each stage of the treatment. Primary treatment intends to reduce the solid content of the wastewater. However, the secondary treatment is focused in removing organic matter and/or nutrients with aerobic or anaerobic systems. The most common secondary treatment used is the conventional activated sludge (CAS). In the final step, sometimes a tertiary treatment is applied to remove phosphorus. In some wastewater treatment plants the effluent is also disinfected before it is released into the environment, typically by chlorination or ultraviolet irradiation (see figure 3).

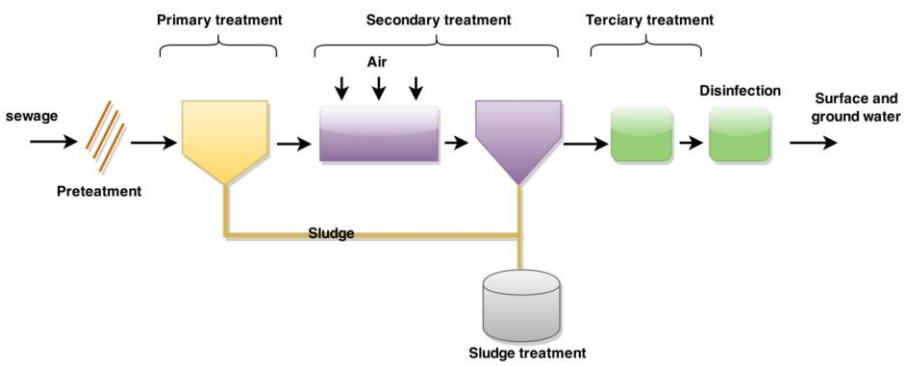

Figure 3: Conventional wastewater treatment plant.

With regard to a specific wastewater plant in Valencia City and its metropolitan area, the processes included are: pretreatment (grating thick, sieving, sand trap and degreaser), primary treatment (physical-chemical and decantation), secondary treatment (activated sludges and nitrogen removal), tertiary treatment (coagulation flocculation and filtration) and disinfection treatment by UV.

Disinfection reduces the microbiological risks, but not the chemical risks. In fact, many substances recently known as emerging contaminants, which are new and not regulated (Deblonde *et al*., 2011), are released through the urban water cycle (as it is mentioned in section 1.4.1), even reaching the drinking water effluents, because most of the wastewater treatments are conventional technologies that were not originally designed for the elimination of these novel substances (Carmona *et al*., 2014).

Regarding water purification, this consists in the removal of contaminants from untreated water to produce drinking water for human consumption. A combination selected from the following processes is used for municipal drinking water treatment worldwide: pre-chlorination, aeration, coagulation, polyelectrolytes, sedimentation, filtration, desalination and disinfection (see figure 5).

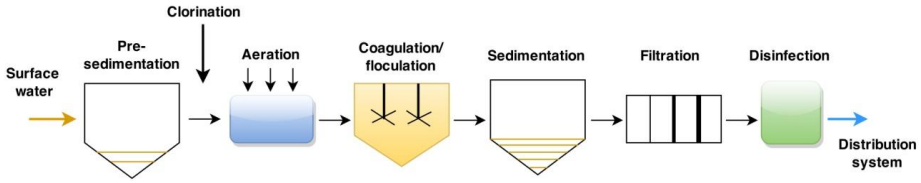

Figure 4: Conventional drinking water treatment plant.

Chlorination is the most common method worldwide for the disinfection of drinking water. However, the identification of potential toxic products from

this method has led to develop other techniques. Thus, ozone, ultraviolet light, advanced oxidation process and electrochemical disinfection, between others, are considered some alternatives to chlorination.

It is important to mention that in both, wastewater treatment and drinking water treatment, there are no unique solutions, since the choice of the water treatment is related to the water features, the country legislation and, mainly, the operating costs.

2.4. – Wastewater discharge to the environment

- ***Legislation***

The standards for water quality are typically set by governments or by international standards. These standards will typically set minimum and maximum concentrations of contaminants for the use that is to be made of the water.

At European level the Council Directive 91/271/CEE defines the collection, treatment and discharge of urban wastewater and the treatment and discharge of wastewater from certain industrial sectors. This Directive has been transposed into Spanish normative by the Royal Decree 2116/1998 of 2 October, by amending the Royal Decree 509/1996 of 15 March, of development of the Royal Decree-law 11/1995 of 28 December, where are laid down the rules for urban wastewater treatment. At regional level, the law 2/1992 of wastewater sanitation develops the Royal Decree 2116/1998 in the area of Valencia.

- ***Presence of contaminants***

Emerging organic pollutants may enter the water cycle through multiple entrances, treated or not, including industrial, agricultural and urban channels. Focusing on treated effluents, the primary entry routes is by discharge of treated wastewater effluents to surface waters (Fawell and Ong, 2012). In wastewater treatment, biological processes will degrade many of these substances; however, some will be broken down only partially or not at all, reaching in this case the surface waters and finally the water supply. This is because the functions of wastewater treatment plants (WWTP) are to reduce the contamination from typical components, such as: coarse solids, settleable solids, suspended solids, fats, biodegradable matter, nitrogen, phosphorus and pathogens, but not emerging contaminants at trace level. The components which are the most effectively eliminated in a wastewater treatment plant including an activated sludge system are phthalates with a removal efficiency above 90% (Bendz *et al*., 2005) and psycho- stimulants with about 97% removal (Ying *et al*., 2009). Bisphenol A is eliminated at about 70% (Gómez *et al*., 2007). However, the molecules of the therapeutic classes like analgesic, anti-inflammatory and beta-blockers are the least effectively removed (30-40%) (Deblonde *et al*., 2011).

Regarding drinking water treatment plants, there is an important variation in different treatments or combinations of treatments to remove emerging contaminants and some drinking-water treatment may be inadequate to completely eliminate some of these substances, particularly some pharmaceutical compounds. This has been demonstrated due to trace levels of substances have been found in drinking water (Kuster *et al*., 2008; Benotti *et al*., 2009; Cooney, 2009). The reason is that drinking water

treatments are focused, mainly, in the elimination of natural organic matter, microorganisms, turbidity, chemicals (as arsenic, antimony, cyanide, etc.), between others.

2.5. – Contaminant substances

During the past three decades, research on the impact of chemical pollution has focused almost exclusively on the conventional "priority pollutants". Today, these compounds are less relevant for many first world countries because emissions have been substantially reduced through the adoption of appropriate legal measures and the elimination of many of the dominant pollution sources. The focus has consequently switched to compounds present in lower concentrations but which nevertheless might have the ability to cause harm (Larsen *et al*., 2004), these substances are so-called emerging organic pollutants. One of the interesting characteristics of many of the chemicals that might cause this type of pollution is that they do not need to be persistent in the environment to cause negative effects (Ayscough *et al*., 2000). This is because their high transformation and removal rates can be offset by their continuous introduction into the environment, often through sewage treatment works (Suter and Giger, 2001). This is one reason why there is an increasingly widespread consensus that this kind of contamination might require legislative action sooner rather than later (Petrović *et al*., 2003).

- ***Risks to human health and the environment***

The major exposure route both for humans and animals is by ingestion of emerging contaminants via food/drink intake which leads to bioaccumulation and biomagnification, especially towards species at top level of food chain. However, little is known about potential health effects

associated with long term chronic ingestion of low concentrations through drinking water (Kümmerer, 2001). Moreover, drinking water criteria currently are based on the toxicity of individual compounds and not combinations of compounds (Nourizadeh-Lillabadi *et al*., 2009).

The effects of these contaminants toward animals are well reported, although the direct effects to humans are still debated and require more studies (Fawell and Ong, 2012). However, few studies suggest that the effect of these compounds on human reproductive health includes a decrease in male sperm count, an increase in testicular, prostate, ovarian and breast cancer and reproductive malfunctions (Toft *et al*., 2006; Vested *et al*., 2014a). Moreover, the major concern is toward fetal and childhood states since they are the most vulnerable periods (Vested *et al*., 2014a). Currently, most researchers are focusing on persistent, bio-accumulating, toxic substances (also termed as priority pollutants), that were still found in birds, fish and mammals, even though these substances (i.e., dioxins, PCBs, organochlorine pesticides) have been reduced or banned as it has been mentioned above. On the other hand, more attention goes to substances that are persistent and also discharged or widespread in the environment although they occur at low levels. Typical examples are pharmaceuticals and personal care products. These substances can affect growth, reproduction and development of organisms in the ecosystem (Galus et al., 2013). Potential risks of emerging contaminants to humans and aquatic animals are summarized in table 1.

Table 1: Ecotoxicological risk of emerging organic pollutants to humans and animals. Adapted from: Pal *et al*., 2014.

Categories	Chemicals	Humans	Animals	References
Plasticizers	Bisphenol A	Possible carcinogenic effect, EDC	Estrogenic and reproductive effect	Flint et al., 2012; Wu et al., 2012
Perfluorinated Compounds	PFOS	Possibility of thyroid disease and low sperm count	Mussel mortality observed	Lindstrom et al., 2011; Post et al., 2012
Pesticides	Fipronil	May pose mild temporary health effect; possible carcinogen but no data so far	Potential adverse effect on endocrine and neuromuscular systems of larval fish	Herin et al., 2011; Beggel et al., 2012
Antiobiotics	Sulfamethoxazole	Mixture with 12 other pharmaceuticals could potentially inhibit the growth of human embryonic kidney cells at ng/l	Mutagenic; acute, chronic toxicity at low mg/l; chronic effect on freshwater microalgae *p. subcapitata;* acute effect on cyanobacterium *S. leopoliensis*	Zhang et al., 2010; Zheng et al., 2012
Pharmaceuticals	Acetaminophen	Alters steroidogenic pathway, increases estrogenicity in adrenal cell H295R high above aquatic concentrations	Affects embryonic development of zebrafish; survival of *Daphnia* and fish (*Oryziaslatipes*) affected at ppm	Kim et al., 2012; Galus et al., 2013
Hormones	Estrone	—	Fathead minnows: abnormal testicular growth in male at low ng/l; chronic exposure causes feminization	Hamid & Eskicioglu, 2012
Personal care products	DEET N, N-Diethyl-m-toluamide	Not carcinogenic, developmentally toxic, or mutagenic; sizures at high dose	Chronic and acute toxic effects on *Daphnia* and algae at concentrations much higher than aquatic concentrations	Costanzo et al., 2007; Aronson et al., 2012

- ***Legal regulations***

Several laws at European and Spanish level have been developed with the aim to cope the water contamination in a complex and effective way.

At European level, it was developed the Directive 2000/60/EC with the purpose to establish a framework for the protection of inland surface waters, transitional waters, coastal waters and groundwater. As a further step in the water protection, it was approved the Directive 2008/105/EC, where is laid down the environmental quality standards (EQS) to priority pollutants and others contaminants. Currently, after having revised the priority substances list, the European Council has made the conclusion that it is appropriate to change this list partially and to determine new substances which must be taken into account when taking priority actions in their regard at the Union level. Furthermore, the environmental quality standards should be updated too. That conclusion was accepted and legitimized on August 12, 2013 by the Directive 2013/39/EU of the European Parliament and Council that has partially replaced the provisions of Directives 2000/60/EC and 2008/105/EC on the priority substances in the field of aquatic policy. This new Directive shall establish a watch list of emerging pollutants that may pose a significant risk. So, the list of 33 priority substances presented in the Directive has been supplemented with additional 15 substances. For the first time due to their toxicological significance, emerging contaminants as pharmaceutical substances, such as Diclofenac, 17-beta-estradiol (E2) and 17-alpha-ethinylestradiol, were proposed to be included in the *Monitoring list*. The Commission shall review the adopted list at the latest four years after the date of entry into force of this Directive and at least every six years thereafter, and come forward with new proposals as appropriate.

The Directive 2000/60/EC and 2008/105/EC have been transposed into Spanish normative by the Royal Decree 60/2011 of 21 January, on the norms of environmental quality in the field of the politics of waters. In the annex of this Royal Decree is laid down the environmental quality standards

for priority substances and preferred. In such annex pharmaceutical substances are not regulated yet.

- ***List of contaminants***

In this point we are going to focus only in the most representative emerging contaminants listed in table 2, because it is almost impossible to cover all of them, since new substances are constantly being introduced in the environment. These substances have been chosen by supposing a risk to humans and the environment. These compounds range from cosmetics and personal care products, to the wide range of medicines as pharmaceutical substances, antibiotics and hormones. Other emerging contaminants that have been included in the list are plasticizers, pesticides and perfluorinated compounds (POPs). Thus, potential emerging contaminants release to the surface waters are summarized in table 2.

Table 2: Emerging organic pollutants release to surface waters. Adapted from: Pal *et al*., 2014.

Categories	Chemicals	Annual production, year, ref.	Origin contaminants	Annual release to water, year, ref.
Plasticizers	Bisphenol A. (EDC)	5,216,312.3 t (global), 2008, (Dow, 2012)	Plastic manufacture from plastic, combustion of domestic waste, WWTP effluents, etc.	2.8 t, (global), 2007, (USEPA, 2010)
Perfluorinated compounds	PFOS (POP)	73-162 t (global), 2005, (OECD, 2006)	Manufacturing wastes, food-packaging, WWTP effluents, etc.	1.25 t, (global), 2008, (OECD, 2011)
Pesticides	Fipronil	480 t (France), 1997, (Tingle et al., 2000)	Pest-control and crop protection, irrigation runoff, etc.	——
Antibiotics	Sulfamethoxazole	10.9 t (Spain), 2009, (Ortíz de García et al., 2013)	Manufacturing wastes, domestic and farm disposal of unused, expired antibiotics, WWTP effluents, etc.	2.1 t (Spain), 2009, (Ortíz de García et al., 2013)
Pharmaceuticals	Acetaminophen	1,460.2 t (Spain), 2009, (Ortíz de García et al., 2013)	Manufacturing wastes, unused and expired drugs disposed from household, WWTP effluents, etc.	23.3 t (Spain), 2009, (Ortíz de García et al., 2013)
Hormones	Estrone (E1)	——	Hormones injected to livestock and fishes, released from animal farms and aquacultures respectively, human and animal excreta via WWTP effluents.	28.2 kg E1 metabolite, (Spain), 2009, (Ortíz de García et al., 2013) 14.8 t (Spain), 2009, (Ortíz de García et al., 2013)
Personal care products	N,N-diethyl-m-toluamide (DEET)	1,814.4 t, (USA), 1990, (USEPA, 1998)	Mosquito repellants: released through showers, WWTP effluents, etc.	80%released to water, (Aronson et al., 2012)

2.6. – Analytical methods for emerging contaminants

The identification and quantification of emerging contaminants has gone in parallel with the improvement in the analytical capabilities, especially in the mass spectrometry field. However, despite to these improvements the development of a proper analytical treatment to identify and quantified emerging contaminants and their transformation products, continues to be one of the hottest trends (Carmona *et al*., 2014).

The first difficulty in developing an analytical approach is determining what emerging contaminants actually are, and which compounds should be used as target analytes. This is because there are several thousand compounds that can be classified as emerging contaminants along with their metabolites, which represent a wide range of physicochemical properties, making it almost impossible to develop analytical methods for all of these compounds. In fact, to our knowledge, no analytical methods for determining simultaneously these types of contaminants have been reported. Furthermore, the analysis of these compounds it is typically very expensive and time consuming.

In order to limit the study of the most common analytical methods for the emerging contaminants and to follow the structure of the document, we are going to focus in pharmaceutical substances, persistent organic pollutants and endocrine disruptors, due to their presence in environmental waters (especially drinking waters) and concern about their adverse effects, both to wildlife and humans (Lyche *et al*., 2011; Galus *et al*., 2013; Vested *et al*., 2014a).

Regarding pharmaceutical substances, the method most common is LC/ MS-MS (LC: liquid chromatography and MS: mass spectrometry) for the determination of all classes of pharmaceuticals in aqueous samples at the ng/l range. Mainly electrospray ionization (ESI) and sometimes atmospheric pressure chemical ionization (APCI) are the ionization methods used (Carmona et al., 2014). Major innovations have been made in modern hybrid mass spectrometry systems coupled to liquid chromatography, providing accurate masses of the analytes and information for mass fragments, which can be used to identify the chemical structures down to detection limits of pg/l and pg/kg (Richardson and Terns, 2014).

In persistent organic pollutants (POPs) such as perfluorinated compounds (PFCs) the method most common is LC/MS/MS with 0.83-10 ng/l detection limits. However, Ullah *et al*. (2011) used the same method with 0.014 to 0.17 ng/l detection limits. The reason to low detection limits was the use of 1-methyl piperidine in the mobile phase, which provided better chromatographic resolution. New methods continue to be developed for PFCs, including a highly sensitive total fluorine method, which can be used to determine how much of the total environmental fluorine contamination is accounted for by measured PFCs. This method developed by Qin *et al*. (2012) uses LC with continuum source-molecular absorption spectrometry (CS-MAS) and can be used to detect non-targeted fluorinated organic compounds in environmental and biological samples.

Finally, about endocrine disruptors the analytical method most common is also LC/MS-MS. The three main types of ionization used are electrospray ionization, atmospheric pressure chemical ionization, and atmospheric pressure photoionization (Jung *et al*., 2015). Other method was developed to analyze endocrine disruptors and pharmaceutical substances using ultra-high performance (UHP) LC-MS/MS (Anumol *et al*., 2013). This method was used to achieve optimum sensitivity and reducing sample analysis time.

Despite that LC/MS has facilitated the analysis of many compounds and is the most common method employed in all of these substances mentioned, it has several limitations. These systems are expensive, time consuming and specialized training and experience are necessary (Jung *et al*., 2015).

3. – ZEBRAFISH AS BIOINDICATOR OF WATER QUALITY

Zebrafish has been established as one of the most important vertebrate model organisms for biological research (Nüslein-Volhard *et al*., 2002). It

has been used predominantly in developmental biology and molecular genetics due to their embryological and genetic manipulation. However, many Scientifics have recognized the increasingly value of this vertebrate in other areas as: chemical toxicity, pharmacology, human diseases (Hill *et al*., 2005), between others. Moreover, and paying particular attention due to the goal of the current work, zebrafish has been used as an ecotoxicological test species to determine effects of specific chemicals on fish survival, growth and reproduction for deriving water quality criteria (Bresch *et al*., 1990; Nagel, 1993).

In this context, zebrafish has been extensively used as a model species to study the presence, the modes of action and the environmental and/or human risk assessment of endocrine disruptors (Segner, 2009), pharmacological substances (Galus *et al*., 2013) and POPs (Lyche *et al*., 2013) presents in the aquatic environment at ng–µg L^{-1} concentrations (Metcalfe *et al*., 2003) and, as well as, in drinking waters (Benotti *et al*., 2009).

Moreover, their easy culture and maintenance under laboratory conditions, their high reproductive rate and their ability to yield reproducible data under controlled laboratory conditions, makes the zebrafish be an ideal bioindicator.

The main parameters used in zebrafish embryos for water quality studies include survival and mortality, developmental delays and developmental failures such as curly tail, pericarditis, spinal deformation (mainly scoliosis) and delay or failure of the hatching rate (see figure 6) (Hallare *et al*., 2005). With regard to zebrafish adults, fertility, morphological development (underdeveloped rate) and sex ratio are the main parameters evaluated (Galus *et al*., 2013; Liu *et al*., 2014).

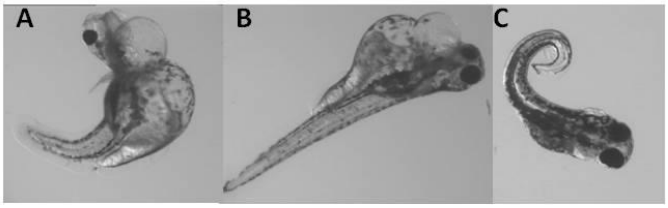

Figure 5: Mainly developmental failures observed in larval zebrafish:

A) pericarditis and scoliosis; **B)** pericarditis and **C)** curly and scoliosis.

OBJECTIVES

Taking into account the hypothesis raised, the aim of this thesis was to validate the zebrafish (*Danio rerio*) as a bioindicator to detect the possible presence of epigenetic substances (endocrine disruptors, persistent organic pollutants (POPs) and pharmaceutical substances) in drinking waters with effects on development and, especially, on reproduction. To this end, the specific objectives of the thesis were as follows:

- ✓ In the study I, the objective was to clarify the dilemma of keeping intact the chorion and assume the loss of information on the initial reproductive effects (genital ridge formation and the migration of primordial germ cells), or to permeabilize the chorion with pronase and, therefore, introduce a distortion factor.

- ✓ In the study II, the objective was to define and to narrow down those parameters that may be useful to detect the possible effects of environmental pollutants, especially on the development and reproduction of zebrafish when they were cultured in drinking waters throughout their life cycle.

- ✓ In the study III, the objective was to analyze the possible cumulative effect between two generations and/or the possible reversibility of the effects, from environmental pollutants in zebrafish reared in drinking waters.

- ✓ Finally, the last objective was to assess the different pathways through which effects are produced on zebrafish reared in two drinking waters: male, female or water where the in vitro fertilization took place.

EXPERIMENTAL PLANNING

In zebrafish, chorion contains pores that have a size of 0.17 μm² (Cheng *et al*., 2007). These pores size may act as a natural barrier for the intake of chemicals. However, it could not be clarified unequivocally whether the chorion represents an effective barrier and, thus, protects the embryo from exposure to distinct chemicals. Hence, as our long-term aim is to validate the zebrafish as a bioindicator, in our first study presented under the title of "**Zebrafish (Danio rerio) as a possible bioindicator of epigenetic factors present in drinking water that may affect reproductive function: is chorion an issue?**" we consider relevant, as a first step, clarify the dilemma of keeping the chorion intact or, on the contrary, permeabilize it with pronase.

Following the research line of our global aim, in our second study published as "**Zebrafish as a possible bioindicator of organic pollutants with effects on reproduction in drinking waters"** we define and narrow down the most sensitive parameters that may be useful to detect the effects of environmental pollutants, especially on the development and reproduction of zebrafish when they were cultured in drinking water throughout their life cycle.

In our third study titled as "**Zebrafish as a possible bioindicator of organic pollutants in drinking waters with effects on reproduction: Are effects cumulative or reversible?**" we assess whether effects possibly from environmental pollutants were cumulative between generations and/or reversible in zebrafish reared in drinking waters.

Finally, in our last study published (accepted) as "**Discrimination of the effects on zebrafish reproduction from pollutants in drinking water via female, via male and/or via fecundation water**" we assess the different pathways through which effects are produced in zebrafish: male, female or water where the in vitro fertilization took place.

STUDY I

Zebrafish (*Danio rerio*) as a possible bioindicator of epigenetic factors present in drinking water that may affect reproductive function: is chorion an issue?

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Zygote 2014; 23 (03): 447-452

Abstract

Emerging organic contaminants have been monitored in stream waters, raw and finished waters and wastewater effluents. Most of these contaminants, such as epigenetic substances, have been detected at very low levels. Unfortunately, their complete monitoring and/or removal are very difficult, given the increasing presence of new contaminants and due to analytical and economic considerations. For this reason, bioindicators are used as an alternative to monitor their presence. To this end, zebrafish is being used to assess certain contaminants in water quality studies.

As our long-term aim is to determine if zebrafish (*Danio rerio*) can be used to detect environmental epigenetic factors in drinking waters with effects on human reproduction, an initial question is whether the chorion could interfere with the possible action of epigenetic factors in two reproductive events: genital ridge formation and migration of the Primordial germ cells (PGCs) to these genital ridges.

In the first experiment, we attempted to partially degrade the chorion of mid blastula transition (MBT) embryos with pronase, with acceptable survival rates at 5 dpf, with the group exposed for 15 minutes giving the best survival results. Since denuded early embryos require a specific culture medium, in the next experiment embryo survival was evaluated when they were cultured until 5 dpf in drinking waters from six different sources. Results showed a negative effect on embryo survival at 5dpf from several waters but not in others, thus distorting the survival outcomes. These results suggest using embryos with the chorion intact from the outset when drinking waters from different sources are to be tested.

Introduction

A wide variety of organic compounds is frequently detected in streams that receive agricultural, domestic and (or) industrial wastewater effluent. These contaminants include antibiotics, other prescription drugs, non-prescription drugs, animal and plant steroids, reproductive hormones, personal care products, detergent metabolites and other extensively used chemicals. If the drugs, their metabolites and transformation products are not eliminated during sewage treatment, they may enter the aquatic environment and eventually reach drinking water (Kümmerer, 2009). In this sense, medical substances (Heberer, 2002), some active pharmaceutical ingredients (Kallenborn *et al*., 2008) and some Persistent Organic Pollutants (POPs) (Ericson *et al*., 2008; Wilhelm *et al*., 2010; Ullah *et al*., 2011; Eschauzier *et al*., 2012), have been detected in drinking water. Unfortunately, the complexity of the mixtures of these substances, their interactions and, in general, their variable and random presence at even relatively low levels in drinking water, make their routine chemical detection and control difficult or even impossible. In consequence, bioindicators are used as an alternative to monitor their presence and zebrafish is currently being used to assess certain contaminants in water quality studies (Ansari *et al*., 2009; Molinari *et al*., 2009).

In recent years there has been a growing body of evidence that exposure to chemicals in the environment poses a serious threat to human and animal reproduction (Gregoraszczuk *et al*., 2013). Moreover, it has been widely demonstrated that different chemical compounds, such as endocrine-disrupting chemicals and reproductive toxicants (Diethylstilbestrol (DES), Bisphenol A (BPA), POPs, etc.), are able to modify the epigenetic marks and induce persistent and in some cases transmissible changes of epigenetic

states (Baccarelli and Bollati, 2009), but without changes in DNA sequence (Wolffe and Guschin, 2000), which is the main epigenetic feature. As our long term aim is to detect the possible presence of epigenetic factors in drinking water with effects on reproduction, using the zebrafish as bioindicator, an initial aspect to be considered is the possible barrier effect of the chorion to the passage of these substances into the embryo until hatching.

As in other fish species, the zebrafish (*Danio rerio*) embryo is surrounded by an acellular envelope, the chorion, between 1.5 and 3.5 µm in thickness (Bonsignorio *et al*., 1996; Rawson *et al*., 2000). The chorion contains pores 0.17 μm^2 in size (Cheng *et al*., 2007). These pores were reported to be abundant at a rate of approximately 7.2 X 10^5 pores per chorion (Hart and Donovan, 1983). The pores might be responsible for size-dependent restrictions on the uptake of chemicals that have been reported for some large compounds exceeding 3 kDa (atomic mass unit), such as fluorescein dextrans (Creton, 2004). Nevertheless, despite ongoing speculation it is not clear if these pores are permeable or what their exact function is, and, consequently, whether the chorion is an effective barrier or not (Henn, 2011).

In the case of zebrafish, hatching occurs between 48-72 hours post fertilisation (hpf), when organogenesis is almost complete (Kimmel *et al*., 1995). Before that, two relevant reproductive events take place: the first is genital ridge formation, followed by the migration of the Primordial Germ Cells (PGCs) to these genital ridges (Yamaha *et al*., 2007). Moreover, it must be taken into account that the germ-cell-specific marker was detected in the cleavage planes in 2- and 4-cell-stage embryos (Yoon *et al*., 1997).

Obviously, the presence and permeation to the chorion of chemicals could influence their effects on these two reproductive events.

The immediate solution to a possible permeation restriction would be to remove the chorion completely by pronase treatment and/or mechanically. Although removal of the embryo chorion is efficient at epiboly or later stages (Truong *et al*., 2011) even with automated dechorionation systems (Mandrall *et al*., 2012), unfortunately at the mid blastula transition (MBT) stage (3 hpf) the efficiency of complete dechorionation with pronase treatment is low and, moreover, time consuming (Simão *et al*., 2010 a,b). Taking into account that a reproducible technique to avoid the barrier effect of the chorion with a high survival rate is required, in the present work the chorion will be partially degraded instead of eliminated. Moreover, early dechorionated embryos require a specific culture medium to reach the fry stage (Westerfield, 2000), so it is essential, in our case, to assess the survival of chorion-degraded embryos when they are cultured in different drinking waters.

Material and Methods

Care and maintenance of zebrafish colony

Adult zebrafish were kept in 20 L tanks in a 3:2 ratio (females: males) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão *et al*., 2010 a). The light cycle was regulated at 14h light/ 10h dark (Matthews *et al*., 2002; Brand *et al*., 2002).

Fertilised embryos at the MBT stage (phase in which the MZT (maternal zygotic transition) has been completed (Westerfield, 2000)) were selected under a stereo microscope and left in a Petri dish with dechlorinated and

decalcified tap water (Westerfield, 2007). The reason embryos were used in MBT stage is because it is the onset of zygotic phase (Dahm, 2002). Damaged embryos were discarded and only intact embryos were used in the experiment. No bleach treatment was applied, but sterilised media and materials (pipettes) in aseptic conditions were used. All chemicals and culture media were from Sigma-Aldrich (Madrid, Spain).

Experimental design

Two consecutive experiments were carried out. The first attempted to establish the pronase treatment duration required for maximum partial degradation of the chorion but with a low mortality rate. Pronase solution was 1.5 mg mL-1 in H10, i.e. Hanks buffered salt solution (HBSS) diluted 10% (v/v) in ultrapure water (Millipore) (Simão, 2010 b; Westerfield, 2007). Each vial used contained 1.5 ml of this solution. Throughout the experiments we used three different commercial batches of pronase.

In each assay, batches of embryos in MBT stage were treated with pronase solution, previously tempered to 28.5ºC, for 5, 10 and 15 minutes respectively, whereas control group embryos were not treated with pronase. After pronase treatment, embryos were washed twice, for 5 minutes each wash, in 50 ml of H10 (35 mOsm) to 28.5 ºC to remove any pronase residue. Next, the chorion of the embryos was recorded with a camera connected to the stereomicroscope (Nikon SMZ 800, Japan) at x15. Pictures of the chorion were captured with the VLC media player program to assess the presence of holes according to the treatment duration. Embryos were kept in H10 at 28.5ºC for 5 days, because by this time the fry are already able to feed. Survival rates were evaluated at 2 hours after pronase treatment (pronase lethal effects), and at 5 days post fertilisation (dpf). At least four replicates were performed in all experimental groups.

Once the pronase treatment duration was established, the second experiment tested the effect of the different drinking waters on embryo survival rates when they were cultured in these waters for five days after the pronase treatment. This second experiment is justified because it is not exactly clear if all types of drinking water will satisfy the culture requirements of dechorionated embryos.

The selected waters were intended to cover the range of water types to be studied in future assays. Except for H10 and mineral bottled water, sampling of tap water from the distribution network was performed on at least three different dates throughout the experiment.

Thus, pronase-treated and non-treated embryos at the MBT stage were cultured until 5 dpf at 28.5ºC in six different waters depending on their source: H10 stored either in a Pyrex glass (A) or plastic bottle (B), bottled spring water (C) and, finally, three waters (D, E, F) from different tap water distribution networks. Type D was tap water from a city located in a region with intensive agricultural activity from the hydrological basin of the river Xúquer; type E was tap water from a city also located in a region with intensive farming activity, but from the Túria river hydrological basin. Type D and E came from groundwater prospecting. Finally, type F was from the tap water distribution network of a medium-sized city supplied from both the Túria and Xúquer rivers. Survival rates were evaluated at 2 hours after pronase treatment and 5 dpf. At least eight replicates were performed in all experimental groups. All water samples whatever the water types were left in open containers prior their use, letting the water stand for several days to eliminate the chlorinated volatile substances used in purification water process, before to be tempered to 28.5ºC.

In both experiments, the results were analysed using Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved.

Results

Results from the first experiment (see table 1) show that at 2 hours after pronase treatment, the time of exposure to this enzyme (5, 10 or 15 minutes) progressively penalised the survival rates. The worst result was reached with the group exposed for 15 minutes. From 2 hours after pronase treatment to 5 dpf, ruling out the direct immediate effect of pronase treatment evaluated at two hours, there were no significant differences between the treated groups, although all of them differed significantly from the non-treated (control) group.

Table 1: Survival rates at 2h after pronase treatment and until 5 dpf of mid blastula transition (MBT) embryos from zebrafish (*Danio rerio*) treated with pronase for 5, 10 and 15 minutes. The control group was not treated with pronase.

		Survival rate 2 h post-treatment	Survival rate 2 h- 5 d post-fertilization
Control		61/61 (100 %)^{a}	60/61 (98 %)^{a}
Pronase	5 min	55/61 (90%)^{a}	43/55 (78%)^{b}
	10 min	47/62 (76%)^{ab}	39/47 (83%)^{b}
	15 min	44/61 (72%)^{b}	32/44 (73%)^{b}

In each column, rows with different superscripts are statistically different ($p<0.05$)

With regard to images captured, although at 10 minutes of pronase treatment holes were observed in some cases, the most evident presence of holes, in all cases, was at 15 minutes.

Based upon results from the first experiment, pronase treatment for 15 minutes was selected to ensure partial degradation of the chorion but without an excessive reduction in embryo survival.

In the second experiment, survival rates at 5 dpf were compared between embryos treated or not with pronase when cultured in each water type. The initial number of embryos was considered at 2 h after pronase treatment in the treated groups (See table 2).

Table 2: Survival rates of embryos from zebrafish (*Danio rerio*) treated and not treated with pronase for 15 minutes and cultured in different waters according to their source until 5 dpf (days post fertilization).

		A	B	C	D	E	F
Pronase treated	2 h- 5d	78/92 (85 %)^{b}	84/104 (81 %)^{b}	116/126 (92 %)^{a}	104/113 (92 %)^{a}	94/109 (86 %)^{b}	97/98 (99 %)^{a}
non-treated	2 h- 5d	140/144 (97 %)	131/133 (98 %)	130/133 (98 %)	129/134 (96 %)	121/124 (97 %)	119/124 (95 %)

In each row, columns with different superscripts are statistically different ($p<0.05$)

According to the results obtained in the second experiment, when survival rates were compared between the six types of water, but independently in treated (chorion degraded) and untreated groups, in the treated groups comparison the worst statistically significant ($p< 0,05$) results were obtained in waters A, B and E. This differential effect of type of water on chorion-degraded embryos can distort the experimental results.

On the contrary, in non-treated groups there were no differences between waters (p=0.8257). This latter result can be explained because all types were drinking water (A and B types were H10) in which no acute toxicity was expected.

Overall, in all types of waters except F type, degradation of the chorion with pronase reduced the embryo survival rates at 5 dpf, reaching levels of significance in embryos cultured in Type A, Type B and Type E waters.

The absence of differences between water type A (stored in Pyrex glass) and B (stored, like the other waters, in plastic bottles suitable for commercial waters), would indicate that the container does not affect embryo survival rates in any case.

With regard to abnormality rates until 5 days post fertilisation (dpf), it was noted that these rates were low in all groups (1-2%), either treated or non-treated, except in Type D water, where the abnormality rates in the pronase treated group reached 4%.

Discussion

Similar observations to those obtained in the first experiment on the immediate detrimental effect of pronase on embryos were already made by Henn *et al*. (2011) and others (Simão *et al*. 2010a) in toxicity and chimaerism studies.

In any case, results show that the effects caused by the pronase treatment, without complete dechorionation, affect embryo survival at 5 dpf, whatever the treatment duration.

The survival results of dechorionated embryos at 5 dpf were relatively low in H10 medium in both experiments. This medium was initially proposed by Westerfield (2000), although other authors later incorporated modifications to the same (Truong *et al*., 2011).

On the other hand, results obtained in the second experiment indicate that the type of water affects chorion-degraded embryo survival at 5 dpf. In toxicity substance assessment studies, dechorionated embryos are used (Truong *et al*., 2011) and a very efficient automated dechorionation system has even been developed (Mandrall *et al*., 2012) for denudation with pronase. However, in this type of studies the culture medium is always the same and only the concentration of the substance to be assessed is changed (Henn et al. 2011; Truong *et al*., 2011). Furthermore, embryo dechorionation is performed in epiboly (6 hpf) (Truong *et al*., 2011) or more advanced stages. These may therefore be the two biggest differences between our case and toxicology studies: the different culture media (drinking water) where the embryos are cultured and the dechorionation of the embryos at an earlier stage (3 hpf).

The dilemma is thus whether to keep the chorion intact and assume the loss of information on the initial reproductive effects, or, alternatively, to permeabilise the chorion and in doing so introduce a distortion factor in the assessment of subsequent damage to embryos and, perhaps, in reproduction, depending on the water where the embryos with the degraded chorion are cultured.

A possible solution to avoid such waters distortion would be to remove the chorion at 24 hours post fertilisation (hpf), with high survival rates at this time (90%) (Henn & Braunbeck 2011). However, it has been stated that before the 20 somites (19 hpf) stage PGCs move to the genital ridge,

whereas those after that stage mostly do not (Yamaha *et al*., 2007). So, removing the chorion at 24 hpf will not prevent the barrier effect at earlier stages on PGCs and on differentiation of the genital ridge.

On the other hand, leaving the chorion intact will prevent any water distortion on embryo survival rates (and, perhaps, on embryo organogenesis and cell differentiation) depending on the tap water source, as was detected in the second experiment. It must be remarked that in water quality studies are used embryos with the chorion intact because the hatching success of embryo is an endpoint assessed (Hallare *et al*., 2005; Wu *et al*., 2014).

It seems that the chorion is not a major barrier for simple chemicals; however, there are exceptions like cationic polymers, heavy metals and large molecules (e.g. polymers) (Henn, 2011), although, in any case, the chorion retards the free exchange of substances (Harvey *et al*., 1983). On the other hand, it is suspected that the chorion pores potentially restrict the uptake of compounds depending on their size (Creton, 2004). Nevertheless, a reasonable molecular size cut-off value for fish embryo testing cannot be set (Henn, 2011). Whatever the case, natural hatching in zebrafish occurs within 48-72 hpf, so the chorion barrier effect, if any, will only be present before that time.

It must be emphasised that in this stage, and in addition to the possible barrier effect of the chorion on the permeability, fish embryos also have low membrane permeability, with the presence of cell layers that act as osmotic barriers making the permeation of substances (e.g. cryoprotectants) extremely difficult (Hagedorn *et al.,* 1997; Cardona-Costa and García-Ximénez, 2007). Obviously, this barrier effect of embryos does not depend on the presence or otherwise of the chorion.

With regard to the following reproductive milestones, these occur much later after hatching, and therefore will not be affected by the previous presence or absence of the chorion, at least directly. Thus, in zebrafish, the first sign of sex differentiation is initiated at 10 to 12 days post fertilisation (dpf) (Tong *et al*., 2010) and is completed at 35 dpf in females and 45 dpf in males (Weiting *et al*., 2013). It should be highlighted that the initial number of primordial germ cells (PGCs) in the gonad may be related to the subsequent sex differentiation (Lo *et al*., 2011).

In conclusion, the results obtained in this work advise against the partial or total denudation of early embryos when they are used to detect substances in different drinking waters and assume the possible limitation to the passage of any likely epigenetic factor which could affect the genital ridge or PGC migration.

Acknowledgements

The authors would like to thank Mr. Javier Rubio Rubio for his valuable technical support and Mr. Neil Macowan for improving the English of this manuscript.

STUDY II

Zebrafish (*Danio rerio*) as a possible bioindicator of organic pollutants with effects on reproduction in drinking waters

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Journal of Environmental Science 2015; 33 (1): 254-260.

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.

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 (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.

Material and Methods

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 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão *et al*., 2010a). The light cycle was regulated at 14 hr light/10 hr dark (Matthews *et al*., 2002; Brand *et al*., 2002).

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 (Martínez-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. 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 28.5ºC 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.

Results

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 (see 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 ($p=0.0617$) (see table 1).

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 (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 ($p<0.05$).

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.

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.

Onset of spawning

Adult zebrafish reach sexual maturity within three months after hatching (Ma *et al*., 2001), 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.

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.

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.

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 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 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.

Acknowledgements

The authors would like to thank Mr. Javier Rubio Rubio for his valuable technical support and Mr. Neil Macowan for improving the English of this manuscript.

STUDY III

Zebrafish (*Danio rerio*) as a possible bioindicator of organic pollutants in drinking waters with effects on reproduction: are effects cumulative or reversible?

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Paper accepted in "Zygote"

Abstract

Due to inefficient detection and removal treatments, organic pollutants are present in drinking waters. For this reason, zebrafish is proposed as a complementary indicator in conventional potabilization treatments.

According to the most sensitive parameters detected in our previous work, in the current work we attempt to study the possible cumulative effect between generations, of environmental pollutants likely present in drinking waters, when specimens are cultured in the same water and/or the possible reversibility of these effects when cultured in control water. To this end, embryos with the chorion intact were cultured in 3 drinking waters from different sources and in one control water up to 5 months, in 20 l tanks. Four replicates were performed in all water groups.

Results in water C revealed a non-reversible effect on fertility rate, and also in water C an alteration of sex ratio towards females, although in this case the alteration was reversible. A transgenerational alteration in the germline via epigenetic mechanism from the previous generation is proposed as the most plausible explanation to this effect.

Introduction

Emerging organic pollutants such as pharmaceutical and medical substances, persistent organic pollutants (POPs) and endocrine disruptors have been dispersed worldwide and as a result are emerging in surface, groundwater and even in drinking waters, in this case due to inefficient removal treatments (Ikehata *et al*., 2008; Benner *et al*., 2013). The concentrations of these substances are low but increasingly numerous and variable over time (Khetan and Collins, 2007; Rodil *et al*., 2012). These substances can exert toxicological but also epigenetic effects on many

functions, operating on somatic cells and in the germ line, in this case promoting transgenerational effects (Rusiecki *et al*., 2008; Skinner, 2011).

Contrasting with toxicological studies where the substances concentration effects to be evaluated have been previously established (Galus *et al*., 2013; Dai *et al*., 2014), in the current paper there is no intention to detect specific pollutants, but the effect of the mixture of pollutants possibly present in drinking water on phenotypic characters of the zebrafish (Martínez-Sales *et al*., 2015). In fact, the option to develop an analysis waters study comes as a very difficult alternative, since emerging organic pollutants are new products without regulatory status and, therefore, without a specific control (Deblonde *et al*., 2011). Moreover, the vast quantity of these new substances in the environment makes very difficult to limit them (von der Ohe *et al*., 2011; Nikolaou, 2013). Hence, due to the complexity of their detection and removal, bioindicators can be used as an alternative to monitor their presence.

In our previous work (Martínez-Sales *et al*., 2015), we defined and narrowed the most sensitive developmental and reproductive parameters in zebrafish, with the long-term aim of establishing the zebrafish as a bioindicator of the possible presence of environmental pollutants. Specifically, the assessment was carried out in three drinking waters from different tap water sources. The most sensitive parameters detected were: hatching rate, fertility rate and underdeveloped specimens. So, in the present work we focused on these parameters in order to study the possible cumulative effect and/or possible reversibility of the effects, between generations, of these environmental pollutants in the same three drinking waters (A, B and C), despite the fact that there are other sensitive parameters, for example sex ratio.

Materials and methods

Zebrafish maintenance

Both F0 obtained from the original wild zebrafish colony and F1 generations were reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão *et al*., 2010a) twice a day. The light cycle was regulated at 14h light/ 10h dark (Matthews *et al*. 2002; Brand *et al*. 2002). The aquariums had water recirculation systems but without active carbon filters to avoid removal of chemical pollutants possibly present in water. According to the Westerfield (2007) recommendations, a quarter of the total aquarium water was removed weekly and replaced by clean water to avoid ammonia, nitrite and nitrate toxic concentrations.

It must be stated that all environmental conditions were identical to all aquariums and the spatial distribution of the aquariums was randomized in the housing room.

The experimental procedures and animal care in this work fully comply with the standards for use of animals established by the Ethical Committee of the Polytechnic University of Valencia, which specifically approved this study.

Water sources

The four different drinking waters used in the present study (the same than in our previous work) were classified depending on their source in the region of Valencia (Spain), into: three waters from different tap water

distribution networks (A, B and C) and one bottled spring water which was established as a control. 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 river Xúquer. Type A and C came from groundwater prospecting.

Before filling the aquariums with water, recipients (where the water was stored) were kept open for at least a week, with a large exchange surface to favour chlorine elimination (Westerfield, 1995).

It should be mentioned that all the waters are potable and also that the chemical parameters defined for tap water for human consumption in Royal Decree 140/2003 of 7 February, which establishes the health criteria for the quality of water intended for human consumption, are suitable for zebrafish breeding and maintenance (Westerfield, 2007). Furthermore, the drinking waters used meet the physical and chemical requirements set by this Royal Decree.

Specimen management

Fertilized embryos were obtained by siphoning. Batches of 20 fertilized and normal developing embryos at the Mid Blastula Transition (MBT) stage with the chorion intact (Martínez-Sales *et al*., 2014; Martínez-Sales *et al*., 2015) were selected under a stereo microscope. These embryos were left in Petri dishes and cultured until 5 dpf (days post fertilization) at 28, 5ºC in dishes with the same water type where their progenitors were reared (same water origin and water destination: A-A; B-B; C-C; Control-Control) and, on the

other hand, in dishes with control water (different water origin and water destination: A-control; B-control; C-control).

Next, from 5 dpf to complete adulthood (5 months post fertilization) larvae were left in aquariums (20 L) in the same type of water as that in which their progenitors were reared and in aquariums with control water, to assess either the possible cumulative effect when specimens are cultured in the same water or the possible reversibility effect when are cultured in control water. From these combinations, four replicates were established with a total of 28 aquariums and with a maximum of 20 specimens per aquarium.

After three months, marbles were placed in each aquarium with the aim of siphoning all aquariums 2 or 3 times a week throughout the 4^{th} and the 5^{th} month, to evaluate the onset of spawning and the fertility rate. Sex ratio of the surviving adults, underdeveloped specimens and survival and abnormality rates at 5 mpf (months post fertilization) were also evaluated. Moreover, in the F1 offspring (F2 larvae) we evaluated the survival and abnormality rates at 5 dpf and the hatching rate at 72 hpf (hours post fertilization).

Experimental design

Two different analyses were carried out on the most sensitive parameters obtained in our previous work: hatching rate, fertility rate and underdeveloped specimens. The first analysis studied the possible cumulative effect between generations. To this end, fertility rate and underdeveloped specimens (runts) were compared in the F0 and F1 generation. In turn, the hatching rate at 72 hpf was compared in the F1 and F2 generation (see figure 1). The second analysis studied the possible

reversibility of the effects in fertility rate and in underdeveloped specimens in the F1 generation, and hatching rate in the F2 generation.

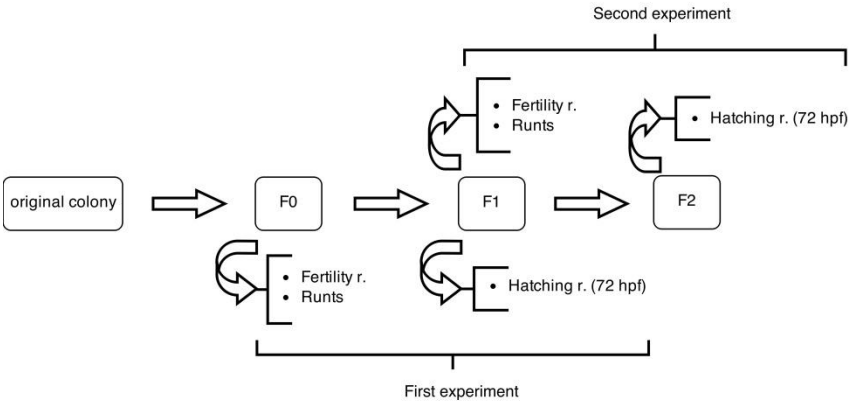

Figure 1: Two different analyses were carried out on the most sensitive parameters. In the first experiment were compared the parameters from F0 (Generation 0) and F1 (Generation 1). The same comparison was carried out in the parameters from the F1 and the F2 (Generation 2).

Statistical analysis

The possible cumulative and reversible effects in all parameters were analysed using Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved. Values were considered statistically different at $p<0.05$.

Results

As stated in material and methods, four replicates were performed in all water groups with a total of 28 aquariums at the outset. However, 8 aquariums were discarded due to total mortality of the larvae cultured in

Petri dishes until 5dpf for reasons unknown and uncontrolled. This mortality cannot be associated to a water type, as the mortality was random between groups. So, the minimum number of replicates per group was two, with a total of 20 aquariums. In the first group (control-control) the final number of replicates was three, in the second group (A-A) the final number of replicates was two, in the third group (A-control) the final number of replicates was also two, in the fourth group (C-C) the final number was three, in the fifth group (C- control) the final number was four, in the sixth group (B-B) the final number was two and in the seventh group (B-control) the final number was four.

Hatching rate

Hatching rate was evaluated at 72 hpf (Martínez-Sales *et al*., 2015) in the F1 and F2 generations during 4^{th} and 5^{th} mpf.

Cumulative effect

The analysis showed statistically significant differences (p<0.05) between the F1 and the F2 generations in all waters studied (see table 1). In all cases, the worst results were obtained in the second generation. These results reveal a cumulative effect in all waters, even in the control water. The negative cumulative effect in the case of water B should be highlighted.

Table 1. Hatching rate of zebrafish (*Danio rerio*) embryos cultured in control water, water A, B and C at 72 hpf in F1 and F2 generations.

Hatching rate at 72 hpf		
Type of water	F1	F2
Control	1215/1221 (99.50%)[a]	994/1082 (92%)[b]
A	473/490 (96.53%)[a]	189/207 (91.30%)[b]
B	761/880 (86.47%)[a]	60/160 (37.5%)[b]
C	828/850 (97.41%)[a]	399/444 (90%)[b]

Columns with different superscripts are statistically different ($p<0.05$)

Reversible effect

The analysis showed statistically significant differences ($p<0.05$) between data from the specimens reared in waters with the same origin and destination and data from the specimens reared in control water in all waters studied (see tables 2, 3 and 4). The worst result was obtained in all waters with the same origin and destination. These results reveal that there was a reversible effect in all waters when specimens were cultured in control water.

Table 2. Hatching rate of zebrafish (*Danio rerio*) embryos cultured in water A-A and in water A-Control at 72 hpf in the F2 generation.

F2	A-A	A-control
Hatching rate at 72 hpf	189/207 (91.30%)[b]	1178/1229 (96%)[a]

Columns with different superscripts are statistically different ($p<0.05$)

Table 3. Hatching rate of zebrafish (*Danio rerio*) embryos cultured in water B-B and in water B-Control at 72 hpf in the F2 generation.

F2	B-B	B-control
Hatching rate at 72 hpf	60/160 (37.5%)[b]	740/835 (88.62%)[a]

Columns with different superscripts are statistically different ($p<0.05$)

Table 4. Hatching rate of zebrafish (*Danio rerio*) embryos cultured in water C-C and in water C-Control at 72 hpf in the F2 generation.

F2	C-C	C-control
Hatching rate at 72 hpf	399/444 (90%)[b]	361/377 (95.75%)[a]

Columns with different superscripts are statistically different ($p<0.05$)

Fertility rate

Fertility rate was evaluated through 4^{th} and 5^{th} mpf in the F0 and F1 generations.

Cumulative effect

The analysis showed statistically significant differences (p<0.05) between the F0 and the F1 generations in all waters studied (see table 5). The worst results were obtained in the second generation (F1). These results reveal a cumulative effect in all waters, including the control water.

Table 5. Fertility rate of adult zebrafish (*Danio rerio*) cultured in control water, water A, B and C in F0 and F1 generations.

	Fertility rate	
Type of water	F0	F1
Control	1274/1713 (74.37%)[a]	1608/2516 (64%)[b]
A	490/1150 (42.60%)[a]	386/1899 (20.32%)[b]
B	895/2608 (34.31%)[a]	211/862 (24.5%)[b]
C	857/1494 (57.36%)[a]	677/1573 (43.03%)[b]

Columns with different superscripts are statistically different (p<0.05)

Reversible effect

The analysis showed statistically significant differences (p<0.05) between data from specimens reared in waters with the same origin and destination and data from specimens reared in control water in all waters studied (see table 6, 7 and 8). In the case of waters A and B, the worst result was obtained in waters with the same origin and destination (A-A and B-B), whereas in water C the result did not improve when specimens were cultured in control water. These results revealed that there was a reversible

effect in waters A and B when specimens were cultured in control water, but a non-reversible effect in water C.

Table 6. Fertility rate of adult zebrafish (*Danio rerio*) cultured in water A-A and in water A- Control in the F1 generation.

F2	A-A	A-control
Fertility rate	386/1899 (20.32%)[b]	1811/2465 (73.46%)[a]

Columns with different superscripts are statistically different (p<0.05)

Table 7. Fertility rate of adult zebrafish (*Danio rerio*) cultured in water B-B and in water B- Control in the F1 generation.

F1	B-B	B-control
Fertility rate	211/862 (24.5%)[b]	1882/3145 (60%)[a]

Columns with different superscripts are statistically different (p<0.05)

Table 8. Fertility rate of adult zebrafish (*Danio rerio*) cultured in water C-C and in water C-Control in the F1 generation.

F1	C-C	C-control
Fertility rate	677/1573 (43.03%)[a]	559/1805 (31%)[b]

Columns with different superscripts are statistically different (p<0.05)

Underdeveloped specimens (runts)

In this work, specimens evaluated at 5 mpf in the F1 generation were all sexes clearly identifiable, and morphologically were also similar. Hence, there were no underdeveloped specimens.

Sex ratio

Even though in the previous work sex ratio was not a sensitive parameter, in the present work, water C displayed a feminization process. Therefore, sex ratio in water C was analysed at 5mpf in the F0 and in the F1 generations.

Cumulative effect

The analysis showed statistically significant differences (p<0.05) between water C from F0 and water C from F1. The worst result was obtained in water C from F1, where the sex ratio was skewed towards females (males 25%: females 75%) (see table 9). No significant difference (p>0.05) was obtained in the other waters (A and B) whose sex ratio percentages were within the normal range in zebrafish in both generations (60 males: 40 females) (Fenske *et al*., 1999).

Table 9. Sex ratio of zebrafish (*Danio rerio*) adults cultured in water C at 5mpf in F0 and F1 generations.

F0-F1		C (F0)	C (F1)
Sex ratio	Males	82/118 (69%)[a]	6/24 (25%)[b]
	Females	36/118 (31%)[a]	18/24 (75%)[b]

Columns with different superscripts are statistically different (p<0.05)

Reversible effect

The feminization detected in specimens cultured in water C, disappeared when were reared in control water (see table 10).

Table 10. Sex ratio of zebrafish (*Danio rerio*) adults cultured in water C-C and in water C- Control at 5mpf in F1 generation.

F1		C-C	C-control
Sex ratio	Males	6/24 (25%)[b]	13/22 (59.09%)[a]
	Females	18/24 (75%)[b]	9/22 (40.90%)[a]

Columns with different superscripts are statistically different ($p<0.05$)

Discussion

The reasons that prevent to develop an analytical water study of emerging organic pollutants are mostly the lack of information regarding their occurrence and toxicity, the lack of appropriate analytical methods for their determination, or both (Nikolaou, 2013).

Based upon results from our previous work (Martínez-Sales *et al*., 2015), hatching rate, fertility rate and underdeveloped specimens were the most sensitive parameters to detect the possible presence of environmental pollutants in drinking waters from different tap water distribution networks (A, B and C). These parameters were selected considering the full life-cycle (from development to reproduction) of zebrafish specimens.

The same waters were used in the present work, but it should be taken into account that although these waters have the same original source, the

physical and chemical conditions of the water may have changed due to seasonal variations in quality at the water source (Ouyang *et al*., 2006), although in order to be drinkable it should meet legal strict limits. Nonetheless, differences between waters also appeared in the same parameters in this experiment, except in the rate of underdeveloped specimens.

The period around hatching is a critical stage during embryogenesis (Henn, 2011), which is why the hatching rate has been extensively used as a parameter in many toxicological studies (Han *et al*., 2011; Galus *et al*., 2013) as well as a parameter for reproductive toxicity assessment (Simon *et al*., 2011). Our results for hatching rate revealed that although the results were high in all waters in both generations, except in water B (86.47% in F1 and 37.5% in F2), there was a negative cumulative effect in the second generation in all waters tested, even in the control water. Surprisingly, water B reached the worst results in both generations compared to the control water, decreasing to 48.97% (86.47%-37.5%) in the second generation compared to the first. These outcomes may suggest either the possible increasing presence of pollutants such as pharmaceutical substances (David and Pacharatna, 2009), endocrine disruptors (Han *et al*., 2011) and insecticides (Mandrell *et al*., 2012), among others (Duan *et al*., 2008) in waters in both experiments (generations) which affect the hatching process and/or the possible transmission of these negative effects to the next generation via epigenetic mechanisms (Skinner *et al*., 2010; Skinner, 2011). However, it should be stated that when specimens were cultured in control water, this cumulative effect disappeared, which rules out a possible transgenerational transmission via epigenetic mechanisms.

Fertility rate has also been used in many toxicological studies as a good parameter (Ankley and Johnson, 2004; Liu *et al*., 2014). Results from fertility show that there was a negative cumulative effect in the second generation compared to the first in all waters, even in the control water. The most pronounced reduction between generations was obtained in water A, 22.28% (42.60%-20.32%), as this water reached the lowest rate (20.32%), followed by water B (24.5%) in the second generation. These outcomes may suggest either the possible increasing presence of the same pollutants in waters in both experiments (generations), which affected the fertility rate and/or the possible transgenerational transmission of these negative effects to the next generation via epigenetic mechanisms (Skinner *et al*., 2010; Skinner, 2011). It should be noted that when specimens were cultured in control water, there was a reversible effect in waters A and B, which ruled out a possible transgenerational transmission via epigenetic mechanism in these waters, although the cumulative effect remained in water C, the fertility rate decreasing to 12.03% (43.03% -31%) when specimens were cultured in control water.

So, on the basis of these findings we posit the possible presence of environmental pollutants in water A and B that affect fertility rate in both generations without transgenerational transmission, due to the reversibility process in these waters. Nevertheless, in water C the non-reversible effect also leads us to consider the possible presence of environmental pollutants in water C that affect fertility rate in both generations, but in this case with a possible transgenerational transmission due to the maintenance of the cumulative effects when specimens were cultured later in control water. This could be explained because early exposure during critical periods of development to environmental pollutants, such as endocrine disruptors (Braw-Tal, 2010), can promote an adult-onset alteration (in this case a

reduction in fertility rate) long after the compound is removed, even in subsequent generations if the germ line is affected through epigenetic mechanisms (Skinner *et al*., 2010; Skinner, 2011).

Regarding the non-reversible effect of the fertility rate in water C, although we are unable to describe the mechanism of action behind this effect, a plausible explanation could be an early exposure to some pollutant in water C during a critical period of embryo development (Braw-Tal, 2010), such as the MBT stage in our case, without a germline alteration via epigenetic mechanism. The crucial period for epigenetic regulation and modification of the germline is during the period of primordial germ cell migration and gonadal sex determination (Skinner *et al*., 2010), events that take place after the MBT stage (3 hpf) (Dahm, 2002), at the early gastrulation stage (from 6 hpf) (Yoshizaki *et al*., 2002). So, taking this argument into account, the most likely explanation could be an alteration in the germline transgenerational transmitted from the previous generation (parents) via epigenetic mechanisms to this generation.

Sex ratio is a relevant parameter used in many toxicological studies (Hill and Janz, 2003; Baumann *et al*., 2013; Liu *et al*., 2014). However, in our previous work, it was not classified as a sensitive parameter because in all drinking waters tested sex ratios were within the normal ranges. Thus, all percentages of females were around 40%, which agreed with our current results and with other studies on zebrafish (60 males: 40 females) (Fenske *et al*. 1999), (68:32) (Örn *et al*., 2003), (56:44) (Vaughan *et al*., 2001; Hsioa and Tsai, 2003). However, in this second experiment in water C there was an alteration of sex ratio towards females (75%), although this feminization changed towards normal values in zebrafish when specimens were cultured in control water.

These results suggests the possible presence of some environmental pollutants, only in water C, such as endocrine disrupting chemicals (17-ethinylestradiol, even at ng/l) that can disrupt sexual differentiation in fish (Larsen *et al*., 2009) and cause feminization and retardation of sexual maturation in zebrafish. These substances may trigger disruption of sex hormones during sexual development and alter female sex, male sex or even both sexes. In fish, the hormonal balance between estrogens and androgens appears to be an important factor in the course of sexual differentiation (Liu *et al*., 2014).

It must be highlighted that all environmental factors were rigorously controlled to avoid any external alteration of our sex differentiation in zebrafish, as this is known to be a difficult process in fish (Liew *et al*., 2014) that can be affected by several environmental factors in a very complex way (Baroiller *et al*., 1999).

Evidence from our results gathered to date corroborates that zebrafish is a suitable model for use as a bioindicator to detect environmental pollutants in drinking water. The complexity of detecting these substances in conventional potabilization treatments, due to their interactions and their variable and random presence even at low levels in drinking water, makes their routine chemical detection and control difficult or even impossible (Khetan and Collins, 2007; Benner *et al*., 2013). For this reason, bioindicators could be used as backup control measures to conventional potabilization treatments.

Finally, the detection in our previous (Martínez-Sales *et al*., 2015) and current works of the negative effects on reproductive parameters in zebrafish reared in drinkable water is cause for alarm, as the presence of emerging organic pollutants in drinking water may be one of the reasons

behind the decline in human reproduction in metropolitan areas (Toft *et al*., 2006; Jurewicz *et al*., 2009; Braw-Tal, 2010; Vested *et al*., 2014).

Acknowledgements

The authors would like to thank Mr. Javier Rubio Rubio for his valuable technical support and Mr. Neil Macowan for improving the English of this manuscript.

STUDY IV

Discrimination of the effects on zebrafish (*Danio rerio*) reproduction from pollutants in drinking water via female, via male and/or via fecundation water.

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Paper accepted in "Zygote"

Abstract

The lack of preventive policy legislation and the low removal rate of organic pollutants in conventional potabilization treatments lead to some of them being present in drinking water. The problem arises because some of these substances have detrimental effects on human reproduction health, via females, via males or even both. In this work, we established the zebrafish as a bioindicator of these types of substances with the goal of discriminating the effects through three different pathways: male, female or water where the fertilization took place.

For this purpose, four parameters were analysed: fertility rate, hatching rate and survival and abnormalities rates. So, for each parameter two groups were formed, according to whether adult males or females were reared in bottled spring water (Z) or tap water (B) and if the in vitro fertilization took place in water Z or B.

Results revealed a decline in the fertility and hatching rate in water B, due to a water effect. The most plausible explanation could be the presence of substances which affect the micropyle and chorion. Moreover, a decrease in the fertility rate due to an effect over the female was also observed, but in this case by an alteration of the oocyte quality.

Introduction

Several emerging organic pollutants (endocrine disruptors, pharmaceutical substances and personal care products) are released mostly through urban wastewater and many of them can spread through the water cycle, even reaching drinking water, due to their low removal rate (Rodil *et al*., 2012). The problem is exacerbated by the fact that many emerging pollutants are

non-regulated (Richardson and Ternes, 2011) or newly introduced, or have only recently been regulated, as is the case with some pharmaceutical substances. Furthermore, although concentrations are generally low (ng/l) and some individual chemicals are not dangerous to human health (Schriks *et al*., 2010), there are worries about the potential and unknown risks of exposure to mixtures (Silva *et al*., 2002), especially in human reproduction, where the alteration could be via female or male or even both.

The detection of organic pollutants in drinking water through the study of the most sensitive developmental and reproductive parameters in zebrafish, particularly the latter, was the aim of our last work (Martínez-Sales *et al*., 2015). In our current work, we attempt to elucidate the origin of the effects on survival, abnormality, hatching and fertility rate in zebrafish adults reared in two waters (Z and B) also tested in our previous works, from three different pathways: male origin, female origin or the water where the in vitro fertilization took place, with the aim of establishing the zebrafish as a bioindicator in water quality study.

Materials and methods

Zebrafish maintenance

The F1 colony was reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão et al. 2010 a) twice a day. The light cycle was regulated at 14h light/ 10h dark (Matthews *et al.,* 2002; Brand *et al*., 2002). The aquariums had water recirculation systems but without active carbon filters. According to the Westerfield (2007) recommendations, a quarter of

the total aquarium water was removed weekly and replaced by clean water to avoid ammonium concentrations.

It must be stated that all environmental conditions were identical to all aquariums and the spatial distribution of the aquariums was randomized.

The experimental procedures and animal care in the present work fully comply with the standards for use of animals laid down by the Ethical Committee of the Polytechnic University of Valencia, which specifically approved this study.

Water origin

Two different waters were used in this work. Bottled spring water (Z) that was used as control in our previous works, and water B also tested in previous works from the tap water distribution network of a medium-sized city, supplied from the Túria and Xúquer rivers. Water B was selected to manifest the most harmful effects on the sensitive parameters studied in our previous works (Martínez-Sales *et al*., 2015).

It should be noted that water B is potable and also that the chemical parameters set forth for tap water for human consumption in Royal Decree 140/2003 of 7 February, whereby the health criteria for the quality of water intended for human consumption are established, are suitable for zebrafish breeding and maintenance (Westerfield 2007). Furthermore, the drinking waters used meet the physical and chemical requirements set by this Royal Decree.

Obtaining inactivated gametes

Gametes extraction was carried out following the method describe by Westerfield (2007). Zebrafish adults (5 months post fertilization) were carefully selected and separated from the colony after having manifested courtship behaviour at dawn. Before any extraction, specimens were sedated in a clove oil solution (100µl oil in 1L of decalcified and dechlorinated water: system water) for a few minutes, then were cleaned in clear water. Eggs were extracted and deposited in a plastic spoon after gentle but firm pressure with plastic forceps on the belly previously dried. Only good eggs (yellow and translucent colour) were kept in Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA (Bovine serum albumin) and 0.1 g of NaCl/100 cc of Hanks' medium (egg medium (F_1); ph: 7.4; osmolarity: 310-320 mOsm) in a 35 mm Petri dish.

For semen extraction, males were placed belly up in a slit of a damp sponge. The genital pore was gently dried to avoid sperm activation. The sides of the fish were gently but firmly pressured with plastic forceps to collect the sperm with a microcapillary (1 x 90 mm, Narishige Scientific Instrument Lab.), which were kept on ice until use. Sperm from 2-3 males was diluted in 100µl of F_1 and kept inactivated in a Petri dish of 35 mm.

In vitro fertilization (IVF)

The IVF and solutions used were at room temperature. Non activated eggs and sperm were mixed in F_1 for a couple of minutes. Two groups were carried out depending on the water (Z or B) used to activate both gametes. So, 1 mL of Z or B water, depending on the experimental group, was added to the egg-sperm mixture. After 2-3 min, the time required for fertilization in zebrafish, the 35 mm Petri dish was fully filled with the corresponding

water. The Petri dish was left in the incubator at 28.5ºC until the 5th day post fertilization.

Experimental design

The following combinations were carried out:

- Sperm from males reared in water B were mixed with oocytes from females reared in water B, and the egg-sperm mixture (fertilization) was cultured in water B.
- Sperm from males reared in water B were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water B were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water B were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water Z.

All these combinations are summarized in the following diagram:

Water (males)	Water (females)	Water (IVF)
B	B	B
B	B	Z
B	Z	B
B	Z	Z
Z	B	B
Z	B	Z
Z	Z	B
Z	Z	Z

In each of these combinations we analysed the following parameters: fertility rate at mid blastula transition (MBT) stage, hatching rate at 72 hours post fertilization (hpf) and survival and abnormalities rates at 5 days post fertilization (dpf). Results were grouped according to the water origin, B or Z, when male effect, female effect or water effect were studied.

Statistical analysis

Results were analysed using Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved. Values were considered statistically different at $P<0.05$.

Results

Fertility rate at MBT stage

Significant differences ($p<0.05$) appeared between all groups regardless of the effect analysed (see table 1). When the male effect was analysed, water B presented better rates than water Z (64.87% vs. 57.51%). However, when the female effect and the water where the in vitro fertilization took place were analysed, the worst result was obtained in water B.

Table 1: Fertility rate of zebrafish (*Danio rerio*) embryos, from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Fertility rate	Z	B
Water (males)	180/313 (57.51%)[b]	314/484 (64.87%)[a]
Water (females)	301/446 (67.48%)[a]	193/351 (54.98%)[b]
Water (IVF)	291/408 (71.32%)[a]	203/389 (52.18%)[b]

Columns with different superscripts are statistically different ($p<0.05$)

Hatching rate at 72 hours post fertilization

Embryo hatching rates were evaluated at 72 hpf (Martínez-Sales *et al*., 2015). No statistically significant differences appeared when the male or female effects were assessed. However, significant differences ($p<0.05$) appeared when the water effect, where the in vitro fertilization took place, was analysed (see table 2). Water B presented the worst result (7.83%).

Table 2: Hatching rate of zebrafish (*Danio rerio*) embryos at 72 hpf, from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Hatching rate	Z	B
Water (males)	109/166 (65.66%)	180/299 (60.20%)
Water (females)	184/287 (64.11%)	105/178 (58.98%)
Water (IVF)	232/252 (92.06%)[a]	13/166 (7.83%)[b]

Columns with different superscripts are statistically different ($p<0.05$)

Survival and abnormality rate at five days post fertilization

Embryo survival rates evaluated at 5 dpf were high in all groups, with no statistically significant differences (p<0.05) between waters, except when the female effect was studied, where significant differences appeared (p=0.0342) (see table 3). Water B obtained the worst result (89.11%) compared to water Z (94.68%).

Table 3: Survival rate of zebrafish (*Danio rerio*) embryos at 5dpf from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Survival rate	Z	B
Water (males)	164/180 (91.11%)	293/314 (93.31%)
Water (females)	285/301 (94.68%)[a]	172/193 (89.11%)[b]
Water (IVF)	273/291 (93.81%)	184/203 (90.64%)

Columns with different superscripts are statistically different (p<0.05)

In the case of abnormalities at 5 dpf, pericardial edema, curled tails and skeletal deformities (lordosis, scoliosis, and abnormal skeletal development) were the main malformations observed. No differences were observed in the abnormality rate evaluated at 5 dpf in any group (see table 4).

Table 4: Abnormality rate of zebrafish (*Danio rerio*) embryos at 5dpf, from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Abnormality rate	Z	B
Water (males)	5/164 (3.05%)	3/293 (1.02%)
Water (females)	5/285 (1.75%)	3/172 (1.74%)
Water (IVF)	6/273 (2.19%)	2/184 (1.08%)

Columns with different superscripts are statistically different ($p<0.05$)

4. Discussion

Based upon results obtained in the current work, it can be stated that the effects of pollutants on the sensitive parameters are caused by three different non-exclusive routes: affecting oogenesis in females, spermatogenesis in males and even by a direct effect of the water during the fertilization process. As these effects operate by different pathways and have also been demonstrated in mammals and in humans, especially via sperm (Toft *et al*., 2006; Vested *et al*., 2014), the value of zebrafish as a bioindicator is confirmed.

As mentioned in material and methods, in our previous work (Martínez-Sales *et al*., 2015) water B manifested the most harmful effects on reproductive parameters, which is the reason we have focused on this water in the present work.

Regarding hatching rate, the male or female direct effects were not the origin of the decrease with respect to the results obtained in the control water. However, this effect was exclusively observed in water B when it was

used in the in vitro fertilization process. Pollutants with effects on chorion seem to be the source of this decrease without an alteration of the female gametes or male gametes. Certainly, some substances found in drinking waters have decreased or even inhibited the hatching process in zebrafish, such as ibuprofen or acetaminophen (Galus *et al*., 2013). In our case it has not been determinate if the reduction in the hatching rate has been due to an effect on embryo or on chorion structure, or even both.

Survival rates at 5dpf were high in all cases studied. In many toxicological studies, a delay in the hatching process entails a decrease in the survival rate (Shi *et al*., 2008; Zhu *et al*., 2008), related with the toxic concentration used. The lower the concentration, the lower the mortality (Powers *et al*., 2010). In our work, the waters employed are drinkable, so the concentration levels (ng/l or µg/l) of emerging contaminants expected are low (Khetan and Collins, 2007; Rodil *et al*., 2012) and thus a high survival rate is predictable.

With respect to fertility rate, there was a decrease when the in vitro fertilization took place in water B, but in this parameter there was also an effect on the quality of oocytes through the oogenesis from female adults reared in water B. However, sperm fertility from male adults reared in water B was not affected. So, despite the water effect, a female effect in this parameter also seems to be the origin of this decrease. The female effect could be explained by the possible presence in water of substances like endocrine disruptors (17α-ethinylestradiol) which could decrease the number and quality of the female gametes produced (Santos *et al.*, 2007) and/or pharmaceutical substances (carbamazepine and gemfibrozil) which have been shown to reduce fecundity (total embryos produced) (Galus *et al*., 2014). Regarding the water effect, this decrease could be explained by

the presence of substances which affect the chorion structure in the micropyle, altering the sperm entry through it. Moreover, this effect can also alter the overall structure of the chorion, which could explain the decrease in the hatching rate previously described. No references to the possible substances which alter the chorion structure were found in the literature reviewed. However, it could be substances that affect directly the chorion in the same way that the bleach.

It is known that human reproduction can be affected by a wide variety of pollutants (Sharpe and Irvine, 2004; Vested *et al*., 2014) via male or female or even both, due to the continual occurrence of emerging or newly identified contaminants in the water resources (Bolong *et al*., 2009) and the lack of preventive policy legislation (Braw-Tal, 2010). For this reason, due to the complex detection and removal of these substances, in our current work and with the support to our previous works (Martínez-Sales *et al*., 2014; Martínez-Sales *et al*., 2015), we verify the use of the zebrafish as a bioindicator of emerging contaminants in drinking water with the possibility, in this case, of discriminating the effects through three different pathways: male, female or water where the in vitro fertilization took place.

Acknowledgements

The authors would like to thank Mr. Javier Rubio Rubio for his valuable technical support and Mr. Neil Macowan for improving the English of this manuscript

GENERAL DISCUSSION

The chemical detection and control of emerging organic pollutants in drinking waters has become the focus of much interest and research, due to the complexity of mixtures of these numerous substances, their interactions, their variable and random presence at low concentrations, and, in general, their difficulty in establishing a proper analytical treatment.

Nowadays, many works justified the use of bioindicators, such as fishes, as a complementary alternative to assess water quality (De Andrade Brito *et al*., 2012; Young *et al*., 2014; Barišić *et al*., 2015) due to their quick response and their low costs. However, these works are focus either in toxicological studies, where the toxic concentration is previously defined (Xiao *et al*., 2015) or in aquatic environments, such as rivers, where fishes are used to identify contaminants (Barišić *et al*., 2015; Jürgens *et al*., 2015; Khandaker *et al*., 2015). But, little information is available about the use of the zebrafish as a bioindicator of emerging contaminants in drinking waters, especially to those substances whose effects can be transmitted to the next generation via epigenetic mechanisms.

In the first step to establish the zebrafish as a bioindicator, results revealed us that the culture media (drinking water) affected the survival rate of embryos with the chorion partially degraded and that the pronase affected the survival rate of embryos, so taking into account that a high survival rates were required to ensure the continuity of our experiments, that our last objective was to detect substances in drinking water and, therefore, that the use of pronase could be introducing a distortion factor, the enzyme was discarded assuming the loss of information on the initial reproductive effects. This result contrast with several works in toxicological studies (Henn and Braunbeck, 2011; Truong *et al*., 2011) where dechorionation with pronase is proposed as the best option, mainly because in this case the

culture medium (artificial water) is always the same and because dechorionation is performed in epiboly stage.

The most relevant parameter obtained to detect emerging contaminants during the critical early stage of development (Henn, 2011) was the hatching rate. This result agrees with other authors where hatching rate in zebrafish is also considered as a relevant endpoint to assess emerging contaminants (Crane *et al*., 2010; Han *et al*., 2011). Regarding adult reproduction, fertility rate was the most sensitive parameter to detect emerging contaminants. These results also agree with other studies where fertility rate in zebrafish is an essential endpoint to study, mainly in multigenerational studies (Liu *et al*., 2014; Coimbra *et al*., 2015). Concerning later stages of development, underdeveloped specimens were also a suitable parameter to detect emerging contaminants in drinking water. However, this parameter did not show differences in the other studies. On the other hand, despite that in the second study the sex ratio was not established as a sensitive parameter, in the third study the sex ratio manifested an alteration of sex towards females. Both parameters have also been used as relevant parameter in several studies (Galus *et al*., 2013; Liu *et al*., 2014).

It is important to note that both the hatching rate and the fertility rate have always obtained the lowest rate in water B, maybe because of the presence of substances such as endocrine disruptors, pharmaceutical substances or POPs that affect the chorion composition and the micropyle in this drinking water. This could be explained because water B is the only one which comes from the surface, while the others waters from groundwater prospecting. However, in the third study water C obtained also a low fertility rate, but in this case when specimens were reared in control water

the negative effects remained. This could be clarified due to an epigenetic modification transmitted from the previous generation.

In the last step to elucidate if zebrafish was a proper bioindicator, we discriminated the effects through three different pathways. So, results indicated us that there was a decrease in the fertility rate due to an effect over the female by an alteration of the oocyte quality.

In conclusion, and taking into account the results obtained, we verify the use of the zebrafish as a bioindicator of epigenetic factors in drinking water. Furthermore, the results obtained, especially in water B and in water C, warn us of the possible presence of these substances in drinking water and in consequence of their possible negative effects over the human beings reproduction, due to the direct use of this resource.

Nevertheless, despite to the positive contribution of the zebrafish in the quality control of drinking water, its full scale use along their complete life-cycle (5 months) it would be a disadvantage in terms of management and control. So, the alternatives could be to limit the use of the zebrafish until the 5^{th} day after fertilization or until the 72 hours after fertilization, moment when take places the hatching process.

On the other hand, it should be remarked that since we have focused on phenotypic effects of the zebrafish, we are unable to describe the molecular mechanism of action behind of our results. For that reason and taking into account that the waters with the most harmful effects on the sensitive parameters have been limited in the present thesis, future works could be focused in these waters and in clarifying the modified genetic pathways through genetic and molecular studies.

Finally, future works could be focused in performing this study in mammals such as rodents in an attempt to make the data more applicable to humans.

CONCLUSIONS

The conclusions arisen from this work are the following:

1st. Chorion of zebrafish embryos must be kept intact when they are used to detect substances in different drinking waters.

2nd. From all the developmental and reproductive parameters studied during the full life-cycle of zebrafish, the hatching, fertility and underdeveloped rates appear to be the most sensitive parameters to detect environmental pollutants in drinking water.

3rd. Effects are cumulative in all parameters from one generation to the next, but only in water C effects are non-reversible when the fertility rate is analysed. A transgenerational alteration in the germline via epigenetic mechanism from the previous generation is proposed as the most plausible explanation to this effect.

4th. Despite not being classified as a sensitive parameter in the study II, sex ratio was skewed towards females in the study III, although effects were reversible.

5th. The fertility decline is produced via female and via water, while the decline of the hatching rate via water.

According to the main aim of the thesis and taking into account the above statements, it can be concluded that zebrafish is a suitable bioindicator to detect epigenetic substances in drinking water.

REFERENCES

Andersen AN, Erb K. 2006. Register data on assisted reproductive technology (ART) in Europe. Including a detailed description of ART in Denmark. *International Journal of Andrology*, 29: 12–16.

Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *Institute for Laboratory Animal Research*, 45: 469–483.

Ansari BA, Sharma DK. 2009. Toxic Effect of Synthetic Pyrethroid Deltamethrin and Neem Based Formulation Achook on Zebrafish, Danio rerio (Cyprinidae). *Trends in Biosciences*, 2(2): 18–20.

Anumol T, Merel S, Clarke BO, Snyder SA. 2013. Ultra high performance liquid chromatography tandem mass spectrometry for rapid analysis of trace organic contaminants in water. *Chemistry Central Journal*, 7: 104.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308: 1466–1469.

Anway MD, Skinner MK. 2006. Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology*, 147: S43-49.

Ayscough NJ, Fawell J, Franklin G. 2000. Review of human pharmaceuticals in the environment. *Environment Agency*.

Baccarelli A, Bollati V. 2009. Epigenetics and environmental chemicals. *Current Opinion in Pediatrics*, 21(2): 243–251.

Barišić J, Dragun Z, Ramani S, Marijić VF, Krasnići N, Čož-Rakovac R, Jordanova M. 2015. Evaluation of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis Karaman*) as an indicator of river pollution. *Ecotoxicology and Environmental Safety*, 118: 158–166.

Baroiller JF, Guiguen Y, Fostier A. 1999. Endocrine and environmental aspects of sex differentiation in fish. *Cellular and Molecular Life Sciences,* 55: 910–931.

Baumann L, Holbech H, Keiter S, Kinnberg KL, Knörr S, Nagel T, Braunbeck T. 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. *Aquatic Toxicology,* 128-129: 34–42.

Bendz D, Paxeus NA, Ginn TR, Loge FJ. 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. Journal of Hazardous Materials, 122: 195–204.

Benner J, Helbling DE, Kohler HPE, Wittebol J, Kaiser E, Prasse C, Boon N. 2013. Is biological treatment a viable alternative for micropollutant removal in drinking water treatment processes? *Water research*, 47(16): 5955–5976.

Benotti MJ, Trenholm RA, Vanderford BJ, Holady JC, Stanford BD, Snyder SA. 2009. Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environmental Science and Technology*, 43: 597–603.

Bolong N, Ismail AF, Salim MR, Matsuura T. 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. *Desalination*, 239(1): 229–246.

Bonsignorio D, Perego L, Del Giacco L, Cotelli F. 1996. Structure and macromolecular composition of the zebrafish egg chorion. *Zygote*, 4: 101–108.

Botella B, Crespo J, Rivas A, Cerrillo I, Olea-Serrano MF, Olea N. 2004. Exposure of women to organochlorine pesticides in Southern Spain. *Environmental Research*, 96: 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). *Aquatic Toxicology*, 90: 29–36.

Boyko A, Kathiria P, Zemp FJ, Yao Y, Pogribny I, Kovalchuk I. 2007. Transgenerational changes in the genome stability and methylation in pathogen-infected plants (virus-induced plant genome instability). *Nucleic Acids Research*, 35: 1714–1725.

Brand M, Granato M, Nüslein-Volhard C. 2002. Keeping and raising zebrafish. In: Nüslein-Volhard C, Dahm R, editors. Zebrafish: A Practical Approach, Oxford University Press, Oxford, England; pp. 16.

Braw-Tal R. 2010. Endocrine disruptors and timing of human exposure. *Pediatric Endocrinology Reviews: PER*, 8(1): 41–46.

Breder CM, Rosen DE. 1966. Modes of reproduction in fishes. New York, Natural History Press.

Bresch H, Beck H, Ehlermann D, Schlaszus H, Urbanek M. 1990. A long-term toxicity test comprising reproduction and growth of zebrafish with 4-chloroaniline. *Archives of Environmental Contamination and Toxicology*, 19: 419–427.

Brion F, Tyler CR, Palazzi X, Laillet B, Porcher JM, Garric J, Flammarion P. 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*). *Aquatic Toxicology*, 68: 193–217.

Bromer JG, Wu J, Zhou Y, Taylor HS. 2009. Hypermethylation of homeobox A10 by in utero diethylstilbestrol exposure: an epigenetic mechanism for

altered developmental programming. *Endocrinology*, 150(7): 3376–3382.

Brossa L, Marcé RM, Borrull F, Pocurull E. 2005. Occurrence of twenty-six endocrine-disrupting compounds in environmental water samples from Catalonia, Spain. *Environmental Toxicology and Chemistry*, 24(2): 261–267.

Canonica S, Meunier L, Von Gunten U. 2008. Phototransformation of selected pharmaceuticals during UV treatment of drinking water. *Water Research*, 42: 121–128.

Cardona-Costa J, García-Ximénez F. 2007. Vitrification of zebrafish embryo blastomeres in microvolumes. *CryoLetters*, 28(4): 303–309.

Carmona E, Andreu V, Picó Y. 2014. Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: From waste to drinking water. *Science of The Total Environment*, 484: 53–63.

Carreño J, Rivas A, Granada A, Lopez-Espinosa MJ, Mariscal M, Olea N, Olea-Serrano F. 2007. Exposure of young men to organochlorine pesticides in Southern Spain. *Environmental Research*, 103: 55–61.

Cheng J, Flahaut E, Cheng SH. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environmental Toxicology and Chemistry*, 26(4): 708–716.

Coimbra AM, Peixoto MJ, Coelho I, Lacerda R, Carvalho AP, Gesto M, Santos MM. 2015. Chronic effects of clofibric acid in zebrafish (*Danio rerio*): A multigenerational study. *Aquatic Toxicology*, 160: 76–86.

Cooney CA, Dave AA, Wolff GL. 2002. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. Journal of Nutrition, 132 (8): 2393S–2400S.

Cooney CM. 2009. Study detects trace levels of pharmaceuticals in US drinking water. *Environmental Science and Technology*, 43(3): 551–551.

Corrales J, Fang X, Thornton C, Mei W, Barbazuk WB, Duke M, Willett KL. 2014. Effects on specific promoter DNA methylation in zebrafish embryos and larvae following benzo [a] pyrene exposure. Comparative Biochemistry and Physiology Part C. *Toxicology & Pharmacology*, 163: 37–46.

Crane M, Gross M, Matthiessen P, Ankley GT, Axford S, Bjerregaard P, Wheeler J. 2010. Multi-criteria decision analysis of test endpoints for detecting the effects of endocrine active substances in fish full life cycle tests. *Integrated Environmental Assessment and Management*, 6(3): 378–389.

Creton R. 2004. The calcium pump of the endoplasmic reticulum plays a role in midline signaling during early zebrafish development. *Developmental Brain Research*, 151: 33–41.

Csaba G, Inczefi-Gonda A. 1998. Transgenerational effect of a single neonatal benzpyrene treatment on the glucocorticoid receptor of the rat thymus. *Human and Experimental Toxicology*, 17: 88–92.

Dahm R. 2002. Atlas of embryonic stages of development in the zebrafish. In: Nüslein-Volhard C, Dahm R, editors. Zebrafish: A Practical Approach, Oxford University Press, Oxford, England; pp. 222.

Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB. 2014. Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry*, 33(1): 11–17.

Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect*, 107: 907–938.

David A, Pacharatna K. 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology*, 27(3): 390–395.

De Andrade Brito I, Freire CA, Yamamoto FY, de Assis HCS, Souza-Bastos LR, Cestari MM, de Oliveira Ribeiro CA. 2012. Monitoring water quality in reservoirs for human supply through multi-biomarker evaluation in tropical fish. *Journal of Environmental Monitoring*, 14(2): 615–625.

Deblonde T, Cossu-Leguille C, Hartemann P. 2011. Emerging pollutants in wastewater: a review of the literature. International Journal of Hygiene and Environmental Health, 214(6): 442–448.

De Coster S, van Larebeke N. 2012. Endocrine-disrupting chemicals: associated disorders and mechanisms of action. *Journal of environmental and public health*, 2012: 2012.

Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. 2006. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect*, 114: 567–572.

Duan Z, Zhu Lin, Zhu L, Yao K, Zhu X. 2008. Individual and joint toxic effects of pentachlorophenol and bisphenol A on the development of zebrafish (*Danio rerio*) embryo. *Ecotoxicology and Environmental Safety*, 71: 774–780.

Ericson I, Nadal M, Van Bavel B, Lindström G, Domingo JL. 2008. Levels of perfluorochemicals in water samples from Catalonia, Spain: is drinking water a significant contribution to human exposure? *Environmental Science and Pollution Research*, 15: 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. *Environmental Science & Technology*, 46: 1708–1715.

Fawell J, Ong CN. 2012. Emerging contaminants and the implications for drinking water. *International Journal of Water Resources Development*, 28(2): 247–263.

Fenske M, Maack G, Ensenbach U, Segner H. 1999. Identification of estrogens-sensitive developmental stages of zebrafish, *Danio rerio*. *Proceedings of the Ninth Annual Meeting of Society of Environmental Toxicology and Chemistry*, 25:29.

Ficociello B, Sturchio E, Minoia C, Casorri L, Imbriani P, Signorini S. 2010. Epigenetics and environmental exposure to xenobiotics. *Giornale italiano di medicina del lavoro*, 32(1): 13–22.

Fonnum F, Mariussen E. 2009. Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants. *Journal of Neurochemistry*, 111: 1327–1347.

Galus M, Kirischian N, Higgins S, Purdy J, Chow J, Rangaranjan S, Wilson JY. 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquatic Toxicology*, 132: 200–211.

Galus M, Rangarajan S, Lai A, Shaya L, Balshine S, Wilson JY. 2014. Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring. *Aquatic Toxicology,* 151: 124–134.

Gómez MJ, Martínez Bueno MJ, Lacorte S, Fernández-Alba AR, Agüera A. 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere*, 66 (6): 993–1002.

Gregoraszczuk E, Ptak A. 2013. Endocrine-Disrupting Chemicals: Some Actions of POPs on Female Reproduction. *International Journal of Endocrinology*, 2013: 2013.

Gros M, Petrović M, Ginebreda A, Barceló D. 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. *Environment International*, 36(1): 15–26.

Guerrero-Bosagna C, Skinner MK. 2014. Environmentally induced epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genetics & Development*, 26: 79–88.

Hagedorn M, Kleinhans FW, Wildt DE, Rall WF. 1997. Chill sensitivity and cryoprotectant permeability of dechorionated zebrafish embryos, Brachydanio rerio. *Cryobiology*, 34: 251–263.

Hallare A, Pagulayan R, Lacdan N, Köhler H, Triebskorn R. 2005. Assessing water quality in a tropical lake using biomarkers in zebrafish embryos: developmental toxicity and stress protein responses. *Environmental Monitoring and Assessment*, 104: 171–187.

Han Z, Jiao S, Shan Z, Zhang X. 2011. Effects of β-endosulfan on the growth and reproduction of zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry*, 30 (11): 2525–2531.

Hart NH, Donovan M. 1983. Fine Structure of the Chorion and Site of Sperm Entry in the Egg of *Brachydanio. Journal of Experimental Zoology*, 227: 277–296.

Harvey B, Kelly RN, Ashwood-Smith MJ. 1983. Permeability of intact and dechorionated zebra fish embryos to glycerol and dimethyl sulfoxide. *Cryobiology*, 20: 432–439.

He J, Yang D, Wang C, Liu W, Liao J, Xu T, Bai C, Chen J, Lin K, Huang C. 2011. Chronic zebrafish low dose decabrominated diphenyl ether (BDE-209) exposure affected parental gonad development and locomotion in F1 offspring. *Ecotoxicology,* 20: 1813–1822.

Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters,* 131: 5–17.

Heindel JJ, McAllister KA, Worth LJ, Tyson FL. 2006. Environmental epigenomics, imprinting and disease susceptibility. *Epigenetics*, 1(1): 2-7.

Heindel JJ. 2006. Role of exposure to environmental chemicals in the developmental basis of reproductive disease and dysfunction. *Seminars in Reproductive Medicine*, 24: 168–177.

Henn K. 2011. Limits of the embryo toxicity test with *Danio rerio* as an alternative to the acute fish toxicity test. Ph Thesis. University of Heidelberg, Germany.

Henn K, Braunbeck T. 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*).

Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 153(1): 91–98.

Hill R, Janz D. 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*). I. Effects on sex ratio and breeding success. *Aquatic Toxicology,* 63: 417–429.

Hill AJ, Teraoka H, Heideman W, Peterson RE. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*, 86(1): 6–19.

Holmstrup M, BindesbØl AM, Oostingh GJ, Duschl A, Scheil V, Köhler HR, Loureiro S, Soares AM, Ferreira AL, Kienle C. 2010. Interactions between effects of environmental chemicals and natural stressors: a review. *Science of the Total Environment,* 408: 3746–3762.

Houtman CJ. 2010. Emerging contaminants in surface waters and their relevance for the production of drinking water in Europe. *Journal of Integrative Environmental Sciences*, 7(4): 271–295.

Hsioa CD, Tsai HJ. 2003. Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo. *Developmental Biology*, 262: 313–323.

Huang Y, Wang XL, Zhang JW, Wu KS. 2015. Impact of Endocrine-Disrupting Chemicals on Reproductive Function in Zebrafish *(Danio rerio*). *Reproduction in Domestic Animals*, 50(1): 1–6.

Ikehata K, Gamal El-Din M, Snyder SA. 2008. Ozonation and advanced oxidation treatment of emerging organic pollutants in water and wastewater. *Ozone: Science and Engineering*, 30(1): 21–26.

Jirtle RL, Skinner MK. 2007. Environmental epigenomics and disease susceptibility. *Nature Reviews Genetics*, 8(4): 253–262.

Jobling S, Coey S, Whitmore JG, Kime DE, Van Look KJW, McAllister BG. 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction*, 67(2): 515-524.

Jurewicz J, Hanke W, Radwan M & Bonde J. 2009. Environmental factors and semen quality. *International Journal of Occupational Medicine and Environmental Health*, 22(4): 305–329.

Jürgens MD, Chaemfa C, Hughes D, Johnson AC, Jones KC. 2015. PCB and organochlorine pesticide burden in eels in the lower Thames River (UK). *Chemosphere*, 118: 103–111.

Kallenborn R, Fick R, Lindberg R, Moe M, Nielsen K.M, Tysklind K, Vasskoog T. 2008. Pharmaceutical residues in Northern European environments: consequences and perspectives. In: Kümmerer K, editor. Pharmaceuticals in the Environment. Sources Fate Effects and Risks, Berlin Heidelberg: third ed, Springer. pp. 61–74.

Kaminen-Ahola N, Ahola A, Maga M, Mallitt KA, Fahey P, Cox TC, Whitelaw E, Chong S. 2010. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet*, 6(1): e1000811.

Kazazian HH. 2004. Mobile elements: drivers of genome evolution. *Science*, 303: 1626–1632.

Keiter S, Baumann L, Färber H, Holbech H, Skutlarek D, Engwall M, Braunbeck T. 2012. Long-term effects of a binary mixture of

perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish (*Danio rerio*). *Aquatic Toxicology*, 118–119: 116–129.

Khandaker MU, Asaduzzaman K, Nawi SM, Usman AR, Amin YM, Daar E, Okhunov AA. 2015. Assessment of Radiation and Heavy Metals Risk due to the Dietary Intake of Marine Fishes (*Rastrelliger kanagurta*) from the Straits of Malacca. *PloS one*, 10(6): e0128790.

Khetan SK, Collins TJ. 2007. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. *Chemical Reviews,* 107: 2319–2364.

Kim S, Cho J, Kim IS, Vanderford B, Snyder S. 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Research*, 41: 1013–1021.

Kime DE. 1998. Endocrine disruption in fish. Springer: US.

Kimmel C, Ballard W, Kimmel S, Ullmann B, Schilling T. 1995. Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics*. 203: 253–310.

Kortenkamp A. 2007. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environmental Health Perspectives*, 115: 98–105.

Kraugerud M, Doughty RW, Lyche JL, Berg V, Tremoen NH, Alestrøm P, Aleksandersen M, Ropstad E. 2012. Natural mixtures of persistent organic pollutants (POPs) suppress ovarian follicle development, liver vitellogenin immunostaining and hepatocyte proliferation in female zebrafish (Danio rerio). *Aquatic Toxicology*, 116–117: 16–23.

Kümmerer K. 2001. Introduction: pharmaceuticals in the environment. In Pharmaceuticals in the Environment, pp. 1-8. Springer: Berlin Heidelberg.

Kümmerer K. 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. *Journal of Environmental Management*, 2354–2366.

Kuster M, Alda MJL, Hernando MD, 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). *Journal of Hydrology*, 358: 112–123.

La Vignera S, Condorelli RA, Balercia G, Vicari E & Calogero AE. 2013. Does alcohol have any effect on male reproductive function? A review of literature. *Asian Journal of Andrology*, 15(2): 221.

Lang-Mladek C, Popova O, Kiok K, Berlinger M, Rakic B, Aufsatz W, Jonak C, Hauser MT, Luschnig C. 2010. Transgenerational inheritance and resetting of stress-induced loss of epigenetic gene silencing in Arabidopsis. *Molecular Plant*, 3: 594–602.

Larsen TE, Lienert J, Josse A and Siegrist H. 2004. How to avoid pharmaceuticals in the aquatic environment. *Journal of Biotechnology*, 113: 295–304.

Larsen MG, Bilberg K, Baatrup E. 2009. Reversibility of estrogenic sex changes in zebrafish (*Danio Rerio*). *Environmental Toxicology and Chemistry*, 28: 1789–1785.

Lawrence C, Ebersole J, Kesseli R. 2007. Rapid growth and outcrossing promote female development in zebrafish (*Danio rerio*). *Environmental Biology Fish*, 81: 239–246.

Li E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics*, 3(9): 662–673.

Liew WC, Orbán L. 2014. Zebrafish sex: a complicated affair. *Briefings in Functional Genomics*, 13(2): 172–187.

Liu SY, Jin Q, Huang XH, Zhu GN. 2014. Disruption of zebrafish (*Danio rerio*) sexual development after full life-cycle exposure to environmental levels of triadimefon. *Environmental Toxicology and Pharmacology,* 37: 468–475.

Lo KH, Hui MNY, Yu RMK, Wu RSS, Cheng SH. 2011. Hypoxia Impairs Primordial Germ Cell Migration in Zebrafish (*Danio rerio*) Embryos. *Plos One*, 6(9): e 24540.

Loucks E, Ahlgren S. 2012. Assessing teratogenic changes in a zebrafish model of fetal alcohol exposure. *Journal of visualized experiments: JoVE*, 61.

Lyche JL, Nourizadeh-Lillabadi R, Almaas C, Stavik B, Berg V, Skåre JU, Ropstad E. 2010. Natural mixtures of persistent organic pollutants (POP) increase weight gain, advance puberty, and induce changes in gene expression associated with steroid hormones and obesity in female zebrafish. *Journal of Toxicology and Environmental Health, Part A*, 73(15): 1032–1057.

Lyche JL, Nourizadeh-Lillabadi R, Karlsson C, Stavik B, Berg V, Skåre JU, Alestrøm P, Ropstad E. 2011. Natural mixtures of POPs affected body

weight gain and induced transcription of genes involved in weight regulation and insulin signaling. *Aquatic Toxicology*, 102: 197–204.

Lyche JL, Grześ IM, Karlsson C, Nourizadeh-Lillabadi R, Berg V, Kristoffersen AB, Ropstad E. 2013. Parental exposure to natural mixtures of POPs reduced embryo production and altered gene transcription in zebrafish embryos. *Aquatic Toxicology*, 126: 424–434.

Ma C, Fan L, Ganassin R, Bols N &Collodi P. 2001. Production of zebrafish germ-line chimeras from embryo cell cultures (sex maturity). *Proceedings of the National Academy of Sciences,* 98(5): 2461–2466.

Mandrell D, Truong L, Jephson C, Sarker M, Moore A, Lang C, Simonich M, Tanguay R. 2012. Automated Zebrafish Chorion Removal and Single Embryo Placement: Optimizing Throughput of Zebrafish Development Toxicity Screens. *Journal of Laboratory Automation*, 17:66–74.

Marsit CJ, Eddy K and Kelsey KT. 2006. MicroRNA responses to cellular stress. *Cancer Research*, 66(22): 10843–10848.

Martínez-Sales M, García- Ximénez F & Espinós FJ. 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*, 23(03):447–452.

Martínez-Sales M, García- Ximénez F & Espinós FJ. 2015. Zebrafish as a possible bioindicator of organic pollutants with effects on reproduction in drinking waters. *Journal of Environmental Sciences*, 33: 254–260.

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

Metcalfe CD, Miao XS, Koenig BG, Struger J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry*, 22: 2881–2889.

Molinari A, Troisi S, Fallico C, Paparella A, Straface S. 2009. Broom fibre PRB for heavy metals groundwater remediation. *EGU General Assembly*, 8075.

Monosson E. 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. *Environmental Health Perspectives*, 113: 383–390.

Muñoz I, Milà-i-Canals L & Fernández-Alba AR. 2010. Life cycle assessment of water supply plans in Mediterranean Spain. *Journal of Industrial Ecology*, 14(6): 902–918.

Nagel, R. 1993. Fish and environmental chemicals—a critical evaluation of tests. *Fish ecotoxicology and ecophysiology. VCH, Weinheim*, 174–178.

Namihira M, Kohyama J, Abematsu M and Nakashima K. 2008. Epigenetic mechanisms regulating fate specification of neural stem cells. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1500): 2099–2109.

Nash JP, Kime DE, Van der Ven LT, Wester PW, Brion F, Maack G, Tyler C. R. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives*, 112(17): 1725–1733.

National Research Council. 1998. Issues in potable reuse—the viability of augmenting drinking water supplies with reclaimed water, National Academy Press, Washington, DC, p. 263.

Newbold RR, Padilla-Banks E, Jefferson WN. 2006. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*, 147: S11–17.

Nikolaou, A. 2013. Pharmaceuticals and related compounds as emerging pollutants in water: analytical aspects. *Global NEST Journal*, 15(1): 1–12.

Njiwa JRK, Müller P, Klein R. 2004. Life cycle stages and length of ZF (*Danio rerio*) exposed to DDT. *Journal of Health Science*, 50(3): 220–225.

Nourizadeh-Lillabadi R, Lyche JL, Almaas C, Stavik B, Moe SJ, Aleksandersen M, Ropstad E. 2009. Transcriptional regulation in liver and testis associated with developmental and reproductive effects in male zebrafish exposed to natural mixtures of persistent organic pollutants (POP). *Journal of Toxicology and Environmental Health, Part A*, 72(3-4): 112–130.

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

Örn S, Holbech H, Madsen TH, Norrgren L & Petersen GI. 2003. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyl testosterone. *Aquatic Toxicology*, 65: 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. *Archives of Environmental Contamination and Toxicology,* 51: 445–451.

Ouyang Y, Nkedi-Kizza P, Wu QT, Shinde D & Huang CH. 2006. Assessment of seasonal variations in surface water quality. *Water Research*, 40(20): 3800–3810.

Pal A, Gin KYH, Lin AYC & Reinhard M. 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. *Science of the Total Environment*, 408(24): 6062–6069.

Pal A, He Y, Jekel M, Reinhard M, Gin KYH. 2014. Emerging contaminants of public health significance as water quality indicator compounds in the urban water cycle. *Environment International*, 71, 46–62.

Petrović M, Gonzalez S, Barceló D. 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. *TrAC Trends in Analytical Chemistry*, 22(10): 685–696.

Petrović M, Hernando MD, Díaz-Cruz MS, Barceló D. 2005. Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. *Journal of Chromatography*, 1067 (1–2): 1–14.

Pogribny IP and Rusyn I. 2013. Environmental toxicants, epigenetics, and cancer. In Epigenetic Alterations in Oncogenesis, pp. 215–232. Springer: New York.

Pozo K, Harner T, Wania F, Muir DC, Jones KC & Barrie LA. 2006. Toward a global network for persistent organic pollutants in air: results from the GAPS study. *Environmental Science & Technology*, 40(16): 4867–4873.

Powers CM, Yen J, Linney EA, Seidler FJ, Slotkin TA. 2010. Silver exposure in developing zebrafish (*Danio rerio*): Persistent effects on larval behavior and survival. *Neurotoxicology and Teratology*, 32(3): 391–397.

Qin Z, McNee D, Gleisner H, Raab A, Kyeremeh K, Jaspars M, ... & Feldmann J. 2012. Fluorine speciation analysis using reverse phase liquid chromatography coupled off-line to continuum source molecular absorption spectrometry (CS-MAS): identification and quantification of novel fluorinated organic compounds in environmental and biological samples. *Analytical Chemistry*, 84(14): 6213–6219.

Rawson DW, Zhang T, Kalicharan D, Jongebloed WL. 2000. Field emission scanning electron microscopy and transmission electron microscopy studies of the chorion, plasma membrane and syncytial layers of the gastrula-stage embryo of the zebrafish (*Brachydanio rerio):* a consideration of the structural and functional relationships with respect to cryoprotectant penetration. *Aquaculture Research*, 31: 325–336.

Richardson SD, Ternes TA. 2011. Water analysis: emerging contaminants and current issues. *Analytical Chemistry*, 83: 4614–4648.

Richardson SD, Ternes TA. 2014. Water analysis: emerging contaminants and current issues. *Analytical Chemistry*, 86(6): 2813–2848.

Rodil R, Quintana JB, Concha-Graña E, López-Mahía P, Muniategui-Lorenzo S & Prada-Rodríguez D. 2012. Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain). *Chemosphere*, 86(10): 1040–1049.

Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. 2008. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. *Environmental Health Perspectives*, 116(11): 1547–1552.

Ryökkynen A, Käyhkö UR, Mustonen AM, Kukkonen JV and Nieminen P. 2005. Multigenerational exposure to phytosterols in the mouse. *Reproductive Toxicology*, 19(4): 535–540.

Santos MM, Micael J, Carvalho AP, Morabito R, Booy P, Massanisso P, Lamoree M, Reis-Henriques MA. 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 142: 151–155.

Santos EM, Paull GC, Van Look KJ, Workman VL, Holt WV, Van Aerle R, Tyler CR. 2007. Gonadal transcriptome responses and physiological consequences of exposure to oestrogen in breeding zebrafish (*Danio rerio*). *Aquatic Toxicology*, 83(2): 134–142.

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*. *Journal of Toxicology and Environmental Health*, 70: 768–779.

Scholz S, Mayer I. 2008. Molecular biomarkers of endocrine disruption in small model fish. *Molecular and Cellular Endocrinology*, 293(1): 57–70.

Schriks M, Heringa MB, van der Kooi MM, de Voogt P, van Wezel AP. 2010. Toxicological relevance of emerging contaminants for drinking water quality. *Water Research*, 44: 461–476.

Schwarzenbach RP, Escher BI, Fenner K, Hofsteltter TB, Johnson CA, Von Gunten U, Wehrli B. 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. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 149(2): 187–195.

Sharpe RM, Irvine DS. 2004. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? *BMJ: British Medical Journal*, 328(7437): 447–451.

Shi X, Du Y, Lam P, Wu R, Zhou B. 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicology and Applied Pharmacology*, 230: 23–32.

Simão M, Cardona-Costa J, Pérez Camps M, García-Ximénez F. 2010a. Ultraviolet Radiation Dose to be Applied in Recipient Zebrafish Embryos for Germ-line Chimaerism is Strain Dependent. *Reproduction in Domestic Animals*, 45: 1098–1103.

Simão M, Cardona-Costa J, Pérez M, García-Ximénez F. 2010b. Ultraviolet radiation and handling medium osmolarity affect chimaerism success in zebrafish. *Zygote*, 19: 67–70.

Simon O, Mottin E, Geffroy B & Hinton T. 2011. Effects of dietary uranium on reproductive endpoints—fecundity, survival, reproductive success—of the fish *Danio rerio*. *Environmental Toxicology and Chemistry*, 30(1): 220–225.

Silva E, Rajapakse N, Kortenkamp A. 2002. Something from "nothing"—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environmental Science & Technology*, 36: 1751–1756.

Skakkebæk NE, Jørgensen N, Main KM, Meyts ERD, Leffers H, Andersson AM, Toppari J. 2006. Is human fecundity declining? *International Journal of Andrology*, 29(1): 2–11.

Skinner MK, Manikkam M, Guerrero- Bosagna C. 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology & Metabolism*, 21: 214–222.

Skinner MK. 2011. Role of epigenetics in developmental biology and transgenerational inheritance. *Birth Defects Research Part C: Embryo Today: Reviews*, 93(1): 51–55.

Skinner MK, Manikkam M and Guerrero-Bosagna C. 2011. Epigenetic transgenerational actions of endocrine disruptors. *Reproductive Toxicology*, 31(3): 337–343.

Soares J, Coimbra AM, Reis-Henriques MA, Monteiro NM, Vieira MN, Oliveira JMA, Guedes-Dias P, Fontaínhas-Fernandes A, Silva Parra S, Carvalho AP. 2009. Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol. *Aquatic Toxicology,* 95: 330–338.

Soeteman-Hernández LG, Bos PM, Talhout R. 2013. Tobacco Smoke–Related Health Effects Induced by 1, 3-Butadiene and Strategies for Risk Reduction. *Toxicological Sciences*, 136(2): 566–580.

Sørmo EG, Larsen HJ, Johansen GM, Skaare JU, Jenssen BM. 2009. Immunotoxicity of polychlorinated biphenyls (PCB) in free-ranging gray seal pups with special emphasis on dioxin-like congeners. *Journal of Toxicology and Environmental Health Part A*, 72: 266–276.

Stackelberg P, Furlong E, Meyer M, Zaugg S, Henderson A, Reissman D. 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *Science of the Total Environment*, 329: 99–113.

Stearns V, Zhou Q and Davidson NE. 2007. Epigenetic regulation as a new target for breast cancer therapy. *Cancer Investigation*, 25(8): 659–665.

Suter MJF, Giger W. 2001. Trace determinants of emerging water pollutants: endocrine disruptors, pharmaceuticals, and speciality chemicals. *Chimia (Aarau)*, 54: 13–16.

Szyf M. 2007. The dynamic epigenome and its implications in toxicology. *Toxicological Sciences*, 100(1): 7–23.

Talsness CE. 2008. Overview of toxicological aspects of polybrominated diphenyl ethers: a flame-retardant additive in several consumer products. *Environmental Research*, 108: 158–167.

Toft G, Rignell-Hydbom A, Tyrkiel E, Shvets M, Giwercman A, Lindh CH, et al. 2006. Semen quality and exposure to persistent organochlorine pollutants. *Epidemiology*, 17(4): 450–458.

Tong SK, Hsu HJ, Chung BC. 2010. Zebrafish monosex population reveals female dominance in sex determination and earliest events of gonad differentiation. *Developmental Biology*, 344 (2): 849–856.

Truong L, Harper S, Tanguay R. 2011. Evaluation of embryotoxicity using the zebrafish model. *Methods in Molecular Biology*, 691: 271–279.

Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Montgomery B, Churchwell MI, Pogribny IP. 2006. Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. *Carcinogenesis*, 27(8): 1713–1720.

Turner BM. 1998. Histone acetylation as an epigenetic determinant of long-term transcriptional competence. *Cellular and Molecular Life Sciences,* 54: 21–31.

Ullah S, Alsberg T, Berger U. 2011. Simultaneous determination of perfluoroalkylphosphonates, carboxylates and sulfonates in drinking water. *Journal of Chromatography*, 1218: 6388–6395.

Vandegehuchte MB, Lemiere F, Vanhaecke L, Vanden Berghe W, Janssen CR. 2009. Direct and transgenerational impact on Daphnia magna of chemicals with a known effect on DNA methylation. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 151: 278–285.

Van der Ven L, Wester PW, Vos JG. 2003. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry*, 22(4): 908–913.

Vaughan M, Van Egmond R, Tyler CR. 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.

Vested A, Giwercman A, Bonde JP, Toft G. 2014. Persistent organic pollutants and male reproductive health. *Asian Journal of Andrology* 16(1): 71.

Voelker D, Vess C, Tillmann M, Nagel R, Otto GW, Geisler R, Schirmer K, Scholz S. 2007. Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. *Aquatic Toxicology*, 81: 355–364.

Von der Ohe PC, Dulio V, Slobodnik J, De Deckere E, Kühne R, Ebert RU, Brack W. 2011. A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific pollutants under the European Water Framework Directive. *Science of the Total Environment*, 409(11): 2064–2077.

Walker DM, Gore AC. 2011. Transgenerational neuroendocrine disruption of reproduction. *Nature Reviews Endocrinology*, 7: 197–207.

Wan LB and Bartolomei MS. 2008. Regulation of imprinting in clusters: noncoding RNAs versus insulators. *Advances in Genetics*, 61:207–223.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. 2004. Epigenetic programming by maternal behavior. *Nature Neuroscience*, 7: 847–854.

Weiting C, Wei G. 2013. Gonad differentiation and puberty onset in the zebrafish: evidence for the dependence of puberty onset on body

growth but not age in females. *Molecular Reproduction and Development*, 80 (5): 384–392.

Westerfield M. 1995. The zebrafish book. University of Oregon, Eugene, OR.

Westerfield M. 2000. The zebrafish book: A guide for the laboratory use of zebrafish (*Brachydanio rerio*). 4th Ed. University of Oregon Press, Eugene, OR, USA.

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. *Environmental Science & Technology*, 39: 6649–6663.

Whitelaw NC and Whitelaw E. 2008. Transgenerational epigenetic inheritance in health and disease. *Current Opinion in Genetics & Development*, 18(3): 273–279.

Wilhelm M, Bergmann S, Dieter HH. 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. *International Journal of Hygiene and Environmental Health*, 213: 224–232.

Wolff GL, Kodell RL, Moore SR, Cooney CA. 1998. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *The FASEB Journal*, 12: 949–957.

Wolffe AP, Guschin D. 2000. Review: chromatin structural features and targets that regulate transcription. *Journal of Structural Biology*, 129(2–3): 102–122.

Wu L, Jiang Y, Zhang L, Chen L, Zhang H. 2014. Toxicity of urban highway runoff in Shanghai to Zebrafish (*Danio rerio*) embryos and luminous bacteria (Vibrio qinghaiensis. Q67). *Environmental Science and Pollution Research*, 21(4): 2663–2676.

Xiao G, Feng M, Cheng Z, Zhao M, Mao J, Mirowski L. 2015. Water quality monitoring using abnormal tail-beat frequency of crucian carp. *Ecotoxicology and Environmental Safety*, 111: 185–191.

Yamaha E, Saito T, Goto–Kazeto R, Arai K. 2007. Developmental biotechnology for aquaculture, with special reference to surrogate production in teleost fishes. *Journal of Sea Research*, 58: 8–22.

Ying GG, Kookana RS, Koplin DW. 2009. Occurrence and removal of pharmaceutically active compounds in sewage treatment plants with different technologies. *Journal of Environmental Monitoring*, 11: 1498–1505.

Yoon C, Kawakami K, Hopkins N. 1997. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cells-stage embryos and is expressed in the primordial germ cells. *Development*, 124: 3157–3165.

Yoshizaki G, Takeuchi Y, Kobayashi T, Ihara S, Takeuchi T. 2002. Primordial germ cells: the blueprint for a piscine life. *Fish Physiology and Biochemistry*, 26(1): 3–12.

Young SS, Yang HN, Huang DJ, Liu SM, Huang YH, Chiang CT, Liu JW. 2014. Using benthic macroinvertebrate and fish communities as

bioindicators of the Tanshui River basin around the greater Taipei area—multivariate analysis of spatial variation related to levels of water pollution. *International Journal of Environmental Research and Public Health*, 11(7): 7116–7143.

Zhang C, Willett C, Fremgen T. 2003. Zebrafish: An Animal Model for Toxicological Studies. *Current Protocols in Toxicology*,1:7.

Zhu X, Zhu L, Duan Z, Qi R, Li Y, Lang Y. 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering*, 43: 278–284.