Universidad del País Vasco
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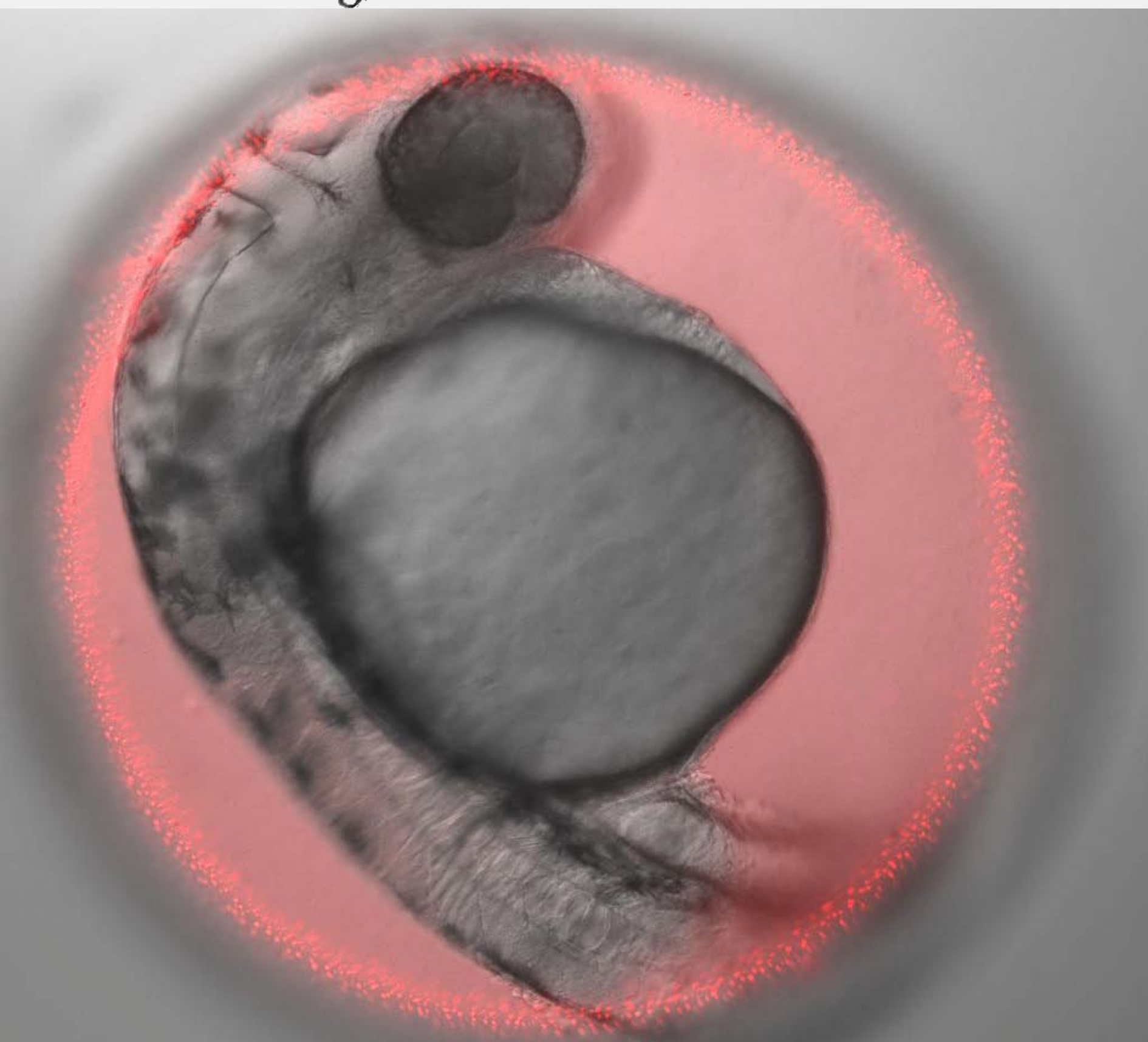
Evaluation of the toxicity of Metal and metal bearing nanoparticles in aquatic organisms using Zebrafish as model



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International Ph.D. Thesis 2016

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Evaluation of the toxicity of metal and metal bearing nanoparticles in aquatic organisms using zebrafish as model

International Ph. D. Thesis submitted by

José María Lacave Lena

for the degree of

Philosophiae Doctor

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Uno no escoge el país donde nace;
pero ama el país donde ha nacido.

Uno no escoge el tiempo para venir al mundo;
pero debe dejar huella de su tiempo.
(Gioconda Belli)

À toujours Sego

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I. INTRODUCTION

ABBREVIATIONS

AChE, Acetyl cholinesterase

CAT, Catalase

CPI, Consumer products inventory

EC_{50}, Effective concentration to 50% of the population

FET, Fish embryo toxicity

FSNPs, Fluorescent SiO_2 NPs

GO, Gene ontology

GSH, Reduced glutathione

hpf, Hours post fertilization

hph, Hours post hatch

LC_{50}, Lethal concentration to 50% of the population

LMS, Lysosomal membrane stability

MN, Micronuclei

NMs, Nanomaterials

NPs, Nanoparticles

QDs, Quantum dots

RAPD-PCR, Random amplified polymorphic DNA-PCR

ROS, Reactive oxygen species

SOD, Superoxide dismutase

STP, Sewage treatment plant

TEM, Transmission electron microscopy

UPP, Ubiquitin-proteasome pathway

1 NANOTECHNOLOGY

Nanotechnology is defined by the United States National Nanotechnology Initiative as "*the understanding and control of matter at dimensions between approximately 1 to 100 nanometers, where unique phenomena enable novel applications not feasible when working with bulk materials or even with single atoms or molecules*" (http://www.nanotechproject.org/). Nanotechnology has provided a basis for innovation in a wide range of fields and has resulted in an exponential increase in both the deployment of nanotechnologies in products along with the development of novel nanomaterials (NMs) (Gottschalk and Nowack, 2011; Etheridge et al., 2013). The European Commission has defined NMs as "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%"* (EU COM, 2011). Among NMs, nanoparticles (NPs) are defined as a material with at least two dimensions between 1 and 100 nm (Klaine et al., 2008).

1.1 Engineered NPs

Though NMs from volcanoes, forest fires, products of bacteria, etc have occurred naturally in the environment since the beginning of the life (Oberdörster et al., 2005), in the last few decades the production and use of engineered NPs has spiked (Maurer-Jones et al., 2013). Engineered NPs include metals, metal oxides and alloys, carbon based materials such as fullerenes, nanotubes and fibres, silicates, quantum dots (QDs) and polymer composites (Tiede et al., 2009). Their global production has increased exponentially, being estimated thousands of tons in 2004 and projected to increase over a half million of tons by 2020 (Maurer-Jones et al., 2013). Engineered NPs are present a high variety of products such as personal care products, clothing, cosmetics, sporting goods and electronics, among others (Fig 1, Vance et al., 2015). The number of potential applications of engineered NPs, especially metal bearing NPs (including

metal oxides NPs and QDs), is growing rapidly because of their unique electronic, optical, magnetic and catalytic properties (Klaine et al., 2008; Zhou et al., 2009).

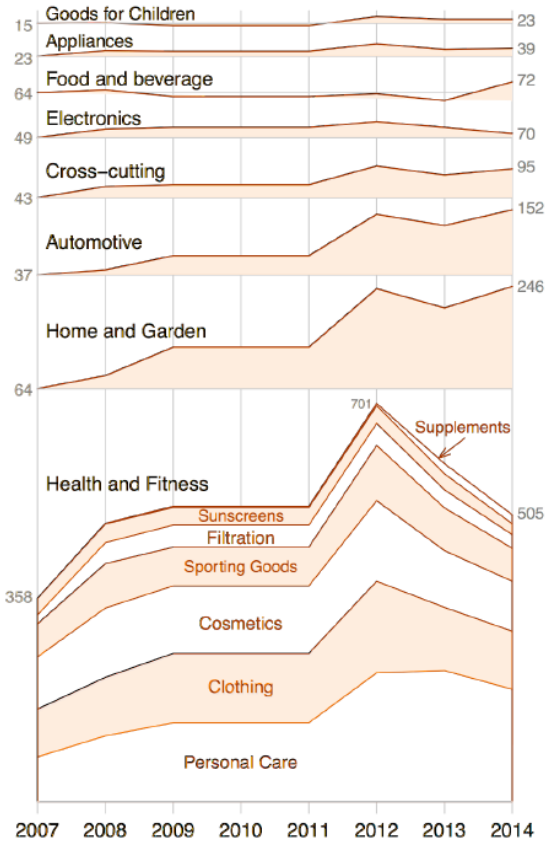

Figure 1.- Number of available products over time in different categories presenting engineered NPs (Vance et al., 2015).

The differential properties of NMs in comparison with the bulk form of the same substances arise from two primary sources. On the one hand, the small *size* of the NP itself implies that the surface atoms dominate the chemistry and physics of the NP. On the other hand, the compression of NP electrons into unusually small spaces results in electron cloud overlaps and altered orbitals, thereby changing the wavelength of the light emitted by the NP and the chemical reactivity of the nano-cluster (Fako and Furgeson, 2009). Along with the size, the *shape* can influence the optical, mechanical and electrical properties of NPs, resulting in significant changes in the specific surface area and affecting particle solubility (Misra et al., 2013).

Among the variety of products containing engineered NPs registered in the Nanotechnology Consumer Products Inventory (CPI), the majority (37%) correspond to those containing metal NMs, followed by those containing carbonaceous NMs and

silicon-based materials (Fig 2, Vance et al., 2015). The NMs grouped in the metal category include different types of metal (e.g. silver and gold) and metal-bearing NPs (e.g. zinc oxide). Other metal-containing NMs of increasing use are cadmium-based QDs (e.g. cadmium sulphide).

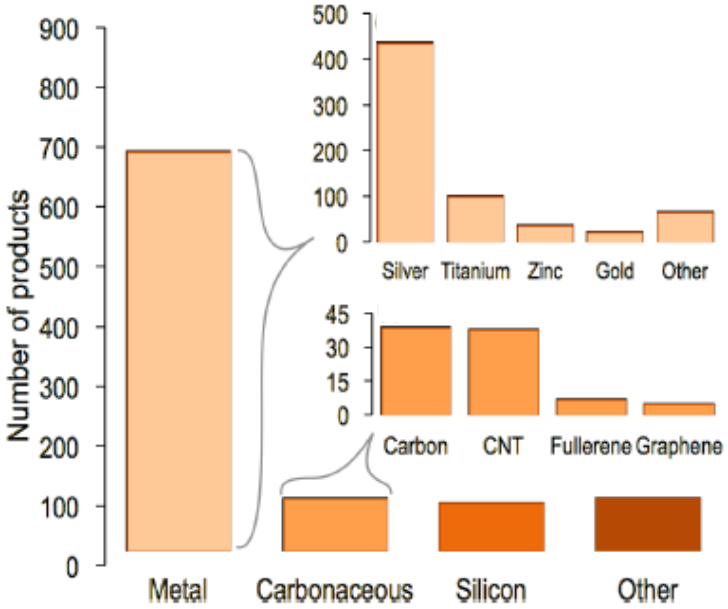

Figure 2.- Claimed composition of NMs listed in the CPI, grouped into four major categories: metal (including metals and metal oxides), carbonaceous NMs (carbon black, carbon nanotubes, fullerenes, graphene), silicon-based NMs, and other (organics, polymers, ceramics, etc.) (Modified from Vance et al., 2015).

Silver NPs (Ag NPs) have gained high commercial and scientific interest because of their unique optical, catalytical and disinfectant properties (Haase et al., 2011; Kvitek et al., 2011). Ag NPs show efficient antimicrobial activity (Franci et al., 2015), because of the antibacterial properties of silver (Zawadzka et al., 2014), along with the large surface area of the NPs, which provides better contact with microorganisms. NPs get attached to the cell membrane and also penetrate inside the bacteria (Rai et al., 2009), inhibiting the growth and multiplication of the bacteria, including multiresistan bacteria such as methicillin-resistant *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Guzmán et al., 2009). This makes this material very suitable as coating in medical devices (Rai et al., 2009), for water treatment (Gong et al., 2007), in burn treatments, socks, detergents and soaps, washing machines, wet wipes, bedding and other industrial textiles (Buzea et al., 2007).

Gold NPs (Au NPs) have been one of the most reported nano-system in the literature (Bohnsack et al., 2012). The use of Au NPs is very extensive, being the medical field one of their most important applications (Azzazy and Mansour, 2009), providing non-toxic carriers for drug and gene delivery applications (Ghosh et al., 2008). Moreover, Au NPs are widely used in consumer products, including cosmetics, sunscreens, food packaging, beverages, toothpaste, automobiles, and air handling units (Sung et al., 2011; Borase et al., 2014).

Zinc oxide NPs (ZnO NPs) have a wurtzite crystal structure that provides to these NPs specific piezoelectric and pyroelectric properties, which make them particularly useful for applications in optoelectronics, sensors, transducers and biomedical sciences (Wang, 2004). Furthermore, due to their optical properties and particle properties, such as cristallinity and morphology, ZnO NPs are widely applied in ceramics, pigments, cosmetics, sunscreens, etc (Kuo et al., 2010).

Silica NPs (SiO_2 NPs) are being used for biomedical applications such as drug delivery and imaging and diagnostic agents (Nelson et al., 2010). These biomedical applications have increased due to the recent improvements in regulating the geometry, porosity, and surface characteristics of SiO_2 NPs (Yu et al., 2012). Moreover, optical absorption and emission properties, concentration of silanol groups, specific surface area and density are some of the key parameters that govern the utilization of SiO_2 NPs (Rahmna et al., 2009).

Cadmium-containing NPs are commonly synthesized as quantum dots (QDs), which are fluorescent semiconductor crystals with a size ranging from 2 to 100 nm used in biomedical research, microelectronics and solar panel technology. The materials are usually composed of a semiconductor core (i.e. CdSe, CdTe) and can be encapsulated by a shell (i.e. ZnS) to enhance the electronic and optical properties (King-Heiden et al., 2009; Louis et al., 2010). For biomedical applications, QDs are often coated with organic molecules, such as thioglycolic acid, to increase the dispersion in water and to direct them to biological targets (King-Heiden et al., 2009; Louis et al., 2010).

1.2 NPs in the aquatic environment

The spread use of manufactured NPs in different consumer products (e.g., sunscreens and cosmetics) has raised concerns about their release into the environment and the potential effects on ecosystem health that NMs could provoke (Klaine et al., 2008). Despite there are not direct measurements of NP concentration in the environment, because of the absence of analytical methods able to quantify trace concentration of NPs (von der Kammer et al., 2012), several authors have developed theoretical models in order to estimate the environmental NP concentrations (Table 1). These mathematical models are based on production and emission data, on the fate and on behavior of NPs into the environment. Most of the studies have reported metal concentrations values in the range of ng/L for different NPs.

For silver, several studies have predicted metal concentration in different environmental compartments, being in effluents of sewage treatment plants (STP) where in general the highest values have been estimated, up to 18 µg/L in STP effluents of Rhine river (Blaser et al., 2008). Only a higher value has been estimated in major Taiwanese rivers, with a median predicted environmental concentration value of 40 µg/L (Chio et al., 2012). On the contrary, the lowest predicted value has been reported in surface water of Europe, with an estimated NP concentration of 0.002 ng/L (Dumont et al., 2015). Although, most of the studies have been focused on Ag NPs, other metal bearing NPs have also been analyzed. Tiede et al. (2009) calculated a concentration of 140 ng/L for Au NPs and 0.7 ng/L for SiO_2 NPs. In the case of ZnO NPs, the concentration value of 76 µg/L predicted by Tiede et al. (2009) is much higher than the concentration estimated by other authors. In other studies, the highest reported concentration was 2300 ng/L in STP effluents (Sun et al., 2014) and the lowest values have been estimated as 1 ng/L in surface water (Gottschalk et al., 2009). Differences in the concentration values estimated between STP effluents and surface water could be due to the material-flow model of engineered NPs, which begins with the incorporation into products, followed by the release of engineered NPs from products during their use and their transport to STP. After the landfill and recycling processes, these wastes are finally transferred to different aquatic compartments (Sun et al.,

2014). Therefore, the engineered NP concentration at the end of the process may be lower than in the STP effluents.

Table 1.- Predicted concentrations in water bodies for nanoparticles used in this work, according to different models. There are not available data for CdS NPs. Concentrations are given in ng/L.

Matrix	Area	Predicted concentration	Reference
Ag			
STP effluent	Rhine river	2000-18000	Blaser et al. (2008)
River water		40-320	
Interstitial sediment water		9-70	
Water	Switzerland	30-80	Mueller and Nowack (2008)
Surface water	Europe/USA/Switzerland	0.764/0.116/0.717	Gottschalk et al. (2009)
STP effluent		42.5/21/38.7	
Surface water	Switzerland	0.0033	Gottschalk et al. (2010)
STP effluent		38.7	
Water	ND	10	Tiede et al. (2009)
Rivers	Taiwan	40000	Chio et al. (2012)
STP effluent	USA	19-89	Hendren et al. (2013)
Water	Europe	10	Gottschalk et al. (2013)
Rivers	Netherlands	5-20	Markus et al. (2013)
Surface water	Europe/Switzerland	0.66/0.45	Sun et al. (2014)
STP effluent		0.17/0.32	
Surface water	Europe	0.002	Dumont et al. (2015)
Surface water	Denmark	0.015	Gottschalk (2015)
STP effluent		0.5	
WMS	Germany	0.217-0.708	Wigger et al. (2015)
Au			
Water	ND	140	Tiede et al. (2009)
ZnO			
Surface water	Europe/USA/Switzerland	10/1/13	Gottschalk et al. (2009)
STP effluent		432/300/441	
Water	ND	76000	Tiede et al. (2009)
STP effluent	Singapore	1780	Majedi et al. (2012)
Water	Europe	4770	Gottschalk et al. (2013)
Surface water	Europe/Switzerland	90/120	Sun et al. (2014)
STP effluent		2300/5300	
Surface water	Europe	1.5	Dumont et al. (2015)
SiO_2			
Water	ND	0.7	Tiede et al. (2009)

STP: sewage treatment plants; WMS: Waste management systems; ND: no data.

NPs may enter in the aquatic environment either directly (through aerial deposition) or indirectly via effluents, dumping and run-off (Baker et al., 2014). Once in the aquatic systems, physico-chemical properties of NPs may be altered depending on the characteristics of the receiving media, such as pH or the ionic strength. NPs can undergo a variety of processes as sorption, oxidation, dissolution, etc, which determine their fate in the abiotic environment and their interaction with biota. These processes are of relevance to both the abiotic and biotic environment. It is necessary to understand how the interaction of metal bearing NP with the abiotic environment is, in order to know how the interaction into the organism may be (Fig 3, Schirmer et al., 2013).

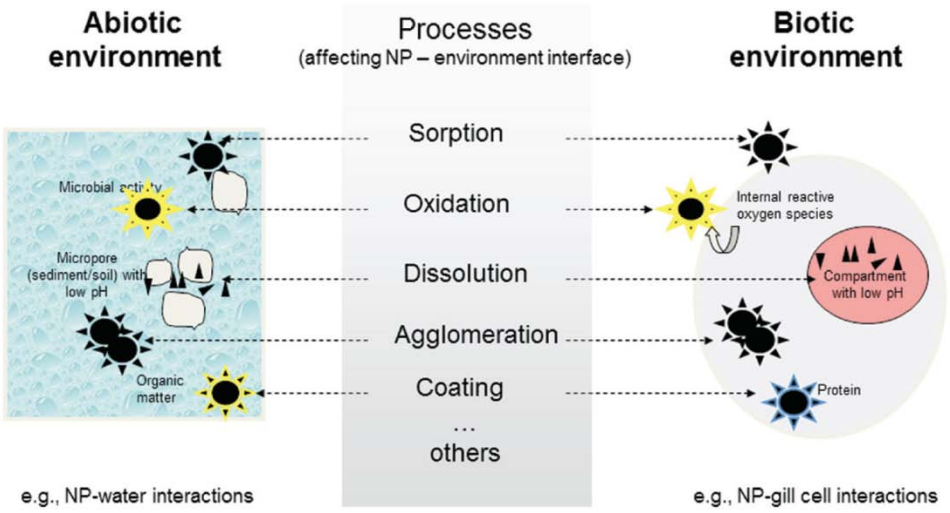

Figure 3.- Some of the processes that engineered NPs can undergo in the environment, which determine their fate in the abiotic environment and interaction with biota (Schirmer et al., 2013).

The dissolution of the NPs in the aquatic environment is an important property to understand their fate. This parameter can be influenced by different intrinsic and extrinsic factors (Fig 4). The thermodynamic parameter that controls this process is described as *solubility* (Misra et al., 2012). The solubility is size-dependent, the nanoparticulated materials dissolve more quickly than materials with a greater size (Borm et al., 2006). Surface characteristics can affect the NP solubility since agglomeration in dispersions and the NP hydrodynamic size distributions can be altered by a small change of the particle surface charge (Jiang et al., 2009). Moreover, changes in the surface characteristics can be produced by the presence of a *coating agent* which may protect the surface, leading a change of size or shape of NPs which

can bring out changes in the NP dissolution behavior (Misra et al., 2012). Linked to the presence of a coating agent in the NP suspension is the *aggregation*, since the presence of these agents keeps single particles suspended in a liquid and facilitate the synthesis process. Aggregation reduces surface area and, therefore, the dissolution potential, especially at high particle concentration (Baker et al., 2014).

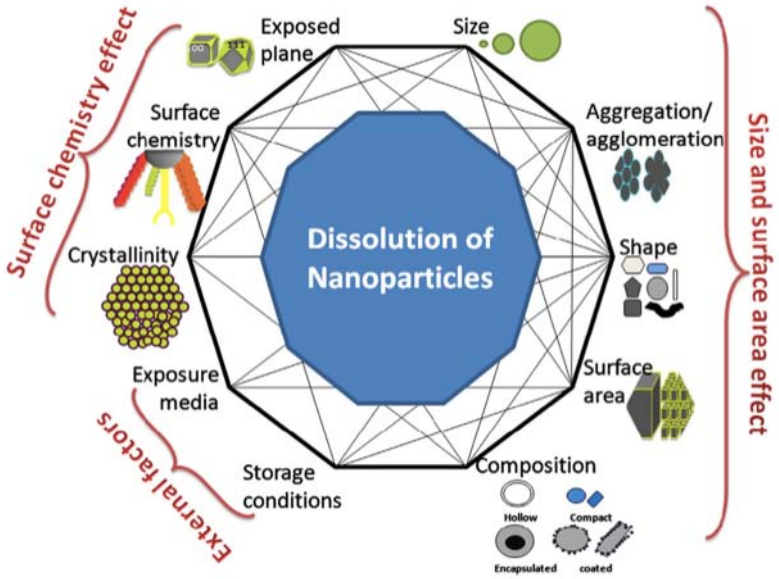

Figure 4.- A simplistic representation showing the factors that can affect dissolution of NPs and the possible interconnectivity among the factors, themselves (Misra et al., 2012).

The *chemical composition* is related to the persistence of the NPs in the medium. Some NPs, such as organic ones, can be biodegradable, while others, such as metal or metal bearing NPs, can be more or less prone to weathering by oxidation and/or dissolution similarly to their corresponding bulk materials (Xu et al., 2004; Dorn et al., 2013).

The route of entrance of NPs into the organisms depends on their habitat. In fish, as illustrated in Fig 5, NPs can be directly adsorbed by the skin, scales and fins, being specially marked in those zones which present mucus that covers the fish body and may embed NPs (Ma and Lin, 2013). Through the respiratory system, fish maintain a continuous entrance of water. The gills are in contact with NPs suspended in the water column being, therefore, a transfer point into organisms. Another route of NP entrance into the organisms is through the diet. Animals can feed suspended matter or other organisms previously exposed to NPs (Schrimer et al., 2013).

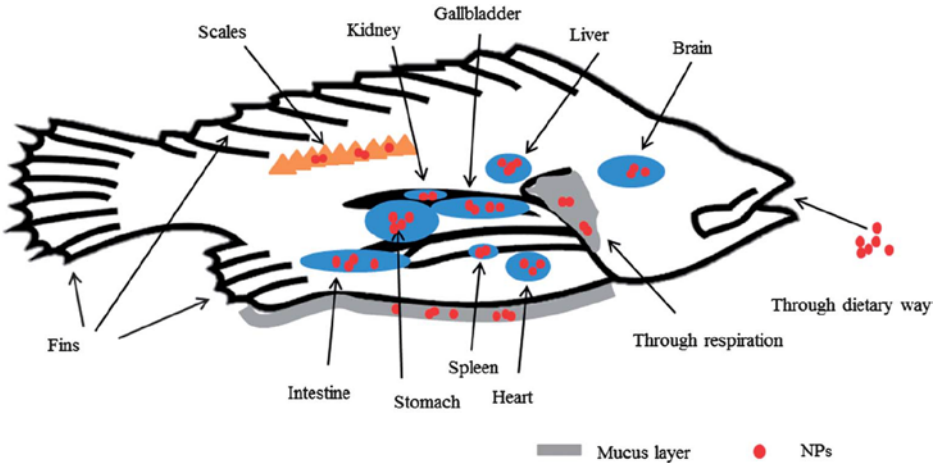

Figure 5.- Adsorption of NPs on the outer surfaces and distribution in the main internal organs of fish. The involved outer surfaces included scales, mucus layer, fins and skin. Main routes of entrance of NPs into the organism are the respiratory and digestive systems (Ma and Lin, 2013).

2 ASSESSMENT OF NANOPARTICLE TOXICITY IN AQUATIC ORGANISMS

Data on sublethal effects show that, although in general NMs provoke a similar toxic effect in fish to those provoked by the soluble or bulk forms of metals, it is necessary to know whether the nanoform can induce any additional or differential hazard compared to the dissolved metal (Shaw and Handy, 2011).

As previously mentioned, the exposure to NMs provokes their entrance into the organisms, distribution and accumulation in different tissues and organs (Ma and Lin, 2013). Chemical analyses in whole fish tissue or in selected organs provide accurate information on the metal bioavailability and accumulation capacity of fish, as well as on metal distribution among the different organs. Nevertheless, chemical analyses do not allow knowing the distribution of the metal among different cell types or cell compartments. Autometallographical detection of metals in tissue sections (Danscher, 1984) provides information not only on the amount of metal accumulated in the tissues, but on the metal localization within the cellular compartments. This approach has been successfully employed to study the fate of soluble metals in aquatic organisms including mollusks and fish (Soto and Marigómez, 1997; Alvarado et al., 2005). More recently, autometallography has been applied to the localization of metals in organisms exposed to metal NPs (Vicario-Parés et al., 2014; Jimeno-Romero et al., 2016). Along with the localization and quantification of metal in different cell types and cell compartments, the study of the NP fate once inside the cell is also important. For this purpose, transmission electron microscopy (TEM) is a suitable tool

to determine whether the NPs enter into the cells and, in that case, if they remain in the nanoparticulated form. Several studies have reported the presence of NP aggregates in liver and gills cells of different fish species, including zebrafish, after waterborne exposure. In gills, aggregates of ZnO NPs have been detected on the mucus of chloride cells or in the cytoplasm of cells of the filament and lamella of common carp (*Cyprinus carpio L.*) (Lee et al., 2014). Moreover, aggregates of Ag NPs have been detected in the cytoplasm or in the nuclear membrane of hepatocytes of zebrafish (Choi et al., 2010; Krishnaraj et al., 2016). The waterborne exposure of invertebrates to metal bearing NPs can also provoke the entrance of these particles into different compartments, as in the endolysosomal system in mussels (Ruiz et al., 2015; Jimeno-Romero et al., 2016). Moreover, TEM can be used in nanotoxicology for the evaluation of ultrastructural changes in cells and tissues provoked by the exposure to nanoscale toxicants (Heinlaan et al., 2011). The waterborne exposure of fish to NPs provoked an increase of the rough endoplasmic reticulum and cytoplasmic inclusion bodies or vesicles in hepatocytes (Lee et al., 2014; Massarsky et al., 2014a). In addition, congestive enlargement of lysosomes has been reported after the exposure to ZnO NPs, provoking a vacuolar degeneration in liver sinusoids of common carp (Hao et al., 2013). In gill cells, ultrastructural changes were detected after the exposure to ZnO NPs, such as a collapse of the apical region structure or an increase in the vacuolization in the cytoplasm in chloride cells and hypertrophic pavement in epithelial cells of the gill lamellae of common carp (Lee et al., 2014). Ultrastructural changes have been also observed after the exposure of aquatic invertebrates to diverse metal NPs. Gill cells of oysters exposed to ZnO NPs presented loss of mitochondrial cristae, and an increase in the number of endocytic vesicles in the cytoplasm, near of the cell membrane (Trevisan et al., 2014). These ultrastructural changes were also evident in the digestive gland cells of these organisms.

Gene transcription can be altered during the exposure to a contaminant, as either a direct or indirect result of the exposure (Nuwaysir et al., 1999). As the whole genome of several environmentally relevant organisms are already sequenced, gene expression microarrays can be used to define the transcriptional response to NM exposure in order to assess the mechanistic basis of their toxicity (Kahru and Dubourguier, 2010;

Fröhlich et al., 2014). The main goals of toxicogenomics are to understand the mechanisms of action of the toxicant, and to identify gene transcription patterns that are representative of adverse outcomes (Coverdale et al., 2004). In addition, the transcription level of selected genes can be also analyzed by quantitative real time PCR, which grant an accurate quantification of the mRNA transcription level useful for the assessment of the transcription profile of genes of toxicological interest in organisms exposed to contaminants, as NPs (Bebianno et al., 2015). Several studies have already focused their effort on the study of the effects provoked by NP exposure at molecular level in diverse aquatic organisms. The exposure to different metal NPs has been reported to alter the transcription of genes involved in oxidative stress, energy metabolism, DNA damage and metal detoxification, among others (Chae et al., 2009; Scown et al., 2010; Griffitt et al., 2013; Bebianno et al., 2015). Moreover, the study of the gene transcription levels can be used to analyze the distinct modes of action of NPs and their ionic counterparts, since the transcription pattern obtained after the exposure to both forms of the same metal may be different. For instance, exposure of zebrafish for 28 days to different concentrations of Ag NPs (5-50 μg/L) resulted in a larger number of genes significantly regulated (ranging from 319 to 624 genes) than exposure to 5 μg/L of ionic silver which significantly altered only 95 genes (Griffitt et al., 2013). Exposure of medaka (*Oryzias latipes)* to Ag NPs or ionic silver also resulted in different genes significantly altered. The exposure to NPs provoked the alteration of genes involved in cellular and DNA damage, carcinogenesis, oxidative stress or metal detoxification, while after the exposure to ionic silver mainly genes involved in inflammatory response or metal detoxification were significantly altered (Chae et al., 2009). The exposure of freshwater invertebrates, such as *Daphnia magna*, to metal NPs, such as ZnO NPs, or to their ionic counterpart resulted in a different transcription pattern of genes involved in cellular metabolism and reproduction, which were significantly regulated after exposure to both forms of the metal (Poynton et al., 2011).

As previously mentioned, at cellular level, lysosomes are one of the main targets of metal and metal NP exposure (Köhler et al., 2002; De Matteis et al., 2015). NPs can be internalized in the cells by endocytosis (Fig 6) and move into the lysosomes (De

Matteis et al., 2015). The NP-lysosome interaction can induce the production of reactive oxygen species (ROS) when the NPs are exposed to the acidic environment of lysosomes (Chang et al., 2012). The generation of ROS, which are extremely reactive, induces lipid peroxidative chain reaction that can produce the destabilization of the lysosomal membrane (Nohl and Gille, 2005; Terman et al., 2006). In addition, the degradation of the NPs inside the lysosomes leads to the release of ions (e.g. Cd^{2+}, Ag^{+}) that cross the organelle membrane spreading into the cytosol and inducing further ROS production through the Fenton reaction (Buzea et al., 2007; Sabella et al., 2014). The damage provoked in the lysosomal membrane by the exposure to metal and metal bearing NPs has been previously analyzed in diverse aquatic organisms and compared with the effect provoked by the same nominal concentration to their ionic counterparts. A significant decrease in the time necessary to desestabilize the membrane was observed after the exposure to different NP types (TiO_2 and CuO NPs) analyzed in mussels and fish (Jimeno-Romero et al., 2016; Vicario-Parés, 2016).

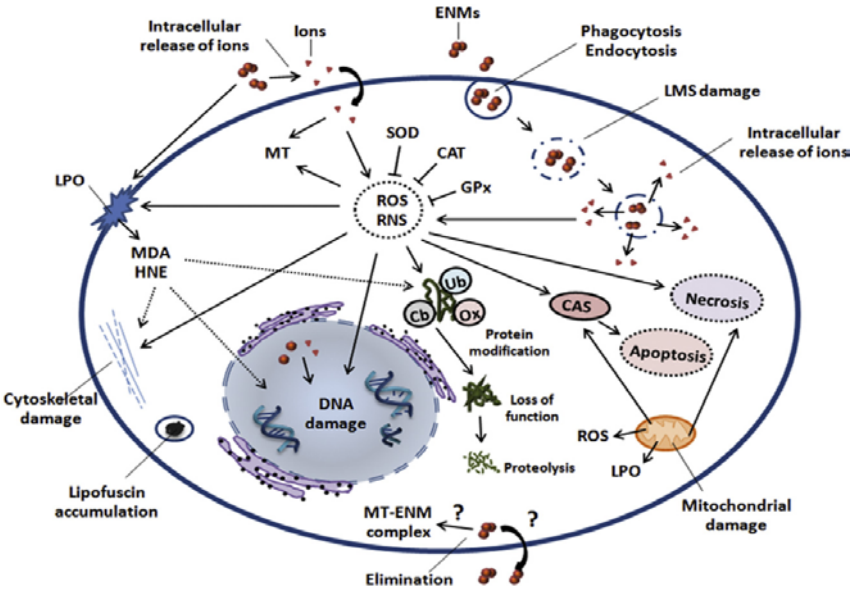

Figure 6.- General scheme illustrating the mode of action of metal-based engineered NMs in cells (Rocha et al., 2015). LMS: Lysosomal membrane stability.

Abundance of ROS can lead to potentially damaging biological effects resulting in oxidative stress phenomenon. Oxidative stress results from an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage. The hierarchical model of oxidative stress, which posits that low levels of oxidative stress induce protective effects while

higher levels of oxidative stress produce damaging effects (Li et al., 2008), has been proposed to illustrate mechanism for NP-mediated oxidative stress (Manke et al., 2013). According to this model, cells and tissues respond to increasing levels of oxidative stress via activation of antioxidant enzyme systems (Sies, 1997). The presence of ROS and, therefore, oxidative stress have shown to damage cells by peroxidizing lipids, altering proteins, disrupting DNA, interfering with signaling functions, and modulating gene transcription (Buzea et al., 2007).

An increase in the presence of ROS is known to cause a range of reversible and irreversible covalent modifications of amino acid side-chains of proteins (Ghezzi and Bonetto, 2003). Among the main protein redox lesions caused by ROS, formation of aldehyde/ketones, oxidation of S-containing residues (such as methionine and cysteine), ubiquitination and effects on disulphide patterns, like glutathionylation, and on protein thiol status have been reported (McDonagh et al., 2005; Biswas et al., 2006; McDonagh and Sheehan, 2006; 2007; 2008). These modifications have different effect in proteins, since some of them can lead to inactivation of proteins, while other are protective of the protein's structural integrity and some can be viewed as a means of the cell "sensing" changes in redox status (McDonagh et al., 2005).

Among these protein modifications, those that can cause the loss of protein function, such as carbonylation and ubiquitination have been mainly analyzed to determine the oxidative damage provoked by the exposure of aquatic organism to metal and metal bearing NPs (Fig 6, Rocha et al., 2015). Carbonylation is an irreversible modification of amino acid residue side-chains into aldehyde or ketone groups, which can lead to protein aggregation, inactivation or degradation (Levine et al., 2000; Costa et al., 2002). Damaged proteins are removed from cells by proteolysis, mainly via the ubiquitin-proteasome pathway (UPP) (Marques et al., 2004). Ubiquitin is a highly-conserved protein of 20 kDa that flags damaged cytosolic and nuclear proteins for transport to the proteasome for degradation. UPP is responsible for selective degradation of short-lived intracellular regulatory proteins or abnormal cytosolic and nuclear proteins (Marques et al., 2004). Significant increases of carbonylation and ubiquitination have been measured in gill and digestive gland of aquatic invertebrates,

such as mussel, which may confirms the oxidative damage provoked by the exposure to metal NPs (Tedesco et al., 2008; Hu et al., 2014).

An important aspect in the study of the NPs toxicity is their capacity to induce genotoxicity, as damage to the genetic material may result in the induction or promotion of carcinogenesis (Doak et al., 2012). NMs can lead to DNA damage (Fig 6) through two different mechanisms, namely primary and secondary mechanisms. Primary mechanisms are those produced by the NMs themselves at the level of the single-cell and may be either the result of direct or indirect interaction between the NMs and DNA. Also, the induction of genotoxicity can be provoked indirectly by the interaction of NMs with nuclear proteins involved in DNA replication, transcription or repair (Magdolenova et al., 2013). Secondary mechanisms are those which induce genotoxicity as a result of the excessive generation of ROS (Doak et al., 2012). Different genotoxic effects are caused by the exposure to NMs, such as chromosomal fragmentation (clastogenic effects), DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles (Singh et al., 2009). The genotoxicity caused by the exposure to different metals and NMs has been amply studied in diverse aquatic organisms. Several studies have reported significant differences in genotoxic effects on fish, including zebrafish, waterborne or dietary exposed to metal and metal bearing NPs, such as gold, cadmium or cadmium sulphide NPs using the random amplified polymorphic DNA-PCR (RAPD-PCR) methodology (Cambier et al., 2010; Geffroy et al., 2012; Ladhar et al., 2014; Dedeh et al., 2015). Clastogenic effects have been also detected using the micronuclei (MN) frequency test in fish, although the increased presence of micronucleated in erythrocytes was not statistically significant (Filho et al., 2014; Vicario-Parés, 2016). Aquatic invertebrates, such as mussels or *Daphnia magna*, have been also used to determine the genotoxic effect provoked by the exposure to different NPs. In mussels exposed to CuO NPs a significant increase in the presence of MN was detected after 21 days of treatment (Ruiz et al., 2015). The comet assay in diverse aquatic species, such as mussels, daphnia or fish (*Piaractus mesopotamicus*), also provides evidences of oxidative damage to DNA caused by exposure to metal bearing NPs (TiO_2, CuO, Ag, CeO_2, SiO_2) (Lee et al., 2009; Clemente et al., 2013; Gomes et al., 2013).

Alterations in the cell physiology provoked by the above mentioned cellular and molecular effects can be also reflected at tissue level. Gills are one of the main organs of entrance of NPs into the organisms since the water and air would pass through this organ (Ma and Lin, 2013). Therefore, gills are the primary tissues that make contact with exogenous toxicants in the aquatic environment. Thus, branchial impairment provoked by the exposure to toxicants may influence oxygen consumption and disrupt osmorregulation (Wu and Zhou, 2013). The exposure to NPs (e.g. Cu, Ag) can affect the activity of the gill Na^+/K^+-ATPase, which plays a key role in ionoregulatory processes (Griffitt et al., 2007; Katuli et al., 2014). Changes in the activity of this enzyme can alter the plasma electrolyte levels, inducing stress in aquatic organisms (Katuli et al., 2014). These biochemical changes may derive in morphological changes in gills. Two types of gill injuries have been reported after exposure to metal and metal bearing NPs (e.g. Ag, ZnO, TiO_2). The first type of injury results from a defense response and includes hyperplasia of the gill filament epithelium and oedema of gill lamellae; the second type is the direct injury and includes necrosis and shedding of gill epithelium (Griffitt et al., 2009; Chen et al., 2011; Govidansamy and Rahuman, 2012; Lee et al., 2012a; 2012b; Wu and Zhou, 2013).

The intestine is other important target organ of dietary and waterborne exposure to NMs. Intestine is where absorption of nutrients occur which, in turn, may lead to incorporate contaminants and to distribute them to other organs (Zhu et al., 2010). Moreover, freshwater fish usually drink a few amount of water, being increased under a stress situation (Best et al., 2003; Smith et al., 2007). Thus, the ingestion of water or food containing NPs may provoke a direct effect on the tissue structure of the digestive system, such as inflammation or erosion, which affects the absorption of these materials by endocytosis (Handy et al., 2008). Necrosis and inflammation of the intestine are histopathological alterations detected after the exposure to sublethal concentrations of Ag NPs in rainbow trout (*Oncorhynchus mykiss*) (Johari et al., 2014a). Also, erosion and fusion of the intestinal epithelium and increase of areas of vacuolization have been reported after the exposure of rainbow trout to TiO_2 NPs (Federici et al., 2007).

The liver, commonly used for histopathological analysis, is an important organ of active metabolism and detoxification and extremely sensitive to pollutants (Hao et al., 2009). Histopathological lesions in liver, as disruption of hepatic cell cords or apoptotic changes, have been associated to the oxidative stress provoked by the exposure to Ag NPs in zebrafish (Choi et al., 2010). Moreover, other histopathologies as extensive cell death, necrosis and degenerative changes are associated to the ability of metal NPs, such as Ag NPs, to alter the biochemical functions associated with the liver (Devi et al., 2015). Histopathological alterations described in the liver of fish, such as medaka, common carp or zebrafish, after the exposure to sublethal concentrations of metal and metal bearing NPs (ZnO or Ag NPs) are pyknotic nuclei, dilated sinusoids, focal necrosis, narrowing of sinusoids, fatty degeneration, hypertrophy of hepatocytes, irregular shaped nucleus, nuclear degeneration, cytoplasmic degeneration and apoptosis, among others (Choi et al., 2010; Wu and Zhou, 2013; Subashkumar and Selvanayagam, 2014).

3 ZEBRAFISH ANIMAL MODEL

Danio rerio or zebrafish (Fig 7) is a freshwater cyprinid of 5-6 cm length, original from the subtropical South of Asia. It has the capacity to adapt to live in drastic weather changing areas, as well as the capacity to survive in factor fluctuating environments, where salinity, pH or temperature are continuously changing. This may explain its wide range of tolerance that facilitates the culture of this species in captivity (Harper and Lawrence, 2011). Since the late 1970's, zebrafish have been used in developmental biology and for the study of the effects of a high number of chemicals (Lele and Krone, 1996). Nevertheless, it is from the late 1990´s when the zebrafish has been established as an animal model for the study of vertebrate development, as a model in toxicology or for the study of cancer and drug discovery (Hill et al., 2005). The use of zebrafish in toxicogenomics studies is also increasing, due to the high level of conservation of genomic and functional pathways between fish and mammals (Williams et al., 2014). Moreover, the fact of the zebrafish genome project is completed (Howe et al., 2013), along with the available zebrafish commercial microarrays, make it an ideal model organism (Ung et al., 2010). This species was the first fish species in which experiments

carried out in a laboratory confirmed that carcinogens active in mammals cause neoplasia in fish (Spitsbergen and Kent, 2003).

Figure 7.- Adult zebrafish: male (left) and female (right). Source: http://news.stanford.edu/news/2007/october17/med-fishsleep-101707.html, retrieved on July 8th 2015.

The small size of the adult zebrafish is the main advantage of this animal model, because it offers some technical advantages, such as ease of maintenance, observation and manipulation and possibility to maintain a large number of fish in relatively small facilities with reduced costs. Their relatively short generation time of three to four months to mature is another advantage (Segner, 2008). One female produces 200-300 eggs per week, which are fertilized externally, and have rapid embryonic development (in 2-4 days) with a beating heart and visible erythrocytes by 24 h (Bahary and Zon, 1998). The zebrafish eggs and embryos are small and transparent (Fig 8), which allows reasonable sample sizes to be tested together using a simple cell-culture plate to provide several experimental replicates at one time. The zebrafish is perfectly known morphological, biochemical, physiological and genetically at all stages of development from eggs to adults, in both sexes (Hill et al., 2005).

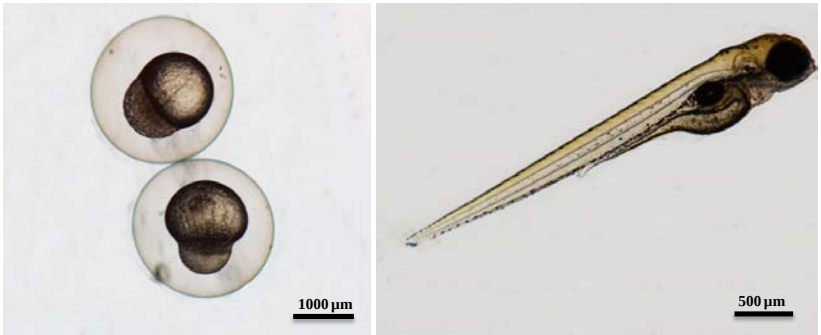

Figure 8.- (A) Zebrafish embryos at 3 hours post fertilization (hpf). (B) Zebrafish embryo at 120 hpf.

3.1 Brine shrimps as live food for zebrafish

Brine shrimps (*Artemia sp*, Fig 9) are small crustaceans with a very wide geographical distribution, which can be found in salt lakes, coastal lagoons and man-made salt

ponds. They are characterized by their adaptability and tolerance to wide ranges of salinity (5-250 g/L) and temperature (6-35ºC) and also to a great variety of nutrients that they filter in a non-selective way (Nunes et al., 2006). Different easily distinguishable life stages can be found in the lifecycle of brine shrimp: “first instar” during the first 15-24 hours post hatch (hph), “second instar” between 24-72 hph, “metanauplius” after 72-96 hph, “zoea” are considered after 6-7 days post hatch and, finally, adults are considered after 15 days post hatch (Rajasree et al., 2011).

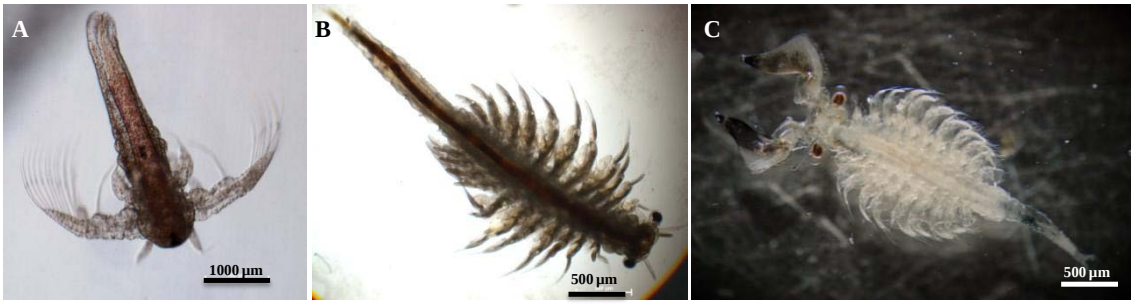

Figure 9.- (A) Brine shrimp in “second instar” life stage; (B) Female adult brine shrimp; (C) Male adult brine shrimp (Courtesy of Álvaro Fanjul).

Brine shrimps are commonly used to feed fish in aquaculture, due to the advantages they offer. Brine shrimp cultures are easy and cheap to maintain and can be produced in massive quantities in short periods of time (Sorgeloos et al., 2000). Moreover, brine shrimps present a high nutritional value to be used as food for fish, with high content of proteins, fatty acids, lipids and carbohydrates, especially in the first life stages (Léger et al., 1987).

The tolerance to large changes in different parameters such as salinity, ionic composition, temperature, and oxygen tension, and the fact that they are able to adapt to changeable nutrient resources as they are non-selective filter feeders, make brine shrimps an advantageous species in ecotoxicological studies (Kalčíková et al., 2012). Despite standardized toxicity testing protocols for brine shrimps are only available with soluble reference chemicals (Kos et al., 2016), the number of studies analyzing the effects of different NMs in these species is increasing. Brine shrimps might be a suitable biological model, at least for screening purposes, due to its cost-effectiveness (Libralato, 2014). Therefore, it is necessary to harmonize all the steps in the test procedure to standardize the protocol for the analysis of the effect provoked

by the exposure of brine shrimps to NMs in order to obtain reproducible toxicity data (Kos et al., 2016).

In addition to the screening experiments, the fact that brine shrimps are used to feed fish, along with their capacity to accumulate NMs (Wang and Wang, 2014), make them a suitable organism to investigate the transfer of NMs through the food chain (Libralato, 2014).

3.2 The fish embryo toxicity (FET) test

The fish acute toxicity test was proposed as a test to study the water quality (OECD TG203, 1992). In this test mortality was the unique endpoint and fish suffered severe distress and pain (Nagel, 2002; Braunbeck et al., 2005). This is in conflict with current animal rights legislation (Strähle et al., 2012). Moreover, the environmental significance of individuals death after a short-term exposure to high concentration, except in the case of accidental spills, is low (Nagel, 2002).

Since the implementation of the Animal Welfare Guideline 86/609/EC in 1986, the declared policy of the EU institutions is to support the development and use of alternative methods, which are defined as *"any method that can be used to replace, reduce or refine the use of animal experiments in biomedical research, testing or education"* (Lilienblum et al., 2008). The "alternative" concept is attributed to Russell and Burch (1959), who defined three types of alternatives, the replacement, reduction or refinement of animal tests, the so called RRR (or 3R) principle (Lammer et al., 2009a). According with the concept of 3R, new toxicity tests were proposed to replace acute toxicity test, the fish embryo toxicity (FET) test and related fish eleutheroembryo toxicity test (Nagel, 2002; Lammer et al., 2009b; Embry et al., 2010; OECD TG236, 2013).

The FET test is considered as an alternative to the acute toxicity test (Schulte and Nagel, 1994; Nagel, 2002; Braunbeck et al., 2005) because in the case of fish the European Directive 2010/63/EU on the protection of animals used for scientific purposes only applies to independently feeding larval forms, which in the case of zebrafish is considered to happen after 5 days post-fertilization (Braunbeck et al.,

2014). In this test four apical observations are recorded as indicators of acute lethality in fish: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heart-beat (ISO, 1996; OECD TG236, 2013). But, mortality is not the unique endpoint; a variety of sublethal endpoints can be incorporated into the test protocol (Lammer et al., 2009b).

Moreover, the FET may serve as a model system in other fields of ecotoxicology such as sediment toxicity assessment, genotoxicity and mutagenicity testing, histopathological analysis and induction studies based on microarray techniques (Braunbeck et al., 2005). During the last years, the FET test has been amply used in nanotoxicology, in order to analyze the effect provoked by different NMs (Bohnsack et al., 2012).

3.3 Zebrafish as a model in nanotoxicology

In zebrafish, most of the studies on the effects provoked by the exposure to metal and metal bearing NPs have been carried out with embryos, while a limited number of studies have been carried out with adults. Waterborne exposure to NMS has been the main exposure route selected in these studies, while few studies have addressed dietary exposure. The main results reported up to day using similar NMs as the ones selected in the present study have been summarized in tables 2-7.

Table 2 summarizes studies reporting toxic effects of Ag NPs, which is by far the most studied nanomaterial, to zebrafish embryos. Overall, results show that the exposure to Ag NPs affect the development and survival of fish embryos, in a concentration-dependent way (Lee et al., 2007; Asharani et al., 2008; 2010; Yeo and Kang, 2008; George et al., 2011; Kannan et al., 2011; Lee et al., 2012c; 2012d; George et al. 2014; Ribeiro et al., 2014; Yoo et al., 2016). Ag NP toxicity has been mainly calculated as LC_{50} values, ranging from 0.02 mg/L (Kannan et al., 2011) to 25-50 mg/L (Asharani et al., 2008). The presence of a coating agent can reduce the toxicity of the NP suspension. Osborne et al. (2013) reported that uncoated Ag NPs were more toxic than citrate-coated Ag NPs of a similar size. Nevertheless, differences in the toxicity have been also reported depending on the additive used, being Ag NPs stabilized with polyvinyl pyrrolidone (PVP) which presented the highest toxicity (Cunningham et al., 2013; Kim

et al., 2013a; Kim and Tanguay, 2014). Moreover, differences in the toxicity have been detected depending on the NP size. Most of the studies have described a higher toxic effect for the small NPs than for those with a larger size (Lee et al., 2007; Bar-Ilan et al., 2009; Asharani et al., 2010; Cowart et al., 2011; Powers et al., 2011; Bowman et al., 2012; Kim et al., 2013b; Kim and Tanguay, 2014). Nevertheless, Osborne et al. (2013) reported higher toxicity for larger NPs (35 nm) than for the smaller NPs (10 nm). As in the case of the size, the exposure to NMs with different shape provoked differences in the toxicity to zebrafish embryos. George et al. (2012) exposed zebrafish embryos to Ag nanoplates, spheric Ag NPs and Ag nanowires, being the nanoplates which presented the highest toxicity and nanowires were the least toxic. The Ag NP toxicity in zebrafish embryos can also vary with the ionic strength of the media, increasing the NP toxicity in low ionic strength media (Levard et al., 2013; Olasagasti et al., 2014). Depending on the composition of the media the toxicity of the Ag NPs can be altered, since oxidized Ag^{+} from the dissolution of Ag NPs can react with different elements present in the media as inorganic sulfide or chloride ions to form Ag_2S NPs or AgCl species, reducing the toxicity of the Ag NPs (Levard et al., 2013).

The embryonic stage at which the organism is exposed has been described as an important factor in the Ag NPs toxicity, but different results have been reported. On the one hand, some authors detected an increase in the mortality and in malformation prevalence in the embryos when the exposure was carried out during the first embryonic stages, being the cleavage stage (2 hpf) the most sensitive stage (Browning et al., 2013a; Groh et al., 2014). The increase in the toxicity during the exposure of younger zebrafish embryos may be due to the disruption of important developmental processes particularly susceptible to the Ag NPs occurring at earlier stages (Groh et al., 2014). On the other hand, Lee et al. (2013a) exposed embryos for 2 h at different stages and then transferred them to clean water up to 120 h. They detected an increase in the NP toxicity in the last-segmentation stage (21 hpf) embryos respect to the early-segmentation stage embryos. These results suggest that disruption of cell differentiation by the NPs causes the most toxic effects on embryonic development.

The presence of the chorion during the exposure is also an important factor determining the Ag NP toxicity. Park et al. (2013) reported an increase in the mortality

of embryos surrounded by the chorion, while dechorionized embryos presented an increase in malformation prevalence. Nevertheless, Kim and Tanguay (2014) detected an increase in mortality and malformation prevalence in embryos without chorion respect to unhatched embryos exposed to Ag NPs of different sizes (20 and 110 nm), coatings (PVP and citrate) and exposure media (embryo medium and $CaCl_2$). The size- and surface coating-dependent toxicity of Ag NPs was controlled by the permeability function of the chorion, which serves as a barrier to avoid Ag NP contact with the embryos.

In addition to the effects of Ag NPs on embryo survival and malformation prevalence, other sublethal effects have been reported. The exposure to Ag NPs can alter their behavior. Observation of abnormal swimming, pectoral fin gyrations, or mouth movements were detected in zebrafish embryos after the exposure to Ag NPs of 20 nm in a concentration ranging from 0.5 to 1 mg/L (Bowman et al., 2012). These effects are related to neurobehavioral disruption, provoked by the exposure to Ag NPs which causes a disruption in neural cell replication and differentiation, affecting to the normal behavior of the zebrafish (Powers et al., 2011). At molecular level, the alteration in the transcription of genes involved in the neuronal cell differentiation (ELAV like neuron-specific RNA binding protein 3 (*huC*)*, glial fibrillary acidic protein* (*gfap*) and *neurogenin 1* (*ngn1*)) has been also detected in zebrafish embryos exposed for 96 h to Ag NPs of 4 and 10 nm, in a concentration ranging from 0.48 to 23.1 mg/L. These alterations in gene transcription have been related to the presence of head malformations in zebrafish embryos (Xin et al., 2015). The exposure for 24 h to 0.5 and 1 mg/L of Ag NPs (average size 120 nm) provoked the up-regulation of genes involved in endoplasmatic reticulum stress, which can have several consequences including the activation of apoptotic (e.g. *NADPH oxidase activator* (*Noxa), cyclin-dependent kinase inhibitor 1* (*p21*), *tumor protein p53* (*p53*)) and inflammatory (e.g. *beta interacting protein* (*BiP*)*, synovial apoptosis inhibitor* (*Synv*)) pathways (Christen et al., 2013). Finally, Van Aerle et al. (2013) examined the effects of the exposure for 48 h to 5 μg/L of Ag NPs of 10 nm, resulting in an inhibition of the oxidative phosphorylation pathway after 24 h, potentially due to the increase in ROS within mitochondria. These results were attributed partially to the dissolution of silver in Ag^+, although not all changes

observed in gene transcription can be explained by the effects of Ag^{+} alone. Genes related to chryptochrome (e.g. *cry1a*) were strongly down-regulated after 24 and 48 h. These genes are induced by light exposure available and, therefore, it was suggested that the light availability to embryos exposed to Ag NPs was lower than the light available for control organism, since suspended NPs were adsorbed at the surface of the embryos acting as a barrier to the light (Van Aerle et al., 2013). Moreover, the regulation of genes involved in detoxification of metals (e.g. *metallothionein* (*mt*), *ATP-binding cassette c* (*abcc*)), response to inflammation and immune response (e.g. interleukin-1 beta (*Il1β*)) and oxidative stress (e.g. *heat shock protein7* (*hsp7*)) has been also reported in the zebrafish embryos exposed to Ag NPs (Christen et al., 2013; Osborne et al., 2013; Park and Yeo, 2013; Olasagasti et al., 2014; Gao et al., 2015; Park and Yeo, 2015; Xin et al., 2015).

As for embryos, differences in the toxicity provoked by the exposure to Ag NPs synthesized by different methods and containing different capping agents have been reported in adult zebrafish (Table 3). Despite their smaller size, the exposure for 96 h to PVP-coated Ag NPs of 9.2 nm synthesized using the top-down (physical method) provoked lower toxicity in adult zebrafish (LC_{50} = 0.54 mg/L) than the exposure to citrate-coated Ag NPs of 63.45 nm synthesized by means of a bottom-up method (LC_{50} = 0.014 mg/L). The differences in the LC_{50} values can be related to the toxic effect provoked by the chemical precursors used in bottom-up approaches for reducing silver ions to Ag NPs (Johari et al., 2014b). A higher LC_{50} value (1.42 mg/L) has been reported after the exposure for 96 h to a range of concentrations (0.23-3.31 mg/L) of Ag NPs of 24.1 nm using plant extract mediated synthesis (Krishnaraj et al., 2016). In this study, 100% of mortality was registered at 2.84 and 3.31 mg/L. Moreover, genotoxicity evaluated as the presence of MN and nuclear abnormality in erythrocytes, cytological changes in gills and intrahepatic localization of NPs after the exposure for 14 days to 0.71 mg/L were also reported.

Alterations in the liver gene transcription, especially in genes related to DNA damage, oxidative stress, and apoptosis have been reported after a short-term exposure (24 h) of zebrafish up to 120 mg/L of Ag NPs of 5-20 nm as well as after long-term exposure (36 d) to lower concentrations (0.4 and 4 mg/L) of Ag NPs of 10-20 nm (Yeo and Pak,

2008; Choi et al., 2010). In addition, after the exposure for 14 d to 0.71 mg/L of the above mentioned Ag NPs of 24.1 nm, oxidative stress and immunotoxicity were confirmed studying the hepatic transcription of different stress related- (metal transcription factor 1 (*mtf-1*), *hsp70*) and immune response related- (*toll like receptor4* (*tlr4*), *nuclear factor kβ* (*nfkb*), *CCAAT/enhancer binding protein* (*cebp*), *transferrin* (*tlr22*), *toll like receptor22* (*trf*)) genes (Krishnaraj et al., 2016). Short-term exposure to 10 mg/L of Ag NPs of 26.6 nm also resulted in significantly regulated genes in zebrafish gill (148 genes at 24 h of exposure and 462 genes at 48 h exposure) as detected through a microarray analysis (Griffitt et al., 2009). After a longer term period (28 days) of water-borne exposure to 5-50 μg/L of Ag NPs of 3.1 nm, the microarray analysis in gills revealed that the number of regulated genes was higher (3019 genes) than after the short-time period. These genes differentially transcribed were associated to gene ontology (GO) terms involved in both extracellular and intracellular processes, indicating that Ag NPs were exerting effects both inside and outside the cell. GO terms involved in DNA damage and repair were significantly over-represented after the exposure (Griffitt et al., 2013). Higher concentration of silver was reported in gills than in the carcass, although this accumulation did not provoked pathological alterations in the gills (Griffitt et al., 2013).

In fact, gills appear as a target organ of Ag NP toxicity in zebrafish. A prolonged exposure (up to 3 weeks) to two different concentrations (2 and 4 mg/L) of Ag NPs (16.6 nm) inhibited gill Na^+/K^+-ATPase and acetyl cholinesterase (AchE) activities, and induced the stress response, evidenced by the increased plasma glucose and cortisol levels (Katuli et al., 2014). Osborne et al. (2015) also reported effects in gills of zebrafish exposed for 4 days to 1 mg/L of two different citrate-coated Ag NPs (20 and 110 nm). These authors found a significantly higher content of silver in fish exposed to Ag NPs of 20 nm than in those exposed to Ag NPs of 110 nm. Moreover, the histopathological analysis revealed fusion, hyperplasia and inflammation in the secondary lamellae of fish treated with Ag NPs of 20 nm, while after the exposure to the large NPs only hyperplasia was detected (Osborne et al., 2015). In order to determine the fate of the Ag NPs in the gills a silver staining was performed, detecting most of the Ag NPs adhered to the secondary filaments, especially after the exposure

to the small NPs. Finally, Mansouri and Johari (2016) analyzed gill ultrastructure and histopathological changes after the exposure for 4 days to a range of concentration (1.5-15 μg/L) of citrate-capped Ag NPs (63.45 nm). At the highest tested concentration (15 μg/L), an increase in the diameter of gill filaments and secondary lamellae and reduced length of the secondary lamellae were detected. Moreover, exposure to all concentrations caused different injuries including vacuolization, dilated and clubbed tips, aneurism, hyperplasia, edema, fusion, swollen mucocytes, hypertrophy, and necrosis.

In liver, histopathological alterations such as extensive cell death, necrosis and degenerative changes were detected after the exposure to 0.1 mg/L of Ag NPs for 15 days (Devi et al., 2015). In adult zebrafish fed with a diet containing Ag NPs (500 mg/kg food) no effects were detected in the histology of the intestine nor in the liver. Only changes in the intestine microbiome were observed after 14 days of treatment (Merrifield et al., 2013). Nevertheless, in the above referenced study, Osborne et al. (2015) reported histopathological changes in the intestine of zebrafish. The intestine of fish exposed to the small Ag NPs for 4 days presented an increase in the number of goblet cells in the epithelial layer, some reduction in microvilli and partial damage to the lamina propria. The intestine of zebrafish exposed to the larger Ag NPs showed evidence of vacuolization and partial damage to lamina propria with abundant microvilli. The silver staining revealed Ag NPs in the basolateral membrane of the intestinal mucosa, especially after the exposure to citrate-coated Ag NPs of 20 nm.

Table 4 summarizes studies reporting toxic effects of CdS NPs and other cadmium-containing QDs with different cores and shells to zebrafish embryos after water-borne exposure and to adults after dietary exposure. In these studies, differences in the toxicity of $CdSe_{core}/ZnS_{shell}$ QDs have been detected depending on the capping agent, with LC_{50} values ranging from 0.787 mg/L (capped with poly-L-lysine) to 4.72 mg/L (capped with poly-(ethyleneglycol)-OCH_3) (King-Heiden et al., 2009). Differences in the chemical composition of the core or of the shell also provoked differences in the toxicity to embryos, since the presence of metals, as selenium, in the core increases the toxic effect of these NPs (Wiecinski et al., 2013). An increase in mortality and in malformation prevalence, as well as a decrease in the hatching rate have been

reported in all the studies (George et al., 2011; Zhang et al., 2012a; 2012b; 2012c; 2013; Wiecinsky et al., 2013; Ong et al., 2014). Moreover, accumulation of cadmium in embryos has been analyzed after treatment with two different QDs (5 nm). Zebrafish embryos were exposed to CdSe/ZnS-COOH functionalized QDs (QDs-C) and CdSe /ZnS QDs in toluene (QDs-P) for 48 h at two different concentrations (0.5 and 1 mg/L), followed by 24 h of depuration in clean water. Exposure to the highest concentration provoked 100% mortality after 9 h of exposure. In embryos exposed to 0.5 mg/L, cadmium accumulation was not detected. The distribution of cadmium into the embryos was analyzed after the exposure to QDs-P. QDs-P appeared distributed along the larvae surface indicating that QDs-P were not assimilated by the organism (Zarco-Fernández et al., 2016). Zhang et al. (2012c) also studied the bioaccumulation after simultaneous exposure to mercaptopropionic acid-CdSe QDs (3 nm) and to Cu^{2+}. The presence of QDs facilitated the accumulation of copper into the organism, since Cu^{2+} might be adsorbed onto mercaptopropionic acid-CdSe QDs as a result of large surface area and electrostatic attraction (Zhang et al., 2012c).

In adult zebrafish, the toxic effect of the dietary exposure to cadmium-containing QDs has been analyzed in two studies. Ladhar et al. (2013) fed adult zebrafish for 36 and 60 days with two different doses (40 and 100 ng NPs/day/g body weight) of CdS QDs of two different sizes (8 and 50 nm). Results showed a significant accumulation of cadmium in the liver at both periods of time and, also, in the brain after 60 days of exposure. Moreover, after 60 days of dietary exposure to both concentrations of QDs genotoxic effects were reported using RAPD-PCR genotoxicity test. The gene transcription analysis in liver showed a general downregulation of genes involved in mitochondrial metabolism, DNA repair, apoptosis and antioxidant defenses after 36 days of exposure to the smallest NPs (8 nm). After 60 days of exposure, these genes appeared repressed in the intestine and over expressed in the brain (Ladhar et al., 2013). Lewinski et al. (2011) simulated a simply food chain using a primary consumer (*Artemia sp*) exposed to 0.6 mg/L of CdSe/ZnS QDs to feed a secondary consumer (zebrafish) for 14 days. Uptake of QDs was detected in zebrafish, although biomagnification was not recorded after the dietary exposure.

In Table 5, studies carried out using zebrafish embryos and adults exposed to Au NPs are summarized. The low toxicity of different Au NPs has been demonstrated in most of the studies, where significant increases in mortality, malformation prevalence or hatching rate have not been observed in zebrafish embryos exposed up to 120 hpf to Au NPs of different sizes (Bar-Ilan et al., 2009; Asharani et al., 2010; Wang et al., 2010; George et al., 2011; Ganeshkumar et al., 2013; García-Cambero et al., 2013; Kovrižnych et al., 2013). Nevertheless, some authors have reported toxic effects of Au NPs, with an increase in mortality and presence of malformations in embryos exposed for 120 h. Browning et al. (2009), using citrate-capped Au NPs of 11.6 nm, detected an increase in mortality and malformation prevalence at the highest concentration tested (12.6 mg/L). Rizzo et al. (2013), using non-capped Au NPs of 1.4 nm, reported toxic effects at very high concentrations (100 and 1000 mg/L), suggesting that Au NPs toxicity depends on the size and capping agent. Harper et al. (2011) detected differences in the toxicity of Au NPs, in terms of mortality and malformation prevalence, depending on the capping agent which conferred different charge properties. Positively charged Au NPs were more toxic than negatively charged Au NPs. Effects at molecular level have been also reported upon exposure to Au NPs. A significant increase in cell death in the eyes and an increase of *p53* and *bcl2-associated X protein* (*bax*) gene transcription has been reported in embryos exposed to 10 and 30 mg/L of Au NPs of 1.3 nm (Kim et al., 2013b).

The dietary exposure to Au NPs (36-106 ng gold/fish/day) of two sizes (12 and 50 nm) for 36 and 60 days (Geffroy et al., 2012) and the exposure for 20 days to sediment spiked with two different concentrations (16 and 55 mg/g dry weight) of Au NPs of 14 nm (Dedeh et al., 2015) provoked similar toxic effects in adult zebrafish. The over transcription of genes involved in DNA repair, detoxification processes, apoptosis, mitochondrial metabolism and oxidative stress, as well as the genotoxicity detected according to the RAPD-PCR test, caused by the exposure to Au were the main effects reported in both studies. In a shorter-term experiment (96 h), Kovrižnych et al. (2013) did not detect toxic effect in adult zebrafish waterborne exposed up to 200 mg/L of Au NPs.

Regarding the studies addressing the toxicity of ZnO NPs in zebrafish embryos (Table 6), a dose-dependent increase in mortality and malformation prevalence has been observed (Zhu et al., 2008; 2009; Bai et al., 2010; George et al., 2011; Xiong et al., 2011; Wehmas et al., 2015). Different LC_{50} values have been reported, ranging from 1.793 mg/L in zebrafish embryos exposed for 96 h to ZnO NPs of 20 nm (Zhu et al., 2008) to 9.1 mg/L after the exposure of zebrafish embryos to ZnO NPs of 8.35 nm for 120 h (Wehmas et al., 2015). Moreover, a delay in the hatching time has been detected in zebrafish embryos exposed at concentrations of ZnO NPs ≥5 mg/L (Xia et al., 2011; Lin et al., 2012; Brun et al., 2014; Chen et al., 2014; Ong et al., 2014; Vicario-Parés et al., 2014). This effect has been related to the inactivation of the hatching protease activity provoked by the exposure to ZnO, since the presence of high concentrations of Zn^{2+} resulting from the ZnO NPs dissolution interfere with the zebrafish hatching enzyme (ZHE1) producing a delay in the embryo hatching (Xia et al., 2011; Lin et al., 2012). Other effects appearing in zebrafish embryos exposed to ZnO NPs include reduced superoxide dismutase (SOD) and catalase (CAT) activities, and reduced glutathione (GSH) concentration in the liver after the exposure to 5 mg/L of ZnO NPs. In the gut, at the same exposure concentration, SOD and CAT activities and GSH concentration increased (Xiong et al., 2011). Zhao et al. (2013) also detected oxidative stress related effects, SOD activity and malondialdehyde content increased significantly, while CAT activity was decreased.

To our knowledge, only Skjolding et al. (2014) have studied the effect provoked by different ZnO NPs (uncoated NPs, ZnO-OH NPs and ZnO-octyl NPs) in adult zebrafish using *Daphnia magna* exposed to different concentrations as food for the zebrafish during 14 days. Only after the exposure to uncoated ZnO NPs and ZnO NPs-octyl a trophic transfer of zinc from *Daphnia magna* to zebrafish was detected.

Few studies have addressed the issue of SiO_2 NP toxicity (Table 7). Most of them reported no toxic effects in zebrafish at early stages in terms of increased mortality and malformation prevalence after waterborne exposure (Fent et al., 2010; George et al., 2011; Kovrižnych et al., 2013; Ong et al., 2014) or after injection into the yolk sac (Nelson et al., 2010; Sharif et al., 2012). Nevertheless, an increase in mortality and malformation prevalence has been registered by other authors in zebrafish embryos

after waterborne exposure to high concentrations (100-200 mg/L) of Si NPs (Duan et al., 2013a; 2013b), after 24 h of intravenous injection of high concentrations (1000-12000 mg/L) of Si NPs of 62 nm (Duan et al., 2015) or when SiO_2 nanowires were injected. In this latter case, a significant increase of mortality and presence of malformations was detected at 132 hpf in embryos injected with SiO_2 nanowires at 1- or 2- cell stage, related to the shape effect, since materials with large surface areas and elongated shapes may generate mechanical disturbances in animal tissues that spherical materials do not (Nelson et al., 2010). Moreover, toxicity was detected in zebrafish embryos after the exposure to maghemite@SiO_2 rattle type microspheres at relatively low concentrations (0.25-2.5 mg/L), with a high incidence of malformations (Liu et al., 2012). In adult zebrafish no toxic effects have been reported after the exposure to SiO_2 NPs for 96 h (Kovrižnych et al., 2013).

Table 2.- Summary of main effects reported in zebrafish embryos water-borne exposed to Ag NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
Citrate	5-46	up to $7.7x10^{-5}$	120 h	Increased prevalence of deformed embryos up to $2.1x10^{-5}$ mg/L and, then, increased number of dead embryos	Lee et al. (2007)
Starch BSA	5-20	5, 10, 25, 50 and 100	72 h	Malformations provoked by both NPs at 50 mg/L Dose-dependent and growth stage-dependent (64–128 cell) toxicity LC_{50} = 25-50 mg/L	Asharani et al. (2008)
Uncoated	26.6 ± 8.8	up to 10	48 h	LC_{50} = 7.2 mg/L	Griffitt et al. (2008)
Uncoated	10-20	10^{-5} and $2x10^{-5}$	72 h	Mortality, malformations and hatching delay at both concentrations, especially at the highest one	Yeo and Kang (2008)
Citrate	3, 10, 50 and 100	10.78 0.027, 0.27, 2.7 and 26.7	120 h	Significantly increase of malformations at 10.78 mg/L. LC_{50} (Ag3) = 10.06 mg/L, LC_{50} (Ag10) = 13.55 mg/L, LC_{50} (Ag50) = 13.7 mg/L, and LC_{50} (Ag100) = 14.8 mg/L	Bar-Ilan et al. (2009)
PVA	5-35	10, 25, 50, 75 and 100	72 h	Malformations and mortality recorded at 25, 50, 75 and 100 mg/L Detectable levels of metal accumulation at 25 and 50 mg/L	Asharani et al. (2010)
Uncoated	12 and 21	10^{-9} to 10^{-5}	120 h	Based on estimations: LC_{50} (12) = 15.8 mg/L, EC (12) = 12.6 mg/L; LC_{50} (21) = 50.1 mg/L, EC (21) = 5 mg/L.	Cowart et al. (2011)
Uncoated	10	1, 5, 15 and 25	120 h	Malformations, mortality and inhibition of hatching at 15 and 25 mg/L	George et al. (2011)
Citrate	430	0.001-0.025	120 h	Malformations at 0.014 and 0.02 mg/L LC_{50} = 0.02-0.022 mg/L	Kannan et al. (2011)
Citrate PVP	10 10 and 50	0.0011-10.08	120 h	Hatching delay at ≥ 1.08 mg/L (C). Ag NP toxicity: PVP > C, 10 nm > 50 nm AgNPs-PVP altered neurodevelopment at 3.084 mg/L	Powers et al. (2011)
Uncoated	20, 50 and 100	0.1, 0.5, 1 and 5	120 h	Ag NPs-50 were the most toxic, inducing 100% of mortality at 0.5 mg/L Abnormal motility in embryos dosed with Ag NPs-20 at 0.5-1 mg/L	Bowman et al. (2012)
PVP Uncoated Uncoated	20, 40 and 100 45x10 (nanoplates) 20000x65 (nanowires)	1.25, 2.5, 5 and 10	120 h	Nanoplates were the most toxic, 100% mortality and increased malformations at 10 mg/L Nanowires were the least toxic	George et al. (2012)
Uncoated	95.4 ± 16	$5.7x10^{-4}$, $5.7x10^{-3}$, 2.8, 5.7	120 h	Mortality and malformations at 2.8 and 5.7 mg/L Ag NPs passively entered into the embryos through chorion pores	Lee et al. (2012c)

Table 2 (continued)

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
Citrate	41.6 ± 9.1	5, 12, 24, 48, 95, 119 and 146	120 h	100% mortality at ≥ 48 mg/L Malformations from 5 to 24 mg/L	Lee et al. (2012d)
DIS PVP Bare	15 80 35	0.005-100.8	96 h	Toxicity ranking: Ag NPs-Dis>Ag NPs-PVP> Ag NPs-Bare EC_{50} (Ag NPs-Dis) = 0.088 mg/L; EC_{50} (Ag NPs-PVP) =0.161 mg/L and EC_{50} (Ag NPs-Bare) = 0.211 mg/L	Wang et al. (2012)
Citrate	97 ± 13	6.03, 12.06, 24.12, 48.24 and 72.36	2 h	Ag NPs incited distinctive stage-dependent toxicity and create stage-specific phenotypes Cleavage stages (2 h) were the most sensitive, followed by gastrula stage (4 h), early (12 h) and late segmentation stage (21 h)	Browning et al. (2013a)
Uncoated	120	0.01, 0.1, 1 and 5	120 h	Hatching delay at 5 mg/L; ER stress related gene transcripts up-regulated (0.1, 5 mg/L) after 24 h, pro-apoptotic gene transcripts up-regulated (0.1-1 mg/L) and down-regulated (5 mg/L) after 48 h *cat* (0.1, 5 mg/L) and *mt2* (1 mg/L) up-regulated during the exposure period	Christen et al. (2013)
TSC PVP Thiol	40-180	0.001-100	72 h	Toxicity ranking: PVP > Thiol > TSC LC_{50} values were lower after 48 h than after 24 h Hatching delay especially marked after the exposure to Ag NPs-PVP	Cunnigham et al. (2013)
PVP Citrate	20 and 110	0.8, 4, 10, 20 and 50	120 h	Size-dependent (20 nm > 110 nm) and coating-dependent (PVP > C) toxicity	Kim et al. (2013a)
Uncoated	ND	1.25, 2.5, 5 and 10	96 h	LC_{50} (96 h) = 2.7 mg/L, malformations at 2.5 mg/L	Kovrižnych et al. (2013)
Uncoated	13.1 ± 2.5	0-7.55x10^{-5}	2 h (at different stages)	Malformations and mortality, stage-dependent at ≥ 2.16x10^{-6} mg/L. The last-segmentation stage (21 h) was the most sensitive	Lee et al. (2013a)
Positively charged Negatively charged More negatively charged	11.7 ± 2.7	up to 6.47x10^{-6}	120 h	Toxicity dependent on the surface charge, surface functional groups and chemical composition of the NPs	Lee et al. (2013b)

Table 2 (continued)

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
PVP	37	0.08-50	120 h	Lower mortality in higher ionic strength media than in lower ionic strength media	Levard et al. (2013)
PS	8.39 ± 0.98	0.03, 0.16, 0.31, 0.78 and 1.55	120 h	Malformations and mortality at 0.78 and 1.55 mg/L. Significantly reduction of TGSH levels at 0.78 and 1.55 mg/L LC_{50} (96 hpf) = 1.18 mg/L	Massarsky et al. (2013)
NM-300k	20	(1) 0.01, 0.1, 1 and 10 (2) 0.5, 0.66, 0.87, 1.14 and 1.5 (3) 0.25, 0.35, 0.5, 0.71 and 1	48 h	(1) ISO water, LC_{50} (48 h) = 1.26 mg/L Sewage treatment process, (2) LC_{50} (48 h) = 0.173 mg/L; (3) LC_{50} (48 h) = 0.225 mg/L	Müth-Kohne et al. (2013)
Uncoated	20	0.001-100	48 h	Mortality increased with concentration, 100% at 100 mg/L Malformations at ≥ 0.1 mg/L AchE significantly inhibited and PChE significantly different at 0.01 and 0.1 mg/L	Myrzakhanova et al. (2013)
Citrate Uncoated	10 and 35	0.005, 0.05, 0.5, 5 and 25	48 h	Toxicity 35 nm> 10 nm and uncoated > C Cell necrosis in early life stage and significantly up-regulation of *mt2* 0.5 mg/L (35 nm)	Osborne et al. (2013)
Uncoated	20 and 30	$20x10^{-5}$	72 h	278 genes up-regulated and 36 down-regulated, (apoptosis, immune response and heat shock protein activity) Apoptosis was the function with the highest proportion of differentially transcribed genes	Park and Yeo (2013)
Citrate	10 and 100	5, 10, 20 and 40	120 h	Mortality, hatching decrease and malformations at ≥ 10 mg/L Mortality: chorionized > dechorionized Malformations: chorionized < dechorionized	Park et al. (2013)

Table 2 (continued)

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
Uncoated	10	0.005	48 h	Down-regulation of the oxidative phosphorylation pathway at 24 h and up-regulation at 48 h	Van Aerle et al. (2013)
Uncoated	53.1 ± 4.1	0.5	ND	No significant overall delay in developing embryos in the early stage of embryogenesis	Beasley et al. (2014)
PVP	12	1.25, 2.5 and 5	120 h	Mortality at ≥ 1.25 mg/L. Malformations: short body axis, pericardial edema, not yolk depletion, and tail malformation	George et al. (2014)
Carbonated	28	1.07, 4.32 and 21.6	48 h	Toxicity depended on the time exposure starts, with young embryos (2 hpf) being the most sensitive LC_{50} (2 hpf) = 2.8 mg/L, LC_{50} (4 hpf) = 7.8 mg/L, LC_{50} (6 hpf) = 18.3 mg/L	Groh et al. (2014)
Citrate	20 ± 10	0.1 and 1	48 h (24 h depuration)	Negligible silver accumulation (BCF 0.6-1)	López-Serrano et al. (2014)
Uncoated	8.39 ± 0.98	0.5	96 h	Mortality and hatching delay increased significantly at 96 h	Massarsky et al. (2014b)
Uncoated	18	0.01, 0.5, 1, 2.5, 5, 10 and 15	120 h	Up-regulation of *hsp70, Il1β, mt* LC_{50} (embryo water) = 6.24 mg/L LC_{50} (deionized water) = 0.94 mg/L	Olasagasti et al. (2014)
Citrate	80 ± 2	up to 100 mg/L	120 h	Hatching delay at 10 mg/L	Ong et al. (2014)
PVP	61-70	5, 10, 25, 50, 75 and 100	72 h	Malformations, mortality and hatching delay at ≥ 25 mg/L	Pavagadhi et al. (2014)
Uncoated	7.5 ± 1.7	0.01, 0.025, 0.05, 0.1 and 0.25	96 h	Malformations and mortality at ≥ 0.1 mg/L LC_{50} (96 hpf) = 0.13 mg/L	Ribeiro et al. (2014)

Table 2 (continued)

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
PVP Citrate	20 and 110	0.8, 4, 10, 20 and 50	120 h	Mortality and malformations: at 10 mg/L in dechorionized embryos and at 50 mg/L in chorionized embryos 20 nm > 110 nm and PVP > C	Kim and Tanguay (2014)
sock-Ag NP solution (*) spun-Ag NPs (**)	(1) 78 ± 11 (2) 39 ± 17	(1) 0.01-0.83 (2) up to 1.2	72 h	(1) LC_{50} (socks) = 0.26 mg/L, LC_{50} (spun) = 0.14 mg/L (2) *sod* was significantly up-regulated at the highest concentration	Gao et al. (2015)
Uncoated (NC) Uncoated nanotubes (NT)	20-50 nm 20-30 nm/1 µm long	Up to 0.01 Up to 0.001	72 h	NC, 264 genes significantly regulated (98 up, 166 down) NT, 175 genes significantly regulated (36 up, 139 down)	Park and Yeo (2015)
Uncoated	4 and 10	0.48, 0.96, 1.93, 3.85, 7.7, 11.55 and 23.1	96 h	Altered neurological development and transcription of genes related to neural development, metal-sensitive metallothioneins and *abcc* transporters	Xin et al. (2015)
Uncoated	10	0.003, 0.006, 0.012 and 0.024	120 h	72 h, mortality and malformations at 0.024 mg/L 120 h, apoptotic hair cell damage in the neuromasts induced at 0.024 mg/L	Yoo et al. (2016)

(*) Ag NP solution was removed from socks coated with Ag NPs and (**) centrifugated to separate Ag NPs from the solution. AChE: acetylcholinesterase; BCF: bioaccumulation factor; BIO: biocompatible gelatine preparations; BSA: bovine serum albumin; *cat*: catalase; DIS: dispersant; ER: endoplasmic reticulum; *hsp70*: heat shock protein 70; *Il1β*: interleukin-1 beta; *mt*: metallothionein; NM-300k: NM-300k Dis stabilized agent; ND: no data; PChE: Pseudocholinesterase; PVA: polyvinyl alcohol; PS: polyacrylate sodium; PVP: polyvinyl pyrrolidone; *sod*: superoxide dismutase; TGSH: total glutathione; Thiol: 16-mercaptohexacanoic acid; TSC: trisodium citrate.

Table 3.- Summary of main effects reported in adult zebrafish exposed to Ag NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
Citrate	20-30	up to 10	48 h	LC_{50} = 7.07 mg/L	Griffitt et al. (2008)
Uncoated	10-20	0.4 and 4	36 d	Alteration of *p53* gene pathway at 0.4 and 4 mg/L Defects in fin regeneration. Penetration into organelles and cell nucleus at 0.4 and 4 mg/L	Yeo and Pak (2008)
Uncoated	26.6 ± 8.8	10	48 h	Slight changes in the fill filaments Increase in the number of expressed genes from 148 at 24 h to 462 at 48 h	Griffitt et al. (2009)
Uncoated	5-20	30, 60, 120	24 h	DNA damage, oxidative stress and apoptosis induced in the liver at 120 mg/L	Choi et al. (2010)
PVP	81	0.018-0.143	48 h	100% mortality after 24 h (0.125 and 0.143 mg/L). Respiratory toxicity, increased rate of operculum movement and surface respiration from 0.072 mg/L. LC_{50} (48 hpf) = 0.084 mg/L	Bilberg et al. (2012)
Uncoated	ND	1.25, 2.5, 5 and 10	96 h	LC_{50} (96 h) = 2.9 mg/L	Kovrižnych et al. (2013)
Uncoated	3.1 ± 2.23	0.005, 0.015, 0.025 and 0.05	28 d	Silver accumulation in gills > carcass 3019 genes identified as differentially expressed in gills	Griffitt et al. (2013)
Uncoated	58.6 ± 18.6	500 mg/Kg food	14 d (dietary)	Changes in intestine microbiome	Merrifield et al. (2013)
PVP Citrate	9.2 63.45	0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 1, 3 and 5	96 h	PVP (physical method, top-down), LC_{50} = 0.54 mg/L C (chemical method, bottom-up), LC_{50} = 0.014 mg/L	Johari et al. (2014b)
Uncoated	16.6	16.76 2 and 4	4 d 21 d	AChE was significantly decreased in both exposure periods Gill Na^+/K^+-ATPase activity decreased significantly from the day 14^{th} Increased plasma glucose and cortisol levels indicating induce stress response	Katuli et al. (2014)

Table 3 (continued)

Coating/additive	Size	Concentration	Exposure	Main effects	Reference
Uncoated	8.39 ± 0.98	0.5	96 h (10 months in clean water)	The acute exposure in embryos did not impact the ability of adults to elevate cortisol when stressed	Massarsky et al. (2014b)
PVP	22-26	0.1	15 d	Liver oxidative stress, altered detoxification enzymes and affected brain AChE activity	Devi et al. (2015)
Citrate	20	1	4 h, 4d	Silver content in gills: AgC-20 > AgC-110; Reduction of Na^+/K^+-ATPase activity in gills and intestine: AgC-20 > AgC-110 Gills: fusion, hyperplasia and inflammation; Ag NPs adhered to the secondary filaments (AgC-20 > AgC-110) Intestine: increased number of goblet cells in the epithelial layer, microvilli reduction and partial damage to the lamina propria; Ag NPs adhered to the basolateral membrane of the mucosa (AgC-20)	Osborne et al. (2015)
	110			Vacuolization and partial damage to the lamina propria with abundant microvilli; Ag staining occurring on the apical membrane of the intestinal epithelial cells (AgC-110)	
Malva crispa Linn., leaves extract	24.1	0.23, 0.47, 1.42, 2.37, 2.84, 3.31	96 h	100% mortality at 2.84 and 3.31 mg/L; LC_{50} = 1.422 mg/L	Krishnaraj et al. (2016)
		0.71	14 d	Genotoxicity: micronuclei and nuclear abnormality in erythrocytes Cytological changes in gills and intrahepatic localization of Ag NPs Regulation of stress related (*mtf-1, hsp70*) and immune response (*tlr4, nfkb, il1β, cebp, trf, tlr22*) genes	
Citrate	63.45	0.0015, 0.00375, 0.0075 and 0.015	4 d	Increased diameter of gill filament and secondary lamellae, reduced length of secondary lamellae at 0.015 mg/L Gill injuries as fusion, necrosis, vacuolization, hyperplasia at all concentration tested	Mansouri and Johani (2016)
Uncoated	16.6	0.5, 1 and 2 mg/L (in *Artemia*) per day	14 d	Dose-dependent accumulation, greater accumulation at higher exposure concentrations; BMF < 1, not potential of trophic transfer	Rahmany et al. (2016)

AchE: acetylcholinesterase; *BMF:* biomagnification factor*; cebp:* CCAAT/enhancer binding protein (C/EBP)*; hsp70*: heat shock protein 70; *il1β:* interleukin-1 beta*; mtf-1*: metal transcription factor 1; ND: no data; *nfkb: Nuclear factor kβ;* PVP: polyvinyl pyrrolidone; *trf: transferrin; tlr22: toll like receptor22; tlr4:* toll like receptor4.

Table 4.- Summary of main effects reported in zebrafish exposed to different cadmium containing NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
embryos	CdSe/ZnS-PLL (QDs1) CdSe/ZnS-PEG5000-NH_2 (QDs2) CdSe/ZnS-PEG5000-COO- (QDs3) CdSe/ZnS-PEG5000-OCH_3 (QDs4)	9 14 14 14	0.0225 - 22.48	120 h	Malformations at 0.22 mg/L of QDs2, QDs3 and QDs4 LC_{50} (QDs1) = 0.787 mg/L; LC_{50} (QDs2) = 2.36 mg/L; LC_{50} (QDs3) = 3.15 mg/L, LC_{50} (QDs4) = 4.72 mg/L	King-Heiden et al. (2009)
	CdSe/ZnS (QDs1) MPA-CdSe/ZnS (QDs2) MPA-CdSe (QDs3)	6.5 7.5-8 6.5	1-25	120 h	Mortality, malformations and hatching rate decrease at ≥ 5 mg/L Toxicity ranking: QDs1>QDs3>QDs2	George et al. (2011)
	TGA-CdTe QDs	3.5	0 - 44.96	120 h	Mortality at ≥ 11.24 mg/L, malformations and hatching rate decreased at 22.48 mg/L LC_{50} = 0.0209 mg/L	Zhang et al. (2012a)
	MPA-CdSe QDs	3.5	0.05-31.25	120 h	Mortality, malformations ≥ 12.15 mg/L and hatching rate decreased ≥ 0.45 mg/L. LC_{50} = 1.98 mg/L	Zhang et al. (2012b)
	MPA-CdSe QDs + Cu^+	3.5	0.5	120 h	Mortality, malformations and hatching rate decreased. Joint exposure to QDs and Cu^+ facilitated the accumulation of copper	Zhang et al. (2012c)
	CdSe-undecylenic acid	181 ± 12	up to 100 mg/L	120 h	Hatching delay at 10 mg/L; totally inhibited at 100 mg/L	Ong et al. (2013)
	$CdSe_{core}/ZnS_{shell}$ QDs	3.6	0.02 – 22.48	120 h	Malformations increased at 2.25 mg/L. LC_{50} = 4.72 mg/L	Wiecinski et al. (2013)
	TGA-CdTe QDs + Cu^+	3.5	0.12-48	120 h	Mortality ≥ 0.01 mg/L (QDs + Cu^+), malformations and hatching rate decreased ≥ 0.1 mg/L (QDs + Cu^+) LC_{50} = 22.31 mg/L	Zhang et al. (2013)
	CdSe/ZnS-COOH (QDs-C) CdSe/ZnS, in toluene (QDs-P)	5	0.5 and 1	48 h (24 h depuration)	100% mortality after 9 h at 1 mg/L; Not accumulation at 0.5 mg/L. Distribution of QDs-P along the larvae surface, QDs-P not assimilated in the organism	Zarco-Fernández et al. (2016)
adults	CdSe/ZnS- PAA	ND	0.6 mg/L (in *Artemia*) per day	14 d (dietary)	Trophic transfer of Cd from brine shrimps to zebrafish. A low percentage of QDs were absorbed and transported to other tissues	Lewinski et al. (2011)
	CdS NPs	8 and 50	40 and 100 ng NPs/day/g body	36 and 60 d (dietary)	Gene expression level modifications, mutations and mitochondrial impairment	Ladhar et al. (2013)

MPA: mercaptopropionic acid; ND: no data; PAA: poly (acrylic acid)-octylamine copolymer; PLL: poly-L-lysine; PEG: poly (ethylene glycol); TGA: thioglycolic acid.

Table 5.- Summary of main effects reported in zebrafish exposed to Au NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
embryos	Citrate	3, 10, 50 and 100	0.027, 0.27, 2.7 and 26.7	120 h	No toxic effects detected	Bar-Ilan et al. (2009)
	Citrate	11.6	0.25, 0.53, 1.1, 2.1, 4.2, 6.3, 8.4, 10.5, 12.6	120 h	30% of mortality and 6% of malformation prevalence at 12.6 mg/L	Browning et al. (2009)
	PVA	15 and 35	10, 25, 50, 75 and 100	120 h	High levels of metal accumulation, but no toxic effects	Asharani et al. (2010)
	MBA	25 and 40	ND	96 h	No toxic effects detected	Wang et al. (2010)
	Uncoated	10	1, 5, 15 and 25	120 h	No toxic effects detected	George et al. (2011)
	TMAT MEEE MEE MES	0.8 and 1.5	0.016, 0.08, 0.4, 2, 10, 50 and 250	120 h	MEE- and MEEE- (neutral charge) no toxic effect detected TMAT-Au NPs (positively charged) more toxic than MES-Au NPs (negatively charged) in terms of mortality and malformations	Harper et al. (2011)
	Citrate	86.2	2.6, 5.1, 9.5, 20, 39 and 78	120 h	No toxic effects detected	Browning et al. (2013b)
	Uncoated	70.9	$4x10^{-4}$, $4x10^{-4}$, $8x10^{-4}$, 10^{-3}	72 h	No toxic effects detected	Ganeshkumar et al. (2013)
	Uncoated HA	12.8	20 and 100	96 h	No toxic effects detected	García-Cambero et al. (2013)
	TMAT	1.3	0.08, 0.4, 2, 10, 20, 30, 40 and 50	ND	Significant increase in cell death in the eye, and increase of *p53* and *bax* transcription at 10 and 30 mg/L. Mortality, malformations and abnormal behavior activity at ≥ 30 mg/L	Kim et al. (2013b)
	Uncoated	1.4	0.1-1000	120 h	Acute (24 h, 1000 mg/L) and delayed (120 h, 100 mg/L) toxicity with mortality, hatching delay and malformations	Rizzo et al. (2013)
	Uncoated	ND	up to 200	96 h	No toxic effects detected	Kovrižnych et al. (2013)

Table 5 (continued)

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
adults	Citrate	12 and 50	36 and 106 ng Au/fish/day	36 and 60 d (dietary)	Up-regulation of genes involved in DNA repair, detoxification processes, apoptosis, mitochondrial metabolism and oxidative stress. Alteration of genome composition using a RAPD-PCR genotoxicity test	Geffroy et al. (2012)
	Uncoated	ND	up to 200	96 h	No toxic effects detected	Kovrižnych et al. (2013)
	Citrate	14.3	16.5 and 55 µg/g dried sediment weight; $2.5x10^{-4}$ and $8x10^{-4}$ released in water column	20 d (sediment/ water)	Gold accumulation. Over-expression of genes involved in DNA repair, detoxification processes, mitochondrial metabolism and oxidative stress. Significant increase in brain and muscle AchE activity	Dedeh et al. (2015)

HA: hyaluronic acid functionalized; MBA: mercaptobenzoic acid; MEE: 2-,2-mercaptoethoxyethanol; MEEE: 2-,2-mercaptoethoxyethoxyethanol; MES: 2-mercaptoethanesulfonate; ND: no data; PVA: polyvinyl alcohol; TMAT: N,N,N-trimethylammoniumethanethio-ligand.

Table 6.- Summary of main effects reported in zebrafish exposed to ZnO NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
embryos	Uncoated	20	0.1, 0.5, 1, 5, 10 and 50	96 h	Mortality at ≥ 0.5 mg/L, effects on hatching rate and malformations ≥ 5 mg/L LC_{50} (96 hpf) =1.793 mg/L	Zhu et al. (2008)
	Uncoated	20	0.1, 0.5, 1.5, 10, 50 and 100	96 h	EC_{50} (84 h) = 23.06 mg/L (hatching) Malformations increased significantly at 50 and 100 mg/L Hatching delay, EC_{50} = 23.06 mg/L	Zhu et al. (2009)
	Uncoated	30	1, 5, 10, 25, 50 and 100	96 h	Mortality effects at 50 and 100 mg/L Malformations at ≥ 10 mg/L and effects on hatching rate at ≥ 1-10 mg/L	Bai et al. (2010)
	Uncoated	10	1, 5, 15 and 25	120 h	Hatching delay at 1 mg/L. Mortality was detected at 25 mg/L	George et al. (2011)
	Uncoated	23 ± 7	0.05, 0.5, 5, 25, 50, 100 and 200	120 h	Increased *hsp70* gene transcription attributed to NP dissolution	Lin et al. (2011)
	Uncoated Fe-doped	8.3-15 20.2	up to 50 mg/L	120 h	ZnO NPs inhibited the hatching protease activity	Xia et al. (2011)
	Uncoated	30	1, 2, 5, 10, 30 and 50	96 h	Mortality; LC_{50} (96 hpf) = 4.92 mg/L SOD and CAT activity, and GSH concentration reduced in the liver and increased in the gut at 5 mg/L	Xiong et al. (2011)
	Uncoated	30	1, 2, 5, 10, 30 and 50	96 h	Mortality; LC_{50} (96 hpf) = 3.969 mg/L	Yu et al. (2011)
	Uncoated	22.6 ± 5.1	0.05, 0.5, 5, 25, 50 100 and 200	ND	Exposure to ZnO NPs provoked ZHE1 inactivation and, therefore, hatching delay	Lin et al. (2012)
	Polymer Uncoated Uncoated leaf	20 ± 2 96 ± 123 169 ± 31	up to 100	120 h	Inhibition of hatching protease activity; Mortality at 10 and 100 mg/L of uncoated NPs and uncoated leaf	Ong et al. (2013)
	Uncoated	< 100	1, 5, 10, 20, 50 and 100	144 h	Malformations and hatching rate decrease ≥ 10 mg/L. SOD activity increase at ≥ 1 mg/L, MDA content increased and CAT activity decreased at ≥ 20 mg/L, ROS production increased ≥ 10 mg/L	Zhao et al. (2013)

Table 6 (continued)

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
embryos	Uncoated	9.4	0.2, 1 and 5	96 and 168 h	Hatching delay at 1 and 5 mg/L. *cat* and *Cu/Zn-sod* genes up-regulated after 96 h in embryos, and down-regulated after 48 h of exposure in eleuthero-embryos, *mt2* up-regulated after 48 h at 5 mg/L	Brun et al. (2014)
	Uncoated	50-70	0.1, 0.5, 1, 5 and 10	144 h	Hatching delay at ≥ 0.5 mg/L; Larval activity level, mean velocity, and maximum velocity altered at 5 and 10 mg/L	Chen et al. (2014)
	Uncoated	< 100	0.01, 0.1, 1, 5 and 10	120 h	Hatching delay at 5 and 10 mg/L	Vicario-Parés et al. (2014)
	Uncoated	8.35	0.08, 0.4, 2, 10 and 50	120 h	LC_{50} = 3.5-9.1 mg/L, mortality and malformations at 10 and 50 mg/L EC_{50} = 0.5-3.5 mg/L	Wehmas et al. (2015)
adults	Uncoated	30 ± 17	7690 ± 3580 mg Zn/kg dry weight (*D. magna*)	14 d (dietary)	ZnO transfer from *D. magna* exposed to uncoated and ZnO-octyl NPs to zebrafish	Skjolding et al. (2014)
	ZnO-OH NPs		37230 ± 2560 mg Zn/kg dry weight (*D. magna*)			
	ZnO-octyl NPs		287 ± 91 mg Zn/kg dry weight (*D. magna*)			

cat: catalase; GSH: reduced glutathione; *hsp70*: heat shock protein 70; MDA: malondialdehyde; *mt2*: metallothionein2; ND: no data; ROS: reactive oxygen species; SOD: superoxide dismutase.

Table 7.- Summary of main effects reported in zebrafish exposed to SiO_2 NPs. NMs sizes are shown in nm. Concentrations are shown in mg/L.

Stage	Coating/additive	Size	Concentration	Exposure	Main effects	Reference
embryos	FSNPs	60 and 200	0.25, 2.26 and 25.6 0.2, 2 and 200	96 h	No toxic effects detected	Fent et al. (2010)
	SiO_2 NPs Nanowires	50 and 200 55 nm x 2.1µm	10^{-5}, 10^{-4} and 10^{-3}	132 h (injected into yolk sac)	No toxic effects detected with SiO_2 NPs Nanowires provoked mortality and malformations in embryos injected during the 1- or 2- cell stage (1 mg/L, corresponding with 3 pg of material)	Nelson et al. (2010)
	Uncoated	30	1, 5, 15 and 25	120 h	No toxic effects detected	George et al. (2011)
	MSRMs	ND	0.25, 2.5, 25, 100 and 200	144 h	Malformations at ≥ 2.5 mg/L	Liu et al. (2012)
	MSNP	200	10000	120 h (injected into yolk sac)	No toxic effects detected	Sharif et al. (2012)
	Si NPs	62	25, 50, 100 and 200	96 h	Mortality and malformations at 100 and 200 mg/L	Duan et al. (2013a)
	Si NPs	62	25, 50, 100 and 200	96 h	Mortality and malformations at 100 and 200 mg/L	Duan et al. (2013b)
	UA	15 ± 2	up to 100	120 h	No toxic effects detected	Ong et al. (2013)
	Uncoated	ND	up to 1600	96 h	No toxic effects detected	Kovrižnych et al. (2013)
	Si NPs	62.14±7.16	1000, 2000, 3000, 6000 and 12000	24 h (intravenous microinjection)	Pericardial edema and bradycardia at ≥ 6000 mg/L Inhibition of calcium signaling pathway, induction of cardiac dysfunction via the neutrophil-mediated cardiac inflammation and cardiac contraction at all tested concentrations	Duan et al. (2015)
adults	Uncoated	ND	up to 1600	96 h	No toxic effects detected	Kovrižnych et al. (2013)

FSNPs: fluorescent SiO_2 NPs; MSNPs: mesoporus SiO_2 NPs; MSRMs: Maghemite@SiO_2 Rattle Type Microspheres; ND: no data; UA: undecylenic acid.

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II. STATE OF THE ART, OBJECTIVES & HYPOTHESIS

STATE OF THE ART

The development of nanotechnology and the massive production of nanomaterials are increasing rapidly, being nanomaterials currently applied in different consumer and industrial products of everyday use. The nanomaterials containing wastes produced from these processes and products have the aquatic environment as their ultimate fate, being necessary to improve the information about their behavior and hazard once they have been released into the environment. Nowadays, it is not feasible to know the real concentration of nanomaterials in the different environmental compartments because of the limitations of the analytical techniques, being the use of predictive models the most used approach. However, these predictive models do not always take into account the environmental fate, persistence and bioavailability of nanomaterials. Due to their "nano" size, nanomaterials present specific physico-chemical properties in comparison with their bulk form or soluble (ionic) counterparts, especially regarding the aggregation or dissolution behavior. Moreover, these properties can be modified by abiotic characteristics of the exposure media, such as pH, ionic strength or organic matter content. Once in the environment, nanomaterials can enter into the aquatic organisms by different routes, as by the gills and skin in the case of suspended particles or by the intestine in the case of particles ingested with the diet. Thus, studies of the potential toxic effect and risk assessment of metal and metal bearing nanoparticles for aquatic organisms are required. In addition, to determine whether these effects vary depending on the form of the metal (nano, bulk or ionic) is of great concern for regulatory purposes.

Zebrafish, the selected organism for this study, is an animal model increasingly used to study the nanotoxicity of different metal and metal bearing nanoparticles, due to the advantages that both embryos and adults present. The toxicity test with zebrafish embryos (up to 120 hours post fertilization) has been standardized by the OECD, and has been already applied to evaluate the toxicity of a high amount of chemicals in short periods of time, being considered as an alternative method to animal testing and a high throughput methodology for toxicity screening. This approach has allowed testing different combinations of nanomaterials, with diverse shapes, sizes, coatings, etc. Moreover, this test can be a useful tool to compare the effects provoked by the

nanomaterials and those provoked by their corresponding ionic and bulk forms in a broad range of concentrations, allowing to determine the specific effects produced by the nano-sized materials.

In zebrafish, exposure to metal and metal bearing nanoparticles may result in metal uptake and bioaccumulation into the different organs of the organism. Once inside, nanoparticles can cause toxicity through cellular mechanism involving oxidative stress, genotoxicity or damage to the lysosomal compartment. Therefore, a battery of biomarkers covering these cellular processes can help to understand the effects provoked by the nano-size in comparison to those provoked by the ionic form of the metals. Toxicogenomics is an useful tool to assess the mechanisms of action of diverse contaminants, enabling to screen a large set of genes or even entire transcriptome for genes differentially transcribed. Diverse studies have shown that exposure to different nanomaterials affects the transcription of genes involved in oxidative stress, DNA damage and genotoxicity. Therefore, the assessment of the differential response of the whole zebrafish genome to the nanoparticle exposure in comparison to the ionic counterpart can be useful to understand the toxicity of the nanomaterials.

A wider knowledge on the effect caused by metal and metal bearing nanoparticles to the aquatic organisms in comparison to the effect provoked by other metal forms is necessary for a proper risk assessment of these materials and to understand and prevent the consequences for the aquatic environment of their release.

HYPOTHESIS

The exposure to metal and metal bearing nanoparticles can provoke toxic effects on developing zebrafish embryos. These effects can vary depending on the intrinsic physicochemical characteristics of the nanoparticles and may differ from those provoked by other forms of the metal. In adult zebrafish, the waterborne and dietary exposure to metal and metal bearing nanoparticles can lead to bioaccumulation and effects related to oxidative stress, genotoxicity or damage at different levels of biological organization which can be assessed using a battery of biomarkers ranging from the molecular to the tissue level.

OBJECTIVES

In order to prove this hypothesis true and in order to improve our understanding of the environmental effects of nanoparticles in aquatic environment, the following general objectives were addressed:

1- To study the acute and sublethal toxicity, in comparison to that of the ionic and bulk form of the metal, of a set of metal and metal bearing NPs (Ag, Au, CdS, ZnO and SiO_2) combining several properties, such as different sizes, shapes and coatings in zebrafish embryos using the fish embryo toxicity (FET) test.

2- To study the sublethal effects to adult zebrafish produced by the aqueous exposure to maltose-coated Ag NPs of 20 nm for 21 days in comparison with those provoked by the same nominal concentration of ionic silver, as well as to evaluate the potential long-term effects or the recovery after the cease of the exposure.

3- To decipher the metabolic pathways altered by the exposure to Ag NPs of 20 nm in comparison to those altered by ionic silver through the analysis of the whole liver transcriptome of adult zebrafish after 3 and 21 days of exposure).

4- To study the acute and sublethal effects to adult zebrafish produced by the aqueous exposure to CdS NPs of 3.5-4 nm for 21 days in comparison with those provoked by the same nominal concentration of ionic silver, as well as to evaluate the potential long-term effects or the recovery after the cease of the exposure.

5- To decipher the metabolic pathways altered by 3 and 21 days of exposure to CdS NPs compared with results obtained after the exposure to the same nominal concentration of ionic cadmium, through the analysis of the whole liver transcriptome of adult zebrafish.

6- To test the acute toxicity of PVP/PEI-coated Ag NPs of 5 nm to larvae of the crustacean *Artemia sp* and to zebrafish embryos as a previous step to assess bioaccumulation and effects of dietary exposure for 21 days of adult zebrafish to Ag NPs using a simplified food web of two trophic levels.

III. RESULTS & DISCUSSION

CHAPTER I

Effects of the exposure to different metal bearing nanoparticles (Ag, Au, CdS, ZnO and SiO_2) on developing zebrafish embryos

This chapter has been published as:

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Parts of this chapter have been presented at:

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23rd Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC Europe). Glasgow (Scotland), 12th-16th May 2013. **JM Lacave**, A Retuerto, D Gilliland, MP Cajaraville, A Orbea. "Interactions between (fluorescent) silica nanoparticles and developing zebrafish embryos". Poster.

7th Special Science Symposium "Fate and ecotoxicity of nanoparticles in the environment (SETAC Europe). Brussels (Belgium), 2nd-3rd October 2013. A Orbea, U Vicario-Parés, **JM Lacave**, D Berhanu, P Reip, M Oron, D Gilliland, E Valsami-Jones, MP Cajaraville. "Toxicity ranking and sublethal effects of metal nanoparticles using zebrafish as model organism". Poster.

3rd Edition of the Largest European Event in Nanoscience & Nanotechnology (ImagineNano). Bilbao (Spain), 10th-13th March 2015. MP Cajaraville, A Katsumiti, A Jimeno-Romero, **JM Lacave**, I Marigómez, M Soto, A Orbea. "An integrated multispecies two-tiered approach for the environmental risk assessment of nanomaterials: a case study with Ag NPs". Oral presentation.

ABBREVIATIONS

FET, Fish embryo toxicity

FSNP, Fluorescent silica nanoparticles

hpf, Hours post fertilization

LC_{50}, Lethal concentration to 50% of the population

NMs, Nanomaterials

NPs, Nanoparticles

PVP, Polyvinylpyrrolidone

QDs, Quantum dots

ZHE1, Zebrafish hatching enzyme

ABSTRACT

Due to the increasing commercialization of consumer and industrial products containing nanoparticles (NPs), an increase in the input of these materials in the environment is expected. NP toxicity to aquatic organisms depends on multiple biotic and abiotic factors resulting in an unlimited number of combinations impossible to test in the practice. The zebrafish embryo model offers an useful screening tool to test and rank the toxicity of nanomaterials depending on those diverse factors. This work aims to study the acute and sublethal toxicity of a set of metal bearing NPs displaying different properties, in comparison to that of the ionic and bulk forms of the metals, in order to establish a toxicity ranking. Soluble NPs (Ag, CdS and ZnO) showed the highest acute and sublethal toxicity, with LC_{50} values as low as 0.529 mg Ag/L for Ag NPs of 20 nm, and a significant increase in malformation prevalence in embryos exposed to 0.1 mg Cd/L of CdS NPs of ~4 nm. For insoluble NPs, like SiO_2 NPs, acute effects were not observed during early embryo development due to the protective effect of the chorion. But effects on larvae could be expected since deposition of fluorescent SiO_2 NPs over the gill lamella and excretion through the intestine were observed after hatching. In other cases, such as for gold NPs, toxicity could be attributed to the presence of additives (sodium citrate) in the NP suspension, as they displayed a similar toxicity when tested separately. Overall, results indicated that toxicity to zebrafish embryos depends primarily on the chemical composition and, thus, solubility of the NPs. Other characteristics, such as size, played a secondary role. This was supported by the observation that ionic forms of the metals were always more toxic than the nano forms and bulk forms were the least toxic to developing zebrafish embryos.

Key words: metal bearing nanoparticles, embryo toxicity, zebrafish

LABURPENA

Nanopartikulak (NPak) dauzkaten produktu industrial eta kontsumo-produktuen komertzializazioa geroz eta handiagoa denez, material hauen sarreraren emendioa espero da ingurunean. NPek organismo urtarretan duten toxikotasuna faktore biotiko eta abiotiko askoren menpekoa da, konbinazio anitzak sortarazten dituztenak eta praktikan aztertzea ezinezkoa gertatzen direnak. Zebra arrainaren enbrioiaren ereduak faktore desberdinen araberako nanomaterialen toxikotasuna testatzeko eta sailkatzeko tresna erabilgarria eskaintzen du. Lan honen helburua ezaugarri desberdinak azaltzen dituzten NP metaldun multzo baten toxikotasun azkar eta subletala ikertzea da, metalen aldaera ioniko eta masiboarekin konparatuz, toxikotasun araberako sailkapena ezartzeko. NP disolbagarriek (Ag, CdS eta ZnO) toxikotasun azkar eta subletal handiena erakutsi zuten. 20 nm-ko Ag NPek 0.529 mg Ag/L-ko LC_{50} balio baxua erakutsi zuten eta ~4 nm-ko CdS NP-en 0.1 mg Cd/L pean egondako enbrioietan malformazioen prebalentziaren igoera behatu zen. SiO_2 bezalako NP disolbagaitzen kasuan ez ziren efektu azkarrik ikusi enbrioien garapen goiztiarrean, korionaren efektu babeslea dela eta. Hala ere, epe luzeko ondorioak espero zitezkeen larbetan, eklosioaren ondoren SiO_2 NP fluoreszenteak zakatzen lamelen gainean eta heste bidezko eskrezioa ikusi baitziren. Beste kasu batzuetan, urre NPetan esaterako, toxikotasuna NPen suspentsioko gehigarrien (sodio zitratoa) presentziari egotzi dakioke, aparte testatutakoan antzeko toxikotasuna erakutsi zuenez. Orokorrean, emaitzek adierazten dute zebra arrainetan toxikotasuna konposaketa kimikoaren menpekoa dela nagusiki, beraz, NPen disolbagarritasunaren menpekoa. Beste ezaugarri batzuek, tamainak esaterako, bigarren mailako papera jokatzen dute. Hori, ondorengo behaketek sostengatu zuten: metalen aldaera ionikoak nano aldaerak baino toxikoagoak ziren eta aldaera masiboa zebra arrainaren garapen enbrionarioan zehar toxikotasun baxuenekoa izan zen.

Gako-hitzak: metaldun nanopartikulak, enbrioiaren toxikotasuna, zebra arraina

RESUMEN

Debido al incremento en la comercialización de productos industriales y de consumo que contienen nanopartículas (NPs), se espera un incremento en la entrada de estos materiales en el medio ambiente. La toxicidad de las NPs en los organismos acuáticos depende de múltiples factores bióticos y abióticos que resultan en un número ilimitado de combinaciones que son imposibles de testear en la práctica. El modelo de embriones de pez cebra ofrece una valiosa herramienta de cribado para testear y establecer una clasificación de la toxicidad de los nanomateriales dependiendo de diversos factores. El objetivo de este trabajo fue estudiar la toxicidad aguda y subletal de un grupo de NPs metálicas y que presentaban diversas propiedades, en comparación con las formas iónicas y masivas de los mismos metales, para clasificarlas según su toxicidad. Las NPs solubles (Ag, CdS, ZnO) presentaron la mayor toxicidad aguda y subletal, con valores de LC_{50} tan bajos como 0.529 mg Ag/L para las NPs de Ag de 20 nm y un incremento significativo de la prevalencia de malformaciones en embriones expuestos a 0.1 mg Cd/L de NPs de CdS de ~4 nm. En el caso de las NPs insolubles, como el SiO_2, no se observaron efectos agudos durante el desarrollo temprano del embrión debido al efecto protector del corion, aunque se podrían esperar efectos a largo plazo ya que tras la eclosión se observaron NPs fluorescentes de SiO_2 depositadas sobre las laminillas de la branquia y su excreción a través del intestino. En otros casos, como en el de las NPs de oro, la toxicidad se puede atribuir a la presencia de aditivos (citrato de sodio) en la suspensión de las NPs, el cual provocó una toxicidad similar cuando se testeó por separado. En general, estos resultados indican que la toxicidad depende principalmente de la composición química y, por tanto, de la solubilidad de las NPs. Otras características, como el tamaño, juegan un papel secundario. Esta conclusión se basa también en la observación de que la forma iónica de los metales fue siempre más tóxica que la forma nano y la forma masiva fue la menos tóxica para el desarrollo embrionario del pez cebra.

Palabras clave: nanopartículas metálicas, toxicidad embrionaria, pez cebra

INTRODUCTION

Nanoparticles (NPs) have been present in the environment from millions of years; they originate naturally from combustion processes such as forest fires and volcanoes (Oberdöster et al., 2005). However, the interest on the study of NP toxicity is growing due to the increasing use of manufactured nanomaterials (NMs) in consumer and industrial products (Baker et al., 2014). The number of their potential applications is growing rapidly due to the unique electronic, optical, magnetic and catalytic properties of NMs compared with the corresponding bulk materials (Bohnsack et al., 2012). As consequence, an increasing input into the environment is expected. Soil and water compartments represent the ultimate fate of these materials, whether they have been released directly into them or indirectly, for instance, via sewage treatment plants, waste handling or aerial deposition (Nowack et al., 2007). The small size and, thus, the large specific surface area of NPs confer them specific properties. Increased surface reactivity predicts that NPs exhibit greater biological activity per given mass than larger particles, which could lead to undesired effects, such as increased toxicity (Oberdöster et al., 2005).

Among all the classes of manufactured NMs, metal bearing NPs, including metal oxide NPs, have received special attention due to their use in a variety of applications (Klaine et al., 2008). Metal and metal oxide NPs are easily synthesized from many metals as silver, gold, cadmium, zinc or silicon. Some of these metals are toxic to aquatic organisms in their soluble and/or bulk form. In addition, a specific toxic effect derived from the nanoparticulated size of these metals is expected due to their properties mentioned above (Griffitt et al., 2009).

The nano-specific toxicity of NPs to aquatic organisms depends on multiple factors, some intrinsic to the NP including chemical composition, size, shape, surface functionalization and presence of coatings or additives that influence physicochemical properties driving NP behavior (Baker et al., 2014; Katsumiti et al., 2014a; Katsumiti et al., 2015a). Other factors include the physicochemical characteristics of the receiving media and the target organisms (Batley et al., 2013). This means an unlimited number of combinations impossible to test in the practice. Recently, it has been highlighted the

need of high throughput experimental methods able to generate large volumes of data that in a near future could feed *in silico* methods to estimate NP toxicity based on their properties (Winkler et al., 2013).

Zebrafish (*Danio rerio*) embryos are good candidates for a high-throughput *in vivo* testing system (George et al., 2011; Lin et al., 2011; 2012). Among other characteristics, zebrafish have a rapid embryonic development (4-5 days) with a beating heart and visible erythrocytes by 24 h (Bahary and Zon, 1998). Zebrafish eggs and embryos are small and transparent, allowing reasonable sample sizes to be tested together using a simple cell culture plate to provide several experimental replicates at one time (Hill et al., 2005). Recently, zebrafish embryos have been successfully used as *in vivo* sensors for the detection of metal ions by dye-assembled upconversion NPs (Peng et al., 2015), which can suppose a step forward in the study of metal NPs toxicity. Thus, in this work we have used the zebrafish embryo model (OECD TG236, 2013) to study the acute and sublethal toxicity of a set of metal-bearing NPs (Ag, Au, CdS, ZnO and SiO_2) displaying different properties, such as different sizes, shapes and coatings in comparison to that of the ionic and bulk form of the metals. In addition, fluorescent SiO_2 NPs were used to study the interaction of these NPs with developing zebrafish embryos. Results were used to establish a toxicity ranking depending on those properties and to compare with the toxicity of the ionic and bulk forms of the metals. Comparisons between ionic and nanoparticulated metals have been addressed in several works, but comparisons with the bulk counterparts are scarce. Filling this knowledge gap is of upmost importance in order to identify possible nano-specific effects that could be relevant for regulatory purposes (Duester et al., 2014), since usually nano and bulk forms, but not ionic forms, share the same molecular formula.

MATERIALS AND METHODS

Nanoparticles and other metal compounds

Maltose-coated Ag NPs, citrate-coated Au NPs, glutathione-capped CdS quantum dots and L-arginine stabilized SiO_2 NPs were synthesized by wet chemistry at Joint Research Centre (Ispra). Ecodis-P90 stabilized ZnO NPs were produced by milling at Dead Sea Laboratories (Israel). A summary of the main characteristics of the NP suspensions

used in this study is shown in Table 1. Further details on synthesis and characterization have been already published by Katsumiti et al. (2014b; 2015a; 2015b).

Silver nitrate solution (1 g Ag/L in 0.5 M HNO_3 matrix) was obtained from Spectrosol, BDH chemical Ltd Poole (England). Remaining bulk materials and metal salts were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Fish maintenance and breeding

Zebrafish (wild type AB Tübingen) were maintained in a temperature-controlled room at 28 ºC with a 14-hour light/10-hour dark cycle in 100 L tanks provided with mechanic and biological filters following standard protocols as described in Vicario-Parés et al. (2014). Fish were fed Vipagran baby (Sera) and *Artemia* nauplii twice per day.

Breeding fish were selected and separated in a tank. Females and males were maintained separately, in order to avoid continuous spawning. The day prior to the beginning of the exposures, one female and one male zebrafish were placed separately in each breeding tramp which had previously been located in a 2 L tank. Fish were left overnight and, just before the light switched on in the morning, the separation was removed. The resulting eggs were collected in a Petri dish and fertilized viable eggs were selected under a stereoscopic microscope (Nikon smz800, Kanagawa, Japan). During the procedure of embryo selection, water salinity was reduced gradually. Finally, fertilized eggs were transferred to the exposure microplates.

Fish Embryo Toxicity (FET) test

Working suspensions at the selected concentrations were prepared the day of the beginning of the test by diluting stock solutions with deionized water and stirring. In all the cases, pH ranged from 6.5 to 7.5.

Table 1.- Summary of the characterization data of the NPs used in this study.

NP	Size (nm)	Stock solution concentration (g/L)	Zeta Potential	pH	Additive	Aggregate formation	Ions release in milliQ/ $NaNO_3$	Reference
Ag20-Mal	24	0.107	- [30-35]	7.4	Maltose (0.25 mM)	M/S	Yes	Katsumiti et al. (2015b)
Ag40-Mal	44		- 49					
Ag100-Mal	96		- [30-35]					
Au5-Cit	4.4	0.1	-35	6.3	Sodium citrate (2.5 mM)	M/S	No	Katsumiti et al. (2015a)
Au15-Cit	13.5			6.1				
Au40-Cit	40.4			6.9	Sodium citrate (0.5 mM)			
CdS5-GSH	3.5–4	4.7	N/C		---	N/S	Yes	Katsumiti et al. (2014)
ZnO<130-EcoP90	20-70	1000	- 39.9		Ecodis P90 (10 g/L)	N/C	Yes	Katsumiti et al. (2015a)
ZnO<280-EcoP90	500x260x10-100		- 54.5					
SiO_2-15	15	3.8	N/C		---	N/S	No	Katsumiti et al. (2015a)
SiO_2-30	30	1.13	N/C					
SiO_2-70	70	3.7	N/C					
Fluorescent SiO_2 Ru-27	27	2.4	N/C		---	N/S	No	Katsumiti et al. (2015a)

N/C: Not calculated. N/S: no significant aggregation detected. M/S: minor/slow aggregation with formation of limited number of larger particles.

The test was carried out in covered 24-well polystyrene microplates placing one embryo per well in 2 mL of test solution. In each microplate two different concentrations were tested (10 embryos in each concentration). In the remaining wells, four control embryos were placed in deionized water. For each compound three replicates were prepared, resulting in 30 embryos exposed to each concentration and 36 control embryos. For each compound, five concentrations (Table 2) were selected according to the expected toxicity based on literature data. Exposure started just after embryo selection and lasted up to 120 hours post fertilization (hpf). The test was considered valid only when survival rate in the control group was ≥ 90% (OECD 236, 2013).

Daily and up to the end of the test, embryos were examined to determine survival rate (as the percentage of alive embryos at 120 hpf), hatching rate (as the percentage of embryos that have hatched during the 120 h exposure period though some of them could have died by 120 hpf), hatching time (as the time that embryos need to hatch) and malformation prevalence (as the percentage of malformed embryos over surviving embryos at 120 h). Normal embryo morphology was based on Kimmel et al. (1995). Malformations were recorded and photographed under a stereoscopic microscope (Nikon AZ100, Kanagawa, Japan).

Exposure to fluorescent SiO_2 NPs (FSNP)

In the case of the exposure to FSNPs, three concentrations (0.1, 10 and 100 mg Si/L) were selected based on the results of the FET experiments. 50 newly fertilized eggs per concentration were placed in a Petri dish with 50 mL of NP solution. 50 control embryos were maintained in deionized water. Exposed and control larvae were examined under a confocal microscope (Olympus Fluoview FV500, Tokyo, Japan) at 6, 30, 54, 78, 102 and 126 hpf.

Statistical analyses

Statistical analyses were done using the SPSS statistical package v20.0 (SPSS Inc, Microsoft Co, WA, USA). According to the Kolmogorov-Smirnov test (p<0.05) data did not follow a Normal distribution. Thus, data on survival and hatching rates and

malformation prevalence were analyzed by Fisher´s exact test (p<0.05). For hatching time, the non-parametric Kruskal-Wallis test was applied followed by the Dunn's post hoc test (p<0.05). LC_{50} values were calculated through a Probit model (p<0.05). Estimation of parameters was performed using the Firth method (Firth, 1993) in R 3.1.0, whenever convergence was not obtained using the maximum likelihood method (Kosmidis, 2013).

RESULTS

Silver

The smallest Ag NPs (Ag20-Mal) resulted the most toxic of the three sizes (LC_{50}= 0.529 mg Ag/L), causing 93.64% mortality at 1 mg Ag/L. Ag100-Mal NPs provoked 100% mortality at 5 mg Ag/L (LC_{50}= 1.973 mg Ag/L), whereas Ag40-Mal were the least toxic NPs (LC_{50}= 3.94 mg Ag/L) causing 66.67% mortality at this concentration (Fig 1A). Ionic silver was the most toxic silver form for developing zebrafish embryos. 100% mortality of exposed embryos was observed at 0.1 mg Ag/L (LC_{50}= 0.047 mg Ag/L). In all cases embryos died before hatching, which may indicate that the metal passed through the chorion pores. Bulk Ag and maltose did not cause significant mortality on the test organisms at any of the tested concentrations (Fig 1A).

The results obtained for hatching rate at 120 h followed a similar pattern compared to that obtained for the survival rate (data not shown), indicating that all surviving embryos had hatched at 120 hpf. No significant effects were observed for hatching time, except in the case of the exposure to Ag20-Mal NPs, which at 1 mg Ag/L anticipated hatching (Fig 1B). Nevertheless, this observation should be considered carefully, since only two embryos survived to this treatment.

Exposure to Ag40-Mal NPs and Ag100-Mal NPs at 1 and 5 mg Ag/L, respectively, caused a significant increase in malformation prevalence (Fig 1C). Some malformations were also observed in embryos exposed to Ag20-Mal NPs, although in this case the prevalence increase was not statistically significant due to the low number of surviving embryos. Yolk sac edema was the most frequently recorded malformation. Eye abnormality, pericardial edema, tail and/or spinal cord flexure, and finfold abnormality

were other malformations observed (Table 3). Malformations were not observed in surviving embryos exposed to bulk or ionic silver or to maltose.

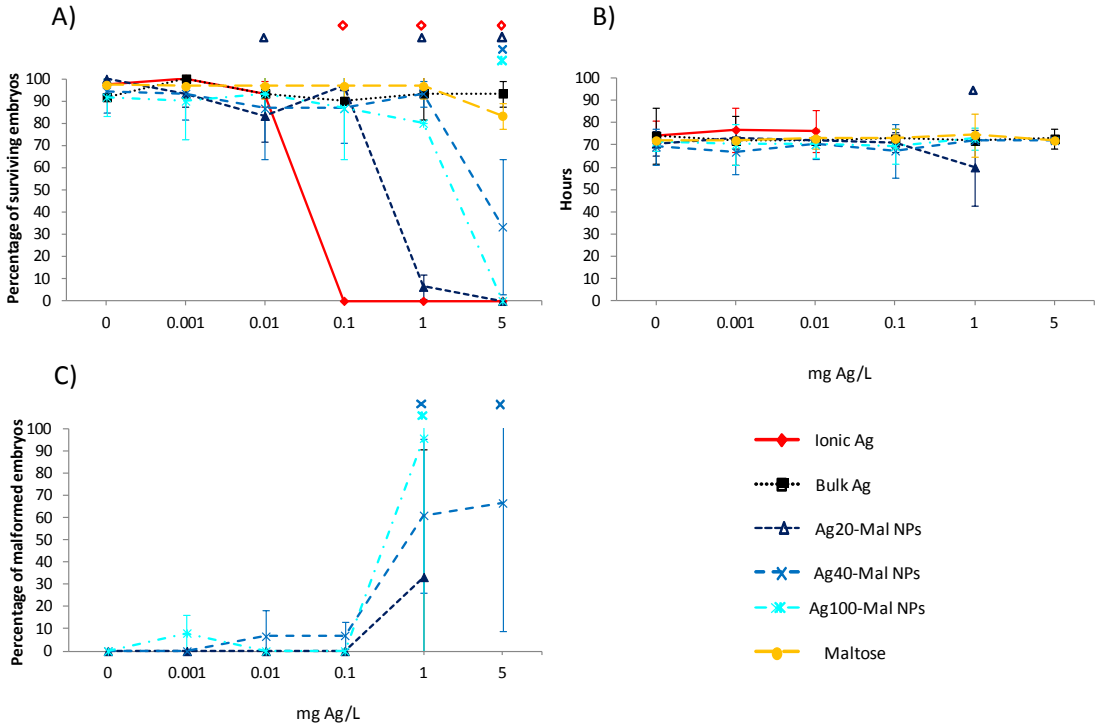

Figure 1.- Effects of the exposure of zebrafish embryos to Ag NPs and related compounds for 120 h. A: survival rate; B: hatching time; C: malformation prevalence. The empty symbols indicate significant differences ($p < 0.05$) with respect to the control group.

Gold

Under exposure to Au NPs of three sizes, significant effects on survival rate were registered only at the highest concentrations (50 and 100 mg Au/L, Fig 2A). At these concentrations, individuals died after hatching. The LC_{50} values obtained for the three NP suspensions (24.655 mg Au/L for Au5-Cit NPs; 24.61 mg Au/L for Au15-Cit NPs; 34.717 mg Au/L for Au40-Cit NPs) indicated a slight size-dependent effect, as the largest NPs were the least toxic. Similar effects were recorded when embryos were exposed only to the equivalent concentration of sodium citrate present in the NP suspension. As described for silver, ionic gold was the most toxic form of the metal for the test organisms (LC_{50}= 4.619 mg Au/L). At concentrations ≥ 10 mg Au/L, 100% of the individuals died by the first day of the exposure. Bulk Au did not cause mortality in the organisms (LC_{50} >> 100 mg Au/L).

The results obtained for hatching rate showed that embryos exposed to high concentrations of Au NPs and sodium citrate hatched, although they did not survive at the end of the exposure time. Thus, hatching rate was higher than survival rate (Fig 2B). Exposure to high concentrations (≥ 10 mg Au/L) of Au5-Cit and Au15-Cit NPs produced significant effects in the time that embryos needed to hatch, since embryos hatched earlier than in the control group. Exposure to the highest concentration (100 mg Au/L) of Au40-Cit NPs caused the same effect. In the case of sodium citrate exposed embryos, significant differences were only observed at low concentrations (equivalent to that present in the NP suspensions containing 0.1 and 1 mg Au/L), since embryos hatched earlier than in the control group (Fig 2C). Exposure to ionic gold at concentrations of 0.1 and 1 mg Au/L produced hatching delay in the embryos. Under exposure to bulk Au, significant differences were not observed in the time needed to hatch at any of the tested concentrations.

Malformations were not observed in surviving embryos exposed to different Au compounds or to sodium citrate.

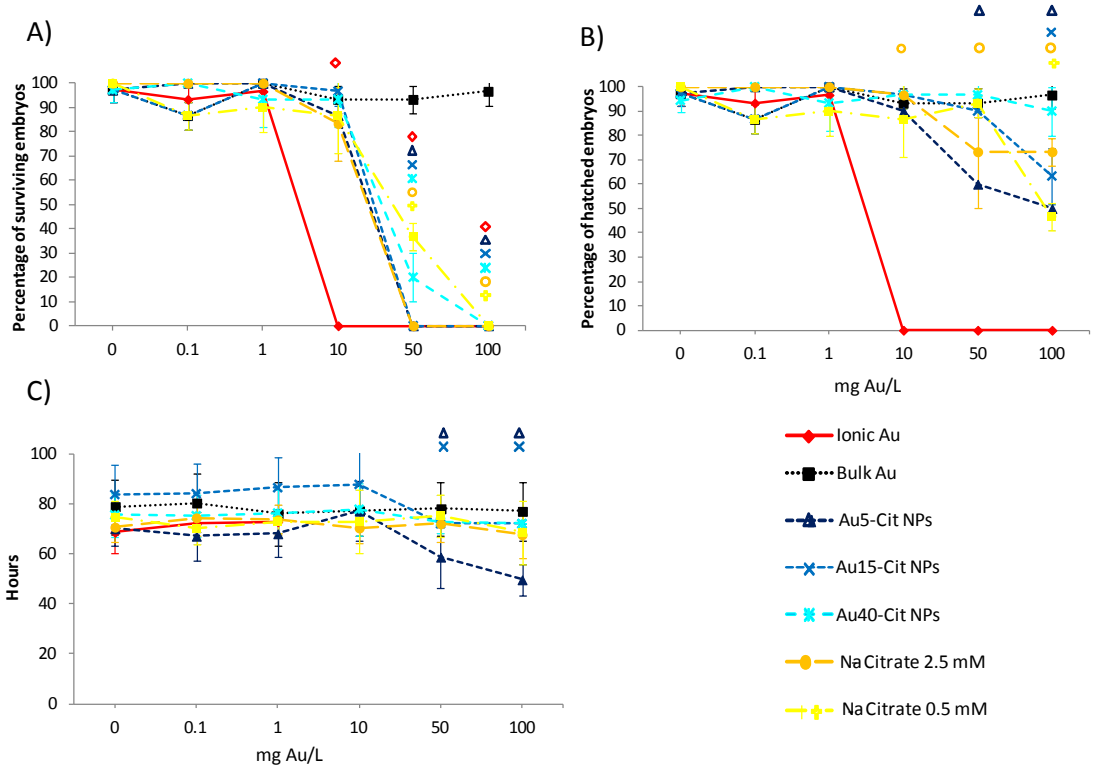

Figure 2.- Effects of the exposure of zebrafish embryos to Au NPs and related compounds for 120 h. A: survival rate; B: hatching rate; C: hatching time. The empty symbols indicate significant differences ($p < 0.05$) with respect to the control group.

Cadmium

Significant effects on the survival rate of embryos exposed to CdS NPs were observed at lower cadmium concentrations (0.1 mg Cd/L) than in the case of embryos exposed to the ionic form of the metal (5 mg Cd/L) (Fig 3A). Nevertheless, in terms of LC_{50} value, ionic cadmium resulted the most toxic cadmium form for developing zebrafish embryos (LC_{50}= 3.082 mg Cd/L for ionic cadmium and LC_{50} = 7.036 mg Cd/L for CdS NPs). At 10 mg Cd/L, ionic cadmium provoked mortality to 100% of the embryos. Bulk CdS was the least toxic form (LC_{50}= 7.868 mg Cd/L). Significant effects on survival rate were only registered at the highest concentration tested (10 mg Cd/L). In general, at the highest concentrations of exposure (5 and 10 mg Cd/L) embryos died after hatching.

Embryos exposed to low concentrations of cadmium (up to 1 mg Cd/L) presented similar patterns of hatching rate and survival rate. At higher concentrations (5 and 10 mg Cd/L), hatching rate was higher than survival rate because embryos died after hatching (Fig 3B), as mentioned previously. In embryos exposed to 10 mg Cd/L of CdS NPs a significant hatching delay was observed, but at 0.01 and 0.1 mg Cd/L embryos hatched significantly earlier (Fig 3C) than control embryos. For embryos exposed to ionic cadmium no significant differences were observed in the time needed to hatch. Exposure to bulk CdS induced a significant hatching delay at concentrations ≥ 0.1 mg Cd/L (Fig 3C).

Exposure to CdS NPs and bulk CdS at 0.1, 1 and 5 mg Cd/L caused a significant increase of malformation prevalence (Fig 3D). Observed malformations were yolk sac edema, pericardial edema, spinal cord flexure and fin fold abnormality (Table 3). No significant differences were observed at 10 mg Cd/L due to the high mortality recorded at this concentration. In the case of ionic cadmium, no significant differences in malformation prevalence were observed.

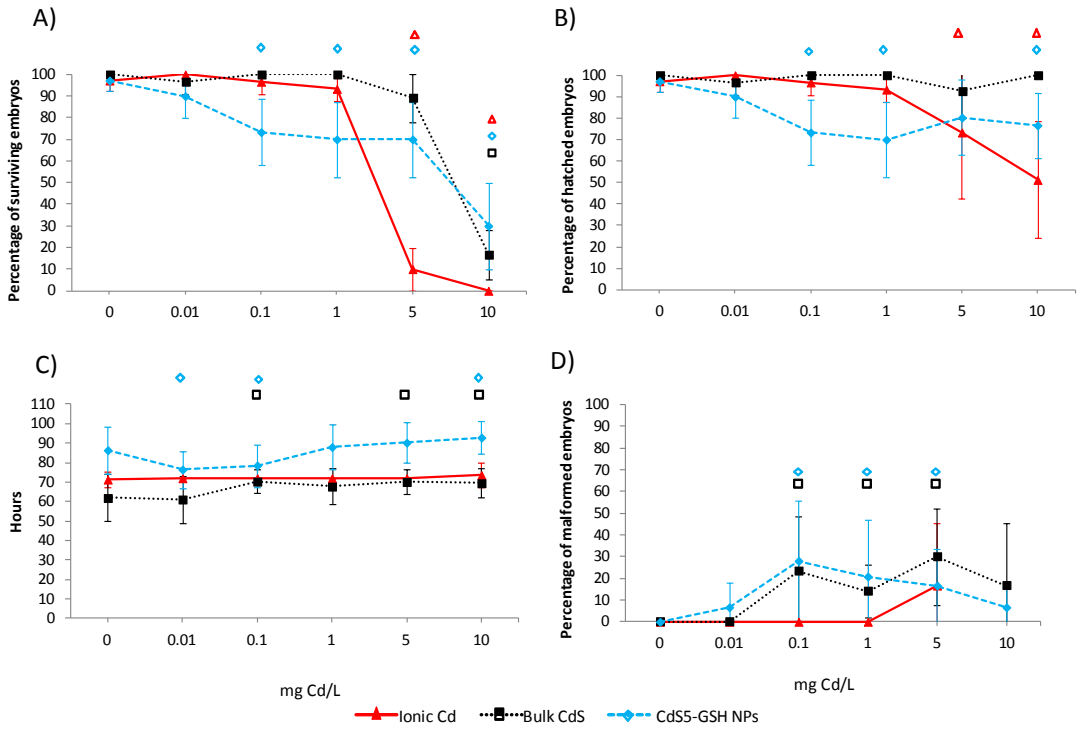

Figure 3.- Effects of the exposure of zebrafish embryos to CdS NPs and related compounds for 120 h. A: survival rate; B: hatching rate; C: hatching time; D: malformation prevalence. The empty symbols indicate significant differences ($p < 0.05$) with respect to the control group.

Zinc

Exposure to the different zinc forms caused a significant increase in mortality at 5 and 10 mg Zn/L (Fig 4A). Similarly, all embryos exposed to the highest concentration of Ecodis P-90 died. The lowest LC_{50} value was obtained for ZnO<130-EcoP90 NPs (LC_{50}= 4.289 mg Zn/L), followed by ionic zinc (LC_{50}= 4.616 mg Zn/L), ZnO<280-EcoP90 NPs (LC_{50}= 5.538 mg Zn/L) and, finally, bulk ZnO (LC_{50}= 6.565 mg Zn/L). In general, at high concentrations of zinc (5 and 10 mg Zn/L) mortality of embryos was observed after hatching, as previously reported for embryos exposed to cadmium and gold.

At low zinc concentrations, a similar pattern was obtained for hatching rate and for survival rate. However, differences were observed at high zinc concentrations (5 and 10 mg Zn/L) where hatching rate was higher than survival rate, because embryos died after hatching (Fig 4B).

A significant hatching delay was observed in embryos exposed to all zinc forms. Under exposure to ZnO NPs, hatching delay was detected at 5 mg Zn/L for ZnO NP<130-EcoP90 NPs and at 1 and 5 mg Zn/L for ZnO NP<280-EcoP90 NPs. For embryos exposed

to ionic zinc, hatching delay was observed at 5 and 10 mg Zn/L, and for those exposed to bulk ZnO at 10 mg Zn/L. Finally, in the case of embryos exposed to Ecodis P-90 a significant delay was observed at the equivalent concentration present in the suspension of ZnO NPs containing 5 mg Zn/L (Fig 4C).

For embryos exposed to ionic zinc and ZnO NP<280-EcoP90 NPs, a significant increase in malformation prevalence was observed at 5 mg Zn/L (Fig 4D). In the case of Ecodis P-90, 100% of embryos presented malformations at 120 h of exposure at the equivalent concentration present in the suspension of ZnO NPs containing 5 mg Zn/L. The malformations observed were yolk sac edema, eye abnormality, pericardial edema, spinal cord and tail flexure and fin fold abnormality (Table 3). Exposure to ZnO NP<280-EcoP90 NPs caused head malformations in some individuals, a malformation type that has not been observed in embryos exposed to other NPs.

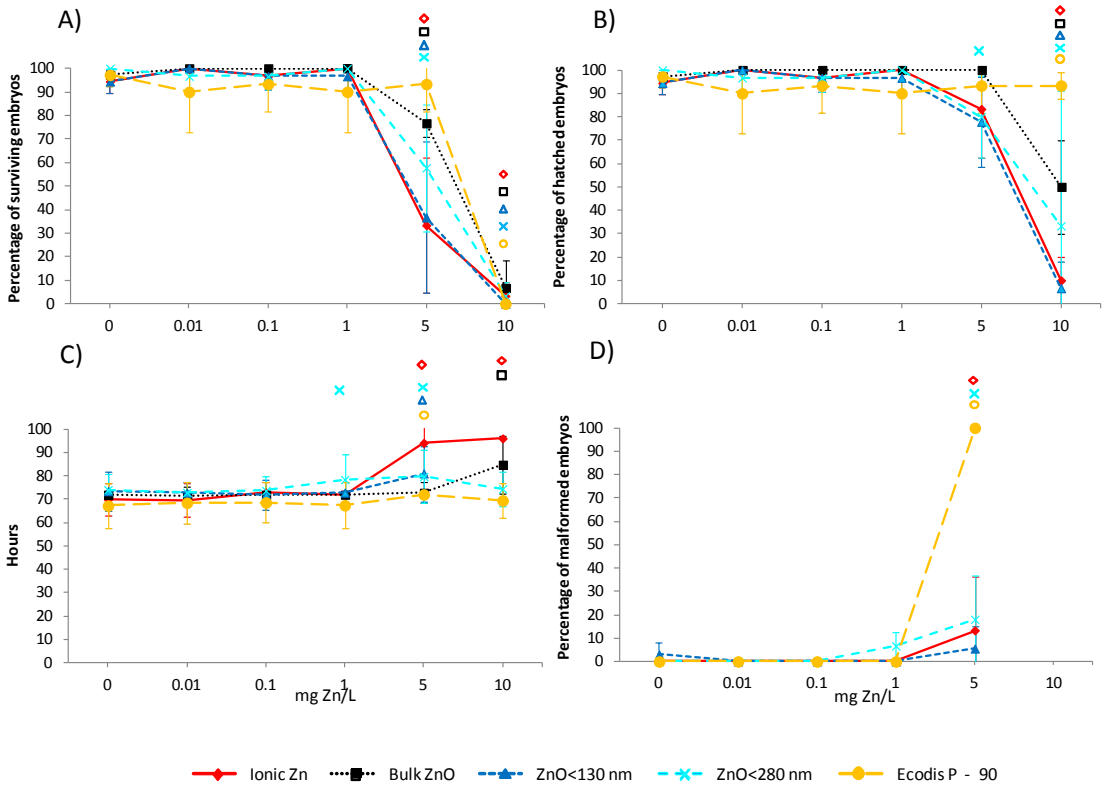

Figure 4.- Effects of the exposure of zebrafish embryos to ZnO NPs and related compounds for 120 h. A: survival rate; B: hatching rate; C: hatching time; D: malformation prevalence. The empty symbols indicate significant differences ($p < 0.05$) with respect to the control group.

Silica

LC_{50} values for all tested silicon forms were above the highest tested concentrations (100 mg Si/L), except for SiO_2-70, for which a LC_{50} value of 83.329 mg Si/L was calculated. Significant effects on survival rate were observed only in the case of SiO_2-70 NPs at the two highest exposure concentrations (Fig 5A). At these exposure concentrations embryos died before hatching.

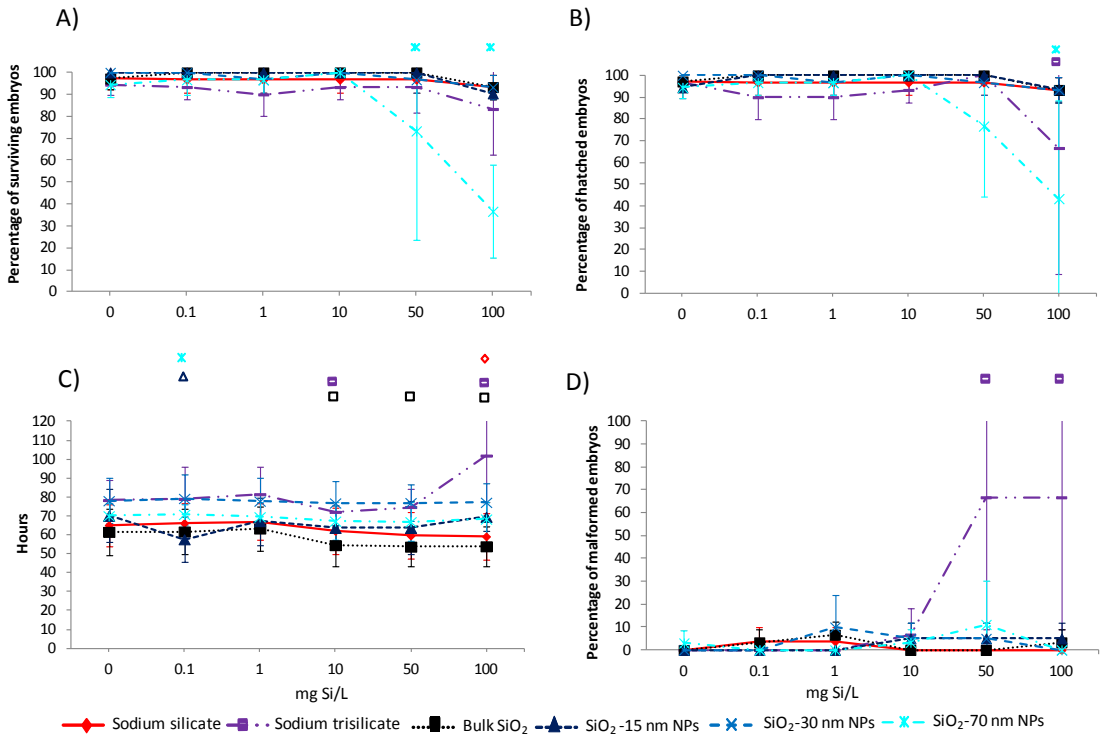

Figure 5.- Effects of the exposure of zebrafish embryos to SiO_2 NPs and related compounds for 120 h. A: survival rate; B: hatching rate; C: hatching time; D: malformation prevalence. The empty symbols indicate significant differences ($p < 0.05$) with respect to the control group.

At 120 hpf, all surviving embryos had hatched, except in the case of some individuals exposed to sodium trisilicate at 100 mg Si/L (Fig 5B). Embryos exposed to SiO_2-15 NPs at 0.1 mg Si/L, to sodium trisilicate at 10 mg Si/L, to sodium silicate at 100 mg Si/L and to bulk SiO_2 at 10, 50 and 100 mg Si/L (Fig 5C) hatched significantly earlier than control embryos. In contrast, a significant hatching delay was observed for embryos exposed to SiO_2-70 at 0.1 mg Si/L and to sodium trisilicate at 100 mg Si/L.

Exposure to sodium trisilicate at 50 and 100 mg Si/L caused a significant increase in malformation prevalence (Fig 5D) which consisted of yolk sac edema, pericardial

edema or spinal cord flexure. The other silicon forms did not provoke significant increase in malformation prevalence at any of the tested concentrations.

Table 2.- LC_{50} values for the compounds tested in this study.

Metal	Compound	Range of tested concentration (mg metal/L)	LC50 (mg metal/L)
Ag	Ag20-Mal	0.001, 0.01, 0.1, 1, 5	0.529
	Ag40-Mal		3.94
	Ag100-Mal		1.973
	Ionic silver		0.047
	Bulk Ag		> 5
Au	Au5-Cit	0.1, 1, 10, 50,100	24.655
	Au15-Cit		24.61
	Au40-Cit		34.717
	Ionic gold		4.619
	Bulk Au		> 100
Cd	CdS5-GSH	0.01, 0.1, 1, 5, 10	7.036
	Ionic cadmium		3.082
	Bulk CdS		7.868
Zn	ZnO<130-EcoP90	0.01, 0.1, 1, 5, 10	4.289
	ZnO<280-EcoP90		5.538
	Ionic zinc		4.616
	Bulk ZnO		6.565
Si	SiO_2-15	0.1, 1, 10, 50,100	> 100
	SiO_2-30		> 100
	SiO_2-70		83.329
	Sodium silicate		> 100
	Trisodium silicate		> 100
	Bulk SiO_2		> 100

Embryo exposure to FSNP

Unexposed control embryos did not show fluorescence signal at any time (Fig 6A, E, G, I, K). In exposed embryos, fluorescence signal intensity was time- and concentration-dependent. At 10 and 100 mg Si/L, FSNP were seen attached to the chorion surface from the beginning of the exposure (6 h, Fig 6C, D) until hatching at approximately 48-72 hours (Fig 6F). After hatching, fluorescence was observed on the surface of the fish body (Fig 6J). Also, presence of FSNP was observed under the gill opercle covering the surface of the gill lamellae, in the gut tract and in the cloacal chamber of the posterior intestine at 126 hpf (Fig 6J, L).

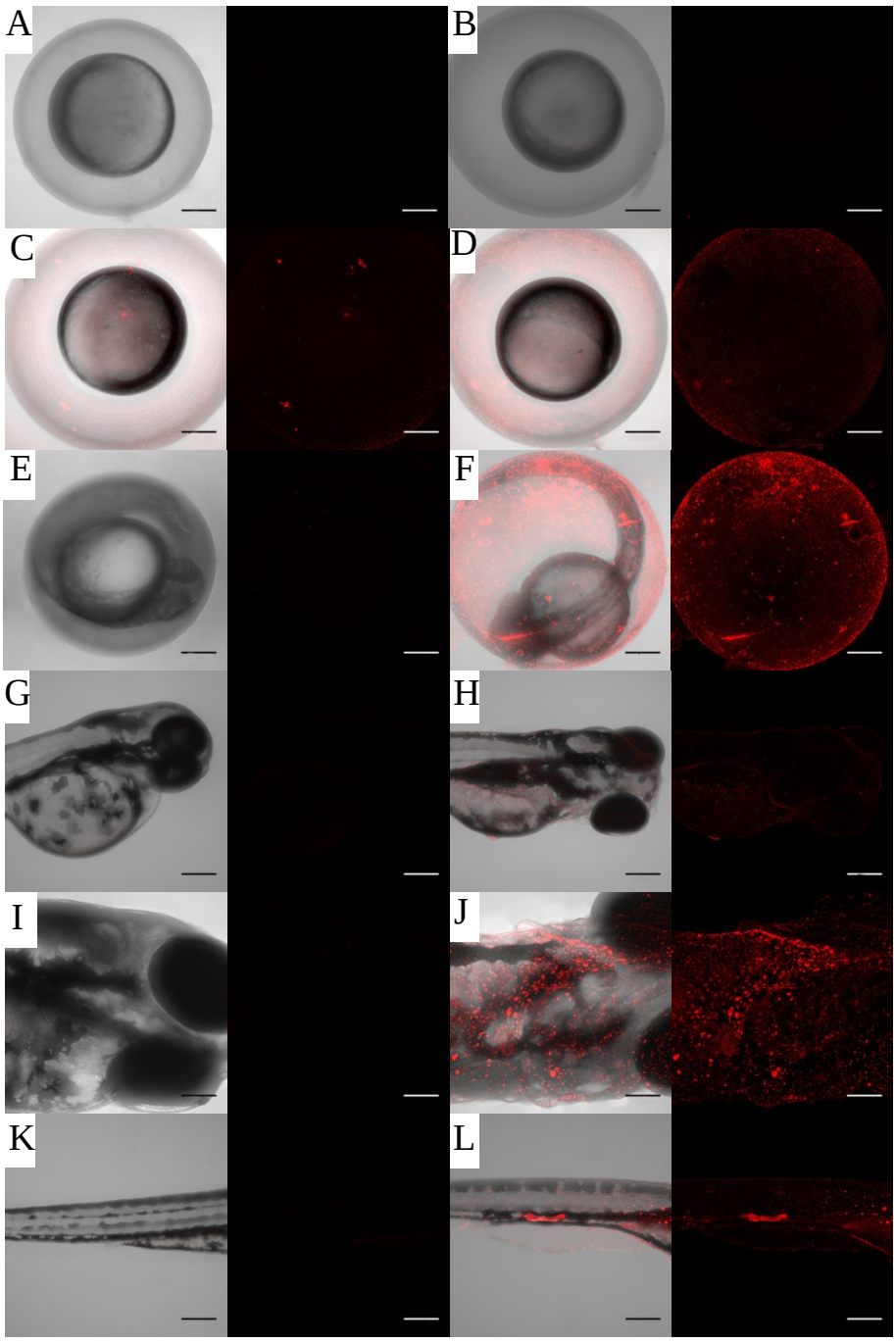

Figure 6.- Micrographs of zebrafish embryos at different stages of development obtained by confocal microscopy. A: control unexposed embryo at 6 hpf; B: embryo exposed to 0.1 mg Si/L of *fluorescent SiO_2 NPs* at 6 hpf; C: embryo exposed to 10 mg Si/L of *fluorescent SiO_2 NPs* at 6 hpf; D: embryo exposed to 100 mg Si/L of *fluorescent SiO_2 NPs* at 6 hpf; E: control unexposed embryo at 30 hpf; F: embryo exposed to 10 mg Si/L of *fluorescent SiO_2 NPs* at 30 hpf; G: control unexposed embryo at 102 hpf; H: embryo exposed to 100 mg Si/L of *fluorescent SiO_2 NPs* at 102 hpf; I, K: control unexposed embryos at 126 hpf; J, L: embryos exposed to 100 mg Si/L of *fluorescent SiO_2 NPs* at 126 hpf. Scale bars: 100 µm (I, J) and 200 µm (A-H, K, L).

Table 3.- Malformation prevalence. The concentration at which malformation prevalence is significantly increased is indicated in the second column. The percentage of total malformed embryos and specific malformations is calculated over surviving embryos at 120 hpf. A: control embryo showing normal morphology. B: embryo exposed to 5 mg Ag/L of Ag40-Mal NPs showing yolk sac edema, eye abnormality, spinal cord and tail flexure, and finfold abnormality. C: embryo exposed to 1 mg Ag/L of Ag100-Mal NPs showing yolk sac edema, pericardial edema, spinal cord and tail flexure, and finfold abnormality. D: embryo exposed to 5 mg Zn/L of Zn<280-EcoP90 NPs showing yolk sac edema, eye abnormality, pericardial edema and finfold abnormality E: embryo exposed to 0.1 mg Cd/L of CdS5-GSH NPs showing yolk sac edema, tail flexure, spinal cord flexure and finfold abnormality. Scale bar: 500 µm.

	Concentration (mg/L)	Micrographs	Total malformed embryos (%)	Specific malformations (%)						
				Yolk sac edema	Head edema	Eye abnormality	Pericardial edema	Spinal cord flexure	Tail flexure	Finfold abnormality
Control	--		0	0	0	0	0	0	0	0
Ag40-Mal NPs	1		60.7	57.1	0	0	3.6	10.7	0	7.1
	5		100	100	0	30	20	50	20	60
Ag100-Mal NPs	1		95.8	95.8	0	0	16.7	12.5	8.3	8.3
CdS5-GSH NPs	0.1		31.8	4.5	0	0	18.7	13.6	0	0
	1		23.8	23.8	0	0	0	0	0	0
	5		19.1	0	0	0	0	14.3	0	4.9
ZnO<280 -EcoP90 NPs	5		22.3	22.3	5.6	5.6	5.6	11.2	11.2	5.6

DISCUSSION

A ranking of toxicity was established for the different NP suspensions based on the obtained LC_{50} values, being Ag-Mal NPs the most toxic ones, followed by ZnO-EcoP90 NPs, CdS5-GSH NPs, Au-Cit NPs and, finally, SiO_2 NPs.

For Ag-Mal NPs of 20 nm, significant effects on survival rate were recorded at concentrations ≥ 0.01 mg/L, resulting in the lowest LC_{50} value (0.529 mg Ag/L) of all NP suspensions tested. A wide range of LC_{50} values have been reported in the literature for zebrafish embryos exposed to different types Ag NPs. To the best of our knowledge, the lowest LC_{50} value of 0.011 mg Ag/L was reported by Ribeiro et al. (2014) in zebrafish embryos exposed to alkane-coated Ag NPs of 7.5 nm. On the contrary, Griffitt et al. (2008) reported a value of 7.2 mg Ag/L in zebrafish embryos exposed to sodium citrate-stabilized Ag NPs of 20-30 nm. An increase in the mortality prevalence, hatching delay or an increase in the presence of malformations (non depleted yolk, bent tail, malformed spine, edema, etc) were the most common toxic effects reported (Lee et al., 2007; Griffitt et al., 2008; Asharani et al., 2010; Massarsky et al., 2013; 2014; Pavagadhi et al., 2014; Ribeiro et al., 2014). Capping agents used during manufacture of Ag NP suspensions can increase or contribute to the toxicity of Ag NPs, as in the case of NPs capped with polyvinylpyrrolidone (PVP) or citrate (Kim et al., 2013). Maltose used as capping agent for the Ag NPs tested in this study did not affect the toxicity of the NP suspensions. The chemical composition of the exposure medium can also modulate NP toxicity, since the concentration of chloride ions affects the solubility and, thus, the bioavailability of Ag^+ ions (Olasagasti et al., 2014). Deionized water used in the present study may contribute to the low LC_{50} value estimated, since the lack of chloride ions that can interact with Ag^+ ions avoids the formation of insoluble AgCl which could reduce silver availability to the embryos. Christen et al. (2013) have demonstrated that Ag NPs are able to induce the endoplasmatic reticulum stress response in zebrafish embryos altering the transcription level of marker genes, such as *BiP* and *Synv*, and other genes related to pro-apoptotic and oxidative stress processes. Nevertheless, these responses have shown to be stronger in zebrafish liver cells.

A high toxicity was also observed for the two ZnO NPs tested, with LC_{50} values of 4.289 mg Zn/L for ZnO<130-EcoP90 and 5.538 mg Zn/L for ZnO<280-EcoP90. Furthermore, a significant increase in the presence of malformations in surviving embryos was recorded after the exposure to both ZnO NPs at a concentration of 5 mg Zn/L. In this case, the contribution of the additive Ecodis P-90 to observed toxicity must be taken into account. Previous studies with mussel cells also reported that Ecodis P-90 contributed partly to the observed cytotoxicity of the same ZnO NPs used in this study (Katsumiti et al., 2015a). The exposure to Ecodis P-90 alone, at the equivalent concentration present in the NP suspension containing 5 mg Zn/L, caused malformations in the 100% of surviving embryos. All individuals exposed to Ecodis P-90 showed yolk sac edema and 60% of the embryos presented pericardial edema, among other malformations. Appearance of malformations in zebrafish embryos has also been observed in other studies of exposure to ZnO NPs. Zhao et al. (2013) reported a significant increase in malformation prevalence (pericardial edema, tail deformity, spinal curvature and hyperaemia) after exposure to 10 to 100 mg/L of commercial uncapped ZnO NPs (< 100 nm). Moreover, Vicario-Parés et al. (2014) did not find a significant increase in the prevalence of malformations after embryo exposure to commercial uncapped ZnO NPs.

The LC_{50} value estimated in this study for CdS NPs was 7.036 mg Cd/L, which is higher than the lethal concentration reported in other studies with zebrafish embryos exposed to different Cd-containing NPs (CdSe NPs of 9 and 14 nm, and CdTe NPs of 3.5 nm) with diverse capping agents. LC_{50} values ranged from 0.021 mg/L for $CdSe_{core}/ZnS_{shell}$ NPs capped with poly-L-lysine to 4.72 mg/L for CdTe NPs capped with thioglycolic acid (King-Heiden et al., 2009; Zhang et al., 2012a). Thus, the capping agent and the synthesis process, producing NPs with different core and shells, are important in determining NPs toxicity, since the presence of metals, as selenium, in the core increases the toxic effect of these NPs (Wiecinski et al., 2013). The LC_{50} value obtained in the present study is also much higher than the LC_{50} value reported for CdTe NPs using zebrafish hepatocytes exposed *in vitro* (Tang et al., 2013). These authors report an *in vitro* LC_{50} value of 0.112 mg/L after 24 h of exposure, which is also much lower

than the LC_{50} value obtained after the exposure of zebrafish embryos (120 hpf) to similar NPs (CdTe NPs capped with thioglycolic acid) (Zhang et al., 2012).

For Au-Cit NPs and SiO_2 NPs, significant effects were observed at concentrations much higher than those for the above discussed NPs. In the case of the exposure to Au-Cit NPs, LC_{50} values ranged from 24.61 to 34.717 mg Au/L and first significant effects appeared in embryos exposed to 50 mg Au/L. As for ZnO NPs, the effect of the capping agent sodium citrate present in the Au NP suspensions must be considered, since similar results were observed after the exposure to sodium citrate alone. In accordance with our study, Browning et al. (2009) reported that the survival rate scored in zebrafish embryos exposed to Au NPs was similar to that observed in zebrafish embryos exposed to the sodium citrate dilution used to stabilize the NP suspensions. Bar-Ilan et al. (2009) did not find toxicity for Au NPs nor for sodium citrate, but the highest concentration tested in their study was equivalent to our lowest concentration, at which we did neither observe adverse effects. For SiO_2 NPs, embryo mortality was observed only after exposure to SiO_2 NPs of 70 nm, resulting in a LC_{50} value of 83.329 mg Si/L and in a significant effect on survival at 50 and 100 mg Si/L. For the other sizes (15 and 30 nm), the estimated LC_{50} value was higher than the maximum concentration used in our experiments. In a previous study, Duan et al. (2013) also observed a significant increase in the mortality only in embryos exposed to high concentrations of SiO_2 NPs of 60 nm (100-200 mg/L). Other authors did neither reported effects after the exposure to SiO_2 NPs, at concentrations up to 200 mg/L (Fent et al., 2010; George et al., 2011).

Overall, the results obtained herein are in agreement with other studies comparing the toxicity of a set of metal and metal bearing NPs. George et al. (2011) exposed zebrafish embryos for 120 hours to different NPs (Au, SiO_2, Pt, $CdS_{shell}/ZnSe_{core}$ QDs, ZnO and Ag) in a range of concentrations from 0 to 25 mg/L. These authors only reported toxic effects after exposure to $CdS_{shell}/ZnSe_{core}$ QDs, ZnO and Ag NPs, but at higher concentrations (from 5 mg/L) than those observed in our experiments (from 0.01 mg/L). The toxicity of silver, cadmium and zinc containing NPs has been partly attributed to the release of ions into the exposure medium, as these are highly soluble metals (Misra et al., 2012; Schirmer et al., 2013; Ivask et al., 2013; Katsumiti et al.,

2014b; 2015a; 2015b). It has been shown that metal ions, such as Zn^{2+} resulting from ZnO NPs dissolution, interfere with the zebrafish hatching enzyme (ZHE1) and produce a delay in embryo hatching (Lin et al., 2012) as we observed in this work. Ong et al. (2013) also reported interaction of the ions released from the NPs with the ZHE1, resulting in complete hatching inhibition in zebrafish embryos exposed to 10 and 100 mg/L of ZnO NPs and hatching delay in embryos exposed to 10 mg/L of CdSe and of Ag NPs, a concentration at which embryos did not survive in the present study. Therefore, toxic metal ions released from Ag, ZnO and CdS NPs can contribute to the higher toxicity elicited by these soluble NPs in comparison to more insoluble compounds, such as Au and SiO_2.

The above conclusion is also supported by differences recorded in toxicity when the metal forms of the compounds were compared. In general, the ionic form of each metal was the most toxic, followed by the NPs and, finally, the bulk form, which is in fact the least soluble form of the metals. These results agree well with those reported in several studies on the toxicity of metal NPs already published using zebrafish as test organism (Zhu et al., 2008; Vicario-Parés et al., 2014), being silver the most studied metal (Powers et al., 2011; Massarsky et al., 2013; Ribeiro et al., 2014). The difference in toxicity between the ionic and NP forms of silver was attributed to the fact that the $AgNO_3$ (ionic form when dissolved in water) dissociates completely releasing a large portion of free Ag^+ ions very rapidly, whereas Ag^+ ions from Ag NPs are released more slowly, which results in lower concentrations in the medium (Laban et al., 2010). Although we did not observe toxic effects after exposure to bulk silver, in a previous study, Osborne et al. (2013) reported a dose-dependent toxicity for bulk silver, with a significant increase in embryo mortality at concentrations ≥ 5 mg/L, occurring mainly during the gastrulation period.

While ionic cadmium was more toxic than CdS NPs and bulk CdS in terms of LC_{50} value, CdS NPs caused significant effects on zebrafish embryos at lower concentrations (0.1 mg Cd/L) than ionic cadmium (5 mg Cd/L). Other studies using different Cd-containing NPs (CdSe QDs of 9 and 14 nm, and CdTe QDs of 3.5 nm) and soluble cadmium have also reported higher acute toxicity to developing zebrafish embryos for NPs (LC_{50} from 0.0209 to 4.72 mg Cd/L) than for ionic cadmium, with LC_{50} values of 45.98 and 17.26

mg Cd/L King-Heiden et al., 2009; Zhang et al., 2012a). These differences in cadmium toxicity among studies could be due to the different chemical composition of the test media, especially Ca^{2+} concentration. Calcium ions compete with cadmium ions for binding sites on the embryo (Meinelt et al., 2001). In the previously mentioned studies, the concentration of Ca^{2+} in the exposure medium was higher than in our study, where deionized water was used for the test solutions.

Similar LC_{50} values were obtained during the exposure of zebrafish embryos to all forms of zinc. As discussed above, the release of Zn^{+}, along with the use of the stabilizer Ecodis P-90, provoke partly the toxic effect of ZnO NPs and, their combined effect appear to be similar to those produced by the ionic form of the metal. In a previous study, Vicario-Parés et al. (2014) reported toxic effects for ionic zinc at 5 mg Zn/L, whereas no significant effects on mortality were found for uncapped ZnO NPs or for the bulk counterpart at the tested concentrations (up to 10 mg Zn/L). The different ZnO NPs used in both studies easily explain these results, as Vicario-Parés et al. (2014) used commercial ZnO NPs. Zhu et al. (2008) also compared bulk ZnO and ZnO NPs, reporting similar toxic effects after both exposures (LC_{50} values after 96 h of exposure: 1.793 mg/L for ZnO NPs and 1.550 mg/L for bulk ZnO). On the contrary, Yu et al. (2011) obtained higher toxicity, in terms of LC_{50}, for the bulk form than for ZnO NPs, because at high concentrations NPs tended to aggregate and, therefore, availability and, in turn, toxicity to zebrafish embryos was reduced.

As to the influence of NP size, overall data reported in the literature point out that NP toxicity is size-dependent, especially for Ag and Au NPs (Bar-Illan et al., 2009; Powers et al. 2011; Kim et al., 2013; Katsumiti et al., 2015b). This phenomenon has been attributed to the higher surface-to-volume ratio of the smaller NPs, which increases the proportion of atoms in the NP surface in contact with the test solution. Moreover, small sized NPs show greater ability to passively diffuse into developing embryos via chorion pore channels which, in turn, may also block the pores by the aggregation of NPs (Lee et al 2007) increasing their toxicity in comparison to larger NPs.

During the present study, embryo mortality occurred at different exposure times depending on the NP tested. Exposure to Au, CdS and ZnO NPs caused mortality mainly

after hatching. Similarly, ionic cadmium and zinc, and additives as sodium citrate and Ecodis P-90 caused mortality after embryos had hatched. During the first stages of development (up to 48-72 hpf), zebrafish embryos are surrounded by the chorion, which acts as a barrier limiting the access of molecules to the embryo (Braunbeck et al., 2014) and, therefore, protecting them from the toxic effect of the NP suspensions. This protective role of the chorion has been reported for different nanomaterials, such as SiO_2 NPs (Fent et al., 2010) and carbon nanotubes (Cheng et al., 2007). But for Ag NPs, Lee et al. (2013) observed the entry of NPs into the embryo through the chorion pore channels during the exposure, resulting in accumulation of NPs into the organism that produce a toxic effect (mortality and increase in the presence of malformations) directly to the embryo. Accordingly, in our study, embryos exposed to Ag NPs also died before hatching.

In embryos exposed to different concentrations of fluorescent SiO_2 NPs of 27 nm, SiO_2 NPs were only observed attached to the chorion surface while embryos remained unhatched. Therefore, for this NP size and type the chorion acted as a barrier to protect against SiO_2 NPs entrance into the embryos. Similarly, Fent et al. (2010) also observed that fluorescent SiO_2 NPs of 60 and 200 nm NPs (0.0025-200 mg Si/L up to 96 hpf) were too large to penetrate the chorion as they formed larger agglomerates.

Only after a longer exposure (102 hpf), once the embryos had hatched, fluorescent NPs were observed under the gill opercle covering the surface of the gill lamellae, in the digestive tract and in the cloacal chamber of the posterior intestine of the larvae. The presence of NPs in the intestine is in agreement with the findings reported by Zhao et al. (2014). These authors have demonstrated a novel NP excretion pathway in zebrafish through the intestinal tract after NP injection directly into the yolk sac. We have previously reported that waterborne exposure of zebrafish embryos to NPs results in tissue bioaccumulation of metals. Also, Olasagasti et al. (2014) reported the presence of agglomerates of Ag NPs in the digestive tract of zebrafish embryos exposed during 120 hpf after the hatching (95-120 hpf). Thus, fluorescence detected in embryo digestive tract and cloacal chamber could result from direct NP uptake through the digestive tract and from excretion of NPs incorporated into the fish by other routes as gills (Griffitt et al., 2009; 2013).

CONCLUSIONS

A ranking of toxicity for different metal bearing NPs has been established, being Ag NPs the most toxic of the tested NPs, followed by CdS and ZnO NPs, and finally by Au and SiO_2 NPs. The toxic effects of NPs to zebrafish embryos depended on their chemical composition (and, thus, solubility) and size, being the former the most relevant characteristic involved in the toxic effect according to our results. Accordingly, the soluble forms of the metals were more toxic than the nanoparticulated form, being the bulk form the least toxic. Additives present in the NP suspensions, such as sodium citrate and Ecodis P-90, are also an important factor contributing to the toxicity. The interaction between the chorion and the NPs must be taking into account when the effect of the exposure to different NPs is studied, especially when data obtained at different exposure times are compared, since the chorion acts as a protector barrier against the entrance of SiO_2 NPs into the organism during the first developmental stages.

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CHAPTER II

Waterborne exposure of adult zebrafish to silver nanoparticles results in silver accumulation and sublethal effects at cellular level

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X Edition Nanoforum. Rome (Italy), 22th-25th September 2014. **JM Lacave**, U Vicario-Parés, E Bilbao, D Gilliland, M Rossi, L Dini, MP Cajaraville, A Orbea. "Waterborne exposure of zebrafish to Ag NPs results in silver internalization leading to sublethal effects from changes in liver transcriptome to gill histopathologies". Oral presentation.

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ABBREVIATIONS

BSA, Bovine serum albumin

BSDs, Black silver deposits

CAT, Catalase

FESEM, Field emission scanning electron microscopy

LMS, Lysosomal membrane stability

LP, Labilization period

MN, Micronuclei

NPs, Nanoparticles

PBS, Phosphate buffered saline

PVP, Polyvinyl pyrrolidone

ROS, Reactive oxygen species

SOD, Superoxide dismutase

TEM, Transmission electron microscopy

ABSTRACT

Due to their bactericide properties, silver nanoparticles (NPs) are among the most common nanomaterials currently used. Ag NPs are incorporated in domestic and medical products as well as in industrial processes, resulting in silver containing wastes that can reach aquatic environments and could potentially affect aquatic organisms. In order to study the effects of waterborne exposure of aquatic organisms to Ag NPs, adult zebrafish were exposed for 21 days to 10 µg Ag/L of maltose-coated Ag NPs of 20 nm (Ag20-Mal) or to the same nominal concentration of ionic silver and, subsequently, maintained up to 6 months in clean water. Silver accumulation was measured by chemical analyses in the whole organism, and metal accumulation was specifically detected in liver and intestine by autometallography. Field emission scanning electron microscopy used to analyze the presence of silver in gill, liver and intestine tissues corroborated that these organs are targets for silver accumulation. Ag NPs subcellular fate was determined by transmission electron microscopy, detecting Ag NPs in the cytoplasm of epithelial cells from the primary lamellae of gills, and in the nucleus and mitochondria of hepatocytes. A battery of biomarkers was used to study the sublethal effects provoked by the exposure to both forms of the metal. In the assayed conditions, evident effects on hepatocyte lysosomal membrane stability, oxidative stress and genotoxicity were not found, but the histopathological analysis showed a variety of alterations in the gills. Thus, the presence of nanomaterials in the environment could result in metal accumulation in aquatic organisms that could lead to toxic effects, being the gills one of the main target organs after waterborne exposure.

Keywords: Ag NPs, zebrafish, bioaccumulation, biomarkers, electron microscopy

LABURPERNA

Beraien ezaugarri bakterizidak direla eta, zilarrezko nanopartikulak (NPak) gehien erabiltzen ari diren nanomaterialen artean daude. Ag NPak etxe- eta medikuntza-produktuetan gehitzen dira, baita prozesu industrialetan ere, ingurune urtarrera iritsi eta bertako organismoei eragin ditzaketen zilardun hondakinak sortarazten direlarik. Ur esposizioaren bidez, Ag NPek organismo urtarretan eragiten dituzten efektuak ikertzeko, zebra arrain helduak maltosaz estalitako 20 nm-ko Ag NPen (Ag20-Mal) 10 ug Ag/L pean edo zilar ionikoko kontzentrazio nominal berean mantendu ziren 21 egunez eta, ondoren, 6 hilabetez mantendu ziren ur garbitan. Zilar metaketa, organismo osoan egindako analisi kimikoen bidez neurtu zen eta metal metaketa autometalografia bidez neurtu zen espezifikoki gibelean eta hestean. Zakatz, gibel eta hesteetan zilarraren agerpena aztertzeko erabilitako eremu igorpeneko ekorketazko mikroskopio elektronikoak, organo horiek zilarraren metaketarako itu organoak direla berretsi zuen. Ag NPen patua transmisio mikroskopia elektroniko bidezko ikerketekin zehaztu zen, Ag NPak zakatzetako lamela primarioetako epitelio-zelulen zitosolean eta hepatozitoetako nukleo eta mitokondrietan antzemanez. Biomarkatzaile multzo bat erabili zen metalaren bi aldaerek eragindako efektu subletalak ikertzeko. Testatutako egoeretan ez zen gibeleko lisosomen mintzaren egonkortasunean efekturik, estres oxidatiboaren areagotzerik edo genotoxizitaterik aurkitu, nahiz eta ikerketa histopatologikoak zakatzetan zenbait alterazio erakutsi zituen. Beraz, ingurumenean gertatzen den nanomaterialen agerpenak organismo urtarretan metaketa eta efektu toxikoak eragin ditzake, zakatzak izanik ur bidezko esposizioaren itu-ehun nagusia.

Gako-hitzak: Ag NPak, zebra arraina, biometaketa, biomarkatzaileak, mikroskopia elektronikoa

RESUMEN

Debido a sus propiedades bactericidas, las nanopartículas (NPs) de plata se encuentran entre los nanomateriales que más se están utilizando. Las NPs de Ag se han incorporado en productos de uso doméstico y médico así como en procesos industriales, lo que da lugar a residuos que contienen plata y que pueden llegar al medio acuático y afectar a los organismos. Para estudiar los efectos de la exposición a través del agua de organismos acuáticos a NPs de Ag, peces cebra adultos se expusieron durante 21 días a 10 µg Ag/L de NPs de 20 nm cubiertas de maltosa (Ag20-Mal) o a la misma concentración nominal de plata iónica y, a continuación, se mantuvieron 6 meses en agua limpia. La acumulación de plata se midió a través de análisis químicos en el organismo completo y la acumulación de metales se detectó específicamente en el hígado y en el intestino por autometalografía. La microscopía electrónica de barrido de emisión de campo que se utilizó para analizar la presencia de plata en las branquias, hígado e intestino corroboró que estos órganos son dianas para la acumulación de plata. El destino subcelular de las NPs de Ag se determinó mediante microscopía electrónica de transmisión, detectándose en el citosol de las células epiteliales de las laminillas primarias de las branquias y en el núcleo y en las mitocondrias de los hepatocitos. Se utilizó una batería de biomarcadores para estudiar los efectos subletales provocados por la exposición a ambas formas del metal. En las condiciones ensayadas, no se encontró un efecto evidente en la estabilidad de la membrana de los lisosomas hepáticos, incremento del estrés oxidativo o genotoxicidad, aunque el análisis histopatológico mostró diversas alteraciones en las branquias. Por tanto, la presencia de nanomateriales en el medio ambiente puede resultar en la acumulación en los organismos acuáticos pudiendo provocar efectos tóxicos, siendo las branquias uno de los principales órganos diana tras la exposición a través del agua.

Palabras clave: NPs de Ag, pez cebra, bioacumulación, biomarcadores, microscopía electrónica

INTRODUCTION

The antibacterial and antimicrobial properties of Ag nanoparticles (NPs) are increasing their use in different consumer products (Whiteley et al., 2011), including textiles, respirators, household water filters, antibacterial sprays, cosmetics and detergents, among others (Marambio-Jones and Hoeck, 2009). The wastes derived from the industrial processes and releases from consumer products will enter into the environment directly from atmospheric deposition, water run-off or through the wastewater treatment plants, introducing Ag NPs into the aquatic ecosystem (Benn and Westerhoff, 2008). Thus, the increase in the use of Ag NPs is inexorably linked to an increase in the environmental input (Massarsky et al., 2014a). Although available analytical methods are not sensitive enough to distinguish between natural materials or dissolved substances and manufactured NPs in complex environmental matrices (Markus et al., 2013), several studies have estimated the concentration of Ag NPs in different environmental compartments, based on production and emission data (Fabrega et al., 2011; Chio et al., 2012; Hendren et al., 2013; Markus et al., 2013; Dumont et al., 2015). Most studies report silver concentration values in the range of ng/L for surface waters from USA and Europe, while Chio et al. (2012) predicted values up to 40 µg/L in effluents of Taiwanese rivers.

The entrance of Ag NPs into the aquatic environment could produce deleterious effects in the organisms. Experimental studies have already shown a variety of toxic effects provoked by the exposure to Ag NPs in different aquatic organisms, including algae (Navarro et al., 2008), freshwater and marine invertebrates (Gomes et al., 2013; Arulvasu et al., 2014; Oliver et al., 2014), and different species of fish, as recently reviewed by Massarsky et al. (2014a). Among fish species, zebrafish has received special attention as a model organism. Several authors have reported toxic effects in embryos after the exposure to Ag NPs of different sizes and with different capping agents, being the decrease in the survival rate and the increase in the prevalence of malformations the most common effects observed (Kim et al., 2013; Lee et al., 2013; Massarsky et al., 2013; Ribeiro et al., 2014; Chapter I). In adult zebrafish, both acute and sublethal toxic effects have also been described after the exposure to Ag NPs. Considerable differences are found in the literature regarding acute toxicity of Ag NPs

to adult zebrafish. Choi et al. (2010) reported a LC_{50} value as high as 250 mg/L after 24 h of exposure to Ag NPs of 5-20 nm. Nevertheless, Bilberg et al. (2102) reported a much lower LC_{50} value (84 µg/L) after 48 h of exposure to polyvinyl pyrrolidone (PVP)-coated Ag NPs of 81 nm. In this latter study, acute toxicity was not observed after PVP exposure alone. Regarding sublethal effects significant silver burden increase in the gills and alteration of the gill transcriptome but not histopathological alteration of gill tissue have been previously reported after acute exposure (48 h) to 1 mg/L of Ag NPs of about 26 nm (Griffitt et al., 2009). Similar results were obtained after a longer exposure (28 days) to lower concentrations (5 to 50 µg/L) of much smaller Ag NPs (3.1±2.2 nm), being the number of genes with significantly altered transcription level in the gill increased in a dose-dependent way (Griffitt et al., 2013). Metal bioaccumulation has also been reported in the liver. The presence of NPs has been detected in hepatocytes after exposure to 30 and 120 mg/L of Ag NPs of 80 nm for 24 h, causing induction of apoptosis and oxidative stress (Choi et al., 2010). Similar results were reported after exposure for 15 days to 100 µg/L of PVP-coated Ag NPs of 22-26 nm (Devi et al., 2015). These authors found extensive cell death in the liver, associated with diverse indications of oxidative stress, such as enhancement of nitric oxide production, reduced catalase (CAT) and superoxide dismutase (SOD) activities and increased sulphydryl groups.

The mechanisms of toxicity of Ag NPs are not totally clear, although most studies carried out *in vitro* with mammalian cells and *in vivo* with a variety of aquatic organisms, point out to oxidative stress and related effects such as mitochondrial damage, DNA damage (genotoxicity) and cell death in the liver by both apoptotic and necrotic pathways, as the main cause of toxicity (Fu et al., 2014; Massarsky et al., 2014a; McShan et al., 2014). In fish, disruption of the Na^+/K^+ ATPase activity in the gills and the inhibition of the acetylcholinesterase activity in various tissues, which interfere with the ionoregulation and neuroregulation capabilities, have also been described (Schultz et al., 2102; Katuli et al., 2014; Devi et al., 2015). Nevertheless, whether the ultimate responsible for these observed effects are the NPs themselves or the extracellularly or intracellularly released silver ions from the NPs is currently an open debate (McShan et al., 2014; De Matteis et al., 2015).

Studies comparing the effect of ionic silver with that provoked by the exposure to Ag NPs describe, in general, a stronger effect caused by the ionic form than by the nanoparticulated form. In the above mentioned study of Bilberg et al. (2012), ionic silver resulted 3.4 times more toxic than the Ag NPs by mass of silver added to the tanks (LC_{50} value of 25 μg/L and 84 μg/L, respectively) in adult zebrafish. This observation has also been extensively reported in zebrafish embryos (Powers et al., 2011; Massarsky et al., 2013; Ribeiro et al., 2014; Chapter I).

A recent study performed with human cells shows that Ag NPs are internalized by endocytosis and moved into the lysosomes, where they undergo a degradation process, releasing ions that cross the organelle membrane spreading into the cytosol, while the ions released in the culture medium played a negligible effect (De Matteis et al., 2015). Accordingly, Griffitt et al. (2009) state that the biological effect of Ag NP exposure on adult zebrafish does not appear to be driven solely by release of soluble metal ions into the water column.

The aim of this work was to study the sublethal effects to adult zebrafish produced by the aqueous exposure to maltose-coated Ag NPs of 20 nm (Ag 20-Mal NPs) for 21 days in comparison with those provoked by the same nominal concentration (10 μg Ag/L) of ionic silver. After exposure, zebrafish were maintained in clean water for 6 months to evaluate the potential long-term effects or the recovery after the cease of the exposure. In a previously performed short-term experiment with zebrafish embryos (Chapter I), the acute toxicity of maltose-coated Ag NPs of different sizes (20, 40 and 100 nm) was tested, being the smallest NPs (Ag 20-Mal NPs) the most toxic size. Nevertheless, embryo mortality was not recorded at the concentration used in this work with adult fish, which is lower than the concentration used in most of the toxicological studies reported in the literature. Although it is above most predicted environmental concentrations, it is still lower than the concentration reported by Chio et al. (2012) for Taiwanese rivers. Thus, this silver concentration could be environmentally relevant in hot spots, such as water masses receiving effluents from water waste treatment plants.

Accumulation of silver in the organisms after the 21 days of exposure was measured by chemical analyses in the whole zebrafish body and the fate of silver in various organs was specifically assessed using different techniques. Autometallography, transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) were employed to determine the tissue distribution and intracellular presence of silver in exposed organisms. After being established for other aquatic species (Soto et al., 1998), autometallography has been successfully employed to study metal fate in fish exposed to different ionic metals (Alvarado et al., 2005; 2006) and, more recently, metal accumulation has been determined by autometallography in zebrafish embryos exposed to metal oxide NPs (Vicario-Parés et al., 2014). TEM is a widely used method to study the entrance of NPs in the cells (Choi et al., 2010) while, to our knowledge, up to date FESEM has only been applied in zebrafish embryos to study the fate of Ag NPs (Olasagasti et al., 2014), being this the first study reporting the application of this technique in adult fish tissues.

Different biomarkers covering the main mechanisms of toxicity described for Ag NPs were applied to determine the cellular effects of silver in zebrafish. Oxidative damage in the liver was assessed by western blot analysis using specific antibodies against protein carbonyls and ubiquitin, since interaction of Ag NPs with cellular proteins leading to oxidative damage and protein unfolding has been reported (McShan et al., 2014). Likewise, the micronuclei test used in previous works to study the genotoxic effect caused by the exposure to different metals and nanomaterials in diverse aquatic organisms (Yadav Trivedi, 2009; Domingues et al., 2010; Filho et al., 2014; Vicario-Parés, 2016) was applied in zebrafish erythrocytes. Cellular uptake of Ag NPs by endocytosis converts lysosomes into major target organelles (Wei et al., 2015). The exposure of Ag NPs to the lysosomal acidic environment can enhance the NP dissolution and, in turn, silver ion release and production of reactive oxygen species (Massarsky et al., 2014a; De Matteis et al., 2015; Wei et al., 2015). Thus, the lysosomal membrane stability test was used to assess lysosomal damage. This test, widely used in diverse aquatic species to analyze the general health status of the organisms and used as prognostic biomarker of toxic liver injury in fish (Köhler et al., 2002), has been also previously applied to detect the toxic effect provoked by metals and metal NPs

(Jimeno-Romero, 2014; Vicario-Parés, 2016). Finally, at tissue level, the presence of histopathological alterations in gills and liver as target organs for toxicity has been evaluated. Up to date, controversial results have been published regarding histopathological effects in fish exposed to Ag NPs (Griffitt et al., 2009; 2013; Farmen et al., 2011).

MATERIALS AND METHODS

Silver compounds

Maltose-coated Ag NPs of roughly 20 nm (Ag20-Mal NPs) were synthesized using the Tollens method (Kvítek et al., 2005) resulting in a suspension containing 107.87 mg Ag/L and maltose 0.25 mM. Full characterization data for these NPs have been published by Katsumiti et al. (2015). Dynamic light scattering analysis showed that Ag NPs were monodispersed and with a negative zeta potential value ranging from -30 to -35 mV (Katsumiti et al., 2015). Solution of silver nitrate (1 g Ag/L in 0.5 M HNO_3 matrix) was obtained from Spectrosol, BDH Chemical Ltd Poole (England).

Maintenance and experimental exposure of adult zebrafish

The experimental procedure described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. Zebrafish (wild type AB Tübingen) individuals were specifically produced and grown in our facility at the UPV/EHU. Adult fish of approximately 4 months old were exposed to Ag NPs and to ionic silver at a concentration of 10 µg Ag/L for 21 days. An unexposed control group was run in parallel in identical experimental conditions.

The exposures were carried out in 35 L aquaria containing 50-60 fish. During the exposure period, approximately 2/3 of the aquarium water was changed by siphoning every three days and the corresponding volume of contaminated or clean water was redosed. Fish were fed with Vipagran baby (Sera, Heinsberg, Germany) and live *Artemia* (INVE Aquaculture, Salt Lake City, Utah, USA) twice per day. Samples were taken after 3 and 21 days of exposure after euthanasia by overdose of anesthetic (benzocaine, Sigma-Aldrich, St. Louis, Missouri, USA). After 21 days of exposure, remaining fish were transferred to clean water and maintained up to 6 months to

evaluate the appearance of long-term effects or the potential reversibility of the effects detected. During this period, fish were fed only with Sera Vipagran baby twice per day. Water was maintained in continuous movement through an air pump to assure an appropriate aeration to the tank. Biological and physical filters were used to maintain the chemical parameters of the water (nitrate, nitrite and ammonium) that were controlled once per week using Sera ammonium, nitrite and nitrate kits. Water was changed if the values were higher than zero mg/L for ammonium or nitrite and 50 mg/L for nitrate. At 6 months post-exposure fish samples were collected as described above.

Metal accumulation in whole organisms: chemical analysis

After 21 days of exposure, 20 individuals per experimental group were collected, frozen individually in liquid nitrogen and stored at -80 ºC until processed for chemical analysis. Whole zebrafish were dried in an oven at 130 ºC for 24 h. Dry tissues were weighted and pooled (five pools of four zebrafish each). Each pool was placed into 25 mL Erlenmeyer flasks and 2 mL of 65% nitric acid (extra pure quality, Scharlau, Barcelona, Spain) was added for tissue digestion. The mouth of the Erlenmeyer flask was blocked with a crystal ball to minimize evaporation. After finishing the digestion of the sample, the remnant liquid was evaporated in an 80 ºC hot plate inside an exhaust hood. Then, 2.5 mL of nitric acid 0.1 M were added to each Erlenmeyer flask, and left for 1 day. The content of each flask was then put into tubes and centrifuged for 4 min at 2000 rpm (Heraeus Labofuge 200 centrifuge, Hanau, Germany). The supernatants were moved to clean tubes, and stored at 4 ºC. Finally, silver content was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES, 7700x, Agilent Technologies, California, USA) following the US-EPA 6010D direction. Detection limit was established at 0.007 μg/L.

Histological preparations

The visceral mass and gills of 10 individuals per experimental group were dissected after 21 days of exposure and after 6 months in clean water. Tissues were placed in histological cassettes and immersed in 10% neutral buffered formalin for 24 h at 4 ºC. Then, samples were transferred to 70% ethanol and stored at 4 ºC until complete

tissue processing under vacuum conditions in an ASP300 Tissue Processor (Leica Microsystems, Nussloch, Germany). Paraffin blocks were done using plastic molds. Sections (5 µm thick) were cut in a RM2125RT microtome (Leica Microsystems) for autometallography, FESEM and histopathological analysis. For the histopathological analysis and for localization of tissues of interest before the autometallographical staining, slides were stained with hematoxylin/eosin (H/E) in an Auto Stainer XL (Leica Microsystems) and mounted in DPX (Sigma-Aldrich) by means of a CV5030 Robotic Coverslipper (Leica Microsystems). H/E stained histological sections of the visceral mass and gill tissue were examined under a BX51 light microscope (Olympus, Tokyo, Japan).

Metal accumulation in tissues: autometallography

Autometallographical staining was applied on paraffin sections of visceral mass of ten individuals per experimental group sampled after 21 days of exposure using a method modified from Soto et al. (1998). Briefly, sections were dewaxed, hydrated in a graded series of ethanol and left until they were completely dry. Then, sections were covered with the photographic emulsion (Ilford nuclear emulsion L4, Norderstdedt, Germany) and left in total darkness for 30 min. Reaction was developed in the developer bath (1:4.5 dilution in deionized water of B&W Negative developer Tetenal, Norderstdedt, Germany) for 15 min and, then, rinsed in the stop bath (1% solution of acetic acid, Panreac, Barcelona, Spain) for 1 min. Sections were fixed in a 10% solution in deionized water of B&W Film/Paper Fixer AGFA (Mortsel, Belgium) for 10 min. Finally, sections were washed in deionized water and mounted in Kaiser's glycerine gelatine (Merck, Darmstadt, Germany). Once the slides were dried, the presence of black silver deposits (BSDs) indicating the presence of metals in the tissue was semiquantitatively analyzed under an Olympus BX51 light microscope at a magnification of 20x using the criteria previously published: (-) Tissue without presence of BSDs; (+) presence of homogeneously distributed small BSDs; (++) presence of homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size and (+++) tissues with a greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits (Vicario-Parés et al., 2014).

Tissue localization of silver: field emission scanning electron microscopy (FESEM)

Paraffin sections of gill and visceral mass of organisms exposed to Ag NPs for 21 days and of the control fish were mounted on aluminium SEM stubs. Sections were dewaxed in Bio-Clear (Bio-Optica, Milan, Italy) and left to the air until the liquid excess was evaporated. Then, samples were analyzed in a Field Emission Microscope Zeiss Auriga 405, 1 nm nominal resolution (Oberkochen, Germany) equipped with a Bruker Quantax energy dispersive X-ray spectroscope (EDS) (Energy Resolution: 123 eV K_α of the Mn). Samples were observed under high vacuum (10^{-5}- 10^{-6} mbar) at variable kV.

Subcellular localization of Ag NPs: transmission electron microscopy (TEM) and X-ray analysis

Gills and liver from control fish and fish exposed to Ag NPs for 3 and 21 days were dissected and fixed for 1 h at 4 ºC in sodium cacodilate (Sigma-Aldrich) buffer 0.1 M, pH 7.2, containing 2.5% glutaraldehyde (Panreac). Then, samples were washed twice for 15 min in sodium cacodilate buffer, postfixed for 1 h in 1% osmium tetroxide (Sigma-Aldrich) with 1.5% potassium ferrocianure (Sigma-Aldrich) containing sodium cacodilate buffer 0.1 M and washed twice for 30 min in deionized H_2O. Then, samples were dehydrated in a graded series of ethanol, cleared twice for 10 min with propylene (Sigma-Aldrich) and embedded for several hours and, then, overnight in a mixture (1:1) of propylene and Epon resin (Sigma-Aldrich) prepared according manufacturer's instructions. Finally, samples were embedded in Epon resin for several hours and encapsulated for polymerization for 48 h at 60 ºC.

Semithin sections of 1500 nm in thickness were cut using a Reichert Ultracut S ultramicrotome (Leica Microsystems), stained with 1% tolulidine blue (Sigma-Aldrich) and observed under a light microscope to determine the presence of the tissues of interest. Ultrathin sections of 50 nm in thickness were then cut from selected blocks. Sections were picked up in 150 mesh copper grids, contrasted with 1% uranyl acetate (Fluka, Steinheim, Germany) for 3 min and with 0.3% lead citrate (Fluka) for 4 min and, finally, examined and photographed under a Hitachi HT7700 transmission electron microscope (Tokyo, Japan) at 60 kV. Selected samples with electrodense structures resembling NPs were analyzed by X-ray microanalysis, with the scanning-TEM module

of the Auriga 405 microscope in order to corroborate whether observed structures corresponded to Ag NPs. Sections intended for X-ray microanalysis were not contrasted with uranyl acetate and lead citrate in order to minimize interferences.

Oxidative stress: Western Blot analysis of ubiquitin and carbonylated actin

The visceral mass of 5 fish per experimental group sampled after 3 and 21 days of exposure and at 6 months post-exposure was dissected out, frozen immediately in liquid nitrogen and stored at -80 ºC for quantification of immunoreactive bands corresponding to ubiquitin and carbonylated actin using a method modified from McDonagh and Sheehan (2006). Each sample was homogenized in 10 mM Tris-HCl pH 7.2 containing 1 mM EDTA and 2% protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 2500 rpm (Precellys 24-Dual homogenizer, Bertin Technologies, Montigny le Bretonneux, France) for 5 min and the resulted aqueous phase was removed. Protein concentration was determined by measuring the optical density at 280 nm (Harris, 1989) and adjusted to 2 µg/µL with the buffer used previously. Samples were mixed 1:1 with Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 0.1 M dithiothreitol, 30% glycerol and 2 mg/mL bromophenol blue) in order to obtain a final concentration of 1 µg protein/µL.

One-dimensional electrophoresis was performed on 12.5% polyacrylamide gels containing sodium dodecyl sulfate. 10 µL of sample or 5 µL of Precision Plus Protein Standards Dual-colour (Bio-Rad, Hercules, CA, USA) were loaded in each lane. The electrophoresis was run at 200 V for 40 min (Power Pac^{TM}, Bio-Rad). Separated proteins were transferred to a PVDF membrane (Bio-Rad) by a trans-blot turbo transfer system (Bio-Rad) for 30 min up to 1 A and 25 V. For ubiquitin detection, the membrane was blocked in phosphate buffered saline (PBS) containing 1% of bovine serum albumin (BSA) for 1 h and washed in PBS. Then, it was incubated for 1 h at room temperature with the polyclonal rabbit anti-ubiquitin antibody (Dako, Glostrup, Denmark) diluted 1:1000 in PBS, followed by several washes in PBS and incubation for 1 h at room temperature with the secondary antibody (peroxidase-conjugated anti rabbit Ig G, whole molecule, Sigma-Aldrich) diluted 1:2000 in PBS. Finally, the membrane was washed several times in PBS. For the determination of protein

carbonylation level, samples were derivatized with 2,4-dinitrophenylhydrazine (DNP, Sigma-Aldrich) following the method by Conrad et al. (2001) and, then, processed as described above. The rabbit anti-DNP antibody (Sigma-Aldrich) was diluted 1:1000 in PBS and the secondary antibody was diluted 1:10000 in PBS. Peroxidase activity was visualized using an enhanced chemiluminescence kit (Thermo Scientific, Illinois, USA).

Photographic films were scanned using a GS-800 calibrated densitometer (Bio-Rad). The average optical density of the immunoreactive bands corresponding to free ubiquitin and carbonylated actin was quantified using the Quantity One image analysis software (v. 4.6.5, Bio-Rad). All the data were transformed into percentages respect to the average value of the control group at 3 days.

Genotoxicity: Micronuclei frequency

Blood samples of ten individuals per experimental group were collected after 3 and 21 days of exposure and at 6 months post-exposure by tail cutting and direct blood smear on clean microscope glass slides. Blood smears were left to air-dry and, then, fixed for 15 min in cold methanol. Once again, slides were left to air-dry and smears were stained with 6% Giemsa (Sigma-Aldrich) for 15 min. Afterwards, slides were rinsed in tap water and left to air-dry overnight and mounted in DPX (Sigma-Aldrich). 2000 erythrocytes were scored per individual fish under a Olympus BX51 light microscope at a magnification of 100x. The criteria used to determine the presence of micronuclei was: size not bigger than a 1/3 diameter of the main nucleus, same texture and colour, clearly separated from the main nucleus and with oval or circular shape (Baez-Ramirez and Prieto-García, 2005). Micronuclei frequency was expressed in ‰.

General health status: Lysosomal membrane stability (LMS)

The visceral mass of 5 individuals per experimental group was dissected after 3 and 21 days of exposure and at 6 months post-exposure, embedded in Cryo-M-Bed (Jung, Heidelberg, Germany) and frozen in liquid nitrogen. Frozen tissue sections (10 µm) were obtained in a CM3050S cryotome (Leica Microsystems) at a cabinet temperature of -24 ºC. The determination of LMS was based on the method used by Bröeg et al. (1999) as the time of acid labilization treatment required to produce the maximum

staining intensity in hepatocyte lysosomes after demonstration of acid phosphatase activity. Time intervals used for acid labilization were 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min according to Bilbao et al. (2010).

Labilization period (LP) was determined under an Olympus BX51 light microscope as the maximal accumulation of reaction product associated with lysosomes (Bröeg et al., 1999). Four determinations were made for each individual liver by dividing each section in the acid labilization sequence into 4 approximately equal segments. A mean value was then obtained for each section, corresponding to an individual fish.

Statistical analyses

Statistical analyses were performed using the SPSS statistical package v22.0 (SPSS Inc, Microsoft Co, WA, USA). Previous to the analysis, data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). Data on silver content followed a Normal distribution and were analyzed by one way ANOVA followed by the Duncan post-hoc test. For LMS, western blot and micronuclei frequency data, the non-parametric Kruskal-Wallis test was applied followed by the Dunn's post hoc test. For prevalence of histopathological alterations, Fisher's exact test was applied. In all cases, significance was established at $p<0.05$.

RESULTS

Metal bioaccumulation in whole organisms

Accumulation of silver in the whole body of fish compared to the control fish was detected after 21 days of exposure of adult zebrafish to both forms of silver, Ag NPs or ionic silver, being the increase of silver content statistically significant only after the exposure to ionic silver (Fig 1). High variability in silver content between individuals was found in fish exposed to Ag NPs. A bioaccumulation factor of 88 was calculated after the exposure to Ag NPs and of 101 after exposure to ionic silver.

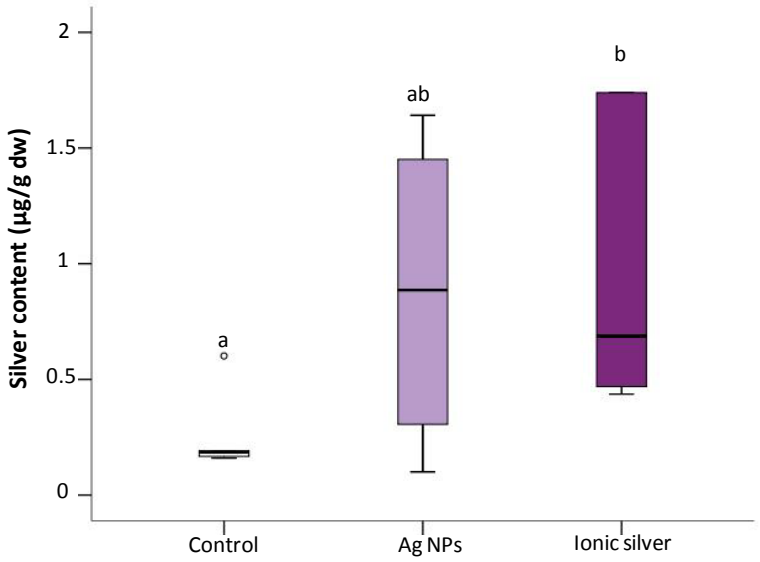

Figure 1.- Box-plot for silver accumulation (µg Ag/g dry weight) in whole fish tissue after 21 days of exposure. Boxes represent the percentages data value in between the 25th and the 75th percentile, median indicated by a line in the middle of the box. Whiskers are the data values in up to the 5th percentile and 95th percentile. Outliers are represented by circles. Different letters indicate statistically significant differences (p<0.05) according to the Duncan's test after one way ANOVA.

Metal accumulation in tissues

Presence of BSDs was not observed in tissues of control fish, neither in the intestine (Fig 2A) nor in the liver (Fig 2B). In individuals exposed to Ag NPs, the presence of BSDs was found in the intestinal epithelium (Fig 2C), with especially intense staining in the cytoplasm of enterocytes and in the microvilli. The cytoplasm of the secretory goblet cells was lack of BSDs. No noticeable staining was detected in the subjacent connective tissue. In the liver, more discrete and uniformly distributed BSDs than in the intestine were observed in hepatocytes after exposure to Ag NPs (Fig 2D). After the exposure to ionic silver, 60% of the samples presented discrete BSDs into the secretory vesicles of the goblet cells of the intestine (Fig 2E). Moreover, in the 30% of the samples, the intestinal epithelium showed a similar staining to that observed after exposure to NPs. In the case of the liver, a general staining in all the tissue was observed with a high number of BSDs (Fig 2F). The results of the semi-quantitative analysis of the presence of BSDs indicating metal accumulation in intestine and liver are shown in Table 1. The most intensely stained tissue after the exposure of both forms of the metal was the intestine of individuals exposed to Ag NPs, followed by the liver of individuals exposed to the ionic silver (Table 1).

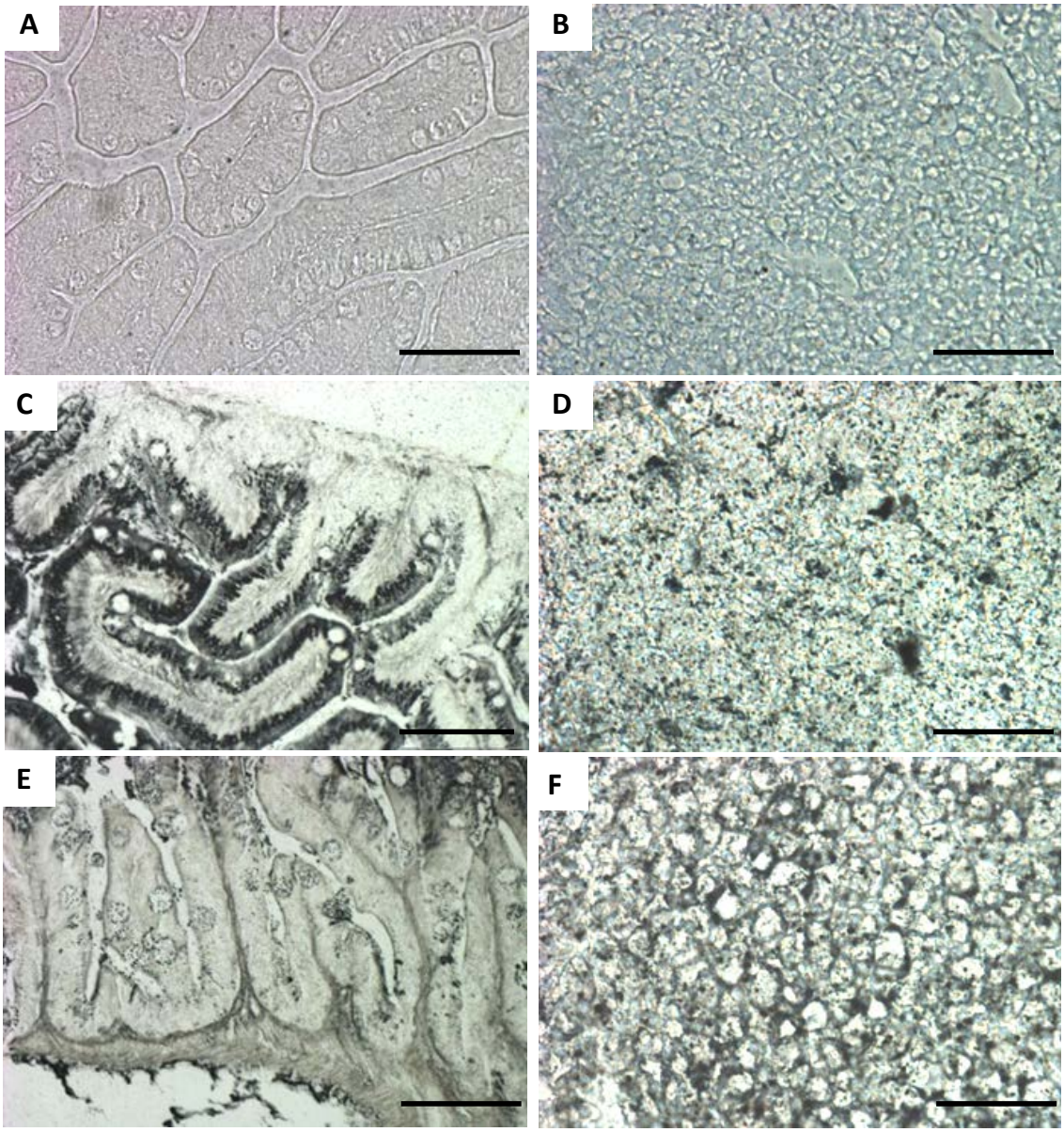

Figure 2.- Micrographs of paraffin sections of the visceral mass of adult zebrafish after autometallographical staining. (A) Intestine of control adult zebrafish. (B) Liver of control adult zebrafish. (C) Intestine of adult zebrafish exposed for 21 days to Ag NPs. (D) Liver of adult zebrafish exposed for 21 days to Ag NPs (E) intestine of adult zebrafish exposed for 21 days to ionic silver. (F) Liver of adult zebrafish exposed for 21 days to ionic silver. Scale bars in A, C, E 100 µm and in B, D, F 50 µm.

Table 1.- Semiquantification of BSDs in the intestine and liver of adult zebrafish after 21 days of exposure.

Organism	Control		Ag NPs		Ionic silver	
	Intestine	Liver	Intestine	Liver	Intestine	Liver
1	-	-	++	++	+	++
2	-	-	+	+	++	+
3	NT	NT	++	++	++	++
4	-	-	+	+	+	NT
5	-	-	+++	++	++	++
6	NT	NT	+++	++	+	++
7	-	-	NT	+	+	++
8	-	-	+++	NT	+	++
9	-	-	++	+	++	++
10	-	-	+	-	++	NT
Average number of +	0	0	2	1.3	1.5	1.9

Semiquantification criteria according to Vicario-Parés et al. (2014): (-) Tissue without presence of BSDs; (+) presence of homogeneously distributed small BSDs; (++) presence of homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size and (+++) tissues with a greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits; NT: No specific tissue in the sample.

Samples of gills (Fig 3A), liver (Fig 3B) and intestine (Fig 3C) were also analyzed by FESEM to specifically detect the presence of silver into the organs. After the X-ray analysis of the paraffin sections, a spectrum showing the elementary composition of each sample was obtained. The spectra of samples of gill (Fig 3D), liver (Fig 3E) and intestine (Fig 3F) of the control group did not show peaks in the energy range corresponding to silver. The analysis of the samples corresponding to fish exposed to Ag NPs revealed the presence of a peak at 2,9 KeV in the energy range corresponding to silver. This peak was detected in the gill (Fig 3G, J), liver (Fig 3H, K) and intestine (Fig 3I, L) samples. The energy dispersive spectroscope spectrum confirms that these organs are target for silver accumulation after the waterborne exposure to Ag NPs.

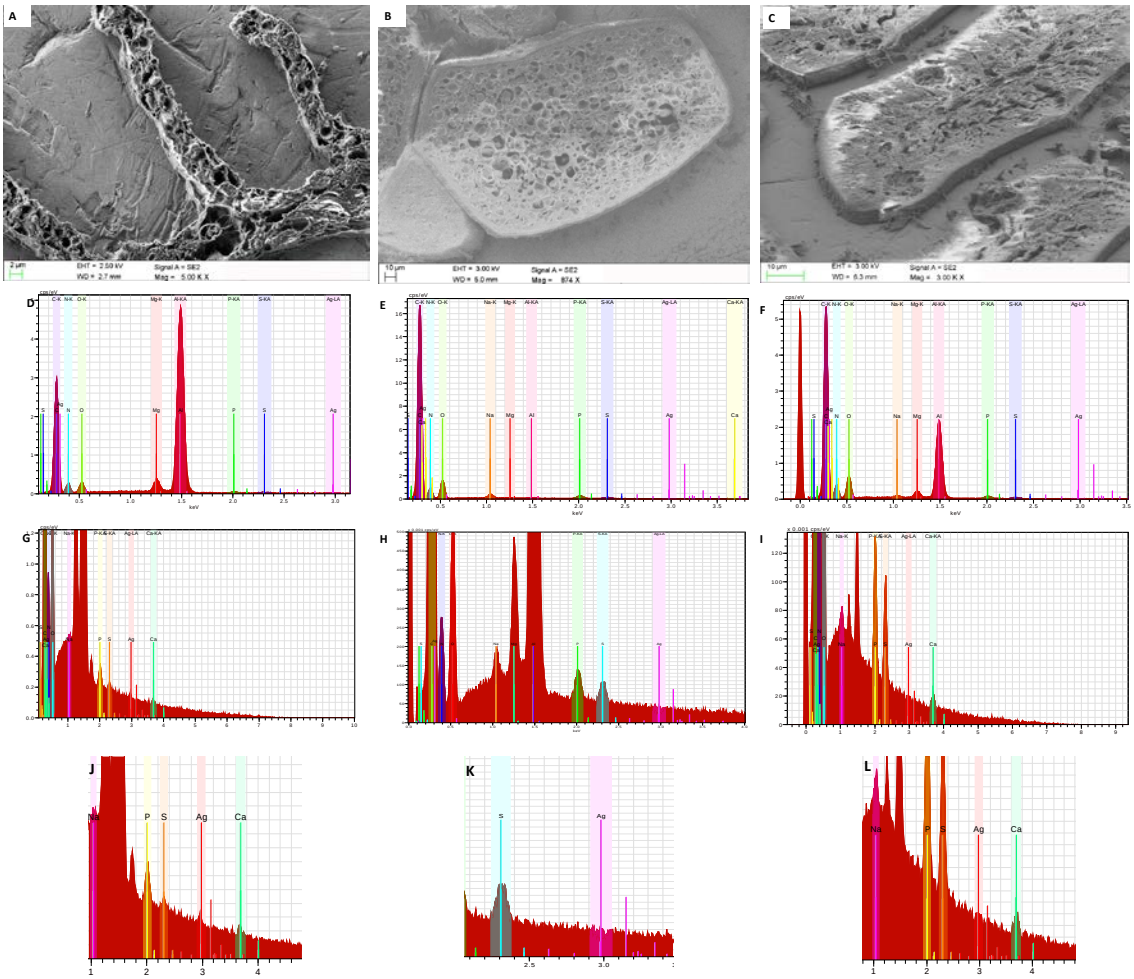

Figure 3.- Silver detection in zebrafish tissues by FESEM. Micrographs of paraffin sections of gills (A), liver (B) and intestine (C) of fish exposed to Ag NPs. Scale bar: 10 µm. Spectra obtained after X-ray analysis of gills (D), liver (E) and intestine (F) of control zebrafish. Spectra obtained after X-ray analysis of gills (G), liver (H) and intestine (I) of zebrafish exposed to Ag NPs for 21 days. Detailed view of energy range corresponding to silver in the spectra for gills (J), liver (K) and intestine (L) of zebrafish exposed to Ag NPs for 21 days.

Subcellular localization of Ag NPs

The presence of Ag NPs in gill and liver cells and their intracellular distribution were studied through TEM. Electrodense structures resembling NPs were analyzed by X-ray microanalysis in order to corroborate their chemical composition.

Gill cells (Fig 4A) of control fish presented well preserved cell structure, with intact cell membranes and well structured organelles. The different gill cell types of individuals exposed to Ag NPs did not present any histological damage (Fig 4B). Regarding the presence of NPs in the gill, individual Ag NPs were observed in the cytoplasm of epithelial cells from the secondary lamellae after 3 days of exposure (Fig 4C). The

chemical composition of the electrondense structures identified as NPs was corroborated as silver after the X-ray analysis (Fig 4D).

Hepatocytes of control fish also presented intact cell membrane and well structured organelles (Fig 5A), while hepatocytes of zebrafish exposed to Ag NPs presented an increased presence of lipid droplets and glycogen (Fig 5B). In liver, individual Ag NPs were observed in the nucleus (Fig 5C) and in mitochondria (Fig 5D) of hepatocytes after 3 and 21 days of exposure. The chemical composition of the electrondense structures identified as Ag NPs was corroborated as silver after the X-ray analysis (Fig 5E).

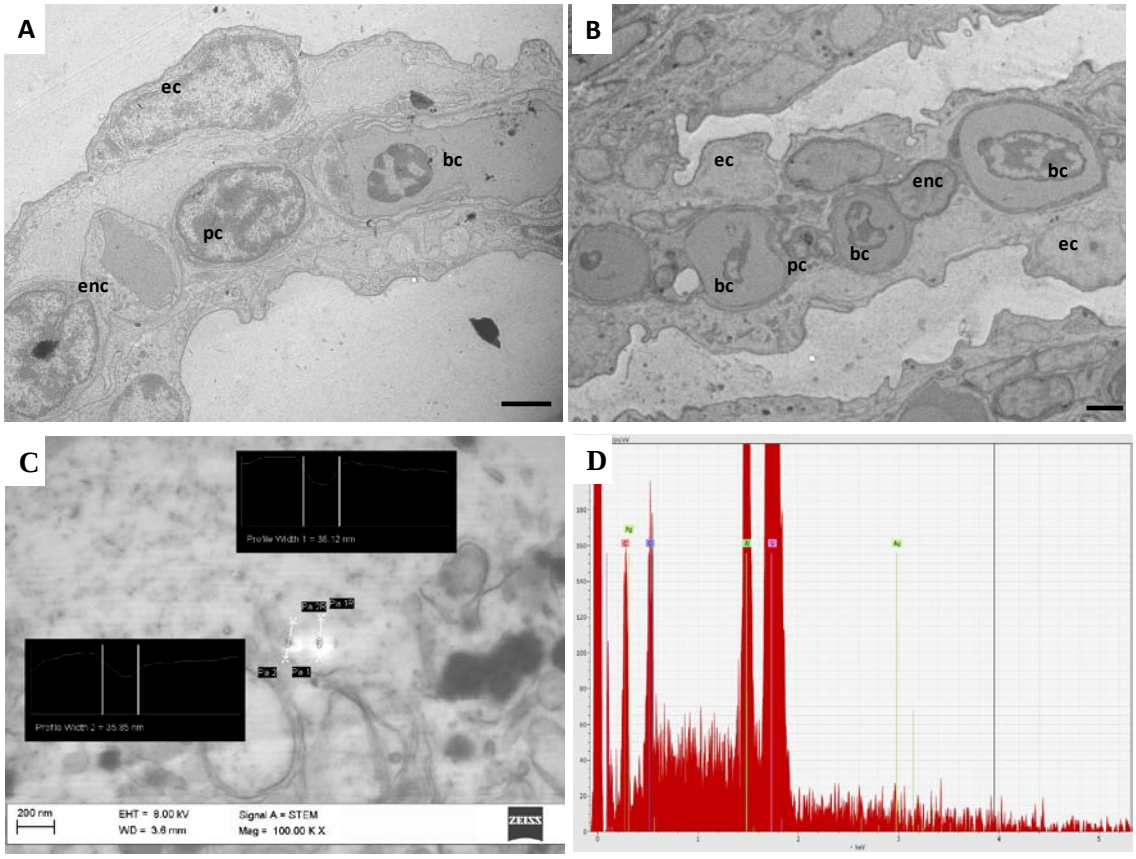

Figure 4.- Micrographs of ultrathin sections of zebrafish gill. (A) Secondary lamella of the gill from a control zebrafish. (B) Secondary lamella of the gill from a zebrafish exposed to Ag NPs for 21 days. (C) Cytoplasm of an epithelial cell in the secondary lamellae of the gill from a zebrafish exposed to Ag NPs for 3 days, Ag NPs are visible as electrondense structures. (D) X-ray energy spectrum confirming the presence of silver in the electron-dense particles shown in C. Pillar cell (pc), blood cell (bc), epithelial cell (ec), entothelial cell (enc). Scale bar: (A) 1.5 µm, (B) 2 µm, and (C) 200 nm.

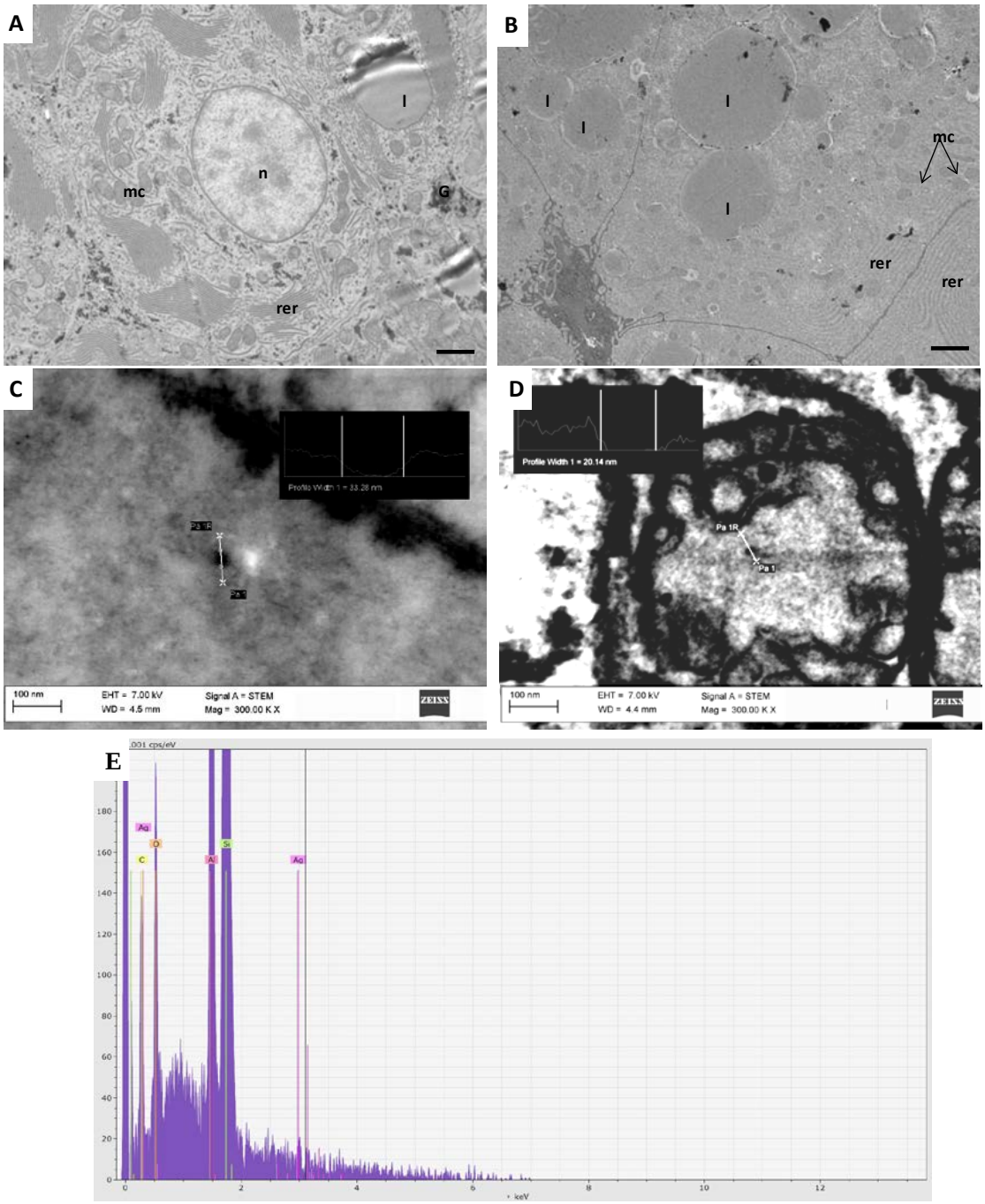

Figure 5.- Micrographs of ultrathin sections of zebrafish liver: (A) Hepatocyte of a control zebrafish. (B) Hepatocyte of a zebrafish exposed to Ag NPs. (C) Nucleus of a hepatocyte of a zebrafish exposed to Ag NPs for 21 days, Ag NPs are visible as electrondense structures. (D) Mitochondria of a hepatocyte of a zebrafish exposed to Ag NPs for 3 days, Ag NPs are visible as electrondense structures. (E) X-rat energy spectrum confirming the presence of silver in the electron-dense particles shown in C. Nucleus (n), mitochondria (mc), rough endoplasmic reticulum (rer), lipid droplet (l). Scale bar: (A) 1 µm, (B) 2 µm and (C, D) 100 nm.

Oxidative stress

With the antibody anti-ubiquitin used in this study, only one immunoreactivite band at a molecular weight of 8 kD was detected (Fig 6A). This band corresponded to free ubiquitin used as a reservoir to provide the cell with monomeric ubiquitin when is

needed. Moreover, protein carbonylation in liver after electrophoretical separations of proteins was analyzed. Different bands corresponding to several carbonylation proteins appeared in the blot (Fig 6B). Thus, for quantification purposes the immunoreactive band at a molecular weight of 40 kD corresponding to actin was selected, as actin has been described as a target protein for carbonylation.

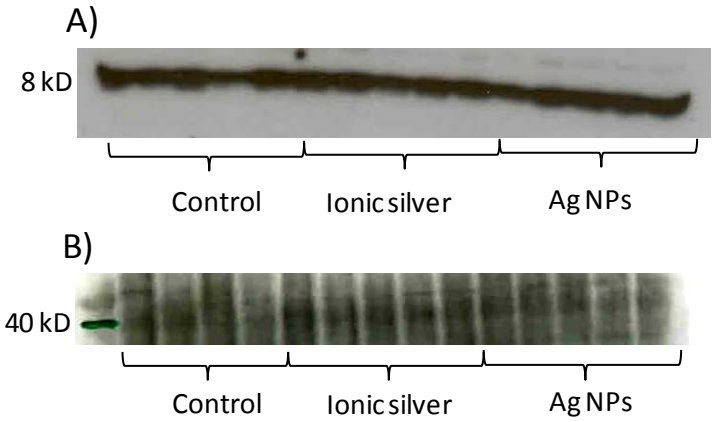

Figure 6.- Immunoreactive band for (A) ubiquitin free after 21 days of exposure and (B) carbonylated actin after 21 days of exposure.

In both cases, no significant effects were observed when the groups exposed to Ag NPs or to ionic silver were compared with the control group (Fig 7). Nevertheless, a significant increase in the level of free ubiquitin was recorded in fish after 3 days of exposure to Ag NPs, compared to fish exposed to ionic silver (Fig 7A). For actin carbonylation, a significant decrease was observed at the post-exposure period in the group exposed to Ag NPs respect to the fish exposed to ionic silver.

Genotoxicity

Exposure of zebrafish to 10 µg Ag/L of Ag NPs or to ionic silver did not cause genotoxic effects at any of the measured time points, according to the micronuclei test performed in zebrafish erythrocytes. Only one micronucleated cell was observed in one individual fish after the exposure to Ag NPs for 3 days (data not shown).

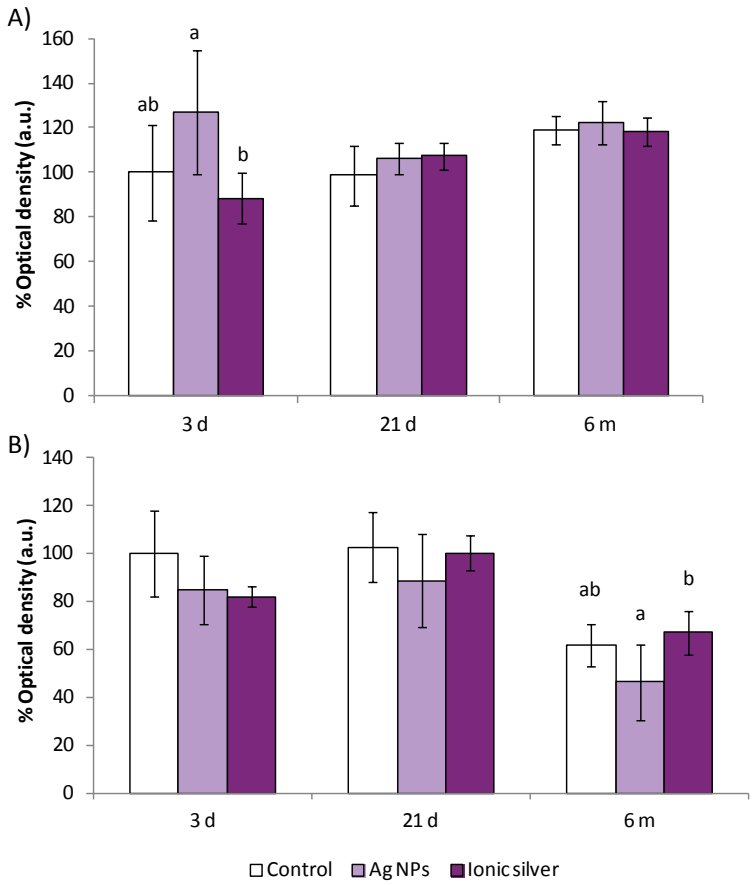

Figure 7.- Relative quantification of the optical density of the immunoreactive band for (A) free ubiquitin and (B) carbonylated actin. Data are shown as percentages respect to the average value of the control group at 3 days. Different letters indicate statistically significant differences (p<0.05) within each sampling time according to the Kruskall-Wallis test followed by the post hoc Dunns test. a.u.: arbitrary units.

Lysosomal membrane stability

The general health status of the fish was studied through the LMS test. Although not significantly, a decrease in the mean value of the LP was observed after 3 and 21 days of exposure to Ag NPs, being noticeable the high variability in the LMS recorded in these groups. Exposure to ionic silver for 3 days also caused a slight decrease of the LP (Fig 8). After 21 days, a small increase in the LP was measured in fish exposed to ionic silver, resulting in a significant difference respect to fish exposed to Ag NPs. At the end of the post-exposure period, a significant decrease in the LP was observed in fish previously exposed to Ag NPs. Desestabilization of the lysosomal membrane was observed from the first time measured and, thus, a LP value of zero was calculated for this group.

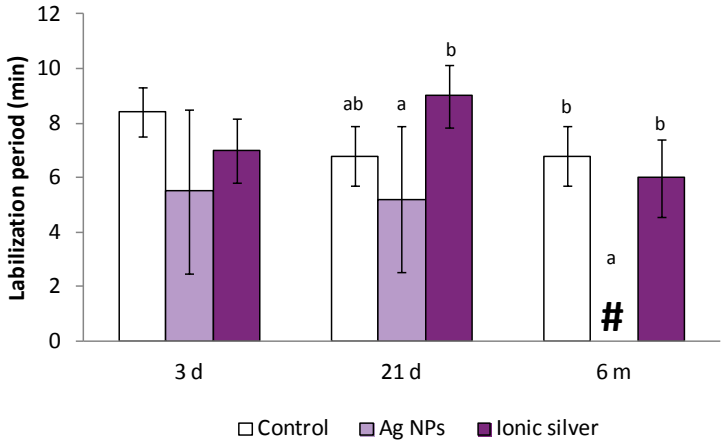

Figure 8.- Labilization period (in minutes) of the lysosomal membrane in liver cells. Different letters indicate statistically significant differences (p<0.05) within each sampling time according to the Kruskall-Wallis test followed by the post hoc Dunns test. # At 6 months, fish previously exposed to Ag NPs showed desestabilization of the lysosomal membrane from the first time point measured.

Histopathological analysis of liver and gills

In liver, histopathological alterations were neither observed after 21 days of exposure to Ag NPs or to ionic silver nor at the end of the post-exposure period.

In the gills, different pathological conditions, such as aneurism, inflammation and fusion of the secondary lamellae and hyperplasia in cells of the primary lamellae, were observed in individuals exposed to both forms of silver. The prevalence of the histopathological alterations found in gills is shown in Table 2. Control fish showed in general a normal arquitecture of the gill (Fig 9A). At 21 days of experiment only one individual showed hyperplasia in the primary lamellae. At 6 months, the control group presented an increase in the prevalence of pathologies respect to those recorded at 21 days, appearing some cases of hyperplasia, inflammation and aneurism. At 21 days of exposure to Ag NPs, all the individuals presented histopathological alterations; the prevalence of inflammation in secondary lamellae of these individuals was significantly higher than in the control group. Other pathologies appearing in the individuals were aneurisms (in the 30% of the individuals), hyperplasia (in the 20% of the organisms) (Fig 9B) and fusion of the secondary lamellae (in the 10% of the individuals). Exposure to ionic silver for 21 days also caused severe histopathological alterations. All individuals presented inflammation in secondary lamellae (Fig 9C) and 30% of them also showed aneurisms. 60% of the analyzed fish showed hyperplasia of the secondary lamellae, being this prevalence higher than after the exposure to Ag NPs. At the post-

exposure period, same histopathological alterations were observed in the previously exposed fish (Fig 9D), although always in a lower prevalence than after 21 days of exposure.

Table 2.- Prevalence of histopathological alterations in gills of zebrafish. Data are shown in percentages. Asterisks indicate statistically significant differences (p<0.05) between control and exposed groups according to the Fisher's exact test.

		n	Secondary lamellae			Primary lamellae	Total
			Inflammation	Fusion	Aneurism	Hyperplasia	
Control	21 d	8	0	0	0	12.5	12.5
	6 m	8	12.5	0	12.5	25	37.5
Ag NPs	21 d	10	90*	10	30	20	100
	6 m	8	50	0	25	12.5	50
Ionic silver	21 d	10	100*	0	30	60	100
	6 m	9	22.5	0	0	44.45	66.7

n: number of individuals per experimental group (in some cases n < 10 because the gill tissue was not always present in the histological sections used for the histological analysis).

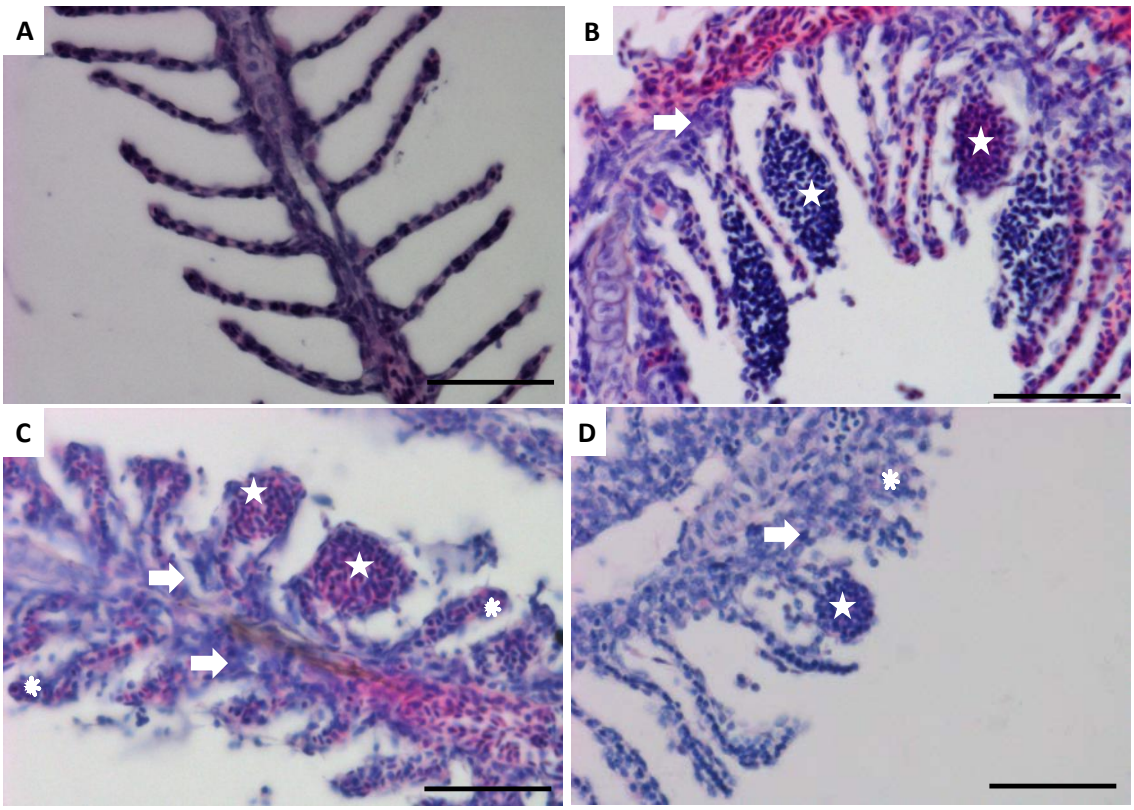

Figure 9.- Micrographs of paraffin embedded and hematoxylin/eosin stained section of zebrafish gills. (A) Gill of a control zebrafish showing normal morphology. (B) Gill of a zebrafish exposed to Ag NPs for 21 days, showing aneurisms (stars) in the secondary lamellae and hyperplasia (arrow) in the primary lamellae. (C) Gill of a zebrafish exposed to ionic silver for 21 days, showing aneurisms (stars) and inflammation (asterisks) of the secondary lamellae and hyperplasia (arrow) in the primary lamellae. (D) Gill of a zebrafish at 6 months post-exposure, previously exposed to Ag NPs for 21 days, showing aneurisms (stars) and inflammation (asterisks) in the secondary lamellae and hyperplasia (arrow) in the primary lamellae Scale bar: 50 µm.

DISCUSSION

In the present study, adult zebrafish were exposed to a sublethal concentration (10 µg Ag/L) of maltose-coated Ag NPs of 20 nm, as well as to the same nominal concentration of ionic silver for 21 days. After exposure, fish were maintained in clean water up to 6 months in order to detect potential long-term effects derived from the previous exposure or the recovery of recorded alterations. Different endpoints, ranging from silver accumulation in the organism to cellular effects in different organs, were measured in order to assess the effects provoked by the exposure to both forms of silver. Overall, our results indicate that silver was accumulated after 21 days of exposure to both, Ag NPs and ionic silver. Silver was specifically detected in gills, liver and intestine. Relevant cellular effects were not observed in the liver after the exposure to both forms of silver in the assayed conditions. Nevertheless, the histopathological analysis of gills revealed several severe histopathological alterations, such as inflammation and aneurisms in the secondary lamellae and hyperplasia in the primary lamellae, indicating that gills are the primary target organ for silver toxicity after waterborne exposure to low concentrations of silver.

According to the chemical analysis, exposure to both forms of silver, Ag NPs and ionic silver, for 21 days resulted in silver accumulation in the whole organism, with bioaccumulation factors of 88 and 101, respectively. In adult zebrafish exposed for 28 days to different concentrations of Ag NPs (from 5 to 50 µg/L), a dose dependent accumulation of silver in the gills and eviscerated carcass of the organisms was reported (Griffitt et al., 2013). At similar experimental conditions to those assayed in the present study (21 days of exposure to 15 µg Ag/L), the accumulation measured by these authors in gills and eviscerated carcass was lower than in the present study, where the whole organism was used to measure the accumulation. Some studies have reported that organs as liver and intestine of fish can accumulate more silver after the exposure to Ag NPs, than gills (Gaiser et al., 2012; Scown et al., 2010) and, therefore, the higher concentration measured in our study may be due to the higher Ag concentration accumulated in other organs.

Metal accumulation in liver and intestine was specifically observed through autometallography. This technique has been applied in zebrafish embryos exposed for 5 days to CuO and ZnO NPs and their ionic counterparts, detecting metal accumulation in different organs, such as brain, gills, liver, yolk sac and tail (Vicario-Parés et al., 2014). The analysis showed higher concentration of metals in the intestine than in the liver of fish exposed to Ag NPs, being specially marked in the cytoplasm of enterocytes and in the microvilli. This observation suggests that the digestive tract is likely a route of metal entrance into the organisms after waterborne exposure to the Ag NPs. Previous studies have also demonstrated that, along with the gills, the intestine is one of the main entrances of metals in the organism (Gaiser et al., 2012; Schirmer et al., 2013). Osborne et al. (2015) have shown that waterborne exposure of zebrafish to citrate-coated Ag NPs of 20 and of 110 nm for 4 days provoked differential pathological alterations in the intestine. The intestine of fish exposed to the smaller Ag NPs presented an increase in the number of goblet cells in the epithelial layer, some reduction in microvilli and partial damage to the lamina propria. The intestine of zebrafish exposed to the larger Ag NP presented evidence of vacuolization and partial lamina propria damage with abundant microvilli. Moreover, the intestine of fish exposed to ionic silver showed almost obliteration of the lamina propria, as results of inflammatory infiltrates, epithelial vacuolization and loss of microvilli. After autometallographical staining, these authors detected Ag NPs in the basolateral membrane of the intestinal mucosa, especially after the exposure to citrate-coated Ag NPs of 20 nm. Nevertheless, silver staining occurred on the apical membrane of the intestinal epithelial cells after the exposure to the largest NPs and to ionic silver. They indicated that the particle size and surface area could be responsible for a larger number of Ag NPs of 110 nm adhered to the microvilli. Small NPs could be more rapidly taken up by endocytosis in the epithelial layer than larger NPs, thereby acting as a delivery vehicle for increased Ag deposition in the basolateral membrane of intestinal tissues. In our study, most of the BSDs were also detected after the exposure to Ag NPs in the apical membrane. During the characterization process performed with the Ag NPs used herein (Katsumiti et al., 2015) the formation of NP aggregates was detected, indicating that the behavior of these aggregates may be similar to the behavior of the largest Ag NPs used during their work. After the aqueous exposure of

rainbow trout to TiO_2 NPs, histopathological alterations of the intestine as erosion of the villi, fusion and vacuolization of the mucosa were observed, indicating the entrance of NPs into the intestine (Federici et al., 2007). The intestine has also been reported as a Novel NP excretion pathway in zebrafish after activated carbon NP (30-200 nm) injection directly into the yolk sac (Zhao et al., 2014). Nevertheless, after the exposure to ionic silver a higher accumulation of metals was detected in liver than in the intestine. In the intestine of zebrafish exposed to ionic silver, the largest number of BSDs was detected into the secretory vesicles of the goblet cells, where the mucus is produced and promotes the elimination of gut contents and, therefore, the accumulation of metal was lower than after the exposure to Ag NPs.

The analysis carried out by FESEM in gills, liver and intestine corroborated the presence of silver in these organs after the exposure to Ag NPs, since a peak was detected at 2,9 KeV, a energy value corresponding to silver. These results further supported that these organs are target organs for silver accumulation after the exposure to Ag NPs, but whether the accumulated silver correspond to soluble silver or Ag NP was unresolved. By TEM, Ag NPs were observed in the cytoplasm of cells of the secondary lamellae, as well as in the nucleus and mitochondria of the hepatocytes. The entrance of Ag NPs into the nucleus and mitochondria of the cell was also detected after *in vitro* exposure of human cells to Ag NPs (6-20 nm), suggesting their direct involvement in the mitochondrial toxicity and DNA damage (Asharani et al., 2009). Accordingly, Krishnaraj et al. (2016) detected the presence of Ag NPs in various parts of the cytoplasm in particular between nucleus and plasma membrane in liver cells after the *in vivo* exposure for 14 days of adult zebrafish to 0.071 mg/L of Ag NPs of 24.1 nm. Also, in studies with zebrafish exposed to a high concentration (120 mg/L) of Ag NPs for 24 h, Ag NPs have been detected in the cytoplasm and in the nuclear membrane of hepatocytes of adult zebrafish (Choi et al., 2010). Moreover, in zebrafish embryos exposed to 25 mg/L for 48 h, Ag NPs were observed mainly in the nucleus of cells near the trunk and the tail, as well as in cells of the heart and brain, showing a higher affinity for the nucleus than for the cytoplasm of the cells (Asharani et al., 2008).

Oxidative stress has been suggested as one of the main mechanisms of toxicity of Ag NPs, being proteins target biomolecules of oxidative damage (McShan et al., 2014; Wei

et al., 2015). Thus, in this work we investigated the level of carbonylated proteins, measuring carbonylation of actin as target protein (McDonagh et al., 2005), and the level of free ubiquitin (Mott et al., 2001). Although the ubiquitination of proteins has been mainly used as an indicator of oxidative stress along with the carbonylation of proteins (McDonagh and Sheehan, 2006; Chora et al., 2008; Tedesco et al., 2008), levels of free ubiquitin are also measured as oxidative stress indicator as it has been suggested that free ubiquitin acts as a reservoir that can rapidly provide the cell with monomeric ubiquitin when is needed, such as under stress conditions (Kimura et al., 2009). At the experimental conditions of this study, no significant effects were observed in exposed fish in comparison with the control fish for these two parameters. The exposure concentration used (10 µg Ag/L) was much lower than the concentration reported in other studies where oxidative stress has been detected. Exposure of adult zebrafish to a high concentration (30 and 120 mg/L) of Ag NPs for a short period (24 hours) provoked a significant increase of reduced glutathione content, which was related to a physiological response against the oxidative damage caused by the exposure to Ag NPs (Choi et al., 2010). The exposure of carps for 96 h to lower concentrations (0.1 and 0.2 mg Ag/L) of Ag NPs than the concentration of the previous study provoked a significant increase in glutathione-S-transferase activity and a decrease in CAT activity (Lee et al., 2012). Also, in medaka liver the exposure to Ag NPs (0.05-0.5 mg/L) for 14 days provoked a dose-dependent decrease of SOD and CAT suggesting an excessive consumption of these antioxidants in the liver (Wu and Zhou, 2013). In the present study, despite the longer exposure period (21 days) compared to the previously mentioned studies, the low concentration used for exposure does not seem to provoke a significant oxidative stress resulting in protein damage. Accordingly, genotoxic effects were not detected by the micronuclei test in erythrocytes. The frequency of micronuclei in exposed fish was not increased in comparison with control fish. Similarly, after the exposure to CuO NPs and ionic copper (10 µg Cu/L) for 21 days, MN frequency was not increased in zebrafish erythrocytes (Vicario-Parés, 2016). Apparently, much higher metal concentrations are needed to induce MN formation in zebrafish erythrocytes. Krishnaraj et al. (2016) reported a significant increase in the frequency of MN and nuclear abnormalities after exposure for 14 days to 0.071 mg/L of Ag NPs (24.1 nm). Therefore, other more sensitive biomarkers should be considered

to analyze the effect of Ag NPs at environmentally relevant concentrations before genotoxicity can be discarded as a potential deleterious effect of fish exposure to Ag NPs. For instance, random amplified polymorphic DNA has been suggested to replace the MN test at low relevant concentrations of metals (Cambier et al., 2010).

Although we have not found NPs in the endolysosomal compartment during the ultrastructural analysis of gill and liver samples, lysosomes have been described as a target organelle for NPs (De Matteis., 2015). Destabilization of the hepatic lysosomal membrane provoked by the exposure of zebrafish to 10 µg Cu/L of CuO NPs for 3 and 21 days have been previously reported (Vicario-Parés, 2016). In the present study, at similar experimental conditions, no significant differences in the time necessary to desestabilize the lysosomal membrane were observed in fish exposed to Ag NPs. Only at 6 months post-exposure, fish previously exposed to Ag NPs showed a significant decrease in the labilization period of hepatic lysosomal membrane.

After 21 days of exposure to both forms of silver, some histopathological alterations, such as aneurism and inflammation of the secondary lamella, and hyperplasia in cells of the primary lamella were observed in the gills. According to Richmonds and Dutta (1989), gill pathologies are divided in two groups: (1) the direct deleterious effects of the irritants and (2) the defense responses of the fish. The inflammation and fusion of the lamellae have been associated to the protection of the gill against the contaminants and gill hyperplasia as a defensive mechanism leading to a decrease in the respiratory surface (Cengiz and Unlu, 2006). Despite all the organisms exposed for 21 days to both forms of silver presented any pathological alteration, the prevalence of pathologies was higher in individuals exposed to ionic silver than in fish exposed to Ag NPs. In previous studies with adult zebrafish, no pathologies in gills were observed after the exposure for 48 h to a much higher concentration (1 mg/L) of Ag NPs (Griffitt et al., 2009). Nevertheless, they observed a significant increase in the thickness of the filaments of the secondary lamella after the exposure to ionic silver at the concentration present in the NP suspension, suggesting that the NP size must inhibit or prevent the morphological changes produced by the ionic silver exposure. Similar results were also obtained after a longer exposure (28 days) to a lower concentration of Ag NPs (50 µg/L) (Griffitt et al., 2013). Accordingly, histopathological alterations in

gills were not detected after the exposure to Ag NPs (10 and 35 nm) at 10 and 100 μg/L for 10 days (Scown et al., 2010). Nevertheless, during the same experimental period, slight damage with deformation of the secondary lamellae were detected after the exposure to ionic silver (0.1 μg/L). Differences between both exposures were related to the dissolution of NPs in very small zero-valent silver NPs, which are less toxic than silver ions (Scown et al., 2010). On the contrary, Mansouri and Johari (2016) detected several histopathological alterations in gills of adult zebrafish exposed for 4 days to a range of concentrations (1.5 to 15 μg/L) of Ag NPs (63.45 nm). The effects were dose-dependent, being the greatest damage detected at the highest concentration. Also, histopathological alterations in gills of zebrafish exposed to a high concentration (1 mg/L) of citrate-coated Ag NPs (20 and 110 nm) or to ionic silver have been reported by Osborne et al. (2015). After the exposure to Ag NPs for 4 days, fusion, hyperplasia and inflammation of the secondary filaments were detected, especially after the exposure to the smallest NPs. Nevertheless, the highest toxic effect was observed in gills of zebrafish exposed to ionic silver with the most prominent inflammation and fusion of the secondary lamellae.

Although silver accumulation was detected by autometallography and electron microscopy in the liver of organisms exposed to both forms of the metal, histopathological alterations were not observed. In accordance with our results, after the exposure to a much higher concentration of Ag NPs (30 and 120 mg/L) for a shorter time period (24 hours) liver pathology was neither recorded (Choi et al., 2010). Nevertheless, at ultrastructural level, some ultrastructural changes were observed in the hepatocytes of fish exposed to Ag NPs, as an increase of lipids droplets and glycogen. In accordance with this result, a significant effect in genes involved in glycogen production was detected in rainbow trout after the exposure to Ag NPs (Gagné et al., 2012).

CONCLUSIONS

Under the experimental conditions described herein, silver accumulation was observed in zebrafish after the exposure to both forms of silver, Ag NPs and ionic silver, being the gills, liver and the intestine target organs for accumulation. Moreover, the

presence of NPs in the cytoplasm of cells from the secondary lamella of gill and the histopathological alterations registered in this organ indicate that this is one of the main entrances of NPs in the organism. The intestine, where silver has been detected, has also to be considered as an important route of uptake, as NPs can easily attach to food particles. In the assayed conditions, no evident effects on hepatic lysosomes, oxidative stress or genotoxicity were found. Therefore, at the assayed concentration, close to environmentally relevant concentrations, Ag NP exposure may produce slight toxic effects in aquatic organisms. Nevertheless, in sites where this silver concentration can be found, such as water bodies receiving effluents of waste water treatment plants, many other contaminants will be present and combined effects should be considered.

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CHAPTER III

Transcriptomic response in zebrafish liver under exposure to silver nanoparticles and ionic silver

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ABBREVIATIONS

COA, Correspondence analysis

DTGs, Differentially transcribed genes

GO, Gene ontology

LIMMA, Linear models for microarray data

NPs, Nanoparticles

PC, Principal component

qPCR, Quantitative real time PCR

ROS, Reactive oxygen species

ABSTRACT

Changes on gene transcription levels could provide an early warning of the potential toxicity of nanoparticles (NPs) due to the rapid response of genes to environmental alterations. In order to study the effect of Ag NPs in comparison with the effect provoked by ionic silver, adult zebrafish (*Danio rerio*) were waterborne exposed to 10 µg Ag/L of maltose-coated Ag NPs of 20 nm or to the same nominal concentration of ionic silver. After 3 and 21 days of exposure, the liver of 20 males was dissected out and the hepatic transcriptome was analyzed using the Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k. After 3 days of exposure, Ag NPs significantly regulated (adj *p* value < 0.05) 219 different transcripts, while the ionic form regulated 410 transcripts. After 21 days, the opposite trend was found: the ionic form regulated a lower number of transcripts (291) than at 3 days, while the effect of Ag NPs was more marked, altering the transcription level of 799 different transcripts. According to the Bast2GO analysis, all treatments especially enriched metabolic processes, while immune system or reproductive processes were especially enriched after the exposure to Ag NPs. The functional analysis identified effects on the energy metabolism with the significant regulation of "glycolysis/gluconeogenesis" after the exposure to ionic silver at 3 days and to the NP form at 21 days. Moreover, the "pyruvate metabolism" was significantly altered after the exposure to ionic silver for 3 days and the KEGG pathway "steroid biosynthesis" was significantly altered after the exposure to Ag NPs for 3 days. Other KEGG pathways detected were involved in DNA damage (purine metabolism and pyrimidine metabolism) after the exposure to Ag NPs and ionic silver at 3 days and Ag NPs at 21 days. The correspondence analysis separated the four treatments, being the exposure time the factor that explained most of the variability. Overall, exposure to both forms of silver significantly altered the zebrafish liver transcriptome, being the response to each compound different depending on the exposure time. Ionic silver exerted a stronger effect at the shortest exposure time, while Ag NPs affected at the longest exposure time, but exclusive effects could also be detected under exposure to Ag NPs suggesting that the toxicity of the Ag NPs may not be solely related to the release of ions, but also to the NP form.

Keywords: zebrafish, Ag NPs, liver transcriptome, microarray

LABURPENA

Gene transkripzio mailan gertatzen diren aldaketek, nanopartikulen (NP) toxikotasunaren abisu goiztiarra eman dezakete, geneek ingurumenaren aldaketen aurrean duten erantzun azkarra dela eta. Ag NPen efektua zilar ionikoak eragiten duen efektuarekin konparatzeko asmoz, zebra arrain (*Danio rerio*) helduak maltosaz estalitako 20 nm-ko 10 µg Ag/L NPen edo zilar ionikoko kontzentrazio berdinaren eraginpean mantendu ziren 3 eta 21 egunez. Ondoren, 20 arren gibelak disekzionatu ziren eta transkriptoma aztertu zen, Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k mikrotxip komertziala erabiliz. 3 egunetara, Ag NPek modu esanguratsuan 219 transkrito erregulatu zituzten (adj *p* balioa < 0.05), aldaera ionikoak aldiz, 410 transkrito erregulatu zituen. 21 egunen ostean, kontrako joera ikusi zen: zilar ionikoak, 3 egunetara baino transkrito gutxiago erregulatu zituen (291) eta Ag NPen efektua berriz, nabariago izan zen, 799 transkrito desberdinen transkripzio maila aldatu zituelarik. Blast2GO analisiaren arabera, tratamendu guztiek prozesu metabolikoak aberastu zituzten bereiziki; sistema immunea eta ugalketa prozesuak aldiz, Ag NPen eraginaren ondorioz aberastu ziren bereiziki. Analisi funtzionalak energia metabolismoaren gaineko efektuak identifikatu zituen, “glukolisi/glukoneogenesi”-aren erregulazio esanguratsua gertatu zelarik 3 egunez zilar ionikoaren eta 21 egunez NPen eraginpean egon ondoren. Bestalde, “pirubato metabolismoa” esanguratsuki aldatu zen 3 egunez zilar ionikoaren eraginpean egondako arrainetan eta “esteroide biosintesia” KEGG bidezidorra esanguratsuki aldatu zen 3 egunez Ag NPen eraginpean egondako animalietan. Antzemandako beste KEGG bidezidorrak DNA kaltearekin erlazionatuta zeuden (purinen eta pirimidinen metabolismoa) 3 egunez Ag NP eta zilar ionikoaren eta 21 egunez Ag NPen eraginpean mantendu ondoren. Korrespondentzia analisiak lau tratamenduak bereizi zituen, esposizio-denbora aldakortasun gehiena azaltzen zuen faktorea izan zelarik. Orokorrean, zilarraren bi aldaerek esanguratsuki aldatu zuten zebra arrainaren gibeleko transkriptoma, konposatu bakoitzaren aurrean emandako erantzuna esposizio-denboraren araberakoa izan zelarik. Zilar ionikoak efektu nabarmenagoa eragin zuen esposizio denbora laburrenean, Ag NPek aldiz esposizio denbora luzeenean eragina izan zuten, baina Ag NPen efektu bereizgarriak ere aurkitu ziren, Ag NPen toxikotasuna ioien askapenarekin lotuta egoteaz gain, NParen aldaerarekin erlazionatuta ere badagoela iradokiz.

Gako-hitzak: zebra arraina, Ag NPak, gibel transkriptoma, mikrotxipa

RESUMEN

Los cambios en los niveles de transcripción de los genes pueden advertirnos de manera temprana del potencial tóxico de las nanopartículas (NPs), debido a la rápida respuesta de los genes ante alteraciones en el medio ambiente. Para estudiar el efecto de las NPs de Ag en comparación con el efecto producido por la exposición a plata iónica, peces cebra (*Danio rerio*) adultos se expusieron vía agua a 10 µg Ag/L de NPs de Ag de 20 nm cubiertas de maltosa o a la misma concentración nominal de plata iónica. Tras 3 y 21 días de exposición, se diseccionó el hígado de 20 peces macho y se analizó el transcriptoma hepático utilizando el microchip comercial Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k. Tras 3 días, la exposición a NPs de Ag alteró significativamente (valor de *p* ajustado < 0.05) el nivel de transcripción de 219 transcriptos diferentes, mientras que la forma iónica reguló 410 transcriptos. Tras 21 días, se observó la tendencia contraria: la forma iónica reguló un menor número de transcriptos (291), mientras que el efecto de las NPs de Ag fue mucho más marcado, ya que se alteró el nivel de transcripción de 799 transcriptos diferentes. El análisis Blast2GO mostró que los distintos tratamientos enriquecieron de manera significativa los procesos metabólicos. Además, tras la exposición a las NPs de Ag también aparecieron especialmente enriquecidos los procesos relacionados con el sistema inmune o con procesos reproductivos. El análisis funcional evidenció una alteración del metabolismo energético con la regulación significativa de la "glicolisis/gluconeogénesis" tras la exposición a la forma iónica (3 días) y a las NPs de Ag (21 días). Además, el "metabolismo del piruvato" y la ruta "biosíntesis de esteroides" se alteraron tras 3 días de exposición a la forma iónica y a las NPs de Ag, respectivamente. Otras rutas KEGG detectadas estaban relacionadas con el daño en el ADN (metabolismo de purinas y de pirimidinas) tras la exposición a plata iónica (3 días) y NPs de Ag (3 y 21 días). El análisis de correspondencia separó los cuatro tratamientos, siendo el factor "tiempo de exposición" el que explicaba la mayor parte de la variabilidad. En general, la exposición a ambas formas de plata alteró de manera significativa el transcriptoma hepático del pez cebra, siendo la respuesta a cada compuesto diferente dependiendo del tiempo de exposición. La plata iónica ejerció un efecto mayor en un tiempo de exposición más corto, mientras que las NPs de Ag afectaron tras una exposición más prolongada, pero también se detectaron efectos específicos tras la exposición a NPs de Ag, sugiriendo que la toxicidad de las NPs de Ag no estaría solo relacionada con la liberación de iones de plata, sino también con la forma nanoparticulada.

Palabras clave: pez cebra, NPs de Ag, transcriptoma del hígado, microchip

INTRODUCTION

Silver nanoparticles (NPs) are among the most concerning nanomaterials for aquatic wildlife due to their potential widespread use in domestic and medical products as well as in industrial processes. In consequence, concentrations of silver in the environment could increase, since the wastewater treatment systems may have difficulty to remove the silver present in the water, remaining in the treated effluent stream (Benn and Westerhoff, 2008).

Several studies have been carried out in aquatic organisms in order to analyze acute and sublethal effects provoked by the exposure to Ag NPs (Navarro et al., 2008; Farmen et al., 2011; Pham et al., 2011; Gomes et al., 2013; Massarsky et al., 2014). Studies with zebrafish embryos have mainly focused on establishing acute toxicity levels provoked by the exposure to Ag NPs of different size, shape and coating, and on comparing such effects to the effects provoked by the exposure to ionic silver. Increased mortality and prevalence of malformations are among the most reported effects in embryos (Powers et al., 2011; Massarsky et al., 2013; Ribeiro et al., 2014; Chapter I). In adults, studies have mainly focused on the sublethal effects provoked by the exposure to both, Ag NPs and ionic silver. Oxidative stress (Katuli et al., 2014; Devi et al., 2015), bioaccumulation in different organs such as liver, gills and intestine and in the whole organism (Griffitt et al., 2013; Osborne et al., 2015; Chapter II) and presence of histopathological lesions in liver and gills (Devi et al., 2015; Chapter II) are among the alterations widely reported in zebrafish waterborne exposed to Ag NPs. Moreover, increased mortality has also been described after waterborne exposure to Ag NPs (Griffitt et al., 2008; Choi et al., 2010; Bilberg et al., 2012; Kovrižnych et al., 2013). Less information is available regarding the effects of Ag NPs at molecular level in zebrafish. Alteration of gene transcription could precede effects at cellular and physiological level caused by different pollutants and, thus, it may be a sensitive parameter to investigate the mechanisms of action of different toxic elements (Zucchi et al., 2011). Further, the use of genomic/transcriptomic techniques presents a significant advantage over traditional single endpoint methods (Piña and Barata, 2011). In fact, whole genome expression arrays provide information on a variety of cellular effects and, thus, arrays have been used to obtain specific information on significantly regulated pathways in

cells and tissues after the exposure to NPs (Fröhlich et al., 2014). The adverse outcome pathways for NPs are still unknown and, therefore, the utilization of microarrays may provide this information (Lee et al., 2015).

The transcriptomic response in zebrafish gills has been analyzed after exposure to different metal and metal bearing NPs. Griffitt et al. (2007) studied the response of the gill transcriptome of zebrafish exposed to 0.1 mg/L of 80 nm Cu NPs for 48 h or to the concentration of soluble copper matching that present in the NPs solution, using a commercial Agilent 1x22k zebrafish microarray. Exposure to copper resulted in 82 differentially transcribed genes. Cluster analysis of these genes demonstrated that the transcriptional response induced by nanocopper was highly divergent from that generated by only the soluble fraction of nanocopper exposures (Griffitt et al., 2007). In a similar experiment where TiO_2, Ag and Cu NPs (0.1 mg metal/L) were tested, the exposure to TiO_2 NPs (20.5 nm) resulted in no differentially transcribed genes after 24 h and in 413 differentially transcribed genes after 48 h; the exposure to Ag NPs (26.6 nm) regulated 148 and 462 genes after 24 and 48 h, respectively, and the exposure to Cu NPs (26.7 nm) altered the transcription level of 126 genes, which increased to 413 genes after 48 h of exposure (Griffitt et al., 2009). Exposure to Cu NPs significantly affected pathways involved in apoptosis, cell proliferation and differentiation, while Ag NPs or TiO_2 NPs did not affected any specific pathway. Effects caused on zebrafish gill transcriptome by the exposure to Ag NPs (3.1 nm) for a longer period of time (28 days) have also been studied (Griffitt et al., 2013), being the number of regulated genes concentration-dependent. Exposure to 5 µg Ag/L resulted in 366 differentially regulated genes while exposure to 50 µg Ag/L regulated 624 genes. In this latter case, differentially regulated genes were involved in DNA damage repair and cellular and developmental processes, such as cell growth, cell migration, anatomical structure morphogenesis, organ morphogenesis, cell morphogenesis, embryonic development, embryonic organ development, and skeletal development (Griffitt et al., 2013).

The hepatic transcriptomic response of zebrafish has also been analyzed after the exposure for 21 days to CuO NPs (10 µg Cu/L) or to the same nominal concentration of ionic copper. Gene transcription profiles were found to be mainly altered in liver after 3 days of exposure to CuO NPs and after 21 days of exposure to ionic copper (Vicario-

Parés, 2016). After 3 days of exposure to CuO NPs, upregulated “nucleotide biosynthesis”, “glycine biosynthesis”, “response to oxidative stress” and “oxidation and reduction process” related transcripts were identified. Moreover, down-regulation of genes related to “one carbon metabolism”, “fatty acid elongation”, “developmental processes” and different transmembrane transport processes was also observed. After 21 days of exposure to CuO NPs, most of the regulated transcripts were linked to DNA damage, while after the exposure to ionic copper the transcripts regulated were involved in DNA repair (Vicario-Parés, 2016).

Some other studies have focused on quantitative real time PCR (qPCR) measurements of specific gene transcription levels after fish exposure to Ag NPs. In zebrafish, a short exposure (24 h) to a high concentration (30 to 120 mg/L) of approximately 5-20 nm Ag NPs caused significant up-regulation of liver genes involved in DNA damage, oxidative stress and apoptosis (Choi et al., 2010). In medaka liver, transcripts coding for metallothioneins (MT), heat shock protein 70 (HSP70) and glutathione S-transferase (GST) showed a significant up-regulation after 2 days of exposure to ionic silver (1.58 and 39.46 µg/L). Nevertheless, exposure to Ag NPs of 49.6 nm (1 and 25 µg/L) resulted in the up-regulation of genes coding for MT, HSP70, GST, P53 and cytochrome p4501A (CYP1A) and in the down-regulation of the gene coding for transferrin (tf) from the first day of exposure (Chae et al., 2009). The significantly higher transcription levels seen for the *cyp1a*, *hsp70* and *p53* suggest that the exposure to NPs results in cellular damage, DNA damage and repair. In liver and gills of rainbow trout, genes related to metabolism and response to xenobiotics were studied after the exposure to Ag NPs of 10 and 35 nm (10 and 100 µg/L). Only the transcription of *cytochrome P4501A2* (*cyp1a2*) was significantly induced in gills after the exposure to Ag NPs of 10 nm at the highest tested concentration, suggesting a possible increase in oxidative stress in this tissue (Scown et al., 2010).

In the present study, adult zebrafish were exposed to 10 µg/L of Ag NPs or to the same nominal concentration of ionic silver for 3 and 21 days. Cell and tissue level effects caused by Ag NPs have been already described in Chapter II. Exposure to Ag NPs or to ionic silver provoked accumulation of silver in gills, liver and intestine as revealed by autometallography and field emission scanning electron microscopy. Moreover, using

transmission electron microscopy, presence of NPs was detected in epithelial cells from the primary lamellae of gills, as well as, in the nucleus and mitochondria of hepatocytes. In the assayed conditions, evident effects on hepatocyte lysosomal membrane stability, increased oxidative stress or genotoxicity were not found, but the histopathological analysis showed a variety of relevant alterations in the gills (Chapter II). In this work, in order to decipher the metabolic pathways altered by the exposure to Ag NPs, the whole liver transcriptome was analyzed and results were compared with results obtained after the exposure to ionic silver.

MATERIALS AND METHODS

Silver compounds

Maltose-coated Ag NPs of roughly 20 nm were synthesized using the Tollens method (Kvítek et al., 2005) resulting in a suspension containing 107.87 mg Ag/L and 0.25 mM maltose. Full characterization data have been published by Katsumiti et al. (2015) and are given in Chapter II.

Solution of silver nitrate (1 g Ag/L in 0.5 M HNO_3 matrix) was obtained from Spectrosol, BDH Chemical Ltd Poole, England.

Maintenance and experimental exposure of adult zebrafish

The experimental procedure described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. Zebrafish (wild type AB Tübingen) individuals were specifically produced and grown for the experiment in our facility at the UPV/EHU. Adult fish of approximately 4 months old were exposed to Ag NPs or to ionic silver at a concentration of 10 µg/L for 21 days. An unexposed control group was run in parallel in identical experimental conditions. The exposures were carried out in 35 L aquaria containing 50-60 fish. During the exposure period, approximately 2/3 of the aquarium water was changed by siphoning every three days and the corresponding volume of contaminated or clean water was redosed. Fish were fed with Vipagran baby (Sera, Heinsberg, Germany) and live *Artemia* (INVE Aquaculture, Salt Lake City, Utah, USA) twice per day.

After 3 and 21 days of exposure, fish were euthanized by overdose of anesthetic (benzocaine, Sigma-Aldrich, St. Louis, Missouri, USA) and the liver of 20 male zebrafish per experimental group was dissected out, pooled in 5 groups of 4 livers each and placed in cryovials containing TRIzol® (ThermoFisher Scientific, California, USA) and zirconia/silica beads (Biospec, Bartlesville, USA). Then, samples were immediately frozen in liquid nitrogen and stored at -80ºC.

RNA extraction

Total RNA was extracted following the TRizol® extraction method (ThermoFisher Scientific). Concentration of RNA was measured in a Biophotometer (Eppendorf, Hamburg, Germany). RNA was purified with RNeasy mini kit (Qiagen, Venlo, The Netherlands). In addition, RNA quality was assessed in an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Only RNA samples with a RIN value above 8.1 were used for microarray and qPCR analysis.

Microarray analysis and data treatment

Microarray analysis was carried out in the General Genomic Service – Gene Expression Unit (SGiKer) of the University of the Basque Country. Labeling of samples was carried out following the Agilent Technologies "One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling)" Version 6.5 protocol. 100 ng of total RNA were retrotranscribed and labelled using the Low imput Quick Amp Labeling kit, One color (Agilent Technologies) following the manufacturer's instructions. Hybridizations were performed on zebrafish 4x44k full genome microarrays (version V3, AMADID 026437 Agilent Technologies) containing 43,803 unique probes using the SuperHyb hybridization chamber (Agilent Technologies). Finally, slides were scanned using a G2565CA DNA microarray scanner (Agilent Technologies). Feature Extraction software v. 10.7.3.1 was used to feature signal intensity extraction and quantile normalization was applied to the raw intensities (log2 values) using the Agilent GeneSpring GX software (v 11.2).

Gene transcription profiles were compared using the LIMMA analysis in the MultiExperiment Viewer (tMeV) vs. 4.7.1 (http://www.tm4.org/mev/) software.

Benjamin-Hochberg method (FDR) for multiple test correction was employed to obtain the corrected *p* value. Significant differences were set at an adjusted *p* value $p<0.05$ and based on $\log_2FC< -1$ or $\log_2FC> 1$ ($\log_2$ fold change). Venn diagrams were obtained using the free application developed by Oliveros (2007) (http://bioinfogp.cnb.csic.es/tools/venny/) to represent the number of significantly regulated transcripts after each specific treatment as well as the number of significantly regulated common transcripts when comparing different treatments and/or exposure times. Then, significantly regulated transcripts were studied using Blast2GO (Conesa et al., 2005) and a KEGG pathway summary was performed in order to decipher biological processes altered after each treatment. Fisher exact test ($p<0.05$) was used to find statistically overrepresented functions. The significant regulation ($p<0.05$) of the KEGG pathways respect to the whole genome of *Danio rerio* was performed using DAVID online tool (Huang et al., 2009a; 2009b). In addition, a correspondence analysis (COA) was performed in order to visualize the association between the experimental groups.

Quantitative Real Time PCRs (qPCRs)

Six genes differentially regulated in the exposed groups in comparison with the control group were selected to validate microarray results by qPCRs. Genes were selected based on two criteria: fold change ($\log_2FC< -1$ or $\log_2FC> 1$) and genes of toxicological interest. Therefore, selected target genes were: *ubiquitin specific peptidase 37* (*usp37,* NM_001077343), *tumor protein p63 regulated 1* (*tprg1,* NM_001089528), *novel protein similar to vertebrate pim oncogene family* (*pim-like oncogene,* XM_005170111.2), *peroxisome proliferator-activated receptor alpha a* (*pparαa,* NM_001161333), *fibroblast growth factor 19* (*fgf19,* NM_001012246) and *superoxide dismutase 2* (*sod2,* NM_199976). *18S rRNA* (FJ915075) was selected as reference gene, since its transcription level did not vary significantly in the microarray. Available commercial TaqMan® assays (ThermoFisher Scientific) were used to amplify *usp37* (Dr03136596_m1), *tprg1* (Dr03430145_m1), *fgf19* (Dr03090227_m1) and *sod2* (Dr03100019_m1). Assays for the amplification of *pim-like oncogene, pparαa* and *18S rRNA* were not commercially available and, therefore, specific primers and probes

were designed using the Primer Express 3.0 software (ThermoFisher Scientific) (Table 1).

Table 1.- Specific primers and probes used to amplify each target gene by TaqMan qPCRs.

Gene	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
pim-like oncogene	GCTTGGAGGATGGCCTTGA	TTTCATGTTCGATGGCTTTCTG	TGGCAGTAAAAATTG
18S rRNA	CGGAGGTTCGAAGACGATCA	GGGTCGGCATCGTTTACG	ATACCGTCGTAGTTCCG
pparαa	TGCCGATTCCGCAAGTG	GCCCAAAACGAATAGCCGTTGT	CTTGCAGTGGGCATGT

Pim-like oncogene was selected to validate results obtained after the exposure to Ag NPs at 3 days. This gene, together with *usp37* and *tprg1*, was also used for the validation of the results obtained after exposure to ionic silver for 3 days. *tprg1* and *sod2* were selected to validate results obtained after 21 days exposure to both metal forms. In addition, *fgf19* and *pparαa* were used in the case of Ag NP and ionic silver exposures, respectively.

Total RNA (1 µg) was retrotranscribed to cDNA using the AffinityScript multi temperature cDNA synthesis kit (Agilent Technologies) following manufacturer's conditions in a 2720 Thermal Cycler (ThermoFisher Scientific). qPCRs were run in 25 µL reactions containing 2 µL of cDNA on a 7300 Applied Biosystems thermocycler (ThermoFisher Scientific). Universal PCR conditions were used in all the cases. Relative transcription levels were calculated based on the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) using the lowest value in the control group as calibrator and *18S rRNA* transcription levels as reference gene, with a coefficient of variation of 1,46%.

Statistical analyses were performed using the SPSS statistical package v20.0 (SPSS Inc, Microsoft Co, WA, USA). According to the Kolmogorov-Smirnov test (p<0.05), data did not follow a Normal distribution. Thus, significant differences among groups were based on the non-parametric Kruskal-Wallis test followed by the Dunns post hoc test (p<0.05) or the Mann-Whitney *U* test (p<0.05).

RESULTS

LIMMA analysis showed that a total of 1379 different transcripts were significantly regulated after the exposure to Ag NPs or ionic silver for 3 or 21 days. At 3 days of exposure to Ag NPs, 219 different transcripts coding for 129 different genes were

significantly regulated, while after the exposure to the ionic form 410 transcripts coding for 242 different genes were regulated. After 21 days, the opposite trend was found, 291 transcripts coding for 165 different genes were significantly regulated after the exposure to the ionic form, while 799 different transcripts coding for 432 different genes were significantly regulated after the exposure to Ag NPs (Fig 1A). As it can be observed in the Venn diagram, 724 transcripts were exclusively regulated as a response to Ag NPs, most of them (611) after 21 days of exposure, while 94 were specifically regulated at 3 days of exposure and 19 transcripts were significantly regulated by Ag NPs at both exposure times (Fig 1A). Transcripts exclusively regulated by the exposure to the ionic silver were 428, most of them (248) at 3 days, 175 after 21 days and only 5 transcripts were significantly regulated at both times. Except for the group exposed to ionic silver for 21 days, most of the transcripts significantly regulated were up-regulated. Finally, 21 transcripts were up-regulated in all the treatments (Fig 1B).

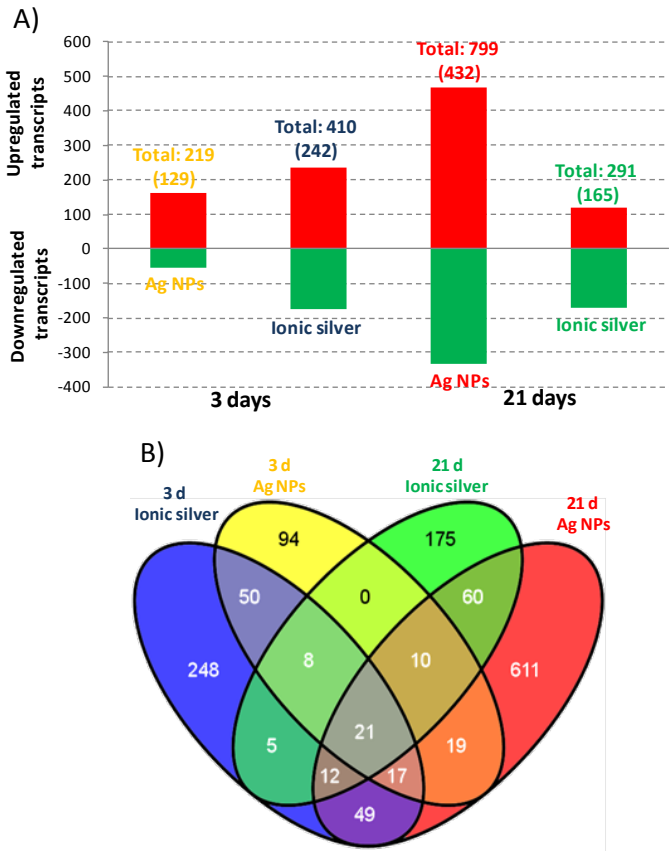

Figure 1.- (A) The bar graph shows the number of significantly up and down regulated transcripts for each treatment. The total number of significantly regulated transcripts and genes (in brackets) are indicated in each case. (B) Venn diagram showing the number of significantly regulated transcripts after each specific treatment as well as the number of significantly regulated transcripts shared among treatments.

According to the Bast2GO analysis, the GO terms enriched after the exposure to both silver forms at both exposure times belong to biological processes mainly grouped in functional categories such as “response to stimulus” and “metabolic process” (Table 2). The functional category “immune system process“, which presented two different GO terms enriched, showed a higher number of DTGs after the exposure to Ag NPs than after the exposure to ionic silver at both exposure times (Table S1). GO terms involved in the functional category “reproductive process” appeared enriched exclusively after the exposure to Ag NPs for 21 days (Table 2). The Fisher exact test did not identified GO terms differentially enriched, neither between exposures times nor between forms of silver.

A KEGG pathway summary was performed to identify the pathways affected by the different treatments (Table 3). “Purine metabolism” was affected after 3 days of exposure to both forms of silver and after 21 days of exposure to Ag NPs while “pyrimidine metabolism” was affected only after 3 days of exposure to ionic silver. The energetic metabolism appeared affected after the exposure to both silver forms, “glycolysis/gluconeogenesis” was affected after the exposure to ionic silver for 3 days and to Ag NPs for 21 days and “thiamine metabolism” pathway was affected by the exposure to Ag NPs at both exposure times. KEGG pathways related to the xenobiotic metabolism such as “aminobenzoate degradation” and to the immune system “T cell receptor signaling” appeared only after exposure to Ag NPs for 21 days. Exposure to ionic silver for 21 days did not alter any KEGG pathway at the cut off level established.

A functional analysis was performed to determine the significantly regulated pathways respect to the whole genome of zebrafish (Table 4). After 3 days of exposure to ionic silver “glycolysis/gluconeogenesis” and “pyruvate metabolism” were significantly affected. After 3 days of exposure to Ag NPs, only “steroid biosynthesis” was significantly affected, and after 21 days of exposure to Ag NPs “glycolysis/gluconeogenesis” appeared significantly affected.

Table 2 .- Gene Ontology (GO) term list of biological processes over-represented after the exposure to Ag NPs and ionic silver for 3 and 21 days.

Functional category	GO ID	GO term	Nº of DTGs			
			3 days		21 days	
			Ag NPs	Ionic silver	Ag NPs	Ionic silver
response to stimulus	GO:0009719	response to endogenous stimulus		2	4	2
	GO:0009628	response to abiotic stimulus	3	2	4	
	GO:0009607	response to biotic stimulus	3	5	4	
	GO:0009605	response to external stimulus	3	7	13	
	GO:0006950	response to stress	6	9	14	
	GO:0051716	cellular response to stimulus	10	23	36	7
	GO:0042221	response to chemical		5	16	2
	GO:0051606	detection of stimulus			2	
immune system process	GO:0006955	immune response	6	3	10	2
	GO:0019882	antigen processing and presentation	5	2	6	1
metabolic process	GO:0044238	primary metabolic process	24	57	98	33
	GO:0044237	cellular metabolic process	23	54	92	29
	GO:0006807	nitrogen compound metabolic process	13	31	53	14
	GO:0044710	single-organism metabolic process	16	29	46	11
	GO:0071704	organic substance metabolic process	26	58	103	34
	GO:0009056	catabolic process	3	8	11	5
	GO:0009058	biosynthetic process	8	27	47	13
cellular component organization or biogenesis	GO:0016043	cellular component organization	4	7	18	5
	GO:0044085	cellular component biogenesis	2	2	5	3
localization	GO:0051234	establishment of localization	12	17	30	8
	GO:0033036	macromolecule localization	2	2	5	3
	GO:0051674	localization of cell	1	6	8	2
	GO:1902578	single-organism localization	9	11	25	7
	GO:0051641	cellular localization	3		5	2

Table 2 (Continued)

Functional category	GO ID	GO term	Nº of DTGs			
			3 days		21 days	
			Ag NPs	Ionic silver	Ag NPs	Ionic silver
single-organism process	GO:0016265	death	1	2	5	3
	GO:0008283	cell proliferation		3	4	
developmental process	GO:0048856	anatomical structure development	6	20	37	11
	GO:0044767	single-organism developmental process	7	22	38	12
cellular process	GO:0044763	single-organism cellular process	32	61	102	30
	GO:0044707	single-multicellular organism process	7	22	41	12
biological adhesion	GO:0007155	cell adhesion		2	4	
biological regulation	GO:0050789	regulation of biological process	16	39	68	19
	GO:0065008	regulation of biological quality	6	5	13	2
	GO:0065009	regulation of molecular function	1	4	4	1
reproductive process	GO:0003006	developmental process involved in reproduction			1	
	GO:0032504	multicellular organism reproduction			3	
	GO:0044703	multi-organism reproductive process			3	
	GO:0044702	single organism reproductive process			3	
rhythmic process	GO:0007623	circadian rhythm				1
signaling	GO:0044700	single organism signaling	8	22	32	7

DTGs: Differentially transcribed genes.

Table 3.- Significantly transcribed genes (DTGs) contributing to the KEGG pathways highlighted after the different treatments. Arrows indicate ↑ up- and ↓ down-regulation of detailed DTGs.

KEGG pathway	Enzyme	Enzyme ID	DTGs		
			3 days		21 days
			Ag NPs	Ionic silver	Ag NPs
Purine metabolism	cyclo-ligase	ec:6.3.3.1		*gart*↑	
	DNA polymerase	ec:2.7.7.7		*polm*↑, *pole*↓	
	RNA polymerase	ec:2.7.7.6		*zgc:110727*↓	
	adenylpyrophosphatase	ec:3.6.1.3		*crbn*↑	
	kinase	ec:2.7.1.40		*pklr*↓	*pklr*↑, *pkm2a*↓
	formyltransferase	ec:2.1.2.2		*gart*↑	
	kinase	ec:2.7.4.8	*zgc:86776*↑	*gart*↑	
	ligase	ec:6.3.4.13		*gart*↑	
	phosphatase	ec:3.6.1.15	*crbn*↑, *irgf1*↑, *zgc:171695*↓, *abcc4*↓	*crbn*↑, *irgf1*↑	*abcb3l1*↓, *rasl11b*↑, *tdrd9*↑, *zgc:153738*↑, *zgc:193690*↑, *irgf1*↑, *abcc4*↑, *zgc_171695*↓
	adenylpyrophosphatase	ec:3.6.1.3	*crbn*↑, *abcc4*↓		*abcb3l1*↓, *tdrd9*↑, *zgc:193690*↑, *abcc4*↑
	phosphoribosyltransferase	ec:2.4.2.8			*hprt1l*↑
	hydrolase	ec:3.5.2.17			*urah*↑
	synthase	ec:6.3.5.3			*si:dkey-183n20.16*↑
	phosphodiesterase	ec:3.1.4.17			*pde6h*↑
	synthase	ec:6.3.2.6			*paics*↑
	allantoinase	ec:3.5.2.5			*zgc:103559*↑
	carboxylase	ec:4.1.1.21			*paics*↑
Pyrimidine metabolism	DNA polymerase	ec:2.7.7.7	*polm*↑, *pole*↓		
	RNA polymerase	ec:2.7.7.6	*zgc:110727*↓		
	deoxyribosyltransferase	ec:2.4.2.6	*si:ch211-117c8.4*↓		
	synthase (glutamine hydrolysing)	ec:6.3.4.2	*ctps*↑		

Table 3 (Continued)

KEGG pathway	Enzyme	Enzyme ID	DTGs		
			3 days		21 days
			Ag NPs	Ionic silver	Ag NPs
Glycolysis/Gluconeogenesis	hydratase	ec:4.2.1.11		*eno1*↓	*eno1*↓
	kinase	ec:2.7.1.40		*pklr*↓	*pklr*↑, *pkm2a*↓
	dehydrogenase (NAD+)	ec:1.2.1.3		*aldh9a1a*↓	
	ligase	ec:6.2.1.1		*acss1*↑	
	isomerase	ec:5.3.1.9			*gpib*↑
	glucose 6-phosphate phosphatase	ec:3.3.1.9			*si_ch211-215l11.5*↓
Thiamine metabolism	phosphatase	ec:3.6.1.15	*crbn*↑, *irgf1*↑, *zgc:171695*↓, *abcc4*↓		*abcb3l1*↓, *rasl11b*↑, *tdrd9*↑, *zgc:153738*↑, *zgc:193690*↑, *irgf1*↑, *abcc4*↑, *zgc:171695*↑
Aminobenzoate degradation	nitrophenyl phosphatase	ec:3.1.3.41			*dusp6*↓, *pfkfb4l*↓, *pptc7*↓, *ptpro*↑, *zgc:154055*↓, *si_ch211-215l11.5*↓, *ptpn22*↓
T-cell receptor signaling	phosphatase	ec:3.1.3.16			*dusp6*↓, *pptc7*↓, *ptpn22*↓, *ptpro*↑
Steroid biosynthesis	delta-isomerase	ec:5.3.3.5	*ebp*↓, *msmo1*↓		
Pyruvate metabolism	kinase	ec:2.7.1.40		*pklr*↓	
	dehydrogenase (NAD+)	ec:1.2.1.3		*aldh9a1a*↓	
	ligase	ec:6.2.1.1		*acss1*↑	

Table 4.- List of significantly altered KEGG pathways after functional analysis of different treatments (p<0.05).

KEGG pathway	3 days				21 days	
	Ag NPs		Ionic silver		Ag NPs	
	p value	FDR	*p* value	FDR	*p* value	FDR
Glycolysis / Gluconeogenesis			6.90E-04	0.044	0.0077	0.478
Pyruvate metabolism			0.0099	0.276		
Steroid biosynthesis	2.15E-06	6.02E-05				

Finally, the correspondence analysis separated the four treatments (Fig 2A, B). Three principal components explained the existing variability among all the treatments (PC1: 62%, PC2: 27% and PC3: 11%). PC1 separated samples according to the exposure time and explained most of the data variability, PC2 separated the Ag NPs 21 days treatment from the rest of the treatments and PC3 separated samples according to the metal form.

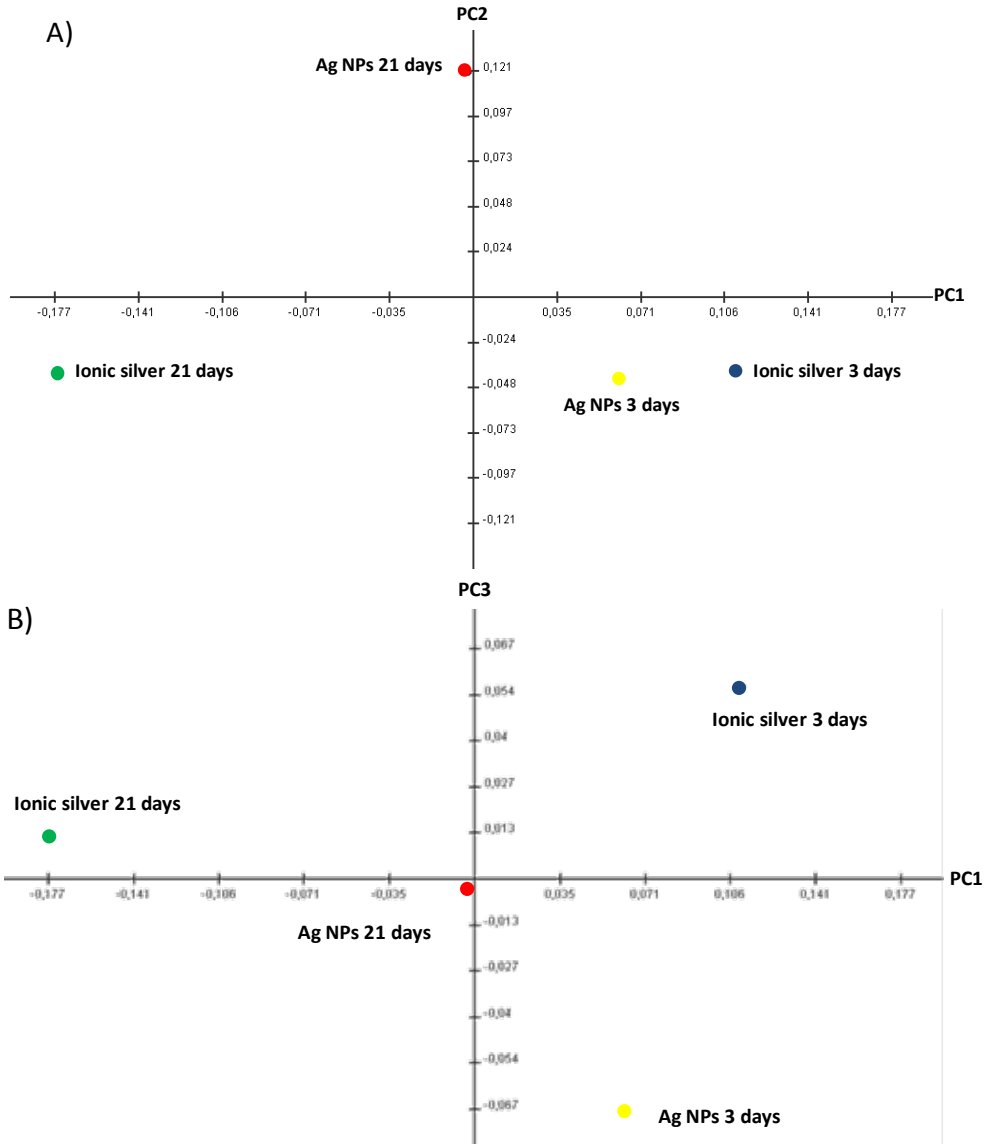

Figure 2.- Correspondence analysis (COA) axes showing the distribution of each treatment (dots) according to their transcription profile.

Quantitative PCR analysis

Selected target genes, *pim-like oncogene, fgf19, pparαa, sod2, tprg1* and *usp37,* showed similar transcription profiles both in the microarray and by qPCR analysis (Table 5).

DISCUSSION

In order to compare the effect provoked by the exposure to Ag NPs and to ionic silver, alterations in the zebrafish liver transcriptome were studied. The hepatic transcriptome of male zebrafish was analyzed after 3 and 21 days of exposure to a sublethal concentration (10 μg Ag/L) of both silver forms using a commercial microarray.

Although previous studies have already reported alterations in gills after the exposure to Ag NPs (Griffitt et al., 2009; 2013), as far as we know, this is the first time in which the hepatic transcriptome of adult zebrafish is analyzed after exposure to Ag NPs. A total of 1379 different transcripts were significantly regulated after the exposure to Ag NPs or ionic silver for 3 and 21 days. Despite the number of transcripts significantly regulated was low compared with the total amount of probes present in the array, overall, previous studies have also reported a low number of transcripts significantly regulated in comparison with transcripts present in the array used after zebrafish exposure to metal-bearing NPs. The exposure to 0.1 mg/L of Cu NPs of 80 nm for 48 h resulted solely in the differential regulation of 82 transcripts of the gill transcriptome (Griffitt et al., 2007), although the exposure to smaller Cu NPs or to TiO_2 NPs of 20.5 nm resulted in a higher number of sequences regulated (up to 413 transcripts) (Griffitt et al., 2009). Exposure to Ag NPs for 48 h resulted in 462 transcripts differentially regulated in the gill (Griffitt et al., 2009). Griffitt et al. (2013) also analyzed the gill transcriptome after exposing (28 days) zebrafish to 5 times higher silver concentration (50 μg/L) than the concentration used in the present study and found 624 transcripts altered, while exposure to ionic silver (5 μg/L) resulted in 95 transcripts differentially regulated.

Table 5.- Regulation and transcription level of selected target genes after zebrafish exposure to Ag NPs or ionic silver for 3 and 21 days, measured both in the microarray and by qPCRs. Fold change and *p* values in microarray results were based on the LIMMA analysis while the non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test or *U*-Mann Whitney ($p<0.05$) were used for qPCRs. Asterisks indicate statistically significant differences respect to the control group.

Treatment	Exposure time (days)	Accession number	Probe	Microarray			qPCR		
				Regulation	log_2 FC	Adjusted *p*-value	Regulation	log_2 FC	Adjusted *p*-value
Ag NPs	3	XM_005170111.2	novel protein similar to vertebrate pim oncogene family	up	2.52	0.048	up	1.52	0.021*
Ionic silver		NM_001089528	tumor protein p63 regulated 1 (*tprg1*)	down	-1.44	0.025	down	-0.45	0.014*
		NM_001077343	ubiquitin specific peptidase 37 (*usp37*)	down	-1.41	0.006	down	-0.13	0.014*
		XM_005170111.2	novel protein similar to vertebrate pim oncogene family	up	2.91	0.027	up	0.70	0.127
Ag NPs	21	NM_001089528	tumor protein p63 regulated 1 (*tprg1*)	down	-1.13	0.002	down	-0.37	0.049*
		NM_001012246	fibroblast growth factor 19 (*fgf19*)	down	-6.01	0.035	down	-1.18	0.009*
		NM_199976	superoxide dismutase 2 (*sod2*)	up	0.64	0.0001	up	0.18	0.011*
Ionic silver		NM_001089528	tumor protein p63 regulated 1 (*tprg1*)	down	-1.23	0.049	down	-0.36	0.071
		NM_001161333	peroxisome proliferator-activated receptor alpha a (*pparαa*)	up	0.75	0.035	up	0.12	0.251
		NM_199976	superoxide dismutase 2 (*sod2*)	up	0.45	0.034	up	0.08	0.537

FC: Fold change.

In the case of Ag NPs, the number of regulated transcripts increased with exposure time, but after 21 days of exposure to ionic silver a lower number of regulated transcripts than after exposure for 3 days was observed. This decrease in the number of significantly regulated transcripts may be due to an adaptative response of fish to extended silver exposure. Adaptative responses at transcriptional levels have been previously reported in fish chronically exposed to several metals (e.g. Zn, Cu) (Bougas et al., 2016). At biochemical and physiological level, fish are also able to adapt to silver exposure as result of compensatory changes in Na^+ transport in the gill, as observed in rainbow trout exposed for 23 days to 5 µg/l of ionic silver (Galvez and Wood, 2002).

In both treatment groups, a large number of transcripts involved in "metabolic processes" appeared significantly regulated. GO terms such as "cellular metabolic process" (GO:0044237), "primary metabolic process" (GO:0044238) or "organic substance metabolic process" (GO:0071704), presented the largest number of genes significantly altered (Table S1). Among them, *glucose phosphate isomerase b* (*gpib), phosphoribosylglycinamide formyltransferase (gart), enolase 1 (alpha) (eno1), aldehyde dehydrogenase 9 family, member A1a (aldh9a1a) or acyl-CoA synthetase short-chain family member 1 (acss1)* were significantly altered. This alteration provoked in the metabolic processes is also reflected in the functional analysis. The KEGG pathway "glycolysis/gluconeogenesis" also appeared significantly regulated after the exposure to ionic silver for 3 days and to Ag NPs for 21 days, while "pyruvate metabolism" appeared significantly regulated after the exposure to ionic silver for 3 days. Among the DTGs contributing to this pathway, *pyruvate kinase*, which is the key enzyme linking glycolysis with the massive ATP production through oxidative phosphorylation, showed different regulation after the exposure to ionic silver for 3 days and after the exposure to Ag NPs for 21 days. After the exposure to ionic silver for 3 days the isozyme *pyruvate kinase LR* (*pklr*) was down-regulated, while after the exposure to Ag NPs for 21 days this isozyme was up-regulated. In this latter case, the isozyme *pyruvate kinase, M2* (*pkm2a*), that catalyzes the rate limiting ATP producing step of glycolysis also appeared down-regulated and it is specifically oxidized by reactive oxygen species (ROS).

Alterations of the metabolic processes have been previously described in fish exposed to several metals. Bougas et al. (2016) reported over-transcription of genes involved in energy

metabolism, the gluconeogenesis pathway, and β-oxidation in caged yellow perch, suggesting an increase in energy metabolism induced by stress provoked by the chronic exposure to metals. Effects in the metabolism of fish such as *Oreochromis niloticus, Perca flavescens* or *Danio rerio* have been also detected after the exposure to metals (Ag, Cd, Cr, Cu, Zn) at different biological levels caused by the accumulations of metals in the tissues (Öner et al., 2009; Scown et al., 2010; Bougas et al., 2013).

Previous studies have attributed the toxicity of Ag NP to the release of ions (Luoma and Rainbow, 2008; Bilberg et al., 2012; McShan et al., 2014; De Matteis et al., 2015). Nevertheless, other factors related to their nanoparticulated form may be also partially involved in their toxicity (Chae et al., 2009; Griffitt et al., 2009; Scown et al., 2010, Gagné et al., 2012). As mentioned, the regulation of KEGG pathways related to the alteration of the metabolism such as "glycolysis/gluconeogenesis", or to DNA damage as "purine metabolism" were detected after the exposure to both forms of silver at different time points.

DNA damage provoked by the exposure to Ag NPs has been suggested as one of the main mechanisms of toxicity of Ag NPs (McShan et al., 2014). The exposure to Ag NPs can provoke an overproduction of ROS that may cause oxidative stress (Christen et al., 2013). Elevated concentrations of ROS provoke DNA damage, resulting in the up regulation of genes involved in DNA repair processes (Griffitt et al., 2013). In fish, DNA damage has been previously associated with the exposure to Ag NPs, along with cellular damage and oxidative stress observed in different organs such as liver and gills (Chae et al., 2009; Choi et al., 2010; Griffitt et al., 2013). In the present study DNA damage related pathways, such as "pyrimidine metabolism", appeared altered after the exposure to Ag NPs for 3 days. In addition, "purine metabolism" presented several transcripts altered after the exposure to ionic silver for 3 days and after the exposure to Ag NP for 3 and 21 days. The presence of silver ions inside the cell can also stimulate the production of ROS leading to provoke serious cellular injuries such as DNA damage (De Matteis et al., 2015). Nevertheless, these effects provoked at molecular level, were not detected at higher biological levels with the biomarkers used in this study (Chapter II).

Harmful effects of ROS can be prevented by antioxidant enzymes, such as superoxide dismutase, that can neutralize ROS (Andreyev et al., 2005; Hanukoglu, 2006). In this study, s*uperoxide dismutase* 2 (*sod2*), which was used for the validation of the microarray results, was significantly up-regulated after the exposure of zebrafish to both forms of silver for 21 days.

After the exposure to Ag NPs for 3 days, the KEGG pathway "steroid biosynthesis" appeared significantly altered, despite only two genes were significantly down-regulated, *methylsterol monooxygenase 1 (msmo1)* and *emopamil binding protein (sterol isomerase)* (*ebp*), which are involved in cholesterol biosynthesis. The liver is the primary organ for cholesterol production in vertebrates, and reduction in the transcription of genes involved in cholesterol biosynthesis has potentially major implications for health status, since cholesterol is required for membrane stability, formation of billiary acids, or biosynthesis of steroid hormones (Santos et al., 2010). In agreement with our results, Lee et al. (2012) reported a decrease in the total cholesterol concentration in common carp (*Cyprinus carpio*) after the exposure to Ag NPs (12 nm) for 4 days, especially at the lowest concentration tested (0.025 mg/L), which is close to the concentration used in the present study.

The GO analysis reflected a large number of DTGs regulated under the functional category "response to stimulus", especially after the exposure to the ionic silver for 3 days and to the Ag NPs for 21 days. Also related to this term, the immune system was affected after the exposure to both Ag NPs and ionic silver after 3 and 21 days as reflected in the GO analysis, with the GO terms "immune response" and "antigen processing and presentation" which presented a higher number of DTGs after the exposure to Ag NPs in both times than after the exposure to ionic silver. Moreover, the exposure to Ag NPs for 21 days mainly provoked the down-regulation of transcripts involved in the KEGG pathway "T cell receptor signaling pathway". The immune functions of different organisms, including invertebrates and vertebrate animals, may be affected after the exposure to metal containing NPs (Luo et al., 2015). The main function of the immune system is to protect the host from foreign materials; however, inadvertent recognition of NPs as foreign by the immune cells may result in a multilevel immune response against the NPs and eventually lead to toxicity in the host (Zolnik et al., 2010). The exposure to Ag NPs has been previously described as a factor

which provoke the regulation of several transcripts (e.g. *interleukin-1 beta (Il1β)*), involved in the immune response in adult zebrafish and embryos (Park and Yeo, 2013; Olasagasti et al., 2014; Park and Yeo, 2015; Krishnaraj et al., 2016).

The COA analysis separated the groups depending on the silver form and exposure time, being the group exposed to Ag NPs for 21 days separated from the other groups, which could indicate a stronger effect in the hepatic transcriptome after the exposure to this treatment in comparison to the other treatments. The exposure to Ag NPs for 21 days exclusively regulated the functional category "reproductive processes", presenting different GO terms significantly enriched. Among genes significantly altered in this process, *chemokine (C-X-C motif) ligand 12a (stromal cell-derived factor 1)* (*cxcl12a*) and *chemokine (C-X-C motif) receptor 4a* (*cxcr4a*) appeared down-regulated. These transcripts are involved in the migration of germ cells, critical for conveying the genetic information to the next generation, especially during the early gastrulation stage (Nishimura and Tamaka, 2014; Zou et al., 2015). Effects provoked by the exposure of Ag NPs in the regulation of these genes and their consequences have not been adequately investigated. Therefore, a deeply analysis of the toxic effects provoked by the exposure to Ag NPs in zebrafish reproduction and in the regulation of these genes deserves further investigation.

CONCLUSIONS

Overall, results show that ionic silver and Ag NPs, under the experimental conditions described herein, provoked similar effects in the zebrafish liver transcriptome, but at different exposure times. Exposure to ionic silver for 3 days provoked significant alteration of transcripts mainly involved in the energetic metabolism, while such pathways were significantly altered after 21 days of exposure to Ag NPs. Moreover, the stronger effect in the immune system and DNA damage-related transcripts along to the effects in reproductive processes exclusively detected after the exposure of Ag NPs for 21 days suggest that the effects of Ag NPs at transcriptomic level in zebrafish liver is not solely due to the release of ions but also to the NP form.

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SUPPLEMENTARY DATA

Table S1.- Gene Ontology (GO) processes over-represented after the exposure the different treatments. Arrows indicates ↑ up- and ↓ down-regulation of DTGs.

Treatment	GO ID	GO term	DTGs
Ag NPs 3 days	GO:0065007	biological regulation	*zgc:152808*↑, *rhoua*↑, *jag1a*↑, *selt1a*↑, *gata1a*↑, *si:ch211-206a7.2*↑, *arhgap12*↑, *sepw2a*↑, *nr1h3*↑, *adrb3a*↑, *syn1*↓
	GO:0002376	immune system process	*mhc1uea*↑, *mhc1uda*↓, *mhc1uaa*↑, *gata1a*↑, *mhc1ufa*↓, *zgc:153138*↓
	GO:0023052	signaling	*rhoua*↑, *jag1a*↑, *arhgap12*↑, *adrb3a*↑, *syn1*↓
	GO:0044699	single-organism process	*zgc:153681*↑, *gata1a*↑, *sepw2a*↑, *zgc:171695*↓, *dclre1c*↑, *zgc:171957*↑, *selt1a*↑, *abcc4*↓, *adrb3a*↑, *cahz*↑, *zgc:65857*↑, *syn1*↓, *sept9a*↑, *si:ch211-5k11.6*↑, *zgc:92880*↑, *jag1a*↑, *cry5*↓, *cry3*↓, *hsd17b7*↓, *trappc2l*↑, *arhgap12*↑, *msmo1*↓, *si:ch211-197g15.10*↑, *zgc:152808*↑, *slc6a13*↑, *rhoua*↑, *ebp*↓
	GO:0008152	metabolic process	*hadhb*↑, *zgc:153681*↑, *cry5*↓, *zgc:110346*↑, *cry3*↓, *psmb9b*↑, *gata1a*↑, *si:ch211-206a7.2*↑, *hsd17b7*↓, *msmo1*↓, *zgc:152808*↑, *zgc:163044*↑, *ebp*↓, *dclre1c*↑, *zgc:171957*↑, *nr1h3*↑, *cahz*↑
	GO:0051179	localization	*si:ch211-5k11.6*↑, *zgc:92880*↑, *slc6a13*↑, *zgc:153681*↑, *zgc:162560*↓, *zgc:171957*↑, *abcc4*↓, *syt5a*↑, *trappc2l*↑, *zgc:65857*↑, *syn1*↓
	GO:0050896	response to stimulus	*mhc1uea*↑, *mhc1uda*↓, *jag1a*↑, *cry5*↓, *mhc1uaa*↑, *cry3*↓, *arhgap12*↑, *mhc1ufa*↓, *zgc:153138*↓, *rhoua*↑, *dclre1c*↑, *adrb3a*↑, *cahz*↑
	GO:0051704	multi-organism process	*mhc1ufa*↓
	GO:0071840	cellular component organization or biogenesis	*zgc:15416*↑*4*, *zgc:171937*↑
	GO:0032502	developmental process	*jag1a*↑, *gata1a*↑
	GO:0032501	multicellular organismal process	*jag1a*↑, *gata1a*↑
	GO:0009987	cellular process	*psmb9b*↑, *gata1a*↑, *si:ch211-206a7.2*↑, *sepw2a*↑, *zgc:171695*↓, *zgc:163044*↑, *dclre1 c*↑, *zgc:171957*↑, *selt1a*↑, *zgc:171937*↑, *abcc4*↓, *adrb3a*↑, *cahz*↑, *zgc:65857*↑, *syn1*↓, *sept9a*↑, *jag1a*↑, *zgc:154164*↑, *cry5*↓, *zgc:110346*↑, *cry3*↓, *arhgap12*↑, *msmo1*↓, *si:ch211-197g15.10*↑, *zgc:152808*↑, *rhoua*↑, *nr1h3*↑

Table S1 (Continued)

Treatment	GO ID	GO term	DTGs
Ionic silver 3 days	GO:0002376	immune system process	*si:ch211-287j19.6*↓
	GO:0023052	signaling	*stmn1a*↓, *rgs7bpb*↓, *csrp1a*↓, *rhoae*↑, *adam8a*↑, *rabl2*↓, *socs1*↑, *rangap1*↑, *prkcd*↑, *zgc:100963*↓, *nlk1*↑, *angpt1*↑, *arf1l*↑, *s1pr1*↓, *adrb2b*↑
	GO:0050896	response to stimulus	*stmn1a*↓, *rgs7bpb*↓, *si:ch211-234p6.13*↓, *csrp1a*↓, *hspa4a*↑, *rhoae*↑, *si:ch211-287j19.6*↓, *adam8a*↑, *rabl2*↓, *socs1*↑, *rangap1*↑, *mpx*↓, *hspb8*↑, *ca2*↑, *prkcd*↑, *c1ql4l*↑, *zgc:100963*↓, *nlk1*↑, *angpt1*↑, *arf1l*↑, *s1pr1*↓, *adrb2b*↑
	GO:0040007	growth	*ctgf*↓
	GO:0040011	locomotion	*lama5*↓, *csrp1a*↓, *pard3*↑, *s1pr1*↓
	GO:0032502	developmental process	*sox3*↓, *csrp1a*↓, *pard3*↑, *foxe3*↓, *acta1b*↓, *usp37*↓, *ankhb*↓, *lama5*↓, *ved*↑, *c1ql4l*↑, *angpt1*↑, *ldb1b*↑, *s1pr1*↓, *ctgf*↓
	GO:0032501	multicellular organismal process	*sox3*↓, *csrp1a*↓, *pard3*↑, *foxe3*↓, *acta1b*↓, *usp37*↓, *ankhb*↓, *lama5*↓, *ved*↑, *c1ql4l*↑, *angpt1*↑, *ldb1b*↑, *s1pr1*↓, *ctgf*↓
	GO:0009987	cellular process	*csrp1a*↓, *zgc:153725*↑, *taf6*↑, *pole*↓, *zgc:173738*↓, *lama5*↓, *ved*↑, *ca2*↑, *prkcd*↓, *c1ql4l*↑, *hmga1b*↓, *ctps*↑, *pes*↑, *sox3*↓, *rgs7bpb*↓, *zgc:136639*↑, *rhoae*↑, *foxe3*↓, *gart*↑, *sc5dl*↓, *zgc:158450*↑, *ccne*↓, *ctgf*↓, *stmn1a*↓, *irf2*↑, *zgc:172145*↑, *si:ch211-117c8.4*↓, *adam8a*↑, *zgc:153268*↑, *usp37*↓, *tnikb*↓, *calrl2*↑, *dscc1*↓, *bmi1b*↓, *zgc:100963*↓, *nlk1*↑, *angpt1*↑, *orc6*↓, *zgc:153296*↓, *fut9*↓, *slc2a3*↓, *pard3*↑, *elovl5*↑, *cdk10*↑, *rabl2*↓, *socs1*↑, *zgc:153454*↑, *rangap1*↑, *mfsd2ab*↑, *arf1l*↑, *zgc:110727*↓, *taldo1*↓, *s1pr1*↓, *zgc:123275*↑, *adrb2b*↑, *cideb*↑
	GO:0065007	biological regulation	*stmn1a*↓, *irf2*↑, *csrp1a*↓, *zgc:172145*↑, *taf6*↑, *adam8a*↑, *ankhb*↓, *lama5*↓, *ved*↑, *ca2*↑, *prkcd*↓, *c1ql4l*↑, *zgc:100963*↓, *nlk1*↑, *angpt1*↑, *hmga1b*↓, *arfgap3*↓, *sox3*↓, *rgs7bpb*↓, *zgc:136639*↑, *rhoae*↑, *foxe3*↓, *rabl2*↓, *socs1*↑, *zgc:153454*↑, *rangap1*↑, *arf1l*↑, *s1pr1*↓, *ctgf*↓, *adrb2b*↑
	GO:0044699	single-organism process	*stmn1a*↓, *csrp1a*↓, *zgc:172145*↑, *si:ch211-117c8.4*↓, *adam8a*↑, *acta1b*↓, *usp37*↓, *zgc:173738*↓, *ankhb*↓, *lama5*↓, *ved*↑, *dscc1*↓, *ca2*↑, *prkcd*↓, *c1ql4l*↑, *zgc:100963*↓, *nlk1*↑, *angpt1*↑, *ctps*↑, *ldb1b*↑, *pes*↑, *sox3*↓, *zgc:153296*↓, *rgs7bpb*↓, *fut9*↓, *plcb4*↓, *slc2a3*↓, *pard3*↑, *elovl5*↑, *rhoae*↑, *aldh9a1a*↓, *foxe3*↓, *gart*↑, *rabl2*↓, *socs1*↑, *sc5dl*↓, *rangap1*↑, *mfsd2ab*↑, *mpx*↓, *ccne*↓, *zgc:163057*↑, *arf1l*↑, *taldo1*↓, *s1pr1*↑, ↓*zgc:123275,*↑ *ctgf*↓, *adrb2b*↑, *cideb*↑, *fgf19*↓
	GO:0022610	biological adhesion	*lama5*↓, *pcdh2ac*↓
	GO:0008152	metabolic process	*irf2*↑, *csrp1a*↓, *zgc:172145*↑, *zgc:153725*↑, *si:ch211-117c8.4*↓, *taf6*↑, *adam8a*↑, *pole*↓, *zgc:153268*↑, *usp37*↓, *tnikb*↓, *zgc:173738*↓, *csrp2bp*↑, *calrl2*↑, *ved*↑, *dscc1*↓, *ca2*↑, *prkcd*↓, *nlk1*↑, *zgc:114081*↓, *hmga1b*↓, *ctps*↑, *pes*↑, *arfgap3*↓, *sox3*↓, *orc6*↓, *zgc:153296*↓, *fut9*↓, *plcb4*↓, *zgc:136639*↑, *elovl5*↑, *acaa1*↑, *acss1*↑, *cdk10*↑, *aldh9a1a*↓, *foxe3*↓, *gart*↑, *zgc:153454*↑, *sc5dl*↓, *mpx*↓, *acss2*↓, *zgc:158450*↑, *ccne*↓, *taldo1*↓, *zgc:110727*↓, *pcyt2*↓, *zgc:123275*↑
	GO:0051179	localization	*fabp7a*↑, *slc2a3*↓, *csrp1a*↓, *pard3*↑, *zgc:172145*↑, *mfsd2ab*↑, *ankhb*↓, *ap2m1b*↑, *lama5*↓, *ca2*↑, *api5*↑, *zgc:163057*↑, *s1pr1*↓
	GO:0051704	multi-organism process	*si:ch211-234p6.13*↓, *mpx*↓, *c1ql4l*↑
	GO:0071840	cellular component organization or biogenesis	*bmi1b*↓, *ctgf*↓, *pes*↑

Table S1 (Continued)

Treatment	GO ID	GO term	DTGs
Ag NPs 21 days	GO:0032502	developmental process	*agxt2l1↑, pacsin3↑, copeb↓, quo↓, rbpjb↓, cebpa↓, tdrd9↑, oc90↑, cdc73↑, paics↑, mych↓, rasl11b↑, ihha↑, msna↓, tbx2b↑, cxcr4b↓, grna↓, extl3↓, fzd8a↓, dusp6↓, spi1↓, cxcl12a↓, prickle1a↓, musk↓, gsc↓, cav1↓, alcamb↓, igfbp2a↓, nadl1.2↓, nkd3↓*
	GO:0032501	multicellular organismal process	*crfb7↓, agxt2l1↓, pacsin3↓, copeb↓, quo↓, rbpjb↓, trpa1a↓, cebpa↓, tdrd9↓, parvb↓, oc90↓, cdc73↓, paics↓, mych↓, ihha↓, tbx2b↓, cxcr4b↓, grna↓, extl3↑, fzd8a↑, dusp6↓, spi1↓, cxcl12a↓, opn1sw2↑, musk↑, prickle1a↓, gsc↑, cav1↓, alcamb↓, igfbp2a↑, pde6h↑, nadl1.2↑*
	GO:0009987	cellular process	*zgc:154055↓, camk2n1a↓, pacsin3↑, zgc:113984↓, dnajb1b↓, rbpjb↓, fkbp5↓, lhx9↑, trpa1a,↑ got1↑, hif1al↓, asb11↑, mych↓, eno1↓, si:ch211-220i15.2↑, calrl↓, tbx2b↑, bzw1b↓, spi1↓, zgc:171801↓, musk↑, zgc:153759↓, mycb↓, araf↑, zgc:77060↓, slc25a21↑, dnase2↓, ptpro↑, tsc22d3↓, rab6b↑, slc25a27↑, sfxn1↑, atf7a↑, psmb10↓, acsf2↑, si:ch211-35n24.1↓, zgc:65857↑, paics↑, si:ch211-10j20.1↓, rfx1a↑, mthfsd↑, dcdc2b↑, ihha↓, zgc:103559↑, chac1↓, foxo3b↓, abcb3l1↓, agpat2↑, cxcl12a↓, cacna1sb↓, cbx4↓, etv5a↓, cav1↓, glulb↓, rnd1l↑, brf1a↓, nadl1.2↑, slc25a43↓, zgc:113944↑, stk35↓, zgc:112970↑, zgc:100906↓, tubgcp2↑, cebpa↓, dbx2↑, pkm2a↓, hsp90b1↓, bhlhe40↑, zgc:162824↑, ca4a↓, cry5↑, ttll1↑, cmklr1↓, st6galnac3↓, wasb↓, ttll7↑, urah↑, opn1sw2↑, alcamb↓, igfbp2a↑, adra2b↑, arhgef3l↓, arl4d↓, quo↓, zgc:112356↓, socs3a↓, nr1d2a↓, tdrd9↑, hmgcs1↓, tagap↓, si:ch211-171o19.1↑, cdc73↑, aptx↑, slc25a1↓, rhogb↓, arl5c↓, si:dkey-52h23.1↓, ucp2↓, rpl15↑, zgc:153537↑, rasl11b↑, cxcr4b↓, cdo1↑, zgc:92903↑, grna↓, extl3↑, fzd8a↑, si:ch211-215m21.17↑, dusp6↓, pfkfb4l↓, hprt1l↑, gpib↑, prickle1a↑, dync2i1↑, pklr↑, gsc↑, fth1a↓, si:dkey-183n20.16↑, gnmt↑, zgc:162641↑, stxbp1b↑*
	GO:0044699	single-organism process	*pacsin3↑, rbpjb↓, zgc:162591↑, trpa1a↑, got1↑, hif1al↓, asb11↑, mych↓, eno1↓, tbx2b↑, cyp11b2↑, spi1↓, zgc:171801↓, musk↑, hsd11b2↓, zgc:153759↓, araf↑, slc25a21↓, rab6b↑, slc25a27↑, sfxn1 , parvb↑, acsf2↑, si:ch211-35n24.1↓, zgc:65857↑, paics↑, mthfsd↑, dcdc2b↑, ihha↑, chac1↓, abcb3l1↓, agpat2↑, cxcl12a↓, cacna1sb↓, mical2b↓, cav1↓, glulb↓, rnd1l↓, nadl1.2↑, zgc:152990↑, dhrs3a↓, slc25a43↓, crfb7↓, copeb↓, zgc:100906↓, tubgcp2↑, cebpa↓, pkm2a↓, ca4a↓, cry5↑, cmklr1↓, st6galnac3↓, wasb↓, urah↑, opn1sw2↑, alcamb↓, igfbp2a↑, adra2b↑, arhgef3l↓, agxt2l1↑, arl4d↓, quo↓, socs3a↓, tdrd9↑, hmgcs1↓, oc90↑, tagap↓, zgc:165649↓, plod2↑, cdc73↑, aptx↑, slc25a1↓, p4ha1b↓, rhogb↓, arl5c↓, ucp2↓, zgc:153537↑, rasl11b↑, cxcr4b↓, zgc:92903↑, cdo1↑, grna↓, extl3↑, fzd8a↑, dusp6↓, pfkfb4l↓, hprt1l↑, gpib↑, prickle1a↑, dync2i1↑, pklr↑, gsc↑, fth1a↓, pde6h↑, si:dkey-183n20.16↑, gnmt↑, zgc:162641↑, stxbp1b↑, nkd3↑, fgf19↓, cdk5↑, mcl1b↓*
	GO:0022610	biological adhesion	*parvb↑, hapln3↓, tbx2b↑, nadl1.2↑*

Table S1 (continued)

Treatment	GO ID	GO term	DTGs
Ag NPs 21 days	GO:0008152	metabolic process	*zgc:154055↓, camk2n1a↓, dnajb1b↓, rbpjb↓, fkbp5↓, lhx9↑, zgc:162591↑, got1↑, hif1al↓, mych↓, eno1↓, si:ch211-220i15.2↑, ugt1b4↑, ugt1b3↑, calrl↓, tbx2b↑, cyp11b2↑, bzw1b↓, ugt5b6↑, zgc:162184↑, spi1↓, zgc:171801↓, musk↑, hsd11b2↓, mycb↓, araf↑, zgc:77060↓, gcat↑, dnase2↓, ptpro↑, tsc22d3↓, atf7a↑, psmb10↓, acsf2↑, paics↑, si:ch211-10j20.1↑, rfx1a↑, mthfsd↑, slc27a6↑, zgc:103559↓, ihha↑, zgc:112302↓, foxo3b↓, agpat2↑, agpat4↓, zgc:136461↓, mical2b↓, etv5a↓, glulb↓, brf1a↓, dhrs3a↓, zgc:113944↑, gpt2l↑, stk35↓, zgc:112970↑, zgc:100906↓, cebpa↓, dbx2↑, pkm2a↓, hsp90b1↓, bhlhe40↑, zgc:162824↓, ca4a↓, cry5↑, ttll1↑, st6galnac3↓, ttll7↑, urah↑, opn1sw2↑, igfbp2a↑, zgc:64119↑, adra2b↑, zgc:112356↓, nr1d2a↓, tdrd9↑, hmgcs1↓, oc90↑, zgc:165649↓, si:ch211-171o19.1↑, acsl4l↓, plod2↑, cdc73↑, aptx↑, p4ha1b↓, si:dkey-52h23.1↓, rpl15↑, zgc:153537↑, zgc:92903↑, cdo1↑, si:ch211-215m21.17↑, extl3↑, dusp6↓, ctssb.1↓, pfkfb4l↓, hprt1l↑, gpib↑, pklr↑, gsc↑, fth1a↓, si:dkey-183n20.16↑, gnmt↑*
	GO:0051179	localization	*zgc:152990↑, slc25a21↑, slc25a43↓, rab6b↑, slc25a27↑, sfxn1↑, trpa1a↑, lmbr1l↑, zgc:65857↑, slc25a1↓, ipo8↓, rbp1a↑, ucp2↓, tbx2b↑, cxcr4b↓, wasb↓, abcb3l1↓, cxcl12a↓, urah↑, cacna1sb↓, prickle1a↑, musk↑, dync2i1↑, cav1↓, alcamb↓, fth1a↓, zgc:162641↑, stxbp1b↑*
	GO:0022414	reproductive process	*cxcl12a↓, tdrd9↑, cxcr4b↓*
	GO:0071840	cellular component organization or biogenesis	*zgc:113984↓, cxcr4b↓, wasb↓, grna↓, extl3↑, tubgcp2↑, cxcl12a↓, musk↑, dync2i1↑, cbx4↓, tdrd9↑, cav1↓, alcamb↓, igfbp2a↑*
	GO:0000003	reproduction	*cxcl12a↓, tdrd9↑, cxcr4b↓*
	GO:0002376	immune system process	*spi1↓, zgc:110349↑, cxcl12a↓, cebpa↓, si:busm1-160c18.3↑, agxt2l1↑, zgc:153759↓, tnfaip8l2a↓, cdc73↑, wasb↓*
	GO:0023052	signaling	*arhgef3l↓, arl4d↓, quo↓, rab6b↑, socs3a↓, hif1al↓, tagap↓, si:ch211-35n24.1↓, asb11↑, rhogb↓, arl5c↓, rasl11b↑, dcdc2b↑, cmklr1↓, ihha↑, cxcr4b↓, extl3↑, fzd8a↑, dusp6↓, opn1sw2↑, prickle1a↑, musk↑, zgc:153759↓, igfbp2a↑, rnd1l↑, araf↑, adra2b↑*
	GO:0007610	behavior	*cxcl12a↓, cxcr4b↓*
	GO:0050896	response to stimulus	*arhgef3l↓, crfb7↓, arl4d↓, keap1b↑, quo↓, rab6b↑, socs3a↓, trpa1a↑, hif1al↓, tagap↓, si:ch211-35n24.1↓, asb11↑, aptx↑, zgc:110349↑, hsp90b1↓, rhogb↓, arl5c↓, cry5↑, hsp47↓, rasl11b↑, dcdc2b↑, cmklr1↓, hspa4a↓, chac1↓, tnfaip8l2a↓, cxcr4b↓ wasb↓, extl3↑, abcb3l1↓, fzd8a↑, dusp6↓, cxcl12a↓, opn1sw2↑, prickle1a↑, si:busm1-160c18.3↑, musk↑, zgc:153759↓, igfbp2a↑, alcamb↓, rnd1l↑, araf↑, adra2b↑*
	GO:0040007	growth	*cxcl12a↓, musk↑, copeb↓, igfbp2a↑, cxcr4b↓, grna↓, extl3↑*
	GO:0040011	locomotion	*cxcl12a↓, prickle1a↑, musk↑, alcamb↓, tbx2b↑, cxcr4b↓, wasb↓, extl3↑*
	GO:0065007	biological regulation	*zgc:113944↓, crfb7↓, camk2n1a↓, rbpjb↓, zgc:100906↓, lhx9↑, cebpa↓, dbx2↑, hif1al↓, asb11↑, bhlhe40↑, mych↓, cmklr1↓, tbx2b↓, spi1↓, opn1sw2↑, musk↑, zgc:153759↓, igfbp2a↑, mycb↓, araf↑, zgc:77060↓, adra2b↑, arhgef3l↓, arl4d↓, tsc22d3↓, quo↓, zgc:112356↓, rab6b↑, atf7a↑, socs3a↓, nr1d2a↓, parvb↑, tdrd9↑, tagap↓, si:ch211-35n24.1↓, cdc73↑, rfx1a↑, rhogb↓, arl5c↓, si:dkey-52h23.1↓, rasl11b↑, dcdc2b↑, cxcr4b↓, foxo3b↓, zgc:92903↑, extl3↑, fzd8a↑, dusp6↓, cxcl12a↓, prickle1a↑, etv5a↓, gsc↓, fth1a↓, rnd1l↑, brf1a↓*
	GO:0051704	multi-organism process	*cxcl12a↓, rhogb↓, tdrd9↑, cxcr4b↓*

Table S1 (continued)

Treatment	GO ID	GO term	DTGs
Ionic silver 21 days	GO:0065007	biological regulation	*ar*↑, *birc5b*↓, *socs9*↑, *rock2b*↑, *nfil3*↓, *zorba*↓, *si:dkey-167i21.2*↓, *sepw2b*↓, *pou5f1*↓, *gpr56*↑, *zgc:193933*↓
	GO:0002376	immune system process	*zgc:173545*↓
	GO:0023052	signaling	*ar*↑, *socs9*↑, *rock2b*↑, *gpr56*↑
	GO:0044699	single-organism process	*slc25a42*↑, *slc16a3*↓, *socs9*↑, *rock2b*↑, *nfil3*↓, *si:dkey-167i21.2*↓, *sepw2b*↓, *st3gal4*↑, *zgc:110783*↑, *gpr56*↑, *spna2*↓, *fah*↑, *zgc:110251*↓, *enpp6*↑, *ar*↑, *birc5b*↓, *zgc:56231*↓, *kcnc2*↑, *zgc:63694*↓, *cyp2aa4*↓, *pou5f1*↓, *zar1*↓, *ca15b*↓, *ccna1*↓, *cdk5*↑, *mcl1b*↓
	GO:0008152	metabolic process	*zgc:77816*↓, *slc27a2*↑, *rock2b*↑, *nfil3*↓, *usp2a*↓, *st3gal4*↑, *zgc:55413*↓, *zgc:110783*↑, *qdprb2*↓, *zgc:153038*↑, *fah*↑, *zgc:77118*↓, *zgc:193933*↓, *zgc:110251*↓, *enpp6*↓, *plk3*↓, *ar*↑, *zgc:171517*↓, *zgc:152769*↑, *zorba*↓, *cyp2aa4*↓, *pou5f1*↓, *zgc:101791*↑, *ca15b*↓
	GO:0051179	localization	*slc25a42*↑, *slc16a3*↓, *kcnc2*↑, *si:dkey-167i21.2*↓, *zgc:63694*↓, *ap1s2*↓
	GO:0050896	response to stimulus	*ar*↑, *socs9*↑, *rock2b*↑, *gpr56*↑, *zgc:173545*↓
	GO:0071840	cellular component organization or biogenesis	*zgc:165551*↓, *si:dkey-167i21.2*↓, *spna2*↓
	GO:0032502	developmental process	*birc5b*↓, *rock2b*↑, *nfil3*↓, *pou5f1*↓, *spna2*↓, *zar1*↓
	GO:0032501	multicellular organismal process	*birc5b*↓, *nfil3*↓, *rock2b*↑, *pou5f1*↓, *spna2*↓, *zar1*↓
	GO:0009987	cellular process	*slc25a42*↑, *zgc:77816*↑, *slc16a3*↓, *rock2b*↑, *nfil3*↓, *sepw2b*↓, *st3gal4*↑, *fah*↑, *zgc:77118*↓, *zgc:193933*↓, *zgc:110251*↓, *plk3*↓, *zgc:56231*↓, *kcnc2*↑, *zgc:63694*↓, *pou5f1*, *ccna1*↓, *socs9*↑, *usp2a*↓, *si:dkey-167i21.2*↓, *zgc:55413*↓, *gpr56*↓, *spna2*↓, *zgc:165551*↓, *ar*↑, *birc5b*↓, *zorba*↓, *ca15b*↓
	GO:0048511	rhythmic process	*nfil3*↓

CHAPTER IV

Bioaccumulation and cellular effects in adult zebrafish under exposure to cadmium sulphide quantum dots and ionic cadmium

This chapter is being prepared for publication as:

JM Lacave, E Bilbao, D Gilliland, F Mura, L Dini, MP Cajaraville, A Orbea. Bioaccumulation, cellular and molecular effects in adult zebrafish under exposure to CdS QDs and ionic cadmium.

Parts of this chapter have been presented at:

XVII Congress on Pollutant Responses In Marine Organisms (PRIMO). Faro (Portugal), 5^{th}-8^{th} May 2013. **JM Lacave**, U Vicario, D Gilliland, P Reip, MP Cajaraville, A Orbea. "Bioaccumulation and cellular effects in adult zebrafish under exposure to CuO, Ag and CdS nanoparticles". Oral presentation.

7th Special Science Symposium "Fate and ecotoxicity of nanoparticles in the environment (SETAC Europe)". Brussels (Belgium), 2^{nd}-3^{rd} October 2013. A Orbea, U Vicario-Parés, **JM Lacave**, D Berhanu, P Reip, M Oron, D Gilliland, E Valsami-Jones, MP Cajaraville. "Toxicity ranking and sublethal effects of metal nanoparticles using zebrafish as model organism". Poster.

ABBREVIATIONS

BCF, Bioaccumulation factor

BSA, Bovine serum albumin

BSDs, Black silver deposits

GSH, Glutathione

H/E, Hematoxylin/eosin

LMS, Lysosomal membrane stability

LP, Labilization period

MN, Micronuclei

NPs, Nanoparticles

PBS, Phosphate buffered saline

QDs, Quantum dots

RAPD-PCR, Random amplified polymorphic DNA-PCR

ROS, Reactive oxygen species

TEM, Transmission electron microscopy

ABSTRACT

Among engineered nanoparticles (NPs), quantum dots (QDs) are fluorescent semiconductor crystals with special optical and electrical properties that make them very suitable for many industrial and biomedical applications. QDs may contain metallic components such as cadmium that are highly toxic to aquatic organisms. Adult zebrafish (*Danio rerio*) were selected to analyze the bioaccumulation and the potential toxic effects provoked by waterborne exposure to CdS NPs. For this purpose, zebrafish were exposed to 10 µg Cd/L of CdS NPs of ~4 nm or to the same nominal concentration of ionic cadmium for 21 days and, then, maintained up to 6 months in clean water. By day 4 of exposure, high mortality was detected in both exposure groups and after 21 days, a significant cadmium accumulation was measured in the whole fish. The autometallographical analysis showed more abundant black silver deposits, indicating higher accumulation of metal, in the intestine than in the liver. In the liver, higher content of metal was observed after exposure to ionic cadmium, which could be causing the stronger oxidative damage to proteins measured as altered free ubiquitin and carbonylated actin levels. However, a stronger destabilization of the hepatocyte lysosomal membrane was recorded under exposure to CdS NPs. By transmission electron microscopy, the presence of NPs was detected in the cytoplasm of epithelial cells of the secondary lamellae of gills, attached to the nuclear envelope and into small membrane vesicles of hepatocytes. No genotoxic effects were detected in the assayed conditions according to the micronuclei frequency test in erythrocytes. Increased prevalence of vacuolization was found in the livers of exposed animals and relevant histopathological alterations, such as inflammation, aneurism and fusion of the secondary lamellae and hyperplasia in the primary lamellae, were detected in the gills, indicating that gills are one of the main entrances of cadmium into the organisms. Therefore, at the concentration tested, exposure to both cadmium forms resulted in cadmium accumulation and exerted an acute toxic effect to zebrafish, which could be mediated by oxidative stress.

Keywords: CdS NPs, zebrafish, biomarkers, bioaccumulation, cellular effects

LABURPENA

Nanopartikulen (NP) artean, puntu kuantikoak (PK) oso baliagarriak dira hainbat erabilera industrial eta biomedikoetarako, beraien ezaugarri optiko (fluoreszentzia) eta elektrikoak direla eta. PKek konposatu metalikoak eduki ditzakete, kadmioa esaterako, organismo urtarrentzako oso toxikoak izan daitezkeenak. CdS NPek ur esposizioaren bidez eragindako efektu toxikoak eta biometaketa aztertzeko zebra arrain (*Danio rerio*) helduak aukeratu ziren. Helburu horrekin, zebra arrainak 21 egunez ~4 nm-ko CdS NPen 10 µg Cd/L-ren edo Cd ionikoko kontzentrazio nominal berdinaren eraginpean jarri ziren eta, ondoren, ur garbitan mantendu ziren 6 hilabetez. 4 egunetara hilkortasun tasa handia behatu zen bi taldeetan eta 21 egunetara, kadmio metaketa esanguratsua neurtu zen arrain osoan. Autometalografia bidezko analisiak zilar metakin beltz kopuru handiagoa erakutsi zuen, metalen metaketa handiagoa erakutsiz hestean gibelean baino. Gibelean, metalen metaketa handiagoa behatu zen kadmio ionikoaren eraginpean egondako arrainetan, honek proteinei eragindako kalte oxidatibo handiagoa azaldu dezake, ubikitina askearen eta aktinaren karbonilazio mailen alterazioren neurketa bidez ebaluatuta. Hala ere, CdS NPen eraginaren ondorioz hepatozitoen lisosomen mintzaren ezegonkortze handigoa neurtu zen. Transmisiozko mikroskopio elektroniko bidez, NPen presentzia zakatzetako lamela sekundarioen epitelioko zelulen zitoplasman, nukleoaren gaineztadurari erantsita eta hepatozitoen mintz-besikula txikietan aurkitu zen. Testatutako egoeretan ez zen efektu genotoxikorik antzeman. Tratamendu pean egondako animalien gibeleko bakuolizazioaren prebalentziaren emendioa eta zakatzetan alterazio histopatologiko nabarmenak behatu ziren, inflamazioa, aneurismak, lamela sekundarioen fusioa eta lamela primarioaren hiperplasia; zakatzak, organismoan kadmioaren sarrera nagusienetarikoa direla adieraziz. Beraz, testatutako kontzentraziora, kadmioaren bi aldaeretara egindako esposizioak kadmio metaketa eragin zuen, eta efektu toxiko azkarra izan zuen zebra arrainetan, estres oxidatibo bidez eragindakoa izan daitekeena.

Gako-hitzak: CdS NPak, zebra arraina, biomarkatzaileak, biometaketa, efektu zelularrak

RESUMEN

Los puntos cuánticos (PCs) son un tipo de nanopartículas (NPs) que por sus propiedades ópticas (fluorescencia) y eléctricas son muy útiles para diversas aplicaciones industriales y biomédicas. Los PCs pueden contener elementos metálicos, como el cadmio, que son altamente tóxicos para los organismos acuáticos. Con el objetivo de analizar la bioacumulación y el posible efecto tóxico de la exposición vía agua a NPs de CdS, peces cebra (*Danio rerio*) adultos se expusieron a 10 µg Cd/L de NPs de CdS de ~4 nm o a la misma concentración nominal de cadmio iónico durante 21 días y, a continuación, se mantuvieron hasta los 6 meses en agua limpia. Al cuarto día de exposición se detectó mortalidad alta en ambos grupos de exposición y una acumulación significativa de cadmio tras 21 días de exposición. El análisis autometalográfico mostró un mayor número de depósitos negros de plata, indicando una mayor acumulación de metal, en el intestino que en el hígado. En el hígado, la mayor acumulación de metales se observó tras la exposición a cadmio iónico, lo cual puede explicar el mayor daño oxidativo a las proteínas evaluado como alteración de los niveles de ubiquitina libre y carbonilación de actina. Sin embargo, se registró una mayor desestabilización de la membrana lisosómica de los hepatocitos tras la exposición a las NPs de CdS. Mediante microscopía electrónica de transmisión, se detectaron NPs en el citoplasma de células epiteliales de la laminilla secundaria de las branquias, adheridas a la envoltura nuclear y dentro de pequeñas vesículas de la membrana de los hepatocitos. No se detectaron efectos genotóxicos en las condiciones testeadas. Se observó un incremento en la prevalencia de vacuolización en el hígado de animales expuestos y alteraciones histopatológicas relevantes en las branquias, como inflamación, aneurismas y fusión de las laminillas secundarias e hiperplasia de la laminilla primaria, indicando que las branquias son una de las principales entradas de cadmio en el organismo. Por lo tanto, a la concentración testeada, la exposición a ambas formas resultó en acumulación de cadmio y supuso un efecto tóxico agudo en el pez cebra, el cual puede ser mediado por estrés oxidativo.

Palabras clave: NPs de CdS, pez cebra, biomarcadores, bioacumulación, efectos celulares

INTRODUCTION

Cadmium is a non-essential metal whose use has increased in the last decades in several industrial products due to its widespread use as a colour pigment in paints, in electroplating and galvanizing, in batteries, etc (Gonzalez et al., 2006). The Water Framework Directive (2000/60/EC) included cadmium as a priority hazardous substance and, therefore, its emission may cease and phased out (European Union, 2000). Measured cadmium concentrations in surface waters range from 0.002 to 0.015 µg/L in clean rivers, increasing up to 2-3 µg/L in surface waters of impacted environments (EPA, 2016). Very high values of cadmium in surface water, up to 30 µg/L, have been measured in sampling sites receiving effluents of zinc manufacturing industries (Andres et al., 2000). Cadmium is one of the most toxic metals in the environment (Soares et al., 2008) and can be accumulated by aquatic organisms through different routes including respiration, adsorption and ingestion and its toxicity has been thoroughly studied (Perera et al., 2015).

Among engineered nanoparticles (NPs), quantum dots (QDs) are semiconductor crystals ranging from 2 to 100 nm that present unique optical and electrical properties. These nanomaterials may contain metallic components such as cadmium being appropriate for several applications in biomedical devices and industrial processes. QDs present a fluorescence spectrum, which renders them optimal fluorophores for biomedical imaging. Moreover, QDs can be used for a variety of information and visual technologies (Hardman, 2006). QDs can be synthesized with different formulations. They are composed by a semiconductor core (e.g. CdS, CdSe, CdTe) and, in most of the cases, they are also encapsulated by a shell (e.g. ZnS) (King-Heiden et al., 2009; Louis et al., 2010). Due to their increasing production and use during the last decade, an input in the aquatic environment from the wastewater effluents or from industries could be provoked and, therefore, a negative impact in the aquatic organisms is to be expected (Munari et al., 2014). Several studies have been carried out in order to analyze the toxic effects of QDs in microalgae (Morelli et al., 2012) and in aquatic invertebrates such as *Daphnia magna* or freshwater and marine mussels (Gagné et al., 2008; Jackson et al., 2009; Peyrot et al., 2009; Kim et al., 2010; Feswick et al., 2013; Rocha et al., 2014). In fish, most of the studies have been carried out in zebrafish embryos exposed

to QDs of different composition (e.g. CdS, CdTe/ZnS core shell, CdSe/ZnS core shell) with several coating agents in order to analyze the effect on survival rate, hatching parameters and malformation prevalence (King-Heiden et al., 2009; George et al., 2011; Zhang et al. 2012a; 2012b; 2012c; 2013; Wiecinski et al. 2013; Chapter I). The toxicity of cadmium containing NPs has been partially attributed to the release of ions into the exposure medium, since cadmium is a highly soluble metal (Misra et al., 2012). LC_{50} values have been reported ranging from 0.0209 mg/L in embryos exposed to thioglycolic acid-CdTe QDs of 3.5 nm (Zhang et al., 2012a) to 4.72 mg/L in embryos exposed to CdSe/ZnS- poly (ethylene glycol) 5000-OCH_3 QDs of 14 nm (King-Heiden et al., 2009). Differences in the LC_{50} values have been related to differences in the coating used and to the size of the NPs. The lowest LC_{50} values have been reported after the exposure to small QDs (King-Heiden et al., 2009; George et al., 2011). Moreover, the presence of other metals (e.g. selenium) and their dissolution can increase the toxicity of QDs in the embryos (Wiecinski et al., 2013).

Few studies have analyzed the effects provoked by the exposure to QDs in adult fish. Sanders et al. (2008) waterborne exposed sticklebacks (*Gasterosteus aculeatus*) for 21 days to different concentrations (0.005, 0.05 and 0.5 mg/L) of CdS NPs (4.2 nm). In this study, fish exposed at the highest concentration displayed hepatocellular nuclear pleomorphism. Moreover, at the two highest concentrations tested elevated levels of oxidized glutathione were detected. In rainbow trout, the exposure for 96 h to 1, 2 and 6 μg/L of CdS/CdTe QDs provoked a significant regulation of 25 genes involved in inflammation, xenobiotic biotransformation and endocrine system, including the induction of vitellogenin and its receptor (Gagné et al., 2010). Through dietary exposure, the effect provoked by the exposure to QDs was analyzed using adult zebrafish fed for 36 and 60 days with food containing CdS NPs of two different sizes (8 and 50 nm) in two different doses (40 and 100 ng NPs/day/g body weight). Results showed a significant accumulation of cadmium in the liver at both periods of time and, also, in the brain after 60 days of exposure. Moreover, after 60 days of dietary exposure to both concentrations of NPs, genotoxic effects were reported using random amplified polymorphic DNA-PCR (RAPD-PCR) genotoxicity test. The gene transcription analysis in liver showed a general downregulation of genes involved in

mitochondrial metabolism, DNA repair, apoptosis and antioxidant defenses after 36 days of exposure to the smallest NPs (8 nm). After 60 days of exposure, these genes were found repressed in the intestine and over expressed in the brain (Ladhar et al., 2013). Trophic transfer of poly (acrylic acid)-octylamine copolymer-coated CdSe/ZnS from brine shrimps to zebrafish have been also analyzed. Brine shrimps exposed to 0.6 mg/L were used to feed zebrafish for 14 days and a low percentage of QDs was absorbed by fish and transported to different tissues (Lewinski et al., 2011).

To the best of our knowledge, no *in vivo* studies have been performed in adult zebrafish to assess the toxic effects of the waterborne exposure to CdS NPs. Nevertheless, several studies have reported toxic effect after waterborne exposure to soluble cadmium on adult zebrafish. Noticeable differences in cadmium toxicity have been reported in these studies, because cadmium toxicity can vary greatly depending on the hardness of the exposure medium (Hollis et al., 1997). An increase in the concentration of calcium ions can decrease the bioavailability and, thus, the toxicity of cadmium, since calcium ions compete with cadmium ions for the same binding sites on the organisms (Meinelt et al., 2001). After the exposure to adult zebrafish for 96 h to a set of concentrations ranging from 4.256 to 48.72 mg Cd/L a LC_{50} value of 11.46 mg Cd/L was obtained (Vergauwen et al., 2013a). In a longer-term cadmium exposure to a lower concentration (560 μg Cd/L for 21 days), Vergauwen et al. (2013b) reported a 20% of mortality and cadmium tissue accumulation, in the following order: muscle < brain < gonads < carcass < liver < gills < gut. Exposure for 21 days to 1.9 and 9.6 μg Cd/L, provoked genotoxic effects according to the RAPD-PCR test and a high percentage of mortality (56%) was observed after the exposure to the highest concentration (Cambier et al., 2010). After 96 h of exposure to 5 and 8.3 mg Cd/L, Ling et al. (2010) reported a decrease in the activity of superoxidase dismutase, catalase and acetylcholinesterase, which was also detected after a longer-term period (21 days) to a lower concentration (0.4 mg/L) (Banni et al., 2011).

Thus, the present investigation was aimed to study the bioaccumulation, cell localization and cellular effects of waterborne exposure to 10 μg Cd/L of CdS NPs (3.5-4 nm) in comparison with those effects provoked by the same nominal concentration of ionic cadmium in adult zebrafish. The selection of the exposure concentration was

based on the results reported in the literature (Cambier et al., 2010) and on the previously performed short-term experiments with embryos where mortality was not recorded at the concentration used in this work (Chapter I). After 21 days of exposure, fish were maintained in clean water up to 6 months in order to observe the effects provoked in the organisms after a long-term period recovery. Different biomarkers (previously described in Chapter II) were applied to determine the cellular and tissular effects of CdS NPs and ionic cadmium in zebrafish.

MATERIALS AND METHODS

Cadmium compounds

Glutathione (GSH) capped CdS NPs of 3.5-4 nm were synthesized by wet chemistry following a method modified from Zou et al. (2009) as described in Katsumiti et al. (2014). After the synthesis, CdS NPs were washed using centrifugal ultrafiltration, resulting in CdS NPs with no residual free GSH. CdS NPs were provided as suspension at a concentration of 4.7 g Cd/L. CdS NPs were characterized in deionized water by transmission electron microscopy (TEM), and isolated particles or very small aggregates (5–10 nm) were observed. In a 1 mM solution of $NaNO_3$, 1.1% of cadmium was dissolved from the CdS NPs after 24 h, 6.6% after 72 h and 13% after 7 days (Katsumiti et al., 2014). Ionic cadmium solution was prepared from $CdCl_2$ purchased to Sigma-Aldrich (St. Louis, Missouri, USA). In both cases, a solution of 1 mg Cd/L was prepared by dissolving the original stocks in deionized water. The final exposure concentration (10 µg/L) was achieved by adding a given volume of the stocks solution to the exposure aquaria.

Maintenance and experimental exposure of adult zebrafish

The experimental procedure described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. Zebrafish (wild type AB Tübingen) individuals were specifically produced and grown in our facility at the UPV/EHU. Adult fish of approximately 4 months old were exposed to CdS NPs and to ionic cadmium at a concentration of 10 µg Cd/L for 21 days. An unexposed control group was run in parallel in identical experimental conditions.

The exposures were carried out in 35 L aquaria with conditioned water of 600 µsΩ containing 50-60 fish. During the exposure period, approximately 2/3 of the aquarium water was changed by siphoning every three days and the corresponding volume of contaminated or clean water was redosed. Fish were fed with Vipagran baby (Sera, Heinsberg, Germany) and live *Artemia* (INVE Aquaculture, Salt Lake City, Utah, USA) twice per day. Samples were taken after 3 and 21 days of exposure after euthanasia by overdose of anesthetic (benzocaine, Sigma-Aldrich). After 21 days of exposure, remaining fish were transferred to clean water and maintained up to 6 months to evaluate the appearance of long-term effects or the potential reversibility of the effects detected. During this period, fish were fed only with Sera Vipagran baby twice per day. Water was maintained in continuous movement through an air pump to assure an appropriate aeration to the tank. Biological and physical filters were used to maintain the chemical parameters of the water (nitrate, nitrite and ammonium) that were controlled once per week using Sera ammonium, nitrite and nitrate kits. Water was changed if the values were higher than zero mg/L for ammonium or nitrite and 50 mg/L for nitrate. At 6 months post-exposure fish samples were collected as described above.

Metal accumulation in whole organisms: chemical analysis

After 21 days of exposure, 20 individuals per experimental group were collected, frozen individually in liquid nitrogen and stored at -80 ºC until processed for chemical analysis. Whole zebrafish were dried in an oven at 130 ºC for 24 h. Dry tissues were weighted and pooled (five pools of four zebrafish each). Each pool was placed into 25 mL Erlenmeyer flasks and 2 mL of 65% nitric acid (extra pure quality, Scharlau, Barcelona, Spain) was added for tissue digestion. The mouth of the Erlenmeyer flask was blocked with a crystal ball to minimize evaporation. After finishing the digestion of the sample, the remnant liquid was evaporated in an 80 ºC hot plate inside an exhaust hood. Then, 2.5 mL of nitric acid 0.1 M were added to each Erlenmeyer flask, and left for 1 day. The content of each flask was then put into tubes and centrifuged for 4 min at 2000 rpm (Heraeus Labofuge 200 centrifuge, Hanau, Germany). The supernatants were moved to clean tubes, and stored at 4 ºC. Finally, cadmium content was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES,

7700x, Agilent Technologies, California, USA) following the US-EPA 6010D direction. Detection limit was established at 0.005 μg/L.

Histological preparations

The visceral mass and gills of 10 individuals per experimental group were dissected after 21 days of exposure and after 6 months in clean water. Tissues were placed in histological cassettes and immersed in 10% neutral buffered formalin for 24 h at 4 ºC. Then, samples were transferred to 70% ethanol and stored at 4 ºC until complete tissue processing under vacuum conditions in an ASP300 Tissue Processor (Leica Microsystems, Nussloch, Germany). Paraffin blocks were done using plastic molds. Sections (5 μm thick) were cut in a RM2125RT microtome (Leica Microsystems). For the histopathological analysis and for localization of tissues of interest before the autometallographical staining, slides were stained with hematoxylin/eosin (H/E) in an Auto Stainer XL (Leica Microsystems) and mounted in DPX (Sigma-Aldrich) by means of a CV5030 Robotic Coverslipper (Leica Microsystems). H/E stained histological sections of the visceral mass and gill tissue were examined under a BX51 light microscope (Olympus, Tokyo, Japan).

Metal accumulation in tissues: autometallography

Autometallographical staining was applied on paraffin sections of visceral mass of ten individuals per experimental group sampled after 21 days of exposure using a method modified from Soto et al. (1998). Briefly, sections were dewaxed, hydrated in a graded series of ethanol and left until they were completely dry. Then, sections were covered with the photographic emulsion (Ilford nuclear emulsion L4, Norderstdedt, Germany) and left in total darkness for 30 min. Reaction was developed in the developer bath (1:4.5 dilution in deionized water of B&W Negative developer Tetenal, Norderstdedt, Germany) for 15 min and, then, rinsed in the stop bath (1% solution of acetic acid, Panreac, Barcelona, Spain) for 1 min. Sections were fixed in a 10% solution of B&W Film/Paper Fixer AGFA (Mortsel, Belgium) in deionized water for 10 min. Finally, sections were washed in deionized water and mounted in Kaiser's glycerine gelatine (Merck, Darmstadt, Germany). Once the slides were dried, the presence of black silver deposits (BSDs) indicating the presence of metals in the tissue was semiquantitatively

analyzed under an Olympus BX51 light microscope at a magnification of 20x using the criteria previously published: (-) Tissue without presence of BSDs; (+) presence of homogeneously distributed small BSDs; (++) presence of homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size and (+++) tissues with a greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits (Vicario-Parés et al., 2014).

Subcellular localization of CdS NPs: transmission electron microscopy (TEM) and X-ray analysis

Gills and liver from control fish and fish exposed to CdS NPs for 3 and 21 days were dissected and fixed for 1 h at 4 ºC in sodium cacodilate (Sigma-Aldrich) buffer 0.1 M, pH 7.2, containing 2.5% glutaraldehyde (Panreac). Then, samples were washed twice for 15 min in sodium cacodilate buffer, postfixed for 1 h in 1% osmium tetroxide (Sigma-Aldrich) with 1.5% potassium ferrocianure (Sigma-Aldrich) containing sodium cacodilate buffer 0.1 M and washed twice for 30 min in deionized H_2O. Then, samples were dehydrated in a graded series of ethanol, cleared twice for 10 min with propylene (Sigma-Aldrich) and embedded for several hours and, then, overnight in a mixture (1:1) of propylene and Epon resin (Sigma-Aldrich) prepared according manufacturer's instructions. Finally, samples were embedded in Epon resin for several hours and encapsulated for polymerization for 48 h at 60 ºC.

Semithin sections of 1500 nm in thickness were cut using a Reichert Ultracut S ultramicrotome (Leica Microsystems), stained with 1% tolulidine blue (Sigma-Aldrich) and observed under a light microscope to determine the presence of the tissues of interest. Ultrathin sections of 50 nm in thickness were then cut from selected blocks. Sections were picked up in 150 mesh copper grids, contrasted with 1% uranyl acetate (Fluka, Steinheim, Germany) for 3 min and with 0.3% lead citrate (Fluka) for 4 min and, finally, examined and photographed under a Hitachi HT7700 transmission electron microscope (Tokyo, Japan) at 60 kV. Selected samples with electrodense structures resembling NPs were analyzed by X-ray microanalysis, with the scanning-TEM module in a Field Emission Scanning Electron Microscope Zeiss Auriga 405, 1 nm nominal resolution (Oberkochen, Germany) in order to corroborate whether observed

structures corresponded to CdS NPs. Sections intended for X-ray microanalysis were not contrasted with uranyl acetate and lead citrate in order to minimize interferences.

Oxidative stress: Western Blot analysis of ubiquitin and carbonylated actin

The visceral mass of 5 fish per experimental group sampled after 3 and 21 days of exposure and at 6 months post-exposure was dissected out, frozen immediately in liquid nitrogen and stored at -80 ºC to quantify immunoreactive bands corresponding to ubiquitin and carbonylated actin using a method modified from McDonagh and Sheehan (2006). Each sample was homogenized in 10 mM Tris-HCl pH 7.2 containing 1 mM EDTA and 2% protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 2500 rpm (Precellys 24-Dual homogenizer, Bertin Technologies, Montigny le Bretonneux, France) for 5 min and the resulted aqueous phase was removed. Protein concentration was determined by measuring the optical density at 280 nm (Harris, 1989) and adjusted to 2 µg/µL with the buffer used previously. Samples were mixed 1:1 with Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 0.1 M dithiothreitol, 30% glycerol and 2 mg/mL bromophenol blue) in order to obtain a final concentration of 1 µg protein/µL.

One-dimensional electrophoresis was performed on 12.5% polyacrylamide gels containing sodium dodecyl sulfate. 10 µL of sample or 5 µL of Precision Plus Protein Standards Dual-colour (Bio-Rad, Hercules, CA, USA) were loaded in each lane. The electrophoresis was run at 200 V for 40 min (Power Pac^{TM}, Bio-Rad). Separated proteins were transferred to a PVDF membrane (Bio-Rad) by a trans-blot turbo transfer system (Bio-Rad) for 30 min up to 1 A and 25 V. For ubiquitin detection, the membrane was blocked in phosphate buffered saline (PBS) containing 1% of bovine serum albumin (BSA) for 1 h and washed in PBS. Then, it was incubated for 1 h at room temperature with the polyclonal rabbit anti-ubiquitin antibody (Dako, Glostrup, Denmark) diluted 1:1000 in PBS, followed by several washes in PBS and incubation for 1 h at room temperature with the secondary antibody (peroxidase-conjugated anti rabbit Ig G, whole molecule, Sigma-Aldrich) diluted 1:2000 in PBS. Finally, the membrane was washed several times in PBS. For the determination of protein carbonylation level, samples were derivatized with 2,4-dinitrophenylhydrazine (DNP,

Sigma-Aldrich) following the method by Conrad et al. (2001) and, then, processed as described above. The rabbit anti-DNP antibody (Sigma-Aldrich) was diluted 1:1000 in PBS and the secondary antibody was diluted 1:10000 in PBS. Peroxidase activity was visualized by means of an enhanced chemiluminescence kit (Thermo Scientific, Illinois, USA).

Photographic films were scanned using a GS-800 calibrated densitometer (Bio-Rad). The average optical density of the immunoreactive bands corresponding to free ubiquitin and carbonylated actin was quantified using the Quantity One image analysis software (v. 4.6.5, Bio-Rad). All the data were transformed to percentages respect to the average value of the control group at 3 days.

Genotoxicity: Micronuclei frequency

Blood samples of ten individuals per experimental group were collected after 3 and 21 days of exposure and at 6 months post-exposure by tail cutting and direct blood smear on clean microscope glass slides. Blood smears were left to air-dry and, then, fixed for 15 min in cold methanol. Once again, slides were left to air-dry and smears were stained with 6% Giemsa (Sigma-Aldrich) for 15 min. Afterwards, slides were rinsed in tap water and left to air-dry overnight and mounted in DPX (Sigma-Aldrich). 2000 erythrocytes were scored per individual fish under an Olympus BX51 light microscope at a magnification of 100x. The criteria used to determine the presence of micronuclei was: size not bigger than a 1/3 diameter of the main nucleus, same texture and colour, clearly separated from the main nucleus and with oval or circular shape (Baez-Ramirez and Prieto-García, 2005). Micronuclei frequency was expressed in ‰.

General health status: Lysosomal membrane stability (LMS)

The visceral mass of 5 individuals per experimental group was dissected after 3 and 21 days of exposure and at 6 months post-exposure, embedded in Cryo-M-Bed (Jung, Heidelberg, Germany) and frozen in liquid nitrogen. Frozen tissue sections (10 µm) were obtained in a CM3050S cryotome (Leica Microsystems) at a cabinet temperature of -24 ºC. The determination of LMS was based on the method used by Bröeg et al. (1999) as the time of acid labilization treatment required to produce the maximum

staining intensity in hepatocyte lysosomes after demonstration of acid phosphatase activity. Time intervals used for acid labilization were 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min according to Bilbao et al. (2010).

Labilization period (LP) was determined under an Olympus BX51 light microscope as the maximal accumulation of reaction product associated with lysosomes (Bröeg et al., 1999). Four determinations were made for each individual liver by dividing each section in the acid labilization sequence into 4 approximately equal segments. A mean value was then obtained for each section, corresponding to an individual fish.

Statistical analyses

Statistical analyses were performed using the SPSS statistical package v22.0 (SPSS Inc, Microsoft Co, WA, USA). Previous to the analysis, data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). Data on cadmium content followed a Normal distribution and were analyzed by one way ANOVA followed by the Duncan post-hoc test. For LMS, western blot and micronuclei frequency data, the non-parametric Kruskal-Wallis test was applied followed by the Dunn's post hoc test. For prevalence of histopathological alterations, Fisher's exact test was applied. In all cases, significance was established at $p<0.05$.

RESULTS

Mortality

At the concentration used during the experimental procedure, mortality was not detected in embryos (Chapter I). Nevertheless, in adults exposed to both forms of cadmium, mortality was observed by the fourth day of exposure. At the end of the exposure period, 72% of fish exposed to CdS NPs and a 78% of fish exposed to ionic cadmium had died. During the post-exposure period, mortality was not longer registered.

Metal bioaccumulation in whole organism

A significant accumulation of cadmium in the whole body of fish compared to the control fish was observed after the exposure for 21 days to both forms of cadmium,

CdS NPs and ionic cadmium (Fig 1). A bioaccumulation factor (BCF) of 619 was calculated after the exposure to CdS NPs and of 729 after exposure to ionic cadmium.

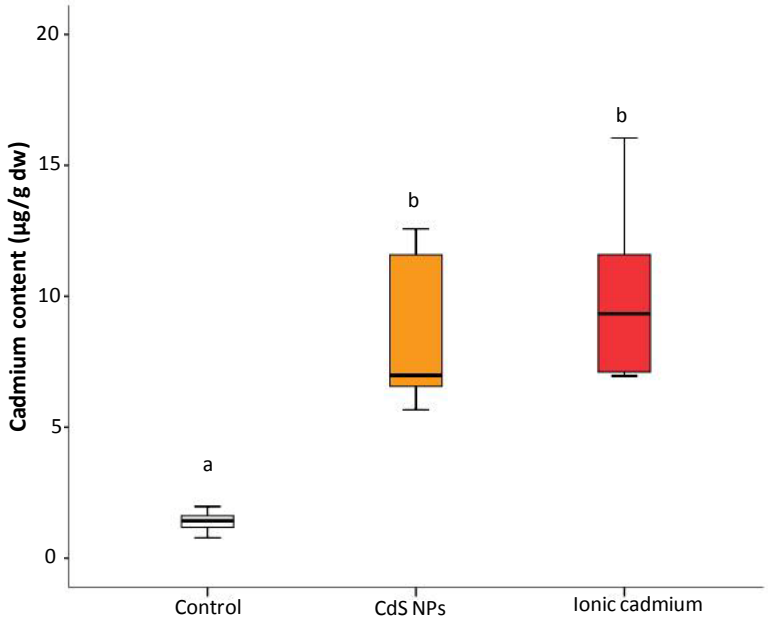

Figure 1.- Box-plot of cadmium accumulation (µg Cd/g dry weight) in whole fish tissue after 21 days of exposure. Box-plot boxes represent the percentages data value in between the 25th and the 75th percentile, median indicated by a line in the middle of the box. Whiskers are the data values in up to the 5th percentile and 95th percentile. Outliers are represented by circles. Different letters indicate statistically significant differences (p<0.05) according to the Duncan's test after one way ANOVA.

Metal accumulation in tissues

Results of the semiquantification of autometallographical BSDs indicating the presence of metal in the intestine and liver of zebrafish are shown in Table 1. After autometallographical staining, presence of BSDs was not detected in tissues of control fish, neither in the intestine (Fig 2A) nor in the liver (Fig 2B). The apical end of the enterocytes was the most stained part of the intestine after exposure to CdS NPs. BSDs were not observed into the secretory goblet cells (Fig 2C). Sixty six percent of the individuals exposed to CdS NPs did not present BSDs in the liver and, when present, they were scarce (Fig 2D). The staining pattern observed in the intestine of fish exposed to ionic cadmium was similar to that obtained after exposure to CdS NPs, being the apical end of the enterocytes the most stained zone. Nevertheless, in the case of ionic cadmium exposure, some BSDs were found in the cytoplasm of the secretory cells (Fig 2E). In the case of the liver, 70% of the individuals exposed to ionic cadmium presented BSDs, being the density of the BSDs high in some of the examined

individuals (Fig 2F). Overall, the semiquantitative analysis showed that the intestine was the most intensely stained tissue and metal content was similar after the exposure of both forms of cadmium, while the liver of zebrafish showed bigger differences between animals exposed to CdS NPs or to ionic cadmium (Table 1).

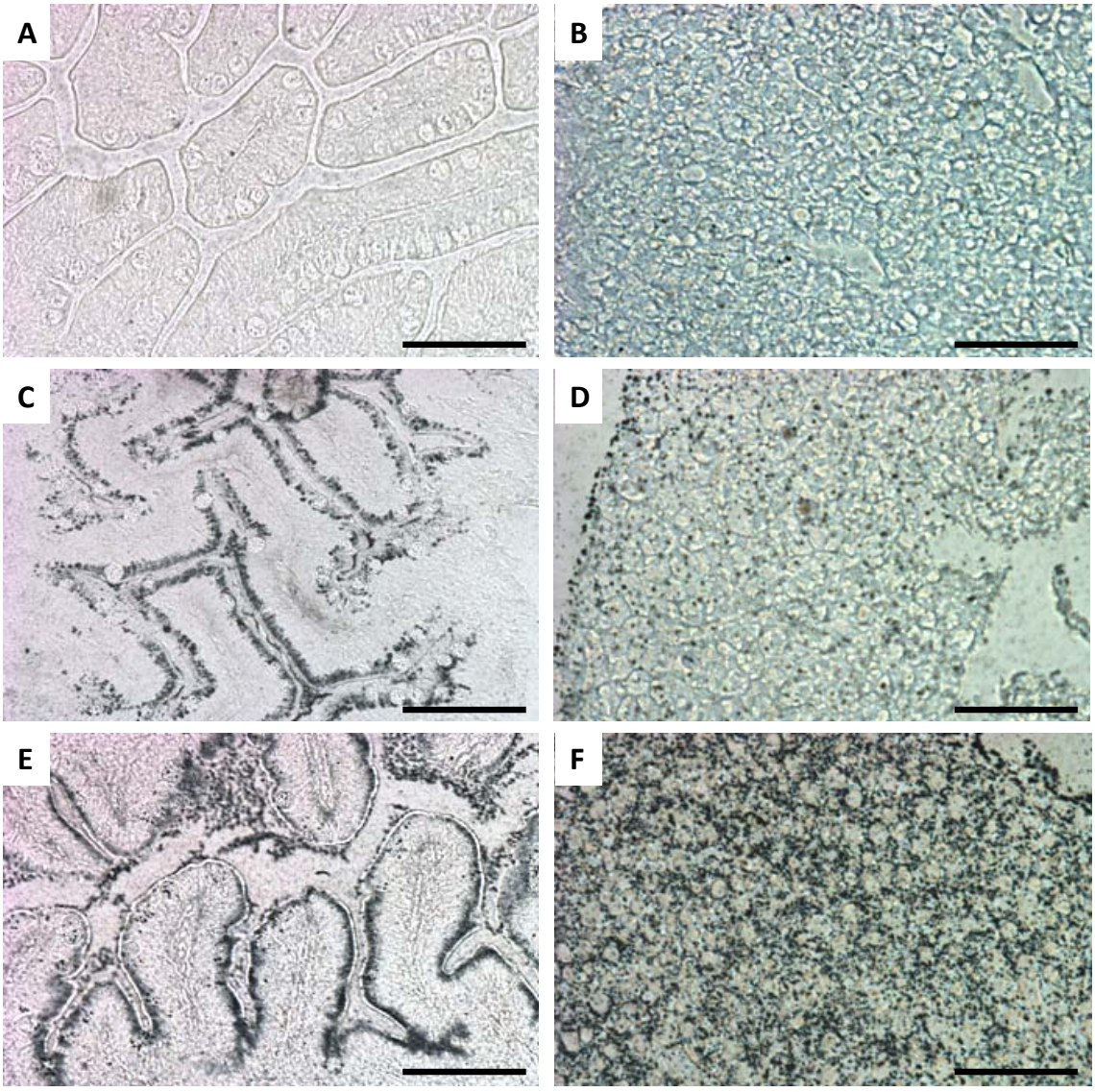

Figure 2.- Micrographs of paraffin sections of the visceral mass of adult zebrafish after autometallographical staining. (A) Intestine of control adult zebrafish. (B) Liver of control adult zebrafish. (C) Intestine of adult zebrafish exposed for 21 days to CdS NPs. (D) Liver of adult zebrafish exposed for 21 days to CdS NPs. (E) Intestine of adult zebrafish exposed for 21 days to ionic cadmium. (F) Liver of adult zebrafish exposed for 21 days to ionic cadmium. Scale bars: (A, C, E) 100 µm and (B, D, F) 50 µm.

Table 1.- Semiquantification of BSDs in the intestine and liver of adult zebrafish at 21 days of exposure.

Organism	Control		CdS NPs		Ionic cadmium	
	Intestine	Liver	Intestine	Liver	Intestine	Liver
1	-	-	+	+	+	+
2	-	-	++	+	++	+
3	NT	NT	+	NT	+	-
4	-	-	+++	-	++	+
5	-	-	+	-	NT	+
6	NT	NT	++	-	++	++
7	-	-	+	NT	+	+
8	-	-	+	NT	-	-
9	-	-	-	-	+	-
10	-	-	NT	NT	++	++
Average number of +	0	0	1.3	0.3	1.3	0.9

Semiquantification criteria according to Vicario-Parés et al. (2014): (-) Tissue without presence of BSDs; (+) presence of homogeneously distributed small BSDs; (++) presence of homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size and (+++) tissues with a greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits; NT: No specific tissue in the sample.

Subcellular localization of CdS NPs

A TEM analysis was carried out in order to analyze the fate of the CdS NPs in gill and liver cells of zebrafish. The electrodense structures resembling NPs were analyzed by X-ray microanalysis in order to corroborate their chemical composition.

Gill cells (Fig 3A) of control fish presented well preserved cell structure, with intact cell membranes and well structured organelles. The different gill cell types of individuals exposed to CdS NPs did not appear to present any relevant ultrastructural damage (Fig 3B-C). Regarding the presence of NPs in the gills, aggregates of structures containing Cd (Fig 4A-B) according to the X-ray analysis (Fig 4C) were observed in the cytoplasm of the epithelial cells in fish exposed for 3 and 21 days.

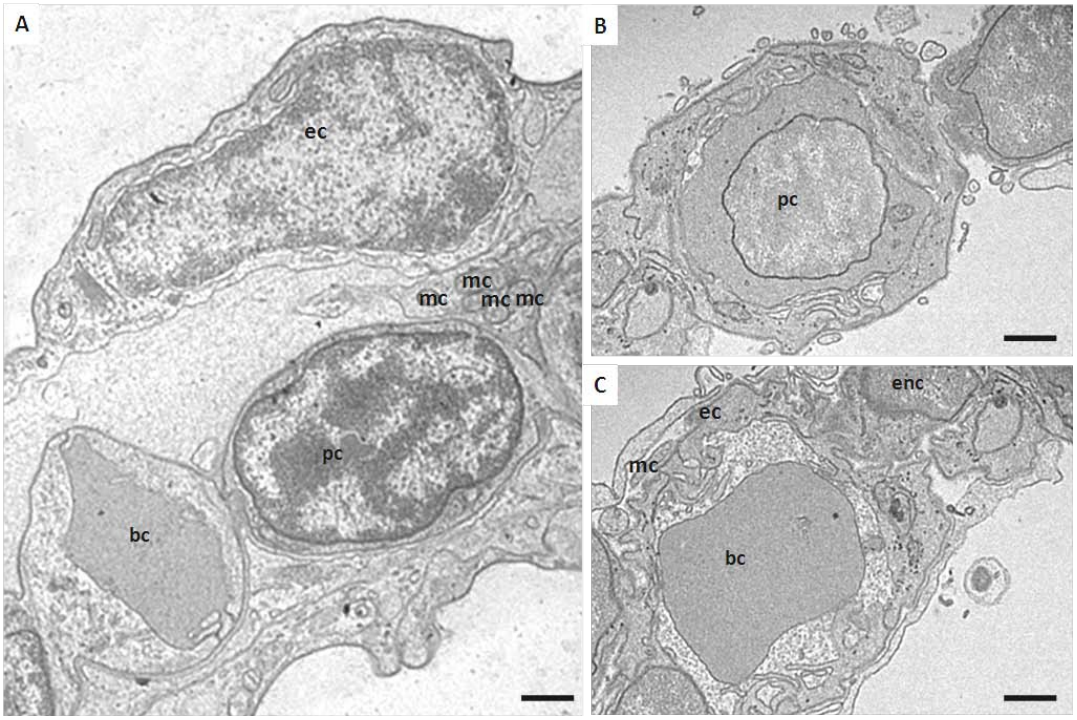

Figure 3.- Micrographs of ultrathin sections of zebrafish gill. (A) Secondary lamellae of the gill from a control zebrafish. (B,C) Secondary lamellae of the gill from a zebrafish exposed to CdS NPs for 21 days. Pillar cell (pc), blood cell (bc), epithelial cell (ec), entothelial cell (enc), (mc) mitochondria. Scale bars: 1 µm.

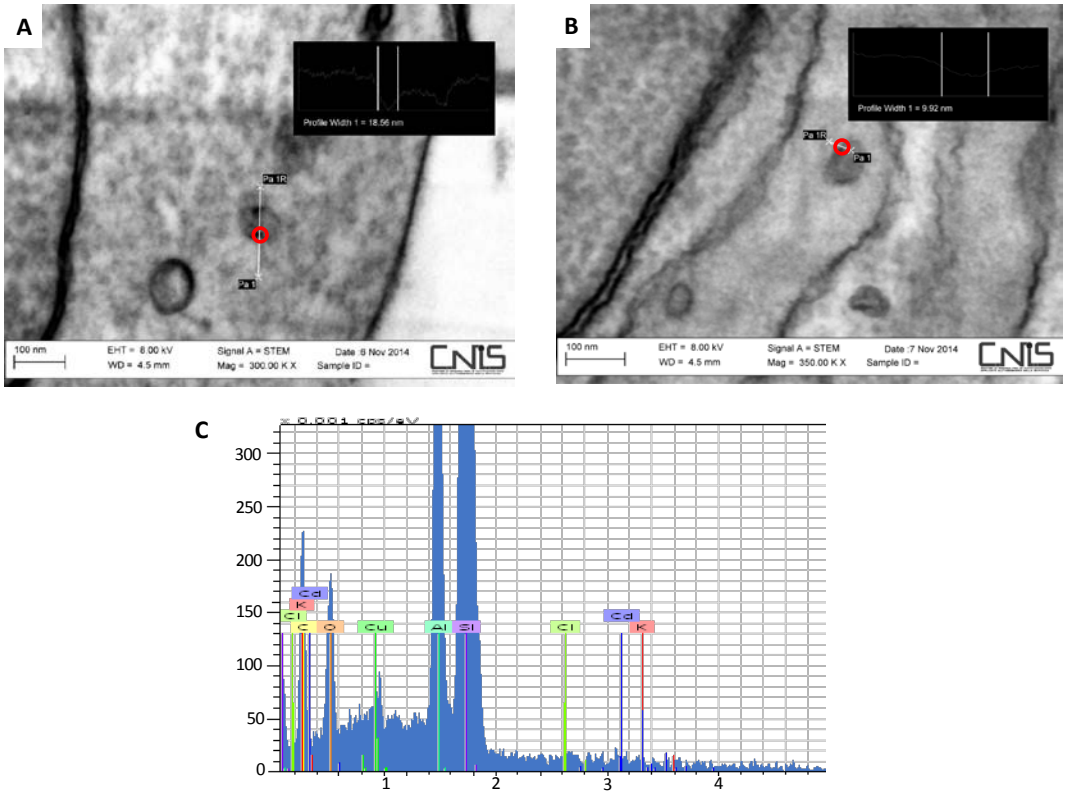

Figure 4.- (A, B) Cytoplasm of epithelial cells of the secondary lamellae of the gill from zebrafish exposed to CdS NPs for 3 and 21 days, respectively. CdS NPs are visible as electrondense structures. The red circle and the white line indicate the presence of structures containing Cd as measured by X-ray. (C) X-ray energy spectrum confirming the presence of cadmium in the electron-dense particles shown in B.

As in the case of the gills, no ultrastructural damage was observed in the hepatocytes of fish exposed to CdS. Hepatocytes of both control (Fig 5A, B) and treated (Fig 5C, D) zebrafish showed intact cell membranes and well structured organelles, although an increased presence of glycogen was observed in exposed fish (Fig 5D). The presence of putative CdS NPs was detected in hepatocytes inside membrane-surrounded vesicles (Fig 6A) and attached to the nuclear envelope (Fig 6B) after 3 days of exposure. The chemical composition of these electrondense structures resembling NPs was corroborated as cadmium after the X-ray analysis (Fig 6C).

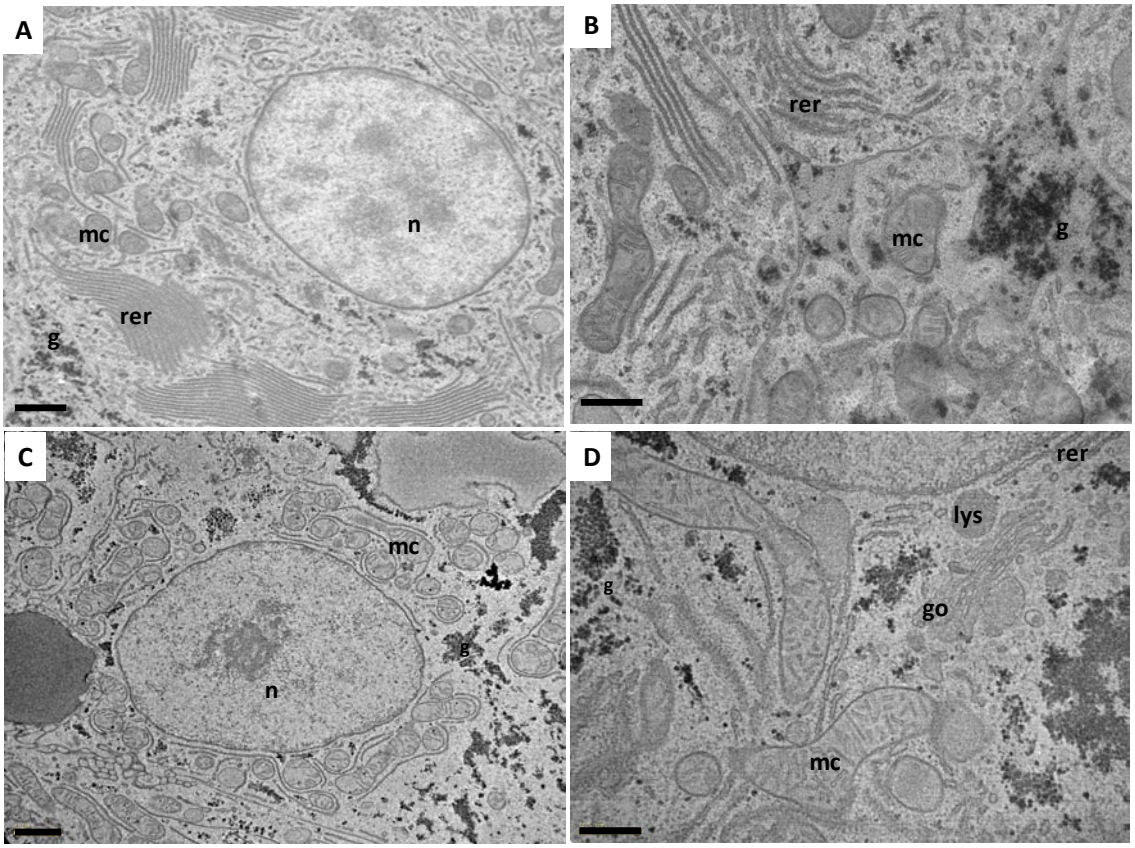

Figure 5.- Micrographs of ultrathin sections of zebrafish liver: (A, B) Hepatocytes of a control zebrafish. (C, D) Hepatocytes of a zebrafish exposed to CdS NPs for 3 days. Nucleus (n), mitochondria (mc), rough endoplasmic reticulum (rer), lysosome (lys), Golgi apparatus (go). Scale bar: (A, C) 1 µm, (B, D) 2 µm.

Oxidative stress

Oxidative damage to proteins was assessed by quantification of the immunoreactive band corresponding to free ubiquitin, detected at a molecular weight of 8 kD (Fig 7A) and of the immunoreactive band corresponding to carbonylated actin appearing at a molecular weight of 40 kD (Fig 7B).

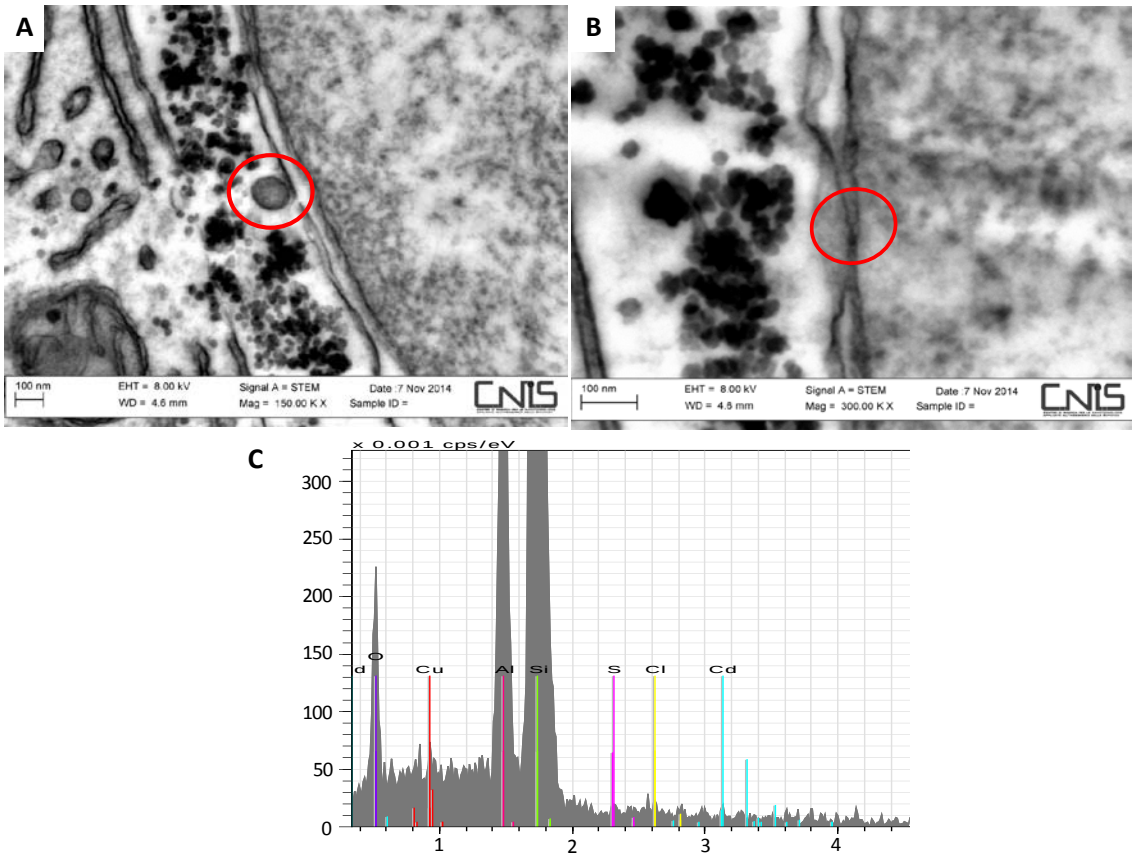

Figure 6.- (A) Membrane-surrounded vesicles containing electrodense particles that resemble NPs in an hepatocyte of a zebrafish exposed to CdS NPs for 3 days. (B) Perinuclear region of a hepatocyte of a zebrafish exposed to CdS NPs for 3 days, NPs were detected attached to the nuclear envelope. The red circles indicate the area measured by X-ray. (C) X-ray energy spectrum confirming the presence of cadmium in the electron-dense particles found in the region encircled in B.

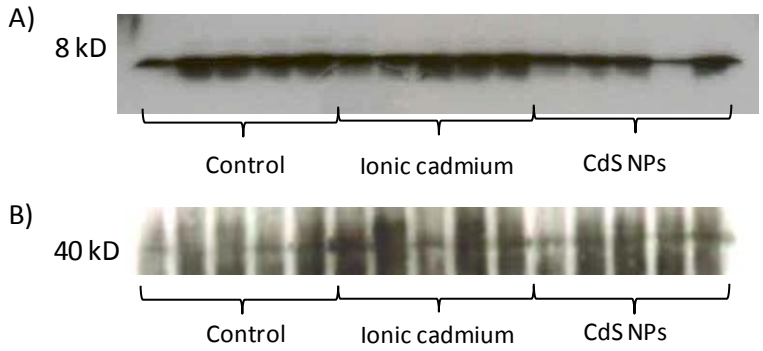

Figure 7.- Immunoreactive band for (A) ubiquitin free after 21 days of exposure and (B) carbonylated actin after 3 days of exposure.

A significant increase in the level of free ubiquitin was recorded in the liver of fish after 3 days of exposure to ionic cadmium in comparison to fish of the control group and fish exposed to CdS NPs, while a significant decrease respect to the control group was recorded after 21 days of exposure. No significant effects were measured after the exposure to CdS NPs respect to the control group (Fig 8A). For actin carbonylation, a significant decrease compared to the control group was detected after 3 days of

exposure to CdS NPs, while for fish exposed to ionic cadmium only a slight decrease was measured. After 21 days, a significant increase in the level of carbonylated actin was recorded in fish exposed to ionic cadmium respect to the control fish (Fig 8B). At 6 months post-exposure, no differences were found between animals from the control group and fish exposed to CdS NPs. Due to the high mortality recorded during the exposure to ionic cadmium, samples of this treatment group, could not be collected at the post-exposure period.

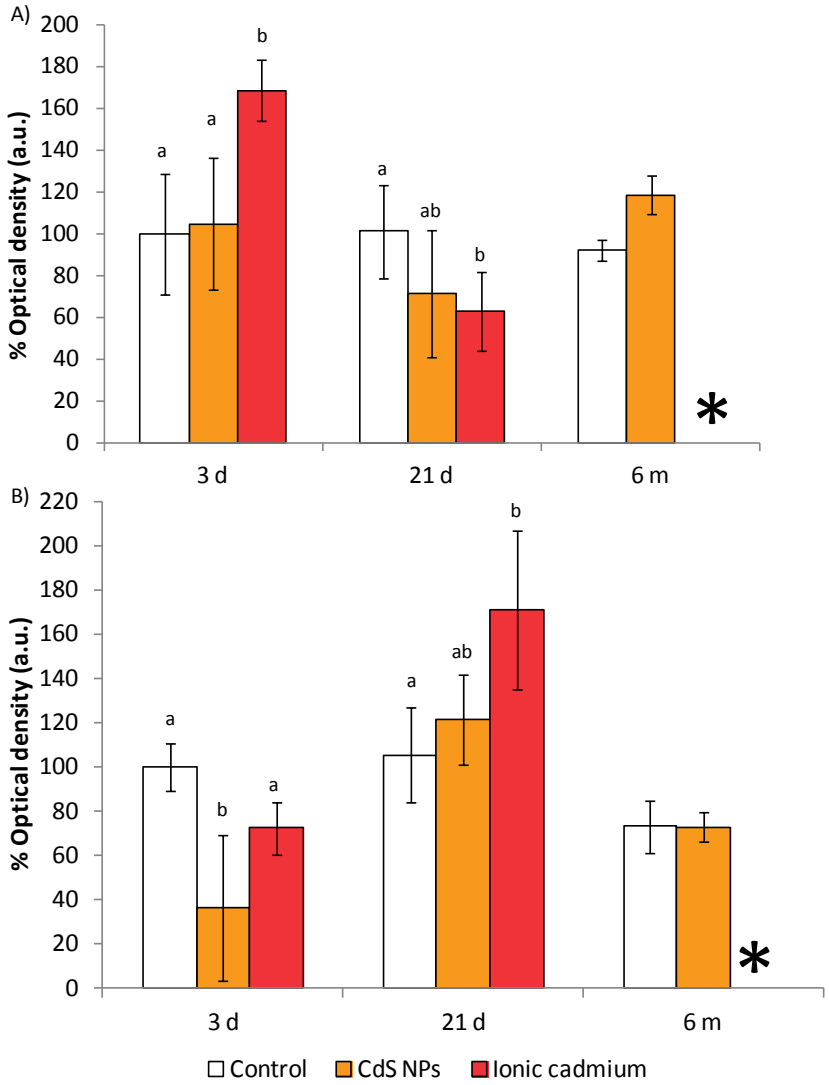

Figure 8.- Relative quantification of the optical density of the immunoreactive band for (A) free ubiquitin and (B) carbonylated actin. Data are shown as percentages respect to the average value of the control group at 3 days. * At 6 months, samples for the ionic group could not collected due to the mortality registered in this group during the exposure period. Different letters indicate statistically significant differences (p<0.05) with each sampling time according to the Kruskall-Wallis test followed by the post hoc Dunns test. a.u.: arbitrary units.

Genotoxicity

Exposure of zebrafish to 10 µg Cd/L of CdS NPs or to ionic cadmium did not cause significant genotoxic effects at any of the measured time points, according to the micronuclei test performed in zebrafish erythrocytes. Only three individual after 3 days of exposure to ionic cadmium and one individual after exposure for 21 days showed each one micronucleated cell (data not shown).

Lysosomal membrane stability

The general health status of the fish after 3 and 21 days of exposure was studied through the LMS test. Due to the lack of individuals provoked by the high mortality, samples for the different exposure treatments were not collected at 6 months. Low mean LP values were measured in all the groups, even in the control groups. A significant decrease in the mean LP value was observed after 3 and 21 days of exposure to CdS NPs, being the desestabilization of the lysosomal membrane observed from the first time measured (Fig 9). After 21 days, fish exposed to ionic cadmium also showed significant decrease in the mean LP value compared to control fish.

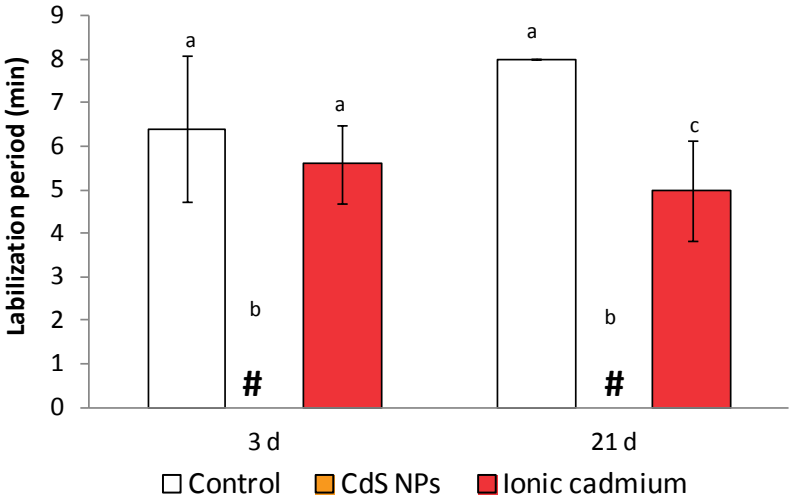

Figure 9.- Labilization period (in minutes) of the lysosomal membrane in liver cells. Different letters indicate statistically significant differences (p<0.05) within each sampling time according to the Kruskall-Wallis test followed by the post hoc Dunns test. # At 3 and 21 days, fish previously exposed to CdS NPs showed desestabilization of the lysosomal membrane from the first time point measured.

Histopathological analysis

In gills, different pathological conditions, such as aneurism, inflammation and fusion of the secondary lamellae and hyperplasia of cells of the primary lamellae, were recorded

in individuals exposed to both forms of cadmium. The prevalence of the histopathological alterations in gills is shown in table 2. Control fish showed in general a normal arquitecture of the gill (Fig 10A), only one individual sampled at 21 days of experiment showed hyperplasia in the primary lamellae. At 6 months, the control group presented a slight increase in the number of individuals with any pathology appearing some cases of hyperplasia, inflammation and aneurism. After the exposure to CdS NPs the most common pathologies were inflammation of the secondary lamellae (Fig 10B) and hyperplasia of the primary lamellae, being the prevalence of inflammation in these individuals (50%) significantly higher than in the control group. Fusion of secondary lamellae was also observed with a much lower prevalence (10%). Exposure to ionic cadmium provoked a significant increase in the number of fish presenting pathologies (Fig 10C-D), being the prevalence of inflammation of the secondary lamellae (78%) and aneurism (45%) significantly higher than in the control group. Other pathologies detected were hyperplasia of the secondary lamellae (55%) and fusion of the secondary lamellae (23%). After the post-exposure period, same histopathological alterations were recorded in the previously exposed fish, with higher prevalence respect to the samples analyzed after 21 days in fish previously exposed to CdS NPs, and with lower prevalence in fish previously exposed to ionic cadmium.

Table 2.- Prevalence of histopathological alterations in gills of zebrafish. Data are shown in percentages. Asterisks indicate statistically significant differences between control and exposed groups (p<0.05) according to the Fisher's exact test.

		n	Secondary lamellae			Primary lamellae	Total
			Inflammation	Fusion	Aneurism	Hyperplasia	
Control	21 d	8	0	0	0	12.5	12.5
	6 m	8	12.5	0	12.5	25	37.5
CdS NPs	21 d	10	50*	10	0	50	60
	6 m	9	88.89*	22.23	33.34	11.12	88.9
Ionic cadmium	21 d	9	77.78*	22.23	44.45*	55.56	88.9*
	6 m	9	44.45	11.12	33.34	44.45	88.9

n: number of individuals per experimental group (in some cases n < 10 because the gill tissue was not always present in the histological sections used for the histological analysis).

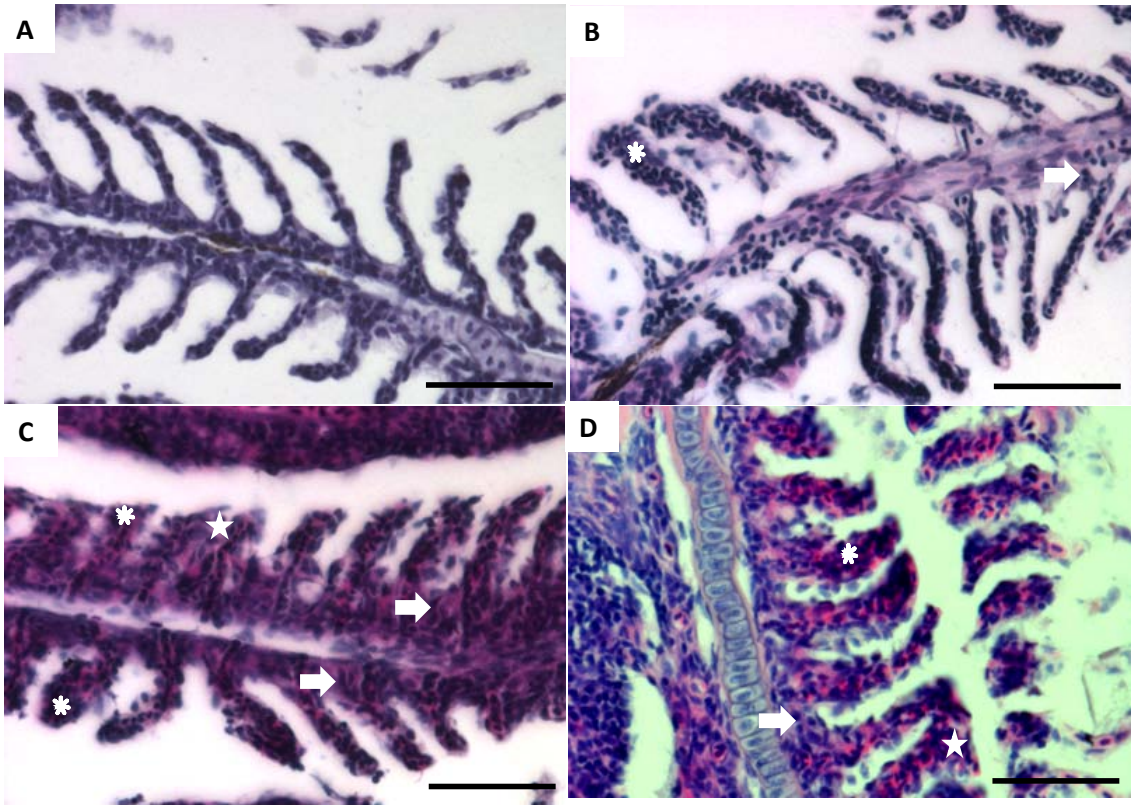

Figure 10.- Micrographs of paraffin embedded and hematoxylin/eosin stained section of zebrafish gills. (A) Gill arquitecture of a control fish, showing normal morphology. (B) Gill of a zebrafish exposed to CdS NPs for 21 days, showing inflammation (asterisks) in the secondary lamellae and hyperplasia (arrow) in the primary lamellae. (C) Gill of a zebrafish exposed to ionic cadmium for 21 days, showing fusion (stars) and inflammation (asterisks) of the secondary lamellae and hyperplasia (arrow) in the primary lamellae. (D) Gill of a zebrafish exposed to ionic cadmium for 21 days, showing fusion (stars) and inflammation (asterisks) of the secondary lamellae and hyperplasia (arrow) in the primary lamellae. Scale bars: 50 μm.

After the histopathological analysis of the liver, different pathologies were detected in the liver of zebrafish exposed to both forms of cadmium after 21 days, as well as at the post-exposure period. The prevalence of the histopathological alterations found in liver is shown in table 3. Control fish showed in general a normal liver (Fig 11A) at 21 days of experiment, while at 6 months two individuals showed vacuolization. After the exposure for 21 days to CdS NPs, the number of individuals presenting vacuolization (Fig 11B) was significantly higher (87.5%) than in the control group. Also, one individual (12.5%) presented megalocytosis (Fig 11C). Exposure to ionic cadmium also provoked a significant increase in the prevalence of fish presenting vacuolization. 100% of the individuals of this group presented this pathological condition. After the post-exposure period, the prevalence of individuals presenting vacuolization decreased respect to the individuals sampled at 21 days of exposure, but remained higher than in control fish at

the same sampling time. One fish previously exposed to ionic cadmium presented an eosinophilic focus (Figure 11D).

Table 3.- Prevalence of histopathological alterations in liver of zebrafish. Data are shown in percentages. Asterisks indicate statistically significant differences between control and exposed groups (p<0.05) according to the Fisher's exact test.

		n	Vacuolization	Megalocytosis	Eosinophilic focus	Total
Control	21 d	8	0	0	0	0
	6 m	10	20	0	0	20
CdS NPs	21 d	8	87.5*	12.5	0	87.5*
	6 m	9	55.6*	0	0	55.6
Ionic cadmium	21 d	10	100*	0	0	100*
	6 m	7	57.1	0	14.3	71.4

n: number of individuals per experimental group (in some cases n < 10 because the liver tissue was not always present in the histological sections used for the histological analysis).

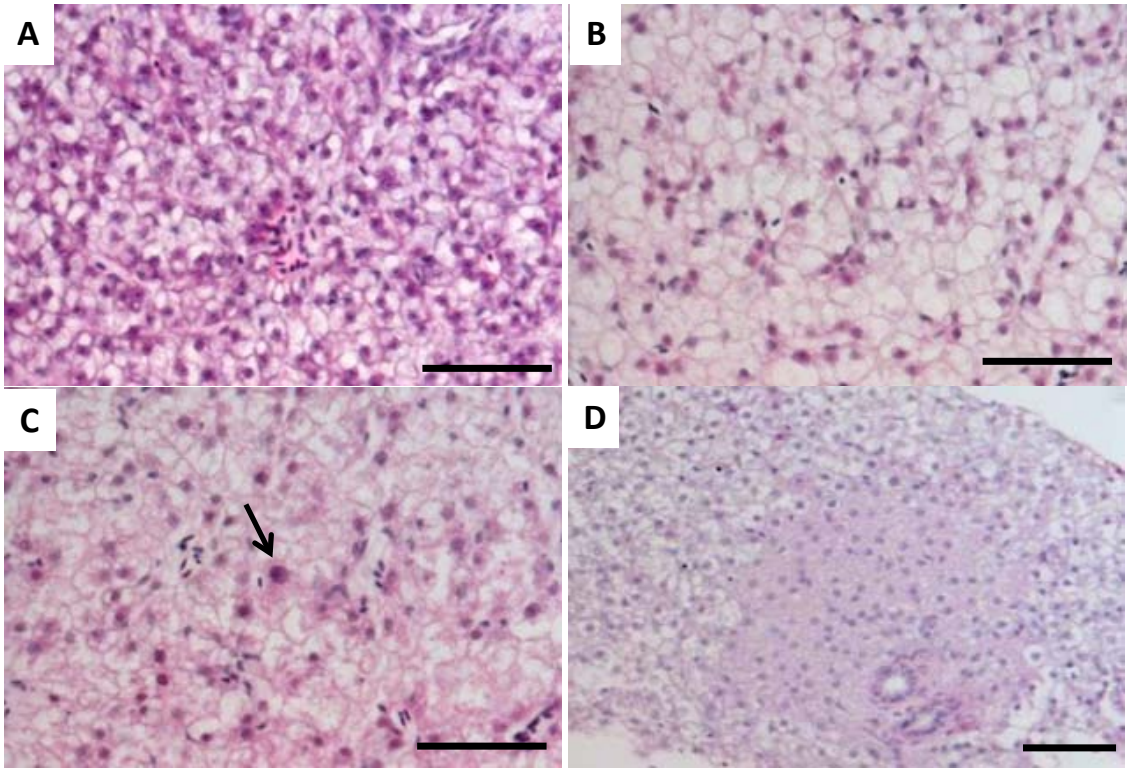

Figure 11.- Micrographs of paraffin embedded and hematoxylin/eosin stained section of zebrafish liver. (A) Liver of a control adult, showing normal morphology. (B) Liver of a zebrafish exposed to CdS NPs for 21 days, showing vacuolization. (C) Liver of a zebrafish exposed to CdS NPs for 21 days, showing hepatic megalocytes (arrow). (D) Liver of a zebrafish exposed to ionic cadmium at the post-exposure period, showing an eosinophilic focus. Scale bars: 50 μm.

DISCUSSION

In this study, adult zebrafish were exposed for 21 days to 10 µg Cd/L of CdS NPs, as well as to the same nominal concentration of ionic cadmium. Then, fish were maintained in clean water for 6 months in order to detect potential long-term effects derived from the previous exposure or the recovery of recorded alterations. Different endpoints, including cadmium accumulation in the organism and cellular effects in different organs, were measured in order to assess the effects provoked by the exposure to both forms of cadmium. Although the cadmium concentration used during the exposure period did not cause embryo mortality (Chapter I), a high mortality was recorded from the fourth day of exposure to both forms of cadmium (72% in fish exposed to CdS NPs and 78% in fish exposed to ionic cadmium at 21 days). This mortality is higher than the mortality reported previously in studies with adult zebrafish exposed to cadmium. Cambier et al. (2010) registered a 56% of mortality after 21 days of exposure to 9.6 µg Cd/L, while Vergauwen et al. (2013b) registered a much lower mortality (20%), despite having exposed fish to a much higher concentration (560 µg/L) of ionic cadmium for 21 days. These differences in mortality may be related to differences in the hardness of the exposure medium employed in the experiments. Previous studies have demonstrated that a high concentration of calcium in the exposure medium decreases bioavailability and, thus, the toxicity of cadmium for the organisms (Meinelt et al., 2001; Pellet et al., 2009). Calcium concentration in the water used by Vergauwen et al. (2013b) was much higher than in our study resulting in a BCF for ionic cadmium of 7.5, while we calculated a BCF of 619 after the exposure to CdS NPs and of 729 after the exposure to ionic cadmium. The analysis of metal accumulation by autometallography showed a higher metal content in the intestine than in the liver, being the apical end of the enterocytes the most stained zone. In agreement with our results, some authors have reported that in fish, including zebrafish, the intestine is the organ which accumulated cadmium at the highest extent followed by liver and gill, while muscle and brain presented the lowest accumulation capacity (Souid et al., 2013; Vergauwen et al., 2013b).

The gastrointestinal tract of the fish can be a route of entrance of cadmium into the organism after waterborne exposure (Olsson et al., 1998), since the metal can be

attached to the food or dissolved in the water drunk by the fish. Moreover, the intestine has been reported as the initial organ where metals are accumulated after a waterborne exposure (Souid et al., 2013), being the non-essential metals retained in mucosal layer of intestine (Clearwater el al., 2005).

In addition to the intestine, gills have been identified as the main entrance of cadmium in the organism after waterborne exposure, playing a role in metal uptake and transfer to other organs via blood transport (Thophon et al., 2003; Alvarado et al., 2006; Costa et al., 2013). During the present study, aggregates of CdS NPs were detected in the cytoplasm of epithelial cells after 3 and 21 days of exposure. Cadmium is taken up in the gill across the epithelial layer via calcium channels in the apical cell membrane, provoking an imbalance in calcium concentration, which induces damage in the gill structure of fish (Glynn, 1996; Thophon et al., 2003). The elimination of cadmium from the gill has been described through the chloride cells (Costa et al., 2013). This route of elimination of cadmium can increase the gill damage, resulting in hyperplasia of chloride cells, or the fusion of two neighboring secondary lamellae (Alvarado et al., 2006), a pathological condition detected during our study after the exposure to both forms of cadmium for 21 days. The damage provoked in the gills by the exposure to cadmium, causes a reduction in the oxygen consumption and disruption of the osmoregulatory function in aquatic organisms (Liu et al., 2011), which could explain the increase in the mortality detected during our experimental conditions. At the end of the 6 month period in clean water, the pathologies previously described were also detected even in a higher prevalence for inflammation, fusion and aneurism of the secondary lamellae, indicating that the toxic effect of cadmium may be sustained after the cease of the exposure. The presence of histopathologies even after 6 months post exposure may be related to the fact that high concentrations of cadmium can be maintained in the tissues after long periods in clean water (Arini et al., 2015).

After the entrance and accumulation in organs such as gills and intestine, cadmium is transferred to the liver, which is well known as an organ involved in storage and detoxification of metals (Handy, 1993). During our study, aggregates of CdS NPs were detected in the nuclear envelope and surrounded vesicles of hepatocytes. Moreover, the accumulation of metal in the liver after the exposure to both forms of cadmium

was corroborated by autometallography. Higher accumulation was detected after the exposure to ionic cadmium than after the exposure to CdS NPs. Autometallography is a technique that requires the presence of metal atoms in the tissue to catalyze the deposition of metallic silver around them (Danscher, 1994). Therefore, the higher signal detected in the liver after exposure to ionic cadmium compared to the exposure to CdS NPs may be due to the solubility rate showed by the CdS NPs used in this study. Although CdS is soluble, only 13% of the metal dissolved in a 1 mM solution of $NaNO_3$ after 7 days (Katsumiti et al., 2014). The exposure to cadmium for 21 days and the resulting accumulation provoked diverse histopathological lesions in the liver. The most common pathology observed was the vacuolization of hepatocytes. Vacuolization, which has been previously related to the response of hepatocytes to toxicants and metals, is associated with the inhibition of protein synthesis, energy depletion or disaggregation of microtubules, or shifts in substrate utilization (Hinton and Laurén, 1990). In previous studies, vacuolization of hepatocytes has been also amply detected on different fish species waterborne exposed to soluble cadmium (Thophon et al., 2003; Liu et al., 2011; Costa et al., 2013). Other pathologies present during the experimental period were the presence of hepatic megalocytosis, associated with DNA damage (Spitsbergen and Kent, 2003) and eosinophilic focus, which has been also detected in *Solea senegalensis* exposed for 28 days to ionic cadmium at the same exposure concentration (10 μg/L) (Costa et al., 2013). This latter pathology has been potentially related to a pre-neoplastic condition (Costa et al., 2013).

Along with the histopathologies detected in the liver, other analysis were carried out in the liver in order to study the effect provoked by the waterborne exposure to both forms of cadmium. The endolysosomal system of the cells has been described as a target of metal exposure, both in soluble and NP form (Alvarado et al., 2005; Broeg et al., 2005; Schultz et al., 2015). Metals are known to induce alterations in the lysosomal structure and membrane desestabilization. The stability of the lysosomal membrane is considered an integrative marker that reflects the breakdown of the adaptative capacity of the fish liver to toxic injury (Broeg et al., 2005). Although the mean LP values measured in all experimental groups were lower than those previously recorded

for zebrafish (Vicario-Parés, 2016; Chapter II), the exposure to both form of cadmium significantly decreased the mean LP value respect to the control. Exposure to ionic cadmium caused a slight decrease of LP value after 3 days and a significant decrease after 21 days. Effects after the exposure to ionic cadmium have been also reported in turbot, although at higher cadmium concentration (10 mg/L) for 14 days (Alvarado et al., 2005). In fish exposed to CdS NPs, the desestabilization of the lysosomal membrane was observed from the first time measured, suggesting that the entrance of NPs may provoke the early desestabilization of the lysosomal membrane. This desestabilization can be provoked by the release of the ions after the dissolution of the NPs due to the acidic characteristics of the lysosomes (Rocha et al., 2015).

Under exposure to cadmium, oxidative stress plays an important role in Cd poisoning of the organisms (Liu et al., 2009). The generation of free ubiquitin chains has been previously determined as marker of oxidative stress in a response to a variety of stressors, such as a high increase of temperature, exposure to methyl methanosulfonate, exposure to H_2O_2 or to $CdCl_2$ (Braten et al., 2012). It has been suggested that free ubiquitin chains can play a role in signal transduction and serve as storage for a large amount of free monomeric ubiquitin for utilization under stress (Braten et al., 2012). A significant increase in the level of free ubiquitin was reported after 3 days of exposure to ionic cadmium, while after 21 days a significant decrease was measured. A decrease in the level of ubiquitin free has been related to oxidative damage, since monomeric ubiquitin is required by the cell under oxidative stress conditions (Kimura et al., 2009). Surprisingly, no effects on the levels of free ubiquitin were measured after exposure to CdS NPs. Along with the levels of free ubiquitin, the carbonylation of proteins and especially carbonylation of actin as target protein has also been used as an indicator of oxidative stress caused by different pro-oxidant treatments, including H_2O_2 or $CdCl_2$ (McDonagh and Sheehan, 2006; Chora et al., 2010). In the present study, a significant increase in the optical density of the immunoreactive band corresponding to carbonylated actin was observed only after the exposure to ionic cadmium for 21 days, while after the exposure to CdS NPs only a slight increase was detected. These results indicated a significant increase of oxidative

stress reflected in the oxidative damage observed in the proteins in fish exposed to ionic cadmium but not in fish exposed to CdS NPs.

Cadmium has been also previously documented as a genotoxic substance in aquatic organisms, both directly by the interaction of Cd^{2+} with DNA and indirectly through ROS-mediated lesions to DNA that cause various dysfunctions in cells under oxidative stress (Dabas et al., 2012). In the present study, no clastogenic effects were detected in zebrafish erythrocytes after the exposure to ionic cadmium or CdS NPs using the micronuclei test. Accordingly, Cambier et al. (2010) did neither detected genotoxic effect after the exposure to 9.6 µg Cd/L of ionic cadmium for 21 days using the MN test and comet assay, but, they reported genotoxicity using a RAPD-based methodology. Increased MN frequency was reported in erythrocytes of carps exposed for 14, 21 and 28 days to a higher concentration of cadmium (0.65 mg/L) (Witeska et al., 2010), indicating that the MN test in erythrocytes, could not to be sensitive enough at low concentrations of cadmium.

CONCLUSIONS

Under the experimental conditions analyzed in the present study a significant cadmium accumulation was measured in the whole organism after the exposure of zebrafish to both forms of cadmium. The localization of CdS NPs in the cytoplasm of epithelial cells of gills and the histopathological alterations corroborated that this organ is one of the main entrances of NPs in the organism. The autometallographical analysis showed a higher extent of metal accumulated in the intestine than in the liver. Both forms of cadmium provoked different effects as reflected in the lysosomal membrane stability test and in the oxidative damage to proteins, indicating that the toxicity may vary depending on the form of the exposure compound. Despite cadmium has been previously reported as a genotoxic substance, the MN test in erythrocytes at the concentration tested did not detect clastogenic effects.

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CHAPTER V

A microarray study reveals differences in the hepatic transcriptome of zebrafish waterborne exposed to CdS quantum dots and ionic cadmium

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ABBREVIATIONS

COA, Correspondence analysis

DTGs, Differentially transcribed genes

FDR, False discovery rate

GO, Gene ontology

LIMMA, Linear models for microarray data

MHC, Mayor histocompatibility complex

NPs, Nanoparticles

PC, Principal component

QDs, Quantum dots

qPCR, Quantitative real time PCR

ROS, Reactive oxygen species

TEM, Transmission electron microscopy

ABSTRACT

Quantum dots (QDs) are nanoparticles (NPs) with special optical and electrical properties that make them very suitable for consumer and industrial products. QDs can contain metallic components such as cadmium that are highly toxic. In the present study, zebrafish (*Danio rerio*) was selected as model organism to study the effects of CdS NPs in comparison with the effects of ionic cadmium in the liver transcriptome using the Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k. Zebrafish were waterborne exposed for 3 and 21 days to the same nominal concentration (10 μg Cd/L) of CdS NPs (~4 nm) or to ionic cadmium. CdS NPs significantly regulated (adj *p* value<0.05) 15 and 4128 different transcripts after 3 and 21 days, respectively, while the ionic form significantly regulated 47 and 15802 transcripts, respectively. GO terms involved in different biological processes such as immune response, or mitochondrial calcium ion homeostasis, cellular components such as intermediate filament or molecular functions such as actin binding were over-represented after the exposure to the ionic cadmium respect to the CdS NPs for 21 days (Fisher analysis). No KEGG pathways were found altered at 3 days, but after 21 days several KEGG pathways appeared significantly affected. Exposure to CdS NPs for 21 days caused a significant effect in the immune response and oxidative stress, while the exposure to ionic cadmium affected significantly those pathways involved in DNA damage and repair and in the energetic metabolism. The correspondence analysis separated all the treatments, being most of the variability explained by the exposure time. In summary, both ionic cadmium and CdS NPs elicited strong effects on the zebrafish liver transcriptome at medium-term exposure, being the effects provoked in the liver of zebrafish different depending on the cadmium form.

Keywords: CdS NPs, ionic cadmium, zebrafish, liver transcriptome, microarray

LABURPENA

Puntu kuantikoak (PKak) ezaugarri optiko eta elektriko bereziak dituzten nanopartikulak (NPak) dira, horri esker oso baliagarriak dira kontsumo- eta industria-produktuetan erabiltzeko. PKek kadmioa bezalako osagai metalikoak izan ditzakete, oso toxikoak direnak. Ikerketa honetan, zebra arraina (*Danio rerio*) organismo eredu modura hautatu zen, CdS NPek eta kadmio ionikoak gibelaren transkriptoman duten efektua konparatzeko; horretarako, Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k mikrotxip komertziala erabili zen. Zebra arrainak 3 eta 21 egunez ~4 nm-ko Cd NPen eta kadmio ionikoko kontzentrazio nominal berdinaren (10 µg Cd/L) eraginpean mantendu ziren. CdS NPek modu esanguratsuan (*p* balioa adj < 0.05) 15 eta 4128 transkrito desberdin erregulatu zituzten 3 eta 21 egunetara, hurrenez hurren; aldaera ionikoak aldiz, 47 eta 15802 transkrito erregulatu zituen, hurrenez hurren. GO terminoen analisiak, erantzun immunea edo mitokondrioen kaltzio ioien homeostasia bezalako prozesu biologikoak, tarteko piruak bezalako zelulen osagaiak edo konposatu molekularrak diren aktinarekiko loturak, kadmio ionikoaren eraginpean, CdS NPen eraginpean egondako animaliekin aldenduz esanguratsuki emendatu zirela erakutsi zuen (Fisher Testa). Analisi funtzionalak ez zuen KEGG bidezidorren alteraziorik antzeman 3 eguneko tratamenduen eraginez, 21 egunetan aldiz, KEGG bidezidor desberdinak esanguratsuki alteratuta zeudela ikusi zen. 21 egunez CdS NPen eraginpean egon ondoren, sistema immunean eta estres oxidatiboan efektu esanguratsua antzeman zen, kadmioaren aldaera ionikoraren eraginpean egon ondoren aldiz, DNAren kalte eta konponketarekin erlazionaturiko bidezidorrak eta metabolismo energetikoarekin erlazionaturikoak erasan zituen. Korrespondentzia analisiak tratamendu guztiak banatu zituen, esposizio-denbora aldakortasun gehiena azaltzen zuen faktorea izan zelarik. Laburbilduz, kadmioaren aldaera ionikoak zein CdS NPek eragin handiak eragiten dituzte zebra arrainaren gibeleko transkriptoman epe ertainean, eragindako efektuak esposizioan erabilitako kadmio aldaeraren araberakoak izanik.

Gako-hitzak: CdS NPak, kadmio ionikoa, zebra arraina, gibeleko transkriptoma, mikrotxipa

RESUMEN

Entre las nanopartículas (NPs), los puntos cuánticos (PCs) presentan una serie de propiedades ópticas y eléctricas que los hacen muy útiles para su uso en productos de consumo e industriales. Los PCs contienen componentes metálicos como cadmio que son altamente tóxicos. En el presente estudio, se seleccionaron peces cebra (*Danio rerio*) adultos para estudiar el efecto de la exposición vía agua a NPs de CdS en comparación con el efecto producido por el cadmio iónico en el transcriptoma del hígado mediante el microchip comercial Agilent technology Zebrafish (v3) Gene Expression Microarray, 4x44k. Para ello, se expusieron peces cebra durante 3 y 21 días a la misma concentración nominal (10 µg Cd /L) de NPs de CdS (~4 nm) o de cadmio iónico. Las NPs de CdS regularon de manera significativa (valor de *p* ajustado < 0.05) 15 y 4128 transcriptos diferentes tras 3 y 21 días de exposición, respectivamente, mientras que la forma iónica reguló 47 y 15802 transcriptos. El análisis de los términos GO mostró que procesos biológicos como la respuesta inmune o la homeostasis mitocondrial de los iones de calcio, componentes celulares como los filamentos intermedios o funciones moleculares tales como las uniones a actina se enriquecieron de manera significativa tras la exposición a cadmio iónico respecto de la exposición a NPs de CdS (test de Fisher). El análisis funcional no detectó rutas KEGG significativamente alteradas tras 3 días de exposición, mientras que tras 21 días diversas rutas KEGG aparecieron significativamente afectadas. La exposición a NPs de CdS durante 21 días causó un efecto significativo en el sistema inmune y estrés oxidativo, mientras que tras la exposición a la forma iónica del cadmio alteró significativamente rutas relacionados con el daño y la reparación del ADN, así como con el metabolismo energético. El análisis de correspondencia separó los cuatro tratamientos, siendo el tiempo de exposición el factor que explicaba la mayor parte de la variabilidad. En resumen, ambas formas de cadmio ejercen un fuerte efecto sobre el transcriptoma hepático del pez cebra tras una exposición a medio plazo, siendo los efectos provocados en el hígado del pez cebra diferentes dependiendo de la forma del cadmio a la que se les expone.

Palabras clave: NPs de CdS, cadmio iónico, pez cebra, transcriptoma hepático, microchip

INTRODUCTION

Cadmium containing quantum dots (QDs) are engineered nanoparticles (NPs) with unique optical and electrical properties making them especially appropriate for biomedical research, microelectronics and solar panel technology (Magarian et al., 2013). The interest in the study of the NPs toxicity has increased exponentially during the last years because of their use in the manufacturing of industrial and consumer products (Maurer-Jones et al., 2013).

Zebrafish (*Danio rerio*) is a model organism commonly used in toxicogenomics, whose earliest whole-genome sequence is available since 2002 (Howe et al., 2013). Few studies have been carried out in order to analyze the effect provoked by the exposure to cadmium containing QDs in the hepatic gene transcription levels of fish. *In vitro* exposure of zebrafish hepatocytes to CdTe QDs of 3.4 nm (0.0012 to 0.72 mg/L) provoked changes in the transcription of several genes related to stress response and DNA repair mechanisms at the highest concentration tested. Significant enhanced transcription levels of *superoxidase dismutase 1* (*sod-1), xeroderma pigmentosum complementation group A* and C (*XPA and XPC), lupus ku autoantigen protein p80 (Ku80), xeroderma pigmentosum and 8-oxoguanine glycosylase (Ogg1)* were reported, while significantly lower *tumor suppressor protein p53 (p53)* transcription was observed in cells exposed to CdTe QDs than in controls (Tang et al., 2013). Ladhar et al. (2013) exposed zebrafish thorough the diet to two concentrations (40 and 100 ng NPs/day/g body weight) of CdS NPs of two different sizes (8 and 50 nm) for 36 and 60 days. Genes involved in mitochondrial metabolism, DNA repair, apoptosis and antioxidant defense in the liver, brain and muscle were down-regulated after 36 days of exposure to the smallest NPs (8 nm), while after 60 days these genes were down-regulated in the intestine and up-regulated in the brain. Also, after 60 days of exposure to both NPs (8 and 50 nm), genotoxic effects were reported using random amplified polymorphic DNA-PCR genotoxicity test.

More studies have focused on analyzing the effect of ionic cadmium in zebrafish gene transcription levels. After 21 days of exposure to cadmium (1.9 and 9.6 µg/L), Gonzalez et al. (2006) studied the regulation of 14 genes involved in antioxidant defense, metal

chelation, active efflux of xenobiotics, mitochondrial metabolism, DNA repair and apoptosis in zebrafish, and reported significant up-regulation of *metallothionein 1* (*mt1)* and *jun protoncogen c* (*c-jun)* in the liver. Also, Arini et al. (2015) exposed zebrafish to 35 µg/L of ionic cadmium for 15 days and reported that the transcription level of genes involved in oxidative stress response (*sod*, *sodmt*) and in detoxification mechanisms (*mt1, mt2*), in mitochondrial mechanisms (*cytochrome c oxidase subunit 1*, *cox1*) and DNA repair (*rad51 homolog*, *rad51* and *growth arrest and DNA-damage-inducible*, *gadd45*) were significantly up-regulated. Also, after the exposure to a higher concentration (560 µg Cd/L) of ionic cadmium for different periods of time (4 and 28 days), up-regulation of diverse genes such as *mt1*, *heat shock protein 70 (hsp70)*, the cytosolic and the mitochondrial isoform of *superoxide dismutase* (*Cu/Zn-sod* and *Mn-sod*) and *glutathione reductase* (*gr*) was reported (Vergauwen et al., 2013).

Other fish have also been used to analyze the effect of the exposure to ionic cadmium. The hepatic transcriptome of carp was analyzed after waterborne exposure (9, 105 and 408 µg/L) and dietary exposure (9.5, 122 and 144 µg/g) to ionic cadmium for 28 days (Reynders et al., 2006). At low exposure concentrations, energy metabolism-related genes were affected. Among them, up-regulation of genes coding for pancreatic proteins that play a key role in the digestion of proteins (elastase, chymotrypsinogen) and carbohydrates (amylase*)* was observed, which could reflect a compensatory mechanism for the inhibition of enzyme activity. On the other hand, induction of *glucokinase* and *malic enzyme*, and inhibition of *cytochrome c oxidase* and *cytochrome b* suggested a stimulation of anaerobic metabolism and a decreased energy production in the citric acid cycle. At middle exposure concentrations, several stress-related genes were induced (e.g. *hemopexin, cytochrome P450 2F2*), suggesting a general stress response (Reynders et al., 2006). The hepatic transcriptome has been also analyzed in European flounder (*Platichthys flesus*) after 3 days of injection with 2 mg/Kg body weight of cadmium. Up-regulation of 27 transcripts, involved in oxidative stress such as *Cu/Zn sod*, or genes as *heat shock protein 90* (*hsp90*), *glucose regulated protein 170* (*grp170*) and down-regulation of 14 genes, such as *cytochrome P4501A* (*cyp1a)* was reported (Sheader et al., 2006). Auslander et al. (2008) administered 57.25 µg Cd/g food for 57 days or injected 2.5 mg Cd/kg body weight to striped seabream

(*Lithograthus mormyros)* and sacrificed the organisms at 8 days post injection. 31 significantly regulated transcripts from a whole hepatic genome microarray were reported after both experimental approaches. Among them, 23 transcripts such as *elastase 4, carboxypeptidase B, trypsinogen, perforin, complement C31, cytochrome P450 2K5 and carboxyl ester lipase* were down-regulated, while *ceruloplasmin, metallothionein or glutathione peroxidase* were up-regulated (Auslander et al., 2008). Mehinto et al. (2014) intraperitoneally injected adult largemouth bass (*Micropterus salmoides*) with 20 µg/kg of ionic cadmium and a microarray analysis was conducted in the liver 48 h after injection. Gene ontology (GO) analysis indicated an over-representation of hepatic transcripts implicated in various terms, such as rRNA processing, carbohydrate and cholesterol metabolism, translation elongation factor activity and DNA/RNA polymerase activity. Specific transcripts affected in the liver included a significant up-regulation of the antioxidant *catalase* and the cytoprotective chaperone *DnaJC7*. Genes involved in DNA replication, DNA repair and transcriptional pathways were suppressed. Among those, *SET nuclear oncogene, MYB binding protein 1A, SAP30 binding protein* and a number of DNA-directed and RNA-directed polymerases were down-regulated. Genes of the immune response pathways were disrupted including an up-regulation of *complement factor b* and of *complement component 8 beta* and down-regulation of *immunoglobulin binding protein 1*. The results obtained in this study indicated that down-regulation of DNA repair, transcriptional pathways and the induction of oxidative stress pathways are among the initial molecular changes caused by low levels of cadmium in the liver (Mehinto et al., 2014).

The aim of this study was to decipher the metabolic pathways altered by 3 and 21 days of exposure to CdS NPs compared with results obtained after the exposure to ionic cadmium, through the analysis of the whole liver transcriptome of zebrafish. To the best of our knowledge, this is the first time in which the whole adult zebrafish liver transcriptome has been studied after exposure to CdS NPs. Cell and tissue level effects caused by CdS NPs have been already described in Chapter IV. Exposure to CdS NPs or to ionic cadmium provoked accumulation of cadmium in liver and in intestine as revealed by autometallography. By transmission electron microscopy (TEM), presence

of NPs was detected in the cytosol of gill epithelial cells of the secondary lamellae, attached to the nuclear envelope and into small membrane vesicles of hepatocytes. In the assayed conditions, significant effects on hepatocyte lysosomal membrane stability and increased oxidative stress were detected, along with a variety of relevant histopathological alterations in gills and liver (Chapter IV).

MATERIALS AND METHODS

Cadmium compounds

Glutathione (GSH) capped CdS NPs of 3.5-4 nm were synthesized by wet chemistry following a method modified from Zou et al. (2009) as described in Katsumiti et al. (2014). After the synthesis, CdS NPs were washed using centrifugal ultrafiltration, resulting in CdS NPs with no residual free GSH. CdS NPs were provided as a suspension at a concentration of 4.7 g Cd/L. CdS NPs were characterized in deionized water by TEM, and isolated particles or very small aggregates (5–10 nm) were observed. In a 1 mM solution of $NaNO_3$, 1.1% of cadmium was dissolved from the CdS NPs after 24 h, 6.6% after 72 h and 13% after 7 days (Katsumiti et al., 2014). Ionic cadmium solution was prepared from $CdCl_2$ purchased to Sigma-Aldrich (St. Louis, Missouri, USA). In both cases, a solution of 1 mg Cd/L was prepared by dissolving the original stocks in deionized water. The final exposure concentration (10 µg/L) was achieved by adding a given volume of the stocks solution to the exposure aquaria.

Maintenance and experimental exposure of adult zebrafish

The experimental procedure described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. Zebrafish (wild type AB Tübingen) individuals were specifically produced and grown for the experiment in our facility at the UPV/EHU. Adult fish of approximately 4 months old were exposed to 10 µg Cd/L of CdS NPs or of ionic cadmium for 21 days. An unexposed control group was run in parallel in identical experimental conditions. The exposures were carried out in 35 L aquaria containing 50-60 fish. During the exposure period, approximately 2/3 of the aquarium water was changed by siphoning every three days and the corresponding volume of contaminated or clean water was

redosed. Fish were fed with Vipagran baby (Sera, Heinsberg, Germany) and live *Artemia* (INVE Aquaculture, Salt Lake City, Utah, USA) twice per day.

After 3 and 21 days of exposure, fish were euthanized by overdose of anesthetic (benzocaine, Sigma-Aldrich) and the liver of 20 male zebrafish per experimental group was dissected out, pooled in 5 groups of 4 livers each and placed in cryovials containing TRIzol® (ThermoFisher Scientific, California, USA) and zirconia/silica beads (Biospec, Bartlesville, USA). Then, samples were immediately frozen in liquid nitrogen and stored at -80ºC.

RNA extraction

Total RNA was extracted following the TRizol® extraction method (ThermoFisher Scientific). Concentration of RNA was measured in a Biophotometer (Eppendorf, Hamburg, Germany). RNA was purified with RNeasy mini kit (Qiagen, Venlo, The Netherlands). In addition, RNA quality was assessed in an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Only RNA samples with a RIN value above 8.1 were used for microarray and qPCR analysis.

Microarray analysis and data treatment

Microarray analysis was carried out in the General Genomic Service – Gene Expression Unit (SGiKer) of the University of the Basque Country. Labeling of samples was carried out following the Agilent Technologies "One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling)" Version 6.5 protocol. 100 ng of total RNA were retrotranscribed and labelled using the Low imput Quick Amp Labeling kit, One color (Agilent Technologies) following the manufacturer's instructions. Hybridizations were performed on zebrafish 4x44k full genome microarrays (version V3, AMADID 026437 Agilent Technologies) containing 43,803 unique probes using the SuperHyb hybridization chamber (Agilent Technologies). Finally, slides were scanned using a G2565CA DNA microarray scanner (Agilent Technologies). Feature Extraction software v. 10.7.3.1 was used to feature signal intensity extraction and quantile normalization was applied to the raw intensities (log2 values) using the Agilent GeneSpring GX software (v 11.2).

Gene transcription profiles were compared using the LIMMA analysis in the MultiExperiment Viewer (tMeV) vs. 4.7.1 (http://www.tm4.org/mev/) software. Benjamin-Hochberg method (FDR) for multiple test correction was employed to obtain the corrected *p* value. Significant differences were set at an adjusted *p* value $p<0.05$ and based on $\log_2FC< -1$ or $\log_2FC> 1$ ($\log_2$ fold change). Venn diagrams were obtained using the free application developed by Oliveros (2007) (http://bioinfogp.cnb.csic.es/tools/venny/) to represent the number of significantly regulated transcripts after each specific treatment as well as the number of significantly regulated common transcripts when comparing different treatments and/or exposure times. Then, significantly regulated transcripts were studied using Blast2GO (Conesa et al., 2005) and a summary of the KEGG pathways was performed in order to decipher biological processes altered after each treatment. Fisher exact test ($p<0.05$) was used to find statistically over-represented functions. The significant regulation ($p<0.05$) of the KEGG pathways respect to the whole genome of *Danio rerio* was performed using DAVID online tool (Huang et al., 2009a; 2009b). In addition, a correspondence analysis (COA) was performed in order to visualize the association between the experimental groups.

Quantitative Real Time PCRs (qPCRs)

Six genes differentially regulated in the exposed groups in comparison with the control group were selected to validate microarray results by qPCRs. Genes were selected based on two criteria: fold change ($\log_2FC< -1$ or $\log_2FC> 1$) and genes of toxicological interest. Therefore, selected target genes were: *metallothionein 2 (mt2,* NM_001131053*), ATP-binding cassette, sub-family G member 2b (abcg2b,* NM_001039066.1*), glutathione peroxidase 1b (gpx1b,* NM_001004634.2*), heat shock protein 47 (hsp47,* NM_131204*), jun dimerization protein 2a (jdp2a,* AL927014*)* and *member RAS oncogene family (rab15,* NM_001002318*). 18S rRNA* (FJ915075) was selected as housekeeping, since its transcription level did not vary significantly in the microarray. Available commercial TaqMan® assays (ThermoFisher Scientific) were used to amplify *hsp47* (Dr03150230_g1)*, rab15* (Dr03101758_g1) and *gpx1* (Dr03121558_m1). Assays for the amplification of *jdp2a, abcg2a, mt2* and *18S rRNA*

were not commercially available and, therefore, specific primers and probes were designed using the Primer Express 3.0 software (ThermoFisher Scientific) (Table 1).

Table 1.- Design of specific primers and probes used to amplify each target gene by TaqMan qPCRs.

Gene	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
jdp2a	TGCCACCACACTCTCTGAACA	ACAGCATGGCCCAATGT	CTGAAATATGCAGACCTC
mt2	CCCATCTGGTTGCAGCAAGT	AGCCACAGGAATTGCCTTTG	TGCCTCTGGCTGCG
abcg2a	GCGCTTAACCAAGGGAACAGTT	TCGATGATGTCTCCACTTTTTT	TCCAGTGATAAGAA
18S rRNA	CGGAGGTTCGAAGACGATCA	GGGTCGGCATCGTTTACG	ATACCGTCGTAGTTCCG

hsp47 was selected to validate results obtained after the exposure to ionic cadmium at 3 days, and *jdp2a* was selected to validate results obtained 3 days after CdS NP exposure. *gpx1, abcg2a, mt2* and *rab15* were selected for the ionic cadmium exposure for 21 days, while *gpx1, abcg2a* and *mt2* together with *jdp2a* were used after the exposure to CdS NPs for 21 days.

Total RNA (1 µg) was retrotranscribed to cDNA using the AffinityScript multi temperature cDNA synthesis kit (Agilent Technologies) following manufacturer's conditions in a 2720 Thermal Cycler (ThermoFisher Scientific). qPCRs were run in 25 µL reactions containing 2 µL of cDNA on a 7300 Applied Biosystems thermocycler (ThermoFisher Scientific). Universal PCR conditions were used in all the cases. No template controls were run for quality assessment. Relative transcription levels were calculated based on the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) using the lowest value in the control group as calibrator and *18S rRNA* transcription levels as reference gene, with a coefficient of variation of 3,78%.

Statistical analyses were performed using the SPSS statistical package v20.0 (SPSS Inc, Microsoft Co, WA, USA). According to the Kolmogorov-Smirnov test (p<0.05), data did not follow a Normal distribution. Thus, significant differences among groups were based on the non-parametric Kruskal-Wallis test followed by the Dunns post hoc test (p<0.05) or the Mann-Whitney *U* test (p<0.05).

RESULTS

After 3 days of exposure to CdS NPs 15 transcripts coding for 9 different genes were significantly regulated, 11 transcripts were significantly down-regulated and 4 were up-

regulated. After exposure to ionic cadmium 47 transcripts coding for 37 different genes were significantly regulated, 41 transcripts were significantly down-regulated and 6 were up-regulated. After 21 days of exposure, the number of regulated transcripts increased greatly, especially as results of exposure to ionic cadmium, which regulated 15802 transcripts coding for 8570 genes, 7802 transcripts were up-regulated and 8000 were down-regulated (Fig 1A). Exposure to CdS NPs for 21 days regulated 4128 transcripts coding for 2650 different genes, 1636 transcripts were significantly up-regulated and 2492 were down-regulated. As shown in the Venn diagram (Fig 1B), 486 transcripts were exclusively regulated by CdS NPs, almost all after 21 days of exposure. Similarly, ionic cadmium significantly altered 12177 transcripts, most of them also after 21 days of exposure (Fig 1B).

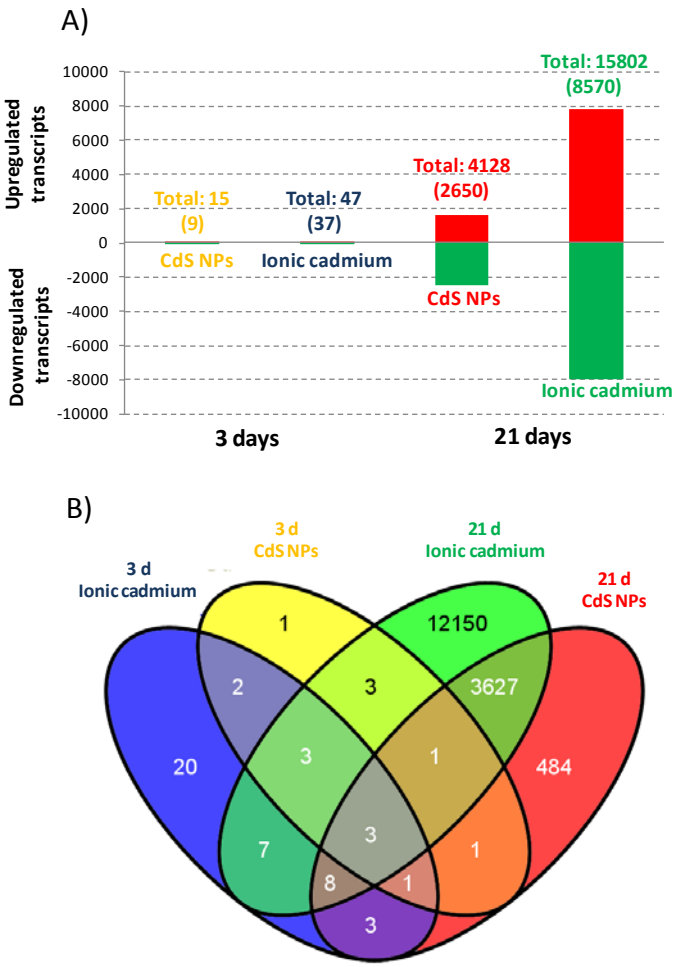

Figure 1.- (A) The bar graph shows the number of significantly up and down regulated transcripts for each treatment. The total number of significantly regulated transcripts and genes (in brackets) are indicated in each case. (B) Venn diagram showing the number of significantly regulated transcripts after each specific treatment as well as the number of significantly regulated transcripts shared among treatments.

According to the Bast2GO analysis, after 3 days of exposure to both forms of cadmium no GO terms appeared enriched. Nevertheless, after the exposure for 21 days several GO terms belonging to the biological processes, molecular functions and cellular components were enriched (Fig 2). Both treatments resulted in similar GO terms enriched, such as “intracellular”, “primary metabolic processes”, “organic substance metabolic processes”, “intracellular parts”, “single organism cellular processes”, “organic cyclic compound binding”, “heterocyclic compound binding”, “cellular metabolic processes” or “ion binding” were mainly enriched (Fig 2).

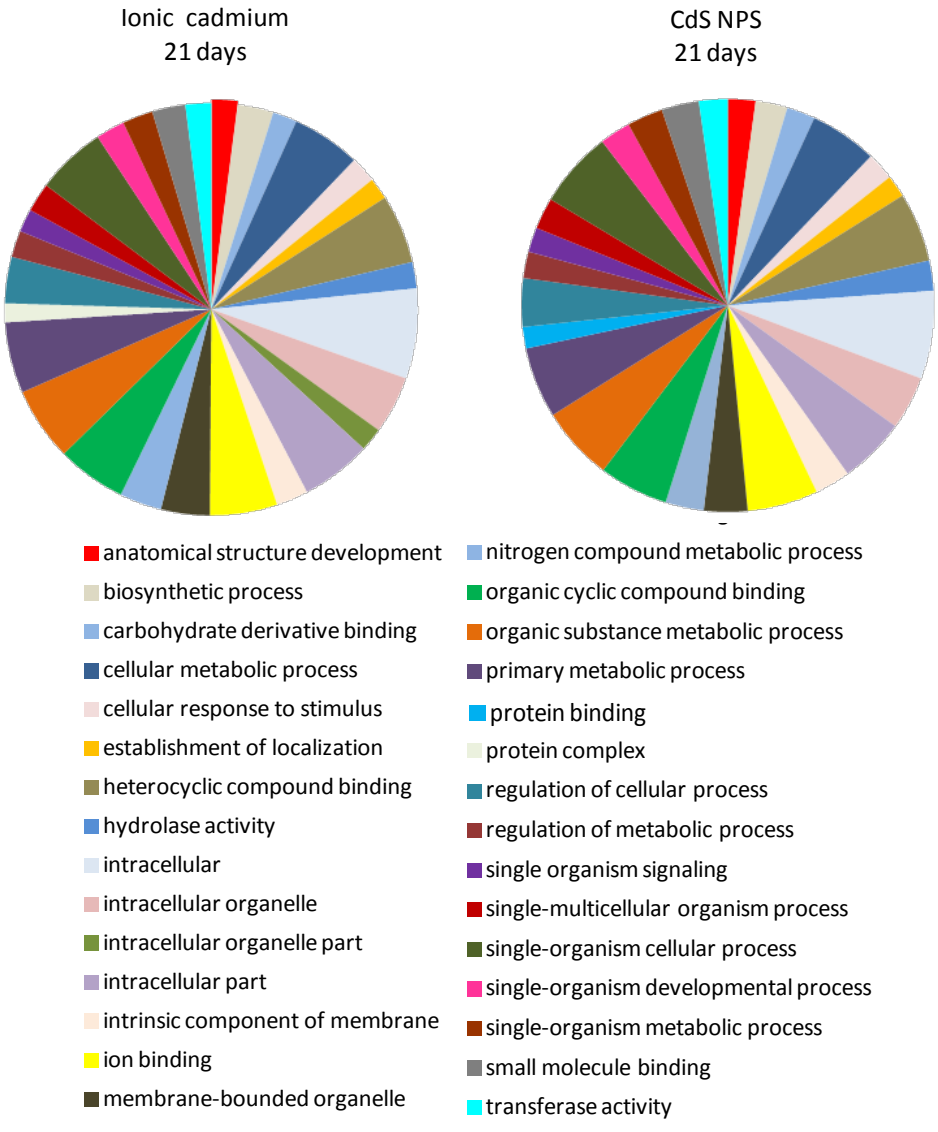

Figure 2.- Multilevel pie graphs showing the GO terms distribution in an ontology level 3 of significantly regulated transcripts after the exposure to ionic cadmium and CdS NPs for 21 days.

According to the Fisher analysis, GO terms involved in immune system processes, mitochondrial calcium ion homeostasis or actin binding were differentially enriched for the ionic cadmium over the NP form (Table 2).

Table 2.- Results obtained after the Fisher exact test performed between treatments in order to indentify GO terms differentially enriched by ionic cadmium over CdS NPs after 21 days.

GO ID	GO terms	GO namespace	*p* value	FDR
GO:0006955	immune response	P	6.43E-07	2.21E-03
GO:0002376	immune system process	P	6.04E-06	5.93E-03
GO:0019882	antigen processing and presentation	P	2.13E-07	1.46E-03
GO:0042611	MHC protein complex	C	2.85E-06	3.34E-03
GO:0042612	MHC class I protein complex	C	1.19E-06	2.72E-03
GO:0051561	positive regulation of mitochondrial calcium ion concentration	P	6.19E-05	3.54E-02
GO:0046686	response to cadmium ion	P	1.31E-05	1.09E-02
GO:0045111	intermediate filament cytoskeleton	C	2.91E-06	3.34E-03
GO:0005215	transporter activity	F	2.30E-05	1.58E-02
GO:0022804	active transmembrane transporter activity	F	7.52E-05	3.98E-02
GO:0003779	actin binding	F	1.43E-05	1.09E-02

P: Biological process, F: Molecular function, C: Cellular component

The functional analysis performed using DAVID in order to identify the pathways significantly altered after each treatment against the whole zebrafish genome resulted in no KEGG pathways significantly affected after 3 days of exposure. After 21 days of exposure several pathways appeared significantly affected. In case of the exposure to CdS NPs, KEGG pathways such as “natural killer cell mediated cytotoxicity”, “retinol metabolism”, “D-arginine and D-ornithine metabolism” or “adherens junction” were significantly affected (Table 3). All these KEGG pathways showed a larger number of up-regulated genes than down-regulated genes. On the other hand, after the exposure to ionic cadmium KEGG pathways such as “cell cycle”, DNA related pathways (“purine metabolism”, “pyrimidine metabolism”, “mistmach repair”, “nucleotide excision repair”, “DNA replication”), “RNA polymerase”, “spliceosome” or “homologous recombination” appeared significantly altered. In this case, a larger number of genes were down-regulated than up-regulated. However, KEGG pathways belonging to amino acids metabolism (“glycine, serine and threonine metabolism”, “alanine, aspartate and glutamate metabolism”), to energetic metabolism (“glycolysis/gluconeogenesis”, “pentose phosphate pathway”, “biosynthesis of

unsaturated fatty acids", "citrate cycle (TCA cycle)") or "dorso-ventral axis formation" contained a larger number of genes up-regulated than down-regulated (Table 4).

Finally, the exposure to both forms of cadmium commonly significantly altered two KEGG pathways, "selenoamino acid metabolism" and "drug metabolism" which had a larger number of significantly up-regulated genes than down-regulated genes (Table 3 and 4).

The correspondence analysis (COA) built to have an overall view of the transcription differences resulting from each treatment, separated the four experimental groups. Most of the variability was explained by the first two principal components (Fig 3), PC1 or exposure time explained 84.12% of the variability, and PC2 or metal form explained 14.02% of the variability.

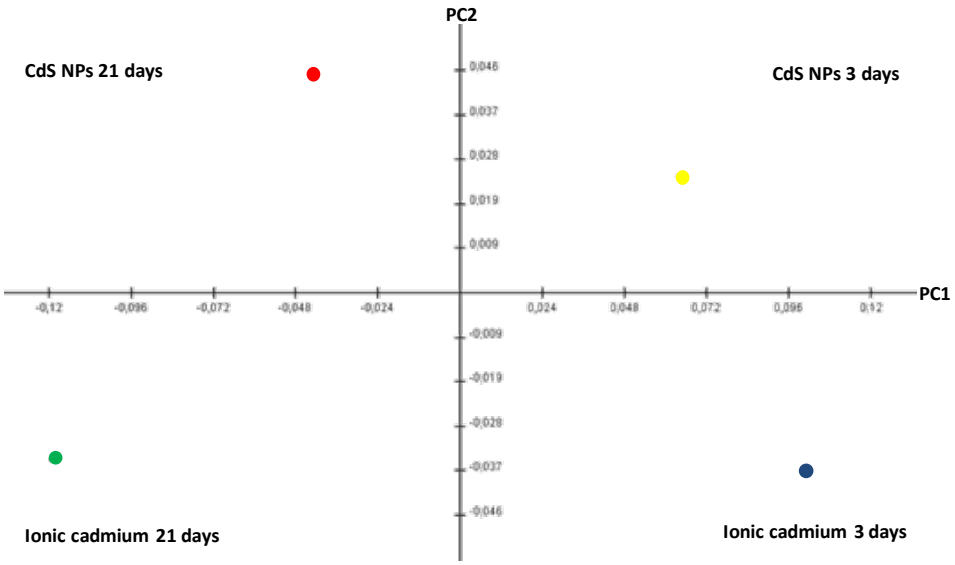

Figure 3.- Correspondence analysis (COA) axes showing the distribution of each sample (dots) according to their transcription profile.

Quantitative real time PCR

In general, microarray and qPCR showed similar results for most of the selected genes. *jdp2a* was an exception, since according to the microarray it was down-regulated after 3 days of exposure and up-regulated after 21 days of exposure and according to the qPCR analysis it was up-regulated after 3 days and down-regulated after 21 days of exposure (Table 5).

Table 3.- Significantly regulated genes contributing to the KEGG pathways identified by DAVID analysis in zebrafish exposed to CdS NPs for 21 days (p<0.05).

KEGG pathways	*p* value	FDR	DTGs	
			up-regulated	down-regulated
Natural killer cell mediated cytotoxicity	0.012	0.404	*prkca, ptpn6, hrasa, crfb5, casp3b, cd247, ptk2bb, kras, rac2, grb2a, plcg2, lck, nfat5, zap70, pak1, zgc:101809, fas*	*fyna*
Retinol metabolism	0.005	0.293	*bcmo1, aldh1a2, dhrs3b, cyp3a65, zgc:172315, adh5, dgat1a, bco1l*	*dhrs2*
D-Arginine and D-ornithine metabolism	0.031	0.498	*dao.1, dao.2, dao.3, pfkla, aldocb, pfkfb4*	--
Adherens junction	3.05E-04	0.039	*ptpn6, crebbpa, wasb, lmo7a, rac2, wasa, tcf7l2, tcfl1a, cdc42l, rhoab, src, rhoaa, egfra, LOC559111*	*fyna, ptpn1, ctnna2, wasf3, acvr1b, zgc:109889, igfr1rb, smad2m, lef1*
Drug metabolism	0.023	0.534	*cyp3a65, mgsta3, aox1, zgc:172315, adh5, mao, gstp2*	--
Selenoamino acid metabolism	0.013	0.182	*ggt1b, ahcyl1, ahcyl2, cbsb*	*mat1a, tyw3, papss1, sephs1*

DTGs: Differentially transcribed genes; FDR, False discovery rate.

Table 4.- Significantly regulated genes contributing to the KEGG pathways identified by DAVID analysis in zebrafish exposed to ionic cadmium for 21 days (p<0.05).

KEGG pathways	*p* value	FDR	DTGs	
			up-regulated	down-regulated
Cell cycle	8.89E-04	0.031	*ywhag2, crebbpa, mcm6, mycb, cdkn1bl, gadd45aa, ywhaqb, mcm3, cdkn2c, LOC557882, ywhah, wu:fc17g12, gsk3b, ccnd1, LOC559111*	*rab43, mcm4, wee1, mdm2, dbf4, rbx1, mcm2, orc2, hdac1, mcm7, si:dkey-23c22.2, anapc11, bub3, cdc23, anapc4, cdc27, anapc5, mcm5, mad1l1, LOC569473, rb1, anapc7, pcna, cdc16, orc4, cdk7, ttk, smad2, cdkn1c, ccnh, cdk2, mcm3l, chek1,bub1bb, cdk1, cdc25, cdc45, chek2, ccna1, cdc6, ccnb1, ccnb2, plk1, mad2l1, ccna2, wee2, skp2, mcm6l, cdc7, cdc20*
Purine metabolism	0.049	0.35	*ada, loc568219, nt5e, adss, entpd2a.1, cant1b, pklr, adcy8, ak2, nt5c2a, ak1, allc, si:dkey-28b4.7, xdh, adcy2b, polr2a, LOC556578, papss2b, nt5c2b. adka. nme4, adssl1*	*prps1a, adsl, nme6, polr3d, polr2d, nme7, polr1c, polr2j, polr2l, atic, polr3c, prps1b, ppat, hprt1, nudt5, polr1b, impdh2, entpd4, pole3, polr3f, polr3k, pola1, fhit, prim1, polr2gl, polr1e, pole, pola2, pole2, papss1, dguok, polr2i, dck, aprt, prim2, polr2h, pold2, pold1, pde6d, rrm1, POLR3G, ampd1, znrd1, rrm2*
Pyrimidine metabolism	2.92E-05	0.002	*LOC559958, nt5e, ak3, cant1b, dpys, nt5c2a, si:dkey-28b4.7, polr2a, nt5c2b, nme4, upp1, cmpk*	*nme6, dhodh, polr3d, polr2d, nme7, polr1c, polr2j, polr2l, polr3c, polr1b, cad, entpd4, pole3, tk2, uck2b, polr3f, polr3k, pola1, prim1, polr2gl, polr1e, dut, pole, dtymk, pola2, pole2, polr2i, dck, prim2, tyms, uck2a, polr2h, pold2, pold1, rrm1, POLR3G, znrd1, tk1, rrm2, dctd*
Mismatch repair	0.01	0.151	*si:dkey-28b4.7*	*rfc5, mlh1, rpa1, lig1, rpa2, pcna, rfc4, msh2, rfc2, pms2, pold2, pold1, MSH3, rpa3*
Nucleotide excision repair	0.003	0.07	*cul4a, gtf2h2, si:dkey-28b4.7*	*ercc3, rbx1, rfc5, gtf2h3-2, rpa1, ercc4, ercc1, lig1, pole3, mnat1, gtf2h5, rpa2, pcna, rfc4, ercc2, rfc2, pole, cdk7, pole2, ccnh, pold2, pold1, rpa3*
DNA replication	4.70E-09	6.82E-07	*mcm4, rnaseh1, mcm2, rfc5, rpa1, mcm7, rnaseh2a, lig1, pole3, mcm5, rpa2, rnaseh2b, pola1, pcna, rfc4, prim1, rfc2, pole, pola2, pole2, prim2, pold2, pold1,mcm3l, rpa3, dna2,fen1, mcm6l*	*mcm6, mcm3, si:dkey-28b4.7, rnaseh2c*

Table 4 (Continued)

KEGG pathways	*p* value	FDR	DTGs	
			up-regulated	down-regulated
RNA polymerase	0.009	0.157	*polr2a*	*polr3d, polr2d, polr1c, polr2j, polr2l, polr3c, polr1b, polr3f, polr3k, polr2gl, polr1e, polr2i, , polr2h, POLR3G, znrd1*
Spliceosome	0.004	0.088	*hsc70, hnrnpm, sf3b1, hspa8, hnrnpua*	*prpf8, hnrpkl, snrpb, rab43, prpf38b, cherp, u2af1, prp19, sf3b3, puf60a, rbm17, cwc15, ppil1, dhx16, ctnnbl1, hnrnpa1a, sf3b5, prpf31, ppie, ccdc12, snrpg, sf3a3, thoc1, ncbp2, prpf3, snrnp27, snrpd2, zgc:55733, tra2b, rbmx, xab2, plrg1, snrpa, snrpd1, prpf18, snrpc, usp39, snrpd3l, smx5, mcm5, snrpe, lsm8, bud31, prpf4, LOC798846, lsm5, prpf38a, phf5a, lsm6, magoh, ppih, thoc3, snrpb2*
Homologous recombination	0.009	0.165	*si:dkey-28b4.7*	*mre11a, rpa1, brca2, top3a, rpa2, rad54l, rad51c, mus81, pold2, pold1, xrcc3, rpa3, rad51d, rad51, rad52, xrcc2*
Glycine, serine and threonine metabolism	4.06E-04	0.019	*dao.2, dao.1, cbsb, mao, dao.3, cthl, alas2, gatm, agxt2, aoc2, gnmt, dldh, tdh, alas1, cth, tha1, psat1, cbsa, shmt2, shmt1, gldc*	*phgdh*
Alanine, aspartate and glutamate metabolism	0.024	0.23	*adss, glud1b, acy3.2, agxt2, got1, aldh4a1, gfpt1, glsb, gpt2l, adssl1*	*adsl, gpt2, glula, ppat, cad, asl, glulb, asns, LOC100148522*
Glycolysis/ Gluconeogenesis	0.016	0.19	*ldha, pfkla, loc560944, pklr, aldob, pck1, adh5, aldocb, acss2, pgm2, pgam2, dldh, fbp1a, dlat, hk1, aldh9a1a, pdhb, pck2, aldoaa, aldh2a, gpia, aldh2b, pgm1, pgk1, acss1, erlin2*	*bpgm, akr1a1b, hk2, zgc:153973, pfkma, tpi1a*
Pentose phosphate pathway	0.043	0.35	*pfkla, loc560944, aldob, taldo1, aldocb, pgm2, fbp1a, dera, aldoaa, gpia, pgm1, erlin2*	*prps1a, rpe, rpia, prps1b, pfkma*

Table 4 (Continued)

KEGG pathways	*p* value	FDR	DTGs	
			up-regulated	down-regulated
Biosynthesis of unsaturated fatty acids	0.021	0.22	*elovl2, fads2, acox1, elovl6, ptplb, acox3, acot7, tecrb, acaa1, elovl5*	*yod1, tecra*
Citrate cycle (TCA cycle)	0.017	0.19	*pck1, idh1, aclya, sdhdb, sdhb, sdha, dldh, cs, dlat, pdhb, pck2, dlst, aco1, suclg2, idh3g, mdh1a, mdh2*	*idh3a, mdh1b*
Dorso-ventral axis formation	0.013	0.182	*etv7, kras, notch1 , notch3, ets2, ets1a, notch2, egfra, grb2, spire1a, etv6, mapk3, mapk1*	*notch1a, piwil2, zorba, piwil1*

DTGs: Differentially transcribed genes; FDR, False discovery rate.

Table 5.- Regulation and transcription level of selected target genes after zebrafish exposure to CdS NPs or ionic cadmium for 3 and 21 days, measured both in the microarray and by qPCRs. Fold change and *p* values in microarray results were based on the LIMMA analysis while the non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test or *U*-Mann Whitney ($p<0.05$) were used for qPCRs data analysis. Asterisks indicate statistically significant differences respect to the control group.

Treatment	Exposure time (days)	Accession nº	gene	Array			qPCR		
				Regulation	log_2 FC	Adjusted p-value	Regulation	log_2 FC	Adjusted p-value
CdS NPs	3	AL927014	jun dimerization protein 2a (*jdp2a*)	down	-2.97	2.85E-03	up	0.02	0.933
Ionic cadmium		NM_131204	heat shock protein 47 (*hsp47*)	down	-2.41	0.004478	down	-0.54	0.003*
CdS NPs	21	NM_001004634.2	glutathione peroxidase 1b (*gpx1b*)	down	-3.61	0.048536	down	-0.93	0.099
		NM_001039066.1	ATP-binding cassette, sub-family G member 2b (*abcg2b*)	down	-4.87	0.041821	down	-0.70	0.012*
		NM_001131053	metallothionein 2 (*mt2*)	up	1.38	7.43E-06	up	0.97	0.01*
		AL927014	jun dimerization protein 2a (*jdp2a*)	up	1.71	0.000735	down	-0.30	0.194
Ionic cadmium		NM_001004634.2	glutathione peroxidase 1b (*gpx1b*)	down	-3.96	0.023058	down	-0.32	0.099
		NM_001039066.1	ATP-binding cassette, sub-family G member 2b (*abcg2b*)	down	-5.76	0.009183	down	-0.51	0.012*
		NM_001131053	metallothionein 2 (*mt2*)	up	4.84	3.30E-08	up	1.81	0.01*
		NM_001002318	member RAS oncogene family (*rab15*)	up	5.01	0.000255	up	2.21	0.03*

DISCUSSION

Previous studies have documented the toxicity of soluble cadmium in zebrafish through the analysis of the transcription level of specific genes involved in known mechanisms of toxicity, such as oxidative stress and DNA damage (Gonzalez et al., 2006; Vergauwen et al., 2013; Arini et al., 2015). In the present work we have studied the whole hepatic transcriptome of zebrafish in order to analyze the effects provoked by exposure to CdS NPs in comparison to those caused by exposure to ionic cadmium at different time points. LIMMA analysis evidenced a great increase in the number of transcripts significantly regulated at 21 days respect to 3 days of exposure to both forms of cadmium. This strong alteration of the hepatic transcriptome is in agreement with the results described in the previous chapter. At the end of the 21 days of exposure to both forms of cadmium, a significant metal accumulation was measured in whole organisms and high mortality was registered in both groups (78% in fish exposed to ionic cadmium and 72% in fish exposed to CdS NPs). Moreover, the effect on the hepatic transcriptome was stronger in animals exposed to ionic cadmium than in those exposed to the NPs, which was also reflected in biomarker responses described in the previous chapter. For instance, the quantification of the immunoreactive bands corresponding to free ubiquitin or to carbonylated actin showed a significant effect only after the exposure to ionic cadmium, especially at 21 days of exposure (Chapter IV).

According to the Bast2GO analysis, after 3 days no GO terms were enriched, but after the exposure to both, CdS NPs and ionic cadmium, for 21 days similar GO terms were enriched respect to the control group. The Fisher exact test analysis performed with the GO terms enriched after each treatment showed some over-represented terms in fish exposed to ionic cadmium in comparison to animals exposed to CdS NPs for 21 days. This result could be related to the higher concentration of dissolved cadmium in the exposure to ionic cadmium than in the exposure to CdS NPs. Characterization data of the CdS NPs used in this study showed that only 13% of cadmium was dissolved after 7 days (Katsumiti et al., 2014). Among the enriched terms when both treatments were compared “mitochondrial ion homeostasis” appeared over-represented. The entrance of cadmium in the cytosol may generate free radicals, through the depletion

of endogenous intracellular radical scavengers, such as protein sulfhydryls, as a consequence of their reaction with Cd^{2+}, affecting the redox status of the cell and hence the cellular levels of redox active species, or can lead to damage in organelles such as mitochondria (Thévenod, 2009). Moreover, the hereby altered term may indicate a disruption of the mitochondrial calcium equilibrium. It has been previously reported that the exposure to cadmium can produce an increase in calcium ion levels in the cells which can lead to apoptosis by stimulating the generation of reactive oxygen species (ROS), suggesting that calcium ion play important roles in cadmium-induced toxicity through the generation of ROS (Son et al., 2010). Moreover, the increase in the generation of ROS may also contribute to the disruption of the cytoskeleton after the exposure to cadmium (Williams et al., 2006). The internal cytoskeleton of eukaryotic cells is composed of actin microfilaments, microtubules, and intermediate filaments and the exposure to cadmium may provoke dysregulation of actin microfilaments and intermediate filaments. In the present study, GO processes involved in cytoskeleton such as “intermediate filament cytoskeleton” or “actin binding” were also over-represented after the exposure to ionic cadmium compared with the exposure to CdS NPs. Cd competes with intracellular Ca^{2+} for protein binding sites and may affect actin filaments (DalleDonne et al., 1997). As previously mentioned, increased oxidative stress provoked by the exposure to ionic cadmium was also detected by western blot analysis. A significant increase in the optical density of the immunoreactive band corresponding to carbonylated actin was observed after the exposure to ionic cadmium for 21 days (Chapter IV). Carbonylation of proteins, especially carbonylation of actin as a target protein, have been thoroughly reported in the literature for the exposure to cadmium (McDonagh and Sheehan, 2006; Chora et al., 2010).

Along with the mentioned GO processes, Fisher exact test also reported immune response related terms over-represented after the exposure to ionic cadmium respect to the CdS NPs exposure for 21 days. Cadmium is known to provoke the dysregulation of the immune system in different fish species such as yellow perch, European flounder or carp, even after the exposure to low concentrations of cadmium (Reynders et al., 2006; Williams et al., 2006; Auslander et al., 2008; Bougas et al., 2013). GO

terms corresponding to biological processes such as “immune system process” or “antigen processing and presentation”, along with the cellular component “MHC protein complex” and “MHC class I protein complex” appeared over-represented in the present study. Mayor histocompatibility complex (MHC) I glycoproteins are present in almost every cell of the body and are involved in the response to endogenous antigens originating from the cytoplasm (Hewitt, 2003). In rare minnow (*Gobiocypris rarus*) waterborne exposed to ionic cadmium (75 µg/L) for 35 days, up-regulation of genes involved in MHC I complex was detected by a gill microarray (Wang et al., 2016). In addition, they detected down-regulation of MHC II complex, which also conducts antigen processing signaling other than MHC I complex, although in an opposite manner. The regulation of both complexes might suggest that the anti-virus ability of fish cells declined after the exposure to cadmium (Wang et al., 2016).

The COA analysis showed all the treatments separated, being the variability mainly explained by the exposure time and, in a lower extent, by the metal form. This significant time effect was reflected in the amount of KEGG pathways significantly altered after 21 days of exposure to any of the treatments, but especially after the exposure to ionic cadmium, since at 3 days of exposure no KEGG pathways were affected. Differences in the response of the hepatic transcriptome to cadmium depending on the exposure time have been also reported by Williams et al. (2006) in European flounder (*Platichthys flesus*) injected with 50 µg Cd/Kg body weight. Injection of cadmium provoked the induction of genes involved in the response to oxidative stress, protein synthesis, transport and degradation terms according to the Blast2GO gene ontology analysis. Moreover, they detected disruption of the cell cycle and apoptotic processes, as well as repression of cytokines genes involved in immune responses. Major transcription changes were reported after 1 day, less at middle time points (2, 4 and 8 days) and increased alteration at 16 days post-injection (Williams et al., 2006). In the present work, few KEGG pathways appeared significantly altered after the exposure to CdS NPs for 21 days. Among them, “adherens junction” term presented most of the genes significantly up-regulated, such as *ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2) (ras2), cell division cycle 42 like 2 (cdc421l), ras homolog gene family, member Aa (rhoaa) and ras*

homolog gene family, member Ab (*rhoab*). Rho proteins are a subfamily of Ras proteins, involved in several pathways such as cell adhesion and, therefore, a dysregulation of their activities provoke diverse aberrant cellular phenotypes (Malliri and Collard, 2003). Cadmium is known to provoke the up-regulation of important proteins of the RAS signaling pathway implicated in cell proliferation, as well as the differentiation and the induction of diverse proto-oncogenes, which promote the proliferation of cells and development of tumors (Bertin and Averbeck, 2006).

The Fisher exact test analysis showed that GO terms related to immune response were differentially enriched after the exposure to ionic cadmium in comparison to the exposure to the nanoparticulated form, but the functional analysis showed that the KEGG pathway "natural killer cell mediated cytotoxicity" was significantly regulated after the exposure to CdS NPs for 21 days. Most of the genes of this pathway were up-regulated. Effects of the immune response after exposure to Cd containing QDs have previously reported in rainbow trout exposed to 1, 2 and 6 μg/L of CdS/CdTe QDs for 48 h (Gagné et al., 2010), who observed a depression of the immune system determined by leukocyte counts, viability and resting/active phagocytic activity. Moreover, using a cDNA microarray analysis after the exposure to CdS/CdTe QDs they found 25 genes significantly affected, which were involved in the immune responses mentioned above (Gagné et al., 2010).

The functional analysis performed also showed diverse KEGG pathways related to the increased oxidative stress caused by the exposure to CdS NPs for 21 days. As previously mentioned, the exposure to cadmium may provoke an increase in the oxidative stress in the liver cells of fish (Liu et al., 2009). As a mechanism to neutralize it, organisms increase hepatic vitamin A concentrations, which may provide a protection against the oxidative stress (Defo et al., 2012). The functional analysis revealed that the pathway "retinol metabolism" appeared significantly regulated after the exposure to CdS NPs for 21 days. In a previous study, using a cDNA microarray on adult yellow perch (*Perca flavescens*) exposed to ionic cadmium (0.8 and 3.65 μg/L) for 6 weeks, the photoreceptor associated *retinol dehydrogenase type 2*, gene involved in retinoid metabolism, was up-regulated at the highest cadmium exposure. Furthermore, a global analysis of gene expression patterns revealed that genes

involved in vitamin binding were up-regulated in the liver of Cd-exposed fish (Bougas et al., 2013).

In the present study the KEGG pathway "selenoamino acid metabolism" appeared significantly altered by the exposure to ionic cadmium for 21 days. Most of the genes contributing to the regulation of this pathway were significantly up-regulated, such as *seleno-phosphate synthetase 2* (*sps2*), which is the key component of the selenoamino acid metabolic pathway, producing the selenocysteine necessary for some antioxidant proteins (Williams et al., 2006). This pathway appeared significantly altered also after the exposure to CdS NPs, presenting similar significant gene regulation. Also, "drug metabolism" pathway was significantly regulated after the exposure to both forms of cadmium for 21 days, with a large number of genes significantly up-regulated after the exposure to ionic cadmium and only one gene significantly down-regulated. Among them, *cytochrome P450, family 3, subfamily A, polypeptide 65* (*cyp3a65*) or *glutathione S-transferase pi 2* (*gstp2*), which are key genes for the detoxification of xenobiotics, or alcohol dehydrogenase 5 (*adh5*), a member of a family of dehydrogenase enzymes that metabolizes a wide variety of alcohols, appeared significantly up-regulated. The up-regulation of these genes has been previously reported in studies performed in aquatic environments polluted by metals and organic compounds using diverse fish species such as *Catostomus macrocheilus* or *Anguilla anguilla* (Pujolar et al., 2013; Christiansen et al., 2014).

In addition to the KEGG pathways mentioned above, the exposure to ionic cadmium for 21 days mostly down-regulated transcripts that significantly affected KEGG pathways involved in DNA damage and repair. The pathways "purine metabolism" and "pyrimidine metabolism" are involved in the synthesis of the nucleotides required for DNA replication. Similarly, genes contributing to the KEGG pathways significantly affected such as "nucleotide excision repair", "mismatch repair" or "homologous recombination", which are involved in DNA repair processes, or "RNA polymerase" and "spliceosome", which are involved in RNA processes, were significantly down-regulated. The exposure to cadmium has been associated with the inhibition DNA repair-related proteins, which could lead to mutagenic and carcinogenetic effects in diverse organisms (Giaginis et al., 2006; Candéais et al., 2010). Previous studies have

reported the carcinogenicity of cadmium in zebrafish through the analysis of genes involved in these processes (Gonzalez et al., 2006; Vergauwen et al., 2013; Arini et al., 2015). In addition to DNA repair processes, other response to DNA damage is the regulation of cell cycle progression by activation of DNA damage checkpoints (Hartwing et al., 2002). In this study, genes contributing to "cell cycle" were significantly affected, being most of them significantly down-regulated. Inhibition of DNA repair processes and alterations in the cell cycle process were previously detected after exposure to low concentrations of ionic cadmium (Hartwing et al., 2002; Williams et al., 2006). Although the study of the liver transcriptome detected this alteration, clastogenic DNA damage in erythrocytes was not detected by the micronuclei frequency test, but one individual exposed to ionic cadmium for 21 days presented megalocytosis in the liver (Chapter IV).

Different pathways involved in the energetic metabolism also appeared significantly enriched after the exposure to ionic cadmium for 21 days. "Glycolysis/gluconeogenesis", "pentose phosphate pathway", "citrate cycle (TCA cycle)", "biosynthesis of unsaturated fatty acids" were significantly affected, presenting most of the genes significantly up-regulated. An increase in energetic metabolic processes has been previously reported after the exposure to environmentally relevant or low concentrations of cadmium in different fish species such as *Perca flavescens* or *Cyprinus carpio* (De Smet and Blust, 2001; Levesque et al., 2002; Reynders et al., 2006). An increase in the energetic metabolism is associated to an increase of the participation of proteins in the energy metabolism in response to an increased energy demand to cope with the stress situation (De Smet and Blust, 2001). Alteration of the energetic metabolism was also observed at histological level. Increase of vacuolization in liver samples after exposure to ionic cadmium for 21 days and at the end of the 6 months recovery period have been described in the previous chapter (Chapter IV).

CONCLUSIONS

The exposure to CdS NPs and to ionic cadmium provoked alteration of the zebrafish liver transcriptome. The effect greatly increased with exposure time and was more

pronounced after the exposure to ionic cadmium than after exposure to CdS NPs. The functional analysis showed a significant effect in the immune response and oxidative stress after the exposure to CdS NPs for 21 days, while the exposure to ionic cadmium affected significantly those pathways involved in DNA damage and repair and in the energetic metabolism. Therefore, the analysis of the liver transcriptome under our experimental conditions reveals a different effect in zebrafish depending on the cadmium form.

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CHAPTER VI

Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimps exposed to PVP/PEI-coated silver nanoparticles to zebrafish

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- E Bilbao, **JM Lacave**, A Fanjul, MP Cajaraville, A Orbea "Transcriptomic and cellular effects of dietary exposure of zebrafish to silver nanoparticles". Poster.
- A Orbea, **JM Lacave**, N Gonzalez-Soto, I Barrio, MP Cajaraville "Low concentrations of silver nanoparticles impair zebrafish reproduction and embryo development". Platform presentation.

ABBREVIATIONS

BSDs, Black silver deposits

CI, Confidence intervals

EC_{50}, Effective concentration to 50% of the population

FET, Fish embryo toxicity

HD, High dose

hpf, Hours post fertilization

hph, Hours post hatch

H/E, Hematoxylin/eosin

LC_{50}, Lethal concentration to 50% of the population

LD, Low dose

LMS, Lysosomal membrane stability

LP, Labilization period

NPs, Nanoparticles

OR, Odd ratio

PEI, Polyethylenimine

PVP, Poly N-vynil-2-pirrolidone

QDs, Quantum dots

Vv, Volumen density

ABSTRACT

Silver nanoparticles (NPs) are extensively used due to their antimicrobial activity and, therefore, their input into the ecosystem will increase. Silver can be bioaccumulated by low trophic level organisms and, then, incorporated into the food chain, reaching high level predators. The objectives of this study were to test the acute toxicity of poly N-vynil-2-pirrolidone/polyethylenimine (PVP-PEI) coated Ag NPs of 5 nm to brine shrimp (*Artemia sp*) larvae and to zebrafish (*Danio rerio*) embryos and to assess bioaccumulation and effects of silver transferred by the diet. For the later, brine shrimps were exposed to two different concentrations of Ag NPs, 100 ng/L as an environmentally relevant concentration and 100 µg/L as a likely effective concentration, in parallel with an unexposed control group and, then, used to feed zebrafish during 21 days in order to simulate two trophic levels of a simplified food web. For brine shrimp larvae, EC_{50} values ranged from 7.39 mg Ag/L (48 h post hatch larvae (hph) exposed for 48 h) to 19.63 mg Ag/L (24 hph larvae exposed for 24 h), while for zebrafish embryos LC_{50} value at 120 h was 0.052 mg Ag/L. Silver accumulation was measured in brine shrimps exposed to 0.1 and 1 mg/L of Ag NPs for 24 h. In zebrafish fed with brine shrimps exposed to Ag NPs, intestine showed higher metal accumulation than liver, although both organs presented the same pattern of dose and time-dependent metal accumulation as revealed by autometallography. Feeding of zebrafish for 3 days with brine shrimps exposed to 100 ng/L of Ag NPs was enough to impair fish health as reflected by the significant reduction of the lysosomal membrane stability and the presence of several histopathological conditions in the liver. Overall, results showed that Ag NPs were able to exert toxic effects on zebrafish through dietary exposure, even at an environmentally relevant concentration, which should act as concern of the need of studies in further detail about real impact of nanomaterials in the environment.

Keywords: Ag NPs, zebrafish, brine shrimp, dietary transfer, bioaccumulation, cellular effects

LABURPENA

Zilarrezko nanopartikulak (NPak) zabalki erabiliak izaten ari dira erakusten duten mikrobioen kontrako jarduerari esker eta, ondorioz, ekosistemetarako sarrera areagotzen ari da. Zilarra, maila trofiko baxueneko organismoetan metatu eta kate trofikoan sar daiteke, maila altuagoetako harraparietara iritsiz. Lan honen helburuak poli-N-vinil-2-pirrolidona/polietileniminaz (PVP-PEI) gaineztatutako 5 nm-ko Ag NPek artemia (*Artemia sp*) larbetan eta zebra arrain (*Danio rerio*) enbrioietan duten toxikotasun azkarra aztertzea, biometaketa neurtzea eta dietaren bidezko zilarraren transferentziaren ondorioak aztertzea izan ziren. Horretarako, artemiak bi Ag NP kontzentrazio desberdinen eraginpean jarri ziren, 100 ng/L, ingurumeneko kontzentrazioa izan daitekeena eta 100 µg/L kontzentrazio eraginkora izan daitekeena eta, paraleloki, esposatu gabeko kontrol talde bat jarri zen. Gero, artemiak arrainak 21 egunez elikatzeko erabili ziren bi mailako kate trofiko sinple bat itxuratuz. Artemia larbentzako, EC_{50} baloreak 7.39 mg Ag/L (48 orduz esposaturiko 48 ordutako larbak) eta 19.63 mg Ag/L (24 orduz esposaturiko 24 ordutako larbak) artekoak izan ziren. Zebra arrain enbrioietan aldiz, 120 ordutara LC_{50} balorea 0.052 mg Ag/L izan zen. Zilar metaketa, 24 orduz 0.1 eta 1 mg/L Ag NP-en pean jarritako artemietan neurtu zen. Autometalografia bidez ikus zitekeenez, Ag NPen eraginpean jarritako artemiez elikatutako zebra arrainetan, hesteak gibelak baino metal metaketa altuagoa erakusten zuen, nahiz eta bi organoek dosi eta denbora menpeko patroi berdina erakutsi zuten. Zebra arrainak, 3 egunez 100ng/L Ag NPen eraginpean izandako artemiez elikatzea nahikoa izan zen arrainen osasuna kaltetzeko, lisosomen mintzaren egonkortasunaren murrizketa esangarriak islatu zuenez. Oro har, gure lanaren emaitzek agerian uzten dute, Ag NPek eragin toxikoak izan ditzaketela janariaren bidez esposaturiko zebra arrainetan, ingurumenean aurki daitezkeen kontzentrazioetan. Guzti honek, nanomaterialek ingurugiroan duten inpaktuari buruzko ikerketa xeheen beharra islatzen du.

Gako-hitzak: Ag NPak, zebra arraina, artemia, dieta bidezko transferentzia, biometaketa, zelulen gaineko efektuak

RESUMEN

Las nanopartículas (NPs) de plata están siendo ampliamente utilizadas debido a su actividad antimicrobiana y, por tanto, aumentará su entrada en los ecosistemas. Los organismos de los niveles tróficos más bajos pueden bioacumular plata e incorporarla en la cadena trófica, llegando a los depredadores de los niveles más altos. Los objetivos de este estudio fueron testear la toxicidad aguda de NPs de Ag de 5 nm cubiertas de poli N-vinil-2-pirrolidona/polietilenimina (PVP-PEI) en larvas de artemia (*Artemia sp*) y en embriones de pez cebra (*Danio rerio*) y evaluar la bioacumulación y los efectos de la transferencia de plata a través de la dieta. Para ello, se expusieron las artemias a dos concentraciones diferentes de NPs de Ag, 100 ng/L como una concentración ambientalmente relevante y 100 µg/L como una concentración potencialmente efectiva, en paralelo con un grupo control no expuesto. Estas artemias se utilizaron para alimentar a los peces cebra durante 21 días simulando una cadena trófica simple de dos niveles. Para las larvas de artemia, los valores de EC_{50} obtenidos oscilaron entre 7.39 mg Ag/L (larvas de 48 h expuestas durante 48 h) y 19.63 mg Ag/L (larvas de 24 h expuestas durante 24 h), mientras que para los embriones de pez cebra el valor de LC_{50} a las 120 h fue de 0.052 mg Ag/L. Se registró acumulación de plata en artemias expuestas a 0.1 y 1 mg/L de NPs de Ag durante 24 h. Mediante autometalografía se observó que los peces cebra alimentados con artemias expuestas a NPs de Ag acumularon mayor cantidad de metal en el intestino que el hígado, aunque en ambos órganos se observó el mismo patrón de acumulación de metal dependiente de la dosis y del tiempo. Tres días de dieta con artemias expuestas a 100 ng/L de NPs de Ag fue suficiente para alterar la salud general de los peces, según se reflejó en la reducción significativa del tiempo necesario para desestabilizar la membrana lisosómica y por la presencia de diversas histopatologías en el hígado. En general, los resultados de este trabajo mostraron que la exposición a NPs de Ag a través de la dieta fue capaz de provocar efectos tóxicos en el pez cebra, incluso a concentraciones ambientalmente relevantes, lo cual indica la necesidad de llevar a cabo estudios más detallados sobre el impacto real de los nanomateriales en el medio ambiente.

Palabras clave: NPs de Ag, pez cebra, artemia, transferencia por la dieta, bioacumulación, efectos celulares

INTRODUCTION

Due to their antibacterial activity, silver nanoparticles (Ag NPs) are one of the most widely nanomaterials used, with a continuously growing production. This will lead to an increasing entry of silver into the environment (Yin et al., 2015). The current concentration of Ag NPs in the environment remains unknown, because of the lack of sensitive analytical methods to distinguish different metal forms in complex environmental matrices (Sun et al., 2014). Nevertheless, several studies have estimated the potential concentration of Ag NPs in diverse environmental compartments, using mathematical models (Blaser et al., 2008; Gottschalk et al., 2009; Tiede et al., 2009; Fabrega et al., 2011; Chio et al., 2012; Hendren et al., 2013; Markus et al., 2013; Dumont et al., 2015). These studies have reported values ranging from 0.002 ng/L in the surface water of European rivers (Dumont et al., 2015) up to 40 μg/L in effluents of Taiwanese rivers (Chio et al., 2012).

Despite our limited knowledge on the fate and impact of Ag NPs in the environment, previous data on environmental and physiological implications of exposure of aquatic organisms to different silver compounds provides a baseline for the assessment of the potential effects of Ag NPs to the aquatic ecosystem (Fabrega et al., 2011). Two different routes of entrance of Ag NPs into the organisms have been defined, through the respiratory system since the gills are directly exposed to the NP suspended in the water column, and through the diet in animals that feed suspended matter or other organisms previously exposed to NPs (Schirmer et al., 2013). Previous studies have mainly focused on the effects provoked by waterborne exposure to Ag NPs (Aruvalsu et al., 2014; Katuli et al., 2014; Massarsky et al., 2014; Osborne et al., 2015), while few studies have been carried out addressing dietary exposure. Merrifield et al. (2013) fed zebrafish (*Danio rerio*) with artificial food containing Ag NPs for 14 days and reported a toxic effect in the zebrafish microbiome which provoked changes in the digestive system function and organism health. Other authors have also reported toxic effects in fish fed with artificial food containing nanomaterials. Blickley et al. (2014) fed the estuarine fish *Fundulus heteroclitus* with diets containing 1 or 10 μg of lecithin-encapsulated CdSe/ZnS QD (Quantum dots)/day for 85 days detected cadmium bioaccumulation in the liver. Ladhar et al. (2014) also detected cadmium

bioaccumulation in the liver of zebrafish after 36 and 60 days and in brain and muscle after 60 days of exposure through contaminated artificial food with CdS NPs. These authors also reported genotoxicity and oxidative stress after the experimental period. The dietary transfer of metal can also occur from one organism to another, which may lead to bioaccumulation and biomagnification along the food web and to provoke a long term negative impact on the ecosystem functions (Pakrashi et al., 2014). In order to study a possible transfer of NPs among aquatic organisms, diverse studies have tried to simulate a simply food chain using only two trophic levels. In some cases, the primary producer (phytoplankton) is exposed to NPs and used to feed the primary consumer, such as zooplankton (Pakrashi et al., 2014; Lee et al., 2015). In both studies an effective NP transfer, Al_2O_3 and Au NPs respectively, between species was detected. In the other cases, the primary consumer (a crustacean) is exposed to NPs and used to feed the secondary consumer (fish), allowing to study biomagnification and toxic effects in the predator (Zhu et al., 2010; Lewinski et al., 2011). In these studies TiO_2 NPs transfer and uptake of CdSe/ZnS QDs, were also detected, although biomagnification was not recorded after the dietary exposure.

In the present study, brine shrimps (*Artemia sp*), as the primary consumer, and zebrafish, as the secondary consumer, were selected with the aim of studying silver bioaccumulation and effects provoked by the exposure to Ag NP through the food web. Brine shrimps, which serve for feeding many different fish species in culture, have been commonly used in ecotoxicological testing because of their capacity to adapt to different environments, and they are starting to be considered as a new biological model in nanoecotoxicology (Libralato, 2014). Zebrafish is a well established animal model for testing toxicological effects (Hill et al., 2005), being thoroughly used as a model for assessing the toxicity of nanomaterials (Bohnsack et al., 2012).

The acute toxicity of the Ag NP suspension was analyzed in embryo/larvae stages of both species, according to standardized OECD test guidelines. For brine shrimps, the acute toxicity test was based on the standardized OECD test guidelines for *Daphnia sp* (OECD TG202, 2004) where immobilization is used as mortality criteria. In zebrafish, the fish embryo toxicity test (FET) OECD TG236 (2013) was used. In this test, four apical endpoints are recorded as indicators of acute lethality in fish: coagulation of fertilized

eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heart-beat (OECD TG236, 2013).

Previous to the dietary transfer experiment, silver bioaccumulation was measured in brine shrimps exposed to different Ag NP concentration and results were used to select the Ag NP concentration to expose brine shrimps. Then, the potential metal accumulation and effects of silver transfer through dietary exposure was analyzed using brine shrimps exposed to two different Ag NP concentrations, an environmentally relevant concentration and a likely effective concentration. A battery of biomarkers was selected to elucidate the effects provoked in zebrafish by the dietary exposure for 21 days to Ag NPs. Accumulation of silver in zebrafish was measured through chemical analysis and metal distribution was assessed in different organs quantifying the volume density of black silver deposits (Vv_{BSDs}) after autometallography. This method has been successfully applied to study the metal fate in fish exposed to metal salts (Alvarado et al., 2005; 2006), as well as to metal bearing NPs (Vicario-Parés et al., 2014; Chapter II; Chapter IV). General fish health status was study through the lysosomal membrane stability test, as lysosomes are the central site for sequestration of toxic metals (Köhler et al., 2002). Exposure to metals can result in an increased radical generation, resulting in oxidative damage to the membranes. This test has been previously applied in diverse aquatic organisms to detect the toxic effect provoked by different metal nanoparticles (Vicario-Parés, 2016; Jimeno-Romero, 2014; Chapter II; Chapter IV). The observation of histopathological lesions in target tissues, such as intestine and liver, is a direct indicator of contaminant effect and provides an appropriate indicator of the general health of individuals and populations exposed to contaminants (Davies and Vethaak, 2012). The intestine is one of the major target tissues as it is the main entrance of NPs into the organisms during the dietary exposure (Zhu et al., 2010; Piccinetti et al., 2014). In addition, the liver, as the principal organ involved in the detoxification of xenobiotics (Feist et al., 2004), is the other main target tissue for NP toxicity.

MATERIALS AND METHODS

Silver nanoparticles

Ag NPs were purchased to NANOGAP (A Coruña, Spain) as an aqueous dispersion containing 10 g/L of Ag NPs stabilized with poly-N-vynil-2-pirrolidone (PVP, Sigma-Aldrich, St. Louis, Missouri) and polyethylenimine (PEI, Sigma-Aldrich), being the concentration of the PVP-PEI mixture 104 g/L in a proportion of 77% PVP and 23% PEI. The NP size distribution measured by transmission electron microscopy was 5.08 ± 2.03 nm, the Z potential in distilled water measured by dynamic light scattering was +18.6 ± 7.9 mV at pH 8.43, according to the information provided by the supplier. Dissolution of the Ag NPs in artificial seawater has been previously described in Schiavo et al. (*in prep*). After 24 hours, PVP-PEI coated Ag NPs released around 20% of silver ions, increasing to 29.6% at 72 hours.

Brine shrimp culture and acute toxicity test

Cysts of brine shrimps (INVE Aquaculture, Salt Lake City, Utah, USA) were hatched and grown in reactors with artificial salt water (33‰ salinity). Brine shrimp cultures were maintained with continuous aeration and illumination in a temperature controlled room at 28ºC. After 24 h of hydratation, most cysts hatched and, then, they were maintained for other 24 or 48 h. Brine shrimp nauplii were collected using a mesh of 150 µm.

The acute toxicity of the Ag NP suspension to brine shrimps was tested following a procedure based on the standardized OECD TG 202 (2004) for *Daphnia magna*. The test was carried out in covered 24-well polystyrene microplates placing 5-7 brine shrimp nauplii of 24 or 48 h post hatch (hph) per well in 2 mL of exposure medium, at a temperature of 18.5 ºC and continuous illumination. Brine shrimps were exposed to five different dilutions of the Ag NP suspension containing 1, 2.5, 5, 7.5 and 10 mg Ag/L. The toxicity of the PVP-PEI mixture alone was assayed in parallel by exposing the brine shrimps in the same conditions to the equivalent concentrations present in the dilutions of the NP suspension. An unexposed control group was also run. The test was considered as valid only when survival rate in the control group was ≥ 90% (OCDE TG

202, 2004). At 24 and 48 hours of exposure brine shrimps were examined to determine mortality (percentage of immobilized larvae).

Zebrafish culture and embryo toxicity test

Zebrafish (wild type AB Tübingen) individuals were grown in a temperature-controlled room at 28ºC with a 12-hour light/12-hour dark cycle in 100 L tanks provided with mechanic and biological filters. Water was in continuous movement triggered by the action of an aeration siphon. Water was previously conditioned by passage through a deionization system and then buffered to pH 7.2 with Sera pH plus (Sera, Heinsberg, Germany) and to 600 µsΩ with commercial marine salt (Sera). Fish were fed twice a day with 24 hph live brine shrimps and Vipagran baby (Sera). Water chemical parameters were controlled once per week using Sera ammonium, nitrite and nitrate kits and water changed if the values were higher than zero mg/L for ammonium or nitrite and 50 mg/L for nitrate.

Breeding fish were selected and separated in a tank. Females and males were maintained separately, in order to avoid continuous spawning. The day prior to the beginning of the exposures, one female and one male zebrafish were placed separately in each breeding tramp which had previously been located in a 2 L tank containing conditioned water. Fish were left overnight and, just before the light switched on in the morning, the separation was removed. The resulting eggs were collected in a Petri dish and fertilized viable eggs were selected under a stereoscopic microscope (Nikon smz800, Kanagawa, Japan). During the procedure of embryo selection, water salinity was reduced gradually up to MilliQ water. Finally, fertilized eggs were transferred to the exposure microplates.

The FET test was carried out following the OECD TG236 (2013) and the experimental setup described in Chapter I. Zebrafish embryos were exposed to different dilutions of the Ag NP suspension containing from 0.01 to 10 mg/L, as well as to the equivalent concentration of the PVP-PEI mixture present in the dilutions of the NP suspension. These concentrations were selected according to the expected toxicity based on previous studies carried out in our laboratory (Chapter I). Exposure started just after selection of the fertilized embryo and lasted up to 120 h post fertilization (hpf). The

test was considered as valid only when survival rate in the control group was ≥ 90% (OECD TG236, 2013).

Daily and up to the end of the test, embryos were examined to determine survival rate, hatching rate, hatching time and malformation prevalence. Normal embryo morphology was based on Kimmel et al. (1995). Malformations were recorded and photographed under a Nikon AZ100 (Tokyo, Japan) stereoscopic microscope.

Exposure of zebrafish through contaminated brine shrimps

For the establishment of the brine shrimp exposure concentrations to be used in the dietary experiment, Instar I brine shrimps (24 hph) cultures were previously waterborne exposed to Ag NP suspension for 24 h to five different concentrations of Ag NPs (0.1, 1, 10, 100 and 1000 µg Ag/L). Once the two concentrations were selected, new brine shrimp (24 hph) cultures were daily exposed to obtain a continuous stock to be used to feed the zebrafish during the experimental period.

The experimental procedure involving adult zebrafish described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. During the experimental period, zebrafish of approximately 1 year old were kept in 35 L aquaria containing 55-75 fish. These aquaria were equipped with biological filters and air pumps for water aeration and recirculation. Water chemical parameters were controlled as indicated above. Adult zebrafish were fed daily for 21 days with the brine shrimps exposed for 24 h to the Ag NP concentrations selected. A control group fed with unexposed brine shrimps was run in parallel in identical experimental conditions. The daily amount of feeding was set to 2.5% of fish body weight (Blanco-Vives and Sánchez-Vázquez, 2009; Lawrence et al., 2012) distributed in two doses.

During the experimental period, fish samples were taken at 3 or 21 days of exposure depending on the endpoint, after euthanasia by overdose of anesthetic (ethyl 4-aminobenzoate, Fluka, Steinheim, Germany). In addition, during the experimental period samples from the brine shrimps cultures were collected for chemical analysis at

four different days (1st, 7th, 14th, and 21st), and were used to quantify the silver content.

Metal accumulation: chemical analyses

Brine shrimp nauplii were collected from the cultures using a 150 μm mesh and the resulting samples were introduced into pre-weighted 25 mL Erlenmeyer flasks and weighted again. Then, flasks were placed into a 130 ºC oven overnight, and weighted again in order to calculate the dry weight of the brine shrimp samples. Then, samples were digested using 6 mL of aqua regia, prepared as 25% nitric acid (65% extra pure quality, Scharlau, Barcelona, Spain) and 75% hydrochloric acid (36%, Tracepur®, Scharlau). The mouth of the Erlenmeyer flasks was blocked with a crystal ball to minimize evaporation. After finishing the digestion of the sample, the remnant liquid was evaporated in an 80 ºC hot plate inside an exhaust hood. After evaporation, 2.5 mL aqua regia was added to each flask and stored at 4 ºC. Finally, silver content was measured by inductively coupled plasma mass spectrometry (ICP-MS, 7700x, Agilent Technologies, California, USA) following the US-EPA 6020A directions Detection limit was established at 0.01 μg/L.

For chemical analysis of zebrafish tissue, 20 individuals per experimental group were collected, frozen individually in liquid nitrogen and stored at -80 ºC. Whole zebrafish were dried in an oven at 130 ºC for 24 h. Dry tissues were weighted, pooled (five pools of four zebrafish each) and placed into 25 mL Erlenmeyer flasks, and processed as described for brine shrimps. The content of each flask was then transferred into tubes and centrifuged for 4 min at 2000 rpm (Heraeus Labofuge 200 centrifuge, Hanau, Germany). The supernatants were moved to clean tubes and stored at 4 ºC. Finally, silver content was measured by ICP-MS (7700x, Agilent Technologies) following the US-EPA 6020A directions. Detection limit was established at 0.01 μg/L.

Histological preparations

The visceral masses of 10 fish per experimental group were dissected after 21 days of dietary exposure. Tissues were placed in histological cassettes and immersed in 10% neutral buffered formalin for 24 h at 4 ºC. Then, samples were transferred to 70%

ethanol and stored at 4 ºC until complete tissue processing under vacuum conditions in an ASP300 Tissue Processor (Leica Microsystems, Nussloch, Germany). Paraffin blocks were done using plastic molds. Sections (5 µm thick) were cut in a RM2125RT microtome (Leica Microsystems) for autometallography and histopathological analysis. For the histopathological analysis and for localization of tissues of interest for the autometallographical staining, slides were stained with hematoxylin/eosin (H/E) in an Auto Stainer XL (Leica Microsystems) and mounted in DPX (Sigma-Aldrich) by means of a CV5030 Robotic Coverslipper (Leica Microsystems).

H/E stained histological sections of the visceral mass were examined under a BX51 light microscope (Olympus, Tokyo, Japan). Samples were specifically analyzed for the determination of the presence of histopathological alterations, such as inflammatory responses.

Cellular localization: autometallography

Paraffin sections were dewaxed in xylol (Fluka), hydrated in decreasing concentrations of ethanol and left until they were completely dry. A silver enhancement kit for light and electron microscopy (BBI Solutions®, Cardiff, UK) was used according to the manufacturer instructions. The reaction was stopped by rinsing the slides in tap water. Slides were mounted with Kaiser's glycerol gelatin (Sigma-Aldrich). The quantification of the volume density (Vv) of the developed black silver deposits (BSDs) in the intestine and liver tissues was done over five different sections of each sample. The observation was made using a Laborlux S microscope (Leica Microsystems) and quantification of Vv_{BSDS} was made by means of the Biological Measure System (BMS) software (Sevisan, Leioa, Spain).

Volume density (Vv) of goblet cells

Paraffin sections were obtained as described previously. Samples were immersed into an 1% Alcian blue (Sigma-Aldrich) pH 2.5 solution for 30 min, washed 2x30 s in deionized water, rinsed in tap water for 3 min, and dehydrated in a graded series of ethanol. Samples were mounted in DPX. Using a BX61 microscope (Olympus) equipped with a camera, two different microscopic fields on each sample were photographed at

10x magnification. Afterwards, using the Olympus CELL^D Software, a 50x50 μm^2 squared grid was superimposed onto the image. The number of the grid intersections over goblet cells and over intestine tissue was counted to calculate the Vv of the goblet cells over the entire intestine using the following stereological formula: nº of intersections over goblet cells / (nº of intersections over goblet cells + nº of intersections over intestine).

General health status: Lysosomal membrane stability (LMS)

The liver of 5 individuals per experimental group was dissected after 3 and 21 days of dietary exposure, embedded in Cryo-M-Bed (Jung, Heidelberg, Germany) and frozen in liquid nitrogen. Frozen tissue sections (10 µm) were obtained in a CM3050S cryotome (Leica Microsystems) at a cabinet temperature of -24 ºC. The determination of LMS was based on the method used by Bröeg et al. (1999) as the time of acid labilization treatment required to produce the maximum staining intensity in hepatocyte lysosomes after demonstration of acid phosphatase activity. Time intervals used for acid labilization were 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min according to Bilbao et al. (2010).

Labilization period (LP) was determined under an Olympus BX51 light microscope as the maximal accumulation of reaction product associated with lysosomes (Bröeg et al., 1999). Four determinations were made for each individual liver by dividing each section in the acid labilization sequence into 4 approximately equal segments. A mean value was then obtained for each section, corresponding to an individual fish.

Statistical analyses

Data recorded in both species from the acute toxicity tests were statistically analyzed by binomial logistic regression. This analysis allowed calculating the odds ratios in order to estimate and to compare the risk associated to silver exposure for the two species. EC_{50}/LC_{50} values were calculated using a Probit model. Estimation of parameters was performed using the penalized maximum likelihood proposed by Firth (Firth, 1993), whenever convergence was not obtained using the maximum likelihood method (Kosmidis, 2013). Data from the dietary transfer experiment were statistically

analyzed by multivariate general linear regression models. All the analyses were performed using R 3.1.0.

RESULTS

Acute toxicity test: brine shrimps

After 24 h of exposure, significant effects on the surviving rate were recorded only for the 48 hph brine shrimps exposed to 10 mg Ag/L (Fig 1C), while no significant effects were recorded for the 24 hph brine shrimps (Fig 1A). After 48 h of exposure to the Ag NP suspension, a concentration dependent effect was detected for both the 24 hph and the 48 hph brine shrimps (Fig 1B, 1D). Significant effects were recorded at 5, 7.5 and 10 mg Ag/L for 48 hph brine shrimps and at 7.5 and 10 mg Ag/L for 24 hph brine shrimps. At 10 mg Ag/L, the percentage of surviving individuals decreased to 55% for the group of 24 hph brine shrimps and to 21.7% for the group of 48 hph brine shrimps. This decrease was also reflected in the odd ratios values which indicate the increase in the risk of mortality in brine shrimps exposed to 10 mg Ag/L respect to the control group, with a value of 54.457 (confidence interval at 95% (CI) 6.628 - >999.99) in 24 hph brine shrimps and a value of 70.239 (CI: 19.445 - 384.71) in 48 hph brine shrimps. Exposure to the PVP-PEI mixture alone did not cause any significant effect in any of the assayed conditions (Fig 1). Calculated EC_{50} values and their confidence intervals at 95% are shown in Table 1.

Table 1.- EC_{50} and LC_{50} values and confidence intervals at 95% (in brackets) for the compounds tested in this study with brine shrimp nauplii and zebrafish embryos.

	Brine shrimps nauplii (EC_{50})				**Zebrafish embryos (LC_{50})**
	24 hph		**48 hph**		**120 hpf**
	24 h of exposure	**48 h of exposure**	**24 h of exposure**	**48 h of exposure**	
Ag NP suspension (mg Ag/L)	19.63 (3.81 - 35.45)	10.24 (8.96 - 11.52)	16.52 (10.83 - 22.21)	7.39 (6.63 - 8.15)	0.057 (0.051 - 0.062)
PVP-PEI mixture (mg PVP-PEI/L)	277.07 (24.13 - 530)	500.73 (-486.77 - 1488.23)	206.73 (75.5 - 337.95)	212.7 (69.16 - 356.24)	5.42 (3.93 - 6.70)

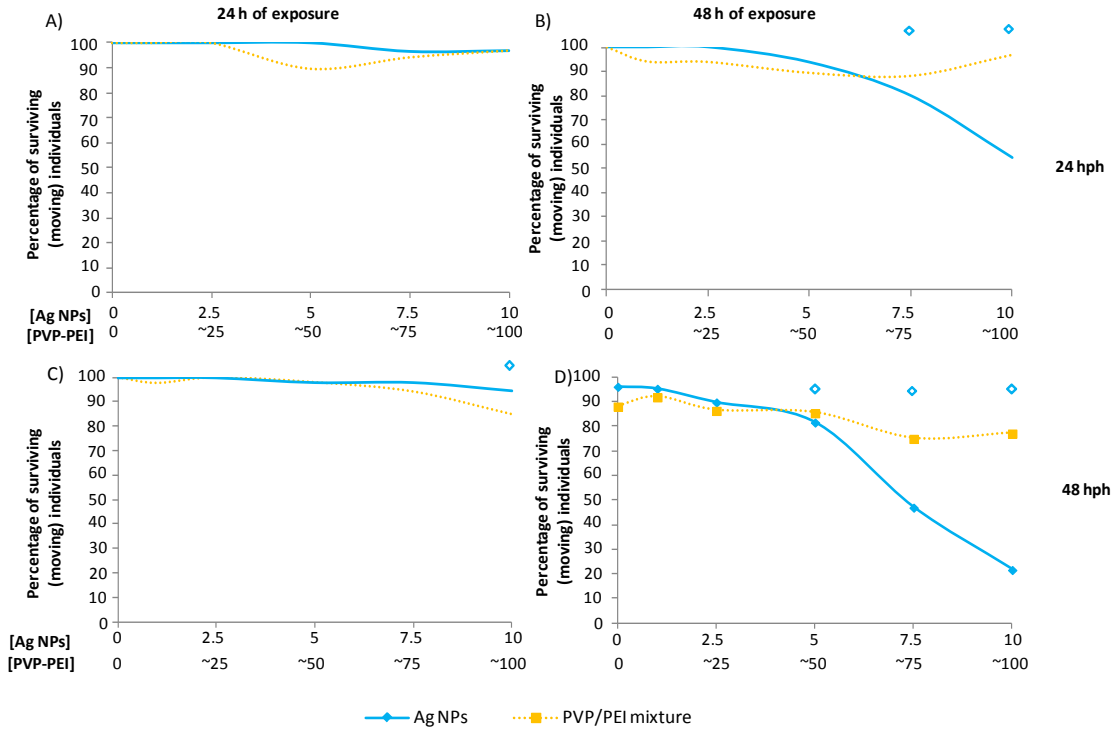

Figure 1.– Effects on survival of the exposure of brine shrimp nauplii to different dilutions of the Ag NP suspension and the equivalent concentration of the PVP-PEI mixture present in the dilutions of the NP suspension. (A) 24 hph brine shrimps exposed for 24 h; (B) 24 hph brine shrimps exposed for 48 h; (C) 48 hph brine shrimps exposed for 24 h; (D) 48 hph brine shrimps exposed for 48 h. The empty symbols indicate significant differences (p<0.05) respect to the control group.

Acute toxicity test: zebrafish

The Ag NP suspension at concentrations≥ 0.1 mg Ag/L caused 100% of mortality after 24 h (Fig 2A). These results did not allow calculating the LC_{50} value, since only zebrafish exposed to the lowest concentration survived. In order to be able to obtain a LC_{50} value, a new test was carried out in the range of concentrations between 100% and 0% survival (0.01-0.1 mg Ag/L) in the previous test. Significant effects in the survival rate at 120 hpf were observed after the exposure to ≥ 0.05 mg Ag/L (Fig 2B). 26.7% of the embryos exposed to 0.05 mg Ag/L and 76.7% of the embryos exposed to 0.075 mg Ag/L died after 48 hours, before hatching. The calculated LC_{50} value was 0.057 mg Ag/L (Table 1).

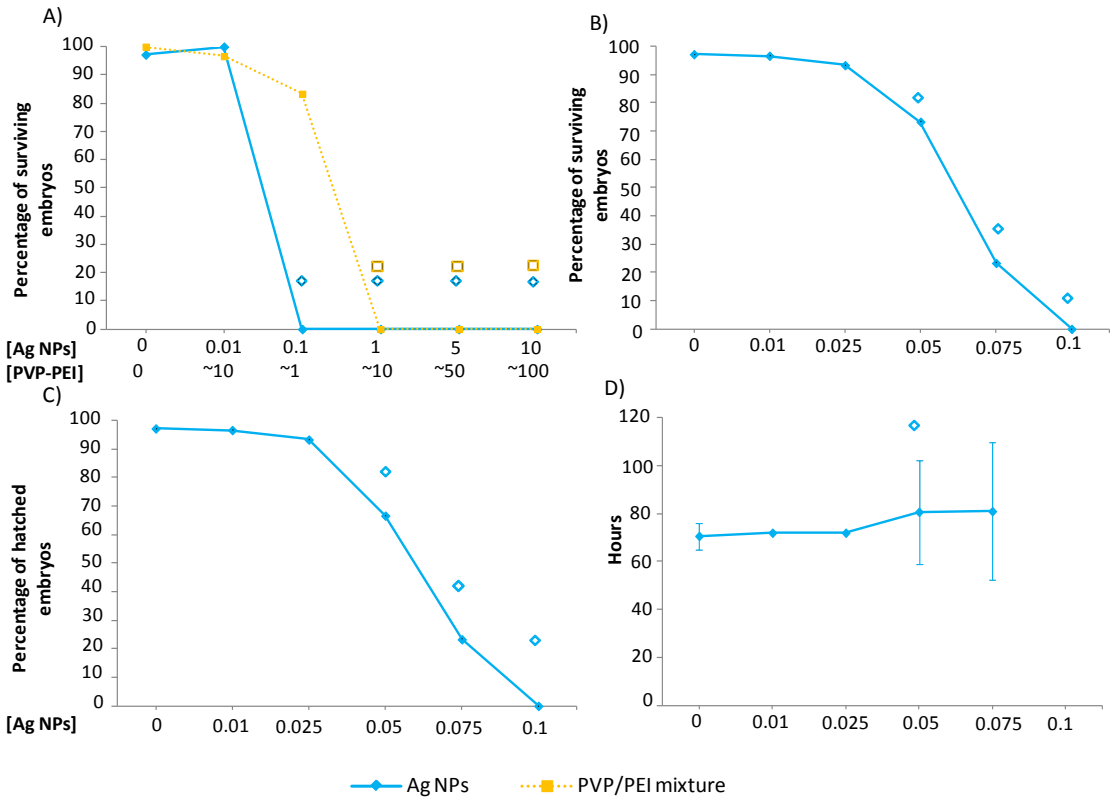

Figure 2 - Effects of the exposure of zebrafish embryos to different dilutions of the Ag NP suspension and the equivalent concentration of the PVP-PEI mixture present in the dilutions of the NP suspension for 120 h. (A and B) Survival rate; (C) hatching rate; (D) hatching time. The empty symbols indicate significant differences (p<0.05) respect to the control group.

Exposure to the PVP-PEI mixture at concentrations up to ~1 mg PVP-PEI/L, equivalent to the concentration present in the dilution of the NP suspension containing 0.1 mg Ag/L, did not cause any effect on embryos survival rate at 120 hpf. Exposure to concentrations ≥ 10 mg/L of the PVP-PEI mixture resulted in 100 % embryo mortality at 120 hpf. The survival time of embryos exposed to PVP-PEI was concentration-dependent. At ~10 mg PVP-PEI/L embryos died after hatching (> 72 hpf), at ~50 mg PVP-PEI/L embryos died at 48-72 hpf always before hatching, and at ~100 mg PVP-PEI/L embryos died at 24 h of exposure. The LC50 value obtained at 120 hpf was 5.42 mg PVP-PEI/L (Table 1).

Significant differences for hatching rate were observed in embryos exposed to 0.05 0.075 and 0.1 mg Ag/L (Fig 2C) and significant differences for hatching time were observed only in embryos exposed to 0.05 mg Ag/L, when a hatching delay respect to the control embryos was registered, as some embryos did not hatch during the exposure period (Fig 2D).

Exposure to the Ag NP suspension at 0.05 and 0.075 mg Ag/L provoked malformations in surviving embryos (Fig 3). Yolk sac edema, pericardial edema, tail flexure, spinal cord flexure and finfold abnormality were detected after the exposure to the Ag NP suspension (Table 2). Moreover, some malformations were observed in unhatched embryos of 72-96 hpf exposed to 10 and 50 mg PVP-PEI/L, but these embryos died before hatching (Fig 3F). Malformations present in zebrafish embryos exposed to PVP-PEI were yolk sac edema, pericardial edema and tail flexure.

Table 2.- Malformation prevalence. The percentage of total malformed embryos and specific malformations is calculated over all the embryos during the experimental period.

	Conc. (mg/L)	Total malformed embryos (%)	Specific malformations (%)				
			Yolk sac edema	Pericardial edema	Tail flexure	Finfold abnormality	Spinal cord flexure
Ag NPs	0.05	20	16.7	13.3	-	6.67	3.3
	0.075	13.3	13.3	6.7	3.3	-	9.9
PVP-PEI	10	3.3	3.3	3.3	3.3	-	-
	50	13.3	13.3	-	-	-	-

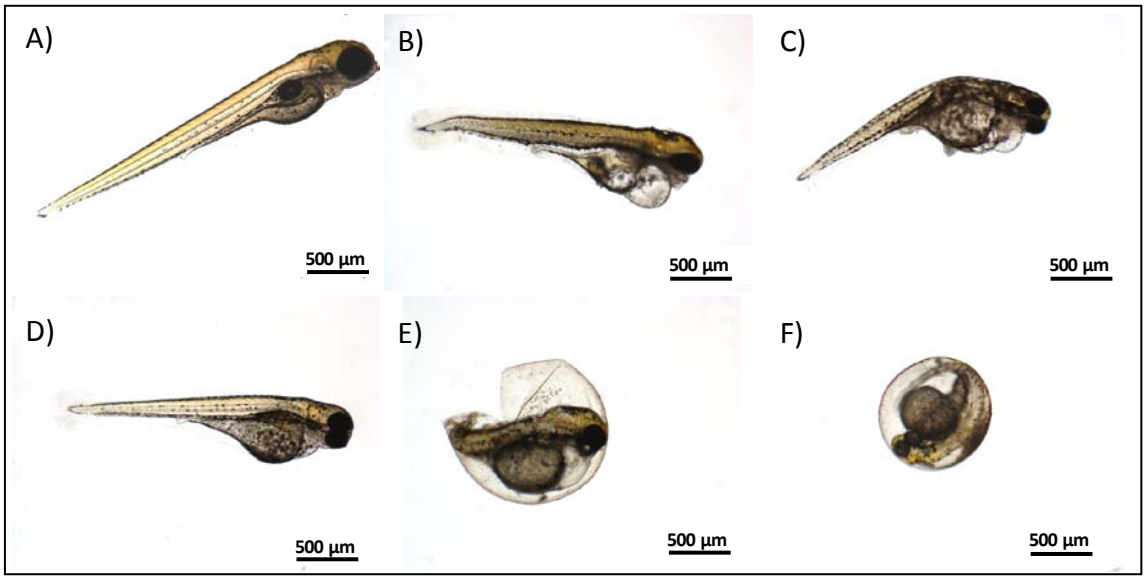

Figure 3.- Micrographs of zebrafish embryos: (A) 120 hpf control embryo showing normal morphology; (B) 120 hpf embryo exposed to 0.05 mg Ag/L, presenting yolk sac edema, pericardial edema and finfold abnormality; (C) 120 hpf embryo exposed to 0.075 mg Ag/L, presenting yolk sac edema, pericardial edema and spinal cord flexure; (D) 120 hpf embryo exposed to 0.075 mg Ag/L, presenting yolk sac edema; (E) 96 hpf unhatched embryo exposed to 1 mg/L of the PVP-PEI mixture, presenting cardiac malformation, yolk sac edema and malformation of the tail; (F) 96 hpf unhatched embryo exposed to 5 mg/L of the PVP-PEI mixture, presenting yolk sac edema.

Zebrafish embryos were significantly more sensitive to the exposure to both, the Ag NP suspension and the PVP-PEI mixture alone, at the same nominal concentration than brine shrimp larvae. At concentrations of Ag NPs≥ 1 mg/L zebrafish embryo mortality happened during the first 48 h of exposure. Therefore, the comparison between both acute tests was carried out at the same experimental conditions. The odd ratio (OR) values were calculated to determine the increase in the risk of mortality for zebrafish embryos respect to the brine shrimps larvae of 24 and 48 hph exposed during 24 and 48 h, being statistically significant different at all the concentrations compared (Table 3).

Table 3.- Odd ratio (OR) values indicating the increase in the risk of mortality for zebrafish embryos in comparison with brine shrimp larvae of 24 or 48 hph, exposed for 24 or 48 h to the Ag NP suspension or to the PVP-PEI mixture alone. In brackets the confidence intervals at 95%. Asterisks indicate statistically significant differences (p<0.05) according to the binomial logistic regression.

[Ag NPs] mg Ag/L	**Ag NP suspension**			
	24 hph, 24 h of exposure	**48 hph, 24 h of exposure**	**24 hph, 48 h of exposure**	**48 hph, 48 h of exposure**
1	>999.99* (199.86 - >999.9)	>999.99* (140.30 - >999.9)	>999.99* (199.86 - >999.9)	>999.99* (96.36 - >999.9)
5	>999.99* (193.75 - >999.9)	>999.99* (160.57 - >999.9)	744.15* (70.17 - >999.9)	260.19* (31.47 - >999.9)
10	>999.99* (106.52 - >999.9)	>999.99* (37.78 - >999.9)	72.81* (8.86 - >999.9)	17.56* (2.1 - >999.9)
[PVP-PEI] mg PVP-PEI/L	**PVP-PEI mixture**			
	24 hph, 24 h of exposure	**48 hph, 24 h of exposure**	**24 hph, 48 h of exposure**	**48 hph, 48 h of exposure**
~ 10	>999.99* (205.98 - >999.9)	>999.99* (224.33 - >999.9)	792.99* (74.93 - >999.9)	601.28* (61.79 - >999.9)
~ 50	467.66* (50.57 - >999.9)	>999.99* (157.19 - >999.9)	467.66* (50.57 - >999.9)	337.53* (39.76 - >999.9)
~ 100	>999.99* (103.15 - >999.9)	817.38* (77.31 - >999.9)	>999.99* (103.15 - >999.9)	197.35* (23.25 - >999.9)

Metal accumulation: chemical analyses in brine shrimps and zebrafish and selection of exposure concentrations for the zebrafish feeding

Silver accumulation was measured in brine shrimps of 24 hph after exposure for 24 h to five different concentrations of the Ag NP suspension (Fig 4A). At the two highest exposure concentrations (0.1 and 1 mg Ag/L) similar and significantly higher values of silver accumulation than in the control fish were recorded, with a bioconcentration factor (BCF) of about 78 for the exposure to 0.1 mg Ag/L. Exposure to 10 µg Ag/L resulted in an average accumulation of 1.8 µg Ag/g dw with a BCF for this concentration of 3.5, although no significant differences were found in comparison with the control group. At lower Ag NP concentrations, similar values were measured in exposed and control animals.

Based on these results, the selected concentrations to expose the brine shrimps for zebrafish feeding were 100 µg Ag/L as the high dose (HD) and 100 ng Ag/L as the low dose (LD) which is an environmentally relevant concentration of silver. From these accumulation data and the selected zebrafish diet of 2.5% body weight per day, a nominal exposure concentration of 2.1817 ng Ag/fish/day, in the case of the HD exposure group, and 0.17025 ng Ag/fish/day, in the case of the LD exposure group, was estimated.

During the dietary exposure experiment, a sample of the brine shrimp cultures was collected at four different days (days 1, 7, 14 and 21), for chemical analysis of silver in order to corroborate previous accumulation data. Accumulation of silver in brine shrimps was lower than in the previous experiment, but accumulation pattern was maintained (Fig 4B). An average silver accumulation of 3.9 µg Ag/g dw was recorded for the HD exposure group, while a mean value of 0.3 µg Ag/g dw was obtained for the LD exposure group. Significant differences were found between de HD exposure group and the control group, as well as, between the HD and the LD exposure groups.

Chemical analysis of control zebrafish fed with unexposed brine shrimps and zebrafish fed for 21 days with brine shrimps exposed to the low concentration of the Ag NP suspension showed similar silver content. Zebrafish fed for 21 days with brine shrimps exposed to the high concentration of the Ag NP suspension showed higher silver

content, although this increase was not statistically significant due to the high variability recorded between individuals (Fig 4C).

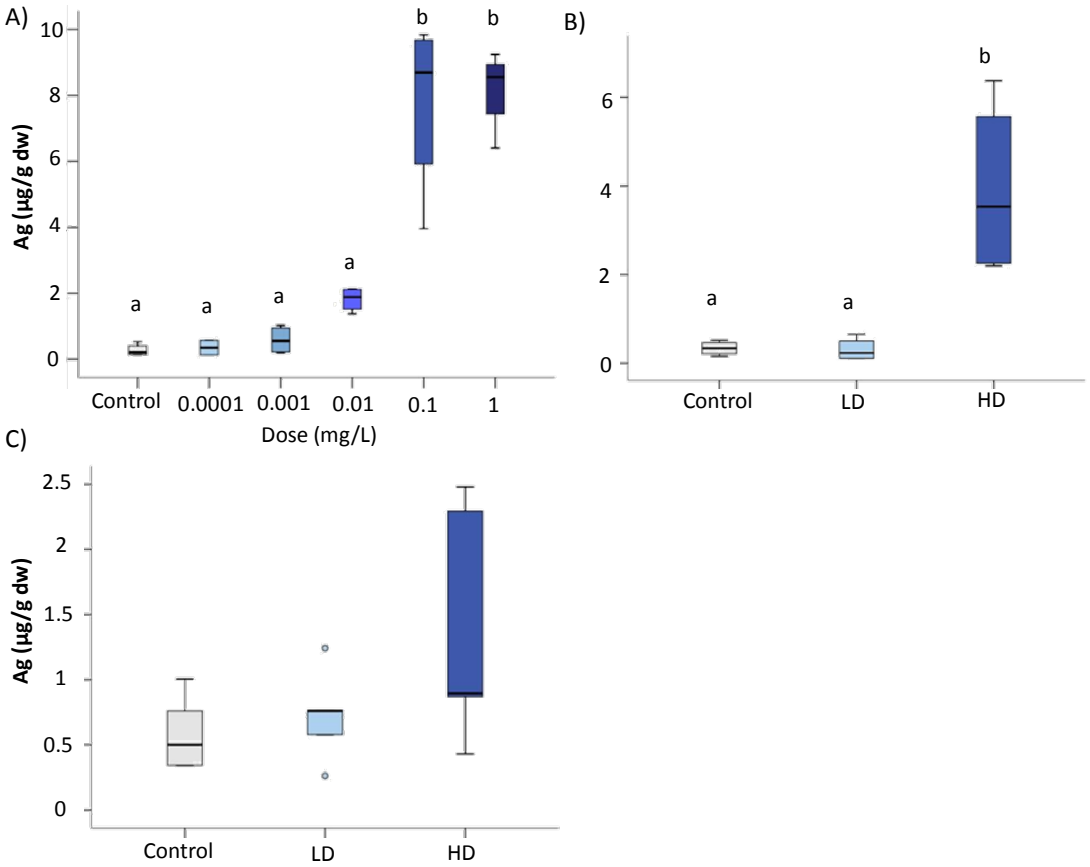

Figure 4.- Box-plot displaying silver accumulation levels (µg Ag/g dry weight) measured by ICP-MS; (A) brine shrimps of 24 hph exposed to five different concentrations for 24 h; (B) silver accumulation in brine shrimps cultured for the dietary exposure experiment and collected at four different days; (C) silver accumulation in whole zebrafish tissue after 21 days of dietary exposure. Different letter indicate statistically significant differences (p<0.05) according to the multiple regression models. Circles (o) indicate outlier sample within a group.

Autometallography: volume density of BSDs

Intestine tissue showed higher metal accumulation (higher values of Vv_{BSDs}) than liver, although both organs showed the same pattern of dose- and time-dependent metal accumulation.

In the intestine, significant differences were not detected at any exposure time, despite the high differences between both exposure concentrations, due to the high variability among individuals measured in zebrafish fed with brine shrimps exposed to the HD (Fig 5A). In the control group, no BSDs were detected at any time (Fig 5B). In the intestine of fish fed with brine shrimps exposed to the LD, few BSDs were detected

at three days (Fig 5C), increasing their amount after 21 days (Fig 5D). In the intestine of fish fed with brine shrimps exposed to the HD, a high number of BSDs were observed at three (Fig 5E) and at 21 days (Fig 5F). High density of deposits was found in the epithelial cells and especially in the apical end.

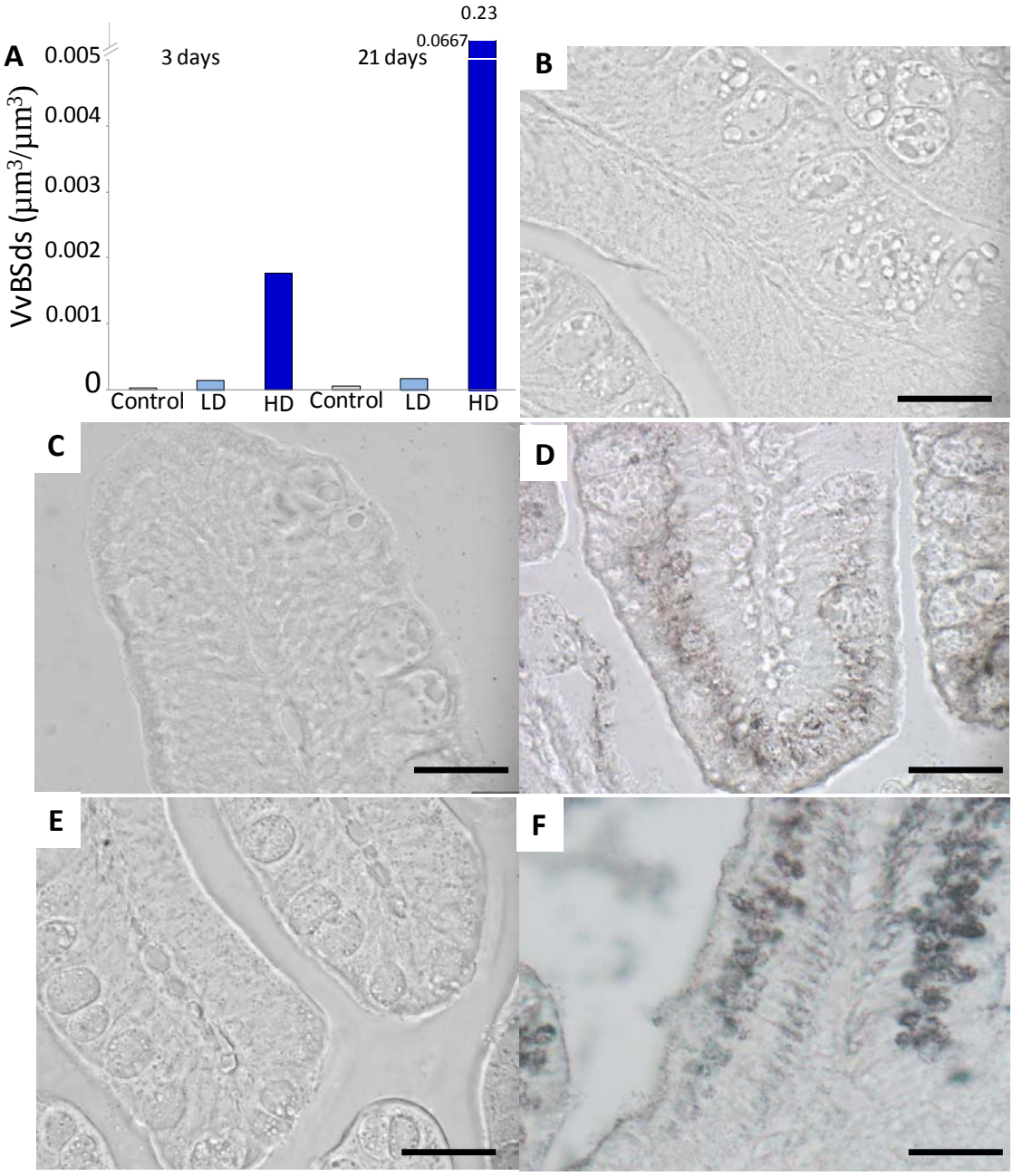

Figure 5.- (A) Metal accumulation (Vv_{BSDs}) in the intestine of zebrafish fed with brine shrimps exposed to different concentrations of the Ag NP suspension. (B-F) Micrographs of paraffin sections of the intestine after autometallographical staining. (B) Zebrafish fed with unexposed control brine shrimps. (C) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 3 days. (D) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 3 days. (E) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 21 days. (F) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 21 days. Scale bars: 20 µm.

In the liver, differences between treatments were found after both exposure times, but only after 21 days these differences were statistically significant in fish fed with brine shrimps exposed to the HD of the Ag NP suspension (Fig 6A). As in the case of the intestine tissue, a high variability was observed in the HD treatment. No BSDs were detected in the liver of control fish (Fig 6B). Few BSDs were detected after 3 days (Fig 6C), increasing after 21 days in the liver of fish fed with brine shrimps exposed to the LD (Fig 6D). In fish fed with brine shrimps exposed to the HD, few BSDs were present after 3 days (Fig 5E), appearing large and abundant BSDs homogeneously distributed thorough hepatocytes after 21 days (Fig 6F).

General health status: Lysosomal membrane stability (LMS)

The general health status of the fish was studied through the LMS test. A significant decrease in the mean value of the labilization period was reported after both treatments at both exposure times (Fig 7). No significant differences were found between fish fed with brine shrimps exposed to the LD and those exposed to the HD.

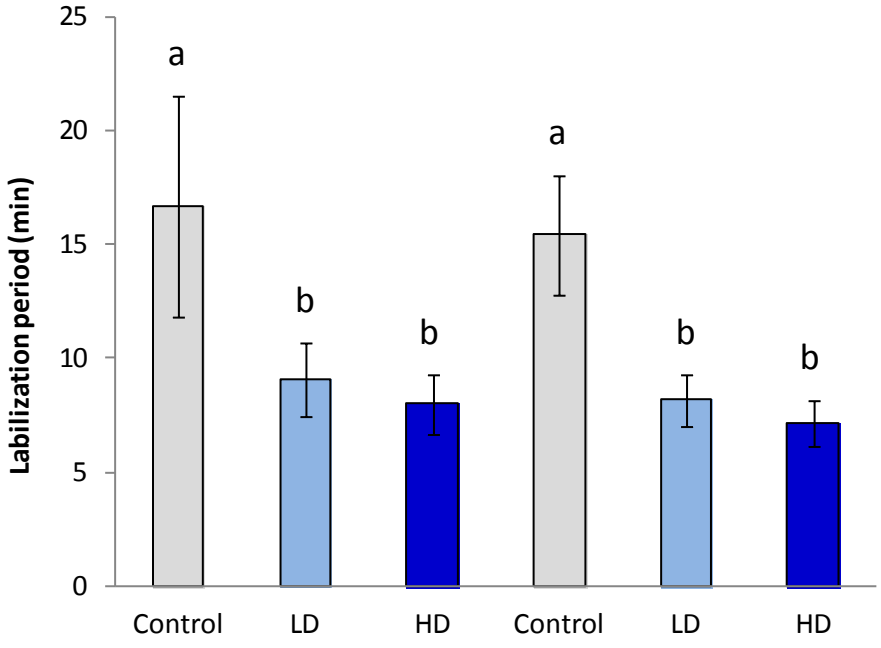

Figure 7.- Labilization period (in minutes) of the lysosomal membrane in liver cells. Different letters indicate statistically significant differences (p<0.05) according to the multiple regression models.

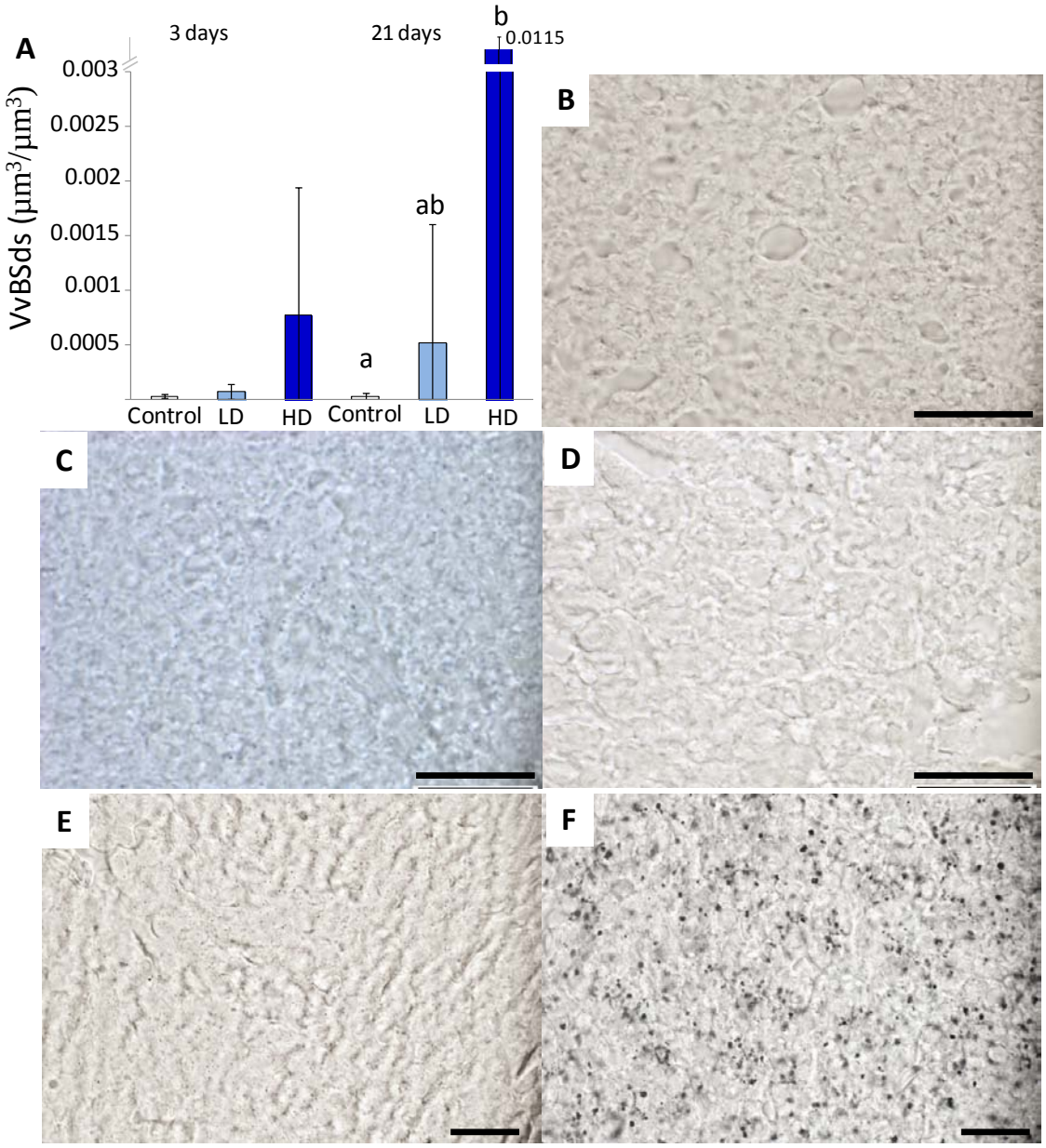

Figure 6.- (A) Metal accumulation (Vv_{BSDs}) in the liver of zebrafish fed with brine shrimps exposed to different concentrations of the Ag NP suspension. Different letters indicate statistically significant differences (p<0.05) respect to the control according to the multiple regression models. (B-F) Micrographs of paraffin sections of the liver after autometallographical staining. (B) Zebrafish fed with unexposed control brine shrimps. (C) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 3 days. (D) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 3 days. (E) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 21 days. (F) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 21 days. Scale bars: 20 µm.

Histological assessment

No significant differences among groups were detected in the volume density of intestinal goblet cells after Alcian blue staining of paraffin sections (data not shown). In the liver, different histopathological conditions, such as vacuolization and necrosis, were detected in individuals fed with brine shrimps exposed to both doses of Ag NPs. The prevalence of the histopathological alterations in liver is shown in Table 4. Control fish showed in general a normal liver at both exposure times (Fig 8A-B), only one individual sampled at 21 days of experiment showed vacuolization. After dietary exposure to both doses of Ag NPs, a higher prevalence of histopathological alterations was detected at 3 days than at 21 days, being this prevalence significantly higher at 3 days respect to the control in both treatment doses; the dietary exposure of zebrafish through brine shrimps treated with Ag NPs, even at environmentally relevant concentrations, provoked a vacuolization of the liver, increasing the prevalence significantly after 3 days for the HD (Fig 8C). Also, necrotic foci were observed in the liver of zebrafish fed with brine shrimps exposed to both doses of Ag NPs (Fig 8D).

Table 4.- Prevalence of histopathological alterations in liver of zebrafish. Data are shown in percentages. Asterisks indicate statistically significant differences between control and exposed groups (p<0.05) according to the Fisher's exact test.

Group	Sampling	n	Vacuolization	Necrosis	Total
Control	3 days	10	0	0	0
	21 days	10	10	0	10
Low dose	3 days	10	40	10	50*
	21 days	10	0	20	20
High dose	3 days	10	60*	10	70*
	21 days	10	30	0	30

n: number of individuals per experimental group. Total: number of individuals per group presenting any histopathological alteration.

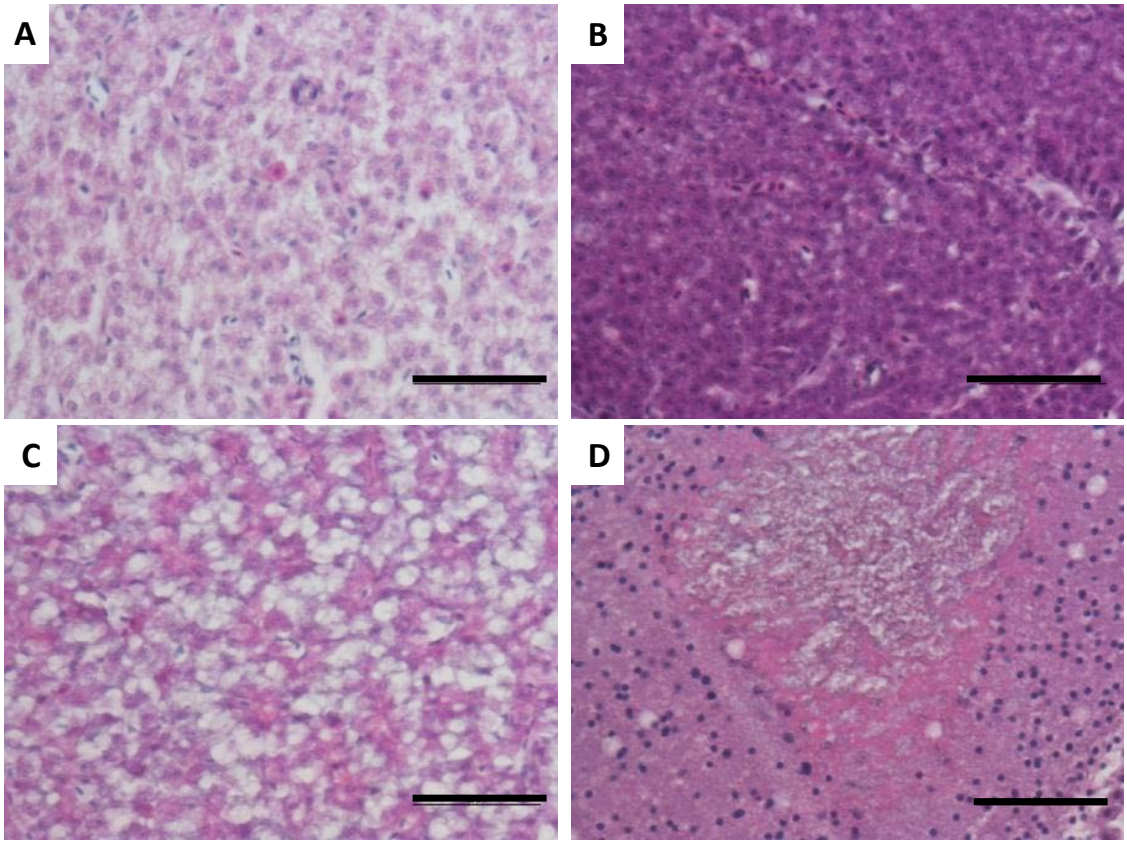

Figure 8.- Micrographs of paraffin sections of the zebrafish liver after H/E staining. (A) Liver of control zebrafish at 3 days; (B) Liver of control zebrafish at 21 days. (C) Liver of zebrafish fed for 3 days with brine shrimps exposed to the HD of Ag NPs, presenting vacuolization. (D) Liver of zebrafish fed for 21 days with brine shrimps exposed to the LD of Ag NPs, presenting a necrotic focus. Scale bar: 50 µm.

DISCUSSION

In the present study, transference of silver through the food web and derived effects were studied using brine shrimp larvae and adult zebrafish as a simple trophic chain. Brine shrimps were exposed to a Ag NP suspension and, then, used to feed zebrafish. Previously, acute toxicity of the Ag NP suspension and the PVP-PEI mixture present in the suspension was tested in both species. For brine shrimps, the PVP-PEI mixture was found to be nontoxic at any of the assayed exposure times or concentrations, while the Ag NP suspension showed significant acute toxic effects at the highest tested concentrations (≥ 5 mg Ag/L, depending on the exposure conditions). As exposure time increased, the silver concentration causing significant effects decreased. Aruvalsu et al. (2014) also reported an increase in the mortality rate of brine shrimp nauplii as exposure time to Ag NPs increased. They detected a higher amount of Ag NPs aggregates into the gut of brine shrimps exposed for 48 h than in the gut of brine shrimps exposed for 24 h, which provoked a lack of food uptake in the organisms.

Moreover, during the present study, for the same exposure period brine shrimps of 48 hph were more sensitive to Ag NPs than brine shrimps of 24 hph. The increased toxicity at more advanced stages of brine shrimp development, in comparison with the earlier stages, has been related to the fact that more developed brine shrimps feed more voraciously and, therefore, the ingestion of NPs may increase (Rajasree et al., 2011). In comparison with other crustaceans, such as *Daphnia magna*, brine shrimps seem to be less sensitive to the exposure to Ag NPs. The EC_{50} values at 24 h of exposure determined in the present study were 19.63 mg Ag/L (24 hph) and 16.52 mg Ag/L (48 hph). In *D. magna,* EC_{50} values at 24 h of exposure to Ag NPs prepared with different dispersion methods ranged between 0.004 and 3.844 mg Ag/L (Jo et al., 2012). This variability in the EC_{50} values for silver concentration reported for *D. magna* can be explained by the wide variety of treatments that Ag NPs can be put through, the diversity of coatings and sizes and how it all affects their toxicity (Jo et al., 2012; Römera et al., 2013; Silva et al., 2014). Nevertheless, although Ag NPs assayed in the present work are much more toxic than other formulations, according to the results obtained in zebrafish embryos (Chapter I), EC_{50} values obtained in brine shrimps are considerably lower than those reported for *D. magna*. Differences between the toxicity of Ag NPs to both species can be related to several biotic and abiotic factors being likely the habitat and, therefore, the exposure medium using in the laboratory test a key factor. *D. magna* is a freshwater organism and brine shrimp is a salt water organism. Salinity of the exposure medium can be a determinant factor for the Ag NP toxicity. It is well described that the higher ionic strength may result in aggregation of NPs and create links between free silver cations and anions present in the salt water, which neutralizes the toxicity of the silver ions in the Ag NP suspension (Kalbassi et al., 2011; Zhao and Wang, 2012). Moreover, brine shrimp as euryhaline specie has a great osmoregulatory capacity, which contributes to a greater resistance to the toxic effect of the metal cations and allows living in environments with a high salt concentration (Gajardo and Beardmore, 2012).

In zebrafish embryos, the toxicity of the Ag NP suspension was also due to the Ag NPs rather to the PVP-PEI mixture present in the suspension. The PVP-PEI mixture was not toxic at the concentration present in the dilution of the Ag NP suspension that caused

100% of mortality. The LC_{50} value for the Ag NP suspension (0.057 mg Ag/L) was similar to that reported for ionic silver (0.047 mg Ag/L) in a previous study carried out at the same experimental conditions and much lower than for maltose-coated Ag NPs of larger size (0.529-3.94 mg Ag/L, Chapter I). Many data found in the literature indicate an inverse relationship between the size of the NPs and their toxicity (Powers et al., 2011; Kim et al., 2013; Chapter I), which is attributed to the higher surface-to-volume ratio of the smaller particles that increases the proportion of atoms in the NP surface in contact with the test solution. Moreover, small NPs show greater ability to passively diffuse into developing embryos via chorion pore canals, which may increase the bioavailability and block the pores by the aggregation of NPs causing the mortality of the embryos before hatching (Lee et al., 2007).

Results indicate very different sensitivity of both species to the Ag NP suspension, being zebrafish embryos significantly more sensitive than the brine shrimp nauplii. Differences in the sensitive may be mainly explained by the nature of the environment and the biology of the species, although in the case of brine shrimps nauplii still exists a lack of knowledge about the toxicity provoked by Ag NPs (Libralato et al., 2014). Differences in the exposure medium are evident, zebrafish are freshwater organisms and brine shrimps live in high salinity environments. As previously mentioned, the concentrations of ions in the exposure media is a determinant factor for the Ag NP toxicity, as well as the high osmoregulatory capacity of the brine shrimps which may prevents from the entrance of metals into the organisms.

Although no acute toxicity was found, exposure of brine shrimps to 0.1 mg Ag/L led to significant silver accumulation and similar to that obtained after exposure to 1 mg Ag/L. Thus, 0.1 mg Ag/L, along with an environmentally relevant concentration (100 ng Ag/L) according to literature data, was selected to expose brine shrimps to be used as food for zebrafish. The low dose or environmentally relevant concentration was selected from the three lowest concentrations tested in the bioaccumulation experiment. No significant differences were found among them and, therefore, the lowest one was selected. Blaser et al. (2008) estimated that the Ag NP concentration in European rivers could be up to 320 ng/L. This value is higher than the values calculated by Gottschalk et al. (2009) in surface waters (up to 127 ng/L). Hendren et al. (2103)

reported similar values to the LD used in the present study, estimating that the Ag NP concentration present in the European rivers is around 89 ng/L.

According to the results obtained, dietary exposure resulted in a silver transference from the brine shrimps exposed to the Ag NP suspension to zebrafish. Metal transference through the food chain has been also detected in zebrafish fed with *D. magna* exposed to Ag NPs, causing changes in the microbiome structure of the zebrafish gut (Merrifield et al., 2013). Also, with other metal containing NPs, as TiO_2 NPs, effective metal transference has been measured from crustaceans to zebrafish in a simplified food web (Zhu et al., 2010). In this later study, metal accumulation was significantly higher in zebrafish exposed through the diet than in zebrafish waterborne exposed to the same original exposure concentration of TiO_2 NPs. In a previous study performed in our laboratory, waterborne exposure of zebrafish for 21 days to 10 µg Ag/L of maltose-coated Ag NPs of 20 nm resulted in an accumulation value of 0.88 µg Ag/g dw (Chapter II). In the present study, dietary exposure for 21 days through brine shrimps exposed to 100 µg Ag/L resulted in an accumulation of 1.39 µg Ag/g dw. Therefore, zebrafish can take up silver directly from the medium and through the food, being necessary to take into account both routes in order to assess the biological effects provoked by the exposure to Ag NPs in the environment.

The autometallographical staining performed in intestine and liver tissues of zebrafish manifested an effective dose- and time-dependent accumulation of metal in the tissues. Autometallographical staining has been already employed to evidence the deposition of metals as appearance of BSDs, in the tissues of zebrafish after waterborne exposure to metals and metal bearing nanoparticles (Vicario-Parés et al., 2014; Chapter II; Chapter IV). In the present work, higher Vv_{BSD} values were found for the intestine than for the liver in agreement with the exposure route used. Thus, the intestine seems to be the gate of entrance of the metal in zebrafish, as it was the site where the digestion of the contaminated brine shrimps took place. Many of the BSDs found in the intestine were located in the goblet cells, may be due to the strong affinity that glycoproteins and proteoglycans present in the mucous exhibit for metals and other xenobiotics (Pawert et al., 1998), and their excretion function into the gut lumen. This novel excretion pathway of nanomaterials through the intestinal goblet

cells has been proposed by Zhao et al. (2013) who injected zebrafish embryos with 30-200 nm activated carbon NPs directly into the yolk sac reporting that NPs can be excreted directly through intestinal tract without involving the hepato-biliary system. In our study, despite the BSDs were detected in goblet cells, no significant differences were detected in the Vv of intestinal goblet cells among groups. In the intestine of zebrafish waterborne exposed to Ag NPs, BSDs were also mainly detected in the intestinal epithelium, although the most of them were seen in the microvilli of enterocytes, while after the waterborne exposure to ionic silver, BSDs were detected into the goblet cells (Chapter II). Despite the exposure was carried out through waterborne, Ferry et al. (2009) suggested that NPs can pass from the water to the food web, resulting in bioaccumulation in the gut content of different organisms. Accordingly, Osborne et al. (2015) reported histopathological injuries in the intestine of zebrafish waterborne exposed to citrate-coated Ag NPs of two sizes (20 and 110 nm) for 4 days. The intestine of fish exposed to the smaller Ag NPs presented an increase in the number of goblet cells in the epithelial layer, some reduction in microvilli and partial damage to the lamina propria. The intestine of zebrafish exposed to the larger Ag NP presented evidence of vacuolization and partial lamina propria damage with abundant microvilli (Osborne et al., 2015). Moreover, the same nominal concentration of ionic silver caused complete obliteration of the lamina propria, inflammatory infiltrates, epithelial vacuolization and loss of microvilli. After silver staining, they detected Ag NPs in the basolateral membrane of the intestinal mucosa, especially after the exposure to citrate-coated Ag NPs of 20 nm. The size of the Ag NPs used during the present study (5 nm) was smaller than the used during the waterborne exposure (20 nm), as previously mentioned. The small NP size is related to a high solubility (Borm et al., 2006) and, therefore, their effect may be more similar to that provoked by the ionic form of the metal.

The amount (Vv_{BSDs}) of metal within the liver was lower than in the intestine. Similar results were also found in the liver of zebrafish waterborne exposed to Ag NPs (Chapter II). The presence of metal in the liver is mediated by the blood vessel transport after absorption through the intestine (Hadrup and Lam, 2014). The liver is the main organ involved in the detoxification of xenobiotics (Feist et al., 2004) and it is

highly irrigated. Therefore, the transport through the blood vessels may be the main route for metals to access and to be accumulated in the liver. Yeo and Pak (2008) observed nanosilver accumulation in blood vessels after waterborne exposure zebrafish to Ag NPs.

Lysosomes have been described as a target organelle of metals and metal NP exposure (Köhler et al., 2002; De Matteis et al., 2015). The characteristic acidic environment of lysosomes can provoke the NP dissolution and, in turn, the release of silver ions to the cell cytoplasm increasing the production of oxyradicals (Wei et al., 2015). Vicario-Parés (2016) observed a desestabilization of the lysosomal membrane after 3 and 21 days of waterborne exposure of zebrafish to 10 µg Cu/L of ionic copper and CuO NPs. Also, waterborne exposure of zebrafish to 10 µg Ag/L of ionic silver and Ag NPs caused the decrease of the time necessary to desestabilize the lysosomal membrane, although in a lesser extent than in the case of the exposure to CuO NPs (Chapter II). During the present study, although zebrafish fed for 3 days with brine shrimps exposed to the LD of Ag NPs did not accumulate silver significantly and few BSDs were detected in liver by autometallography, a significant decrease was measured in the stability of the lysosomal membrane, which can induce the formation of ROS when the NPs are exposed to the acidic environment of lysosomes (Chang et al., 2012). Thus, the dietary exposure of Ag NPs to zebrafish, even at environmentally relevant concentrations, and for a short-time provokes a toxic effect in the organisms.

Along with the accumulation of metal detected by autometallography and the decrease in the stability of the lysosomal membrane reported, some histopathological alterations were detected in the liver. Feeding with brine shrimps exposed to both Ag NP concentrations provoked similar alterations at both times of exposure. Fat vacuolization in the liver of fish has been proved to be provoked by the exposure to toxic compounds (Wester and Canton, 1987; Köhler et al., 2002; McHugh et al., 2011). This histopathological condition is a symptom of metabolism disruption, which has been demonstrated to be produced after the waterborne exposure (0.01 mg/L) to Ag NPs for 21 day after the analysis of the liver transcriptome (Chapter III), although that disruption was not detected after the histopathological analysis of the liver (Chapter II). Necrosis was also detected in liver of zebrafish fed with brine shrimps exposed to

both concentrations, being in agreement with the results obtained by Devi et al. (2015), who detected extensive cell death, necrosis and degenerative changes in liver of adult zebrafish waterborne exposed to 0.1 mg/L of Ag NPs for 15 days. Similarly, in zebrafish exposed to higher concentrations (30 and 120 mg/L) for a shorter period of time (24 hours), histopathological lesions such as disruption of hepatic cells cords and apoptotic changes (chromatin condensation and pyknosis) have been reported (Choi et al., 2010). Devi et al. (2015) indicated the ability of Ag NPs to alter the biochemical functions associated with the liver which could provoke the toxic effect in the organism. Other authors have suggested that the exposure to Ag NPs provoke oxidative stress in liver of different fish, which may provoke the apparition of histopathological lesions in the liver (Chae et al., 2009; Choi et al., 2010; Wu and Zhour, 2013).

CONCLUSSIONS

The acute toxicity of the Ag NP suspension used in this study depends on the species, being zebrafish embryos much more sensitive than brine shrimp nauplii. Bioaccumulation of silver was detected in brine shrimps exposed to sublethal concentrations of Ag NPs, being effectively transferred through the diet to adult zebrafish. The silver transfer can cause toxic sublethal effects and act in detriment of the health of the fish as indicated by the significant reduction of the stability of the hepatocyte lysosomal membrane and the presence of histopathological alterations in the liver. Therefore, the potential risks to which predators in high levels of the food chain are exposed by the release of NPs into the natural environment is envisaged.

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IV. GENERAL DISCUSSION

ABBREVIATIONS

BSDs, Black silver deposits

FET, Fish embryo toxicity

GO, Gene ontology

LC_{50}, Lethal concentration to 50% of the population

MN, Micronuclei

NPs, Nanoparticles

qPCRs, Quantitative real time PCR

ROS, Reactive oxygen species

TEM, Transmission electron microscopy

Among nanomaterials, nanoparticles (NPs) are defined as a material with at least two dimensions between 1 and 100 nm (Klaine et al., 2008). Engineered NPs are one type of manufactured NPs including metals, metal oxides and alloys, carbon based materials such as fullerenes, nanotubes and fibres, silicates, quantum dots (QDs) and polymer composites (Tiede et al., 2009). Engineered NPs present a variety of special physico-chemical properties and chemical reactivity, which do them substantially different from their respective bulk materials of the same composition (Handy et al., 2008).

The entrance of engineered NPs into the environment is increasing because their use in commercial products is growing, and their wastes are being spilled into the aquatic environment (Baker et al., 2014). Once in the aquatic systems, physico-chemical properties of NPs may be altered depending on the characteristics of the receiving media, such as pH or the ionic strength. NPs can undergo a variety of processes such as sorption, oxidation, dissolution, etc, which determine their fate in the abiotic environment and their interaction with biota (Fig 1). These processes are of relevance to both the abiotic and biotic environment, understanding how the interaction of metal bearing NP with the abiotic environment is, we will know how the interaction into the organism may be (Schirmer et al., 2013).

Once into the aquatic environment, the interaction between NPs and aquatic organisms may cause a toxic effect, since these NPs can remain in the water column or in sediments, being uptaken by the organisms through different routes (Moore, 2006). This effect begins at the molecular level and is connected with the effect shown at the biochemical, subcellular, cellular, tissue, organ and individual levels (Lee et al., 2015).

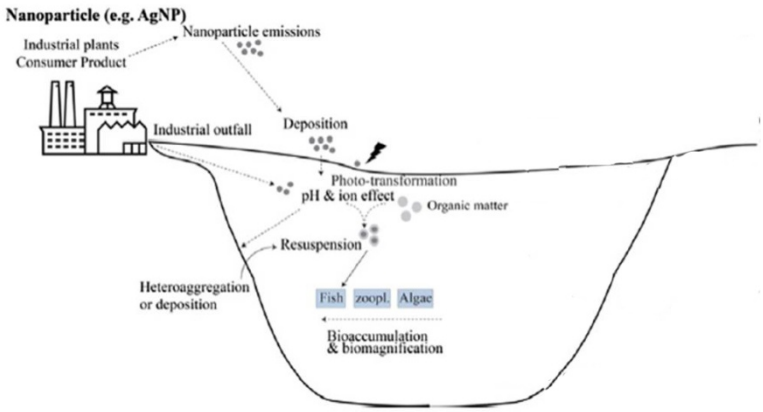

Figure 1.- Fates of nanoparticles in the aquatic environment. Modified from Lee et al. (2015).

In this PhD thesis, using zebrafish (*Danio rerio*) as animal model, different approaches were used with the aim of analyzing the toxic effect provoked by the exposure to metal and metal bearing NPs in this organism.

The toxic effect of metal and metal bearing NPs in zebrafish can vary depending on intrinsic characteristics of the NPs. Moreover, the toxic effect provoked by the exposure to NPs may be different of the toxicity provoked by the ionic and bulk forms of the metal (Shaw and Handy, 2011). To establish a ranking of toxicity depending on the characteristics of the NPs, a set of NPs with different characteristics was assessed in **Chapter I** using the fish embryo toxicity (FET) test (OECD TG236, 2013). Zebrafish embryos were exposed to a range of concentrations of a set of metal and metal bearing NPs (Ag, Au, CdS, ZnO and SiO_2) displaying different properties, such as different sizes, shapes and additives. Exposures to their ionic and bulk counterparts were run in parallel. Also, in case that the NP solution presented any additive, zebrafish embryos were exposed to the corresponding concentration of the additive matching with that present in dilutions of the NP solution tested. The test was carried out for 120 h and the survival rate, hatching rate and time, and the presence of malformations were evaluated. According to the results obtained in **Chapter I**, the ionic form was the most toxic, followed by the NP form and, finally, the bulk form of the metals. Among the different metal and metal bearing NPs assayed, Ag NPs resulted the most toxic ones in terms of LC_{50}, expressed as mg/L, followed by ZnO NPs, CdS NPs, Au NPs and, finally, SiO_2 NPs (Fig 2A). These results were in accordance with previous data found in the literature (George et al., 2011), where the toxic effect of NPs has been mainly related to the chemical composition and, thus, solubility of the metals. For soluble compounds, such as silver, cadmium and zinc, toxicity has been mainly attributed to the release of ions into the exposure medium (Misra et al., 2012; Schirmer et al., 2013; Ivask et al., 2013). Therefore, toxic metal ions released from the NPs can cause or contribute to the higher toxicity elicited by soluble NPs in comparison to more insoluble compounds, such as Au and SiO_2. Moreover, the toxic effect of the NP assayed in zebrafish embryos was influenced by factors such as the size or shape. The smallest NPs were the most toxic ones, as observed in the case of the exposure to Ag NPs. Ag20-Mal presented a lower LC_{50} value than the other Ag NPs assayed, in

agreement with the previous results provided in the literature (Bar-Ilan et al., 2009; Powers et al., 2011; Kim et al., 2013). The presence of additives such as stabilizers can also vary the toxicity of the NP formulations. Sodium citrate or Ecodis P-90 present in the Au NP and ZnO NP formulations, respectively, were responsible for the toxicity found in zebrafish embryos. In the literature data has been reported that the presence of the chorion in embryos can also alter the interaction between the embryo and the NP suspension (Lee et al., 2007). Using fluorescent NPs we observed that the chorion acted as a barrier for the entrance of the SiO_2 NPs in accordance with the results previously described in the literature (Fent et al., 2010). Nevertheless, the chorion does not always act as a barrier. In some cases, NPs can penetrate through the pores of the chorion provoking a toxic effect in the embryos (Lee et al., 2007). This could explain the mortality detected in the present study after the exposure to Ag NPs, which was observed before embryos hatched. In the present study, a ranking of toxicity for different NP solutions was established, although, further studies would be necessary to understand the mechanisms of action of each specific NP and the influence of each intrinsic factor.

According to the ranking based on results obtained in **Chapter I**, Ag NPs resulted the most toxic NPs to the zebrafish embryos in terms of LC_{50} values, followed by ZnO NPs, CdS NPs, Au NPs and, finally, SiO_2 NPs (Fig 2A). In this work, as in the majority of the studies described in the literature, the NP concentrations are given in milligrams of metal per liter. The main reasons for the choice of this concentration units are: 1) it is the most usual expression of chemicals concentration in ecotoxicology; 2) it is the form that best matches with results of chemical analyses to assess bioaccumulation; and 3) it is the most usual expression used for regulatory purposes. Nevertheless, the metrics that should be used to express NP concentration, especially for comparison purposes, is an open debate (Shang and Gao, 2014). When metal concentration is expressed in molar concentration, the number of metal atoms per unit is comparable among different compounds. Some publications have already used this concentration expression, mainly for *in vitro* studies (Coradeghini et al., 2013; Tang et al., 2013a), but also using zebrafish embryos as test model (Lee et al., 2007; Browning et al., 2013). If we convert the LC_{50} values expressed as mg/L obtained in **Chapter I** to molar concentrations of metals, the ranking of toxicity changed slightly, although Ag NPs

were still the most toxic NPs followed by CdS NPs, ZnO NPs, Au NPs and, finally, SiO_2 NPs (Fig 2B).

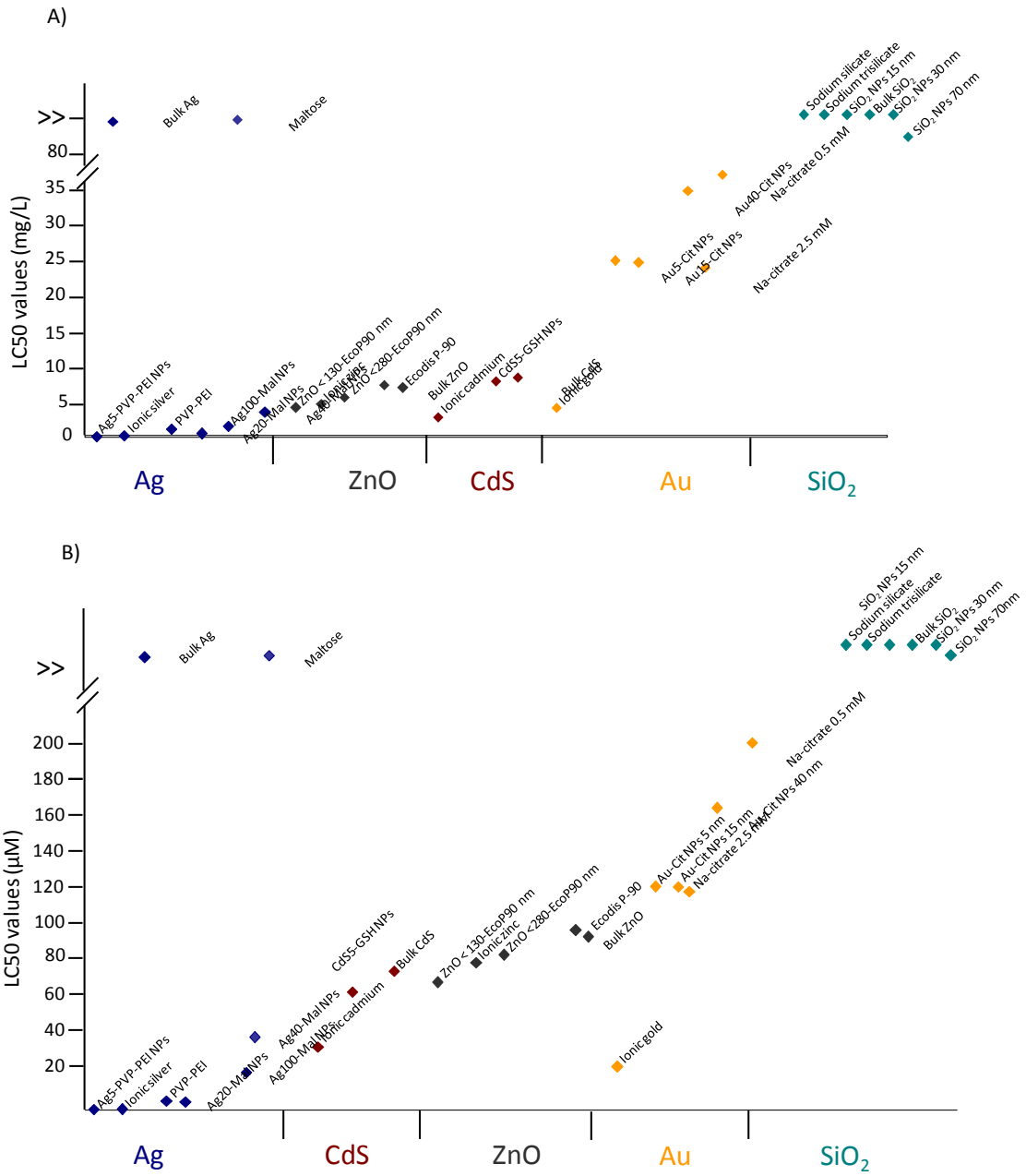

Figure 2.- LC50 values in (A) mg metal/L and in (B) µM obtained in the FET tests for the different NPs, for their ionic and bulk counterparts, and for the additives present in the NP solutions. LC_{50} values were calculated using Probit model (p<0.05). The different metals are represented from the most toxic to the least toxic.

Based on the toxicity rankings established in **Chapter I**, in the following chapters we analyzed the toxic effect of metal and metal bearing NPs in adult zebrafish, in comparison with the toxicity of the same nominal concentration of the ionic form of the metal. For this purpose, Ag NPs and CdS NPs were selected to analyze the effects

provoked after the waterborne exposure for 21 days. After the exposure period, zebrafish were maintained in clean water up to 6 months to evaluate the potential long-term effects or the recovery after the cease of the exposure. Ag NPs were selected since they clearly resulted the most toxic NPs to the zebrafish embryos. CdS NPs were selected because they were the second most toxic NPs to embryos when molar concentrations were used and, interestingly, despite the LC_{50} value estimated for CdS NPs was higher than the LC_{50} value for ionic cadmium, according to the results obtained in the FET test, CdS NPs provoked a significant effect on embryo survival at lower concentration than ionic cadmium. Moreover, cadmium is classified among the most toxic metals and is found among the priority substances (EPA, 2016) besides from being a carcinogenic compound (IARC, 1993). Several studies have been performed to study the toxicity of the waterborne exposure to ionic cadmium in adult zebrafish (Cambier et al., 2010; Banni et al., 2011; Vergauwen et al., 2013a; 2013b), but no studies have addressed the toxicity of Cd containing NP in zebrafish through waterborne exposure. Only two studies have reported toxicity to zebrafish after dietary exposure to Cd-containing NPs (Lewinski et al., 2011; Ladhar et al., 2013).

Thus, in **Chapters II** and **III** the toxic effect provoked by 21 days of waterborne exposure to Ag NPs or to ionic silver in adult zebrafish was analyzed. Remaining zebrafish previously exposed were maintained up to 6 months in clean water. In **Chapter II** a set of different biomarkers covering the main mechanisms of toxicity described for NPs were determined to assess the toxicity of Ag NPs of 20 nm, which resulted the most toxic size according to results reported in the previous chapter, and ionic silver in the adult zebrafish. In **Chapter III**, the whole hepatic transcriptome of male adult zebrafish exposed for 21 days to both forms of silver was analyzed.

In **Chapters IV** and **V**, we followed the same experimental approach to evaluate the toxicity of CdS NPs and ionic cadmium in adult zebrafish.

Despite adult zebrafish were exposed to the same nominal concentration of each metal in both studies, metal accumulation detected in fish exposed for 21 days to both forms of cadmium was much higher than in those exposed to both forms of silver, especially after the exposure to ionic cadmium. The high value of accumulated

cadmium in the organisms could explain the high mortality observed in the individuals from the fourth day up to the day 21 of exposure. The exposure to CdS NPs or to ionic cadmium provoked mortality to 72% and 78% of the zebrafish, respectively, after 21 days. This mortality level was much higher than the mortality registered in previous studies in similar exposure conditions (Cambier et al., 2010; Vergauwen et al., 2013a). Calcium concentration in the exposure medium has been described as an important factor to reduce the cadmium toxicity in organisms, since calcium ions compete with cadmium ions for binding sites (Meinelt et al., 2001; Pellet et al., 2009). Thus, calcium concentration in the exposure media, which was lower in our study, could easily explain the different mortality level described.

Metal accumulation was specifically detected in liver and intestine using autometallography, a technique successfully used in fish exposed to different soluble and nanoparticulated metals (Alvarado et al., 2005; 2006; Vicario-Parés et al., 2014). In individuals exposed to Ag NPs, the presence of black silver deposits (BSDs) was found in the intestinal epithelium, with especially intense staining in the cytoplasm of enterocytes and in the microvilli. After the exposure to ionic silver, discrete BSDs were detected into the secretory vesicles of the goblet cells of the intestine. Osborne et al. (2015) also reported differences in the localization of silver in the intestine depending on the silver form. Ag NPs of 20 nm were detected in the basolateral membrane of the intestinal mucosa, which was attributed to the small size of the NPs as they could be more rapidly taken up by endocytosis in the epithelial layer, increasing the silver deposition in the basolateral membrane of intestinal epithelium. Larger Ag NPs (110 nm) were detected in the apical membrane (Osborne et al., 2015). Thus, the localization described by these authors for the Ag NPs o 110 nm matches better with our observations than the results described for the Ag NPs of 20 nm, besides the reported size of the primary particles of the Ag NPs used in Chapter II was 20 nm. This disagreement can be explained considering the characterization data of the Ag NPs used in our study (**Chapter I**) which reflected the tendency of the NPs to form aggregates. Thus, the fate of these aggregates may be similar to the fate of the largest Ag NPs used by Osborne et al. (2015). Previous studies have also demonstrated that, along with the gills, the intestine is one of the main entrances of metals in the

organism (Gaiser et al., 2012; Schirmer et al., 2013), being specifically reported as a target organ for accumulation after waterborne exposure to cadmium (Souid et al., 2013). From the intestine, metals are distributed to other organs (Gaiser et al., 2012).

After the entrance of metals in organs such as gills and intestine, they are transferred to the liver, which is well known as a key organ involved in storage and detoxification of metals (Handy, 1993). The presence of BSDs in the liver of zebrafish exposed to silver was higher in individuals exposed to the ionic form than in those exposed to the NP form, indicating a higher transference of metal from the intestine. In the case of the exposure to both NPs, the higher amount of BSDs in the intestine revealed a higher accumulation of metal in the intestine than in the liver. By transmission electron microscopy (TEM), aggregates of CdS NPs and Ag NPs were detected in different compartments of the hepatocytes. Aggregates of CdS NPs were detected attached to the nuclear envelope and in vesicles, while aggregates of Ag NPs were observed in the nucleus and mitochondria of the hepatocytes. Previous studies have reported differences in the target organelles after the exposure to metal and metal bearing NPs. Ag NPs have been previously detected into the nucleus and mitochondria after *in vitro* exposure of human cells (Asharani et al., 2009), *in vivo* exposure of adult zebrafish (Choi et al., 2010) or *in vivo* exposure of zebrafish embryos (Asharani et al., 2008), suggesting their direct involvement in the mitochondrial toxicity and DNA damage (Asharani et al., 2009).

Oxidative stress has been suggested as one of the main mechanisms of toxicity of metal and metal bearing NPs, being proteins main target biomolecules of oxidative damage (McShan et al., 2014; Wei et al., 2015). The level of carbonylated actin, used as target protein, and the level of free ubiquitin measured by western blotting did not evidence oxidative damage in zebrafish exposed to both forms of silver compared with the control group. The exposure to CdS NPs for 21 days produced a slight decrease and increase in the intensity of the immunoreactive bands corresponding to free ubiquitin and carbonylated actin, respectively, while after the exposure to ionic cadmium these differences were statistically significant. Previous studies have detected oxidative stress in fish exposed to higher concentrations of Ag NPs than those used in this work (Choi et al., 2010; Lee et al., 2012; Wu and Zhou, 2013). Nevertheless, the exposure to

cadmium at the same nominal concentration than the used during the exposure to silver was enough to provoke oxidative stress in the liver of zebrafish. Cadmium has been previously reported to play an important role in the oxidative stress detected in organisms (Liu et al., 2009).

Clastogenic effects were not detected according to the analysis of micronuclei (MN) frequency in erythrocytes after the exposure to Ag NPs or ionic silver. The exposure to both cadmium forms neither provoked clastogenic effects in erythrocytes, despite cadmium has been previously considered as a genotoxic substance (Dabas et al., 2012). At similar experimental conditions, Cambier et al. (2010) neither detected genotoxic effects in adult zebrafish exposed to environmentally relevant concentrations (9.6 µg/L) using the MN test. These results suggest that the MN test is not a sensitive method to be used at low concentrations of metals. Other methods such as random amplified polymorphic DNA, which has been previously successful used to measure the genotoxic effect provoked after the exposure to metals (Cambier et al., 2010), could be used to replace the MN test at low and environmentally relevant concentrations of metals.

The endolysosomal system of the cells has been described as a target of exposure to metals, both in soluble and NP form (Alvarado et al., 2005; Broeg et al., 2005; Schultz et al., 2015). Metals are known to induce alterations in the lysosomal structure and membrane desestabilization (Broeg et al., 2005). The presence of CdS NPs in hepatocytes could be linked with the desestabilization of the lysosomal membrane observed from the first time measured. In fish exposed to Ag NPs only a slight decrease in the time necessary to desestabilize the lysosomal membrane was detected. Differences in the effect provoked by both NPs could be partially due to the differences in their size, the smaller size of CdS NPs could facilitate their uptake and transfer to the liver resulting in the early desestabilization of the lysosomal membrane.

Despite metal bioaccumulation was observed, histopathological alterations were not recorded in the liver of zebrafish exposed to both, Ag NPs and ionic silver, for 21 days. On the contrary, the accumulation of cadmium in the liver provoked an increase in the prevalence of histopathological alterations, being the prevalence of hepatic

vacuolization significantly increased after the exposure to both forms of cadmium for 21 days. Other pathology found after exposure to CdS NPs was hepatic megalocytosis, which has been associated to DNA damage (Spitsbergen and Kent, 2003). Eosinophilic foci, which have been potentially related to a pre-neoplastic condition (Costa et al., 2013), appeared at the end of the post-exposure period after the exposure to ionic cadmium.

In gills, several pathological conditions such as aneurism and inflammation of the secondary lamellae, and hyperplasia in cells of the primary lamellae were detected since this organ is one of the main entrances of metals after the waterborne exposure to NPs (Ma and Lin, 2013). In a previous study where zebrafish were exposed for 4 days to a higher concentration (1 mg/L) of Ag NPs of a similar size (20 nm), similar pathologies were detected in the gills (Osborne et al., 2015). They proposed that the tissue injury along with the mucus hypersecretion produced by the exposure to silver could accentuate the adherence of smaller particles to secondary filaments provoking the increase of diverse histopathologies. Exposure to CdS NPs also caused diverse pathologies whose prevalence was increased after the exposure to ionic cadmium for 21 days. Cadmium damage to gill could, in turn, result in reduced oxygen consumption and disruption of the osmoregulatory function of aquatic organisms (Liu et al., 2011). This could also contribute to the increased mortality detected in fish exposed to cadmium. These pathologies were also detected after the 6 months recovery period in clean water, indicating that the toxic effect of cadmium may be sustained after the cease of the exposure.

As mentioned above, in **Chapters III** and **V** the whole hepatic transcriptome was analyzed after the waterborne exposure for 3 and 21 days to both forms of silver and cadmium. Up to date, most of the studies have focused on the analysis of the effect provoked by waterborne exposure of Ag NPs in some selected genes involved mainly in DNA damage, oxidative stress or apoptosis (Yeo and Pak, 2008; Choi et al., 2010; Katuli et al., 2014). In addition, the gill transcriptome of zebrafish exposed to Ag NPs has been also analyzed in several works (Griffitt et al., 2009; 2013). Some studies have been performed to analyze the whole hepatic transcriptome of several fish species exposed to ionic cadmium (Reynders et al., 2006; Sheader et al., 2006; Auslander et al.,

2008; Mehinto et al., 2014), but to the best of our knowledge, no information was available regarding the effect of Ag NPs and CdS NPs on the zebrafish liver transcriptome.

According to the results obtained in **Chapter III**, the exposure to all silver treatments altered the transcription profile of genes involved in metabolic processes, especially after the exposure to ionic silver for 3 days and to Ag NPs for 21 days. This effect was also detected by the functional analysis performed, which evidenced a significant regulation of the "glycolysis/gluconeogenesis" pathway. Moreover, the "pyruvate metabolism" was significantly altered after the exposure to ionic silver for 3 days and the KEGG pathway "steroid biosynthesis" was significantly altered after the exposure to Ag NPs for 3 days. Other KEGG pathways detected after the exposure to Ag NPs and ionic silver at 3 days and Ag NPs at 21 days were involved in DNA damage ("purine metabolism" and "pyrimidine metabolism"). In agreement, previous studies have reported toxic effects due to the exposure to Ag NPs with the alteration of the transcription of genes involved in DNA damage (Yeo and Pak, 2008; Choi et al., 2010; Katuli et al., 2014; Massarsky et al., 2014). Some works have attributed the toxicity of Ag NPs to the release of ions (Luoma and Rainbow, 2008; Bilberg et al., 2012; McShan et al., 2014; De Matteis et al., 2015). Nevertheless, other factors related to their nanosize may be also partially involved in their toxicity (Chae et al., 2009; Griffitt et al., 2009; Scown et al., 2010; Gagné et al., 2012). According to our results, alteration of reproductive processes or of the immune system were exclusively detected after the exposure to Ag NPs, indicating that the toxicity provoked by Ag NPs might not be related exclusively to the dissolution of ions, but also to the NP form.

Nevertheless, results obtained in **Chapter V,** where the effect of cadmium was analyzed, differed from those obtained as results of the exposure to Ag NPs or to ionic silver. CdS NPs and ionic cadmium also altered the hepatic transcriptome of zebrafish, although this alteration was especially detected after 21 days of exposure. Several works have addressed the effect provoked by cadmium containing NPs and ionic cadmium in the transcription of different genes. Up-regulation of genes involved in mitochondrial metabolism, DNA repair, apoptosis, antioxidant defenses or detoxification mechanisms was reported (Gonzalez et al., 2006; Sheader et al., 2006;

Auslander et al., 2008; Ladhar et al., 2013; Tang et al., 2013b; Vergauwen et al., 2013a; Arini et al., 2015). The Bast2GO analysis performed with the transcripts significantly regulated after exposure to CdS NPs or to ionic cadmium showed that at 21 days similar GO terms were enriched for both treatments. According to the Fisher exact test, some terms were over-represented in fish exposed to ionic cadmium in comparison to animals exposed to CdS NPs. Among them, diverse GO terms involved in immune system processes or mitochondrial calcium ion homeostasis were differentially enriched. The functional analysis performed showed different KEGG pathways significantly altered after the exposure to both forms of cadmium. While the exposure for 21 days to CdS NPs affected significantly pathways such as “natural killer cell mediated cytotoxicity”, “retinol metabolism”, “D-Arginine and D-ornithine metabolism” or “adherens junction”, the exposure to ionic cadmium significantly altered several pathways related to DNA damage and repair (“purine metabolism”, “pyrimidine metabolism”, “mistmach repair”, “nucleotide excision repair”), energetic metabolism (“glycolysis/gluconeogenesis”, “pentose phosphate pathway”, “biosynthesis of unsaturated fatty acids”, “citrate cycle (TCA cycle)”) or amino acids metabolism (“glycine, serine and threonine metabolism”, “alanine, aspartate and glutamate metabolism”). The exposure to cadmium may provoke disturbs of physiological cellular functions, gene transcription and regulation, resulting in cell death or stress-induced adaptation (Thévenod, 2009).

As previously mentioned, the functional analysis evidenced that exposure to both metals, silver and cadmium, provoked a significant alteration of the energetic metabolism. Metals are known to provoke the alteration of metabolic processes in fish such as *Oreochromis niloticus, Perca flavescens* or *Danio rerio* (Öner et al., 2009; Scown et al., 2010; Bougas et al., 2013). For instance, in caged yellow perch chronically exposed to diverse metals over-transcription of genes involved in energy metabolism, the gluconeogenesis pathway and β-oxidation was detected, suggesting an increase in energy metabolism induced by stress (Bougas et al., 2016). In **Chapter IV**, the alteration of the metabolism was also detected during the histopathological analysis which showed a significant increase of vacuolization in the liver of fish exposed to both forms of cadmium.

Differences in the effects provoked by the exposure to both metals can be also observed in results of the COA analysis performed considering all data together (Fig 3). Unlike silver exposure, which produced similar toxicity at 3 and 21 days of exposure, the exposure to cadmium exerted a stronger effect after 21 days than after 3 days of exposure. Despite the COA analysis performed in **Chapter III** separated the groups exposed to different silver treatments, especially the group corresponding to Ag NPs for 21 days, when the results obtained in **Chapter V** were included, all silver treatments were clustered together. Nevertheless, Fig 3 shows that groups exposed to cadmium remained separated depending on the exposure time, which was previously detected in **Chapter V** when these data were analyzed separately. Therefore, taking into account all the results obtained after the analysis of the whole hepatic transcriptome of zebrafish exposed to both metals, we may conclude that the effects exerted by the exposure to cadmium over the transcriptome were stronger than the effects provoked by silver at the same experimental conditions.

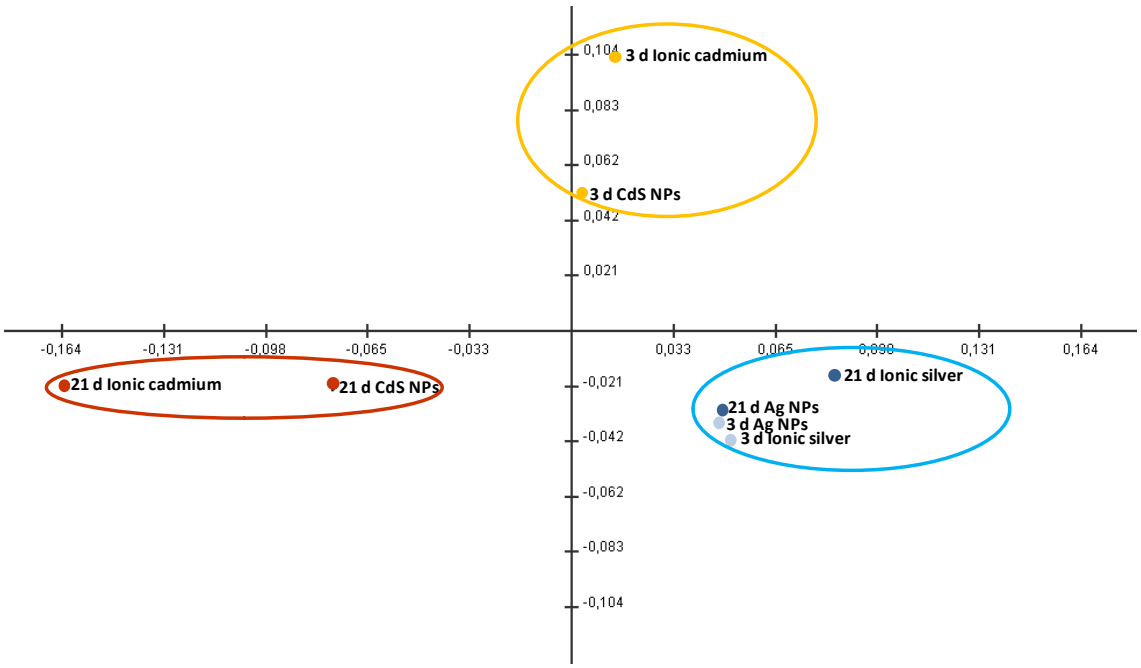

Figure 3.- Correspondence analysis (COA) axes showing the distribution of each treatment (dots) according to their transcription profile. The COA clearly separated treatments according to the metal type. All silver treatments grouped together, while in the case of the cadmium exposure, treatments were separated depending on the exposure time.

Despite the waterborne exposure is an important route of entrance of NPs into the aquatic organisms, other routes must be taken into account (Maurer-Jones et al., 2013). The diet is also an important entrance of NPs into the organisms, since animals

can feed suspended matter or other organisms previously exposed to NPs (Schrimer et al., 2013). For this reason, in **Chapter VI** brine shrimps (*Artemia sp*) larvae were exposed to two different concentrations of Ag NPs and, subsequently, used to feed adult zebrafish for 21 days. Before performing the dietary transfer experiment, the acute toxicity of these silver NPs was tested in zebrafish embryos and brine shrimp larvae. Ag NPs used in this chapter were smaller (5 nm) than those used in previous chapters and coated with a mixture of poly N-vynil-2-pirrolidone and polyethylenimine (PVP-PEI). Following the same approach as in **Chapter I,** zebrafish embryos were exposed to a range of concentrations of PVP/PEI-coated Ag NPs. The LC_{50} value for the Ag NP suspension (0.057 mg Ag/L) was similar to that reported in **Chapter I** for ionic silver (0.047 mg Ag/L) and much lower than for maltose-coated Ag NPs of larger size (0.529-3.94 mg Ag/L). Results obtained in this chapter were in agreement with the results obtained in **Chapter I** regarding size-dependence toxicity, being the smallest NPs the most toxic ones. The acute toxicity of Ag NPs was also analyzed in brine shrimp larvae, and the odd ratios were calculated to determine the increase in the risk of mortality for zebrafish embryos respect to the brine shrimps larvae. Zebrafish embryos were significantly more sensitive to the Ag NP exposure than the brine shrimp larvae.

Brine shrimps were daily exposed to an environmentally relevant concentration (100 ng/L) and a likely effective concentration (100 µg/L) of PVP/PEI-coated Ag NPs and used to feed adult zebrafish for 21 days, in order to analyze the trophic transfer of silver and the effects provoked by the dietary exposure. Silver accumulation in brine shrimps was analyzed during the dietary exposure and significant higher silver content was measured in brine shrimp larvae exposed to the high dose than in the control and in larvae exposed to the low dose. Metal transfer from brine shrimp larvae to adult zebrafish was corroborated by autometallography in the liver and intestine of zebrafish fed with brine shrimps exposed to Ag NPs, being the presence of BSDs especially detected in the intestine. The distribution of BSDs in the intestine followed a similar pattern to that described in **Chapter IV** after waterborne exposure to CdS NPs. This could be related with the fact that the size of the Ag NPs used for the dietary exposure (5 nm) was similar to the size of the CdS NPs (3.5-4 nm) used in **Chapter IV** for waterborne exposure, indicating than the distribution of NPs in the intestine may be

mainly driven by their size, which has been also suggested by Osborne et al. (2015) after the exposure of adult zebrafish for 4 days to Ag NPs of different sizes. We detected a significant increase in the presence of BSDs in the liver of zebrafish fed for 21 days with brine shrimps exposed to the high dose (0.1 mg/L), with a homogeneous BSD distribution. The metal transferred from brine shrimps to adult zebrafish was distributed to the liver leading to its accumulation into the lysosomes, as demonstrated by the presence of BSDs (Amaral et al., 2002), which in turn provoked the significant reduction in the time necessary to desestabilize the lysosomal membrane detected even at the environmentally relevant silver concentration used during the experimental process. Moreover, the dietary exposure provoked histopathological alteration in liver of zebrafish as vacuolization and necrosis, which were observed by the third day even at the lowest concentration. Previous studies had demonstrated the toxic effect in the liver at histopathological level after waterborne exposure to NPs (Choi et al., 2010; Devi et al., 2015). Now we have reported a toxic effect through dietary exposure even at environmentally relevant concentration, therefore, the diverse routes of entrance of NPs into the organisms must be taken into account to have an overall picture of the effect provoked by the NP exposure.

This PhD Thesis attempted to fill some gaps in our limited understanding on the effects elicited by exposure to metal and metal bearing NPs in zebrafish. Data presented herein suggest that the toxicity of metals depends mainly on the metal form to which the organism is exposed, being especially marked after the exposure to the ionic form of the metals. Toxicity of metal and meta-bearing NPs seems to depend on the chemical composition of the NPs and on their physicochemical characteristics such as size and the additives present in the NP suspension, but other factors such as the exposure time or the route of entrance in the organisms may have an important role in their toxicity. Thus, waterborne exposure to different NPs mainly affected the gills, which presented several histopathological alterations, along with the liver which presented in general a reduction of the stability of the lysosomal membrane, oxidative stress and a significant regulation of transcripts involved in DNA damage and repair and in the energetic metabolism, especially after the exposure to both forms of cadmium. Dietary exposure also led to a significant reduction of the stability of the

lysosomal membrane and to histopathological lesions in the liver by the third day of feeding with brine shrimps exposed to an environmentally relevant concentration, which also provoked an increase of BSDs in the intestine. In summary, differences in the characteristics of the metal and metal-bearing NPs, as well as different routes of exposure are determinant to assess the environmental risk of these new materials. For future studies, the interaction of nanomaterials with different emergent and legacy pollutants already present in the aquatic environment should also be taking into account (Wiesner et al., 2009; Rocha et al., 2015).

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V. CONCLUSIONS & THESIS

CONCLUSIONS

I. A ranking of toxicity for different metal bearing NPs has been established, being Ag NPs the most toxic of the tested NPs, followed by CdS and ZnO NPs, and finally by Au and SiO_2 NPs. The toxic effects of NPs to zebrafish embryos depended on their chemical composition (and, thus, solubility) and size, being the former the most relevant characteristic involved in the toxic effect according to our results. Accordingly, the soluble forms of the metals were more toxic than the nanoparticulated form, being the bulk form the least toxic. Additives present in the NP suspensions, such as sodium citrate and Ecodis P-90, are also an important factor contributing to the toxicity.

II. The interaction between the chorion and the NPs must be taking into account when the effect of the exposure to different NPs is studied, especially when data obtained at different exposure times are compared, since the chorion acts as a protector barrier against the entrance of SiO2 NPs into the organism during the first developmental stages.

III. Silver accumulation was observed in zebrafish exposed to Ag NPs of 20 nm and to ionic silver, being the gills, liver and the intestine target organs for accumulation. The histopathological alterations observed in gills indicate that this organ is one of the main entrances of NPs into the organism. The intestine, where silver has been detected, has also to be considered as an important route of uptake, as NPs can easily attach to food particles.

IV. No evident effects on hepatic lysosomes, oxidative stress or genotoxicity were found in zebrafish exposed to Ag NPs of 20 nm. Therefore, at the assayed concentration, close to environmentally relevant concentrations, Ag NP exposure may produce slight toxic effects in aquatic organisms.

V. The analysis of the whole hepatic transcriptome showed that exposure to Ag NPs of 20 nm or to ionic silver provoked similar effects but at different exposure times. Exposure to ionic silver for 3 days provoke significant alteration of transcripts mainly involved in the energetic metabolism, while such pathways were significantly altered after 21 days of exposure to Ag

NPs. Moreover, the Ag NP exposure provoked strong effects in the immune system and in reproductive processes, indicating that the Ag NP effects at transcriptomic level is not solely due to the release of ions but also to the NP form.

VI. The waterborne exposure of adult zebrafish to CdS NPs and to ionic cadmium produced a significant cadmium accumulation in the whole organism, being the gills and the intestine the main entrances of NPs in the organism. Differences in the results obtained after the exposure to both forms of cadmium were reflected in the lysosomal membrane stability test and in the oxidative damage to proteins, indicating that the toxicity of cadmium may vary depending on the form of the exposure compound.

VII. The exposure to CdS NPs and to ionic cadmium provoked alteration of the zebrafish liver transcriptome. The effect greatly increased with exposure time and was more pronounced after the exposure to ionic cadmium than after exposure to CdS NPs. The functional analysis showed a significant effect in the immune response and oxidative stress after the exposure to CdS NPs for 21 days, while the exposure to ionic cadmium affected significantly those pathways involved in DNA damage and repair and in the energetic metabolism. Therefore, the analysis of the liver transcriptome under our experimental conditions reveals a different effect in zebrafish depending on the cadmium form.

VIII. The acute toxicity of PVP/PEI coated Ag NPs of 5 nm varies depending on the species, being zebrafish embryos much more sensitive than brine shrimp nauplii. Silver accumulated in brine shrimps exposed to sublethal concentrations of Ag NPs was effectively transferred through the diet to adult zebrafish. The silver transfer caused toxic sublethal effects and acted in detriment of the health of the fish as indicated by the significant reduction of the stability of the hepatocyte lysosomal membrane and the presence of histopathological alterations in the liver. Therefore, the potential risks to which predators in high levels of the food chain are exposed by the release of NPs into the natural environment is envisaged.

THESIS

The toxicity of metal and metal bearing nanoparticles to zebrafish embryos vary depending on the characteristics of the nanoparticles, such as chemical composition and size, and is strongly affected by the presence of additives in the nanoparticle suspension. Moreover, the nanoparticles toxicity differs from the toxicity provoked by the ionic or bulk counterpart of the metal. The exposure of adult zebrafish to metal and metal bearing nanoparticles and to the ionic form of the metal leads to bioaccumulation and to several effects related to oxidative stress, lysosomal membrane stability or tissue damage. The whole hepatic transcriptome of adult zebrafish shows specific responses to the exposure to different nanoparticle types (Ag *versus* CdS) and metal forms (nanoparticle *versus* ionic), but common responses, such as alteration of the energy metabolism, are also envisaged.

VI. APPENDIX

ABBREVIATIONS

AP, Ammonia persulfate

BSA, Bovine serum albumin

BSDs, Black silver deposits

DDSA, Dodecenylsuccinic anhydride

DMSO, Dimethyl sulfoxide

DNP, 2-4-dinitrophenyl hydrazine

dpf, Days post fertilization

DTT, Dithiothreitol

EC_{50}, Effective concentration to the 50% of the population

EDTA, Ethylenediaminetetraacetic acid

FET, Fish embryo toxicity

H/E, Hematoxylin/Eosin

hpf, Hours post fertilization

hph, Hours post hatch

LC_{50}, Lethal concentration to the 50% of the population

LMS, Lysosomal membrane stability

LP, Labilization period

MNA, Methylnorbornene 2,3-dicarboxylic acid anhydride

PAA, Polyacrylamide

PBS, Phosphate buffered saline

PVDF, Polyvinylidene fluoride

qPCR, Quantitative real time polymerase chain reaction

RIN, RNA integrity number

RT, Room temperature

SDS, Sodium dodecyl sulfate

TEM, Transmission electron microscopy

TEMED, Tetramethylethylenediamine

Vv, Volume density

Protocols of experimental procedures

1. Animal culture

1.1 Zebrafish (*Danio rerio*)

- Maintenance
- Reproduction and growth

1.2 Brine shrimp (*Artemia sp.*)

- Culture set up
- Performance counting

2. Acute toxicity test

2.1 Fish embryo toxicity (FET) test

2.2 Brine shrimp immobilization test

2.3 Statistics

3. Experimental exposure

3.1 Water-borne exposure of zebrafish

3.2 Water-borne exposure of brine shrimp

3.3 Dietary exposure of zebrafish

3.4 Sample collection

4. Chemical analysis of silver content

4.1 Zebrafish

- Protocol 1
- Protocol 2

4.2 Brine shrimps

5. Genotoxicity assessment: micronuclei frequency

6. Molecular biology techniques

6.1 Microarray analysis

6.2 qPCR

6.3 Western blot analysis

- Ubiquitination
- Carbonylation

7. Lysosomal membrane stability

8. Histological procedures

8.1 Histological preparations

8.2 Hematoxylin/Eosin (H/E) staining and histopathology

8.3 Autometallographical staining and quantification of black silver deposits

- Protocol 1
- Protocol 2

8.4 Alcian blue staining and quantification of volume density of globet cells

9. Electron microscopy

9.1 Transmission electron microscopy (TEM) and X-ray microanalysis

9.2 Field emission scanning electron microscopy (FESEM)

1. Animal culture

1.1 Zebrafish (*Danio rerio*)

❖ Maintenance

- Keep zebrafish in a temperature-controlled room at 28 ºC with a 14-hour light/10-hour dark cycle in a fish density of 1 fish each 2 L in tanks provided with mechanic and biological filters (Figure 1);

- Maintain water in continuous movement through an air triggered siphon or continuous water flow to provide an appropriate aeration to the tank;

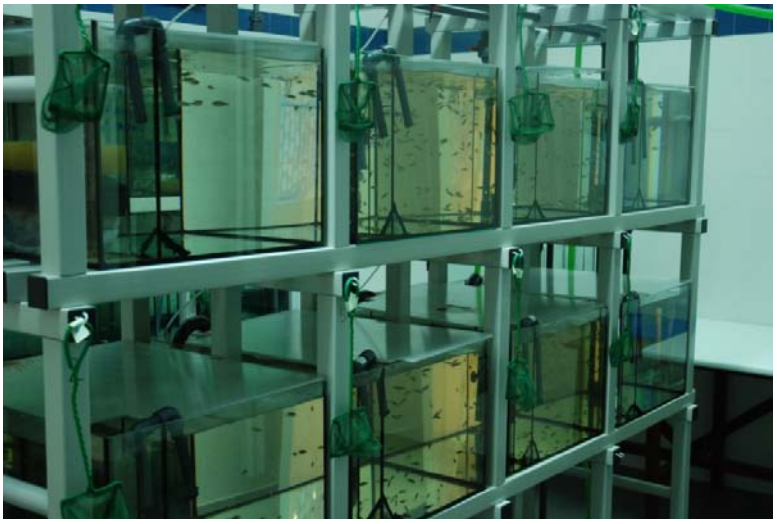


Figure 1- Tanks used to maintain the zebrafish stock

- Condition the water by passage through a deionization system and, then, buffer to pH 7.2 with Sera pH plus (Sera, Heinsberg, Germany) and to 600 µsΩ with commercial marine salt (Sera marin Salt);

- Feed fish twice per day with Sera Vipagran baby and/or brine shrimp larvae (INVE Aquaculture, Salt Lake City, Utah, USA) hatched in the laboratory;

- Measure physico-chemical parameters (nitrate, nitrite and ammonium concentrations) once per week using Sera ammonium, nitrite and nitrates kits and change the water totally or partially if the values are higher than zero mg/L for ammonium or nitrite, and 50 mg/L in the case of nitrate.

❖ Reproduction and growth

- Clean 2 L tanks and breeding tramps with water and disinfect them with H_2O_2 diluted in osmotic water (600 µL H_2O_2 in 1 L), dry completely before use;

- Select breeding fish and place females in small separate baskets hanging from the upper part of the tank in order to avoid continuous spawning;

- Place, the day before embryos are required, one female and one or two male zebrafish in breeding tramps previously located in a 2 L tank containing conditioned water (Figure 2);

- Leave the fish overnight and collect the resulting embryos the next morning;

Figure 2- Breeding tramps placed in 2 L tanks with one or two males and one female zebrafish per tramp.

- Collect the embryos with a plastic Pasteur pipette or with a siphon and a 400 µm mesh and place them in Petri dishes with 600 µS Ω water;

- Maintain embryos for 120 h in Petri dishes and clean them daily removing dead embryos and replacing water;

- Place 50-60 embryos in a small tank with an open water system to grow up them for 3 months (Figure 3);

- Feed newly born zebrafish twice per day in accordance with their food requirements: from 6 to 14 days post fertilization (dpf) only with Micron (Sera), from 14 to 30 dpf with Micron and brine shrimp larvae, from 30 to 60 dpf with Vipan baby (Sera) and brine shrimp larvae, and from 60 dpf with Vipagran baby and brine shrimp larvae;

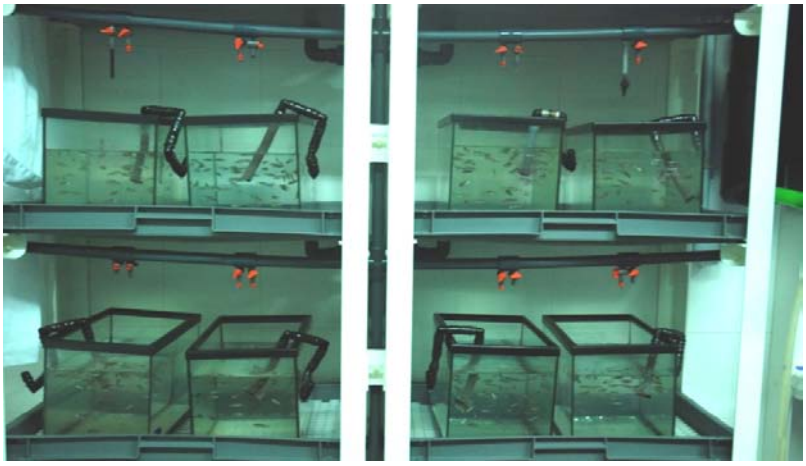


Figure 3- Nursery with tanks used to grow up zebrafish.

- Transfer zebrafish to larger tanks, in the same conditions and maintain them as previously described.

1.2 Brine shrimps (*Artemia sp.*)

❖ Culture set up

- Weight 35 g of commercial marine salt (Sera) and add them to a reactor made from 1.5 L plastic bottles (Figure 4) containing 1 L of deionized water;

- Weight 2 g of brine shrimp cysts (INVE Aquaculture) and add them to the artificial salt water;

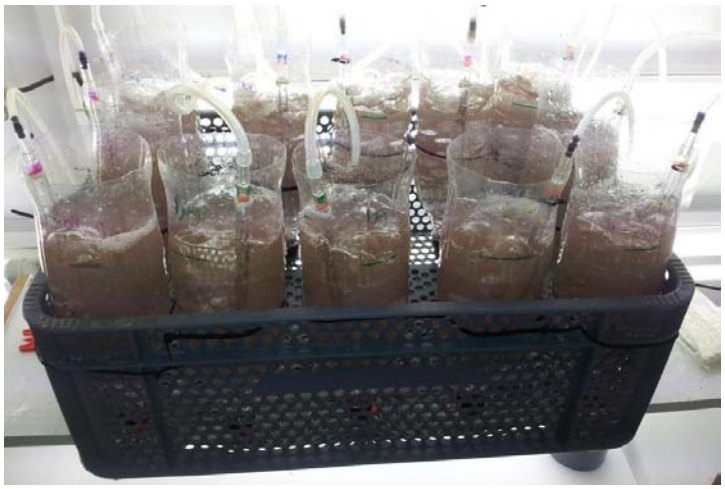


Figure 4- Reactors made from 1.5 L plastic bottles with the brine shrimp culture and continuous aeration.

- Maintain brine shrimp cultures for 48 h with continuous aeration in a room with illumination and controlled temperature (28 ºC);

- Stop the aeration and allow non-hatched cysts to settle down for some minutes;

- Discard the first few mL of the bottom of the culture, corresponding to the non-hatched cysts, and collect brine shrimp nauplii at Instar I stage (24 h post hatch – hph) with the aid of a mesh of 150 µm. Discard the last few mL of the top of the culture, corresponding to empty cysts.

❖ **Performance counting**

- Set 10 cultures as described previously and, calculate the performance in 5 replicates for each culture;

- Estimate the number of hatched brine shrimps per liter as the mean value obtained in the last dilution step x 10000 as shown in Figure 5.

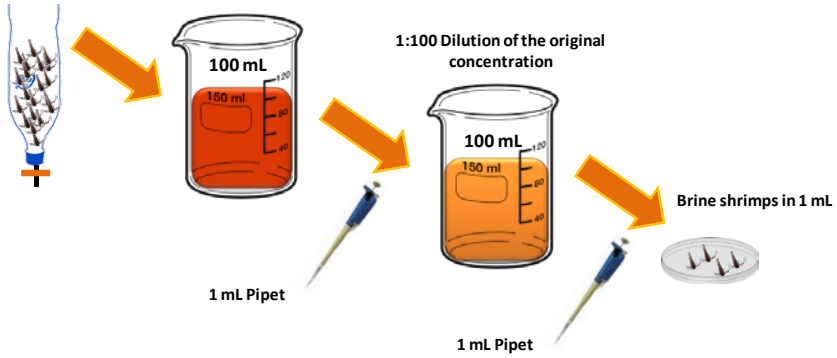


Figure 5- Steps of dilution from the culture to the 1 mL over which the count of hatched nauplii takes place (based on Treece, 2000).

2. Acute toxicity test

2.1 Fish embryo toxicity test (FET)

- FET experiments are performed according to the current standard guidelines (OECD TG236, 2013; ISO 15088, 2007);

- Place eggs in Petri dishes with 400 µS Ω water;

- Conduct the selection of the fertilized eggs under a stereoscopic microscope;

- Transfer fertilized eggs to Petri dishes with 200 µS Ω water (Figure 6) and, afterwards, transfer them to the exposure chambers;

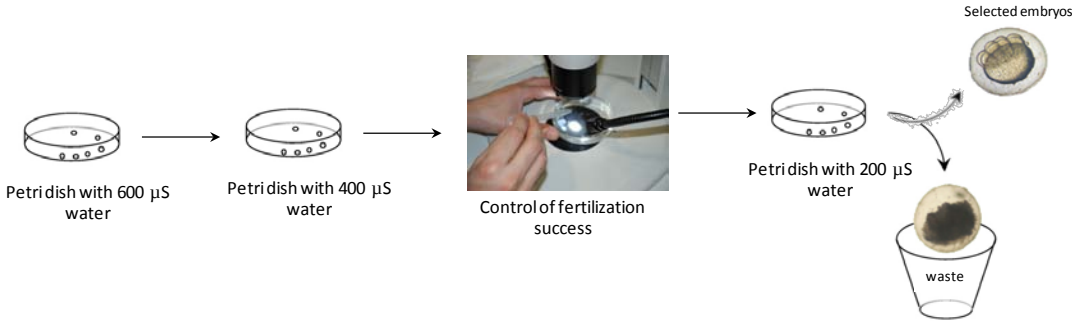


Figure 6- Selection of fertilized eggs under a stereoscopic microscope.

- Carry out the test in covered 24-well polystyrene microplates placing one embryo per well in 2 mL of test solution in deionized water. Test in each microplate two different concentrations (10 embryos in each concentration) and place four control embryos in deionized water (Figure 7);

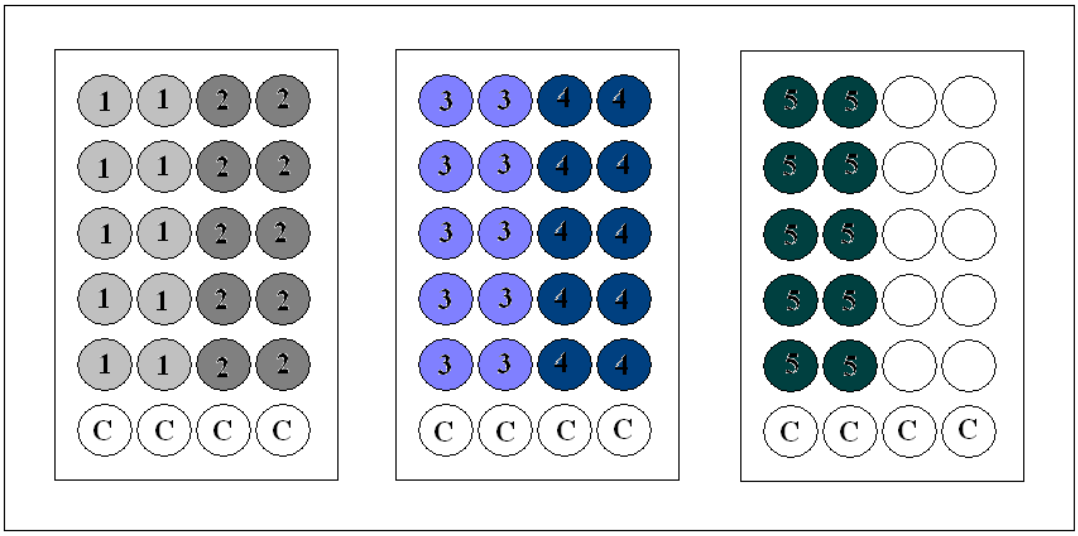


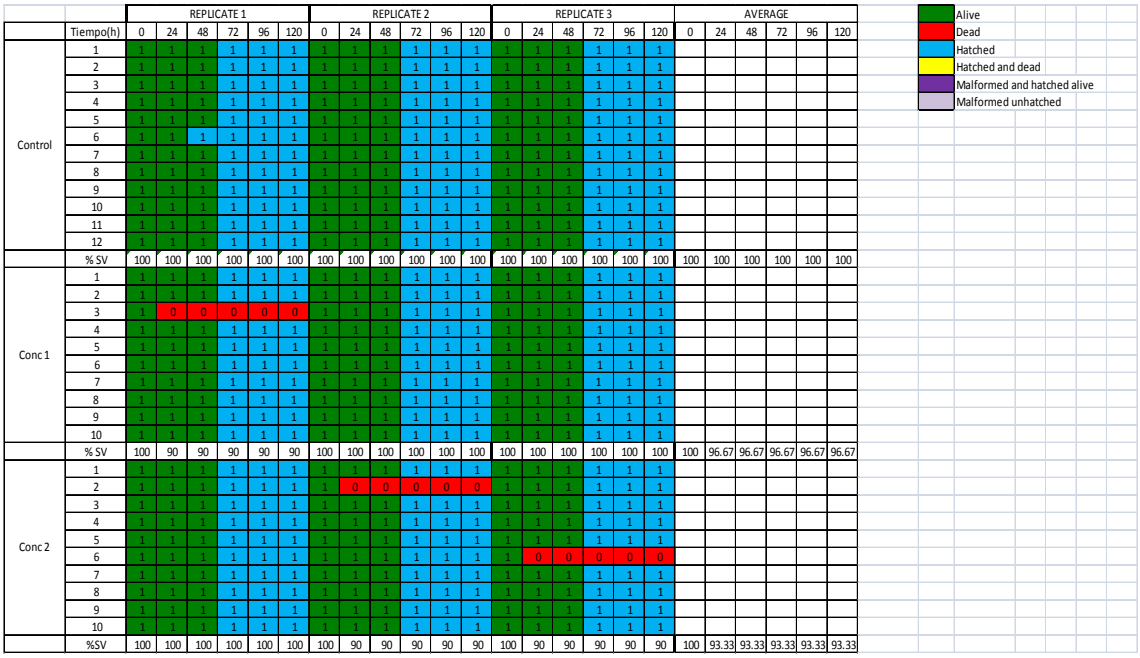
Figure 7- Schematic representation of the 24-well polystyrene microplates showing the sample distribution. Numbers indicate the different concentrations tested and C indicates the wells containing the control embryos.

- Prepare three replicates for each compound, resulting in 30 embryos exposed to each concentration and 36 control embryos. Start the exposure just after embryo selection and maintain to 120 hours post fertilization (hpf).

- Daily and up to the end of the test, examine the embryos under a stereoscopic microscope to determine:

- ✓ Survival rate: percentage of alive embryos at 120 hpf;
- ✓ Hatching rate: percentage of embryos that have hatched during the 120 h exposure period though some of them could have died by 120 hpf;
- ✓ Hatching time: time that embryos need to hatch;
- ✓ Malformation prevalence: percentage of malformed embryos over surviving embryos at 120 h. Expected malformations are: yolk sac edema, head edema, eye abnormality, pericardial edema, spinal cord flexure, tail flexure and finfold abnormality;

- Register the results in Excel files used as registration tables designed for that purpose (Figure 8);



		REPLICATE 1						REPLICATE 2						REPLICATE 3						AVERAGE					
	Tiempo(h)	0	24	48	72	96	120	0	24	48	72	96	120	0	24	48	72	96	120	0	24	48	72	96	120
Control	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	% SV	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Conc 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	3	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1						
	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	% SV	100	90	90	90	90	90	100	100	100	100	100	100	100	100	100	100	100	100	100	96.67	96.67	96.67	96.67	96.67
Conc 2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	2	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1						
	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	6	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0						
	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
	%SV	100	100	100	100	100	100	100	90	90	90	90	90	100	90	90	90	90	90	100	93.33	93.33	93.33	93.33	93.33

Figure 8- Example of Excel sheets designed for the daily registration of data during the FET. 1: embryo alive; 0: dead embryo.

- Use criteria described by Kimmel et al. (1995) to determine the normal morphology of the embryos. By means of a photographic camera, attached to a stereoscopic microscope, take photographs of malformed larvae;

- Consider the test as valid only when survival rate in the control group was ≥ 90% (OECD TG 236, 2013).

2.2 Brine shrimp immobilization test

- Use the procedure based on the standard OECD TG 202 (2004) for *Daphnia magna* to test the acute toxicity to brine shrimp larvae;

- Carry out the test at a temperature of 18.5 ± 0.5 ºC and continuous illumination;

- Select brine shrimp nauplii at instar I and II (approximately 24 and 48 hph) under a stereoscopic microscope and place 5-7 in each well (four replicate well per concentration) with 2 mL of test solution in covered 24-well polystyrene microplates. Run an unexposed control group in parallel (Figure 9);

- Score the number of immobilized individuals (consider immobilized brine shrimps as dead, while consider actively swimming ones as alive) at 24 and 48 h post exposure;

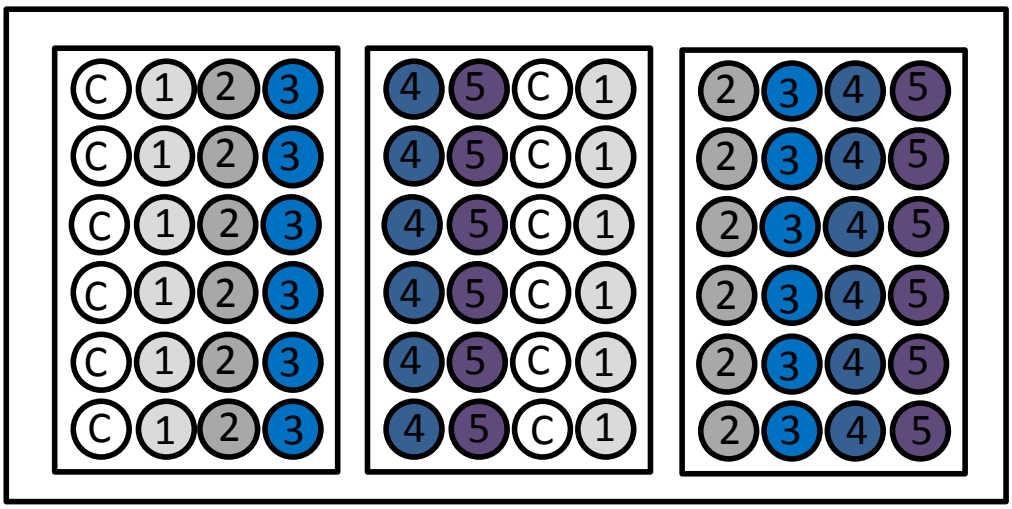


Figure 9.- Schematic representation of the 24-well polystyrene microplates showing the sample distribution. Numbers indicate the different concentrations tested and C indicates the wells containing the control nauplii.

- Consider the test as valid only in the case that more than 90% of the control brine shrimps survived during the exposure period (OCDE TG 202, 2004).

2.3 Statistics

- Calculate the LC_{50}/EC_{50} values through a Probit model ($p<0.05$). Estimate the parameters using the Firth method (Firth, 1993) in R, whenever convergence is not obtained using the maximum likelihood method (Kosmidis, 2013).

3. Experimental exposure

3.1 Water-borne exposure of zebrafish

- Place fish in the aquaria in an optimal density of 1 fish each 2 L (Figure 10);

Figure 10- Experimental room equipped with glass tanks used to carry out the experimental exposures.

- Maintain water in continuous movement through an air pump to assure an appropriate aeration to the tank;
- During the exposure period, change approximately 2/3 of the aquarium water by siphoning every three days and redose the corresponding volume of contaminated or clean water;
- Feed fish with Vipagran baby (Sera) and live brine shrimps twice per day;
- After the exposure period, if applicable, maintain remaining fish in clean water. During this period, feed fish only with Sera Vipagran baby twice per day;
- During post-exposure period, use biological and physical filters to maintain the chemical parameters of the water (nitrate, nitrite and ammonium). Control water chemical parameters once per week using Sera ammonium, nitrite and nitrate kits and change the water if the values are higher than zero mg/L for ammonium or nitrite and 50 mg/L for nitrate.

3.2 Water-borne exposure to brine shrimp

- Previous to the water-borne exposure, determine the test concentrations through a bioaccumulation test as following:
- Set cultures of brine shrimps containing 2 g cysts/L;
- Clean the cultures as described in **1.2**
- Expose two cultures of Instar I brine shrimps to each concentration for 24 h and, then, split each culture into two samples. Maintain an unexposed control culture;
- Collect brine shrimps using a mesh of 150 µm.
- For the water-borne exposure, set cultures containing 2 g cysts/L of brine shrimps;
- Clean the cultures as described in **1.2**
- Expose a culture of Instar I brine shrimps to each concentration selected for 24 h;
- Collect a half culture of brine shrimps using a mesh of 150 µm.

3.3 Dietary exposure of zebrafish

- Transfer the adult zebrafish to experimental tanks equipped with biological filters and air pumps for water aeration and recirculation keeping an optimal fish density of 1 fish each 2 L;
- Control water chemical parameters as indicated above;
- Feed zebrafish twice per day with brine shrimps of 24 hph exposed for 24 h prepared as indicated in 3.2;

- Adjust to the amount of feeding to 2.5% of body weight (Blanco-Vices and Sánchez-Vázquez, 2009; Lawrence et al., 2012);

- Keep a control fed group with unexposed brine shrimps in parallel in identical experimental conditions.

3.4 Sample collection

Equipment and reagents:

- Anesthetic (Benzocaine E1501, Sigma-Aldrich, St. Louis, Missouri, USA)
- Stereoscopic microscope (Nikon smz800, Kanagawa, Japan or similar)
- Eppendorf-type tubes (A142632G, Eppendorf, Hamburg, Germany)
- Cryovials (Thermo Scientific, Braunschweis, Germany)
- Dissection material: scissors, scalpels, tweezers, Petri dishes with solid paraffin
- Embedding cassettes (M503-3 Simport Qc, Canada)
- RNA later (ThermoFisher Scientific, California, USA)
- Zirconia/silica beads (Biospec, Bartlesville, USA)
- Formaldehyde 40% (Panreac 211328, Barcelona, Spain)
- $Na_2HPO_4.12H_2O$ (Panreac 131678)
- $NaH_2PO_4.H_2O$ (Panreac 13965)
- 25% Glutaraldehyde solution (Sigma-Aldrich G5882)
- Sodium cacodilate trihydrate (Sigma-Aldrich C0250)
- Liquid nitrogen
- Microscope glass slides (Thermo-Scientific or similar)
- Cryo-M-Bed (Jung, Heidelberg, Germany)

Procedure:

- Euthanize the fish by overdose of anesthetic (250-500 mg/L);

- For chemical analyses of metal content, place 20 whole zebrafish individually in cryovials and freeze in liquid nitrogen. Store at -80 ºC until analysis;

- For genotoxicity assessment, collect blood samples of 10 individuals by tail cutting and direct blood smear on clean microscope glass slides;

- For transcriptomics, dissect out the liver of 20 male individuals, pool in groups of 4 livers each one, place them in cryovials with RNA later and zirconia/silica beads and freeze in liquid nitrogen;

- For Western Blot analysis, collect the visceral mass of 10 individuals, place them individually in cryovials and freeze them in liquid nitrogen;

- For lysosomal membrane stability test, dissect out the liver of 5 individuals, embed in Cryo-M-Bed and freeze in liquid nitrogen;

- For histological preparations, dissect out the gills and visceral mass of 10 individuals, place them in histological cassettes and immerse them in freshly prepared fixative (10% neutral buffered formalin) for 24 h at 4 ºC. These samples are also used for autometallography and field emission scanning electron microscopy (FESEM);

Preparation of fixative (10% neutral buffered formalin):

57.84 g $Na_2HPO_4.12H_2O$
5.12 g $NaH_2PO_4.H_2O$
200 mL 40% formaldehyde
1700 mL H_2O adjust pH 7
up to 2000 mL with dH_2O

- For transmission electron microscopy (TEM), collect gills and liver samples of 5 individuals and immerse them in freshly prepared fixative (glutaraldehyde 2.5% in cacodilate 0.1 M), pH 7.2 for 1 h at 4 ºC;

glutaraldehyde 2.5% in cacodilate 0.1 M:

Sodium cacodilate 0.1 M: 5.35 g Sodium cacodilate trihydrate in 250 mL dH_2O
Glutaraldehyde 2.5% in cacodilate buffer: 5 mL glutaraldehyde + 45 mL cacodilate 0.1 M, adjust pH to 7.2.

4. Chemical analysis

4.1 Zebrafish

Equipment and reagents:

- Micropipette set and tips
- 130 ºC oven
- Precision electronic balance (CP225D-OCE Sartorius, Göttingen, Germany)
- Erlenmeyer flasks of 25 mL
- 65% nitric acid (extra pure quality 143255.1611, Scharlau, Barcelona, Spain)
- 69% nitric acid (Tracepur® 1.5187.1000, Merck Millipore, Amsterdam, Netherlands)
- 37% hydrochloric acid (Tracepur® 1.00317.1000, Merck Millipore)
- Crystal balls
- 80 ºC hot plate (Plactronic, JP Selecta, Barcelona, Spain or similar)
- Heraeus Labofuge 200 centrifuge (Hanau, Germany)
- Crystal tubes

Procedure:

❖ **Protocol 1**

- Dry 20 whole zebrafish individually in a 130 ºC oven for 24 h;
- Weight and pool dry tissues (pools of similar weight containing four zebrafish each);
- Place each pool into 25 mL Erlenmeyer flasks containing 2 mL of 65% nitric acid for tissue digestion (24-48 h);
- Block the mouth of the Erlenmeyer flask with a crystal ball to minimize evaporation;
- Evaporate the remnant liquid in an 80 ºC hot plate inside an exhaust hood;
- Add 2.5 mL of 0.1 M nitric acid to each Erlenmeyer flask, and leave for 1 day at 4 ºC;
- Transfer each sample into a clean tube and centrifuge in a Heraeus Labofuge 200 centrifuge for 4 min at 2,000 rpm at 4 ºC;
- Remove the supernatants to clean tubes, and store at 4 ºC until analysis;
- Measure the metal content by inductively coupled plasma atomic emission spectrometry (ICP-AES);
- Calculate the metal content (µg/g) using the equation: [Metal]*2.5*(1/Pool weight) = µg/g, where [Metal] = metal concentration in µg/mL; 2.5 = mL nitric acid added to each Erlenmeyer flask; Pool weight = Weight obtained in each pool in g.

❖ **Protocol 2**

- Dry 20 whole zebrafish individually in a 130 ºC oven for 24 h;
- Weight and pool dry tissues (pools of four zebrafish each);
- Place each pool into 25 mL Erlenmeyer flasks containing 2 mL of aqua regia (25% nitric acid (69%) + 75% hydrochloric acid (36%)) for tissue digestion (24-48 h);
- Block the mouth of the Erlenmeyer flask with a crystal ball to minimize evaporation;
- Evaporate the remnant liquid in an 80 ºC hot plate inside an exhaust hood;
- Add 2.5 mL aqua regia 0.1 M to each Erlenmeyer flask, and keep for 1 day at 4 ºC;
- Transfer each sample into a clean tube and centrifuge in a Heraeus Labofuge 200 centrifuge for 4 min at 2,000 rpm at 4 ºC;
- Remove the supernatants to clean tubes, and store at 4 ºC until analysis;
- Measure the metal content by inductively coupled plasma mass spectrometry (ICP-MS);

- Calculate the metal content (µg/g) using the equation: [Metal]*2.5*(1/Pool weight) = µg/g, where [Metal] = metal concentration in µg/mL; 2.5 = mL aqua regia added to each Erlenmeyer flask; Pool weight = Weight obtained in each pool in g.

4.2 Brine shrimps

Equipment and reagents:

- Erlenmeyer flasks of 25 mL
- 130 ºC oven
- Micropipette set and tips
- Precision electronic balance (CP225D-OCE Sartorius or similar)
- 69% nitric acid (Tracepur® 1.5187.1000, Merck Millipore)
- 37% hydrochloric acid (Tracepur® 1.00317.1000, Merck Millipore)
- Crystal balls
- 80 ºC hot plate (Plactronic, JP Selecta o similar)
- Heraeus Labofuge 200 centrifuge (Hanau)
- Crystal tubes

Procedure:

- Introduce the sample in pre-weighted 25 mL Erlenmeyer flasks

- Dry the sample in an oven at 130 ºC for 24 h;

- Weight dry sample plus the Erlenmeyer flasks in order to calculate the dry weight of the brine shrimp sample;

- Add 6 mL of aqua regia (25% nitric acid (69%) + 75% hydrochloric acid (36%)) for sample digestion (24-48 h);

- Block the mouth of the Erlenmeyer flask with a crystal ball to minimize evaporation;

- Evaporate the remnant liquid in an 80 ºC hot plate inside an exhaust hood;

- Add 2.5 mL aqua regia 0.1 M to each Erlenmeyer flask, and keep for 1 day at 4 ºC;

- Transfer each sample into tubes and centrifuge in a Heraeus Labofuge 200 centrifuge for 4 min at 2,000 rpm at 4 ºC;

- Remove the supernatants to clean tubes, and store at 4 ºC until analysis;

- Measure the metal content by inductively coupled plasma mass spectrometry (ICP-MS).

- Calculate the metal content (µg/g) using the equation: [Metal]*2.5*(1/weight) = µg/g, where [Metal] = metal concentration in µg/mL; 2.5 = mL aqua regia d added to each Erlenmeyer flask; Pool weight = Weight obtained in each sample in g.

5. Genotoxicity assessment: micronuclei frequency

Equipment and reagents:

- Microscope slides and cover slides (Thermo Scientific or similar)
- Methanol (Panreac 211081 or similar)
- Giemsa (Sigma-Aldrich GS-500)
- DPX mounting media (Sigma-Aldrich 06522 or similar)
- Light microscope (Olympus BX51)

Procedure:

- Leave the blood smears to air-dry;

- Fix them for 15 min in cool methanol at 4 ºC;

- Stain with 6% Giemsa for 15 min;

- Rinse in tap water several times and leave the slides to air-dry overnight;

- Mount the slides with DPX;

- Score 2000 erythrocytes per individual fish under a light microscope at a magnification of 100x;

- Use the following criteria to determine the presence of micronuclei: size not bigger than a 1/3 diameter of the main nucleus, same texture and colour, clearly separated from the main nucleus and with oval or circular shape (Baez-Ramirez and Prieto-García, 2005).

6. Molecular biology techniques

6.1 Microarray analysis

- RNA extraction

Equipment and reagents:

- Micropipette set and tips
- 1 mm Zirconia/Silica beds (BioSpec products, Inc, Oklahoma, USA)
- Ethanol (Panreac 131086)
- DNase-RNase free water (Life Technology 10977-035, California, USA)
- Chloroform (Scharlau CL01981000, Ciudad, País)
- Isopropyl alcohol (Scharlau AL03101000)
- Eppendorf tubes (1.5-2 ml)
- Refrigerated centrifuge (Eppendorf Centrifuge5415 R, Hamburg, Germany)
- Tissue homogenizer (Hybaid Rybolyser^{TM} cell disruptor, Ashford, UK)
- TRIzol® (15596018, ThermoFisher Scientific)

Procedure:

- Homogenize the samples in 0.5 mL of TRIzol® using a Hybaid Ribolyser™ cell disrupter at 4 m s^{-1} for 40 s;
- Incubate the homogenized samples at room temperature (RT) for 5 min to allow complete dissociation of nucleoprotein complexes;
- Add 0.1 mL of chloroform and hand shake the tubes vigorously for 15 s;
- Incubate the tubes at RT for 2 min;
- Centrifuge in a refrigerated Eppendorf centrifuge the samples at 12,000 g for 15 min at 4 ºC. By centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase, RNA remains exclusively in the aqueous phase;
- Transfer the aqueous phase to a new tube;
- Add 0.25 mL of isoproponyl alcohol to precipitate the RNA, shake by hand 4/5 times;
- Incubate the samples at RT for 10 min;
- Centrifuge at 12,000 g for 10 min at 4 ºC. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the bottom of the tube;
- Remove the supernatant and wash the RNA pellet with 0.5 mL of 75% ethanol;
- Mix the samples by vortexing and centrifuge at 7,500 g for 5 min at 4 ºC;
- Dry the RNA pellet for 5 min, but not completely since complete drying would decrease its solubility (in darkness);
- Dissolve the pellet in 100 µL of RNase free water.

- Measurement of the concentration and purity of nucleic acids

Equipment and reagents:

- Micropipette set and tips
- RNase-/DNase-/protein-free microtube tube (Eppendorf 4092.3N)
- UV spectrophotometer (Eppendorf BioPhotometer)
- RNase-/DNase-/Protein-free disposable single sealed cuvette (Eppendorf 952010051)
- RNase-/DNase-/Protein-free water (Thermo Scientific HyClone SH30538.02)

Procedure:

- Dilute the RNA sample (2:98 or 1:60) in RNase-/DNase-/Protein-free water inside a RNase-/DNase-/protein-free microtube tube;

- Select RNA function in the spectrophotometer;

- Add RNase-/DNase-/Protein-free water to a RNase-/DNase-/Protein-free disposable single sealed cuvette (100 µL) and establish the "blank" value;

- Change the cuvette and add the dilution previously prepared (100 µL);

- Enter data in the spectrophotometer regarding the dilution and the nucleic acid type to be measured (the total RNA isolated should have an A_{260}/A_{280} ratio of 1.8–2.2).

- RNA cleanup

Equipment and reagents:

- Micropipette set and tips
- Eppendorf tubes (Eppendorf 4092.3N)
- Reffrigerated centrifuge (Eppendorf Centrifuge 5415 R)
- Vortex (Yellowline TTS2, IKA, Staufen Germany)
- Mini-centrifuge (Sprout™ Heathrow Scientific, Illinois, USA)
- RNeasy® MinElute® Cleanup Kit (Qiagen, Venlo, Netherlands)
  - Buffer RTL
  - Buffer RPE
  - RNase free water
- Ethanol (Panreac 131086)
- Agilent RNA Nano LabChips (Agilent Technologies, California, USA)

Procedure:

- Adjust the volume of each sample to 100 µL with RNase-free water and mix;

- Add 350 µL of Buffer RLT and mix;

- Add 250 µL of ethanol (96-100%) and mix well by pippeting;

- Transfer the sample (700 µL) to an RNase Mini spin column placed in a 2 mL collection tube. Centrifuge for 15 s at 10,000 rpm;

- Add 500 µL of buffer RPE to the RNeasy spin column. Centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through;

- Add 500 µL of buffer RPE to the RNeasy spin column. Centrifuge for 2 min at 10,000 rpm to wash the spin column membrane;

- Place the RNeasy spin in a new 2 mL collection tube. Centrifuge at full speed for 1 min;

- Place the RNeasy spin in a new 1.5 mL collection tube. Add 30-50 µL of RNase-free water. Centrifuge for 1 min at 10,000 rpm to elute the RNA;

- Measure the purity of RNA with an Agilent RNA Nano LabChips in order to confirm the quality of the samples for further steps (samples with a RIN greater than 8.0 are considered of a good quality);

- Store at -80 ºC until analysis.

- Microarray analysis

Equipment and reagents:

- Micropipette set and tips
- NanoDrop ND-8000 UV-VIS spectrophotometer (Thermo Scientific)
- NanoDrop ND-1000 spectrophotometer (Thermo Scientific)
- One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) (Agilent Technologies)
- Low imput Quick Amp Labeling kit, One color (Agilent Technologies)
- AffinityScript Reverse Transcriptase (AffinityScript RT, Agilent Technologies)
- Silica-based RNeasy spin columns (Qiagen)
- Zebrafish 44 k full genome microarrays (version V3, AMADID 026437 Agilent Technologies)
- DNA microarray scanner (Agilent Technologies G2565CA)
- Ozone-barrier slide covers (Agilent P/N G2505-60550)
- Feature Extraction software (Agilent Technologies)
- GeneSpring GX software (Agilent Technologies)

Procedure:

- Quantify RNA samples using a NanoDrop ND-8000 UV-VIS spectrophotometer;

- Label samples following the Agilent Technologies protocol: "One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling)";

- Retrotranscrib 100 ng of purified RNA with AffinityScript Reverse Transcriptase, using Oligo dT primers coupled to T7 promoter;

- *In vitro* transcribe the double stranded cDNA synthesized by AffinityScript RT by T7 RNA polymerase in the presence of Cy3-CTP fluorophore to generate amplified and labeled cRNA;

- Purify the labeled samples with silica-based RNeasy spin columns;

- Quantify cRNA with a NanoDrop ND-1000 spectrophotometer in order to determine the yield and specific activity of each reaction;

Yield (µg of cRNA) should be > 1.65 µg
Specific activity (pmol Cy3 per µg of cRNA) should be >6

- Perform the hybridizations on zebrafish 44 k full genome microarrays (version V3, AMADID 026437) containing 43,803 unique probes using the SuperHyb hybridization chamber;

- Scan using a G2565CA DNA microarray scanner, with ozone-barrier slide covers (Agilent P/N G2505-60550) and default parameters;

- Use the Feature Extraction software to feature signal intensity extraction and apply quantile normalization to the raw intensities (log2 values) using Agilent GeneSpring GX software.

6.2 qPCR

- Reverse transcription

Equipment and reagents:

- Micropipette set and tips
- AffinityScript multi temperature cDNA synthesis kit (Agilent Technologies)
- Thermal Cycler (ThermoFisher Scientific 2720)
- SuperCycler (Agilent Technologies 8800)
- 0.5 mL and 0.2 mL tubes
- RNase-free water (Thermo Scientific)

Procedure:

- Mix and centrifuge briefly each sample;

- Prepare RNA/primer mixture in sterile 0.5 mL tubes;

1 µg total RNA
3 µL random hexamers (0.1 µg/µL)
up to 15.7 µL RNase-free water

- Incubate the samples in the SuperCycler at 65 ºC for 5 min and cool the reaction at RT for 10 min;

- Add 8.6 µL of the following mixture to each sample, mix gently and spin down;

2 µL 10x AffinityScript RT buffer
0.8 µL of dNTP mix (25 mM of each dNTP)
0.5 µL of RNase Block Ribonuclease Inhibitor (40 U/µL)
1 µl AffinityScript Multiple Temperature Reverse Transcription

- Incubate the tubes in the termocycler at 25 ºC for 10 min;

- Mix gently and incubate again in the SuperCycler at 49 ºC for 60 min, at 70 ºC for 15 min and maintain at 4 ºC until subsequent PCR amplification.

- qPCRs

Equipment and reagents:

- Micropipette set and tips
- Thermo cycler (ThermoFisher Scientific 7300)
- Primer Express 3.0 software (ThermoFisher Scientific)
- TaqMan Reverse Transcription Reagent (TaqMan, New Jersey, USA)
- MicroAmp® Optical 96-Well Reaction Plate (ThermoFisher Scientific N8010560)
- MicroAmp™ Optical Adhesive Film (ThermoFisher Scientific 4311971)
- Heraeus Labofuge 200 centrifuge (Hanau, Germany)

Procedure:

- Use available commercial TaqMan® assays or design primers and probes using the Primer Express 3.0 software to amplify selected genes;

- Develop TaqMan qPCR amplifications in 20 µL reactions (per triplicate) containing

10 µL TaqMan master mix
1 µL TaqMan assay
2 µL of cDNA template at appropriate dilution
7 µL RNase-free water

- Use the universal PCR conditions for all genes

1 cycle at 50 °C for 2 min
1 cycle at 95 °C for 10 min
40 cycles at 95 °C for 15 s
40 cycles at 60 °C for 1 min

- Run a control without template for quality assessment and use a housekeeping gene for normalization of transcription levels of target genes;

- Calculate relative gene transcription with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) relative to the lowest value for each gene in the whole study:

$$\Delta\Delta Ct = \Delta Ct \text{ (experimental sample)} - \Delta Ct \text{ (selected reference value)}$$
$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$
$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

6.3 Western blot analysis

Equipment and reagents:

- Micropipette set and tips
- Trizma (Sigma-Aldrich T1503)
- HCl (Panreac 141020 o similar)
- Ethylendiamine tetracetic acid (EDTA, Sigma-Aldrich E6511)

- Protease inhibitor cocktail (Sigma-Aldrich P8340)
- Centrifuge Precellys 24-Dual homogenizer (Bertin Technologies, Montigny le Bretonneux, France)
- Epoch Biotek Spectrophotometer (Biotek France, Colmar, Cedex, France)
- Take3 Multi-Volume Plate (Biotek France)
- Sodium dodecyl sulfate (SDS, Sigma-Aldrich L3771)
- Dithiothreitol (DTT, 161-0301 Bio-Rad, Hercules, CA, USA)
- Glycerol (Sigma-Aldrich G8898)
- Bromophenol blue (Merck 8122)
- Precision Plus Protein Standards Dual-colour (Bio-Rad #1610374)
- Mini-PROTEAN electrophoresis chamber Power Pac™ (Bio-Rad #1658027FC)
- Polyvinylidene fluoride membrane (PVDF, Bio-Rad 1620177)
- Trans-blot turbo transfer system (Bio-Rad 1704155)
- Running buffer with SDS (Bio-Rad 1610732)
- Polyacrylamide (PAA, Bio-Rad 161-0156)
- Ammonia persulfate (AP, Sigma-Aldrich A3678)
- Tetramethylethylenediamine (TEMED, Sigma-Aldrich T9281)
- Phosphate buffered saline (PBS, Sigma-Aldrich P4417)
- Bovine serum albumin (BSA, Sigma-Aldrich A-9647)
- Polyclonal rabbit anti-ubiquitin antibody (Dako Z0458, Glostrup, Denmark)
- Peroxidase-conjugated anti rabbit Ig G, whole molecule (Sigma-Aldrich A-9169)
- 2,4-dinitrophenylhydrazine (DNP, Sigma-Aldrich D199303)
- Rabbit anti-DNP antibody (Sigma-Aldrich D9656)
- Methanol (Panreac 211091)
- GS-800 calibrated densitometer (Bio-Rad)
- Quantity One image analysis software (v. 4.6.5, Bio-Rad)
- Enhanced chemiluminescence kit (Thermo Scientific 32106)
- Photographic films (Biomax films 829 4985, Carestream, Madrid, Spain)

Procedure:

- Homogenize each sample with 500 µL of 10 mM Tris-HCl pH 7.2, 1 mM EDTA;

- Mix samples (previously homogenized in 500 µL of buffer solution) with protease inhibitor cocktail (200 µL cocktail in 9.8 mL homogenization buffer);

- Centrifuge at 2,500 rpm for 5 min in Centrifuge Precellys 24-Dual homogenizer;

- Remove the supernatant and measure total protein concentration as described by Pineiro et al. (1999), in an Epoch Biotek Spectrophotometer, using the Take3 Multi-Volume Plate, following manufacturer´s directions;

- Mix with buffer Tris/HCl pH 7.2 in order to obtain a sample concentration of 2 µg/µL;

- Mix 1:1 with Laemmli sample buffer;

Preparation of Laemmli buffer:

62.5 mM Tris-HCl, pH 6.8
8% SDS,
0.1 M DTT,
30% glycerol
2 mg/mL bromophenol blue

- Cook the samples at 95 ºC for 15 min;

- Prepare gels for the electrophoresis;

Charging gel (for 2 gels):
2.5 mL PAA stock
2.5 mL 1.5 M tris pH 8.8
1 mL running buffer with SDS
50 µl AP
up to 10 ml dH_2O
10 µL TEMED
Separating gel (for 2 gels):
625 µL PAA stock
1.25 mL 0.5 M tris pH 6.8
500 mL running buffer with SDS
up to 5 mL dH_2O
5 µL TEMED

- Place the separating gel between the glasses and use methanol in order to smooth the surface of the gel, allow to polymerize, remove the methanol and clean the surface with running buffer;

- Place the charging gel and place the comb up to polymerization;

- Charge 20 µg protein of sample into each lane and 5 µL of Precision Plus Protein Standards Dual-colour in one lane;

- Run the gel at 150 V for 5 min and, then, at 175 V for approximately 1 h (until the bromophenol blue front line reaches the bottom of the gel cassette) in a Bio-Rad Mini-PROTEAN electrophoresis chamber at RT;

- Transfer the separated proteins to a PVDF membrane by a trans-blot turbo transfer system for 30 min up to 1 A and 25 V;

- Place the membrane in a container and proceed as follows,

- **Immunodetection of ubiquitinated proteins** (all the steps in continuous shaking and RT):

Steps	**Time**
Wash in PBS	5 min
Block in PBS/BSA 1%	1 h
Wash in PBS (x2)	5 min
Incubate with polyclonal rabbit anti-ubiquitin antibody (1: 1000)	1 h
Wash in PBS (x4)	5 min
Incubate with peroxidase-conjugated anti rabbit Ig G, whole molecule (1:2000)	1 h
Wash in PBS (x4)	5 min

- **Immunodetection of carbonylated proteins** (all the steps in continuous shaking and RT):

Previous to the incubation with the specific antibody, some steps of derivatization are necessary

Dry completely the membrane at RT

Steps	**Time**
Ethanol 100%	15 sec
Dry completely	
20% ethanol-80% PBS	5 min
HCl 2 N	5 min
2-4- DNP (0.5 mM) in HCl 2 N	5 min
HCl 2 N (x3)	5 min
Ethanol 100% (x5)	5 min
Wash in PBS	5 min
Block in PBS/BSA 1%	1 h
Wash in PBS (x2)	5 min
Incubate with rabbit anti-DNP antibody (1:1000)	1 h
Wash in PBS (x4)	5 min
Incubate with peroxidase-conjugated anti rabbit Ig G, whole molecule (1:10000)	1 h
Wash in PBS (x4)	5 min

- Visualize the reaction with an enhanced chemiluminescence system (ECL). Incubate the membrane for 1 min in a 1:1 mixture of the two solutions of the ECL kit;

- Place the membrane between two plastic sheets;

- Expose the film on the membrane in the dark as long as necessary;

- Then, develop and fix the film following manufacturer´s directions;

- Scan photographic films using a GS-800 calibrate densitometer (Bio-Rad);

- Quantify the average optical density of the immunoreactive bands corresponding to free ubiquitin (8 kD) and carbonylated actin (40 kD) using the Quantity One image analysis software;

- Measure the band, adjusting the conditions as necessary (dark contrast, background);

- Set the value of the control group as 100%. Normalize the other groups data respect to the control value.

7. Lysosomal membrane stability

Equipment and reagents:

- Microscope slides and cover slides (Thermo Scientific 10144633B)
- Cryotome (CM3050S Leica Microsystems, Nussloch, Germany)
- Hellendal jars
- Sodium-citrate (Sigma-Aldrich S4641 or similar)
- PHmeter (Crison micropH 2001, Barcelona, Spain)
- NaCl (Sigma-Aldrich S9888 or similar)
- Napthtol AS-Bi phosphate (Sigma-Aldrich N4006)
- Dimethyl suphoxide (DMSO, Sigma-Aldrich D5819)
- POLYPEP (Sigma-Aldrich P5115)
- $Na_2HPO_4.12H_2O$ (Panreac 131678)
- $NaH_2PO_4.H_2O$ (Panreac 13965)
- Fast violet (Sigma-Aldrich F1631)
- $CaCl_2$ (Probus 049810)
- Formaldehyde 40% (Panreac 211328)
- Kaiser glycerine gelatin (Merck 1.09242.0100, Madrid, Spain)
- Shaking water-bath (JP Selecta, Barcelona, Spain)
- Light microscope (Olympus BX51)

Procedure:

- Cut 10 µm thick sections of frozen liver in a cryotome at a cabinet temperature of –24 ºC;

- Transfer the sections to microscope slides brought from RT and store at -40 ºC until staining;

- Place the slides in a Hellendal jar containing lysosomal membrane labilizing buffer for different times (0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min, according to Bilbao et al., 2010) at 37 ºC;

Preparation of lysosomal membrane labilizing buffer (Solution A)

2.352 g Na-citrate in 80 mL dH_2O
fix pH 4.5
fill up to 100 mL dH_2O
2.5 g NaCl

- Transfer the set of slides to substrate incubation medium and incubate for 15 min at 37 ºC in a Hellendal jar in a shaking water-bath;

Preparation of substrate incubation medium (to be prepared just 5 min before use)

10 mg of naphtol AS-BI phosphate dissolved in 1 mL DMSO
fill up to 50 mL with solution A
3.5 g POLYPEP

- Wash the slides in a saline solution (3% NaCl) at 37 ºC for 5 min;

- Transfer the slides to a diazodium dye for 9 min at RT;

Preparation of diazodium dye (in a dark room)

2.892 g of $Na_2HPO_4.12H_2O$ in 80.8 mL dH_2O
0.265 g of $NaH_2PO_4.H_2O$ in 19.2 mL dH_2O
mix both solutions and fix pH 7.4
2.5 g of NaCl and 100 mg fast violet, dissolve for 30 min

- Rinse the slides in running tap water for 10 min;

- Fix the sections in Baker's fixative at 4 ºC, rinse in dH_2O and mount in aqueous mounting medium with Kaiser glycerine gelatin;

Preparation of Baker's fixative:

1 g of calcium chloride in 10 mL of 40% formaldehyde
2.5 g NaCl
fill up to 100 mL with dH_2O

- Allow to dry before examining the slides under a light microscope;

- Determine the labilization period (LP) as the maximal accumulation of reaction product associated with lysosomes (Bröeg et al., 1999);

- Make four determinations for each individual liver by dividing each section in the acid labilization sequence into 4 approximately equal segments. Obtain a mean value for each section, corresponding to an individual.

8. Histological procedures

8.1 Histological preparations

Equipment and reagents:

- Tissue processor (Leica Microsystems ASP300)
- Paraffin wax dispenser (Electrothermal MH8523B, Staffordshire, UK)
- Base moulds disposable (Kaltek 2781, Padova, Italy)
- Microtome (Leica RM2125RT)
- Cold plate (PF100 Bio Optica, Milano, Italy)
- PHmeter (Crison micropH 2001)
- Disposable microtome blades (Leica Microsystems 818)
- Thermostatic water bath (Termofin, JP Selecta)
- Microscope slides (Thermo Scientific)
- Drying oven (37 ºC) (Selecte)
- Ethanol (Panreac 131086)
- Xylene (Fluka 95690, Steinheim, Germany)
- Paraffin (Panreac 253211)

Procedure:

- Replace the fixative (previously described, see **3.4**) by 70% ethanol for storage for not longer than one week until processing;

- Embed individual tissue samples in paraffin using an automatic tissue processor with the following embedding sequence under vacuum conditions:

Reagent	**Time and temperature**
70% ethanol	60 min at RT
96% ethanol (x2)	60 min at RT
100% ethanol (x2)	60 min at RT
100% ethanol:xylene (1:1)	60 min at RT
Xylene (x2)	60 min at RT
Paraffin (x3)	120 min at 56 ºC

- Place tissues in a plastic mould, oriented and totally covered with melted paraffin. Use the tissue cassette as holder;

- Leave the mould at RT for paraffin to harden for at least one day before removing the mould;

- Cut paraffin blocks in sections of 5 µm thickness using a microtome. Place the section on the surface of water at 45-50 ºC and allow expanding. Pick up sections on an albumin coated slide;

- Place the slides in a drying oven at 37 ºC overnight.

8.2 Hematoxylin/Eosin (H/E) staining and histopathological analysis

Equipment and reagents:

- Cover slides (Thermo Scientific)
- Robotic stainer (Leica autostainer XL) and coverslipper (Leica CV5030)
- Light microscope (Olympus BX51)
- Ethanol (Panreac 131086)
- Xylene (Fluka 95690)
- Harris hematoxylin solution (Sigma-Aldrich HHS32)
- Eosin yellowish hydroalcoholic solution 1% (Panreac 251301.1611)
- DPX mounting media (Sigma-Aldrich 06522)

Procedure:

- Use one slide and stain with the H/E protocol;

- Stain using an automatic stainer with the following sequence:

Reagent	**Time**
Xylene (x2)	10 min
100% ethanol (x2)	2 min
96% ethanol	2 min
70% ethanol	2 min
dH_2O	5 min
Harris hematoxylin	30 sec
Tap water (x2)	4 min
Eosin Yellowish	2 min
Tap water	30 sec
70% ethanol	2 min
96% ethanol	2 min
100% ethanol (x2)	2 min
Xylene (x2)	5 min

- Mount the slides with Kaiser's glycerine gelatin;

- Examine the histological sections of the visceral mass and gill tissue under an Olympus BX51 light microscope using the different objectives for closer examination of suspected pathologies.

8.3 Autometallographical staining and quantification of black silver deposits

❖ Protocol 1 (modified from Soto et al., 1998)

Equipment and reagents:

- Robotic stainer (Leica Microsystems autostainer XL)
- Ethanol (Panreac 131086)
- Xylene (Fluka 95690)
- Photographic emulsion (Ilford nuclear emulsion L4, Norderstdedt, Germany)

- B&W Negative developer (Tetenal, Norderstdedt, Germany)
- Acetic acid (Panreac 211008)
- B&W Film/Paper Fixer (AGFA, Mortsel, Belgium)
- Kaiser's glycerine gelatin (Merck 1.09242.0100)
- Timer
- Red light

Procedure:

- Use one slide per sample previously cut;

- Dewax and hydrate the tissues using an automatic stainer with the following sequences:

Reagent	**Time**
Xylene (x2)	10 min
100% ethanol (x2)	2 min
96% ethanol	2 min
70% ethanol	2 min
dH_2O	5 min

- Dry the slides in a drying oven at 37 ºC for 24 h;

- In a dark room, with red light, cover the slides with the photographic emulsion and keep in total darkness for 30 min;

- Develop the reaction in a bath containing a B&W Negative developer dilution 1:4.5 in deionized water for 15 min;

- Stop the reaction in 1% solution of acetic acid for 1 min;

- Fix the samples in a 10% solution of B&W Film/Paper Fixer in deionized water for 10 min;

- Wash the sections in deionized water;

- Mount the slides with Kaiser's glycerine gelatine;

- Allow to dry before performing the semiquantitative analysis of black silver deposits (BSDs) under a light microscope at a magnification of 20x;

- Use the following criteria (Vicario-Parés et al., 2014): no presence of BSDs (-), presence of homogeneously distributed small BSDs (+); homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size (++) and greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits (+++).

❖ Protocol 2:

Equipment and reagents:

- Timer
- Silver Enhancement Kit for Light and Electron Microscopy (BBI Solutions®, Cardiff, UK).
- Kaiser's glycerine gelatine (Merck 1.09242.0100)
- Laborlux S microscope (Leica)
- Biological Measure System (BMS) Software (Sevisan, Leioa, Biscay)
- IBM SPSS Software (Armonk, New York) MS-DOS version

Procedure:

- Use one slide per sample previously cut;

- Dewax, hydrate and dry the slides as mentioned above;

- In total humidity atmosphere cover the sample with the mixture (1:1) of the Silver Enhancement Kit for 20 min (this staining time should be adapted for different samples);

- Stop the reaction rinsing the slides in tap water;

- Mount the slides with Kaiser's glycerine gelatine;

- Examine slides under a 100x magnification objective at a Leica Laborlux S microscope;

- Measure 5 different sections of each sample for the quantification of the volume density of the developed black silver deposits (Vv_{BSDs}) in the tissue. Use for this purpose the Biological Measure System Software to quantify Vv_{BSDs};

- Calculate Vv_{BSDs} values according to the formulae presented by Lowe et al. (1981) as $Vv_{BSD} = V_{BSD}/V_{ti}$

V_{BSD} is the volume of BSDs
V_{ti} is the volume of the tissue

8.4 Alcian blue staining and quantification of volume density of globet cells

Equipment and reagents:

- Alcian blue (Sigma-Aldrich A5268)
- pHmeter (Crison micropH 2001)
- DPX mounting media (Sigma-Aldrich 06522)
- Light microscope (Olympus BX51) equipped with a camera (Nikon)
- CELL^D Software (Olympus)

Procedure:

- Use one slide per sample previously cut;

- Dewax, hydrate and dry the slides as mentioned above;

- Immerse the slides in 1% Alcian Blue pH 2.5 solution for 30 min;

- Clean in two baths of 30 sec with deionized water and two baths of 2 min of 100% ethanol;

- Mount the slides in DPX by means of a robotic coverslipper;

- Photograph two different fields of each sample at 10x magnification with a microscope equipped with a camera;

- Using the Olympus CELL^D Software, superimpose a 50x50 μm^2 squared grid over the micrographs;

- Count the number of intersections over globet cells, and the number of the intersections over intestine tissue;

- Calculate the Vv of the globet cells over the entire intestine using the formula: nº of intersections over globet cells / (nº of intersections over globet cells + nº of intersections over intestine).

9. Electron microscopy

9.1 Transmission electron microscopy (TEM) and X-ray microanalysis

Equipment and reagents:

- Sodium cacodilate trihydrate (Sigma-Aldrich C0250)
- pHmeter (Crison micropH 2001)
- Osmium tetraoxide (Sigma-Aldrich O5500)
- Potassium ferrocianure (Sigma P3289)
- Ethanol (Panreac 131086)
- Propylene (Sigma-Aldrich 295663)
- Epoxy embedding medium kit (Sigma-Aldrich 45359)
- EPON812 (Fluka 45345)
- DDSA (Fluka 45346)
- MNA (Fluka 45347)
- DMP30 (Fluka 45348)
- Syringe 50 mL (4616502F Braun, Melsungen, Germany or similar)
- Embedding polyethylene capsules (C052 TAAB, Aldermaston, England)
- Drying oven (60 ºC) (Selecte)
- Reichert Ultracut S ultramicrotome (Leica)
- Toluidine blue (Sigma-Aldrich 89640)
- Light microscope (Olympus BX51)
- Mesh copper grids (SPI supplies, PA, USA)

- Lead citrate (Fluka 15326)
- Uranyl acetate (Fluka 94260)
- NaOH (Fluka 71689)
- HT7700 transmission electron microscope (Hitachi, Tokyo, Japan)
- Field Emission Microscope Zeiss Auriga 405, 1 nm nominal resolution (Oberkochen, Germany) equipped with a Bruker Quantax energy dispersive x-ray spectroscope (EDS) (Energy Resolution: 123 eV K_α of the Mn)
- STEM module for Field Emission Microscope Zeiss Auriga 405

Procedure:

- After 24 h in fixative (previously described, see **3.4**), wash the samples 2x15 min in cacodilate buffer 0.1 M, pH 7.2;

- Fix samples for 1 h in a solution containing osmium tetraoxide 1% and potassium ferrocianure 1.5% in sodium cacodilate 0.1 M;

Preparation of solutions:

Osmium tetraoxide (2%): 1 g in 50 mL dH_2O
Potassium ferrocianure (3%): 3 g in 100 mL sodium cacodilate 0.2 M buffer (4.28 g sodium cacodilate trihydrate in 100 mL dH_2O)
mix 1:1

- Dehydrate and embed the samples in epoxy resin following the sequence:

Reagent	**Time**
dH_2O (x2)	30 min
Ethanol 30% (x2)	10 min
Ethanol 50% (x2)	10 min
Ethanol 70%	Overnight
Ethanol 96% (x2)	10 min
Ethanol 100% (x2)	30 min
Propylene (x2)	10 min
Propylene + resin (1:1)	Several hours

Resin preparation (resin can be stored frozen in syringes of 50 ml):

EPON812	9 mL
DDSA	6 mL
MNA	5 mL
mix all the components	
DMP30	0.3 mL

Propylene + resin (1:1)	Overnight (in uncovered containers)
Resin	Several hours (4-5 h)

- Encapsulate the samples and polymerize in resin in an oven at 60 ºC for 48 h;

- Cut semithin sections of 1.5 µm in thickness using a Reichert Ultracut S ultramicrotome;

- Stain the semithin sections with 1% toluidine blue and observe under a light microscope to determine the presence of tissues of interest;

- Cut ultrathin sections of 50 nm in thickness using a Reichert Ultracut S ultramicrotome and;

- Pick up sections in 150 mesh copper grids;

- Immerse the grids for 3 min in 1% uranyl acetate in dH_2O;

- Immerse the grids for 4 min in lead citrate in a saturated atmosphere of NaOH;

Lead citrate: 0.03 g in 10 mL dH_2O
0.1 mL NaOH 0.1 M

- Wash the grids with dH_2O twice and dry them completely;

- Examine and photograph the samples under a HT7700 TEM at 60 kV;

- Analyze the selected grids without contrast by microanalysis, with the STEM module of the Auriga 405 microscope;

- Photograph the selected fields.

9.2 Field emission scanning electron microscopy (FESEM)

Equipment and reagents:

- Microtome (Leica RM2125RT)
- Aluminium SEM stubs (AGG301, Agar scientific, Essex, UK)
- Bio-Clear (Bio-Optica, Milan, Italy)
- Field Emission Microscope Zeiss Auriga 405, 1 nm nominal resolution (Oberkochen, Germany) equipped with a Bruker Quantax energy dispersive x-ray spectroscope (EDS) (Energy Resolution: 123 eV K_α of the Mn)

Procedure:

- Cut 5 µm thickness gills and visceral mass samples with the microtome and mount them on aluminium SEM stubs;

- Dewax the sections in Bio-Clear and leave to the air until the liquid excess is evaporated;

- Analyze the samples in an Auriga 405 microscope. Observe the samples under high vacuum (10^{-5}- 10^{-6} mbar) at variable kV;

- Photograph the selected field.

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