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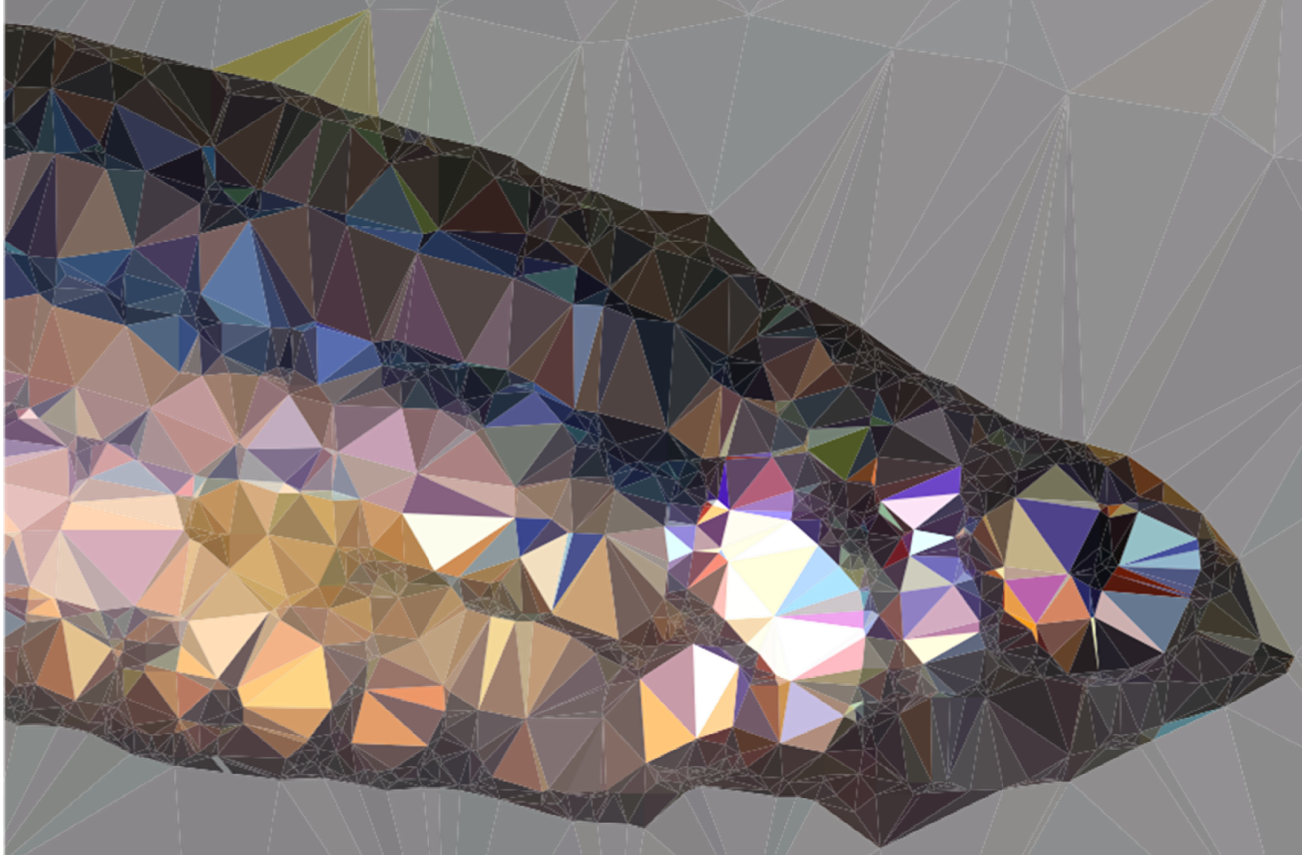
Universidad del País Vasco

Euskal Herriko Unibertsitatea

Cellular and molecular responses of zebrafish to legacy and emerging pollutants: the specific cases of PAHs and metal oxide nanoparticles



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Cellular and molecular responses of zebrafish to legacy and emerging pollutants: the specific cases of PAHs and metal oxide nanoparticles

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“A la família. Per mantenir-me dempeus quan no volia caminar”

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I. INTRODUCTION

Most relevant abbreviations

7,12-dimethylbenz(*a*)anthracene, DMBA

Adverse outcome pathway, AOP

Aldo-keto reductase, AKR

Aryl hydrocarbon receptor, AHR

Aryl hydrocarbon receptor repressor, AHRR

Aryl hydrocarbon receptor nuclear translocator, ARNT

American society for testing and materials, ASTM

Benzo(*a*)pyrene, B(*a*)P

Catalase, CAT

Days post-fertilization, dpf

Emerging pollutants, EPs

Environmental risk assessment, ERA

Epoxide hydrolase, EH

Estrogen receptor, ER

Glutathione, GSH

Glutathione S-transferase, GST

Hours post-fertilization, hpf

International agency for research on cancer, IARC

International organization for standardization, ISO

Metallothionein, MT

Mode of action, MOA

Molecular initiating event, MIE

Molecular weight, MW

NADP(H)-quinone oxidoreductase 1, NQO1

Nanomaterial, NM

Nanoparticle, NP

Organization of economic co-operation and development, OECD

Polycyclic aromatic hydrocarbon, PAH

Registration, evaluation, authorization and restriction of chemicals, REACH

Reactive oxygen species, ROS

Sulfotransferase, SULT

Superoxide dismutase, SOD

UDP-glucuronosyltransferase, UGT

Xenobiotic response element, XRE

A. LEGACY AND EMERGING POLLUTANTS

Historically chemical wastes generated through industrial processes have been disposed of through flagrant release into the environment (Leblanc 2004). Consequently, at the present time there are approximately 100,000 chemicals in the environment, with an additional 500-1,000 added each year (Laws 2012). Many of those anthropogenic residues have found their ultimate fate in water bodies allowing their dilution and efficient transport away from the site of generation (Leblanc 2004). Potential adverse effects of the introduction of such anthropogenic chemicals into the environment were viewed as insignificant relative to the benefits bestowed by such practices (Leblanc 2004). The potential damaging capacity of all those substances is not always known. Thus, we have historically assumed a risk to the human and environmental health that is continuously threatened by the presence of both the pre-existing and the newly generated pollutants (Sauvé and Desrosiers 2014). Since the early sixties, mankind has become aware of the potential long-term adverse effects of these chemicals in general and their potential risks for aquatic and terrestrial ecosystems in particular (Van der Oost et al. 2003). Luckily, awakening of the general public to the hazards of chemicals have led public organizations and governments to regulate and limit the release of chemicals to the environment (Leblanc 2004). The European regulation (EC) No. 1907/2006 (EU 2006) concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) together with the establishment of an European Chemicals Agency (ECHA), supposed a turning point in the protection of human and environmental health. In order to implement effective and sustainable regulations, extensive knowledge on the effects of chemicals to the environment and the human health is essential (Deblonde et al. 2011).

Environmental toxicology aims to assess the adverse effects of environmental chemicals through different scientific disciplines. The ultimate goal of these assessments is elucidating the adverse effects of chemicals that are present in the environment (retrospective hazard assessment) and predicting any adverse effects of new chemicals before they are discharged into the environment (prospective hazard assessment) (Leblanc 2004). In this context, the adverse outcome pathways (AOPs) are of great use in ecotoxicology (Lee et al. 2015). This term embraces two clasic terms that have been widely employed up today, mechanism of action and mode of action (MOA), but which are less useful for risk assessment purposes. AOPs provide a linkage within a direct molecular initiating event (MIE) and an adverse outcome at a biological level of organization (Fig. 1) (Ankley et al. 2010). The central concept of the AOP approach is that while every mechanistic detail of toxicity may be unclear for a given chemical class, the principal acute or chronic MOA(s) can provide a qualitative guide towards optimal bioassay selection. Key

elements to be considered in such a framework include the chemical toxicant´s overall pattern of absorption, distribution, metabolism, excretion and target sites (Hutchinson et al. 2013).

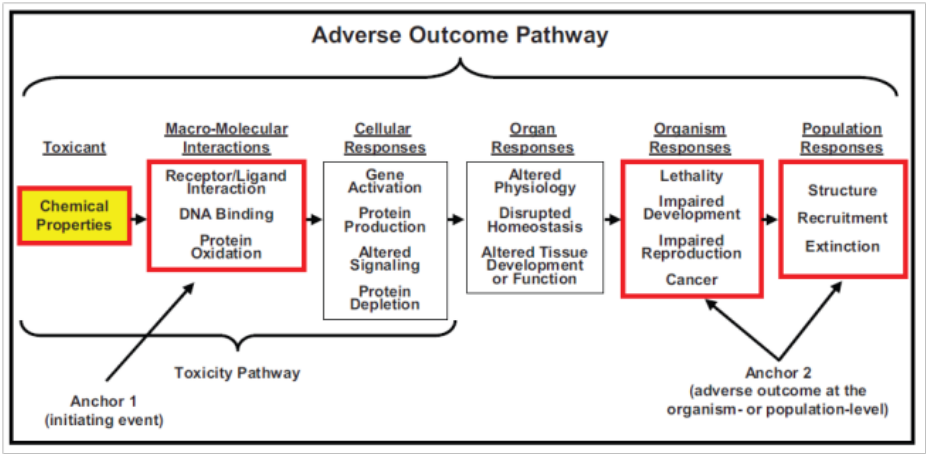


Fig. 1. Schematic representation of an adverse outcome pathway (Ankley et al. 2010).

A.1. Legacy persistent organic pollutants. A specific case: PAHs

Persistent organic pollutants (POPs) are chemical substances that persist in the environment, biomagnify through the food web, and pose a risk of causing adverse effects to human health and the environment (Jones and Voogt 1999). They are characterized by low water solubility and high lipid solubility, leading to bioaccumulation in fatty tissues. Short-term exposures to high concentrations of POPs may result in illness and death, while chronic exposures may also be associated with a wide range of adverse health and environmental effects (IPCS 1995). Some POPs have been present in the environment for decades, and consequently they have been largely considered by water quality regulations (Eljarrat and Barceló 2003).

Legacy pollutants refer to those pollutants that are already being considered by environmental policies. Polycyclic aromatic hydrocarbons (PAHs) are an extraordinarily large and diverse class of legacy POPs originating from both synthetic and natural sources (Fig. 2) (Albers 1995; Harvey 1997). In 1775, the English physician Percivall Pott described an unusually high incidence of scrotal cancer among London chimney sweeps and suggested this was due to their exposure to soot and ash (Brown and Thorton 1957). This way, substances containing high amounts of PAHs became the first environmental agents related to cancer development. Since then,

PAHs have been the focus of great attention and, consequently, information on their characteristics and effects is abundant.

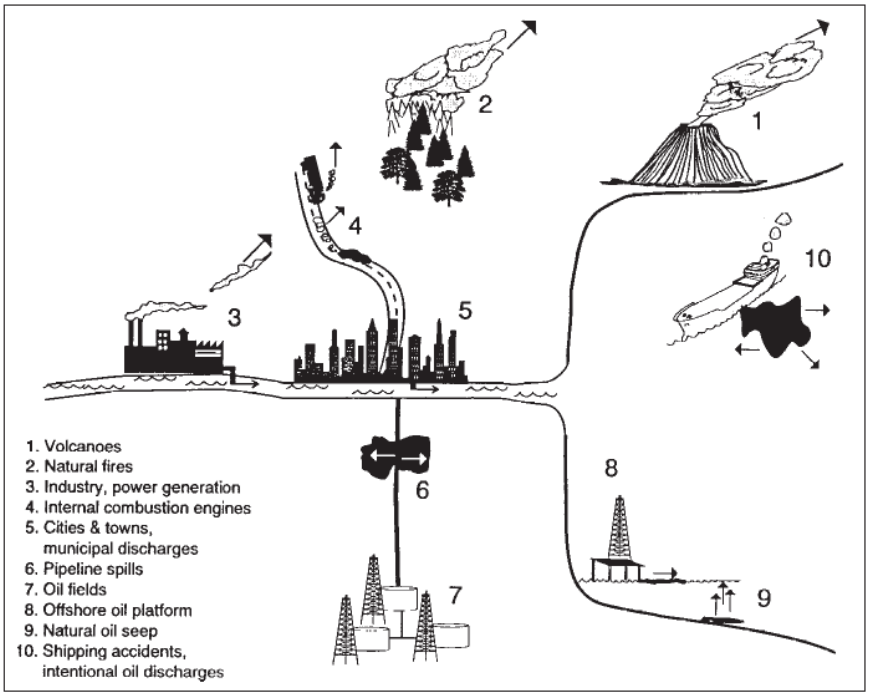


Fig. 2. Schematic view of PAH sources in the environment. Arrows indicate initial movements of PAHs from different sources into the air, soil and waters (Albers 1995).

PAHs consist of conjoined aromatic rings without heteroatoms (Stogiannidis and Laane 2015). They range from the low molecular weight (MW) PAHs with two-three rings structure (naphthalene) up to high MW structures with more than five rings (benzo(g,h,i)perylene) (Fig. 3). In addition to the unsubstituted PAHs (parent compounds), the PAH family also includes substituted derivatives, bearing one or several alkyl groups, like the potent carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA) or other sulfur, or oxygens (Cousin and Cachot 2014). There are thousands of PAH compounds, each differing in the number and position of aromatic rings, and in the position of substituents on the basic ring system (Eisler 1987).

These compounds have been present in the environment for decades and are expected to be produced and spilled in important amounts in future years (Shen et al. 2013). Their ubiquitousness is a matter of great concern since it is well established that some PAHs, such as DMBA, are among the most carcinogenic substances known (Harvey 1997). The International Agency for Research on Cancer (IARC) classifies many of them as carcinogens and the US Environmental Protection Agency (EPA) has identified 16 PAHs as particularly important due to their toxicity to

mammals and aquatic organisms (Fig. 3) (Pampanin and Sydnes 2013). In fact, levels of PAHs commonly found in many marine and freshwater environments have been considered important risk factors for various aspects of fish health (Payne et al. 2003). Field studies in different polluted areas such as Puget Sound (USA) or German Bight (North Sea) reveal the existence of a relationship between PAH pollution and increased tumour presence in fish (Johnson et al. 2008; Vethaak et al. 1992).

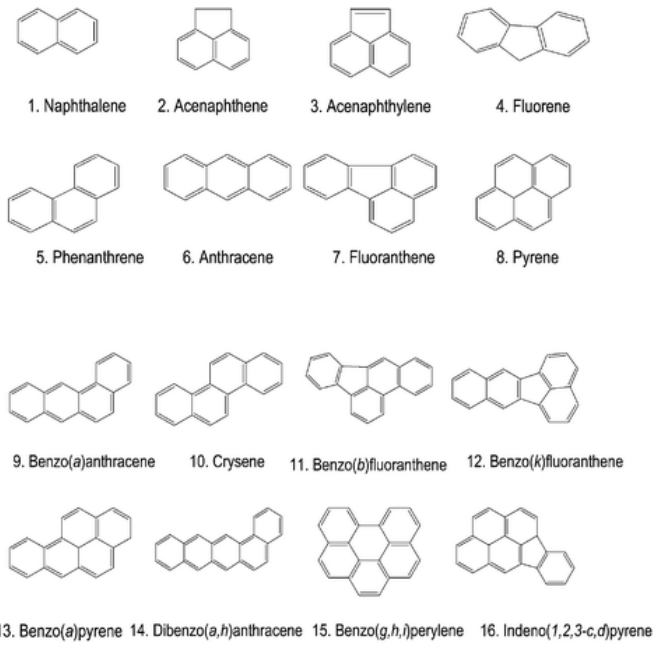


Fig. 3. 16 priority parental PAHs for the US EPA (Bruzzoniti et al. 2010).

PAHs of different MW and structure vary substantially in their behavior and distribution in the environment as well as in their biological effects (Baird et al. 2005; Eisler 1987). Physical and chemical characteristics of PAHs generally vary with MW (Neff 1979). With increasing MW, aqueous solubility decreases, and melting point, boiling point, and the logarithmic octanol/water partition coefficient (log Kow) increase. Decreased solubility of PAHs suggests reduced bioavailability while increased log Kow has been related to higher toxicity and suggests increased solubility in fats and a decrease in resistance to oxidation and reduction (Incardona et al. 2004). But their toxicity is not solely related to their MW (Table 1). Chemical features, including the position of substituent methyl groups, play also an important role in their toxicity (Skupinska et al. 2004). PAHs with bay regions like benzo(*a*)pyrene (B(*a*)P) are likely to be potent carcinogens, while those with fjord regions or sterically hindered bay regions, such as in DMBA, are even more potent (Baird et al. 2005). Moreover, their chemical structure is related to their capacity to induce their metabolic transformation through the xenobiotic metabolism (Barron et al. 2004).

Organisms have been exposed to naturally existing substances of vegetal origin through evolutionary history and have consequently been adapted to the exposure to xenobiotics of natural origin by evolving chemical defence/protective/resistance mechanisms (Kennedy and Tierney 2012). Today, most organisms rely on these constituent chemical defense mechanisms for protection against the human origin pollution (Kennedy and Tierney 2012). Most of the tissues and organs are well equipped with diverse and various drug metabolizing enzymes, including phase I and phase II metabolizing enzymes, as well as phase III transporters (Xu et al. 2005). These enzymes are found in some procariotes and in all eukaryotes (Boelsterli 2007).

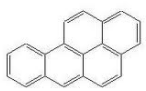
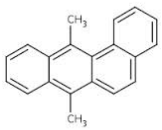
Although the major role of biotransformation metabolism is detoxification, it can also act as an “intoxication” process (Park et al. 2005). PAHs require metabolic activation to elicit their carcinogenic outcome (Lee et al. 2015; Luch 2005). The initial step during conversion of organic xenobiotics into hydrophilic and excretable derivatives is mainly catalyzed by CYP enzymes (Xu et al. 2005).

These enzymes play key roles in the initial step of oxidation, reduction, hydrolysis or hydration of PAHs (Testa et al. 2012). When PAHs are bioactivated by the P450 enzyme system (phase I), highly reactive compounds such as diol epoxides, quinones, radical anions and benzylic carbenium ions are produced (Fig. 4) (Henkler et al. 2012; Shimada 2006; Xu et al. 2005).

The resulting reactive species are able to damage cellular biomolecules, including DNA (Luch 2005; Xue and Warshawsky 2005). These electrophilic PAH metabolites are capable of forming stable DNA adducts or to promote depurination at damaged nucleotide sites which, in turn, may provoke tumour initiation (Henkler et al. 2012).

To eliminate the damaging metabolites arising from phase I activation of PAHs, cells are provided with phase II metabolism enzyme superfamilies including, sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT) and enzymes like glutathione S-transferase (GST), NADP(H)-quinone oxidoreductase 1 (NQO1), and aldo-keto reductase (AKR) (Fig. 4) (Shimada 2006; Xu et al. 2005). In general, phase II enzymes mediated conjugations increases hydrophilicity, and thereby enhance excretion in the bile and/or the urine and consequently a detoxification effect is exerted (Xu et al. 2005). Finally, phase III transporters like ABC transporters do also participate in cell protection, providing formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion (Xu et al. 2005).

Table 1. PAHs employed in this study and their properties.

Compound	Structure	MW	MP (ºC)	Log (Kow)	RP	CC	Risk statements	Hazard symbol
B(*a*)P		252	175	6.04	0.00024375	++++	May cause cancer. Very Toxic to aquatic organisms may cause long-term adverse effects in the aquatic environment.	
DMBA		256.34	122	6.36	0.000418	+++++	May cause cancer. Harmful if swallowed.	

B(*a*)P, benzo(*a*)pyrene; DMBA, 7,12-dimethylbenz(*a*)anthracene. MW, molecular weight; MP, melting point; S, water solubility; Kow, octanol-water partition coefficient; RP, relative potency compared to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as aryl hydrocarbon receptor agonist; CC, comparative carcinogenicity based on mouse skin tumor data (Barron et al. 2004; Latimer and Zheng 2003; Cavalieri and Rogan 1984). Data from risk assessment and the hazard symbols were obtained from the data sheet provided by a commercial dealer (Sigma-Aldrich, Missouri, USA).

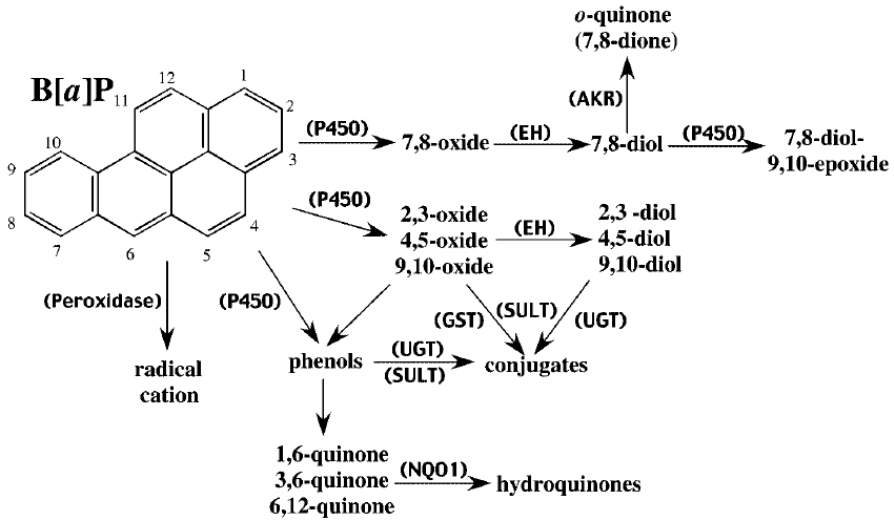


Fig. 4. Metabolism of B(*a*)P by xenobiotic-metabolizing enzymes: P450, cytochrome P450; EH, epoxide hydrolase; GST, glutathione S-transferase; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; NQO1, NADP(H)-quinone oxidoreductase 1; AKR, aldo-keto reductase (Shimada 2006).

A concomitant effect of the bioactivation of PAHs is the increase in reactive oxygen species (ROS) production (Miller and Ramos 2001). The intracellular production and mitigation of oxidation-reduction reactions is referred as redox balance (Trachootham et al. 2008). Increased ROS presence arising from the xenobiotic metabolism can, in turn, result in the alteration of this equilibrium inducing oxidative damage to DNA and other cell components, such as membranes and proteins (Miller and Ramos 2001).

Consequently, PAHs can produce cancer to the organisms through genotoxic and non genotoxic mechanisms. The former include the mentioned formation of DNA adducts and chromosome damage, the latter include mechanisms such as induction of inflammation, immunosuppression, formation of ROS, activation of receptors such as arylhydrocarbon receptor (AHR) or estrogen receptor (ER). Together, these genotoxic and non-genotoxic mechanisms can alter signal-transduction pathways that finally result in hypermutability, genomic instability, loss of proliferation control, and resistance to apoptosis (Fig. 5) (Luch 2005; IARC 2010).

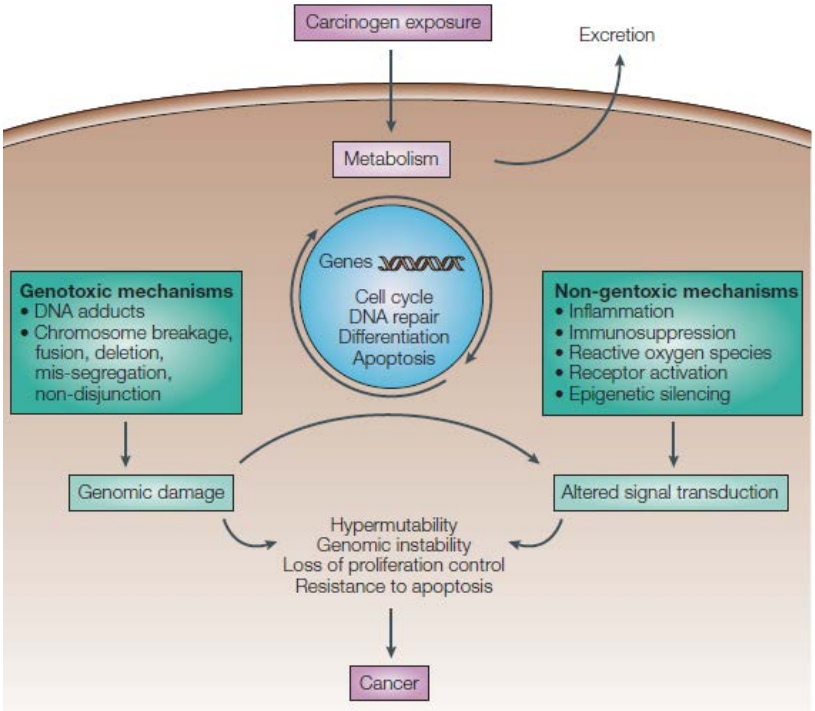


Fig. 5. Genotoxic and non-genotoxic effects of carcinogens (Luch 2005).

A.2. Emerging pollutants. A specific case: metal oxide nanoparticles

Until very recently, attention on chemicals affecting environmental health has been focused on nutrients, metals, "traditional" active ingredients in pesticide products and persistent organic pollutants like PAHs. However, in recent years, there has been increasing concern over the environmental risks of the so called emerging pollutants (EPs) (Boxall 2012). EPs are defined as compounds that are not currently covered by existing water-quality regulations, have not been studied before, and are thought to be potential threats to ecosystems and human health and safety (La Farré et al. 2008). Thus, EPs are not necessarily new chemicals; they may be substances that have been present in the environment for a long time but whose presence and significance are only now being recognized (Boxall 2012). The term has come to encompass a wide variety of chemicals-pharmaceuticals and household chemicals such as fragrances, antimicrobials, surfactants, and fluorescent whitening agents as well as newer classes of compounds, such as nanomaterials (NMs) and genetically modified food items (Glassmeyer 2007). In Europe various protocols have been developed to assess the risk of emerging new substances since environmental risk

assessment was introduced in 1980. According to the European REACH legislation, authorities should pay special attention to any chemical that may enter into the environment as a result of the industrial activity as well as to ensure safety for the population (Deblonde et al. 2011).

Among all those EPs, NMs have been the focus of substantial attention. Over the past two decades, the clear advantages in using NMs for consumer products have led to a new stage in nanotechnology development (Oberdöster et al. 2005). Nanotechnology offers rapid advances across many areas of science and engineering that are expected to help solving crucial issues to society (Roco and Bainbridge 2005). Today nanotechnology is already a reality for an incredible variety of innovations that are being incorporated into a wide range of applications including cosmetics, car parts, drugs, food packaging, sport equipments, electronics, etc… (Maynard et al. 2006). This, in turn, has fueled a dramatic growth in the nanotechnology industry, from a $10 billion enterprise in 2012 to an anticipated up to $1 trillion by 2015 (Yang and Westerhoff 2014). This huge market of nano based products has been estimated to require over half a million tons of manufactured NMs by 2020 (Maurer-Jones et al. 2013). The increasing presence and consequent release of NMs has brought safety concerns to broad public attention and, thus, prepared the ground for the acknowledgment of the need of scientific research on their potential hazardous effects (Kahru et al. 2008; Kahru and Savolainen 2010).

According to the British Standards Institution, the America Society for Testing Materials (ASTM) and the Scientific Committee on Emerging and Newly-Identified Health Risks, NMs are defined as materials with at least one dimension under 100 nm (Klaine et al. 2008). The European Commission provides a more specific definition and defines NMs as follows: "Nanomaterial" means 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 (EU 2011).

There are a few major classes of NMs including metal and metal oxide NMs and carbon-based NMs like nanotubes and fullerenes (Fent 2010). Within this group of materials, according to the ASTM, nanoparticles (NPs) are defined as a sub-classification of ultrafine particles with lengths in three dimensions greater than 1 nanometer (nm) and smaller than about 100 nm, and which may or may not exhibit size-related intensive properties.

Due to their small size and consequent large number of surface atoms per mass unit, some NPs possess unique mechanical, catalytic and optical properties as

well as electrical conductivity when compared with their bulk counterparts (Niemeyer 2001; Oberdörster et al. 2005). Consequently, the vast body of knowledge on metal pollution may not suffice to predict the environmental effects caused by metal and metal-bearing NPs, since key factors such as persistence of NPs both in the environment and in organisms may differ from those of conventional metals. As part of the regulatory management of manufactured NPs, the key question is whether nanosize imparts a different fate and toxicity from that of the equivalent aqueous counterparts and macroforms (Handy et al. 2008; Moore 2006; Nel et al. 2006). These same unique properties have prompted concern that unique physiological responses will be elicited in living systems by interaction with these materials (Maurer-Jones 2013). Thus, understanding the critical factors that contribute to differences in behaviour of NPs such as aggregation, solubility, as well as the effect of coatings used in specialized formulations is important for regulatory purposes (Angel et al. 2013). All these concerns leaded Donaldson and co-workers (2004) to propose "nanotoxicology" as a new branch in toxicology to specifically assess the toxicity of NMs. In recent years, a great amount of work has been done to assess the ecotoxicological risk of new engineered NMs in aquatic environments, but this research is still at an initial stage of development and several issues need to be resolved (La Farré et al. 2008).

The largest share of manufacture and application for both industrial and household applications among different NMs belongs to metal oxide NPs, which hold promise for future applications (Chang et al. 2012; Djurisić et al. 2015). Metal based NPs are attractive for a large variety of products including personal-care products (TiO_2 and ZnO are included in toothpaste, beauty products, sunscreens) or environmental remediation (groundwater remediation with nanoscale Fe). Metal oxide based NPs are also increasingly used in fillers, opacifiers, ceramics, coatings, catalysts, semiconductors, microelectronics, prosthetic implants and drug carriers (Kahru et al. 2008; Oskam 2006).

According to Kahru and co-workers (2008), despite the increasing research on metal oxide NPs there is a lack of information regarding the toxicity and ecotoxicity of metal oxide NPs. This is due to the existing widespread controversies and ambiguities with respect to the toxic effects and mechanisms of metal oxide NPs (Chang et al. 2012; Djurisić et al. 2015). Unlike many organic substances that need to be metabolized to become toxic or to be detoxified and excreted, metals do not. Key factors in hazard evaluation after exposure to bulk materials are chemical composition, dose, and exposure route. However, for NPs, additional factors are to be considered (Djurisić et al. 2015; Yan et al. 2011). As mentioned before, size is an important parameter to be considered when NPs toxicity is evaluated (Buzea et al. 2007; Djurisić et al. 2015). Particle size determines the routes of internalization into

the cells and affects other relevant parameters affecting their toxicity. Moreover, the universal occurrence of aggregation of most NPs in aqueous media can lead to a range of sizes (size of agglomerates) and consequent size related effects (Li et al. 2014).

One characteristic strongly related to size is the surface area. Surface area determines the interaction between NPs and other surfaces such as biological membranes, and sorption capacities for other aqueous species such as natural organic matter (Fukushi and Sato 2005; Heinlaan et al. 2008). NPs possess a very large surface area and high particle number per unit mass (Buzea et al. 2007). Increased surface area promotes not only the accumulation of NPs, but also an increase of reactivity and enhanced interactions between NPs and biomolecules (Chang et al. 2012).

Solubility is another important property that explains the reasons of toxic effects on many organisms. Dissolution of NPs influences their MOA and, consequently, their fate, availability and environmental impact. Both solubility and the speed at which this phenomenon occurs (dissolution rate) are dependent on the chemical and surface properties of each particle. Moreover, both solubility and dissolution kinetics can be influenced by the particle size (Bian et al. 2011; Mudunkotuwa and Grassian 2011). Together with the size of the particle, other factors such as surface area, surface morphology, crystallinity, and crystal structure might also influence solubility and need to be taken into consideration (Misra et al. 2012).

There are different ways through which NPs can lead to adverse outcomes (Fig. 6). Some recurrent observations include oxidative stress, interaction with cellular elements, and metal ion toxicty (Chang et al. 2012).

Firstly, the toxicity of metal oxide NMs is frequently attributed to ROS and ROS-induced damage (Djurisić et al. 2015). Metal oxide NPs can induce ROS through their interaction with proteins of oxidative organelles such as mitochondria, or in the case of NPs containing transition metals (iron, copper, chromium, vanadium, etc.) through the generation of ROS by acting as catalysts in Fenton type reactions (Buzea et al. 2007; Chang et al. 2012). Consequently, damage to DNA and other cell components, such as membranes and proteins can occur (Miller and Ramos 2001). Secondly, metal oxide NPs can get attached to cell surface or other cellular components allowing cellular toxicity, and protein missfunctions or inactivation to occur (Handy et al. 2008). Finally, the toxicity of metal oxide NPs which are partially soluble (i.e. release metal ions in aqueous solutions) is often attributed to the metal ion release. Metal based NPs could deliver very high free metal ion concentrations locally in/on the cell, resulting in latent metal toxicity (Handy et al. 2008).

Approximately 40% of all known proteins contain metal cations. Free ions can interact whith cellular components and can consequently disrupt cellular homeostasis to result in cell toxicity. When homeostasis variation exceeds the range of physiological tolerance, toxicity occurs (Chang et al. 2012; Djurisić et al. 2015).

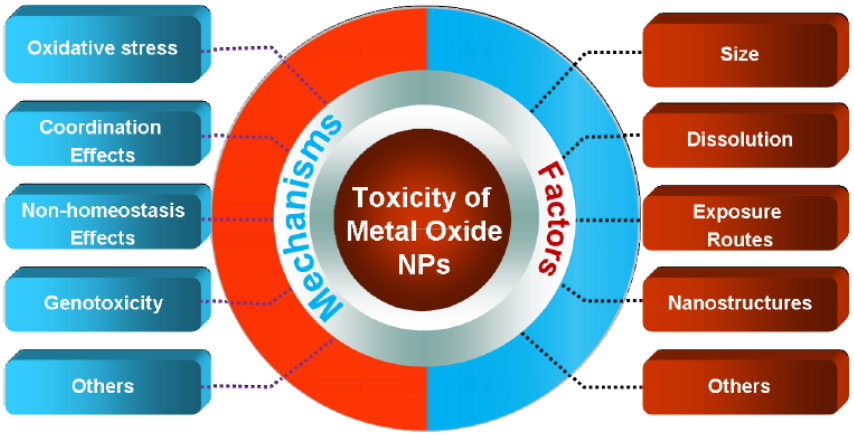


Fig. 6. Mechanisms through which metal oxide NPs may produce toxic effects and factors affecting their damaging capacity (Chang et al. 2012).

B. Effects of PAHs to fish

The effects of PAHs to fish have been studied since 1960s. Reports from many coastal harbors, as well as inland rivers and lakes, showed the worrying reality of these areas where varying levels of PAHs were detected and more attention began to be placed on assessing their ecotoxicological potential (Payne et al. 2003). Important observations were made on tumors and other pathologies in fish from contaminated environments, like the Black River (Ohio, USA), German Bight (North Sea) or the Kumaru River (Japan), with effects being strongly linked in some instances to PAHs (Baumann 1998; Vethaak and Rheinallt 1992).

Since then, a large number of publications have reported the toxic effects of PAHs observed on wild-caught fish after oil spills as well as after experimental exposures (Cousin and Cachot 2014). Studies looking at the former revealed the toxic potential of PAHs and possible mechanisms, while work examining the latter confirmed the toxicity of PAHs, and provided information on underlying molecular mechanisms of PAHs (Cousin and Cachot 2014). Today, fish are the most widely used non-mammalian vertebrates in toxicity testing of individual substances and effluents for risk assessment and regulation (Braunbeck and Lammer 2006; Schirmer 2006). Consequently many studies have been conducted addressing the toxicity of PAHs in fish.

The damaging capacity of these compounds can be detected at different steps (levels of biological organization) in the sequence of events leading to an adverse outcome. Fish allow measuring those effects, favoring the development of early-warning biomarker signals of effects at later response levels (Sanchez and Porcher 2009). Thus, studying the effects produced by PAHs in fish can lead to the better understanding of their AOPs as well as to the development of early warning biomarkers.

It is generally accepted that PAHs, in fish, produce their deleterious effects through either of two MOA: (1) “dioxin-like” toxicity mediated by activation of AHR, which controls a battery of genes involved in both phase I and phase II xenobiotic metabolism, such as *cytochrome P4501A* (*cyp1a*) and *gst*, and (2) “nonpolar narcosis”, in which PAH uptake by tissues is dependent solely on hydrophobicity and toxicity is mediated through non-specific partitioning into lipid bilayers (Dimitrov et al. 2003; Incardona et al. 2006).

B.1. Bioaccumulation of PAHs

Aquatic organisms may accumulate persistent hydrophobic chemicals, like some PAHs, through different mechanisms: via the direct uptake from water by gills or skin (bioconcentration), via uptake of suspended particles (ingestion) and via the consumption of contaminated food (biomagnification) (Van der Oost et al. 2003). PAHs can, therefore, be readily absorbed by fish reaching levels higher than those in the medium (Zhang et al. 2015). However, since some PAHs can be rapidly metabolized depending on their chemical properties, such as MW, they do not necessaryly accumulate in fish tissue to any extent (Eisler 1987; Payne et al. 2003). Bioaccumulation of low MW PAHs in aquatic biota is higher than bioaccumulation of high MW PAHs (Zhang et al. 2015). This may be related to lower capacity of low MW PAHs to induce the AHR mediated biotransformation metabolism (Barron et al. 2004).

B.2. Transcriptomic effects of PAHs

PAHs can produce altered gene transcription through the direct interaction with different transcription factors like the AHR, or indirectly as a part of the biologic response to the toxic stimulus including genes related to oxidative stress or damage repair mechanisms (Fujii-Kuriyama and Mimura 2005; Luch 2005).

The AHR is a ligand activated transcription factor, which enhances the transcription of target genes in response to PAHs. Normally, AHR exists in a dormant state within the cytoplasm in association with a complex of HSP90, XAP2 and p23 (Fujii-Kuriyama and Mimura 2005). Upon PAH binding, AHR in the complex is activated. The ligand-activated AHR in the complex translocates into the nucleus and forms a heterodimer with the closely related ARNT (aryl hydrocarbon receptor translocator) protein already present in the nucleus by dissociating from the complex. The activated AHR/ARNT heterodimer complex binds to its cognate DNA sequences, termed xenobiotic response elements (XREs), and activates the expression of AHR target genes (Fig. 7). The AHR-dependent gene battery includes *cyp1a, gst, ugt* and *nqo1* among others (Nebert et al. 2004). However, not all of them are equally responsive. In the presence of PAHs, *cyp1a* is especially sensitive (Della Torre et al. 2010; Hawliczek et al. 2012). The *AHR repressor* (*AHRR*) is also located downstream of the XRE. The proteic product of this gene is involved in the regulation of the AHR mediated response. The AHRR can bind to the ARNT to replace the AHR/ARNT complex in association with the XRE sequence (Fujii-Kuriyama and Mimura 2005).

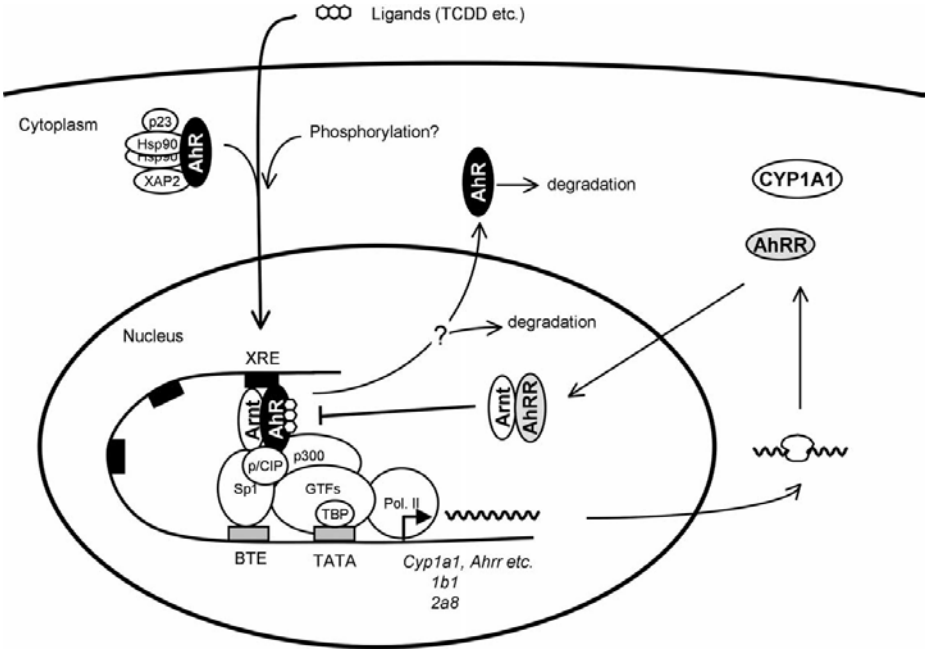


Fig. 7. A model for AHR signalling pathway (Fujii-Kuriyama and Mimura 2005).

Nevertheless, a full understanding of the effects of PAHs at the transcriptomic levels must not only consider the AHR downstream response, but a wider perspective including the transcriptomic response to the effects produced by the activated PAHs and the non specific narctotic effect. Consequently, some works have already analyzed the whole transcriptomic response after fish exposure to different PAHs, which are susceptible of producing different responses (Song et al. 2012). Nevertheless, some usual responses include the altered transcription of genes related to: biotransformation, oxidative stress, immune response, bioenergetics, transport or ion homeostasis (Bui et al. 2012; Hook et al. 2006; Krasnov et al. 2005).

B.3. Enzymatic effects of PAHs

Responses at the enzymatic level are not solely produced through the embodiment of responses occurring at the transcriptomic level, but through altered enzymatic activity (Stegeman and Hahn 1994). Overall, enzymes related to the xenobitoc metabolism are sensitive to PAHs presence. Changes in their abundance or activity can be measured and, therefore, they are susceptible of being employed as biomarkers (Setegeman and Lech 1991; Van der Oost et al. 2003). Among them, the increased activity of CYP1A-associated ethoxyresorufin O-deethylase (EROD) or GST are considered good markers of PAH exposure (Au et al. 1999; Lu et al. 2009; Van der Oost et al. 2003).

Together with GST, the increased activities of other antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are usually measured after fish exposure to PAHs or in field studies conducted in polluted areas. Nevertheless, SOD and CAT are not so responsible to the presence of PAHs and the increased activity of GST is not always accompanied by an increase in their activities (Livingstone et al. 1993; Stephensen et al. 2003; Van der Oost et al. 2003; Vasanth et al. 2012).

B.4. Genetic effects of PAHs

Damage to the genetic material is a common occurrence when fish are exposed to PAHs (Tuvikene 1995). Firstly, electrophilic PAH metabolites are capable of forming stable DNA adducts or to promote depurination at damaged nucleotide sites (Henkler et al. 2012). These alterations can be fixed as mutations during replication. Consequently, metabolic activation of PAHs leading to the formation of DNA adducts is considered as a crucial event in chemical carcinogenesis, involving covalent binding between the chemical carcinogen and the DNA (Miller and Miller 1981). Secondly, PAHs can also confer DNA damage through the ROS arising from their metabolism. ROS are highly reactive and capable of oxidizing diverse biological macromolecules. The oxidative nucleoside 8-oxo-7,8-dihydro-2-deoxyguanosine is known to be a major form of oxidative damage in DNA exposed to ROS and closely related with mutation and carcinogenesis (Kryston et al. 2011). Moreover, both activated metabolites and ROS can produce DNA single- and double-strand breaks (Mitchelmore and Chipman 1998). These DNA breaks have been recorded as increased prevalences of micronuclei in the erythrocytes of fish exposed to PAHs (Cheikyula et al. 2009).

As a consequence of the genetic damage induced by PAHs, other downstream effects are produced. Tumpor protein 53 mediated protective responses, such as apoptosis or cell cycle arrest, and the induction of DNA repair mechanism are switched on after exposure to PAHs (Marlowe and Puga 2005).

B.5. Immunological effects of PAHs

Increasing number of studies have demonstrated immune effects in fish after exposure to environmental pollutants (Cuesta et al. 2011; Huang et al. 2015; Ribas et al. 2015; Skupniska et al. 2004). Immune dysfunctions are also related with a higher susceptibility to cancer development (Zaccaria and McClure 2013). The immune system of fish is very sensitive to PAHs as they can affect both non-specific and specific immunity. However, the effects observed are depend on the type of PAH, the route of administration, the concentration used and the fish species studied. The capacity of PAHs to induce immunotoxicity implies different intracellular

mechanisms, such as PAH metabolism by cytochrome P450, binding to AHR and intracellular calcium mobilization (Reynaud and Deschaux 2006).

B.6. Cellular effects of PAHs

Peroxisome proliferation and reduced stability of the lysosomal membrane are other consequence of fish exposure to PAHs (Cajaraville et al. 2003; Zorita et al. 2008).

Peroxisomes are cellular organelles characterized by the presence of H_2O_2-generating flavin oxidases and H_2O_2-scavenging CAT. PAHs are able to interact with the peroxisome proliferator activated receptor (PPAR) and to produce peroxisome proliferation, a phenomenon which has been related with cancer development in rodents (Reddy et al. 1980; Reddy and Lalwani 1983). Peroxisomal proliferation may result in increased lipid metabolism such as β-oxidation of very long chain fatty acids and ROS production arising from the stronger induction of oxidases compared to the antioxidant enzymes like CAT (Cancio and Cajaraville 2000). Consequently, peroxisome proliferation is considered a non-genotoxic mechanism of carcinogenesis. Changes in the activity of the peroxisomal enzymes like acyl-CoA oxidase or CAT, together with volume density of peroxisomes are used as exposure biomarkers (Cajaraville et al. 2003).

Alteration of the stability of the lysosomal membrane is a cellular effect that has also been widely used as a general biomarker of effect of different chemicals (Köhler et al. 1992; Köhler et al. 2002). Lysosomal membrane stability is reduced in fish from PAH polluted areas (Holth et al. 2008). This effect has also been described to occur concomitantly with tumor development (Köhler and Pluta 1995).

Besides the previously mentioned peroxisome proliferation, laboratory exposure of fish to B(*a*)P causes increases in abundance of lipofuscin granules, mitochondria, lipids, lysosomes, slight proliferation of the endoplasmatic reticulum system, and depletion of glycogen (Au et al. 1999).

B.7. Histopathological effects of PAHs

Many relevant observations have been made over the past 25 years linking histopathological effects including the development of tumors in fish to polluted waters containing elevated levels of PAHs (Eisler 1987; Payne et al. 2003). Lesions associated with genotoxins like PAHs include skin tumors, such as papillomas and squamous carcinomas, liver tumors, both hepatic and biliary; and pigmented cutaneous lesions such as chromatophoromas (Baumann 1998).

Results obtained in experimental studies with fish chronically exposed to sediments containing PAHs, as well as various studies with extracts of PAHs from contaminated sediments, or specific PAHs, provide additional support for PAHs being a likely cause for some of the pathological effects found in fish in highly contaminated environments (Payne et al. 2003). Spitsbergen et al. (2000) described several tumors after exposing zebrafish (*Danio rerio*) to DMBA. In that work, the liver together with gills and blood vessels were identified as a targets for PAH produced neoplasia.

As summarized by Myers and co-workers (1998), the hepatic lesions that may be produced after exposure to PAHs include:

1. Neoplasms: hepatocellular adenoma, hepatocellular carcinoma, cholangioma, cholangiocellular carcinoma, and mixed hepatobiliary carcinoma (Fig. 8).
2. Putatively preneoplastic loci of cellular alteration: eosinophilic focus, clear cell focus, basophilic focus.
3. Nonneoplastic proliferative lesions: hepatocellular regeneration, biliary hyperplasia or proliferation, 'oval cell' proliferation, cholangiofibrosis, and increased mitotic activity in hepatocellular/biliary epithelial cells.
4. Specific or unique degenerative/necrotic conditions: hepatocellular nuclear pleomorphism, megalocytic hepatosis, and rarely, spongiosis hepatis.
5. Nonspecific necrotic lesions unassociated with visible infectious agents: necrosis, hepatocellular or biliary coagulative necrosis, hydropic degeneration, hyalinization, pyknosis and karyorrhexis.
6. Hydropic vacuolation of biliary epithelial cells or hepatocytes.

B.8. Developmental effects of PAHs to fish

While there is an extensive literature describing the effects of PAHs on adult or juvenile animals, few studies have addressed the effects of PAHs on embryonic and early larval development in fish (Incardona et al. 2004). Developmental stages display greater sensitivity to xenobiotics than adult organisms and are therefore of special concern (Payne et al. 2003). Fish embryo exposures to either individual or mixtures of PAHs have shown the teratogenic potential of PAHs (Barjhoux et al. 2014; Incardona et al. 2004; Le Bihanic et al. 2014a, b). PAHs can produce a variety of deleterious effects on developing individuals including delayed hatching, developmental abnormalities, disruption of larvae swimming activity as well as DNA damage. Moreover, effects at early stages of development may subsequently affect the health and survival of adult organisms. Fish embryo exposures to PAHs are able to produce carcinogenic lesions in adult organisms (Lam et al. 2006; Wang et al. 2010).

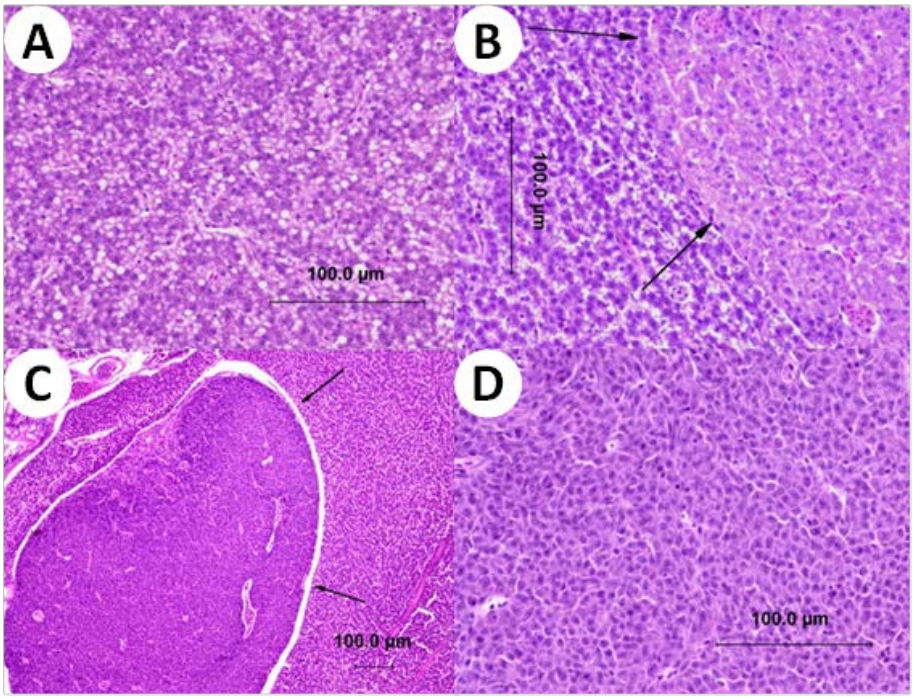


Fig. 8. Micrographs of hematoxylin eosin stained samples of zebrafish normal liver and liver tumors induced by exposure to PAHs. A: Histology of normal zebrafish liver. B: Hepatocellular adenoma in liver of zebrafish developed one year after an acute exposure (24 h) during embryogenesis to 2.5 mg/L DMBA. Arrows point to neoplasm. C and D: Hepatoblastoma in liver of uma line fish developed one year after an acute exposure (24 h) during embryogenesis to 0.6 mg/L dibenzo(*a,l*)pyrene. Arrows point to neoplasm. Modified from Lam et al. (2006).

However, different PAHs own the capacity to produce distinct and specific effects on fish at early life history stages (Incardona et al. 2004). Developmental toxicity mediated through the interaction with the AHR includes increased cardiovascular dysfunction, pericardial and yolk sac edemas, subcutaneous hemorrhages, craniofacial deformities, reduced growth, and increased mortality rates (Billiard et al. 2006). On the other hand, narcosis dependent embryo toxicity has been related to the development of the blue sac disease and increased embryo mortality (Barron et al. 2004; Billiard et al. 2008).

C. Effects of metals and metal oxide NPs to fish

The aquatic environment is a sink for pollutants from both the atmosphere and soil and for those entering the water system directly. This suggests that the aquatic environment is particularly vulnerable to metal oxide NPs (Scown et al. 2010). Consequently, fish result suitable models to study the toxicity of metal NPs and their underlying mechanisms of toxicity. Research with fish provides a conceptual framework and evolutionary reference point for other vertebrate studies (Powers 1989). In fact, according to Scown and co-workers (2010), understanding the effects of NPs on fish is an important aspect when considering the overall knowledge on NPs toxicity. A central question in acute toxicity is whether or not nanometals are more, or less toxic than the equivalent metal salt. Such studies have yet to be done for most NMs and species of fish (Handy et al. 2011).

Metals have been historically spilled to the environment in dissolved or ionic form or in their bulk form –conventional metals- and, more recently, together with the development of the nanotechnologies, as metal NPs (Maynard et al. 2006; Zeitoun and Mehana 2014). Thus, metals are among the batch of legacy pollutants whose presence in the environment has been strongly spread as a result of anthropogenic activities (Glassmeyer 2007). Many of them are of concern because of their toxic properties; however, at certain concentration ranges some metals are also essential for health and survival of animals and humans (Nordberg et al. 2002). Metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) are essential nutrients that are required for various biochemical and physiological functions (WHO 1996).

C.1. Effects of “conventional” metals to fish

Metal toxicity to fish has been studied for decades. Data on the effects of conventional metal exposure to fish behavior, survival and growth have been reported since the 70s (Atchison et al. 1987; Pascoe et al. 1986; Sauter et al. 1976). Today, we know that fish waterborne exposure to metals can lead to a variety of alterations at different levels of biological organization (Sabullah et al. 2015). These effects are often dependent on the metal or metal mixture to which fish are exposed, as well as to the species in which the study is carried on. Anyhow, in order to produce a biological effect, be it beneficial or harmful, the metal must cross the cell membrane and enter the cell where it then can bind a cellular target and thereby alter specific biochemical processes (Hollenberg 2010). Common mechanisms by which toxic metals may produce an adverse outcome include inhibition of enzymes, disruption of the structure and/or function of organelles, interaction with DNA

leading to mutagenesis or carcinogenesis, covalent modification of proteins, displacement of other metals in various metals dependent proteins, and inhibitory or stimulatory effects on the regulation of expression of various proteins (Hollenberg 2010). Moreover, metals are inducers of oxidative stress in aquatic organisms, promoting the formation of ROS (Sevcikova et al. 2011).

C.1.1. Bioaccumulation of metals

Fish can bioaccumulate metals leading to increased concentrations of the metals in body tissues compared to those present in the environment (Zeitoun and Mehana 2014). Polesky and co-workers (2010) stated that the accumulation of cadmium (Cd), lead (Pb), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), mercury (Hg) and arsenic (As) in Danube Basin (Hungary and Serbia) in Danube sterlet (*Acipenser ruthenus*) liver, gills and skin ocurred in response to the presence of this pollutants in the environment. Moreover, metal accumulation in fish is a time dependent process and thus prolonged exposures to metals can lead to toxic concentrations in the organisms able to produce the toxic effects described in the following sections (Jezierska and Witeska 2006). Bioaccumulation is a point of special relevance as fish are often at the top of the aquatic food chain and can concentrate large amounts of some metals (Parvathi et al. 2011). Studies on different fish species (*Atherina hepsetus, Cyprinus carpio, Mugil cephalus, Sardina pilchardus, Sparus auratus,* and *Trigla cuculus*) showed that metals (Cd, Cr, Cu, Fe, Pb, Zn) accumulate mainly in the liver, as a metabolic organ that stores metals for detoxification by producing metallothioneins (MTs) (Canli and Atli 2003; Kargin and Erdem 1991). Nevertheless, being the first organ to which the pollutant comes in contact in waterborne exposures, gills are also especially vulnerable to metal accumulation (Au 2004). For instance, it has been reported that Fe was mainly accumulated in the gills of Atlantic saury (*Scomberesox saurus*) (Canli and Atli 2003). In fact, in zebrafish exposed to Cd, metal accumulation in gills and liver has been described as a time dependent process in which, after an initial bioaccumulation in gills, Cd is transferred to liver as the main storage and excretion organ (Arini et al. 2015).

C.1.2. Transcriptomic and protein level effects of metals

Like many other pollutants, metals can alter the transcription and expression of genes in fish. Global transcriptomic analyses after rainbow trout (*Oncorhynchus mykiss*) exposure to Zn or yellow perch (*Perca flavescens*) to Cd and Cu have identified energy production, protein synthesis, and inflammatory responses as frequently altered processes in fish exposed to metals (Hogstrand et al. 2002; Pierron et al. 2011).

Altered transcription and expression of a number of metal binding peptides and proteins that serve to detoxify and store metal ions through binding and removal of their redox potential are also usual effects of exposures to metals (Arini et al. 2015; Chan 1995; Heikkila et al. 1982; Hogstrand and Haux 1990; Vergauwen et al. 2013; Webster et al. 2013). Glutathione (GSH) and MTs act as buffers for metal ions entering cells; both have very high affinity for most metals and GSH is generally present in the cells at high concentrations (Srikanth et al. 2013; Webster et al. 2013). The majority of proteins and peptides that function in the uptake, distribution, storage or detoxification of metal ions possess one or several metal binding sites (Flora and Pachauri 2010). These sites are often formed by aminoacids like cysteine which are rich in sulfhydryl groups able to chelate metals (Flora and Pachauri 2010; Klaassen et al. 1999).

MT mediated metal chelation is the most consistent, and sometimes the unique, mechanism of metal tolerance in fish (Van der Oost et al. 2003; Webster et al. 2013). This response is mediated through the pluripotent transcriptional regulator mtf1, which in the presence of metals binds metal response elements in its promoter region, stimulating transcription (Günter et al. 2012). The induction of MT synthesis is depedent on the metal binding affinity and thus it is not always observed in response to metal pollution (Sevcikova et al. 2013; Wang et al. 2014). Induction has been shown to occur for example in rainbow trout injected with Cd as well as in gudgeon (*Gobio gobio*), roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) from pollutend areas correlating with Zn accumulation (Bervoets et al. 2013; Price-Haughey et al. 1986).

Metals can also affect the enzymatic activity through direct interaction with the enzymes. They can bind directly to sulfhydryl groups of proteins and lead to protein inactivation and denaturation, they can bind to intracellular GSH or antioxidant enzymes (e.g. SOD, CAT, GSH reductase/peroxidases) and reduce the antioxidant ability of cells; or they can compete for metal-cofactor binding of metallo-enzymes and lead to their inactivation (Regoli et al. 2005; Sparta and Alexandrova 2012). Moreover, redox-active metals like Cu, Fe, V, Co and Cr contribute to increase oxidative stress in fish that may result in protein/enzymatic damage, lipofuscin induction and the activation of redox-sensitive transcription factors such as AP-1, p53, and NF-κB (Sevcikova et al. 2011). These transcription factors control the expression of protective genes which repair DNA and influence apoptosis, cell differentiation, and cell growth (Guarino et al. 1995; Sevcikova et al. 2011; Valko et al. 2005).

C.1.3. Genetic effects of metals

ROS arising from redox cycling, the above mentioned impairment of oxidative defenses or Fenton reactions catalized by redox active metals such as Fe, Cu or Cr can

also react with DNA (Ercal et al. 2001; Srikanth et al. 2013). In turn, some of the products of DNA-oxidative damage can induce mutations, which may lead to carcinogenic effects (Bal and Kasprazk 2002; Koedrith et al. 2013). Such products include modified bases (primarily 8-oxoguanine), abasic sites (primarily depurinated), base adducts of carbon-centered radicals (including DNA-crosslinked proteins), and single and double breaks in the phospho-sugar backbone of DNA (Aust and Eveleigh 1999). According to the IARC, metals classified as carcinogenic substances to humans include: Antimony (Sb) trioxide and trisulfite, arsenic (As) and its compounds, beryllium (Be) and its compounds, cadmium (Cd) and its compounds, chromium (Cr), Cr (VI) compounds and Cr (III) compounds, cobalt (Co) and its compounds, gallium (Ga) arsenide, indium (In) phosphide, mercury (Hg) and its compounds, nickel (Ni) compounds and selenium (Se) and its compounds, vanadium (Va) pentoxide (Beyersmann and Hartwig, 2008). Metals can therefore produce clastogenic effects on exposed fish that may be recognized by increased MN frequency. Increased MN frequencies have been observed in the erythrocytes of common carp, prussian carp (*Carassius gibelio*) and peppered cory (*Corydoras paleatus*) exposed to Cu, Cd, Cr, as well as in zebrafish exposed to Cr (Cavas et al. 2005; Domingues et al. 2010).

C.1.4. Cellular effects of metals

Lysosomes play key roles in metal sequestration and detoxification in cells of many organisms (Ahearn et al. 2010). Alterations in lysosomes are used as indicators of toxicity produced by several environmental pollutants in fish (Köhler 1991; Köhler et al. 1992). Changes in the stability of the lysosome membrane together with changes in the appearance of morphological alterations have been observed in European seabass (*Dicentrarchus labrax*) injected with Cu or Cd (Roméo et al. 2000). Similarly, waterborne exposures of turbot (*Scophthalmus maximus*) to Cd and Cu produced enlarged lysosomes and reduced lysosomal membrane stability that were not observed after exposure to Zn (Alvarado et al. 2005a). In European flounder (*Platichthys flesus*) from polluted areas correlation between Cd exposure and effects on the lysosomal membrane stability have also been described while that correlation could not be established for Hg and Pb (Köhler et al. 2002).

C.1.5. Histopathological effects of metals

Histopathological effects have been observed in several organs such as liver, gills, brain and skin as a result of waterborne exposure of fish to metals (Fatima et al. 2014; Sabullah et al. 2015; Zeitoun and Mehana 2014). In the liver, common lesions arising from those exposures include alteration on the lipid/glycogen content of the hepatocytes, loss of liver structure and necrosis. All or some of those pathologies have been identified for example in mrigal carp (*Cirrhinus mrigala*) exposed to Hg and Pb, common carp (*C. carpio*) exposed to Pb or Cd, and Senegalese sole (*Solea*

senegalensis), zebrafish and roach exposed to Cu (Arellano et al. 1999; Chavan and Muley 2014; Paris-Palacios and Biagianti-Risbourg 2006; Patnaik et al. 2011). Gill histological alterations include lamellar hyperplasia, edema, necrosis, separation and fusions as well as expansion of the cartilaginous base of the gill arches. All or some of those pathologies have been identified for example in zebrafish exposed to Cu, mrigal carp exposed to Hg and Pb and Mozambique tilapia (*Tilapia mossambica*) exposed to Hg and Cr (Campagna et al. 2008; Chavan and Muley 2014; Dwivedi et al. 2012). Moreover, changes in the cellular composition of the gills (increased presence of chloride cells) have been observed in turbot exposed to Cu and Zn (Alvarado et al. 2005b).

C.1.6. Developmental effects of metals

Many studies have shown that the early life stages of fish are especially sensitive to metals (Shaw and Handy 2011). Metals may affect various developmental processes. Together with reduced survival rate, a variety of non-lethal physiological effects can affect fish embryos which might results in a reduction of offspring quantity and quality (Jezierska et al. 2009). Non lethal adverse outcomes include delayed hatching process, premature hatching, and deformations such as craniofacial anomalies, yolk sac malformation, vertebral shortening and curvatures, and cardiac malformations (Jezierska et al. 2009). All or some of those effects have been observed in zebrafish after exposure to Cu, Zn, Ni, Cr, Co, Hg, Pb, in ide (*L. idus*) after exposure to Cu or Cd or in common carp exposed to Cd or Zn, among other fish species (Ansari and Ansari 2015; Dave and Xiu 1991; El-Greisy et al. 2015; Johnson et al. 2007; Küçükoglu et al. 2013; Witeska et al. 2014). Anyhow, the protective role of the chorion (through metal accumulation on its surface) makes fish embryo more resistant to metals than early larval stages, where the exposures may produce effects at lower concentrations (Guadagnolo et al. 2000; Johnson et al. 2007). Different fish species (*Salvelinus fontinalis, O. mykiss, Salmo trutta, Salvelinus namaycush, Esox lucius , Catostomus commersonii, Clupea harengus, Micropterus dolomieu*) *exposed* to Cu or rainbow trout exposed to Ag have been probed to be more resistant during the embryonic stages than in the larvae stage (Ansari and Ansari 2015; Guadagnolo et al. 2000; McKim et al. 1978).

C.2. Effects of metal oxide NPs to fish

The amount of knowledge on the effects of NPs has increased considerably during the past few years thanks to the springing up of toxicity studies with this focus. However, the lack of correlation between in vitro and in vivo studies when their toxicity has been analyzed makes necessary to perform in vivo research to fully describe possible effects and mechanisms of NPs toxicity (Fischer and Chan 2007).

One concern is that some metal NPs could undergo dissolution and deliver locally high concentrations of free metals onto the surface of the gills, the digestive tract, etc… thereby releasing free ions directly into the organism in situ (Baker et al. 2014; Handy et al. 2011). Considering the batch of effects resulting from fish exposure to metals described in the previous section, concerns on the possible toxic effects of metal based NPs are well justified. But there are other points to be considered when the toxicity of metal NPs to fish is analyzed.

According to Handy and co-workers (2008), the main concerns in fish nanotoxicology include:

(i) The physical and chemical behaviour of the toxic substance in the external medium will influence bioavailability and therefore toxicity. Some selected abiotic factors (temperature, salinity, dissolved oxygen, type of organic matter, etc.) should influence bioavailability or toxicity in a predictable manner.

(ii) The chemical reactivity of the toxic substance with the molecular components of the tissue (e.g., SH-residues, lipids, nucleic acids, etc.) is predictive of the types of toxic effects and mechanisms involved (e.g., an oxidizing chemical is expected to cause oxidative stress).

(iii) Particle size or shape is important in toxicity. Different sizes (e.g., TiO_2 NPs versus TiO_2 bulk powder) as well as differences in the size or shape of NPs with the same chemical composition should have different toxic effects.

(iv) There is some evidence or indication that the NP follows the general rules for absorption, distribution, metabolism, and excretion. These include uptake or effects at sites of entry into the fish (gills, gut or skin), identification of some target organs and predictions of how the material is carried in the blood, the possibilities for metabolism or storage, and likely routes of excretion.

(v) Similar to bulk chemicals, manufactured NPs with different chemistries should have different toxic effects.

Moreover, the effects of certain metal NPs can also differ among fish species. A comparative study on the acute toxicity of Cu NPs to juveniles of three fish species (rainbow trout, fathead minnow *Pimephales promelas* and zebrafish) revealed similar effects but differences in sensitivity (Song et al. 2015). The exposure caused gill injury and mortality to the three species, however zebrafish was the most sensitive followed by fathead minnow and rainbow trout (Song et al. 2015).

Although data sets on the adverse outcomes that exposure to NPs may produce on fish are still limited, emerging studies on the acute toxicity of nanometals

have so far shown that these materials can be lethal to fish in the µg-mg/L range, depending on the type of material (Bondarenko et al. 2013; Shaw and Handy 2011). Considering nanotoxicology as a newborn science and the still limited information on the effects of NPs to fish is logical to assume that data on metal oxide NPs is even scarcer. Anyhow, during the past few years a number of studies have started to bring some light to the existing knowledge gap on the effects of metal oxide NPs to fish (Table 2). Information comprising from the LC50 values to the transcriptomic effects is now available for a number of species and metal oxide NPs.

C.2.1. Bioaccumulation

Metal bioaccumulation is also a common effect observed in different fish species exposed to metal oxide NPs such as MnO, CuO, TiO_2, ZnO, etc... (Ates et al. 2014; Hao et al. 2013; Ramsden et al. 2009; Vijayakumar et al. 2014). Metal uptake to fish tissues has been documented after waterborne, dietary and injection-mediated exposures to metal oxide NPs (Isani et al. 2013; Ramsden et al. 2009; Zhao et al. 2011). Studies suggest that at least some of the internal target organs for metal oxide NPs are similar to those for traditional chemicals and include the gills, gut, liver and sometimes the brain. Cu accumulation in juvenile common carp after waterborne exposure to CuO NPs has been observed in intestine, gills, muscle, skin , liver and brain (Zhao et al. 2011). Similarly, Ti accumulation was observed in several organs (gill, gut, liver, brain and spleen) of rainbow trout after dietary exposure, as well as in zebrafish after waterborne exposure to TiO_2 NPs (Ramsden et al. 2009; 2013). Bioaccumulation of other metal oxide NPs has shown to be more tissue specific. Significant metal uptake was found only in the liver of zebrafish waterborne exposed CeO_2 NPs (Johnston et al. 2010). Finally, zinc accumulation has also been reported in juvenile common carp exposed to ZnO NPs (Hao et al. 2013).

Thus, bioaccumulation of metal after exposure to metal oxide NPs is a phenomenon affecting different developmental stages and fish species.

Once in the organism, NPs can enter the cells through different pathways, once inside, NPs are stored (accumulated) within lysosomes, the endoplasmatic reticulum, or the Golgi apparatus and can serve as focal points in inducing oxidative damage and lipid peroxidation (Moore et al. 2006).

C.2.2. Transcriptomic and enzymatic effects of metal oxide NPs

Different nanometals are known to produce different responses at the molecular level. Transcriptomic analysis after zebrafish exposure to Cu NPs indicated that many of the genes whose trasnscription is altered in response to the NPs are involved in apoptosis, cell proliferation and differentiation, while exposure to TiO_2 NPs altered

the transcription of a number of genes involved in ribosomal function (Griffitt et al. 2008). Transcriptomic analysis of the full genome in zebrafish embryos exposed to TiO_2 identified significant effects on the regulation of genes related to circadian rhythm, kinase activity, vesicular transport and immune response (Jovanovic et al. 2011a).

However, common responses to different metal oxide NPs (TiO_2, ZnO, Cu_2O) were observed in different fish species. Among others, common effects of different metal oxide NPs included altered transcription or expression of enzymes related to the antioxidant response such as SOD, CAT or GST (Chen et al. 2011a; Varela-Valencia et al. 2014; Zhao et al. 2013).

C.2.3. Genetic effects of metal oxide NPs

Data on the genotoxic effects of metal oxide NPs in fish are still scarce. However, this is a point to be considered as metal oxide NPs have been reported to cause DNA damage indirectly through increased ROS production in different organisms, which may suggest carcinogenic capacity (Chang et al. 2012; Singh et al. 2009). Zhao and coworkers (2013) found increased DNA damage after zebrafish embryo exposure to ZnO NPs and related the appearance of DNA damage with the increased presence of ROS arising from the exposure to NPs. TiO_2 NPs have also been reported to produce DNA damage in goldfish (*Carassius auratus*) skin cells as well as in a rainbow trout gonad cells, in both cases DNA damage was also related with altered ROS production (Reeves et al. 2008; Vevers and Jha 2008). Similarly, in vitro exposure of rainbow trout erythrocytes to a suspension of CuO NPs produced DNA damage which was again attributed to the ROS producing capacity of these NPs (Isani et al. 2013).

C.2.4. Immunological effects of metal oxide NPs

Most of the NPs present in the environment can produce sublethal effects on the immune system of fish with serious implications (Jovanovic et al. 2012). The very first line of fish immune defensive mechanisms against invading pathogens and parasites is the mucus membrane layer of the gills, skin and intestines. Kaya and co-workers (2016) found increased secretion of mucus and swelled globet cells in the gills of tilapia (*Oreochromis niloticus*) exposed up to 10 mg/L of ZnO NPs. In that work reduced potential killing activity of phagocytic cells was detected. Phagocytic cell count (white cell) was also reduced in Indian major carp (*Labeo rohita*) exposed to Fe_2O_3 (500 mg/L) (Remya et al. 2015).

As reviewed by Jovanovic et al. (2012), metal oxide NPs can lead to inflamatory responses and reduced lysozime activity which, in turn, could make organisms more prone to bacterial infection. In fact, reduced resistance to bacterial

infection was observed in fathead minnows exposed up to 10 mg/L TiO_2 (Jovanović et al. 2015). In the same species reduced functionality of neutrophyls and regulation of immune response related genes has also been observed after intraperitoneal injection of 10 μg/g TiO_2 (Jovanović et al. 2011b). Finally, in zebrafish embryos micronjected with 2 μg/g TiO_2 reduced transcription of genes related with the immune function has been observed (Jovanović 2011a).

C.2.5. Histopathological effects of metal oxide NPs

Being the first barrier when fish are waterborne exposed to NPs, gill histology has been the focus of much attention. Their morphological alteration may affect osmoregulatory and respiratory functions of the fish making them a key organ to study effects of aquatic toxicants (Evans, 1987). Dose dependent gill injuries, such as aneurism, dilated and clubbed tips, hyperplasia, edema, curvature, fusion of lamellae, increase of mucous secretion, have been observed in zebrafish exposed to Co_2O_3 NPs (Mansouri et al. 2015). Semi-static exposure of rainbow trout to TiO_2 NPs for 14 days also resulted in gill edema and thickening of the gill lamellae (Federici et al. 2007). Similar alterations (hyperplasia of epithelial cells, lamellar fusion, aneurism, lamellar disorganization, curling) have also been reported in carps exposed to ZnO NPs for 21 days (Subashkumar and Selvanayagam 2014).

As mentioned above, fish liver is also a target organ for NPs toxicity. Hepatocytes with condensed nuclei have been observed in fish exposed to TiO_2 NPs (Federici et al. 2007). Similarly, high concentrations (100, 200 mg/L) of TiO_2 NPs have also been reported to produce necrotic and apoptotic cells in the liver of juvenile carp (Hao et al. 2009).

C.2.6.Developmental effects of metal oxide NPs

Effects on early life stages of fish are also emerging, with suggestions that the nano-forms of some metals (ZnO) may be more toxic to embryos or juveniles, than the equivalent metal salt (Shaw and Handy 2011). The effects that metal oxide NPs produce on developing fish embryos include altered gene transcription, effects on hatching, increased malformation prevalence (finfold abnormalities, tail and spinal cord flexure/truncation, cardiac malformation, yolk sac edema, etc…), tissue ulceration, impairment of antioxidative defence, effects on larval activity level and death among others (Bai et al. 2010; Chen et al. 2014; Hall et al. 2009; Jovanovic et al. 2011a; Xia et al. 2011; Zhao et al. 2011, 2013; Zhu et al. 2012). However, no all metal oxide NPs are equally toxic to fish embryos. Kovrižnych et al. (2013) analyzed the acute toxicity of 10 metal oxide NPs and 5 oxygen containing metal compounds to zebrafish embryos and found that only CaO, CuO, $CuZnFe_4O_4$ and MgO produced cumulative mortality in a 96 h test. Metal oxide NPs do also differ in the sublethal

effects produced to fish embryos. Lin and co-workers (2012) tested the effects of 24 metal oxide NPs on the hatching of zebrafish embryos and found out that only in 4 of 24, the metal oxide NPs (CuO, ZnO, Cr_2O_3, and NiO) could interfere with embryo hatching. They also demonstrated that hatching inhibition was mediated by a mechanism involving ligation of critical histidines in the zebrafish hatching enzyme center by the shed metal ions.

Table 2. Summary of the publications reporting effects of metal oxide NPs to fish.

Metal oxide NPs	Species (D.S.)	Exposure	Analyzed endpoint	Effects	Reference
Cu_2O	*Danio rerio* (Larvae)	**Route:** Waterborne **NP:** 10-20 nm **Concentration:** 30, 60, 121 µg/L **Duration:** 96 h	-Mortality -Transcription -Bioaccumulation	-LC50: 242.4 µg/L -Altered transcription of Cu related genes. Uregulated transcription of *mt*, *Cu/Zn sod*, *mtf1*, *atp7a*, *atp7b*. Downregulated *gst* -NOEL (transcription): 30 µg/L -LOEL (transcription): 121 µg/L -Bioaccumulation at the highest concentrations	Chen et al. (2011a)
CuO	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** 193 ± 90 nm **Concentration:** 0.02, 0.1 0.25, 0.5, 1, 1.5, 2, 2.5, 12.5 mg/L **Duration:** 96 h	-Hatching -Bioaccumulation	-Inhibition of ZHE1 -Concentration dependent hatching delay -Cu accumulation in the perivitelline space	Muller et al. (2015)
CuO	*Cyprinus carpio* (Juveniles)	**Route:** Waterborne **NP:** 394 ± 35 nm **Concentration:** 10, 50, 100, 200, 300, 500, 1000 mg/L **Duration:** 96 h	-Mortality	-Mortality below 30% in all the exposure groups	Zhao et al. (2011)
CuO	*Cyprinus carpio* (Juveniles)	**Route:** Waterborne **NP:** 394 ± 35 nm **Concentration:** 100 mg/L **Duration:** 30 days	-Growth -Bioaccumulation -Neurotoxicity	-Growth inhibition -Cu bioaccumulation in intestine > gill > muscle > skin and scale > liver > brain -ChE activity inhibited (potential neurotoxicity)	Zhao et al. (2011)
CuO	*Cyprinus carpio* (Adults)	**Route:** Waterborne **NP:** 40 nm **Concentration:** 5, 10, 50, 100, 150 µg/L **Duration:** 24, 48, 72, 96 hours.	-Hematological parameters	-Incresased presence of AST, ALT, glucose, cortisol, urea in all the exposure groups	Miri and Rhadari (2015)
CuO	*Oncorhynchus mykiss* (Adults)	**Route:** Intraperitoneal injection **NP**: 20-200 nm **Concentration:**1 mg/g bodyweight	-Bioaccumulation -DNA damage	-Bioaccumulation of Cu ion in gills>kidney>liver -No DNA damage detected	Isani et al. (2013)

CuO	*Oncorhynchus mykiss* (Adults)	**Route:** Waterborne (270 mg/L water hardness) **NP:** CuO particle size range: 5-50 nm **Concentration:**1, 5, 20, 100 mg/L **Duration:** 96 h	-Mortality -Hematological parameters	-No mortality -Affected counts of white blood cells, lymphocytes, eosinophils, neutrophils, hematocrits, MCH, MCHC, MCV -No effects on monocytes, hemoglobins	Khabbazi et al. (2014)
CuO	*Cyprinodon variegates* (Adults)	**Route:** Waterborne -Half strength SW: 1.5% -Full strength SW: 3% **NP:** 30-75 nm in stock, 530 nm in SW **Concentration:** 5, 10 mg/L **Duration**: 7 days	-Mortality -Behaviour -Oxidative stress -Bioaccumulation	**Both water strengths** -No mortality -Behavioral changes : increased mucus secretion, less general activity and loss of equilibrium -Bioaccumulation **Half strength** -Lipid peroxidation in liver and gills -Higher Cu contents in the tissues: intestine > gills > liver	Ates et al. (2014)
CuO	*Oreochromis mossambicus* (Adults)	**Route** :Waterborne -FW -SW **NP:** 21.2 ± 11.8 nm. Variable sizes at diffetent timepoints **Concentration:** 0.5, 5 mg/L **Duration:** 6 days	-Respiration -Oxidative stress -Transcription -Bioaccumulation	-Increased opercular ventilation rate (5 mg/L SW); milder response in FW **Both FW and SW** -Altered activity of antioxidant enzymes CAT, SOD, GR -Increased transcription of metal-responsive genes, GSH:GSSG ratio, and Cu content in fish gill and liver	Villareal et al. (2014)
CuO	*Oreochromis niloticus* (Adults)	**Route** :Waterborne **NP:** < 50 nm **Concentration:** 0, 200, 400, 600, 800, 1000 mg/L **Duration:** 96 h	-Mortality	-LC50 96h: 150 mg/L	Abdel-Khalek et al. (2015)

CuO	*Oreochromis niloticus* (Adults)	**Route** :Waterborne **NP:** < 50 nm **Concentration:** 7.5, 15 mg/L **Duration:** 30 days	-Hematological parameters -Oxidative stress	-Reduced presence of serum glucose, liver AST, ALT, ALP -Increased presence of creatinine, uric acid -Reduced presence of serum total proteins, albumin, globulin, total lipids -In gills, reduction of GSH content -In gills,elevation in MDA and GPx activities -In liver, reduced activity of MDA and GPx	Abdel-Khalek et al. (2015)
ZnO	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** ZnO aggregates, 2196 (1037-4650 nm), 3144 (1421-6823 nm), 2540 (1227-5158 nm) and 2497 (1150-4935 nm) at 0, 0.5, 12 and 48 h **Concentration:** 0.1, 0.5, 1, 5, 10, 50, 100 mg/L **Duration:** 96 h	-Mortality -Hatching -Teratogenicity -Oxidative stress	-Mortality of 10% of unhatched embryos -Dose-dependent hatching inhibition -Pericardial edema (≥5 mg/L) -84 h EC50 (hatching rate) 23.06 mg/L -Increased ROS presence -Downregulation of anti-oxidant genes *Gstp2* and *Nqo1*	Zhu et al. (2009)
ZnO	*Danio rerio* (Embryos)	**Route**: Waterborne **NP:** ZnO aggregates, 142.4-517.7 nm. **Concentration:** 1, 5, 10, 25, 50, 100 mg/L **Duration:** 96 h	-Mortality -Hatching	-Mortality 28.3 ± 14.5% (50 mg/L) and 65.0 ± 8.9% (100 mg/L) -Hatching inhibition (>25 mg/L)	Bai et al. (2010)
ZnO	*Danio rerio* (Adults)	**Route**: Waterborne **NP:** 80-49 nm **Concentration:** 1, 2, 5, 10, 30, 50 mg/L **Duration:** 96 h	-Mortality -Bioaccumulation	-LC50 96 h: 3.969 mg/L -Increased zinc presence with increasing exposure concentration	Yu et al. (2011)
ZnO	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** Iron doped-ZnO NPs. A range of sizes depending on the doping level. **Concentration:** Up to 50 µg/L **Duration:** 5 days	-Mortality -Hatching -Teratogenicity	-No mortality -Delayed hatching -No effects on malformation rate	Xia et al. (2011)

ZnO	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** 50-100 nm **Concentration:** 1, 5, 10, 20, 50, and 100 mg/L **Duration:** 96 and 144 h	-Mortality -Hatching -Teratogenicity -Oxidative stress -DNA damage	-No mortality **96 h** -Dose-dependent effect on hatching and malformation rates (>10 ng/L) -Malformations: hyperaemia, pericardial edema, tail deformity, spinal curvature **144 h** -ROS induction at 50 and 100 mg/L -Oxidative damage/response related genes downregulated: *bcl-2, Nqo1, Gstp2* -Up-regulated ROS production related *Ucp2* -Increased antiox enzyme activities: SOD, CAT, GPx -Lipid peroxidation: Increased MDA concentration -Concentration dependent DNA damage	Zhao et al. (2013)
ZnO	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** 79.51 ± 6.17 nm **Concentration:** 0.1, 0.5, 1, 5, 10 mg/L **Duration:** 144 h	-Mortality -Teratogenicity -Hatching -Behaviour	-No mortality -No malformations -Dose dependent hatching delay -Larval activity level, mean velocity, maximum velocity altered (5-10 mg/L)	Chen et al. (2014)
ZnO	*Cyprinus carpio* (Adults)	**Route:** Waterborne **NP:** <100 nm **Concentration:** 0, 2, 4, 8, 16 mg/L **Duration:** 96 h	-Mortality	-LC50 96 h: 4.897 mg/L	Subashkumar and Selvanayagam (2014)
ZnO	*Cyprinus carpio* (Adults)	**Route:** Waterborne **NP:** <100 nm **Concentration:** 5, 10, 20 % of the LC50 **Duration:** 21 days	-Histological effects in gill	**5% exposure group** -Lamellae with marginal channel dilation, epithelial lifting, desquamation and necrosis, alteration in secondary structure, loss of secondary lamellae **10 % exposure group** -Acute cellular swelling, blood congestion **20 % exposure group** -Gill histology: hyperplasia of epithelial cells, lamellar fusion, aneurism, lamellar disorganization, curling	Subashkumar and Selvanayagam (2014)

ZnO	*Oreochromis niloticus* (Adults)	**Route**: Waterborne **NP:** <100 nm. **Concentration:** 1, 2, 4, 8, 16 mg/L **Duration:** 96 h	-Mortality	-LC10 24 h:3.23 ± 0.4 mg/L; LC10 48 h: 2.81 ± 0.3 mg/L; LC10 96 h: 2.3 ± 0.5 mg/L -LC50 24 h: 4.2± 0.7 mg/L; LC50 48 h: 3.9 ± 0.5 mg/L; LC50 96 h: 3.1 ± 0.4 mg/L -LC90 24 h: 6.1 ± 0.3 mg/L; LC90 48 h: 5.1 ± 0.4 mg/L; LC90 96h: 4.4 ± 0.2 mg/L	Alkaladi et al. (2015)
ZnO	*Oreochromis niloticus* (Adults)	**Route**: Waterborne **NP:** <100 nm **Concentration:** 1, 2 mg/L **Duration:** Up to 15 days	-Hematological parameters -DNA damage	-Leukocytosis, heterophilia, lymphopenia and monocytopenia -Altered blood biochemical parameters -Nuclear aberrations in erythrocytes -Morphological aberrations in erythrocytes	Alkaladi et al. (2015)
ZnO	*Oreochromis niloticus* (Adults)	**Route**: Waterborne **NP**: 10-30 or 100 nm **Concentration:** 1, 10 mg/L **Duration:** Up to 14 days	-Histology -Osmoregulatory effects -Immune effects	**All exposure groups** -Kidney: melanomacrophage aggregates, tubular deformations, necrosis, cytoplasmic vacuolations -Liver: edema, mononuclear cell infiltrations, fatty changes, pyknotic nuclei, hepatocellular vacuolations -Gills: hyperplasia, aneurysms, epithelial liftings -Intestine: hyperplasia, swelled of goblet cells, villus deformations -Inhibition of Na^{+},K^{+}-ATPase -Increased Ca^{2+} and Cl^{-} presence in serum -Increased mucus secretion in skin and gills **1 mg/L** -Damaged immune system: increased respiratory burst and potential killing activity of phagocytic cells **10 mg/L** -Damaged immune system: reduced respiratory burst and potential killing activity of phagocytic cells	Kaya et al. (2016)

ZnO	*Oryzias melastigma* (Embryos)	**Route**: Waterborne **NP**: 26.2 ± 5.1 nm **Concentration:** 4, 40 mg/L **Duration:** 96 h	-Expression of stress related enzymes	-No effects on the expression of SOD and MT -Induction of HSP70 expression	Wong et al. (2010)
ZnO	*Cyprinus carpio* (Juveniles)	**Route**: Waterborne **NP:** 30 nm **Concentration:** 50 mg/L **Duration** 30 days	-Bioaccumulation -Ionoregulation -Oxidative stress -Histology	-Increased Zn contents in gill, liver, intestine and brain -Decreased Na^+/K^+-ATPase activity in liver and gill -Reduced SOD activity -Reduced GSH levels in liver and gill tissues -Increased lipid peroxidation (MDA content) -Protein oxidation in liver and gills -Gill histopathology: damaged cell membrane, swollen and distorted organelles, disruption of cells -Liver histopathology: vacuolization, apoptosis, cell membrane rupture, desquamation of hepatocytes, severe cellular necrosis	Hao et al. (2013)
TiO_2	*Danio rerio* (Adults/Juveniles)	**Route:** Waterborne **NP:** 220.8 and 687.5 nm **Concentration:** up to 10 mg/L **Duration:** 48 h	-Mortality -Transcription -Histology	-LC50 48 h >10 mg/L both in adult and juveniles -Altered transcription of ribosomal genes -No histological effects on gills	Griffit et al. (2008)
TiO_2	*Danio rerio* (Adults)	**Route**: Waterborne **NP:** 14.10 ± 1.52 nm **Concentration:** 10, 100 mg/L **Duration:** 14 days	-Biochemical constituents	-Changes in the structures of gill proteins -Changes in the relative composition of biomolecules	Palaniappan and Pramod (2010)
TiO_2	*Danio rerio* (Adults)	**Route**: Waterborne **NP:** Anatase, 14.10 ± 1.52 nm **Concentration:** 10 mg/L **Duration:** 14 days	-Hepatic micro-environment (Raman spectroscopy)	-Changes in the structure of the liver: proteic conformational changes, decreased water domain size, disruption of salt bridges, variation in the hydrogen bonding of the phenolic hydroxyl group	Palaniappan and Pramod (2011)
TiO_2	*Danio rerio* (Embryos)	**Route:** Microinjection in the otic vesicle **NP:** Anatase, 86 and 409 nm **Concentration:** 2 µg/g	-Transcription	-Downregulation of genes involved in circadian rhythm, cell signaling through kinase-related activities, exocytosis and trafficking of Golgi vesicles, immune function	Jovanovic et al. (2011a)

TiO_2	*Danio rerio* (Adults)	**Route**: Waterborne **NP:** <10 nm **Concentration:** 1, 2, 4, 5, 7 mg/L **Duration:** 6 months	-Growth -Histology	-Concentration dependent and time-dependent inhibition of growth and decrease of the liver weight ratio -Gill injury: thickening of edema and gill lamellae	Chen et al. (2011b)
TiO_2	*Danio rerio* (Adults)	**Route**: Waterborne **NP**: 25% rutile and 75% anatase, 24.1 ± 2.8 nm **Concentration:** 0.1, 1 mg/L **Duration:** 14 days	-Bioaccumulation -Hematological parameters -Ionoregulation -Oxidative stress -Histology -Reproduction	-Significantly increased Ti concentrations (1 mg/L) -No effects on erythrocyte counts -Decline in leukocyte counts -No ionoregulatory effects -Increased GSH levels in brain, gill, liver -No histological effects -Reduced viable progeny (1 mg/L)	Ramsden et al. (2013)
TiO_2	*Danio rerio* (Embryos)	**Route**: Waterborne **NP**: 220 nm **Concentration:** 0.01, 10, 1000 mg/L **Duration:** 130 h	-Hatching rate	-Adsorption of NPs to the chorion **1000 mg/L** -Premature hatching at 62 hpf -EC 10 (hatching): 0.073 mg/L -EC 10 (hatching): 107.2 mg/L	Samaee et al. (2015)
TiO_2	*Oncorhynchus mykiss* (Adults)	**Route:** Waterborne **NP:** 75% rutile/25% anatase, 21 nm. **Concentration:** 0.1, 0.5, 1 mg/L **Duration:** 14 days	-Gill histology -Hematological parameters -Oxidative stress -Ionoregulation -Histology	-Gill pathologies: edema, thickening of the lamellae -No major hematological disturbances -Increased TBARS in the gill, intestine and brain -Increased GSH in gills, depletion in liver -Decreased Na^+K^+-ATPase activity in the gills and intestine -Liver cells: minor fatty changes and lipidosis, condensed nuclear bodies	Federici et al. (2007)
TiO_2	*Oncorhynchus mykiss* (Juveniles)	**Route**: Dietary **NP:** 75% rutile/25% anatase, 21 nm **Concentration:** 10, 100 mg/kg **Duration:** 8 weeks followed by a 2 week recovery period	-Growth -Behaviour -Hematological parameters -Bioaccumulation -Ionoregulation -Oxidative stress	-No effects on growth, nutritional performance and hematological parameters -Ti accumulation in gill, gut, liver, brain, spleen that remained after the recovery period -Cu, Zn levels and Na^+K^+-ATPase activity altered in brain -No effects on GSH content -TBARS reduction in gill and intestine	Ramsden et al. (2009)

TiO_2	*Cyprinus carpio* (Juveniles)	**Route**: Waterborne **NP:** Rutile, 50 nm **Concentration:** 10, 50, 100, 200 mg/L **Duration:** 8 and 20 days	-Oxidative stress -Histology	**8 days** -Decreased SOD, CAT, POD activities (100, 200 mg/L) -Increased lipid peroxidation in liver (MDA) (100, 200 mg/L) **20 days** -Gill pathologies: edema, thickening of gill lamellae and of gill filaments (100, 200 mg/L) -Liver pathologies: necrotic hepatocytes and apoptotoic bodies (100, 200 mg/L)	Hao et al. (2009)
TiO_2	*Carassius auratus* (Adults)	**Route:** Waterborne **NP:** Rutile, 100-1000 nm **Concentration:** 10, 100 mg/L **Duration:** 5 days	-Mortality -Oxidative stress -Bioaccumulation -Growth	-No mortality -Lipid oxidation in liver (MDA) -Dose dependent bioaccumulation in intestine and gills -Growth inhibition	Ates et al. (2013)
TiO_2	*Oreochromis niloticus* (Adults)	**Route**: Intraperitoneal injection **NP:** Anatase and rutile, 7, 14, 21 nm **Concentration:** 0.1, 1, 10 mg/L **Duration:** 3, 6, 12, 24 h	-Trasncription -Oxidative stress	-1 mg/L exposures induced CAT, GST, SOD transcription at 6 hpe, after which transcript levels decreased -7, 14 nm NPs induced CAT, GST, SOD transcription at 6 hpe -Stronger GST induction by 21 nm rutile NPs at 6 hpe	Varela-Valencia et al. (2014)
TiO_2	*Pimephales promelas* (Embryos)	**Route:** Waterborne **NP:** Anatase, 10 nm **Concentration**: Not specified **Duration:** 96 h and 7 days	-Mortality -Growth	-LC50 96 h: 500 mg/L -IC25 (growth) values 7 days: >340 mg/L	Hall et al. (2009)
TiO_2	*Pimephales promelas* (Embryos)	**Route:** Waterborne **NP:** Anatase, 43 nm **Concentration:** 0.01, 0.1, 1, 10, 100, and 1000 mg mL **Duration:** 7 days	-Mortality	-No mortality was observed	Jovanović et al. (2011b)

TiO_2	*Pimephales promelas* *(*Adults)	**Route:** Intraperitoneal injection **NP:** Anatase, 43 nm **Concentration:** 10 µg/g **Duration:** 48 h	-Hematological parameters -Neutrophil functionality -Transcription	-No effects in the number of circulating lymphocytes, monocytes, neutrophils, thrombocytes -Decreased neutrophil functionality: increased respiratory burst -Upregulated transcription of: *interleukin 11* (4 fold increase), m*acrophage stimulating factor 1* (2.5 fold increase) and *neutrophil cytosolic factor 2* (2 fold increase)	Jovanović et al. (2011b)
TiO_2	*Pimephales promelas* (Adults)	**Route**: Intraperitoneal injection **NP:** Anatase, 220 and 585 nm **Concentration:** 2 and 10 mg/g **Duration:** 48 h	-Bioaccumulation -Immunotoxicity -Histology	-TiO_2 accumulation in kidney > spleen > liver -Reduced resistance to bacterial infection -Changes in glomerular and endocardial architecture of the kidney	Jovanović et al. (2015)
Fe_2O_3	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** 30 nm **Concentration:** 0.1 mg/L **Duration:** 168 h	-Mortality -Hatching -Teratogenicity	-168 h LC50: 53.35 mg/L -Hatching delay -EC50 hatching rate: 36.06 mg/L -Tissue ulceration, pericardial edema, body arcuation	Zhu et al. (2012)
Fe_2O_3	*Oreochromis mossambicus* (Adults)	**Route:** Oral administration **NP:** 19-33 nm **Concentration:** 0.5, 5, 50 µg/mL **Duration:** 96 h	-Hematological parameters -Biochemistry	-Lower erythrocytes and hemoglobin levels (48, 96 h) at all concentrations -Increased hematocrit values (24, 48 h) -Serum transaminase enzymes: elevated level of GOT and GPT enzymes till 48 h (hepatic damage)	Karthikeyeni et al. (2013)
Fe_2O_3	*Labeo rohita* (Adults)	**Route:** Waterborne **NP:** <100 nm **Concentration:** 1, 25 mg/L **Duration**: 96 h	-Hematological parameters -Biochemistry -Ionoregulation	-Decreased hemoglobin content, hematocrit value, mean cellular volume, mean cellular hemoglobin, protein level -Decreased Na^+, K^+, Cl^- levels -Decreased gill Na^+/K^+-ATPase activity -Increased white blood cell, mean cellular hemoglobin concentration -Increased glucose levels -No significant changes in red blood cell count and gill (25 mg/L)	Saravanan et al. (2015)

Fe_2O_3	*Labeo rohita* (Adults)	**Route:** Waterborne **NP:** 100-200 nm **Concentration:** 500 mg/L **Duration**: 25 days	-Hematological parameters -Biochemistry -Ionoregulation	-Increased hemoglobin content, red blood cell count, hematocrit value -Decreased MCV, MCH, MCHC, WBC -Altered gill Na^+/K^+-ATPase activity -Decreased Na^+, Cl^-, K^+ levels	Remya et al. (2015)
Co_2O_3 NPs	*Danio rerio* (Adults)	**Route:** Waterborne **NP:** 50 nm **Concentration:** 10, 40, 70, 100 mg/L **Duration:** 8 days	-Mortality -Gill histology	-LC50 > 100 mg/L -Dose dependent gill injuries: aneurism, dilated and clubbed tips, hyperplasia, edema, curvature, fusion of lamellae, increase of mucus secretion, hypertrophy, and necrosis	Mansouri et al. (2015)
MgO	*Oreochromis mosssambicus* (Adults) *Danio rerio* (Adults)	**Route:** Waterborne **NP:** <50 nm **Concentration:** 10, 50,100, 150 and 200 mg/L **Duration**: 120 h	-Mortality -Oxidative stress -Bioaccumulation	-No mortality -Gradual and sporadic increase in CAT activity -Concentration dependent increased activity of GST, SOD -Bioaccumulation	Vijayakumar et al. (2014)
$ZnO/TiO_2/Al_2O_3$	*Danio rerio* (Embryos)	**Route:**Waterborne **NP:** ZnO 180 nm /TiO_2 (anatase) 230 nm /Al_2O_3 930 nm **Concentration:** -ZnO: 0.1, 0.5, 1, 5, 10, 50 mg/L -TiO_2: 1, 10, 50, 100, 500 mg/L -Al_2O_3: 1, 10,100, 1000 mg/L **Duration:** 96 h	-Mortality -Hatching -Teratogenicity	-96 h LC50 ZnO: 1.793 mg/L -84 h EC50 (hatching rate) 2.065 mg/L -Tissue ulceration in larvae exposed to nZnO -TiO_2/Al_2O_3: no toxicity	Zhu et al. (2008)
TiO_2/Cu_xTiO_y	*Danio rerio* (Embryos)	**Route**: Waterborne **NP**: TiO_2 (anatase) 30-50 nm/ Cu_xTiO_y >50 nm **Concentration:** 10, 20 µg/L **Duration:** 72 h	-Hatching -Teratogenicity -Oxidative stress	-Reduced hatching in all exposure groups -Malformations included inflammatory phenotype, edema and abnormal notochords -Increaesd GSH amount, GSR, GST, CAT activities in the hatched larvae	Yeo and Kang (2009)
CeO_2/ZnO	*Danio rerio* (Adult)	**Route:** Waterborne **NP:** CeO_2 10.2 ± 0.78 nm/ ZnO 68.7 ± 3.35 nm **Concentration:** 500, 5000 µg/L **Duration:** 7 days	-Bioaccumulation	-Significant uptake of cerium in zebrafish liver -No other significant uptakes of Ce or Zn in liver, brain, gill, skin	Johnston et al. (2010)

TiO_2	*Oncorhynchus mykiss* (Adult)	**Route:** Waterborne **NP:** 34.2 ± 1.73 nm **Concentration:** 500, 5000 µg/L **Duration:**14 days	-Bioaccumulation	-TiO_2 presence detected in gill, gut, liver, brain -No significant metal concentration increase detected in liver, brain, gill, skin, blood, gut	Johnston et al. (2010)
TiO_2	*Oncorhynchus mykiss* (Adult)	**Route:** Diet **NP:** 34.2 ± 1.73 nm **Concentration:** 0.01%, 0.1% of the diet weight **Duration:** 21 days	-Bioaccumulation	-TiO_2 presence detected in gut, gill -No significant metal concentration increase detected in liver, brain, gill, skin	Johnston et al. (2010)
CuO/ZnO/NiO/ Co_3O_4	*Danio rerio* (Embryo)	**Route**: Waterborne **NP:** CuO 18 ± 5 nm/ ZnO 23 ± 7 nm/ NiO, 40 ± 12 nm/ $Co_3O_4$12 ± 7 nm **Concentration** 0.05, 0.5, 5, 25, 50, 100, 200 mg/L **Duration:** 5 days	-Hatching effects -Cellular stress	-CuO, ZnO, NiO dose-dependent hatching interference -CuO, ZnO, NiO induced *hsp70* transcription	Lin et al. (2011)
TiO2	*Danio rerio* (Adults)	**Route:** Waterborne **NP:** Anatase, 200-500 nm **Concentration:** 2, 5, 10, 30, 50 mg/L **Duration:** 96 h	-Mortality -Oxidative stress -Histology	-LC50 96h: 124.5 mg/L -50 mg/L decreases in SOD activity in liver, increased SOD activity in gut, CAT inihibition in liver -Cell membrane damage, irregular cell outlines, pyknotic nuclei, trend of complete disruption of gill cells	Xiong et al. (2011)
ZnO	*Danio rerio* (Adults)	**Route:** Waterborne **NP:** Six-wurtzite, 400-1400 nm **Concentration:** 10, 50, 100, 150, 200, 300 mg/L **Duration:** 96 h	-Mortality -Oxidative stress -Histology	-LC50 96h: 4.92 mg/L -5 mg/L increased lipid peroxidation, CAT stimulation, reduced SOD activity in liver, increased SOD activity in gut tissue -Loss of cell cytoplasm, abnormal nuclei shape	Xiong et al. (2011)

$CuO/Cr_2O_3/ZnO$ $/NiO/Yb_2O_3/$ $SiO_2/CeO/$ $Gd_2O_3/Y_2O_3/$ $ZrO_2/La_2O_3/$ $Ni_2O_3/Fe_3O_4/$ $Mn_2O_3/Sb_2O_3/$ $WO/In_2O_3/$ $Fe_2O_3/Co_3O_4/$ $CoO/HfO/TiO_2/$ Al_2O_3/SnO_2	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** see reference **Concentration:**0.05, 0.5, 5 and 50 mg/L **Duration:** 5 days	-Hatching -ZHE1 activity	-Hatching affected: CuO > 0.5 mg/L, ZnO,Cr_2O_3 > 5 mg/L, NiO > 50 mg/L -Hatching interfered due to dissolved ions interaction with ZHE1	Lin et al. (2012)
$Al_2O_3/$ $Al_2O_3.TiO_2/$ $BaFe_{12}O_{19}/Bi_2O_3$ $/Cr_2O_3/CuO/Cu$ $ZnFe_4O_4/$ $NiFe_2O_4/Fe_2O_3/$ $Mg(OH)_2/MgO/$ $NiO/SnO_2/TiO_2/$ $WO_3/CaO/SiO_2$	*Danio rerio* (Adults/Embryos)	**Route:** Waterborne **NP:** see reference **Concentration:** Different range for each NP **Duration:** 96 h	-Mortality -Hatching -Teratogenicity	-CaO, CuO, $CuZnFe_4O_4$, MgO: cumulative mortality (96 h). -LC 48/ 96 for each NP: see reference	Kovrižnych et al. (2013)
Metal doped TiO_2: Cu-TiO_2/ Mn-TiO_2/ Fe-TiO_2 /Ni-TiO_2	*Danio rerio* (Embryos)	**Route**: Waterborne **NP**: Cu-TiO_2/ Mn-TiO_2/ Fe-TiO_2/Ni-TiO_2 (anatase): 10-15 nm **Concentration:** 4 mg/L **Duration:** 72 h	-Mortality -Teratogenicity	-Mortality: Cu-TiO_2: 53% Mn-TiO_2: 44% Fe-TiO_2: 63% Ni-TiO_2:62% -Malformation rates: Cu-TiO_2: 8.51% Mn-TiO_2: 8.93% Fe-TiO_2: 5.41% Ni-TiO_2: 3.95%, -Abnormal morphologies regarding: heart, edema, kidney, axis, tail, pigment, and otolith organs	Park et al. (2014)
$ZnO/TiO_2/CeO_2$ $/SnO_2$	*Danio rerio* (Embryos)	**Route:** Waterborne **NP:** ZnO 8.35 nm/ TiO_2 11.64 ± 0.26 and 10.47 ± 0.13 nm/ CeO_2 2.87 ± 0.12 and 2.77 ± 0.12 nm/ SnO_2 2.99 ± 0.08 and 2.87 ± 0.06 nm **Concentration:** 0.08, 0.4, 2, 10, 50 mg/L **Duration:** 120 h	-Mortality -Hatching -Teratogenicity	-Only ZnO toxic LC50 96 h: 3.5-9.1 mg/L -LC50 24 h: >50 mg/L -EC50(mortality+malformation): 0.5-3.51 mg/L	Wehmas et al. (2015)

ZnO	*Danio rerio* (Larvae)	**Route:** Waterborne **NP:** 8.35 nm **Concentration:** 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 mg/L **Duration:** 24 h	-Mortality	-LC50 24 h: 2.20 mg/L	Wehmas et al. (2015)
CuO/ZnO/CeO_2	*Carassius auratus* (Juveniles)	**Route:** Waterborne **NP:** CuO 51.09 ± 9.98 nm/ ZnO 48.16 ± 13.56 nm/CeO_2 32.24 ± 10.12 nm single particles and mixtures **Concentration:** 20, 40, 80, 160, 320 mg/L **Duration:** 4 days	-Oxidative stress -Nervous system -Osmoregulation	-SOD and CAT inhibited by CuO and ZnO (≥160 mg/L) -AChE inhibited by CuO and ZnO -Concentration-dependet Na^+/K^+-ATPase induction -IBR: Synergistic effects of CuO and ZnO mixtures -IBR: Additive toxic effect for ternary mixture -IBR: Antagonistic toxic effects in CeO_2 containing binary mixtures	Xia et al. (2013)

Table abbreviations: ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; *atp7a: ATPase alpha polypeptide; atp7b: ATPase beta polypeptide; bcl-2: B-cell lymphoma 2*; DS: Developmental stage; CAT: Catalase; ChE: Cholinesterase; EC: Effect concentration 50; FW: Fresh water; GOT: Glutamic oxaloacetic transaminase; GPT: Glutamic pyruvic transaminase; GPx: Glutahione peroxidase; GR: Glutathione reductase; GSH: Glutathione; *gst: glutathione-S-transferase* Hpe: Hours post exposure; Hpf: Hours post fertilization; HSP70: Heat shock protein 70; IBR: Integrated biomarker response; LC50: Lethal concentration 50; LOEL: Lowest observed effect level; MCH: Mean cell hemoglobin; MCHC: Mean cell hemoglobin concentration; MCV: Mean corpuscular volume; MDA; Malondialdehyde; MT: Metallothionein; *mtf1: metal regulatory transcription factor 1* NOEL: Non observed effect level; *nqo1: NAD(P)H quinone oxidoreductase 1* POD: Peroxidase; ROS: Reactive oxygen species; SOD: Superoxide dismutase; SW: Salt water; TBARS: Thiobarbituric acid reactive substances; *ucp2: mitochondrial uncopling protein 2*; WBC: White cell count; ZHE1: Zebrafish hatching enzyme 1

D. THE ZEBRAFISH MODEL

D.1. Characteristics and natural distribution

The zebrafish (*Danio rerio*) is a small subtropical freshwater fish which was first described by the Scottish surgeon Francis Hamilton in 1822 (Spence et al. 2008). An adult individual rarely exceeds 4 cm in length, its body posses a fusiform shape and it is laterally compressed with a terminal oblique mouth directed upwards (Spence et al. 2008). Although both genders are similar, males tend to be gold coloured in the belly and fins and slenderer than females (Wixon 2000). They have two pairs of barbells and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal vein (Spence et al. 2008). These characteristic stripes give its name to this species, which belongs to the family of Cyprinidae (Bopp et al. 2006). Despite, more than 450 millions of phylogenic separation between cyprinids and mammals (Fig. 9), basic biology remains very much the same throughout vertebrates and the zebrafish has proved to be a sweeping useful vertebrate model for multiple studies (Lam and Gong 2006; Metscher and Ahlberg 1999). At higher phylogenetic levels, the zebrafish shares the infraclass teleostei with other model organisms, such as medaka (*Oryzias latipes*), the pufferfish (*Takifugu rubripes, Tetraodon nigroviridis*), and the three-spined stickleback (*Gasterosteus aculeatus*), whose common ancestor lived about 100 million years ago (a similar distance as that between humans and the mouse) (Mayden et al. 2009).

Native to South Asia, they are distributed primarily around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, Nepal and northen Myanmar (Fig. 10) (Engeszer et al. 2007; Spence et al. 2006). This geographic region has a monsoon climate, with pronounced rainy and dry seasons that have a profound effect on habitat parameters, including water chemistry and resource abundance (Lawrence 2007). Water quality in these habitats can therefore vary depending on the geographic distribution and seasonality. Zebrafish have been found in waters with pH, conductivity, and temperature ranging between 5.9-8.5, 10-2000 µS, and 6-38ºC, respectively (Engeszer et al. 2007; McClure et al. 2006; Spence et al. 2006; 2008).

They are primarily a floodplain species, most typically encountered in shallow, standing, or slow-moving bodies of water near rice cultivations, but they have also been found in rivers and small streams with a low flow regime (Engeszer et al. 2007; Spence et al. 2008).

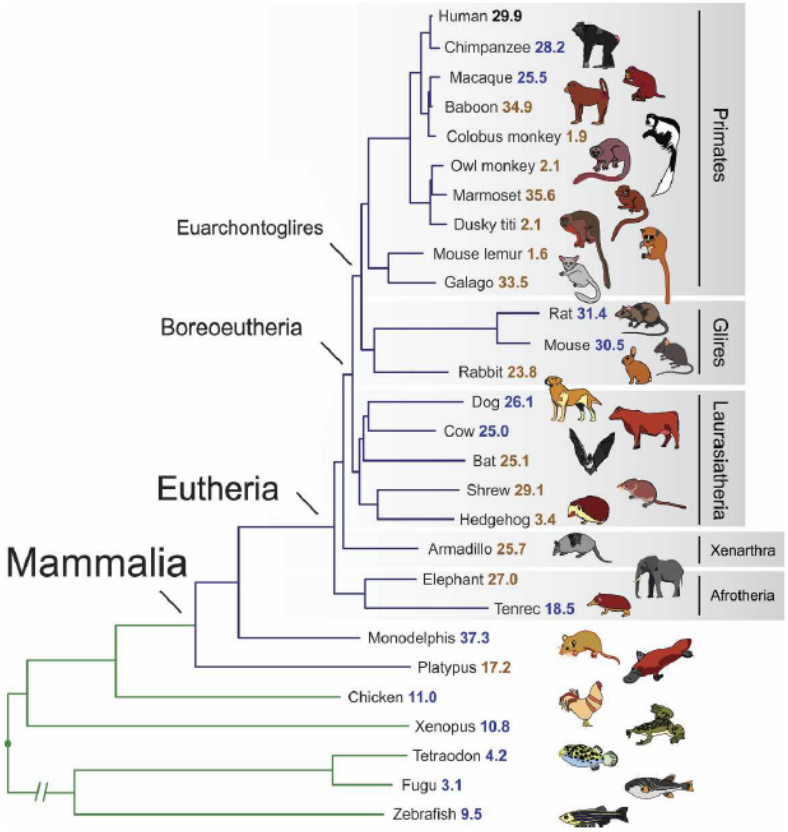


Fig. 9. Zebrafish phylogeny in relation to other fish and mammals (Margulies et al. 2007).

In those natural habitats, a variety of zooplankton and insects (both aquatic and terrestrial) are found that together with algae, detritus, and various other organic materials constitute the basis of their diet (McClure et al. 2006).

D.2. Reproduction and development

The zebrafish lives in small schools of 5-20 individuals (Pritchard et al. 2001). They are batch spawners, females scatter clutches of eggs over the substratum, during the early morning along the margins of flooded water bodies, often in shadows, still, and heavily vegetated areas (Engeszer et al. 2007). Eggs, which are externally fertilized, are demersal and transparent during the first few days of development (Teraoka et al. 2003; Wixon 2000). Adult individuals breed year-round, and natural mating generate large and synchronous batches of embryos; average clutch size is about 200 (Barut and Zon 2000).

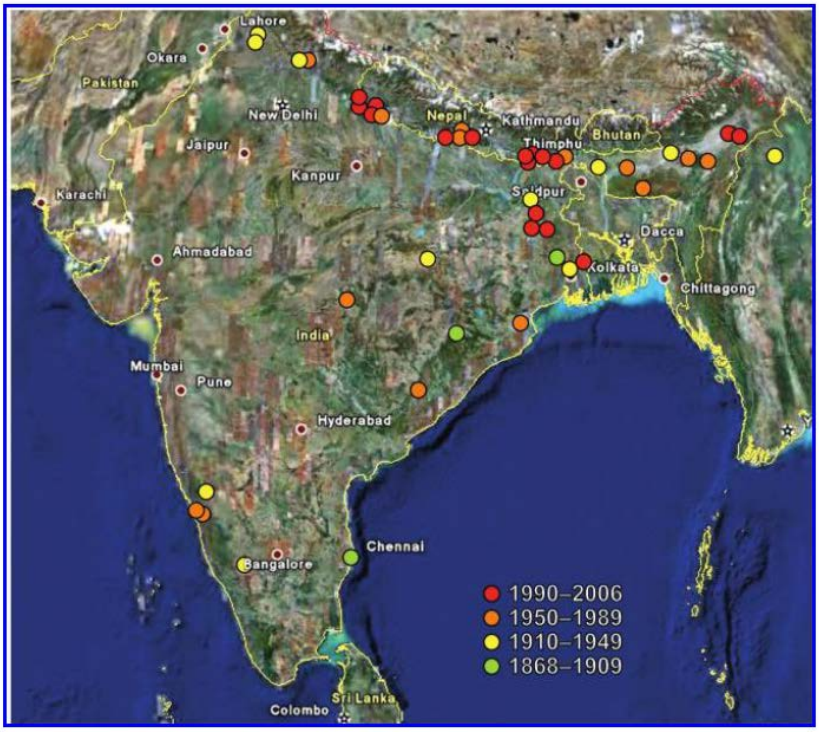


Fig. 10. Historical collections of zebrafish in India and neighboring countries since 1868 (Engeszer et al. 2007).

From the moment an egg is fertilized until it becomes a reproductive adult individual, a zebrafish embryo overcomes many changes and passes through different developmental stages which are named as follows: embryos are 0 to 72 hours post fertilization (hpf), early larvae are 72 hpf to 13 days post fertilization (dpf), mid larvae are 14 to 29 dpf, juveniles are 30 dpf to 3 or 4 months (when they reach sexual maturity), and adults are sexually mature from 3 or 4 months old (Fleming 2007).

Embryo and larvae development have been carefully described by Kimmel and co-workers (1995) (Fig. 11). Under laboratory conditions, the zebrafish embryonic development is spectacularly fast: it generates a typical vertebrate body plan with a neural tube, muscles, a vascular system and a beating heart within 24 hours of fertilization at 28 °C. Soon after fertilization, cytoplasmic streaming movements in the egg generate an area of yolk-free cytoplasm (the blastodisc) at the top (animal) pole of the embryo. Early cleavages occur every fifteen minutes. They divide the blastodisc, but not the yolk, generating a mound of cells on top of, and in cytoplasmic contact with, the ball of yolky cytoplasm below. The embryo is now termed a blastula (2 $^{1/4}$ hours).

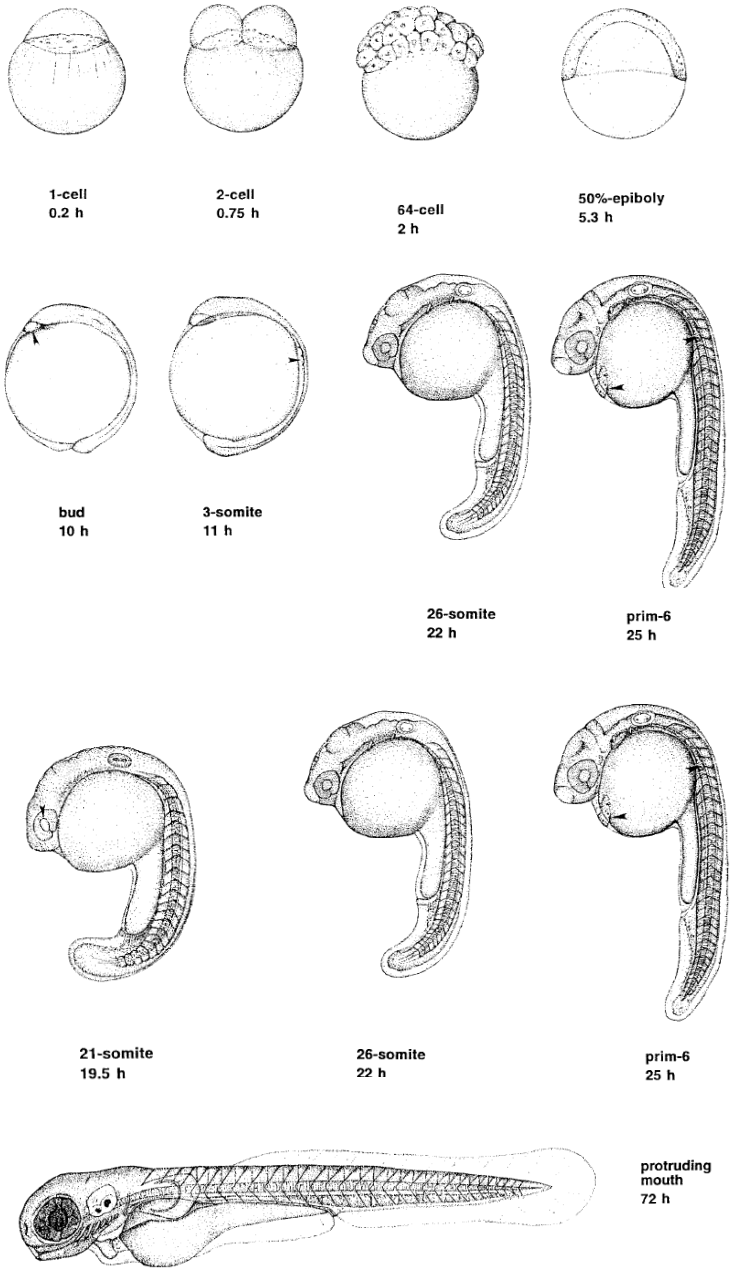


Fig. 11. Zebrafish embryonic development. Modified from Kimmel et al. (1995).

In the tenth cell cycle, the embryo undergoes a characteristic mid-blastula transition: the cell cycle slows and becomes asynchronous, cells become motile and transcription from the zygotic genome begins. In the late blastula, the blastodisc thins and forms a blastoderm that spreads over the yolk, eventually completely engulfing it. This spreading movement, termed epiboly, provides a useful marker of the stage

of development, which can be measured by the percentage of yolk covered, or "percent epiboly". Gastrulation occurs by 5¼ hours after fertilization. At 50% epiboly, the leading edge of the blastoderm margin thickens (generating the germ ring), and migrating cells begin to converge on the prospective dorsal side of the embryo, generating the embryonic shield. These thickenings are the first sign of the gastrulation movements that now begin to build the body plan; this is evident by the 18 somite stage, a mere 18 hours after fertilization. Muscular contractions now begin, and the rudiments of several organs (brain, eye, ear) become visible. In the next few hours, the tail extends, the heart begins to beat and the embryo takes on a more fish-like appearance.

24 hours after fertilization pigmentation and fins develop and just 24 hours later organogensis and morphogenesis are completed and hatching can occur. 72 hours after the initial zygote stage the swim bladder inflates and the new larvae start food seeking (Kimmel et al. 1995).

In natural areas, larval fish remain in the quiet margins of flooded areas as they develop, and move into deeper, open water as they mature and as floodwaters recede (Engeszer et al. 2007).

D.3. Advantages of zebrafish as model organism

Since early 1970's, when George Streisenger started to use the zebrafish as a vertebrate model for development and genetics, the zebrafish has been increasingly employed in research. The revolutionary, pioneering work performed by Dr. Streisinger's team at the University of Oregon allowed scientists to employ techniques that had previously been feasible only in invertebrates to study the genetics of development in higher organisms (Harper and Lawrence 2011). Today the zebrafish is extensively used worldwide, a basic search at any of the international scientific browsers returns thousands of articles where this organism has been used as a model to seek for diverse answers. Fish models cannot replace mammals for research into mammalian physiology, but they can offer an inexpensive and, in some instances, more acceptable alternative for chemical carcinogen testing (Powers 1989). By now, the zebrafish is a popular model in several areas including developmental toxicology, genetics, carcinogenesis, drug discovery, disease modeling, biomedical research, pharmaceutics, toxicology and ecotoxicology among others (Langheinrich 2003; Parichy 2015; Spitbergen and Kent 2003; Sprague et al. 2003; Stern and Zon 2003; Teraoka et al. 2003).

The reason for such popularity is related to the several advantages it offers as a model. According to Leader and Padgett (1980) a good animal model should accomplish the following characteristics:

1. It should accurately reproduce the disease or lesion under study.
2. It should be available to multiple investigators.
3. It should be exportable to other laboratories.
4. If genetic, it should be in a polytocous species.
5. It should be large enough for multiple biopsies of samples.
6. It should fit into available animal facilities of most laboratories.
7. It should be easily handled by most investigators.
8. It should be available in multiple species.
9. It should survive long enough to be usable.

The zebrafish meets most of those features. The tiny size of the larval and adult zebrafish greatly reduces costs as it enables the reduction of housing space and husbandry costs (Bopp et al. 2006; Hill et al. 2005). The short life cycle and generation time (2-3 months for eggs to become reproductive adult) described above are also key characteristics that allow the performance of many bioassays. This high reproductive output allows the operation of tests in high-throughput screenings, i.e. in multi-well plates, and thus a sufficient database from many replicate samples can be gained for statistical evaluation and validation of results (Bopp et al. 2006). The use of embryos is also attractive for other reasons. Fish embryos (before the stage of independent feeding) are considered an alternative to animal testing and their use is not restricted by regulations for animal welfare (Embry et al. 2010). Moreover, the optical clarity of their embryos allows direct observation of their development and alterations (Hill et al. 2005). By contrast, whole-animal assays provide data that are more easily extrapolated to humans and allow complex organismal functions (e.g. behavior and development) to be studied (Barnes 1986).

Today, the zebrafish has been well characterized morphological, physiological and genetically providing an additional advantage when toxicological studies have to be carried out. Finally, the availability of different new technologies as well as the spreading of their application in different fields has greatly supported the prevalence of zebrafish as a model (Bopp et al. 2006). In this sense, the development of zebrafish microarrays in different platforms, allows the use of zebrafish in genomic and transcriptomic studies. In September 2015, Geo DataSets library at NCBI displayed the existence of 168 platforms for zebrafish. Up today, microarray technology has been applied in zebrafish with different purposes like studying the embryonic development or the effects of different chemicals like Cu or 17alpha-ethinylestradiol (Craig et al. 2009; Martyniuk et al. 2007; Mathavan et al. 2005).

The increasing use of zebrafish as a model has been paralleled by an increase in the knowledge on this organism. Consequently available tools and databases have

sprung up on the internet. An interesting review on the zebrafish related online resources was published by Smith (2012), with links to general information, resources, specific information (sequencing, images and imaging, videos, laboratories with primary zebrafish focus and facilities) and zebrafish journals, associations, and societies.

D.4. Zebrafish in ecotoxicology

Ecotoxicology is one of the traditional fields for the application of the zebrafish model at which its potential is still being developed (Busch et al. 2011; Seok et al. 2008). Both zebrafish embryos and adults are extensively employed to assess the toxicity of chemicals (Hill et al. 2005). Zebrafish characteristics make it a unique system to analyze sublethal toxic effects that only can be studied applying holistic, in toto approaches (Raldúa et al. 2012)

In this context, the zebrafish has already been employed as a useful model to test diverse pollutants including metals, endocrine disruptors and organic pollutants (Dai et al. 2014). Moreover, the zebrafish is also considered appropriate to assess the toxicity of emerging pollutants (Raldúa et al. 2012). In fact, the zebrafish is already a considered a model organism to evaluate the toxicity and modes of action of NPs (Fako and Furgeson 2009; Orbea et al. 2015). Thus, in coming years the zebrafish might reinforce its role as reference model species in ecotoxicology due to its usefulness for nanotoxicological assessments (Rizzo et al. 2013).

The use of an entire vertebrate organism allows testing effects of compounds on overall viability, organs and tissues (Lessman 2011). Due to the new restrictions for the in vivo tests and the need to fulfil the 3 Rs principle, zebrafish embryos are of great interest when toxicity of hundreds of chemical has to be evaluated (Fleming 2007; Lessman 2011). One of the first applications for the zebrafish embryo in environmental sciences was promoted by the aim to develop an alternative to the 96-h acute fish toxicity test (Nagel 2002). However, this model offers many other opportunities beyond acute toxicity testing, particularly for the elucidation of mechanisms of toxicity. According to Dai and co-workers (2014), the zebrafish could be used for studies on eco-environmental monitoring and multitudinous pollutant evaluations, such as toxic metals, endocrine disruptors, organic pollutants, as well as newly emerged pollutants like NMs.

Due to the several advantages as animal model and the wide range of applications in ecotoxicology, zebrafish is one the most frequently model organisms described in standardized guidelines, developed for ecotoxicological evaluations (Bopp et al. 2006). The International Organization for Standardization (ISO) first published the zebrafish toxicity test in 1984 (ISO 1996). Thereafter, other

international organizations like the OCDE promulgated their own toxicity test standards by using zebrafish. Protocols for ecotoxicological assessment using zebrafish are summarized in Table 3.

Table 3. Protocols for ecotoxicological assessment of chemicals using zebrafish proposed by the OECD and ISO.

Guideline	Reference
Test No. 203: Fish, acute toxicity test	OECD (1992)
Test No. 212: Fish short term toxicity test on embryo and sac-fry stage	OECD (1998)
Test No. 215: Fish juvenile growth test	OECD (2000)
Test No. 230: 21-day fish assay	OECD (2009)
Test No. 234: Fish sexual development test	OECD (2011)
Test No. 229: Fish short term reproduction assay	OECD (2012)
Test No. 210: Fish, early-life stage toxicity test	OECD (2013)
Test No. 236: Fish embryo acute toxicity (FET) test	OECD (2013)
Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]	ISO 7346 (1996)
Determination of toxicity to embryos and larvae of freshwater fish	ISO 12890 (1999)
Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)	ISO 15088 (2007)

Besides the application of embryos, juvenile or adult zebrafish, the use of cell lines to completely avoid animal testing is gaining importance (Bopp et al. 2006). Fish cell lines in general are in vitro systems emerging throughout the last decade (Fent 2001). In this area several contributions have also been done using zebrafish (Eide et al. 2014; Spitsbergen and Kent 2003).

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II. State of the art, Hypothesis and Objectives

State of the art

Human activities have historically been linked to the production of wastes that have been spilled into the environment without any consideration to the consequences they could have on the human and environmental health. Today the damaging capacity of many extensively used compounds is not still completely understood. It is therefore important to certify that the benefits obtained through a given compound are worth the risk of disclosing it to the environment. In this context determining the toxic potential of both legacy and emerging environmental pollutants, as well as understanding the mechanisms underlying their damaging capacity is a business of interest.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous carcinogenic pollutants that have largely been present in the environment. These compounds can produce cancer through genotoxic and non-genotoxic mechanisms. Their toxic effects can be elicited through the interaction with the aryl hydrocarbon receptor (AHR) or the nonspecific narcotic effect. The AHR is a transcription factor that, after binding to PAHs or other organic chemicals, activates the downstream transcription of xenobiotic metabolism genes. Xenobiotic metabolism involves the transformation of the compounds for their excretion. However, this detoxification mechanism can produce activated reactive species and increased oxidative stress that may ultimately produce DNA and cellular damage. Compared to the well known PAHs, metal oxide nanoparticles (NPs) are new emerging pollutants. Their extensive use in consumer products together with the growing perspectives for future applications makes them a target for toxicity studies. Their toxic and carcinogenic potential still remains unclear. Due to their small size, they own specific characteristics that may lead to differential toxic effects when compared with their equivalent ionic and bulk forms. Anyhow, some metal oxide NPs are known to produce damage through the production of reactive oxygen species, the direct interaction with cellular components or through the metal ions arising from their dissolution.

In order to unravel the damaging mechanisms of PAHs and metal oxide NPs is important to understand the effects they may produce on the organism before the

appearance of adverse outcomes. In this scenario, the zebrafish (*Danio rerio*) is a suitable model widely employed in toxicological studies. Both the embryonic and adult stages of zebrafish offer several advantages to assess the short and long term effects of these compounds.

Hypothesis

PAHs and metal oxide NPs can produce detectable toxic effects at different levels of biological organization in the zebrafish. Different PAHs may produce similar but differentiable responses at the cellular and molecular levels at embryo and adult stages. The toxicity of metal oxide NPs can vary depending on the chemical compound and may differ from their bulk and ionic counterparts. Despite the different chemical compositions, different environmental pollutants, like PAHs and metal oxide NPs, can share cellular and molecular mechanisms of toxicity.

Objectives

In order to proof this hypothesis true, the present work attempts to address the following **general objective**:

- To provide deeper insight on the effects and carcinogenic potential of different legacy (PAHs) and emerging (metal oxide NPs) environmental pollutants using the zebrafish as a model organism in order to contribute to the existing knowledge on their adverse outcome pathways.

The general objective has been subdivided into a series of **specific objectives** that are shown below and each objective is addressed in a different chapter:

1. To identify and compare the cellular and molecular responses produced in zebrafish by short- and medium-term exposures to benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA), using a battery of cell and molecular biomarkers.

2. To compare the cellular processes altered by two model carcinogenic PAHs, B(*a*)P and DMBA, and to compare their effects at two different time points in order to gain knowledge about the mode of action underlying their carcinogenic capacity.

3. To analyze the long-term transcriptional responses of key genes related to xenobiotic metabolism and cell cycle control and the histopathological effects produced by an early acute exposure to B(*a*)P and DMBA during zebrafish embryogenesis.

4. To contribute to the existing knowledge on metal oxide NPs (CuO, ZnO and TiO_2) toxicity using the zebrafish embryo model and to compare their relative toxicity with the ionic form, the equivalent bulk form of the metal and the additives present in NPs suspensions.

5. To assess and compare the effects of waterborne exposure of adult zebrafish to an environmentally relevant concentration of copper in form of CuO NPs or dissolved copper at different levels of biological organization in order to gain deeper knowledge on the mode of action of these two forms of the metal.

III. Results and Discussion

CHAPTER I

Cellular and molecular effects of waterborne exposure of adult zebrafish to carcinogenic PAHs

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Abbreviations

Aryl hydrocarbon receptor, AHR

Acyl-CoA oxidase, AOX

Benzo(*a*)pyrene, B(*a*)P

3,3'-diaminobenzidine tetrahydrochloride, DAB

7,12-dimethylbenz(*a*)anthracene, DMBA

Dimethyl sulfoxide, DMSO

Labilization period, LP

Lysosomal membrane stability, LMS

Micronucleus, MN

Polycyclic aromatic hydrocarbon, PAH

Reactive oxygen species, ROS

2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD

Gene abbreviations

cyclin, ccn

cytochrome P450, cyp

jun B proto-oncogen, junb

ribosomal protein S18, rps18

tumor protein 53, p53

Abstract

Several environmental pollutants, especially organic compounds such as polycyclic aromatic hydrocarbons (PAHs), are potent carcinogenic chemicals. In previous studies in which zebrafish (*Danio* rerio) have been exposed to PAHs, a high prevalence and variety of tumoral lesions have been reported. In order to analyze the effects produced at cellular and molecular levels by the model carcinogenic PAHs benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA), adult zebrafish were exposed for 3 days to different concentrations of DMBA (0.3, 0.6 and 1 mg/L) and for one and two weeks to 0.3 mg/L of DMBA or B(*a*)P. Exposure to 1 mg/L DMBA for 3 days resulted in high mortality (80%), whereas no lethal effects were observed at lower concentrations. Both PAHs altered the transcription level of genes related to cancer development, cell cycle control and phase I metabolism. Significant upregulation of *cyp1a and cyp1b* was observed after the first week of exposure to both PAHs. After two weeks of exposure, *cyp1b* upregulation was even stronger. DMBA also upregulated the transcription of *junb* after 2 weeks of exposure. Moreover, both PAHs elicited a significant impairment of the general health status of fish reflected in a decrease of the lysosomal membrane stability in hepatocytes. No significant changes were registered for the peroxisomal parameters at any of the DMBA concentrations tested for 3 days, neither after one week of exposure to 0.3 mg/L B(*a*)P or DMBA. Nevertheless, peroxisome proliferation was observed after 2 weeks of exposure to both compounds. Genotoxic effects, measured as increased micronucleus frequency in erythrocytes, were only observed at the highest DMBA concentration after 3 days of exposure. Finally, exposures did not provoke relevant histopathological alterations. Overall, our results indicate that PAHs impaired general health status likely through the activated metabolites arising from the xenobiotic metabolism and highlight the importance of the exposure time to study PAHs effects at low levels of biological organization.

Key words: Zebrafish, benzo(*a*)pyrene, 7,12-dimethylbenz(*a*)anthracene, gene transcription, lysosomal responses, peroxisome proliferation, genotoxicity.

Laburpena

Ingurunean topa daitezkeen hainbat kutsatzaile, bereziki hidrokarburo aromatiko poliziklikoak (HAPak) bezalako konposatu organikoak, kartzinogeno indartsuak dira. HAPen pean izan diren zebra arrainetan (*Danio rerio*), lesio tumoralen prebalentzia altuak ikusi izan dira. Bentzo(*a*)pirenoak (B(*a*)P) eta 7,12-dimetilbentzo(*a*)antrazenoak (DMBA) zelula eta molekula-mailan sortutako eraginak aztertzeko asmoz, zebra arrain helduak 3 egunez DMBA-ren kontzentrazio ezberdinen (0.3, 0.6 eta 1 mg/L) pean eta aste betez edo bi astez B(*a*)P edo DMBA-ren 0.3 mg/L pean mantendu ziren. Kontzentrazio baxuen pean izandako arrainetan hilkortasunik antzeman ez zen bitartean, 1 mg DMBA/L kontzentrazioak hilkortasun altua (%80a) eragin zuen. Bi HAP-ek kantzer garapenarekin, zelularen zikloaren kontrolarekin eta I-faseko metabolismoarekin erlazionatutako geneen transkripzio maila aldatu zuten. Bi HAPen pean aste betez egondako arrainetan *cyp1a* eta *cyp1b*-ren transkripzioaren emendatze esanguratsua ikusi zen. Bi aste ondoren *cyp1b*-ren transkripzio maila are gehiago igo zen eta DMBAren pean egondako arrainetan *junb*-ren transkripzioaren emendio esanguratsua ere gertatu zen. Gainera, bi HAP-ek hepatozitoen lisosomen mintzaren egonkortasuna esanguratsuki murriztu zuten, osasun egoera orokorraren andeatzea adieraziz. Peroxisomekin erlazionaturiko parametroetan ez ziren aldaketarik aurkitu 3 egunez DMBA pean egondako arrainetan, ez eta aste betez 0.3 mg/L-ko B(*a*)P edo DMBA pean egondako arrainetan ere. Hala eta guztiz ere, peroxisomen proliferazioa antzeman zen bi astez PAHen pean egondako arrainetan. Eritrozitoen mikronukleoen prebalentziaren emendio giza neurtutako eragin genotoxikoak, DMBAren kontzentrazio altuenen pean 3 egunez izandako animalietan besterik ez ziren antzeman. Azkenik, aztertutako kontzentrazioek ez zuten alterazio histopatologiko esanguratsurik eragin. Oro har, gure emaitzek HAP-ak osasun egoera orokorra endekatzeko gai direla erakusten dute, ziur aski xenobiotikoen metabolismotik sortzen direneko aktibaturiko metabolitoen eragina medio. Gainera, HAP-en eraginak antolakuntza biologikoaren maila baxuetan aztertzerakoan tratamenduaren iraupena duen garrantzia azpimarratzen dute.

Gako hitzak: Zebra arraina, bentzo(*a*)pireno, 7,12-dimetilbentzo(*a*)antrazeno, geneen transkripzioa, lisosomen erantzunak, peroxisomen proliferazioa, genotoxizitatea.

1.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants produced as a result of natural and industrial processes (Burgess et al. 2003). In fish, exposure to PAHs can produce a wide range of toxic effects due to their direct interaction with hydrophobic sites of the cell or due to the appearance of even more reactive species arising from their metabolism (Tuvikene 1995). Benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA) are two members of the PAH family with a well known carcinogenic potential (Baird et al. 2005). These compounds are metabolized and consequently bioactivated by cytochrome P450-dependent oxidases mainly present in the smooth endoplasmic reticulum of hepatocytes (Shimada et al. 2006; Tuvikene 1995). During this process, highly reactive compounds such as diolepoxides, quinones, radical anions and benzylic carbenium ions are produced, which are able to bind covalently macromolecules like proteins or DNA and provoke tumor initiation (Shimada and Fuji-kuriyama 2003; Stegeman and Lech 1991). Moreover, a concomitant increase in reactive oxygen species (ROS) production occurs as a result of the oxidases activity, which induces oxidative damage to DNA and other cell components, such as membranes and proteins (Miller and Ramos 2001). Consequently, PAHs can produce cancer by directly reacting with DNA causing adduct formation (genotoxic mechanism), or without direct DNA interaction due to altered gene expression and the oxidative stress arising from their biotransformation metabolism which are considered non-genotoxic mechanisms of carcinogenesis (Luch 2005).

The present work aimed to identify and compare the cellular and molecular responses produced in zebrafish (*Danio rerio*) by short- and medium-term exposures to B(*a*)P and DMBA, using a battery of cell and molecular biomarkers. To achieve this objective, two experiments have been conducted with adult zebrafish. Firstly, the toxicity of DMBA at different concentrations was screened after 3 days of exposure. Based on the results of this initial study, a second experiment was designed to test and compare the sublethal effects of B(*a*)P and DMBA after one and two weeks of exposure.

The zebrafish is a model organism that is often used in toxicological and cancer studies (Spitsbergen and Kent 2003). The effects of several environmental pollutants, including carcinogenic contaminants like PAHs, have been already assessed using zebrafish (Beckwith et al. 2000; Hsu and Deng 1996; Moore et al. 2006; Spitsbergen et al. 2000). Moreover, zebrafish was the first fish species in which laboratory experiments confirmed that carcinogens active in mammals cause neoplasia in fish (Stanton 1965), but it was not until the past decade when zebrafish was massively used in carcinogenesis studies (Spitsbergen and Kent 2003).

The biological effects of PAHs can be assessed at different levels of biological organization. Biomarkers are defined as measurements carried out on body fluids, cells or tissues that indicate, in biochemical or cellular terms, the presence of contaminants or the magnitude of the host response (McCarthy and Shugart 1990). Effects at higher hierarchical levels are preceded by changes in early biological processes (Ankley et al. 2010; Arukwe and Goksøyr 1998). Thus, transcriptional responses occur early after chemical exposure and can be detected before effects appear at cellular and tissue levels.

In zebrafish, exposure to a mixture of pollutants including different PAHs at concentrations ranging from 0.54 to 5.4 µg/L has shown to alter the transcription of genes related to xenobiotic metabolism, cell cycle control and cancer (Holth et al. 2008). Thus, in this chapter we specifically analyzed by qPCR the transcription level of six target genes related to these processes that appeared regulated after exposure to B(*a*)P and DMBA according to the microarray analysis presented in **Chapter II**. The proteic products of the cytochrome p450 family members *cyp1a* and *cyp1b* are involved in the metabolic activation of PAHs to reactive species (Shimada 2006). In fish, these genes are induced in response to PAHs. Exposure to 0.1 mg/L of B(*a*)P for 14 days induced the transcription of *cyp1a* in different fish species, such as medaka (*Oryzias latipes),* zebrafish*,* carp (*Cyprinus carpio*) and freshwater minnow (*Zacco platypus*) (Lee et al. 2015). Similarly, channel catfish (*Ictalurus punctatus*) injected with 20 mg/Kg B(*a*)P showed increased transcription of *cyp1b* (Willet et al. 2006). Tumor protein p53 (*p53*) is a tumor suppressor gene whose transcription is altered in many cancerous processes. Thus, we analyzed the transcription of p53 and one of its transcriptional target genes *cyclin g1* (*ccng1*) which is involved in cell cycle arrest in response to DNA damage (Kimura et al. 2001). Together with *ccng1*, we analyzed the transcription of *cyclin b1* (*ccnb1*) which is also involved in cell cycle regulation and whose upregulated expression has been detected in DMBA induced fish tumors (Johnson and Walker 1999; Lam et al. 2006). Moreover, we measured the transcription level of *jun B proto-oncogen* (*junb*), a transcription factor that might play both as tumor suppressor and proto-oncogen and that has been involved in different cancers. In addition, *junb* is known to play a role in tissue regeneration (Shaulian 2010).

Together with the transcription level analysis, we analyzed the effects produced by these model carcinogens using different cellular biomarkers in order to provide information that may contribute to fill the remaining knowledge gaps on the chain of events leading to the adverse outcome of PAHs. The general fish health status was evaluated using the lysosomal membrane stability (LMS) test and histopathological analysis of liver. LMS reduction in fish liver is considered a general stress biomarker (Moore et al. 2004) and it has been reported in different fish species

after exposure to PAHs containing mixtures (Bilbao et al. 2010; Holth et al. 2011). Lysosomal responses are closely linked to toxicopathological alterations and have clear prognostic value for cell death (ICES 2010; Köhler et al. 2002). Histolopatological changes, in particular liver disease, are indicators of chronic effects of PAH exposure (ICES 2010). Histological evaluation of adult zebrafish (9 months) after waterborne exposure to DMBA at fry stages for 24 h (up to 5 mg/L) as well as diet exposure of juveniles for 4 months revealed a great spectrum of neoplasic lesions, being the liver the primary target organ where those lesions were produced (Spitsbergen et al. 2000). Regarding non-neoplasic lesions, Zodrow et al. (2004) observed lipidosis, hepatocyte hypertrophy, decreased number of hepatocyte nuclei, glycogen depletion and bile duct hypertrophy in zebrafish injected with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which, as in the case of PAHs, is a potent agonist of the aryl hydrocarbon receptor (AHR) pathway (Boelsterli 2007).

Peroxisome proliferation, which is indicative of exposure to organic contaminants, was analyzed as possible non-genotoxic mechanism of carcinogenesis underlying PAHs carcinogenicity (Cajaraville et al. 2003a). Peroxisome proliferation has been demonstrated in different fish species exposed to crude and lubricant oils containing PAHs (Cajaraville et al. 2003b; Cancio and Cajaraville 2000). This phenomenon is characterized by increased hepatic peroxisomal volume density, which in rodents is usually accompanied by the transcriptional upregulation of enzymes involved in lipid homeostasis, such as the acyl-CoA oxidase (AOX) that participates in peroxisomal β-oxidation (Qi et al. 2000).

Finally, micronucleus (MN) frequency was used to detect genotoxicity (Siu et al. 2004). Since teleost erythrocytes are nucleated, MNs have been scored in fish erythrocytes as a measure of clastogenic activity (Al-Sabti and Metcalfe 1995). Increased MN frequency in erythrocytes of different fish species have been observed after exposure to different pollutants, both under field and laboratory conditions (Cheikyula et al. 2009; Domingues et al. 2010; Rocco et al. 2012). Waterborne exposure for 10 days of red sea bream (*Pragus major*) to a mixture of PAHs (93 µg PAH/L) containing phenanthrene, pyrene, chrysene and B(*a*)P caused a significant increase of MN frequency of the peripheral erythrocytes (0 ‰ in controls vs 0.36 ‰ in exposed fish) (Cheikyula et al. 2009).

1.2. Materials and Methods

1.2.1. Experimental organisms

Fish were maintained and raised in a controlled room at 27 ± 1 ºC with a 14-hour light / 10-hour dark cycle in 100 L tanks. Tank water was prepared by conditioning osmotic water with Sera marin basic salt and Sera KH/pH plus (Sera Gmbh, Heinsber, Germany) up to 600 µS and pH 7.4, before mechanical filtering (1 µm) and sterilization (ultraviolet light). Water aeration and filtration was produced by an airlift pump in each tank. Residual metabolites were controlled using Sera GmbH ammonium, nitrite and nitrate kits, maintaining water at 0-0.5 mg/L, 0-0.5 mg/L and 5-10 mg/L, respectively. When the highest concentration values were surpassed, water was partially replaced. Fish were fed with *Artemia* nauplii (INVE, Dendermonde, Belgium) and commercial dry food (Sera Microgran).

1.2.2. Exposures

The experimental procedures employed in this work were approved by the committee for animals' welfare of the University of the Basque Country.

Fish were exposed for 3 days (short-term exposure) to 0, 0.3, 0.6 and 1 mg/L DMBA (Sigma-Aldrich, St. Louis, USA). Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was used as a carrier for the exposure such that a final concentration of 0.1% DMSO was present in the control group as well as in the different DMBA exposure groups.

Medium-term exposures to 0.3 mg/L B(*a*)P (Sigma-Aldrich) or 0.3 mg/L DMBA were conducted for 2 weeks. DMSO was used as a carrier for the exposure such that a final concentration of 0.01% DMSO was present in the control group as well as in the different PAH exposed groups. Samples were collected after 1 and 2 weeks.

In both experiments, fish were euthanized by overdose of MS-222 (tricaine methane-sulfonate, Sigma-Aldrich), and samples were collected for the different endpoints as explained below.

1.2.3. Gene transcription levels

Liver samples from medium-term exposures were collected for microarray analysis (**Chapter II**) and qPCR analysis. Animals were dissected and 3 biological pseudoreplicates (Nikinmaa et al. 2012) consisting of pools of 5 livers each were prepared from each treatment at each sampling time. Samples were immersed in RNA Later® (Sigma-Aldrich) and stored at -80ºC. For RNA extraction, samples were

defrosted and homogenized in a RNAse free 1.5 mL tube (Eppendorf, Hamburg, Germany) using a MHX/E motorized hand tool (Xenox, Wecker, Luxemburg). Total RNA extraction was performed following the TRIzol® extraction method (Invitrogen Life-Technologies, Merelbeke, Belgium) by adding 1 mL of TRIzol® to homogenized liver tissue. RNA extracts were purified using DNAse I and RNAse inhibitor (Fermentas, St. Leon-Rot, Germany) and subsequent phenol/chloroform extraction. RNA purity and integrity were evaluated by measuring 260/230 nm and 260/280 nm absorbance ratios (NanoDrop Technologies, Rockland, DE, USA) and by denaturing formaldehyde-agarose gel electrophoresis, respectively. The resulting samples of total hepatic RNA were stored at −80 °C and used for real time qPCR.

The genes *cytochrome P450 1A1* (*cyp1a*, ID: Dr03112441_ml)*, cytochrome P450 1B1* (*cyp1b1,* ID: Dr03181453_gl), *cyclin b1* (*ccnb1*, ID: Dr03105834_ml)*, cyclin g1* (*ccng1,* ID: Dr03132065_ml)*, tumor protein 53* (*p53*, *ID*:Dr03112082_gl)*,* and *jun B proto-oncogen* (*junb,* ID: Dr03204057_sl) which are involved in processes well known to be affected by PAH exposure, such as xenobiotic metabolism, cell cycle regulation and carcinogenesis were selected for qPCR analysis. *Ribosomal protein S18* (*rps18,* ID: Dr03144509_ml) was used as housekeeping gene. Taqman probes were purchased from Applied Biosystems (Carlsbad, California). Triplicate PCR reactions were carried out as indicated by manufacturer's protocol using an ABIS 7300 cycler (Applied Biosystems) under the following conditions: 2' at 50 °C, 10' at 95 °C followed by 40 cycles of 15'' at 95 °C and 1' at 60°C. Data were normalized against *rps18* and the corresponding time point control by means of the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

1.2.4. Histochemistry of lysosomal acid phosphatase and assessment of lysosomal membrane stability (LMS)

The liver of 5 individuals per exposure group was dissected out and individually placed in a plastic mould containing Jung tissue freezing medium (Leica Microsystems, Nussloch, Germany). Then, samples were immediately frozen in liquid nitrogen and stored at -80 ºC until analyses.

Tissue sections (10 µm) were obtained in a Leica CM3000 cryotome at a cabinet temperature of -24 ºC. The determination of LMS was based on the time of acid labilization treatment required to produce the maximum staining intensity in lysosomes according to Broeg et al. (1999) after in situ demonstration of acid phosphatase activity. Slides were brought to room temperature for 5 min prior to staining. Serial cryotome sections were subjected to acid labilization in intervals of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min in 0.1 M sodium citrate buffer (pH 4.5 containing 2.5% NaCl) in a shaking water bath at 37º C, in order to find out the pre-treatment time needed to completely labilize the lysosomal membrane. Then,

samples were transferred to the substrate incubation medium, consisting of 10 mg naphthol AS-BI-phosphate (Sigma-Aldrich) dissolved in 1 mL DMSO (Sigma-Aldrich), and made up to 50 mL with 0.1 M sodium citrate buffer (pH 4.5) containing 2.5 % NaCl and 3.5 g Polypep® (Sigma-Aldrich) to act as a section stabilizer. Sections were incubated for 15 min at 37 ºC, rinsed in a saline solution (3 % NaCl) at 37 ºC for 5 min and then transferred to 0.1 M phosphate buffer (pH 7.4) containing 1 mg/mL of diazonium dye Fast Violet B salt (Sigma-Aldrich), for 10 min at room temperature. Slides were then rinsed in running tap water for 5 min, fixed in Baker´s formol calcium containing 2.5% NaCl at 4 ºC for 15 min and rinsed in deionized water. Finally, slides were mounted in Kaiser´s glycerin gelatine (Merck, Darmstadt, Germany) and sealed with nail varnish.

The labilization period (LP) was determined under an BX51TF light microscope (Olympus, Tokyo, Japan) as the maximal accumulation of reaction product associated with lysosomes as explained in Moore et al. (2004).

1.2.5. Catalase histochemistry and assessment of the peroxisomal volume density

In the short-term experiment, 8 µm cryotome liver sections were obtained as described for the LMS test and employed for the determination of peroxisomal volume density after catalase histochemistry according to Fahimi (1969). Slides were brought to -20 ºC 10 min before fixation with 1.5% glutaraldehyde (Fluka, Buchs, Switzerland) in cacodylate (Sigma-Aldrich) buffer (0.1 M, pH 7.2) at 4 ºC for 10 min. Then, slides were rinsed in 0.1 M cacodylate buffer (pH 7.2) 2 x 5 min at room temperature. Incubation was performed in darkness in a shaking water bath at 37 ºC for 1 h in a reaction medium containing 0.01 M Teorell-Stenhagen buffer, 0.2% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) and 0.15% hydrogen peroxide (Sigma-Aldrich). Slides were washed in 0.01 M Teorell-Stenhagen buffer for 5 min before dehydration in a series of increasing concentrations of ethanol (2 x 2 min in each concentration). Finally samples were submerged in xylene (VWR Chemicals, Fontenay-sous-Bois, France) 2 x 2 min and mounted in DPX (Fluka).

Peroxisomes were visualized as dark brownish spherical particles. A lattice of 168 test points (multipurpose test system P168; Weibel 1979) was superimposed onto the preparations using a camera lucida attached to a Laborlux S (Leica Microsystems) light microscope. Peroxisomal volume density was calculated as the ratio between the number of test points falling onto peroxisomes and the total number of test points falling onto hepatocytes. All the measurements were made with a 100x magnification objective.

In samples from the medium-term experiment, catalase cytochemistry was performed before samples were embedded in Epon 812 resin (Fluka) according to

Orbea et al. (1999). Briefly, livers were dissected and immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h. Then, livers were washed in cacodylate buffer and preincubated in 0.01 M Teorell-Stenhagen buffer at pH 10.4 containing 5 mM DAB for 30 min at 37ºC. Incubation was performed in the same medium plus 0.15% H_2O_2 for 1 h at 37 °C in darkness. In control experiments, specimens were incubated without H_2O_2 or in complete incubation media containing 0.2 M of the inhibitor 3-amino-1,2,4-triazole (Sigma-Aldrich). The reaction was stopped by washing the tissue in Teorell-Stenhagen buffer. Finally livers were postfixed in 1% osmium tetroxide (Sigma-Aldrich) and 1.5% potassium ferrocyanide (Sigma-Aldrich) in 0.1 M cacodylate buffer for 1 h at 4ºC. After fixing, samples were dehydrated in a series of alcohol of increasing concentration and finally embedded in resin.

Semithin sections were obtained with a Leica Ultracut microtome and used for the assessment of peroxisomal volume density as described before.

1.2.6. Peroxisomal acyl-CoA oxidase (AOX) activity

The visceral masses of 12 fish per experimental group from the short-term experiment were dissected and frozen in liquid nitrogen in pools of 3 animals and stored at -80ºC for the spectrophotometric measurement of the peroxisomal AOX activity.

Samples were homogenized in 1:5 mass/volume ratio in 60 mM Tris buffer (Sigma-Aldrich) (pH 8.3) containing 0.25 M sucrose (Sigma-Aldrich) using a Hybaid ribolyzerTM (Hybaid, Ashfold, UK) and centrifuged at 600 g at 4 ºC for 15 min. Resulting supernatant was diluted 1:1 in homogenization buffer and AOX activity was measured using a Shimadzu UV-1603 spectrophotometer (Duisburg, Germany) by monitoring the H_2O_2-dependent dehydrogenation of 2´,7´-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, California) at 502 nm in the presence of 12 U/mL horseradish peroxidase (Sigma-Aldrich) and using 30 µM palmitoyl-CoA (Sigma-Aldrich) as substrate in 0.01 M potassium-phosphate buffer (Small et al. 1985).

Protein concentration of each sample was assayed in a 96 well plate using the Bio-Rad DC protein assay (Bio-Rad, Hercules, California) based on Lowry's method following the instructions of the manufacturer. The activity is given as mU AOX mg-1 protein.

1.2.7. Micronucleus assay

Blood from 5 animals per experimental group was obtained by tail cutting and direct blood smear on clean microscope slides. Blood smears were left to air-dry and cells were 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 (Fluka).

Small, non-refractile, circular or ovoid chromatin bodies showing the same staining pattern as the main nucleus and with an overall area not bigger than a 1/3 of the main nucleus were considered as MN (Baez and Prieto 2005). 2000 erythrocytes were examined in each individual and data are expressed in ‰.

1.2.8. Liver histopathology

At the end of the exposures, animals were ventrally opened and tail was removed. Samples were fixed in 10% neutral buffered formalin (pH 7), and decalcified in 3.5 g/L citric acid-citrate buffer for 3 h. Then, animals were longitudinally sliced in two symmetrical parts and dehydrated in a series of increasing concentrations of ethanol (20 min in each bath). After dehydration, samples of the short-term experiment were embedded methacrylate (Technovit 7100, Kuzler, Germany) and samples of the medium-term experiment were embedded in paraffin. The embedded material was cut into 5 µm thick sections using a 2065 Supercut microtome (Leica Microsystems) in the case of methacrylate embedded tissues or a Leica 2125RT microtome (Leica Microsystems) in the case of paraffin embedded samples. Finally, samples were stained with hematoxylin-eosin.

1.2.9. Statistical analysis

Statistical analyses were performed using the SPSS for Windows (SPSS Chicago, IL) software. Bootstrap resampling techniques (Efron and Tibshirani, 1993) were used to assess the effect of the treatments on the different cellular and molecular parameters. For each experiment, N=2000 repetitions were selected by bootstrap resampling method. After that, Bonferroni's correction was used for multiple comparisons between pairs of groups. For histopathological data Chi-square test was used. In all cases, significance was established at $p< 0.05$.

1.3. Results

1.3.1. Mortality

After 3 days of exposure, 80% of mortality was registered in the group of fish exposed to 1 mg/L DMBA. Among surviving animals lateral swimming was observed in some of them, indicating an imminent dead. In the medium-term experiment, no mortality was recorded during the two weeks of exposure to 0.3 mg/L of B(*a*)P or DMBA.

1.3.2. Gene transcription levels

rps18 was used as housekeeping gene. A coefficient of variability of 5.36% was obtained for the transcription level values of this gene. Transcription data of target genes were normalized to the housekeeping gene and the control group of each sampling time. Results are shown in Fig. 1. Exposure to PAHs significantly altered the transcription of *cyp1a1*, *cyp1b1* and *junb.* In the case of *ccnb1, ccng1* and *p53*, no statistically significant differences were found although alterations of the transcription levels were observed mainly after two weeks of exposure. The loss of a sample of the control group at two weeks of exposure might have influenced these results.

Significant differences were observed for *cyp1a1* after one week of exposure and for *cyp1b1* after one and two weeks of exposure to both PAHs (Fig. 1A; 1B). Genes related with cell cycle regulation showed different transcription patterns. No changes were detected for *ccnb1* after one week exposure to both compounds or after two weeks exposure to DMBA, but upregulated and very variable transcription was measured after two weeks exposure to B(*a*)P (Fig. 1C). DMBA exposed fish showed very slight upregulation of *ccng1* at both sampling times (Fig. 1D). Similarly, slight up-regulation of *p53* was detected after two weeks of exposure to both PAHs (Fig. 1E), while a significant up-regulation of *junB* was detected after two weeks of exposure to DMBA (Fig. 1F).

1.3.3. Lysosomal membrane stability (LMS)

Exposure to 0.3 mg/L DMBA for 3 days produced a significant reduction of the stability of liver lysosomal membrane compared to that of the control group (Fig. 2A). This parameter could not be measured after exposure to 1 mg/L of DMBA, due to the high mortality recorded in this group.

Both PAHs reduced the LMS after 1 and 2 weeks of exposure. This reduction was statistically significant after 2 weeks (Fig. 2B).

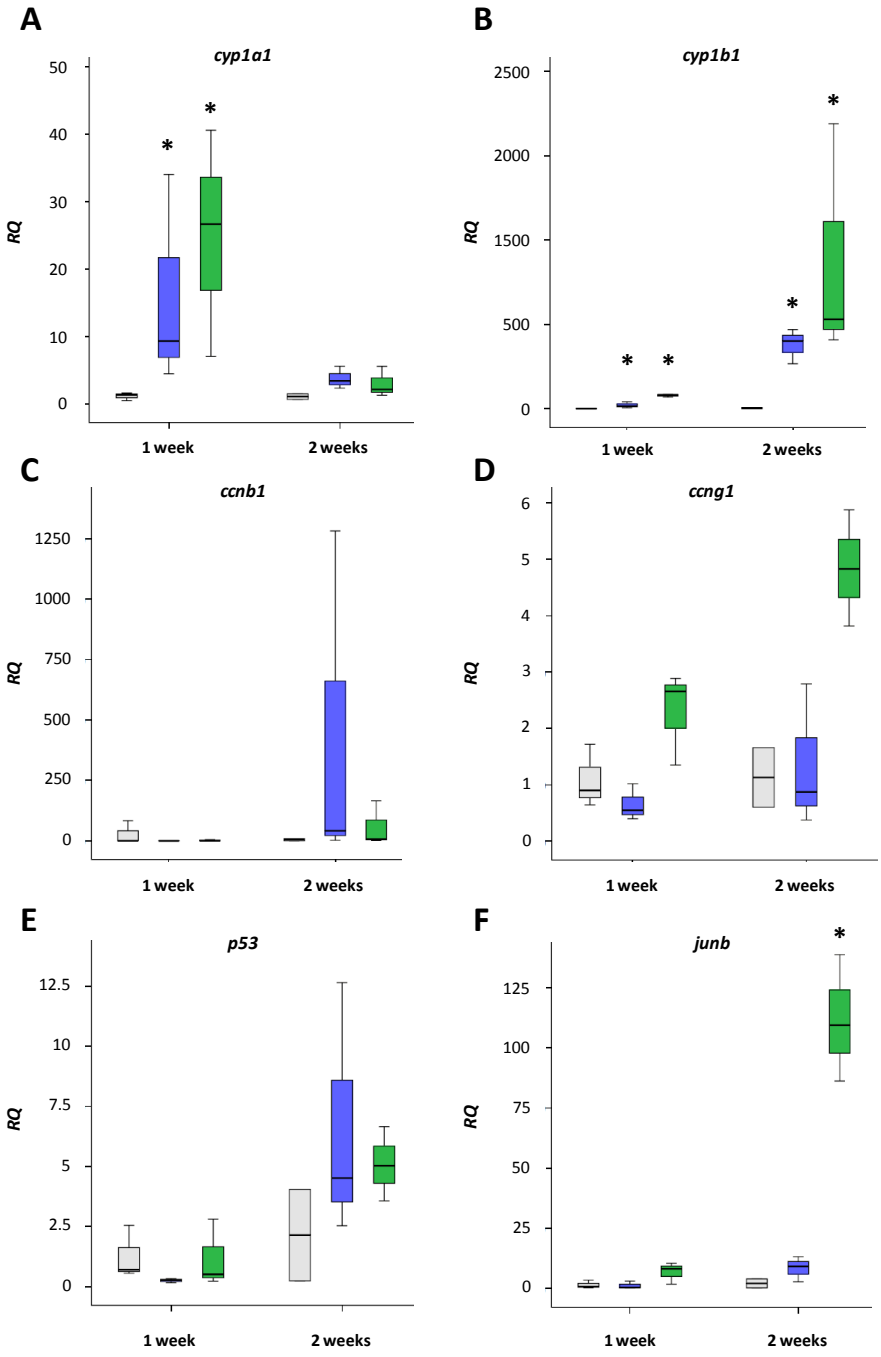

Fig.1. Relative transcription values (RQ) of the analyzed cancer related genes in animals from the medium-term experiment. Results show the transcription of each gene normalized to *rsp18* and the corresponding time point control in fish exposed to B(*a*)P (blue) or DMBA (green). Box-plot boxes represent the percentage 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.

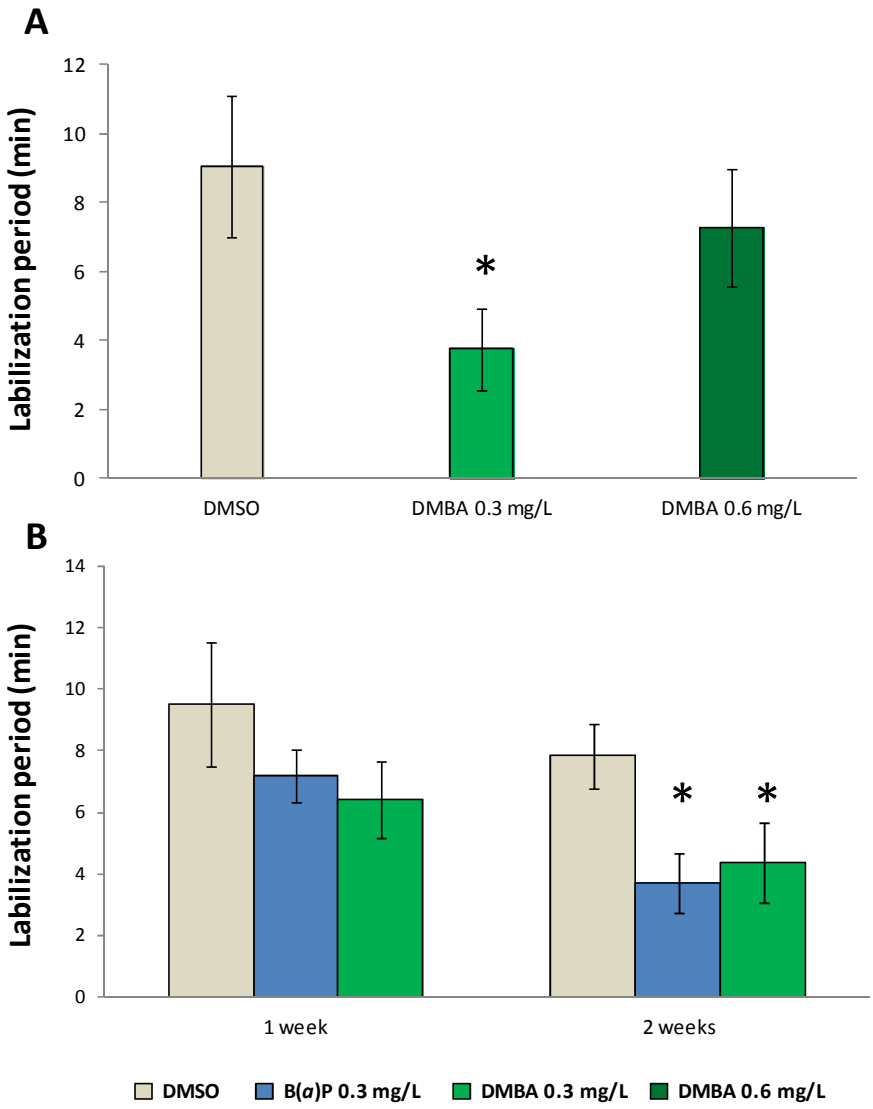

Fig. 2. Results of the LMS test in the liver of adult zebrafish. Bars represent mean values and vertical segments correspond to standard deviations. A: Results from the short-term experiment showing the LP values obtained for zebrafish exposed to different doses of DMBA for 3 days. Asterisk indicates significant ($p<0.05$) differences towards the control. B: Results from the medium-term experiment showing the LP values obtained for zebrafish exposed for one or two weeks to 0.3 mg/L of B(*a*)P or DMBA. Asterisks indicate significant differences with respect to the control group of each exposure week.

1.3.4. Peroxisome proliferation

In both experiments, peroxisome proliferation was assessed by means of the peroxisome volume density. After DAB histochemistry for the localization of catalase activity, peroxisomes were visible as dark brown spherical organelles in the cytoplasm of hepatocytes (Fig. 3). Additionally, in the short-term experiment AOX

activity was measured as a biochemical marker of peroxisome proliferation in zebrafish exposed for 3 days to different concentrations of DMBA.

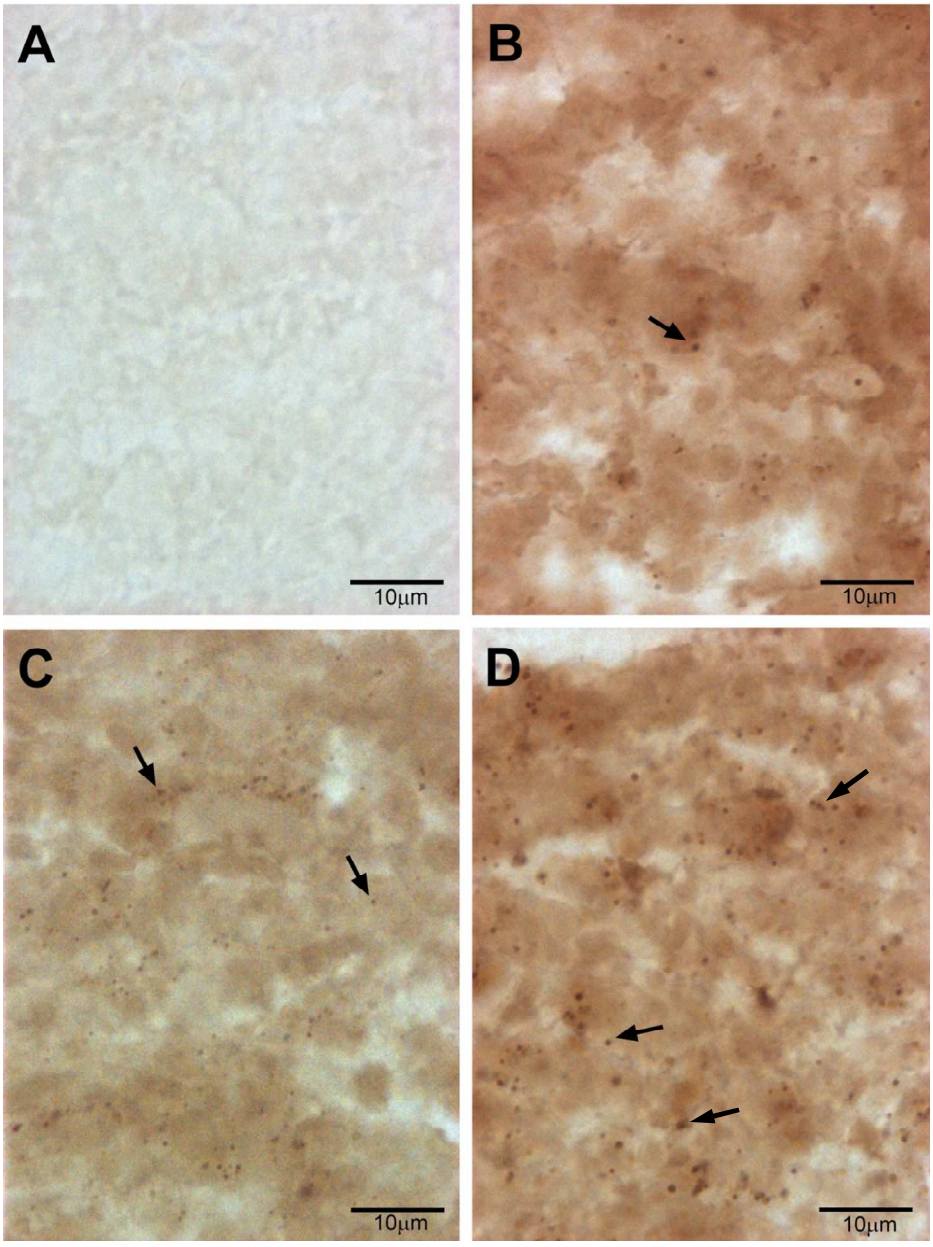

Fig. 3. Micrographs of the liver tissue after catalase histochemistry in cryostat sections from the short-term experiment (3 days). A: Control staining without H_2O_2 showing no positive reaction. B: Catalase histochemistry in DMSO exposed control fish. C: Catalase histochemistry in fish exposed to 0.3 mg/L DMBA exposed fish. D: Catalase histochemistry in fish exposed to 0.6 mg/L DMBA. Arrows point to peroxisomes.

Exposure to DMBA did not produce significant differences in AOX activity between exposed animals and non-exposed ones (Fig. 4A). Accordingly, peroxisomal volume density did not differ significantly in animals exposed for 3 days to DMBA from that measured in the control group, but a trend to increase with DMBA concentration was observed (Fig. 4B).

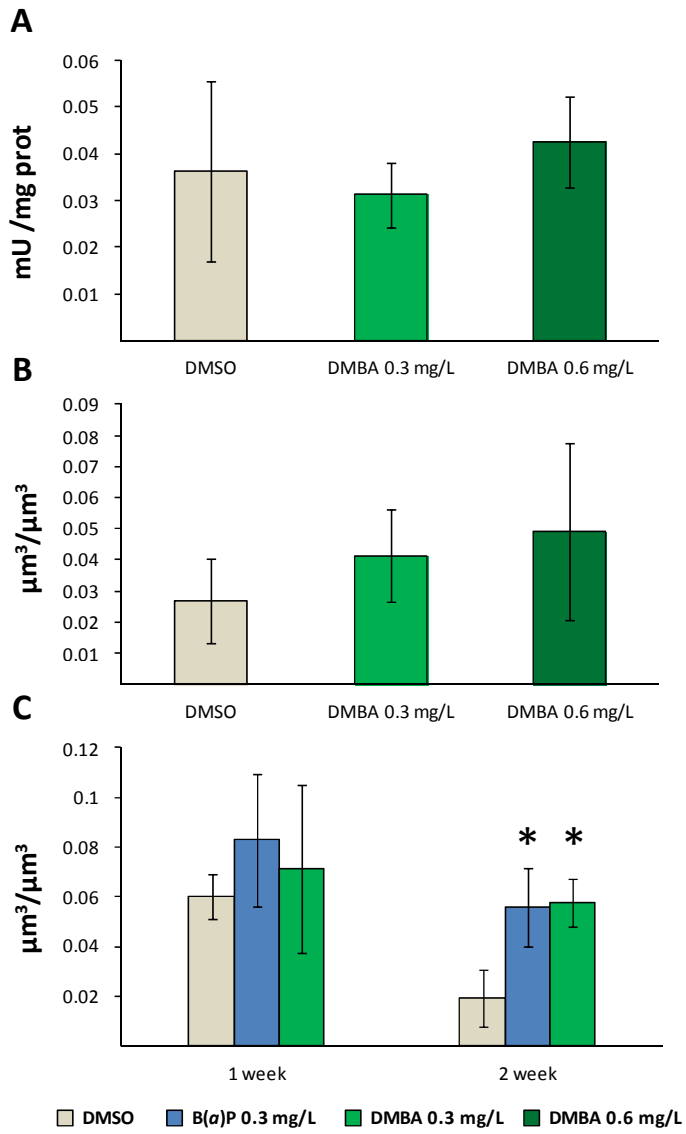

Fig. 4. Results obtained for the peroxisome proliferation related parameters. Bars represent mean values and vertical segments correspond to standard deviations. A: AOX activities obtained in the short-term experiment (3 days). B: Peroxisomal volume density obtained in the in the short-term experiment (3 days). C: Peroxisomal volume density obtained in the medium-term experiment (1 or 2 weeks). Asterisks indicate significant differences with respect to the control group of each exposure time.

In the medium-term experiment, exposure to 0.3 mg/L of both PAHs did not affect peroxisomal volume density after one week of exposure, but significant differences towards the control were observed after 2 weeks (Fig. 4C). Nevertheless, it must be noted that, in fish exposed to DMSO, lower values were recorded after 2 weeks of exposure than after 1 week of exposure.

1.3.5. MN frequency

Only nuclear aberrations fulfilling the established criteria were considered as MN (Fig. 5). In the short-term experiment, animals exposed to 1 mg/L of DMBA showed a significant increase in MN frequency (Fig. 6) in comparison to the control group as well as to the other DMBA exposure groups. In spite of a slight increase in the MN frequency was also recorded in the group exposed to 0.6 mg/L of DMBA, this was not as high as the value obtained in fish exposed to 1 mg/L of DMBA. No significant genotoxic effects were detected after DMBA or B(*a*)P treatment in animals from the medium-term experiment (data not shown).

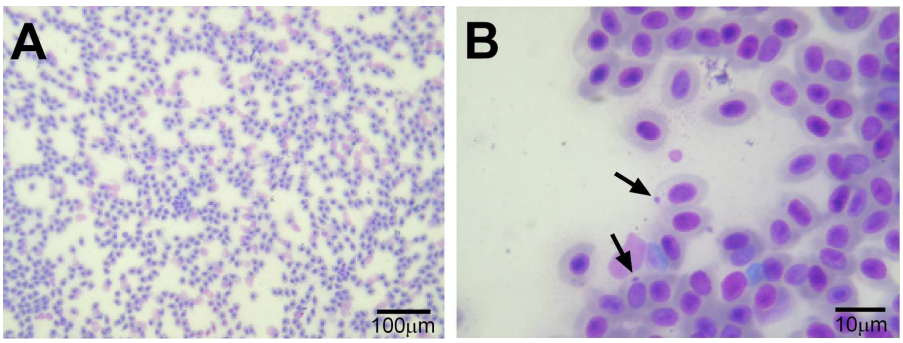

Fig. 5. Blood smears of adult zebrafish. A: Erythrocyte dispersion on the slide obtained by tail cutting and direct blood smear seen at low magnification. B: Two MN (arrows) in adult zebrafish erythrocytes treated with 1 mg/L DMBA for 3 days.

1.3.6. Liver histopathology

No histopathological alterations were observed in animals exposed to different DMBA concentrations for 3 days. In samples from the medium-term experiment, vacuolization was observed after 2 weeks of exposure, although no significant differences were found between the control and exposed fish. These changes were registered in 28.5% of the control fish, in 12.5% of the DMBA treated animals and in 44.4% of the B(*a*)P exposed individuals. Interestingly, one of the DMBA treated fish showed hepatic megalocitosis after two weeks of exposure (Fig. 7).

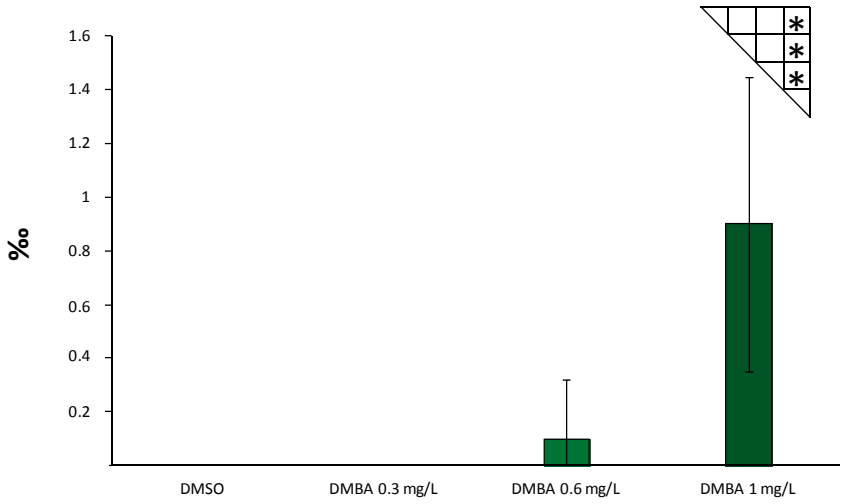

Fig. 6. MN frequency in erythrocytes from the short-term experiment (3 days). Asterisks in the upper triangular matrix indicate significant differences between the 1 mg/L exposed group and the rest of the treatments ($p<0.05$).

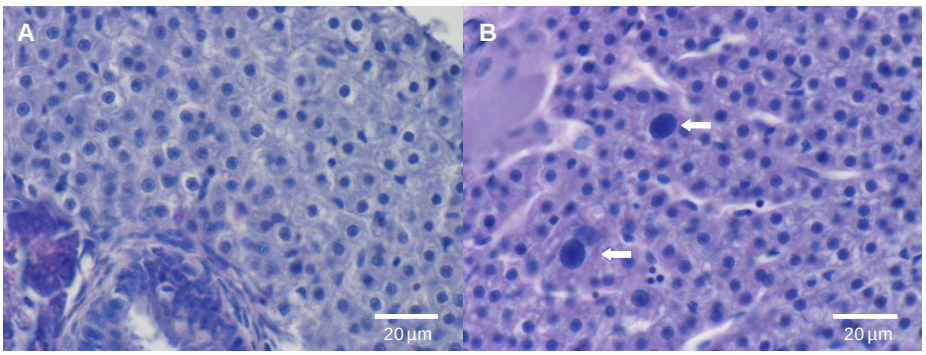

Fig. 7. Micrographs of hematoxylin/eosin stained sections of zebrafish liver form the medium term experiment after 2 week of exposure. A: Normal zebrafish liver corresponding to a control fish. B: Hepatic megalocytosis (arrows) corresponding to a fish exposed to 0.3 mg/L DMBA for 2 weeks.

1.4. Discussion

PAHs are ubiquitous environmental pollutants which damaging capacity is mainly revealed after being bioactivated by the monooxygenases of the cytochrome P450 system (Baird et al. 2005). The strong carcinogenic potential of these compounds and their metabolites arises both from their capacity to directly interact with DNA and the oxidative stress produced by the overproduction of ROS during the phase I

metabolism (Tuvikene 1995). When an organism is exposed to a chemical, the biological response depends on the physicochemical properties of the compound, but also on the concentration and duration of the exposure (Boelsterli 2007). Thus, in this study we analyzed the cellular and molecular responses produced on zebrafish liver after waterborne exposure to a series of DMBA concentrations (0.3, 0.6, and 1 mg/L) for a short period of time (3 days), as well as the effects produced after two weeks of exposure to a single concentration (0.3 mg/L) of B(*a*)P or DMBA.

When an organism is not able to cope with a chemical insult, toxicity occurs producing damage that might lead to an adverse outcome like death. Mortality is therefore a basic parameter employed in aquatic toxicology studies (Wester et al. 2002). The exposures carried out in this work allowed studying the range from lethal to sublethal effects, as far as, the exposure for 3 days to the highest tested concentration of DMBA (1 mg/L) produced an 80% of mortality, while no lethal effects were recorded at lower doses of DMBA or after B(*a*)P exposure for up two weeks. Few data are available on the acute toxicity of DMBA to fish. In adult individuals of the viviparous fish *Poeciliopsis lucida* waterborne exposure to 5 mg/L DMBA for 24 h produced 60% of mortality (Schultz et al. 1989). In tilapia *Oreochromis niloticus*, intraperitoneal injection of 75 mg/kg DMBA for 5 consecutive days also caused mortality (Hart et al. 1998). As far as we know, no works have previously waterborne-exposed adult zebrafish to DMBA. Nevertheless, Spitsbergen et al. (2000) run several experiments in which zebrafish were exposed at earlier developmental stages or through the diet. In three week old zebrafish (fry stage) waterborne exposure for 24 h to 2.5 and 5 mg/L DMBA caused 47% and 68% mortality, respectively, whereas in juvenile fish exposure 1 mg/kg through the diet for 12 weeks also produced up to 41% mortality.

Medium-term exposure (2 weeks) to the lowest concentration employed in the short-term experiment (0.3 mg/L) of B(*a*)P and DMBA did not produce lethal effects. Although, to the best of our knowledge, no LC_{50} values have been reported after waterborne exposure of adult zebrafish to B(*a*)P or DMBA, in previous works where zebrafish were long-term exposed (56 days) to low doses (1.5 or 3 µg/L) of B(*a*)P no mortality was described (Hoffman and Oris 2006).

It is well known that effects at high levels of biological organization are preceded by changes in early biological processes (Arukwe and Goksøyr 1998). Therefore, we analyzed the cellular and molecular effects produced by B(*a*)P and DMBA exposure to adult zebrafish in order to bring some light about the mode of action underlying the effects produced by these compounds. For these analyses, we focused our attention in liver, as far as, being the main organ that metabolizes and

excretes exogenous chemicals, it is considered a target organ for toxicological studies (Boelsterli 2007; Burczynski 2003; Zhou et al. 2009).

1.4.1. Gene transcription

Despite the loss of a sample from the control group of the second week, which could have interfered with the statistical results, some interesting observations were done. Both PAHs significantly upregulated the transcription level of genes coding for monooxygenases of the cytochrome P450 system (*cyp1a, cy1b1*) that are required for the bioactivation of PAHs (Fig. 1). Significant upregulation of *cyp1b* was observed in fish exposed to any of the two PAHs indicating that *cyp1b1* mediated adaptive response remains longer than the *cyp1a1* mediated response. These genes are known to be responsive to the exposure of AHR agonists in fish (Gao et al. 2013; Jönsson et al. 2010). CYP1B1 overlaps in function with CYP1A; however, it is generally thought to have a greater tendency to metabolize substrates to more toxic products than CYP1A (Billiard et al. 2006). In zebrafish, upregulation of *cyp1a* transcription after 7 days of waterborne exposure to 100 µg/l of B(*a*)P has been observed (Thompson et al. 2010). Thus, our results suggest the formation of highly reactive metabolites that together with the ROS arising from the biotransfromation process could produce damage to several cell components (Miller and Ramos 2001; Shimada et al. 2006).

Both PAHs showed a trend to increase the transcription of the tumor suppressor *p53* after 2 weeks of exposure (Fig. 1E), which could indicate that exposure to 0.3 mg/L B(*a*)P or DMBA may impair hepatic DNA. Moreover, it is worth attending to the transcription patterns observed after 2 weeks of exposure for *ccng1* in animals exposed to DMBA (Fig. 1D), as well as for *ccnb1* in animals exposed to B(*a*)P (Fig. 1C). The former is a p53 downstream target gene able to arrest the cell cycle at the G2 to M transition (Kimura et al. 2001), while *ccnb1* is necessary for the transition of cells into mitosis (Johnson and Walker 1999). These results may suggest the capacity of both PAHs to alter cell cycle, which is a feature of cancer cells. Finally DMBA exposure did also result in a significant upregulation of *junb* (Fig. 1F) which is related to tissue damage and whose altered regulation may be involved in the carcinogenic capacity of this compound, as far as it has been described as a context dependent tumor suppressor and oncogen (Shaulian 2010).

1.4.2. Lysosomal responses

Lysosomal membrane stability is considered a robust general stress biomarker in fish hepatocytes (ICES 2010) and it has proved to be a rapid and sensitive tool to assess the effects of organic xenobiotics and other injurious agents in different aquatic species (Köhler 1991). Previous studies performed with flounder (*Plathichthys flesus*)

have shown that changes in lysosomal membrane integrity can precede the appearance of neoplasms (Köhler and Pluta 1995). As far as we know, this is the first time in which lysosomal membrane stability test has been applied in zebrafish after exposure to PAHs LP values obtained in the control groups are comparable to those obtained in different fish species (anchovy, hake, eelpout), in which lysosomal membrane stability test has been previously assessed (Izagirre 2007). According to Köhler et al. (2002), the first labilization peak at <10 min clearly indicates the onset and progression of liver pathologies (Fig. 2). However, different fish species can have different base LP values. Results reported in this chapter together with the results obtained by Lacave et al. (in prep a,b), who obtained LP values close to 9 min for the control groups, indicate that LP values for control zebrafish may be below 10 min. Moreover, the existence within the same tissue of different lysosomal populations with different LPs is well established (Moore et al. 2004). Nonetheless, in our experiment, reduced LP values were measured in treated fish compared to control animals and similar results were obtained after B(*a*)P and DMBA treatments, suggesting that both carcinogens produce comparable zebrafish health impairment (Fig. 2B). Exposure to 0.3 mg/L DMBA was able to produce significant destabilization of the lysosomal membrane, while both PAHs produced a significant decrease after 2 weeks, indicating that prolonged exposures to any of the assessed compounds can impair zebrafish health status.

1.4.3. Peroxisome proliferation

Peroxisome proliferation is a cellular phenomenon indicative of exposure to different pollutants including organic compounds like PAHs, which has been related to the appearance of liver carcinogenesis in rodents (Lake 1995). This phenomenon is usually accompanied by the induction of certain peroxisomal enzymes which enhance H_2O_2 production (Reddy and Mannaerts 1994). Peroxisome proliferation has been demonstrated as a responsive biomarker in zebrafish. In previous works where zebrafish were exposed to a range of concentrations (0.01 to 0.5 mg/L) of different organic compounds, peroxisome proliferation was observed after 7 and 15 days of exposure (Ortiz-Zarragoitia and Cajaraville 2005).

In this study, neither AOX activity (Fig. 4A) nor the peroxisomal volume density (Fig. 4B) were significantly increased after 3 days of exposure to any of the tested DMBA concentrations indicating that peroxisome proliferation did not occur in response to the short-term DMBA exposure. Moreover, the detected AOX activity levels were low if compared with previous studies in which values around 0.2 mU AOX/mg protein and 1 mU AOX/mg protein were obtained in control and exposed zebrafish, respectively (Ibabe et al. 2005; Ortiz-Zarragoitia and Cajaraville 2005).

Peroxisome proliferation was observed after two weeks of exposure to 0.3 mg/L of both PAHs (Fig. 4C). These alterations suggest that medium term exposures to PAHs are required to produce peroxisome proliferation in zebrafish. It must be noted that, a reduction on the peroxisomal volume density of the control group was observed by the second week (Fig. 4C). Reduction of the activity of peroxisomal AOX has also been described in DMSO controls from an experiment performed with mussels (*Mytillus galloprovincialis*) (Orbea et al. 2002). Peroxisomes are key organelles in lipid metabolism, and thus the lipidic alterations observed in the histological analysis (see below) might be related with the peroxisomal metabolism (Wanders 2004). In fact, DMSO has been described to be no toxic and to enhance lipid synthesis and secretion in cultures of adult rat hepatocytes (De la Vega and Mendoza-Figueroa 1991). Therefore, the carrier effect should also be considered when peroxisome proliferation has to be analyzed in fish. Anyhow, it must be considered that the same DMSO concentration (0.01%) was present in all the exposure groups of the second experiment. And therefore, the observed differences would correspond to the effect produced by the exposures to PAHs.

1.4.4. Genotoxicity

The erythrocyte micronucleus test has demonstrated efficiency and sensitivity in different fish species to monitor aquatic pollutants displaying genotoxic features (Al-Sabti and Metcalfe 1995; Grisolia and Cordeiro 2000). In zebrafish, MN frequency is an exposure time and concentration dependent parameter that has been used to assess the genotoxicity of several substances including metals, biocides and antibiotics (Baez and Prieto 2005; Domingues et al. 2010; Oliveira et al. 2009; Rocco et al. 2012). In those works, MN frequencies in non exposed control zebrafish varied from 0 to 0.6 ‰. Thus, our results are in agreement with the existing bibliography on the baseline MN frequencies for zebrafish. On the other hand, in those works, MN frequency in exposed zebrafish showed great variability depending on the exposure compound ranging from 0.208 to 45‰.

Even if no works have studied PAHs genotoxicity on zebrafish by means of the MN test, this technique has been employed in other fish species to evaluate the impact produced by exposures to PAHs. Waterborne exposure of red sea bream (*Pragus major*) for 10 days to a mixture of phenantrene (30 µg/L), pyrene (30 µg/L), chrysene (30 µg/L) and B(*a*)P (3 µg/L) caused increased MN frequency from the second day of exposure (Cheikyula et al. 2009). In our study, DMBA exposure for 3 days to concentrations below 1 mg/L did not produce increased MN frequency, indicating that in zebrafish PAHs are clastogenic only at high concentrations (Fig. 6). According to our data, zebrafish could be more resistant to PAHs genotoxicty than other fish species. Moreover, the increased exposure time tested in the medium-

term experiment, in which animals were exposed for two weeks to 0.3 mg PAH/L did not produce increased MN frequency.

1.4.5. Histopathological alterations

Up today, zebrafish has shown to be susceptible to various chemical carcinogens which are able to produce many neoplasm types in different tissues showing remarkable histopathological resemblance to human and other mammalian cancers (Amatruda et al. 2002; Lam and Gong 2006; Spitsbergen and Kent 2003). In contrast, very low incidence of spontaneous cancers has been reported in zebrafish (Spitsbergen and Kent 2003).

The exposure concentrations and times tested in this work were enough to produce effects at cellular and molecular levels (gene transcription and LMS). However, the histopathological analysis did not show significant increases in the prevalence of the histopathological lesions. Nevertheless, some histological alterations were observed when longer lasting exposures were performed (Fig. 7). Vacualization (Fig. 7B) was observed in fish from the medium-term experiment; however this alteration was detected in all the groups including the DMSO control fish. Different reasons may be underlying the observed tendency to vacuolization. On the one hand, according to Wolf and Wolfe (2005), fish liver tends to be vacuolated due to a high content of lipid and/or glycogen, being the accumulation of fat or glycogen content a common morphologic response to toxic exposures, due to reduced glycogen breakdown as a result of hepatocellular toxicity. Actually, zebrafish injection with TCDD, which as in the case of the PAH exerts its toxic effects by means of the AHR, has shown to produce lipidosis in liver hepatocytes (Zodrow et al. 2004). Moreover, vacuolization has also been shown to occur in medaka (*Oryzias latipes*) exposed to N-nitrosodiethylamine, which is also an organic carcinogen (Boorman et al. 1997). On the other hand, as mentioned above, DMSO has been described as a non toxic lipid synthesis enhancer in cultures of adult rat hepatocytes (De la Vega and Mendoza-Figueroa 1991).Therefore our data would indicate a possible confounding effect produced by DMSO exposure. Finally, hepatocellular vacuolization has also shown to be especially apparent in the livers of captive fish presumably due to imbalances in energy intake and expenditure caused by artificial feeding and housing conditions (Wolf and Wolfe 2005). Therefore, the hepatic vacuolization observed in our experiment may also have been caused by an excessive energetic intake as far as fish in the control group showed the same morphology.

Interestingly, one of the fish exposed for two weeks to 0.3 mg/L of DMBA showed hepatic megalocytosis (Fig. 7B). Although no significant incidence of this pathology was detected, it must be taken into account that data from many vertebrates, including zebrafish, indicate that hepatocyte megalocytosis is caused by

toxicant damage to DNA or the mitotic apparatus following carcinogenic exposure (Spitsbergen and Kent 2003). Likely, longer exposure times or assessment at longer time periods after exposure would be required to detect more relevant tissue level alterations in zebrafish, such as neoplastic lesions.

1.5. Conclusions

Short- and medium-term exposures to PAHs produce toxic effects on adult zebrafish, visualized as alterations in the studied molecular and cellular level biomarkers. Gene transcription levels indicated upregulation of phase I metabolism that would consequently produce damaging reactive metabolites. Consequently, both PAHs produced impairment of general health status as demonstrated by reduced LP values in exposed fish. Increased oxidative stress arising from peroxisome proliferation produced by both PAHs may also contribute to the observed reduction on the general health status. Relevant tissue level effects were not detected, possibly because the histopathological analysis was performed too early to identify adverse outcomes at the tissular level.

Overall, results indicate that both PAHs own similar toxic capacity even if the results from the gene transcription analysis suggest stronger capacity of DMBA to induce the tissue damage related *junb.* Moreover, exposure to 1 mg/L of DMBA produced high mortality and significant genotoxic effects on adult animals.

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Effects of waterborne exposure to carcinogenic PAHs on adult zebrafish hepatic transcriptome

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Abbreviations

7,12-dimethylbenz(*a*)anthracene, DMBA

Aryl hydrocarbon receptor, AHR

Benzo(*a*)pyrene, B(*a*)P

Correspondence analysis, COA

Dimethyl sulfoxide, DMSO

Estrogen receptor, ER

Estrogen receptor responding element, ERE

Gene ontology, GO

Hierarchical clustering, HCL

Mode of action, MOA

Polycyclic aromatic hydrocarbon, PAH

Principal component, PC

Reactive oxygen species, ROS

Standard deviation of local background intensities, SD

Gene abbreviations

cyclin, ccn

cytochrome P450, cyp

heat shock protein, hsp

jun B proto-oncogen, junb

ribosomal protein S18, *rps18*

tumor protein 53, *p53*

zona pellucida protein, zp

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are potent carcinogenic chemicals. Exposure to PAHs causes a high prevalence and variety of tumor lesions in fish. To study the potential mode of action of two carcinogenic PAHs, benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA), a transcriptomic analysis of adult zebrafish (*Danio rerio*) liver was performed after 1 and 2 weeks of exposure to 0.3 mg/l. Both compounds regulated the transcription of genes involved in similar biological processes and the correspondence analysis identified exposure time as the key factor. Both PAHs downregulated transcripts involved in cell cycle after the first week of exposure while upregulation was observed by the second, suggesting an initial attempt to face DNA damage and the posterior loss of cell cycle control. Gametogenesis related genes were also regulated following the same transcription pattern indicating a possible crosstalk between aryl hydrocarbon receptor and estrogen receptor. The Fisher exact test identified cell cycle as a process especially affected by B(*a*)P and an additional set of transcripts related to this biological process was identified in B(*a*)P exposed animals that was not regulated in DMBA exposed ones. DMBA especially affected sequences involved in proteolysis and blood coagulation. The pathway analysis identified DNA damage, energy metabolism, amino acid metabolism and glutathione metabolism related pathways which are usually altered in proliferative cells. Results show that exposure to PAHs can ultimately provide a suitable environment for the appearance of tumor lesions.

Keywords: benzo(*a*)pyrene, dimethylbenz(*a*)anthracene, liver transcriptome, microarray, zebrafish.

Laburpena

Hidrokarburo aromatiko poliziklikoak (HAPk) konposatu kartzinogeniko indartsuak dira. Arrainetan, HAPek lesio tumoral desberdinen prebalentzia emendatzen dute. Bentzo(*a*)pireno (B(*a*)P) eta 7,12-dimetilbentzo(*a*)antratzeno (DMBA) HPA-en balizko ekintza modua aztertzeko asmoz, transkripzio analisia burutu genuen 0.3 mg/L-ko kontzentrazioaren pean astebetez edo bi astez egondako zebra arrainen (*Danio rerio*) gibelean. Bi konposatuek antzeko prozesu biologikoetan parte hartzen dituzten geneen transkripzioa erregulatu zuten eta korrespondentzia analisiak esposizio denbora identifikatu zuen faktore gakotzat. Bi HAPek zelularen zikloaren erregulazioan parte hartzen duten geneen transkripzioa gutxitu zuten lehendabiziko astearen ondoren, ostera, bigarren astean gene berdinen transkipzioaren emendioa ikusi genuen. Honek DNAn gertatutako kalteei aurre egiteko saiakera eta ondorengo zelularen zikloaren kontrol galtzea ematen du aditzera. Gametogenesiarekin erlazionatutako geneak ere erregulatuak izan ziren transkipzio patroi bedinari jarraituz, arilo taldearen hartzailearen eta estrogenoen hartzailearen arteko balizko komunikazio gurutzatua iradokiz. Fisher-en test zehatzak zelularen zikloa B(*a*)P-ak bereziki eragindako prozesutzat identifikatu zuen, eta DMBA-ren pean izandako animalietan erregulatuak izan ez ziren eta prozesu biologiko horrekiko lotura duten transkriptoen multzo gehigarria identifikatu genuen. DMBAk proteolisian eta odol koagulazioan parte hartzen duten sekuentziak erasan zituen. Bidezidorren analisiak DNA-kalteekin, energia-metabolismoarekin, amino azidoen metabolismoarekin eta glutation-metabolismoarekin erlazionatutako bidezidorrak identifikatu zituen. Prozesu biologiko hauek zelula proliferatzaileetan erasanda egon ohi diren prozesuak dira. Oro har, lortutako emaitzek HAPak lesion tumoralen agerpenerako aproposa den ingurunea bermatzen dutela adierazten dute.

Gako Hitzak: bentzo(*a*)pireno, dimetilbentzo(*a*)antrazeno, gibel transkriptoma, mikroarraia, zebra arraina.

2.1. Introduction

Benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA) are two well studied members of the polycyclic aromatic hydrocarbon (PAH) family which are widespread environmental contaminants produced as a result of natural and industrial processes. It has been reported that PAHs with bay regions, as in the case of B(*a*)P, are likely to be potent carcinogens, while those with fjord regions or sterically hindered bay regions such as DMBA are even more potent (Baird et al. 2005). PAHs are metabolized by cytochrome P450-dependent oxidases mainly present in the smooth endoplasmic reticulum of hepatocytes (Shimada 2006). As a result, they are bioactivated with the concomitant overproduction of highly reactive compounds such as diol epoxides, quinones, radical anions and benzylic carbenium ions, which are able to damage DNA and provoke tumor initiation (Henkler et al. 2012). During this process, a concomitant increase in reactive oxygen species (ROS) production occurs, which induces oxidative damage to DNA and other cell components, such as membranes and proteins (Miller and Ramos 2001). Consequently, organic compounds like B(*a*)P and DMBA can produce cancer by directly reacting with DNA causing adduct formation (genotoxic mechanism), or without direct DNA interaction through different non-genotoxic mechanisms including inflammation, immunosuppression, formation of ROS, activation of transcription factors such as the aryl hydrocarbon receptor (AHR) or the estrogen receptor (ER) and epigenetic silencing (Luch 2005). Toxicological studies using model organisms often focus on responses in liver, since liver is the main organ for metabolism of exogenous chemicals, including PAHs (Boelsterli 2007; Burczynski 2003; Spitsbergen and Kent 2003).

During the last decades, the zebrafish (*Danio rerio*) has emerged as a model organism important for the identification and characterization of genes and pathways involved in development, organ function, behavior, disease and toxicology (Dai et al. 2014; Pichler et al. 2003; Raldúa et al. 2011; Sprague et al. 2003). It is well accepted that the aquatic environment is the major sink for many contaminants and therefore pollutants can pose a threat to aquatic wildlife including fish (Denslow et al. 2007). Simultaneously, advances on molecular biology have contributed to a revolution on toxicity testing. While traditional tests are focused on searching for adverse effects, today understanding toxicity pathways producing those effects is gaining importance (NRC 2007; Zhou et al. 2009). Some studies have already focused their attention on assessing the effects of PAH exposure on fish hepatic transcriptional responses. A common thread in those studies was the finding of transcriptional adjustments related to oxidative stress. Exposure of rainbow trout (*Oncorhynchus mykiss*) to 1 µg/l of B(*a*)P for 7 days upregulated 55 genes out of which 28 were shared between B(*a*)P and the pro-oxidant compound Dignet, suggesting that B(*a*)P exerted its toxicity via

oxidative stress (Hook et al. 2006). In the European flounder (*Platichthys flesus*), an intraperitoneal injection of 3-methylcholanthrene produced the upregulation of oxidative stress–responsive genes and the chaperone *heat shock protein 90* (*hsp90*- Williams et al. 2008). Transcriptional responses related to the production of ROS have been observed after stickleback (*Gasterosteus aculeatus*) exposure to dibenzanthracene, where the transcription of genes related to oxidoreductase activity and the xenobiotic metabolism related *cytochrome P450 1a* (*cyp1a*) were altered (Geoghean et al. 2008; Williams et al. 2009).

Despite the similar responses produced by PAHs and other pro-oxidant compounds, fish exposure to petroleum substances containing PAH mixtures has been shown to result in specific transcriptional responses, that were distinguishable from those responses obtained in fish exposed to other organic (diquat) or metal (Cr IV) pro-oxidants (Hook et al. 2010).

According to Piña and Barata (2011), the zebrafish is the most commonly employed fish species in environmental toxicity studies, employing microarray technology. Holth et al. (2008) analyzed the transcription pattern after exposure for 1 and 7 weeks to produced water arising from oil and gas production containing, among other contaminants, a mixture of seven PAHs accounting for a total PAH exposure concentration of 5.4 µg/L. This resulted in transcriptional adjustments of genes related, among other functional groups, to cancer, cellular growth and proliferation, cell cycle and tumor morphology. Furthermore, Lam et al. (2006) exposed zebrafish fry to 2.5 mg/L DMBA for 24 h in order to produce tumoral lesions and the transcriptome analysis of those lesion revealed decreased transcript levels of genes coding for proteins involved in cell cycle/proliferation, apoptosis, DNA replication and repair, metastasis and cytoskeletal organization, protein synthesis and liver-specific functions. Additionally, there are some studies focused on changes in transcription levels of a limited set of target genes. Bugiak and Weber (2009) injected zebrafish with B(*a*)P (1 mg/kg), resulting in upregulation of *cyp* genes and cyclooxygenases after 24 h. The transcription of reproduction-related genes such as *vitellogenin* has also been shown to be altered in liver of zebrafish after 56 days of waterborne exposure to 3 µg/L of B(*a*)P (Hoffman and Oris 2006).

In spite of all the information available, there is still a lack of knowledge regarding the changes at transcription level occurring during the first two weeks of exposure to specific PAHs on zebrafish hepatic transcriptome, which are of great interest if the mode of action (MOA) of these compounds is to be understood. As recently pointed out by Piña and Barata (2011), the response to stressors follows different stages. The immediate adaptive response reflects the primary response to the stressor through the interaction with its cellular targets, and is followed by the

compensatory response in which the cell activates mechanisms to cope with the damage produced by the stressor. These different toxicity phases are related to different patterns in gene transcription (Fent and Sumpter 2011). Studying the hepatic transcriptional response to model carcinogenic PAHs using different exposure periods is therefore a point of interest that could help to elucidate the MOA of these model toxicants. Thus, in this study we aimed to compare the cellular processes altered by two model carcinogenic PAHs, B(*a*)P and DMBA, and to compare their effects at two different time points in order to gain knowledge about the MOA underlying their carcinogenic capacity.

2.2. Materials and Methods

2.2.1. Experimental organisms

Adult zebrafish (*Danio rerio;* AB Tubingen) were raised and maintained at 27 ± 1 ºC with a 14-hour light / 10-hour dark cycle in 100 l tanks at a density of 1.5 fish/l. Tank water was prepared by conditioning osmotic water with marine basic salt (Sera Gmbh, Heinsberg, Germany) and KH/pH plus (Sera) up to 600 µS and pH 7.4, before mechanical filtering (1 µm) and sterilization by ultraviolet light. Water aeration and biological filtration was achieved by an airlift pump in each tank. Residual metabolites were measured using Sera GmbH ammonium, nitrite and nitrates kit, maintaining water at 0-0.5 mg/L; 0-0.5 mg/L and 5-10 mg/L respectively. When the highest concentration values were surpassed water was partially replaced. Fish were fed *ad libitum* with live *Artemia* nauplii (INVE, Dendermonde, Belgium) and commercial dry food, Microgran (Sera).

2.2.2. Exposure and hepatic tissue collection

The experimental procedure employed in this work was approved by the committee for animals' welfare of the University of the Basque Country.

3 months old fish were exposed to nominal concentrations of 0.3 mg/L B(*a*)P (Sigma-Aldrich, St. Louis, USA) or 0.3 mg/L DMBA (Sigma-Aldrich) for 2 weeks at a density of 1.375 fish/L in 50 L tanks using one tank per experimental group. Test concentrations were selected based on previous pilot experiments in which high mortality was recorded after 3 days exposure to 1 mg/L DMBA (**Chapter I**). During the experiment, fish welfare was daily controlled and no mortality or alterations on swimming and feeding behavior were detected in any of the experimental groups. 20% of the water in each tank was renewed and the corresponding amount of test solution was added twice a week. DMSO (Sigma-Aldrich) was used as a carrier for the

exposure at a final concentration of 0.01%, being present in the control group as well as in the exposure groups. Samples from each treatment were collected after 1 and 2 weeks of exposure. Fish were euthanized by overdose of MS-222 (tricaine methane-sulfonate, Sigma-Aldrich), and livers were dissected, immersed in RNA Later ® (Sigma-Aldrich), immediately frozen in liquid nitrogen, and stored at -80ºC until RNA extraction.

2.2.3. RNA extraction

Samples were homogenized in an RNAse free 1.5 mL tube (Eppendorf, Hamburg, Germany) using a MHX/E motorized hand tool (Xenox, Wecker, Luxemburg). Total RNA extraction was performed following the TRIzol® extraction method (Invitrogen Life-Technologies, Merelbeke, Belgium) by adding 1 mL of TRIzol® to homogenized liver tissue. RNA extracts were purified using DNAse I and RNAse inhibitor (Fermentas, St. Leon-Rot, Germany) and subsequent phenol/chloroform extraction. RNA purity and integrity were evaluated by measuring 260/230 nm and 260/280 nm absorbance ratios (NanoDrop Technologies, Rockland, DE, USA) and by denaturing formaldehyde-agarose gel electrophoresis, respectively. 260/230 ratios were always higher than 1.7, 260/280 ratios were always higher than 1.75. There were no visual signs of RNA degradation. The resulting samples of total hepatic RNA were stored at −80°C and used for microarray analysis as well as for real time PCR.

2.2.4. Microarray analysis

Fluorescently labelled cRNA was constructed starting from total RNA extracts, following Agilent's two-color microarray-based gene transcription analysis protocol (version 5.7, http://www.agilent.com) using the Quick Amp kit (Agilent Technologies, Santa Clara, CA, USA). Briefly, total RNA was reverse transcribed into first and second strand cDNA, after which first-strand cRNA was constructed using the second strand cDNA as a template, in the presence of either Cy3-CTP or Cy5-CTP. The labeled cRNA was purified using the Qiagen RNeasy mini spin column kit (Qiagen, Venlo, The Netherlands). Labeling efficiency was determined at 550 nm (Cy3) and 650 nm (Cy5) using a NanoDrop spectrophotometer. Hybridizations were performed on zebrafish 44k full genome microarrays (version V2, AMADID 019161 Agilent Technologies) containing 43,803 unique probes. For each time point, samples from the different treatments were hybridized in an n+2 (n = 9) A-optimal loop design. Additionally, these two designs were linked by two extra hybridizations to enable comparison between time points (Knapen et al. 2009). 825 ng of both Cy3 and Cy5 labeled cRNA was applied onto every microarray according to the hybridization design. Microarray slides were incubated at 65 °C for 17 h in a rotating Agilent hybridization chamber. Slides were subsequently washed with Agilent wash buffers and acetonitrile and finally submersed in stabilization and drying solution (Agilent Technologies) to

prevent ozone-induced Cy5-degradation. Microarrays were scanned using a Genepix Personal 4100A confocal scanner (Axon Instruments, Union City, CA, USA) at a resolution of 5 µm. The photomultiplier tube voltages for separate wavelengths were adjusted to obtain an overall green/red ratio of one. Images were processed using GenePix Pro 4.1 software (Axon Instruments) for spot identification and quantification of the fluorescent signal intensities.

qPCR was used to independently verify the changes in mRNA levels. Among the genes regulated in the microarray analysis, we selected genes involved in processes well known to be affected by PAH exposure, such as xenobiotic metabolism, cell cycle regulation and carcinogenesis: *cyp1a* (ID: Dr03112441_ml), *cyp1b1* (ID: Dr03181453_gl), *cyclin b1* (*ccnb1*-ID: Dr03105834_ml), *cyclin g1* (ccng1-ID: Dr03132065_ml), *tumor protein 53* (p53-ID:Dr03112082_gl), and *jun B proto-oncogen* (*junb*-ID: Dr03204057_sl). *Ribosomal protein S18* (rps18-ID: Dr03144509_ml) was used as housekeeping gene. The same RNA extracts used for the array experiments were used for the qPCR as it has been described in **Chapter I**. Taqman probes were purchased from Applied Biosystems (Carlsbad, California). Triplicate PCR reactions were carried out as indicated by manufacturer's protocol using an ABIS 7300 cycler (Applied Biosystems, Carlsbad, California) under the following conditions: 2' at 50°C, 10' at 95°C followed by 40 cycles of 15'' at 95°C and 1' at 60°C.

2.2.5. Statistics

From each treatment at each sampling time 3 biological pseudoreplicates (Nikinmaa et al. 2012) consisting of pools of 5 livers each were prepared.

For microarray data, statistical analysis was performed using the LIMMA R package (Smyth 2004) after discarding two arrays (one in each time point) that due to the high dye-biases observed in MA-plots did not reach quality standards. Spots for which red or green FG<BG+2SD (Sclep et al. 2007) on all arrays were deleted before analysis (FG: median foreground intensity; BG: average local background intensity calculated over the full microarray; SD: standard deviation of local background intensities). Median intensity data were background corrected using a normal-exponential convolution model using the function background Correct with method "normexp", offset=50 (Ritchie et al. 2007). Data were loess-normalized using the function normalizeWithinArrays. Between-array normalization, like variance stabilization, was not necessary because of the low inter-array variability of the printed arrays. Linear models were fitted to intensity ratios, after which probes were ranked in order of evidence of differential transcription using an empirical Bayes method (Smyth 2004). The averaged values of both treatments at both time points were contrasted against the averaged values of their respective controls. These contrasts were fitted to the linear models, a false discovery rate of 0.05 and a cutoff

of $log_2FC>1$ or $log_2FC<-1$ (log_2 fold change) were established. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible under the GEO series accession number GSE4375. [Data not yet released; reviewer read-only link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rfkdroeokymayxy&acc=GSE43675].Gene ontology (GO) and KEGG pathways analysis were performed using Blast2GO (http://www.blast2go.com) software in order to identify biological processes and molecular pathways linked to regulated transcripts. Pie charts were constructed to visualize GO distributions. Fisher exact tests were used to compare the representation of GO classes between time points and between compounds ($p<0.05$) in the same web tool.

A correspondence analysis (COA) was performed in order to visualize the association between the transcripts and the contrasts using the MultiExperiment Viewer (tMEV) software (http://www.tm4.org/mev.html; Saeed et al. 2006). For this analysis, log_2FC values were calculated by contrasting each replicate separately with the average of the respective controls. Using the same web tool, heat maps were constructed to visualize the transcription patterns of genes differentially regulated by each compound and genes significantly regulated in all the four studied conditions. For the latter, a self organizing trees analysis (SOTA) of the transcripts and a hierarchical clustering (HCL) of the contrasts was performed using Pearson correlation and average linkage based on all the transcripts which were significantly altered in the four treatments.

Data from the qPCR analysis were normalized against *rps18* by means of the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Statistical analyses were performed using the SPSS for Windows (SPSS Chicago, IL) software to compare the regulation level measured by both methods. Normality was checked using the Kolmogorov-Smirnov test and one-way analysis of variance (ANOVA) was used to compare the qPCR results with the microarray results. Significance was established at $p<0.05$.

2.3. Results

2.3.1. Data overview

A total of 2806 unique probes were differentially expressed in at least one of the exposure groups. The pie charts in Fig. 1 show the GO category distribution of the transcripts for each condition and for up- and downregulated transcripts separately. Exposure of zebrafish to B(*a*)P for one week produced a higher percentage of down

regulated than upregulated sequences, while exposure for two weeks to B(*a*)P produced a higher percentage of upregulated than downregulated transcripts (Fig. 1). For DMBA such pattern was not observed. At both time points, the amount of upregulated sequences was slightly higher than the amount of downregulated ones. Correspondence analysis separated samples according to the four experimental groups (Fig. 2).

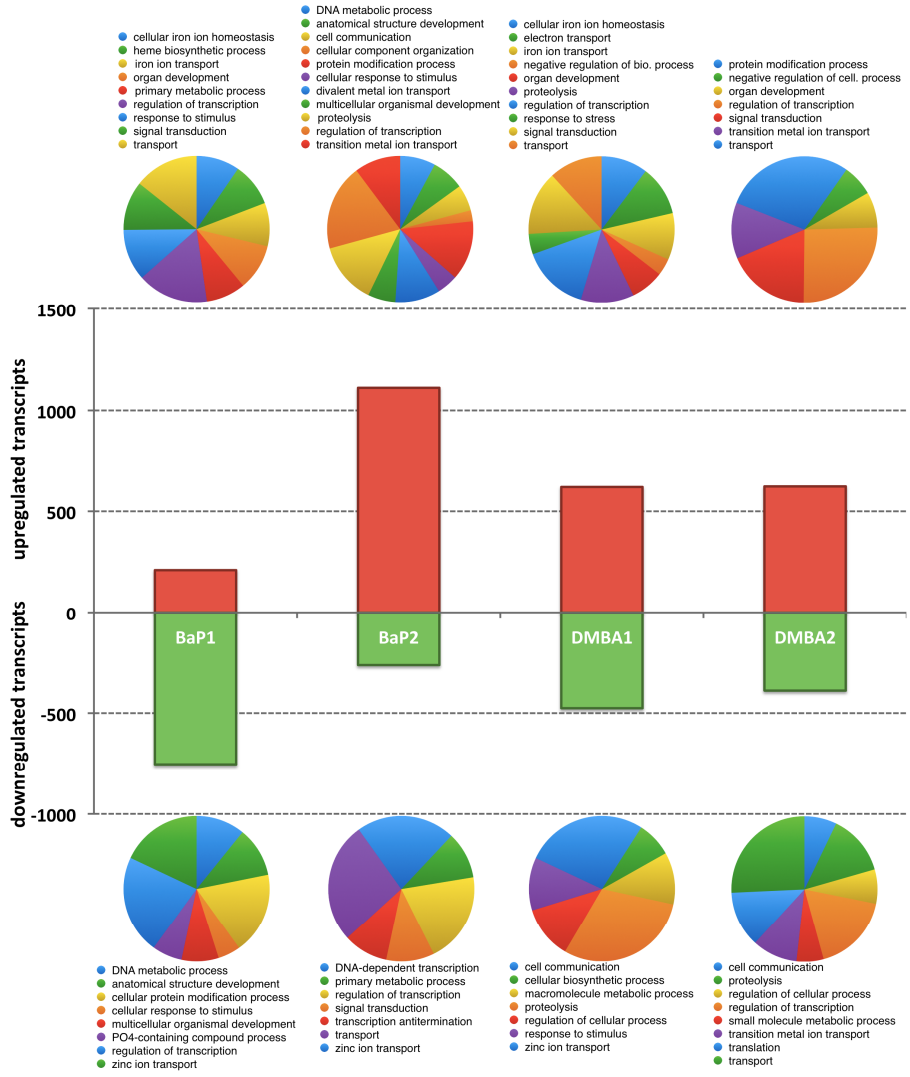

Fig. 1. Number of significantly up and down regulated transcripts for each treatment group. Pie charts over and below each bar denote the GO distribution corresponding to each group of genes.

Samples from fish exposed for one week to any of the two PAHs were separated from those corresponding to fish exposed for two weeks by the first component (PC1, X axis), which explained the 58% of sample variability, indicating that most of the data variability was explained by the exposure time. On the other hand, PC 2 (Y axis) separated samples according to the exposure compound and explained 16% of the variability.

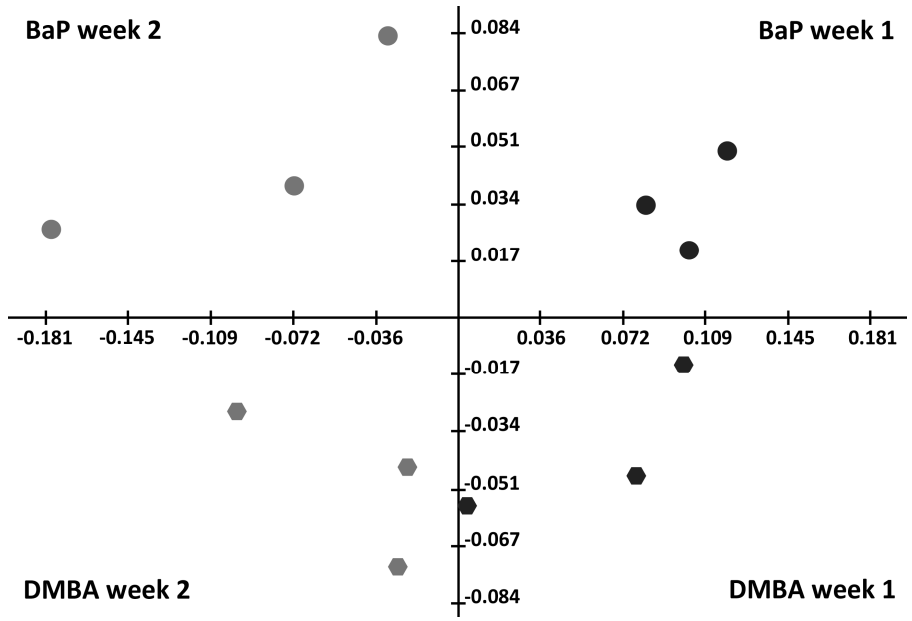

Fig. 2. Correspondence analysis (COA) based on transcription profiles in liver of zebrafish exposed to B(*a*)P and DMBA. Distribution explaining sample variability is represented in two axes corresponding to the first (x-axis) and the second (y-axis) components of the COA. Circles represent samples of fish exposed to B(*a*)P and hexagons represent samples of fish exposed to DMBA. Black was used for fish exposed for 1 week, while grey represents 2 weeks of exposure.

2.3.2. Similarities between the responses to B(*a*)P and DMBA

To investigate similarities between the responses to both PAHs a SOTA analysis and a hierarchical clustering were conducted with all the 108 genes that were significantly regulated in all the four tested conditions (Fig. 3). Four main transcription patterns were identified. Time points were clustered together; an 80% of the genes were clustered in groups which transcription pattern was opposite from the first to the second week of exposure. The differential transcription was stronger in B(*a*)P exposed fish. The 72.2% of the genes were downregulated after the first week of exposure and upregulated after the second week. Transcripts belonging to this group included *cyclins* (*ccna2, ccnb1*, *ccnb2*), *granulin1*, *granulin2*, *influenza virus NS1A*

binding protein b, or *H2A histone family X* that are related to cell cycle and genes like *zona pellucida proteins* (*zp*), *cth1* and *daz-like gene* that are gametogenesis related genes (Fig. 4). The 9.25 % of the genes were upregulated after the first week of exposure to both PAHs and downregulated after the second week, including the *chromosome 20 open reading frame 149 homolog*. Only the 3.7 % of the transcripts were significantly upregulated in the four conditions; these included two members of the cytochrome p450 family (*cyp1c1* and *cyp1b1*).

There were also some genes (18.51 %) of which the transcription did not fit the temporal distribution. For example, *DNAJ homolog* was upregulated in fish exposed to B(*a*)P for one week and downregulated in the rest of exposure groups, while *hsp70* followed the opposite transcription pattern.

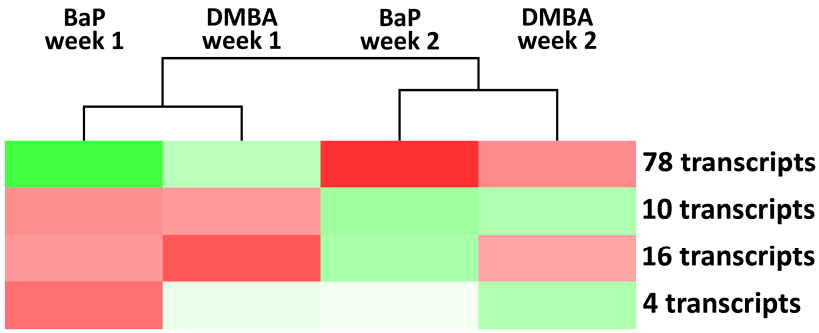

Fig. 3. SOTA analysis and hierarchical clustering based on genes differentially transcribed in the four treatments. Four main transcription patterns are identified. Transcripts significantly upregulated towards the controls are represented in red while those significantly downregulated are shown in green.

2.3.3. Differences between the responses to B(*a*)P and DMBA

Even though exposure time was identified as the main parameter determining variance between treatment groups, the existence of a chemical dependent effect was also revealed by the COA. Fisher exact tests were performed between conditions to identify GO classes differentially enriched by each compound compared to the other. Significant differences between B(*a*)P and DMBA were only observed after the first week of exposure. Enriched GO classes were grouped into 3 main biological classes (proteolysis, cell cycle and blood coagulation) and heatmaps were constructed with the genes contributing to those classes (Fig. 5).

Fig. 4. Heatmap constructed with those transcripts underlying the SOTA analysis and hierarchical clustering in Fig. 3.

GO categories involved in proteolysis were mainly enriched after one week of DMBA exposure (Fig. 5A). Secondly, GO classes involved in cell cycle were enriched after one week of B(*a*)P exposure compared to one week of DMBA exposure. B(*a*)P exposure produced a general downregulation after the first week of exposure that was not observed in DMBA exposed animals (Fig. 5B). Finally, zebrafish exposure to DMBA produced strong upregulation of transcripts involved in blood coagulation after the first exposure week, which was still visible after the second week, while this was not the case after B(*a*)P exposure (Fig. 5C).

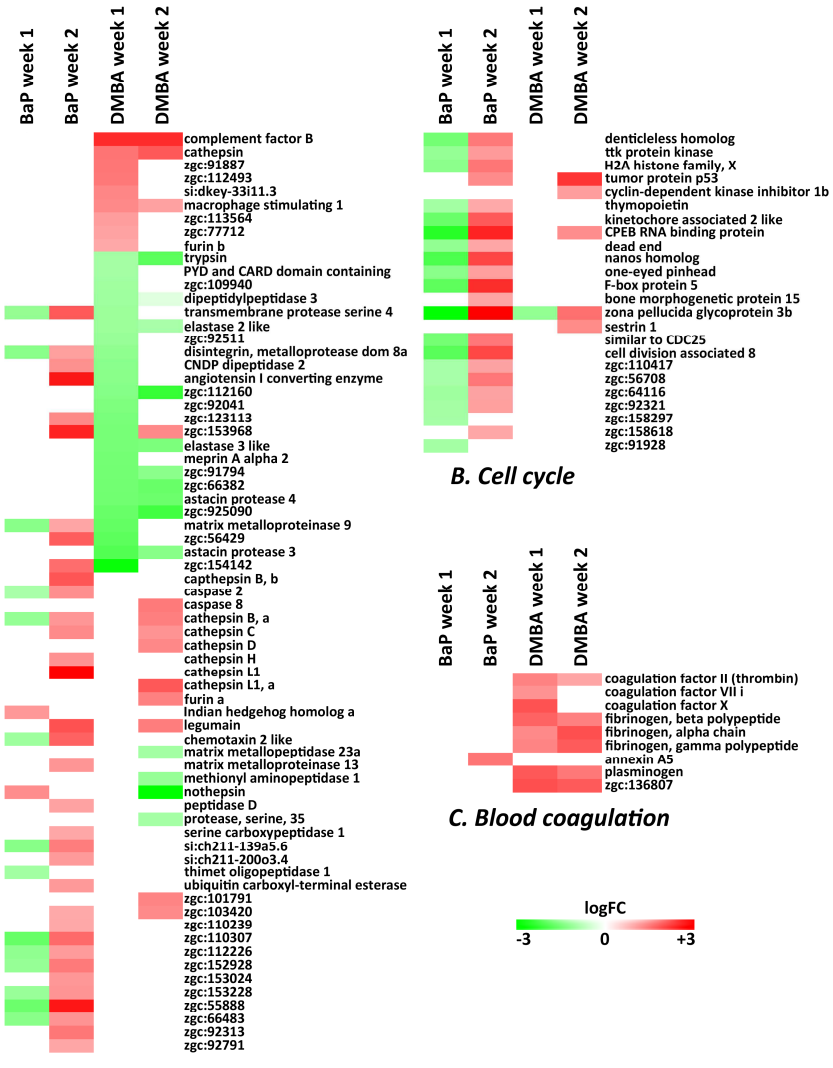

Fig. 5. Heatmaps constructed with those transcripts involved in the biological processes (proteolysis, cell cycle, blood coagulation) identified as enriched after the first week of exposure to B(*a*)P vs DMBA.

2.3.4. Pathway analysis

KEGG map analysis was performed to identify the potential pathways affected by each of the PAHs irrespective of time (Fig. 6). For both compounds, pathways related to DNA damage were strongly regulated; “purine metabolism” and “pyrimidine metabolism” were especially affected in both cases. Pathways related to altered cellular energetic balance (“glycolysis/gluconeogenesis”, “fructose and mannose metabolism”, “pentose phosphate”), and amino acid metabolism (“cysteine, methionine metabolism”, “serine, glycine, threonine metabolism”) were also identified. Finally, oxidative stress related “glutathione metabolism” was also strongly regulated by both PAHs. The transcription pattern of the genes contributing to those pathways is shown in Fig. 7.

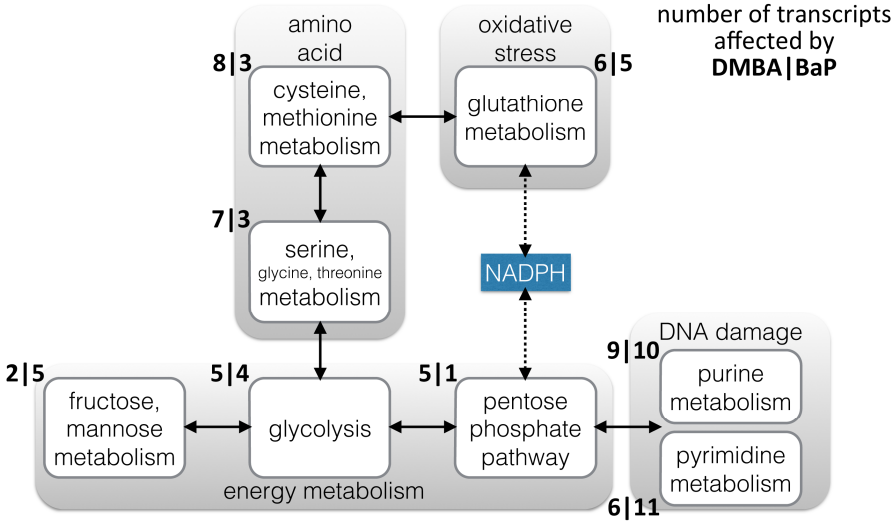

Fig. 6. KEGG pathway analysis. Significantly enriched pathways according to the KEGG pathway analysis. Numbers present in the top left corner of each pathway correspond to the number of transcripts regulated by DMBA/B(*a*)P.

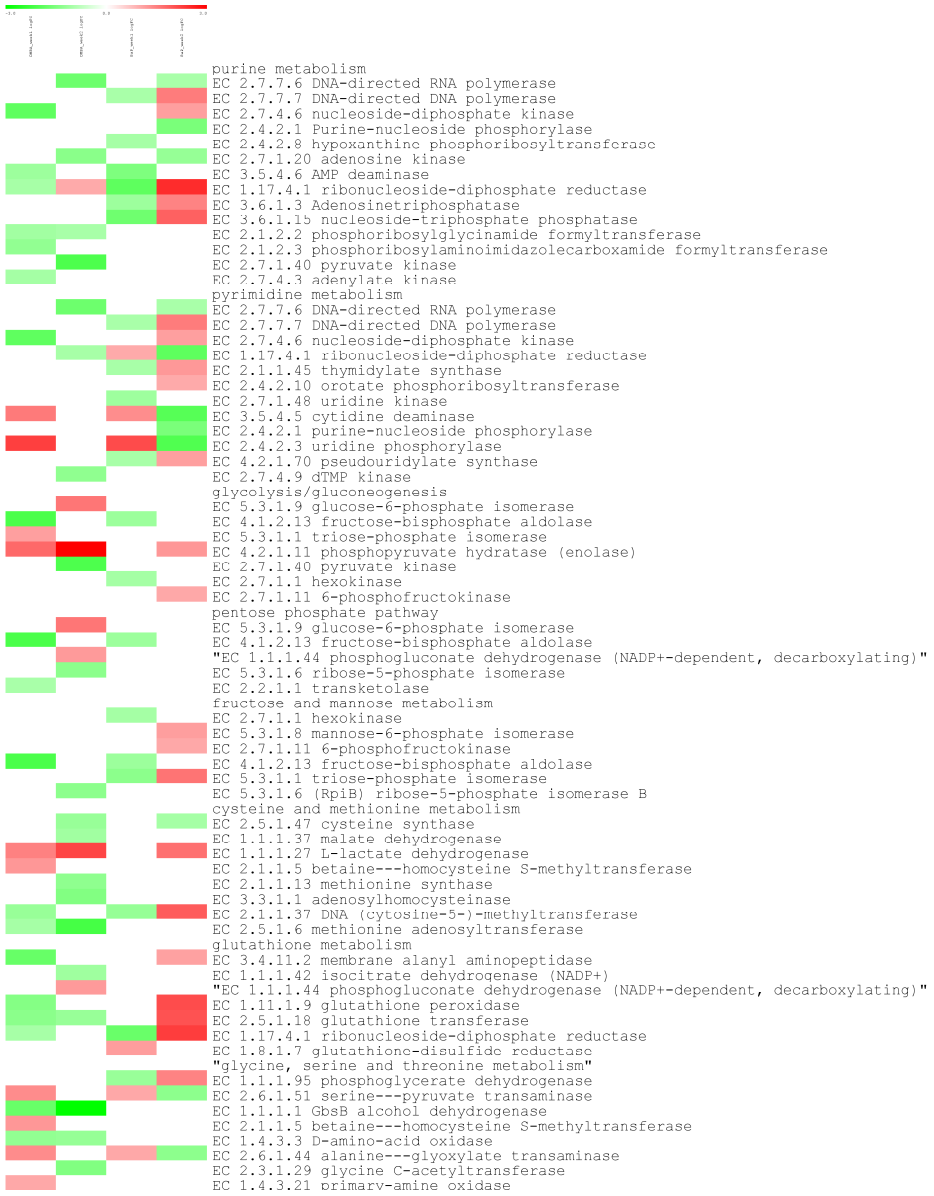

Fig. 7. Heatmaps constructed with those transcripts contributing to the pathways identified in the KEGG analysis (Fig. 6).

2.3.5. qPCR

For a selected set of genes that appeared regulated according to microarray results and that were considered of special toxicological interest, mRNA transcription levels were also analyzed by qPCR and results (specifically described in **Chapter I**) were

compared to those obtained by microarray analysis. In all the cases except in *ccng1* for B(*a*)P exposures, and *ccnb1* in the second week of exposure to DMBA, the relative transcription level in comparison with the corresponding control groups, observed by qPCR followed the same trend determined by the microarray analysis. In the cases where the transcription patterns were different (down-regulation according to qPCR and up-regulation according to the microarray analysis), no significant differences were obtained.

A similar fold change was maintained with no significant differences in the case of the transcription level shown by *cyp1a*, *ccng1*, and *junb* at both sampling times (Fig. 8).

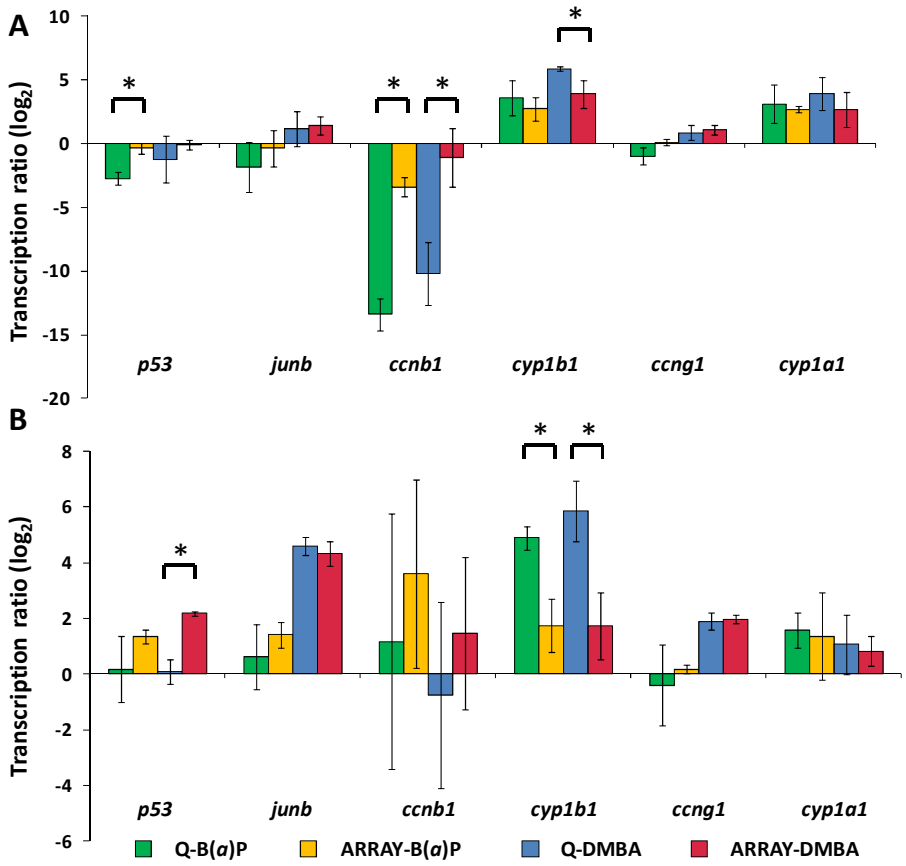

Fig. 8. Comparison between mRNA transcription levels of B(*a*)P and DMBA treated fish with respect to their respective control groups assessed by microarray analysis or qPCR. A: first week of PAH exposure. B: second week of exposure. All values represent the mean log2 ± standard deviation

The transcription level of *ccnb1* in the second week of exposure showed great variability both in qPCR and in microarray analysis. Significant differences were observed for *p53* in animals exposed for one week to B(*a*)P and in those exposed for 2 weeks to DMBA, for *ccnb1* in animals exposed either to B(*a*)P or DMBA for one week and for *cyp1b1* in animals exposed for two weeks to B(*a*)P and at both time points in fish exposed to DMBA. When significant differences were observed, stronger transcription was always recorded by qPCR analysis.

2.4. Discussion

With the aim of studying and comparing the transcriptomic response produced by two different carcinogenic PAHs as well as of analyzing the early time-course response of zebrafish liver, the whole hepatic transcriptome after one and two weeks of exposure to a sublethal (0.3 mg/L) concentration of B(*a*)P and DMBA was analyzed. Data herein provided is exclusively related to gene transcription and cannot therefore be interpreted as a trusty reflect of the alterations occurring in the cells until functional findings supports them (Nikinmaa and Rytkönen 2012). Nevertheless, the analysis and interpretation provided in this study may be a useful prediction of the processes altered after the exposure to these model PAHs.

2.4.1. Data overview

The disrupting capacity of PAHs has been mainly explained in literature by biotransformation processes, involving xenobiotic metabolizing enzymes belonging to the cytochrome P450 family, rendering highly mutagenic and carcinogenic metabolites (Miller and Ramos 2001; Shimada 2006). The expression these enzymes is mediated by the aryl hydrocarbon receptor (AHR) (Irigaray and Belpomme 2011). As reviewed by Aardema and MacGregor (2002), compounds that induce toxicity through similar mechanisms will induce similar changes in gene trasncription patterns. The COA identified exposure time as the key factor contributing to the variability between groups and attributed only 16% of the variability to the differences between both PAHs (Fig. 2). These results indicate that overall comparable transcriptomic responses were produced by B(*a*)P and DMBA. Similar amounts of genes related to a variety of fundamental cellular processes were regulated by both compounds. Many of those processes were recurrent terms between conditions (Fig. 1). When an organism is exposed to a chemical insult, it is commonly accepted that gene transcription is one of the first actions prior to the onset of changes at physiological and other levels of biological organization (Fent and Sumpter 2011). Correspondingly, transcripts involved in mediating the transcriptomic response, like “regulation of transcription”, “signal transduction”, “DNA dependent

transcription" and "cell communication" were regulated (Fig. 1). Recurring GO categories also included "response to stimulus" and "response to stress". These terms are linked to transcripts coding for xenobiotic metabolism enzymes and may reflect the existence of an adaptive response to PAH exposure (Boelsterli 2007). These processes were also enriched in the human hepatic cell line HepG2 exposed to a mixture of PAHs for 24 hours (Castorena-Torres et al. 2008). Accordingly, differential transcription of genes involved in "iron ion transport" after the first week of exposure reflects an increase in the transcription of genes coding for hemoproteins of the phase I metabolism. Altered "iron ion homeostasis" and "heme group production" have previously been reported after exposure to different PAHs in fish (Krasnov et al. 2005; Williams et al. 2009). In fact, exposure to TCDD, which is an AHR agonist as in the case of PAHs, was shown to regulate iron homeostasis and heme biogenesis in zebrafish embryos (Alexeyenko et al. 2010).

2.4.2. Similarities between the responses to B(*a*)P and DMBA

Four main transcription patterns were identified by the SOTA analysis and a HCL performed with those genes significantly regulated in all the four conditions (Fig. 3). The time effect previously identified by the COA was noticeable again due to the high resemblance between the effects produced by both compounds. This resemblance was sustained when the transcription pattern changed from the first to the second week. Consequently, the HCL clustered the conditions according to the exposure time. These differences between time points indicated that cells underwent similar dynamic changes to face the exposure to both compounds along the time.

2.4.2.1. Resemblances holding a temporal pattern: Time effect

Genes differentially regulated in all the four conditions included genes related to cell cycle control and gametogenesis which were downregulated in the first week of exposure and upregulated in the second week of exposure to both carcinogens (Fig. 5). Altered cell cycle and cyclin expression are known to be common features of cancer (Johnson and Walker 1999). Cancer initiation occurs when a normal cell is transformed into an initiated cell in which the cellular cycle controls fail and as a result, a growth advantage is acquired (Gutierrez and Salsamendi 2001). In fact, "cell cycle" has been shown to be a GO category enriched in rat tumoral cells at initiation stage after treatment with azoxymethane, which is also a potent carcinogen (Cho et al. 2011). A general downregulation of genes related to cell cycle (*ccna2, ccnb1, ccnb2, granulin1, granulin2, myristoilated alanine rich protein kinase C substrate, ribonucleotide reductase M2 polypeptide*) occurred during the first week of exposure to both PAHs. This suggests that progression in the cell cycle may have been slowed likely to cope with the damage produced by the chemical insult, which would be part of the compensatory response (Johnson and Walker 1999). AHR mediated cell cycle

arrest has already been observed in 3T3 fibroblasts after exposure to B(*a*)P (Vaziri and Faller 1997). Moreover, other AHR agonists have previously shown to produce cell cycle arrest and apoptosis (Marlowe and Puga 2005). However, this effect was inverted by the second week of exposure, when cell cycle related transcripts were upregulated (Fig. 5). Time dependent cell cycle regulation has already been identified in in-vitro studies in which the transcriptomic response was analyzed after PAH exposure (Hockley et al. 2006; Lu et al. 2010; Van Delft et al. 2010). The ability of PAHs to both promote and inhibit cell cycle proliferation has been considered by Puga et al. (2002). According to these authors, AHR dependent cell cycle arrest might occur in the presence of DNA damaging AHR exogenous ligands, while the persistent exposure could produce an increase of ROS that would provoke DNA damage to the extent that proliferative barriers would be bypassed and responsiveness to the environmental signal compromised. The transcription pattern followed by cell cycle related genes would therefore suggest a loss of cell cycle control as a result of extensive DNA damage produced by the activated carcinogens. The existence of DNA damage after two weeks of exposure to both PAHs was inferred by the increased transcription of *H2A histone family, member X* and *influenza virus NS1A binding protein b* (Osada et al. 2006; Paull et al. 2000). Thus, results indicate that B(*a*)P and DMBA exposures could lead to cancer initiation through deregulation of cell cycle.

Gametogenesis related genes were also regulated by both carcinogens following the same transcription pattern observed for cell cycle related genes (Fig. 5. We hypothesize that this could be attributed to cross talk between AHR and estrogen receptor (ER) pathways. Even if the exact mechanisms underlying these interactions remain unknown, exposure to AHR ligands can produce both transactivation and transrepression of the ER mediated response (Matthews and Gustafsson 2006; Nicolas 1999; Ohtake et al. 2003). Zebrafish exposure to B(*a*)P has already been reported to alter the transcription of genes involved in reproduction (Hoffmann and Oris 2006). However, we report the regulation of several *zps* (Fig.5), which in zebrafish have been described to be non inducible by estradiol as no estrogen receptor responding element (ERE) has been identified in their sequence (Liu et al. 2006; Mold et al. 2001). In spite of that, regulated ZP proteins have been identified in zebrafish exposed to different estrogens (De Wit et al. 2010; Holth et al. 2008; Kausch et al. 2008). Thus, our results suggest that PAHs might produce endocrine disrupting effects in zebrafish liver through ER-mediated and ERE independent genomic regulation (Hall et al. 2001). The capacity of PAHs to activate the ER is considered a non-genotoxic mechanism of carcinogenesis (Luch 2005; Sandonato 1997). Therefore the crosstalk between AHR-ER pathways might also contribute to B(*a*)P and DMBA carcinogenicity.

2.4.2.2. Time independent resemblances

There were also genes regulated by both carcinogens in all the four conditions that did not follow a time dependent transcription pattern. Those genes included chaperones like the heat shock proteins (*DnaJ, hsp70*), and *protein disulfide isomerase associated 4,* as well as, the cochaperon *FK506 binding protein,* all together indicating the existence of stress (Richter et al. 2010; Touma et al. 2011; Wilkinson and Gilbert 2004). Few genes, including members of the cytochrome P450, usually used as markers of exposure to PAHs, were upregulated in all the exposure groups, further suggesting that xenobiotic metabolism mediated carcinogen activation (adaptive response) occurred (Fig. 5).

2.4.3. Compound specific response

Gene transcription profiling can discriminate carcinogens with differences in their MOA (Van Delft et al. 2004). In fact, chemical-specific signatures may be sufficient to identify faint differences induced even by chemicals belonging to a specific class of toxicants (Afshari et al. 1999). Thus, each PAH may induce a distinguishable gene transcription profile (Song et al. 2012). Despite the high resemblance of the response produced by B(*a*)P and DMBA, the second component of the COA distinguished between PAHs, 16% of the total variability corresponded to the characteristic transcription pattern produced by each of the PAHs (Fig. 2). The preferential induction of genes linked to a specific biological class could be due to the relative potency of PAHs as AHR agonists. In fish, it has been shown that different PAHs possess different potency as AHR agonists and, thus, the magnitude of the AHR mediated response may be different for different PAHs (Barron et al. 2004). Using Fisher exact tests we compared effects of both compounds at the two time points. Significant differences were only observed after the first week of exposure to both compounds. We identified proteolysis, cell cycle, and blood coagulation as enriched biological classes between fish exposed for one week to B(*a*)P and DMBA (Fig. 4). Song and co-workers (2012) exposed human hepatocellular carcinoma cells (HepG2) to nine different PAHs and a group of 430 genes was seen to be able to discriminate among PAHs with different carcinogenic potencies. Interestingly, the major biological process ontology categories in that study included, among others, “proteolysis” and “cell cycle”. Among the transcripts identified by Song et al. (2012) we only identified c*athepsin H* in our list of genes related to proteolysis and *H2A histone family X* in those related to cell cycle, both of them exclusively regulated in B(*a*)P exposed animals.

Proteolytic processes are often required to face stress scenarios (Richter et al. 2010). Actually, increased proteolysis has been reported as a response to protein damage arising from the stress scenario produced by PAH exposure (Carvalho et al.

2011). Our transcriptome analysis indicates that *cathepsins* mediated lysosomal protein degradation may have occurred after 2 weeks of exposure, however, only DMBA exposed animals showed increased *cathepsin* transcription after the first week of exposure while B(*a*)P downregulated *cathepsinb* (Fig. 4A). Proteases are also known to play an important role in cancer (Koblinski et al. 2000). Among them, proteases playing a role in extracellular matrix remodeling are involved in tumor development (Duffy 1992; Gialeli et al. 2011; Lapis and Timar 2002). Even though some *matrix metalloproteinases* were regulated by both compounds, DMBA specifically downregulated some members of the astacin family (*astacin proteases 3* and *4, meprin A alpha2*), which are known to participate in tumor progession (Bond et al. 2005; Murphy and Gavrilovic 1999; Noël et al. 1997). These results suggest that members of the astacyn family could be downregulated in response to damage arising from DMBA exposure. Interestingly, *astacin protease 4* was also downregulated after soft coral (*Scleronephthya gracillimum*) exposure to a mixture of PAHs (Woo et al. 2014). DMBA also regulated transcripts that may be related with its carcinogenicity; *macrophage stimulating 1* and *furin b*, which have been shown to be upregulated in cancer, were upregulated, while other transcripsts that may function as tumor suppressors, like *trypsin* and *CNDP dipeptidase 2* were downregulated (Bassi et al. 2005; Yamashita et al. 2003; Zalcenstein et al. 2006; Zhang et al. 2014). Some proteolysis related transcripts that could contribute to B(*a*)P carcinogenicity were also specifically regulated after first week of exposure. The transcription of *indian hedgehog homolog*, which is a well known promoter of the cell cycle progression, was upregulated while the pro-apoptotic agent *caspase2*, *chemotaxin 2 like,* which is known to be downregulated in hepatocellular carcinomas, and *thimet olipopeptidase,* whose increased expression leads to decreased antigen presentation through the major histocompatibility complex I, were downregulated (Elmore 2007; Harris et al. 2011; Uchida et al. 1999; York et al. 2003).

Cell cycle was the second biological process identified as differentially regulated in B(*a*)P and DMBA exposed fish. Although, as described above, both carcinogens regulated some transcripts related to cell cycle (Fig. 5), the Fisher exact test revealed the existence of B(*a*)P enriched cell cycle related GO classes. Thus, additional down regulated transcripts related to cell cycle, that were not significantly regulated in DMBA exposed fish, were specifically identified in B(*a*)P exposed animals after the first week of exposure (Fig. 4B). Genes involved in G1/S transition (*thymopoietin*), G2/M transition (*denticleless homolog*, *cdca8*), and in both checkpoints (*similar to CDC25*, *ttk protein kinase*) were downregulated, further supporting the before mentioned cell cycle arrest mediated compensatory response (Boutros et al. 2007; Brosh and Rotter 2010; Date et al. 2007; Hogg et al. 1994; Sansam et al. 2006; Yang et al. 1997). Moreover, transcripts involved in cell proliferation (*CPEB binding protein*) and DNA packaging (*H2A histone family X*) were

also downregulated (Bava et al. 2013; Paull et al. 2000). All together, this may indicate a stronger disruption of cell cycle in the case of B(*a*)P exposed animals. Even if no significant differences were observed between B(*a*)P and DMBA exposed animals after two weeks of exposure, the heatmaps constructed to visualize cell cycle related genes reflected the before mentioned time effect in B(*a*)P regulated transcripts. Moreover, *tumor protein 53, sestrin1* and *cyclin dependent kinase inhibitor 1b* were identified as DMBA regulated transcripts, indicating the possible existence of p53 and p27 mediated response to DNA damage (Budanov 2011).

Thirdly, DMBA exposure upregulated transcription of genes related to blood coagulation, while B(*a*)P did not. PAHs are known to alter haematological parameters in fish (Tuvikene 1995). Genetic lesions responsible for cancer onset and progression regulate the transcription of genes involved in blood coagulation (Boccaccio and Medico 2006). These authors proposed that fibrin clots produced by growing cells would provide an adhesive support for cell anchoring that, together with associated coagulation factors, would provide a highly pro-angiogenic environment. DMBA upregulated genes involved in fibrin production (*fibrinogen, thrombin, coagulation factor X*) together with other factors that have been directly related with cancer (*coagulation factor III, plasminogen*) (Kasthuri et al. 2009; Kwaan and McMahon 2009; Palumbo et al. 2000). Thus, our results indicate that the altered gene transcription by the exposure to DMBA may provide an appropriate environment for cancer development.

All together our results suggest the existence of slight differences at transcription levels in the MOA of B(*a*)P and DMBA after the first week of exposure, regulating different genes involved in similar biological processes. The lack of significant differences between compounds after two weeks of exposure might indicate that the response to PAHs tends to homogenize when exposure time increases.

2.4.5. KEGG pathway analysis

According to the KEGG analysis, pathways related to DNA damage, energy metabolism, amino acids and oxidative stress may have been regulated. These pathways are well interconnected and their regulation correlates well with the response to the chemical insult (Fig. 5). Results suggest that both PAHs altered mainly metabolism related pathways, which has already been described as a common response to PAHs exposure (Castorena-Torres et al. 2008; Song et al. 2012). “Purine metabolism” and “pyrimidine metabolism”, involved in the synthesis of the nucleotides required for DNA replication, were the most strongly affected pathways. All the transcripts contributing to the purine and pyrimidine metabolism pathway were downregulated after the first week of exposure, except *cytidine deaminase* and

uridine phosphorylase (Fig. 7). These latter transcripts are involved in the recovery of bases and nucleosides formed during DNA degradation through the salvage pathway, known to be induced in response to DNA damage (Danilova et al. 2014). In zebrafish exposed to PAHs and oxo-PAHs, metabolomic analysis revealed that purine metabolism pathway was the most affected pathway and authors hypothesized that purine bases are salvaged and reutilized during times of oxidative stress (Elie et al. 2015). In B(*a*)P exposed fish, transcription of *ribonucleoside diphosphate*, which is important in DNA repair, was also upregulated (Elledge and Davis 1990). These results are relevant to the cell cycle mediated compensatory response observed after the first week of exposure. In B(*a*)P exposed fish, some of those transcripts were upregulated after the second week of exposure further supporting the strong effect of B(*a*)P on cell cycle (Fig. 5). Some of the transcripts involved in purine metabolism (*AMP deaminase, adenosine triphosphatase, phosphoribosylglycinamide formyltransferase, phosphoribosylaminoimidazolecarboxamide formyltransferase, pyruvate kinase, adenylate kinase*) are also related to the cellular energetic balance as far as GTP and ATP act as energy source in many reactions. Actually, three pathways related to the energetic metabolism were also significantly affected after the exposure to both PAHs. Fructose and mannose metabolism, glycolysis and the pentose phosphate pathway were differentially regulated at the transcriptomic level (Fig. 5). Altered energy metabolism is a hallmark of cancer cells, in which glycolysis is enhanced to cope with the rapid ATP demand of dividing cells (Zhang and Yang 2012). The fructose and mannose metabolism transforms these sugars into metabolites that can be used in glycolysis. This pathway was upregulated in fish exposed for two weeks to B(*a*)P and may also be related to the strong induction of *cyclins* observed in B(*a*)P exposed fish. Accordingly, glycolysis related transcripts followed the above mentioned time dependent effect in B(*a*)P exposed animals (Fig. 7). Interestingly, DMBA exposure produced a different response in glycolysis related transcripts. Transcripts coding for enzymes involved both in glycolysis and in the pentose phosphate pathway (*glucose-6-phosphate isomerase, triose-phosphate isomerase*) were upregulated. The pentose phosphate pathway, which was mainly differentially regulated in DMBA exposed fish, plays a role in the antioxidant cellular defence, providing NADPH necessary for glutathione regeneration, as well as in DNA synthesis providing the riboses necessary for DNA duplication and repair (Riganti et al. 2012). However, downregulated transcription of *pyruvate kinase,* which is the key step in glycolysis before the massive ATP production through oxidative phosphorylation, was observed after 2 weeks of exposure to DMBA (Fig. 7). It has been proposed that in some proliferative and cancer cells, glycolysis may be regulated to fulfil the need to incorporate biomolecules such as amino acids and nucleotides required for cellular duplication (Lunt and Vander Heiden 2011; Vander Heiden et al. 2009). In this scenario, termed the “Warburg effect”, downregulated transcription of *pyruvate kinase* (Fig. 7) may contribute to redirect glucose metabolites into the pentose

phosphate pathway, as well as into amino acid biosynthesis (Kang et al. 2015; Vander Heiden et al. 2009). Accordingly, the serine, glycine, threonine and the cysteine, methionine metabolisms were mainly regulated in DMBA exposed fish. Moreover, glycine and serine have been suggested to be involved in ATP production in cells under the influence of the Warburg effect; this alternative ATP source would produce lactate as a side product (Vaquez et al. 2011). In this context, increased expression of *lactate dehydrogenase* would be required to excrete excess of carbon (Vander Heiden et al. 2009). This transcript was upregulated at both time points by DMBA and in the second week of exposure to B(*a*)P (Fig. 5). However, most of the transcripts involved in these pathways were downregulated. These results indicate that DMBA exposure may induce the Warburg effect primarily to produce nucleotides and to cope with the energetic requirements necessary for DNA repair, rather than to increase amino acid synthesis.

"Glutathione metabolism" was also a strongly regulated pathway at the transcription level. Glutathione, which can be synthesised from cysteine and glycine, plays an important role in different cellular processes including the protection against oxidative stress (Lu 2013; Wu et al. 2003). Both DMBA and B(*a*)P exposures enriched glutathione metabolism related transcripts (Fig. 5). These pathways have also been identified in zebrafish exposed to different PAHs and oxo-PAHs demonstrating that the transcription level regulation of these pathways is a common initial step in the sequence of events regulated by exposure to different PAHs (Elie et al. 2015). In fish exposed for 2 weeks to B(*a*)P, glutathione related transcripts were upregulated (Fig. 7). This may be related to the phase II metabolism of xenobiotics or to the response given to the presence of ROS arising from the increased oxidative potential produced during the phase I biotransformation metabolism (Lu 2013). On the other hand, DMBA exposure resulted in downregulated transcription of glutathione metabolism related genes (Fig. 7). Reduced glutathione presence is essential for apoptosis and may lead to necrosis due the uncontrolled oxidative damage (Lu 2013).

Overall both PAHs regulated transcripts involved in similar pathways, however, the response to B(*a*)P may be mainly related to the before mentioned effect on the cell cycle while results obtained after DMBA exposure may reflect extensive DNA damage.

2.5. Conclusions

Zebrafish exposure to B(*a*)P and DMBA produced highly similar gene transcription profiles in the liver. Due to the scarce differences between the effects produced by

both chemicals, exposure time was the major factor determining transcriptomic profiles. The differences observed in the transcription profiles are, therefore, mainly linked to the toxicity stage in which the analysis has been performed. Our results indicated that PAHs may have been activated to carcinogenic compounds through the xenobiotic metabolism producing DNA damage that affected the transcription of genes related to DNA metabolism and cell cycle, which were strongly downregulated after the first week of exposure and upregulated after the second week. Estrogenic effects may have been also produced by both PAHs following the same transcription pattern suggesting the existence of AHR-ER crosstalk which is considered a non-genotoxic carcinogenic mechanism.

Compound-specific responses were only observed after the first week of exposure suggesting that extensive damage produced by longer exposures leads to higher similarity in the transcriptomic profiles. Specific pre-translational responses inferred a stronger effect of B(*a*)P on the cell cycle, while DMBA exposure affected to a higher extent proteolysis and blood coagulation indicating that both compounds could ultimately provide a suitable environment for cancer development through slightly different MOA.

Finally, the KEGG analysis identified pathways related to DNA damage and proliferative requirements further supporting the strong effect that B(*a*)P may have on the cell cycle and revealing the need of DMBA exposed animals to face DNA repair.

Thus, both PAHs showed potential carcinogenicity according to their transcriptional effects; our results indicate that after the second week of exposure the toxicity exerted by B(*a*)P and DMBA could have overtaken the proliferative barriers as a result of extensive DNA damage.

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CHAPTER III

Long-term transcriptional and histopathological effects in zebrafish exposed to B(*a*)P and/or DMBA during embryogenesis

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Abbreviations

7,12-dimethylbenz(*a*)anthracene, DMBA

Aryl hydrocarbon receptor, AHR

Benzo(*a*)pyrene, B(*a*)P

Dimethyl sulfoxide, DMSO

Hours post fertilization, hpf

Hours post exposure, hpe

Micronucleous, MN

Mode of action, MOA

Octanol-water partition coefficient, K_{ow}

Polycyclic aromatic hydrocarbon, PAH

Reactive oxygen species, ROS

Weeks post exposure, wpe

Gene abbreviations

cyclin g1, ccng1

cytochrome P450 family 1 subfamily a, cyp1a

jun B proto-oncogene, junb

tumor protein 53, p53

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental carcinogens that require metabolic activation to reveal their carcinogenic activity. Short exposures to PAHs at early life stages can result in tumor formation in adult organisms. Thus, this work aimed to analyze the long-term effects on the transcription level of key genes related to cancer development after an early acute exposure to benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA) during zebrafish (*Danio rerio*) embryogenesis. In addition, genotoxicity and liver histopathology were assessed at 12 weeks post exposure (wpe). Embryos at 24 hours post fertilization were exposed for 24 h to 0.3, 0.6 or 1 mg/L of B(*a*)P, DMBA and to both PAHs consecutively. Embryos exposed to both compounds sequentially and to DMBA alone did not survive by the 2nd and 6th wpe, respectively. At 24 hours post exposure (hpe), all treatments significantly up-regulated the transcription of the xenobiotic metabolism related *cyp1a* in a dose dependent manner. The transcription of *cyp1a* remained upregulated at 3 and 6 wpe in individuals exposed to the DMBA. Moreover, at 3 wpe, the transcription patterns observed in DMBA treated individuals for *p53*, *ccng1* and *junb* followed the same trend observed for *cyp1a* which could be indicative of the genotoxic capacity of this compound. Thus, DMBA exerted a stronger effect than B(*a*)P in agreement with its solubility and aryl hydrocarbon receptor agonistic strength. By the end of the study (12 wpe), none of the analyzed genes was regulated and DNA damage measured as micronuclei presence in erythrocytes was not detected in any of the exposure groups. Some liver histopathologies were detected in all groups but, overall, higher prevalence was recorded after PAH treatment. In conclusion, short-term exposure of zebrafish to carcinogenic PAHs at early life stages may produce long lasting effects on the transcription profiles of target genes and cause hepatic alterations in adult individuals.

Key words: Polycyclic aromatic hydrocarbons, zebrafish, early exposure, cancer-related genes.

Laburpena

Ingurunean aurkitzen diren hidrokarburo aromatiko poliziklikoak (HAPak) kartzinogenoak dira, baina jarduera kartzinogenikoa erakusteko metabolikoki aktibatuak izan behar dira. Garapen fase goiztiarretan HAPen pean egondako organismoek tumoreak garatu ditzakete helduaroan. Hau dela eta, lan honetan kantzerrarekin erlazionaturiko geneen transkripzioan gertatzen diren epe luzeko efektuak ikertu nahi izan genituen bentzo(*a*)pireno (B(*a*)P) edo/eta 7,12-dimetilbentzo(*a*)antrazeno (DMBA) pean egondako zebra arrainetan (*Danio rerio*). Honetaz gain, genotoxizitatea eta gibel-histopatologia aztertu ziren 12 asteren buruan. Ernaldu osteko 24 ordutako enbrioiak 24 orduz egon ziren 0.3, 0.6 edo 1 mg/L B(*a*)P, DMBA edo, modu sekuentzialean, bi konposatuen pean. Azken tratamendu honen pean hala nola 1 mg/L DMBA pean egondako enbrioiak hilik suertatu ziren 2 eta 6 asteren buruan hurrenez hurren. 24 orduen ostean, tratamendu guztiek xenobiotikoen metabolismoarekin erlazionatutako *cyp1a*-ren transkripzioa emendatu zuten kontzentrazio menpeko moduan. DMBA pean egondako enbrioietan c*yp1a*-ren transkripzioa emendatuta mantendu zen 3 eta 6 aste ondoren. Gainera, DMBA pean 24 orduz egondako animalietan, 3 asteren buruan *p53*, *ccng1* eta *junb-k* agertutako transkripzio ereduek konposatu honen ahalmen genotoxikoa iradoki zuten, *cyp1a*-k jarraitutako joera berdina erakutsi baitzuten. Beraz, DMBAk eragin zituen efekturik nabarmenenak bere disolbagarritasunarekin eta hidrokarburoen arilo taldearen hartzailearekiko duen afinitatearekin bat etorriz. Esperimentuaren amaieran (12 aste), esposizio taldeetako batean ere ez zen erregulatutako generik antzeman. Era berean, DNA kalterik ez zen ikusi eritrozitoen MN-en prebalentzia aztertu zenean. Talde guztietan gibeleko histopatologiaren bat antzeman bazen ere, prebalentziarik altuenak HAP-en pean izandako organismoetan ikusi ziren. Laburbilduz, zebra arrainaren bizitzako hasierako faseetan gertatutako epe-laburreko esposizioek epe-luzean dirauten efektuak sortaraz ditzakete arrain helduen gene gakoen transkripzio profiletan eta gibelean kalteak eraginez.

Gako hitzak: Hidrokarburo aromatiko poliziklikoak, zebra arraina, esposizio goiztiarra, kantzer geneak.

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that enter the environment through natural sources such as oil seeps and forest fires and through a variety of anthropogenic activities (Billiard et al. 2006, 2008). Living organisms exposed to PAHs can develop a wide range of toxic effects due to the non-specific interaction of PAHs with hydrophobic sites of the cell (narcosis) or due to the action of the highly reactive intermediates arising from their metabolism (Cousin and Cachot 2014; Incardona et al. 2006; Jayasundara et al. 2014). Among PAHs, there are varying structures based on the number of rings (2-6), bonding patterns of those rings and heteroatom substitutions (Timme-Laragy et al. 2007). This structural heterogeneity is reflected in key characteristics determining PAHs toxicity, such as hydrophobicity and the relative potency as agonists of the aryl hydrocarbon receptor (AHR) (Barron et al. 2004a; Incardona et al. 2006). Benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA) are two model carcinogenic PAHs owning 5 and 4 aromatic rings, respectively (Baird and Mahadevan 2004; Shimada 2006). It has been reported that PAHs with bay regions, as in the case of B(*a*)P, are likely to be potent carcinogens, while those with fjord regions or sterically hindered bay region, such as DMBA, are even more potent (Baird and Mahadevan 2004).

Cancer development is a multistage process. Cellular initiation, promotion and progression are therefore necessary steps for the appearance of a cancerous focus with acquired growth advantage and genetic instability (Poirier 2004). In order to be an effective carcinogen, a compound must therefore act as initiator and as promoter (Potter 1980). During the phase I metabolism, PAHs are bioactivated to reactive metabolites by enzymes of the cytochrome P450 system whose expression is dependent on AHR. Many activated PAHs are complete carcinogens, they can produce DNA damage and they may also act as promoters (Baird et al. 2005). Together with the appearance of highly reactive species, increased oxidase activity leads to overproduction of reactive oxygen species (ROS) that cause oxidative damage to different cell components, including DNA (Miller and Ramos, 2001). This ROS overproduction can also contribute to PAHs carcinogenicity as increased presence of oxygen species may lead to the formation of newly initiated preneoplastic cells and/or enhance the selective clonal expansion of latent initiated preneoplastic cells (Klaunig et al. 1998).

Water bodies are often the ultimate destination of pollutants arising from anthropogenic activities. Consequently, fish have been extensively used in toxicological studies. In this context, zebrafish (*Danio rerio*) embryos and larvae are recognized as a reference model to study the effects of xenobiotics, including PAHs (El-Amrani et al. 2013; Hill et al. 2005; López-Serrano et al. 2011; Nagel 2002; Weigt

et al. 2011). PAH exposure produces a variety of effects in fish embryos ranging from altered gene transcription to developmental defects and increased mortality (Barron et al. 2004b; Billiard et al. 2008; Carls et al. 2008; Wassenberg and Di Giulio 2004; Zhang et al. 2012). Regulation of genes related to the phase I metabolism is a common observation in different fish species exposed to PAHs (Bugiak and Weber 2009; Hook et al. 2006; Williams et al. 2009). Consequently the expression of these genes is often employed as marker of PAH exposure. Waterborne exposure of newly fertilized zebrafish embryos up to 4 days post fertilization (dpf) to 5 µg/L B(*a*)P significantly induced *cyp1a* transcription after 5 and 10 dpf (Bugiak and Weber, 2010). This response has also been observed in adult zebrafish after 7 days of exposure to 100 µg/L B(*a*)P (Thompson et al. 2010). Fish exposure to PAHs has also been related to altered transcription of genes related to DNA damage and cell cycle. Together with *cyp1a,* waterborne exposure of medaka (*Oryzias latipes*) embryos to pyrene and methylpyrene significantly induced the transcription of the cell cycle regulator *wingless integration site 1,* and the oxidative DNA damage repair related *8-oxoguanine glycosylase 1* (Barjhoux et al. 2014). Moreover, in **Chapter II** we reported that the transcriptomic analysis revealed cell cycle arrest and regulation of pathways related to DNA damage in adult zebrafish exposed to B(*a*)P and DMBA. *p53*, which is involved in those pathways, codifies for the tumor supressor protein 53 whose misregulation is described in many cancer types. This transcription factor is able to modulate cell cycle in response to DNA damage through a number of pathways, including the regulation of *ccng1* transcription which may result in cell cycle arrest at G2 to M transition (Kimura et al. 2001). In early developmental stages of zebrafish, the increased presence of p53 in response to stressors such as overexpression of oncogenes, DNA damage, chromatin dysregulation, hypoxia, X-ray and UV irradiation, defects in ribosome biogenesis, etc. leads to embryo death (Danilova et al. 2008). Similarly *junb,* is known to be involved in cancerous processes. This early response gene, necessary for tissue regeneration, is induced in zebrafish embryos in response to fin amputation and its transcription has been proposed as a key step in zebrafish tissular regeneration (Ishida et al. 2001; Shaulian 2010).

As mentioned above, the activation of PAHs leads to the increased production of reactive metabolites and ROS that can produce DNA damage (Baumann and Harshbarger 1995). In adult zebrafish, increased micronuclei (MN) frequency has been recorded after DMBA exposure (1 mg/L) for 72 h (**Chapter I**). Presence of MN in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle et al. 1991). As reviewed by Brown and Steinert (2003), in addition to a linkage with cancer, increases in DNA damage precede or correspond with reduced growth, abnormal development, and reduced survival of adults, embryos, and larvae. In humans, increased MN frequency is known to precede other effects at tissular level including cancer development (Bonassi et al. 2011).

Exposure to PAHs can also result in different histopathological alterations; therefore, histopathological analysis is a useful tool that contributes to the characterization of tissue level effects produced by the exposure to toxic compounds (Wester et al. 2002). Different histological lesions including necrosis, inflammation and altered glycogen storage have been described in liver after fish exposure to PAHs (Ortiz-Delgado et al. 2007; Santos et al. 2011; Tuvikene 1995). Moreover, in zebrafish, punctual exposures to B(*a*)P and DMBA at early developmental stages produce different carcinogenic lesions in adult organisms (Lam et al. 2006; Spitsbergen et al. 2000; Wang et al. 2010).

However, despite the load of information on the effects of PAHs, there is still a lack of information regarding the time-through effect of acute embryo exposure to PAHs. Thus, in this study, we aimed to analyze the long-term transcriptional and histopathological effects produced by an early acute exposure to two model PAHs (B(*a*)P and DMBA) during zebrafish embryogenesis. Exposure concentrations (0.3-1 mg/L) were selected based on the studies by Spitsbergen et al. (2000), who demonstrated neoplasia development in 1 year-old zebrafish treated with up to 1 mg/L of DMBA during embryogenesis. With this objective we combined the analysis transcription levels in liver of key genes related to xenobiotic metabolism (*cyp1a*) and cell cycle control (*p53, ccng1, junb*) with a biomarker of genotoxicity (MN frequency in erythrocytes) and the histopathological analysis of the liver.

3.2. Materials and Methods

3.2.1. Fish maintenance and breeding

Adult zebrafish (*Danio rerio;* AB Tübingen) were maintained at 27 ± 1 ºC with a 14-hour light / 10-hour dark cycle in 100 L tanks. Tank water was prepared by conditioning osmotic water with marine basic salt (Sera Gmbh, Heinsberg, Germany) and KH/pH plus (Sera) up to 600 µS and pH 7.4 before mechanical filtering (1 µm) and sterilization by ultraviolet light. Water aeration and filtration was achieved by an airlift pump in each tank. Residual metabolites were controlled using ammonium, nitrite and nitrates kits (Sera), maintaining water at 0-0.5 mg/L, 0-0.5 mg/L and 5-10 mg/L, respectively. Fish were fed twice per day with *Artemia* nauplii (INVE Aquaculture, Dendermonde, Belgium) and commercial dry food, Microgran (Sera).

The day prior to the beginning of the experiment, one female and two male adult zebrafish were placed separately in the same breeding tramps which had previously been located in a 2 L tank containing conditioned water. Fish were left

overnight and just before turning on the light they were allowed to gather. The resulting embryos were collected and maintained in conditioned water. At 24 hpf viable eggs were selected under a stereoscopic microscope (Nikon smz800, Tokyo, Japan) and used for the exposures.

3.2.2. Exposures and sample collection

The experimental procedure described below was approved by the committee for animals' welfare of the University of the Basque Country. Embryos were waterborne exposed in baker glasses to 0.3, 0.6 and 1 mg/L B(*a*)P or DMBA dissolved in dimethyl sulfoxide (DMSO) whose final concentration in the exposure media was 0.01%. A control group exposed to the same DMSO concentration was run in parallel. Two additional groups were exposed to 0.3 mg/L DMBA for 24 h, maintained in clean water for 24 h, and newly exposed to 0.6 or 1 mg/L of B(*a*)P for another 24 h. Then, embryos were transferred to tanks containing clean water and samples were collected at 24 hours post exposure (hpe), and at 3, 6 and 12 weeks post exposure (wpe). Samples for histology and micronucleus (MN) test were collected at the end of the experiment (12 wpe).

3.2.3. Gene transcription analysis

5 samples per exposure group and sampling time were collected, immersed in RNA Later® (Sigma-Aldrich, St. Louis, USA), frozen in liquid nitrogen and stored at -80 ºC until processing. Whole individual larvae were collected at 24 hpe, larvae without the head and tail were sampled at 3 wpe and visceral mass without the swim bladder was taken 6 and 12 wpe. Individual samples were transferred to tubes containing 1 mL TRIzol® (Invitrogen Life-Technologies, Merelbeke, Belgium) and homogenized using a rybolizer (Hybaid, Middlesex, UK). Total RNA extraction was performed following the TRIzol® extraction method and subsequent phenol/chloroform extraction. RNA purity and integrity were evaluated by measuring 260/230 nm and 260/280 nm absorbance ratios in a spectrophotometer (Eppendorf, Hamburg, Germany). cDNA was constructed and the whole amount of cDNA resulting from each sample was stored at -80ºC and used for real time qPCR analysis. Due to the small size of the larvae RNA concentration was not measured and total cDNA was used for comparisons in the case of larvae collected at 24 hpe and 3 wpe.

Standard Taqman probes for *cyp1a* (ID: Dr03112441_ml)*, cyclin g1* (*ccng1*-ID: Dr03132065_ml)*, tumor protein 53* (*p53-ID*:Dr03112082_gl)*, jun B proto-oncogen* (*junb*-ID: Dr03204057_sl) and *ribosomal protein S18* (*rps18*-ID: Dr03144509_ml) were purchased to Applied Biosystems (Carlsbad, California). Triplicate PCR reactions were carried out as indicated by manufacturer's protocol using an ABIS 7300 cycler (Applied Biosystems) under the following conditions: 2' at 50 °C, 10' at 95 °C followed

by 40 cycles of 15'' at 95 °C and 1' at 60 °C. Data of the four target genes were normalized against rps18 (Vanhauwaert et al. 2014), whose transcription level did not vary among experimental groups (CV<10%), by means of the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

3.2.4. Micronuclei assay

Blood was obtained from 5 animals per experimental groups by tail cutting and direct blood smear on clean slides. Blood smears were left to air-dry and cells were 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). Small, non-refractive, circular or ovoid chromatin bodies showing the same staining pattern as the main nucleus and with an overall area not bigger than a 1/3 of the main nucleus were considered as MN (Baez and Prieto 2005). 2000 cells were examined per individual and MN frequency was expressed in ‰.

3.2.5. Sample processing for histological analysis

Ten animals were ventrally opened and tail was removed. Samples were fixed in 10% buffered formalin (pH 7). Then, animals were longitudinally sliced in two symmetrical parts and dehydrated in a series of increasing concentrations of ethanol (20 min in each bath). After dehydration, samples were embedded in paraffin. The embedded material was cut into 5 µm thick sections using a Leica 2125RT microtome (Leica Instruments, Nussloch, Germany). Finally samples were stained with hematoxylin-eosin.

3.2.6. Statistical analysis

Statistical analyses were performed using the SPSS for Windows (SPSS Chicago, IL) software. Bootstrap resampling techniques (Efron and Tibshirani 1993) were used to assess the effect of the treatments on gene transcription. For each experiment, N=2000 repetitions were selected by bootstrap resampling method. After that, Bonferroni's correction was used for multiple comparisons between pairs of groups. For histopathological data Chi-square test was used. In all the cases significance was established at $p< 0.05$.

3.3. Results

None of the embryos sequentially exposed to DMBA and B(*a*)P regarded alive by the second wpe. Similarly, embryos exposed to the highest concentration of DMBA (1 mg/L) died by the 6 wpe. Embryos in this group showed reduced mobility as fast as 72 hpe and at 96 hpe first dead larvae were registered.

3.3.1. Gene transcription level

At 24 hpe, *cyp1a* transcription was significantly upregulated in all the exposure groups (Fig. 1A). Although no significant differences were observed among the different concentrations of each PAH, *cyp1a* induction showed a dose dependent trend to increase, being more marked in DMBA treated larvae than in B(*a*)P treated ones. It must be noted that the highest induction in B(*a*)P exposed larvae was lower

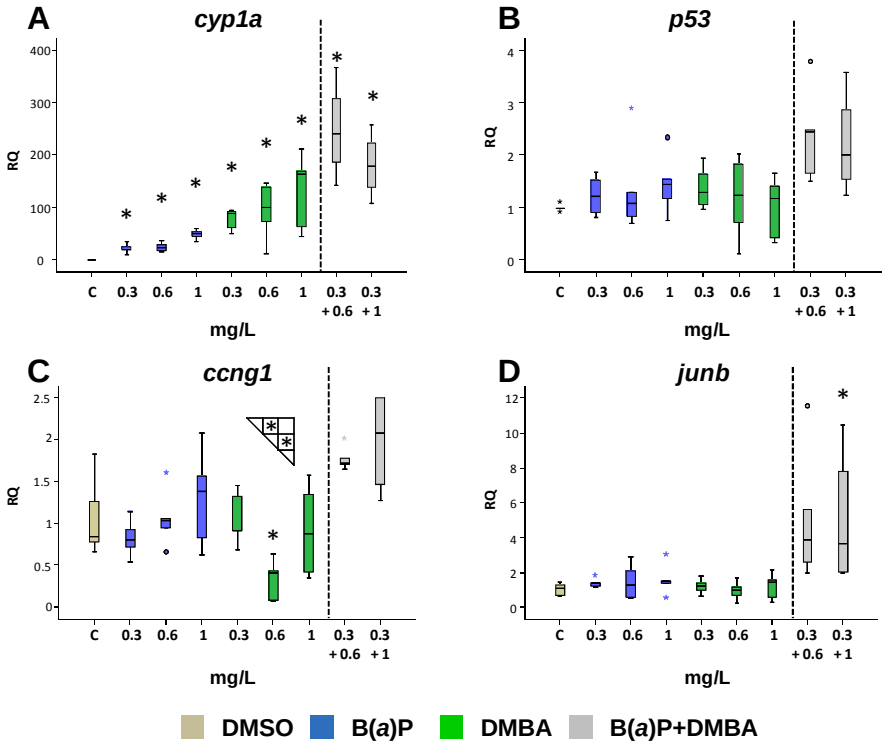

Fig. 1. Relative gene transcription (RQ) levels for *cyp1a* (A), *p53* (B), *ccng1* (C) and *junb* (D) in whole zebrafish embryos at 24 hpe. Box-plot boxes represent the percentage data value in between the 25th and the 75th percentile, median indicated by a line in the middle of the box. Whiskers are the data values in up to the 5th percentile and 95th percentile. Outliers are represented by small asterisks and circles. Significant differences towards the control are represented by a large asterisk. When present in the triangular matrix asterisk indicate differences between animals exposed to different concentrations of the same PAH.

than the lowest observed induction in DMBA exposed embryos. Individuals sequentially exposed to DMBA and B(*a*)P showed the strongest induction of *cyp1a.*

For *p53* and *junb*, there were not significant differences between exposed and control larvae at any of the tested concentrations. The transcription of *ccng1* was significantly decreased in embryos exposed to 0.6 mg/L DMBA. The highest induction of *p53, ccng1* and *junb* (Fig. 1B; 1C; 1D) were also observed in fish sequentially exposed to both PAHs, but the increase in the transcription was only significant for *junb* in animals sequentially exposed to the highest concentration of DMBA.

At 3 wpe, only the exposure to DMBA produced significant sustained upregulation of *cyp1a* (Fig. 2A) compared to control fish except in the case of animals exposed to 0.6 mg/L, whose *cyp1a* transcription level was significantly lower than in organisms treated with the other DMBA concentrations. As observed at 24 hpe, exposure to DMBA produced stronger upregulation of *cyp1a* than exposure to B(*a*)P.

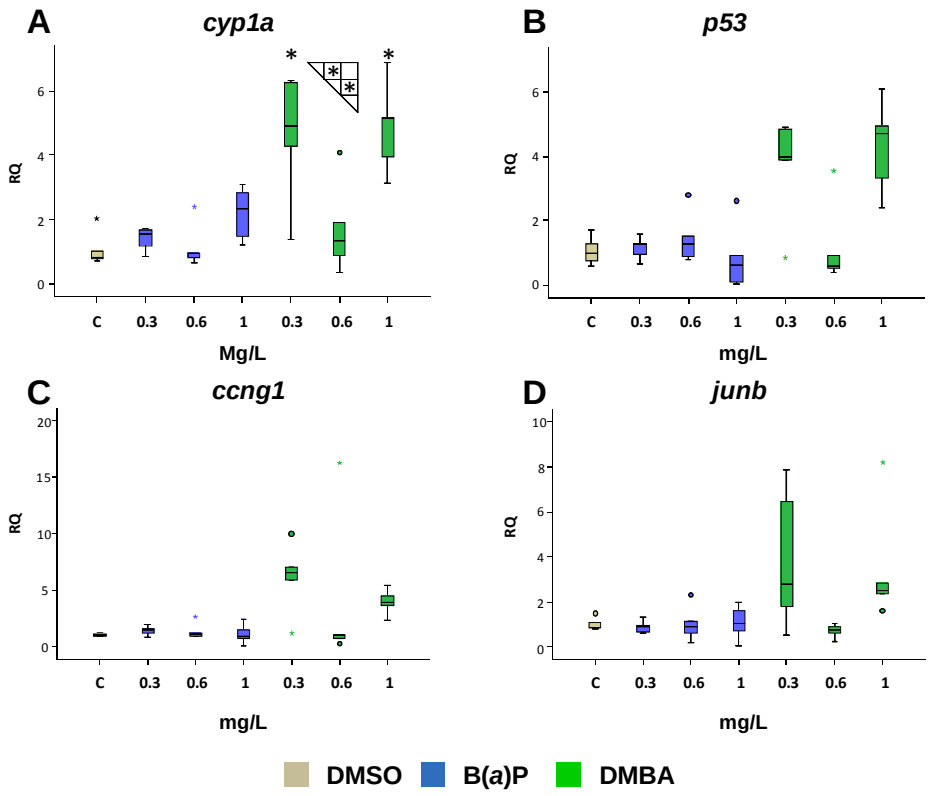

Fig. 2. Relative gene transcription (RQ) levels for *cyp1a* (A), *p53* (B), *ccng1* (C) and *junb* (D) in zebrafish embryos at 3 wpe. Representation and statistics as in Fig. 1.

Nor individuals exposed to B(*a*)P neither those treated with DMBA showed altered *p53, ccng1* or *junb* transcription levels. However, in the case of animals exposed to DMBA, the transcription of *p53, ccng1* and *junb* followed a transcription pattern similar to that observed for *cyp1a* (Fig. 2B; 2C; 2D). Unexpected low values were recorded for all the tested genes in embryos exposed to 0.6 mg/L of DMBA (Fig. 2).

At 6 wpe, surviving animals treated with DMBA (0.3 and 0.6 mg/L) showed upregulated *cyp1a* transcription (Fig. 3A). Only embryos exposed to 0.3 mg/L of B(*a*)P showed upregulated *p53* transcription level (Fig. 3B) and significant upregulation of *junb* was observed in animals exposed 0.6 mg/L of B(*a*)P (Fig. 3D). Exposure to DMBA did not alter *p53, cycng1,* or *junb* transcription levels at 6 wpe.

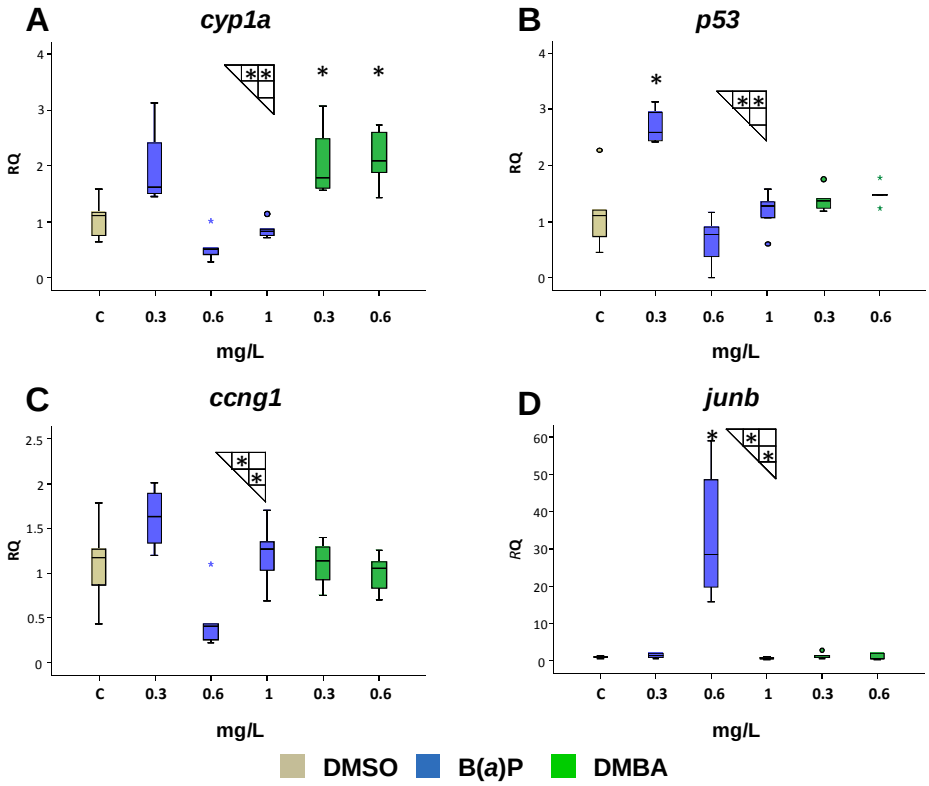

Fig. 3. Relative gene transcription (RQ) for *cyp1a* (A), *p53* (B), *ccng1* (C) and *junb* (D) in 6 wpe larvae with respect to carrier control group (DMSO) assessed by qPCR. Representation and statistics as in Fig. 1.

Finally, at 12 wpe, no significant transcription alterations were observed for any of the tested genes in any of the exposure groups (Fig. 4).

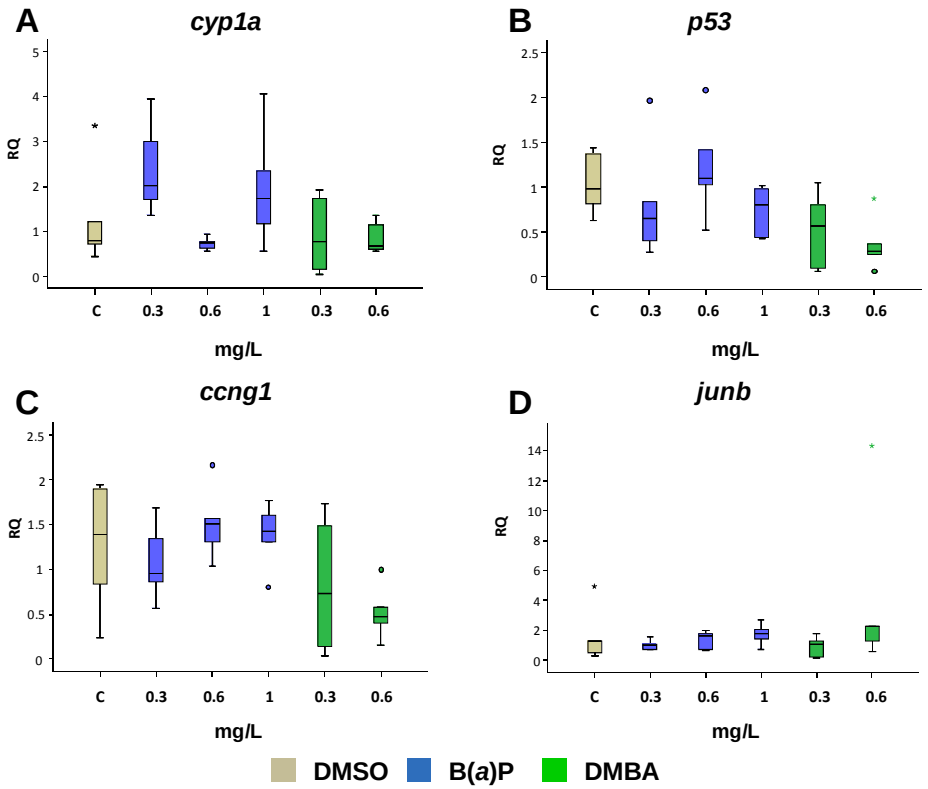

Fig. 4. Relative gene transcription (RQ) levels for *cyp1a* (A), *p53* (B), *ccng1* (C) and *junb* (D) in 12 wpe zebrafish with respect to carrier control group (DMSO) assessed by qPCR. Representation as in Fig. 1.

3.3.2. Micronuclei test

At 12 wpe, MNs were not found in the DMSO control fish. Exposure to both PAHs resulted in a slight increase of the MN frequency, but significant differences were not detected between exposed animals and control fish. Exposure to 0.3, 0.6 and 1 mg/L of B(*a*)P resulted in a MN frequency of 0.1‰, 0.7‰ and 0.5‰, respectively. Exposure to 0.3 and 0.6 mg/L of DMBA resulted in a MN frequency of 0.2‰ and 0.1‰, respectively.

3.3.3. Histopathology

Histopathological alterations, and specially megalocytosis, were observed in all experimental groups including the control group (Table 1; Fig. 5A). In fish exposed to B(*a*)P, a concentration dependent increasing trend in the prevalence of megalocytosis was observed (Fig. 5B). Animals exposed to 0.3 mg/L of DMBA showed higher prevalence than those exposed to 0.6 mg/L. Prevalence of hepatocyte vacuolization (Fig. 5C) was significantly higher in fish exposed to 0.6 mg/L of B(*a*)P. Necrotic foci

(Fig. 5D) were also observed in most of the treatment groups, being fish exposed to 0.3 mg/L of DMBA which showed the highest prevalence.

Table 1: Prevalence of histopathological alterations (in %) in the liver of fish sampled at 12 wpe. Asterisk indicates statistical significant differences respect to the DMSO control group according to Chi-square test ($p<0.05$).

Treatment	PAH (mg/L)	n	Megalocytosis	Vacuolization	Necrosis
DMSO	0	10	50	10	10
B(*a*)P	0.3	11	40	20	10
B(*a*)P	0.6	11	72.72	54.54*	9.09
B(*a*)P	1	10	80	40	0
DMBA	0.3	11	72.72	27.27	18.18
DMBA	0.6	10	40	10	10

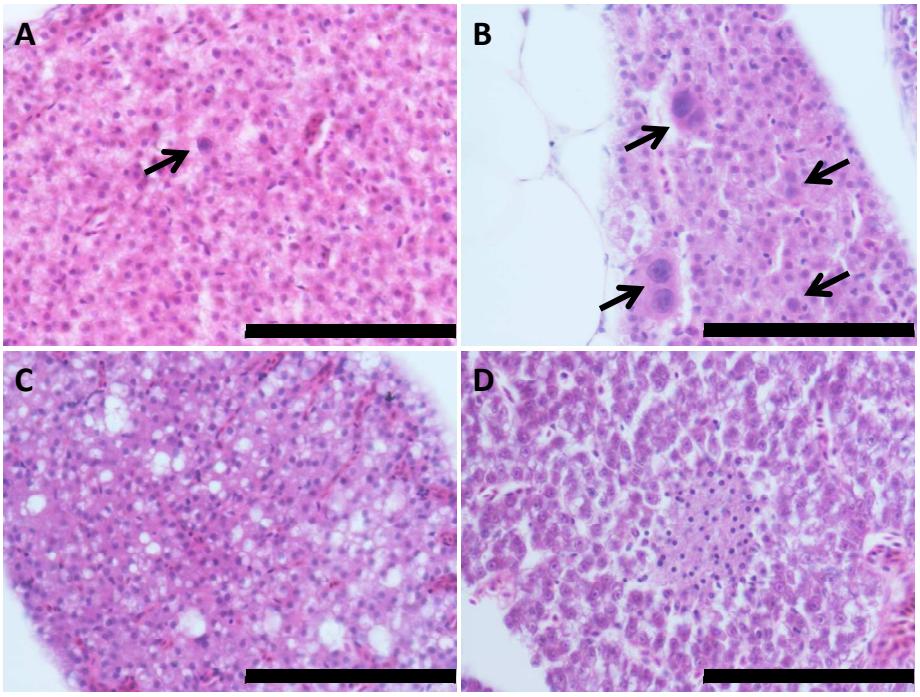

Fig. 5. Micrographs of zebrafish liver sampled at 12 wpe. A: DMSO control fish showing slight megalocytosis (arrow). B: Fish exposed to 1 mg/L of B(*a*)P showing megalocytosis (arrows). C: Fish exposed to 1 mg/L of B(*a*)P showing hepatocyte vacuolization. D: Fish exposed to 0.6 mg/L of DMBA showing a necrotic focus surrounded by disorganized hepatic tissue. Scale bars: 100 µm.

3.4. Discussion

It has been well established that PAH exposure produces several toxic effects to zebrafish embryos, including malformations, increased oxidative stress and mortality (El-Amrani et al. 2013; Goodale et al. 2013; Incardona et al. 2004; Jones et al. 2010). As reviewed by Incadrona and co-workers (2004), for compounds with log octanol-water partition coefficients (log K_{ow}) higher than 5, toxicity declines with increasing lipophilicity in association with the lower water solubility and bioavailability. The relative toxicity of PAHs to zebrafish and the Japanese medaka has previously been described to be dependent on the log K_{ow} of each compound (Perrichon et al. 2014). log K_{ow} for B(*a*)P and DMBA are above 5 (6.13 and 5.8, respectively) and, therefore, according to their water solubility, DMBA should be more bioavailable than B(*a*)P (Incardona et al. 2004). Our results showed that exposure to the highest DMBA concentration resulted lethal to 100% of exposed zebrafish embryos by 6th wpe, while embryos exposed to 1 mg/L B(*a*)P survived the whole experimental period despite the natural mortality registered in all groups. Accordingly, up to 88% of mortality was registered one year after the end of the exposures in zebrafish exposed at 60 hpf for 24 h to 1 mg/L DMBA (Spitsbergen et al. 2000). Thus, our results are in accordance with existing literature regarding PAHs toxicity in relation with their solubility and consequent bioavailability. Increased PAH bioavailability could explain the results obtained in organisms exposed sequentially to both PAHs. This treatment resulted lethal to all embryos by the end of the second week of exposure. In those cases, the longer exposure time (24 h of exposure to 0.3 mg/L of DMBA plus 24 h of exposure to either 0.3 or 0.6 mg/L of B(*a*)P) and consequent increased bioavailability may have played a critical role in the overall effect. Increased presence of PAHs in the organism should results in a stronger narcotic effect as well as a bigger interaction with the AHR receptor. The latter resulting in the transcription of the AHR downstream genes and PAH activation to reactive damaging species (Incardona et al. 2006). Activated PAHs can produce cellular damage to a point in which DNA damage leads to alterations in the cell cycle that may produce a wide variety of cancers (Marlowe and Puga 2005; Spitsbergen et al. 2000). In order to contribute to the understanding of this process, it is critical to investigate whether early life PAH exposure can persistently affect gene transcription that might lead to disease in later life (Fang et al. 2013). Thus, in this work we analyzed the transcription level of *cyp1a1, p53, ccng1* and *junb*, together with the genotoxic and histopathological effects after an acute exposure to B(*a*)P and DMBA during embryogenesis.

3.4.1. Gene transcription

At 24 hpe, a strong induction of the xenobiotic metabolism related *cyp1a* was observed (Fig. 1A). In zebrafish embryos, transcription of *cyp1a* has been detected as

soon as 8 hpf (Otte et al. 2010). c*yp1a* mRNA concentration is dependent on the developmental stage and inducible in a concentration dependent manner under exposure to AHR agonists (Jones et al. 2010; Jönsson et al. 2007). In fact, it has been demonstrated that zebrafish embryos are able to activate procarcinogenic substances, including B(*a*)P, through the phase I enzyme activity as early as 3 days post fertilization (Mattingly and Toscano 2001; Weigt et al. 2011). These data are in agreement with the results obtained in this work that show that both, B(*a*)P and DMBA, significantly induced the transcription of *cyp1a* in zebrafish embryos at 24 hpe in a concentration dependent manner (Fig. 1A). It is generally accepted that PAHs must be activated to produce tumor initiation (Miller 1970). Therefore, according to our transcriptomic results both compounds may have the capacity to produce tumor initiation at any of the concentrations tested as fast as 24 hpe. Exposure to DMBA resulted in a stronger induction of *cyp1a* transcription than exposure to B(*a*)P, which relates well with the relative potency of each PAH as AHR agonists described by Barron et al. (2004a) and the previously mentioned lower K_{ow} coefficient of DMBA compared to that of B(*a*)P. These results suggest that DMBA owns stronger damaging capacity to zebrafish embryos and explain the high mortality observed in embryos exposed to 1 mg/L DMBA. Accordingly, DMBA has been described as a potent carcinogen, while B(*a*)P is considered a moderately carcinogenic substance (Baird et al. 2005). At 24 hpe, the strongest induction of *cyp1a* was detected in embryos sequentially exposed to 0.3 mg/L DMBA and 0.6 or 1 mg/L B(*a*)P. Fish embryos and larvae are very sensitive to PAHs mixtures (Bui et al. 2012). However, as mentioned above, *cyp1a* mRNA concentration depends on the developmental stage and therefore the longer time of exposure and the consequent larger larvae size of the individuals analyzed in these exposure groups are to be taken into account (Jones et al. 2010). Anyhow, additive effects have previously been described in the transcription of *cyp1a* after exposure to two PAHs. Co-exposure to B(*a*)P and α-naphtoflavone caused additive agonistic effects on transcription of *cyp1a* in 10 dpf larvae exposed for 4 days after fertilization (Bugiak and Weber 2010). Similarly, exposure of 24 hpf embryos for 24 h to 1µg/L β-naphtoflavone and 100 µg/L α-naphtoflavone produced synergistic induction of *cypa1* (Timme-Laragy et al. 2007).

Fish sequentially exposed to DMBA and B(*a*)P also showed the highest transcription level of *p53* and *junb* at 24 hpe (Fig. 1C; 1D). As reviewed by Storer and Zon (2010), in zebrafish p53 plays a key function in the response to DNA damage and DNA damage induced apoptosis. Thus, the transcription of *p53* suggested the existence of DNA damage, which could enhance the accumulation of mutations necessary for cell initiation. This would be supported by the transcription pattern of *ccng1,* which is a downstream target of *p53* (Fig. 1C). According to these results, 24 hpe may not be enough time to produce significant effects on *p53* or its downstream targets, but it was enough to detect a trend. However, the increased transcription of

junb supported the existence of cellular damage. Junb can act as a tumor suppressor arresting the cell cycle through the downregulation of cyclin D and upregultaion of p16 (Shaulian 2010). These results agree well with the mortality registered in sequentially exposed groups. Due to the premature death of all the larvae exposed to both carcinogens, the promoter capacity of B(*a*)P after DMBA exposure could not be further analyzed. Thus lower doses should be considered for further studies on the promotion mechanisms of PAHs.

Although the relative induction was lower compared to the results obtained at 24 hpe, induced transcription of *cyp1a* was observed 3 and 6 wpe in DMBA exposed groups (except at 3 wpe in embryos exposed to 0.6 mg/L), and this effect completely disappeared at 12 wpe (Fig. 2A; 3A; 4A). A non significant increase in the transcription of *cyp1a* was also observed in B(*a*)P exposed embryos at 6 wpe. It has been reported that, in fish, the transcription level of *cyp1a* rapidly decreases within the first hours after the exposure to B(*a*)P or DMBA (Wang et al. 2010). Thus, the temporal transcription pattern observed in our study can hardly be explained by the AHR mediated *cyp1a* upregulation. Gene transcription is not solely dependent on the PAH-AHR interaction. PAH mediated gene transcription is also regulated by the access of the AHR to its promoter, which is in turn controlled by epigenetic mechanisms such as DNA methylation (Bollati and Baccarelli 2010). Actually, B(*a*)P is known to decrease global methylation and to specifically affect the methylation pattern of cancer related genes like *cyp1b* or *p53* in zebrafish embryos (Corrales et al. 2014; Fang et al. 2013). Abnormal methylation patterns in cancer related genes have been shown to be involved in cancer development in zebrafish (Mirbahai et al. 2011). Thus, one possible mechanism underlying the time-course transcription patterns observed for *cyp1a* could be related to alterations in the DNA methylation produced by PAHs. Although the differences towards the control groups were not significant, the same transcription pattern observed for *cyp1a* transcription were observed for *p53*, *ccng1* and *junb* (Fig. 2B; 2C; 2D). These results could be related to the existence of DNA damage produced by DMBA metabolites or the ROS arising from its activation as well as to the above mentioned epigenetic regulation that may occur after exposure to PAHs. Thus, we hypothesize that cell cycle arrest at the G2 to M transition mediated by *p53* and *ccng1* may be occurring in response to DNA damage (Kimura et al. 2001). Moreover, Junb is required for tissue regeneration in zebrafish larvae, suggesting the existence of damaged cellular environment (Ishida et al. 2010). At 6 wpe upregulated *p53* transcription level was observed in animals exposed to 0.3 mg/L of B(*a*)P (Fig. 3B) and a significant upregulation of *junb* was achieved in animals treated with 0.6 mg/L of B(*a*)P (Fig. 3D). Overall, these data suggest the existence of a favorable cellular environment for tumor initiation (Cheah and Loo 2001; Muñoz and Albores 2011; Shaulian 2010). Finally, results obtained at 12 wpe did not suggest the existence of DNA damage as none of the tested genes was significantly upregulated

suggesting that after acute exposure to PAHs, mutations leading to initiation could be fixed before 12 weeks. Moreover, epigenetic changes might be affecting the transcription pattern. Thus, together with the accumulation of mutations, future investigations on the effects of PAHs should address the influence of epigenetic processes in the temporal transcription pattern of cancer related genes.

3.4.2 Genotoxicity

The exposure of an organism to genotoxic substances may alter DNA structure and function (Anderson and Wild 1994). At 12 wpe we did not detect significant MN increase after B(*a*)P and DMBA exposures, suggesting that 24 h of exposure to the tested hydrocarbons owns no permanent clastogenic capacity. Nevertheless, it must be considered that the gene transcription analyses indicated the possible existence of DNA damage at previous sampling points. Our results agree with those obtained by Barjhoux and co-workers (2014) who exposed Japanese medaka embryos to sediments spiked with environmental concentrations of pyrene and methylpyrene. They did not detect DNA damage in 2 dph larvae (exposed from 24 hpf until hatching) but they did detect increased *p53* transcription and explained this phenomenon hypothesizing that DNA repair mechanism may be properly functioning preventing the clastogenic activity of the pollutants (Barjhoux et al. 2014). Anyhow, 12 wpe might be too late to detect clastogenicity as DNA damage leads to apoptosis (Roos and Kaina 2006). Moreover, the sensitivity of the MN test to detect pollutants induced DNA damage has proved to be limited (Cambier et al. 2010). Thus, attending to transcription patterns observed for the analyzed cancer related genes and the results from **Chapter I**, in future studies the analysis should be performed at earlier time points and, if possible, substituted by more sensitive techniques.

3.4.3 Histopathology

Exposure of zebrafish embryos to 0.25, 0.5 and 1 mg/L DMBA for 24 h has demonstrated to produce cancer development with a prevalence ranging 2-16% 12 months after the end of the exposure (Spitsbergen et al. 2000), being the liver the primary target organ for cancer development. In the present work, we did not detect any clear carcinogenic lesion in the analyzed samples. This could be due to the fact that tumor development after a single exposure to B(*a*)P or DMBA could require longer than 12 weeks to fully develop. In addition, the limited amount of samples investigated could have not been enough to detect tumor appearance taking into account reported prevalence (Spitsbergen et al. 2000).

Although no carcinogenic foci were identified, diverse pathologies were detected during the histopathological analysis (Table 1). Hepatic megalocytosis was the most common of the alterations identified being present also in the control

group. Mild to moderate hepatocyte megalocytosis with karyomegaly is detected in about 50% standard husbandry systems feeding commercial diets (Spitsbergen and Kent 2003). Although no significant differences were observed, higher prevalence were observed in embryos exposed to 0.6 or 1 mg/L B(*a*)P and in those exposed to 0.3 mg/L DMBA. Interestingly, existing bibliography indicates that hepatocyte megalocytosis may be induced by damage to DNA or the mitotic apparatus following carcinogenic exposure (Spitsbergen and Kent 2003). Some damaged necrotic foci were also identified in all the exposure groups. Finally, hepatocyte vacuolization was also observed. Vacuolization may occur due to lipid or glycogen accumulation. It is challenging to identify vacuolization as pathology as it can be a dietary effect; however, it has been related to the toxic effect of different compounds including AHR ligands (Wolf and Wolfe 2005; Zodrow et al. 2004). Compared to the control group, higher prevalence of vacuolization was observed in all the exposure groups (except in embryos exposed to 0.3 mg/L DMBA). This difference was significant in fish exposed to 0.6 mg/L B(*a*)P. Thus, the histopathological analysis suggested the capacity of PAHs to produce alterations on lipid homeostasis.

3.5. Conclusions

At the concentrations and exposure times assayed, sequential exposures to DMBA and B(*a*)P were lethal to zebrafish embryos and, thus, lower concentrations should have been employed to test the carcinogenic and promoter capacity of these PAHs. Both B(*a*)P and DMBA were able to strongly induce the transcription of *cyp1a* at 24 hpe, suggesting the appearance of damaging metabolites. The sustained effects on this gene transcription may be related to the bioavailavility and AHR affinity of each PAH. Other mechanisms, such as epigenetic modifications produced by PAHs should be further explored.

Although transcriptomic analysis suggested the existence of DNA damage, cancer related genes were not any longer upregulated 12 wpe, and the MN test did not show the existence of DNA damage. Thus earlier analysis and more sensitive techniques should be employed in future works. Anyhow, higher percentages of hepatic megalocytosis and vacuolization were observed at 12 wpe in exposed groups suggesting that exposures at the embryo stages may lead to tissular alterations in the adulthood.

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Comparative toxicity of metal oxide nanoparticles (CuO, ZnO, TiO_2) to developing zebrafish embryos

PUBLICATION

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Abbreviations

Black silver deposit, BSD

Disodium laureth sulfosuccinate, DSLS

Dynamic light scattering, DLS

Nanomaterial, NM

Nanoparticle, NP

Scanning electron microscopy, SEM

Transmission electron microscopy, TEM

Abstract

Increasing use of nanomaterials is resulting in their release into the environment, making necessary to determine the toxicity of these materials. With this aim, the effects of CuO, ZnO and TiO_2 nanoparticles (NPs) on zebrafish development were assessed in comparison with the effects caused by the ionic forms (for copper and zinc), bulk counterparts and the stabilizer used for rutile TiO_2 NPs. None of the NPs caused significant embryo mortality. CuO NPs were the most toxic affecting hatching and increasing malformation prevalence (≥1 mg Cu/L), followed by ZnO NPs that affected hatching at ≥5 mg Zn/L and stabilized TiO_2 NPs that caused mortality and decreased hatching at 100 mg Ti/L. Exposure to the stabilizer alone provoked the same effect. Thus, toxicity of the TiO_2 NP suspension can be linked to the surfactant. For all the endpoints, the greatest effects were exerted by the ionic forms, followed by the NPs and, finally by the bulk compounds. By autometallography, metal-bearing deposits were observed in embryos exposed to CuO and ZnO NPs, being more abundant in the case of embryos exposed to CuO NPs. The largest and most abundant metal-bearing deposits were detected in embryos exposed to ionic copper. In conclusion, metal oxide NPs affected zebrafish development altering hatching and increasing the prevalence of malformations. Thus, the use and release of metal oxide NPs to the environment may pose a risk to aquatic organisms as a result of the toxicity caused by NPs themselves or by the additives used in their production.

Key Words: Zebrafish development, metal oxide nanoparticles, fish embryo test, nanotoxicology.

Laburpena

Nanomaterialen erabileraren areagotzearekin batera, material hauen isurketek ingurunera gora egin dute. Ondorioz, nanomaterialek izan dezaketen toxizitatea ezagutzea beharrezko zeregin bihurtu da. Helburu honekin, CuO, ZnO eta TiO_2 nanopartikulek (NPek) zebra arrainaren garapenean dituzten efektuak aztertu genituen beraien aldaera ionikoen (kobre eta zinkaren kasuetan), aldaera gordinen eta TiO_2 errutilo NPen egonkortzailearen efektuekin alderatuta. Aztertutako NPek ez zuten enbrioien heriotza tasa handitu modu esanguratsuan. CuO NPak izan ziren toxikoenak, eklosio tasa murriztu eta malformazioen prebalentzia handitu baitzuten (≥1 mg Cu/L). Ondoren, ZnO NPak izan ziren toxikoenak eklosio tasan eraginak sortuz ≥5 mg Zn/L-ko kontzentrazioetan. Azkenik egonkortutako TiO_2 NPek hilkortasuna areagotu eta eklosio tasaren murriztu zuten 100 mg/L-ko kontzentrazioan. Egonkortzaile hutsaren pean izandako enbrioietan efektu berberak behatu ziren. Beraz, TiO_2 NP-en suspentsioaren toxizitatea surfaktantearen eraginaren menpekoa litzateke. Aztertutako parametro guztietan efekturik indartsuenak aldaera ionikoek eragin zituzten, NPek tarteko efektuak eragin zituzten eta azkenik konposatuen aldaera gordinek izan zituzten eraginik ahulenak. Autometalografiaren bitartez metal metaketak antzeman ziren CuO eta ZnO NPen pean egondako enbrioietan. Metaketa hauek kopuru handiagoan agertu ziren CuOren pean egondako enbrioietan. Metaketarik handienak eta ugarienak kobre ionikoaren pean egondako enbrioietan antzeman ziren. Ondorioz, metal oxidoen NPek zebra arrainaren garapenean eraginak sortarazten dituzte eklosio tasa murriztuz eta malformazio prebalentzia areagotuz. Beraz, metal oxidoen NPen erabilerak eta hauen isurtzeak arriskua izan dezakete organismo urtarrentzat, NPek beraiek edo/eta NPen produkzioan erabiltzen diren gehigarrien toxizitatearen ondorioz.

Gako Hitzak: Zebra arrainaren garapena, metal oxidoen NPak, arrain enbrioien testa, nanotoxikologia.

4.1. Introduction

In recent years, our ability to engineer matter at the nanoscale has been developed to a point in which new products or enhanced materials arising from nanotechnology have been introduced in the markets worldwide (Maynard et al. 2006).

Due to their small size and consequent large number of surface atoms per mass unit, nanoparticles (NPs) possess unique mechanical, catalytic and optical properties as well as electrical conductivity when compared with their bulk counterparts (Niemeyer 2001; Oberdörster et al. 2005). These unique properties make them suitable for many industrial processes and consequently, manufactured NPs are currently used in different areas such as electronics, biomedicine, pharmaceuticals, cosmetics, environmental analysis and remediation, catalysis and material sciences (Nowack and Bucheli 2007; Ju-Nam and Lead 2008). Concomitantly with the increasing integration of nanomaterials (NMs) in outstanding technological applications and their introduction in mass produced commercial goods, concerns are rising due to nano-specific properties potentially leading to unforeseen health or environmental hazards (Maynard et al. 2006). The distinctive behaviour of nanometer-scale particles when compared to their larger counterparts has led to the development of nanotoxicology, the branch of toxicology responsible for assessing the effects of these NMs on living organisms (Donaldson et al. 2004). It would be appropriate to ensure that when novel chemical substances, such as NPs, are produced for commercial use, a risk assessment is carried out prior to mass production and use, in case such applications bring forth negative impacts on the environment (Lee et al. 2010; Thomas et al. 2011; Warheit et al. 2008). Toxicological evaluation of the biological effects plays a key role in the assessment of chemicals safety.

As the most numerous and phylogenetically diverse group of vertebrates, fish have been valuable models for the understanding of fundamental processes in vertebrate evolution, development and disease (Spitsbergen and Kent 2003). In this context, over the past decades fish have become model organisms for toxicological studies (Spitsbergen and Kent 2003; Teraoka et al. 2003). Among fish species, the zebrafish (*Danio rerio*) is one of the most used models. Studies using zebrafish have increased exponentially due to the multiple advantages it offers as a toxicological model (Hill et al. 2005). The deleterious effects of different substances, including manufactured NPs have been assessed in studies conducted with adult zebrafish (Griffitt et al. 2007; Hill et al. 2005; Xiong et al. 2011; Yu et al. 2011). In this scenario, the increasing efforts on seeking alternatives to traditional animal tests, makes zebrafish embryos even more of a suitable choice when toxicity screenings have to be performed (Busch et al. 2011; Lin et al. 2011; Yang et al. 2009). Their high

reproductive output and the optical clarity of zebrafish embryos allow the performance of different bioassays and the direct observation of embryo development and alterations (Teraoka et al. 2003). Several toxicity assays have been developed using zebrafish embryos (Hallare et al. 2006; Lammer et al. 2009; Nagel 2002). Viability and morphological assessment are the typical endpoints used in these studies to assess developmental toxicity (Augustine-Rauch et al. 2010). Interestingly, zebrafish embryos have been proposed as a predictive model for NMs toxicity assessment (Fako and Furgeson 2009) and several studies have already been conducted (Asharani et al. 2008; Lee et al. 2007; Zhu et al. 2007; 2008). Results indicate that nano-sized metals can cause both lethal and sublethal effects on developing fish, which include increased mortality, abnormal development and hatching rate reduction (Shaw and Handy 2011). Specifically, metal oxide NPs cause different toxic effects including tissue damage, acute lethality or induction of ROS production (Lin et al. 2011; Zhao et al. 2013; Zhu et al. 2008; 2009). Among the range of nanotechnology products, in this work we have focused our attention to three metal oxide NPs (CuO, ZnO and TiO_2) that are widely implemented in consumer products. The soluble and bulk forms of these metals are toxic to aquatic organisms (Bernardeschi et al. 2010; Clearwater et al. 2002; Grosell et al. 2007; Larsson et al. 1980; Stohs and Bagchi 1995), implying that the potential adverse effects over biota could also exist for the nanoparticulate forms; additionally, such effects, given NPs unique properties, could be different from those caused by the ionic and bulk forms. Thus, to further characterize the toxicity of metal oxide NPs, in this study we employed zebrafish embryo testing to analyze the toxic effects of CuO, ZnO and TiO_2 NPs in comparison with their bulk and ionic counterparts (in the case of copper and zinc) and when necessary with other substances present in the NPs suspensions.

CuO NPs are used as additives in lubricants, in computer processors, conductive coatings, printer inks, cosmetics, and as antimicrobial agent (Ren et al. 2009; Shaw and Handy 2011). These NPs have already been shown to be toxic to different organisms producing oxidative stress and DNA damage (Lee et al. 2010). In zebrafish, embryo exposure to CuO NPs reduces hatching rate at concentrations higher than 0.5 mg CuO/L (Lin et al. 2012).

ZnO NPs are applied in electronic sensors, solar voltaic devices as well as in the production of sunscreens, cosmetics, paints, ceramics, and fungicides or in wastewater treatment (Lee et al. 2010; Vaseem et al. 2010). Previous studies in zebrafish embryos have shown that ZnO toxicity is dose dependent and similar for the nanoparticulate and bulk forms (Zhu et al. 2008), although inconsistent results appear in the literature. Specifically, Zhu and co-workers (2008) reported LC50 values of 1.793 mg/L and 1.55 mg/L for the ZnO NPs and bulk ZnO, respectively. However, in other works, mortality was only observed at concentrations higher than

50 mg/L (Bai et al. 2010). Effects on embryo hatching have usually been reported at concentrations higher than 1-5 mg/L (Bai et al. 2010; Lin et al. 2012; Zhao et al. 2013; Zhu et al. 2008).

Finally, TiO_2 NPs, which are among the most widely used NPs, are very useful in photocatalysis, in environmental technology for the treatment of waste water and ground water and for the degradation of air pollutants and are often used in the production of sunscreens and cosmetics (Fries and Simko 2012; Macwan et al. 2011). Even though studies with adult zebrafish have shown that TiO_2 NPs seem to be less toxic than other metal oxide NPs, toxic effects have been reported after exposure to high doses and prolonged periods (Chen et al. 2011a; Wang et al. 2011). TiO_2 exists in different structural forms with different properties and consequent environmental impacts. Among them, anatase and rutile are considered the most likely to be found in the environment (Ju-Nam and Lead 2008). As reviewed by Auffan et al. (2009), anatase has proved to be biologically more active in terms of cytotoxicity or DNA damage. However, according to existing literature, exposures of up to 500 mg/L of anatase TiO_2 either as NP or in its bulk form do not produce toxic effects on developing zebrafish embryos (Zhu et al. 2008).

Taken into account that the properties and, therefore, the potential toxicity of NPs may vary depending on many factors, such as synthesis method, size, coatings, etc., with this study we aimed to contribute to the existing knowledge on metal oxide NPs toxicity using the zebrafish embryo test. Endpoints assessed were survival, hatching and malformation prevalence. In addition, autometallography (Soto et al. 1996) has been applied for the first time as a potentially useful technique to determine the fate of metals in zebrafish embryos after exposure to metal oxide NPs.

4.2. Materials and Methods

4.2.1. Fish maintenance and breeding

Adult zebrafish (*Danio rerio;* AB Tubingen) were maintained at 27 ± 1 ºC with a 14-hour light / 10-hour dark cycle in 100 L tanks following standard protocols. The day prior to the beginning of the experiment one female and two male adult zebrafish were placed separately in the same breeding tramp which had previously been located in a 2 L tank containing conditioned water. Then fish were left overnight and just before turning on the light they were allowed to gather. The resulting embryos were collected with Pasteur pipettes and fertilized viable eggs were selected under a stereoscopic microscope (Nikon smz800, Kanagawa, Japan).

4.2.2. Metal compounds used for experimental exposures

CuO (CuO-poly) and rutile/anatase TiO_2 (TiO_2-RUAN-poly) NPs were provided by Intrinsiq Materials. Disodium laureth sulfosuccinate (DSLS) stabilized rutile TiO_2 (TiO_2-60-DSLS) NPs were produced at Dead Sea Laboratories. DSLS was provided by Zschimmer & Schwarz Italiana S.p.A. (Tricerro, Italy). Commercial rutile/anatase TiO_2 (TiO_2-RUAN-P25) NPs were obtained from Evonik Industries (Dusseldorf, Germany). ZnO NPs, bulk CuO, bulk ZnO, bulk rutile TiO_2 (TiO_2-BRU), bulk anatase TiO_2 (TiO_2-BAN), $CuCl_2$ and $ZnCl_2$ were purchased from Sigma Aldrich (Madrid, Spain).

4.2.3. Characterization

The main characteristics of NPs are shown in Table 1. For the CuO and TiO_2 NPs used in the present study, characterization data have been previously reported in the literature (Buffet et al. 2011; 2013; Katsumiti et al. 2015). For ZnO NPs, transmission electron microscopy (TEM) was used to characterize the size and shape of the nanoparticles (Fig. 1), using a Hitachi H7100 TEM (Tokyo, Japan) for Fig. 1A and a JEOL 1200EX TEM (Tokyo, Japan) for Fig. 1B, both operating at 100 kV. Dynamic light scattering (DLS) measurements were performed in a Malvern Zetasizer Nano ZS instrument (Worcestershire, United Kingdom).

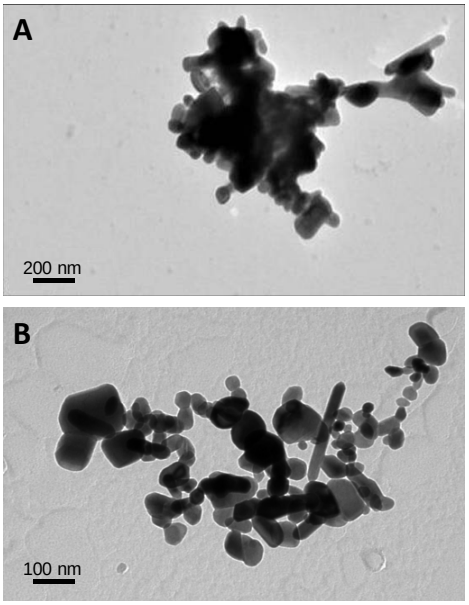

Fig. 1. TEM micrographs of bulk ZnO (A) and ZnO nanoparticles (B).

4.2.4. Preparation of exposure suspensions

In order to minimize particle aggregation and sedimentation, deionized water was used to prepare NP suspensions and used as control media, as it has previously been done in similar studies (Zhu et al. 2008), after corroborating that deionized water did not affect embryo survival and development. For the experimental exposure of zebrafish embryos, stock solutions (or suspensions in the case of more insoluble forms) of the chloride and oxide forms of copper and zinc were prepared at the highest nominal concentration evaluated (10 mg Cu or Zn/L). For that purpose, metal

Table 1. Main characteristics of NPs used in the present work.

NPs	Crystal structure	Synthesis Method	Size in nm		Z potential in mV	Original form	Reference
			DLS	TEM SEM			
CuO-poly	Tenorite	Plasma	40-500 Average 197	---	26.3	Powder	Buffet et al. (2011, 2013)
ZnO	---	Plasma	150-1000	<100 (TEM)	-22.2	Powder	This work
TiO_2-RUAN-poly	55% rutile 45% anatase	Plasma	---	<100 (TEM)	-19.8	Powder	Katsumiti et al. (2015)
TiO_2-60-DSLS	100% rutile	Milling	---	10-400 Average 60 (SEM)	-24.6	Suspension	Katsumiti et al. (2015)
TiO_2-RUAN-P25	10% rutile 70% anatase (20% unidentifiable)	Unknown	---	Majority 10-20 Also >100 (TEM)	-25.1	Powder	Katsumiti et al. (2015)

DLS: dynamic light scattering; DSLS: disodium laureth sulfosuccinate; SEM: scanning electron microscopy; TEM: transmission electron microscopy.

salts and metal oxide forms were added to deionized water, stirred for 3 h and sonicated for 10 min in a Selecta Ultrasons H 3000840 (50 Hz; Barcelona, Spain) sonication bath at 25 ºC. The day of the beginning of the test, dilutions (0.01, 0.1, 1, 5 mg/L) were prepared. Once ready, they were manually shaken and sonicated again for 10 min. Prior to expose the organisms pH and temperature were measured.

In the case of titanium dioxide, all the stock suspension and dilutions (0.1, 1, 10, 50, 100 mg Ti/L) were prepared the day of the experiment. Different protocols were used to prepare the stock suspensions. TiO_2-BAN was suspended in deionized water and hand shaken before preparing the dilutions as well as before starting the exposures. In the case of TiO_2-RUAN-P25 NPs, TiO_2-RUAN-poly NPs and TiO_2-BRU, powders were gently hand disaggregated using a pestle and mortar and adding a few drops of deionized water. Deionized water was then added until a homogeneous suspension was obtained. Afterwards, samples were diluted and sonicated for 1 min before starting the exposures. Finally, TiO_2-60-DSLS NPs suspension was vortexed before preparing the dilutions. Exposure media containing the equivalent concentration of surfactant present in the TiO_2-60-DSLS NPs suspensions (0.00834, 0.0834, 0.834, 4.17, 8.34 mg/L) were also prepared.

4.2.5. Embryo toxicity study

For each compound and concentration, eight newly fertilized embryos were exposed for 120 h in 24 well plates. Two embryos were placed in each well containing 2 mL of the corresponding medium or deionized water (control) at a constant room temperature of 27º C. Microplates were covered to avoid evaporation. Three replicates were run for each of the tested compounds and concentrations.

Autometallography has been previously employed to successfully localize copper and zinc in the tissues of aquatic species exposed to the soluble form of these metals (Soto et al. 1996). Thus, in the case of the exposures to the three forms of copper and zinc, at the end of the experiment (120 hpf), five unexposed larvae and five larvae exposed to 10 mg metal/L, as the highest concentrations tested of each compound, were fixed and processed for histology and further localization of metal by autometallography in paraffin sections.

4.2.6. Histological processing and autometallography

Larvae were fixed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding and hematoxylin/eosin (H/E) staining. For autometallography, the next sections coming after those stained with H/E were dewaxed and processed for automethallography as described by Soto and co-workers (1996). Abundance of

autometallographical black silver deposits (BSDs) in the tissues was semiquantified using the following criteria: 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 (+++). All the observations were done under the 100x objective.

4.2.7. Data analysis

Statistical analyses were done using the SPSS/PC statistical package (SPSS Inc, Microsoft Co, WA, USA). For survival and hatching rates and malformation prevalence, significant differences were studied by Fisher's exact test ($p<0.05$). In the case of hatching time, significant differences between pairs of means were studied by means of two-independent samples Mann Withney test ($p<0.05$).

In order to determine the lethal concentration for the 50% of the organisms exposed (LC_{50}), Probit analysis was performed employing the same statistical package.

4.3. Results

During the exposures all the NPs tested tended to aggregate and sediment. In the case of CuO-poly NPs, whose brownish colour made it more evident, 24 h after adding the test suspension, a layer of sedimented NPs could be observed at the bottom of the wells as well as attached to the surface of the organisms (Fig. 2C).

Table 2. LC_{50} values for tested substances.

Metal	Tested Forms	Exposure concentration	LC 50 (mg/L)
Copper	CuO-poly NPs	0.01-10 mg Cu/L	>10
	Bulk CuO		>10
	Ionic Cu ($CuCl_2$)		3.083
Zinc	ZnO NPs	0.01-10 mg Zn/L	>10
	Bulk ZnO		>10
	Ionic Zn ($ZnCl_2$)		3.004
Titanium dioxide	TiO_2-60-DSLS NPs	0.1-100 mg Ti/L	84.095
	TiO_2–RUAN-poly NPs		>100
	TiO_2-RUAN-P25 NPs		>100
	TiO_2-BAN		>100
	TiO_2-BRU		>100
	TiO_2-MIX		>100
	DSLS	0.0083-8.3 mg/L (*)	4.825 (60.252)

(*)Surfactant concentration present in the TiO_2-60-DSLS NPs suspension. For DSLS, LC50 value is given in mg/L and in the equivalent metal concentration of the hypothetical NP suspension containing that DSLS concentration (in brackets).

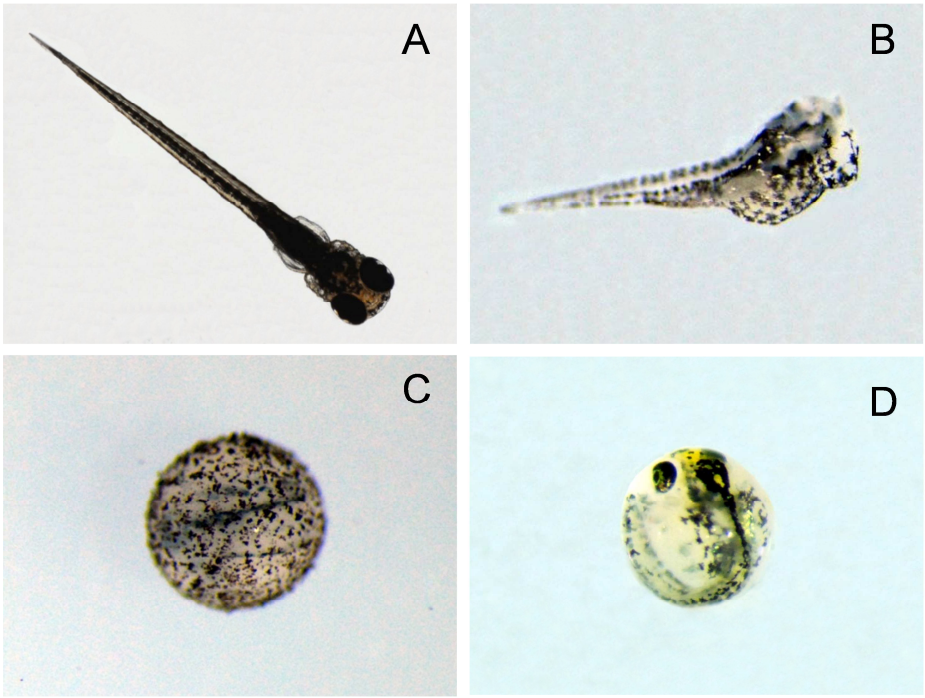

Fig. 2. Zebrafish embryos at 120 hpf. A) Control embryo. B) Anencephalic embryo exposed to CuO NPs (5 mg Cu/L). C) Non-hatched embryo covered by CuO NPs (10 mg Cu/L). D) Non-hatched embryo exposed to ZnO NPs (0.01 mg Zn/L).

4.3.1. Embryo toxicity of copper

Exposure to CuO-poly NPs or bulk CuO up to 10 mg Cu/L did not produce any significant decrease on embryo survival (Fig. 3A) and their LC_{50} values were estimated to be above the highest tested concentration (Table 2). The ionic form was the most toxic for all the parameters analyzed. A LC_{50} value of 3.083 mg/L was calculated for ionic copper which increased significantly embryo mortality at 5 and 10 mg/L (Fig. 3A). According to the other parameters analyzed, CuO-poly NPs showed a higher toxicity than bulk CuO. Significantly decreased hatching rate was observed in embryos treated with CuO-poly NPs at 10 mg Cu/L (Fig. 3B). At this concentration 73.34 % of the surviving embryos had not hatched, and a significant hatching delay was registered at concentrations of 1, 5 and 10 mg/L (Fig. 3C). Ionic copper affected hatching in a more severe manner, reducing the percentage of hatched embryos and delaying hatching at concentrations in the range 0.1-10 mg/L (Fig. 3B; 3C). At 0.1 mg Cu/L only around 40% of the embryos could hatch, while at higher concentrations none of the surviving embryos were able to hatch. Bulk CuO exerted the lowest effect, affecting only the hatching time (Fig. 3C). CuO-poly NPs produced significant

increase in the malformation prevalence (evaluated as the percentage of surviving embryos) in embryos exposed to 1, 5 and 10 mg Cu/L (Fig. 3D). Again, ionic copper provoked malformations at a lower concentration (0.1 mg Cu/L) than the other copper forms and affected 100% of the surviving embryos at concentrations of 1 and 5 mg Cu/L. Yolk sac oedema and tail flexures were the predominant abnormalities. Even if the malformation prevalence did not show such a notable increase in the case of embryos exposed to CuO-poly NPs, severe malformations such as anencephaly (embryo exposed to 5 mg Cu/L) were observed in CuO-poly NPs exposed embryos (Fig. 2B). No significant incidence on the malformation prevalence was observed in the case of bulk CuO exposed embryos (Fig. 3D).

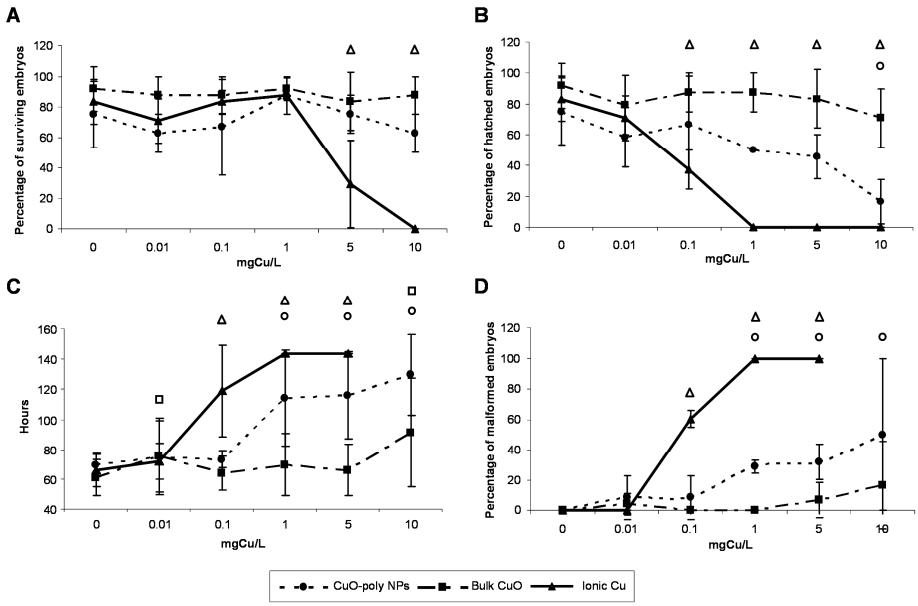

Fig. 3. Results obtained for embryos exposed to CuO NPs (circles), ionic copper (triangles) and bulk CuO (squares). A) Percentage of surviving embryos after 120 h of exposure. B) Percentage of hatched embryos after 120 h. C) Embryo hatching time. D) Percentage of malformed embryos over surviving embryos after 120 h. Significant effects towards the unexposed controls are identified for each tested compound by empty forms.

4.3.2. Embryo toxicity of zinc

As described for copper, ZnO NPs were less toxic (LC50>10 mg Zn/L, Table 2) than the ionic form of zinc, which exerted the highest toxicity (LC50=3.004 mg Zn/L). None of the tested concentrations of ZnO NPs or bulk ZnO produced significantly increased mortality, while significantly reduced survival was observed in all the exposures to ionic zinc (Fig. 4A). Regarding the hatching parameters, ZnO NPs produced significant reduction of the percentage of hatched embryos only at 10 mg Zn/L (Fig. 4B)

although this effect was also registered at lower concentrations (Fig. 2D). Again, ionic zinc was the most toxic form, exerting a significant impairment in the hatching rate at all the concentrations tested. This impairment was attributable to mortality except in the case of 10 mg/L exposed embryos. In this group 50% of the surviving embryos did not hatch. Surprisingly, bulk ZnO produced a significant reduction of hatching only in embryos exposed to 0.1 mg Zn/L (Fig. 4B). Exposure to the three tested metal forms caused hatching delay at the highest concentrations (5 and 10 mg Zn/L). Moreover, bulk ZnO also increased the time required to hatch at 0.1 mg Zn/L (Fig. 4C).

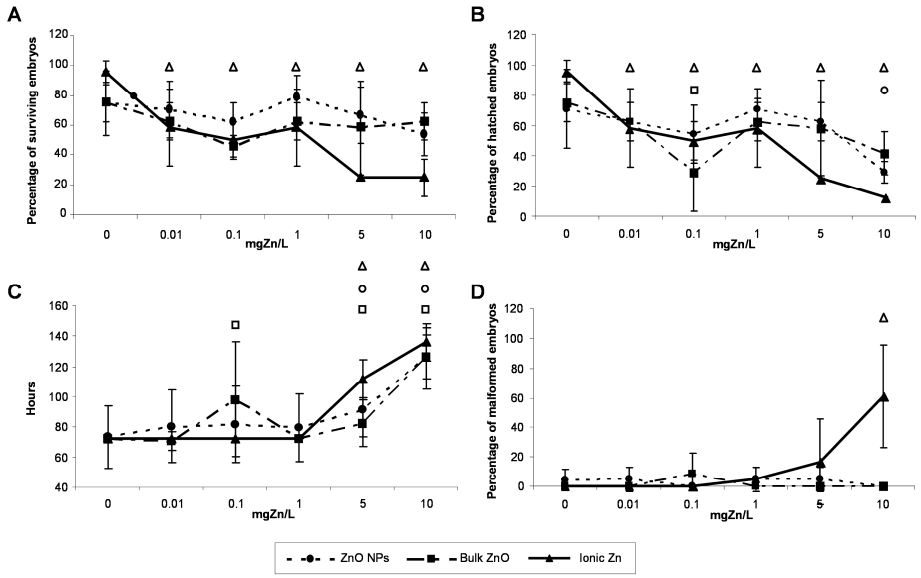

Fig. 4. Results obtained for embryos exposed to ZnO NPs (circles), ionic zinc (triangles), and bulk ZnO (squares). A) Percentage of surviving embryos after 120 h of exposure. B) Percentage of hatched embryos after 120 h. C) Embryo hatching time. D) Percentage of malformed over surviving embryos after 120 h. Significant effects towards the unexposed controls are identified for each tested compound by empty forms.

The malformation prevalence increased significantly only at the highest tested concentration of ionic zinc. Yolk sac oedema and tail flexures were the predominant abnormalities. Malformations were rare under bulk ZnO or ZnO NPs exposures (Fig. 4D).

4.3.3. Embryo toxicity of titanium dioxide

A LC50 value of 84.095 mg Ti/L was calculated for TiO_2-60-DSLS NPs, while for the rest of treatments LC50 values were either estimated above the highest tested concentration or could not be calculated (Table 2). TiO_2-60-DSLS NPs exerted a significant effect on survival and hatching rates at the highest tested concentration

(100 mg Ti/L), when compared to their control groups (Fig. 5A; 5B). However, the same effect was observed when the surfactant was tested alone. Neither TiO_2-RUAN-poly NPs (55% rutile and 45% anatase) nor TiO_2-RUAN-P25 NPs (10% rutile, 70% anatase, 20% unidentifiable material) produced any toxic effect (data not shown). Similarly, no mortality was observed after exposure to any of the bulk TiO_2 tested forms and concentrations. None of the TiO_2 compounds produced a significant increase in the malformation prevalence. Only in the case of embryos exposed to TiO_2-BRU some malformations such as pericardial oedema and tail curvature were recorded, but the effect was not statistically significant.

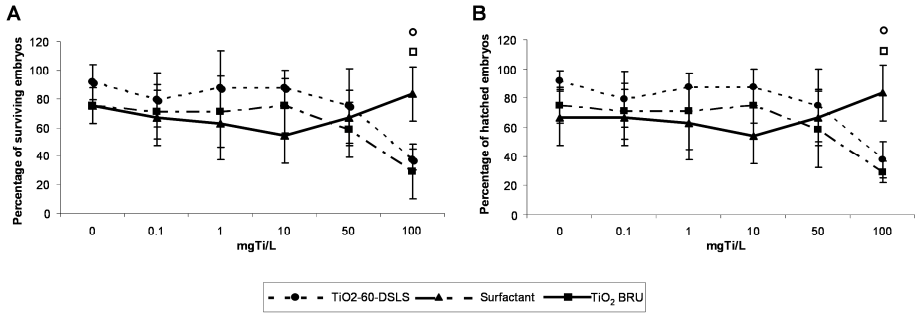

Fig. 5. Results obtained for embryos exposed to TiO_2-60-DSLS NPs (circles), the surfactant DSLS (squares) and bulk TiO_2 (triangles). A) Percentage of surviving embryos after 120 h of exposure. B) Percentage of hatched embryos after 120 h. Significant effects towards the controls are identified for each tested compound by empty forms.

4.3.4. Autometallography

The results for the semi-quantitative analysis of the presence of BSDs are shown in Table 3. BSDs were detected in several organs such as brain, gill, liver, yolk sac and tail (Fig. 6). Control zebrafish embryos exhibited scarce small uniformly distributed BSDs in their tissues. Embryos exposed to bulk CuO, bulk ZnO, ZnO NPs and ionic zinc showed almost the same amount of homogenously disseminated small BSDs throughout the tissues studied. Sections of organisms exposed to CuO-poly NPs and ionic copper exhibited the highest amount of BSDs among the experimental groups.

Fig. 6. Micrographs of a 120 hpf zebrafish embryo exposed to 10 mg Cu/L of CuO-poly NPs. A) H/E stained section. B) Section of the same embryo shown in A subjected to autometallographical detection of metals. C) BSDs (arrows) in the liver tissue seen at higher magnification.

Table 3. Semi–quantitative evaluation of the presence of BSDs in whole embryo sections of organisms exposed to different forms of Cu and Zn.

Organism	Control	CuO-poly NPs	Bulk CuO	Ionic Copper	ZnO NPs	Bulk ZnO	Ionic Zinc
A	-	+	++	++	+	-	+
B	-	++	+	++	-	+	+
C	+	++	+	+++	+	-	+
D	+	+++	++	++	+	++	++
E	-	++	+	+++	+	+	+
Total number of +	2	10	7	13	4	4	6
Average number of +	0.4	2	1.4	2.6	0.8	0.8	1.20

(-) Tissues 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.

4.4. Discussion

The increasing production, use and consequent release of metal oxide nanoparticles into the environment makes it necessary to assess the environmental and health hazards that these compounds could exert (Lee et al. 2010; Wang et al. 2013). In the present study, we evaluated the toxic effects of different metal oxide nanoparticles (CuO-poly, ZnO, TiO_2-60-DSLS, TiO_2-RUAN-poly, and TiO_2-RUAN-P25) compared to their bulk and ionic (for copper and zinc) counterparts using the zebrafish embryo test.

4.4.1. Differential toxicity of metal oxide nanoparticles

Except in the case of TiO_2-60-DSLS NPs, whose effect on survival could be attributable to the surfactant present in the NP suspension, for the other NPs studied in this work, the LC50 values were estimated above the highest tested concentration or it could not be calculated (Table 2). In agreement with these results, Lin et al. (2012) did not observe a significant increase in embryo mortality after 72 h of exposure to 50 mg/L CuO, ZnO or TiO_2. Zhu and co-workers (2008) found no toxicity for TiO_2 NPs after exposing embryos up to 500 mg/L, but they established a LC50 value of 1.793 mg/L (96 h) for ZnO NPs exposed embryos. The higher toxicity observed by Zhu et al. (2008) for ZnO NPs, could be due to the differences in the employed particle size. According to the DLS analysis, NPs employed by Zhu et al. (2008) were smaller (50 to 360 nm) than the ZnO NPs assayed here (150-1000 nm).

A toxicity ranking for the studied NPs could be established based on the sublethal parameters analyzed in this study; CuO-poly were the most toxic NPs exerting significant effects on embryos (hatching delay, increased malformation prevalence) at concentrations of 1 mg Cu/L, followed by ZnO NPs that caused deleterious effects at concentrations of 5 mg Zn/L (hatching delay). TiO_2 NPs were the least toxic. From the three different TiO_2 NPs assayed (Table 1), only in the case of TiO_2-60-DSLS NPs, effects (reduced survival and hatching rates) were observed at 100 mg Ti/L, but as mentioned, these effects can be attributed to the surfactant present in the NP suspension that exerted the same effect when assessed alone (Fig. 5).

Solubility is a fundamental parameter to be considered when NPs toxicity is assessed as it can lead to the delivery of highly toxic ions (Auffan et al. 2009; Misra et al. 2012; Shaw and Handy 2011). Accordingly, CuO-poly and ZnO NPs, which were the most toxic, have been shown to be more soluble than TiO_2 NPs (Johnston et al. 2010; Shaw and Handy 2011). Hatching impairment was the main effect shared by CuO-poly and ZnO NPs exposed embryos. Both CuO-poly and ZnO NPs reduced the hatching rate in embryos exposed to 10 mg/L and delayed hatching at lower concentrations

(Figs. 3B; 4B). It has been demonstrated that, due to their chemical characteristics, metal ions released from relatively soluble metal oxide NPs like CuO and ZnO can fit the active site of the zebrafish hatching enzyme (metalloprotease ZHE1) responsible for the degradation of the chorionic membrane and, consequently, inhibit zebrafish embryo hatching, while exposure to the sparingly soluble TiO_2 does not interfere (Lin et al. 2012). Our results are in good agreement with those obtained by Lin and co-workers (2012), who observed reduced hatching rate at 72 h in embryos exposed to concentrations over 0.5 mg/L and 5 mg/L of CuO and ZnO NPs, respectively. In our study effects on hatching were registered as delayed hatching (Figs. 3C; 4C) as far as hatching rate was calculated at the end of the test (120 h). These results indicate that even if the soluble fraction arising from the exposure to CuO and ZnO NPs can produce delayed hatching by inhibiting the hatching enzyme at concentrations over 0.5 and 5 mg/L, respectively, this inhibition is not sustained after 120 h and embryos are able to hatch at concentrations below 10 mg/L (Figs. 3B and 4B).

Aggregation is another NP property to be considered when NPs toxicity is assessed, since it leads to changes in NPs size distribution and to differential presence of NPs along the water column (Bai et al. 2010; Johnston et al. 2010). In the present study, even if deionized water was used to run the exposures, NPs aggregated and precipitated as exposure time increased. This effect has previously been reported in other studies with similar NPs (Griffitt et al. 2007; Hund-Rinke and Simon 2006; Yu et al. 2011). There is evidence that the presence of NPs attached to the chorion surface resulting from NP precipitation could interfere with the regular transport through the chorion pores (Lee et al. 2007). This blockage would result in reduced oxygen supply to the embryo and, consequently, the produced hypoxia would be another factor impairing normal hatching (Bai et al. 2010). The zeta potential values obtained for the tested NPs (Table 1), were all moderate (positive or negative) indicating that they are all likely to have a similar tendency to aggregate, and therefore aggregation and precipitation should not be parameters explaining the differential toxicity of studied NPs. Thus, even if aggregation is a parameter affecting distinct metal oxide NPs toxicity, the differential toxicity observed for the metal oxide NPs tested in this study depends on their chemical composition and solubility.

The induction of malformations in developing embryos is another common effect observed after zebrafish embryo exposure to metal NPs (Asharani et al. 2008; King-Heiden et al. 2009; Zhao et al. 2013). In our study, only CuO-poly NPs produced increased malformation prevalence at concentrations equal or above 1 mg Cu/L. Nevertheless, it must be considered that the low hatching rate of embryos exposed to ZnO NPs may have acted as a confounding factor when the malformation prevalence was calculated due to the difficulties to register slight anomalies in non-hatched embryos.

For NPs of the same chemical composition, crystal structure has also been considered a parameter affecting NPs toxicity (Warheit et al. 2008). In this study, different forms of TiO_2 NPs, containing different proportions of anatase and rutile TiO_2 were tested and only TiO_2-60-DSLS NPs (100% rutile) produced effects at 100 mg Ti/L. It has been reported that anatase is more cytotoxic than rutile (Auffan et al. 2009; Katsumiti et al. 2015), but it must be considered that toxicity of TiO_2-60-DSLS NPs appeared to be caused by the surfactant agent present in the suspension, indicating that special attention should be paid to the chemicals that are used as additives for nanoparticle formulations. In fact, when the toxicity of TiO_2-60-DSLS NPs was tested in mussel haemocytes and gill cells, the surfactant was suggested to be responsible of the TiO_2-60-DSLS NPs cytotoxicity (Katsumiti et al. 2015).

Overall, our results indicate that in the concentration range tested in this study and for the particular TiO_2 NPs and experimental conditions used, no significant toxicity is observed by any rutile/anatase combination. Indeed a major conclusion from this study is that the chemical composition of the NPs, along with additives used to disperse them are the key factors affecting NPs toxicity.

4.4.2. Nanoparticles toxicity compared to their ionic and bulk counterparts

Several works have studied and compared the relative toxicity of metallic NPs and their ionic counterparts, in terms of equivalent soluble fraction, reaching the conclusion that NPs are more toxic than their soluble ionic counterpart (Bai et al. 2010; Kasemets et al. 2009; Yu et al. 2011; Zhu et al. 2008). In order to provide straight comparable data in terms of the toxicity arising from equal metal quantities, in this work we exposed zebrafish embryos to identical nominal concentrations of each metal form. When NPs toxicity is considered in comparison to their ionic counterparts in these terms, the ionic forms of copper and zinc resulted significantly more toxic than the CuO-poly and ZnO NPs, respectively (Figs. 3; 4). The ionic counterpart for titanium was not tested in this study since, as mentioned previously, TiO_2 is a sparingly soluble compound. Ionic copper caused mortality, reduced the percentage of hatched embryos, delayed hatching and increased malformation prevalence always at lower concentrations than NPs (Fig. 3). The lower toxicity of copper bearing NPs when compared to their ionic form has previously been described in different zebrafish developmental stages. Chen and co-workers (2011b) exposed 5 days post fertilization zebrafish larvae to Cu_2O and $CuCl_2$, obtaining a lower LC50 value in the groups of larvae exposed to $CuCl_2$ (0.24 mg/L and 0.085 mg/L, respectively). The higher toxicity of soluble copper has also been described by Griffitt et al. (2007, 2008), who after exposing adult zebrafish to both ionic and Cu NPs (0.1 mg Cu/L) registered a higher mortality in the case of fish exposed to the former. Regarding zinc exposed embryos, no significant effect on survival was exerted neither

by the ZnO NPs nor by the bulk form while significantly increased mortality was observed at all ionic zinc concentrations. The lower toxicity to zebrafish embryos of ZnO NPs in comparison with ionic zinc (regarding nominal concentrations) has also been described by Bai et al. (2010), who reported delayed hatching at lower doses in embryos exposed to ionic zinc at concentrations higher than 2.152 mg Zn/L.

When NPs toxicity was considered in comparison to their bulk counterparts, different results were obtained for each metal. CuO-poly NPs exerted sublethal toxicity at lower concentrations than bulk CuO, while ZnO NPs produced similar effects to bulk ZnO on the studied endpoints.

CuO NPs have already shown to be more toxic than their bulk counterparts in human epithelial cells (Wang et al. 2012), bacteria and crustaceans (Heinlaan et al. 2008). In the case of ZnO, different results have been reported. Yu et al. (2011) showed that the bulk form of the metal resulted more toxic to adult zebrafish than ZnO NPs, and argued that this was a direct consequence of reduced availability of NPs, as a result of their tendency to precipitate. However, in the case of zebrafish embryo exposures, NPs precipitation resulted in an increased concentration around the embryo and consequent increased contact between the embryo and the NPs, which could therefore explain the toxicity recorded for CuO-poly and ZnO NPs. In fact, Zhu et al. (2008) reported a similar toxicity for bulk ZnO and ZnO NPs both producing delayed embryo development and decreased survival and hatching rates. After 96 h, they recorded lower LC_{50} values for both nano ZnO (1.793 mg Zn/L) and bulk ZnO (1.55 mg Zn/L) compared to our study. These differences could be due to the different methodologies employed during exposures, since the study by Zhu et al (2008) involved shaking of the incubation plates every 12 hours.

Assuming that the toxic effects observed in this study for TiO_2-60-DSLS NPs were produced by the surfactant present in the NPs suspension, none of the assayed TiO_2 NPs and bulk compounds produced any toxic effect to zebrafish embryos at the concentrations tested. TiO_2 NPs have been considered innocuous and used as non-toxic control NPs for zebrafish (Griffitt et al. 2008). Our results are in agreement with previous works in which the toxicity of TiO_2 has been assessed using zebrafish embryos. Zhu and co-workers (2008) did not find any toxic effect on zebrafish embryos exposed to concentrations of up to 500 mg/L (96 h) of TiO_2 NP and bulk forms. However, toxic effects have been reported after longer exposures of adult zebrafish (Chen et al. 2011a; Xiong et al. 2011) and, thus, attention should be paid to the possible effects of TiO_2 NPs after longer exposures or different exposure conditions, such as exposure under UV radiation, that could increase their toxicity (Clemente et al. 2013), as well as to other developmental stages which could be affected through different modes of action.

Overall, our results indicate the lower toxicity of NPs when compared to the ionic forms when nominal concentrations are compared, while the relative toxicity of the NPs compared to the bulk forms is dependent on the metal oxide employed. These results are consistent with those obtained in other model organisms, such as yeast (*Saccharomyces cerevisiae*; Kasemets et al. 2009), bacteria (*Vibrio fischeri*; Heinlaan et al. 2008) and mussel cells (Katsumiti et al. 2015). However, in the case of crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*), CuO NPs were also less toxic than the ionic form while ionic zinc resulted less toxic than the NPs (Heinlaan et al. 2008).

4.4.3. Metal detection in tissue sections

In order to assess the toxicity of a compound it is important to establish the final destination that it will have in the organism. To date, the body distribution of nanometals is poorly understood and hampered by a lack of methods for measuring NPs in tissues (Shaw and Handy 2011). Determining whether NPs exposure produces increased metal uptake, and the consequent increased bioaccumulation, is an initial step to allow a better understanding of toxicity mechanisms (Johnston et al. 2010). Autometallography allows to localize metallic deposits in tissue sections of organisms (Soto et al. 1996). This technique has previously been successfully used to determine the existence of metallic deposits in the gills of turbot (*Scophthalmus maximus*) (Alvarado et al. 2006) or to describe subcellular distribution of metals in molluscs (Marigómez et al. 2002). In adult zebrafish, autometallography has been used to determine methylmercury distribution in the retina after trophic exposure (Mela et al. 2010).

The semi-quantitative results obtained for metal accumulation in embryos exposed to the different metal forms of copper and zinc (Table 3) match the toxicity pattern reported in previous sections. Embryos exposed to the ionic form of copper and zinc showed the highest presence of BSDs, reinforcing the idea that solubility is a key parameter determining NPs toxicity. Autometallography results showed lower accumulation of metal in embryos exposed to zinc than in those exposed to copper.

The variety of organs and tissues in which BSDs were detected (brain, gills, liver, yolk sac, tail) and the existing literature about NPs fate (Lee et al. 2007; Handy et al. 2008), in which gills, gut, liver and brain have been referenced as targets for nano-compounds, indicate that the distribution pattern inside the organisms is wide, and that to some extent, the fate of metallic compounds in fish is related to the developmental stage and the uptake pathway metals follow. Results suggest that autometallography can be successfully utilized to localize metallic compounds (which may have entered the organism either as dissolved ions or as suspended NPs) in zebrafish embryo tissue sections. The application of this technique could therefore

fulfil the lack of simple, routine methods for the direct measurement of metals in tissues. Thus, autometallography could allow a better knowledge of body distribution, or, serve as a complement for other techniques such as the labour-intensive electron microscopy of dissected tissues.

4.4.4. Environmental relevance

Even though little information is available about the current environmental concentrations of NPs, some estimations have been reported. The environmental concentration for Cu NPs in major Taiwanese rivers has been estimated to be 0.06 mg/L (Chio et al. 2012). This concentration is within the range of concentrations tested for CuO NPs (Fig. 3). We did not observe any effect at concentrations lower than 1 mg/L suggesting that pollutant levels estimated by Chio and co-workers should not be a cause for concern. The estimated average concentrations for ZnO and TiO_2 NPs in European surface and waste waters are 10 ng/L and 430 ng/L for ZnO NPs and 15 ng/L and 3.47 µg/L for TiO_2 NPs, respectively (Gottschalk et al. 2009). As in the case of CuO NPs, considering that toxic effects were only observed at higher concentrations (Figs. 4; 5), we could conclude that the current environmental concentrations of ZnO and TiO_2 NPs should not pose a risk to the aquatic environment if each metal is considered separately. Nevertheless, usually metals appear in the environment as complex mixtures, and therefore, the total toxicity risk should be taken into account. Furthermore, other organisms may display higher sensitivities than zebrafish.

Moreover, it must be considered that the results presented in this study correspond to acute exposures, and therefore, further investigations are needed before discarding long-term effects as consequence of chronic exposures. It must also be taken into account, that an increased production and release of NPs is expected to occur in future years. In fact, it has been estimated that the production of engineered NMs will increase to 58,000 tons before 2020 (Maynard et al. 2006). Therefore, even if the concentrations tested in this work are over the currently estimated environmental concentrations, our results indicate that a future increase in CuO, ZnO or TiO_2 NPs environmental concentrations may turn into an environmental issue.

4.5. Conclusions

The present study shows that the toxic effect of NPs on developing zebrafish embryos is a function of their chemical composition; CuO NPs are the most toxic of the NPs tested in this study and TiO_2 the least toxic. Of all TiO_2 NPs tested, toxic effects were only produced by TiO_2-60-DSLS NPs (100 mg Ti/L). However, in this case toxicity was linked with the presence of DSLS surfactant in NPs suspensions. Thus, special attention should be paid to the chemicals used in NP formulations. Finally, when assessing NPs toxicity using the zebrafish embryo model, it is important to consider not only mortality, but the sublethal effects produced by the exposures, otherwise, NPs toxicity could be underestimated.

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Cellular and molecular responses of adult zebrafish after exposure to CuO nanoparticles or ionic copper

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Abbreviations

Correspondence analysis, COA

Dynamic light scattering, DLS

Linnear model for microarray analysis, LIMMA

Lysosomal membrane stability, LMS

Months post-exposure, mpe

Nanomaterial, NM

Nanoparticle, NP

Self organizing tree analysis, SOTA

Significant analysis of microarray, SAM

Abstract

Concerns are increasing about the effects that the growing presence of engineered nanoparticles (NPs) in the aquatic environment could produce to biota. In this context, zebrafish (*Danio rerio*) has shown to be a useful model to study the biological effects of NPs. The aim of this work was to study the effects produced by CuO-poly NPs in adult zebrafish in comparison to those produced by the dissolved metal prepared from $CuCl_2$ at the same nominal concentration (10 µg Cu/L). Samples were collected at 3 and 21 days of exposure and at 6 months post-exposure. After 21 days of exposure, metal accumulation was detected by chemical analyses. The lysosomal membrane stability test showed significantly impaired general health status in fish exposed to both metal forms. Histopathological alterations of gill structure were detected in animals from both exposure groups in all the analyzed time points suggesting that exposure to CuO-poly and ionic copper might reduce gill functionality. No relevant histological alterations were detected in liver. Genotoxic effects were not observed using the micronucleus test in erythrocytes. The analysis of the hepatic transcriptome identified 97 regulated transcripts. Data suggested that after 3 days of exposure CuO-poly NPs could produce oxidative stress and reduce metabolism and transport processes, while ionic copper seemed to produce DNA damage after 21 day of exposure. Results indicated differences on the effects produced by the two metal forms after 3 days of exposure and a trend to become similar after 21 days. The significance analysis of microarrays identified genes of the circadian clock as the main responsible of those time-dependent differences. We observed that exposures to both copper forms led to impaired health status and metal accumulation that might result in oxidative stress and DNA damage.

Key words: CuO nanoparticles, zebrafish, bioaccumulation, cellular biomarkers, transcriptomics

Laburpena

Ingurune urtarrean, diseinatutako nanopartikulen presentziak biotan eragin ditzaketen efektuen inguruko kezkak sortzen ari dira. Testuinguru honetan, zebra arrainak (*Danio rerio*) nanopartikulek eragiten duten efektu biologikoak aztertzeko modelo erabilgarria dela erakutsi du. Lan honen helburua, zebra arrain helduetan eta kontzentrazio nominal berdinetan (10 µg Cu/L), CuO-poly NPek eta $CuCl_2$-tik prestatutako metal solugarriak sortutako efektuak konparatzea izan zen. Laginak, 3 eta 21 eguneko tratamenduaren ondoren eta tratamendu osteko 6 hilabete ondoren jaso ziren. 21 egun eta gero, metalen metaketa neurtu zen analisi kimikoen bidez. Lisosomen mintzaren egonkortasunaren testak, bi metalen aldaeren pean egondako arrainen osasun orokorra kaltetua zegoela adierazi zuen. Denboran zeharreko laginketa guztietan, bi taldeetako animalietan zakatzetako egituraren aldaketa histopatologikoak ikusi ziren, aditzera emanez CuO-poly NPek eta kobre ionikoak zakatzen funtzionaltasuna murriztu zezaketela. Gibelak ez zuen aldaketa histopatologikorik azaldu. Eritrozitoetan egindako mikronukleoen testak ez zuen efektu genotoxikorik erakutsi. Gibeleko transkriptomaren azterketak 97 transkrito identifikatu zituen. Esperimentua hasi eta 3 egunetara lorturiko datuek CuO-poly NPek metabolismo eta garraio prozesuen gainbehera eta estres oxidatiboa sor zezaketela aditzera eman zuten. Datuek, 3 egun ostean bi metal motek eragindako efektuak ezberdinak zirela eta 21 egun eta gero berdintzeko joera zegoela eman zuten aditzera. Mikroarraien esangarritasun analisiek denboraren menpeko aldaketen eragile nagusitzat erloju zirkadianoko geneak identifikatu zituen. Datuen arabera bi kobre motek metalen metaketa eta osasun egoeraren endekatzea eragiten dute, estres oxidatiboa eta DNA mailako kalteak sor ditzaketenak.

Gako Hitzak: CuO nanopartikulak, zebra arraina, biometaketa, zelula mailako biomarkatzaileak, transkriptomika

5.1. Introduction

During the last decades nanotechnology has rapidly developed to a point at which our capacity to engineer matter at the nanoscale offers the opportunity to solve many industrial and societal problems (Bystrzejewska-Piotrowska et al. 2009; Roco and Baindbridge 2005). Due to their nano size and consequent large number of surface atoms per mass unit, nanomaterials (NMs) possess unique mechanical, catalytic and optical properties (Niemeyer 2001; Oberdörster et al. 2005). These properties make them suitable in different areas such as electronics, biomedicine, cosmetics, environmental analysis and remediation, catalysis and material sciences (Ju-Nam and Lead 2008; Nowack and Bucheli 2007). Consequently, a dramatic growth of the "nano" industry has occurred, and NMs have been introduced in a wide range of consumer products present in worldwide markets. According to Roco and co-workers (2011), the worldwide market for nanoproducts may represent over 200 billion dollars by 2020. In fact, it has been estimated that by 2020 the global production of engineered NMs will reach 10^{16} tonnes per year (Borm et al. 2006).

Concomitantly with the spread of nanotechnology and the industrial scale production of "nano" products, concerns arose about the possible effects that release of considerable amounts of NMs into the environment could have on living organisms. Due to the need of studying the differential effects of nanometer-scale particles when compared to their larger counterparts, Donaldson and co-workers (2004) proposed "nanotoxicology" as a new subcategory of toxicology in order to address the specific effects of NMs. Since then, wide knowledge about nanoparticle (NP) toxicity has been produced. However, these efforts have not been equally distributed among existing NPs and lesser attention has been paid to those used in smaller quantities (Bohnsack et al. 2012; Kahru and Dubourgier 2010; Kahru and Savolainen 2010). CuO NPs are among the latter and, thus, there is a need to provide wider knowledge about their toxic effect (Bondarenko et al. 2013).

CuO NPs have many industrial applications. They show efficient antioxidant activity and bactericidal effect, being promising antioxidants in industrial polymer processing and as disinfectant agents for textiles and public spaces (Das et al. 2013; Ren et al. 2009). Other applications of CuO NPs and Cu NPs include lubrication oil additive, electronics and computer processors, conductive coatings, printer inks, anti-ageing creams and skin conditioners and mineral supplements (Shaw and Handy 2011).

Being water bodies the ultimate destination of many of the human released pollutants, it is important to determine the amount of contaminants present in the aquatic media. Some investigations are starting to provide data about the current

environmental presence of NMs in water bodies (Gottschalk et al. 2013; Nam et al. 2014). However, little information is available regarding CuO NPs presence in the environment. Predicted environmental concentration of Cu NPs for major Taiwanese rivers is 0.06 mg/L with 95% confidence interval being 0.01-0.92 mg/L (Chio et al. 2012).

The presence of pollutants in water bodies has often made fish target organisms to study the toxicity of chemicals (Carvan 2007). Fish were the first non-human, non-rodent model in which nanotoxicity was studied (Oberdörster et al. 2006). Among fish species, zebrafish (*Danio rerio*) has emerged as a powerful model in biomedicine and especially robust to assess NMs toxicity (Bohnsack et al. 2012; Fako and Furgeson 2009). Zebrafish embryos offer a rapid and cost effective tool to screen the effects of a wide variety of NMs (**Chapter IV**), meanwhile experiments with adults allow to elucidate more specific modes of action of NMs and to identify target organs.

For living organisms copper is an essential micronutrient that can lead to disease when present in excess (Gravato et al. 2006; Grosell et al. 2007). Until now, some studies have addressed the toxicity of Cu, CuO or Cu_2O NPs to fish at different levels of biological organization (Al-Bairuty et al. 2013; Ates et al. 2014; Bohnsack et al. 2012; **Chapter IV**, Chen et al. 2011; Fako and Furgeson 2009; Griffitt et al. 2007; 2008; 2009; Isani et al. 2013; Lin et al. 2012; McNeil et al. 2014; Villarreal et al. 2014; Zhao et al. 2011).

When assessing the toxicity of NPs, their physicochemical characteristics are of crucial importance to understand their biological effects and, thus, it has been proposed that nanotoxicology should be seen as an interactive process between chemistry and biology (Bohnsack et al. 2012). Among physicochemical characteristics, solubility of NPs is a key property to understand their environmental fate, behavior and toxicity mechanisms (Misra et al. 2012). Soluble copper is toxic to fish, especially for those living in freshwater environments, as far as copper disrupts the ability to sustain the Na^+ gradient from the blood plasma to the water (Grosell et al. 2007). CuO NPs are found among the most dissolvable metal oxide NPs (Lin et al. 2012). It has been shown that exposure of zebrafish embryos to CuO NPs interferes with hatching process due to the inhibitory effect of copper ions on the hatching enzyme ZHE1 (Lin et al. 2011; 2012). Accordingly, both CuO-poly NPs and ionic copper reduce embryo hatching and increase the malformation rate in zebrafish embryos, being the ionic form the most toxic of the two (**Chapter IV**).

In zebrafish, CuO NPs have been generally described to be less toxic than ionic copper (**Chaper IV**; Isani et al. 2013; Lin et al. 2011; 2012; McNeil et al. 2014). Exposure to 0.05 mg/L of Cu NPs produces toxic effects to zebrafish embryos

reducing the number of functional lateral neuroblasts and affecting their behavior, being these effects stronger in fish exposed to soluble $CuSO_4$ (McNeil et al. 2014). Nevertheless, the acute toxicity of metal NPs is not always explained, or only partially explained, by the presence of free metal ions. Juvenile zebrafish have a 48-h LC50 of about 0.71 and 1.78 mg/L for nano- and dissolved forms of Cu, respectively (Griffitt et al. 2008). Griffitt et al. (2007; 2009) also reported specific "nano" effects at both tissue-level (gill histology) and molecular level (gill transcriptome) in adult zebrafish. These authors conclude that responses to the NPs exposure are not driven solely by the release of soluble metal ions into the water column. Anyhow at the transcriptional level similarities have been described in zebrafish exposed to ionic or nano copper. The transcription of metal responsive genes such metallothioneins and copper transporters has been identified after exposures to CuO NPs and soluble copper at concentrations ranging 7.6-15 µg/L (Chen et al. 2011; Craig et al. 2009). Together with genes directly related to copper, transcripts related to oxidative stress, gene expression and transmembrane transport have often been identified in zebrafish among genes altered after exposures to copper (Chen et al. 2011; Craig et al. 2009; 2010).

In other fish species, such as rainbow trout (*Oncorhynchus mykiss*), controversial results have been also published regarding the relative toxicity of the different metal forms. Isani et al. (2013) reported the stronger toxicity of the ionic form in rainbow trout, in which significant DNA damage was only detected after $CuSO_4$ injections. On the contrary, Al-Bairuty and co-workers (2013) found that equal exposures of rainbow trout to $CuSO_4$ and Cu-NPs (20-100 µg/L) caused similar histopathological alterations, including hyperplasia, aneurisms and necrosis in the secondary lamellae of the gills and hepatitis like injury and cells with pyknotic nuclei in the liver. However, the metal form affected the severity or incidence of injuries, being Cu-NPs produced pathology in the intestine, liver and brain worse than that produced by the same concentration of $CuSO_4$.

The aim of this study was to assess and compare the effects of waterborne exposure of adult zebrafish to an environmentally relevant concentration of copper (10 µg/L) in form of CuO NPs or dissolved copper at different levels of biological organization in order to gain deeper knowledge on the mode of action of these two forms of the metal. With this objective, analyses were conducted at two different exposure times (3 and 21 days) as well as at 6 months post exposure (mpe). Metal bioaccumulation in whole fish, changes in the hepatic transcriptome, together with cell-level effects in liver and histopathological alterations both in liver and in gills were analyzed in adult zebrafish exposed to ionic or CuO-poly NPs.

5.2. Materials and Methods

5.2.1. CuO NPs and preparation of exposure media

CuO (CuO-poly) NPs were synthesized by plasma and provided as powder by Intrinsiq Materials, Ltd. Main characterization data for these NPs have been previously reported in the literature (Buffet et al. 2011; 2013). Briefly, crystal structure analysis showed that the sample contained tenorite copper oxide and no other material was detected. Particle size of the powder suspended in deionized water ranged from roughly 40 nm to 500 nm with a majority at ~ 100 nm as indicated by dynamic light scattering (DLS). Particles were positively charged with an average zeta potential value of 26.3 mV and dispersed well. Once the sample was diluted, a brown transparent solution was obtained. Electron microscopy imaging confirmed the results obtained by DLS and revealed that particles were agglomerated, but individual particles could be observed, suggesting a good dispersion in solution.

To prepare the exposure media, a stock suspension of NPs containing 27 mg Cu/L was prepared in conditioned water for zebrafish (600 µS). NPs were added to deionized water, stirred for 3 h and sonicated for 10 min in an Ultrasons H 3000840 (50 Hz) sonication bath at 25 ºC. Ionic copper solution was prepared from $CuCl_2$ (Sigma-Aldrich, Madrid, Spain). An intermediate stock solution was prepared by dissolving the product in conditioned water for zebrafish. Exposure suspensions (10 µg Cu/L) were achieved by adding a given volume of the stock solutions to a reservoir tank used to fill the aquaria.

5.2.2. Maintenance and experimental exposure of adult zebrafish

The experimental procedure described herein was approved by the Ethics Committee of the UPV/EHU according to the current regulations. Zebrafish (wild type AB Tübingen) individuals were specifically produced and grown up to 4 months old for the experiment in the Aquatic Biology Experimental Service of the University of the Basque Country (UPV/EHU). Fish were maintained in a temperature-controlled room at 28º C with a 14-hour light/10-hour dark cycle in 100 L tanks provided with mechanic and biological filters. Water was maintained in continuous movement through an air triggered siphon to provide an appropriate aeration to the tank. Water was conditioned, by passage through a deionization system and then buffered to pH 7.2 with Sera pH plus (Sera, Heinsberg, Germany) and to 600 µs Ω with commercial marine salt (Sera marin Salt). Fish were fed Sera Vipagran baby and *Artemia* twice per day. Live *Artemia* was produced from encapsulated eggs (INVE Aquaculture, Salt Lake City, Utah, USA) in continuously aerated sea water maintained at 25º C. Water chemical parameters (nitrate, nitrite and ammonium) were measured once per week using Sera ammonium, nitrite and nitrates kits to verify water quality.

The exposures were carried out in 35 L aquaria containing 50-60 fish. During the 21 days of the exposure period, 2/3 of the aquarium water was changed by siphoning every three days and the corresponding volume of contaminated or clean water was redosed. During this period water movement was produced with an electric pump to allow oxygenation and to avoid NPs sedimentation. No filters were used during this period. After exposure, fish were maintained in clean water up to 6 months to evaluate the potential reversibility of the effects detected and the possible development of carcinogenic lesions. 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 and filters were restored. Chemical parameters were measured as described above and water renewed when the values were higher than zero mg/L for ammonium or nitrite and 50 mg/L for nitrate. Samples were taken after 3 and 21 days of exposure and after 6 mpe. Before fish were sacrificed, they were euthanized by overdose of MS-222 (tricaine methane-sulfonate, Sigma-Aldrich).

5.2.3. Chemical analysis of copper content

Twenty fish per experimental group were collected after 21 days of exposure, placed in cryotubes, frozen in liquid nitrogen and stored at -80 ºC until processing. Whole zebrafish were dried at 130 ºC for 24 h and weighted. Then, fish were pooled (five pools of four zebrafish each) and placed into 25 mL Erlenmeyer flasks. Nitric acid (65%, extra pure quality, Scharlau, Barcelona, Spain) was added until samples were covered to digest the tissue and the mouth of the Erlenmeyer was blocked with a crystal ball to minimize evaporation. After finishing the digestion of the sample, the remnant liquid was evaporated in an 80º C hot plate inside an exhaust hood. Finally, after evaporation, 2.5 mL nitric acid 0.1 M were added to each flask, and left for 1 d. The content of each flask was then put into tubes and centrifuged for 4 min at 2,000 rpm (Heraeus Labofuge 200 centrifuge; Hanau, Germany). The supernatants were moved to clean tubes, and stored at 4º C until required for chemical analysis. Analyses of metal content in whole zebrafish body were performed in the General Research Services of the University of the Basque Country (SGIKER) by inductively coupled plasma atomic emission spectrometry (ICP-AES).

5.2.4. Lysosomal membrane stability test in liver

The visceral mass of 5 individuals was dissected at 3 and 21 days of exposure and at 6 mpe, embedded in Cryo-M-Bed (Jung, Heidelberg, Germany), frozen in liquid nitrogen and stored at -40 ºC until staining. Frozen tissue sections (10 µm) were obtained in a Leica CM3000 cryotome (Leica Mycrosystems, Nussloch, Germany) at a cabinet temperature of -24 ºC. The determination of lysosomal membrane stability (LMS) was based on the time of acid labilization treatment required to produce the maximum

staining intensity in lysosomes after demonstration of acid phosphatase activity in hepatocyte lysosomes as described in Bilbao et al. (2010). Labilization period was determined under an Olympus BX51TF light microscope (Olympus, Tokyo, Japan) as the maximal accumulation of reaction product associated with lysosomes. Four determinations were made for each individual by dividing each section in the acid labilization sequence into 4 approximately equal segments and assessing the second labilization peak in each. A mean value was then obtained for each section, corresponding to an individual liver.

5.2.5. Micronuclei frequency test in erythrocytes

Blood samples of 5 individuals per experimental group were analyzed at 3 and 21 days of exposure and at 6 mpe. Blood was obtained by tail cutting and direct smear on clean microscope glass slides. Blood smears were left to air-dry and then cells were fixed for 15 min in cold methanol (Scharlau). Once again, slides were left to air-dry and then 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). Small, non-refractile, circular or ovoid chromatin bodies showing the same staining pattern as the main nucleus and with an overall area not bigger than a 1/3 of the main nucleus were considered as MN (Baez and Prieto 2005). 2000 erythrocytes were scored per individual fish using a light microscope at a magnification of 100x. Micronuclei frequency was expressed in ‰.

5.2.6. Histopathological analysis

Samples for histological analysis were collected after 3 and 21 days of exposure and at 6 mpe. The visceral mass of 10 individuals were dissected, placed in histological cassettes and immersed in 10% buffered formalin for 24 h at 4ºC. Then, samples were transferred to 70% ethanol and stored at 4ºC until complete tissue processing under vacuum conditions in a Leica ASP300 Tissue Processor. Then, paraffin blocks were done using plastic molds. Sections (5 µm thick) were cut in a Leica RM2125RT microtome. For histopathological analysis, sections were stained with hematoxylin/eosin in a Leica Auto Stainer XL and mounted in DPX by means of a Leica CV5030 Robotic Coverslipper. Slides were specifically examined under a Olympus BX51 light microscope for the determination of the presence of carcinogenesis-related histopathological alterations.

The gills of 5 individuals per exposure group were dissected and immediately fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h. Gills were postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 1 h at 4ºC. After fixing, samples were dehydrated in a series of alcohol of increasing concentration and finally embedded in Epon 812 resin (Sigma-

Aldrich). Semithin sections were obtained with a Leica Ultracut microtome, stained with 1% Toluidine blue (Sigma-Aldrich) and used for histopathological analysis. A semiquantitative scoring of pathologies was performed in each exposure group.

5.2.7. Transcriptomics

The liver of 20 male individuals was dissected out after 3 and 21 days of exposure. The livers of 4 individuals were pooled together in 2 mL cryovials containing 1 mL Trizol® (Invitrogen Life-Technologies, Merelbeke, Belgium) and zirconia/silica beads (Biospec, Bartlesville, USA). The cryovials were immediately frozen in liquid nitrogen and stored at -80º C until total RNA extraction following the Trizol® extraction method according to the manufacturer's instructions. Brieftly, pooled livers were homogenized using a Hybaid RyboliserTM (Hybaid, Ashford, UK) at shaking speed of 4 m/s for 20 s. Phase separation step was performed after incubation for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes, and then 0.2 mL of chloroform (Sigma-Aldrich) were added to each pool. Tubes were shacked vigorously for 15 s and incubated at room temperature for 3 min. After that, samples were centrifuged (Heraeus Labofuge 200 centrifuge) at 12,000 x g for 15 min at 4ºC. Aqueous phase was placed in a new RNAse free tube for RNA precipitation and 0.5 mL of isopropyl alcohol (Sigma-Aldrich) were added. Samples were incubated for 5 min at room temperature and centrifuged at 12,000 x g for 10 min at 4ºC. Finally, supernatants were removed and the pellets washed with 1 mL of 75% ethanol (Sigma-Aldrich). Samples were centrifuged at 7,500 x g for 5 min at 4ºC. Supernatants were removed and pellets dried for 5 min at room temperature. After that, pellets were dissolved in 100 µL of RNAse/DNAse free water. Samples were diluted 50 times (2 µL sample + 98 µL RNAse/DNAse free water) and concentration was measured in a Biophotometer (Eppendorf, Hamburg, Germany). The same equipment was used to assess purity through the 260/230 nm and 260/280 nm absorbance ratios. RNA quality was assessed using RNA Nano LabChips (Agilent Technologies, Carslbad, California, USA) and the Agilent 2100 Bioanalyzer.

Microarray hybridization and scanning were performed at the university SGIKER services as follows: 100 ng RNA per sample were labeled following Agilent Technologies´ protocol "One-Colour Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling)" Version 6.5, May 2010. Samples were retrotranscribed (first strand synthesis) and labeled using Low input Quick Amp Labeling kit, One color (Agilent Technologies) following manufacturer's instructions. Briefly, total RNA was reverse transcribed into first and second strand cDNA, after which first-strand cRNA was constructed using the second strand cDNA as a template, in the presence of Cy3-CTP. Labeled cRNA was purified with silica-based RNeasy spin columns (Qiagen, Venlo, The Netherlands). After labeling and purification, cRNA was quantified with a

NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Wilmington, USA) in order to determine the yield and specific activity of each reaction. Yield should be > 1.65 µg of cRNA. Specific activity should be > 6 pmol Cy3 per µg of cRNA. Hybridizations were performed on zebrafish 44 k full genome microarrays (version V3, AMADID 026437 Agilent Technologies) containing 43,803 unique probes using SuperHyb hybridization chamber (Agilent Technologies). 1.65 µg labeled cRNA was added in 100 µL for hybridization and incubated at 65ºC for 17 hours at 10 rpm in the hybridization oven.

Hybridized microarrays were scanned on a G2565CA DNA microarray scanner (Agilent Technologies), with ozone barrier slide covers (Agilent Technologies P/N G2505-60550). Obtained images were quantified using Agilent Feature Extraction Software (ver.10.7.3.1) (Agilent Technologies). Raw data were log base 2 transformed and quantile intra-chip normalization was performed to avoid systematic error due to technical variations. After this, means were centred to 0 to obtain the normalized signal. Finally spot filtering was performed to remove marginal signals.

5.2.8. Statistics

For data on metal accumulation and LMS, statistical analyses were performed using the SPSS for Windows (SPSS Chicago, IL) software. Bootstrap resampling techniques (Efron and Tibshirani 1993) were used to assess the effect of the treatments. For each experiment, N=2000 repetitions were selected by bootstrap resampling method. After that, Bonferroni's correction was used for multiple comparisons between pairs of groups. In all the cases significance was established at $p<0.05$.

For the microarray data, the statistical analysis was performed using MultiExperiment Viewer (MeV) vs. 4.7.1 (http://www.tm4.org/mev/) application. The significance analysis LIMMA package was used and the Benjamini-Hochberg method (FDR) for multiple test correction was employed to obtain the corrected p value. The results obtained for significantly regulated sequences correspond to the average values of the two treatments (ionic and nanoparticle forms) at both time points (3 days and 3 weeks) contrasted against the average values of their respective controls for both metals. The cut off $p<0.05$ (adjusted p value) was established.

Using the same software, a correspondence analysis (COA) was performed in order to visualize the association between the transcripts and the contrasts and a significance analysis of microarrays (SAM) was performed to identify genes whose transcription significantly changed between time points. Finally, heat maps were constructed to visualize the expression patterns of genes differentially regulated in any of the four exposure groups after a self organizing tree analysis (SOTA). Gene ontology (GO) analysis were performed using Blast2GO (http://www.blast2go.com)

software in order to identify biological processes and molecular pathways linked to regulated transcripts

5.3. Results

5.3.1. Metal accumulation

Chemical analyses performed in whole fish tissue showed significantly higher accumulation of copper in fish exposed for 21 days to ionic copper than in control fish (Fig. 1). In fish exposed for 21 days to CuO-poly NPs higher copper content than in the control fish was also detected, but this increase was not statistically significant.

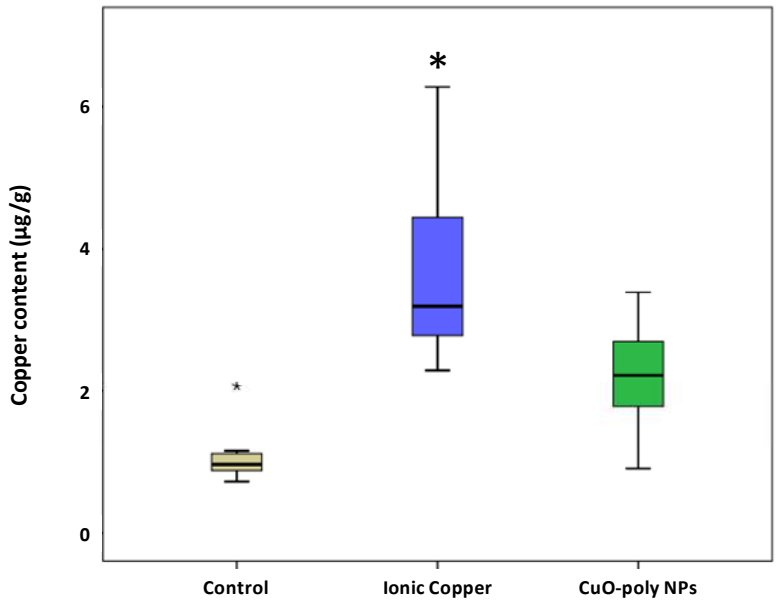

Fig. 1. Results of the chemical analysis of metal content in different treatment groups after 21 days of exposure. Box-plot boxes represent the percentage data value in between the 25th and the 75th percentile, median indicated by a line in the middle of the box. Whiskers are the data values in up to the 5th percentile and 95th percentile. Outliers are represented by small asterisks. Asterisk represents significant differences compared with the control group.

5.3.2. Lysosomal membrane stability in liver

The LMS test showed a strong health impairment of fish exposed to CuO-poly NPs and to ionic copper, as reflected by the decrease of the lysosomal labilization period

after 3 and 21 days of exposure, as well as at 6 mpe (Fig. 2). This effect was significant for both treatments at 3 days of exposure and at 6 mpe. After 21 days of exposure the labilization periods of the lysosomal membrane in both exposure groups were very similar to those recorded after 3 days of exposure, however a decrease in the LMS of control fish was observed. The lysosomal membrane labilization periods measured in exposed fish were always below 10 min.

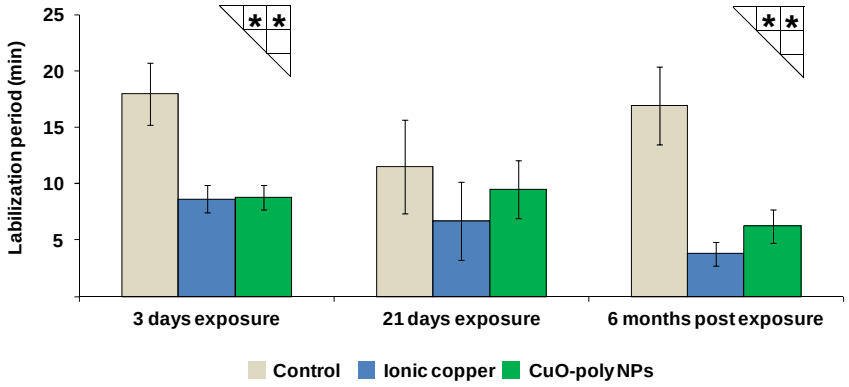

Fig. 2. Results of the LMS test after 3 and 21 days of exposure and at 6 mpe. Asterisks in the upper triangular matrix indicate significant differences between groups within the same sampling time.

5.3.3. Histopathological analysis

No relevant histopathological alterations were recorded in the hepatic tissue after histological analysis at any of the exposure groups or assessed time points.

The results of the histological examination of the gills are summarized in Table 1. Five samples were collected for all the groups, however the histological analysis of some of the samples was not possible due to the inappropriate orientation of the embedded sample and the consequent reduced amount of suitable tissue for histopathological evaluation. Some of the lesions found are shown in Fig. 3. Lesions such as lamellar hyperplasia and epithelial lifting (edema) were present in fish exposed to ionic copper and CuO-poly NPs, but also in the control group. Nevertheless, in exposed fish the prevalence of the histopathological alterations was higher or they were present in more than one time point. Exposure to both ionic copper and CuO-poly NPs produced increased presence of aneurisms and necrosis that were not detected in the control group. Exposure for 3 days to ionic copper produced the highest prevalence of some of the pathologies such as lamellar congestion or aneurism, while lamellar hyperplasia was more frequent in animals exposed to CuO NPs. At 6 mpe, a general trend to reduce the prevalence of the

pathologies previously found was observed. Nevertheless, animals exposed to CuO-poly NPs showed higher prevalence of lamellar fusion and necrosis at 6 mpe than during exposure.

Table 1. Semi-quantitative histopathological analysis of the gills.

Pathology	Time point	Control		Ionic copper		CuO-poly NPs	
		n	AI	n	AI	n	AI
Lamellar hyperplasia	3 d	5	1	4	1	4	2
	21 d	2	0	5	1	4	0
	6 mpe	4	0	5	0	5	0
Lamellar fusion	3 d	5	1	4	1	4	0
	21 d	2	0	5	1	4	0
	6 mpe	4	0	5	0	5	2
Lamellar congestion	3 d	5	0	4	2	4	0
	21 d	2	0	5	0	4	0
	6 mpe	4	0	5	0	5	0
Epithelial lifting	3 d	5	2	4	2	4	1
	21 d	2	1	5	3	4	3
	6 mpe	4	0	5	2	5	2
Aneurism	3 d	5	0	4	3	4	1
	21 d	2	0	5	1	4	1
	6 mpe	4	0	5	0	5	1
Necrosis	3 d	5	0	4	2	4	0
	21 d	2	0	5	1	4	1
	6 mpe	4	0	5	0	5	2

n: number of examined samples in each group. AI: number of affected individuals

5.3.4. Micronuclei frequency test in erythrocytes

The micronucleus test performed in erythrocytes showed that, under assayed conditions, in vivo exposure of zebrafish to sublethal concentrations (10 µg Cu/L for 21 days) of CuO-poly NPs or ionic copper did not cause genotoxic effects in terms of increased micronuclei frequency neither after 21 days exposure, nor at 6 mpe (data not shown).

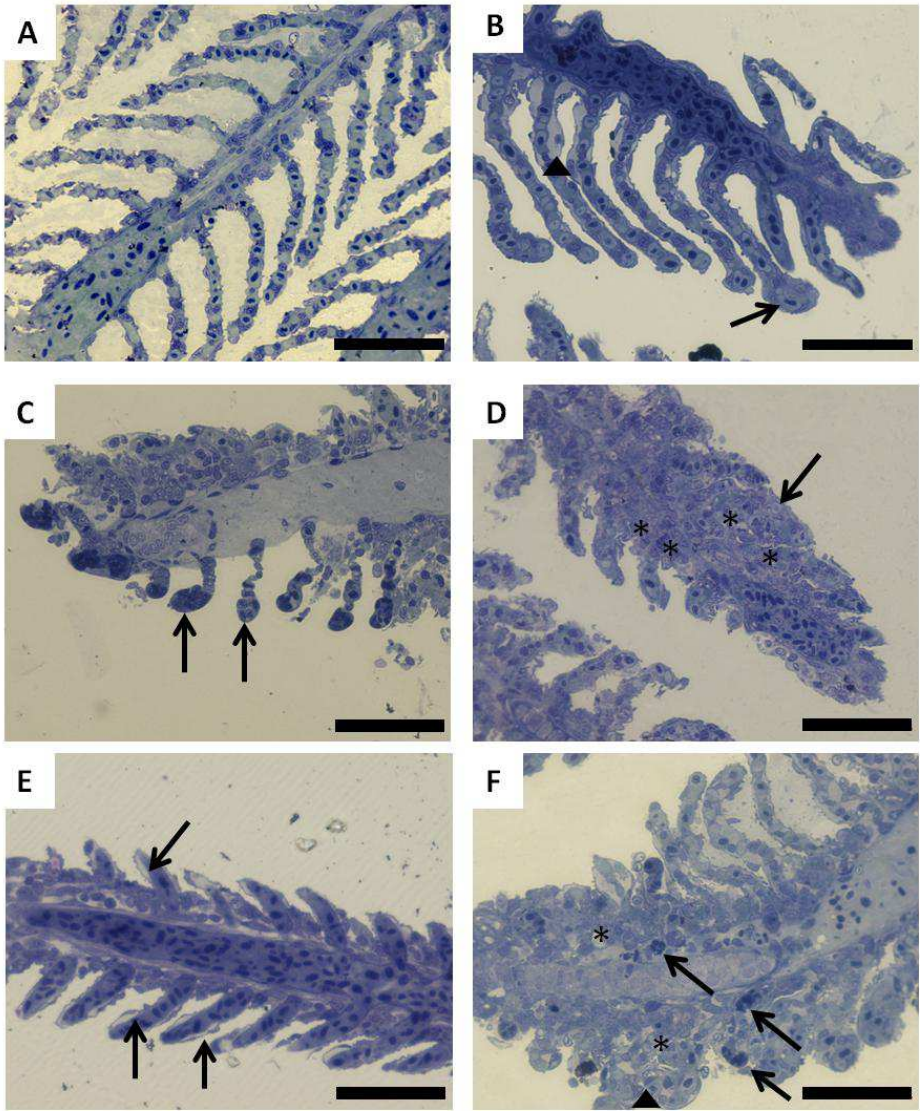

Fig. 3. Micrographs of semithin sections of zebrafish gills. (A) Gills from a control fish. (B) Gills from a fish exposed for 3 days to ionic copper showing epitelial lifting (arrow head) and aneurism (arrow). (C) Gills from a fish exposed for 3 days to CuO-poly NPs with multiple aneurisms (arrows). (D) Gills from a fish exposed for 21 days to ionic copper showing hyperplasia (asterisks) and lamellar fusion (arrow). (E) Gills from a fish exposed for 21 days to ionic copper showing epithelial lifting. (F) Gills from a fish exposed for 21 days to CuO-poly NPs at 6 mpe with necrosis (arrow), hyperplasia (asterisk) and lamellar fusion (arrow head). Scale bars: 50 μm.

5.3.5. Transcriptomics

97 unique sequences were significantly regulated in any of the exposure groups. Exposure to CuO-poly NPs for 3 days resulted in 69 regulated transcripts and 3 weeks of exposure to ionic copper significantly altered the transcription of 30 transcripts. It is important to notice that due to the reduced number of samples employed in the transcriptomic analysis, and the restrictive statistical analysis used, no transcript was significantly regulated (adjusted p value < 0.05) in those groups in which quality standards required to remove outlier samples (one of the arrays for 3 days ionic copper and one of the arrays for 3 weeks CuO-Poly NPs). Similar amount of up and down regulated transcripts were recorded after 3 days of exposure to CuO-poly NPs. The correspondence analysis (COA) performed with all the significantly regulated genes at any of the two treatments clustered together animals exposed for 3 week to ionic copper and to CuO-poly NPs (Fig. 4). First component (PC1, X axis) explained the 80.55% of sample variability, while the second component explained 14.64 % of the total variability.

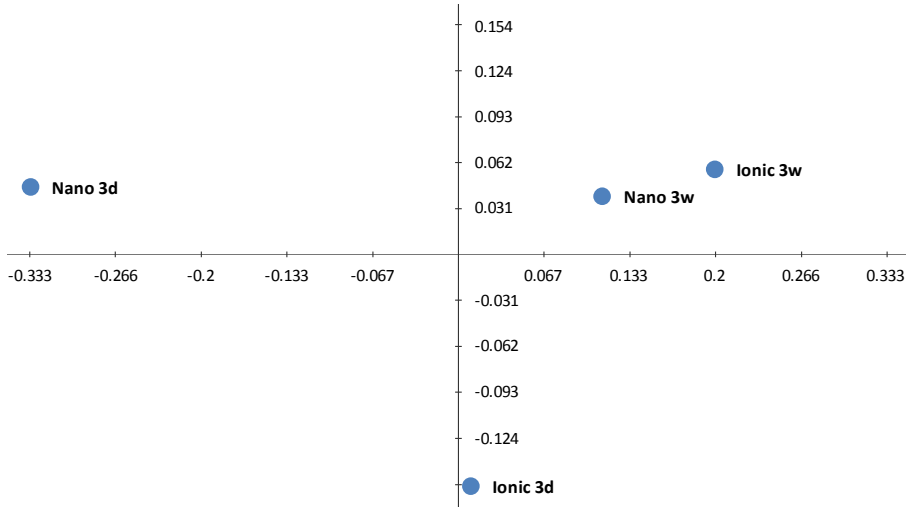

Fig. 4. Results of the correspondence analysis based on transcription profiles in liver of zebrafish exposed to CuO-poly NPs or ionic copper during 3 or 21 days. Distribution explaining sample variability is represented in two axes corresponding to the first (x-axis) and the second (y-axis) components.

In order to identify genes that significantly contributed to the differences between time points, a significant analysis (SAM) was performed irrespective of the exposure compound and 4 genes were identified. Two of the sequences corresponded to transcripts codifying for *cryptochrome 2a* (*cry2a*) (NM_131791, BC095305), another one was identified as a predicted sequence for the same gene (CN319073) and the last one coded for the period homolog 2 (*per2*) (NM_182857).

According to the results obtained by the self organizing tree algorithm (SOTA), average data diversity could be explained by means of 3 clusters. Thus, to further study the transcriptional responses produced by CuO-poly NPs and ionic copper, 3 heatmaps were constructed with all the 97 transcripts that were significantly regulated in the tested conditions (Fig. 5). The first cluster (A) grouped together sequences mainly upregulated after 3 days of exposure to CuO-poly NPs. According to the gene ontology, genes in this cluster were linked to “nucleotide biosynthetic process”, “responses to oxidative stress”, “glycine biosynthetic process” and “oxidation and reduction process”. Cluster B grouped transcripts mainly downregulated aftes 3 days of exposure to CuO-poly NPs. These genes were related to “one carbon metabolic process”, “oxalate transport”, “ion transmembrane transport”, “regulation of membrane potential”, “sulfate transport”, “chloride transport”, “fatty acid elongation and developmental process”. Finally, the third cluster (C) grouped together transcripts that were mainly upregulated after 3 weeks of exposure to ionic copper. Genes in this cluster were related to “DNA repair”, “signal transduction”, “response to hydrogen peroxide”, “regulation of cell growth”, “photoperiodism” and “entrainment of circadian clock”. In this last cluster, transcripts of animals exposed to CuO-poly NPs for 3 weeks showed the same expression pattern observed for those exposed to ionic copper. However, in fish exposed to NPs the transcription level of these genes was not significantly regulated according to the LIMMA analysis.

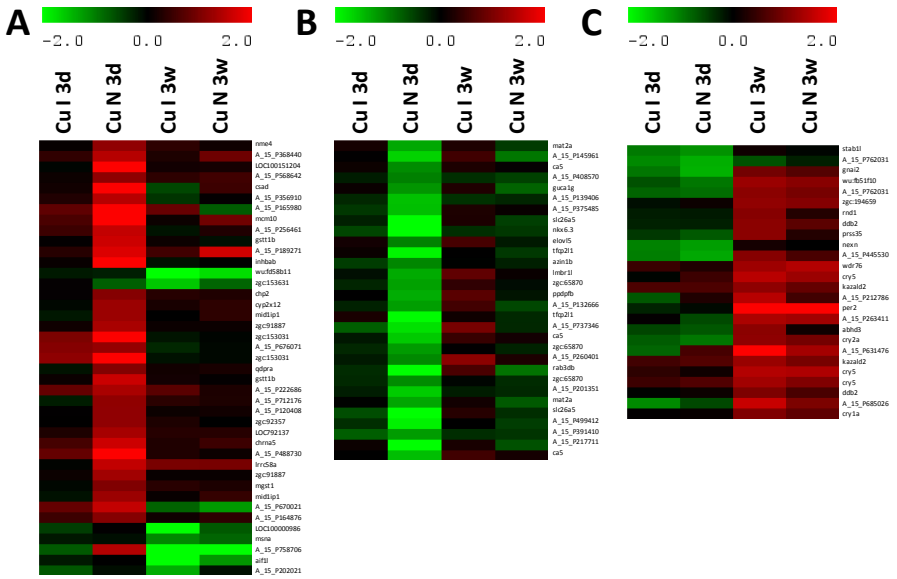

Fig. 5. Heatmaps constructed with all the significantly regulated sequences at any of the exposure groups.

5.4. Discussion

CuO NPs have been incorporated in a variety of products and processes (Das et al. 2013; Ren et al. 2009; Shaw and Handy 2011). Consequently, these NPs together with other Cu based NPs are likely to be released into the environment. Currently few works have assessed the effects CuO NPs. However, when their toxicity has been assessed in comparison to other metal oxide NPs, such as TiO_2 NPs, CuO NPs resulted specially harmful (**Chapter IV;** Karlson et al. 2008). These considerations make CuO NPs a target case of study for the environmental risk assessment. When assessing NMs toxicity many works have focused on the relative toxicity of the soluble fraction arising from NPs in comparison to the toxicity of NPs themselves. However, from the point of view of environmental risk assessment, total metal content should be considered, as far as discriminating the metal form (nanoparticulate versus soluble) in complex biotic or environmental samples is not a trivial issue (Maurer-Jones et al. 2013). Some studies are starting to provide the estimated environmental concentrations and information is often supplied as the estimated amount of NPs present in water in terms of mass/volume or tons of NPs expected to be spilled (Chio et al. 2012; Gottschalk et al. 2009; Maynard et al. 2006). Therefore, according to the scarce published data regarding environmental water concentration of CuO NPs (Chio et al. 2012), we exposed fish to equal nominal concentrations of Cu (10 µg/L) provided as CuO-poly NPs or soluble copper, which is also a concentration matching the environmental concentration of ionic copper (Santos et al. 2010).

5.4.1. Metal accumulation

Chemical analyses by ICP-AES indicated that metal uptake occurred both in animals exposed to CuO-poly NPs and to ionic copper, and in the latter occurred at a higher and significant extent (Fig. 1).

Our results are in agreement with previous works reporting copper uptake in different fish species after exposure to both metal forms (Al-Bairuty et al. 2013; Isani et al. 2013). The higher presence of copper in animals exposed to soluble copper could be due to the higher bioavailability of dissolved copper. Griffitt et al. (2009) showed that gill-through copper uptake corresponded mainly to the ionic form of the metal. Moreover, it has been shown that fish injected with soluble copper accumulate copper in different organs at a higher extent than those injected with CuO NPs (Isani et al. 2013). There are also some studies describing exposures to Cu based NPs where similar (Griffitt et al. 2007), or even higher copper accumulation, was observed (Chen et al. 2011) when compared to exposures to soluble copper. Nevertheless, it must be considered that in those studies the exposures were performed using lower Cu NP concentrations in order to match the equivalent

soluble fraction of Cu or to perform the exposures according to the LC50 values of the nano and soluble forms of copper, which was higher for the latter (Chen et al. 2011; Griffitt et al. 2007).

5.4.2. General health status

General health status of fish was evaluated using the LMS test which is considered a general stress marker (Moore et al. 2004) and histopathological analysis which is often employed to assess the effects of chemicals on aquatic organisms (Bernet et al. 1999). Analyses were performed in liver as it is the main route for metal excretion in fish (Shaw and Handy 2011). For histopathological analysis, gills were also analyzed as they are a main uptake route and target organ for environmental toxicants and could act as such also for NPs (Griffitt et al. 2009).

Exposure to different metals including copper can produce destabilization of the lysosomal membrane in aquatic animals (Köhler 1991). Control animals showed values over 10 min in all the groups, while none of the exposed groups reached that value (Fig. 2), indicating the onset of liver pathology (Köhler et al. 2002). Significant differences were found after 3 days of exposure and at 6 mpe, indicating the existence of toxicity produced by the exposures at environmentally relevant concentrations of copper (Chio et al. 2012). Despite the higher copper content observed in fish exposed to ionic copper after 21 days of exposure (Fig. 1), no differences were observed in the labilization period between exposed and control animals (Fig. 2). However this was due to a slight reduction on the labilization period recorded in the control fish group while the labilization period observed in fish from both treatment groups remained similar to that observed after 3 days of exposure. Even when the exposure ended by the third week, no increased labilization period was observed at 6 mpe, supporting the existence of time persistent toxic effect (Fig. 2). These results indicate that a similar toxicity is exerted by both metal forms.

Exposure to copper is known to produce histological damage in fish, including altered lipid content, necrosis and edemas in liver as well as different structure alterations in gill epithelia, such as hypertrophy, necrosis and fusion of secondary lamellae (Arellano et al. 1999; Griffitt et al. 2007; 2009; Pandey et al. 2008; Paris-Palacios et al. 2000). Zebrafish has been shown to be sensitive to ionic copper and Cu NPs. Griffitt and co-workers (2007) reported gill pathologies in zebrafish characterized by proliferation of epithelial cells as well as edema of primary and secondary gill filaments after exposure to 0.25 and 1.5 mg/L for 48 h, while no hepatocellular alterations were recorded, being necrosis and eosinophilic vacuolation minimal in the liver of control or treated fish. In agreement with the results obtained by Griffitt and co-workers, we only observed histological alterations in the gills (Fig. 3).

Gills play a primer role in respiration, and their direct contact with water make them an important tissue for histopathological alterations. The histological examination showed that exposure to both metal forms disrupted the gill epithelium (Table 1). Copper is a well-known respiratory and ionoregulatory toxicant in fish (Grosell et al. 2007). Actually, some studies have already pointed the possibility that Cu based NPs may lead to similar effects (Al-Bairuty et al. 2013). We observed hyperplasia, fusion of the lamellae, epithelial lifting (edema) among other pathologies. All together, these alterations contribute to reduce the surface and to increase the diffusion distance for gas exchange (Al-Bairuty et al. 2013; Hao et al. 2013). Consequently, CuO-poly NPs and ionic copper could compromise oxygen uptake and contribute to a systemic hypoxia. We did also detect branchial aneurisms, which according to Federici and co-workers (2007) may reflect either some local vascular wall injury in the branchial capillary bed or interruptions of capillary flow. Not all the alterations were equally induced by both treatments, suggesting that CuO-poly NPs and ionic copper might affect gill morphology at different extent. In fish exposed to ionic copper, stronger effects have already been reported than in fish exposed to Cu NPs (Al-Bairuty et al. 2013). While the prevalence of some pathologies, such as lamellar hyperplasia, decreased at 6 mpe, the presence of other pathologies did not decrease indicating that long lasting effects may be expected after medium-term exposures.

These results demonstrate that LMS test and histopathological analysis of gill in zebrafish are useful sensitive indicators to assess fish health impairment under exposure to low doses of CuO-poly NPs and ionic copper. Thus, the estimated environmental concentrations of CuO-poly NPs can affect the general health of aquatic organisms.

5.4.3. Genotoxicity

The micronucleous test in fish erythrocytes has demonstrated sensitivity and efficiency to assess genotoxicity in different fish species (Al-Sabti and Metalcafe 1995). In zebrafish, it has been successfully employed to assess the genotoxicity of different compounds including metals (Domingues et al. 2010; Oliveira et al. 2009; Rocco et al. 2012). Copper is known to be genotoxic to fish, although different fish species own different sensitivity to metal treatment (Cavas et al. 2005). We did not detect genotoxic effects after any of the exposures. In agreement with our results, the exposure to soluble copper (10 µg/L) for 21 days in three different fish species did not produce increased presence of MN (Cavas et al. 2005). Nevertheless, the reliability of this assay to test NPs genotoxicity at environmental relevant concentrations should be validated before discarding their genotoxic capability. The use of more sensitive techniques able to detect non-clastogenic damage to evaluate

genotoxicity in zebrafish has already been suggested (Cambier et al. 2010; Orieux et al. 2011). As matter of fact, we detected increased transcription of genes related to DNA damage (see below). We, therefore, suggest being cautious when employing the MN assay in erythrocytes to test the genotoxicity caused by exposure to low concentrations of metal NPs.

5.4.4. Microarray analysis

A total of 97 transcripts were significantly regulated in fish exposed to CuO-poly NPs for 3 days and to ionic copper for 3 weeks. Although this is a small amount considering the whole genome analysis, similar results (82 regulated genes) were reported by Griffitt and co-workers (2007) in the transcriptome analysis of the gill after exposing zebrafish to 10 times higher copper concentration (0.1 mg/L). The expression of hepatic genes in fish after exposure to copper is dependent on the exposure concentration (Santos et al. 2010). Thus, our results indicate that low copper concentrations provided either as NPs or as soluble form own limited capacity to alter zebrafish hepatic transcriptome. Even if no significantly regulated genes were detected after 3 days of exposure to ionic copper or 3 weeks of exposure to CuO-poly NPs, the COA suggests that in zebrafish, the hepatic transcriptomic patterns after exposure to CuO-poly NPs or ionic copper tend to become more similar as exposure time increases. A central assumption of toxicogenomics is that chemicals that produce toxicity by the same mechanism will produce similar transcription responses under a given set of conditions (Fent and Sumpter 2011; Griffitt et al. 2009). Therefore, the similarities observed in the COA after 21 days of exposure could be related to the soluble fraction arising from CuO-poly NPs that after 21 days of exposure may produce a similar response to that obtained for soluble copper.

The transcriptomic response is one the first biological replies when an organism is exposed to a toxic insult (Fent and Sumpter 2011). After 3 days of exposure to CuO-Poly NPs, upregulated “nucleotide biosynthesis”, “glycine biosynthesis”, “response to oxidative stress” and “oxidation and reduction process” related transcripts were identified (Fig. 5A). *Peroxiredoxin 6* (TC378547) contributed to the oxidative stress term. *Peroxiredoxins* have also been upregulated after zebrafish exposure to copper (Craig et al. 2009). Other transcripts, such as *gluthatione-S-transferases,* which are known to play a role in the response to the oxidative stress, were also upregulated (Craig et al. 2007; 2010). Copper is known to produce reactive oxygen species (ROS) in living organisms through Fenton reactions (Prousek 2007). Thus, the increased transcription of genes related to nucleotide synthesis could be explained by the transcription requirements of the biological response to CuO-poly NPs and the oxidative stress arising from the increased ROS presence. Moreover, the upregulation of genes involved in glycine biosynthesis may

also be related to an increased oxidative stress, since glycine is required for glutathione synthesis (Lu 2013). All together, these results indicate that 3 days of exposure to CuO-poly NPs upregulate genes involved in cell protection, that may be responding to a possible oxidative stress.

On the other hand, dowregulation of genes related to "one carbon metabolism", "fatty acid elongation", "developmental processes" and different transmembrane transport processes was observed after 3 day in fish exposed to CuO-poly NPs (Fig. 5B). This could be related to reduction of nonessential processes in favor of essential functions to face the toxic insult (Craig et al. 2010). Depletion of energy reserves has also been reported in other model fish after copper exposure (Santos et al. 2010). Downregulated transcripts, included *carbonic anhydrase 5* and *methionine adenosyltransferase II* (one-carbon metabolism), a member of the *elongation of very long chain fatty acid* family (fatty acid elongation) and *solute carrier 26* (transmembrane transport), which have been reported to be downregulated in presence of copper (Huster et al. 2007; Le et al. 2014). Under external stress, reduction in ion leakage across membrane constitutes a major source of bioenergetic saving (Hand and Hardewig 1996).

Finally, transcripts significantly upregulated in animals exposed for three weeks to ionic copper (Fig. 5C) were mainly linked to DNA damage, indicating the genotoxic capacity of sustained copper exposures. Although in fish exposed to NPs the transcription level of these sequences was not significantly regulated according to the LIMMA analysis, these genes showed the same transcription trend observed in fish exposed to the ionic form (Fig. 5C). Considering that these transcripts were also related to the term "response to hydrogen peroxide", data suggest the feasible presence of DNA damage produced by increased oxidative stress. Other general terms like "signal transduction" and "regulation of cell growth" could be related to the specific transduction requirements and exhaustive control over cell growth provided in response to DNA damage. Interestingly, exposure to ionic copper significantly regulated *cryptochrome 2a* and *period homolog 2,* which are related to "photoperiodism" and "entrainment of circadian clock". Cryptochrome (*Cry*) genes participate in DNA repair, by regulating nucleotide excision mediated repair and by coordinating the circadian clock with DNA damage checkpoints (Fu et al. 2002; Sancar et al. 2010), while period 2 has been proposed to function as tumor suppressor in response to DNA damage (Fu et al. 2002). Thus, our data suggest the implication of circadian genes in the response to DNA damage produced after exposure to environmentally relevant concentrations of copper.

These same transcripts were identified as significant contributors to differences between time points. Disruption of circadian rhythms in freshwater fish

following exposure to some trace metals may provide early warning of their toxicity at realistic environmental concentrations (Styrishave et al. 1995). In fact, loss of circadian rhythm in fish has been described among the effects produced by chronic exposure to copper (Handy 2003). The regulation of circadian rhythm related transcripts after exposure to NPs was first described by Jovanović and co-workers (2011). In zebrafish embryos, *period homolog 2* and several *cry* genes were downregulated 48 h after larvae injection with titanium dioxide NPs (Jovanović et al. 2011). To our knowledge, no studies involving zebrafish and CuO NPs have previously described effects on circadian rhythm related transcripts, and therefore this point should be further verified.

5.5. Conclusions

Zebrafish exposure to CuO-poly NPs and ionic copper at an environmentally relevant concentration lead to copper accumulation and general health impairment identified as reduced labilization periods of hepatocyte lysosomal membrane and disrupted gill morphology. Alhough both metal forms own limited capacity to alter the hepatic transcriptome at the tested concentration, according to the microarray analysis, CuO-poly NPs can produce damage to exposed animals through increased oxidative stress and exposure to ionic copper leads to DNA damage. Genes from the circadian clock are involved in the response given to the exposure to both compounds, being therefore an interesting target for future research focused on the modes of action of metal bearing NPs.

The MN test was not sensitive enough to detect the genotoxicity suggested by the microarray analysis. Therefore, more sensitive techniques should be employed in order to evaluate the genotoxic capacity of metal oxide NPs.

All things considered, our results indicate that CuO-poly NPs could affect aquatic organisms even at current environmental concentrations.

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IV. General discussion

Abbreviations

7,12-dimethylbenz(*a*)anthracene, DMBA

Adverse outcome pathway, AOP

Aryl hydrocarbon receptor, AHR

Benzo(*a*)pyrene, B(*a*)P

Dimethyl sulfoxide, DMSO

Estrogen receptor, ER

Fish embryo test, FET

Labilization period, LP

Lysosomal membrane stability, LMS

Molecular initiating event, MIE

Nanoparticle, NP

Polycyclic aromatic hydrocarbon, PAH

Reactive oxygen species, ROS

Weeks post exposure, wpe

Gene abbreviations

cyclin g1, ccng1

cytochrome P450 family 1 subfamily a, cyp1a

junb proto-oncogene, junb

tumor protein 53, p53

The aquatic environment is continuously loaded with diverse xenobiotics such as organic compounds and metals in different forms, including metal and metal-bearing nanoparticles (NPs), arising from diverse anthropogenic activities. All these substances are potentially toxic to organisms (Scown et al. 2010, Stegeman and Hahn 1994). Some of those pollutants have been present in the environment for decades and considered in water-quality regulations (legacy pollutants), while others (new or historical pollutants) are just starting to be considered (emerging pollutants) (Boxall 2012). The potential damaging capacity of all those substances is not always known and it is therefore necessary to study the impact they may have on the human and environmental health (Deblonde et al. 2011). The effects of toxicants begin at the molecular level and then progress to the biochemical, subcellular, cellular, tissue, organ, individual, and population levels (Van der Oost et al. 2003). In ecotoxicology the application of adverse outcome pathways (AOPs) providing linkage between a direct molecular initiating event (MIE) and an adverse outcome at a biological level of organization has been proposed for risk assessment purposes (Ankley et al. 2010). The development of environmental pollutant-mediated AOPs may enable a better understanding of the effects of contaminants (Lee et al. 2015a). This term comes to provide a framework within which data and knowledge collected at many levels of biological organization can be synthesized in a way that is useful to risk assessors and the ecotoxicologists (Ankley et al. 2010).

The present work aimed to gain a deeper understanding on the effects and the mechanisms underlying the toxicity/carcinogenicity of two major environmental contaminants: polycyclic aromatic hydrocarbons (PAHs) and metal oxide NPs (Fig. 1).

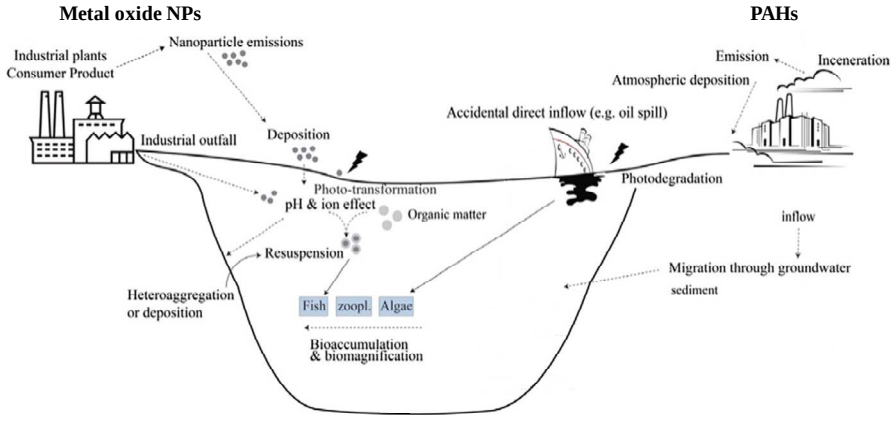

Fig. 1. Fates of NPs and PAHs in the aquatic environment. Modified from Lee et al. (2015a).

PAHs are legacy organic pollutants whose adverse outcome is well known as they are potent carcinogens, while nanomaterials in general and metal oxide NPs in

particular are new emerging pollutants whose potential toxicity is not still completely understood. AOPs for PAHs and certain metal NPs have already been proposed (Fig. 2, Lee et al. 2015a).

In both cases, gaps on the events through which they produce toxic effects still remain. Thus, in order to contribute to gain knowledge on the AOPs of these environmental pollutants, we carried a batch of experiments to analyze their effects on zebrafish (*Danio rerio*), which is considered a reference model organism for toxicology studies (Busch et al. 2011). The objective was addressed at different levels of biological organization, different developmental stages, as well as after acute or longer lasting exposures to obtain a wide view of the effects that these pollutants are likely to produce.

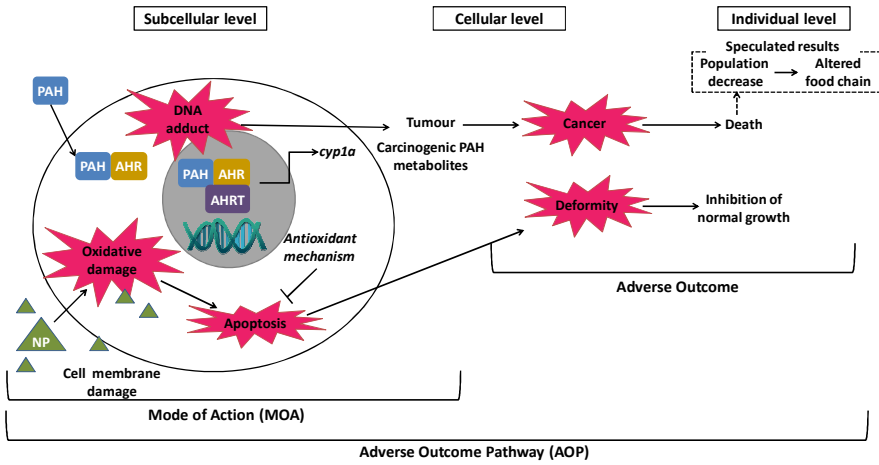

Fig.2. AOPs proposed for PAHs and metal NPs. Modified from Lee et al. (2015a).

It is a matter of fact that PAHs are potent carcinogens able to induce tumor formation through genotoxic and non-genotoxic mechanisms (Luch 2005). In this research work we used two model PAHs, benzo(*a*)pyrene (B(*a*)P) and 7,12-dimethylbenz(*a*)anthracene (DMBA) whose toxicity is known to be directly produced through the non specific interaction with non-polar sites of the cells (narcosis) or by means of their interaction with the aryl hydrocarbon receptor (AHR). Despite the existing information on the toxicity and carcinogenicity of PAHs, there are non-answered questions regarding the processes altered by these compounds and the time-course of these alterations.

When an organism is exposed to a chemical, the biological response depends on the physicochemical properties of the compound, but also on the concentration and duration of the exposure (Boelsterli 2007). In **Chapter I,** we exposed adult zebrafish to different concentrations (0.3, 0.6 and 1 mg/L) of DMBA for 72 h to analyze the acute effects of this strong carcinogen and to find an appropriate concentration for longer term exposures. The acute exposure of adult zebrafish to 1 mg/L of DMBA resulted in a fatal outcome for an 80% of the exposed fish and strongly affected the surviving individuals producing clastogenic DNA damage, as shown by the significantly increased frequency of micronuclei in erythrocytes. Moreover, lower concentrations affected the general health status as shown by the lysosomal membrane stability (LMS) test. These results together with existing literature reporting that 24 h of exposure of zebrafish at larval stage to 0.25 mg/L DMBA produced a variety of tumors after 8 months (Spitsbergen et al. 2000), indicated the suitability of a dose of 0.3 mg DMBA/L for a longer lasting exposure in order to study the early time-course response before the appearance of a tumoral lesion. Thus, in a second experiment we studied and compared the cellular and molecular effects of 0.3 mg/L of B(*a*)P or DMBA after 1 and 2 weeks of exposure (**Chapters I and II**).

It is well known that the strong carcinogenic potential of PAHs arises both from their capacity to directly interact with DNA and from the oxidative stress produced by the overproduction of reactive oxygen species (ROS) during the phase I metabolism (Luch 2005). According to our results, both B(*a*)P and DMBA induced the transcription of cytochrome P450 genes (*cyp1a, cyp1b*), whose proteic products are responsible for PAH bioactivation (Shimada 2006; Xu et al. 2005). Induction of *cyp* genes trough the AHR is considered a MIE that may ultimately result in cancer development (Lee et al. 2015a). In zebrafish, the induction of *cyp1a* transcription after 1 and 2 weeks of exposure to 0.1 mg/L B(*a*)P has already been described, although the strongest upregulation was detected 4 days after the start of the exposure (Lee et al. 2015b). These results indicated that the adaptive response remains active up to 2 weeks of exposure, despite the strongest response occurs earlier in the time course (Maronpot et al. 2010). Nevertheless, PAHs toxicity is determined by the cumulative toxicity with the increasing exposure time (Lee et al. 2002). After 2 weeks of exposure, B(*a*)P and DMBA reduced the general health status identified as a significant reduction of the lysosomal membrane stability (LMS) and provoked peroxisome proliferation in the liver of adult zebrafish. Peroxisome proliferation is an early occurring process that has been related to cancer development in rodents and which is known to precede hepatocyte proliferation (Ockner 2007; Reddy et al. 1980; Reddy and Lalwani 1983), meanwhile lysosomal responses, like reduction of the LMS, have demonstrated prognostic value as early biomarkers of carcinogenesis in fish (Köhler et al. 2002). Laboratory studies have

demonstrated that both B(*a*)P and DMBA leads to the development of carcinogenic lesions in fish 8-9 months after acute exposures (Lam et al. 2006; Spitsbergen et al. 2000; Wang et al. 2010). As expected, we did not detect any carcinogenic lesion after 2 weeks of continued exposure, as cancer needs longer periods of time to develop. However, in fish exposed to DMBA the expression of *junb,* which is related to tissue regeneration, was upregulated. Moreover, results indicate that alterations on the lysosomal membrane stability and peroxisome proliferation could precede more relevant tissue level alterations in zebrafish exposed to any of the two PAHs tested after longer post exposure time periods (Köhler et al. 2002; Reddy et al., 1980; Reddy and Lalwani, 1983; Spitsbergen et al. 2000). Thus, longer lasting experiments could serve to clarify whether reduced LP values and peroxisome proliferation could be incorporated to the AOP leading to cancer development in zebrafish after the MIE of *cyp* transcription induction. According to our results, both PAHs own similar toxic capacity even if the results from the gene transcription analysis suggest stronger capacity of DMBA to induce tissue damage.

A precise understanding of the effects of toxicants on the molecular or biochemical level can provide valuable early warning signals, as opposed to higher level adverse effects that occur later in this chain of progression (Lee et al. 2015a). Thus, to further analyze the early-time course events occurring after 1 or 2 weeks of exposure to B(*a*)P and DMBA, in **Chapter II,** we performed a full genome transcriptional analysis**.** Microarray technology can reproducibly measure changes in the transcriptome of fish exposed to environmental pollutants and has been proved useful in aquatic systems to identify the MOA of toxicants (Hook et al. 2006; 2010). A central assumption of toxicogenomics is that chemicals that produce toxicity by the same mechanism will produce similar expression responses under a given set of conditions (Fent and Sumpter 2011; Griffitt et al. 2009). We found that both PAHs produced similar transcriptomic response that changed from the first to the second week of exposure. Thus, our results agreed with the occurrence of similar initial events after the exposure to both PAHs and reflected the existence of a strong time dependent effect (Lee et al. 2002). Initial events after exposure to both PAHs included the altered transcription of genes involved in the adaptive response to the chemical insult including the previously mentioned *cyp*s. Moreover, cell cycle related transcripts were downregulated after the first week of exposure suggesting cell cycle arrest as part of the compensatory response to the damaging effects of PAHs (Johnson and Walker 1999; Reed and Waters 2005). The arrest was reverted by the second week of exposure. It has been reported that AHR dependent cell cycle arrest might occur in the presence of DNA damaging AHR exogenous ligands, while the persistent exposure could produce an increase of ROS that would provoke DNA damage to the extent that proliferative barriers would be bypassed and responsiveness to the environmental signal compromised (Puga et al. 2002). Our

results also revealed that PAHs alter gametogenesis related genes following the same expression pattern observed for cell cycle related transcripts. We hypothesized that this could be attributed the cross talk between AHR and estrogen receptor (ER) pathways, which might produce endocrine disrupting effects through ER-mediated and ERE independent genomic regulation (Hall et al. 2001). Binding the ER is considered a non-genotoxic mechanism of carcinogenesis and could therefore contribute to the carcinogenic outcome of these compounds (Luch 2005; Sandonato 1997).

Despite the similarity of the response produced by B(*a*)P and DMBA, PAHs are known to induce distinguishable gene transcription profiles depending on their carcinogenic potential and affinity to the AHR (Goodale et al. 2013; Song et al. 2012). These properties are directly related to their chemical structure. DMBA has been described as stronger carcinogen and AHR inducer than B(*a*)P (Baird et al. 2005; Barron et al. 2004). Accordingly, a 16% of the total variability observed in the transcriptomic responses was attributable to the compound specific effect produced by each PAH and significant differences in the transcriptomic responses were detected after the first week of exposure. DMBA affected protein degradation and blood coagulation, while B(*a*)P affected in a deeper way cell cycle regulation described for both compounds. All these processes are known to be altered in cancerous processes (Burger and Seth 2004; Hartwell and Kastan 1994; Monroe and Hoffman 2009). By the second week of exposure, significant differences were not observed between the transcriptomic responses to B(*a*)P and DMBA. The higher resemblance of the responses could be related to a common response to a similar damage that would agree with the results for LMS test obtained in **Chapter I** (Hook et al. 2006).

The pathway analysis indicated that B(*a*)P and DMBA altered mainly metabolism related pathways, which has already been described as a common response to PAH exposure (Castorena-Torres et al. 2008; Song et al. 2012). Effects on DNA and energetic metabolism are known to be a hallmark of cancer cells (Zhang and Yang 2012). In our study, the metabolism of nitrogenous bases required for DNA synthesis and repair together with energetic balance related pathways were affected by both compounds. These pathways further supported the observed compensatory effect and posterior cell cycle upregulation in B(*a*)P exposed fish. Meanwhile, the pathways affected in DMBA exposed fish brought to mind the Warburg effect, a phenomenon occurring in normal proliferating cells and cancerous cells (Lunt and Vander Heiden 2011; Vander Heiden et al. 2009). We suggested that after DMBA exposure, the glucose metabolites could be redirected into the pentose phosphate pathway primarily to produce nucleotides necessary for DNA repair and to cope with the energetic and antioxidant requirements (Fig. 3, Kang et al., 2015; Vander Heiden

et al. 2009). Finally, both PAHs altered glutathione metabolism. In the case of B(*a*)P exposed fish, this could be related with the antioxidant properties of this peptide (Lu 2013; Wu et al. 2003). In DMBA exposed fish, this pathway was downregulated which could be related with apoptotic processes (Lu 2013). All together, data from **Chapter I and II** indicate that both PAHs could contribute to produce suitable biochemical environment for cancer development affecting cell cycle, and disrupting DNA metabolism and energetic balance related pathways in animals exposed for 2 weeks.

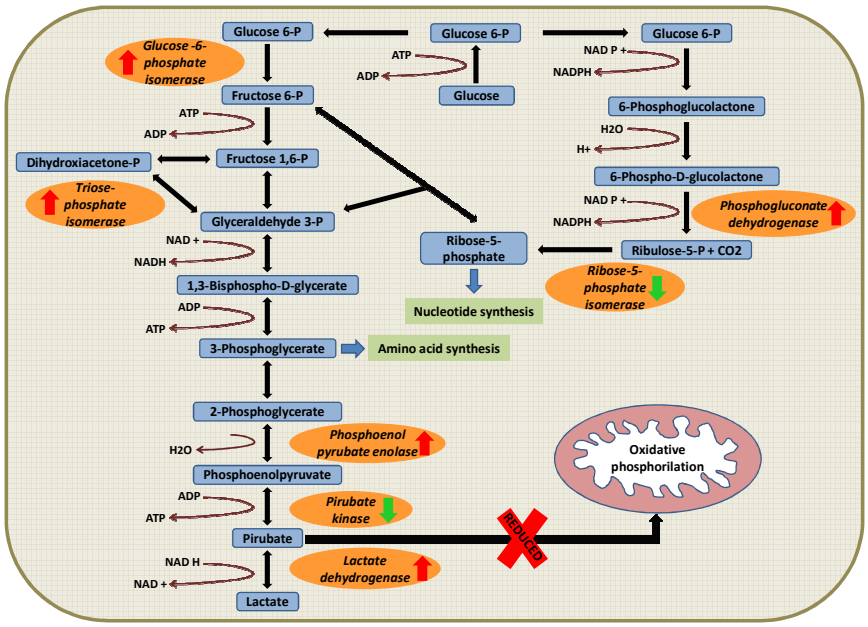

Fig. 3. Glycolysis and pentose phosphate pathways related transcripts regulated after 2 weeks of exposure to DMBA. Red arrows indicate upregulated transcription. Green arrows indicate downregulated transcription.

To further contribute to the knowledge on the effects produced by B(*a*)P and DMBA in zebrafish, in **Chapter III**, we investigated the effects of the exposure of zebrafish embryo (24 h post fertilization) for 24 h to different concentrations (0.3, 0.6 or 1 mg/L) of the individual compounds and to a sequential exposure to B(*a*)P (0.3 mg/L) for 24 h followed by 24 h of exposure to DMBA (0.6 or 1 mg/L). The time-through transcription level of selected cancer related genes together with the clastogenic and histological outcome of those exposures were analyzed up to 12 weeks after the end of the exposures. In agreement with the results from the previous chapters, the transcription of *cyp1a* was strongly upregulated suggesting that B(*a*)P and DMBA may have the capacity to produce tumor initiation at any of the concentrations tested as fast as 24 h post exposure (Luch 2005). The damaging effects of AHR ligands in zebrafish are concentration dependent (Bugiak and Weber 2010). Accordingly, we observed a concentration dependent induction of *cyp1a.* As

reported for adult individuals in **Chapter I**, the exposure to 1 mg DMBA/L was lethal for embryos after 24 h of exposure. B(*a*)P, which induced lower levels of *cyp1a* transcription, appeared to be less toxic as embryos exposed to the same concentration survived. PAHs are complete carcinogens able to produce cell initiation and progression; however repeated exposure to these carcinogens increases the incidence of carcinogenic lesions (Baird et al. 2005). Sequential exposures to B(*a*)P and DMBA could have contributed to the understanding of the initiation and promotion capacities of these compounds. Sequential exposures produced the strongest induction of *cyp1a* as well as the induction of *p53* and *junb* after 24 h, however both treatments resulted lethal by the second week of exposure. Thus the induction of *cyp1a* and the transcription pattern observed for cancer related genes (*p53*, *ccng1,* and *junb*) could be related to the mortality observed in zebrafish embryos. Our data suggested that after an acute exposure to PAHs, the bioavailability (concentration and exposure time) and AHR agonistic strength of each PAH are factors determining *cyp1a* transcription. Moreover, epigenetic changes occurring due to PAHs exposure may have influenced the time-through transcription pattern of the analyzed cancer related genes.

The transcription pattern observed at 3 weeks post exposure (wpe) in DMBA exposed animals for analyzed cancer related genes followed the same transcription pattern observed for *cyp1a* suggesting the existence of DNA damage (Fig. 4). In agreement with results from **Chapter II**, *p53* and *ccng1* transcription suggested that in DMBA exposed fish cell cycle arrest at the G2 to M transition may have occurred in response to DNA damage (Kimura et al. 2001). We did also detect *p53* and *junb* induction at 6 wpe to B(*a*)P, which might also have led to cell cycle regulation (Shaulian et al. 2010).

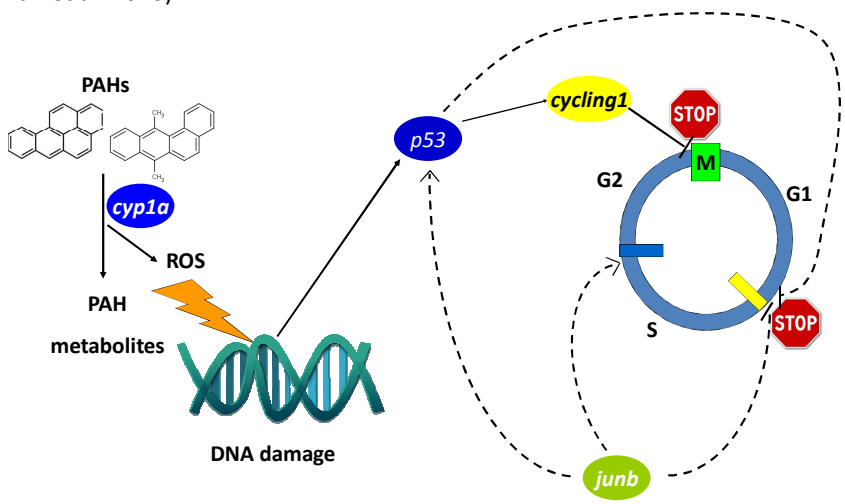

Fig. 4. Molecular interactions of the proteic products of the analyzed transcripts. Direct interactions are represented with solid arrows. Indirect interactions are represented with lined arrows.

In any cell, despite the action of repair processes, some DNA damage may remain, which may lead to disease (Loeb et al. 2003). DNA reactive chemicals like PAHs can directly induce DNA lesions that have the potential to be fixed as point mutations or chromosomal aberrations (Marticorena and Campbell 2005). At 12 weeks after the end of the acute exposure, there were no significantly upregulated transcripts and clastogenic damage was not detected. Thus according to data obtained at 24 hours post exposure and 3 and 6 wpe, the existence of fixed mutations that might contribute to cell initiation could not be discarded although cellular repair mechanism might have acted avoiding the formation of MN (Barjhoux et al. 2014). Moreover, in zebrafish, tumor development after dietary exposure to PAHs has been reported to occur without genotoxic effects being detected (Larcher et al. 2014). In that work, authors argued that a multistage carcinogenic process can result from increased cellular proliferation or through epigenetic alteration like altered DNA methylation (Larcher et al. 2014).

Adverse histological outcomes were detected. The histological analysis revealed the existence of non-carcinogenic tissue alterations. Megalocytosis which is caused by toxicant damage to DNA or the mitotic apparatus following carcinogenic exposure but which has also a baseline presence in untreated fish was present in all the exposure groups but higher prevalence was observed in embryos exposed to 0.6 or 1 mg/L B(*a*)P and in those exposed to 0.3 mg/L DMBA (Spitsbergen and Kent 2003). Hepatocyte vacuolization was also observed supporting the long term damaging capacity of exposures produced at the embryo stages. Thus, data suggest that acute exposure to PAHs at embryo stages may lead to late adverse outcomes that might be due to their non-specific effect or the non-clastogenic DNA damage arising from their bioactivation.

Together with legacy pollutants like PAHs, water bodies are continuously supplied with new pollutants arising from anthropogenic activities (Hansen 2007). During the last decades, nanomaterials have been incorporated into a number of products for industrial and household applications. Metal oxide NPs are particularly attractive for a number of products and are consequently produced and spilled into the environment in a considerable amount (Chang et al. 2012; Djurisić et al. 2015). When a novel chemical is produced and disposed to the natural media, its toxicity must be analyzed in case such spills bring forth negative impacts on the environment (Lee et al. 2010; Thomas et al. 2011). The soluble and bulk forms of these metals are toxic to aquatic organisms (Bernardeschi et al. 2010; Clearwater et al. 2002; Grosell et al. 2007), implying that potential adverse effects over biota could also exist for the NP forms. Such effects, given NPs unique properties, could be different from those caused by the ionic and bulk forms (Maurer-Jones 2013). **Chapters IV and V** were intended to analyze the toxicity of metal oxide NPs, whose AOPs are still to be

completely characterized. Metal oxide NPs are known to cause different adverse outcomes, including tissue damage, acute lethality or induction of ROS production (Lin et al. 2011; Zhao et al. 2013; Zhu et al. 2008, 2009). To contribute to the existing knowledge on metal oxide NPs, a two tier approach was designed.

In the first step of our strategy (**Chapter IV**), three different metal oxide NPs of wide use in consumer products (CuO, ZnO and TiO_2) were analyzed in comparison to their equivalent soluble and bulk counterparts using zebrafish embryos. When AOPs for pollutants toxicity assessment have been developed, increased lethality and malformation rates are among the considered outcomes (Volz et al. 2011). The fish embryo test (FET) allowed establishing a toxicity ranking for the studied NPs based on the sublethal parameters analyzed. CuO-poly were the most toxic NPs. Their deleterious outcome (hatching delay and increased malformation prevalence) was observed at concentrations of 1 mg Cu/L, followed by ZnO NPs that caused deleterious effects (hatching delay) at concentrations of 5 mg Zn/L, while TiO_2 NPs were the least toxic and their toxicity was attributed to the surfactant present in the NP suspension. We identified solubility as a key parameter driving the toxicity of these metal oxide NPs. CuO-poly and ZnO NPs, have been shown to be more soluble than TiO_2 NPs (Johnston et al. 2010; Shaw and Handy 2011). According to Muller and co-workers (2015), CuO NPs solubility and bioaccumulation are key parameters affecting the AOP leading to hatching impairment in zebrafish embryos. Both CuO-poly and ZnO NPs reduced the hatching rate in embryos exposed to 10 mg/L and delayed hatching at lower concentrations. Metal oxide NPs ability to affect hatching has been demonstrated to be dependent on their solubility, since metal ions released from relatively soluble metal oxide NPs such as CuO and ZnO can fit the active site of the zebrafish hatching enzyme (metalloprotease ZHE1) (Lin et al. 2012). All together, data indicated that NPs chemical composition, which determines parameters like solubility, along with the additives present in the suspensions are key factors affecting NPs toxicity.

When nominal exposure concentrations were considered, the ionic forms of copper and zinc resulted significantly more toxic than the CuO-poly and ZnO NPs, respectively. Thus, ions arising from NPs could be the major responsible for their toxicity. The lower toxicity of copper-bearing NPs when compared to their ionic form has previously been described in zebrafish at different developmental stages (Chen et al. 2011). CuO-poly NPs exerted sublethal toxicity at lower concentrations than bulk CuO, while ZnO NPs produced similar effects to bulk ZnO on the studied endpoints. The higher toxicity of metal oxide NPs compared to their bulk counterparts could be related to precipitation, which in the case of embryos, leads to the accumulation of NPs in the surface of the chorion (Yu et al. 2011). Metal accumulation in tissues detected by autometallography matched the toxicity pattern described above,

further supporting the idea that solubility is a key parameter determining NPs toxicity. Overall we concluded that at the currently estimated environmental concentrations (Chio et al. 2012; Gottschalk et al. 2009), none of the tested NPs should pose a risk to the aquatic environment. Nevertheless, before concerns on metal oxide NPs toxicity can be discarded, there are other several factors to be considered. Longer lasting exposures may produce stronger effects as copper accumulated in tissues. Moreover increased production and release of NPs are expected to occur in future years (Maynard et al. 2006). Therefore, although the concentrations tested in this work were over the currently estimated environmental concentrations, our results indicated that a future increase in CuO, ZnO or TiO_2 NPs environmental concentrations may turn into an environmental issue. In addition, usually metals appear in the environment as complex mixtures, and therefore, the total toxicity risk should be taken into account.

Based on the results from **Chapter IV** we selected CuO-poly NPs for the second step of our approach to further analyze their effects in a longer-term experiment with adult organisms (**Chapter V**). CuO NPs are relatively new pollutants in the environment. Thus, in this case we selected an environmentally relevant concentration (Chio et al. 2012) to test the effects of CuO-poly NPs in adult zebrafish in comparison with the effects of ionic copper. We employed a nominal concentration of 10 µg Cu/L in the exposures and samples were collected at 3 and 21 days of exposure and at 6 months post-exposure in order to study the possible reversibility of the effects found during exposure or the potential development of long-term effects such as histopathological alterations, including cancer. As it was observed in **Chapter IV**, animals exposed to ionic copper accumulated higher amount of metal. This finding has been previously described in other studies indicating higher bioavailavility of the ionic form than of the NP form, and the risk of prolonged exposures (Griffitt et al. 2009). As it occurred with exposures to PAHs, the LMS test indicated the existence of significantly impaired general health status in fish exposed to both metal forms. Reduction on the LMS is therefore a common occurrence in zebrafish after exposure to organic and metallic compounds (**Chapert I and V)**. We did not detect liver toxicity at the histological level, but histopathological alterations on gill structure were detected in animals from both exposure groups in all the analyzed time points. Consequently, exposure to CuO-poly and ionic copper may reduce gill functionality. Copper is a well-known respiratory and ionoregulatory toxicant in fish (Grosell et al. 2007). Actually, some studies have already pointed the possibility that Cu based NPs may lead to similar effects (Al-Bairuty et al. 2013). Gill alterations contribute to reduce the surface and increase the diffusion distance for gas exchange (Al-Bairuty et al. 2013, Hao et al. 2013). Consequently CuO-poly NPs and ionic copper could compromise oxygen uptake and contribute to a systemic hypoxia.

In agreement with the absence of histological effects in liver, the exposures altered slightly the hepatic transcriptome. The analysis suggested that CuO-poly NPs may produce oxidative stress and a reduction on metabolism and transport processes after 3 days, while ionic copper seems to produce DNA damage after 21 day of exposure. The analysis suggested that the responses produced by the ionic and the nano forms tended to become similar after 21 days. Results also suggested the existence of DNA damage that was not visualized as clastogenic outcome. The absence of genotoxic effects is in agreement with similar studies conducted with other fish species (Cavas et al. 2005). Nevertheless, the reliability of this assay to test NPs genotoxicity at environmental relevant concentrations should be validated before discarding genotoxic potential (Cambier et al. 2010). Overall, data indicated that exposure to environmental concentrations of copper led to impaired health status and metal accumulation that may result in adverse outcome after longer lasting exposures.

This PhD study attempted to fill the gaps in our limited understanding on the effects elicited by exposure to PAHs and metal oxide NPs in zebrafish and served to gain knowledge on molecular and cellular mechanisms that could lead to different adverse outcomes. Moreover, data presented herein indicate that some of the effects observed after PAH and CuO NP exposures are similar, suggesting that these pollutants may share some of the stages in their respective AOPs. Common observations included reduction of the stability of the lysosomal membrane, and the upregulated transcription of DNA damage related transcripts that were not turned into clastogenic damage. Anyhow, unraveling the mechanism responsible of the effects produced by these pollutants is only a first step to understand the environmental impact they may pose. Today environmental pollution is a complex reality where thousands of contaminants of different origin and chemical characteristics coexist (Geissen et al. 2015). Consequently, the effects that a given substance might produce into an organism is not solely determined by its characteristics, but by the interactions with the surrounding pollutants (Fan and Lin 2011). As recently highlighted by Rocha et al. (2015), the research focus on nanomaterials toxicity has changed to a more environmentally realistic point of view, taking into consideration the interaction of nanomaterials with other contaminants. Thus, future works should address the AOPs and effects of combined exposures to different classes of pollutants.

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IV. CONCLUSIONS

AND THESIS

Conclusions

In view of the results obtained during this investigation the following conclusions have been obtained:

I. Short- and medium-term exposures to PAHs produced toxic effects on adult zebrafish, visualized as alterations in the studied molecular and cellular level biomarkers. Medium-term exposure to both PAHs produced impairment of general health status likely caused by the activated PAHs and the reactive oxygen species arising from the phase I xenobiotic metabolism. Increased oxidative stress arising from peroxisome proliferation may also have contributed to the impairment of the general health status. At the tested concentrations, two weeks of exposure to the tested PAHs did not produce relevant tissue level effects. Longer lasting experiments are required to identify adverse outcomes at the tissular level.

II. Zebrafish exposure to B(*a*)P and DMBA produced highly similar gene transcription profiles in the liver. Due to the scarce differences between the effects produced by both PAHs, exposure time was a major factor determining transcriptomic profiles after exposure to these PAHs. According to the transcriptomal analysis, both PAHs may have been activated to carcinogenic compounds through the xenobiotic metabolism producing DNA damage that affected the transcription of genes related to DNA metabolism and cell cycle, which were strongly downregulated after the first week of exposure and upregulated after the second week.

III. PAH-specific responses were only detectable after the first week of exposure suggesting that damage produced by longer exposures leads to higher similarity in the transcriptomic profiles. B(*a*)P affected specially genes related to the cell cycle, while DMBA exposure affected to a higher extent proteolysis and blood coagulation. These results together with results from the KEGG analysis which identified pathways related to DNA damage and proliferative requirements in B(*a*)P and revealed the need of DMBA exposed animals to face DNA repair indicated that both compounds could ultimately provide a suitable environment for cancer development through slightly different MOA.

IV. At the concentrations and exposure times assayed, sequential exposures to DMBA and B(*a*)P were lethal to zebrafish embryos and, thus, lower concentrations should have been employed to test the carcinogenic and promoter capacity of these PAHs. Both B(*a*)P and DMBA were able to strongly induce the transcription of *cyp1a* at 24 hpe embryos, suggesting the appearance of damaging metabolites. The sustained effects on this gene transcription may be related to the bioavailability and

AHR affinity of each PAH. Other mechanisms, such as epigenetic modifications produced by PAHs should be further explored.

V. Higher percentages of hepatic megalocytosis and vacuolization were observed at 12 weeks post exposure suggesting that PAH exposures at the embryo stages may lead to tissular alterations in the adulthood.

VI. The toxic effect of NPs on developing zebrafish embryos was a function of their chemical composition; CuO NPs were the most toxic of the NPs tested in this study and TiO_2 the least toxic. In the case of TiO_2 NPs, toxicity was linked with the presence of DSLS surfactant in NPs suspensions. Thus, special attention should be paid to the chemicals used in NP formulations.

VII. When assessing NPs toxicity using the zebrafish embryo model, it is important to consider not only mortality, but the sublethal effects produced by the exposures, otherwise, NPs toxicity could be underestimated.

VIII. Zebrafish exposure to CuO-poly NPs and ionic copper at an environmentally relevant concentration lead to copper accumulation and general health impairment identified as reduced labilization periods of hepatocyte lysosomal membrane and disrupted gill morphology.

IX. Both metal forms owned limited capacity to alter the hepatic transcriptome at current environmental concentrations. Genes from the circadian clock were involved in the response given to the exposure to both compounds, being therefore an interesting target for future research focused on the modes of action of metal bearing NPs.

X. The MN test was not sensitive enough to detect the genotoxicity suggested by the microarrays analyses after exposures to PAHs or metal oxide NPs. Therefore, more sensitive techniques should be employed in order to evaluate the genotoxic capacity of environmental pollutants in zebrafish.

Thesis

PAHs and metal oxide NPs are toxic to zebrafish both at embryo and adult stages, causing similar but distinguishable responses at molecular level. Altered transcription of xenobiotic metabolism and cell cycle related genes is a common feature of animals exposed to B(*a*)P or DMBA. Moreover, PAHs produce mortality at high doses and impair the general health status in adult zebrafish. Metal oxide NPs toxicity is different depending on the chemical compound and differs from their bulk and ionic counterparts. Overall at equal nominal concentrations, metal oxide NPs are less toxic than the equivalent soluble forms. Both classes of pollutants share some of the stages in their respective adverse outcome pathways. Common observations included reduction of the general health status and the upregulated transcription of DNA damage related transcripts.