The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: from morphological to molecular approach

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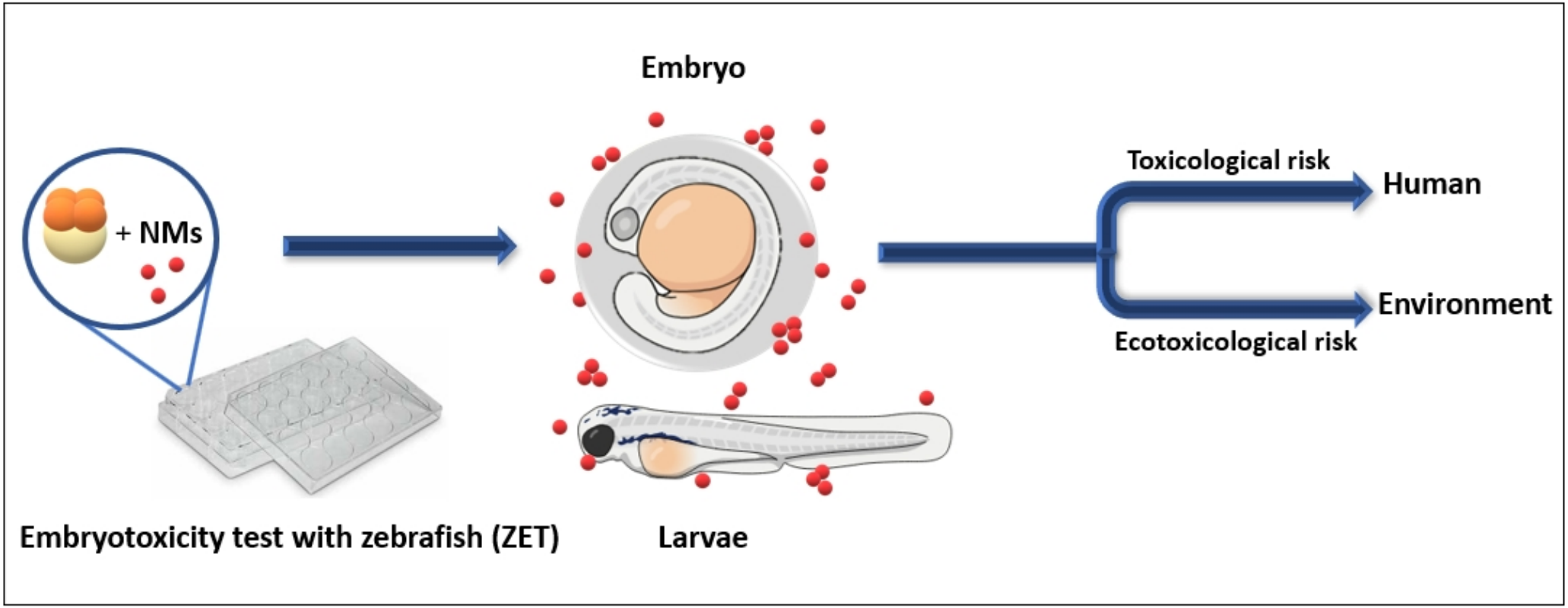
Highlights

- Systematic review on the use of the ZET in NM toxicity assessment.
- ZET shown to be an excellent test to assess NM toxicity.
- Inorganic NMs more studied (90 %) than organic NMs (10 %).
- Morphological alterations induced by NM were classified in reaction patterns.
- Further studies on NM toxicity under environmental conditions are suggested.


Embryo
+ NMs
Toxicological risk
Human
Environment
Ecotoxicological risk
Embryotoxicity test with zebrafish (ZET)
Larvae

ABSTRACT

Nanotechnology and use of nanomaterials (NMs) improve life quality, economic growth and environmental health. However, the increasing production and use of NMs in commercial products has led to concerns about their potential toxicity on human and environment health, as well as its toxicological classification and regulation. In this context, there is an urgent need to standardize and validate procedures for nanotoxicity testing. Since the zebrafish embryotoxicity test (ZET) has been indicated as a suitable approach for the toxicity assessment of traditional and emergent pollutants, the aim of this review is to summarize the existing literature on embryotoxic and teratogenic effects of NMs on zebrafish. In addition, morphological changes in zebrafish embryos induced by NMs were classified in four reaction models, allowing classification of the mode of action and toxicity of different types of NM. Revised data showed that the interaction and bioaccumulation of NMs on zebrafish embryos were associated to several toxic effects, while the detoxification process was limited. In general, NMs induced delayed hatching, circulatory changes, pigmentation and tegumentary alterations, musculoskeletal disorders and yolk sac alterations on zebrafish embryos. Recommendations for nanotoxicological tests are given, including guidance for future research. This review reinforces the use of the ZET as a suitable approach to assess the health risks of NM exposure.

A capsule: A critical review about the use of the zebrafish embryotoxicity test (ZET) on nanotoxicity assessments.

Key words: *Danio rerio*; nanoecotoxicity; teratogenicity; nanoparticles.

1. Introduction

The development of nanotechnology allowed the use of nanomaterials (NMs) in several products, and consequently their release in more than 10.000 tons per year. By 2050 a significant increase in the NM concentration is estimated in fresh and marine waters, sediments and soils (Giese et al., 2018). Despite the increasing production and use of NMs, their toxic effects on the aquatic environment and human health remain unclear (Kahn et al., 2017; Kobayashi et al., 2017). Furthermore, there is a lack of toxicological and ecotoxicological data for commercial NM-enable products, as well an increased concern regarding their toxicological classification and regulation (Bundschuh et al., 2018).

Aquatic toxicity testing is stipulated for environmental hazard and risk assessment by regulatory frameworks [e.g. Organisation for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO)], being the fish embryo toxicity (FET) test (OECD 236) one of the examples indicated in the regulatory context of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) (Busquet et al., 2014). The most commonly fish species used for FET is the zebrafish *Danio rerio*. Zebrafish have many characteristics that make it favourable to serve as model organisms in nanotoxicity tests, such as small size, easy to keep in laboratory conditions, high egg production and rapid development. Furthermore, zebrafish´s genetic material is similar to humans, which ensures a similarity between developmental processes, cell signalling, cell structure, anatomy and physiology with vertebrate species (Hill et al., 2005; Howe et al., 2013; Bambino and Chu et al., 2017).

The literature provides an increasing number of studies about the use of zebrafish adults as model system in the nanotoxicology (Griffitt et al., 2013; Bugel et al., 2014; Chakraborty et al., 2016; Haque et al., 2018; Hou et al., 2018). However, most of these studies do not account the differential toxicity during the early development, the use of molecular and genetics technologies associated to embryotoxicity tests, as well as the classification of morphological alterations on zebrafish embryos to support both environmental risk assessment and hazard classification. In this context, to better understand the effect of NMs on environmental and human health, recent studies have tented to use of zebrafish embryos for nanotoxicity assessment (George et al., 2011; Lin et al., 2013; Chakraborty et al., 2016; Haque and Ward et al., 2018).

The zebrafish embryotoxicity test (ZET) has been indicated as an excellent model to evaluate the toxicity of chemicals (Lammer et al., 2009; Beekhuijen et.al., 2015; Sobanska et al., 2018), such as NMs (Hanque and Ward, 2018). However, the ZET was not initially designed to assess the toxicity of NMs, generating concerns regarding the validity and accuracy of its results. Thus, it is essential to determine the parameters for the execution and determination of nanotoxicity using the ZET as a model. Accordingly, the aim of the present review was to summarize the embryotoxic and teratogenic effects induced by the different NM types using ZET as a model. Test conditions, such as exposure time, exposure medium, exposure chambers were considered, as well as types of NMs, physicochemical properties and concentrations used. Hatching rate, teratogenicity, LC_{50} (Median lethal concentration), EC_{10} (Effect Concentration 10 %) and EC_{50} (Effect Concentration 50 %) were also taken into consideration. In addition, changes in zebrafish development stages induced by exposure to NMs were classified into reactional models, as a means to characterize the mechanisms of action (MoA) and toxicity of NMs.

2. Methodology

A literature review was performed in February to December 2018 using the Web of Science, Science Direct and PubMed database, in which papers published between 2007 and 2019 were considered. The keywords “embryotoxicity”, “embryo” and “zebrafish” were combined with “nanoparticle” or “nanomaterial”, in both singular and plural forms, to retrieve data records in the database. Technical reports, academic theses or abstracts in scientific events were not included. A total of 78 papers were compiled in terms of year of publication, type of NMs, physical and chemical properties, experimental design (i.e. exposure time, concentration and exposure system) and endpoints used. The morphological alterations on zebrafish embryos and larvae induced by NMs were classified into four reactional pattern (Rp): circulatory changes (Rp_1), pigmentation and tegumentary changes (RP_2), musculoskeletal disorders (Rp_3) and yolk sac alterations (RP_4), such as described in the Table 1.

3. The use of ZET in nanotoxicological research

The first paper about the toxic effects of NMs on zebrafish embryos was published in 2007 (Cheng et al., 2007), which described the toxicity of carbon nanotubes (CNTs) during 96 h of exposure. The CNT accumulation in the zebrafish chorion was associated to delayed hatching at 120 mg L^{-1}. After this, the annual production of papers about the use of ZET on nanotoxicological research showed an increasing trend, especially after 2013 (Fig. 1). The revised data showed that this growth was directly linked to the increase in knowledge about the molecular biology of *D. rerio*. The sequencing of the zebrafish genome, initiated by the Sanger Institute in 2001 (Howe et al., 2013) has enabled increased research on zebrafish genes similar to those of humans and other vertebrates (Rubinstein, 2003; Kelkar et al., 2014). The development of OMIC techniques has led to an increase in the application of the ZET in nanotoxicological research, especially after 2000 (Dooley and Zon, 2000; Rubinstein, 2003; Moro et al., 2007; Deng et al., 2009; Meyer et al., 2018). Furthermore, in 2013 the OECD recognized the use of the FET as an official guideline to assess the effects of chemicals (OECD, 2013).

According to OECD test guideline 236 (OECD, 2013), zebrafish embryos at the blastula stage (≤ 3 hpf) are exposed to five increasing concentration of the test chemical and control during 96 h. Every 24 h, the toxicity is recorded in terms of coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. At the end of exposure (96 h), the LC_{50} is estimated, the frequency (%) of endpoints recorded, jointly with physical-chemical properties of the exposure medium. To validate the test, some criteria are needed, such as fertilisation rate of all eggs collected ≥ 70 %, water temperature (26 ± 1 ºC) and dissolved oxygen concentration (≥ 80 %) maintained constants, survival rate and hatching rate of negative control ≥ 90 and ≥ 80 %, respectively after 96 h of exposure. However, the initial ZET protocol does not include endpoints about the sublethal effects. In this context, several modifications on ZET have been proposed, such as spontaneous contraction frequency and heart rate, sublethal endpoints associated to growth, neurodevelopment, cardiovascular development and functions (Babić et al., 2017; Krzykwa et al., 2018), and transgenic fish with fluorescent proteins and toxicogenomic approaches (Li et al., 2018),

In addition to including the 3 Rs (Reduction, Substitution and Refinement), the revised data indicated that the ZET has several advantages in nanotoxicity assessment, such as good reproduction captivity (production of a large number of embryos in a single reproduction), external fertilization, low cost, the need for small amounts of NMs,

reduced exposure time, optically transparent embryos which facilitates the visualization of their development, short life cycle and rapid embryonic development, rapid phenotype discovery, genetic tractability, and cost-effective and ethically acceptable animal models for NM screening. In addition, the revised data showed that the ZET allows the evaluation of chronic responses, teratogenicity, cardiotoxicity, genotoxicity, muscle and bone disorder, phenotypic screens to identify gene function, ototoxicity, developmental genetics, neurobehavioral toxicity, organ specific toxicity (i.e. hepatotoxicity and nephrotoxicity), reproductive toxicity, endocrine disruption, oxidative stress and environmental risk assessment (Fig. 2).

Initially, the nanotoxicological studies mainly addressed morphological aspects, hatching delay and mortality evaluation throughout the early developmental stages of the zebrafish (Asharani et al., 2008; Bar-llan et al., 2009). However, the advancement of molecular biology allowed the mapping of mammalian homologous using zebrafish genes for the identification of molecular biomarkers. This allowed a better understanding of alterations in gene expression and biological responses induced by NM exposure. Among the molecular techniques applied to nanotoxicological studies, the following stand out: electrophoresis, RT-PCR (Barilan et al., 2011; Zhao et al., 2013; Wang et al., 2014; Massarsky et al., 2014; Miao et al., 2015; Gao et al., 2015, Cui et al., 2016; Zhao et al., 2016; Du et al., 2016; Ramachandran et al., 2017; Duan et al., 2017; Nikapitiya et al., 2018; Li et al., 2018), Enzyme Linked ImmunonoSorbent Assay - ELISA (Zhao et al., 2013), inductively coupled plasma mass spectrometry - ICP-MS (Muth-Kohne et al., 2013; Zhang et al., 2018) , RNA-Seq, qRT-PCR, Whole-Mount *In Situ* Hybridization - WISH (Cheng et al., 2007; Cui et al., 2016; Zhang et al., 2018), Intracellular Reactive Oxygen Species (ROS) Assay (Wang et al., 2014; Faria et al., 2014; Fang et al., 2014; Ganesan et al., 2015; Ahmad et al., 2015; Yuan et al., 2016; Duan et al., 2016; Thit et al., 2017; Zhang et al., 2018; Li et al., 2018), Western-blot (Wang et al., 2014; Duan et al., 2017), radioimmunoassay (Du et al., 2016), reporter genes and cloning (Barilan et al., 2011)

Advances in molecular biology and genetics have provided an understanding of the initial responses and mechanisms of action of NMs in zebrafish, especially in adults (Griffitt et al., 2013; Chakraborty et al., 2016; Haque et al., 2018; Hou et al., 2018), while the association between genotypic and phenotypic data from early developmental stages is scarce. The recent advances in the development of OMICs technologies associated to

ZET, such as genomics, transcriptomics, proteomics and metabolomics, have provided rapid nanotoxicity screens with zebrafish embryos (Fako and Furgeson, 2009; Choi et al., 2016; Della Torre et al., 2018) (Fig. 3). In this context, the use of OMICs technologies to assess the ecotoxicological and health impacts induced by NMs is an emergent research in the environmental OMICs.

4. Types of NMs assessed through the ZET

The ZET has been in majority applied to study the ecotoxicity of inorganic NMs (89 %) in contrast to organic NMs (11 %) (Fig. 4). Among the inorganic NMs, the most studied were metal oxides (34 %) and metal NPs (31 %), followed by SiO_2 NPs (19 %) and quantum dots - QDs (5 %) (Fig. 4). Metal and metal oxide NPs have been studied most frequently due to its numerous applications in the food industry (Singh et al., 2017), pharmacy (Mody et al., 2010), biomedicine (Salata, 2004), development of biomaterials (Hamouda, 2012), groundwater and soil remediation, among others (Rajan, 2011). Similar nanoecotoxicological data was reported for other fish species (Kashiwada, 2006), bivalves (Rocha et al., 2015, 2017), microcrustaceans (Castro et al., 2018) and algae (Becaro et al., 2015), indicating that studies about the ecotoxicity of organic NMs on aquatic organisms are still needed.

Among the inorganic NMs studied, Ag NPs (24 %) and SiO_2 NPs (19 %) were the commonly used for the ZET (Fig. 4). Ag NPs have aroused interest of the scientific community due to their suitable technological properties, such as high conductivity, high catalytic degree, high surface area, and antimicrobial and anti-inflammatory activity (Tian et al., 2007). Ag NPs are also widely used in commercial products used in biomedicine, such as tissues, implants, prostheses, surgical instruments, catheters, bandages and hydrogels (Xu et al., 2012). SiO_2 NPs have also been extensively studied, since these particles are used in the biomedical area due to their specific surface characteristics, porosity and functionality. These NPs are used as drug delivery systems, acting as contrast agents in the detection and separation of biomolecules (Bitar et al., 2012).

TiO_2 NPs (13 %) and ZnO NPs (12 %) represent the second group of metal oxide NPs most studied (Fig. 4). Because TiO_2 NPs are more efficient in UVB and ZnO in the UVA range, both NPs are commonly combined for the manufacture of sunscreens that guarantee greater UV protection (Smijs and Pavel, 2011; Lu et al., 2015). ZnO NPs also have antimicrobial activity, being used in the production of paints, fabrics and sprays

(Padmavathy and vijayaraghavan, 2008), while TiO_2 NPs are widely used in the removal of micropollutants in water treatment (Mahmoud et al., 2017).

From the remaining metal and metal oxide NPs, Au NPs (5 %), CuO NPs (4 %) and QDs (5 %) represent the least studied. The same can be said for other types of NMs, which account for only 13 % of the published studies (Fig. 4). Although the OECD acknowledges aluminium oxide NPs, dendrimers and nanoclays on the priority list of manufactured NMs for the assessment of toxicology and risk to human and environmental health (OCED 2010), there is currently not data available about its toxicity using the ZET (Fig. 4; Table S1), reflecting a significant gap of knowledge regarding this priority NMs.

5. Experimental design

Existing OECD guidelines on toxic testing of chemicals are not always suitable for NMs assessment. Thus, standard ecotoxicity testing with NMs often require modifications in experimental design (e.g. exposure media modification) to address specific NMs behaviour, which can have tremendous and unpredictable impacts on the results obtained. However, the modifications incorporated in these studies are not always clearly stated, and in conjunction with varying conditions, NMs types, size and surface functionalization, make comparisons between studies very difficult (Petersen et al., 2015).

Revised data showed a lack of standardization in the experimental protocols applied for nanotoxicity assessment using the ZET (Table S1; Fig. 5), which difficult the consistency in interpreting and comparing results and drawing conclusions. Several factors can interfere in toxic assessment of NMs using the ZET, such as exposure time, exposure chamber, exposure medium, temperature, pH, concentrations, exposure method (static and semi-static) and use of solvent, as previously reported for other pollutants (Lammer et al., 2009; Beekhuijen et al., 2015; Truong et al., 2011). For this reason, the development of standards protocols is required for the use of the ZET in a nanotoxicological context to maximize test consistency.

The exposure time in the ZET varies greatly in the revised studies (Table S1), from 48 (4 %) to 336 h (1 %), with the majority of the works (32 %) using 96 h (Fig. 5A). Zebrafish has a rapid development, and the early stages of embryonic development are completed within the first 24 hours post-fertilization (hpf), while the larvae is formed after 120 hpf (Kimmel et al., 1995; Giannaccini et al., 2014). In this context, an exposure

length between 120 and 144 hpf is indicated as optimal and covers the development stages as organogenesis, yolk consumption and swimming behaviour.

The selection of exposure chambers can also impact the ecotoxicological outcome of testing with NMs. Increasing the consistency of the exposure chamber dimensions (material, size, aspect ratio, internal surface area) is known to reduce differences in the rate of MN agglomeration, settling, dissolution, or sorption, however, a single type of test vessel may not always be suitable for all types of MNs (Petersen et al., 2015). Regarding the revised data, 29 % of the studies used 24-well microplates with one embryo in each well, such as was recommended by Lammer et al. (2009) and Beekhuijzen et al. (2015). Many studies used 96-wells microplates (25 %) and 6-wells microplates (12 %), while 17 % used petri dishes (25 mL) containing the embryos (10 to 20) or beakers (200 to 500 mL) containing a large number of embryos (≈400) (Fig. 5B). However, these exposure chambers are not recommended, because unviable embryos can affect viable embryos if kept together (Beekhuijzen et.al., 2015).

Another modification in ecotoxicity testing using NMs is related to the composition of test medium. The FET OECD guideline 236 allows for a flexibility in the selection of dilution water, as long as properly characterized and incorporated in the test as negative controls and internal plate controls (OECD 2013). However, for MNs toxicity testing, this flexibility can difficult comparison between test results, especially for studies that use the same basic test method (Petersen et al., 2015). Regarding the exposure medium reported in literature (Table S1, Fig. 5C), 65 % of the studies did not mention which medium was used, while the E3 medium was used by only 30 % of the studies, as recommended by the OECD guideline 236 (OECD, 2013). Few studies (5 %) have used other media types, such as dechlorinated water. It is widely recognized that NMs can interact with different components of the exposure media, such as proteins, metal ions, lipoproteins and coagulation factors (Saptarshi et al., 2013), making the choice of a suitable exposure medium one of the critical points to consider when conducting this type of studies.

The physico-chemical properties of the medium (e.g. temperature, pH, oxygen, etc.) are of importance to the toxic assessment in the ZET. According to OECD test guideline 236, water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test for it to be considered valid (OECD, 2013). However, according to the published studies, the temperature used varied between 25 and 30 °C, with 53 % of studies conducted a temperature of 28 ± 1 °C (Fig. 5D). Beekhuijen et al. (2015) showed

that an increase in temperature causes an accelerated development of the zebrafish embryos, in which temperatures higher than 28 °C have been associated with an increase in the number of malformations. An increase in temperature could also induce evaporation of the test solutions, causing interference in the maintenance of the nominal test concentrations during the exposure period. Another important factor to consider is the pH of the exposure medium, since pH and ionic strength combined with NMs characteristics (such as area and surface charge), considerably affect NMs behaviour in the exposure medium and consequently its toxic potential (Clement et al. al., 2017). In addition, reducing the ionic strength or adjusting the pH of the dilution water may reduce the rate of aggregation and deposition for many MNs but may be physiologically stressful for the zebrafish (Petersen et al., 2015). Published studies describe a pH range of 6.5 to 7.5, which is in accordance with the range stated in OECD test guideline 236 (OECD, 2013). The impact of modifications in the test medium when using the ZET further highlights the need for standardization of this test for nano-toxicological evaluations.

The principles of the FET test are based on four apical observations recorded as indicators of lethality during the exposure period, after which an LC_{50} is calculated (OECD, 2013). For this reason, at least 5 concentrations by a constant factor not exceeding 2.2 should be tested to obtain a reliable dose-response curve, especially for data associated with lethality (OECD, 2013). Nonetheless, the revised data showed that 52 % of the studies used less than 5 concentrations of NMs (Fig. 5E). Another potential modification to standard ZET test procedures is the frequency on which test media should be changed during a test with NMs. Three types of exposure method have been employed in the ZET studies published: static, semi-static and flow (Fig. 5F). The choice of the exposure method depends on the stability of the concentrations tested during exposure, which for NMs has been a highly discussed parameter in terms of experimental design (e.g. Handy et al., 2012). The primary objective of frequent media changes is to ensure that exposure and nominal test concentrations are maintained by increasing stability of NMs, as several NMs are known to change particle size/shape through aggregation, dissolve or sediment within short periods of time. The most used exposure method in literature was the static one (52 %), i.e. without renewal of the medium, and only 48 % of the studies renewed the medium every 24 h, 45 % of which renewed the whole medium and 3 % renewed only half (1 mL). In short acute tests, replacing the test media is optional, however this should need to be done more frequently when using NMs in comparison with traditional chemicals. Nonetheless, this can be overcome if a thorough

characterization of the NMs is performed in the test medium beforehand to confirm alterations in particle stability, in addition to a proper chemical characterization of test media.

As for the use of solvents for example, 99.9 % de ethanol (Manjunatha et al., 2018), DMSO (Whemas et al., 2015; Li et al., 2018; Tian et al., 2019), aquatic toxicology does not recommend the use of solvents due to potentially secondary toxic effects towards the target organism, but when used a proper positive control should be included in the test (Beekhuijen et al., 2015). The final solvent concentration in the stock solution should not exceed 100 µl L^{-1} and should be the same in all test vessels (OECD, 2013). Several MNs are not stable in aqueous media without the addition of dispersants/stabilizing agents (e.g. citrate) or surface coatings (e.g. polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP)). When commercial MNs are synthesized with these additional characteristics, they should be considered an integral part of the MN, as they will vary in state in behaviour, depending largely on the testing media. Therefore, additional control experiments should be conducted to elucidate the impact (stimulatory or inhibitory) of the dispersant or capping agent on the overall results (OECD, 2013; Petersen et al., 2015).

6. Interaction of NMs with chorion, uptake and accumulation

The NM accumulation in zebrafish embryos and larvae is shown in Fig. 6 and Table S2. Revised data indicated that NMs accumulate in zebrafish embryos mainly in the region of the chorion, with accumulation being dependent on NM type and size. After uptake, NM can be transported to different organs, mainly the gastrointestinal system, heart, brain, yolk and liver (Asharani et al., 2008; Chen et al., 2017; Pitt et al., 2018). On the other hand, in zebrafish larvae NMs were observed mainly in the gastrointestinal tract, indicating that absorption, bioaccumulation and distribution of NMs in tissues is dependent on the development stage of zebrafish. The zebrafish chorion is a barrier that covers the embryo up to 48 - 72 h and has pores (diameter = 500 – 700 nm) that are important for the transport of oxygen, nutrients and excretion (Rawson et al., 2001). However, these pores can facilitate the entry of NMs that diffuse through the chorion membrane and may be toxic to embryo development during the period of organogenesis (Cheng et al., 2007). To date, little is known about the interaction of NMs with the chorion, and how this structure interacts and affects the absorption, accumulation and distribution of NMs in the embryos (Table S2).

Fent et al. (2010) described the interaction of fluorescent silica NPs (FS NPs) with the chorion, determining its absorption capacity and biodistribution in zebrafish embryos. Large FS NPs (60 nm – 200 nm) did not cross the chorion and did not induced malformations during zebrafish development. In addition, these NPs did not interfere in gas exchange processes of embryos that are essential for their development. Similar results were seen for CNTs (11 nm; 360 mg L^{-1}), in which the chorion also prevented the passage and toxicity of these NMs, and consequently no alterations in the gas exchanges was observed during 96 h of exposure (Cheng et al., 2007). In opposite, Ag NPs (5 – 20 nm) crossed the zebrafish chorion and accumulated in the brain, heart, yolk and blood of embryos, leading to the several morphological changes, such as pericardia edema, deformities mouth, notochord malformations, decaying tail tissue and blood accumulation heart region (Asharani et al., 2008). Similar results have also been reported in other studies (Asharani et al., 2011; Chen et al., 2017; Pitt et al. 2018).

In environmentally relevant exposure conditions, the formation of NM aggregates in aqueous suspensions increase the hydrodynamic diameters of NMs and may reduce its uptake by zebrafish chorion (Chao et al., 2018; Cheng et al., 2007). Furthermore, the NM interaction with others macromolecules present in the test medium also changes its interaction and uptake by zebrafish embryos. The NM interaction with natural organic matter (NOM) can reduced its toxicity on zebrafish embryos, such as reported by Kteeba et al. (2017) using ZnO NPs (10 – 30 nm) and NOM isolated from Milwaukee-WI, Yukon-AK and Suwannee River-GA rivers. The NOM was able to mitigate toxic effects induced by ZnO NPs, resulting in reduced delays in hatch rate, mortality, and malformations.

After hatching (72 hpf), the zebrafish embryo loses its protective barrier (the chorion) and is susceptible to NM exposure by other routes during the larval period (Fig. 6). Pomeren et al. (2017) investigated the different uptake routes (via chorion, dermal and oral exposure) of polystyrene (PS) NPs (25, 50, 250 and 700 nm) in three phases of zebrafish development in order to investigate the influence of size in NP route of exposure and whether the uptake route determines the target organ to be reached. The three stages consisted of: the first stage when the embryo is still protected by the chorion, the second stage when the embryo's mouth is still closed and the third stage which the embryo is fully formed, and the functions of absorption and excretion are functioning. In this study, during the period of 24 hpf, the NPs were adsorbed by the chorion, and only after hatching (72 hpf), uptake of small NP (25 and 50 nm) were detected in the embryos through the

oral and dermal routes, following by distribution in the body and accumulation in the eye of the larvae, while the larger NPs (250 and 700 nm) were found in the digestive tract and absorbed by the epidermis.

7. Mortality

The LC_{50} values reported in the literature indicate that inorganic NMs present high toxicity compared to organic ones (Table S1). Among the inorganic NMs, lower LC_{50} values were reported for CuO NPs (0.00139 mg L^{-1}; Chen et al., 2011), PVP-functionalized Ag NPs (0.11 mg L^{-1}; Goss et al., 2018), TiO_2 NPs (3.5 mg L^{-1}; Welmas et al., 2015), Ecodis-P-90 functionalized ZnO NPs (4.289 mg L^{-1}; Lacave et al., 2016), QDs CdS (7.036 mg L^{-1}; Lacave et al., 2016), Au NPs (24.61 mg L^{-1}; Lacave et al., 2016), $Fe_2 O_3$ NPs (53.35 mg L^{-1}; Zhu et al., 2012), SiO_2 (83.329 mg L^{-1}; Lacave et al., 2016), MgO NPs (428 mg L^{-1}; Ghobadian et al., 2015). Theses LC_{50} values are above the concentrations reported in environmental water samples, which are in the order of ng L^{-1} (Gottschalk et al., 2009, 2013), indicating low effects of NMs on mortality at environmentally relevant concentrations. Furthermore, two studies estimated the LC_{50} for organic NMs, which showed that the Tween 80 -functionalized CS NPs has high toxicity (25.06 mg L^{-1}) (Yuan et al., 2016) when compared to uncoated CS NPs (270 mg L^{-1}) (Wang et al., 2016), confirming the role of the NM functionalization on nanotoxicity.

8. Morphological alterations on zebrafish embryos induced by NMs

A total of 33 distinct morphological alterations induced by NM exposure were reported in the revised studies (Table S3). To facilitate the comparison and discussion of the nanotoxicological data, the zebrafish alterations induced by NMs were grouped in the following four reaction patter (Rp): Rp_1 (circulatory changes), Rp_2 (pigmentation and tegumentary changes), Rp_3 (musculoskeletal disorders) and Rp_4 (yolk sac alterations) (Table S3).

Among the teratogenic effects observed in the zebrafish exposed to NMs, inorganic NMs induced mainly pericardial edema (18 %), followed by spinal curvature (14 %), flexure tail (10 %), edema of the yolk sac (9 %), absence or irregular eye size (7 %), swimming bladder deformity (4 %), notochord malformations (4 %), growth

retardation (4%), abnormal circulation or vasculature (4 %) and other malformations represent (26 %). In accordance, inorganic NMs induced mainly musculoskeletal disorders (Rp_3), circulatory changes (Rp_1) and yolk sac alterations (Rp_4) when compared to pigmentation and tegumentary changes (Rp_2). As for organic NMs, there is no general trends in data regarding morphological alterations due a lack of studies using this type of NMs (Table S3). A summary of the embryotoxicity of both types of NMs in terms of morphological alterations are summarized in the sections below.

8.1. Embryotoxicity of inorganic NMs

8.1.1 Ag NPs

Ag NPs induced a high number of morphological alterations in the zebrafish embryos (Table S3), especially those of Rp_1 and Rp_3, such as heart edema (19 %), yolk edema (13 %), spinal curvature (13 %) and tail flexure (11 %). In addition, the mouth deformities (2 %) and bubble-like formations in the yolk sac (2 %) were found only after exposure to Ag NPs. The citrate-functionalized Ag NPs (15 – 50 nm; 0.2 to 1 mg L^{-1}; 96 h) induced yolk edema and heart malformation on zebrafish embryos (Cui et al., 2016). Similar effects were reported in embryos exposed to citrate- and PVP-functionalized Ag NPs (2 – 110 nm, 0.8 to 50 mg L^{-1}) for 120 h (Kim and Tanguay, 2014). However, the citrate-functionalized Ag NPs (10 nm; 3 – 30 µM; 120 h) increased the frequency of abnormal swim bladder development and atrophic growth (Powers et al., 2011), while the uncoated Ag NPs (5 – 20 nm; 5 – 100 µg L^{-1}; 72 h) induced the notochord malformations, heart oedema, body degradation, blood accumulation heart region and decaying tail tissue (Asharani et al., 2008). These revised data indicated that the embryotoxic and teratogenic effects of Ag NMs are dependent on size and functional groups.

8.1.2. SiO_2 NPs

SiO_2 NPs induced five types of malformations during embryo development (Table S3), which belong to Rp_1, Rp_2 and Rp_3, mainly pericardial edema (21 %), bradycardia (16 %), spinal curvature (16 %), yolk sac edema (11 %), abnormal circulation or vasculature (11 %). The heart edema is caused by the swollen atrium and ventricle, and this abnormal accumulation of fluid in the pericardial cavity generates intrapericardial pressure. When cardiac function is completely blocked, the formation of yolk edema was observed (Chao et al., 2017).

441 SiO_2 NPs (300 nm; 3 mg L^{-1}) induced bradycardia in zebrafish embryos after 72
442 hpf (Duan et al., 2016). Similarly, embryos exposed to SiO_2 NPs (107 nm; 1 to 12 mg L^{-1}) for 72 h showed bradycardia, pericardial edema, abnormal vascular circulation and
444 reduction of the area of sub-intestinal vesicles (Duan et al., 2017). SiO_2 NPs (20 – 80 nm;
445 12.5 to 200 mg L^{-1}) after 120 h of exposure induced pericardial edema, yolk sac edema,
446 decreased growth, changes of the spine curvature and deformities in the yolk (Phan et al.,
447 2016). These results confirm that exposure to SiO_2 NPs induced mainly circulatory
448 changes (Rp_1) in zebrafish embryos.
449
450 **8.1.3 ZnO NPs**
451 ZnO NPs induced morphological changes, especially those of Rp_1 and Rp_3, such
452 as pericardial edema (19 %), yolk sac edema (17 %), spinal curvature (14 %) and tail
453 flexure (14 %) (Table S3). The chitosan and PEG-functionalized ZnO NPs (16 nm, 1 to
454 100 mg L^{-1}) did not induce any type of malformation after 144 h of exposure
455 (Girigoswami et al., 2015). On the other hand, the embryo exposed to uncoated ZnO NPs
456 (100 nm, 1 to 100 mg L^{-1}) for 144 h induced pericardial edema, hyperemia, curvature of
457 the vertebral column and malformation of the axial region of the head (Zhao et al., 2013).
458 Similar toxicity of uncoated ZnO NPs was reported by Du et al. (2016) and Zhao et
459 al.,2016, indicating that the NP functionalization with chitosan or PEG decreased the
460 toxicity of NMs during the early developmental stages of the zebrafish, as well as
461 confirms the role of functionalization in the nanotoxicological potential.
462
463 **8.1.4 TiO_2 NPs**
464 The TiO_2 NP toxicity to zebrafish embryo was associated with morphological
465 alterations in the category Rp_1, Rp_2, Rp_3 and Rp_4, mainly pericardium edema (25 %),
466 absence or irregular eyes (10 %) and notochord malformation (10 %) (Table S3). TiO_2
467 NPs functionalized with organically coated; 99.5% trace metal basis, (15 – 25 nm; 10 to
468 100 μg mL^{-1}) showed low toxicity when compared to PVP-functionalized NPs (61 – 70
469 n; 10 to 100 μg mL^{-1}) after 72 h of exposure (Pavagadhi et al., 2014). The embryos
470 exposed to TiO_2 NPs (33.4 ± 1.9 nm; 0.1 to 10 μg mL^{-1}) for 96 h induced pericardial
471 edema, fluid accumulation in the pericardium and decreased locomotor activity (Hu et al.
472 2017). Similar results were observed in embryos exposed to TiO_2 NPs (9.83 nm; 0.1 mg
473 L^{-1}) for 144 h (Miao et al., 2015), confirming the TiO_2 NP embryotoxicity.
474

8.1.5 Au NPs

Despite the reduced number of studies, the AuNPs induced few morphological changes in zebrafish embryos, such as yolk sac edema (100 %), which belongs to category Rp4. The citrate-functionalized Au NPs (4.4, 13.5, 40.4 nm, 0.1 to 100 mg L^{-1}) were the only NPs that did not induce any type of malformation in the embryos when compared to CdS, ZnO NPs and SiO_2 NPs (Lacave et al., 2016). Similar results were found by Asharani et al. (2011), which showed low embryotoxicity of Au NPs (15 – 35 nm; 10 to 100 mg L^{-1}) when compared to Ag NPs and Pt NPs. Only Ramachandran et al. (2017) indicated that Au NPs without functionalization (5 - 50 nm; 5 to 100 µg mL^{-1}) induced morphological changes in the yolk sac of embryos. Thus, the revised results indicate that Au NPs present low toxicity to the early stages of zebrafish development.

8.1.6 CuO NPs

The CuO NPs showed to be extremely toxic to the embryos, leading to serious teratogenic effects. Alterations of Rp_1, Rp_2 and Rp_3 were observed after CuO NP exposure, among them yolk edema (10 %), tail flexion (10 %), head malformation (10 %), curvature of the spine (10 %) and pericardium edema (10 %). The notochord malformations (5 %) and malformation of the sacrum and otolith (5 %) were found only after exposure to CuO NPs (Zhang et al., 2017) (Table S3). The exposure of zebrafish embryos to CuO NPs (40 – 60 nm; 0.15 to 1 mg L^{-1}) for 96 h induced several morphological alterations, including tail flexure, spinal curvature, yolk sac edema, malformation of the head, irregular absence and size of the eyes, swimming bladder deformity and reduction of the area of sub-intestinal vesicles (Zhang et al., 2017). Similar results were found after the exposure to CuO NPs (50 nm; 5 to 120 ppm) for 48 h (Ganesan, 2015), confirming that CuO NPs induce several teratogenic effects on zebrafish.

8.1.7 QDs

The zebrafish embryos exposed to QDs showed changes in Rp_1, Rp_3 and Rp_4, mainly pericardial edema (21 %), spinal curvature (21 %), yolk sac edema (21 %) and tail flexion (17 %). The carboxyl-QDs (340-390 nm; 1, 4 and 8 nM; 120 h) can accumulate in various regions of the embryo, and may penetrate the epithelium or be ingested through the mouth and gills and reach internal organs through the cardiovascular system (Chen et al., 2017). Furthermore, the embryos exposed to CdS QDs (3.5 – 4 nm; 0.01 to 10 mg L^{-}

509 ^{1}) for 120 h showed pericardial edema, yolk sac edema, spinal curvature, yolk deformity
510 (Lacave et al., 2016). Graphene QDs (2 – 5 nm; 12.5 to 200 μg mL^{-1}) induced pericardial
511 edema, spinal curvature, yolk sac edema and tail flexion after 96 h of exposure (Guo et
512 al. 2015).
513
514 **8.2. Embryotoxicity of organic NMs**
515 Organic NMs have few studies when compared to inorganic ones. The carbon
516 nanotubes (CNTs) (2.5 %), fullerenes (2.5 %) and NPs of chitosan (CS NPs) (2.5 %) were
517 the most studied organic NMs when compared with PS NPs (1.5 %). Amphiphilic
518 Nanoparticles of Resveratrol-Norcantharidin (RES-NCTD) (1 %) and pristine graphene
519 (PG) (1 %) (Table S3). The CNTs induced a low rate of morphological alterations in the
520 zebrafish embryos, indicating low toxicity. Only alterations in the Rp_3 were observed,
521 such as reduction of growth (67 %) and reduction of locomotor activity (33 %). The PEG-
522 coated CNTs (20 – 40 nm, 0.01 to 1 mg L^{-1}) reduced growth and the locomotor activity
523 after 96 h of exposure (Cordeiro et al., 2018). Similarly, the CNTs exposure (10 – 20 nm;
524 1 to 100 mg L^{-1}; 96 h) reduced the zebrafish embryo growth (Tong et al., 2014),
525 confirming that the CNTs interfere in the growth and behaviour of zebrafish.
526 CS NPs induced changes in zebrafish in categories Rp_1 and Rp_3, such as
527 pericardial edema (25 %), spinal curvature (25 %), swimming bladder deformity (25 %),
528 hyperemia (12.5 %) and head malformation (12.5 %). The CS NPs (84 – 86 nm; 100 to
529 400 mg L^{-1}) induced pericardium edema, axial head malformation and swimming bladder
530 deformity of zebrafish embryos after 120 h (Wang et al., 2016). CS NPs (181.2 nm; 5 up
531 to 30 μg mL^{-1}) also induced similar alterations, such as pericardial edema, hyperemia and
532 curvature of the spinal column after 120 h exposure (Nikapitiya et al., 2018). Although
533 the high concentrations of CS NPs are toxic to the embryos leading to the appearance of
534 malformations, these NPs at 5 μg mL^{-1} did not cause effects during embryo development
535 and are able to increase the larvae resistance to *Aeromonas hydrophila*, because it has
536 strong immunomodulatory activities, which promote immune defence functions *in vivo*.
537 In this sense, the toxicity of these NPs depending on their physic and chemical properties,
538 indicating their potential biotechnological applications. In relation to fullerenes, they
539 induced changes in the categories Rp1, Rp2 and Rp3, such as pericardial edema (50 %),
540 tail flexure (25 %) and yolk sac edema (25 %). The PG also caused alterations in the same
541 categories of fullerenes, with pericardial edema (17 %), blood accumulation (17 %),

spinal curvature (17 %), head malformation (17 %), absence and eye size (17 %) and yolk of the yolk sac (17 %).

PS NPs (34.5 - 10.8 nm, 0.1 to 10 ppm) did not induce embryo alterations, although they accumulated in the gastrointestinal tract, gallbladder, liver, pancreas, heart and brain after 24 h of exposure (Pitt et al., 2018), confirming their systemic distribution and accumulation. Similarly, Yan et al. (2016) observed that the accumulation of RES-NCTD NPs in the stomach and intestine were not associated with morphological alterations, indicating that future studies are necessary to understand the cellular and molecular responses of zebrafish embryos exposed to organic NMs, as well as their effects on gastrointestinal microbiome.

9. Effect of NMs on hatching rate

Several studies showed that the Ag NPs reduced the hatching rate of zebrafish embryos (Table S1). The exposure to Ag NPs (8.39 ± 0.98 nm; 0.03 – 1.55 μg mL $^{-1}$) for 48 h decreased the hatching rate (80 %) (Massarsky et al., 2013), while the Ag NPs (10 – 20 nm; 0.5 and 0.05 μg mL^{-1}) inhibited 40 – 50 % after 56 h of exposure. Similar results were reported by Asharani et al. (20080, Powers et al. (2010) and Orbea et al. (2017).

SiO_2 NPs (40 nm; 50 – 200 mg L^{-1}) (Chao et al., 2017) reduced 39.6 % of the hatching rate of zebrafish, respectively. However, the SiO_2 NPs (20, 50 e 80 nm, 12.5 – 200 mg L^{-1}) accelerated the hatching rate (Pham et al., 2016). The premature hatching of zebrafish embryos can be explained due to the blockage of the pores of the corion that causes a hypoxic condition and hinders the excretion of metabolites. These conditions may facilitate the release of enzymes that facilitate chorion rupture (Silva et al., 2018).

ZnO NPs functionalized with chitosan and PEG (16 nm; 1 – 100 mg L^{-1}) for 76 h (Girigoswami et al., 2015) and ZnO NPs (100 nm; 1 – 100 mg L^{-1}) for (Zhao et al., 2013) reduced 62 and 25.72 % of the hatching rate, respectively. The effects of ZnO NPs on hatching rate also were reported by Chen et al. (2014), Hua et al. (2014), Zhao et al. (2016) and Kteeba et al. (2017). It was also observed that ZnO NP (20 nm; 1-100 mg L^{-1}) in the highest concentration did not hatch embryos causing embryos to die inside the chorion (Ong et al., 2013). The delay in hatching rate may be caused by interference in the expression of genes related to the hatching process. Hgg1 (*Cathepsin L*, *ctslb*), a well-established incubator enzyme gene, is expressed abundantly during the hatching process. This gene acts as a transcriptional factor in the intracellular environment and in the

extracellular environment in the migration of cancer cells, matrix degradation and cell digestion. Thus, changes in hatching rate interfere with gene expression (Zhang et al., 2018).

Inhibition of hatching of zebrafish embryos have also been reported for different types of inorganic and organic NMs, such as TiO_2 NPs (21 nm; 0.01 – 1000 mg L^{-1}) (Samare et al., 2015), TiO_2 NPs (33.4 ± 1.9 nm; 0.1 – 10 μg L^{-1}) (Hu et al., 2017), TiO_2 NPs (9.83 ± 0.55 nm; 0.1 mg L^{-1}) (Miao et al., 2015), CuO NPs (6 nm; 0.1 – 200 μM) (Thit et al.,2017), CuO NPs (50 nm; 5 – 120 ppm) (Ganesan et al., 2015), graphene-functionalized QDs (2 – 5 nm; 12.5 – 200 μg mL^{-1}) (Chen et al., 2017), PEG-functionalized CNTs (20 – 40 nm; 0.01 – 1 mg L^{-1}) (Cordeiro et al., 2018), CNT (11 nm; 20 – 360 mg L^{-1}) (Cheng et al., 2007), CNT (10 – 20 nm; 1 – 100 mg L^{-1}) (Tong et al., 2014), PS NPs (25 – 700 nm; 5 – 25 mg L^{-1}) (Pomeron et al., 2017), $COFe_2O_4$ NPs (40.1 nm; 10 – 500 μM) (Ahmad et al., 2015), MgO NPs (20 nm; 50 – 400 mg L^{-1}) (Ghobadian et al., 2015), Au NPs (5 – 25 nm; 0.32 – 2.6 mg L^{-1}) (Ganeshkumar et al., 2012), Fe_2O_3 NPs (30 nm; 0.1 – 100 mg L^{-1}) (Zhu et al., 2012) and Au NPs (5-25 nm; 0.325 – 2.6 mg L^{-1}).

The effect of NMs on hatching success of zebrafish depend on their surface composition, concentration and exposure period (Table S1), because no effects were reported for TiO_2 NPs (27.73 ± 0.98 nm; 0.1 mg L^{-1}) (Fang et al., 2014), CdSe carboxyl-functionalized whit carboxyl (340 – 390 nm; 1 – 8 nM) (Chen et al., 2017), CeO_2 NPs (0.3-10 nm; 0.08 – 50 mg L^{-1}) (Welmas et al., 2015), SnO_2 NPs (0.3-10 nm; 0.08 – 50 mg L^{-1}) (Welmas et al., 2015), Pt NPs (3 – 10 nm; 10 – 100 mg L^{-1}) (Asharani et al., 2011) and RES-NCTD NPs (231,96-18,68 nm; 10 – 50 mg L^{-1}) (Yan et al., 2016).

10. Interactive effects of NMs with other pollutants

Although some NMs alone did not induce toxicity in organisms, several studies indicate that the NM interaction with other pollutants may potentiate its toxic effects, alerting about the potential risks of releasing these NMs into the environment (Fang et al., 2014; Li et al. 2018). After interaction with other molecules, the NMs may undergo changes in their properties, such as ion dissolution, aggregation state and redox reactions (Bundschuh et al., 2018; Lei et al., 2018). Due their physicochemical properties, NMs can act as carriers of other molecules to the cells, including contaminants ("Trojan horse effect"; Limbach et al., 2007), inducing changes in their bioavailability and toxicity. The co-exposure of NMs can induce additive, synergistic or antagonistic responses in different

types of organisms (Hartmann and Baun, 2010), such as observed in the early developmental stages of zebrafish (Table S1).

The co-exposure of TiO_2 NPs (434 ± 15 nm; 1 mg L^{-1}) and the pesticide cypermethrin (0.4 to 10 μg L^{-1}) for 120 h increased the bioaccumulation of cypermethrin and induced several morphological alterations in the zebrafish embryos, such as pericardial edema, body curvature, decrease in body length, besides inducing neurotoxicity due to the reduction of neurotransmitters (i.e. serotonin, dopamine and γ-aminobutyric acid - GABA) that caused a reduction of locomotor activity (Li et al. 2018), indicating that TiO_2 NPs may potentiate the effects of cypermethrin on zebrafish embryos.

The co-exposure of TiO_2 NPs (7.04 nm; 0.1 mg L^{-1}) with the flame retardant polybrominated diphenyl ethers (BDE-209) (0.38 mg L^{-1}) induced increasing in the BDE-209 bioaccumulation, changes in the gene and protein expression of thyroid hormones and reduced the locomotor behavior of the zebrafish larvae, potentiating the effect of endocrine thyroid disorders and developmental neurotoxicity in the zebrafish embryos (Wang et al., 2014). Similarly, the co-exposure of TiO_2 NPs (27.73 ± 0.98 nm; 0.1 mg L^{-1}) with the insecticide and herbicide pentachlorophenol (0, 3, 10, and 30 μg L^{-1}) for 144 h increased the reactive oxygen species (ROS) production, DNA damage and morphological alterations on zebrafish embryos (Fang et al., 2014).

The interactive effects of ZnO NPs (40 nm; 50 mg L^{-1}) and the fluorosurfactant perfluorooctane sulfonate (0.2 to 0.8 mg L^{-1}) induced thyroid dysfunction in zebrafish by increasing the triiodothyronine (T3) and changes in the expression of thyroglobulin (TG), transthyretin (TTR) and thyroid receptors, as well as reduced growth and increased the embryo malformations, such as pericardial edema, yolk sac edema, spinal curvature and swimming bladder deformity (Du et al., 2016). A literature overview showed that more studies about the interactive effects of NMs with other pollutants during the early developmental stages of zebrafish are need, especially in environmental relevant conditions.

11. Conclusion

NMs are being produced and used on a large scale. However, with the increased release of these NMs into the environment, new toxicological studies are needed. In this

context, ZET proved to be a promising test in assessing the toxicity of NMs. However, a specific protocol should be created for the use of TET in the nanotoxicity assessment, due to their specific physicochemical properties. In addition, studies are required to take into account the behavior of these NMs under relevant environmental conditions due their transformations into environment. In addition, the use of molecular techniques will enable the understanding of nano-specific mechanisms of action, as well as the discovery of new biomarkers

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Figure captions

Figure 1. Timeline of the number (black) and cumulative number (white) of papers published *per* year about the zebrafish embryotoxicity test (ZET) applied in nanotoxicity assessment.

Figure 2. Approaches of the zebrafish embryotoxicity test (ZET) for nanotoxicity assessment.

Figure 3. The use of OMICs technologies (genomic, transcriptome, proteomic and metabolomic) in the zebrafish embryotoxicity test (ZET).

Figure 4. Number of papers published *per* year about the type of nanoparticle (organic and inorganic) analyzed by zebrafish embryotoxicity test (ZET) until May, 2018.

Figure 5. Experimental design of papers published about the zebrafish embryotoxicity test (ZET) applied in nanotoxicity assessment. A) Exposure time. B) Exposure chamber. C) Exposure medium. D) Concentration ranges.

Figure 6. General scheme of the accumulation of nanomaterials in the zebrafish embryo (A) and larvae (B).

Table captions

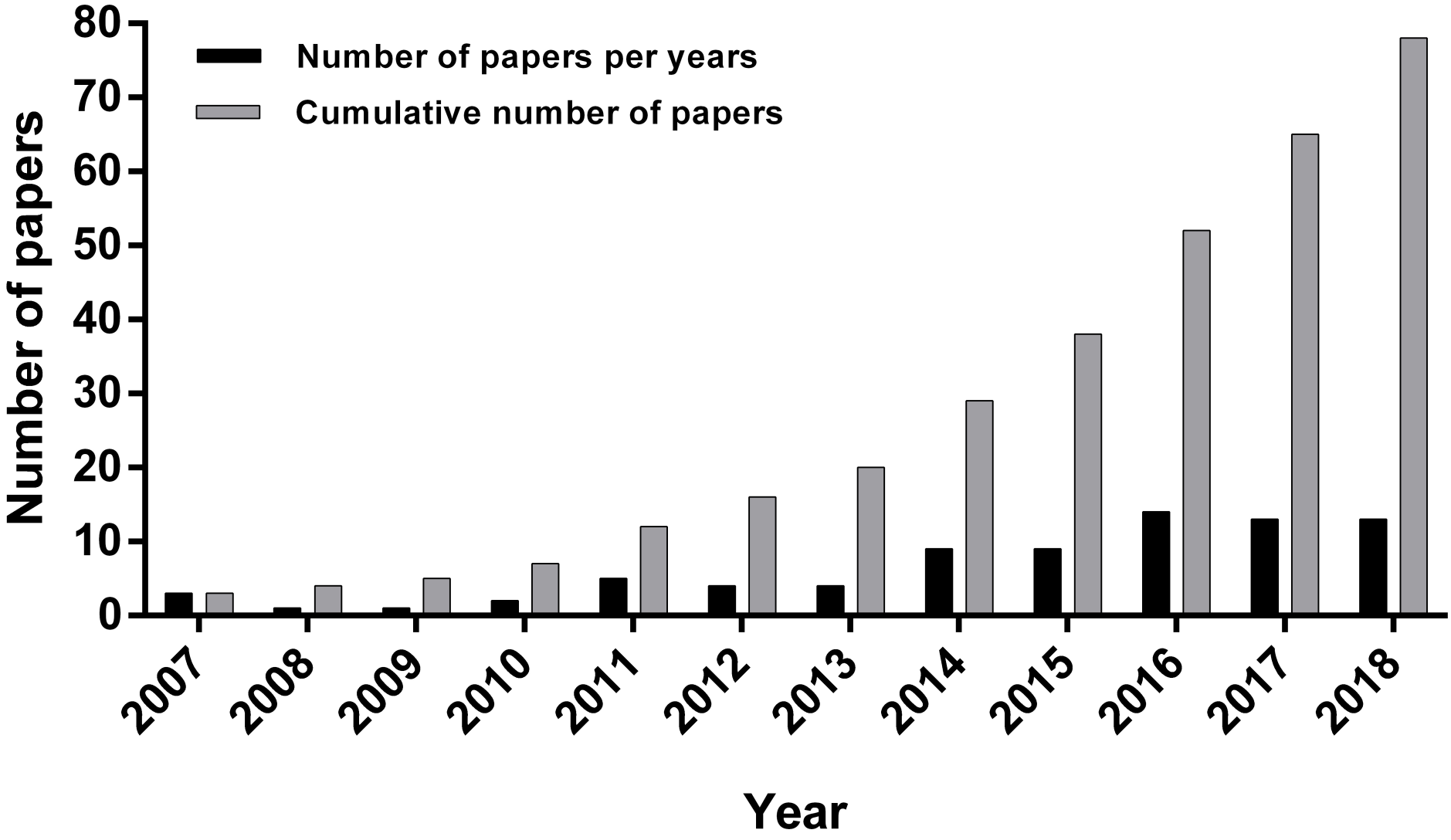
Table 1. Reaction models of morphological changes in zebrafish induced by nanomaterials during the zebrafish embryotoxicity test (ZET).

Supplementary materials

Table S1. Overview of reported toxicity of nanomaterials in the zebrafish using the zebrafish embryotoxicity test (ZET).

Table S2. Number of papers published related to accumulation of nanomaterials in the zebrafish using the zebrafish embryotoxicity test (ZET).

Table S3. Morphological changes in zebrafish induced by nanomaterials using the zebrafish embryotoxicity test (ZET).


Number of papers per years
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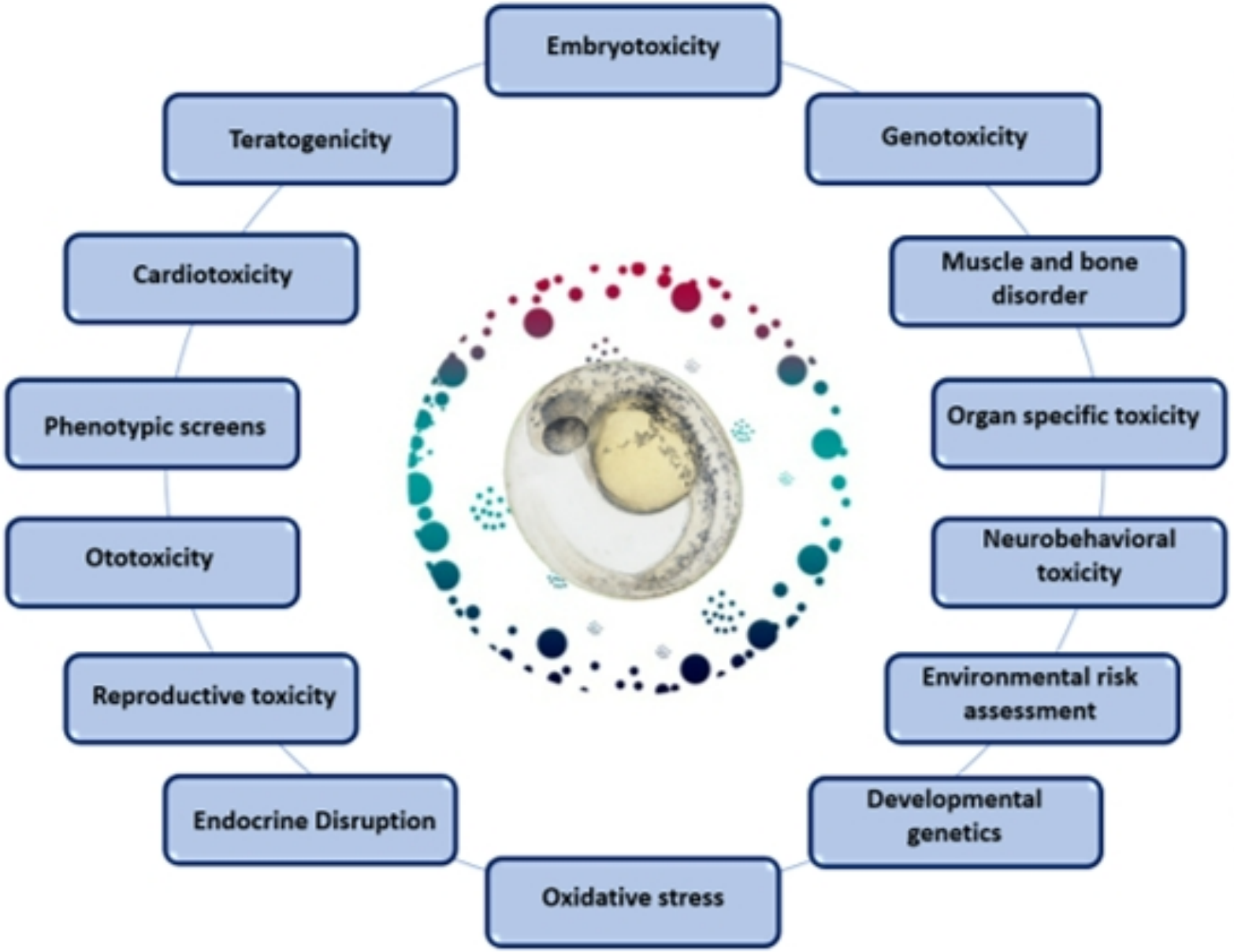
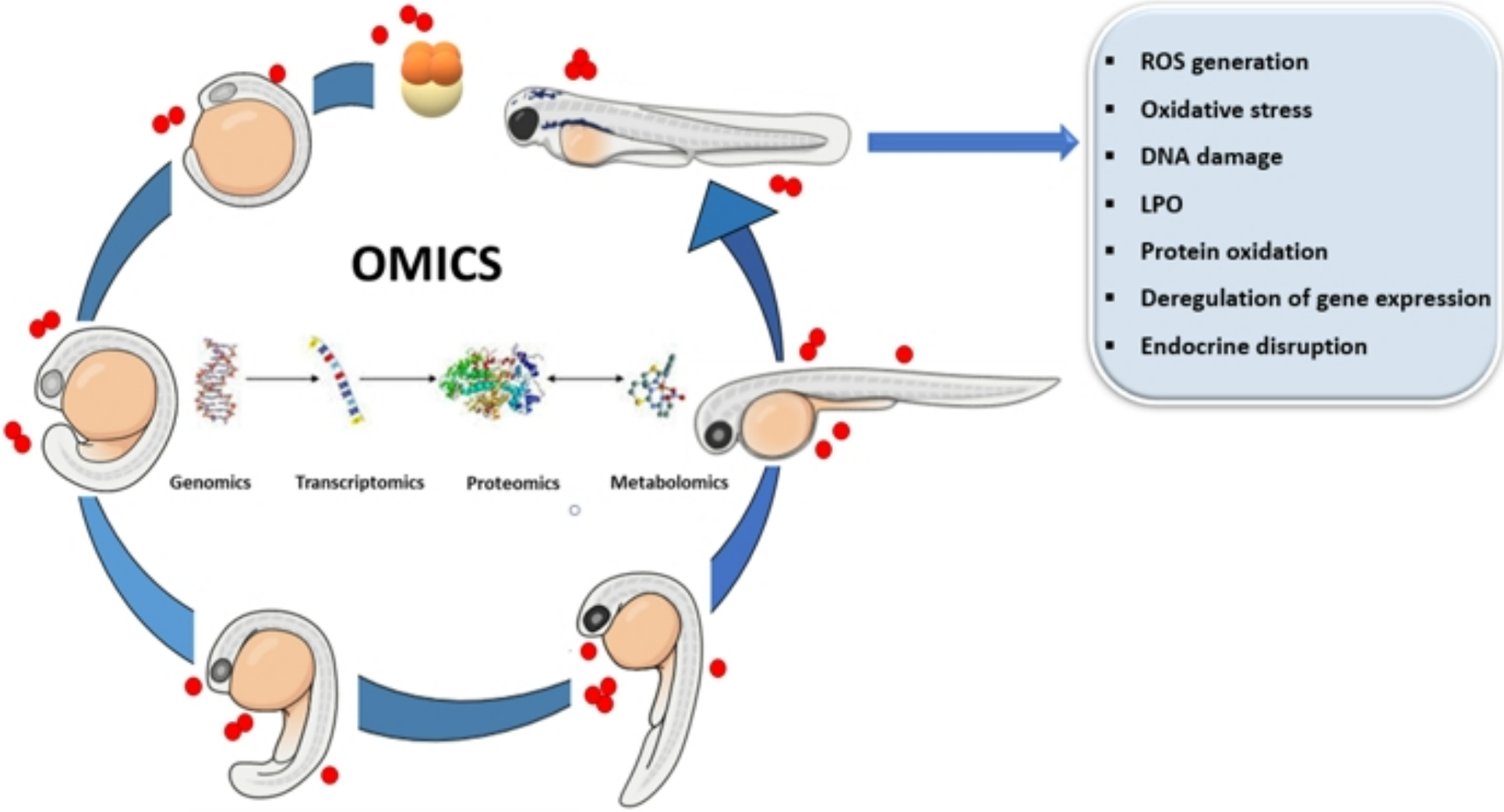
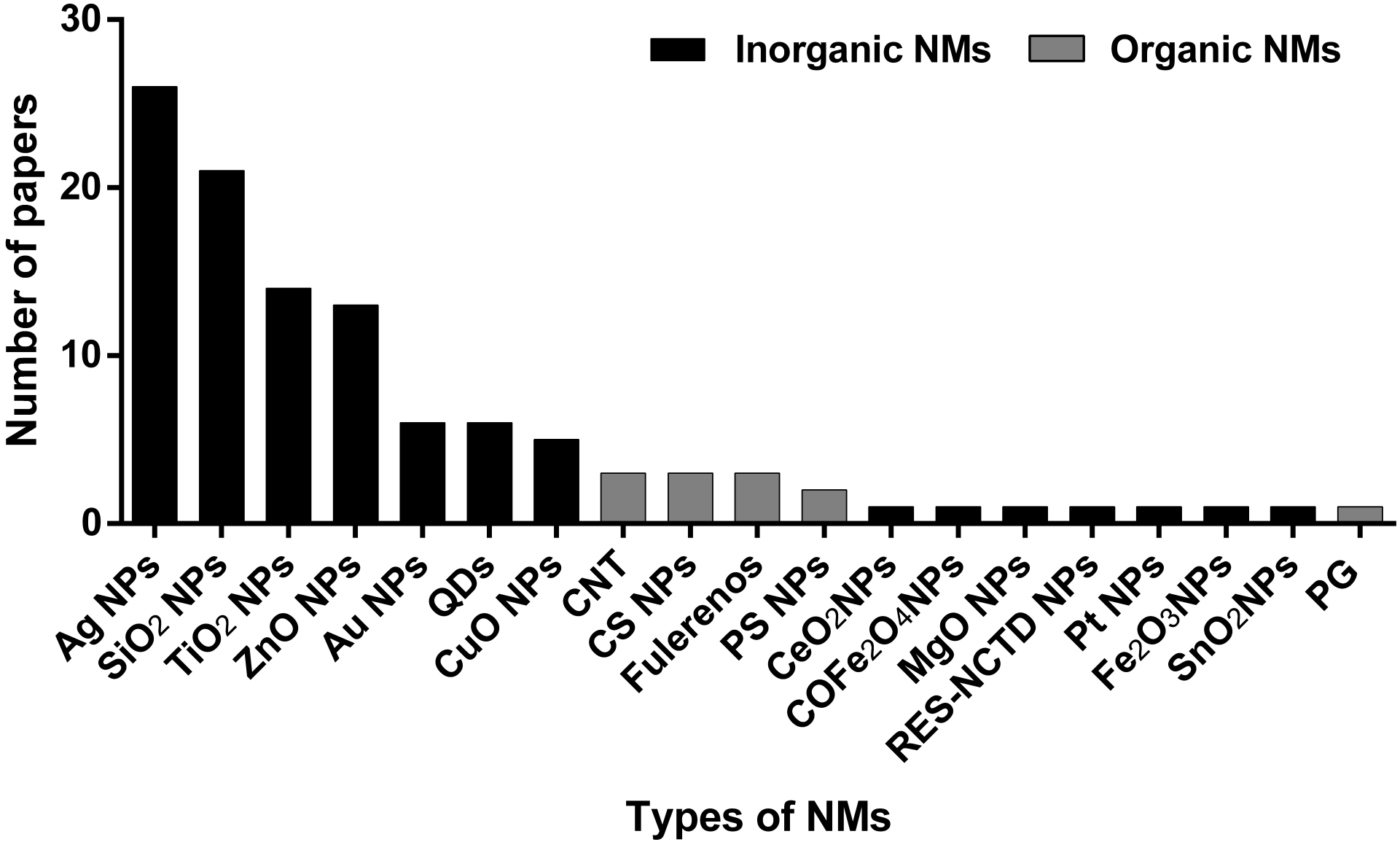
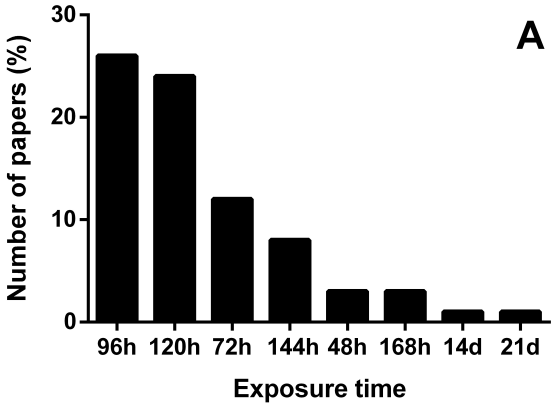
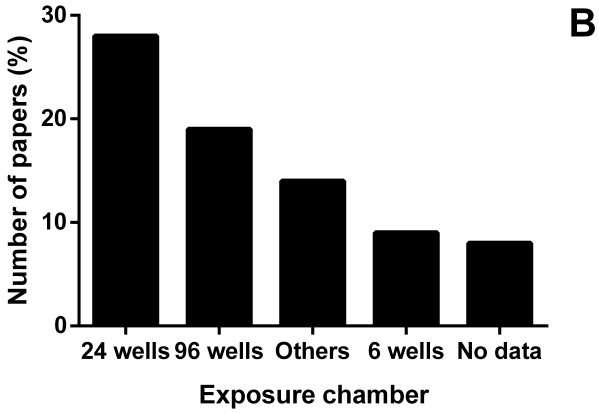
2018
Year

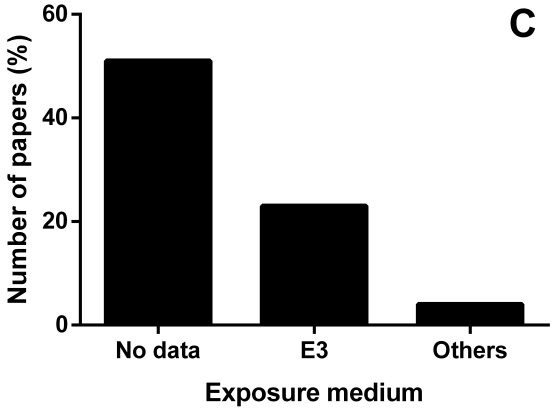

Embryotoxicity
Teratogenicity
Genotoxicity
Cardiotoxicity
Muscle and bone disorder
Phenotypic screens
Organ specific toxicity
Ototoxicity
Neurobehavioral toxicity
Reproductive toxicity
Environmental risk assessment
Endocrine Disruption
Developmental genetics
Oxidative stress

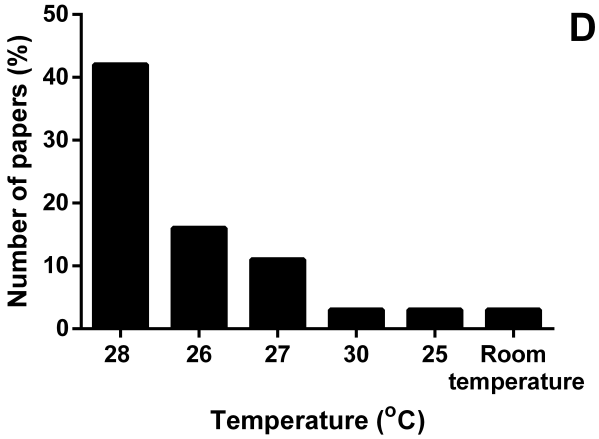

OMICS
Genomics
Transcriptomics
Proteomics
Metabolomics
ROS generation
Oxidative stress
DNA damage
LPO
Protein oxidation
Deregulation of gene expression
Endocrine disruption

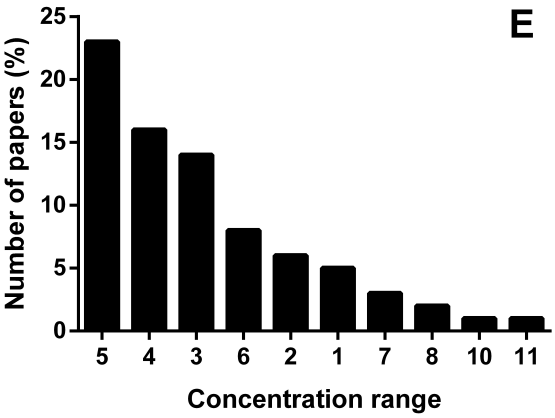

Inorganic NMs
Organic NMs
Number of papers
30
20
10
0
Ag NPs
SiO2 NPs
TiO2 NPs
ZnO NPs
Au NPs
QDs
CuO NPs
CNT
CS NPs
Fulerenos
PS NPs
CeO2NPs
COFe2O4NPs
MgO NPs
RES-NCTD NPs
Pt NPs
Fe2O3NPs
SnO2NPs
PG
Types of NMs

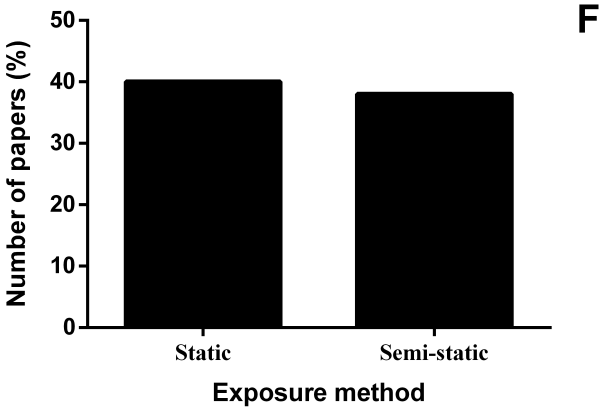

A
Number of papers (%)
30
20
10
0
96h 120h 72h 144h 48h 168h 14d 21d
Exposure time


B
Number of papers (%)
30
20
10
0
24 wells 96 wells Others 6 wells No data
Exposure chamber


C
Number of papers (%)
60
40
20
0
No data E3 Others
Exposure medium


D
Number of papers (%)
50
40
30
20
10
0
28 26 27 30 25 Room temperature
Temperature (°C)


E
Number of papers (%)
25
20
15
10
5
0
5 4 3 6 2 1 7 8 10 11
Concentration range


F
Number of papers (%)
50
40
30
20
10
0
Static Semi-static
Exposure method

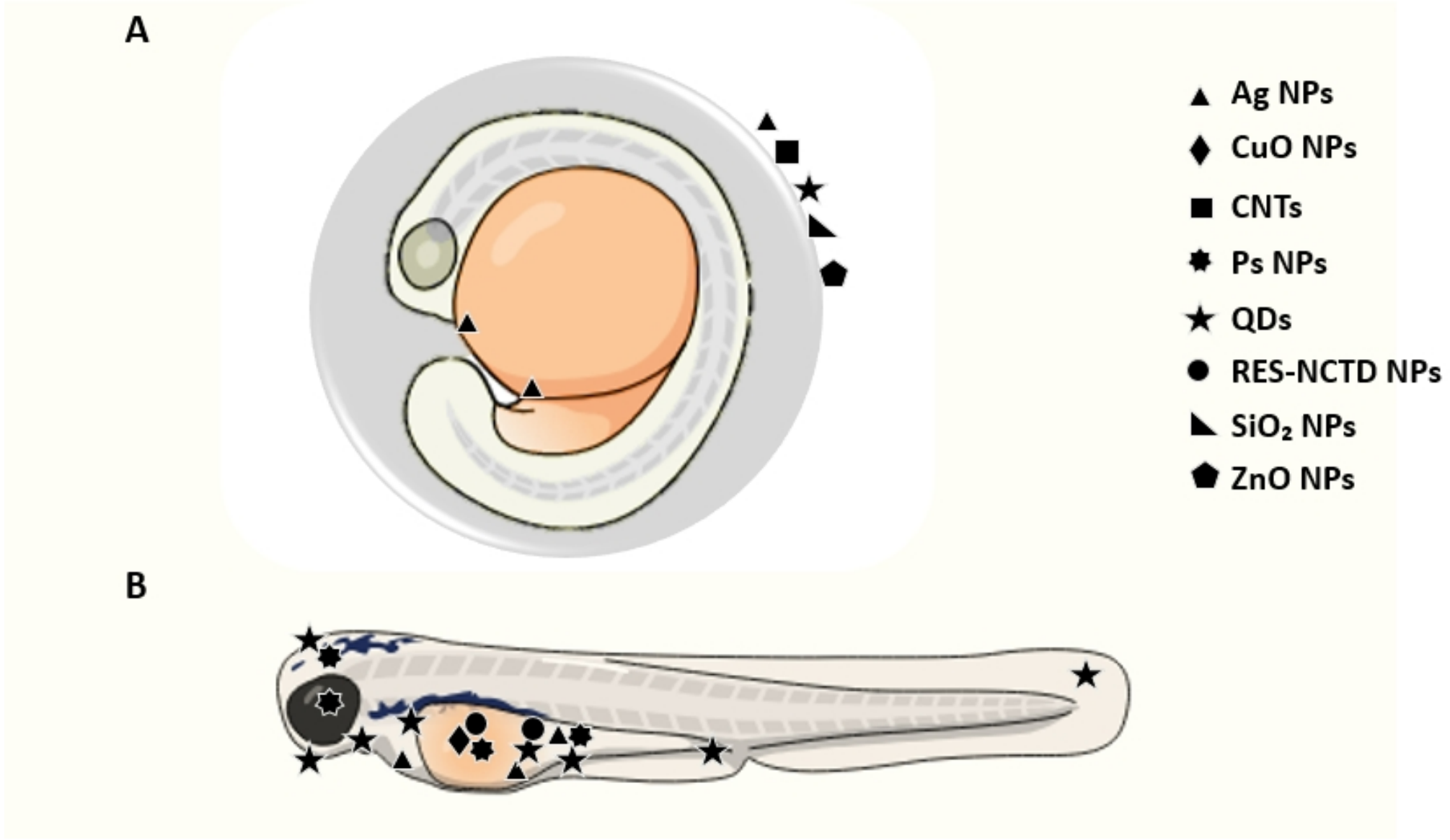

A
Ag NPs
CuO NPs
CNTs
Ps NPs
QDs
RES-NCTD NPs
SiO_2 NPs
ZnO NPs
B

Table 1.

Reaction pattern (Rp)	Alteration
Circulatory changes	Pericardial edema
	Heart malformation
	Bradycardia
	Hyperemia
	Body arch edema
	Anormal circulation or vasculature
	Blood accumulation
Pigmentation and tegumentary changes	Changes of pigmentation of the head
	Changes of pigmentation of the eyes
	Changes of pigmentation of the tail
	Body ulceration
Musculoskeletal disorders	Scoliosis
	Rachischisis
	Notochord malformations
	Spinal curvature
	Defects in the somites
	Tail flexure
	Decaying tail tissue
	Growth retardation
	Reduction of locomotor activity
	Craniofacial
	Axial
	Head malformation
	Absence or irregular size of eyes
	Reduced area of sub-intestinal vessels
	Swimming bladder deformity
	Pectoral fin malformatios
	Deformities mouth
	Changes in the sacculi/otoliths
Yolk sac alterations	Yolk sac edema
	Yolk deformity
	Bubble-like formations on the yolk sac

Table S1.

Nanomaterials			Exposure conditions				Accumulation[c]	EC_{10} (mg L^{-1})	EC_{50} (mg L^{-1})[d]	LC_{50} (mg L^{-1})	Hatching rate	Effects [e]	Ref.
Type	**Capping layer** [a]	**Size (nm)**	**Concentration (mg L^{-1})**	**Time (hpf)**	**Exposure chambers** [b]	**Medium**							
Fullerenes	–	–	100, 200, 250 and 1000 ppb	96	96 well MP	–	–	–	–	200 ppb	↓	PE, YSE, TM	Usenko et al., 2007
	Uncoated	100	1,5 and 50	96	24 well MP	E3	–	–	–	1,5	↓	PE	Zhu et al., 2007
	–	35.6 ± 10.9 nm	0.5 and 20	96	Petri plates (4 mL)	–	GIT	–	–	–	–	No malformatios	Della Torres et al., 2018
CS NPs	–	181.2	5, 10, 20 and 30 µg mL^{-1}	5 d	6 well MP	–	–	–	–	–	↓	CS, PE, H	Nikapitiya et al., 2018
	–	84-86	100, 150, 200, 250, 300 350 and 400	120	96 well MP	EM	–	–	–	270	↓	AM, PE, SWIM	Wang et al., 2016
	Tm	251-15	5, 10, 20, 30, 40 and 50	96	6 well MP	Fish culture medium	–	–	–	25.06	–	SWIM, CS	Yuan et al., 2016
CNTs	PEG	20-40	0.01, 0.1 and 1	96	96 well MP	–	CHO	–	–	–	↓	AG, DLA	Cordeiro et al., 2018
	–	10-20	1, 5, 10, 50, 100	96	24 well MP	–	CHO	–	–	–	↓	AG	Tong et al., 2014
	–	11	20, 40, 60, 120, 240 and 360	96	Petri plates (6 mL)	–	CHO	–	–	–	↓	–	Cheng et al., 2007
RES-NCTD	–	231,96 -18,68	10, 25 and 50 ppm	7 d	–	–	STO, INTV	–	–	–	–	–	Yang et al., 2016
PS NPs	–	25, 50, 250 and 700	25 nm: 25; 50 nm: 25; 250 nm: 5 700 nm: 5	120	24 well MP	–	25 and 50nm - eyes 250 and 750 nm - GIT	–	–	–	↓	–	Pomeren et al., 2017
PS NPs	–	34,5-10,8	0,1, 1 and 10 ppm	120	glass containers	ASW	YO, GIT, PA, BR, GAL, HE	–	–	–	–	FFF	Pitt et al., 2018
PG	–	170-390	1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/L	96	24 well MP	–	–	–	–	–	↓	PE, SPC, YSE, HM, EYE, CIRC, TM	Manjunatha et al., 2018
Ag NPs	CT –	10-40	0, 0.2, 0.4, 0.75 and 1	96	*	–	–	–	–	–	–	YSE, HTM	Cui et al., 2016
	Uncoated	20	0.01, 0.1, 0.5, 1 and 10	21 d	–	–	LV, GIT	–	–	–	–	–	Caambier et al., 2018

	CT and PVP	15	0.1, 0.2, 0.5, 0.8, and 1 µg/mL	96	24 well MP	Deionized water	–	–	–	–	↓	PE, SPC, PFM, TM	Qiaoshu et al., 2017
	COO-	20	0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.75, 6.5, 8.5 and 10	96	–	–	–	–	–	1.19	↓	PE, YSE, TM, SPC	Cáceres-Vélez et al., 2018
	PVP	10-125	0.5, 1 and 10	120	6 well MP	E3	CHO	–	–	0.11	–	–	Boyle et al., 2018
	–	30 ±16 nm	13.6, 21.6, 42.4, 64, 128 µgL^{-1}	72	12 well MP	–	–	–	–	–	–	TM, NCM, SOMI	Sarkar et al., 2018
	–	14-50	0.5 and 100	72	Petri plates (25 mL)	–	–		0.12	0.14 0.14	–	PE, YSE, CS	Gao et al., 2015
	–	100	0.5, 5, 10 and 25	120	6 well MP	E3	YO−	–	8.8e61 mg Ag/L	1.7	–	TM, YND, PE	Gupta et al., 2016
	–	5-10	10, 100, 1, 2, 5, 10, 20, 30, 40 and 50 µg mL^{-1}	96	24 well MP	–	FPE	–	–	23.63 µg mL^{-1}	–	HM, YSE, PFA, BH, DCH, DM	Iniyan et al., 2017
	–	20-10	1000 and 100	72	Petri plates (20 mL)	–	–	–	–	–	–	–	Serrano et al.,2014
	–	8.39 ± 0.98	0.03, 0.16, 0.31, 0.78 and 1.55 µg/mL $^{-1}$	5 d	Petri plates 14 mL)	–	–	–	–	1.18 µg mL ^{1}	↓	FCE, YND, CS, PE	Massarsky et al., 2013
	SP	10-20	0.5 and 0.05 µg / mL	4 d	Petri plates (14 mL)	E3	–	–	–	–	↓	–	Massarsky et al., 2014
	PVP and PEI	5.08 ± 2.03	0.01, 0.025, 0.05, 0.075 and 0.1	120	24 well MP	–	–	–	–	50 µg L^{-1}	↓	YSE, PE, FCE, SPC	Orbea et al., 2017
			0.3, 1, 3, 10 and 100 µM	5 d	96 well MP	–	CHO	–	–	–	↓	AG	Powers et al., 2010
	CT and PVP	10-50 10	3, 10 and 30 µM	5 d	96 well MP	–	–	–	–	–	No effect	SWIM, AG	Powers et al., 2011
	–	10	30, 60, 120 and 240 nM	120	*	–	–	–	–	50 nM	–	TM, CS, YND	Yoo et al., 2016
	–	20	0.5, 0.66, 0.87, 1.15, 1.5, 4, 8 and 16	48	96 well MP	–	LI, ERY	–	1.09	1.26		AG, TM, HM, PE	Muth kohne et al., 2013
	CT and PVP	20-110	0.8, 4, 20, 10 and 50	120	96 well MP	–	EMB	–	–	–	–	YSE	Kim and Tanguay, 2014
	–	5-20	5, 10, 25, 50 and 100 µg mL $^{-1}$	72	24 well MP	–	BR, HE, YO, BEMB	–	–	50 µg ml^{-1}	↓	NCM, PE, BD, BAH, DTT	Asharani et al., 2008
	–	20-50	0.4, 0.6, 0.7, and 0.8 mmol L^{-1}	96	.24 well MP	E3	–	–	–	Nanospheres: 0.0415 Nanoplates: 0.0169	↓	SC, AG	Abramenko et al., 2018
Ag NPs and	–	2-20	5, 10, 20, 40, 60, 80	96	24 well MP	–	–	–	–	–	No effect	YSE	Ramachandran

Au NPs		and 5-50	and 100 µg mL^{-1}										et al., 2017
Ag NPs and Au NPs	–	3, 10, 50 and 100	250, 25, 2.5 and 0.25 µM	120	–	–	–	–	–	93.31 µM 125.66; cAg50: 126.96; cAg100: 137.26 µM	–	AgNPs: YND, HM, CIRC, BAH, TM, BYS, DTT, PE, CS, YSE; AuNPs: No malformation	Bar-lian et al., 2009
Ag NPs and TiO_2 NPs	PVP	61-70 and 15-25	10, 25, 50, 75 and 100 µg mL^{-1}	72	24 well MP	–	–	–	–	–	↓ ↓	↓ Heart rate	Pavagadhi et al., 2014
Ag NPs, Au NPs and Pt NPs	–	15-35, 5-35, and 3-10	10, 25, 50, 75 and 100	72	Petri plates (60 mL)	–	EMB	–	–	–	Ag-NP and Pt-NP: ↓	Ag-NP PE, HTM, EYE, CIRC Au-NP: no effect Pt-CS	Asharani et al., 2011
Ag NPs, Au NPs, CdS, ZnO NPs and SiO_2 NPs	Ag: MAL Au: CIT ZnO: ECOP90	24, 44, 96 4.4,13. 5, 40.4 3.5-4 20, 70 27 15, 30, 70	0.001, 0.01, 0.1, 1.5; 0.1, 1, 10, 50, 100 0.01, 0.1, 1.5, 10 0.01, 0,1, 1, 5, 10 0.1, 1, 10, 50, 100	120	24 well MP	–	–	–	–	0.529 3.94 1.973 24.655 24.61 34.717 7.036 4.289 5.538 83.329	Ag- Died Au- Died after hatching CdS - delay ZnO - delay SiO2- delay (0.1, 1) and died (10, 50, 100)	Ag: YSE, EYE, PE, TM, SPC CdS- YSE, PE, FFF, SCP; ZnO: YSE, EYE, PE, SPC, FCE SiO_2: YSE, PE, SPC	Lacave et al., 2016
Au NPs		5-25	0.325, 0.65, 0.97, 1.3, 1.62, 1.95, 2.27 and 2.6	4 d	*	E3	–	–	–	–	↓	No malformatios	Ganeshkumar et al., 2012
	MES or MEEE	1.5	10 µg mL^{-1} 50 µg mL^{-1} 50 µg mL^{-1}	120	96 well MP	–	–	–	–	–	–	Behavioral abnormality	Truong et al., 2012
$CoFe_2O_4$ NPs	SA	40.1	10, 62.5, 125, 250 and 500 µM	96	–	E3	–	–	–	–	↓	EYE, TM, SPC, YSE, DLA, BAH	Ahmad et al., 2015
CuO NPs	–	10-20	0, 30, 60 and 121 ppb	96	96 well MP	–	LC	–	–	1.34 µM,	–	–	Chen et al., 2011
	–	6	0.1, 0.5, 2, 10, 50 and 200 µM	120	96 well MP	–	–	–	–	≈30 µM,	↓	–	Thit et al.,2017
	–	40-60	0, 0.15, 0.25, 0.5,	96	*	E3	–	–	–	–	–	FCE, SPC,	Zhang et al.,

			1									YSE, AF, HY, HM, EYE, SWIM, SIVs	2017
	–	30 ± 9 and 40 ± 2	50, 125, 250 and 500	72	–	–	–	–	–	175	↓	PE, NCM	Kumari et al., 2017
	–	50	0, 5, 10, 20, 40, 60, 80, 100 and 120 ppm	48	24 well MP	E3	EMB	–	–	64 ppm	↓	AM, HM, SO HTM, YSE, AG, SC, TM, CS, RA, PE	Ganesan et al., 2015
Fe_2O_3 NPs	Uncoated	30	100, 50, 10, 5, 1, 0.5 and 0.1	168	24 well MP	Fish culture medium	–	–	10 and 36.06	53.35	↓	PE, BA, BU	Zhu et al., 2012
MgO NPs	–	20	50, 100, 200 and 400	144	24 well MP	–	–	–	174	428	↓	PE, AG, EYE, CS, YND, CFM, PIG, YSE	Ghobadian et al., 2015
QDs	Carboxyl		1, 4 and 8 nM	5 d	*	–	M, GI, F, I, GO, CH, OP, ASL, CHO, B	–	–	–	No effect	PE, YSE, TM	Chen et al., 2017
	Graphene	2-5	0, 12.5, 25, 50, 100, and 200 μg mL^{-1}	96	96 well MP	E3	–	–	–	–	↓	PE, YSE, FCE, CS, DLA	Guo et al., 2015
	CdTe	3,5	1, 25, 50, 100, 300, 200, 400 nM	120	–	–	–	–	–	185,9 nm	↓	PE, YSE, PIG, EYE, CS, TM	Zhang et al., 2012
SiO_2 NPs	–	40	0, 50, 100 and 200	96	96 well MP	E3	CHO, EMB		59, 40.2 and 28.2	–	↓	PE, CS, BDC	Chao et al., 2017
	–	300	3 and 0,01	72	6 well MP	–	–	–	–	–	–	BDC	Duan et al., 2016
	–	107	1, 3, 6, 12	72	*	–	–	–	–	–	–	PE, BDC, CIRC, SIVs	Duan et al., 2017
	G	570	0, 0.1, 0.3, 0.5 and 1	120	*	E3	–	–	–	–	–	DLA, BAH	Dumitrescu et al., 2017
	–	40	50, 100 and 200	96	96 well MP	E3	CHO	–	–	–	↓	PE, CS	Chao et al., 2018
	–	20, 50 and 80	12.5, 25, 50, 100 and 200	120	–	–	CHO	–	–	–	Premature hatch rate	PE, YSE, AG, CS, OY	Pham et al., 2016
	[Ru (bpy) 3] Cl_2	~ 60	0.0025 and 200	96	24 well MP	–	CHO	–	–	–	↓	No malformation	Fent et al., 2010
SiO_2 NPs, CdSe NPs, AgNPs and ZnO NPs	AUE, UA, CT,P	3-6, 703 ± 13, 6–35, 3–9	1, 10, 100	120	6 well MP	–	–	–	–	–	↓	CdSe: CS	Ong et al., 2013

TiO_2 NPs	–	27,73 ± 0.98	0.1	6 d	Becker (500 ml)	–	–	–	–	–	No effect	↑malformation	Fang et al., 2014
	–	9.83 ± 0.55	0.1	6 d	Glass containers (600 mL)	–	–	–	–	–	↓	HTM, PE, AM	Miao et al., 2015
	–	21	0, 0.01,10 and 1000 mg mL^{1}	120	24 well MP	–	–	0.073	107.2	–	↓	–	Samaee et al.,2015
	–		0, 10, 20, 60, 120 mg L^{-1}	96	–	–	–		60	–	↓	–	Shih et al.,2016
	–	7.04	0.1	7 d	Glass containers (500 mL)	–	–	–	–	–	No effect	No malformation	Wang et al., 2014
	C		0, 1, 10, 100, 500, 1000 µg ml^{-1}	120	24 well MP	–	–	–	–	300 µg mL^{-1}	–	AG, CFM, PE, TM	Bar-llan et al., 2012
	–	33.4 ± 1.9	0, 0.1, 1, 10 µg mL^{-1}	96	96 well MP	–	–	–	–	–	↓	PE, FCE, DLA	Hu et al., 2017
	–	434 ± 15	1	120	Becker (200 ml)	–	–	–	–	–	No effect	PE, CS, BD	Li et al., 2018
TiO2-MWCNT	–	20-50	30.0, 100.0, 130.0, and 110.0	96	24 well MP	E3	–	–	–	–	↓	No malformation	Silva et al., 2018
TiO_2 NPs and ZnO NP	–	19 ± 4	0, 1.5, 3, 6, 12, 24	120	96 well MP	–	–	–	1.3 and 7.3ZnO NP(total) + TiO2 NPs 3	7.1 and 9.5	↓	–	Hua et al., 2016
TiO_2 NPs SnO_2 NPs ZnO NPs CeO_2 NPs	Uncoated	0.3-10	50, 10, 2, 0.4 and 0.08	120	96 well MP	–	–	–	0.5 and 3.51	3.5 and 9.1	–	NCM, YSE, CS, SC, EYE, PE, OTM, SOMI, PFM, PIG, CMS, SWIM, NCM	Welmas et al., 2015
ZnO NPs	–	50-70	0.1, 0.5, 1, 5 and 10	144	24 well MP	–	CHO	–	–	–	↓	No malformation	Chen et al., 2014
	–	40	50	14 d	–	–	–	–	–	–	–	SWIM, PE, CS, TM, YSE	Du et al., 2016
	CTS and PEG	16	1, 5, 10, 25, 50 and 100	144	6 well MP	E3	CHO	–	–	–	↓	–	Girigoswami et al., 2015
	–	10-30	10, 20, 50 and 100 ppm	96	Petri plates (3 mL)	–	–	–	–	–	↓	YSE, PE, CS, AM	Kteeba et al., 2017
	–	100	1, 5, 10, 20, 50 and 100	144	24 well MP	Fish culture medium	–	–	–	–	↓	H, PE, TM, CS	Zhao et al., 2013
	–	30, 40, 60	10, 30, 60, 90 and 120	96	Glass (2000 mL)	–	–	–	–	–	↓	PE, H, YSE, CS, TM,	Zhao et al., 2016

												SWIM	
–	20–30	0.01, 0.1, 1 and 10	96	96 well MP	–	–	–	–	–	–	TM, YSE, PE	Choi et al., 2016
–	27, 32, 202	2, 4, 8, 16, and 32	120	96 well MP	–	–	–	2.2	9.6	↓	TM, YSE, PE	Hua et al., 2014

[a] Tm (Tween 80), PEG (Polyethylene glycol), CT (Citrate), SP (Sodium polyacrylate), PVP (Poly-N-vinyl-2-pyrrolidone), PEI (Polyethyleneimine), Maltose (MAL), ZnO- Ecodis P90 (ECOP90) MES (Negatively charged 2 mercaptoethanesulfonic acid), MEEE (Neutral 2-(2- mercaptoethox) ethoxy) binders of ethanol), SA (Secondary Amines), G (Glycine), AUE (Undecanoic Acid in Ethane), UA (Undecylenic acid), C (Carbon), CTS (Chitosan), Au-Sodium azide (CIT), P (Polymer), PG (pristine graphene)

[b] MP (Microplate), *(does not specify which microplate)

[c] CHO (Chorion), STO (Stomach), INTV (Intestinal Villi), GIT (Gastrointestinal Tract), YO (Yolk), FPE (Fluid in the Pericardium), LI (Lumen of the intestine), ERY (Erythrocytes), EMB (Embryos), BR (Brain), HE (Heart), BEMB (Blood of Embryos), LC (Liver Cell), GO (Genital Openings), CH (Cheek), OP (Operculum), ASL (Abdominal Skin of Larvae), GI (Gill), M (Mouth), F (Fins), I (Intestine), LV (Liver), GAL (gallbladder)

[d]Ht (hatching), Mf (malformation), PA (Pancreas)

[e]AM (Axial Malformation), AG (Atrophic Growth), AF (Fin Abnormality), BAH (Blood Accumulation Heart Region), BA (Body Arch), BDC (Bradycardia), BD (Body Degradation), BYS (Bubble-like Formations on the Yolk Sac), BU (Body ulceration),CS (Curvature of the Spine), CMS (Abnormal Circulation or Vasculature), CFM (Craniofacial Malformations), CIRC (Abnormal Circulation or Vasculature), DM (Deformities Mouth), DCH (Deformities of Chamber), DLA (Disturbed Locomotive Activity), DTT (Decaying Tail Tissue),EYE (Eye Malformations Such as Large or Small Eyes), FFF (Flap Flexing Fold), FCE (Tail Flexure), GB (Gallbladder), H (Hyperemia), HTM (Heart Malformation), HM (Head Malformation), HY (Hypoplasia), NCM (Notochord malformations), OTM (Otic vesicle malformations), OY (0), PE (Pericardia Edema), PFA (Pericardial fluid is accumulated), PFM (Pectoral fin malformations), PIG (Abnormal pigmentation - hypo or hyper pigmentation), RA (Rachischisis), SWIM (Abnormal swim bladder development), SC (Scoliosis), SOMI (Abnormal somite), SO (Sacculi or Otolith), SPC (Spinal Cord), SIVs (Reduced area of sub-intestinal vesels), TM (Malformations Tail), TU (Tissue ulceration),YSE (Yolk sac edema), YND (Nondepleded)

Table S2.

Accumulated region	Number of articles	Types NMs
Abdominal skin of larvae	1	QDs
Blood of embryos	2	Ag NPs
Brain	3	Ag NPs, PS NPs, QDs
Chorion	12	CNTs, Ag NPs, ZnO, SiO_2 NPs, QDs
Cheek	1	QDs
Embryos	4	Ag NPs, CuO NPs, SiO_2 NPs
Eye	1	PS NPs
Fins	1	QDs
Gallbladder	1	PS NPs
Gastrointestinal tract	7	RES-NTCDs NPs , PS NPs, Ag NPs, QDs
Gill	1	QDs
Genital openings	1	QDs
Heart	3	Ag NPs, PS NPs
Liver	3	Ag NPs, CuO NPs
Mouth	1	QDs
Operculum	1	QDs
Pancreas	1	PS NPs
Stomach	1	RES-NTCDs NPs
Yolk	3	Ag NPs, PS NPs

Morphological malformations		Types of NPs																			
		SiO_2 NPs	Ag NPs	ZnO NPs	TiO_2 NPs	Au NPs	Cu_2O NPs	QDs	CS NPs	PS NPs	SnO_2 NPs	$COFe_2O_4$ NPs	MgO NPs	RES-NCTD NPs	Pt NPs	Fe_2O_3 NPs	CeO2 NPs	CNTs	PG	Fullerenes
Circulatory changes	Pericardial edema	4	12	8	4		2	4	2		1		1			1	1		1	2
	Heart malformation		2		1		1													
	Bradycardia	3																		
	Hyperemia			2					1											
	Blood accumulation	1	2									1								
	Anormal circulation or vasculature	2	2	1	1						1						1		1	
	Body arch edema															1				
Pigmentation and tegumentary changes	Changes of pigmentation of the head																			
	Changes of pigmentation of the eyes			1	1			1			1		1				1			
	Changes of pigmentation of the tail																			
	Body ulceration															1				
Musculoskeletal disorders	Scoliosis		1	1	1		1				1						1			
	Rachischisis						1													
	Notochord malformations		2	2	2		1				1						2			
	Spinal curvature	3	8	6	1		2	4	2		1	1	1		1		1		1	
	Defects in the somites		1	1	1						1						1			
	Tail flexure		7	6	2		2	3				1								1
	Decaying tail tissue		2																	
	Growth retardation	1	4		1		1						1					2		
	Reduction of locomotor activity	1			1			1				1						1		
	Craniofacial				1								1							
	Axial			1	1		1		1											
	Head malformation		3				2												1	
	Absence or irregular size of eyes		2	3	2		1	1			1	1	1				2		1	
	Reduced area of sub-intestinal vessels	1					1													
	Swimming bladder deformity		1	3	1		1		2		1						1			
	Pectoral fin malformatios			1	1						1						1			
	Deformities mouth		1																	
	Changes in the sacculi/otoliths						1													
Yolk sac alterations	Yolk sac edema	2	8	7	1	1	2	4			1	1	1				1		1	1
	Yolk deformity	1	4					1					1							
	Bubble-like formations on the yolk sac		1																	