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Graphene-based nanomaterials toxicity in fish

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Abstract

Due to their unique physicochemical properties, graphene-based nanoparticles (GPN) constitute one of the most promising types of nanomaterials used in biomedical research. GPN have been used as polymeric conduits for nerve regeneration, carriers for targeted drug delivery, and in the treatment of cancer via photo-thermal therapy. Moreover, they have been used as tracers to study the distribution of bioactive compounds used in healthcare. Due to their extensive use, GPN released into the environment would probably pose a threat to living organisms and ultimately to human health. Their accumulation in the aquatic environment creates problems to aquatic habitats as well as to food chains. Until now the potential toxic effects of GPN are not properly understood. Despite agglomeration and long persistence in the environment, GPN are able to cross the cellular barriers successfully, entered into the cells and able to interact with all most all the cellular sites including the plasma membrane, cytoplasmic organelles, and nucleus. Their interaction with DNA creates more potential threats to both the genome and epigenome. In this brief review, we focused on fish, mainly zebrafish (*Danio rerio*), as a potential target animal of GPN toxicity in the aquatic ecosystem.

Keywords

Graphene oxide; graphene-based nanoparticles; single layer graphene; few-layer graphene; reduced graphene oxide; graphene nanosheets; graphene ribbons; graphene quantum dots; reduced graphene quantum dots; zebrafish; Japanese medaka fish; common carp; aquatic environment; zebrafish embryos; zebrafish larvae; neurodegenerative disorders; Parkinson's disease; sleeping disorder; genome; epigenome; oxidative stress; apoptosis; bioaccumulation; toxicity; biomedical research; agglomeration

1. Introduction

Graphene (GP) is the new allotrope of carbon and is defined as a single layer of monocrystalline graphite with carbon atoms tightly packed in a two-dimensional honeycomb lattice, resulting in a large surface area on both sides of the planar axis (Li et al. 2017). GP has been used extensively in chemical industry, healthcare, electronic devices, as well as in biomedical research. Graphene oxide (GO) was first discovered in 1859 via harsh oxidative treatment of graphite (Brodie 1859) and then modified via the Hummers method (Hummers

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and Offeman 1958). The production and the application of GP-based materials throughout the world promotes the release of graphene-based nanoparticles (GPN) into the environment including single-layer GP, few-layer GP (2–10 layers), GP nanosheets, GP ribbons, GP oxide (GO; normally single layer), reduced graphene oxide (rGO; normally single layer), GP quantum dots (GQD) and reduced GQD (rGQD). GPN, once entered into the ecosystem, can pose a threat to living organisms and ultimately to humans. Several *in vivo* studies indicated that GPN accumulate in the living organism and produce potential threats to the developing embryos and fetuses (Chong et al. 2014; Wen et al. 2015; Ema et al. 2016; Szmidi et al. 2016). Because of their poor solubility, high agglomeration, long-term retention, and relatively long circulation time in the blood (Begum et al. 2011), there exists a need to further investigate the toxic potentials of GPN. The UK government body, the Medicines and Healthcare Products Regulatory Agency (MHRA), and the US Food and Drug Administration (U.S.FDA) are now reviewing the toxic effects of all forms of GPN and functionalized GO.

Due to variations in shape, size, surface area, charge, and aggregation chemistry, it is challenging to evaluate the toxic effects of GPN in a straight-forward way. Transportation of GPN into the aquatic environment can occur either through physical processes or food chains. When released into the aquatic environment, GPN interact with inorganic ions and natural organic matters (NOM) that modulates its toxic effects (Chowdhury et al. 2013, 2015). Depending on pH, ionic strength, the concentration of dissolved organic matter, GPN can aggregate or agglomerate in the aquatic environment. The aggregation and stability of GPN in the aquatic environment follow the colloidal theory. Compared with pristine graphene (pG), GO contains many oxygen groups and is relatively dispersible in water. Because aggregation or agglomeration of GO can change its size, effective surface area, and other physicochemical properties, it may modulate its toxicity to aquatic organisms including fish. Although several excellent reviews on the toxic effects of GPN are currently available on aquatic organisms (Zhao et al. 2014; De Marchi et al. 2018), to our knowledge, a comprehensive review focusing on fish is lacking. A significant impediment for writing a comprehensive review on GPN toxicity targeting fish is the lack of extensive literature in this area. In most of the reviews on GPN toxicity that include fish, the effects were briefly summarized either as a separate section or in a comparative approach with other organisms (De Marchi et al. 2018). Moreover, the possible mechanisms of interaction of GPN with the biological systems in fish are not well elucidated. However, the use of fish and especially the fish embryo toxicity test (FET) forms an integral part of hazard identification in ecological risk assessment. FET test is included in the guidelines of Food and Drug Administration (FDA) to perform toxicity test, in the International Council for Harmonisation of Technical requirements (ICH) for pharmaceutical products, in the Environmental Protection Agency (EPA) and Organization for Economic Co-Operation and Development (OECD) for chemical substances (OECD 2013). Moreover, FET fits well with the traditional studies employing *in vitro* cell cultures and those are used in mammalian models (Lin et al., 2013). Unfortunately, to date, there are few studies that have used FET for nanoecotoxicological assessment. Due to excellent morphological and biological features, Lin et al (2013) proposed to use zebrafish (*Danio rerio*) as an *in vivo* model for the study of environmental health and safety (EHS) aspects of engineered nanomaterials and nano-related products.

Since then several excellent studies based on accumulation, morphology, behavior, neurological disorders, molecular mechanisms, and other potential targets of GPN have been conducted on embryos, larvae, adults, and transgenic zebrafish (Liu et al. 2014; Chen et al. 2015a,b; Jiang et al. 2015; Mu et al. 2015; Wang et al. 2015; Ren et al. 2016; d'Amora et al. 2017; Li et al. 2017; Lu et al. 2017; Soares et al. 2017; Souza et al. 2017; Zhang et al. 2017a; Zhang et al., 2017b). Studies have also been carried out with other fish species (Japanese medaka; *Oryzias latipes*) (Mullick Chowdhury et al. 2014; Li et al. 2014). In this review, we have focused on fish, especially on zebrafish (*Danio rerio*) as a potential target species of GPN toxicity. We have made efforts to include all the available information on the toxic effects of GPN on fish, highlighting the possible mechanisms at the molecular level.

Fish constitute a major group of the aquatic food chain and are most potentially exposed to nanoparticles either through food chain or by direct absorption/adsorption from the aquatic environment or both. During the past decades, investigators have tried to standardize ecotoxicological protocols for the assessment of the effects of nanoparticles on the environmental health (Clemente et al. 2014). Several investigations have focused on ecotoxicity addressing the mechanisms related to the transport and intake of nanoparticles at different growth stages of embryo, larvae and mature fish (Zhu et al. 2010; Chen et al. 2011; Xiong et al. 2011). In hazard assessment of nanoparticles, several major issues such as availability of low amounts of testing materials, or novel experimental design should be addressed. As such, zebrafish have been used as an appropriate *in-vivo* model for comparative studies of nanoparticle toxicity (King-Heiden et al. 2009; Zhu et al. 2009; Brundo et al. 2016). This fish shows remarkable similarities in molecular signaling processes, cellular structure, anatomy and physiology with other high-order vertebrates including human (Hill et al., 2005). The embryo-larval zebrafish model has proved useful in quickly identifying and prioritizing the screening of promising nanomaterials (Harper et al. 2011) like GPN. The small size, high fecundity, and transparency of the embryo-larval zebrafish make it feasible to conduct exposures in multiwell plates and noninvasively studying exposure-dependent toxic effects of nanoparticles using microscopy. Moreover, the embryo-larval zebrafish lack a fully functional adaptive immune system until ~28 day post fertilization (Lam et al. 2004) making it possible to study the effects of nanoparticles without the interference of adaptive immune system during embryo-larval development. Therefore, focusing on zebrafish as the model animal for reviewing toxic effects of GPN is very appropriate. Based on the observations on zebrafish, for confirmation, it is also necessary to extend more studies in different fish models like *Japanese medaka*, fathead minnow, stickleback, which are small fish, easily maintained in the laboratory with lower maintenance costs compared to other *in vivo* models including rats or mice.

Characterization techniques such as nuclear magnetic resonance (NMR), ultraviolet-visible spectroscopy (UV-Vis), scanning electron microscopy (SEM), or transmission electron microscopy (TEM), which have been used during nanoparticle assay, are unable to provide critical information on the uptake and dose of GPN that entered into the human body from the surrounding environment. For these reasons, *in vivo* experiments with animal or plant models or in *in vitro* cell culture models are necessary. Zebrafish development has been described well and could be used as a nano-toolkit (Fako and Ferguson 2009) that allows several parameters to quantify the toxic potentials of GPN including its uptake/accumulation

from the aquatic environment. This fish species has been largely used to test developmental toxicity of different kinds of nanoparticles (NPs), spanning from quantum dots (QDs) to silver, gold, platinum, titanium and silica NPs (Bar-Ilan et al. 2009, 2012, 2013; King-Heiden et al. 2009; Asharani et al. 2011; Duan et al. 2013; Brundo et al. 2016; Pecoraro et al. 2017). Based on the phenotypic changes in the zebrafish embryos, a modified scoring spectrum developed by Heiden et al (2007) which is used by several laboratories to evaluate the toxic effects of chemicals released into the environment. In this review, we have described the accumulation and toxic effects of GPNs in different developmental stages (embryos, larvae, and adults) of zebrafish. Moreover, available information on GPN toxicity on Japanese medaka and common carp (*Cyprinus carpio*), although very limited, are also included.

2. Uptake and biodistribution of GPN in embryos, larvae, and adult fish.

The accumulation and distribution of GPN in the embryos and adult zebrafish have been investigated by many investigators. Zebrafish embryos are covered by a transparent acellular membrane, known as chorion, which is composed of glycosylated proteins (Mold et al. 2009), organized in three layered structures (Bonsignorio et al. 1996), and pierced by cone-shaped pore canals which are approximately 500–700 nm in diameter (Rawson et al. 2000). The average surface area of these pores is about $0.17 \mu\text{m}^2$ (Cheng et al. 2007). The chorion is the first barrier that prevents the entry of exogenous materials including GPN from the external environment into the embryonic body. For studying GPN toxicity, most investigators have used both microinjection and continuous exposure approaches (Fako and Furgeson 2009). Fertilized eggs were either microinjected with GPN in nanoliter volumes within 4 h post fertilization (hpf) (Goallavelli and Ling 2012; Jeong et al. 2015; Zhu et al. 2016) or continuously exposed in a medium containing GPN from 2 hpf until 72–168 hpf with change of the medium once every 12–24 h interval (Chen et al. 2015a; Wang et al. 2015; d'Amora et al., 2017) and assessed the larvae on 72–168 hpf, depending upon the nature of the experiments. Larvae and adult fish were mostly on continuous exposure to GPN for various time points (Buccheri et al. 2016; Lu et al. 2017; Zhang et al. 2017b). Another fish, Japanese medaka (*Oryzias latipes*), considered as a complementary model to zebrafish, has prolonged embryonic development than zebrafish. Investigators exposed the Japanese medaka embryos to GPN in ovo for 6 days (1–7 dpf) (Mullick Chowdhury et al. 2014) while the 1–2 day post-hatch free-swimming larvae (ex-ovo) were only for 4–48 h (Li et al. 2014).

Both conditions have many advantages as well disadvantages. First of all, during microinjection, the developmental stage and the site of the embryos where GPNs are delivered is very important. Most of the investigators injected GPN into the upper part of the yolk materials when the embryos were in 1–4-cell stage, expecting that GPN could be adsorbed into the embryonic body and transduced inside the cell either by passive diffusion or by any signal transduction-mediated mechanisms. So far we did not find any report that GPNs are directly injected into the body of the developing embryos. Moreover, the volume of the injected material should be kept minimum probably 10 nL. In this technique, GPNs are available to the embryos only once and diluted over time as the number of dividing cells increased with the advancement of development. In case of continuous exposure, the

developmental stage of the embryo and the duration of exposure are very critical. Several investigators exposed the embryos starting from 2–168 hpf with the static renewal of media (50–100%) every day (Zhang et al. 2017b). In Japanese medaka, the renewal of the media was on every alternate day (Mullick Chowdhury 2014). In these experiments, even though the availability of the nanomaterial to the developing embryos/larvae remained constant, the agglomeration of GPN outside or on the chorion can impair oxygen supply to the developing embryos (Chen et al. 2015a). Therefore, the toxic effects of the nanomaterials should be an indirect effect (hypoxia) on the embryo development rather than the direct interaction of the GPN at the cellular level. Several GPN molecules were able to cross the chorion and interact with developing embryos. Therefore, the actual concentration of GPN that induced the biological effects was not the same as the waterborne concentration.

In general, zebrafish embryos grew rapidly; based on the morphological changes during development, the embryos have passed through seven broad periods during embryogenesis; these are zygote (0–0.75hpf), blastula (2.25–5.25hpf), gastrula (5.25–10hpf), segmentation (10–24 hpf), pharyngula (24–48 hpf), and hatching periods (48–72 hpf) (Kimmel et al. 1995). The modified scoring spectrum developed by Heiden et al (2007) is based on the phenotypic changes, ranging from 0 (normal phenotype), 1 (minor phenotypic changes), 2 (moderate alterations), 3 (severe embryo deformation), and 4 (death of the embryo) scoring points (Fako and Furgeson 2009). Morphological changes in different developmental stages are correlated with the expression and functions of specific genes. Therefore, those genes which are active in *ovo* (development inside the chorion) may not be functional in *ex ovo* (hatched larvae). The *in ovo* development period in zebrafish is approximately 72 hpf and the larvae can swim actively in 4 dpf (96 hpf). By exposing the embryos from 2 hpf to 7 dpf (168 hpf) covers the entire period of development both in *ovo* and *ex ovo*. Therefore, it made difficult to find the appropriate developmental time points when GPN successfully interact with the cellular events of the embryos/larvae and disrupt the normal phenotypic morphology. So, the impact of GPN either in genome and epigenome at specific stages of development cannot be understood by simply exposing the fertilized embryos for the entire period of development (both in *ovo* and *ex ovo*). For larvae (Ren et al. 2016) and adults (Lu et al. 2017) the duration of exposure ranges from 1–14 day depending on the nature of the experiment. Although the absorption sites are mainly through gills in adults, accumulation of GPN may occur in the liver, brain, intestine and other organs of the body (Lu et al. 2016).

2.1. Distribution in embryonic development:

2.1.1. GPN administration into the embryos by microinjection—Goallavelli and Ling (2012) microinjected fluorescein-labeled multifunctional graphene (MFG; 0.1 ng/nL; 10 nL volume)- into the pole region of two-cell stage embryos of zebrafish (AB strain) and the fluorescence was monitored in larvae hatched on 72 hpf using confocal laser scanning microscope (Table 1). The larvae exhibited excellent fluorescence signal from head to the tail, especially in the yolk sac, blood vessels, and in brain ventricles; which was suggested that MFG entered into the embryonic body and distributed all over the embryos. Jeong et al (2015) microinjected approximately 1 nL of 0.25 mg/mL of nanographene oxide (NGO) or Alexa568-labeled NGO (NGO-A568) to one cell stage of endothelial cell-specific transgenic zebrafish [*Tg (kdrl:egfp)*] at 250pg (single injection), 500pg (two consecutive injection), and

750pg (3 consecutive injections) doses. The embryos were imaged for red fluorescence by confocal microscopy at 30 and 52 hpf. It was observed that NGO-A568 was distributed throughout the body including the head region and in developing vasculature both in 30 and 52 hpf embryos. In another experiment, zebrafish embryos at single cell stage were microinjected with 0.5 nL TPE-TPA-FN-nanographene oxide nanoparticles (TTF-NGONP) into the yolk and then cultured for 48 h (Zhu et al. 2016). The TTF-NGONP was distributed uniformly in the zebrafish as observed in 3 photon luminescence (3PL) imaging. Even after allowing the zebrafish to grow further, no obvious aggregation of TTF-NGONP was observed; which indicated that the nanoparticles are chemically and optically stable in zebrafish (Zhu et al. 2016) and distributed in the entire body of the larvae. From all these studies, it was understood that although the GPN was delivered in the yolk by microinjection, the nanoparticle was successfully transduced into the embryonic body and distributed in specific organs during the development.

2.1.2. GPN administration by waterborne exposure—Zebrafish embryos were exposed to fluorescently labelled GQD (12.5 µg/mL-200 µg/mL) 4–96 hpf waterborne (Table 1). Signals were mainly localized in intestine and heart (Wang et al., 2015). These observations suggest that GQD were able to cross the chorion and accumulated in specific regions of the body. Chen et al (2015a) exposed zebrafish embryos older than 24 hpf to FITC-labeled GO (GO-F) and viewed under laser scanning confocal microscope (LSCM). It was observed that after 8 min incubation, the fluorescence signal was detected in the yolk sac. TEM studies of these embryos on 96 hpf indicated that GO aggregated around the eyes, heart, yolk sac and tail. These observations suggest that GO permeated through the chorion and selectively localized in the yolk and distributed in other developing organs as appropriate. In another experiment, wild type zebrafish embryos and transgenic *Tg(cyp1a:gfp)* zebrafish embryos at 4hpf were exposed in reduced graphene oxide quantum dots (rGOQD; 10 nm lateral size and 1 nm height) until 7dpf with the static renewal of the media every day. Initially the fluorescence (GFP) was observed in whole embryos; however, at 48 hpf, the fluorescence was only distributed in the abdominal region (Zhang et al., 2017a), probably due to the differences in tissue affinities for rGOQD in zebrafish embryos which seem to be specific to the developmental stages. The distribution of ¹⁴C-labeled few layer graphene (FLG; four graphene layers) and small FLG (sFLG; 25–75 nm) in zebrafish embryos (2–48 hpf) was shown to vary with lateral sizes. When the embryos were exposed to FLG for 12–48 h, 85–98% of the total mass of the accumulated FLG was in the chorion and only a smaller percentage was detected in the yolk. However, for embryos exposed to sFLG, the accumulation was increased over time and 16–21% of the accumulated sFLG passed the chorion and entered into the yolk (Su et al. 2017). In Japanese medaka, the increase in probe sonication time (from 1 min to 10 min) significantly decreased the size of GPN and enhanced the mortality of the embryos (Mullick Chowdhury et al. 2014). All these observations indicate that, a decrease in size greatly enhanced the membrane penetration ability of FLG and the microstructure of the chorion may be a decisive factor affecting the transmembrane transport of FLG.

2.2. Distribution in larvae.

Zebrafish larvae on 4 dpf (no chorion) were incubated with GO-FITC (fluorescein isothiocyanate) complex (0.1, 1.0, and 10 mg/L) for 1 h and the fluorescence intensity was evaluated under a fluorescence microscope (Zhang et al. 2017b). The study indicated that GO appeared mainly near the fish mouth, yolk sac, cardiac region, and tail blood (Table 1). In another experiment, 72 hpf larvae (no chorion) exposed to 0.01, 0.1, and 1 µg/L GO for 24 h and used for distribution analysis on 96 hpf (Ren et al., 2016). The larvae were sectioned in an ultra-microtome to get a section of 70 nm thick which were stained with uranyl acetate and lead citrate to get a positive signal. The brain and intestinal tissues of the larvae have GO (0.01–1.0 µg/L) as dark dots. These observations indicated that despite agglomeration, the ultralow concentration of GO were able to cross the cell membrane and accumulated on the target organs.

2.3. Distribution in adults

Adult zebrafish (AB strain, > 3 months old) were exposed to ¹⁴C-labeled FLG for 48 h with two different sizes; larger (L-FLG; 300–700 nm) and smaller (S-FLG; 20–70 nm) with a concentration of 250 µg/L (Table 1) (Lu et al., 2017). The amount of L-FLG uptake was found to be 170-fold greater than S-FLG in the whole body mass. However, the addition of NOM increased the uptake of S-FLG to 16-fold compared to L-FLG which was only 2.5-fold. L-FLG was mainly found in the gut, but S-FLG was found in both gut and liver with or without NOM. Moreover, S-FLG was able to pass through the intestinal wall and enter into the intestinal epithelial cells and blood. This study indicated that the size, as in embryos (Su et al., 2017), and the interaction of GP with NOM has a significant impact on the accumulation and distribution of GPN in the body of zebrafish.

Taken together, we predict that exposure to GPN waterborne is the better method for the incorporation of GPN into the embryonic body, larvae, or adult fish. Although the microinjection of GPN to zebrafish embryos in early stages of development (1–4 cells) showed incorporation and distribution in almost the entire body of the embryo/larva, due to small volume of the injected materials, the developing cells of the embryos might not be able to reach the optimum concentrations to produce a desired toxicological effect. However, the incorporation by immersion either in embryos, larvae, or adults, the nanomaterial, even at the ultralow concentration (0.01–1 µg/L) is able to reach equilibrium and can show organ specific distribution rather than the uniform distribution in the entire body as observed by microinjection. On the other hand, incorporation through waterborne exposure depends on the size, concentration of GPN, time of exposure, developmental stage, as well as the presence of different types of NOM in the environment (Lu et al. 2017; Su et al., 2017) (Table 1). The s-FLG is more potent in crossing the chorion in the embryos (Su et al. 2017) or in the organs of adult zebrafish (Lu et al., 2017). In Japanese medaka, the survivability of the embryos was dependent on the size of the nanoparticle; smaller size enhanced mortality (Mullick Chowdhury et al., 2014). Presence of NOM in the environment was also able to significantly increase the accumulation of both s-FLG and l-FLG in adult zebrafish (Lu et al., 2017) which suggests that if GPNs are released into the environment with larger sizes and stayed sufficiently longer period of time, NOM has the potential to modulate the size and make them feasible for bioaccumulation. Moreover, the accumulation of GPN in

zebrafish is found to be dependent on the developmental stage of the fish. In embryos (24 hpf), the FITC-labeled GO (GO-F) appeared near the yolk sac after 8 min of exposure (Chen et al., 2015a). In larvae, at 96 hpf, GO-FITC reached fish mouth, yolk sac, cardiac region, and tail blood within 1 h of incubation (Zhang et al., 2017b). However, in adult fish, maximum accumulation of s-FLG and l-FLG was reached at 48 h of exposure (Lu et al., 2017). These studies indicate that the developmental stage (age) of fish has a significant influence on GPN accumulation. Furthermore, the physicochemical properties of GPN such as surface energy, surface composition, or surface charge, can modify the attachment of fluorescent dyes to GPN. It is also possible that the attached dye may be released from the nanoparticles in the biological fluids of the fish, giving erotic results. Therefore, all the probable factors need to be considered while evaluating the accumulation of nanoparticles in embryos, larvae, and adult fish through waterborne exposure.

3. Toxic effects of GPN:

The toxic potential of GPNs in embryos, larvae, and adults of zebrafish, embryos and larvae of Japanese medaka, and adults of common carp, is summarized below and also listed in Table 2.

3.1. Effects on embryo:

3.1.1. Administration by microinjection: The toxicity of GO was evaluated in zebrafish embryos by different approaches. One-four cell zebrafish embryos from wild type AB strain were microinjected with GO (average area distribution 40–60 nm, thickness 1–3 nm) or MFG (40–60 nm size, thickness ~4–6 nm) in 10 nL volume with concentrations 0.05–0.1 ng/nL into the pole region and evaluated for toxicological end points at 72 hpf (Goallavelli and Ling, 2012). It was observed that although fluorescein-labelled MFG showed excellent signal in the entire body of the larvae, microinjection of GO or MFG showed morphological abnormalities only in limited number of individuals (4–6% cases showed yolk sac edema and 6–12% showed tail or spinal cord flexure, 2% showed cardiac malfunction for 0.5–1 ng GO/ embryo; 9–12% showed yolk sac edema, 8% showed tail or spinal cord flexure, 6% showed cardiac malfunction for 0.5–1 ng MFG/embryo). Moreover, there were no potential toxic effects on larval survivability. We predict that although GO or MFG can successfully transfer into the developing embryos by microinjection, due to the lack of optimal concentrations of GO and MFG (0.5–1.0 ng/ embryo) toxic effects observed only in small populations (< 15%).

In contrast to the studies by Goallavelli and Ling (2012), microinjection of NGO (250, 500, 750 pg/embryo, which is < 1 ng) (lateral size 100–200 nm, height 1–1.5 nm) to 1–2 cell stage embryos of zebrafish in upper part of the yolk caused several distinct morphological defects including curved spine, shortened body stature, pericardial edema, lowered yolk consumption, underdeveloped brain and retinas, and occasional disrupted circulation. Moreover, injection of polyethylene glycol-coated NGO (NGO-PEG) also showed similar morphological defects, but the severity of toxicity was attenuated by PEG coating or by labeling with A568 (NGO-A568) (Jeong et al. 2015). In addition, several NGO-injected cells (500 pg) in the head region of 30 hpf embryos were undergoing apoptosis as evaluated

by immunostaining. In transgenic zebrafish [*Tg(kdr1:egfp)*], injection of 500 pg NGO caused blood vessel sprouting at 52 hpf when the intersomitic vessels (ISV) in the trunk vasculature were formed. NGO-A568 injection (500 pg) also showed similar effects without any significant variation in ISV number. Gene expression of *vegfaa*, the zebrafish homolog of mammalian vascular endothelial growth factor A (*VEGF α*), was upregulated, while notch-regulated Ankyrin repeat protein a and b (*nrarpa/nrarpb*) genes were downregulated in 30 hpf embryos by NGO in a dose-dependent manner. These studies documented that NGO (0.25–0.75 ng/embryo) probably due to size difference with the GPN used by Goallavelli and Ling (2012), specifically disrupted zebrafish development targeting genes responsible for angiogenesis.

GP (1, 10, 50 μ g/mL) was microinjected into the yolk of the 1–4-cell stage zebrafish embryos (4nL/embryo) and used for sleep/wake behavioral profile analysis on 4.5 dpf larvae (Li et al., 2017). The mean particle size of the injected GP was 1456.8 ± 16.1 nm for 1 μ g/mL, 2012.0 ± 18.5 nm for 10 μ g/mL, and 5808.2 ± 50.2 nm for 50 μ g/mL. Dimethyl sulfoxide (DMSO) at a concentration of 0.3% was used as vehicle. Although 0.3% DMSO slightly affected the rest and waking activities of the zebrafish larvae on 4.5 dpf, no significant alterations in these activities (waking and rest) were observed in zebrafish larvae injected with GP (1–50 μ g/mL which is equal to 4–200 pg/embryo) during development. Moreover, gene expression profiles of the hypocretin (*hcrt*) system and sleep/wake regulators, such as *hcrt*, *hcrt*-G protein coupled receptor (*hcrtR*) and arylalkylamine N-acetyltransferase 2 (*aanat2*) by *in situ* hybridization (ISH) or by quantitative real-time PCR (RT-qPCR) techniques did not show any significant alterations in the expression patterns in these genes in larvae at 108 hpf (4.5 dpf). These studies documented that GP (4–200 pg/embryo) is unable to induce any sleeping disorder in zebrafish larvae probably because the dose of GP (amount of microinjected material) did not reach the toxic level.

From these three studies based on microinjection it is evident that the toxic effects of GPN were evaluated in various aspects of zebrafish development including morphological disorders (body length, tail flexure, yolk sac and cardiac edema, heart and blood vessels) behavior (sleeping disorder), biochemical endpoints (apoptosis) and gene expression/regulation analyses (*nrarpa*, *nrarpb*, *hcrt*, *hcrtR*, *aanat2*, *vegfaa*). However, the concentration of GPN used, and the time of observation on the animals after GPN administration were different and the resulting effects were not well characterized. For proper justification and confirmation of these observations, further studies are necessary.

3.1.2. Administration of GPN by waterborne exposure—Zebrafish embryos at 4hpf were exposed to different concentrations (1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μ g/L) of pG (size 170–390 nm) in E3 medium (5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM calcium chloride, 0.33 mM magnesium sulphate, pH 7.4) and evaluated for developmental disorders by assessing morphological features including cardiac and yolk sac edema, hatching, and mortality at different time points of development (24, 48, 72, and 96 hpf) (Manjunatha et al., 2018). It was observed that pG was able to induce embryonic mortality, and hatching delay (Table 2). Embryos exposed to 30 μ g/L or above pG died within 30 min–2 h of treatment. Moreover, pericardial and yolk sac edema were also observed in embryos exposed to lower concentrations (5–25 μ g/L) of pG. Pericardial toxicity

and bradycardia (slow heart rate) were also observed. These data indicate that pG is able to induce adverse effects in zebrafish development targeting heart and other morphological features.

Zebrafish embryos were exposed to GQD (size ranged from 2.3–6.4 nm), a zero dimensional carbon-based material, at 12.5, 25, 50, 100, and 200 µg/mL concentrations, 4–120 hpf in E3 medium containing 10–15% methylene blue (Jiang et al., 2015). The data indicate that GQD was mainly incorporated in the myocardial cytoplasm and the heart beats were reduced only at higher concentrations of GQD (50–200 µg/mL) from 50–120hpf (Jiang et al., 2015). In another study, zebrafish embryos exposed to GQD (12.5–200 µg/mL) 4–96h showed reduction in the heart rate and hatching efficiencies of the embryos in a concentration-dependent manner (Wang et al. 2015). At the highest concentration, the toxic effects of GQD (200 µg/mL) were more pronounced in the larvae showing pericardial edema, bent spine, vitelline cyst and curved tail (Wang et al. 2015). The swimming behavior of the larvae was also affected by GQD during zebrafish development. Spontaneous movement of the larvae was decreased in a concentration-dependent manner; however, the embryos were hyperactive at 12.5 µg/mL concentration, but became hypoactive in 25, 50, 100 and 200 µg/mL GQD (Wang et al. 2015). Zebrafish embryos exposed to thiolated GQD (SH-GQD) (prepared by using thermal treatment of 20% citric acid in presence of 20% GSH) for various time points (0– 7dpf) with several concentrations (0.02–1 mg/mL) in 30% Danieau's solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM Hepes, pH 7.4] increased the larval mortality in a concentration-dependent manner (Oh et al. 2017). Morphological deformities in head, vent tail, pericardial edema, and yolk sac edema were observed when the concentration of SH-GQD increased to 0.8 mg/mL. Although the inhibition of ROS generation due to oxidative stress was inhibited by SH-GQD in a concentration-dependent manner in zebrafish embryos, at higher concentrations, SH-GQD is toxic to the larva (46% for 1 mg/mL).

Zebrafish embryos 4–120 hpf were treated with 5, 10, 50, 100 µg/mL GO (Graphene Inc, New York, USA) in E3 (2 embryos in 1 mL medium /well) and evaluated at 4, 24, 48, 72, 96, and 120 hpf (d'Amora et al. 2017) for toxicological endpoints. The test solution was refreshed every 12 h. The toxicity of GO with regard to hatching and developmental delay showed a concentration-dependent effect with 50 µg/mL and above than the controls and lower concentrations (5–10 µg/mL). At higher concentrations (50–100 µg/mL), the survival rate and the hatching of the embryos decreased significantly between 72 and 120 hpf. The heart beats of the larvae at 72 hpf and the frequency of movements at 96 hpf were reduced significantly in the embryos treated with 50–100 µg/mL GO (d'Amora et al. 2017). In a separate study, zebrafish embryos were exposed to GO flakes (40 and 80 mg/L) in freshwater for 4–96 h and evaluated for morphological and behavioral features and also the expression of heme-oxygenase (HO) and inducible Nitric Oxide synthase (iNOS) enzymes in larvae. No alteration in mortality, growth, brain morphology, pharyngeal arches and jaw structures, heart, fins, notochord, somites, body shape, cardiovascular function, yolk sac and locomotor functions were observed. However, both HO and iNOS mRNA and proteins levels were enhanced by GO at 96 hpf (Pecoraro et al. 2018) which indicate that the generation of reactive nitrogen species (RNS) in zebrafish larvae can be induced by GO flakes during development.

Zebrafish embryos 2hpf were exposed to 5, 10, 50, and 100 mg/L GO suspended in 1% pluorenic F (PF68) solution (Soares et al. 2017) until 6 dpf with a static renewal of media every day. Parallel control groups were run in system water and 1% pluronic F 68 (PF68) side by side. Mortality and hatching rates were found to be unaltered by GO. Spontaneous movements was also unaltered in 24 hpf embryos by all concentrations of GO (5–100 mg/L). Heart rates of 48 hpf embryos remained unaltered except in 10 mg/L concentration where it was significantly increased. With regard to larval morphology, no significant difference was noticed in eye areas of the larvae on 5 dpf, however, the total body length was reduced significantly only in larvae exposed to 100 mg/L GO. Expression analysis of genes responsible for nervous system development showed that *synapsin IIa* gene was upregulated and *dat* gene was downregulated in 6 dpf larvae exposed to 5 mg/L GO. Moreover, larvae of 10 mg/L group, showed upregulation in *neurogenin 1* and *dat* genes; expression of other genes such as *gap43*, *gfap*, and *nestin* remained unaltered. Analysis of larval locomotion in 6 dpf indicate that the average speed and the total distance travelled by the larvae exposed to 10 mg/L group was found to be significantly higher than the larvae maintained in 1% PF68. The turn angle of the larvae during locomotion was found to be increased in 10 mg/L group. While acetylcholinesterase enzyme (AChE) activity in these larvae remained unaltered on 4.5 dph larvae of zebrafish, dopamine levels were found to be decreased in 10 mg/L group. Further analysis of genes responsible for apoptosis in these larvae indicated that the antiapoptotic gene B-cell lymphoma 2 (*bcl2*) and the apoptotic gene *caspase 3* were increased significantly in larvae exposed to 10 mg/L when compared with the larvae exposed to 1% PF68. Histological evaluation of the brain of the 6 dpf larvae exposed to 10 mg/L GO showed autophagosome formation, vacuoles, and partial loss of nuclear membrane in specific regions of the ventral diencephalon which indicate impairment of brain development in zebrafish by GO (Soares et al. 2017).

Chen et al (2015a) demonstrated that zebrafish embryos exposed from 2.5–96 hpf with 100 mg/L GO (size range 0.8–1.2 nm) in E3 medium showed adherence and interaction of GO on the surface of the chorion after forming a layer approximately 50–600 nm in thickness. Moreover, GO interacts via hydroxyl radical that resulted blockage of the pore canals on the chorionic membrane and induced hypoxia and hatching delay. Furthermore, GO also spontaneously penetrated the chorion, entered into the embryo via endocytosis, and primarily translocated to the eye, heart, and yolk sac regions. Entry of GO into the embryo damaged the mitochondria, induced excessive generation of ROS, and increased oxidative stress that resulted in DNA damage and apoptosis. Moreover, GO also induced malformed eyes, cardiac and yolk sac edema, tail flexure and altered heart rates (Chen et al. 2015a). Studies in Japanese medaka fish embryos exposed to oxidized graphene nanoribbons (O-GNRS, 20 µg/mL, 1dpf-7 dpf) indicated that O-GNRS can enter the chorion and enhanced hatching efficiency by 2 days than the control embryos which were generally hatched by 12 dpf (Mullick Chowdhury et al. 2014).

Blastula stages of zebrafish embryos were exposed to different concentrations (5, 25, and 50 µg/mL) of GO (height ~1.5 nm; lateral size 1500 nm) for 12, 24, 48, and 72 hpf (Lu et al., 2017). The survival rates of the embryos were decreased with all GO concentrations within first 24 h of exposure and remained constant thereafter (from 48–72 h). With the increase of GO concentration and exposure time, hatching rates were not significantly affected.

Transcription levels of base excision repair (BER) enzyme genes such as *apex 1*, *ogg1*, *polb*, and *creb 1* were upregulated in 24 hpf embryos exposed to 50 µg/mL GO. These observations indicate that although genomic DNA can be disrupted by GPN, activation of BER enzymes have the potential to repair/recover the DNA damage, if any, by enhancing the functions of BER enzymes.

Zhang et al (2017b) exposed zebrafish embryos to GO (thickness 1.01 ± 0.05 nm, lateral length 300–2600 nm) in E3 medium with concentrations 0.001–100 mg/L from 2.5–168 hpf (7dpf). The test solution was renewed every day. The developmental toxicity (hatching rate, malformation, mortality, oxidative damage, organ development, gene expression, and metabolite disruption) was found to be nonmonotonic. As the mortality was not concentration-dependent between 0.001 mg-100 mg/L, the lethal concentration (LC_{50}) of GO during embryonic development of zebrafish was difficult to calculate (Zhang et al. 2017b). The embryos exposed to trace/ultralow concentration of GO (1–100 µg/L) showed DNA alteration, protein carbonylation, and excessive generation of ROS (especially, the superoxide radicals). Transcriptomic analysis also indicate that GO at ultralow concentrations (1–100 µg/L) disrupted the expression of collagen and metalloproteinase-regulated genes that affected the cardiac and skeletal development in zebrafish. Moreover, metabolomics analysis in the larvae exposed to ultralow concentrations of GO (1–100 µg/L) on 7 dpf showed inhibition of amino acid metabolism that impaired the ratios of unsaturated to saturated fatty acids (Zhang et al., 2017b). Low concentration (20 µg/mL) suspension of graphene nanoribbons (GNRs) prepared by as low as 1 min probe sonication, compared to bath-sonicated or nonsonicated suspension, can significantly increase embryo/larval mortality of Japanese medaka embryos (Mullick Chowdhury et al 2014). Moreover, 80% of the embryos incubated with oxidized bath sonicated (20 min) GNR (O-GNR) solutions, hatched by 10 dpf which is about 2 days earlier than the control embryos (hatched on 12 dpf). All these studies confirmed that the toxic potential of GO is more prominent in µg levels (1–100 µg/L) than in mg levels (1–100 mg/L).

In the presence of 10 mg/L humic acid (HA) in the medium, adverse effects of GO on zebrafish embryos were markedly reduced with regard to larval morphology, ROS, and expression of oxidative enzymes at the transcription level (Chen et al. 2015b). HA, in the presence of 100 mg/L GO, covered the chorion by a layer of GO approximately 800–1200 nm thickness which is comparatively higher than the 100 mg/L GO alone (which is 50–600 nm thickness). Moreover, HA also altered the uptake and deposition and decreased the aggregation of GO in embryonic yolk cells and deep layer cells. In contrast to the protective effects of HA documented by Chen et al (2015b), in a similar study by Clemente et al (2017) found contradictory results. They (Clemente et al. 2017) have exposed zebrafish embryos in 1, 10, and 100 mg/L GO (width 394.21 ± 215.05 nm, height 0.89 ± 0.01 nm) from 1hpf to 96 hpf with or without HA (20 mg/L); also 1hpf embryos were exposed to 100 mg/L base-washed GO (bwGO; width 286.53 ± 104.42 nm, height 0.94 ± 0.02 nm) with or without HA (20 mg/L). They (Clemente et al. 2017) observed that GO at 100 mg/L concentration was able to significantly reduce the body length but 20 mg/L HA supplementation to the medium was unable to attenuate the reduction of larval body length at 96 hpf. Moreover, bwGO (100 mg/L) alone was unable to reduce the body length; but in presence of HA (20 mg/L) in the medium, the body length was significantly reduced as observed in larvae exposed to GO

(100 mg/L) only. This group further observed that GO (1, 10, 100 mg/L) alone was also unable to alter catalase, GST, or acid phosphatase (AP) enzyme activities in 96 hpf larvae; but HA (20 mg/L) alone was able to increase AP activity and that increase was significantly reduced in presence of GO (1, 10, 100 mg/L) in a concentration-dependent manner. Also compared to control larvae, bwGO (100 mg/L) alone was able to significantly reduce GST enzyme activities; supplementation of HA ameliorated GST enzyme reduction and restored the activity to the control level. Moreover, AChE activity was found to be significantly reduce in the larvae exposed to 100 mg/L GO and that reduction was also observed after HA (20 mg/L) supplementation. Therefore attenuation of the toxic effects of GO by HA is controversial and needs further study.

Mu et al (2015) was able to covalently immobilize L-cysteine into GO to form L-cysteine-GO hybrids (cysGO; 1.42 ± 0.34 nm sheet thickness, lateral length 78–590 nm) and used to reduce the adverse effects of GO (thickness is 0.87 ± 0.157 nm, lateral length 50–250 nm) using zebrafish embryos as the model. Fertilized eggs of zebrafish were exposed to GO or cysGO (0.01–10 mg/L), 0–120 hpf in E3 medium, replaced every day. Cardiac edema and the suppression of cell nucleus development in the inner eye tissue were observed in the 120 hpf larvae exposed to GO. In contrast, in cysGO exposure which exhibited remarkable uptake in vivo, no tissue defects, malformation, death or any significant hatching delay were observed. Moreover, cysGO was unable to induce any significant DNA damage, or reduction in Na^+/K^+ -ATPase activity, or decreased in mitochondrial membrane potential as observed in embryos exposed to GO only (Mu et al. 2015). Also, cysGO protected the embryos from arsenic toxicity. Therefore, immobilization of cysteine, an inexpensive biomolecule, onto GO is able to reduce the toxic effects and make it more biocompatible (Mu et al. 2015) to the embryonic body.

Embryos of zebrafish (0–120 hpf) were exposed to GO nanosheets (GONS) and GONS coated with biological secretion (GOBS) (Mu et al. 2016); GOBS are small organic molecules containing, proteins, nucleotides, and mucopolysaccharides released from the body of zebrafish into the culture medium and bound to GONS; The thickness of GOBS is approximately 10 nm and the lateral length ranged from 19.5 to 282 nm. The concentrations of GONS and GOBS used in these experiments were 0.01, 0.1, and 1 mg/L (media replacement every 24 h). Mortality (based on movement, heartbeats, and blood circulation), malformation (tail flexure and pericardial edema), and heartbeats, were evaluated every day until 5 dpf. ROS, β -galactosidase activity, mitochondrial membrane potential, oxygen concentration, calcium influx, and DNA methylation were also determined in 5 dpf embryos. GOBS triggers higher toxicity than GONS with regard to death, malformation, loss in mitochondrial membrane potential, and upregulation of β -galactosidase probably by inhibiting ion exchange and oxygen uptake in the embryos (Mu et al. 2016). Therefore, biological secretions released in the aquatic environment can modulate the toxic potential of GPN. Further investigations are also needed in this research area.

Zebrafish embryos were exposed to GO and rGO from 2hpf to 96hpf with 1, 5, 10, 50, and 100 mg/L concentrations. It was observed that rGO significantly inhibited hatching and decreased the length of the hatched larvae at 96 hpf (Liu et al., 2014). Other evaluated

parameters such as spontaneous movement, heart rate, mortality, and morphological malformations were remained unaltered.

Wild type AB strain zebrafish and transgenic (*Tg(cyp1a:gfp)*) zebrafish were treated with 25, 50, and 100 µg/mL rGOQD at 4 hpf in deionized (DI) water and maintained for 7 days with the media changed every day (Zhang et al., 2017a). It was observed that rGOQD (25–100 µg/mL) has no effect on body length, mortality, hatching rates, however, the heartbeats (96 hpf; 100 µg/mL) were significantly reduced compared to control larvae. Moreover, the pericardial edema, vitelline cyst, and bent spine were induced in larvae exposed to 100 µg/mL rGOQD (Zhang et al., 2017a). The expression of AhR pathway genes such as *cyp1a*, *cyp1c*, *cyp7a1* and *hsp70* genes were enhanced in larvae (7 dpf) exposed to 100 µg/mL rGOQD (only *cyp1a* and *cyp1c*, showed significant difference). In transgenic zebrafish (*Tg(cyp1a:gfp)*), the expression of *gfp* was enhanced in a concentration-dependent manner which indicate that GPNs can disrupt AhR pathway.

It is evident from all these studies that the embryo-larval development of zebrafish is a unique model to evaluate the toxic potential of GPNs in the aquatic environment. Although exposure of the embryos to GPN waterborne (media used is mostly E3, other media are also used) is the only similarity among all these experiments designed by various investigators, the variabilities in concentration, shape, size, thickness of GPN molecules, time of exposure, condition of exposure, make it very complicated to develop a unique hypothesis regarding the mechanisms of GPN toxicity to aquatic organisms at the molecular level. The embryos were exposed to GPN either for a short period of time while they are inside the chorion, or the treatment was prolonged for longer period starting from the beginning of embryonic life (within the chorion) to the early phases of larval life when they are no longer inside the chorion. The concentration of the nanomaterials used in these studies is also widely variable starting from µg/L to mg/L (Table 2). The investigations also examined others important endpoints including morphology (body length, eye, craniofacial structures, tail/spinal flexure, yolk sac and cardiac edema, malfunction of heart and blood vessels, hatching and mortality), behavior (swimming and movement; sleeping disorders), cell signaling mechanisms (ROS, RNS and oxidative stress), metabolic disorders (*cyp1a*, *cyp1c*, *cyp7a1*); apoptosis (*bcl2*, caspase; autophagosome), nervous system deregulation (acetylcholinesterase, dopaminergic neurons; neurotrophin, synapsin IIa), genetic and epigenetic mechanisms (*colla1a*, *coll1a2*, *col2a1a*, *col11a1a*, *dat*, *hcrt*, *hcrtr*, *aanat2*, *gap43*, *gfp*, *nestin*, *vegfaa*, *apex1*, *ogg1*, *polb*, *creb1*, *IIa*, *hsp70*), and others (organs). Despite all these variations, it is conceivable that GPN are toxic to many stages of zebrafish embryo-development which provides a unique model for the evaluation of GPN toxicity in the aquatic environment.

3.2. Effects on larvae

Zebrafish larvae 72 hpf (no chorion) were exposed to 0.01, 0.1, 1.0 µg/L GO (thickness 1.02±0.15 nm; lateral length 0.5 µm to several microns) for 24 h and the mortality and malformation were observed in 120 hpf (Ren et al., 2016). The malformation including pericardial edema, yolk sac edema, tail flexure, and spinal curvature in the 120 hpf larvae were induced in a concentration-dependent manner (Table 2). Moreover, at 96 hpf, the

distribution of dopaminergic neurons in the diencephalic region of the brain of zebrafish larvae was reduced significantly in a concentration-dependent manner (0.01–1 µg/L). The enhancement of ROS by GO exposure significantly increased *α-synuclein* and *ubiquitin* gene transcriptions in 96 hpf larvae followed by a decrease in locomotor activities in 7 dpf larvae. These observations suggest that even at ultralow concentrations (µg/L), GO is a potential inducer of Parkinson's disease (PD) like phenotypes in zebrafish. Increase in ROS and upregulation of several amino acids and some fatty acids (dodecanoic acid, hexadecanoic acid, octadecenoic acid, nonanoic acid, arachidonic acid, eicosanoic acid, propanoic acid, benzenedicarboxylic acid) and downregulation of other fatty acids (butanoic acid, phthalic acid, and docosenoic acid) as observed by metabolomics study could be linked to the generation of PD phenotypes in zebrafish larvae (Ren et al. 2016).

Twenty four–48 hour post-hatch (hph) larvae of Japanese medaka fish were exposed to graphene nanoparticles (GNP) and graphene-TiO₂ nanoparticles composites (GNP-TiO₂) at concentrations of 2, 5, 8, 10, 14, 17, and 20 mg/L under simulated solar radiation (SSR) for 4, 24, 28, and 48 h and evaluated the rate of mortality during the exposure period. In another experiment, 24–48 hph larvae were exposed to 167 and 500 mg/L GPN-TiO₂ for identical periods (4, 24, 28 and 48 h) under dark and evaluated the mortality at 4, 24, 28 and 48 h. The calculated LC₅₀ values were 11 mg/L for GPN-TiO₂ under SSR, and more than 500 mg/L in dark (Li et al. 2014). The authors (Li et al. 2014) suggested that the toxic effects of GPN-TiO₂ was more pronounced under SSR because of the generation of more ROS leading to a rapid death of the larvae in SSR than in the dark.

Compared to embryos, the use of zebrafish larvae in the evaluation of GPN toxicity is very limited. The exposure was initiated when the embryos were no longer inside the chorion and evaluated for morphological (tail flexure; cardiac and yolk sac edema), behavioral (swimming), biochemical (metabolic disorders and apoptosis) and gene expression (*α-synuclein*, *ubiquitin*) analyses (Table 2). Moreover, Japanese medaka fish larvae have been documented GPN-TiO₂ toxicity under specific conditions. With these limitations, we can conclude that more studies are needed on zebrafish larvae before accepting this larval model as a reference in the evaluation of GPN toxicity in the aquatic environment.

3.3. Effects on adults.

Two month old zebrafish were exposed to 1, 5, 10, and 50 mg/L GO for 14 days with a 50% static renewal of the medium every day (Chen et al. 2016). No obvious acute toxicity was observed; however, histological analysis indicated that in liver and intestine, cellular vacuolation, loose arrangement, histolysis, and disintegration of cellular boundaries occurred (Chen et al. 2016). Further analysis showed that enzyme activities related to oxidative stress such as superoxide dismutase and catalase (CAT) were increased that enhanced malondialdehyde (MDA) and decreased glutathione (GSH) content of liver after GO treatment (Table 2). Moreover, in the spleen, the transcription of proinflammatory cytokine genes such as tumor necrosis factor- α (*tnf- α*), interleukin-1 β (*il-1 β*), and interleukin-6 (*il-6*) were increased. Although any significant toxic effects of GO on zebrafish morphology were not observed, modulation of enzymes related to oxidative stress and inflammation

suggested that GO has the potential to target biochemical pathways such as oxidative stress that induced immune-mediated toxicity.

Six months old adult zebrafish (both male and female) were exposed to sub-lethal concentrations (2, 10, and 20 mg/L) of GO (thickness 0.1 nm, average area 0.58 μm^2) either for 24 or 72 h as short-term exposure (for apoptosis, necrosis, DNA damage and oxidative stress) or for 14 days for chronic assay (histopathology) (Souza et al. 2017) in fasting conditions. In short-term exposure, as a result of significant induction of ROS, the number of apoptotic and necrotic cells in gills were increased. However, no significant damage in blood cell DNA was observed. In chronic exposure, structural analysis of gills revealed injuries and necrosis, including a dilated marginal channel, lamellar fusion, clubbed tips, swollen mucocytes, epithelial lifting, and aneurysms. In liver, the lesions also appeared in hepatocytes with peripherally located nuclei; moreover, hepatocytes exhibited a non-uniform shape, picnotic nuclei, vacuole formation, cell rupture and necrosis (Table 2). The gut of the GO-exposed fish was filled with unidentified brown spots, probably the accumulation of GO in gut tissues, even though the fish were in fasting conditions during the entire period of exposure (14 days).

The effects of GO (hydrodynamic size of GO nanoplates 246–257 nm and thickness 0.7–1.8 nm) in common carp (*Cyprinus carpio*), were evaluated in combination with other organic pollutants such as perfluorooctanesulfonate (PFOS), an organic pollutant that persists in the aquatic environment (Qiang et al. 2016). Juvenile common carps of 3 months old were exposed to PFOS (500 ng/L) and GO (1mg/L) for 28 days. The blood, liver, kidney, gill, intestine, and muscle were used for PFOS analysis. GO significantly enhanced PFOS bioaccumulation in the tissues of common carp, especially in liver, kidney and intestine (Table 2).

Although most of the current toxicity assays of GPN using embryos, larvae and adults of zebrafish, have been based on morphological end points, the vast genomic resources in this fish have also been utilized to target the molecular mechanisms associated with GPN toxicity (Lu et al. 2017; Ren et al. 2016; Zhang et al. 2017a; Zhang et al. 2017b). Besides zebrafish, no other aquatic organisms have been utilized so extensively to study the molecular mechanisms of GPN toxicity. While the experimental design, developmental stage, GPN types used in zebrafish models have been extensively considered, the embryo-larval development provides a better opportunity to develop as a unique model to evaluate and determine the toxic limits of GPN in the aquatic environment.

4. Discussion and perspectives:

With the rapid increase in the production and application of GPN, significant amounts are released into the environment, posing a potential threat to human health. When released into the aquatic environment, GPN interact with the existing inorganic ions and NOM that potentially cause significant adverse effects on the ecosystem. To our knowledge, the information regarding the overall concentration, or the environmentally relevant concentrations (ERC) of GPN, in the aquatic environment is unavailable. However, the concentrations of engineered nanomaterials (ENM), based on a stochastic/probabilistic

material-flow computer model, are in the range of ng/L to µg/L (Sun et al., 2016). We expect that the disposition of GPNs in the aquatic environment is also within the similar range (ng/L to µg/L) which will be able to induce significant toxic effects.. Moreover, investigations on GPN toxicity in aquatic organisms including bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*), algae (*Raphidocelis subcapitata*, *Chlorella vulgaris*), invertebrates (*Daphnia magna*, *Artemia salina*, *Tubifex tubifex*), and vertebrates (*Danio rerio*; *Oryzias latipes*) (Mullick Choudhury et al. 2014; Qiang et al. 2016; De Marchi et al., 2017) have been conducted; however, the published literature is still insufficient to allow the selection of a unique model organism for testing the toxic potential of GPN or to establish guidelines for an ERC limit which seems to be safe for the aquatic organisms. Zebrafish have been proposed as an *in vivo* vertebrate model for mechanistic studies of nanoparticles of interest, including GPN (Lin et al., 2013; ORCD 2013). However, the ERC limit of GPNs in the aquatic environment is yet to be determined. More studies with this fish model and other fish species are necessary for the universal acceptance of zebrafish as the unique model for the evaluation of toxicity or the estimation of ERC limit of GPN in the aquatic environment.

Even though the published literature is very limited, from the available reports it is clear that GPN has the potential to induce developmental, respiratory, neurobehavioral, inflammatory, and metabolic disorders in fish, especially in zebrafish. Our literature search, in addition to zebrafish, found only two other fish species, Japanese medaka and common carp, which were used for the evaluation of GPN-induced toxicity. The route of administration, duration, age of the fish (embryo, larvae, adult), and culture conditions, are the important determinants that impacts the toxicity of GPNs. Both microinjection and waterborne exposure were used for zebrafish embryos, however, for larvae and adults only waterborne exposure was adopted and studies were mostly on embryo-larval development rather than adult fish (Table 1). Among GPN, pG, GQD, GO, GO nanosheets, GO flakes, single layer GO, rGO and rGOQD were used; in addition, several NOM, including HA, CYS, bwGO, and GOBS potentially modulate the effects of GBNs on zebrafish (Table 2). Although in this review, we have restricted our observations on embryos, larvae, and adult fish, several fish cell lines, including the hepatocellular carcinoma cell line PLHC-1 (derived from topminnow fish, *Poeciliopsis lucida*) (Lammel and Navas, 2014), cardiac cell lines (SICH) of *Catla catla* (Xing et al. 2016) and bluegill sunfish cells (BF-2) (Srikanth et al. 2018) have been used for the evaluation of GPN toxicity (GO and RGO for SICH cell lines and PLHC1 and BF-2 for GO). From the available literature we reviewed we found that the size, shape, surface properties and chemistry, concentration, agglomeration, dose, and method of preparation of GPN, are the major determinants that affect the various biological activities in zebrafish (Chen et al. 2015a, b; 2016; Zhu et al., 2015; Ren et al. 2016; Lu et al. 2017; Zhang et al. 2017a; Zhang et al. 2017b; De Marchi et al. 2018). As mentioned above, presence of NOM, thiol, or other compounds such as polyethylene glycol in the media or coating/labeling of GPN (functionalization) by fluorescence emitters or others, were also able to substantially modulate the toxic effects of GPN (Chen et al. 2015b; Mu et al. 2015;2016; Clemente et al. 2017; Oh et al. 2017). Most of the methods used for the assessment of toxicity in fish did not consider the various physicochemical properties including shape, size, surface charges and others observed in GPNs which may interfere with the interpretation of the results. Even

though OECD described standard experimental guidelines for the evaluation of the toxic potential of various chemicals in zebrafish (OECD 2013), the conditions used for GPN studies by various investigators are widely variable which can also interfere during data interpretation (Table 2).

The most needed information about the acute toxicity of a chemical is its lethal concentration (LC_{50} or IC_{50}) that kills 50% of test organisms. To our knowledge there is no published data available on LC_{50} or IC_{50} values of GPN on zebrafish. However, it has been documented that the higher concentrations of pG (30 mg/L and above) are highly toxic to the zebrafish embryos at 4hpf (Manjunatha et al. 2018). The major problem for LC_{50} calculation in fertilized zebrafish embryos exposed to GO (0.001–100 mg/L for 7 days) is the lack of a concentration-dependent mortality after GO exposure (Chen et al. 2015a; Zhang et al. 2017b). For Japanese medaka, the LC_{50} of GNP-TiO₂ is photo-sensitive (11 mg/L under SSR, and greater than 500 mg/L in dark) (Li et al. 2014). Studies on other aquatic models such as *Daphnia magna* have documented that the LC_{50} for GNP-TiO₂ is 90 mg/L under SSR and 138 mg/L in dark (Li et al. 2014). Report indicate that the 72h LC_{50} of GO in *D. magna* is 45.4 mg/L (Lv et al. 2018) which seems to be 3-fold lower than GNP-TiO₂. Therefore, to evaluate the toxic effects of GPN in zebrafish, studies on morphological, neurobehavioral, biochemical, or molecular biological features (Supplementary file 1) are necessary.

Despite all these limitations, one of the significant endpoints that was affected by GPN is the reduction in hatching efficiencies of the embryos. In standard culture conditions (E3 medium) zebrafish embryos generally hatched within 48–72 hpf. However, Japanese medaka embryos cultured in ERM initiate hatching ~175 hpf (Wu et al. 2008). In zebrafish, a higher concentration of GPN (generally mg/mL) was able to delay hatching (Manjunatha et al. 2018), however, in Japanese medaka the hatching was enhanced by GPN (20 mg/L) (Mullick Chowdhury et al. 2014). Hatching in both zebrafish and medaka are regulated by hatching enzymes. The zebrafish genome consists of two hatching enzyme homologs; zhe1, a zinc metalloprotease stored in glands before being released into the perivitelline space (Okada et al. 2009). The expression of zhe1 was observed by 11.5 hpf and a strong signal was found in 24 hpf and no expression was observed after hatching. The other enzyme zhe2 is rarely expressed. In medaka, there are two hatching enzymes, high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE) (Yamagami 1996). HCE swells the egg envelope, and LCE completely dissolves it. Although expression of hatching enzyme genes in embryos of zebrafish and Japanese medaka by GPN was not studied, Chen et al (2015a) commented that hatching enzyme genes are not responsible for hatching delay in zebrafish; however, we expect that even though more studies are needed, modulation of hatching enzyme gene expression by GPN in both zebrafish and Japanese medaka is a potential cause of hatching delay.

From all these studies it is evident that GPN can successfully accumulate in embryos, larvae, and adult fish from the surrounding environments (Table 1) and enhance mortality by targeting the morphological, neurobehavioral, biochemical, cell signaling, metabolomics, genetic mechanisms at specific organs (liver, heart, gut, brain, spleen) of the zebrafish (Table 2). The primary mechanism of toxicity caused by GPN currently proposed is the induction

of oxidative stress (Chen et al. 2015a, b; 2016; Ren et al. 2016; Zhang et al. 2017) which subsequently triggers apoptosis and cell death. Normal cellular homeostasis reflects a balance between the generation of ROS (as prooxidants) and elimination/or reduction of ROS by antioxidant enzymes (Stone et al. 2009; Sanchez et al. 2012; Seabra et al. 2014). Studies indicate that GPN administration to embryos, larvae, and adults induced ROS that target mitochondrial pathways (Li et al. 2012). GO can damage the mitochondrial membrane and induce ROS via direct physical process. It has been reported that GO has sharp edges that can directly rupture the mitochondrial membrane and induce ROS (Chen et al. 2015a; Ren et al. 2016). In addition, hypoxic environments especially in embryos exposed to GPN waterborne may also induce ROS as well as alter energy metabolism (Chen et al. 2015a). It was observed that agglomeration of GO on the chorion of zebrafish embryos was able to reduce the oxygen content in the interchorionic space and create a hypoxic state to the developing embryos and induce apoptosis (Chen et al. 2015a).

ROS generation by GPN in the embryos, larvae, and some specific organs such as eye, heart, tail, brain, gill and liver of embryos, larvae and adult zebrafish were increased in a concentration and time-dependent manner (Chen et al. 2015a; 2016; Ren et al. 2016; Zhang et al. 2017b). The generation of ROS can potentially activate the antioxidant enzymes to eliminate ROS from the cellular environment. However, the major antioxidant enzymes responsible for ROS elimination are catalase (CAT), glutathione peroxidase type I, copper/zinc superoxide dismutase, superoxide dismutase (SOD), and glutathione s-transferase (GST) which were down-regulated by GO (Zhang et al. 2017b). Moreover, MDA and GSH, the indicators of oxidative stress at the cellular level, were increased significantly by GO in larvae at 72 hpf (Chen et al. 2015a). MDA is produced as a result of the reaction between the free radicals and unsaturated fatty acids in the cell membranes. Enhancement of MDA by GPN indicated a probable increase in lipid peroxidation which was attributable to ROS production. The GSH followed a nonenzymatic mechanism to scavenge free radicals generated from oxidative metabolism those were not decomposed by antioxidant enzymes (El-Shenawy 2010). Moreover, SOD is crucial in converting superoxide radicals to hydrogen peroxides (H_2O_2) and CAT facilitates removal of hydrogen peroxide (H_2O_2), metabolizing it to molecular oxygen and water (Daoud et al. 2012). GO also down-regulated SOD activity in a concentration-dependent manner and increased the concentration of malondialdehyde (MDA) (Chen et al. 2015a). Furthermore, it was also observed that GO at different concentrations did not change GST or CAT activities in embryos of zebrafish (Clemente et al. 2017); in adult fish (2-month old) SOD, CAT and MDA were increased and GSH content was decreased in liver exposed to GO (1, 5, and 10 mg/L, 1–4 days) (Chen et al. 2016). In contrast to these observations, no obvious oxidative stress with regard to alteration in the activities of SOD, CAT, GSH and MDA enzymes was observed in the liver of adult zebrafish exposed to 250 μ g/L sFLG for 24, 48, and 72 h (Lu et al., 2017). Moreover, the enhancement of iNOS mRNA and protein in zebrafish larvae exposed to GO (Pecoraro et al. 2018) indicated that reactive nitrogen species (RNS)–mediated pathways were also involved in GO-induced toxicity. HO, the enzyme catalyzes the degradation of heme, is also enhanced by GO (Pecoraro et al., 2018). Although the data are contradictory, all these studies indicate that generation of ROS is able to modulate the expression and functions of antioxidant

enzymes either directly or indirectly and have a potential impact on cellular MDA and GSH concentrations.

Studies also indicated that GPNs exhibit several unique properties by which they interact with biomolecules such as nucleic acids, lipids and fatty acids, protein and peptides and also with sugars (Sanchez et al., 2012) and able to generate ROS and oxidative stress at the cellular level. Damage of DNA and induction of apoptosis by oxidative stress can disrupt embryo-larval development as well as cellular functions in larvae and adult fish exposed to GPN. Studies documented that caspase enzyme genes (*caspase 3* and *caspase 8*) and anti-apoptotic gene *bcl2* were increased significantly in 6 dpf larvae exposed continuously to 10 mg/L GO (Soares et al., 2017) and caspase 8 protein in 72 hpf larvae exposed to GO for 24 h (Ren et al. 2016). In adults (6 month old) short-term exposure (24 h) to 2 or 20 mg/L GO enhanced the number of apoptotic cells in the gills. These data suggests that oxidative stress induced by GPN can activate apoptosis and thus cell death.

Despite morphological anomalies in embryos and larvae, oxidative stress induced by GPN can impair angiogenesis (Jeong et al. 2015), neurobehavior (Wang et al. 2015; Clemente et al. 2017; Li et al. 2017; Ren et al. 2016; Soares et al. 2017), and inflammatory responses (Chen et al. 2016). The significant observation in abnormalities in angiogenesis induced by GPN (NGO) is the sprouting of ISV in the trunk vasculature of the 52 hpf zebrafish embryos which were microinjected with NGO (500 pg) at 1–2 cell stages of development (Jeong et al. 2015). Gene analysis at 30 hpf indicate that GO disrupted the expression of VEGF (*vegfaa*), and Notch (*nrarpa* and *nrarpb*) signaling pathway genes which can regulate growth and branching of blood vessels by interdependent mechanisms. Neurobehavioral analysis of zebrafish larvae, exposed to ultralow concentration of GO (0.01, 0.1, and 1.0 µg/L) for 24h (72–96 hpf) showed Parkinson's disease like symptoms (Ren et al., 2016). However, microinjection of GP (4, 40 or 200 pg/embryo) to 1–4 cell stage embryos and evaluation of sleep/wake activities in 4.5 dpf larvae failed to establish any significant effect (Li et al. 2017). Moreover, gene expression patterns of hcrt system (*aanat2*, *hcrt*, and *hcrttr*) in larvae pretreated with GPN, remained unaltered which indicate ineffectiveness of GPN in sleep/wake activities of zebrafish. Continuous exposure to 10 mg/L GO during embryo-larval development and the assessment of locomotor activities on 6 dpf zebrafish larvae were found to be increased significantly; while increase in the exposure concentrations of GO to 50 and 100 mg/L were unable to establish any significant effects, probably due to agglomeration (Soares et al. 2017). Another study with GQD (12.5–200 µg/mL, 4–96 hpf exposure) showed significant reduction (hypoactive) in the spontaneous movement (total swimming distance and speed) of the larvae in a concentration-dependent manner (25–200 µg/mL). However, the embryos were hyperactive if exposed to low concentration (12.5 µg/mL) GQD (Wang et al. 2015). Taken together, it was suggested that oxidative stress-mediated neuronal damage by GPN was more effective in larvae exposed to ultralow concentrations and able to target brain and induced movement-related disorders than the higher concentrations (mg/L) which have a tendency to agglomerate in the environment.

GPN-induced oxidative stress also showed immunomodulatory effects in spleen of zebrafish adults. Although the modulation of oxidative stress-related events such as enhancement of MDA, CAT, and SOD reduction of GSH content, was persistent only for short periods of

time (1–4 day exposure), immunomodulatory response was observed in spleen of zebrafish adults after two weeks of GO treatment, which suggest that oxidative stress in earlier treatment can activate inflammatory responses in later time points (Stone et al. 2009; Moller et al. 2010). Since the data on immunomodulation in GO exposure at earlier time points are unavailable in zebrafish, further studies are needed to confirm the concept.

Oxidative stress mediated toxicities were elucidated extensively by transcriptomics and metabolomics analysis. Transcriptomics analysis by RNA-seq of 7 dpf zebrafish larvae continuously exposed to GO (100 µg/L) from 2.5 hpf to 7 dpf revealed that the expression of collagen- and matrix metalloproteinase (MMP)-related genes that affect the skeletal and cardiac development of zebrafish were disrupted (Zhang et al. 2017b). Metabolomics analysis also showed that GO exposure inhibited amino acid metabolism and altered the ratios of unsaturated to saturated fatty acid levels that contributed to the above developmental toxicity (Zhang et al., 2017b). However, metabolomics analysis of 96 hpf zebrafish larvae exposed to GO for 24 h (72–96 hpf) with ultralow concentrations (0.01–1.0 µg/L) upregulated some amino acids and altered the ratios of fatty acids that lead to the development of Parkinson's disease like phenotypes (Ren et al. 2016).

In addition to oxidative stress, aryl hydrocarbon receptor (AhR) pathway is also induced in zebrafish larvae exposed to rGOQD. Due to aromatic hydrocarbon skeleton in GP, it is expected that GPN can target AhR pathway in zebrafish development. AhR pathway is responsible for regulating the activity of xenobiotics (Go et al. 2014). It mediates the regulation of cytochrome P450 enzymes leading to the transcriptional activation of CYP1A, which plays a key role in physiological immunity (Nebert and Karp 2008). It was observed that continuous exposure of the zebrafish embryos to rGOQD (100 µg/mL) from 4 hpf –168 hpf significantly induced *cyp1a* and *cyp1c* gene transcriptions that probably promotes gfp protein expression in these larvae as evidenced in *Tg(cyp1a:gfp)* transgenic zebrafish (Zhang et al. 2017). Therefore, the immunomodulatory response as observed in zebrafish larvae and adults by GPN could be a combined effects of oxidative stress as well as activation of AhR pathway.

Because of the wide ranging applications in various fields, further studies are needed to elucidate the environmental and health risks of GPN (Fojtu et al., 2017). Until now there is a lack of strong scientific evidence regarding the potential hazards generated due to the release of nanomaterials into the aquatic environment. Also no threshold level has been established for the onset of novel nanomaterial-specific hazards, and novel pathogenic pathways that are specifically activated by nanomaterials (Donaldson and Poland 2013; Gebel et al. 2014). Because of the release of considerable amounts nanoparticles into the environment almost every day, and for the ERC guidelines, investigations on the effect of GPN on aquatic organisms are critical. Some studies have found that GPN exhibited high toxicity to marine and freshwater algae, damaged organelles, enhanced ROS generation, induced nutrition depletion, and reduced photosynthetic pigment concentration (Hazeem et al. 2016; Zhao et al. 2017; De Marchi et al., 2018). We have also documented from the published literature included in this review that GPN was able to penetrate the chorion (both zebrafish and Japanese medaka) and reach the embryonic body in fish during development and causing DNA damage. However, there are multiple forms of GPN (single-layer GP, few-layer GP,

GO; rGO, GP nanosheets, GP ribbons, GQD and rGQD) with unique properties (size, shape, surface properties) that exert toxicological effects at multiple endpoints. Although the traditional toxicological testing protocols were applied, based on the physicochemical properties, more extensive research is also needed to develop new methods for identifying GPN toxicity at the molecular level. Moreover, GPN toxicity studies in fish were mostly restricted on zebrafish as proposed by Lin et al (2013) or by OECD (2013); another fish species, such as Japanese medaka which is recognized as complementary to zebrafish and showed potential for the evaluation of GPN toxicity (Mullick Chowdhury et al., 2014; Li et al., 2014), should be more extensively used for the confirmation of the data generated on zebrafish. Otherwise, a fish living in marine or estuarine environment, for example *Fundulus heteroclitus*, should be used for the evaluation of GPN toxicity. Moreover, the studies were restricted only for short period; long-term and transgenerational studies are needed to establish the toxic effects of GPN on aquatic organisms at the molecular level as well as for ERC guide lines which is extremely important for the organisms living in the aquatic environment.

Conclusion:

The extensive use of GPN in biomedical applications have increased their release into the aquatic environment and impose a threat to the aquatic organisms. The accumulation of GPN in organisms living in the aquatic environment can occur either through food chain or direct absorption/adsorption from the surrounding environment. So far, the potential toxicity of GPN has been studied in various aquatic models including bacteria, algae, invertebrates and fish, however, none of the organisms is considered as unique to evaluate the potential toxic effects of GPN. Due to its excellent morphological and biological features, zebrafish has been utilized as a potential model for the evaluation of GPN toxicity. Although zebrafish is a freshwater laboratory fish used extensively in biomedical research, to understand the molecular targets of GPN more efficiently and effectively in aquatic models, studies on other fish species, especially on a fish living in marine or estuarine environment, is necessary. With regard to mechanisms, the GPN toxicity are mainly focused on the generation of ROS and induction of oxidative stress. Few studies are focused on the genomic level. Moreover, the methods used for the evaluation of GPN toxicity in fish were mainly designed without considering the unique physicochemical properties of GPN, such as size, surface composition, surface energy, or shape, which can interfere with the interpretation of the biological effects of nanoparticles on fish or to establish ERC of nanomaterials in the aquatic environment. To resolve these issues, in the future, extensive studies on diverse fish models applying advance techniques and considering all the properties of GNP molecules are necessary.

Summary:

This review focused on the importance of fish, especially zebrafish, as an aquatic animal model in the evaluation of the GPN toxicity at the molecular level. As a result of human activities, GPNs are accumulated in the aquatic environment and imposed a potential threat to the aquatic organisms, food chain, and finally to human health. Although more scientific studies are needed, GPN are found to be toxic to the cellular environment even at ultralow

concentrations. Moreover, the size of the GPN has a significant impact on its toxicity. Smaller nanoparticles are more toxic than the larger particles. The surface properties of the GPN molecules also have a significant impact on toxicity. Presence of humic acid, L-cysteine, or biological secretion in the environment is also able to modulate the toxic effects of GPN. The use of embryos, larvae, and adult zebrafish and embryos and larvae of Japanese medaka indicate that the toxic effects are mainly induced by oxidative stress due to the generation of ROS. Other mechanisms, such as apoptosis, metabolic disorders, neurodegeneration, and immunomodulation by GPN are primarily mediated by ROS. More studies are needed to establish zebrafish as a unique model for the evaluation of toxic potential of GPN or to determine the ERC limit which seems to be safe for aquatic organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviation:

AChE	Acetylcholinesterase
AhR	aromatic hydrocarbon receptor
AP	Acid Phosphatase
BER	base excision repair
bwGO	base-washed graphene oxide
CAT	catalase
cysGO	L-cysteine GO hybrids
DNA	Deoxyribonucleic Acid
dpf	days post-fertilization
ERM	embryo-rearing medium
FLG	few layered graphene
GO	graphene oxide
GO-FITC	graphene oxide-fluorescein isothiocyanate
GOBS	graphene oxide nanosheets coated with biological secretion
GONS	graphene oxide nanosheets

GP	graphene
GPN	graphene-based nanomaterials
GQD	graphene quantum dots
GST	Glutathione s-transferase
HA	Humic Acid
HCE	High choriolytic enzyme
HO	heme oxygenase
hpf	hours post-fertilization
iNOS	inducible Nitric Oxide Synthase
ISV	intersomitic vessels
LSCM	laser scanning confocal microscope
LCE	low choriolytic enzyme
MDA	Malondialdehyde
MFG	Multifunctional graphene
MHRA	Medicines and Healthcare Products Regulatory Agency
NGO	nanographene oxide
NOM	natural organic matter
NPs	nanoparticles
O-GNRS	oxidized graphene nanoribbons
POFS	perfluorooctanesulfonate
QDs	quantum dots
rGO	reduced graphene oxide
rGQD	reduced graphene quantum dots
RNA-seq	RNA sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
TEM	Transmission electron microscope
UK	United Kingdom

U.S. FDA	United States Food and Drug Administration
VEGF	vascular endothelial growth factor

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Table 1:

Uptake and biodistribution of GPN in fish

Species	Developmental stage	Probe	Application method	Concentration/volume	Detection Method	Detection time	Distributions	References
Zebrafish (AB strain)	Two cell embryo	Fluorescein-labeled multifunctional graphene	Microinjection (single injection)	0.1 ng/L; 10 nL	Confocal laser scanning microscopy	72 hpf	Head, tail, yolk sac, blood vessels, brain ventricles	Goallavelli and Ling (2012)
Zebrafish (transgenic) <i>Tg(kdrl:egfp)</i>	One cell stage embryo	Alexa568-labeled nanographene oxide (NGO-A568)	Microinjection (one, two and three injections)	250, 500 and 750 pg	Confocal microscopy	30 and 52 hpf	Head and developing vasculature	Jeong et al. 2015
Zebrafish (wild type AB)	Single cell stage embryo	TPE-TPA-FN-nanographene oxide nanoparticles (TTF-NGONP)	microinjection	0.5 nL (calculated number of TTF-NGONP in 1 ml suspension is 1.47X10 ¹⁰)	3photon luminescence (3PL) imaging	48 h embryos and larvae	Entire body	Zhu et al. 2016
Zebrafish	4–96 hpf	Fluorescently labeled graphene quantum dots (GQD)	waterborne	12.5 µg/mL-200 µg/mL	Fluorescence microscopy	larvae	Intestine and heart	Wang et al. 2015
Zebrafish (AB strain)	Older than 24 hpf	GO hybridized with flourescein isothiocyanate (GO-FITC) (GO-F)	waterborne	GO (100 µg/mL) hybridized with FITC (1 mg/mL)	Laser scanning confocal microscopy (LSCM) and Transmission electron microscopy (TEM)	After 8 min and 96 h exposure	Accumulated in the yolk sac (8 min); aggregated around the eyes, heart, yolk sac and tail (96 hpf)	Chen et al. 2015a
Zebrafish (wild type)	4 hpf-7 dpf	Reduced graphene oxide quantum dots (rGOQD) (10 nm lateral size and 1 nm height)	waterborne	25, 50 and 100 µg/mL	Fluorescence microscopy	Before and after 48 hpf	Fluorescence distributed in whole embryos during early period of development; after 48 hpf the fluorescence was distributed only in the abdominal region	Zhang et al. 2017a
Zebrafish (transgenic) <i>Tg(cyp1a:egfp)</i>	4–120 hpf	Reduced graphene oxide quantum dots (rGOQD)	waterborne	100 µg/mL	Fluorescence microscopy	24–120 hpf	Whole body (trunk, tail, spine and head)	Zhang et al. 2017a
zebrafish	2 hpf	¹⁴ C-labeled few layer graphene (FLG) and sFLG	suspension	75 µg/L	Radioactivity using LSC; TEM	12, 24 and 48 h	Size dependent accumulation in the chorion and yolk sac (Higher accumulation of sFLG in both chorion and yolk sac than FLG)	Su et al. 2017
Japanese medaka	1 dpf	Oxidized graphene nanoribbons (O-GNRs)	suspension	20 µg/mL	TEM	6 days	Inside chorion	Mullick Chowdhury et al., 2014
zebrafish	4 dpf larvae	GO-FITC	1h Suspension	0.1,1.0 and 10 mg/L	Fluorescence microscopy	4 dpf	Mouth, yolk sac, cardiac region, tail blood	Zhang et al. 2017b

Species	Developmental stage	Probe	Application method	Concentration/volume	Detection Method	Detection time	Distributions	References
Zebrafish	72 hpf larvae	graphene	waterborne	0.01–1.0 µg/L	High resolution TEM (HRTM) and Raman Spectra	96 hpf	Brain and intestine	Ren et al., 2016
Zebrafish (AB strain)	Adult (3 months old)	S-FLG; L-FLG (¹⁴ C labeled)with and without natural organic matter (NOM)		50, 75 and 250 µg/L	Body burden and Histology	4, 12, 24, 48, and 72 h	250 µg/L showed that maximum accumulation was reached at 48h for both L-FLG and S-FLG. Accumulation of L-FLG occurred in the gut; S-FLG in both gut and liver as well as in the intestinal epithelial cells and blood.	Lu et al. 2017

Table 2:

GPN toxicity in zebrafish and other fish species.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
Zebrafish wild-type (AB strain)	Embryos [2-cell stage]	Microinjection (10 nL total volume)	graphene oxide (GO) : area= 40–60 nm; thickness 1–3 nm) Multi-functional graphene (MFG) : size 40–60 nm, thickness 4–6 nm)	0.05 and 0.1 ng/nl; [2 cell stage-72 hpf]	GO induced yolk sac edema in 4–6% larvae 6–12% showed tail or spinal cord flexure 2% showed cardiac malfunction MFG Induced yolk sac edema in 9–12 % 8% showed tail or spinal cord flexure	Gollavelli and Ling 2012.
Zebrafish wild-type (AB strain) and endothelial cell-specific transgenic [<i>Tg(kdrl:egfp)</i>] embryos	1–2 cell stage embryos	Microinjection; (1 nL volume by single/two/three injections of 0.25 mg/L);	Nanographene oxide [NGO; lateral size= 100–200 nm; height 1–1.5 nm] Polyethylene glycol-coated NGO (NGO-PEG) [hydrodynamic diameter: 310.3±87.23 nm] NGO-Alexa568 (NGO-568) [hydrodynamic diameter: 184.3±8.13 nm]	250 pg; 500 pg 750 pg; [30 hpf and 52 hpf]	NGO : (i) curved spine (ii) shortened body stature (iii) pericardial edema (iv) lowered yolk consumption (v) underdeveloped brain and retinas (vi) occasional disrupted circulation (30 hpf) Apoptotic positive cells in the head region (500 pg, 30 hpf) Blood vessel sprouting with irregular positioning of growing intersomitic vessel (ISV) trunk vasculature of transgenic [<i>Tg(kdrl:egfp)</i>] zebrafish (500pg; 52 hpf) Vascular endothelial growth factor A (<i>vegfaa</i>) gene expression was upregulated. Notch-regulated Ankyrin repeat protein a and (<i>nrarpa/narapb</i>) gene expression was downregulated NGO-PEG Toxic effects by NGO only were ameliorated by NGO-PG. NGO-A568 Toxic effects by NGO only were ameliorated by NGO-A568. Elicited the angionic defects	Jeong et al. 2015.
Zebrafish wild-type (AB strain)	2-cell stage embryos (1–4 cell stage)	microinjection;	Graphene (used 0.3% DMSO as solvent) [particle size: 1456.8±16.1 nm for 1µg/mL; 2012.0±18.5 nm for 10 µg/mL; 5808.2±50.2 nm for 50 µg/mL]	1, 10, 50 µg/mL [2 cell stage-4 and 4.5 dpf]	No significant effect on waking and rest activity on 4 dpf larvae Regulator of hypocretin system genes, such as <i>hcrtr</i> , <i>hcrtr:aaanat2</i> remained unaltered.	Lu et al. 2017
Zebrafish embryos	4hpf	Immersion (10 embryos in 2 mL) in E3 medium (5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM calcium chloride, 0.33 mM magnesium sulphate, pH 7.4); media renewed every 24h	Pristine graphene (pG) [size 170–390 nm]	1, 5, 10, 15, 20, 25, 30, 35, 40,45, 50 µg/L [4–96 hpf]	Induce embryo mortality; All the embryos exposed to 30 µg/L pG or above shows 100% mortality of the embryos within 30 min-2h of exposure. Delayed hatching Pericardial disorder Bradycardia Yolk sac and pericardial edema	Manjunatha et al., 2018
Zebrafish embryos (AB strain)	(4 hpf-120 hpf)	Immersion in E3 medium containing 10–15% methylene blue.	Graphene quantum dots (GQD) size: 2.3–6.4 nm; average lateral dimension 3.4 nm (n=200)	12.5, 25, 50, 100, 200 µg/mL	(i) Distributed on myocardial cell cytoplasm (100–200 µg/mL) as observed in 96 hpf (ii) Reduction in heart rate in a concentration-dependent manner (50–200 µg/mL) from 48 hpf- 120hpf.	Jiang et al. 2015.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
Zebrafish embryos	4–96 hpf	immersion	GQD	12.5, 25, 50, 100 and 200 µg/mL	(i) fluorescence intensity was mainly localized in intestines and heart (ii) heart rate decreased with the increase in GQD concentrations Hatching rates decreased with increasing concentrations of GQD Pericardial edema, vitelline cyst, bent spine and bent tail was observed in the larvae exposed to 200 µg/mL GQD. Spontaneous movement decreased significantly at GQD concentrations 50, 100, and 200 µg/mL. Visible light test (behavior) indicate that the total swimming distance and speed decreased depending on GQD concentration; Embryos exposed to 12.5 µg/mL GQD shows hyper activity and those exposed to 25, 50, 100 and 200 µg/mL shows hypoactivity in visible light (light-dark) test.	Wang et al. 2015
Zebrafish (wild type; locally breed and reared short-fin)	embryos (0–7 dpf)	Immersion; in 2 mL of 30% Danieau's solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM Hepes, pH 7.4]	Thiolated GQD (SH-GQD)	0.02, 0.05, 0.1,0.2, 0.3, 0.6, 0.8, and 1 mg/mL	(i) High concentration of SH-GQD (1 mg/mL) enhanced mortality, however, low concentration of SH-GQD (0.1 mg/mL) did not. (ii) SH-GQD has shown protective effects against oxidative stress SH-GQD induced head deformation, vent tail, pericardial edema, and yolk sac edema in a concentration-dependent manner SH-GQD did not affect the hatching rates, however, 46% of the larvae died on 7 dpf exposed to 1 mg/mL SH-GQD	Oh et al. 2017
Zebrafish (wild-type)	4 hpf	Immersion in E3 medium; [media refreshed every 12 h]	GO (Particle size: 600 nm); Graphenea, New York, USA	5, 10, 50, 100 µg/mL [4–120 hpf]	The survival rate exhibited a time and concentration-dependent behavior between 72–120 hpf with 50–100 µg/mL showed significant effects. Significant reduction in hatching rates between 72–120 hpf in 50–100 µg/mL groups. The heart rates at 72 hpf were found to be significantly reduced in larvae exposed to 50–100 µg/mL GO. The frequency of movement in 96 hpf embryos were significantly reduced in larvae exposed to 50–100µg/mL GO.	d' Amora et al., 2017.
Zebrafish	embryo 4hpf-96 hpf;	Immersion; 10 embryos in 5 mL freshwater/well in 24 well culture plates. Media renewed every 24h.	GO flakes	40 and 80 mg/L [every 24 h]	No embryo mortality was observed. No alteration in growth, brain morphology, pharyngeal arches and jaws, heart, fins, notochord, somites, body shape, cardiovascular function, yolk sac and locomotion. Increase in heme oxygenase 1 (HO-1) mRNA and protein expression by GO Increase in inducible Nitric Oxide synthases (iNOS) mRNA and protein expression by GO.	Pecoraro et al. 2018.
Zebrafish (wild type)	Embryos/larvae	Immersion in system water; buffer containing 1% pluronic F 68; Media changed every 24h until 6 days	GO. Single layer; thickness 5.3nm. [suspended in 1% pluronic F 68 water suspension]	5, 10, 50, 100 mg/L [2–168 hpf]	No change in mortality, hatching rate, and spontaneous movement Heart rates remained unaltered (increased in 10 mg/L dose) No change in eye areas in 120 hpf larvae. Reduction in body length (100 mg/L) in 6 dpf larvae No change in <i>gap43</i> , <i>grfp</i> , <i>nestin</i> gene transcriptions (genes associated with nervous system development) in 6 dpf larvae. <i>Neurogenin1</i> and <i>dat</i> gene transcriptions (genes associated with nervous system development) were increased at 10 mg/L in 6 dpf larvae Upregulation of <i>synapsinIIa</i> and down regulation of <i>dat</i> genes (genes associated with nervous system development) in 5 mg/L groups in 6 dpf larvae Increase in distance travel, the speed, and the turn angle of the 6 dpf larvae exposed to 10 mg/L. AChE activity remained unaltered in 6 dpf larvae. Dopamine level was reduced in 6 dpf larvae exposed to 10 mg/L	Soares et al. 2017.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
Zebrafish AB strain	2 hpf-96 hpf	Immersion; E3 medium; Media replaced every day	GO nanosheets 0.8–1 nm; diameter 101–258 nm	0.01, 0.1, 1.0, 10, 100 mg/L [48, 72 and 96h]	<i>bcl2</i> and <i>caspase3</i> genes were increased in 6 dpf larvae exposed to 10 mg/L Autophagosome formation, vacuoles, and partial loss of nuclear membrane architecture at specific regions of ventral diencephalon in 6 dpf larvae exposed to 10 mg/L. Mortality did not increase significantly Accumulation of GO in the chorion and severe hatching delay in embryos (both at 72 and 96 hpf) exposed to 100 mg/mL GO. Heart rate significantly decreased in 48 hpf embryos exposed to 1 mg/L GO, however, significantly increased in embryos exposed to 100 mg/L GO. Spontaneous movement of the embryos exposed to 100 mg/L GO were significantly reduced at 48 hpf Incidence of yolk sac edema in 96 hpf embryos were more pronounced and found to be concentration-dependent. Significantly high incidence of Tail/spinal cord flexure was evident in larvae at 96 hpf exposed to 1 mg/mL GO Significantly higher percentage of 96 hpf larvae without eyes/head were observed when the embryos were exposed to 10 and 100 mg/L GO. ROS generation in the whole body of the 96 hpf larvae was increased by GO in a concentration-dependent manner. GO down-regulated SOD activity in a concentration-dependent manner and increased the MDA concentration. GO at 1mg/L suppressed the level of global DNA methylation, while at 100 mg/L promoted methylation	Chen et al. 2015a.
Zebrafish (wild- type)	Blastula	Immersion	GO nanosheets (height ~1.5 nm; lateral size: 1.5µm	5, 25, 50 µg/mL [12, 24, 48 and 72 hpf]	Survival rates were decreased with the increase in exposure time (12–24 h); remained constant 48–72h. Hatching rates slightly decreased (5 mg/mL) and increased at higher concentrations (25 and 50 µg/mL; not significant) Up regulation of <i>apex1</i> , <i>ogg1</i> , <i>polb</i> , <i>creb1</i> (genes involved in base excision repair pathways) in embryos exposed to GO (50 µg/mL) for 24 h	Lu et al. 2017.
Zebrafish (AB strain);	embryo (2.5hpf–168hpf (7dpf);	Immersion in E3 medium	GO nanosheets (thickness1.01±0.05 nm; lateral length: 0.3–2.6 µm) dispersed in E3 medium	1µg (0.001 mg) /L- 100 mg/L,	The embryomortality was not concentration-dependent; so the LC50 for GO in zebrafish embryos was remained undetermined. heart rate increased (100 µg/L) at 96 hpf; heart rate decreased, hatching delayed (1–100 mg/L) spontaneous movement inhibited (10 µg/L; 100 µg/L; 100 mg/L) trunk curved, tail malformed, pericardial edema, yolk sac edema, craniofacial malformation, reduced body length (10 µg/L, 100 µg/L, 1 mg/L, 100 mg/L) ROS intensity (8-OHdG) increased (100 µg/L) Carbonyl protein content increased (1, 10, 100 µg/L) Super oxide radical, MDA, increased (1, 10 and 100 µg/L) Catalase, glutathione peroxidase type I, copper/zinc superoxide dismutase, SOD, GST were down regulated Downregulated (100 µg/L) <i>coll1a1a</i> , <i>coll1a2</i> , <i>col2a1a</i> , and <i>col11a1a</i> genes. (these genes played key roles in the development of cartilage and notochord) Inhibition of parasphenoid development (100 µg/L); affects Meckel’s cartilage; reduced the length of lower jaw and intercranial distance (cranial deformities) 87.18% decrease in erythrocyte content (100 µg/L) Cardiac output declined, heart rate increased (100 µg/L)	Zhang et al. 2017b.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
Zebrafish AB-strain	embryos 2.5 hpf-72hpf	Immersion in E3 medium; media replaced every day	GO nanosheets (thick ness 0.8–1.2 nm) Humic acid (diameter 2–12 nm)	(i) GO = 0–100 mg/L in E3 medium (ii) HA= 0.01–100 mg/L in E3 medium (iii) GO-HA= 100 mg/L GO+10 mg HA	GO100 (100 mg/L GO): hatching rate reduced; rate of pericardial edema enhanced; heart beat increased HA (0.01–100 mg/L) did not induce adverse effects on embryo development GO-HA (100 mg/L GO + HA (10 mg/L) recovered hatching rate, pericardial edema, heart beats. Mitochondria became swollen and loose, and the integrity of the membrane and cristae was damaged by GO100. GO100 significantly increased GSH, MDA, ROS and inhibited SOD. HA reduced the lipid protein, and DNA damage induced by GO	Chen et al. 2015b.
Zebrafish (AB strain)	embryos 2–120 hpf;	Immersion in E3 medium; Media changed every 24 h	GO nanosheets (thickness: 0.87±0.157 nm; lateral length 50–200 nm) CysGO (thickness 1.42±0.234 nm; lateral length 78–590 nm	GO (0.01–10 mg/L) CysGO (0.01 –10 mg/L+ Arsenic 1mg/L) in E3 medium;	Pericardial edema, tail flexure, eye malformation in 5–9% larvae (120 hpf) exposed to GO (0.01–10 mg/L);No toxic effects were observed with CysGO Hatching rates were 10–30% in embryos exposed to GO at 72 hpf in contrast to 75% in control embryos; no hatching delay for CsyGO (0.01–1 mg/mL) were observed; however, embryos exposed to 10 mg/mL CysGO only 70% of the embryos hatched at 72 hpf. At 96 hpf all embryos were hatched (control and treated) 5–8% death occurred in GO-exposed larvae; however, CysGO did not induce embryonic death. GO suppressed cell nucleus development in the inner eye tissue while CysGO did not. Pericardium development in embryos exposed to GO were ill -developed; but pericardial development is normal in embryos exposed to CysGO. No notable oxidative damage to DNA occurred in larvae exposed either to GO or CysGO at concentrations 0.01–10 mg/L. GO reduced the Na+K+ATPase activity in a concentration-dependent manner; CysGO (0.01–10 mg/L) did not significantly alter the activity. Mitochondrial membrane polarization lost by GO; however, the damage was relatively less in larvae exposed to CsyGO CysGO protects the embryos from arsenic poisoning.	Mu et al., 2015.
Zebrafish	embryos (2 hpf-120 hpf)	Immersion in E3 medium; media replaced every 24h until 120 hpf	GO nanosheets in biological secretions (GOBS) (thickness 10 nm; lateral length 19.5–282 nm) GO nanosheets (GONS) (thickness 0.83±0.12 nm; lateral length is 0.5µm- several microns)	exposed to 0.01, 0.1,1 mg/L)	GONS is more readily taken up by the embryos than GOBS. GOBS showed increased mortality, malformation, faster heart beats, upregulation of β-galactosidase and loss of mitochondrial membrane potential than GONS; Both GOBS and GONS induced stronger adverse effects than controls (embryos exposed to bulk-activated carbon powders with an average diameter of 147±41 µm) Pericardial edema and tail flexure were also observed in juvenile fish, especially in larvae exposed to GOBS. Embryonic death and faster heart beat was more pronounced in larvae exposed to GOBS than GONS. No alteration in ROS DNA methylation enhanced by both GOBS and GONS. Both GOBS and GONS enhanced β-galactosidase levels Both GOBS and GONS inhibited calcium exchange in the embryos	Mu et al., 2016.
Zebrafish	embryos 1hpf-96 hpf	Immersion	GO [width 394.21 ±215.05 nm; Height 0.89±0.01 nm] base washed GO (bw GO) [width 286.53 ±104.42 nm	GO=1, 10, 100 mg/L in medium (96 mg/L NaHCO3; 60 mg/L MgSO4; 4 mg/L KCl;	Mortality below 17% independent of exposure conditions. Reduced body length in GO (100 mg/L), GO (100 mg/mL)+HA (20 mg/mL), and in bwGO (100 mg/mL)+ HA (20 mg/mL) No alteration in Catalase enzyme activity by GO, bwGO either alone or in presence of HA	Clemente et al 2017.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
			Height 0.94±0.02 nm GO+ 20 mg/L humic acid (HA) bwGO+ 20 mg/L HA	60 mg/L CaSO ₄ , 2H ₂ O; pH 7.4) at 27 °C bwGO= 100 mg/L in medium HA=20 mg/L in medium	GST remained unaltered by GO, GO+HA20 mg/L, but significantly decreased by bwGO (100 mg/L) and increased by bwGO (100 mg/L)+HA (20 mg/L) Acid phosphatase (AP) remained unaltered by GO (1,10, or 100 mg/L); HA (20 mg/L) increased AP; GO+HA reduced AP in a concentration-dependent manner; bwGA alone was unable to alter AP, however, in presence of HA (20 mg/mL), AP enzyme activities reduced significantly Significant reduction in AChE activity by GO (100 mg/L GO) alone or in presence of 20 mg/mL HA (GO+HA)	
Zebrafish	Embryos [2–96 hpf]	suspension	GO and rGO	1.5,10, 50, 100 mg/L [2–96 hpf]	rGO inhibited hatching rGO decreased the length of the hatched larvae at 96 hpf no mortality or morphological malformation were induced by GO and rGO	Liu et al. 2014.
Zebrafish (wild-type and transgenic <i>Tg (cyp1a:egfp)</i> ;	4 hpf -168 hpf (7 dpf)	Immersion in deionized water (DI); media renewed everyday	Reduced graphene oxide quantum dots (rGOQDs) (10 nm lateral size; 1 nm height)	25, 50, 100 µg/mL	No effect on hatching rate (72 hpf), body length, and mortality in wild-type fish Heart beats (96 hpf) reduced (100µg/mL) in wild-type larvae Pericardial edema, vitelline cyst, bent spine (100 µg/mL) observed in wild-type larvae Upregulation of <i>cyp1a</i> , <i>cyp1c</i> , <i>cyp7a1</i> , <i>hsp70</i> (100 µg/mL) (7 dpf) in wild-type larvae Green fluorescence protein expression was significantly increased in wild-type and transgenic <i>Tg(cyp1a:egfp)</i> larvae exposed to 50 and 100 µg/mL rGOQD on 7 dpf.	Zhang et al., 2017a
Japanese medaka embryo	(1 dpf-7 dpf);	suspension in embryo rearing meium (17.1 mM NaCl, 272 mM CaCl ₂ .2H ₂ O, 402 mM KCl, 661 mM MgSO ₄ , 7H ₂ O; pH 6.3); Media replaced every alternate day	Sonicated or unsonicated oxidized Graphene nanoribbons (O-GNR) ; Diameter: 250–400 nm; Average length 744±178 nm (bath sonicated); 323±50nm (sonicated for 1 min); 201±28 nm (sonicated for 5 min); 100±10 nm (sonicated for 10 min)	20 µg/mL	Probe sonicated O-GNR increased embryo-larval mortality depending upon the sonication time; however, no significant effects on mortality was observed in embryos exposed to bath-sonicated o-GNR (20 min) O-GNR is able to enter inside the chorion Bath-sonicated O-GNR induced hatching of the embryos 2 days earlier than control embryos.	Mullick Chowdhury et al 2014.
Zebrafish	larvae (72 hpf-96 hpf)	Cultured in E3 medium	GO (thickness 1.02±0.15 nm; lateral length 0.5µm-several microns)	0.01, 0.1 and 1µg/L GO in E3 medium for 24 h	(i) Larval zebrafish incubated with 0.01 µg/L GO (72hpf-96 hpf) at 120 hpf exhibited tail flexure and spinal curvature; Pericardial edema seen in larval zebrafish exposed to 0.1 µg/mL GO; pericardial and yolk edema coexists in larval zebrafish exposed to 1µg/mL GO. Distribution of dopaminergic neurons in the diencephalon was reduced by ~70% in larvae exposed to GO (0.01–1.0 µg/L) at 96 hpf. 222–522% increase in α-synuclein and 69–179% increase in ubiquitin occurred in 96 hpf larvae exposed to 0.01–1.0µg/mL GO (72–96 hpf) The swim speed of 7 dpf larvae was decreased by 19–57% following GO exposure (0.01–1 µg/L; 72–96 hpf) Other movement-related disorders such as nearest neighbor distance (distance between a given fish and its nearest neighbor, NND) decreased by 22–49%, and inter individual distance (average distance of a given fish from its nearest neighbor, IID) increased by 31–91% in 7 dpf larvae exposed to GO (0.01–1µg/L; 72–96 hpf) Upregulation of caspase 8 (38–152%) protein was occurred in larvae exposed to GO (0.01–1.0 µg/L; 72–96 hpf) Increase in β-galactosidase activity (41–83%) was observed in larvae exposed to GO (0.01–1.0µg/L; 72–96 hpf).	Ren et al. 2016.

Species	Developmental stage	Method of application	Graphene based nanomaterials	Concentration and duration	Effects	References
					GO exposure resulted in metabolic disturbance in 96 hpf larvae.	
Japanese medaka	larvae (24–48 hph)	in moderately hard-constituted water; live brine shrimp was given as food.	Graphene and graphene-TiO2 nanoparticle composites (GNP-TiO2)	2, 5, 8, 10, 14, 17, and 20 mg/L GNP-TiO2 under simulated solar radiation (SSR) exposure; 167 and 500 mg/L GNP under dark conditions ; exposure period is 4–48 h	Graphene exhibited no toxicity in both dark and SSR conditions. Under dark conditions LC50 for GNP-TiO2 was greater than 500 mg/L. Under (SSR) the LC50 for GNP-TiO2 was 11 mg/L.	Liu et al. 2014.
Adult zebrafish	(2-month-old).	10 males and 10 females per tank (6-L glass tanks); fed with brine shrimp naupli. Half of the exposure water was renewed every day.	GO	1, 5, and 10 mg/L for 14 days	No apparent damage to gill histology by GO (1, 5 and 10 mg/L). Vacuolation, loose arrangement of cells, histolysis, and disintegration of cell boundaries were seen in both liver and intestine of fish exposed to GO in a concentration-dependent manner. Number of goblet cells increased with higher GO concentrations. Malondialdehyde (MDA) content in liver was increased in day 1 by 1,5 and 10 mg/L and in day 4 by 1mg/L GO. GSH was decreased in liver in day 1 by 1, 5, and 10 mg/L and in day 4 by 1 and 10 mg/l GO. SOD and catalase was increased in liver by 1, 5 and 10 mg/L GO only in day 4. Expression of tumor necrosis factor α (TNF- α), interleukin-1 β , and interleukin -6 was increased in the spleen of zebrafish exposed to GO for 14 day in a concentration-dependent manner.entration-depenobserved in a concentration-dependent manner.	Chen et al. 2016.
Adult zebrafish local commercial source	male and female; 6 months old;	Fed with Tetra Color-tropical flakes.	GO Thickness: 1nm; area: 0.58 μm^2	2, 10, 20 mg/L; Exposure: short-term: either 24h or 72 h Long-term: 14 days	The number of apoptotic and necrotic cells in gills were increased in both 24 or 72 h exposure to GO (2 or 20 mg/L) ROS increased significantly in gill cells after 24 h of GO exposure (2, 10, or 20 mg/L). No DNA damage in blood cells was observed in any concentration of GO used in this study after 72 h of exposure. Gill morphology with regard to dilated marginal channel, lamellar fusion, clubbed tips, aneurysms, and necrosis was disrupted in fish exposed to 2, 10 and 20 mg/L GO for 14 days (chronic exposure). The liver of fish exposed to 2 mg/L of GO for 14 days showed peripheral nucleus; those with 10 mg/L showed pyknotic nuclei and those with 20 mg/L showed necrosis in the liver. The lumen of the GUT exposed to GO (2, 10, or 20 mg/L; 14 days) filled with unidentified brown spots.	Souza et al. 2017.
Common carp (<i>Cyprinus carpio</i>)obtained from local fish market.	3 months old (Juvenile)	water	GO Thickness 0.7–1.8 nm Surface area: 208.6 m ² /g	1 mg/L GO+500 ng/L perfluorooctanesulfonate (POFS) [GO+POFS] 1mg/L GO + 500 ng/L perfluorooctanesulfonate (POFS) +2 mg C/L Fulvic acid. [GO+POFS+FA] Exposure period 28 days; recovery period 29, 30, 34, 38, 44, 50, 56, 62, and 82 (considering the date of exposure is 0 day)	The accumulation of POFS was enhanced by GO in blood, intestine, liver, kidney, gill, and muscle tissues of common carp. Large amount of black residues were found in the intestine of common carp exposed to either GO+POFS or GO+POFS+FL. FA reduced the accumulation of PFOS in tissues of common carp by reducing the bioavailability of GO and PFOS	Qiang et al. 2016.