Review

An Update Report on the Biosafety and Potential Toxicity of Fullerene-Based Nanomaterials toward Aquatic Animals

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Received: date; Accepted: date; Published: date

Abstract: Fullerene molecules are composed of carbon in forms of a hollow sphere, ellipsoid, or tube. Fullerenes have attracted considerable attention in different fields of science since their discovery in 1985. The unique carbon cage structure of fullerene provides immense scope for derivatization, rendering potential for various industrial applications. The prospective applications of fullerenes thus have led to assorted fullerene derivatives. The unique chemical structure also provides ease for fullerene to be synthesized through various kinds of conjugating techniques, where fullerene can be located either on the backbone or the branch chain. Here in this review, we have compiled the toxicity and biosafety aspects of fullerene in aquatic organisms. The frequent use of fullerene is likely to come in contact and interact with the aquatic environment and aquatic organisms. According to the current understanding, waterborne exposure to fullerene-based nanomaterials indeed triggers toxicities at cellular, organic, molecular as well as neurobehavioral levels.

Keywords: fullerene; fish; daphnia; toxicity; aquatic animal; nanomaterial

Introduction of fullerene and its application

Carbon is known to exist in two allotropes, viz., diamond and graphite. Another allotropic form of carbon is fullerene, which was first discovered in 1985 by Kroto, Curl, and Smalley [1]. In 1996 they were awarded the Nobel Prize for the discovery and their pioneering efforts. Fullerenes are regarded as three-dimensional analogues of benzene. They are composed of carbon atoms connected by single and double bonds formimg a closed or partially closed mesh, with fused rings of five to seven carbon atoms. A fullerene molecule having only carbon atoms in various shapes may be a hollow sphere, ellipsoid, tube, or of many other shapes and sizes [2,3]. The discovered fullerenes consist of various n numbers of carbon atoms, obeyed a specific rule. The most famous of the known fullerenes (C60) is composed of n = 60 carbon atoms and known as buckminsterfullerene, named in honor of R.

 doi:10.20944/preprints202009.0376.v1

Buckminster Fuller, who invented the geodesic domes in the 1960s [2]. The C60 fullerenes are also informally called buckyballs for their resemblance to a soccer ball shape.

The most abundant form of fullerenes is C60, with 60 carbon atoms arranged in a spherical cage structure of about 7 Å in diameter. There are two types of C-C bonds with distinct lengths in C60: C5-C5 single bonds in the pentagons and C5-C6 double bonds in the hexagons [4]; the C5-C5 bond is 1.45 Å, and the C5-C6 double bond is 1.40 Å in distance. The C60 has a low specific gravity relative to diamond (1.65 compared to 3.51). Chemically, the molecule is very stable owing to the fact that destruction of the cages requires temperatures above 1000°C [4,5]. Other than C60, fullerenes can contain between 30 to 980 carbon atoms forming various structures with different properties and applications

These new forms of carbon's unique physical and chemical properties led many scientists to propose several technological applications. Since C60 molecules have a high electron affinity, they have attracted considerable attention due to their antioxidant and radical scavenging properties, which can absorb many free radicals responsible for aging the skins and graft surfactants or hydrophilic polymers to fullerenes in aqueous environments [6-8]. Fullerenes can be integrated into shafts and frames for strengthening composite materials with very thin-walled, lightweight, robust carbon structures [9]. Therefore, fullerenes are currently applied in cosmetic products and sporting goods industries. Recent developments suggest that many of the proposed fullerene applications are practical technologies in a wide range of areas such as information technology devices, lubricants, catalysts, diagnostics, pharmaceuticals, polymer modifications, environmental fields, and energy applications [2,3,6,10]. Their unique cage structure, coupled with their immense scope for derivatization, make fullerenes a potential therapeutic agent. Fullerenes have been extensively used for several biomedical applications, including MRI contrast agents, X-ray imaging contrast agents, anti-HIV drugs, photodynamic therapy, and targeted drug delivery systems [11-13]. The production of fullerenes and their use in technical products are expected to increase in the future. However, most fullerenes are non-biodegradable molecules whose potential toxicity has not been thoroughly investigated so far. The increased demand for fullerenes and their mass production have raised biosafety and environmental concerns.

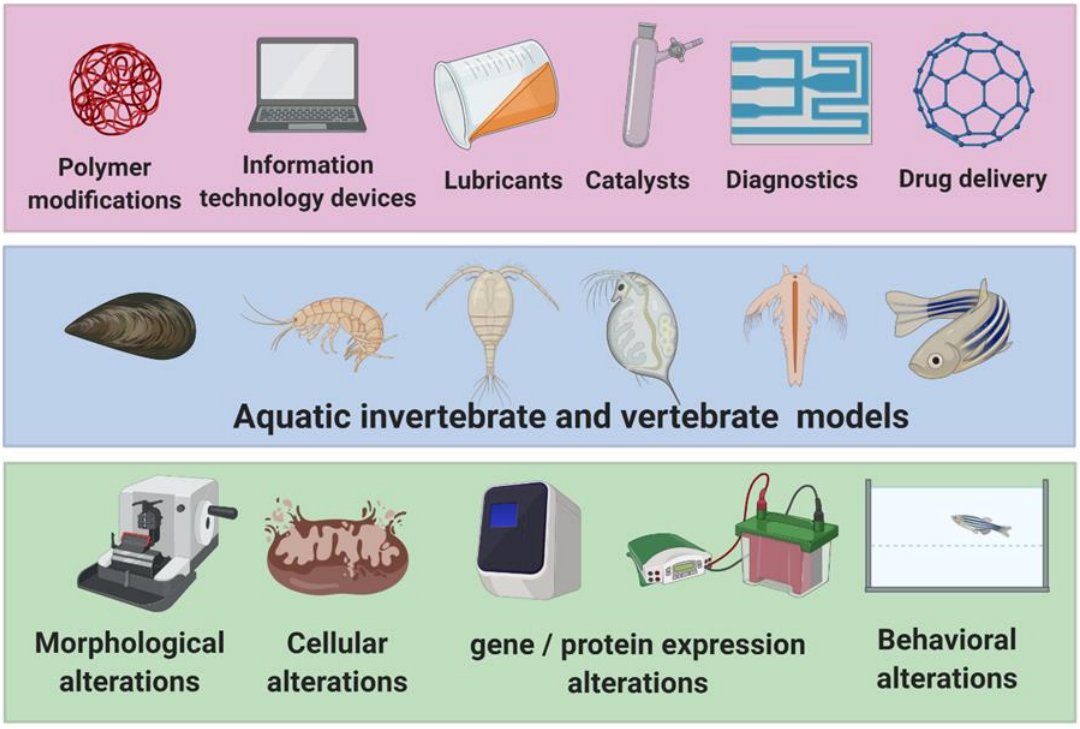

Figure 1. Summary of the application, animal models, and methods for fullerene toxicity assessment in aquatic species. The industrial and biomedical applications of fullerene were summarized in the

 doi:10.20944/preprints202009.0376.v1

upper panel (pink color). The invertebrate and vertebrate animal models used to perform fullerene toxicity assessment were summarized in the middle panel (blue color). The variety of methods used to detect fullerene-induced changes at either morphological, cellular, gene/protein expressional, or behavioural levels were summarized in the bottom panel (green color).

Chemistry of different types of fullerenes

Fullerenes have a similar structure like graphite, except that they may contain pentagonal rings. Fullerenes have been found to occur in nature [14]. Some minute quantities of fullerene in the form of C60, C70, C76, and C84 might be found hidden in carbon soot [15]. In 2010, the C60 and C70 were also detected by NASA's Spitzer infrared telescope in a cloud of cosmic dust surrounding a star [16]. As for the synthesis of these fullerenes, the production generally starts by forming fullerene-rich soot. The original process generates an electric arc between two graphite rods in an inert atmosphere. The vaporizing carbon from the resulting electric arc then cools into sooty residue [1,17]. Carbon soot is also formed by laser ablation of graphite targets or laser pyrolysis of aromatic hydrocarbons [17]. By contrast, combustion is the most efficient method to produce commercial fullerenes in high-temperature, low-pressure premixed flat flames [18]. A solid mixture of various fullerenes and other carbons are formed through these chemical processes. Small amounts of fullerenes are then extracted from the soot using suitable organic solvents and then separated by liquid chromatography [17].

There are two major families of fullerenes, with fairly distinct properties and applications: the closed buckyballs and cylindrical carbon nanotubes. However, hybrid structures also exist between those two classes, such as carbon nanobuds. buckyballs and carbon nanotubes have special properties due to the way their carbon atoms are arranged. Each carbon atom in the closed fullerenes, especially C60, is bonded to three others and is sp2 hybridized. These delocalized electrons on the surface of a three-dimensional structure stabilize the spheroid structure of C60 by resonance [19]. Distorted buckyballs with n = 24, 28, 32, 36, and 50 were also obtained, but they are predicted to be unstable. Other relatively common clusters with n = 70, 72, 74, 76, 80, 82, and 84 exist but are less abundant in the experimentally produced carbon soot [20]. A general rule is observed that the chemical reactivity significantly decreases with increasing size of the fullerene molecule. The closed buckyballs, in contrast to graphite, are not electrically conductive. Buckyballs are suitable lubricants because of their spherical shape. Their unique, hollow cage could make buckyballs useful for delivering medicine in the future. On the other hand, cylindrical fullerenes are called carbon nanotubes or buckytubes. Each nanotube is a single molecule consisting of millions of carbon atoms. This molecule's width is usually only a few nanometers, but it can range from less than a micrometer to several millimeters in length [21]. The nanotubes often have closed ends but can also be open-ended. Carbon nanotubes exhibit higher tensile strength, flexibility, elasticity, and high thermal conductivity [22,23]. They are often utilized to reinforce composite materials with improved mechanical, electrical, and thermal properties. Depending on the hexagonal units' orientation in the tube wall with the tube axis, the nanotube may behave electrically as either a metal or a semiconductor [24].

Because all the fullerenes have the cyclo-hexanes in abundance, they are very aromatic and have stable, inert, carbon bonds. The insolubility in aqueous media and poor miscibility of fullerenes limit the biological applications, and its strong tendency to form self-aggregate also leads to phase separation problems [25,26]. Since pristine fullerenes and carbon nanotubes lack hydrogen atoms or other groups on their surface, they cannot undergo substitution reaction. Because of this reason, they need to carry out surface modification in order to promote the functionalization on their surface [26]. It is worth noting that fullerenes are the only known allotropic carbons that are soluble in common organic solvents (for example, toluene) despite a limited solubility in most solvents [4,26-28]. Once fullerenes are dissolved in organic solvents, various chemical reactions tend to proceed in solution, and thus numerous fullerene derivatives are formed. In producing these derivatives, fullerenes can undergo various chemical reaction, e.g., oxidation, reduction, nucleophilic substitutions, halogenations, hydrogenations, and radical additions, transition-metal-complex formations, and regioselective functionalization reactions [2]. Researchers have been able to increase the reactivity of

 doi:10.20944/preprints202009.0376.v1

fullerenes by attaching active groups to their surfaces and modifying their basic properties to be tailored to specific functions.

Functionalized buckyballs are primarily divided into two classes: endohedral and exohedral fullerenes. Exohedral fullerenes are formed with substituents outside their cages, while endohedral fullerenes with trapped atoms or molecules inside their cages [27]. These endohedral and exohedral derivatives have been demonstrated to possess attractive photonic, electronic, superconducting, lubrication, magnetic and biomedical properties due to their unique structures [25]. Similar studies have shown that the carbon nanotubes also need to be chemically modified by attachment of functional groups for efficiently improving the solubility, processing, and compatibility with host materials in the engineering of multi-functional materials [29,30]. Accordingly, the chemical modifications preserve the interesting chemical, physical and electrochemical properties of pristine fullerenes and make them to be more reactive and versatile for several applications [31].

Fullerene interaction, bioavailability, toxicity and biosafety to aquatic invertebrates

The invertebrates represent a unique target group for nanoparticles (NPs) ecotoxicity, since they have highly developed processes for cellular internalization of nano- and microscale particles (endocytosis and phagocytosis), which are integral to key physiological functions such as intracellular digestion and cellular immunity. The biologic, ecologic, and toxicologic characteristics of invertebrates render them a suitable model to detect chemicals and pollutants in specific habitats, mainly through bioaccumulation potential. Also, invertebrates offer an advantage to evaluate the individual effect of exposure of the tested chemicals. Assumptions have also been made that the invertebrate model might allow for prediction of toxicity effects at population and community levels. Therefore, they can be used as early warning indicators of deterioration or restoration of ecosystem structure and function [32]. We will discuss effects of fullerene in aquatic invertebrate organisms in the following section.

The toxicity of fullerene C60 was tested on Chironomus riparius, also known as the harlequin fly, based on the hypothesis that higher food concentrations reduce toxic response. The test was performed using two concentrations of food, 0.5 and 0.8% Urtica sp., in sediment containing fullerene masses of 0.36 to 0.55 mg/cm2 in a 10-day chronic test. The results demonstrated that at 0.5% food treatment significant difference was found in growth- related endpoints, whereas little effect was observed for higher food concentrations than the control group. Although fullerene agglomerates were observed in the gut, the microvilli were damaged. The work showed potential toxicity of fullerene to C. riparius in terms of morphological changes and inhibiting larval growth [33]. A similar study on the chronic effects C60 on C. riparius at different life stages where larvae and adult midges were investigated in 10-day growth and 42-day emergence tests. The results showed a decrease in body length at concentration of 0.0025-20 mg/kg C60, but effects disappeared at higher concentrations. The study stated that small fullerene agglomerates have more significant effects on C. riparius than large agglomerates, as observed with high doses of C60. The C60 exposure resulted in a bell- shaped dose-response relationship because of the relative growth pattern. This response complicates ecological risk assessment to C60 because some effects are expressed at low concentrations [34].

In another study, marine bivalve Mytilus was investigated for the toxicity of C60 at 1, 5, and 10 µg/ml concentrations. The C60 expressed no significant effect in lysosomal membrane stability, indicating a lack of major toxicity effect. However, C60 suspension induced a concentration-dependent lysozyme release, extracellular oxy-radical, and nitric oxide production. The results supported the hypothesis that the bivalve immune system represents a significant target for NPs [35]. In a similar study with Mytilus sp., the marine mussels were exposed for three days to C60 and polycyclic aromatic hydrocarbon (PAH) fluoranthene alone or in combination. C60 and fluoranthene individually caused a concentration- dependent increase in DNA strand breaks. In contrast, the combined exposure additively increased the levels of DNA strand breaks along with a 2-fold increase in total Mytilus galloprovincialis glutathione level. Moreover, significant accumulation of C60 was observed in all organs, with the highest concentration in the digestive gland up to 24.90 ± 4.91 µg/g ww. This work concluded that the tested concentrations of both C60 and fluoranthene evoked toxic

 doi:10.20944/preprints202009.0376.v1

response and genetic damage, and the combined exposure revealed damage with additive rather than synergistic effects [36]. Further, Mytilus galloprovincialis Lam. were exposed to C60 at 0.01, 0.1, and 1 mg/l concentrations for 72 h. C60 accumulated in the digestive gland induced dephosphorylation of mTOR. No oxidative stress was observed for cellular distribution of C60 in the digestive gland at 0.01 mg/l concentration. The study suggested that mussels' most affected functions were related to energy metabolism, lysosomal activity and cytoskeleton organization [37].

Further, eco-toxicity of fullerenes C60, C70, and C60-phenyl-C61-butyric acid methyl ester (PCBM) were assessed on benthic organisms Lumbriculus variegatus (California blackworm). The standard toxicity test indicated population growth in L. variegatus reduced at 25 to 150 mg/kg C60 and no effect on organism's growth or weight at 25 mg/kg C70, respectively [38]. Similarly, in a study, C60 was exposed to L. variegatus at 10, and 50 mg/kg dry mass for 28 days did not impact survival or reproduction compared to the control. The impairment of feeding activity indicated C60's disruptive effect on worm feeding. Electron and light microscopy also detected C60 agglomerates in fecal pellets but no absorption in gut epithelial cells. This study also reported that L. variegatus transferred fullerenes from sediment to sediment surface through feeding and egestion, potentially increasing C60 bioavailability to epibenthic organisms and facilitating C60 transfer in the food web [39].

C60 acute toxicity tests were performed on Daphnia magna, Hyalella Azteca, and Copepods. Exposure of Daphnia magna to C60 for 21 days at 2.5 and 5 ppm , respectively, resulted in a significant delay in molting and reduced offspring production, producing impacts at a population level [40]. Further toxicity tests were performed in Daphnia magna, and Moina macrocopa with 4 h/d sunlight exposure, photo-toxicity of fullerene by the environmental level of ultraviolet light was tested. Antioxidant enzyme activities were increased by co-exposure to C60 aqueous suspensions and by sunlight. The results demonstrated that fullerene led to oxidative damages to D. magna, which was aggravated by natural sunlight [41]. In a study, C60 NPs were prepared by sonication and ultra-filtration before exposure to Artemia salina for 48 h for acute toxicity testing. Exposure to sonicated C60 showed varied mortalities in different stages of A. salina, whereas filtered solution showed increased mortality with increased C60 NPs concentrations [42].

In a study, analysis of antioxidant and oxidative damage responses in the anterior, middle, and posterior region of Lophiotoma acuta (marbled turrid) and bacteria (feeding on mucus produced by L. acuta) was performed after C60 exposure for 24 h. The anterior region of L. acuta presented lower antioxidant capacity and lipid peroxidation after exposure to 1.0 mg C60/l. The study indicated that complex interactions between estuarine organisms and associated bacteria could be jeopardized by nanomaterials (C60 in this paper), with ecological consequences needed to be properly evaluated [43]. Next, C60 was tested in Daphnia magna for 48 h acute toxicity tests. Results showed an increase in mortality with increased concentration of C60 and higher levels of toxicity at lower C60 concentrations [44]. Similarly, when C60 was evaluated on Daphnia; Daphnia for the amount of C60 stored in the body at a particular time, as a result of exposure. It exhibited uptake of C60 with a body burden of 413 μg/g in wet weight in the 1 mg/LC50 treatment group. C60 accumulated significantly in the gut of Daphnia. The results also demonstrated a reduction in digestion and filtration rates, as well as gut impairments and inhibition of digestive enzymes cellulose, amylase, trypsin, and β-galactosidase. The research work provides evidence for restriction in energy acquisition and an increase in oxidative damage in Daphnia, which might be associated with the bioaccumulation of C60 and finally led to immobility and mortality [45]. In another similar study, the toxicity of Daphnia was assessed by C60 exposure in artificial freshwater. After 24 h exposure to 2 mg/l fullerene solution, the wet weight of the organism's wet mass was 4.5±0.7 g/kg. Elimination of 46% and 74% of accumulated fullerene was found after depuration in clean water for 24 and 48 h, respectively. Also, large aggregates of fullerenes were found in the gut of Daphnids. The study suggested that D. magna may play a role as a fullerene carrier from one trophic level to another because of their significant uptake of fullerene and relatively slow depuration [46]. Taken together, we envisage that the suspended C60 nanoparticles, when exposed to Daphnia magna revealed protection against short term UV and fluoranthene photo-induced toxicity but caused cellular damage in Daphnia magna.

 doi:10.20944/preprints202009.0376.v1

The presence of C60 protected cellular components, such as mitochondria, microvilli, and basal unfolding, as evidenced by transmission electron microscopy in organisms exposed to UV and fluoranthene photo-toxicity in short-term exposure. Whereas, long-term exposure (21 days) of low - level C60 caused significant cellular damage in Daphnia magna alimentary canal [47]. Further, when C60 was analyzed in Perinereis gualpensis (a Polychaete species), no evidence of oxidative damage, GSH, and GCL was observed in any tested concentration. The antioxidant capacity was found to be higher in the C60 group after 2 and 7 days, suggesting a possibility for fullerene acting as an antioxidant [48].

 doi:10.20944/preprints202009.0376.v1

Table 1. Summary of fullerene-based nanomaterial toxicity in aquatic invertebrates.

Fullerene	Model organism	Dosage and time	Toxic effect	LC50	Reference
C_{60}	*Chironomus riparius*	10 g wet artificial sediment and 40 ml C_{60}, food source 0.5 and 0.8% *urtica sp.* 10 days	Morphological changes and inhibiting larval growth. Agglomeration in gut and damage of microvilli.	NA	[33]
C_{60}	*Chironomus riparius*	Artificial sediment 0.0004-80 mg/kg dry weight 10 days and 42 days	C_{60} resulted in a bell-shaped dose- response relationship in view of the relative growth patterns.	NA	[34]
C_{60}	*Mytilus galloprovincialis Lam*	1, 5, 10 ppm 30 minutes to 4 hours	Concentration- dependent lysozyme release, extracellular oxy-radical, and nitric oxide production.	NA	[35]
C_{60}	*Mytilus galloprovincialis Lam.*	0.01, 0.1 and 1 ppm 72 h	C_{60} accumulated in the digestive gland induced dephosphorylation of mTOR.	NA	[37]
C_{60} and fluoranthene alone and combination	*Mytilus sp.*	0.10–1 ppm 32–100 ppb 3 days	C_{60} and fluoranthene evoke toxic responses and genetic damage. The combined exposure produced enhanced damage with additive rather than synergistic effects.	NA	[36]
C_{60}, C_{70}, and C_{60}-PCBM	*Lumbriculus variegatus*	0, 10, 25, 100, 150 ppm 28 days	C_{60} can affect population growth of *L. variegatus* but C_{60}-PCBM and C_{70} effects are lower in comparison.	NA	[38]
C_{60}	*Lumbriculus variegatus*	10 and 50 ppm 28 days	Impairment of feeding activity and C_{60} aggregates presence in feces.	NA	[39]
C_{60}	*Daphnia magna, Hyalella azteca, Copepods*	30 ppm 5 days. 7 ppm 48-96 h, 0, 3.75, 7.5, 15 and 22.5 ppm 96 h	C_{60} 21 day *Daphnia* exposure resulted in a significant delay in molting and reduced offspring production at 2.5 and 5 ppm.	NA	[40]
C_{60}	*Daphnia magna* and *Moina macrocopa*	4 hr/d sunlight C_{60} filtered 0.2 µm (0, 0.462, 0.925, 1.85, 3.70 and 7.40 ppm) and 0.45 µm (0, 0.703, 1.40, 2.81, 5.62 and 11.2 ppm) 24, 48, 72 and 96 h	Fullerene leads to oxidative damage to *D. magna* and it was aggravated by natural sunlight.	0.2 µm – 96 h LC50 – 5.95 ppm 0.45 µm- 96 h LC50; >11.2 ppm (Outdoor) 0.2 µm – 96 h LC50 1.35 ppm 0.45 µm – 96 h LC50 1.58 ppm (Outdoor)	[41]
C_{60}	*Artemia salina*	Filtered C_{60} 40, 180, 260, 350, 440, 510, 700 and 880 ppb Sonicated C_{60} 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ppm for 1, 6, 12, 24, 36, 48 and 96 h	Exposure to sonicated nanoparticles shows varied mortalities in different stages of *A. salina* whereas filtered solutions showed increasing mortality with increase in concentration.	Sonicated C_{60}, the adult LC50 value was 3.17 ppm, whereas it was 617 ppb for the filtered solution.	[42]
C_{60}	*Laeonereis acuta*	0.01; 0.10 or 1.00 ppm 24 h	*L.acuta* anterior region presented lower antioxidant capacity and lipid peroxidation after exposure to 1.0 mg C_{60}/l.	NA	[43]

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C_{60}	*Daphnia magna*	Filtered C_{60} 40, 180, 260, 350, 440, 510, 700, and 880 ppb Sonicated C_{60} 0.2, 0.45, 0.9, 2.25, 4.5, 5.4, 7.2, and 9 ppm 48 h	C_{60} caused an increase in mortality with increase in concentration and higher levels of toxicity at lower concentrations.	Filtered C_{60} 460 ppb Sonicated C_{60} 7.9 ppm	[44]
C_{60}	*Daphnia magna*	1, 5, 10, 20, and 40 ppm 72 h	C_{60} exposure restricted- energy acquisition and induced oxidative damage, which might be the mechanisms underlying the observed acute toxicity of C_{60} to daphnia.	16.3 ± 0.8 ppm	[45]
C_{60}	*Daphnia magna*	Accumulation 0, 0.2, 2, 7, 15, 30, and 50 ppm 24 h Depuration 48 h	*D. magna* may play a role as a carrier of fullerenes from one trophic level to another.	NA	[46]
C_{60}	*Daphnia magna*	Short term 22 ppm and Long term 1 ppm 10 and 21 days.	C_{60} protected cellular components in organisms exposed to UV and fluoranthene photo-toxicity in short-term exposure, whereas long term exposure 21 days of low- level C_{60} caused significant cellular damage in *Daphnia magna* alimentary canal.	NA	[47]
C_{60}	*Perinereis gualpensis*	200 g 14 days	The data indicated an absence of toxic responses mediated by oxidative stress in estuarine worms exposed to C_{60} mixed in sediments.	NA	[48]

 doi:10.20944/preprints202009.0376.v1

Fullerene biological interaction, bioavailability, toxicity and biosafety to aquatic vertebrates

Identification of nanomaterial toxicity is a challenge since the potential usage of nanomaterial exposes them in the environment, eventually harming human health. The assessment of cytotoxicity of nanomaterials is important to understand the actual interaction and interpretation in a biological organism. The quality of NPs depends on the dispersion medium used for their suspension, which may add to their cytotoxicity potential. The main concern of fullerene testing is to identify the associated risk factors. Also, the defined conditions of laboratories play a large part in the toxicology profile. Understanding nanomaterial interaction inside the biological body is essential to understand all aspects of cells, organs and blood systems. The vertebrate system plays an important role in providing cheap, easy and time-efficient animal models to assess toxicity rapidly. In a study, embryonic zebrafish were exposed to fullerenes C60, C70, and C60(OH)24. The results showed that C60 and C70 induced a significant increase in malformations, pericardial edema, and mortality, where C60 induced both necrotic and apoptotic cellular death throughout the embryos. In contrast, C60(OH)24 exposure induced an increase in embryonic cellular death, but it did not induce apoptosis. The study suggested that C60(OH)24 is significantly less toxic than C60 [49]. In a similar study conducted by Henry et al. [50], with the embryonic zebrafish model, the loss of C60 from exposure water solution and embryonic zebrafish uptake was evaluated. The average recovery of C60 from zebrafish embryos extracts was 90%. The toxicological assay revealed the loss of C60 due to sorption to test vials resulted in a decrease of exposure-solution concentration over 6 h to be less than 50% of the initial concentration. The embryo uptake of C60 increased throughout the 12 h exposure. The study suggested that it is necessary to measure the time course of fullerene concentrations to establish the range of concentrations to which the organism is exposed.

Further, in a study conducted by Zhu et al. [51], Danio rerio embryo was exposed to nC60 to analyze the developmental toxicity in a 96 h exposure. The results demonstrated that nC60 at 1.5 mg/L delayed zebrafish embryo and larval development, decreased the survival and hatching rates, and caused pericardial edema, whereas fullerol hydroxylated C60 derivative at 50 mg/L did not exert to zebrafish embryos. The study also showed that toxicity was mitigated by adding an antioxidant (glutathione), suggesting a free radical-induced mechanism or another form of oxidative stress played a role in developmental toxicity.

Similarly, the ability of each fullerene to protect against toxicity induced by three different chemical toxins, gentamicin, cisplatinum, and 6-hydroxydopamine were assessed in this whole-animal system. When both positively and negatively charged water-soluble fullerenes were exposed to zebrafish embryos at 1 and 500 μM for 24-120 hours post- fertilization (hpf), the results indicated that water-soluble fullerenes could protect against chemical toxin-induced apoptotic cell death in a vertebrate. The whole-animal model also may be useful for predicting the efficacy and toxicity of these compounds in mammals. Furthermore, this work suggested that the relative potential for these compounds' pharmacologic use varies significantly with respect to stability [52].

In another in vivo study conducted by Sarasamma et al. [53], where C60 was exposed waterborne to adult zebrafish for 12 days at 1 and 2 ppm concentrations, respectively, and fish's behavioral alterations were measured by phenomic approach. The results showed that locomotion, novel tank exploration, aggression, shoaling, and color preference activities were significantly reduced. Also, the fish displayed dysregulation of the circadian rhythm. The corroboratory biochemical test results showed the induction of oxidative stress, DNA damage, reduced anti-oxidative capacity, and ATP content. The research concluded that adult zebrafish exposure to C60 at low concentration induced multiple behavioral abnormalities. Similarly, in another study also conducted by Sarasamma et al. [54], the potential adverse effects of fullerene C70 exposure were assessed on adult zebrafish. Two different doses (0.5 ppm and 1.5 ppm) were exposed to adult zebrafish for two weeks. Similar to C60 results, the results showed a decrease in locomotion, exploration, mirror biting, social interaction, shoaling, anxiety elevation, and circadian rhythm locomotor activity. Also, biochemical marker tests revealed a significant increase in superoxide dismutase (SOD), reactive oxygen species (ROS), cortisol, Hif 1-α, ssDNA, TNF-α, and IL-1β in brain

 doi:10.20944/preprints202009.0376.v1

and muscle tissue. Therefore, the study concluded several and similar toxic effects and alterations in neurobehavior parameters after zebrafish exposure to fullerene-based nanomaterials [54].

In another study conducted by Oberdörster et al. [55], nC60, when exposed to largemouth bass, resulted in significant lipid peroxidation in the brain after 48 h at 0.5 ppm uncoated nC60. Also, glutathione (GSH) was observed to be marginally depleted in the gills of fish with an increase in water clarity. Further, in another study, stress- induced toxicity of C60 fullerene on reproductive parameters of freshwater fish Anabas testudineus was studied for 60 days at 5 mg/L and 10 mg/L. The results showed reduction in gonadal steroidogenesis with a decrease in steroidogenic enzymes, 3β- and 17β-hydroxysteroid dehydrogenase. The serum testosterone and estradiol showed a significant decrement in male and female fish, respectively, in concentration- and time-dependent manners. The study suggested stress-induced by C60 exposure provoked reproductive toxicity in Anabas testudineus [56]. In a similar study protocol, the toxic effect of C60 was evaluated on behavioral and hematological changes in freshwater fish Anabas testudineus at 5 and 10 mg/L for 96 h and 60 days. The decline in acetylcholinesterase (AChE) enzyme activity in brain tissue showed prominent changes in fish behavior. The hematological parameter showed a significant reduction in blood cells with increased alanine and aspartate aminotransferase in serum. The results concluded that the sublethal concentration of C60 has a toxic impact on fish A. testudineus by affecting normal physiology, which might affect the ecosystem's health status [57].

Next, in a study conducted by Sumi et al. [58], the role of C60 was evaluated on the role of the brain antioxidant system of cichlid fish, Pseudetroplus maculatus. The fish were exposed to 0.1 mg/L concentration for 96 h. The results demonstrated no significant changes in the weight of the brain, whereas the significant decrease in antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) reductase and a significant increase in hydrogen peroxide and lipid peroxidation after 48 h treatment. Also, acetylcholinesterase (AChE), a marker enzyme for the brain, showed a significant reduction after exposure to C60 at the end of 48-96 h. Thus, the work suggested that exposure of C60 have adverse effects in the fish brain. In the next study reported by Henry et al. [59], C60 in two different forms (C60-water) and tetrahydrofuran (THF-C60) were exposed to larval zebrafish Danio rerio to assess the changes in survival and gene expression. The results demonstrated that the survival of larval zebrafish was reduced in THF-C60 and THF-water but not in C60-water. The biggest difference in gene expression was observed in THF-C60. The research indicated that this study linked toxic effects to THF degradation products rather than C60 and may explain toxicity attributed to C60 in other investigations.

The aqueous suspensions of C60 aggregates were studied on marine teleost Fundulus heteroclitus of embryo, larvae, and adult stages. Because the aggregates of C60 precipitated as a result of mixing in natural seawater, and levels of C60 significantly increased in bottom water after 24 h resulted in very low mortality, no median lethal concentrations could be calculated at <10 mg/L. The C60 aggregates adhered to chorion but did not affect the development of embryos on hatching success. With higher exposure levels, the movement of C60 from chorion into the embryo tended to increase. This trend was accompanied by a dose-dependent increase in total glutathione (GSH) and a decrease in lipid peroxidation (LPO) [60]. The Cyprinus carpio (common carp) was exposed to C60 caused no effect to viability, whereas growth was arrested after 3 h of exposure to three concentrations of C60 (0.1, 1, and 10 mg/l). Also, non-reactive colonies to C60 presented high antioxidant competence to peroxyl radicals compared with reactive colonies [61].

Table 2. Summary of fullerene-based nanomaterial toxicity in aquatic vertebrates.

Fullerene	Model organisms	Dosage and time	Toxic effect	LC50	Reference
C_{60}, C_{70}, $C_{60}(OH)_{24}$	*Danio rerio* (embryo)	100 and 500 ppb for C_{60} and C_{70} and 500 to 5000 ppb for $C_{60}(OH)_{24}$.	Exposure to C_{60} induced both necrotic and apoptotic cell death in embryo, while $C_{60}(OH)_{24}$ induced an increase in embryonic cellular death. Results obtained suggest $C_{60}(OH)_{24}$ is significantly less toxic than C_{60}.	C_{60}/C_{70}- 200 ppb $C_{60}(OH)_{24}$ 4000 ppb	[49]
C_{60}	*Danio rerio* (embryo)	100, 200, and 400 ppb 2, 6, 12 h	Concentrations of C_{60} decreased to levels not associated with mortality, <50 ug/L, 100% mortality results when embryos were exposed to concentrations from 250 to 130 ppb.	130 ppb	[50]
nC_{60}, fullerol	*Danio rerio* (embryos)	nC_{60} – 1.5 ppm $C_{60}(OH)_{16–18}$ - 50 ppm 96 hpf	nC_{60} at 1.5 ppm delayed zebrafish embryo and larval development, decreased the survival and hatching rates, and caused pericardial edema, whereas fullerol hydroxylated C_{60} derivative at 50 ppm did not exert to zebrafish embryos.	NA	[51]
Water -soluble fullerenes (1-12)	*Danio rerio (embryos)*	1, 10, 100 and 250 μM for 24 hours	Positively charged water-soluble fullerenes tend to exhibit greater toxicity than negatively charged fullerenes with similar structures; Toxicity varies considerably among negatively charged fullerenes from very low to moderate, depending on structural features.	Cationic fullerenes 120 μM Anionic fullerenes 500 μM	[52]
C_{60}	*Danio rerio (adult)*	1 and 2 ppm 1 days	C_{60} exposure to adult zebrafish at low concentration induces multiple behavioural abnormalities.	NA	[53]
C_{70}	*Danio rerio (adult)*	0.5 and 1.5 ppm for 2 weeks	Toxicity and the alterations observed in several neurobehavior parameters after zebrafish exposure to environmentally relevant amounts of C_{70}.	NA	[54]
nC_{60}	*Micropterus salmoides* (juveniles)	0.5 ppm and 1 ppm 48 h	Increase in lipid peroxidation in brains at 0.5 ppm and marginal depletion of glutathione (GSH) in gills.	NA	[55]
C_{60}	*Anabas testudineus*	5 and 10 ppm for 60 days	Stress-induced by fullerene C_{60} exposure provoked reproductive toxicity in the fish, *Anabas testudineus*.	96 h-LC50 50 ppm	[56]
C_{60}	*Anabas testudineus*	5 and 10 ppm for 96 h and 60 days	Sublethal concentrations of fullerene C_{60} have a toxic impact on the fish *A. testudineus* by affecting the normal physiology.	96 h-LC50 50 ppm	[57]
C_{60}	*Pseudetroplus maculatus*	0.1 ppm for 96 h	Decrease in SOD, CAT, GSH reductase, AChE. Increase in hydrogen peroxide and lipid peroxidation.	NA	[58]
C_{60}	*Fundulus heteroclitus*	0, 1, 2.5, and 10 ppm 96 h	Water-stirred suspensions of nC_{60} are not toxic to embryonic, larval, or adult stages of *F. heteroclitus* at concentrations up to 10 ppm.	NA	[60]
C_{60}	*Cyprinus carpio*	0.1, 1, and 10 ppm 48 h	The results indicated that C_{60} affects bacterial communities that live in mucus secretions of common carp.	NA	[61]

 doi:10.20944/preprints202009.0376.v1

Discussion based on current understanding

The fullerenes are groups of particles characterized by specific physicochemical properties such as surface area, surface charge, degree of agglomeration, particle morphology and surface coating. The various characteristics of fullerene suggest that they show potential applications in present- day technology such as lubricants, catalysts, pharmaceuticals, polymer modifications, environmental fields, and energy applications. Hence, the manufacturing of daily usage products and their continuous increase without toxicity awareness is harmful in many ways. Given the possibility of fullerene's intentional or unintentional release to the environment/aquatic environment, it is important to understand the toxicity caused by fullerene from the base level.

The manufacturing of fullerenes to fit in for a specific task demands changes in their surface chemistries and properties. With the improvisation according to demand, the fullerenes aquire novel physicochemical properties to be assessed for potential toxicological behavior compared to the natural ones. The impact of fullerenes via direct contact with water containing the amount of fullerene through skin and inhalation, and via an indirect consumption of aquatic organisms exposure might pose a serious threat to human health in the long run [62,63]. Until now, the real effects associated with the interaction of fullerenes with the aquatic organisms are still lacking and remains challenging to analyze in the absence of relevant data.

The techniques for evaluation of the toxicity of fullerenes are evolving in the recent years. The current understanding of fullerene toxicity must recognize that data compilation limitation serves as a barrier to understanding the interaction of fullerene inside the organism's body to interpret the results firmly. In reviewing the emerging environmental issue, it is highlighted that important effects on environment fate, transport, and bioavailability of co-contaminants all play a crucial part in understanding fullerene's toxicology.

The toxicity of fullerenes is, to date, poorly understood, and contradictory in some cases. However, experimentation on fullerene toxicity testing has demonstrated that fullerene is toxic in some forms. Studies have shown that reactive oxygen species and free radical production are among the primary mechanisms of nanotoxicity; in turn, they may lead to oxidative stress, inflammation, and consequent damage to proteins, membranes, and DNA [64]. In a study, Oberdoster et al. (2004) [55] showed that fullerenes had been found to cause brain damage in fish. Also, Fortner et al. (2005) [65] demonstrated that fullerenes killed water fleas and showed bactericidal properties. Sayes et al. (2004) [66] stated that toxicity appears to be a sensitive function of surface derivatization. Rouse et al. (2006) [67] reported that the extent of aggregation, emulsion bases, and different solvents are important variables in the formation of aggregates.

The studies discussed here with fullerene toxicity response on invertebrate and vertebrate models depicted contradictory results. Although fullerene C60 has been shown to cause mortality, ROS production, aggregation, and lipid peroxidation on a large basis, some studies have reported conflicting results. Waissi-Leinonen et al. (2015) [34] demonstrated a bell-shaped dose- response relationship of relative growth patterns after C. riparius exposure to C60. Rajasree et al. (2011) [42] showed that exposure of sonicated C60 resulted in varied mortality in different stages of A. salina, whereas the filtered solution of C60 upon exposure revealed increasing mortality with an increase in concentration. Similarly, Blickley et al. (2008) [60] suggested that water-stirred suspensions of C60 are not toxic to embryonic, larval, and adult stages of H. heteroclitus at a concentration up to 10 mg/L.

In another study, Tervonen et al. (2010) [46] demonstrated D. magna to play a role as a fullerene carrier from one trophic level to another. Similarly, Pakarinen et al. (2011) [39] demonstrated L. variegatus to transfer fullerenes from sediment to sediment surface through feeding and egestion. This observation indicated that this transfer might potentially increase the fullerenes' bioavailability to epibenthic organisms that may be more sensitive to fullerene exposure and might further facilitate fullerene transfer in the food web. In the vertebrate model, Beuerle et al. (2007) [52] exhibited that positively charged fullerene exhibited greater toxicity in comparison to negatively charged fullerene, which showed a dependency behavior on the structural features.

Bio-distribution of fullerene after ingested by an aquatic organism

The bio-distribution of fullerene on ingestion by aquatic organisms has been seldom addressed in the literature. The concentration of fullerenes in various environmental matrices is unknown, and quantification techniques are still under development [33,68]. However, NPs within cells may cause alterations in the cytoskeletal network [69]. Waissi-Leinonen et al. (2012) depicted the agglomeration of fullerene C60 in the gut area and damage of microvilli of C. riparius [33]. In a study, Sforzini et al. (2020) [37] reported subcellular distribution of fullerene C60 in mussel digestive gland cells and the possible involvement of mTOR inhibition in pathophysiological perturbation induced by nanoparticle accumulation. Further, the group stated that autophagic induction by fullerene C60 might represent an attempted degradation in lysosomes of material that is recognized by cells as foreign, such as pathogens, damaged intracellular proteins, and membranes. The statement was made based on observations, where C60 accumulation in the lysosomal-vacuolar system of major digestive gland epithelial cells. Overall, the report suggested that dysregulation of mTOR 1 and 2 may reduce the capacity of cells and organisms to grow and reproduce properly. Also, in a study, Pakarinen et al. (2011) [39] reported that fullerene concentration in feces of L. variegatus was high in comparison to the bulk sediments. The study suggested that high fullerene concentration in pellets may partly stem from worms consuming and absorbing some sediment fraction for nutritional purposes while the fullerenes and other particles are excreted. Thus, the various papers and results suggest more research is needed to understand the risks that fullerene may pose in sediments and organisms' bodies in different doses and concentrations under specific environmental conditions. Therefore, the limited information in the fullerene bio-distribution is an important topic for future research.

Future directions

We propose that techniques such as behavioural assay, using phenomics, tissue distribution analysis by MASS spectrum or isotope labelling for in vivo tracking, should be used to study fullerene induced toxicity in aquatic animals, i.e. specific biomarkers study, bio-distribution, and interaction within organism's body, in order to accumulate relevant data required to achieve a clear picture about fullerene induced toxicity.

Author Contributions: Conceptualization, N.M. and C.-D.H.; funding acquisition, T.-R.G., J-S.L. and C.-D.H.; investigation, T.-R.G., J-S.L. and C.-D.H.; project administration, and C.-D.H.; resources, A.L.C., G.A., P.S. and M.J.R.; supervision, T.-R.G. and C.-D.H.; visualization, N.M. and C.-D.H.; writing—original draft, N.M., T.-R.G., J.-S.L., J.-R. Chen and C.-D.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the grants sponsored by the Ministry of Science and Technology MOST107-2622-B-033-001-CC2, MOST108-2622-B-033-001-CC2 and MOST108-2313-B-033-001-MY3 to C.-D.H. and MOST109-2112-M153-002 to J.-S.L.

Acknowledgments: We appreciate the anonymous reviewers and editors for their insightful comments, which improved the quality of this paper.

Conflicts of Interest: The authors declare no conflicts of interest.

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