











Review Article

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

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Fullerene molecules are composed of carbon in the form of a hollow sphere, tube, or ellipsoid. Since their discovery in 1985, they have gained a lot of attention in many science fields. The unique carbon cage structure of fullerene provides immense scope for derivatization, rendering potential for various industrial applications. Thus, the prospective applications of fullerenes have led to assorted fullerene derivatives. In addition, their unique chemical structure also eases them to be synthesized through various kinds of conjugating techniques, where fullerene can be located either on the backbone or the branch chain. In this review, we have compiled the toxicity and biosafety aspects of fullerene in aquatic organisms since 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, and neurobehavioral levels.

1. Introduction and Application of Fullerene

1.1. Introduction of Fullerene. Carbon is known to be found in two allotropes, namely, diamond and graphite. In 1985, Kroto, Curl, and Smalley discovered another allotropic form of carbon, which was fullerene. Because of the discovery and their pioneering efforts, they received the Nobel Prize in 1996 [1]. Fullerenes are regarded as three-dimensional analogs of benzene and composed of carbon atoms that are joined by single

and double bonds. Together with fused rings of five to seven carbon atoms, these bonds form a closed or partially closed mesh. Carbon atoms in a fullerene molecule can be found in a variety of sizes and shapes, including hollow spheres, ellipsoids, and tubes [2, 3]. The discovered fullerenes consist of various n numbers of carbon atoms, which obeyed a specific rule.

C_{60} , one of the most well-known and abundant forms of fullerenes, is composed of $n = 60$ carbon atoms, which are arranged in a spherical cage structure of about 7 Å in diameter.

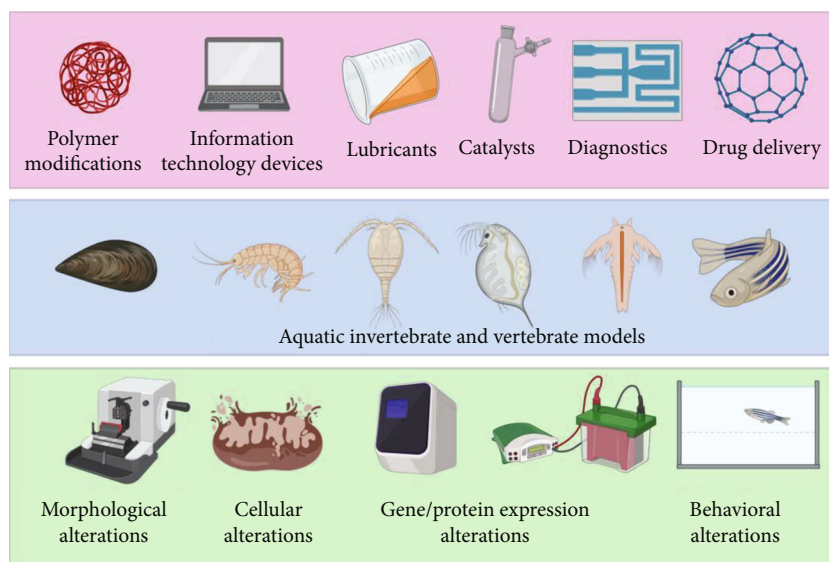


FIGURE 1: Summary of applications, animal models, and methods of fullerene toxicity assessment in aquatic species. The industrial and biomedical applications of fullerene were compiled in the upper panel (pink color). The invertebrate and vertebrate animal models used to perform fullerene toxicity assessment were compiled in the middle panel (blue color). The various methods used to detect fullerene-induced changes at either morphological, cellular, gene/protein expression, or behavioral levels were summarized in the bottom panel (green color).

Furthermore, to honor the inventor of geodesic domes in the 1960s, it also goes by the name of buckminsterfullerene [2]. Informally, C_{60} fullerenes are also known as buckyballs since their shape resembles a soccer ball shape. In C_{60} fullerenes, there are two types of C-C bonds with distinct lengths: C_5-C_5 single bonds in the pentagons and C_5-C_6 double bonds in the hexagons with 1.45 Å and 1.40 Å in distance, respectively [4]. The C_{60} has a low specific gravity relative to the 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]. In addition, besides C_{60} , other fullerenes can consist of numerous carbon atoms, ranging from 30 to 980, creating various structures with different characteristics and applications.

1.2. Applications of Fullerene. These new forms of carbon's distinctive physical and chemical properties have taken numerous scientists to propose several technological applications. Nowadays, C_{60} molecules have attracted a lot of attention since they have a high electron affinity and antioxidant and radical scavenging properties, which can absorb many free radicals responsible for aging the skins and grafting surfactants or hydrophilic polymers 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 that make them currently be applied in cosmetic products and sporting goods industries [9]. Recently, several investigations suggested that a lot of the proposed fullerenes are applicable in many areas, including information technology devices, lubricants, catalysts, diagnostics, pharmaceuticals, polymer modifications, energy applications, and environmental fields [2, 3, 6, 10]. Furthermore, the unique cage structure of fullerenes, coupled with their immense scope for derivatization, opens up the

potential of fullerenes to be a therapeutic agent. In addition, it has also been extensively used in many biomedical applications, including MRI contrast agents, X-ray imaging contrast agents, anti-HIV drugs, targeted drug delivery systems, and photodynamic therapy [11–13]. Therefore, the production and usage of fullerenes are expected to escalate in the future. However, most fullerenes are nonbiodegradable 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. *In vivo* toxicity testing provides intact systems for the prediction of biological responses. Aquatic invertebrates and vertebrates provide cost, labor, and time-effective platforms for these studies. These *in vivo* study models provide results on many cellular, anatomical, and physiological characteristics. Moreover, their small size, speedy development, and short life cycle make them attractive for evaluating nanomaterial's toxicological effects. Various methods of toxicity assessments have been developed at different levels of these *in vivo* model systems, such as morphological alterations assessed under a microscope, cellular alterations, gene/protein expression alteration, biochemical analysis, and behavior alterations. The applications of fullerene, aquatic invertebrate, and vertebrate model systems used to assess the toxicity and methods of toxicity analysis of fullerene are compiled in Figure 1.

2. Types and Chemical Composition of Fullerene

2.1. Types of Fullerenes. Fullerenes occur in nature and have a similar structure to graphite, except that they may contain pentagonal rings [14]. Small quantities of fullerene in the form of C_{60} , C_{70} , C_{76} , and C_{84} might be found hidden in

carbon soot [15]. In 2010, by using Spitzer infrared telescope, NASA also found C_{60} and C_{70} in a cloud of cosmic dust surrounding a star [16]. Generally, the synthesis of these fullerenes starts by forming fullerene-rich soot. The original process generates an electric arc between two graphite rods in an inert atmosphere resulting in a vaporized carbon that is subsequently cooled into sooty residue [1, 17]. In addition, laser ablation of graphite targets or laser pyrolysis of aromatic hydrocarbons can also form carbon soot [17]. By contrast, combustion is the most effective method to produce commercial fullerenes in high-temperature, low-pressure premixed flat flames [18]. Later, through these chemical processes, a solid mixture of various fullerenes and other carbons is formed. Afterward, by using suitable organic solvents, small amounts of fullerenes are extracted from the soot and separated by liquid chromatography [17].

The closed buckyballs and cylindrical carbon nanotubes are two major fullerene classes with distinct properties and applications, although some hybrid structures also exist besides these families, including carbon nanobuds. These classes have unique properties due to their carbon atom arrangement. Each carbon atom in the closed fullerenes, especially C_{60} , bonds to three others and is sp^2 hybridized. These delocalized electrons on the surface of a three-dimensional structure stabilize the spheroid structure of C_{60} 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 the increasing size of the fullerene molecule. The closed buckyballs, in contrast to graphite, are not electrically conductive. Due to their spherical shape, buckyballs are well known as suitable lubricants. Moreover, buckyballs might be useful in medicine deliveries in the future because of their unique and hollow cage. On the other hand, cylindrical fullerenes, another major family of fullerenes, are known as carbon nanotubes or buckytubes. Every nanotube is a single molecule consisting of millions of carbon atoms. Generally, this molecule's width is only a few nanometers; however, the length ranges from less than a micrometer to several millimeters [21]. Most of the time, the nanotubes have closed ends; however, it is also possible to 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. In addition, these nanotubes may act electrically as either a metal or a semiconductor depending on the hexagonal units' orientation in the tube wall with the tube axis [24].

2.2. Chemical Composition of Fullerene. Since every fullerene possesses abundant cyclohexanes, they are very aromatic and have stable and 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 reactions. 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 reactions, e.g., oxidation, reduction, nucleophilic substitutions, halogenations, hydrogenations, radical additions, transition-metal-complex formations, and regioselective functionalization reactions [2]. By attaching active groups to their surface and modifying their basic properties to be adjusted to specific functions, the increment in fullerenes reactivity has been demonstrated.

Functionalized buckyballs are primarily divided into two classes: endohedral and exohedral fullerenes. Exohedral fullerenes are formed with substituents outside their cages, while endohedral fullerenes are formed with trapped atoms or molecules inside their cages [27]. These endohedral and exohedral derivatives have been shown to exhibit attractive photonic, electronic, superconducting, lubrication, biomedical, and magnetic properties due to their unique structures [25]. Similar studies have shown that the carbon nanotubes require chemical modifications by attaching the functional groups to improve the compatibility, processing, and solubility with host materials in the engineering of multifunctional materials [29, 30]. Accordingly, the chemical modifications maintain the interesting pristine fullerenes electrochemical, chemical, and physical properties and make them more applicable and reactive for many applications [31].

3. Interaction of Fullerene with Aquatic Invertebrates in reference to Bioavailability, Toxicity, and Biosafety

The invertebrates are among the target groups for nanoparticle (NP) ecotoxicity due to their highly developed cellular internalization processes, such as phagocytosis and endocytosis, of nano- and microscale particles that are essential for physiological functions (cellular immunity and intracellular digestion). The biologic, ecologic, and toxicologic characteristics of invertebrates render them a suitable model to detect chemicals and pollutants in typical habitats, primarily via bioaccumulation potential. In addition, invertebrates also offer an advantage to evaluate the individual effect of exposure to the tested chemicals. Assumptions have also been made that the invertebrate model can be used to predict the effects of some toxicants at population and community levels. Thus, they can early indicate the ecosystem function and structure's deterioration or restoration [32]. We are going to discuss the effects of fullerene in aquatic invertebrate organisms in the current section.

First, the chronic toxicity of fullerene C_{60} was tested on *Chironomus riparius*, also known as the harlequin fly, and

hypothesized that higher food concentration could reduce the toxic response. This 10-day test was performed by using *Urtica* sp. as food in two different concentrations, which were 0.5 and 0.8%. The test was conducted in sediment that contained fullerene with masses of 0.36 to 0.55 mg/cm². The results demonstrated that at 0.5% food treatment, a significant difference in growth-related endpoints was found, whereas little effect was observed for the higher food concentrations than the control group. Furthermore, although they found agglomerates of fullerene in the gut, the microvilli were damaged. Taken together, this finding demonstrated the fullerene's potential toxicity to *C. riparius* in terms of morphological changes and larval growth inhibition [33]. Next, a similar study on the chronic effects of C₆₀ on different life stages of *C. riparius* in 10-day growth (larvae) and 42-day (adult midges) emergence tests was conducted. The results showed a decrease in body length at a concentration of 0.0025–20 mg/kg C₆₀, but effects disappeared at higher concentrations. The study stated that small fullerene agglomerates more significantly affected *C. riparius* than the large ones, as shown with high doses of C₆₀. Further, a bell-shaped dose-response relationship was observed in the C₆₀ exposure results, which might be caused by the relative growth pattern. This dose-response relationship makes ecological risk assessment of C₆₀ more difficult since several effects occur at low concentrations [34].

Several prior studies had assessed the toxicity of C₆₀ in *Mytilus*. First, the toxicity of C₆₀ at 1, 5, and 10 µg/ml concentrations was investigated in marine bivalve *Mytilus*. From the results, the C₆₀ expressed no significant effect in lysosomal membrane stability, which indicated an absence of a major toxicity effect. However, C₆₀ suspension led to the release of lysozyme and extracellular oxyradical and nitric oxide production in a concentration-dependent manner. Therefore, the results supported the speculation about the bivalve immune system as a key target of NPs [35]. Forward, in a similar study with *Mytilus* sp., a marine mussel, they were exposed for three days to either 0.10–1 mg/l of C₆₀ and 32–100 µg/l of polycyclic aromatic hydrocarbon (PAH) fluoranthene or the combination of both compounds. The observed results depicted a concentration-dependent increase in DNA strand breaks caused by C₆₀ and fluoranthene individually; however, their combined exposure enhanced the level of DNA strand breaks together with a twofold increment in total glutathione (GSH) content, indicating oxidative stress. The work concluded the generation of toxic response and damage with additive effects. Afterward, the research group suggested further analysis of C₆₀ and fluoranthene alone or in combination for a longer duration to establish concrete toxicity results [36]. Further, *Mytilus galloprovincialis* Lam. were also exposed to C₆₀ at 0.01, 0.1, and 1 mg/l concentration for 72 h. Later, the accumulation of C₆₀ in the digestive gland was demonstrated to induce dephosphorylation of mTOR with no oxidative stress for cellular distribution of C₆₀ at 0.01 mg/l concentration. The study suggested that mussels' most affected functions were related to the organization of the cytoskeleton, energy metabolism, and lysosomal activity [36, 37]. Lastly, in another report on *Mytilus galloprovincialis*, the mussels were exposed to C₆₀ (0.01, 0.1, and

1 mg/l), B[a]P (5, 50, and 100 µg/l), and B[a]P (5, 50, and 100 µg/l) in combination with C₆₀ (1 mg/l). Afterward, the uptake of each treatment group was measured by a different set of chromatography techniques. The study supports the hypothesis of an interaction between these two compounds and demonstrated an antagonistic relationship at the genotoxic and proteome expression level. However, as this effect is observed on a single concentration, the research group further suggested that other dosage concentrations should also be investigated [37, 38].

Further, ecotoxicities of several fullerenes (C₆₀, C₇₀, and C₆₀-phenyl-C₆₁-butyric acid methyl ester (PCBM)) on *Lumbriculus variegatus* (California blackworm), a benthic organism, were assessed. The results indicated that 25 to 150 mg/kg of C₆₀ reduce the population growth of *L. variegatus*, even though no effect on the organism's growth or weight was observed after treated with 25 mg/kg of C₇₀ [38, 39]. Similarly, in another study, 10 and 50 mg/kg dry mass of C₆₀ were exposed to *L. variegatus* for 28 days. Later, it was found that the survival rate or reproduction of *L. variegatus* was not affected by C₆₀. However, the impairment of feeding activity observed in the study indicated the C₆₀'s disruptive effect on worm feeding. In addition, electron and light microscopy also detected C₆₀ agglomerates in fecal pellets, and they were not absorbed in gut epithelial cells. This study also reported that through feeding and egestion, fullerene transfer from sediment-to-sediment surface occurred in *L. variegatus*. This phenomenon might increase the bioavailability of C₆₀ to epibenthic organisms and facilitate the C₆₀ transfer in the food web [39, 40].

The acute toxicity of C₆₀ was also assessed on *Daphnia magna*, *Hyalella Azteca*, and *Copepods*. The 21-day exposure of C₆₀ in different concentrations (2.5 and 5 ppm) generated a significant delay in molting and a reduction in offspring production, producing impacts at a population level [40, 41]. Further toxicity tests were performed in *Daphnia magna* and *Moina macrocopa* with 4 h/d sunlight exposure, testing the photo-toxicity of fullerene by the environmental level of ultraviolet light. The neonates were exposed acutely to aqu/nC₆₀ filtered through 0.2 µm (0, 0.462, 0.925, 1.85, 3.70, and 7.40 mg/l) and filtered through 0.45 µm (0, 0.703, 1.40, 2.81, 5.62, and 11.2 mg/l) at 21 ± 1°C, and observations were recorded at 24, 48, 72, and 96 hours. From the results, antioxidant enzyme activities were observed to be increased by coexposure to C₆₀ aqueous suspensions and sunlight. The results demonstrated that fullerene led to oxidative damage to *D. magna*, aggravated by natural sunlight [41, 42]. Next, in another study, C₆₀ NPs were prepared at different concentrations by sonication (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ppm) and ultrafiltration (40, 180, 260, 350, 440, 510, 700, and 880 ppb) before exposure to *Artemia salina* for 1, 6, 12, 24, 36, 48, and 96 h for acute toxicity testing. The results showed that sonicated C₆₀ caused varied mortalities in different chosen stages of this brine shrimp at 15–24 h, 24–48 h, 72–96 h, 6 to 7 days old, and adult. In contrast, filtered solution showed increased mortality with increased C₆₀ NP concentrations [42, 43]. Next, a prior study analyzed the antioxidant and oxidative damage responses in the several regions of *Lophiotoma acuta* (marbled turrid) and a bacterium (feeding on

mucus produced by *L. acuta*) after C_{60} exposure for 24 h. After 1.0 mg of C_{60} /l was administered, low levels of antioxidant capacity and lipid peroxidation were displayed in the anterior region of *L. acuta*, indicating that complex interactions between estuarine organisms and associated bacteria could be compromised by nanomaterials, which is C_{60} in this case. This finding highlights the importance of proper evaluation of C_{60} usage, considering the ecological consequences [43, 44]. Next, C_{60} was tested in *Daphnia magna* for 48 h acute toxicity tests. From the results, C_{60} increased the mortality rate with an increased concentration of C_{60} and caused even more severe toxicity effects at lower C_{60} concentrations [44, 45]. Similarly, the amount of C_{60} stored in the body at a particular time due to the exposure of C_{60} was also evaluated on *Daphnia*. After being treated with 1 mg/l of C_{60} , C_{60} was taken with a body burden of 413 $\mu\text{g/g}$ wet weight. C_{60} was observed to be accumulated significantly in the gut of *Daphnia*. Moreover, gut impairments, reduced digestion and filtration rates, and inhibited several digestive enzymes, such as β -galactosidase, trypsin, cellulase, and amylase, were shown after the exposure. The research work provides evidence for limitation in energy acquisition and increases in oxidative damage in *Daphnia* that might be associated with the C_{60} bioaccumulation, which causes immobility and mortality later [45, 46]. In another similar study with the same animal model, the toxicity of C_{60} in artificial freshwater was investigated. After 2 mg/l of fullerene solution was administered for 24 hours, the wet weight of the organism's wet mass was 4.5 ± 0.7 g/kg. However, after depuration for 24 and 48 h in clean water, 46% and 74% of accumulated fullerene, respectively, were eliminated. Also, large aggregates of fullerenes were found in the gut of *Daphnids*. Taken together, the study suggested that *D. magna* might have contributed to carrying fullerene from one trophic level to another because of their significant uptake of fullerene and relatively slow depuration [46, 47]. Further, another behavior study in *D. magna* also discovered that chronic exposure of nC_{60} (21 days) in different concentrations (0.1 or 1 mg/l) yielded a reduction in their feeding ability, hopping, and heartbeat frequencies. Later, the transcriptome analysis depicted this phenomenon which is possibly due to alterations in some underlying physiological functions, including protein degradation, reproduction, energy metabolism, and cell structure repair [37, 38]. Similarly, C_{60} and PCBM (is a solubilized version of the C_{60}) were not found to be acutely toxic when tested at 5, 10, 25, and 50 mg/l for 21 days, while C_{70} had significant acute toxic effects. However, C_{60} , C_{70} , and PCBM depicted heart rate elevation over time [47, 48]. In addition, another study demonstrated that accumulated C_{60} in *D. magna* could be transferred to zebrafish through dietary exposure and accumulated mostly in the intestines; however, no magnification was found [48, 49].

Taken together, we envisage that when exposed to *Daphnia magna*, the suspended C_{60} nanoparticles revealed protection against short-term UV and fluoranthene photo-induced toxicity, although it caused cellular damage. The cellular components, such as microvilli, mitochondria, and basal unfolding, are protected by C_{60} , which evidenced by transmission electron microscopy in organisms after being

shortly exposed to UV and fluoranthene photo-toxicity, while longer exposure time (21 days) of C_{60} at low concentration led to significant cellular damage in the alimentary canal of *Daphnia magna* [49, 50]. Further, when C_{60} was analyzed in *Perinereis gualpensis* (a Polychaete species), there were no oxidative damage, GSH (glutathione), and GCL (glutamate cysteine ligase) observed in all tested concentrations, even though after 2 and 7 days, the antioxidant capacity was found to be elevated in the treated group, suggesting a possibility for fullerene acting as an antioxidant [50, 51]. The summary of the toxicity of fullerene-based nanomaterial to aquatic invertebrates is described in Table 1.

4. Interaction of Fullerene with Aquatic Vertebrates in reference to Bioavailability, Toxicity, and Biosafety

Identification of nanomaterial toxicity is challenging since the potential usage of nanomaterial exposes them to the environment and eventually harms 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. In addition, the defined conditions of laboratories play a large part in understanding the toxicology profile. The comprehension of nanomaterial interaction inside the biological body is essential to understand all aspects of cells, organs, and blood systems. The vertebrate system plays a significant part in providing cheap, easy, and time-efficient animal models to assess toxicity rapidly. In a prior study, fullerenes (100-500 ppb of C_{60} and C_{70} and 500-5000 ppb of $C_{60}(\text{OH})_{24}$) were administered 24-96 hours of postfertilization (hpf) zebrafish embryo. The results showed that while C_{60} alone led to apoptotic and necrotic cell death, both C_{60} and C_{70} were demonstrated to cause mortality, malformations, and pericardial edema in the embryos. On the other hand, even though $C_{60}(\text{OH})_{24}$ exposure increased embryonic cellular death, it did not lead to apoptosis. Therefore, the study suggested less toxicity of $C_{60}(\text{OH})_{24}$ compared to C_{60} [52]. Furthermore, in a similar study in the embryonic zebrafish model conducted by Henry et al., the depletion of C_{60} from exposure medium and embryonic zebrafish uptake was evaluated [53]. Later, it was found that around 90% of C_{60} could be recovered from zebrafish embryo extracts. The toxicological assay revealed that sorption to test vials caused the loss. After 6 hours, this absorption already resulted a decrease of exposure solution to less than 50% of the initial dose. The embryo uptake of C_{60} increased throughout the 12 h exposure. The study suggested that it is necessary to measure the time course of the C_{60} dose to determine the range of concentrations to which the organism will be exposed. Furthermore, in a prior experiment done by Zhu et al., the *Danio rerio* embryo was exposed to nC_{60} to analyze the developmental toxicity in a 96 h exposure [54]. The results demonstrated that 1.5 mg/l of nC_{60} delayed the development of the zebrafish embryo

TABLE 1: Fullerene-based nanomaterial toxicity in aquatic invertebrates.

Fullerene	Model organism	Dosage and time	Toxic effect	LC50	Reference
C ₆₀	<i>Chironomus riparius</i>	10 g wet artificial sediment and 40 ml C ₆₀ food source 0.5 and 0.8% <i>Urtica</i> sp. 10 days	Morphological changes and inhibiting larval growth. Agglomeration in gut and damage of microvilli.	NA	[33]
C ₆₀	<i>Chironomus riparius</i>	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	<i>Mytilus galloprovincialis</i> Lam.	1, 5, and 10 ppm 30 minutes to 4 hours	Concentration-dependent lysozyme release, extracellular oxyradical, and nitric oxide production.	NA	[35]
C ₆₀	<i>Mytilus galloprovincialis</i> Lam.	0.01, 0.1, and 1 ppm 72 h	C ₆₀ accumulated in the digestive gland-induced dephosphorylation of mTOR.	NA	[36, 37]
C ₆₀ and fluoranthene alone and combination	<i>Mytilus</i> 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, 51]
C ₆₀ , C ₇₀ , and C ₆₀ -PCBM	<i>Lumbriculus variegatus</i>	0, 10, 25, 100, 150 ppm 28 days	C ₆₀ can affect the population growth of <i>L. variegatus</i> but C ₆₀ -PCBM and C ₇₀ effects are lower in comparison.	NA	[38, 39]
C ₆₀	<i>Lumbriculus variegatus</i>	10 and 50 ppm 28 days	Impairment of feeding activity and C ₆₀ aggregate presence in feces.	NA	[39, 40]
C ₆₀	<i>Daphnia magna</i> , <i>Hyalella azteca</i> , 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 <i>Daphnia</i> exposure resulted in a significant delay in molting and reduced offspring production at 2.5 and 5 ppm.	NA	[40, 41]
C ₆₀	<i>Daphnia magna</i> and <i>Moina macrocopa</i>	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 <i>D. magna</i> 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, 42]
C ₆₀	<i>Artemia salina</i>	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 <i>A. salina</i> , whereas filtered solutions showed increased mortality with the increase in concentration.	Sonicated C ₆₀ , the adult LC50 value was 3.17 ppm, whereas it was 617 ppb for the filtered solution.	[42, 43]

TABLE 1: Continued.

Fullerene	Model organism	Dosage and time	Toxic effect	LC50	Reference
C ₆₀	<i>Laonereis acuta</i>	0.01, 0.10, or 1.00 ppm 24 h	<i>L.acuta</i> anterior region presented lower antioxidant capacity and lipid peroxidation after exposure to 1.0 mg C ₆₀ /l.	NA	[43, 44]
C ₆₀	<i>Daphnia magna</i>	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 an increase in concentration and higher levels of toxicity at lower concentrations.	Filtered C ₆₀ 460 ppb Sonicated C ₆₀ 7.9 ppm	[44, 45]
C ₆₀	<i>Daphnia magna</i>	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, 46]
C ₆₀	<i>Daphnia magna</i>	Accumulation 0, 0.2, 2, 7, 15, 30, and 50 ppm 24 h Depuration 48 h	<i>D. magna</i> may play a role as a carrier of fullerenes from one trophic level to another.	NA	[46, 47]
C ₆₀	<i>Daphnia magna</i>	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 <i>Daphnia magna</i> alimentary canal.	NA	[49, 50]
C ₆₀	<i>Perinereis gualpensis</i>	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	[50, 51]

NA: not available.

and larvae, reduced the hatching and survival rates, and caused pericardial edema, whereas 50 mg/l of fullerol hydroxylated C_{60} derivative did not affect the zebrafish embryos. The study also showed that the addition of an antioxidant (glutathione) mitigated the toxicity, suggesting the developmental toxicities are regulated by a free radical-induced mechanism or another form of oxidative stress. Furthermore, in the following study reported by Henry et al., C_{60} in two different forms (C_{60} -water) and tetrahydrofuran (THF- C_{60}) were exposed to larval zebrafish to assess the changes in survival and gene expression [55]. The results demonstrated that the zebrafish larval survivability was compromised in THF- C_{60} and THF-water. However, this phenomenon was not observed in the C_{60} -water treatment group. In addition, in terms of gene expression, the biggest difference was displayed in the THF- C_{60} group. The research indicated that toxic effects found in this study might be associated with the products of THF degradation rather than C_{60} . Additionally, this also may explain the C_{60} toxicity found in other findings.

In another *in vivo* study conducted by Sarasamma et al. [56], waterborne C_{60} was exposed to adult zebrafish for 12 days at 1 and 2 ppm concentrations, respectively, and fish's behavioral alterations were measured by phenomics approach. The results showed the alteration in fish's locomotor activity, response to a novel environment, aggression level, shoal formation, and color preference. Moreover, the fish also displayed dysregulation in the circadian rhythm locomotor activity. The corroboratory biochemical test results showed the induction of oxidative stress and DNA damage, followed by a significant reduction in antioxidative capacity and ATP level. The research group concluded low concentration of C_{60} -induced multiple behavioral abnormalities in adult zebrafish. Similarly, in another prior study also conducted by Sarasamma et al. [57], the potential adverse effects of fullerene C_{70} 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 C_{60} results, the results showed abnormalities in locomotion, explorative behavior, aggressiveness level, conspecific social interaction behavior, shoal formation, 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-1 β in brain and muscle tissue, concluding several and similar toxic effects in altering the neuro-behavior parameters of zebrafish after exposed to fullerene-based nanomaterials [57]. Taken together, the studies performed on *Danio rerio* in the embryos and adult stages indicate the importance of concentration range and exposure time in assessing the toxicity effect of fullerene over vertebrate model since different toxicological effects are observed in different studies under different concentrations and tests performed. To establish a concrete result of the toxicological effect of fullerene, it is necessary to accumulate more data on different parameters.

Next, in a previous study done by Oberdörster, when 0.5 ppm of uncoated nC_{60} was exposed to largemouth bass, *Micropterus salmoides*, for 48 hours, it resulted in significant lipid peroxidation in the brain [58]. In addition, GSH was

observed to be marginally decreased in the fish's gills with an increase in water clarity. Further, in another experiment on *Anabas testudineus*, a freshwater fish, C_{60} was demonstrated to induce toxicity, specifically on reproductive parameters, after being exposed for 60 days at 5 mg/l and 10 mg/l. The results showed a reduction in gonadal steroidogenesis with a decrease in steroidogenic enzymes, 3β - and 17β -hydroxysteroid dehydrogenase. Furthermore, a significant decrement was also observed in the serum testosterone and estradiol in male and female fish, respectively, in concentration- and time-dependent manners. Thus, the study suggested stress induced by administration of C_{60} leads to reproductive toxicity in *Anabas testudineus* [59]. In a similar study protocol on *Anabas testudineus*, the toxic effect of C_{60} was evaluated on their behavior and hematology levels 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. Furthermore, 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 C_{60} generates toxicity by affecting the normal physiology of *A. testudineus*, which might affect the ecosystem's health status [59, 60].

Forward, in another prior study by Sumi and Chitra and Blickley and McClellan-Green., the role of C_{60} was evaluated on the role of the brain antioxidant system of cichlid fish, *Pseudotropheus maculatus* [60, 61]. In their study, 0.1 mg/l of C_{60} was administered to the fish for 96 hours. The results demonstrated no significant alterations in terms of the brain weight, whereas the notable reduction in antioxidant enzymes (like catalase, SOD, and GSH) and a significant increase in hydrogen peroxide and lipid peroxidation were found after 48 h C_{60} treatment. Also, acetylcholinesterase (AChE), a marker enzyme for the brain, showed a significant reduction after exposure to C_{60} at the end of 48-96 h. Thus, the work showed that the administration of C_{60} has adverse effects on the fish brain. The aqueous suspensions of C_{60} aggregates were studied on marine teleost *Fundulus heteroclitus* of an embryo, larvae, and adult stages. In natural seawater, the aggregates of C_{60} are mixed and precipitated in bottom water after 24 hours, resulting in very low mortality. No median lethal doses could be calculated at a concentration below 10 mg/l. In addition, even though the C_{60} aggregates were attached to chorion, no effect in the development of the embryos on hatching success was observed. With higher exposure levels, the movement of C_{60} from chorion into the embryo tended to increase together with a dose-dependent increase in GSH and a decrease in lipid peroxidation (LPO) [61]. Further, the C_{60} exposed to *Cyprinus carpio* (common carp) demonstrated no effect on viability, whereas hampered growth occurred after 3 h of exposure to several concentrations of C_{60} , which were 0.1, 1, and 10 mg/l. Also, higher antioxidant competence to peroxyl radicals was observed in this fullerene than in other reactive colonies [62, 63].

On the contrary, another study exposed three different chemical toxins, which were 6-hydroxydopamine, gentamicin, and cisplatin, to a whole animal system in order to

investigate the fullerenes' ability to protect the animal from the toxins. This model is useful to predict the toxicity and efficacy of this fullerene in mammals. When water-soluble fullerenes in both positive and negative charges were exposed to the zebrafish embryos at 1 and 500 μM for 24-120 hpf, the results indicated that the fullerenes could give protection against the toxins, which can induce apoptotic cell death in a vertebrate. Furthermore, this work suggested that the relative potential for these compounds' pharmacologic use varies significantly with respect to stability [63, 64]. Hence, more studies on different parameters of concentration, time, environmental factors, and vertebrate model are necessary to understand the toxic as well as beneficial properties of fullerene nanomaterials. We have compiled the results of different toxicity studies in Table 2.

5. Biodistribution and Fate of Fullerene after Ingestion by Aquatic Organisms

The biodistribution of fullerene on ingestion by aquatic organisms has been seldom addressed in the literature. Moreover, the concentration of fullerenes in many environmental matrices is still unknown, while quantification methods are under development [33, 65, 66]. However, NPs within cells may cause alterations in the cytoskeletal network [67, 68]. Waissi-Leinonen and colleagues depicted the agglomeration of fullerene C_{60} in the gut area and damaged the microvilli of *C. riparius* [33]. Furthermore, in another study by Sforzini et al. and Barranger et al., C_{60} was reported to be distributed in mussel digestive gland cells [36, 37]. In addition, the inhibition of mTOR might also be involved in pathophysiological perturbation induced by nanoparticle accumulation. Further, they stated that autophagic induction by fullerene C_{60} might reflect the degradation of unrecognized materials attempted by lysosomes. These materials might be identified by cells as damaged intracellular proteins and membranes or pathogens. The statement was made based on observations, where C_{60} accumulation in the lysosomal-vacuolar system of the epithelial cells is in the major digestive gland. Overall, the report suggested that dysregulation of mTOR 1 and 2 might inhibit the growth and reproduction of cells and organisms. Also, in a study, Pakarinen et al. and Oberdörster et al. reported that fullerene concentration in feces of *L. variegatus* was high in comparison to the bulk sediments [39, 40]. The study suggested that high fullerene concentration in the form of pellets might partially stem from worm's consumption and absorption of some sediment fraction for nutritional purposes, whereas the fullerenes and other particles are excreted. Further, Tao et al. and Rouse et al. exposed *D. magna* to 0, 0.01, 0.02, 0.04, 0.06, and 1.0 mg/l for seven days and the results showed an increment in body burden of C_{60} along with a higher dose and bigger particle size [69, 70]. Thus, the various papers and results suggest that 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 biodistribution is an important topic for future research.

6. Discussion Based on Current Understanding

The manufacturing of fullerenes to fit in for a specific task demands changes in their surface chemistries and properties. With the improvement according to demand, the fullerenes acquire 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 [65, 67, 71, 72]. Until now, the real effects associated with the interaction of fullerenes with the aquatic organisms are still lacking and remain challenging to analyze in the absence of relevant data.

The methods to evaluate the toxicity of fullerenes are evolving in recent years. The current understanding of the toxicity of fullerene must acknowledge 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 problem, it is highlighted that all significant effects on environmental fate, transport, and bioavailability of cocontaminants 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 ROS and free radical production are among the main mechanisms of nanotoxicity; in turn, they might lead to inflammation, oxidative stress, and consequent damage to proteins, membranes, and DNA [69, 73]. In a study, Oberdörster showed that fullerenes caused damage in fish brains [58]. Also, Fortner et al. and Howard demonstrated that fullerenes killed water fleas and showed bactericidal properties [66, 71]. Furthermore, Sayes et al. and Daughton stated that toxicity is a sensitive function of surface derivatization [68, 72], while Rouse et al. and Nel et al. reported that the extent of aggregation, emulsion bases, and different solvents are important variables in the formation of aggregates [70, 73].

The studies discussed here with fullerene toxicity response on invertebrate and vertebrate models depicted contradictory results. Although fullerene C_{60} 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. demonstrated the effect of C_{60} in relative growth patterns of *C. riparius* in a bell-shaped dose-response manner [34]. Rajasree et al. and Marques et al. showed that exposure of sonicated C_{60} resulted in varied mortality in different stages of *A. salina*, whereas the filtered solution of C_{60} upon exposure revealed increasing mortality with an increase in concentration [42, 43]. Similarly, Blickley and McClellan-Green and Letts et al. also suggested the nontoxic effect of water-stirred suspensions of C_{60} at a concentration up to 10 mg/l in *H. heteroclitus* at different life stages [61, 62].

Next, in another study, Tervonen et al. and Wang et al. mentioned the role of *D. magna* in carrying fullerene from one trophic level to another [46, 47]. Similarly, Pakarinen

TABLE 2: Fullerene-based nanomaterial toxicity in aquatic vertebrates.

Fullerene	Model organisms	Dosage and time	Toxic effect	LC50	Reference
C_{60} , C_{70} , $C_{60}(\text{OH})_{24}$	<i>Danio rerio</i> (embryo)	100 and 500 ppb for C_{60} and C_{70} and 500 to 5000 ppb for $C_{60}(\text{OH})_{24}$	Exposure to C_{60} induced both necrotic and apoptotic cell deaths in the embryo, while $C_{60}(\text{OH})_{24}$ induced an increase in embryonic cellular death. Results obtained suggest $C_{60}(\text{OH})_{24}$ is significantly less toxic than C_{60} .	C_{60}/C_{70} —200 ppb $C_{60}(\text{OH})_{24}$ —4000 ppb	[52]
C_{60}	<i>Danio rerio</i> (embryo)	100, 200, and 400 ppb 2, 6, 12 h	Concentrations of C_{60} decreased to levels not associated with mortality, <50 $\mu\text{g}/\text{l}$, 100% mortality results when the embryos were exposed to concentrations from 250 to 130 ppb.	130 ppb	[53]
nC_{60} , fullerol	<i>Danio rerio</i> (embryos)	nC_{60} —1.5 ppm $C_{60}(\text{OH})_{16-18}$ —50 ppm 96 hpf	nC_{60} at 1.5 ppm delayed the 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 the zebrafish embryos.	NA	[54]
Water-soluble fullerenes (1-12)	<i>Danio rerio</i> (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	[63, 64]
C_{60}	<i>Danio rerio</i> (adult)	1 and 2 ppm for 1 day	C_{60} exposure to adult zebrafish at low concentration induces multiple behavioral abnormalities.	NA	[56]
C_{70}	<i>Danio rerio</i> (adult)	0.5 and 1.5 ppm for 2 weeks	Toxicity and the alterations were observed in several neurobehavior parameters after zebrafish exposure to environmentally relevant amounts of C_{70} .	NA	[57]
nC_{60}	<i>Micropterus salmoides</i> (juveniles)	0.5 ppm and 1 ppm for 48 h	Increase in lipid peroxidation in the brains at 0.5 ppm and marginal depletion of glutathione (GSH) in the gills.	NA	[58]
C_{60}	<i>Anabas testudineus</i>	5 and 10 ppm for 60 days	Stress induced by fullerene C_{60} exposure provoked reproductive toxicity in the fish, <i>Anabas testudineus</i> .	96 h LC50—50 ppm	[59, 64]
C_{60}	<i>Anabas testudineus</i>	5 and 10 ppm for 96 h and 60 days	Sublethal concentrations of fullerene C_{60} have a toxic impact on fish <i>A. testudineus</i> by affecting normal physiology.	96 h LC50—50 ppm	[59, 60]

TABLE 2: Continued.

Fullerene	Model organisms	Dosage and time	Toxic effect	LC50	Reference
C ₆₀	<i>Pseudotroplus maculatus</i>	0.1 ppm for 96 h	Decrease in SOD, CAT, GSH reductase, AChE. Increase in hydrogen peroxide and lipid peroxidation.	NA	[60, 61]
C ₆₀	<i>Fundulus heteroclitus</i>	0, 1, 2.5, and 10 ppm for 96 h	Water-stirred suspensions of nC ₆₀ are not toxic to embryonic, larval, or adult stages of <i>F. heteroclitus</i> at concentrations up to 10 ppm.	NA	[61, 62]
C ₆₀	<i>Cyprinus carpio</i>	0.1, 1, and 10 ppm for 48 h	The results indicated that C ₆₀ affects bacterial communities that live in mucus secretions of common carp.	NA	[62, 63]

NA: not available.

et al. and Oberdörster et al. demonstrated the fullerenes transfer process from sediment to sediment surface by *L. variegatus* through feeding and egestion [39, 40]. This observation indicated that this transfer might potentially increase the fullerenes' bioavailability to epibenthic organisms, which might be more sensitive to the exposures and might further assist the transfer process in the food chain. Beuerle et al. and Sumi and Chitra stated that positively charged fullerene exhibited greater toxicity in the vertebrate model compared to negatively charged fullerene, which showed a dependency behavior on the structural features [63, 64].

7. Conclusions of Future Directions

Taken together, the toxicity of fullerenes to aquatic animals was clearly demonstrated. Since it has great medical implication potentials, it should be studied further in the future. We propose that techniques such as behavioral assay by phenomics, tissue distribution analysis by MASS spectrum, or isotope labeling for *in vivo* tracking should be used to study fullerene-induced toxicity in aquatic animals (i.e., specific biomarkers study, biodistribution, and interaction within an organism's body) to accumulate relevant data required to achieve a clear picture about fullerene-induced toxicity.

Data Availability

Data is available upon request to authors.

Disclosure

This paper has been submitted as a preprint in Research Square in the below link: <https://www.preprints.org/manuscript/202009.0376/v1>.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

N.M. and C.-D.H. are responsible for the conceptualization. T.-R.G., J.-S.L., and C.-D.H. are responsible for the funding acquisition. T.-R.G., J.-S.L., and C.-D.H. are responsible for the investigation. C.-D.H. is responsible for the project administration. A.L.C., G.A., P.S., J.M.S.R., and M.J.R. are responsible for the resources. T.-R.G. and C.-D.H. are responsible for the supervision. N.M. and C.-D.H. are responsible for the visualization. N.M., T.-R.G., J.-S.L., J.-R.C., and C.-D.H. are responsible for writing the original draft. All authors have read and agreed to the published version of the manuscript. Nemi Malhotra and Gilbert Audira are equal contribution authors.

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