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ORIGINAL ARTICLE

Impact of fullerene C_{60} on behavioral and hematological changes in the freshwater fish, *Anabas testudineus* (Bloch, 1792)

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Abstract
The unique physico-chemical properties of fullerene C_{60} have aided in its application in various fields along with several ecological impacts as an emerging pollutant. So, there is a significant need to demonstrate the ecological risk and toxic impact of fullerene C_{60} in the freshwater ecosystem. The present study focused on the toxic impact of fullerene C_{60} on behavioral and hematological changes in the freshwater fish, *Anabas testudineus*. The acute toxicity of fullerene C_{60} in *Anabas testudineus* determined by Probit analysis was found to be 50 mg/L. For the analysis, the fish were exposed to two sublethal concentrations of 5 mg/L (one-tenth of LC_{50}-96 h) and 10 mg/L (one-fifth of LC_{50}-96 h) for short-term (24, 48, 72 and 96 h) and long-term (7, 15, 30 and 60 days) durations. Fullerene C_{60} exposure showed prominent changes in behavior of the fish that comprises changes in swimming activity, disruption of schooling behavior, air engulping and surfacing along with morphological alterations such as descaling, slight hemorrhage and mucous secretion on the body surface, which was correlated to significant ($P < 0.05$) decrease in the activity of acetylcholinesterase enzyme in brain tissue. Hematological changes includes significant ($P < 0.05$) reduction in the erythrocyte and leukocyte counts, hemoglobin concentration, percentage of the packed cell volume (hematocrit) and the levels of serum albumin, globulin and total protein, whereas serum glucose concentration and the activities of the serum alanine and aspartate aminotransferases were significantly ($P < 0.05$) increased during the sublethal exposures. Severe degeneration in the columnar epithelial cells and lamina propria, along with increased number of mucous cells observed in the intestine of the fish indicates sublethal toxicity of fullerene C_{60}. The present findings led to the conclusion that the sublethal concentrations of fullerene C_{60} have a toxic impact on the fish *A. testudineus* by affecting the normal physiology, and thus the presence of this nanomaterial in the environment may affect the health status of the ecosystem.

Keywords Fullerene C_{60} · *Anabas testudineus* · Median lethal concentration (LC_{50}) · Behavior · Hematology

Introduction

Nanostructured materials are unique groups that have distinctive structural features with at least one dimension of 100 nm or less, which includes zero-dimensional quantum dots or nanoparticles, one-dimensional nanowires, nanotubes, nanorods, nanobelts, and nanoribbons, two-dimensional nanosheets, nanowalls, nanoprisms, nanoplates, and nanodisks and three-dimensional materials such as nanoballs, nanocoils, nanopillars, nanocones, and nanoflowers (Tiwari et al. 2014). Novel carbon-derived nanomaterials such as carbon nanotubes, graphene, crystalline diamond, and diamond-like carbon have gained significant attention in multiple disciplines including physics, chemistry and nanomedicines. One of the carbon nanomaterials, fullerene C_{60}, discovered by Kroto et al. (1985) is one of the most common and stable members in the fullerene family possessing unique properties including a large surface-to-volume ratio, high conductivity, and electron mobility at room temperature that distinguish it from other carbon nanomaterials. Fullerene C_{60} with a truncated icosahedron structure having 12 pentagons and 20 hexagons used its specific features to introduce promising new applications in the fields of material science (Coro et al. 2016), medicine (Pan et al. 2015), personal care products (Lens 2011) and solar cells (Mohajeri and Omidvar 2015). Biomedical applications of fullerene

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ranged from the treatment of the several diseases related to oxidative stress (Injac et al. 2013), neurodegenerative disorders (Dugan et al. 1997), cancer (Shi et al. 2014), HIV (Friedman et al. 1993) and influenza (Shoji et al. 2013).

Humans were exposed to fullerene at low concentrations mainly in geological materials dating back to 85 billion years (Becker et al. 1994). Fullerenes exist naturally in coal deposits (Fang et al. 2006), lava pillows (Jehlicka et al. 2000), shungites (Parthasarathy et al. 1998) and fulgurites (Daly et al. 1993). Recently, the presence of fullerenes in the environment increased drastically due to the anthropogenic activities mainly during emission from the working environment, transportation and use, at the time of disposal, and relatively small amounts of soluble and dispersed fullerenes emit into the water during the manufacture, depending on poor treatment facility (Farre et al. 2010). During sewage treatment, most of the fullerene particles are likely to form deposits, but soluble fullerenes are emitted into the aquatic environment (Dhawan et al. 2006). The release of nanomaterials into the aquatic environment is expected to form unstable colloidal suspensions. The stability of colloid under natural conditions depends upon various factors including pH, ionic strength and the presence of natural organic matter (Lead and Wilkinson 2006). Nanomaterials released into the environment is affected by several environmental factors such as light, oxidants or microorganisms that result in chemical or biological modification, degradation of the surface functionalization or the embedding matrix which may result in the formation of free nanoparticles (Nowack and Bucheli 2007). The potential mobility of nanomaterials under natural conditions depends on the surface modification, while the adsorption of nanomaterials to organic compounds depends on the dispersion state (Cheng et al. 2004).

Nanomaterials are able to cross the cell membrane and become internalized into a variety of mammalian cell types (Rothen-Rutishauser et al. 2006). One of the direct aquatic exposure routes of fullerene C_{60} is from the cosmetic wastewater effluents mainly released from the skin care products at a detectable range from 0.04 to 1.1 µg/g (Benn et al. 2011). Fullerene C_{60} and C_{70} were detected directly in 8 of 11 cosmetic sample products with the concentration of 10–340 ng/g (Zakaria et al. 2018). Fullerenes were noticed in trace amounts in the wastewater treatment plants with the highest concentration reaching µg/L in the suspended solids of effluents (Farre et al. 2010). Thus, domestic wastewater forms the main source that brings fullerene C_{60} into the aquatic environment and the estimated maximum concentration released from the skin care products is about 26 µg/kg body weight (Hansen et al. 2008). Noticeable concentrations of pristine fullerenes such as C_{60} and C_{70} were found in pond water at the range of 9–330 pg/L, while in sediments it ranged between 0.1 and 7.2 ng/Kg, whereas the concentration of fullerene C_{60} derivatives ranged from 1.5 to 8.5 pg/L in pond water samples (Astefanei et al. 2014). Besides these, certain hydrological conditions and heavy rains also contribute to the high concentration of nanomaterial contamination in the aquatic ecosystems.

Aggregation and size-dependent sedimentation are the main parameters that determine the uptake of nanomaterials into the body of the organisms (Limbach et al. 2005). Reports have suggested that fullerenes form stable nanoscale aggregates such as nano-C_{60} by the action of certain natural constituents such as dissolved natural organic matter found in the aquatic bodies (Xie et al. 2008). The suspended form of C_{60} fullerene aggregates exists in water bodies even for months at a concentration of 100 mg/L (Fortner et al. 2005). However, poor solubility and the propensity for aggregation of fullerenes in the water bodies limit the accessibility to living organisms to a certain extent (Brant et al. 2005). Usually, the uptake of nanomaterials occurs through endocytosis or phagocytosis in specialized cells where the coating of nanomaterials by protein in the growth medium causes conformational changes in the protein structure, which stimulates the uptake of nanomaterials into the cell by limiting it below 120 nm (Lynch et al. 2006). Nanomaterials are then stored within the vesicles or mitochondria of the cells to exert a toxic response.

Most of the toxicity studies have been carried out on aquatic organisms including fish (Oberdorster 2004; Oberdorster et al. 2006; Zhu et al. 2006), daphnids (Lovern and Klaper 2006; Lovern et al. 2007), bacteria (Fortner et al. 2005; Lyon et al. 2006; Lyon and Alvarez 2008) and aquatic invertebrates (Pakarinen et al. 2011; Waissi-Leinonen et al. 2015). Information available regarding the toxicity of fullerene C_{60} in fish population seems to depend on several factors that cause potential hazardous effects including generation of reactive oxygen species (Nel et al. 2006; Sumi and Chitra 2017c), cytotoxicity (Oberdorster et al. 2006), respiratory and cardiovascular diseases (Moore 2006), and carcinogenicity (Armstrong et al. 2004). Nanomaterials can be transferred from lowest to the highest trophic levels of the food chain and this has generated significant interest in aquatic toxicology, since fish are the key element of aquatic ecosystems that convey the reliable effects to other vertebrates including humans (Holbrook et al. 2008).

In a study on male Fischer rats, water-soluble fullerenes were reported to penetrate the blood–brain barrier, and 90% of the particles retained in the body for a long period (Yamago et al. 1995). Accumulation of fullerene in kidney, spleen and liver of rats indicated the translocation and accumulation of nanomaterials, which were enabled through the blood vascular system (Chen et al. 1998). A similar study reported that the penetration of fullerene C_{60} nanoparticles from the gastrointestinal tract of rats into the bloodstream occurs after intragastrical administration, which then translocated into liver, lungs, spleen and kidneys without showing

lethality in the exposed animal (Hendrickson et al. 2014). Rats exposed to C_{60} fullerene nano- and microparticles for 10 days showed alteration in hematological parameters along with a prominent increase in the pulmonary deposition of nanoparticles than the microparticles (Baker et al. 2008). However, oral administration of polyvinylpyrrolidone-wrapped fullerene C_{60} for 7 days in mice did not exhibit any effects in hematological parameters (Yamashita et al. 2013). Similarly, a dietary exposure of the rainbow trout fish, *Oncorhynchus mykiss* to fullerene C_{60} for 6 weeks showed no effect on growth or hematology (Fraser et al. 2011). A previous report from our laboratory showed that C_{60} fullerene induced oxidative imbalance in gonads and caused reproductive dysfunction in the freshwater fish, *Anabas testudineus* (Sumi and Chitra 2019). The available literature provides both safety and toxic effects of fullerene C_{60} in various animal models. Although there are several toxicological endpoints to detect the adverse effects of fullerene, studies on hematological parameters are used as a valuable diagnostic tool to assess the health and physiological status of an organism against the injury, lesion, deficit, stress and disease condition (Tavares-Dias and Moraes 2007). As there is lack of knowledge on the effect of fullerene C_{60} nanomaterial in the complete blood count and plasma chemistry profile of teleostian fish, the present study was conducted to address the gap of knowledge in behavioral and hematological parameters of the freshwater fish, *Anabas testudineus*. Blood profiling is considered as an essential tool in establishing the challenges exhibited by fish to overcome toxicant-related stress conditions. Therefore, the study performed provides new insights into the sublethal toxicity of fullerene C_{60} nanomaterials in the aquatic ecosystems.

Materials and methods

Model organism

Healthy freshwater fish, *Anabas testudineus* of weight 11 ± 1 g and length 8 ± 1 cm were collected from Pulimugham hatcheries, Alappuzha district, Kerala, India. Fish were transported to the laboratory in well-aerated polythene bags with least disturbance and acclimatized in the laboratory conditions for 2 weeks prior to the experiment. During acclimatization, fish were properly fed three times a day with standard fish food pellets and maintained in dechlorinated water under a photoperiod of 12 h light: 12 h dark and the health of fish was continuously monitored. The test tanks were covered with mono-filament netting to avoid the fish from jumping out of the experimental tanks. The preliminary tests were carried out in tap water following standardized procedures as prescribed by the APHA guidelines (1998) thus maintaining the water temperature at 28 ± 2 °C, pH 6.5–7.5 and oxygen saturation (70 and 100%) throughout the experiment durations.

Test chemicals

Fullerene C_{60} (purity > 98%, Product Number: 483036, CAS No. 99685-96-8) was purchased from Sigma-Aldrich, Germany and was dispersed in dimethyl sulfoxide (DMSO) (Himedia, CAS No. 67-68-5) by sonicating for 3 h to ensure an even size distribution. Previous studies reported from our laboratory (Asifa and Chitra 2017; Sumi and Chitra 2017c, 2019), and other studies reported in zebrafish in vivo (Usenko et al. 2007) and embryonic stem cells in vitro (Adler et al. 2006) have suggested that the use of 1% DMSO, as vehicle solvent, did not elicit toxic effects in exposed fishes, and therefore, that concentration was used in the present study. The concentration of nanomaterial selected in the present study was chosen to evaluate the sublethal effects of fullerene C_{60} in the fish model, and not to mimic the environmental concentration and its exposure scenario. All other chemicals used in the present study were of analytical grade, which were purchased from local commercial sources.

Characterization

Fullerene C_{60} nanomaterial was characterized by X-ray diffraction (XRD) with Cu Kα radiation exposed at 1.54 α wavelength, 40 kV and 30 mA current. The analysis was done using PanAlytical X'pert-PRO MRD diffractometer system, Eindhoven, Netherlands. Further, the average crystalline size was determined by Scherrer's formula, $D = 0.94\ k\lambda/\beta \cos\theta$, where D, K, λ, β and θ are the average crystal size, Scherrer coefficient (0.94), X-ray wavelength, Bragg's angle and the full width at half maximum in radian, respectively. The size and morphological nature of the fullerene C_{60} nanomaterial were determined using a high-resolution transmission electron microscope (JEOL-JEM-200 CX) having 0.23 nm point-to-point resolution, 0.14 nm lattice resolution and 2000 X–1500000 X magnification.

Acute toxicity test

Acute toxicity of fullerene C_{60} was tested preliminarily to find the median lethal concentration (semi-static; 96 h-LC_{50}) according to the guideline of OECD 203 (OECD 1992). Fish were stopped from feeding 24 h prior to the experiment to reduce the contamination of the test solution with excess food and feces. Different concentrations of the test chemical (20, 30, 40, 50, 60, 70 and 80 mg/L) were added to separate tanks having a capacity of 50 L holding ten healthy fish per tank maintaining triplicates under the same conditions. A control tank without toxicant and solvent, and vehicle tank with 1% DMSO were also maintained along with the test

tanks. The mortality and behavioral changes of fish from the controls and treatment tanks were recorded at every 24 h up to 96 h duration. The concentration at which 50% mortality of fish represent the median lethal concentration (96 h-LC_{50}) was analyzed using the Probit tool of regression analysis with a confidence limit of 5% level (Finney 1971).

Sublethal test

After estimating the median lethal concentration (50 mg/L), two sublethal concentrations, i.e., one-tenth (5 mg/L) and one-fifth of the LC_{50} (10 mg/L) were selected for further analysis and the fish were grouped as follows:

Group I: Negative control group (without solvent and toxicant).

Group II: Vehicle control group (1% DMSO).

Group III: Treatment groups.

Group IIIA: Fullerene C_{60} at 5 mg/L exposed for 24, 48, 72 and 96 h.

Group IIIB: Fullerene C_{60} at 5 mg/L exposed for 7, 15, 30 and 60 days.

Group IIIC: Fullerene C_{60} at 10 mg/L exposed for 24, 48, 72 and 96 h.

Group IIID: Fullerene C_{60} at 10 mg/L exposed for 7, 15, 30 and 60 days.

Monitoring of fish behavior

The behavioral modifications were monitored continuously for 30 min in each experimental tank at every 24 h interval up to 96 h in acute toxicity tests and short-term sublethal exposure groups. Similarly, the changes in the behavioral pattern were observed at 24 h interval for long-term sublethal exposure groups for 60 days. The observation time changed from morning to evening to avoid the changes in the behavior due to the diurnal fluctuations. The behavioral changes observed in the treatment groups were noted and compared with the control groups.

Histomorphology of intestine

After the end of the 96 h and 60 days of both sublethal treatment groups, fish were sacrificed and the intestines of the fish were dissected out, cleaned with physiological saline (0.9%) and finally fixed in buffered formalin (10%) for 24–48 h. The tissues were then dehydrated with ascending grades of alcohol, cleared in xylene and then dipped in molten paraffin wax for an hour for complete infiltration so as to prepare the tissue blocks. Serial sections were made using a rotary microtome with a thickness of 4–6 µm and the sections were double stained with haematoxylin and eosin, and finally mounted with DPX (Roberts and Smail 2001). The slides were observed under the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope and microphotographs were taken using a Canon shot camera fitted to the microscope.

Hematological and biochemical parameters

Fish were exposed to sublethal concentrations of fullerene C_{60} for short-term (24, 48, 72 and 96 h) and long-term (7, 15, 30 and 60 days) durations. At the end of every tenure of treatments, fish were gently removed from the experimental tanks for hematological and biochemical analyses. Anesthesia was not given to fish before collecting the blood samples, because it could affect the blood parameters and also hemolyze the tissues (McKnight 1966).

RBC and WBC counts

Blood was collected by cardiac puncture and the dilutions were made with appropriate diluting fluids such as Hayem's (for RBC) and Turk's fluid (for WBC) and immediately used for the estimation of erythrocyte and leukocyte count using the Neubauer counting chamber (Rusia and Sood 1992). The RBC and WBC counts were expressed as 10^6/cu. mm and 10^3/cu. mm, respectively, which were obtained by the given calculations:

$$\text{RBC count(million/cu. mm blood)} = \frac{\text{Number of RBC counted} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}},$$

where the dilution factor = 200, the area counted = 0.2 square mm and the depth of fluid = 0.1 mm.

$$\text{WBC count(1000/cu. mm blood)} = \frac{\text{Number of WBC counted} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}},$$

where the dilution factor = 20, the area counted = 4 square mm and the depth of fluid = 0.1 mm.

Hemoglobin content and packed cell volume

The hemoglobin content was measured by the cyanmethaemoglobin method according to Blaxhall and Daisley (1973). Briefly, 20 µL of blood was mixed thoroughly with 4 mL of Drabkin's reagent and kept for 10 min to allow complete conversion of hemoglobin into cyanmethaemoglobin, which was measured spectrophotometrically at 540 nm, and was expressed as g/dL. The packed cell volume was measured using a micro-haematocrit according to Hesser (1960), and was expressed in percentage (%).

Collection of blood serum

The blood sample collected from fish, without adding anticoagulant, was kept undisturbed for 30–60 min at room temperature to clot, which was then centrifuged at 1000*g* for 15 min to obtain the serum, and stored at − 80 °C until biochemical analysis were performed.

Total protein

The total protein was analyzed by Lowry et al. (1951) using bovine serum as standard. An aliquot of serum is mixed with 4.5 mL of alkaline copper reagent and kept at room temperature for 10 min. After incubation, the sample was mixed with 0.5 ml of Folin–Ciocalteau reagent and kept at room temperature for 20 min, then a blue color developed which was measured at 620 nm and the result was expressed as mg/mL.

Albumin and globulin

Albumin was estimated with the bromocresol green binding method described by Doumas et al. (1997). Briefly, 5 µL of sample was added to bromocresol green reagent (0.6 mM), and allowed to stand at room temperature for 10 min, and the absorbance was measured at 620 nm using UV–visible spectrophotometer. The concentration of serum albumin was expressed in mg/mL.

Serum globulin was calculated from the known value of protein and albumin concentration using the formula:

Serum globulin = serum total protein−serum albumin.

Glucose

Glucose was estimated according to Trinder (1969). Briefly, the serum sample was mixed with sodium sulfate–zinc sulfate reagent, centrifuged at 800*g* for 5 min, and the supernatant collected was mixed with 5 mL of glucose oxidase reagent. The reagent mixture was incubated at room temperature for 10 min, and absorbance was measured at 520 nm in UV–visible spectrophotometer and the unit was expressed in mg/dL.

Alanine aminotransferase

The activity of serum alanine aminotransferase was measured according to the method of Reitman and Frankel (1957). Briefly, the reaction mixture containing DL-α-alanine (0.2 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37 °C for 1 h. After incubation, 250 µL of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, sodium hydroxide (0.4 N) was added to stop the reaction, mixed and incubated at room temperature for 10 min. The absorbance was read at 510 nm against the blank. A standard calibration was prepared using different concentrations of sodium pyruvate. The results were expressed as µM pyruvate formed per mL.

Aspartate aminotransferase

The activity of aspartate aminotransferase was assayed by the method as described by Reitman and Frankel (1957). The reaction mixture containing aspartate (0.1 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37 °C for 1 h. After incubation, 250 µL of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, to stop the reaction, sodium hydroxide (0.1 N) was added, mixed and incubated at room temperature for 10 min. The absorbance was read at 510 nm against the blank. A standard calibration was prepared using different concentrations of sodium pyruvate and the results were expressed as µM glutamate formed/ml protein.

Acetylcholinesterase in brain

The supernatant of brain tissue homogenate (1% w/v) from all durations of both sublethal concentrations was used to analyze the activity of acetylcholinesterase enzyme (Ellman et al. 1961). Briefly, the supernatant of tissue homogenate in phosphate buffer (0.1 M, pH 8.0) was dissolved in sodium bicarbonate and dithiobisnitrobenzoic acid (DTNB; 0.01 M). The enzyme activity was calculated by measuring the yellow color indicator produced from thiocholine on reaction with dithiobisnitrobenzoate ion. The absorbance was measured at 405 nm against the reagent blank and the activity of enzyme was expressed as nmol acetylthiocholine hydrolysed/min/mg protein.

Statistical analysis

The median lethal concentration was analyzed by Probit regression analysis with 95% confidence limit, and then the correlation between the mortality versus concentration was plotted for the best fit line.

The hematological and biochemical data were analyzed using the statistical package SPSS 21.0 by one-way analysis of variance (ANOVA). The differences between the mean of control versus treatment groups were determined using the Duncan's multiple range as the post hoc test, in which the significance level was denoted in asterisk as $P < 0.05$ against the control groups. Data were expressed as mean ± standard deviation (SD) for ten fish in each group.

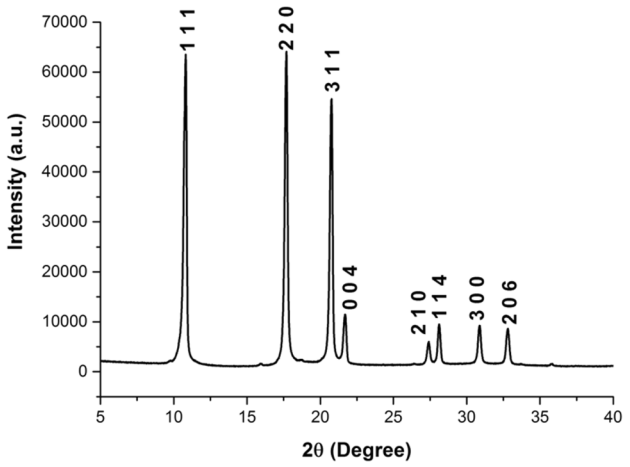

Fig. 1 XRD (PanAlytical X'pert-PRO MRD) image showing structural and crystalline nature of the powdered sample of fullerene C_{60} nanomaterials with particle size of 34.95 nm

Results

Characterization of fullerene C_{60} using X-ray diffraction (XRD) and high-resolution transmission electron microscope (HR-TEM)

The purity of fullerene C_{60}, obtained from the manufacturer was analyzed using the X-ray diffraction technique (Fig. 1). The face-centered cubic (FCC) lattice of the fullerene C_{60} demonstrated three distinct reflections showing diffraction peaks approximately at 10°, 17° and 20° angles corresponding to 111, 220 and 311 crystal planes of FCC, respectively. All the peaks obtained were distinct without any peaks for impurities, and the XRD results of fullerene C_{60} were analogous to that of the standard cards (JCPDS file No.44-0558). The size of the crystalline form of fullerene C_{60} confirmed by XRD was further estimated using the Scherrer's formula measuring the full width at half maximum (FWHM) of the Bragg's angle, and the average size calculated was 34.95 nm.

High-resolution transmission electron microscopy (HR-TEM) was used to analyze the size and morphological structure of fullerene C_{60} (Fig. 2). The average particle size obtained ranged between 30 and 60 nm, and the selected area electron diffraction (SAED) pattern of fullerene C_{60} used to identify the morphology of nanomaterial confirmed the crystalline structure, which was in agreement with the XRD results (Fig. 2).

Median lethal concentration (96 h-LC_{50}) of fullerene C_{60}

No mortality was observed in the control and vehicle control groups throughout the experiment durations. Exposure to fullerene C_{60} at 20 mg/L concentration did not show mortality for 96 h. Fish when exposed to 30, 40, 50 and 60 mg/L concentrations showed 10, 40, 50 and 60% mortality, respectively. When the concentration of fullerene C_{60} was increased to 70 and 80 mg/L, 80% mortality was shown at 96 h and 72 h, respectively (Table 1). The graph of concentration against mortality plotted using MS Excel showed a high degree of positive correlation, $r = +0.978$ (Fig. 3). The median lethal concentration, i.e., 96 h-LC_{50} of fullerene C_{60} calculated using Probit analysis was 50 mg/L in concentration. Two sublethal concentrations, i.e., 5 mg/L (one-tenth of 96 h-LC_{50}) and 10 mg/L (one-fifth of 96 h-LC_{50}) were chosen as sublethal concentrations to evaluate the toxicity of the nanomaterial.

Fullerene C_{60}-induced behavioral modifications

Fish exposed to fullerene C_{60} showed behavioral modifications in both the acute toxicity and sublethal tests when compared with the control groups. In the acute toxicity test, soon after fullerene C_{60} exposure, disruption was seen in the schooling behavior, while surfacing and air engulping increased. After 24 h of exposure, the fish showed vigorous swimming activity, banged with each other and also the walls of the treatment tanks, and the movement gradually declined leading to lethargy. Slight hemorrhage, increased mucous secretion and loss of equilibrium were also observed at the end of exposure period immediately before the mortality. The death of the fish was confirmed by the cessation of the opercular movements and the failure of animal to respond to gentle prodding with a glass rod.

Sublethal exposure groups also showed prominent behavioral modifications particularly in the long-term exposure groups. The main abnormalities included the fish preferring to stay at the bottom of the tank all the time but intermittently surfacing to engulp atmospheric air. The morphological changes observed were slight hemorrhage on the body surface, increased descaling and mucous secretion and interestingly, black aggregates were observed in the intestinal lumen of fullerene C_{60}-exposed fish when dissecting the animal (Fig. 4). However, no mortality was observed throughout the treatment period. Fullerene C_{60}-induced intestinal abnormalities were further confirmed by histological analysis and the changes in the behavioral pattern were compared for alteration in the activity of acetylcholinesterase enzyme in brain tissue of sublethal exposed groups and with the control groups.

Histopathological changes in the intestine of fish

The transverse section of intestine of fish obtained from the control and vehicle control groups showed normal histoarchitecture with serosa, muscularis, lamina propria and mucosal layer. Several villous occupied the mucosal layer

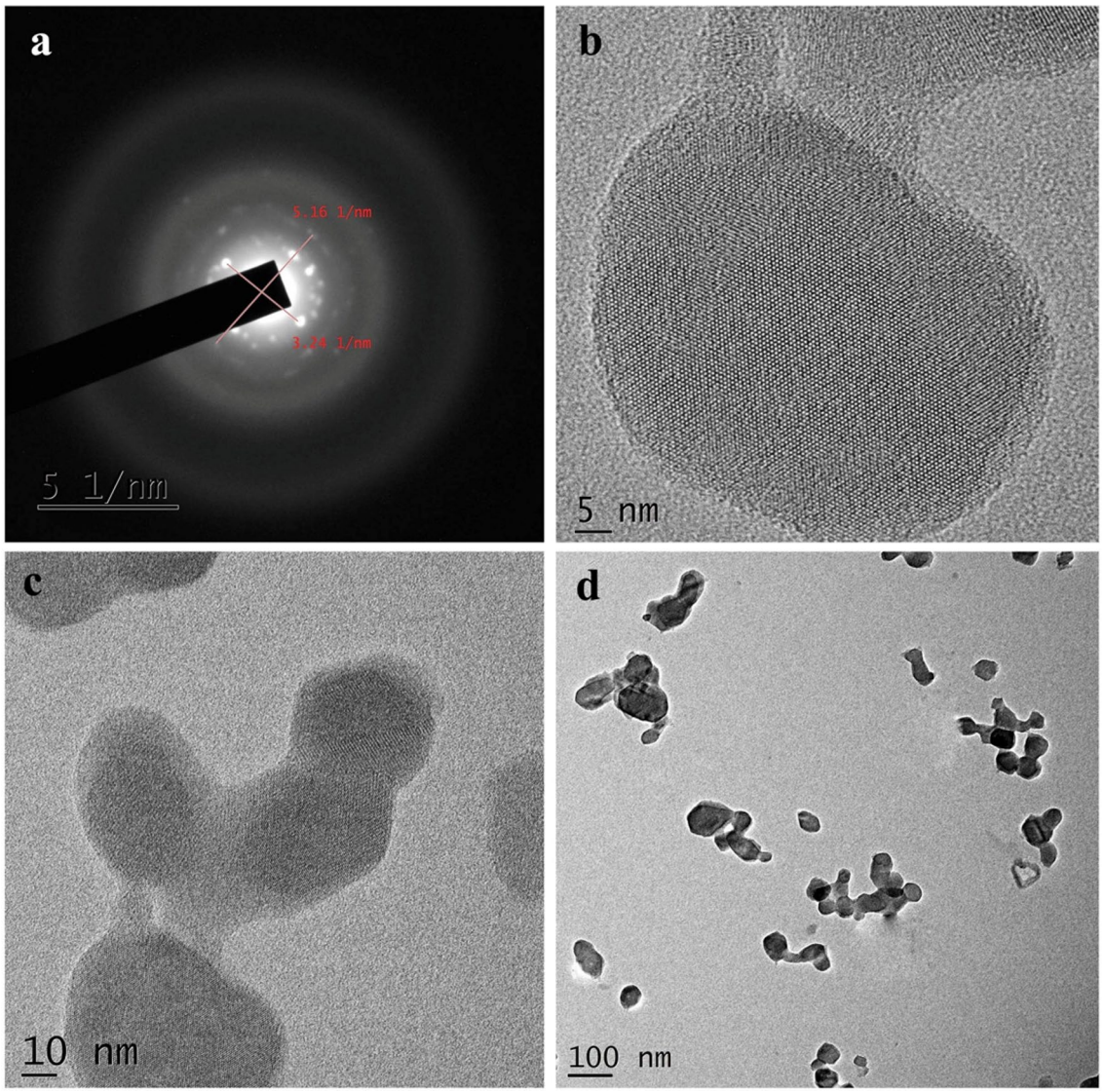

Fig. 2 **a** SAED pattern of fullerene C_{60} nanomaterial; b–d TEM image showing the morphology of fullerene C_{60} aggregates at various nanometers

Table 1 Effect of fullerene C_{60} on the mortality rate of the fish, *Anabas testudineus* exposed for 96 h ($n = 10$)

Concentration (mg/L)	Mortality (number of animals)	Mortality (%)	Hour of mortality (h)
Control	0	0	96
Solvent control	0	0	96
20	0	0	96
30	1	10	96
40	4	40	96
50	5	50	96
60	6	60	96
70	8	80	96
80	8	80	72

with the basal lining of tall columnar cells and centrally located nuclei. Mucous-secreting goblet cells were more prominent in continuity with the intestinal lumen (Fig. 5a). The intestine of fish exposed to sublethal concentrations of fullerene C_{60} for 96 h and 60 days showed significant alterations including severe degeneration of lamina propria, degeneration of columnar epithelial cells and increased number of prominent mucous cells (Fig. 5b). The severity observed in the treatment groups were found to increase with increase in the time and concentration of fullerene.

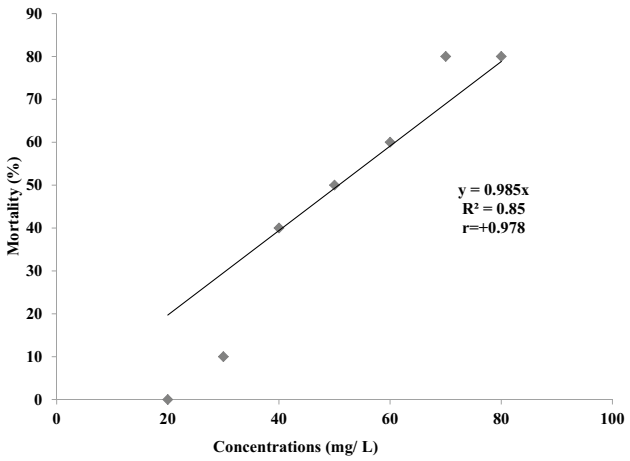

Fig. 3 Median lethal concentration or LC_{50}-96 h of fullerene C_{60} in the freshwater fish, *Anabas testudineus*

Alteration in the brain acetylcholinesterase enzyme activity after sublethal exposure to fullerene C_{60}

Exposure of fish to both sublethal concentrations of fullerene C_{60} in short-term treatment groups showed significant ($P < 0.05$) decrease in the activity of acetylcholinesterase enzyme only after 96 h in the brain tissue (Fig. 6). However, in the long-term exposure groups, there was a significant ($P < 0.05$) decrease in the activity of acetylcholinesterase enzyme in a time-dependent manner when compared to the control brain tissues (Fig. 6).

Hematological changes after sublethal exposure to fullerene C_{60}

Short-term exposure to both sublethal concentrations of fullerene C_{60} did not show any significant changes in the erythrocyte count, packed cell volume and hemoglobin content, except at 10 mg/L concentration exposed for 96 h (Figs. 7, 8, 9). However, long-term exposure to fullerene C_{60} showed a time-dependent significant ($P < 0.05$) reduction in the erythrocyte count, packed cell volume and hemoglobin content in a time-dependent manner (Figs. 7, 8, 9). Fullerene C_{60} exposure significantly ($P < 0.05$) decreased the leukocyte count after 96 h at 5 mg/L concentration, and after 72 h at 10 mg/L concentration in a time-dependent manner (Fig. 10).

Changes in serum biochemistry after sublethal exposure to fullerene C_{60}

Exposure to fullerene C_{60} at 5 and 10 mg/L concentrations showed significant ($P < 0.05$) reduction in serum total protein and serum albumin after 96 h of high sublethal concentration, i.e., 10 mg/L (Figs. 11 and 12). While the serum total protein and serum albumin showed significant ($P < 0.05$) reduction after 7 days at 5 mg/L, i.e., low sublethal concentration (Figs. 11 and 12). Serum globulin showed significant ($P < 0.05$) increase only after 96 h at 10 mg/L concentration, whereas a low sublethal concentration did not show any significant changes (Fig. 13). In the long-term exposure groups, there was a significant ($P < 0.05$) increase in the serum globulin after 7 and 15 days, while it declined significantly ($P < 0.05$) after 30 and 60 days of low sublethal exposure group (Fig. 13). Fullerene C_{60} exposed at 10 mg/L concentration showed a time-dependent significant ($P < 0.05$) reduction in the serum globulin of long-term exposure groups (Fig. 13). Exposure to fullerene C_{60} at 5 mg/L concentration showed a significant ($P < 0.05$) and time-dependent increase in the serum glucose after 96 h onwards, while at 10 mg/L concentration, the level of serum glucose was found to be increased significantly ($P < 0.05$) after 72 h in a time-dependent manner (Fig. 14). The activities of alanine and aspartate aminotransferases showed significant ($P < 0.05$) increase in a time-dependent manner in the long-term exposure groups at both sublethal concentrations (Figs. 15 and 16). However, no significant changes in the activities of enzymes were noted in short-term exposure groups other than a significant ($P < 0.05$) increase after 96 h of fullerene C_{60} exposure at 10 mg/L concentration (Figs. 15 and 16).

Discussion

The present study showed a negative impact of fullerene C_{60} on the behavior and hematology of the freshwater fish, *Anabas testudineus*. The unique physico-chemical properties of fullerene C_{60} inspired researchers in nanotechnology, and simultaneously, the proven toxic impact gained the attention of the nanotoxicologists. The anticipated production of nanomaterials on a large scale and their possible release into the environment has led to widespread concerns about the potential toxicity. Several literatures have stated that fullerenes have an impact on aquatic life such as algae, fungi, bacteria, aquatic invertebrates, fishes and other higher aquatic vertebrates, though relatively small effects were reported at environmentally relevant concentrations. In the natural environment, fullerenes are expected to settle in the bottom sediments even up to 100 mg/L concentration for months or end up in the lipids of aquatic organisms. The present study focused on the toxic effect of fullerene C_{60} in the fish, *Anabas testudineus* by applying a sublethal concentration rather than the environmental relevant concentration. The water solubility of fullerene C_{60} is very peculiar showing a dualistic character of insoluble property to water and other polar solvents having less than 10^{-9} mg/L solubility (Ruoff et al. 1993). However, fullerenes have a characteristic to transform

Fig. 4 Behavioral modifications in the fish, *Anabas testudineus* after fullerene C_{60} exposure

the poor solubility property by modifying as water-soluble colloidal clusters or agglomerates, either by vigorous stirring or sonication in the laboratory, or by natural processes like water flow and mixing in the natural environmental condition (Scharff et al. 2004). Fullerene C_{60} was subjected to sonication in the present study so as to improve homogeneity and stability of the nanosuspension. Homogeneity brings a much narrower particle size distribution, whereas stability prevents from bottom sediment settlement thereby ensuring uniform dispersion of fullerene C_{60} nanomaterial in the exposed experimental tanks.

Prior to the initiation of any toxicological experiments, it is always necessary to understand the characterization of the nanomaterial, since the particle size and structure influence the biological or toxicological effects. In the present study, fullerene C_{60} was characterized by the X-ray diffraction technique, and the size and morphological structure further confirmed using high-resolution transmission electron microscopy was found to be an average size of 34.95 nm, as compared to the manufacturer's data. The toxic nature of fullerene C_{60} nanomaterial was initially determined by the conventional acute toxicity test conducted for 96 h using

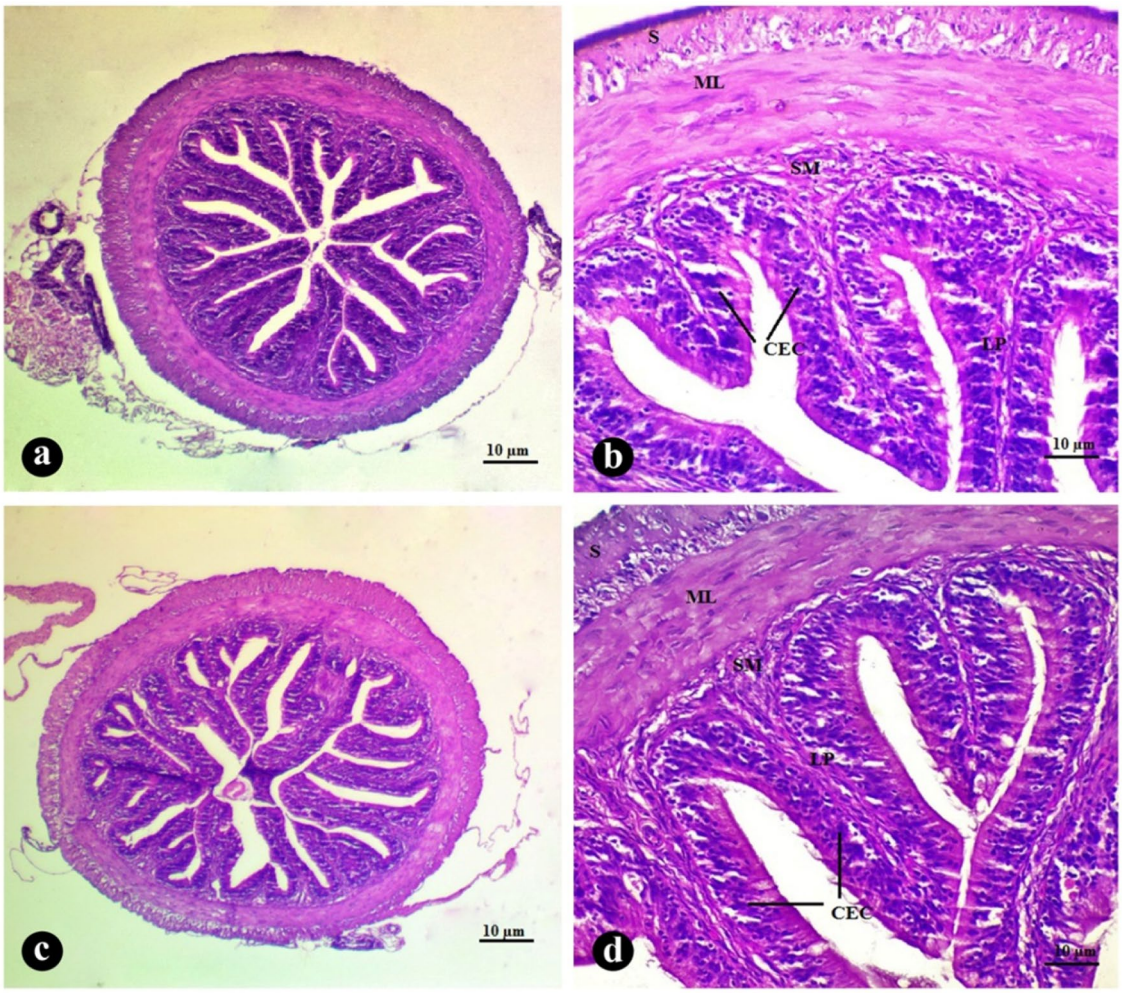

Fig. 5 Photomicrographs of the intestine of *Anabas testudineus* **a**–**d** (H&E). **a**, **b** control; **c**, **d** vehicle control (1% DMSO) showing normal histoarchitecture with serosa (S), muscularis (ML), submucosa (SM), mucosa with lamina propria (LP), columnar epithelial cells (CEC) and intestinal villi. **b** Photomicrographs of the intestine of *Anabas testudineus* exposed to fullerene C_{60} **e**–**l** (H&E). **e**, **f** 5 mg/L-C_{60} fullerene-exposed group for 96 h; **g**, **h** 10 mg/L-C_{60} fullerene-exposed group for 96 h; **i**, **j** 5 mg/L-C_{60} fullerene-exposed group for 60 days; **k**, **l** 10 mg/L-C_{60} fullerene-exposed group for 60 days. The treatment groups showing shortened and fused intestinal villi with disintegration of the submucosa (SM), lamina propria (LP) and columnar epithelial cells (→), increased number of gastric glands (G) and vacuole formation (V)

eight different concentrations of nanomaterial in a semi-static condition maintaining temperature, light intensity and other factors at a constant range. The median lethal concentration that killed 50% of the test animals was 50 mg/L, and the detected concentration was the expected level of concentration that could cause adverse effects in the field population. In another study, the acute toxicity of fullerene C_{60} was determined in *Daphnia magna* by preparing the test solution in two methods like sonication and dissolution in tetrahydrofuran and filtration. The study has reported that acute toxicity (48 h-LC_{50}) of filtered C_{60} in tetrahydrofuran was 460 ppb, whereas the acute toxicity of sonicated C_{60} was only at 7.9 ppm (Lovern and Klaper 2006). Similarly, exposure of tetrahydrofuran-nC_{60} at 0.5 ppm concentration in the adult male fathead minnow, *Pimephales promelas* showed 100% mortality within the initial 6–18 h, while the same concentration when exposed for 48 h after sonication did not show significant toxicity (Zhu et al. 2006) thereby proving the risk of using tetrahydrofuran as solvent. In the present study, dimethyl sulfoxide (DMSO; 1%) was used as vehicle solvent to prepare uniform suspension of the test solution by sonication and the results presented in this study showed no toxicity in the vehicle control group. Acute toxicity of aqueous suspension of fullerene C_{60} has also been reported in other aquatic organisms such as *Oryzias latipes*, *Daphnia magna* and *Pseudokirchneriella subcapitata* at greater than 2.15, 2.25 and 2.27 mg/L concentrations, respectively (Seki et al. 2008). The results of the reported studies were

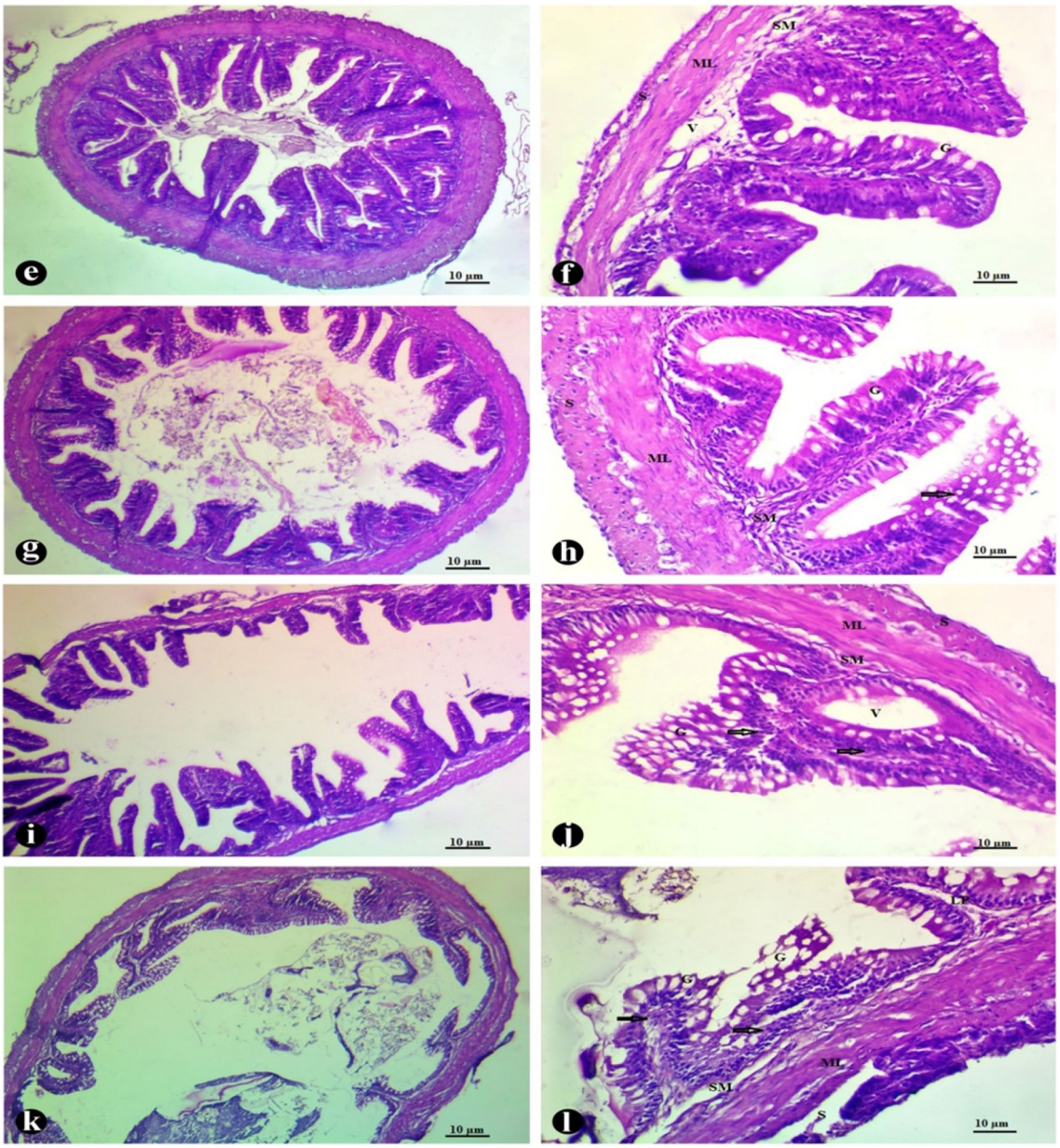

Fig. 5 (continued)

not comparable to the present observation, as the median lethal concentration of sonicated fullerene C_{60} was 50 mg/L, stating that either surface modification by sonication or less toxicity of DMSO could have contributed conflict of nanomaterial toxicity in the test organism, *Anabas testudineus*.

The behavior of an organism serves as a link between the ecology and physiology, hence the behavioral alterations can be used as an end point to study the adverse effects of toxicants. In the present investigation, the fish, *Anabas testudineus* were exposed to fullerene C_{60} which resulted in behavioral alterations including disruption of schooling, increased mucous secretion, descaling, increase in surfacing and altered swimming activity. The disruption in the schooling behavior was observed as the lethal and sublethal effects of nanomaterial which resulted in the change of normal swimming activity (Murthy 1987). Similarly, the increased surfacing behavior of fish during the long-term exposures of fullerene C_{60} might be due to treatment-related hypoxia and decreased oxygen level in the treatment tanks. Fish exposed to fullerene C_{60} also preferred to stay at the bottom of the tank for sometime, which was in agreement with another study when fathead minnow, *Pimephales promelas*, exposed to 20 ppm of hydroxylated fullerene by intraperitoneal injection showed lethargy, clustered schooling behavior and spent

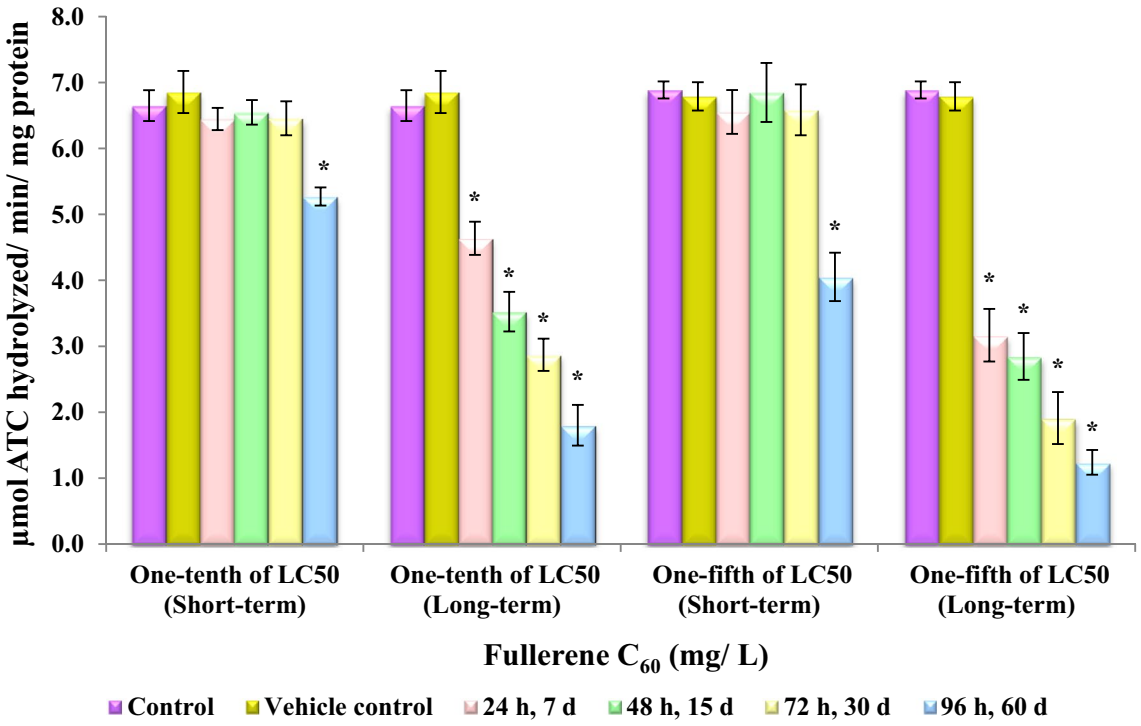

Fig. 6 Effect of fullerene C_{60} on the activity of acetylcholinesterase enzyme in brain of the fish, *Anabas testudineus*

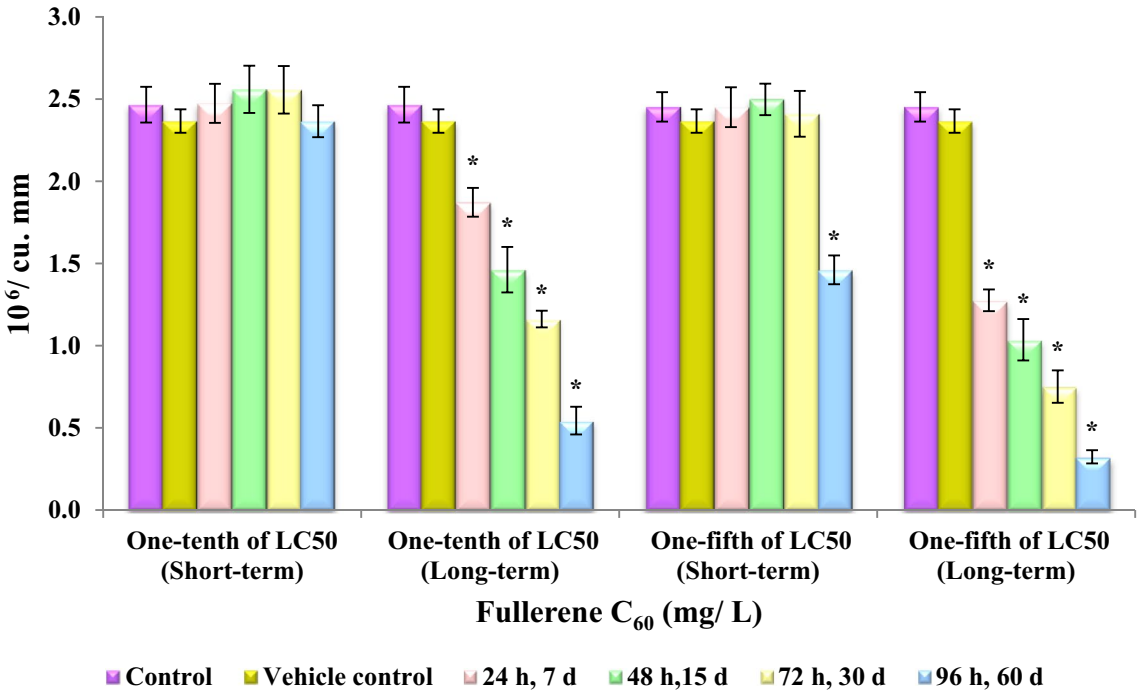

Fig. 7 Effect of fullerene C_{60} on erythrocyte count in the blood of the fish, *Anabas testudineus*

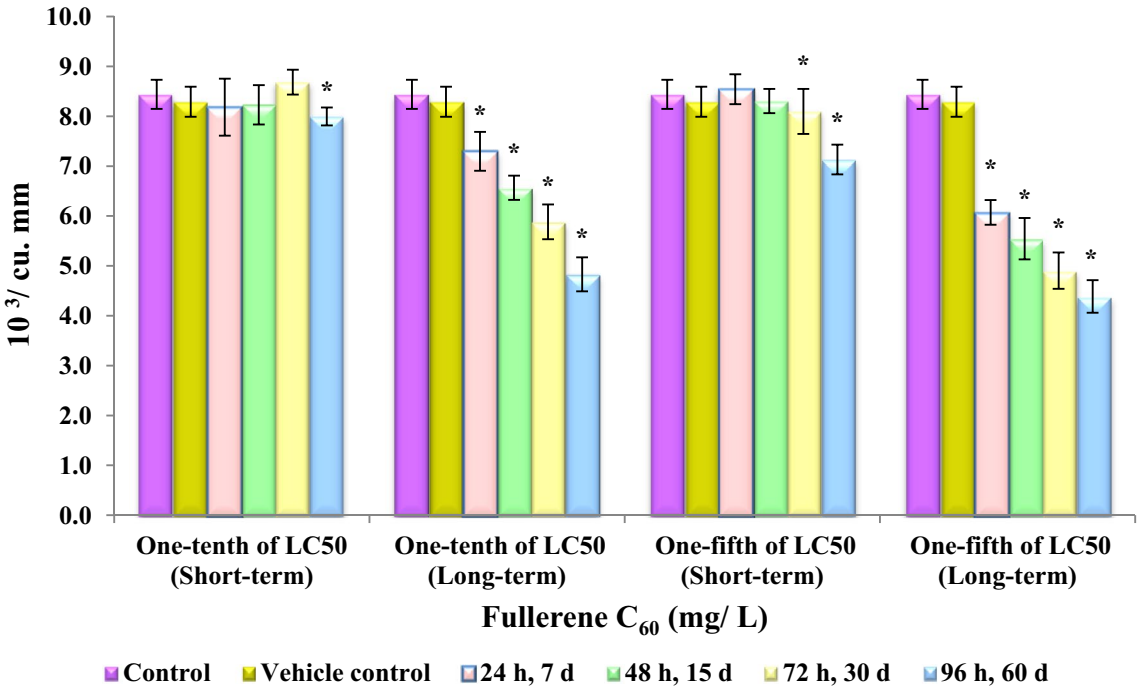

Fig. 8 Effect of fullerene C_{60} on the packed cell volume in the blood of the fish, *Anabas testudineus*

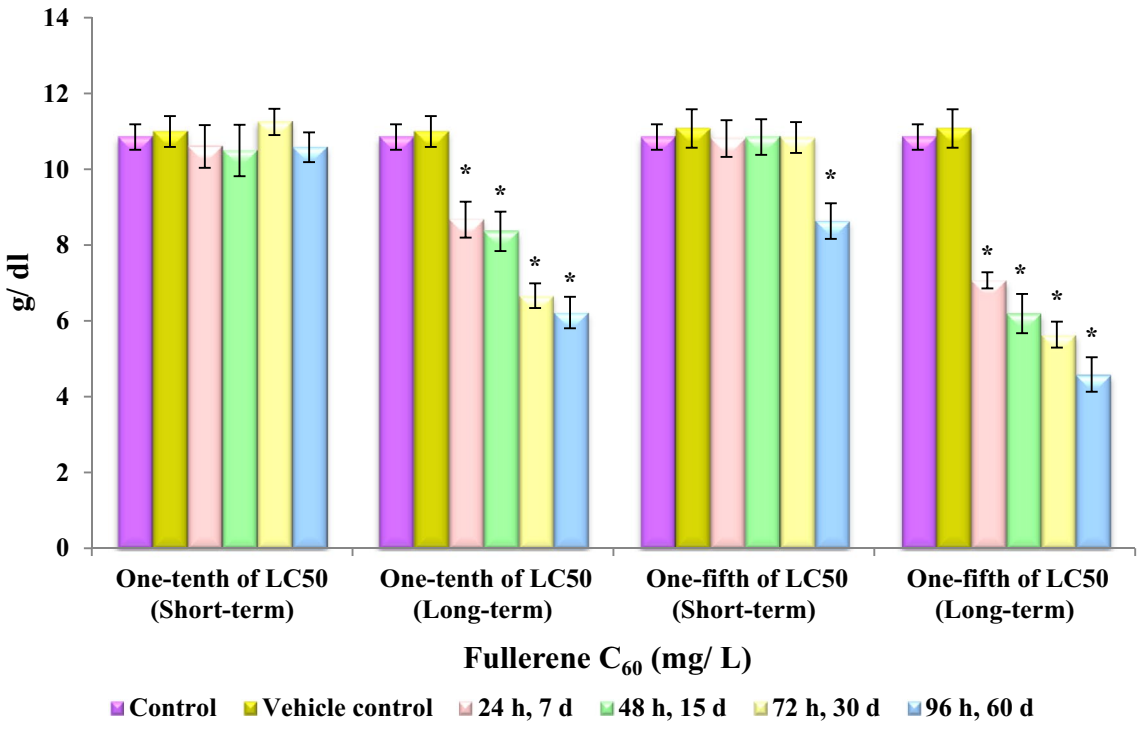

Fig. 9 Effect of fullerene C_{60} on hemoglobin content in the blood of the fish, *Anabas testudineus*

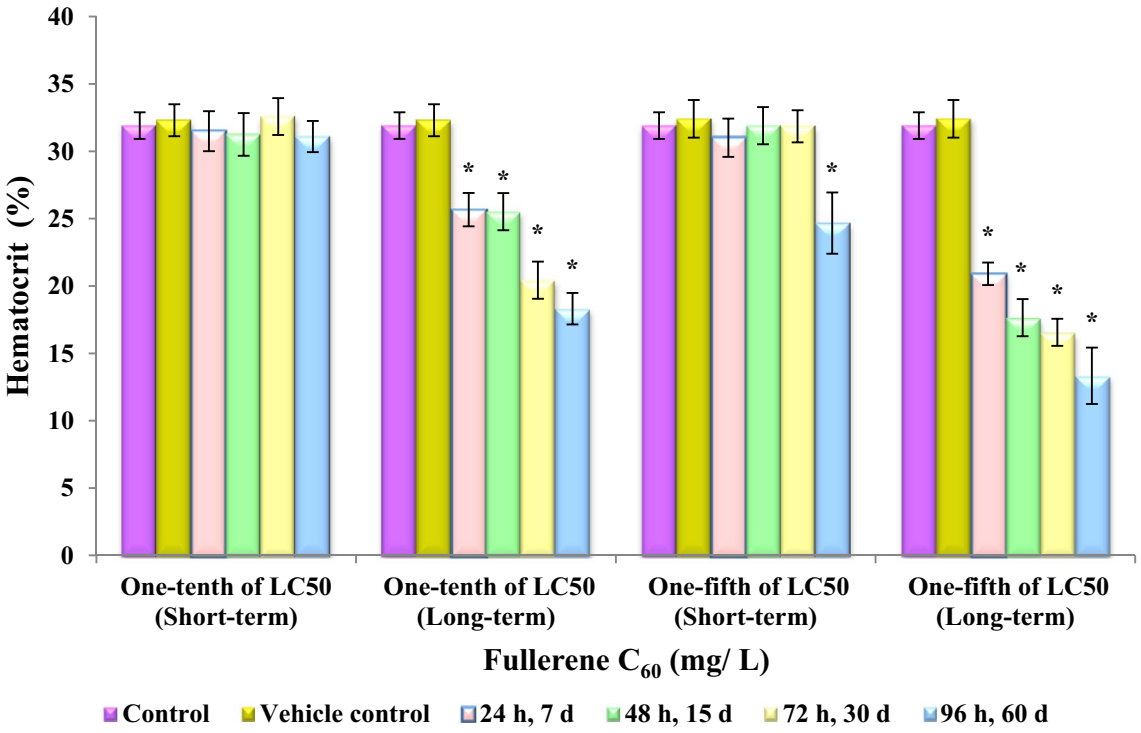

Fig. 10 Effect of fullerene C_{60} on leukocyte count in the blood of the fish, *Anabas testudineus*

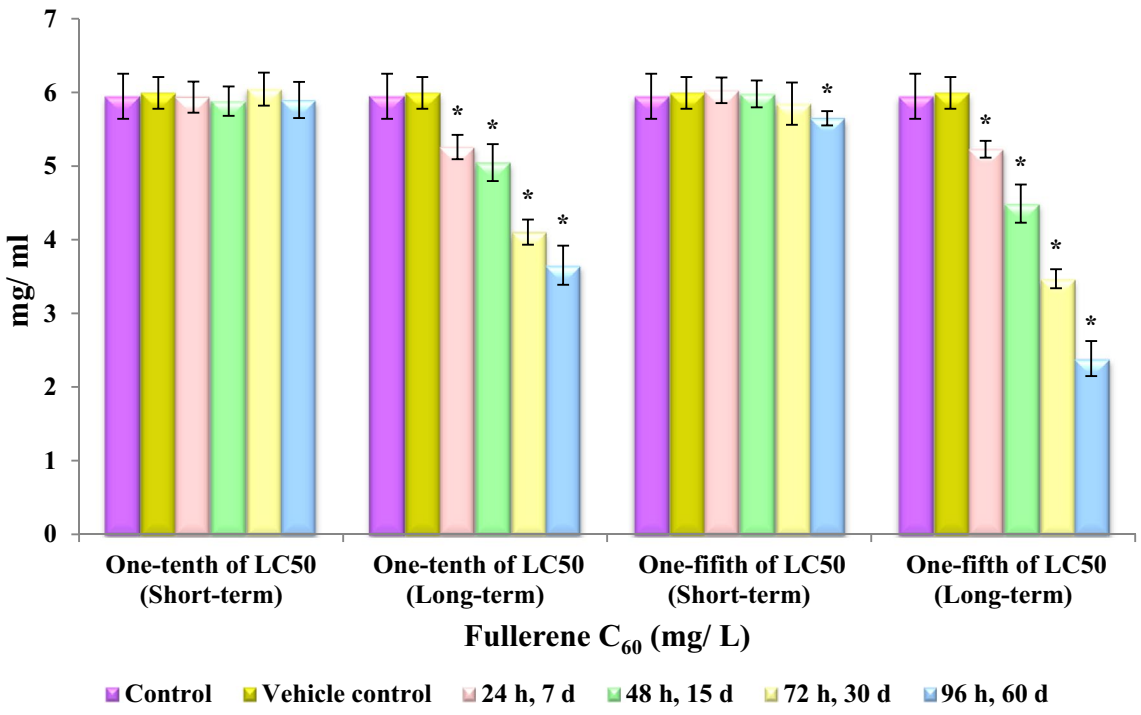

Fig. 11 Effect of fullerene C_{60} on the total protein in serum of the fish, *Anabas testudineus*

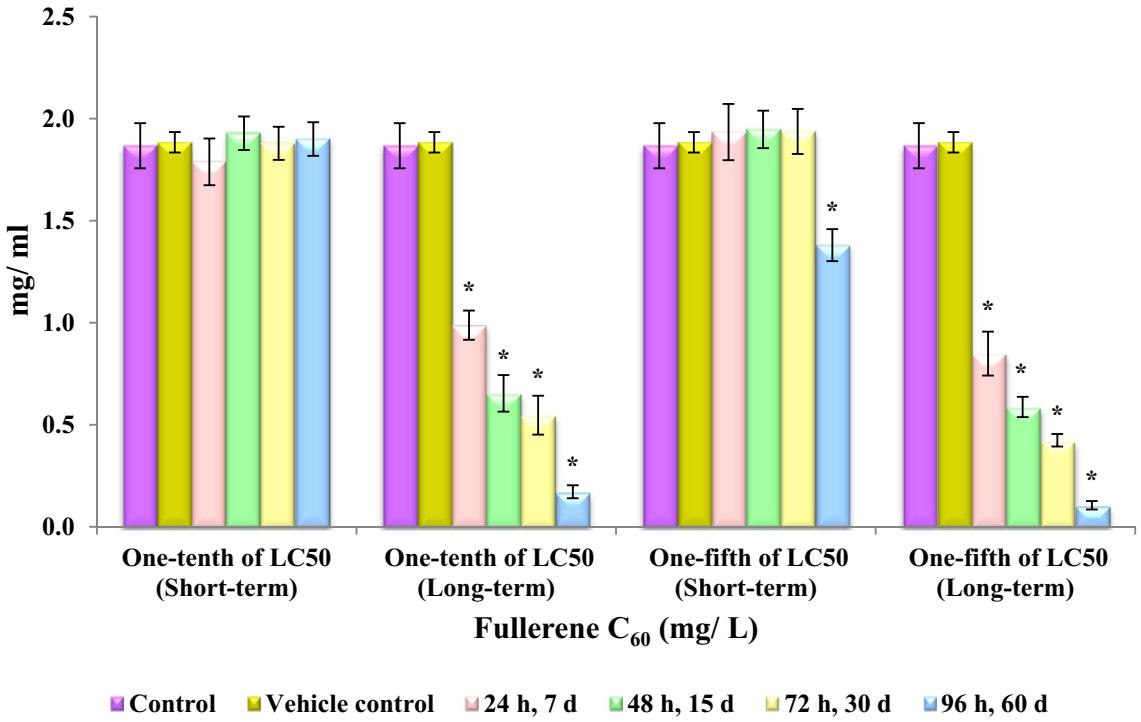

Fig. 12 Effect of fullerene C_{60} on the level of serum albumin in the fish, *Anabas testudineus*

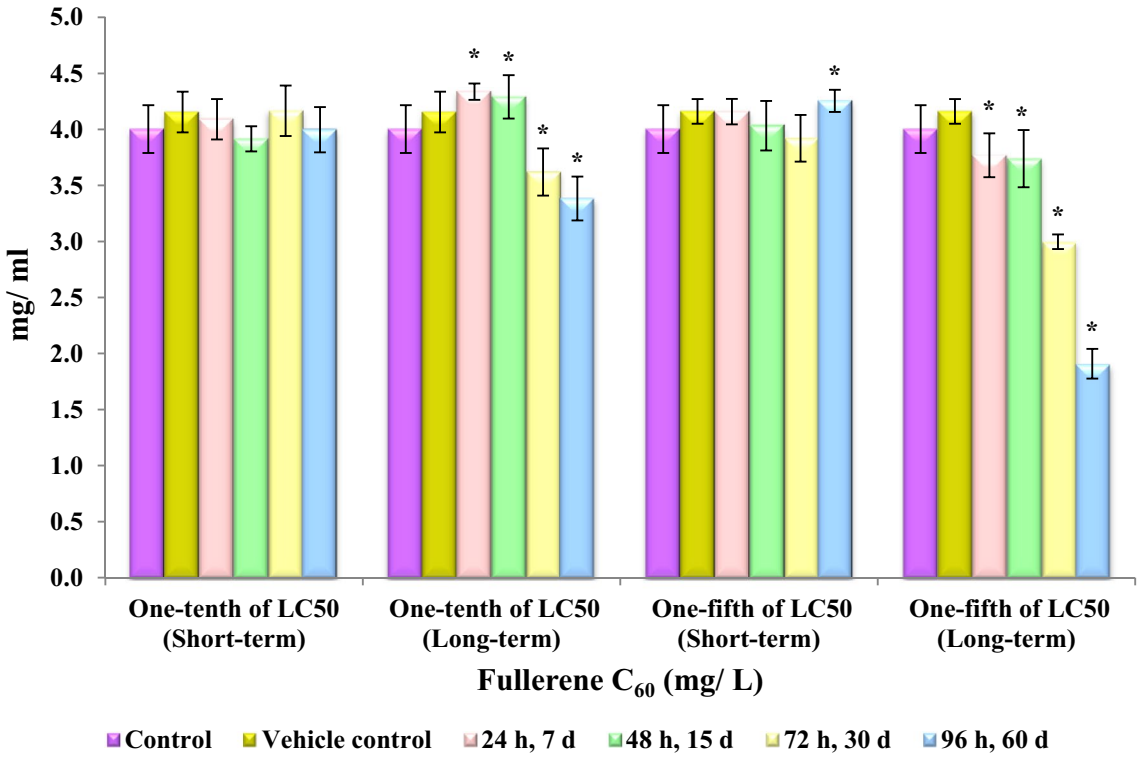

Fig. 13 Effect of fullerene C_{60} on the level of serum globulin in the fish, *Anabas testudineus*

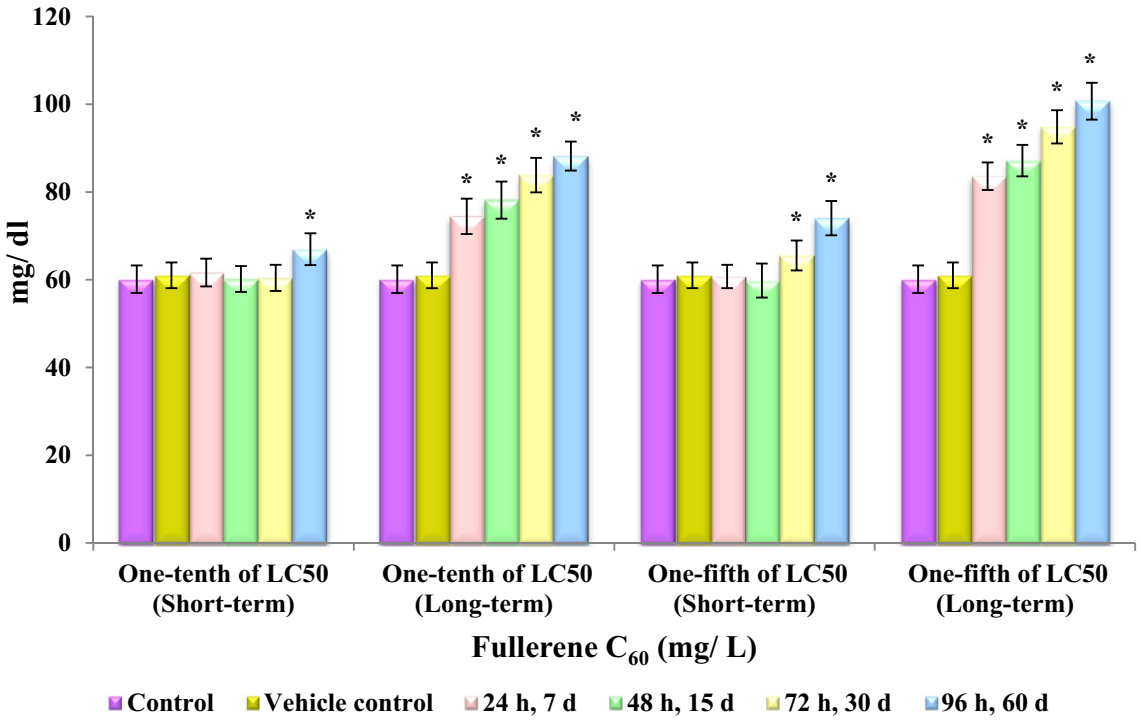

Fig. 14 Effect of fullerene C_{60} on the level of serum glucose in the fish, *Anabas testudineus*

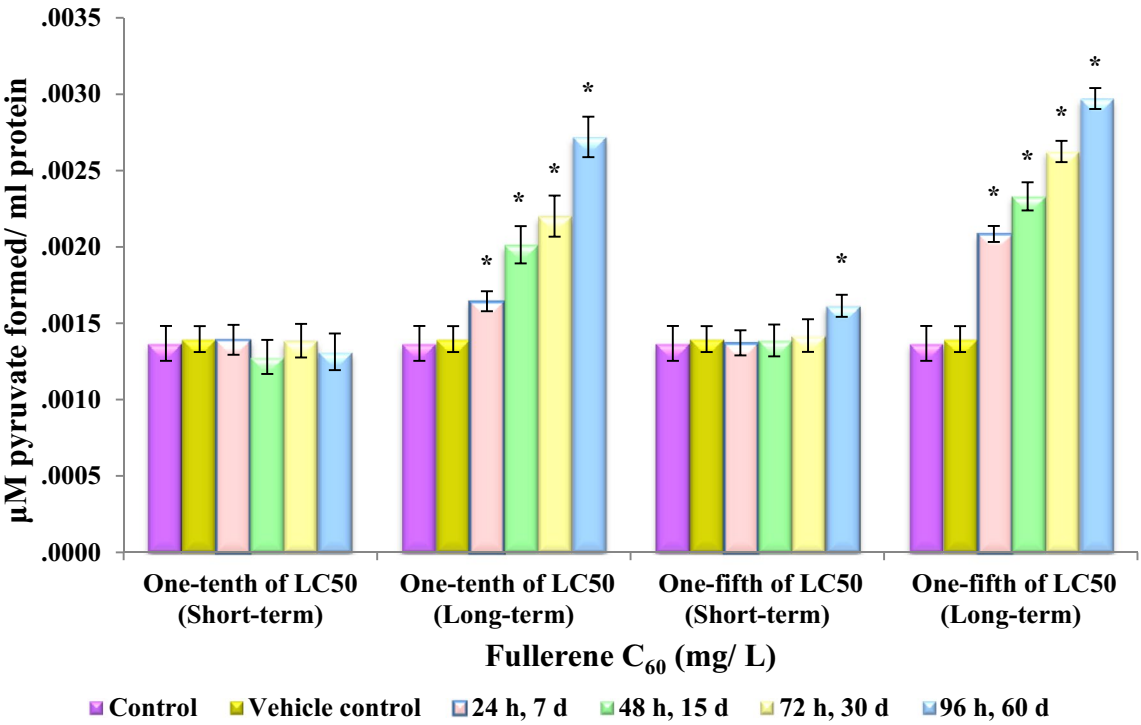

Fig. 15 Effect of fullerene C_{60} on the activity of alanine aminotransferase in serum of the fish, *Anabas testudineus*

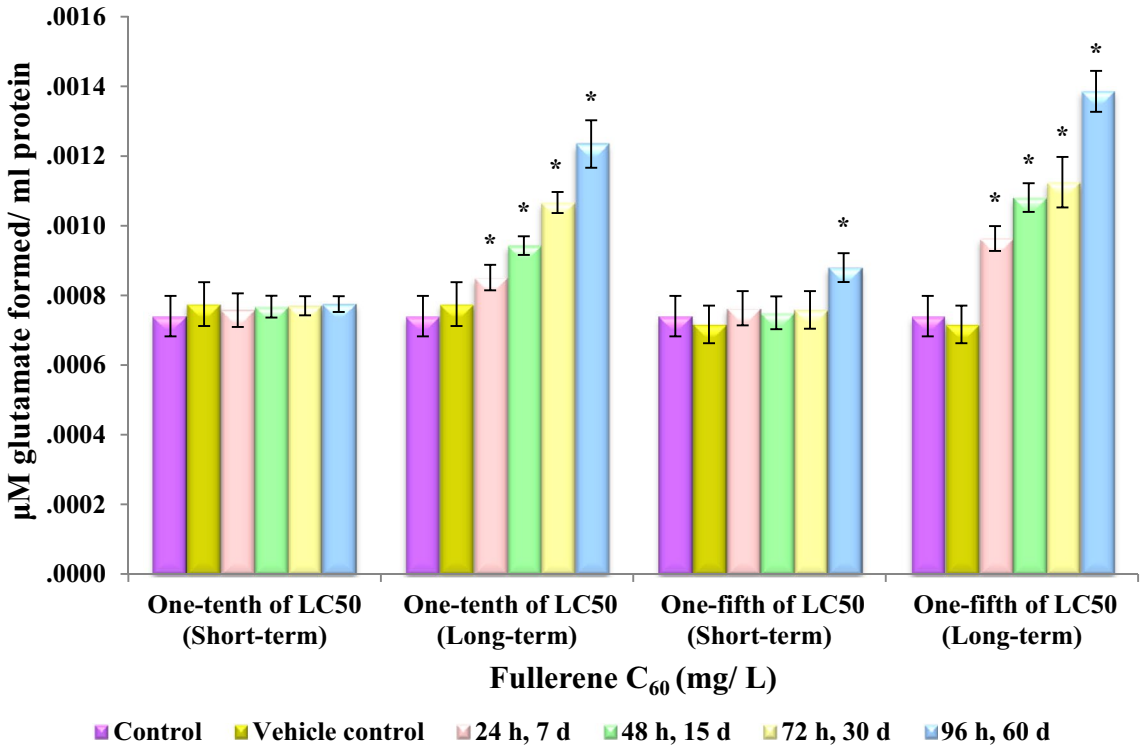

Fig. 16 Effect of fullerene C_{60} on the activity of aspartate aminotransferase in serum of the fish, *Anabas testudineus*

more time at the aquarium's bottom (Jovanovic et al. 2014). Exposure to C_{60} carbon nanoparticles at 1 and 2 mg/L concentrations in adult zebrafish showed behavioral alterations such as hypoactivity, reduced aggression or fear, anxiety and impairment in circadian rhythm (Sarasamma et al. 2018). Fullerene also induced behavioral changes in *Daphnia magna* as observed with increased hopping frequency, heart rate, appendage movement and curling of post-abdominal claw (Lovern et al. 2007). In another study, water-stirred C_{60} and sonicated carboxylic acid-functionalized fullerenes have been shown to alter swimming behaviors which seriously affected other vital behaviors such as predator avoidance and predation risk in *Daphnia magna* (Brausch et al. 2011).

Fullerene C_{60}-exposed fishes showed thick mucous deposition throughout the body surface, which is considered as a protective mechanism to avoid the absorption of nanomaterial through the skin. In addition, descaling was also observed after chronic exposure of the treated fish, and this could be due to the passage of lipophilic nanomaterial through the thick mucous of skin. It may ultimately lead to skin and muscle tissue damage in the fish as observed in our previous study in the fish, *Pseudetroplus maculatus* (Sumi and Chitra 2017a). In the present study brain acetylcholinesterase enzyme activity was used as a biomarker of neurotoxicity which was found to be associated with behavioral alterations. Chronic exposure of fullerene C_{60} inhibited acetylcholinesterase enzyme in a time-dependent manner and this could be the reason for the abnormal locomotion and loss of equilibrium of exposed fish. Accumulation of fullerene C_{60} in the form of black aggregates was observed in the intestinal lumen of exposed fish indicating adverse effects of nanomaterial on the histoarchitecture of intestine. Histopathological alterations such as degeneration in the columnar epithelial cells, lamina propria and stratum compactum along with increased number of mucous cells observed in this study further confirmed sublethal toxicity of fullerene C_{60}, which in turn could obstruct the intestinal absorption. The histomorphological changes were in agreement with another study on exposure to hydroxylated fullerene at 20 ppm concentration for 72 h has been shown to cause lesions in the intestinal lumen of fathead minnow, *Pimephales promelas* (Jovanovic et al. 2014). Accumulation of single walled nanotubes in the gut tract after 45 min and 1 h of exposure, which was followed by precipitated clumps of nanotubes after 20 h of exposure, has been reported in *Daphnia magna* (Roberts et al. 2007).

There arises a serious concern that after accumulation of C_{60} fullerene in intestine, it could cross the intestinal barriers to reach other vital organs through blood circulation, therefore, analysis of hematological parameters is the most significant aspect. Fullerene nanoparticles have been known to penetrate from the gastrointestinal tract into the bloodstream and translocate into secondary organs (Hendrickson et al. 2015). In the present study, hematological

alterations were examined in the fish, *Anabas testudineus* exposed to sublethal concentrations of fullerene C_{60}. A significant reduction in erythrocyte and leukocyte counts, hemoglobin content and packed cell volume observed in a time-dependent manner reflected the anemic state of fish as a result of fullerene C_{60}-induced haemolysis. Changes in the hematological profile indicated the stress condition of fish on exposure to fullerene nanomaterial, which could obviously affect the normal physiology and metabolism (Kaya et al. 2014). In a study, C_{60} nanoparticles prepared by solvent exchange method (nC_{60}THF) caused shrinkage, crenation and, eventually, hemolysis of human RBC and the erythrocyte damage that was mediated through oxidative stress (Trpkovic et al. 2010). Infiltration of C_{60} nanoparticle has reported to affect the mechanical properties of RBC membrane and weakened the tensile resistance of lipid bilayers in human RBC (Zhang et al. 2013).

The haemolytic property of fullerene C_{60} was further confirmed by serum biochemical analysis, which could reflect the physiological state of the fish, and hence was used as an important tool to diagnose the sublethal toxicity of nanomaterial. Fullerene C_{60} exposure at sublethal concentrations showed reduction in serum total protein, albumin and globulin with an increase in the level of glucose, which was concentration and time dependent. The decline in the levels of serum total protein and albumin could occur due to several reasons including protein degradation, utilization for the metabolic processes, inhibited hepatic synthesis of blood protein and the destruction of subcellular protein synthesizing structures (Fontana et al. 1998). The ability of nanoparticle to bind strongly with the protein may contribute to loss of protein function and also lead to protein denaturation (Roach et al. 2005). The binding affinity of one of the nanoparticles, carboxyfullerene with different proteins such as fullerene specific antibody, HIV protease, bovine serum albumin and human serum albumin has been documented using several docking models (Benyamini et al. 2006). The rise in the level of serum glucose observed in the present study indicated the release of glucose from stored glycogen owing to the toxicant-induced high energy demand. A similar observation was reported on inhalation of fullerene nanoparticles in the serum of rat (Baker et al. 2008). The activities of serum transaminases namely alanine and aspartate aminotransferases were recorded as the conventional indicator of liver damage in fish, exposed at two sublethal concentrations of fullerene C_{60} nanomaterial. Serum transaminases activities are found to be in a high level in heart, liver, skeletal muscle, kidney and erythrocytes and any damage to these organs raises the level of enzyme activities in blood serum (Zilva et al. 1992). The elevated activities of transaminase enzymes in the serum reflected membrane damage of any vital organs, which leads to extensive leakage of enzymes into the blood serum. The findings in the present study gain conformity with the subsequent tissue damage reported after fullerene C_{60} exposure in the freshwater fish, *Pseudetroplus maculatus* (Sumi and Chitra 2017b). The overall alteration in the hematological and biochemical parameters indicated toxic metabolic stress induced by fullerene C_{60} in the fish, *Anabas testudineus*.

Conclusions

The present findings proved that sublethal exposure to fullerene C_{60} resulted in adverse effects in the freshwater fish, *Anabas testudineus* as evident by the prominent behavioral, hematological and biochemical changes, and pathological alteration in intestinal tissue. The translocated fullerene from the intestine into blood elicited significant toxicity which advocated changes in the hematology and serum biochemistry of the fish. The release of fullerene C_{60} into the aquatic ecosystem even at a sublethal concentration could seriously affect the health and existence of the aquatic organisms. Therefore, the discharge of fullerene C_{60} from the manufacturing sites or from industries into the environment, especially in aquatic ecosystems, should be strictly monitored.

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