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Effects of stable aqueous fullerene nanocrystal (nC_{60}) on *Daphnia magna*: Evaluation of hop frequency and accumulations under different conditions

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Abstract

We investigated the effects of environmental factors and properties of water-stable crystal fullerene (nC_{60}) on the uptake of nC_{60} by *Daphnia magna* based on known accumulation in our laboratory. This study was performed for seven days using different environmental factors including temperature, pH, water hardness, concentration (density of particle), and particle size. Results demonstrated that body burden of C_{60} increased with time in all experiments. Body burden of C_{60} increased with increasing concentration and particle size, and uptake of particles >100 nm reached their maximums more quickly than those <100 nm. Under high hardness in aqueous systems with lower pH and high temperature, uptake was higher than those under opposite conditions. Uptake in all batch tests reached balance within five days. Both nC_{60} properties and environmental factors influenced uptake of nC_{60} by *D. magna* in an aqueous system. Additionally, environmental factors may have affected accumulation by changing nC_{60} properties, which are critical to understand the accumulation of fullerenes in aqueous systems.

Key words: C_{60}; fullerene; *Daphnia magna*; uptake; bioaccumulation

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Introduction

Fullerenes are used in various ways and fields, including as superconductives, in sports tools, and as catalysts (Kelty et al., 1991; Ruoff and Ruoff, 1991; Tang et al., 2010), and their potential uses are increasing. Most nanoparticle (including fullerene) run-off and waste ultimately enters aqueous systems as highly hydrophobic contaminants (Lynn et al., 2007). Fullerenes can form stable crystal colloids in aqueous solution (termed nC_{60}) in two ways (Andrievsky et al., 1995; Cheng et al., 2004). Although it is not found in high enough concentrations to cause acute or sublethal toxicity in natural aqueous systems, nC_{60} is a hydrophobic chemical that accumulates in organisms (Oberdöster et al., 2006) and causes toxic effects on *D. magna* reproduction and can accumulate even at low concentrations (Tao et al., 2009).

The ecological toxic effects of nanoparticles on many organisms in different aquatic trophic chain levels have been presented individually in previous researches, including on bacteria (Perdrial et al., 2008), algae (Baun et al., 2008), daphnia (Tao et al., 2009), and fish (Oberdörster, 2004). As a producer, algae adsorbed nanoparticle (Renault et al., 2008) onto its surfaces, and its photosynthesis (Baun et al., 2008), growth and viability (Hund-Rinke and Simon, 2006) were damaged. Furthermore, more toxic effects for algae were observed under the conditions of smaller size particles relatively. As a predator of algae, *D. magna* (Oberdöster et al., 2006; Tao et al., 2009; Zhu et al., 2009) could take up nanoparticles through the filter-feed system (Filella et al., 2008), and lethal effects of 50% for different nanoparticles were caused after exposure to 0.46 mg/L nC_{60} to *D. magna* (Lovern and Klaper, 2006), 100 mg/L TiO_2 to *D. magna* (Zhu et al., 2010), and more than 0.46 mg/L C_{60} derivatives to *D. magna* (Lovern et al., 2007). As a higher trophic predator, fish was induced lipid peroxidation (LPO) in the brain, decreased glutathione level in gill of Largemouth Bass juvenile (0.5 mg/L nC_{60}) (Oberdörster, 2004), inhibited the growth of juvenile carp (1 mg/L nC_{60}) (Zhu et al., 2008) and caused fathead minnow (1 mg/L nC_{60}) to die within 18 hr (Zhu et al., 2006). The study of medaka (fish) on nanoparticles accumulation (Kashiwada, 2006) indicated that nanoparticles could be absorbed into the chlorion and taken up into eggs oil droplet, some shifted into yolk and gallbladder with

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the development of embryos; and adult fish were mainly accumulated the nanoparticles in the gills and intestine.

In sublethal concentrations, as typical wide-spread plankton in aqueous system that change significantly from north to south, *D. magna* is sensitive to environmental contaminants (Lopes et al., 2004). Physical behaviors including hops, heart-beat, and appendage movement of *D. magna* have been used as indicators of nanoparticle toxicity (Lovern et al., 2007). Hops, in particular, are important behaviors for escaping danger and responding to toxic environments in sublethal concentrations (Seely and Lutnesky, 1998; Lopes et al., 2004; Baillieul and Blust, 1999). In this experiment, *D. magna* hops were examined for the influence of nC_{60} accumulation.

In the subtle assay of the nanomaterial potential, accumulation is a key factor because uptake condenses the hydrophobic nanoparticles in aqueous organisms from low-concentration water and shows the toxic effects over a period of exposure, and might even cause biomagnifications (Jafvert and Kulkarni, 2008). Bioaccumulation is affected not only by the properties of chemical and biological species, but also by environmental conditions of the aqueous system according to the bioaccumulation assumption (Hamelink et al., 1977). Previous research has suggested that the physical and chemical properties of nanocarriers, such as size, shape, and surface fictionalizations, influence uptake and bio-distribution (i.e., passing through endothelial and epithelial barriers), target-ability (either passively by size control or actively by attaching targeting moieties to the surface of carrier), and bioactive agent release (by control of pore size within the carrier) (McNeil, 2005). In addition, different environmental factors change nanoparticle (Fortner et al., 2005) and physiological properties (Black et al., 1991) of aqueous organisms. While the uptake of nC_{60} has been studied in restrictive laboratory conditions (Baun et al., 2002; Oberdörster et al., 2006; Petersen et al., 2009; Tervonen et al., 2010), little is known about uptakes under different environmental factors.

Long-term sublethal toxicity has been studied in our laboratory (Tao et al., 2009), however, short time sublethal toxicity and accumulation under different conditions is still largely unknown. We selected *D. magna* to study sublethal toxic response and subsequent experiments on the uptakes of nC_{60} under properties of nC_{60} and aqueous system factors. Specifically, concentration, particle-size, water hardness, pH, and temperature were used to study the effect of different conditions on the uptake of nC_{60}. In addition, a first-order, two-compartment equation was used to show accumulation trend.

1 Materials and methods

1.1 Chemicals and materials

The C_{60} (99.9% purified through sublimation) was purchased from the Materials Electronics Research Corporation (USA). Tertrahydrofuran (THF) (spectroanalyzed, > 99.99%) and toluene (HPLC, 99.9%) were obtained from Fisher Scientific (USA). The *D. magna* was obtained from the Carolina Biological Supply Company (USA). The water used was ultra-purified to >18 Ω (Millipore® Synergy system).

1.2 Preparation and size of nano-C_{60}

Preparation of nC_{60} and different particle sizes followed Fortner et al. (2005). The THF and its oxides were removed by YM 20000 membrane under liquid nitrogen pressure and replicated ten times (Fortner et al., 2005). Residual organic concentrations were below the detection limit (< 1 μg/L) of a GC-MS (Agilent 6890/5793 GC/MS equipped with a HP-5MS (a 30 m × 0.25 mm i.d. column) (Zhang et al., 2009). The *Z*-size of nC_{60} was 98 nm (Zetasizer Nano, Malvern Instruments Ltd., USA).

1.3 Culture of *D. magna*

D. magna were kept according to standard operating procedures of US EPA (2004) in a (21 ± 1)°C constant chamber with 16 hr light and 8 hr dark cycle. Dissolved oxygen was 6 mg/L and pH was 6.5–7.5. The *D. magna* were fed on *Paramecium caudatum*, *Enterobacter aerogenes*, and *Chlamydomonas* sp., and they were used in the following experiments for seven days after hatching.

1.4 Precondition of plastic vials

Plastic liquid scintillation vials of 20 mL were prepared by immersing in de-ionic water for six days, with water changed every two days. Then, the bottles were washed and dried in an oven at 80°C for using.

1.5 Sublethal effect on hop frequency

D. magna were exposed to 0, 0.01, 0.02, 0.04, 0.06, and 0.1 mg/L (sublethal concentration was 0.1 mg/L (Tao et al., 2009)) of 10 mL nC_{60} in a disposable dish. After exposure for 60 min, hops of *D. magna* (hops/min) were observed and counted in an optical anatomic microscope for 10 min. The animal was allowed to acclimate for at least 1 hr prior to testing (Wong et al., 1983). C_{60} in all experimental bugs were measured as the following method (Section 1.6). The correlation between hops and uptakes was analyzed by Original 8.0.

1.6 Measurement of C_{60} in daphnids

The C_{60} in daphnids was measured as per previous research (Tao et al., 2009). In detail, three daphnids in each vial were rinsed with 50 mL ultra purified water for 20 min, manually homogenized with 2 mL purified water for 10 min, and then vigorously cyclic shaken for 30 min after adding 0.3 mL of regular bleach and 0.7 mL of 0.1 mol/L $Mg(ClO_4)_2$. Approximately 2 mL toluene was then added to extract C_{60} from the tissue and shaken for 10 hr. The mixture of toluene and other liquids was separated by freezing the water portion under –20°C. The C_{60} with toluene was measured with an ultraviolet-visible spectrophotometer at 337 nm. Wet weight of mother daphnids was measured directly. All the data of accumulations got after minus background and were shown as mg per kg wet weight tissue.

1.7 Effects of different conditions on uptake

1.7.1 Uptake of common conditions and model of accumulation

D. magna were exposed to 0.1 mg/L (100 nm) nC_{60} concentration, under the conditions of pH 7.0, medium hardness, and 21°C. The experimental procedures were: 63 daphnids at each temperature level were distributed evenly into 21 vials. Each vial contained three daphnids and 10 mL of C_{60} medium. Three vials at each temperature level were selected randomly every day and the C_{60} concentrations in daphnids of each vial were measured. All remaining daphnids were picked out of the nC_{60} medium and cultured in original medium for 2 hr every day. Experimental (nC_{60}) medium in the vials were refreshed daily. To investigate uptake rate coefficients and maximums under certain conditions, a first-order, two compartment equation was used (Zhang et al., 2007).

1.7.2 Factor-dependent uptake

D. magna were exposed to various solutions of differing nC_{60} concentrations (0.01, 0.02, 0.04, 0.06, and 0.10 mg/L), as well as differing nC_{60} particle sizes (80, 90, 125, and 284 nm) (Fortner et al., 2005) at a concentration of 0.1 mg/L to study the concentration and size effects of nC_{60}, respectively. Experimental procedures were the same as in Section 1.7.1.

To study the hardness effect, *D. magna* were exposed to 0.1 mg/L nC_{60} solutions using various water hardness of ultra purified water (PW), very soft hardness (VS), soft hardness (S), medium hardness (M), hardness (H), and very hardness (VH) (APHA, 1985) at room temperature. In this experiment, ionic strength (I) of VH, H, M, S, VS and PW were $0.01608I$, $0.00804I$, $0.00402I$, $0.00201I$, $0.000503I$ and $0I$, respectively. The nC_{60} concentration in all hardness solutions was maintained constant for seven days at 21°C. All experimental daphnids were exposed to nC_{60} solutions with various levels of hardness for three days before experiments. Experimental procedures were the same as in Section 1.7.1.

The effect of pH was conducted by exposing *D. magna* to 0.1 mg/L nC_{60} solutions of differing pH levels (6.5, 7.0, 7.5, 8.0, and 8.5) adjusted by 0.001 mol/L NaOH. All experimental daphnids were exposed to the different pH solutions for three days before experiment. Experimental procedures were the same as in Section 1.7.1.

For understanding temperature effect, *D. magna* were exposed to 0.1 mg/L of nC_{60} at different temperatures (18, 21, 24, and 27°C). All experimental daphnids were reproduced in their experimental temperature to acclimate. Experimental procedures were the same as in Section 1.7.1.

1.8 Analysis

The relationship between sublethal hops frequency and uptakes of *D. magna* was analyzed by linear equation. All uptake velocities under the different conditions over three days were also linearly regressed with time.

2 Results

2.1 Sublethal hop frequency and body-burden

Results showed that nC_{60} increased hopping frequency (Fig. 1a), with hops showing a positive relationship with uptake of C_{60} (Fig. 1b). Hops of *D. magna* were (64.33 ± 2.73) per min, and increased by an average of 0, 17, 26.33, 45, 73, and 123.17 per min after exposure to 0, 0.01, 0.02, 0.04, 0.06, and 0.1 mg/L nC_{60}, respectively. Uptake of C_{60} showed a good relationship to increasing hops, with the linear regression equation as follows (Eq. (1)).

$$y = a + bx \qquad R^2 = 0.9857 \tag{1}$$

where, y (mg/kg) is the uptake of C_{60}; x (times/min) is the increasing hops; a is the intercept, with a value of 0; and b is the slope, with a value of 0.25 (hops/min)/(mg/kg).

2.2 Model of uptake

Uptake of nC_{60} by daphnids appeared to follow first-order (exponential) kinetics (Fig. 2), with the concentration in daphnid tissues asymptotically approaching a steady-state value within five days. The equation is as follows (Eq. (2)).

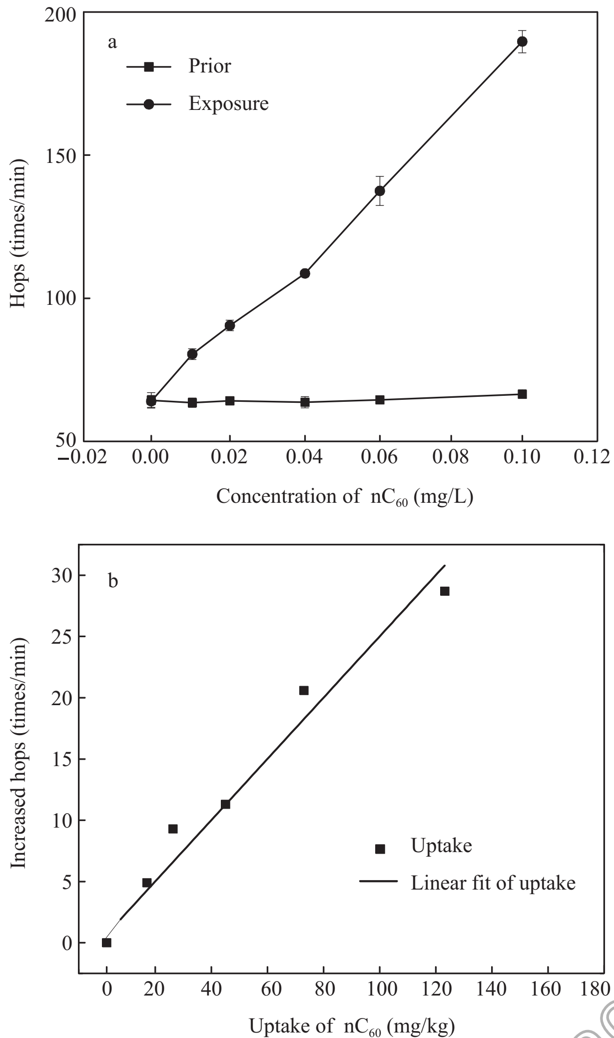

Fig. 1 Sublethal effects of nC_{60} on hops of *D. magna* under various concentrations (a) and correlation between hop and uptake of nC_{60} (b).

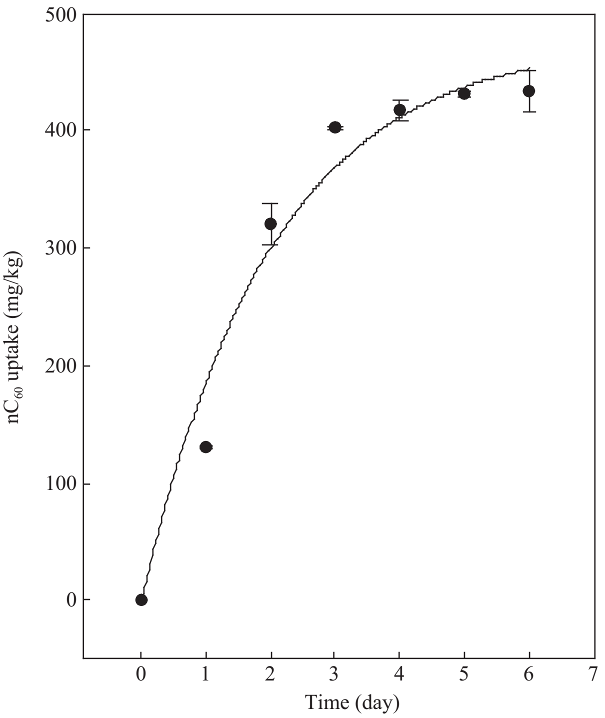

Fig. 2 Nonlinear regression of uptake for six days exposure. Error bars represated standard deviations of results conducted in triplicates.

$$C_b = A(1 - \exp(-K_u t)) \qquad R^2 = 0.9713 \tag{2}$$

where, C_b (mg/kg wet weight) is the C_{60} concentration in the daphnids tissues; A (477.45 mg/kg) is the maximum uptake value; and K_u (0.4933 (mg/(kg·day)) is the uptake rate coefficient. This model had the following assumptions: nC_{60} in solution remained constant over the experimental period; uptake was from water only; biomass remained constant over the experimental period; and initial time, $t = 0$, $C_b = 0$.

2.3 Effects of different conditions on uptake

Uptake in daphnids increased with increasing concentration and time (Fig. 3a). Almost all concentration levels reached their maximum within five days. Among the different concentrations, the concentration of 0.10 mg/L achieved the highest maximum at 433.64 mg/kg. Maximums for 0.06, 0.04, 0.02 and 0.01 mg/L levels were 325.74, 307.71, 247.93, and 192.26 mg/kg, respectively. Within three days, average uptake rates in 0.10, 0.06, 0.04, 0.02, and 0.01 mg/L were 134.23, 100.75, 76.31, 57.98, and 37.54 mg/(kg·day), respectively.

Uptake in daphnids increased with increasing time in all size solutions (Fig. 3b). Uptake of 125 and 284 nm solutions reached their maximums more quickly than those of 80 and 90 nm. The maximum among the different concentrations was achieved by 80 nm (498.31 mg/kg), while maximums for 90, 125, and 284 nm were 462.98, 444.97, 447.20 mg/kg. Within three days, average uptake rates in solutions of 80, 90, 125 and 284 nm were 105.67, 107.47, 117.41, and 137.33 mg/(kg·day), respectively.

Uptake in daphnids increased with increasing hardness and time (Fig. 3c). Uptake of solutions at VH, H, and M reached their maximums more quickly than those of S, VS and PW. The maximums in VH, H, M, S, VS, and PW solutions were 426.56, 418.35, 410.62, 358.62, 347.29, and 327.73 mg/kg. Within three days, average uptake rates in VH, H, M, S, VS, and PW solutions were 134.92, 130.85, 119.56, 69.12, 36.41, and 23.42 mg/(kg·day), respectively.

Uptake in daphnids increased with increasing time in all pH solutions (Fig. 3d). Uptake of solution pH 7.5 reached its maximum (543.39 mg/kg) quickly and also was the maximum among all pH solutions. Maximums for pH 6.5, 7.0, 8.0, and 8.5 were 444.00, 452.37, 506.54, and 516.51 mg/kg. Within three days, average uptake rates in solutions at pH 6.5, 7.0, 7.5, 8.0, and 8.5 were 136.27, 129.68, 163.56, 95.70, and 101.85 mg/(kg·day), respectively.

Uptake in daphnids increased with time under various temperatures (Fig. 3e). Under all temperatures, uptake of experimental solution reached their maximum within five days. The maximum under different temperatures was achieved at 18°C (539.48 mg/kg), with 21, 24 and 27°C reaching 502.12, 467.88, and 442.97 mg/kg, respectively. Within three days, average uptake rates at 18, 21, 24 and 27°C were 91.71, 99.99, 129.81 and 133.16 mg/(kg·day), respectively.

3 Discussion

3.1 Sublethal hop frequency

Hops are an important escape response for organisms such as *D. magna* (Seely and Lutnesky, 1998), and can be an indicator of toxic effects (Lopes et al., 2004). Our results showed that nC_{60} increased the hops of *D. magna*, and this sublethal physical toxic result is similar to previously published results (Lovern et al., 2007). The reason for this may be that the increasing accumulation of C_{60} in *D. manga* (Fig. 1b) increased toxic escape behavior. As a result, other nanoparticles might also cause sublethal physical toxic escape by accumulation.

3.2 Influence of different conditions on uptake of nC_{60}

Equilibrium exists between water and the bodies of daphnids. When accumulation balance of C_{60} between in and out of the body reached, uptake rate equals to that of excretion, the maximum of uptake will reach at the same time. The same scale particles are taken up according to its abundance in the environment (Demott, 1982), and the uptake rate shows a positive correlation to the concentration in solution. In this study, uptake increased with concentration and time. The maximums of all concentrations also increased with exposure concentrations. The maximum uptake of certain concentrations remained balanced with related concentrations. Higher body-burden showed in higher concentrations. The uptake rate also increased with the corresponding concentrations in water.

The feeding appendage of *D. magna* easily filters particles ranging from 0.1 to 35 μm in size, with special emphasis on particle diameters between 0.1 and 1 μm (Gophen and Geller, 1984). Particles larger than the average mesh size are retained very efficiently by daphnids

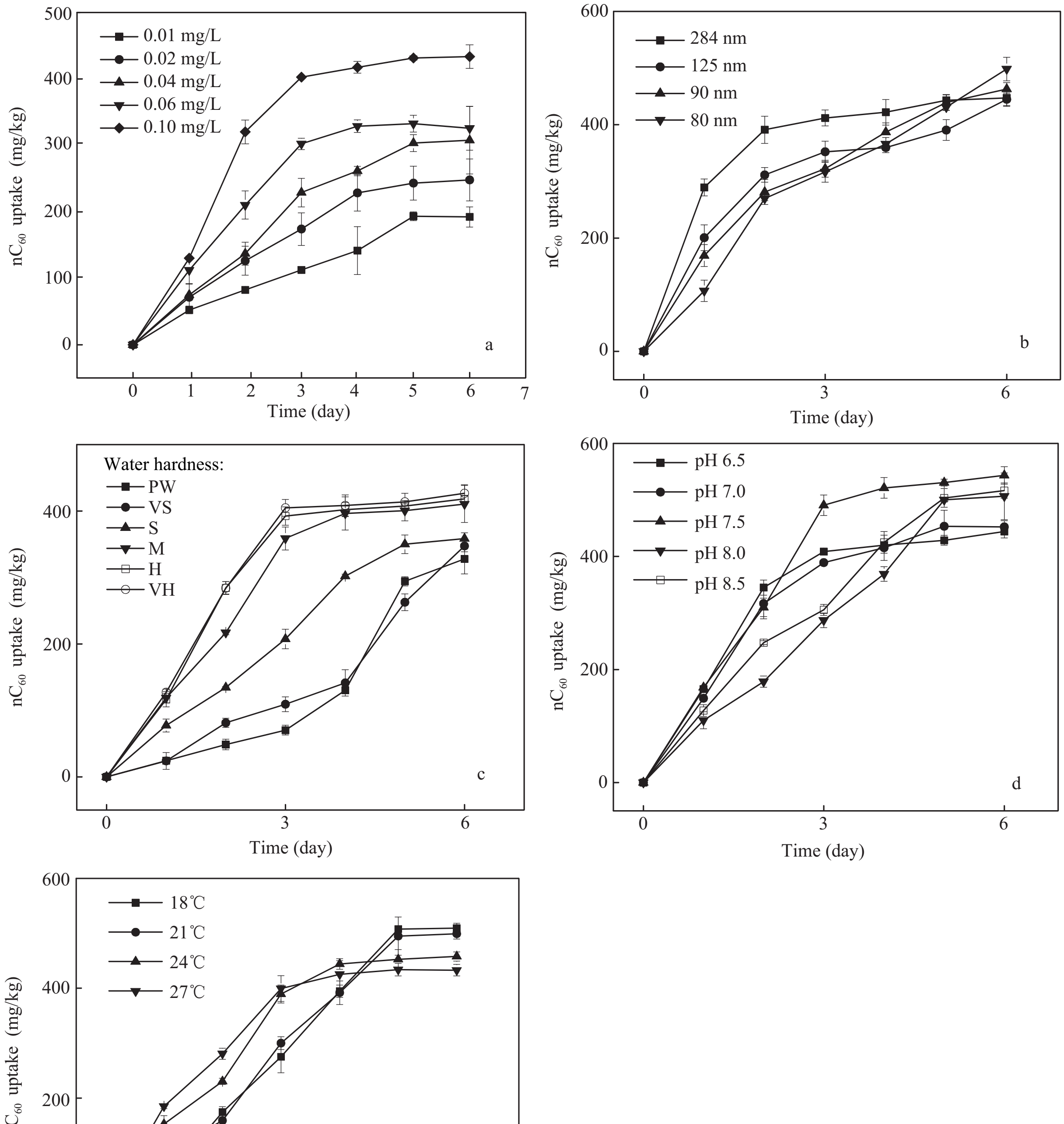

Fig. 3 Uptake in daphnids after exposure to various concentrations of nC_{60} (a), after exposure to 0.1 mg/L nC_{60} with various average diameter (b), water hardness (c), solution pH (d), and temperatures (e). Error bars represent standard deviations of results conducted in triplicates. PW: ultra purified water; VS: very soft hardness; S: soft hardness; M: medium hardness; H: hardness; VH: very hardness.

(Gophen and Geller, 1984; Demott, 1985; Brendelerger, 1991), and the particles are sent to the intestine (Zhu et al., 2010; Kukka et al., 2010) where they aggregate and block the intestine for a long period until death (Zhu et al. 2009). The larger size particles were taken up by daphnia quickly at the beginning of exposure, and reached balance quickly (Fig. 4) because of the filter efficiency of daphnia (Gophen and Geller, 1984). Smaller particles less than 100 nm exhibit less protein absorption than those greater than 100 nm (Fang et al., 2006), therefore smaller particles can be taken up and not only stored in the lipid of the body (Tao et al., 2009) but also stored in protein. When a balance was reached between the daphnia tissue and aqueous system, the maximum uptake of smaller size nC_{60} was more than the uptake of large size nC_{60}. Reports about increased inflammatory activity and epithelia translocation of manmade 20- and 30-nm solid particles appeared recently (Ferin et al., 1990; Oberdöster and Yu, 1990), which revealed that particle size was a key factor in the uptake maximum and uptake rate.

Harder water increased the uptake rate and maximum uptake of nC_{60} in the aqueous system. Hardness of water was positively correlated to ionic strength, with harder water leading to higher ionic strength and, in turn, aggregation of nC_{60} colloids in aqueous systems (Fortner et al., 2005). In this study, ionic strength of VH, H, M, S, VS, and PW were 0.01608I, 0.00804I, 0.00402I, 0.00201I, 0.000503I, and 0I, respectively. The particle size of nC_{60} with higher ionic strength was larger than with lower ionic strength, and larger particles were more effectively filtered by daphnids' thoracic appendages. With high daphnia filter efficiency, as much as 16.6 mL/hr (McMahon and Rigler, 1965), larger particles of colloids in the higher ionic strength solution were able to pass the daphnia filter and enter the body through absorbance in the intestine. In H and VH solutions, uptakes of nC_{60} in daphnids increased quickly at the start, while those in other hardness solutions increased slowly. Uptake speeds of daphnia in higher ionic strength solutions were greater than those in lower ionic strength.

Results showed that pH influenced the uptake of nC_{60} in daphnids by modifying particle size. Fortner et al. (2005) found that nC_{60} particles in aqueous systems disaggregate with pH. Gilbert et al. (2007) also showed that a pH-dependent aggregation and disaggregation with larger aggregate radius at higher pH vice versa. According to the trend seen with changing pH, particle size is adapted to not only be taken up by the intestine but also by the feeding appendages. In this study, the uptake rate of nC_{60} was greater in higher pH solution than in lower pH. The maximum appeared in the solution of pH 7.5, which means the size at pH 7.5 is the proper sizes for both uptake routes mentioned above.

Increasing temperature increased C_{60} uptake from aqueous systems, which was not only caused by the nanoparticle itself but also by physicochemical changes of daphnids. The activity of nanoparticles in aqueous system increased with increasing temperature according to the Brownian motion, where the increased frequency of crashes between colloids and daphnids increased the possibility of being taken up. The filter frequency of daphnia increased with temperature in some range (Kibby, 1971), such that daphnia filter more water and uptake more particles, and thus reach balance quickly. Our results showed, therefore, higher uptake rate at higher temperature and less time to reach the maximum than at lower temperatures, as previously reported (Black et al., 1991; Sijm et al., 1993). The maximums at higher temperature were less than those at lower temperature. The maximum in this experiment is mainly up to the lipid content, because the lipid is the reservoir of some hydrophobic chemicals like C_{60} in body. Total fatty acid of daphnids grown in lower temperature is higher than that of daphnids grown in higher temperature (Schlechtriem et al., 2006). The amount of lipid in the daphnia determines the bioaccumulation of hydrophobic chemicals. Higher lipid levels have greater potential to accumulate more chemicals (Dauble et al., 1985) and C_{60} (Tao et al., 2009). Therefore, the maximums in higher temperatures were less than that in the lower temperatures, when C_{60} bioaccumulation reached the balance between the experimental solution and the body (Hamelink et al., 1977).

In the lower concentrations of nanomaterial, *D. magna* can take up nC_{60} (Tao et al., 2009) and other engineered nanomaterials (Zhu et al., 2010; Petersen et al., 2010), and the uptake rate and maximum can be adjusted by different environmental conditions and particle sizes. The different conditions in this research simulated applied and disposal environmental conditions and showed their effects on nC_{60} potential in aqueous systems, therefore, strict steps should be taken in production, transportation, storage, application, and disposal to avoid inadvertent release and reduce risk potential of environmental factors.

3.3 Influences of different factors on the model parameters

The nonlinear regression equation showed the trend of uptake, similar to other uptake models of nanoparticles (Zhang et al., 2007; Sun et al., 2009). Although the environmental factors in this study influenced the uptake of nC_{60}, the trend of growing to the maximum is the same and fits the equation, with adjustment of the two constant parameters (A and K_u). It is important, therefore, for other nanoparticle uptake studies to include C_{60} uptake. Influences of different factors on the equation parameters are shown l in Table 1.

Table 1 Influences of different factors on the equation parameters of C_{60} uptake for *D. magna*

Parameter	Concentration	Size	Hardness	pH	Temperature
A	+	–	–	+	–
K_u	+	+	+	–	+

A: Maximum uptake, K_u: uptake rate.
+: positive correlation; –: negative correlation.

4 Conclusions

In this study, hopping escape behavior was increased with concentration among the sublethal concentration range of nC_{60}, and uptake of nC_{60} appeared to be a key factor for toxic escape. The uptake of nC_{60} in daphnids was highly dependent on environmental factors and the uptakes under different conditions fitted the exponential kinetics model. This study will provide information for the use and disposal of engineering nanomaterial.

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