Trophic Transfer and Biomagnification of Fullerenol Nanoparticles in an Aquatic Food Chain

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Materials and methods

Preparation of ^{13}C -labelled Fullerenols Solution

^{13}C-labelled fullerenols were prepared as described previously.[1] The ^{13}C-isotope rate of fullerenols were determined by isotope ratio mass spectrometry (IRMS; Delta V Advantage, Thermo, Bremen, Germany). A stock solution of fullerenols was made by adding 10 mg of ^{13}C-labelled fullerenols to 1000 mL of ultrapure water (Merck Millipore, Billerica, MA, USA). The ^{13}C-labelled fullerenols stock solution was diluted with BG11 media to 1 mg/L, the concentration chosen for water exposure. After sonication in an ice bath using an ultrasonic cell pulverizer (VCX800, Sonics & Material, Newtown, CT, USA) for 10 min, the fullerenol nanoparticles were analysed by transmission electron microscopy (TEM; Tecnai F30, Philips-FEI, Eindhoven, Netherlands) at a 120 kV accelerating voltage. The hydrodynamic size of the fullerenol nanoparticles in the exposure solutions was measured using a Zetasizer Nano ZS system (ZEN 3600, Malvern Instruments, Malvern, U.K.).

Experimental Animals

Both *S. obliquus* and *D. magna* were obtained from the State Key Laboratory of Environmental Aquatic Chemistry (SKLEAC), Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. *S. obliquus* was cultured in BG11 medium made with freshwater and maintained in a manual climatic box at 25±

1°C under a 16:8 h light/dark cycle. Conical flasks were shaken three times per day. *D. magna* was cultured in aerated artificial freshwater for 3 d at 20±1°C under a 16:8 h light/dark cycle. *D. magna* was fed once a day with a culture of *S. obliquus*, and 80% of the volume was refreshed once every 2 d. Healthy 4-month-old adult *D. rerio* (length 3.09±0.20 cm; weight 0.16±0.02 g [mean± SD]; 160 individuals) were obtained from the State Key Laboratory of Environmental Criteria and Risk Assessment (SKLECRA), Chinese Research Academy of Environmental Sciences (CRAES). Specimen batches of 80 *D. rerio* individuals were separated into aquaria (60 L) containing aerated artificial freshwater at 25°C and cultured under a 14/10 h light/dark cycle as specified in the "Code for *D. rerio* culture" (China *D. rerio* Resource Centre). *D. rerio* were fed *D. magna* once per day for 1 week. The average feed weight for each fish per day was 0.01 g wet weight of *D. magna* (approximately 17 *D. magna* per *D. rerio*), which was within the feed rate of 3%-5%.[2] Fish faeces were removed from the aquaria each day, and 80% of the aquarium volume was refreshed every 2 d. The aquaria were cleaned once a week.

Quantification of ^{13}C-labelled Fullerenols

^{13}C-labelled fullerenols were quantified as described in our previous studies.[3-5] We determined the carbon isotope ratios (^{13}C/^{12}C) in *S. obliquus, D. magna*, and *D. rerio* using IRMS. The elemental carbon content was measured according to the peak areas obtained from mass spectrometry. Every 12 samples, labelled urea was analysed as a

standard to check the accuracy and precision of the isotope ratios measurements. According to the elemental carbon content, the $^{13}C/^{12}C$ ratio was converted to the ^{13}C concentration and expressed as ω_{13_c}. The concentrations of ^{13}C-fullerenols in *S. obliquus, D. magna*, and *D. rerio* were calculated as below (Eq. 1) and expressed as mg/g dry weight.

$$\omega_{fullerenols} = \frac{\omega_{13_c}(sample) - \omega_{13_c}(control)}{\omega_{13_c}(fullerenols)} \quad (1)$$

The limit of detection of this method was defined as 3.29 times of the standard deviation of ^{13}C concentration of the blank sample (the natural organism) according to IUPAC.[6] For the algae, daphnia and zebrafish, the LODs were 0.043, 0.071, and 0.15 mg/g, respectively. The limits of quantification (LOQ) were 10 times the standard deviation of the blank sample (the natural organism) according to IUPAC.[1] For algae, daphnia and zebrafish, the LOQs were 0.13, 0.21, and 0.45 mg/g, respectively. The recoveries of fullerenols in different organisms were determined by the standard addition method at a concentration level of 10 μg (n=10). For algae, daphnia and zebrafish, the recoveries of fullerenol were 93.2±3.9%, 95.6±2.7%, and 94.6±3.7%, respectively. The recoveries for this method were stable across all samples, so the results of the quantification of ^{13}C-labelled fullerenol were not corrected by the recoveries.

The uptake rate constant (k_u) and depuration rate constant (k_e) were modeled using the first-order kinetic model given in the following two equations.[7]

$$C_{t1} = \frac{C_F k_u}{k_e}\left(1 - e^{-k_e t}\right) \quad (2)$$

$$C_{t2} = C_{t3} e^{-k_e t} \quad (3)$$

where C_{t1} is the ^{13}C-fullerenol concentration in tissues and organisms derived from dietary uptake at the time of the uptake experiment, C_{t3} is the ^{13}C-fullerenol concentration in tissues and organisms at the beginning of the depuration experiment, C_{t2} is the ^{13}C-fullerenol concentration in tissues and organisms at the time of the depuration experiment, t is the period of the uptake or depuration experiment, and C_F is the ^{13}C-fullerenol concentration in the feed.

The bioconcentration factor (BCF) is the ratio of the chemical concentration in an organism to that in water (i.e., initial concentration). This value was calculated for the water exposure at steady state by Eq. 4. The biomagnification factor (BMF) is the ratio of the pollutant concentration in the predator to that in its prey, which was calculated for dietary exposure experiments at steady state using Eq. 5. Here, C_o (mg/g dry weight) is the concentration of fullerenol in the organism, C_w (mg/L) is the concentration of fullerenol in the water (*i.e.*, initial concentration), and C_F (mg/g dry weight) is the concentration of fullerenol in the feed. The fitted bioconcentration factor (BMF_f) was calculated for the dietary exposure experiments at the fitting plateau using Eq. 6, in

which Co_f (mg/g dry weight) is the fitted plateau concentration of fullerenol in the organism. The distribution-based trophic transfer factor (TTF_d, [unitless]) was calculated to evaluate the trophic transfer potential in the specific tissues of zebrafish using Eq. 7, in which C_t (mg/g dry weight) is the fitted plateau concentration of fullerenol in the tissues of the organism.

$$BCF = \frac{C_o}{C_w} \quad (4)$$

$$BMF = \frac{C_o}{C_F} \quad (5)$$

$$BMF_f = \frac{Co_f}{C_F} \quad (6)$$

$$TTF_d = \frac{C_t}{C_F} \quad (7)$$

Statistical Analysis of Experimental Data

Statistical analyses were conducted using GraphPad Prism ver. 7.00. All data are presented as the mean values of three individual observations with the standard deviation (mean ± SD). One-way analysis of variance (ANOVA) followed by Tukey's post- hoc test was performed to evaluate significant differences in the body burden of fullerenol in *S. obliquus* from 12-36 h, *D. magna* form 24-48 h, and between tissues. The criterion for statistical significance was $p < 0.05$.

Evaluation of the Effectiveness of the Washing Step for *S. obliquus*

To evaluate the effectiveness of the "cleaning and centrifugation" method for cleaning fullerenol nanoparticles adsorbed on the algae surface, the concentration of fullerenols in the supernatant was determined by continuous washing and centrifugation after algae exposure had reached steady state (Figure S1). The concentrations of fullerenols during the final centrifugation and in the original fullerenols suspension were determined. According to the differences in the fullerenol concentrations among the samples, the removal of fullerenols through centrifugation could be calculated. Approximately 93.3% of the fullerenols in the exposure solution was removed through the cleaning and centrifugation procedure. Furthermore, to show the effectiveness of this method, we also measured the fullerenol concentration in algae before and after cleaning. The changes in the ratio of the fullerenol concentration in algae after cleaning to that before cleaning were shown in the Table S4. The result indicated that the difference in algae concentration after the second and third washing was only 3‰, which can be neglected. Thus, the washing step for removal of fullerenol nanoparticles adsorbed on the algae surface was effective.

Table S1. Bioaccumulation and Depuration Characteristics of Fullerenols in *D. rerio*

k_u (d^{-1})	k_e (d^{-1})	R^2	Maximum body burden (mg/g)	Fitted plateau (mg/g)	Fitted concentration at 28 d (mg/g)	Half concentration time of depuration (d)	90% concentration time of depuration (d)

D. rerio	0.036	0.067	0.80	3.70±0.27	3.73	3.16	10.35	34.37

Table S2. Modelled Bioaccumulation and Depuration Characteristics of Fullerenol Nanoparticles in *D. rerio* Tissues

	k_u (d^{-1})	k_e (d^{-1})	R^2	Fitted plateau (mg/g)	Fitted concentration at 28 d (mg/g)	Half-life $t_{1/2}$(d)	90% concentration time of depuration (d)
Intestine	0.088	0.091	0.72	6.70	6.18	7.62	25.30
Liver	0.066	0.069	0.81	6.63	5.67	10.05	33.37
Muscle	0.048	0.064	0.79	5.20	4.34	10.83	35.98
Gill	0.054	0.113	0.80	3.31	3.17	6.13	20.38
Brain	0.008	0.037	0.83	1.50	0.97	18.73	62.23

Table S3. Modelled Trophic Transfer Factor (TTF_d) of Fullerenol Nanoparticles in *D. rerio* Tissues

	Fitted plateau (mg/g)	Maximum body burden (mg/g) of *D. magana*	Fitted TTF_d
Intestine	6.70±0.54	6.93±0.56	0.97
Liver	6.63±0.54		0.96
Muscle	5.20±0.42		0.75
Gill	3.31±0.27		0.48

Brain	1.50±0.12	0.22

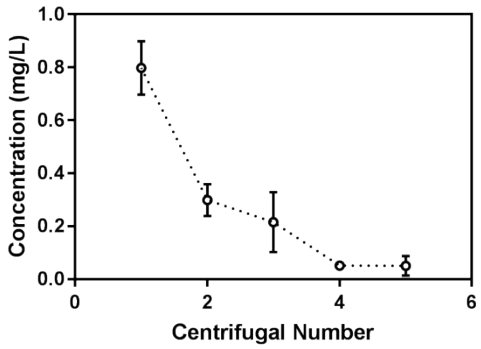

Figure S1. Variation in the fullerenol concentration in the supernatant solution during successive cleaning and centrifugation.

Table S4. Changes in the Ratio of the Fullerenol Concentration in *S. obliquus* after Cleaning to that before Cleaning

	1 time	2 times	3 times
ω_A/ω_B	0.942	0.800	0.797

Notes: ω_A represents the concentration of fullerenols in algae after 1-3 rounds of cleaning and centrifugation; ω_B represents the concentration of fullerenols in algae before cleaning.

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