Bioconcentration and bioaccumulation of C₆₀ fullerene and C₆₀ epoxide in biofilms and freshwater snails (*Radix* sp.)

Josep Sanchís^{a,*}, Anna Freixa^{b,c}, Julio C. López-Doval^{b,c}, Lúcia H.M.L.M. Santos^{b,c}, Sergi Sabater^{b,c}, Damià Barceló^{a,b}, Esteban Abad^a, Marinella Farré^a

^a Water and Soil Quality Research Group, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), C/Jordi Girona, 18-26, 08034, Barcelona, Catalonia, Spain

^b Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, H2O Building, C/Emili Grahit, 101, E17003, Girona, Catalonia, Spain

^c University of Girona, 17071, Girona, Catalonia, Spain

ARTICLE INFO

Keywords:

Fullerenes
Nanomaterials
Bioaccumulation
Biofilms
Radix sp.

ABSTRACT

Fullerenes are carbon nanomaterials that have awoken a strong interest due to their adsorption properties and potential applications in many fields. However, there are some gaps of information about their effects and bioconcentration potential in the aquatic biota. In the present work, freshwater biofilms and snails (*Radix* sp.) were exposed to fullerene C₆₀ aggregates, at concentrations in the low µg/L order, in mesocosms specifically designed to mimic the conditions of a natural stream. The bioconcentration factors of C₆₀ fullerene and its main transformation product, [6,6]C₆₀O epoxide, were studied to the mentioned organisms employing analyses by liquid chromatography coupled to high-resolution mass spectrometry.

Our results show that C₆₀ fullerene and its [6,6]C₆₀O present a low bioconcentration factor (BCF) to biofilms: BCF_{C60} = 1.34 ± 0.95 L/kg_{dw} and BCF_{C60O} = 1.43 ± 0.72 L/kg_{dw}. This suggests that the sorption of these aggregates to biota may be less favoured than it would be suggested by its hydrophobic character. According to our model, the surface of fullerene aggregates is saturated with [6,6]C₆₀O molecules, which exposes the polar epoxide moieties in the surface of the aggregates and decreases their affinity to biofilms. In contrast, freshwater snails showed a moderate capacity to actively retain C₆₀ fullerenes in their organism (BAF_{C60} = 2670 ± 3070 L/kg_{dw}; BAF_{C60O} = 1330 ± 1680 L/kg_{dw}), probably through ingestion. Our results indicate that the bioaccumulation of these carbon nanomaterials can be hardly estimated using their respective octanol-water partition coefficients, and that their colloidal properties, as well as the feeding strategies of the tested organism, play fundamental roles.

1. Introduction

Fullerenes and their derivatives are cage-like polyhedral molecules with unique properties and applications in fields as diverse as nanomedicine, nanoelectronics, the renewable energy industry and the automotive industry (Bakry et al., 2007; Brabec et al., 2010; De Jong and Borm, 2008; Jurkowska et al., 2006; Thompson and Fréchet, 2008). The potential mass production of these nanomaterials has urged the need to assess their environmental risks. In this regard, environmental studies have typically focused their attention on C₆₀ fullerene, the most abundant molecule that is produced by graphitic arc-discharge methods and in common hydrocarbon flames (Anacleto et al., 1992; Howard et al., 1991; Krätschmer et al., 1993; Tiwari et al., 2016). Experimental

studies and environmental models conclude that C₆₀ fullerene aggregates can distribute ubiquitously in multiple environmental compartments. The current emission rates result in concentrations levels in the pg/L – ng/L range (Astefanei et al., 2014; Emke et al., 2015; Encinas and Gómez-de-Balugera, 2018; Gottschalk et al., 2009, 2013; Laitinen et al., 2014; Núñez et al., 2012; Sanchís et al., 2012; Sanchís et al., 2013, 2015b; Sanchís et al., 2015a; Utsunomiya et al., 2002) or lower (Bäuerlein et al., 2017), which according to the current state of knowledge are not expected to pose a threat for the aquatic ecosystems regarding acute toxicity (Freixa et al., 2018a, 2018b). However, the environmental occurrence of fullerenes entails other risks. Notably, fullerene aggregates and other carbon-based nanomaterials can interact with co-occurring micropollutants (i.e. adsorbing them onto their

* Corresponding author.

E-mail address: jsanchis@icra.cat (J. Sanchís).

<https://doi.org/10.1016/j.envres.2019.108715>

Received 21 March 2019; Received in revised form 21 August 2019; Accepted 1 September 2019

Available online 05 September 2019

0013-9351/ © 2019 Elsevier Inc. All rights reserved.

surface) changing their bioavailability, enhancing/reducing their mobility and modulating their toxicity (Baun et al., 2008; Costa et al., 2012; Ferreira et al., 2014; Henry et al., 2013; Naasz et al., 2018; Sanchís et al., 2015c; Santín et al., 2016), which overall may result in synergistic-like or antagonistic-like effects. Also, the chronic exposure of aquatic organisms to trace concentrations of fullerenes has been associated with sublethal effects and to metabolic disorders such as oxidative stress (Oberdörster, 2004; Sanchís et al., 2017; Usenko et al., 2008; Zhu et al., 2008), cell membrane penetration and genotoxicity (Dhawan et al., 2006).

The bioconcentration and bioaccumulation potential of fullerene aggregates has been poorly assessed up to date and, currently, most information is limited to model organisms exposed to fullerene aggregates dispersed by ultrasounds or by solvent transfer methods. These methods produce aggregates with size distributions, morphologies and physicochemical properties that may not be translatable to real-life aggregates. Tao et al. (2011) studied the uptake of C₆₀ fullerene clusters (dispersed by solvent-transfer method) to *Daphnia magna* and Tervonen et al. (2010) studied the dynamics of accumulation and depuration of aggregates (dispersed by continued stirring and filtered) in this filter-feeding microcrustacean. Later, Du et al. (2016) assessed the bioaccumulation of hydroxylated fullerenes (*fullerenols*, dispersed by sonication) in *D. magna*. Sanchís et al. (2018) recently quantified the residue of C₆₀ fullerene (dispersed by continued stirring) that remained in edible tissues of *Mytilus galloprovincialis* after 3 weeks of exposure to fullerenes at low ng/L concentrations. Nevertheless, the role of biofilms in the accumulation of fullerenes has never been assessed. Biofilms are complex and heterogeneous communities of microorganisms adhered onto the water-riverbed interphase that play a fundamental role in the biogeochemical cycles of river ecosystems, regulating the uptake, storage and release of carbon, nitrogen and phosphorous and changing the composition and concentration of organic matter (Freixa et al., 2016; Pusch et al., 1998; Sabater et al., 2007). Given the high hydrophobicity and bactericidal properties of C₆₀ aggregates (Lyon et al., 2005), it is relevant to study their potential impact on the composition and viability of river biofilms (Freixa et al., 2018a, 2018b). The same can be extrapolated to fullerene environmental transformation products (TPs), such as fullerene epoxides and fullerene dimers, which are slowly produced when C₆₀ aggregates are dispersed in water media under environmentally relevant conditions (Sanchís et al., 2018).

In the present work, the settling behaviour and bioconcentration potentials of C₆₀ fullerene and its TPs on river biofilms and freshwater snails were studied in two sets of experiments. An aqueous suspension of C₆₀ fullerene was dispersed and aged under environmentally representative conditions, and organisms were exposed in a series of freshwater mesocosms that emulated the natural conditions of a freshwater stream. Analyses based on liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) were used to determine the bioconcentration of these fullerene species on biofilms and freshwater snails (*Radix* sp.)

2. Methods and materials

2.1. Chemicals and reagents

C₆₀ fullerene (sublimed, 99.9% purity; reference 572500) was purchased from Sigma-Aldrich (Steinheim, Germany). ¹³C-enriched C₆₀ fullerene (> 99% purity; abbrev. ¹³C₆₀; reference MRL613) was purchased from MER Corporation (Tucson, AZ, USA).

Methanol and ultrapure water (Optima® LC/MS grade) were purchased from Fischer Chemical (Loughborough, UK) and toluene (Chromasolv®) was purchased from Merck (Darmstadt, Germany).

Stock solutions of C₆₀ fullerene and ¹³C₆₀ for LC-HRMS analyses were prepared in toluene at 1000 ng/μL, while the final calibration curve was prepared in 90:10 toluene:methanol.

Table 1

Composition and physicochemical parameters of the artificial freshwater employed in these experiments. Physicochemical parameters were checked periodically during the experiment.

Parameter	Value
Conductivity	155–201 μS/cm
pH	8.1 ± 0.2
O ₂	10.2 ± 1.1 mg/L
T	19.4 ± 0.1 °C
Dissolved organic carbon	3.49 ± 0.83 mg/L
[NH ₄ ⁺]	4.48 ± 1.52 μg/L
[NO ₂ ⁻]	23.4 ± 5.0 μg/L
[NO ₃ ⁻]	1.41 ± 0.15 mg/L
[PO ₄ ³⁻]	3.44 ± 0.86 μg/L

2.2. Dispersion of aggregates in water

100 mg of C₆₀ fullerene powder were dispersed in 1.00 L of artificial freshwater. The dispersion was carried out in an amber glass bottle by mechanical agitation, with a PTFE-coated magnetic nucleus for several weeks. During this process, dispersing agents (organic solvents or surfactants) and ultrasounds were avoided. The bottle was covered with aluminium foil, which allowed free air exchange while minimised dust deposition inside the suspension.

2.3. Mesocosms description

Twelve home-made mesocosms were manufactured and located in the Catalan Institute of Water Research (ICRA) for this study. Each mesocosm consisted in an open circular channel, resembling a halved tori of 25 cm diameter radius and 15 cm high (see Fig. S1 in the supporting information). Mesocosms were made of glass, to minimise the sorption of fullerenes to internal walls, and approximately contained 4.5 L of rain water. The rain water had been previously filtered with activated carbon filters and offered a physic-chemical composition that can be found in natural oligotrophic streams (see composition in Table 1). A rotating glass blade allowed the constant circulation of the water during the experiment (estimated flow: ~0.15 L/s; ~3.4 cm/s). Day/night periods was using a Lumina Led 62, 48 W, simulating sun irradiation with 12-h day-night cycles. During the experiments, water loss by evaporation (approx. 4.5 mL/day) was corrected by adding water drop by drop.

2.4. Exposure experiments

A preliminary experiment, with no exposed organisms, was performed to study the behaviour and settlement dynamics of fullerenes (*abiotic tests*). Afterwards, to checking the effects of fullerenes on freshwater organisms, we performed two sets of experiments: a short term experiment to investigate the effect on freshwater biofilm and a long term experiment to study the accumulation in a freshwater snail.

Exp. 1 Abiotic tests: During these experiments (9 days), ~150 mL of water were periodically taken (after 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 9 days) for the characterisation of the aggregates and the quantification of C₆₀ fullerene and their TPs. This experiment was performed in triplicate.

Exp. 2 Exposure of freshwater biofilms to fullerenes: In the second series of tests, a short-term exposure of freshwater biofilms was studied. Biofilms were introduced inside the mesocosms in frosted glass tiles (1.5 × 1.5 cm). Biofilms had been collected from the Llémena stream, a tributary of the Ter river, in a non-polluted site near Sant Gregori (Girona, NE Spain), and had grown in artificial stream channels, as detailed in a previous study (Freixa et al., 2018a, 2018b). Each mesocosm was colonised with 20 tiles of biofilm. Three of the mesocosms were used as controls, and three other mesocosms were spiked with

fullerenes at a concentration of $\sim 1.0 \mu\text{g/L}$. Biofilms were collected after 72 h, and water aliquots ($\sim 150 \text{ mL}$) were also taken at the end of the experiment.

Exp. 3 Exposure of freshwater snails to fullerenes: The following experiment was a long-term exposure mimicking a single food web. Biofilms and freshwater snails (*Radix* sp.) were co-exposed to $\sim 1.0 \mu\text{g/L}$ of C_{60} fullerenes. *Radix* sp. is a freshwater snail common in Mediterranean river ecosystems with a feeding strategy based on scraping on biofilm in freshwater surfaces. A control treatment and exposure treatment (with C_{60}) with 3 mesocosms each treatment were used. Each mesocosm contained 9 snails, which had been collected in the same pristine stream than the biofilms and had been climatized for 48 h before the experiment started. Three mesocosms were exposed to fullerene C_{60} during 21 days. Biofilm was cultured as explained before and was exposed to C_{60} 72 h after being introduced into the mesocosms to saturate biofilm of contaminants and ensure transfer to the snail via food intake. In order to provide fresh food to the snails, which feed on biofilm, colonized tiles were added every 7 days and the grazed ones were removed. Water was wholly renewed every 3–4 days in all the mesocosms to ensure a constant exposition to C_{60} aggregates. Water aliquots ($\sim 150 \text{ mL}$) were taken two times per week to check the concentration of C_{60} , while snails were removed at the end of the experiment frozen in N_2 and freeze-dried.

2.5. Extraction of fullerenes from water

Water samples were extracted immediately after being taken using the method described in Sanchís et al. (2015b). Briefly, 150 mL of water were sequentially filtered with a $0.7 \mu\text{m}$ pore-size glass fiber filter and a $0.45 \mu\text{m}$ pore-size nylon filter (Whatman, Maidstone, UK). The particulate and the filtrate were analysed separately. The filters were dried at 60°C overnight and extracted by ultrasound assisted solvent extraction (UASE) in an ultrasonic bath with two consecutive fractions of 20 mL of toluene. Meanwhile, the filtrated water was salted out (NaCl 1% w/v) and extracted by liquid-liquid extraction (LLE) with two volumes of 15 mL of toluene. Particulate extracts and water phase extracts were concentrated to 1.00 mL with a gentle flow of nitrogen, transferred into a LC vial and stored at -80°C until HPLC-HRMS analysis.

2.6. Analysis of fullerenes from biofilms and snails

For the analysis of biofilms, around $500 \text{ mg}_{\text{ww}}$ ($\sim 290 \text{ mg}_{\text{dw}}$) of biofilm were collected in tared glass vials with the help of an inox steel spatula. Samples were spiked with $10.0 \mu\text{L}$ of a toluene suspension of $^{13}\text{C}_{60}$ ($1.0 \text{ ng}/\mu\text{L}$) and let undisturbed for 3 h at 4°C . Afterwards, 2.00 mL of toluene were pipetted inside the vial, and it was vortexed for 1 min. The vial was extracted by UASE during 30 min and centrifuged at 3000 g for 5 min. Extracting during 60 min instead of 30 min did not improve the extraction efficiency of C_{60} fullerene while the signal intensity worsened because of matrix effect. After the extraction, the supernatant was collected inside an amber LC vial.

For the analysis of snails, organisms were placed individually in 2.0 mL Eppendorf vials and extracted sequentially by two procedures:

- Analysis of those aggregates attached to the organism surface was carried out by adding $500 \mu\text{L}$ of toluene and agitating during 30 s with a vortex at 500 rpm .
- Analysis of total fullerene content was carried out by ultrasound assisted solid-liquid extraction (60 min in an ultrasonic bath at 37 KHz).

After their extraction, Eppendorf® vials were centrifuged for 3 min at 1700 g (4000 rpm). The supernatants were quantitatively transferred to LC vials and stored at -80°C until HPLC-HRMS.

2.7. HPLC-HRMS analyses

The instrumental method had been developed in a previous study (Sanchís et al., 2018). Briefly, $20 \mu\text{L}$ of extract were injected in each run into an Acquity UPLC System (Waters, Milford, USA) equipped with a COSMOSIL™ Buckyprep column ($150 \times 2.0 \text{ mm}$; $5 \mu\text{m}$ particle size) from Nacalai Tesque Inc. (Kyoto, Japan). A mixture of toluene:methanol (9:1) flowing at $0.4 \text{ mL}/\text{min}$ was employed as mobile phase. Mass spectrometric analyses were carried out with a Q Exactive™ (Thermo Fischer Scientific, San Jose, USA). The ionisation was performed with an Ion Max source (Thermo Fischer Scientific, San Jose, USA) working in APPI mode and negative polarity. Sheath gas and auxiliary gas were set at 40 a.u. and 25 a.u.; the capillary and probe heater temperatures, were set at 300°C and 400°C ; S-lens RF were set at 90%. Full scans were acquired from $m/z = 300$ to 1600 with a resolution of 140,000 full width at half maximum.

2.8. Characterisation of fullerene aggregates

The suspensions were characterized at each sampling time in terms of aggregate's hydrodynamic diameter and heterogeneity by means of nanoparticle tracking analysis (NTA) using a NanoSight instrument model LM10-HS (NanoSight, U.K.). Data was recorded in static mode during 90 s and 600–1000 tracks were acquired each run. The mode diameter, Mo , was defined as the size of the most frequently detected aggregate. The mean diameter, $\bar{x}_{\text{NTA}}$, was defined as the number-weighted arithmetic average of aggregates diameters. The dispersity, $\bar{D}$, was defined as the square of the ratio between the standard deviation of the aggregates size and the mean particle size.

In addition, micrographs of fullerene aggregates were obtained by scanning electron microscopy (SEM). A subsample of stock solution was diluted 10 times with filtered rainwater and disposed onto 200-mesh grid Formvar membrane, which was air-dried and observed at 60 KV. Micrographs were recorded by a CCD Gatan Orius 200 camera and showed that fullerene aggregates were well-dispersed and round-shaped (see Fig. 1).

3. Results and discussion

The behaviour of fullerenes in abiotic experiments

C_{60} fullerene and up to eleven of its TPs were included as target compounds (see Table 2), including eight epoxides ($[\text{6,6}]\text{C}_{60}\text{O}$, three diepoxides, three triepoxides and one tetraepoxide) and three dimers (one monoxidised dimer and two dioxidized dimers). These TPs had been detected and identified in a previous work (Sanchís et al., 2018).

The frequency of detection of each TP is detailed in Table 2. As can be seen, eight TPs were spotted, most of them only sporadically but C_{60} and C_{60}O were detected in all the samples. C_{60}O_2 was also detected in most of the water samples but, as expected, at low concentrations, and it was never detected in biota in subsequent experiments.

The behaviour of fullerene C_{60} and its main TPs, $[\text{6,6}]\text{C}_{60}\text{O}$, is presented in Table 3. As can be seen, C_{60} was the dominant compound. Virtually all the fullerenes were contained in the largest fraction ($> 0.45 \mu\text{m}$) and fullerene C_{60} disappeared completely from the finest fraction briefly after being transferred to mesocosms. This suggests that supra-aggregation was a relevant phenomenon in the mesocosms, and while some nanosized aggregates had been stable in the mother dispersion, which was under constant stirring, these small aggregates clustered under environmentally relevant conditions.

The increasing size of the aggregates during time was confirmed by NTA, as can be observed in Fig. 2. Mo increased from 100 nm to 151 nm during the 9 days of the experiment and the average size, $\bar{x}_{\text{NTA}}$, also increased, while the dispersity remained relatively large ($\bar{D} > 0.7$) during the whole experiment (Table 4).

Despite the pieces of evidence indicating supra-aggregation, it should be noticed that the concentration of fullerenes was relatively

Fig. 1. SEM micrographs of a 10 µg/L suspension of fullerenes, showing a sample of well-dispersed nanosized aggregates. Fullerene aggregates typically occurred as discrete nanoparticles and, less frequently, as small (2–6 units) supra-aggregates. Micron-size supra-aggregates were rarely observed. The aggregates did not present sharp edges and appeared as spheres or ovals.

stable during the first 96 h of the experiment. After 9 days, the concentration of C_{60} (250 ± 250 ng/L) was significantly lower than the initial one (674 ± 136 ng/L), which indicates that a large part of the initial aggregates settled to the bed of the mesocosm after that time. According to these results, in the next tests, performed with living organisms, the water was renewed every 3 days in order to obtain relatively stable concentrations.

3.1. Bioconcentration of C_{60} in biofilm

Fullerenes C_{60} and $[6,6]C_{60}O$ were detected in all the exposed biofilms at quantifiable concentrations by HPLC-HRMS. However, because of the low concentrations detected and the intrinsically complex and heterogeneous nature of biofilms, fullerene aggregates could not be unambiguously distinguished in SEM micrographs.

C_{60} was detected at an average concentration of 2.76 ± 2.38 ng/ g_{dw} (1.58 ± 1.47 ng/ g_{ww}), while the level of $[6,6]C_{60}O$ was significantly lower: 5.99 ± 2.56 pg/ g_{dw} (3.32 ± 1.32 pg/ g_{ww}). On the other hand, $C_{60}O_2$, which was repeatedly detected in water, was never detected in biofilm. The non-detection of $C_{60}O_2$ can be explained because of the extremely low concentrations of exposure and/or because of the polar character of this molecule.

Bioconcentration factors in biofilm, $BCF_{biofilm}$, were calculated according to eq (1):

$$BCF_{i,biofilm} = \frac{C_{i,biofilm}}{C_{i,water}}, \quad (1)$$

where $C_{i,biofilm}$ and $C_{i,water}$ are the concentrations of i (C_{60} or $C_{60}O$) in biofilm or water, respectively. The experimental $BCF_{C_{60},biofilm}$ was 1.34 ± 0.95 L/ kg_{dw} and the $BCF_{C_{60}O,biofilm}$ was 1.43 ± 0.72 L/ kg_{dw} , which indicates that these carbon nanomaterials are slightly bioaccumulable in biofilms.

It should be highlighted that the obtained $BCF_{biofilm}$ values of C_{60} and that of $C_{60}O$ are quite similar and they are not distinguishable

according to a conventional two means comparison T-test ($p \gg 0.1$). This result can be justified because of the mixed composition of fullerene aggregates. As it is well known, fullerenes do not occur in aqueous solutions as conventional solvated molecules, but they form aggregates (Andrievsky et al., 1995; Brant et al., 2005). As fullerenes (hetero)aggregate into a cluster in which different species co-occur (in this case, basically, C_{60} and $[6,6]C_{60}O$), the BCFs are not related to individual molecules but to aggregates as a whole. In the present case, the aggregates that were produced by stirring as described present a $BCF_{biofilm}$ of 1.3–1.4 L/ kg_{dw} .

3.2. Bioconcentration and bioaccumulation of fullerenes in snails

All the exposed snails presented quantifiable concentrations of C_{60} , in their surfaces and the homogenised body, while the control snails, which were not exposed to C_{60} , did not.

Exposed freshwater snails presented a mean concentration of C_{60} of 10.2 ± 10.3 ng/ g_{dw} in their surfaces. This resulted in an apparent bioconcentration factor, $BCF_{snail,C_{60}}$, of only 17.9 L/ kg_{dw} . In contrast, the total concentration of C_{60} in the snail homogenate was significantly higher: 1530 ± 1670 ng/ g_{dw} . Considering the concentration level of C_{60} in water, this resulted in an average bioaccumulation factor, $BAF_{snail,C_{60}}$, of 2670 ± 3070 L/ kg_{dw} .

Regarding the TPs, as in the case of biofilms, $[6,6]C_{60}O$ was the only detected one and its concentrations on the surface and the total homogenate of the snails were 0.222 and 34.63 ng/ g_{dw} . When considering the whole organism, the resulting $BAF_{snail,C_{60}O}$ was 1330 ± 1680 L/ kg_{dw} .

As it happened with biofilms, C_{60} and $C_{60}O$ showed a low tendency to attach to the surface of the test organisms ($BCF_{snail,C_{60}} = 17.9 \pm 17.7$ L/ kg_{dw} and $BCF_{snail,C_{60}O} = 61.0 \pm 78.1$ L/ kg_{dw}). However, in the case of exposed freshwater snails, these organisms preferentially accumulated fullerene aggregates inside their organism. Other previous works had observed high bioaccumulation

Table 2
TPs of C_{60} fullerene determined in the present work.

	Empirical formula	Tentative structure	Frequency of detection (t = 0)	Frequency of detection (t = 96 h)
1	$C_{60}O$	C_{60} epoxide ($[6,6]C_{60}O$)	100%	100%
2	$C_{60}O_2$	Diepoxidized C_{60} (<i>cis-I</i> isomer)	94.4%	100%
3	$C_{60}O_3$	Triepoxidized C_{60}	33.3%	66.7%
4	$C_{60}O_3$	Triepoxidized C_{60} (C_{3v} sym.)	11.1%	0%
5	$C_{60}O_4$	Tetraepoxidized C_{60}	16.7%	0%
6	$C_{120}O$	C_{60} dimer linked by 1 furane-like ring	94.4%	11.1%
7	$C_{120}O_2$	C_{60} dimer linked by 2 furane-like rings	66.7%	11.1%

Table 3

Concentration of C₆₀ and their TPs in abiotic controls along the experiments. C₆₀ fullerene was quantified by isotopic dilution using ¹³C-labelled C₆₀ as surrogate, while the concentrations of the two TPs was determined semiquantitatively, assuming the same response factor for these fullerenes than for its precursor.

Compound	Fraction	Time						
		6 h	12 h	24 h	48 h	72 h	96 h	216 h
C ₆₀	< 0.45 μm	0.2 ± 0.3 ng/L	0.1 ± 0.1 ng/L	not detected	not detected	not detected	not detected	not detected
	> 0.45 μm	670 ± 140 ng/L	920 ± 130 ng/L	1200 ± 160 ng/L	650 ± 330 ng/L	790 ± 210 ng/L	930 ± 200 ng/L	250 ± 250 ng/L
[6,6]C ₆₀ O	< 0.45 μm	not detected	not detected	not detected	not detected	not detected	not detected	not detected
	> 0.45 μm	5.5 ± 4.8 ng/L	3.6 ± 0.6 ng/L	4.4 ± 0.6 ng/L	3.9 ± 1.3 ng/L	2.6 ± 1.2 ng/L	2.8 ± 0.5 ng/L	1.3 ± 1.6 ng/L

potential of fullerenes in aquatic organism such as in the filter-feeding organism *Daphnia magna* (Sanchís et al., 2015c; Petersen et al., 2009; Tervonen et al., 2010). In these reports, fullerene aggregates were seen to be packed and depurated cyclically inside the daphnid's digestive tracks and gut. In the case of Sanchís et al. (2017), mussels (*Mytilus galloprovincialis*) exposed during 3 weeks to ultra-trace concentrations

of fullerene aggregates resulted in c_{C60,biota} of 4.61–12.1 ng/g_{dw} and BAF_{mussel,C60} values of ~2390 L/kg_{ww}. Assuming a ~90% of water content of mussel tissues, a BAF_{mussel,C60} of ~2.4 × 10⁴ L/kg_{dw} can be estimated, which is ~130 times higher than the BAF_{snail,C60} experimentally determined in the present work. Such difference can be attributed to the different feeding strategies of both animals: while a

Fig. 2. Results of NTA measurements: (a) Size distribution of fullerene aggregates at t = 0 (blue) and at t = 96 h (red); (b) accumulative abundance of fullerene aggregates depending on their sizes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Description of the heterogeneity of the fullerene aggregates at the beginning of the experiment and after 9 days, obtained from NTA measurements.

Parameter	t = 0 d	t = 9 d
Mo	100 nm	151 nm
$\bar{X}_{NTA}$	162 nm	215 nm
σ	96.8 nm	106 nm
$\bar{D}$	0.773	0.701

mussel typically filtrates water volumes at rates of 0.25–1.5 L/h order, depending on the environmental conditions, freshwater snails are restricted to grazing food in limited surfaces, accumulating much smaller amounts of nanomaterial.

3.3. Environmental implications

The presented results suggest that fullerene bioconcentration potential in biofilms may be lower than expected if we consider the presumable nonpolar character of C_{60} ($\log K_{OW} = 6.67$, according to Jafvert and Kulkarni, 2008). The $BCF_{biofilm}$ values are significantly lower than those of other nonpolar organic molecules such as nonpolar pesticides (Lundqvist et al., 2012) or polychlorobiphenyl ethers (Wang et al., 1999), and lower than those of other nanomaterials (such as Ag nanoparticles (Zhou et al., 2016) or TiO_2 nanoparticles and nanotubes (Yeo and Nam, 2013)). Therefore, the octanol-water partition coefficient of C_{60} is not an adequate method for estimating the bioconcentration of carbon-based nanomaterials in the environment, most likely because the colloidal properties and behaviour of fullerenes have a complex impact on the fugacity of nanomaterials.

We also hypothesise that $BCF_{biofilm}$ is lower than expected because of the weathering process of the nanomaterial and the resultant relative location of the epoxide molecules in the aggregates: Epoxidation reaction is likely to be limited to their surface, so functionalised fullerenes would preferably accumulate in the outer sphere of the clusters. The presence of fullerenes with oxygen moieties in the surface of the aggregates, even at concentrations two orders of magnitude lower than that of their precursor, would favour the stability of fullerenes in the medium because of van der Waals forces of the epoxide functional group, but it would also decrease the affinity of the fullerene clusters to biota.

In this sense, it is noteworthy that the ratio of the concentration of C_{60} and the concentration of $[6,6]C_{60}O$ has the same order of magnitude than the rate of superficial molecules of fullerene and the total number of molecules: Assuming an ideally spherical aggregate, grown with a face-cubic-centered (FCC) crystalline structure (Duncan et al., 2007), and presenting a lattice constant (ℓ) of 14.17 Å (Zhu et al., 1992) the number of fullerene molecules in the aggregate surface ($N_{surface}$) and the number of fullerene molecules in the whole aggregate (N_{total}) can be deduced from geometrical parameters of the FCC unit cell as in eq (2) and (3), respectively:

$$N_{surface} = \frac{A_{aggregate}}{A_{unit\ cells}} \sigma_{face} = \frac{4\pi r^2}{\ell^2} \sigma_{face} = \frac{8\pi r^2}{\ell^2} \quad (2)$$

$$N_{total} = \frac{V_{aggregate}}{V_{unit\ cells}} \sigma_{total} = \frac{\frac{4}{3}\pi r^3}{\ell^3} \sigma_{total} = \frac{16\pi r^3}{3\ell^3} \quad (3)$$

where $A_{unit\ cells}$ is the area of one face of the FCC unit cell, $A_{aggregate}$ is the area of a spherical aggregate, σ_{face} is the number of lattice points in the face of one FCC unit cell ($\sigma_{face} = 2$), r is the radius of a spherical aggregate, $V_{unit\ cells}$ is the volume of a FCC unit cell, $V_{aggregate}$ is the volume of a spherical aggregate and σ_{total} is the number of lattice points in a FCC unit cell ($\sigma_{total} = 4$). Accordingly, the ratio of superficial atoms vs total atoms, χ , in such a spherical aggregate with FCC crystalline structure can be defined as:

$$\chi = 1.5 \frac{\ell}{r} \quad (4)$$

During the experiment, the mean ratio of the concentrations of $[6,6]C_{60}O$ and C_{60} (see Table 3) was $4.75 \times 10^{-3} \pm 1.86 \times 10^{-3}$, which corresponds to the χ of an aggregate presenting a hydrodynamic radius of ~ 890 nm, while most of the aggregates were in the 100–200 nm according to NTA measurements. This fact suggests that the surface of the aggregates were saturated with $[6,6]C_{60}O$ molecules.

Our conclusions are in the same line than those of Bjorkland et al. (2017), which stated that carbon nanotubes present a limited bioconcentration potential according to the reviewed literature. However, this may only be true for primary producers. The moderately high BAFs determined for the gastropod *Radix* sp. and for the bivalve *M. galloprovincialis* suggest that the feeding strategies of some primary consumers favour the uptake of nanomaterials, reaching higher concentrations of fullerenes in biota. It is important to understand the dynamics of accumulation, biotransformation and depuration of fullerene aggregates in primary consumers, as well as to assess their potential transferability to other species, including to human diet.

Finally, the present study highlights the relevance of working with aggregates that contain representative mixtures of fullerenes and their environmental TP's to obtain representative results. Future experiments studying or modelling the properties, fate and behaviour of carbon nanomaterials should be planned with weathered aggregates instead of pure non-functionalised fullerenes.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness through the project NANO-transfer (ERA-NET SIINN PCIN-2015-182-CO2-01) and by the Generalitat de Catalunya (Consolidated Research Groups “2014 SGR 418 – Water and Soil Quality Unit” and “2014 SGR 291 – ICRA”). Lúcia H.M.L.M. Santos acknowledges the Juan de la Cierva program (IJCI-2017-32747) and Julio C. López-Doval thanks the program Juan de la Cierva from the Spanish Ministry of Economy and Competitiveness (IJCI-2015-23644).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2019.108715>.

References

- Anacleto, J.F., Perreault, H., Boyd, R.K., Pleasance, S., Quilliam, M.A., Sim, P.G., Howard, J.B., Makarovskiy, Y., Lafleur, A.L., 1992. C60 and C70 fullerene isomers generated in flames. Detection and verification by liquid chromatography/mass spectrometry analyses. *Rapid Commun. Mass Spectrom.* 6 (3), 214–220.
- Andrievsky, G.V., Kosevich, M.V., Vovk, M., Shelkovsky, V.S., Vashchenko, L.A., 1995. On the production of an aqueous colloidal solution of fullerenes. *J. Chem. Soc., Chem. Commun.* 12, 1281–1282.
- Astefanei, A., Núñez, O., Galceran, M.T., 2014. Analysis of C60-fullerene derivatives and pristine fullerenes in environmental samples by ultrahigh performance liquid chromatography 389 atmospheric pressure photoionization-mass spectrometry. *J. Chromatogr. A* 1365, 61–71.
- Bakry, R., Vallant, R.M., Najam-ul-Haq, M., Rainer, M., Szabo, Z., Huck, C.W., Bonn, G.K., 2007. Medicinal applications of fullerenes. *Int. J. Nanomed.* 2 (4), 639.
- Bäuerlein, P.S., Emke, E., Tromp, P., Hofman, J.A., Carboni, A., Schooneman, F., de Voigt, P., van Wezel, A.P., 2017. Is there evidence for man-made nanoparticles in the Dutch environment? *Sci. Total Environ.* 576, 273–283.
- Baun, A., Sørensen, S.N., Rasmussen, R.F., Hartmann, N.B., Koch, C.B., 2008. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C60. *Aquat. Toxicol.* 86 (3), 379–387.
- Bjorkland, R., Tobias, D.A., Petersen, E.J., 2017. Increasing 399 evidence indicates low bioaccumulation of carbon nanotubes. *Environ. Sci.: Nano* 4 (4), 747–766.
- Brabec, C.J., Gowrisanker, S., Halls, J.J.M., Laird, D., Jia, S., Williams, S.P., 2010. Polymer fullerene Bulk-Heterojunction solar Cells. *Adv. Mater.* 22 (34), 3839–3856.
- Brant, J., Lecoanet, H., Wiesner, M.R., 2005. Aggregation and deposition characteristics of fullerene nanoparticles in aqueous systems. *J. Nanoparticle Res.* 7 (4–5), 545–553.
- Costa, C.L.A., Chaves, I.S., Ventura-Lima, J., Ferreira, J.L.R., Ferraz, L., De Carvalho, L.M.,

- Monserat, J.M., 2012. In vitro evaluation of co-exposure of arsenium and an organic nanomaterial (fullerene, C 60) in zebrafish hepatocytes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 155 (2), 206–212.
- De Jong, W.H., Borm, P.J., 2008. Drug delivery and nanoparticles: applications and hazards. *Int. J. Nanomed.* 3 (2), 133.
- Dhawan, A., Taurozzi, J.S., Pandey, A.K., Shan, W., Miller, S.M., Hashsham, S.A., Tarabara, V.V., 2006. Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity. *Environ. Sci. Technol.* 40 (23), 7394–7401.
- Du, M., Zhang, H., Li, J., Yan, C., Zhang, X., Chang, X., 2016. Bioaccumulation, depuration, and transfer to offspring of 13C-labeled fullereneols by *Daphnia magna*. *Environ. sci. technol.* 50 (19), 10421–10427.
- Duncan, L.K., Jinschek, J.R., Vikesland, P., 2007. 417 J., C60 colloid formation in aqueous systems: effects of preparation method on size, structure, and surface charge. *Environ. Sci. Technol.* 42 (1), 173–178.
- Emke, E., Sanchís, J., Farre, M., Bäuerlein, P., de Voigt, P., 2015. Determination of several fullerenes in sewage water by LC HR-MS using atmospheric pressure photoionisation. *Environ. Sci.: Nano* 2 (2), 167–176.
- Encinas, D., Gómez-de-Balugera, Z., 2018. Fullerene C60 in atmospheric aerosol and its relationship to combustion processes. *Arch. Environ. Contam. Toxicol.* 1–9. <https://doi.org/10.1007/s00244-018-0524-z>.
- Ferreira, J.L.R., Lonné, M.N., Franca, T.A., Maximilla, N.R., Lugokenski, T.H., Costa, P.G., Fillmann, G., Soares, F.A.A., Fernando, R., Monserat, J.M., 2014. Co-exposure of the organic nanomaterial fullerene C 60 with benzo [a] pyrene in *Danio rerio* (zebrafish) hepatocytes: evidence of toxicological interactions. *Aquat. Toxicol.* 147, 76–83.
- Freixa, A., Ejarque, E., Crognale, S., Amalfitano, S., Fazi, S., Butturini, A., Romani, A.M., 2016. Sediment microbial communities rely on different dissolved organic matter sources along a Mediterranean river continuum. *Limnol. Oceanogr.* 61 (4), 1389–1405.
- Freixa, A., Acuña, V., Sanchís, J., Farré, M., Barceló, D., Sabater, S., 2018a. Ecotoxicological effects of carbon based nanomaterials in aquatic organisms. *Sci. Total Environ.* 619, 328–337.
- Freixa, A., Acuña, V., Gutierrez, M., Sanchís, J., Santos, L.H., Rodriguez-435 Mozaz, S., Farré, M., Barceló, D., Sabater, S., 2018b. Fullerenes influence the toxicity of organic micro contaminants to river biofilms. *Front. Microbiol.* 9, 1426.
- Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO₂, ZnO, Ag, CNT, fullerenes) for different regions. *Environ. Sci. Technol.* 43 (24), 9216–9222.
- Gottschalk, F., Sun, T., Nowack, B., 2013. Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. *Environ. Pollut.* 181 (0), 287–300.
- Henry, T.B., Wileman, S.J., Boran, H., Sutton, P., 2013. Association of Hg₂⁺ with aqueous (C60) n aggregates facilitates increased bioavailability of Hg₂⁺ in Zebrafish (*Danio rerio*). *Environ. sci. technol.* 47 (17), 9997–10004.
- Howard, J.B., McKinnon, J.T., Makarovskiy, Y., Lafleur, A.L., Johnson, M.E., 1991. Fullerenes C60 and C70 in flames. *Nature* 352 (6331), 139–141.
- Jafvert, C.T., Kulkarni, P.P., 2008. Buckminsterfullerene's (C60) Octanol – Water partition coefficient (K_{ow}) and aqueous solubility. *Environ. Sci. Technol.* 42 (16), 5945–5950.
- Jurkowska, B., Jurkowski, B., Kamrowski, P., Pesetskii, S., Koval, V., Pinchuk, L., Olkhov, Y.A., 2006. Properties of fullerene- containing natural rubber. *J. Appl. Polym. Sci.* 100 (1), 390–398.
- Krätschmer, W., Huffman, D.R., Jura, M., Leach, S., 1993. Production and discovery of fullerenes: new forms of crystalline carbon [and discussion]. *Philos. Trans. R. Soc. London, Ser. A: Phys. Eng. Sci.* 343 (1667), 33–38.
- Laitinen, T., Petäjä, T., Backman, J., Hartonen, K., Junninen, H., Ruiz-Jiménez, J., Worsnop, D., Kulmala, M., Riekkola, M.-L., 2014. Carbon clusters in 50 nm urban air aerosol particles quantified by laser desorption-ionization aerosol mass spectrometer. *Int. J. Mass Spectrom.* 358 (0), 17–24.
- Lundqvist, A., Bertilsson, S., Goedkoop, W., 2012. Interactions with DOM and biofilms affect the fate and bioavailability of insecticides to invertebrate grazers. *Ecotoxicology* 21 (8), 2398–2408.
- Lyon, D.Y., Fortner, J.D., Sayes, C.M., Colvin, V.L., Hughes, J.B., 2005. Bacterial cell association and antimicrobial activity of a C60 water suspension. *Environ. Toxicol. Chem.* 24 (11), 2757–2762.
- Naasz, S., Altenburger, R., Kühnel, D., 2018. Environmental mixtures of nanomaterials and chemicals: the Trojan-horse phenomenon and its relevance for ecotoxicity. *Sci. Total Environ.* 635, 1170–1181.
- Núñez, Ó., Gallart-Ayala, H., Martins, C.P.B., Moyano, E., Galceran, M.T., 2012. Atmospheric pressure photoionization Mass Spectrometry of fullerenes. *Anal. Chem.* 84 (12), 5316–5326.
- Oberdörster, E., 2004. Manufactured nanomaterials (473 fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environ. Health Perspect.* 112 (10), 1058.
- Petersen, E.J., Akkanen, J., Kukkonen, J.V., Weber Jr., W.J., 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ. sci. technol.* 43 (8), 2969–2975.
- Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B.K., Kaplan, L.A., Lock, M.A., Naegeli, M.W., Traunsperger, W., 1998. The role of micro-organisms in the ecological connectivity of running waters. *Freshw. Biol.* 40 (3), 453–495.
- Sabater, S., Guasch, H., Ricart, M., Romani, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Anal. Bioanal. Chem.* 387 (4), 1425–1434.
- Sanchís, J., Berrojalbiz, N., Caballero, G., Dachs, J., Farré, M., Barceló, D., 2012. Occurrence of aerosol-bound fullerenes in the mediterranean sea atmosphere. *Environ. Sci. Technol.* 46 (3), 1335–1343.
- Sanchís, J., Božović, D., Al-Harbi, N.A., Silva, L.F., Farré, M., Barceló, D., 2013. Quantitative trace analysis of fullerenes in river sediment from Spain and soils from Saudi Arabia. *Anal. Bioanal. Chem.* 405 (18), 5915–5923.
- Sanchís, J., Oliveira, L.F.S., de Leão, F.B., Farré, M., Barceló, D., 2015a. Liquid chromatography–atmospheric pressure photoionization–Orbitrap analysis of fullerene aggregates on surface soils and river sediments from Santa Catarina (Brazil). *Sci. Total Environ.* 505, 172–179 492.
- Sanchís, J., Bosch-Orea, C., Farré, M., Barceló, D., 2015b. Nanoparticle tracking analysis characterisation and parts-per-quadrillion determination of fullerenes in river samples from Barcelona catchment area. *Anal. Bioanal. Chem.* 407 (15), 4261–4275.
- Sanchís, J., Olmos, M., Vincent, P., Farré, M., Barceló, D., 2015c. New insights on the influence of organic Co-contaminants on the aquatic toxicology of carbon Nanomaterials. *Environ. sci. technol.* 50 (2), 961–969.
- Sanchís, J., Llorca, M., Olmos, M., Schirizzi, G.F., Bosch-Orea, C., Abad, E., Barceló, D., Farre, M., 2017. Metabolic responses of mytilus galloprovincialis to fullerenes in mesocosms exposure experiments. *Environ. Sci. Technol.*
- Sanchís, J., Aminot, Y., Abad, E., Jha, A.N., Readman, J.W., Farré, M., 2018. Transformation of C60 fullerene aggregates suspended and weathered under realistic environmental conditions. *Carbon* 128, 54–62.
- Santín, G., Eljarrat, E., Barceló, D., 2016. Bioavailability of classical and novel flame retardants: effect of fullerene presence. *Sci. Total Environ.* 565, 299–305.
- Tao, X., He, Y., Zhang, B., Chen, Y., Hughes, J.B., 2011. Effects of stable aqueous fullerene nanocrystal (nC60) on *Daphnia magna*: evaluation of hop frequency and accumulations under different conditions. *J. Environ. Sci.* 23 (2), 322–329.
- Tervonen, K., Waissi, G., Petersen, E.J., Akkanen, J., Kukkonen, J.V., 2010. Analysis of fullerene- C60 and kinetic measurements for its accumulation and depuration in *Daphnia magna*. *Environ. Toxicol. Chem.* 29 (5), 1072–1078.
- Thompson, B.C., Fréchet, J.M.J., 2008. Polymer-fullerene composite solar cells. *Angew. Chem. Int. Ed.* 47 (1), 58–77.
- Tiwari, A.J., Ashraf-Khorassani, M., Marr, L.C., 2016. C60 fullerenes from combustion of common fuels. *Sci. Total Environ.* 547, 254–260.
- Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2008. Fullerene C60 exposure elicits an oxidative stress response in embryonic zebrafish. *Toxicol. Appl. Pharmacol.* 229 (1), 44–55.
- Utsunomiya, S., Jensen, K.A., Keeler, G.J., Ewing, R.C., 2002. Uraninite and fullerene in atmospheric particulates. *Environ. Sci. Technol.* 36 (23), 4943–4947.
- Wang, H., Kostel, J.A., Amand, A.L.S., Gray, K.A., 1999. 2. The response of a laboratory stream system to PCB exposure: study of periphytic and sediment accumulation patterns. *Water Res.* 33 (18), 3749–3761.
- Yeo, M.-K., Nam, D.-H., 2013. Influence of different types of nanomaterials on their bioaccumulation in a paddy microcosm: a comparison of TiO₂ nanoparticles and nanotubes. *Environ. Pollut.* 178, 166–172.
- Zhou, K., Hu, Y., Zhang, L., Yang, K., Lin, D., 2016. The role of exopolymeric substances in the bioaccumulation and toxicity of Ag nanoparticles to algae. *Sci. Rep.* 6, 32998.
- Zhu, Q., Cox, D.E., Fischer, J.E., Kniaz, K., McGhie, A.R., Zhou, O., 1992. Intercalation of solid C60 with iodine. *Nature* 355 (6362), 712.
- Zhu, X., Zhu, L., Lang, Y., Chen, Y., 2008. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environ. Toxicol. Chem.* 27 (9), 1979–1985.