

# Nanoparticle tracking analysis characterisation and parts-per-quadrillion determination of fullerenes in river samples from Barcelona catchment area

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**Abstract** In the present work, the analysis of seven fullerenes ( $C_{60}$  and  $C_{70}$  fullerenes and five functionalised fullerenes) has been performed in river samples collected in the vicinities of Barcelona (Catalonia, NE of Spain). The results of 48 samples (25 river waters, 12 river sediments and 11 wastewater effluents) are presented. Extracts of river water, river sediments and wastewater effluents were analysed by liquid chromatography (LC), using a pyrenylpropyl group bonded silica based column, coupled to a high-resolution mass spectrometer (HRMS), using a dual ion source, atmospheric pressure photoionisation/atmospheric pressure chemical ionisation source (APPI/APCI). The novel methodology presents good chromatographic separation, excellent selectivity and instrumental limits of quantification (ILOQ) in the femtogram order. Method limits of quantification (MLOQ) ranged from 2.9 to 17 pg/l and from 3.2 to 31 pg/l in surface waters and wastewaters, respectively. In wastewater effluents, the sums of  $C_{60}$  and  $C_{70}$  ranged from 0.5 to 9.3 ng/l. In surface waters,  $C_{60}$  fullerene was the most ubiquitous compound, being detected in 100 % of the samples in concentrations from 31 pg/l to 4.5 ng/l, while  $C_{70}$  concentrations ranged from less than the method limits of detection (MLOD) to 1.5 ng/l. The presence of fullerenes in both the large particulate (diameter  $\varnothing > 450$  nm) and the colloidal ( $\varnothing < 450$  nm) fractions of surface waters should be noticed. In sediments, the concentrations of

fullerenes were between the MLOD and 34.4 pg/g. In addition, nanoparticle tracking analysis (NTA) was used for the characterisation of water samples in terms of nanoparticle number concentration and size distribution. As far as our knowledge is concerned, this is the first time that NTA has been used for the characterisation of complex river waters with an environmental focus.

**Keywords** Fullerenes ·  $C_{60}$  ·  $C_{70}$  · River water · Wastewater · Sediments

## Introduction

Fullerenes are a nanostructured class of carbon allotropes, discovered and structurally elucidated by Kroto et al. in 1985 [1]. Currently, the possibility to manipulate material at the nanoscale and the physicochemical properties exhibited by the fullerenes, such as thermal stability, conductivity, adsorption and catalytic capacity, have driven the exploration of their potential applications in different industrial fields [2]. In the USA, their industrial production was estimated at between 2 and 81 tons, in 2011 [3]. However, in the coming years, their use is expected to increase because of their inclusion in new drug delivery systems [4], medical imaging [5], antitumor agents [6], microelectronics [7], solar panels [8] and new materials [9].

Currently, however, fullerene risk assessment analysis is impaired by the lack of works dealing with their characterisation and analysis in complex environmental matrices [10]. Therefore, there are gaps of information with respect to their environmental occurrence, fate and transport. Moreover, as a consequence of the alteration of their physicochemical properties and aggregation states, toxicity tests appropriated for bulk substances may introduce artefacts during their study [11–13]. Therefore, specific and standardised toxicity tests,

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addressing these limitations and using new endpoints or new toxicity biomarkers for nanomaterials, continue being required in order to obtain more reliable data [11]. In contrast, while potential negative effects, such as antimicrobial activity [14], bioaccumulation [15, 16] and sub-lethal effects to aquatic organisms [17, 18], have been related to high concentrations of fullerenes in surface water, there is still a scarcity of available information about their occurrence and physicochemical states in real environmental scenarios. In addition, during recent years, the potential association of carbon-based nanomaterials with other organic contaminants, and how they may modulate their bioavailability and toxicity, have been subject to scientific attention [19].

In defining real environmental scenarios of exposure, one of the main limitations has been the development of analytical methods sensitive enough for the detection of fullerenes in complex samples. Currently, different sample preparation approaches have been presented, based on liquid–liquid extraction (LLE) [20–24], solid-phase extraction (SPE) [20–24] and filtration followed by ultrasonic-assisted extraction (UAE) with toluene [25, 26]. High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS) has been the technique of choice for their separation and detection [25–31]. Table 1 summarises the main analytical methodologies, which have been applied to water samples. Electrospray ionisation (ESI) [25, 27], atmospheric pressure ionisation (APCI) [32] and atmospheric pressure photoionisation (APPI) [31] have been used as ionisation techniques. However, it is noteworthy that the best performance in terms of sensitivity has been achieved by APPI. Furthermore, few studies have assessed the presence of fullerenes in the aquatic environment [25, 31, 33, 34], to date. In these studies, the concentrations of fullerenes in surface water ranged from the method limits of detection (MLOD) to 130 ng/l of C<sub>60</sub> fullerene [34]. C<sub>60</sub> and C<sub>70</sub> fullerenes were also recently detected in particulates from the Mediterranean Sea atmosphere [28]. In addition, surface waters are natural receptors of fullerenes from wastewater disposal [25] and atmospheric deposition [28].

In order to better understand the environmental behaviour of fullerenes, besides the quantitative analysis, their characterisation in the environment is required.

In this study, a novel combined approach is presented in order to characterise the nanoparticles' content and size and to assess the occurrence of seven fullerenes (C<sub>60</sub>, C<sub>70</sub>, *N*-methylfulleropyrrolidine, [6,6]-phenyl C<sub>61</sub> butyric acid methyl ester, [6,6]-thienyl C<sub>61</sub> butyric acid methyl ester, C<sub>60</sub> pyrrolidinetris-acid ethyl ester and [6,6]-phenyl C<sub>71</sub> butyric acid methyl ester) in environmental water samples and wastewater. The size distribution and content of nanoparticles were assessed by nanoparticle tracking analysis (NTA). In parallel, a method based on UAE with toluene, followed by liquid chromatography coupled to high-resolution mass

spectrometry, using an APPI ionisation source in negative conditions (LC–APPI–HRMS), was applied for the analysis of fullerenes. In comparison with the previous approaches, this analytical method presents mass accuracy, robustness and an improved sensitivity.

This combined approach was used to assess 48 real environmental samples, collected within the vicinities of Barcelona City (Spain): 12 river sediments (4 from the Besòs River and 8 from the Llobregat River) and 36 water samples (11 treated effluents from 6 different wastewater treatment plants (WWTPs), 25 river waters (10 from the Besòs River and 15 from the Llobregat River)).

This study contributes to augmenting data on fullerene occurrence in real surface water.

## Experimental section

### Chemicals and materials

Fullerene standards C<sub>60</sub> fullerene (99.9 % purity, CAS: 99685-96-8), C<sub>70</sub> (99 % purity, CAS: 115383-22-7), *N*-methylfulleropyrrolidine (abbrev. [60]NMFP, 99 % purity, CAS: 151872-44-5), [6,6]-phenyl C<sub>61</sub> butyric acid methyl ester (abbrev. [60]PCBM, >99.5 % purity, CAS: 160848-22-6), [6,6]-thienyl C<sub>61</sub> butyric acid methyl ester (abbrev. [60]ThPCBM, ≥99 % purity, CAS: 925673-03-6), C<sub>60</sub> pyrrolidinetris-acid ethyl ester (abbrev. [60]PTAE, >97 % purity) and [6,6]-phenyl C<sub>71</sub> butyric acid methyl ester (abbrev. [70]PCBM, 99 %, CAS: 609771-63-3) were purchased from Sigma-Aldrich (Steinheim, Germany). <sup>13</sup>C-labelled C<sub>60</sub> fullerene was purchased from MER Corporation (Tucson, AZ, USA).

Sodium chloride (≥99.5 % ACS reagent, reference 71380-500G) was purchased from Sigma-Aldrich (Steinheim, Germany). Toluene and methanol CHROMASOLV<sup>®</sup> were supplied by Merck (Darmstadt, Germany). Nitrogen, used as drying gas with 99.995 % purity, was acquired from Air Liquide (Spain).

### Sampling

A total number of 48 samples (10 surface waters and 4 sediments from the Besòs River, 15 surface waters and 8 sediments from the Llobregat River and 11 wastewater effluents) were collected within the vicinities of Barcelona City (Spain). Details are summarised in Table 2.

River water samples were collected during periods December 2013 and February 2014, after a dry climatic period and shortly after a precipitation period, respectively. The river sediments were collected during the second sampling campaign at the same sampling sites as those of the surface waters (Table 2).

Wastewater effluents were taken from six different WWTPs in the municipalities of Sabadell and Montcada i Reixac, serving

**Table 1** Selected relevant methods for the HPLC–MS analysis of fullerenes in water samples

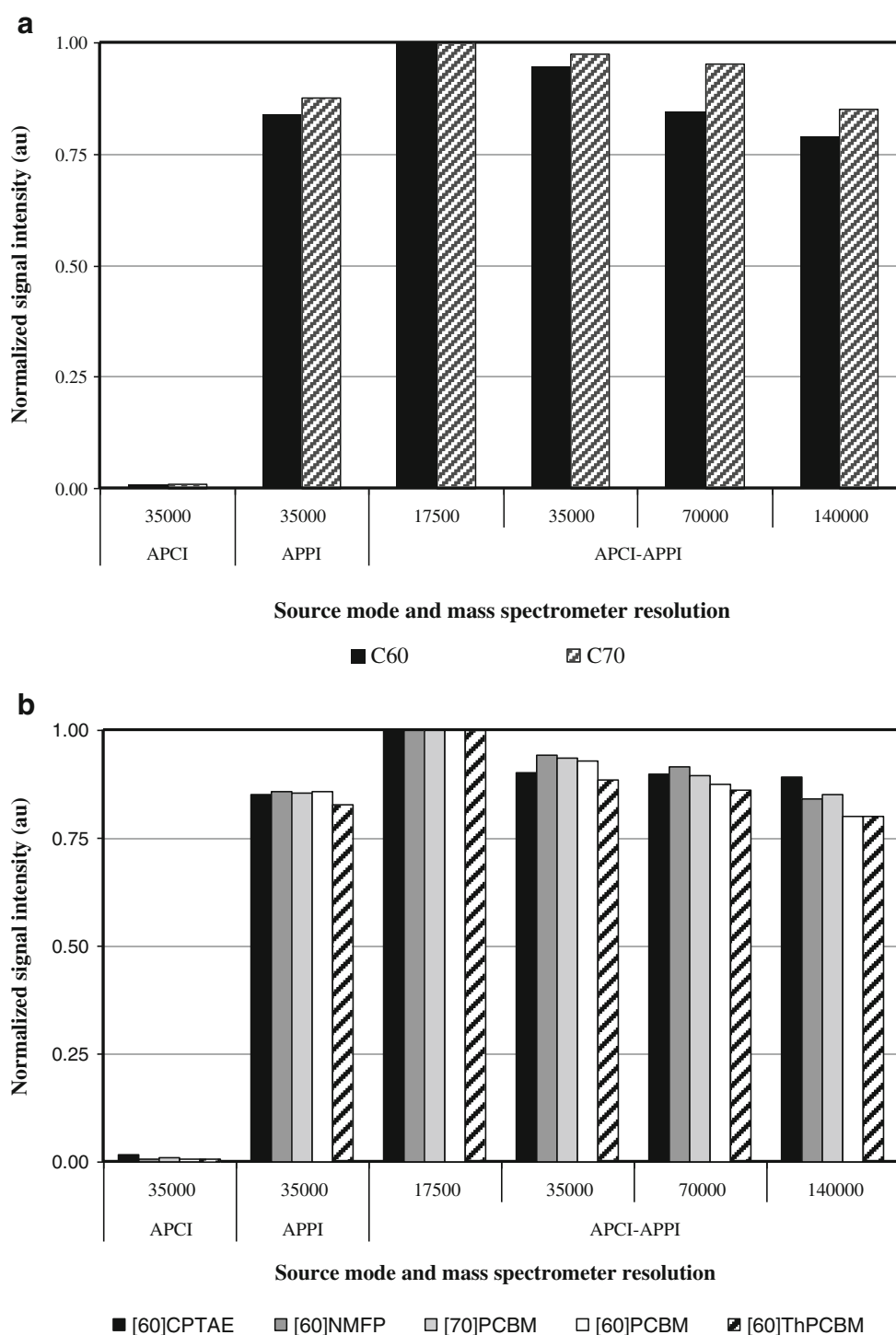
Reference	Analytes	Column (length × diameter, particle size)	Mobile phase	Interface	Mass spectrometer (acquisition mode)	Extraction	P.F.	MLOD
Chen et al. [20]	C <sub>60</sub>	Nova-Pak C18 (150 × 3.9 mm)	Toluene:ACN (55:45)	APCI	Varian 1200 QqQ (SIM mode)	-Evaporation -Residue resuspension LLE SPE	111 55.6 1000	2.8 ng/ml (UPW and TW) 3.3 ng/ml (UPW and TW) 0.3 ng/ml (UPW and TW)
Farré et al. [25]	C <sub>60</sub> , C <sub>70</sub> , NMFP	Purospher STAR RP-18 (12.5 2.0 mm, 5 μm)	Toluene:MeOH (50:50)	ESI	Applied Biosystems API 3200 QqQ (SRM mode)	-Filtration -Ultrasonics with toluene	133	1–10 ng/l (UPW) 2–12 ng/l (SW) 5–20 ng/l (WW)
Wang et al. [22]	C <sub>60</sub>	COSMOSIL Buckyprep (250 4.6 mm, 5 μm)	Toluene	APCI	Agilent 6120 Quadrupole (SIM mode)	LLE SPE	10 1000	4–11 ng/ml (WW) Not detailed
Núñez et al. [31]	C <sub>60</sub> , C <sub>70</sub> , C <sub>76</sub> , C <sub>78</sub> and C <sub>84</sub>	Hypersil GOLD C18 (150 2.1 mm, 1.9 μm)	Toluene	APPI	Thermo TSQ Quantum Ultra AM (H-SIM mode)	-Filtration -Ultrasonics with toluene	500	0.01–5.0 ng/l (SW)
Kolkman et al. [24]	C <sub>60</sub> , C <sub>70</sub> and 6 functionalized fullerenes	COSMOSIL Buckyprep (250 2.0 mm, 5 μm)	Toluene:ACN (75:25)	ESI	Thermo LTQ Orbitrap (full-scan mode)	SPE	1000	0.17–0.28 ng/l (UPW)

P.F. preconcentration factor (initial water volume/final extract volume), ACN acetonitrile, MeOH methanol, SIM single-ion monitoring, SRM single-reaction monitoring, H-SIM high-resolution single-ion monitoring, LLE liquid–liquid extraction, SPE solid-phase extraction, UPW ultrapure water, UPW tap water, SW surface water, WW wastewater

**Table 2** Characteristics of the sediment, wastewater and surface water samples

	Code	Sampling	River basin	River	Town	Coordinates		
Surface water	S01	9 Dec 2013	Besòs	Riu-Sec	Sabadell	41.51441; 2.108685		
	S02	9 Dec 2013	Besòs	Ripoll	Montcada i Reixac	41.494917; 2.151039		
	S03	9 Dec 2013	Besòs	Ripoll	Montcada i Reixac	41.488159; 2.187565		
	S04	9 Dec 2013	Besòs	Besòs	Montcada i Reixac	41.489726; 2.192799		
	S05	9 Dec 2013	Besòs	Besòs	Santa Coloma de Gramanet	41.419668; 2.231822		
	S06	9 Dec 2013	Besòs	Besòs	Sant Adrià del Besòs	41.419817; 2.231927		
	S07	9 Dec 2013	Llobregat	Cardener	Manresa	41.720803; 1.827565		
	S08	9 Dec 2013	Llobregat	Llobregat	Monistrol de Montserrat	41.61217; 1.846304		
	S09	9 Dec 2013	Llobregat	Llobregat	Martorell	41.492474; 1.921399		
	S10	9 Dec 2013	Llobregat	Riera de Rubí	Rubí	41.456371; 2.001271		
	S11	9 Dec 2013	Llobregat	Riera de Rubí	Rubí	41.460988; 2.000863		
	S12	9 Dec 2013	Llobregat	Llobregat	Sant Feliu de Llobregat	41.384444; 2.025893		
	S13	9 Dec 2013	Llobregat	Llobregat	Sant Feliu de Llobregat	41.381574; 2.033401		
	S14	12 Feb 2014	Besòs	Besòs	Santa Coloma de Gramanet	41.419668; 2.231822		
	S15	12 Feb 2014	Besòs	Ripoll	Montcada i Reixac	41.488159; 2.187565		
	S16	12 Feb 2014	Besòs	Besòs	Montcada i Reixac	41.489726; 2.192799		
	S17	12 Feb 2014	Besòs	Riu-Sec	Sabadell	41.51441; 2.108685		
	S18	12 Feb 2014	Llobregat	Riera de Rubí	Les Fonts	41.52172; 2.037274		
	S19	12 Feb 2014	Llobregat	Riera de Rubí	Les Fonts	41.51161; 2.033814		
	S20	12 Feb 2014	Llobregat	Llobregat	Sant Feliu de Llobregat	41.381574; 2.033401		
	S21	12 Feb 2014	Llobregat	Llobregat	Sant Feliu de Llobregat	41.384444; 2.025893		
	S22	12 Feb 2014	Llobregat	Riera de Rubí	Rubí	41.456371; 2.001271		
	S23	12 Feb 2014	Llobregat	Riera de Rubí	Rubí	41.460988; 2.000863		
	S24	12 Feb 2014	Llobregat	Cardener	Manresa	41.720803; 1.827565		
	S25	12 Feb 2014	Llobregat	Cardener	Castellgallí	41.681077; 1.848878		
Sediments	SED01	12 Feb 2014	Besòs	Besòs	Santa Coloma de Gramanet	41.419668; 2.231822		
	SED02	12 Feb 2014	Besòs	Ripoll	Montcada i Reixac	41.488159; 2.187565		
	SED03	12 Feb 2014	Besòs	Besòs	Montcada i Reixac	41.489726; 2.192799		
	SED04	12 Feb 2014	Besòs	Riu-Sec	Sabadell	41.51441; 2.108685		
	SED05	12 Feb 2014	Llobregat	Riera de Rubí	Les Fonts	41.52172; 2.037274		
	SED06	12 Feb 2014	Llobregat	Riera de Rubí	Les Fonts	41.51161; 2.033814		
	SED07	12 Feb 2014	Llobregat	Llobregat	Sant Feliu de Llobregat	41.381574; 2.033401		
	SED08	12 Feb 2014	Llobregat	Llobregat	Sant Feliu de Llobregat	41.384444; 2.025893		
	SED09	12 Feb 2014	Llobregat	Riera de Rubí	Rubí	41.456371; 2.001271		
	SED10	12 Feb 2014	Llobregat	Riera de Rubí	Rubí	41.460988; 2.000863		
	SED11	12 Feb 2014	Llobregat	Cardener	Manresa	41.720803; 1.827565		
Wastewater effluents		Code	Sampling	River basin	Town	Equivalent population	Capacity (m <sup>3</sup> /day)	Coordinates
	Eff-1	9 Dec 2013	Llobregat	St. Feliu de Llobregat	373,333	64,000	41.381096; 2.033433	
	Eff-2	9 Dec 2013	Llobregat	Manresa	196,167	53,500	41.703772; 1.843232	
	Eff-3	9 Dec 2013	Llobregat	Rubí	135,000	27,000	41.461447; 2.003419	
	Eff-4	9 Dec 2013	Besòs	Sabadell-Riu Sec	296,333	33,000	41.517075; 2.101912	
	Eff-5	9 Dec 2013	Llobregat	Montcada	423,500	72,600	41.472330; 2.190766	
	Eff-6	12 Feb 2014	Llobregat	Terrassa	400,000	75,000	41.517907; 2.034551	
	Eff-7	12 Feb 2014	Llobregat	St. Feliu de Llobregat	373,333	64,000	41.381096; 2.033433	
	Eff-8	12 Feb 2014	Llobregat	Manresa	196,167	53,500	41.703772; 1.843232	
	Eff-9	12 Feb 2014	Llobregat	Rubí	135,000	27,000	41.461447; 2.003419	
	Eff-10	12 Feb 2014	Llobregat	Sabadell-Riu Sec	296,333	33,000	41.517075; 2.101912	
Eff-11	12 Feb 2014	Besòs	Montcada	423,500	72,600	41.472330; 2.190766		

**Fig. 1** Intensities ( $n=3$ ) obtained using APPI, APCI and a hybrid APCI/APPI ionisation mode for **a** pristine fullerenes and **b** functionalized fullerenes



about 207,649 and 34,863 inhabitants, respectively, and discharging at the Besòs River, and also from the municipalities of Terrassa, Manresa, Rubí and Sant Feliu de Llobregat, serving about 215,055, 76,170, 74,468 and 43,769 inhabitants, respectively, and discharging at the Llobregat River.

Water samples were collected in amber glass bottles in duplicates, and a metal dredge was employed for sediment sampling. The samples were immediately transported to the

laboratory at a temperature of 4 °C. The sample pre-treatment and extraction were carried out within the 24 h following arrival at the laboratory.

#### Sample preparation

Water samples were extracted according to a previous protocol [25] after some modifications and additions. In brief,

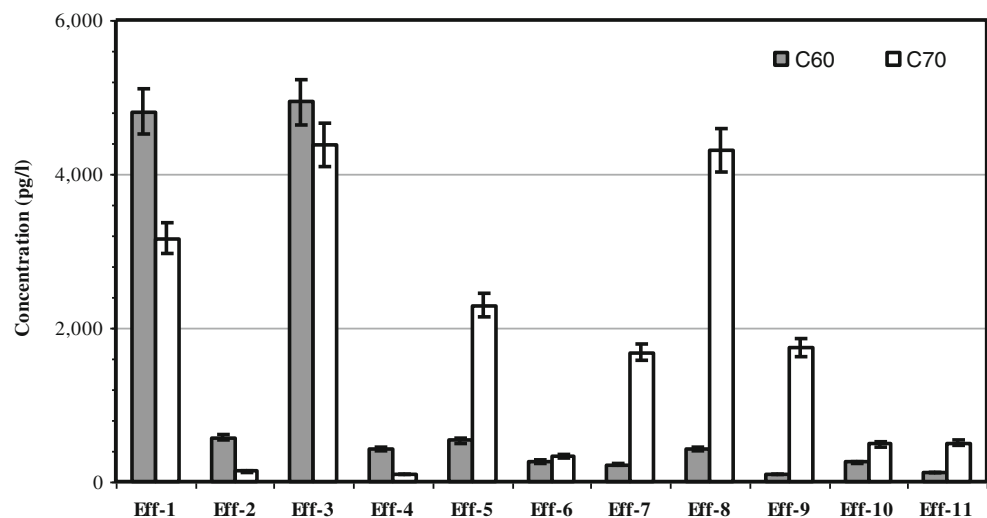
**Table 3** Instrumental quality parameters for the analysis of fullerenes by HPLC–APPI(–)–Q–Orbitrap–MS. Method quality parameters for the analysis of fullerenes in surface waters and wastewaters

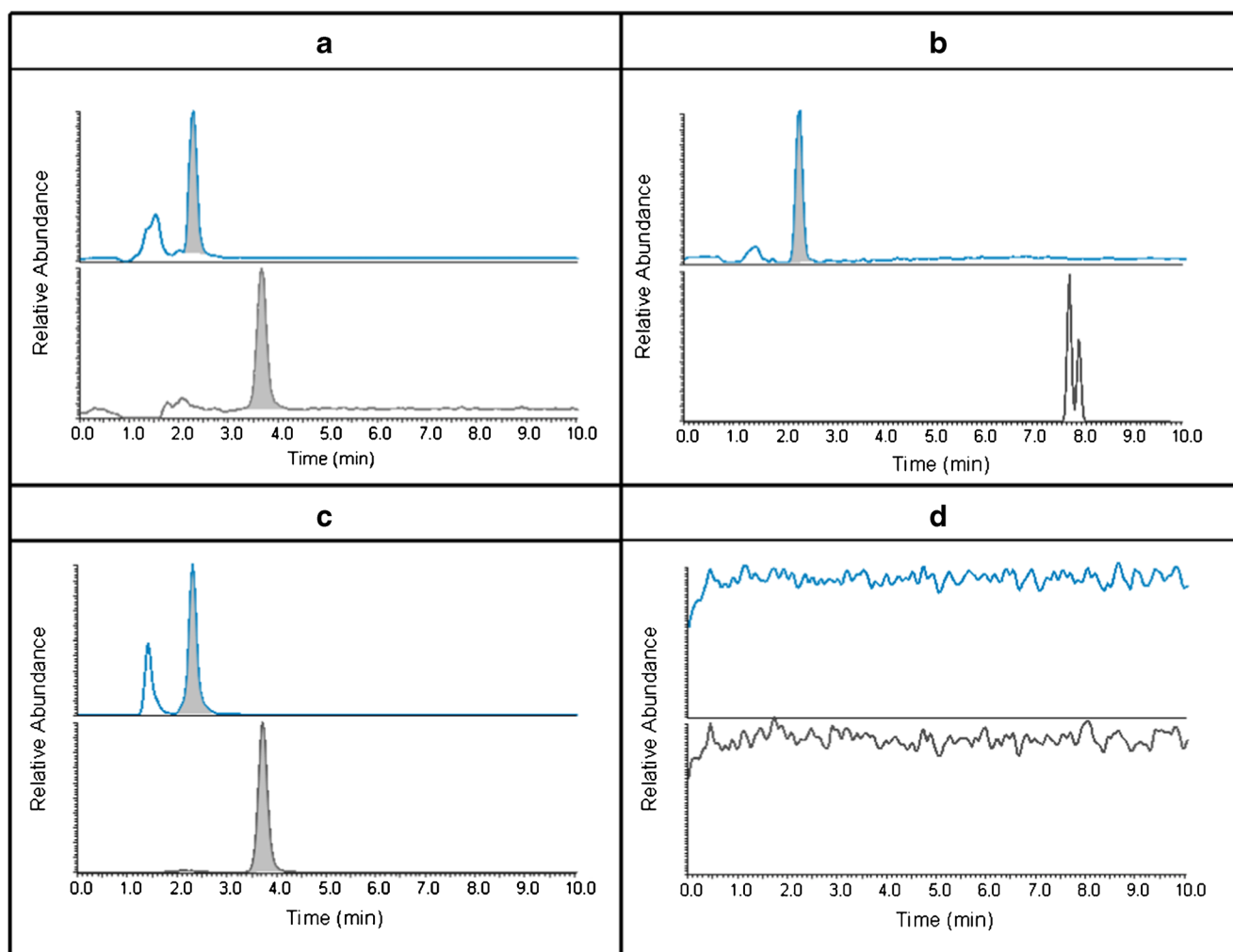
Parameter (units)		C <sub>60</sub>	C <sub>70</sub>	[60]CPTAE	[60]PCBM	[60]ThPCBM	[60]NMFP	[70]PCBM	
Instrumental parameters	ILOD (fg)	1	10	100	1	1	1	100	
	ILOQ (fg)	3	30	300	3	3	3	300	
	Asymmetry factors	1.1	1.1	1.1	0.92	1.0	0.93	0.93	
	Peak tailing factor	0.94	1.0	1.1	0.97	1.1	1.1	1.1	
	Linearity <sup>a</sup> , R <sup>2</sup>	0.9995	0.9982	0.9991	0.9981	0.9980	0.9980	0.9973	
	Sensitivity (a.u.)	4.2	3.3	0.94	2.0	1.8	0.78	0.71	
	Inter-day precision (%)	50 fg/μl	9.0	5.9	9.7	6.0	5.2	8.1	6.6
		100 fg/μl	8.5	5.2	9.3	5.7	4.3	4.0	5.1
		500 fg/μl	8.2	3.6	4.9	5.4	4.0	3.5	3.7
Method parameters	Surface water	MLOD (pg/l)	0.9	1.3	2.2	2.2	2.3	1.4	4.9
		MLOQ (pg/l)	2.9	4.4	7.2	7.2	7.2	4.8	17
	Wastewater	MLOD (pg/l)	2.3	2.4	4.1	1.0	1.1	0.7	9.4
		MLOQ (pg/l)	7.6	8.0	14	3.2	3.3	2.4	31
	Inter-day precision (%)	10 ng/l	12	13	11	13	14	12	12
		50 ng/l	10	8.1	4.2	9.3	9.6	7.3	9.1
		1000 ng/l	3.2	5.3	2.6	4.6	4.4	3.3	2.0
	Recovery (%)	10 ng/l	100	110	68	86	79	85	80
		50 ng/l	102	105	73	93	98	99	89
		1000 ng/l	99	100	88	97	99	100	95
	Matrix effect in surface water (%)	10 ng/l	80	64	94	68	61	60	67
		50 ng/l	86	68	99	74	68	66	72
		1000 ng/l	99	67	102	107	83	111	94
	Matrix effect in wastewater (%)	10 ng/l	55	62	94	60	55	65	70
		50 ng/l	55	64	96	64	57	72	73
		1000 ng/l	75	90	137	87	71	123	135

<sup>a</sup>Linearity is assessed between MLOQ and 5.0 ng injected mass

100 μl of <sup>13</sup>C-labelled C<sub>60</sub> fullerene at 1 pg/μl in toluene was dispersed in 2 ml of ethanol and spiked to 150 ml of surface water or to 100 ml of effluent wastewater samples. Samples were mixed thoroughly and filtered through 0.7 μm glass fibre

and 0.45 μm nylon filters (Whatman, Maidstone, UK), previously tared. Particulate and filtered water were extracted and analysed in parallel for both surface water and wastewater samples.

**Fig. 2** Concentrations in wastewater effluent samples



**Fig. 3** Example of extracted chromatograms of **a** real surface water, **b** a sediment sample, **c** a standard mixture, and **d** a blank sediment

The filters were dried at 60 °C for 24 h and were then weighed (the remaining humidity did not interfere in the analyses). Afterwards, the filters were extracted by UAE with 35 ml of toluene for 45 min. This process was repeated twice more. The combined extracts were collected in an amber spherical flask, and the excess of toluene was eliminated by rotary evaporation to 0.5 ml, approximately. Then, it was quantitatively transferred to a tared amber vial. The final volume was adjusted by weight to 1000 ml with toluene.

To assess the fullerenes in the filtrated samples, a large-volume liquid–liquid extraction (LLE), based on the method described by Bouchard and Ma [21], was performed. In brief, the filtered samples (100 ml of wastewater effluents or 150 ml of river waters) were placed in a separatory funnel and NaCl was added in order to obtain a 1 % saline solution. Then, the extraction was performed with toluene. After centrifugation, the toluene fraction was collected. This process was repeated twice more. Then, the process was continued, as already described.

Sediment samples were extracted after minor modifications, according to the procedure described by Sanchis et al. [26]. Wet sediments were centrifuged at 3000g for 5 min. After water decantation, sediments were dried overnight in a desiccator. Then, the samples were ground with an agate mortar and sieved (<125 μm). Ten grams of sieved sediment was placed into a glass vessel and fortified with 100 μl of <sup>13</sup>C-labelled C<sub>60</sub> fullerene suspension (1.0 pg/μl in toluene) and used as internal standard. Then, sediment samples were homogenised and left in the dark for 3 h at 4 °C in order to reach equilibrium. After this time, 40 ml of toluene was added and sediments were extracted by UAE during a period of 4 h. The suspension was centrifuged at 3000g for 10 min. This process was repeated twice more. Then, the combined extract (80 ml) was introduced into a spherical flask and concentrated by rotary evaporation to 1 ml, approximately. Afterwards, it was transferred to a pre-tared vial and evaporated with a gentle nitrogen stream to 500 μl, approximately. The final volume was adjusted to 1.000 ml by weight.

**Table 4** Fullerenes concentrations in wastewater and freshwater samples

Sample			C <sub>60</sub> (pg/l)	C <sub>70</sub> (pg/l)	C <sub>60</sub> /C <sub>70</sub> ratio							
Wastewater samples	Dec 2013	Sant Feliu de Llobregat	4810	3160	1.5							
			Feb 2014	Manresa	568	138	4.1					
					Rubí	4930	4380	1.1				
						Sabadell-Riu Sec	429	88.4	4.8			
							Montcada	530	2290	0.23		
								Terrassa	259	328	0.79	
	Sant Feliu de Llobregat	220							1680	0.13		
		Manresa	421	4300					0.10			
			Rubí	91	1735				0.05			
				Sabadell-Riu Sec	254	484			0.52			
					Montcada	116	501		0.23			
						Surface water	Dec 2013	Besòs River Basin	S01	$\varnothing > 450$ nm	204	9.62
	$\varnothing < 450$ nm									136	<MLOD	–
S02	$\varnothing > 450$ nm	300							26.8	11		
	$\varnothing < 450$ nm	27.5	<MLOD						–			
S03	$\varnothing > 450$ nm	136	49.9	2.7								
	$\varnothing < 450$ nm	<MLOD	<MLOD	–								
S04	$\varnothing > 450$ nm	312	83.8	3.7								
	$\varnothing < 450$ nm	<MLOD	<MLOD	–								
S05	$\varnothing > 450$ nm	31.2	<MLOD	–								
	$\varnothing < 450$ nm	<MLOD	<MLOD	–								
S06	$\varnothing > 450$ nm	93.9	54.7	1.7								
	$\varnothing < 450$ nm	10.2	<MLOD	–								
Llobregat River Basin	S07	$\varnothing > 450$ nm	223	85.1	2.6							
		$\varnothing < 450$ nm	<MLOD	<MLOD	–							
	S08	$\varnothing > 450$ nm	549	591.4	0.93							
		$\varnothing < 450$ nm	<MLOD	<MLOD	–							
	S09	$\varnothing > 450$ nm	4500	<MLOD	–							
		$\varnothing < 450$ nm	<MLOD	<MLOD	–							
S10	$\varnothing > 450$ nm	1050	1540	0.68								
	$\varnothing < 450$ nm	<MLOD	<MLOD	–								
Feb 2014	Besòs River Basin	S11	$\varnothing > 450$ nm	491	191		2.6					
			$\varnothing < 450$ nm	<MLOD	<MLOD		–					
		S12	$\varnothing > 450$ nm	157	187		0.84					
			$\varnothing < 450$ nm	<MLOD	<MLOD		–					
		S13	$\varnothing > 450$ nm	906	345	2.6						
			$\varnothing < 450$ nm	<MLOD	<MLOD	–						
Llobregat River Basin	S14	$\varnothing > 450$ nm	134	463	0.29							
		$\varnothing < 450$ nm	135	58	2.3							
	S15	$\varnothing > 450$ nm	142	352	0.40							
		$\varnothing < 450$ nm	38	17	2.8							
	S16	$\varnothing > 450$ nm	49	358	0.14							
		$\varnothing < 450$ nm	166	63	2.6							
S17	$\varnothing > 450$ nm	40	383	1.10								
	$\varnothing < 450$ nm	273	82	3.3								
S18	$\varnothing > 450$ nm	104	335	0.31								
	$\varnothing < 450$ nm	<MLOD	<MLOD	–								
S19	$\varnothing > 450$ nm	349	557	0.63								
	$\varnothing < 450$ nm	<MLOD	28	–								



**Table 4** (continued)

Sample			C <sub>60</sub> (pg/l)	C <sub>70</sub> (pg/l)	C <sub>60</sub> /C <sub>70</sub> ratio
S20	Ø>450 nm		235	370	0.63
	Ø<450 nm		<MLOD	<MLOD	–
S21	Ø>450 nm		88	369	0.24
	Ø<450 nm		49	17	2.88
S22	Ø>450 nm		174	587	0.30
	Ø<450 nm		806	214	3.77
S23	Ø>450 nm		38	666	0.06
	Ø<450 nm		392	102	3.8
S24	Ø>450 nm		190	639	0.30
	Ø<450 nm		68	25	2.7
S25	Ø>450 nm		89	617	0.14
	Ø<450 nm		<MLOD	<MLOD	–

### Instrumental analysis

The separation of fullerenes was achieved by non-aqueous LC, using the ACQUITY UPLC System (Waters, Milford, MA, USA), equipped with a Buckyprep (150×2.0 mm, particle size 5 µm) analytical column from Nacalai Tesque (Kyoto, Japan). The mobile phase composition consisted of 100 % toluene, and the flow rate was kept at 0.400 ml/min. The separation of selected fullerenes was achieved in less than 4 min, throughout the total chromatographic run of 10 min. The sample injection volume was set at 10 µl. The LC system was coupled to a Q Exactive (Thermo Fisher Scientific, San Jose, CA, USA) hybrid quadrupole-Orbitrap mass spectrometer with an APPI source, acquiring data in APPI/APCI mode with negative polarity. The acquisition was performed in full scan ( $m/z$  600–1200) with a resolution of 35,000 full width at half maximum (FWHM).

The method parameters were optimised by continuous infusion of a mixture of 1 mg/l standard solution of each fullerene, via syringe pump at a flow rate of 5 µl/min. The optimal APPI source conditions were as follows: sheath gas at 50 a.u., auxiliary gas at 35 a.u., spray voltage at 5.0 kV, spray current set at 10 µA, S-lens RF at 70, capillary T at 350 °C and probe T at 450 °C. In order to improve the sensitivity, the spare gas was closed.

For instrument control and data processing, Xcalibur 2.2 (Thermo Fisher Scientific) software was used. For the unambiguous determination of fullerenes, the molecular ion  $[M]^-$  (in the case of pristine fullerenes,  $[^{12}C_x]^-$ ,  $x=60$  or  $70$ ) was used for quantification and the isotopic signal  $[M+1]^-$  (for pristine fullerenes,  $[^{13}C_1^{12}C_{(x-1)}]^-$ ,  $x=60$  or  $70$ ) was used for confirmation purposes. Overall, the number of identification points required for confirmation of organic contaminants in environmental analyses [35] was fulfilled.

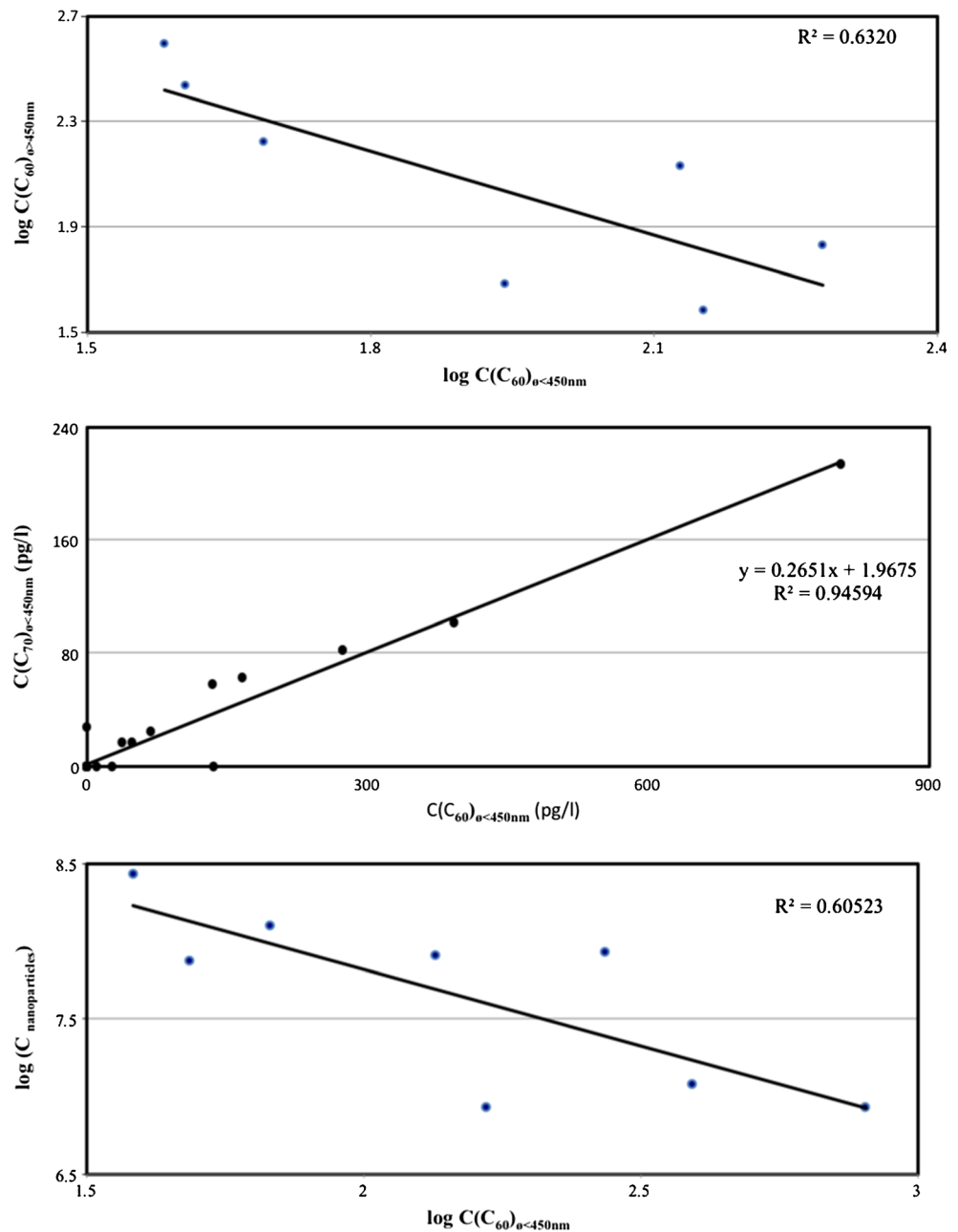
In order to assess the potential carry-over contamination, in each injection batch, instrumental and procedural blanks and a standard mixture were injected. An instrumental blank was introduced after each five injections. In addition, after each batch of samples, a cleaning sequence was performed, consisting of the injection of toluene, using a toluene-acetonitrile gradient to remove polar interference substances from the system.

### Quality control and quality assurance

The analytical method was validated with special attention to linearity, recovery, matrix effects, precision, limits of detection (LOD) and limits of quantification (LOQ). The instrumental quality parameters were calculated using a ten points calibration curve in toluene, ranging from the instrumental LOD to 500 ng/ml. The instrumental limits of detection (ILOD) were calculated by progressive dilution as the lowest concentration at which each compound could be detected. Instrumental linearity and sensitivity were estimated using the Pearson index ( $R^2$ ) and the slopes of the calibration curves, respectively. Instrumental reproducibility (inter-day precision) was calculated as the average percentage of the relative standard deviation (RSD%) of standard solutions (ten replicates) at three concentration levels (50, 100 and 500 fg/µl) on three consecutive days.

The method limits of detection (MLOD) and quantification (MLOQ) were based on matrix-matched calibration curve points. The MLOD and MLOQ of each analyte were defined as the lowest concentrations which fulfilled the criteria: (1) signal-to-noise ratio, at least, 3 and 10, respectively; (2) relative standard deviation of three replicates, below 22 %; (3) Gaussian peak shapes; (4) less than 3 ppm of exact mass error; and (5) adequate molecular isotopic pattern.

**Fig. 4** Relationships between  $C_{60}$  concentrations in river water samples and other parameters. **a**  $C_{60}$  concentrations in the particulate versus  $C_{60}$  concentrations in the aqueous phase ( $p=0.033$ ). **b**  $C_{60}$  concentrations in the aqueous phase versus  $C_{70}$  concentrations in the aqueous phase ( $p<0.00$ ). **c** Relationship between  $C_{60}$  concentrations in the aqueous phase and concentrations of nanoparticles ( $p=0.023$ )



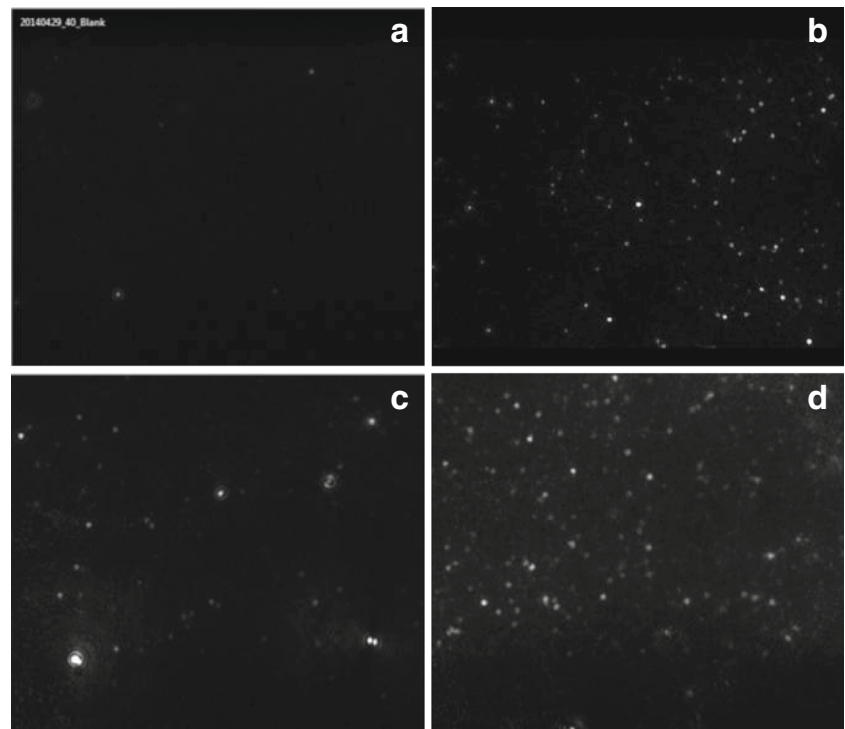
The reproducibility (inter-day precision) was calculated as the average relative standard deviation (in percentage) of the analytes ( $n=10$ ) of blank wastewater effluents fortified with the seven analytes at three concentrations (10, 50 and 1000 ng/l). Recoveries were calculated by the analysis of the same samples.

Matrix effects were assessed by comparing the analytical signals of the standard suspension and the analytical signals of fortified extract at the same concentrations (0.2, 2.0 and 2.0 ng/g). Sediment extraction was previously validated [26].

#### Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) utilises the properties of both light scattering and Brownian motion in order to obtain particle size distributions of samples in liquid suspension. A laser beam is passed through a prism-edged flat glass within the sample chamber. The angle of incidence and refractive index of the flat glass are designed to be such that when the laser reaches the interface between the glass and the liquid sample layer above it, the beam refracts, resulting in a compressed beam with a reduced profile and high power density.

**Fig. 5** Captions from NTA recordings of a HPLC water blank (a), a surface water sample, (b) a WW effluent (c) and a WW influent (d)



The particles in suspension in the path of this beam scatter light which can be detected [36]. NTA was performed in flow mode with a NanoSight LM10-HS instrument (NanoSight Amesbury, UK) equipped with an LM14 nanoparticle viewing unit with temperature control, a CCD monochrome camera and an automated syringe pump.

NTA calibration was carried out with stock suspensions of polystyrene latex nanoparticles with diameters of 100, 200 and 400 nm. A pre-scan was carried out in order to adjust the camera level and data threshold to the characteristics of each sample, prior to sample characterisation. In order to track a significant number of nanoparticles, measurements were carried out in quintuplicate, with recordings of time periods in the range of 60–90 s. The five size population distributions were normalised and averaged in order to obtain the final plot.

In order to avoid modification of the aggregation states of the samples, they were not frozen. Prior to performing the

NTA analysis, to discard micro-sized suspended particles, the samples were filtered through a 450-nm pore size filter and then were shaken, prior to their analysis.

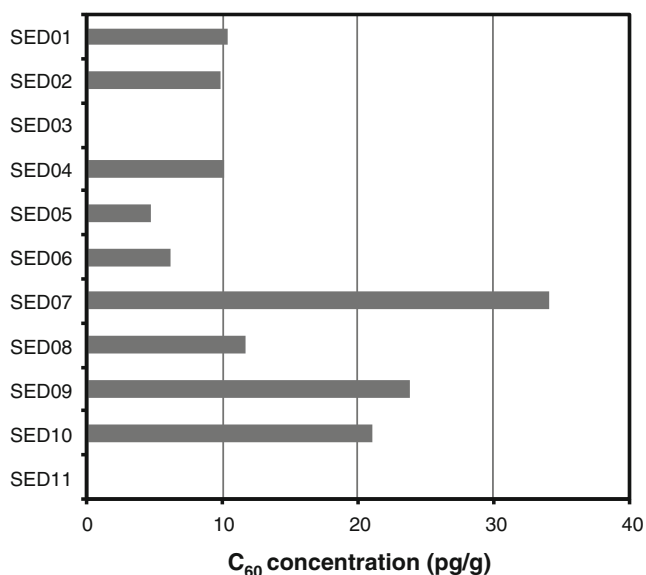
#### Precautions and safety considerations

In order to avoid degradation and oxidation processes, the samples and the extracts were stored in the dark, and exposure to air was minimised. Glass materials were heated to 400 °C, overnight, and rinsed with toluene, prior to their use. To circumvent cross-contamination, only glass was used. In order to minimise the potential losses by adsorption to the containers, the use of plastic materials was avoided during the whole analytical process. For the same reason, complete dryness was prevented during the evaporation of the extracts. Finally, in order to minimise any health risks, all manipulations and the solvent transferring procedures were performed

**Table 5** Concentrations of nanoparticles in water samples obtained by NTA

	Concentrations in river water samples (1/ml)		Concentrations in WWTP effluent samples (1/ml)
	Before WWTP	After WWTP	
Manresa	$3.88 \times 10^8$	$1.26 \times 10^8$	$2.61 \times 10^8$
Sant Feliu	$7.48 \times 10^7$	$7.93 \times 10^7$	$1.86 \times 10^8$
Terrassa	$3.55 \times 10^7$	$3.50 \times 10^7$	$1.36 \times 10^8$
Montcada	$2.35 \times 10^8$	$8.13 \times 10^7$	$2.70 \times 10^8$
Rubí	$8.50 \times 10^6$	$1.20 \times 10^7$	$4.55 \times 10^8$
Sabadell	NA	$8.57 \times 10^7$	$1.88 \times 10^8$

NA not analysed



**Fig. 6** Concentrations in river sediment samples

under a fume hood. Since toluene was used as the mobile phase, the LC waste bottle was vented into the fume hood.

## Results and discussion

### Analytical method

The novel analytical approach for water matrices reported in this work combines UAE with toluene followed by LC, using a pyrenylpropyl group bonded silica based column, coupled to a high-resolution mass spectrometer (HRMS) using a dual-mode atmospheric pressure photoionisation source, in conjunction with atmospheric pressure chemical ionisation (APCI/APPI) ion source (Ion Max™, Thermo Scientific). The hybrid ionisation source allows working in APCI, APPI or combined APCI/APPI mode. In terms of sensitivity, the best performance was obtained with the APPI or the combined APCI/APPI mode. Since slightly better results were obtained using the hybrid source (Fig. 1), it was selected for the optimised method. In contrast, a compromise between mass accuracy and sensitivity was adopted and the acquisition was set at 35,000 FWHM.

Validation was carried out for surface water and wastewater effluents. Good linearity was obtained (Table 3), with calibration curves (in standard solution and fortified blank extracts) having correlation coefficients of  $R^2 > 0.99$  and no significant residual trends. A slight percentage of ion suppression, ranging from 2 to 30 % in wastewater, was observed. However, potential matrix effects were corrected by internal standard addition. The ILOD and instrumental limits of quantification (ILOQ) values were ranging from 1 to 100 fg and from 3 to 300 fg, respectively. In comparison to previous works for

fullerene analysis in environmental waters, this method presents a great improvement in terms of sensitivity [26]. The MLOD ranged from 0.9 to 4.9 pg/l for surface waters, from 2.3 to 9.4 pg/l for wastewater effluents and from 19 to 160 pg/kg for sediments. Recovery yields were compressed between 67.7 and 106 %.

### Results in environmental river samples and WWTP effluents

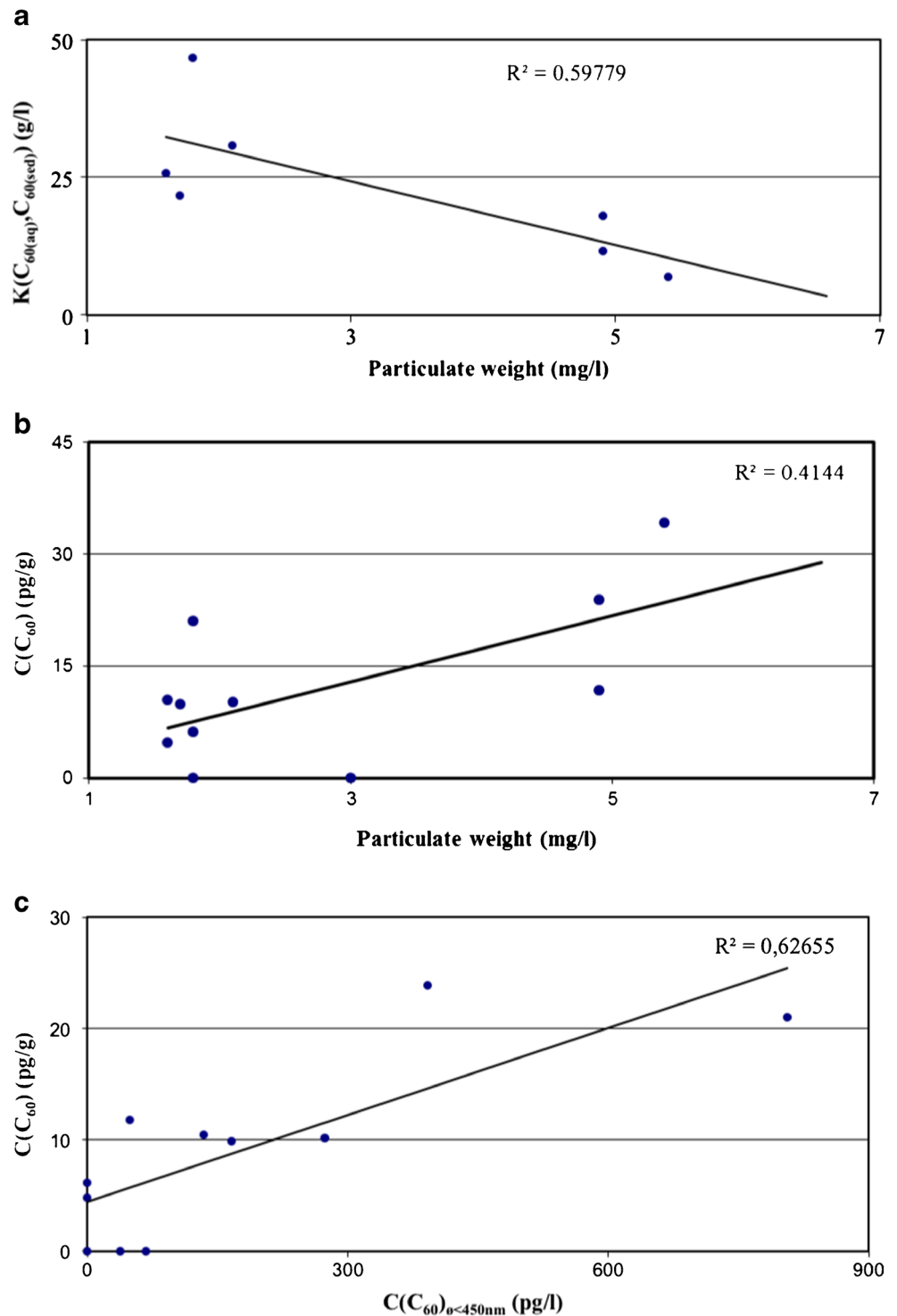
$C_{60}$  and  $C_{70}$  fullerenes were confirmed in all the samples, while functionalised fullerenes were not detected. These results can be related to the present low production and/or the degradation to more stable forms. In Fig. 2, the concentrations of fullerenes in wastewater samples are summarised.  $C_{76}$ ,  $C_{78}$  and  $C_{84}$  fullerenes, which were detected in other works [31], were not detected here. In Fig. 3, chromatograms of each matrix are presented.

The sums of  $C_{60}$  and  $C_{70}$  varied from 0.5 to 9.3 ng/l with mean concentrations of 1.1 and 1.7 ng/l for  $C_{60}$  and  $C_{70}$ , respectively.  $C_{70}$  was quantified at slightly higher concentrations with median concentrations of 0.4 and 1.7 ng/l for  $C_{60}$  and  $C_{70}$ , respectively. These concentrations are consistent with the predictions by the Gottschalk [37] model. The highest levels of fullerenes were from the WWTPs located in Sant Feliu de Llobregat, Rubí and Manresa that are highly populated and industrialised areas. Nevertheless, high variability between WWTPs was observed. Fullerenes were mainly associated with the particulate phase. This is consistent with the DLVO model which, because of the suppression of the electric double barriers in high-ionic-strength media, predicts the supra-aggregation of nanoparticles.

In surface waters,  $C_{60}$  and  $C_{70}$  fullerenes were quantified at much lower concentrations than in wastewater (Table 4), as expected.  $C_{60}$  fullerene was the most ubiquitous compound, being detected in 100 % of the samples in concentrations from 31 pg/l to 4.5 ng/l, while  $C_{70}$  concentrations ranged from <MLOD to 1.5 ng/l. Fullerenes are hypothesised to reach the river water by WWTP discharge and by atmospheric deposition.

A significant difference between the fullerene content in the surface water of the Besòs and Llobregat rivers was found. The median concentrations of  $C_{60}$  fullerene were 0.92 and 0.39 ng/l in the Llobregat and Besòs rivers, respectively. In the Besòs River, the mean concentration was slightly higher than the predictions by Gottschalk et al. [37], while in the Llobregat River, much higher values were detected, with mean concentrations of 0.498 and 0.697 ng/l for  $C_{60}$  and  $C_{70}$ , respectively. In the European Union (EU) and the USA for the scenarios projected by the Gottschalk model, predicted values were 17 and 3 pg/l, and for the particular Switzerland scenario, 40 pg/l. The differences with the Gottschalk model can be attributed to the characteristics of the Mediterranean rivers, with intense differences in seasonal water flows [38, 39].

**Fig. 7** Relationships between  $C_{60}$  concentrations in river sediment samples and other parameters. **a** Amount of suspended matter versus the distribution of fullerenes and the sediment and the water compartments ( $p=0.041$ ). **b** Amount of suspended matter versus the  $C_{60}$  concentrations in sediment ( $p=0.017$ ). **c** Concentrations of  $C_{60}$  in sediment versus concentrations of  $C_{60}$  in water ( $p=0.025$ )



The presence of fullerenes in both the large particulate (diameter  $\varnothing > 450$  nm) and the colloidal (diameter  $\varnothing < 450$  nm) fractions of surface waters is noteworthy (Table 4). In general, fullerenes were mainly found associated to the particulate fraction. But, in some of the samples (e.g. S22 and S23), about 80–90 % of the fullerenes were found in the fraction smaller than 450 nm.

An inverse tendency was found between the levels of  $C_{60}$  fullerene associated with the particulate and in the aqueous phase (Fig. 4a,  $R^2=0.6320$ ,  $p=0.033$ ). This correlation is not consistent with the predictions by Langmuir or Freundlich models, which would typically show a positive slope. Therefore, the fullerene sorption onto particulate surface is not the predominant process, as would

be expected for hydrophobic organic pollutants. Fullerene aggregation with naturally occurring colloids is likely to occur in natural waters, contributing to the stabilisation of fullerene aggregates. In contrast, these findings support the tenet that fullerene aggregate suspensions can be stable in environmental conditions, similarly with those suspensions prepared at lab scale by long-term stirring in Oberdörster et al. [17] and further characterised in Zhu et al. [40].

In the colloidal phase, a significant correlation ( $R^2 > 0.94$ ,  $p < 0.000$ ) was found between the concentrations of  $C_{60}$  and  $C_{70}$  fullerenes (Fig. 4b), suggesting a common origin for both fullerenes. In addition, the concentrations of  $C_{60}$  were, in general, about three times the concentration of  $C_{70}$  and this ratio is consistent with those from combustion flame emissions [41]. Therefore, atmospheric deposition can be considered the main source in river water. However, this ratio was not observed in WWTP effluents, where the influences of the organic matter content, aggregation and supra-aggregation formation, adsorption to suspended material and precipitation processes play an important role in the compartment of fullerenes from the water column to sediments. In addition, this is also an indication of the low influence of WWTP effluents in fullerene concentrations in river water.

NTA analysis (see Fig. 5) provided a wide characterisation of the number and size of nanoparticles and the aggregate sizes in the water samples. The total concentrations of nanoparticles in wastewater effluents were up to 20 times higher than those in river samples (Table 5). However, no significant differences in terms of size distribution or total nanoparticle concentrations were found between river samples, before and after WWTP discharges. Most probably, abrupt changes in salinity, pH and organic material contents when effluents are discharged into the rivers produce flocculation and precipitation of colloids and suspended material.

River samples collected near highways, under the influence high-intensity vehicle traffic, such as Manresa and Montcada i Reixach, presented concentrations of nanoparticles in the same order as wastewater effluents. But, it is noteworthy that samples with a high concentration of nanoparticles in the sub-450 nm phase exhibited lower concentrations of  $C_{60}$  (see Fig. 4c). In these cases, suspended nanoparticles are mainly inorganic (metal nanoparticles, metal oxides and nano-sized mineral grains from natural erosion), while when the total concentration of nanoparticles is higher, carbon-based nanoparticles, such as fullerenes, tend to be associated to humic and fulvic acids, forming large-sized hetero-aggregates that tend towards precipitation, which is consistent with the results obtained in the analyses of sediment samples of the same sites.

Figure 6 shows the concentration of fullerenes in sediments. The only detected fullerene in sediments was  $C_{60}$ ,

occurring in 75 % of the samples. The concentrations of  $C_{60}$  ranged from <MLOD to 32.1 pg/g, with median and average concentrations of 10.0 and 11.0 pg/g, respectively.

A certain regression was found when the partitioning of  $C_{60}$  fullerene (sub-450 nm) between river water and the sediment,  $k_{C_{60}(aq), C_{60}(sed)}$ , versus the weight of suspended material in water, was studied. As can be seen in Fig. 7a, for river waters with higher concentrations of particulates,  $C_{60}$  fullerene tends to partition into the sediment compartment ( $R^2 = 0.60$ ,  $p = 0.023$ ). In addition, the concentration of  $C_{60}$  fullerene in sediments tends to be higher when the particulate weight in water is much higher ( $R^2 = 0.41$ , Fig. 7b).

These two observations stress the major role played by hetero-aggregation processes in the distribution of fullerenes in natural waters and sediments, particularly in these scenarios where concentrations of natural colloids are high, as previously indicated in Quik et al. [42].

However, no correlation was found between the fullerene concentration and large-diameter particulates. Instead, the concentrations of  $C_{60}$  in the aqueous phase ( $\varnothing < 450$  nm) showed a statistically significant correlation with the concentrations of  $C_{60}$  in the sediment ( $R^2 = 0.6265$ ,  $p = 0.025$ , Fig. 7c). The preferential relationship between the sub-450 nm fraction and the sediment concentrations can be explained by the higher surface charge of the smallest aggregates, which allows them to interact further with humic acids, natural matter and clayey particulates.

## Conclusions

A novel analytical approach combining NTA and UAE-LC-APCI/APPI-MS has been presented. The method based on UAE-LC-APCI/APPI-MS has presented an improved sensitivity in comparison with previous methods. The approach was used to study selected fullerenes in 48 samples of river waters, sediments and wastewater effluents.

The main results of this study corroborate that, as a consequence of the current low production of engineered fullerenes and the much higher stability of pristine fullerenes, solely pristine fullerenes were detected in all types of samples (river water, sediments and WWTP effluents) at picogram per litre and picogram per kilogram levels.

In the colloidal phase, a significant correlation was found between the concentrations of  $C_{60}$  and  $C_{70}$  fullerenes, suggesting a common origin. In addition, the ratio  $C_{60}/C_{70}$  was consistent with ratios from combustion processes [41]. On the contrary, this ratio was not maintained in WWTPs, indicating multiple sources.

NTA analysis was used to provide a wide characterisation of the number and size of nanoparticles and the size of aggregates in the water samples, showing that the total concentrations of nanoparticles in wastewater effluents were

about ten times higher than in river samples. However, no differences were observed between river samples, before and after WWTP discharging points, either in terms of size distribution or in terms of total nanoparticle concentrations.

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**Conflict of interest** The authors state that there is no conflict of interest.

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