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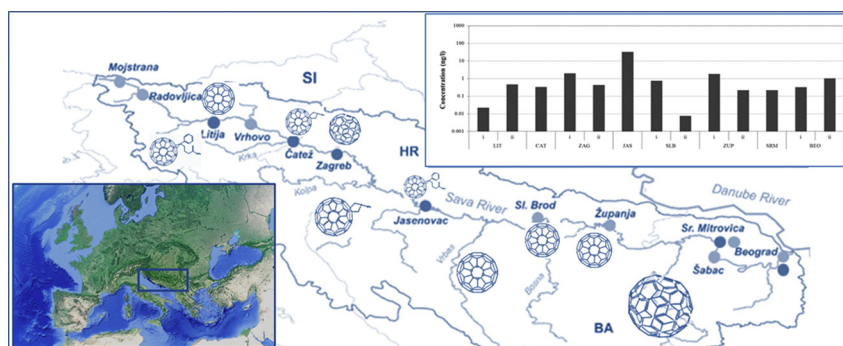
## Science of the Total Environment

journal homepage: [www.elsevier.com/locate/scitotenv](http://www.elsevier.com/locate/scitotenv)Occurrence of C<sub>60</sub> and related fullerenes in the Sava River under different hydrologic conditionsJosep Sanchís<sup>a,\*</sup>, Radmila Milačič<sup>b</sup>, Tea Zuliani<sup>b</sup>, Janja Vidmar<sup>b</sup>, Esteban Abad<sup>a</sup>,  
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## HIGHLIGHTS

- Fullerene aggregates have been detected in the Sava River in two sampling campaigns.
- Concentrations of C<sub>60</sub> fullerene in surface water ranged from 8 pg/l to 59 ng/l.
- During high flow conditions, concentrations were significantly lower.
- The fullerene derivatives [60]PCBM and [60]NMFP were detected in some samples.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The presence of nanomaterials in the environment has caught the attention of the scientific because of the uncertainties in their fate, mobility and potential toxic effects. However, few studies have determined experimentally their concentration levels in aquatic systems up to date, which complicates the development of an adequate risk assessment.

In the present study, the occurrence of ten fullerenes has been assessed in the Sava River (Southeastern Europe): 27 freshwater samples and 12 sediment samples from 12 sampling points have been analysed during two sampling campaigns. C<sub>60</sub> was the most ubiquitous fullerene, with concentrations of 8 pg/l–59 ng/l and 108–895 pg/g<sub>dw</sub> in water and sediments, respectively. Statistically significant differences existed between the levels in 2014 and 2015, which has been attributed to the extreme hydrologic conditions (severe floods and drought, respectively). C<sub>70</sub> fullerene has been detected in most of the samples and the fullerene derivatives [6,6]-phenyl C<sub>61</sub> butyric acid methyl ester and N methyl fulleropyrrolidine have been detected eventually, which highlights that nanotechnology research and development activities are responsible for emitting these emerging contaminants to the environment. The role of diverse potential anthropogenic sources (including oil refinery, general industrial activity, river navigation, urban emissions and nanotechnology) is discussed.

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### 1. Introduction

Fullerenes are carbon allotropes structured as hollow polyhedrons that were discovered and described by Sir H.W. Kroto and coworkers in 1985 (Kroto et al., 1985). These cage-like molecules are defined as nanomaterials, although their dimensions commonly fall below 1.0 nm (European Commission, 2011). C<sub>60</sub> fullerene (buckminsterfullerene, C<sub>60</sub>) is the most studied fullerene. In its most stable form (Sure et al., 2017), C<sub>60</sub> presents sixty carbon atoms organised as a characteristic truncated icosahedron, with twenty hexagonal faces and twelve isolated pentagons, in I<sub>h</sub> symmetry.

Fullerenes are emitted to the environment from both natural and anthropogenic sources. Natural sources are energetic events where organic matter is subjected to high temperature and pressures conditions, such as lightning discharges, volcanic eruptions, chondritic impacts and wildfires. Therefore, their presence has been reported in geologic strata (Becker et al., 2001; Chijiwa et al., 1999; Heymann et al., 1996; Heymann et al., 1994), in lava pillows (Jehlička et al., 2000), in coal (Fang et al., 2006; Fang and Wong, 1997) and in some mineraloids, such as in some fulgurites (Daly et al., 1993) and shungites (Parthasarathy et al., 1998).

Regarding anthropogenic sources, fullerenes have been detected as by-products in different types of hydrocarbon flames (Anacleto et al., 1992; Tiwari et al., 2016). Because of this, C<sub>60</sub> and C<sub>70</sub> fullerenes have been noticed in atmospheric particulate from urban and industrial areas (Encinas and Gómez-de-Balugera, 2018; Laitinen et al., 2014; Wang et al., 2016). Also, fullerenes are manufactured for nanotechnology purposes. Early estimations calculated an annual production of fullerenes of roughly 1500 t/y (Mitsubishi Chemical Corporation, 2001). Nowadays, fullerenes are employed in materials science (Jurkowska et al., 2006) and personal care products formulations (Zakaria et al., 2018), among other applications. Because of this, traces of fullerenes have been detected in wastewater treatment plants (WWTPs) influents (Emke et al., 2015) and effluents (Farré et al., 2010). Therefore, WWTPs can be regarded as one source of fullerenes to the environment.

During the recent years, several sensitive analytical methods have been developed for the extraction and determination of fullerenes in the environment. Fullerenes have been quantified in air particulate (Encinas and Gómez-de-Balugera, 2018; Sanchís et al., 2012; Utsunomiya et al., 2002), wastewater (WW) (Emke et al., 2015; Farré et al., 2010; Wang et al., 2010), WWTP sludge (Zakaria et al., 2018), soils (Carboni et al., 2014; Sanchís et al., 2013; Sanchís et al., 2015b) and surface water (Astefanei et al., 2012; Astefanei et al., 2014; Herrero et al., 2014; Herrero et al., 2015; Sanchís et al., 2015a; van Wezel et al., 2011). High pressure liquid chromatography (HPLC) coupled to mass spectrometers, preferably with an atmospheric pressure photoionization (APPI) source is the technique of choice for

assessing the occurrence of fullerenes in complex environmental samples (Astefanei et al., 2014; Astefanei et al., 2015; Núñez et al., 2012). This instrumentation presents a high sensitivity, selectivity and relatively low signal suppression in different matrices. Also, it is convenient to circumvent potential contamination issues related to fullerene self-generation, which may lead to false positives, as has been observed in laser-desorption-based ionisation sources (Santos et al., 2016).

Despite of the recent advances in the development of robust analytical methods for their analysis, the number of studies reporting the occurrence of fullerenes in the environment is scarce. Tables 1a and 1b summarise the concentrations of the most commonly studied fullerenes, C<sub>60</sub> and C<sub>70</sub>, in surface water and sediments according to the literature. There are still large uncertainties regarding the occurrence, fate and behaviour of fullerenes in the aquatic environment.

In the present study, the primary goal was to study the presence of fullerenes in the Sava River. Ten fullerenes were selected (see Table 2), including 5 pristine fullerenes (C<sub>60</sub>, C<sub>70</sub>, C<sub>76</sub>, C<sub>78</sub> and C<sub>84</sub>, which are the most abundant fullerenes produced in hydrocarbon flames and arc discharge methods) and five engineered fullerenes (with relevant applications in microelectronics and bulk heterojunction solar cells). In addition, several degradation products of C<sub>60</sub> fullerene, previously identified in Sanchís et al. (2018), were analysed. Regarding the Sava River, it is an important tributary of the Danube River located in Southern Europe and, to the best of our knowledge, the present work is the first to assess the presence of fullerenes in this river basin. For this study, 27 surface water samples and 12 sediments from 12 sampling points were collected during two sampling campaigns in two extreme hydrologic conditions: under extremely high flow and drought events.

### 2. Methods and materials

#### 2.1. Studied area and sampling

The Sava River is the third longest (945 km) and the richest-in-water (1700 m<sup>3</sup>/s at its discharge mouth, 1564 m<sup>3</sup>/s average) tributary of the Danube River (Milačić et al., 2017a). It flows through four riparian countries (Slovenia, Croatia, Bosnia-Herzegovina and Serbia) and its basin covers significant areas of up to six countries, where it has historically been a valuable socio-economic motor, a hydropower generator, a relevant communication route, a tourist attraction and a vital freshwater resource for the general population, the agriculture and industrial development (International Sava River Basin Commission, 2008). According to the International Sava River Basin Commission (International Sava River Basin Commission, 2016), it receives a significant anthropogenic pressure and 49% (in length) of the water bodies from the Sava River Basin are estimated to be at risk because of the

**Table 1a**  
Occurrence of C<sub>60</sub> and C<sub>70</sub> fullerenes in surface waters. MLOD refers to method limit of detection and MLOQ refers to the method limit of quantification. N.A. refers to “not analysed”.

Reference	van Wezel et al. (2011)	Núñez et al. (2012)	Sanchís et al. (2013)	Astefanei et al. (2014)	Sanchís et al. (2015a)	Herrero et al. (2015)		
Sampled water bodies	Not detailed (NL)	Besòs River Basin (ES)	Pounds Barcelona airport area (ES)	Llobregat River Basin (ES)	Pounds Barcelona airport area (ES)	Besòs River Basin (ES)	Llobregat River Basin (ES)	Drentsch (NL)
No of samples	Unknown	2	4	6	6	10	15	Undetermined
C <sub>60</sub> Freq.	0	100%	75%	33%	100%	100%	100%	0%
MLOD	10 <sup>6</sup> pg/l	10 pg/l	10 pg/l	127 pg/l	2.3 pg/l	0.9 pg/l	0.9 pg/l	210 ng/l
Range	<MLOD	17 pg/l	<MLOD – 22 pg/l	<MLOD – 7.9 ng/l	<MLOD – 25 pg/l	31.2–340 pg/l	89 pg/l – 4.5 ng/l	<MLOD
Average	<MLOD	17 pg/l	not available	4.42 ng/l	14.3 pg/l	223 pg/l	697 pg/l	<MLOD
C <sub>70</sub> Freq.	N.A.	100%	100%	67%	83.3%	90%	93.3%	N.A.
MLOD		10 pg/l	10 pg/l	60 pg/l	3.4 pg/l	1.3 pg/l	1.3 pg/l	
Range		25 pg/l	MLOQ – 20 pg/l	<MLOD – 1.2 ng/l	<MLOD – 330 pg/l	<MLOD – 521 pg/l	<MLOD – 1.54 ng/l	
Average		25 pg/l	not available	560 pg/l	66.0 pg/l	200 pg/l	498 pg/l	

**Table 1b**

Occurrence of C<sub>60</sub> and C<sub>70</sub> fullerenes in sediments. MLOD refers to method limit of detection. N.A. refers to "not analysed". When not available in the cited reference, the average concentration was calculated considering <MLOD and <MLOQ as MLOD/2 and MLOQ/2.

Reference	Sanchís et al. (2013)	Astefanei et al. (2014)	Sanchís et al. (2015a)	Sanchís et al. (2015b)
Sampled water bodies	Llobregat River Basin (ES)	Pounds Barcelona airport area (ES)	Llobregat and Besòs River basins (ES)	Tubarão River (BR)
No of samples	6	7	11	15
C <sub>60</sub>				
Freq.	33%	85%	73%	93%
MLOD	19 pg/g	0.23 pg/g	0.037 pg/g	0.037 pg/g
Range	<MLOD – 700 pg/g	<MLOD – 1.1 ± 0.1 pg/g	<MLOD – 34.4 pg/g	<MLOD – 28 pg/g
Average	145 pg/g	0.569 pg/g	11.2 pg/g	8.5 pg/g
C <sub>70</sub>				
Freq.	0%	71%	0%	93%
MLOD	8.7 pg/g	0.25 pg/g	0.022 pg/g	0.022 pg/g
Range	<MLOD	<MLOD – 7.2 ± 0.2 pg/g	<MLOD	<MLOD – 48 pg/g
Average	<MLOD	1.49 pg/g	<MLOD	18 pg/g

presence of hazardous substances (i.e., 100% of water bodies from Croatian and 54% of Serbian water bodies).

Twelve sampling sites (see Fig. 1) from three countries (Slovenia, Croatia and Serbia) along the Sava River were selected. Two sampling campaigns were carried out. The first one, in September 2014 was carried out under high-flow conditions after some months of intense precipitations, which resulted in once-in-a-hundred-years floods. In contrast, the sampling campaign of September 2015 was characterised by low-flow conditions after a year of particularly low precipitations. In Table 3, the general characteristics and the locations of each sampling site are summarized.

Regarding the sampling procedure, ~1.0 l of water was collected in amber glass bottles following a Lagrangian approach (i.e. following the water mass during its transport taken in consideration the flow). Samples were frozen and transported to the laboratory. Sediments were sampled according to the recommendation on the implementation of Water Framework Directive (Dulio et al., 2010). In each sampling point, about 3 kg of sediment were collected. Sediments were sieved in situ with 2 mm mesh sieves, placed in aluminium trays and frozen. Samples were shipped to the laboratories for their analysis and they were not thawed until the day of their analysis.

## 2.2. Reagents and materials

Fullerene standards of C<sub>60</sub> fullerene (99.9% purity), C<sub>70</sub> fullerene (99%), N methylfulleropyrrolidine (99%), [6,6]-phenyl C<sub>61</sub> butyric acid methyl ester (>99.5%), [6,6]-thienyl C<sub>61</sub> butyric acid methyl ester ([60]ThPCBM, ≥99%), C<sub>60</sub> pyrrolidinetris-acid ethyl ester (>97%) and [6,6]-phenyl C<sub>71</sub> butyric acid methyl ester (99%) were purchased from Sigma Aldrich (Steinheim, Germany). Table 2 shows their abbreviations, CAS numbers (Cozzi et al., 2005) and formulae.

<sup>13</sup>C-labelled C<sub>60</sub> fullerene (abbrev. <sup>13</sup>C<sub>60</sub>; purity, >90%; <sup>13</sup>C isotopic abundance, 20–30% according to the supplier) was purchased from MER Corporation (reference MR613, Tucson, AZ, USA). Minor traces of

C<sub>60</sub> molecules containing <8 and >23 <sup>13</sup>C atoms were experimentally detected (accounting for <0.6%), but no interferences were noted for ions [C<sub>60</sub>]<sup>−</sup> or [<sup>13</sup>C]<sub>2</sub>[C<sub>59</sub>]<sup>−</sup>.

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

## 2.3. Surface water extraction

Water samples were extracted according to a previous method (Sanchís et al., 2015a). Briefly, 150 ml of water were sequentially filtered through 0.7 μm glass fibre (Whatman, Maidstone, UK) and 0.45 μm nylon (Whatman, Maidstone, UK) tared filters. The filtrate and the filters were extracted separately. The filtrate was salted out with 1.5 g of NaCl, homogenized and extracted by liquid-liquid extraction (LLE) with 50 ml of toluene in a separatory funnel. The filters were dried at 60 °C overnight, fortified with 100 pg of <sup>13</sup>C<sub>60</sub> fullerene (100 μl of standard at 1.0 pg/μl) and placed in glass tubes. Their extraction was carried out in two cycles, employing aliquots of 35 ml of toluene as extractant, in an ultrasonic bath FB15064 (Fisherbrand, Pittsburgh, PA, USA), working at 37 kHz during 45 min. Then, the LLE extract and the filter extracts were combined in an amber-glass spherical flask and they were concentrated by rotatory evaporation to ~1 ml. Finally, the extract was quantitatively transferred into tared amber vials, and its volume was adjusted to 1.000 ml.

## 2.4. Sediment extraction

Sediment samples were extracted after minor modifications, according to the procedure described by Sanchís et al. (2013). Sediments were thawed at room temperature and sieved (<63 μm) in wet conditions using water from the corresponding sampling point. Sieved sediments were then placed in conic Falcon tubes and dewatered by centrifuging

**Table 2**

List of analysed fullerenes.

	Compound/most stable isomer*	Abbreviation	CAS number	Formula	Main molecular ion	m/z	Confirmation ion
1	C <sub>60</sub> fullerene/(C <sub>60</sub> -I <sub>h</sub> )[5,6]fullerene	C <sub>60</sub>	99685-96-8	C <sub>60</sub>	[ <sup>12</sup> C <sub>60</sub> ] <sup>−</sup>	720.00055	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>59</sub> ] <sup>−</sup>
2	C <sub>70</sub> fullerene/(C <sub>70</sub> -D <sub>5h</sub> )[5,6]fullerene	C <sub>70</sub>	11538322-7	C <sub>70</sub>	[ <sup>12</sup> C <sub>70</sub> ] <sup>−</sup>	840.00055	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>69</sub> ] <sup>−</sup>
3	C <sub>76</sub> fullerene/(C <sub>76</sub> -D <sub>2</sub> )[5,6]fullerene	C <sub>76</sub>	142136-39-8	C <sub>76</sub>	[ <sup>12</sup> C <sub>76</sub> ] <sup>−</sup>	912.00055	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>75</sub> ] <sup>−</sup>
4	C <sub>78</sub> fullerene/(C <sub>78</sub> -C <sub>2v</sub> )[5,6]fullerene	C <sub>78</sub>	139707-96-3	C <sub>78</sub>	[ <sup>12</sup> C <sub>78</sub> ] <sup>−</sup>	936.00055	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>77</sub> ] <sup>−</sup>
5	C <sub>84</sub> fullerene/(C <sub>84</sub> -D <sub>2d</sub> )[5,6]fullerene	C <sub>84</sub>	145809-20-7	C <sub>84</sub>	[ <sup>12</sup> C <sub>84</sub> ] <sup>−</sup>	1008.00055	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>83</sub> ] <sup>−</sup>
6	C <sub>60</sub> pyrrolidine tris-acid ethyl ester	[60]CPTAE	–	C <sub>72</sub> H <sub>19</sub> O <sub>6</sub> N	[C <sub>72</sub> H <sub>18</sub> O <sub>6</sub> N] <sup>−</sup>	992.11396	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>71</sub> H <sub>18</sub> O <sub>6</sub> N] <sup>−</sup>
7	[6,6]-thienyl C <sub>61</sub> butyric acid methyl ester	[60]ThPCBM	925673-03-6	C <sub>70</sub> H <sub>12</sub> O <sub>2</sub> S	[C <sub>70</sub> H <sub>11</sub> O <sub>2</sub> S] <sup>−</sup>	916.05635	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>69</sub> H <sub>11</sub> O <sub>2</sub> S] <sup>−</sup>
8	[6,6]-phenyl C <sub>61</sub> butyric acid methyl ester	[60]PCBM	160848-22-6	C <sub>72</sub> H <sub>14</sub> O <sub>2</sub>	[C <sub>72</sub> H <sub>13</sub> O <sub>2</sub> ] <sup>−</sup>	909.09210	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>71</sub> H <sub>13</sub> O <sub>2</sub> ] <sup>−</sup>
9	[6,6]-phenyl C <sub>71</sub> butyric acid methyl ester	[70]PCBM	609771-63-3	C <sub>82</sub> H <sub>14</sub> O <sub>2</sub>	[C <sub>82</sub> H <sub>13</sub> O <sub>2</sub> ] <sup>−</sup>	1029.09210	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>81</sub> H <sub>13</sub> O <sub>2</sub> ] <sup>−</sup>
10	N-methyl fulleropyrrolidine	[60]NMFP	151872-44-5	C <sub>63</sub> H <sub>7</sub> N	[C <sub>63</sub> H <sub>6</sub> N] <sup>−</sup>	776.05057	[ <sup>13</sup> C] <sub>2</sub> [ <sup>12</sup> C <sub>62</sub> H <sub>6</sub> N] <sup>−</sup>
11	<sup>13</sup> C-labelled C <sub>60</sub> (~25% abundance)	<sup>13</sup> C <sub>60</sub>	–	<sup>13</sup> C <sub>x</sub> C <sub>(60-x)</sub> , x = 8–23	[ <sup>13</sup> C] <sub>15</sub> [ <sup>12</sup> C <sub>45</sub> ] <sup>−</sup>	735.05033	[ <sup>13</sup> C] <sub>15</sub> [ <sup>12</sup> C <sub>45</sub> ] <sup>−</sup>

\* Lowest energy isomers among those structures that obey the isolated pentagon rule (Bendale and Zerner, 1995; Ettl et al., 1991; Sabirov et al., 2018).

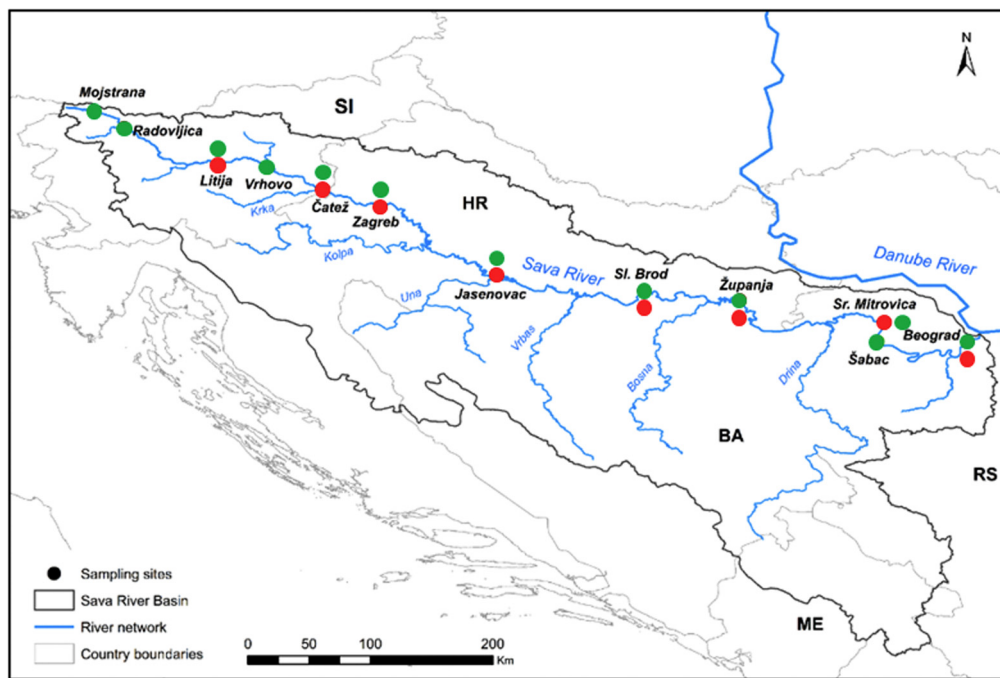


Fig. 1. Sava River sampling locations. Red points refer to the approximate location of samples in 2014, while green points refer to samples from 2015.

at 3000g during 5 min. After water decantation, sediments were dried overnight in a desiccator.

10 g of sieved sediment were fortified with 100 µl of <sup>13</sup>C-labelled C<sub>60</sub> fullerene suspension 1.0 pg/µl in toluene, homogenized and left in the dark for 3 h at 4 °C. After this time, they were combined with 40.0 ml of toluene and they were extracted in an ultrasonic bath during 4 h. The suspension was centrifuged at 3000g during 10 min and the toluene was collected. This process was repeated and both extracts were combined in a spherical flask. Extracts were concentrated to ~1 ml by

rotatory evaporation, transferred to a pre-tared 2 ml amber vials and their final volume were adjusted to 1.000 ml.

2.5. Instrumental method

The analysis of the extracts was carried out using HPLC coupled to high-resolution mass spectrometry (HRMS) with an APPI source, according to a previous method (Sanchís et al., 2018). The chromatograph was an Acquity UPLC System (Waters, Milford, MA, USA), the mass

Table 3

Summary of analysed sample and description of the sampling point. Stressor a: relevant discharge of pollutants ("!" : untreated discharge); b: land-use changes; c: intense agricultural activity; d: navigable section; e: relevant urban nucleus (>40,000 inh.); f: oil refinery; g: metal industry; h: mining; i: relevant industrial activity. W and S refer to the number of water samples and sediment samples that were taken in each point.

#	Name	Country	Samples				Relevant identified stressors (Milačić et al., 2017b)									Description
			2014		2015		a	b	c	d	e	f	g	h	i	
			W	S	W	S										
1	Mojstrana (MOJ)	Slovenia	0	0	1	1	-	-	-	-	-	-	-	-	-	Small alpine village (1200 inh.). Considered as a reference point.
2	Radovljica (RAD)	Slovenia	0	0	1	1	x	-	-	-	-	x	-	-	Small city (5900 inh.). Local workshop activities.	
3	Litija (LIT)	Slovenia	2	0	2	1	-	x	-	-	-	-	-	-	Small city (15,000 inh.).	
4	Vrhovo (VRH)	Slovenia	0	0	1	1	x	-	-	-	-	-	-	x	Potentially impacted by the Vrhovo dam. Near Vrhovo (~250 inh.), a small village.	
5	Čatež (CAT)	Slovenia	1	0	1	1	-	x	-	-	-	-	-	-	Hydropower plant. Close to Hrastnik (5500 inh.). Rural area near Čatež on Sava (~1000 inh.).	
6	Zagreb (ZAG)	Croatia	2	0	1	1	x	x	-	-	x	-	-	x	Located in Zagreb, capital of Croatia (800,000 inh., metropolitan area: 1,100,000 inh.)	
7	Jasenovac (JAS)	Croatia	1	0	1	1	-	x	x	x	-	-	-	-	Rural area near Jasenovac (Jasenovac Municipality, 2000 inh.)	
8	Slavonski Brod (SLB)	Croatia	2	0	1	1	x	x	-	x	x	x	-	x	Close to Slavonski Brod (~60,000 inh.).	
9	Županja (ZUP)	Croatia	2	0	1	1	x	x	x	x	-	x	x	x	Close to Županja (~12,000 inh.).	
10	Sremska Mitrovica (SRM)	Serbia	1	0	1	1	!	x	x	x	x	-	-	x	Close to Sremska Mitrovica (~40,000 inh.), district capital.	
11	Šabac (SAB)	Serbia	0	0	2	1	!	-	x	x	x	-	x	-	Close to Šabac (125,000 inh.), district capital.	
12	Belgrade (BEO)	Serbia	2	0	1	1	!	x	-	x	x	-	-	-	In Belgrade (1200,000 inh., metropolitan area: 1700,000 inh.)	

spectrometer was a Q Exactive™ (Thermo Fischer Scientific, San Jose, CA, USA) and their coupling was carried out with an Ion Max source (Thermo Fischer Scientific, San Jose, CA, USA). The chromatographic separation was achieved using a COSMOSIL™ Buckyprep analytical column (150 × 2.0 mm; particle size, 5 µm; pore size, 120 Å) from Nacalai Tesque Inc. (Kyoto, Japan) and a mobile phase composed of toluene-methanol (9:1). The flow rate of the mobile phase was set at 0.4 ml/min and the total analysis time was 10 min. In Table S1, the optimal parameters of the ionisation source are detailed. The acquisition was performed in full scan mode, from  $m/z = 300$  to  $m/z = 1600$ , with a resolution of 140,000 full width at half maximum (FWHM).

Table 2 shows the molecular ions of the analysed compounds and their exact masses. In addition, several degradation products described in Sanchís et al. (2018) were analysed, including C<sub>60</sub> fullerenes mono-, bi-, tri- and tetraepoxides ( $m/z = 735.9955$ , 751.9904, 767.9853, 783.9802) and the dimers (C<sub>60</sub>)<sub>2</sub>O and (C<sub>60</sub>)<sub>2</sub>O<sub>2</sub> ( $m/z = 1456.9988$  and 1472.9937, respectively).

## 2.6. Method quality parameters

The employed analytical method for sediments and surface waters were validated in two previous works (Sanchís et al., 2015a, 2015b). Recovery percentages and method limits of detection (MLOD) and quantification (MLOQ) are summarized in Table S2 in the Supplementary Information.

In surface water, recovery percentages ranged from 100% to 110% for pristine fullerenes and from 68% to 86% for engineered fullerenes with <15% inter-day variability in all the cases. Limits of detection ranged from 0.9 to 140 pg/l for pristine fullerenes and from 4.8 pg/l to 7.2 pg/l for engineered fullerenes. In the case of sediments, recovery percentages ranged from 68% to 106% and limits of detection ranged from 0.019 pg/g<sub>dw</sub> to 17 pg/g<sub>dw</sub>. As can be seen in Table S2, the MLOD of higher fullerenes (C<sub>76</sub>, C<sub>78</sub> and C<sub>84</sub>) are significantly higher than those of C<sub>60</sub> and C<sub>70</sub> in both matrices, in agreement with other MS works that employed APPI (Astefanei et al., 2014) or other ionisation sources (Dunk et al., 2015; Parker et al., 1991).

## 2.7. Additional analytical precautions and safety considerations

Prolonged light exposure was prevented during the whole experimental work. Amber glass bottles, containers and vials were employed to avoid fullerenes photodegradation. All the glass material had been heated at 400 °C overnight and rinsed with toluene and acetone before its use in order to prevent contamination. Plastic materials were avoided during the whole process to prevent sorption of fullerenes.

Procedural blanks were conducted together with sample extractions to check contamination and cross-contamination. Commercial mineral water and grounded clay soil from a natural park were employed as

matrices for surface water and sediment analyses, respectively. Instrumental blanks, consisting of toluene, were also analysed periodically. No contamination was noticed in any blank.

Finally, the experimental work was carried out in a fume hood to limit the analyst exposure to toluene vapours. For the same reason, the whole HPLC-APPI-HRMS system was fitted inside a home-made fume hood.

## 3. Results and discussion

### 3.1. Pristine fullerenes in surface water

Concentrations of C<sub>60</sub> and C<sub>70</sub> fullerenes are represented in Fig. 2 and summarized in Table 4. An extracted ion chromatogram of C<sub>60</sub> and C<sub>70</sub> fullerenes can be found in the Supporting information (Fig. S1).

C<sub>60</sub> fullerene was the most frequently detected compound, being found in all sampling sites in both campaigns. In 2014, it was detected at concentrations ranging from only 8 pg/l up to 32.4 ng/l (in the Fig. S2 of the supporting information, the concentrations of C<sub>60</sub> fullerene are presented in logarithmic scale to facilitate their visualization). As can be seen, the maximum contamination level was detected in a particular sample (codified as JAS) from Jasenovac (Croatia), while the rest of sites presented significantly lower concentrations, below 1 ng/l. The median level was 430 pg/l. Dixon's Q-test and Grubb's test consider that the concentration in JAS was abnormally high ( $p < 0.002$ ), which suggests a punctual peak of contamination that did not significantly affect the samples downstream.

In 2015, the concentrations were higher than those in the previous campaign, with levels that ranged from 150 pg/l to 59 ng/l, and a median concentration of 4.31 ng/l.

In order to compare both sampling campaigns, a Kruskal-Wallis test was used. This non-parametrical test was employed since concentrations did not follow a normal distribution ( $p = 0.37$  and  $p = 0.77$  according to Shapiro-Wilk normality test), as expectable in datasets composed by complex environmental samples related to multiple stressors and miscellaneous contamination sources. The Kruskal-Wallis test confirmed that the concentrations of C<sub>60</sub> in 2014 and in 2015 were statistically different ( $p = 0.0049$ ). The same was observed in C<sub>70</sub> fullerene, which had not been detected during the first sampling campaign but exhibited quantifiable concentrations up to ~20 ng/l during 2015 (median concentration, 1.3 ng/l).

As it is known, hydrological conditions and heavy rains significantly contribute to the level of contaminants in river water, diluting their concentrations and/or re-suspending some of those pollutants and organic materials from riverbed sediments. This phenomenon is evident in the results of C<sub>60</sub> and C<sub>70</sub> fullerenes in the Sava River. In 2014, several episodes of intense precipitation were registered in South-eastern Europe. Among them, there was a Vb-type cyclone in May 2014 that

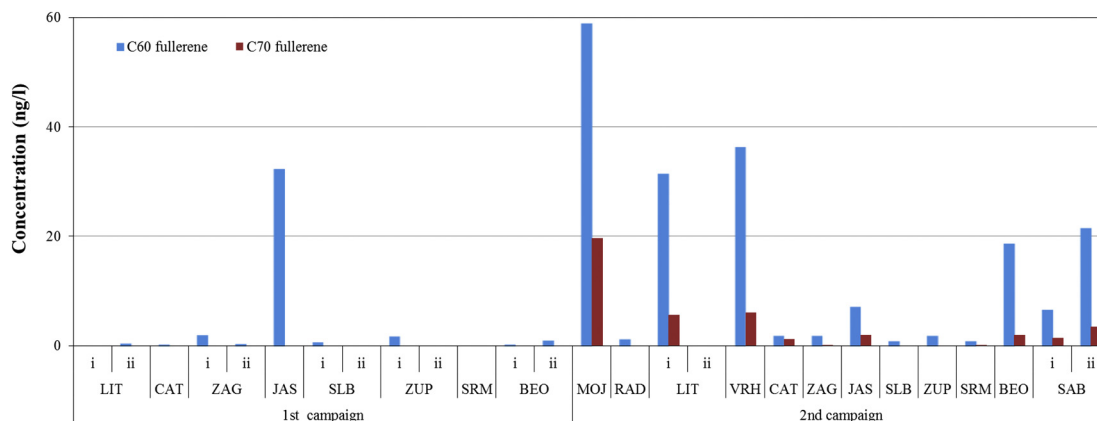


Fig. 2. Concentrations of C<sub>60</sub> and C<sub>70</sub> fullerenes in water samples.

**Table 4**

Summary of concentrations of fullerenes in surface waters from the Sava River. MLOD and MLOQ refer to method limits of detection and quantification, respectively.

		C <sub>60</sub>	C <sub>70</sub>	Higher fullerenes (C <sub>76</sub> , C <sub>78</sub> & C <sub>84</sub> )	[60]PCBM	[60]NMFP, [60]CPTAE, [60]ThPCBM and [70]PCBM
2014	Frequency	100%	0%	0%	7.7%	0%
	Range	0.008–32.4 ng/l	<MLOD	<MLOD	<MLOD – 6.02 ng/l	<MLOD
	Median	0.431 ng/l	<MLOD	<MLOD	<MLOD	<MLOD
	Average	3.08 ng/l	<MLOD	<MLOD	<MLOD	<MLOD
2015	Frequency	100%	71%	0%	0%	0%
	Range	0.150–59.0 ng/l	<MLOD – 19.7 ng/l	<MLOD	<MLOD	<MLOD
	Median	4.31 ng/l	1.33 ng/l	<MLOD	<MLOD	<MLOD
	Average	13.6 ng/l	3.00 ng/l	<MLOD	<MLOD	<MLOD

delivered acute rainfalls and resulted in torrential floods in the Sava River Basin, causing dozens of casualties, thousands of displaced people and losses of 3.5 billion € (Gaál et al., 2014; Haumont, 2015; Koninklijk Nederlands Meteorologisch Instituut, 2014; Stadtherr et al., 2016). In contrast, 2015 did not register such intense precipitations (Table S3 in the Supplementary Information). These observations indicate a higher environmental burden of C<sub>60</sub> fullerenes under drought conditions. Similar results were observed for potentially toxic elements in the Sava River: The soluble part of elemental concentrations was in general appreciably higher during low water discharges than during high water discharges (high dilution factor) (Milačič et al., 2017a).

Regarding higher order fullerenes (C<sub>76</sub>, C<sub>78</sub> and C<sub>84</sub>), none of these compounds was observed in the present work as it happened in previous studies (Sanchís et al., 2015a). This can be justified because of the lower emission rates of higher fullerenes from incidental and nanotechnological sources. Hydrocarbon flames typically generate higher fullerenes at significantly lower rates than C<sub>60</sub> and C<sub>70</sub> (Richter et al., 1997). Similar results have been observed in arch-discharge methods, such as the classical Krätschmer-Huffman graphite arc-discharge reactor (Diederich and Whetten, 1992). Regarding their use in nanotechnology-related products, the synthesis and isolation of relatively large amounts of isomerically pure higher fullerenes is complex and expensive. Subsequently, these compounds are less accessible for nanotechnology applications and their study is hindered (Dresselhaus et al., 1996). In addition, the persistence of higher fullerenes in the environment is expected to be lower than that of C<sub>60</sub> and C<sub>70</sub> because of their faster ozonation (Heymann and Chibante, 1993), which also reduces the expected occurrence levels of higher fullerenes in the real environment.

Finally, none of the environmental transformation products described in Sanchís et al. (2018) was noticed in the present work. According to that study, epoxides and dimers are expected to occur at concentrations at least 10<sup>4</sup> times lower than the parent C<sub>60</sub>, so the required limits of detection may be too demanding for the current instrumentation.

### 3.2. Comparison with the literature

The concentrations of C<sub>60</sub> and C<sub>70</sub> fullerenes quantified along the Sava River in the present work were, in general, higher than previously reported levels in surface waters, as can be seen in Table 1. The average concentrations of C<sub>60</sub> fullerene for both sampling campaigns in the Sava River were 3.08 and 13.6 ng/l, which are in the same order than those observed in the Llobregat River basin in Sanchís et al. (2013). However, these values were significantly higher than those reported for other rivers from the Iberian Peninsula and the Netherlands. In this last case, C<sub>60</sub> fullerene was never detected in surface water, perhaps because of the higher flows of Dutch rivers in comparison with Iberian ones.

On the other hand, fullerenes composed by a higher number of carbon atoms, such as the C<sub>84</sub> fullerene, are expected to be also generated in combustion processes, although at much lower amounts than C<sub>60</sub> fullerene. In the Sava River, higher fullerenes were investigated but in all

cases were found below the limit of detection. These results can barely be contrasted with the current literature since their presence had been previously studied in only two articles (Astefanei et al., 2014; Núñez et al., 2012). In those studies, C<sub>84</sub> fullerene was detected in two river samples at pg/l levels and it reached a concentration of 19.2 ng/l in a pond sample. For this reason, future monitoring studies should still include these larger fullerenes, which are also employed in nanotechnology.

### 3.3. Potential sources of pristine fullerenes

Sampling points along the Sava river were subjected to quite different kinds of stressors, as has been presented in Table 3. Activities which may potentially be responsible for fullerenes emissions include oil refinery, ships propelled with diesel engines, car traffic and nanotechnology wastes.

Our results in the Sava River cannot distinguish a single source of fullerenes in the different sampling points. For instance, the sampling sites with oil refinery activity (SLB, ZUP, SRM) do not present significantly higher concentrations of fullerenes, as had been suggested in other works (Sanchís et al., 2012; Sanchís et al., 2013). Also, the most concentrated sample, MOJ, actually belongs to an Alpine village with a small population and a non-significant industrial activity. However, fuel burning (coal and wood for local heating in smaller towns along the Sava River) and diesel and gasoline engines from traffic and ships may be the primary sources. In agreement with this, Tiwari et al. identified fullerenes in soot from propane grill, diesel-powered generators and wood fire (Tiwari et al., 2016), all of them common combustion sources which are very likely to occur in rural areas and high mountain locations.

Also, it should be taken into account that untreated discharges have been identified near the three last sampling points, which may also be responsible for the comparatively larger concentrations of fullerenes in Šabac and Belgrade.

### 3.4. Pristine fullerenes in sediments

In Table 5, the concentrations in sediments are summarized. As in the case of surface water, C<sub>60</sub> fullerene was the most frequently detected analyte and exhibited the highest concentrations. This fullerene was detected in all the 12 analysed samples at concentrations higher than the MLQ (from 109 to 895 pg/g<sub>dw</sub>), with an average concentration of 337 pg/g<sub>dw</sub> and a median concentration of 219 pg/g<sub>dw</sub>. Regarding C<sub>70</sub> fullerenes, it was quantified in almost 60% of the samples at concentrations up to 3.7 pg/g<sub>dw</sub>, with an average and a median concentration of 0.9 and 1.1 pg/g<sub>dw</sub>, respectively. No correlation was observed between the concentrations of C<sub>60</sub> fullerene in surface water and sediments (R<sup>2</sup> = 0.15, p > 0.2), as in a previous work (Sanchís et al., 2015a).

Overall, the observed concentrations of C<sub>60</sub> and C<sub>70</sub> fullerenes are higher than those previously obtained in the Besòs and Llobregat Rivers (Sanchís et al., 2015a). In that work, C<sub>60</sub> fullerene was widely detected (75% of the samples), but at concentrations 22 times lower, while C<sub>70</sub> fullerene was not detected in any sample. Also, the concentration values

**Table 5**  
Results in sediment samples (<63 µm fraction). MLOD and MLOQ refer to method limits of detection and quantification, respectively. Those values above the MLOD are highlighted in bold font. Average and median concentrations were calculated assigning values under MLOD and MLOQ as MLOD/2 and MLOQ/2, respectively.

Compound	Concentration (pg/g <sub>dww</sub> )													Frequency of quantification	Range	Average (pg/g <sub>dww</sub> )	Median (pg/g <sub>dww</sub> )
	MOJ	RAD	LIT	VRH	CAT	ZAG	JAS	SLB	ZUP	SRM	SAB	BEO					
C <sub>60</sub>	141	895	155	194	196	162	240	486	446	109	523	491	108–895	337	219		
C <sub>70</sub>	3.7	<MLOQ	1.2	1.1	<MLOQ	1.2	1.3	1.3	1.0	<MLOQ	<MLOQ	<MLOQ	<MLOQ-3.70	0.92	1.05		
C <sub>76</sub>	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
C <sub>78</sub>	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
C <sub>84</sub>	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
[60]CPTAE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
[60]ThPCBM	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
[70]PCBM	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD		
[60]NMFP	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOD-<MLOQ	<MLOD	<MLOD		
															8.3%		

of the present study are significantly higher than those in Tubarão, Brazil, where average concentrations of C<sub>60</sub> and C<sub>70</sub> were equal to 8.5 and 18 pg/g<sub>dww</sub> (Sanchís et al., 2015b).

These significant differences may be attributed to the fact that different fraction of sediments were analysed in these works: Analyses in Barcelonan and Brazilian sediments were performed analysing the whole sediment sample, while in the Sava sediments only the clay-silt fraction (<63 mm) was analysed, following the recommendations of the Water Framework Directive (Dulio et al., 2010). This fine fraction presents a higher relative surface area, often coated with organic matter, and it is responsible of the sorption of most of the organic pollutants (this may be particularly true for fullerenes, since they are strong sorbent materials with a high affinity for organic matter). On the contrary, concentrations of organic contaminants in the sandy fraction of the sediments are commonly negligible and, in the previously mentioned works, the introduction of large grains in the analysis resulted in a systematic dilutions of the results. This highlights the relevance of defining the analysed fraction of sediments in analytical works in general and in fullerenes in particular.

None of the higher fullerenes (C<sub>76</sub>, C<sub>78</sub> or C<sub>84</sub>) or the oxidized C<sub>60</sub> fullerenes (epoxides and dimers) was detected in any sediment.

### 3.5. Occurrence of engineered fullerenes in the Sava River

Five engineered fullerenes ([60]PCBM, [70]PCBM, [60]NMFP, [60]ThPCBM and [60]CPTAE) were included in this study. In almost all the samples, these compounds were not detected. However, in the first campaign, one water sample from Zagreb presented a quantifiable concentration of [60]PCBM (6.21 ng/l), which is remarkably higher than other positive samples in the literature (Astefanei et al., 2014). Regarding sediment samples, a single compound ([60]NMFP) was detected in one sample (SAB), at a concentration close to its limit of quantification (S/N ~8).

The detection of these two compounds can be justified because of their emission from R&D activities in the nanotechnology industry or academia: [60]PCBM and [60]NMFP were synthesized for the first time 20 years ago (Hummelen et al., 1995; Maggini et al., 1994) and have been studied in nanotechnology because of its photovoltaic performance, as they can reach remarkable power conversion efficiencies in bulk heterojunction photovoltaic cells (Huang et al., 2015). To the best of our knowledge, currently, fullerene-based solar cells are under development and further improvements in terms of efficiency and thermal stability are required in order to succeed in hypothetical large-scale commercialization (Ma et al., 2005).

According to our results it can be concluded that the influence of nanotechnology as a pollution source of fullerenes is minor, nowadays. However, it should be also taken into account that little is known about the degradation pathways of engineered fullerenes. The lower abundance of these compounds may be justified because of derivatised fullerenes are less stable in the environment than C<sub>60</sub> or C<sub>70</sub>.

Regarding the presence of these analytes in other studies, Astefanei et al. (2014) reported the presence of engineered fullerenes in six water samples from Catalan ponds. In that work, two samples exhibited concentrations of [60]PCBM close to the limit of detection (~1.4 pg/l) and two other samples exhibited concentrations of 5.2 and 8.0 pg/l, much lower than those observed in the present work. Besides, [60]NMFP was detected in 4 of 6 samples at concentrations up to 8.5 ± 0.5 pg/l. This compound was also frequently detected at high concentrations in WWTP effluents (Farré et al., 2010). In contrast, [60]NMFP was not detected in the water samples of the present study, as it was not detected in the Llobregat river Basin (Sanchís et al., 2015a).

In sediments, Astefanei et al. (2014) detected both, [60]NMFP and [60]PCBM. While in the present study [60]NMFP was only detected once, at a concentration close to the MLOQ, in Astefanei et al. (2014) [60]NMFP was ubiquitously distributed in the seven analysed samples at concentrations which ranged from to the limit of quantification

(0.030–0.10 pg/g) to 2.7 pg/g. In other studies near Barcelona and in Brazil (Sanchís et al., 2015a, 2015b) engineered fullerenes were not detected.

Overall, different environmental profiles can be observed in Catalonia, Brazil and in the Sava River. This suggests that the sources of engineered fullerenes are local and significantly different in different regions.

#### 4. Conclusions and future perspectives

The presence of ten fullerenes has been determined in 27 freshwater samples and 12 sediment samples from the Sava River. The results show the presence of C<sub>60</sub> and C<sub>70</sub> at part-per-trillion and part-per-quadrillion concentrations, and these levels were significantly impacted by the hydrological conditions. In contrast, the other pristine fullerenes (C<sub>76</sub>, C<sub>78</sub> and C<sub>84</sub>) were never detected in this work. In some few samples [60]PCBM and [60]NMFP were detected at concentrations close to the current state-of-the-art limits of detection.

It can be concluded that hydrological conditions must be taken into account when comparing the levels of nanomaterials in environmental reports. Also, the present results confirm that the emission of engineered fullerenes from nanotechnology-related sources should not be neglected in the future. More studies should be carried out to unambiguously identify the sources of engineered and pristine fullerenes in other regions, including coastal areas, where the occurrence of fullerenes has been poorly assessed up to date.

The detected concentrations (pg/l-ng/l order) are unlikely to cause environmental problems regarding acute toxicity (Freixa et al., 2018), and they are unlikely to modify the mobility and toxic effects of other co-contaminants (e.g. Trojan Horse effect (Sanchís et al., 2015c)). However, the presence of nanomaterials is known to stress the metabolome of aquatic organisms even at environmental levels (Sanchís et al., 2017), with uncertain consequences after chronic exposure. No information exists regarding engineered fullerenes, but these compounds present several functional groups that may establish an additional series of interactions with small metabolites and proteins. It is important to study the sub-lethal effects of pristine and engineered nanomaterials in the environment at the ultra-trace levels.

#### Conflict of interest

The authors state that there are no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.06.285>.

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