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Beyond nC_{60} : strategies for identification of transformation products of fullerene oxidation in aquatic and biological samples

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Abstract

Owing to their exceptional properties and versatility, fullerenes are in widespread use for numerous applications. Increased production and use of fullerenes will inevitably result in accelerated environmental release. However, study of the occurrence, fate, and transport of fullerenes in the environment is complicated because a variety of surface modifications can occur as a result of either intentional functionalization or natural processes. To gain a better understanding of the effect and risk of fullerenes on environmental health, it is necessary to acquire reliable data on the parent compounds and their congeners. Whereas currently established quantification methods generally focus on analysis of unmodified fullerenes, we discuss in this review the occurrence and analysis of oxidized fullerene congeners (i.e., their corresponding epoxides and polyhydroxylated derivatives) in the environment and in biological specimens. We present possible strategies for detection and quantification of parent nanomaterials and their various derivatives.

Keywords

Polyhydroxylated fullerene; Fullerene epoxide; Biota; Bioaccumulation; Mass spectrometry

Introduction

The unusual properties and versatility of nanomaterials have led to a rapid increase in their production for a wide range of applications. Among these materials are fullerenes, which are especially attractive owing to the many covalent modifications and coatings that can give the compounds modular properties [1]. With its popularity rivaled only by carbon nanotubes (CNTs), the buckyball (C_{60}) is the most commonly investigated closed-shape carbon nanomaterial, and varying forms are under development for an array of applications [2, 3]. Although fullerenes are used in food products, fabrics, and cosmetics, most of these compounds are produced at high purity for high-tech applications or research [4, 5]. With the increase in industrial manufacture of fullerenes (currently exceeding several tons/year) [4], concerns regarding potential adverse effects on human health and the environment have become more pertinent. Biological outcomes of exposure to pristine fullerene aggregates have been observed in vivo and in vitro, mostly in the high parts-per-billion range and beyond [6–13], although the initially reported toxic effects were mainly a result of artifacts from the method for preparation of aqueous fullerene dispersions (nC_{60}) [14, 15]. Although formation of radical oxygen species (ROS) has emerged as the principal cause of the toxicity of fullerenes [11], moderate non-ROS-related toxic effects have also been reported [16]. At the time of the writing of this manuscript, when using < fullerene > as a search term, more than 2,000 manuscripts could be retrieved from the Toxicity Literature Online (TOXLINE) database and more than a dozen from the Developmental Toxicology Literature (DART) database. Entries in both databases also are accessible via the Toxicology Data Network portal (TOXNET, toxnet.nlm.nih.gov).

Fullerene nanotoxicology is a subject of increasing complexity, but few criteria are available to determine which strategies are most relevant for future research. Firstly, there is no consensus in the research community about which *fullerene dispersion methods* are representative of real-life exposure, because these are highly dependent on the application studied and the biological system affected. The lack of uniformity presumably arises from the dearth of information on the chemical properties of different fullerene dispersions. Nonetheless, prudence is advised when interpreting toxicity data on nC_{60} prepared in a carrier solvent, because transformation of the solvent can lead to unexpected toxic effects independent of the fullerene itself [14]. Secondly because industry needs to solubilize and/or functionalize fullerenes for specific uses [17], which *fullerene congeners* dominate current relevant exposures is unclear (with a few exceptions [18, 19]). In all likelihood, the functional groups or coating chemicals change fullerene toxicity and additional research is required to achieve targeted detection and quantification in their respective matrices. Hence, toxicologists and exposure scientists study different types of fullerene dispersion [12, 20, 21] and focus mostly on pristine fullerenes, polyhydroxylated fullerenes (also known as fullerols or PHFs), or complex fullerene derivatives for specific applications [11, 13, 20, 22–24], whereas the toxicology of fullerene size (e.g., C_{60} versus C_{70}) or fullerene particle size is rarely studied [25–28].

Recent efforts have begun to investigate the fate of pristine fullerenes under laboratory and environmental conditions by assessing the impact of organic matter, ionic strength, electromagnetic radiation, or biological systems [27, 29–33]. This has produced valuable information upon the forms into which fullerenes can be transformed upon release into the environment. This expanding range of fullerene diversity creates significant challenges to

their qualitative and quantitative detection. Here, we review current knowledge on the occurrence and fate of fullerenes leading up to and after exposure. We then discuss the challenges of fullerene congener analysis and propose strategies that may enable better monitoring of this group of chemicals.

Fullerene exposure and toxicology

Fullerenes are currently used in consumer products, albeit on a relatively small scale compared with other nanomaterials, for example metallic silver or titanium dioxide [34, 35]. Fullerene toxicology has therefore focused on dermal and pulmonary exposure by simulating exposure from cosmetics, clothing, or the manufacturing process itself. In this field, most research uses pristine fullerenes that are either ground to a microparticle powder or dispersed in ultrapure water, organic solvents, or oils to simulate real-life exposure [21, 36]. Pristine C₆₀ fullerenes have been detected in commercial cosmetics at concentrations ranging from 0.04 to 1.1 µg g⁻¹ [19]; this research, for the first time, enabled calculation of a potential daily exposure dose of approximately 0.6 µg per application of 500 mg cosmetics. In two commercial cosmetic products, pristine C₇₀ fullerenes were detected in addition to C₆₀, which emphasizes the importance of testing different fullerene sizes and coatings in future toxicity research. In the same study, cosmetics labeled to contain fullerenes were characterized by use of transmission electron microscopy (TEM) and the fullerenes found morphologically resembled polyvinylpyrrolidone (PVP)-encapsulated fullerenes. Owing to the complexity of the matrix, whether fullerene derivatives are also used in consumer products will remain uncertain until regulations are updated or new analytical approaches become available. Because of dermal use and the limited ability of nanoparticulate fullerenes to penetrate the skin [21], these nanomaterials are highly likely to be a source of gray water contamination. The fraction of water-soluble fullerenes that the body does absorb will probably mostly not be excreted via urine, assuming murine models are a valid means of providing information about uptake, metabolism, and excretion by humans [37].

The second exposure route, inhalation, is the best-studied in fullerene nanotoxicology. Although a wealth of information has become available on the instillation or inhalation of fullerenes in murine models [38], human exposure can be estimated only from scarce atmospheric data (mostly obtained in the vicinity of manufacturing plants) [39]. In addition to industrial production [40], fullerenes are also formed inadvertently during incomplete combustion [41–43] and are present in the environment as a result of natural processes [44]. Recently, an atmospheric contamination survey of the Mediterranean Sea revealed a high prevalence of fullerenes [18]. This study targeted six pristine and functionalized (i.e., anthropogenic) fullerenes. C₆₀ and C₇₀ fullerenes were detected at median concentrations of 0.06 and 0.48 ng m⁻³, respectively. These results suggest ubiquitous occurrence that is presumably related to urban and industrial activity across Europe. However, no modified fullerenes were detected in any of the samples.

Both instillation and inhalation studies with pristine and polyhydroxylated C₆₀ show limited biological effects only at much higher doses than the environmentally detected concentrations; no long-term adverse lung tissue effects occur [13]. In addition, intratracheally instilled fullerenes were shown to be removed from the lung surface with a half-life of 15–28 days [33]. In this study, a relative increase in fullerene epoxide (C₆₀O) concentrations upon extended exposure of lung tissue was shown, as was the generation of at least two currently unidentified compounds. The mechanisms behind these reactions and the rates of formation of these compounds have not yet been unraveled. Further analytical and toxicological research on these fullerene transformation products is warranted. There is still some uncertainty regarding the biological effects of different fullerenes, although

mounting evidence shows that both pristine and polyhydroxylated fullerenes are relatively benign under the conditions examined. In particular, the beneficial (antioxidative) effects will confuse the assessment of inflammatory responses. In the next section, the environmental fate and effects of fullerenes is discussed, because they are likely to become a waste-water pollutant [45].

Environmental fate of fullerenes

Fullerenes in aquatic systems

Limited data are available on the occurrence of fullerene and fullerene derivatives in real-world aqueous environments. As is the case for small molecules, released engineered carbon nanoparticles are a minute fraction of naturally occurring colloids (e.g., soot [46]). On the basis of monitoring data, it has been suggested that engineered fullerenes will lose their surface modifications and be an additional source of pristine fullerenes [18]. These pristine fullerenes have extremely low solubility in water (for C₆₀ and C₇₀, respectively, 1.3×10^{-11} and 1.3×10^{-10} ng mL⁻¹ at room temperature) [47], but C₆₀ fullerenes can occur in a water-stabilized colloidal form (*n*C₆₀). Even though the preparation of aqueous C₆₀ dispersions is often conducted in the presence of an organic solvent, stable colloids can also form during extensive stirring in water alone. Although this process is very slow, requiring weeks to months [48, 49], it may be an important mechanism for introduction of C₆₀ congeners into aquatic systems despite their overall low water solubility. However, colloidal systems are thermodynamically unstable, and, therefore, the environmental fate and occurrence of fullerenes is likely to be governed largely by their aggregation behavior [50]. Several factors are known to affect the colloidal stability and aggregation behavior of C₆₀, including pH and the presence of electrolytes, surfactants, organic matter, and electrochemical irradiation [51–57]. The chemistry and forces behind *n*C₆₀ particle formation have been scrutinized in detail [58–60]. Recently, a fraction of the fullerenes in these colloidal *n*C₆₀ particles was shown to be enriched in hydroxyl moieties that stabilize the *n*C₆₀ aggregates [59]. Because hydroxylation of fullerenes is achieved even under mild conditions, this reaction is very likely to occur in aquatic environments. We expect all the above-mentioned processes also apply to the C₇₀ fullerenes that are deposited in aqueous systems from aerosols [18]; hence, *n*C₇₀ nanoparticles, C₇₀ epoxides, and polyhydroxylated C₇₀ fullerenes will appear in the environment.

Mass flux considerations expect amounts of fullerenes to be 0.003 ng L⁻¹, 4–310 ng L⁻¹, and 13.1 μg kg⁻¹ in surface water, wastewater effluents, and soil, respectively [61, 62]. Although detected concentrations in wastewater-treatment plant effluents across Europe are much higher than the estimated values [45], no fullerenes or fullerene transformation products have yet been detected in surface waters [45, 63]. This is probably because of the combined effect of low emissions in some regions of the world and high removal efficiency in systems with high organic content (e.g., active sludge and/or biosolids) [61, 64] rather than because of biodegradation [65].

Once released into the environment, pristine fullerenes are highly likely to rapidly partition out of the aqueous phase and to become adsorbed by microbial biomass [66, 67], aquatic submerged plants [68], and soil particles [69]. In environments containing less organic material (e.g., sandy soils), fullerenes are expected to be more mobile, particularly when coated with organics (e.g., humics) [70, 71]. Nevertheless, substantial accumulation of these aggregates is expected near the source of contamination. In environments that are contaminated with xenobiotics or organic nutrients, the bioavailability of fullerenes may be diminished [72], and in environments in which fullerenes remain mobile, they may facilitate chemical transport via shuttling [71]. However, these processes are presumed to be very limited because of their low prevalence and the high probability of physicochemical

transformation of fullerenes in natural and engineered environments. As is true for CNTs, these mechanisms could be useful in engineered processes utilizing fullerenes, e.g., as adsorbents for analytical approaches [73, 74].

Fullerene transformation and degradation in aquatic systems

Colloid formation is not the only process that increases the aqueous stability of fullerenes. Once released into the environment, the fullerenes in the outer shell of nC_{60} particles may undergo extensive chemical transformation that increases their individual water solubility. When the nC_{60} particles break up and/or erode over time (particularly owing to mechanical erosion), the internal fullerite crystal structure will be exposed [59]. Larger C_{60} aggregates are expected to erode more slowly because of their smaller specific surface accessible to water. The erosion of fullerene particles may enable them to decrease in size over time with concurrent release of polyhydroxylated fullerenes from their outer shell. The water solubility of these fullerols can be $>50 \text{ mg L}^{-1}$ and, depending on the number of hydroxyl groups present, can be extremely mobile in porous media [75]. Measurements targeting unmodified C_{60} may thus underestimate the presence of other fullerenes in water samples. The environmental fate of fullerenes is only now being investigated in detail [30]. However, several laboratory experiments suggest that under ambient conditions, oxygenation and hydroxylation reactions are predominant. The best-understood transformations under environmentally relevant conditions include oxidation reactions, as a result of photoactivation or ozonation. Important C_{60} transformation paths are summarized in Fig. 1.

Fullerenes are well known for their propensity to degrade in the presence of light. Early experiments demonstrated that exposure to light of C_{60} dissolved in benzene resulted in eventual cage opening of C_{60} accompanied by formation of aldehydes and ketones [76]. More recently, detailed experiments have examined the phototransformation of C_{60} in aqueous samples. In these studies, water-stabilized nC_{60} particles were irradiated by UV and sunlight, and the level of functionalization was assessed by toluene extraction and UV-visible spectroscopy [77–79]. Depending on the irradiation source, up to 95 % of the initial nC_{60} was photo-transformed in as little as seven days. Almost complete transformation was found after several weeks or months of irradiation with sunlight. This change was accompanied by reduction in nC_{60} cluster size, as assessed by TEM, which indicates an increase in the water solubility of the fullerenes. Although the actual structure of the fullerene progeny in these experiments is not known in detail, Fourier transform infrared (FTIR) spectroscopic analysis indicated the presence of ketone and hydroxyl groups. Qualitative comparisons revealed high similarity between the transformation products and commercial fullerols, which indicates that oxygenation and hydroxylation reactions occurred during phototransformation. One proposed pathway for the initial phototransformation step involves formation of singlet oxygen by energy transfer from UV-excited triplet C_{60} . The singlet oxygen can then react with triplet C_{60} to form C_{60} epoxides [80, 81]. In a subsequent report, the visible spectrum of sunlight also was found to contribute significantly to phototransformation of C_{60} [78]. Furthermore, these experiments suggested resilience of the rate of transformation to both presence of organic matter and changes in pH [77]. However, another study indicated that humic acids reduce the rate of phototransformation of C_{60} , presumably owing to physical shielding of the fullerenes [78].

In addition to phototransformation, extensive chemical transformations of C_{60} occur in the presence of ozone [82]. Ozonation of nC_{60} resulted in significant transformation within hours, as demonstrated by a reduction in fullerene-specific absorbance peaks in UV-visible measurements ($>90 \%$ after 90 h). Subsequent FTIR analysis identified the transformation products as highly oxygenated and hydroxylated fullerenes. X-ray photoelectron spectroscopic (XPS) analysis suggested an average composition of $C_{60}(O)_x(OH)_y$, with the sum of x and y equaling 29. [83]. Radical-initiated surface modifications have also been

investigated as a possible source of C₆₀ modification. However, available data suggest that fullerenes are relatively recalcitrant to hydroxyl radicals [84].

Overall, these findings indicate that polyhydroxylated fullerols are likely transformation products of C₆₀ in aquatic systems. However, fullerols themselves are also known to be photoreactive. In contrast with fullerenes, they readily undergo mineralization during exposure to light [85]. In batch studies, up to 47 % of the fullerols irradiated with artificial sunlight were found to undergo mineralization after approximately 20 days. The remaining transformation products of unknown composition did not absorb UV light. Another removal process that has recently attracted attention is the ability of fungal microorganisms to degrade fullerols biologically [86]. In addition, oxygenation and/or hydroxylation also may occur in the absence of light [59], albeit at a slow and uncertain rate.

Together these findings depict a potential pathway for transformation of water-insoluble C₆₀ into much more soluble fullerol species. These, in turn, can eventually be mineralized by either photodegradation or biological activity. In addition, synthesized fullerols, which future biomedical applications are expected to use [87], could be released directly into the environment. The relative contributions of fullerene oxidation reactions and the direct release of fullerols to the total environmental discharge of fullerenes remain unknown. Even though highly hydroxylated fullerenes could be readily eliminated from the system, the transformation of C₆₀ into water-soluble forms could occur at a much faster rate. This would result in a net mobilization of fullerenes into aquatic systems of unknown proportions. To accurately assess the effect of fullerenes on humans and the environment, water-soluble fullerene congeners and their open-cage derivatives must be considered.

Environmental exposure

Given the fate of fullerenes in the aquatic systems described above, it is of interest to examine the design of ecotoxicity studies of fullerene fate and to compare their outcomes to inform us of future research needs. Particularly in the last two years, numerous ecotoxicological studies have reported on the effect of fullerenes on a wide range of model organisms, e.g., mollusks [88], crustaceans [27, 89–92], oligochaetes [93–95], bony fish [11, 91, 96–101], and aquatic angiosperms [68], and polyhydroxylated fullerenes were found to stimulate the growth of green algae and *Arabidopsis thaliana*, reproduction of *Ceriodaphnia dubia*, and longevity of mice [102].

Despite limited systemic uptake of fullerenes by the bloodstream, pristine particles seem to have a strong tendency to accumulate in some tissues, particularly in the mammalian liver and in mollusk hepatopancreatic cells [88, 103]. They are, however, barely retained in the gut of crustaceans or worms [27, 93]. After ingestion by crustaceans, fullerene particle sizes have been observed to increase from the nanometer range to 10–70 μm [27]. This caused the fullerenes to settle out of suspension after defecation. Apparently, organisms (e.g., soil-dwelling worms) absorb molecular fullerenes more readily than aggregated fullerene nanoparticles. The overall extent of ¹⁴C-labeled C₆₀ uptake is low (biota–sediment accumulation factor (BSAF) = 0.065–0.13) [104] but higher than that of CNTs (BSAF = 0.006–0.02) [105]. The principal property excluding uptake and bioaccumulation of fullerenes is thought to be their large size, i.e., high molecular weight.

For adequate toxicological assessment, proper characterization and quantification of nanomaterials is essential. This cannot be limited to simple measurement of *n*C₆₀, however, owing to the previously discussed known mechanisms of chemical modification that may occur in vivo or during sample preparation in vivo. Ideally, methods for quantification should thus include the full range of closed-cage C₆₀ transformation products as target analytes. Some microbial fungi have been shown to decompose fullerols [86] and use them

as carbon source; in addition, direct photolysis can mineralize fullerols to inorganic carbon [85]. Future research also is needed to better understand the chemical makeup of fullerene constituents after opening of the carbon cage. In the section below, we speculate about potential strategies for quantifying fullerenes and their oxidized derivatives.

Challenges in the analysis of fullerene transformation products

Analytical approach for fullerene detection

Numerous analytical methods have been developed for quantification of C₆₀ in a variety of matrices (Table 1). The typical method for C₆₀ extraction exploits the high solubility of C₆₀ in toluene, with addition of counter-ions to destabilize the *n*C₆₀ particles for quantitative extraction of pristine fullerene and its epoxide. In addition, solid-phase extraction of fullerenes from water has been conducted for samples of environmental and biological origin [106, 107]. HPLC–MS and HPLC–UV–visible strategies have been successfully used to measure the fullerenes content of a variety of matrices [18, 64, 96]. Separation of fullerene is generally achieved by HPLC with a C₁₈ column in conjunction with an eluent consisting of a mixture of organic solvents (e.g., toluene and acetonitrile) with no aqueous component. Other stationary phases include pyrenylpropyl group–bonded silica [108]. For detection and quantification of C₆₀ fullerenes, MS-based detection is more specific than UV-based detection, because it measures the mass-to-charge ratio and, in the case of tandem MS, also yields structural information; it is, generally, also more sensitive. However, strong absorbance of UV by fullerenes results in detection limits comparable with those of atmospheric pressure chemical ionization (APCI)-MS and electrospray ionization (ESI)-MS; tandem MS is even more sensitive [45, 96, 106]. Sensitive detection of fullerenes by UV–visible spectroscopy was achieved for spiked water samples (method detection limit, MDL = 0.02 µg mL^{−1}), but this is very likely to be insufficient to reveal exposure at the currently expected levels of contamination. Toluene-extraction-based MS analysis procedures often also result in the detection of oxygenated species, e.g., C₆₀O, which indicates that these methods are, at least in principle, able to detect slightly modified fullerene species. Unfortunately, no current method can be used to determine whether this extraction and quantification is quantitatively accurate. Changes in chemical makeup (e.g., oxidation) can occur as a result of chemical aging, the method used to prepare the C₆₀ fullerene stock dispersion, and the ionization conditions used. As a point of reference, we propose monitoring and reporting of C₆₀O-to-C₆₀ ratios to assess the importance of these variables and enable qualitative interlaboratory comparison of pristine *n*C₆₀ dispersions (in spiked samples).

Unfortunately, this methodology cannot be easily extended to the analysis of polyoxygenated and polyhydroxylated fullerenes. As already mentioned, one of the distinguishing properties of UV-transformed fullerenes is their insolubility in toluene. For the same reason, existing reversed-phase HPLC-based separation strategies are not appropriate. Moreover, quantification of fullerol has proved challenging. Unlike C₆₀, polyhydroxylated fullerenes do not have any strong absorbance peaks in the UV–visible region [107]. MS-based approaches are also limited, mainly because of the instability of fullerols, even under mild ionization conditions [108]. We recently reported that, despite massive fragmentation, fullerols can be detected and quantified efficiently by use of hydrophilic interaction chromatography in combination with tandem MS (HILIC–LC–MS–MS) [109]. In addition, quantification of C₆₀ fullerols is possible in water samples by detection of a specific fragmentation series by use of ESI–MS [109]. Lower detection limits were achieved by monitoring a specific fragment (*m/z* 73) for each precursor ion (i.e., the major peaks of the initially detected *m/z* 74 series). Whether this method can easily be extended to the large variety of fullerene transformation products expected remains to be tested. However, the commercial fullerol used in that study was not chemically pure but had

an average composition of $C_{60}(OH)_x(ONa)_y$, with $x+y=24$ and y generally in the range of 6–8; these values are close to those expected for a C_{60} transformation product.

Considering their high water solubility and low toluene extractability, oxidized transformation products of fullerenes may occur at higher concentrations than their respective nC_{60} precursors. The plausible diversity of different transformation products of different polarity is a substantial analytical challenge. Moreover, interference from non-target compounds, including dissolved organic matter in aquatic samples, may further complicate accurate quantification. General steps in the detection of fullerenes from aquatic samples include extraction, separation, and detection, each of which presents specific methodological challenges. Considering the vastly different polarity of fullerene and its transformation products, no simple method is likely to enable combined extraction of all transformation species. For the same reason, chromatographic separation could be problematic, because different mobile and/or stationary phases must be used to achieve proper separation. Finally, the specific detection and quantification of the complete range of transformation products is analytically very challenging. Because little is known about their chemical identities, a detection method that integrates mass and structural information, for example, tandem MS, likely will be crucial. However, little research has been conducted on the extraction and MS-based detection of oxidized fullerenes [109, 110].

In summary, we currently have methods for quantification of water-insoluble C_{60} and water-soluble C_{60} fullerol (Fig. 2). We do, however, lack methods applicable to the wealth of transformation products on the chemical continuum between these compounds. Because establishment of a specific method for each possible fullerene congener is impractical, it would be beneficial to use methods resulting in only one or two enrichment fractions that are amenable to analysis for either unmodified C_{60} fullerenes or C_{60} fullerols. In the next section we discuss several pre-analytical methods that may assist in acquiring these samples.

Pre-analytical strategies for detection of fullerene and fullerene transformation products

Defunctionalization—In the simplest case, the modified fullerenes could be defunctionalized to pristine C_{60} , which would then be amenable to established methods of quantification (Fig. 3). Several chemical functionalizations known from organic synthesis are reversible, including Diels–Alder, Bingel, and Prato reactions [111–113]. However, no specific reactions are known for defunctionalization of hydroxylated fullerenes. Nonetheless, thermogravimetric analyses on a variety of hydroxylated fullerene species have shown that temperatures of 150–300 °C can lead to elimination of hydroxyl moieties [114–116]. This thermal instability may thus be exploited for defunctionalization of fullerol species. However, it is not known whether this can lead to a quantitative removal of hydroxyl groups, because highly hydroxylated fullerene cages may open [115]. By analogy, trace detection of $C_{60}O$ fullerene epoxides at the retention time of pristine C_{60} could be indicative of either the instability of dimeric $C_{120}O$ fullerenes at the APCI interface (even though they are expected to elute later) or oxidation of a small fraction of pristine C_{60} fullerene into its epoxide.

Another possible strategy is to use reductive agents at moderately elevated temperatures. Alumina columns have been successfully used to reduce nitrated polycyclic hydrocarbons for quantitative analysis [117]. Moreover, chromatography on alumina resulted in quantitative (91 %) conversion of $C_{60}O$ into C_{60} [118]. This approach has not yet been tested on more highly oxygenated or hydroxylated fullerene derivatives, although quantitative dehydration may occur at least for less hydroxylated C_{60} fullerols. Fullerols have usually been regarded as the desired product of fullerene derivatization rather than the precursor; however, further research into this methodology could significantly improve fullerene analysis in environmental samples.

Functionalization—Instead of reducing the water solubility of oxygenated and hydroxylated fullerenes by defunctionalization, the inverse could be achieved by hydroxylation. This approach has the advantage that well-known chemical reactions could be exploited. This includes direct synthesis of fullerol C_{60} under acidic conditions [119], conversion of C_{60} halogenated fullerene intermediates, or fullerene nitration to nitro-fullerene with subsequent hydrolysis under alkaline conditions. Under laboratory conditions, highly water-soluble fullerols with average compositions $C_{60}O_nH_m$, where $n = 10\text{--}26$ and $m = 14\text{--}30$, were formed [120–123]. Another advantage of this approach is that use of acids or bases for hydroxylation of C_{60} can assist in the degradation of potentially interfering organic material within the sample. A disadvantage is that these reactions are quantitative only under controlled conditions, and without sufficient purification, the efficiency and specificity may be insufficient and result in unintended products. Another possible method of increasing the water solubility of nC_{60} is to simulate accelerated aging of fullerene in the environment with controlled ozone treatment [83]. One of the biggest challenges with the above methods is to achieve controlled addition or removal of hydroxyl groups within environmental water samples as matrix. Moreover, the expected diversity in the degree of hydroxylation and oxygenation may not enable one-step derivatization.

Differential extraction—A simple approach that could be combined with functionalization or defunctionalization strategies is the pre-fractionation of fullerene and fullerol-containing water samples. Ideally, this would result in an aqueous fraction containing water-soluble fullerols and an organic fraction containing unmodified C_{60} or less-oxygenated and/or hydroxylated species. Depending on the complexity and diversity of the fullerene congeners, direct MS-based analysis of many of the fullerene species may be possible. Alternatively, each fraction, or sub-fractions thereof, could be subjected to an optimized functionalization or defunctionalization strategy and then quantified by use of an established method (Fig. 3). For instance, in fractions of low polarity, $C_{60}O_mH_n$ species with low $m+n$ are to be expected, and these may be stable enough to be defunctionalized by heat treatment alone or heating in the presence of alumina. Conversely, separation according to water solubility may enable more controlled functionalization of each fraction, which would simplify subsequent analysis.

Liquid–liquid extraction (LLE) with toluene as organic solvent is routinely used to isolate organic compounds, including fullerenes, from water samples [124]. To acquire C_{60} fractions with different levels of functionalization, existing LLE strategies could be extended by using serial LLEs with increasingly polar solvents. This would enrich less-functionalized and non-functionalized C_{60} in the early fractions and enrich highly hydroxylated fullerols in the later ones. Quantitative extraction of polyhydroxylated fullerols has been less well investigated. However, owing to their high water solubility, ready separation of these water-soluble fullerol species into the aqueous phase is expected, particularly under conditions precluding extensive precipitation or flocculation.

Solid-phase extraction techniques also have been successfully used for fullerenes [19, 106, 125]. Here, different sorbents could be combined with use of differential elution with solvents of staggered polarity to obtain fractions enriched in chemical targets to serve as starting points for further functionalization and/or defunctionalization strategies.

As a tentative analysis scheme, the fractions could be tested with matrix-assisted laser desorption/ionization (MALDI) MS for the presence of the fullerene derivatives. This technique is not quantitative, and, owing to the high stability of the core, the C_{60} ion (m/z 720) yields the strongest signal. Nevertheless, less-derivatized fullerenes for example $C_{60}O$ have also been detected successfully. Even though past attempts to identify highly hydroxylated fullerene ions were unsuccessful [123, 126], the characteristic m/z 720 ion of

the C₆₀ fullerene peak is a suitable qualitative indicator for successful enrichment of C₆₀ and its derivatives.

Filtration—Filtration is another potential method for simultaneous enrichment of fullerenes and fullerols despite their extremely different water solubility. Here, the inherent tendency of fullerenes to aggregate may be exploited to promote aggregation for increased filtration efficiency. In solution, fullerenes have negative zeta potentials [52]. Moreover, their aggregation behavior can be modeled satisfactorily by classic Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, which predicts electrostatic double-layer repulsion as the dominant force [54]. Accordingly, provision of counter-ions may serve to increase aggregation, thereby improving the efficacy of subsequent filtration of fullerene species using porous membranes. The removal efficiency of C₆₀ was tested in the presence of alum (for coagulation) and Ca²⁺, and found to be highly pH-dependent [127]. Similarly, fullerols were removed successfully from solution by filtration in the presence of counter-ions. However, in contrast with *n*C₆₀, the authors found that pressure had a large effect on fullerol filtration efficiency. Overall, the destabilization of C₆₀ fullerenes and fullerols by counter-ions has been shown to be a suitable technique for controlling colloid stability and thus promoting more efficient filtration. The presence of natural organic matter can complicate this process, because it is known to stabilize fullerene colloids via surface adsorption, thereby modifying their steric repulsion behavior [55]. However, if quantitative filtration is possible, samples enriched in fullerene and fullerols could be dried and subjected to the procedures described above. This concentration of fullerene species from dilute samples may greatly enhance the prospect of subsequent successful quantifications.

Conclusions

The current literature reveals the significant potential of fullerenes to undergo transformation in the environment. Therefore, their actual occurrence and environmental concentrations may be significantly greater than those determined experimentally by use of available methods of limited capacity. For a more complete risk assessment of fullerenes, more work will be needed to reveal this currently obscured but putatively substantial fullerene chemistry. One obstacle in the way of this important objective is the lack of reliable procedures for qualitative and quantitative analysis of fullerene derivatives in both environmental and biological matrices. The potential strategies for fullerene separation and detection proposed here are intended as a starting point for further method development and refinement.

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Fig. 1.

Known transformations of fullerenes under environmentally relevant conditions. A significant amount of fullerenes is expected to be oxygenated and hydroxylated

Fig. 2. LC-MS-based quantification of water-soluble and pristine C₆₀ fullerenes. Owing to different stability and polarity, different chromatographic separation techniques and MS-based detection methods must be used. *HILIC* = hy-drophilic interaction chromatography

Fig. 3.

Potential strategies to identify and quantify fullerenes and fullerene transformation products in aquatic samples. (1) Direct functionalization or defunctionalization may result in a homogenous fullerene species that is amenable to established quantification methods. (2) If the sample matrix is too complex to allow a one-step functionalization or defunctionalization of the fullerenes, differential extraction, according to increasing fullerene modification and thus polarity, can be conducted. The individual fractions may be analyzed directly or subjected to different functionalization and/or defunctionalization strategies. (3) Addition of an ultrafiltration step may improve the purity of the samples and enable simplified downstream extraction strategies

Table 1

Summary of methods for sample preparation and analysis applied to environmental samples

Sample preparation	Target	Sample	Range of average recovery	Refs.
Filtration and/or evaporation	C ₆₀ , C ₇₀	<ul style="list-style-type: none"> • UPW, tap water, surface water, wastewater • Aerosols 	<ul style="list-style-type: none"> • 63–77 %/2–35 % • 60–70.6 % (Atmospheric) 	[18, 45, 128]
Liquid–liquid extraction	PVP/C ₆₀ , C ₆₀ , C ₇₀	<ul style="list-style-type: none"> • UPW, plasma, blood, urine • Lung, liver, brain, spleen, skin • Fish embryos • 4.5 % BSA, 5–50 mg L⁻¹-C NOM, 1 % SDS • Wastewater, groundwater, surface water, tap water • Cosmetics 	<ul style="list-style-type: none"> • 13–92 % • 84–102 % • 90 % • 94–100 % • 32–98 % • 78 % 	[19, 33, 36, 45, 64, 96, 106, 124, 127–131]
Solid-phase extraction	PVP/C ₆₀ , C ₆₀	<ul style="list-style-type: none"> • UPW, plasma, blood, urine • Groundwater, surface water, tap water, reclaimed wastewater, secondary effluent • Cosmetics 	<ul style="list-style-type: none"> • 62 % • 9–84 % • 103 % 	[45, 57, 63, 64, 106, 131]
Analysis	Target	Sample	Limits Of Detection	Refs.
LC–UV–Vis or DAD	PVP/C ₆₀ , C ₆₀	<ul style="list-style-type: none"> • UPW, plasma, blood • Liver, spleen, skin, lung • 4.5 % BSA, 5–50 mg L⁻¹-C NOM, 1 % SDS • Groundwater, surface water, wastewater, secondary effluent 	<ul style="list-style-type: none"> • 0.3–5 µg L⁻¹ (liquid) • 8.9 ng g⁻¹ (tissue) 	[36, 63, 64, 124, 130, 131]
LC–ESI–MS	C ₆₀ , C ₆₀ O, C ₆₀ OH, C ₆₀ OCH ₃ , C ₆₀ OCH ₃ OH, MFP, CPTAE, PCBM, ThPBCM, C ₇₀ , Fullerols ^a	<ul style="list-style-type: none"> • UPW • Fish embryo • Secondary effluent • Aerosols 	<ul style="list-style-type: none"> • 0.001–1 µg L⁻¹ (liquid) • 5.4–20.9 pg m⁻³ (atmospheric) 	[18, 45, 96, 109, 124]
LC–APCI–MS	C ₆₀ , C ₆₀ O, C ₇₀ , C ₁₂₀ O	<ul style="list-style-type: none"> • UPW, urine • Tap water, wastewater 	<ul style="list-style-type: none"> • 0.3–11 µg L⁻¹ (liquid) 	[64, 106, 128]
LC–APPI–MS	C ₆₀ , C ₇₀	<ul style="list-style-type: none"> • 0.1–10 mg L⁻¹ humic acids 	<ul style="list-style-type: none"> • Not reported 	[132, 133]

^aWith 24 hydroxyl moieties

APCI, atmospheric-pressure chemical ionization; APPI, atmospheric pressure photoionization; CPTAE, C₆₀ pyrrolidine tris-acid ethyl ester; DAD, photodiode array; ESI, electrospray ionization; LC, liquid chromatography; MFP, *N*-methylfulleropyrrolidine C₆₀; NOM, natural organic matter;

PVP, polyvinylpyrrolidone; PCBM, [6,6]-phenyl-C₆₁ butyric acid butyl ester; SDS, sodium dodecyl sulfate; ThPBCM, [6,6]-thienyl C₆₁ butyric acid methyl ester; UPW, ultrapure water; UV, ultraviolet detector.