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Nanomaterials in the Environment

METHODOLOGICAL CONSIDERATIONS FOR TESTING THE ECOTOXICITY OF CARBON NANOTUBES AND FULLERENES: REVIEW

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Abstract—The recent emergence of manufactured nanoparticles (NPs) that are released into the environment and lead to exposure in organisms has accelerated the need to determine NP toxicity. Techniques for measuring the toxicity of NPs (nanotoxicology) in ecological receptors (nanoecotoxicology) are in their infancy, however, and establishing standardized ecotoxicity tests for NPs are presently limited by several factors. These factors include the extent of NP characterization necessary (or possible) before, during, and after toxicity tests such that toxic effects can be related to physicochemical characteristics of NPs; determining uptake and distribution of NPs within exposed organisms (does uptake occur or are effects exerted at organism surfaces?); and determining the appropriate types of controls to incorporate into ecotoxicity tests with NPs. In this review, the authors focus on the important elements of measuring the ecotoxicity of carbon NPs (CNPs) and make recommendations for ecotoxicology testing that should enable more rigorous interpretations of collected data and interlaboratory comparisons. This review is intended to serve as a next step toward developing standardized tests that can be incorporated into a regulatory framework for CNPs. Environ. Toxicol. Chem. 2012;31:60–72. © 2011 SETAC

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INTRODUCTION

Typically, manufactured nanoparticles (NPs) are defined as having one dimension within the range of 1 to 100 nm [1] and can have unique physicochemical properties that are useful in many applications. Many NPs have already been incorporated into numerous consumer products (see the Woodrow Wilson database: http://www.nanotechproject.org/inventories/consumer/), and the number of products containing NPs and the types of NPs incorporated are expected to increase dramatically in future years. Despite the promise of nanotechnology to revolutionize many sectors of society, major concerns exist regarding the potential for NPs to pose unexpected negative environmental or human health risks [2–4]. To avoid unexpected negative consequences of nanotechnology, proactive research is being conducted a priori to assess the effects of releasing NPs into the environment. This research should help mitigate the potentially harmful consequences of NPs and lead to the sustainable development of this technology. Research is also needed to avoid unsupported negative perceptions of the nanotechnology industry that could impede realizing the full benefits of this technology as a result of a lack of public trust [5].

The ability to manipulate the configuration of carbon atoms within molecular structures is one of the major achievements of nanoscience and is the platform from which many applications of nanotechnology are developing rapidly. Carbon fullerenes and carbon nanotubes (CNTs) are the two most prominent classes of carbon nanoparticles (CNPs) in terms of research investigations and current applications in consumer goods. This is expected to lead to high production of these NPs in future years [6–8]. Carbon nanotubes are one of the most promising classes of new materials to emerge from nanotechnology to date. They are composed of extensive sp^2 carbon atoms arranged in fused benzene rings. Their structures give them exceptional material properties, which are in turn being used in composite materials, sensors, hydrogen-storage fuel cells, and various environmental applications [9–12]. For a more extensive discussion of the unique properties and characteristics of CNTs see Mauter and Elimelech [12]. Single-walled carbon nanotubes (SWNTs) are one-layered graphitic cylinders having diameters of a few nanometers, whereas multiwalled carbon nanotubes (MWNTs) have between two and 30 concentric cylinders with outer diameters commonly between 30 and 50 nm. Carbon molecules arranged into a spherical shape resembling a geodesic dome have become known as fullerenes. There are multiple spherical configurations of fullerenes involving different numbers of carbon atoms (e.g., C_{60}, C_{70}, C_{80}), but C_{60} (Buckminster fullerene, or Bucky ball) is by far the most prominent in terms of production, scientific interest, and research engagement. Numerous researchers have discussed the unique physicochemical properties [13,14] and potential beneficial properties of C_{60} [15,16].

Incorporating CNPs into products indicates that these materials will ultimately be released into the environment either during normal use of the product or when the useful life of the product is completed. Carbon nanoparticles enter the environment through various routes (Fig. 1), with some routes more likely than others and more likely to affect the form (e.g., aggregated, functionalized) of the CNP on arrival. Nowack and colleagues [6–8] recently have made modelling efforts and have

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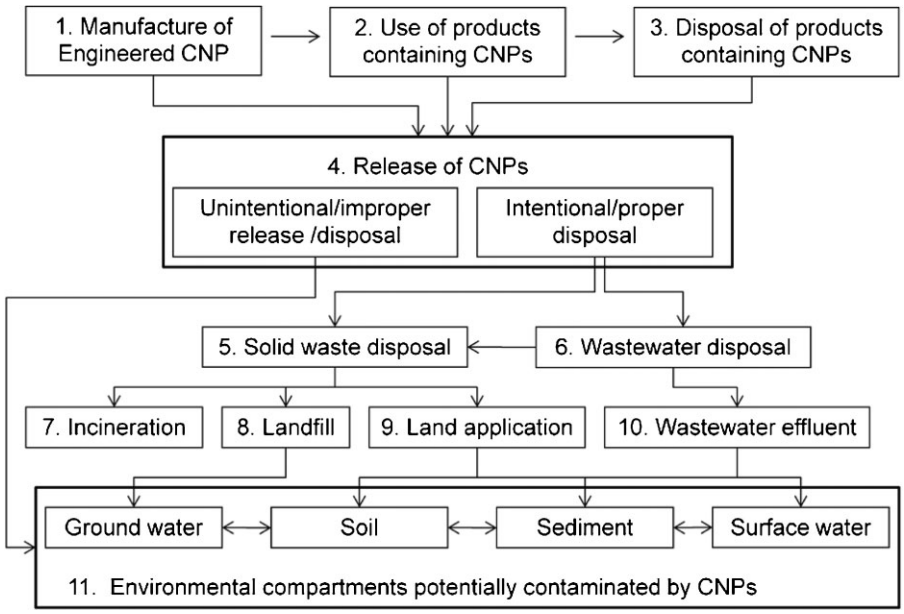

Fig. 1. Diagram of likely pathways for carbon nanoparticles (CNPs) to arrive and contaminate environmental compartments and consequently lead to exposure in organisms. The level of contamination is the outcome of the amount of CNP produced (1); the form of the CNP incorporated (for example, embedded in resin, free particles, functionalization) into products, their use, and disposal (2–4); likelihood of the CNP to pass through waste treatment processes (5–10) or bypass treatment processes (improper disposal); and persistence of CNP within the environmental compartment (11).

conducted detailed analysis of the potential masses of carbon nanoparticles expected to enter the environment through different routes of arrival, and Petersen et al. [17] recently reviewed CNT release pathways from polymer nanocomposites. The life cycle of many NPs is expected to include their release into wastewater treatment plants as a primary route through which they might ultimately enter the environment [6–8]. The potential for different steps in the wastewater treatment plant to remove CNPs, therefore, has also been investigated [18–20]. Based on the likely scenarios for CNPs to arrive in the environment, it is possible to indicate the types of ecosystems likely to be contaminated and the organisms that are of highest priority for testing the negative effects of CNPs (Fig. 2). These scenarios suggest that investigating the ecotoxicity of CNPs present in the aqueous phase (aquatic organisms) is environmentally relevant as is assessing the toxicity of CNPs deposited in soil or sediments. Routes of priority exposure for ecotoxicity testing include respiratory surfaces (e.g., gills via aqueous exposure), dermal (contact between organism surfaces and CNPs), and dietary (either via ingesting CNPs directly or by ingesting organisms that have accumulated CNPs). One major challenge related to the ecotoxicological testing of fullerenes and CNTs is that their expected concentrations in the environment are unknown, but they are estimated to be very small. For example, Gottschalk et al. [6] recently estimated that the average (mode) increase in sediment and sludge-treated soil for fullerenes in the United States was 2.5 ng kg^{-1} $year^{-1}$ and 1.01 ng kg^{-1} $year^{-1}$, respectively, whereas those for CNTs were 46 ng kg^{-1} $year^{-1}$ and 31.4 ng kg^{-1} $year^{-1}$. Similarly, low concentrations in surface water were estimated, 0.001 ng/L and 0.003 ng/L for CNTs and fullerenes, respectively [6]. To our knowledge, these concentrations are all orders of magnitude lower than those needed for all analytical techniques that can be used to quantify CNPs in environmental media, although a liquid chromatography/mass spectrometry procedure has been used to detect fullerene concentrations in complex media as low as 300 ng/L [21]. Currently, these modeled values cannot be corroborated using environmental samples. In addition, it should be noted that the concentrations stated are estimated averages, and those in various hot spots would be expected to be orders of magnitude higher.

Given that organisms in water, sediment, and soil compartments of the ecosystem will potentially be exposed to CNPs, standardized ecotoxicity tests must be developed to evaluate the risks that various types of CNPs may pose to organisms in each of these environmental compartments. Results of toxicity tests of CNPs relevant to ecotoxicology have been reported in the literature since 2004 (see, e.g., Oberdörster [22]). During this initial period, researchers in the developing field of nanoecotoxicology began to recognize the difficulties of evaluating NPs and that techniques used to test other substances were not adequate for NPs. Many early review articles speculated about the needs for assessing ecotoxicity of NPs [2,3] and suggested techniques that should be developed and the aspects of NPs that may contribute to toxicity. It is now appropriate to review nanoecotoxicity testing critically based on the current state of the science rather than a priori expectations. The lack of knowledge about how to test the toxicity of NPs is broadly recognized, and a recent study by Grieger et al. [23] indicated that testing considerations were the greatest area of uncertainty for determining the environmental health and safety of nanoparticles. Baun et al. [24] published a review on recommendations for NP testing using aquatic invertebrates; the present review focuses on important considerations for ecotoxicity testing of CNTs and fullerenes in all environmental compartments, with a focus on specific issues for these two CNPs. Testing the potential ecotoxicology risks of CNTs and fullerenes poses many challenges given the substantial differences between these CNPs and typical organic and inorganic pollutants. After carefully reviewing the available ecotoxicological literature on CNPs, we summarize several methodological considerations to help in avoiding experimental artifacts, to

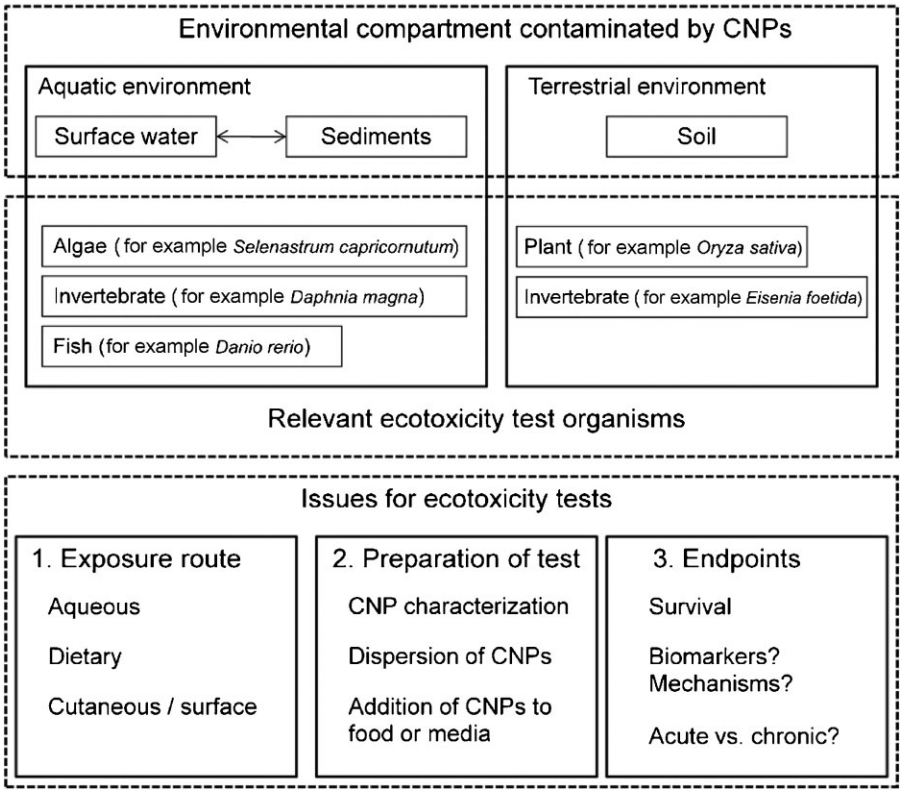

Fig. 2. Environmental compartments contaminated by carbon nanoparticles (CNPs), relevant ecotoxicity test organisms, and issues for ecotoxicity tests that must be taken into account when undertaking investigations to determine the effects of CNPs on organisms in the environment.

facilitate comparisons among studies, and to allow more robust interpretations of the results obtained. Whereas several studies have investigated the ecotoxicological effects of CNPs on co-contaminants [25–32], this review focuses on the risks of the CNPs themselves. First, it is critical to characterize thoroughly the CNP powder and NPs in suspension and, to the extent possible, quantify the NP concentrations to which the organisms are exposed. In addition, several experimental artifacts have to be considered, such as the release of metal catalysts by CNTs or byproducts from certain fullerene dispersion techniques. We also recommend a set of control conditions or experiments. Finally, most studies do not indicate systemic absorption of detectable concentrations of CNTs and fullerenes by a range of ecological receptors; the implications of this observation for ecotoxicity tests are discussed.

STEPS IN CONDUCTING ECOTOXICITY TESTS WITH CNPs

Characterization of CNPs before, during, and after ecotoxicity testing

Characterizing CNPs is an essential component of ecotoxicity testing and can include evaluating the starting materials, CNP characteristics during ecotoxicity tests, and the extent to which the CNPs are altered during the test. Numerous analytical techniques are available to characterize CNPs, but here we focus on techniques related to testing parameters expected to be important for ecotoxicity experiments. A recent panel indicated the following as a priority list for environmental studies: size, dissolution, surface area, surface charge, and surface composition/surface chemistry [33]. Formulating a new list of priority characterization techniques is beyond the scope of this paper, but researchers are encouraged to read other review articles (see, for example, Stone et al. [33]) for a more thorough discussion of this topic. A list of selected characterization techniques that covers techniques to assess these properties is provided in Table 1. Trade-offs certainly exist among the time, costs, and instrument availability associated with full particle characterization and the importance of these measurements for the particular study. Comprehensive NP characterization is desirable, but the current lack of knowledge about the properties that are most toxicologically important indicates that some measurement techniques described (electron energy loss spectroscopy) may be relevant only for specific studies.

Characterizing starting materials is essential, because CNPs can contain substances (including chemicals known to be toxic) in addition to the CNPs themselves [34–36] and because the purity of the CNP material is frequently unreliable, based only on the manufacturer's information. Large differences in reported toxicity of CNPs in the literature are likely a consequence of investigators actually testing different materials, starting materials containing varying amounts of contamination, and variable aggregation of the NPs occurring in the test solution. Carefully characterizing starting materials will enhance comparability of results among laboratories, facilitate interpretations of ecotoxicity results, and help the field to develop standardized ecotoxicity tests for CNPs [37–40]. To support characterizing starting materials and comparability among laboratories, efforts are underway to prepare reference materials for various types of NPs at the National Institute for Standards and Technology (NIST). For example, three gold NP reference materials (10, 30, and 60 nm) have recently been certified by NIST (NIST RM 8011: https://www-s.nist.gov/

Table 1. Selected characterization techniques for carbon nanotubes and fullerenes[a]

Technique	MWNT	SWNT	Fullerenes	What it can analyze	References
Thermal gravimetric analysis	x	x	x	Mass of surface coatings, remaining metal catalysts, carbon impurities (amorphous carbon and graphitic spheres)	[48,124,125]
Transmission and scanning electron microscopy	x	x	x	Size (diameter, length), impurities, aggregation state	[48,125]
Cryotransmission electron microscopy	x	x	x	Properties of the nanoparticles in the aqueous phase	[126]
Liquid chromatography (LC)/mass spectrometry or LC with ultraviolet (UV) detection			x	Can separate and quantify different types of fullerenes (i.e., C_{60} and C_{70})	[21,105]
Spectrofluorimetry		x		Chiralities of semiconducting SWNTs, sample purity	[48,125]
Raman spectroscopy	x	x		SWNT purity, chiralities of SWNTs	[48,125]
X-ray photoelectron spectroscopy	x	x	x	Elemental composition of sample (top 1 to 10 nm), surface chemistry of powders	[68]
Atomic force microscopy	x	x	x	Size (diameter, length) but may be limited for SWNT diameter, properties of nanoparticles in aqueous phase	[48,127]
Brunauer, Emmett, and Teller (BET) surface area analysis	x	x	x	Surface area	[28]
Inductively coupled plasma-mass spectrometry	x	x	x	Metal concentrations	[48]
Instrumental neutron activation analysis	x	x	x	Metal concentrations	[48]
Dynamic light scattering	x	x	x	Size of aggregates in aqueous phase but may not work well for nanotubes as a result of modeling assumptions based on spheres	[60,79]
Centrifugation	x	x		Length distribution of carbon nanotubes and size of nanoparticle aggregates	[128]
Electrospray differential mobility analysis		x	x	Size distribution of nanotubes and small aggregates	[129]

[a] MWNT = multiwalled carbon nanotubes; SWNT = single-walled carbon nanotubes.

srmors/reports/8011.pdf; RM 8012: https://www-s.nist.gov/srmors/reports/8012.pdf; RM 8013: https://www-s.nist.gov/srmors/reports/8013.pdf).

Residual chemical impurities (organic chemicals or metals) from the synthesis process or from subsequent chemical modifications of CNPs are likely to be present especially for CNTs. Carbon nanotubes are often synthesized by chemical vapor deposition, a process in which nanotubes are formed by passing a hydrocarbon gas over a metal catalyst at an elevated temperature [41,42]. Thus, subsequent purification steps such as acid treatments are needed to remove the catalyst materials, but this process may be hindered by encapsulation of metal catalyst particles by carbon [43]. The difficulty of fully removing these catalysts by acid treatment suggests a limited bioavailability of a fraction of the metals given the expected minimal degradation of CNPs during the course of experimental exposures. Nevertheless, unpurified CNTs and to a lesser extent purified CNTs are likely to leach metals in the test media. In addition, byproducts from as-prepared fullerenes and metallofullerenes were shown recently to leach toxic concentrations of metals [36]. Assays such as the toxicity characteristic leaching procedure [44] or bioaccessibility tests [45–47] coupled with relevant analytical techniques can be used to measure ecologically relevant concentrations of the impurities. Researchers are also recommended to estimate the total metal fraction remaining in their CNT mixtures using a combination of thermal gravimetric analysis (TGA), inductively coupled plasma-mass spectrometry (ICP-MS; or ICP-optical emission spectroscopy), and instrumental neutron activation analysis (iNAA) [48]. Given the ease with which very-high-purity fullerenes are obtained (>99% purity), it may not be necessary to assess the concentration of metals in purified fullerenes.

Beyond assessing the purity of CNP starting materials, it is also essential to assess the physicochemical properties of the NPs themselves given the lack of information indicating which properties, if any, dictate toxicity in various receptors. Among the properties that can be assessed are CNP surface area, size/shape, and surface chemistry. Surface area is one of the primary factors initially hypothesized to be related to novel NP toxicity [2,3]. Auffan et al. [49] showed that the toxicity of cerium oxide NPs to human fibroblast cells was higher for NPs compared with bulk particles on a mass basis but similar on a surface-area-normalized basis. Furthermore, surface area was an important predictor for toxicity of silica NPs with algae [50]. However, the importance of NP surface area to toxicity for CNPs in ecotoxicity tests is presently unknown, and surface area has not yet been related to any in vivo ecotoxicological effects for CNPs to our knowledge. Investigations comparing the toxicity of CNPs to bulk hard carbons should consider potential differences in surface area as a possible variable to explain differences in toxicity. The most common analytical approach for surface area analysis is the Brunauer, Emmitt, and Teller (BET) method, which can be readily assessed with surface area analysis equipment by measuring adsorption of nitrogen gas onto powder samples at the temperature of liquid nitrogen. One limitation of this technique for ecotoxicity tests, especially those for which CNPs are sonicated prior to exposure, is that dispersion may influence the surface area of the particles if aggregates are broken apart or the ends of the carbon nanotubes are opened during this process. In addition, although the ends of the CNTs being opened could influence their capacity to adsorb chemicals, it is unlikely that this would influence their ecotoxicological effects. Another potential approach is to calculate the surface area of CNPs in suspension after counting the sizes of a large number of CNPs, but this result would be only an estimate of the actual surface area and would be hindered by the polydispersivity of CNPs in solution and the variable extent to which the NPs are aggregated.

Shape and morphology can differ radically among CNPs and have been identified to have potential consequences for toxicity of CNPs, but only a few studies have investigated this topic. In one study with rainbow trout (*Oncorhynchus mykiss*), dietary

exposure to either C_{60} or SWNTs (500 mg/kg food) did not result in differences in toxicity [51], whereas, in another study, a smaller carbonaceous nanofiber fraction, rods with diameters of 1 nm and lengths less than 18 nm, increased lifetime mortality of the estuarine copepod *Amphiascus tenuiremis* relative to purified SWNTs at the same concentration (10 mg/L) [52]. Further work on the effects of CNP shape related to toxicity is clearly warranted. Characterizing CNP shape should be an important element of CNP ecotoxicity tests; for example, carbon nanotubes with different sizes and physicochemical characteristics have shown varying toxicity to bacteria [53–55]. Methods for determining the size and shape of CNPs include various microscopy techniques—transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Many other detailed reviews of NP characterization have discussed this topic in depth, including Hassellov et al. [56] and Tiede et al. [57].

Surface chemistry, particularly the presence of functional groups on CNPs, can alter the physicochemical properties of CNPs completely and influence environmental fate [58–60] and toxicity of these materials. Functionalized CNPs may be the starting material for ecotoxicity tests, and these characteristics should be verified prior to testing; alternatively, changes in surface functionalization may occur during testing and should be evaluated when possible. Evidence exists that surface chemistry can influence toxicity in test organisms, including *Ceriodaphnia dubia* [61], in which alkyl and amino functional groups on CNTs dramatically increased toxicity, whereas hydrophilic groups made the CNTs less toxic. Likewise, various types of surface-modified fullerenes have also exhibited substantially different toxicities to *Daphnia pulex* and *Daphnia magna* compared with underivatized fullerenes [62,63]. It was shown previously that cellular toxicity varies substantially based on surface coatings or functional groups on the surfaces of CNTs [64,65]. Changes at the cellular level as a result of different surface modifications do not, however, necessarily translate to similar effects in whole organisms. For example, the cytotoxicities of nC_{60} fullerene aggregates (fullerene aggregates produced by stirring in water) and hydroxylated fullerenes varied by several orders of magnitude, but neither was found to have toxic effects when instilled in rat lungs [66].

Techniques that can be used to assess the surface chemistry of CNPs include zeta potential analysis, X-ray photoelectron spectroscopy (XPS), and Fourier transform infrared spectroscopy (FTIR). Laser Doppler velocimetry can be used to assess the electrophoretic mobility of CNPs in solution, and the electrophoretic mobility can then be used to estimate the zeta potential with the Henry equation and the Smoluchowski approximation, but this approximation is only rigorously valid for spherical particles and may be problematic when applied to CNTs. Additionally, XPS can be used to assess the surface chemical composition and functional group distribution of carbon nanomaterials by deconvoluting the C(1s) spectra. However, chemical derivatization using fluorine containing tags/molecules combined with XPS is a far more accurate approach for identification of specific functional groups rather than deconvoluting the C(1s) spectra given the close proximity of different functional groups, the limited resolution of the spectra, and the possibility for different fitting combinations to give equally good results [67,68]. Fourier transform infrared spectroscopy has also been used to analyze the functional groups of CNTs [69], but this approach rarely provides quantitative functional group concentrations, and it may be challenging to differentiate between small peaks and the background [68]. For additional information about characterization of CNTs surfaces, see the recent review article by Wepasnick et al. [68].

CNP dispersion methods for ecotoxicity tests

One of the principal challenges associated with testing the ecotoxicological effects of CNTs and fullerenes is that CNTs are not readily dispersed at a detectable concentration without adding a surfactant and/or a dispersion process (sonication). Perhaps the principal example of an artifact that impacted the interpretation of nanoecotoxicology results was with tetrahydrofuran-dispersed fullerenes (THF-nC_{60}). The hypothesis that THF-nC_{60} can induce oxidative injury in aquatic organisms was supported in early studies [22,70] but has subsequently been refuted as techniques for investigating toxicity of C_{60} have been refined. Toxicity attributed to C_{60} in those studies is instead more likely linked to THF decomposition products, as demonstrated in a study with zebrafish [71] and further confirmed in subsequent research [72]. Results showing that (THF-nC_{60}) does not generate oxidative injury (or any other toxic effects) when THF and THF decomposition products are removed [72] resulted in a convincing rejection of the hypothesis that C_{60} was responsible for the toxicity reported in studies that have used THF-nC_{60}. Despite this evidence, numerous articles continue to cite studies that have used THF-nC_{60} to indicate toxicity of C_{60} (see, for example, Kahru and Dubourguier [73]). Results of THF-nC_{60} investigations demonstrate the challenges of testing the toxicity of NPs but are not acceptable for discussing the toxicity of nC_{60} dispersed in water [72,74].

Assessing the environmental risks of CNPs has to be based on how these NPs are treated during their processing steps before being used in consumer products, how and to what extent they are dispersed in different products, and ultimately how they will likely be dispersed in environmentally relevant matrices. However, it is challenging to gain proprietary information from companies about how CNP dispersions are produced, and current analytical techniques are often unable to distinguish between anthropogenically formed versus incidental NPs in environmental matrices. These factors hinder environmental scientists from knowing how to test environmentally relevant NP dispersions. If companies use THF-nC_{60} in consumer products, then studies using this dispersal approach have yielded important information about the expected environmental consequences. However, results should be discussed carefully to clarify that fullerene mixtures produced using this process are toxic, not that fullerenes themselves are necessarily inherently toxic. This is a particularly important distinction given that findings of high NP toxicity can sway public opinion strongly and that such opinions can influence governmental regulations [5]. Given the serious toxic effects observed for THF-nC_{60}, suspending fullerenes with THF for consumer products should be avoided unless no other alternative approach is available that meets the manufacturers' specifications.

Carbon nanoparticles can also be suspended using a range of natural and synthetic polymers and surfactants such as natural organic matter (NOM), sodium dodecyl sulfate (SDS), polyvinylpyrrolidone (PVP), and polyethyleneimine (PEI). The role that these surface coatings have in CNP toxicity is not yet well understood, but limited numbers of studies have investigated this topic using *Daphnia* [69,75]. Multiwalled carbon nanotubes wrapped with different types of NOM possessed significantly different LC50 values [69]. Grafting PEI coatings onto MWNTs to give the CNTs positive, negative, or neutral surface charges increased the toxicity of the MWNTs compared with

unmodified nanotubes, but the toxicity did not show a clear trend with regard to the surface charge [75]. In addition, it is important to test the toxicity of any polymers or surface coatings themselves, and it may be important to dialyze CNP composites to remove any excess coatings before toxicity experiments [75].

QUANTIFICATION AND CHARACTERIZATION METHODS

Methods of CNP analysis in solutions

Use of carefully characterized starting materials is essential, and further characterizing CNPs is necessary for evaluating exposure preparations and to determine how ecotoxicity tests may cause alterations in CNPs. Unless CNPs are functionalized with sufficient numbers of hydrophilic groups, CNPs will aggregate in aqueous media, a process that is accelerated by the presence of high cation concentrations, especially divalent cations [76,77]. Carbon nanoparticle aggregates in aqueous media can form stable suspensions as complex colloids that interact with other substances in the media, such as NOM, by adsorption and absorption interactions that can influence the size, shape, and stability of the aggregate within the aqueous phase [77]. Associations between CNP aggregates and NOM are particularly environmentally relevant given the ubiquity of NOM in aquatic ecosystems and may be especially challenging to interpret in the context of ecotoxicity tests. The aggregation state and settling of CNPs can also be influenced by organisms. *Daphnia magna*, for example, were shown previously to consume a lipid surface coating on SWNTs, thereby reducing the aqueous stability of the CNT [78]. Suspended fullerenes were also shown to be larger in the guts of *Daphnia* than in the initial solution, and the presence of *Daphnia* increased the fullerene settling rate during a 24-h interval [79]. Therefore, researchers are encouraged to assess the sizes of CNPs before and after experiments. Although such steps are time consuming, this information will allow the researcher to understand the dynamic changes that occur within these experimental systems and to provide information about how interactions with organisms may influence the environmental fate of CNPs.

Although it is relatively straightforward to test the size of suspended fullerene aggregates with dynamic light scattering (DLS), this technique is not well suited for measuring sizes of CNTs. The typical algorithm used to interpret data obtained from DLS measurements operates under the assumption that the particles are monodisperse spheres, a problematic assumption for CNTs and for polydisperse suspensions, which may pose issues for fullerene suspensions too. It is possible to use alternative fitting algorithms for rods in certain light scattering equipment [80], but this approach is uncommon. Instead, data from DLS approaches can yield, at best, qualitative comparisons among the relative sizes of the nanotubes before and after the experiment. An alternative is to use various microscopic techniques to assess the aggregation state of NPs in solution. Most electron microscopic techniques for assessing CNPs require sample drying prior to analysis, so it can become challenging to differentiate between aggregation that occurred during the drying process and aggregation that was present in the aqueous suspension. Although cryo-TEM does not require drying of the sample, it requires extensive sample preparation and uncommon TEM capabilities.

One primary limitation of many ecotoxicity investigations of CNPs in water is a lack of quantification of NP concentrations in the test media. When these measurements are performed, they are typically taken at the beginning and conclusion of experiments and during water renewal when a specific dose is added to maintain a certain aqueous-phase NP concentration. Given that settling of CNPs is common during aqueous exposures, researchers are encouraged to use Organisation for Economic Co-Operation and Development (OECD) methods to indicate a time-weighted mean if substantial settling has occurred [81]. Quantifying NPs in the aqueous phase has been conducted most commonly in experiments with C_{60} fullerenes, because they can be measured readily after toluene extraction and then ultraviolet-visible (UV/vis) spectroscopy [79]. For CNTs, however, concentrations in the aqueous phase during ecotoxicity tests are often limited to nominal concentrations (the CNP mass that was sonicated initially). The mass of CNTs suspended during sonication will vary based on numerous factors, including CNT physicochemistry and dispersion techniques (e.g., sonication power, duration, pulsed sonication vs continuous, use of an ice-water bath, bath vs probe sonication), and only a small fraction of the nanotubes may remain suspended in the aqueous phase. The sonication process may also destroy certain types of CNTs if an ice-water bath is not used [82]; even then, damage to the sidewalls of the CNT is expected. Substantial differences are found among different sonicators, so researchers are encouraged to test their instrument calorimetrically to facilitate comparisons among research groups [83]. Nevertheless, several techniques are available for quantifying CNT in aqueous suspensions: UV/vis spectroscopy [84,85], spectrofluorimetry [86–88], thermal optical transmittance [89], and radioactivity measurements for radioactively labeled nanotubes [75,90]. Perhaps the most straightforward approach is to assess the concentration of suspended nanotubes gravimetrically. In this procedure (modified from Kim et al. [31]), a known mass of CNTs is sonicated in a preweighed beaker, the supernatant is decanted, the beaker is reweighed to determine the volume of liquid remaining, the water is evaporated, the beaker is reweighed, and finally the mass of nanotubes in suspension is calculated by mass balance. This solution can also be analyzed using UV/vis spectroscopy, and changes in the aqueous-phase concentration during subsequent measurements can be assessed using UV/vis spectroscopy given the highly linear calibration curves for absorbance and CNT concentration [84,85]. Given the numerous techniques available for assessing the aqueous-phase nanotube concentrations, researchers are strongly encouraged to make such measurements during their experiments.

Methods of CNP analysis in other matrices

Many relatively straightforward methods are available for quantifying CNTs and fullerenes in simple water solutions in the absence of NOM. However, methods to identify and characterize CNTs and fullerenes in soils, sediments, and organisms are often substantially more challenging. Techniques that can be used to identify both fullerenes and CNTs in these media will be discussed first, followed by methods specific to fullerenes and CNTs. All of these methods are summarized in Table 2.

One of the most common methods used to identify CNPs in cells and, to a much lesser extent, tissues is TEM. Transmission electron microscopy has been used to identify fullerene aggregates in soil extracts from a soil spiked with 154 mg/kg fullerenes [91] and to investigate absorption of CNTs and fullerenes in *Daphnia* [69,79], *Lumbriculus variegatus* [92], and cells [93,94]. Numerous challenges are associated with using TEM to identify CNPs in tissues, including the lack of contrast for CNPs given that they are composed of the same element as the tissue and the resin used to make the samples; the challenge of finding these particles given their extremely low concentrations

Table 2. Selected techniques for identifying or quantifying carbon nanoparticles (CNPs) in environmentally relevant matrices[a]

Technique	MWNT	SWNT	Fullerenes	Function	Strengths	Limitations	References
Radioactive labeling	x	x	x	Measures beta emissions from carbon-14	Detects CNPs in any matrix, quantitative, works to test degradation or transformation	High cost, safety issues, limited availability	[27,41,42,90,96,98]
Toluene extraction and UV/Vis absorbance			x	Measures fullerenes peaks after extraction	Fast, relatively easy and uses common laboratory equipment	May not work in all tissues	[79,102,103]
Spectrofluorimetry		x		Detects absorption and emission of photons in near-IR range	Low detection limit, can examine biodistribution using a microscope	Only tests individually dispersed semiconducting SWNTs	[86,87,109]
Raman spectroscopy	x	x		Detect characteristic CNT Raman peaks	Allows for detection of CNTs in tissues	Qualitative	[78,108]
Liquid chromatography-mass spectrometry			x	Quantifies fullerenes in different matrices	Very low (i.e., ppb) detection limits	May be challenging in certain sample matrices or after changes to fullerenes (i.e., oxidation)	[21,105]
Thermal optical transmittance	x			Combusts aqueous sample and measures mass loss at different temperatures	Quantitative, can be used in presence of dissolved organic matter	Uncommon equipment, aqueous phase only	[89]
Light microscopy	x	x	x	Visually identifies large CNP aggregates	Readily available in many environmental laboratories	Qualitative, nonspecific, only works for very large aggregates	[52,78,90,108,119]
Transmission electron microscopy	x	x	x	Investigate absorption or scattering of an electron beam through a sample	High resolution, can "fingerprint" CNPs using electron energy loss spectroscopy	Aqueous samples typically require drying, identification with EELS requires uncommon expertise, challenging sample preparation for tissues, may be challenging to locate CNPs in samples	[69,79,93,94,108]
UV/Vis absorbance	x	x	x	Measures absorbance of aqueous sample at different wavelengths	Readily available in many environmental laboratories	Potential interference from other sample components	[84,85]
Coherent anti-Stokes Raman scattering		x		Achieves contrast from different vibrational frequencies within a sample	Identifies nanoparticles in tissues without additional sample processing	Not specific to particle type, uncommon equipment	[100]
Chemothermal oxidation at 375°C	x	x		Chemothermal oxidation at 375°C	Quantitative measurement of nanotube concentrations in soils	Low accuracy, only very high CNT concentrations were tested	[110]
Two-photon excitation spectroscopy	x			Excites sample with photons and then measures fluorescence	Detects individual nanotubes in tissues	Equipment is uncommon	[130]
Scanning electron microscopy	x	x	x	Measures backscattered electrons off of a sample	Investigate surface of organisms or cross-sections, faster and more readily available than TEM	Qualitative, lower sample resolution compared with TEM	[18,131]

[a] CNT = carbon nanotubes; MWNT = multiwalled nanotubes; SWNT = single-walled nanotubes; EELS = electron energy loss spectroscopy; TEM = transmission electron microscopy.

in tissues; and distinguishing CNPs from other objects in organisms that are also nanometer-sized and have a similar structure. One technique that has been used successfully to identify fullerenes and CNTs unequivocally in cells is electron energy loss spectroscopy [93,94]. This technique requires a highly skilled operator to be performed successfully, posing a challenge for routine analyses. The importance of using electron energy loss spectroscopy for identifying quantum dots in cells highlights the possibility of false identification of NPs in cells [95]. In addition, characteristic structural information may be identifiable using high-resolution TEM that is unique to the different CNPs.

Another specialized technique used to quantify CNTs in soils, sediments, and biological tissues and fullerenes in biological tissues is radioactive labeling [27,41,42,90,96–99]. Some studies have indicated that biological oxidation of MWNTs and SWNTs in soils, sediments, and tissues was needed to avoid artifacts related to the self-quenching: beta emissions from ^{14}C were absorbed by the NP aggregates [41,42], whereas another study did not find this effect [27]. Therefore, researchers are encouraged to test the possibility of self-quenching of their CNPs because this may differ among CNPs produced by different methods. Despite the high cost and challenge of synthesizing these materials, the use of radiolabeled NPs is a promising technique for CNP quantification in various media and for developing new analytical techniques to quantify CNPs without radiolabeling.

One promising technique that has only recently been used to investigate SWNT uptake in clams is coherent anti-Stokes Raman scattering microscopy (CARS) [100]. Results with this technique indicated that there was no detectable uptake of SWNTs by clams after exposure for 10 d to sediment concentrations of 0.03 g/kg, a result that was confirmed by TEM. Coherent anti-Stokes Raman scattering microscopy was also recently used to investigate the absorption of a range of metal and metal oxide nanoparticles in fish [101].

Several procedures are available to identify fullerenes in *Daphnia* by extracting the fullerenes and then measuring absorbance of their characteristic peaks using UV/vis spectroscopy [79,102,103]. However, these approaches may not work with larger organisms in which these extraction techniques also remove large quantities of other biomolecules such as lipids, which may hinder the detection of the fullerenes by spectroscopic methods. A liquid–liquid extraction procedure has also been used to quantify fullerenes in solutions with protein-containing media and tape-stripped skin samples [104] and from zebrafish [105]. C_{70} has also been detected in plant tissues using FTIR based on the unique characteristics of C_{70} [106]. Isaacson et al. [107] presented a detailed discussion of analytical techniques for quantifying fullerenes in environmental matrices.

Raman spectroscopy is another common technique for qualitatively identifying CNTs in biological systems [78,108]. Spectrofluorimetry has been used to quantitatively identify SWNTs in rabbits [87], cells [86], and fruit flies [109]. However, this approach detects only individually dispersed semiconducting SWNTs and cannot be used to detect MWNTs, metallic SWNTs, or bundles of SWNTs that contain metallic SWNTs. Nevertheless, the quantitative capacities of this approach are promising for environmental studies and detection of SWNTs in soils, sediments, and organisms. Finally, chemothermal oxidation at 375°C (CTO-375) has been used to identify additions of SWNTs and MWNTs added to soils and sediments and yielded recoveries of 66 to 171% for nanotube concentrations of 1 to 20 mg CNT/g soil [110]. However, the capacity of this approach for lower, environmentally realistic concentrations that are many orders of magnitude smaller is unclear, and relatively high naturally occurring concentrations of hard carbons (median ratios of black carbon to total organic carbon are 0.09 and 0.04 for sediments and soils [111]) will likely challenge this approach.

POTENTIAL EXPERIMENTAL ARTIFACTS

One potential artifact for ecotoxicity studies of CNPs is the presence of metal catalysts with resultant toxicity. For example, it was observed recently that yttrium released from CNTs affected the functioning of neuronal Ca channels [34], a large concentration of bioavailable Ni was found in unpurified CNTs [35], and the impact of released metals was suggested as a potential cause of the differing effects of exposure to SWNT and double-walled carbon nanotubes (DWNT) on zebrafish embryos [112]. Metals leached from as-prepared fullerenes and metallofullerenes were previously shown to be highly toxic [36]. One possible method to test for these effects is to monitor the release of potential metal or hydrophobic contaminants after the CNPs are dispersed in the test solution. This can be readily tested in water-only exposures by adding the CNPs to the test media for the duration of the experiment, removing the NPs by filtration, and exposing organisms to the filtrate. Assessing the potential for leached compounds to cause toxicity will help to distinguish whether the observed toxic effects result from exposure to the CNPs themselves or to leached impurities [35,113]. If metals leached from the nanotubes are identified as the source of the toxic responses, additional CNT purification steps may be necessary to allow determination of toxicity attributable to CNTs [35].

A related but distinct impurity in a SWNT sample was a carbonaceous nanofiber fraction composed of rods with diameters of 1 nm and lengths less than 18 nm [52]. These fluorescent nanocarbon byproducts were shown to increase life-cycle mortality of an estuarine copepod (*Amphiascus tenuiremis*) at a concentration of 10 mg/L, whereas purified SWNTs did not have an effect at this same concentration. This suggests that certain fractions of apparently purified SWNTs may have unknown toxic effects and that additional research into the various fractions present in CNTs is a critical future research topic. Similar toxic effects from residual impurities from purified fullerenes have not been shown consistently in the literature.

Additionally, SWNTs have been shown to affect many cytotoxicity assays. One example of this is the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for which the SWNTs caused false-positive results [114]. Therefore, researchers are encouraged to assess the extent to which the concentration of CNPs remaining in the sample could influence the use of toxicity assays and, when possible, to use complimentary assays for an endpoint. Along these lines, it was recently shown that germanium NP attachment directly to DNA during the alkaline Comet assay procedure probably resulted in arbitrary DNA fragmentation, a determination made by observing apparent DNA damage to cells harvested immediately after the NP addition [115]. In addition, fullerene NPs have recently been shown to oxidize in the presence of sunlight, forming a currently not-well-defined set of byproducts [116,117]. Thus, researchers should be cautious of potential artifacts that may occur for fullerenes that are exposed unintentionally to sunlight or room light. Carbon nanotubes have also

been shown to produce reactive oxygen species in the presence of light [118], so the lighting used for ecotoxicity testing is an important topic of concern for experiments with CNPs. One additional potential artifact for microorganism studies is sterilization of CNPs and the extent to which common sterilization procedures might alter CNPs.

RECOMMENDED CONTROLS

We have discussed a broad number of issues related to testing the ecotoxicity of CNPs, many of which can be translated into appropriate experimental controls that should be used in concert with the CNPs to clarify toxic responses. First, it is important to include negative (vehicle only) and positive controls in experiments. Although negative controls are almost always included, including positive controls is infrequent. Positive controls should be selected when possible based on the endpoints studied to ensure that such effects are observed based on chemicals known to elicit such effects. Second, it is important to include control experiments based on the potential for other impurities present in the CNP solution to cause toxic effects. This includes leached metal ions, polycyclic aromatic hydrocarbons, and toxic chemicals included in or formed by the dispersion process (THF byproducts for fullerenes). Many of these potential artifacts can be identified by thoroughly analyzing the materials in the test media prior to initiating ecotoxicity experiments (see Kennedy et al. [61] for an example of a thorough characterization of potential byproducts after CNP modifications). Additionally, it is typically possible to filter solutions used for the toxicity testing to remove the CNPs and then test the toxicity of the filtrate. If toxicity is not observed at the highest nanomaterial test concentration, it is unlikely that chemicals in the filtrate will affect results unless other complex effects of a mixture of contaminants cause toxicity. Third, for experiments conducted in soils or sediments, we recommend also testing the toxicity of activated carbon or carbon black as a *hard carbon* control, as was recently performed in an experiment by Kennedy et al. [119]. Carbon present in soil and sediment organic matter spans a wide range of diagenetic ages, with a harder carbon fraction such as shales, coals, and other highly condensed carbons having substantially stronger sorption behaviors than younger, more amorphous carbon [120]. In the prior ecotoxicological study with CNTs, activated carbon, and carbon black spiked to sediments, the LC50 values for activated carbon and carbon black were actually lower than those for MWNTs for *Leptocheirus plumulosus* and *Hyallela azteca* [119]. Testing these other hard carbon controls allows researchers to investigate whether there is a toxic response unique to the CNPs or whether the observed toxic effects may simply be a result of adding hard carbon materials to the soils or sediments.

LACK OF CNP ABSORPTION INTO TISSUES

Although large numbers of studies have investigated the ecotoxicity of CNTs, CNT absorption has not been observed consistently for organisms tested in terrestrial, sediment, and aquatic conditions [27,41,42,69,90,96,100]. In the four studies that investigated the extent to which SWNTs and MWNTs would accumulate in organisms in sediments, researchers found negligible absorption into tissues of oligochaetes [42,96], two estuarine invertebrates [27], and a lugworm [100]. Changing the properties of the CNTs so that they possess higher octanol–water distribution coefficients, a change that typically corresponds to higher organism accumulation for hydrophobic organic chemicals (HOCs), was not found to increase their bioaccumulation factor values for the oligochaete *Lumbriculus variegatus* [96]. In studies focused on soils, SWNTs and MWNTs did not accumulate in earthworms to a significant extent, even when the MWNTs were modified to be more hydrophilic [41,96]. Studies have also been conducted in the absence of soil and sediment using water-only conditions to assess whether sorption was the factor limiting absorption of the nanotubes. There did not appear to be substantial absorption of MWNTs across the intestines by *Daphnia magna*, but rather a large mass of MWNTs appeared to be compacted in the organisms' guts as measured microscopically and by using radioactively labeled CNTs [90]; a different study investigating MWNT uptake by *Daphnia* using TEM also did not find absorption of the MWNTs across the gut lining [69]. Large masses of CNTs have been found in the guts in many other organisms by using microscopic methods [78,108,119], but no study to our knowledge has shown substantial absorption of CNTs across the gut linings in any aquatic organism. These results for multicellular organisms stand in contrast to those observed for single-celled organisms in which uptake by various cells has been commonly observed [121].

Similarly, evidence for absorption of C_{60} by most ecological receptors is limited, but uptake has been observed for some species. Uptake after oral administration in rats indicated that water-soluble $^{14}C_{60}$ (generated by preparation of C_{60} in saline containing 0.2% Tween 80) was minimal and that the administered C_{60} was voided with feces [122]. Fullerenes were not readily absorbed by *Daphnia magna* based on microscopic examination of microvilli by TEM, and most fullerenes were present as large aggregates within the *Daphnia* gut lumen [79]. Additionally, fullerene accumulations within the gut lumen appeared to be limited by the size of the gut rather than the aqueous-phase concentration, again suggesting minimal systemic absorption into the organism. In one study with fullerenes dispersed using THF and with the THF byproducts subsequently removed, substantial maternal transfer of the fullerenes was reported for *Daphnia magna* [103]. These authors removed excess THF byproducts prior to the *Daphnia* exposures, but it is unclear whether the THF dispersion process influenced the surface chemistry of the fullerene aggregates in some unique way or whether these fullerenes had some toxic effect on the *Daphnia* that allowed for their transfer to the embryos. Transfer of fullerenes across the microvilli was not confirmed by TEM. Thus, future work is needed to investigate maternal transfer with a broader range of dispersed fullerene particles such as those suspended by water mixing (i.e., nC_{60}) and TEM investigations. In plants, however, uptake and translocation of fullerene (C_{70}) coated with NOM was observed, and fullerenes were detected in the roots, stems, leaves, and seedlings of the original plants [106].

Given the lack of evidence of absorption of CNTs and fullerenes in most multicellular organisms, researchers are urged to be cautious when interpreting toxic effects in tissues without documentation of the presence of the CNPs in the tissue. It is possible that toxic effects observed in tissues (e.g., brain of fish) are a consequence of effects of CNPs on external epithelial surfaces without CNP absorption across epithelial membranes and transport to internal organs. For example, in the study by Smith et al. [123], lesions reported in the brains of fish after aqueous SWNT exposure could be a consequence of the occlusion of the gills by SWNTs that was reported in the study and should not necessarily be interpreted to indicate the presence of and toxic effects induced by SWNTs in the brain.

CONCLUSIONS

Given the substantial number of studies investigating the ecotoxicology of CNPs during the past few years, it is now possible to provide general suggestions for testing the toxicity of these materials rather than relying on a priori expectations. This review was intended to help researchers account for the unique considerations involved in the ecotoxicology testing of CNPs. We hope this information will help scientists conduct experiments that avoid major problems of earlier studies and thus provide a scientifically grounded knowledge base for the risk assessment of these materials and for standard nanoecotoxicology methods. Some of the main recommendations of this review article are the following. First, to the extent possible, quantify CNPs concentrations in organisms, which will help link observed toxic effects to CNP concentrations and provide information about the bioaccumulation potential of the CNPs. Second, test for potential artifacts caused by release of chemicals from the CNP powders (heavy metals from CNT catalysts) or from dispersing procedures used to make the nanoparticles stable in solution (THF byproducts with fullerenes). Third, quantify CNP concentrations in solutions before and after exposures for water-only experiments. Fourth, thoroughly characterize nanoparticles and do not rely on manufacturer-provided information. This information will help in comparisons between experiments and the determination of which physicochemical characteristics (length, surface charge, etc.) are related to toxic effects for different organisms. Finally, if toxic effects are observed in specific organs for larger organisms, it is important to test the NP concentration in that tissue to ensure that the observed toxic effect is not the result of an artifact.

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