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Surface Area of Carbon Nanoparticles: A Dose Metric for a More Realistic Ecotoxicological Assessment

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Supporting Information

ABSTRACT: Engineered nanoparticles such as graphenes, nanodiamonds, and carbon nanotubes correspond to different allotropes of carbon and are among the best candidates for applications in fast-growing nanotechnology. It is thus likely that they may get into the environment at each step of their life cycle: production, use, and disposal. The aquatic compartment concentrates pollutants and is expected to be especially impacted. The toxicity of a compound is conventionally evaluated using mass concentration as a quantitative measure of exposure. However, several studies have highlighted that such a metric is not the best descriptor at the nanoscale. Here we compare the inhibition of *Xenopus laevis* larvae growth after in vivo exposure to different carbon nanoparticles for 12 days using different dose metrics and clearly show that surface area is the most relevant descriptor of toxicity for different types of carbon allotropes.

KEYWORDS: Carbon allotropes, graphene, carbon nanotubes, nanodiamonds, metrics comparison, ecotoxicity

Engineered nanoparticles (NPs) such as graphenes, nanodiamonds (NDs), and carbon nanotubes (CNTs) have a number of unique features that make them behave differently from classical chemical products and bulk materials: “small act differently.” The ratio of surface to total atoms or molecules increases exponentially with decreasing particle size. Increased surface reactivity predicts that nanoparticles should exhibit greater biological activity per unit mass compared with larger particles. This suggests that the expressed mass concentration would fail to correctly predict the biological effect of NPs. The aim of this study is to find the most relevant dose metric to quantify the response of exposure to carbon-based nanoparticles (C-NPs) having different structures and morphologies.

For this purpose, an animal model widely recognized in ecotoxicology was used: the larvae of the amphibian *Xenopus laevis*. Exposure of larvae was based on the international standardized bioassay procedure (ISO, 2006). The amphibian model offers numerous advantages, including easy breeding, permeable skin, and gills, and has previously been used for the ecotoxicological assessment of NPs (e.g., cerium dioxide and CNTs). In order to find the most appropriate dose metric, exposures were conducted for four different types of C-NPs: few-layer graphene (FLG), NDs, double-walled CNTs (DWCNTs), and multiwalled CNTs (MWCNTs) (Figure 1). On the basis of previous studies on CNTs, we focused on growth inhibition as an especially sensitive end point. Indeed, growth inhibition represents an integrative toxicological response that includes direct and indirect effects of the different NPs, reflecting the global health status of the living organisms.

*X. laevis* males were injected with 50 IU of pregnant mare’s serum gonadotrophin (PMSG) 500 (Intervet, France, [9002-70-4]) and the females with 750 IU of human chorionic gonadotropin (HCG) (Organon, France, [9002-61-3]) in order to induce spawning. Viable eggs were maintained in a tank filled with tap water until they hatched into larvae. Spawning water was exchanged daily and was used immediately to prepare the larvae bioassay procedure. The larvae were allowed to grow in an incubator until they reached the desired stage for the experiment. For this purpose, an animal model widely recognized in ecotoxicology was used: the larvae of the amphibian *Xenopus laevis*. Exposure of larvae was based on the international standardized bioassay procedure (ISO, 2006).
The exposures were performed under semistatic conditions (ISO, 2006).

The larvae were fed every day on dehydrated and crushed food (Tetraphyll). The larvae were submitted to a 12 h/12 h light/dark cycle. 

Supporting Information. Besides C-NP-exposed larvae, negative controls were also used. The temperature was 22.0 ± 0.5 °C, and the larvae were submitted to a 12 h/12 h light/dark cycle. The larvae were fed every day on dehydrated and crushed fish food (Tetraphyll).

Growth inhibition was evaluated by measuring the length of each larva at the beginning (t0) and the end (t12) of the exposure. Length measurements were performed with the ImageJ software (NIH Image, Bethesda, MD, USA). The length data were standardized as follows:

$$\left( \frac{L_{t_{12}} - M_{t_0} L_{t_0}}{M_{t_0} L_{t_{12}}} \right) \times 100 \times \left( \frac{100}{M_{L_{C_{t_{12}}}}} \right)$$

where Lt12 is the length of one individual larva at 12 days, Mt0 is the mean length of the group at 0 days, and MLt12 is the mean length of the control group at 12 days.

Amphibian dose–response growth inhibition was modeled by predicting the normalized size using the following two-parameter logistic equation:

$$E(\text{Size}_{ijk}) = \frac{100}{1 + \left( \frac{x_{ikj}}{EC_{ij}} \right)^{1/k}}$$

where \( x_{ijk} \) is the dose and \( i, j, \) and \( k \) are the indices over dose metrics, NPs, and concentrations, respectively. EC_{ij} is the value of dose metric \( i \) when the predicted size reaches 50%; the slope at this point is \(-25 \frac{EC_{ij}}{EC_{kj}}\).

Unequal residual variances were taken into account by modeling the observed sizes as independent normally distributed variates:

$$\text{Size}_{ijk} \sim \text{Normal}(E(\text{Size}_{ijk}), \sigma^2_{ijk})$$

where \( \sigma^2_{ijk} \) is the residual variance of the predicted size for NP \( j \) at concentration \( k \). Values for \( \sigma^2_{ijk} \) are issued from within-treatment measurement error variances. Maximum-likelihood (ML) estimates are equivalent to least-squares (LS) regression estimates under the hypothesis of normally distributed residual errors. Consequently, ML estimates were computed by nonlinear weighted LS regression, in which case the regression weights are \( 1/\sigma^2_{ijk} \). Three models were compared, one per dose metric, and their performances were evaluated via their \( R^2 \) and Akaike information criterion (AIC) values, where larger \( R^2 \) and smaller AIC are considered better. The evidence ratio, given by \( \exp((\text{AIC}_i - \text{AIC}_j)/2) \), where \( \text{AIC}_i \) and \( \text{AIC}_j \) are the AIC estimates for dose metrics \( i \) and \( j \), indicates how much more likely dose metric \( j \) is than dose metric \( i \) to be the best predictor for growth inhibition given the set of the three dose metrics and the data. Statistical computations were carried out in R.17

The dose metrics were also measured with errors. Consequently, the data had to be fitted using nonlinear weighted LS errors-in-variables regression, which requires advanced statistical procedures. This was implemented as a hierarchical Bayesian model (HBM), which is presented in the Supporting Information.

The different doses of C-NPs to which Xenopus larvae were exposed and the comparisons between the metrics are summarized in Table 1. The detailed procedures and methods used to obtain these different metrics are provided in the Supporting Information together with the physicochemical characterizations of the C-NPs.

Statistically significant growth inhibition was evidenced after a 12 day exposure to C-NPs. At first sight and on the basis of mass concentrations, growth inhibition seemed to strongly...
Table 1. Corresponding Metrics for Each Dose (A–E) of Nanoparticles to Which *X. laevis* Larvae Were Exposed

<table>
<thead>
<tr>
<th>C-NP</th>
<th>FLG &lt; NDs &lt; MWCNTs &lt; DWCNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>mass concentration (mg L⁻¹)</td>
<td>1.00 × 10⁻²</td>
</tr>
<tr>
<td>number of particles (L⁻¹)</td>
<td>1.00 × 10³</td>
</tr>
<tr>
<td>surface concentration (m² L⁻¹)</td>
<td>1.00 × 10⁻³</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDs</td>
<td>1.00 × 10⁷</td>
<td>1.00 × 10⁷</td>
<td>1.00 × 10⁷</td>
<td>1.00 × 10⁷</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
</tr>
<tr>
<td>DWCNTs</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
<td>1.00 × 10⁸</td>
</tr>
</tbody>
</table>

The growth inhibition mechanism appeared to mostly depend on the surface area of the C-NPs, which can be explained by several hypotheses. Exposure by ingestion was ascertained, and nanoparticles could enter the digestive tract. Guts filled with “black material” were observed for all of the exposed larvae compared with the controls. Furthermore, in earlier studies DWCNTs and MWCNTs were evidenced in the intestine lumen by Raman spectroscopy analysis, high-resolution transmission electron microscopy, or microwave permittivity.
measurements.12,15,20 The presence of C-NPs in the digestive tract could therefore limit the exchange surfaces between the gut lumen and the internal wall, leading to a decrease in absorption of nutrients. Similarly, macro observations revealed the presence of "black material" in the branchial baskets. In X. laevis larvae, buccopharyngeal surfaces have a dual function: they serve in both food particle entrapment21,22 and breathing. If these surfaces become coated with C-NPs, the efficiency of food intake could be reduced. Finally, C-NPs could interact with all of the external respiratory surfaces of X. laevis larvae and may withdraw oxygen from buccopharyngeal respiratory surfaces, skin, or lungs. When aquatic respiratory surfaces are saturated with C-NPs, the absorption of oxygen could be reduced. If the aquatic gas exchangers are not sufficient to extract oxygen from water, the larvae could use its lungs to complete the oxygen supply.23 This aerial respiration has an energetic cost that could lead to reduced growth.24 These two mechanisms may also interact and be jointly responsible for the size reduction of exposed animals.

The results and the hypotheses that were made are specific to the particular mode of exposure used (i.e., raw nanoparticles without any dispersant, semistatic exposure, and reconstituted water under normalized conditions). It is important to note that even with different C-NP physicochemical characteristics (i.e., structure and morphology), growth inhibition mostly depends on the surface area. Finally, it is important to note that the toxic effects with C-NPs were observed at nonenvironmentally realistic doses.25 However, the computed effective area concentration (EC_{50} = 7.47 m^2 L^{-1}) showed that in the case of high-surface-area NPs we could evidence growth inhibition at lower mass concentrations. For example, in the case of "perfect" graphene with a theoretical specific surface area of 2630 m^2 g^{-1}, the predicted mass concentration EC_{50} would be 2.8 mg L^{-1}.

The next step will be to test a new panel of C-NPs (e.g., oxidized) in order to corroborate the model of growth inhibition. The adequacy or inadequacy of the inhibition model would be fruitful and will provide key information to understand the role of surface chemistry but also intrinsic properties of each NP (atomic composition, dispersibility) in their degrees of toxicity.

This study shows that the usual approach based on mass concentrations fails to compare the toxicities of different C-NPs. Apart from papers on aerial nanotoxicology,6,8,26−29 most of published data in nanoeotoxicology are based on mass concentration, and the provided physicochemical characterization is not enough to express the results versus surface area concentration. A full characterization of engineered C-NPs must be provided in every ecotoxicological study in order to allow the toxicity comparison of C-NPs, which should be done on the basis of surface area concentration when it is relevant. The use of this metric would help in the definition of a more realistic risk assessment strategy for carbon-based nanoparticles in the aquatic environment.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.6b00348.

Table 2. Growth in X. laevis Larvae Exposed to the Different C-NPs

<table>
<thead>
<tr>
<th>NC</th>
<th>FLG</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>100.00 ± 4.30</td>
<td>88.90 ± 2.73</td>
<td>93.69 ± 2.86</td>
<td>94.42 ± 4.43</td>
<td>101.30 ± 2.23</td>
<td>nt</td>
</tr>
<tr>
<td>NDs</td>
<td>100.00 ± 2.10</td>
<td>nt</td>
<td>97.02 ± 3.23</td>
<td>84.18 ± 3.26³</td>
<td>73.21 ± 3.00***</td>
<td>59.75 ± 3.11***</td>
</tr>
<tr>
<td>DWCNTs</td>
<td>100.00 ± 11.36</td>
<td>nt</td>
<td>121.80 ± 6.81</td>
<td>91.32 ± 6.24</td>
<td>35.86 ± 5.53**</td>
<td>4.25 ± 2.77***</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>100.00 ± 3.19</td>
<td>nt</td>
<td>94.78 ± 4.47</td>
<td>85.24 ± 2.61</td>
<td>82.19 ± 2.25³</td>
<td>20.30 ± 3.39***</td>
</tr>
</tbody>
</table>

"Results are given as the normalized mean (%) ± Standard Error of the Mean (SEM). "**" corresponds to a significantly different size of larvae compared to the negative control group (mean value) for p ≤ 0.01; "***" corresponds to a significantly different size of larvae compared to the negative control group (mean value) for p ≤ 0.001. NC = negative control; each letter from A to E corresponds to a different concentration of C-NPs.

Figure 2. Growth inhibition in X. laevis larvae after a 12 day exposure to FLG, NDs, DWCNTs, and MWCNTs. Normalized size (%) is plotted vs the base-10 logarithms of three different metrics: mass concentration (mg L^{-1}), number concentration (L^{-1}), and surface area concentration (m^2 L^{-1}). Black dashed lines represent nonlinear regression model predictions, and shaded areas are 95% confidence intervals (CIs) on these. The 95% CIs on the mean sizes, which were computed from the experimental assays, are represented as vertical error bars.
Nanoparticle features, nanoparticle dispersion protocols, protocols for contamination of the exposure media, calculation of particle number and total surface area, and hierarchical Bayesian modeling of errors-in-variables regression (PDF)

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS
AIC, Akaike information criterion; BET, Brunauer–Emmett–Teller; C-NPs, carbon-based nanoparticles; CNTs, carbon nanotubes; DWCNTs, double-walled carbon nanotubes; FLG, few-layer graphene; HBM, hierarchical Bayesian model; HCG, human chorionic gonadotropin; LS, least-squares; ML, maximum-likelihood; MWCNTs, multiwalled carbon nanotubes; NDs, nanodiamonds; NPs, nanoparticles; PMSG, pregnant mare’s serum gonadotropin; SSA, specific surface area

■ REFERENCES

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