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Ecotoxicity of pristine graphene to marine organisms

Carlo Pretti [a,*], Matteo Oliva [a], Roberta Di Pietro [a], Gianfranca Monni [a], Giorgio Cevasco [b], Federica Chiellini [c], Christian Pomelli [d], Cinzia Chiappe [d]

[a] Department of Veterinary Sciences, University of Pisa, San Piero a Grado (PI) 56122, Italy
[b] Department of Chemistry and Industrial Chemistry, University of Genoa, Genoa 16146, Italy
[c] Department of Chemistry and Industrial Chemistry, University of Pisa, Pisa 56126, Italy
[d] Department of Pharmacy, University of Pisa, Pisa 56126, Italy

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ABSTRACT

The ecotoxicity of pristine graphene nanoparticles (GNC1, PGMF) in model marine organisms was investigated. PGMF resulted more toxic than GNC1 to the bioluminescent bacterium *Vibrio fischeri* and the unicellular alga *Dunaliella tertiolecta* on the basis of EC_{50} values (end-points: inhibition of bioluminescence and growth, respectively). No acute toxicity was demonstrated with respect to the crustacean *Artemia salina* although light microscope images showed the presence of PGMF and GNC1 aggregates into the gut; a 48-h exposure experiment revealed an altered pattern of oxidative stress biomarkers, resulting in a significant increase of catalase activities in both PGMF and GNC1 1 mg/L treated *A. salina* and a significant increase of glutathione peroxidase activities in PGMF (0.1 and 1 mg/L) treated *A. salina*. Increased levels of lipid peroxidation of membranes was also observed in PGMF 1 mg/L exposed *A. salina*.

1. Introduction

Graphene, a two dimensional crystalline material constituted by a single layer of sp^2 hybridized carbon atoms arranged in an honeycomb-like lattice structure, has sparked a considerable scientific interest starting from its discovery in 2004 (Novoselov et al., 2004). Fascinated by the exceptional physical properties (high electronic conductivity, good thermal stability, and excellent mechanical strength), the scientific community has immediately envisaged the possibility to apply this material in strategic fields, such as nanoelectronic (Westervelt, 2008), polymers (Stankovich et al., 2006), supercapacitors (Vivekchand et al., 2008), battery electrodes (Paek et al., 2009), printable inks (Wang et al., 2010), antibacterial paper (Dikin et al., 2007) and biomedical technologies (Feng and Liu, 2011). In few years, the number of publications on graphene is increased exponentially and other related materials, including few layer-graphene and ultra thin graphite, graphene oxide, reduced graphene oxide and graphene nanosheets, have been subjected to an intensive investigation. All these materials constituted the composite class of graphene-family nanomaterials (GFN) which, analogously to carbon nanotubes, vary in the layers number, lateral dimensions, surface chemistry, defect density or quality of the individual graphene sheets and purity. Undoubtedly, the structural features of GFN significantly affect the physico-chemical properties of the resulting materials and, although this aspect has not been systematically investigated, probably they determine also the main biological effects. The recent biomedical applications of graphene and derivatives have determined a rapid increase of the studies related to the biological interactions of these materials (Sanchez et al., 2012), however, as for many other nanomaterials, the issue of potential toxicity is related not only to this specific application. If researchers and companies believe in the possibility to apply these chemicals in multiple fields, going from electronics to optics, including mechanics and sensors (Soldano et al., 2010), it is reasonable to suppose a future large-scale production of graphene and graphene-derived materials. GFN dispersed in air might represent a danger to people handling these materials on daily basis, either by contact or inhalation, and studies on this topic are therefore necessary. Nevertheless, accidental spills and effluent discharges can determine an increased risk of release of these exogenous nanoparticles (NPs) into aquatic environments and even though emissions of GFN to aquatic environment should be low (if any), their expected low degradability requires adequate investigation. Investigations on the environmental impact of graphene are therefore mandatory before any large scale application, in agreement to the European regulatory framework for the Registration, Evaluation and Authorization of Chemicals (REACH). In particular, the registration process REACH indicates the requirement of (eco) toxicological assessment for all chemicals produced in or imported into the European Union (today, above one metric tonne per year,

* Corresponding author. Fax: +39 0502210182.
E-mail addresses: cpretti@vet.unipi.it, carlopretti@gmail.com (C. Pretti).

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but in the future the conditions will be more restrictive). The predicted no-effect concentration (PNEC) represents one of the major indicators required for the environmental hazard assessment of chemicals starting from the available chemical and ecotoxicity data, derived from test on organisms, such as bacteria, algae, crustaceans and fish in both acute and chronic toxicity experiments (Pretti et al., 2011).

In this paper we report results about the ecotoxicity of pristine (not-functionalized) graphene. In particular, the effects of different nanometric particles of two commercial formulation of pristine graphene, pristine graphene monolayer flakes (PGMF) and graphene nanopowder grade C1 (GNC1), were studied on model marine organisms, in comparison with the corresponding micrometric bulk material (graphite, GRP).

2. Materials and methods

2.1. Chemicals

Graphite (GRP), the bulk material from which graphene derives and two different commercial forms of graphene, pristine graphene monolayer flakes (PGMF) and graphene nanopowder grade C1 (GNC1), were employed in this study. Graphene was purchased from Graphene Laboratories Inc., Calverton, NY (USA); graphite was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The specifications of these substances, as given by manufacturers, are shown in Table 1. PGMF was ultrapure (no oxidation, no surfactants); > 99.99% carbon content.

GNC1 and GRP were suspended in natural seawater (NSW), milli-Q water, buffers or media, as indicated in specific Materials and methods section; PGMF was suspended in the same manner after ethanol evaporation. NSW (salinity 35 g/L) was oxygen saturated and filtered (0.21 μm). All other chemicals were obtained from Sigma-Aldrich (St.Louis, MO, USA). Freshly opened commercial materials were always employed.

As the hydrophobic nature of pristine graphene derivatives could lead to the formation of aggregates in water, all exposure experiments were carried out under stirring and the use of dispersing agents was avoided (in the case of PGMF ethanol was removed before dispersion in water) in order to reduce eventual interfering effects arising from the presence of co-solvents.

2.2. Vibrio fischeri: inhibition of bioluminescence

The inhibition of bioluminescence test was performed according to standard operating procedure using the Basic protocol (Azur Environmental, 1995), based on the ISO (2007). The same bacterial (*V. fischeri*) lot no. 10J1010, exp. date 09/2012, Ecotox LDS, Pregnana Milanese, MI, Italy) was used for all experiments that were carried out well within the expiration date. Bacteria were obtained as freeze-lyophilized cells in separated vials and always resuspended in NSW.

In order to investigate whether PGMF, GNC1 and GRP (suspended in NSW, maximum concentration of 5 mg/L) could interfere in light emission, several light emission readings with activated bacteria at zero time were performed. Interferences of graphene nanoparticles on light emission readings were evaluated by adding 100 μl of bacteria to each cuvette containing 1 ml of suspension of the considered substance, and reading the light emission every 5 min for 30 min. Relative bioluminescence intensity was expressed in relative bioluminescence units (RBU).

Prior to submit the samples to a full test (identification of ecotoxicological parameters such as $EC_{20/50}$), a screening test (maximum % of effect, I%) at maximum concentration of 5 mg/L was performed. Only substances that showed I% > 20% were submitted to the full test. Bacteria were exposed to a dilution series of the sample and their light emission was determined after incubation. The light emission of the bacteria in the samples was measured after 5, 15 and 30 min and compared to an aqueous control. The tests were performed at 15 °C within the pH operative range (6–8,) by the use of three replicates and four controls. All measurements were performed by using the M500 luminometer equipped with the appropriate cells. The instrument was PC interfaced and acquisition and data handling were performed with the Microtox® Omni 1.16 software. Zinc sulfate eptahydrate was used as the reference toxicant. Mean EC_{50} values (three replicate determinations) were expressed as mg/L together with confidence limits (95%).

2.3. Dunaliella tertiolecta: inhibition of growth

The inhibition of growth of *D. tertiolecta* was evaluated according to the protocol described in ISO procedures (ISO, 1995), with slight modifications. *D. tertiolecta* strain CCAP 19/27 was purchased from the reference center CCAP (Culture Collection of Algae and Protozoa—Scottish Association for Marine Science/SAMS Research Services Ltd). *D. tertiolecta* was cultured in F2-medium (NSW supplemented with a salt mix and a vitamin mix, according with Guillard and Ryther, 1962). Late logarithmic phase algae were inoculated in 25 mL fresh medium (50 mL conical flasks) to an initial concentration of 10^4 cells/mL and were grown at 20 ± 2 °C under cool white fluorescent continuous light of 7000 lx under slow shaking (80 rpm) for 72 h. All cultures were aseptic and bacteria free. Experiments were performed in triplicate. F2-medium acted as control. PGMF and GNC1 were suspended in F2-medium at serial concentrations ranging from 10 mg/L to 0.675 mg/L before inoculum. GRP, used as reference bulk material, was suspended at the concentration of 10 mg/L. Potassium dichromate was used as reference toxicant. The endpoint was the inhibition of growth (*n* cells/mL) at the end of 72 h. Cells were counted by the use of Scepter 2.0 Handheld Automated Cell Counter (Millipore Corporation, Billerica MA USA). $EC_{20/50}$ values of PGMF and GNC1 were calculated with the Linear Interpolation Method for Sublethal Toxicity software (U.S. EPA, 1993).

2.4. Artemia salina acute toxicity test (24 h)

The hatching of *A. salina* cysts (Artemia Gold Argentemia) followed the procedure described in standardised short-term toxicity test (ARC-test) with nauplii (Vanhaecke and Persoone, 1981). The newly hatched nauplii were collected and 5 nauplii were directly transferred into Petri dishes containing 5 ml of NSW that acted as control, and NSW plus PGMF, GNC1 or GRP at different concentration, ranging from 10 mg/L to 0.625 mg/L. The plates were sealed, incubated at 25 °C in the darkness for 24 h under a gentle shaking (80 rpm). The endpoint (immobility/death) was assessed at the end of the test a Zeiss stereomicroscope: a nauplium was considered to be immobile or dead, if it could not move its antennae after slight agitation of the water. Potassium dichromate was used as reference toxicant. Three independent experiments were performed.

2.5. Artemia salina 48 h-exposure: oxidative stress

Nauplii from the parental stock cultures were immersed in the testing solutions. Tests were performed in 250 ml glass flasks covered with parafilm in a volume of 100 ml of testing solution as follow: control (NSW), PGMF (0.1 and 1 mg/L in NSW), GNC1 (0.1 and 1 mg/L in NSW), GRP (0.1 and 1 mg/L in NSW). Tests were performed at 20 ± 1 °C under a photoperiod of 16 h light/8 h darkness, as reported by Nunes et al. (2006). Three replicates were used for each concentration. For all determinations about 2000 nauplii were used for each replicate. Values of pH, temperature and percentage of dissolved oxygen were measured every 24 h, for test validation purposes.

After the exposure period, animals were collected on a mesh and homogenized in ice-cold phosphate buffer (50 mM, pH 7) by sonication (Julabo bath sonicator, 10 s) and homogenization with a potter Elvejem. Aliquots of crude homogenates were stored at −80 °C until the determination of the extent of lipid peroxidation. Crude homogenates were also centrifuged at 15000 × *g* for 10 min and supernatants were divided into aliquots and stored at −80 °C until used for the enzymatic determinations (glutathione peroxidase and catalase). On crude homogenates and supernatants the protein content was measured by the method of Lowry et al. (1951).

Table 1
Commercial graphite and graphene derived materials properties.

	Pristine graphene monolayer flakes **PGMF**	Graphene nanopowder grade C1 **GNC1**	Graphite **GRF**
Purity (% carbon)	99.9	97	99.9
Form	Flakes, dispersed in ethanol.	powder, flakes	powder
Average particle (lateral) size	~550 nm	5–25 μm	> 150 μm
Average flake thickness	0.35 nm (1 monostrate)	5–30 nm	n.a.