2D Materials

TOPICAL REVIEW

Ecotoxicological effects of graphene-based materials

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Abstract

Graphene-based materials (GBMs) are currently under careful examination due to their potential impact on health and environment. Over the last few years, ecotoxicology has started to analyze all the potential issues related to GBMs and their possible consequences on living organisms. These topics are critically considered in this comprehensive review along with some considerations about future perspectives.

1. Introduction

Carbon nanomaterials (CNMs) represent a class of materials that have received intense research interest aimed at a wide range of applications. Human exposure to CNMs is estimated to increase, due to their projected broad use, so that their potential risks must be carefully taken into account.

The most promising and actual CNMs are graphene-based materials (GBMs). This review aims to critically analyze the studies on the effects that this family of CNMs may exert on living organisms and the environment. The possible mechanisms of interaction of CNMs with the environment are highly variable, clearly depending on the type of organism taken into consideration. For instance, unicellular and multicellular organisms could interact and respond differently to CNMs exposure: recently, Mu et al. [1] reviewed the interactions between engineered nanomaterials and various biological systems, however these dynamics may not be generalized and applied completely to GBMs. Also, animal and plant cells differ in the presence of a cell wall, peculiar to plants. This complex barrier could stop the entrance of CNMs, which is instead allowed by the cell membrane. Moreover, the interaction could differ with organism age, because the thickness and complexity of the cell wall increases with growth, whereas the newly synthetized one results easier to be penetrated [2].

2. Graphene-based materials

2.1. General

Graphene is a single layer sheet of sp2-bonded carbon atoms, part of the broader family of GBMs [42, 43]. Since its discovery [3], GBMs have attracted big interest for their innovative nature and their promising industrial/scientific uses. A roadmap has been proposed with the aim of providing future directions for their development in the fields of electronics, photonics, composite materials, energy generation and storage, sensors and metrology, and biomedicine [4]. Nowadays, despite the global production of GBMs is not yet significant for industry, especially compared to CNTs production (120 tons versus 4000 tons, respectively) [5], estimates for GBM market foresee investments for almost $400 million by 2025 [6], and industrial patents applications have increased promptly in recent years [7]. Surprisingly, given the growing worldwide production, potential effects of GBMs on living organisms and the environment are still not sufficiently investigated [8], despite the importance of the subject has been underlined on many occasions [9–12].

A generalization about the toxicity of GBMs can be misleading and therefore should be avoided due to the many differences of structure, chemistry, dimensions and fabrication of GBMs [13], that makes difficult and challenging to compare the possible toxicological
effects [14]. In this context, some efforts have been made in order to standardize first of all the nomenclature, then the tested materials and lastly the toxicological methodologies [15, 16].

GBMs family includes many constituents: graphene (G), graphene oxide (GO), few-layer graphene (FLG), reduced graphene oxide (rGO), and many more [14]. The nomenclature standardization has a double advantage: firstly, the field can move forward with a higher degree of common understanding [14], and secondly it helps to better understand the relationship between physicochemical characteristics and health and environmental risks of any nanomaterial [16]. Although little is known about the possible interactions of different types of graphene with biological components of the ecosystem, several factors might influence GBM ecotoxicity. Recently, three easy-to-measure and quantifiable characteristics have been recognized as a starting point for the categorization of GBMs: thickness (number of layers), lateral size and atomic C/O ratio [16]. The broad range of concentrations used in the experiments is also another point that should be taken into account when comparing various studies and that makes the comparison somehow problematic. Some studies report that most of the GBMs released into the environment may be in the ng l\(^{-1}\) or \(\mu g\) l\(^{-1}\) range [17]. Most of the literature here reported deal with these concentrations, while others, like Kryuchkova et al [18] or Xie et al [19] selected instead a much higher and less realistic range, up to 4000 mg l\(^{-1}\). Although major research has been focused to develop biocompatible GBMs, still very little has been reported about their biodegradation [20]: GBM biodegradability is a key aspect not only for their possible uses in clinical innovations, but it is also mandatory for their safe disposal in the environment.

Few reviews focused lately on GBMs [21, 22]; they are based on articles dating until early 2014, reporting that the focus on the subject in biology is growing but it still remains a small subset of the total literature on these materials. What emerged is that GBMs may be toxic to some of the many organisms studied, but the authors are concerned about the lack of standardized protocols and the absence of certified reference materials for the GBM ecotoxicity testing, which makes somehow difficult a critical comparison of the results. Jastrzębska and Olszyna [8] tried to calculate for the first time a life cycle assessment for GBMs, reporting however many gaps in the literature, to be covered in the near future. As a final suggestion they reported the need of developing methods and tools for the characterization of GBM features (as concentrations, size etc) not only in the lab but also in environmental samples.

Given the fast-growing interest towards GBMs, and the rising number of publications in recent years, there is constant need to gather updated information. Recently, the ecotoxicity of GBMs was evaluated on various model and non-model organisms, from bacteria, to plants and animals. Here we focus on environmental toxicology of GBMs (figure 1) from studies published in recent years (see table 1). Despite the obvious environmental implications of the potential GBM toxicity, it is striking that there is a lack of field studies, with all investigations still carried out in the lab. Out of the 27 studies analyzed, almost 78% tested ecotoxicity of GO, whereas the remaining 22% used a mixed of nanomaterials or just one type. Of the total numbers of studies, 59% showed various negative effects on the target organisms, whereas 19% showed contrasting effects, some positive and some negative; 6% of the studies taken in account did not find any significant effect or just at some peculiar conditions (see table 1).

### 2.2. Graphene in water ecosystems

Due to the expected large scale production of GBMs, it is reasonable to focus on their fate into the environment, which is in certain cases unknown, and could eventually end up into water treating systems [23], affecting and/or modifying microbial community and enhancing ROS production. Nanoparticles are expected to have slow biodegradability and therefore require adequate investigations [24].

Bacteria and protozoa are the main components of the activated sludge, involved in the biological wastewater treatment process. The presence of contaminants in the wastewater influent may adversely affect the functions of these microorganisms. Therefore, GO exposure in the range 10–300 mg l\(^{-1}\) on a wastewater microbial community has been investigated [23], showing that the metabolic activity could be significantly compromised. GO also negatively impacted the effluent quality and sludge
Table 1. Ecotoxicological effects of GBMs on selected organisms.

| Type of GBMs | Size range | Organism | Concentration | Effects | Reference |
|--------------|------------|----------|---------------|---------|-----------|
| GO           | Not given  | Microbial community | 10–300 mg l<sup>−1</sup> | Various | [23] |
| GO           | 0.65 μm<sup>2</sup> average sheet area; 1.4 nm thickness | Escherichia coli | — | — | — |
| Monolayer graphene film | 1 cm<sup>2</sup> | Escherichia coli and Staphylococcus aureus | — | Moderate toxicity | Membrane integrity damage |
| CVD (chemical vapour deposition) graphene GO | 1 cm<sup>2</sup>; 50 μm thick | Escherichia coli and Staphylococcus aureus | — | No effects | — |
| GO           | ~800 nm    | Pseudomonas putida | 0, 50, 100, 250, 50, 1000 mg l<sup>−1</sup> | Moderate toxicity | Membrane integrity damage |
| PGMF (Pristine Graphene Monolayer Flakes) GNC1 (Graphene Nanopowder Grade C1) | PGMF (0.35 thickness, 550 nm average lateral size) GNC1 (5–30 nm thickness, 5–15 μm average lateral size) | Vibrio Fischeri | 0–5 mg l<sup>−1</sup> | PGMF > GNC1 | Bioluminescence inhibition |
| GO           | 0.9 nm height | Phanerochaete chrysosporium | 0, 100, 200, 400, 1200, 2000, 3000, 4000 mg l<sup>−1</sup> | Positive (<1200) negative (>2000) | Morphology changes; ultra-structure disruption; loss of decomposition activity |
| GO           | 1940 ± 90 nm (hydrodynamic diameter): 2000 nm width; 2–10 nm thickness | Paramecium caudatum | 62.5, 125, 250, 1500, 2000, 4000 mg l<sup>−1</sup> | Moderate toxicity | Inhibition of motility; DNA damages in macronucleus |
| GO           | 1–10 μm, 0.7 nm thickness | Euglena gracilis | 0–25.2 mg l<sup>−1</sup> (growth inhibition test) 0–5 mg l<sup>−1</sup> (acute toxicity test) | Negative | Growth inhibition; oxidative stress |
| GO and Cd<sup>2+</sup> | 588 ± 5 nm (hydrodynamic diameter) | Microcystis aeruginosa | 0–50 mg l<sup>−1</sup> | Moderate toxicity if coupled with Cd<sup>2+</sup> exposition; ‘Shading effect’; cell wall integrity damage; oxidative stress | |
| GO           | Average planar dimension: 120–200 nm Thickness: ~3.5 nm | Raphidocelis subcapitata | 0, 0.5, 2, 5, 10, 20, 50, 70, 100 mg l<sup>−1</sup> | Moderate toxicity | Oxidative stress; membrane integrity damage |
| GO           | Thickness: ~0.1–1 nm for GONS ~ 4.8–5.2 nm for GOQD Lateral lengths: 1.5 μm for GONS, 20–50 nm for GOQD | Chlorella vulgaris | 0.01–10 mg l<sup>−1</sup> | Negative | Oxidative stress; inhibition of cell division; internalization of GO; plasmolysis |
| PGMF (Pristine graphene monolayer flakes) GNC1 (Graphene nanopowder grade C1) | PGMF (0.35 thickness, 550 nm average lateral size) GNC1 (5–30 nm thickness, 5–15 μm average lateral size) | Dunaliella tertiolecta | 0.675–10 mg l<sup>−1</sup> | PGMF > GNC1 | |
| G (Pristine Graphene) | 0.8 nm thickness | Triticum aestivum | 200 mg l<sup>−1</sup> | Negative | Oxidative stress | [42] |
Table 1. (Continued.)

| Type of GBMs                        | Size range                  | Organism                  | Concentration | Effects                                | Reference |
|-------------------------------------|-----------------------------|---------------------------|---------------|----------------------------------------|-----------|
| GO                                  | Not given                   |                           |               | Negative                                | Oxidative stress | [39] |
| HGR (Hydrated Graphene Ribbon)      | Ribbon morphology (0.38 nm thickness, 0.4 μm width, 2.0 μm lengths) | Arabidopsis thaliana     | 40, 80 mg l⁻¹ | Positive                                | Increased seed germination |
| Graphene                            | 0.5/0.6 μm–1.5/6.5 μm       | Arabidopsis thaliana     | 0.01–1 mg l⁻¹ | Negative                                | Oxidative stress  | [40] |
| GO                                  | 1 nm (thickness), mostly 40–50 nm (but also 30–70 nm) | Arabidopsis thaliana     | 0.01–1 mg l⁻¹ | Effects only if under drought stress or salt stress | No effects  | [41] |
| GO (Single-bilayer graphene oxide sheets) | 0.5–5 μm                  | Vicia faba                | 0, 100, 200, 400, 800, 1600 mg l⁻¹ | Positive (800 > 400) negative (1600 > 200 > 100) | – Oxidative stress  | [44] |
| GO (Single-bilayer graphene oxide sheets) | 0.5–5 μm                  | Vicia faba                | 0, 100, 200, 400, 800, 1600 mg l⁻¹ | Positive (800 > 400) negative (1600 > 200 > 100) | + Increased seed germination and root elongation; oxidative stress |
| GO                                  | 40–60 nm                    | Caenorhabditis elegans   | 0.5–100 mg l⁻¹ | Negative                                | Oxidative stress; enhanced permeability of biological barrier; suppressed defecation | [49, 50] |
| GO                                  | 0.5–5 μm                    | Amphibalanus amphitrite  | 0.01, 0.1, 0.5 mg l⁻¹ (cyprids larvae)0.001–0.75 mg l⁻¹ (nauplius larvae) | Negative | Swimming inhibition; antisettlement properties | [32] |
| PGMF (Pristine graphene monolayer flakes) GNC1 (Graphene nanopowder grade C1) | PGMF (0.35 thickness, 550 nm average lateral size) GNC1 (5–30 nm thickness, 5–15 μm average lateral size) | Artemia salina          | 0.675–10 mg l⁻¹ | Negative                                | Oxidative stress detected | [24] |
| GO                                  | 0.5–5 μm                    | Artemia salina           | 0, 10, 100, 500, 600, 700 mg l⁻¹ | Negative                                | Swimming inhibition; mortality  | [31] |
| GO                                  | 1 mm height × few μm lateral dimension | Acheta domestica         | 0.1 μl per 100 mg of insect weight | Moderate toxicity | Oxidative stress  | [51] |
| GO                                  | Not given                   | Danio rerio              | 0, 3, 4, 7.6, 12.5, 25, 50 mg l⁻¹ | Moderate toxicity | Cell growth inhibition  | [33] |
| GO and rGO                          | ~500 nm for GO, ~400 nm for rGO | Danio rerio              | 1.5, 10, 50, 100 mg l⁻¹ | Moderate toxicity | Embryos hatching modified; larvae length reduced  | [34] |
| MLG (Multilayer graphene)           | 1.2–5.4 μm                  | Xenopus laevis           | 0.1, 1, 10, 50 mg l⁻¹ | Effects only at high doses | Growth inhibition  | [35] |
| GO                                  | Not given                   | Mus musculus             | 0.1–0.4 mg | Effects only at high doses | Cytotoxicity  | [52] |
dewaterability, which can cause regulatory violations and increase the sludge disposal costs, respectively. A more recent study [25] evaluated the effect of different concentrations (from 0 to 1000 mg l\(^{-1}\)) of GO on the viability and activity of *Pseudomonas putida*, considering this species as a simplified model of an activated sludge biotreatment. The growth of *P. putida* resulted inhibited by the presence of GO concentrations higher that 50 mg l\(^{-1}\), which is thought to cut the cell membranes with the sharp edges of the sheets. Further investigations are needed to unravel the exact contribution of physical and oxidative pathways in the antimicrobial activity of GO. In this effort, the interaction of GO with *E. coli* cell membranes was studied using atomic force microscopy [26]. The results presented suggest that physical interactions are repulsive and that other mechanisms, such as oxidative pathways, should be examined more closely.

*Paramecium caudatum*, a ciliate protozoon model organism, was used to investigate the toxicity of various nanoparticles, including a broad range of GO nanoflakes concentrations (up to 4000 mg l\(^{-1}\)) [18]. The results of the study suggest that GO is severely toxic for *P. caudatum*, accounting the toxicity to an inhibition of motility and the interaction with DNA in macronucleus.

GO toxicity was tested on the unicellular protozoan *Euglena gracilis* [27], a common facultative photoautotroph of freshwater environments, with cultures exposed to GO concentrations of 0–25.2 mg l\(^{-1}\). Significant adverse effects were observed at concentrations exceeding 2.5 mg l\(^{-1}\), as demonstrated by growth inhibition, enhancement of malondialdehyde (MDA) content and antioxidant enzyme activity. ‘Shading effect’ was also detected, caused by the GO covering of the membranes; this effect may inhibit the light use by the protozoan and therefore be responsible for a decreased growth.

Tang et al [28] investigated the freshwater cyanobacterium *Microcystis aeruginosa*, testing combined exposures to Cd\(^{2+}\) and GO (concentrations between 0.2–0.7 mg l\(^{-1}\) and 1–50 mg l\(^{-1}\), respectively); they observed that GO alone at low concentrations below 10 mg l\(^{-1}\) had no significant toxicity. After treatments with GO/Cd\(^{2+}\) system, the mortality was mainly due to the uptake of Cd\(^{2+}\) and the induction of oxidative stress, increased by the increasing concentrations of GO and demonstrated by the changes in ROS and MDA levels. Moreover, scanning and transmission electron microscopy observations reported that GO with Cd\(^{2+}\) easily adhered to the cell walls and entered into the algal cells, surprisingly not causing a significantly visible damage. Finally, they suggest that nanoparticle released in aquatic systems might lead to a potential enhancement of background contaminants toxicity, even at low non-toxic concentrations.

GO effects were studied on the green alga *Raphidocelis subcapitata*, a species broadly used in ecotoxicology [29]; liquid algal cultures were exposed to GO concentrations between 0 and 100 mg l\(^{-1}\) for 96 h, reporting a 50% of growth inhibition starting at 20 mg l\(^{-1}\). A significant increase of oxidative stress levels coupled with membrane damage and confirmed by fluorescence analysis was observed for concentrations starting at 10 mg l\(^{-1}\). The authors hypothesized that the growth inhibition in part could be caused by a ‘shading effect’, since GO aggregates attached to the algae were detected.

The interactions of GO (in the form of nanosheets, GONS, and quantum dots, GOQD) and the model green alga *Chlorella vulgaris* was recently tested by Ouyang et al [30]. GO was added to the liquid algal cultures at concentrations between 0.01 and 10 mg ml\(^{-1}\) and the possible envelopment–internalization synergistic effects were studied with metabolomics. Internalization of GOQD (smaller than GONS), resulted 10–80 times higher than GONS, and ecotoxicity resulted also higher with various effects (e.g. cell division, cell permeability and oxidative stress).

Pristine graphene nanoparticles (pristine graphene monolayer flakes PGMF and graphene nanopowder grade C1 GNC1) toxicity was investigated in model marine organisms by Prettì et al [24]. The range of concentrations varied between 0.675 and 10 mg l\(^{-1}\), resulting in moderately toxic effects to the gram–negative bacterium *Vibrio Fischeri* and the green flagellate alga *Dunalieila tertiolecta*, with smaller particles (PGMF) more toxic than bigger ones (GNC1), thus showing that toxicity increases as nanoparticles size decreases. Another model organism, the brine shrimp *Artemia salina*, resulted not affected by lower graphene concentrations (0.675–5 mg l\(^{-1}\)), even though some oxidative stress biomarkers were altered. More recently, Mesarič et al [31] investigated the effects of three different carbon-based nanomaterials, among which GO, on *A. salina* larval stages. Differently from Prettì et al [24], they exposed the larvae at the nauplius stage to higher GO concentrations (0–700 mg l\(^{-1}\)) and therefore reported acute mortality at the highest concentration. SEM observations confirmed that GO aggregates were attached to the larvae surface and on gills and appendages, causing an alteration on the swimming behavior.

The effects of single-layer GO on settlement of the crustacean Amphibalanus amphitrite cyprid larvae were assessed after 24, 48, and 72 h of exposure at 0.01, 0.1 and 0.5 mg ml\(^{-1}\) concentrations [32]. Additionally, the effects on the mortality and swimming behavior of the nauplius larvae of *A. amphitrite* were determined after 24 and 48 h of exposure to a larger range of concentrations, between 0.001 and 0.75 mg ml\(^{-1}\). Higher concentrations of single-layer GO led to increased mortality and decreased swimming speed, both of which occurred in a concentration-dependent manner, particularly after 48 h long exposure. However, the authors observed a reversibility of the antiseent activity after the rinsing of the cyprids.
Chen et al. [33] investigated the effects of GO towards the model organism zebrafish (*Danio rerio*), finding a moderate toxicity at the high dose of 50 mg l\(^{-1}\). Cytotoxicity resulted lower compared to that of MWCNTs due to the different geometric nanostructures of the materials and their consequent different chemical and physical interaction with the target organism. According to the authors, the flat shape of GO, compared to the tubular shape of CNTs, reduces the capacity to penetrate into cells and thus also toxicity would be reduced. Another study on zebrafish tested the toxicity of GO and rGO from 1 to 100 mg l\(^{-1}\) for 96 h [34]. Neither morphological malformation nor mortality were observed; GO had significant effects on the heart rate while rGO affected the embryos hatching and the length of larvae at high concentrations.

Muzi et al. [35] evaluated the ecotoxicity of MLG (multi-layer graphene, 2–20 sheets) on larvae of another model organism, *Xenopus laevis*. After 12 days of exposure to a broad range of MLG concentrations (from 0.1 to 50 mg l\(^{-1}\)) they concluded that the nanomaterial is substantially not toxic for this aquatic species. They however observed a significant larval size reduction on the larvae exposed to the highest concentrations of MLG, 10 and 50 mg l\(^{-1}\), but the absence of mortality and genotoxicity. Larval observations indicate an uptake of MLG and an accumulation inside the gut and gills leading thus to intestinal and respiratory clogging. The absence of harmful effects is explained by the authors with the failure in the internalization of the aggregated particles.

2.3. Graphene in terrestrial ecosystems

Almost all of the soil systems are very complex, and if GBMs are released into the soil, they may interact with its components. As previously stated by Jastrzębska and Oliszyna [8], there is a huge knowledge gap on GBM fate and transport in soil.

Testing GBM antibacterial activity led to conflicting results. Some authors [36] used *Staphylococcus aureus* and *Escherichia coli* to investigate the antibacterial actions of large-area monolayer graphene film on conductor Cu, semiconductor Ge and insulator SiO\(_2\), SiO\(_2\)-containing surfaces resulted not antibacterial, contrarily to Cu- and Ge-containing surfaces which instead induced the disruption of both species membrane integrity, with the leakage of their cytoplasmic content. They proposed a model to explain their results, accounting the toxicity to the conductivity of the Cu- and Ge-containing underlying substrates. The membrane electrons are supposed to be extracted by the graphene film in a quick and strong way, until the bacterial cell loses its viability. This electron transfer model was later questioned and not accepted by Dellieu et al. [37], who provided reliable evidences in support of their findings. They examined the potential toxicity of chemical vapor deposited (CVD) graphene on conductive substrates of Au and Cu and no antibacterial activity was observed for *S. aureus* and *E. coli*.

White rot fungus *Phanerochaete chrysosporium* was exposed for 14 days to a broad range of GO concentrations inside the liquid culture medium (up to 4000 mg l\(^{-1}\)) [19]. Despite low concentrations stimulated the growth of the fungus, higher ones had an inhibitive effect. Moreover, GO induced morphology changes, ultrastructure disruption and most importantly the complete loss of the decomposition activity, with significant ecological implications.

GBM toxicity was tested against some worldwide-distributed seed plants and their cultured cells, since they are essential base components of all terrestrial ecosystems and are considered as potent media for the transfer of absorbed nanoparticles to the biota through the food chain [38]. The model plant *Arabidopsis thaliana*, extensively studied in many biological fields, has been used to investigate GBM toxicity as well. Begum *et al.* exposed *A. thaliana* T87 cell suspensions to a not better identified GBM graphene at concentrations between 0 and 80 mg l\(^{-1}\), reporting negative effects in term of nuclei fragmentation, membrane damage, mitochondrial dysfunction and ROS increasing and accumulation at the lowest concentration (40 mg l\(^{-1}\)), all leading to induction of cell death [39]. Additionally, graphene endocytosis was observed. Toxicity and translocation of GO in *A. thaliana* plants was also studied under normal and under stress conditions [40, 41]. They cultured *A. thaliana* seeds in plates containing GO in the standard medium (0.1–1 mg l\(^{-1}\)) for 2 weeks, maintaining the plates vertical to allow the roots to grow on the surface of the agar medium. Then, they transferred and cultured for two weeks the two weeks old seedlings, changing the nutritive fluid containing GO every two days. Four weeks exposure to GO did not affect seeds germination nor the development of seed sprouting. TEM observations allowed to examine the translocation patterns of GO through the plant: GO was found largely in all the cell compartments of the cotyledon cells. In the seedlings GO was accumulated in the root system but not in the leaf cells, implying that the plant strongly copes with GO translocation from root to stem or leaves. When the GO exposures were coupled to a preexistent stress, like drought or salt, they induced more severe adverse effects if compared to the stress exposure alone: GO may induce severe oxidative stress and membrane ion leakage, which can further increase GO translocation from roots to leaves.

A study on *Triticum aestivum* showed a differential response to the exposure at 200 mg l\(^{-1}\) concentration of three GBMs: hydrated graphene ribbons (HGRs), graphene (G) and GO. HGRs, unexpectedly, relieved oxidative stress and promoted root elongation and aged seed germination rate (100%) compared to the control treatment (93%), while G and GO inhibited the germination (both 87%) [42]. However, the HGR material, according to the characterization that is
presented in this work, is more like functionalized-doped graphene (hydrophilic material because the amounts of nitrogen and oxygen are higher than the ones from the exfoliated graphene) than the pristine one. The authors indicated that this positive effect is probably due to the molecular features of HGRs, which is in fact more hydrated. The three GBMs are responsible for inducing each one specific metabolic pathways that can differentially regulate the plant metabolism. In another contribution, it was reported that GO can amplify the phytotoxicity of arsenic in wheat, an effect that is dependent on the concentration of GO [43]. This latest contribution, in agreement with the results of Tang et al [26] discussed above, is important because it focuses on the impact of ‘indirect’ nanotoxicity, defined as toxic amplification of other toxicants or pollutants by nanomaterials, which should also be taken into account.

Investigations on Vicia faba reported a purported differential sensitivity toward GO. This plant would be tolerant to 400 and 800 mg L⁻¹, but sensitive to higher (1600) and lower (100–200 mg L⁻¹) concentrations [44, 45]. Increased V. faba sensitivity was apparently due to an increased oxidative stress and a contemporary impaired glutathione metabolism, in term of lower glutathione-regeneration and enhanced glutathione utilization [44]. It is widely accepted that the induction of oxidative stress via generation of reactive oxygen species (ROS) appears as one of the main toxicity mechanisms related to GBM exposure (see e.g. Garza et al [46]). In this context it is necessary to underline that the toxic effect is only indirectly caused by GBMs. GBMs are not affecting cells directly, but primarily induce oxidative stress and hence cells result affected. The tolerance to other GO concentrations was attributed to an elevated glutathione regeneration coupled to a lowered glutathione utilization. More recently, the authors [45] confirmed this behavior towards the exposition to 800 and 400 mg L⁻¹ GO concentrations, optimistically suggesting that it can lead to an improved V. faba health, in terms of an increased seed germination and root elongation.

Only a few studies dealing with GBM toxicity on animal model organisms have been conducted so far. The free-living nematode Caenorhabditis elegans is often used as an assay system because it offers the advantage of a laboratory culture. Toxicity of GBMs has been tested, reporting negative effects after exposures [47, 48]. Wu et al [49] examined the potential adverse effects of GO on C. elegans comparing the in vivo effects of GO between acute and prolonged exposure. The authors found that a prolonged exposure to 0.5–100 mg L⁻¹ of GO caused damage on functions of both primary (intestine) and secondary (neuron and reproductive organ) targeted organs. They also identified molecular signals involved in the control of the translocation and toxicity of GO in C. elegans [50].

A recent study conducted on a further model organism, the house cricket (Acheta domestica), by Dźwięcka et al [51] underlined how GO can provoke oxidative stress, especially after 24 h of exposure, although this insect managed to cope with the derived stress, recovering in short time (concentration used: 0.1 μl for 100 mg of body weight).

On the other hand, in a slightly older study exposure to GO did not cause significant toxicity on mice cells neither at low nor at middle concentrations (0.1–0.25 mg), whereas only the highest dose (0.4 mg) caused the death of almost 50% of the mice cells, thus showing a dose-dependent GO toxicity [52].

3. Conclusions

Despite the efforts and the research developed so far, part of which is reported in this review article, there is still a large knowledge gap, which is necessary to cover in relation to CNM ecotoxicity. There are also other challenges that need to be addressed: is it better to focus only on target organisms for the different component of the ecosystem, both water (freshwater and marine) and terrestrial (above and below ground)? What are the effects on the different components of a population, that is, are young generations more affected than older ones by CNM toxicity (if present)? What is known about the long-term fate of these nanomaterials in the environment? How will long-term exposure affect the environment and the organisms? How important are CNMs in increasing or decreasing the toxicity of well-known pollutants, when they occur together?

For the future, Bussy et al [13] proposed a useful set of general guidelines to improve safety related to use and development of graphene regarding dimension (the smaller the better), surface properties (improve-develop high hydrophilicity) and dispersion (small, single graphene sheets).

A thorough answer to all these questions is highly desired in order for the new materials to be used in practical applications. We look forward to the many new and important contributions that will elucidate the effects of CNMs on health and environment.

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