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Ecotoxicological effects of Graphene-Based Materials

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To Danila,
my silent strength,

and to Checca and Rosetta,
my roots.

"I've been dreaming of things yet to come"

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Riassunto

Negli ultimi anni, il rapido progresso nel campo dei nanomateriali ha portato ad un incremento nel loro sviluppo e di conseguenza nella loro produzione e commercializzazione. Tra i nanomateriali, quelli composti da carbonio sono i più ampiamente studiati a causa del loro potenziale in campi molto diversi, con un ruolo predominante occupato dalla famiglia di materiali composti da grafene.

Il grafene è un materiale bidimensionale, formato da uno strato monoatomico di atomi di carbonio, disposti in un reticolo a nido d'ape. Dalla sua scoperta, l'attenzione dei ricercatori si è focalizzata sulle sue eccezionali proprietà, come la rigidità, la resistenza, l'elasticità e l'elevata conducibilità elettrica e termica, che hanno portato allo sviluppo di numerose applicazioni. I cospicui investimenti economici hanno permesso un incredibile progresso nel campo industriale, accompagnato sfortunatamente da un minore interesse e progresso nello studio dell'impatto del grafene sulla salute umana e sull'ambiente.

Finora, gli effetti dei materiali composti da grafene sono stati studiati su organismi modello, per lo più animali e batteri, indicando che la tossicità dipende da varie proprietà fisico chimiche del materiale, come la forma, le dimensioni, lo stato ossidativo e la presenza di gruppi funzionali.

Lo scopo di questo progetto di dottorato è stato di analizzare gli effetti ecotossicologici di due materiali composti da grafene, few-layers grafene (FLG) e grafene ossido (GO), su microalghe verdi aeroterrestri. Questi tipi di grafene sono stati selezionati come materiale di riferimento dal Working Package 4, Health and Environment, nell'ambito del progetto europeo Graphene-Flagship.

Gli effetti a breve termine (30 e 60 minuti) sono stati studiati sulle specie algali *Coccomyxa subellipsoidea* e *Trebouxia gelatinosa* valutando la permeabilità di membrana, quantificando il potassio rilasciato tramite spettroscopia di emissione atomica.

Gli effetti a lungo termine (4 settimane) sono stati studiati sulle specie algali *Apatococcus lobatus*, *Chlorella vulgaris*, *C. subellipsoidea* e *T. gelatinosa* tramite misure di fluorescenza clorofilliana (parametro F_v/F_m) e quantificazione totale del contenuto di pigmenti fotosintetici.

Dopo l'esposizione a breve termine di grafene sull'alga *T. gelatinosa* è stato valutato tramite microscopia confocale se vi è stata internalizzazione del nanomateriale. I possibili effetti ossidativi causati dal grafene sono stati studiati analizzando l'efficienza dei fotosistemi attraverso misure di emissione di fluorescenza della clorofilla *a*, modificazioni dell'espressione genica di 8 geni di interesse con Real-Time PCR quantitativa e quantificazioni della proteina HSP70 mediante western blot.

Sulla base dei risultati ottenuti, non sono stati osservati effetti negativi provocati da grafene, sia da FLG che GO, sia a seguito di esposizioni brevi che esposizioni lunghe. L'internalizzazione

delle particelle di grafene non è stata verificata chiaramente, nonostante 30 minuti di esposizione a FLG abbiano indotto una sottoespressione del gene che codifica per la proteina HSP70. Questi risultati supportano l'ipotesi che un'interazione innocua avvenga tra grafene e alghe a livello di parete cellulare – membrana plasmatica, coinvolgendo potenzialmente una via di segnale ancora sconosciuta.

Abstract

In the recent years, the rapid advancement in the field of nanomaterials has increased their development and consequently their production and commercialization. Among nanomaterials, the carbon-based ones are the most widely researched because of their potential on the most diverse fields, with a predominant role occupied by Graphene-Based Materials (GBMs).

Graphene is a two-dimensional, single layer sheet of carbon atoms organized in a honeycombed network with six-membered rings. Since its discovery, the attention of researchers was focused on its unique and exceptional properties, such as mechanical stiffness, strength, elasticity, very high electrical and thermal conductivity, which led to the development of numerous applications. The huge investments brought to an incredible advancement in the industrial field, unfortunately accompanied by a slower progress in the understanding of the impact on human health and the environment.

So far, the effects of GBMs have been evaluated mostly on animal and bacterial model organisms, suggesting that GBMs toxicity is dependent on various physiochemical properties such as shape, size, oxidative state and presence of functional groups.

The aim of this PhD project was to assess the ecotoxicological effects of two GBMs, few-layers graphene (FLG) and graphene oxide (GO), on aeroterrestrial green microalgae. These GBMs were selected as reference material by the Working Package 4, Health and Environment, in the framework of the European Project Graphene-Flagship.

Short-term exposure (30 and 60 minutes) effects were evaluated on the algal species *Coccomyxa subellipsoidea* and *Trebouxia gelatinosa* in terms of membrane permeability with the quantification of potassium leakage by inductively coupled plasma-atomic emission spectrometry.

Long-term exposure (4 weeks) effects were evaluated on *Apatococcus lobatus*, *Chlorella vulgaris*, *C. subellipsoidea* and *T. gelatinosa* through chlorophyll *a* fluorescence measurements (F_v/F_m parameter) and quantifications of the total photosynthetic pigments content.

After a short-term exposure of GBMs on the alga *T. gelatinosa*, internalization was investigated with confocal laser scan microscopy. Potential oxidative effects of GBMs were then studied analyzing the efficiency of the photosystems through measurements of chlorophyll *a* fluorescence emission (F_v/F_m parameter), changes of gene expression of eight genes of interest through quantitative Real-Time PCR, and quantification of HSP70 protein through western blot.

According to the final results obtained, no negative effects were observed for either FLG or GO, in both short- and long-term exposures. Internalization was not clearly observed, even though the FLG exposure after 30 minutes induced the downregulation of the gene coding for HSP70 protein.

These results supported the hypothesis that a harmless interaction occurred between GBMs and algae at cell wall – plasma membrane level, involving potentially a yet unknown signaling pathway.

Introduction

Nanotechnology is defined as "the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale" (Yang *et al.* 2008). According to the European Committee for Standardization, nanomaterials are materials with an external dimension at the nanoscale, or that possess nanoscale internal or surface structures (Lövestam *et al.* 2010). Nanoparticles are nanomaterials with one (e.g. nanolayers), two (nanowires and nanotubes) or three (quantum dots, metal nanoparticles, fullerenes) external dimensions of 100 nm or less (Ju-Nam *et al.* 2008). They can be spherical, tubular, or irregularly shaped, and can exist in fused, aggregated or agglomerated forms (Nowack and Bucheli 2007). Other definitions are available but efforts have been made globally to standardize the subject, for instance with norms like the very recent ISO/TS 80004-2:2015.

Nanoparticles are categorized differently on the basis of their nature, composition and origin (see e.g. Ma *et al.* 2010; Peralta-Videa *et al.* 2011; Yadav *et al.* 2014; Sajid *et al.* 2015). Grillo *et al.* (2015) instead divide nanoparticles in non–engineered and engineered. Non-engineered ones are mainly already present in the environment, deriving from natural events such as terrestrial dust storms, erosion, volcanic eruptions and forest fires (Nowack and Bucheli 2007; Cupaioli *et al.* 2014); furthermore, they comprehend incidental nanoparticles, defined as anthropogenic nanoparticle waste (Yadav *et al.* 2014), as a result of human activities like industrial processes, coal combustion and welding fumes (Smita *et al.* 2012). Engineered nanoparticles are the ones intentionally produced by man using many different materials, such as metals, (including Ag, Zn, Au, Ni, Fe, and Cu; Xu *et al.* 2012), metal oxides (TiO_2, Fe_3O_4, SiO_2, CeO_2, and Al_2O_3; Bozon-Verduraz *et al*. 2009), non-metals (silica and quantum dots; Probst *et al.* 2013), carbon (nanotubes and fullerenes; Isaacson *et al*. 2009; Ma *et al.* 2010), polymers (alginate, chitosan, hydroxymethylcellulose, polyhydrox-yalkanoates, and poly-e-caprolactone; Rao and Geckeler 2011; Paques *et al.* 2014), and lipids (soybean lecithin and stearic acid; Wang *et al.* 2012; Kumar and Sawant 2013).

Graphene-Based Materials

Among this wide group of engineered nanoparticles, carbon-based nanomaterials are the most widely researched because of their potential in the most diverse fields (Lalwani *et al.* 2016) with a predominant role occupied by Graphene-Based Materials (GBMs) (Novoselov *et al.* 2012).

Graphene, a material which was presumed not to exist in the free state, is a two-dimensional crystal composed of monolayers of carbon atoms arranged in a honeycombed network with six-membered rings (Geim and Novoselov 2007). Since their discovery, GBMs have attracted big interest

for their innovative nature and their promising industrial and scientific uses, making progresses in the field highly rapid. Graphene exceptionality relies on properties such as mechanical stiffness, strength, elasticity, very high electrical and thermal conductivity, which are considered to be supreme, hence leading to the development of a broad variety of applications: electronics, photonics, composite materials, energy generation and storage, sensors and metrology and biomedicine (Novoselov *et al.* 2012). The fact that these properties are all combined in the same material means that graphene could become, and in part is already happened, the new disruptive technology (Ferrari *et al.* 2015).

Graphene market was estimated to be around US$ 12 million in 2013 (Zurutuza and Marinelli 2014), a number not yet significant for industries, especially if compared with carbon nanotubes production (120 tonnes vs 4000 tonnes, respectively) (Ciriminna *et al.* 2015), reaching at the end of 2015 the tipping point between fundamental research and application (Spasenovic 2016). The market projection indicates that a significant expansion and production is to be expected, driving down the costs and driving up the production scale (Zurutuza and Marinelli 2014).

This growth however needs to be accompanied by an interest in the nanosafety of GBMs, in order to exclude possible risks on health and environment. Nanosafety, defined as all the safety issues associated with nanotechnology, is required to translate any future development into action, from industrial applications to health care approaches (Ferrari *et al.* 2015). Nevertheless, an interest towards nanotoxicity, *i.e.* environmental and life forms toxicity issues, is necessary and mandatory. As the production and the spread of nanomaterials are constantly rising, an uncontrolled release into the environment might be expected in the near future, with unclear consequences for the ecosystems (Gottshalk and Nowack 2011).

"Recurring cycles in the history of civilization"

In the past, humans already faced similar situations, and one of the most famous case is the one of plastic. Since the development of the first modern plastics at the beginning of the 1900, inexpensive manufacturing techniques have been optimized, leading to the massive production of lightweight, durable, inert and corrosion-resistant plastics (Cole *et al*. 2011). These properties, especially the durability, made this material attractive and life-changing, while at the same time it has now became a threat. Plastic waste are accumulated everywhere and their impact is huge on global environments (Barnes *et al*. 2009). It has been estimated that plastic longevity is hundreds to thousands of years, but in the recent years microplastics are the ones most concerning because they are considered the most harmful. They are plastic debris which progressively decrease in size by mechanical and microbial degradation and they are widespread particularly in marine environments. Unexpected implications have been identified: microplastics are bioavailable to the biota throughout

the food-web (Cole *et al.* 2011), they can transport non-native species to new locations (Barnes 2002) and their large surface area to volume ratio makes them highly susceptible to contaminations by waterborne-pollutants, including persistent organic pollutants (Rios *et al.* 2007).

A frightening parallelism with GBMs is the one observed by Bhattacharya *et al*. (2010), who studied the physical adsorption of nanosized plastic beads on the algal species *Chlorella* sp and *Scenedesmus* sp. They reported an inhibition of algal photosynthesis, possibly through the physical blockage of light and air flow by the nanoparticles, and ROS production was also detected. Such algal responses to plastic exposure might have implications on the sustainability of the aquatic food chain.

The Graphene-Flagship experience

In the framework of the Graphene-Flagship, a 1.3 billion euro 10 years long project which deals with quite every aspect of GBMs in each discipline possible, our interest as part of the Working Package 4 - Health and Environment is to assess ecotoxicological effects of GBMs towards living forms and ecosystems. Despite the commitment of the Graphene-Flagship and of other existing programs interested in GBMs ecotoxicity, environment is still understudied, even though it is considered highly important. Studies on the field are missing and we are losing a chance to make the things the right way.

Ecotoxicological studies on GBMs

So far, ecotoxicity of GBMs has been tested on various model and non-model organisms among which bacteria (Combarros *et al.* 2016; Szunerits and Boukherroub 2016), protists (Hu *et al.* 2015; Kryuchkova *et al.* 2016), algae (Nogueira *et al.* 2015; Ouyang *et al*. 2015), plants (Anjum *et al*. 2013, 2014; Hu and Zhou 2014; Zhao *et al.* 2015) and animals (Mesarič *et al.* 2013, 2015; Liu *et al*. 2014; Pretti *et al*. 2014; Dziewięcka *et al.* 2016; 2017). For a comprehensive review focused on the last 3 years see Montagner *et al*. 2017, the first paper reported in this thesis. All the studies herein reported, however, are only a small part of what has been done in the last decade, but there are many questions that still needs to be addressed. In fact, many challenges are faced in GBMs studies, most of which occur during the comparison of the results.

First of all, a worldwide standardization of the nomenclature is lacking, notwithstanding it allows a better common understanding (Bianco *et al*. 2013; Wick *et al.* 2014). GBMs family in fact includes many constituents: graphene (G), graphene oxide (GO), few-layer graphene (FLG), reduced graphene oxide (rGO), graphene quantum dots (GQDs) and many more.

It is well known that toxicity of nanomaterials and in particular of GBMs might depend on various aspects proper of the nanomaterials: fabrication procedures (Chng and Pumera 2013),

morphology of nanomaterials (Novoselov *et al*. 2012; Bianco 2013), size (Guo and Mei 2014), etc. In order to have comparable results, all these factors should be carefully taken into account and standardized. This issue has been considered a priority by many authors (Hu and Zhou 2013; Guo and Mei 2014; Seabra *et al.* 2014; Kulkarni 2015).

In order to make the studies the most environmentally relevant, the concentrations tested should reflect the expected future releases in the environment (Doudrick *et al.* 2012). This is related to the necessity of studies on prolonged timescales, which are so far absent in the literature (Handy *et al.* 2008).

Furthermore, as already mentioned, studies in the field are missing; there are no information available on the combined effects of different pollutants which may potentially enter in contact and for which the resulting interaction might be totally unexpected.

Aeroterrestrial green microalgae as target organisms

Most of the ecotoxicological studies available in the literature are focused on both marine and terrestrial model organisms. Of course the huge previous knowledge on these species is essential to produce reliable results, but most of these species are not ecologically relevant, notwithstanding their importance is mostly economical.

The activities of this PhD were then mainly focused on the evaluation of ecotoxicological effects on aeroterrestrial microalgae. The choice was driven by the thought that special attention should be given to photoautotrophic organisms, being at the basis of the primary production of the ecosystems, both marine and terrestrial. Since they are responsible for a considerable proportion of O_2 production and CO_2 fixation, a negative effect on this component of the ecosystems might have extreme consequences on the total environment. Aeroterrestrial green microalgae are a ubiquitous cluster of organisms naturally occurring on a variety of substrates (wet soils, sandstone, limestone, other plants; Lüttge and Büdel 2010) colonizing the most diverse environments (cities, deserts, mountains; Belnap *et al.* 2001; Freystein and Reisser 2010).

Algal toxicity tests in general are extensively applied to assess the effects of hazardous substances in water (Sadiq *et al*. 2011). OECD guidelines for the testing of chemicals, for example, have been adopted in 1984 for the first time (OECD, 1984) and are currently the most used in many ecotoxicological studies focused on nanoparticles (Aruoja *et al*. 2009; Long *et al*. 2012; Hu *et al*. 2016). This kind of guidelines has risen recently many questions regarding their reliability in nanoparticles investigations (Sørensen 2015). First of all, nanoparticles are suspended rather than dissolved in the test medium, and the assumption of a stable exposure over the duration of the test is violate, probably explaining why toxicity studies are sometimes affected by poor reproducibility

(Hartmann *et al*. 2013). Furthermore, these tests are made to detect direct effects of nanoparticles, which may lead to ignore indirect effects or let them stand out. However, certain considerations and modifications to the test setup are to be considered in the experimental design and a good understanding of the outcomes is recommended (Sørensen *et al.* 2015). More recently, studies on microalgae were focused on the effects of GBMs (Nogueira *et al.* 2015; Ouyang *et al.* 2105; Zhao *et al*. 2017), based mostly on short-term exposure experiments, however with contradictory results leading to an unclear trend in toxicity.

The work of this three PhD years was focused on the understanding of the ecotoxicological effects of two types in particular of GBMs, FLG and GO, on aeroterrestrial green microalgae. These two GBMs were distributed by the University of Castilla-La Mancha (Ciudad Real, Spain) in the framework of the above-mentioned Working Package 4, Health and Environment. Using the same materials and comparable concentrations was a shared decision that allowed – and allows – us to compare results from different studies on different organisms.

In the first full paper reported in this thesis, short-term exposure effects on four species of aeroterrestrial green microalgae (*Apatococcus lobatus, Chlorella vulgaris, Coccomyxa subellipsoidea, Trebouxia gelatinosa*) were evaluated in term of membrane permeability, with the quantification of potassium leakage after 30 and 60 minutes of GBMs exposure at the concentration of 50 μg ml^{-1}. Long-term exposure effects (4 weeks and GBMs concentrations of 0.01, 1, 50 μg ml^{-1}) were evaluated through chlorophyll *a* fluorescence measurements and quantifications of the total photosynthetic pigments content.

The first results obtained were controversial, they were attributed to biases in the experimental procedures and led to a more cautious approach. GBMs were checked for the presence of residual toxic elements potentially coming from the production procedure and they were carefully characterized, underlying the importance of these factors. No negative effects were observed for either FLG or GO, in both short- and long-term exposures.

In the second full paper reported in this thesis, internalization of GBMs and oxidative stress were investigated on the aeroterrestrial green microalga *Trebouxia gelatinosa*. After 10 and 30 minutes of exposure to 50 μg ml^{-1} GBMs, internalization was analyzed with confocal laser scan microscopy, while potential oxidative stress was studied with measures of chlorophyll *a* fluorescence, changes of gene expression of selected genes of interest and quantification of HSP70 protein.

No GBMs were clearly detected inside the cells, despite they were found in close contact with algal cells. GBMs did not induce any oxidative effect, with a unique exception. FLG exposition after 30 minutes induced the downregulation of the expression of the gene coding for HSP70 protein, which however is not reflected in the subsequent quantification of the protein. These results support

the hypothesis that a harmless interaction between FLG and algal cells occurred at cell wall – plasma membrane level, involving potentially a yet unknown signaling pathway.

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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 sp^2–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 vs 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 μg 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 (LCA) 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).

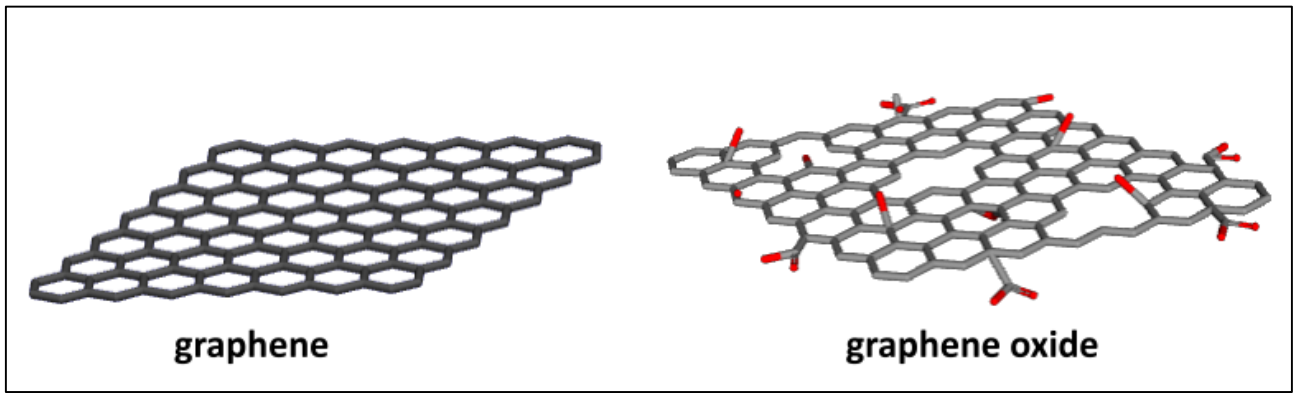

Figure 1. GBMs examined in this review.

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 to 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 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 (AFM)

Type of GBMs	Size range	Organism	Concentration	Effects		Reference
GO	Not given	Microbial community	10-300 mg l^{-1}	Various	Oxidative stress; internalization of GO; reduced activity and viability	[23]
GO	0.65 μm^2 average sheet area; 1.4 nm thickness	*Escherichia coli*	-	-	-	[26]
Monolayer graphene film	1 cm^2	*Escherichia coli* and *Staphylococcus aureus*	-	Moderate toxicity	Membrane integrity damage	[36]
CVD (chemical vapour deposition) graphene	1cm^2; 50 µm thick	*Escherichia coli and Staphylococcus aureus*	-	No effects	-	[37]
GO	~800 nm	*Pseudomonas putida*	0, 50, 100, 250, 500, 1000 mg l^{-1}	Moderate toxicity	Membrane integrity damage	[25]
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^{-1}	PGMF > GNC1	Bioluminescence inhibition	[24]
GO	0.9 nm height	*Phanerochaete chrysosporium*	0, 100, 200, 400, 1200, 2000, 3000, 4000 mg l^{-1}	Positive (<1200) negative (>2000)	Morphology changes; ultrastructure disruption; loss of decomposition activity	[19]
GO	1940 ± 90 nm (hydrodynamic diameter	*Paramecium caudatum*	62.5, 125, 250, 1500, 2000, 4000 mg l^{-1}	Moderate toxicity	Inhibition of motility; DNA damages in macronucleus	[18]
GO	1-10 µm; 0.7 nm thickness	*Euglena gracilis*	0-25.2 mg l^{-1} (growth inhibition test) 0-5 mg l^{-1} (acute toxicity test)	Negative	Growth inhibition; oxidative stress	[27]
GO and Cd^{2+}	588 ± 5 nm (hydrodynamic diameter)	*Microcystis aeruginosa*	0-50 mg l^{-1}	Moderate toxicity if coupled with Cd2+ exposition	"shading effect"; cell wall integrity damage; oxidative stress	[28]
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^{-1}	Moderate toxicity	Oxidative stress; membrane integrity damage	[29]
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^{-1}	Negative	Oxidative stress; inhibition of cell division; internalization of GO; plasmolysis	[30]
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^{-1}	PGMF > GNC1	-	[24]
G (Pristine Graphene)	0.8 nm thickness	*Triticum aestivum*	200 mg l^{-1}	Negative	Oxidative stress	[42]

Type of GBMs	Size range	Organism	Concentration	Effects		Reference
GO	Not given	*Arabidopsis thaliana*	40, 80 mg l^{-1}	Negative	Oxidative stress	
HGR (Hydrated Graphene Ribbon)	Ribbon morphology (0.38 nm thickness, 0.4 µm width, 2.0 µm lengths)			Positive	Increased seed germination	[39]
Graphene	0.5/0.6 µm - 1.5/6.5 µm			Negative	Oxidative stress	
GO	1 nm (thickness), mostly 40-50 nm (but also 30-70 nm)	*Arabidopsis thaliana*	0.01-1 mg l^{-1}	No effects	-	[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	-	[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	[45]
GO	40-60 µm	*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 nm height few × few µm lateral dimension	*Acheta domesticus*	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 l^{-1}	Effects only at high doses	Cytotoxicity	[52]

[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 protozoan 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 Pretti 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 *Dunaliella tertiolecta*, with smaller particles (PGMF) more toxic than bigger ones (GNC1), thus showing that toxicity increases as nanoparticles size decreases. Another model organism, the brime 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 Pretti 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 antisettlement 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.

3.3 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 Olszyna [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 *Phanaerochaete 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 domesticus*), by Dziewięcka 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 to 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 carbon nanomaterials on health and environment.

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Ecotoxicity of Graphene-Based Materials on aeroterrestrial microalgae

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Environmental significance

The importance of Graphene-Based Materials (GBMs) on everyday life is currently rising and hence it is essential to understand the potential effects of this materials-family on living organisms. Special focus needs to be given to photoautotrophs, as responsible for most of the primary production of ecosystems. GBMs tested in our experimentation were produced with highly standardized processes and were carefully characterized. Furthermore, the experimental design and the methods applied, accurately standardized, assure reproducibility and reliability of the results. The observed absence of negative effects contradicts the results of some previous authors, but are in line with those of others, and reinforces the assumption that GBMs are not particularly harmful for unicellular photoautotrophs which are protected by a cellulosic cell wall.

Abstract

The exposure effects of few-layers graphene (FLG) and graphene oxide (GO) have been tested on four species of aeroterrestrial green microalgae (*Apatococcus lobatus*, *Chlorella vulgaris*, *Coccomyxa subellipsoidea* and *Trebouxia gelatinosa*), after a careful chemical-physical characterization of materials. Short-term exposures were carried out on *C. subellipsoidea* and *T. gelatinosa*, whose axenic suspensions were mixed to 50 μg mL^{-1} FLG and alternatively GO, and shaken for 30 and 60 min. Potassium leakage, as a proxy of membrane damage, was quantified by Inductively Coupled Plasma-Atomic Emission Spectrometry against a control shaken for the same time in distilled water. The effects of a four-week-long exposure was tested applying two procedures, namely with algae deposited on a layer of graphene flakes lying on CA or PTFE membranes (0.1 μm mesh) or shaken for 30' with the flakes, and then filtered together on the same type of membranes. Chlorophyll *a* fluorescence (F_v/F_m, as a proxy of photosystems efficiency), and chlorophyll content (as a proxy of biomass growth) in the four-week-old axenic cultures grown on solid medium were measured. Control values of F_v/F_m and chlorophylls varied naturally amongst species but there were no significant differences amongst treatments and concentrations. No negative effects were observed for either types of graphene, in both short- and long-term exposures. The role of the cell wall as a physical barrier against GBMs internalization is shortly discussed.

Introduction

Graphene is a two dimensional, single layer sheet of carbon atoms organized in a hexagon-structure[1], that drew an incredible attention in the research and industrial world since it was firstly described[2–4]: in fact the number of publications studying graphene increased constantly every year, overtaking 20000 in 2016 (Source ISI Web of Science, search: Topic = Graphene). The importance of graphene-based materials (GBMs),[5,6] on everyday life is currently exponentially rising due to their exceptional properties (like e.g. high mechanical strength, electronic and thermal conductivity, and impermeability to gases, etc.[7,8]). The unique characteristics of GBMs are leading to an enhanced production of various materials,[9] which are potentially suitable for developing the most diverse applications.[7] Estimates for GBMs market foresee investments for almost $ 400 million by 2020,[10] and industrial patents applications have increased promptly in recent years.[11] Therefore, an uncontrolled release of GBMs into the environment is expected in the near future, with unclear consequences for the ecosystems.[12] Despite the growing interest, potential effects of GBMs on living organisms and the environment are still not sufficiently investigated,[13] notwithstanding the

importance of the subject has been pointed out on many occasions.[4,14–16] So far, ecotoxicity of GBMs have been tested on various model and non-model organisms.[17] In our opinion, special attention should be given to the interactions between GBMs and photoautotrophic organisms, because they are at the basis of the primary production of the ecosystems, both marine and terrestrial, being responsible of a considerable proportion of O_2 production and CO_2 fixation[18,19]: a negative effect on this component of the ecosystems might have extreme consequences on the environment.

Amongst photoautotrophic organisms, we are focusing on aeroterrestrial microalgae, a ubiquitous cluster naturally occurring on a variety of substrates (wet soils, sandstone, limestone, other plants[20,21]) colonizing distinct environments (cities, deserts, mountains[22,23]). Microalgae in general have been used in recent years as target organisms to test toxicity of different types of engineered nanoparticles,[24–27] resulting one of the most sensitive groups to pollutants in aquatic ecosystems[26]. Nevertheless, toxicity effects of distinct nanomaterials were not always consistent among different studies and contradictory effects were observed.[28–30] More recently, some studies on microalgae were focused on the effects of GBMs, based mostly on short-term exposure experiments,[31–33] but no clear trends in toxicity were observed.

Here we tested the ecotoxicity of two types of graphene, few-layers graphene (FLG) and graphene oxide (GO), selected as reference materials by the Working Package 4, Health and Environment, in the framework of the European Project Graphene-Flagship.[34] The aim of this study was to test short and long-term ecotoxicological effects of FLG and GO: acute effects were evaluated with a short-term exposure, whereas a long-term exposure could represent how the colony would react to a prolonged interaction.

Materials and methods

Algal strains and culture condition

Four aeroterrestrial algal species with different ecology and growth-form (details in Table S1) were chosen for this study: *Apatococcus lobatus* (Chodat) J.B. Petersen, *Chlorella vulgaris* Beijerinck, *Coccomyxa subellipsoidea* E. Acton, and *Trebouxia gelatinosa* Archibald. *A. lobatus* (SAG 2096), *C. vulgaris* (SAG 211-11b) and *C. subellipsoidea* (SAG 216-13) were obtained from SAG Culture Collection at University of Goettingen (Germany). *T. gelatinosa*, which is a lichen photobiont, was isolated from thalli of *Flavoparmelia caperata* (L.) Hale according to Yamamoto *et al*. (2002). The isolated photobiont was inoculated in sterile plastic tubes filled with approximatively 5 mL of slanted solid *Trebouxia* medium (TM;[35] 1.5% agar), and kept in a thermostatic chamber at 18±1 °C and 20±2 μmol photons m^{-2} s^{-1} with a light/dark regime of 12/12 h until abundant biomass

was produced. Algal cultures were subcultured on solid BBM medium (BBM[36] or TM; 1.5% agar) every 30-45 days and kept at the same conditions. Reference material of all the algal species has been cryo-conserved according to Dahmen *et al.* (1983)[37] and is available upon request.

Graphene preparation and characterization

Two types of GBMs were used, FLG and GO (see Fig. 1).

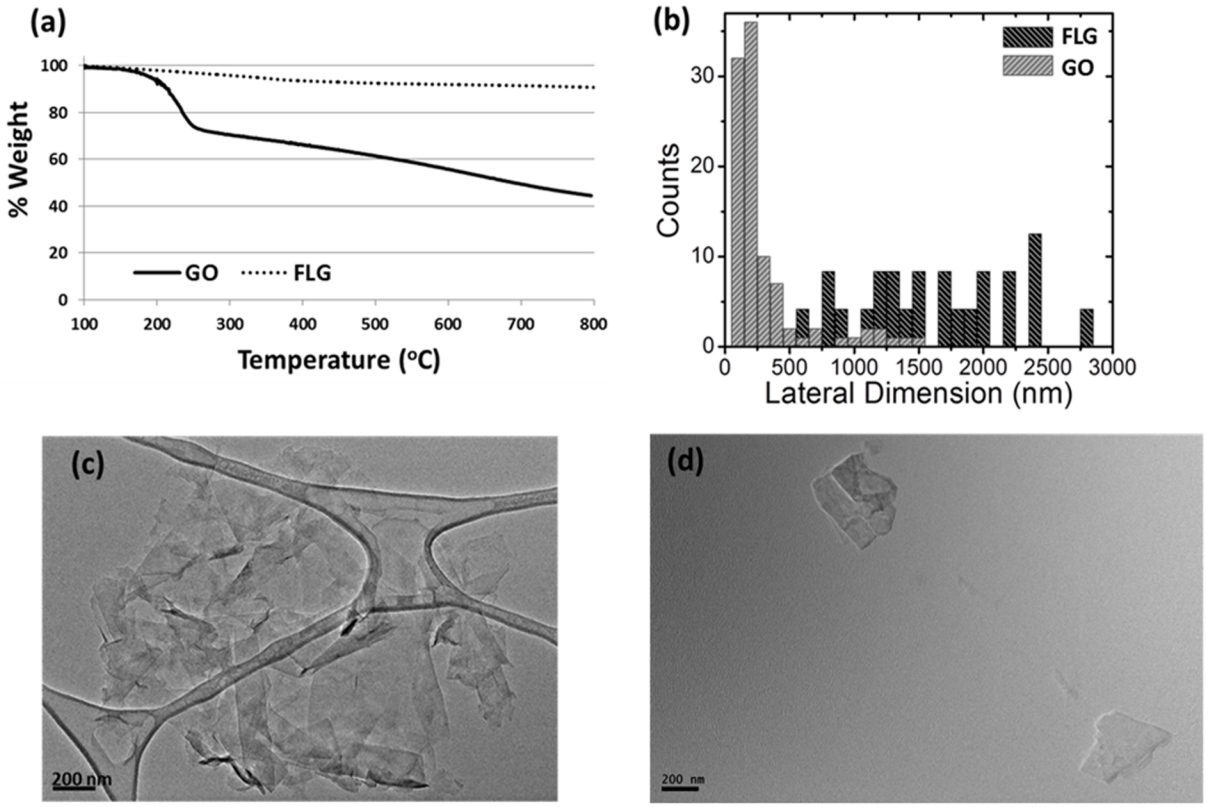

Figure 1 Thermogravimetric analysis of few-layers graphene (FLG), produced by ball milling, and graphene oxide (GO), produced by oxidation of carbon fibres (a); lateral size distribution (n = 100) of FLG and GO (b); representative TEM images of FLG (c) and GO (d).

FLG was prepared by a ball milling treatment following León *et al*. (2014),[38] using a Retsch PM 100 planetary mill under air atmosphere. Graphite (7.5 mg SP-1 graphite powder, purchased from Bay Carbon Inc., USA) and melamine (22.5 mg 1,3,5-Triazine-2,4,6-triamine) were ball milled at 100 rpm for 30 min. After the treatment, the resulting solid mixture was dispersed in 20 mL of water and sonicated for 1 min to produce a black suspension. Melamine was removed by filtering and washing with hot water the suspension and the resulting dispersion was left to settle down for 5 days. The precipitate, consisting in poorly exfoliated graphene, was discarded and the liquid fraction was

carefully extracted and characterized. FLG water dispersions were obtained with a final concentration of 0.09 mg mL^{-1}. Elemental analysis gave an average value of 0.66±0.02 %N, which corresponds with a melamine content in solution of 0.9 ppm.

GO was purchased from Grupo Antolin Ingegnería (Burgos, Spain), which produced it by oxidation of carbon fibres (GANF Helical-Ribbon Carbon Nanofibres, GANF®) and sodium nitrate in sulfuric acid at 0°C.

The concentrations used in our experiments (0.01, 1, and 50 μg mL^{-1}) were chosen in agreement with all the members of the Working Package 4, Health and Environment, in the framework of the European Project Graphene-Flagship.[34] This shared decision allows to easily compare the results from different studies on different organisms, and since these concentrations reflect the expected GBMs release into the environment,[39] it should also be ecologically relevant.

The content of toxic elements was checked in both GBMs. 1.0 mL of FLG (0.09 mg mL^{-1}) suspension or 0.5 mL of a GO (0.5 mg mL^{-1}) suspension was mixed with 5 mL of freshly prepared Aqua Regia in a beaker, heated at boiling point for one hour, then diluted to 10 mL with MilliQ water and filtered through a GHP Acrodisc (Pall Corporation, USA) syringe filter (pore size: 0.45 μm). The solutions were then analyzed for Cd, Cr, Cu, Pb, Mn, K by Inductive Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) with an Optima 8000 Spectrometer (PerkinElmer, USA), equipped with an S10 Autosampler. The Limits of Detection (LOD) are listed in Table S2. Both materials were also analyzed by TEM (Transmission Electron Microscopy). Stable dispersions of GBMs were drop-cast on nickel grids (3.00 mm, 200 mesh) and dried under vacuum. The samples were studied by High-Resolution Transmission Electron Microscopy (JEM 2100, JEOL Ltd, Japan) at an accelerating voltage of 200 kV. Lateral dimension distribution was calculated by using Fiji software. Thermogravimetric analyses (TGA) of FLG and GO were also performed with a TGA Q50 (TA Instruments, USA) at 10 ºC/min under nitrogen atmosphere, from 100ºC to 800ºC.

Experimental design

Assessment of membrane damage after short-term exposure to GBMs

Algal cells of *C. subellipsoidea* and *T. gelatinosa* from approximately 4 weeks old colonies were resuspended in distilled water; the suspensions were firstly filtered with a 40 μm sieve in order to disaggregate the cells, especially those of *T. gelatinosa*, obtaining a homogeneous algal suspension. Algal cells were then gently washed with Milli-Q water through a vacuum filtration system to remove ions derived from the culture medium and a suspension of approximately 3×10^6 mL^{-1} algal cells was prepared. The algal suspension was then subdivided in aliquots. Afterwards FLG or GO suspensions were added to reach a final concentration of 50 μg mL^{-1}. Algal suspensions containing only algal

cells and algal cells plus one of the two GBMs were gently shaken on an orbital shaker for 30 or 60 minutes. All the samples after the treatment (control samples with only algal cells and samples containing algae and GBMs) were filtered by vacuum filtration on membranes [respectively cellulose acetate (CA), 25 mm diameter, pore size 0.45 μm, Sartorius Lab Holding GmbH, D and polytetrafluoroethylene (PTFE), 25 mm diameter, pore size 0.1 μm, Merck KGaA, D] and both the filtrate and the algal material were conserved for the following analyses.

Membrane permeability was evaluated measuring the concentration of potassium (K^+) in the algae and in the filtrates previously obtained. 1.0 mL of pre-exposure samples containing only algal cells was mixed with 1 mL of HNO_3 (69.5% v/v), heated for one hour, diluted to 10.0 mL with Milli-Q water, filtered through a GHP Acrodisc syringe filter (pore size 0.45 μm) and analyzed by ICP-OES. Similarly, 1.0 mL of the filtrate samples was diluted to 4.0 mL with Milli-Q water and analyzed using a calibration curve obtained by diluting a standard solution for ICP-OES analyses (Sigma-Aldrich, USA) in the range 0 – 10 mg L^{-1}. The precision of the measurements as relative standard deviation for the analysis was always less than 5%. LOD at the operative wavelength of 766.490 was 0.010 mg L^{-1}. Potassium concentrations were then normalized for the mass of the silica dried algal material.

SEM observations

A Scanning Electron Microscope Gemini SUPRA 40 (Carl Zeiss NTS GmbH, D) was used to investigate the samples surfaces before and after treatments with FLG and GO. The algal samples were washed in Milli-Q water and fixed for 40 minutes in a water solution 4:1 of 37% formaldehyde and 25% glutaraldehyde, containing also NaH_2PO_4 (11.6% m/v) and NaOH (2.7% m/v). Samples were then washed in Milli-Q water and dehydrated by dipping in water/ethanol solutions at progressively higher alcohol concentrations (50%, 75%, 95% and 100% ethanol for 5 minutes each) and then let dry overnight in 100% ethanol. Prior to SEM imaging samples were gold metalized in a metal sputter coater (SC7620, Quorum Technologies Ltd, UK).

Effect assessment after long-term exposure to GBMs

Algal cells of *A. lobatus*, *C. vulgaris*, *C. subellipsoidea*, *T. gelatinosa* were exposed to GBMs (both FLG and GO) suspensions following two treatments (as explained in Fig. 2): in the first, called "deposited" (D), the graphene suspension was previously filtered and let to dry out on PTFE membranes (25 mm diameter, pore size 0.1 μm, Merck KGaA, D) and afterwards 50 μL of algal suspension were filtered above the layer of graphene deposited by vacuum filtration. In the second treatment, called "shaken" (S), a suspension of algal cells and graphene was prepared, shaken for 30

minutes altogether and then filtered on the PTFE membranes. Before the treatments, algal suspensions (except for *C. vulgaris*) were filtered with a sterile 40 µm sieve with the aim of disaggregate the cells clusters. This filtration allowed to obtain a homogeneous algal suspension, which was constantly kept agitated throughout the preparation of the inocules, necessary to guarantee the repeatability of the inocules and avoid methodological biases. Previous experiments have been performed to standardize all the methods here described in order to exclude over- or underestimations of the eventual ecotoxicity of GBMs.

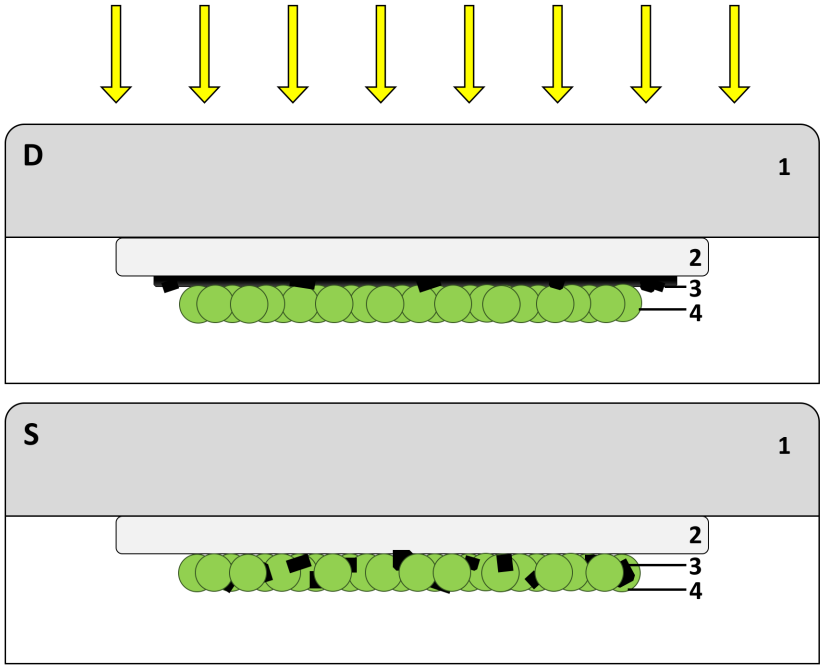

Figure 2 Schematic representation of the treatments "deposit" (D) and "shaken" (S) for the long-term exposure experiments. (1) solid medium; (2) filtering membrane; (3) Graphene-Based Material (FLG or GO); (4) algal cells; arrows: direction of artificial light.

Membranes were laid on solid BBM at the bottom of Microbox Junior 40 vessels (Duchefa Biochemie B.V., NL), equipped with a micro-filter strip on the cover which allows gas exchange while keeping the internal volume in sterile conditions. Seven inoculated membranes corresponding to the six treatments (0.01S, 1S, 50S, 0.01D, 1D, 50D) plus one control (*i.e.* algae without GBMs) were introduced in each vessel, arranged upside down and kept for 4 weeks in growth chambers at the same condition described above. At the end of the cultivation period, membranes were used firstly to measure chlorophyll *a* fluorescence (Chl_aF) emission, and afterwards to measure the total content of photosynthetic pigments (chlorophylls and total carotenoids).

In order to test melamine influence on algal growth, a parallel experiment was performed. 50 µL of an algal cells suspension with melamine were deposited by vacuum filtration on a CA membrane and let grow with control samples as previously described for 4 weeks. Three different concentrations of melamine were tested, corresponding to the same concentrations present in the respective FLG suspensions, i.e. 0.5, 0.01 and 0.0001 µg mL^{-1}. At the end of the cultivation period, the algal colonies were used firstly to measure Chl_aF emission, and afterwards to extract their photosynthetic pigments.

Chlorophyll a fluorescence

Measurements of chlorophyll *a* fluorescence (Chl_aF) were taken on all samples at the end of the experiment. Each sample was dark adapted for 30 minutes before the maximum photochemical efficiency of photosystem II (F_v/F_m) was measured with a pulse–amplitude–modulated fluorimeter Mini-PAM (Walz Heinz GmbH, D), positioning the measuring optic fiber (length: 100 cm; active diameter: 5.5 mm) at 60° and approximately 5 mm from the center of the culture discs, where the cell density is higher. The modulated light was turned on to obtain F_0 (minimal Chl_aF level); a saturating light pulse of ca. 8,000 µmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain F_m (transient maximum Chl_aF level), and thus to calculate F_v (variable Chl_aF level, i.e. $F_m - F_0$) and F_v/F_m (maximum quantum efficiency of PSII photochemistry).[40] Non-photochemical quenching (NPQ), photochemical quenching (qP) and qN,[41,42] were also recorded, as described by Bertuzzi *et al*. (2013)[43] (data not shown).

Total photosynthetic pigments content quantification

Total photosynthetic pigments content was measured spectrophotometrically in crude extracts following Tretiach *et al*. (2007) with some modifications.[44] Each sample was put in a 15 mL tube with DMSO (from 1.2 to 5 mL according to preliminary tests) and kept 1 hour in the dark at 65 °C. Afterwards, samples were cooled down to ambient temperature for 20 minutes in darkness and centrifuged for 20 minutes at 10000 r.p.m.. The absorbance of the supernatant was measured at 750, 665, 658 and 480 nm with a Jenway 7315 UV/VIS spectrophotometer (Bibby Scientific, UK) and chlorophyll *a*, *b* and total carotenoids contents were calculated according to Wellburn (1994).[45]

Statistics

All statistical analyses were run in Microsoft Office Excel 2003 SP3 (Microsoft corporation, USA) and R version 3.2.0 (The R Foundation for Statistical Computing). Statistical differences

among treatments were assessed by nonparametric Kruskal-Wallis and non-paired Mann-Whitney *U* tests ($p < 0.05$).

Results

Graphene characterization

TGA was used to quantify the degree of functionalization of the GBMs (Fig. 1a). The low weight loss observed in FLG (8%) corroborated the low quantity of oxygen groups generated by the exfoliation process in comparison to the values obtained for GO (46%).[46] In both FLG and GO suspensions Cd, Cr, Cu, Pb were below the instrumental resolution threshold. The concentrations of K and Mn were respectively 0.13 mg L^{-1} and 0.03 mg L^{-1} in FLG and 0.44 mg L^{-1} and 1.06 mg L^{-1} in GO. TEM analysis showed a higher lateral size distribution for FLG (< 2760 nm) compared to GO (< 1500 nm) sheets (n = 100) (Fig. 1b). Representative TEM images of FLG and GO can be seen in Fig. 1c and Fig. 1d, respectively.

Potassium leakage after short-term exposure to GBMs

No statistically significant differences were observed between the treatments and the control samples of *C. subellipsoidea* and *T. gelatinosa* (Fig. 3), suggesting that no damage occurred during the short-term exposure to both GBMs.

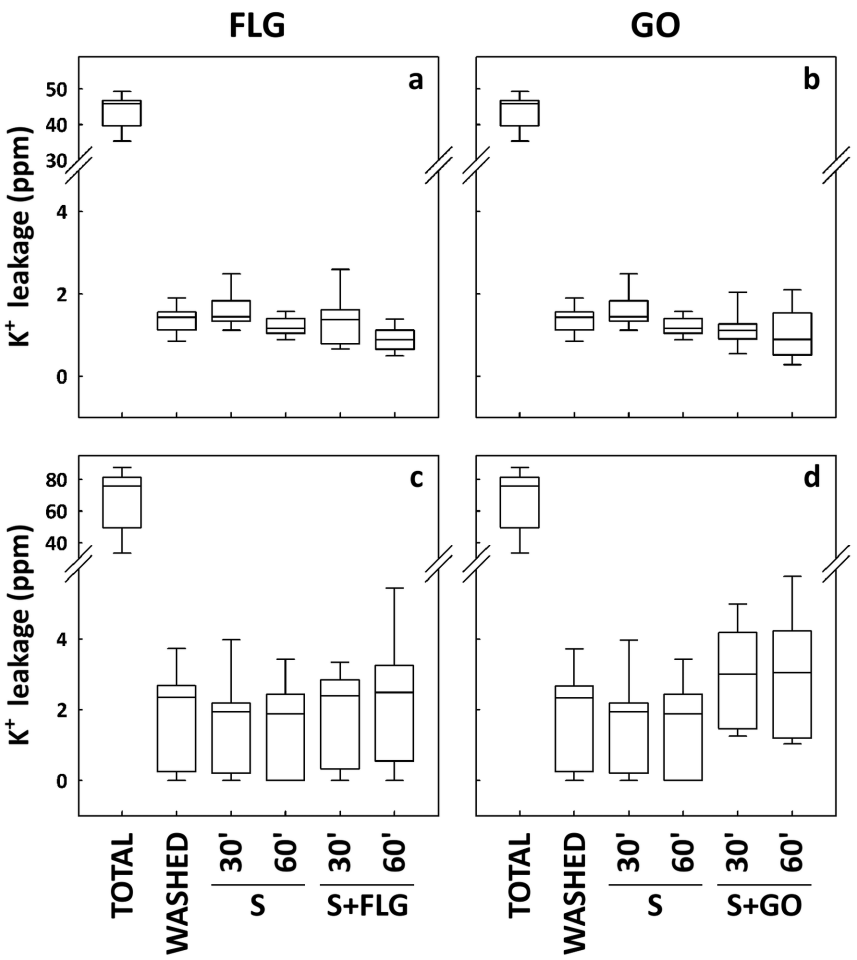

Figure 3 Potassium (K^+) leakage in algal samples. Total K^+ content of pre-exposure *Coccomyxa subellipsoidea* (a, b) and *Trebouxia gelatinosa* (c, d) samples (TOTAL); K^+ leakage from control samples washed but not shaken (WASHED); samples washed and shaken for 30' and 60' without GBMs (S 30' and S 60', respectively) and with FLG (a, c; S+FLG 30' and S+FLG 60', respectively) or GO (b, d; S+GO 30' and S+GO 60', respectively) (a, b: n = 7; c, d: n = 9).

Effect assessment after long-term exposure to GBMs

Mean F_v/F_m values measured in control samples averaged for both treatments varied amongst the species, from 0.668±0.009 (*A. lobatus*) to 0.691±0.005 (*T. gelatinosa*) (see Table 1).

Table 1 F_v/F_m mean plus standard deviation values of control samples after 4 weeks of growth (n = 7).

Species	F_v/F_m mean values of control samples	
	FLG	**GO**
Apatococcus lobatus	0.668±0.009	0.677±0.015
Chlorella vulgaris	0.689±0.013	0.684±0.010
Coccomyxa subellipsoidea	0.675±0.021	0.672±0.018
Trebouxia gelatinosa	0.690±0.006	0.691±0.005

After 4 weeks of cultivation in presence of GBMs, the cultures of *T. gelatinosa* gave very uniform results (see Fig. 4g, h). In the three other species, variability of F_v/F_m was slightly higher within treatments, but again there were no significant differences amongst treatments (see Fig. 4).

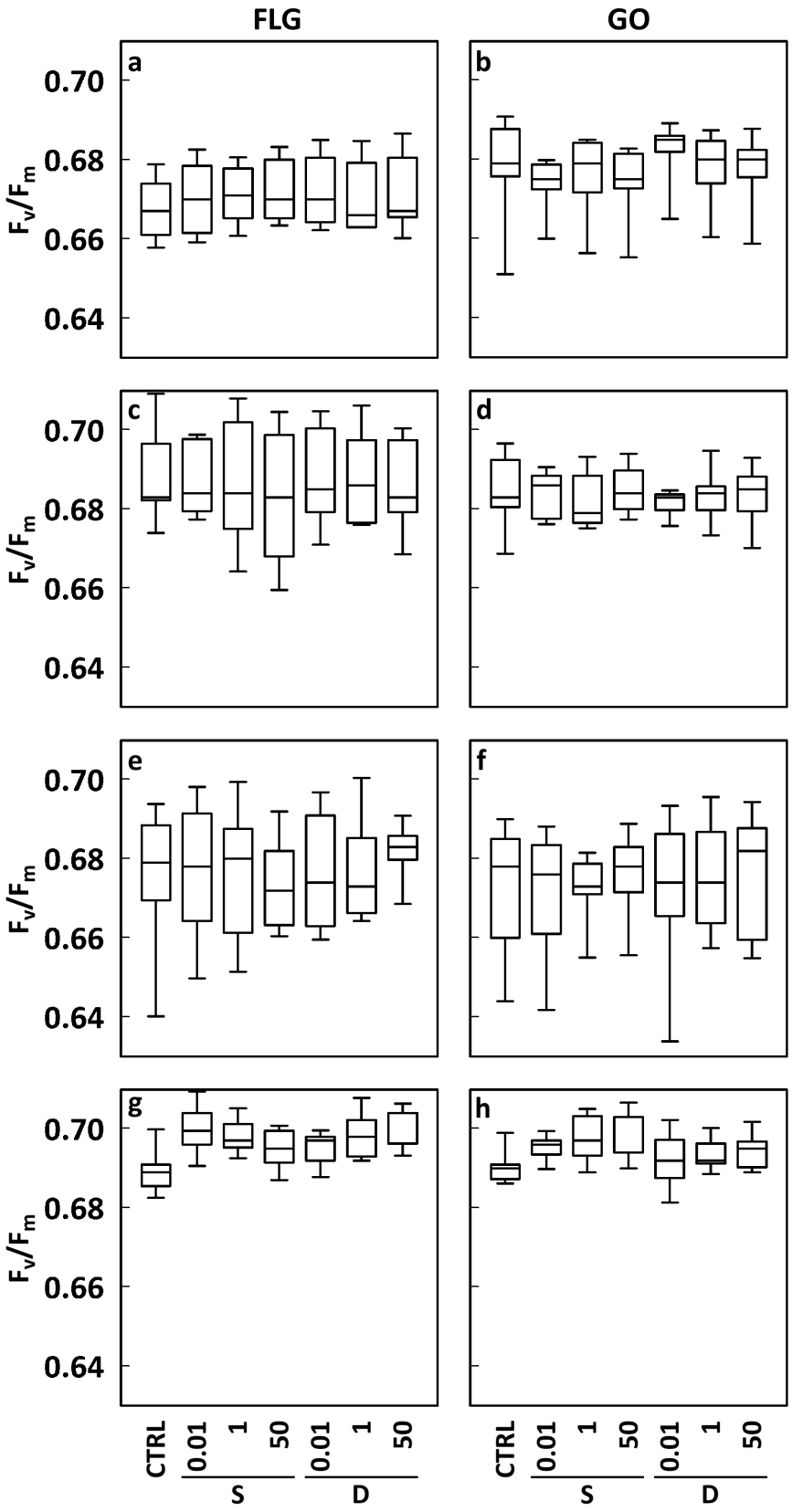

Figure 4 Maximum quantum efficiency of PSII photochemistry (F_v/F_m) measured in 4-week old axenic cultures of *Apatococcus lobatus* (a, b), *Chlorella vulgaris* (c, d), *Coccomyxa subellipsoidea* (e, f) and *Trebouxia gelatinosa* (g, h). The cells were exposed to FLG (a, c, e, g) and GO (b, d, f, h) at 0.01, 1, 50 μg mL^{-1} (0.01, 1, 50 respectively), through the "shaken" and "deposit" treatments (S and D, respectively). For each boxplot median, 25-75° percentiles (boxes), minimum and maximum (whiskers) are reported (n = 7).

Total chlorophylls content (chlorophyll *a* plus chlorophyll *b*) in the 4-week old cultures ranged between 29.7±5.1 μg mL^{-1} (*T. gelatinosa*) and 202.2±14.2 μg mL^{-1} (*C. vulgaris*) (see Table 2), and this depends on various factors, among which the algal growth rate, which varies as well (see Fig. 5).

Table 2 Total chlorophylls (*a* + *b*) content (mean ± standard deviation) of control samples after 4 weeks of growth (n = 7).

Species	**Total chlorophylls content means of control samples (μg mL^{-1})**	
	FLG	**GO**
Apatococcus lobatus	46.6±4.6	49.2±10.6
Chlorella vulgaris	196.6±12.8	202.2±14.2
Coccomyxa subellipsoidea	102.8±17.9	93.9±17.0
Trebouxia gelatinosa	29.7±5.1	32.3±3.9

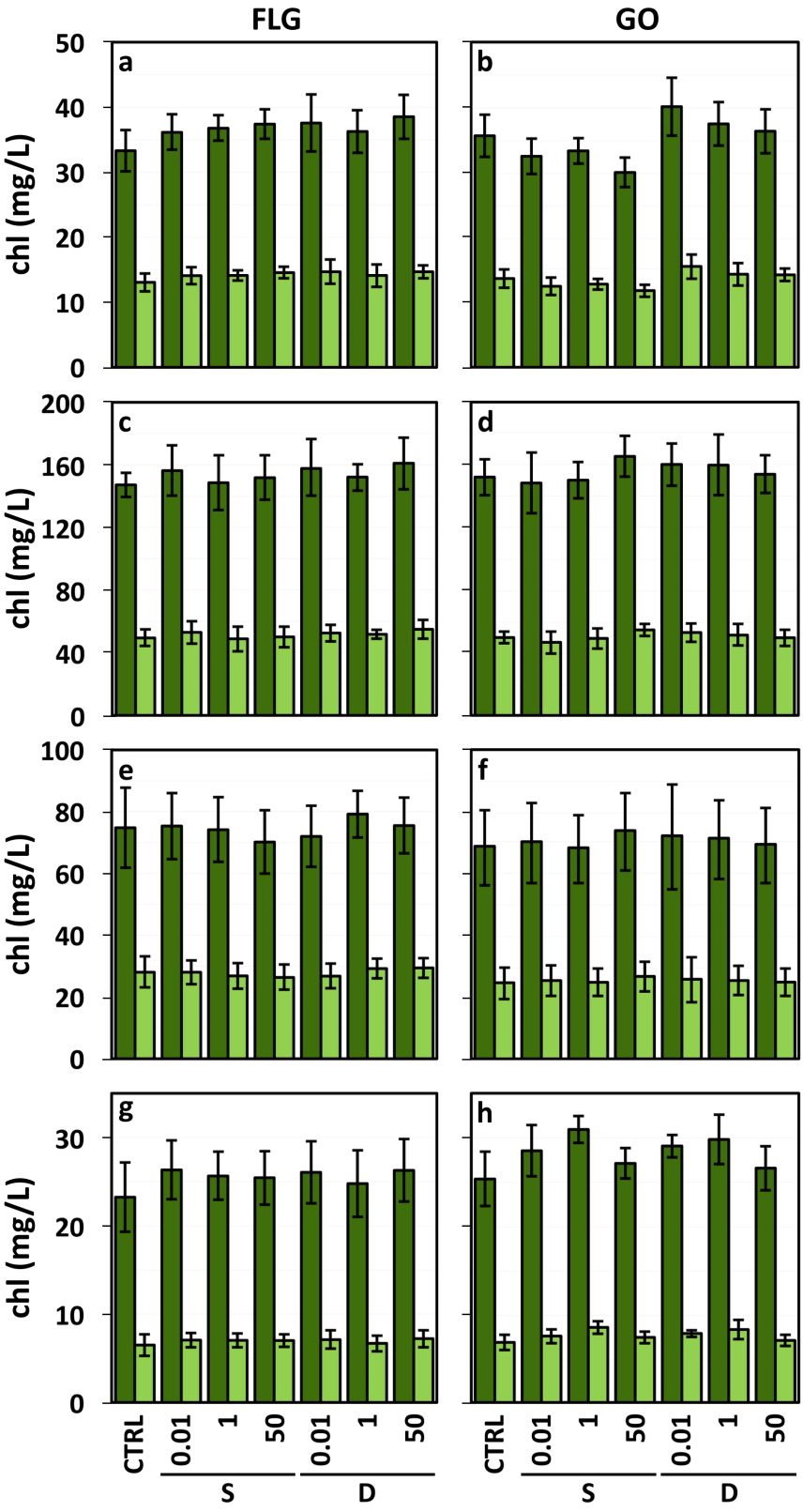

Figure 5 Chlorophyll *a* (dark green bars) and *b* (light green bars) content measured in 4-week old axenic cultures of *Apatococcus lobatus* (a, b), *Chlorella vulgaris* (c, d), *Coccomyxa subellipsoidea* (e, f) and *Trebouxia gelatinosa* (g, h). The cells were exposed to FLG (a, c, e, g) and GO (b, d, f, h) at 0.01, 1, 50 µg mL^{-1} (0.01, 1, 50 respectively), through the "shaken" and "deposit" treatments (S and D, respectively) (n = 7).

Comparing the chlorophyll content of the treatments towards the respective control samples no statistically significant differences were detected for both FLG and GO, independently of the concentrations, suggesting that no damage occurred during the long-term exposure. Total carotenoids contents are reported in Table S3; no significant differences amongst treatments were recorded. Also the total photosynthetic pigments content of samples grown in presence of melamine showed no statistically significant differences towards the respective control samples (data not shown).

Discussion

The GBMs tested in our experiments (FLG and GO) did not cause negative effects to the four target species of aeroterrestrial microalgae. They did not suffer acute toxicity in terms of K^+ leakage in the short-term exposure. Likewise, the results from the long-term exposure suggest that if there was any other kind of damage in the short-term exposure, this was rapidly and easily overcame by the algal populations, and in the meantime the protracted exposure to GBMs did not affect them, because all the vitality parameters were similar to those of the control samples. The careful standardization of the inoculation method allowed us to exclude some biases in the results that at the beginning were interpreted as a proof of the negative effects of FLG and GO on selected species.

Among our four species, only *C. vulgaris* has previously been used as model organism to test GBMs ecotoxicity, but the results collected so far were controversial if not conflictual. Hu *et al.* (2014, 2015)[47,48] and Ouyang *et al*. (2015)[49] studied the effects of the envelopment of *C. vulgaris* cells by GO, and by graphene oxide nanosheets (GONS), respectively. Hu *et al*. (2014)[47] reported decreasing amounts of chlorophyll *a* content in samples treated with increasing GO concentrations with respect to control samples, and referred these differences to a concentration-dependent toxicity. This conclusion, however, was not confirmed by Hu *et al.* (2015)[48], who worked exactly at the same GO concentrations of Hu *et al*. (2014)[47], and could not confirm any variation in the chlorophyll content in dependence to the GO concentration. The latter authors, on the contrary, documented a purported GO influence on cell division, that would be positive after 24 hours of exposure, but negative after 96 hours. This contrasting, time-dependent phenomenon was observed also by Ouyang *et al*. (2015): GONS and GO provided as quantum dots (GOQD) apparently promote cell division up to, 48 and 72 hours of exposure respectively, to inhibit it after longer exposure times.[49] Furthermore, chlorophyll *a* content did not vary for GONS-treated cells, but varied significantly for those exposed to GOQD, whatever the concentration tested. Totally contrasting conclusions were those of Haniff Wahid *et al*. (2013), who treated *C. vulgaris* with GO in order to intentionally coat and confine the

algal cells within GO layers.[50] They reported a reduction of the cell division rate, ascribed to a negative feedback phenomenon caused by the physical contact of the cells with the GO flakes, but not a reduction in viability, as demonstrated by the conserved ability of the cells to normally replicate when they eventually escaped from the GO wrap. The latter authors, in full agreement with our conclusions, considered this as an indirect demonstration that GO is non-toxic.

The measurement of K^+ leakage from plant tissues is a classical method to estimate membrane integrity. In water suspension, GBMs can actually physically damage unicellular organisms, in particular bacteria, due to the sharp edges of the flakes;[51,52] furthermore, GBMs may induce ROS production,[53−55] which causes lipid peroxidation and thus disrupt cell membrane functionality, with consequent K^+ release. The absence of an increased K^+ leakage in the GBMs-exposed algae (Fig. 3) clearly supports the fact that they did not suffer damage to the membranes. This is in good accordance with the results of Hu *et al*. (2015), who observed no evident changes in the ultrastructure of the protozoan *Euglena gracilis*, despite the heavy GO cover of the cells.[56] Conversely, Nogueira *et al*. (2015) documented membrane damage in the green alga *Raphidocelis subcapitata* for GO concentrations higher than 10 μg mL^{-1}.[32] Interestingly, whereas *E. gracilis* lacks a robust cellulosic cell wall, having a pellicle made up of a protein layer internally supported by a substructure of microtubules, *R. subcapitata* develops a cellulosic monolayered cell wall,[57] c. 600 nm thick,[58] which is particularly resistant to destruction.[59] It must be underlined that *R. subcapitata*, which is widely used as a target organism in routinary ecotoxicological tests, is known to be highly sensitive to heavy metals. Therefore, we cannot exclude that some of the negative effects referred to GBMs are actually due not to the physical damage to the cells, but instead to the minimal co-presence of phytotoxic elements such as Mn and Cd. This is a very delicate issue, because there is experimental evidence that the contemporary presence of GO and e.g. Cd enhances the uptake and the consequent toxicity of the heavy metal at increasing GO concentrations.[33] The residual presence of toxic contaminants due to fabrication and handling procedures has never been checked carefully in GBMs ecotoxicological studies, but might explain some of the discrepancies in the results. In our case, Mn was the sole element whose content was higher than the detection limit in our GBMs pristine samples. Derived most probably by the permanganate oxidative treatment, Mn was higher in GO than in FLG of an order of magnitude, but however not at such harmful concentrations to reduce the algal growth, although Mn is known as a phytotoxic element, particularly for green algae.[60]

Size appears to have an important role in the toxicity of GBMs,[6,15] as for other nanomaterials,[61,62] because it influences the uptake by the cells.[33,49] In algae, internalization is a problematic issue for the presence of the cell wall, that is the primary site for the interaction with GBMs, and acts as a more or less robust barrier against both physical damage and GBMs uptake, in

dependence to its molecular composition, thickness, and size of the pores. Navarro *et al*. (2008) in fact reported that only particles smaller than the pore size of the cell wall can effectively enter into a walled cell.[24] From this point of view, our species offer a wide spectrum of peculiar cell wall features, which are typical of aerial green microalgae and might *de facto* play a pivotal role in the observed protection against GBMs. At maturity, the cell wall of *C. vulgaris* is c. 17-21 nm thick, and consists of a chitosan-like microfibrillar layer composed by glucosamine, which accounts for its high rigidity,[63,64] whereas that of *C. subellipsoidea* is three-layered, the inner, rigid layer being made of highly durable sporopollenin.[65,66] The cell wall of *T. gelatinosa* is quite thick, up to 1 μm,[67] and composed by several layers of different chemical composition.[68] The species with the thickest cell wall is *A. lobatus*, which in some cases is more than 2 μm thick.[69] Information about its composition are missing, even though this alga is often reported as a rather resistant species. The cell wall is subjected to important modification with cell development,[70] and therefore nanoparticles might pass through the cell wall when the cells are in the first juvenile stages. At that time the newly synthesized primary wall is still thin, cross-linkages among macromolecules are lax, and the same molecular composition may differ from that of the adult cell wall.[71] From this point of view, the good resistance to GBMs of our four-week-old algal cultures might be explained as the re-establishment of the population from adult cells which, having thick, mature and robust cell walls, were not exposed to the negative effects of GBMs internalization and/or physical damage, to which were eventually exposed the youngest cells of the inoculum, with their immature cell walls.

In our experimental design, the exposure mode applied in the long-term growth experiment assured a tight physical contact between our algae and the two GBMs, as confirmed by the SEM observations (Fig. 6).

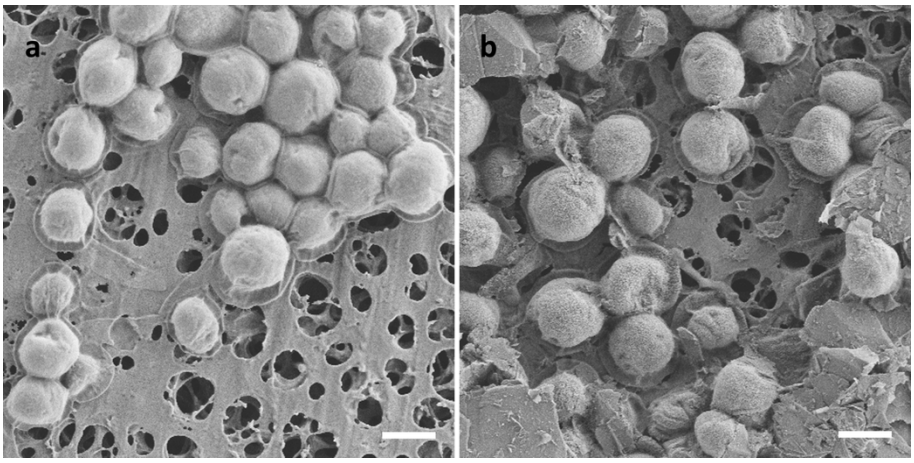

Figure 6 Scanning electron microscope images of *Chlorella vulgaris* cells on cellulose acetate membranes before (a) and after the exposure (b) to FLG (30', 50 μg mL^{-1}). Scale bars = 2 μm.

As suggested by Nogueira *et al.* (2015),[32] the formation of algal-nanocarbon aggregates, when massive, may decrease the light absorbed by the photosystems, with subsequent growth reduction.[72] Some authors considered this as a direct evidence of the toxicity of nanocarbon materials.[73,48,32] This can hardly be the case, otherwise also the shading of a tree should be defined "toxic" to the understorey because it reduces the quantity of light at ground level. More correctly, Cleuvers and Weyers (2003)[74] and Hjorth *et al*. (2016)[75] claimed that shading and toxicity are not additive effects. Regarding our experimental design, the presence of a GBM layer between the light source and the algal layer in the type "D" exposure (Fig. 2) did not influence the algal growth, because we did not observe a statistically significant difference between S and D samples in terms of final biomass (Fig. 5). In our opinion this is not a surprising outcome, since our growth experiments were conducted on solid media, with a different exposure method than liquid cultures, at dim light (20±2 μmol photons m^{-2} s^{-1}), a light regime significantly lower than the one (from 80 to 120 μmol photons m^{-2} s^{-1}) applied by those authors who claimed a shade-induced growth reduction. Cleuvers and Weyers (2003) pointed out that toxicity may be masked and underestimated when the photon flux density is too low, because algae with high growth rate would be more responsive than algae whose growth is low due to dim light.[74] In our opinion, however, also this problem is faced from the wrong side. Microalgae can typically optimize their photosystems to the light environment to which they are exposed, through a phenomenon called "photo-acclimation".[76] Therefore, we cannot exclude that some apparent variations in the growth rates observed by some authors are the result of observation times too short to be compatible with photo-acclimation phenomena. To overcome this problematic issue, Hjorth *et al.* (2016) proposed a new endpoint based on the ratio between chlorophylls and xanthophylls, which is supposed to increase with shading.[75] Since no significant differences in the composition were observed between the controls and the GBMs-exposed cultures, we can exclude that our colonies were affected by a reduction of the light regime, and in the meantime we can also exclude an underestimation of GBMs toxicity, since the light regime applied is appropriate to obtain good growth rates.[77] It must be underlined that shading has been excluded as the main driver for toxicity of metal oxide nanoparticles by several authors, who instead pointed out the attention to other mechanisms, such as the solubilization of the metal oxide nanoparticles themselves (literature cited *supra*),[78,30,79] or the interference with nutrient acquisition. For instance, Zhang *et al*. (2016) reported an increased algal mortality caused by the aggregation of nanoparticles on the cell surface, which was attributed to a reduced uptake of mineral nutrients.[80] In our growth experiment, the presence of a GBMs layer between the solid medium and the algal colonies (type "D", see Fig. 2) did not influence the mineral uptake, since their growth parameters were the same of both control and "S" colonies. Several authors report that the major factor affecting the viability of GBMs exposed cells is an increased oxidative

stress, caused by the altered activity of the enzymes involved in the oxidative balance regulation, which led to a more or less important increase of ROS.[52,81,82] According to Hu *et al*. (2015), however, ROS production might directly be generated by the GO-mediated hypoxia of the algal cells, caused by the perturbation of the mitochondrial redox chain activity,[48] in analogy to what is documented in vascular plants.[83] Also in this case, the effects would be a consequence of GBMs internalization. Both phenomena are under study in *T. gelatinosa* and will be the subject of a forthcoming paper.

Conclusions

Our experiments demonstrated the absence of a direct impact of FLG and GO in short-term exposure conditions in the free-living *Coccomyxa subellipsoidea* and in the lichen photobiont *Trebouxia gelatinosa*, and the globally negligible impact of FLG and GO on four aeroterrestrial green microalgae (the above mentioned *C. subellipsoidea* and *T. gelatinosa*, plus *Apatococcus lobatus*, and *Chlorella vulgaris*) in our long-term exposure conditions.

Acknowledgements

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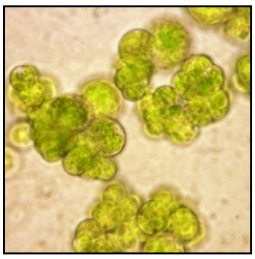
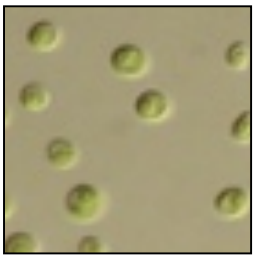
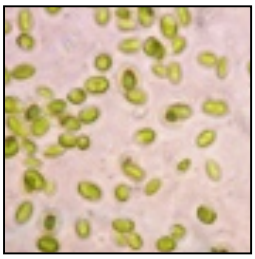
Electronic Supplementary Material

Table S1 Main characteristics of the biology of the four aeroterrestrial microalgae used in these experiments.

Species	***Apatococcus lobatus* (Chodat) J.B. Petersen**	***Chlorella vulgaris* Beijerinck**	***Coccomyxa subellipsoidea* E. Acton**	***Trebouxia gelatinosa* Archibald**
				
Dimension (µm)	6-9	2-10	6-10 × 4-6	5-16
Cell shape	subspherical to spherical	spherical	irregularly elliptical to globular	spherical
Pyrenoid	absent	present	absent	present, *gelatinosa*-type
Cell wall: thickness; composition	up to 2 µm, thickened in older cells	17-21 nm; chitosan-like microfibrillar layer composed by glucosamine	80-90 nm; three-layered, the inner made of sporopollenin	up to 1 µm; several layers of different composition; development of an external gelatinous sheath (1.5-2 µm) around the individual cells
Colonial behavior	sarcinoid; cells aggregates in groups of 4 or more	single coccoid cells	single coccoid cells	single coccoid when free-living, often forming compact aggregates

Reproduction	asexual; formation of autospores, rarely zoospores	asexual; formation of autospores	asexual; formation of 2-4 autospores	asexual; formation of aplanospores, rarely zoospores
Habitat	terrestrial; mostly bark/rock biofilms	freshwater / terrestrial	terrestrial	terrestrial; mostly as lichen photobiont, rarely in biofilms
Distribution	cosmopolitan	cosmopolitan	cosmopolitan	cosmopolitan
Previous ecotoxicological studies (GBMs)	none	[47, 48, 50]	none	none
References	[69, 84]	[63, 64, 85]	[66, 65, 86]	[67, 68, 87]

Table S2 Limits of Detection of the ICP-OES elemental analysis.

	Wavelength	**LOD (mg L^{-1})**
Cd	228.802	0.005
Cr	267.716	0.005
Cu	327.393	0.010
Pb	220.353	0.010
Mn	257.610	0.005
K	766.490	0.010

Table S3 Total carotenoids content (mean ± standard deviation) after 4 weeks of growth (μg mL^{-1}; n = 7).

	Apatococcus lobatus		*Chlorella vulgaris*		*Coccomyxa subellipsoidea*		*Trebouxia gelatinosa*	
	FLG	**GO**	**FLG**	**GO**	**FLG**	**GO**	**FLG**	**GO**
CTRL	10.1±0.7	10.9±1.7	33.8±1.2	35.3±2.0	20.1±2.9	19.1±3.0	5.7±0.8	6.3±0.8
0.01 S	10.7±0.7	9.7±0.5	36.1±2.8	34.5±3.9	20.3±2.4	19.2±2.8	6.5±0.8	7.1±0.7
1 S	11.0±0.7	10.1±0.8	34.5±3.5	34.7±2.0	20.0±2.0	18.5±2.3	6.3±0.6	7.6±0.4
50 S	11.1±0.8	9.0±0.7	34.5±2.7	37.4±2.7	18.9±2.2	19.5±2.4	6.3±0.7	6.6±0.5
0.01 D	11.2±1.2	12.1±0.8	36.4±3.3	34.5±5.6	19.5±2.2	20.1±3.9	6.4±0.7	7.1±0.2
1 D	10.9±0,8	11.5±1.7	34.7±2.1	37.2±3.9	21.3±1.6	19.4±2.9	6.1±0.8	7.2±0.8
50 D	12.0±2.1	10.9±1.0	37.0±3.1	35.0±2.3	19.8±1.7	18.7±2.3	6.5±0.8	6.5±0.7

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Ecotoxicological effects of Graphene-Based Materials on the aeroterrestrial microalga *Trebouxia gelatinosa*: focus on internalization and oxidative stress

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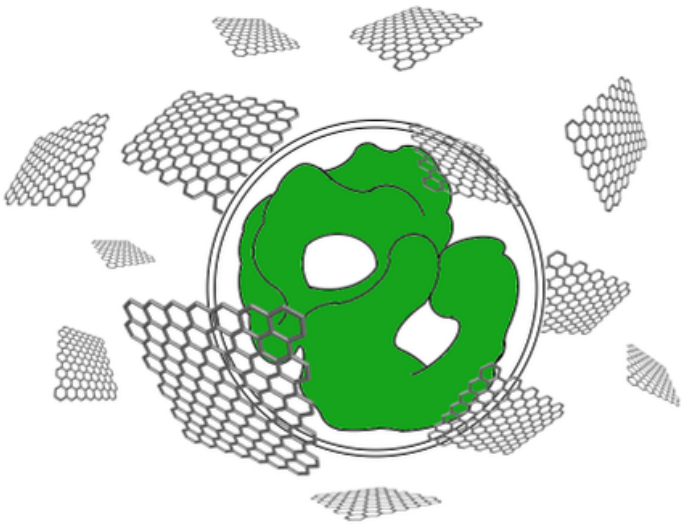


Abstract

The exposure effects of two Graphene-Based Materials (GBMs), few-layers graphene (FLG) and graphene oxide (GO), have been studied on the aeroterrestrial green microalga *Trebouxia gelatinosa* Archibald. Algal suspensions without GBMs and with FLG or GO at the concentration of 50 μg mL^{-1} were shaken for 10 and 30 minutes. After exposure, GBMs internalization was investigated with confocal laser scan microscopy. Potential oxidative effects of GBMs were studied analyzing (*i)* efficiency of the photosystems through measurements of chlorophyll *a* fluorescence emission (F_v/F_m parameter), (*ii)* changes of gene expression of eight genes of interest through quantitative Real-Time PCR, and (*iii)* quantification of HSP70 protein through Western blot. Potential oxidative effects of GBMs were compared with exposures to three different concentrations of H_2O_2, used as positive controls.

No GBMs were clearly detected inside the cells, despite they were observed in close contact with the algal cells. While H_2O_2 treatments produced dose-and time-dependent oxidative effects, GO was ineffective, whereas FLG caused the downregulation of a single gene (HSP70) limited to the 30 minute exposure However, this did not correspond to a decrease in the quantity of the corresponding protein. The results suggest that a harmless interaction might occur between GBMs and plasma membrane, inside the algal cell wall. *T. gelatinosa* defense mechanisms to oxidative stress are briefly discussed.

Introduction

In the recent years, the rapid advancement in the field of nanomaterials has increased their development and consequently their production and commercialization. Among nanomaterials, the carbon-based ones are the most widely researched because of their potential on the most diverse fields,[1] with a predominant role occupied by Graphene-Based Materials (GBMs).[2] Graphene is a two-dimensional crystal composed of monolayers of carbon atoms arranged in a honeycombed network with six-membered rings.[3] Since its discovery, the attention of researchers was focused on its unique and exceptional properties, such as mechanical stiffness, strength, elasticity, very high electrical and thermal conductivity, which lead to the development of multiple applications in electronics, photonics, composite materials, energy generation and storage, sensors and metrology and biomedicine.[2] The huge investments brought to an incredible advancement in the industrial field, unfortunately accompanied by a slower progress in the understanding of the impact on human health and the environment, hence making nanosafety a priority.[4]

So far, the effects of GBMs have been evaluated mostly on animal and bacterial model organisms,[5] highlighting that GBMs toxicity appears to be dependent on various physiochemical properties such as shape, size, oxidative state and presence of functional groups.[6-8] In particular, these properties affect also the graphene ability to cross cell membranes. In animal cells, GBMs were frequently observed being internalized through many different endocytosis pathways. For example, graphene oxide (GO) sheets were found either surrounded by membranes into endosome-like structures or free in the cytoplasm, suggesting that the cell uptake occurs by a combination of active and passive mechanisms.[9] Furthermore, internalization of graphene quantum dots (GQD) by caveolae-mediated endocytosis have been observed in breast cancer MCF-7 cells: GQDs accumulate in the endoplasmic reticulum, in the cytoplasm and even in the nucleus, demonstrating that they can pass through different cell membranes.[10] Despite GBMs uptake in both the previously mentioned studies led to toxicity, a carboxyl functionalization allowed graphene to enter the cells without causing any toxic effect.[11] For this reason, GBMs could also be successfully used as nano-carriers for selective drug delivery.[12]

As opposed to animals, plants and algae generally possess a cell wall, which is the first site of interaction with GBMs but also the primary barrier preventing GBMs uptake.[13] It has been shown that nanoparticles smaller than the pore size of the cell wall are able to enter into the cell[13] and that GO with a lateral dimension of 500 nm is internalized in *Arabidopsis thaliana* T87 cells by a non-energy dependent endocytosis, while larger sheets of about 1 μm by phagocytosis.[14] In both cases, internalization caused decreased mitochondrial function and eventually cell death.[14] By contrast, in another research, GO internalization did not influence *A. thaliana* germination, seed development, shoot and root development of seedlings and flowering time.[15] Obviously, GBMs internalization plays a key role in cell toxicity,[16] although in plant cells this phenomenon has not been investigated in detail.

Aeroterrestrial microalgae are a cosmopolitan cluster naturally occurring on a variety of substrates (wet soils, sandstone, limestone, other plants[17]), colonizing the most diverse environments.[18,19] Some of them (e.g. the genus *Trebouxia* - Chlorophyta) are able to form a stable symbiotic association with fungi (usually ascomycetes) through lichenization.[20,21]

Microalgae in general have nowadays been broadly employed to study nanoparticles toxicity, turning out to be important organisms to study internalization and its effects. The cell walls of these organisms are very different in thickness and composition, often with peculiar species-specific characteristics.[22] Some of them may have very thick cell walls, like the one of *Apatococcus lobatus*, which can be over 2 μm thick.[23] Cell wall has an important role in the control of algal water status, and contributes in their desiccation tolerance.[24]

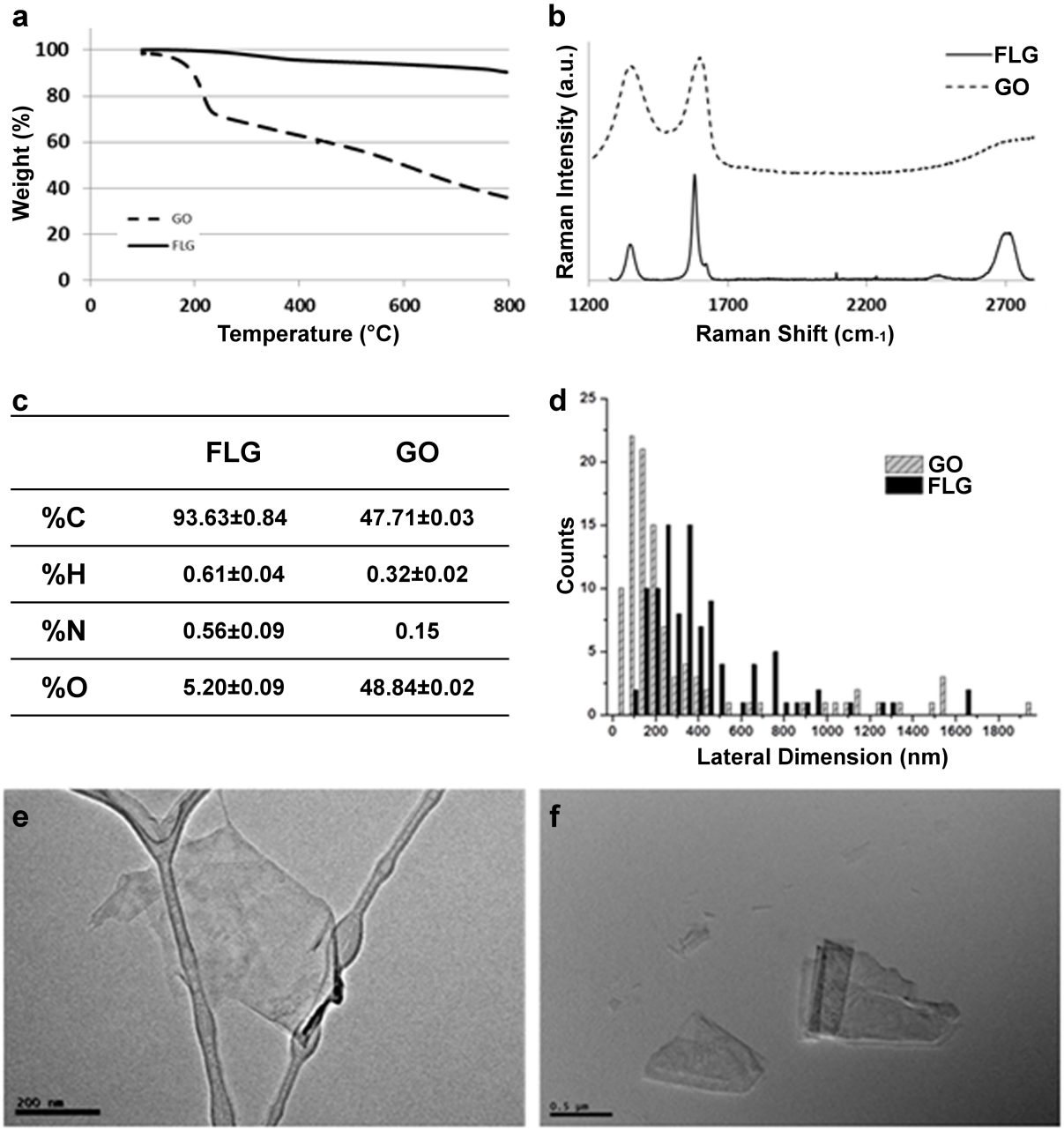
GBMs internalization has been reported in the green algae *Chlorella pyrenoidosa*[25] and *C. vulgaris*.[26-28] In the former study the authors claimed that only multi-layer graphene (MG) and reduced graphene oxide (rGO) entered the cells, while GO did not. Some researchers demonstrated that internalization of GBMs in general is linked to the production of ROS[11] or to the increase of oxidative stress.[28] Oxidative stress is one of the main toxic effects induced by GBMs on microorganisms,[29,30] together with mechanical damage[31] and cell wrapping by the GBMs flakes.[32,33] Normal cellular redox homeostasis is a balance between ROS generation and their elimination or reduction by the antioxidative defenses. Most stress factors have in common that they increase the ROS production/development in organisms, hence unbalancing the cell redox status. Thus, an uncontrolled ROS accumulation can cause membrane peroxidation, protein cleavage, and DNA strand breakage,[30] which in the worst case can lead to cell death. The studies conducted so far had showed that aeroterrestrial microalgae possess a constitutive antioxidant machinery which is able to scavenge the "oxidative burst" in minutes after his insurgence;[34,35] however, oxidative stress response on these ecological important organisms has been still poorly investigated.

The aim of the study was to analyze GBMs internalization and the effects on physiology and gene transcription of a short-term exposure to two GBMs, few-layers graphene (FLG) and graphene oxide (GO), selected as reference materials by the Working Package 4, Health and Environment, in the framework of the European Project Graphene-Flagship. Among photoautotrophic organisms, we selected the aeroterrestrial microalga *Trebouxia gelatinosa* Archibald, which is a member of the most widespread genus of lichenized algae,[36] which can be found also living in the free state in complex communities like algal biofilms on tree barks,[17] demonstrating its relevant ecological importance. *T. gelatinosa* proved to be a resistant organism towards GBMs exposure (unpublished) and to other stresses including desiccation.[35] Moreover, *T .gelatinosa* transcriptome was recently published,[35] allowing to design specific primers for the analysis of gene expression. After a short-term exposure, we evaluated whether (*i*) GBMs are internalized by *T. gelatinosa* cells, (*ii*) GBMs exposure induces oxidative stress response and (*iii*) increases algal mortality, through confocal laser scanning microscopy observations, physiological measurements, gene expression analyses at transcript and protein level.

Results

GBMs preparation and characterization

Elemental analysis was performed to determine carbon, hydrogen, nitrogen and oxygen content in the two GBMs (Fig. 1c). The value of %N in FLG corresponds to a melamine content of



	FLG	GO
%C	93.63±0.84	47.71±0.03
%H	0.61±0.04	0.32±0.02
%N	0.56±0.09	0.15
%O	5.20±0.09	48.84±0.02

Figure 1. Thermogravimetric analysis of few-layer graphene (FLG), produced by ball milling, and graphene oxide (GO), produced by oxidation of carbon fibers (a); average Raman spectra of FLG and GO (b); elemental analysis of FLG and GO, (c); lateral size distribution (n = 100) of FLG and GO (d); representative TEM images of: FLG (e, scale bar = 200 nm) and GO (f, scale bar = 500 nm).

0.84 wt%. The results of the elemental analysis agree with those of the thermogravimetric analysis (TGA) for both materials: a weight loss of 6.4% was observed in the case of FLG, corroborating the low quantity of oxygen groups generated by the exfoliation process, while a 46% of weight loss was obtained from TGA analysis of GO (Fig. 1a). The differences between the Raman spectra of FLG and GO evidence the contrast between these derivatives (Fig. 1b). The Raman spectrum of FLG

shows the two most intense peaks of graphene, the G band and the 2D peak, which appear at around 1580 cm^{-1} and 2700 cm^{-1}, respectively. The average I(2D)/I(G) ratio is 0.49, proving the samples to be few-layer graphene, usually assigned for I(2D)/I(G) < 1.[37,38] When graphene is affected by defects, a peak appears at around 1345 cm^{-1} (D band). In this case, the average spectrum of FLG shows an I(D)/I(G) ratio about 0.36, confirming a low level of defects which are attributed to the edges of the micrometer flakes.[39] The average Raman spectrum of GO, in contrast, shows broad D and G bands. In addition, a bump can be observed in this spectrum instead of the usual 2D band common to graphene structures. Transmission electron microscopy (TEM) analysis showed higher lateral dimensions for FLG sheets compared to GO sheets. Lateral size distributions of both GBMs are shown in Fig. 1d (n = 100), with representative TEM images of FLG and GO in Fig. 1e and Fig. 1f, respectively.

In-vivo *GBMs-algae interaction assessment*

The cell wall of *Trebouxia gelatinosa* observed at the confocal laser scanning microscopy (CLSM) reflects a faint light when illuminated by the laser (Fig. 2a), especially with the setup used for the visualization of GO (Fig. 2b). In autospores (diam. < 7 μm) light was reflected also from a single defined spot which was observed in both controls and treated samples (Fig. 2b, 2d). However, the GBMs flakes with a lateral dimension bigger than 1 μm were clearly distinguishable by the more intense light they reflect (Fig. 2c, 2d). Both FLG and GO flakes were observed adhering to the cell wall whenever they get in contact with the algae (Fig. 2c, 2d). Small FLG flakes (lateral size < 1 μm) were observed within the cell wall (Fig. 3), but never reaching the cytoplasm of the cells.

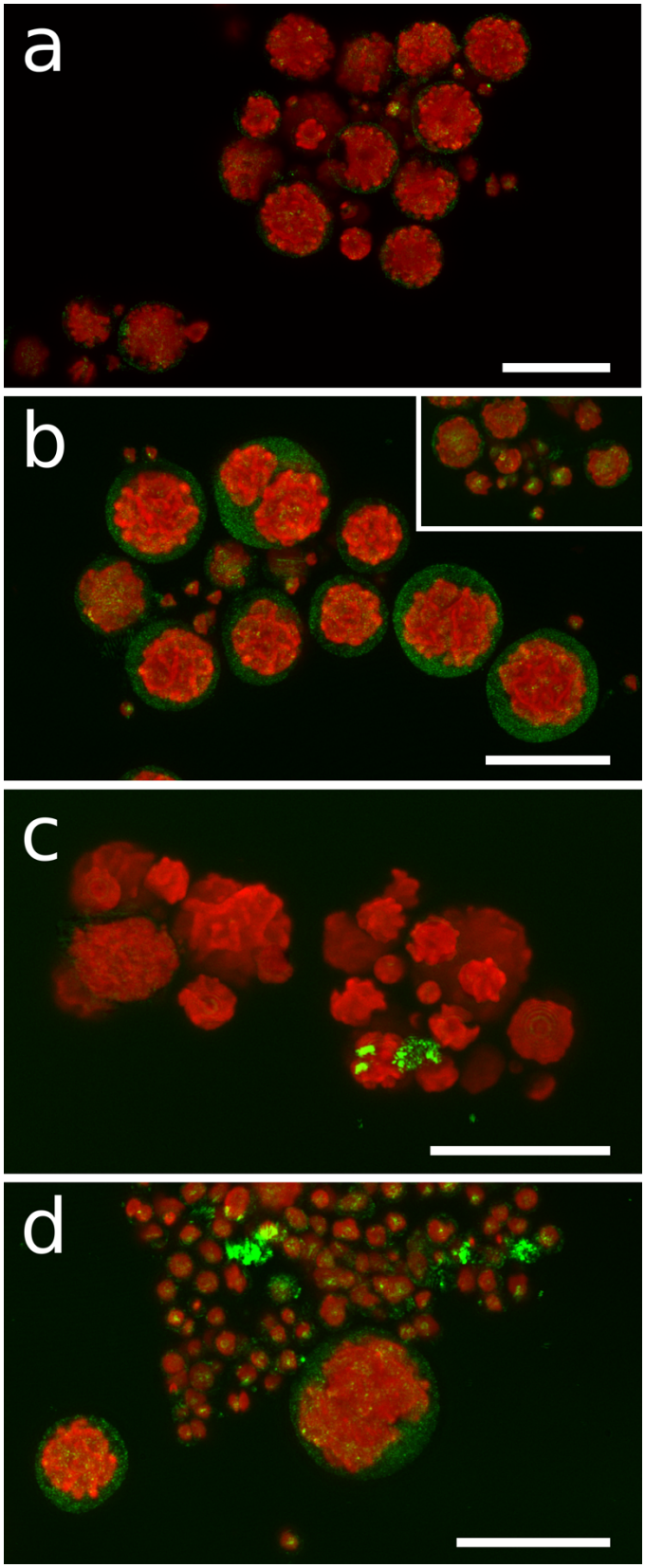


Figure 2. Cells of the green microalga *Trebouxia gelatinosa* observed with confocal laser scan microscopy in reflection mode. Cells before exposure observed with FLG setting (a) and GO setting (b); after exposure to FLG (c) or GO (d). Red signal emitted by chlorophyll *a*; weak green signal reflected by algal cell walls (b, d); strong green signal reflected by FLG (c) or GO (d).

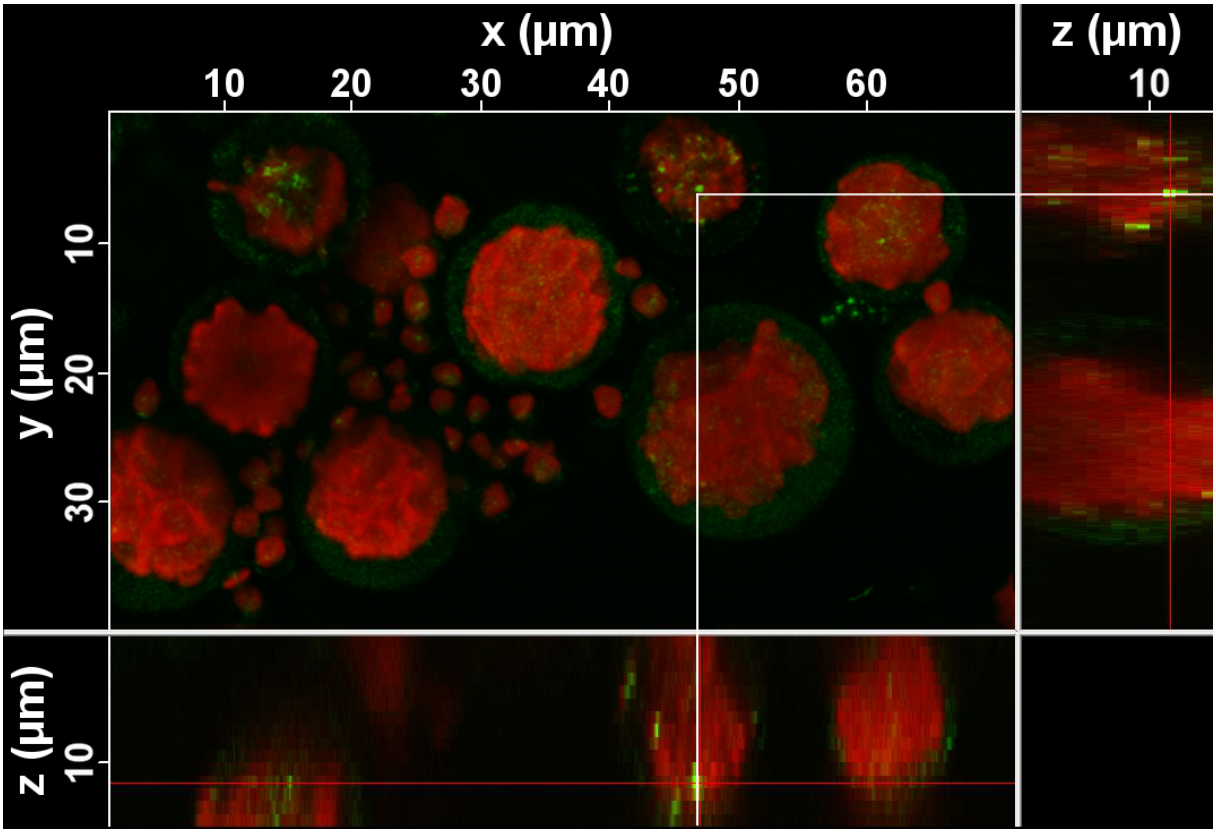


Figure 3. 3D reconstruction of cells of the green microalga *Trebouxia gelatinosa* observed with confocal laser scan microscopy in reflection mode after the exposure to GO. Red signal emitted by chlorophyll *a*; weak green signal reflected by algal cell walls; strong green signal reflected by GO.

Effects of GBMs and H_2O_2 on Chl_aF

F_v/F_m values were consistent in pre-treatment and control samples, suggesting that the shaking treatment did not affect cell viability. F_v/F_m of pre-treatment samples was 0.514±0.068, which slightly increased to 0.522±0.075 and 0.518±0.079 in control samples after 10 and 30 minutes (Fig 4), respectively. The samples exposed to H_2O_2 at concentrations higher than 0.05M had significantly lower F_v/F_m. At 0.5M and 0.8M, after 10 minutes it decreased to 80% (p-val = 0.00132) and 50% (p-val = 4.5e-06) of the control values, respectively, while after 30 minutes F_v/F_m further decreased to 50% (p-val = 6.3e-06) in the former and to 30% (p-val = 6.6e-09) in the latter. No significant differences were observed in samples exposed to FLG or GO with respect to control values.

Effects of GBMs and H_2O_2 on stress-related genes expression at transcript level

Samples exposed to GBMs for 10 minutes did not modify the expression level of any of the genes considered (Fig. 5), whereas after 30 minutes the exposure to FLG significantly affected the transcription level of a single gene, HSP70, which was reduced to 35% of the control value (Fig. 5).

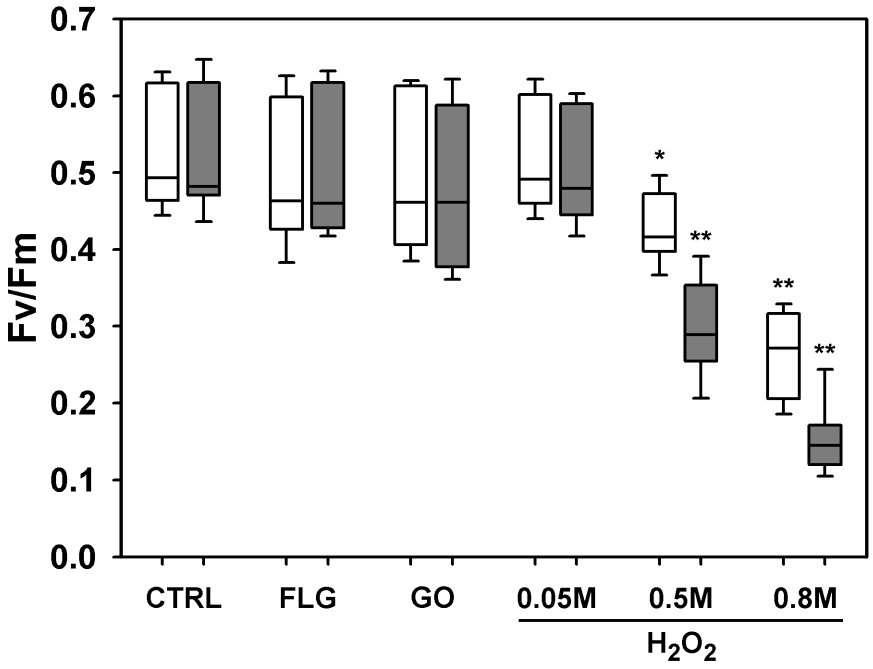


Figure 4. Maximum quantum yield of PSII photochemistry (F_v/F_m) measured in *Trebouxia gelatinosa* resuspended for 10 (a) and 30 (b) minutes in dH_2O (Ctrl), different H_2O_2 solutions (0.05M, 0.5M and 0.8M) and GBMs suspensions (FLG or GO; 50 μg ml^{-1}). * $p \leq 0.05$, ** $p \leq 0.01$, non-parametric Kruskal-Wallis test and non-paired Wilcoxon post-hoc test (n = 18).

Samples exposed to H_2O_2 had their APX and HSP70 transcripts levels significantly reduced after 10 minutes; the former transcript was reduced to ~35% of its control value by the highest H_2O_2 concentration whereas the latter was significantly reduced to 50% at 0.5M and to 15% at 0.8M H_2O_2. Transcripts levels of H_2O_2 treated samples further decreased after 30 minutes of exposure. Among antioxidant enzymes, APX transcription decreased to ~30% and 15% at 0.5 and 0.8M H_2O_2, respectively (Fig. 5). At the same concentrations, the transcription level of CAT was significantly reduced to 70% and 65% whereas that of GR remained steady independently of the H_2O_2 concentrations (Fig. 5). Differently, H_2O_2 had an inverse effect on MnSOD transcription levels, with the strongest decrease (down to ~25%) observed at the lowest H_2O_2 concentration. Among the stress related proteins, the transcription levels of DRP11 and HSC70 remained steady throughout the experiment. Those of HSP70, instead, had the most severe decrease among all the transcripts, i.e. the highest H_2O_2 concentration completely inhibited the transcription of HSP70, reducing it to 1%. Furthermore, LHCII transcript showed a significant dose-dependent reduction (to ~50% up to ~20%) in the expression.

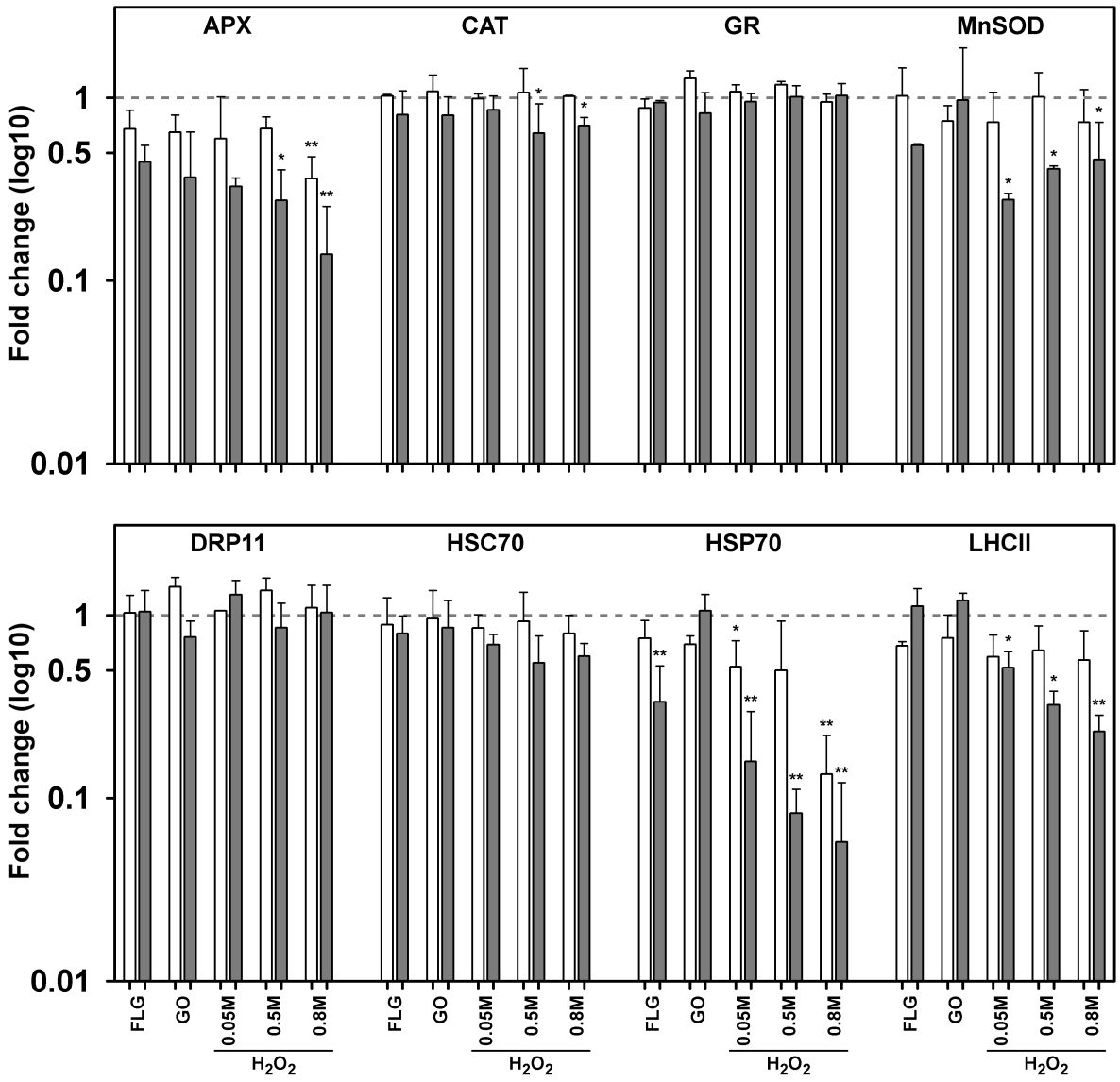


Figure 5. Fold change in the expression of 8 transcripts obtained with qRT-PCR in cultures of the green microalga *Trebouxia gelatinosa* treated with various concentrations of H_2O_2 and GBMs compared to the respective controls (blue line) after 10 (a) and 30 (b) minutes of exposure. *$p \leq 0.05$, **$p \leq 0.01$ (n = 3).

Effects of GBMs and H_2O_2 on HSP70 expression at protein level

HSP70 protein expression was not affected by any GBMs treatment, while a significant decrease in the expression was detected at the highest H_2O_2 concentration after both exposure times (Fig 6).

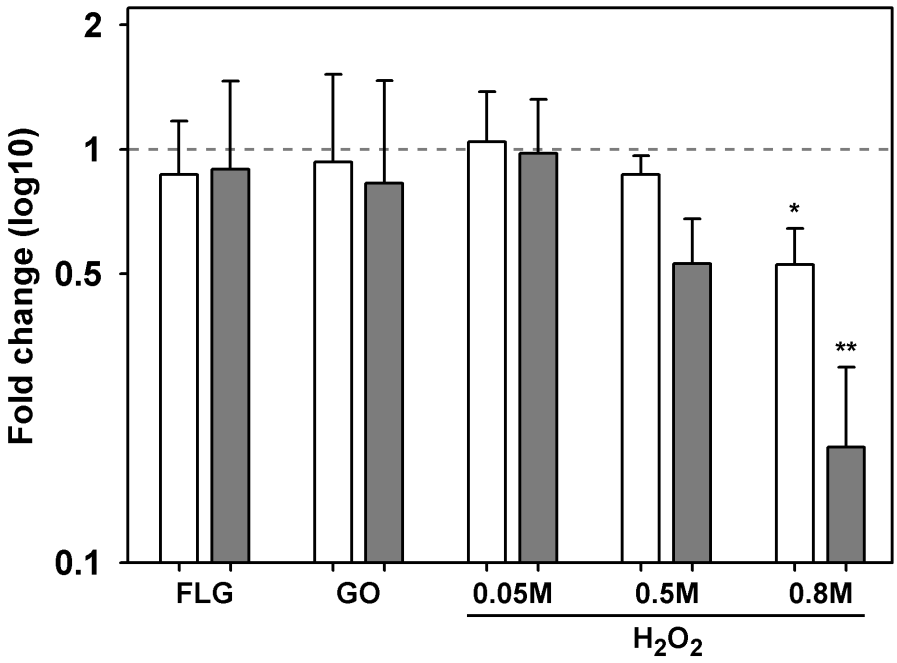


Figure 6. Levels of HSP70 protein in colonies of the green microalga *Trebouxia gelatinosa* exposed to various concentrations of H_2O_2 and GBMs compared to the respective controls after 10 (a) and 30 minutes (b) exposure. HSP70 bands of representative experiments after 10 (c) and 30 minutes (d) exposure. *$p \leq 0.05$, **$p \leq 0.01$ (n = 3).

Discussion

Internalization of GBMs is a controversial issue, depending both on the different characteristics of the material itself[40] and on the investigated organism. In our study, there are no clear evidences of GBMs internalization by the cells of *T. gelatinosa*, despite small FLG flakes were observed at the boundaries among cytoplasm – plasma membrane and plasma membrane – cell wall interfaces. The lateral size of these flakes ranges around 1 μm, which is close to the limit of precise measurability of GBMs flakes (0.5 μm) assessed with the CLSM approach used (Fig. 3). The natural light reflection observed at the cell wall level, especially with the settings needed for the observations of GO (Fig. 2b, 2d), might have prevented the detection of the light reflected by the smaller flakes. Moreover, the small brilliant spots observed in the autospores, which reflect light more intensely than the surrounding cell wall, might be misleadingly recognized as GBMs flakes (Fig. 3). They are provisionally identified here, on the basis of observations in transmitted light microscopy, as the nucleolus. However, considering that both the GBMs batches tested in this study are made of graphene flakes with lateral dimensions that can be as small as 100 nm for FLG and 50 nm for GO (Fig. 1), it is possible that only the fraction of the graphene with the smallest sizes reached and/or crossed the plasma membrane.

This hypothesis is supported by the observation of the downregulation of the HSP70 transcript (Fig. 5), which is interestingly the unique significant change at gene expression level, induced by a 30 minutes FLG exposure. HSP70 proteins can be regulated by different cellular pathways.[41] We hypothesize that the presence of nanoparticles at the interface between plasma membrane and cell wall could activate plasma membrane receptors that, through cellular pathways, lead to changes in the HSP70 expression. One of these pathways can involve the intracellular changes in calcium ions (Ca^{2+}): the induction of Ca^{2+}/calmodulin (CaM) genes followed by transcriptional changes of different HSPs, including HSP70, were already recorded in plants.[42,43] CaM genes can be regulated during abiotic stress by the activation of plasma membrane receptors like the cyclic nucleotide gated channels (CNGCs),[41] or by others, still unidentified,[42] that could respond to the interaction with GBMs at plasma membrane level. Feng and coworkers (2015) found that the depolarizing effect of graphene on voltage-gated Ca^{2+} channels increased calcium concentration inside human neurons, due to the activation of calmodulin kinase pathways.[44] The reason why HSP70 was downregulated is still unknown since HSP70 are considered stress-inducible proteins.[45,46] As chaperones, they are important in protein stabilization, folding, assembly, translocation and degradation especially under stress conditions[46] such as heat, drought, salinity, acidity, and cold.[47] Generally, the overexpression of HSP70 genes results in enhanced tolerance to abiotic stress in plants.[46,48] Interestingly, a downregulation of HSP70 at protein level was instead recorded after cumene hydroperoxide (CuHP) exposure in another member of the genus *Trebouxia*, the lichen photobiont TR1, while in TR9 (an alternative photobiont of the same lichen species, *Ramalina farinacea*), the oxidative treatment caused a strong increase in the amounts of this protein.[49] In our case, instead, the downregulation of HSP70 gene expression did not correspond to a decrease in the quantity of the protein itself (Fig. 6); low correlations between mRNA abundance and protein level are common,[50] and can are usually attributed to post-transcriptional regulation.[51]

Some authors demonstrated that internalization of GBMs in general is linked to the production of ROS[11] or to the increase of oxidative stress,[28] which eventually led to cell death. In our experimental design, we investigated the expression changes of some gene of interest, among which antioxidant enzymes and proteins related to stress. Gene expression changes in general are a major component of stress responses, which in some cases are activate by intracellular signaling pathways. Interestingly, the control of gene expression has fast response kinetics, even within minutes in the presence of stress, and is able to return to basal state after the removal of the stress.[52] The potential changes in gene expression after GBMs exposure were here compared to the ones induced by H_2O_2 treatments, a substance which is known to induce reactive oxygen species production.[9] From the gene expression analysis of antioxidant enzymes (Fig. 5), it can be concluded that these are not involved

in the response to GBMs exposure. Interestingly, *Trebouxia* species are known to be oxidative stress tolerant because they own a strong constitutive antioxidant machinery which is able to scavenge an "oxidative burst" in minutes after his potential insurgence.[35] However, H_2O_2 treatments produced dose- and time-dependent oxidative effects, evidenced by significant changes in the transcripts level of APX, CAT, and MnSOD. This response is in agreement with Chl_aF measurements (Fig. 4), which showed a dose- and time- dependent decrease in F_v/F_m parameter and therefore a corresponding algal vitality decrease after H_2O_2 treatments, while algal vitality after GBMs exposures was not impaired.

Studies focused on microalgae reported GBMs internalization, in particular in *Chlorella pyrenoidosa* and *C. vulgaris*.[26-28] Comparing these species with *T. gelatinosa*, the main differences are thickness and composition of the cell walls. In *T. gelatinosa* the cell wall can be 1 μm thick,[53] while that of both *Chlorella* species is only c. 20 nm thick.[54,55] In comparison, the latter is therefore considerably thinner, and represents a weaker barrier against GBMs, which may easily be internalized, also because the α-cellulose microfibrils are present as an irregular network over the cell wall, lying approximately in two directions at right angles to one another,[54] although a wide variability has been documented in the cell wall composition of the members of the genus *Chlorella*.[56] The cell wall of *T. gelatinosa*, on the contrary, is relatively stable in composition, and highly differentiated.[57] It consists of five different layers, mostly composed by highly packed cellulosic fibrils, non-cellulosic species-specific polysaccharides and a three-dimensional web of sporopollenin, which is considered important because of its high resistance and high chemical stability. Moreover, *T. gelatinosa* develops a gelatinous sheath 1.5 – 2 μm thick outside the cell wall,[53] which is sticky and may support a close contact with GBMs.

Hence, all these evidences reported herewith allow us to hypothesize that a harmless interaction between FLG and the outermost layer of the plasma membrane may occur after 30 minutes of exposure. At this moment, we do not known, however, which pathway is involved in the reaction which led to the downregulation of the HSP70 gene expression, caused interestingly only by FLG. GO, in respect to FLG, is considered to be more toxic, but no generalizations can be made because of the contradictory results available in the literature.[5] In general, however, GO is more stable in suspension thanks to the carboxylate groups on the periphery, which make the sheets more hydrophilic, and probably also more prone to stick on the outer gelatinous sheath of *T. gelatinosa*, which consists of species-specific carbohydrates and proteins, including uronic acid.[58]

Materials and methods

GBMs preparation and characterization

Two types of GBMs were used in this study, few-layers graphene (FLG) and graphene oxide (GO). FLG was prepared by ball-milling treatment, according to previous published procedures.[59] In general, a mixture of graphite and melamine (1,3,5-Triazine-2,4,6-triamine) (7.5 mg of SP-1 graphite powder, purchased from Bay Carbon, Inc. (USA), and 22.5 mg of melamine purchased from Sigma-Aldrich) was ball-milled at 100 rpm for 30 minutes using a Retsch PM 100 (Retsch Technology GmbH, D) planetary mill under air atmosphere. The resulting solid mixture was dispersed in 20 mL of water and sonicated for 1 minute to produce a dark suspension. Melamine was afterwards eliminated by dialysis. The precipitate, consisting in poorly exfoliated graphene, was removed from the liquid fraction after stabilization for 5 days. The FLG water dispersions were lyophilized and the final graphene powder was thoroughly characterized.

GO was purchased from Grupo Antolin Ingeniería (Burgos, S), which produced it by oxidation of carbon fibers (GANF Helical-Ribbon Carbon Nanofibres, GANF®) and sodium nitrate in sulfuric acid at 0°C.

TGA of FLG and GO were performed with a TGA Q50 (TA Instruments, USA) at 10 ºC per minute under nitrogen atmosphere, from 100ºC to 800ºC. In addition, the dispersions of both GBMs were drop-cast onto a Si wafer and dried on a hot plate in order to study the Raman spectra. At least 30 Raman measurements on both materials were collected in different locations using a inVia Reflex Microscope (Renishaw plc, UK) at 532 nm with a 100x objective and an incident power of 1% (1 mW μm^{-2}). Quantitative elemental analyses on FLG and GO were then performed with a LECO CHNS-932 (LECO Corporation, USA) elemental analyzer. Lateral dimension distribution of GBMs was calculated by using Fiji software. GBMs were studied by TEM. Stable dispersions of both materials were drop-cast on nickel grids (3.00 mm, 200 mesh) and dried under vacuum. The samples were studied by a JEM 2100 (JEOL Ltd, JP) high-resolution transmission electron microscope (HRTEM) at an accelerating voltage of 200 kV.

Cultures of Trebouxia *photobiont*

Trebouxia gelatinosa Archibald was isolated following Yamamoto *et al*. (2002)[60] from thalli of *Flavoparmelia caperata* (L.) Hale collected in the Classical Karst plateau (NW Italy). The algal cultures were subcultured on solid Trebouxia Medium (TM; 1.5% agar)[61] every 30-45 days and kept in a thermostatic chamber at 18±1 °C and 20±2 μmol photons m^{-2} s^{-1} with a light/dark regime of 14/10

hours. Reference algal material has been cryoconserved according to Dahmen *et al*. (1983)[62] and is available upon request.

Oxidative stress treatment

Algal cells of *T. gelatinosa* from 4 week-old colonies were resuspended in distilled water and gently pressed with a syringe through a filter net (mesh size = 40 μm). This procedure was used to disaggregate the clusters of cells in order to obtain a homogeneously dispersed algal suspension. Thirteen 1.5 mL Eppendorf tubes (with pierced lids) were filled with algal suspension (samples); one was left untreated representing the pre-treatment whereas the other twelve were spin-centrifuged to separate the cells from the supernatant, of which 1.3 ml were discarded. Thereafter, the treatments were conducted in the dark to induce the complete oxidation of the reaction centers, that allows to measure the maximum quantum efficiency of PSII immediately after the treatments. To the samples were then added, two by two, 1.3 ml of: distilled water (controls), distilled water plus H_2O_2 to reach the final concentrations of 0.05M, 0.5M, 0.8M, and distilled water plus aqueous suspensions of GBMs (FLG or GO) to reach the final concentration of 50 μg mL^{-1}. The samples were then placed on a shaker and one sample of each couple per treatment was taken for the analyses after 10 and 30 minutes, respectively. The procedure was repeated six times and for each repetition three biological replicates were processed.

In-vivo *GBMs internalization assessment*

A sub-aliquot (10 μL) of the algal suspension for each treatment was put on Polysine™ Microscope Adhesion Slides (Thermo Fisher Scientific, USA) immediately after the GBMs treatments, covered with coverslips and then observed *in-vivo* with a CLSM Nikon C1-si (Nikon, JP). To visualize GBMs flakes, the microscope was used in reflection mode: samples were illuminated with a 514 nm laser set at an intensity of 0.2% and 0.5% for the observation of FLG and GO, respectively, since they have different light reflection capacity, higher in the former than in the latter. Light reflected by GBMs was detected by a 525/50 band pass filter. Algal cells were visualized by illuminating samples with a 488 nm laser (12% intensity) and acquiring the autofluorescence of chlorophylls with a 650 long pass filter ($\lambda > 650$ nm). One to three fields were acquired for each replicate (n = 4). A variable number of focal planes (stacks), depending on the algal abundance and the dimension of the GBMs flakes were acquired for each field. In total, more than 1300 cells were analyzed. Acquisitions were elaborated with the Nikon EZ-C1 FreeViewer software (Nikon, JP) and with the freeware suite ImageJ 1.46r (NIH, USA). A unification algorithm (Z-projection) was applied

to merge stacks into bi-dimensional images, and 3D reconstruction were obtained by using the ImageJ 3D viewer plugin.

Chlorophyll* a *fluorescence (Chl_aF) measurements

After the treatments, each sample was collected by vacuum filtration on a cellulose acetate membrane (25 mm diameter, pore size 0.45 µm, Sartorius Lab Holding GmbH, D) and measurements of chlorophyll *a* fluorescence (Chl_aF) emission were taken with a photosynthetic efficiency analyzer fluorimeter Handy-PEA (Hansatech, UK). A modified clip was positioned right over the sample on the membrane. A saturating red light pulse of 1,500 µmol photons m^{-2} s^{-1} for 1 s was emitted to obtain the Kautsky induction and thus F_m (transient maximum Chl_aF level). F_0 (minimal Chl_aF level), which is needed to calculate F_v (variable Chl_aF level, i.e. $F_m - F_0$) and thus F_v/F_m (maximum quantum efficiency of PSII photochemistry), was calculated *a posteriori* by an algorithm that determines a line of best fit through the data points recorded immediately after the start of illumination. Afterwards, each sample was put inside an Eppendorf tube, soaked in liquid nitrogen and stored at -80°C for downstream applications.

RNA isolation and cDNA synthesis

Three replicates per treatment from three distinct experiments were randomly selected and pooled together for three times to obtain three samples for RNA extraction. PowerPlant® RNA Isolation Kit (MO BIO Laboratories Inc., USA) was used to extract total RNA. RNA quality was verified with NanoDrop® 2000 (Thermo Fisher Scientific, USA), followed by a denaturing 1% agarose gel. cDNA was synthesized using IScript cDNA synthesis kit (Bio-Rad, USA).

qRT-PCR

The expression of eight different transcripts, four coding for antioxidant enzymes and four for stress-related proteins was measured by quantitative Real-Time PCR (qRT-PCR). The former were ascorbate peroxidase (APX), catalase (CAT), gluthatione reductase (GR), and manganese superoxide dismutase (Mn-SOD), the latter were desiccation related protein 11 (DRP11), molecular chaperones Heat Shock Cognate 70 (HSC70) and Heat Shock Protein 70 (HSP70), and the chlorophyll a-b binding protein of the light harvesting complex II (LHCII). Primers were designed with Primer3Plus[63] (Table S1) or following Candotto Carniel et al. (2016).[35] Each reaction was performed in three technical replicates in a mix containing 1 µL cDNA (1:10 template dilution), 8 µL SSOAdvanced™ SYBR® Green Supermix (Bio-Rad, USA) and 200 nM of each primer. The PCR amplifications were performed with CFX 96™ Real-Time PCR System (Bio-Rad, USA) using the following cycle: 98 °C

for 30' and 40 cycles at 95 °C for 10' and 60 °C for 20'. A melting curve analysis (65 °C to 95 °C increment 0.5 °C for 5') was performed to verify the absence of non-specific amplification products. Transcript levels were calculated with Bio-Rad CFX Manager software (Bio-Rad, USA), based on the comparative Ct method ($2^{-\Delta\Delta}$ Ct method)[64] and gene expression data were normalized using as housekeeping gene the ribosomal protein L6 (RPL6).[35]

Proteins isolation

Three pooled samples of *T. gelatinosa* frozen cultures prepared as mentioned in the "*RNA isolation and cDNA synthesis*" section, were pulverized in liquid nitrogen, transferred in 1.5 ml Eppendorf tubes and resuspended in 100 µl of 1× Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.2M dithiothreitol (DTT) and 0.1% (w/v) bromophenol blue)[65]. Samples were then vortexed and incubated at 95 °C for 5 minutes. After a 3 minutes centrifugation at 14000 r.p.m., protein extracts were recovered from the upper phase of the tube and transferred in a new Eppendorf. When not immediately used for analysis, the samples were stored at -20°C and incubated 5 minutes at 95 °C before loading on the gel. To check quality and quantity of the total proteins extracted, 12% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970).[65] The gel was stained with Coomassie brilliant blue R250.[66]

HSP70 immunodetection

To perform 12% SDS-PAGE, 15 µg of proteins were used. Proteins were then transferred on a Hybond™ nitrocellulose membrane (Amersham, UK) using the Criterion™ blotter apparatus (Bio-Rad, USA) as explained in Dinakar and Bartels (2012).[67] Transfer was obtained after 1 h at 70 V with pre-chilled buffer. Before immunodetection, the membrane was stained for 30 minutes with Ponceau S red to visualize the samples and check their equal amount. 4 °C overnight incubation with blocking solution [3% (w/v) skimmed milk in Tris-buffered saline] was performed to prevent unspecific binding of antibodies. The membranes were incubated for 1 hour with HSP70/HSC70 primary antibody (1:1000 dilution)[68], and for 45 minutes with secondary antibody (anti-rabbit IgG-peroxidase, 1:5000 dilution, Sigma-Aldrich, USA). Antigen-antibody complexes were detected with the ECL kit (Amersham, UK) and a lumi-imager (LAS 1000, Fujifilm, JP). Densitometry of protein bands was with Image J software 1.37 V (National Institute of Health, USA).

Statistics

Descriptive statistics were performed with Microsoft Office Excel 2010 (Microsoft Corporation, USA) and R version 0.99.441 (The R Foundation for Statistical Computing). The non-parametric Kruskal-Wallis test and Wilcoxon non-paired test were applied to verify the significance of differences for Chl_aF measurements.[69,70] A one-way ANOVA followed by a Fisher's LSD post-hoc test was applied to verify significant differences between the relative abundancy of transcripts and HSP70 protein content in treated versus control samples. Figures were produced with Sigmaplot 10.0 (Systat Software, USA).

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Supplementary Materials

Table S1. Primers custom designed for quantitative Real-Time PCR analysis.

Gene	Primer ID	Forward sequence	Reverse sequence
Ascorbate peroxidase	APX	CAGGGTTCACAAGGACAGGT	TCAGCAAACAGGCACTCATC
Glutathione reductase	GR	TTCGAACAGCAGACATCGAC	CCTCCAGTCTTTTCGTCAGC
Mn-superoxide dismutase	MnSOD	CACCCAGCTTGCTGACTACA	GGTCAAACTGTGCCTGGAAT
Catalase	CAT	ACTACTTCCCATCCCGCTTT	CCTGGTGATGAACCTGTCCT
Light Harvesting Complex II	LHCII	CTGATGACCCAGATGCCTTT	GGTCCTTTGCCTGTCACAAT
Desiccation Related Protein 11	DPR11	CATATGGCGAGGGTATTGCT	TGTGCGATTTCATTCTCAGC
Heat Shock Protein 70	HSP70	CAGTCACCACTGCCTTCTCA	CAAGTCAGCCAATGCAAAGA
Heat Shock Cognate 70	HSC70	AGGAGCAGACCTTCTCCACA	GACCACAATTTGGGGAACAC
Ribosomal protein L6	RPL6	AGGAGCTAGCTAGGGGCATC	TCTCGTGCTTTGGGAACTCT

Conclusions

The main aim of the present PhD project was to assess the ecotoxicological effects of GBMs, with a particular focus on aeroterrestrial green microalgae.

Four aeroterrestrial green microalgal species (*Apatococcus lobatus, Chlorella vulgaris, Coccomyxa subellipsoidea, Trebouxia gelatinosa*) were subjected to short- and long-term exposures to two GBMs, FLG (few-layers graphene) and GO (graphene oxide). Different approaches have been applied to determine the effects of GBMs.

Short-term effects after the exposure to FLG and GO at the concentration of 50 μg mL^{-1} were evaluated in terms of: *i)* chlorophyll *a* fluorescence measurements, modifications of the expression of selected genes through quantitative Real-Time PCR and HSP70 immunodetection with Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (10' and 30'); *ii)* membrane permeability, with the quantification of potassium leakage (30' and 60').

Long-term (up to 4 weeks) effects after the exposure to FLG and GO at the concentrations of 0.01, 1, 50 μg mL^{-1} were evaluated through chlorophyll *a* fluorescence measurements and quantifications of the total photosynthetic pigments content.

Furthermore, GBMs internalization was analyzed with confocal laser scan microscopy, but no GBMs were clearly detected inside the cells, despite they were found in close contact with algal cells. According to the final results obtained, no negative effects were observed for either FLG or GO, in both short- and long-term exposures. However, a harmless interaction between FLG and algal cells occurred at cell wall - plasma membrane level, involving potentially a yet unknown signaling pathway.

On the basis of the researches here presented, aeroterrestrial green microalgae proved to be interesting and useful organisms to study the ecotoxicity of GBMs, due to their ubiquitous nature and their easy handling in laboratory procedures. The experimental designs were accurately standardized to assure reproducibility and reliability of the results. This was accompanied by an accurate production procedure of the GBMs and their characterization, which led to an overall complete knowledge of GBMs behavior towards the studied species of aeroterrestrial microalgae.

Therefore, the examined GBMs are considered in this framework as safe, at least for the ecotoxicological point of view. However, this statement must not be generalized and not applied to all the organisms, especially if pertaining to different compartments of the ecosystems which are still too less studied.

For the future, there is the necessity to *i)* study more ecologically relevant organisms and not only model species; *ii)* study different GBMs and different concentrations, their interaction between

them and with other substances or pollutants environmentally available; *iii)* use different and new approaches, including studies in the field as well; *iv)* standardize the production procedures and characterize accurately the nanomaterials.

Appendix

Other papers published between 2014 and 2016

Candotto Carniel F., Gerdol M., **Montagner** A., Banchi E., De Moro G., Manfrin C., Muggia L., Pallavicini A., Tretiach M. **2016**. New features of desiccation tolerance in the lichen photobiont *Trebouxia gelatinosa* are revealed by a transcriptomic approach. Plant Mol. Biol. 91: 319-339

Banchi E., **Montagner** A. **2016**. Lichens and the omics. Not. Soc. Lich. Ital. 29: 66-75

Conferences and workshops contributions

Montagner A., Candotto Carniel F., Gerdol M., Banchi E., Manfrin C., Muggia L., Pallavicini A., Tretiach M. Transcriptomic analysis of the lichen photobiont *Trebouxia gelatinosa* subjected to dehydration and rehydration processes. 1st Meeting of the *Trebouxia-Working* group. Trieste, 26-28 September **2016**. (oral communication)

Montagner A., Candotto Carniel F., Gerdol M., Banchi E., Manfrin C., Muggia L., Pallavicini A., Tretiach M. Transcriptomic analysis of the lichen photobiont *Trebouxia gelatinosa* subjected to dehydration and rehydration processes. 8th Symposium of the International Association for Lichenology. Helsinki, Finland, 1-5 August **2016**. Abstract Book, p. 79. (oral communication)

Banchi E., Gerdol M., **Montagner** A., Candotto Carniel F., Muggia L., Pallavicini A., Tretiach M. Potential horizontal gene transfer (HGT) of Desiccation Related Proteins in the lichen photobiont *Trebouxia gelatinosa*. 8th Symposium of the International Association for Lichenology. Helsinki, Finland, 1-5 August **2016**. Abstract Book, p. 92. (poster presentation)

Montagner A., Bidussi M., Bosi S., León V., Vázquez E., Tretiach M., Prato M. Ecotoxicity of graphene-based nanomaterials on aeroterrestrial microalgae. Graphene Week 2016 (From ideas and pioneering experiments towards new technologies and innovation industry). Warsaw, Poland, 13-17 June **2016**. Book of Abstract, p. 82. (oral communication)

Banchi E., Gerdol M., **Montagner** A., Candotto Carniel F., Muggia L., Pallavicini A., Tretiach M. The desiccation-related proteins in *Trebouxia*: a family to discover. XXVIII Convegno annuale della Società Lichenologica Italiana, Lanciano, 9-11 settembre **2015**, Riassunti (a cura di Caporale S.), Notiziario della Società Lichenologica Italiana 28: 34. (poster presentation)

Bidussi M., **Montagner** A., Bosi S., León V., Vázquez E., Tretiach M., Maurizio Prato M. Ecotoxicity of graphene-based nanomaterials on aeroterrestrial microalgae. nano2015, 10th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Wien, Austria, 6-10 September **2015**, Abstract Program Guide, p. 67. (oral communication)

Petruzzellis F., Cauduro F., Bertuzzi S., Savi T., Craighero T., **Montagner** A., Muggia L., Tønsberg T., Tretiach M., Nardini A. Water relations in lichens: a case study with the tripartite *Peltigera britannica* and its isolated photobionts. 8th Congress of the International Symbiosis Society, Lisbon, Portugal, 12-18 July **2015**. (poster presentation)

Montagner A., Bidussi M., Bosi S., León V., Vázquez E., Tretiach M., Prato M. Ecotoxicity of graphene-based materials on aeroterrestrial microalgae. SETAC Europe 25th Annual Meeting, Barcelona, Spain, 3-7 May **2015**, Abstracts Book, p. 441. (poster presentation)

Montagner A., León V., Bosi S., Vázquez E., Tretiach M., Prato M. Evaluation of graphene-based materials effects on the coccoid green alga *Trebouxia gelatinosa* Archibald. Graphene Week 2014 (Science, technology and emerging applications of Graphene). Gothenburg, Sweden, 23-27 June **2014**, Abstracts Book, p. 209. (poster presentation)

Montagner A., Gerdol M., Candotto Carniel F., De Moro G., Manfrin C., Muggia L., Pallavicini A., Tretiach M. Transcriptomic analysis of the lichen-forming alga *Trebouxia gelatinosa* subjected to dehydration and rehydration processes. New Frontiers in Anhydrobiosis. Pornichet, France, 23-27 March **2014**, Book of Abstracts, p. 78. (poster presentation)

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