Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2018.22476

117 (2018) 249–256
June

Effects of environmental factors on graphene oxide ecotoxicity towards crustacean *Daphnia magna*

Radosław Kalinowski[a,*], Beata Tomczyk[a], Magdalena Trzcińska[a], Radosław Walkowiak[a], Marcin Kaźmierczuk[a], Szymon Paczkowski[a], Barbara Gworek[a], Michał Woluntarski[b]

[a]*Institute of Environmental Protection—National Research Institute, 5/11D Krucza Street, 00-548 Warsaw, Poland, Tel. +48 22-3750635; emails: radoslaw.kalinowski@ios.edu.pl (R. Kalinowski), beata.tomczyk@ios.edu.pl (B. Tomczyk), magdalena.trzcinska@ios.edu.pl (M. Trzcińska), radoslaw.walkowiak@ios.edu.pl (R. Walkowiak), marcin.kazmierczuk@ios.edu.pl (M. Kaźmierczuk), szymon.paczkowski@ios.edu.pl (S. Paczkowski), barbara.gworek@ios.edu.pl (B. Gworek)*
[b]*Institute of Electronic Materials Technology, 133 Wólczyńska Str., 01-919 Warsaw, Poland, Tel. +4822-6395827; email: michal.woluntarski@itme.edu.pl (M. Woluntarski)*

Received 20 December 2017; Accepted 4 May 2018

ABSTRACT

Growing popularity of graphene-based nanomaterials raises awareness about its environmental behavior and impact. Literature provides evidence on the influence of environmental factors on nanomaterials ecotoxicity. The purpose of this study was to evaluate the different medium composition and lighting effects on the acute ecotoxicity of graphene oxide on crustacean *Daphnia magna*. Standard Organisation for Economic Co-operation and Development 202 methodology [5] was used. Experiments were performed with four different artificial waters according to American Society for Testing and Materials (very soft, soft, hard, and very hard) and under two different lighting regimes—illuminated and in darkness. An increase of the toxic effect with incubation time was observed. Effective concentrations EC50 after 48 h of incubation were nearly two times lower than that after 24 h. It was found that in media with lower hardness the toxicity of graphene oxide increases when compared with harder water. Calculated EC50-48 h values were 159.17 and 167.79 mg/L in very soft water and 500.40 and 470.89 mg/L in very hard water, in illuminated and non-illuminated assays, respectively. No significant differences were found when comparing illuminated and non-illuminated variants of the experiment.

Keywords: Graphene oxide; *Daphnia magna*; Ecotoxicity

1. Introduction

Nanomaterials (NMs) are defined as a natural, incidental, or manufactured materials containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range of 1–100 nm. The huge increase of NMs utilization alongside the propagation of nanotechnology as a scientific discipline that occurred in last decades has led to the formulation of different sizes, shapes, and compositions of NMs [1]. Among all NMs, carbon-based nanomaterials (CNMs) are one of the groups widely developed due to their valuable properties. Apart from carbon nanotubes and fullerenes, graphene and graphene oxide (GO) are the most popular forms of NMs in diverse CNMs group. The whole family of graphene NMs has unique electric, magnetic, and mechanical properties that cause an increase of fields of application of this NM. Knowledge is still limited about the ecotoxic properties of nanoparticles and the ways of their fate

* Corresponding author.

Presented at the 13th Conference on Microcontaminants in Human Environment, 4–6 December, Czestochowa, Poland, 2017.

1944-3994/1944-3986 © 2018 Desalination Publications. All rights reserved.

and behavior [2,3]. Our previous experiments indicated that graphene oxide stability depends on the composition of the ecotoxicity test medium [4]. It was found that the graphene oxide was the most stable in a very soft medium (pH, 8.14; ionic power, 0.86 mM/L; conductivity, 43.55 μS/cm; and total hardness as $CaCO_3$, 11.2 mg/L) and the least stable in the hardest medium (pH, 7.94; ionic power, 10.84 mM/L; conductivity, 671.5 μS/cm; and total hardness as $CaCO_3$, 250.3 mg/L). Therefore, a new question raised is if graphene oxide behavior in test media alters its ecotoxicity.

2. Materials and methods

2.1. Graphene oxide

The experiments were carried out using 2.0 g/L suspension of graphene oxide Delta-1 (Department of Chemical Technologies, Institute of Electronic Materials Technology, Warsaw, Poland). Graphene oxide was obtained from natural flake graphite by modified Hummers method. The reaction mixture contained sulfuric acid of 95%, phosphoric acid of 65%, and graphite. Potassium permanganate was used as an oxidizer. Reagents were stirred at a temperature of 50°C for 10 h. The reaction was stopped by dilution of a mixture with deionized water. In the next step, hydrogen peroxide was added. This reagent facilitates further the purification of graphene oxide that was conducted using a microfiltration device. The obtained material is a water suspension of graphene oxide flake. The measured thickness of the flake indicates the presence of one to three atomic layers. The lateral size of flakes is in the range of 2–6 μm. The main ingredients of dried suspension are as follows: carbon (40%–42%), oxygen (45%–52%), sulfur (1%–3%), nitrogen (<0.3%), and hydrogen (2.5%–3%). Due to their chemical nature, graphene oxide is stable in water suspension. This behavior results from the presence of many chemical groups including carbonyl, epoxyl, and hydroxyl. Scanning electron microscope photography of graphene flakes used in the experiments is presented in Fig. 1.

2.2. Bioassay

The ecotoxicity tests were conducted with the freshwater crustacean *Daphnia magna* (clone A, originated from INERIS, France). Daphnids are sensitive bioindicators commonly used in ecotoxicity evaluation. Standard test methodologies (including toxkit type) of this acute crustacean assay are implemented all around the world, and dedicated ISO and Organisation for Economic Co-operation and Development (OECD) test guidelines are available. An immobilization test with *Daphnia* is one of three needed for basic substance classification and is a part of the so-called "base test battery." Organisms are successfully cultured at the Department of Ecotoxicology of the Institute of Environmental Protection—National Research Institute since 2014. The culture is continuously maintained at 20 ± 2°C, with 1,000–1,500 lx illumination (16:8 light:dark) in Elendt M4 medium. Organisms are fed daily with green algae (*Chlorella vulgaris*) in suspension at a rate of 0.1–0.2 mg C/organism. Sensitivity and reproduction efficacy of crustaceans are regularly verified in toxicity tests with reference substances.

Acute immobilization assay was conducted according to the OECD 202 method [5] with a reduced number of replicates (two instead of four). A total of 20 neonates (<24 h) of test organisms were introduced into the test vessels containing graphene oxide solutions in four different reconstituted test medium according to American Society for Testing and Materials (ASTM) (very soft water, soft water, hard water, and very hard water) [6]. Test mediums were aerated overnight to maintain a sufficient amount of dissolved oxygen in solutions during the incubation period. No aeration was provided during the exposure. The composition and measured parameters of each medium are given in Table 1.

Geometric series of six dilutions with a dilution factor equal to 2 were prepared starting from a graphene oxide concentration 1,000 mg/L. Test tubes were incubated for 48 h at 20 ± 1°C. Two separate bioassays were prepared

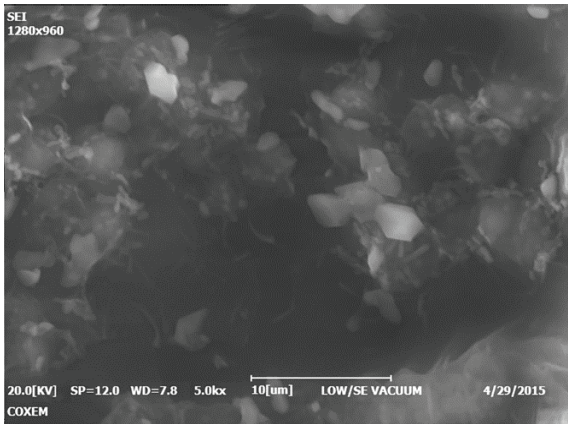

Fig. 1. Graphene oxide Delta-1 sheets from scanning electron microscope.
Source: Courtesy of LabSoft (Warsaw, Poland).

Table 1
Composition and basic chemical parameters of artificial media used in the bioassay

Medium		Very soft water	Soft water	Hard water	Very hard water
Composition (mg/L)	$NaHCO_3$	12	48.0	192	384
	$CaSO_4 \cdot 2H_2O$	7.5	30	120	240
	$MgSO_4$	7.5	30	120	240
	KCl	0.5	2	8	16
pH		7.95	8.02	8.34	8.48
Conductivity (μS/cm)		84.0	165.0	562.9	935.3
Total hardness (mg $CaCO_3$/L)		28.2	45.2	165.6	259.7

for incubation in light (continuous cool white fluorescence illumination with intensity 1,500 lx) and in darkness. Immobilization of neonates was defined according to OECD 202 [5], that is, those animals that are not able to swim within 15 s after gentle agitation of the test vessel are considered to be immobilized (even if they can still move their antennae). Immobile daphnids were counted after 24 and 48 h.

2.3. Statistical analysis

Bioassays were evaluated using ToxRat Professional version 3.2.1 (ToxRat Solutions GmbH, Alsdorf, Germany). Effective concentrations (EC50-*t*) values were calculated by Weibull analysis using linear maximum likelihood regression, and no observable effective concentrations (NOEC-*t*) and lowest observable effective concentrations (LOEC-*t*) values were determined by the step-down Cochran–Armitage test procedure.

3. Results

Different testing media imitating different natural waters were used in the acute toxicity bioassay with *D. magna*. Detailed immobility of crustacean at each test combination as well as dose response curves is presented in supplementary materials – Appendix A and Appendix B, respectively. It was found that graphene oxide ecotoxicity varies among different test mediums.

The highest toxicity was observed in softer waters compared with hard waters. The 48 h EC50 values ranged from 159.17 (very soft water) to 500.40 mg/L (very hard water) and from 164.79 to 470.89 mg/L in darkness and in the continuous illumination, respectively (Table 2). The time-dependent increase of toxic response of crustaceans was found. In extreme case (illumination and very hard water), the EC50 after 48 h was nearly two times lower than that after 24 h.

No statistically significant differences between EC50s obtained in bioassays conducted in complete darkness and continuous fluorescent illumination were found. It was found that NOECs and LOECs in hard and very hard water were lower in an experiment conducted under continuous illumination (Table 3).

4. Discussion

Growing interests of NMs (especially belonging to the graphene family) in different applications including removal of toxic pollutants from wastewater, biomedical usage, etc. [7–9] will definitely lead to an increase in its environmental emissions. Apart from that NMs are widely used and there is still a huge gap in the knowledge of their ecotoxicity to living organisms. Recent papers on GO ecotoxicity report similar endpoint values for daphnids to those presented in this paper. Liu et al. [10] estimated EC50-48 h for graphene oxide at 150.75 (±19.37) in the simplified Elendt M7 medium. Slightly longer exposure of daphnids (72 h) in experiments conducted by Lv et al. [11] decreased EC50 values to the level of 45.4 mg/L. Another commonly used crustacean species in ecotoxicity tests is *Ceriodaphnia dubia* which was exposed by Souza et al. [12] to GO solutions in soft reconstituted water (hardness 40–48 mg of $CaCO_3$/L). EC50-48 h values in this experiment were estimated on a level of 1.25 mg/L. Studies

Table 2
Graphene oxide EC50 values and its 95% confidence intervals (mg/L) for *Daphnia magna*

Lighting	Very soft water	Soft water	Hard water	Very hard water
24 h				
Darkness	164.79	354.67	565.68	631.82
	136.95–193.03	313.85–395.52	490.95–888.88	496.74–802.23
Illumination	186.14	344.57	515.19	841.74
	166.38–205.44	303.20–386.80	417.02–617.60	595.72–1468.61
48 h				
Darkness	159.17	241.28	500.45	500.40
	132.47–187.96	203.40–298.21	407.22–600.81	391.52–623.57
Illumination	164.79	344.57	416.77	470.89
	136.95–193.03	303.20–386.80	329.79–511.51	369.18–583.42

EC, effective concentrations.

Table 3
Graphene oxide NOEC and LOEC values (mg/L) for *Daphnia magna*

Lighting	Endpoint	Very soft water	Soft water	Hard water	Very hard water
Darkness	LOEC-48 h	125.00	250.00	250.00	250.00
	NOEC-48 h	62.50	125.00	125.00	125.00
Illumination	LOEC-48 h	125.00	250.00	125.00	125.00
	NOEC-48 h	62.50	125.00	62.50	62.50

NOEC, no observable effective concentrations; LOEC, lowest observable effective concentrations.

on graphene-family materials to freshwater algae *Chlorella pyrenoidosa* were conducted by Zhao et al. [13]. They calculated 96 h EC50 for graphene oxide at the level of 37 mg/L. Authors analyzed mechanistic factors connected to algae response and found that for graphene oxide, shading effect (~16%), oxidative stress-induced membrane damage, and nutrient depletion (~53%) were responsible for the observed toxicity. About 10 times lower EC50-96 h was found for graphene oxide by Hu et al. [14] in their experiments with protozoan *Euglena gracilis*. The growth of these test organisms was affected in 50% at a concentration of graphene oxide equal to 3.76 mg/L, but significant adverse effects were present at concentrations exceeding 2.5 mg/L. Authors stated that graphene oxide released into the aquatic environment may interact with protozoa and thus it presents environmental risks. Mesarič et al. [15] found that graphene oxide alters mobility and survival of saltwater crustacean—*Artemia salina* at similar concentrations that are found in this study. The EC50-48 h for swimming inhibition was estimated at a level of 160 mg/L (110–240 mg/L), while LC50-48 h was estimated at a level of 650 mg/L. Authors also found some indication of biochemical responses of test organisms while exposed to graphene oxide (an increase of cholinesterase activity and a decrease of glutathione S-transferase activity) that support the thesis of oxidative stress as one of the graphene oxide toxic mode of action. Similar findings were presented by Souza et al. [16] in their study on graphene oxide effects on fish—*Danio rerio* at concentrations of up to 20 mg/L. Short-term exposure resulted in cell apoptosis and necrosis as well as oxidative stress responses, probably due to the strong graphene oxide interaction with and accumulation on cell membranes. Authors did not find any evidence on genotoxic properties of graphene oxide in the comet assay. As the cellular mechanism of graphene oxide toxicity is recognized, some studies about its antimicrobial activity were also published. Combarros et al. [17] found that graphene oxide has a negative effect on the bacterial growth and viability of *Pseudomonas putida*. The growth of *P. putida* was inhibited by the presence of graphene oxide at concentrations higher than 0.05 mg/mL. Their results suggest that the main impact of graphene oxide on bacterial cells was the loss of the membrane integrity, as a result of the sharp edges of nanosheets, which probably act as "blades" in the solution [17]. Similar findings published by Brandeburová et al. [18] indicate that an antimicrobial effect was observed only against gram-positive bacteria exposed to graphene oxide. This phenomenon connected with the limited biodegradation possibility of graphene oxide may indicate problems in wastewater treatment plants affected by this NM. Nguyen et al. [19] confirmed that in batch reactors significant effects on the chemical and biological parameters of these reactors were found in the presence of graphene at 10 mg/L or higher concentrations. Authors also showed that the graphene acute toxicity caused significant reduction of the microbial community metabolic activity, which in return reduced biological oxygen demand, nitrogen, and phosphorus removals. Numerous studies indicate changes in NM's behavior under different environmental conditions that may cause an impact on bioavailability and therefore on the toxicity of these compounds. Aggregation and agglomeration are usually connected to physical properties of nanoparticles (size and shape, surface coatings), chemistry of the mediums (pH, ionic strength, electrolyte patterns, and organic matter content), and various environmental conditions (temperature and dissolved oxygen) [20]. It was also found that solar irradiation alters the chemistry and behavior of graphene oxide in the aquatic environment. Zhao and Jafvert [21] proved that upon exposure to light, electron transfer reactions occur from graphene oxide to O_2, forming O_2, and significant quantities of H_2O_2 that support the thesis of oxidative stress as one of the graphene oxide toxic modes of action. Literature provides numerous environmental factors affecting GO stability in water solutions including temperature, high ionic strength, presence of divalent cations, pH, and natural organic matter (especially humic acids) [22,23]. It was found that GO dispersions remain stable in the pH range of 4–11 [24], and a slightly narrower stability range of pH was estimated by Chowdhury et al. [25]. Authors found that the hydrodynamic diameter of GO remained nearly constant (about 250 nm) from pH 4 to 10, while the colloidal size significantly increased at lower pH levels [25]. Not so clear effects of temperature on GO stability were investigated by Wang et al. [26]. Authors found that without the presence of humic acids, increasing temperature enhanced GO aggregation in both monovalent and divalent cation solutions. The temperature effect on GO aggregation was more complicated in the presence of natural organic matter. For monovalent electrolytes, the lowest temperature (6°C) destabilized GO most efficiently while the aggregation kinetics at 25°C and 40 °C were close to each other and were notably weaker than that of 6°C [26].

5. Summary

Authors realize that emissions of nanoparticles became common nowadays. Placing on the global market products like biocides, plant protection products, drugs, and numerous devices leads to exposure of living organisms to nanoparticles. Widespread usage of products containing NMs gains on researches on nanoparticles fate and behavior in the environment which is a final destination of all NMs released by human activities. Current status of knowledge about NMs ecotoxicity requires numerous basic researches not only on effects caused by NMs but also on their behavior under different environmental conditions. Literature on environmental impacts of CNMs is still very limited. Lack of scientific data on ecotoxicity of NMs causes that proper environmental risk assessment of these substances including all trophic levels in the ecosystems is practically impossible. Graphene oxide toxicity is not fully described and explained in the literature. Ou et al. [27] in their comprehensive review of the origins and mechanisms of graphene-family materials indicate that several typical mechanisms underlying graphene toxicity have been identified, these include physical destruction, oxidative stress, DNA damage, inflammatory response, apoptosis, autophagy, and necrosis. Authors also pointed out that various factors determine the toxicity of graphene NMs including the lateral size, surface structure, functionalization, charge, impurities, aggregations, and corona effect [27]. Our study showed clearly that also testing conditions represented by the composition of test media in ecotoxicity bioassays alters the graphene oxide impact on living organisms. This is why it should be clearly defined what type of medium is used in the experiments to allow the

reproduction test results between laboratories. In this study, it was found that specifically for immobilization endpoints in acute daphnids bioassay test results may vary from 159.17 up to 500.40 mg/L depending on composition of test medium. It was found that the limited hardness of aqueous environment increases the toxic effects of graphene oxide, therefore hardness of standard Elend test medium (about 250 mg of $CaCO_3$/L) used in immobilization assay with daphnids may lead to underestimating the risk associated with graphene oxide presence in the environment. No significant differences between illuminated and non-illuminated assays were found in this study. Data presented in this paper fill one of the gaps in the knowledge of graphene oxide ecotoxicity—impact of test medium composition on the ecotoxicological test results. It was shown that environmental behavior of CNMs under different conditions may be one of the important factors limiting risk associated with its presence in the environment.

Conflicts of interest:

The authors declare no conflict of interest.

Acknowledgment

Some of the equipments used in experiments described above are part of Special Research Infrastructure called μEcotox partially financed by The Polish Ministry of Science and Higher Education.

References

[1] M. Maliszewska-Mazur, Nanotechnology—new challenges, new possibilities and new questions, Environ. Prot. Nat. Resour., 45 (2010) 153–161.
[2] M. Pierścieniak, A. Barański, B. Gworek, Nanoparticles: an environmental threat?, Przem. Chem., 91 (2012) 564–568.
[3] X. Hu, D. Li, Y. Gao, L. Mu, Q. Zhou, Knowledge gaps between nanotoxicological research and nanomaterial safety, Environ. Int., 94 (2016) 8–23.
[4] J. Rybak, R. Kalinowski, S. Paczkowski, B. Tomczyk, M. Kaźmierczuk, K. Librant, M. Łepecki, Graphene stability under quasi-environmental conditions, Przem. Chem., 3 (2016) 536–540.
[5] OECD, Test No. 202: *Daphnia* sp., Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals/Section 2, OECD Publishing, Paris, 2004, pp. 1–12.
[6] ASTM Standard, Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians, ASTM International, West Conshohocken, PA, 2007.
[7] S. Chowdhury, R. Balasubramanian, Recent advances in the use of graphene-family nanoadsorbents for removal of toxic pollutants from wastewater, Adv. Colloid Interface Sci., 204 (2014) 35–56.
[8] C. Chung, Y.K. Kim, D. Shin, S.R. Ryoo, B.H. Hong, D.H. Min, Biomedical applications of graphene and graphene oxide, Acc. Chem. Res., 46 (2013) 2211–2224.
[9] A. Khan, J. Wang, J. Li, X.X. Wang, Z. Chen, A. Alsaedi, T. Hayat, Y. Chen, X.X. Wang, The role of graphene oxide and graphene oxide-based nanomaterials in the removal of pharmaceuticals from aqueous media: a review, Environ. Sci. Pollut. Res., 24 (2017) 7938–7958.
[10] Y. Liu, W. Fan, Z. Xu, W. Peng, S. Luo, Comparative effects of graphene and graphene oxide on copper toxicity to *Daphnia magna*: role of surface oxygenic functional groups, Environ Pollut., 236 (2018) 962–970.
[11] X. Lv, Y. Yang, Y. Tao, Y. Jiang, B. Chen, X. Zhu, Z. Cai, B. Li, A mechanism study on toxicity of graphene oxide to *Daphnia magna*: direct link between bioaccumulation and oxidative stress, Environ. Pollut., 234 (2018) 953–959.
[12] J.P. Souza, F.P. Venturini, F. Santos, V. Zucolotto, Chronic toxicity in *Ceriodaphnia dubia* induced by graphene oxide, Chemosphere, 190 (2018), 218–224.
[13] J. Zhao, X. Cao, Z. Wang, Y. Dai, B. Xing, Mechanistic understanding toward the toxicity of graphene-family materials to freshwater algae, Water Res., 111 (2017) 18–27.
[14] C. Hu, Q. Wang, H. Zhao, L. Wang, S. Guo, X. Li, Ecotoxicological effects of graphene oxide on the protozoan *Euglena gracilis*, Chemosphere, 128 (2015) 184–190.
[15] T. Mesarič, C. Gambardella, T. Milivojević, M. Faimali, D. Drobne, C. Falugi, D. Makovec, A. Jemec, K. Sepčić, High surface adsorption properties of carbon-based nanomaterials are responsible for mortality, swimming inhibition, and biochemical responses in *Artemia salina* larvae, Aquat. Toxicol., 163 (2015) 121–129.
[16] J.P. Souza, J.F. Baretta, F. Santos, I.M.M. Paino, V. Zucolotto, Toxicological effects of graphene oxide on adult zebrafish (*Danio rerio*), Aquat. Toxicol., 186 (2017) 11–18.
[17] R.G. Combarros, S. Collado, M. Díaz, Toxicity of graphene oxide on growth and metabolism of *Pseudomonas putida*, J. Hazard. Mater., 310 (2016) 246–252.
[18] P. Brandeburová, L. Bírošová, M. Vojs, A. Kromka, M. Gál, J. Tichý, J. Híveš, T. Mackuľak, The influence of selected nanomaterials on microorganisms, Monatshefte fuer Chemie., 148 (2017) 525–530.
[19] H.N. Nguyen, S.L. Castro-Wallace, D.F. Rodrigues, Acute toxicity of graphene nanoplatelets on biological wastewater treatment process, Environ. Sci. Nano., 4 (2017) 160–169.
[20] C. Peng, W. Zhang, H. Gao, Y. Li, X. Tong, K. Li, X. Zhu, Y. Wang, Y. Chen, Behavior and potential impacts of metal-based engineered nanoparticles in aquatic environments, Nanomaterials, 7 (2017) 21.
[21] Y. Zhao, C.T. Jafvert, Environmental photochemistry of single layered graphene oxide in water, Environ. Sci. Nano., 2 (2015) 136–142.
[22] Z. Hua, Z. Tang, Z. Bai, J. Zhang, L. Yu, H. Cheng, Aggregation and resuspension of graphene oxide in simulated natural surface aquatic environments, Environ Pollut., 205 (2015) 161–169.
[23] K. He, G. Chen, G. Zeng, M. Peng, Z. Huang, J. Shi, T. Huang, Stability, transport and ecosystem effects of graphene in water and soil environments, Nanoscale, 17 (2017) 5370–5388.
[24] F. Abbasi, J. Karimi-Sabet, C. Ghotbi, Z. Abbasi, S.A. Mousavi, N. Amini, The effect of pH on lateral size and surface chemistry of graphene oxide, Scientia Iranica, 24 (2017) 3554–3559.
[25] I. Chowdhury, M.C. Duch, N.D. Mansukhani, M.C. Hersam D. Bouchard, Colloidal properties and stability of graphene oxide nanomaterials in the aquatic environment, Environ. Sci. Technol., 47 (2013) 6288–6296.
[26] M. Wang, B. Gao, D. Tang, H. Sun, X. Yin, C. Yu, Effects of temperature on aggregation kinetics of graphene oxide in aqueous solutions, Colloids Surf., A, 538 (2018) 63–72.
[27] L. Ou, B. Song, H. Liang, J. Liu, X. Feng, B. Deng, T. Sun, L. Shao, Toxicity of graphene-family nanoparticles: a general review of the origins and mechanisms, Part. Fibre Toxicol., 13 (2016) 57.

Supplementary materials

Appendix A

Detailed immobilization of daphnids.

Table S1
Raw data on immobilization of daphnids in very soft ASTM water, in darkness

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	1	10	10	10
2		0	0	1	1	10	10	10
1	48	0	0	0	2	10	10	10
2		0	0	1	1	10	10	10

Table S2
Raw data on immobilization of daphnids in very soft ASTM water, in light

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	0	10	10	10
2		0	0	0	0	10	10	10
1	48	0	0	1	2	10	10	10
2		0	0	0	0	10	10	10

Table S3
Raw data on immobilization of daphnids in soft ASTM water, in darkness

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	0	1	10	10
2		0	0	0	0	0	10	10
1	48	0	0	0	0	4	10	10
2		0	0	0	1	7	10	10

Table S4
Raw data on immobilization of daphnids in soft ASTM water, in light

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	0	1	10	10
2		0	0	0	0	1	10	10
1	48	0	0	0	0	1	10	10
2		0	0	0	0	1	10	10

Table S5
Raw data on immobilization of daphnids in hard ASTM water, in darkness

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	0	0	2	10
2		0	0	0	0	0	4	10
1	48	0	0	0	0	2	3	10
2		0	0	0	0	2	5	10

Table S6
Raw data on immobilization of daphnids in hard ASTM water, in light

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	1	2	4	10
2		0	0	0	0	1	3	10
1	48	0	0	0	1	3	6	10
2		0	0	0	2	1	5	10

Table S7
Raw data on immobilization of daphnids in very hard ASTM water, in darkness

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	1	1	6	8
2		0	0	0	0	0	4	7
1	48	0	0	0	1	2	8	9
2		0	0	0	0	1	5	8

Table S8
Raw data on immobilization of daphnids in very hard ASTM water, in light

Replicate	Time (h)	Control	31.25 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
1	24	0	0	0	1	2	5	6
2		0	0	0	0	2	3	4
1	48	0	0	0	1	3	6	9
2		0	0	0	1	2	3	10

Appendix B

Dose–response relationships for 48 h.

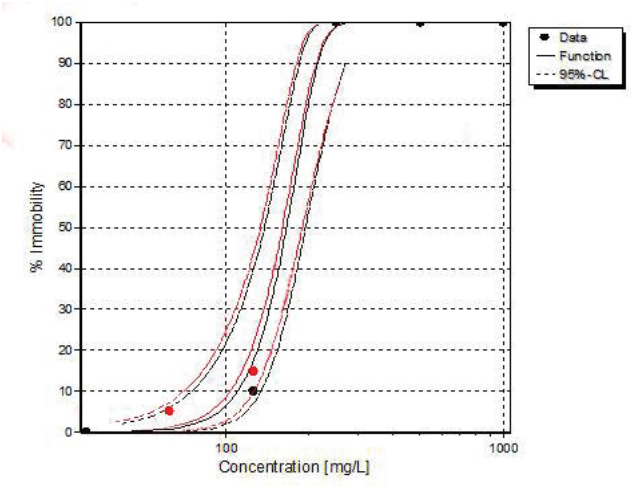

Fig. S1. 48 h dose–response curve for daphnids immobilization in very soft ASTM water, in light (black line) and in darkness (red line).

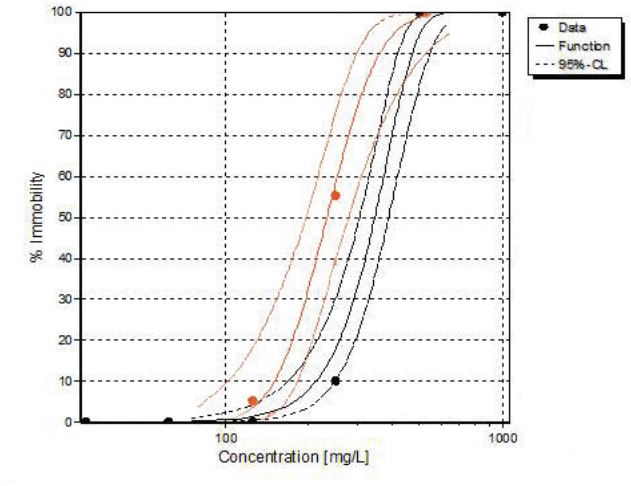

Fig. S2. 48 h dose–response curve for daphnids immobilization in soft ASTM water, in light (black line) and in darkness (red line).

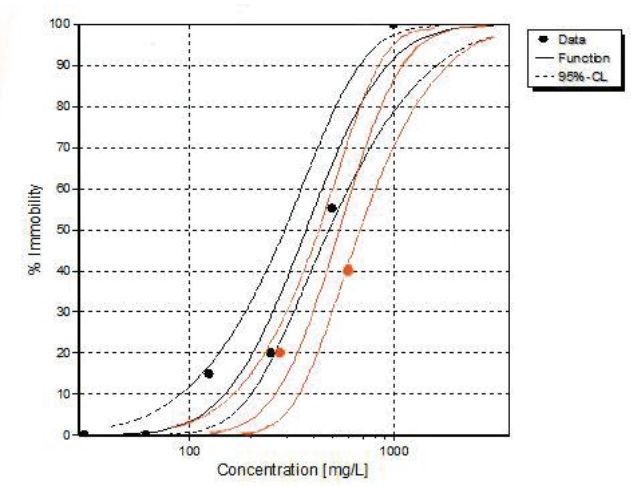

Fig. S3. 48 h dose–response curve for daphnids immobilization in hard ASTM water, in light (black line) and in darkness (red line).

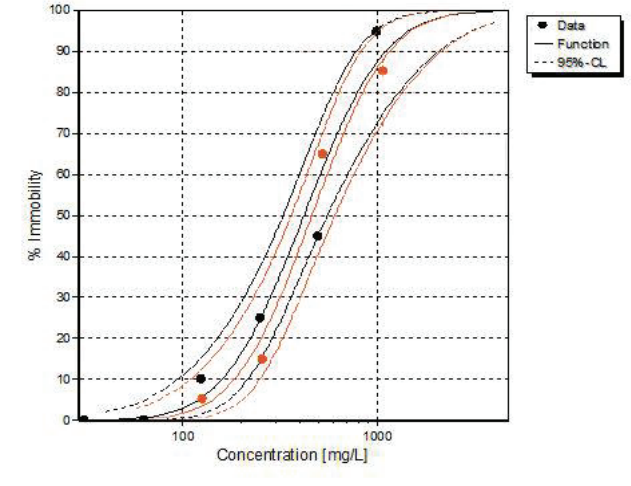

Fig. S4. 48 h dose–response curve for daphnids immobilization in very hard ASTM water, in light (black line) and in darkness (red line).