High surface adsorption properties of carbon-based nanomaterials are responsible for mortality, swimming inhibition, and biochemical responses in *Artemia salina* larvae

Tina Mesarič\textsuperscript{a}, Chiara Gambardella\textsuperscript{b}, Tamara Milivojević\textsuperscript{a}, Marco Faimali\textsuperscript{b}, Damjana Drobne\textsuperscript{a,c,d}, Carla Falugi\textsuperscript{e}, Darko Makovec\textsuperscript{f}, Anita Jemec\textsuperscript{a}, Kristina Sepčić\textsuperscript{a,*}

\textsuperscript{a}Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia (tina.mesaric84@gmail.com; milivojevietamara@gmail.com; damjana.drobne@bf.uni-lj.si; kristina.sepcic@bf.uni-lj.si; anita.jemec@bf.uni-lj.si)

\textsuperscript{b}Institute of Marine Sciences, National Research Council, Genova, Italy (chiara.gambardella@ge.ismar.cnr.it; marco.faimali@ismar.cnr.it)

\textsuperscript{c}Centre of Excellence in Nanoscience and Nanotechnology (CO Nanocentre), Ljubljana, Slovenia

\textsuperscript{d}Centre of Excellence in Advanced Materials and Technologies for the Future (CO NAMASTE), Ljubljana, Slovenia

\textsuperscript{e}Department of Earth, Environment and Life Sciences, University of Genova, Genova, Italy (carlafalugi@hotmail.it)

\textsuperscript{f}Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia (darko.makovec@ijs.si)

\textsuperscript{*}Corresponding author:

Kristina Sepčić, Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia; Tel: +386-1-3203419; Fax: +386-1-2573390; E-mail address: kristina.sepcic@bf.uni-lj.si
Abstract

We investigated the effects of three different carbon-based nanomaterials on brine shrimp (*Artemia salina*) larvae. The larvae were exposed to different concentrations of carbon black, graphene oxide, and multiwall carbon nanotubes for 48 h, and observed using phase contrast and scanning electron microscopy. Acute (mortality) and behavioural (swimming speed alteration) responses and cholinesterase, glutathione-S-transferase and catalase enzyme activities were evaluated. These nanomaterials were ingested and concentrated in the gut, and attached onto the body surface of the *A. salina* larvae. This attachment was responsible for concentration-dependent inhibition of larval swimming, and partly for alterations in the enzyme activities, that differed according to the type of tested nanomaterials. No lethal effects were observed up to 0.5 mg/mL carbon black and 0.1 mg/mL multiwall carbon nanotubes, while graphene oxide showed a threshold whereby it had no effects at 0.6 mg/mL, and more than 90% mortality at 0.7 mg/mL. Risk quotients calculated on the basis of predicted environmental concentrations indicate that carbon black and multiwall carbon nanotubes currently do not pose a serious risk to the marine environment, however if uncontrolled release of nanomaterials continues, this scenario can rapidly change.

**Keywords:** *Artemia salina*; biochemical biomarkers; carbon-based nanomaterials; mortality; swimming inhibition.

Abbreviations:
CB, carbon black; GO, graphene oxide; FNSW, filtered natural sea water; MWCNTs, multiwall carbon nanotubes; PEC, predicted environmental concentration; PNEC, predicted no-effect concentration; RQ, risk quotient; SEM, scanning electron microscopy.
1. Introduction

Naturally occurring and industrially derived carbon-based nanomaterials are one of the most abundant nanomaterials in the environment (Hussain et al., 2010). However, some carbon-based nanomaterials have potentially toxic effects. It is therefore important to understand their effects on the biota, especially of aquatic environments, where they can finally accumulate.

Carbon black (CB) is a form of amorphous carbon with extensive uses in industrial applications (e.g., rubber products, paint, plastic, ink). It is manufactured by controlled vapour-phase pyrolysis of hydrocarbons (Sorahan et al., 2007; Screening Assessment for the Challenge, 2013), and it should not be confused with black carbon, which is a product of incomplete combustion of fossil fuels and biomass (Goldberg, 1985; Masiello and Dreffel, 1998). Graphene oxide (GO) is a single planar layer of carbon atoms that is arranged into a honeycomb-like lattice (Norwegian Pollution Control Authority, 2008). This has recently attracted great interest for its potential applications in electronics, energy, materials and biomedical areas. Carbon nanotubes (CNT) are ‘rolled-up’ graphene sheets that can be capped or not at both ends by a half fullerene (another allotrope of carbon). CNTs can be formed of single (single-wall CNT; SWCNTs) or multiple (multiwall CNTs; MWCNTs) carbon layers (Norwegian Pollution Control Authority, 2008). With their unique electrical, thermal, chemical, mechanical and optical properties, CNTs are one of the most interesting, prospective and used nanomaterials (Park and Ruoff, 2009).

Most of the available data on the toxicity of carbon-based nanomaterials towards aquatic invertebrates have been reported for freshwater model crustaceans, such as *Daphnia magna* (Arndt et al., 2013a; Baumerte et al., 2013; Lovern and Klaper, 2006; Oberdörster et al., 2006; Petersen et al., 2009; Roberts et al., 2007), *Daphnia pulex* (Klaper et al., 2009), *Ceriodaphnia dubia* (Li and Huang, 2011) and *Hyallela azteca* (Kennedy et al., 2008). These studies have
provided evidence that acute and chronic exposure to carbon-based nanomaterials at concentrations from 0.4 mg/mL up to more than 35 mg/mL can cause adverse effects for aquatic organisms. Some studies on carbon-based nanomaterials have also been carried out on marine organisms; e.g., crustaceans, bivalves and polychaetes. Canesi et al. (2010a, 2010b) reported adverse effects of CB and C$_{60}$ fullerenes (0.05-10 µg/mL) on *Mytilus galloprovincialis* after 24 h of exposure. The C$_{60}$ fullerenes and CNTs (1-10 µg/mL) were also shown to induce cytotoxicity in immune cells of *M. galloprovincialis* (Moore et al., 2009). Effects of CB and MWCNTs on the marine crustacea *Artemia salina* after 48 h of exposure were studied by Baumerte et al. (2013), with reported LC$_{50}$ of 0.03 mg/mL and 0.013 mg/mL, respectively. Miglietta et al. (2011) also showed that these two nanomaterials can be toxic for *A. salina*. In their study, exposure of *A. salina* to suspensions of CB and MWCNTs (66 µg/mL) induced mortalities of 50% and >95%, respectively, after only 24 h. In a study by Pretti et al. (2014), no toxicity of different types of pristine graphene to *A. salina* was recorded up to 10 mg/L after a 24 h of exposure. However, after a 48 h exposure, pristine graphene monolayer flakes up to the concentration of 1 mg/L increased the biomarkers of oxidative stress (catalase and glutathione peroxidase activity), as well as the levels of lipid peroxidation. Templeton et al. (2006) showed an increase in mortality and a delay in development for the marine copepod *Amphiascus tenuiremis* after 35-day exposure to 10 µg/mL SWCNT. The recent study by Sohn et al. (2015) reveals that SWCNTs have no short-term acute toxicity against *D. magna* up to 100 µg/mL, but they can affect freshwater microalgae. On the other hand, carbon-based NMs including SWCNT and MWCNT were found to exert a multigenerational effect on *D. magna* survival, reproduction, and growth as exposure of the parent population (Arndt et al., 2013b). In our previous study (Mesarić et al., 2013a), we reported the adverse effects of GO and CB (up to 1 and 5 mg/mL, respectively) on the behaviour and survival of the barnacle *Amphibalanus amphitrite* after 24 h and 48 h of exposure.
The literature data have reported high surface adsorption potential of carbon-based nanomaterials in comparison with metal-based ones (Ruh et al., 2012; Xia et al., 2011, Li et al., 2013). This potential is postulated to be the primary driving force behind all of the interactions and dynamic changes between nanomaterials and biological systems (Xia et al., 2011). Therefore, it can be expected that compounds with high surface-adsorption potential will strongly attach onto the surface of aquatic invertebrates, and will affect their activities, like swimming, filtration, breading and moulting. In the present study, we selected three different carbon-based nanomaterials and studied their effects on the larvae of the crustacea A. salina: CB, GO, MWCNTs. This organism is a suitable and widely used model of acute toxicity (Ates et al., 2009). Here, we measured the different ecotoxicological end-points of mortality and behavioural response (swimming speed alteration), and some selected biochemical biomarkers that are frequently analysed in toxicological studies, as the activities of the cholinesterase, glutathione-S-transferase and catalase enzymes (Boldina-Cosqueric, et al., 2010; Buffet et al., 2011; Garaventa et al., 2010).

We hypothesised that the high surface-adsorption potential of these carbon-based nanomaterials might be the main factor that is responsible for the expected responses (i.e., mortality, alteration of enzyme activities), and would also affect the swimming behaviour of this model organism. We aimed to use the data from this study for environmental risk assessment of carbon-based nanomaterials in marine environments, by assessing the ratio between their predicted environmental concentration (PEC) and predicted no-effect concentration (PNEC).

2. Material and methods
2.1. Characteristics of carbon-based nanomaterials

Carbon black was purchased from PlasmaChem GmbH (Germany). The mean size of the primary particles was estimated from transmission electron microscopy (TEM) analysis (TEM Jeol 2010 F operated at 200 kV) to be 13 nm. Single-layer GO was from Graphene Supermarket, as dry flakes with an average size of 0.5 µm to 5 µm, as determined from the TEM. At least 80% of the GO was composed of single-layer molecules. MWCNTs were obtained through the EU FP7 large-scale integrating project known as NanoValid and were supplied by Nanocyl (http://www.nanocyl.com/). According to the TEM analysis the MWCNTs had an outer diameter ranging from 5.7 to 15 nm and were several µm long. (Supplementary information Fig.S1).

All three carbon-based nanomaterials were freshly prepared as suspensions in dH2O to provide stock suspensions at the final concentration of 1 mg/mL, which were sonicated for 30 min in an ultrasonic bath sonicator (model LBS1, Falc; Italy), as described in Canesi et al. (2010a). The stock dispersions of nanomaterials were further diluted to toxicity test concentrations in FNSW.

2.1.1 Characteristics of the carbon-based nanomaterials in distilled water

ζ-potential of the carbon-based nanomaterials dispersed in distilled water (0.01 mg/mL) was measured using a Brookhaven Instruments Corp., ZetaPALS. In distilled water, the carbon-based nanomaterials showed a relatively high, negative ζ-potential at neutral pH (-32 mV, -36 mV, and -21 mV for CB, GO, and MWCNT, respectively). Although the ζ-potential of the CB suspension exceeded the absolute value of 30, which is considered to be high enough to electrostatically prevent the particles from agglomeration (Tadros, 1987); substantial agglomeration was observed by dynamic light scattering. The size of the CB agglomerates in
the distilled water suspension ranged between 60 nm and 170 nm, and around 300 nm (Mesarič et al., 2013a). The dynamic light scattering analyses of the GO and MWCNT suspensions were not possible because of the extremely anisotropic shape of the primary particles.

2.1.2 Characteristics of the carbon-based nanomaterials in FNSW

The $\xi$-potential (Brookhaven Instruments Corp., ZetaPALS) of nanomaterials suspended in the FNSW is much lower to that in the distilled water because of electrostatic screening effects related to the very high ionic strength. The $\xi$-potentials of approximately -14 mV were measured for the all three types of carbon-based nanomaterials in the FNSW. Due to the decreased effective electrostatic repulsions between the particles in the FNSW they quickly flocculate. Under strong mechanical shearing, i.e., during sonication, the soft flocules brake into smaller ones. However, when the sonicator is turned off, the flocules grow to the size visible to a naked eye in just few minutes. This is also the reason why reliable measurements of the hydrodynamic size of the FNSW-suspended nanomaterials using dynamic light scattering were not possible, even at the lowest tested concentration (0.01 mg/mL). Similar sedimentation of carbon-based nanomaterials in salt water (15 ppt) was also observed by Baumerte et al. (2013).

Carbon-based nanomaterials were also inspected under SEM. The samples for SEM were prepared by injection of the nanomaterial-FNSW suspensions (0.1 mg/ml) onto 0.2-µm filter papers (Millipore, Merck, Germany). After drying, the filter papers were mounted on the SEM stubs using self-adhesive carbon discs, and prepared and investigated in the same way as the $A$. salina larvae (see section 2.4.2). EDS was used to confirm the identity of carbon nanomaterials. The SEM micrographs of carbon nanomaterials are shown in Figure 2 (upper panels). CB preserves an amorphous powdery consistency, GO monolayers are irregular-shaped pieces, and MWCNTs appear like tangled string structures. In carbon nanomaterials
FSNW suspensions, also cubic crystals were identified. Using EDS, they were confirmed to be composed of salts from the seawater. (Supplementary information Fig.S2).

2.2. Model organism
Commercially available dehydrated cysts (Blue Line, Italy) of brine shrimp (*A. salina* Linnaeus, 1758; Anostraca, Brachiopoda, Crustacea) were used in this study. To obtain Instar I stage larvae, approximately 500 mg dehydrated cysts were put into FNSW and incubated for 24 h at 28 °C, under a 16 h light: 8 h dark cycle. The newly hatched larvae were separated from non-hatched cysts using a Pasteur pipette, and transferred into a new beaker with FNSW, by exploiting their positive phototaxis, before their use in the ecotoxicological bioassays.

2.3. Acute toxicity and swimming speed alteration test
The toxicity bioassay was performed by adding 10-15 *A. salina* larvae to each well of a 24-well polystyrene plate containing 1 mL FNSW (according to previous studies of Garaventa et al., 2010 and Gambardella et al., 2014) and the different concentrations of the carbon-based nanomaterials. The test concentrations of CB, GO and MWCNTs were chosen on the basis of the results of a preliminary screening test using an order-of-magnitude dilution series (Supplementary Table S1), with which we assessed swimming speed inhibition and mortality as ecotoxicological end-points. Five definitive concentrations for CB and GO, and three concentrations for MWCNTs were tested, as given in Table 1. Concentrations of MWCNTs >0.1 mg/mL were not tested due to the formation of a very unstable suspension, which was impossible to manage by pipetting. Controls without added nanomaterials were performed in parallel for each of the carbon-based nanomaterials. The plates were incubated at 20 °C for 48 h with a 16 h: 8 h light/dark cycle. During the exposure, the test larvae were not fed. All of the test concentrations were performed in triplicate. After 48 h, the larvae were collected from the
wells, rinsed, and transferred into new plates with clean FNSW. The numbers of dead and living larvae were counted under a stereomicroscope. The median lethal concentration (LC$_{50}$) and the related confidence limits were calculated, where possible as the concentration of the suspended carbon-based nanomaterials that causing 50% mortality of the A. salina larvae after 48 h of exposure. The larvae were not analysed after 24 h as it was not possible to observe them due to the strong black colouring of the solution.

The swimming speed alteration test was performed on the survived larvae according to Garaventa et al. (2010). Swimming was registered by using the Swimming Behavioural Recorder experimental set-up. This recorder consists of a video camera with a macro-objective, which records the swimming paths of a sample of larvae. The apparatus was positioned inside a black box (60 × 60 × 100 cm) to exclude external sources of light, and the recording chamber was monitored under infrared light. The A. salina larvae were dark-adapted for 2 min before the video recording (the time was fixed in preliminary tests, to reach a steady speed and uniform spatial distribution). The swimming behaviour was digitally recorded for about 3 s at 25 frames per second, and the images were analysed using advanced image processing software, to reconstruct the tracks of the individual swimming paths, for the average swimming speed (mm/s) for each sample (10-15 individuals). The data are finally referred to as swimming inhibition, normalised to the average swimming speed (S) of the control sample, according to Equation (1):

\[
\text{Inhibition (\%)} = \left( \frac{S_{\text{treated}} - S_{\text{control}}}{S_{\text{control}}} \right) \times 100 \tag{1}
\]

The median concentrations effective for swimming alterations (EC$_{50}$) and their confidence limits were calculated, where possible as the concentrations of the suspended carbon-based nanomaterial that had a 50% effect on the A. salina larvae after 48 h of exposure.
2.4. Microscopy investigation of *A. salina* exposed to nanomaterials - ingestion and attachment study

2.4.1. Light microscopy

The uptake of the carbon-based nanomaterials by the *A. salina* larvae that were exposed to CB, GO and MWCNTs for 48 h (see section 2.3) was observed under the microscope (Olympus, Japan). The images were visualised through a colour-view camera (Olympus, Japan) and acquired using the AnalySIS software (Soft Imaging System, USA).

2.4.2. Scanning electron microscopy

The *A. salina* larvae exposed for 48 h to the carbon-based nanomaterials (0-1 mg/mL for CB, 0-0.7 mg/mL for GO, and 0-0.1 mg/mL for MWCNTs; Table 1), were additionally investigated using SEM to investigate the attachment of nanomaterials on the surface of *A. salina*. The larvae were fixed according to the preparation technique for crustacea larvae suggested by Mayer and Melzer (2004), and reported by Nation (1983). Specimens were put into 2.5% glutaraldehyde (Merck, Germany) with 50 mM potassium phosphate, pH 7.0, for 2 h at 4 °C. After rinsing several times with phosphate-buffered saline (15 min) and being taken through a graded ethanol (Merck, Germany) series (25%-70%; 2× 15 min each), the larvae were dehydrated through an acetone (Merck, Germany) series (70%-90%, 2× 100%; 10 min each). The larvae were then dried in hexamethyldisilazane (Merck, Germany) and left overnight so that the fluid dried out. After mounting the larvae on SEM stubs with self-adhesive carbon discs, the dried specimens were coated with a thin layer of gold (<10 nm; Gatan-682, Germany) and investigated using a field emission scanning electron microscope (JEOL JSM-6500F, Japan) at the Institute of Metals and Technology (Ljubljana, Slovenia).
2.5. Assessment of enzyme activities

The activities of cholinesterase, glutathione S-transferase and catalase were examined after the 48 h exposure of *A. salina* larvae to these carbon-based nanomaterials. For these tests, about 200 larvae were exposed to 50 mL suspension of each carbon-based nanomaterial at the different ranges of concentrations used (CB, 0-1 mg/mL; GO, 0-0.5 mg/mL; MWCNTs, 0-0.1 mg/mL; Table 1), for 48 h, as described in Rajasree et al. (2010). Three replicates were prepared for each concentration of carbon-based nanomaterials and for the control without nanomaterials. After 48 h of exposure, the larvae were placed into FNSW, rinsed four times in 50 mM potassium phosphate buffer, pH 7.0, and homogenised for 3 min in 0.5 ml homogenisation buffer (50 mM phosphate buffer, pH 7.0), using a glass-glass Elvehjem-Potter homogeniser. The homogenates were centrifuged for 15 min at 15,000× g and 4 °C, as described in Jemec et al. (2007). Supernatants were stored at -80 °C until all of the tests were performed, but no longer than one week. Protein concentrations in these supernatants were determined using BCA™ Protein Assay kits, which use a modification of the bicinchoninic acid protein assay (Pierce, Rockford, USA).

2.5.1. Cholinesterase activity analyses

Cholinesterase activity was analyzed by the method of Ellman et al. (1961), using a VIS microplate reader (Anthos, UK). The reaction mixture was prepared in 100 μL of 100 mM potassium phosphate buffer (pH 7.4) containing acetylthiocholine chloride and 5,5′ dithiobis-2-nitrobenzoic acid (both Sigma Aldrich, Germany) at final concentrations of 1 mM and 0.5 mM, respectively. Protein supernatant (100 μL) was added to start the reaction, which was followed spectrophotometrically at 405 nm and 25°C for 1 h. A blank reaction was performed by replacing the protein supernatant with 100 μL of 100 mM potassium phosphate buffer (pH
Cholinesterase activity was expressed in nmoles of hydrolysed acetylcholine chloride/min/mg protein (extinction coefficient, $\varepsilon_{405}=13,600 \text{ M}^{-1} \text{ cm}^{-1}$).

2.5.2. Glutathione-S-transferase activity analyses

Glutathione-S-transferase activity was analyzed by the method described by Habig et al. (1974) using a VIS microplate reader (Anthos, UK). 1-chloro-2,4-dinitrobenzene (Sigma Aldrich, Germany) was dissolved in ethanol to obtain a 50 mM solution, which was afterwards diluted with 100 mM potassium phosphate buffer (pH 6.5) to a final concentration of 4 mM. This solution was used to prepare a reaction mixture containing 1 mM of 1-chloro-2,4-dinitrobenzene and 1 mM of reduced glutathione (Sigma Aldrich, Germany). Protein supernatant (50 μL) was added to start the reaction. A blank reaction was performed by replacing the protein supernatant with 50 μL of 100 mM potassium phosphate buffer (pH 7.4). The final concentration of ethanol in the mixture was 2%, a concentration at which the activity of glutathione-S-transferase was not inhibited (Jemec et al., 2007). The reaction was followed spectrophotometrically at 340 nm and 25 °C for 20 min. Glutathione-S-transferase activity was expressed in nmoles of conjugated glutathione/min/mg protein (extinction coefficient, $\varepsilon_{340}=9600 \text{ M}^{-1} \text{ cm}^{-1}$).

2.5.3. Catalase activity analyses

The catalase activity was determined according to Aebi (1984). Here, 50 μL protein supernatant were combined with 950 μL hydrogen peroxide (Sigma Aldrich, Germany) solution (10.8 mM) prepared in 50 mM potassium phosphate buffer (pH 7.0). The final concentration of hydrogen peroxide was 10 mM. The reaction was followed spectrophotometrically for 2 min at 25 °C and 240 nm using a Shimadzu UV-2101PC spectrophotometer (Japan). The catalase
activity is expressed as µmoles of degraded hydrogen peroxide/min/mg protein (extinction coefficient, $\varepsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$).

2.6. Statistical analysis
Data are expressed as means ± standard error (SE) of two experiments. The LC$_{50}$ for mortality and EC$_{50}$ for swimming speed alteration, and the related 95% confident limits were calculated using Trimmed Spearman-Karber analysis (Finney et al., 1978). Significant differences between controls and treated samples were determined using one-way ANOVA followed by the Bonferroni nonparametric post hoc tests, where $p<0.05$ is considered to be significantly different. In addition, the significant differences among concentrations of each treatment were determined as described above.

3. Results

3.1. Ingestion of carbon-based nanomaterials by A. salina larvae
After 48 h of exposure to these carbon-based nanomaterials, the guts of the A. salina larvae were investigated under light microscopy. The images obtained after exposure of the A. salina larvae to the lowest concentration of the carbon-based nanomaterials (0.01 mg/mL) are shown in Figure 1. As can be seen in comparison to the control, as a consequence of the ingested carbon-based nanomaterials, within 48 h the A. salina larvae showed a dark-coloured gut. This confirms that the nanomaterials were ingested and concentrated in the gut already at the lowest exposure concentrations.

3.2 Attachment of carbon-based nanomaterials on the body surfaces of A. salina larvae
SEM imaging was performed on the *A. salina* larvae treated with the suspensions of the different concentrations of the carbon-based nanomaterials. Representative images of the *A. salina* larvae gills and abdomen after 48 h exposure to 0.1 mg/mL of these different nanomaterials are shown in the middle and lower panels of Figure 2, respectively. All of the treated specimens had unequally distributed carbon-based nanomaterials attached onto their body surfaces. The carbon-based nanomaterial agglomerates were extensively attached to the gills, which caused the gill branches to fuse together, as seen in the middle panels of Figure 2.

### 3.3. Swimming speed alteration and mortality

The swimming behaviour of the *A. salina* larvae was affected by the 48-h exposure to all of the tested carbon-based nanomaterials (Figure 3), with dose-dependent inhibition of their motion. The swimming speeds decreased as much as 70% in a concentration-dependent manner after exposure of the *A. salina* larvae to the CB suspensions. The calculated EC$_{50}$ for CB was 0.93 mg/mL. GO led to a decrease in the swimming speeds by up to 68% at 0.6 mg/mL, with a calculated EC$_{50}$ of 0.16 mg/mL. Exposure to MWCNTs caused a gradual decrease in the swimming speeds at all of the tested concentrations (0.01, 0.05 and 0.1 mg/mL). At the highest tested concentration of MWCNTs (0.1 mg/mL), the swimming speed was decreased by 48.4%, with the EC$_{50}$ for these swimming speed alterations calculated as 0.23 mg/mL. Table 2 gives the EC$_{50}$ for swimming speed inhibition, and the LC$_{50}$ for mortality after 48 h for all of the tested carbon-based nanomaterials. GO apparently acts in a threshold-like manner, with no mortality at 0.6 mg/mL, and more than 90% mortality at 0.7 mg/mL. Exposure to MWCNTs did not cause mortality up to a concentration 0.1 mg/mL.

### 3.4. Cholinesterase, glutathione S-transferase and catalase activities
The activities of cholinesterase, glutathione S-transferase and catalase in the larvae of *A. salina* that had been previously exposed for 48 h to these different carbon-based nanomaterials are shown in Figure 4. All of these carbon-based nanomaterials resulted in noticeable effects on these enzyme activities, although the levels varied and the different nanomaterials did not affect all of the enzymes in the same way.

CB induced a significant and dose-dependent increase in cholinesterase activity at all of the tested concentrations (0.1, 0.1, 1.0 mg/mL) compared to the control (*p*<0.05), but no significant increase in catalase activity was observed. A dramatic increase in glutathione S-transferase activity was shown at the highest tested concentrations of CB (0.1, 1.0 mg/mL). GO induced an inhibitory effect on glutathione S-transferase, but no significant differences were observed against catalase at any of the tested concentrations. GO also induced a significant increase in the cholinesterase activity (*p*<0.05), although only at the highest concentration tested (0.5 mg/mL). MWCNTs also showed significant inhibitory effects on the glutathione S-transferase and catalase activities, although with no such effects on cholinesterase. A decrease in glutathione S-transferase activity was observed at all of the concentrations of MWCNTs, while a decrease in catalase appeared only at the lower MWCNT concentrations tested. At the highest concentration (0.1 mg/mL), there was no difference from the control. No significant differences (*p*<0.05) were found by comparing concentrations of each treatment, with the exception of glutathione S-transferase, where significant differences between 0.01 and 0.1 mg/mL, as well as between 0.01 and 1 mg/mL carbon black were found.

4. Discussion

In this study, we provide experimental evidence that 48-h exposure of *A. salina* larvae to three tested carbon-based nanomaterials leads to: (i) filling of the gut with the nanomaterials and
attachment of the nanomaterials onto the body surfaces, including the appendages and gills, at all of the exposure concentrations (0.1 to 1 mg/mL); (ii) dose-dependent inhibition of the swimming activities; (iii) no mortality except at high, and environmentally unrealistic, concentrations; and (iv) altered activities of at least one of the three measured enzymes, as e.g. cholinesterase, glutathione-S-transferase, and catalase.

Interactions of nanomaterials with organisms can be external, as attachment of the nanomaterials onto the skin or exoskeleton, or internal, via food intake, or both. All of these interactions can cause different physiological disturbances. Ingestion of nanomaterials has been documented in many studies (Garaventa et al., 2010; Kennedy et al., 2008; Li et al., 2011; Pérez et al., 2009; Pretti et al., 2014; Sohn et al. 2015). Ingestion has thus been observed for CB or SWCNT with *D. magna* (Fernandes et al., 2007; Petersen et al., 2009; Sohn et al. 2015), and for MWCNTs with *C. dubia* (Hussain et al., 2010; Li et al., 2011), and for different types of pristine graphene nanoparticles for *A. salina* (Pretti et al., 2014). Kennedy et al. (2008) reported a connection between MWCNTs in the gut and mortality in *C. dubia*. They explained the mortality as a result of physical stress, as the *C. dubia* struggle to eliminate the nanomaterials, or oxidative stress, which leads to toxicity, or direct damage of the lipid membranes, due to the high affinity of MWCNTs for lipid membranes. Our results confirm that, after 48 h of exposure, *A. salina* larvae ingested all three types of carbon-based nanomaterials at all exposure concentrations, but we did not find any link between the presence of nanomaterials in the gut and mortality. Furthermore, the effects of carbon-based nanomaterials have been discussed in terms of these various organisms moving through the water column, altering their feeding abilities (Baumerte et al., 2013; Baun et al. 2008; Roberts et al., 2007) and their reproduction and moulting (Oberdörster et al., 2006).

SEM images in the present study confirm that aggregates of all three of these carbon-based nanomaterials were attached over the entire body surface of these *A. salina* larvae,
including the gills and the appendages. When the nanomaterials are attached onto the gills, the gill branches can become fused together, which will be reflected as swimming alterations. Indeed, all of the tested carbon-based nanomaterials in the present study induced dose-dependent swimming inhibition of these A. salina larvae, with comparable EC50 values. Baumerte et al. (2013) also reported swimming inhibition of A. salina and D. magna by carbon-based nanomaterials; however, EC50 were not reported for this parameter. The EC50 obtained in this study are comparable to those obtained by Mesarić et al. (2013a) for swimming inhibition obtained after exposing A. amphitrite larvae for 48 h to CB (EC50, 0.48 mg/mL) and GO (EC50, 0.31 mg/mL), which indicate non-specific surface adsorption attachment of these carbon-based nanomaterials regardless to their shape and structure. On the contrary, under the same experimental set-up with A. salina larvae exposed for 48 h to comparable final concentrations of Fe3O4, SnO2 (Gambardella et al., 2014), and TiO2 nanomaterials (T. Mesarić, C. Gambardella, personal communication), their swimming patterns were not significantly altered. We explain these data according to the low adsorption potential of the metal-based nanomaterials (Xia et al., 2011). Another difference between carbon-based and metal-based nanomaterials is the formation of floccules in the suspensions of the carbon-based nanomaterials. These floccules result in mechanical disturbance to the swimming of the exposed larvae.

In the present study, the swimming alteration responses in these A. salina larvae were not in agreement with the variations in the enzyme activities. The cholinesterase and glutathione S-transferase activities were significantly enhanced after exposure to CB, while after exposure to GO, there was an increase in cholinesterase activity that was accompanied by a decrease in glutathione S-transferase activity. The catalase activity was not significantly affected, except with MWCNTs, although this effect was not dose-dependent. Overall, MWCNTs did not induce significant alterations in the activities of any of the tested enzymes. These data suggest that
different nanomaterials induce different effects on these different enzyme biomarkers, as was also observed in another study with metal oxide nanomaterials (Gambardella et al., 2014).

Furthermore, mortality of *A. salina* larvae did not directly correlate to any of the other responses measured. After 48 h of larval exposure to the different concentrations of CB, GO and MWCNTs, no significant mortality was observed, except at the highest exposure concentrations of CB (1 mg/mL) and GO (0.7 mg/mL), where the respective LC50 of 1.89 mg/mL and 0.65 mg/mL were calculated. Similarly, a lack of mortality has been observed after 48-h exposure of *A. salina* larvae to comparable concentrations (up to 1 mg/mL) of metal oxide nanomaterials (Gambardella et al., 2014). The lowest LC50 in the case of GO is in line with other studies on different organisms, which indicated that graphene nanomaterials are more toxic than the conventional carbon nanomaterials like carbon black (Zhao et al., 2014). Baumerte et al. (2013) also investigated the effects of CB and MWCNTs on *A. salina* larvae after 48-h exposure, and they reported LC50 of 0.013 mg/mL for MWCNT and 0.03 mg/mL for CB, respectively. These differences might derive from the different characteristics of the suspended nanomaterials. Indeed, the toxicity of carbon-based nanomaterials on freshwater crustaceans was reported to be dependent both on the nanomaterial preparation (Lovern and Klaper, 2006; Zhu et al., 2006) and on the nanomaterial diameter (Li et al., 2011). These findings suggest the need for a thorough characterisation of these nanomaterials, and also for the standardisation of experimental protocols for the safety evaluation of these compounds.

Finally, the goal of eco(nano)toxicity studies is to provide data for environmental risk assessment. Here, the PECs and PNECs for each environmental compartment (i.e., air, water, soil) are required (Aschberger et al., 2011). The ecological risk for a certain compound is expressed by the risk quotient (RQ) of the PEC and PNEC (Muller and Nowack, 2008; Quik et al., 2008). Based on the PECs for CB that were calculated in heavily polluted fresh waters (0.08 to 7.5 µg/mL (Screening Assessment for the Challenge, 2013)), and considering the lowest
effective concentration of *A. salina* larvae swimming inhibition as the PNEC, we calculated the RQ for CB to be between 0.008 and 0.75. As aggregation in marine environments is more intensive than in freshwater, the toxic potential and risk in marine environments is expected to be even lower. Similarly, when the PECs calculated in fresh waters for CNT (up to 67 pg/mL, (Gottschalk et al., 2013a; Gottschalk et al., 2013b)) are applied also as PECs in the marine environment, our calculated RQ is lower than 0.0067. Different studies, including the present, have shown that CNTs are not of immediate concern and need to be considered only when new toxicity data become available, or when significant increases in CNT discharge become evident. The PECs for GO are not available in the literature yet; however, these nanomaterials are considered as suitable materials for pollution clean-up, and are likely to be synthesised on large scales and at low prices in the near future (Zhao et al., 2011).

5. Conclusions

Together with data published by Gambardella et al. (2014), the present study provides experimental confirmation of the differences in the adsorption potentials of the different nanomaterials, where as reported by Xia et al. (2011), these carbon-based nanomaterials have the highest, and metal-based nanomaterials the lowest, surface adsorption energies. We show here that all three of the tested carbon-based nanomaterials (i.e., CB, GO, MWCNTs) affect the swimming of these *A. salina* larvae in dose-dependent manners and with comparable EC$_{50}$, which indicates nanomaterial-independent attachment onto their external surfaces, or mechanical disturbance due to the formation of large flocculates in the marine water.

The enzyme biomarkers were also affected by these nanomaterials, and their responses differed according to the type of tested nanomaterials.
Finally, the PECs for the two tested nanomaterials that are reported in the literature, and the PNECs obtained in the present study give RQs in range of 0.008 to 0.75 for CB, and <0.0067 for CNTs. This indicates that these carbon-based nanomaterials currently do not present any serious risk for the marine environment.

Acknowledgements

The authors gratefully acknowledge Dr. Chris Berrie for critical reading and appraisal of the manuscript, Mr. Matej Hočevar for SEM imaging, and the Slovene Human Resources Development and Scholarship Fund, for financial support of Tina Mesarič. The research underlying this study has received funding from the European Community Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 263147 (NanoValid - Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials).
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Figure captions

**Figure 1. Ingestion of carbon-based nanomaterials by* A. salina* larvae.** Representative microscopy images show *A. salina* larvae after 48 h exposure to the lowest concentration (0.01 mg/mL) of the carbon-based nanomaterials. The guts are empty in the control group of larvae, but filled with material in the exposed larvae. CB, carbon black; GO, graphene oxide; MWCNTs, multiwall carbon nanotubes. Bar, 200 µm.

**Figure 2. Attachment of carbon-based nanomaterials onto the* A. salina* body surface.** Upper panels: Representative SEM of the carbon-based nanomaterials on the membrane surface. Middle and lower panels: Representative SEM of the carbon-based nanomaterials on gills (middle) and ventral abdominal side (lower) of the *A. salina* larvae after 48 h of exposure (0.1 mg/mL). CB, carbon black; GO, graphene oxide; MWCNTs, multiwall carbon nanotubes.

**Figure 3. Effect of carbon-based nanomaterials on mortality (black bars) and swimming speed alteration (white bars) of* A. salina* larvae after 48 h of exposure.** Data are means ±standard error (n = 3). CB, carbon black; GO, graphene oxide; MWCNTs, multiwall carbon nanotubes.

**Figure 4. Effects of carbon-based nanomaterials on enzyme biomarkers in the* A. salina* larvae.** Cholinesterase (upper panels), glutathione S-transferase (middle panels) and catalase (lower panels) activities in *A. salina* larvae exposed to the different concentrations of the nanomaterials, as indicated. Data are means ±SE (n = 3), as analysed by ANOVA followed by Bonferroni post-hoc tests. * p<0.05. CB, carbon black; GO, graphene oxide; MWCNTs, multiwall carbon nanotubes.