Title:

Effects of nano carbon black and single-layer graphene oxide on settlement, survival and swimming behaviour of *Amphibalanus amphitrite* larvae

Authors:

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Abstract

The effects of two carbon-based nanomaterials, nano-sized carbon black (nCB) and single-layer graphene oxide (GO), on settlement of *Amphibalanus amphitrite* (Cirripedia, Crustacea) cypris larvae (cyprids) were assessed after 24, 48 and 72 h of exposure. Additionally, the effects of these nanomaterials on the mortality and swimming behaviour of the nauplius larvae (nauplii) of the same organism were determined after 24 and 48 h. The data indicate that nCB is more effective as a potential antisettlement agent than single-layer GO; moreover, nCB did not show any adverse effects on the larvae. The swimming behaviour of II stage nauplii of *A. amphitrite* exposed to a suspension of nCB was inhibited only at very high nCB concentrations (≥0.5 mg/mL). Single-layer GO, on the contrary, showed lower antisettlement effects and was more active in altering the survival and inhibiting the swimming behaviour of the nauplii. An indication of the toxic or non-toxic mechanisms of the antisettlement properties of both of these nanomaterials is provided by the reversibility of the antisettlement activity. In conclusion, we propose nCB as an innovative antifouling nanomaterial that shows low toxicity towards the model organism (crustaceans) used in this study.

Keywords: *Amphibalanus amphitrite*; antifouling; nanotoxicity, nanomaterials; nano carbon black; single-layer graphene oxide.

Word count: 3,041.

1. Introduction

From the initial adsorption of organic molecules, through the colonisation by microorganisms, to the development of complex sessile assemblages, biofouling affects most man-made surfaces, such as ship hulls, oilrigs, mariculture cages, pipelines, heat exchangers and seawater intakes in general, which results in significant economic costs [1]. Biofouling is a problem that can affect any artificial device in contact with water, so different antifouling strategies are being designed to prevent the attachment and growth of microfouling and macrofouling organisms on man-made submerged surfaces.

Most of the commonly used antifouling paints contain biocides. The key property of a good antifouling biocide with respect to the environment is that it is effective in preventing fouling without causing persistent adverse environmental effects. The effects and behaviour of biocides used in antifouling paints have been extensively studied [2,3]. Currently, a wide range of chemicals are used as antifouling biocides, which are governed by different regulations, and which depend on the legislation in each country. In the search for innovative and less harmful antifouling materials, several metal nanomaterials are being tested, and these have shown promising results to date for the prevention of biofilm formation [4-8]. Studies on the antisettlement effects of carbon-based nanomaterials have also been reported recently; e.g., synthetic carbon nanotubes have been shown to be very efficient for the prevention of the formation of microbial biofilms [9], as well as against fouling by zooplanktonic and phytoplanktonic larvae [10]. It was also recently shown that graphene oxide (GO) can be used for the prevention of protein adsorption in microfluidic systems [11]. To our knowledge, no data on antisettlement effects of other carbon nanomaterials towards microfouling and macrofouling have been reported. Furthermore, although some different nanomaterials have shown good potential as antifouling agents, present knowledge relating to their adverse

effects in aquatic environments is very limited [12-14]. It is therefore very important to gain more data about the effects of these materials before they are used globally, and before different ecosystems are exposed to them.

The aim of the present study was therefore to evaluate the effects of two different carbon nanomaterials, nano-sized carbon black (nCB) and single-layer GO, on the settlement of *Amphibalanus amphitrite* cypris larvae (cyprids), combined with an assessment of their toxicity towards the naupliar larval stage (nauplii) of the same organism. The barnacle, *A. amphitrite*, is a cosmopolitan cirriped crustacean that represents one of the main species of biofouling organisms. Indeed, it is reared and used mainly as a model organism for efficacy and toxicity testing of antifouling products. The ease of obtaining and rearing its larval stages has extended its use also as a model for ecotoxicological studies [15,16].

Nano-sized carbon black is a form of amorphous carbon that has a high surface area-to-volume ratio. It is a product of the incomplete combustion of fossil fuels and vegetation [17], and it is used in rubber production, as a black pigment in printing inks, as electrodes in batteries, and in leather production [18]. Graphene oxide is a two-dimensional nanomaterial that has single, double or a few (≤10) layers of carbon atoms arranged in six-membered rings [19]. It has unique electrical, chemical and mechanical properties, such as a large surface area, excellent conductivity, high mechanical strength, and ease of functionalisation and mass production [20]. For this reason, GO is one of the most exciting materials under investigation. In the present study, we used single-layer GO; e.g., a graphene sheet with additional oxygen functional groups attached to its edges (carboxylic groups) or to its basal plane (phenol hydroxyl and epoxide groups) [21].

2. Materials and methods

2.1. Materials

The nCB powder was provided by PlasmaChem GmbH (Berlin, Germany), with the average size of the primary particles given as 13 nm. Dry flakes of single-layer GO were purchased from Graphene Supermarket (USA). According to the producer, this GO is composed of carbon (79%) and oxygen (20%). The flake size was assessed as between 0.5 μm and 5 μm. Stock suspensions were prepared of 1 mg/mL nCB or single-layer GO, in 0.22-μm-filtered natural sea water (FNSW); these were sonicated for 15 min using a 50% on/off cycle. These stock suspensions were kept on ice during the preparation. Further characterisation of the secondary characteristics of these nanomaterials (e.g., dynamic light scattering, ζ-potential) was not possible as they did not form stable suspensions in FNSW.

2.2. Methods

2.2.1. Settlement test

The cypris larval phase of *A. amphitrite* is competent for metamorphosis and settlement, and these cyprids were obtained from laboratory cultures of a brood stock. Before being used in settlement assays, the larvae were filtered and maintained in FNSW at 4 °C for 3 days [22]. After 3 days, the settlement tests were performed by adding 15-20 cyprids to each well of a 24-well polystyrene plate that contained 2 mL of different concentrations of the suspended nCB (0.01, 0.1, 0.2, 0.3 mg/mL) or single-layer GO (0.01, 0.1, 0.5 mg/mL). The plates were stored at 28 °C for 72 h, under 16-h:8-h light/dark cycles. All of the settlement tests were performed in triplicate. After 24, 48 and 72 h, the number of settled and non-settled cyprids was examined under a stereomicroscope. The concentration of the nanomaterial that resulted in 50% settlement inhibition of these exposed organisms ($EC_{50[cyprids]}$) was calculated at 72 h. Additionally, after 72 h, the settlement recovery of the cyprids was tested: the unsettled larvae

were collected from the wells at 72 h, and they were rinsed and transferred into new plates with clean FNSW. After an additional 24 h at 28 °C, the settlement percentage of these transferred larvae was determined again.

2.2.2. Naupliar toxicity test

The nauplii were obtained from laboratory cultures of a brood stock of *A. amphitrite.* This toxicity test was performed by adding 15 to 25 II stage nauplii to each well of a 24-well polystyrene plate that contained 1 mL of different concentrations of the suspended nCB (0.1, 0.5, 1, 5 mg/mL) or single-layer GO (0.001, 0.01, 0.1, 0.5, 0.75, 1 mg/mL). The plates were stored at 20 °C for 24 h and 48 h, under 16-h:8-h light/dark cycles. All of the toxicity tests were performed in triplicate. After 24 h and 48 h, the nauplii were collected from the wells, rinsed, and transferred into new plates with clean FNSW. The number of dead larvae was determined by examination under a stereomicroscope. The concentration of the nanomaterial that resulted in 50% mortality of these exposed organisms ($LC_{50[nauplii]}$) was calculated after 24 h and 48 h of exposure.

2.2.3. Naupliar swimming speed alteration test

The naupliar swimming speed alteration test was performed according to [15, 23]. Briefly, 15 to 25 II stage nauplii were exposed to the nanomaterials as described in paragraph 2.2.2. After 24 h and 48 h, the swimming of the nauplii was recorded using a Swimming Behavioural Recorder experimental set-up. A Swimming Behavioural Recorder consists of a video camera with a macro-objective that records the paths of a sample of larvae swimming. The apparatus is caged inside a black box (60 × 60 × 100 cm) to exclude external sources of light, and the recording chamber is monitored under infrared light. The nauplii were dark-adapted for 2 min before starting the video recording (a time that was fixed in preliminary tests, to allow their

steady speed and uniform spatial distribution). The swimming behaviour was digitally recorded for about 3 s at 25 frames/s, and the images were analysed using advanced image processing software (the Swimming Behavioural Recorder system developed by e-magine IT, Genova, Italy). This analysis provided reconstructions of individual nauplius path-tracks, and measurements of the average swimming speed (mm/s) for each sample (of 15-20 individuals). The data are finally referred to as the swimming induction or inhibition, which was normalised to the average swimming speed of the control (where S is the average swimming speed), and Inhibition (%) = $((S_{Treated} - S_{Control}) / S_{Control}) \times 100$). After the image analysis, the presence/ absence of individual tracks of each sub-sample of larvae (control and treated groups) provided the estimation of the swimming induction or inhibition.

2.2.4. Statistical analysis

The $EC_{50[cyprids]}$, as the settlement inhibition of the cyprids after 72 h, the $EC_{50[nauplii]}$, as the swimming speed effects on the nauplii after 24 h and 48 h, and the $LC_{50[nauplii]}$ mortality, and their related 95% confident limits after 24 h and 48 h, were calculated using trimmed Spearman–Karber analysis [24].

3. Results

3.1. Settlement test

The results of the settlement inhibition test are shown in Figure 1. **[insert Figure 1 near here]** Both of these carbon nanomaterials showed dose-dependent antisettlement effects on the *A. amphitire* cypris larvae. The antisettlement effect of nCB on these cyprids was prominent at all exposure times (24 h, 48 h, 72 h) at nanomaterial suspensions ranging from 0.01 mg/mL to 0.3 mg/mL. After 24 h of exposure to 0.01 mg/mL nCB, complete settlement

inhibition was observed. At 48 h and 72 h, the inhibition effect of nCB decreased. In the concentration range from 0.1 mg/mL to 0.3 mg/mL, nCB completely inhibited the settlement of the cyprids at all exposition times. The calculated $EC_{50[cyprids]}$ (after 72 h) was 0.03 mg/mL. Single-layer GO had slightly lower effects in terms of settlement inhibition. The data show a more gradual decrease in the settlement inhibition at all of the exposure times (24 h, 48 h, 72 h) and complete inhibition at 0.5 mg/mL of single-layer GO only after 24 h and 48 h, while after 72 h, the settlement percentage was around 40%. The calculated $EC_{50[cyprids]}$ (after 72 h) was 0.15 mg/mL.

Interestingly, in both cases, the inhibition of settlement showed a reversible effect after rinsing the cyprids and exposing them for 24 h to FNSW without added nCB or single-layer GO. In the case of nCB, the reversibility was tested at all of the concentrations used, while single-layer GO was assayed only at the highest concentration of 0.5 mg/mL, due to the very high cost of the graphene particles. In both cases, at the highest tested concentration (0.3 mg/mL nCB, 0.5 mg/mL single-layer GO), the recovery from the settlement activity was complete.

3.2. Naupliar toxicity and swimming test

The stage II nauplii of *A. amphitrite* behaved in different ways when they were exposed to the two nanomaterials tested.

The data showing the adverse effects of exposure of the nauplii of *A. amphitrite* to nCB for 24 h and 48 h are illustrated in Figure 2. **[insert Figure 2 near here]** Here, following small increases in the swimming behaviour at both 24 h and 48 h, higher concentrations of nCB induced increasing inhibition of the swimming behaviour. Also, although after 24 h of nCB there was no mortality of the nauplii at any of the nCB concentrations (Fig. 2A), after the 48-h exposure time, there was naupliar mortality at 5 mg/mL Ncb (Fig. 2B).

Similar effects, although at lower concentrations of nanomaterial, were observed after exposure of the nauplii to single-layer GO for 24 h (Fig. 3A) and 48 h (Fig. 3B). **[insert Figure 3 near here]** These indicate that increasing concentrations of single-layer GO and increasing exposure times lead to decreases in their swimming speed and to increases in their mortality. Again, as with nCB, although more pronounced, at the lower single-layer GO concentrations, there was induction of an increase in the swimming behaviour. This phenomenon was observed at up to 0.5 mg/mL and 0.01 mg/mL single-layer GO after 24-h and 48-h exposure, respectively. Higher concentrations of single-layer GO led to increased mortality and decreased swimming speeds, both of which occurred in a concentration-dependent manner particularly after the 48-h exposure time.

Table 1 gives the calculated $LC_{50[nauplii]}$, the swimming speed inhibition $EC_{50[nauplii]}$ and settlement inhibition $EC_{50[cyprids]}$ after 24 h and 48 h. The $LC_{50[nauplii]}$ and swimming speed inhibition $EC_{50[nauplii]}$ were not calculable after 24 h of nCB exposure, conversely to the single-layer GO exposure. **[insert Table 1 near here]**

The therapeutic ratio is the ratio between the amount of a therapeutic agent that provides a benefit and the amount that causes harm (i.e., death or toxicity). Thus, here the therapeutic ratio is defined as $LC_{50[nauplii]}$/ settlement inhibition $EC_{50[cyprids]}$, and as indicated in Table 1, the calculated therapeutic ratios are 61.3 for nCB and 3.7 for single-layer GO.

4. Discussion

The carbon-based nanomaterials used in this study show antifouling effects on *A. amphitrite* larvae, causing 50% inhibition of settlement ($EC_{50[cyprids]}$) at concentrations of 0.03 g/mL nCB and 0.15 g/mL single-layer GO. These calculated EC_{50} values are higher with respect to the antifouling booster biocides, such as zinc-pyrithione, with an EC_{50} of 2×10^{-5} mg/mL [25],

223 although particularly in the case of nCB, the settlement inhibition $EC_{50[cyprids]}$ is comparable to
224 the EC_{50} obtained for antifouling compounds of natural origin [26].

225 Both nCB and single-layer GO induced increases in naupliar mortality, and decreases
226 in the swimming speed of the nauplii in concentration-dependent manners. In addition, the
227 nauplii exposed to both of these carbon-based nanomaterials show hormetic dose-response
228 curves at 24 h and 48 h, whereby there is a stimulatory effect at low concentrations, and an
229 inhibitory effect at higher concentrations.

230 The antisettlement activities induced by nCB and single-layer GO appear not to be due
231 to the toxicity of these selected nanomaterials, since the naupliar mortality at concentrations
232 that completely inhibited settlement was very low (*ca*. 2%) for nCB and about 20% for single-
233 layer GO. To define this further, we also calculated the therapeutic ratio (or index), which is
234 generally given as the ratio between the amount of a therapeutic agent that provides a benefit
235 and the amount that causes harm (i.e., death or toxicity). Here, the therapeutic ratio
236 ($LC_{50[nauplii]}$/ settlement inhibition $EC_{50[cyprids]}$) is thus an index used to estimate whether
237 settlement inhibition is due to toxicity or other mechanisms, as has also been calculated across
238 a number of similar studies [22, 27, 28]. A review from Qian et al. [29] on antifouling agents
239 underlined how the therapeutic ratio is commonly used as a yardstick of the potential
240 antifouling activity of a compound. In this review, it was suggested that a compound with a
241 therapeutic ratio >15 can be considered as a non-toxic antifouling compound, even if a much
242 higher therapeutic ratio is recommended when selecting candidate compounds. Furthermore,
243 Qian et al. [29] suggested that only a therapeutic ratio >50 and an EC_{50} <5 mg/L for the
244 inhibition of both hard and soft fouling organisms should allow the antifouling compound to
245 be considered as environmentally friendly. Following these recommendations, only nCB
246 shows both of these requisites; conversely, single-layer GO does not satisfy these criteria.
247 Moreover, an indication of the non-toxicity of the antifouling mechanism is provided by

consideration of the reversibility of the antisettlement activity: after the 72-h incubation with nCB or single-layer GO, when the cyprids were rinsed and transferred to clean FNSW, they settled again, thus completely regaining their settlement ability after 24 h, when the percentage of settlement was comparable to that obtained in the control settlement assay.

The exact mechanism of the antisettlement activity of the nanomaterials used in the present study remains to be clarified, and will be one of the main subjects of our future studies. The antisettlement effects of both of these nanomaterials used might be due to a simple mechanical interaction of these nanomaterials with the *A. amphitrite* cypris larvae, as both of these nanomaterials were not stable in FNSW. Such nanomaterials might alter the points of contacts of the cyprid antennular discs with the surface during the exploration phase before the settlement, which might therefore inhibit the whole process. In other words, the nanomaterials that precipitated to the bottom of the each well of the 24-well polystyrene multiwell plates (and on other submerged surfaces in natural environments) might alter the roughness of the surface, thus decreasing drastically the possibility of larval attachment. The effect of a nanostructured surface on the settlement of various representatives of fouling marine macroorganisms has also been shown recently by Scardino et al. [30]. In their study, superhydrophobic coatings with different physical architectures were prepared by bonding fumed silica nanomaterials to polysiloxane and its derivatives. Scardino et al. [30] showed a clear correlation between the settlement inhibition and the presence of “nanoroughness”.

Finally, very little or nothing is known about the adverse effects of nCB or single-layer GO on marine organisms. Rosenkranz et al. [31] reported that the LC_{50} of nCB to freshwater *Daphnia magna* after 96 h is 0.4 mg/L. Our data show lower effectiveness of nCB versus *A. amphitrite* after 24 h and 48 h, where the $LC_{50[nauplii]}$ was 1.84 mg/mL, while the single-layer GO showed a lower $LC_{50[nauplii]}$ (0.56 mg/mL). The secondary characteristics of these nanomaterials, such as those expressed by dynamic light scattering and ζ-potential,

might contribute to an understanding their bioactivity. Thus, their detailed particle characterisation is needed to have a better view towards an understanding the results of the present study.

5. Conclusions

Two carbon-based nanomaterials were tested here to determine their effects on the settlement, mortality and swimming behaviour of *A. amphitrite* larvae, to investigate the possibility of finding new compounds for antifouling applications that show low toxicity. Our data indicate that nCB has less pronounced effects on the behaviour and survival of *A. amphitrite* larvae while having a considerable antisettlement effect. Single-layer GO shows more pronounced adverse effects, together with a lower antisettlement effects compared to nCB. Both of these nanomaterials can still be considered as potentially suitable as antifouling nanomaterials that might be incorporated into innovative paint formulations, although further investigations of their secondary characteristics, their toxicities towards other classes of organisms, and their behaviour in marine environments are required.

Acknowledgements

The authors would like to gratefully acknowledge the Slovene Human Resources Development and Scholarship Fund, for financial support of Tina Mesarič, and for her stay in Genova, Italy. The authors acknowledge the Italian Sea Research (RITMARE) Flagship Project, a National Research Programme funded by the Italian Ministry of Universities and Research, and Dr. Chris Berrie for editing and appraisal of the manuscript.

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389 **Table 1**. Calculated $LC_{50[nauplii]}$, swimming speed inhibition $EC_{50[nauplii]}$, settlement inhibition $EC_{50[cyprids]}$, and therapeutic ratio from the present

390 study.

391

Nanomaterial	**$LC_{50(nauplii)}$**		**Swimming speed inhibition $EC_{50(nauplii)}$**		**Settlement inhibition $EC_{50(cyprids)}$**	**Therapeutic ratio***
	(24 h)	**(48 h)**	**(24 h)**	**(48 h)**	**(72 h)**	
nCB	>1	1.84 (1.66-2.03)	nc	0.48 (0.42-0.55)	0.03 (0.03-0.04)	61.3
Single-layer GO	<1 (nc)	0.56 (0.51-0.61)	0.84 (0.82-0.86)	0.31 (0.27-0.35)	0.15 (nc)	3.7

392

393 nCB, nano-sized carbon black

394 GO, graphene oxide

395 Data are means (95% confident limits)

396 nc, not calculable; SSA, swimming speed alteration**.**

397 *, $LC_{50[nauplii]}$ (48 h)/ settlement inhibition $EC_{50[cyprids]}$

398

Figure captions

Figure 1. Settlement of *A. amphitrite* cypris larvae after 24, 48 and 72 h with different concentrations of nCB (**A**) and single-layer GO (**B**). Data are means ±standard error (n = 3). The settlement reversibility (after 96 hours) is also shown for these cyprids (nCB, at all tested concentrations; single-layer GO, only at 0.5 mg/mL).

Figure 2. Effect of nCB on mortality (black bars) and swimming speed (white bars) of *A. amphitrite* nauplii after 24 h (**A**) and 48 h (**B**). Data are means ±standard error (n = 3).

Figure 3. Effects of single-layer GO on mortality (black bars) and swimming speed (white bars) of *A. amphitrite* nauplii after 24 h (**A**) and 48 h (**B**). Data are means ±standard error (n = 3).