An integrated approach to determine interactive genotoxic and global gene expression effects of multiwalled carbon nanotubes (MWCNTs) and benzo[a]pyrene (BaP) on marine mussels: evidence of reverse ‘Trojan Horse’ effects

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ABSTRACT
The interactions between carbon-based engineered nanoparticles (ENPs) and organic pollutants might enhance the uptake of contaminants into biota. The present integrated study aimed to assess this potential ‘Trojan Horse’, probing the interactive effects of purpose-made multi-walled carbon nanotubes (MWCNTs), a representative ENP, and benzo[a]pyrene (BaP), a ubiquitous polycyclic aromatic hydrocarbon (PAH) pollutant, on the marine mussel Mytilus galloprovincialis. Mussels were exposed to MWCNTs and BaP either alone or in various combinations. The co-exposure of BaP with MWCNTs revealed that the presence of MWCNTs enhanced the aqueous concentrations of BaP, thereby reducing the uptake of this pollutant by mussels as evidenced by lowering BaP concentrations in the tissues. Determination of DNA damage (comet assay) showed a concentration-dependent response for BaP alone which was absent when MWCNTs were present. Global gene expression using microarray analyses indicated that BaP and MWCNTs, in combination, differentially activated those genes which are involved in DNA metabolism compared to the exposures of BaP or MWCNTs alone, and the gene expression response was tissue-specific. Mechanisms to explain these results are discussed and relate primarily to the adsorption of BaP to MWCNTs, mediated potentially by van der Waals interactions. The use of a novel approach based on gold-labeled MWCNTs to track their uptake in tissues improved the traceability of nanotubes in biological samples. Overall, our results did not indicate the ‘Trojan Horse’ effects following co-exposure to the contaminants and clearly showed that the adsorption of BaP to MWCNTs modified the uptake of the pollutant in marine mussels.

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Introduction
The production of manufactured or engineered nanoparticles (ENPs) and nanomaterials (NMs) has grown extensively over the last few years and they are entering into the environment (Giese et al. 2018). Both academic and industrial researchers are extensively exploring their unusual, size-dependent properties to develop the next generation of functional materials. The research effort has led to the exploration of a number of promising applications of ENPs and NMs, particularly in the health sector, where their potential utilization as targeted drug delivery agents are being investigated (Cho et al. 2008). However, to date, most of the actual applications of ENPs and NMs are associated with consumer products, e.g. in cosmetics, food, and food packaging, paints, and coatings (Foss...
Hansen et al. 2016). In fact, in January 2019, it was reported that more than 3000 products in Europe alone contained NPs and NMs, with the database having grown by 1000 products over the course of just 18 months (Foss Hansen et al. 2016; http://nanodb.dk/). Yet, whilst the benefits and improvements offered by nanotechnology are well established, significant concern regarding the potential risks have been raised. This stems from the fact that nanoscale materials, in common with other pollutants, can enter the aquatic environment through different routes (Giese et al. 2018) with very little research to assess their potential impact on human health and the natural environment established.

Among ENPs, carbon-based nanoparticles, especially carbon nanotubes (CNTs), are of major commercial interest, currently incorporated in a diverse range of commercial products, from rechargeable batteries and automotive parts to sporting goods and in water filters (De Volder et al. 2013). CNTs have the highest production volumes among engineered carbonaceous nanomaterials worldwide (Gottschalk, Sun, and Nowack 2013), exceeding 730 tonnes per year in Europe (Sun et al. 2016). Based on this production data, the latest predictions estimate the environmental concentrations of CNTs in surface water at 0.28 ng L\(^{-1}\) (\(Q_{0.15} = 0.04\) ng L\(^{-1}\); \(Q_{0.85} = 0.65\) ng L\(^{-1}\)) (Sun et al. 2016). With a substantial rise in the production and use of CNTs expected over the coming years, the concentrations of CNTs in all environmental compartments, including the aquatic environment, will inevitably increase. Of particular concern are multi-walled carbon nanotubes (MWCNTs) as they are presently used to reduce the biofouling of ship hulls by discouraging the attachment of algae and barnacles (Beigbeder et al. 2008) and hence are directly released into the marine environment.

Whilst the presence of nanoscale materials in seawater represents a significant current issue, it is, in fact, their potential combination with other ubiquitous contaminants that represents a far more pressing concern. Carbon nanotubes, possessing a large hydrophobic surface area, have a high adsorption capacity for hydrophobic molecules and thus a high affinity for co-released environmental pollutants. Among these contaminants, polycyclic aromatic hydrocarbons (PAHs) represent a significant concern. One of them, benzo[a]pyrene (BaP), is a known mutagenic and carcinogenic contaminant, present in human foods as well as being ubiquitous in environmental samples (Sogbanmu et al. 2016; Acevedo-Whitehouse et al. 2018; Di et al. 2011). It is on the list of the priority pollutants of the European Water Framework Directive (2000/60/CE) and is also a monitored PAH of the United States Environmental Protection Agency (USEPA). To date, few studies have investigated the interactive effects of carbon-based nanoparticles with different environmental pollutants on aquatic organisms, especially marine organisms, with significant inconsistencies in the results reported (reviewed in Canesi, Ciacci, and Balbi 2015; Barranger et al. 2019). Indeed, available data on the combined effects of CNTs and other contaminants in aquatic organisms are inconclusive, if not conflicting. Some studies indicate that the adsorption of pollutants to CNTs reduces their bioaccumulation in organisms (Ferguson et al. 2008), whereas others highlight that co-exposure with CNTs may amplify the toxic effects of other compounds by increasing their cellular uptake and accumulation (Sun et al. 2014). These conflicting results could be explained by current evidence suggesting that the variability in bioavailability of CNT-adsorbed organic contaminants is largely driven by the size, configuration, and surface area coverage of the contaminant on the nano-material (Linard et al. 2017).

In addition to conflicting genotoxicological studies, one of the major challenges that has limited the investigation of CNT uptake in environmental studies is the lack of a method to quantify them in biological or environmental media. A recent review (Bjorkland, Tobias, and Petersen 2017) highlighted that CNTs have been detected in environmental matrices and organisms using a broad range of analytical techniques, including optical spectroscopies, electron microscopy, thermal methods, and radio-labeling. However, these methods are generally qualitative or at best quantitative with some bias and do not precisely determine the concentration of CNTs in organisms.

With knowledge of the above information, in this study, we evaluated the interactions between MWCNTs and the ubiquitous environmental pollutant BaP on marine mussels. Firstly, we analyzed the aggregation properties and genotoxicity of MWCNTs using light scattering and two different
assays (the comet and the micronucleus assays), respectively. Secondly, in a series of experiments where mussels were co-exposed to MWCNTs and BaP at different concentrations, we measured the uptake, expression of genes related to DNA metabolism as well as DNA damage and bulky DNA adduct formation in mussels. Finally, we probed the novel application of gold-labeled MWCNTs as a method to improve the tracking of carbon nanotubes in mussel tissues, utilizing both spectroscopy and electron microscopy approaches. This integrated and interdisciplinary strategy allowed us to test whether the combination of BaP with MWCNTs induces molecular pathways different to those observed for the pollutant alone and thus the viability of MWCNTs as a potential ‘Trojan Horse’ effects.

Materials and methods

Mussel collection and maintenance

Mussels (Mytilus galloprovincialis; 45–50 mm) collected from the intertidal zone at Trebarwith Strand, Cornwall, a reference site (50°38’ 40” N, 4°45’ 44” W) were maintained under laboratory conditions prior to experimentations as described previously (Dallas et al. 2013; D’Agata et al. 2014; Vernon and Jha 2019).

Preparation and characterization of MWCNTs

MWCNTs were purchased from NanoLab, USA (PD30L520, synthesized by chemical vapor deposition). In the absence of a single Organisation for Economic Cooperation and Development (OECD) standard for MWCNTs (OECD Environmental, Health and Safety Publications Dossier No. 68), these MWCNTs were selected owing to their structural and physicochemical similarities to other MWCNTs commonly reported in the wider literature. Moreover, the study of MWCNTs, rather than single-walled carbon nanotubes (SWCNTs), was deemed more imperative given their greater commercial application and thus propensity to be released into the natural environment. To homogenize the nanotube length distribution, MWCNTs were shortened and purified using a multi-step strategy based on site-selective catalytic oxidation (La Torre et al. 2010; Miners et al. 2014). In studies on bivalve molluscs, ENPs and NMs are typically characterized in seawater without animals (Gomes et al. 2013; D’Agata et al. 2014), which is not strictly speaking representative of the experimental conditions. In order to better replicate the conditions of the experiment during analysis, mussels (4.5 mussels L⁻¹) were maintained in 2-L glass beakers for 24 h with natural seawater from Plymouth Sound (filtered at 10 μm). Subsequently, MWCNTs (1 mg) were added to the mussel-exposed seawater (10 mL) and the suspension homogenized by ultrasonication (Langford Sonomatic 375, 40 kHz) for 1 h at room temperature. The suspension was allowed to settle for at least 4 h at room temperature prior to analysis of the aggregate size. Control measurements of aggregate size in (i) seawater, in the absence of mussels and (ii) mussel-exposed seawater in the presence of BaP (1 mg L⁻¹) were additionally performed. Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano-ZS at room temperature. Quoted values are the average of 2–3 measurements. Bright field transmission electron microscopy (TEM) was performed using the JOEL 2100+ microscope operated at 200 keV. Energy dispersive X-ray (EDX) spectra were acquired using an Oxford Instruments INCA X-ray microanalysis system and processed using Aztec software. Samples were prepared by casting several drops of the respective suspensions onto copper grid-mounted lacey carbon films.

Exposure of Mytilus galloprovincialis to MWCNTs

Experimental design

After depuration, the mussels were transferred to 2-L glass beakers containing 1.8 L of the same seawater as above and allowed to acclimatize for 48 h. Two mussels were used per beaker. A photoperiod of 12 h light, 12 h dark was maintained throughout the experiment. Good seawater oxygenation was provided by a bubbling system. The seawater quality was monitored in each of the beakers by measuring the salinity (36.65 ± 0.18‰), pH (7.98 ± 0.04), percentage of dissolved oxygen (97.09 ± 1.28%) and temperature (14.89 ± 0.13 °C). Mussels were exposed to different treatments for 7 days: a seawater control (12 mussels); a positive control (0.04 mg L⁻¹ CuSO₄; 12 mussels); 0.01 mg L⁻¹ MWCNTs (12 mussels);
0.1 mg L⁻¹ MWCNTs (12 mussels); and 1 mg L⁻¹ MWCNTs (12 mussels). MWCNTs were pre-weighed in glass vials for each beaker according to the required final concentration (0.018 mg, 0.18 mg and 1.8 mg to reach 0.01, 0.1 and 1 mg L⁻¹ respectively) and directly tipped in the beakers. Mussels were not fed and did not spawn during the experiment. At the end of the exposure, 10 mussels were sampled for each treatment. Two different tissues, gills and digestive gland (DG) were used to perform the comet assay and the micronucleus assay. Gill and digestive gland cells were chosen to unravel the potential interactive toxic effects of MWCNTs and BaP. In mussels, feed particles are filtrated first through gills and transported up to the digestive gland (DG) to be metabolized. This leads to high bioaccumulation of contaminants in this tissue. It is to be noted that as an analogue to mammalian liver, enzymes involved in the biotransformation of both endogenous and exogenous substrates are mainly localized in the digestive gland of mussels (Livingstone and Pipe 1992; Akcha et al. 1999).

Comet assay to determine DNA damage
The comet assay on gill and digestive gland (DG) cells (n = 10) was performed as previously described by us (Dallas et al. 2013; Banni et al. 2017; Vernon and Jha 2019).

Micronucleus assay
For the micronucleus assay, tissues (i.e. gill and DG) were digested with the same protocol as for the comet assay. The cell suspension was spread gently across the slide with a clean cover slip, allowed to adhere onto the slides by placing in the fridge for 1 h. To fix the cells, the dried slides were immersed in a coupling jar containing Carnoy’s fixative for 20 min. Following fixation, the slides were stained with 20 μl of ethidium bromide (20 μg mL⁻¹). Slides were randomized and scored under the microscope for the induction of micronuclei. At least 1000 cells were scored from each slide (two slides per individual mussel) according to the detailed criteria described elsewhere (Bolognesi and Fenech 2012; Dallas et al. 2013; Vernon and Jha 2019).

Exposure of Mytilus galloprovincialis to BaP and MWCNTs
Experimental design
In a separate experiment, mussels were exposed for 3 days (with no water changes) to BaP alone (5, 50 and 100 μg L⁻¹), MWCNTs alone (1 mg L⁻¹) and a combination of BaP and MWCNTs (1 mg L⁻¹ MWCNTs + 5 μg L⁻¹ BaP; 1 mg L⁻¹ MWCNTs + 50 μg L⁻¹ BaP; 1 mg L⁻¹ MWCNTs + 100 μg L⁻¹ BaP). Concurrently, mussels were also exposed to a solvent control (0.02% DMSO). Due to the chemical composition of seawater, the occurrence of PAHs (very hydrophobic compounds), is at very low levels (<1 ng/L), in contrast to concentration in other aqueous matrices. For example, in marine sediments, the corresponding values could be in the range of 1 ng/g d.w. to >10000 ng/g d.w. (Nikolaou et al. 2009). In marine mussels, depending on the study area, PAHs can be found at concentrations ranging from 25 to 3 900 ng/g d.w. (Baumard, Budzinski, and Garriguess 1998; Baumard et al. 1999), corresponding to our lowest exposure concentration (5 μg L⁻¹). Regarding the selection of higher BaP exposure concentrations (50 and 100 μg L⁻¹), previous studies have reported that the selected concentration-range induced biological or biomarker responses in mussels (Halldörsson et al. 2008; Di et al. 2011; Banni et al. 2017). In the present study, for each treatment, 26 mussels were used. As previously described, MWCNTs were pre-weighed in glass vials for each beaker according to the final concentration (1.8 mg to reach 1 mg L⁻¹) and directly tipped into the beakers. Seawater quality was monitored in each of the beakers by measuring the salinity (36.42 ± 0.05‰), pH (7.72 ± 0.11), percentage of dissolved oxygen (94.58 ± 2.40%) and temperature (15.55 ± 0.32 °C).

Chemical analysis of BaP in water and tissue
Water and tissue extracts were analyzed using an Agilent Technologies (Stockport, UK) 7890 A Gas Chromatography (GC) system interfaced with an Agilent 5975 series Mass Selective (MS) detector as described previously by us (Banni et al. 2017).

DNA metabolism gene expression
Microarray hybridization and analysis.
Competitive dual-colour microarray hybridization
was performed with the STREM (Stress Response Microarray in Mytilus sp.) platform (Banni et al. 2017); fluorescence-labelled cDNA probes were obtained by direct labelling in the presence of modified Cy3- and Cy5-dCTP (Perkin Elmer). The procedure was carried out as described previously (Banni et al. 2011, 2017). MIAMI-compliant microarray data, including a detailed description of the experimental design and each hybridization experiment, were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/).

**Functional genomics analysis**

Functional characterization of mussel genes represented on a microarray was based on Gene Ontology (GO) annotation and carried out in Blast2GO (Conesa et al. 2005) using default parameters. However, in the case of the STREM platform, target genes were putatively annotated and ranked under established biological processes, making the generation of robust processes easier and faster.

**qRT-PCR.** qRT-PCR analysis was carried out using the same RNA extracted for microarray hybridization. Relative mRNA abundance of the mussel genes encoding 4 Probes and primer pairs (Table S2) were designed using Beacon Designer v3.0 (Premier Biosoft International, Inc.). The procedure is described in Banni et al. (2011, 2017).

**Comet assay**

The comet assay was performed as reported above (section “Comet assay to determine DNA damage”) with gills and DG tissues from 10 mussels for each treatment.

**DNA adducts**

DNA from 10 mussels for each treatment was isolated from gills and DG tissues using a standard phenol-chloroform extraction procedure. We used the nuclease P1 enrichment version of the thin-layer chromatography (TLC) $^{32}$P-postlabelling assay to detect BaP-derived DNA adducts (i.e. 10-[(deoxyguanosin-N$^2$-yl)7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP [dG-N$^2$-BPDE]). The procedure was essentially performed as described previously (Phillips and Arlt 2014).

### Evaluation of the uptake of gold-labelled MWCNTs by mussels

#### Synthesis of labelled nanotubes

To probe the uptake of carbon nanotubes by the mussels, it was necessary to label the nanotubes with a diagnostic marker. Gold was selected for this purpose due to its relatively low abundance in the natural environment (Goldberg 1987), thus providing an excellent spectroscopic handle facilitating ease of detection by both bulk and local-probe spectroscopy approaches. Confinement of nanoparticles within the internal void of MWCNTs is a highly efficient process allowing retention of the metal label in the system (Miners, Rance, and Khlobystov 2016) and simultaneously excluding any interference of the label with measurements on nanotubes. Gold nanoparticles were synthesized using a modified Brust–Schiffrin reduction (Rance, Marsh, and Khlobystov 2008) followed by insertion into MWCNTs utilizing a thermally-assisted Ostwald ripening procedure yielding AuNP@MWCNTs (Scheme S1).

#### Experimental design

Mussels were exposed to labelled MWCNTs only (1 mg L$^{-1}$ AuNP@MWCNTs) and in combination with BaP as the same concentration as above (1 mg L$^{-1}$ AuNP@MWCNTs $+$ 5 µg L$^{-1}$ BaP; 1 mg L$^{-1}$ AuNP@MWCNTs $+$ 50 µg L$^{-1}$ BaP; 1 mg L$^{-1}$ AuNP@MWCNTs $+$ 100 µg L$^{-1}$ BaP). Four mussels were used for each treatment.

#### Bulk spectroscopic analysis

For the determination of tissue-specific gold concentration, 4 mussels per treatment were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Each individually dissected tissue (gills, DG, all other tissues pooled together) was washed with distilled water, blotted dry and transferred to a pre-weighed acid washed vial. Samples were dried overnight at 60 °C and re-weighed. Tissue digestion was achieved by addition of 1 mL concentrated nitric acid (trace analysis grade) and incubation for 2 h at 70 °C. Digested tissue samples were diluted to a final volume of 5 mL with Millipore Milli Q water and stored at room temperature until analysis. An internal standard of 115-In was added, to a final concentration of 10 µg L$^{-1}$. This verified that
instrumental drift was not the cause of sample variation. Indium was selected based on its minimal occurrence in marine samples and low polyatomic interference with seawater. Samples were analyzed using an X Series II ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) with PlasmaLab software (Thermo Fisher Scientific Inc., Waltham, MA, USA).

**Mussel sectioning and electron microscopy analysis**

To probe the spatial location of the labelled MWCNTs with respect to the mussel DG, samples of whole tissues and cross-sections were analyzed by environmental scanning electron microscopy (ESEM) and scanning transmission electron microscopy (STEM), respectively. In either case, after the exposure experiments detailed above, a small piece (~5 mm²) was dissected out of the center of the digestive gland and fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 2.5% NaCl, 2 mM CaCl₂ in 0.1 M PIPES, pH 7.2 for 3 h. The tissue was then stored in 2.3 M sucrose (in 0.1 M PIPES) until analysis. Two mussels were analyzed per treatment. Whole tissue samples were washed with deionized water (four times), transferred onto aluminum specimens stubs and imaged at 3°C (cooled using the Peltier stage) in ESEM mode with the backscatter detector using the FEI Quanta 650 ESEM. Electron transparent sections for STEM analysis were prepared by cutting ~1 mm² pieces from the washed whole tissues and sectioning to a thickness of ~180-200 nm at ~80°C using the RMC Products PowerTome with the CR-X cryochamber. The cross-sections were transferred onto copper-grid mounted graphene oxide films using the Tokuyasu technique and imaged in dark field STEM using the JOEL 2100+ microscope operating at 200 keV.

**Statistical analyses**

Statistical tests were conducted using R software. Normality was checked using Lilliefors’ test and variance homogeneity was evaluated using Bartlett’s test. When necessary, raw data were mathematically transformed (Ln) to achieve normality before proceeding with an ANOVA. When significant, a posteriori Tukey test was performed.

**Analysis of interactions**

The non-parametric Mann–Whitney U-test was used to compare the data from treated mussels with those of the controls (Sforzini et al. 2018a). Further analysis of the combined effects of MWCNTs and BaP on DNA Damage (based on Comet Assay) was performed by calculating the Interaction Factor (IF) in order to test for evidence of additivity, synergism and antagonism (Schlesinger et al. 1992; Katsifis et al. 1996; David, Ebbels, and Gooderham 2016; Zhang et al. 2019):

$$
IF = \left( G_{\text{MWCNT}} - C \right) - \left( \left[ G_{\text{MWCNT}} - C \right] + \left[ G_{\text{BaP}} - C \right] \right) \\
= G_{\text{MWCNT}} + \text{BaP} - G_{\text{MWCNT}} - G_{\text{BaP}} + C
$$

$$
SEM_{\text{IF}} = \sqrt{\left( \text{SEM}^2_{\text{MWCNT}} + \text{BaP} \right) + \text{SEM}^2_{\text{MWCNT}}} + \text{SEM}^2_{\text{BaP}} + \text{SEM}^2_{C}
$$

where, IF is the interaction factor: negative IF denotes antagonism, positive IF denotes synergism, and zero IF denotes additivity. G is the mean cell pathological reaction to toxicants (BaP, MWCNTs and BaP+MWCNTs), C is the mean cellular response under control conditions. SEM(x) is the standard error of the mean for group X. Results were expressed as IF, and the 95% confidence limits were derived from the SEM values.

Equation In order to test the mixture IF values against predicted additive values (assumed to have an IF = 0), the predicted additive mean values (A) were calculated:

$$
A = \left( G_{\text{MWCNT}} - C \right) + \left( G_{\text{BaP}} - C \right)
$$

The Pythagorean Theorem method for combining standard errors was used to derive combined standard errors for the predicted mean additive values (A) of MWCNTs and BaP (http://mathbench.org.au/statistical-tests/testing-differences-with-the-t-test/6-combining-sds-for-fun-and-profit/). The standard errors for the three C₆₀ and BaP treatments (predicted additive) were derived using the following equation:

$$
\text{SEM}_{\text{add}} = \sqrt{\left( \text{SEM}^2_{\text{MWCNT}} + \text{SEM}^2_{\text{BaP}} + \text{SEM}^2_{C} \right)}
$$

This enabled the 95% confidence limits to be derived for the predicted additive values. The confidence limits were used to test the predicted additive values having an IF = 0 against the IF values for the mixtures.
Results

Preparation and characterization of MWCNTs in seawater

Light scattering and electron microscopy analyses of MWCNTs, shortened and purified by the site-selective, silver-catalyzed nanotube oxidation procedure developed previously (Miners et al. 2014) which yielded nanotubes ~600 nm in length (Figure 1(b)), in mussel-exposed seawater indicated the formation of micron-sized aggregates (Figure 1(c); Figure S1 and S2). The mean aggregate size determined by DLS (1541 ± 193 nm) showed minimal variation relative to those measured in seawater in the absence of mussels (1666 ± 198 nm) and in the presence of BaP in mussel-exposed seawater (1642 ± 431 nm).

Genotoxicity of MWCNTs in gill and digestive gland cells

At the end of the exposure period, a subtle increase in DNA strand breaks was observed in both the gills and the DG of mussels exposed to the highest concentrations of MWCNTs (0.1 and 1 mg L\(^{-1}\)) relative to the seawater-only control (Figure 2). No effect was noted at the lowest concentration (0.01 mg L\(^{-1}\)). Furthermore, no increase in micronuclei was observed after MWCNTs exposure in the gills and DG (Figure 3).

![Figure 1](image-url)
Co-exposure to BaP and MWCNTs

**BaP seawater concentration and uptake in mussel DG**

At one hour after dosing, the BaP concentrations in seawater were very close to the nominal concentrations of 64, 81 and 129% for the 5, 50 and 100 μg L⁻¹ BaP-only exposures and 48, 78 and 113% for the co-exposures to MWCNT + BaP 5, 50 and 100 μg L⁻¹, respectively. At the end of the exposure, a strong decrease was observed to approximately 95 and 85% of the initial value for exposure to BaP alone and the co-exposure, respectively (Table S1).

As expected, the concentration of BaP in DG in the solvent and MWCNT-only control treatments was below the limit of detection and found to increase with concentration in both the single and co-exposure experiments. However, BaP uptake was significantly lower in mussels co-exposed to MWCNTs and BaP (p < 0.05), with a 78 and 44% decrease in the uptake for mussels exposed to MWCNTs and BaP at concentrations of 50 and 100 μg L⁻¹, respectively, compared to mussels exposed to BaP alone at the same concentrations (Table 1).

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**Figure 2.** Levels of DNA damage as measured by the Comet assay in the mussel tissues after the 7-day exposure to MWCNTs. Asterisks indicate the statistical differences observed between control and exposed groups. (*) p < 0.05, (***) p < 0.01, (****) p < 0.001.

**Figure 3.** Levels of micronuclei in the mussel tissues after the 7-day exposure to MWCNTs.
**DNA metabolism gene expression**

The transcriptomic approach performed in this work is based on a new platform developed by our group to investigate gene expression profiling of *Mytilus sp.* to environmental stressors. DNA metabolism was the main investigated process over the 15 processes present on the array. Our data revealed that over the 36 targets present on the array covering the DNA metabolism process, 27 were involved in the response to BaP and MWCNTs in DG and 24 in the gills, in at least one condition. In DG, the number of differentially expressed genes (DEGs) involved in the stress response was maximal at 5 μg L⁻¹ BaP and 100 μg L⁻¹ BaP with 14 DEGs and 12 DEGs respectively (Table 2). However, in the gills the highest number of DEGs was observed in mussels exposed to BaP at 5 μg L⁻¹ and 12 DEGs respectively (Table 2). In particular, p53, caspase 3, p63/73 and alkaline phosphatase targets were found to be the most present in all conditions in the DG and gill tissues showing a distinct pattern between conditions. Indeed, while, the maximum p53 expression level in digestive gland (M value 1.88) was observed in mussels exposed to 5 μg L⁻¹ BaP, it was maximum in gills (M value 2.21) for mussels exposed to nanotubes alone. Moreover, caspase 3 gene expression was maintained down regulated in digestive gland tissues exposed to 5 μg L⁻¹ BaP (M value −2.30) and nanotubes (M value −3.08). However, the same target was observed to reach a maximum up-regulated level in digestive gland tissues for mussels exposed to MWCNTs and BaP at concentrations of 50-100 μg L⁻¹ (M value 2.29). A similar trend was observed in gills. Gene expression analysis by qPCR of selected targets (e.g. p53, caspase3, DNA ligase and topoisomerase) in the same tissues was in trend with the array data (Figure S4).

**DNA damage in gills and digestive gland after co-exposure**

Regarding the comet assay, a tissue-specific response was observed, the DG being more prone to DNA damage compared to the gills. In DG, BaP-induced genotoxicity was concentration-dependent whereas BaP + MWCNTs co-exposure reduced the genotoxic effects. As observed in the previous experiment, exposure to MWCNTs induced DNA strand breaks. In the gills, BaP was only genotoxic at the highest concentration 100 μg L⁻¹, and the co-exposure induced DNA damage only at the intermediate concentration (Figure 5). No significant effect was observed in mussels exposed to MWCNTs only. No bulky BaP-DNA adducts (i.e. dG-N²-BPDE) were detected in any tissue at any of the treatment condition (data not shown).

**Interactions.** Interactions between MWCNTs and BaP on DNA damage are shown in Table 3. There was evidence of an antagonistic interaction between MWCNTs and BaP for DNA damage (Comet assay) at the 5 and 100 μg L⁻¹ BaP + MWCNT combination treatment (Table 3).

**Analysis of MWCNT uptake in mussels**

The presence of gold in the mussel tissues (from the DG, gills and all other tissues pooled together), as diagnostic of the uptake of labelled MWCNTs,

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**Table 1.** Chemical analyses of the mussel digestive gland after the 3-day exposure to BaP and MWCNTs.

| Treatments                  | Water content (% wet weight) | BaP concentration (μg g⁻¹ dry weight) |
|-----------------------------|------------------------------|--------------------------------------|
| Solvent control             | 77.1 ± 2.2                   | <0.5                                 |
| Solvent control + MWCNT 1 mg L⁻¹ | 78.8 ± 2.1                   | <0.5                                 |
| BaP 5 μg L⁻¹                | 78.7 ± 2.6                   | 6.9 ± 2.1                            |
| BaP 50 μg L⁻¹              | 76.1 ± 1.8                   | 163.2 ± 68.4                         |
| BaP 100 μg L⁻¹             | 75.7 ± 3.8                   | 475.0 ± 45.7                         |
| MWCNT + BaP 5 μg L⁻¹       | 78.5 ± 3.5                   | 6.0 ± 2.6                            |
| MWCNT + BaP 50 μg L⁻¹      | 77.6 ± 3.2                   | 36.1 ± 23.1                          |
| MWCNT + BaP 100 μg L⁻¹     | 77.9 ± 3.2                   | 267.9 ± 64.4                         |

Asterisks indicate the statistical differences observed between treatments exposed to BaP only and treatments exposed to BaP + MWCNTs. (*) p < 0.05.

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**Table 2.** Number of DEGs depicted in mussels exposed to BaP alone and with MWCNTs against control (DMSO).

| Treatments                  | Gills | DG |
|-----------------------------|-------|----|
| BaP 5 μg L⁻¹                | 2     | 7  |
| BaP 50 μg L⁻¹               | 8     | 4  |
| BaP 100 μg L⁻¹              | 6     | 3  |
| MWCNT                      | 1     | 7  |
| MWCNT + BaP 5 μg L⁻¹       | 8     | 5  |
| MWCNT + BaP 50 μg L⁻¹      | 6     | 4  |
| MWCNT + BaP 100 μg L⁻¹     | 6     | 3  |

Shown are numbers of up- and down-regulated DEGs.
was quantified using ICP-MS. The highest amount of gold was found in DG with a mean concentration of 66.73 \( \mu \)g/g determined, approximately one order of magnitude higher than that observed in the gills (9.42 \( \mu \)g/g) and all other tissues (5.52 \( \mu \)g/g). No significant difference was observed in gold level between the different treatments (with or without BaP) in all of the tissues (Figure 6). However, despite an exhaustive electron microscopy investigation of whole and cross-sectioned DG tissues, no direct visualization of nanotubes was afforded (Figures S5–S8).

**Figure 4.** Gene expression profile of gill (a) and DG (b) tissues in animals exposed to increasing BaP concentrations (5, 50 and 100 \( \mu \)g L\(^{-1}\)) and their combination with MWCNTs (1 mg L\(^{-1}\)). The heat map reports log2 relative expression level with respect to the reference condition. 24 and 27 DEGs were generated in at least one condition for gill and DG respectively. Microarray data was analysed using the Linear Model for Microarray Analysis (LIMMA) software as described in Banni et al. (2011). B statistics with adjusted \( p \)-value, 0.05 and B.0 were used as a threshold for rejection of the null hypothesis (no variation).

**Figure 5.** Levels of DNA damage as measured by the Comet assay in mussel tissues after 3-day exposure to BaP with and without co-exposure to 1 mg L\(^{-1}\) MWCNT. Data are expressed as % control values (not shown) in order to standardize across two different sampling days. Asterisks indicate significant differences from the same tissues in control mussels.
Discussion

Characterization of MWCNTs in relevant environmental media

The size, shape and surface chemistry of nanoscale materials is known to affect their dispersion in environmental media (Gottschalk, Sun, and Nowack 2013). Effective understanding of these physicochemical parameters is the key to their potential uptake and consequent biological impact on the marine biota. For carbon-based nanoparticles, such as MWCNTs, this is particularly critical as their surfaces are inherently hydrophobic. MWCNTs therefore have a strong tendency to form aggregates in water, the size and stability of which depends on the properties of both the nanotube and the specific aqueous environment. Indeed, our light scattering measurements (Figure S1) confirm the formation of stable micron-sized agglomerates of nanotubes in mussel-exposed seawater, consistent with analogous studies of MWCNT aggregates in both natural (Anisimova et al. 2015) and synthetic seawaters (Xu et al. 2011; Cerrillo et al. 2015). Interestingly, there was no significant difference in the mean MWCNT aggregate size observed in the mussel-exposed seawater, relative to nanotube aggregates formed in seawater in the presence of BaP. This suggests that enhancing the propensity for interactions of MWCNTs with either the proteins secreted by mussels or small aromatic molecules does not, in these instances, aid the solvation of individual MWCNTs or affect the observed aggregate sizes formed in seawater. This is however not expected to be a general phenomenon. We therefore recommend that all future ecotoxicological studies on ENPs and NMs feature analysis in the species of interest to account for any possible effects of this nature.

Genotoxicity of MWCNTs

Despite their environmental relevance, little information is available regarding the genotoxicity of

Table 3. Analysis of combined effects of MWCNT and BaP on DNA damage (Comet assay) based on interaction factors (IF).

| Treatments                        | Interaction factors for DNA damage (Comet assay) |
|-----------------------------------|--------------------------------------------------|
| MWCNT 1 mg L⁻¹ + BaP 5 µg L⁻¹    | −396.42 ± 174.57                                  |
| MWCNT 1 mg L⁻¹ + BaP 50 µg L⁻¹   | −438.38 ± 297.56                                  |
| MWCNT 1 mg L⁻¹ + BaP 100 µg L⁻¹  | −505.92 ± 166.69                                  |

Interaction Factor ± 95% Confidence limit/2 (Moore et al. 2018). *indicates significance at the 5% level. A negative IF indicates antagonism; an IF of 0 indicates additivity; and a positive IF indicates synergism.

Figure 6. Gold concentration in mussel (a) gill, (b) DG and (c) other tissues (mean ± SE) after the 3-day exposure to 1 mg L⁻¹ gold-labelled MWCNT (AuNP@MWCNT), with and without co-exposure to BaP. Regarding DG, the highest Au level in the absence and presence of BaP at a concentration of 5 µg L⁻¹ are due to single outliers. Non-parametric statistical test (Kruskal–Wallis) indicated no significant differences (p-value = 0.3256).
carbon nanoparticles and in particular carbon nanotubes in marine invertebrates. In our study, DNA strand breaks were observed in mussel gills and DG after 3-days exposure to 0.10 and 1 mg L$^{-1}$ to MWCNTs and in DG after 3-days exposure to 1 mg L$^{-1}$ MWCNTs. However, no effect was observed in gills after 3-days exposure to 1 mg L$^{-1}$ MWCNTs suggesting a tissue specific genotoxic effect of MWCNTs in mussels. In the marine polychaete Arenicola marina, no significant effect on DNA strand breaks was observed after 10 days of exposure to single-walled carbon nanotubes (SWCNTs) through natural sediment (0.003 and 0.03 g/kg) (Galloway et al. 2010). Regarding other carbon nanomaterials, increased DNA strand break formation was observed in hemocytes of the marine mussels after exposure to C$_{60}$ at 0.10 and 1 mg L$^{-1}$ for 3 days (Al-Subiai et al. 2012; Di et al. 2016).

The mechanism of genotoxicity of CNTs in bivalve cells remains unknown. Genotoxic responses of CNTs may arise via direct mechanical injury or as a secondary result of CNT-mediated reactive oxygen species (ROS) generation and, therefore, oxidative stress (Hutchison et al. 2010). Among these mechanisms, oxidative stress is indicated as the key factor of genotoxicity induced by ENMs in bivalve species and their accumulation associated with exposure time is also an important factor in induced-genotoxicity (Rocha et al. 2015). Studies by De Marchi et al. (2017a, 2017b) in two polychaete species (Diopatra neapolitana and Hediste diversicolor) and in a marine bivalve (Ruditapes philippinarum) showed the induction of oxidative stress after 28 days of exposure to MWCNTs (0.10 and 1.00 mg L$^{-1}$). Elevated ROS levels may activate cellular stress-dependent signaling pathways. It can directly damage mitochondria, cause DNA fragmentation in the nucleus, cell cycle arrest, apoptosis, and/or inflammatory responses (Nel et al. 2006; Maurer-Jones et al. 2013). In mammalian cells, it has been shown that CNTs are able to induce a range of different genotoxic effects. Among these damages, CNTs can also impair the functionality of the mitotic apparatus inducing micronuclei and chromosomal aberrations (VanBerlo et al. 2012). However, in our study, no potential aneugenic effect was studied with the micronucleus assay due to lack of species specific centromeric probes. Currently, the genotoxic potential of CNTs in marine invertebrates is not clear. Various factors could influence this phenomenon including differences in experimental design among studies, experimental models used, exposure routes, type of CNTs examined and their preparation procedures, concentrations used and assessed endpoints.

**Co-exposure to BaP and MWCNTs**

The main objective of this study was to determine if a ‘Trojan Horse’ effect could be observed when mussels were exposed to MWCNTs in combination with BaP. Mussels accumulated BaP in gills and DG in a manner consistent with previous studies (Canova et al. 1998; Banni et al. 2017). It was however interesting to observe a decrease in BaP uptake when mussels were co-exposed to MWCNTs. In the Japanese Medaka (Oryzias latipes), it has been shown that the coexistence of SWCNTs facilitated the accumulation of phenanthrene in the digestive track of fish and therefore enhanced the whole-body phenanthrene concentration (Su et al. 2013). In the earthworm (Eisenia foetida), the addition of nanotubes in soil significantly decreased pyrene uptake (Petersen et al. 2009b). The same contradictory results were observed with other carbon nanoparticles as for example fullerenes. Della Torre et al. (2017) demonstrated that carbon nanopowder facilitated BaP uptake by zebrafish embryos and also affected the distribution of the pollutant in the organism. However, in the same species (i.e. zebrafish larvae), it has been shown that bioavailability of 17$\alpha$-ethynylestradiol (EE2) was reduced with increasing concentration of $n$C$_{60}$ nanoparticles (Park et al. 2011). Interestingly, in the digestive gland of M. galloprovincialis, comparable BaP tissue concentrations in the presence or absence of C$_{60}$ were observed indicating that, despite the expected strong sorption of BaP on C$_{60}$, no ‘Trojan horse’ effect was observed and C$_{60}$-sorbed BaP also remained bioavailable (Barranger et al. 2019). In our study, this decrease in BaP uptake when mussels were co-exposed to MWCNTs could be due to high adsorption properties of MWCNTs towards organic compounds and in our case BaP, as widely described for carbon-based ENPs (Yang et al. 2006; Hu et al. 2008, 2014). Our results showed that BaP is more present in the seawater and less in the DG at the end of the exposure period when MWCNTs are present. Our results suggest that adsorption of
BaP on MWCNTs through van der Waals interactions (Figure 1(a)) in seawater prevent BaP from reaching the mussel. Genotoxic effects measured by the Comet assay confirmed these results showing less DNA damage (i.e. antagonistic interaction, Table 3) in DG when mussels are co-exposed to BaP and MWCNTs, as a result of a lower bioavailability of BaP adsorbed on MWCNTs (Zhang et al. 2019). Previous studies demonstrated that hydrophobic interactions largely drive adsorption of PAHs to MWCNTs (Linard et al. 2017). Bioavailability appears to be more influenced by the ability of PAH molecules to access the available adsorption sites, as a function of molecular size and morphology, rather than the type of carbon nanomaterial (Xia et al. 2012; Linard et al. 2017).

MWCNTs are relatively large supra-molecular structures but may have limited access into the DG cells by endocytosis (Maruyama et al. 2015). However, BaP will probably enter the DG cells through a combination of direct transfer (Plant, Benson, and Smith 1985) and through endocytosis in bound form mediated by Van der Waals interaction with food proteins and lipids; or else bound to cell surface proteins (Rashid, Horobin, and Williams 1991; Moore et al. 2004; Sayes et al. 2004). Most of the observed DNA damage will probably result from oxidative injury to DNA by ROS generated from futile cycling of BaP; as well as being produced by the MWCNTs and by intra-lysosomal lipofuscin associated with iron (Brunk and Terman 2002; Zangar, Davyдов, and Verma 2004; Moore et al. 2007; Sforzini et al. 2018). The antagonistic interaction observed for highest test concentration of MWCNT + BaP combinations probably result from the consequences of an intracellular limitation of oxidative damage in the mixture treatment (Della Torre et al. 2018). Such limitation may be caused by binding of BaP to externalized non-endocytosed MWCNTs resulting in reduced entry and bioavailability of BaP as evidenced by reduced BaP in DG (Table 1), hence, reducing ROS generation.

In addition to this adsorption effect, according to the literature, various nanoparticles suspended in seawater form aggregates of nano- and microsizes (Canesi et al. 2010a, 2010b, 2014). In the presence of bivalves, the aggregates associate with mucus are suggested to be deposited along the byssus thread and settle on the bottom of the aquarium (Canesi et al. 2010b). Thus, the amount of nanoparticles, and in our case of BaP adsorbed on it, captured by molluscs is significantly less than that expected on the basis of the initial concentration of the suspension. These results could lead to the hypothesis that the high sedimentation rate observed within aquarium highlighted that MWCNTs would transport PAHs mainly in sediments (Della Torre et al. 2017, 2018).

Regarding other genotoxic effects, in our study no bulky BaP-DNA adducts were detected in any of the treatments. This result differs with a previous experiment (Banni et al. 2017), where bulky DNA adducts (i.e. dG-N²-BDDE) were detected after exposure to BaP using the same experimental design (same concentrations and exposure duration). One explanation could be the physiological state of the mussels. It is known that mussels from the species *M. galloprovincialis*, are spawning all year around compared to *Mytilus edulis* which has a specific period of spawning in May and October. Even if we performed our experiment in November, gonads of mussels were mature, and it is known that during maturation there is a drop in the bio-transformation process that could explain the lack of bulky BaP-DNA adducts in DG (Solé, Porte, and Albaiges 1995; Shaw et al. 2004).

In addition to the biomarkers of genotoxic effects, the transcriptomics data provided interesting information regarding the expression of genes involved in DNA metabolism. We noticed a marked regulation of genes involved in DNA metabolism in both tissues in mussels exposed to BaP alone or in combination with MWCNTs. However, exposure to MWCNTs alone resulted in a very low number of DEGs in both tissues. Interestingly, mussels exposed to the mixtures of BaP and nanotubes manifested a significant increase in the number of DEGs in gills while no marked increase was observed in DG. In the last decade, transcriptomics has proved to be a reliable tool, increasing our understanding of many important physiological processes in marine organisms in response to environmental stressors such as chemicals (Negri et al. 2013; Sforzini et al. 2018b) as well as to physiological parameters such as annual cycle (Banni et al. 2011). Moreover, transcriptional control can allow stressed organisms to cope with the alteration of cellular functions and to avoid cellular damage.
A significant alteration was recorded by the Comet assay in DG cells from animals exposed to BaP alone, and co-exposed to BaP and MWCNTs but to lesser extent. Transcriptomic data corroborated these results showing less DEGs in DG of mussels co-exposed to the mixture. In gills however mussels exposed to the mixture of BaP and MWCNTs exhibited a significant increase in the number of DEGs. These results are not correlated with the genotoxic response measured by Comet assay showing no additional effect when mussels are co-exposed to both contaminants. The relatively short exposure period (3 days) may explain the absence of cellular alterations where gene expression occurs.

The investigation of DNA damage response genes coding for caspase (HQ424451.1) revealed a marked up-regulation of apoptotic genes in gills of mussels exposed to the highest BaP concentration alone or in combination with nanotubes. DNA repair-related enzymes (DNA ligase: AJ624686.1; p53: KC545827.1) were however markedly up-regulated in animals exposed to lower BaP concentration and to nanotubes (data confirmed by qPCR). Mussel cells may react to DNA alterations via p53-mediated cell cycle arrest or apoptosis, upon high or irreparable DNA damage, p53 promotes the cells towards apoptosis (Schwartz and Rotter 1998).

Overall, these results highlight that, once inside the organism, BaP and MWCNTs activate genes involved in DNA metabolism in a different way to the activation by BaP or MWCNTs alone. Genotoxic and transcriptomic data may suggest a tissue-specific response and the occurrence of a DNA repair or apoptosis events with respect to the applied BaP/nanotubes concentrations. In our case, it is difficult to conclude the exact mechanisms of this response. Despite the fact that less BaP is uptaken when mussels are co-exposed, gills displayed higher DEGs in the co-exposure treatment, which is not the case for DG. It has been shown that BaP accumulates differently in tissue when zebrafish embryos are co-exposed to carbon nanopowder (CNPW) and BaP (Della Torre et al. 2017). In our study, only one tissue (i.e. DG) has been analyzed for BaP uptake making it difficult to make a conclusion.

**Tracking MWCNTs in the mussel DG**

One of the most significant challenges when probing the uptake of nanoscale materials, including carbon nanotubes, in marine biota is developing reliable and quantitative methods for their detection. In this respect, CNTs represent a particular problem as their inherent structural polydispersity (i.e. broad range of diameters and lengths) hinders conventional analytical approaches, such as chromatographic separation. Moreover, methods based on elemental analysis and spectroscopic techniques are generally not feasible because of the presence of organic matter. Spectrofluorometric analysis is one approach that has been used successfully to quantify nanotubes in mouse cells and rabbits (Cherukuri et al. 2004, 2006). However, as this technique is only applicable to semiconducting SWCNTs. Insensitive to either individual metallic SWCNTs or bundles containing metallic SWCNTs, the electronic properties and aggregation characteristics of nanotubes in environmental systems is often unknown. This approach has therefore limited potential (O’Connell et al. 2002). Raman spectroscopy was used to detect SWCNTs qualitatively in the aquatic organism *D. magna* (Roberts et al. 2007); yet, this approach cannot provide quantitative results and is best suited for SWCNTs owing to the effects of resonant signal enhancement. A method used recently to detect carbon nanotubes in biological systems is tagging them with molecules that are either bonded to radioactive isotopes or are themselves fluorescent (Kam et al. 2004; Kam, Liu, and Dai 2006; Singh et al. 2006). The use of such probes however depends on the stability of its attachment to the nanotubes and, for radioactive labelling, attachment of the isotope to the polymer. The addition of such bulky tags likely influences the physicochemical properties of the nanotubes and thus their environmental behaviors.

The novel approach explored in our study was the utilization of gold-labelled MWCNTs to improve the traceability of nanotubes in biological samples. Indeed, ICP-MS results indicated the presence of gold in mussel tissues, particularly in the digestive gland. Thus, bioaccumulation of MWCNTs in mussels at the whole tissue level was confirmed, consistent with analogous previous studies using other aquatic species (Templeton et al. 2006; Roberts et al. 2007; Smith, Shaw, and Handy 2007; Ferguson et al. 2008; Petersen et al. 2009a). In an attempt to probe the spatial location of labelled MWCNTs at the cellular level, we analyzed samples using
electron microscopy. It is important to note that it has been shown previously that incorrect sample preparation and subsequent image interpretation may lead to the generation of false positives and inaccurate conclusions (Edgington et al. 2014). The most common issue of this nature is that all high-contrast material presented in bright-field images of the sections (where dark features correspond to structures comprising high atomic number elements) are assumed to be from the specific artificial nanomaterial under examination. However, these may, in fact, be related to unknown organic or inorganic matter or artefacts induced during the staining preparation that is standard protocol for producing high quality transmission electron microscopy images. It is for this reason that we elected to use gold-labelled nanotubes, in the absence of stains, in our studies. This provided a uniquely diagnostic handle enabling us to correlate the presence of any foreign materials with their exact elemental composition. Indeed, our combined electron microscopy and in situ spectroscopy approach enabled us to show the presence of numerous metals in nanoparticle form in both ESEM images of the whole tissues and STEM images of the digestive gland cross-sections from control samples in the absence of labelled MWCNTs (Figures S5 and S7). Yet, despite an extensive electron microscopy examination we were not able to visualize AuNP@MWCNTs in any of the samples appraised (Figures S5 and S7). The absence of evidence from STEM/EDX analysis of the cross-section is not unexpected given the large dimensions of the mussel (~5 mm²) relative to the size of the sections (~180-200 nm in thickness). The fact that no evidence was observed in images of the whole tissue and that gold is detected from bulk ICP-MS analysis means that MWCNTs must be located within the internal structures of the DG. It suggests that the local probe nature of STEM confers a sampling issue, though visualization of their location at the cellular level remains elusive. These results emphasize the challenge of direct imaging of nanostructures in biological samples and the importance of using a range of different analytical techniques in order to obtain a good understanding of these complex systems.

It should be noted that in our experiment the results were obtained at concentrations well above environmentally realistic concentrations, especially for MWCNTs. As stated earlier, the predicted environmental concentration for CNTs is at low ng/L. In a review, the assembled results from different experiments indicate that carbon nanomaterials (CNMs) are not expected to be toxic to aquatic organisms at environmentally relevant concentrations (Freixa et al. 2018). Toxic effects of CNMs to aquatic organisms occur under high concentrations in short-term experiments (Jackson et al. 2013). As stated in the review however (Freixa et al. 2018), in the near future numerous commercial products are expected to include nanoparticles in their formulations (De Volder et al. 2013), indicating that, the concentration of CNMs in aquatic systems could increase to which organisms could be chronically exposed. It should also be noted that the antagonistic response observed in the current study between BaP and MWCNTs is based on a single concentration of the carbon nanotubes and as such represents a general overview of potential toxicological behavior. It is possible that this antagonistic interaction could change when another concentration range is selected. Future studies must include more realistic exposure scenarios to estimate accurately toxicity of nanomaterials.

**Conclusion**

Our findings highlight that impacts of the co-exposure of organic pollutants and CNTs on the bioavailability and accumulation of the contaminants are mixed and species specific, indicating that in the case of BaP the accumulation of this PAH is not facilitated by the presence of MWCNTs in *M. galloprovincialis*. Environmentally realistic experiments are mandatory in order to identify and explain the possible threat of ENMs to organisms. Many variables can modify the fate and consequently toxic effects of ENPs and adsorbed pollutants. Fine characterization of nanoparticles in (sea)water (including characterization of the adsorbed contaminants) and localization are the main challenge to understand the toxic effect of this emerging contaminant in the environment. Overall, our study demonstrate the challenges for hazard and risk assessments posed by ENMs and contribute to improve our understanding of their potential interactive effects with
other ubiquitous pollutants on an ecologically important marine organism.

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