Phenanthrene bioavailability and toxicity to *Daphnia magna* in the presence of carbon nanotubes with different physicochemical properties

Florian Zindler[1§], Berit Glomstad[1], Dag Altin[2], Jingfu Liu[3], Bjørn M. Jenssen[1], Andy M. Booth[4,*]

[1] Department of Biology, Norwegian University of Science and Technology, Trondheim NO-7491, Norway

[2] BioTrix, Trondheim NO-7022, Norway

[3] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China

[4] SINTEF Materials and Chemistry, Trondheim NO-7465, Norway

Abstract

Studies investigating the effect of carbon nanotubes (CNTs) on the bioavailability and toxicity of hydrophobic organic compounds in aquatic environments have generated contradictory results, and the influence of different CNT properties remains unknown. Here, the adsorption of the polycyclic aromatic hydrocarbon phenanthrene (70-735 µg/L) to five types of CNTs exhibiting different physical and chemical properties was studied. The CNTs were dispersed in the presence of natural organic matter (nominally 20 mg/L) in order to increase the environmental relevance of the study. Furthermore, the bioavailability and toxicity of phenanthrene to *Daphnia magna* in the absence and presence of dispersed CNTs was investigated. Both CNT dispersion and adsorption of phenanthrene appeared to be influenced by CNT physical properties (diameter and specific surface area). However, dispersion and phenanthrene adsorption was not influenced by CNT surface chemical properties (surface oxygen content), under the conditions tested. Based on nominal phenanthrene concentrations, a reduction in toxicity to *D. magna* was observed during co-exposure to phenanthrene and two types of CNTs, while for the others, no influence on phenanthrene toxicity was observed. Based on freely dissolved concentrations, however, an increased toxicity was observed in the presence of all CNTs, indicating bioavailability of CNT-adsorbed phenanthrene to *D. magna*.

Keywords: Nanomaterials, polycyclic aromatic hydrocarbons, adsorption, dispersion, aquatic organisms, aquatic ecotoxicity.

INTRODUCTION

The production of carbon nanotubes (CNTs) is increasing due to their incorporation into a growing number of applications, including in composite materials and environmental technologies.[1, 2] Due to their hydrophobicity and tendency to aggregate and settle, a significant proportion of CNTs released into the environment are assumed to accumulate in sediments.[3] However, the adsorption of colloidal and dissolved natural organic matter (NOM) to CNT surfaces has been found to increase the dispersion stability and water column residence time of CNTs in aqueous media, significantly influencing their environmental fate and behavior.[4-8]. CNTs are potent adsorbents of hydrophobic organic compounds (HOCs), making them interesting e.g. in remediation of contaminated sites or in wastewater treatment.[2, 9, 10] This raises questions with respect to their effect on the fate, bioavailability and toxicity of HOCs, including polycyclic aromatic hydrocarbons (PAHs) which are common pollutants in natural environments. CNT adsorption of HOCs is influenced by several factors, including the CNT physicochemical properties, CNT dispersion state, the presence of NOM and media properties.[11-15]

Several studies have observed ingestion of CNTs by aquatic organisms, including daphnids and fish.[16-19] Moreover, adverse effects as a direct result of CNT exposure have been observed in algae, daphnids and fish.[4, 17, 18, 20-23] Further studies have investigated the adsorption of HOCs by CNTs or other carbon-based nanomaterials (CNMs), such as fullerene C_{60}, and the subsequent bioavailability and toxicity of these contaminants to aquatic organisms. Only freely dissolved HOCs are considered bioavailable to aquatic organisms, and the adsorption to CNMs has been suggested to reduce HOC bioavailability. However, studies have shown contradictory findings.

Some studies found that CNM adsorption of HOCs led to a reduction in their bioavailability and toxicity to aquatic organisms.[24-26] In contrast, other studies have reported that HOCs adsorbed by CNMs remain bioavailable to both pelagic and benthic organisms.[11, 19, 25, 27, 28] For example, in Japanese Medaka (*Oryzias latipes*) it was demonstrated that a CNT-adsorbed PAH (phenanthrene) was released after ingestion of the particles, resulting in increased body concentrations in the fish.[19] Our previous study revealed that a range of CNTs with different physicochemical properties readily adsorbed phenanthrene in the presence of environmentally relevant concentrations of NOM and that it was bioavailable to freshwater algae (*Pseudokirchneriella subcapitata*).[11] These results show that CNMs can facilitate the transport of co-adsorbed contaminants into organisms through alternative uptake routes, indicating that they remain bioavailable. The bioavailability of CNT-adsorbed HOCs is thought to be influenced by factors such as CNT aggregation behavior and the presence of NOM. Aggregation behavior is believed to affect CNT retention/elimination rates in organisms, whilst the presence of NOM has been suggested to impede PAH desorption from CNTs.[19, 24] The contradictory results from previous studies highlight the complexity of combined studies with CNTs and HOCs, and how the results may be influenced by CNT properties or environmental factors (e.g. NOM). Despite the fact that CNT properties can influence CNT aggregation behavior[6] and their adsorption of organic pollutants,[11-13] few studies have addressed the effect of CNT properties on co-contaminant bioavailability and toxicity to aquatic organisms. More knowledge regarding CNT effects on bioavailability and toxicity of HOCs in the presence of NOM is of importance for the evaluation of their environmental effects.

In this study, we used phenanthrene as a model PAH to evaluate the bioavailability and toxicity to *D. magna* in the presence of CNTs and environmentally relevant concentrations of NOM. To investigate the influence of the physicochemical properties of CNTs on the bioavailability and toxicity of phenanthrene, five types of CNTs were used in the study, including one single-walled CNT (SWCNTs), two non-functionalized multi-walled CNTs (MWCNT-15 and MWCNT-30) with different diameter and lengths and two functionalized MWCNTs (hydroxyl: MWCNT-OH and carboxyl: MWCNT-COOH). Adsorption of phenanthrene to the different types of CNTs in the presence of NOM was determined, and the subsequent influence on bioavailability and toxicity to *D. magna* assessed through acute toxicity tests.

MATERIALS AND METHODS

Chemicals. The carbon nanotubes (SWCNT, MWCNT-15, MWCNT-30, MWCNT-OH and MWCNT-COOH; >95%) were obtained from Timesnano (Chengdu Organic Chemicals Co., Ltd, Chinese Academy of Sciences). Characterization of the CNTs was performed to determine outer diameter, purity, specific surface area (SSA) and surface oxygen content. Details on the characterization techniques has been previously published.[11] Phenanthrene (≥99.5%, Sigma Aldrich) was dissolved in methanol (analytical grade, Fluka Analytical) for the preparation of stock solutions (100 mg/L). Suwannee River natural organic matter (SR-NOM) was purchased from the International Humic Substance Society (IHSS).

Preparation of CNT dispersions. Elendt M7 medium[29] containing 20 mg/L SR-NOM (M7-NOM) was used as medium for the preparation of CNT dispersions throughout the study. SR-NOM was dissolved in M7 medium by magnetic stirring for 24 h. Undissolved material was

100 subsequently removed by filtration through a 0.2 μm Nalgene filter unit (Thermo Fisher
101 Scientific, Inc.). The pH of the M7-NOM was 7.74 ± 0.08 and needed no adjustment after
102 preparation. CNT stock dispersions in M7-NOM (100 mg/L) were prepared using bath
103 sonication according to a previously published method.[11] After sonication, the stock dispersions
104 were mixed into larger volumes of the same media to achieve an initial CNT concentration of 10
105 mg/L. The prepared dispersions were left for 24 h to allow any non-dispersed CNT aggregates to
106 settle out of the water column before the supernatant containing the remaining dispersed CNTs
107 was withdrawn and used in the further experiments. The CNT concentration in the supernatant
108 was determined by UV-vis absorbance measurements at 800 nm (Hitachi U-2000
109 Spectrophotometer), as no interference from NOM, the media or phenanthrene was observed at
110 this wavelength. Quantification was performed using calibration curves prepared for individual
111 CNTs as previously described.[11] The calibration curves are presented in Figure S1 in the
112 Supplementary Information (SI). For MWCNT-15, MWCNT-OH and MWCNT-COOH the
113 concentration was determined in single replicates which were subsequently used throughout the
114 adsorption and toxicity studies. Due to an oversight, the concentration of MWCNT-30 and
115 SWCNT was not measured directly in the dispersions used in the adsorption and toxicity studies.
116 The concentration of SWCNT and MWCNT-30 was therefore determined independently in
117 seven and six replicates, respectively. The average concentrations of these were used to
118 confidently estimate the CNT concentration in dispersions used in adsorption and toxicity
119 studies. The coefficients of variation (CV) of the replicates were 15% and 27% for SWCNT and
120 MWCNT-30, respectively.
121

Phenanthrene toxicity to daphnids. *D. magna* were cultivated in Elendt M7 medium at 20 ± 2 °C. Prior to testing, they were kept under a 16-hour light and 8-hour dark cycle and fed with the freshwater algae *P. subcapitata*. A modified version of the OECD *Daphnia* sp., Acute Immobilisation Test[29] was used to assess phenanthrene toxicity to daphnids, using 3-4 days old daphnids. Pre-tests showed that for newborn organisms (< 24 h, as recommended in the OECD test guidelines) attachment of CNT aggregates to the animals could cause mechanical immobilization. Although mechanical immobilization could be considered as a physical mechanism of toxicity, it is suggested this would not be of significant relevance in natural fresh waters where CNT concentrations are expected to be low. To minimize the risk of mechanical influence of the CNTs, larger 3-4 day old animals were used.

The daphnids were exposed to phenanthrene spiked into M7-NOM at five different concentrations (70 µg/L, 126 µg/L, 227 µg/L, 408 µg/L and 735 µg/L) in order to determine the effect concentrations. Five animals were carefully pipetted into 20 mL exposure solution contained in 22 mL glass vials with PTFE lined screw caps (Agilent Technologies, Inc.), maintaining a headspace of 1-2 cm. Four replicates were included at each phenanthrene concentration. The animals were exposed for 48 hours in darkness at a temperature of 19.6 ± 0.3 °C, after which the number of immobilized animals was counted. Abnormalities, including mortality and disoriented animals, were also recorded. Negative controls for M7 (n=8), M7-NOM (n=32) and methanol dissolved in M7-NOM (which was used as a solvent for phenanthrene, 7.35 µg/L; n=8) were included in the study. Dissolved oxygen (dO_2) and pH was determined in the sample solutions at the start and end of the exposure.

A total of six tests with phenanthrene-only (i.e. without the presence of CNTs) were performed in order to investigate the variability of the test. Four of the tests were performed using the same batch of daphnids, while two tests were performed with different batches of the daphnids, giving very good control of test variability both within one batch and between different batches of daphnids. Based on these data, effect concentrations causing 50% immobilization of *D. magna* (EC_{50}) resulting from exposure to phenanthrene-only were calculated. Furthermore, the EC_{30} and EC_{70} values were calculated and used as a control for biological variability throughout the rest of the study (i.e. in the tests subsequently performed with CNTs). This was done by exposing daphnids to phenanthrene-only at the concentrations corresponding to the calculated EC_{30} and EC_{70} values, and verifying that the responses were similar in all tests. The calculations and statistical analysis were performed using GraphPad Prism 6.0. EC values were calculated from nonlinear fit/concentration-response stimulation (log(agonist) vs. response), using an ordinary fit (least squares) and variable slope, with bottom and top constrain set as 0% and 100%, respectively. Statistical differences between groups were tested using analysis of variance (ANOVA) with Tukey's post hoc test.

Phenanthrene toxicity in the presence of CNTs. The CNT dispersions were prepared as described, spiked with five nominal concentrations ($C_{nominal}$) of phenanthrene (70-735 µg/L) and divided into aliquots of 20 mL contained in 22 mL glass vials. The phenanthrene $C_{nominal}$ range used was identical to that of the test with pure phenanthrene (no CNTs). The samples were left on an orbital shaker (165 rpm) in darkness for five days to ensure adsorption equilibrium.[11, 30] Once equilibrium had been achieved, five daphnids were placed in each sample vial and exposed for 48 h under identical conditions as those used in the phenanthrene-only toxicity test described above. Four replicates at each $C_{nominal}$ were used. Immobilization and abnormalities (CNT

attachment to the animals, mortality and disoriented animals) were recorded after the 48 hours. Controls containing only CNTs (CNT dispersions without phenanthrene) were included to account for any toxicity due to the CNTs themselves. In addition, animals were exposed to phenanthrene-only at the calculated EC_{30} and EC_{70} from the phenanthrene-only tests to control for any biological variability (as previously described).

Microscopy analysis. Visual inspections of the exposed daphnids, in the absence and presence of CNTs, were performed using light microscopy (Leica MZ-APO microscope; Leica Microsystems, with a DS-5M-U1 camera; Nikon).

Chemical analysis. The concentration of freely dissolved phenanthrene (C_{free}) was determined at the start of the exposure for all phenanthrene concentrations, both in the absence and presence of CNTs. Three replicates were analysed in the absence of CNTs, while single replicates were analysed in the presence of CNTs. Duplicate measurements of each replicate were applied in all cases. In studies where CNTs were present, filtration using a hydrophilic polytetrafluoroethylene (PTFE) filter membrane (0.1 μm pore size, Merck Millipore) was used to isolate and remove the dispersed CNTs from the water phase prior to determination of C_{free}. Details on the filtration technique have been previously published.[11, 31] Briefly, a highly reproducible sorption of phenanthrene to the filter membrane of approximately 18% was observed at all phenanthrene concentrations.[31] This allowed for the calculation of phenanthrene loss from the dissolved phase during the filtration process. C_{free} in the presence of CNTs was then used to calculate the phenanthrene adsorption to CNTs. C_{free} was determined by direct injection using high-performance liquid chromatography with photodiode-array UV detection (HPLC-UV; Agilent Technologies, Inc.) as previously described.[31] The Dubinin-Ashtakhov model (DAM), which was successfully used for

describing phenanthrene adsorption to CNTs in our previous study, was fitted to the adsorption data.[11]

RESULTS AND DISCUSSION

CNT properties and dispersion behavior. A detailed summary of the CNT physicochemical properties determined have been previously published[11] and is available in Table S1. Briefly, transmission electron microscopy analysis indicated that the SWCNT had an average diameter of ~2 nm whilst the four MWCNTs had average diameters that ranged between 14-20 nm. The SWCNT exhibited a SSA of 483.7 m^2/g, significantly higher than the MWCNTs with SSA values <177.4 m^2/g. Surface oxygen content was 5.7% and 3.9% for MWCNT-COOH and MWCNT-OH, respectively, while the non-functionalized CNTs all had similar surface oxygen contents of <2%.

The concentration of the five different CNTs remaining dispersed in the supernatants after the 24 h settling period was determined and these values were used to calculate the available surface area in each dispersion (**Table 1)**. The available surface area in each dispersion is calculated based on the individual SSA values determined for the dry CNTs rather than dispersed CNTs, and can therefore only be considered as estimates. However, as surface area is likely to be an important factor in controlling adsorption and to have a potentially decisive effect on co-contaminant bioavailability and toxicity,[11, 13, 32] these estimates offer a useful measure for interpreting the results generated in the subsequent toxicity studies.

The SWCNT and MWCNTs exhibited large differences in dispersibility, with the percentage of the original 10 mg/L of each CNT remaining in dispersion being 5 % SWCNT, 25 % MWCNT-

15, 26 % MWCNT-30, 23 % MWCNT-OH, and 24 % MWCNT-COOH. The SWCNT exhibited a lower dispersed concentration than the MWCNTs, consistent with the observations made in our previous study[11] and in accordance with studies reporting a positive relation between CNT diameter and dispersibility.[6] The dispersion concentrations for the four functionalized and non-functionalized MWCNTs were all very similar, being in the range 2.29-2.62 mg/L (23-26%). Interestingly, this is in contrast to our previous study using the same CNTs in algae medium with NOM, where higher dispersed concentrations were observed for the functionalized MWCNTs than for the non-functionalized MWCNTs.[11] Previous studies have shown a positive relationship between surface oxygen content and dispersibility.[6] However, artificial freshwater containing high concentrations of divalent cations and NOM has been shown to reduce the effect of CNT surface oxygen on dispersibility.[33] The Elendt M7 medium used in the current study is more complex and has a higher ionic strength and concentration of divalent cations than the algae medium used in our previous study.[11] The specific conductivity (measure of ionic strength) for Elendt M7 and the algae medium is 658 and 161 μS/cm, respectively, while the Ca^{2+} concentration (calculated from the media composition[29, 34]) is 2.0 and 0.1 mM, respectively. As the same CNTs, dispersion method and NOM concentration was employed in both studies, it is suggested that the Elendt M7 medium composition is causing the comparable dispersion concentrations for functionalized and non-functionalized MWCNTs. It is therefore important to consider the role of aquatic ecotoxicity media recommended for different species in standard tests. This also highlights the importance of such parameters in natural waters and the role they will have on CNT behavior in the aquatic environment.

Phenanthrene adsorption to CNTs. The determination of phenanthrene C_{free} showed significant phenanthrene adsorption to all five types of CNTs. When present at their individual maximum dispersion concentrations shown in **Table 1**, the five CNTs adsorbed 25-50% of the $C_{nominal}$ phenanthrene over the concentration range tested (70-735 µg/L; Figure S2). The adsorption isotherms are presented in **Figure 1**, and adsorption capacity and adsorption affinity for each of the CNTs, described by the DAM parameters log Q^0 and E, respectively, are presented in Table S2. Furthermore, C_{free} as a function of nominal phenanthrene concentrations ($C_{nominal}$) is presented in Figure S3. As the adsorption data used for model fitting was based on single data points, no statistical tests of the data were performed. The use of single data points was based upon (i) high reproducibility was observed between the replicates from adsorption studies in our previous study employing the same method[11], (ii) the DAM provided a strong fit for the single data points generated in the current study, and (iii) the calculated maximum adsorption capacities were highly comparable between the current and previous study for the non-functionalized MWCNTs. The generated adsorption isotherms and the fitted parameters contribute valuable information for comparison of adsorption behavior between the different CNT types in the M7 Elendt media.

The data showed a higher Q^0 of SWCNT compared to the MWCNTs. This is in accordance with our previous study using the same CNTs with algae medium and NOM,[11] and with other reports demonstrating a positive correlations between CNT SSA and Q^0.[32] Interestingly, no relationship between CNT surface oxygen content and Q^0 was observed in the current study. This was further supported by the adsorption isotherms, which were similar for both functionalized and non-functionalized MWCNTs (**Figure 1A**). These data are in contrast to previous observations using

262 the same CNTs in algae medium and NOM[11] and to other studies where a decrease in Q^0 with
263 increasing surface oxygen content has been observed.[12, 32] The Q^0 of the non-functionalized
264 MWCNTs was very similar in both our current (8.28 μg/kg and 8.37 μg/kg for MWCNT-15 and
265 MWCNT-30, respectively) and previous studies (8.22 μg/kg and 8.39 μg/kg for MWCNT-15
266 and MWCNT-30, respectively). However, a higher Q^0 was observed for the functionalized
267 MWCNTs in the current study, with values being similar to those of the non-functionalized
268 MWCNTs. For example, the Q^0 of MWCNT-COOH in the current study was 8.20 μg/kg,
269 compared to 7.64 μg/kg in the previous study. Normalizing the data against the estimated
270 available surface area allows for further investigation of the effect of surface chemistry. Using
271 data from our previous study, surface area normalized adsorption isotherms of the functionalized
272 MWCNTs, in particular for MWCNT-COOH, differed from those of the non-functionalized
273 MWCNTs (Figure S4). This indicated a suppression of phenanthrene adsorption due to the
274 presence of oxygen containing surface functional groups. In the current study, normalizing the
275 adsorption isotherms against the estimated available surface area resulted in very similar
276 adsorption isotherms for all MWCNTs irrespective of surface chemistry (**Figure 1B**). In the M7-
277 NOM medium, the presence of functional groups does not appear to influence phenanthrene
278 adsorption to CNTs significantly. We have already demonstrated that media properties (ionic
279 strength, concentration of divalent cations) can influence the role of CNT surface chemistry on
280 CNT dispersibility. The current study also indicates that media properties are of importance with
281 respect to the role of CNT surface chemistry on adsorption behavior. Media properties have been
282 reported to affect adsorption of NOM to CNTs,[33] but there is limited knowledge regarding the
283 effect of media properties on the adsorption of HOCs, such as phenanthrene, to CNTs. Further
284 studies are required to assess the role of aquatic media properties on adsorption behavior, as it

would also have implications for the further effect of CNTs on co-contaminant bioavailability and the process of HOC adsorption in different aquatic environments.

In the current study, no clear relationship was observed between CNT properties and *E*. Similar observations were made in our previous study with the same CNTs in algae medium.[11] It has been previously reported that *E* of PAHs (pyrene) to CNTs varied with the type functionalization when dispersed by shaking. However, treatment with sonication removed differences in *E* between different CNT types, but also resulted in an overall increase in *E* for all CNT types.[13] The presence of NOM has been shown to reduce *E*.[14] Despite the identical sonication treatment and SR-NOM concentration used in both our current and previous studies[11], slightly lower *E* values were observed for all CNTs in the current study, in particular for the MWCNTs. Again, this suggests an influence of media properties on CNT adsorption behavior, which should receive attention in future studies.

Phenanthrene toxicity to daphnids. In all of the control exposures (pure Elendt M7, M7-NOM and M7-NOM with methanol) <10% of the animals were immobilized, meeting with the OECD test criteria. This confirmed there was no toxicity that could be attributed to the M7 media, the presence of NOM or from the methanol used for dissolving phenanthrene. There were no variations in pH outside the recommended range in any of the tests, and the dO_2 concentration at the end of the experiment was well above the validation criteria (>3 mg/L) of the test guideline.

The percentage immobilization of daphnids as a function of phenanthrene concentration for the six individual tests performed is presented, together with the calculated EC_{50} values, in Figure

S5. Although the C_{free} (i.e. the measured phenanthrene concentration in the water phase) varied by <10% from $C_{nominal}$ at all phenanthrene concentrations tested in the absence of CNTs, dose-response curves were calculated both for $C_{nominal}$ and C_{free}. This was done as the response of *D. magna* to phenanthrene exposure was subsequently compared in the absence and presence of CNTs, both for $C_{nominal}$ and C_{free}. The CV of the calculated EC_{50} values for the six tests was <6%. As the variability between the tests was low, the data from the six tests were pooled in order to create an average dose-response curve and determine EC_{50} values for phenanthrene. The average dose-response curves for phenanthrene-only are presented in **Figure 2** (together with dose-response curves for phenanthrene in the presence of CNTs), while the average 48 h EC_{50} value is shown in **Table 2**. Based on $C_{nominal}$ and C_{free}, the EC_{50} values were 342.1 µg/L ($EC_{50,nominal}$) and 310.9 µg/L ($EC_{50,free}$), respectively. The 48 h EC_{50} for 3-4 day old daphnids exposed to phenanthrene was comparable to the 48 h EC_{50} values for <24 h old daphnids previously reported (range from 230 µg/L to 550 µg/L).[25, 35-37] Although we observed comparability with literature values for <24 h old daphnids, a detailed assessment of the implications of using 3-4 day old daphnids was not tested in the current study.

Phenanthrene toxicity in the presence of CNTs. In control exposures containing CNTs-only (no phenanthrene), the immobilization of daphnids was <10% in all cases, indicating there was no acute toxicity from any of the five CNT types at the concentrations tested (**Table 1**). Previous studies have reported a 50% mortality (LC_{50}) of *D. magna* (<24 h) exposed to MWCNTs dispersed in NOM at concentrations of 2-2.5 mg/L.[18, 38] These LC_{50} concentrations are comparable to the CNT concentrations used in the current study. However, they were achieved after a 96 h exposure time rather than the recommended 48 h exposure time in the test guideline.

One of these studies also investigated *D. magna* (<24 h) mortality after 48 h, observing no mortality even at 20 mg/L MWCNT exposure concentrations.[38] The absence of an acute toxic response after 48 h and at the CNT concentrations employed in the current study therefore appears comparable to previous studies despite the difference in age of the *D. magna* used. The positive control samples contained daphnids exposed to phenanthrene-only (no CNTs) at concentrations representing the calculated EC_{30} and EC_{70} from the phenanthrene-only tests. These exhibited similar responses as those obtained in the phenanthrene-only tests, indicating no biological variability between the tests.

D. magna immobilization as a function of phenanthrene concentration in the presence of CNTs is presented in **Figure 2**, and the calculated EC_{50} values are presented in **Table 2**. When using the $EC_{50,nominal}$ values, a significant reduction in phenanthrene toxicity (p<0.003) was observed in the presence of SWCNT and MWCNT-30 compared to phenanthrene-only (**Figure 2A**, **Table 2**). For the three other CNTs (MWCNT-15, MWCNT-OH and MWCNT-COOH), no significant difference in phenanthrene toxicity was observed in exposures with and without the presence of CNTs. Owing to the slightly higher dispersion concentration (2.62 mg/L) and available surface area (0.46 m^2/L) of MWCNT-30 compared to the other MWCNTs (2.29-2.45 mg/L and 0.32-0.34 m^2/L, respectively; **Table 1**), MWCNT-30 adsorbed a higher quantity of the added phenanthrene. As a result, C_{free} was lower in the presence of MWCNT-30 than for the other MWCNTs (Figure S3). In the SWCNTs dispersions, the C_{free} was also lower than the C_{free} values determined for MWCNT-15, MWCNT-OH and MWCNT-COOH for most of the $C_{nominal}$ phenanthrene values used in the study. As it was only the SWCNT and MWCNT-30 that reduced phenanthrene toxicity through adsorption, this could indicate that increased adsorption of

354 phenanthrene by CNTs (C_{CNT}) causes a reduction in phenanthrene bioavailability to *D. magna*.
355 Importantly, similar EC_{50} values were observed for both the phenanthrene-only exposures and
356 the combined phenanthrene-CNT exposures for MWCNT-15, MWCNT-OH and MWCNT-
357 COOH despite a reduction in C_{free} of 25-40% in these dispersions. These results indicate that
358 phenanthrene adsorbed to CNTs remains bioavailable to *D. magna*. This bioavailability was
359 further investigated by calculating dose-response curves for each CNT type based on their
360 corresponding C_{free} values (**Figure 2B**, **Table 2**). The data show a significant increase in
361 phenanthrene toxicity in the presence of all CNT types when compared to phenanthrene-only.
362 Therefore, the study strongly indicates that the phenanthrene adsorbed to all five different CNT
363 types remained at least partially bioavailable and contributed directly to the observed toxicity.
364 Similarly, it has been observed that phenanthrene adsorbed to the fullerene C_{60} and to suspended
365 sediment remain bioavailable and contribute to toxicity when exposed to *D. magna*.[25, 37]
366 Moreover, phenanthrene absorbed to the same CNTs as used in the current study remained
367 bioavailable to the algae *P. subcapitata*.[11]
368
369 No significant difference in toxicity was observed between any of the CNT types based on C_{free}
370 values. Thus, at similar C_{free} values, the contribution to toxicity from CNT-adsorbed
371 phenanthrene was comparable for all types of CNT. This indicates that CNT physicochemical
372 properties did not significantly influence the mechanisms by which CNT-adsorbed phenanthrene
373 was bioavailable or toxic to *D. magna*. It has been previously suggested that aggregation,
374 expected to be higher for SWCNTs than MWCNTs due to the smaller diameter of SWCNTs,
375 influences CNT elimination rates from the organisms and therefore affects PAH bioavailability
376 to fish (*Oryzias latipes* and *Pimephales promelas*).[19, 24] Although the similar $EC_{50,free}$ values

377 determined for each CNT type in the current study does not support this hypothesis, the
378 importance of CNT aggregation behavior could vary for different organisms depending on their
379 exposure and uptake routes. The data for *D. magna* are comparable to our previous study with
380 freshwater algae (*P. subcapitata*), where no clear relationship was observed between any of the
381 CNT physicochemical properties evaluated and the $EC_{50,free}$ values determined.[11] It is interesting
382 to note that in both studies the hill slope of the dose-response curves were somewhat steeper for
383 MWCNT-15 compared to the other CNTs and phenanthrene-only.[11] This indicates there may be
384 some variation in the bioavailability of phenanthrene in the presence of the different CNTs,
385 despite the similar $EC_{50,free}$ observed for all CNTs. However, this would need further
386 investigation. The same observation regarding hill slope was made both for algae and for
387 daphnids, suggesting this possible variation in phenanthrene bioavailability is independent of the
388 organism exposed to the CNT/phenanthrene mixture, and more likely related to one or more of
389 the physicochemical differences between the test CNTs.
390
391 Optical microscopy images showed that CNTs were present in the digestive tracts of the
392 daphnids (**Figure 3**). In some cases, CNT aggregates were observed attached to the surface of
393 the daphnids; however, these were easily removed or dislodged by slight movement of the
394 organisms with a pipette. As these surface-attached CNT aggregates were only occasionally
395 observed, their influence on phenanthrene bioavailability and toxicity to *D. magna* is considered
396 negligible when compared to the large quantity of ingested CNTs (observed in the gut). Uptake
397 of PAHs through biological membranes and by pelagic invertebrates by passive diffusion has
398 previously been reported.[39, 40] In the absence of CNTs, passive diffusion is considered the main
399 uptake route of dissolved phenanthrene by *D. magna* in the current study. In the presence of

CNTs, uptake by passive diffusion would be lower due to the lower C_{free}. Thus, data from the toxicity study indicate that another uptake route contributed to the toxicity. Ingestion of CNTs by daphnids appears to represent an alternative uptake route for phenanthrene, and the data suggest that CNT-adsorbed phenanthrene must be desorbing from the CNTs when passing through the digestive tract. Dissolved PAHs in aqueous systems will undergo adsorption or desorption to CNTs in order to establish equilibrium.[41] When CNTs with adsorbed phenanthrene are ingested by *D. magna*, a lower (or most likely zero) phenanthrene concentration in the digestive tract will therefore promote desorption of phenanthrene from the CNTs into the dissolved phase where it is known to be bioavailable. Furthermore, the altered chemical environment in the digestive tract of *D. magna* might also promote desorption of phenanthrene from CNTs (e.g. pH which is 6.0-7.2 in daphnids[42]). The high Q^0 of CNTs implies that their presence could significantly alter the exposure routes of phenanthrene or other HOCs, increasing the likelihood of dietary exposure. A increased negative impact on endpoints such as reproduction and egg production in daphnids has been observed following dietary exposure and uptake to silver nanoparticles when compared to uptake of dissolved silver through passive diffusion from the water.[43] However, the importance of dietary uptake with respect to uptake by passive diffusion for PAHs in *D. magna* is not known.

In the current study, no acute toxicity (immobilization) was observed due to the presence of the CNTs at any of the dispersion concentrations employed. However, sublethal effects caused by CNTs, and which may be compounded in the presence of phenanthrene, cannot be excluded. Furthermore, the presence of CNTs in the exposure system may facilitate an increased uptake or toxicological response to the dissolved phenanthrene fraction. Synergistic effects arising from

423 the mixture of CNTs and phenanthrene could therefore represent another mechanism responsible
424 for the increased toxicity based on C_{free} observed in the current study. An insight into the role of
425 mixture toxicity could be gained by investigating other, more sensitive, endpoints for CNT
426 toxicity, or by employing different CNT concentrations in a study where phenanthrene
427 concentration remains constant. In the current study, no difference in toxicity was observed
428 between the different types of CNTs when evaluating C_{free}. This implies that the contribution
429 from the CNTs to any mixture toxicity effect should be similar for all CNT types, irrespective of
430 their different properties and different dispersion concentrations (e.g. SWCNTs compared to
431 MWCNTs). This would be in contrast to previous studies where a higher toxicity for non-
432 functionalized MWCNTs compared to functionalized MWCNTs has been reported for daphnids
433 (*Ceriodaphnia dubia*).[44] Furthermore, comparable observations were made in our previous study
434 where no difference in toxicity (based on C_{free}) was observed between CNT types. Although the
435 combined effects of CNTs and phenanthrene could be a mechanism of toxicity, the data of our
436 current and previous study suggest that this alone cannot explain the increased toxicity observed
437 based on C_{free}.
438
439 There are several similarities between the toxicity data in our current study and our previous
440 study with freshwater algae (*P. subcapitata*).[11] Most importantly, in the previous study a
441 significant reduction in algal toxicity was only observed when using $C_{nominal}$ values in the
442 presence of SWCNT. However, when considering C_{free}, an increase in algal toxicity compared to
443 phenanthrene-only control samples was observed in the presence of all CNTs, similar to the
444 observations made in the current study.[11] These similarities were observed despite the fact that
445 the exposure routes of CNT-adsorbed phenanthrene are significantly different for algae (e.g.

446 dietary exposure is not relevant). Attachment of algal cells to CNT aggregates with adsorbed
447 phenanthrene was believed to be of importance. In addition, a measurable reduction in C_{free} due
448 to the presence of algae was observed, implying that desorption of phenanthrene from CNTs
449 could contribute to maintaining a higher C_{free}.[11] In contrast, the current study found there was no
450 significant difference in C_{free} in the presence and absence of *D. magna* in solutions with
451 phenanthrene-only (Figure S6). Thus, desorption of phenanthrene from CNTs in the water phase
452 is not believed to significantly influence C_{free}. It has been previously suggested that the presence
453 of NOM impedes desorption of PAHs from CNTs, thus reduces the PAH bioavailability to fish.[24]
454 However, our current and previous studies[11] suggest that the presence of NOM does not in
455 general prevent bioavailability of PAHs adsorbed to CNTs, as the CNT-adsorbed phenanthrene
456 was bioavailable to both algae and daphnids despite the presence of NOM in these studies.
457 However, the mechanisms of uptake and toxicity of CNT-adsorbed PAHs are different in
458 different species and trophic levels (e.g. algae, daphnids and fish). Both the mechanisms by
459 which CNT-adsorbed phenanthrene contributes to toxicity for different organisms and the role of
460 NOM in affecting the bioavailability of HOCs adsorbed by CNTs is of high environmental
461 relevance and should be further investigated.
462
463
464 ACKNOWLEDGMENT
465 The work reported here has been undertaken as part of the Research Council of Norway (RCN)
466 funded project 'NanoSorb' (Grant Agreement number 209685/E50). The authors wish to thank
467 the RCN for their financial support. We also wish to thank the External Cooperation Program of
468 Chinese Academy of Sciences (Grant number GJHZ1206) for financial support. The authors

469 acknowledge the essential technical assistance of Kristin Bonaunet, Lisbet Støen, Inger
470 Steinsvik, Marianne Rønsberg, Kjersti Almås, Calin D. Marioara, John Walmsley and Aud
471 Spjelkavik (SINTEF Materials and Chemistry). We would also like to thank Gurvinder Singh for
472 creating SEM images of the CNT materials.

473

474 ASSOCIATED CONTENT

475 **Supporting Information**. Tables showing physicochemical properties of the CNTs and fitted
476 parameters of the Dubinin-Ashtakhov model. Figures showing CNT calibration curves, C_{CNT} (%)
477 and C_{free} as a function of $C_{nominal}$, adsorption isotherms from a previous study for comparison,
478 dose-response curves from six tests addressing phenanthrene toxicity to *D. magna* and measured
479 phenanthrene concentration after 48 hours exposure. This material is available free of charge via
480 the Internet at http://pubs.acs.org.

481

482 AUTHOR INFORMATION

483 * Corresponding author
484 E-mail address: andy.booth@sintef.no.
485 Tel.:+47 93089510
486 Address: SINTEF Materials and Chemistry, Trondheim NO-7465, Norway

487

488 **Current address**

489 § Aquatic Ecology and Toxicology Section, Centre for Organismal Studies (COS), University of
490 Heidelberg, Im Neuenheimer Feld 504, D-69120 Heidelberg, Germany.

491 REFERENCES

492 1. De Volder, M. F. L.; Tawfick, S. H.; Baughman, R. H.; Hart, A. J., Carbon Nanotubes:
493 Present and Future Commercial Applications. *Science* **2013,** *339*, (6119), 535-539.
494 2. Yu, J. G.; Zhao, X. H.; Yang, H.; Chen, X. H.; Yang, Q.; Yu, L. Y.; Jiang, J. H.; Chen, X.
495 Q., Aqueous adsorption and removal of organic contaminants by carbon nanotubes. *Sci. Total*
496 *Environ.* **2014,** *482*, 241-251.
497 3. Koelmans, A. A.; Diepens, N. J.; Velzeboer, I.; Besseling, E.; Quik, J. T. K.; van de
498 Meent, D., Guidance for the prognostic risk assessment of nanomaterials in aquatic ecosystems.
499 *Sci. Total Environ.* **2015,** *535*, 141-149.
500 4. Cerrillo, C.; Barandika, G.; Igartua, A.; Areitioaurtena, O.; Uranga, N.; Mendoza, G.,
501 Colloidal stability and ecotoxicity of multiwalled carbon nanotubes: Influence of select organic
502 matters. *Environ. Toxicol. Chem.* **2016,** *35*, (1), 74-83.
503 5. Hyung, H.; Fortner, J. D.; Hughes, J. B.; Kim, J. H., Natural organic matter stabilizes
504 carbon nanotubes in the aqueous phase. *Environmental Science & Technology* **2007,** *41*, (1),
505 179-184.
506 6. Schwyzer, I.; Kaegi, R.; Sigg, L.; Smajda, R.; Magrez, A.; Nowack, B., Long-term
507 colloidal stability of 10 carbon nanotube types in the absence/presence of humic acid and
508 calcium. *Environmental Pollution* **2012,** *169*, 64-73.
509 7. Peijnenburg, W. J. G. M.; Baalousha, M.; Chen, J.; Chaudry, Q.; Von der kammer, F.;
510 Kuhlbusch, T. A. J.; Lead, J.; Nickel, C.; Quik, J. T. K.; Renker, M.; Wang, Z.; Koelmans, A. A.,
511 A Review of the Properties and Processes Determining the Fate of Engineered Nanomaterials in
512 the Aquatic Environment. *Critical Reviews in Environmental Science and Technology* **2015,** *45*,
513 (19), 2084-2134.
514 8. Petersen, E. J.; Zhang, L. W.; Mattison, N. T.; O'Carroll, D. M.; Whelton, A. J.; Uddin,
515 N.; Nguyen, T.; Huang, Q. G.; Henry, T. B.; Holbrook, R. D.; Chen, K. L., Potential Release
516 Pathways, Environmental Fate, And Ecological Risks of Carbon Nanotubes. *Environmental*
517 *Science & Technology* **2011,** *45*, (23), 9837-9856.
518 9. Mauter, M. S.; Elimelech, M., Environmental applications of carbon-based
519 nanomaterials. *Environmental Science & Technology* **2008,** *42*, (16), 5843-5859.
520 10. Ren, X.; Chen, C.; Nagatsu, M.; Wang, X., Carbon nanotubes as adsorbents in
521 environmental pollution management: A review. *Chemical Engineering Journal* **2011,** *170*, (2-
522 3), 395-410.
523 11. Glomstad, B.; Altin, D.; Sørensen, L.; Liu, J.; Jenssen, B. M.; Booth, A. M., Carbon
524 Nanotube Properties Influence Adsorption of Phenanthrene and Subsequent Bioavailability and
525 Toxicity to Pseudokirchneriella subcapitata. *Environmental Science & Technology* **2016,** *50*, (5),
526 2660-2668.
527 12. Wu, W. H.; Chen, W.; Lin, D. H.; Yang, K., Influence of Surface Oxidation of
528 Multiwalled Carbon Nanotubes on the Adsorption Affinity and Capacity of Polar and Nonpolar
529 Organic Compounds in Aqueous Phase. *Environmental Science & Technology* **2012,** *46*, (10),
530 5446-5454.
531 13. Kah, M.; Zhang, X. R.; Hofmann, T., Sorption behavior of carbon nanotubes: Changes
532 induced by functionalization, sonication and natural organic matter. *Science of the Total*
533 *Environment* **2014,** *497*, 133-138.

14. Zhang, X.; Kah, M.; Jonker, M. T. O.; Hofmann, T., Dispersion State and Humic Acids Concentration-Dependent Sorption of Pyrene to Carbon Nanotubes. *Environmental Science & Technology* **2012,** *46*, (13), 7166-7173.
15. Pan, B.; Xing, B., Adsorption Mechanisms of Organic Chemicals on Carbon Nanotubes. *Environmental Science & Technology* **2008,** *42*, (24), 9005-9013.
16. Petersen, E. J.; Akkanen, J.; Kukkonen, J. V. K.; Weber, W. J., Biological Uptake and Depuration of Carbon Nano-tubes by Daphnia magna. *Environmental Science & Technology* **2009,** *43*, (8), 2969-2975.
17. Roberts, A. P.; Mount, A. S.; Seda, B.; Souther, J.; Qiao, R.; Lin, S. J.; Ke, P. C.; Rao, A. M.; Klaine, S. J., In vivo biomodification of lipid-coated carbon nanotubes by Daphnia magna. *Environmental Science & Technology* **2007,** *41*, (8), 3025-3029.
18. Edgington, A. J.; Roberts, A. P.; Taylor, L. M.; Alloy, M. M.; Reppert, J.; Rao, A. M.; Mao, J. D.; Klaine, S. J., The Influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environmental Toxicology and Chemistry* **2010,** *29*, (11), 2511-2518.
19. Su, Y.; Yan, X. M.; Pu, Y. B.; Xiao, F.; Wang, D. S.; Yang, M., Risks of Single-Walled Carbon Nanotubes Acting as Contaminants-Carriers: Potential Release of Phenanthrene in Japanese Medaka (Oryzias latipes). *Environmental Science & Technology* **2013,** *47*, (9), 4704-4710.
20. Alloy, M. M.; Roberts, A. P., Effects of suspended multi-walled carbon nanotubes on daphnid growth and reproduction. *Ecotoxicology and Environmental Safety* **2011,** *74*, (7), 1839-1843.
21. Long, Z. F.; Ji, J.; Yang, K.; Lin, D. H.; Wu, F. C., Systematic and Quantitative Investigation of the Mechanism of Carbon Nanotubes' Toxicity toward Algae. *Environmental Science & Technology* **2012,** *46*, (15), 8458-8466.
22. Schwab, F.; Bucheli, T. D.; Lukhele, L. P.; Magrez, A.; Nowack, B.; Sigg, L.; Knauer, K., Are Carbon Nanotube Effects on Green Algae Caused by Shading and Agglomeration? *Environmental Science & Technology* **2011,** *45*, (14), 6136-6144.
23. Smith, C. J.; Shaw, B. J.; Handy, R. D., Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Toxicology* **2007,** *82*, (2), 94-109.
24. Linard, E. N.; van den Hurk, P.; Karanfil, T.; Apul, O. G.; Klaine, S. J., Influence of carbon nanotubes on the bioavailability of fluoranthene. *Environ. Toxicol. Chem.* **2015,** *34*, (3), 658-666.
25. Baun, A.; Sorensen, S. N.; Rasmussen, R. F.; Hartmann, N. B.; Koch, C. B., Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C-60. *Aquatic Toxicology* **2008,** *86*, (3), 379-387.
26. Ferguson, P. L.; Chandler, G. T.; Templeton, R. C.; Demarco, A.; Scrivens, W. A.; Englehart, B. A., Influence of sediment-amendment with single-walled carbon nanotubes and diesel soot on bioaccumulation of hydrophobic organic contaminants by benthic invertebrates. *Environmental Science & Technology* **2008,** *42*, (10), 3879-3885.
27. Schwab, F.; Bucheli, T. D.; Camenzuli, L.; Magrez, A.; Knauer, K.; Sigg, L.; Nowack, B., Diuron Sorbed to Carbon Nanotubes Exhibits Enhanced Toxicity to Chlorella vulgaris. *Environmental Science & Technology* **2013,** *47*, (13), 7012-7019.
28. Shen, M.; Xia, X.; Zhai, Y.; Zhang, X.; Zhao, X.; Zhang, P., Influence of carbon nanotubes with preloaded and coexisting dissolved organic matter on the bioaccumulation of

polycyclic aromatic hydrocarbons to Chironomus plumosus larvae in sediment. *Environmental Toxicology and Chemistry* **2014,** *33*, (1), 182-189.
29. OECD, Test No. 202: Daphnia sp. Acute Immobilisation Test. In *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing: Paris, 2004.
30. Yang, K.; Zhu, L. Z.; Xing, B. S., Adsorption of polycyclic aromatic hydrocarbons by carbon nanomaterials. *Environmental Science & Technology* **2006,** *40*, (6), 1855-1861.
31. Glomstad, B.; Sørensen, L.; Liu, J.; Shen, M.; Zindler, F.; Jenssen, B. M.; booth, A. M., Evaluation of methods to determine adsorption of polycyclic aromatic hydrocarbons to dispersed carbon nanotubes. **2016,** *Submitted*.
32. Yang, K.; Xing, B., Adsorption of Organic Compounds by Carbon Nanomaterials in Aqueous Phase: Polanyi Theory and Its Application. *Chemical Reviews* **2010,** *110*, (10), 5989-6008.
33. Smith, B.; Yang, J.; Bitter, J. L.; Ball, W. P.; Fairbrother, D. H., Influence of Surface Oxygen on the Interactions of Carbon Nanotubes with Natural Organic Matter. *Environmental Science & Technology* **2012,** *46*, (23), 12839-12847.
34. OECD, Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. In *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing: Paris, 2011.
35. Smith, K. E. C.; Dom, N.; Blust, R.; Mayer, P., Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. *Aquatic Toxicology* **2010,** *98*, (1), 15-24.
36. Wu, J.; Liu, Z.; Yan, Z.; Yi, X., Derivation of water quality criteria of phenanthrene using interspecies correlation estimation models for aquatic life in China. *Environmental Science and Pollution Research* **2015,** *22*, (12), 9457-9463.
37. Zhang, X.; Xia, X.; Dong, J.; Bao, Y.; Li, H., Enhancement of toxic effects of phenanthrene to Daphnia magna due to the presence of suspended sediment. *Chemosphere* **2014,** *104*, 162-169.
38. Kim, K.-T.; Edgington, A. J.; Klaine, S. J.; Cho, J.-W.; Kim, S. D., Influence of Multiwalled Carbon Nanotubes Dispersed in Natural Organic Matter on Speciation and Bioavailability of Copper. *Environmental Science & Technology* **2009,** *43*, (23), 8979-8984.
39. Bugg, T.; Foght, J. M.; Pickard, M. A.; Gray, M. R., Uptake and active efflux of polycyclic aromatic hydrocarbons by Pseudomonas fluorescens LP6a. *Applied and Environmental Microbiology* **2000,** *66*, (12), 5387-5392.
40. Meador, J. P.; Stein, J. E.; Reichert, W. L.; Varanasi, U., Bioaccumulation of Polycyclic Aromatic Hydrocarbons by Marine Organisms. *Reviews of Environmental Contamination and Toxicology <D>* **1995,** *143*, 79-165.
41. Yang, K.; Xing, B. S., Desorption of polycyclic aromatic hydrocarbons from carbon nanomaterials in water. *Environmental Pollution* **2007,** *145*, (2), 529-537.
42. Ebert, D., Introduction to Daphnia Biology. In *Ecology, Epidemiology, and Evolution of Parasitism in Daphnia*, National Center for Biotechnology Information,: Bethesda (MD), USA, 2005.
43. Hook, S. E.; Fisher, N. S., Sublethal effects of silver in zooplankton: Importance of exposure pathways and implications for toxicity testing. *Environ. Toxicol. Chem.* **2001,** *20*, (3), 568-574.
44. Kennedy, A. J.; Hull, M. S.; Steevens, J. A.; Dontsova, K. M.; Chappell, M. A.; Gunter, J. C.; Weiss, C. A., Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. *Environmental Toxicology and Chemistry* **2008,** *27*, (9), 1932-1941.

625

Table 1. CNT dispersed concentration in M7-NOM and calculated available surface area.

	Dispersed concentration (mg/L)	Percentage dispersed (%)	Available surface area (m^2/L)
SWCNT	0.46	4.6	0.22
MWCNT-15	2.45	24.5	0.34
MWCNT-30	2.62	26.2	0.46
MWCNT-OH	2.29	22.6	0.32
MWCNT-COOH	2.41	24.1	0.34

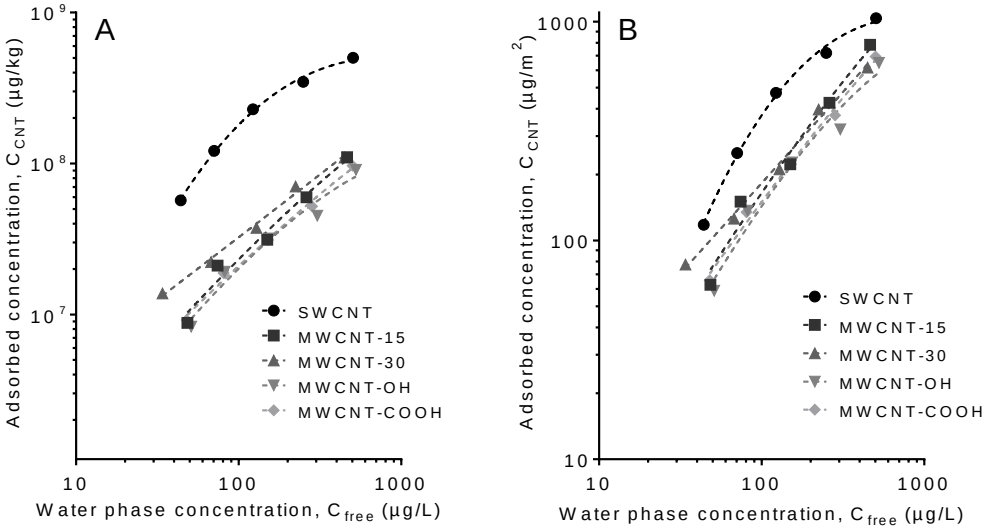
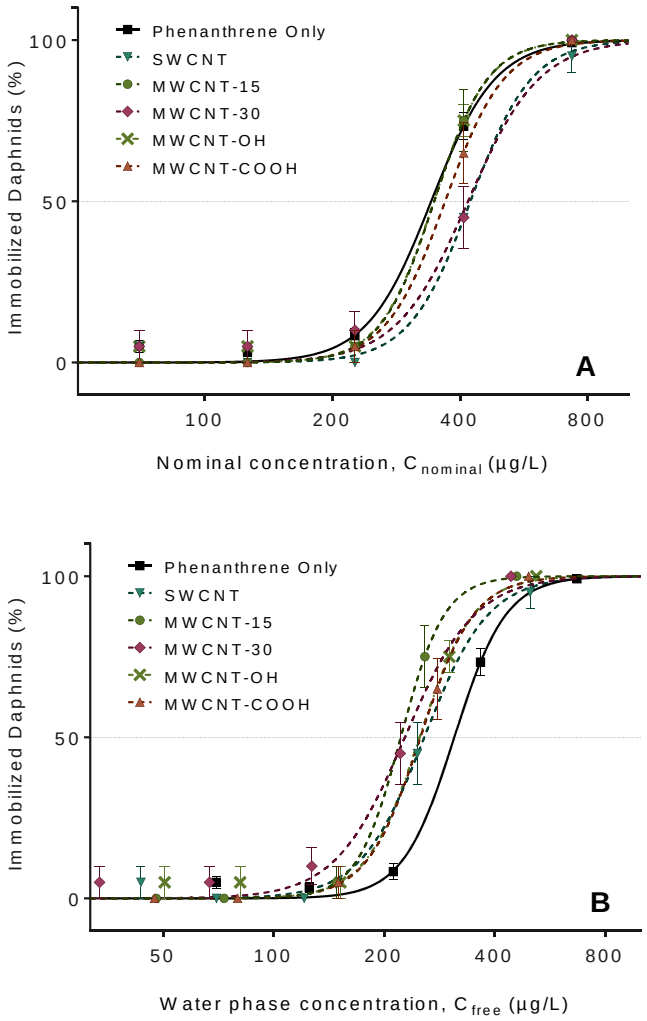


Figure 1. Adsorption isotherms of phenanthrene adsorbed to five types of CNTs calculated based on A) CNT mass and B) CNT available surface area.



638

639 **Figure 2.** Immobilization of D. magna after exposure to phenanthrene in the absence and
640 presence of CNTs. Phenanthrene concentrations are presented as A) nominal concentrations
641 ($C_{nominal}$), and B) freely dissolved concentrations (C_{free}). Error bars represent the standard error of
642 mean.
643

644

Table 2. Calculated EC_{50} values for both $C_{nominal}$ ($EC_{50,\ nominal}$) and C_{free} ($EC_{50,\ free}$) following exposure to phenanthrene-only and phenanthrene in the presence of CNTs. Variations are presented as the 95% confidence interval (CI) and the goodness of fit described by the coefficient of determination (R^2). The EC_{50} values for phenanthrene-only are based on pooled data from six individual toxicity tests. Differing letters indicate statistical significant difference (p<0.05) within columns.

CNT	$EC_{50,nominal}$ (µg/L)	95% CI	R^2	$EC_{50,free}$ (µg/L)	95% CI	R^2
Phenanthrene-only	342.1[a]	327.3-357.5	0.9207	310.9[a]	298.5-323.6	0.9207
SWCNT	423.2[b]	393.1-455.7	0.9373	257.5[b]	235.6-281.4	0.9373
MWCNT-15	347.8[ab]	316.6-382.0	0.9632	222.1[b]	203.4-242.6	0.9632
MWCNT-30	417.9[b]	379.9-459.6	0.9119	227.9[b]	205.1-253.3	0.9163
MWCNT-OH	347.2[ab]	316.4-381.0	0.9602	250.0[b]	224.4-278.5	0.9601
MWCNT-COOH	369.0[ab]	340.8-399.5	0.9601	251.3[b]	231.1-273.3	0.9599

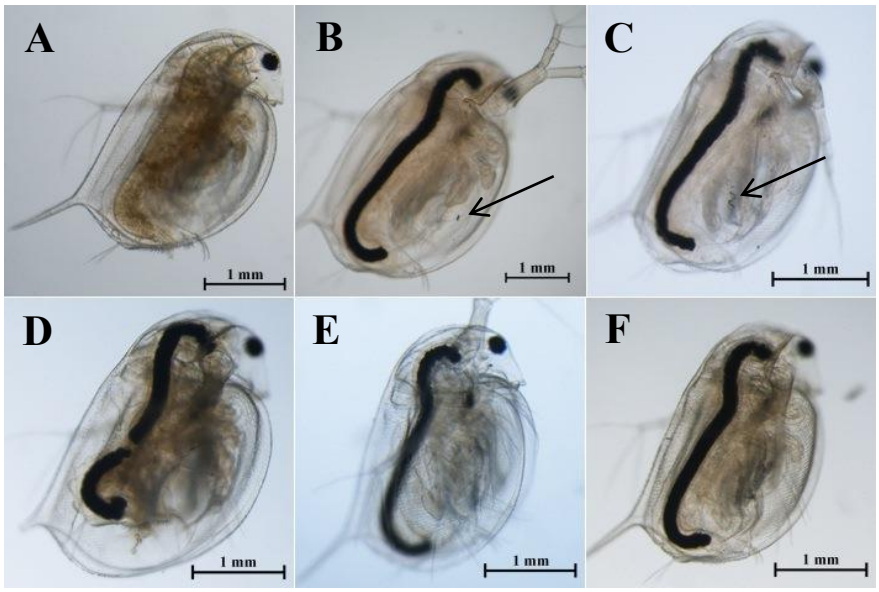
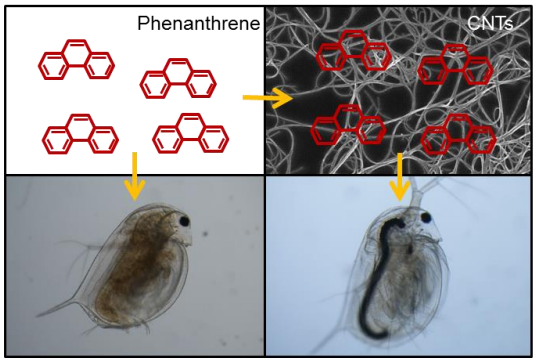


Figure 3. Light microscopy images (x40) of *D. magna* after 48 hours with A) no exposure or B-F) exposed to CNTs. (SWCNT, MWCNT-15, MWCNT-30, MWCNT-OH and MWCNT-COOH for B, C, D, E and F, respectively.) The presence of CNTs in the digestive tract was clearly visible in exposed animals. Black arrows show CNT aggregates attached to the surface of the organisms.

659 **TOC/Abstract art**

660



661