

1 **Dispersibility and dispersion stability of carbon nanotubes in**
2 **synthetic aquatic growth media and natural freshwater**

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17

18 ABSTRACT

19 The dispersion behavior of carbon nanotubes (CNTs) is influenced by both their
20 physicochemical properties and by the aqueous media properties (e.g. ionic strength, presence of
21 divalent cations and natural organic matter) in which they are dispersed. In the current study, the
22 dispersibility and dispersion stability of four multi-walled CNTs (MWCNT) and a single walled
23 CNT (SWCNT) with different physicochemical properties were investigated in three freshwater
24 growth media (with and without natural organic matter; NOM) used in algae and daphnia
25 ecotoxicity studies. CNT dispersion behavior was also investigated in a natural freshwater for
26 comparison. SWCNTs and non-functionalized MWCNTs showed similar dispersibility
27 irrespective of the media type (SWCNTs = 0.5-0.9 mg/L; MWCNTs = 1.5-2.8 mg/L).
28 Functionalized MWCNTs exhibited higher dispersion concentrations, but were more dependent
29 upon the ionic strength and divalent cation concentration of each media (MWCNT-COOH = 3.0-
30 6.6 mg/L). In contrast, CNT surface oxygen content had no influence on CNT dispersibility in
31 the natural water (all MWCNTs = 0.9-1.4 mg/L). Functionalized MWCNTs were affected more
32 by the differences in media properties than non-functionalized MWCNTs. The dispersed CNT
33 concentration decreased over time for all CNT types and in all media due to sedimentation, but
34 was influenced by both CNT and media properties. The study shows how a complex interplay
35 between CNT and media properties can influence the environmental fate of CNTs. Furthermore,
36 the study demonstrates how different CNT types and/or ecotoxicological media in aquatic tests
37 influences the dispersion behavior of the CNTs, and thus their exposure and toxicity to aquatic
38 organisms.

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40

41 KEYWORDS

42 Carbon nanotubes, fate, dispersion, stability, sedimentation, ecotoxicology

43 INTRODUCTION

44 Carbon nanotubes (CNTs) in their pristine form are hydrophobic and prone to aggregation in
45 aqueous media. The dispersibility (dispersion concentration) and dispersion stability
46 (agglomeration and aggregation) of CNTs plays a key role in their fate in aquatic environments
47 (Petersen et al., 2011). This in turn affects their potential for exposure and toxicity to pelagic and
48 benthic organisms (Schwab et al., 2011; Du et al., 2013; Parks et al., 2013; Cerrillo et al., 2016b).
49 Furthermore, the dispersion state of CNTs can influence their interaction with other environmental
50 contaminants and thus potentially affect the environmental fate and effects of such environmental
51 contaminants (Zhang et al., 2012; Su et al., 2013; Glomstad et al., 2016; Zindler et al., 2016;
52 Glomstad et al., 2017).

53
54 Both the physicochemical properties of CNTs and environmental factors, including the presence
55 of natural organic matter (NOM) and the ionic strength of the aquatic media, are known to
56 influence their dispersibility and dispersion stability (Hyung et al., 2007; Kennedy et al., 2008;
57 Peijnenburg et al., 2015). The presence of NOM has been found to increase the stability of CNTs
58 in water through electrostatic and/or steric repulsion (Hyung et al., 2007; Lin et al., 2012;
59 Peijnenburg et al., 2015). In contrast, increased ionic strength, and in particular the presence of
60 divalent cations, induces aggregation and sedimentation of CNTs (Saleh et al., 2008; Li and
61 Huang, 2010; Lin et al., 2012). A wide range of CNTs with different physicochemical properties
62 are being produced, including single-walled (SWCNTs), multi-walled CNTs (MWCNTs), and
63 surface functionalized CNTs. These physicochemical properties can significantly influence CNT
64 fate in aqueous media, with increased diameter and surface oxygen content leading to enhanced
65 stability (Smith et al., 2009; Li and Huang, 2010; Schwyzer et al., 2012). However, the relationship

66 between CNT properties, media characteristics and NOM, and their overall effect on CNT
67 dispersibility and dispersion stability, has proven to be complex. Increased stability due to the
68 presence of oxygen containing surface functional groups is diminished in the presence of Ca^{2+} in
69 the aqueous media, possibly due to specific interactions between Ca^{2+} and carboxyl groups causing
70 a neutralization of the CNT surface charge (Yi and Chen, 2011). Ca^{2+} can also act as bridges
71 between carboxyl groups on oxidized CNTs and NOM, influencing their agglomeration (Smith et
72 al., 2012). For the C_{60} fullerene, the presence of NOM was reported to enhance agglomeration due
73 to bridging mechanisms at high concentrations of Ca^{2+} (Chen and Elimelech, 2007; Shen et al.,
74 2015). In contrast, intermediate concentrations of Ca^{2+} were reported to temporarily increase CNT
75 dispersion stability in the presence of humic acids (Schwyzer et al., 2013).

76
77 A thorough understanding of the influence of aquatic media characteristics and CNT properties on
78 the fate of CNTs is necessary for evaluating their environmental effects in aquatic ecosystems. It
79 is also important for improving the accuracy of models used to estimate environmental
80 concentrations of CNTs, as CNT concentrations in the aquatic environment cannot be readily
81 measured due to a lack of simple and cost effective approaches and analytical techniques
82 (Gottschalk et al., 2013; Petersen et al., 2016). Furthermore, an understanding of CNT
83 dispersibility and dispersion stability in different media, together with settling behavior, is of
84 importance when conducting ecotoxicological tests as it may significantly influence exposure and
85 the subsequent outcome of the tests (Chang et al., 2015). As there are currently no optimized
86 guidelines for conducting aquatic ecotoxicological tests with CNTs (or other nanomaterials), there
87 are large variations in the experimental approaches implemented with respect to the
88 ecotoxicological media and CNT types used, together with different dispersion techniques and any

89 dispersants applied (Menard et al., 2011; Cerrillo et al., 2015; Cerrillo et al., 2016a; Hund-Rinke
90 et al., 2016). An incomplete understanding of how aquatic ecotoxicological media composition
91 affects the dispersion behavior of different CNT types makes comparison between studies difficult,
92 and hampers our understanding of their potential for environmental exposure and effects.
93 Furthermore, increasing knowledge of CNT dispersibility and dispersion stability under different
94 representative conditions would be valuable in environmental fate modelling.

95

96 In the current study, we investigated the dispersibility and dispersion stability of CNTs with
97 different physicochemical properties in a selection of commonly used standard aquatic ecotoxicity
98 media and a natural water. It is hypothesized that both CNT physicochemical properties and media
99 properties will influence CNT dispersion behavior. As the dispersibility and dispersion stability of
100 CNTs is important for the exposure conditions and subsequent outcomes of ecotoxicological tests,
101 three commonly used ecotoxicological aquatic growth media were utilized, including OECD algal
102 growth medium (TG201) (OECD, 2011), US EPA moderately hard reconstituted water (MHRW)
103 (U.S. Environmental Protection Agency, 2002) and OECD daphnia growth medium (M7) (OECD,
104 2004). NOM was added to the growth media to facilitate dispersion of the CNTs. In addition, the
105 CNTs were dispersed in a natural freshwater for comparison of their behavior in synthetic and
106 natural waters. Five types of CNTs, including one SWCNT, two non-functionalized MWCNTs
107 (MWCNT-15 and MWCNT-30) and two functionalized MWCNTs (MWCNT-OH and MWCNT-
108 COOH) were used to study the effect of CNT physicochemical properties on their dispersion
109 behavior under the different media characteristics. This study addresses a current knowledge gap
110 concerning the combined effects of different media properties and CNT physicochemical
111 properties on their dispersion behavior in standard aquatic ecotoxicity test media.

112

113 MATERIALS AND METHODS

114 **Chemicals.** The CNTs, purchased from Timesnano (>95%, Chengdu Organic Chemicals Co., Ltd,
115 Chinese Academy of Sciences), included one SWCNT, two non-functionalized MWCNTs
116 (MWCNT-15 and MWCNT-30; numbers refer to the maximum outer diameter as given by the
117 manufacturer) and two functionalized MWCNTs (MWCNT-OH and MWCNT-COOH).
118 Characterization techniques, (transmission electron microscopy (TEM), X-ray photoelectron
119 spectroscopy (XPS) and analysis of nitrogen adsorption/desorption isotherms) used to determine
120 CNT outer diameter, surface chemistry, and specific surface area (SSA), has been previously
121 described (Glomstad et al., 2016). Suwannee River NOM (SR-NOM) was provided by the
122 International Humic Substance Society (IHSS).

123

124 **Preparation of synthetic media-NOM and natural freshwater.** Three different commonly used
125 ecotoxicological growth media were included in the study; TG201 (OECD, 2011), MHRW (U.S.
126 Environmental Protection Agency, 2002) and M7 (OECD, 2004). SR-NOM (nominally 20 mg/L)
127 was dissolved in the media by magnetic stirring for 24 h. The natural freshwater was collected in
128 the lake Haukvatnet (Trondheim, Norway). No NOM was added to the natural freshwater. Media-
129 NOM (TG201-NOM, MHRW-NOM and M7-NOM) and the natural water was filtered through a
130 0.2 µm Nalgene® filter unit (Thermo Fisher Scientific, Inc.). Following their preparation, pH and
131 specific conductivity (Malvern Zetasizer Nano-ZS ZEN3600) were determined. The total organic
132 carbon (TOC) was determined in TG201-NOM, MHRW-NOM and the natural water using a
133 Sievers 900 TOC Analyzer (GE Analytical Instruments).

134

135 **Preparation of CNT dispersions.** The preparation of CNT dispersions has been previously
136 described (Glomstad et al., 2016). Briefly, stock CNT dispersions were prepared in the media-
137 NOM and natural water by bath sonication (Bandelin Sonorex Super 109 RK 510H, 4 × 15 min,
138 640W, 35 kHz) and then added into larger volumes of the corresponding media (Glomstad et al.,
139 2016). Dispersions were also prepared in TG201 and MHRW in the absence of NOM. The initial
140 CNT concentration was 10 mg/L in all dispersions. Following preparation of the dispersions, a 24
141 h settling period was applied to allow settling of non-dispersed CNTs. The supernatant was then
142 transferred to clean bottles and used for further studies.

143
144 **CNT dispersibility.** An overview of the combinations of CNT types and media tested is presented
145 in **Figure 1**. CNT dispersibility refers to the concentration of CNTs remaining in dispersion after
146 the initial 24 h settling period, referred to as day 0 in the following text. Dispersed CNT
147 concentration was determined by UV-vis absorbance measurements at 800 nm and related to CNT
148 concentration through calibration curves prepared for the individual CNT types (Glomstad et al.,
149 2016). The calibration curves were prepared in MHRW-NOM, but were applied for CNTs
150 dispersed in all types of media, media-NOM and the natural water, as no significant absorbance
151 from any of the solutions themselves was observed at the wavelength used. For dispersions
152 prepared in media-NOM a minimum of three replicate samples of each CNT type were used
153 (MHRW-NOM n=3, M7-NOM n=4; TG201-NOM n=6). For the natural water, single samples
154 were used. Dispersions prepared in the absence of NOM (TG201 and MHRW) were also prepared
155 as single samples. Triplicate measurements (i.e. for each replicate, three aliquots were sampled
156 and measured) were used throughout the study.

157

158 **CNT dispersion stability.** The stability of CNT dispersions in all media-NOM and the natural
159 water was monitored over a period of 14 days in static conditions. Dispersion stability was
160 investigated by measuring CNT concentration in the supernatants by UV-vis absorbance at 800
161 nm as described in the previous section. For TG201-NOM and M7-NOM (the media-NOM
162 exhibiting the lowest and highest specific conductivity and Ca^{2+} concentration), the zeta potential
163 was determined parallel to the concentration measurements using a Malvern Zetasizer Nano-ZS
164 ZEN3600. Three replicates were used for zeta potential measurements at day 0 while single
165 replicates were used for the following days. All CNT dispersions were contained in glass flasks
166 and stored in the dark at room temperature over the course of the experiment. Care was taken not
167 to move the bottles to avoid disturbance of any settled CNTs. Dispersions in TG201-NOM and
168 M7-NOM were contained in flasks of 1 L, while those in MHRW-NOM and natural water were
169 kept in 0.25 L flasks. Three replicate samples were used for TG201 and M7 while single replicates
170 were used for MHRW and natural water.

171

172 RESULTS AND DISCUSSION

173 **CNT characterization.** CNT properties determined by in-house characterization have been
174 previously published along with properties supplied by the manufacturer (Glomstad et al., 2016),
175 and a summary is presented in Table S1 in the Supplementary Information (SI). Importantly, the
176 SWCNT exhibited a lower average diameter (2.4 nm) and a significantly higher SSA (483.7 m^2/g)
177 compared to the MWCNTs (diameter > 14.3 nm; SSA < 177.4 m^2/g). MWCNT-15, MWCNT-OH
178 and MWCNT-COOH had very similar SSA (~140 m^2/g) while it was somewhat higher for
179 MWCNT-30 (177.4 m^2/g). The surface oxygen content was the highest for MWCNT-COOH
180 (5.7%), followed by MWCNT-OH (3.9%). The surface oxygen content of the non-functionalized

181 CNTs was <2%. According to the manufacturer, SWCNT and MWCNT-30 had lengths of 5-30
182 μm and 10-30 μm , respectively. The length of MWCNT-15, MWCNT-OH and MWCNT-COOH
183 was $\sim 50 \mu\text{m}$.

184

185 **Properties of media-NOM and natural water.** Properties of the synthetic media and the natural

186 water are presented in **Table 1**. The pH was similar in all three media-NOM and the natural water

187 with values ranging from 7.71-7.83. The specific conductivity of the synthetic media decreased in

188 the order M7-NOM > MHRW-NOM > TG201-NOM, while the specific conductivity of the natural

189 water was similar to that of TG201-NOM. Specific conductivity can be used as an indication of

190 the ionic strength of the media, but provides no information regarding the types of ions in the

191 media. As divalent cations have been found to influence CNT dispersion and stability in aqueous

192 media (Yi and Chen, 2011; Lin et al., 2012; Schwyzer et al., 2012) the concentration of Ca^{2+} and

193 Mg^{2+} in each of the synthetic media was calculated from the media compositions (**Table 1**) (U.S.

194 Environmental Protection Agency, 2002; OECD, 2004, 2011). The Ca^{2+} concentration increased

195 in the same order as the specific conductivity with concentrations of 0.12, 0.41 and 2.00 mM in

196 TG201-NOM, MHRW-NOM and M7-NOM, respectively while the Mg^{2+} concentration was

197 lowest in TG201-NOM (0.12 mM) and similar in MHRW-NOM and M7-NOM (0.5 mM).

198 Although, the media used in the current study are characterized by high concentrations of Ca^{2+} and

199 Mg^{2+} divalent cationic species, TG201 and M7 also contain very low concentrations of multivalent

200 trace elements (e.g. Fe^{+2} , Fe^{+3} , Zn^{+2} , Mn^{+2}). It is suggested that these trace element species will

201 also contribute to the overall effect on CNT behaviour dominated by the high concentrations of

202 Ca^{2+} and Mg^{2+} . The nominal concentration of SR-NOM was identical in all synthetic media-NOM

203 (20 mg/L). The TOC of the media-NOM, determined based on measurements in TG201-NOM and
204 MHRW-NOM, was 8.5 ± 0.4 mg/L, while in the natural water it was 5.5 ± 0.1 mg/L.

205
206 **CNT dispersibility.** Although bath sonication delivers lower energy to a dispersion than probe
207 sonication, and is therefore less efficient at dispersing CNTs, it is considered to significantly
208 decrease the risk of CNT damage (Taurozzi et al., 2011; Cerrillo et al., 2015). In order to limit
209 CNT damage, bath sonication was applied in the current study to prepare CNT stock dispersions
210 before they were mixed into larger volumes of the corresponding media. Complete CNT dispersion
211 was not achieved, as non-dispersed aggregates could be observed in the stock dispersions after
212 sonication. After mixing the stock dispersions into the bulk volume of the samples, the 24 h settling
213 period allowed the non-dispersed and large CNT aggregates to sediment. The CNTs remaining
214 suspended in the water column after 24 h were considered to be dispersed. Between 34-98% of the
215 initial CNTs were removed from the water column during the 24 h settling period (Table S2; SI).
216 The removal of larger CNT aggregates from the dispersion also improved the accuracy of UV-vis
217 absorbance measurements, as those significantly larger than the wavelength of the light used have
218 been found to influence the measured absorbance (Li et al., 2006; Cerrillo et al., 2015). Removal
219 of any large aggregates during the 24 h settling period was evidenced by multiple measurements
220 of each replicate sample showing very little variation in absorbance values (three measurements
221 from each replicate sample).

222
223 *CNT dispersibility in the absence of NOM.* CNT dispersibility in TG201 and MHRW in the
224 absence of NOM is presented in Figure S1 (SI). In the absence of NOM, the SWCNTs could not
225 be quantified, as the dispersion concentration was below the limit of quantification (LOQ = 0.007

226 a.u.). MWCNT-15, MWCNT-30 and MWCNT-OH were present at low concentrations in both
227 media (< 0.5 mg/L), whilst MWCNT-COOH was present at 4.7 mg/L and 1.1 mg/L in TG201 and
228 MHRW, respectively. The higher dispersibility of MWCNT-COOH is considered to be related to
229 the increased surface oxygen content relative to the other CNTs (Kennedy et al., 2008; Schwyzer
230 et al., 2012). Furthermore, carboxyl groups have a greater stabilizing effect upon CNTs in aqueous
231 media than other oxygen containing groups (i.e. hydroxyl and carbonyl) (Smith et al., 2009), which
232 is consistent with the differences observed between MWCNT-COOH and MWCNT-OH in the
233 current study. The higher dispersibility of MWCNT-COOH in TG201 compared to MHRW may
234 be due to the lower ionic strength and divalent cation concentration of TG201 (Saleh et al., 2008;
235 Lin et al., 2012). For the other CNTs no clear relationship between a lower ionic strength of the
236 media and increased dispersibility was observed.

237
238 *CNT dispersibility in the presence of NOM – effect of media properties.* The dispersibility of the
239 five CNTs in different media-NOM and natural water is presented in **Figure 2**, while images of
240 the CNT dispersions in selected media (MHRW-NOM and Natural Water) can be seen in **Figure**
241 **3**. The presence of NOM clearly resulted in increased dispersibility for all CNT types. However,
242 differences in dispersibility were observed both between CNT type and between media type. The
243 non-functionalized MWCNTs showed very similar dispersibility in the three synthetic media-
244 NOM (1.5-1.8 mg/L), with the exception of MWCNT-30 in M7-NOM (2.8 mg/L). Similarly,
245 SWCNTs exhibited no significant differences between the three synthetic media-NOM (0.5-0.9
246 mg/L). In contrast, MWCNT-COOH dispersibility was 6.6 mg/L, 4.3 mg/L and 3.0 mg/L in
247 TG201-NOM, MHRW-NOM and M7-NOM respectively. A similar pattern was observed for
248 MWCNT-OH, although this was not statistically significant (**Figure 2**). Increasing ionic strength

249 of aqueous media due to the presence of divalent cations has been shown to induce CNT
250 aggregation and reduce CNT dispersibility (Saleh et al., 2008; Saleh et al., 2010; Schwyzer et al.,
251 2012). The results from the current study, indicate that the dispersibility of non-functionalized
252 MWCNTs is largely unaffected by changes in media composition, whereas the dispersibility of
253 functionalized MWCNTs varies significantly with the divalent cation concentration.

254

255 *CNT dispersibility in the presence of NOM – effect of CNT properties.* SWCNTs appear to exhibit
256 lower dispersibility than MWCNTs, although this was not statistically significant in any media-
257 NOM (**Figure 2**). The smaller diameter of SWCNTs relative to MWCNTs has previously been
258 related to lower dispersibility (Lin and Xing, 2008; Schwyzer et al., 2012; Cerrillo et al., 2016b).
259 It has been suggested that larger diameter CNTs exhibit looser entanglement, improving adsorption
260 of NOM to CNTs and causing steric and/or electrostatic stabilization (Lin and Xing, 2008).
261 Furthermore, TEM images revealed SWCNTs were aligned parallel to each other, while MWCNTs
262 were cross-linked (**Figure 4**). Breaking apart the parallel aligned SWCNT aggregates would
263 require more energy than separating the MWCNT aggregates (Huang and Terentjev, 2012),
264 consistent with the lower dispersibility of SWCNTs than MWCNTs in the current study. The non-
265 functionalized MWCNTs exhibited very similar dispersion behaviors in TG201-NOM, MHRW-
266 NOM and the natural water. Although the measured concentration of MWCNT-30 was higher than
267 for MWCNT-15 in M7-NOM, this was not statistically significant. Differences in physicochemical
268 properties that influence CNT dispersibility (e.g. diameter, length and surface oxygen) (Marsh et
269 al., 2007; Huang and Terentjev, 2012; Schwyzer et al., 2012) are minimal between the two non-
270 functionalized MWCNTs and support the similar dispersibility observed.

271

272 MWCNT-COOH dispersed at a significantly higher concentration than the non-functionalized
273 MWCNTs in both TG201-NOM and MHRW-NOM. MWCNT-OH exhibited a similar trend to
274 MWCNT-COOH in both media, but was only statistically different from the non-functionalized
275 MWCNTs in TG201-NOM. MWCNT-COOH was significantly more dispersible than MWCNT-
276 OH in TG201-NOM, but not in MHRW-NOM. This is consistent with previous studies reporting
277 greater CNT dispersibility with increasing surface oxygen (Smith et al., 2009; Li and Huang, 2010;
278 Schwyzer et al., 2012). The current study indicates that CNT dispersibility in TG201-NOM and
279 MHRW-NOM was most influenced by surface oxygen content than by any other physicochemical
280 property. However, no clear relationship between CNT surface oxygen content and dispersibility
281 was observed in M7-NOM. This suggests that the influence of CNT surface oxygen content on
282 dispersibility depends on the properties of the media in which the CNTs are dispersed.

283
284 Increased CNT surface oxygen content led to increased dispersibility in media with low ionic
285 strength and a low concentration of divalent cations (e.g. TG201-NOM), while this was less
286 pronounced in media with higher ionic strength and divalent cation concentration (e.g. M7-NOM).
287 In contrast, non-functionalized MWCNTs appear insensitive to such differences in media
288 properties. Previous studies have shown that functionalized CNTs exhibit increased stability
289 relative to non-functionalized CNTs in the presence of monovalent cations (both in the absence
290 and presence of NOM), while the stability of functionalized and non-functionalized CNTs were
291 similar at higher ionic strengths and in the presence of divalent cations (Yi and Chen, 2011; Smith
292 et al., 2012). A similar degree of NOM adsorption to different CNTs in media with higher ionic
293 strength and divalent cation concentration has been suggested to occur due to (i) screening of
294 negative charges on the functional groups of both CNTs and NOM at higher ionic strengths, and

295 (ii) Ca^{2+} bridging between functionalized CNTs and NOM.(Smith et al., 2012) As NOM
296 adsorption to CNTs strongly influences their dispersibility (Figure S1; SI and **Figure 2**), enhanced
297 NOM adsorption to functionalized CNTs with increasing ionic strength and divalent cation
298 concentration could explain the observed variation in functionalized MWCNT dispersibility in the
299 different media-NOM. The data in the current study indicate that increased NOM adsorption to
300 functionalized CNTs (e.g. in M7-NOM compared to TG201-NOM) causes a reduction in
301 dispersibility. It is suggested that NOM coating of the functional groups reduces their effect on
302 CNT dispersibility, and while NOM increases CNT dispersibility, the influence of NOM is lower
303 than that of the functional groups. As charge screening and Ca^{2+} bridging would mainly affect
304 functionalized CNTs (Smith et al., 2012), it would also explain the insensitivity of the non-
305 functionalized MWCNTs to the differences in media properties in the current study.

306
307 *CNT dispersibility in natural fresh water.* The dispersibility of the group of five CNTs in natural
308 water was quite different to any of the synthetic media (**Figure 2, Figure 3**). SWCNTs exhibited
309 the lowest dispersion concentration, with very similar dispersibility observed for all MWCNTs.
310 The dispersibility of functionalized MWCNTs was lower in natural water (0.9-1.4 mg/L) than in
311 synthetic media-NOM, while the dispersibility of the non-functionalized MWCNTs was fairly
312 similar (1.3 mg/L). The comparable concentration of non-functionalized MWCNTs in natural
313 water and synthetic media-NOM appears to provide further support for the relative insensitivity of
314 these particles to differences in media properties. The natural water had a similar specific
315 conductivity as TG201-NOM, however, detailed information about the ionic composition (e.g.
316 concentration of divalent cations) is not available. The TOC was lower in the natural water than
317 the synthetic media-NOM, and the NOM composition in the natural water will differ from that of

318 the standard SR-NOM. Importantly, the similar dispersibility of functionalized and non-
319 functionalized MWCNTs in the natural water indicates there was no influence from CNT surface
320 oxygen content. This questions the importance of surface chemistry on the fate of CNTs in natural
321 freshwater environments. Furthermore, it suggests that synthetic aquatic ecotoxicity media that
322 suppress the role of CNT surface chemistry, i.e. high ionic strength and divalent cation
323 concentration, may be considered more relevant models for studying CNT fate and effects under
324 laboratory conditions.

325

326 **CNT dispersion stability over time.** In addition to the initial dispersibility of CNTs in aquatic
327 ecotoxicity media, it is also important to understand how exposure concentration might vary over
328 the duration of a test. The concentration exposure to test organisms will vary as a function of
329 individual CNT dispersion stability and the experimental conditions. The dispersed concentration
330 of the CNTs over a 14 day period in all media-NOM and the natural water is presented in **Figure**
331 **5**. It should be noted that the variation in CNT dispersion concentrations in the water phase could
332 itself be a factor affecting the sedimentation process, i.e. a higher CNT concentration could be
333 expected to result in an increased collision rate between particles and a corresponding increase in
334 aggregation and sedimentation. Although the use of different initial CNT dispersion concentrations
335 represents a potential bias, clear trends in dispersion stability were observed using the current
336 approach, which could be related to specific physicochemical properties of the different CNT types
337 in different media. The results show that the concentration of CNTs remaining in dispersion
338 decreased over time for all types of CNTs in all media. This indicates that the CNT exposure
339 concentration in the water phase will decrease correspondingly over the duration of both acute and
340 chronic toxicity tests, assuming no renewal of the medium is performed. As the CNTs exhibited

341 highly different dispersibility (i.e. different settling during the first 24 hours after preparation of
342 the dispersions), comparison of their dispersion stability over the 14 day period cannot be achieved
343 by simply comparing the CNT concentration remaining in dispersion. The concentrations of the
344 individual CNTs that had sedimented over time is presented in Figure S2 and Figure S3 (SI). The
345 data is presented in two figures for clarity; Figure S2 describes the sedimentation of different CNT
346 types in each media, allowing comparison between CNT types, while Figure S3 shows the
347 sedimentation of individual CNT types in different media, allowing for comparison between
348 different media.

349
350 Despite the large difference in dispersibility of the CNTs in TG201-NOM (**Figure 2**), **Figure 5**
351 shows that the slope of the curves in this media is similar for all CNT types, indicating a
352 comparable sedimentation behavior. This was confirmed by looking at the sedimented
353 concentrations (Figure S2; SI), which presents a very similar pattern for all CNT types. In MHRW-
354 NOM, it appears that sedimentation of the functionalized MWCNTs was slightly faster than for
355 the non-functionalized MWCNTs. A similar, but more pronounced decrease in sedimentation time
356 and dispersion concentration was observed for the functionalized MWCNTs in M7-NOM. The
357 data suggests that CNT dispersion stability is similar for all CNT types in media with lower ionic
358 strength and divalent cation concentration (TG201-NOM). In contrast, increasing the ionic
359 strength and divalent cation concentration of the media (MHRW-NOM and M7-NOM) appears to
360 result in decreased stability of functionalized MWCNTs compared to non-functionalized
361 MWCNTs. Similar observations of decreased CNT dispersion stability with increasing surface
362 oxygen content has been previously reported in an artificial groundwater (Smith et al., 2012). For
363 fullerenes, destabilization of C₆₀ suspensions has been observed in the presence of high

364 concentrations of Ca^{2+} and NOM due to bridging mechanisms (Chen and Elimelech, 2007; Shen
365 et al., 2015). As NOM adsorption to functionalized MWCNTs is expected to increase with
366 increasing ionic strength and divalent cation concentration of the media, it is possible that similar
367 mechanisms facilitate the increased aggregation and sedimentation of functionalized MWCNTs
368 observed in M7-NOM. It also appears that MWCNT-30 exhibited higher sedimentation rates than
369 MWCNT-15, particularly in M7-NOM. Furthermore, sedimentation of the SWCNTs was lower
370 than for all MWCNTs in both MHRW-NOM and M7-NOM. However, for MWCNT-15 and
371 SWCNT the data could be influenced by their low dispersion concentrations, as sedimentation
372 cannot be evaluated when this approaches zero.

373

374 A relatively similar sedimentation behavior was observed for both functionalized and non-
375 functionalized MWCNTs in the representative natural water employed in the current study (**Figure**
376 **5**). The similar dispersion stability and sedimentation behavior exhibited by the five CNT types in
377 the natural water was most comparable to their observed behavior in TG201-NOM. However, the
378 dispersibility of all five CNT types in the natural water was low. This low dispersibility could have
379 affected their sedimentation behavior and masked any potential differences between CNT types.

380 It is also important to note that the natural water used in the current study represents a single
381 example of natural freshwaters and that water from other locations may exhibit very different
382 properties, which might cause the suite of CNTs to behave differently. It is recommended that
383 further studies using a suite of well-characterized natural waters representing a range of different
384 ionic strengths and NOM contents are needed to understand CNT behavior more thoroughly.

385

386 A comparison of how the individual CNT types behave in the different synthetic media-NOM
387 (Figure S3; SI) indicates the highest sedimentation of MWCNT-30, MWCNT-OH and MWCNT-
388 COOH in M7-NOM, followed by MHRW-NOM and finally TG201-NOM. The decreased
389 dispersion stability and increased sedimentation of these three CNT types appears to be linked
390 with increasing ionic strength and divalent cation concentration of the synthetic media-NOM.
391 Again, the functionalized MWCNTs appear to be influenced more significantly by changes in ionic
392 strength and divalent cation concentration, as their increased sedimentation in the media-NOM
393 with higher ionic strength and divalent cations was higher than for MWCNT-30. In contrast, no
394 relationship between ionic strength and divalent cation concentration and the degree of
395 sedimentation was observed for SWCNT and MWCNT-15. However, this could be a result of the
396 low initial dispersibility of these CNTs, e.g. in M7-NOM (**Figure 5**). The sedimented
397 concentrations in natural water were similar to those of the suite of CNTs when dispersed in
398 TG201-NOM (Figure S3; SI). The zeta potential for CNTs dispersed in TG201-NOM and M7-
399 NOM is presented in **Figure 6**. For all CNT types and at all time points, greater zeta potential
400 values were measured in TG201-NOM compared to M7-NOM. This supports the observation of
401 greater dispersion stability of the CNTs in TG201-NOM compared to M7-NOM determined using
402 UV-vis absorbance. It is also interesting to note that the absolute zeta potential value of the
403 functionalized MWCNTs typically decreased over time in M7-NOM while remaining generally
404 constant in TG201-NOM. This also supports our previous observations of increased sedimentation
405 of the functionalized MWCNTs in M7-NOM.

406

407 **Environmental and ecotoxicological implications.** The current study shows that the fate of CNTs
408 in aqueous environments is influenced both by CNT properties and media properties (ionic

409 strength and divalent cation concentration). The influence of CNT physicochemical properties,
410 especially surface oxygen content, on their dispersibility and dispersion stability was dependent
411 upon media properties and vice versa. This makes it challenging to simply describe the effect of
412 specific properties or parameters (e.g. CNT surface oxygen concentration or media ionic strength)
413 on the general fate of CNTs in aquatic systems. Furthermore, predicting the environmental fate of
414 CNTs can be demanding and will vary based on CNT type and environmental conditions. This has
415 implications with respect to environmental fate modelling, where it is suggested that CNT
416 dispersibility and dispersion stability should be tested under a range of different, but representative
417 conditions as part of any model development. In addition to synthetic media, studying CNT
418 dispersion behavior in well-characterized natural waters can help increase our understanding of
419 their fate in aquatic environments.

420

421 Another implication of the data presented in the current study is that the choice of medium in
422 aquatic ecotoxicological tests can significantly influence the dispersibility and dispersion stability
423 of CNTs. This potentially has an important impact on the outcome of the ecotoxicological tests.
424 Best on the results from the present study, it can be predicted that when using the same CNTs and
425 test organisms but with different media, differences in the results of the test will occur due to the
426 variation in dispersion behavior and the subsequent exposure concentrations available to the
427 organisms. This increases the difficulty of comparing data from different studies. For instance,
428 both MHRW and M7 are media recommended for toxicity tests using daphnids (U.S.
429 Environmental Protection Agency, 2002; OECD, 2004), but significant differences in the
430 dispersibility and dispersion stability of specific CNT types can be expected in these two media.
431 The dispersion stability influences the exposure concentration, especially in static exposure

432 systems, and sedimentation of CNTs implies that organisms will be subjected to exposure
433 concentrations that change over time. As a result, derived effect concentrations (e.g. EC₅₀) based
434 on the initial exposure concentration might not accurately describe the toxicity of the CNTs to
435 aquatic organisms. The applicability of OECD test guidelines to nanomaterials (NMs) has been
436 evaluated by the OECD Working Party on Manufactured Nanomaterials and found in many cases
437 to be suitable to NMs (OECD Working Party on Manufactured Nanomaterials (WPMN), 2009).
438 A critical review also recently addressed issues regarding the adaptation of OECD aquatic toxicity
439 tests specifically, where the NM behavior in test media was one of the challenges discussed
440 (Petersen et al., 2015). Further research on how to maintain NMs in dispersion during toxicity
441 tests, but also on how sedimentation of NMs influences the results and the variability of
442 ecotoxicological tests, was recommended.

443

444 CONCLUSIONS

445 Both CNT physicochemical properties and synthetic media properties significantly influence the
446 dispersibility and dispersion stability of CNTs in aquatic ecotoxicity tests. Importantly, media
447 properties determine the degree of influence exhibited by certain CNT physicochemical properties.
448 For example, CNT surface oxygen content plays a key role in the dispersibility of CNTs in
449 synthetic media with the low ionic strength and divalent cation concentration, while it had no
450 influence on CNT dispersibility in a natural water. Functionalized MWCNTs were more sensitive
451 to changes in media properties than non-functionalized MWCNTs. Sedimentation over time
452 occurred for all CNT types and in all media, decreasing the concentration of CNTs remaining in
453 the water phase. As the influence of CNT properties on CNT behavior in aqueous media depends
454 on media properties and vice versa it is challenging to describe the effect of specific properties on

455 CNT fate in general terms. This complex interplay between media properties and CNT
456 physicochemical properties also has significant implication in the application of standard aquatic
457 ecotoxicity tests and the interpretation of resulting data. It is therefore important not to draw
458 general conclusions about all CNTs from the assessment of single CNT types, but rather utilize a
459 range of CNTs in such tests. Furthermore, the varying behavior and exposure of a specific CNT
460 type in different standard ecotoxicity test media suggests the need for caution when comparing
461 results from ecotoxicity tests with different aquatic species.

462

463 CONFLICT OF INTEREST

464 There are no conflicts to declare.

465

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470

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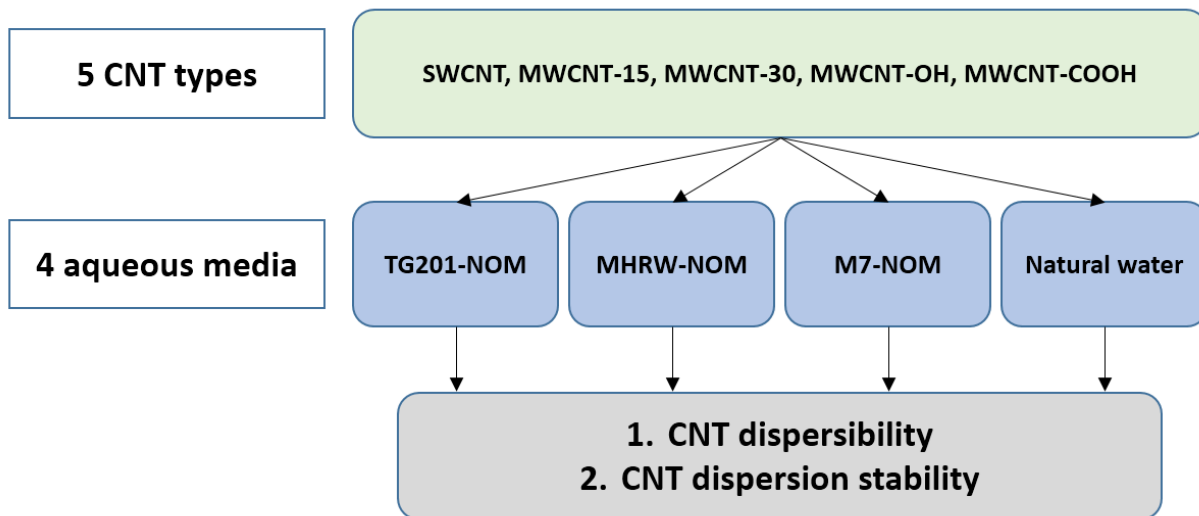
605 **Table 1.** Overview of the pH and specific conductivity (variation represent the standard deviation;
 606 n=3) and calculated concentrations of the primary divalent cations in the media-NOM and natural
 607 water.

Media	pH	Specific conductivity ($\mu\text{S}/\text{cm}$)	Ca²⁺ (mM)*	Mg²⁺ (mM)*
TG201-NOM	7.82	161.3 \pm 2.5	0.12	0.12
MHRW-NOM	7.71	308.7 \pm 0.6	0.41	0.50
M7-NOM	7.83	658.0 \pm 18.8	2.00	0.50
Natural water	7.71	162.6 \pm 0.3	-	-

608 * Calculated from descriptions of the media preparations in the relevant test guidelines (U.S. Environmental
 609 Protection Agency, 2002; OECD, 2004, 2011).

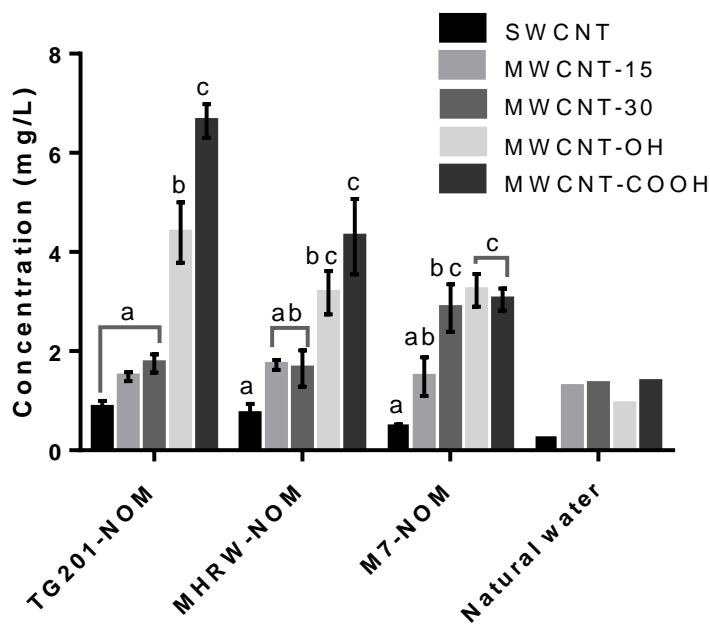
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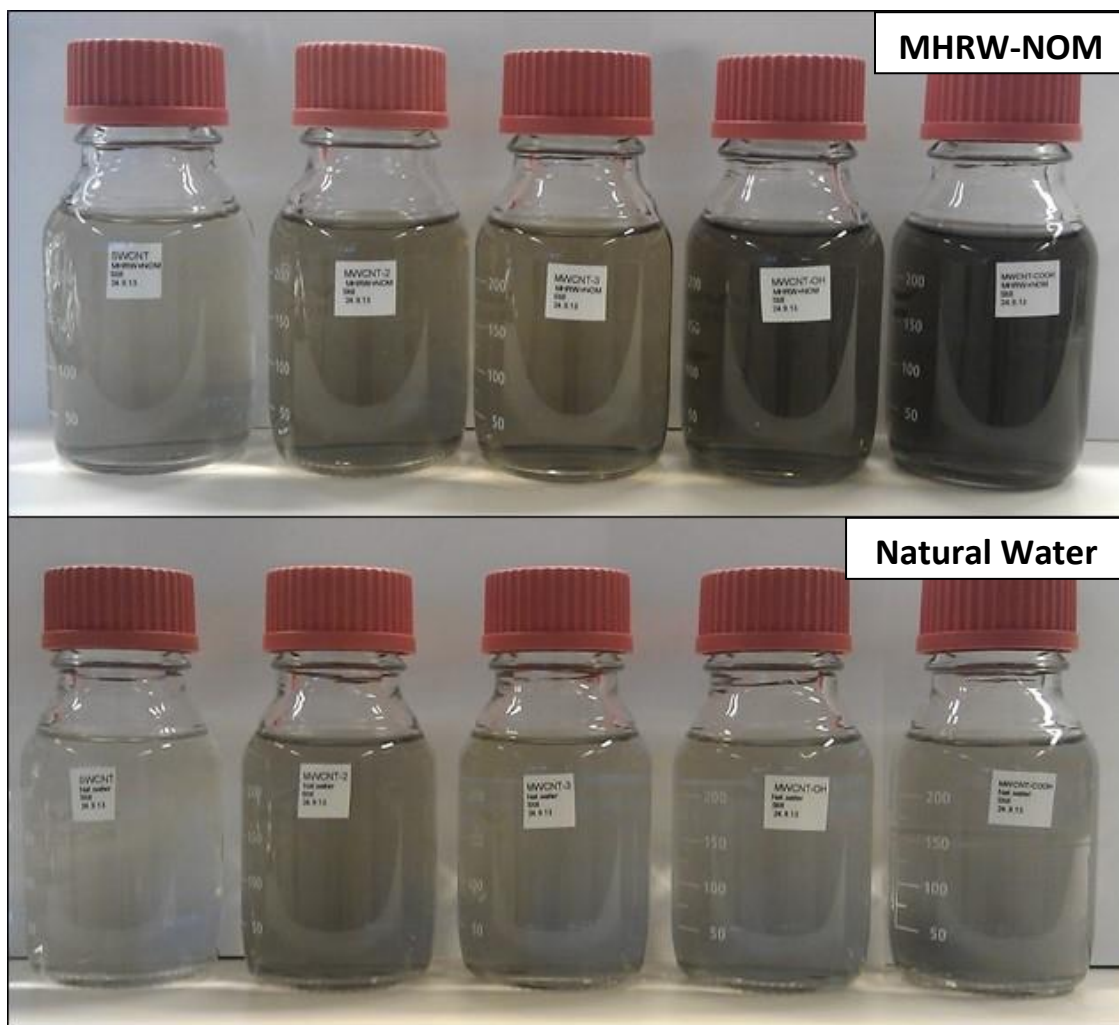
612
 613 **Figure 1.** Overview of the CNT and media types included in the study and the aspects of CNT
 614 dispersion behavior tested.

615
 616
 617



618
 619
 620 **Figure 2.** The concentration of CNTs remaining dispersed in the water phase following bath
 621 sonication and 24 h of settling. Error bars represent the standard errors of mean. The same letters
 622 above the bars within each group indicate no significant difference. Significant difference between
 623 groups (different media-NOM types) is not indicated in the figure.

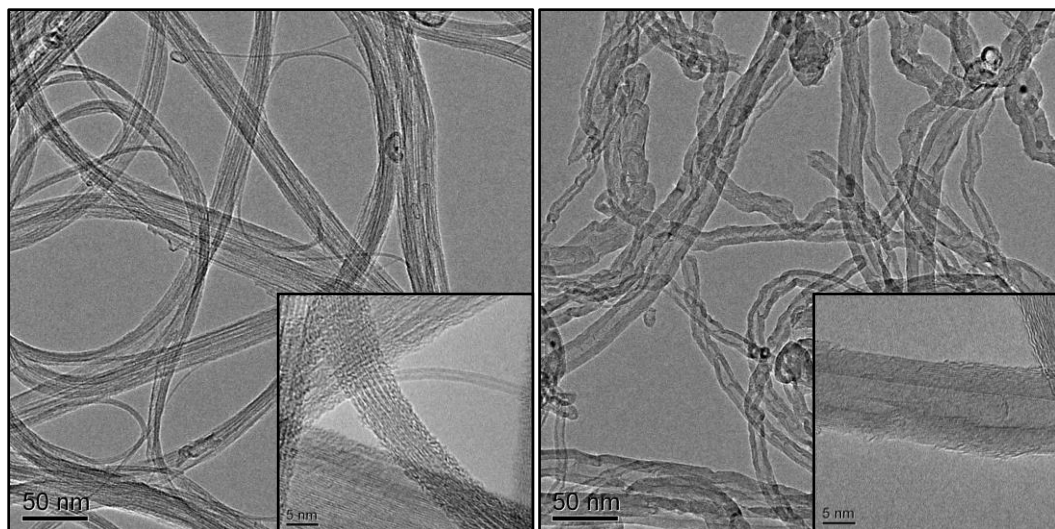
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626 **Figure 3.** Different dispersibility among CNT types and between different media, illustrated by
627 CNTs dispersions in MHRW-NOM (top) and natural water (bottom). From the left: SWCNT,
628 MWCNT-15, MWCNT-30, MWCNT-OH and MWCNT-COOH.

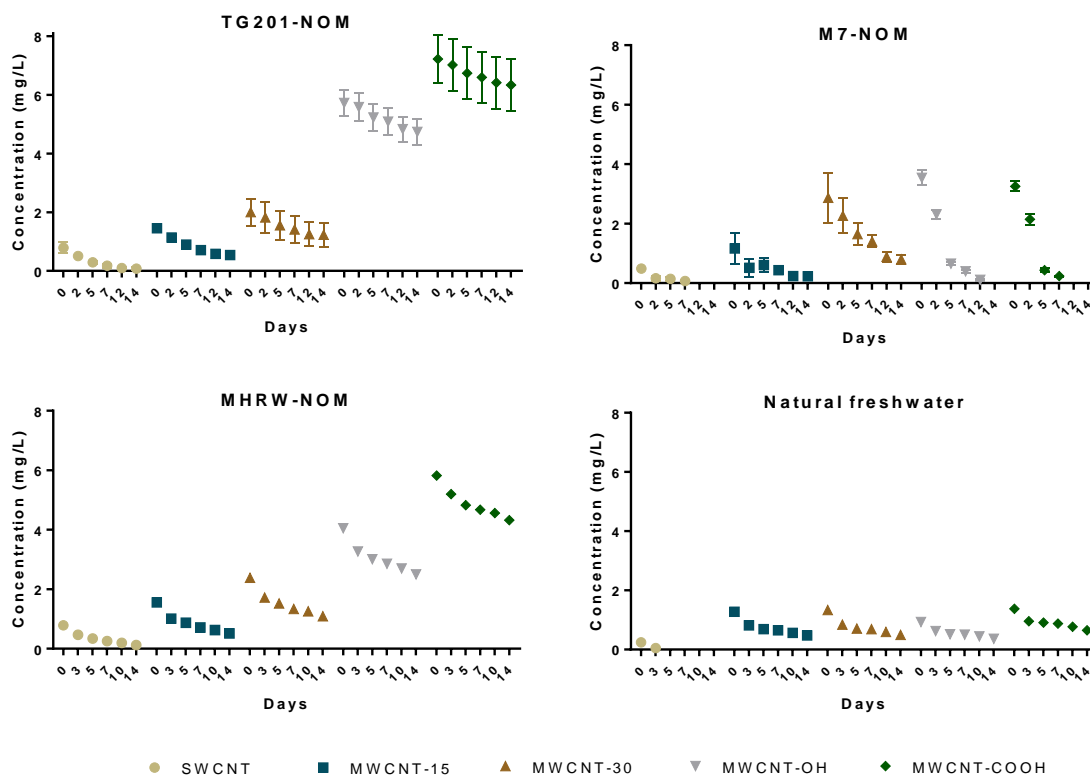
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631 **Figure 4.** TEM images of SWCNT (left) and MWCNT-15 (right). The SWCNTs were parallel
 632 aligned to each other while the MWCNTs were cross linked. The other MWCNTs showed similar
 633 aggregation morphology as MWCNT-15.

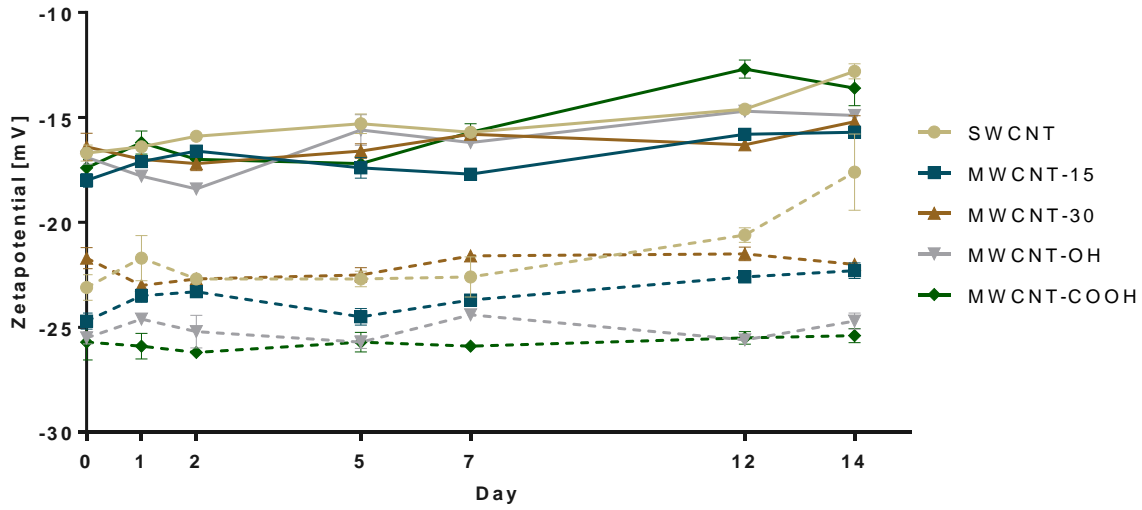
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636 **Figure 5.** The concentration of CNTs remaining in dispersion over a period of 14 days. Error
 637 bars show the standard deviations (n=3; only for TG201-NOM and M7-NOM). Data points
 638 below the LOQ are not included in the figure.

639



640

641

642 **Figure 6.** Zeta potential of MWCNT-30, MWCNT-OH and MWCNT-COOH over a time period
643 of 14 days in two different media-NOM. Symbols connected with solid lines are measured in M7-
644 NOM, while symbols connected with dotted lines are in TG201-NOM.
645