1

2 **Corresponding author**

3 Andy M. Booth

4 SINTEF Materials and Chemistry, Marine Environmental Technology, N-7465 Trondheim, Norway

5 Phone: +47 93089510

6 Email: andy.booth@sintef.no

7

Uptake and toxicity of methylmethacrylate-based nanoplastic particles in aquatic organisms

ANDY M. BOOTH[*†], BJØRN HENRIK HANSEN[†], MAX FRENZEL[†], HEIDI JOHNSEN[‡] and DAG ALTIN[§]

[†] *SINTEF Materials and Chemistry, Environmental Technology Dept., N-7465 Trondheim, Norway*

[‡] *SINTEF Materials and Chemistry, Biotechnology and Nanomedicine Dept., N-7465 Trondheim, Norway*

[§] *BioTrix, N-7022 Trondheim, Norway*

Ecotoxicity and uptake of nanoplastic particles

*Address correspondence to andy.booth@sintef.no

Abstract: The uptake and toxicity of two poly(methylmethacrylate)-based plastic nanoparticles (PNPs) with different surface chemistries (medium and hydrophobic) was assessed using aquatic organisms selected for their relevance based on the environmental behaviour of the PNPs. Pure poly(methylmethacrylate) (medium; PMMA PNPs) and poly(methylmethacrylate-*co*-stearylmethacrylate) copolymer (hydrophobic; PMMA-PSMA PNPs) of 86-125 nm were synthesised using a mini emulsion polymerisation method. Fluorescent analogues of each PNP (FPNPs) were also synthesised using monomer 7-[4-(trifluoromethyl)coumarin]acrylamide and studied. *Daphnia magna*, *Corophium volutator* and *Vibrio fischeri* were employed in a series of standard acute ecotoxicity tests, being exposed to the PNPs at three different environmentally realistic concentrations (0.01, 0.1, and 1.0 mg L^{-1}) and a high concentration 500-1000 mg L^{-1}. In addition, sublethal effects of PNPs in *C. volutator* were determined using a sediment reburial test whilst the uptake and depuration of FPNPs was studied in *D. magna*. The PNPs and FPNPs did not exhibit any observable toxicity at concentrations up to 500-1000 mg L^{-1} in any of the tests except for PMMA-PSMA PNPs and FPNPs following 48 h exposure to *D. magna* (LC50 values of 879 and 887 mg L^{-1}, respectively). No significant differences were observed between labelled and non-labelled PNPs, indicating the suitability of using fluorescent labelling. Significant uptake and rapid excretion of the FPNPs was observed in *D. magna*.

Keywords: Plastic nanoparticle, Toxicity, Uptake, *Daphnia magna*, *Corophium volutator*.

41 ## INTRODUCTION

42 As with many other pollutants, aquatic systems have emerged as the primary sink for micron-
43 sized plastic particles (PMPs) and nano-sized plastic particles (PNPs) [1-3], with sediments identified
44 as potential environmental sinks and concentration hot spots [4]. PMPs and PNPs in the environment
45 can be derived from both primary particles (e.g. personal care and cosmetic products) and secondary
46 particles which result from degradation of larger plastic items [5]. Whilst most focus has been on
47 PMPs in the marine environment, very little is known about the fate and effects of PNPs. PNPs are
48 easy and cheap to synthesise, and have almost unlimited potential for physical and chemical
49 modification for targeted application. Already, PNPs have been demonstrated to have application in a
50 wide variety of technologies, including targeted drug and vaccine delivery diagnostics and
51 bioimaging in nanomedicine [6-9], protein purification and immobilisation matrices [10], shell
52 structures for nanosized containers encapsulating dyes, lubricants and other chemicals [11], and
53 material surfaces and coatings [12].
54
55 Recently, PNPs in both freshwater and marine environments have become the subject of an
56 increasing number of studies [1, 4, 13-18]. Many of the available studies have employed polystyrene
57 PNPs (PS PNPs). Polystyrene is one of the five main high production-volume plastics, amounting to
58 approximately 90% of the total demand [19], and commonly found in the marine environment [20].
59 PS PNPs have been shown to adsorb to the surface of algal cells, reducing photosynthesis through
60 possible shading effects and also enhancing production of reactive oxygen species (ROS) [21]. PS
61 PNPs have been found to be taken up by *D. magna* and to translocate from the gut to other body
62 tissues [17]. Besseling et al [1] studied the effects of PS PNPs exposure on the growth and
63 photosynthesis of the green alga *Scenedesmus obliquus* and the growth, mortality, neonate
64 production, and malformations of *D. magna* at concentrations between 0.22 and 103 mg/L. Reduced
65 population growth and chlorophyll concentrations were observed in the algae, consistent with the

results of Bhattacharya et al [21]. *D. magna* showed a reduced body size and severe alterations in reproduction. The effects of PS PNPs on the feeding behaviour of the blue mussel (*Mytilus edulis*) have also been studied, with production of pseudofeces and a reduction in filtering activity reported [18]. It has also been shown that PS PNPs can be transported through an aquatic food chain from algae, through zooplankton to fish, affecting lipid metabolism and behaviour of the top consumer [13, 14]. Amine functionalised PS PNPs have been found to cause severe developmental defects in sea urchin embryos (*Paracentrotus lividus*), whilst carboxyl functionalised PS PNPs exhibited no effects [22].

The toxicity of other PNPs types and co-polymers have also been studied [15, 16]. The acute toxicity of Poly *N*-isopropylacrylamide (NIPAM) and *N*-isopropylacrylamide/*N*-*tert*-butylacrylamide (NIPAM/BAM) co-polymer PNPs was assessed using a battery of acute aquatic tests (*Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Thamnocephalus platyurus*), and significant ecotoxicological responses were observed at particle concentrations of up to 1000 mg L^{-1} [15]. The ecotoxicological response was seen to correlate well with the ratio of BAM monomer but not with particle size. The sensitivity of the test species was seen to vary depending on copolymer composition. A similar study investigated the ecotoxicity of polyethyleneimine polystyrene PNPs (PS-PEI PNPs) to the same battery of freshwater species representing different trophic levels (*V. fischeri*, *P. subcapitata*, *D. magna* and *T. platyurus*) [16]. Significant toxicity was detected after exposure to PS-PEI PNPs at concentrations from 0.40 mg L^{-1} to 416.5 mg L^{-1}, with differing sensitivities for each of the different organisms.

In a previous study, we showed that environmental fate assessment of poly(methylmethacrylate)-based PNPs (PMMA-based PNPs) is an important step in the identification and selection of relevant ecotoxicity tests and organisms [4]. The study indicated that

PNP surface chemistry and environmental parameters such as salinity and dissolved organic material (DOM) concentration had a significant effect on PNP fate in aquatic environments. PMMA-based PNPs with medium and hydrophobic surface chemistries remained freely dispersed for prolonged periods of time in freshwater environments under environmentally realistic PNPs concentrations, but agglomerated and sedimented rapidly under weakly saline conditions. These studies indicated that in freshwater environments PNPs will be exposed to pelagic organisms whilst in estuarine and marine environments benthic organisms are those most at risk to exposure. However, in low energy freshwater environments (e.g. lakes and reservoirs) the presence of natural colloids and suspended solids is likely to result in heteroaggregation and settling, leading to exposure of benthic species. Furthermore, processes such as biofouling and aging may influence PNP fate.

In the present study we used this information as the basis for selecting relevant aquatic organisms to assess the ecotoxicity of the PMMA-based PNPs and their fluorescently labelled analogues (FPNPs). To assess surface chemistry-dependent ecotoxicity, PMMA-based PNPs were synthesised with and without a co-monomer to allow variation in surface chemistry from medium to hydrophobic. FPNPs were produced by incorporation of the fluorescent dye 7-[4-(trifluoromethyl)coumarin]acrylamide. Investigation of the acute ecotoxicological effects of the PMMA-based PNPs and FPNPs was conducted using bioassays representing different trophic levels. The tests employed included the Microtox® bacterial species (*Vibrio fischeri*), a pelagic filter feeding freshwater crustacean (*D. magna*) and a benthic sediment re-working marine crustacean (*C. volutator*). Sublethal effects (sediment reburial) were assessed in *C. volutator* and qualitative uptake and excretion of FPNPs assessed in *D. magna*. We investigated the effects of a broad range of expected environmentally relevant and elevated concentrations of PMMA-based PNPs and FPNPs in the toxicity studies.

METHODS

Synthesis of PNPs

Two types of poly(methylmethacrylate)-based plastic nanoparticles (PNPs) were synthesised with hydrophobic and medium surface chemistries (Figure 1A and 1B). The medium chemistry PNPs were comprised of pure poly(methylmethacrylate) polymer (PMMA PNPs) and the hydrophobic PNPs were comprised of poly(methylmethacrylate-*co*-stearylmethacrylate) copolymer (PMMA-PSMA PNPs). It should be noted that the detailed structure of PMMA-PSMA (Figure 1B) is unknown and may consist of alternating PMMA and PSMA units, blocks of PMMA and PSMA units or a fully random distribution. The PNPs were synthesised using a standard miniemulsion polymerisation method described previously [4]. Briefly, a stabilising solution of water containing sodium dodecyl sulphate (SDS) and the liquid monomer containing a polymerisation initiator (V-59) are mixed together and sonicated to form an emulsion of nano-sized monomer droplets in water. The monomer droplets are then polymerised to form the final nano-sized particles which are suspended in the aqueous medium. Following synthesis, the PNPs were isolated and purified by dialysis in deionised water to remove any residual monomer and the stabiliser. The final PNP in water dispersions were stored in a glass bottle, in the fridge until required. Immediately before use in the ecotoxicity studies, all samples were sonicated for 30 min to ensure any agglomerates were broken down and that the PNPs were fully dispersed in the media. Whilst no significant aggregation was observed in any of the PNPs samples prior to sonication, the presence of freely dispersed nanoplastic particles in aquatic environments may be unlikely due to heteroaggregation with natural particulates.

In order for the PNPs to be determined in biological samples from uptake studies, fluorescent analogues of each type of PNP were synthesised (Figure 1D and 1E). These analogues (PMMA FPNPs and PMMA-PSMA FPNPs, respectively) were synthesised to contain the fluorescent dye/marker 7-[4-(trifluoromethyl)coumarin]acrylamide which is an acrylamide derivative of

coumarin (Figure 1C). The fluorescent dye was used as a co-monomer in the polymerisation process, which allowed it to be linked chemically to the PNPs and thus eliminate potential problems associated with leakage (and therefore any potential toxicity) of the dye from the final PNPs. The synthesis method was the same as that described above for the non-labelled analogues. It should be noted that Figures 1D and 1E represent just one example of how the polymer structures could look. The fluorescent label has a double bond that will participate in the polymerization, however the amount of fluorescent label is very small compared to the other monomers. It is likely that the final polymers would form a chain predominantly composed of PMMA or PMMA-PSMA units interspersed with occasional molecules of the fluorescent label.

PNP characterisation

The particle shape and size of the synthesised PNPs and FPNPs was characterised by transmission electron microscopy (TEM) and dynamic light scattering (DLS). A Phillips CM30 Transmission electron microscope (TEM) equipped with a LaB6 electron filament was used to investigate PNP shape and size. The average particle size of the synthesised PNPs was determined by dynamic light scattering (DLS) using a Malvern ZetaSizer™. A SpectraMax Gemini XS plate reader fluorescence spectrometer was used to quantify the amount of fluorescent dye in the FPNP analogues. A detailed description of these methods is provided by Booth et al., [4].

Ecotoxicity tests

The aim of the ecotoxicity tests was to assess the potential for toxicological responses to the PNPs when present in environmentally realistic concentrations and to see if a very high concentration also resulted in an effect. In recent studies, PNP effects have been studied at concentrations ranging from 0.22−1100 mg/L depending on the test species and experimental set up employed [1, 13, 14]. In the current study, PNPs and FPNPs were tested at concentrations of 0.01,

166 0.1 and 1.0 mg L^{-1}, which is are considered to represent realistic environmental concentrations and
167 consistent with concentration ranges employed in other studies. In addition, the PNP and FPNPs
168 were tested at 1000 mg L^{-1} (500 mg L^{-1} in the *C. volutator* tests) to determine if the test materials
169 elicited a response to PNPs at very high concentrations. This is again consistent with the upper
170 concentrations used in other studies [1]. The nominal exposure concentrations used for each test
171 species are summarised in Table 1.
172
173 *Microtox® test.* The acute toxicity of each PNP and FPNP analogue to the bioluminescent
174 marine bacterium *Vibrio fischeri* was determined using the 90% basic test for aqueous extract
175 protocol [23]. All Microtox® reagents and lyophilised *V. fischeri* bacteria (NRRL B-11177) were
176 obtained from SDI Europe. Tests were carried out at 15 °C in the supplied Microtox® diluent. Phenol
177 was used as a reference. X and Y minute EC50 tests were performed using the Microtox® Toxicity
178 Analyser (SDI, Newark, U.S.A.) following the instructions of the manufacturer. Toxicity data were
179 obtained and analysed using the MicrotoxOmni software. The effective concentration, EC50, is
180 defined as the concentration that produces a 50% light reduction. EC50 was measured after 15 min
181 contact time.
182
183 *Daphnia magna immobilisation*. A *Daphnia magna* starter culture, originating from Denmark
184 (purchased via a Norwegian distributor), consisted of approximately 100 pregnant females which
185 were transferred to M7 medium, as described in the OECD 202 Guideline [24]. The culture was kept
186 for at least 3 generations before neonates were used in exposure experiments. The culture was kept at
187 20-22°C with a light:dark regime of 16:8, and fed green algae (*P. subcapitata*) in excess daily.
188 Exposure studies were conducted according to the OECD standard procedure. Four PNP and FPNP
189 concentrations (0.01, 0.1, 1 and 1000 mg L^{-1}) plus blank controls were tested. The exposure solutions
190 (25 mL) were added to 50 mL Erlenmeyer flasks, and five neonates (<24 h old) from a brood were

191 added. Neonates were not fed for the duration of the experiment. After 24 and 48 h, immobility
192 (mortality) of the individuals within the container was recorded. All exposure concentrations were
193 performed in triplicate and 6 controls containing only M7 medium were used. At the end of the
194 experiment, exposure solutions were analysed for O_2 and pH to verify that they were within the
195 acceptable range reported in the OECD guideline. Animals unable to swim within 15 s of gentle
196 agitation of the test vessel are considered immobile. The DEBtox software (v2.0.1), freely available
197 on the internet (http://www.bio.vu.nl/thb/), was used for calculations of effect concentrations (EC)
198 and no effect concentrations (NEC) from the data generated in the *Daphnia magna* acute
199 toxicity/immobilisation test.
200
201 *Corophium volutator acute toxicity and reburial*. The test procedure followed in the present
202 study is outlined in NS-EN ISO 16712:2005 (Water Quality - Determination of acute toxicity of
203 marine or estuarine sediment to amphipods) [25]. As with freshwater systems, the presence of natural
204 colloids in seawater is likely to influence the aggregation behaviour and settling of PNPs. Natural
205 colloids were not present in the current exposure system as the seawater used is filtered prior to use,
206 however, our previous study indicated rapid aggregation and setting of both PMMA-PSMA and
207 PMMA PNPs under common seawater salinity levels [4]. To allow a 'natural' aggregation of the
208 PNPs when introduced into seawater they were diluted and sonicated for 15 min in full strength
209 seawater at the different exposure concentrations and introduced in the test vessels together with the
210 overlying water. The test animals were introduced 5-6 hours later when visual inspection confirmed
211 the PNPs and FPNPs had precipitated to the sediment. To test for viability and sublethal effects after
212 10 days of exposure, the post exposure reburial test suggested for *Corophium* sp. by Bat & Raffaelli
213 [26] was performed by transferring the animals to beakers with clean sediment and 1 cm of overlying
214 seawater. The recommended duration of the reburial test to be able to discriminate between normal
215 and moribund individuals is set to 1 hour.

Uptake and depuration study

In order to investigate potential uptake and depuration of PMMA PNPs, sub-adult *D. magna* were exposed to the FPNP analogues for 48 h. The conditions used were the same as those described for the 48 h immobilisation test. Organisms were exposed to 1 mg L^{-1} concentrations of the FPNPs in order to ensure that sufficient amounts of the test materials were available for filtration and subsequent detection by fluorescence microscopy, but also to ensure no mortality of the daphnids occurred. Exposure flasks containing 5 organisms were used in uptake and depuration studies, and each test was completed in triplicate. Control samples not containing any FPNPs were also used. Any mortality of the organisms was recorded after 24 h and 48 h of exposure. After 48 h, the organisms from the uptake study flasks were collected and studied qualitatively under the fluorescence microscope (Nikon eclipse TE2000 with Omega Optical XF-03 filter cube [ex: 330WB80, dichroic mirror: 400DCLP and em: 450DF65] and x-cite 120 metal halide arc lamp) for evidence of FPNP ingestion. After 48 h, the organisms in depuration study flasks were transferred to new flasks containing fresh media. After 24 h, depuration was assessed by studying the organisms under the fluorescence microscope. Faecal pellets excreted by the organisms were also collected and analysed under the fluorescence microscope.

RESULTS

Synthesis and characterisation of PNPs

The PMMA PNPs (Figure 1A) and PMMA-PSMA PNPs (Figure 1B) particles were successfully synthesised by mini-emulsion polymerisation and cleaned using dialysis. Fluorescently labelled homologues (FPNPs) of each of these particles were also successfully synthesised by incorporation of the fluorescent dye 7-[4-(trifluoromethyl)coumarin]acrylamide (Figure 1C-E). A detailed description of the PNP and FPNP characterisation has been previously reported [4]. Briefly,

241 transmission electron microscopy (TEM) showed that individual particles were spherical in nature
242 and exhibited slight differences in particle size, typically within the range 86-125 nm (see Booth et
243 al., 2013 for images [4]). DLS analysis showed that the PMMA-PSMA PNPs exhibited an average
244 particle size of 86 nm and the PMMA PNPs an average particle size of 125 nm. No significant
245 difference in average particle size was observed between the non-labelled and fluorescently labelled
246 analogues. Single, narrow peaks were observed for the PMMA and PMMA-PSMA PNPs and
247 FPNPs, indicating a very narrow size distribution and no measurable occurrence of agglomeration.
248 Fluorescence was confirmed by measuring the emission spectra of the FPNPs, which also shows that
249 approximately the same amount of the dye has been incorporated into both types of PNP.
250
251 *Ecotoxicity tests*
252 *Microtox® tests.* Under the experimental conditions used, none of the PNPs and FPNPs
253 suspensions resulted in toxic effects (Table 2). In each case, the toxic concentration (EC50) was
254 above the range of concentrations studied (0.001-1000 mg L^{-1}).
255
256 *Daphnia magna.* The percentage of *D. magna* immobilised after 24 and 48 h in the acute
257 toxicity tests with the PNPs and FPNPs is shown in Figure 2, whilst the calculated LC50 and NEC
258 data are summarised in Table 2. The PMMA PNPs and FPNPs did not cause significant mortality
259 even at the highest concentration tested (1000 mg L^{-1}) and so LC50 and NEC values could not be
260 determined. In contrast, the PMMA-PSMA PNPs and FPNPs both exhibited significant toxicity at
261 some or all of the concentrations tested (Figure 2). DEBtox calculations of the test data indicated
262 both PMMA-PSMA PNPs and FPNPs appeared to have normal kinetics, with calculated NECs being
263 similar for both materials at 524 and 407 mg L^{-1}, respectively. In addition, the calculated 48 h
264 exposure EC50 values of the PMMA-PSMA PNPs and FPNPs were 879 and 887 mg L^{-1}
265 respectively, both below the maximum exposure concentration studied (Table 2).

266

267 *Corophium volutator.* The percentage immobilisation and percentage reburial of *C. volutator*
268 after a 10 d exposure to the PMMA and PMMA-PSMA PNPs and FPNPs are shown in Figure 3. The
269 data show that none of the PNPs or FPNPs tested resulted in significantly increased immobilisation
270 of the organisms at any of the test concentrations compared to the control samples. As a result, EC50
271 and NEC values could not be determined for any of the PNPs and FNPNs tested, and must therefore
272 be at concentrations above 500 mg L^{-1} (Table 2). In the reburial test conducted after the 10 d
273 exposure period, there was no significant difference observed between PNP and FPNP exposed
274 organisms and control organisms. Exposure to concentrations of PNPs and FPNPs ≤500 mgL^{-1}
275 appeared to have no effect upon reburial rates. Successful reburial is defined as occurring within 1 h
276 of the test organisms being transferred to clean sediment and seawater. All organisms in the present
277 study completed reburial within 1 h. No difference was observed between non-labelled and
278 fluorescently labelled PNP analogues in either test.

279

280 *Uptake studies*

281 Analysis of *D. magna* from the uptake study showed an intense blue fluorescence in the gut
282 of the organisms after only 24 h exposure (Figure 4A). Control organisms also exhibit a low-level
283 natural blue fluorescence generally distributed across the organism, but lacked the intense response
284 from the gut region observed in organisms exposed to FPNPs (Figure 4B). The presence of
285 fluorescent material in the gut of the exposed daphnids indicates rapid filtration of the FPNPs. After
286 the standard 48 h exposure period, strong fluorescence was still observed in the gut of organisms
287 exposed to the FPNPs (data not shown). However, after a recovery period of 24 h in clean media, no
288 fluorescence was observed in the gut of organisms exposed to the FPNPs (Figure 4C) and the
289 organisms appear the same as the control organisms after 72 h (48 h exposure and 24 h depuration)
290 in clean media (Figure 4D). This indicates that the FPNPs are quickly excreted by *D. magna*. The

presence of fluorescent faecal material (Figure 4E) in the recovery flasks of those organisms which had been exposed to the FPNPs confirms the rapid depuration through excretion. No significant mortality of the daphnids used in these studies was observed, indicating that the exposure concentration of 1 mg L^{-1} represented a sublethal concentration.

DISCUSSION

PMMA PNPs do not appear to be toxic to standard test species in either freshwater or marine ecosystems at environmentally relevant concentrations or even at very high concentrations. However, PMMA-PSMA PNPs appear to exhibit acute toxic effects at high concentrations. Naha et al, [15] investigated the acute ecotoxicity of NIPAM and 3 different ratios (85:15, 65:35 and 50:50) of NIPAM/BAM copolymer PNPs to *V. fischeri*, *D. magna*, the freshwater algae *Pseudokirchneriella subcapitata* and the freshwater shrimp *Thamnocephalus platyurus*. The PMMA and PMMA-PSMA PNP and FPNP EC50/LC50 and NEC values determined for *V. fischeri* in the current study are very similar to those observed for NIPAM and NIPAM/BAM 85:15 (>1000 mg L^{-1}), indicating that PMMA-based PNPs and NIPAM are not acutely toxic. The PMMA PNP and FPNP EC50/LC50 and NEC values determined for *D. magna* in the current study are all >1000 mg L^{-1}, whilst the PMMA-PSMA PNP and FPNP exhibited 48 h EC50/LC50 and NEC values in the range 879-887 mg L^{-1} and 407-524 mg L^{-1}, respectively. All NIPAM and NIPAM/BAM PNPs exhibited 48 h EC50/LC50 and NEC values in the range 413.6-60.6 mg L^{-1} and <250-50 mg L^{-1} respectively [15]. Increasing toxicity was observed with an increasing amount of BAM. The PMMA-PSMA PNP and FPNP EC50/LC50 and NEC values are comparable to those determined for NIPAM indicating that these two PNPs have a similar effect on *D. magna*.

There appears to be a significant influence from the PNP physicochemical properties on the potential for toxicity. It appears as though hydrophobicity plays a role, with the more hydrophobic

316 PMMA-PSMA PNPs eliciting a response in *D. magna* whilst the medium PMMA PNPs do not. The
317 increased hydrophobicity of the PMMA-PSMA PNPs could be increasing the uptake rate. Whilst, it
318 was not possible to quantify the uptakes rates for either PMMA-PSMA PNPs or PMMA PNPs, both
319 appeared to be readily taken up and filled the gut of *D. magna*. It is therefore suggested that the
320 presence of the stearylmethacrylate copolymer could be directly responsible for the toxic response
321 offering an alternative surface chemistry to that of the PMMA PNPs. Furthermore, it appears there is
322 no hindrance effect on toxicity from the presence of the large alkyl chain in the PMMA-PSMA
323 PNPs, again supporting a chemical source for the observed toxicity. However, it should be noted that
324 the observed toxicological differences may also be related to PNP size, with PMMA-PSMA being
325 smaller (86 nm) than PMMA (125 nm).
326
327 In contrast to the current study and that of Naha et al, [15], Casado et al, [16] report that 55
328 and 110 nm PS-PEI PNPs exhibited a strong toxic response for most of the species studied, except
329 for *V. fischeri*, where similar values of >1000 mg L^{-1} were observed. PS-PEI PNPs exhibited 48 h
330 EC50/LC50 values for *D. magna* in the range 0.66-0.77 mg L^{-1}, with large particles (110 nm)
331 exhibiting slightly higher responses than smaller particles (55 nm). These data indicate that PS-PEI
332 PNPs are considerably more toxic to *D. magna* than any of the PNPs and FPNPs used in the present
333 study or the NIPAM and NIPAM/BAM copolymers studied by Naha et al, [15]. Naha et al, suggest a
334 species sensitivity order for NIPAM as *D. magna* > *T. platyurus* > *V. fischeri* > *P. subcapitata* [15].
335 This is consistent with the findings of the current study using PNPs and FPNPs where the sensitivity
336 order is *D. magna* > *V. fischeri/C. volutator*. Similarly to the current study and that of Naha et al., *D.*
337 *magna* was identified as one of the most sensitive species, although Casado et al, [16] found that *P.*
338 *subcapitata* was the most sensitive species in their studies. The higher sensitivity of *D. magna* may
339 be related to a different uptake route (filter feeding) than either *V. fischeri* (direct contact) and *C.*
340 *volutator* (deposit feeder), or possibly a higher uptake rate through filter feeding. As no toxicity

341 towards *D. magna* was observed for the PMMA PNPs but was observed for the PMMA-PSMA PNPs
342 it seems that the mode of toxic action is not related to a nutritional problem. Instead, the clear
343 difference between the two PNP types indicates there is an intrinsic toxicity associated with the
344 physicochemical properties of the PMMA-PSMA PNPs.
345
346 Nano-sized particulate materials are often stabilised in aqueous dispersion using a range of
347 stabilising agents [4, 6, 27, 28]. In the present study, the test PNPs were synthesised using SDS as a
348 stabilising agent. As a result, the surface of the PNPs and FPNPs will be coated with the SDS and
349 there is potential for excess SDS to be present in the exposure solutions. Therefore, the direct toxicity
350 of SDS must be considered within the context of the results obtained. Whilst the concentration of
351 free SDS in the exposure solutions is unknown, even at the highest concentrations of PNPs and
352 FPNPs used in the present study (500-1000 mg L^{-1}) a toxic effect was only observed for the PMMA-
353 PSMA PNPs and FPNPs and then, only for *D. magna*. A previous study reports a 48 h LC50 value
354 for SDS with *D. magna* of 19.129 mg L^{-1} [29]. Bessling et al., [1] also provide toxicity data for SDS
355 to *D. magna* and the freshwater alga *Scenedesmus obliquus*. As the corresponding PMMA PNPs and
356 FPNPs did not result in a toxic effect, this indicates that the presence of any free SDS is not
357 influencing the observed toxicity and is therefore below the reported LC50 value of 19.129 mg L^{-1}.
358 This should be the case as the PNPs and FPNPs were carefully dialysed after synthesis to remove as
359 much free SDS as possible. The observed acute toxicity in the current study appears to be related
360 directly to differences in physicochemical properties of the PMMA and PMMA-PSMA PNPs.
361
362 Rapid uptake of the FPNPs into the gut of *D. magna* was observed after only 24 h (
363 A) and was still present after 48 h. This confirms that PNPs in the size range studied (86-125
364 nm diameter) can be rapidly filtered by filter feeding aquatic organisms such as *D. magna*. Filtration

was followed by a corresponding rapid depuration period of 24 h when the organisms were transferred to clean systems and evidenced by fluorescent faecal pellets (

E). The most likely route of uptake of PNPs and other ENPs by *Daphnia magna* is through filtration, including active selection by the feeding apparatus, as well as passive diffusion or uptake alongside larger particles [17]. For an adult *D. magna*, the largest ingestible particles are considered to be approximately 70 µm [30]. The minimum size is believed to be dependent on the distances between the setulae on the thoracic limbs of *D. magna*, which is independent from age or size due to the gap being constant [31]. *Daphnia magna* is able to actively filter particles as small as 200 nm, although this is an estimate based upon the size of the gap between the setulae [17]. In the current study, no significant mortality of *D. magna* was observed, indicating that the exposure concentration of 1 mg L^{-1} represented a sublethal concentration. Whilst uptake and excretion of FPNPs were both rapid, it is unclear from the resolution of the imaging technique employed if any of the FPNP materials was able to cross the gut wall and into the organisms. As there was no clear fluorescent response from the organisms following the depuration period, it is assumed that any transport of FPNPs across the gut wall is limited. Whilst the use of fluorescent labelling to study uptake of PNPs may be more limited for organisms without translucent bodies, the study of faecal material after transferral to clean media may offer a method for their assessment. Carbon-based nanomaterials have previously been shown to efficiently adsorb hydrophobic organic pollutants (e.g. PAHs and PCBs) in aquatic systems [32-34]. Similar adsorption has also been observed for PMPs [35-37] and PNPs [34], with adsorption to PNPs typically being 1−2 orders of magnitude stronger than to PMPs [34]. Whilst such adsorption has not been investigated for the PNPs used in the present study, it is likely that a similar process would occur. This means that PNPs could potentially offer an alternative uptake route for organic pollutants in filter feeding organisms and that during transport through the gut, these compounds may be desorbed from the particle surface and taken up by the organism.

The identification of PNPs, PMPs and other ENPs of interest in complex biological and environmental matrices remains a challenging task. Matrices such as soils and sediment contain mixtures of solids of biotic and abiotic origin in the nano-size range, making identification of exogenous PNPs, PMPs and ENPs difficult. One potential method of overcoming this is to fluorescently label the test material particles. Particles with intrinsic fluorescent properties or specifically labelled with fluorescent dyes or markers offer the potential for detection during environmental fate studies (e.g. sedimentation studies) and for monitoring movement, uptake and accumulation within organisms in ecotoxicological experiments [17, 38-40]. In uptake studies the use of fluorescent particles are best suited to organisms with translucent bodies such as the freshwater cladoceran, *Daphnia magna* and the freshwater fish Medaka (*Oryzias latipes*) [17, 39]. However, there is concern that chemical modification of PNPs, PMPs and ENPs to generate fluorescence may result in changes in the environmental fate and effects of the particle from the non-labelled analogue. In a previous study, we showed that the incorporation of the fluorescent dye 7-[4-(trifluoromethyl)coumarin]acrylamide into poly(methylmethacrylate)-based PNPs had no effect upon the environmental behaviour compared to non-labelled analogues [4]. The present study included an assessment of the fluorescent dye on the ecotoxicity of the PNPs compared to the non-labelled analogues. The data show that there is no significant difference between the fluorescently labelled and non-label analogues, indicating that the proportion of the fluorescent label in these particles does not influence their ecotoxicity to the species studied. These results support the use of fluorescent labelling as a non-invasive tracking approach for PNPs in environmental samples.

CONCLUSIONS

The least sensitive model systems were the marine bacterium *V. fischeri* and the amphipod *C. volutator*, whilst the most sensitive was the 48 h immobilisation of *D. magna*. In terms of response, the PMMA-PSMA PNPs and FPNPs appeared to show the greatest toxicity in the present study.

Here we observe some differences in ecotoxicity between two differently functionalised PNPs suggesting that surface chemistry may play an important role in influencing ecotoxicity. The results indicate that that the ecotoxicity of PNPs cannot be reliably assessed using a single PNP type. Furthermore, ecotoxicity of the PNP materials assessed in the present study varied between test species, indicating that conclusions regarding the ecotoxicity of PNPs must be drawn from a comprehensive assessment based on multi-trophic approach. Importantly, the results in the present study indicate that none of the PNPs appear to illicit significant acute ecotoxicological responses to representative test species in freshwater and marine compartments at concentrations considered to be environmentally realistic. Further work investigating the potential sublethal effects of PNPs and PMPs is necessary to fully understand their environmental impacts.

Acknowledgment: The present study was partially funded under the Norwegian Research Council (NFR) Miljø2015 Programme through the 'NanoSORB' project (Grant Agreement number 209685/E50). The authors wish to thank NFR for their financial support. The authors acknowledge the essential technical assistance of K. Bonaunet, K. Almås and I. Steinsvik at SINTEF Materials and Chemistry. The authors declare that there is no conflict of interest regarding the contents of the present study.

References

1. Besseling, E., et al., *Nanoplastic Affects Growth of S. obliquus and Reproduction of D. magna.* Environmental Science & Technology, 2014. **48**(20): p. 12336-12343.
2. Cole, M., et al., *Microplastic Ingestion by Zooplankton.* Environmental Science & Technology, 2013. **47**(12): p. 6646-6655.
3. Cole, M., et al., *Microplastics as contaminants in the marine environment: A review.* Marine Pollution Bulletin, 2011. **62**(12): p. 2588-2597.
4. Booth, A., et al., *Influence of salinity, dissolved organic carbon and particle chemistry on the aggregation behaviour of methacrylate-based polymeric nanoparticles in aqueous environments.* International Journal of Environment and Pollution, 2013. **52**(1/2): p. 15 - 31.

5. Sivan, A., *New perspectives in plastic biodegradation.* Current Opinion in Biotechnology, 2011. **22**(3): p. 422-426.
6. Garay-Jimenez, J.C., et al., *Physical properties and biological activity of poly(butyl acrylate-styrene) nanoparticle emulsions prepared with conventional and polymerizable surfactants.* Nanomedicine: Nanotechnology, Biology and Medicine, 2009. **5**(4): p. 443-451.
7. Kamaly, N., et al., *Targeted polymeric therapeutic nanoparticles: design, development and clinical translation.* Chemical Society Reviews, 2012. **41**(7): p. 2971-3010.
8. Rice-Ficht, A.C., et al., *Polymeric particles in vaccine delivery.* Current Opinion in Microbiology, 2010. **13**(1): p. 106-112.
9. Valo, H., et al., *Electrospray encapsulation of hydrophilic and hydrophobic drugs in poly(L-lactic acid) nanoparticles.* Small, 2009. **5**(15): p. 1791-1798.
10. Lu, X.-Y., et al., *Chapter 7 - Polymer Nanoparticles*, in *Progress in Molecular Biology and Translational Science*, V. Antonio, Editor. 2011, Academic Press. p. 299-323.
11. Chavez, J.L., J.L. Wong, and R.S. Duran, *Core-Shell Nanoparticles: Characterization and Study of Their Use for the Encapsulation of Hydrophobic Fluorescent Dyes.* Langmuir, 2008. **24**(5): p. 2064-2071.
12. Dai, S., P. Ravi, and K.C. Tam, *Thermo- and photo-responsive polymeric systems.* Soft Matter, 2009. **5**(13): p. 2513-2533.
13. Cedervall, T., et al., *Food chain transport of nanoparticles affects behaviour and fat metabolism in fish.* PloS one, 2012. **7**(2): p. 22.
14. Mattsson, K., et al., *Altered Behavior, Physiology, and Metabolism in Fish Exposed to Polystyrene Nanoparticles.* Environmental Science & Technology, 2015. **49**(1): p. 553-561.
15. Naha, P.C., et al., *Preparation, characterization of NIPAM and NIPAM/BAM copolymer nanoparticles and their acute toxicity testing using an aquatic test battery.* Aquatic Toxicology, 2009. **92**(3): p. 146-154.
16. Casado, M.P., A. Macken, and H.J. Byrne, *Ecotoxicological assessment of silica and polystyrene nanoparticles assessed by a multitrophic test battery.* Environment International, 2013. **51**(0): p. 97-105.
17. Rosenkranz, P., et al., *A comparison of nanoparticle and fine particle uptake by Daphnia magna.* Environmental Toxicology and Chemistry, 2009. **28**(10): p. 2142-2149.
18. Wegner, A., et al., *Effects of nanopolystyrene on the feeding behavior of the blue mussel (Mytilus edulis L.).* Environmental Toxicology and Chemistry, 2012. **31**(11): p. 2490-2497.
19. Zarfl, C. and M. Matthies, *Are marine plastic particles transport vectors for organic pollutants to the Arctic?* Marine Pollution Bulletin, 2010. **60**(10): p. 1810-1814.
20. Moore, C.J., *Synthetic polymers in the marine environment: A rapidly increasing, long-term threat.* Environmental Research, 2008. **108**(2): p. 131-139.
21. Bhattacharya, P., et al., *Physical Adsorption of Charged Plastic Nanoparticles Affects Algal Photosynthesis.* Journal of Physical Chemistry C, 2010. **114**(39): p. 16556-16561.
22. Della Torre, C., et al., *Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos Paracentrotus lividus.* Environmental Science and Technology, 2014. **48**(20): p. 12302-12311.
23. Azur Environmental Ltd, *Microtox® Acute Toxicity Basic Test Procedures, Report.* 1998: Carlsbad, CA. p. 1-13.
24. OECD, *Daphnia sp., Acute Immobilisation Test*, in *OECD Guideline for the Testing of Chemicals*. 2004, Organisation for Economic Cooperation and Development (OECD): Paris.
25. ISO, *Water quality - Determination of acute toxicity of marine or estuarine sediment to amphipods*. 2005, International Organisation for Standardisation.
26. Bat, L. and D. Raffaelli, *Sediment toxicity testing: a bioassay approach using the amphipod Corophium volutator and the polychaete Arenicola marina.* Journal of Experimental Marine Biology and Ecology, 1998. **226**(2): p. 217-239.

27. Booth, A., et al., *Freshwater dispersion stability of PAA-stabilised cerium oxide nanoparticles and toxicity towards Pseudokirchneriella subcapitata.* Science of the Total Environment, 2015. **505**(0): p. 596-605.
28. Taniguchi, T., et al., *Preparation of highly monodisperse fluorescent polymer particles by miniemulsion polymerization of styrene with a polymerizable surfactant.* Journal of Colloid and Interface Science, 2008. **327**(1): p. 58-62.
29. Guilhermino, L., et al., *Acute Toxicity Test with Daphnia magna: An Alternative to Mammals in the Prescreening of Chemical Toxicity?* Ecotoxicology and Environmental Safety, 2000. **46**(3): p. 357-362.
30. Burns, C.W., *The relationship between body size of filter feeding cladocera and the maximum size of particle ingested.* Limnology and Oceanography, 1968. **13**(4): p. 675–678.
31. Geller, W. and H. Müller, *The filtration apparatus of Cladocera: Filter mesh-sizes and their implications on food selectivity.* Oecologia, 1981. **49**(3): p. 316-321.
32. Chen, W., L. Duan, and D.Q. Zhu, *Adsorption of polar and nonpolar organic chemicals to carbon nanotubes.* Environmental Science & Technology, 2007. **41**(24): p. 8295-8300.
33. Kah, M., et al., *Measuring and Modeling Adsorption of PAHs to Carbon Nanotubes Over a Six Order of Magnitude Wide Concentration Range.* Environmental Science & Technology, 2011. **45**(14): p. 6011-6017.
34. Velzeboer, I., C.J.A.F. Kwadijk, and A.A. Koelmans, *Strong Sorption of PCBs to Nanoplastics, Microplastics, Carbon Nanotubes, and Fullerenes.* Environmental Science & Technology, 2014. **48**(9): p. 4869-4876.
35. Teuten, E.L., et al., *Transport and release of chemicals from plastics to the environment and to wildlife.* Philos. Trans. R. Soc., B, 2009. **364**: p. 2027.
36. Rochman, C.M., et al., *Long-Term Field Measurement of Sorption of Organic Contaminants to Five Types of Plastic Pellets: Implications for Plastic Marine Debris.* Environmental Science & Technology, 2013. **47**(3): p. 1646-1654.
37. Rochman, C.M., et al., *Polystyrene Plastic: A Source and Sink for Polycyclic Aromatic Hydrocarbons in the Marine Environment.* Environmental Science & Technology, 2013. **47**(24): p. 13976-13984.
38. Fent, K., et al., *Assessment of uptake and toxicity of fluorescent silica nanoparticles in zebrafish (Danio rerio) early life stages.* Aquatic Toxicology, 2010. **100**(2): p. 218-228.
39. Kashiwada, S., *Distribution of Nanoparticles in the See-through Medaka (Oryzias latipes).* Environ Health Perspect, 2006. **114**(11).
40. Ward, J.E. and D.J. Kach, *Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves.* Marine Environmental Research, 2009. **68**(3): p. 137-142.

532 **Figure & Table Legends**

533 *Figures*

534 Figure 1. Chemical structures of the two types of PNP and the two types of FPNP synthesised in this
535 study using a mini-emulsion polymerisation method. (A) medium poly(methylmethacrylate) polymer
536 (PMMA PNP), (B) hydrophobic poly(methylmethacrylate-*co*-stearylmethacrylate) copolymer
537 (PMMA-PSMA PNP), (C) fluorescent dye 7-[4-(trifluoroethyl)coumarin]acrylamide, (D) PMMA
538 polymer with fluorescent label copolymer (PMMA FPNP), and (E) PMMA-PSMA polymer with
539 fluorescent label copolymer (PMMA-PSMA FPNP).

540

541 Figure 2. Effect of PMMA PNP, PMMA FPNP, PMMA-PSMA PNP and PMMA-PSMA FPNP on
542 the immobilisation of *Daphnia magna* after 24 h and 48 h. Data are presented as the mean percentage
543 ± SD (n=3) except for the control sample where n=6.

544

545

546 Figure 3. Effect of the PMMA PNP, PMMA FPNP, PMMA-PSMA PNP and PMMA-PSMA FPNP
547 on immobilisation and reburial of *Corophium volutator* after a 10 d exposure period. Data are
548 presented as the mean ± SD where n=3 except for the control sample where n=6.

549

550 Figure 4. Fluorescence microscope images of *Daphnia magna*. Images A-D are all the same scale.

551

552

553

554

555

556 *Tables*

557 Table 1. Concentration of nanoparticles (mg L^{-1}) used in the toxicity assays and uptake/depuration
558 studies.

559

560 Table 2. Summary of EC50/LC50 and no effect concentration ecotoxicity data for the PMMA and
561 PMMA-PSMA PNPs and FPNPs for selected test species and endpoints.

562