Plastic Debris in the Aquatic Environment

UPTAKE AND TOXICITY OF METHYLMETHACRYLATE-BASED NANOPLASTIC PARTICLES IN AQUATIC ORGANISMS

ANDY M. BOOTH,†* BJØRN HENRIK HANSEN,† MAX FRENZEL,† HEIDI JOHNSEN,† and DAG ALTIN‡
†Environmental Technology Department, Foundation for Scientific and Industrial Research (SINTEF) Materials and Chemistry, Trondheim, Norway
‡Biotechnology and Nanomedicine Department, Foundation for Scientific and Industrial Research (SINTEF) Materials and Chemistry, Trondheim, Norway

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Abstract: The uptake and toxicity of 2 poly(methylmethacrylate)-based plastic nanoparticles (PNPs) with different surface chemistries (medium and hydrophobic) were assessed using aquatic organisms selected for their relevance based on the environmental behavior of the PNPs. Pure poly(methylmethacrylate) (medium; PMMA PNPs) and poly(methylmethacrylate-co-stearyl methacrylate) copolymer (hydrophobic; PMMA–PSMA PNPs) of 86 nm to 125 nm were synthesized using a miniemulsion polymerization method. Fluorescent analogs of each PNP were also synthesized using monomer 7-[4-(trifluoromethyl)coumarin]acylamide 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 3 different environmentally realistic concentrations (0.01 mg/L, 0.1 mg/L, and 1.0 mg/L) and a high concentration 500 mg/L to 1000 mg/L. In addition, sublethal effects of PNPs in C. volutator were determined using a sediment reburial test, and the uptake and depuration of fluorescent PNPs was studied in D. magna. The PNPs and fluorescent PNPs did not exhibit any observable toxicity at concentrations up to 500 mg/L to 1000 mg/L in any of the tests except for PMMA–PSMA PNPs and fluorescent PNPs following 48-h exposure to D. magna (median lethal concentration values of 879 mg/L and 887 mg/L, respectively). No significant differences were observed between labeled and nonlabeled PNPs, indicating the suitability of using fluorescent labeling. Significant uptake and rapid excretion of the fluorescent PNPs was observed in D. magna. Environ Toxicol Chem 2016;35:1641–1649. © 2015 SETAC

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INTRODUCTION

As for many other pollutants, aquatic systems have emerged as the primary sink for micron-sized plastic particles (PMPs) and plastic nanoparticles (PNPs) [1–3]. Sediments have been specifically identified as potential environmental sinks and concentration hotspots [4]. Micron-sized plastic particles and PNPs in the environment can be derived from both primary particles (e.g., personal care and cosmetic products) and the secondary particles that result from degradation of larger plastic items [5]. Most research has focused on PMPs in the marine environment, and very little is known about the fate and effects of PNPs. Easy and inexpensive to synthesize, PNPs have almost unlimited potential for physical and chemical modification for targeted application. They have already been demonstrated to have applications in a wide variety of technologies, including targeted drug and vaccine delivery diagnostics and bioimaging in nanomedicine [6–9]; protein purification and immobilization matrices [10]; shell structures for nanosized containers encapsulating dyes, lubricants, and other chemicals [11]; and material surfaces and coatings [12].

Recently, PNPs in both freshwater and marine environments have become the subject of an increasing number of studies [1,4,13–18]. Many of the available studies have employed polystyrene PNPs. Polystyrene is 1 of the 5 main high production–volume plastics, amounting to approximately 90% of the total demand [19], and is commonly found in the marine environment [20]. Polystyrene PNPs have been shown to adsorb to the surface of algal cells, reducing photosynthesis through possible shading effects and also enhancing production of reactive oxygen species [21]. Polystyrene PNPs have been found to be taken up by Daphnia magna and to translocate from the gut to other body tissues [17]. Besseling et al. [1] studied the effects of polystyrene PNP exposure on the growth and photosynthesis of the green alga Scenedesmus obliquus and the growth, mortality, neonate production, and malformations of D. magna at concentrations between 0.22 mg/L and 103 mg/L. Reduced population growth and chlorophyll concentrations were observed in the algae, consistent with the results of Bhattacharya et al. [21]. Daphnia magna showed a reduced body size and severe alterations in reproduction. The effects of polystyrene PNPs on the feeding behavior 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 polystyrene PNPs can be transported through an aquatic food chain from algae, through zooplankton to fish, affecting the lipid metabolism and behavior of the top consumer [13,14]. Amine-functionalized polystyrene PNPs have been found to cause severe developmental defects in sea urchin embryos (Paracentrotus lividus), whereas carboxyl-functionalized polystyrene PNPs exhibited no effects [22].

The toxicity of other PNP types and copolymers have also been studied [15,16]. The acute toxicity of poly N-isopropylacrylamide and N-isopropylacrylamide/N-tert-butyacylamide copolymer PNPs was assessed using a battery of acute aquatic tests (with Vibrio Fischeri, Pseudokirchneriella subcapitata, D. magna, and Thamnocephalus platyurus), and significant ecotoxicological responses were observed at particle concentrations of up to 1000 mg/L [15]. The ecotoxicological response was seen to correlate well with the ratio of N-tet-tert-butyacylamide 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 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 polystyrene-polyethyleneimine PNPs at concentrations from 0.40 mg/L to 416.5 mg/L, 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]. That study indicated that PNP surface chemistry and environmental parameters such as salinity and dissolved organic material concentration had a significant effect on PNP fate in aquatic environments. The PMMA-based PNPs with medium and hydrophobic surface chemistries remained freely dispersed for prolonged periods in freshwater environments under environmentally realistic PNP concentrations but agglomerated and sedimented rapidly under weakly saline conditions. These studies [4] indicated that in freshwater environments PNP will be exposed to pelagic organisms whereas in estuarine and marine environments benthic organisms are those most at risk to exposure. In low-energy freshwater environments (e.g., lakes and reservoirs), however, 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 labeled analogues (fluorescent PNPs). To assess surface chemistry-dependent ecotoxicity, PMMA-based PNPs were synthesized with and without a comonomer to allow variation in surface chemistry from medium to hydrophobic. The fluorescent PNPs 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 fluorescent PNPs was conducted using bioassays representing different trophic levels. The tests employed included the Microtox® bacterial species (V. fischeri), a pelagic filter feeding freshwater crustacean (D. magna), and a benthic sediment reworking marine crustacean (C. volutator). Sublethal effects (sediment reburial) were assessed in C. volutator, and qualitative uptake and excretion of fluorescent PNPs were assessed in D. magna. We investigated the effects of a broad range of expected environmentally relevant and elevated concentrations of PMMA-based PNPs and fluorescent PNPs in the toxicity studies.

MATERIALS AND METHODS

Synthesis of PNPs

Two types of PMMA-based PNPs were synthesized with hydrophobic and medium surface chemistries (Figure 1A and B). The medium-chemistry PNPs were comprised of pure 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 synthesized using a standard miniemulsion polymerization method described previously [4]. Briefly, a stabilizing solution of water containing sodium dodecyl sulfate and the liquid monomer containing a polymerization initiator (V-59) were mixed together and sonicated to form an emulsion of nano-sized monomer droplets in water. The monomer...
droplets were then polymerized to form the final nano-sized particles, which were suspended in the aqueous medium. Following synthesis, the PNP s were isolated and purified by dialysis in deionized water to remove any residual monomer and the stabilizer. The final PNP s in water dispersions were stored in a glass bottle in the refrigerator until required. Immediately before use in the ecotoxicity studies, all samples were sonicated for 30 min to ensure that any agglomerates were broken down and that the PNP s were fully dispersed in the media. No significant aggregation was observed in any of the PNP samples prior to sonication; the presence of freely dispersed nanoplastic particles in aquatic environments may be unlikely because of heteroaggregation with natural particulates.

For the PNP s to be determined in biological samples from uptake studies, fluorescent analogs of each type of PNP s were synthesized (Figure 1D and E). These analogues (PMMA fluorescent PNP s and PMMA–PSMA fluorescent PNP s, respectively) were synthesized 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 comonomer in the polymerization process, which allowed it to be linked chemically to the PNP s; thus potential problems associated with leakage (and therefore any potential toxicity) of the dye from the final PNP s were eliminated. The synthesis method was the same as that described above and in Booth et al. [4] for the nonlabeled analogs. It should be noted that Figures 1D and E represents just 1 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 with 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 characterization

The particle shape and size of the synthesized PNP s and fluorescent PNP s was characterized by transmission electron microscopy (TEM) and dynamic light scattering. A Phillips CM30 TEM equipped with a LaB6 electron filament was used to investigate PNP shape and size. The average particle size of the synthesized PNP s was determined by dynamic light scattering using a Malvern ZetaSizer™. A SpectraMax Gemini XS plate reader fluorescence spectrometer was used to quantify the amount of fluorescent dye in the fluorescent PNP analogs. 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 PNP s when present in environmentally realistic concentrations and to see whether a very high concentration also resulted in an effect. In recent studies, PNP effects have been studied at concentrations ranging from 0.22 mg/L to 1100 mg/L depending on the test species and experimental setup employed [1,13,14]. In the present study, PNP s and fluorescent PNP s were tested at concentrations of 0.01 mg/L, 0.1 mg/L, and 1.0 mg/L, which are considered to represent realistic environmental concentrations and are consistent with the concentration ranges employed in other studies. In addition, the PNP and fluorescent PNP s were tested at 1000 mg/L (500 mg/L in the C. volutator tests) to determine whether the test materials elicited a response to PNP s at very high concentrations. This is again consistent with the upper concentrations used in other studies [1]. The nominal exposure concentrations used for each test species are summarized in Table 1.

Microtox test. The acute toxicity of each PNP and fluorescent PNP analog to the bioluminescent marine bacterium V. fischeri was determined using the 90% basic test for aqueous extract protocol [23]. All Microtox reagents and lyophilized V. fischeri bacteria (NRRL B-11177) were obtained from SDI Europe. Tests were carried out at 15°C in the Microtox diluent supplied. Phenol was used as a reference. Minute X and Y median effective concentration (EC50) tests were performed using the Microtox Toxicity Analyzer (SDI) following the manufacturer’s instructions. Toxicity data were obtained and analyzed using MicrotoxOmni software. The effective concentration, EC50, is defined as the concentration that produces a 50% light reduction. The EC50 was measured after 15 min of contact time.

Daphnia magna immobilization. A D. magna starter culture originating from Denmark (purchased from the Norwegian Institute of Water Research; NIVA) consisted of approximately 100 pregnant females, which were transferred to M7 medium as described in Organisation for Economic Co-operation and Development guideline 202 [24]. The culture was kept for at least 3 generations before neonates were used in exposure experiments. The culture was kept at 20°C to 22°C with a 16:8-h light:dark regime and fed green algae (P. subcapitata) in excess daily. Exposure studies were conducted according to the Organisation for Economic Co-operation and Development’s standard procedure. Four PNP and fluorescent PNP concentrations (0.01 mg/L, 0.1 mg/L, 1 mg/L, and 1000 mg/L) plus blank controls were tested. The exposure solutions (25 mL) were added to 50-mL Erlenmeyer flasks, and 5 neonates (<24 h old) from a brood were added. Neonates were not fed for the duration of the experiment. After 24 h and 48 h, immobilization (mortality) of the individuals within the container was recorded. All exposure concentrations were performed in triplicate, and 6 controls containing only M7 medium were used. At the end of the experiment, exposure solutions were analyzed for O2 and pH to verify that they were within the acceptable range reported in the Organisation for Economic Co-operation and Development guideline. Animals unable to swim within 15 s of gentle agitation of the test vessel were considered immobile. Version

Table 1. Concentration of nanoparticles (mg/L) used in the toxicity assays and uptake/depuration studies

| Sample          | Vibrio fischeri Microtox™ test | Daphnia magna immobilization test | Corophium volutator acute toxicity and reburial | Daphnia magna uptake and depuration test |
|-----------------|---------------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------|
| PMMA PNP        | 0.01, 0.1, 1, and 1000          | 0.01, 0.1, 1, and 1000            | 0.01, 0.1, 1, and 500                         | N/A                                     |
| PMMA FFPNP      | 0.01, 0.1, 1, and 1000          | 0.01, 0.1, 1, and 1000            | 0.01, 0.1, 1, and 500                         | 1                                      |
| PMMA–PSMA FFPNP| 0.01, 0.1, 1, and 1000          | 0.01, 0.1, 1, and 1000            | 0.01, 0.1, 1, and 500                         | N/A                                     |
| PMMA–PSMA FFPNP| 0.01, 0.1, 1, and 1000          | 0.01, 0.1, 1, and 1000            | 0.01, 0.1, 1, and 500                         | 1                                      |

PMMA PNP = poly(methylmethacrylate) plastic nanoparticle; FFPNP = fluorescent analog of plastic nanoparticles; PNP = plastic nanoparticles; PMMA–PSMA = poly(methylmethacrylate-co-stearylmethacrylate) copolymer.
2.0.1 of the DEBtox software was used for calculations of effect concentrations (ECs) and no-effect concentrations (NECs) from the data generated in the *D. magna* acute toxicity/immobilization test.

*Corophium volutator* acute toxicity and reburial. The test procedure followed in the present study is outlined in NS-EN ISO 16712:2005 [25]. As with freshwater systems, the presence of natural colloids in seawater is likely to influence the aggregation behavior and settling of PNPs. Natural colloids were not present in the present study’s exposure system as the seawater used is filtered prior to use; however, our previous study indicated rapid aggregation and settling of PMMA–PSMA and PMMA PNPs under common seawater salinity levels [4]. To allow a natural aggregation of the PNPs when introduced into seawater, they were diluted and sonicated for 15 min in full-strength seawater at the different exposure concentrations and introduced in the test vessels together with the overlying water. The test animals were introduced 5 h to 6 h later when visual inspection confirmed that the PNPs and fluorescent PNPs had precipitated to the sediment. To test for viability and sublethal effects after 10 d of exposure, the postexposure reburial test suggested for *Corophium* sp. by Bat and Raffaelli [26] was performed by transferring the animals to beakers with clean sediment and 1 cm of overlying seawater. The recommended duration of the reburial test to be able to discriminate between normal and moribund individuals is set to 1 h.

**Uptake and depuration study**

To investigate potential uptake and depuration of PMMA PNPs, subadult *D. magna* were exposed to the fluorescent PNP analogs for 48 h. The conditions used were the same as those described for the 48-h immobilization test. Organisms were exposed to 1 mg/L concentrations of the fluorescent PNPs to ensure that sufficient amounts of the test materials were available for filtration and subsequent detection by fluorescence microscopy, but also to ensure that 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 fluorescent PNPs 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; em: 450DF65] and x-cite 120 metal halide arc lamp) for evidence of fluorescent PNP 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. Fecal pellets excreted by the organisms were also collected and analyzed under the fluorescence microscope.

**RESULTS**

**Synthesis and characterization of PNPs**

The PMMA PNP (Figure 1A) and PMMA–PSMA PNP (Figure 1B) particles were successfully synthesized by mini-emulsion polymerization and cleaned using dialysis. Fluorescently labeled homologs (fluorescent PNPs) of each of these particles were also successfully synthesized by incorporation of the fluorescent dye 7-[4-(trifluoromethyl)coumarin]acrylamide (Figure 1C–E). A detailed description of the PNP and fluorescent PNP characterization has been previously reported [4]. Briefly, TEM showed that individual particles were spherical in nature and exhibited slight differences in particle size, typically within the range 86 nm to 125 nm (see Booth et al. for images [4]). Dynamic light scattering analysis showed that the PMMA–PSMA PNPs had an average particle size of 86 nm and the PMMA PNPs an average particle size of 125 nm. No significant difference in average particle size was observed between the nonlabeled and fluorescently labeled analogs. Single narrow peaks were observed for the PMMA and PMMA–PSMA PNPs and fluorescent PNPs, indicating a very narrow size distribution and no measurable occurrence of agglomeration. Fluorescence was confirmed by measuring the emission spectra of the fluorescent PNPs, which also showed that approximately the same amount of the dye was incorporated into both types of PNPs.

**Ecotoxicity tests**

**Microtox tests.** Under the experimental conditions used, none of the PNP and fluorescent PNP suspensions resulted in toxic effects (Table 2). In each case, the toxic concentration (EC50) was above the range of concentrations studied (0.001–1000 mg/L).

*Daphnia magna*. The percentage of *D. magna* immobilized after 24 h and 48 h in the acute toxicity tests with the PNPs and

| Species          | End point                     | PMMA PNP EC50/LC50 (mg/L) | PMMA PNP NEC (mg/L) | PMMA FPNP EC50/LC50 (mg/L) | PMMA FPNP NEC (mg/L) | PMMA–PSMA PNP EC50/LC50 (mg/L) | PMMA–PSMA PNP NEC (mg/L) |
|------------------|-------------------------------|---------------------------|---------------------|----------------------------|----------------------|-------------------------------|---------------------------|
| Vibrio fischer   | 15-min inhibition             | >1000                     | –                   | >1000                      | –                    | >1000                         | –                         |
| *Daphnia magna*  | 24-h immobilization           | >1000                     | >1000               | >1000                      | >1000                | 1550                          | 524                       |
| *Daphnia magna*  | 48-h immobilization           | >1000                     | >1000               | >1000                      | >1000                | 879                           | 524                       |
| *Corophium volutator* | 10-d immobilization     | >500                      | >500                | >500                       | >500                 | >500                          | >500                       |
| *Corophium volutator* | 10-d reburial            | >500                      | >500                | >500                       | >500                 | >500                          | >500                       |

EC50 = median effective concentration; LC50 = median lethal concentration; NEC = no-effect concentration; PNP = plastic nanoparticle; PMMA PNP = poly(methylmethacrylate) plastic nanoparticle; FPNP = fluorescent analog of PNP; PMMA–PSMA = poly(methylmethacrylate-co-stearimethylacrylate) copolymer.
fluorescent PNPs is shown in Figure 2, and the calculated median lethal concentration (LC50) and NEC data are summarized in Table 2. The PMMA PNPs and fluorescent PNPs did not cause significant mortality even at the highest concentration tested (1000 mg/L) and so LC50 and NEC values could not be determined. In contrast, the PMMA–PSMA PNPs and fluorescent PNPs both exhibited significant toxicity at some or all of the concentrations tested (Figure 2). Calculations of the test data (DEBtox) indicated that both PMMA–PSMA PNPs and fluorescent PNPs appeared to have normal kinetics, with calculated NECs being similar for both materials at 524 mg/L and 407 mg/L, respectively. In addition, the calculated 48-h exposure EC50 values of the PMMA–PSMA PNPs and fluorescent PNPs were 879 mg/LL and 887 mg/L, respectively, both below the maximum exposure concentration studied (Table 2).

Corophium volutator. The percentage of immobilization and percentage of reburial of C. volutator after a 10-d exposure to the PMMA and PMMA–PSMA PNPs and fluorescent PNPs are shown in Figure 3. The data show that none of the PNPs or fluorescent PNPs tested resulted in significantly increased immobilization of the organisms at any of the test concentrations compared with the control samples. As a result, EC50 and NEC values could not be determined for any of the PNPs and FNPNs tested and must therefore be at concentrations above 500 mg/L (Table 2). In the reburial test conducted after the 10-d exposure period, there was no significant difference observed between PNPs and fluorescent PNPs-exposed organism and control organisms. Exposure to concentrations of PNPs and fluorescent PNPs ≤500 mg/L appeared to have no effect on reburial rates. Successful reburial is defined as occurring within 1 h of the test organisms being transferred to clean sediment and seawater. All organisms in the present study completed reburial within 1 h. No difference was observed between nonlabeled and fluorescently labeled PNP analogs in either test.

Uptake studies

Analysis of D. magna from the uptake study showed an intense blue fluorescence in the gut of the organisms after only 24 h of exposure (Figure 4A). Control organisms also exhibited a low-level natural blue fluorescence generally distributed...
across the organism but lacked the intense response from the gut region observed in organisms exposed to fluorescent PNPs (Figure 4B). The presence of fluorescent material in the gut of the exposed daphnids indicates rapid filtration of the fluorescent PNPs. After the standard 48-h exposure period, strong fluorescence was still observed in the gut of organisms exposed to the fluorescent PNPs (data not shown). However, after a recovery period of 24 h in clean media, no fluorescence was observed in the gut of organisms exposed to the fluorescent PNPs (Figure 4C), and the organisms appeared the same as the control organisms after 72 h (48-h exposure and 24-h depuration) in clean media (Figure 4D). This indicates that the fluorescent PNPs are quickly excreted by D. magna. The presence of fluorescent fecal material (Figure 4E) in the recovery flasks of those organisms that had been exposed to the fluorescent PNPs 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 represented a sublethal concentration.

**DISCUSSION**

The 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 have acute toxic effects at high concentrations. Naha et al. [15] investigated the acute ecotoxicity of N-isopropylacrylamide and 3 different ratios (85:15, 65:35, and 50:50) of N-isopropylacrylamide/N-tert-butylacrylamide copolymer PNPs to V. fischeri, D. magna, the freshwater algae P. subcapitata, and the freshwater shrimp T. platyurus. The PMMA and PMMA–PSMA PNP and fluorescent PNP EC50/LC50 and NEC values determined for V. fischeri in the present study are very similar to those observed for N-isopropylacrylamide and N-isopropylacrylamide/N-tert-butylacrylamide 85:15 (>1000 mg/L), indicating that PMMA-based PNPs and N-isopropylacrylamide are not acutely toxic. The PMMA PNP and fluorescent PNP EC50/LC50 and NEC values determined for D. magna in the present study are all >1000 mg/L, whereas the PMMA–PSMA PNP and fluorescent PNP exhibited 48-h EC50/LC50 and NEC values in the ranges of 879 mg/L to 887 mg/L and 407 mg/L to 524 mg/L, respectively. All N-isopropylacrylamide and N-isopropylacrylamide/N-tert-butylacrylamide PNPs exhibited 48-h EC50/LC50 and NEC values in the ranges of 413.6 mg/L to 60.6 mg/L and <250 mg/L to 50 mg/L, respectively [15]. Increasing toxicity was observed with an increasing amount of N-tert-butylacrylamide. The PMMA–PSMA PNP and fluorescent PNP EC50/LC50 and NEC values are comparable to those determined for N-isopropylacrylamide, indicating that these 2 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 PMMA–PSMA PNPs eliciting a response in D. magna, whereas the medium PMMA PNPs do not. The increased hydrophobicity of the PMMA–PSMA PNPs could be
increasing the uptake rate. Although it was not possible to quantify the uptakes rates for either PMMA–PSMA PNPs or PMMA PNPs, both appeared to be readily taken up and filled the gut of *D. magna*. It is therefore suggested that the presence of the stearyl methacrylate copolymer could be directly responsible for the toxic response offering an alternative surface chemistry to that of the PMMA PNPs. Furthermore, it appears there is no hindrance effect on toxicity from the presence of the large alkyl chain in the PMMA–PSMA PNPs, again supporting a chemical source for the observed toxicity. However, it should be noted that the observed toxicological differences may also be related to PNP size, with PMMA–PSMA being smaller (86 nm) than PMMA (125 nm).

In contrast to the present study and that of Naha et al. [15], Casado et al. [16] report that 55 nm and 110 nm of polystyrene-polyethyleneimine PNPs exhibited a strong toxic response for most of the species studied, except for *V. fischeri*, in which similar values of >1000 mg/L were observed. The polystyrene-polyethyleneimine PNPs exhibited 48-h EC50/LC50 values for *D. magna* in the range of 0.66 mg/L to 0.77 mg/L, with large particles (110 nm) exhibiting slightly higher responses than smaller particles (55 nm). These data indicate that polystyrene-polyethyleneimine PNPs are considerably more toxic to *D. magna* than any of the PNPs and fluorescent PNPs used in the present study or the *N*-isopropylacrylamide and *N*-isopropylacrylamide/N-tert-butylacrylamide copolymers studied by Naha et al. [15]. Naha et al. suggest a species sensitivity order for *N*-isopropylacrylamide as follows: *D. magna > T. platyurus > V. fischeri > P. subcapitata* [15]. This is consistent with the findings of the present study using PNPs and fluorescent PNPs, in which the sensitivity order is *D. magna > V. fischeri/C. volutator*. Similarly to the present study and that of Naha et al., *D. magna* was identified as one of the most sensitive species, although Casado et al. [16] found that *P. subcapitata* was the most sensitive species in their studies. The higher sensitivity of *D. magna* may be related to a different uptake route (filter feeding) than either *V. fischeri* (direct contact) and *C. volutator* (deposit feeder), or possibly a higher uptake rate through filter feeding. As toxicity toward *D. magna* was not observed for the PMMA PNPs but was observed for the PMMA–PSMA PNPs, it seems that the mode of toxic action is not related to a nutritional problem. Instead, the clear difference between the 2 PNP types indicates there is an intrinsic toxicity associated with the physicochemical properties of the PMMA–PSMA PNPs.

Nanoparticles are often stabilized in aqueous dispersion using a range of stabilizing agents [4,6,27,28]. In the present study, the test PNPs were synthesized using sodium dodecyl sulfate as a stabilizing agent. As a result, the surface of the PNPs and fluorescent PNPs were coated with the sodium dodecyl sulfate and there is potential for excess sodium dodecyl sulfate to be present in the exposure solutions. Therefore, the direct toxicity of sodium dodecyl sulfate must be considered within the context of the results obtained. Although the concentration of free sodium dodecyl sulfate in the exposure solutions is unknown, even at the highest concentrations of PNPs and fluorescent PNPs used in the present study (500–1000 mg/L), a toxic effect was only observed for the PMMA–PSMA PNPs and fluorescent PNPs and then only for *D. magna*. A previous study reports a 48-h LC50 value for sodium dodecyl sulfate with *D. magna* of 19.129 mg/L [29]. Bessling et al. [1] also provide toxicity data for sodium dodecyl sulfate to *D. magna* and the freshwater alga Scenedesmus obliquus. Because the corresponding PMMA PNPs and fluorescent PNPs did not result in a toxic effect, this indicates that the presence of any free sodium dodecyl sulfate is not influencing the observed toxicity and is therefore below the reported LC50 value of 19.129 mg/L. This should be the case, as the PNPs and fluorescent PNPs were carefully dialyzed after synthesis to remove as much free sodium dodecyl sulfate as possible. The observed acute toxicity in the present study appears to be related directly to differences in physicochemical properties of the PMMA and PMMA–PSMA PNPs.

Rapid uptake of the fluorescent PNPs into the gut of *D. magna* was observed after only 24 h (Figure 4A) and was still present after 48 h. This confirms that PNPs in the size range studied (86–125 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 fecal pellets (Figure 4E). The most likely route of uptake of PNPs and other engineering nanoparticles (ENPs) by *D. 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 setae on the thoracic limbs of *D. magna*, which is independent from age or size because the gap is constant [31]. *Daphnia magna* are able to actively filter particles as small as 200 nm, although this is an estimate based on the size of the gap between the setae [17]. In the present study, no significant mortality of *D. magna* was observed, indicating that the exposure concentration of 1 mg/L represented a sublethal concentration. Although uptake and excretion of fluorescent PNPs were both rapid, it is unclear from the resolution of the imaging technique employed if any of the fluorescent PNP materials were able to cross the gut wall into the organisms. Because there was no clear fluorescent response from the organisms following the depuration period, it is assumed that any transport of fluorescent PNPs across the gut wall is limited. Although the use of fluorescent labeling to study uptake of PNPs may be more limited for organisms without translucent bodies, the study of fecal material after transfer 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., polycyclic aromatic hydrocarbons and polychlorinated biphenyls) 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 to 2 orders of magnitude stronger than to PMPs [34]. Although 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 labeled 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 is best suited to organisms with translucent bodies such as the freshwater cladoceran D. 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 nonlabeled analog. 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 on the environmental behavior compared with nonlabeled analogs [4]. The present study included an assessment of the fluorescent dye on the ecotoxicity of the PNPs compared with the nonlabeled analogs. The data show that there is no significant difference between the fluorescently labeled and nonlabeled analogs, 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 labeling as a noninvasive tracking approach for PNPs in environmental samples.

CONCLUSIONS

The least sensitive model systems were the marine bacterium V. fischeri and the amphipod C. volutator, whereas the most sensitive was the 48-h immobilization of D. magna. In terms of response, the PMMA–PSMA PNPs and fluorescent PNPs appeared to show the greatest toxicity in the present study. We observe some differences in ecotoxicity between 2 differently functionalized PNPs, suggesting that surface chemistry may play an important role in influencing ecotoxicity. The results indicate that the ecotoxicity of PNPs cannot be reliably assessed using a single PNP type. Furthermore, the 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 a multitrophic approach. Importantly, the results in the present study indicate that none of the PNPs appear to elicit 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.

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Data availability—Data and calculation tools are available on request. Please contact A. Booth (andy.boo@sinnef.no).

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