

## Nanoplastic Affects Growth of *S. obliquus* and Reproduction of *D. magna*

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### Supporting Information

**ABSTRACT:** The amount of nano- and microplastic in the aquatic environment rises due to the industrial production of plastic and the degradation of plastic into smaller particles. Concerns have been raised about their incorporation into food webs. Little is known about the fate and effects of nanoplastic, especially for the freshwater environment. In this study, effects of nano-polystyrene (nano-PS) on the growth and photosynthesis of the green alga *Scenedesmus obliquus* and the growth, mortality, neonate production, and malformations of the zooplankter *Daphnia magna* were assessed. Nano-PS reduced population growth and reduced chlorophyll concentrations in the algae. Exposed *Daphnia* showed a reduced body size and severe alterations in reproduction. Numbers and body size of neonates were lower, while the number of neonate malformations among neonates rose to 68% of the individuals. These effects of nano-PS were observed between 0.22 and 103 mg nano-PS/L. Malformations occurred from 30 mg of nano-PS/L onward. Such plastic concentrations are much higher than presently reported for marine waters as well as freshwater, but may eventually occur in sediment pore waters. As far as we know, these results are the first to show that direct life history shifts in algae and *Daphnia* populations may occur as a result of exposure to nanoplastic.



### INTRODUCTION

Pollution with plastic is a growing concern in the marine environment.<sup>1</sup> However, emissions from land-based sources reach rivers first, and freshwaters provide an important source of marine plastic pollution through riverine transport.<sup>2</sup> Therefore, the occurrence of plastic in the freshwater environment receives increasing attention.<sup>3–6</sup> Special concerns exist with respect to nanoplastics because of their large surface area and hypothesized ability to penetrate cells.<sup>7–11</sup> Both primary particles from personal care and cosmetic products and secondary particles from degradation of larger plastic items are expected to contribute to pollution of the environment with nanoplastic.<sup>12</sup> Recent reports showed the importance of physical abrasion as a source of secondary micro- and nanoplastic.<sup>13,14</sup> Yet there are hardly any proven life history effects of micro- and nanoplastic on marine organisms, and effect data for freshwater organisms are lacking. For microplastic, the first reported data on effects on invertebrates relate to survival, feeding, oxidative status, and PCB uptake in lugworms (*Arenicola marina*).<sup>15–17</sup> In marine zooplankton, decreased feeding<sup>18</sup> and reduced survival and fecundity have been observed.<sup>19</sup> Even less is known about the effects of nanoplastic. For mussels (*Mytilus edulis*), an increased

pseudofeces production and reduced filtering activity have been reported.<sup>20</sup> For algae, nanoplastic has been shown to reduce CO<sub>2</sub> uptake and enhance the production of reactive oxygen species (ROS).<sup>21</sup>

As the interaction of organisms with pollutants in particulate form is completely different from that with conventional dissolved chemicals, there is a potential high risk associated with particles.<sup>7,22</sup> Given the limited data, there is an urgent need to quantify the effects of nanoplastic on freshwater organisms. Effects of nanoplastic may be related to particle toxicity, toxicity of plastic-associated chemicals, or both and will depend on the characteristics of the nanoplastic, such as particle size, polymer type, and age. However, previous research on nanoparticle behavior and effects was often conducted using pristine particles,<sup>23</sup> whereas aged and naturally altered particles are of higher importance considering environmental relevance, which will therefore be addressed in the present study.

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Plastic interacts with man-made organic compounds,<sup>24</sup> as studied for several kinds of pollutants and additives.<sup>16,24–26</sup> Recently, an exceptionally strong sorption of PCBs to nanoplastic was observed, which might imply a strong transport capacity including increased exposure upon penetration of cells or tissues.<sup>27</sup> Effects of nanoplastic might also be caused by direct particle toxicity, attachment to algae, reduction of light penetration, reduced food quality, release of additives, or interference with chemical communication. Here we hypothesize that nanoplastic might also interact with natural organic molecules such as kairomones, which may yield unforeseen effects on the interactions among species. *Daphnia* are known to express life history traits such as altered adult and/or neonate body size and altered neonate quantity in response to the presence of predator kairomones.<sup>28,29</sup> Sorption of kairomones to nanoplastic might disturb these life history traits.<sup>30</sup>

The aim of the present study was to investigate effects of nanoplastic at the first two trophic levels of the freshwater aquatic food chain; algae, represented by *Scenedesmus obliquus*, and zooplankton, represented by *Daphnia magna*. Both species are widely used for ecotoxicity tests. Nanosized polystyrene (nano-PS) spheres were used as the test substance, as polystyrene is one of the most widely used commercial plastics in the world and was used in earlier toxicity tests.<sup>15,20,21</sup> We investigated direct and indirect effects of a broad range of expected environmentally relevant and elevated concentrations of nano-PS in fresh water bioassays. The bioassay we present here is the first interaction bioassay of nano-PS combined with an interspecific organic molecule: fish kairomone. We took the interaction time between plastic particles and algae into account by using both pristine and aged dispersions of nano-PS, thereby providing novel information about the potential role of particle aging.

## ■ EXPERIMENTAL PROCEDURES

Bioassays were performed with algae (*S. obliquus*), and with *D. magna* fed with these algae.

**Organisms.** *Scenedesmus obliquus* SAG 276/3A was obtained from the University of Göttingen, Germany and was maintained in modified algal growth medium (WC-medium).<sup>31</sup> Stock cultures and the *Scenedesmus* bioassay were maintained similar to previous procedures at 20 °C in a climate chamber with 24 h continuous light ( $\sim 100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and 100 rpm rotational shaking.<sup>32</sup> Algae inoculum was prepared 3 days ahead of the *Scenedesmus* bioassay, to obtain exponential growth at the start of the test. *Daphnia magna* originated from lake Zwemlust, Nieuwesluis,<sup>33</sup> The Netherlands and were cultured in artificial growth medium (RT medium<sup>33</sup>) with a pH of 7.7–8.1. The *Daphnia* cultures and bioassay were kept at a temperature of  $21 \pm 1$  °C with the natural spring daylight regime (low beam day conditions  $<20 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). In the *Daphnia* bioassay two generations were used: (1) *Daphnia* of age  $<24$  h at the start, maturing during the bioassay, and (2) their offspring, i.e., neonates hatched while being in the bioassay. In all dispersions used in the *Daphnia* bioassay, *S. obliquus* served as food at approximately 0.36 mg carbon/*Daphnia*.

**Nano-PS Beads.** Polystyrene nanoparticle stocks were supplied as 20% nano-PS dispersion by AVT-PCC, Wageningen UR. The particles were synthesized from styrene monomers with sodium dodecyl sulfate (SDS) as surfactant and potassium persulfate as initiator.<sup>34</sup> SDS concentrations

were kept far below toxicity thresholds of *Daphnia*<sup>35,36</sup> and *Scenedesmus*. Absence of toxicity to *Scenedesmus* was confirmed in separate pilot tests with SDS, which are provided as Supporting Information (SI). Similarly, because of its hydrophobicity and high volatility with reported half-lives of 1–3 h in lake water,<sup>37</sup> presence of styrene monomers in the aqueous phase can be assumed negligible. The polystyrene beads had a primary nominal size of  $\sim 70$  nm (confirmed by transmission electron microscopy) and contained 0.01% on mass basis of the hydrophobic fluorescent dye (Nile Red), which was immobilized by the polymer matrix. Consequently, presence of Nile Red in the aqueous phase can also be assumed negligible, which is consistent with the use of Nile Red as a tracer in numerous studies of biological systems.<sup>38–40</sup> Furthermore, even if all Nile Red in the polystyrene would have been bioavailable, the concentration would still have been a factor  $1.5 \times 10^4$  below the effect concentration reported by Wu et al.<sup>41</sup> (Calculation provided as SI). To better represent nano-PS occurring in products and in the environment,<sup>42</sup> the spheres were functionalized with carboxylic acid groups. As the glass–liquid transition temperature of polystyrene<sup>43</sup> is much higher than the maximum temperature in our bioassay (21 °C), leaching of chemicals from the polymer matrix and therewith their occurrence in the exposure dispersions is negligible. The form of nano-PS in aqueous suspension was extensively characterized before (see SI Figure S1).<sup>27</sup>

**Scenedesmus Bioassay.** *Scenedesmus obliquus* were exposed to 44–1100 mg nano-PS/L in 80 mL of WC medium in a 72-h bioassay. Details about the used concentration range are provided as SI. Algae populations with an initial density of approximately  $3 \times 10^6$  cell/mL were used. A growth inhibition test was performed three times with controls in 6-fold and nano-PS treatments in triplicate.<sup>35,44</sup> Cell densities were determined using a CASY counter (CASY model TT, INNOVATIS) at the start and after every  $24 \pm 1$  h. At the end of two of the bioassays, Chlorophyll-a (Chl-a) was extracted and determined by spectrophotometry (Beckman Coulter, DU 730 Life Science UV/vis) to assess photosynthetic capacity and biomass following a hot ethanol extraction method with phaeopigment correction.<sup>45</sup>

**Daphnia Bioassay.** *Daphnia magna* were exposed individually to 80 mL nano-PS test dispersion in a 21-day bioassay, according to OECD guidelines.<sup>46,47</sup> Four types of nano-PS test dispersions were tested, which are referred to as (1) pristine, (2) pristine-kairomone, (3) aged, and (4) aged-filtered (Figure 1). (1) Pristine refers to the treatment where the exposure of the *Daphnia* started immediately after mixing algae and nano-PS. Nano-PS dispersions were dilutions of nano-PS stock in RT medium to which algae were added just before use in the bioassay. Pristine exposures were applied at ten nanoplastic concentrations in the range of 0.22–150 mg nano-PS/L. Details about the used concentration ranges are provided as SI. (2) For the pristine-kairomone dispersions, the only difference from the pristine dispersions was the presence of fish kairomones in the initial RT medium. Fish kairomones were kindly obtained from a parallel study at our university, where three individuals of *Perca fluviatilis* (total overall length  $\pm 12$  cm) were inhabited in 20 L of aerated RT medium for a week. *Perca fluviatilis* is a predator known to induce life history responses in *Daphnia*.<sup>48,49</sup> Three times a week, the fish were fed with *Daphnia*. Before use in the *Daphnia* bioassay, the RT medium with fish kairomones was filtered over a 0.45-μm membrane filter (Whatman cellulose nitrate membrane, grade

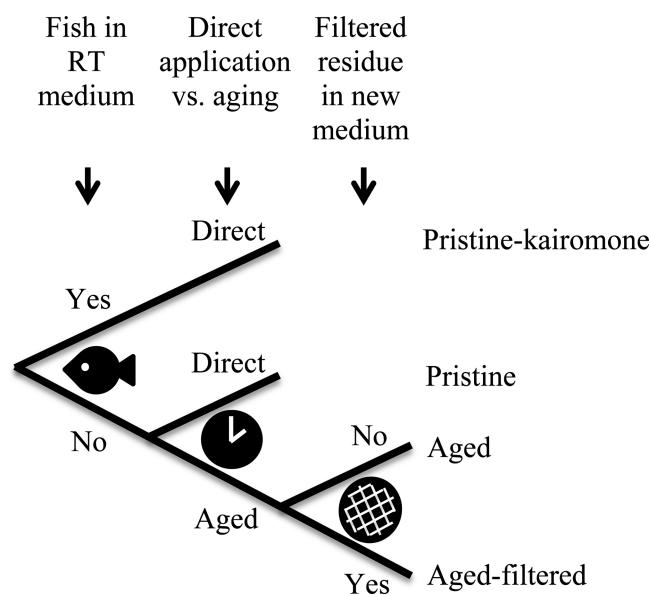


Figure 1. Visualization of the four different types of test dispersions. All dispersions contain RT medium with nano-PS and algae.

NC45). The pristine-kairomone dispersions were applied at concentrations of 0.88 and 1.8 mg nano-PS/L. (3) The aged dispersion was prepared in the same way as the pristine dispersions, the only difference was that the aged dispersions were not used immediately after addition of the algae, but instead aged at the conditions used for stock cultures (see Organisms) for 5 days. The aged treatment was applied at one concentration; 32 mg nano-PS/L. (4) The aged-filtered dispersion was made in the same way as the aged dispersion at the same nanoplastic concentration. Thereafter, it was further processed as follows: the algae were separated from the water phase by filtering over a 1.2- $\mu\text{m}$  glass fiber filter (Whatman GF/C). The residue was rinsed from the filter with new RT medium and the new RT medium was applied in the bioassay. As controls, original RT medium and RT medium with fish

kairomones were included, both with algae, but without nano-PS. The control treatment with original RT medium without nano-PS was replicated 16-fold, and all other treatments were replicated 12-fold. The four dispersion types enabled us to make various mechanistic comparisons. For instance, comparison of pristine with pristine-kairomone enabled revealing interaction between plastic and kairomones. Comparison of pristine with aged allowed showing the consequences of aging of the nano-PS dispersions on the *Daphnia*. The difference in preparation between aged and aged-filtered reduced the relative importance of aquatic exposure to plastic of the *Daphnia*. Although it can be assumed that the functionalized nano-PS stays dispersed,<sup>27</sup> the replacement of the aged aquatic phase by fresh RT medium in the aged-filtered dispersion allows a check on the relative importance of the (nano-PS absorbed to) aged algae being an exposure route. *Daphnia* were transferred to glass tubes with 80 mL of new medium three times a week. *S. obliquus* is known as a good food source for *Daphnia*.<sup>50</sup> Survival of *Daphnia* was checked and reproduction was counted on a daily basis. Body size<sup>51</sup> of both adult and neonate *Daphnia* was measured and number of malformed neonates was counted using a stereobinocular (Nikon SMZ-10, magnification 0–40). During the bioassay, three times a week, water quality was measured in a randomly chosen replicate of each treatment. On average the pH was  $7.80 \pm \text{SE } 0.015$ , oxygen concentration was  $8.80 \pm \text{SE } 0.012 \text{ mg/L}$ , and conductivity was  $296.92 \pm \text{SE } 0.71 \mu\text{S/cm}$ , thereby being within the range of the guideline.

**Data Analysis.** Algae growth inhibition rates were derived from cell density over time according to ISO guidelines,<sup>52</sup> by using nominal initial cell densities. *Daphnia* population growth rates ( $r$ ) were estimated from Euler–Lotka's equation.<sup>51</sup> Statistical analyses were performed with R statistical software (R Development Core Team) by 2-way ANOVA, (multiple) Linear regression, Kruskal–Wallis and Nemenyi–Damico–Wolfe–Dunn (NDWD) tests with  $\alpha = 0.05$ .

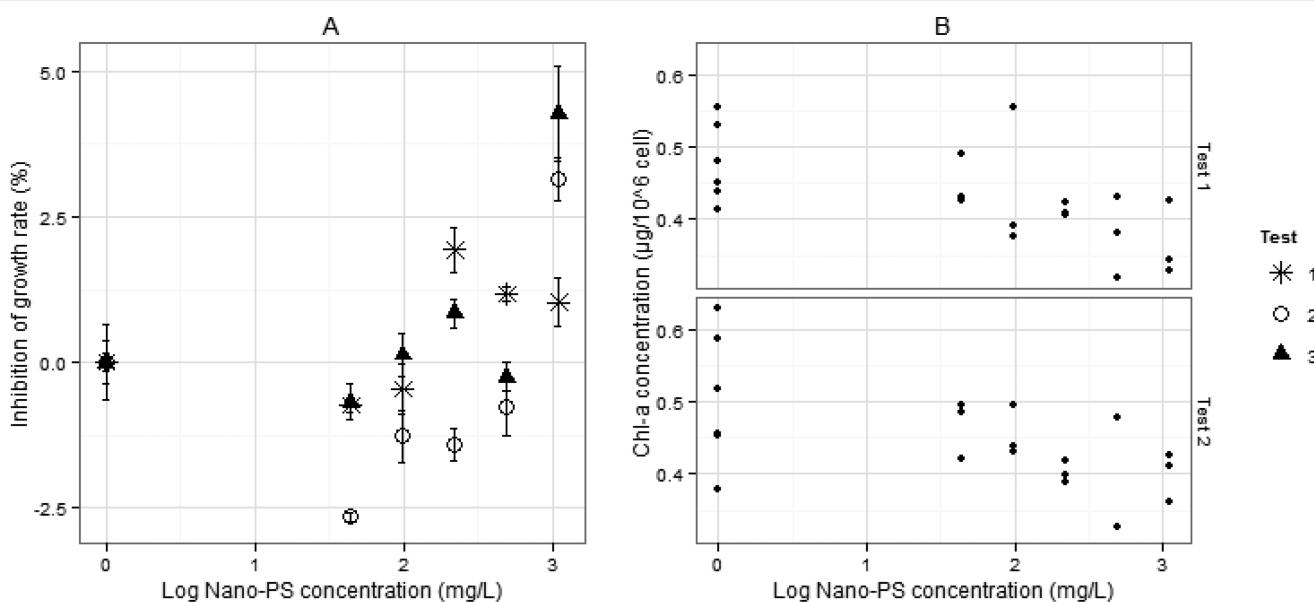
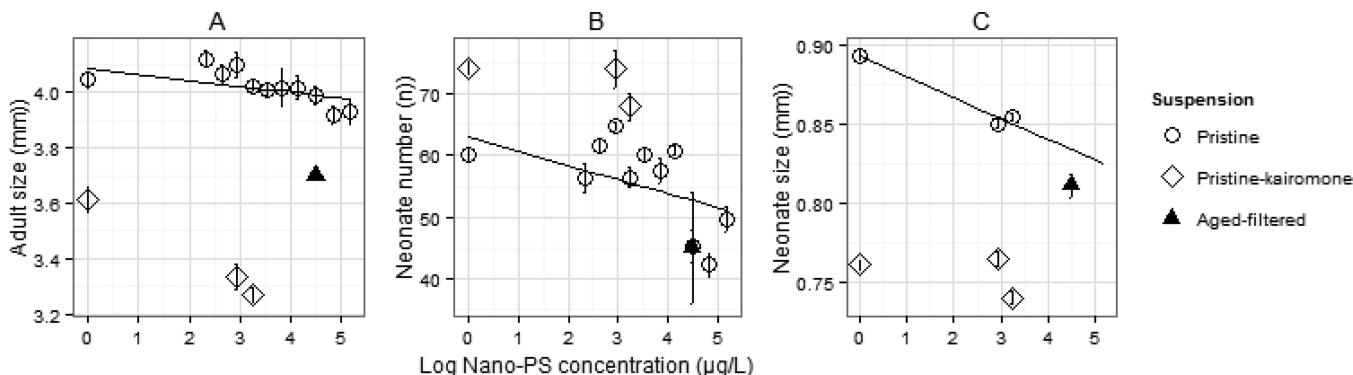


Figure 2. Nano-PS effects on *Scenedesmus obliquus*. Panel A: Inhibition of the growth rate (%) as a function of nano-PS concentration after 72 h of exposure. Panel B: Upper part: test 1, lower part: test 2. Chl-a concentration/ $10^6$  cell as a function of the 72-h nano-PS exposure.



**Figure 3.** Nano-PS effects on *Daphnia magna*. Panel A: Body size of adult *Daphnia* after 21 days exposure to nano-PS. B: Reproduction quantity, number of neonates produced by *Daphnia* that were exposed to nano-PS. C: Body size of neonates produced by *Daphnia* that were exposed to nano-PS. Controls without added nano-PS are depicted on the x-axis as log plastic concentration = 0.

## RESULTS AND DISCUSSION

**Effect of Nano-PS on Growth and Chlorophyll-*a* of *S. obliquus*.** We performed three bioassays with the green alga *Scenedesmus obliquus* and show that exposure to nano-PS leads to inhibition of growth (Figure 2A) and to reduced Chl-*a* levels in the cells (Figure 2B). As far as we know, these are the first direct negative effects of nanoplastic on algal populations established. The growth inhibition had limited magnitude, yet the increased growth inhibition with increasing nano-PS concentration was statistically significant and did not differ among the three tests (2-way ANOVA, plastic treatment significant, *p*-value = 0.013). At a high nano-PS concentration of 1 g/L there was approximately 2.5% growth inhibition of *S. obliquus*. The negative relationship between nano-PS concentration and Chl-*a* concentration is similar for both tests and statistically significant, although the variability within controls and nano-PS treatments is high, and below 100 mg nano-PS/L no reduced Chl-*a* concentration is expected to occur. The negative relationships of growth and Chl-*a* with nano-PS concentration are independent, as after correction for cell density, Chl-*a* concentration remains significantly negatively related to nano-PS concentration (2-way ANOVA, plastic treatment significant, *p*-value =  $5.1 \times 10^{-5}$ ). Previous research showed absorption of nanoplastic by algae and indications of reduced algal health, i.e. reduced CO<sub>2</sub> uptake observed at concentrations higher than 1.8 mg/L and promoted production of ROS.<sup>21</sup> Our present results add that also direct effects of nanoplastic on algal growth and Chl-*a* levels may occur. Before, it was suggested that shading by plastic might cause the observed effects on CO<sub>2</sub> uptake and production of oxygen species.<sup>21</sup> However, as shading is known to cause an upward correction of the Chl-*a* level in cells,<sup>53</sup> our findings of a Chl-*a* reduction with increasing nano-PS concentration contradict this suggestion. Thereby, although at relatively high concentrations, our novel observed reduction in Chl-*a* implies that another mechanism is at work, which may help to direct further mechanistic effect research. Note that we do not fully distinguish here between the possible mechanisms explaining the toxicity of nano-PS, to which direct nanoparticle toxicity and effects of nano-PS associated-chemicals such as styrene, may contribute.

**Effects on Survival and Body Size of *D. magna* by Nano-PS. Effects of Pristine and Aged Nano-PS Dispersions.** Across the treatments, the *Daphnia* mortality ranged from 0 to 100%, with an average of 27%. The mortality of 18.8% in the

control groups was within the limit set by the OECD guidelines 2008.<sup>47</sup> Pristine suspensions of nano-PS were not lethal to *D. magna*, but the aged dispersions were (Kruskal-Wallis; NDWD test: aging sign, *p*-values  $\leq 0.014$ ). Aging of algae with plastic caused a 4.4–6 times higher mortality in *Daphnia* as compared to a diet without pre-exposure of the algae. Several explanations for this difference in mortality can be considered. First, in the aged-filtered treatment, after the exposure of the algae the plastic was removed from the water phase using a glass fiber filter. Some release of glass fibers into the aged-filtered treatment was observed and it may be speculated that this contributed to the mortality in this treatment. However, in the aged treatment no glass fiber filter was used and a similar mortality was observed, which renders the speculation less likely. Second, the higher mortality could relate to a plastic treatment effect implying that the pre-exposed algae adsorbed nano-PS, thereby being the route for exposure of the *Daphnia* resulting in an elevated mortality. An enhancement of the uptake via food might be the explanation for the six times higher mortality compared to pristine exposure when the aged dispersion was used. Whereas in pristine dispersions, nano-PS mainly resided in the water medium, nano-PS might be absorbed to the *S. obliquus* in aged dispersions, thereby changing the main uptake route or degree of exposure. A third explanation could be that although the presence of aqueous-phase styrene is unlikely, aging may enhance the transfer of styrene monomers from the nano-PS<sup>54</sup> into algae, thus increasing the bioavailability of styrene. It is very important to take the effect of aging and plastic associated chemicals into account in the risk identification of nanoplastic, as this affects the outcomes of bioassays as well as the comparability with environmental conditions.

**Interacting Effects of Kairomones and Nano-PS on *Daphnia*.** A 10.7% reduction in *Daphnia* body size due to kairomones was observed in our bioassay (Figure 3A). The reduction in *Daphnia* body size due to kairomones only was also observed by Hanazato and Dodson<sup>28</sup> and Riessen<sup>29</sup> and was explained by differences in survival strategy with/without predator presence. From the *Daphnia* that were treated with aged dispersions, not enough individuals survived to consider body size as a representative end point. The presence of plastic also had a negative effect on body size with up to 3.1% reduction in length. The significance of the term accounting for interaction between nano-PS and kairomones implies that with kairomones present, the body size reduction with nano-PS concentration is stronger than without kairomones (Figure 3A)

(Multiple linear regression:  $\log(\text{nano-PS})$ , kairomones as well as the interaction between them were significant,  $R^2_{\text{adj}} = 0.80$ ,  $p$ -values  $<1.7 \times 10^{-3}$ ). At a concentration of 1.8 mg nano-PS/L, interaction with kairomones reduced the body size by up to 18.9%. This might constitute an additive negative effect of both kairomones and nano-PS or an interaction between nano-PS and kairomones. For example, the presence of nano-PS could change the exposure concentration of kairomones in water, the uptake route for kairomones, or the susceptibility of *Daphnia*, resulting in an altered growth reduction. This possible nano-PS interference with kairomones is the first report of an effect of plastic on chemical communication among organisms. This hypothesis of an increased kairomone effect might be less relevant for other, for instance more hydrophobic, kairomones, which implies that more studies on these interactions are recommended.

**Effects on Reproduction and Neonate Malformations of *D. magna*.** Effects on life history traits of aquatic organisms often provide sensitive metrics for ecological stress or chemical toxicity. To determine the effect of nano-PS on reproduction, we investigated the neonates produced by the exposed adult *Daphnia*. Only those replicates where the adult *Daphnia* survived the bioassay were included in the analyses of neonate number and size. The total number of neonates produced in the first three broods in the control without kairomones was  $53.4 \pm \text{SE } 18.9$  and in the control with kairomones was  $55.7 \pm \text{SE } 33.7$ . Exposure to nano-PS in the pristine treatments reduced the cumulative number of neonates in the first three broods (Figure 3B). A slightly lower decrease in *Daphnia* neonate quantity was observed in the presence of kairomones (multiple linear regression, nano-PS, kairomones and interaction significant,  $R^2_{\text{adj}} = 0.52$ ,  $p$ -value  $<10^{-16}$ ). The overall neonate number per surviving adult was 19.4% higher with kairomones present, which is consistent with previous findings.<sup>29,48</sup> Neonate number was also significantly related to adult body size, although it had a lower significance than nano-PS and kairomones. Multiple linear regression performed with the explanatory variables nano-PS, kairomone (interaction), and adult body size had an adjusted  $R^2$  of 0.53 ( $p$ -value  $<10^{-16}$ ). Population growth rates ( $r$ ) were in the range of 0.23–0.55 day<sup>-1</sup>, with  $r = 0.23$ –0.42 day<sup>-1</sup> for aged dispersions, 0.44–0.45 day<sup>-1</sup> for dispersions with kairomones, and 0.46–0.55 day<sup>-1</sup> for pristine dispersions. Replication of the bioassay would allow calculations of significant differences in population growth rates between treatments.

From the first three broods, a random selection of 16 neonates per treatment was subjected to body size measurements. The body size of the neonates was negatively affected by the nano-PS concentration, and aging of dispersions enhanced this effect. Neonates of *Daphnia* exposed to kairomones were much smaller, but further reduction in body size caused by nano-PS was smaller than that in the pristine treatment (Figure 3C) (multiple linear regression,  $\log(\text{plastic})$ , kairomones and interaction significant ( $R^2_{\text{adj}} = 0.93$ ,  $p$ -value = 0.035). Former research on *Daphnia* showed that a trade-off between clutch size and neonate body size exists. Low food availability results in fewer but larger neonates, whereas small mature *Daphnia* or the presence of fish kairomone causes a greater number of smaller neonates,<sup>48</sup> the latter mechanism being confirmed in our bioassay. It has been demonstrated that the overall maternal investment often decreases by exposure to pollutants.<sup>55</sup> Here we show that nano-PS reduces both clutch and

neonate body size, thereby acting as a stressor similar to other contaminants.

Although low numbers (< 2.5%) of malformations were observed in the control treatment and the lower range of Pristine treatments, from 32 mg nano-PS/L onward elevated numbers of malformed neonates were observed (Figure 4) and

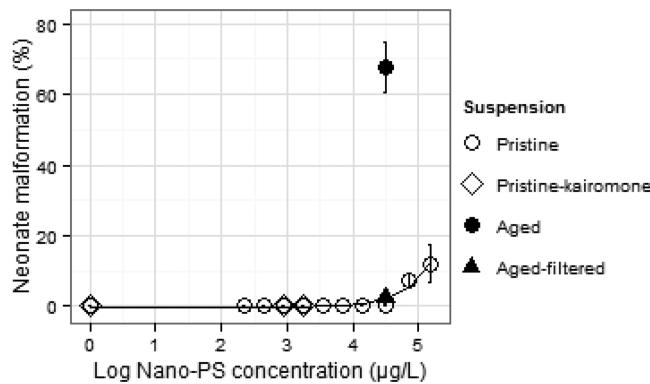


Figure 4. Percentage occurrence of malformations in neonates that were produced by *Daphnia* during 21 days exposure to nano-PS.

a factor 2.2–4.9 increase in plastic caused a 7–12% increase in the occurrence of malformations. When exposed to aged dispersions the occurrence of malformations increased radically, by 67% compared to the pristine treatment at 32 mg nano-PS/L (multiple linear regression plastic, aging and interaction significant ( $R^2_{\text{adj}} = 0.81$ ,  $p$ -value  $<10^{-16}$ )). This increase also implies once more that the malformations are not due to any initially present cocontaminant (e.g., styrene, SDS, or Nile Red) because these were used in the pristine treatment, too. For the analyses of malformation occurrence all neonates were taken along, including those of adult *Daphnia* that did not survive the exposure. Nano-PS affected several developmental stages of *Daphnia* neonates, as different malformation types were observed (Figure 5). In order of decreasing occurrence they were internal vacuoles, shortened antenna and lump in carapace, altered tail spine. The normal embryonic development of *Daphnia* consists of six stages, i.e. cleavage, gastrulation, early embryonic maturation, midembryonic maturation, late embryonic maturation, and fully developed neonate.<sup>56</sup> In our study we observed a considerable number of neonates with incomplete developed second antennae setae and curved tail spines (Figure 5). These malformed neonates were mainly observed in the high nano-PS exposures, especially in the aged treatment. This indicates disruption of one or more embryonic development stages from the stage of midembryonic maturation onward, as the second antenna (including setae) and tail spine are developed and extended in these stages.<sup>56</sup> Malformed tail spines and incomplete developed antennae setae have been reported to occur in *D. magna* exposed to cyanobacterial toxins, mercury, and a mixture of clofibric acid and fluoxetine.<sup>57–59</sup> We did not find any report of styrene or related compounds causing such malformations. Also, to exceed the 1.9 mg/L NOEC of styrene for *Daphnia*,<sup>37</sup> in the treatment with highest malformation occurrence, 6% of the nano-PS in that aged treatment would need to be monomer styrene in suspension. That is an unlikely high percentage. As mentioned before, given the rapid volatilization of styrene,<sup>37</sup> it is even less likely that such concentrations occurred during our bioassay. In contrast to the aged treatment, effects of the aged-filtered



**Figure 5.** Malformations in different developmental stages of *Daphnia* neonates. Top-right: incomplete developed antenna setae, curved shell spine and vacuoles around ovary. Top-middle: lump in the carapace. Top-left: normal developed neonate. Bottom-right: short antenna setae. Bottom-left: normal developed antenna setae. The arrows depict malformed body parts.

suspension did not significantly differ from those of the pristine and pristine-kairomone treatments. Thereby, it validates our assumption that the nano-PS did not aggregate with the algae to such an extent that only exposure via algae (aged-filtered) would cause effects equal to combined aquatic and algal exposure (aged). Although not irreversibly bound to algae, a change in allocation of the nano-PS in the dispersion with algae might however have changed due to aging and thereby influenced the extent of exposure. The occurrence of a lump in neonates' carapaces (Figure 5, top-middle), has not been observed in our laboratory before and we are not aware of any other publications on this type of malformation. The color of the observed lumps was similar to the nano-PS color and might indicate accumulation of nano-PS in neonates. Solubilization of carbon nanotubes with polymers and by fouling is thought to enhance the uptake in biological systems,<sup>60,61</sup> implying that polymer nanoparticles are likely to reach these systems, too. The observed malformations might relate to the alteration of membrane properties found by Rossi et al.<sup>62</sup> We recommend further, detailed histological study for direct evidence of uptake and transfer of nanoplastic from *Daphnia* adults to offspring.

## IMPLICATIONS

These bioassays are the first to show that direct life history responses in algae and *Daphnia* populations can occur as a result of exposure to nanoplastic. We observed 67.7% malformed offspring at exposure to 32 mg nano-PS/L aged nano-PS. For pristine nano-PS, 0.3% of the offspring malformed at a concentration of 32 mg nano-PS/L and 12.1% malformed at 155 mg nano-PS/L. These thresholds are a factor  $10^6$  higher than the 0.04–34 ng/L microplastic concentrations found in fresh water in Europe and USA, and a factor 100 higher than the highest reported microplastic concentration in marine water,<sup>3,39,63–65</sup> based on reported densities of  $7.9 \times 10^{-5}$  n/L to  $6.8 \times 10^5$  n/km<sup>2</sup>, an estimated trawling depth of 0.01 m, and an average particle weight of 5

µg/particle.<sup>66</sup> Environmental concentrations of microplastics in sediment reach up to 81 mg/kg dry weight,<sup>67</sup> which, with a sediment density of 2 kg/L and a water content of 50% on mass basis, would equate to a concentration in pore water of 162 mg/L. Assuming that microplastics degrade into nanosized plastic particles in the environment, for organisms inhabiting porewater or the sediment–water interface<sup>68</sup> these environmental concentrations exceed the observed effect thresholds for nano-PS. Furthermore, effects of plastic should not be considered in isolation. Other anthropogenic stressors are known to cause similar effects on reproduction, including malformations.<sup>57–59,69</sup> The relevance of the present findings therefore does not only follow from the environmentally relevant plastic concentrations or those anticipated in the near future, but merely from the joint effects of multiple stressors per category of responses. Plastic simply adds to the stress already existing from traditional contaminants and therefore make organisms less tolerant and more vulnerable to additional stressors. This implies that the effects identified in this study may in general reduce the resilience of aquatic ecosystems.

## ASSOCIATED CONTENT

### Supporting Information

Background information on the bioassays and pilot SDS tests, calculation on availability of Nile Red in the bioassay, and TEM image of the nano-PS. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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### Notes

The authors declare no competing financial interest.

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