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Environmental impact of nanoplastics from fragmentized consumer plastics

Final project report

Mikael T. Ekvall, Jing Hua,
Egle Kelpsiene, Martin Lundqvist,
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Preface

This report entitled *Environmental impact of nanoplastics from fragmented consumer plastics* presents the results of a research project on the effect of nanoplastics which is one of five funded projects within the call Microplastics from 2018. The research results from this call aim to increase knowledge about microplastics and their sources, transmission routes, ecological effects and consequences and how measures can help reduce them.

The project has been funded with funds from the Swedish Environmental Protection Agency's environmental research grant to support the Swedish Environmental Protection Agency's and the Swedish Agency for Marine and Water Management's knowledge needs.

This report is written by Mikael T. Ekvall, Jing Hua, Egle Kelpsiene, Martin Lundqvist, and Tommy Cedervall at Lund University. The authors are responsible for the content of the report.

The Swedish Environmental Protection Agency, July 2022

Maria Ohlman
Head of Sustainable Development Department

Förord

Denna rapport med titeln *Environmental impact of nanoplastics from fragmentized consumer plastics* presenterar resultaten av ett forskningsprojekt om effekter av nanoplast, som är ett av fem beviljade projekt inom utlysningen Mikroplaster från 2018. Forskningsresultaten från denna utlysning syftar till att öka kunskapen om mikroplaster och deras källor, spridningsvägar, ekologiska effekter och konsekvenser och hur åtgärder kan bidra till att reducera dem.

Projektet har finansierats med medel från Naturvårdsverkets miljöforskningsanslag till stöd för Naturvårdsverkets och Havs- och vattenmyndighetens kunskapsbehov.

Denna rapport är författad av Mikael T. Ekvall, Jing Hua, Egle Kelpsiene, Martin Lundqvist, och Tommy Cedervall på Lunds universitet. Författarna ansvarar för rapportens innehåll.

Stockholm, juli 2022

Maria Ohlman
Chef för Hållbarhetsavdelningen

Content

Abbreviations

ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
BDVF	breakdown sample, containing molecules that are larger than 10 kDa
DCS	Differential Centrifugation Sedimentation
DLS	Dynamic Light Scattering
FT	molecules (smaller than 10 kDa) after filtration with the VivaFlow device
HDPE	High Density Polyethylene
LDPE	Low Density Polyethylene
MWCO	Molecular Weight Cut Off
NTA	Nanoparticle Tracking Analysis
PE	Polyethylene
PEBD	Breakdown material after filtered through 0.8 µm syringe filters
PET	Polyethylene Terephthalate
PEVF	concentrated nanoparticles (using a 10 kDa cut off filter in Vivaflow device)
PLA	Polylactic acid
PS	Polystyrene

Summary

Misplaced plastics is an ongoing environmental problem. The breakdown of plastics into smaller pieces, microplastics, likely cause additional environmental burdens as they affect animals and plants at the beginning of the food chain. This may be even more true for the smallest of microplastics: the nanoplastics as they will behave differently in nature and interact in new ways with organisms and potentially be taken up by the organisms and affect internal organs. The small size of nanoplastics and their chemical resemblance with the surrounding environment makes them difficult to find, isolate and study. Most of what is known about nanoplastics behaviour in nature and their effect on nature derives from studies using commercially available polystyrene nanoparticles. These are probably different in many ways, such as structure, surface chemistry, and size distribution, compared to nanoplastics broken down in nature from plastic debris. Despite this, we have used polystyrene nanoparticles to study knowledge gaps. The toxicity to zooplankton *Daphnia magna* (*D. magna*) of small positively charged amine-modified polystyrene nanoparticles is not affected by protein-induced aggregation. All tested polystyrene nanoparticles were toxic to *D. magna* regardless of their toxicity in acute tests. Proteins bound to polystyrene nanoparticles after filtration by *D. magna* were different on acutely and non-acutely toxic particles which may imply different mechanisms behind the toxicity. In order to study the effect of nanoplastics that resemble what can be expected in nature we have mechanically broken down 8 different plastics and rubbers from 14 different consumer products and isolated the nanoplastics. Careful characterization shows that the nanoplastics are irregular in shape, have a slightly negative surface charge, and often have a strongly oxidized surface compared to the starting material. The nano-sized fractions are not toxic to *D. magna* in the used concentrations. In contrary, for at least two plastics High Density Polyethylene (HDPE) and Polylactic acid (PLA) the nanoplastics increase the lifetime of the *D. magna* probably because the nanoplastics can be utilized by bacteria which in turn serve as additional food for the zooplankton. However, leached additives and/or smaller polymers from HDPE are toxic to *D. magna*. We have also seen that UV irradiation further degrade polystyrene nanoparticles. The bacterial growth and the UV breakdown may imply that the nanoplastics break down faster than believed in nature and that they with time may disappear.

Sammanfattning

Plastskräp i naturen är ett växande miljöproblem. Nedbrytningen av plast till mindre bitar, mikroplaster, orsakar sannolikt ännu större miljöpåverkan eftersom små plastbitar påverkar djur och växter långt ner i ekosystemet. De allra minsta mikroplasterna, nanoplasterna, är mindre än cirka 100 nm. De beter sig annorlunda i naturen. De interagerar på nya sätt med organismer och kan potentiellt tas upp av organismerna och påverka inre organ. Nanoplasternas ringa storlek och deras kemiska likhet med den omgivande miljön gör dem svåra att hitta, isolera och studera. Vi har därför delvis använt oss av modellpolystyrennanopartiklar för att studera kunskapsluckor. Alla testade nanopartiklar av polystyren var giftiga för djurplanktonen *D. magna* i livstidstester oavsett deras toxicitet i akuta tester, vilket tyder på olika mekanismer för akut toxicitet jämfört med långtidstoxicitet. Aggregering av nanoplaster skulle kunna minska den storleksberoende påverkan men små positivt laddade aminmodifierade polystyrennanopartiklar var lika akut toxiska för *D. Magna* före och efter proteininducerad aggregering. Olika proteiner band till partiklar som var akut toxiska jämfört med partiklar som inte var toxiska. Detta skulle kunna vara en ledtråd till vilka mekanismer det är som ligger bakom toxiciteten. För att studera effekten av nanoplaster som bättre liknar vad som kan förväntas finnas i naturen har vi mekaniskt brutit ner 8 olika plaster och gummin från 14 olika konsumentprodukter och isolerat nanoplasterna. Noggrann karakterisering visar att nanoplasterna är oregelbundna i formen, har en ytladdning som är något negativ och ofta har en starkt oxiderad yta jämfört med utgångsmaterialet. Fraktionerna som innehåller partiklar i nanostorlek är inte giftiga för *D. magna* i de använda koncentrationerna, varken i akuta eller livstidstester. Tvärtom, för minst två plaster HDPE (high density polyethylene) och PLA (polylactic acid) ökar närvaron av nanoplasten livslängden på *D. magna*, förmodligen för att nanoplasten kan utnyttjas av bakterier som i sin tur fungerar som extra mat för djurplanktonen. Urlakade tillsatser och/eller mindre polymerer från HDPE är dock giftiga för *D. magna*. Vi har också sett att UV-bestrålning ytterligare bryter ned polystyrennanopartiklar. Bakterietillväxten och UV-nedbrytningen kan innebära att nanoplasterna bryts ner snabbare än man trott i naturen och att de med tiden kan försvinna.

1. Introduction

1.1 Breakdown of plastics in nature

Today, plastics are considered the most used and versatile materials of the modern age (Bakir, et al., 2012). Plastic is a great material to work with because it is durable and strong. But due to these properties, plastic degrades quite slowly. Most plastics do not degrade but rather fragment into smaller (micro- and nano-sized) particles from larger plastic pieces, (Zhang, et al. 2021), which is a continuous process in nature. But information on how this happens is incomplete. Plastic waste, misplaced in nature, is likely to become a potential source of smaller and smaller pieces and even degrade into molecular components. For example, styrene oligomers, probably from polystyrene waste, are found in many places around the world (Kwon et al., 2014, 2015, 2017). Small plastic pieces below 5 mm are defined as microplastics and the smallest fraction of the microplastics are sometimes called nanoplastics. There are two different definitions of nanoplastics. Either they should be below 1 µm or 100 nm in diameter (Gigault et al., 2021). The latter definition adheres to the broadly accepted definition of a nanoparticle. Furthermore, size dependent effects are often more distinctive at sizes below 100 nm. Microplastics are divided into two groups – primary and secondary microplastics. Primary microplastics are the ones that are intentionally manufactured, whereas secondary microplastics result from the breakdown of larger plastic pieces. Nanoplastics can be divided in the same way, but it has been suggested that nanoplastics are only from degraded plastics (Gigault et al., 2021), but in accordance with microplastics rubber could be included in the definition. The degradation of plastic is a combination of physical and chemical processes, which involve photodegradation, oxidation, hydrolytic degradation, biodegradation, and mechanical disintegration (Luengo et al., 2006, Eubeler et al., 2010, Yousif et al., 2013, Cao et al., 1999). Nanoplastics, in contrast to larger microplastics, are due to their small size and their chemical composition difficult to detect in nature. Although, they have been found in the Atlantic Ocean (Ter Halle, et al., 2017).

1.2 Polystyrene nanoparticles as a model for nanoplastics

Due to the small size of nanoplastics, they will likely have a different biological effect than larger microplastics. These size dependent features have been reviewed, (see for example Mattsson et al., 2015, Chae and An, 2017, Larue et al., 2021). However, almost all we know of the effect of nanoplastics derive from studies using commercial polystyrene nanoparticles. These polystyrene nanoparticles are in many ways not as we expect fragmentized nanoplastics to be. Most obviously nanoplastics will come from many different plastics not only polystyrene. Moreover, the polystyrene nanoparticles are almost perfect spheres, and they are often surfaced modified with carboxyl or amine groups. Especially, the later positively charged group is unlikely formed in natural processes. The polystyrene nanoparticles are often

delivered in solutions containing stabilizing compounds such as Tween and anti-bacterial compounds as sodium azide. If not handled with care these compounds will affect the biological response which has been reviewed (see Pikuda, et al., 2019, Heinlaan, et al., 2020, Kelpsiene et al., 2022). Even so, studies with polystyrene nanoparticles have given us an insight into what nanoplastics can do. The toxicity to zooplankton is dependent on size and surface charge, as small and positively charged particles are acutely more toxic (Mattsson et al., 2017, Saavedra et al., 2019, Pochelon et al., 2021). The increased toxicity of positively charged polystyrene nanoparticles may be due to a strong interaction between a positively charged nanoparticle surface and a negatively charged Daphnia cell membrane (Nasser and Lynch 2016). Although size and charge effect may not be as important in life-time toxicity tests (Kelpsiene et al., 2020). The polystyrene nanoparticle can also, for example, accumulate in the gut (Jemec, et al. 2016), reduce reproduction (Besseling et al., 2014, Rist, et al. 2017), and travel through a food web from algae to zooplankton to fish and affect the metabolism and behaviour of the fish (Mattsson et al, 2017). Trophic transfer and behavioural changes are also shown in a four species food chain with an additional predatory fish as the end consumer (Chae et al., 2018)

1.3 Nanoplastics in the literature

The difficulties with trying to find or collect nanoplastics in nature have led to several studies in which plastics are artificially broken down to nano-size. Another approach is to try to isolate nanoplastics leached from plastic products. Polystyrene (PS), Polyethylene Terephthalate (PET), and Polyethylene (PE) microplastics have been broken down into nanoplastics by different kinds of mechanical forces (Ekvall, et al., 2019, Mekaru, 2020, Ji et al., 2020), PET has also been broken down by laser ablation (Magri et la., 2018), and PS nanoplastics has been observed after UV irradiation (Lambert and Wagner, 2016). A combination of thermal oxidation and soft mechanical forces on expanded PS resulted in nanosized particles (Mattsson et al., 2021) Another approach is to isolate nanoplastics from leachate which has been done on nylon tea bags (Hernandez et al., 2019) and on a mix of daily used plastic products (Xu et al, 2020). The characterization of the nanoplastics is challenging also in an experimental environment and only in a few cases the chemistry and shape of the observed nanoplastics have been asserted (Magri et al., 2018, Ekvall et al., 2019, Hernandez, et al., 2019). In general, the nanoplastics are chemically different from the starting material due to oxidation and the shape is irregular differing them from the commercially available polystyrene nanoparticles described above.

2. Methods

2.1 Model polystyrene nanoparticles Toxicity testing on *Daphnia magna*

Polystyrene model particles are from bangs Inc., USA. We have used three different particles with different surface modifications, sulfonated (PS- OSO_3H), carboxylated (PS-COOH) and aminated (PS-NH_2). The carboxylated and aminated particles are based on the sulfonated. The sulfonated and carboxylated particles have a negative Z-potential at neutral pH. The amine group is positively charged. However, the level of functionalization varies. Therefore, as the particle is based on the sulfonated particles, the Z-potential varies from being positive to negative. In general, small aminated particles are more positive than larger ones. We have used particles with a diameter from 25 to 330nm. Polystyrene nanoparticles (PS-NH_2, PS-COOH and PS-OSO_3H) with sizes ranging from 25 to 330 nm at different concentrations (0.005 g/L to 0.150 g/L) were exposed to *D. magna* for 24 h. During the short-term exposure, *D. magna* individuals were exposed in tap water and not fed during the experiment.

During the life-time exposure, *D. magna* individuals (one individual per replicate) were placed into 100 mL uncovered glass jars with 80 mL of total volume, with (treatment) or without (control group) PS-NH_2 and PS-COOH nanoparticles. *D. magna* were exposed to nanoparticles with sizes ranging from 26 to 62 nm at concentrations of 0.32 to 7.6 mg/L. Every third day, alive *D. magna* were gently transferred to fresh tap water, with or without PS nanoparticles, and containing 2.5 mL of food (algae, *Scenedesmus* sp.).

2.2 Material for breakdown

The plastic and rubber material for breakdown were chosen using two criteria, it should be 1) commonly used plastics and rubbersand 2) different types of plastics and rubbers. Table 1 shows the different products that were used and what material they are made of.

Table 1. The products and material used in breakdown experiments

Products	Plastic/rubber							
	PS	HDPE	LDPE	PET	PLA	Silicone	Latex	Unknown[1)]
Expanded foam	X							
Coffee cup lids	X				X			
Bags		X	X		X			X
Granules					X			
Bottles		X		X	X			
Pacifiers						X	X	
Sealing agent (food grade)						X		

[1)] We have not been able to determine the material even after contact with the company.

2.3 Mechanical breakdown, fractionation and concentration

2.3.1 Mechanical breakdown

The mechanical breakdown was done using a by us previously developed method (Ekvall et al., 2019). Shortly, two grams of plastic (e.g., PS, PE, etc.) was broken down into small pieces (approximately 1 cm^2), which makes the next breakdown step easier. The small plastic pieces were placed in a glass beaker containing around 100 mL of water (i.e., 115 mL for PS, 200 mL for PE). An immersion blender (Bosch ErgoMixx 600W) were fully cleaned, and background levels of particles produced by the mixer itself was established. Then, the blender was used to mix the plastic and water for 2–5 min (depending on the type of plastic being broken down) at the highest operating speed. After the blending, half the volume of the solution (i.e., 100 mL for PE) was withdrawn from the beaker using a syringe and then filtered through syringe filters into a separate glass bottle. The aim of filtering the sample is to exclude microplastics from the breakdown solution. To the remaining plastic and water mix, another 100 mL of water (i.e., the same volume as the withdrawn volume) was added and again disintegrated by the immersion blender. This procedure was repeated until enough volume of filtered breakdown plastic water (BD) was obtained (i.e., 500 mL for PE).

2.3.2 Fractionation and concentration

The breakdown samples were further fractionated by filtration and concentration using a crossflow filtration device (VivaFlow 50R, HY, Sartorius) with a 10 kDa filter and ran at a flow rate of approximately 250 mL/min. The total volume of the breakdown sample (BDVF), which contains molecules that are larger than 10 kDa, was concentrated several times (i.e., 100 mL, a fifth of the original, for PE). The smallest particles, short chains of plastic that have been broken off from the particles, and potentially solved additives (molecules that are smaller than 10 kDa) will go through the VivaFlow device into the flow through fraction (FT). In some cases, reducing the concentration of FT in the fraction is needed. The concentration step was repeated by diluting the water and then concentrating again with the VivaFlow device. The final concentrated fraction was collected for toxicity testing.

2.4 Breakdown with UVC and UVB

For the ultraviolet (UV) radiation experiments we bought Exo-Terra Reptile UV-B 200 lamps and JBL PRO CRISTAL UV-C lamps. The lamps light spectrum was measured and can be seen in Figure 1. We built our own exposure setups for the two different lamps which are described in more detail in section 2.4.1, UV-C, and section 2.4.2, UV-B.

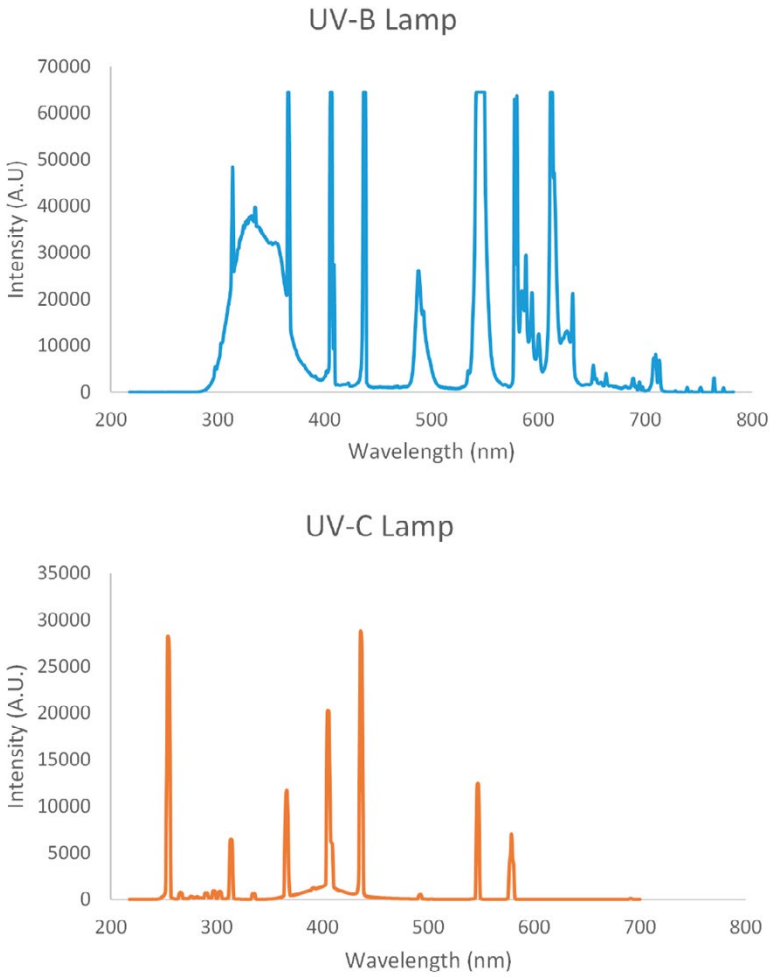

Figure 1. Spectrum for the UV-B, upper panel, and UV-C lamps, lower panel.

2.4.1 UV-C

The UV-C lamps were setup, so it was submerged into the solution containing the nanoparticles, see Figure 2. The particle solution was circulated using a peristaltic pump in order to cool the solution. The setup was placed in a cold room. Samples were treated for 24 hours with the UV-C lamp shining all the time and samples were taken for analysis at different time points during the 24 hours treatment. The stock solutions of the model polystyrene nanoparticles were diluted 1:10 with MilliQ H_2O. The solution was placed in a dialysis tube, Millipore with MWCO 6000, and dialysed against MilliQ H_2O, 10 mL against 5 L and the water was changed three times, in order to significantly reduce the amount of any additives that the manufacturer had added to the nanoparticles. The dialysed particle solution was diluted 76 times with tap water from the Ecology building at Lund university and then treated with UV-C for 24 hours.

Figure 2. The left image shows how the lamp was submerged into the solution in a 600 mL glass beaker. The glass beaker was placed in a cardboard box and two glass pipettes were placed inside the beaker. The two glass pipettes were connected to each other via a peristaltic pump with rubber tubing. Finally, a thermometer was connected in order to measure the temperature of the liquid while it was circulated with help of the pump. The lamp and beaker were covered in aluminum foil before the UV-C lamp was turned on to limit the risk of exposure to UV-C light for the person conducting the experiment. The whole setup was placed in a cold room.

2.4.2 UV-B

The UV-B lamps were setup in a cardboard box which was placed in a refrigerator, see Figure 3. Here the lamps are not submerged into to the particle solution, instead they are located above the samples. The nanoparticles were loaded into Quartz petri dishes with quarts lids to allow the UV-B radiation to reach the particle solution and at the same time limit the loss of water due to evaporation.

80 mL of the same particle solution described above for the UV-C samples, dialysed and diluted, was added to the Quartz petri dish and placed in the fridge. The UV-B lamps were turned on and samples were taken every day to be analysed with NTA and ATR-FTIR. The temperature within the fridge with all three lamps turned on was ~22 °C. The degrading process was followed for four months.

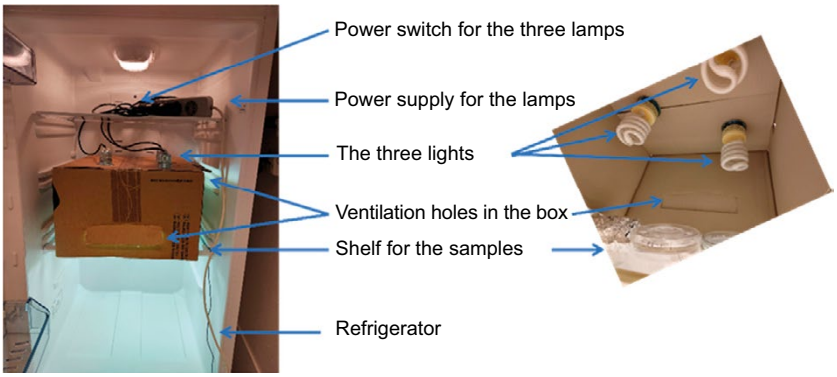

Figure 3. The homebuilt UV-B treatment facility. The lamps were installed in a cardboard box. Two ventilation holes was cut into the cardboard box sides. The box were placed on a shelf in a refrigerator on maximal cooling. about the lamps were placed approximately 10–15 cm from the shelf where the samples were placed.

2.5 Characterization of size

Model polystyrene nanoparticles, model PLA nanoparticles, and the breakdown nanoplastics of different materials were characterized for size using NTA using NanoSight LM10 (Amesbury, UK) and analysed with software NanoSight NTA 3.1 with a standard analysis setting. All reported data is an average of 3 to 5 separate recordings. NTA is suitable for plastic nanoparticles larger than 50 nm. The average particle size was also determined by dynamic light scattering (DLS) using a DynaPro Plate Reader II, Wyatt Technology Corp, USA. The dynamic light scattering were, for each sample, recorded 10 times at 23 °C for 10 s, and the data was analysed using the Dynamics V7 program. A third method, Differential Centrifugation Sedimentation (DCS) using DC24000 UHR Disc Centrifuge (CPS Instruments Europe, Oosterhout, Netherlands) was used to estimate the size of larger aggregates. The sucrose gradient was 4 to 12 percent and the centrifugation speed 24 000 rpm.

2.6 Characterization of surface chemistry

The particles surface chemistry has been characterized by three methods.

2.6.1 Zeta potential

The particle zeta potential (Z-potential), when in a MilliQ-H_2O dispersion, has been determined using a Malvern ZetaSizer. In practise, 1 mL sample was added to a DTS1070 cuvette (Malvern). The solution was allowed to equilibrate, inside the instrument's cuvette holder, for 2 minutes to reach 25 °C (the samples were kept at room temperature). Three individual measurements were conducted for each sample and then analysed.

2.6.2 ATR-FTIR

ATR-FTIR spectrometry was used to identify materials and changes to the materials after they had been treated with either mechanical forces or UV-C or UV-B radiation.

Three different ATR-FTIR instruments have been used during the project:

- PerkinElmer Spectrum 1
- Thermo Scientific
- PerkinElmer Spectrum 2

All three were equipped with ATR-units with a diamond crystal surface. Depending on the instrument, the setup was to record spectra between 450–4 000 or 550–4 000 cm^{-1}, with 64 scans and a resolution of 4 cm^{-1} for each spectrum. 2 µL of the sample was applied to the diamond surface and the water was allowed to evaporate for 30 min. After 30 min the dried sample was set under pressure using the ATR-units pressure plunger and a spectrum was recorded.

2.6.3 Titration of functional groups

We have also used a ProbeDrume spectrophotometer (Probation Labs, Sweden) to titrate titratable groups on the particle surface. The particles were either titrated with 1M HCl or 0.25M NaOH under an atmosphere of nitrogen. 1 mL sample was pipetted to a quartz cuvette with 1 cm pathlength, a magnetic stirrer bar was added, and the cuvette was placed in the cuvette holder in the ProbeDrum. Thereafter, small aliquots of the base or acid were repeatedly added to the samples with the ProbeDrum syringe while the pH, sample temperature, absorbance, and light scattering were monitored over time.

2.7 Toxicity testing on *Daphnia magna*

We used four treatments for long-term toxicity testing. After the mechanical breakdown procedure, 70 mL of the breakdown solutions (BD) was distributed into 20 uncovered glass beakers. After fractionation and concentration by VivaFlow, 70 mL of the breakdown sample (BDVF) and 70 mL of fraction (FT) were distributed into 20 uncovered glass beakers, respectively. A control was made from only tap water. One *D. magna* (less than 24 h old) and 10 mL of green algae (Scenedesmus sp.) were added to each beaker. Resulting in a total volume of 80 mL in each jar, the final algal concentration was 450 µg/L (AlgaeLabAnalyser, bbe Moldaenke, GmbH). The *D. magna* in the jars were maintained at a constant temperature of 18 °C at a 12:12 h light/dark photoperiod. Animals that died within the first 48 h were replaced and their deaths were not included in the final statistical analysis. This was performed to not include deaths from possible injuries attained in the pipette transfer of organisms from the culture aquaria to the test vessels. The animals were fed with 5 mL of green algae once per week. The beakers were checked every weekday. Mortality was recorded, and the offspring were counted and removed using a Pasteur pipette as soon as detected. The experiment proceeded until all *D. magna* were immobilized.

In the study of identifying adsorbed proteins on the surface of polystyrene nanoparticles after they had been filtrated by *D. magna* we used acutely toxic (53 nm PS-NH2) and non-toxic (200 nm PS-NH2, 62 nm PS-COOH and 200 nm PS-COOH) at concentrations of 16 and 224 mg/L, for the small and large particles, respectively. These concentrations were used as they approximately provide the same particle surface area. Firstly, *D. magna* individuals were left to filtrate the clean tap water in order to remove as much algae from the gut as possible. Then, 15 *D. magna* individuals were placed in 15 mL Falcon tubes (four replicates per group) containing a total volume of 5 mL tap water with (treatment group) and without (control group) nanoparticles and left to filtrate the water for 4 h. After incubation time, all individuals were removed, and nanoparticle-protein complexes were collected by centrifugation step. The content of the pellet was dissolved in SDS-PAGE loading buffer and proteins were separated by a 12 % premade SDS-PAGE gels. Finally, samples (visible bands from the gel and pellets containing nanoparticle-protein complexes) were further analysed by MS and MS/MS.

3. Results

3.1 The effect of polystyrene nanoparticle aggregation on toxicity

In biological systems polystyrene nanoparticle aggregation can be induced by other substances in the surrounding media. Aggregation of the particles will change the size of the particles introduced to *D. magna* and affect the stability of the particle dispersion which may further change the exposure scenario. We evaluated if aggregation of 50 nm PS-NH_2 affected their acute toxicity on *D. magna* by creating aggregates of a specific size using a human serum protein, immunoglobulin G, as a linker between the particles (Frankel et al, 2020). This was based on an earlier article describing protein driven polystyrene nanoparticle aggregation (Cukalevski, et al., 2015). The toxicity of the 50 nm PS-NH_2 aggregates was compared to non-aggregated 50 nm PS-NH_2 and to polystyrene particles of similar size as the aggregates. The toxicity was the same whether they were aggregated or non-aggregated 50 nm PS-NH_2, whereas the larger particles, 200 nm, did not show any toxicity.

3.2 Life-time toxicity of model polystyrene nanoparticles

We wanted to test our breakdown nanoplastics in life-time tests to maximize the exposure time and exposure material. Previously, the acute (24 h) toxicity studies using model polystyrene nanoparticles showed that only 50 to 60 nm PS-NH_2 polystyrene nanoparticles are toxic to *D. magna,* whereas 200 nm PS-NH_2 and different sizes of PS-COOH and PS-OSO_3H polystyrene nanoparticles (Mattsson, et al. 2017) are not toxic within the used concentration range, Table 2. Later the LD50 values for many polystyrene nanoparticles have been determined (Saveedra et al, 2019, Pochelon et al., 2021). Differences in toxicity are probably due to that younger *D. magna* were exposed in the later studies, and that these may be more sensitive. To compare the breakdown nanoplastics with the polystyrene nanoparticles we performed life-time experiments on two different sizes of PS-COOH and one PS-NH_2 (Kelpsiene et al., 2020). The particles were chosen for three reasons: 1) the 53 nm PS-NH_2 is the only particle that is acutely toxic and the smallest commercially available PS-NH_2, 2) the 62 nm PS-COOH is not acutely toxic but comparable in size with the 53 nm PS-NH_2, and 3) the 26 nm PS-COOH as smaller particles were more toxic when comparing different PS-NH_2.

Life-time exposure to the 53 nm PS-NH_2 and 26 and 62 nm PS-COOH polystyrene nanoparticles revealed increased mortality in *D. magna*. The increased mortality at as low concentrations as 0.32 mg/L is almost 100 times lower than in previous acute toxicity tests for 53 nm PS-NH_2, Table 2. That the PS-COOH, which is not toxic in acute tests, is toxic in life-time exposure, at the same concentration as 53 nm PS-NH_2 indicates that there are different mechanisms behind the acute toxicity and life-time toxicity.

Table 2. The acute and life-time toxicity of different polystyrene nanoparticles

Particles	Surface charge	Diameter (nm)	Conc. (mg/L)	Acute toxicity[1)]	LT toxicity[2)]
PS-OSO_3H	Neg[3)]	23 and 200	25 to 400	No	
PS-COOH	Neg	26 to 220	25 to 400	No	
PS-NH_2	Pos[4)]	52, 53, 57,58	5 to 25	No	
PS-NH_2	Pos	52, 53, 57,58	50 to 50	Yes	
PS-NH_2	Pos	120, 180	5 to 150	No	
PS-COOH	Neg	26	0.32 to 7.6		Yes
		62	0.32 to 7.6		Yes
PS-NH_2	Pos	53	0.32		Yes
		53	0.032		No
		53	0.0032		No

[1)] From Mattsson et al., 2017
[2)] From Kelpsiene et al., 2020
[3)] Negatively charged
[4)] Positively charged

3.3 Life-time toxicity of breakdown high density polyethylene nanoplastics

During the project we have broken down many different plastic items, see section 3.5 and characterized the breakdown fraction and estimated the toxicity, if any. The most detailed study is on HDPE from package bags (Ekvall et al., 2022). The rationale is that HDPE is one of the most common plastics produced and found in our waters and packaging bags is a product we all encounter.

3.3.1 Sizes after breakdown, fractionation and filtration

The bags were broken down with an immersion blender as described in section 2.3.1. Particles larger than 0.8 µm were removed by filtration. The filtrate was concentrated on Vivaflow device using a filter with a 10kDa cut off. The filtrate contains everything smaller than about 3 nm in diameter. This could be for example, additives or polymer chains. The remaining fluid contains nanoplastics and everything that is in the filtrate. The nanoplastics were purified from smaller molecules by three rounds of dilution/concentration using the Vivaflow. The different fractions are described in Table 3.

Table 3. The different fractions from PE breakdown characterized and tested for toxicity

Fraction	Procedure	Possible contents
PEBD: polyethylene breakdown	Breakdown material after filtered through 0.8 µm	Nanoplastics, polymers, and additives
PEVF: polyethylene Vivaflow	Concentration using a 10 kDa cut off filter in Vivaflow	Purified nanoplastics. 20 % Polymers and additives compared to PEBD
FT: flowthrough	Flow through from concentration	Nothing larger than about 3 nm. Polymers and additives at the same concentration as in PEBD
3rd PEVF: third polyethylene Vivaflow	Purification (3 times) and concentration of PEVF1 using a 10 kDa cut-off filter in Vivaflow	Concentrated nanoplastics (2 times) compared to PEVF. Less than 1 % of polymers and additives
3rd FT: third flow through	Flow through from the 3rd round of purification in Vivaflow	Less than 1 % of polymers and additives

The particle sizes were measured in the fractions. Some examples are given in Table 4. The size was around 110 nm as determined by NTA. The size limit for detecting polymeric nanoparticles by NTA is around 50–60 nm. DLS potentially measures smaller particles, but the polydispersity in PEBD and PEVF was too high. Therefore, PEVF was concentrated 10 times and subsequently filtered through filters with a cut off of 450, 200, and 100 nm. The size decreased for each step but was around 100 nm after the last filtration with indications of smaller particles.

Table 4. The sizes measured in the fractions described in Table 3 by NTA and DLS

Fractions	Diameter (nm)	
	NTA	DLS
PEBD	112	
PEVF	111	
PEVF concentrated 10 times		200
PEVF concentrated 10 times and filtered through 450 nm filters		150
PEVF concentrated 10 times and filtered through 200 nm filters		140
PEVF concentrated 10 times and filter through 100 nm filters		100

3.3.2 Surface chemistry and charge

The surface charge was evaluated by measuring the Z-potential of PEVF concentrated 10 times in water. The Z-potential is slightly negative (–11mV). The chemical signature was evaluated by ATR-FTIR. The starting material have an ATR-FTIR spectrum clearly representing HDPE. In the PEVF (concentrated 10 times) all minima, characteristic of HDPE, could be identified. In addition, there were changes in the spectra suggesting strong oxidation of the PE, Table 5.

Table 5. Additional signals in breakdown PE nanoplastics and the chemical groups they may represent

Wavelength (cm^{-1})	Chemical groups
3 300 to 3 500	Hydroxyl
1 600 to 1 800	Carboxyl
1 100 to 1 200	Carbon-oxide bonds
875	Possibly carbonate

3.3.3 Toxicity of different fractions

The toxicity on *D. magna* was tested for the different fractions in two different life-time experiments. In the first test, the breakdown fractions were added at the beginning of the test. In the second test, the breakdown fractions were added to *D. magna* every week to optimize the effective exposure concentration. Furthermore, in the second test, the PEVF concentration was approximately doubled compared to in the first test. Adding nanoplastics every week may also mimic a more realistic continuous exposure scenario. The result is summarized in Table 6. In the first toxicity test, PEBD and FT, but not PEVF, exhibited increased mortality. In the second test, 3rd FT, that has less than 1 % of the contents in FT, exhibited no toxicity. In 3rd PEVF, with twice the concentration nanoplastics compared to PEVF, the *D. magna* lived significantly longer than in the control, probably due to bacterial growth on the

nanoplastics, which can provide additional food for the *D. magna*. In conclusion, the observed toxicity for PEBD is due to smaller molecules *i.e.*, less than 3 nm in diameter, and not the nanoplastics.

Table 6. The toxicity on *D. magna* of the different PE breakdown fractions.

Fraction	Toxicity test	Life-time Toxicity
PEBD	1st	Yes
PEVF	1st	No
FT	1st	Yes
3rd PEVF	2nd	No, longer survival[1)]
3rd FT	2nd	No

[1)] Compared to the control group.

3.4 Size, chemistry, and toxicity changes after UV breakdown

Exposure of polystyrene nanoparticles to UV radiation resulted in changes of the measured particle size after exposure. Analysing UV exposed 200 nm polystyrene nanoparticles using DLS and NTA revealed that the size of the particles was reduced after exposure. However, measuring the same exposed 200 nm polystyrene particles using DCS revealed an increase in particle size. The three techniques utilize different methods for calculating the particle size, DLS and NTA calculate particle size based on the Brownian motions of the particles while DCS calculates the particle size based on the sedimentation of the particle through a sucrose gradient. Combining the results from the three techniques we conclude that UV radiation acts on the particles by breaking up the material. The DCS results also imply that the particle is less compact after UV exposure which would cause larger friction once the particle moves through the sucrose gradient. The less compact particle would then, by DCS analysis, be interpreted as having increased in size while in fact it has started to break up.

Exposure of the particles to UV also resulted in changes in the particle surface chemistry. Analysing the particles using FTIR showed oxidation of the material compared to the particles un-exposed to UV, Figure 4. Changes in particle surface chemistry may also result in changes in the interactions between the particle and its surrounding environment. For example, particles going from having a net positive charge to a net neutral or negative charge may have a weaker interaction with negatively charged macromolecules in the environment. This may also give rise to changes in the toxicity of the material. Amine modified polystyrene nanoparticles with a diameter of 50 nm have previously been proven very toxic to the freshwater zooplankter *D. magna* (Mattsson et al, 2017). Exposing these particles to UV radiation rendered the particles less toxic as compared to un-exposed particles when evaluating the toxicity using acute toxicity tests with *D. magna*.

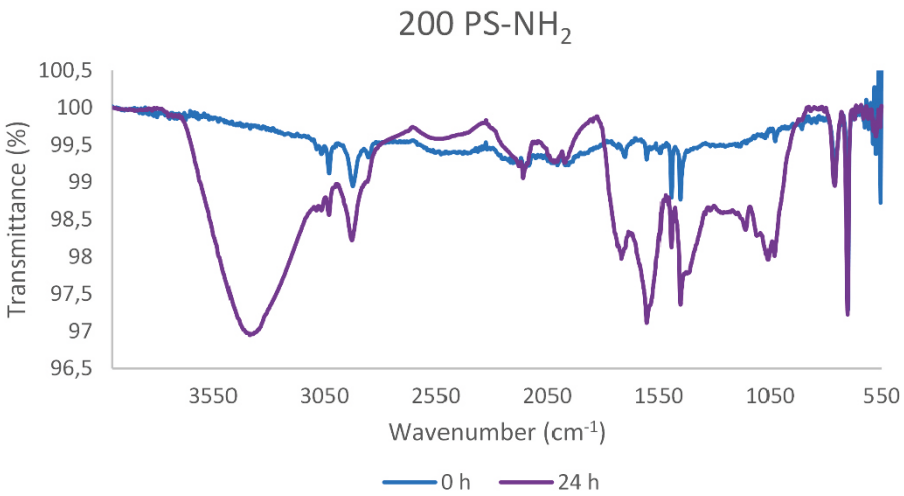

Figure 4. The figure shows the surface chemistry, determined with ATR-FTIR, on 200 nm aminated polystyrene particles before, in blue, and after, in purple, 24 hours UV-C treatment.

3.5 Breakdown, characterization, and toxicity of other materials

The same methodology used to break down polystyrene and polyethylene products (Ekvall et al., 2019, 2022) into nanoplastics was applied to two other plastics and two rubbers from in total 11 different products. All plastic products tested generated nanoplastics, Table 7, in a size range between 104 and 180 nm. The z-potential is in general slightly negative with two exceptions, the PLA 3D printer filament and bags. These two products generated two populations one with negative and one with positive Z-potential. Similar to PS and PE, the latex and silicone nanoplastics could by ATR-FTIR be shown to be the same material as the starting material but oxidized. The determination of the PLA breakdown products is more difficult, and no conclusions could be made. The starting material, although labelled as PLA, is different from each other. Two products, the plastic bag and the coffee lid, seem to be of another material than PLA.

Acute toxicity was only observed for PE breakdown products (as described above), latex pacifiers, and for un-dialysed PLA nanoparticles. Latex has previously been shown to be toxic to *D. magna* (Hubschman and Engel, 1965). The toxicity of PLA nanoparticles disappeared after dialysis probably due to the removal of low molecular additives. Interestingly, the purified PE nanoplastics, all the tested PLA products, including the PLA nanoparticles after dialysis, increased the *D. magna* survival compared to the control group. A possible explanation is that the nanoplastics are a food source for bacteria that in turn can be used by *D. magna*.

Table 7. Characterization of nanoplastics formed after mechanical breakdown.

Product	Plastic	Diameter (nm)[1]	Z-potential (mV)	ATR-FTIR	*D. magna* toxicity
Expanded foam[2]	PS	135±12	–14	Yes	**0**[3]
Coffee lid[2]	PS	104±6	–7	Yes	**0**
Nanoparticles	PLA	250	–15	*ND*	**X**[4]/**0**[5]
Soup lid	PLA	132	–22	*ND*	++[6]
3D-printer filament	PLA	129	+43 and –44	*ND*	*ND*[7]
Mug	PLA	134	–10	*ND*	*ND*
Coffee lid	PLA	140	–6	*ND*	*ND*
Bags	PLA	133	+3 and –16	*ND*	+++
Bottles	PET	180	–25	No	*ND*
Bottles	PE	126	–12	No	**0**
Bag[8]	PE	112	–11	Yes	**0**
Pacifier	Latex	143 ± 59	–6	Yes	**X**
Pacifier	Silicone	129 ± 47	–17	Yes	**0**
Sealing agent	Silicone	140 ± 55	*ND*	Yes	*ND*

[1] Measured by NTA, [2] From Ekvall et al., 2019, [3] No toxicity observed, [4] Toxicity observed, [5] No toxicity observed after dialysis of particles, [6] *D. magna* exhibited longer life than control, [7] Not determined, [8] From Ekvall et al., 2022.

3.6 Protein binding after filtration of polystyrene nanoparticles

Adverse effects at sub-lethal concentrations of nanoplastics can be very relevant from an enviromentally point of view. Examples of effects are reduced growth, reduced reproduction, and behavioural changes. Other parameters such as e.g., RNA arrays may give more information about the mechanisms behind the observed effects. We explored two pathways that, to our knowledge, has not previously been explored using *D. magna*; determination of bound proteins to the nanoplastics during filtration by *D. magna* and Nuclear magnetic resonance (NMR) based metabolomics after acute and long-term nanoplastics exposure to *D. magna*.

The protein binding was determined to acutely toxic 53 nm PS-NH_2 and to not toxic 200 nm PS-NH_2, and 62 nm and 200 nm PS-COOH (Kelpsiene et al., 2022). The data reveal that 53 nm PS-NH_2 binds different proteins compared to the not acutely toxic polystyrene nanoparticles. To develop the methodology, we used polystyrene nanoparticles at high concentrations i.e., 16 and 224 mg/L, for the small and large particles, respectively, concentrations which approximately provide the same particle surface area. Proteins that are bound to 53 nm PS-NH_2 can be divided into two groups. One group of proteins is related to the digestive system, such as carboxypeptidase B, serine protease and chymotrypsin elastase family member 2A. Whereas the other group is involved in functions related to epithelium, and intracellular structures and processes, for example, beta-klotho, actin, tubulin, elongation factors and histones. Proteins that bound to 200 nm PS-NH_2 are involved in various functions. The most abundant proteins were vitellogenin-1, hemocyte protein-glutamine gamma-glutamyltransferase, and putative hemocyte protein-glutamine gamma-glutamyltransferase. Similarly, vitellogenin-1, hemocyte protein-glutamine gamma-glutamyltransferase, and actin, alpha skeletal muscle proteins were the main proteins that bound to 62 and 200 nm PS-COOH nanoparticles (Kelpsiene et al., 2022).

As the polystyrene nanoparticles not only bind to proteins, but also to lipids (Lima, et al., 2020), we measured the total triglyceride concentration of each experimental fraction. We found out that triglycerides effectively bind to 200 nm PS-COOH nanoparticles but not to the other tested nanoparticles (Kelpsiene et al., 2022).

4. Discussion

Plastics are fantastic materials that have revolutionized our life situation. However, plastics in the wrong places (i.e. misplaced plastic waste) have during the last decades manifested itself as a huge problem. Plastic waste in the environment is problematic from several different points of view:

- High persistence in the environment
- Waste of resources
- Documented severe impact on wildlife by macro- and microplastics
- Possible severe impact on the environment and wildlife by nanoplastics
- Aesthetically ugly

Some of the severe effects caused by macro- and microplastic on wildlife and the environment are easy to see, for example, plastic bags in the stomach of dead whales, birds and turtles entangled in plastics and so on, and are well documented. However, during the last decade, the research society has asked the question of what happens when plastic waste fragmentise into even smaller sizes, nanoparticles. Nanoparticles are so small that they can, for example, enter an aquatic food web already at plankton or zooplankton level and then be transported up in the food chain. We have shown that polystyrene particles, in a laboratory experiment, can be transported up in a food chain and clearly affect the top predator (Mattsson et al., 2015, 2017). This raises the question if this process also occurs right now in the natural environment where an enormous amount of plastic waste has accumulated. We do not have an answer yet to this question. However, we have shown that everyday used plastic products generate nanoparticles if they are subjected to mechanical wear. Breakdown of plastics due to mechanical wear is also something we can expect out in nature e.g., waves moving plastics back and forward on a sandy beach where the sand then may mimic a “natural sandpaper”.

4.1 Plastics, microplastics, nanoplastics, and the environment

Historically there has been a discussion if nanoplastic can exist or not in the environment. The difficulties in sampling and detecting nanoplastics in the environment have so far made it impossible to conclusively argue that nanoplastics exist and even less to evaluate their possible impact on the environment. Recently attempts have been made to detect nanoplastics in leachate from ordinary plastics (Hernandez et al., 2019, Xu et al., 2020) and to breakdown plastics with various methods into nanosized particles (Lambert and Wagner, 2016, Magri et al., 2018, Ekvall et al., 2019, Mekaru, 2020, Ji et al., 2020, Mattsson et al., 2021). However, although it is reasonably easy to fractionate and detect nanosized particles, it is more difficult to determine their chemistry and link the chemistry to a specific nanosized particle. Furthermore, it is known from analyses of microplastics weathered in nature that the surface chemistry is changed due to oxidation, which will further complicate the analyses. Only a handful of studies provide a chemical analysis of the nanoplastics after breakdown (Magri et al., 2018, Ekvall et al., 2019, Hernandez, et al., 2019).

We have previously developed a method to mechanically break down expanded polystyrene and take-away coffee cup lids made of polystyrene into nanosized particles in a quantity that allowed for chemical analysis by ATR-FTIR (Ekvall et al., 2019). This method has been used to break down six different plastics and rubbers, from 14 different consumer products to nanoplastics (see Table 7). The chemistry of the nanoplastics can be linked back to the original plastics by ATR-FTIR and in many cases, strong oxidation of the material can be seen. This clearly indicates that mechanical processes can, in general, result in nanosized particles from plastic and rubber products.

It is not only mechanical processes that will break down plastics in nature. UV-radiation will also change the plastics which are well known for macro- and microplastics. Irradiation with UV-B and UV-C of polystyrene nanoparticles resulted, as expected, in chemical changes suggesting oxidation of the polymers. Furthermore, the size of nanoparticles significantly decreased, suggesting that they could with time disappear (i.e., be broken down). Theoretically, the oxidation process could continue until all plastics are turned into CO_2. Judged from the oxidation of break-down nanoplastics from polystyrene and polyethylene the polystyrene nanoplastics is more stable. It is possible that the oxidation process of polyethylene is much faster, which needs to be further investigated.

Plastic waste that has ended up in the environment will experience mechanical wear, UV-B radiation, microbial organisms, and different chemical environments. Taking this into consideration, we are convinced that macro- and microplastic will degrade to objects on the nanoscale. However, the faith of these nano objects is still uncertain.

4.2 How to test environmentally relevant toxicity? – Acute and long-term toxicity

What is the best way to test the potential toxicity of a man-made material that is disintegrated by mechanical wear, UV-B radiation, and chemistry in nature? This is not a question with an easy answer. The closer to nature we design our studies, the more complicated our test systems become. We are currently using two different methods, acute and life-time toxicity tests. Both with their own advantages and disadvantages.

Acute toxicity tests are often performed during 24 or 48 h which may not represent environmentally relevant conditions. According to us, life-time toxicity tests, in which the whole life span of the tested organism is followed, are probably closer to what may happen in the environment during the process when plastic is disintegrated by natural forces. Here the test organism is constantly exposed to a low concentration of the tested material.

We, and other research groups, have used manufactured polystyrene nanoparticles with different surface modifications as model particles when performing toxicity studies. Of these model polystyrene particles, the small, 50–60 nm, positively charged (aminated) polystyrene particles have been shown to be acutely toxic to *D. magna*, while all the other tested model polystyrene nanoparticles were not, see Table 2. However, in life-time toxicity tests, polystyrene nanoparticles that showed no toxicity in acute tests were as toxic as the acutely toxic 50–60 nm aminated polystyrene particles, see Table 2. This suggests that the mechanisms behind acute and life-time toxicity are different.

One explanation for the observed acute toxicity may be that the proteins bound to particles after filtration by *D. magna* differ between acutely toxic 52 nm PS-NH2 and non-toxic larger PS-NH2. On the 52 nm PS-NH2 there are for example cytosolic proteins detected, which may indicate a rupture of the intestinal cell walls of *D. magna*. In life-time exposure, the particle concentration is lower with a lower impact on the cell wall. Instead, life-time exposure may allow for uptake of particles and a slow accumulation within *D. magna* or in the intestine that with time increases the burden for the organism. However, no direct conclusion can be made, and further experiments are needed to explain the mechanisms behind the toxicity.

Nanoplastics are expected to interact with organic materials in nature. A possible outcome is that the particles aggregate which in turn can affect the toxicity. However, protein-induced aggregation of acute toxic 52 nm PS-NH2 was observed to not affect the toxicity (Frankel et al., 2020). Although the results are surprising especially as the aggregation process does not only change the size but also the surface charge as the bound protein has a negative net charge. The results can be explained by several hypotheses. For example, the aggregates may be more fractal than spherical minimizing the difference in surface area between aggregated and non-aggregated particles. Furthermore, if the curvature of the interacting surface, rather than the particle size is important for toxicity both fractal and spherical aggregates will still expose surfaces with high curvature. Regarding the charge, a change of the average particle charge may not be evenly distributed on the particle surface and positively charged amine groups can still be exposed. Another explanation is that the proteins aggregating the particles rapidly are digested by the *D. magna* which may cause the aggregates to break up. However, further experiments are needed to fully understand what happens with the formed nanoparticle aggregates when they enter the *D. magna*.

Our results that are discussed here illustrate some of the complexity in determining if a certain product is toxic or not to the environment. The results also demonstrate that there are benefits of evaluating both acute and life-time toxicity.

4.3 Breaking down and characterizing plastic from everyday used products

Table 1 shows all the everyday used plastic products that we have broken down into nanoparticles with the help of mechanical wear. We argue that plastic waste in nature will be subjected to mechanical forces, for example, rolling, with help of waves, over stones and sand at shorelines. All plastic materials we tested so far, in laboratory experiments, release nanoparticles during mechanical wear. We have characterized the surface chemistry of the formed nanoparticles with ATR-FTIR and most of them show that the surface has undergone oxidation.

We have also used UV-C or UV-B irradiation to break down plastic particles. Both UV-C and UV-B affect plastic particles and the process is much faster for UV-C. UV treatment of particles also seems to lead to, among other things, oxidation of the particle surface.

Table 7 summarises the data that we so far have acquired for the nanoparticles from everyday used plastic items regarding the characterization and the observed toxicity.

4.4 What part of a broken-down plastic is toxic?

Plastic is a collective name for materials that are built up by connecting a specific molecule to a copy of itself into long chains of the same molecule, a polymer, and is, in its simplest form, a uniform chemical entity. However, many plastic products contain, in addition to the polymer material, additives that give the plastics different properties. Many of these additives are known to be toxic to *D. magna*, and they may be released or leached out from the plastic polymer when it is broken down. If a sample of broken-down plastic shows toxicity in a test using *D. magna*, the observed toxicity could potentially arise from the additives and not from the actual polymer nanoparticles.

In our experiments with HDPE, from packing bags, the PE breakdown (PEBD in which just particles larger than 0.8 µm had been removed from the sample) was toxic to *D. magna* in life-time experiments. However, after the separation of smaller molecules and nanoplastics, only the fraction with the small molecules remained toxic to *D. magna*, which may indicate that additives are a more severe environmental concern than the actual nanoplastics. The fraction with small molecules contains molecules that are smaller than 3–4 nm, 10 000 MWCO, which means that it probably contains additives to plastics and small chains of the plastic polymer that has been broken off from the bulk material in the breakdown process, see Table 3 for more information about what the different fractions contain and Table 6 for information about which fraction was toxic and which was not.

4.5 Nanoplastic, a food source for bacteria?

One of the most interesting observations that we have registered during the project is that some of the fractions of nanoparticles from broken down plastic actually increase the survival time of *D. magna* in long-term toxicity tests compared to the control group, see Table 6. This phenomenon was also observed for nanoplastics from two PLA products even without separation in acute tests without any added food, see Table 7.

We speculate that the increased survival can be due to bacterial growth on the nanoplastics. If the nanoplastics can, in general, be broken down by bacteria, it would speed up the overall breakdown of the plastics. This is something that we currently are going to start to investigate further.

4.6 Conclusions

We have shown that everyday used plastic products generate nanoparticles if they are subjected to mechanical wear. The produced nanoparticles differ from manufactured particles since they have irregular shapes and often have an oxidized surface. We have also shown that acute and life-time toxicity can differ for the same material.

- Nanoplastic is formed during breakdown of macro and microplastic
- Nanoplastic produced in a breakdown process differs from manufactured nanoplastic
- The nanoparticles formed in a breakdown process often have an oxidized surface
- Both acute and long-term toxicity should be studied
- The toxicity of different size fractions of the breakdown product should be studied

No severe toxicity was observed from any of the produced nanoplastics from consumer products that we have been testing. The nanoplastics are strongly oxidized after the mechanical process. The oxidation continues in UV-B and UV-C radiation which further breaks up the nanoplastics into smaller particles. Based on what is known from the toxicity of the model polystyrene, oxidized nanoplastics should be less acutely toxic. The same may not be true in lifetime tests. The oxidized surface has a different charge and hydrophobicity compared to the starting material and may therefore interact differently with macromolecules in the environment. Different macromolecules adsorbed to the surface of the particles may or may not change the toxicity of nanoplastics (Naseer and Lynch, 2016, Frankel et al., 2020). Nanoplastics from some plastics seem to provide the basis for bacterial growth. More studies are of course needed, but the results indicate that the breakdown of plastics into nanoplastics and the processes thereafter may result in the complete oxidation of the plastics. Earlier studies mapping the occurrence of styrene oligomers around the world (Kwon et al., Kwon et al., 2014, 2015, 2017) support that idea as the oligomers probably are derived from misplaced broken down polystyrene. However, styrene is itself toxic and it is possible that breakdown products from other plastics are toxic. Future studies should focus on the kinetics of these processes to evaluate if nanoplastics accumulate in the environment or if the breakdown process into microplastics is much slower and on detailed characterization of the toxicity of the breakdown products during the process.

5. Outcome

5.1 Published papers

Kelpsiene, E., Torstensson, O., Ekvall, M.T., Hansson, L-A., and Cedervall, T. (2020) Long-term exposure to nanoplastics reduces life-time in *Daphnia magna*. *Sci Rep* 10, 5979

Frankel, R., Ekvall, M.T., Kelpsiene, E., Hansson, L-A., and Cedervall, T. (2020) Controlled protein mediated aggregation of polystyrene nanoplastics does not reduce toxicity towards *Daphnia magna*. *Environ. Sci.: Nano*, 2020,7, 1518-1524

Kelpsiene, E., Ekvall, M.T., Lundqvist, M., Torstensson, O., Hua, J. and Cedervall, T. (2021) Review of ecotoxicological studies of widely used polystyrene nanoparticles *Environ. Sci.: Processes Impacts*, 24, 8-16

Ekvall, M.T., Gimskog, I., Hua, J., Kelpsiene, E., Lundqvist, M., and Cedervall, T. (2022) Size fractionation of high-density polyethylene breakdown nanoplastics reveals different toxic response in *Daphnia magna*. *Sci Rep* 12, 3109

Kelpsiene, E., Brandts, I., Bernfur, K., Ekvall, M.T., Lundqvist, M., Teles, M., and Cedervall, T. (2022) Protein binding on acutely toxic and non-toxic polystyrene nanoparticles during filtration by *Daphnia magna*. Accepted by *Environ. Sci.: Nano*

5.2 Manuscript under preparation

Breakdown nanoplastics from latex and silicone pacifiers. Ekvall, M.T., Mellring, A., Månsson, A. Lundqvist, M., and Cedervall T.

Breakdown of polystyrene nanoparticle with UV radiation and its effect on toxicity on *D. magna*. Ekvall, M.T., Lundqvist, M., and Cedervall, T.

5.3 Master and Bachelor thesis

Mellring, Alice (2019) The characteristics of plastic nanoparticles and their effect on zooplankton, Lund University

Månsson, Alma (2019) Degradation of Commonly Used Plastics into Nanoparticles, Lund University

Golovtchenko, Erik (2021) A Mega Impact on a Nanoscale – Finding out what proteins “dress” polyethylene and polystyrene nanoparticles in bovine blood serum

Rydberg, Melinda (2022) Characterization of PLA Nanoplastics, and Their Effects on *Daphnia magna*

Abbas, Ramy (2022) Isolation of polystyrene-protein corona complex from bovine serum using size exclusion chromatography and expression of *Daphnia magna’s* proteins using EDDIE fusion technology

Abrar Snowbar (2022) A study of nanoparticles interaction with plasma membrane proteins.

5.4 Other participants in the project

Isabella Gimskog: Laboratory assistant

Shanti Naidu: Laboratory assistant

Katja Bernfur: Researcher, Mass spectrometry specialist

Björn Persson: Researcher

5.5 Outreach

Outreach projects was difficult to organize during the COVID-19 pandemic. The project described below describe what we have done before and after and in some cased during the pandemic.

5.5.1 Popular Science Lectures

The popular science lectures address the plastic problem in general, what is special about nanoparticles and nanoplastics, how nanoplastics can be generated, and how nanoplastics affect the environment. The lectures are adapted to the audience and for each situation.

TetraPak World Wide, Lund, but people working at TetraPak around the world can participate on line.

Landskrona Rotary, Lund

Kattegatts kustvattenråd, Falkenberg

Nanoplast – små partiklar, stora risker, Lunds Stadsbibliotek

5.5.2 Other Outreach Projects

Den rätta vägen: Theatre, workshops, lecture project together with theatre Sagohuset, performed for about 1 500 students at 9th to 12th grade from Ystad in the south of Sweden to Kiruna in the north of Sweden during 2019, 2021 and 2022. The play was about how to handle all new research news in your ordinary day. The workshops were about how to create nanoplastics from ordinary plastics, and how to study nanomaterial and its effect on the environment (Cedervall, et al., 2019) The project was financed by Formas. However, all people active in the research group was invited to join the project as it was an excellent route for nanoplastics information and an effective way to boost creativity and performance in the research group.

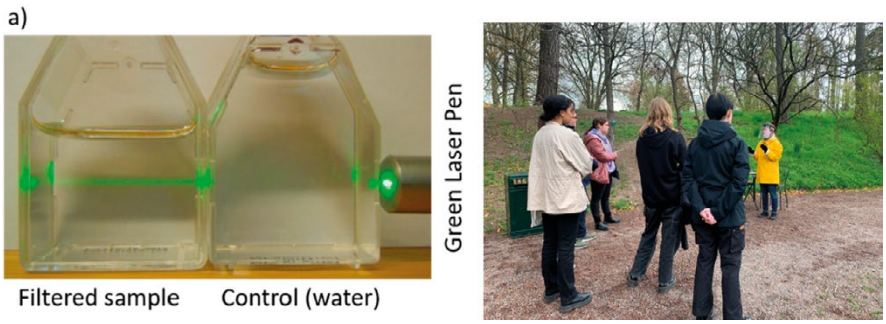

Figure 5. Left panel: Workshop showing that newly made broken down PS nanoplastics are scattering the laser beam. Outdoor workshop showing how nanoplastics can affect the organisms in the environment.

Outdoor workshops: We showed four different workshops in the botanical garden in Lund late spring 2021 to 9th grade student. The workshops were adapted to the corona pandemic restrictions, i.e., outdoor, small groups and protective gears. It also meant that a significant part of the workshops took place in heavy rain. However, both we and the student longed to be engaged outdoor and it was overall a great experience. The workshops were about how to breakdown plastics and how nanoplastics can affect the environment.

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Environmental impact of nanoplastics from fragmentized consumer plastics

Final project report

The smallest microplastics are called nanoplastics. Due to the small size, they are difficult to find and study, but have different biological impact then larger pieces of plastics. The project has used model polystyrene nanoplastics to fill knowledge gaps about life-time toxicity to filtrating zooplankton *D. magna* and to identify what proteins from the gut of *D. magna* bind to toxic and non-toxic nanoplastics. Nanoplastics in nature will come from break-down of larger plastics. The project has mechanically mimicked this process and produced nanoplastics from 8 different plastics and rubbers using 14 different daily used products. The nanoplastics are irregular in shape and highly oxidized compared to the starting material. Nanoplastics from HDPE and PLA are not toxic to *D. magna*. On the contrary *D. magna* exhibit increased lifetime probably due to that the nanoplastics can support bacterial growth which can be used as food by *D. magna*. UV-irradiation further degraded polystyrene nanoplastics. This, together with the bacterial growth, may imply faster plastic degradation in nature than believed today.

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