Master's Thesis in Engineering Nanoscience

Characterization of PLA Nanoplastics, and Their Effects on *Daphnia magna*

Author: Melinda Rydberg
Examiner: Patric Jannasch
Supervisor: Tommy Cedervall
Co-supervisor: Egle Kelpsiene
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Abstract

As plastic pollution in aquatic environments keeps happening it has become more and more important to learn more about nanoparticles. Bio-based and biologically degradable plastics, such as polylactic acid (PLA), are gaining popularity and it is therefore important to determine whether they break down into nanoplastics or not. Should this be the case, it is of interest to characterize their size, morphology, surface charge, chemical composition, and determine if they are toxic to aquatic organisms.

The approach was to break down five different consumer PLA plastic products in Milli-Q water with an immersion blender. These products were a soup cup lid, coffee cup lid, plastic cup, plastic bag and 3D printer filament. The soup cup lid and coffee cup lid were also exposed to UV-C to see if that made any difference when characterizing them. Manufactured PLA nanoparticles were also purchased and studied. They were then characterized by using tools such as NTA, DLS, FTIR and TEM. To determine the toxicity of the particles, the zooplankton *Daphnia magna* was exposed to them.

The results showed that nanoplastics were formed, with an average size 145 - 230 nm in diameter depending on the product. The particles seemed to have different shapes, there were particles with sharp edges, and particles with round edges. The surface charge differed between the different products, all values indicated they should be unstable in Milli-Q water. However, when the stability was studied with the NTA, they seemed stable. The chemical composition of the soup cup lid, plastic cup and 3D printer filament matched that of pure PLA, while the coffee cup lid and the plastic bag seemed to be made of polystyrene and polyester, terephtalic acid (PET), respectively.

The toxicity was tested with the soup cup lid, the UV-C treated soup lid, the plastic bag and the manufactured nanoparticles in a short-term test where the plankton were not fed. They showed no sign of being toxic. On the contrary, the plankton seemed to live longer when exposed to breakdown PLA nanoplastics in the water. When exposed to the manufactured PLA, there was a toxic effect on the plankton.

Contents

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1 Introduction

Each year massive amounts of plastic end up in the environment and about 8 million tons makes its way to the ocean. It is well known that plastic garbage can cause strangulation and starvation in animals, and that they break down into microplastics.

Microplastics have an ability to be transported long distances and have been found all over the world; at the top of Mount Everest, in the deep sea, along coast lines and in the Polar regions, to name a few places. These microplastics are then ingested by animals, wherein they could potentially cause tissue and cell damage. Another negative impact they can have is that they bioaccumulate in the guts of the animal, so that they don't feel hungry and therefore starve, or the animal is eaten and the microplastics move further up the food chain. [1, 2]

Microplastics in the environment has been extensively studied and it is easy to find articles on this topic. However, it has been proven that microplastics continue to break down into the even smaller nanoplastics [3]. Nanoplastics can be defined as particles smaller than 100 nm in at least one dimension. Articles on these are much harder to find, although they are becoming a more popular topic to write about. Even though the ways nanoplastics spread around the world are not researched, they have been found in remote places such as polar ice, and alpine snow [4].

Conventional plastic products, made from fossil resources, are often made to last a long time. Some are estimated to take thousands of years to degrade in marine environments [5]. To combat the long degradation times, biodegradable and compostable polymers have started to make a larger appearance on the market. These are often also bio-based, and one such example is PLA.

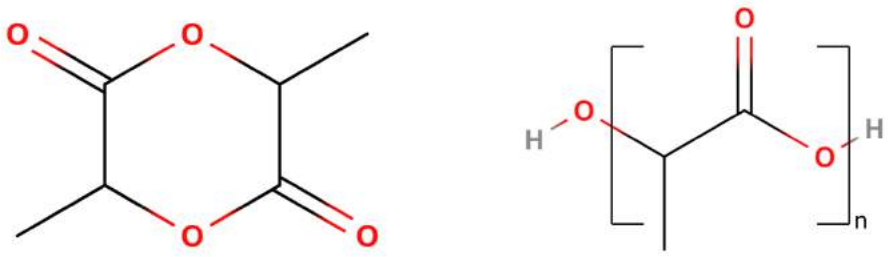
The purpose of this study is to determine whether consumer PLA products break down into nanoplastics, and characterize these if they do. The characteristics of interest are their size, morphology, chemical composition and surface charge of the particles. Further, the toxicity of these particles to the zooplankton *Daphnia magna* will be examined.

2 Theory

2.1 Polylactic acid

PLA is a bio-based, compostable polymer. Bio-based means that the raw material for the polymer is based on biological matter, as opposed to the oil that fossil-based polymers are made of. A biodegradable polymer is one that will break down into smaller fragments with the help of microorganism action [6]. Some groups of bacteria that can produce PLA degrading enzymes are the *Chryseobacterium* sp., *Sphingobacterium* sp. and *Amycolatopsis* sp., among others [7]. A compostable polymer is always biodegradable, but has some extra criteria to fulfill. These are that no negative effects on the quality of the resulting compost are allowed, and the rate of degradation has to be consistent with other composting materials [8].

PLA is built by ring-opening polymerization of the lactide monomer, Figure 1a, into long chains, Figure 1b.[9]



(a) (b)

Figure 1: (a) Lactide monomer. (b) Lactic acid as a polymer. The n describes how many monomers are present in the polymer.

2.1.1 Biodegradation and breakdown mechanisms

PLA is compostable in industrial composting facilities, where they can control the temperature, humidity and environment. The temperature must be at least 60 ℃ and the humidity high for hydrolysis to occur. After this the polymers can be degraded by microorganism action [9]. In these conditions the plastic can be degraded within three months [10]. These conditions are not likely to occur in the environment, especially not marine or other aqueous environments. This means that in nature, PLA is not biodegradable and

will degrade through other mechanisms.

These mechanisms are photo-, thermal, mechanical and chemical degradation. Through these mechanisms micro, and nanoplastics may be formed and released into the environment. One example of mechanical breakdown would be plastic rubbing against sand on a beach.

2.2 Characterization of nanoplastics

2.2.1 Size, morphology and stability

To measure the sizes of nanoparticles one can use techniques such as Nanoparticle Tracking Analysis (NTA), or Dynamic Light Scattering (DLS).

The NTA is a tool that can view and track multiple nanoparticles in the size range 30 - 1000 nm. The lower limit changes a bit depending on the refractive index of the material. A higher refractive index lowers the lower limit. The instrument illuminates the particles with a laser and captures videos of these with a camera. The instrument then tracks individual particles, and is able to tell the diameter and concentration of particles in a solution. When making measurements with the NTA, the user gets to decide the duration of each video capture, as well as the number of captures per measurement. The measurements acquired are the size of the particles (diameter) and the concentration (particles/ml). [11]

The DLS also uses a laser on the particles so they scatter the light. The intensity of the scattered light is recorded and used to calculate the velocity of the Brownian motion, which is used to calculate the size of the particles. The size range of a DLS is 0.3 nm - 10 µm, depending on the specific machine used. The DLS cannot determine the concentration of the samples. [12]

In certain DLS machines it is also possible to measure the surface charge, or zeta potential, of colloidal particles. A colloid is another name for particles dispersed in a solution, and the stability of it can oftentimes be determined by measuring the zeta potential. According to the DLVO theory, the stability of a colloid system is determined by the attractive van der Waals forces, and the repulsive forces of the electric double layer on the particle's surface. In a stable solution the repulsive forces are greater than the attractive forces, and this will keep the particles from agglomerating. The zeta potential is a part of what makes up the strength of the electric double layer, and a value above ±30 mV is considered stable. [13]

The morphology of particles at the nanometer scale can be imaged in a

Transmission Electron Microscope (TEM). These work by sending out electrons in a focused beam towards the sample. Most electrons will pass through the sample grid and be detected with their full energy. A small portion of the electrons will interact with the sample, and reach the detector with lower energy. In "mass-thickness" mode, a sample with a larger mass and/or thickness will make the image appear darker due to larger loss of energy in the electrons. In a sample with homogeneous mass, a darker color will most likely indicate a thicker part of the sample. [14]

2.2.2 Chemical composition

The chemical composition of carbon based materials can be measured with Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy. A laser with wavennumbers from 450 to 4000 cm^{-1} scans the surface of the material, and the absorbance is measured. Different types of chemical bonds and atoms will absorb the energy of the laser at different wavenumbers, which results in very specific spectra acquired for different materials. The region between the wavenumbers 500 and 1500 cm^{-1} is called the fingerprint region, and this can be used to identify samples. The individual peaks here are difficult to connect to specific functional groups, but if the spectra has the same peaks in this region for two samples, they are the same material. Above 1500 cm^{-1} the peaks are more specific to functional groups. [15]

2.3 Toxicity

When testing chemicals for toxicity it is advised to follow guidelines developed by the Organisation for Economic Co-operation and Development (OECD). Test No. 202: *Daphnia* sp. Acute Immobilisation Test and Test No. 211: *Daphnia magna*. Reproduction Test are developed to measure toxic effects in *Daphnia magna*. In Test No. 202 the duration is 48 hours and the time at which the *D. magna* become immobile is recorded. In Test No. 211 the duration is 21 days, the *D. magna* are fed, the offspring produced is counted and eventual mortality recorded. [16, 17]

2.3.1 *Daphnia magna*

Daphnia magna, or *D. magna* is a freshwater zooplankton often used in experiments for determining toxicity. As adults they are 2-5 mm in size, and

this is an advantage when working with them as they can be seen with the naked eye. *D. magna* is a filter feeding plankton that mainly feeds on algae, but can also survive on microorganisms such as bacteria and fungal spores. As the *D. magna* is transparent their guts are visible when full and the plankton are well fed. *D. magna* has an average lifespan of around 56 days if kept at 20 ℃and are fed properly. The females become sexually mature within 6-10 days of hatching. They may then reproduce either sexually, or asexually. Asexual reproduction is most common when living conditions are good. The female plankton will then produce 6-10 eggs, which will hatch in her carapace and the offspring is released the next time the female molts. Usually within three days. Sexual reproduction only happens when conditions are unfavorable, and these eggs are encased in an ephippia where they will hatch when the conditions become favorable. [18]

D. magna has been shown to be able to ingest particles of sizes as small as 20 nm up to 40 µm. [19, 20]

2.3.2 Previous studies on nanoplastic toxicity

Previous experiments with nanoplastics and *D. magna* have been made, where the plastics used were 100 nm fluorescent polystyrene (PS) beads [21], 53 nm aminated PS particles [22, 23], 26 and 62 nm carboxylated PS particles [23] and polyethylene (PE) breakdown particles [24].

The 100 nm PS beads were used to measure uptake and changes in filtration rates, and comparing these to 2 µm fluorescent PS beads. The experiment was carried out both short term (24h) and long term (21 days). In the long term experiment the filtration rate went down by 21% when the 100 nm PS beads had been ingested. [21]

The aminated and carboxylated PS particles were tested in both short term (24 h) and long term (103 days) exposures. The aminated PS particles were toxic in both, while the carboxylated only showed signs of toxicity in the long term experiments. [22, 23]

The breakdown PE particles were broken down with an immersion blender and showed toxic effects in a long term experiment (134 days). However, when fractionated through a 10 kDa filter, they lost their toxic effects. The fraction with particles under 10 kDa in size were also tested for, and showed signs of toxicity. [24]

3 Experimental

The different types of PLA products examined in this study were soup cup lids [25], coffee cup lids [26], 3D printer filaments [27], plastic cups (Duni, bought at ICA) and plastic bags [28]. A sample of 250 nm manufactured nanoparticles of PLA (hereby referred to as PLA NPs) were purchased from CD bioparticles [29], and amorphous PLA powder was purchased from Sigma-Aldrich [30]. The PLA NPs came in DI water, and a 1.0% w/v ratio. The states of these products is unknown, apart from the powder, which was amorphous. The other products may have been either amorphous or crystalline. Images of the products can be found in the appendix.

3.1 Preparation of nanoparticles

The PLA nanoparticles were prepared by using an immersion blender (Bosch ErgoMixx 600W (E-nr: MSM66020/1), Robert Bosch Hausgeräte GmbH, Germany) and above mentioned products made from PLA. The standard breakdown procedure was to weigh 2 g of PLA product and cut it into small pieces (ca 1 x 1 cm) into a beaker. The beaker was then filled with 150 ml Milli-Q water and the blender was turned on at maximum speed for 2 minutes. A 50 ml syringe was used to remove 75 ml of the water, which was filtered through a 1.0 µm filter cloth into another, smaller, beaker. This water was then filtered through a 0.8 µm cellulose acetate syringe-filter (Whatman, GE) into a glass bottle storage container. If more particle solution was needed, the larger beaker was filled with another 75 ml Milli-Q water, and the blending process and filtering was repeated.

3.2 Characterization

3.2.1 Size and stability

The stability of the PLA nanoparticles in water was tested by making particles according to the standard breakdown procedure in section 3.1, making a total of 150 ml. The particle size and concentration were then measured with NTA using NanoSight LM10 (Amesbury, UK) and the software NanoSight NTA 3.1 on the day of breakdown, and again after 6 days to test if the particles had agglomerated. All PLA products were tested like this. The settings used with the NTA were: 5 video captures, each 60 seconds long.

The sizes were also measured using DLS in a Zetasizer Nano S (Malvern Instruments, Worcestershire, UK), where the same sample was measured three times consecutively, and the result was presented as an average.

3.2.2 Surface charge

The zeta potential was measured for all the PLA products and the PLA nanoparticles. This was done by breaking down the particles according to the standard breakdown procedure. The soup cup lid, plastic bag and 3D printer filament particles were then concentrated using a VivaFlow (VIVAFLOW 50, Sartorius) to improve the data collection. 1 ml of the particle solution was then inserted into a cuvette, and measured in a Zetasizer Ultra or Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The PLA NPs were diluted 1 000x and then measured. As in the DLS size measurement, each sample was measured three times consecutively, and the results presented as the average of these.

3.2.3 TEM

The soup lid, coffee lid, and 16 h UV-C exposed coffee lid were broken down according to the standard breakdown procedure. 2 µl of the sample was added to a pioloform-coated single slot grid (Ted Pella, Cu, Pelco Slot Grids, USA), and left to air dry over night. The samples were then inserted into a JEOL JEM-1400 PLUS TEM operated at 100 kV (JEOL Ltd., Japan), where micrographs were obtained using TEM Centre for JEM1400 Plus software.

3.2.4 FTIR

All PLA products, a pure amorphous PLA granules and the PLA nanoparticles were analyzed in an FTIR Spectrum Two (PerkinElmer) using the software PerkinElmer Spectrum IR. The products were analyzed both before and after breakdown. The acquired spectra were then compared to the spectra in the software library.

3.3 UV light exposure

2 g samples of soup cup lids were exposed to UV-C (254 nm) light in a UV chamber (CL-1000 Ultraviolet Crosslinker (UVP/Analytik Jena UV Crosslinkers)) for 0, 1, 2, 4, 8, 16 and 32 hours. The lids were analyzed in

the FTIR to take note of any changes. 2 g samples of coffee cup lids were also exposed to UV-C, for 0, 1, 2, 4, 8 and 16 hours. These broken down according to the standard breakdown procedure and the particles were imaged in the TEM.

3.4 Toxicity

3.4.1 0.8 µm filtered water

In the first toxicity study, the plastics used were the plastic bag, the soup cup lid, and the soup cup lid that had been exposed to UV-C for 16 hours. The plastic bag was broken down using the standard breakdown procedure, for a total of 300 ml nano particle solution. This was done on day -5 of the experiment. The soup lid and UV-C treated soup lid were broken down in the same way, with the difference that all 150 ml of the water was filtered after breaking it down with the blender for two minutes. This way two lids were necessary to make 300 ml nano particle solution. This was done on day -3 of the experiment. All particle samples were filtered through cloth filters and 0.8 µm filters as described in the standard breakdown procedure. The water used was regular tap water from the ecology building at Lund University instead of Milli-Q water. This was not filtered through any filters. The concentrations and sizes of the particles were measured in the NTA, and are presented in Table 1. The plastic bag particles were measured in the NTA on day -5, and both types soup cup lid particles were measured on day 0.

Table 1: The sizes and concentrations of nanoplastics used in toxicity experiment where all particles were filtered through a 0.8 µm spin filter.

	NTA mean (nm)	NTA mode (nm)	Concentration (particles/ml) (10^8)
Soup lid	237	177	1.54
16h UV-C soup lid	179	145	2.45
Plastic bag	214	149	74.3

The particle solution was then allocated to 50 ml Falcon tubes. There were ten replicates, with 25 ml water in each tube for each type of plastic. In total there were 40 tubes; 30 with nanoparticles and 10 as control.

On day 0, one *D. magna* was added to each tube randomly. All *D. magna* used were less than 24 hours old. They were checked on daily, with a few

exceptions on weekends, until all of them were immobile. The *D. magna* were not fed once placed in their respective test tubes.

3.4.2 0.2 µm filtered water

The above experiment was performed once again, as the *D. magna* exposed to nanoplastics lived longer than the control. It was suspected that there may be bacteria in the water, that the *D. magna* could eat. Therefore all the water was filtered through 0.8, 0.45 and 0.2 µm syringe-filters so that any bacteria would be filtered out. Nanoparticles were prepared from the plastic bag and the soup lid in the same way as the previous toxicity test. These were filtered after the breakdown. The PLA NPs were also used, but since they were already nano-sized, they did not need to be broken down. 300 µl of these were added to 299.7 ml filtered water to reach a similar concentration as the plastic bag particles. The control was also filtered, but did not contain any particles. The water used was again from the ecology building. All particle solutions, and control samples were prepared on day -1 of the experiment. The final concentrations used are presented in Table 2, and were measured on day 1.

Table 2: The sizes and concentrations of nanoplastics used in toxicity experiment where all particles were filtered through a 0.2 µm spin filter.

	NTA mean (nm)	NTA mode (nm)	Concentration (particles/ml) (10^8)
Soup lid	185	140	2.25
PLA NPs	298	173	2.59
Plastic bag	202	257	30.5

The water was then transferred to 50 ml Falcon tubes with 25 ml water in each tube. Again, there were ten replicates per type of plastic and control. *D. magna* less than 24 hours were added randomly to the tubes, one per tube, on day 0. They were checked on daily until day 23. After this they were checked on again on day 28, 30, 32 and 34. Once again the *D. magna* were not fed during the experiment period.

3.4.3 Statistical analysis

Statistical analysis of the survival was performed using the statistical computing software GraphPad Prism version 9.3.1 (471) for Windows (GraphPad Software, Inc., `www.graphpad.com`). The analysis performed were the Log Rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. These tests were performed to find a significant difference between the control and each of the particle samples.

4 Results and discussion

4.1 Preparation of nanoparticles

By breaking down the PLA products mechanically with an immersion blender and then analyzing the water, it was proven that PLA nanoplastics were formed during breakdown. The breakdown process was intended to be used to prove that nanoplastics could be formed, as well as being a convenient way of producing nanoplastics for characterization and toxicity studies. For these purposes it worked very well. It is impossible to say if the nanoplastics produced have the same morphology or sizes as ones possibly existing in nature.

Since this breakdown procedure is rather violent for the plastic, it is possible that chemical changes occur. If, for example, the starting PLA material was crystalline, the particles could become amorphous, and vice versa.

There are some limitations to the breakdown process, one being that the concentration of particles will never be the same for two different break downs. It is therefore not possible to compare small differences in concentration. Should a comparison be made, the difference should be fairly large, or the certain products should be broken down multiple times to acquire an average.

Another point of note is that the blender itself releases nanoparticles during use. The amount is not nearly as much as the nanoplastics formed, but still worthy of taking into account. Efforts were made to find a blender that released as few particles as possible.

4.2 Characterization

4.2.1 Sizes and stability

The sizes of the PLA products were measured in the NTA and the DLS after breakdown in Milli-Q water, Table 3. The majority of the particles were around 130 nm in diameter measured by the NTA (mode), regardless of product type. The mean value showed more differences in size. For the products with a much larger mean value than mode value, there were some larger particles present as well, but most particles were the size of the mode value. Measurements by the DLS were also a mean value, and most results were in between the mean and mode values of the NTA. The true value of the particles is impossible to know, especially since they are all different in size. However, since all measurements are around the same values, it is possible to get a rough idea of what sizes may be present.

It is also possible that there are much smaller particles present, and that they could not be detected. Both the NTA and DLS use lasers to visualize the particles. Since larger particles scatter more light, it is possible that the light scattered from smaller particles would drown out, like the stars behind a street light.

Table 3: Sizes measured in the NTA and DLS on the day of breakdown, day 0. The measurements of the PLA NPs are included as well.

	NTA mean (nm)	NTA mode (nm)	DLS (nm)	DLS PDI
Soup lid	155	132	169	0.22
Coffee lid	200	140	158	0.21
Plastic bag	230	133	193	0.24
Plastic cup	154	134	134	0.22
3D printer filament	145	129	137	0.25
PLA NPs	276	264	289	0.08

To determine if the particles would stay stable in Milli-Q water, similar sizes and concentrations were expected on the day 0 and day 6 measurements. The sizes could be compared in Tables 3 and 4, and they seemed to be similar to each other. The sizes of the coffee lid particles were smaller after one week.

Table 4: Sizes of the breakdown particles measured in the NTA 6 days after breakdown. These were the same particles as in Table 3.

	NTA mean (nm)	NTA mode (nm)
Soup lid	152	123
Coffee lid	171	130
Plastic bag	221	132
Plastic cup	140	121
3D printer filament	147	129

The concentrations of the particles were compared in Table 5, and these also seemed similar to each other. Some differences were to be expected due to how the measurement is made in the NTA, and the samples were therefore deemed stable in Milli-Q water for the time period.

Table 5: Concentration of the breakdown particles on both day 0 and day 6, measured in the NTA.

	Day 0 (particles/ml) (10^8)	Day 6 (particles/ml) (10^8)
Soup lid	7.14	7.01
Coffee lid	4.22	5.34
Plastic bag	67.3	75.2
Plastic cup	6.25	6.85
3D printer filament	15.6	14.1

4.2.2 Surface charge

The surface charge of the PLA products and the PLA NPs were measured in the DLS, Table 6.

Table 6: The surface charge of the breakdown particles and the PLA NPs. The soup cup lid, plastic bag and 3D printer filament were concentrated 10x to be able to obtain results.

Product	Zeta potential (mV)
Soup lid	-22.13
Coffee lid	-5.89
Plastic bag	-16.25 & 3.25
Plastic cup	-9.59
3D printer filament	-44.41 & 42.67
PLA NPs	-14.94

As mentioned in section 2.2.1, a colloid system is considered stable if the charge is greater than ± 30 mV. The only product that measured greater than this was the 3D printer filament. However, this sample had two values in the measurement. This could indicate that there are two different species of particles in the solution. Since they have opposite charge they should attract each other, and the colloid should therefore be unstable. The same is true for the plastic bag, which also had two values. Though the 3D printer filament and plastic bag particles should be unstable in water, they showed no sign of agglomeration in the NTA measurements. This could mean there were not two different particle species in these samples and the measurements were wrong. It could also mean that one of the particle types were too small for the NTA to measure, and they did in fact agglomerate after one week. Then they would be stable enough for the zeta potential to be measured right after breakdown, but agglomerate soon after. However, this does not seem very likely as the agglomerates would be measured as larger as they would be within the size range of measurement for the NTA. A way to test this would be to measure the zeta potential after one week of breakdown.

The other particles should also be unstable according to the zeta potential. For the PLA NPs, this should not be true, as the manufacturer guarantees that they should stay stable for at least six months if stored correctly. As for the soup lid, coffee lid and plastic cup particles, they seemed stable in the NTA, but should not be stable according to the zeta potential. The reason for this could be that they need more time to agglomerate than one week. However, the coffee lid particles had a particularly small value of the zeta potential and should therefore agglomerate quickly.

These results with low zeta potential, and possibly stable particles, sug-

gest that perhaps the zeta potential alone cannot determine stability of particles.

4.2.3 TEM

Soup lid

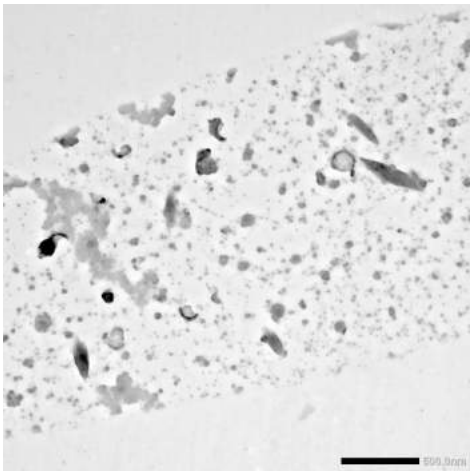
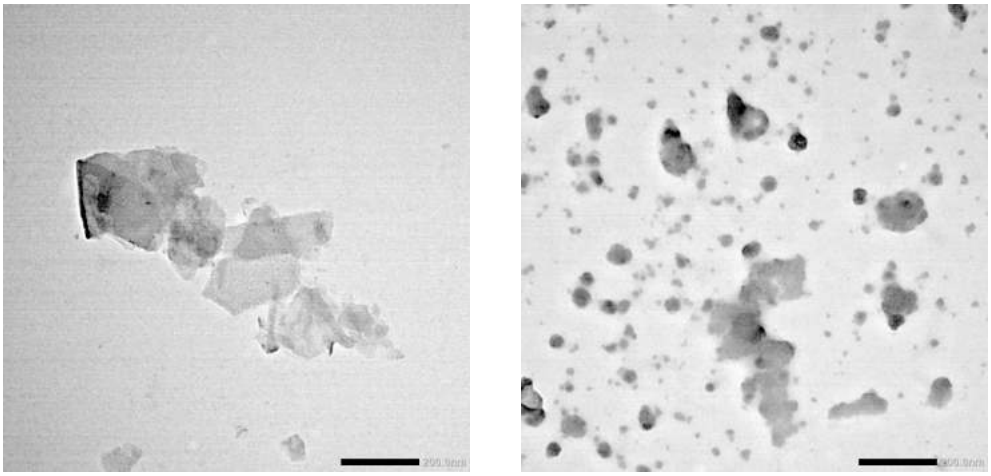


Figure 2: Overview of the soup lid particles. The scale bar is 500 nm. Particles of multiple sizes and shapes are depicted.

In the overview of the soup lid particles, Figure 2, many different shapes and sizes could be observed. Some of the particles seemed to have sharp edges, and some seemed to have rounder edges.



(a) (b)

Figure 3: (a) depicts a particle that has sharp edges. The scale bar is 200 nm, which is about the same as the width of the particle. (b) shows multiple particles of different sizes. They all seem to have rounded edges. The scale bar is 200 nm, so the smaller particles are around 50 nm.

When zooming in on the particles, Figure 3 a and b, it was easier to distinguish the morphology and sizes of the particles. The particles with sharp edges seemed to be larger, and the particles with rounder edges seemed to be smaller. Many of the particles in Figure 3b seem to be smaller than the scale bar of 200 nm. A majority seem to be smaller than 100 nm as well.

Coffee lid

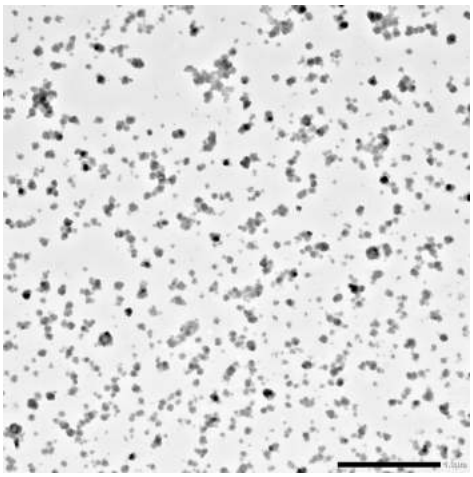
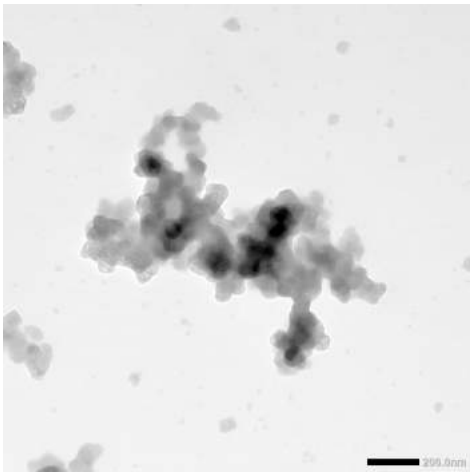
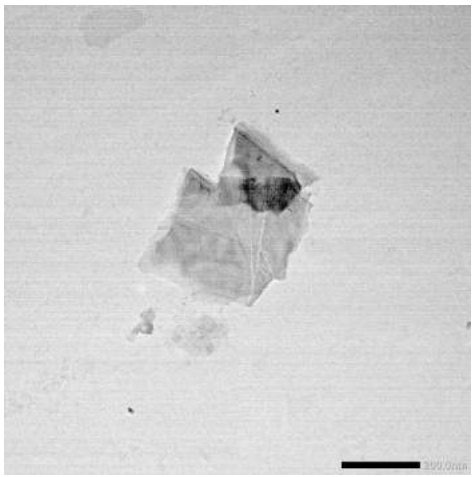


Figure 4: An overview of the coffee lid particles, imaged in a TEM. The scale bar is 1 µm. Most particles seem to look similar to each other.

In Figure 4 an overview of the coffee lid nanoplastics could be seen. Most of these particles seem to be small with rounded edges, or clusters of these.



(a)



(b)

Figure 5: (a) Particle found with rounded edges. The scale bar is 200 nm, so the imaged particles is quite large. (b) shows a particle with sharp edges, that is closer to 300 nm in size.

There were also some particles with sharper edges, as in Figure 5b. These, like the soup lid particles, seemed to be on the larger end. The particles with rounder edges seemed to have a larger size diversity, as seen in Figure 5a. There, a large particle was in the center and a smaller particle could be found on the left edge.

UV-C treated coffee lid

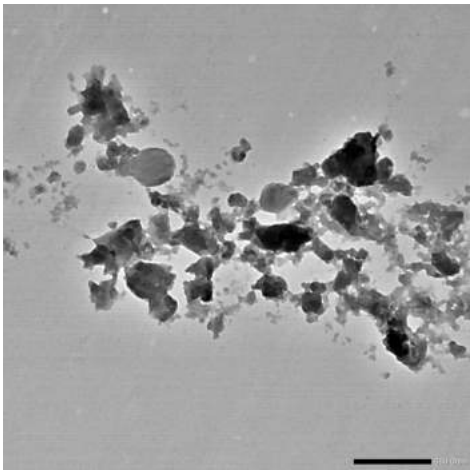
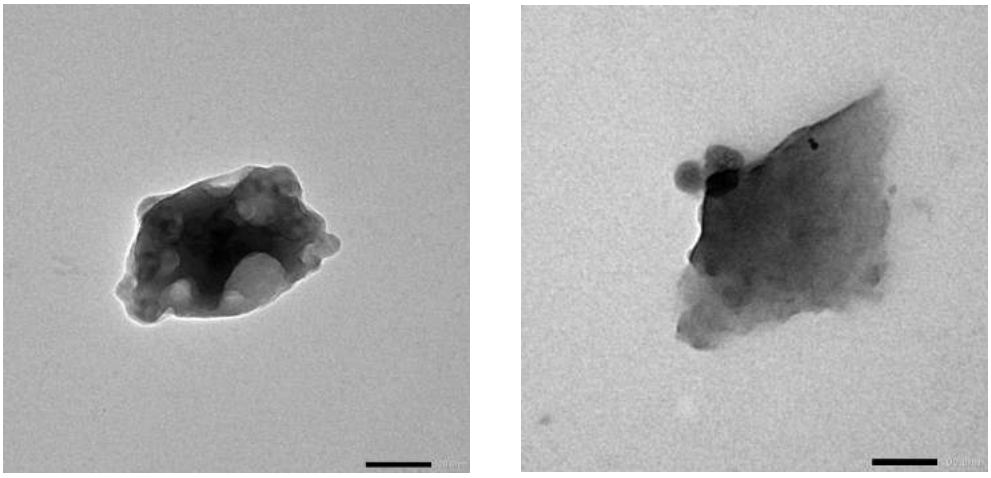


Figure 6: Overview of UV-C exposed coffee cup lid particles. The scale bar is 500 nm. Multiple particles seem to be present, and they are darker than previous particles.

The UV-C treated coffee lid particles were a bit more difficult to distinguish. In the overview, Figure 6 most of the particles were clustered together. They were also darker than the previous particles, which should mean that they were more dense, or thicker.



(a) (b)

Figure 7: Particles with both round (a) and sharp (b) edges. In both images the scale bar is 100 nm. The particles are darker than previous particles, and may therefore be thicker.

The particles again had both rounded and sharp edges, as seen in Figure 7. However, the sharp edges were not as sharp as the previous two samples.

All the different lids imaged had particles of different sizes, with both rounded and sharp edges. The fragments with sharp edges look as if they have been cut with something sharp, like scissors or knives. This would make sense since the breakdown was performed with an immersion blender. The same logic could not be applied to the particles with rounded edges, and these may instead be a component of the plastic which had the round shape when it was fabricated. When considering the toxicity of these two types, there may be a difference. This would need to be studied more specifically.

4.2.4 FTIR

All PLA pruducts, as well as the amorphous PLA powder and PLA NPs were characterized in the FTIR, Figure 8. When compared to each other in the software, the amorphous PLA and PLA NPs had a 92% similarity. When compared to spectra of pure PLA in other articles, the peaks seemed to appear at the same wavenumbers as well [31, 32, 33, 34]. It is therefore highly likely that these samples were pure PLA with no additives, although there is always the possibility that an additive has been used in a very small

amount. The spectrum would likely not look very different, as the peaks from a small percentage of something else would be too small to be seen. The reason that the spectrum of the PLA NPs has a small dip at the 3 500 - 3 000 cm^{-1} region could be due to the particles being in a water solution. All peaks found are listed in the Appendix, in Table 7. When searching for similar spectra in the software library, the closest match was of (S)-(+)-Alpha-Methoxyphenyl Acetic Acid with a 78% similarity. It is therefore likely that a PLA spectra does not exist in the library.

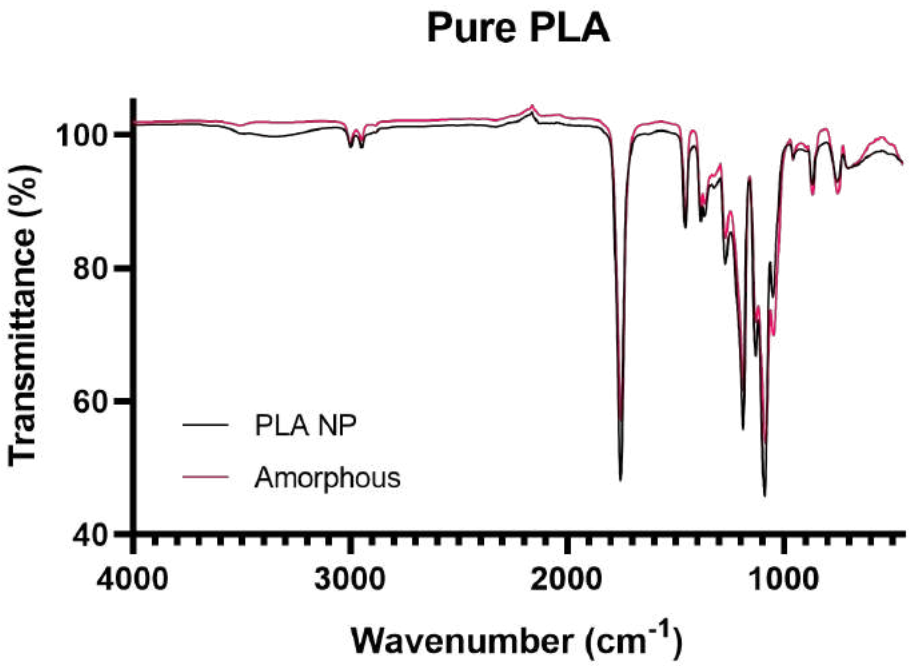


Figure 8: FTIR spectra of pure PLA products. The black line shows the spectra of the PLA NPs, and the pink line shows that of the amorphous PLA powder.

When comparing the PLA products to the PLA NP in the FTIR software, the plastic cup, soup cup lid and the 3D printer filament were the most similar, with 92%, 90% and 86% similarity, respectively. The spectra are presented in Figure 9. The closest fit was the plastic cup with no additional or missing peaks. The soup lid also had a close fit, apart from in the 500

cm^{-1} region. The 3D printer filament was very close to having all the same peaks as well, with a small difference in the 3 000 cm^{-1} region.

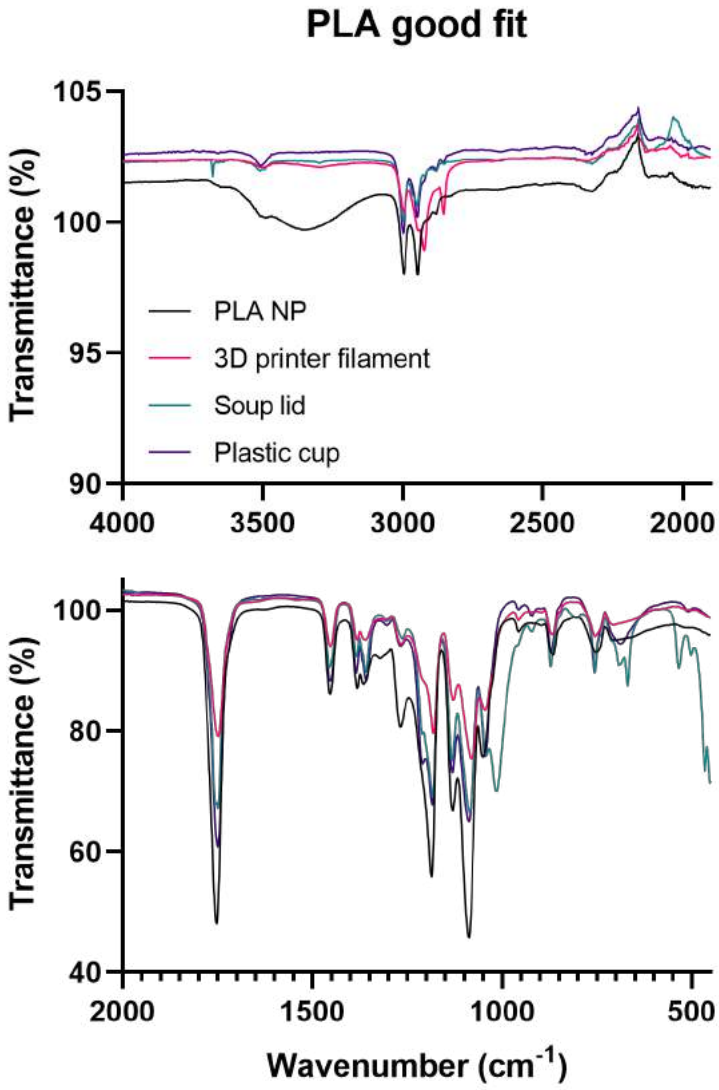


Figure 9: FTIR spectra of the products with the closest resemblance to the PLA NPs which were pure PLA. These were the 3D priter filament, the soup cup lid and the plastic cup. Upper panel: 4000 - 1900 cm^{-1}, lower panel 2000 - 450 cm^{-1} (fingerprint region). Note the difference in the y-axis.

The products which were the most different from pure PLA were the plastic bag and the coffee cup lid, Figure 10. These had a similarity of 15% for the plastic bag, and 5% for the coffee cup lid. The plastic bag had a two peaks that overlapped, at 1454 and 1268 cm^{-1}, with the pure PLA in the fingerprint region, but there were also many additional and missing peaks. The coffee lid peaks were much more different even though two peaks at 1454 and 753 cm^{-1} overlapped with the pure PLA. Almost all existing peaks were at different wavenumbers from the pure PLA. When searching for similar spectra in the software library, the plastic bag got several matches with different types of polyesters. The closest was of 'Polyester, Terephtalic Acid Film/MTC/CSI' with an 87% similarity. Several of the other matches were also of terephthalic acid films. The coffee cup lid got a 99% match with 'Polystyrene, Monocarboxy Terminated', and all other matches were also of polystyrene. Before making these searches, one could believe that the different peaks would come from different additives. However, for almost all the peaks to change position, the additives would probably have to be the main component, as the PLA peaks should still be visible. It would therefore be more likely that the main component of the material is something other than PLA.

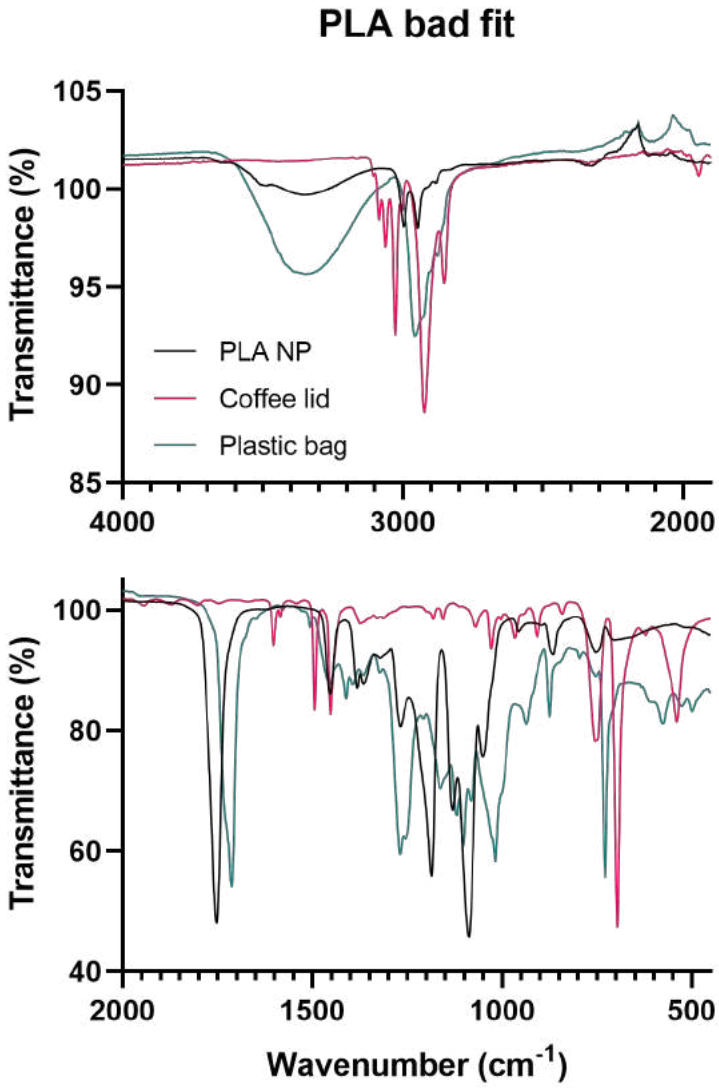


Figure 10: FTIR spectra of the products least similar to the PLA NPs. These were the coffee cup lid and the 3D printer filament. Upper panel: 4000 - 1900 cm^{-1}, lower panel 2000 - 450 cm^{-1} (fingerprint region). Note the difference in the y-axis.

Breakdown spectra

The nanoplastics acquired from breaking down the PLA products were characterized in the FTIR where they had some peaks at the same wavenumbers, Figure 11. These peaks were found at 2923, 1740 and 1657 cm^{-1}. The spectra of the 3D printer filament could have some more peaks that are not visible, since the baseline of it was not stable at 100%.

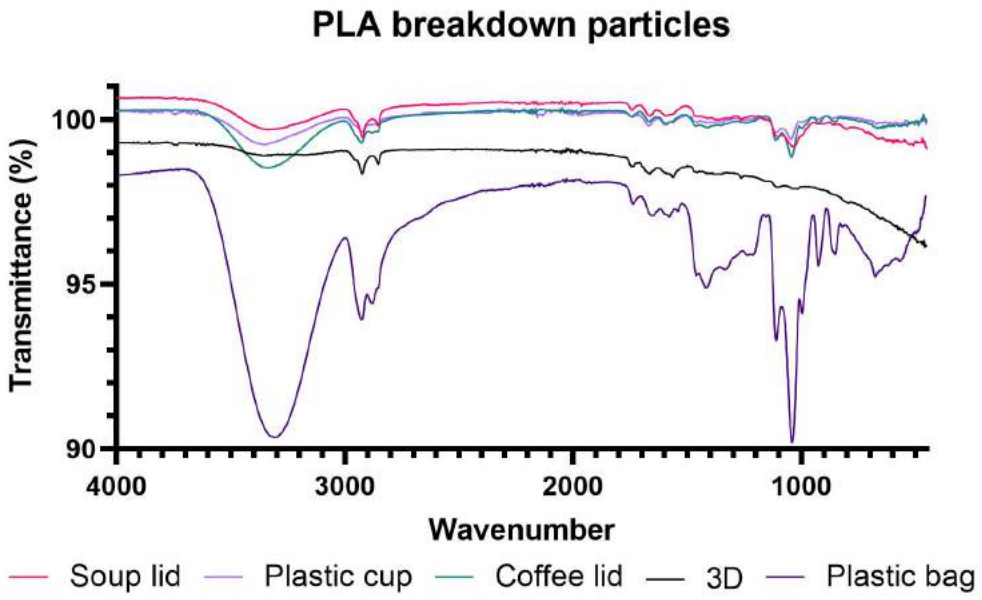


Figure 11: FTIR spectra of the breakdown particles.

Comparing the breakdown spectra with their respective standard samples, the soup cup lid had no matching peaks, the plastic cup had one matching peak at 2854 cm^{-1}, the 3D printer filament and coffee cup lid had one matching peak at 2923 cm^{-1} and the plastic bag had a matching peak at 3336 cm^{-1}. There were some peaks in the breakdown spectra that almost overlapped with the standards, but were shifted a few wavenumbers higher or lower. These should not count as matches, as the FTIR is very specific.

What is interesting is that the spectra of the coffee cup lid particles looks almost identical to the spectra of the soup cup lid and plastic cup breakdown spectra when it seemed to be made of a different material. What happens during the breakdown procedure that changes the spectra so drastically is

unknown. Either there is something happening to the particles, or perhaps it is the filters that release particles during the filtering step. There is no spectra of water that has been filtered, but could be made in the future to compare and analyze. It is not very likely that it is the filter that changes the spectra though, as there should be more filter particles than PLA particles in the water had that been the case.

UV-C exposed soup lids

The soup cup lids were exposed to UV-C, and then the changes were recorded in the FTIR. As can be seen in Figure 12, the transmittance at the peaks goes down the longer the lid has been exposed. This with the exception of 4 hour exposure, as these peaks are the smallest.

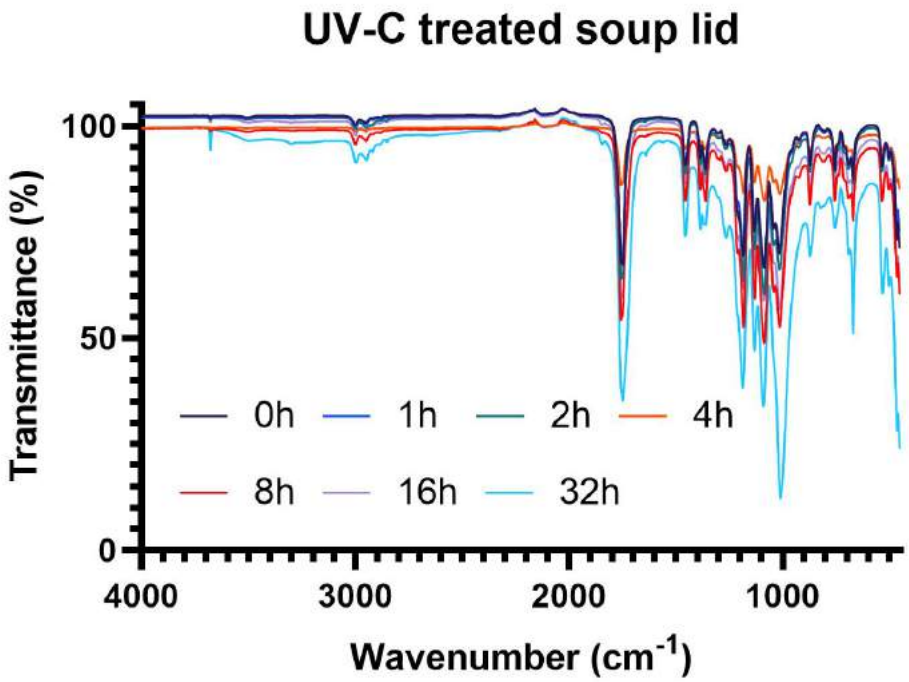


Figure 12: Spectrum of soup lids which have been exposed to UV-C for 0, 1, 2, 4, 8, 16 and 32 hours. As the lids oxidize, the transmittance gets lower and lower at the peaks.

4.3 Toxicity

The parameters which were considered in the toxicity experiments were the survival, and the reproduction.

4.3.1 0.8 µm filtered water

As seen in Figure 13, the control *D. magna* were the first to become immobile. There is a clear difference in the survival between the different samples. The first control became immobile on day 4, the first *D. magna* in soup lid particles on day 7, the first in UV-C exposed soup lid particles on day 8, and the first in plastic bag particles on day 16. The *D. magna* in soup lid particles, and the UV-C exposed soup lid particles had a very similar survival. All *D. magna* in particle solution lived longer than the control, with a statistical significance of four stars.

As the days went on, the surviving *D. magna* grew and became larger than they were in the beginning. They were not measured, but the size was more like that of an adult plankton than a neonate.

None of the *D. magna* showed any signs of reproducing.

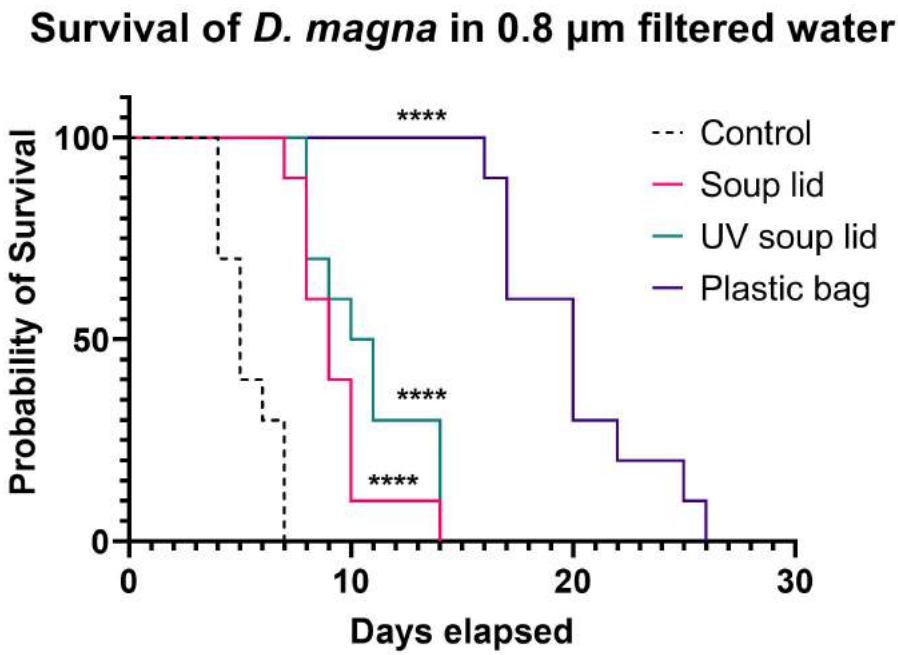


Figure 13: Survival of *D. magna* in water with PLA nanoplastics, and control. There is a significant statistical difference between the control and all particles, denoted with ****. Here, $P < 0.0001$ in both statistical tests.

As there was no algae in the water for the plankton to eat, they were expected to die quickly. Since all plankton with nanoplastics in the water survived longer than the control, it is likely they had something to eat. This was further supported by looking at their guts during the experiment. As the guts of the control *D. magna* turned transparent, the guts of the other *D. magna* were still somewhere between black and transparent. This indicates that there was something in the water they could feed on.

As the particles were in tap water, they were not stable and should have formed aggregates. One hypothesis was that bacteria may attach to and proliferate on these plastic particles, and the *D. magna* could feed on the bacteria. These bacteria could either come from the breakdown process, where no gloves were used, the tap water, or the *D. magna* themselves, as they are covered in bacteria. To test this hypothesis, the experiment was performed once again, with all water filtered through 0.2 µm filters.

There are many other variables that could contribute to differences in

survival. The concentration of particles were different for the plastic bag and the soup lids, and as the *D. magna* survived for different amounts of time in these different samples, this could be a reason. This was, however, a deliberate difference between the two samples as it was easier to get many particles with the plastic bag. Therefore it was used despite having a different chemical composition. The chemical composition is another variable that may have affected the results.

4.3.2 0.2 µm filtered water

Here, the *D. magna* were exposed to particles from a soup cup lid, plastic bag, and the PLA NPs that were 250 nm in size. The first *D. magna* to become immobile were the ones exposed to the PLA NPs, the ones exposed to the soup lid particles, and the control on day 4. The first of these where all the *D. magna* had become immobile was the PLA NPs, on day 9. After that all control had become immobile on day 15, and the ones exposed to soup lid particles were found immobile on day 28. Since the plankton were not checked on from day 23 to 28, they may have become immobile any of these days. The *D. magna* exposed to plastic particles started to become immobile on day 11, and all were immobile on day 34.

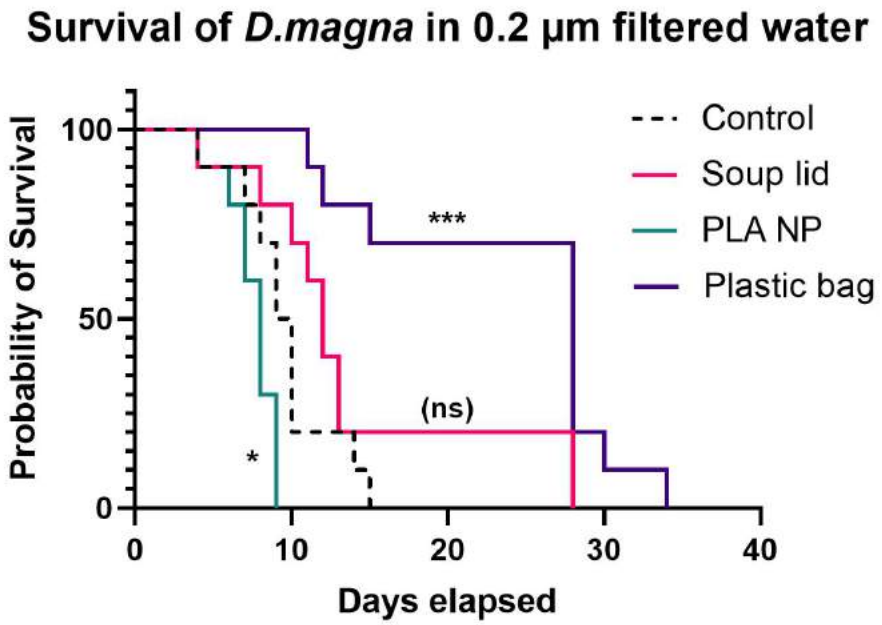


Figure 14: Survival of *Daphnia magna* in tap water with nanoparticles, and control. There was a statistical significance between the control and the PLA NPs (*) and between the control and plastic bag particles (***). There was no significant difference between control and the soup cup lid (ns). * indicates that $P < 0.05$. *** indicates that $P \leq 0.0002$.

All bacteria apart from those living on the *D. magna* should have been filtered out with the 0.2 µm filter in this experiment. Still the plankton lived longer when exposed to the plastic bag particles. As with the previous experiment, the concentration of the plastic bag particles was higher than the soup lid particles so more particles should have been filtered through the plankton. Since PLA does not degrade in nature, it seems unlikely that the plankton should be able to get any sustenance from the particles. This would mean that either the bacteria on the *D. magna* proliferate enough that they can sustain the plankton for multiple days, or there is something else in the water for the plankton to feed on.

The PLA NPs, on the other hand, had a slightly toxic effect on the *D. magna*. They were added in a similar concentration as the soup lid particles, but the sizes of these particles differed. The PLA NPs were larger, which

could have an effect on the toxicity. Another reason could be that the surface of the particles are different. A particle that has been manufactured would probably has a smoother surface than one that has been torn apart with a blender. If the surface is smooth, and it is bacteria keeping the plankton alive, then perhaps the bacteria could not adhere to the nanoparticle surface. If this is not the case, it is also possible that the particles get filtered into the guts of the *D. magna*, where they may block nutritional food.

4.3.3 Comparison to other nanoplastics

The short term experiments previously performed had showed some toxicity, depending on the size and concentration of the particles. These could be compared to the first 24 hours of the PLA toxicity experiments, as they would not have any food in the water. However, all *D. magna* used in this experiment survived longer than 24 hours, and the concentrations used would not be considered toxic in this time span. The long term experiments mentioned in section 2.3.2 should not be compared to this experiment as the *D. magna* were fed with algae, although it is still interesting to see that they show toxic effects, as the breakdown PLA particles showed an opposite toxic effect. The closest to showing a toxic effect were the PLA NPs, and to determine this more clearly, they should be tested in multiple concentrations.

5 Conclusions

What has been determined in this report is that all conventional PLA plastic products tested do break down into nanoparticles. The average sizes of these were between 145 - 230 nm while the mode size was around 130 nm for all of them. When imaging the particles in the TEM, they had different shapes and sizes. Some particles had sharp edges, while other particles were more rounded.

All breakdown particles were deemed stable when the sizes and concentrations were compared a week apart, but should be unstable judging by the zeta potential.

Out of the products tested, the plastic cup, soup cup lid and 3D printer filament were the ones with a chemical composition closest to pure PLA, and the coffee lid and plastic bag turned out to be different plastics. The coffee cup lid seemed to be made of polystyrene and the plastic bags of terephtalic

acid (PET).

After breakdown, the particles lost resemblance of their chemical composition to their whole counterparts. They did, however, look similar to each other regardless of whether they were PLA or not from the start. What this depends on is unclear. It could be due to the forces that the particles are subjected to during the breakdown, some component being soluble in water which may remove some peaks, or some other reason.

The breakdown particles showed no signs of toxicity in tests where the *D. magna* were not fed. On the contrary, the plankton seemed to live longer when there were breakdown particles in the water regardless of the size of the filters used. The reason that they lived longer is still unknown, one hypothesis is that there may be bacteria growing on the plastic particles that the plankton could feed on. When using the PLA NPs of the size 250 nm, there was a small toxic effect. The reason for this is also unknown, though it may be due to their sizes or surface chemistry.

5.1 Future work

When performing experiments such as this, it is inevitable to learn along the way and want to improve and further investigate the results. In the toxicity test, only short term tests were made, where the *D. magna* were not fed. These did go on for quite a long time, so instead it would be interesting to do a 24 hour test with multiple different concentrations. To do this one would need to break down a lot of plastic, and concentrate. Concentration was done in the PE particle experiment, where the particles were concentrated and then diluted to remove small particles from the water [24]. By doing this it would also be possible to determine if it is the PLA particles, or even smaller molecules that contribute to the longer life. Testing different concentrations could, and should also be done with the PLA NPs. These would not need to be concentrated as the stock solution was already very concentrated.

It would also be interesting to perform long term experiments where the *D. magna* are fed with algae. If the *D. magna* lived longer due to there being bacteria on the particles to feed on, this variable may be able to be removed. Then it would be possible to see eventual toxic effects, although an opposite effect would probably not be seen as all plankton would live out their whole life. An experiment like this would also show any differences in reproduction.

Regarding the coffee cup lid and the plastic bag, it would be interesting to find out if there are any common additives used in PLA, and test the

toxicity of these in both short and long term experiments.

When measuring the zeta potential of the 3D printer filament and the plastic bags, there were two peaks. Since this could indicate that there are two different species in the samples, this would be interesting to investigate further as well. A way to separate the particles would be needed.

6 Appendix

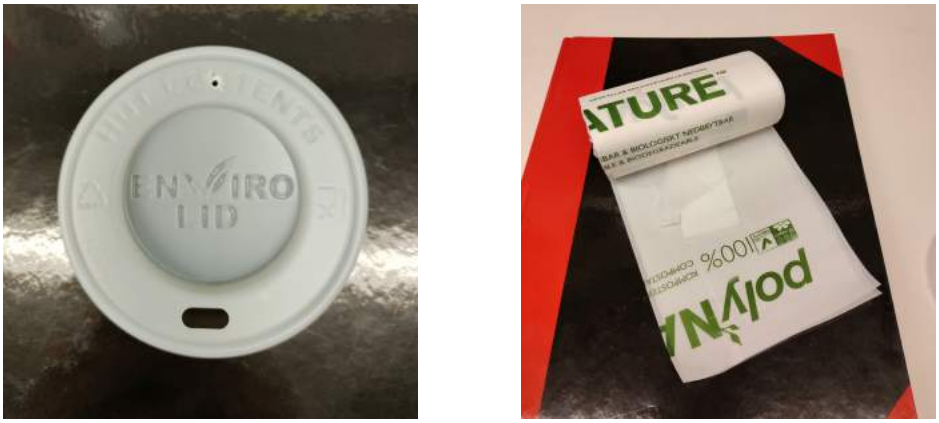
A PLA products

(a) (b)

Figure 15: (a) amorphous PLA powder and (b) PLA NPs.

(a) (b) (c)

Figure 16: (a) plastic cup, (b) soup cup lid and (c) 3D printer filament.



(a) (b)

Figure 17: (a) coffee cup lid and (b) plastic bag.

B FTIR Peak Tables

Table 7: Peaks found in the FTIR spectra of the amorphous PLA powder, PLA NPs, soup cup lid, 3D printer filament and plastic cup.

Wavenumber (cm-1)	Amorphous PLA	PLA NP	Soup cup lid	3D printer filament	Plastic cup
2997	x	x	x		x
2945	x	x	x		x
2923				x	
2854					x
1752	x	x	x	x	x
1454	x	x	x	x	x
1382	x	x	x	x	x
1358					x
1268	x	x		x	
1186	x	x	x	x	x
1130	x	x	x	x	x
1088	x	x	x	x	x
1051	x	x		x	x
1015			x		
956	x	x		x	
865	x	x	x	x	x
752	x	x	x	x	x
704	x	x	x	x	
687					x
533			x		
465			x		

Table 8: Peaks found in the FTIR spectra of the coffee cup lid and the plastic bag.

Wavenumber (cm-1)	Coffee lid	Plastic bag
3336		x
3060	x	
3026	x	
2954		x
2923	x	
2850	x	
1713		x
1601	x	
1494	x	
1454	x	x
1410		x
1373	x	
1268		x
1162		x
1155	x	
1119		x
1102		x
1081		x
1069	x	
1028	x	
1015		x
965	x	
936		x
907	x	
874		x
752	x	
727		x
695	x	
576		x
539	x	
499		x

C FTIR breakdown peak table

Table 9: Peaks found in the FTIR spectra of the breakdown PLA particles.

Wavenumber (cm-1)	Soup cup lid	Plastic cup	Coffee cup lid	3D printer filament	Plastic bag
3336	x	x	x		x
2923	x	x	x	x	x
2881			x		x
2854	x	x		x	
1740	x	x	x	x	x
1657	x	x	x	x	x
1592	x	x	x		
1577					x
1560				x	
1420			x		x
1110	x	x	x		x
1040	x	x	x		x
996					x
924		x	x		x
851		x			x

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