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Polymers of low concern? Assessment of microplastic particles used in 3D printing regarding their toxicity on *Raphidocelis subcapitata* and *Daphnia magna*

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Abstract

The potential effects of microplastic particle exposure on environmental organisms has sparked intense research activities. Various studies have been conducted, however on a limited set of mostly pristine polymer materials. In parallel to the ongoing research activities, it is discussed to include non-natural polymers into the registration process under REACH. Currently, non-natural polymers are exempted from registration, based on the general assumption of being non-hazardous due to their high molecular weight. In addition, the extensive number of polymers exceeding the registration capacities was mentioned as a reason for exemption. Hence, relevant polymers requiring a registration shall be selected according to specific criteria that help to identify those with a concern for hazardous effects. In the line of these developments we here present the results of a systematic ecotoxicity testing of 16 microplastic particles of different polymer composition, part of which are micronized polymer powders used in 3D printing (intentionally produced primary microplastic, losing particle shape by the 3D printing). All polymer materials were systematically varied and extensively characterised with regard to their properties (e.g. particle size, cross-linking, molar mass distribution, end groups, reactivity). Most of the polymers exerted toxicity in 48 h acute *Daphnia magna* immobilisation assay and 72 h chronic *Raphidocelis subcapitata* growth inhibition assay, except one PA and one HDPE material showing toxicity in *D. magna*, and one HDPE material showing toxicity in green algae. From these results we conclude that none of the microplastic particles studied here, independent of their polymer properties, give raise to concern for hazardous effects.

Keywords Primary microplastic, 3D printing, Selective laser sintering (SLS), Tire rubber particles, Chronic toxicity, Polymer of low concern

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Introduction

While being regulated in many other countries [1, 2], currently, non-natural polymers are exempted from registration and evaluation under the European chemicals legislation REACH (Restriction, Evaluation, Authorisation and Restriction of Chemicals) [3]. According to guidance provided by the European Chemicals Agency (ECHA) [3] the exemption bases on the assumption of polymers being non-hazardous due to their high molecular weight, while at the same time existing data gaps due to limited availability of fate and toxicity data are pointed out [3, 4]. The formation of secondary microplastic particles from larger polymer items is considered an inherent step of polymer degradation, and plastic particles and fibers in the size range of 1 μm to 1–5 mm are called microplastic [5, and references therein]. Primary microplastic particles are used in a number of industrial applications, e.g. as an industrial intermediate product in 3D printing. Accordingly, the registration and evaluation of non-natural polymers is anticipated. Due to the extensive number of non-natural polymers already at the market or under development, the preselection of relevant polymers based on criteria indicating a low concern is discussed. According to the OECD (https://www.oecd.org/env/ehs/oecddefinitionofpolymer.htm) the Polymers of Low Concern (PLC) concept defines criteria that if met, imply that a polymer has a low potential to exert hazardous effects. These criteria include, among others, a molecular weight below 1000, or the presence of epoxy and anhydride groups. However, these criteria were set based on a limited database, and for some of them no agreement was reached and no threshold values defined. Nevertheless, the PLC concept has been implemented in various jurisdictions around the globe, but has not been applied in the EU under the REACH legislation [1, 2, 6]. A proposal for inclusion of polymers into REACH, targeting primarily soluble polymers, has also been made by an ECETOC taskforce [7, 8]. An extensive review of the ecotoxicity of insoluble and solid polymer particles, i.e. microplastic, identified food dilution as most important mode of action by particles above 100 μm, and limited effects by smaller particles [9].

With regard to the REACH legislation, criteria for polymers requiring a registration (PRR) have been specified in the Wood/PFA report [10]. So the PRR concept aims to provide scientifically sound justifications for selecting polymers that will require a registration, and criteria indicating a hazard, as opposed to the PLC concept, which aims at identifying criteria indicating a low concern. Besides specific groups of polymers, e.g. cationic polymers, polymers with reactive functional groups (among others), the Wood/PFA report also proposed to consider criteria such as bioavailability and aspects of the lifecycle such as degradation or fragmentation.

Due to the data gaps in the field, and to contribute to the scientific understanding of potential connections between physico-chemical properties and potential hazardous effects of polymers, we conducted a hazard study assessing the aquatic ecotoxicity of a range of non-natural polymers according to predefined hypotheses. For this study solid, insoluble polymer particles were selected, specifically crosslinked polyurethane (PU), thermoplastic polyurethane (TPU, i.e. not crosslinked) and polyamide (PA). They are intentionally micronised by cryomilling to be then consumed during Selective Laser Sintering (SLS), which is an industrially relevant version of additive manufacturing (3D printing). The materials used in SLS represent a specific class of intentionally produced primary microplastics that is derogated from the upcoming ECHA restrictions due to the loss of particle shape during processing [11]. The Selective Laser Sintering (SLS) process fuses the powder intermediate into the final macroscopic plastic part, thereby losing the particle nature. Further, low density polyethylene (LDPE), high density polyethylene (HDPE), poly-methyl methacrylate PMMA and micronized tire rubber particles were included in the study as benchmark materials. All polymer microparticles were extensively characterised before conducting 48 h acute *Daphnia magna* immobilisation assays and 72 h chronic *Raphidocelis subcapitata* growth inhibition assays, with additional focus on particle internalisation by daphnia and particle attachment to algae. The results on polymer properties as well as ecotoxicity are aimed to inform on relevant solid polymer particle properties potentially affecting their hazard and their potential suitability for PLC and PRR concepts.

Materials and methods

Microplastic particles and material characterisation

For this study, a number of primary microplastic particles (PA, PU, TPU) applied for consumption during Selective Laser Sintering (SLS) were selected. In addition, PMMA, LDPE, HDPE, and micronized tire rubber particles were selected as benchmark materials (Table 1). The noncrosslinked TPU (elastomer), crosslinked PU (duromer) and PA particles were supplied by BASF SE (Ludwigshafen am Rhein, Germany). (LD)PE particles were obtained from LyondellBasell (Frankfurt, Germany) and Cospheric (Santa Barbara, USA). PMMA particles were purchased from Polysciences (Warrington, USA), and micronized Tire Rubber was obtained from MRH (Mulsener Rohstoff und Handelsgesellschaft mbH, Mulsen, Germany). None of the materials was modified, see also [12]. All types of polymer particles were of irregular spheroidal shape (Fig. 1). The polymers contain metallic impurities (see Table 1) and in addition a given additive content (relevant for their commercial application), which was not further specified in the scope of this study. All test materials
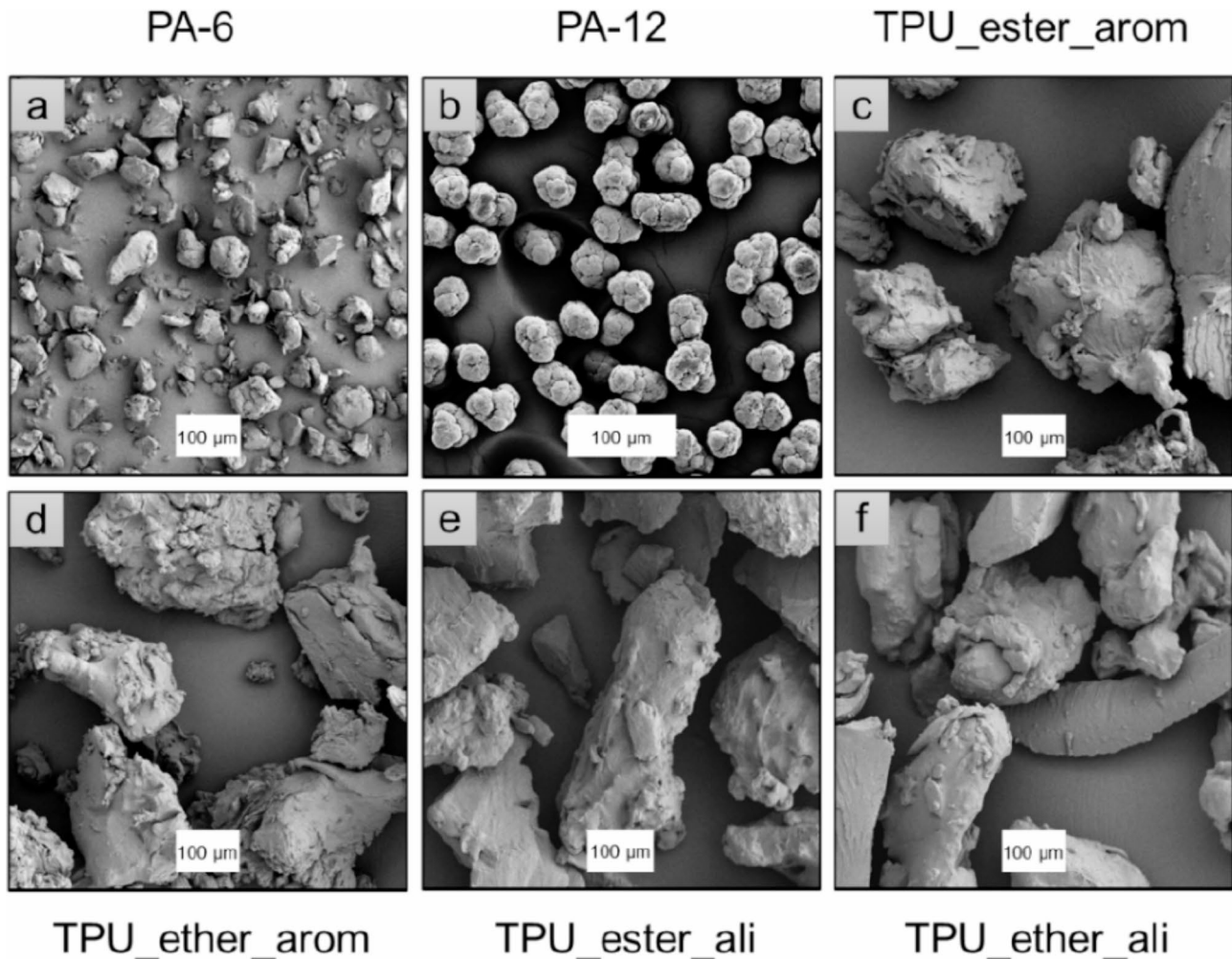
Fig. 1 SEM micrographs of the test materials that are commercially most relevant for the SLS process of 3D printing. Reproduced with permission from the Supporting Information of Pfohl, Wagner [15]

intended for the use in SLS (3D printing) contain anti-oxidant additive, specifically of the class of sterically hindered phenolic amide, which serves to prevent degradation during the high temperatures by the SLS process. The micronized tire rubber is a technical grade material commercially traded from tire recycling facilities, and consist of cryo-milled tread of truck tires, which is specified as natural rubber matrix, with unspecified additives. Leaching from the specific material was investigated in parallel and will be reported elsewhere [13].

The basic characterization of the test materials was reported earlier [14, 15]. In short, the size distribution was determined by laser diffraction (Malvern Mastersizer 3000) after deagglomeration of the powders by sonication in water with surfactant. Morphology was determined on the powders by Scanning Electron Microscopy (SEM) with sputtering. Inorganic impurities were determined on the powders by X-Ray Fluorescence (XRF). The results are reported in decadic bands above the Limit of Detection at 0.1% (g/g). The specific surface area was determined by N_2 adsorption isotherms with BET evaluation, using alumina reference materials (BAM, Berlin) as control in the range of BET values between 0.1 and 1 m²/g.

For representative microplastic materials the surface reactivity was assessed by measurement of the hydroxyl radical generation using the spin trap 5,5-dimethyl-1-pyrroline-N-oxid (DMPO) in water, in daphnia medium (ADaM) and OECD medium used for the tests with the algae. A description of the EPR Method can be found in Hellack et al. [16]. The detection of particle induced reactive oxygen species (ROS) and/or "surface reactivity" was done by spin trap/probe based electron paramagnetic resonance (EPR) spectroscopy technique (EPR Spectrometer Mini Scope 400, Fa Magnettech, Berlin). Two different complementary approaches were used. According to the method described by Shi et al. [17], hydroxyl radical generation (OH•) was measured

using the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). In the second approach a kind of surface reactivity (redox-activity) was detected, using the spin probe 1-hydroxy-3-carboxy-pyrrolidine [18].

The polymer portfolio was selected in a way to test the hypotheses of whether different chemical identities, polydispersity, types of chemical bonds, crosslinking degree, functional groups and amount of aromatic moieties have an impact on ecotoxicity in daphnia (all materials tested) and algae (all SLS materials except PU foam, and selected benchmark materials tested). For algae, in addition the hypothesis of an impact of hetero-agglomeration (meaning the formation of aggregates of algae cells and polymer particles) was assessed.

To assess the effect of chemical identities, the following materials were investigated: TPU, PU, HDPE, LDPE, PMMA, and PA. For conclusions regarding the influence of chemical identity, a variety of polyurethanes (PU, TPU) differing in the polymer backbones, aromaticities, and crosslinking degrees were available. Only the non-crosslinked TPU version is used as primary microplastic in 3D printing, whereas cross-linked PU is environmentally relevant due to potential releases of secondary microplastics during the use as insulation foam and as stone-composite in dyke stabilisation. The influence of polydispersity was assessed for PA and LDPE. Micronised tire rubber particles were added as benchmark material, allowing comparison to the growing literature of this type of microplastic. The respective physical-chemical properties that were hypothesised to be influential on ecotoxicity are listed in Table 1.

Daphnia magna
Dispersion
All polymer particle powders were dispersed according to the same protocol. For the preparation of the stock dispersion, 25 µL/L Tween 40 were first added to the daphnia test medium ADaM [19] and mixed throughout by shaking. To 50 mL of the Tween 40-ADaM mixture, 50 mg of any type of polymer powder was added and mixed by continuous stirring (vortexing). In case of the already dispersed PMMA materials, stock dispersion was prepared by diluting and vortexing the supplied particles in ADaM after 12 h of overhead shaking and short ultrasonic treatment. This stock dispersion was used for preliminary testing at 1 g/L, which was performed for all test materials to assess immobilisation as well as uptake and physical effects. For those polymers showing an effect on immobilisation at 1 g/L, aliquots were taken to prepare additional test concentrations (10, 100 µg/L, 1, 10, 100, 500 mg/L) (see Table S1), with continuous stirring after each step to avoid bias in the test concentration due to flotation or sedimentation issues. As a negative control, ADaM medium containing 25 µL/L Tween 40 was used.

Immobilisation assay
The *Daphnia magna* acute immobilisation test was performed according to OECD TG 202, in a miniaturized format [20, 21], requiring lower sample volumes and facilitating microscopic inspection of daphnia movement. In 24-well microtiter plates, in each well at least 5 neonates not older than 24 h were placed. Each polymer material was tested using at least 5 concentrations with at least 3 biological replicates performed on different days. Per concentration, 4 wells (at least 20 neonates per concentration in total), each containing 1.5 mL of microplastics suspended in ADaM were prepared. The endpoint immobilization was determined after 24 and 48 h by microscopic observation of Daphnia movement. Test validity was confirmed by applying the following criteria: immobilization < 10% in negative control, pH of medium after the test between 6 and 9, dissolved oxygen concentration in the test medium ≥ 3 mg/L, and the EC50 48 h value of the reference substance potassium dichromate ($K_2Cr_2O_7$) within range of 0.6 mg/L to 2.1 mg/L. For those polymers with several concentrations tested, EC50 values were calculated by fitting a sigmoidal curve (Table S1). The EC50s as well as the respective 95% confidence intervals were determined through probit analysis (own Python code). As no immobilization was observed at the tested concentrations for the microplastic particles, no further statistical analysis was performed.

Uptake into the gut
After 48 h, attachment and uptake of the test materials were visually assessed by light microscopy for all microplastic particles at an exposure concentration of 1 g/L. Selected daphnids were placed in petri dishes in a small drop of ADaM and positioned to make the gut available for inspection. Subsequently the petri dishes were placed under a stereomicroscope (Olympus SZX7) equipped with a camera (Olympus DP21). Pictures were taken in dark field mode at 5.6 x magnification.

Raphidocelis subcapitata
Dispersion
The preparation of the test dispersions was based on the behaviour of the polymers in the test medium. PE_broad_distribution and PA-6 were available as powders, and a stock dispersion was prepared by mixing 100 mg with 50 mL of the OECD test medium and stirring with a magnetic bar. No surfactants had to be added for the distribution of the materials in the test medium. To achieve the test concentrations, appropriate aliquots of the stock dispersion were pipetted under continuous stirring and diluted with additional test medium. PU floated on the surface of the test medium and Tween 40 was added to achieve mixture. For the preparation of the stock dispersion, 25 µL/L Tween 40 and 100 mg PU were added to

Table 1 Physical-chemical characteristic of microplastic particles used for SLS and the benchmark materials, respective hypotheses for testing, and tested organisms

Polymer (Purpose)	Hypotheses for testing	State	Size [geometric diameter detected by laser diffraction]	Surface area (BET) [m²/g]	Impurities	Reactivity DMPO[a] Sample to blank ratio						Test organism
						Water (dH$_2$O)		ADaM (D medium)		OECD (A medium)		
						MW±SD	>3*SD Blank	MW±SD	>3*SD Blank	MW±SD	>3*SD Blank	
TPU_ester_arom (SLS)	Thermoplastic vs. crosslinked Ester vs. Ether backbone aromatic vs. aliphatic soft segment foam vs. solid	Powder	Dx10 (µm): 142 Dx50 (µm): 254 Dx90 (µm): 418	0.027	--	1.02±0.14	no	2.11±0.91	no	1.05±0.17	no	A, D
TPU_ether_arom (SLS)		Powder	Dx10 (µm): 128 Dx50 (µm): 246 Dx90 (µm): 413	0.030	<0,1% : Fe	1.28±0.38	yes	1.70±0.12	yes	0.74±0.02	no	A, D
TPU_ester_aliph (SLS)		Powder	Dx10 (µm): 143 Dx50 (µm): 262 Dx90 (µm): 440	0.034	<0,1% : Si Sn	1.37±0.11	yes	1.46±0.13	yes	0.92±0.02	no	A, D
TPU_ether_aliph (SLS)		Powder	Dx10 (µm): 152 Dx50 (µm): 267 Dx90 (µm): 442	0.033	<0,1% : Mg Si Cl Fe Bi	1.45±0.42	yes	1.55±0.48	no	1.12±0.07	no	A, D
PU_binder_aromatic 1 C (stone composite on dykes)		Powder	Dx10 (µm): 82.8 Dx50 (µm): 200 Dx90 (µm): 354	0.1454	0,1–1% : Cl <0,1% : Si Fe Ni Zn	--	--	1.06±0.32	no	--		A, D
PU_binder_aromatic 2 C (stone composite on dykes)		Powder	Dx10 (µm): 77.2 Dx50 (µm): 201 Dx90 (µm): 368	0.1586	0,1–1% : Al Si K <0,1% : S Cl Ca Fe	--	--	1.13±0.27	no	--		A, D
PU Foam (insulation)		Powder	Dx10 (µm): 33.1 Dx50 (µm): 92.8 Dx90 (µm): 211	1.1833	<0,1% : Al Si P K Fe	1.06±0.12	no	--	--	--		D
PA-6 (SLS)	Size small vs. large	Powder	Dx10 (µm): 13.7 Dx50 (µm): 42.2 Dx90 (µm): 75.3	0.366	<0,1% : Mg	1.03±0.09	no	1.13±0.21	no	--		A, D
PA-6 inhalable (SLS)		Powder	--	1.85	--	1.21±0.04	no	--	--	--		D
PA-12 (SLS)		Powder	In progress	0.726	0,1–1% : Si <0,1% : S Fe	1.03±0.05	no	--	--	--		D

Table 1 (continued)

| Polymer (Purpose) | Hypotheses for testing | State | Size [geometric diameter detected by laser diffraction] | Surface area (BET) [m²/g] | Impurities | Reactivity DMPO[a] Sample to blank ratio | | | | | | Test organism |
| | | | | | | Water (dH$_2$O) | | ADaM (D medium) | | OECD (A medium) | | |
						MW ± SD	> 3*SD Blank	MW ± SD	> 3*SD Blank	MW ± SD	> 3*SD Blank	
HDPE_broad_distribution (Benchmark)	Size small vs. large branching (LDPE vs. HDPE)	Powder	0.2–9.9 µm (by manufacturer)	--	--	1.17 ± 0.03	no	1.05 ± 0.38	no	--		A, D
LDPE 250 (Benchmark)		Powder	Dx10 (µm): 96.2 Dx50 (µm): 215 Dx90 (µm): 380	0.243	< 0,1% : Fe	1.07 ± 0.11	no	--	--	--		D
LDPE 80 (Benchmark)		Powder	Dx10 (µm): 19.1 Dx50 (µm): 84.4 Dx90 (µm): 188	0.326	< 0,1% : Fe	1.2 ± 0.07	no	1.49 ± 0.42	no	--		D
PMMA (monodisperse) (Benchmark)	Broad size distribution vs. monodispers	dispersion with 2.5 wt% solid content	1–10 µm (by manufacturer)	15.0	--	--	--	--	--	--		A, D
PMMA_broad_distribution (Benchmark)		dispersion with 5 wt% solid content	0.3 µm (by manufacturer)	1.6	--	--	--	--	--	--		D
Tire rubber (Benchmark)	Complex composition of additives (recycled tire)	Powder	Dx10 (µm): 61.7 Dx50 (µm): 130 Dx90 (µm): 233	0.298	1–10% : Si S Zn 0,1–1% : Ca Fe < 0,1% : Al Cl K Ti Co Cu Br	1.03 ± 0.24	no	1.10 ± 0.07	no	--		D

[a]Hydroxyl radical generation measured according to Shi et al. [17] using electron paramagnetic resonance spectroscopy; SLS – Selective laser sintering, A – algae, D – daphnia, "--" no data

the OECD test medium [22]. During continuous stirring with a magnetic bar, aliquots were removed to prepare the individual test concentrations. Every test concentration and the control were adjusted with Tween 40 to achieve a concentration of 25 µL/L, which is below the maximum allowed dispersant concentration of 100 µL/L as described in OECD TG 201. PMMA was already available as a dispersion (2.5 wt%), and 2 mL of the dispersion were added to 498 mL of the OECD test medium to achieve a final stock concentration of 100 mg/L. The stock was gently shaken for ~ 1 min, resulting in a homogeneous and stable dispersion which was immediately used to prepare the remaining test dispersions. The stock dispersion, corresponding to the highest test concentration, was diluted with OECD test medium as described above to prepare test concentrations. Test validity was confirmed by the criteria listed in the OECD TG 201 [23] applying to the control set-ups: biomass increase

cultures > factor 16, mean coefficient of variation for section-by-section specific growth rates ≤ 35%, coefficient of variation of the average specific growth rates ≤ 7%.

Growth inhibition assay

The inhibition of algal growth (green algae *R. subcapitata*) was determined as described in OECD TG 201 [22] and Hund-Rinke, Schlich [24] and Hund-Rinke, Broßell [21]. Algae biomass was determined via chlorophyll fluorescence, the endpoint growth rate was determined after 72 h. Every material was tested in several concentrations, based on geometric series with a factor of 10. The highest test concentration was 100 mg/L, which corresponds to the requirements of the guideline. The validity criteria listed above were applied.

ToxRat (ToxRat Solutions, Germany) was used to evaluate the effect concentrations and confirm fulfilment of the validity criteria. We calculated the percent inhibition

of growth rate [r] compared to controls for the exposure period, as this is the relevant regulatory parameter. Biological data were analysed to determine EC50 values together with 95% confidence intervals when possible. For the calculations, the following settings were used. Pretesting: normal distribution - Shapiro−Wilk´s; significance level – 0.01; variance homogeneity - Levene; significance level −0.01; Final testing (EC50): test procedure - Williams; significance level – 0.05; test direction - one-sided smaller; ECx computation: selected method - non-linear regression; optimisation - Levenberg−Marquardt (IRLS); Dose/response function metric −3-parametric normal; calculation of confidence limits - Monte-Carlo simulation.

HDPE was tested three times with about 5 months between the assays. For every test, the test dispersions were freshly dispersed. In the second investigation, to tackle the potential effects of leachates, three different set-ups were investigated. Besides the testing according to the guideline, (Variant 1: testing of the freshly prepared dispersion; was used for comparison with Test 1 and with Variant 2) the particles were pre-incubated in test medium without algae at test conditions for 72 h. After this incubation period, the dispersion was tested (Variant 2). In addition, the particles and test medium were separated by centrifugation (10,000 g, 5 min) and the latter investigated in the growth inhibition test (Variant 3; for comparison with Variant 2). To identify substances potentially leached into the medium, a non-target analysis was carried out in the dispersions and the centrifuged

medium, with a Shimadzu GC-MS, for which the sample was mixed with n-hexane and added to the system.

Attachment to algae

The attachment efficiency of the particles to the algae cells was determined via light microscopy in a short-term assay and at test end as described in Hund-Rinke, Sinram [25] and Hund-Rinke, Broßell [21]. While for the short-term assay one set-up with a particle concentration of 100 mg/L and an algae concentration of about 2 Mio cells/mL was investigated, all test concentrations were investigated in the growth test. In case of PU, Tween 40 was present in the growth test as well as in the short-term assay.

Results

Daphnia magna

Immobilisation

The test validity criteria were fulfilled for all tests, with 0% immobilisation in the negative controls, a pH of 7 to 7.8, sufficient oxygen content of the medium after all tests. The EC50 values for the reference substance potassium dichromate ($K_2Cr_2O_7$) were observed to range between 1.1 and 1.9 mg/L.

In general, no ecotoxicity (effects observed up to 100 mg/L) for any of the tested polymer materials was observed in *Daphnia magna*, independent on the material/chemical composition, size, type of chemical bond, crosslinking degree, functional groups and amount of aromatic moieties (see Table 2, Table S1, Suppl. S2)

Table 2 Acute ecotoxicity of microplastic particles towards *D. magna* in the 48 h immobilisation assay

Application / Purpose	Polymer	Hypotheses for testing	Uptake	EC50 48 h	Result
Selective Laser Sintering (SLS) and variations of the same polymer backbone	TPU_ester_arom	• Thermoplastic vs. cross-linked • Ester vs. Ether backbone • aromatic vs. aliphatic soft segment • foam vs. solid	Yes	> 1 g/L	No impact of linking, backbone, aromaticity, reactivity and state on *D. magna* toxicity
	TPU_ether_arom		Yes	> 1 g/L	
	TPU_ester_aliph		Yes	> 1 g/L	
	TPU_ether_aliph		Yes	> 1 g/L	
	PU_binder_aromatic 1 C		Yes	> 1 g/L	
	PU_binder_aromatic 2 C		Yes	> 1 g/L	
	PU Foam		Yes	> 1 g/L	
	PA-6	• Size small vs. large	Yes	> 1 g/L	Size distribution had no impact on *D. magna* toxicity
	PA-6 inhalable		Yes	~ 1 g/L	
	PA-12		Yes	> 1 g/L	
Benchmark	HDPE_broad_distribution	• Size small vs. large • branching (LDPE vs. HDPE)	Yes	~ 1 g/L	Different particle size and branching had no impact on *D. magna* toxicity
	LDPE 250		Yes	> 1 g/L	
	LDPE 80		Yes	> 1 g/L	
	PMMA (monodisperse)	• Broad size distribution vs. monodispers	Yes	> 1 g/L	Size distribution had no impact on *D. magna* toxicity
	PMMA_broad_distribution		Yes	> 1 g/L	
	Micronized Tire rubber	• Complex composition of additives (recycled tire)	Yes	> 1 g/L	Additive content had no impact on *D. magna* toxicity

Except for PA-6 inhalable and HDPE, for which at 100 g/L 35% and 20% immobilisation were observed, respectively. The EC50 values calculated for these materials are around or above 1 g/L, and the values are subjected to high uncertainties. The surface reactivity of selected polymer materials was determined with most of the materials being non-reactive (Table 1). In the case of TPU_ether_arom and TPU_ester_aliph a low reactivity was measured in ADaM, which had no impact on toxicity.

Internalisation of polymer particles

The microscopic inspection of exposed animals after 48 h revealed that all polymer particles, irrespective of their size distribution, were visible in the guts of the animals (Fig. 2; Table 2). The presence of microplastic particles was evident by a white appearance of the gut, compared to control animals without any treatment (microplastic or Tween 40) (Fig. 2). Control experiments with only Tween40 in the medium showed, however, the same characteristic coloration of the gut. In order to exclude an artefact by the dispersant, experiments with polymer particles without the Tween40 were conducted. Due to the mobility of daphnids, they also came into contact with particles not well dispersed in the medium. Among

the microplastic particles with relevance for the 3D printing, the particles with the smallest size, PA-6_inhalable, were selected for this experiment. It was confirmed by this control experiment that the microplastic particles were indeed taken up by the daphnids. Verification of uptake by RAMAN microscopy failed due to the high organic background in the daphnia samples. Uptake of the tire rubber particles, however, was confirmed by HIM-EDX (not shown). None to moderate attachment of the polymer particles to the carapaces of the animals was observed.

Raphidocelis subcapitata
Growth inhibition

With exception of HDPE_broad distribution none of the tested polymer materials showed ecotoxicity up to the highest test concentration of 100 mg/L (Table 3, Table S2). HDPE was tested three times with about 5 months between the assays. Over time, the growth inhibition decreases with 40% at the highest test concentration (100 mg/L) in the first test and 20% and 10% inhibition in the subsequent assays. In the second investigation, besides the testing according to the guideline, the particles were pre-incubated in test medium at test conditions for 72 h. The dispersion as well as the supernatant after
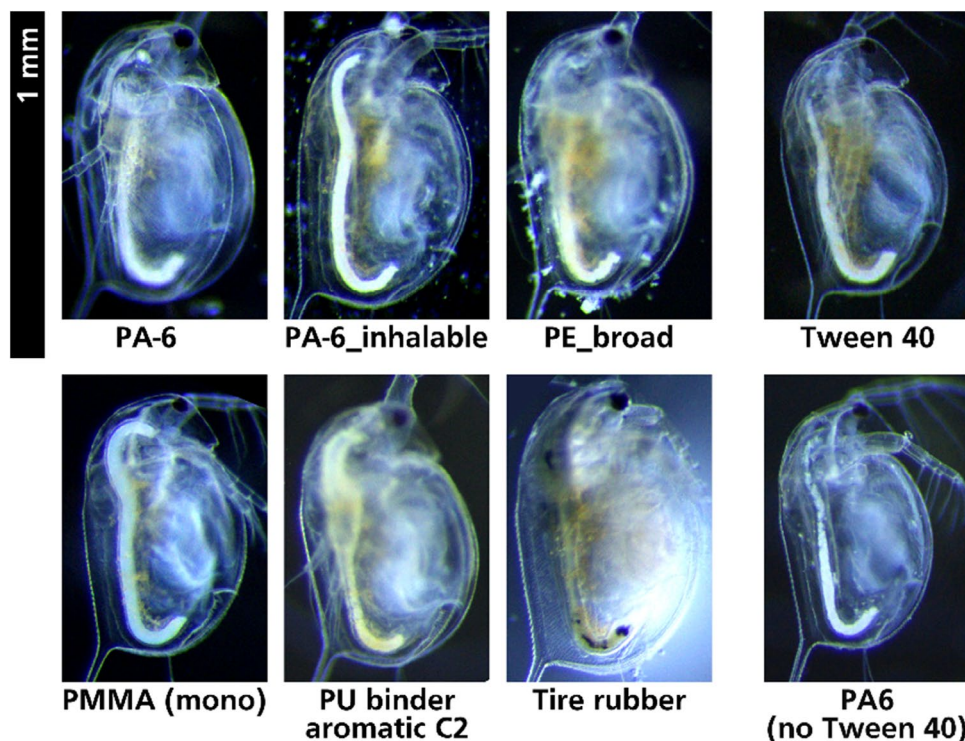

Fig. 2 Uptake of polymer particles into the gut of exposed daphnids on the example of selected polymers (as indicated below each picture) with different characteristics. All were internalised as visible by the white coloration of the gut. In addition, negative control Tween 40 only is shown (top right). In the lower middle right panel, a daphnia exposed to positive control micronized tire rubber is shown, here black particles are visible, as well as the light green coloration typical for control animals. PA6 exposure without Tween 40 (lower right panel) confirmed that the white coloration is indeed a result of microplastic exposure

Table 3 Chronic ecotoxicity of microplastic particles towards *R. subcapitata* in the 72 h growth inhibition assay

Application / Purpose	Polymer	Hypotheses for testing	EC50 72 h [mg/L]	Agglomeration	Result
Selective Laser Sintering (SLS) and variations of the same polymer backbones	TPU_ester_arom	•Thermoplastic vs. cross-linked	>100	No	No impact of linking, backbone, aromaticity and state on *R. subcapitata* toxicity
	TPU_ether_arom	•Ester vs. Ether backbone	>100	No	
	TPU_ester_aliph	•aromatic vs. aliphatic soft segment	>100	No	No impact of particle size in the µm-range on *R. subcapitata* toxicity
	TPU_ether_aliph	•Influence of particle size as well as of polydispersity	>100	No	
	PU_binder_aromatic 1 C		>100	No	
	PU_binder_aromatic 2 C		>100	No	
	PA-6		>100	No	
Benchmark	HDPE_broad_distribution	•Influence of particle size as well as of polydispersity	>100	Minor	No impact of polydispersity on *R. subcapitata* toxicity
	PMMA (monodisperse)		>100	No	No impact of particle size in the nm-range on *R. subcapitata* toxicity

separation of the particles were tested. In none of these fractions, growth inhibition was observed.

Furthermore, in these fractions no remarkable concentrations of unknown substances above the limit of quantification could be detected. The TPU materials reactive in water did not show any reactivity in algae test medium (see Table 1).

Attachment to algae
The materials showed neither homo- nor hetero-agglomeration. Only the polydisperse PE particles formed small agglomerates comprising of particles differing in their size (smaller and larger than algae). Nearly no association of algae to the agglomerates was observable. By far the majority of the algae were free floating.

Discussion
To add to the growing body of knowledge regarding the effects of microplastic particles to aquatic organisms, we conducted a hazard study comparing the effects of several micronized particles applied in Selective Laser Sintering (SLS) and benchmark materials towards daphnia and unicellular green algae *R. subcapitata*. Specific hypotheses were formulated beforehand regarding the influence of specific microplastic properties on ecotoxicity. The present study followed the research recommendations by a recent expert workshop organised by the SCCWRP (Southern California Coastal Water Research Project) [26, specifically Recommendation 1 Identify microplastic characteristics that best predict hazard] and others [27]. The portfolio of SLS and benchmark test materials was selected according to their properties to be able to confirm or reject the test hypotheses (see Tables 1 2 and 3).

All microplastic particles employed in this study did not show acute toxic effects at test concentrations of 100 mg/L in *D. magna* nor in the chronic algae growth inhibition tests with *R. subcapitata*, and hence all test hypotheses were rejected. The polymer properties compared among the different particles were degree of cross-linking, type of polymer backbone, aromaticity, branching, size, reactivity and size distribution. Based on these toxicity results in acute *D. magna* immobilisation and chronic *R. subcapitata* test, no concern is indicated for any of the microplastic particles tested in this study.

Daphnia
The results for *D. magna* are well in line with previous findings, showing that the toxicity of various nanomaterials as well as microscaled particles of various compositions is mainly driven by the release of toxic ions [21]. Inert, or non-toxic ion releasing materials (as microplastic particles are), on the other hand, are of low toxicity [21, 24, 28].

For the polymers applied in selective laser sintering, PU, TPU and PA, only little data on acute daphnia toxicity are available from the literature. For TPU, no studies were found reporting on *D. magna.* For PA, no acute nor chronic effects were detected in *D. magna* [29]. For PU, a chronic 21 d *D. magna* test was conducted, showing that exposure to PU microplastics reduced the reproduction significantly compared to the control at 500 mg/L with EC_{50} 236 mg/L [30]. This indicates the need to consider potential chronic and long-term effects of plastic exposure.

For the benchmark materials, PE, LDPE, PMMA and tire rubber, more information was available. A low acute toxicity (48 h) of PE microplastic particles of broad size distribution (mean size 140 μm) towards *D. magna* was demonstrated [31]. No chronic toxicity (21 d) for PE was observed by Canniff and Hoang [32]. However, studies considering longer exposure times showed different results. After 96 h exposure, 1 and 100 μm PE particles acted differently, as the larger particles were floating on top of the exposure solution and were not available for daphnids. The 1-μm particles on the other hand, led to immobilisation with an EC_{50} of 57.43 mg/L [33]. In exposures over 96 and 168 h (with feeding every 2nd day), respectively, PE microplastic particles were able to induce toxicity in *D. magna* [34, 35]. When comparing fasting and feeding conditions, a reduction of PE toxicity was observed to the feeding group [34]. In a chronic 21 day study, a dependence of survival on PE particle shape was demonstrated, with fragmented particles being more toxic than regular shaped PE beads [36]. The small-sized PE fragments were found to reduce algal feeding, body length, and the number of offspring when compared with PE beads, likely due to their longer retention time in the daphnia digestive tract, pointing to a potential role of microplastic particles on energy budget. For LDPE particles, no acute data were found, and minor long term effects in *D. magna* were reported [37]. No acute toxicity was reported for nanoscaled PMMA (86–125 nm) [38] as well as microscaled PMMA [39]. However, both studies reported PMMA particle uptake in the gut. Also for PMMA, prolonged exposures in chronic tests led to the observation of increasing mortality [40]. For the nanoscaled PMMA particles a clear size effect was observed, with a significant effect on mortality rates of individuals for the 25 nm plastics. On the other hand, no mortality was observed for the 50 nm exposures. Growth and reproductive output was unaffected by both types of particles. A couple of studies assessed the effects of tire wear particles on *D. magna*, indicating a strong effect of additive content and of the origin of the tire materials [41, 42]. This may explain the non-toxicity of the tire rubber material used in this study.

In addition, the internalisation of particles into the gut of daphnids is in line with observations made for a number of polymer particles, as well as microscaled particles composed of other materials. It is apparent for many particulate materials due to the non-selective filter feeding mechanism of *D. magna*, which is also observed for the polymer particles investigated in this study. There is no definite size limit for particle internalisation reported in the scientific literature, one paper showed for example the internalisation of 50 μm prey [43] by daphnids. According to Ebert [44], *D. magna* ingests algae from 1 to 70 μm. There are some indications that prey shape influences uptake, e.g. spherical or oval shaped. Uptake of 63–75 μm fluorescent PE particles in *D. magna* was demonstrated, without acute toxic effects [32]. No uptake was demonstrated for 90 μm plastic particles [45] as well as for 100 μm PE particles [33]. However, it was stated that uptake is not only influenced by particle size, but also by particle availability (e.g. if floating) as well as life stage of animals. For our assays, only neonates aged less than 24 h were used, but even for these animals already differences in body size are evident. Further growth takes place within the 48 h test duration.

Roughly, there seems to be a cut-off value for filtration at around 100 μm diameter [46] but one has to keep in mind that most of particles studied here have a broad size distribution, and hence it may be possible that only the fraction of particles below ~100 μm is internalized.

Algae

For toxicity on algae, particle size and hetero-agglomeration behaviour are the main drivers for ecotoxicity. Our findings for polymers are based on the investigation of mainly large particles comprising different chemical identities. With a size of 40 to 250 μm, TPU, PU and PA-6 exceeded the size of the test algae which is about 8 to 14 μm in length and 2–3 μm in diameter [47]. Only, the two polydisperse materials HDPE and PMMA contained also smaller fractions with particle sizes down to 200 nm (HDPE) and 1000 nm (PMMA).

Various published studies show that the size of the plastic particles is an important factor with regard to ecotoxicity. Nano-sized polystyrene particles (~70 nm) have been demonstrated to have adverse effects on the freshwater algae *Scenedesmus obliquus* [48]. Furthermore, the adverse effects of polystyrene particles (0.05, 0.5 and 6 μm) on the marine algae *Dunaliella tertiolecta* decreased with increasing particle size [49]. The impact of microplastic particle size was evaluated by comparing the ratio of the algae cell size and particle size with the determined effects [50], independent of the chemical identity of the plastic particles and of the test algae. Marine and freshwater algae were included. Despite this diversity, the results show that the probability of toxic

effects is larger for smaller particles compared to micro-sized particles. Adverse effects were observed when the cell to particle size was in the range of 0.75 to 3.07 (log scale), while in the range of -1.85 to 1.33 (log scale) no effects where observed.

Besides size, also the hetero-agglomeration efficiency has to be considered. For small sized polymer particles such as nano-polystyrene spheres (100 nm) and poly-vinylchloride particles (~1 µm), hetero-agglomeration was identified as cause for growth inhibition [51, 52]. Our results of neither agglomeration nor ecotoxicity (growth inhibition) are in line with previous investigations for inorganic materials. While metal oxides in the nanometer range could attach to algae resulting in a coating and in ecotoxicity [25], investigations of further inorganic materials showed that ecotoxicity due to agglomeration of particles and algae is more pronounced for particles obviously smaller than algae [21]. Physical blockage of the gas-transfer through the cell membrane, reduced

illumination and a reduced nutrient availability are suggested as factors affecting algal growth [51, 53].

Furthermore, the chemical identity has to be considered. From long-term experiments with *Chlamydomonas reinhartii* it is hypothesised that plastic particles induce the induction of specific types of exopolysaccharides with different cohesive and sticking properties depending on the chemical identity of the particle [54]. The focus of this study was on the colonization of surfaces, still the observations provide important information on the general interaction of algae and plastic surfaces and thus also on the potential toxicity of the particles due to hetero-agglomeration. Colonization studies also show that the composition of the biofilm differs significantly from the population in the surrounding medium [55] and it can be concluded that besides particle size and hetero-agglomeration behaviour, also the algal species has to be considered in the toxicity assessment of microplastic particles.

For the toxicity assessment, the release of additional substances has to be considered as well. Canniff and Hoang [32] observed stimulating effects of microplastic particles indicating the release of trace concentrations of chemicals. Studies on the effect of four chemicals leaching from the investigated microplastic particles (BPA, DEHP, DBP, UV-326) confirmed their stimulatory effect on the growth of *Dunaliella salina* [50], but these specific IAS and NIAS (intentionally and non-intentionally added substances) have relevance only for specific plastics (epoxy, PVC, PC), not for the PU and PA materials that were in focus here. We observed low toxicity for the HDPE-particles which decreased over time. According to the material safety data sheet, the material contained 30% undeclared substances. After 5 months the test was repeated. No unknown substances could be observed by non-target analyses and much lower toxicity was observed (data not shown). The toxicity continued to decrease over time. Therefore, it cannot be excluded that the unknown substances induced the toxicity of the raw material and have been degraded due to aging. Due to the time-varying behaviour, in future studies one potentially may need to consider volatile components (monomers or oligomers), or instable components, such as biocides.

Table 4 PLC criteria and their relevance for 3D printing materials

PLC criterion	Specification according to[a]	Classification of the tested polymers
No reactive functional groups	**amino groups**, epoxide groups, unsubstituted positions ortho- to phenolic hydroxyl (ecotoxicological concern only for amino groups)	The molecular structure of polymers used in 3D printing powders do not include any of these groups. The measured reactivity is very low
Molar mass	Molecular weight above 10.000 g/mol	Product specification of the thermo-plastics typically used for 3D printing
Extractivity in water	10 mg/L is seen as acceptable	Such test was not performed in the present project.
Metal content	No fixed limit is given by the OECD concept	The analysis of the polymer powders by XRF confirmed metal content below 0.1%, containing metals such as Al Si P K Fe. One material had Al Si K up to 1%, most probably from cryo-milling.
Cationic charge density	not more than one cationic charge in 5000 monomer units (EPA definition)	The molecular structure of PA and PU does not contain cationic groups
Stability	Stable under the conditions of use	PU materials are stable under aquatic conditions, as intended. PUs are susceptible to partial biodegradation during the harsher conditions of industrial composting [14]. Consideration of degradation products from marine conditions is challenging via identification and toxicity prediction [56].
Swelling	100% water uptake	Not relevant for 3D printing powders

[a]https://www.oecd.org/env/ehs/oecddefinitionofpolymer.htm

Conclusions: implications for the polymer of low concern concept

In summary, the polymers studied here with regard to their acute (*D. magna*) and chronic ecotoxicity (*R. subcapitata*), representing innovative materials used as intermediate material for 3D printing, and as benchmark materials, adhere to OECD PLC (Polymers of low concern) criteria (https://www.oecd.org/env/ehs/oecd-definitionofpolymer.htm) (Table 4). The microplastic particles used here consisted of less studied polymers, with systematic variation of the molecular structure of the PU

backbone, including a comparison of a variety of backbones (PA, PU, PE). Further, physical-chemical properties were acquired beyond the best practices recommended and enabled an evaluation of the OECD concept of Polymers of Low Concern (PLC). For the materials tested in our study, no indication of further physical chemical properties relevant for toxicity were observed based on the two organism tests used in this study.

For the microplastic particles used in 3D printing, the intended use leads to the loss of particle shape, as the particles are consumed during the printing process and sinter together to form bigger parts. Additionally, the purely industrial use triggers derogations from the planned ECHA restriction of intentionally produced primary microplastic [11]. Similarly to conventional plastic pellets, for which the same derogations apply, the producers will have to label the intermediate (powder) products, and will have to report uses, but are allowed to commercialize the materials.

For secondary microplastic particles that form via fragmentation of larger plastic items in the environment, however, additional considerations are needed.

Here, the global environmental plastic pollution and its irreversibility have been characterised as planetary boundary threat, and resulting potential for long-term effects for organisms are expected [57–59]. In daphnia tests employing microplastic particles of various polymer types, chronic effects have indeed been detected [30, 36, 40]. Further, there are indications of an influence of microplastic particle ingestion on energy budget, with irregular shaped microplastic having greater impact than spherical, uniform microplastic [60] which again points to the potential for secondary microplastic particles to cause long term effects in daphnids. Food dilution was identified as main mode of action on aquatic organisms [9]. In these lines, the prevention of plastic pollution, and instead a circular economy of plastic materials, is the priority policy option [61]. If emission has to be anticipated, an improved consideration of the environmental consequences of polymer release, use and subsequent formation of microplastics have been proposed recently [4].

In addition, the issue of leaching of associated chemicals (e.g. additives) as specifically relevant for long-term exposures needs further consideration in the future. In *D. magna*, a contribution of chemicals to the toxicity of PVC microplastic was shown, while to the toxicity of PU and PLA microplastic no chemicals contributed, and the effect was mainly driven by the particles [30]. Our study on algae showed that aging of PE can significantly alter the toxicity of a material. While for PE aging led to a decrease in algal toxicity, for other materials a potential increase cannot be ruled out. Therefore, the consideration of stability of a material with respect to long-term behaviour is recommended, by e.g. considering different

life cycle stages, as demonstrated previously by deliberate aging of the microplastics [62]. As secondary microplastic particles were not considered in this study, future studies should include other life cycle stages such as the use phase or the end of life.

List of abbreviations

ADaM	Aachener Daphnien Medium
BET	Brunauer-Emmett-Teller
DMPO	Dimethyl-Pyrroline-Oxide
EC_{50}	half maximal effective concentration
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EPA	Environmental Protection Agency
HIM-EDX	Helium ion microscopy – Energy dispersive X-ray spectroscopy
IAS	intentionally added substances
LDPE	Low density polyethylene
NIAS	non-intentionally added substances
OECD	Organisation of Economic and Cultural Development
PA	Polyamide
PC	Polycarbonate
HDPE	High density polyethylene
PLA	Polylactic acid
PLC	Polymers of low concern
PMMA	polymethylmethacrylate
PRR	polymers requiring a registration
PU	polyurethane
PVC	Polyvinylchloride
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SLS	selective laser sintering
SCCWRP	Southern California Coastal Water Research Project
TG	test guideline
TPUs	thermoplastic polyurethanes
XRF	X-ray fluorescence spectroscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43591-023-00078-y.

> Supplementary Material 1: Summary of observed immobilisation (Daphnia magna) and growth inhibition (Raphidocelis subcapitata)
>
> Supplementary Material 2: Raw data for Daphnia magna immobilisation assay

Author Contributions
K.H.R. and D.K. conceptualized and supervised the study. The experiments for this study were carried out by T.S. (daphnia tests), with contributions from C.W. (measurement of particle reactivity). W.W. micronized and characterized the MNPs. D.K. wrote the manuscript with contributions from K.H.R., C.W., T.S., K.S. and W.W. The authors read and approved the final manuscript.

Data Availability
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors read and approved the final version of the manuscript.

Competing interests
The authors declare no competing financial interest. W.W. is employee of BASF SE, a company producing and marketing polymers, including some of those investigated in this study.

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