Release and intestinal translocation of chemicals associated with microplastics in an in vitro human gastrointestinal digestion model

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Abstract

The global production of plastic currently exceeds 300 million tonnes per year. The extensive use of plastics and bad waste management has resulted in the presence of microplastics at different levels in the food production chain. From a chemical perspective, these microplastics are complex mixtures that contain multiple additives, such as plasticizers, flame retardants, stabilizers and pigments. Also other chemicals can be present in microplastics, including unreacted monomers, starting substances, and non-intentionally added substances. Finally, the microplastics may have adsorbed environmental contaminants. In this study we have used several types of microplastics, either from grind edible or from frequently used food packing materials and pre-production samples. We quantified the chemical and metal release from these microplastics in worst case and physiological scenarios. We use a chemical extraction as worst case, and for the physiological scenarios we used an in vitro model of the human digestion and an in vitro model of the human intestinal epithelium. Subsequently all samples were analysed with sensitive ICP-MS, GCMS methods. We quantified 68 chemicals and 29 metals associated with a diversity of microplastics, some of these chemicals were also released in the luminal content of the human digestive tract under physiological conditions simulated in vitro. Only 22 chemicals reached the basolateral compartment of an in vitro intestinal epithelial model. From the ToxCast dataset we extracted 18 AOPs that were associated with the chemicals, that included AOPs associated with endocrine disruption. For a risk assessment of chemicals associated with microplastics more detailed data on oral microplastics exposure is needed, as well as more detailed toxicological studies on the hazards of both the individual and complex mixtures of the quantified chemicals.

Keywords: Microplastics, Gut barrier, Organic chemicals, Endocrine disruption, In vitro digestion

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Introduction

Worldwide more than 320 million tonnes of plastic are produced annually, and production is growing exponentially [1, 2]. From a materials perspective, plastics are cheap and versatile and thus are an integral part of our everyday lives. However, microscopic particles are released from plastic throughout its lifecycle, via abrasion and/or photochemical degradation and subsequent fragmentation during use, disposal or systems leakage [3, 4]. The particles are known as microplastic and measure less than 5 mm in their maximum dimension, whilst those particles measuring between 1 μm and 100 nm are defined as submicron plastics [5] or those smaller than 100 nm according to the European Food Safety Authority (EFSA) [5–8]. The broad range of applications for plastic material means there is a wide variety of sources of microplastic, such as plastic debris, synthetic clothing, personal care products containing plastic microbeads, industrial scrubbing agents and the leakage of virgin pellets, each which are diverse in their polymer composition. The most commonly produced types of polymers are polyethylene (PE), polypropylene (PP) and polystyrene (PS) however the polymer composition of microplastics in realistic (environmental) samples is very heterogeneous [9, 10].

For humans, the oral route represents an important exposure pathway for microplastics [11, 12], evidenced by the presence of microplastics in human stool [13]. Microplastic contaminates seafood [14], and other food commodities [15, 16]. In the European Commission’s Rapid Alert System for Food and Feed (RASFF), microplastics (classified as foreign bodies) are reported to not only be present in seafood, but for instance also in oligo fructose powder, pickled products and frozen vegetable spring rolls [17]. Microplastics are also found in drinking water from plastic bottles, glass bottles, beverage cartons and in tap water from different countries [18, 19]. Several analytical approaches have been developed to study the presence of microplastics in different matrices, yet given the complexity of the polymer composition and size ranges of microplastics accurately characterising the occurrence and thus predicting human exposure remains challenging [20]. Recently the human intake of microplastics from food was estimated to be 39,000 to 52,000 particles per year depending on age and gender, from different food groups, which together accounted for 15% of the daily average caloric intake consumed by adults in the US, including seafood, water, sugar, salt and honey [21, 22]. Airborne microplastics also provide an important source of microplastics to which humans eventually can be orally exposed. Inhaled microplastics can be swallowed after lung clearance via the mucociliary escalator and end up in the gut [11]. Alternatively airborne microplastics may settle on food and beverages during consumption [23]. Together this might increase the human body burden of microplastics.

Plastic products and thus the microplastics degraded from these products are complex chemical mixtures containing multiple additives, such as plasticizers, flame retardants, stabilizers, antioxidants, fillers and pigments to improve the polymers functionality and characteristics [24, 25]. Chemicals in plastics include phthalates, bisphenol A (BPA), polybrominated diphenyl ethers (PBDE), tetrabromobisphenol A (TBBPA), alkylphenols and organotin compounds [26, 27]. In addition to these additives, other chemicals are present in plastics, including unreacted monomers and non-intentionally added substances (NIAS) [28]. Moreover, and due to their relatively large specific surface area and inherent hydrophobicity, microplastics can adsorb and concentrate pollutant chemicals from the ambient environment [29, 30]. Well-known examples of these are persistent organic pollutants (POP) such as polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), dioxin-like chemicals, polybrominated diphenyl ethers (PBDE), pharmaceuticals. In addition metals can be present in microplastics as some of them are used as additive, or adsorb to microplastics from the environment [31–38].

As most of these chemicals are not covalently bound to the polymer, they are susceptible to leaching to the surrounding medium at all stages of the plastics’ lifecycle. Such leaching is enhanced at physiological temperature, low pH [39] and in lipid-rich environments [40]. Hence, there is potential for the environmental conditions of the human digestive tract to facilitate chemical leaching from microplastic, contributing to daily intakes and body burdens. The relative importance of such physiological digestion is yet to be determined.

The aim of this study is to identify whether chemicals leach out of different types of microplastics. We have selected a range of different microplastics, ranging from microplastics derived from plastic beach litter [9], different pre-production microplastics to which humans might be exposed and that are frequently used in toxicological studies (i.e. polystyrenyl chloride (PVC), PE, PS, PP and polyamide (PA)) [41]. Lastly we used post production microplastics derived from a PP food container [42] and polyethylene terephthalate (PET) from a single use plastic water bottle [43]. We have used two different approaches in our studies. First, an in vitro model of the human digestive system and sensitive ICP-MS and GC-MS methods are used to identify which chemicals can leach out of different types of plastics before and after in vitro digestion. Secondly, an in vitro transport study using a well-established intestinal Caco-2 Transwell model is used to evaluate the in vitro translocation of the microplastic associated chemicals (see Fig. 1).
addition, the potential toxicity of the translocated chemicals was investigated using effect studies from the TOXcast programme resulting in an initial risk assessment of the human oral exposure to these microplastics.

Material and methods

Samples

Nine types of microplastics were used in this study. Two types of environmentally relevant microplastics were prepared by Kühn et al. (2018) from plastic litter collected on Dutch beaches, which were cryomilled to particle sizes of 1 and 3 mm (ENV1 and ENV2) [9].

The following pre-production plastic powders were used: polyvinylchloride, unplasticized (PVC); polyethylene (PE); polystyrene (PS); and polyamide (PA), all purchased from Goodfellow Cambridge Ltd. (UK). All but PVC had been cryo-milled before use in clean zirconium oxide grinding jars (25 mL) with approximately 200 zirconium oxide grinding balls (5 mm) at 30 Hz for 20–60 min, depending on the polymer, with automatic precooling. Pre-production polypropylene (PP1) powder was kindly provided as a sample from Diamond Plastics GMBH (Germany). Post-production plastic powders were fabricated from a brand-new polypropylene (PP2) takeaway food container and brand-new polyethylene terephthalate (PET) single-use plastic water bottle, the contents of which were first discarded. Pieces had similarly been cryo-milled before use in a 50 mL jar with a steel ball (25 mm) using the following programme: 6 min precool, 4 × 2 min milling cycles, 45 s intermediate cooling between cycles. Collectively, these powders represent a) polymers commonly used and encountered as microplastic in daily life and b) use cases of polymers which may contribute to oral microplastic exposure and contain a representative additive profile. All powders were stored and provided dry in glass vials with white plastic (PP) caps. The vials were rinsed in MeOH, rinsed three times with ultrapure water, then oven dried. The caps were rinsed in MeOH and left to air dry (face down). The vials were further individually wrapped in aluminium foil to keep the contents in the dark. The powders were also sized. Briefly, an ad hoc mass was combined with 1 mL of EtOH in an Eppendorf tube to create a suspension. Five-microlitre drop casts were then prepared on clean, plain microscope slides. The drop cast was viewed under a microscope (OLYMPUS BX53). Following a north-south transect through the middle of the drop cast, intercepted particles were viewed at 100-500x magnification and sized in OLYMPUS Stream Start software (Table 1).

Assessment of worst case chemical leachates from microplastics

For the determination of organic compounds 50 mg of microplastic material was ultrasonically extracted for 10 min with 2 mL of ethyl acetate (ENV1, ENV2, PE, PP1, PP2, PET, PA) or acetonitrile (PS and PVC). Acetonitrile was used for the extraction of PS and PVC since these...
tend to dissolve in ethyl acetate. $^{13}$C-PCB-209 was added as an internal standard. The extracts were filtered through a small column of glass wool and sodium sulphate to remove the plastic particles. 100 μL of the extracts were analysed with GCxGC-TOFMS as described above. A sequential extraction was carried out on the extracted microplastic residue of the environmental microplastics which are believed to contain absorbed additives as well as adsorbed contaminants. The results showed that > 90% of the organic compounds were recovered in the first extraction. The filtration step was tested with a suite of representative organic compounds and showed that no absorption occurred during filtration of the extract. Procedural blanks were included and showed low levels of interfering phthalates. Results were corrected for these blank findings.

For the determination of elements 50 mg of plastic was combined with 3 mL of nitric acid (HNO$_3$, 70%) in a glass microwave digestion tube. The samples were digested in a Discover microwave system and the temperature program was as follows: 1600 W power from 20 to 120 °C in 15 min, then to 160 °C in 10 min, and then to 210 °C in 30 min and hold for 1 min. Following digestion and cooling to room temperature, rhodium was added as an internal standard and the extract was diluted with ultrapure water to a concentration of 3% HNO$_3$ (and further with 3% HNO$_3$ to fit the calibration line) and the extract was analysed with ICP-MS. Reference materials BCR-680 and -681 [44] were analysed using the same method and showed good recoveries of the target elements in the range of 89–107%. Procedural blanks were included and showed no interfering elements.

**Assessment of chemical leachates from microplastics during in vitro human intestinal digestion**

An in vitro human digestion model was used to determine the potential release of chemicals from microplastics during human digestion. The in vitro digestion model consists of three steps: saliva, gastric and intestine digestion. Artificial digestive juices for the digestion experiments were prepared on the day before the actual digestions. The constituents and concentrations of these juices are listed in Table 2 (from [45, 46]). The in vitro digestion starts by introducing 2 mL of artificial saliva to 100 mg of microplastic in amber 15 mL glass tubes closed with a screw cap with a Teflon insert. This mixture is rotated (head-over-heals) for 5 min at 55 rpm at 37 °C. For the next step, 4 mL of gastric juice is added to the mixture and the pH is adjusted to pH 2.0 ± 0.5 with hydrochloric acid (HCl, 37% w/w). The mixture is rotated for 2 h. Finally, 4 mL of duodenal juice, 2 mL of bile juice, and 0.5 mL of NaHCO$_3$ solution are added to the mixture.
the mixture and the mixture pH is adjusted to pH 6.5 ± 0.5 with NaOH (1 M). The mixture is rotated for another 2 h. After this period a subsample of 4 ml is collected for the in vitro translocation experiment while the remainder of the mixture is filtered over a 0.45 μm filter to remove the microplastics.

A subsample of 8 ml of the filtrate is extracted with 4 ml of ethyl acetate after addition of the 13C-PCB-209 internal standard, the extract is dried with sodium sulphate and 100 μl of the extract is analysed with GCxGC-TOFMS as described above to determine the organic chemicals released by the microplastics during digestion. For identification a dedicated library was used that was compiled from the compounds that were identified in the initial chemical analysis of the microplastics. The aqueous phase is left over night for the residue ethyl acetate to evaporate. Next, a subsample of 4 ml of the aqueous phase is collected, acidified with nitric acid to 3% HNO3, and after addition of rhodium as the internal standard analysed with ICP-MS as described above to determine the elements released by the microplastics during digestion.

**Determination of translocation of chemical leachates from microplastics using intestinal Caco-2/HT29-MTX cell layers**

Caco2/HT29-MTX cells were used to prepare an in vitro monolayer of intestinal cells in a 12-well plate with Transwell inserts. The human colonic adenocarcinoma (Caco-2) cell line was obtained from the American Type Culture Collection. The human colon adenocarcinoma mucus-secreting (HT29-MTX) cell line was obtained from the European Collection of Cell Cultures. Cells were cultured in Dulbecco’s Modified Eagle Medium with GlutaMAX (DME) (Gibco, Waltham MA, USA) supplemented with 10% fetal calf serum (Gibco), 1% penicillin-streptomycin (Sigma) and 1% non-essential amino acids (Gibco). The cells were subcultured every 2–3 days. For the translocation experiments, the cells were seeded at a density of 40,000 cells per cm2 in Transwell polyester inserts (3 μm pore size, 1.12 cm2 surface area, Corning, Amsterdam, The Netherlands), with a 3:1 ratio of Caco-2/HT29-MTX cells and grown for 21 days. During this period of growing the medium was refreshed every 2–3 days. To assess the integrity of the cell barrier the TEER (Trans Epithelial/Endothelial Electrical Resistance) values were measured using a MERSSTX01 electrode (Millipore, USA) connected to the Millicell ERS-2 Epithelial Volt-Ohm meter (Millipore). Only inserts with initial TEER values above 200 Ωcm2 were used [47]. After the exposure, TEER values of the microplastic exposed wells were not lower than the TEER values detected in control wells. In addition fluorescein transport was studied after exposure to the microplastics. For this fluorescein (Sigma), was added apically to the inserts at a concentration of 10 μM in HBSS (500 μl/insert). Following 1 h incubation at 37 °C basolateral samples were collected. And analysed for fluorescence at 494/525 nm, and compared to a calibration curve. Fluorescence translocation following exposure of cell layers was not increased compared to that in control wells (i.e. 6.1% ± 1.1 and 5.6 ± 1.3 respectively) and in line with previous observations in our laboratory [48].

The cells in these inserts were exposed for 24 h to the exposure medium which was prepared from the unfiltered fluids that resulted from the simulated in vitro digestion. For the final exposure chyme samples were diluted towards non-cytotoxic chyme concentrations as shown before [46, 49]. These samples were vortexed and diluted 10 times with a mixture of HBSS containing 30 mg BSA and 0.01 ml penicillin and streptomycin/ml HBSS to keep the sample sterile. 0.5 ml of the exposure medium was added apically to the inserts with cells. 1.5 ml of a mixture of 10 mg BSA and 0.01 ml P/S /ml HBSS was added to the basolateral compartment per insert.

Typically, 75 μl subsamples were taken at the basolateral compartment at t = 0 and t = 24 h while the plate was kept in the incubator at 37 °C between these time points. The samples were stored in glass vials at ~ 80 °C before further analysis. To determine the organic chemicals that were translocated, the basolateral samples were extracted with 200 μl of ethyl acetate after addition of the internal standard 13C-PCB-209. The extract was dried with sodium sulphate and 100 μl of the extract was analysed with GCxGC-TOFMS as described above. For identification, a dedicated library was used that was compiled from the compounds identified in the in vitro digestion samples. To determine the elements that were translocated during the experiment, a 200 μl aqueous subsample was diluted to 4 mL and acidified with nitric acid to 3% HNO3 and after addition of rhodium as the internal standard analysed with ICP-MS as described above.

**Chemical analysis**

For the identification and quantification of chemicals that are associated with the microplastics, chemical analyses were performed. For the determination of the organic compounds sample extracts were analysed with two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GCxGC-TOFMS). The GCxGC-TOFMS was a combination of a Pegasus-III TOFMS system (LECO, Corporation, St. Joseph, USA) and an Agilent 6890 gas chromatograph (GC, Agilent technologies, Santa Clara, USA) equipped with CIS-4 PTV injector (Gerstel, town, country). The GC was equipped with a 10 m × 0.25 mm, inner diameter 0.25 μm, Rtx-Cl
Pesticide column (Restek, Mülheim an der Ruhr, Germany) in the first dimension and a 1 m x 0.1 mm, film thickness 0.1 μm BPX-50 column (SGE, Austin, USA) in the second dimension. The temperature program was as follows; 1st dimension 60 °C (2 min) to 320 °C (6 min) increasing the temperature with 15°/min; 2nd dimension 125 °C (5 min) to 270 °C with increasing the temperature 15°/min; to 345 °C with 25°/min and to 365 °C (7 min) with 15°/min). A two-step modulator cooled with liquid nitrogen was used and the run time in the second dimension was 4.5 s. The scan speed was 200 scans/s with a mass range of 50–1000 m/z. Peaks were identified using a dedicated in house library database that contained 650 compounds (mainly environmental contaminants) and a NIST database. Only identifications of peaks for which the correlation between the mass spectrum of the unknown component in the chromatogram and the reference spectrum in the database is > 80% were accepted. Compounds identified with the dedicated library database were quantitatively detected while for the other compounds the detection was semi-quantitative based on an averaged response factor.

For the determination of elements aqueous samples or sample digests are analysed with inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS was a Perkin Elmer 350D equipped with an autosampler and a conical glass concentric nebulizer and operated at an RF power of 1600 W. Rhodium was added as an internal standard and data acquisition was performed in the scanning mode with KED (helium 4.1 mL/min) using m/z values in the range of m/z 7 to 238 for the selected elements. Quantification was based on an external standard containing 70 individual elements.

**Data analysis**

GraphPad Prism 5 was used to prepare the graphs. We assessed the ToxCast database [50] online, selected the end-points from the high-throughput assay matching our compounds and extracted the respective activity values 50 (AC50, concentration at 50% of maximum activity), and the indicated Adverse Outcome Pathways. The IARC database [51] was also assessed online.

**Results and discussion**

Chemical toxicity following ingestion of microplastics may occur as they can act as vectors to transfer associated chemicals into the body [52]. Using sensitive GC-MS and ICP-MS we here show firstly that 68 chemicals leached from the microplastics using stringent chemical extraction (Tables 3 and 4) and secondly that some of these chemicals can be released in vitro under conditions representing the luminal content of the human digestive tract (i.e. chyme). For this the in vivo digestive tract conditions were simulated using an in vitro digestion model and the concentration of chemicals in the chyme were quantified. Next the potential translocation of the chemicals in the chyme was evaluated using the in vitro Caco-2 Transwell intestinal epithelial cell model. Of the detected chemicals, 58 chemicals were present in the ToxCast database of which 31 were screened (to some extent). From those we identified AC50 from high-throughput assays targeting endocrine activity. We observed that estrogenic potency is the most often involved Adverse Outcome Pathway (AOP) of the chemicals listed in the ToxCast database. Next to the analysis of the sample for the presence of organic chemicals, samples were analysed for the presence of elements using ICP-MS. In total 28 elements were identified leaching from the microplastics. More details of the chemical presence will be discussed per microplastics type.

**Environmental microplastics < 1 mm**

The environmental microplastics originate from plastic litter collected on Dutch beaches that was subsequently grinded down to two size fractions (i.e. 1 mm and 3 mm) [9]. In the chemical leachate of the 1 mm environmental microplastics 42 organic compounds were identified with typical flame retardants and phthalates being the most prominent (see Fig. 2). Although low concentrations of hexabromocyclododecane and pentabromocyclododecane were found, the major flame retardants were organophosphates with a highest leachate concentration of 7.6 mg/kg for tris (1-chloro-2-propyl)phosphate. Other prominent flame retardants were tri-tylphosphates and bis (3-chloro-1-propyl)(1-chloro-2-propyl)phosphate. Of the phthalates bis (2-ethylhexyl) and di-isoctyl phthalate were identified as the most prominent with the highest concentration being 8.9 mg/kg. Another prominent phthalate was identified as phthalic acid, hex-3-yl nonyl ester with a concentration of 6.1 mg/kg. A number of other compounds were identified with 2,4-di-tert.butyl phenol being the most prominent with a concentration of 6.7 mg/kg. Finally, a series of polycyclic aromatic hydrocarbons (PAH) were identified in concentrations ranging from 0.9 mg/kg for naphthalene to 0.1 mg/kg for chrysene. Some differences with the previous chemical analysis are noted, but the for the most abundant chemicals we confirm the previous analyses [9].

After the in vitro digestion of the 1 mm environmental microplastics 27 organic compounds were identified in the chyme (Fig. 2). The flame retardants and phthalates identified in the chyme sample were also observed in the chemical leachates. In fact, the concentrations of the chlorinated organophosphates were in general comparable to those in the chemical leachates, probably because they are relatively water soluble and are therefore easily released during the in vitro digestion into the chyme.
| Compound                                         | CAS nr   | Present in sample type | Present in Toxcast | AC50 (uM) | AHR OS (NFE2L2) | AR ER (ESR1) |
|--------------------------------------------------|----------|------------------------|---------------------|-----------|-----------------|--------------|
| **Flame retardants**                             |          |                        |                     |           |                 |              |
| Bis(1-chloro-2-propyl)-3-(3-chloropropoxy)propylphosphate | 137,888-37-0 | ENV-1, ENV-3           | no                  |           |                 |              |
| Bis(3-chloro-1-propyl)(1-chloro-2-propyl)phosphate | 137,888-35-8 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.d.         |
| Hexabromocyclododecane                           | 25,637-99-4 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.d.         |
| Pentabromocyclododecane                          | 26,657-83-0 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.d.         |
| Triethylphosphate                                | 78-40-0  | ENV-1                  | yes                 | n.d.     | n.a.            | 90.5         |
| Tris(1-chloro-2-propyl)phosphate                 | 13,674-84-5 | ENV-1, ENV-3, PA, PE, PP-1, PP-2, PS, PVC | yes            | n.d.     | n.d.            | n.d.         |
| Tris(2-chloroethyl)phosphate                     | 115-96-8  | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.a.         |
| Tris(2-chloroisobutyl)phosphate                  | 6145-73-9 | ENV-1, ENV-3, PA, PE, PP-1, PP-2, PVC | yes             | n.a.     | n.a.            | n.a.         |
| **Plasticiser**                                  |          |                        |                     |           |                 |              |
| 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 6422-86-2 | ENV-1, ENV-3           | yes                 | 68.9     | n.d.            | n.a.         |
| Bis(2-ethylhexyl) phthalate                      | 117-81-7 | ENV-1, ENV-3, PA, PE, PET, PP-1, PP-2, PS, PVC | yes             | n.d.     | 52.1            | 59.5         |
| Dibutyl phthalate                                | 84-74-2  | ENV-1, ENV-3, PA, PP-1, PP-2, PVC | yes             | n.d.     | 50.9            | 58.0         |
| Diisooctyl adipate                               | 1330-86-5 | ENV-1                  | yes                 | n.d.     | n.d.            | 3.38         |
| Diisooctyl phthalate                             | 27,554-26-3 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.d.         |
| Phthalic acid, 2-ethylbutyl nonyl ester          | n.a.     | ENV-3                  | no                  |           |                 |              |
| Phthalic acid, 6-methylhept-2-yl nonyl ester     | n.a.     | ENV-1                  | no                  |           |                 |              |
| Phthalic acid, di(2-propylpentyl) ester          | 70,910-37-1 | PA, PET, PP-1, PP-2, PVC | yes             | n.d.     | n.d.            | n.d.         |
| Phthalic acid, di(oct-3-yl) ester                | n.a.     | ENV-3, PE              | no                  |           |                 |              |
| Phthalic acid, hept-3-yl nonyl ester/phthalic acid hexyl nonyl ester | 88,216-56-2 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | n.d.         |
| Phthalic acid, hept-4-yl isobutyl ester          | n.a.     | PA, PVC                | no                  |           |                 |              |
| Phthalic acid, nonyl 2-propylpentyl ester/ phthalic dinonyl ester | 84–76-4 | ENV-1, ENV-3           | yes                 | n.d.     | n.d.            | 3.4          |
| 2-Ethylhexyl methyl isophthalate                 | 2,135,327-80-7 | ENV-1                  | yes                 | n.d.     | n.d.            | n.d.         |
| Tributyl acetylcitrate                           | 693-71-0  | PET                    | yes                 | n.d.     | n.d.            | n.d.         |
| **Monomers and other compounds**                 |          |                        |                     |           |                 |              |
| (Z)-(Z)-Hex-3-en-1-yl 2-methylbut-2-enoate       | 84,060-80-0 | ENV-1                  | no                  |           |                 |              |
| 1,15-Pentadecanediol                             | 14,722-40-8 | PE                     | yes                 | n.d.     | n.d.            | n.d.         |
| 1,8-Dioxaicyclotetradecane-2,9-dione (=nylon-6 cyclic oligomer) | 56,403-09-9 | PA                     | yes                 | n.d.     | n.d.            | n.d.         |
| 1H-Imidazole                                     | 288-32-4  | ENV-1                  | yes                 | n.d.     | n.d.            | n.d.         |
| 2,4-Di-tert-butylphenol                          | 96–76-4  | ENV-1, ENV-3           | yes                 | n.d.     | 36.9            | 12.4         |
| 2-Ethyl-1-hexanol                                | 104–76-7  | ENV-1                  | yes                 | n.d.     | n.d.            | n.d.         |
| 3-(Benzoylthio)-2-methylpropanoic acid           | 74,431-50-8 | ENV-3                  | no                  |           |                 |              |
| 3,3-Dimethylheptanoic acid                       | 67,061-30-7 | PVC                   | no                  |           |                 |              |
| 3,5-di-tert-Butyl-4-hydroxybenzaldehyde          | 1620-98-0 | ENV-1                  | yes                 | n.d.     | n.d.            | n.d.         |
The alkyl- and arylphosphate flame retardants were also present in the chyme but in much lower concentrations than in the chemical leachates (on average 20%). The far more apolar hexabromocyclododecane and pentabromocyclododecane were not detected in the chyme. Phthalates were also found in the chyme and may therefore become available for uptake via the intestinal epithelium. However, the observed concentrations in chyme were about half of those in the chemical leachates. Of the polycyclic aromatic hydrocarbons only naphthalene was identified in the chyme.

Only a few compounds were detected in the basolateral fluids of the translocation study (Fig. 2). Again, these were mainly the chlorinated organophosphate

### Table 3 Overview of all identified plastic associated chemicals and in which type of microplastics sample they were detected (Continued)

| Compound                                             | CAS nr  | Present in sample type | Present in Toxcast | AC50 (µM) | AHR (NFE2L2) | AR | ER (ESR1) |
|-------------------------------------------------------|---------|------------------------|---------------------|-----------|--------------|----|-----------|
| 3-tert-Butyl-4-hydroxyanisole                         | 121–00-6| PVC                    | yes                 | n.d.      | 42.4         | 13.1| 2.46      |
| Alkylated benzenes                                    | n.a.    | PS                     | no                  |           |              |     |           |
| Benzaldehyde                                          | 100–52-7| PS                     | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| Benzene, 1-(chloromethyl)-2-methyl                    | 552–45-4| PVC                    | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| Benzene, 1,3-bis(1,1-dimethylethyl)-                   | 1014-60-4| ENV-1, ENV-3          | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| Benzoic acid, octyl ester                            | 94–50-8 | PVC                    | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| Borane, diethyl (decyloxy)                            | 25,015–63-8| PP-1                | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| Butanediol acid, dimethyl ester                       | 106–65-0| PVC                    | yes                 | 1.49      | n.d.         | n.d.| n.d.      |
| Butylated Hydroxytoluene                              | 128–37-0| ENV-1, ENV-3          | yes                 | 49.2      | 0.112        | 21  |           |
| Caprolactam (=nylon-6 monomer)                        | 105–60-2| PA                     | yes                 | n.d.      | 68.0         | 30.8|           |
| Hexanediol acid, bis(2-ethylhexyl) ester              | 103–23-1| PVC                    | yes                 | n.d.      | 93.0         | 10.9|           |
| Hexanediol acid, bis(2-methylpropyl) ester            | 141–04-8| ENV-3                  | yes                 | n.d.      | n.d.         | n.d.| 8.25      |
| Hexanediol acid, dimethyl ester                       | 627–93-0| PVC                    | yes                 | n.d.      | n.d.         | n.d.|           |
| Lincosadiacid                                          | 506–21-8| ENV-1                  | yes                 | n.d.      | n.d.         | n.d.| n.d.      |
| n-Hexadecanionic acid                                  | 57–10-3 | PP-1, PP-2             | yes                 | n.d.      | 7.46         |     |           |
| Octadecane, 1-isocyanoanionic acid                    | 112–96-9| PA                     | yes                 | n.d.      | n.d.         | n.d.|           |
| Octadecanionic acid                                   | 57–11-4 | PP-1, PP-2             | yes                 | n.d.      | 12.1         | 2.3 |           |
| Octanal, 2-(phenylmethylene)-                         | 101–86-0| ENV-1                  | yes                 | 56.6      | 30.9         | 2.97| 35.5      |
| Oxalic acid, allyl butyl ester                        | 91,915–04-7| PVC               | yes                 | n.d.      | n.d.         | n.d.|           |
| Pentanediol acid, dimethyl ester                      | 1119-40-0| PVC                    | yes                 | n.d.      | n.d.         | n.d.|           |
| Styrene                                               | 100–42-5| PS                     | yes                 | n.d.      | n.d.         | n.d.|           |
| Tetrazolo(1,5-b)1,2,4-triazine, 5,6,7,8-tetrahydro-6,7-| 384,814–90-8| PA                | no                  |           |              |     |           |
| dimethyl-                                             |                                                   |                       |           |              |     |           |

Environmental contaminants

- Acenaphthene                                          | 83–32-9 | ENV-1, ENV-3          | yes                 | n.d.      | n.d.         | n.d.| 24.9      |
- Acenaphthyline                                        | 208–96-8| ENV-1                 | yes                 | 91.5      | n.d.         | n.d.|           |
- Antracene                                             | 120–12-7| ENV-1, PP-1, PP-2     | yes                 | n.d.      | 22.3         | 24.1|           |
- Benz[a]anthracene                                     | 56–55-3 | ENV-1, ENV-3          | yes                 | n.d.      | 1.17         | 5.77| 1.45      |
- Chrysene                                              | 218–01-9| ENV-1, ENV-3          | yes                 | 7.86      | 28.2         | n.d.|           |
- Fluoranthene                                          | 206–44-0| ENV-1, ENV-3          | yes                 | 67.0      | 5.83         | 46.2|           |
- Fluorene                                              | 86–73-7 | ENV-1, ENV-3, PE      | yes                 | n.d.      | 70.1         | 18.1| 0.436     |
- Naphthalene                                           | 91–20-3 | ENV-1, ENV-3, PP-1, PP-2, PVC | yes                   | n.d.      | 20.7         | 0.002|           |
- Phenanthrene                                          | 85–01-8 | ENV-1, ENV-3, PP-1, PP-2, PVC | yes                   | n.d.      | 3.32         | n.d.|           |

Legend: ENV-1 environmental microplastic < 1 mm, environmental microplastic < 3 mm, PA polyamide, PE Polyethylene, PET Polyethylene terephthalate, PP-1/PP-2 Polypropylene, PS Polystyrene, PVC Polychloroethylene.

AHR Aryl hydrocarbon receptor, OS oxidative stress: activation of NFE2L2 (Nuclear factor erythroid 2-related factor 2) gene, AR activation of androgen receptor, ER estrogenic activity, activation of Estrogenic receptor 1, n.d. not determined, n.a. not available.
flame retardants bis (3-chloro-1-propyl)(1-chloro-2-propyl) phosphate, tris (1-chloro-2-propyl) phosphate and tris (2-chloropropyl)phosphate. The concentrations of these flame retardants in the chyme and after in vitro translocation in the basolateral fluids were in the range of 0.1 to 0.9 mg/kg. Of the phthalates, di-isooctyl phthalate was found in a relatively high concentration of 2.2 mg/kg in the basolateral fluids after the in vitro translocation experiment while other phthalates (mainly bis (2-ethylhexyl)phthalate) were also detected but at concentrations < 1 mg/kg. Of the PAHs only naphthalene was found in the basolateral fluids, in a concentration of 0.1 mg/kg. The in vitro translocation experiments were, obviously, performed in plastic Transwells, therefore absorption and adsorption of chemicals to the plastic material need to be considered. The equilibrium between chemicals in medium and absorbed to plastic take several hours or days for high log K<sub>ow</sub> chemicals, and several days for neutral chemicals. As recently reviewed, absorption rates of chemicals to plastics are slower than adsorption rates of chemical to plastics [53]. These processes might result in an underestimation of the chemicals present after in vitro translocation.

A large number of elements were also found in the 1 mm environmental microplastics, in total 28 ranging from lithium to bismuth (Fig. 3). It should be kept in mind that, different from the organic compounds, these elements were not leached from the microplastics but liberated after a complete digestion of the plastic matrix so the concentrations will be accordingly higher. Highest concentrations were found for iron (2099 mg/kg) and barium (1544 mg/kg). Barium sulfate is being used as a

| Name | CAS nr | Present in sample | chronic oral PoD (mg/kg day) | Point of Departure | Species |
|------|--------|-------------------|-----------------------------|-------------------|---------|
| Li 7 | 7439-93-2 | ENV-1, ENV-3, PA, PE, PET, PP-1, PP-2, PVC | 1.2 | NOAEL | human |
| Ti 47 | 7440-32-6 | ENV-1, ENV-3, PA, PE, PP-1, PP-2, PS, PVC | 24,000 | NOEL | rat |
| V 51 | 7440-62-2 | ENV-1, ENV-3, PA, PE, PP-1, PVC | 0.22 | NOAEL | rat |
| Cr 52 | 7440-47-3 | ENV-1, ENV-3, PA, PE, PVC | 1216 | NOAEL | rat |
| Mn 55 | 7439-96-5 | ENV-1, ENV-3, PA, PE, PET, PP-1, PP-2, PS, PVC | 0.14 | NOAEL | human |
| Fe 56 | 7429-89-6 | ENV-1, ENV-3, PA, PE, PP-1 | 1 | LOAEL | human |
| Co 59 | 7440-48-4 | ENV-1, ENV-3, PA, PE, PET | 1 | LOAEL | human |
| Ni 60 | 7440-02-0 | ENV-1, ENV-3 | 5 | NOAEL | rat |
| Cu 65 | 7440-50-8 | ENV-1, ENV-3, PA, PEPS | 5.3 | LOAEL | human |
| Zn 66 | 7440-66-6 | ENV-1, ENV-3, PVC | 0.9 | NOAEL | human |
| As 75 | 7440-38-2 | ENV-1, ENV-3, PVC | 0.0009 | NOAEL | human |
| Se 78 | 7782-49-2 | ENV-1, ENV-3 | 0.015 | NOAEL | human |
| Rb 85 | 7440-17-7 | ENV-1, ENV-3, PVC | 5.3 | LOAEL | not mentioned |
| Sr 88 | 7440-24-6 | ENV-1, ENV-3, PA, PE, PP-1, PVC | 60 | NOAEL | human |
| Zr 91 | 7440-67-7 | ENV-1, ENV-3, PA, PE, PET, PP-1, PP-2, PVC | 3156 | NOAEL | rat |
| Mo 98 | 7439-98-7 | ENV-1, ENV-3, PA, PE, PP-1, PVC | 5 | NOAEL | human |
| Ag 107 | 7440-22-4 | ENV-1, ENV-3, PA, PE, PVC | 0.014 | LOAEL | not mentioned |
| Cd 111 | 7440-43-9 | ENV-1, ENV-3 | 0.0003 | NOAEL | human |
| Sn 118 | 7440-31-5 | ENV-1, ENV-3, PE, PET, PS, PVC | 30 | NOAEL (subacute) | human |
| Sb 121 | 7440-36-0 | ENV-1, ENV-3, PE, PET, PP-1 | 0.06 | NOAEL (subacute) | human |
| Ba 138 | 7440-39-3 | ENV-1, ENV-3, PA, PE, PP-1 | 0.21 | NOAEL | human |
| Ce 140 | 7440-46-2 | ENV-1, ENV-3, PA, PE, PP-1, PVC | n.d. | n.d. | n.d. |
| Ta 181 | 7440-25-7 | ENV-1, PA, PET, PS | n.d. | n.d. | n.d. |
| W 184 | 7440-33-7 | ENV-1, PA, PE | 2 | NOAEL | rat |
| Re 187 | 7440-15-5 | ENV-1, PP-1 | n.d. | n.d. | n.d. |
| Ti 205 | 7440-28-0 | ENV-1, ENV-3, PA, PE,PP-1 | 0.04 | NOAEL | not mentioned |
| Pb 208 | 7439-92-1 | ENV-1, ENV-3, PA, PE,PP-1, PS, PVC | 0.5 | BMDL01 | human |
| Bi 209 | 7440-69-9 | ENV-1, ENV-3, PA, PE,PP-2, PS | 25 | NOAEL | rat |

For each metal it is indicated if a point of departure (PoD) was listed in the TOXCAST database. 

ENV-1 environmental microplastic < 1 mm, environmental microplastic < 3 mm, PA polyamide, PE Polyethylene, PET Polyethylene terephthalate, PP-1/PP-2 Polypropylene, PS Polystyrene, PVC Polyvinylchloride
filler in the plastic industry, for instance in the production of polyethylene sewage tubes and may therefore be present in this sample. Iron has been found before in high concentrations in plastic waste from electrical and electronic equipment [54]. Relatively high concentrations were also found for lead, zinc and chromium.

In the in vitro digestion only a limited number of elements, 15 of the 28 observed in the chemical analysis, are present in concentrations which are in general much lower than in the plastic chemical digest (Fig. 3). Highest concentrations were found for iron (33 mg/kg), zinc (26 mg/kg) and strontium (25 mg/kg). Zinc is used in the plastic industry in the form of zinc stearate ("zinc soap"), a release agent and lubricant [55, 56], and may therefore be found in this sample and in the bioaccessible fraction in a relatively high concentration. No information was found on the origin of strontium in the chemical digest and its relatively high concentration in the bioaccessible fraction.

Only two elements were found to translocate across the Caco-2 cell layer in vitro. These were iron, which was present in high concentrations in the chemical digest and in the in vitro digestion, and strontium which was also present in relatively high concentrations in the in vitro digestion (Fig. 3). Concentrations of iron and strontium in the basolateral fluids were 14.5 and 1.8 mg/kg, respectively.

Environmental microplastics < 3 mm

The results for the 3 mm environmental microplastics are comparable to that of the 1 mm microplastic material which is to be expected since it is the same material only grinded to a different particle size. In total 33 different compounds were identified and as before the flame retardants and phthalates are the most prominent (Fig. 2). In general, the concentrations are somewhat lower than for the 1 mm material, as to be expected because of the smaller surface area to volume ratio. Some other compounds like 2,4-di-tert.butyl phenol and butylated hydroxytoluene were also found in this sample. The PAHs were also found in this sample but, as with the others, in somewhat lower concentrations.

The flame retardants, especially the chlorinated organophosphates, and the phthalates were also found in the in vitro digestion in concentrations which were about half of the concentrations in the chemical leachate (Fig. 2). Of the PAHs only naphthalene was found in a concentration of 0.4 mg/kg which was equal to what was
found in the in vitro digestion of the 1 mm microplastics.

Only a limited number of compounds translocated across the Caco-2 cell layer in vitro (Fig. 2). As for the 1 mm microplastics this was limited to the chlorinated organophosphates and a number of phthalates. Typical concentrations of compounds following in vitro digestion and basolateral fluids ranged from 0.1 to 1.8 mg/kg. Of the PAHs on naphthalene was found in a concentration of 0.1 mg/kg.

A total number of 25 elements were found after chemical digestion of the 3 mm environmental microplastics (Fig. 3). The nature and concentration of these elements is comparable with that of the 1 mm microplastics with iron, barium and lead as the highest. Differences between the 1 and 3 mm material probably originate from the inhomogeneity of the microplastic materials.

The results for the in vitro digestion are also comparable with that for the 1 mm material (Fig. 3). 13 of the 25 elements were found and iron, zinc and strontium are found in the highest concentration. It should be noted that the concentration of lead, 11 mg/kg, in this sample was comparable with that of strontium. Lead is sometimes present in paints which are used as surface coatings for plastic materials and may therefore be found in the investigated microplastics [57]. For the same reason lead may also end up in recycled plastic materials. In the in vitro translocation experiment no elements were detected in the basolateral fluids (Fig. 2). Taking into account the dilutions in the translocation
experiment the limit of detection for the elements is 0.3 mg/kg.

Polyamide microplastics
For these and the other pre-production powders, the source of the chemicals detected in our chemical leachates (and chyme and following translocation) could either be intentionally to the plastics added additives, impurities from the production process, whereas for the environmental microplastics contaminants sorbed from the ambient environment is an additional source. In the pre-production polyamide microplastics 12 compounds were identified in the chemical leachate (Fig. 4). Some flame retardants and phthalates were found but only in low concentrations in the range of 0.1 to 0.7 mg/kg. Most noteworthy are the monomer caprolactam that was found in a concentration of 70 mg/kg and 1,8-diazacyclotetradecane-2,9-dione, which is a nylon-6 cyclic oligomer, which was found in a concentration of 42 mg/kg. Also found was 1-isocyanato octadecane and 5,6,7,8-tetrahydro-6,7-dimethyl-tetrazolo[1,5-b]1,2,4-triazine in concentrations of 5.3 and 2.6 mg/kg, respectively.

Most of the compounds identified in the chemical leachate were also detected in the in vitro digestion be it in lower concentrations (Fig. 4). An exception is caprolactam which is water soluble and is found in a relatively high concentration of 46 mg/kg in the in vitro digestion. Caprolactam is also found in a relative high concentration of 25 mg/kg after in vitro digestion and translocation indicating that there is a potential uptake of this compound following ingestion of polyamide microplastics. Other compounds found after in vitro digestion and translocation are a few phthalates (Fig. 4).

A surprisingly high number of 19 elements were detected in the polyamide microplastics (Fig. 2). While most concentrations are < 10 mg/kg, concentrations for iron, zirconium, barium and bismuth were somewhat higher. Zirconium could be an artefact of the milling process, which was performed in a zirconium oxide jar with zirconium oxide grinding balls. Bismuth pigments have been incorporated into plastics such as polyamide as alternatives for difficult to replace lead chrome pigments which may explain the presence of bismuth [58]. Only four of these elements were also found in the in vitro digestion (Fig. 2). These are vanadium, which may be related to bismuth since it are bismuth-vanadium pigments which exert the yellow color, molybdenum, tungsten and bismuth, with the exception of bismuth in concentrations < 1 mg/kg. Finally, none of the elements was detected after the translocation experiment following the in vitro digestion (Fig. 2), as mentioned before potential adsorption of the elements to the plastic labware used for the translocation experiment cannot be excluded [53].

Polyethylene microplastics
In the polyethylene microplastics 6 compounds were identified in the chemical leachate (Fig. 5). Two flame retardants, both tris (chloro-propyl) phosphates, were found but only in low concentrations of 0.2 and 0.1 mg/kg. Bis (2-ethylhexyl) phthalate was found in a higher concentration of 4.2 mg/kg while phthalic acid, di (oct-3-yl) ester was found in a concentration of 0.5 mg/kg. Other compounds found in low concentrations were 1,15-pentadecane-diol and fluorene.

![Fig. 4](image-url) Overview of chemicals (concentrations in mg/kg microplastics) quantified in leachates of the polyamide (PA) microplastics. P: chemicals in chemical leachate from microplastics; D chemicals in intestinal chyme following in vitro digestion; T chemicals in basolateral compartment following exposure to in vitro epithelial Caco-2 cell layer. Red: value out of scale range.
Four of the compounds identified in the chemical leachate were also detected in the in vitro digestion although in concentrations < 1 mg/kg (Fig. 5). Bis (2-ethylhexyl) phthalate, Tris (1-chloro-2-propyl) phosphate and 1,15-pentadecanediol were also found after in vitro digestion and translocation at concentrations of 0.3 and 0.1 mg/kg indicating only minor transfer from the microplastic material (Fig. 5).

In total 18 elements were detected in the polyethylene material (Fig. 2). While most were detected in concentrations < 10 mg/kg, titanium, iron, zirconium and barium were detected at higher concentrations up to 62 mg/kg for iron. The diverse presences of metals has also been observed in a study analyzing PE plastics used for different applications [59]. Titanium dioxide coated with zirconium oxides are sometimes mixed with polyethylene to increase its strength against environmental conditions which may explain its presence in this material. Only 2 of the 18 elements, tungsten and bismuth, were also detected in the chyme following in vitro digestion be it in low concentrations (Fig. 2). None of the elements were detected after the translocation experiment following the in vitro digestion (Fig. 2).

**Polyethylene terephthalate microplastics**

As expected only very few organic compounds were detected in polyethylene terephthalate (PET) (Fig. 6). These were limited to low concentrations of bis (2-ethylhexyl) phthalate (1.7 mg/kg), bis (2-propylpentyl) phthalate (0.4 mg/kg) and tributyl acetylclitate (0.6 mg/kg). Tributyl acetylclitate is a popular plasticizer that is used as a replacement for phthalates although no information was found about its presence in PET.

All three plasticizers were also found in the in vitro digestion, again at low concentrations < 1 mg/kg (Fig. 6). For tributyl acetylclitate the concentration in the in vitro digestion is almost equal to that in the chemical leachate which may be explained by its moderate water solubility. Two of the plasticizers, bis (2-ethylhexyl) phthalate and tributyl acetylclitate were also found after the translocation experiment following the in vitro digestion at concentrations of 0.1 and 0.3 mg/kg, respectively (Fig. 6).

Only 8 elements were detected in PET, all in low concentrations with the exception of antimony (Sb) which was found at a high concentration of 234 mg/kg (Fig. 2). Since antimony is used as a catalyst in the form of
compounds such as antimony trioxide ($\text{Sb}_2\text{O}_3$) or antimony triacetate in the production of PET, its presence in this material is not surprising [60]. Of the other elements zirconium is found in a concentration of 1.3 mg/kg while the others are all < 1 mg/kg. Again, this is likely an artefact of the milling process.

Antimony is the only element which is found in the in vitro digestion be it in an already low concentration of 1.3 mg/kg (Fig. 2). None of the elements are found after the translocation experiment following the in vitro digestion (Fig. 2). This suggests that for PET microplastic particle exposure from drinking plastic bottled water, the potential for effects to manifest due to chemical leaching is low.

**Pre-production polypropylene microplastics**

In the pre-production sample of polypropylene microplastics (PP1) 11 organic compounds were detected in the chemical leachate (Fig. 7). These included low concentrations of the flame retardants tris (1-chloro-2-propyl) phosphate and tris (2-chloropropyl) phosphate and three plasticizers, bis (2-ethylhexyl) phthalate, dibutylphthalate and di (2-propylpentyl)phthalate. These industrial plasticizers may have been used to improve the mechanical properties of the polypropylene material. Other compounds that were detected were hexadecanoic acid and octadecanoic acid at higher concentrations of 2.1 and 7.6 mg/kg. Fatty acids are used in plastic moulding as external lubricants and release agents and may therefore be present in this material. Surprisingly, the material also contained three polycyclic aromatic hydrocarbons, naphthalene at a low concentration and higher concentrations of anthracene and phenanthrene. It is presently unclear why these compounds are found in the polypropylene microplastics.

Nine of the 11 detected compounds were also found in the in vitro digestion (Fig. 7). The highest concentrations are found for the two alkanoic acids, hexadecanoic acid at 2.6 mg/kg and octadecanoic acid at 5.4 mg/kg, while the other compounds were found in concentrations of 1 mg/kg or lower. The two alkanoic acids are also found after translocation following the in vitro digestion (Fig. 7), indicating that a part of these alkanoic acids will be taken up in the body following ingestion of the microplastic material. Bis (2-ethylhexyl) phthalate was also found in a low concentration after translocation following the in vitro digestion.

In total 13 elements were detected in the polypropylene microplastics (Fig. 2). In the chemical digestion of the material a relatively high concentration of iron (116 mg/kg) was found while for barium a concentration of 16 mg/kg was found. In a detailed elemental analysis of different types of PP-based plastic also iron and barium was present a relatively high concentrations, while in that study also zinc was detected [59], which was absent in our PP microplastics. For the other elements concentrations were of 1.7 and 2.0 mg/kg were found for manganese and titanium respectively while concentrations < 1 mg/kg were found for the other elements.

Only iron was found in the in vitro digestion in a concentration of 4.7 mg/kg (Fig. 2). None of the elements were found after translocation following the in vitro digestion.

**Food container polypropylene microplastics**

The results for the organic compounds in the second polypropylene sample (PP 2; food container) are more or less identical to that of the first sample. 9 compounds
are found including the 2 chlorinated phosphate flame retardants, the 3 phthalate plasticizers and the two alkanoic acids, hexadecanoic acid and octadecanoic acid (Fig. 7). Anthracene and phenanthrene were also found in this sample in concentrations of 2.9 and 2.4 mg/kg, respectively. As mentioned before it is unclear where these PAHs come from and why they would be found in the plastic material. They were, however, excluded as contaminants in the analytical determination and identified based on retention time and full-scan mass spectrum.

All the identified compounds were also found in the in vitro digestion be it in lower concentrations (Fig. 7). The highest concentration of around 1 mg/kg were found for bis (2-ethylhexyl) phthalate and the two PAHs. Low concentrations of the hexadecanoic acid, octadecanoic acid, bis (2-ethylhexyl) phthalate and tris (2-chloropropyl) phosphate were found after translocation following the in vitro digestion indicating that some uptake of these compounds following ingestion of the microplastic material may take place (Fig. 7).

The elemental signature of this material is different from the first polypropylene sample (Fig. 2). Only 5 elements were detected with titanium in the highest concentration of 1.3 mg/kg. The four other elements were found at concentrations < 1 mg/kg. Higher concentrations of iron and barium, as found in the first polypropylene sample, were not found in this sample.

In the in vitro digestion and after the translocation following the in vitro digestion none of the elements were found. Element detection limits are 0.1 mg/kg in the in vitro digestion and 0.3 mg/kg in the translocation experiment.

Polystyrene microplastics

In the chemical leachate 31 organic compounds were identified (Fig. 8). These include the flame retardants tri-o-tolylphosphate and tris (1-chloro-2-propyl) phosphate in concentrations of 6.0 mg/kg, the plasticizers bis (2-ethylhexyl) phthalate and dibutylphthalate in concentrations of 4.6 and 0.1 mg/kg, and a large group of alkylbenzenes (not specified in the tables). The latter group consists of 24 alkylbenzenes and biphenylalkanes like 2-methylstyrene, isopropyltoluene, 1,3-diethylbenzene and 2,2',5,5'-tetramethyl-1,1'-biphenyl in concentrations ranging from 0.6 to 3.2 mg/kg and a total concentration of 30 mg/kg. The presence of these alkylbenzenes and biphenylalkanes in the monomer styrene was already shown in 1977 [61]. They detected over 100 impurities and identified 60 of these compounds many of which were alkylbenzenes. Other compounds identified were benzaldehyde in a concentration of 3.9 mg/kg and the monomer styrene itself in a concentration of 3.6 mg/kg. A low concentration of naphthalene, 0.2 mg/kg, was also found.

Many of the identified compounds were also found in the in vitro digestion (Fig. 8). This was especially true for the flame retardants tri-o-tolylphosphate and tris (1-chloro-2-propyl) phosphate which were found in concentrations of 4.2 and 1.9 mg/kg in the in vitro digestion. As mentioned before it is probably the water solubility of these compounds that results in their relatively high concentrations in the in vitro digestion. Bis (2-ethylhexyl) phthalate was also found at a high concentration in the in vitro digestion and this is the only time that the concentration of a compound is higher in the in vitro digestion than in the chemical leachate. Of the
alkylbenzenes, 30 mg/kg in the chemical leachate, only a small fraction, 2.5 mg/kg, is found in the in vitro digestion. Benzaldehyde and the monomer styrene were found in the in vitro digestion in concentrations of 2.0 and 1.0 mg/kg, respectively. The monomer styrene was also found after the translocation following the in vitro digestion in a concentration of 0.7 mg/kg (Fig. 8). Tri-o-tolylphosphate was also present after the translocation and at a surprisingly high concentration of 2.3 mg/kg. Low concentrations were found for tris (1-chloro-2-propyl) phosphate and bis (2-ethylhexyl) phthalate.

In total 7 elements were found in the chemical digestion of the polystyrene microplastics (Fig. 2). The highest concentrations were found for titanium (11 mg/kg) and copper (5.3 mg/kg). Lead was found in a concentration of 1.4 mg/kg while the other elements, manganese, tin, tantalum and bismuth were found at concentrations < 1 mg/kg.

In the in vitro digestion and after the translocation following the in vitro digestion none of the elements were found. Element detection limits are 0.1 mg/kg in the in vitro digestion and 0.3 mg/kg in.

**Polyvinylchloride microplastics**

In the chemical leachate of the polyvinylchloride (PVC) microplastics 17 compounds were identified. These include the flame retardants tri-isobutylphosphate at a concentration of 1.0 mg/kg and traces of the chlorinated phosphates (Fig. 9). Bis (2-ethylhexyl) phthalate was found at a concentration of 4.2 mg/kg and di (2-propylpentyl) phthalate at a concentration of 1.2 mg/kg. Two more phthalates were found at low concentrations. Dicarboxylic acid esters are also used as plasticizers and were also found in the chemical leachate, hexanedioic acid, bis (2-ethylhexyl) ester at a concentration of 6.7 mg/kg, pentanedioc acid, dimethyl ester at a concentration of 3.0 mg/kg, butanedioc acid, dimethyl ester at a concentration of 2.6 mg/kg and hexanedioc acid, dimethyl ester at a concentration of 1.0 mg/kg. A chlorinated alkylbenzene, 1-(chloromethyl)-2-methylbenzene was found at a concentration of 2.0 mg/kg in the chemical leachate. Naphthalene was found at a concentration of 0.5 mg/kg.

A number of the compounds identified in the chemical leachate were also found in the in vitro digestion.
digestion (Fig. 9). These include tri-isobutylphosphate, bis (2-ethylhexyl) phthalate and di (2-propylpen-tyl)phthalate. Of the dicarboxylic acid esters only hexanedioic acid, bis (2-ethylhexyl) ester was found in the in vitro digestion at a low concentration. Naphthalene was also found in the in vitro digestion in a concentration of 0.5 mg/kg. Bis (2-ethylhexyl) phthalate was the only compound that was found after the translocation following the in vitro digestion at a concentration of 0.9 mg/kg.

In total 15 elements were detected in the chemical digestion of the PVC microplastics (Fig. 2). The highest concentration was 260 mg/kg and was found for zinc, which confirms earlier findings were zinc was present in PVC most abundantly, followed by high concentrations of iron [59], which was not detected in our PVC sample. Zinc stearate is used as a heat stabilizer in PVC so its appearance is not unexpected. The concentrations of all other elements were <2 mg/kg. In the in vitro digestion and after the translocation following the in vitro digestion none of the elements were found. Element detection limits are 0.1 mg/kg in the in vitro digestion and 0.3 mg/kg in the translocation experiment.

Initial hazard assessment of chemicals in leachates from microplastics

Microplastics released into the environment during the product life cycle and degraded from plastic waste and items during use are complex chemical mixtures, that can have different sizes, various shapes, can have different polymeric composition and can contain mixtures of chemicals. Human exposure to microplastics might therefore represent both particle and chemical related hazards [52]. In this work we determined the chemical composition of chemical leachates from 9 types of microplastics under stringent chemical digestion conditions and physiologically realistic conditions simulated in vitro. Microplastics samples collected from Dutch beaches contained the largest number of different chemicals (i.e. 32 and 25 different chemicals for the <1 mm and <3 mm microplastics respectively), while PE microplastics contained 5 different chemicals (number of chemical in different microplastics: Env-1 > Env-3 > PVC > PA > PP > PE > PET). Only a few studies have assessed the chemical composition of different types of plastic [24, 62]. The environmental microplastics have been previously characterized [9], and the chemical analysis of plastic consumer products reported that PVC contained more chemicals than PP and PET plastics [24]. In our study a comparable trend was observed, while we analysed pre-production PVC.

It is still not possible to perform a comprehensive risk assessment of human exposure to microplastics [52]. Of the chemicals found to be associated with microplastics that translocated basolaterally using an in vitro model of the intestinal epithelium, only for some detailed exposure and toxicity data is available. Only 50% of the identified chemicals were listed in the ToxCast database, and of these only a limited number have been screened for endocrine disrupting potency using in vitro screening assays (i.e. activation of the estrogen receptor, activation of the androgen receptor, aryl hydrocarbon receptor of induced oxidative stress; see Table 3). This is also reflected by an analysis of the adverse outcome pathways that might be activated by the chemicals associated with the microplastics. From the ToxCast dataset we extracted 18 AOPs that were associated with the chemicals (Fig. 10), that included AOPs associated with endocrine disruption (i.e. AOP 150: Ah-receptor activation; AOP 200: Estrogen receptor activation, and several AOP that involve the activation of other nuclear receptors AOPs (i.e. AOP 58, 107, 163). 50% of the chemicals that were shown to pass the epithelial intestinal cell layer in vitro are associated with activation of the estrogen receptor (AOP 200). Recent toxicological screening studies indicated that microplastics (as particle or in combination with associated chemicals) induce oxidative stress and endocrine activity pathways in reporter gene assays [24, 62]. Recently the hazards of plastic packaging associated chemicals have been reviewed in detail [63]. These authors indicate that 906 chemicals can be associated with plastic packaging materials, of which 34 are recognized as endocrine disrupting chemicals [63]. While local exposure of the gastrointestinal epithelium might be high, systemic availability of metals associated with microplastics following oral exposure seems to negligible, as only iron (Fe) from ENV microplastics was detected in the basolateral compartment of the in vitro human epithelial cell layer model.

Clearly, from this work and that of others it can be concluded that microplastics should be regarded as a potential source of human exposure to chemicals that have an endocrine disrupting potency and might trigger AOPs related to nuclear receptor activation. For a risk assessment of the microplastics associated chemicals the contribution of chemical exposure via microplastics is needed, that is differentiated from the background exposure to these chemicals. Results from a study in fish larvae fed with PE and PS microplastics to which PCBs were sorbed under experimental conditions, indicate a minimal contribution of the PCB exposure via microplastics [64]. It is not yet known if this can be extrapolated to other microplastics associated chemicals. Yet, combination effects of the microplastics potentially affecting the barrier properties of the intestinal epithelium, resulting in an increase uptake need to be studied in more detail [65]. An important knowledge gap to improve the exposure assessment of chemical associated
Fig. 10 Overview relevant AOPs of chemicals according to the TOXCAST database. Note that not all chemicals are present in the toxcast database and not always AOPs have been identified.
microplastics is the extent to which humans are exposed to microplastics. Recently it was predicted that adults human consume 553 microplastics /person/day resulting in an accumulation of $5.01 \times 10^4$ particles for adults until the age of 70 (re-scaled microplastics 1–10 μm) [22]. In this study microplastics were re-scaled limiting the possibility to link the chemical data per polymer type microplastics to this. Others have estimated an annual human exposure to microplastics through food of $1.42 \times 10^5$–$1.54 \times 10^5$ particles/capita for general population which represent 0.13–2.04 g microplastic/capita/day (average 1.08 microplastic/capita/day) [41], this could result in an exposure in the low μg/capita/day range to individual chemicals detected in chyme.

Conclusion
Using sensitive GC-MS methods we quantified 68 chemicals associated with a diversity of microplastics ranging from pre-productions materials to microplastics derived from plastic beach litter. Some of these chemicals were released to the lumen of the human digestive tract under physiological conditions, simulated in vitro. Only 22 chemicals reached the basolateral compartment of an in vitro intestinal epithelial model, suggesting that only these 22 chemicals could reach systemic circulation. Of the quantified chemicals, only 50% has been evaluated in the ToxCast program. From the ToxCast dataset we extracted 18 AOPs that were associated with the chemicals, that included AOPs associated with endocrine disruption. A rough estimation of the potential human oral exposure to chemical leachates present in chyme can be made, and for individual compounds to oral exposure to individual chemicals in leachates from microplastics is in the low μg/capita/day range. However, for a risk assessment of chemicals associated with more detailed data on oral microplastics exposure is needed, as well as more detailed toxicological studies on the hazards of both the individual chemicals, complex chemical mixtures of the quantified chemicals as well as mixtures of microplastics.

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Authors’ contributions
RP, HB and SW developed the idea for this study, NdJ and LdH performed the experiments, RP and HB analysed the data and together with SW drafted the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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