



Marine microplastic: Preparation of relevant test materials for laboratory assessment of ecosystem impacts



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HIGHLIGHTS

- Beached macroplastic litter was collected for impact assessment studies.
- Cryogenic milling provided homogenous microplastic mixture.
- Common inorganic additives used as colorants, fillers and stabilisers were detected.
- GC-MS identified organic plasticisers, stabilisers, antioxidants and flame retardants.

ARTICLE INFO

Article history:

Received 24 April 2018

Received in revised form

3 August 2018

Accepted 4 September 2018

Available online 7 September 2018

Handling Editor: Tamara S. Galloway

Keywords:

Reference material

Microplastic

Characterisation

Cryo-milling

Impact assessment

Marine debris

ABSTRACT

Studies investigating the effects of plastic litter on marine biota have almost exclusively utilised pristine plastic materials that are homogeneous in polymer type, size, shape and chemical composition. This is particularly the case for microplastics (<5 mm), where collecting sufficient quantities from the marine environment for use in laboratory impacts studies is simply not feasible. Weathered plastics collected from the marine environment show considerable physical and chemical differences to pristine and post-production consumer plastics. For this study, macroplastic litter was collected on a Dutch beach and cryo-milled to create a microplastic mixture for environmental impact assessments. The sample composition followed proportions of marine plastic litter types observed in an earlier large beach clean-up. Polymer composition of the sample was assessed by infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry analysis (DSC). The particle size distribution of the cryo-milled microplastics showed that particles 0.5–2.0 mm represented 68% of mass, but smaller sizes (<2 mm) strongly dominated numerically. Inductively coupled plasma spectroscopy (ICP-MS and ICP-OES) analysis of the microplastic mixture revealed a broad range of metals and other elements (e.g. Al, Cd, Cr, Fe, Mg, Pb, S and Zn), representing common inorganic additives used as colorants, fillers and stabilisers. GC-MS analysis identified a broad range of organic plasticisers, stabilisers, antioxidants and flame retardants. Comparison of different analytical approaches showed that creation of a homogeneous microplastic mixture is possible, representing a first step in closing the gap between laboratory studies with pristine materials and realistic scenarios with weathered microplastic.

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1. Introduction

Widely acknowledged as a global phenomenon, marine litter is known to negatively affect the environment in several ways. In

addition to the economic costs of clean-ups and potential danger of interference with ships (Mouat et al., 2010), the potential hazard to marine organisms has raised concerns and led to marine litter being identified as an emerging issue by the United Nations Environmental Program (UNEP, 2011). Although impacts such as entanglement or the ingestion of marine litter have been recorded for thousands of individuals representing many species (Kühn et al., 2015; Gall and Thompson, 2015), linking negative changes in a

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population to marine debris has been proven to be a difficult task (Browne et al., 2015).

Evaluating the potential impacts of exposure to marine litter in the natural environment is nearly impossible, and therefore laboratory-based studies are necessary to generate such knowledge. However, reported studies investigating the possible effects of plastic litter on marine biota have almost exclusively utilised pristine plastic materials that are homogeneous in polymer type, size, shape and chemical composition. This is particularly the case for microplastics (marine litter < 5 mm; Arthur et al., 2009), as collecting sufficient quantities from the marine environment for use in laboratory impacts assessment is simply not feasible. As a result, most of the currently available data does not reflect environmentally realistic exposure scenarios (Phuong et al., 2016), and there is an urgent need to conduct studies with more relevant plastic test materials from a wide range of consumer products weathered by exposure to the conditions in the marine environment (Jahnke et al., 2017).

Once released into the marine environment, plastic litter immediately begins to undergo a variety of weathering processes that can significantly change the physical and chemical properties of the pristine material (Fotopoulou and Karapanagiotti, 2012; Gewert et al., 2015). It is these very processes that are known to contribute to the formation of micro- and nanoplastic particles. The type and degree of weathering is highly dependent upon the marine environmental compartment the plastic enters and the length of time it has been in the marine environment. The process is complex and influenced by polymer type and the presence of additive chemicals that are added to individual raw plastic materials to create a product with specific physical and chemical properties (e.g. flexibility, colour, function and protection against degradation processes). These additives include, but are not restricted to, plasticisers, flame retardants, colorants, lubricants, antioxidants and UV stabilizers.

Crucially, weathered plastics collected from the marine environment show considerable physical and chemical differences to pristine and post-production consumer plastics (Jahnke et al., 2017). For example, exposure of plastic litter to UV light decreases the stability of polymers leading to fragmentation and changes in chemical composition (Singh and Sharma, 2008), may enhance adsorption of hazardous substances to the plastic from surrounding seawater (Teuten et al., 2009; Mato et al., 2001), and promote release of additive chemicals (Artham and Doble, 2009). Potential endocrine disruption as well as mutagenic and toxic effects of a variety of additive chemicals has been reported by several previous studies (reviewed and summarized recently by Hermabessiere et al., 2017). However, detecting the effects of these substances on wildlife has been difficult (Werner et al., 2016). Experimental effect studies that use pristine materials without additive chemicals may well underestimate the overall impacts of plastic litter exposure to marine organisms.

In the framework of the Joint Programme Initiative on Oceans (JPI Oceans) project PLASTOX, the preparation of a more environmentally relevant sample of microplastic was considered important for allowing more relevant impact assessments. To achieve an environmentally realistic sample of marine plastic litter, the proportions of different types of plastic litter were derived from those documented in an earlier large-scale beach clean-up on the island of Texel, The Netherlands (Van Franeker, 2005). The generated microplastic reference materials provide more insight on the diversity of beached plastics on beaches and at the same time the sample can be used for future experimental work on the effects of marine plastic debris ingestion on wildlife. Given the complexity of the final microplastic mixture and the potential presence of a broad range of organic and inorganic additives, a battery of analytical

techniques has been applied to fully characterize the physico-chemical properties of the final reference material.

2. Methods

2.1. Background data

The composition of the marine litter-derived (MLD) microplastic mixture produced is based on a beach clean-up of the beach of the island of Texel (Van Franeker, 2005; summarized in English in: Van Franeker and Meijboom, 2006), the westernmost Wadden Sea island of the Netherlands (53°1'N, 4°8'E). The western coast of Texel has a 30 km sandy beach facing the North Sea, with predominantly western winds occurring all year round. Texel was selected as plastics collected during the 2005 beach clean-up were extensively quantified in terms of number of items and mass. During the 2005 study, approximately 15 tonnes of plastic debris were collected, with an average of 508 plastic litter items per kilometre. The plastic litter collected was weighed in two main categories: threadlike materials (ropes/nets) as compared to all other plastic consumer debris. The ropes and nets category dominated with an average mass of 229 kg/km, all types of other plastic debris had a combined average mass of 169 kg/km. In counts of other plastic debris, a range of further categories and subcategories of plastic consumer wastes was specified. By weighing a range of individual items belonging to the various sub-categories, their known numerical abundance could be used to estimate the weight of each (sub)category per km of beach (Table 1). Some of the subcategories from the 2005 collection appear to have high estimated average mass values, but these result from the occurrence of a very small number of large and heavy items which skew the contribution from the much larger number of small objects in each subcategory. For example, foam average mass was high because of e.g. some big mattresses, the average for sheet-like materials was increased by large transport- or agricultural sheets, and in the other 'miscellaneous' subcategory, average mass was elevated by infrequent odd but large items such as building materials, car parts, big baskets or buoys.

2.2. Plastic litter collection

In April and August 2016, plastic litter was collected from the same beaches on Texel, with the goal of obtaining source materials matching the composition of the plastic litter collected in the 2005 study. These items were randomly collected over all the beach areas, including the tidal zone and older strandlines. Items of uncertain provenance (e.g. beach toys and drink bottles that may have been brought recently to the beach) were only collected when there was clear evidence of degradation or when the occurrence of biofouling indicated they had been in seawater for a considerable time. Items heavily coated with sand were carefully rinsed with fresh water, otherwise they were simply dried at 18–20 °C for several weeks before residual sand was gently removed with a hand brush to minimise potential changes to the surface.

2.3. Sample preparation

2.3.1. Mixture components

Aiming for the broadest possible diversity of plastic objects in the MLD microplastic mixture, a wide range of different items for all subcategories of plastic was collected from the beach on Texel in 2016. As much as possible different items were used to compose each subcategory of the mixture. This meant that in some of the subcategories only small parts of individual items were used. Where possible equal mass from each object were taken.

Table 1

The average number of items per kilometre, the average item mass and estimated total mass of each beach plastic litter category and sub-category as collected during the 2005 beach clean-up on Texel, The Netherlands.

	Category		Sub-category	Average n/km	Estimated item mass (g)	Estimated mass per km (g)
1	Foam	1.1	Foams	28.1	712	20013
2	Plastic bottles	2.1	Drink bottles	69.9	23	1583
		2.2	Other bottles	34.6	32	1109
3	Fish boxes, jerrycans	3.1	Fish boxes	2.3	2236	5165
		3.2	Jerrycans	17.2	642	11067
4	Other packaging	4.1	Sheets (bags & industrial)	55.0	888	48834
		4.2	Sixpack, strapbands	9.3	3	25
5	Gloves	5.1	Working gloves	4.2	99	419
6	Other plastic materials	6.1	Balloons	6.0	5	31
		6.2	Other fishery equipment	1.7	28	48
		6.3	Silicone/grease tubes	2.6	32	81
		6.4	Food packaging	42.2	12	527
		6.5	Miscellaneous	130.4	614	80098
7	Threadlike	7.1	Ropes and nets	103.9	2204	229000
			Totals	507.5		398000
			<i>plastic consumer debris</i>	404		169000
			<i>nets and ropes</i>	104		229000

2.3.2. Cryogenic milling

An ultra-centrifugal stainless steel mill (Retsch ZM 200 with a combined cyclone) was used to grind the raw material. To avoid overheating of the sample and possible changes to the polymer composition, liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) was added for 30 min to increase brittleness and suitability for milling. The particle size specification of the milling chamber was 3 mm. Pre-tests revealed macroplastic mixtures containing all the different materials did not mill successfully. Therefore, the final milling process was applied to individual sub-samples of nets & ropes (7), foam (1), sheets (4.1) and rigid plastics (2, 3, 4.1, 5, 6), which were subsequently recombined to the original composition. The samples were stored in HDPE Kautex bottles.

2.4. Physical characterisation

The milling process generated a MLD microplastic mixture with a very broad size distribution. To facilitate the process of assessing the particle size distribution of the sample, the bulk microplastic mixture was first sorted by size using a sieve tower comprising sieves of six different size classes: 4.5, 3.15, 2.0, 1.0, 0.5 and 0.2 mm (Retsch AS200 Basic). Determination of the particle size distribution (PSD) of the un-sieved MLD microplastic mixture was attempted using a laser diffraction. However, no reliable results could be achieved due to the irregular shape of the particles, the low density of foam, and aggregation at the media surface.

Two sub-samples of each fraction of the MLD microplastic mixture were manually counted, the results combined, and an average PSD determined. Sub-samples from the larger size fractions (>4.5, 3.15, 2.0 and 1.0 mm) were counted under a light microscope. In addition to counting the total number of particles, they were categorized into fragments, foam, sheets, threads and dust (small fibres that often aggregated to bigger 'dust balls') to see possible variations per size class in relation to the original composition of the sample (For pictures see Online Supplement 3, Fig. 3.2.). For the smaller size fractions (0.5 and < 0.2 mm), sub-samples were placed into a Bogorov counting chamber with 70% ethanol (to reduce aggregation). Pictures of each Bogorov compartment were taken with a camera mounted on a microscope (AxioCam MRc with AxioVision 40 V 4.8.2.0 software; Zeiss, Germany), and the number of particles recorded (see pictures in Online Supplement 3, Fig. 3.3.).

To visualise the different particle types and physical properties, the microplastic mixture was also subjected to scanning electron microscopy (SEM). SEM imaging was performed using a Hitachi S-

3400N electron microscope equipped with an Oxford Instruments Aztec EDS system. Samples were dispersed onto an aluminium sample holder and lightly sputter-coated with gold to improve conductivity and stability. To image a representative portion of the sample, a montage of 48 images was created using the Aztec software.

2.5. Chemical characterisation

A comprehensive chemical profiling was conducted of the MLD microplastic mixture to determine the polymer composition and the organic chemical and metal profile and concentrations associated with the particles (either as additives or pollutants adsorbed to the surface). To determine the polymer distribution of the macroplastic litter items collected from the beach, each item was analysed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR; Shimadzu Prestige 21, 10 Scans, 4 libraries with 325 spectra) and differential scanning calorimetry (DSC; Mettler STARE System DSC3 Basic). While FTIR-ATR was used to describe the plastic composition of each of the individual samples, DSC was only used to analyse the homogenized sample mixture. Due to the low concentration of LDPE and sensitivity of the instrumentation used, no distinction has been made between HDPE and LDPE.

DSC analysis of MLD microplastic mixture was used to verify that the same polymer composition as the original macroplastic mixture had been achieved. DSC identifies polymer types through detection of either endothermic or exothermic reactions. The temperature of crystallisation (CP) is measured during the cooling processes. During the first run, samples were heated to $200\text{ }^{\circ}\text{C}$ and cooled again. In a second run, samples were heated to $300\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$. As DSC measurements on polymers are strongly influenced by the thermal history and the morphology of the sample, it is important to have a previous heating cycle completed while the measurements are carried out at the second heating stage. In the current study, three sub-samples of the MLD microplastic mixture were analysed using DSC so that average values could be determined.

The metals associated with the MLD microplastic mixture were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS) and energy dispersive x-ray spectrometry (EDX). The three different methods were evaluated and compared to identify which represents the most accurate and efficient option for future routine analysis of metals in plastics. For ICP-OES analysis,

10 mL of nitric acid (HNO₃ 65%; Merck) was added to sub-samples (3 g each; triplicate) of the MLD microplastic mixture. After 30 min, the samples were placed in a microwave oven (CEM Mars 5 Digestion) at 800 W and heated to 230 °C. The digests were diluted in deionised water (50 mL) prior to triplicate analysis for 34 elements (Shimadzu ICPE-9000 fitted with a 10UES Nebulizer and a plasma Mini Torch).

For ICP-MS analysis, triplicate MLD microplastic mixture samples were diluted in HNO₃ and hydrogen fluoride (HF) and heated at 220 °C for 20 min in a microwave (Milestone Ethos). The digested samples were analysed in triplicate using an Agilent 8800 Triple Quadrupole ICP-MS fitted with a SPS 4 Autosampler. ⁸⁹Y and ¹⁸⁵Re were used as internal standards and quantified against standards from Inorganic Ventures. A smaller number of target elements were screened using this method (13 different elements), as the main goal was to investigate the sensitivity of the method compared to ICP-OES. To provide an alternative, non-destructive method to ICP-based analyses, the MLD microplastic mixture was also subjected to EDX analysis (Shimadzu EDX-720). Internal calibration using standard reference materials and blank measurements were performed daily before each use. In principle, all elements heavier than carbon can be measured using EDX but is most effective for elements heavier than magnesium. In contrast to the two ICP-based methods, EDX is also able to measure halogens such as chlorine and bromine. In the current study, calibration and quantification was only possible for seven elements using EDX owing to the high costs involved with calibrating individual elements. Four elements (Ba, Cd, Cr and Pb) were quantified by all three methods. They were statistically compared to see whether there are significant differences between the three methods for each of the elements. An ANOVA followed by a Tukey test was performed using R (R version 3.3.1 (2016-06-21); R Core Team 2014).

The presence of organic chemical additives and pollutants was investigated using gas chromatography–mass spectrometry (GC-MS). Analysis comprised single samples without any replicates. Organic chemicals were extracted by adding 1 mL of ethyl acetate solvent to 100 mg of the MLD microplastic mixture and ultrasonicated for 10 min (Llompert et al., 2013). The sample was then centrifuged (4000 rpm) to settle any remaining particles before the supernatant was removed for analysis. Non-target screening was performed on an Agilent GC-MS-5977 instrument. A 1 µL aliquot was injected on a 15m HP-5MS column with an inner diameter of 250 µm and a film thickness of 0.25 µm. Injection was performed in the splitless mode at a detector temperature of 250 °C. The column temperature program was: 60 °C for 4 min, then ramped to 250 °C at a rate of 15 °C/minute, hold time 4 min, and a second ramping up to 315 °C at 15 °C/minute. The mass spectrometer was operated in the EI mode with a scan range from *m/z* 30 to *m/z* 800. Identification of organic chemicals was based on NIST library matching of mass spectra.

3. Results

3.1. Microplastic mixture preparation

In total, 351 items of plastic litter were used for preparation of the microplastic mixture. The target mass required for each sub-category, the number of items collected within each sub-category, and the mass of individual litter items required to produce 1 kg of the target MLD microplastic mixture composition is presented in Table 2. Pictures and the polymer type of all individual items are presented in the Online Supplements 1, 4 and 5.

A mass decrease was observed following cryo-milling, possibly due to the removal of sand during the sample preparation, where items had to be cut into smaller pieces to fit into the cryo-mill.

(details of the rate of return are shown in the Online Supplement 2, Table 2.1.). To determine the recovery efficiency, the mass of the individual milled sub-categories was compared to the mass of macroplastic used at the start of the process. As differences in recovery efficiency affect the composition of the MLD microplastic mixture, the material generated from milling the individual sub-categories was homogenized through combining the three sub-categories by mass to achieve the target mass composition.

3.2. Physical characterisation

The mass contribution of each size fraction to the total microplastic mixture is shown in Fig. 1. Most of the mass of the MLD microplastic mixture is in the size range 0.5–2.0 mm, accounting for ~68% of the total mass. The 0.2–0.5 mm size fraction accounts for ~18%, while particles <0.2 mm account for ~9%. No significant amount of material was present in the 3.15–4.5 mm and >4.5 mm fractions. The total particle number in each size fraction was determined by manual counting (Table 3). Using the total number of particles counted in each fraction, a simplified PSD was produced (Fig. 1, Online Supplement 3). The number of particles belonging to the different sub-categories varies across the different size fractions generated, but 1 g of the complete MLD microplastic mixture contains an estimated 410000 particles.

SEM imaging of the microplastic mixture revealed a broad range of particle shapes and morphologies (Fig. 2), confirming that most particles in the MLD microplastic mixture are highly irregular in nature.

3.3. Chemical characterisation

All individual plastic litter items (*n* = 351, Table 3) used in the preparation of the MLD microplastic mixture were analysed using FTIR-ATR prior to milling. In terms of mass contribution, the main polymers are polyethylene (PE) and polypropylene (PP), which together represent 89% of the marine litter (Fig. 3, Online Supplement 5, Table 5.1.). Much smaller contributions to the plastic litter come from a range of other polymer types (<4% each), with approximately 3% remaining unidentifiable. Semi-quantitative DSC analysis of the MLD microplastic mixture confirmed these results, with PE, PP and polyamide (PA) being the most abundant polymers. However, an accurate quantification of each polymer is not possible with this method as different polymers in a complex mixture will interact during the melting process, changing or eliminating the crystallisation and melting temperatures.

Results of the elemental analysis of the MLD microplastic mixture, conducted by the three different approaches, are presented in Table 4. The most extensive screening of elements present in the MLD microplastic mixture was conducted using ICP-OES. Data showed that 21 of the 34 elements screened were present at concentrations above the instrumental limits of detection. Concentrations of the detectable elements ranged from 4639.2 µg/g (±1665.9 µg/g) for calcium to 1.2 µg/g (±0.1 µg/g) in the case of gallium. In total, 13 heavy metals were detected (Cd, Cr, Cu, Fe, Ga, In, Mn, Mo, Ni, Pb, Pd, Sn, Zn), with their concentrations ranging from 1591.2 µg/g (±163.2 µg/g) for iron to 1.2 µg/g (±0.1 µg/g) for gallium. The heavy metals Ag, Bi, Co, Hg, Rh, and V were not present at detectable concentrations. For light metals (Al, Ba, Ca, K, Mg, Na, Sr, Ti), the concentration ranged from 4639.2 µg/g (±1665.9 µg/g) for calcium to 28.0 µg/g (±6.8 µg/g) for strontium. No metalloid elements (As, B, Sb, Se) were present at detectable concentrations. A range of 13 heavy and light metal elements (Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V and Zn) were also determined using a combination of HF digestion and ICP-MS analysis (Table 4). Finally, a small number of elements (Ba, Br, Cd, Cr, Pb, Sb, Se) were quantified

Table 2

Target mass and percentage contribution of different subcategories in the MLD microplastic mixture as derived from mass per km in Table 1, with the number of different beach litter items used to compose the samples.

	Category	Sub-category	Target mass (g/kg)	% of mass	Nr. items used to produce microplastic mixture
1	Foam	1.1 Foams	50	5.0%	29
2	Plastic bottles	2.1 Drink bottles	4	0.4%	5
		2.2 Other bottles	3	0.3%	12
3	Fish boxes, jerrycans	3.1 Fish boxes	13	1.3%	4
		3.2 Jerrycans	28	2.8%	13
4	Other packaging	4.1 Sheets (bags & industrial)	123	12.3%	67
		4.2 Sixpack, strapbands	0.1	0.0%	12
5	Gloves	5.1 Working gloves	1.1	0.1%	3
6	Other plastic materials	6.1 Balloons	0.1	0.0%	3
		6.2 Other fishery equipment	0.1	0.0%	7
		6.3 Silicone/grease tubes	0.2	0.0%	2
		6.4 Food packaging	1.3	0.1%	33
		6.5 Miscellaneous	201.3	20.1%	52
7	Threadlike	7.1 Ropes and nets	575.4	57.5%	109
		Total	1000	100.0%	351

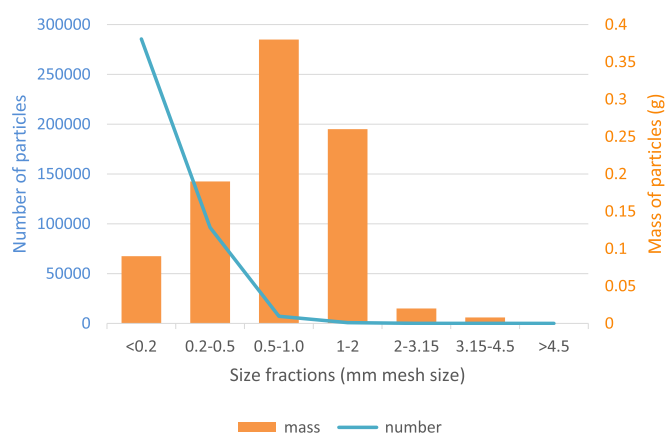


Fig. 1. Particle size distribution of 1 g of the bulk MLD microplastic mixture (blue line; left y-axis) is heavily skewed to the smallest particle sizes, where the mass contribution of the individual size fractions reflects an approximately normal distribution around the 0.5–1.0 mm fraction (orange bars; right y-axis). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

using EDX.

In total, 13 common elements (Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V and Zn) were studied using both ICP-OES and ICP-MS instrumentation. The concentrations determined are similar with both methods, although concentrations are always higher with ICP-MS than ICP-OES. Furthermore, elements identified at very low concentrations by ICP-MS (e.g. Co and V; 3.0 and 3.2 $\mu\text{g/g}$, respectively), were below the detection limits of ICP-OES. Additionally, 4

elements quantified by ICP-based analysis (Ba, Cd, Cr, Pb) were also quantified in the microplastic mixture using EDX. Comparison of the concentrations determined by the three different techniques is presented in Fig. 4. Generally, EDX analysis was able to provide elemental concentration data comparable to that of ICP-based techniques, only Cr showed a significant difference between all the three methods respectively ($p < 0.05$).

GC-MS screening analysis of the MLD microplastic mixture identified the presence of >50 different organic compounds. Of these, 22 were identified with a >80% confidence match by the NIST mass spectral library and are summarized in Table 5. A detailed online and literature search of these chemicals indicated most were common plastic additive chemicals such as flame retardants, plasticisers, colorants and inks. As this is a non-target screening approach, no standards were available for quantification, and the data presented are qualitative. Based on the chromatographic peak areas, compounds present at the highest concentrations were diisooctyl phthalate, tris(2-chloro-1-methylethyl) phosphate, dioctyl terephthalate, bis(3-chloro-1-propyl)(1-chloro-2-propyl)phosphate, and dibutyl phthalate.

4. Discussion

Collection of microplastic from the marine environment in quantities sufficient for use in laboratory studies is not feasible, and would involve extraction and clean up processes that may change physicochemical properties. Creating a representative mixture of marine microplastic from macroplastic marine litter offers an alternative approach to providing more environmentally relevant test materials. However, this approach is challenging and may be the reason why environmental samples have rarely been

Table 3

Number of particles present in each size fraction of the MLD microplastic mixture (subsamples counted: 2; standard deviation given in parentheses). Larger size fractions are counted by sub-category, but this was not possible for the smaller size fractions (n/a).

Size fraction (mm)	No. of particles per class					Total no. particles (g-1)	% no. of particles
	Sheets	Threads	Foam	Fragments	Dust		
>4.5	0.0	0.0	0.0	0.0	3.6 (± 5.1)	3.6 (± 5.1)	0.000
3.15–4.5	114.2 (± 55.5)	0.0	1088.5 (± 160.5)	6.4 (± 9.0)	56.7 (± 25.9)	1265.8 (± 199.2)	0.003
2–3.15	190.8 (± 37.7)	27.7 (± 12.7)	1069.0 (± 42.9)	127.6 (± 21.4)	9.6 (± 12.9)	1424.5 (± 16.4)	0.007
1–2	1133.1 (± 9.2)	365.7 (± 29.9)	558.8 (± 73.6)	737.9 (± 89.0)	6.1 (± 5.8)	2801.5 (± 189.1)	0.187
0.5–1.0	n/a	n/a	n/a	n/a	n/a	18800.0 (± 212.1)	1.833
0.2–0.5	n/a	n/a	n/a	n/a	n/a	506750.0 (± 32173.4)	24.702
<0.2	n/a	n/a	n/a	n/a	n/a	3173100.0 (± 290196.6)	73.268
					Total	411587	100

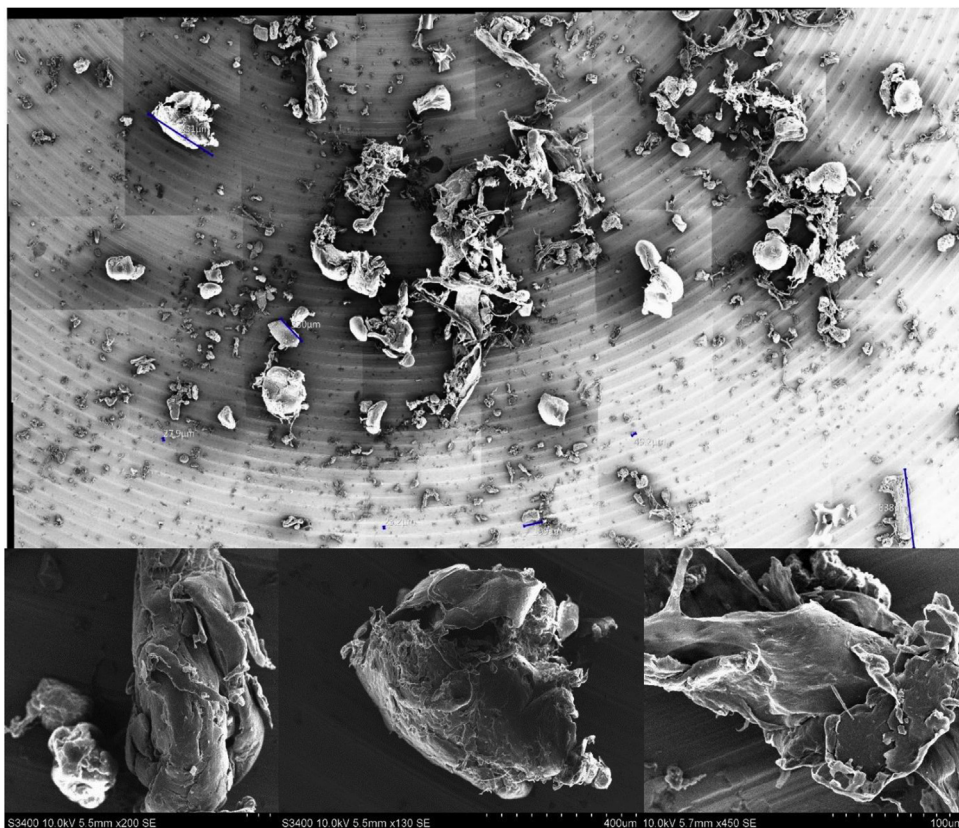


Fig. 2. Scanning electron microscope images of the cryogenically milled MLD microplastic mixture. Top: a composition of 48 pictures. Bottom: detailed images of selected individual particles.

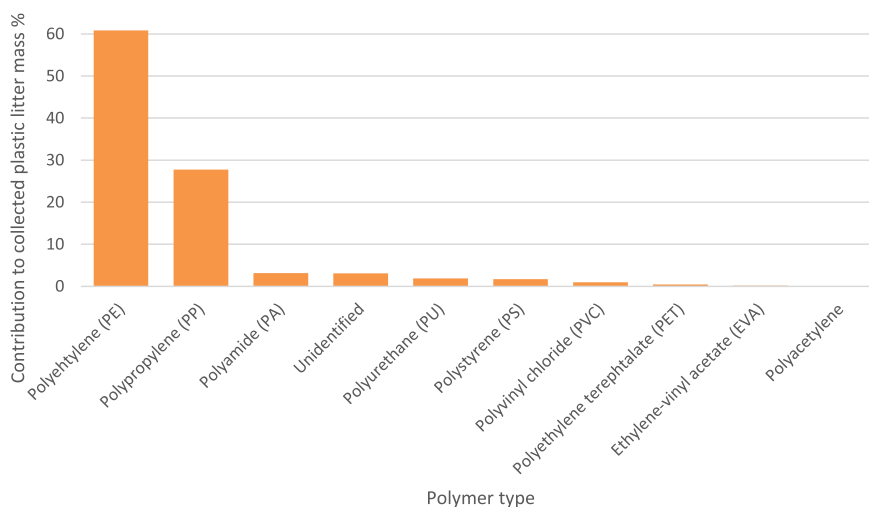


Fig. 3. Percentage contribution by mass of different polymer types to the collected plastic litter (based on 1 kg and 351 individual items). Polymer type was identified using ATR-FTIR analysis.

used in microplastic impact assessments (Phuong et al., 2016). Many factors play a confounding role, with the most prominent being the untraceable history of each marine litter item. Biological, mechanical and chemical degradation will differ for individual items, influencing their behaviour and composition (Gewert et al., 2015; Suhrhoff and Scholz-Böttcher, 2016; Booth et al., 2017). Local currents and bathymetry determine the type of marine plastic litter that arrives on a specific beach, which may be influenced by seasonal weather patterns (Schulz et al., 2013; Galgani et al., 2015;

Watts et al., 2017). Furthermore, specific regions are likely to exhibit a unique composition of plastic litter reflecting the main local sources. For example, the percentage of fisheries-related plastic has been shown to vary per location, with the size of beached plastic also differing (Galgani et al., 2015).

The number of variables influencing plastic litter at specific locations has so far prevented its use in experimental research. However, the use of macroplastic litter for the generation of more environmentally relevant microplastic reference materials appears

Table 4

Elemental profile of the MLD microplastic mixture conducted by ICP-OES (n = 6), ICP-MS (n = 3) and EDX (n = 6), with standard deviation presented in parentheses. The abbreviation n.d. stands for 'not detected', whilst '-' corresponds to elements that were not measured using a specific technique. 'P' is used to denote EDX measurements where an element was detectable but not quantifiable.

Element	ICP-OES	ICP-MS	EDX
	µg/g	µg/g	µg/g
Silver (Ag)	n.d.	—	—
Aluminium (Al)	1006.2 (±100.9)	1273.1 (±218.6)	—
Arsenic (As)	n.d.	—	—
Boron (B)	n.d.	—	—
Barium (Ba)	777.3 (±57.2)	786.9 (±521.5)	783.0 (±258.6)
Bismuth (Bi)	n.d.	—	—
Bromine (Br)	—	—	30.0 (±4.9)
Calcium (Ca)	4639.2 (±1665.9)	—	P
Cadmium (Cd)	15.5 (±3.2)	27.3 (±7.0)	20.5 (±12.1)
Chlorine (Cl)	—	—	P
Cobalt (Co)	n.d.	3.0 (±0.2)	—
Chromium (Cr)	116.3 (±24.0)	179.6 (±19.7)	58.8 (±10.5)
Copper (Cu)	24.3 (±2.9)	51.9 (±4.3)	P
Iron (Fe)	1591.2 (±163.2)	1835.2 (±472.0)	P
Gallium (Ga)	1.2 (±0.1)	—	—
Mercury (Hg)	n.d.	—	—
Indium (In)	7.4 (±3.6)	—	—
Potassium (K)	392.8 (±35.9)	—	P
Lithium (Li)	n.d.	—	—
Magnesium (Mg)	563.8 (±26.1)	—	—
Manganese (Mn)	27.3 (±2.9)	35.2 (±11.8)	P
Molybdenum (Mo)	9.1 (±0.5)	9.6 (±1.4)	P
Sodium (Na)	1043.0 (±66.6)	—	—
Nickel (Ni)	12.7 (±2.8)	49.4 (±8.6)	P
Phosphorus (P)	166.0 (±9.8)	—	—
Lead (Pb)	170.8 (±16.7)	175.0 (±13.0)	156.5 (±35.3)
Palladium (Pd)	19.3 (±6.7)	—	—
Rubidium (Rb)	n.d.	—	P
Rhodium (Rh)	n.d.	—	—
Sulphur (S)	681.7 (±49.8)	—	P
Antimony (Sb)	n.d.	—	14 (±2.3)
Selenium (Se)	n.d.	—	n.d.
Silicon (Si)	n.d.	—	P
Tin (Sn)	14.7 (±1.8)	—	P
Strontium (Sr)	28.0 (±6.8)	—	P
Titanium (Ti)	65.8 (±12.6)	—	P
Vanadium (V)	n.d.	3.2 (±0.6)	—
Zinc (Zn)	70.0 (±6.8)	111.6 (±15.2)	P
Zirconium (Zr)	n.d.	—	P

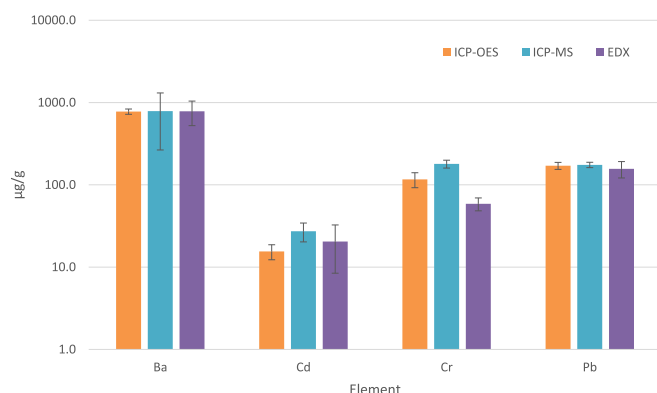


Fig. 4. Comparison of elements measured in the MLD microplastic mixture with three different methods (ICP-OES, ICP-MS and EDX) with standard deviation.

to be a necessary step in closing the current gap between experimental lab studies and observations made in the natural environment (Jahnke et al., 2017). Despite these potential challenges, this study serves as a first step and a suggested method for creating and

characterising a MLD microplastic mixture is presented. The current study shows there are still issues of concern throughout all stages of the process.

The use of macroplastic litter data from a previous study (Van Franeker, 2005) enabled collection of new plastic litter items that reflected the original composition determined in the 2005 study (Table 1). However, generation of a microplastic mixture reflecting the composition of marine litter requires a detailed assessment and categorisation of marine litter in the sampling area. It is important to note that care needs to be taken to ensure items exhibit some degree of weathering or biofouling that suggests a prolonged residence time in the marine environment. Furthermore, any resulting microplastic mixture represents only the area from which the source litter was collected and may not be representative globally. In the current study, PE and PP were the most abundant polymer types in the collected beach litter (Fig. 3), possibly reflecting the lightweight nature of these materials and that they are the highest volume production plastics globally (Geyer et al., 2017). Many studies have shown that PE and PP are the dominant polymers in global marine debris (Hidalgo-Ruz et al., 2012), in North Atlantic sediments (Fries et al., 2013; Sadri and Thompson, 2014; Claessens et al., 2011) and in different species ingesting plastics in the North Sea (Foekema et al., 2013; Rummel et al., 2016; Van Franeker et al., 2017).

The cryogenic milling approach employed in the current study can successfully produce microplastic from macroplastic litter. However, laboratory-scale cryogenic milling is only suitable for small samples, so the raw materials were cut to an acceptable size (<25 mm). Furthermore, some loss of the material was observed during the milling process, with rigid marine litter items exhibiting higher recoveries than materials with a small volume/mass ratio (e.g. sheets and foams). As foil and foams were observed to become statically charged under milling, it was necessary to mill specific categories of materials individually before combining and homogenising into a final microplastic mixture with the target polymer composition. Each step of the microplastic mixture preparation was implemented in a way to try and minimise any change to the chemical composition of the sample (e.g. use of cryogenic system), therefore ensuring that the chemical profile of the final microplastic mixture resembled that of the source marine litter as closely as possible.

By subjecting the bulk microplastic mixture to a serial sieving process, a mass distribution of the different size fractions could be determined (Fig. 1). Results showed that cryogenic milling successfully reduced the macroplastic litter into microplastic, with 95% of the recovered particulates by mass being <2.0 mm in size. The PSD of the microplastic mixture showed that the number particles present in each of the size fractions increased significantly as particle size decreased. Fitting to a basic exponential function: ($y = a * x^{-b}$) and to the logarithm of the same function suggests the relationship between particle size and particle number is exponential (but break down for the highest size categories; Online Supplement 3, Fig. 3.1.). Interestingly, the particle distribution of the different categories varies for the different size fractions (Table 3). The number of sheet, thread and fragment particles increases as the diameter of the size fraction decreases. Conversely, the number of foam and dust particles decreases with decreasing size fraction, suggesting that individual materials behave differently during the milling process. In the largest size category (>4.5 mm) only dust balls and aggregated conglomerates were observed, showing that cryo-milling can be useful to create a microplastic mixture (<5 mm). However, the variation in composition observed in the different size fractions may restrict the use of specific size fractions in certain studies, as the composition does not reflect the original composition on beaches.

Table 5
Summary of organic chemicals present in the MLD microplastic mixture detected with GC-MS screening. Where available, CAS numbers, common names or abbreviations are given, otherwise n/a is stated.

Compound Name	CAS No.	Common Name/ Abbreviation	Percentage match (NIST)	Comments
1,2,4-trimethylbenzene	95-63-6	Pseudocumene	80	Possible source from printing inks
1-Pentene, 4,4-dimethyl-1,3-diphenyl-1-(trimethylsilyloxy)-	n/a	n/a	83	
1-(4-methylphenyl)pentan-1-one	1671-77-8	4'-methyl valerophenone	81	
1-(1'-pyrrolidinyl)-2-butanone	n/a	n/a	80	
2,4-Di-tert-butylphenol	96-76-4	2,6-DTBP	91	UV stabiliser and antioxidant
2,4-dimethyldecane	2801-84-5	n/a	84	Linked to production of tributylphosphate plasticiser
Tri(2-chloroethyl) phosphate	115-96-8	TCEP	84	Flame retardant, plasticiser and viscosity regulator
3,7-dimethylnonane	17302-32-8	n/a	81	
Tris(2-chloro-1-methylethyl) phosphate	13674-84-5	TCCP	93	Flame retardant
Bis(3-chloro-1-propyl)(1-chloro-2-propyl) phosphate	137888-35-8	n/a	86	Flame retardant
Dibutyl phthalate	84-74-2	DBP	94	Plasticiser or possible printing ink
Triphenyl phosphate	115-86-6	TPhP	87	Plasticiser and flame retardant
2-Ethylhexyl diphenyl phosphate	1241-94-7	Octicizer	80	Plasticiser and flame retardant
Phthalic acid, di(6-methylhept-2-yl) ester	n/a	n/a	80	Plasticiser
Dicyclohexyl phthalate	84-61-7	Morflex® 150	84	Plasticiser and UV stabiliser
Bis(2-ethylhexyl) phthalate	117-81-7	DEHP	96	High volume production plasticiser
Diisooctyl phthalate	27554-26-3	DIOP	93	High volume production plasticiser or possible printing ink. Also used as glue in PU- PVA-based colour binders
5-methyl-2-phenyl-1H-indole	13228-36-9	n/a	80	Indoline substances are used as colorants
2-tert-Butyl-6-(5-chloro-2H-benzotriazol-2-yl)-4-methylphenol	3896-11-5	Bumetrizole	86	Antioxidant used to slow the oxidation process of the polymer exposed to UV light.
1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	6422-86-2	Dioctyl terephthalate	92	Non-phthalate plasticiser used in PVC, PMMA and PS.
(Z)-9-Octadecenamamide	301-02-0	Oleamide	80	Lubricant in PE and PP manufacture

SEM images of the microplastic mixture indicate all particles exhibited an irregular shape (Fig. 2). Brittle plastic litter items fragmented into irregular shaped particles, while other particles showed evidence of stretching, tearing and crushing rather than 'clean' fragmentation. Despite the use of cryogenic cooling, specific material types clearly respond differently in the milling process. The irregular shape of the particles also influenced the efficiency of the size fractionation processes. High aspect ratio particles (e.g. fibres) were found to distribute between different size fractions as a small number could pass through the smaller sieves if the particle was orientated the correct way. Nevertheless, the results of comparable but independent chemical analyses (FTIR-ATR and DSC) indicate that a satisfactory homogenisation of the mix is possible. The majority of plastic items analysed consisted of PE and PP (61 and 28% respectively). PE and PP are the most commonly produced polymer materials globally (Geyer et al., 2017), which appears to be reflected in the results of the current study. Both polymer types have a lower density than seawater and tend to float on the water surface as long as they are not heavily bio-fouled or vertically mixed by wind and wave action (Kooi et al., 2017; Kukulka et al., 2012). Thiel et al. (2013) observed a comparable plastic litter compositions in coastal waters and on beaches, suggesting beached material can be used as a proxy for that in surface waters. The MLD microplastic mixture may also be a suitable reference material for plastic below the surface, as plastic ingested by fish in the same region consisted mainly of PE and PP (Rummel et al., 2016; Collard et al. 2015, 2017).

Elemental analysis of the microplastic mixture indicated a broad range of heavy metal, light metal and metalloid elements

associated with the particles. Reproducibility was good between replicate sub-samples of the microplastic mixture, indicating that the final homogenisation step had been successful. The elemental distributions and concentrations determined in the current study were broadly similar with all three techniques employed, indicating successful homogenisation and suggesting the 3-method design reduces the need for using certified reference materials. HF digestion combined with ICP-MS analysis offers the most accurate and sensitive approach, but is more time consuming and costly. EDX offers a good screening method for the presence of many elements, including halogens such as bromine and chlorine which are common in flame retardants, but quantification is difficult as crystallisation and polymer composition affect the result and detection limit. For routine identification and quantification of microplastic elemental composition, ICP-OES appears to offer the best combination of sensitivity and cost.

Many elements are well-known inorganic plastic additives and their industrial sources can be postulated. Other elements may be residual contaminants from the polymerisation process (Crompton, 2007; Brennecke et al., 2016; Turner, 2016). Inorganic plastic additives are typically utilised as fillers (Ba, Ca, Mg), colorants (Cd, Fe, Mn, Pb, Ti) or stabilisers/catalyst left overs/inks (Cu, Mo, P, Pb, Sn, Sr, Zn). Al is commonly added to plastic foils to improve the barrier function in products such as food packaging or non-latex helium balloons, whilst Ba is also used to prevent discoloration or yellowing in PE foils. The presence of such elements in microplastic is rarely considered, and the relationship between plastics and metals remains unclear (Brennecke et al., 2016). For example, Fries et al.

(2013) suggest that Al and Zn present in their samples was assumed to be sorbed from the surrounding environment. Kedzierski et al. (2018) observed only few heavy metals adsorbing to the surface of PVC after being exposed to seawater a prolonged period. Importantly, certain elements are known to be toxic to a wide range of marine organisms, so quantifying their presence in microplastic test materials is necessary when used in ecotoxicity studies.

Using analysis techniques that include a digestion step (e.g. ICP-OES and ICP-MS) mean it is not possible to distinguish between elements from the manufacturing process and those adsorbed to the surface from the environment. As the microplastic mixture in the current study is derived from weathered macroplastic litter, it is possible that the elemental composition represents a combination of sorbed and additive substances. However, it is not possible to accurately identify the sources of the different elements identified within the microplastic mixture.

Organic chemical screening by GC-MS indicated a range of additive chemicals were present in the microplastic mixture, with phthalates and chlorinated alkyl phosphate esters (organophosphorus compounds) being two of the most dominant chemical classes (Table 5). Diisooctyl phthalate (DIOP) and dibutyl phthalate are plasticisers mainly related to PP and PE products, and have previously been identified in marine debris (Fries et al., 2013; Rani et al., 2015). DIOP and dibutyl phthalate are known endocrine disruptors (Oehlmann et al., 2009). Plasticisers are often related to polyvinyl chloride where up to 60% of the material mass may originate from this type of additive. However, they have also been found in printing inks and lacquers used in the production of many other polymer types (Fierens et al., 2012), and is suggested as their most likely source in the MLD microplastic mixture. Dicyclohexyl phthalate (DCHP) has been found in low concentrations in food packaging (Fierens et al., 2012; Schecter et al., 2013). Tris(2-chloro-1-methylethyl) phosphate (TCPP), together with many other organophosphorus compounds, is regularly used as an additive flame retardant and is a suspected carcinogen (Andresen et al., 2004). High levels of Bis(2-ethylhexyl) phthalate (DEHP) and phthalic acid, di(6-methylhept-2-yl) ester have been found in artificially aged rubber ducks, in contrast to low or no levels in unaged ducks (Cambridge Polymer Group, 2016). These substances might be breakdown products of the weathering process, although the exact mechanisms behind the changed levels remain unclear.

The toxicity of many additive organic compounds to marine and freshwater species has been studied previously (Adams et al., 1995; Chen et al., 2014). For example, the acute toxicity of a group of 14 phthalate esters to representative aquatic organisms was found to range from 0.21 to 377 mg/L depending on the ester and the solubility of the test chemical in water (Adams et al., 1995). A general trend was found showing that lower-molecular-weight phthalate esters become more toxic with decreasing water solubility, but there were only minor differences in species sensitivity to each of the phthalate esters. Although the production, sources and environmental occurrence of organophosphorus compounds has been reviewed extensively, there is currently limited data available regarding the toxicity this class of compounds to aquatic organisms (Van der Veen and de Boer, 2012; Wei et al., 2015). The limited data available suggests there may be significant differences in the toxicity of different compounds within the class (Van der Veen and de Boer, 2012).

EDX identified the presence of bromine and chlorine in the microplastic mixture (Table 4), but the technique could not determine if the signal derived from sea salt residues or from additive chemicals (e.g. flame retardants). Although halogenated flame retardants were not specifically studied owing to the non-target GC-MS screening approach employed, the analysis confirmed the presence of chlorinated compounds (Table 5). Brominated

compounds were not observed, suggesting they are present at very low concentrations or absent from the sample. In a study of marine litter items by Turner (2016), comparable values for bromine (and most other elements) were detected in non-porous fragments and ropes, but were much higher in foam (20, 26 and 244 µg/g, respectively). It remains unclear whether these substances were added during production processes or were adsorbed when exposed to seawater, as demonstrated in a long-term exposure experiment by Rochman et al. (2014). Regardless the origin, some of the substances have been described as hazardous (Lithner et al., 2011), and therefore pose a potential risk to marine organisms (Rochman et al., 2014; Turner and Lau, 2016).

It is important to note that the concentrations of inorganic and organic substances associated with the cryo-milled microplastic mixture in the current study may differ from those in true microplastic particles that are the result of natural degradation and subject to potential desorption-adsorption processes in the marine environment. In the milled sample, much of the surface of the resulting microplastic will not have been subjected to the marine environment and may not contain the same levels of adsorbed metals and organics. Conversely, the fresh surface area generated has not had the opportunity to release any additive chemicals to the surrounding marine environment. However, marine organisms as the Northern Fulmar that ingest and grind plastic particles in their muscular stomach (Van Franeker et al., 2011) might be exposed to both additives and adsorbed pollutants.

5. Conclusion

There is a need to move towards using more environmentally relevant microplastic reference materials in laboratory-based effects assessment. The microplastic mixture generated in the current study represents a significant step forward from the use of pristine spherical materials typically used in laboratory studies, especially in terms of physical properties. The generated mixture will be distributed between partners of the JPI Oceans PLASTOX project for laboratory effects assessment studies. As the collection, preparation and characterisation of the MLD microplastic mixture is time consuming, and true reproducibility is impossible, it is currently not feasible to supply the MLD microplastic mixture commercially. However, this study should act as a guide for others to generate relevant MLD microplastic mixtures that are representative of specific marine environments.

There will always be some differences in the chemical profile of the milled material relative to naturally degraded microplastic particles present in the marine environment. Irrespective of the origin of the inorganic and organic substances associated with microplastic in the marine environment (additives or sorbed), it represents a cocktail of substances that may elicit impacts in marine organisms. As we move more towards sublethal effects studies to understand the environmental impacts of plastic pollution, the need to study the role and possible contribution of additive chemicals is crucial. It is therefore recommended that any microplastic test material used in effects studies, regardless of its origin, is subjected to a detailed physicochemical characterisation so that effects data may be interpreted more accurately. The application of multiple, complimentary analytical chemical and imaging techniques appears essential for characterising microplastic test materials.

Acknowledgements

Susanne Kühn is funded through the Joint Program Initiative (JPI) Oceans PLASTOX (Direct and indirect ecotoxicological impacts of microplastics on marine organisms) project by the Netherlands

Organisation for Scientific Research (NWO) under the project number ALW-NWO 856.15.001. SINTEF Ocean was funded through the JPI Oceans project PLASTOX by Research Council of Norway (RCN; Grant Agreement number 257479/E40). The authors wish to thank the NWO and the RCN for their financial support. We are very grateful for the assistance in the labs provided by Markus Ortlieb (Shimadzu), Marianne Kjos and Lisbet Sørensen (SINTEF) as well as Andre Bouwmeester, Stephanie Westerhoff and Erwin Jansen (Carat GmbH). Two anonymous reviewers provided valuable comments to improve earlier versions of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.chemosphere.2018.09.032>.

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