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New techniques for the detection of microplastics in sediments and field collected organisms

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ABSTRACT

Microplastics have been reported in marine environments worldwide. Accurate assessment of quantity and type is therefore needed. Here, we propose new techniques for extracting microplastics from sediment and invertebrate tissue. The method developed for sediments involves a volume reduction of the sample by elutriation, followed by density separation using a high density NaI solution. Comparison of this methods' efficiency to that of a widely used technique indicated that the new method has a considerably higher extraction efficiency. For fibres and granules an increase of 23% and 39% was noted, extraction efficiency of PVC increased by 100%. The second method aimed at extracting microplastics from animal tissues based on chemical digestion. Extraction of microspheres yielded high efficiencies (94–98%). For fibres, efficiencies were highly variable (0–98%), depending on polymer type. The use of these two techniques will result in a more complete assessment of marine microplastic concentrations.

1. Introduction

Plastic marine debris has been an environmental concern for decades (Derraik, 2002; Gregory, 2009; Hammer et al., 2012; Moore, 2008; Thompson et al., 2009). Despite the increased international attention, the build-up of these materials in the environment is considered problematic due to an increasing global plastic production and the continuing improper disposal of plastic waste. The impacts of plastic debris on marine species are widely reported (Derraik, 2002; Gregory, 2009). Up to now, over 660 marine species worldwide are known to be affected in by plastic waste one way or another (GEF, 2012). Relatively large items will, however, eventually undergo fragmentation under the influence of UV radiation, the oxidative properties of the atmosphere and hydrolytic properties of seawater (Andrady, 2005, 2011; Webb et al., 2013). Combined with the mechanical forces exerted by wave action, plastic items will break up into smaller particles (Barnes et al., 2009). Because of the large residence times of plastic debris in our seas and oceans, most plastic present in the marine environment fits in the smaller size classes. For instance, 72% of the plastics recovered from beaches in Portugal belonged to a size class $\leqslant$5 mm (Martins and Sobral, 2011). Similarly, plastic particles <1 mm accounted for 65% of total marine debris collected on beaches in the Tamar Estuary (UK) (Browne et al., 2010). These small items of plastic debris are commonly referred to as microplastics. Many authors have defined microplastics as particles smaller than 5 mm (e.g. Arthur et al., 2009) while other have set the upper size limit at 1 mm (e.g. Costa et al., 2010). While the value of 5 mm is more commonly used, 1 mm is a more intuitive value (i.e. 'micro' refers to the micrometer range). Moreover, once particles are smaller than 1 mm they can potentially be ingested by a range of aquatic invertebrates. Bivalves for instance will preferably ingest and process particles less than 40 μm, but larger particles (up to 600 μm) can be ingested and processed as well (Cefas, 2008).

Microplastics have been detected on beaches and in subtidal sediments worldwide (Table 1). The extraction method used by the majority of these authors was developed by Thompson et al. (2004). This technique, which is currently the most widely used (Hidalgo-Ruz et al., 2012), relies on the density of a concentrated NaCl solution (1.2 kg L^{-1}) to separate sediment from microplastic particles. Indeed, when this salt solution is added to the sediment sample, low density microparticles float to the surface. However, this method is only effective for polymers with a density lower than that of the saturated saline concentration, i.e. 1.2 g cm^{-3}, and not suitable for the extraction of high density polymers. Plastics such as polyvinylchloride (density 1.14–1.56 g cm^{-3}) or polyethylene terephthalate (density 1.32–1.41 g cm^{-3}) will not float in this concentrated NaCl solution. These two polymers, however, represent 18% of the European plastic demand (PlasticsEurope, 2012) and as such could represent an important proportion of the microplastics present in the marine environment. Especially in marine sediments, the proportion of these high density plastics

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Table 1
Maximum concentrations of microplastics found in sediments worldwide. All concentrations are expressed as either number of particles kg^{-1} dry sediment or mg kg^{-1} dry sediment.

Country	Location	Maximum concentration	Unit	Refs.
India	Ship-breaking yard	89	mg kg^{-1}	Reddy et al. (2006)
UK	Beach[a]	9	# kg^{-1}[b]	Thompson et al. (2004)
UK	Estuarine[a]	35	# kg^{-1}[b]	Thompson et al. (2004)
UK	Subtidal[a]	86	# kg^{-1}[b]	Thompson et al. (2004)
Singapore	Beach	16	# kg^{-1}	Ng and Obbard (2006)
UK	Sewage disposal site	15	# kg^{-1}[b]	Browne et al. (2011)
Belgium	Harbour	391	# kg^{-1}	Claessens et al. (2011)
Belgium	Continental shelf	116	# kg^{-1}	Claessens et al. (2011)
Belgium	Beach	156	# kg^{-1}	Claessens et al. (2011)

[a] Only fibre concentrations were reported.
[b] Original unit (# fibres 50 mL^{-1} sediment) converted using an average sediment density of 1600 kg m^{-3} (Fettweis et al., 2007) and 1.25 as average wet sediment/dry sediment ratio.

could be higher: because of their high density, these plastic types will tend to sink more easily than lighter plastics. Since the techniques currently used to extract microplastics from sediments are not efficient in extracting all types of plastics, the concentrations reported in these studies may be underestimates.

To date, vertebrates have been the primary focus concerning plastic ingestion (e.g. Denuncio et al., 2011; do Sul et al., 2011; Laist, 1997; Lazar and Gračan, 2011; Poppi et al., 2012; van Franeker et al., 2011). However, as the plastic breaks down, it becomes available for ingestion by a much wider range of (smaller) organisms (Barnes et al., 2009; Betts, 2008). Recently, it has been shown that invertebrates, such as polychaete worms, barnacles, amphipods and sea cucumbers, can ingest microscopic plastics particles during laboratory trials (Graham and Thompson, 2009; Thompson et al., 2004). In these experiments, the presence of microplastics in the gut, and hence ingestion of these particles, was demonstrated using analysis of casts and dissection of the intestinal tract (Graham and Thompson, 2009; Thompson et al., 2004) as well as histological techniques (Browne et al., 2008). It has also been demonstrated that very small plastic particles (<10 μm) can translocate to the circulatory system of the bivalve *Mytilus edulis* (Browne et al., 2008). Although no significant adverse effects of ingestion and translocation of microplastics have been observed during these laboratory trials (Browne et al., 2008), the presence of microplastics in the environment still raises toxicity concerns, since plastics are known to contain and/or adsorb high concentrations of organic contaminants (Hirai et al., 2011; Mato et al., 2001; Rios et al., 2007; Teuten et al., 2007, 2009). The fate of these contaminants is, however, poorly understood, and recently Gouin et al. (2011) suggested that microplastics are "likely of limited importance" as vectors of the pollutants to marine organisms. Lack of supporting studies, identification of critical data-gaps (Gouin et al., 2011) and the lack of appropriate techniques to extract plastic particles from (soft) organic tissue justify the on-going interest in the presence of microplastics in marine organisms.

In this study, two new techniques to determine the presence and abundance of microplastics in natural samples are described. For sediments, data currently available on the concentrations of microplastics may be biased, since it is not possible to detect high density plastics using a saturated salt solution as frequently used (Hidalgo-Ruz et al., 2012) and initially described by Thompson et al. (2004). Using high density chemicals like sodium iodide (NaI) could resolve this. However, these are expensive to use: 1 kg of NaCl costs less than €1, while 1 kg of NaI costs approximately €70. In order to improve the cost efficiency of microplastics extraction from sediments, a new method using a fluidized sand-bath and a small volume of NaI is proposed. Also, whether or not organisms from natural populations contain microplastics is not known, as ingestion by invertebrates has only been demonstrated in laboratory trials. Determining the plastic body burden of resident, marine organisms is thus important for our understanding of the effects of microplastics. Therefore, a new technique was developed for detecting microplastics in tissue, involving a depuration phase followed by chemical digestion of the tissue.

2. Materials and methods

2.1. Extracting microplastics from sediments: elutriation and flotation

A device was developed to extract microplastics from sediment based on the principle of elutriation. Elutriation is a process that separates lighter particles from heavier ones using an upward stream of gas or liquid. This principle has, for example, been used extensively in marine biology for separating meiofauna from sand with an apparatus called "Barnett's fluidized sand-bath" (Southwood and Henderson, 2000).

Based on this design, a new apparatus was developed, represented schematically in Fig. 1. A PVC column (147 cm length with an internal diameter of 15 cm) is fitted with a 1 mm sieve on top and a 35 μm mesh screen (supported by a strong 1 mm mesh screen to support the weight of the sediment) at the bottom. A sediment sample of 500 mL is transferred into the column by washing it through the 1 mm sieve to remove all large debris. A sieve cover is used to prevent contamination with particles or fibres transported through the air. An upward water flow is then created by forcing tap water through the column from below. At this point the sediment becomes fluidized. At the bottom of the column, aeration is provided to ensure efficient separation of plastic and sediment particles. In order to avoid the creation of dead zones (without aeration) an aeration system using three large air stones ($50 \times 25 \times 25$ mm, Dohse Aquaristik) was constructed. The water flow, combined with the aeration, separates the lighter particles, including microplastics, from the heavier sand particles, and the rising water takes them to the top where they eventually flow over the edge and are retained on a 35 μm sieve (a smaller mesh size can be used if desired, or a series of sieves with decreasing mesh size to avoid clogging). The flow rate of the water is adjusted to achieve a maximum extraction efficiency and minimal contamination of the sample with sand: it was experimentally determined that this flow rate should be set at approximately 300 L h^{-1}, for 15 min. This rate was adequate to keep sand in the pipe while other material, including microplastics, flowed over the edge.

After this first clean-up step, the material collected on the 35 μm sieve subsequently undergoes a sodium iodide extraction (NaI-extraction). The solids are transferred to a 50 mL centrifuge tube, and 40 mL of a NaI-solution (3.3 M, with a density of approximately 1.6 g cm^{-3}), is added. This is followed by vigorous (manual) shaking and centrifugation for 5 min at 3500*g*. After centrifugation,

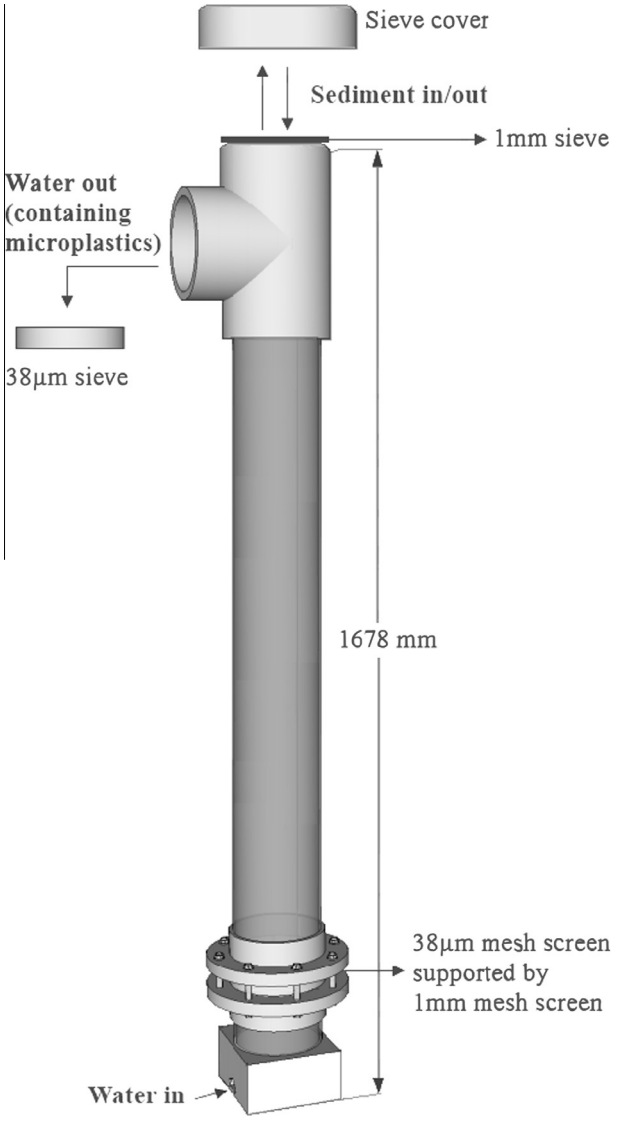

Fig. 1. Elutriation: schematic representation of the elutriation column, used for separating microplastics from the heavier sand particles.

the top layer containing the microplastics is vacuum filtered over a 5 μm membrane filter (Whatman AE98). This NaI-extraction is repeated two to three times to ensure that all plastic particles are removed from the sediment sample. Visual inspection of the filter is performed using a dissection microscope.

2.2. Extracting microplastics from sediments: method validation

A validation phase was included in the development of this new technique to determine the extraction efficiency and to compare this efficiency with that of the method pioneered by Thompson et al. (2004). The evaluation of both techniques involved spiking clean sediment with known concentrations of fibres and granules (PVC or polyethylene (PE)). This sediment was then subjected to the sequential extraction steps described above.

The clean sediment, i.e. sediment without microplastics, was obtained by subjecting a field sample of sediment to several subsequent elutriations, which resulted in the removal of all microplastics present. This sediment was then spiked with 50 particles or fibres per 500 mL of sample. PVC and PE granules were shavings of plastic tubing, and hence had different shapes and sizes (±250 μm). The fibres originated from previously extracted sediment and were of different sizes and materials.

2.3. Extracting microplastics from field organisms: depuration and acid digestion

The blue mussel *M. edulis* was selected as a model species, representing filter feeding species, for the development of a technique for extracting microplastics from animal soft tissue. Mussels with a size ranging from 4 to 4.5 cm were collected along the Belgian coast.

The development of the extraction technique involved chemical digestion of the soft tissue, using either an acid, base, oxidizer or a specific mixture of both, followed by filtration and microscopic analysis. Before the organisms were subjected to the digestion procedures they were kept in filtered artificial seawater (0.8 μm membrane filter, Supor®800, GelmanSciences) for at least 24 h after sampling to allow them to clear their gut. In this way, any material present in the gut such as sand particles, that could hinder the visual inspection for microplastics later on, is removed.

Several digestion protocols were explored: organisms were transferred to a 200 mL conical flask and 20 mL of an acid, base, oxidizer or a specific mixture thereof was added. The chemicals used for the digestions were nitric acid (HNO_3, 22.5 M), hydrogen peroxide (H_2O_2, 32.6 M) and sodium hydroxide (NaOH, 52.5 M). The specific mixtures were prepared by mixing HNO_3 with either hypochloric acid (HCl, 32.3 M) or H_2O_2 in a 3:1 v/v ratio. Each protocol consisted of digestion at a specific temperature and time, followed by dilution (1:10 v/v) either with hot (i.e. at the same temperature as the digestive substances) or cold (i.e. room temperature) filtered deionised water (0.8 μm membrane filter, Supor®800, GelmanSciences) and immediate filtration over a preweighed 5 μm cellulose nitrate membrane filter (Whatman AE98).

The digestion method resulting in the best digestion efficiency was further fine-tuned and validated. The final digestion procedure was performed in 250 mL conical flasks and consisted of overnight destruction of the organisms (3 mussels per flask) in 20 mL of HNO_3 (22.5 M) at room temperature, followed by 2 h of boiling (~100 °C). The resulting mixture was then diluted to 200 mL with warm (~80 °C) filtered deionised water and vacuum filtered over a 5 μm cellulose nitrate membrane filter (Whatman AE98) immediately after dilution.

It is important to note that all materials used for sampling, extraction and analysis were rigorously cleaned with filtered deionised water (0.8 μm membrane filter, Supor®800, GelmanSciences) to prevent any contamination. While this cleaning step was not absolutely necessary for the validation of this technique as plastic particles of known type, size and number were added, this is crucial when analysing field samples. In this case, appropriate procedural blanks should also be regularly included to account for any possible contamination that may occur during extraction.

2.4. Extracting microplastics from field organisms: method validation

To study the digestion efficiency, organisms were weighed prior to digestion. After filtration of the digested tissue, the membrane filters were dried at 60 °C for 24 h, and weighed. This allowed calculation of the tissue fraction remaining on the filters, and hence the efficiency of the digestion procedure applied.

The procedure resulting in the smallest tissue fraction remaining on the filters was then further validated, by subjecting different types of microplastics to the digestion procedure and assessing whether the particles could be recovered after the procedure. This was done by assessing the impact of the digestion procedure on the plastic material (i.e. is it affected by the acid/base/oxidizer and can it be recovered?).

Microplastic particles (polystyrene) and fibres (nylon) were spiked either directly into the conical flask or into mussel tissue. Using a syringe, 20 μL of a solution containing 10 μm polystyrene (PS) spheres (Coulter Standard Latex Beads, Analis) with a concentration of $3.10 \times 10^5 \pm 7.66 \times 10^3$ particles mL^{-1} was injected into mussel tissue. Similarly, other soft tissue samples were injected with 20 μL of a solution containing 30 μm PS spheres (Coulter Standard Latex Beads, Analis) ($8.65 \times 10^4 \pm 4.26 \times 10^3$ particles mL^{-1}). Same amounts of PS spheres were spiked directly into a conical flask. To test the extraction efficiency of this digestion technique for fibres, mussel soft tissue was spiked with 20 fibres. Two types of fibres were used: the first type (dimensions: 100 × 400 μm) was cut from fishing line collected on a local beach, the second type (dimensions: 30 × 200 μm) was obtained from a nylon rope.

3. Results

3.1. Extracting microplastics from sediments

The extraction efficiency for PVC particles using the newly developed technique was 100% after one extraction in the fluidized sand-bath followed by three subsequent extractions with 10 mL of NaI solution. For fibres, a 98% (49 out of 50 fibres) recovery was obtained after one extraction in the elutriation tube, followed by three subsequent NaI extractions. The last fibre was recovered after a third extraction in the fluidized sand-bath (Table 2).

3.2. Extracting microplastics from field organisms

The tissue fractions remaining on the filters differed greatly between different combinations of digestion temperature, filtration temperature and chemicals (Table 3). Irrespective of the chemical or mixture tested, the procedure involving a 1 h heating at 60 °C followed by 1 h boiling at 100 °C yielded the highest recovery efficiencies. Different chemicals reacted differently to filtration temperature: some had higher efficiencies when filtered warm, others when filtered cold. The most efficient digestion was obtained with HNO_3 heated for 1 h at 60 °C and 1 h at 100 °C. The obtained digest was then diluted with warm filtered water (approx. 80 °C) and filtered over a 5 μm membrane filter (Whatman AE98).

Polystyrene (PS) spheres (10 μm diameter) and fibres originating from nylon rope were subjected to this (most efficient) digestion procedure. Fibres (dimensions: 30 × 200 μm) could not be recovered after either direct digestion or digestion in mussel tissue (Table 4). The PS spheres on the other hand could be recovered (Table 4) after both treatments. However, after the direct digestion the spheres had apparently melted and clumped together. This did not occur when the particles were embedded in tissue. Since PS particles with a diameter as small as 10 μm could be recovered with an efficiency of 65.8% from the digested tissue without any visible change in physical structure, this digestion method was further fine-tuned to obtain even better extraction efficiencies.

The modified technique resulted in an additional decrease of the remaining tissue (assessed visually: decreased colouration of the filter with increased digestion efficiency) and was then validated as described earlier: i.e. known amounts of 10 μm and 30 μm PS spheres, and nylon fibres were added to mussel soft tissue, followed by chemical digestion and subsequent determination of the extraction efficiencies. The obtained efficiencies for extracting different types of microplastics (granules and fibres) are represented in Table 4. For 30 μm and 10 μm particles extraction efficiencies of 98% and 94% were obtained. Moreover, using this technique nearly all fishing line fibres were recovered (efficiency of >98%). However, none of the fibres of the nylon rope were detected on the filters with digested tissue.

Table 2
Comparison of two methods for extracting microplastics from sediments. Extraction efficiencies were obtained for three different types of microplastics (i.e. fibres, granules and PVC particles).

	Saturated salt solution (1.2 kg L^{-1}) (%)	Elutriation + NaI solution (1.6 kg L^{-1}) (%)
Fibres	75	98[a]
Granules	61[b]	100
PVC particles	0	100

[a] 100% efficiency was obtained after a third extraction in the fluidized sand-bath.
[b] An efficiency of 83% was achieved after a second extraction and 93% after the third.

Table 3
Development of an extraction technique for microplastics in animal soft tissue: values represent the percentage of mussel soft tissue remaining after digestion, and hence is a measure of the digestion efficiency of the applied procedure. Values in brackets represent the standard deviation (n.d.: standard deviation not determined).

Filtration temperature	Digestion temperature	
	24 h at 60 °C	1 h at 60 °C/1 h at 100 °C
HNO_3		
Cold	0.48 (±0.07)	0.26 (±0.06)
Warm	0.35 (±0.07)	0.15 (±0.05)
HNO_3/HCl		
Cold	1.62 (±0.24)	0.89 (±0.14)
Warm	1.22 (±0.21)	1.10 (±0.15)
NaOH		
Cold	4.05 (n.d.)	0.46 (n.d.)
Warm	0.52 (n.d.)	0.64 (n.d.)
H_2O_2		
Cold	1.49 (n.d.)	0.45 (n.d.)
Warm	1.43 (n.d.)	1.35 (n.d.)
HNO_3/H_2O_2		
Cold	High foam production during heating, resulting in an extensive loss of material	
Warm		

4. Discussion

Recently, the interest in microplastic contamination has increased and concentrations of microplastics present in the marine environment are being reported worldwide. A recent literature review (Hidalgo-Ruz et al., 2012) found that out of 68 studies 44 (i.e. 65%) focused on microplastics in sediments. However, when analysing the results of these studies, a number of limitations are noted. First, often different sampling methods are used, resulting in different units being reported. When sampling beaches for instance, one can opt for reporting microplastic concentrations expressed per m^2 or m^3. Other studies report the observed abundances of microplastics per weight of sediment sampled (Claessens et al., 2011; Reddy et al., 2006). This makes comparison between studies and hence between regions difficult. Second, the method most often applied in these studies for processing the sediment samples is density separation: 65% of all studies extracting microplastics from sediments use density separation (Hidalgo-Ruz et al., 2012). This technique is based on the difference between the density of plastic particles and the (higher) density of the sediment (Hidalgo-Ruz et al., 2012; Thompson et al., 2004). The density of plastic polymers, however, can vary considerably. Depending on the type of polymer, density values can range from 0.9 to 1.6 g cm^{-3}. When using a saturated sodium chloride solution, as is done in the majority of studies (77% of all studies that use density separation (Hidalgo-Ruz et al., 2012)), plastics with a density

Table 4
Validation/evaluation of the new digestion technique for the extraction of different types of microplastics (i.e. polystyrene spheres and nylon fibres of different dimensions) from animal soft tissue. Validation results, i.e. extraction efficiencies, of both the initially approved technique and the fine-tuned technique are represented. The respective extraction efficiencies are in brackets (n.a.: no digestion performed).

Digestion technique		Polystyrene spheres		Nylon fibres	
		10 μm	30 μm	100 × 400 μm	30 × 200 μm
Initial	Direct	Melted together	Melted together	n.a.	0.0%
	Embedded in tissue	65.8%	77.0%	n.a.	0.0%
Final	Embedded in tissue	93.6%	97.9%	98.3%	0.0%

higher than 1.2 g cm^{-3} are not extracted from the sediment sample. Hence, some important high density plastic types such as PVC and PET are overlooked resulting in an underestimation of the total microplastic concentration present in the sample.

To overcome this shortcoming, we developed the described new technique that extracts all types of microplastics present in environmental samples, i.e. including the high-density plastics. As an alternative to the conventional saturated NaCl solution, a sodium iodide solution (NaI), with a density of approximately 1.6 g cm^{-3}, is proposed and evaluated. However, this high-density salt is approximately 70 times more expensive than the common kitchen salt (used to prepare the saturated NaCl solution). To overcome the high costs associated with the use of large volumes of NaI, an additional step in the extraction procedure was introduced to reduce the volume of the sample. An elutriation column was developed in which the lighter particles (microplastics and other light materials) are separated from the heavier (sediment) particles by creating an upward water flow. This pre-treatment of the sample results in a significant decrease in volume of the sediment sample: i.e. a 500 mL sediment sample is reduced to only 10 mL of sediment. Instead of using 3 L of the salt solution for extracting microplastics from 1 kg of sediment sample (Claessens et al., 2011), only 40–80 mL of the high density NaI solution is needed for a similar amount of sediment (when assuming average sediment densities ranging from 1088 kg m^{-3} to 2600 kg m^{-3} (Fettweis et al., 2007)). By introducing this elutriation step, the volume of NaI needed is hence reduced at least by 97%. Additionally, the recovery of the NaI solution is higher through this sample volume reduction: previously only 70% of the salt solution could be reused; with the sediment pre-treatment recovery is increased to 90%. This reduces the cost of lost NaI solution from €63 to only €0.56 per kilogram of sediment sample.

Evaluation of this newly developed technique showed that using a NaI solution, preceded by an elutriation step, is more efficient in extracting different types of microplastics. We evaluated the flotation method described by Thompson et al. (2004): using a saturated salt solution it appeared impossible to extract the heavier PVC particles. For polyethylene particles an extraction efficiency of only 61% was achieved after the first extraction, 83% after the second, and 93% after the third extraction. For fibres, 75% was recovered after one extraction (Table 2). The extraction efficiencies (Table 2) obtained with the newly developed extraction technique are higher than those obtained with the NaCl extraction for microplastic fibres and granules. Moreover, this new extraction method yielded a 100% efficiency for extracting PVC particles from sediment after one extraction in the fluidized sand-bath followed by three NaI-extractions, whereas PVC, due to its high density (1.4 g cm^{-3}), cannot be extracted with a NaCl solution (1.2 g cm^{-3}). Extracting fibres using the newly developed technique achieves a 98% extraction efficiency. This is an important increase in efficiency compared to the 75% extraction efficiency of the NaCl extraction and is acceptable for reporting realistic fibre concentrations.

Ingestion of microplastics under laboratory conditions has been demonstrated with mussels, polychaete worms, barnacles and sea cucumbers (Browne et al., 2008; Graham and Thompson, 2009; Thompson et al., 2004). Only one of these studies, however, has also reported translocation of these ingested particles to the circulatory system of the mussel (*M. edulis*) (Browne et al., 2008). Ingestion in all these studies was demonstrated by dissection (Thompson et al., 2004) or analysis of faeces (Graham and Thompson, 2009); translocation with the use of fluorescent plastic spheres (Browne et al., 2008). When studying the presence of microplastics in tissue of animals exposed to microplastics in the field, these techniques are not very suitable as fluorescent plastics are not present in the environment and concentrations of microplastics in the environment are very low compared to the concentrations used in laboratory experiments. For instance, in laboratory experiments organisms are often exposed to several grams of microplastics (Graham and Thompson, 2009; Thompson et al., 2004). Claessens et al. (2011), on the other hand, reported on average less than 1 mg microplastics per kg dry sediment for Belgian intertidal shores. Hence, the microplastic test concentrations used in laboratory trials are several thousand times higher than those occurring in the environment.

A straightforward technique for detecting microplastics in the tissue of field-collected organisms is thus needed to investigate the impact of microplastic pollution on marine organisms. Here, we developed such a method based on chemical digestion of the soft tissue of marine invertebrates. Four digestion procedures were tested: two different digestion temperatures combined with two different filtration temperatures. All four combinations were tested with different "digestion" chemicals or mixtures thereof.

The tissue fractions remaining on the filter after filtration of the diluted digest were used to quantify the efficiency of the procedure. From the initial results, digestion of mussel tissue was most efficient when treated for 1 h at 60 °C with nitric acid (HNO_3, 22.5 M) followed by 1 h of boiling and warm filtration (Table 3). For some of the tested protocols, tissue fractions remaining on the filter were low, even though the digestion procedure was not optimal. This was caused by extensive loss of material during digestion or filtration. Performing the digestion with the mixture of H_2O_2 and HNO_3 produced a lot of foam while boiling. This foam spilled over the flask's edge, resulting in the loss of material. During filtration of material digested with H_2O_2, a lot of the material stuck to the filtration funnel, which could not be removed by rinsing. Additionally, diluting the digests with cold water resulted in the formation of a precipitate, making subsequent filtration and microscopic analysis more difficult.

Polystyrene (PS) spheres digested directly with the most efficient method seemed to be melted together, even though the melting temperature of PS (240 °C) was not reached during the procedure. The glass transition temperature of PS is 95 °C, which was exceeded during the procedure. The glass transition temperature is the critical temperature at which a non-crystalline material changes its behaviour from being "glassy" (hard and easy to break) to being "rubbery" (soft and flexible): it will become soft upon heating or brittle upon cooling. This could have caused the particles to seemingly melt together. When these spheres were subjected to the digestion procedure while embedded in soft tissue, they did not melt together. This may be due to the presence of

the tissue reducing the amount of acid directly affecting the plastic material, subsequently reducing the degree of melting/clumping together.

Using the method with HNO_3 and heating at 60 °C followed by boiling (cf. above), with subsequent warm filtration, fibres could not be retrieved, even when embedded in soft tissue (Table 4).

Further fine-tuning of this destruction technique (i.e. overnight destruction with HNO_3, followed by 2 h of boiling (~100 °C) and warm filtration (~80 °C)) led to a final digestion protocol which resulted in an even better extraction efficiency than that obtained with the original method. With the final method the remaining amount of tissue on the filter was further reduced, making the detection of extracted microplastics easier and increasing the extraction efficiencies (Table 4). The extraction efficiency of the 30 μm particles increased by over 20% (from 77.0% to 97.9%), while for the smallest particles (i.e. 10 μm diameter) efficiency increased by almost 30% (from 65.8% to 93.6%) when using this technique. Extraction of fibres was not as successful as for the polystyrene spheres: only the fishing line fibres were recovered after digestion (extraction efficiency of 98%). The 0% extraction efficiency of the nylon rope fibres, however, shows that not all of the fibres present in animal tissue will be recovered from that tissue. This could be due to the size of the fibres (too thin) or the material they are made of. Since the fibres used for validating the newly developed digestion technique had a minimal diameter of 30 μm and the smallest polystyrene particles that could be extracted only had a 10 μm diameter, it is more likely that the polymer type of the rope fibres was the limiting factor in extracting these fibres.

In conclusion, two methods for extracting microplastics from environmental samples (sediment and biota) were developed. For sediments, samples are subjected to an upward water flow in an elutriation tube to separate the lighter plastic particles from the heavier sand particles. A sodium iodide solution is then added to the material containing the microplastics. Plastic particles will float in this high density solution, other heavier particles are removed from the solution by centrifugation. The supernatants containing the microplastics are filtered over a 5 μm filter. The technique developed for extracting microplastics from biota consists of overnight destruction of the organisms in HNO_3 at room temperature, followed by 2 h of boiling. The resulting mixture is then diluted with warm filtered deionised water and immediately filtered over a 5 μm filter.

Since both techniques resulted in high extraction efficiencies (from 93% to 98%) for different types of microplastics these standardised and validated methods can help to obtain a more comprehensive understanding of the presence of microplastics in the marine environment, including biota.

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