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Evaluation and optimisation of sample preparation protocols suitable for the analysis of plastic particles present in seafood

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ABSTRACT

Small plastic particles are found ubiquitously in marine and freshwater ecosystems and consequently, their inhabitants. When aquatic animals are consumed as seafood, human exposure to those plastic particles is possible. However, only a few studies, applying largely different analytical procedures, assessed microplastics content in the edible part of seafood. In this study, ten protocols for the extraction of microplastics from biota were chosen and tested for their suitability to digest the edible part of a broad range of seafood species. The following criteria were used for this assessment: 1) feasibility to filtrate the entire digested sample with one filter of approximately 1 μm pore size, 2) effect of reagents applied during the sample preparation on the polymer integrity as assessed by means of infrared and Raman spectroscopy as well as pyrolysis gas chromatography mass spectrometry, 3) total sample preparation time and the possibility to avoid the use of expensive reagents. The most suitable protocol was found to be an enzymatic-alkaline approach, consisting of a quick hydrolysis of proteins with pepsin for 2 h and a consecutive alkaline hydrolysis for 4 h, both at 37 °C for most of the tested seafood species. Digestion efficiency of the optimised protocol was tested with fish fillets and soft tissues of commercially relevant molluscs and crustacean species. Compared to most other protocols described in literature, an adequate digestion of the seafood matrix was achieved faster without being significantly more cost- or labour-intensive. Moreover, only negligible degradation of eleven commercially relevant polymers excluding polyacrylonitrile was observed. Polymer integrity was assessed by a change in particle weight or surface as well as spectroscopic and chromatographic data. The optimised sample preparation protocol aims to support future method standardisation efforts in order to assess the dietary uptake of microplastics in humans.

1. Introduction

First findings of small plastic particles in the marine environment were already reported in the 1970's (Carpenter & Smith, 1972), but only recently, this topic received broad public attention at global level. Although being the subject of many studies over the last years, there is no generally accepted definition of the terms "microplastics" (MP) and "nanoplastics" (NP) yet (Frias & Nash, 2019). According to the most widely used definitions, MP are solid plastic particles with a diameter between 1 μm and 5 mm and NP are solid plastic particles with a diameter below 1 μm (Arthur, Baker, & Bamford, 2009; Frias & Nash, 2019; Hartmann et al., 2019).

Many studies described the presence of small plastic particles in the gastrointestinal tracts of mussels, crustaceans, and fishes from the marine as well as the freshwater environment as summarised by Hantoro, Löhr, Van Belleghem, Widianarko, and Ragas (2019) and O'Connor et al. (2019). The particles, especially those in the lower micrometre to nanometre scale, can translocate from the gut into other body regions,

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such as the haemolymph of mussels (Browne, Dissanayake, Galloway, Lowe, & Thompson, 2008) and crabs (Farrell & Nelson, 2013) or liver (Avio, Gorbi, & Regoli, 2015), brain (Ding, Zhang, Razanajatovo, Zou, & Zhu, 2018), blood (Kashiwada, 2006), and fillet (Zeytin et al., 2020) of fishes.

Especially when the entire organism is eaten (e.g., mussels, oysters, or small fishes such as sprats), consumers can be exposed to plastic particles through seafood products (Santillo, Miller, & Johnston, 2017; Smith, Love, Rochman, & Neff, 2018; Toussaint et al., 2019). These particles can also act as vectors for potentially hazardous chemicals like additives, component monomers or adsorbed environmental pollutants (Wright & Kelly, 2017) occurring in the human food chain. Therefore, plastic particles in seafood may pose a potential risk for human consumers health (Barboza, Cunha, Monteiro, Fernandes, & Guilhermino, 2020). However, for risk assessment, further studies regarding the human exposition towards MP and NP via consumption of seafood are required (EFSA, 2016).

Whereas the MP burden of bivalves was outlined in many studies, e.g., summarised by Toussaint et al. (2019), only few studies investigated MP present in the edible part of fishes, often applying different analytical methodologies (Abbasi et al., 2018; Akhbarizadeh, Moore, & Keshavarzi, 2019, 2018; Barboza, Lopes, et al., 2020; Collard et al., 2018; Ali Karami, Abolfazl Golieskardi, Yu Bin Ho et al., 2017; Su et al., 2019; Zitouni et al., 2020). Apart from the limited number of studies regarding the presence of MP in the edible part of seafood, studies often cannot be compared among each other due to a lack of standardised methods (EFSA, 2016).

The most commonly used methods for MP analysis are microscopic, spectroscopic (especially Fourier-transformation infrared- (FTIR) and Raman-spectroscopy), or thermoanalytical methods (Braun, Jekel, Gerdts, Ivleva, & Reiber, 2018) such as pyrolysis gas chromatography mass spectrometry (py-GC/MS). Each method has its intrinsic benefits and disadvantages regarding reliability, sensitivity for certain polymers, or detection limits. Furthermore, either information on particle number or polymer mass in the sample can be obtained depending on the chosen method (Zarfl, 2019). Therefore, a combination of different identification methods is necessary, when detailed information on particle number, shape and type are required (Braun et al., 2018; Shim, Hong, & Eo, 2017).

These methods require thorough removal of sample matrix for MP analysis in order to enable detection and identification of synthetic polymers (Löder et al., 2017). Larger sample amounts and increased sample size are proposed to be required for statistically reliable results regarding MP occurrence in fishes (Collard et al., 2018, 2019). Consequently, efficient protocols for digesting seafood matrix, suitable for large scale monitoring, are required (Dehaut et al., 2016). For isolating MP from biota, the matrix commonly is removed by acidic, alkaline, oxidative, or enzymatic treatment. Digestion protocols applying acidic, alkaline, or oxidative reagents are often among the most cost-effective protocols (Bessa et al., 2019) and suitable for a broad range of sample matrices such as fish fillets and alimentary tracts, crustaceans and mussels (Dehaut et al., 2016). However, application of these reagents including nitric acid, sodium hydroxide or hydrogen peroxide, may lead to discolouration (Dehaut et al., 2016), degradation (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017; Nuelle, Dekiff, Remy, & Fries, 2014) or destruction (Avio et al., 2015; Cole et al., 2014; Dehaut et al., 2016) of plastic particles. Especially, protocols requiring high temperatures should be avoided due to damaging effects on certain polymer types (Bessa et al., 2019). Whereas enzymatic protocols have negligible impact on polymer integrity (Cole et al., 2014; Löder et al., 2017), these protocols are often expensive and thus, being only suitable for smaller samples (Bessa et al., 2019). Enzymatic digestion is limited to a specific substrate (depending on the chosen enzyme), so previously described methods often are limited in their application regarding sample matrix (Catarino, Thompson, Sanderson, & Henry, 2017). Furthermore, they are often not tested regarding suitability for a broad range of sample matrices (Löder et al., 2017).

This study aimed at evaluating protocols suitable for the extraction of plastic particles present in the edible parts of seafood (especially fish fillets, but also soft tissue of crustaceans, and molluscs). Another aim was to optimise the extraction procedure regarding MP analysis, applying the most common MP detection and identification methods. Finally, the suitability of the optimised sample preparation protocol for the analysis of plastic particles present in seafood was preliminarily assessed by an in-house validation.

2. Material and methods

In order to evaluate and optimise sample preparation methods for the isolation of MP from the edible part of seafood a literature study on extraction protocols was carried out. Based on a catalogue of criteria, ten protocols described in literature for digesting marine biota were selected (2.2.1). These protocols were carried out with a relatively simple matrix (homogenised fish fillet). Suitable methods were further tested in the second step on more complex matrices (mussels, crustaceans) (2.2.2). The most suitable protocol was chosen for further optimisation. The polymer integrity was assessed for all polymer types used during this study (2.1). Therefore, polymer particles were treated according to the chosen protocol without matrix. Mass recovery, morphological (light microscopy) changes, and chemical changes (FTIR, Raman, py-GC/MS) were evaluated. The protocol was then optimised regarding digestion temperature and time. Further optimisation was applied for the filtration procedure, depending on sample matrix or analytical technique (2.3). Digestion efficiency of the optimised protocol was tested regarding its suitability for application on a broad range of commercially relevant seafood species differing e.g., in their fat or collagen content (2.4.1). Furthermore, it was tested if small polymer particles (Ø $<$ 50 µm) were recovered quantitatively applying this protocol on spiked samples (2.4.2). Finally, it was tested, if the matrix was removed sufficiently for enabling polymer identification with py-GC/MS and exemplarily with µ-Raman spectroscopy (2.4.3). In anticipation of further studies, PS nanoparticles were used to test whether and to which extent a nanoparticulate plastic fraction is adsorbed on matrix, MP particles, or the filter material after digestion and filtration or passes the filtrate and can be detected or quantified there.

2.1. Reagents & material

Reagents and materials used within this study as well as more details on the polymer standards are listed in the supplementary information (SI; chapter 1). Due to their good visibility to the naked eye, red polypropylene particles (PP; Ø $\geq$ 500 µm) were used during the evaluation of protocols in order to estimate, if particle loss occurred, e.g., due to insufficient rinsing of the glassware. These particles were obtained by cutting a red PP bottle cap with a scalpel. The most commercially relevant polymer types were chosen as test materials for polymer integrity and identification. Most polymers were purchased from Goodfellow Cambridge Ltd (Lille, France), including polycaprolactam microbeads Ø ≈ 3 mm (PA6), polycaprolactam powder Ø ≈ 25–30 µm (PA12), polyacrylonitrile powder Ø ≈ 50 µm (PAN), polycarbonate microbeads Ø ≈ 3 mm (PC), polyethylene terephthalate microbeads Ø ≈ 4 mm (PET), polypropylene microbeads Ø ≈ 4 mm (PP), crosslinked polystyrene rods Ø ≈ 6.35 mm (PS), polysulfone microbeads Ø ≈ 3 mm (PSu), polytetrafluoroethylene powder Ø ≈ 675 µm (PTFE), polyurethane microbeads Ø ≈ 3 mm (PU) and non-plasticised polyvinyl chloride powder Ø ≈ 250 µm (PVC). Low density polyethylene powder Ø ≈ 500 µm (PE) was purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA). For achieving particles of a more relevant size for MP analysis, smaller particles were generated by centrifugal milling according to Kühn, van Oyen, Booth, Meijboom, and van Franeker (2018) or precipitation, roughly based on the protocol described by Balakrishnan, Déniel, Nicolai, Chassenieux, and Lagarde (2019). PA6, PC,

PET, PS, PSu, PTFE, PU and PVC were cooled with liquid nitrogen and ground in an ultra-centrifugal mill (ZM100, Retsch GmbH, Haan, Germany) (SI, 1.4.1). PP was dissolved in boiling toluene and precipitated by cooling the solution (SI, 1.4.2). After milling or precipitation, the polymers were sieved with stainless steel sieves (mesh sizes 200 μm and 100 μm). For the polymer integrity test, polymers of a size that could be grasped with tweezers (approximately ≥ 100 μm) were chosen. A suspension of 1% PS in water (Ø ≈ 100 nm, nano-PS) was purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA) for exemplarily testing separation of NP from MP during the filtration procedure.

2.1.1. Sample material

Commercially relevant seafood species were chosen as sample material. As already indicated by Collard et al. (2019), lipids are a critical component regarding the performance of sample preparation protocols. Therefore, fish fillets with fat contents in the range of 0.4%–43% were chosen from the species *Lophius piscatorius*, *Pollachius virens*, *Oncorhynchus keta*, *Salmo salar*, *Salmo trutta*, *Thunnus albacares*, *Clupea harengus*, *Reinhardtius hippoglossoides* and *Anguilla anguilla* for assessing differences in the protocol's performance regarding fat contents of samples. Molluscs and crustaceans generally have low fat contents, so the main components influencing the performance are assumed to be of proteinogenic origin. The soft tissue of *Mytilus edulis* and *Pecten maximus* (roe and muscle) were chosen, as they are commonly consumed species of the families of Mytilidae and Pectinidae. *Loligo* sp. was chosen for exemplarily testing digestion of cephalopods. Further samples were the tails of six crustaceans of the commonly consumed species *Lithopenaeus vannamei, Macrobrachium rosenbergii, Nephrops norvegicus, Penaeus monodon, Penaeus semisulcatus, Pleoticus muelleri* and *Procambarus clarkii.*

2.2. Development of an efficient extraction protocol

2.2.1. Identification of sample preparation protocols

A literature survey was conducted for identifying protocols applied for the isolation of MP from aquatic biota, especially fishes, bivalves, or crustaceans, including their gastrointestinal tracts. A total of 94 studies published between 2011 and 2018 were evaluated. Protocols that seemed to be promising for an application in the context of routine analysis were chosen. Criteria including negligible impact on the polymer integrity, low costs of required reagents as well as overall simplicity, and time required for the conduction of the protocol were considered. Ten extraction protocols were chosen that matched at least one of the above-mentioned criteria (Table 2).

2.2.2. Evaluation of sample preparation protocols

The protocols identified in 2.2.1 as well as combinations thereof were applied for digesting seafood as listed in Table 2. To be selected for further optimisation, the protocols had to meet the following criteria:

a) adequate digestion of 10 g seafood matrix to make filtration with one filter (pore size 1 μm) feasible
b) negligible impact on polymer integrity
c) ideally complete sample preparation within approximately 24 h and low costs of reagents

An initial screening for digestion with all methods were made using homogenised fish fillets. It was assumed that adequate digestion can be achieved easier with fish fillets than with the soft tissue of crustaceans and mussels due to the less complex composition of muscle tissue as opposed to the complete soft tissue including gastrointestinal tracts and their contents. Consequently, each protocol was primarily tested with *C. harengus* fillet (with skin) and *S. salar* (without skin). In case of adequate digestion, the experiments were extended to mussels (*M. edulis*) and crustaceans (*M. rosenbergii*, *P. monodon*).

Ten grams of homogenised fish fillet were weighed into an Erlenmeyer flask. Fifteen to twenty red PP particles (Ø ≥ 500 μm) were spiked to each sample by manually transferring the particles into the flask with tweezers. The respective digestion solution was added, and the samples were digested under constant stirring (heated in an oven) or shaking (heated in a water bath) applying temperature and time as listed in Table 2. Afterwards the digested samples were filtered, starting with cellulose nitrate filters (CN-filter) with a diameter of 47 mm (large filters) and a pore size of 8 μm. The flask and the glass funnel of the filtration apparatus were rinsed thoroughly, first two times with approximately 10 mL 0.5% SDS-solution and afterwards with deionised water (DI water) (at least 3 × 30 mL) until no foam remained on the glassware or the filter and all particles were transferred. When filtration was feasible with CN-filters (8 μm pore size), the protocol was repeated using large glass fibre filters (pore size 1.2 μm) and large polycarbonate filters (pore size 1.0 μm; PC-filter). In case filtration of the entire digested fish samples with glass fibre filters (1.2 μm pore size) was feasible, the protocol was tested again with the soft tissue of crustaceans and mussels directly using glass fibre filters. A detailed description of each digestion protocol listed in Table 2 is provided in the SI (SI, 2.1).

In order to evaluate the protocols' suitability, digestion efficiency was estimated qualitatively. Therefore, the total time required for filtration (Ø 47 mm) was assessed and the filter surface inspected visually for the presence of matrix residues and red PP-particles. The total time required for the digestion procedure (excluding filtration) was determined and the costs of reagents were estimated. Polymer integrity was estimated based on data published by Cole et al. (2014), Dehaut et al. (2016), Enders, Lenz, Beer, and Stedmon (2017), Nuelle et al. (2014), (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017), Lusher, Welden, Sobral, and Cole (2017), and Löder et al. (2017).

Table 1
Instruments and their technical parameters used in the study.

instrument	parameters
light microscope	Motic BA210 microscope (Motic, Xiam, China) KL1500 LCD lamp (Schott, Mainz, Germany)
fluorescence microscope	Leica TCS SP8 inverse confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) EL6000 fluorescence lamp (Leica) FITC-Filter (excitation 467 nm–498 nm; emission 513 nm–556 nm)
ATR-FTIR	Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany) LN-MCT Photovoltaic detector (Bruker Optics), cooled with liquid nitrogen Platinum ATR measuring head (Bruker Optics), scan number: 32 spectrometer-software OPUS Version 7.5 (Bruker Optics)
μ-Raman	DXRxi Raman imaging microscope (Thermo Fischer Scientific, MA, USA) DXR532 nm Laser (Thermo Fischer Scientific), 5.0 mW–6.0 mW exposure time: 6 Hz–3 Hz, scan number: 1000 libraries: HR Aldrich Raman, HR FT-Raman Polymer Library, HR Nicolet Sampler Library, Organics by Raman Sample Library, Polymers & Additives Packaging, Raman, Raman Vol. 1 2 cm^{-1}, Raman, Raman Vol. 2 2 cm^{-1}, Raman Sample Library
py-GC/MS	Shimadzu GCMS-TQ8040 (Shimadzu, Kyoto, Japan) pyrolysis in 30 μL-DMI sample insert (GL-Science, Tokyo, Japan) with OPTIC IV injector (GL-Science) at 600 °C, split 1:15 or 1:500 RXI-5Sil MS w/Integra-Guard (30 m x 0.25 ID x 0.25 μm) column flow: 1 mL/min He; temperature program: 30 °C (3 min), ramp to 180 °C (10 min), ramp to 280 °C (5 min), ramp to 320 °C (20 min, hold for 16 min), interface temperature 280 °C mass spectra: ion source 250 °C, electron impact ionisation 70 V, range 50–500 m/z, 2000 Hz scan speed
scanning electron microscopy	Quanta 250 field emission gun, Everhart-Thornley-Detector (10 kV) working distance: 8 mm, chamber pressure: 0.0003 Pa platinum coating with sputter coater (Q150T ES, Quorum Technologies Ltd, UK)

Table 2
Overview of sample preparation protocols tested for their suitability for isolating MP from seafood matrices using filters with a pore size of approximately 1 μm. At least one of the following criteria had to be met for selecting the protocol for further testing: negligible impact on the polymer integrity, low costs of required reagents, overall simplicity, total time required for the conduction of the protocol. T – temperature in °C, t – time in hours; contents in brackets are the parameters of the original method.

	No.	Reagents	process	T [°C]	t [h]	based on
alkaline-acidic	1	50 mL 2.5 mol/L NaOH or KOH	stirred	50	0.25	Roch and Brinker (2017)
		6 mL deionised (DI) water	thinned	–	–	
		144 mL 65% HNO_3	acidified	50	0.25	
			& stirred	80	0.25	
		200 mL hot DI water	thinned	80	–	
alkaline	2a	50 mL 10% KOH (w/v)	stirred	~20	168 (>336)	Foekema et al. (2013)
	2b			37 (40)	72	(Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017); Ali; Karami, Golieskardi, Ho, Larat, and Salamatinia (2017)
	2c			60	16–72 (24)	Dehaut et al. (2016)
alkaline-oxidative	3a	sample preparation	dried	60	24	Grigorakis, Mason, and Drouillard (2017)
		50 mL 10% KOH (w/v)	stirred	~60	1	
		5 mL 30% H_2O_2 (v/v)	stirred	~60	2	
	3b	25 mL 10% KOH (w/v)	stirred	~60	24	derived from 3a
		25 mL 30% H_2O_2 (v/v)	stirred	~20	24	
	4	21.4 mL 10% KOH (w/v)	shaken	~20	24	Fischer (2019)
		3.6 mL 12% NaClO (w/v)				
oxidative	5	60 mL 9% NaClO (w/v)	stirred	~20	16	Collard et al. (2015)
	6	100 mL 33% NaCl (w/v)	dispensed	–	0.17	Avio et al. (2015)
			& settled			
		sample filtration	decanting	–	–	
		10 mL 15% H_2O_2 (v/v)	filter treatment	50	16	
Enzymatic	7	100 mL 10% SDS (w/v)	stirred	50	24	Löder et al. (2017)
		after filtering: 20 mL protease (>1100 U/mL) in 100 mL 1 mol/L TRIS HCl buffer (pH 9.0)	stirred	50	24	
		after filtering: 5 mL lipase (>18000 U/mL) in 100 mL 1 mol/L TRIS HCl buffer (pH 9.0)	stirred	37	24	
		after filtering: 25 mL cellulase (>70 U/mL) in 100 mL sodium acetate buffer (pH 4.7)	stirred	40	72	
		30 mL 30% H_2O_2 (v/v)	filter treatment	37	24	
	8	100 mL 0.32% trypsin (>1500 U/mg) in PBS-buffer (pH 9.0)	stirred	37	1	Courtene-Jones et al. (2017)
	9	90 mL 0.5% pepsin (2000 U/g) in 0.063 mol/L HCl	shaken	37	2	Karl et al. (2014)
		4 mL 10% KOH (w/v)	alkalised	–	–	Dehaut et al. (2016)
		1 mL lipase at pH 9.0	shaken	40	16	
enzymatic-oxidative or enzymatic-alkaline	10a	90 mL 0.5% pepsin (w/v) in 0.063 mol/L HCl	stirred	37	2	derived from 9 & 5
		10 mL 12% NaClO (w/v)	stirred	~20	16	
	10b	90 mL 0.5% pepsin in 0.063 mol/L HCl	stirred	37	2	derived from 9 & 2c
		10 mL 50% KOH (w/v)	stirred	60	4	

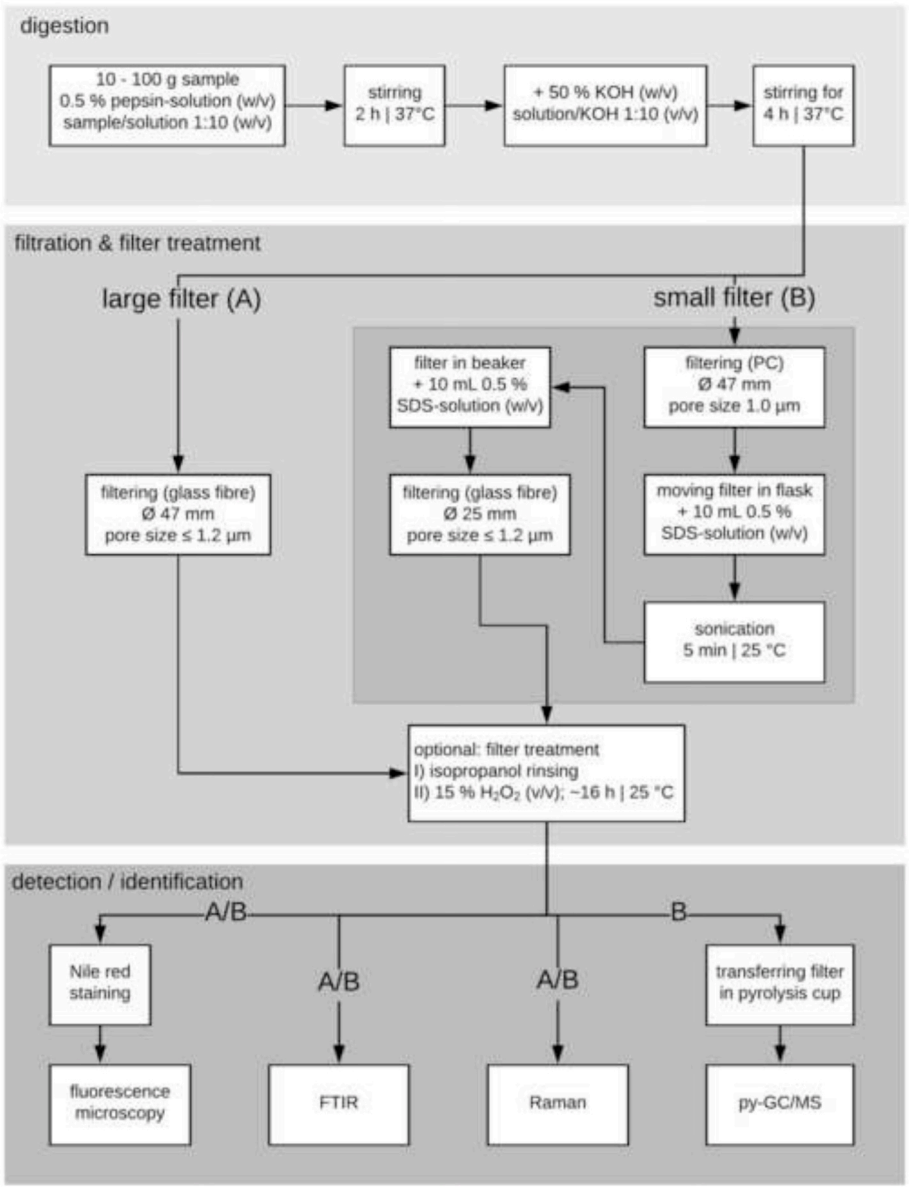

Fig. 1. Optimised enzymatic-alkaline method for the extraction of MP from seafood. Necessity of filter treatment depends on matrix. Option B is recommended for imaging techniques and was required in this study for py-GC/MS analysis. Smooth filters are filters with a plane surface that enable quantitative removal of micron sized particles, such as PC-filters or silver filters.

Moreover, polymer integrity of protocol No. 10b was assessed with FTIR, µ-Raman and py-GC/MS analysis (instrument parameters are listed in Table 1) as well as by determination of changes in weight and particle size (expressed as the area of individual photographed particles).

2.3. Optimised sample preparation protocol

The most promising protocol for seafood digestion according to 2.2.2 was optimised (protocol No. 10b). The application of different proteases (SI, 2.2) was tested. Digestion time and temperature were adjusted depending on polymer integrity (SI, 2.3). Depending on different identification methods, the filtration procedure and choice of filter materials was optimised. Post-filtration treatment was implemented depending on the sample matrix (SI, 2.4). The optimised protocol is illustrated in Fig. 1.

In detail, 90 mL of a solution of 0.5% pepsin (w/v) in 0.063 M HCl were added to the 10 g sample. The sample was digested under constant stirring for 2 h at 37 °C. Afterwards, 10 mL of a 50% solution of KOH (w/v) were added resulting in a concentration of approximately 10% KOH in the sample solution. The sample was hydrolysed at the same temperature for another 4 h. The digested sample was filtered with large glass fibre filters or PC-filters as visualised in Fig. 1, option A. For isolating MP on small filters (Ø 25 mm) instead, the sample was pre-filtered with large PC-filters. Afterwards, the particles were rinsed off onto small filters using sonication and detergent solution, as visualised in Fig. 1, option B (compare to SI 2.4.3 for a detailed description of the pre-filtration procedure). In case of residual fat adhering to the filter, the sample was rinsed with 10 mL isopropanol. For isolating MP from the PC-filter, sonication in 0.5% SDS-solution (w/v) for 5 min (Collard, Gilbert, Eppe, Parmentier, & Das, 2015) was applied. The isolated particles were then filtered onto small glass fibre filters or Al_2O_3 filters. To further reduce the residues of sample matrix on the filter, when necessary 1–3 mL of 15% H_2O_2-solution (v/v) were added dropwise to the filter in order to avoid rinsing particles off the filter surface. The sample was stored in a glass Petri dish and dried subsequently at room

temperature overnight.

As the protocol is supposed to be used for real samples that may contain MP as well as NP particles, a potential influence of the filter material on NP was tested here in anticipation of further studies. Therefore, 20 µL nano-PS were pipetted onto 10 g homogenised *C. harengus* fillet (with skin), digested with the optimised protocol (option A) and filtered with large glass fibre filters (1.2 µm pore size), CN-filters (1.2 µm pore size), silver filters (1.2 µm pore size), PC-filters (1.0 µm pore size), or cellulose acetate filters (0.8 µm pore size; CA-filter), respectively. The surface of glass fibre filters, silver filters, CN-filters and PC-filters were examined with scanning electron microscopy (SEM) (instrument parameters are listed in Table 1).

2.4. Characterisation of the optimised protocol for suitability to analyse microplastics in seafood samples (fish, mussels, crustaceans)

2.4.1. Evaluation of the digestion efficiency for different matrices

As the digestion of seafood was only tested exemplarily with fish fillet for the protocols described in 2.2.1, a broad range of seafood species was digested with the optimised protocol (No. 10b optimised). Therefore, the homogenised edible tissues of commonly consumed seafood species (listed in 2.1.1) were digested according to the optimised protocol (option A) and filtered with large glass fibre filters. The species were chosen based on differences in fat or collagen content as these factors were assumed to be limiting the protocol's effectiveness. In cases of filter clogging, e.g., due to high fat or insoluble collagen content (*A. anguilla, P. maximus* roe, and *L. vannamei*), the alkaline hydrolysis step was conducted at 60 °C (cf. protocol No. 10b). Furthermore, digestion of larger amounts of sample matrix (50 g–100 g) was tested with *L. piscatorius*, *C. harengus*, *P. monodon*, and *S. salar* for which the duration of the alkaline hydrolysis step had to be prolonged to 16 h. The digestion efficiency was expressed as the ratio between matrix residue and sample weight (Catarino et al., 2017). The weight of the matrix residue was determined by the weight difference of the dried filter before and after filtration.

2.4.2. Preliminary in-house validation

For estimating if plastic particles were extracted quantitatively from the seafood matrix, recovery rates of plastic particles spiked to fish fillet and reproducibility of the extraction procedure were determined. As the pre-filtration procedure was more complex than direct filtration and particle loss was more likely, the preliminary in-house validation was conducted with the optimised protocol option B (SI, 2.4.3). PA12 was chosen as a particle reference due to its easily recognisable shape (smooth, rounded particles) and small size range (10 µm–50 µm). PA12 was dyed by dispensing the particles in a solution of 1 mg/mL Nile red according to Tamminga, Hengstmann, and Fischer (2017) with the modification of using isopropanol instead of chloroform. The solvent was left to evaporate. A stock solution of stained PA12-particles was prepared by dispersing the polymer particles in a 0.5% SDS solution (w/v). The particle number of the stock solution was determined by counting with a FUCHS-ROSENTHAL counting chamber. Spiking solutions with particle numbers (n) of approximately 10, 10^2, and 10^3 particles per mL were created. To determine the exact number of particles in the respective spiking solution, 1 mL solution was applied onto a small glass fibre filter. Five replicates were created for each spiking solution for particle number determination. For each spiking solution, five replicates of 10 g homogenised fish fillet (*P. virens*) were weighed into a glass flask and spiked with 1 mL of the respective solution. The samples already containing approximately ten PA12-particles were additionally spiked with approximately 1 µg of each polymer type suited for py-GC/MS analysis (PA6, PA12, PC, PE, PET, PP, PS, PSu, PU) for polymer identification (compare to 2.4.3). In order to spike the samples with a reproducible amount of polymer the plastic particles were dissolved in appropriate solvents (ASTM International, 2018; Vidyarthi & Palit, 1980). More details on the preparation of polymer solutions are given in the SI (SI, 2.5.1).

The samples were digested with the optimised protocol option B (cf. Fig. 1). To avoid bleaching of the pre-stained PA12-particles, no H_2O_2-treatment was applied. The small glass fibre filters were retained between two microscope slides. The slides were fixed with adhesive tape and characterised with a confocal microscope (see Table 1 for instrument parameters). All particles with a regular, oval, or round shape, and bright orange fluorescence were counted either manually (n = 10, n = 100) or with a scan of the complete filter surface (n = 1000) and using the image software "Image J" (Anger, Prechtl, Elsner, Niessner, & Ivleva, 2019; Mukhanov et al., 2019). Following fluorescence microscopy, filters were transferred into pyrolysis cups for py-GC/MS analysis.

2.4.3. Polymer identification

To test whether the sample matrix was removed sufficiently for enabling polymer identification, 10 g of *P. virens* were spiked with approximately 1 µg of each polymer type (cf. 2.4.2). Chemical identification of synthetic polymers was achieved by py-GC/MS (instrument parameters are listed in Table 1). Characteristic pyrolysis products of each polymer type were determined by pyrolysis of individual plastic particles and according to data obtained by Shin, Jahime, and Chuichi (2011). In order to unambiguously identify the polymer type, at least three characteristic pyrolysis products of the respective polymer were detected. Pyrolysis products were identified based on their retention time (≤5% deviation) and the mass of at least three, preferably specific, fragment ions. Qualitative analysis with spectroscopic methods was tested exemplary with individual PA12 particles by µ-Raman spectroscopy. The identity of the particles was confirmed with spectra libraries and by comparison with the spectrum of the virgin polymer standard.

Table 3
Comparison of tested methods regarding filtration, effect on polymer integrity, sample preparation effort (time (t) and number of steps (e.g. adding another solution, filtering) required for complete sample preparation) and estimated costs per 10 g sample. The effect on the polymer integrity was estimated based on findings described in literature or tested in this study (protocol No. 10b).

no.	digestion principle	filtration	integrity	effort		costs
				t [h]	steps	
1a	alkaline-acidic (NaOH, HNO_3)	++	–	0.75	3	+
1b	alkaline-acidic (KOH, HNO_3)	++	–	0.75	3	+
2a	alkaline (KOH), 25 °C	+	+	168	1	–
2b	alkaline (KOH), 40 °C	+/–	+	72	1	–
2c	alkaline (KOH), 60 °C	+	+/–	16	1	–
	alkaline (KOH), 60 °C	++	+/–	72	1	–
3a	alkaline-oxidative (KOH, H_2O_2)	+/–	+/–	3	2	+/–
3b		++	+/–	48	1	+/–
4	alkaline-oxidative (KOH, NaClO)	++*	+	24	1	–
5	oxidative (NaClO)	–	+	16	1	+/–
6	oxidative (H_2O_2)	–	+	16	2	+/–
7	Enzymatic (subtilisin, lipase, cellulase)	–	+	96	9	+
8	enzymatic (trypsin)	–	+	1	1	–
9	enzymatic (pepsin, lipase)	–	+	18	2	–
10a	enzymatic-oxidative (pepsin, NaClO)	–	+	16	2	–
10b	enzymatic-alkaline (pepsin, KOH), 60 °C	++	+/–	6	2	–
	enzymatic-alkaline (subtilisin, KOH), 60 °C	++*	+/–	16	2	–
	enzymatic-alkaline (pepsin, KOH), 40 °C optimised	++	+	≤16	3 (A) 6 (B)	–

*Insufficient for crustaceans (here: *M. rosenbergii*) costs - low among all tested methods±medium among all tested methods + high among all tested methods integrity - (severely) degrading effects on a broad range of synthetic polymers±degrading effects on some polymer types, e.g. polyamides or polyesters + no degrading effects on tested polymers (exception: PAN for alkaline approaches) filtration - no filtration with filters of a pore size of 8 µm could be achieved±slow filtration with pore size of 8 µm, clogging of filters with 1 µm pore size + quick filtration with pore size 8 µm, > 1 filter of 1 µm pore size necessary ++ filtration with one filter of a pore size of 1 µm could be achieved.

2.5. Contamination control

All glassware was rinsed with DI water and dried at 60 °C. Glass flasks, beakers, Petri dishes, and glass fibre filters were covered with aluminium foil, closed or stored in petri dishes respectively and heated to 500 °C for 6 h. All solutions were filtered directly into the flask or onto the filter using glass fibre filters (0.7 μm pore size), a stainless-steel filter holder and a glass syringe. A cotton lab coat and nitrile gloves were worn during the complete process.

All solutions used for the sample preparation were pre-filtered with glass fibre filters (pore size 0.7 μm) and observed individually regarding fluorescent particles after Nile red staining. Special attention should be given with regard to the purity of pepsin, as large numbers of MP-suspect particles were observed in some cases (Figure S1).

3. Results and discussion

3.1. Evaluation and optimisation of sample preparation

A summary of the evaluation of the protocols is given in Table 3. The evaluation and optimisation of sample preparation procedures was based on protocols described as being successfully applied for the extraction of MP present in aquatic biota. However, only a minority of the protocols was optimised for the extraction of MP present in the edible part of seafood, especially in fish muscles. The main criterium for the optimisation of the sample preparation protocol was digestion efficiency as insufficient digestion of the matrix led to clogging of the filter. Especially the filterability of the sample with only one filter with an approximate pore size of 1 μm was considered as very important, because most MP identification methods rely on a preliminary visual examination of the filter surface. Consequently, the overall sample preparation time required for a complete MP assessment increases with the number of filters required per sample. The filter pore size of 1 μm was chosen based on the resolution of most commonly applied identification methods (1 μm–10 μm) (Braun et al., 2018) and the particle size limit regarding the term "microplastics" (ISO/TR 21960:2020). For avoiding misinterpretations of MP findings due to the degradation or loss of polymers such as acid-sensitive polyamides or alkali-sensitive polyesters (Dehaut et al., 2016), the protocols should have negligible effects on the integrity of the tested polymers. As the optimised protocol should be applicable for routine analysis, the total time required for complete digestion and the number of preparation steps should be low. Ideally, only a few expensive reagents should be required.

All oxidative (protocols Nos. 5 & 6), enzymatic (Nos. 7, 8 & 9) as well as enzymatic-oxidative (Nos. 10a) approaches led to insufficient degradation of the sample matrix, resulting in either large amounts of matrix residues on the filter or a rapid clogging of the filter. The results were in accordance with previous findings by Dehaut et al. (2016) using pepsin for the digestion of seafood. Filter clogging might occur due to insufficient digestion of the sample material, especially when the corresponding protocol was originally developed for a different matrix. For instance, the oxidative approach described by Collard et al. (2015) was successfully applied to digestion of fish stomach, which is lower in fat compared to the fish fillet (*C. harengus* with approximately 20% fat). The enzymes applied for the respective protocol itself might also contribute to filter clogging. This was observed e.g., for pancreatin or pepsin solution of different suppliers, even without matrix. Therefore, even though these approaches had the least deleterious effects on MP, their suitability for complete digestion of seafood samples was limited.

The alkaline-acidic approaches (Nos. 1a & 1b) were able to digest the samples sufficiently and were the fastest of all investigated protocols requiring only about 45 min. However, due to the large amount of strong concentrated acids required for digesting the sample, the comparably high costs and the deleterious effects of fuming nitric acid on certain plastic types such as PA (Dehaut et al., 2016), further optimisation was not considered.

All alkaline approaches were confirmed as being simple and effective for digesting the edible part of seafood when high temperatures (60 °C) and prolonged digestion periods (3–8 days) were applied (protocols Nos. 2a & 2c). As some polymers, such as PET, are hydrolysed in alkaline solutions at elevated temperatures already starting at 30 °C (Haghighatkish & Yousefi, 1992; Spaseska & Civkaroska, 2010), reducing temperature or digestion time was necessary to avoid polymer loss (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017). Combining the alkaline hydrolysis with oxidative reagents (NaClO or H_2O_2) enabled further reduction of digestion time or temperature. However, the addition of H_2O_2 (protocols Nos. 3a & 3b) with a concentration that led to a significant improvement of the digestion also led to massive formation of foam potentially resulting in particle loss. Using NaClO as oxidising agent instead of H_2O_2 (protocol No. 4) led to sufficient digestion of fish fillet and bivalves. Moreover, high temperatures or a prolonged digestion period as applied in protocol No. 2c could be avoided. However, digestion of crustacean samples was not successful. Consequently, this protocol could not be applied universally.

The integration of an enzymatic digestion step prior to alkaline hydrolysis enabled complete digestion of *C. harengus and S. salar* fillet as well as the soft tissue of *M. edulis, M. rosenbergii,* and *P. monodon* within 6 h (protocol No. 10b). The duration of the alkaline step was reduced to 4 h instead of 24 h (Dehaut et al., 2016) or 72 h (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017) respectively. In comparison to protocol No. 4, crustaceans were also digested sufficiently (>98%), proving the optimised protocol applicable to a broader range of seafood products. The much shorter time required for sufficient digestion is assumed to be based on partial hydrolysis of collagenous proteins during enzymatic digestion. Non-collagenous proteins in seafood are generally alkali-soluble (Kimura & Tanaka, 1986; Sato, Yoshinaka, Sato, & Shimizu, 1987), whereas collagenous proteins remain insoluble. Pepsin hydrolyses the peptide bonds of proteins, preferentially those involving the imino group of L-tyrosine or L-phenlyalanine (Fruton, Fujii, & Knappenberger, 1961). Consequently, this leads to a loosening of crosslinked molecules increasing the solubility of collagenous proteins (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). Overall, the enzymatic-alkaline approach (protocol No. 10b) resulted in a digestion efficiency of 99.6%. Other studies reported efficiencies ranging between 88% (enzymatic approach by Courtene-Jones, Quinn, Murphy, Gary, and Narayanaswamy (2017)) and 95%–99.8% (alkaline approaches by Dehaut et al. (2016), (Ali Karami, Abolfazl Golieskardi, Cheng Keong

Table 4
Overview of polymer integrity after enzymatic-alkaline digestion according to method 10b. The alkaline step was conducted at 60 °C if not noted otherwise.

polymer	recovery		identification		
	weight [%]	area [%]	FTIR	Raman	py-GC/MS
PA6	95.6 ± 1.5	104.3 ± 1.5	+	+	+
40 °C	94.7 ± 1.0	not tested			
PA12	98.0 ± 1.5	/	+	+	+
PAN	–	–	–	$^{+}/_{-}$	$^{+}/_{-}$
PC	96.1 ± 2.2	97.4 ± 1.4	+	+	+
40 °C	94.5 ± 0.2	not tested			
PE	99.7 ± 2.1	103.1 ± 5.4	+	+	+
PET	90.8 ± 0.1	91.8 ± 5.5	+	+	+
40 °C	91.6 ± 1.5	100.1 ± 3.5			
PP	98.1 ± 0.1	99.9 ± 5.0	+	+	+
PS	99.3 ± 1.9	101.1 ± 2.4	+	+	+
PSu	101.1 ± 1.3	101.1 ± 3.2	+	+	+
PTFE	100.1 ± 2.4	100.1 ± 5.5	+	+	/
PU	98.6 ± 1.9	99.8 ± 4.1	+	+	+
PVC	99.4 ± 1.2	/	+	+	/

/ polymer was not suited for testing with this method-polymer - could not be analysed/identified $^{\pm}$ spectrum/pyrogram indicated polymer identity + polymer could be identified, and no significant differences were observed.

Choo et al., 2017) and Thiele, Hudson, and Russell (2019)). Regarding the digestion of seafood matrix, the enzymatic-alkaline approach described in the present study is comparable to the most efficient protocols described in literature.

3.2. Optimisation of the enzymatic-alkaline protocol

The enzymatic-alkaline approach (protocol No. 10b) was further optimised in terms of minimal polymer degradation and applicability for different methods of MP analysis.

3.2.1. Optimisation of the enzymatic-alkaline protocol regarding polymer integrity

As summarised in Table 4, most polymers were not affected by the enzymatic-alkaline approach (protocol No. 10b) applying 60 °C during the alkaline treatment. No significant changes were observed in the chemical structure of the tested polymers as assessed by ATR-FTIR, μ-Raman or py-GC/MS except for PAN. As no significant difference was observed for spectra and pyrograms of most polymers in this study compared to data already reported in literature, only data regarding PAN are shown exemplarily in the SI (S2 – S4).

The change in polymer weight might not only be caused by chemical decomposition but could also be based on particle loss during filtration. Therefore, morphological changes of individual plastic particles were assessed for confirming degradation. Degradation due to alkaline hydrolysis at 60 °C was only confirmed for polyesters (PET, PC). In alkaline solutions, PET and PC are hydrolysed to their monomers terephthalic acid and bisphenol A, respectively (Pavlov, Khokhlov, Rudakova, & Kuleva, 1976; Rudakova, Moiseyev, Chalykh, & Zaikov, 1972).

For PET, weight loss of 9.2% ± 0.1% was observed which was confirmed by a significant reduction ($\alpha \leq 0{,}05\%$) of the projected area of the particles of 8.2% ± 5.5%. The degradation of PET due to alkaline digestion was already shown by Dehaut et al. (2016) and Karami, Abolfazl Golieskardi, Cheng Keong Choo et al. (2017). PET is one of the dominating polymer types of MP present in the environment (Ajith, Arumugam, Parthasarathy, Manupoori, & Janakiraman, 2020) and was reported in 23 out of 43 studies finding MP in aquatic organisms, in some studies making up to 40% of all found MP (Catarino, Macchia, Sanderson, Thompson, & Henry, 2018). Loss of PET particles might therefore result in a significant bias regarding MP occurrence in seafood samples. Consequently, the protocol was optimised in order to avoid PET loss. No significant decrease ($\alpha \leq 0{,}05\%$) of the particle area of PET-particles was observed when reducing the temperature of the alkaline treatment to 40 °C confirming the observations of Karami, Abolfazl Golieskardi, Cheng Keong Choo et al. (2017). Therefore, low temperature of the alkaline hydrolysis is important for maintaining the morphological integrity of the plastic particles.

PC was less susceptible towards chemical degradation than PET. In detail, weight loss of 3.9% ± 2.2% and reduction of the projected area of the particles of 2.6% ± 1.4% were observed. Chemical hydrolysis of PC in aqueous potassium hydroxide solutions is assumed to take place in a thin surface layer (Pavlov et al., 1976) and minimal impact of KOH on PC was observed by Dehaut et al. (2016), Kühn et al. (2017), and Enders, Lenz, Beer, and Stedmon (2016). Therefore, alkaline hydrolysis at 40 °C is expected to have negligible impact on this polymer.

While PAN was not altered during the enzymatic treatment, the colour of the suspension changed from white to a bright orange/brown already within 10 min after the addition of KOH. The identification of that polymer type was not possible. PAN is known to hydrolyse to poly (sodium acrylate-*co*-acrylamide) in sodium hydroxide solution, while forming brown coloured intermediate stages due to cyclisation (Litmanovich & Platé, 2000). As the alkaline hydrolysis step is essential for digestion of the sample material, PAN cannot be analysed when using this extraction protocol. Even though PAN is not listed among the most

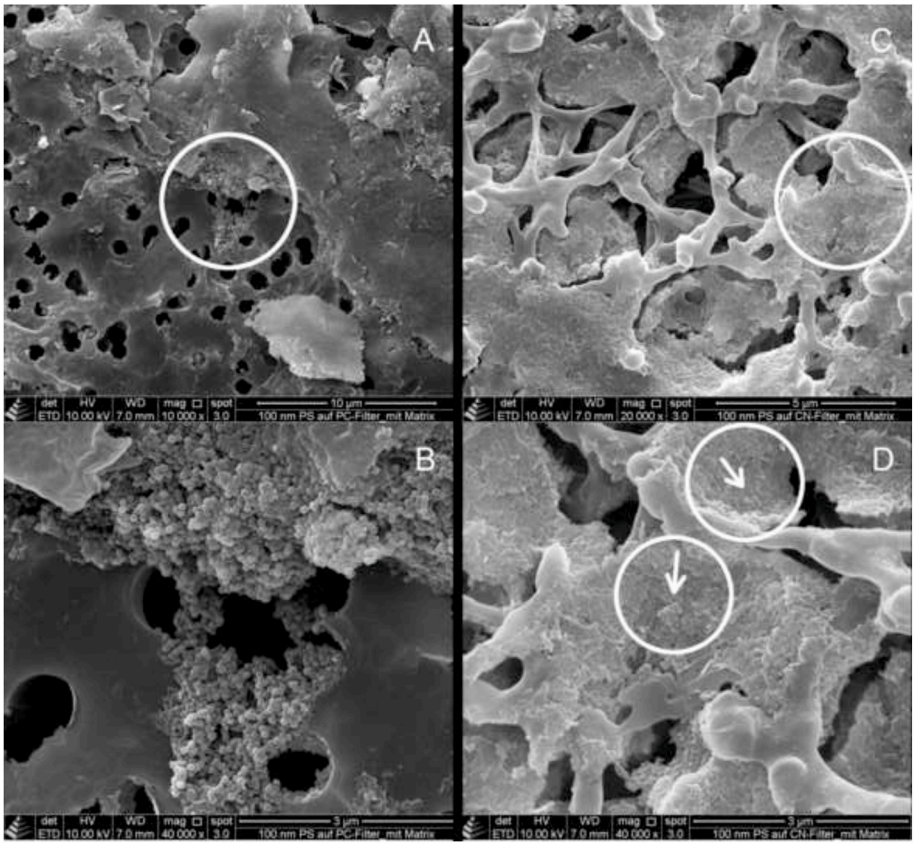

Fig. 2. Scanning electron images of the filter surface after extraction of nano-PS (Ø 100 nm, round shape) from spiked *P. virens*. The white circles in picture A and C indicate the zoom area. The arrows highlight nano-PS retained on the filter or on matrix residues. A & B – PC-filter; C & D – CN-filter.

commonly used plastic types (PlasticsEurope, 2018) in five out of 43 studies PAN was reported as a minor plastic component (<1%–12%) observed in aquatic organisms (Alomar et al., 2017; Baalkhuyur et al., 2018; Collard et al., 2018; Markic et al., 2018; McGoran, Clark, & Morritt, 2017; Mohsen et al., 2019). Other approaches such as sole enzymatic digestion must be considered in case of special interest in PAN.

Even though degradation of PVC was described at 40 °C (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017), this was not observed in this study, neither at 40 °C nor at 60 °C. However, the alkaline treatment was only applied for 4 h in this study, whereas Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al. (2017) applied alkaline treatment for 72 h–96 h. It is assumed under kinetic aspects that PVC degrades slowly, so it was not affected to a significant degree within 4 h of alkaline treatment.

3.2.2. Optional isolation of particles on small (Ø 25 mm) filters

For detection or identification methods based on imaging the filter surface for MP analysis such as FPA-FTIR (Olesen et al., 2017), the overall size of the filter surface determines the time and processing power required for MP analysis. Using filters with a smaller diameter consequently enables a quicker MP detection. Additionally, large glass fibre filters could not be transferred to the sample inserts for py-GC/MS analysis completely, so small filters (Ø ≤ 25 mm) had to be applied for py-GC/MS analysis. However, complete filtration of the sample using only one small filter was not achieved. Therefore, pre-filtration with PC-filters and subsequent rinsing off the particles onto small filters was necessary (Fig. 1, option B). SEM-images showed that PC filters had a smooth surface with pores of 1 µm diameter (Fig. 2). Matrix residues covered the filter surface partially. In contrast, the CN-filter consisted of a three-dimensional structure with matrix residues adhering to several layers of the structure. The three-dimensional structure (e.g., also of glass fibre filters) reduced particle loss during sample handling (such as transferring the filter with tweezers from the filtration apparatus into petri dishes or microscopic slides) as small particles were retained better on the filter. However, a smooth filter surface, such as of the PC-filter, enabled an almost quantitative removal of micron-sized particles from the filter (recovery ≥ 88%, c.f. Table 5) and particles could be detected more easily with optical methods (fluorescence microscopy, µ-Raman).

3.2.3. Optional post-filtration treatment for reducing matrix residues

Although oxidative treatment was not suitable for digesting the complete amount of sample material, matrix residues adhering to the filter surface were destroyed with 15% H_2O_2 solution (v/v) efficiently. Oxidative filter treatment reduced the background fluorescence of the sample thus simplified the detection of polymers with a weak fluorescence (e.g., PA6, PET). In addition, background noise in py-GC/MS analysis was also reduced, thus increasing the sensitivity for plastic pyrolysis products. Oxidative treatment was optional, though, as especially for fishes low in fat, such as *P. virens* or the soft tissue of molluscs

Table 5
Recovery of pre-stained PA12 from spiked *P. virens* using enzymatic-alkaline extraction with pre-filtration over PC-filters and detection with fluorescence microscopy (FITC-filter).

particle number		recovery	CV	corr. mass	requirements*	
spiking solution	sample				CV	recovery
17 ± 3 (CV = 17%)	15 ± 3	88%	18%	25 µg/kg	as low as possible	≥80% ≤110%
191 ± 13 (CV = 7%)	169 ± 23	89%	14%	282 µg/kg	≤23%	
1246 ± 180 (CV = 14%)	1380 ± 136	103%	13%	2.30 mg/kg	≤16%	

*According to Commission Decision 2002/657/EC.

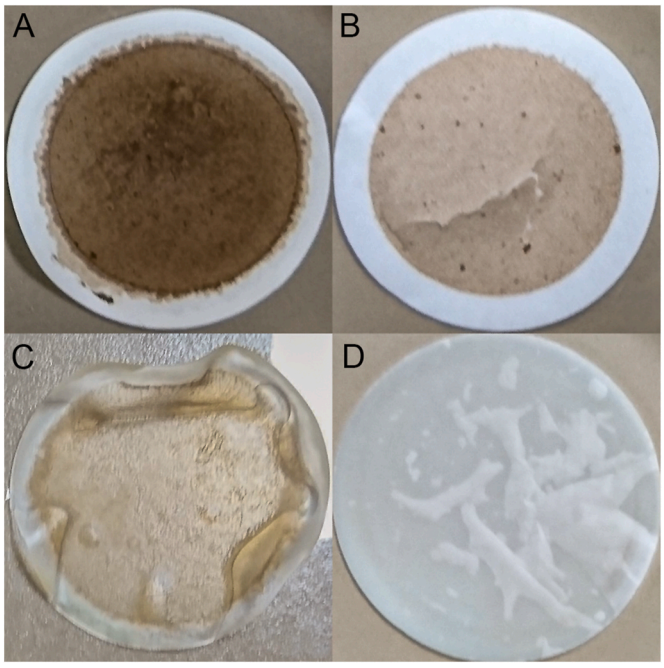

Fig. 3. Residues of digested fish matrix (10 g homogenised fillet of *C. harengus*) on different filters with a common diameter of 47 mm. A – 1.2 µm CN-filter, B – 1.2 µm glass fibre filter, C – 0.8 µm CA-filter, D – 1.0 µm PC-filter. All samples were digested with the optimised enzymatic-alkaline protocol (option A).

negligible amounts of matrix residues remained on the filter. Digestion with H_2O_2 was described as being damaging to some polymer types (e.g., PA, PE, PET, PP), with damages including changes in colour and shape (Nuelle et al., 2014) and degradation of the particles (Ali Karami, Abolfazl Golieskardi, Cheng Keong Choo et al., 2017). Regarding the findings of the present study, H_2O_2 should not be applied with concentrations exceeding 15 wt-% or above room temperature in order to avoid polymer degradation (especially PA6 and PU) confirming the proposals for MP extraction by Bessa et al. (2019). In case of samples with a high fat content, saponification might be incomplete after alkaline hydrolysis at 40 °C for 4 h. Consequently, residual lipids might adhere to the filter. Rinsing with organic solvents removed these residues sufficiently. Regarding dissolution of synthetic polymers in organic solvents, ethanol, and isopropanol were found to have negligible effects on all polymer types tested in this study.

3.2.4. Evaluation of the filter material regarding particle and residue adherence

Apart from incomplete sample digestion, another reason for filter clogging might be based on the choice of filter material and pore size. Löder et al. (2017) successfully applied 10 µm mesh stainless-steel filters after enzymatic digestion of biota. In contrast, filtration with 8 µm CN-filters was not feasible for the same protocol in the present study. For example, CN membranes have a strong protein adsorption due to the negative electric charge of their surface (Přistoupil, Kramlova, & Štěrbíková, 1969) and in the present study, they were significantly more susceptible to clogging than glass fibre filters, or PC-filters, thus, having larger amounts of matrix residues adhering to the filter surface (Fig. 3). Preliminary tests indicated that silver filters had the least adherence of matrix residues compared to other filter materials. However, their application was not tested for other protocols than the optimised protocol due to their high costs (approximately 100× or 20× more expensive than glass fibre or PC-filters, respectively). Sufficient purification of these filters for reuse them was not achieved, limiting their use for routine analysis.

Due to their large relative surface area, NP might interact differently

with the filter material or matrix residues than MP. Samples spiked with 100 nm PS-nanobeads were used to test whether NP can be separated from a MP-containing sample by filtration. In general, NP do not even pass filters with pores in the micrometre size range quantitatively, as they adsorb and agglomerate at the edges of the pores or in the pores. The adherence of more than 80% of the administered particles to filters with a significantly larger pore size (10 μm) than the particle diameter (Ø 500 nm) was already observed for PS-NP by Zierdt (1979). The nano-PS applied in this study showed similar behaviour (Fig. 2). Further studies are required regarding the potential presence of NP in seafood and suitable methods to separate NP from matrix residues other than filtration.

3.3. Application of the sample preparation protocol on seafood samples

3.3.1. Evaluation of digestion efficiency for different matrices

Digestion efficiency for most of the analysed fish, mollusc, and crustacean samples was greater than 98.5% (Figure S6). Digestion efficiencies exceeding 99% were achieved when digesting 50 g–100 g sample matrix, however, more than one filter had to be used per sample to avoid filter clogging. Residual fat on the filter was observed for fish fillets with a high fat content (>10%; e.g., *C. harengus*), indicating incomplete saponification. Residual fat of 50 g–100 g sample matrix led to clogging of the filter, so alkaline hydrolysis was prolonged to 16 h. Differences in fat content of the samples was not the only factor influencing digestion efficiency. Crustacean species of the *Peneaidae* family for example often required significantly more time for complete filtration than other crustaceans regardless of their fat content. It is therefore assumed that the efficacy of pepsin digestion depended on the collagen composition and configuration (Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011), e.g., due to differences in intermolecular crosslinks (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). This is supported by the findings described by Sriket, Benjakul, Visessanguan, and Kijroongrojana (2007), who reported an amount of 0.1% insoluble collagen in *P. monodon* and of 0.8% *L. vannamei*. In the present study, filtration of *L. vannamei* was only feasible when applying 60 °C for the alkaline hydrolysis and still digestion efficiency was considerably lower (98.29 ± 0.19) as compared to *P. monodon* (99.86 ± 0.04) or other crustacean species (Figure S6).

3.3.2. Preliminary in-house validation

The recovery of the MP (Nile Red-dyed PA12-particles, Ø10 μm – 50 μm) spiked to *P. virens* reached 88%–103% with a coefficient of variation (CV) ranging from 13% to 18% (Table 5). As only minor differences were observed in the recovery of different polymer types prepared without sample matrix (c.f. Table 4), assessment of the protocol's suitability with only one polymer type was assumed to be acceptable. However, for validating qualitative and quantitative MP analysis, further studies are required considering the influence of different polymer types, their shape, colour and size and aging on the recovery.

For estimating the suitability of the optimised sample preparation protocol for extracting MP present in the edible part of seafood regarding MP recovery, criteria specified in the European Commission Decision 2002/657/EC article 3 (b) in conjunction with annex Part 2 were considered (Table 5). As the criteria are supposed to be applied for a mass fraction, the mass fraction of the respective particle number in the sample was estimated based on their volume and density. The particles used for spiking had roughly a spherical shape, a median diameter of 25 μm and a density of approximately 1.02 g/cm^3 according to the manufacturer data. The corresponding mass (corr. mass) is listed in Table 5. The recovery data comply with the recommendations of the Commission Decision 2002/657/EC indicating suitability of the optimised sample preparation protocol.

3.3.3. Polymer identification

The polymer identity of PA12 extracted from the matrix *P. virens* was confirmed by Raman spectroscopy (data not shown) as well as py-GC/MS.

Individual PA12 particles extracted from matrix were identified by μ-Raman with spectra match of approximately 80%.Virgin PA12 particles were identified with spectra matches of 82–83% after a similar measurement time. The low deviation of particles extracted from matrix in comparison to virgin, untreated PA12-polymer indicated sufficient matrix removal for the identification of synthetic polymers with microspectroscopic methods. The detection of particles with μ-Raman on glass fibre filters was difficult though, as the surface of the filter was rough, and the particles tended to be hidden between the fibres. In contrast, particles could easily be detected on Al_2O_3 filters. However, Al_2O_3 filters used in this study had an outer PP-ring resulting in significant PP-contamination (>1000 fluorescent particles in blank samples, identity confirmed with py-GC/MS) so their application was not further considered.

Natural as well as synthetic organic polymers generate significant amounts of pyrolysis products. Larger amounts of matrix residues consequently hinder polymer identification, even though they differ in characteristic pyrolysis products. All polymer types spiked to the sample were identified based on characteristic pyrolysis products with py-GC/MS (Table S1), thus confirming sufficient matrix removal for qualitative analysis with thermoanalytical methods. PAN, PTFE, and PVC were not analysed with py-GC/MS, as the first one was decomposed during alkaline hydrolysis and the two latter were not suitable for py-GC/MS analysis. PVC and PTFE form hydrogen halides during pyrolysis. Both acids led to a significant damage of the silica based column (Rood, 1996). Consequently, for analysis of PVC and PTFE, spectroscopic methods seem to be advantageous.

However, the ratio of the pyrolysis products of MP extracted from seafood and pyrolysed in the presence of glass fibre filters did not match the ratio of pyrolysis products observed with virgin plastic particles pyrolysed without glass fibre filters. This effect also occurred, when virgin polymer (tested with PET) was pyrolysed in the presence of glass fibre directly. This might be based on the large surface of the glass fibres which could catalyse pyrolytic reactions or to which pyrolysis products could adsorb to. Further studies are required regarding quantitative analysis by py-GC/MS in the presence of inorganic matrix such as glass fibres.

4. Conclusions

Efficient and validated extraction protocols suitable for routine analysis are required for the analysis of microplastic particles present in seafood enabling the assessment of the dietary intake of these particles in humans. An enzymatic-alkaline approach based on protocols applied for MP present in aquatic biota (mostly digestive tracts) was optimised. Besides being suitable for a broad variety of seafood species and having a negligible impact on most plastic polymers' integrity the developed protocol is simple, fast to conduct and cost efficient. The results of the preliminary in-house validation suggested the suitability of the optimised protocol for the isolation of MP present in the edible part of seafood. Nevertheless, further validation is required including quantitative analysis of microplastic particles present in the edible part of seafood. Additionally, further development on sample preparation protocols for the analysis of NP present in seafood is required.

CRediT authorship contribution statement

Julia Süssmann: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Torsten Krause:** Conceptualization, Methodology, Resources, Data curation, Writing - review & editing. **Dierk Martin:** Methodology, Resources, Data curation, Writing - review & editing. **Elke Walz:** Writing - review & editing. **Ralf Greiner:** Writing - review & editing. **Sascha Rohn:** Writing - review & editing,

Supervision. **Elke Kerstin Fischer:** Methodology, Resources, Data curation, Writing - review & editing, Supervision. **Jan Fritsche:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.107969.

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