Towards reliable data: validation of a machine learning-based approach for microplastics analysis in marine organisms using Nile red staining.

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Abstract

Microplastic research faces challenges due to costly, time-consuming, and error-prone analysis techniques. Additionally, the variability in data quality across studies limits their comparability. This study addresses the critical need for reliable and cost-effective MP analysis methods through validation of a semi-automated workflow, where environmentally relevant MP were spiked into and recovered from marine fish gastrointestinal tracts (GITs) and blue mussel tissue, using Nile red staining and machine learning automated analysis of different polymers. Parameters validated include trueness, precision, uncertainty, limit of quantification, specificity, sensitivity, selectivity, and method robustness. For fish GITs a 95 ± 9% recovery rate was achieved, and 87 ± 11% for mussels. Polymer identification accuracies were 76 ± 8% for fish GITs and 80 ± 13% for mussels. Polyethylene terephthalate fragments showed more variability with lower accuracies. The proposed validation parameters offer a step toward quality management guidelines, as such aiding future researchers and fostering cross-study comparability.

1. Introduction

With worldwide plastics production nearly reaching 390.7 million tonnes in 2021, environmental pollution from plastic waste is a major concern (PlasticsEurope, 2022). Due to abundant use and subsequent disposal, it is estimated that around 3% of the plastic makes its way into the marine environment through land-based (80%) and marine sources (20%) (Jambeck et al., 2015; Li et al., 2016). Microplastics (MP) are plastic particles between 1 µm and 5 mm in size (Hartmann et al., 2019; Arthur et al., 2009). They can be divided into MP that enter the environment directly, e.g. plastic pellets from industrial manufacturing or fibres from synthetic textiles, or indirectly through progressive deterioration, e.g. through exposure to UV radiation from sunlight, or wave abrasion (Barnes et al. 2009; Lambert et al. 2014). Numerous studies on the occurrence and effects of MP in the marine environment have been carried out in recent years. Studies have shown that a variety of marine species ingest MP, including mussels, crustaceans and fish, as well as

seabirds, potentially leading to adverse effects at individual level (Van Cauwenberghe et al., 2015; Wang et al., 2020; D'Costa et al., 2022; Davison et al., 2011; Caldwell et al., 2022; Foekema et al., 2013; Lusher et al., 2013) or at population level (Everaert et al., 2022). Most of these laboratory studies only showed potential effects to occur at concentrations that are orders of magnitude above the recorded environmental MP concentrations (Everaert et al. 2020; Koelmans et al. 2022). Moreover, a disparity exists between MP shapes and polymer types used in effect studies and those commonly found in environmental samples. For instance, De Ruijter et al., 2020 demonstrated that while only 6.5% of MP detected in water and sediment are spherical in shape, 58.1% of the 105 reviewed effect studies tested this shape. Similarly, fibres, which are among the most prevalently found shapes, were only tested within 8.1% of the effect studies. Most studies were also limited to the commonly found polymers PS and PE, while the incorporation of PP, another frequently encountered polymer, was restricted to 5.5% of all studies. Due to this considerable gap between MP concentrations and composition used in laboratory studies vs. those encountered in natural environments, and consequently the lack of environmental relevance, current findings on MP pollution and its impacts are rather inconclusive (De Ruijter et al., 2020).

Accurately measuring MP concentrations in the natural environment and marine organisms is key to assess the associated risks. A multitude of MP analysis techniques have been developed, ranging from light microscopy to spectroscopy and thermal degradation techniques, to meet the monitoring and research needs (Mariano et al., 2021). Many of these methods are considered costly, time-consuming, tedious (Cowger et al., 2020), and prone to human error (LV et al., 2021). As a result, the development of trustworthy, high-throughput, and cost-effective MP analysis methods is recognised as a priority activity (ICES, 2021). Recent advancements in MP identification have introduced innovative and non-destructive approaches that focus on the optical properties of MP combined with machine learning techniques. Some of these methods integrate multi-dimensional features such as polarisation states, holographic images,

and texture, enabling efficient and precise discrimination of MP. By utilising machine learning algorithms, the methods can accurately distinguish between different polymer types and natural materials, offering a rapid and reliable MP analysis of environmental samples (Valentino et al., 2022; Zhu et al., 2024). Similarly, the fluorescence readout of Nile red (NR)-stained particles photographed under multiple filters combined with machine learning techniques has proven promising for the cost- and time-efficient characteristation of MP (Meyers et al., 2022).

The development and optimisation of cost-and time-efficient analysis methods is challenging, as these techniques should, compared to the state-of-the-art benchmarking methods, combine savings in time and cost whilst reaching a similar or at least acceptable level of accuracy and precision. Key criteria to assess this relate to a reliable detectability of a large variety of polymers, and a low contamination probability. To improve effectiveness in terms of both time and cost, efforts have been undertaken to automate MP analysis. Automation increases speed and reduces labour, cost and human bias (Song et al., 2021), but standardised and trustworthy approaches need to be developed (Hermsen et al., 2018). Progress is being made towards harmonisation (e.g. Frias et al., 2018; Gábor et al., 2022), although the wide variety of existing analysis methods nowadays, even when using similar instrumentation, still hampers the comparability of obtained data, because of the inevitable variation in resolution, focus, and most importantly, quality (Hermsen et al., 2018). As with analytical methods, method validation is one approach to ensure the quality control of MP research (Konieczka, 2007). Analytical method validation is a means to demonstrate that the method is suitable for its intended purpose, and that it complies with the applicable standards. It allows to ascertain the reliability of a developed method as well as its ability to produce accurate findings (ISO/IEC 17025:2017). However, as of today, method validation is not established for MP analysis. Despite MP being an important emerging global contaminant in a large variety of environmental matrices, universal MP analysis guidelines are only recently being developed, e.g. for

drinking water and water with a low content of suspended solids (ISO 16094-2). For marine matrices, universal guidelines are non-existent.

To respond to the need for a complete, cost-effective yet reliable MP analysis workflow, as part of the multidisciplinary ANDROMEDA project, we performed a validation of a semi-automated MP analysis method relying on the quantification of fluorescence spectra emitted by NR-stained particles under multiple microscope filters (Meyers et al., 2024a). By using MP of environmental relevance in terms of concentration, composition, size, shape, and degradation level, we overcome the huge discrepancy that often exists between laboratory research and the real environment. By validating the MP analysis method, we provide potential end-users with performance data, and transparently report on the method's limitations and uncertainties. As such, we enable an informed choice on the implementation of the developed method. The validation parameters proposed in this study are a next step towards quality management guidelines to help future researchers in ascertaining the reliability of the methods they are using. This in turn contributes to the harmonisation and quality of published data, to enable cross-study comparability, and consequently the advancement of MP research.

2. Materials & Methods

The study employed a comprehensive five-step protocol for method validation. Firstly, both environmentally relevant MP in terms of composition, size, shape, and degradation level; and marine biological tissues (fish GITs and blue mussel tissue) suitable for MP biomonitoring were selected. Pristine MP as well as MP weathered in the natural environment under semi-controlled conditions were used. Secondly, environmentally meaningful concentrations of either the pristine or weathered MP were added to biota samples, under both standard conditions and varying conditions. In a third step, MP were

extracted and stained with NR. The fourth step involved acquiring fluorescent images of the stained MP with a fluorescence stereomicroscope under different filters, and determining the number of recovered MP in total as well as their respective polymer types using predictive machine learning models. Model accuracy was also validated through µ-FTIR analysis. The fifth and final step focused on determining and evaluating different validation parameters based on the recovery results, to ensure the accuracy and reliability of the developed workflow.

2.1. Preparation of spiked samples

Five of the most abundantly produced plastic polymers, which are commonly found in marine environmental samples (Geyer et al., 2017; Suaria et al., 2020) were chosen to spike the biota samples. Aside from their omnipresence and prevalence in the environment, which was utilised as the first criterion, a wide variety of densities was covered (Frias et al., 2018). Cryomilled, heterogeneously shaped polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) fragments were provided by Carat GmbH and used for spiking the biota samples. Furthermore, as one of the main polymers used in textile production worldwide, also polyacrylonitrile (PAN) fibres were used to spike the biota samples (Barrows et al., 2018; Hurley et al., 1963). In terms of terminology, we use 'fragments' when referring to fragmented MP, while 'particles' encompass both fragments and fibres. To obtain spiked samples representative of environmental samples, 20-35 particles were added per biota sample, belonging to two size classes, where size was determined based on the maximum Feret diameter for fragments, and on length for fibres (Schneider et al., 2012). Selected sizes for analysis in fish GITs were 500-1000 µm (here called "larger-sized MP") and 100-300 µm (here called "smaller-sized MP") for fragments and 200-1,500 µm for fibres.

For blue mussels, spiking included the larger-sized MP and additionally recovery tests with two types of weathered fragments. To produce these fragments, a mix of the aforementioned polymers (500-1,000 µm) were placed into stainless steel tubes (316 mesh) (Inoxia Ltd, UK), which were placed in stainless steel containers (Fig. S1). The containers were deployed in subsurface, coastal waters in the Norwegian Sea (Tromsø, Norway; 69.642730, 18.950389) in February 2021 (Fig. S2) and in deep-sea waters (2380 m depth) (Fig. S3 and S4) of the Mediterranean Sea (off the coast of Marseille, France; 42.807683, 6.043867) in April 2021. The stainless steel containers placed in surface waters during the exposure at sea were manually cleaned at regular intervals to remove bivalves and other attached organisms. The containers were immersed in the water for 12 months. Once retrieved, the stainless steel tubes were removed and placed under a fume hood to dry. Next, the MP were transferred into burnt (450 °C, 6 h) glass vials and shipped to the laboratory. The Attenuated Total Reflectance (ATR)-spectra of each polymer acquired in its pristine, surface water-weathered and deep-sea water-weathered form are freely available in the open-access repository Marine Data Archive (Meyers et al., 2024b).

Two types of biological tissues were considered as matrix, i.e. blue mussel tissue and marine fish GITs. For blue mussel *Mytilus edulis*, whole soft tissue of a) commercial and non-commercial organisms, b) depurated and c) non-depurated mussels were used, while for GITs of fish (a) Common dab - *Limanda limanda*, b) Whiting - *Merlangius merlangus*, c) Plaice - *Pleuronectes platessa*, and d) European flounder - *Platichthys flesus*) were selected. Commercial Zeeland Jumbo mussels were bought in a Belgian supermarket. Non-commercial mussels were collected in a stainless steel container at a breakwater in Ostend, Belgium (51°14'30.0"N 2°56'00.7"E). Fish were caught in spring 2022 during a research campaign in the Belgian Part of the North Sea (BPNS) with the research vessel Belgica. They were stored in freezers (-20°C) on board, followed by lab freezers, and dissected in the lab between March and June 2022. The dissection of fish GITs was performed in the lab using a developed protocol (De witte et al., 2021b), where

metadata such as fish species, full weight, GI weight, length, and sex were recorded (Table S1). Following this, samples were stored in closed metal containers and stored back in the freezer until further analysis.

The validation procedure was performed under both standard and varying conditions, where a single GIT of common dab and 20 g of commercial mussels were considered standard conditions, and all other fish species as well as non-commercial mussels and deviating mussel sample masses were considered varying conditions. In the latter case, the robustness of the method was tested as an extra parameter, i.e. the reliability of the workflow to remain unaffected by deliberate variations in method parameters. For each biota type, under standard conditions, an analysis batch consisted of three independent spiked replicates analysed in one week. Within each batch, three procedural blanks and three unspiked matrix samples were also prepared and analysed, to account for laboratory contamination and matrix contamination, respectively (cfr. Table S2). Under standard conditions, for mussels, three batches with pristine MP were analysed, as well as two batches with weathered MP. For fish GITs, five batches of pristine MP were analysed. During varying conditions, three batches were analysed for fish GITs. For mussels, two batches were considered, which consisted of four and six replicates.

The procedural blanks were acquired to enable quantification and identification of any background contamination arising from the laboratory environment, equipment, reagents, and handling procedures, after extensive contamination measures were put in place (cfr. '2.3. Validation procedure'). To do so, per matrix type, for each batch, three samples that did not contain the matrix were brought through the entire processing procedure and were analysed in the same manner as the spiked samples. To quantify this type of contamination, the limit of quantification (LOQ) was determined for fragments and fibres separately, which is the lowest MP concentration that can be quantified with reasonable repeatability and accuracy using this method. To be able to account for matrix contamination, i.e. plastics already present in the

matrices before spiking, the MP content of three unspiked matrix samples per matrix batch was analysed (cfr. '2.3 Validation procedure' for further details on the considered validation parameters).

To spike MP into the samples, a portion (<0.5 mg) of each reference material and size class was selected and transferred to separate hourglasses. Next, partly based on the method employed by Ourgaud et al., 2022, a stainless steel dissection needle point was dipped in Milli-Q water to ensure particles adhered to it, before being used to select particles one by one using a stereomicroscope, and to transfer the particles to a small 10 mL-glass beaker containing 10 mL of Milli-Q water. The proper transfer of all particles to the respective beakers was verified under a stereomicroscope to ensure no particle went missing. After adding the content of each beaker containing Milli-Q water and reference MP to the appropriate biota sample, the small beaker was thoroughly rinsed with a total of 40 mL Milli-Q water, which was then transferred to the sample as well.

2.2. Processing and analysing of spiked samples

Sample processing was conducted using a double digestion step (cfr. The Andromeda protocol bundle for more details: De Witte et al., 2024). First, potassium hydroxide (KOH) was used to digest animal tissue in the spiked sample, where 10 mL of 10% KOH per 1 g of wet tissue weight was added (1:10 w/v). For the mussel samples, mussels were pooled so that 20 g wet weight per sample was obtained. After adding the spiked MP in 10 mL of Milli-Q water, a KOH solution was added so that a final volume of 200 mL 10% KOH was obtained. For fish GITs, one GIT per sample was used. For both biota types, stirring rods were then added, after which samples were covered with aluminium foil and left to digest for 48 h at 50 °C on a hot stirring plate (150 rpm). Following this, samples were vacuum filtered onto a 105 mm stainless filter (20 µm mesh), constructed using a metal wire mesh made of stainless steel (product number 1.4401, Haver & Boecker) and two stainless steel rings, which was placed above a filtration apparatus (United Scientific

Supplies). When organic material was not entirely digested, the filter was sonicated to detach material adhering to the filter. Next, the digested sample was transferred to a new beaker, and 1 mL of hydrogen peroxide (H_2O_2) was added per mL of Milli-Q water needed for the transfer (1:1 v/v). Samples were again covered with aluminium foil and left to digest on a stirring plate for 24h in the same manner as for the first KOH digestion step. In case sediment was present in the samples, e.g. GITs of some bottom feeding fish species and non-commercial, non-diluted mussels, a density separation step was performed prior to filtration. In the absence of sediment, samples were directly filtered over separate PTFE filters (47 mm diameter, 10 µm pore size, Millipore Ltd.) using a manifold filtration system (3 + 3 workstations, Merck Millipore). Filters and particles present were subsequently stained with 1 mL of a NR solution (10 µg mL−1 in acetone), dosed with a glass pipette, while filters laid on the filter head. After 15 min, filters were rinsed with Milli-Q water and vacuum filtered to discard any remaining liquid. Next, filters were carefully transferred onto labelled glass slides, placed within covered petri dishes, and left to dry in a dark environment for min 24-72 h. Lastly, image series of each complete PTFE filter with their respective MP were taken under a blue, green, and UV filters (Fig. 1). Image acquisition settings of the used Leica M205 FCA fluorescence stereomicroscope are described in Table S3.

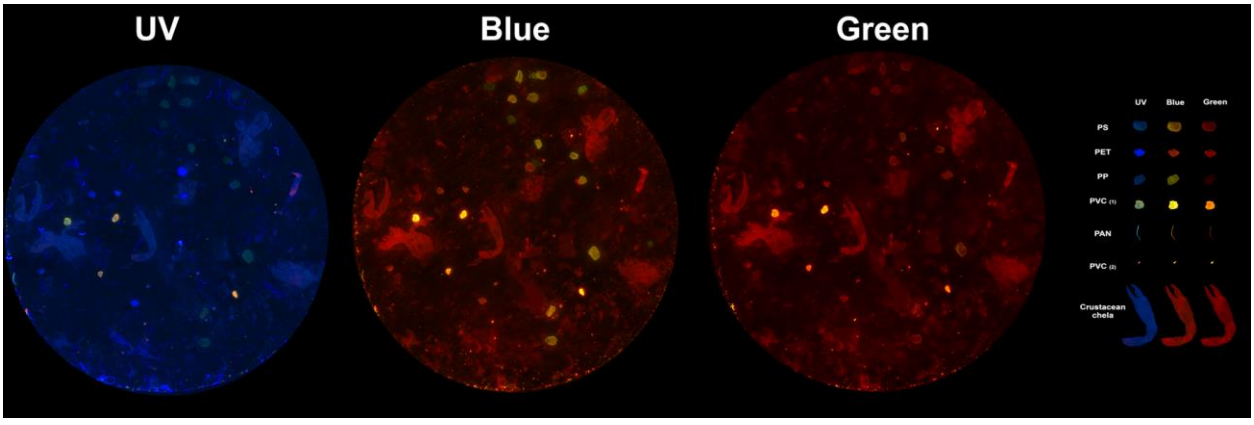

Fig. 1. Image series of recovered microplastics. Image series of recovered microplastics that were spiked in fish gastrointestinal tracts, and photographed on a PTFE filter under a UV, blue and green filter using a fluorescence stereomicroscope. The

different microscope filters are used by the developed machine learning models to detect and identify microplastics based on their respective fluorescent colouration.

Microplastic recovery and polymer identification was performed in two ways, i.e. visually with µ-FTIR validation, and using machine learning (ML) models. Sample processing was performed by two trained lab technicians who were unaware of the spiked MP content per sample, while visual detection and identification of MP was done by a trained researcher, based on the distinct fluorescent colouration of particles present on the acquired image series (Fig. 1). Additional µ-FTIR analyses of subsamples of particles were performed to verify the polymer types of the particles (See Methods Supplement. µ-FTIR analysis). A two-step semi-automated MP analysis was performed to analyse all fragments using two random forest models. The Plastic Detection Model (PDM) can discriminate plastic from non-plastic fragments, while the Polymer Identification Model (PIM) can distinguish between four polymer type categories: PE/PP, PS, PET and PVC, where classification is performed based on the Nile red-based fluorescent colouration of particles. The PDM dataset included RGB data from 420 plastic and 420 organic particles. Similarly, the PIM dataset contained RGB data from 135 particles per polymer (PS, PET, and PVC), along with 67 PE and 68 PP particles pooled within one class due to their similarity in fluorescence. For both models, an 80/20 split for training and testing the models was used, respectively. For the PDM, a percentage of correctly classified instances (CCI %) of 100 ± 0% was obtained for both plastics and for organic material, while for the PIM an overall CCI % of 99 ± 0% was obtained (PE/PP: 99 ± 0%; PET: 99 ± 0%; PS: 98 ± 1%; PVC: 99 ± 0%). For comprehensive information on model construction and application and access to the training datasets, see Meyers et al., 2024ac.

A subset of randomly selected fragments from five image series (n = 30) which were recognised as particles by the ImageJ software (Schneider et al., 2012), and were identified as being of organic origin

through μ-FTIR analysis, were identified by the PDM to verify the rate of false positives. To do so, RGB-statistics datasets were created of the fluorescent colouration of the dyed fragments present on the filters. Using the developed random forest models, these datasets were then ultimately used to predict the identity of each recovered fragment (plastic/non-plastic), and the polymer type (PE-PP/PET/PS/PVC) in case the particle was of plastic origin. Lastly, the difference in recovery between smaller-sized and larger-sized MP in fish GITs was tested for all fragment polymer types. As conditions of normality, evaluated by a Q-Q plot of the residuals (Fig. S5), were not fulfilled, an aligned rank transformation ANOVA (ART ANOVA) (Wobbrock et al., 2011) was performed, as a non-parametric approach to the factorial ANOVA. A significance level of $\alpha = 0.05$ was considered.

2.3. Validation procedure

As background control measures to avoid MP contamination, the use of plastic materials was avoided for sample collection, dissection and analysis, plastic/rubber material present in the sampling and dissection area as well as its colour was registered, a 100 % cotton lab coat and cotton trousers were worn while wearing of synthetic clothes underneath was avoided as much as possible, maximum coverage of sample storage containers was ensured, dissection material and glassware was pre-cleaned before use with Milli-Q water and soap, and all dissections and sample processing were performed in a laminar flow hood, a recommended controlled air environment for MP analysis (Noonan et al., 2023). Laboratory contamination was quantified and accounted for by procedural blanks. Matrix background was determined by analysis of unspiked matrix samples.

The validation of the MP analysis method further enclosed the determination of the **trueness (bias b) (1)** based on repeated analyses, as well as the **precision (2)** including **repeatability** and **within-laboratory reproducibility**, determined per batch analysis and for the repeated analysis, respectively. Acceptable

deviation limits depend on the measurements considered, and since validations are uncommon in MP research, specific limits are not yet established. A deviation limit of ±20% from the spiked value was set for trueness and precision, based on limits used in earlier analytical validations (e.g. Critchley et al., 1999; Aronhime et al., 2014; Vashist et al., 2018). Accuracy in this study was defined and evaluated as a combination of trueness, which refers to the closeness of the measured value to the true value (mean recovered number of MP vs. the spiked number of MP), and precision, which indicates the reproducibility or consistency of the method (ISO 5725-11). Trueness considers the systematic error of a method, caused by e.g. improper equipment functioning. This type of error is consistent in terms of value and proportion and diverges from the true value. Precision considers the random error of a method, caused by unpredictable changes in the environment of the experiment, and arises because of variability found within repeated measurements. The greater the precision, the more predictable the measurement becomes. This type of error clusters around the true value but is not consistent. Random errors are unavoidable but can be decreased through repeated measurements (Stanford & Vardeman, 1994).

Precision was determined as a combination of repeatability and within-laboratory reproducibility. Repeatability here demonstrates precision under the same operating circumstances over a short period of time (all replicates within one analysed batch), and is expressed as the variation coefficient CVr. Within-laboratory reproducibility represents the precision measured within the same laboratory over a longer period and takes into account changes in time, instruments, reagent batches, the matrix, etc., and is expressed as the within-lab reproducibility variation coefficient WLR CVR. In this study, the repeatability and within-lab reproducibility were considered as a measure of random error. The combined evaluation of both trueness and precision ensures that the results obtained with the Nile red-based method are both correct (trueness) and consistent (precision), i.e. they have a high accuracy. The **measurement uncertainty (3)** was also taken into account, including a coverage factor of k = 2 and limit = 50%, as is often

done for environmental monitoring of contaminants (Ballesta et al., 2014; OSPAR 2013; De Witte et al., 2019). This parameter quantifies the margin of doubt that exists for the results of any performed measurement. Measurement uncertainty was determined based on the recovered MP from the processed samples (see Table S4 for all parameter specifications, formulas, and set limits). Furthermore, the **limit of quantification (LOQ) (4)** was determined, based on three procedural blanks per batch, and defined as three times the obtained SD + the average contamination (De Witte et al., 2014). This limit determines the lowest MP concentration that can be quantified with reasonable repeatability and accuracy using this method. Additionally, background MP fragments already present in the unspiked matrix samples (i.e. fish and blue mussels acquired at multiple locations in the BPNS (Table S1 for geographical coordinates) were quantified. Finally, the **specificity, sensitivity and selectivity (5)** of the predictive models were determined (Table S4).

The validation procedure was performed both under standard and varying conditions, where a single GIT of common dab and 20 g of commercial mussels were considered standard conditions and all other fish species as well as non-commercial mussels and deviating mussel sample masses were considered varying conditions. In the latter case, the **robustness (6)** of the method was tested as an extra parameter, i.e. the reliability of the workflow to remain unaffected by deliberate variations in method parameters.

3. Results

3.1. Accuracy, precision, trueness, measurement uncertainty, and robustness

After visual inspection of the fluorescent images, an overall accuracy of 95 ± 9 % for fish GITs, and 87 ± 11 % for mussels was obtained under standard conditions (commercial mussels/GITs of common dab) (Fig. 2) (Table S5 and S6 for more details). When considering just the weathered fragments in the mussel

329 samples, recovery rates were 98 ± 3% and 89 ± 11% for surface- and deep sea-weathered fragments,
330 respectively (Fig. 2). Recovery rate for the smaller-sized MP in the fish GITs was 98 ± 22%. Overall,
331 fragments showed good recoveries: 95 ± 9% and 86 ± 12 % for fish GITs and mussels, respectively.
332 However, PET fragments showed varying recovery rates in the larger size class over the different batches
333 (33 ± 58% - 100 ± 0%), and a recovery > 100% in a limited number of samples (e.g. 133 ± 153% for smaller-
334 sized fragments in batch 4, see Table S5). The latter phenomenon of recovery efficiencies > 100% was also
335 observed for PVC in certain samples (Fig. 2).
336
337 Polymer type, MP size and their interaction significantly affected fragment recovery within fish GITs (Table
338 S7). Recoveries of PET, PVC and PS were higher for smaller-sized fragments than for larger-sized
339 fragments, while for PP recovery was significantly higher for the larger-sized fragments (Fig. S5; Table S8
340 for post-hoc comparisons). For fish GITs, **precision** and **trueness** calculated over all batches was 10% and
341 95%, respectively, while for mussels this was 12% and 88%. All values were within the set 20% limit (Table
342 S4). **Measurement uncertainty** was 24% and 39% for fish GITs and mussels respectively, which is within
343 the set 50% limit (k = 2). Microplastic recovery values under varying conditions for both biota types (i.e.
344 method robustness) were similar and within the set limit (within [75.78% - 115%] for fish GITs and within
345 [64% - 111%] for mussels – calculation details see Table S4), which indicates that reproducibility and bias
346 are independent of sample type (overall recovery of 95 ± 9% and 94 ± 7 % for fish GITs, and 89 ± 19% and
347 98 ± 12% for mussels, under standard and varying conditions, respectively) (Table S9 and S10).
348
349 3.2. Limit of quantification
350 A mean contamination rate of 0.08 ± 0.41 for fragments and 0.29 ± 0.55 for fibres was identified in the
351 procedural blanks for the MP analysis protocol for fish GITs (n=24). For the MP protocol in mussels, the
352 mean MP contamination rate was 0.17 ± 0.38 (n = 21) for both fragments and fibres. This resulted in a

LOQ of 1.3 fragments and 2.1 fibres in fish GITs. In mussels, the obtained LOQ was 1.4 MP for both fragments and fibres. Hence, MP concentrations down to 2 fragments or 3 fibres can be reliably quantified in fish GITs using this method. Similarly, for mussels, an accurate MP quantification can be done from a concentration of 2 fragments/fibres onwards.

3.3. Environmental microplastic contamination in unspiked matrix samples

In both mussels and fish GITs, sampled at multiple locations in the BPNS (fish GITs/non-commercial mussels), background MP fragments already present in the unspiked matrix samples were notably limited: 1.05 ± 1.24 and 0.67 ± 0.89 fragments in fish GITs (n = 24 GITs) and mussels (n = 21 samples), respectively. Similarly, 2.38 ± 2.27 and 0.67 ± 0.89 fibres were found in fish GITs and mussels. Moreover, the spiked PAN fibres were bright fluorescent orange in colour, and could therefore easily be distinguished from fibres already present in the matrix through a stereomicroscope in brightfield modus. The fibres in the matrices could therefore easily be excluded for further analysis.

3.4. Automated microplastic detection and identification

All visually detected fragments were also detected using the automated method, i.e. **sensitivity** was high. The correct identification of MP fragments as plastics in fish GITs was 100 ± 0% under both standard and varying conditions, and 98 ± 4% and 99 ± 1% under standard and varying conditions in mussels, respectively (Tables S11-12). For randomly selected organic material particles **specificity** was 81 ± 16. For the automated polymer identification in fish GITs, i.e. the **selectivity** of the method, 76 ± 8% and 82 ± 14% of all spiked fragments were correctly identified under normal and varying conditions, respectively, as well as 80 ± 13% and 81 ± 10% of fragments spiked in mussels. When considering only the weathered plastics, 89 ± 19% of surface-weathered plastics were correctly classified as plastics, and 82 ± 16% of these polymers were correctly identified. Similarly, 100 ± 0% of deep sea-weathered plastics were accurately

classified, of which the correct polymer type was identified correctly in 77 ± 4% of the cases. The obtained accuracies of these three parameters were all within the set error limit of 30% (Table S4).

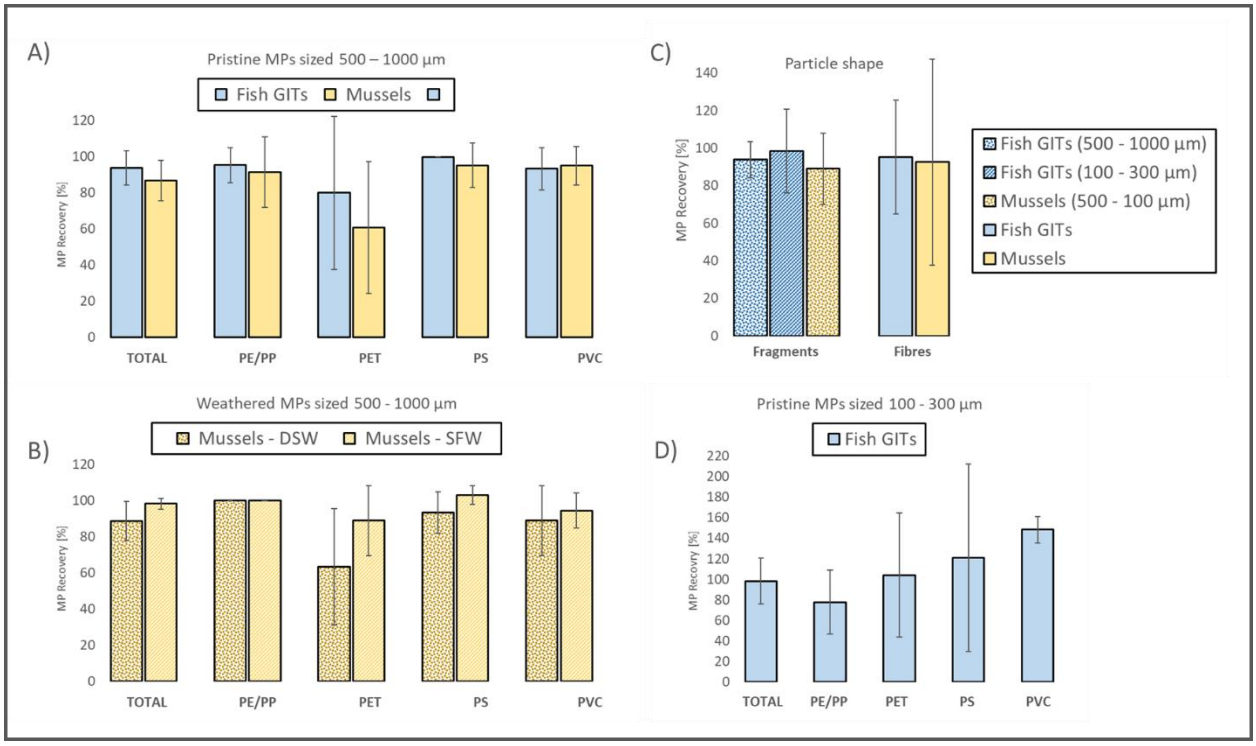

Fig. 2a, b, c and d. Microplastic recovery rates. Mean recovery rates (±SD) for (a) pristine microplastics sized 500 - 1000 µm; (b) surface and deep sea weathered MP sized 500 - 1000 µm; (c) particle shape (fragment/fibre), and (d) pristine microplastics sized 100 - 300 µm. Recovery experiments were performed in biota, i.e. in commercial mussels and GITs of common dab. Microplastics used were a mix of heterogeneously shaped fragments of PE, PP, PET, PS, PVC, and PAN fibres.

4. Discussion

4.1. Microplastic recoveries

Determining varying levels of MP in complex matrices is challenging and requires the utilisation of a good QA/QC validation procedure. Sample processing, which is necessary for the isolation of MP from various environmental matrices, often includes transferring MP back and forth from filters to liquids, which

inevitably results in MP loss to some degree (Hagelskjær et al., 2023). To determine this loss and consequently produce reliable recovery results, positive quality control samples in environmental MP studies are essential, in which a known mix of MP is spiked into the matrix, the MP analysis protocol is performed, and % MP loss is determined. Ideally, a statistically significant number of spiked MP, which are morphologically heterogeneous and of various size ranges, densities, and hydrophobicities are used for such experiments. For practical considerations however, these tests are simplified in many studies, producing environmentally less relevant results (Rozman et al., 2022). In most MP recovery experiments, PE, PS and/or PET are the most frequently used polymers (Way et al., 2022), while fragment-shaped MP are most often used. Many studies also still use spherical MP, which are environmentally less relevant MP shapes (De Ruijter et al., 2020). Additionally, some studies do not report MP shape nor sizes used (Way et al., 2022). Including parameters such as associated functional additives and dyes, as well as different stages of MP degradation renders the positive control samples even more environmentally relevant. Moreover, the interplay between the different plastic polymers, the reagents, and the equipment/materials used should also be considered (Hagelskjær et al., 2023) (cfr. '4.4. Microplastics in the marine environment').

The validation results of our developed semi-automated workflow for the analysis of MP in marine biota demonstrated reliable recovery rates of MP of various shapes and size ranges for the majority of spiked polymers, and this in various biota matrices, for pristine plastics as well as plastics that underwent extensive weathering under surface water and deep-sea conditions. Environmental weathering processes such as UV radiation and mechanical abrasion may interfere with the MP analysis (Dong et al., 2020; Phan et al., 2022) as well as the step preceding the analysis, where MP are extracted from sample matrices. Research has shown changes in the densities of specific polymers due to environmental degradation

(Kowalski et al., 2016), and consequently, extraction methods that depend on density separation processes to isolate MP from sediment may encounter challenges in effectively extracting all MP present.

The obtained accuracies, determined by the obtained values for the parameters precision and trueness of both models, confirm that the complete workflow is suitable for the accurate and reliable, cost-and time-efficient quantification of MP in biota. Using a fluorescence stereomicroscope which enables capturing a whole PTFE filter on one image (⌀ 47 mm), detection, analysis, and quantification of 20 MP took around 13 min. As long as the maximum filter coverage is not exceeded, and particles do not overlap (cfr. '4.2. Automated microplastic detection and identification'), analysis time remained unaffected by an increase in the number of MP analysed. The obtained trueness values indicate a high agreement between the recoveries obtained during repeated analysis and the actual spiked values. The precision values on the other hand demonstrate consistent and reproducible results, which is essential within scientific research and to formulate reliable conclusions. Together, these two quality control measures enhance the trustworthiness of the method. In addition, producing reliable and reproducible results and ensuring consistent data quality decreases the likelihood of errors and the need for repeated analyses, which saves time and resources. Similarly, it prevents costly discrepancies and the need for extensive quality control.

Based on our results, considering recovery rates of each individual polymer separately is nevertheless essential for full comprehension. Variation in particle recovery in fish GITs as a function of size were significantly dependent on polymer type (tested for pristine MP, Table S7). However, for PET and PVC, the observed higher recovery rates of smaller-sized fragments than of larger-sized fragments (Fig. S5) are in part a consequence of fragmentation as mentioned below. Positive quality control experiments within earlier studies showed no evidence of increased MP loss with decreasing particle size (Hagelskjær et al., 2023) although other studies contradict this (Way et al., 2022).

While most batches had high recovery rates for large-sized PET fragments, a small number of batches showed a lower recovery rate (e.g. 33 ± 58% and 28 ± 35, see Tables S5-6, respectively). These results are in agreement with earlier studies where lower recoveries for PET particles than for other polymers were observed (Rivoira et al., 2020). At the same time, recovery rates of PET fragments < 300 µm were sometimes > 100%, which is likely a consequence of degradation and fragmentation of the larger-sized fragments (Tables S7-S8, Fig. S5). Similarly, for some samples recovery rates > 100 % were obtained for smaller-sized PVC fragments. Given that PVC, a polymer with a density range similar to that of PET (both 1.38 - 1.41 $g.cm^{-3}$, Bessa et al., 2019), produced large-sized fragments with good recovery rates, decreased recovery rates as a consequence of the relatively high density of PET is unlikely. Previous studies demonstrated the susceptibility of PET particles to high temperatures and degradation by alkaline solutions through surface erosion mechanisms, leading to a reduction in size and mass (Schrank et al., 2022). This degradation is a heterogeneous process where saponification of ester linkages occurs at the particle surface, affecting the surface but not the core. However, this effect has not, or only to a limited extent, been observed for diluted 10% KOH solutions, nor have temperatures of 50°C been found to be destructive for PET particles (Hurley et al., 2018; Schrank et al., 2022). The use of diluted KOH solutions is therefore universally accepted and used for MP extractions (Dehaut et al., 2016; Lusher et al., 2018). Hurley et al. (2018) measured size and mass loss in PET particles (322-395 µm) treated with 10% KOH and 30% H_2O_2 at 60°C, but noted only minimal reductions in mass and size for PET treated with H_2O_2 (0.25% ± 0.24% and -0.68% ± 5.32%, respectively) and with KOH (-0.86% ± 0.05% and -3.13% ± 5.53%, respectively). In addition, when both PET and PVC fragments were spiked into Milli-Q water and immediately filtered without processing, particle fragmentation was also observed within this study. This indicates that particle breakdown is likely caused by particle brittleness following cryomilling rather than by the processing step. For the other polymers considered, such as PVC, higher recovery rates of smaller particles compared to

larger ones may have been partly influenced by fragmentation. Conversely, the higher recovery of large PP particles suggests that PP is less prone to fragmentation. This indicates that smaller PP particles may either have experienced greater loss during processing, or that the models perform less optimally for this size range.

The obtained results demonstrate that obtained environmental data of especially PET particles should be carefully interpreted when implementing this analysis workflow. The observed varying recovery rates of fibres in some samples (69 ± 30% - 120 ± 35% for fish GITs and 56 ± 51% - 122 ± 69% for mussels) could be explained by the diameter of the spiked fibres (< 20 µm), which allowed them to pass through the mesh of the stainless steel filter lengthwise. Prior research also indicated effects of pore size on the recovery of MP of different shapes (Cai et al., 2020). Retrievals of > 100% could be attributed to fraying and fragmentation of fibres. Our data emphasises the importance of using relevant reference materials for positive quality control samples, where besides MP size and shape, the material strength and associated degree of fragmentation as well as the processes carried out to obtain the required fragment sizes are important parameters to consider.

4.2. Automated microplastic detection and identification

Based on the obtained results for both fish GITs and mussels, the developed models prove to be reliable for the automated detection and identification of the majority of plastic polymers considered, through determination of the parameters sensitivity, selectivity and specificity. Random forest classifiers are an ensemble learning method that combines multiple decision trees to enhance classification accuracy and robustness (Svetnik et al., 2003). This classifier reduces the risk of overfitting and is less sensitive to noise, effectively handling large, high-dimensional datasets. Although such models can be computationally intensive, they were efficient for the analyses performed (see ‘4.1. Microplastic recoveries’). Potential

challenges with these classifiers however may include issues with generalisation to different sample types, and the complexity of interpreting the model due to the large number of trees.

The reliability of the models for the detection and identification of MP within samples obtained during monitoring campaigns is of paramount importance to generate accurate data as it allows for a comprehensive understanding of MP distribution, abundance, sources and pathways, in this way facilitating the development of targeted and effective solutions. At the same time, as the method is cost- and time-effective (cfr. '4.1. Microplastic recoveries'), it opens avenues for more large-scale monitoring in the long term and may contribute to an accelerated pace of data collection. Moreover, the method could lower the barrier for scientists working with limited financial resources, in this way supporting a more collaborative and widespread effort to combat MP pollution.

The developed model for polymer identification (PIM) showed a lower predictive accuracy for PET fragments compared to other polymer types (Tables S11-12), in addition to varying recovery rates. Assumably, this may be a consequence of the digestive reagents used to break down organic material. This could be visibly verified as differences in fluorescence colouration and intensity were observed between NR-stained, processed and unprocessed PET particles. Furthermore, during the image processing step within the semi-automated analysis workflow, some degree of overlap between fragments was observed on certain sample filters, which may also have affected particle identification. To count fragments, the ImageJ plugin 'watershed' (Rishi et al., 2015) and alternative approaches (Jacob et al., 2023) have been used to cut apart connected fragments into separate ones. However, previous research (Hagelskjær et al., 2023) showed that the percentage of filter covered by particles had a significant impact on automated recovery, as well as on analysis time. To prevent excessive particle loss (< 500 µm) and to keep analysis time from increasing, a maximum of 5% filter coverage was recommended. Some of the

polymer misidentifications in our results (e.g. PS in batch 2 for both fish GITs and mussels, Tables S11-12) could be explained by the close proximity of certain fragments to each other, as this also affects their individual fluorescent colouration, with diverging RGB values and incorrect classification of the models as a consequence.

Future advancements of the semi-automated technique to improve the model's predictive accuracy include expanding the training dataset of the polymer identification model with RGB data of PET fragments that underwent processing, so that trustworthy results are obtained for all polymers considered. Additionally, it is recommended to avoid particle agglomeration as much as possible for particle detection and identification purposes, by spreading out particle-rich samples over multiple PTFE filters, and by filtering the samples in such a way that fragments are as homogeneously spread out as possible on the filters.

4.3. Limit of quantification

Despite substantial effort to avoid MP contamination and although limited, some of the blanks showed contamination. Similar results were observed in other research (e.g. Johnson et al., 2020) which demonstrates the importance of contamination resulting from air deposition on samples, equipment and tools used, reagents, etc. as a parameter within MP QA/QC (Verded et al., 2021). Establishing an accurate and trustworthy method to correct for such accidental contamination has proven challenging, especially for low MP sample concentrations (Dawson et al., 2023). Moreover, earlier research found that inclusion of blank controls in experiments is often overlooked by researchers: 20% of all MP studies failed to incorporate blank controls in their experiments, and only few studies (34%) used a controlled air environment to process their samples (Noonan et al., 2023). Of the studies that do include controls, many do no use them for the correction of sample data (Dawson et al., 2023). Of the studies that do account

for accidental contamination, varying strategies have been implemented, e.g. based on subtraction (Lindeque et al., 2020) and spectral similarity (Kroon et al., 2018), however many were deemed unfit because of their inflexibility towards MP data, which is often highly heterogeneous in concentration and composition (Dawson et al., 2023). Using a LOQ to analyse MP contamination data has been recommended, because of its flexibility and ability to account for data variability without compromising on resolution. However, particles can change shape and size following environmental weathering and sample processing, therefore including categorical variables like shape and size into the correction method is not advised (Dawson et al., 2023). Besides this, regardless of the strategy employed, a sufficient number of control blanks is crucial to produce reliable results, with a minimum number of controls per sample batch. Imposing quality control measurements in the laboratory environment (see '2.3 QA/QC') will theoretically reduce the LOQ and hence allow MP trace quantities to be detected, which is particularly relevant for samples where low concentrations of MP are expected (Dawson et al., 2023).

4.4. Microplastics in the marine environment

The limited number of MP found in the unspiked fish GITs sampled in the BPNS were considered negligible in this study, which was consistent with findings in previous studies of the same area. After correction for laboratory contamination, 0.26 ± 0.64 MP per fish GIT were found by De Witte et al., 2022, where only 21% of the sampled fish contained MP. Similarly, other studies reported MP in only 1.8% of a total of 4,389 North Sea fishes from 15 species (Kühn et al., 2020), and a total of 2 MP in 1 out of 400 sampled North Sea fish (Hermsen et al., 2017). Few MP were also found in both commercial and non-commercial mussel tissue. Similarly, a prior study on MP concentration in commercial *M. edulis* from the North Sea showed on average 0.36 ± 0.07 particles g^{-1} wet weight (w.w.) (Van Cauwenberghe & Janssen, 2014). While MP concentrations spiked in fish GITs within this validation were higher than those typically found in North Sea fish, higher concentrations have been reported in more polluted areas, e.g. by Jaafar et al.,

2021 and Jabeen et al., 2017. The concentrations of MP used for spiking mussels in the method validation (1 - 1.75 particles per g w.w.) were also higher than typically found in North Sea mussels. However, Lusher et al., 2017 reported an average of 1.85 ± 3.74 particles per g w.w. for non-depurated mussels in Norwegian coastal waters, which aligns with the concentrations used in this study. Whether a mussel is depurated or not affects particle retention, with non-commercial, non-depurated mussels potentially serving as better bioindicators of environmental MP pollution (Shumway et al., 2023). On the other hand, commercially available mussels, which have undergone depuration, offer insights into human exposure to MP through seafood consumption. The relatively high MP concentrations used in this validation were nevertheless necessary to reliably assess plastic detection, identification, and the effects of both matrices on ML model performance through the determination of the different validation parameters.

The determined LOQ for fibres and particles in fish GITs and mussels closely aligns with the obtained MP background contamination in unspiked samples, which has implications for future MP studies. Due to the low MP detection, larger samples are needed to ensure quantifiable and reliable results, e.g. by pooling fish GITs per sample and using larger mussel sample masses. The findings also highlight the need for larger sample sizes for reliable MP detection, requiring large-scale sampling efforts. De Witte et al., 2022 determined that 109 to 370 fish would be needed to detect statistically significant differences in MP concentrations between different sampling areas, which could be facilitated through collaborations between fisheries and MP experts. Quantifying MP in marine samples is challenging due to their low concentrations, raising the question of whether methods should be able to detect negligible numbers of MP. Since one or two particles are unlikely to cause adverse effects, the inability to quantify such low concentrations might not be problematic. This approach is in parallel to the reporting of potentially harmful chemicals in food, where results often indicate levels that are "below the LOQ", and action is only taken when levels are above the LOQ.

Particles sizes used to spike mussels were larger than the sizes of their preferred food particles, which is typically around 40 µm according to Beecham et al., 2008. However, diatoms and dinoflagellates up to 200 µm in size have been found in the gut of *Mytilus edulis*. The protocol, designed for bivalves in general rather than specifically for mussels, accommodates a size range of 500 – 1000 µm. Studies by Naji et al., 2018 and Sfriso et al., 2020 detected particles ranging from 10 to 5000 µm in clams and other bivalves, validating the chosen size range for these species. Furthermore, analysing a variety of particle sizes was essential to confirm the robustness of the methods, particularly due to variations in fluorescent colouration observed in polymers like PET based on their size.

Plastics in the marine environment often undergo weathering due to e.g. UV radiation and wave action, potentially altering their physicochemical properties by introducing new chemical groups like oxygen functional groups (Campanale et al., 2023). These changes can affect the fluorescent colouration of MP following NR staining, which has implications for the RF model performance. Additionally, weathered plastic particles are more prone to erosion and fragment detachment (Resmerita et al., 2018), and digestive treatments may further enhance this degradation, potentially leading to reduced recovery rates. For this reason, it was essential to incorporate both pristine and weathered MP within the validation experiments. We aimed to produce particles weathered under semi-natural surface water and deep-sea conditions, ensuring they were as representative as possible while keeping the process realistic in terms of time and cost.

4.5. Importance of method validation

Validating a newly developed MP analysis method is of paramount importance in the field of environmental science and pollution management. A first reason is because it ensures the accuracy of

617 data generated during MP analysis. By calculating the parameters trueness and precision (ISO 5725-
618 1:2023), the accuracy and reproducibility of the method can be ensured, reflecting the true concentration
619 of MP in biota matrices. Measurement uncertainty quantifies the confidence in the results, indicating the
620 range within which the true value of the measured MP concentration is expected to lie, while the LOQ
621 defines the minimum number of MP from which accurate quantification is possible, ensuring the
622 effectiveness of the methods at low concentrations. Sensitivity, the ability to correctly identify plastics,
623 along with selectivity, the correct classification of plastic polymers, and specificity, the accurate
624 identification of organic particles, are essential parameters to assess the ability of the method to
625 distinguish MP of various polymer types from other matrix components (Rao et al., 2018). Lastly,
626 calculating the robustness guarantees that the method remains reliable under varying conditions, making
627 it indispensable for consistent and trustworthy MP analysis.
628
629 Although the procedure may be perceived as time-consuming, it represents an imperative step to increase
630 the accuracy and reliability of results obtained in studies, thereby improving cross-study comparability.
631 An intercomparison exercise report by Van Mourik et al., 2021 revealed significant variability in the
632 recovered number of MP among 34 European laboratories using standard methods like µ-FTIR, µ-Raman,
633 and GC-MS (29 - 91%). This underscores the necessity for standardised method validation to ensure
634 consistent QA/QC, even for the more established methods. By validating a method, its performance is
635 thoroughly tested to confirm its reliability (Konieczka, 2007), which is essential to estimate the true extent
636 of MP contamination levels in the oceans. These results create the foundation upon which informed
637 decisions and policies to mitigate adverse effects of MP are built.
638
639 A second reason for validation is its critical role in comparing data across laboratories and studies
640 (Hermsen et al., 2018). With a lack of quality control, variations in methods used can lead to inconsistent

and unreliable results, which in turn impedes assessing potential trends, identifying temporal and spatial changes, and evaluating the effectiveness of implemented mitigation efforts. To create a comprehensive understanding of MP pollution and its potential effects, data that is consistent and comparable is vital. Thirdly, results generated by a validated method generate trust and credibility among the scientific community, policymakers, and the public (Provencher et al., 2020). This confidence is necessary to tackle the current issue of MP pollution effectively, requires transformations across all sectors as well as behavioural changes of the public (Deng et al., 2020; Garcia-Vazquez et al., 2020), and starts with stricter regulations and policies. A fourth reason is choice of method. Once a method is validated, its fit-for-purpose for certain research questions can be assessed more easily, e.g. its suitability for analysing a certain sample or matrix type, detecting specific MP size ranges, or specific MP concentrations. Furthermore, method validation allows to identify potential difficulties of the developed method, as well as error and bias sources. By transparently reporting a method's limitations and uncertainties, future research can focus on overcoming these constraints, and on fine-tuning of the method. As a final important point, method validation contributes to the development of standardised protocols for MP analysis (Mari et al., 2021), which is essential to harmonise research efforts, to facilitate sharing and comparison of trustworthy data, to advance our understanding of MP pollution in the marine environment, and ultimately, to implement effective mitigation strategies related to environmental and human health impacts.

5. Conclusion

In the rapidly evolving field of MP research, the multitude of analysis techniques introduces challenges such as the labour intensiveness and its associated costs, as well as variation in data quality and consequently a limited comparability between all studies. To address these issues, a method validation procedure becomes essential to ensure the reliability and accuracy of the analysis methods. The current

study validated a semi-automated workflow for MP in biota, based on fluorescence spectra emitted by Nile Red-stained fragments for automated particle analysis. Method validation involved spiking MP into fish GITs and blue mussel tissue, extracting the MP by using the recently developed protocols, analysing the number and type of MP polymers by means of predictive ML models, and finally, determining six key validation parameters based on the MP recovery results.

The developed workflow demonstrated reliable recovery rates for most polymers, contributing to an accurate and cost-effective MP detection and identification. Through method validation, potential end-users are provided with performance data, as well as the method's limitations and uncertainties, e.g. for PET particles. Furthermore, by focusing on environmentally significant MP in validation experiments, the study circumvented existing disparities between laboratory and environmental conditions. The proposed validation parameters offer a step towards quality management guidelines, as such aiding future researchers and enhancing cross-study comparability. The study also discussed the potential of fish GITs and blue mussel tissue as valuable matrices for cost-effective MP biomonitoring.

6. CRediT authorship contribution statement

Nelle Meyers: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gert Everaert**: Conceptualisation, Methodology, Project administration, Supervision, Funding acquisition, Writing – review & editing. **Kris Hostens**: Supervision, Funding acquisition, Writing – review & editing. **Natascha Schmidt**: Conceiving, designing and performing of weathering experiments, Writing – review & editing. **Dorte Herzke**: Conceiving, designing and performing of weathering experiments, Funding acquisition, Writing – review & editing. **Jean-Luc Fuda**: Conceiving, designing and performing of weathering experiments, Writing – review & editing. **Colin Janssen**: Supervision, Funding acquisition,

Writing – review & editing. **Bavo De Witte**: Conceptualisation, Methodology, Project administration, Supervision, Funding acquisition, Writing – review & editing.

7. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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10. Appendix A. Supplementary data

Supplementary data to this article can be found online at x

11. References

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