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Abstract

The Mediterranean Sea water bodies are ones of the most polluted, especially with microplastics. As the seafloor is the ultimate sink for litter, it is considered a hotspot for microplastic pollution. We provide an original analytical development based on the coupling of tandem mass spectrometry to pyrolysis-gas chromatography to improve the detection of plastic contamination in marine organisms. Due to the high selectivity of the mass spectrometer, a straightforward sample preparation consists uniquely of potassium hydroxide digestion. The quantification of six common polymers is possible in one run. The method was applied to analyze the plastic content from 500 µm down to 0.7 µm in the whole body of seven benthic species with variable feeding modes. Plastic was detected in

all samples, with an almost systematic detection of polypropylene and polyethylene. Our method presents a major development in determining the levels of plastic contaminations in samples with rich organic matter content.

Keywords

Plastic pollution, marine litter, polymer, bioaccumulation, biodiversity, ingestion, benthic macrofauna

Highlights

- A method for quantifying microplastics in marine organisms down to 0.7 µm was developed using pyrolysis coupled to tandem gas chromatography–mass spectrometry.
- A one-step sample preparation consisting of chemical digestion was utilized.
- Six distinct polymer contents were determined in one run.
- The total polymer contents varied greatly from one organism to the other and was between 105 and 7780 µg/g dw.
- The PE, PET and PS polymers were detected the most often.

Introduction

Plastic loads are increasing in marine ecosystems worldwide (Barnes et al. 2009), and the Mediterranean Sea is one of the most affected marine basins (Consli et al., 2020; (Galgani et al., 1996). Macrolitter densities that exceeded 10^5 items per km² were recorded near metropoles (Galgani et al., 2000). Microplastic concentrations (less than 5 mm) on the seafloor, which are considered hotspots of accumulation, can reach up to 1.9 million pieces per m^2 (Kane et al., 2020).

An initial explanation for microplastic littering is that the litter is transported to the seafloor by vertical settling from surface accumulations and is driven by the density of microplastics. With biofouling, the buoyancy of microplastics is altered, and all types of plastic can sink—whether they are initially buoyant or not (Kooi et al., 2017). Whereas macrolitter sinking may be associated with dense downcanyon flows in the Mediterranean

(de Madron et al., 2017; (Tubau et al., 2015), microplastic sedimentation in the deep sea is driven more by near-bed thermohaline currents (Kane et al., 2020). In coastal areas, seasonal changes in river flow rate and related turbidity currents also considerably impact the spatial dispersion of litter (Angiolillo et al., 2021).

Microplastic hotspots of are also likely hotspots for marine life, as has been shown from the sea surface microlayer (Ghiglione and Laudet, 2020) to deep-sea sediment (Hall, 2002; (Kane et al., 2020). Marine biota interact with microplastics in several ways, and this leads to a reduction in feeding and depletion in energy stores but also causes toxicity, carcinogenesis, endocrine disruption and physical harm with knock-on effects for fecundity and growth (Galloway et al., 2017). After sedimentation, microplastics are available for many benthic species to feed on, such as detritivores and filter-feeding species (Valente et al., 2020). This potentially impacts the biodiversity throughout marine life, as the benthic community plays an important role in providing resources and ecosystem services (Danovaro et al., 2020; (Manea et al., 2020). The extent of the impacts of plastic pollution on Mediterranean ecosystems is poorly estimated, whereas the Mediterranean Sea is a biodiversity hotspot with high levels of endemism (Coll et al., 2010). Monitoring litter-benthic community interactions is largely hampered by difficulties in sampling and the necessary costs (Angiolillo et al., 2021; (Valente et al., 2020), which is why the interactions are poorly described even if all reported studies declare that a quasi-systematic of plastic occurs in individuals (Anastasopoulou et al., 2013).

In general, microplastics that are larger than 500 µm are visually detected and identified by Fourier Transform Infrared Spectroscopy (FT-IR). There are very few publications that compare microplastics that are smaller than 150 µm. The latest spectroscopic developments allow limits of tens of microns to be reached (Schwaferts et al., 2019), but the detection of the particles is strongly impacted by residual organic matter. This is solved by intensive sample preparations, which are time-consuming and costly forms of analysis that involve risks including altering and losing some microplastics and increasing cross contamination. In this context, pyrolysis-gas chromatography–mass spectrometry (Py-GC–MS) appears to be a very promising technique, even if its developments are very recent (Yakovenko et al., 2020). The use of Py-GC–MS does not have size limitations, and the selectivity of the mass spectrometry offers the possibility to simplify the sample preparation. The use of Py-GC–MS is promising in terms of reducing the time of analysis because several polymers are detected in one run.

In addition to all these promising aspects, there are some consequent obstacles with the use of Py-GC–MS (Pico and Barcelo, 2020; (Yakovenko et al., 2020). Two recent studies with important developments resulted, for the first time, in achieving the following robust methods: one for the analysis of biosolids (Okoffo et al., 2020) and the other for seafood samples (Ribeiro et al., 2020). Even if a less intensified purification of the sample is obtained through the use of Py-GC–MS, this step is still important. Okoffo et al. (2020) opted for pressurized liquid extraction, and the remaining organic matter was eliminated during Py-GC–MS analysis using a two-step pyrolysis program (organic matter removal at 300 °C followed by pyrolysis at 650 °C). Ribeiro et al. (2020) proposed a more intensified sample purification that involved alkaline digestion followed by pressurized liquid extraction, and they skipped the decomposition step at 300 °C. Here, we introduce the use of tandem mass spectrometry (Py-GC–MS/MS) to enhance the detection performance, thus permitting a simpler sample preparation using alkaline digestion alone. This study aimed to demonstrate that Py-GC–MS/MS is a fast and reliable tool for microplastic quantification down to 0.7 µm in marine organisms. Here, we provide the first assessment of microplastic content in Mediterranean benthic organisms for a selection of 6 different polymers.

2. Materials and Methods

2.1. Chemicals and Reference Materials.

A total of six polymers were targeted. They were chosen among the most abundant polymers in the marine environment, namely, high density polyethylene (PE), poly(methyl methacrylate) (PMMA), polyethylene terephthalate (PET), polycarbonate (PC), polystyrene (PS), and polypropylene (PP). The first three polymers were purchased from Sigma–Aldrich (St. Louis, MO, USA) and the three others were from Goodfellow Group (Huntingdon, United Kingdom). These polymer standards were used to optimize the mass spectrometry conditions and to prepare standards for external calibration. The external calibration was performed with a mix of polymers diluted in a calcined powdered glass microfiber filter (GF/D diameter 47 mm; Whatman® Sigma–Aldrich, St. Louis, MO, USA).

2.2. Sample Collection and Processing.

All glassware was calcined at 550 °C for 2 hours before use in an incinerator oven (Nabertherm™ LV052K1RN1). Glass fiber filters were calcined at 600 °C for 2 hours before use. Benthic organisms were sampled on the northwestern Mediterranean seafloor from the R/V Nereis II. Specimens sampled with a van veen grab were sorted and stored in a clean metallic bowl on board. At the laboratory, the specimen were identified and placed in calcined glass vials that were closed with a cap, which was equipped with a polytetrafluoroethylene (PTFE) opercula. The details of the GPS location and sampling depth of each organism are given in Table 1. A sampling control consisted of opening a calcined glass vial that contained calcined quartz fiber for approximately the same period of time it took to manipulate the animals both onboard and at the laboratory. The quartz fiber was analyzed by Py-GC–MS/MS similar to the samples. In the laboratory, all animals were freeze-dried and weighed. Under the wood, the animals were transferred to 30 mL glass flasks equipped with glass caps. A ratio of 80 mL per gram of dry animal of 10% potassium hydroxide aqueous solution prefiltered was added. The solution was previously filtered in a closed glass unit from Vagner Glasses Company (Toulouse) on a calcined 47 mm diameter membrane with a porosity of 0.45 µm (PTFE Omnipore™, from Sigma–Aldrich, St. Louis, MO, USA) to remove any potential plastic contamination. For the chemical digestion, the flasks were placed in a shaker incubator (Eppendorf® ThermoMixer® C, Sigma–Aldrich, St. Louis, MO, USA) for 48 h at 40 °C with continuous agitation (500 rpm). A similar flask with potassium hydroxide solution and no sample was used as a procedural blank. Once the digestion was completed, the samples were removed from the incubator and prefiltered on 500 µm stainless steel filter grids (Negofiltre, Moret Loing Et Orvanne, France). The solution was then filtered under vacuum with a closed glass unit onto a calcined glass microfiber filter, GF/F diameter 47 mm or 21 mm Whatman® (Sigma–Aldrich, St. Louis, MO, USA). Filters were stored in glass Petri dishes before cryogrinding using the SPEX® SamplePrep 6775 Freezer/Mill cryogenic Grinder (Delta Labo, Avignon) with the program: precool 2 min ; run 1min ; cool 2 min ; cycles 15 ; cps 15. A sub-sample of 2 mg was precisely weighted in a microscale with a 10-5 g precision (Micro Balance from Sartorius, MCE225P-2S00-A Cubis®-II Semi) on quartz tubes that were freshly calcined at 1000°C with the pyrolysis probe using the “clean” program. A sub-sample of 2 mg was precisely weighted in a microscale with a 10-5 g precision (Micro Balance from Sartorius,MCE225P-2S00-A Cubis®-II Semi) on quartz

tubes that were freshly calcined at 1000°C with the pyrolysis probe using the "clean" program.

Table 1. List of the benthic organisms that were sampled in the northwestern Mediterranean and analyzed for microplastic contents. The corresponding feeding modes, sampling depths and coordinates are also given.

Taxa	Phylum	Feeding modes	Depth (m)	Coordinates (WGS84)
Glandiceps talaboti	Enteropneusta	Surface and/or Subsurface deposit feeder	43	42°30.50'N 3°09.11'E
Amphiura chiajei	Echinodermata	Surface deposit feeder	43	42°30.50'N 3°09.11'E
Amphiura filiformis	Echinodermata	Surface deposit and/or suspension feeder	43	42°30.50'N 3°09.11'E
***Notomastus* sp.**	Annelida	Subsurface deposit feeder	43	42°30.50'N 3°09.11'E
Fustiaria rubescens	Molluska	Carnivorous	80	42°30.00'N 3°11.40'E
***Acanthocardia* sp.**	Molluska	Suspension feeder	80	42°30.00'N 3°11.40'E
Lanice conchilega	Annelida	Surface deposit feeder and/or suspension feeder	90	42°30.00'N 3°12.60'E

2.3 Py-GC–MS/MS Analysis.

The method parameters for analysis by pyrolysis were achieved using a CDS Pyroprobe® 6150 from Quad service (Acheres, France) interfaced with a GC–MS/MS triple quadrupole TSQ® 9000, GC Trace 1310 from Thermo Fisher Scientific (Villebon sur Yvette, France). The gas chromatography column was a TraceGOLD TG-5SilMS from Thermo Fisher Scientific. Samples were pyrolyzed at 600 °C for 30 s. The pyrolysis products were transferred at 300 °C at the interface and were injected at 300 °C with a split ratio of 15:1 (additional data Table SI 1). Multiple reaction monitoring (MRM) optimizations for

collision energy were obtained using Auto SRM 4.0 for Chromeleon software in liquid injection with a Thermo Scientific™ AI/AS 1310 autosampler. The MS acquisition/detection parameters are listed in Table SI 2. Chromatograms were integrated using the Cobra detection algorithm from Chromeleon 7.2.8 software. The external calibrations were achieved between 25 ng and 1.4 µg with 6 calibration points (Table 2 and SI 3). The range of the calibration depends greatly on the polymer because the intensity of the indicator compound could vary greatly. The confirmation/quantification ratios were established with the external standards. For the external calibration preparation the polymers were fist cryo-milled using the SPEX® SamplePrep 6775 Freezer/Mill cryogenic Grinder (Delta Labo, France) with the program: precool 2 min ; run 1 min ; cool 2 min ; cycles 15 ; cps 15. This inert matrix was prepared from glass microfiber filters (GF/D diameter 47 mm from Whatman®) cryo-milled (precool 1 min; run 1 min; cool 1 min; cycles 6; cps 15) and calcined.

2.4 Method Validation and Performance

For each polymer analyzed, an indicator compound was selected for quantification. The analytical limit of detection (LOD) and limit of quantification (LOQ) were determined for each polymer and were defined as S/N of 3 and of 10 respectively. This limit was only reached within the calibration range for PE (130 ng). We selected the following criteria to assess the possibility of determining a peak concentration: 1) the retention time was within a window of 0.05 min compared to that of the standards, 2) the peak was above the analytical LOQ, and 3) there was 30% tolerance in the ratio of the ion transitions. The interday variability will not discussed as the external calibration standards and the samples were all analyzed in the same sequence on the same day. Finally, a polymer was quantified only if the signal was ten times superior to the procedural and field sampling blanks (Table SI 4) and we did not subtract the signal of the blank to the determined concentration. If any of the above cited criteria were not respected, it was specified that the concentration was not determined (n. d.). The extraction efficiency of the sample preparation was estimated with a positive control that consisted of the 6 polymers in concentrations ranging from 940 to 4800 ng/ml of KOH and proceeded with the same steps as those of the preparation and analysis (Table SI 5). To evaluate matrix interferences during pyrolysis or mass spectrometry detection, we proceeded to perform the standard addition method after cryo-

grinding was performed for the filters, and the samples were spiked at concentrations of 50 to 300 µg/g depending on the polymers.

2.5. Quality Assurance and Quality Control (QA and QC)

A need for stricter QA and QC during method development for microplastic analysis in biota was discussed earlier, and we integrated the criteria proposed in the present study (Hermsen et al., 2018). We took special care to minimize contamination during sampling and during sample preparation in the laboratory. Only glass and metal were used. The only plastic that was in contact with the sample was the opercula in the cap PTFE for sample storage, and this opercula is a polymer that does not interfere with the mass detection of the polymer targeted here. Glass and inox materials were cleaned thoroughly three times with Milli-Q water and ethanol and then systematically calcined prior to use. Laboratory coats that were made of 100% cotton were always worn during the analysis procedures. The work was performed in a fume hood to minimize contamination by airborne microplastics. Whenever the samples were not processed, they were stored in closed glass units. The glass fiber filters were also calcined and stored in glass petri dishes that were wrapped in aluminum foil before use. The quartz tubes that were used for access into the pyrolysis chamber were cleaned at 1000 °C for 30 s immediately before being used and were not stored. The samples in the quartz tube were weighed to minimize airborne contamination, as the tubes were placed in a metal sample holder that was stored in a glass unit with a glass cover. All solvents (water, ethanol, or potassium hydroxide solution) were prefiltered on PTFE (0.45 µm, Omnipore™, from Sigma–Aldrich, St. Louis, MO, USA). The glass microfiber filters were prepared via an optimized calcination (from room temperature to 500 °C at a rate of 80°C/hour with hold of 30 hours at 500°C using a LV 5/11 furnace from Nabertherm®).

3. Results and Discussion

3.1. Indicator compound detection and quantification

The method proposed for the identification and quantification of the six targeted polymers (PMMA, PP, PE, PET, PS and PC) is new as it is the first development of tandem mass spectrometry coupled to pyrolysis. The high selectivity of the triple quadrupole allows to shorten the number of steps of the sampling preparation compared to what was proposed with a simple quadrupole (Ribeiro et al., 2020). Because PVC products of pyrolysis are aromatic molecules (like benzene, naphthalene …) and because they are not specific (there are interferences from organic matter): we excluded this polymer from the study. For PS,

we selected the PS trimer as an indicator compound, as in most recent studies (Yakovenko et al., 2020). Styrene cannot be used because it is often a product of natural organic matter pyrolysis (Dierkes et al., 2019; (Fischer and Scholz-Bottcher, 2017; (Zhou et al., 2019). The specific detection of PE has already been discussed considerably because biogenic materials such as natural fats (e.g., fish protein) and waxes are rich in long alkyl chains. They produce n-alkanes and n-alkenes during pyrolysis (Dierkes et al., 2019; (Fischer and Scholz-Bottcher, 2017; (Scholz-Bottcher et al., 2013), which are common products in the pyrolytic decomposition of PE. The selection of an indicator compound among these two families was excluded if there was no intensive sample purification (Okoffo et al., 2020; (Ribeiro et al., 2020). Thus, we opted to use an indicator compound among the n-alkadienes, which are very specific to PE pyrolysis but formed to a much lesser extent (Yakovenko et al., 2020). In this study we selected the congener with 13 carbon atoms (Table 2). In the samples analyzed, we systematically detected PE in the MS/MS mode. The presence of PE was effective because all 3 congeners (the succession of n-alkadienes, n-alkene and n-alkane) were present with an n between 8 and 17. As a demonstration, we reported the signal in the full scan of one sample (Figure 1). The pyrochromatogram is very complex, but the characteristic shape of PE appears in the full scan with the n-alkenes signal (marked with a star in Figure 1). Some fatty acid esters were also present in important proportions and originated from residual organic tissues after chemical digestion. In the inset of Figure 1, the detection of the indicator compound, the n-alkadiene with 13 carbon atoms, is possible with the use of MS/MS. In this insert, we can see that in addition to the indicator compound, we detected many other peaks in MS/MS. Many peaks can be detected in MS/MS (MRM mode) because they have the same transitions as the ones monitored for the C13 target compound which is rather common among hydrocarbon derivatives but the transition ratios are distinct even for structural isomers. All those peaks are hydrocarbons with various unsaturated components and ramifications and are always formed during PE pyrolysis (Sojak et al., 2007). The interference of PE pyrolysis from organic matter, especially with regards of lipids, is a very complex problem which was recently investigated in details (Rauert et al., 2022). In the present study we have considered the ratio C13/C14 as a validation criterion with a tolerance of 30% compared to the ratios recorded for the external standards. Work is under progress to further understand PE pyrolysis and interferences with the matrix investigating several indicator compounds (the ratios recorded for the samples are presented in figure SI 1). In a recent review paper, we

argued for the choice of indicator compound selections for the other polymers (Yakovenko et al., 2020).

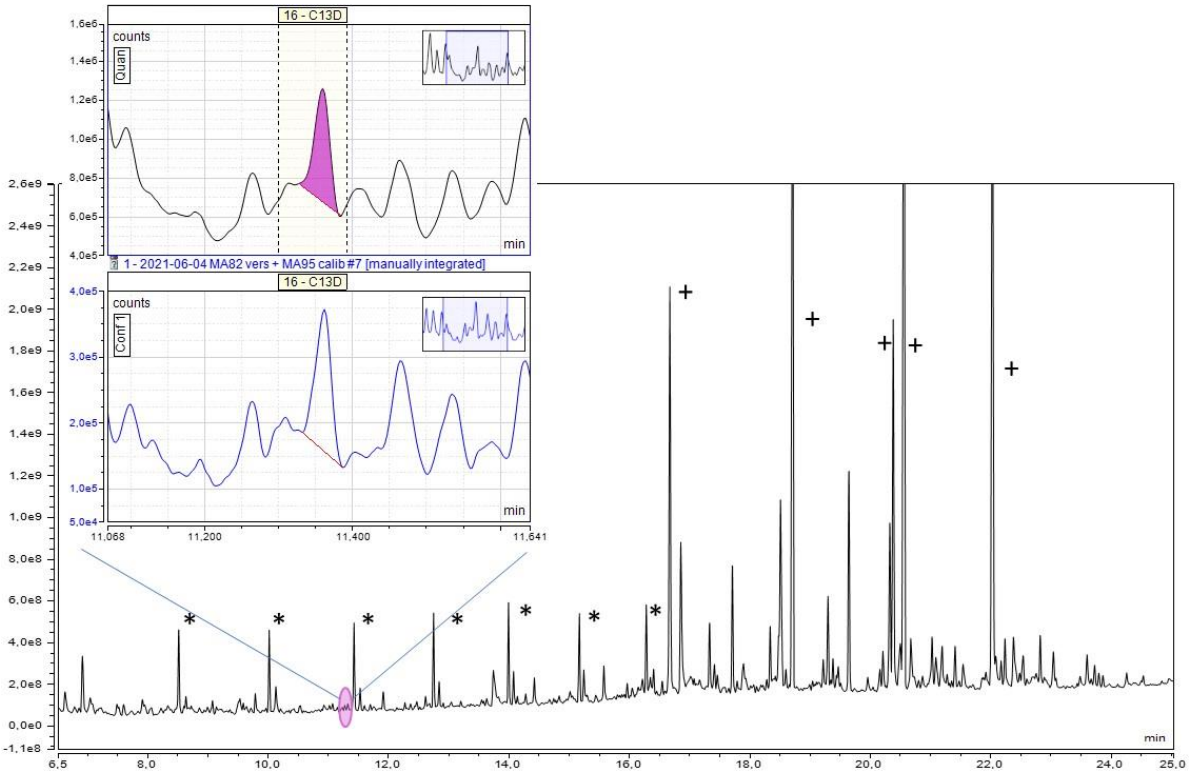

Figure 1: Full scan analysis of the *Amphiura filiformis* sample. The stars mark the peaks of the n-alkene congeners; they are the main products of pyrolytic PE decomposition. The peaks marked with a cross are fatty acid esters and remains of the tissues of the animals after chemical digestion. In the inset box, the signal of the selected indicator compound of PE is presented in the MS/MS; we chose the alkadiene congener with 13 carbon atoms because its signal was the highest.

3.2 Sample digestion efficiency and evaluation of polymer integrity

We selected a chemical digestion protocol using potassium hydroxide to remove the organic tissues. The efficiency of this protocol was discussed considerably, and potassium hydroxide appeared to be a good compromise for obtaining an efficient purification and preserving the polymers (Dehaut et al., 2016). We observed that even if the organisms sampled were very distinct in terms of their taxonomic species, size, weight and feeding modes, the protocol was well adapted to this diversity. The digestion efficiency was estimated by mass balance; we determined that between 97 and 80% of the samples weight was eliminated. The elementary analysis of the remaining matter showed less than 0.3 % of organic carbon; we are assuming that the material left after chemical digestion was

mainly inorganic. This is in accordance with the fact that some organisms were deposit feeders and that they are ingesting sediment particles. The samples with the lowest digestion efficiencies corresponded to *Glandiceps talaboti* and *Notomastus* sp., which are subsurface deposit feeders. Such organisms typically process at least one body weight of sediment daily. As a consequence, their alimentary tract contains large volumes of sediment that are not eliminated during chemical digestion (Lopez and Levinton, 1987). These results underlined the importance of the weight-specific feeding rates to be considered when characterizing the plastic that is ingested by benthic species.

Compared to enzymatic digestion, chemical digestion offers many advantages since it is very efficient and not expensive, but a disadvantage is the possible alteration of some polymers. It was recently reported that even if PET was resistant to digestion when potassium hydroxide was used at 60 °C, smaller particles, such as PET fibers, did not resist such temperatures; thus, lower temperatures are recommended (Treilles et al., 2020). For instance, the digestion of seafood samples at 60 °C resulted in 32% recoveries for PET (Ribeiro and al. 2020). For this reason, chemical digestion was performed at 40 °C. We obtained an extraction procedure efficiency for the six spiked polymers between 82 and 129%, which was within the precision margin of the MS/MS method, so we estimated that the recoveries were acceptable (Table SI 5).

3.3. Method Validation and Performance

To proceed to the fabrication of the external calibration we first cryo-milled the polymer separately. They were then mixed in an inert glass fiber matrix also previously grinded and calcined to remove any trace of polymers. The external standards were first prepared at concentrations ranging from 1 $mg.g^{-1}$ to 5 $mg.g^{-1}$ and the powder was then diluted by a factor 10. To obtain the external calibration we prepared 5 dilutions to reach the calibration range detailed Table SI 3. The repeated injection of an external standard (N=12) showed a standard deviation below 20% for all polymers considered. We thus consider the homogenization of the powders was satisfactory. The response was linear within the calibration range for each polymer with a correlation value (R^2) greater than 0.85 (Table 2). After digestion and filtration of the samples on glass fiber filters, the filters were cryo-ground to present good homogeneity, as only a fraction, typically 2 mg, was introduced in the pyrolysis chamber. After cryo-grinding, a sample analyzed in triplicate showed a standard deviation below 35% for all polymers considered (Table SI 6). We estimated that

cryo-grinding was efficient and that the sample was sufficiently homogeneous. The other samples were analyzed once.

The procedural and field blank polymer concentrations are presented in Table SI 4. The amount of PMMA in the samples was not determined because the concentrations in the sampling control blank were rather important (4.7 µg/g filter, table SI 4). Further studies are needed to determine the potential source of contamination and to improve the QA/QC for this polymer.

The potential impact of the remaining matter after chemical digestion on the polymer analysis was assessed with the standard addition method. The pyrolytic fingerprint of all the polymers was identical when the polymers were injected as a pure sample or within the matrix, indicating that the presence or residual organic or inorganic matter did not interfere with the polymer pyrolysis or the MS/MS detection. The case of PE is remarkable because some natural organic molecules (like lipids) could thermally decompose into dienes as it was recently reported (Rauert et al., 2022). In order to ensure that the remaining matter after sample preparation did not enhance the signal of PE we used an additional validation criterion based on the recording of two indicator compounds (the analogues with 13 and 14 carbon atoms). The ratios recorded and compared to the external standards are reported in Figure SI 1.

Table 2: Polymers targeted together with the indicator compound selection and external calibration characteristics.

Polymer	**Indicator compound**	**Quantification transition (*m/z*)**	**External calibration range (µg)**	**Numbers of point**	**r^2**
PMMA	methyl methacrylate	100>41	35 to 380 ng	6	0.98
PP	2,4-dimethylhept-1-ene	70>55	30 to 300 ng	5	0.90
PE	1,12-tridecadiene	95>67	130 to 1360 ng	6	0.88
PET	dimethyl terephthalate	163>135	25 to 265 ng	6	0.96
PS	5-hexene-1,3,5-triyltribenzene (styrenetrimer)	207>129	50 to 385 ng	5	0.99

PC	2,2-bis(4'-methoxy-phenyl)propane	241>133	27 to 280 ng	6	0.95

345

346 **3.4. Polymer content in the samples**

347 In general, we systematically detected plastic in the marine benthic animals analyzed. The
348 total polymer contents (Figure 2 and Table SI 7) varied from one specimen to another and
349 were between 105 and 7780 µg/g dry weight. These margins are within those recently
350 determined by Py-GC–MS for seafood (Ribeiro et al., 2020). There is not yet an established
351 pattern between the content of plastic in marine organisms and the feeding modes, marine
352 habitat or trophic position, even with a large sample set. Microplastic accumulation in the
353 marine food chain has been supported by some authors (Carbery et al., 2018), while a recent
354 critical review concluded that no plastic biomagnification occurred (Walkinshaw et al.,
355 2020). The authors argued that microplastics do not translocate from the digestive system
356 into tissues or into circulatory fluid and that microplastics are only transitory contaminants
357 with a limited residence time within organisms. Nevertheless, the mechanisms of plastic
358 particle ingestion, egestion or excretion are still not well understood (Cole et al., 2016). In
359 general, authors assume that the residence time of plastic particle in the digestive system
360 is deeply correlated with the particle size, shape and rugosity, which are very
361 heterogeneous in the environment and could explain the great variations obtained between
362 specimens, in addition to ecological or environmental factors.

363 In our study, we collected species with variable feeding modes. *Glandiceps talaboti*,
364 *Notomastus* sp. and *Amphiura chiajei* are strict deposit feeders (Buchanan, 1964), while
365 *Lanice conchilega* is both a suspension feeder and deposit feeder, depending on the
366 environmental conditions (Word, 1990; (Zarkanellas and Kattoulas, 1982). *Amphiura*
367 *filiformis* is known to have a main filtering activity (Buchanan, 1964). *Acanthocardia*
368 *paucicostata* is a strict suspension feeder, and *Fustiaria rubescens* is a carnivorous feeding
369 mainly on foraminifers from sediment surfaces (Gofas et al., 2011). These species are
370 known to be good integrators of environmental variation because of their reduced mobility.
371 Therefore, their plastic content may be considered a good proxy of the plastic content of
372 the environment in the same region, with the limit of the spatial heterogeneity of
373 microplastics on the sea floor. Overall, our results agreed with this hypothesis by indicating
374 that the type of polymer recovered from benthic animals with different feeding modes
375 corresponds to the distribution of the polymer in the oceans. Nonetheless we observed

important variation among individuals; this variability was often reported and is not yet explained (Ribeiro et al., 2020).

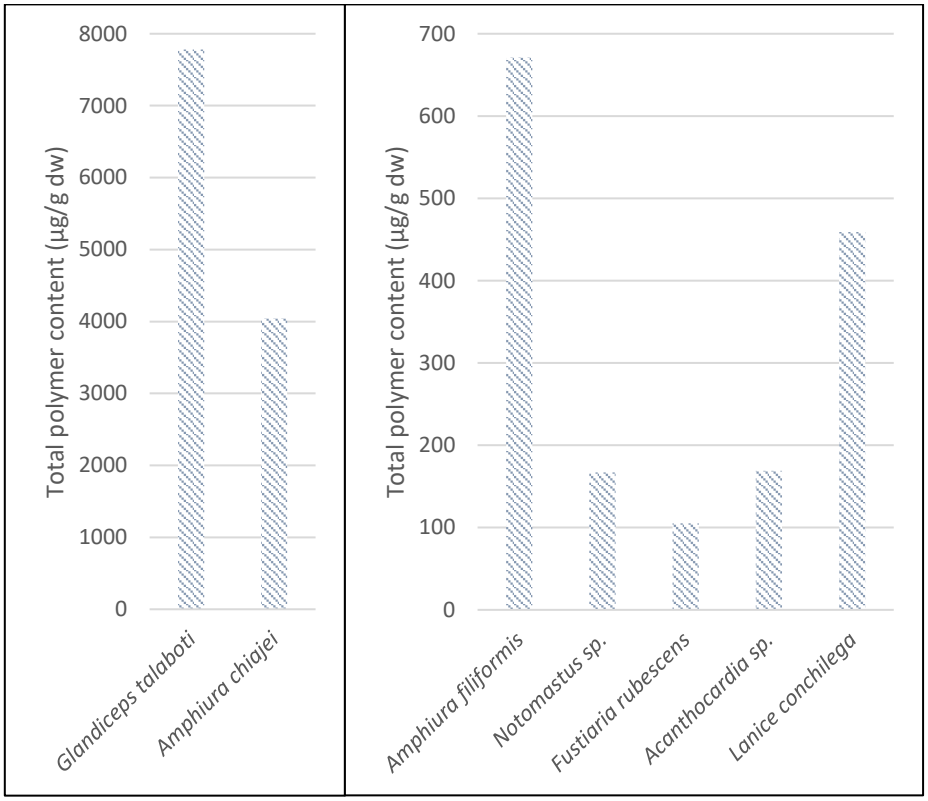

Figure 2: Total polymer content expressed in µg per gram of dry weight (µg/g dw).

PE was detected in six samples of the seven analyzed samples (Figure 3) at concentrations up to 670 µg/g dw for the *Glandiceps talaboti* individual. We noticed that PE was often present in the largest proportions, often superior to 80% of the total polymer content. It has been reported that PE was dominant in marine samples with an average proportion of 42% at the sea surface and with a decrease in abundance through the water column (Erni-Cassola et al., 2019). Our results agreed with those of Missawi et al., who reported that PE was dominant in the polychaete worm *Hediste diversicolor* on the Tunisian coast in the Mediterranean Sea, with important variations among individuals and sites, whereas PP was detected in lesser proportions than those of PE (Missawi et al., 2020), which is in accordance with the reported concentrations at sea (Erni-Cassola et al., 2019).

Notomastus sp. et *Lanice conchilega* presented high contents of PET, which are likely to be associated with deposit feeders because it is a polymer with a higher density than that of sea water. Previous studies using spectroscopic characterization emphasized that a high

proportion of PETs were detected in detritivores, which corresponds to plastic fibers (Renzi et al., 2020). PET fibers have also been detected in high proportions in seafloor samples (Kane et al., 2020). Figure 3 also shows that *Amphiura chiajei* and *Notomastus sp.* exhibited a high content of PS. They are both deposit feeders and are likely exposed to denser polymers such as PS. Along the same line, PMMA, which is also known to be more abundant in the sediment than at its surface (Renzi et al., 2020), has not been detected in suspension feeders such as *A. filiformis* and *A. paucicostata.* This could be explained by the relatively high limit of detection for PMMA under our conditions.

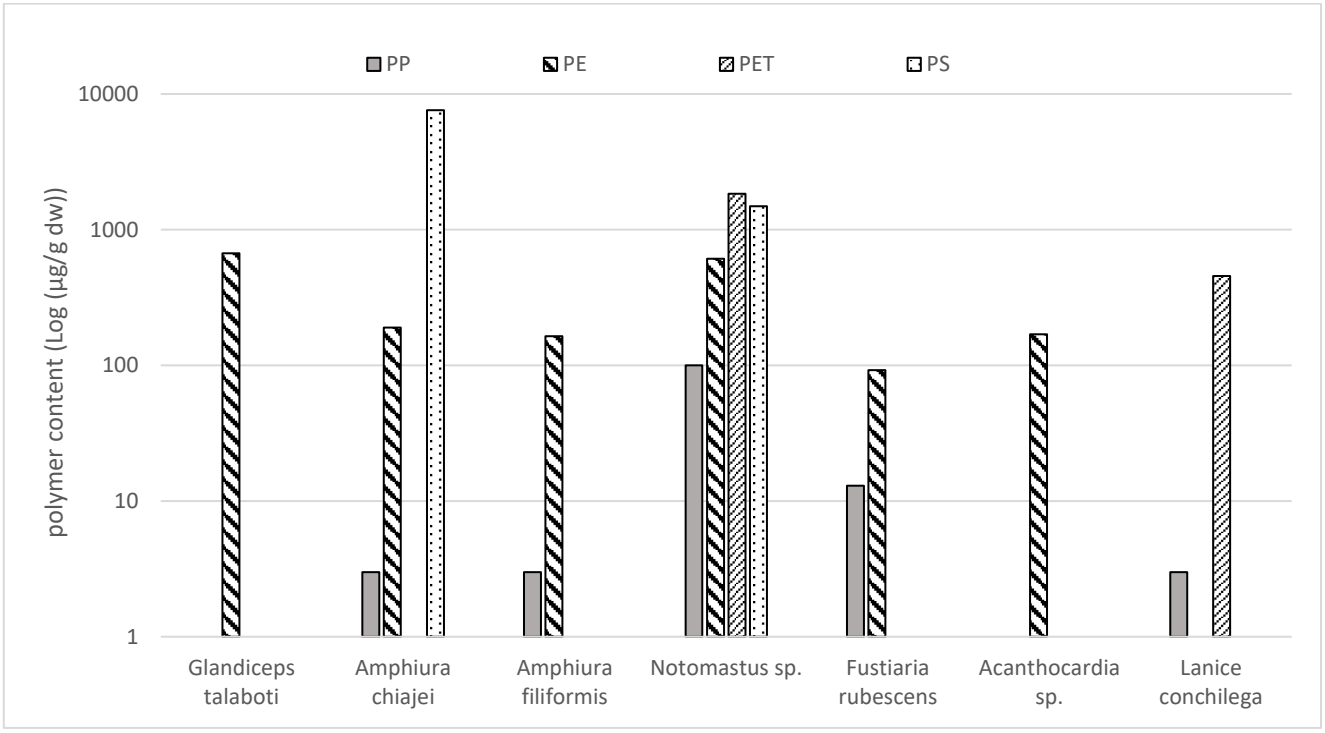

Figure 3: Polymer content in the benthic individuals (expressed in µg/g dry weight).

Concluding remarks

The study demonstrates that a method based on Py-GC–MS/MS leads to a simplified sample purification and enables microplastic contents down to 0.7 µm to be determined with good reliability in organisms. Py-GC–MS does not provide information on the color, shape, or size of microplastics and is complementary to methods that are based on spectroscopy (Primpke et al., 2020). The use of pyrolysis to quantify microplastics still involves limitations and areas of improvement that need to be considered before it becomes a standardized technique. As a first glance, the use of internal standards will certainly

improve the precision of the measurements even if the developments in this direction present some technical difficulties (Lauschke et al., 2021) that are challenging because very few isotopic analog resins are commercially available. Other important undertakings involve achieving a better understanding of matrix interference and the effect of polymer weathering on the pyrolytic response (Ainali et al., 2021; (Biale et al., 2021; (Toapanta et al., 2021). The most appealing aspect of Py-GC–MS is that it does not have size limitations, as there is still very little known about the behavior of small microplastics in the environment and their interaction with organisms. We emphasize the promising potential for the use of Py-GC–MS as it involves straightforward sample preparation, even with complex samples, and the possibility of increasing our capacity to analyze larger sample sets for environmental assessments. To gain a better understanding of the interactions of benthic community with plastic pollution, the variation in plastic concentrations with sediment depth at different locations should be investigated, and this could be first explored by focusing on a single species with a strict feeding mode.

Supporting material

Determination of the microplastic content in Mediterranean benthic organisms by pyrolysis-gas chromatography-tandem mass spectrometry

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446 **Table SI**1: Optimized conditions for Pyrolysis-GCMS/MS

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Pyrolyzer	
Carrier gas	helium
Pyrolysis temperature	600°C
Pyrolysis time	30 s
Gas chromatogram	
Initial temperature	40°C
Flow	1.25 mL.min $^{-1}$
Temperature program	40°C (2 min) => 300°C (5 min) at 10°C.min $^{-1}$
Transfer line temperature	280°C
Mass spectrometer	
Mode	Multiple Reaction Monitoring
Scan time	0.15 s
Source temperature	300°C

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Table SI 2: Additional information for polymer detection and quantification after MS/MS development.

Indicator compound	Retention time	Quantification transition (Tq)	Confirmation transition (Tc)	Tc/Tq (%)*
Methyl-methacrylate	2.73	100>41 (15eV)	100>69 (10eV)	76.1
Dimethyle-heptene	4.47	70>55 (10 eV)	126>83 (5 eV)	13.9
1,12 tridecadiene	11.36	95>67 (10 eV)	109>67 (10 eV)	43.1
Dimethylterephtalate	14.33	163>135 (10 eV)	163>103 (15 eV)	69.9
StyreneTrimere	23.4	207>129 (10 eV)	207>91 (15 eV)	46.7
Methyl-bis-phenol A	20.26	241>133 (15 eV)	256>241 (10 eV)	81.3

*The ratio Tc/Tq was determined over the first 3 most concentrated external standards injected for the calibration

** Typically, we introduce 2 mg of standard or sample for pyrolysis analysis

457 **Table SI 3:** External standards amount injected range and MS/MS peak intensity.

Polymer	Range of concentration of the 6 calibration points (ng)	Range of corresponding peak area for the transition of quantification
PMMA	37 - 384	1E+05 - 2E+06
PP	30 - 309	1E+05 - 8E+05
PE	133 - 1357	8E+03 - 9E+04
PET	26 - 264	6E+04 - 3E+06
PC	50 - 508	7E+04 - 1E+06
PS	27 - 280	1E+06 - 2E+07

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Table SI 4: Amount of polymer detected in the sampling control and the procedural control.

Polymer targeted	**Sampling control blank**	**Procedural control**
PMMA (µg/g)	4.7	2.7
PP (µg/g)	n.d	n.d.
PE (µg/g)	n.d	n.d.
PET (µg/g)	40.0	0.8
PS (µg/g)	4.0	0.7
PC (µg/g)	n.d	n.d.

n.d.: not determined. The indicator compound peak was not detected, and the Tq/Tc ratio was not confirmed.

465 Table SI 5: Digestion recoveries for the polymers with the standard addition method

Polymer targeted	Concentrations of the polymer in the inert matrix (µg.g^{-1})	Amount of polymer added for the recovery test (ng)	Proportion of polymer recovered after chemical digestion (%)
PMMA	607	6182	107
PP	489	4978	82
PE	2147	4260	111
PET	418	4513	129
PS	804	8196	122
PC	443	9036	116

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Table SI 6: The *Lanice conchilega* sample was analyzed in triplicate. The relative standard deviations were reported for all polymers and did not exceed 35%.

Polymer targeted	Indicator compound	SD - %
PMMA	Methyl-methacrylate	19
PP	Dimethyle-heptene	23
PE	1,12 tridecadiene	24
PET	Dimethylterephtalate	29
PS	StyreneTrimere	34
PC	Methyl-bis-phenol A	10

473 Table SI 7: Concentration of the polymer targeted expressed in µg per gram of dry wet
474 (µg/g dw). The results are presented with a standard deviation of 35%.
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Sample N°	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	Glandiceps talaboti	**Amphiura chiajei**	**Amphiura filiformis**	**Notomastus sp.**	**Fustiaria rubescens**	**Acanthocardia sp.**	**Lanice conchilega**
PMMA (µg/g)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PP (µg/g)	1	3	3	100	13	n.d.	3
PE (µg/g)	670	190	164	610	92	169	n.d.
PET (µg/g)	n.d.	n.d.	n.d.	1845	n.d.	n.d.	456
PS (µg/g)	n.d.	7587	n.d.	1487	n.d.	n.d.	n.d.
PC (µg/g)	n.d.	n.d.	n.d.	23	n.d.	n.d.	n.d.

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477 n.d.: the concentrations were not determined.
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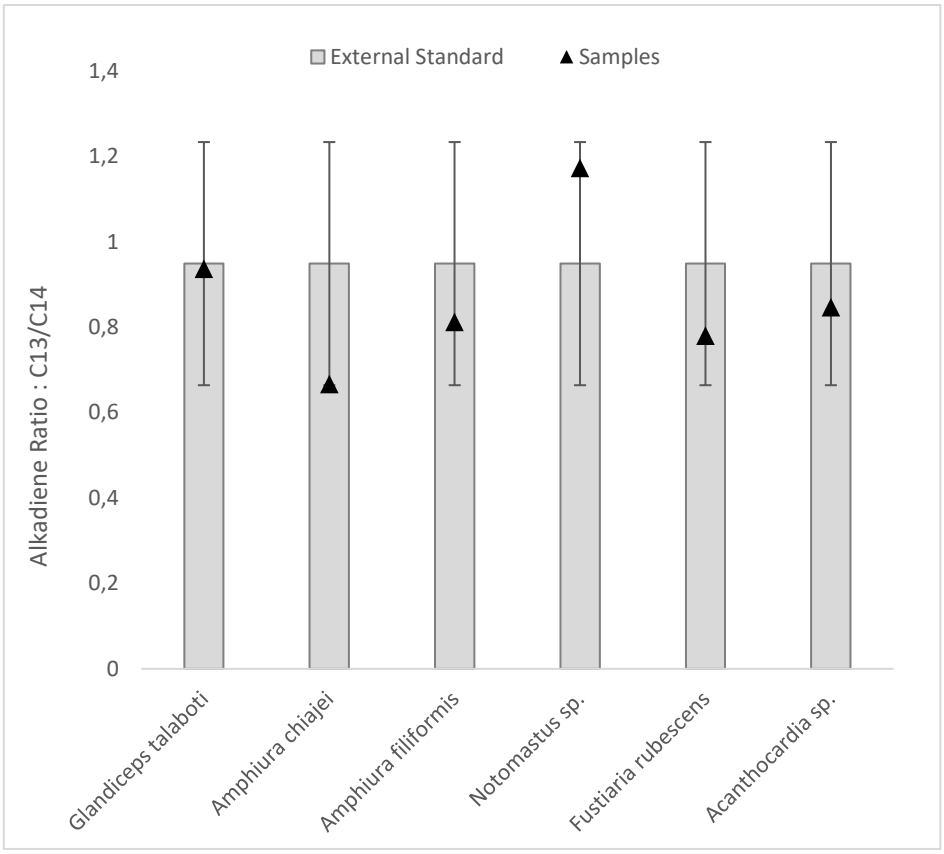

Figure SI 1 : Ratio of the peak areas for the two indicator compounds selected to monitor PE: the alkadienes congeners with 13 and 14 carbons atoms. The grey bars represent the mean values calculated for the external standards (within the calibration range presented) and the black triangles represent the values obtained for the samples. The error bars set at 30% represent the validation criterion adopted to ensure that PE quantification exclude the signal of natural organic matter.

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