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Development and optimisation of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues

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**DEVELOPMENT AND OPTIMISATION OF A STANDARD METHOD FOR EXTRACTION OF
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**DEVELOPMENT AND OPTIMISATION OF A STANDARD METHOD FOR EXTRACTION OF
MICROPLASTICS IN MUSSELS BY ENZYME DIGESTION OF SOFT TISSUES**

Running title: Standard method for microplastics extraction from mussels

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Abstract: We compared procedures for digestion of mussel soft tissues and extraction of microplastics (MPs). Complete tissue digestion was achieved with 1M NaOH, 35% HNO₃ and by 0.1 UHb/mL protease, but use of HNO₃ caused unacceptable destruction of some MPs. Recovery of MPs spiked into mussels was similar (93±10%) for NaOH and enzyme digestions.

We recommend use of industrial enzymes based on digestion efficiency, MP recovery and avoidance of caustic chemicals. This article is protected by copyright. All rights reserved

Keywords: Enzymatic digestion, FT-IR, Microplastics, Mussels, Emerging pollutants,

INTRODUCTION

The presence and accumulation of plastic debris within marine environments has become an issue of high priority for environmental policy [1]. Microplastics [(MPs), particles between 5 mm [2] and 1 μm [3]], are reported as the most abundant pieces of plastics found in the marine environment [4] and these particles have accumulated at the sea surface on shorelines and in sediments [5]. They are also present in organisms [6] and are of toxicological concern [3].

Standardised methods for detection of MP accumulation in organisms are necessary to establish levels of exposure, facilitate comparison among studies, and to enable robust assessments of MPs risks in the environment.

Mussels are particularly good candidates for assessment of MP exposure, in the same way as they are used as indicator species for other environmental contaminants in monitoring programmes such as Mussel Watch (The National Oceanic and Atmospheric Administration, NOAA, USA). If standardised methods for extraction and quantification of MPs in tissues are established and based on good laboratory practice, mussels can act as sentinel species of MP contamination applicable over a wide range of geographical scales. For instance, *Mytilus* spp. are intertidal mussels with a large geographical distribution, they filter large volumes of water, are relatively immobile, and are easily accessible for collection throughout the year. Laboratory studies have demonstrated ingestion of MPs by *Mytilus* spp [7–9], and gut retention times for MPs can be above 72 h [8]. Microplastics have also been found in both wild and cultured *Mytilus* spp., but different soft tissue digestion and quantification methods make comparison of results challenging. For example, particles were found at concentrations between 5 - 75 particles per mussel in Nova Scotia [10], but in other studies the reported concentrations were 0.36 particles g^{-1} wet weight from North Sea coasts [11]) and up to 0.34 particles g^{-1} wet weight from various

European specimens [12].

A standardised and specific method for extraction and quantification of MPs from mussels is necessary to provide the data needed to assess levels of exposure of organisms to MPs, and to provide support for environmental monitoring programmes and management decisions. Recently, Vandermeersch et al. [12] reviewed and compared acid digestion procedures used for soft tissue digestion of *Mytilus* spp., but their evaluation did not consider approaches for tissue digestions that use strong bases or enzymes. Some methods used for extraction of MPs from mussels may not enable accurate quantification of MP abundance. Techniques used for extraction of MPs can alter the shape or destroy of particles present in samples. Extraction of MPs from bivalves in general, and *Mytilus* spp. mussels in particular, has been accomplished by chemical digestion with simple and/or mixtures of strong acids (HCl, HNO₃, HClO₄) [6,11,13] and bases (NaOH, KOH) [14–16]; however, some of these methods can damage and/or destroy pH-sensitive polymers [11,13]. Hydrogen peroxide (H₂O₂) has also been used to digest tissue prior to extraction of MPs, but limitations including incomplete soft tissue digestion and production of foam was indicated to cause lower MP recovery from samples [10,13]. Although not previously used to digest mussel tissues, enzyme digestion has been applied to extract MPs from plankton-rich seawater samples, with reported high digestion rates (up to 97.7 %) and no damage of particles [17]. Similarly, in forensic studies, enzyme digestion (industrial proteases and lipases by Novozymes) has been used as a method for soft tissue digestion, and which additionally does not cause bone damage [18], indicating the potential use of industrial enzymes (used in washing powder and food industry, for instance) to digest soft tissue in other organisms.

The need for a standardised method to assess MPs from organisms, including mussels, has been highlighted by the International Council for the Exploration of the Sea (ICES) advice

provided to the OSPAR Commission on plastic monitoring in organisms [19] and more recently by Vandermeersch et al. [12]. Our goal was to describe a procedure for extraction and quantification of MPs in marine mussels. This method was developed specifically for *Mytilus* species, as the digestion of soft tissue from other organisms will differ in methodological requirements (e.g. chitinous tissues in crustaceans and gut content analysis in large fish [20]). Our approach was first to optimise the digestion efficiency of *Mytilus edulis* soft tissues by 3 different methods: strong acid, strong base, and a new enzyme procedure. This new enzyme procedure uses industrial enzymes that are less expensive than other enzymes used for tissue digestion in laboratory research. Rates of soft tissue digestion were compared and the effects of each digestion method on polymer integrity were assessed by FT-IR analysis of extracted MPs from spiked samples. In addition, spike recovery rates of MPs were determined, airborne fibre contamination assessed, and the industrial enzyme digestion procedure was applied to quantify MPs in *M. edulis* exposed in the field to waters containing MPs. To enable reproducibility, a more detailed standard operating procedure (SOP) based on Good Laboratory Practice (GLP) guidelines [21], is provided in the supplement section.

METHOD DEVELOPMENT

Development of soft tissue digestion of mussels

Three tissue digestion agents were tested under the same conditions to determine the method that provided the most complete digestion of soft tissue with the least damage to plastics. The 1st method used a strong acid (HNO₃: 0, 9, 18, 35, 50 % (v/v); # 10050270 Fisher Scientific) and the 2nd method a strong base (NaOH: 0.25, 0.5, 1.0, 2.5, 5.0 M; # 10142590 Fisher Scientific). Both methods were based on previous procedures used for digestion of mussels for MP quantification using strong acids [6,11,19] or strong acids or bases[13,17]. Selection of

NaOH was because it is a strong base and its base dissociation constant (pK_b) is representative of other strong bases (e.g. KOH). The 3rd method, enzymatic digestion, used an industrial protease, Corolase 7089 (AB Enzymes), obtained from *Bacillus subtilis* cultures (activity of 840 UHb) at volumes of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1 mL to 100 mL of Milli-Q water (Millipore, filtered at 0.22 μm). This enzyme is available commercially and is considerably less expensive than enzymes offered by scientific supply companies. Corolase 7089 is active at pH 6 – 9, allowing its use in water without addition of a buffer, as required by other proteases.

With the exception of the agent used, all conditions for tissue digestion were constant. Blue mussels *Mytilus edulis* were obtained from local consumer fish markets (September and December 2014), and specimens were frozen at $-20\text{ }^\circ\text{C}$ prior to digestion. Mussels were defrosted at room temperature (up to 2 h); all soft tissues were removed from the shell, weighed (wet weight to 0.01 g), and placed in a 250-mL glass Erlenmeyer flask for digestion (one mussel per flask). A 100 mL volume of the digestion agent at the indicated concentrations (see above, current section) was added to each Erlenmeyer flask, which were covered with aluminium foil and placed on a magnetic multi-stirrer plate (up to 10 flasks simultaneously), and stirred for approximately 1 h at $60\text{ }^\circ\text{C}$. The $60\text{ }^\circ\text{C}$ temperature was selected as it is within the range of action of the industrial enzymes (optimum at $55\text{ }^\circ\text{C}$, activity up to $65\text{ }^\circ\text{C}$) and this temperature was unlikely to affect plastics [22]. After digestion, the final product was vacuum filtered [Whatman filters of cellulose nitrate $0.8\text{ }\mu\text{m}$ or glass microfiber $1.6\text{ }\mu\text{m}$, when MPs were to be analysed by FTIR (see section *Effect of digestion procedure on polymers*)] and remaining intact soft tissue on the filter membrane was weighed ($< 0.01\text{ g}$). Digestion efficiency (%) was calculated as the percent of tissue that remained after digestion [i.e., $(1 - \text{final weight} / \text{initial weight}) \times 100\%$]. Each digestion procedure was tested with 2 independent replicates per

concentration. To avoid contamination of samples by airborne fibres and other particles throughout all procedures, the recommendations of ICES [19] and Woodall et al. [23] were followed. Samples were covered to avoid air exposure, vials were capped with aluminium foil during digestion, personnel used protective cotton lab coats, equipment was thoroughly rinsed using Milli-Q water, and glassware was acid-washed prior to use. Procedural blanks, ie, positive controls, to account for airborne fibres contamination, were conducted simultaneously during soft tissue digestions.

Effect of digestion procedure on polymers

To determine the effects of digestion on MPs, MPs of a single polymer type spiked into *M. edulis* samples (one individual per flask) were evaluated after digestion of mussel tissue. Particles were selected based on commonly found particles in marine litter [24] and include polyethylene terephthalate (PET), PET flakes, high-density polyethylene (HDPE), polyvinyl chloride (PVC), all between 500 - 125 μm , and Nylon, between 1,000 - 500 μm . The particles used were obtained from Plastic Industry Development Center, Taiwan, (PET, PVC), Dow Chemical Co. (HDPE) and PET Processors LLC (PET flakes). Nylon particles were cut under a dissection microscope from Nylon thread (obtained from efco), and resulting particles were sorted by size ranges using stainless steel sieves.

Based on the previous observations, overnight (> 12 h) digestions of soft tissue were performed with Corolase 7089 (1 mL to 100 mL Milli-Q water), 1 M NaOH and 35 % HNO_3 . For each digestion agent, 2 replicates were used per type of polymer. Soft tissue was weighed to the nearest decimal (WW) and digestion efficiency was calculated as a percentage of soft tissue digested. After digestion and subsequent filtration, all filters were placed in covered plastic petri dishes and oven dried for approximately 24 h at 60 °C. Filters were observed with a dissection

microscope and particles were stored in closed vials until further use (cork lid, to avoid crossed contamination by other polymers).

Transmittance FT-IR (Brüker IFS 66 Spectrometer with a Bruker Hyperion 1,000 microscope) was used to determine if integrity of polymers was altered during the digestion procedures (i.e. if the polymer would still be identifiable after digestion). Prior to the analysis, MP specimens were placed into a Specac DC-2 diamond compression cell and flattened using manual pressure, reducing the thickness to allow for suitable absorbance. For each particle, the type of polymer was identified by generating a spectrum (after 32 scans) and comparing it against a spectral database of synthetic polymers (Brüker I26933 Synthetic fibres ATR-library) [24]. An FT-IR analysis was also performed on particles from the original stock of each polymer not subjected to any digestion procedure.

Recovery rate of particles and assessment of airborne fibres contamination

The recovery rates of MPs that were spiked into water samples prior to digestion were assessed to determine the ability of the digestion method to quantify MPs in unknown samples. In each vial, 30 particles of a single type of MP [PET, HDPE, or Nylon (all particles < 500 µm)] were added and there were 3 independent replicates for each type of MP. Two procedures, enzyme digestion (Corolase 7089, activity 840 UHb, dilution 1:100) and 1 M NaOH, were selected based on results of experiments described in the section *Effect of digestion procedure on polymers* and tested separately, only in Milli-Q water. The digestion procedure was as described in the section *Effect of digestion procedure on polymers* (overnight digestion in 30 mL of Milli-Q water) and samples were filtered (0.8 µm filters) before being oven dried at 60 °C for approximately 24 h. A stereomicroscope was used to count the number of MPs on the dried filter, and particle recovery rate (%) is expressed as the number of MPs counted divided by the

number of MPs spiked into the sample (i.e., MPs counted / 30×100 %). As selected particles were easily identifiable and distinct from possible contamination sources, the same samples were further examined for the presence of airborne fibres, i.e. the number of fibres present in the filters were quantified. Differences in recovery of MPs (i.e., percent recovery) were tested by two-way ANOVA with particle type and digestion procedure as independent factors along with the particle type \times digestion procedure interaction term. The number of plastic particles (not spiked MPs) that contaminated samples (i.e., the particles that entered during digestion method or by airborne contamination) was compared between the 2 digestion methods by t-test. For all statistical tests, normality and homoscedasticity were tested and a probability level of $p < 0.05$ was used to determine if differences were statistically significant, and all analyses were done using the software Statistica (StatSoft, Inc).

Application of mussel digestion method to quantify MPs in wild mussels

Live *M. edulis* (obtained from a commercial supplier) were held in the intertidal zone for 18 days (January/February 2015) in cylindrical stainless steel mesh cages (10 x 8 cm, height and diameter respectively) in the estuary of the Forth River, Edinburgh, UK, in Port Edgar (N 55°, 59'42", W 3°,24'30"). Eight mussels from 2 cages (4 mussels per cage) were digested overnight (60 °C) with Corolase 7089 enzyme (activity 840 UHb, dilution 1:100), and MPs quantified according the methods described in the section *Effect of digestion procedure on polymers*. To assess airborne fibre contamination during this procedure, 2 Milli-Q water control samples (100 mL) were submitted to the same procedure, and 4 damp filters were held in plastic petri dishes under the same conditions as other filters and oven dried (60 °C for ~ 24 h). All filters were examined with a stereomicroscope for enumeration of particles. Differences in the number of MPs found in mussel samples were compared between deployed cages by t-test and considered

significant at a probability level of $P < 0.05$ (Statistica, StatSoft, Inc).

RESULTS AND DISCUSSION

Each of the three procedures, acid, base and enzymatic digestion, digested all of the mussel soft tissue present in the tested samples. The minimum concentrations required to achieve complete digestion of soft tissue after 1 h at 60 °C, were 1 M for NaOH, 35 % (v/v) HNO₃, and 0.5 mL of Corolase 7089 to 100 mL of water. Although a 100 % digestion efficiency occurred, visual inspection of the filters revealed the presence of tissue residues (less than 0.01 g) in all tested methods. These residues were very small pieces of soft tissue that were below the range of conventional balance (< 0.01 g) and considered not to interfere with MPs quantification. To guarantee complete digestion of soft tissue, overnight (~12 h) digestion is recommended and was used in all subsequent digestions. An additional 1 mL of enzyme solution to 100 mL of water was used for mussels weighting between 2 - 5 g, to ensure complete digestion. For larger mussel samples (e.g., 8 - 21 g WW of *Modiolus modiolus*) a similar complete digestion was achieved by increasing the enzyme volume to 2 mL of Corolase in 100 mL of Milli-Q water (data not shown).

We obtained higher mussel soft tissue digestion efficiencies using lower concentrations of HNO₃ and NaOH after a shorter period of time, than those reported by other authors. For instance, mussel samples were reported not to have been 100 % digested using 52.5 M NaOH (during 1 h at 60 °C plus 1 h at 100 °C) [13], while complete digestion occurred in the present study at 1 M NaOH after 1 h. Similarly, for HNO₃ digestion, Claessens et al. [13] reported lower digestion efficiency for *M. edulis* after use of 22.5 M HNO₃ (98.9 % at 1 h at 60 °C plus 1 h at 100 °C). Our samples were fully digested at 35 % (v/v) HNO₃ after 1 h and it is possible that higher digestion efficiencies obtained in our method can be explained by use of frozen samples (increased destruction of cells) and mild stirring during the procedure (increased mechanical

disaggregation of tissue and contact with chemical agents).

The integrity of plastics spiked into mussels was affected by digestion method. Visual inspection of samples revealed that HNO₃ digestion induced melding (i.e. fusing and/or merging) of some PET and HDPE particles, and that all Nylon fibres were no longer present at the end of overnight digestion. Plastic polymers have viscoelastic properties that can be altered by temperature and chemical action, but that will not necessarily affect their chemical composition/optical properties[25]. Despite possible changes in particle morphology, all particles present were able to be identified using FT-IR (Figure 1). However, loss of material and melding of particles done by chemical digestion may lead to erroneous quantification of MPs. Loss of nylon fibres due to strong acid digestion of mussels has been reported previously [13], and likely leads to underestimation of MPs in wild mussel samples [11]. Digestion of tissues by strong acids has also been reported to meld and/or damage MPs in the digestion of soft tissue from fish [26] and plankton [17], and was not recommended by these authors. Therefore, we discontinued further use of acid digestion and agree that acid digestion should not be used for MPs extraction and quantification.

The recovery of particles spiked in to mussel samples did not differ among particle types tested [HDPE, PET and Nylon particles ($p = 0.06$)] or digestion method [NaOH, Coralase, ($p = 0.74$)]. Although not statistically significant, the mean recovery of the Nylon particles was lower than for the other polymers, as well as more variable: mean recovery of Nylon was 85 % \pm 13.2 SD ($n = 6$) compared to 97 % \pm 6.3 SD ($n = 6$) for PET and 98 % \pm 2.0 SD ($n = 6$) for HDPE. This was likely due to difficulties in working with Nylon particles because they appeared to have higher static electricity and prevention of particle loss was more difficult. The mean recovery rate for enzymatic digestion was 93 % \pm 10.8 SD ($n = 9$) and for 1 M NaOH digestion was 94 %

(± 10.0 SD, $n = 9$), with a total mean recovery rate of $93 \% \pm 10.1$ SD ($n = 18$). These spike recovery values are consistent with the $93.6 - 98.3$ % recovery rates after acid digestion of mussels reported by Claessens et al. [13].

The suitability of the enzymatic digestion protocol was verified in *M. edulis* live specimens placed in the field, showing practical applicability of the method. Fibres, films and particles (spherules, spongious and other particles) were extracted and quantified (Table 1).

Specimens from 2 different cages placed in the field did not present any difference in amount or type of particles extracted ($p > 0.40$). The number of particles is within the expected range with fibres reaching 10.4 per g mussel WW ± 3.42 SD), particles detected were 0.9 ± 0.99 g⁻¹ mussel WW \pm while films was 1.3 ± 2.38 g⁻¹ mussel WW. Mussels from the North Sea collected in Belgium were reported to have 0.36 particles g⁻¹ wet weight soft tissue [11].

Airborne fibres contamination did not differ between tested digestion methods NaOH and Corolase ($p = 0.15$) and the mean number of fibres observed per sample was of 5 ± 6.4 SD fibres per sample ($n = 18$). In the enzymatic digestion of field samples, the observed airborne fibre contamination within the same range (3 and 6 fibres). This level of airborne fibre contamination is consistent with reported contamination of the method used by De Witte et al. [6] for acid extraction of MPs of mussels (limit of detection of airborne fibres between $1.5 - 4.7$ fibres per analysis). Some earlier studies, eg [16] on MPs quantification in mussels failed to report on airborne fibres contamination. Other studies, such as Mathalon and Hill [10], reported up to 100 plastic fibres per digested sample that were attributed to airborne contamination. The use of procedural blanks for systematic monitoring of fibre contamination and the application of good laboratorial practices is essential for quality assurance for quantification of MPs in tissues.

Contamination from airborne fibres can occur at any time during the digestion procedure, but

samples are possibly more vulnerable during initial stages, such as open-air dissection and weighting of samples. According to our results, in the filters only submitted to oven drying ($n = 4$), when samples were covered, the mean airborne fibre contamination was low, 0.8 ± 1.5 SD fibres per filter. Therefore, special care should be taken during initial procedural stages (e.g. dissection, sample digestion, and processing), and the manipulation of samples in a confined and clean room and/or laboratorial hood is recommended. The use of procedural blanks during the entire procedure is essential to monitor the presence of airborne fibres contamination and to assure a more accurate quantification of observed MPs from field samples. Quality assurance and data reliability of data are important considerations, and must be consistent with international recommendations for good laboratory practice (GLP) as recommended by the EFSA Panel on Contaminants in the Food Chain (CONTAM) [20].

Based on soft tissue digestion efficiency, ability to maintain MP integrity during digestion, and high MP spike recovery, the enzyme digestion method described in the present research is offered to become a standard method for MP quantification in mussels (see supplemental data for Standard Operating Procedure, SOP). We believe that our method provides not only a high MP recovery rate, but it also enables recovery of sensitive MPs without damage, and increases the utility of this extraction method for a more accurate estimation of the number of MPs present in bivalves. Furthermore, the use of industrial enzymes is a safer procedure than use of NaOH, and can be conducted without use of a fume cupboard. Compared to other enzymes used in laboratory procedures, industrial proteases have the advantage that they are supplied in a liquid form that does not need to be buffered, and they present lower hazard problems compared to powder forms. In summary, the method advocated here consists of soft tissue digestion overnight (~ 12 h) at $60\text{ }^{\circ}\text{C}$, in a stirred preparation of water and industrial

proteases. After digestion, the preparation is filtered, the filter paper is dried in a covered plastic petri dish at 60 °C (~ 24 h), and quantification of MPs is conducted by examination with a stereomicroscope (60 - 310 x magnification). This method is relatively inexpensive, can be applied in most laboratories, and if employed as described will enable direct comparison of MPs quantification among future studies. The application of this method as a standardised procedure will enable MP assessment to be integrated into existing environmental monitoring programmes, such as the Mussel Watch (The National Oceanic and Atmospheric Administration, NOAA, USA).

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (catarino.anai@gmail.com).

REFERENCES

1. Galgani F, Fleet D, Franeker J van, Katsavenakis S, Maes T, J JM, Oosterbaan L, Zampoukas N, Poitou I, Hanke G, Thompson R, Amato E, Birkun A, Janssen C. 2010. *Marine Strategy Framework Directive Task Group 10 Report Marine litter*.
2. Arthur C, Baker J, Bamford H. 2009. Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris. *NOAA Tech. Memo. NOS-OR&R-30.*, p 47.
3. Browne MA, Galloway T, Thompson R. 2007. Microplastic—an emerging contaminant of potential concern? *Integr. Environ. Assess. Manag.* 3:559–561.
4. Eriksen M, Lebreton LCM, Carson HS, Thiel M, Moore CJ, Borerro JC, Galgani F, Ryan PG, Reisser J. 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS One.* 9:e111913.
5. Claessens M, De Meester S, Van Landuyt L, De Clerck K, Janssen CR. 2011. Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Mar. Pollut. Bull.* 62:2199–204.
6. De Witte B, Devriese L, Bekaert K, Hoffman S, Vandermeersch G, Cooreman K, Robbens J. 2014. Quality assessment of the blue mussel (*Mytilus edulis*): comparison between commercial and wild types. *Mar. Pollut. Bull.* 85:146–55.
7. Browne M a, Dissanayake A, Galloway TS, Lowe DM, Thompson RC. 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L). *Environ. Sci. Technol.* 42:5026–31.
8. Ward JE, Kach DJ. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Mar. Environ. Res.* 68:137–142.

9. von Moos N, Burkhardt-Holm P, Köhler A. 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ. Sci. Technol.* 46:11327–11335.
10. Mathalon A, Hill P. 2014. Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. *Mar. Pollut. Bull.* 81:69–79.
11. Van Cauwenberghe L, Janssen CR. 2014. Microplastics in bivalves cultured for human consumption. *Environ. Pollut.* 193:65–70.
12. Vandermeersch G, Van Cauwenberghe L, Janssen CR, Marques A, Granby K, Fait G, Kotterman MJJ, Diogène J, Bekaert K, Robbens J, Devriese L. 2015. A critical view on microplastic quantification in aquatic organisms. *Environ. Res.* 143:46–55.
13. Claessens M, Van Cauwenberghe L, Vandegheuchte MB, Janssen CR. 2013. New techniques for the detection of microplastics in sediments and field collected organisms. *Mar. Pollut. Bull.* 70:227–33.
14. Rochman CM, Tahir A, Williams SL, Baxa D V, Lam R, Miller JT, Teh F, Werorilangi S, Teh SJ. 2015. Anthropogenic debris in seafood : Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. *Nat. Publ. Gr.*:1–10. doi:10.1038/srep14340.
15. Dehaut A, Cassone A-L, Frère L, Hermabessiere L, Himber C, Rinnert E, Rivière G, Lambert C, Soudant P, Huvet A, Duflos G, Paul-Pont I. 2016. Microplastics in seafood: Benchmark protocol for their extraction and characterization. *Environ. Pollut.* 215:223–233.
16. Claessens M, Van Cauwenberghe L, Vandegheuchte MB, Janssen CR. 2013. New techniques for the detection of microplastics in sediments and field collected organisms. *Mar. Pollut. Bull.* 70:227–233.
17. Cole M, Webb H, Lindeque PK, Fileman ES, Halsband C, Galloway TS. 2014. Isolation of

microplastics in biota-rich seawater samples and marine organisms. *Sci. Rep.* 4:4528.

18. Simonsen KP, Rasmussen AR, Mathisen P, Petersen H, Borup F. 2011. A fast preparation of skeletal materials using enzyme maceration. *J. Forensic Sci.* 56:480–484.

19. ICES. 2015. *OSPAR Request on Development of a Common Monitoring Protocol for Plastic Particles in Fish Stomachs and Selected Shell fish on the Basis of Existing Fish Disease Surveys.*

20. CONTAM. 2016. Presence of microplastics and nanoplastics in food, with particular focus on seafood. *EFSA J.* 14:4501–4531.

21. World Health Organization. 2009. *Handbook: Good Laboratory Practice.* Department for International Development, Geneva. doi:DOI 10.2471/TDR.09. 978-924-1547550.

22. dos Santos WN, de Sousa JA, Gregorio Jr R. 2013. Thermal conductivity behaviour of polymers around glass transition and crystalline melting temperatures. *Polym. Test.* 32:987–994.

23. Woodall LC, Gwinnett C, Packer M, Thompson RC, Robinson LF, Paterson GLJ. 2015. Using a forensic science approach to minimize environmental contamination and to identify microfibrils in marine sediments. *Mar. Pollut. Bull.* 95:40–46.

24. Thompson RC, Olsen Y, Mitchell RP, Davis A, Rowland SJ, John AWG, McGonigle D, Russell AE. 2004. Lost at sea: where is all the plastic? *Science (80-)*. 304:838.

25. Benedek I. 2004. *Pressure-Sensitive Adhesives and Applications.* CRC Press.

26. Avio CG, Gorbi S, Regoli F. 2015. Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from Adriatic Sea. *Mar. Environ. Res.* 111:18–26.

Table 1. The number (mean \pm SD, $n = 18$) of microplastics (fibres, particles and films) per mussel and per g wet weight (WW) of mussel

	Fibres	Particles	Films
MPs / mussel	10.4 \pm 3.42	0.9 \pm 0.99	1.3 \pm 2.38
MPs / g (ww)	2.0 \pm 0.42	0.2 \pm 0.21	0.3 \pm 0.59

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Figure 1. FT-IR spectra of tested polymers (PVC, HDPE, PET and Nylon) spiked in mussel samples: 1) not submitted to digestion (original particles) and 2) after Corolase digestion (post dig.)

