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Review

A review on methods for extracting and quantifying microplastic in biological tissues

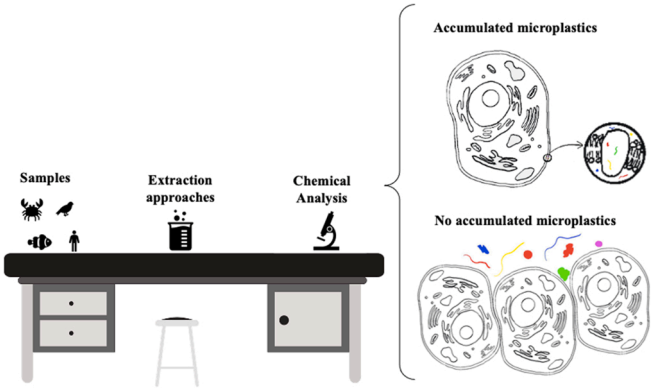
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HIGHLIGHTS

- Digestion methods depends on the chemical substrates of the matrix.
- Accumulation of microplastics in tissues depends on their size.
- Application of analytical techniques depends on the size range of microplastics.
- Large microplastics (> 100 μm) cannot reach internal tissues.

GRAPHICAL ABSTRACT



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ABSTRACT

Literature about the occurrence of microplastic in biological tissues has increased over the last few years. This review aims to synthesis the evidence on the preparation of biological tissues, chemical identification of microplastic and accumulation in tissues. Several microplastic's extraction approaches from biological tissues emerged (*i.e.*, alkaline, acids, oxidizing and enzymatic). However, criteria used for the selection of the extraction method have yet to be clarified. Similarly, analytical methodologies for chemical identification often does not align with the size of particles. Furthermore, sizes of microplastics found in biological tissues are likely to be biologically implausible, due to the size of the biological barriers. From this review, it emerged that further assessment are required to determine whether microplastic particles were truly internalized, were in the vasculature serving these organs, or were an artefact of the methodological process. The importance of a standardisation of quality control/quality assurance emerged. Findings arose from this review could have a broad implication, and could be used as a basis for further investigations, to reduce artifact results and clearly assess the fate of microplastics in biological tissues.

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1. Introduction

Recently, World Health Organisation (WHO) confirmed that human populations are exposed to microplastic pollution [1]. This is due to its ubiquity in the environment and hence the range of exposure matrices it contaminates. Microplastic pollution is transmitted through food chains and air following release, including from everyday plastic materials such as clothes and food packaging [2]. The release of microplastic particles from food packaging [2] and their presence in household dust [3] highlight the plausibility for accidental ingestion or inhalation [4]. Ingestion represents an exposure pathway for humans [5], supported by observations of microplastic in digested human stool samples [4]. Animals are also exposed to microplastics [6–9]. However, whilst there have been increasing publications on internal microplastic exposure in biological tissues in recent years, analysis is still hampered by several factors, such as the lack of harmonized and standardized protocols and technical challenges in the extraction, quantification and qualification of microplastic [10], especially in human tissues. In addition, analytical techniques are complicated, and compromised by matrix effects that have potential to interfere with analysis, including after the pre-treatment/purification of the sample [11]. Pre-treatment methods for biological samples mainly consist of chemical or enzymatic digestions for the removal of organic matter. Chemical digestions can be acidic (*e.g.*, nitric and/or hydrochloric acid [12,13], basic (*e.g.*, potassium hydroxide [14] or sodium hydroxide [15]), or oxidizing (*e.g.,* hydrogen peroxide [16], Fenton's reagent [17], sodium hypochlorite [18]). Biological matrix removal can also be achieved through enzymatic digestions, such as trypsin, papain, collagenase [19] and proteinase-K [20]. Agents used for the pre-treatment of biological samples are evaluated in terms of their impact on microplastic particles and efficiency in both the time resource needed and the relative mass of matrix removed [20]. An appropriate digestion agent should be able to guarantee a high efficiency in removing the organic matter as well as having the least destructive impact on polymer integrity [21]. Quantification of microplastic particles extracted from biological tissues is still challenging. This step is commonly performed by a visual identification and subsequent micro-spectroscopic analysis [14,22,23]. However, recently pyrolysis gas chromatography mass spectrometry (Py-GC-MS) has been used for the chemical identification and indicative quantification of microplastic in biological samples [24]. The bioavailability and biodistribution of microplastic particles have been widely studied in biota and experimental animal models [10,11,25–40], mostly in marine organisms [26,36,37], albeit for restricted microplastic types (*i.e.*, mainly polystyrene (PS) spheres). The location and level of such microplastic accumulation strongly depends on the species, as well as particle physicochemical properties, such as size [36]. Polystyrene microplastic particles have been observed in gut, liver and kidney tissues of mice following experimental exposure, but any related adverse effects and whether microplastic follows the same fate in the human body are unclear [41]. Studies on the accumulation of microplastic in the human body are scarce due to significant analytical difficulties and those that have been published challenge what is known regarding the biological plausibility and likelihood of translocation relative to particle size. More evidence is needed to better to understand the phenomenon. Given the rapidly increasing interest in microplastic research, the aim of this study is to provide a synthesis of current knowledge for microplastic quantification in biological tissues (animal and human) and to serve as a basis for optimizing the available methodologies. It will focus on: *i)* sample preparation methodologies for organic matrix removal for microplastic analysis, *ii)* the analytical methods used for microplastic quantification in biological tissues, and *iii)* the biodistribution of microplastics in biological (organismal) tissues.

This study aims to build on existing reviews on microplastic accumulation, *e.g.*, in humans, by analysing the occurrence of microplastic in biological tissues in relation to particle size. In doing so, we attempt to distinguish which particles were truly accumulated through a biological process. This clarification could be useful for understanding the fate of microplastic particles in living organisms.

2. Materials and methods

This review was conducted following the Prisma approach and guidelines, detailed in the Supplementary Materials. Briefly, the research question (Supp. Mat.) was constructed following the PICO (Population, Intervention, Comparison, Outcomes) model, which informed inclusion and exclusion criteria.

2.1. Information sources

Medline, Scopus and PubMED databases were used in this review, and were consulted up to 14 February 2022.

2.2. Search strategy

For this review, the search was conducted using the databases reported above. Hence, the "research question" identified the key concepts and free-text terms. Key words used for each key concept are reported in Table S1. The development of search terms was carried out using the Medline Ovid database. The first concept indicated the subject of the research, while the second and third ones are related to the target (*i.e.,* organismal tissue) and methodologies, respectively. Some search terms were "exploded" in order to include all narrower and more specific terms in the search. The terms were then combined using the Boolean operator "OR" and "AND", following the protocol reported in Table S1. Studies were thus selected according to the inclusion and exclusion criteria by means of the review software Covidence, detailed in Supp. Mat.

2.3. Synthesis methods

The studies were divided into two categories: studies focusing mainly on the methodological aspect, *i.e.*, sample preparation and qualitative and quantitative analysis, and studies focusing mainly on the biological aspect, *i.e.*, uptake modes, accumulation of microplastic in biological tissues, potential translocation between tissues and/or organs and adverse effects on organisms (Fig. 1). If the studies expressed the results in different units, the results were harmonized by comparing microplastic content in tissue.

3. Results

3.1. Overall summary

Two hundred and seventy-six studies were initially retrieved before the screening began. Following the title and abstract screens, 137

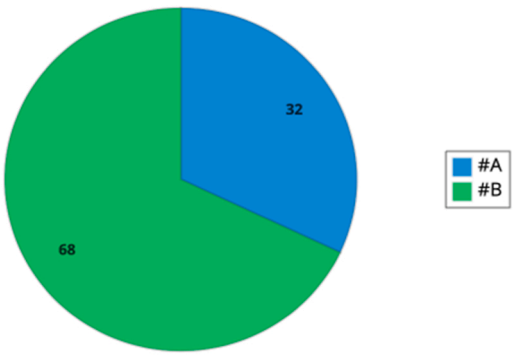


Fig. 1. Graphical representation of the topics covered by the studies considered in the present review; A) analytical studies (32%) and B) biological studies (68%).

articles remained. Full-text screening excluded a further 52 studies, resulting in 84 studies being included in this review. The proportion (%) of resulting studies on microplastic by geographical region is reported in Fig. S1. The studies included in the review split across several themes concerning the microplastics research field (Fig. 1). Concerning the type of organism, 69.8% of studies focused on marine vertebrates, 24.7% focused on marine invertebrates. Just under 2% dealt with the determination of microplastic in mammalian models/tissues and 3.5% concerned human tissues (Fig. 2) whereas the type of tissue investigated in included studies are detailed showed in Fig. 2. These studies have been summarized by how biological tissues were prepared and analysed, with particular regard to extraction (digestion) protocols, and what was found in terms of polymer distribution in tissues, microplastic particle uptake, accumulation and potential for translocation.

3.2. Sample preparation of biological tissues for microplastic analysis

Microplastic quantification in biological tissues typically requires an efficient extraction step. Particularly, biological matrix can mask microplastic particles, and thus it has to be removed generally by digestions [42]. Efficient digestion solutions should eliminate a high majority of biological tissues without damaging the target microplastic analytes [13,43]. Due to the increasing interest on the topic in the scientific community, simple approaches for analysing microplastic in biological tissues are available. These consist of the dissection and physical disruption of biological tissues followed by direct visual observation of potential microplastic particles in it. However, using this technique alone, recovery rates of target microplastic does not exceed 60% [45], resulting in an underestimation of microplastic, *e.g.*, in marine organisms [42]. At present, purifying the sample by removing organic matter seems to be the optimum approach for microplastic identification in tissues [46]. Several methods for this have been published, and are predominantly based on the use of acids, bases, oxidizing agents and, lastly, enzymes (Table 1). Firstly, by synthesising the published data, digestion agents were evaluated by their digestion efficiency. In terms of visual assessment, a digestion can be considered efficient if there is no debris and organic matter (*e.g.*, debris that vary according to the type of digested tissue, for example shell or cartilage in the case of seafood) remaining, which could obstruct the subsequent detection of microplastic particles [12]. Typically, digestion efficiency is empirically measured as a % reduction of the original sample mass. In addition, the recovery rate of microplastic particles (*e.g.*, assessed *via* an internal control/spiking test), adverse effects on polymers (*e.g.*, corrosivity test) [47], and time required to complete the digestion are evaluated in a digestion protocol. However, in the scientific literature there is a lack of studies evaluating the digesting agents as a function of the chemical composition of the tissue to be digested. Such an evaluation would make the application of digestion protocols more systematic.

3.3. Digestion efficiency of biological tissues: bases, acids and enzymatic agents

Digestion efficiency determines the choice of reagent for a biological matrix [42]. Digestion reagents can be divided into two categories: chemical and enzymatic (Table 1). Chemical digestion is conducted through alkaline (*i.e.*, potassium hydroxide, KOH; sodium hydroxide, NaOH), acidic (*i.e.*, nitric acid, HNO_3; hydrochlorid acid, HCl) and oxidizing (*i.e.*, hydrogen peroxide, H_2O_2; Fenton's reagent, H_2O_2 and iron (II); sodium hypochlorite, NaClO) reagents. In terms of alkaline reagents, method development studies found satisfactory digestion efficiencies ($98 \pm 0.33\%$) of fish muscle and skin using KOH (10% aqueous solution at 50 °C for 48–72 h). Furthermore, experimental evidence suggested that temperatures of 40–50 °C facilitate this, with efficiencies of $98.6 \pm 0.1\%$ and $98.9 \pm 0.33\%$, while temperatures of 25 and 60 °C achieve slightly lower digestion efficiencies ($97.1 \pm 1.3\%$ and $97.6 \pm 0.2\%$) [42]. Acid agents were applied to digest digestive gland and gills of Pacific oyster *Magallana gigas*. For instance, HNO_3 (65%) showed satisfactory digestion efficiency (97.8–100%) at 40 °C [56]. Fish muscle and skin digestions have also been carried out using acid. Particularly, both concentrated (37%) and diluted (10%) HCl have been used. Application of HCl (10%) to fish muscle and skin at room temperature did not lead to an optimal digestive efficiency (< 73%) within 96 h, while the same time interval was optimal (> 95%) when using concentrated HCl (37%). For oxidizing agents, oxidizing agents were used to digest digestive gland and gills of Pacific oyster *Magallana gigas*. For example, H_2O_2 showed digestion efficiencies up to 88% and 92.1% for digestive gland and gills, respectively [42]. Fish muscle and skin have been digested using NaClO at room temperature overnight. Under these experimental conditions, digestion efficiency reached $82.0 \pm 1.70\%$. Furthermore, it emerged that the increase in temperature reduced digestive capacity: at 40, 50 and 60 °C, digestion efficiency was $77.7 \pm 3.49\%$, $80.7 \pm 0.94\%$ and $67.9 \pm 1.57\%$, respectively [42]. Fish muscle treated with H_2O_2 (30%, at 50 °C for 6 h) had a digestion efficiency of 73.8% [58]. Finally, enzymatic digestion has found application to digest soft tissue of mussel. For example, an evaluation of trypsin, collagenase and papain for the digestion of soft tissues of mussels found that trypsin was optimum, leading to a removal of $88 \pm 2.5\%$ of biological tissue, compared to collagenase (12%) and papain (16%) [19]. Pancreatic enzymes have been used to digest marine invertebrate tissues. Overnight treatment resulted in a digestion efficiency of $97.7 \pm 0.2\%$ [42].

3.4. Methodological differences in digestion protocols

Alkaline and oxidizing agents were found to be the most common agents used for digesting biological tissues prior to microplastic analysis (Table 2). However, from the analysis of the studies considered, some different methodological approaches emerged. Firstly, digestion temperature/time combinations for digesting the same tissue type (*e.g.*, gastrointestinal tract, GIT) from different species or animals [43,44], vary. For instance, 10% aqueous solution KOH at 60 °C for 24 h [44] and 10% aqueous solution KOH at 50 °C for 12 h were both used for GIT digestion of fish [43]. Differences emerged for other tissue types. For example, viscera and gills were digested using a lower temperature (40 °C) and longer incubation times (72 h) compared to other tissues (*i.e.*, 60 °C for 24 h) and were not fully efficient (*i.e.*, digestion efficiency

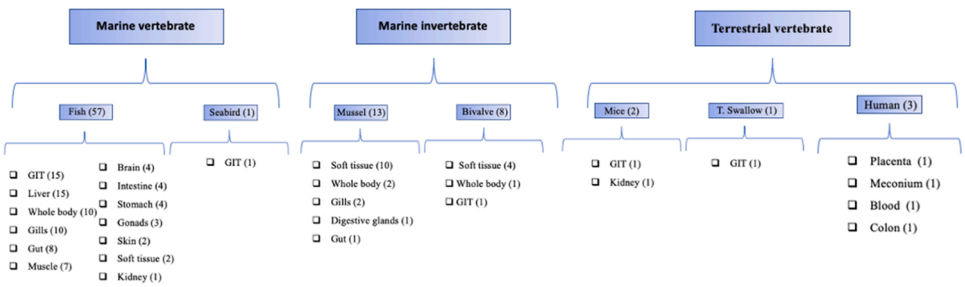


Fig. 2. Number of studies included classified by type of organism and tissue.

Table 1
Summary of the digestion methods applied to biological tissues used in the resulting articles. The concentration of digestion agents, temperatures used, digestion time and type of tissues have been reported. N/A indicates that digestion efficiency is missing.

TISSUE	*AGENT*	*AGENT CONCENTRATION (%)*	*TEMPERATURE (°C)*	*TIME (h)*	*EFFICIENCY (%)*	*REFERENCES*
Fish tissues						
Whole body	Sodium hydroxide	8	55	72	N/A	[55]
	Nitric acid	6	70	2	N/A	[13]
	Enzymatic digestion	-	-	-	N/A	[23]
Muscle	Potassium hydroxide	10	60	24	N/A	[2],[37],[47–50],[69],[75]
					99.8	[14]
	Hydrogen peroxide	30	65 – Room Temp.	24–24/48	N/A	[51]
	Hydrogen peroxide	30	50	6	73.8	[58]
	Potassium hydroxide	10	50	48–72	98.6 ± 0.1	[42]
GI tract	Potassium hydroxide	10	60	24	N/A	[43,44],[48,49],[75]
			50	12–48	N/A	[43]
	Hydrogen peroxide	30	65 – Room Temp.	24–24/48	N/A	[51]
	Mixture	-	-	-	-	[37,40]
	Potassium hydroxide	20	60	24	N/A	[6]
	Potassium hydroxide	10	Room Temp.	2–3 weeks	N/A	[54]
	Potassium hydroxide + Sodium hypochlorite	30	40	72	N/A	[52]
	Potassium hydroxide	10	60	24	N/A	[48]
	Sodium hydroxide	40	Room Temp.	72	N/A	[15]
Stomach	Potassium hydroxide	10	60	24	N/A	[27]
	Potassium hydroxide	10	Room Temp.	One week	N/A	[54]
	Hydrogen peroxide	30	65	24–48	N/A	[16]
Gills	Potassium hydroxide	10	40	72	N/A	[50]
	Potassium hydroxide	10	60	24	N/A	[29]
	Potassium hydroxide	10	40	72	N/A	[48]
	Potassium hydroxide	10	40	48	N/A	[33]
	Hydrogen peroxide	30	65 – Room Temp.	24–24/48	N/A	[51]
	Hydrogen peroxide	30	65	24–48	N/A	[50][59]
	Nitric acid	65	70	2	N/A	[25]
	Mixture	-	-	-	N/A	[37]
	Hydrogen peroxide	15	22	7 days	92.1	[57]
Intestine	Hydrogen peroxide	30	65	24–48	N/A	[16]
	Nitric acid	65	70	2	N/A	[25]
Gut	Potassium hydroxide	10	Room Temp.	One week	N/A	[53]
	Potassium hydroxide	10	40	48	N/A	[33]
	Potassium hydroxide	10	60	24	N/A	[29]
	Nitric acid/Hydrogen peroxide	65–30	70–85	2–2	N/A	[36]
	Fenton's reagent	-	< 60 °C	-	N/A	[62]
	Hydrogen peroxide	30	65	24–48	N/A	[59]
Viscera	Potassium hydroxide	10	40	72	N/A	[50]
Skin	Potassium hydroxide	10	60	24	N/A	[50]
	Potassium hydroxide	10	40	48	N/A	[33]
	Mixture	-	-	-	N/A	[37]
	Potassium hydroxide	10	50	48–72	98.9 ± 0.3	[42]
Liver	Hydrogen peroxide	30	65	24	N/A	[35]
	Potassium hydroxide	10	60	24	N/A	[49][29]
	Sodium hypochlorite	9	RT	overnight	N/A	[39]
	Enzymatic digestion	-	-	-	N/A	[7]
	Mixture	-	-	-	N/A	[37]
	Nitric acid	6	70	2	N/A	[25]
Gonad	Hydrogen peroxide	30	65	24	N/A	[35]
	Potassium hydroxide	10	60	24	N/A	[69]
Brain	Hydrogen peroxide	30	65	24	N/A	[35]
	Potassium hydroxide	10	60	24	N/A	[48]
	Sodium hydroxide	10	55	72	N/A	[55]
Seabird tissues						
GIT	Potassium hydroxide + enzymes	5	60	12	N/A	[70]
Mussel tissues						
GIT	Hydrogen peroxide	30	50	72	N/A	[66]
Gills	Potassium hydroxide	10	40	48	N/A	[67]
Gut	Potassium hydroxide	10	40	48	N/A	[67]
Muscle	Potassium hydroxide	10	40	48	N/A	[67]
Soft tissue	Nitric acid	65	RT	overnight	N/A	[77]
	Potassium hydroxide	10	60	25	N/A	[76]
	Enzyme	-	-	-	88.3	[19]
	Potassium hydroxide	10	50	48–72	97.7 ± 0.2	[42]
Whole body	Potassium hydroxide + enzymes	10	50	6	N/A	[82]
Bivalve tissues						
GIT	Potassium hydroxide	10	60	24	N/A	[75]
Soft tissue	Potassium hydroxide	10	60	24	N/A	[75]

(*continued on next page*)

Table 1 (*continued*)

TISSUE	*AGENT*	*AGENT CONCENTRATION (%)*	*TEMPERATURE (°C)*	*TIME (h)*	*EFFICIENCY (%)*	*REFERENCES*
	Potassium hydroxide	10	60	24	N/A	[65]
	Potassium hydroxide	10	60	24	N/A	[8]
	Hydrogen peroxide	30	60	24	N/A	[57]
Whole body	Potassium hydroxide	10	60	24	N/A	[26]
Gills	Nitric acid	65	40	24	97.6 ± 0.2	[56]
Tree swallow tissue						
GIT	Potassium hydroxide	10	60	24	N/A	[6]
Human tissues						
Colon	Potassium hydroxide	10	60	7–10	N/A	[78]
Placenta	Hydrogen peroxide	30	25	5 weeks	N/A	[4]
Meconium	Hydrogen peroxide	30	25	5 weeks	N/A	[4]
Blood	Potassium hydroxide	10	50	7–10	N/A	[5]

Table 2
Reported sample: reagent ratios (10% KOH) of studies which considered this parameter. Each ratio is reported against the type of tissue digested and the specie.

Ratio sample/ reagent	Tissues and Species	References
1:50	Gastrointestinal tract (*Serranus scriba, fish*)	[44]
1:10	Fillet (*Dicentrarchus labrax, fish*)	[14]
1:3	Gastrointestinal tract (*Scomber colias, fish; Trigla lyra, fish; Merluccius merluccius, fish*), spiral valve (*Lamna nasus, fish*), soft tissues (*Magallana gigas, oyster; Ostrea edulis, oyster; Ruditapes philippinarum, clam*)	[20,46, 47]
1:5	Gastrointestinal tract (*Mullus barbatus, fish*), soft tissues (*Mytilus galloprovincialis, mussel*)	[43]
1:60	Muscle and skin (*Claris gariepinus, fish*)	[42]
1:20	Gut, skin and gills (*Thryssa kammalensis, fish; Amblychaeturichthys hexanema, fish; Odontamblyopus rubicundus, fish; Cynoglossus semilaevis, fish; Chaeturichthys stigmatias, fish; Collichthys lucidus, fish*)	[33]

was lower than 95%) [42,48,50].

Likewise, another uncertain aspect is the volume of reagent to use and resulting ratio with the tissue (mass or volume), even for digesting the same tissue type (*e.g.*, GIT) (Table 2) [1,14,20,33,42,43,46,47].

Among the different methodological approaches, the most common for the digestion of marine vertebrates, invertebrates and animal tissues is 10% aqueous solution KOH at 60 °C for 24 h [1,2,14,27,48–50]. The different methodological approaches are likely due to the digestive capacity of KOH, potentially influenced by the chemical composition of the substrate being digested [46]. Furthermore, it is reasonable to assume that species level differences may influence the chemical composition of different tissue types and therefore digestion efficiency.

Differences in the experimental approaches also emerged for H_2O_2 digestions. For example, fish gills have been digested using H_2O_2 at 15% at 22 °C for 7 days [57] and 30% at 65 °C for 24 h [35,58], in both cases which enabled microplastic detection. Furthermore, different combinations of reagents have been used for digesting biological tissues. For example, Roch *et al.* (2017) [60] used HNO_3 (65%) in combination with NaOH (1 M) for an "accelerated" digestion of fish GIT. The digestion took 1 h to complete, with only a negligible trace of tissue remaining following filtration, visually determined. NaClO has also been used in combination with other agents, such as KOH and H_2O_2. For example, Enders *et al.* (2017) [18] used 7% NaClO aqueous solution combined with 3% KOH aqueous solution for fish stomach digestion. The digestive mixture consisted of a mixture of aqueous solutions of KOH: NaClO, tested in different ratios, such as: 1:1, 2:1, 1:2. A ratio of 1:1 resulted in the complete digestion of fish stomach tissues. Ratios of 1:2 and 2:1, however, showed undigested components and significant foaming, due to the higher proportions of NaClO. Otherwise, excessive reduction of NaClO concentrations reduced the effectiveness of the digestion [18].

3.4.1. Section summary

The most common method for digestion of biological tissues is 10% KOH aqueous solution at 60 °C for 24 h, which has been applied to several biological tissues (*i.e.*, fillet, GIT, stomach, skin, brain of fish) [1,6,27,48,50,55]. However, even though the use of 60 °C for KOH digestion is widespread, some consideration should be given as agglomeration of microplastic particles can occur (see section "Discussion"). However, studies included in this review have proposed various methodological approaches for digestion of biological tissues. Variations have been found in the temperature/time combinations (Table 1) as well as in the sample-to-reagent ratio (Table 2). These variations in methods can be attributed to the digestive capacity of the reagents, which is potentially influenced by the chemical composition of the substrate being digested.

2. The effect of digestion reagents on microplastic particles

Digestion reagents can degrade microplastic polymers [56]. Therefore, they should be efficient in eliminating biological materials while, at the same time, being inert to microplastic particles [42]. Regarding KOH, microscopic observations revealed changes in the shape of polyethylene terephthalate (PET) fragments (~ 300 μm). Cellulose acetate (CA) fragments (~ 300 μm) were completely degraded. Furthermore, 10% KOH at 50 °C and 60 °C altered the colour of nylon-66 (NY66, fragments, ~ 300 μm), determined by visual observations. However, no impact on the recovery rate was observed [42]. As for NaOH, it was found that polymers have a lower resistance to it than to KOH [55]. PET, PC and cellulose are damaged when treated with NaOH aqueous solution (10 M) heated to > 60 °C [12,44]. Virgin PET pellets (0.3–1.0 mm) showed a 4.7% reduction in the mass, following a treatment with NaOH at 50–70 °C whereas post-treatment size changes were not specified [55]. The same digestion treatment degrades cellulose, with a reported mass reduction of nearly 50% [12,44,55]. Acid treatments are well-known for their potential to damage some polymers [12,60]. For instance, polyamide (PA) is particularly sensitive to acid digestion. Microscopic observations found that PA microplastic particles (fragments, ~3 mm) were degraded following treatment with HNO_3 (65%, 60 °C for 2 h). This methodological approach also led to the fusion of PS microplastics (fragments, ~3 mm), but not those embedded in the gut of *M. edulis*. Therefore, the amount of acid directly in contact with microplastic particles is potentially important. Furthermore, LDPE microplastic (fragments, ~3 mm) became discoloured (yellow) when treated with HNO_3 (65%, 60 °C for 2 h), assessed by microscopic observations [12]. Concentrated HNO_3 (65%) at room temperature changed the colour of microplastic particles (*i.e.*, LDPE, HDPE, PP, PS, PET, PVC, nylon-6 and NY66, fragments, ~300 μm) and melted LDPE and PP fragments (~300 μm) [42]. In addition, HNO_3 > 60 °C does not guarantee nylon fiber (< 200 μm) and PS sphere (10–30 μm) recovery

because they were found to agglomerate [61]. Furthermore, a HNO_3 digestion reduced the relative intensities of specific Raman peaks (1288 and 1432 cm^{-1} 627 and 684 cm^{-1}) characteristic of PVC and HDPE, respectively. Likewise, HCl treatment (37%) changed the molecular arrangements of PET, reducing the peak intensity of the C═C ring, stretching at 1610 cm^{-1} and C═O stretching at 1722 cm^{-1}. This indicated depolymerization of the polymer structure [42]. For enzymes, one of the main advantages is their minimal impact on microplastic particles [19,20]. However, corolase 7089 damaged (mainly consisting in surface modification) polymers such as HDPE, PA and PET (fibers, 80 μm). Fresh trypsin, unlike frozen trypsin and trypsin preserved in formaldehyde, was found to damage PA, PE, PET, PP, PS and PVC (fragments, 80 μm) [19,20]. Digestion agents, when used in combination, can affect microplastic polymers. For instance, HNO_3 (65%)/NaOH (1 M) led to colour modifications of PET and PVC (fragments, 1–2 mm), assessed by microscopic observations. PS fragments (1–2 mm) showed corrosion at the edges, while LDPE fragments (1–2 mm) showed a statistically significant weight loss of 15.8 ± 3.3%. However, the most significant damage was observed for PA fragments (1–2 mm), which were completely dissolved [60]. In contrast, no damages were reported for low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyamide-6 (PA6), polypropylene (PP), PS, polyvinyl chloride (PVC) (~300 μm) following KOH treatment (10%, 60 °C for 24 h) when treated with KOH 10% at 50 °C and 60 C° [42], Fenton's reagent [17, 61] and microplastic particles (PET, HDPE, PVC, PP, PS, PA, fragments, <0.5 mm and 0.5–5.0 mm) when treated with trypsin (0.31%) for 30 min [19]. However, it should be noted that studies focused on large microplastics. The smallest microplastics where digestive agents were tested measured 80 μm [20] whilst effects on small microplastics (<10 μm) have not been investigated. The smaller the microplastics the larger the surface area to volume ratio. Therefore, smaller microplastics (<10 μm) could be more susceptible to degradation. Modifications in their size/shape could affect the final results in bioaccumulation studies (*i.e.,* actual concentration in tissues, identification of the sources of contamination). Therefore, it recommended to investigate the effects of digestion procedures on small microplastics to fill a gap microplastic particles which are more relevant to tissue uptake.

3. Analytical methodologies

Following sample preparation, usually purification by digestion, the resulting sample is analysed for microplastic (presence, size, shape, and/or polymer mass) *via* one or multiple of several analytical methods. Generally, accurate and reliable data is difficult to achieve due to the influence of background contamination, and often data is expressed as one of several metrics or units, depending on the method, which can complicate comparisons [11]. Furthermore, expression of results for microplastics concentration in biological tissues is not normalised. For instance, microplastic concentrations in muscle of wild mussels were expressed both as number of microplastic particles per gram tissue [3] and number of microplastic particles per individual [8]. Data should be normalised (*i.e.,* per gram of tissue) to eliminate the variable related to the size of the analyzed organism, which could influence the number of microplastic particles in their bodies. Furthermore, for biological tissues, dry weight should be used since it is much more constant than wet weight.

3.1. Visual analysis

After sample preparation (*e.g.*, digestion), identification and quantification of microplastic particles is sometimes carried out through visual inspection, using a stereoscope or microscope in brightfield, fluorescence or polarised modes. However, underestimates arise due to visual analysis [23]. The selection of potential microplastics can be facilitated using automatic microscopy technology. For example, Zeytin *et al.* (2020) [14] used automatic fluorescence microscopy to validate the visual assessment of autofluorescent microspheres (1–5 μm) in the fillet of European seabass [14]. The average abundance of microspheres from manual counting was 40% lower than from the automatic count. Distinguishing between synthetic and non-synthetic fibers and fragments (PP, PS and PE < 300 μm) using visual identification alone is not optimal. Without the confirmation of the polymer, visual identification in the muscle of *M. edulis* led to an underestimation of fragmented microplastics and an overestimation of microfibers [8]. Confirmation of the polymer thus increases the accuracy of the results [24]. Visual inspection is useful in evaluating the effects of sample preparation methodologies (*e.g.*, digestions) on microplastics, such as changes in shape and size or their fusion [64]. Visual analysis of MP has proved unsuccessful for the quantification of microplastics in biological tissues. Avio *et al.* (2015) [10] visually sorted microplastic fibers from the stomach of fresh fish (*i.e.*, *Sardina pilchardus, Squalus acanthias, Merlucius merlucius, Mullus barbatus and Chelidonichthys lucernus*). The protocol provided an extraction yield of < 60% for spiked polyethylene and polystyrene microplastic particles (fibers, 1–0.5 mm), confirming the potential underestimation of microplastic concentration. Microscopic visualisation is affected by the presence of organic matter/residues in the sample, that can interfere and hide microplastics [10]. Furthermore, visual observations of microplastic particles are subject to bias and inaccuracies. Visual analyses cannot identify the type of polymer, which is fundamental for understanding sources and impacts of microplastics [15,19, 23,47,62].

3.2. Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC-MS)

In the scientific literature reviewed, only two of the studies included have used Py-GC-MS for chemical identification of microplastics. Compared to spectroscopic techniques, this approach is more time-efficient and generates quantitative data in terms of mass per type of polymer present [11,65]. There are some limitations, as Py-GC-MS is a destructive method, details like the shape, colour, and size of microplastics cannot be acquired [24]. Py-GC-MS was used to analyse digested fish muscle which had been extracted by accelerated solvent extraction (ASE). Detected concentrations of PS, PP and PE were 0.1, 0.3 and 2.5 mg g^{-1}, respectively. It is worthy to specify that this methodology was previously tested with spiked samples to determine a limit of detection (LOD) and limit of quantification (LOQ), expressed as μg g^{-1} of tissue for each polymer investigated (*i.e.*, LODs; 0.51 for PS, 0.62 for PP, 5.85 for PE, 7.86 for PVC; LOQs, 0.70, 1.77, 6.79, and 7.90 for PS, PP, PE and PVC, respectively) [24]. Furthermore, Py-GC-MS was also used in a laboratory exposure experiment where the model organism *M. galloprovincialis* was exposed to PS microbeads (3 and 45 μm; exposure concentrations 5, 50, 500 μg L^{-1} and 0.015, 0.15 and 1.5 μg L^{-1}, respectively; exposure time 4 days) [11]. The methodological approach described reported the use of a liquid-liquid extraction (LLE) using toluene, after the alkaline digestion of the digestive glands of exposed *M. galloprovincialis*. However, in the study, the authors did not determine whether microplastics were present in the digestive gland lumen or absorbed in the tissue. The toluene solutions (10 mL of extract) were transferred to a 50 mL flask where 100 mg quartz wool combusted in a muffle furnace was placed. A rotary evaporation to dryness was conducted. Fabbri *et al.* (2020) [11] reported that this step allowed PS to form a thin layer of highly dispersed film on the surface of the quartz. This should minimize the parallel reactions between radicals generated by pyrolysis. Finally, the quartz wool was removed with clean tweezers and introduced into the sample holder of the pyrolysis quartz tube.

Fabbri *et al.* (2020) [11] reported concentrations of PS microbeads (45 μm) extracted from digestive glands (not clear if they were embedded in the tissue) of 1.0, 8.2 and 14 μg g^{-1} for the exposure concentrations of 5, 50, 500 μg L^{-1}. Indeed, concentrations detected for 3 μm PS microbeads ranged between 1 and 2 μg g^{-1} (no significant differences related to the three exposure concentrations emerged) [11].

3.3. Vibrational spectroscopy

There is a wealth of literature about the use of spectroscopic techniques for the identification of different types of microplastics. Fourier-transform infrared microspectroscopy (FTIR), Attenuated Total Reflection-FTIR (ATR-FTIR), and Raman microspectroscopy have been used to identify and classify the chemical nature of particles. Since various studies have reported in-depth the application of these techniques in this field, an overview of the applications of these spectroscopic techniques is outlined below.

3.3.1. Fourier-transform infrared (FTIR) spectroscopy

In the scientific literature reviewed, 46.7% of the studies used FTIR for the chemical identification of microplastic particles. FTIR microspectroscopy (μ-FTIR) has been used to analyse microplastic particles ranging in size from 50 to 5000 μm, extracted from several biological tissues. For instance, microplastic particles extracted from gut [19,33,36,40,62,66,67] and gills [17,29,33,36,51,62,67], ranging in size from 50 μm (fibers, fragments) [62] to 5000 μm (fibers) [17,29,33] have been confirmed using μ-FTIR. Microplastic fibers extracted from fish skin [33], fish digestive tract, muscles [51], bivalve soft tissue [8,63,68] and mussel soft tissue [3], ranging in size of 864–973 μm [33], 100–1000 μm [8,63,68] and 200–2000 μm [3], respectively, have been chemically analysed by μ-FTIR. Likewise, μ-FTIR has been applied to analyse fibers extracted from human tissue, such as placenta, meconium and human stool [4] and blood [5], in a size range of 200–1000 μm and 20 μm, respectively. Identification of microplastic particles of a smaller size becomes increasingly difficult [5,65]. For this reason, other spectroscopy techniques like μ-ATR-FTIR are a good option, *e.g.*, for particles smaller than 20 μm [36].

3.3.2. Raman spectroscopy

In the scientific literature reviewed, 12.5% of the studies used micro-Raman spectroscopy (μ-Raman) for the chemical identification of particles. μ-Raman is also used to assess digestion-induced changes in the molecular integrity of plastic polymers [42]. μ-Raman has been used for classifying the composition of fibers and fragments from 15 to 572 μm, extracted from intestinal tract of bivalves [47,65]. Fibrous microplastics, with a size range of 130–22 400 and 63–5000 μm, extracted from fish livers and seabird GIT, were analysed using μ-Raman [6,39,49]. Published studies reported the suitability of μ-Raman for smaller microplastics (*i.e.*, > 0.45 μm) [44]. For instance, fragments extracted from biological tissues (real samples, tissue type not specified), between > 0.45 and > 3 μm were chemically identified as plastic by μ-Raman spectroscopy [44]. Hence, in comparison to FTIR techniques, μ-Raman spectroscopy has the potential to detect microplastic particles measuring down to 0.5 μm, substantially widening the range of particle sizes that can be analysed. Modifications of microplastic particles, due to pre-treatment (*i.e.*, digestion) can be identified with μ-Raman spectroscopy. In the case of polymer alteration/modification due to digestion agents, band shifts occur [12].

4. The biodistribution of microplastics in biological tissues

Extrapolated data from the studies included in this review showed that sixty eight percent (68%) of studies investigated the biodistribution of microplastic in biological tissues (*i.e.*, marine, terrestrial and humans) [4–6,16,27,36,39,40,47–49,52,55,59,66,67,69,70]. Thirty six percent (36%) of these studies were field studies whereas 64% were laboratory based (*i.e.*, organisms were experimentally exposed to microplastics). For an in-depth analysis of the absorption, distribution, metabolism, and elimination (ADME) of microplastic particles in biological tissues, accumulation and biodistribution need to be clarified. Microplastic accumulation refers to the process by which they build up and concentrate in the tissues and organs of a living organism over time [2,27,58,70]. Therefore, accumulation should imply the embedding of microplastic particles in the cells of tissues/organs [38,49–51,66,67,72]. Therefore, whether microplastic particles are passing through or are within tissues should be clearly distinguished, otherwise there will be repercussions for the interpretation of results (See Table S2 Suppl. Mat.).

Distribution refers to the pattern of microplastic particles within the tissue or organs of a living organism after exposure [29]. Accumulation and distribution mainly depend on the size of microplastic particles [12,29,32,33,59,66,70,73] as this influences their ability to enter biological tissues [5,29,32,69,70,74]. Tissues could be grouped as points of exposure (*i.e.*, outer biological tissues such as gut, skin, GIT, intestine, stomach) and points of accumulation (*i.e.*, more internal biological tissues, plausibly reachable by small microplastics such as gonads, brain, muscle and liver).

Microplastic fragments and fibers in fish gut and skin samples ranged from < 100 to > 1000 μm in size as reported by different studies [17,37,62]. Particularly, microplastic fibers up to 2.01 mm in fish gut were detected [55]. Smaller microplastic fibers (23–75 μm) were detected in fish gut [59]. Microplastic fragments from > 0.45 μm to > 3.0 μm were detected in fish, confirmed by μ-Raman [44]. Furthermore, microplastic fibers from 20 μm [69], 83 μm [46,48], 100 μm [15], 300 μm [66,67], 1–2 mm [47,60,75] to 5 mm [49] were detected. Fibrous microplastics were also found in reported fish stomach tissue (not content), with a size range of 25–1000 μm [27]. Regarding tissues considered as points of accumulation, studies have reported the presence of microplastics fibers and fragments ranging from 50 to 200 μm [48] and > 0.45 to > 3.0 μm [44], respectively, in fish muscle tissue. Microplastic fragments were also found in muscle tissue ranging from < 100 μm to > 1000 μm [37], similar in size to microplastic particles found in gut and skin tissue. Fish liver has been found to contain microplastic particles of 124–438 μm, which have been confirmed through μ-Raman, but the shape of the particles was not mentioned. Furthermore, the presence of suspected microplastic particles with a size range of 71 to > 5000 μm was reported [39]. Finally, fibrous microplastic less than 1 mm (50–200 μm) were reported in fish brain [48,55]. However, due to the particle sizes reported, it is reasonable to assume that the detected microplastics were passing through or contaminating the tissues from the laboratory environment, but not accumulated into them (See Section "Discussions). The concentration of microplastic in biological tissues is crucial when determining the level of exposure to microplastic. For instance, in soft tissue of *Mytilus spp,* average fibrous microplastic concentrations ranged within 1.05 ± 0.66–4.44 ± 3.03 (~ 1.22 mm) microplastic particles per gram tissue w.w., confirmed by FTIR [3,19]. A study by Marques *et al.* (2021) [76] reported a concentration 0.54–3.0 fibrous microplastic particles (30–5000 μm) per gram tissue w.w.in the soft tissue of *Mytilus spp*, confirmed by FTIR [76]. In another study, *Mytilus spp* had an average concentration of 0.2 ± 0.3 microfibers (20–90 μm) per gram tissue [77]. Fibrous microplastic (~ 1 mm) were reported in fish gut. The concentrations ranged from 0.18 to 2.9 particles per individual, chemically confirmed by μ-FTIR analyses [55]. Fish GIT were found to contain concentrations of fibrous microplastic particles, 0.3 ± 0.1 (~ 300 μm) to 3.9 ± 2.2 (20–4800 μm) per individual [66,69]. A concentration of 2.0 spherical microplastic particles (300–400 μm) per individual was detected in the GIT of fish in another study, confirmed by FTIR [53]. Additionally, fish stomach concentrations ranged from 4.1 (25 – 1000 μm) to 12 (> 250 μm) fibrous microplastic particles/individual [27,52], confirmed by FTIR. Regarding fish brain, 0–0.28 microplastic fibers (0.22–2.01 mm) per individual were found [55]. However, the size of the microplastic particles reported above are too large for it to be plausible they were embedded in tissues (See Section "Discussion" and Table S2 Suppl. Mat.).

5. Relation to humans

In the present review, three of the retrieved studies focused on the presence of microplastic in human tissues. However, one was specifically devoted to the advancement of analytical methodological

approaches. The human tissues investigated were colon [78], placenta, meconium and stool [4]. A study conducted by Ibrahim *et al.* (2020) [78] examined the presence of microplastics within the human colon. The experimental design involved the selection of patients (n = 11, age mean age 45.7 ± 17.9 years, age range 34–88 years,) who had not previously consumed drugs containing microplastics. A colectomy was performed, with precautions taken to prevent potential exposure to common plastics within the operating theatre. The analysis revealed that all 11 samples examined contained microplastics, with an average of 28.1 ± 15.4 particles gram^{-1} of fibrous and fragmented microplastics per tissue. The size of these particles ranged from 800 to 1600 μm. μ-FTIR analysis indicated that the microplastic particles were predominantly comprised of polycarbonate, polyamide, and polyethylene. However, the size of the fragments and fibers detected in human colon samples means it is unlikely they were in tissue (See Section "Discussion"). Microplastic fragments were detected in digested placenta, meconium and stool, collected from two (n = 2) women who underwent cesarean partum following the medical guidelines. Negative controls for preparation tools and storage containers but without real samples (*i.e.*, placenta, meconium and stool), were included to perform quality assurance/quality control (*i.e.*, QA/QC). Furthermore, all stages of samples preparation and manipulations were performed under clean-room conditions. To investigate the airborne fallout of microplastic during the cesarean partum, a 10 min air probe from the operating theatre was analysed, which was positive for PU (fragments, >50 μm). Therefore, before placenta samples were chemically digested, they were carefully rinsed with MilliQ water and the external surface of the placenta was carefully removed with a metal scalpel and only the placenta core was screened for microplastic. PE, PP and PU fragments (>50 μm) were detected in digested placental tissues, whereas no microplastic was detected in the relative negative control (*i.e.*, sample preparation instruments and storage containers without the actual placenta) excluding contamination during the tissue preparation process. For PU, authors reported that prevention measures taken before the chemical digestion of the placenta suggest that the PU found in the placenta did not come from airborne contamination. Meconium tissues contained PE fragments, which were not found in the relative negative control (*i.e.*, sample preparation instruments and storage containers without the actual placenta). In the maternal fecal sample, 2 microplastic fragments of PE (> 50 μm) and 1 fragment of PS (> 50 μm) were found per 20 g of feces. However, the negative control for maternal feces revealed the presence of PE, PP, and PS (> 50 μm). Polymer identification was carried out using μ-FTIR [4]. Overall, the presence of microplastic in the placenta, meconium and stool is questionable due to the particle sizes observed (> 50 μm). The particle sizes are not compatible with the patterns of kinetics and transport through bloodstream relative to particle size, and thus, even though severe measures for airborne contamination prevention were taken, these results should be considered with caution (See Suppl. Mat.).

8. Discussions

8.1. Overall summary

The main aim of this review was to systematically investigate the methodological approaches for sample preparation and chemical analyses of microplastic, and the accumulation and distribution of microplastic particles in biological tissue relative to size, polymer type, and shape. This revealed substantial discrepancies in the reported sizes of microplastic particles and their capacity to accumulate in biological tissues. Specifically, a classification of tissues into "accumulation points" (*i.e.*, internal tissues) and "exposure points" (*i.e.*, tissues in contact with the external environment) showed poor clarity about the behaviour of microplastic in tissues. This is highlighted by the similarity in size of microplastic particles discovered in both accumulation and exposure tissues, suggesting the urgent need for methods to prevent external contamination. The resulting methods for sample preparation and analysis of microplastic showed disparate approaches. Therefore, the findings of the review highlight the importance of standardizing methodological approaches, beginning with the pre-treatment of the sample. Implementing consistent preparation methods can minimize the challenges associated with interpreting results. For example, the use of different methods for digesting the same tissue (*i.e.*, fish muscle) at the same temperature led to different digestion efficiencies (KOH, 60 °C, 97.6 ± 0.17%; H_2O_2, 60 °C, 100 ± 0.26%). This could influence the isolation of microplastic particles from the tissue and, thus, the final results (*i.e.*, overestimation or underestimation of microplastic concentration). Furthermore, different methodological approaches influenced recovery rates of microplastic. For instance, digestion of fish muscles at 50 °C by 10% KOH and H_2O_2 led to comparable digestion efficiencies (*i.e.*, 98.9 ± 0.33%; 98.3 ± 0.11%) but different percentage recovery rates for spiked microplastics (*i.e.*, PET (80–300 μm), 84.7 ± 2.88% for KOH; 99.5 ± 11.4% for H_2O_2; NY-66 (80–300 μm), 95.3 ± 4.55% for KOH; 87.1 ± 2.98% for H_2O_2; HDPE, (80–300 μm), 100–0 ± 1.89% for KOH; 99.5 ± 7.37% for H_2O_2; PP (80–300 μm), 95.4 ± 0.75% for KOH; 102.5 ± 9.20% for H_2O_2) [42].

8.2. Selection of the digestion reagent: an issue in microplastic analyses

Choosing the right digestive reagent is a major challenge in biological sample digestion. The suitability of a reagent depends on the chemical composition and organic matter content of the sample, making digestion efficiency matrix-dependent [56]. However, from reviewing the scientific literature, the identification of a criterion for the choice of the digestive reagent as a function of the chemical composition of organic matter is quite complex since the information reported in the studies is often unclear or unspecified. Studies to systematically assess digestion reagents for each type of matrix are needed, however, studies mainly focused on the assessment of the occurrence of microplastic particles in biological tissues. Throughout the included studies, fish GIT was digested using different reagents (*i.e.*, KOH, H_2O_2, NaClO) and mixtures, without explanation about the strengths/weaknesses of the reagent or what informed the choice. So far, it is possible to suggest the use of alkaline agents (KOH and NaOH) for lipid-rich tissues or tissues that have a substantial content of proteins and lipids [46]. This is based on the nature of the chemical reaction. On the other hand, oxidation reactions appear to be more versatile, interacting with multiple chemical substrates, meaning that these types of reactions can also be applied to tissues such as chitinous exoskeletons of crustaceans and carbonate shells of gastropods, which are otherwise difficult to digest with alkaline treatments [46]. The accurate identification and quantification of microplastic strongly depends on the degree of organic matter removal, especially for complex matrices such as biological tissues [31–33,60,70,79]. However, among non-methodological studies, only one of the included ones reported digestion efficiency [14]. Furthermore, it is not clear what criteria were used in the studies to determine when digestion was complete. Similarly, the digestion process can be influenced by the temperature used during the procedure. Consequently, any change in temperature whilst using the same digestion reagent for a particular type of biological tissue (*i.e.*, the stomach) [27,53] must be clearly documented. Karami *et al.* (2017) [42] investigated the temperatures used for digesting fish tissue (*i.e.*, muscle and skin) by 10% aqueous solution KOH. Experimental design was clearly described, and the digestion efficiencies reported suggest there is an impact of temperature on the reactions. Even though temperatures 40–50 °C guaranteed higher digestion efficiencies (*i.e.*, 98.6 ± 0.1% and 98.9 ± 0.33%) than 60 °C (*i.e.*, 97.6 ± 0.2%) [42], studies published after this considered in this review digested muscle and skin in KOH 10% at 60 °C [48,50]. However, the use of 60 °C in digestion of organic matter should be reassessed. Some plastic polymers (*i.e.*, PVC, PA, PET) have a glass transition temperature (Tg) around 60 °C (*i.e.*, 65 °C; 60 °C; 70 °C). When these polymers are heated and reach their Tg temperatures, molecules of

polymers can start to move and thus the polymer becomes sticky. In these conditions, microplastic agglomeration can occur. The use of a lower temperatures for the digestion (*i.e.*, $\leq$ 50 °C) could avoid this phenomenon. Another understudied parameter is the sample/reagent ratio to be used. None of the studies included in this review investigated this. Indeed, the analysis of the studies showed the use of different sample/reagent ratios (Table 2). It would be beneficial to analyse the impact of the volumes of digestive solution to be used according to the grams of sample to be digested in order to evaluate their impact on digestion efficiency. Identifying the optimal sample/reagent ratio would substantially reduce digestion times, limiting the contact time between microplastics and the digestion reagent and, consequently, minimizing any potential damage. The latter is another under investigated aspect. One study found NaOH substantially impacted microplastic particles at temperatures higher than 50 °C, particularly fibrous microplastics smaller than 100 μm [56]. However, studies that have used NaOH at 55 °C do not mention any damage on fibrous microplastics or validation tests for the effect on microplastics.

8.3. Analytical methodologies for microplastic

Quantification of microplastic is a challenge [56,63]. Regarding μ-FTIR, spatial limits of detection present an important limitation (generally it is > 20 μm). However, μ-FTIR has been applied to confirm the chemical nature of standard PS microbeads with a size of 5 μm [34]. From reviewing the included literature, it is clear that the choice of analytical technique should be related to the target particle size. It emerged that μ-FTIR can be used for those bigger than 20 μm and μ-ATR-FTIR for microplastics smaller than 20 μm, with a suggested lower limit of 7 μm [75]. If microplastic particles smaller than 7 μm are a target, they should be identified using μ-Raman [75]. Py-GC-MS has limitations in relation to qualitative and morphological data such as color, size and shape [24]. Furthermore, pyrolysis temperature influences the peaks areas of characteristic compounds and, thus, the detection of plastic polymers [11]. In the considered studies, pyrolysis temperatures used for PS were different (*i.e.*, 650 °C; 500 °C) [11,24] but none of the studies investigated this parameter. Pyrolysis temperatures for polymers should be investigated to identify the best method conditions for their detection. Py-GC-MS is influenced by the presence of organic material in biological tissues, which can be observed in the spectra through the appearance of peaks associated with such materials even after a KOH digestion [11,24]. Moreover, the susceptibility of pyrolysis products of plastic polymers to organic matter appears to vary depending on the polymer type. For instance, Fabbri *et al.* (2020) [11] reported that pyrolysis products for polystyrene (*i.e.*, styrene, styrene dimer (2,4-diphenyl-1-butene) and styrene trimer (2,4,6- triphenyl-1-hexene) showed different levels of interference from organic matter, particularly to biogenic polymers such as proteins. This may be attributed to secondary reactions occurring during pyrolysis, giving rise to pyrolysis products, and their interactions with biogenic constituents [11]. An in-depth investigation into this is needed.

6. Microplastics in biological tissues

Size affects the ability of microplastic particle to enter biological tissues [5,29,32,69,71,74]. This review found that the size range of microplastic particles in tissues that serve as points of accumulation and points of exposure were the same in field studies. For instance, microplastic fibers 100 μm in size were found in both the fish liver (*i.e.*, accumulation point) and fish GIT (*i.e.*, exposure point) [15,39,47,49]. It has been reported that, in laboratory experiments, PVC microplastic fragments of a wide size-range (*i.e.*, 0.1–1000 μm) were able to reach fish liver [62,67,80] after four days of exposure. Laboratory studies have also indicated a size-dependent relationship with microplastic accumulation in tissues. Specifically, larger PS microplastics with a diameter of 8 μm were less able enter fish liver and intestine. In contrast, smaller microplastics with a diameter of 0.25 μm can enter these tissues [25,71]. This is also supported by experiments whereby fish were exposed to two size-groups of PE microplastic (*i.e.*, 4–6 μm and 125–150 μm) for 21 h. Only microplastic particles sized 4–6 μm were present in fish intestine, suggesting bigger microplastic particles (*i.e.*, 125–150 μm) were unable to enter [7], clarifying that studies which reported that microplastics bigger than 6–8 μm can reach fish livers and thus, the assumption that microplastics of 1000 μm reached fish liver should be reassessed [80]. Evidence suggests that fibrous PP microplastic particles in a range size of 10–60 μm can enter fish gills, accumulating along water outflow tracts. However, this should be investigated in-depth. Fish gill cells range from 10 to 20 μm in size. Internalization in gills cells *via* phagocytosis could occur for 1 μm (See Suppl. Mat.). Hence, microplastic fibers of 10–60 μm could be associated with the outer membrane, rather than the inside of cells and tissue of the gill. Discrepancies related to microplastic in fish brain are reported. For instance, it has been stated that fibrous microplastic particles sized 50–200 μm reached the brain of the fish *Oreochromis niloticus* [36]. However, contrasting experimental evidence found that PS microplastic particles greater than 0.1 μm were unable to reach the brain in *O. niloticus* along with blood circulation [36]. PS microbeads sized 0.10–0.12 μm were also found to reach the brain in *Danio rerio* [71]. Results following the experimental exposure of fish (*Oncorhynchus mykiss*) to PS microspheres (100 – 400 μm) for 4 weeks histopathologically found that, because of their dimensions, the PS microspheres did not cross biological barriers and become internalised by cells (See Table 2 Suppl. Mat.). Furthermore, they did not reach and enter internal tissues (*e.g.*, liver) through the bloodstream [9]. It could be suggested that these phenomena (*i.e.*, crossing biological barriers, cellular internalization, transport *via* blood circulation) are likely for nanoscale particles (and not for large microplastics, as it emerged from studies included in this review). Therefore, it is necessary to consider particle size thresholds relevant to biological uptake. Finally, it is reasonable to assume that microplastic particles (from 100 to >5000 μm) detected in studies considered for this review were not internalized by tissues and were possibly due to background or cross-contamination. Aside from size, the concentration of microplastic is important [31,38,76]. Experimental evidence from fish exposure studies suggests that if the microbeads (0.1 μm) exposure concentration is increased from 1 to 10 μg L^{-1}, the percentage increase of microplastic accumulated in the fish gut lumen is approximately 252% (*i.e.*, 25.5 $\times 10^4$ to 89.9 $\times 10^4$ μg kg^{-1} tissue), after 14 days of exposure [36]. Similarly, an increase in microplastic (raw powder with irregular shape, 100 nm in size) exposure concentration from 1 to 10 mg L^{-1} results in a 200% increase of microplastic accumulation in whole fish body after 15 days of exposure [22], supporting the hypothesis that the delivered dose of microplastic influences the internal dose [22,32,36]. Internal dose is rarely reported, for instance, exposure experiments often report the exposure concentration (*i.e.*, 1% w.v. [7], 8 μg L^{-1} [79], 1, 10, 100, 1000 μg L^{-1} [26], 10 mg/fish [9], 200 μg L^{-1} [81], 0.1 and 0.5 mg L^{-1} [80], 10, 000 microplastic L^{-1} [82], 40, 000 microplastic L^{-1} [83]), but do not assess internal dose. The focus of these studies was mainly on the toxic effects of microplastics. However, the degree of toxicity depends on the concentration of microplastics in the organism [73] or at the target tissue/pathological site. Solely examining toxicological effects without accurately determining the internal microplastic dosage could lead to misleading results for certain endpoints. Furthermore, to assess a "realistic" internal dose, egestion should be taken into account [57,83]. Experiments conducted on the mussel *M. galloprovincialis*, considered as model organism in microplastic studies, suggest that PE microsphere particles sized 40–48 μm were totally egested. Particularly, after 14 days of exposure, with exposure concentrations of 1, 10, 100 and 1000 μg L^{-1} no accumulation in soft tissues was observed [26]. After 7–49 days exposure, fish (*Diplodus sargus, Seriolella violacea*) can egest all microplastics fragments (500 μm, 1.2 mm) ingested [73,84]. Therefore, to robustly investigate the fate of microplastic particles in organisms, aside from the concentration of exposure, microplastic egestion should be

carefully quantified. In vitro studies are necessary to identify clear mechanisms of interaction between microplastics and living organisms that can be useful for normalizing results obtained from *in vivo* experiments.

6.1. Relation to humans

The papers included in this review imply that microplastic exposure in human tissues is plausible. However, an improvement in quality assurance and quality control procedures is deemed imperative. As exemplified in Braun *et al.* (2021) [4], the analytical process entails the risk of contamination, despite all precautionary measures, as evidenced by the procedural blanks [4].

In a study by Ibrahim *et al.* (2020) [78], microplastic fibers ranging from 800 to 1600 μm (~1.1 ± 0.3 mm) were detected in colon tissue. However, evidence suggests that the predominant pathway for microplastic to enter the intestinal tissue is through the lymphatics, where non-digestible particles ranging from 0.1 to 15 μm can enter. Hence, the discovery of microplastic fibers exceeding 800 μm requires further scrutiny. Furthermore, human colon cells (*i.e.*, 15–20 μm) are about 100 times smaller than the microfibers found in human colon tissue. This could indicate that uptake did not take place, but that microplastic particles had been in the gastrointestinal lumen. Alternatively, whilst in place, the measures to prevent airborne contamination may not have been enough. Additionally, the most abundant polymer type was PC and the most abundant morphology was fiber. There is limited evidence available on the production of PC fibers specific to fireproof and chemical resistant fabrics. The presence of polymers common in the diet would have been anticipated. Therefore, a misclassification of particles and/or external contamination potentially occurred. However, it seems that the microplastic particles detected were either "passing through" the human tissues analysed (*i.e.*, colon, placenta) but were not inside cells, or they were artefacts of background contamination. Both are plausible due to the lower detected microplastic fiber sizes (50, 800, 1600 μm). Scientific evidence indicates that smaller (*i.e.*, 2.0 μm) microplastic particles can affect cells. For instance, *in vitro* and *in vivo* laboratory experiments found that PS microbeads (2.0 μm) can cause direct damage to human kidney cells and male mice. More precisely, *in vitro* tests conducted on a human kidney-derived cell line, HK-2, demonstrated the capacity of polystyrene microbeads (2 μm) to enter cells and reduce their viability [41], meaning that accumulation is strongly related to microplastic particle size. Understanding whether microplastic accumulates rather than coming in close contact with tissue during passage and elimination is critical to understanding possible long-term effects.

7. Conclusions

This review aimed to systematically organise the scientific evidence on microplastic accumulation in biological tissues, including challenges for their determination.

Firstly, digestion methods for preparing biological tissues prior to microplastic analyses could be improved. The evaluation of the type of reaction needed according to the chemical composition of the tissue to be digested is essential. This, could result in a reduction of the digestion time and, consequently, of the negative impacts that digestive agents can have on microplastic particles. An optimum preparation technique could also improve the quantification and qualification of microplastic, reducing the interference produced by the presence of organic matter.

Regarding the chemical identification of microplastic, identifying and choosing an analytical method which is fit for purpose for the likely target particle size is recommended. Spectroscopy techniques (μ-Raman, μ-FTIR) have limitations mainly related to microplastic particle size.

From a biological point of view, the literature review found unclear and sometimes conflicting evidence in relation to the accumulation of microplastic in biological tissues. Studies considered in this review reported the accumulation of large microplastic particles (*i.e.*, > 100 μm, mainly fibers) in biological tissues (*i.e.*, liver, muscle, GIT of fish). This should be further assessed with systematic toxicokinetic studies. It is important that studies clearly distinguish between microplastic particles which are only passing through and those which are accumulated in tissues and thus embedded/internalised into cells. Mostly, studies which focused on pre-treatments on samples (*i.e.*, effects of digestion on microplastics) considered large microplastic particles (> 100 μm). Therefore, a gap emerged in relation to the translation of results to sizes more relevant to target tissues. Similarly, the majority of microplastic accumulation results reported the occurrence of large microplastics in biological tissues. As the majority of studies (81 out of 84) reported particle sizes higher then to the limit for translocation, their presence could be likely due to false positive results. Eliminating false positives is a challenge, even with the inclusion of blanks and blank correction, and there are already many reviews with recommendations of how to do this, as noted by Shruti *et al.* [85]. However, it is worthy to note that alongside this practice, an element of logic and critical thinking should be used when interpreting data. Finally, it could be recommended that we as researchers consult the wider literature and expertise, crossing into other disciplines such as particle toxicokinetics and therapeutics, as well as other types of solid, persistent particles, to aid interpretation of findings in relation to biological plausibility. This will enable better judgement. Equally, uncertainties or caveats could be reported in manuscripts. Some suggestions for future research are: *i)* the effects of digestion reagents on microplastic particles of a bioavailable size; *ii)* the refinement of analytical techniques for chemical identification of microplastic particles/polymers to avoid errors in final results; *iii)* systematic toxicokinetic experiments using a variety of small microplastic particles (*i.e.*, < 10 μm) to distinguish accumulation of microplastic in tissues *versus* their close contact with tissues during passage; *vi)* the development of validated methodologies, with a particular focus on QA/QC, which can led to a reduction in false positive results.

Environmental implication

The systematic review we would like to submit to this journal analysed some of the papers which reported the occurrence of microplastics in biological tissues in relation to their size, clearly trying to distinguish between microplastics that are "passing through" a tissue and those which are accumulated in it. This differentiation is quite important as all the investigation which aims to the analyses of adverse effects are based on the accumulation of microplastics in tissues. This clarification could be useful for understanding the fate of microplastics in living organisms.

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.

Data Availability

This is a systematic review. Therefore, data used are available in published papers.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132991.

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