Microplastics in vacuum packages of frozen and glazed icefish (*Neosalanx* spp.): A freshwater fish intended for human consumption

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Abstract

It is widely accepted that human is exposed to microplastics through food consumption, however data occurrence in foodstuffs are still little and basically limited to seafood. In this study, the presence of microplastics was investigated in icefish (*Neosalanx* spp.) samples sourced from various mass-market retailers in Italy, supplied as frozen, glazed and vacuum-packed product. Icefish is a small freshwater fish widely imported in Europe from China as surrogate of other fish species subjected to commercial restriction, consumed whole after cooking in several culinary preparation. The samples (~10 g of icefish from each of the 40 packs tested) were digested using a solution of 10% potassium hydroxide and filtered through a 5 µm pore-size filter. Filters of the samples were observed under a stereomicroscope and the chemical composition of the items detected were analysed by FT-IR spectroscopy. A total of 163 items were counted in 37 (92.5%) samples with a mean value of 0.42±0.28 items/g w.w. Fibers were the most detected morphotype and several plastic polymers, such as polypropylene, polyethylene, polyethylene terephthalate and polystyrene, were identified by FT-IR analysis. As store-bought samples, the sources of microplastics could be substantially related to contamination during food processing. However, an intravital exposure to microplastics present in the surroundings waters cannot be ruled out. More foodstuffs need to be investigated for microplastic presence. In this study, microplastic occurrence was reported in freshwater biota intended for human consumption sampled directly from supermarket contributing to the risk assessment of human exposure to microplastics via food consumption.

Introduction

Nowadays, the environmental and human health risks posed by plastic pollution have aroused concerns from the scientific community and public opinion. Understanding and managing the impact of plastic on the health of ecosystems has become an undisputed priority, as highlighted by the growing number of national and international surveillance frameworks and research projects on this topic (Li *et al*., 2019). According to PlasticsEurope (2020), global plastic production reached 368 million tonnes in 2019, 9 million tonnes more than in 2018. The increase in plastic production led to an increase in plastic wastes which persist and accumulate in the environment due to the high stability of their polymer constituents (Zhang *et al*., 2021). During the last years, particular attention has been paid to the issues of microplastics (MPs): plastic fragments in the size range of 1 µm to 5 mm (GESAMP, 2019) which can be originally manufactured of such size (primary MPs) or resulted from fragmentation of larger plastic items (secondary MPs) (EFSA, 2016). MPs have been widely detected in both marine and terrestrial ecosystems and their wide occurrence and bioavailability have aroused concerns especially for the interaction with biota (Hartmann *et al.*, 2017; Arab *et al*., 2021). MPs are small enough to be ingested by biota at all trophic levels (Wesch *et al*., 2016), entering the food chain and reaching humans through the biomagnification process (van Raamsdonk *et al*., 2020). Once ingested, MPs may determine mechanical damages and inflammatory responses or release chemicals additives and contaminants with effects not yet fully understood (Ogonowski *et al*., 2018). There is still too little evidence on the fate, distribution and outcomes of MPs following human oral exposure (Prata *et al*., 2020). While there is a body of evidence regarding the sources, distribution and effects on natural ecosystems, little is known about MP occurrence in foods and relative implications for human health (Barboza *et al*., 2018; Danopoulos *et al*., 2020a). As with environmental studies, most of the data available regarding MPs and foods have basically focused on the marine ecosystem. Many commercially relevant fishery products, such as different species of fishes and shellfishes, were reported to be contaminated by MPs (De-la-Torre, 2020). On the other hand, data occurrence of MPs in terrestrial and freshwater ecosystems and related foodstuff have been less researched (Panebianco *et al.*, 2019; Wang *et al*., 2019). This might seem a paradox considered that plastic pollution in the marine environment is mainly of terrestrial origin and freshwater systems are among the main vehicles of contamination (Auta *et al*., 2017; Arab *et al*., 2020). Furthermore, the presence of MPs in biota intended for human consumption has been mostly reported in organism sampled from the environment while only a few data are available for samples directly collected from markets and supermarkets (Nalbone *et al*., 2021a). Data occurrence in store-bought foodstuffs and edible organisms from lees researched ecosystems are certainly desirable for a comprehensive risk assessment posed by MPs to human health via food consumption.

On this background, icefish could be an interesting target organisms. Species of the Salangidae family, commonly known as icefish, consist of several small (adults ~6 cm long) and anadromous marine and freshwater fishes, endemic to the seacoasts, rivers and lakes of Southeast Asia (CITES, 2020). Adult fishes are neotenic and characterized by an opaque white and smooth body that becomes milky after dead, females are scale-less while males have a few near the origin of the caudal fin. The dorsal fin is small in the latter part of the back while the anal fin is relatively large (Liang, 1991). The juvenile nourishes

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almost exclusively on planktonic crustaceans and copepods whereas adults also feed on other tiny fishes (Liu, 2001; Tang *et al.*, 2012). According to Eschmeyer's Catalog of Fishes, the Salangidae family includes a total of 8 genera (*Hemisalanx*, *Leucosoma*, *Neosalangichthys*, *Neosalanx*, *Parasalanx*, *Protosalanx*, *Salangichthys*, *Salanx*) and eighteen species. Some species are widespread but others, such as *Neosalanx* and *Parasalanx*, have been recorded at low abundance relative to other icefish due to habitat loss, pollution and overfishing (Wang *et al*., 2005). Icefish has been traditionally exploited in China for commercial harvesting, representing one of the most important sources of income for the export of fishery products to third countries (Huang *et al.*, 2020). Not by chance, (Kang *et al.*, 2015) in order to meet market demands, there have been several attempts to introduce non-native species into lakes or reservoirs in China, not always successfully (Kang *et al*., 2015). In Europe, icefish has been widely imported as a surrogate of the juvenile forms of *Sardina pilcardus* and *Engraulis echrasiculus* and *Aphia minuta*, species of higher commercial value traditionally consumed in the areas of the Mediterranean basin marketed in Italy under the name of "bianchetto" and "rossetto", respectively (Armani *et al*., 2011). Due to the morphological similarity, these species are fraudulently replaced with icefish since the fishing and marketing of bianchetto is banned in Europe and the fishing of rossetto is constrained by stringent Community regulations (Regulation EC, 1967/2006). Icefish are generally imported and sold as a frozen fishery product and consumed after cooking in various culinary preparation, boiled or fried with or without other ingredients. In addition, icefish is consumed whole unlike other fishes in which gutting is expected to decrease human dietary exposure to MPs (EFSA, 2016).

In order to extend the state of knowledge on MPs in food, this study aimed to investigate the MP occurrence in frozen, glazed and vacuum-packed icefish samples (*Neosalanx* spp.) sourced in several mass-market retailers in Italy.

Materials and methods

Sampling

The abundance and composition of MPs were investigated in a total of 40 samples of icefish (*Neosalanx* spp.) collected from several mass-market retailers in Calabria and Sicily regions in Italy. Samples were directly sourced as sales units: polyethylene (PE) plastic packages weighing between 250 – 1000 g of frozen, glazed and vacuum-packed icefish inside a paper case. As reported on the label, icefish of all the samples had been caught from the Taihu Lake (China), fished with trawls, gillnets or lift nets. Once collected, samples were brought to the laboratory and stored at -30 °C directly inside their original packaging until processing. The mouth size of 40 specimens randomly selected among the samples was observed with a stereomicroscope (Leica M205C, Germany) and measured Leica's software (Leica LAS X version 3.0.14).

Sample preparation and microplastics' extraction

To exclude any accidental contamination with MPs, samples were processed in a restricted access room and all the materials, equipment and laboratory surfaces used for the analyses were rigorously washed and rinsed with double-filtered deionized water, obtained by sterile syringe filters (cellulose acetate) with 0.22 µm pore size (VWR International). Sample processing was performed under a clean airflow cabinet and a procedural blank, consisting of the entire procedure without icefish, was included for each session of analysis (4 samples at a time for a total of 10 sessions) to evaluate the external contamination, especially from fibres, that is considered a recurring phenomenon (Prata *et al*., 2020). The samples were exposed to air only when strictly necessary and for the shortest possible period. The entire analysis procedure was performed at room temperature.

Soft tissue digestion of the samples was performed according to a modified protocol of Thiele *et al.* (2019). Once defrosted, plastic package of each sample was gently opened using a steel scissors and a fraction of ~10 g was randomly sampled and placed into a 250 ml conical glass flask and then covered with aluminium foil. Samples were digested overnight in a 10% potassium hydroxide (KOH) (Sigma-Aldrich, USA) solution (40 g/mL w.v.) under constant stirring at 40 °C inside an oscillating incubator (Vdrl Asal 711/CT, Bioltecnical service, Italy). Digested samples were successively filtered through white mixed cellulose ester filters having 5 µm pore size and 47 mm diameter (Biosigma, Italy) using a vacuum system (Biosigma, Italy). The flasks were accurately washed with water which was subsequently filtered to ensure the filtration of any possible residues. Membrane filters were placed in Petri glass dishes and left to dry for some minutes.

Filter observation

Filter of each sample and procedural blank were three times observed under a stereomicroscope (Leica M205C, Germany) by two different operators to record and categorized MPs and other anthropogenic items according to their shape, size and color (Hidalgo-Ruz *et al*., 2012). Stereomicroscope observation was performed outside the clean airflow cabinet; therefore, two blank filters were placed next to the samples during the analysis and checked for any occurred air-borne contamination. Any items found on the blank filters were morphologically compared with those detected in the filters of the samples. If too similar to each other, items found in the filter samples were not considered for the final count.

Based on their shape, the identified items were classified according to

Table 1. Morphological descriptors for MPs according to GESAMP (2019).

Filed description	Alternative descriptor	Characteristics	Abbreviation
Fragment	Granule, flake	Irregular shaped hard particles having appearance of being broken down from a larger piece of litter	MP-fr
Foam	Expanded polystyrene or polyurethane	Near spherical or granular particle, which deforms readily under pressure and can be partially elastic, depending on weathering state	MP-fo
Film	Sheet	Flat, flexible particle with smooth or angular edges	MP-fi
Line	Fibre, filament, strand	Long fibrous material that has a length substantially longer than its width	MP-li
Pellet	Resin bead; Mermaids' tears	Hard particle with spherical, smooth or granular shape	MP-pe

GESAMP (2019) in: i) "fragment" (MP-fr); ii) "foam" (MP-fo); iii) "film" (MP-fi); iv) "line" (MP-li); "pellet" (MP-pe) (Table 1). Leica's software (Leica LAS X version 3.0.14) for the imaging analysis was used to measure the size of the items which were then classified into dimensional classes.

Chemical identification of MPs

About 20% of the isolated items were randomly selected according to their morphological prevalence and analyzed for the chemical identification of the constituent polymer by Fourier-transformed infrared (FT-IR) spectroscopy. Items were sampled from the filter surface under the stereomicroscope using a small tweezers or a little brush, washed in double-filtered deionized water and stored in glass vials. FT-IR analysis was performed using an IRAffinity-1S spectrophotometer (Shimadzu, Japan) equipped with a sealed and desiccated interferometer, a DLATGS (Deuterated Triglycine Sulphate Doped with L-Alanine) detector and a Specac Quest ATR accessory (Specac Ltd, London, England). The IR spectra of the selected items were recorded in the range from 4000 cm^{-1} to 400 cm^{-1} (45 scans, 4 cm–1 resolution) with Happ–Genzel apodization and elaborated using Lab Solution IR v. 2.16 software. The IR spectra of the identified items was compared with Shimadzu ATR polymers Library and an index of at least 70% match was considered acceptable. A background reading was collected before each test and two readings were taken from each analyzed item.

Positive controls and validation of the analytical protocol

Since no data are available in literature regarding the use of KOH in icefish, digestive efficacy was assessed by evaluating the appearance and quantity (in terms of weight) of the post digestion organic residues.

Different size ranges (40 – 48 µm, 63 - 90 µm; 91 - 125 µm; 126 - 180 µm; 181 - 355 µm and 356 - 510 µm) of white low-density (0.924 g/cm^3) PE (LDPE) particles (Sigma-Aldrich, USA) were individually spiked in icefish samples to establish the minimum detectable particle size with our analytical protocol and the recovery rate of the used method.

Minimum detectable particle size with our protocol was established by spiking 20 individual LDPE particles of a given size range onto representative icefish samples (~10 g each) that were processed as described above. Particles were tested in order of decreasing class size (starting with the largest size range ie 356 - 510 µm) until they were no more easily detectable in the filter surface under the stereomicroscope or not identifiable with our FT-IR device (Nalbone *et al.*, 2021b).

The recovery rate was evaluated by spiking individually 20 LDPE particles of the minimum detectable size range onto nine icefish samples (~10 g each) which were analyzed three at a time on three different days as described before. The particle recoveries are determined by counting the numbers of retrieved particles by the amounts added. The 10% of the counted particles were confirmed to be LDPE by FT-IR analysis.

Data processing and statistical analysis

The generic term "item" used throughout the text refers to the particles detected in the filters at the stereomicroscope, but their plastic nature has not been confirmed by FT-IR analysis.

The size of particles tested was calculated considering the length of the MPs-li and the longest side for MPs-fr, MPs-pe, MPs-fo and MPs-fi.

The obtained data were presented as mean ± standard deviation and parts to whole expressed as percentage.

The normality distribution of data was tested by D'Agostino-Pearson test and, if necessary, proper transformation was also used. One-way analysis of variance (ANOVA) was used to evaluate any significant prevalence among the different morphological types of items detected. The post hoc Tukey's test was performed for the multiple comparisons within the obtained ANOVA data. The critical significance level (p) was set at 1% (0.01), and all tests were performed two-sided. All the statistical analyses were carried out by Graph Pad Prism 9 software (San Diego, CA, USA).

Results

Abundance and characteristics of microplastics detected in icefish

External contamination with airborne fibers was prevented during sample preparation, digestion and filtration considering the low mean value of 0.17±0.39 fibers/filter detected in the procedural blanks. The number of fibers observed in the blank filters (0.25±0.45 fibers/filter) also allows to look with confidence at the sample results. Most of the contaminations were represented by blue fibers >3000 µm in length. The mouth size of the icefish analyzed was 1.37±0.12 mm.

Overall, a total of 163 items were tallied in 37 (92.5%) samples with a mean value of 0.42 ± 0.28 items/g w.w. with a maximum number of 1.02 items/g w.w. (Table 2). The number of items counted per sample ranged from 0 to 10 with a mean value of 4.08±2.63 items/sample.

As concern the shape, out of 163 items detected, MPs-li were the most observed type (88,34%; n=144), following by MPs-fr (9,82%; n=16), MPs-fo (1,23%; n=2) and MP-fi (0,61%; n=1). Among the samples, a significant difference was observed regarding the number of items/g and shape type. In detail, the mean amount of MPs-li (0.37±0.28 items/g w.w.) was significantly higher than that calculated for all other morphological types (p<0.01). While no signif-

Table 2. Distribution of samples and relative abundance and composition of items detected in frozen, glazed and vacuum-packed icefish sourced from different retails in Italy.

Parameter	Data
Total weight (g) of icefish examined	391.65
N. of samples examined*	40
Weight (g) of a sample*,**	9.79±0.71
Total weight (g) of positive samples*	362.93
N. of positive samples*	37
Percentage of positive samples*	92.5
Size range of the items detected (µm)	50-6000
Total N. of items	163
N. of MPs-li	144
N. of MPs-fr	16
N. of MPs-fo	2
N. of MPs-fi	1
MP abundance	
N. of items per sample*,**	4.08±2.63
N. of items per positive samples*,**	4.41±2.44
N. of items per weight (g)**	0.42±0.28
N. of items per weight (g) of positive samples*,**	0.45±0.26

*Each sample consisted of 10g of icefish. **Values are expressed as mean ± standard deviation. N=number.

icant differences (p>0.01) were observed between the mean amount of the MPs-fr (0.04±0.07 items/g w.w.), MPs-pe (0.005 ± 0.022 items/g w.w.) and MPs-fi (0.002±0.016 items/g w.w.).

Overall, items ranging in size from ~201 µm to ~500 µm were the most detected followed by those between ~500 - 1000 µm and ~1000 - 1500 µm (Figure 1). The MPs-li were between 100 and 6000 µm in length and those in the range of ~200 - 500 µm were the most detected. The size of the MPs-fr ranged from ~50 µm to ~500 µm with a prevalence of those in the size range of ~100 - 200 µm. Both the MPs-fo and the MP-fi were between ~100 - 200 µm in size.

As concern the color, MPs-li were blue (n=101) and black (n=43), MPs-fr were blue (n=12) and yellow (n=4) while the MPs-fo and MP-fi were white.

The polymer composition of 34 items (22 MPs-li, 9 MPs-fr, 2 MP-pe and 1 MP-fi) were investigated by FT-IR analysis (Figure 2). A total of 28 items, ranging in size between ~150 µm and ~1000 µm, were confirmed to be MPs. In detail, 9 MPs-li were identified as polypropylene (PP), 7 MPs-li as rayon (RY), 5 MPs-fr as PE, 4 MPs-fr as polyethylene terephthalate (PET), 2 MPs-fo as polystyrene (PS), 1 MP-fi as PE and 6 as cotton fibers.

Validation of the analytical protocol

The digestion obtained from the 10% KOH solution was effective enough to allow easy filtration of the samples through filters in mixed cellulose ester with 5 µm pore size. The mean weight of the organic residues obtained after the digestion was 0.49±0.35 g/sample/filter. The undigested organic residue was characterized by a homogeneous veil of curled scales, probably bone or cartilage, which can complicate the filter observation for an untrained operator.

Among the different size classes tested, the 63-90 µm range was the smallest in which potential LDPE particles could be easily detected under the stereomicroscope and subsequently identified with our FT-IR device. The smaller LDPE particles tested were also detectable under the stereomicroscope, however, our FT-IR device did not allow correct identification of the polymer composition.

A total of 167 potential LDPE particles were counted on the nine spiked samples with a recovery rate of 92.8%. The 10% of the potential LDPE particles detected were tested with FT-IR analysis which confirmed their polymer composition.

Discussions

Our results show that vacuum packs of icefish regularly marketed as frozen and glazed fishery product can be contaminated with MPs. Sources of contamination in the analysed samples can be several and groupable into two main groups: from the environment or during food processing. It is important to stress that studies on MPs in store-bought samples are suitable to evaluate human exposure via food consumption while they are not significantly reliable for environmental studies (Barboza *et al*., 2018). This because food processing might substantially contribute to the occurrence of plastic particles in foods (EFSA, 2016). Plastic is extensively present in the food industry in structures, equipment, utensils and staff's clothes as well as widely used for packaging. The wear and ageing of facilities might determine particle realise in the workplace that can contaminate food through aerial deposition or by direct contact with worn surfaces (Prata, 2018). MPs were observed in atmospherics fallouts, as well as in indoor and outdoor environments (Gasperi *et al*., 2018). Zhang *et al.* (2020) have reported high levels of MPs in indoor environments (up to 9.9×10^3 MPs/m^2/d) recognizing textile quantity as a main factor affecting their abundance whereas airflow turbulence induced by air conditioners prone to MP migration. In the food industries, temperature-controlled work environments are often needed; therefore, ventilation and air conditioning systems are always present and could play a key role in the spatial distribution of air-borne MPs falling in foods. Particles could also shape from food contact materials (FCM) because of mechanical stimuli that cause abrasions (*e.g*., closing the cap of plastic bottles or rubbing in easily fragmentable surfaces such as expanded PS). In this regard, recycling and reuse might increase particle release from FCM as result of a greater material fragility (Giese *et al*., 2021).

During the production of frozen and glazed vacuum-packed icefish, there are several steps in which MP contamination is possible. As already well documented, the nets used for icefish fishing, usually made of nylon or PE, could be a first important source of plastic fibres (Montarsolo *et al*., 2018). During processing, in all those moments when the fish were exposed to the air, contamination could potentially have occurred. Vacuum packaging could also be a source of contamination, as the packaging machines usually blow air from the outside towards the inside of the packages before creating the vacuum, thus being able to contaminate the packages with air-borne MPs. Contact with scratched plastic surfaces,

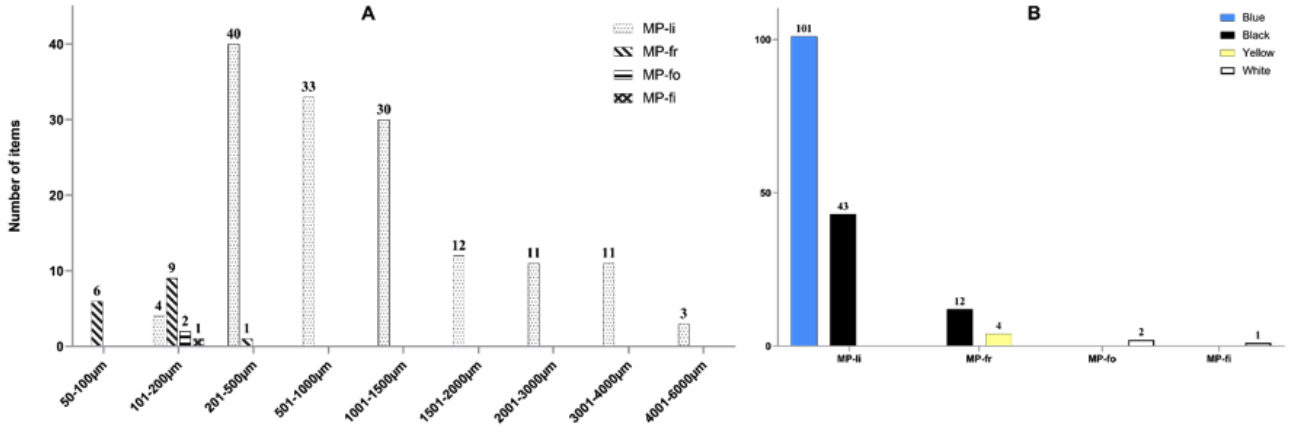

Figure 1. Number, size (A) and color (B) of the different morphological types of items detected in icefish (*Neosalanx* spp.) samples sourced from mass-market retailers in Italy as frozen, glazed and vacuum-packed product.

especially if made of easily fragmentable materials such as expanded PS, could have contaminated the sample. Also, the water, used for a possible post-capture washing or during glazing, can be a source of MPs considering that several Authors have reported the presence of MPs in potable water samples (Danopoulos *et al*., 2020b).

On the other hand, contamination intravital cannot be ruled out. Icefish can accumulate MPs present in the surrounding water through two main pathways: ingestion or adherence. Our samples had all been fished in Taihu Lake (China), the third-largest freshwater lake in China located in the Yangtze River Delta, a heavily industrialized area well known for its tourism and fishing activity. Due to the development of the local economy and industry, Taihu Lake has become one of the most severely polluted lakes in China (Yan *et al*., 2014). In a survey conducted by Su *et al*. (2016) on the presence of microplastics in the waters, sediments, plankton and bivalve molluscs of Taihu Lake, contamination levels are among the highest in freshwater lakes worldwide, with values of 0.01×10^6 - 6.8×10^6 items/km^2 in plankton net samples, 3.4-25.8 particles/L in surface waters, 11.0-234.6 items/kg dw in sediments and 0.2-12.5 items/g ww in Asian clams (*Corbicula fluminea*). The Authors find mainly fibres whose source is attributed to contaminants from domestic or industrial drains carried by influent rivers connecting the Yangtze River area to Taihu Lake. Icefish can accidentally ingest MPs from the waters, mistaking them for prey, or eating other contaminated organisms, as reported for other fishes (Wesch *et al*. 2016).

MP ingestion is conditioned by some physical characteristics of the particles such as size or the three-dimensional arrangement in space. Fibers, for example, can be tangled and easily ingestible despite their length (Ward *et al*., 2019) while the other MPs might be ingested only if smaller than ~1500 µm, considering the observed mouth size of icefish analysed.

However, it is also possible that the particles in the aquatic environment adhered to the surface of the fishes as reported by Kolandhasamy *et al*. (2018) in a contamination study of mussels where adherence contributed about 50% of the MP uptake.

Given the many and several possible sources of contamination for store-bought samples, should not be surprising to found MPs of different polymers and physical characteristics. In the present study, particles consisting of synthetic polymers such as PP, PE, PET and PS as well as semi-synthetic and natural fibres such as RY or cotton were identified. However, considering the above, establishing the exact origin of the particles based on their chemical composition could be speculative. In this regard, more in-depth studies are needed to evaluate, step by step, the possible contaminations along the entire production chain analysing, for each phase, the sources of contamination and the effects of food processing. Even today, there is little evidence of the effects of food treatments, such as cooking, on MPs that could exacerbate the associated chemical hazards (EFSA, 2016). For example, ice fish is usually consumed boiled or fried then exposed to high temperatures that could determine the formation of toxic compounds such as dioxins from MPs (Smith *et al*., 2018).

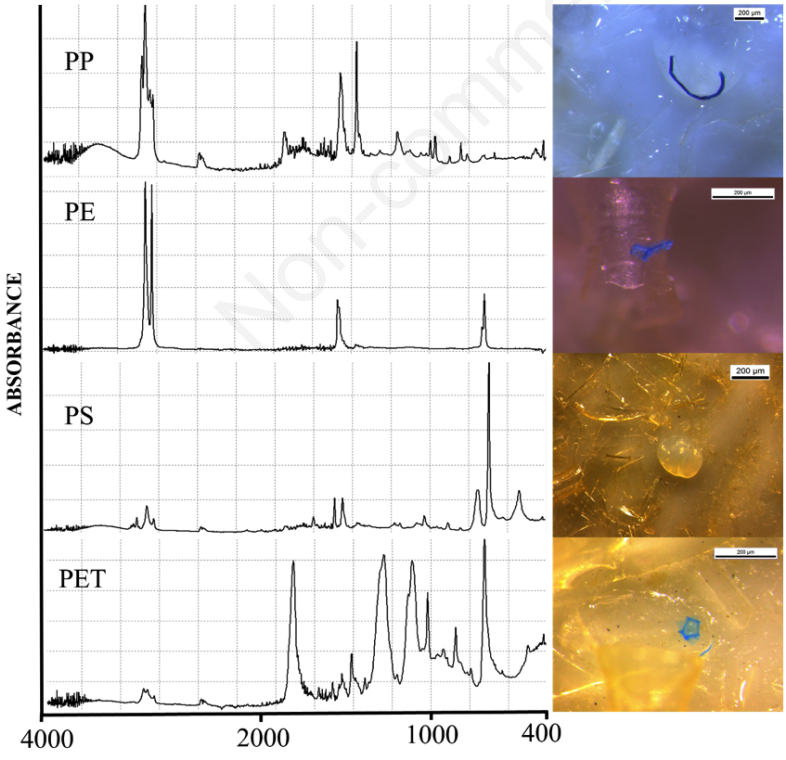

Figure 2. IR spectra of the plastic polymers (PP: polypropylene; PE: polyethylene; PS: polystyrene; PET: polyethylene terephthalate) identified in icefish (*Neosalanx* spp.) samples sourced from mass-market retailers in Italy as frozen, glazed and vacuum-packed product.

Conclusions

To be able to perform a comprehensive risk assessment posed by MPs to human health via the food chain, data on dietary exposure are needed. Despite there have been several efforts by the scientific community, it is still unclear whether MPs are a public health problem. While it is widely accepted that humans are exposed to MPs through food consumption, the occurrence data in food are small and basically limited to seafood whereas still too little is known about other foodstuffs and store-bought samples. Therefore, investigating the presence of MPs in food not yet tested is certainly desirable. In the present study, MPs were detected in a new food matrix, the icefish, collected directly from supermarket as processed product contributing to increase the number of data available about human exposure to MPs through food consumption.

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