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Microplastics in livers of European anchovies (*Engraulis encrasicolus*, L.)☆

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ABSTRACT

Microplastics (MPs) are thought to be ingested by a wide range of marine organisms before being excreted. However, several studies in marine organisms from different taxa have shown that MPs and nanoplastics could be translocated in other organs. In this study, we investigated the presence of MPs in the livers of commercial zooplanktivorous fishes collected in the field. The study focuses mainly on the European anchovy *Engraulis encrasicolus* but concerns also the European pilchard *Sardina pilchardus* and the Atlantic herring *Clupea harengus*. Two complementary methodologies were used to attest the occurrence of MPs in the hepatic tissue and to exclude contamination. 1) MPs were isolated by degradation of the hepatic tissue. 2) Cryosections were made on the livers and observed in polarized light microscopy. Both methods separately revealed that MPs, mainly polyethylene (PE), were translocated into the livers of the three clupeid species. In anchovy, 80 per cent of livers contained relatively large MPs that ranged from 124 μm to 438 μm, showing a high level of contamination. Two translocation pathways are hypothesized: (i) large particles found in the liver resulted from the agglomeration of smaller pieces, and/or (ii) they simply pass through the intestinal barrier. Further studies are however required to understand the exact process.

1. Introduction

Since the 1950s, plastics have been produced in huge quantities, polluting oceans (Cózar et al., 2014), rivers (Gasperi et al., 2014), lakes (Free et al., 2014), and beaches (Turner and Holmes, 2011). Plastics are even found in isolated regions with very small human populations, such as in the Arctic (Bergmann and Klages, 2012; Lusher et al., 2015). Previous studies have shown that these plastics are fragmented by prolonged exposure to UV and by physical abrasion (Andrady et al., 2003; Barnes et al., 2009), leading to smaller particles, such as microplastics (<5 mm) (Arthur et al., 2009) and nanoplastics (<1 μm) (Cole and Galloway, 2015). Microplastics (MPs) are then ingested by various organisms, from zooplankton (Cole et al., 2013) to cetaceans (Fossi et al., 2016), including fishes (Anastasopoulou et al., 2013; Foekema et al., 2013; Lusher et al., 2013; Collard et al., 2015; Romeo et al., 2015, 2016; Neves et al., 2015). Once ingested, it is assumed that MPs are egested (Van Cauwenberghe and Janssen, 2014; Watts et al., 2014), although in *Mytilus edulis* smaller MPs have shown a shorter gut retention time than larger MPs (Van Cauwenberghe and Janssen, 2014).

Several experimental studies have shown that some MPs and nanoplastics can be translocated outside the digestive tract. MPs were, for example, found in different body compartments in two different crab species, *Uca rapax* (MPs of 180–250 μm) and *Carcinus maenas* (0.5 μm in diameter) (Brennecke et al., 2015; Farrell and Nelson, 2013 respectively), where they were found in the hepatopancreas, gills and ovaries. Two studies also focused on plastic translocation after ingestion by the bivalve *Mytilus edulis* (Browne

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et al., 2008; Von Moos et al., 2012). In two different experiments, Browne et al. (2008) showed that (i) MPs (2 and 4–16 μm) were accumulated in the gut lumen and digestive tubules and (ii) MPs (3 and 9.6 μm) were translocated in the hemolymph and hemocytes. Von Moos et al. (2012) aimed to determine whether MPs enter the digestive system and negatively impact mussels' health. They found MPs (<80 μm) on gills and inside the digestive system (intestine and digestive tubules of the hepatopancreas), and that granulocytomas, a non-neoplastic inflammatory cellular condition whose dominant cell type is granulocytes (Lowe and Moore, 1979), occurred in the digestive gland as a consequence of MP ingestion. Another effect was the instability of the lysosomal membrane. These deleterious effects are indicators that the health status of the mussel is impacted by MP ingestion. In fishes, 39 nm polystyrene (PS) particles were detected in the liver, intestine and gonads of the medaka *Oryzias latipes* (Kashiwada, 2006), which were most probably transported through the bloodstream, assuming entry by gills and/or gut epithelium. Mullets (*Mugil cephalus*) have been exposed to particles of different sizes (0.1–1 mm), and one size category (0.2–0.6 mm) was found to be translocated into the liver (Avio et al., 2015). Another experimental study on fish, which focused on impacts on the intestine after MPs exposure (<300 μm), found that virgin MPs and polluted MPs induced moderate to pronounced alterations of the distal intestine, respectively, after a 30-day period exposure (Pedà et al., 2016). Impacts were exacerbated when the exposure reaches 90 days, highlighting the role played by the exposure time and the adsorbed pollutants.

MPs have been previously described at the level of the gut in wild populations of different Clupeiformes (Foekema et al., 2013; Collard et al., 2015, 2017; Rochman et al., 2015). In *Engraulis encrasicolus*, particle sizes ranged from 220 μm to 22 mm (Collard et al., 2017). The fate of ingested particles is, however, not known; they can simply be egested, and/or they can be translocated in the body—thus contaminating different organs. Our objective was to mention and to prove that translocation of MP particles in the liver of commercial species of European anchovies (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*) and the European pilchard (*Sardina pilchardus*) does exist, and to confirm it by cryostatic sections, excluding the contamination as an explanation.

2. Materials and methods

2.1. Sampling

Thirteen anchovies (*E. encrasicolus*) were sampled in the Mediterranean Sea (Gulf of Lions, 41.38°N to 41.81°N and 2.27°E to 3.20°E) in July 2013, during the Pélagiques Méditerranée (PELMED) survey (Bigot, 2013) using a pelagic net. The mean total length (TL) of *E. encrasicolus* was 12.4 cm ± 1.0 cm (standard deviation).

In addition, two sardines (*S. pilchardus*) and two Atlantic herrings (*C. harengus*) were sampled during the International Bottom Trawl Survey (IBTS) (Verin, 2013, 2014). *C. harengus* were sampled in January 2013 in the North Sea (55.27°N to 55.30°N and 7.38°E to 7.40°E). *S. pilchardus* were sampled in the English Channel (50.30°N to 50.63°N and 0.09°E to 0.46°E) in January 2014. All sampling surveys were organized by the IFREMER. Sardines measured 24 cm and 25.5 cm (TLs) and herrings measured 22.5 cm and 23.5 cm (TLs).

Individuals were measured and dissected on board and the livers were directly put into a −20 °C freezer.

2.2. Preventing contamination and blank samples

Three blank samples were run, to identify possible contamination in the laboratory. Moreover, to avoid contamination, all solutions were filtered with the same filter membranes (5 μm porosity) than those which were used for particles' isolation from livers. All dissection materials and work surfaces were cleaned with ethanol (70 per cent). The isolation process was performed under an air-flow cabinet, except for the drying of the stainless-steel plate. During drying, plates were put under a metal sifter (36 μm mesh). Subsequently, particles smaller than 36 μm were not analyzed. No polymer was found in the blank samples.

2.3. Liver digestion and MP isolation

The methodology used to isolate MPs from livers is the same than the one used by Collard et al. (2015). Briefly, frozen livers were put into a 9 per cent NaClO solution overnight. The solution was filtered and the filter membrane was put into a methanol bath. An ultrasonic bath was performed on the methanol and filter membrane for 5 min. The filter membrane was then removed and the methanol solution centrifuged at 5000 rpm for 5 min. Two milliliters of the methanol solution were collected from the bottom of the centrifuge tube using a pipette and deposited on a stainless-steel plate. After 2 h of drying, the plate could be used directly as a mount for Raman analyses. Among the 13 *E. encrasicolus* sampled in the Mediterranean Sea, ten were used for liver digestion and particles isolation.

Before Raman analyses, all particles on the plate were photographed using a MOC-510 Mueller-Optronic 5 megapixel CMOS camera set on a stereo microscope and with a maximum magnification of 45x.

2.4. Cryosections

Livers used for cryosections were kept frozen from the sampling to the cutting. Livers were thawed and embedded in an Optimum Cutting Temperature (OCT) resin, mainly made of water soluble glycols. Frozen sections 16-μm thick were performed from three livers using a Microm HM 500 O cryostat (Microm, Heidelberg, Germany). These were mounted on Super Frost glass slides (VWR, Leuven, Belgium) to be observed under polarized light in a Olympus Provis AX-70 microscope fitted with a VisiCam 5.0 megapixels camera (VisiCam software, VWR, Leuven, Belgium). Particles of interest were then analyzed by Raman spectroscopy. The laser spot was focused on three different points of the particles, to determine whether they were single particles or aggregates of several smaller ones.

2.5. Raman spectroscopy

MPs were analyzed using a LabRam 300 spectrometer (Jobin Yvon) provided with an Olympus confocal microscope and an Andor BRDD Du401 CCD detector. A Spectraphysics argon–ion laser (514.5 nm) and two Olympus objectives (magnification x50 and x100) were used. The maximum beam laser power on the sample was 5 mW. During the Raman measurements, settings were as follows: the confocal hole was 300 μm, the slit was set to 100 μm and the integration times ranged from 10 to 40 s. The instrument was calibrated considering the position and the intensity of the silicon crystal Raman line at 521 cm^{-1}. Matchings between recorded spectra and references from commercially available or homemade libraries were performed using the Thermo Specta 2.0 software. To check the homogeneity of the sample, the laser spot was focused successively on different regions of the particle and, in the case of similar measurements, the best spectrum was retained.

If the spectra shown were location dependent, imaging experiments were performed. The microscope was equipped with an xy computerized mobile stage, which enables imaging by scanning the

sample at defined locations. With a x100 objective, the system allows for reaching a lateral resolution of 0.5 μm and a focusing-depth of about 2 μm. Measurements were made step-by-step every 5 μm, with an integration time of 10 s, and accumulated twice for each step. The size of the recorded area was 75 μm × 75 μm (corresponding to 225 spectra in total). Given the strong overlap of the components' spectra in all frequency ranges, we decided to analyze the spectra from the 2900 cm^{-1} region, since it is the most intense. By using LabSpec software, a spectral image was built from the respective intensities measured at each point, choosing as boundaries 2872–2887 cm^{-1} for component 1 and 2901–2921 cm^{-1} for component 2. Despite the strong overlap, these boundaries were chosen as being the most representative of each component. The results given by this method were confirmed by a mathematical model (see Supporting Information).

Raman data and images were treated using the LabSpec software (Horiba Jobin Yvon, v.5.78) and a lab–made software which allowed both the evaluation and the subtraction of the spectrum background.

2.6. Particles weights and lengths

After Raman analyses, particles extracted with the NaClO treatment were individually weighed with an analytical balance (AX105, Mettler-Toledo, Switzerland). The software ImageJ (v1.48, National Institutes of Health, USA) was used to measure the maximum length of each particle. Results are expressed in values ± standard deviation (SD).

3. Results and discussion

Nine MPs were found in eight of the ten analyzed livers of *E. encrasicolus*. They ranged from 124 μm to 438 μm and averaged 323 μm (±101 μm). Eight MPs were made of polyethylene (PE, Fig. 1), and one was a copolymer of styrene and acrylonitrile (Fig. 2). Three spectra, made on three different zones of the same particle, showed that each sample was made with a single kind of polymer.

Two livers from *S. pilchardus* and two from *C. harengus* were also analyzed. MPs were found in three out of the four analyzed livers. They were also made of PE and were of the same size class than those found in *E. encrasicolus*.

MPs can thus be found in livers of marine vertebrates collected in the field and provides two important questionable results that warrant further studies: the high percentage of MP occurrence and the relatively large size of these MPs. Despite the small number of samples, a high proportion of wild anchovies had MP(s) within the liver, supporting the statement that MPs impact a high proportion of the wild *E. encrasicolus* found in the Mediterranean Sea. Under experimental conditions, *Mugil cephalus* have been fed with PE and PS particles ranging from 0.01 μm to 5 mm at an approximate concentration of 2500 particles/L (Avio et al., 2015), which is much higher than that found in the environment. In the Mediterranean Sea, concentrations of MPs reported in surface layers range between $1.16 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ particle/L (Collignon et al., 2012; de Lucia et al., 2014). Despite lower concentrations in seawater, wild fishes also accumulated MPs in their liver, meaning that translocation is not necessarily related to the pressure of high concentrations.

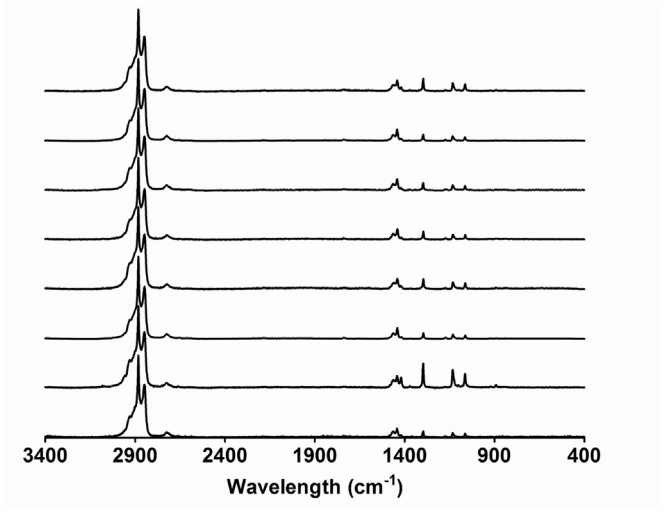

Fig. 1. Raman spectra of PE obtained by the analysis of eight particles extracted from the liver of anchovies.

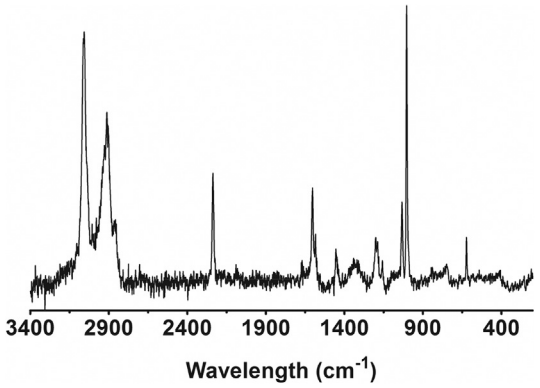

Fig. 2. Raman spectrum of the styrene/acrylonitrile copolymer obtained on the ninth particle from an anchovy liver.

Some studies have reported the occurrence of plastic particles in another tissue than stomach contents of marine organisms maintained in laboratories (Kashiwada, 2006; Browne et al., 2008; Von Moos et al., 2012), but particle sizes were much smaller than the MPs found in the present study. In the mussel *Mytilus edulis*, 3.0 μm and 9.6 μm PS microspheres were found in the circulatory system three days after exposure (Browne et al., 2008). In the fish *Oryzias latipes*, Kashiwada (2006) found nanoparticles (39.4 nm) in the liver, blood, gallbladder, and kidney after seven days of exposure; two translocation pathways were suggested: by gills and by gut epithelium. In a freshwater fish, *Danio rerio*, PS microplastics (5 μm) were translocated into the liver within two days (Lu et al., 2016).

Cryostatic sections revealed that two livers out of three each contained two PE particles, ranging between 39 μm and 90 μm in maximal length (Fig. 3). Three Raman spectra, corresponding to three different zones, were recorded on three of the four particles. These spectra corresponded to PE only, to a cellulose only, or to a mix of these two components (Fig. 4). Unfortunately, cryosections did not allow for the identification of the tissues that shelter MPs.

The Raman image performed on one of the three particles confirmed the 'composite' nature of MPs and showed the areas of distribution of the two components (Fig. 5a): PE (red zones) and cellulose (green zones).

During our study, it was unfortunately not possible to precisely localize MPs in the liver because of the conservation and the cryosections preparation which altered the tissue structure. Consequently, it couldn't be assessed whether particles were in or near a blood vessel, among hepatic cells, etc. This means that we can only hypothesize about the translocation process. Two assumptions, which are not mutually exclusive, are proposed: (1) that the particles found in the liver resulted from the agglomeration of smaller

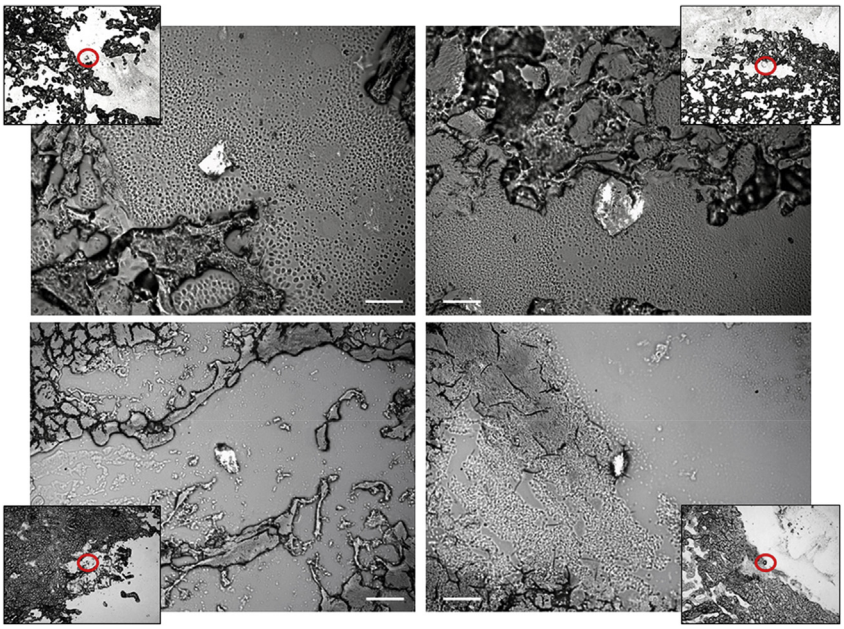

Fig. 3. Polarized light images (objectives x5 and x20) obtained from MPs in cryosections of livers from three anchovies. MPs are shown with red circles in smaller pictures. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

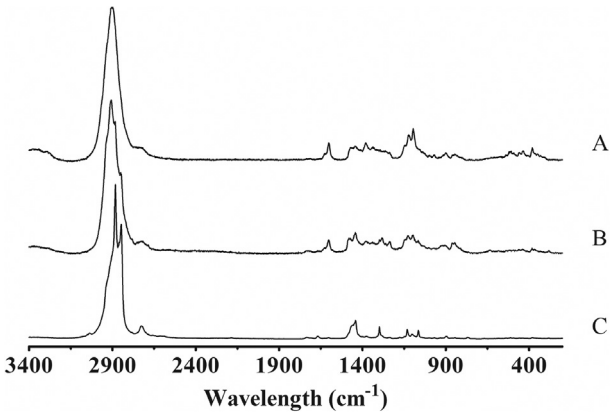

Fig. 4. Raman spectra of three different zones on the same particle in an anchovy liver: (A) cellulose, (B) mix of PE and cellulose, (C) PE.

pieces, and/or (2) that these large particles were directly taken up from the gut lumen by endocytosis, phagocytosis, or another mechanism allowing the particle to pass through the intestinal barrier. Our results and literature support both assumptions, which are both described below.

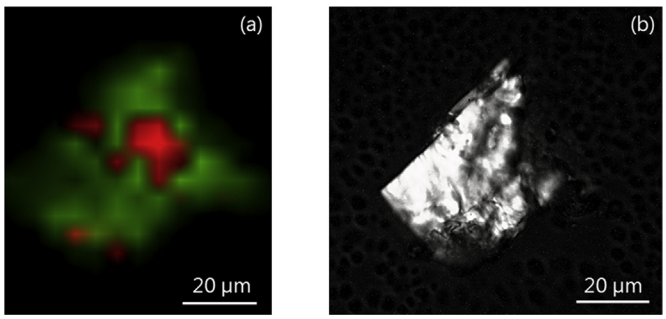

Fig. 5. (a) Raman imaging, (b) picture of the corresponding particle in polarized light microscopy.

The Raman map of a particle found in a cryosection revealed at least two components in the analyzed particle, PE and cellulose, which were distributed differently in space, as shown in Fig. 5. Consequently, the particles analyzed could be an agglomerate of smaller particles made of the two components. Particular aggregates have already been described in the digestive gland of mussels, *M. edulis* (Von Moos et al., 2012). The present results thus suggest that aggregation of 'plastic' microparticle or nanoparticle aggregates could occur in the liver of fishes under natural conditions.

On the other hand, the three analyzed zones of all particles extracted from livers by the degradation process gave the same spectrum, which strongly suggests that the particles have a homogenous composition and constitute a single piece. Avio et al. (2015) and Brennecke et al. (2015) have also shown that 180–600 μm particles could reach the liver or the hepatopancreas, supporting our second assumption that such large microparticles can be transported in full to the liver.

The processes that could be involved in the translocation of microparticles to the liver are unclear, because different parameters such as particle size (Szentkuti, 1997; Mittal et al., 2007), charge (Florence, 1997), composition (Urakami et al., 1994) or molecular weight (Mittal et al., 2007) might influence the process. Also, permeability of the colon could depend on a particle's dye—for example, in rats, particles of the same size but dyed with Methylene Blue penetrated faster and deeper into the epithelial tissue than particles dyed with Light Green Yellowish (Szentkuti, 1997).

In marine organisms, a few studies have detailed how particles have reached the liver or hepatopancreas. In the gastrointestinal tract of *M. edulis*, MP particles were taken up and internalized into cells by endocytosis and granulocytomas containing MP particles occurred in the digestive gland (Von Moos et al., 2012). Still in *M. edulis*, Browne et al. (2008) have suggested that specialized enterocytes called 'microfold cells' could be implicated. In rodents, these cells are involved in the transport of macromolecules, microorganisms and plastic particles (Smith et al., 1995; Buda et al., 2005). They deliver particles from the lumen of the gut to the

organized lymphoid tissue. However, Volkheimer (1975) has noted that large particles (5–110 μm) cannot pass through the enterocytes, but instead pass between them in a paracellular manner (i.e. between enterocytes of the epithelial layer). Avio et al. (2015) fed fish with particles ranging from 0.1 to 1 mm, and found one size class in the gut (0.5–1 mm) and in the liver (0.2–0.6 mm) using cryostatic sections. However, MP particles were randomly localized in the hepatic tissues, hindering the proposal of one or several explanations for this translocation.

Impacts of these translocations in fish are still unknown. In mussels, MPs in liver led to the formation of granulocytomas (Von Moos et al., 2012), and another study conducted by Browne et al. (2008) did not find any changes in the oxidative status of hemolymph, the viability and phagocytic activity of hemocytes, or filter feeding activity. Our main concern should probably be the fate of adsorbed pollutants and additives on MPs; if MPs may reach the circulatory system, pollutants could leach in the hemolymph or blood and be directly transferred through several organs. Moreover, since PS particles have been shown to enhance vascular thrombosis in hamsters (Nemmar et al., 2003), similar impacts could also occur in fish.

The three species studied belong to the Clupeiforme order, which is also the most fished group in the world (FAO, 2014). In addition, they are given to predator fish such as Bluefin tuna (Vita et al., 2004; Mylonas et al., 2010) and *Clupea harengus*, *Sardina pilchardus*, and *Engraulis encrasicolus* can also be used to produce fish meal (Miles and Chapman, 2006; Tacon and Metian, 2009). In laboratories, MPs are transferred from a trophic level to another level (Cedervall et al., 2012; Farrell and Nelson, 2013; Setälä et al., 2014). Fish meals are produced with whole fish, including liver (Windsor, 2001). As our results have confirmed through the presence of MPs in the liver of common forage fish, which are used as food for farmed fish, which are in turn directly consumed by humans, it is possible that we could accumulate MPs deriving from fish meal. Our study raises a lot of questions that need to be answered, regarding the impacts of such translocation, the species concerned, and the possible exposure of humans through consumption of farmed fish.

4. Conclusion

Our study does not only report the occurrence of translocation of plastic in the liver of wild commercial fishes but highlights also the high rate of impacted fishes with MPs of large sizes. These findings should open a new field of research since the translocation pathway(s) and impacts of this process in fish are not known.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.07.089.

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