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Detection of Anthropogenic Particles in Fish Stomachs: An Isolation Method Adapted to Identification by Raman Spectroscopy

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Abstract Microplastic particles (MP) contaminate oceans and affect marine organisms in several ways. Ingestion combined with food intake is generally reported. However, data interpretation often is circumvented by the difficulty to separate MP from bulk samples. Visual examination often is used as one or the only step to sort these particles. However, color, size, and shape are insufficient and often unreliable criteria. We present an extraction method based on hypochlorite digestion and isolation of MP from the membrane by sonication. The protocol is especially well adapted to a subsequent analysis by Raman spectroscopy. The method avoids fluorescence problems, allowing better identification of anthropogenic particles (AP) from stomach contents of fish by Raman spectroscopy. It was developed with commercial samples of microplastics and cotton along with stomach contents from three different Clupeiformes fishes: *Clupea harengus*, *Sardina pilchardus*, and *Engraulis encrasicolus*. The optimized digestion and isolation protocol showed no visible impact on microplastics and cotton particles while the Raman spectroscopic spectrum allowed the precise identification of microplastics and textile fibers. Thirty-five particles were isolated from nine fish stomach contents. Raman analysis has confirmed 11 microplastics and 13 fibers mainly made of cellulose or lignin. Some particles were not completely identified but contained artificial dyes. The novel approach developed in this manuscript should help to assess the presence, quantity, and composition of AP in planktivorous fish stomachs.

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Plastic world production is estimated to be 299 megatons (Mt) in 2013, with 20 % contributed from European sources (Plastics Europe 2015). It is estimated that 10 % of this production ends up in the seas (Thompson 2006). The North Atlantic Gyre is a dramatic example of plastic accumulation with a maximal concentration of 20,328 pieces per km^2 (Law et al. 2010). Among marine plastic debris, two size classes are commonly defined: macroplastics and microplastics (MP). Marine macroplastics comprise items larger than 5 mm. Their size, concentration, and origins often have been described since the late sixties (Merrell 1980; Horsman 1982; Dixon and Dixon 1983; Pruter 1987; Ryan and Moloney 1993; Derraik 2002). Macroplastics mechanically harm marine vertebrates and invertebrates in several ways such as entanglement, external wounds, ingestion, and gut blockage. They also impact them chemically by leaching organic pollutants adsorbed at sea or initially present on plastic matrix (plasticizers) (Thompson 2006). At the opposite, microplastics, which are smaller than 5 mm (Arthur et al. 2009), are less known because they are more elusive. They have been identified since the seventies (Carpenter and Smith 1972) but have been thoroughly studied since the early 2000s, when Moore et al. (2001) found that the

majority of plastics in the North Pacific were, on average, smaller than 4.76 mm and when Thompson et al. (2004) suggested environmental implications. It also is known that MP are widespread in the marine environment (Cózar et al. 2014; Eriksen et al. 2014; Law and Thompson 2014). Impacts caused by MP received less attention, likely due to the difficulty to isolate and identify them accurately (Cole et al. 2011). However, there are recent concerns about their toxicity on humans and wildlife (Wright et al. 2013). Due to their small size, they have the potential to be ingested by a wider range of marine organisms (Barnes et al. 2009), some of them being at the first levels of marine food chains (Thompson et al. 2004). The majority of MP is found at the surface layer (Ivar do Sul and Costa 2014) where they follow currents and accumulate in gyres or beaches (Ivar do Sul et al. 2009; Howell et al. 2012). Moreover, some plastic pieces sink and reach the benthos level due to their high density and also the biofouling by invertebrates or bacteria (Lobelle and Cunliffe 2011; Woodall et al. 2014). Consequently, they may affect both benthic (Murray and Cowie 2011; Van Cauwenberghe and Janssen 2014) and pelagic species (Possatto et al. 2011; Besseling et al. 2015).

In many studies, color, size, and shape are criteria used to identify MP particles ingested by marine organisms (Boerger et al. 2010; Choy and Drazen 2013). Visual examinations are by definition highly subjective and using them to discriminate the nature of the particles can be hazardous. As a result, specific analytical methods are required to identify the chemical composition of the particles (Hidalgo-Ruz et al. 2012), because information on the chemical composition of polymers is crucial to understand the identity and potential effects of these anthropogenic particles (Lusher et al. 2013).

Methods to monitor the levels of anthropogenic debris (including plastics) often vary considerably between countries and organizations, adding difficulties in interpreting trends. As a consequence, the United Nations Environment Programme and the OSPAR Commission are currently taking steps to introduce standardized protocols (OSPAR 2007; Cheshire et al. 2009). Currently, several methods to isolate microplastics from bulk samples (stomach contents or soft tissue) have been reported in the literature (Claessens et al. 2013; Foekema et al. 2013; Cole et al. 2014). Nevertheless, few studies use chemical methods to identify or isolate microplastic particles. The most common, but rarely used, is the digestion of organic matter by a 10 % solution of potassium hydroxide (KOH) first described by Foekema et al. (2013). Other isolation methods are used to identify microplastics from biological samples. For examples, acid digestion of mussels by nitric acid (Claessens et al. 2013) or staining stomach contents by a rose bengal solution (Davison and Asch 2011). As these methods show several pitfalls, such as polyamide destroying or time-consuming process,

alternative methods are needed for a better monitoring of MP ingestion by marine fauna.

The main objective of this study is to propose a new method to isolate anthropogenic particles (AP) (MP and textile fibers of natural origin) from stomach contents of fish and use Raman spectroscopy for precise chemical identification. When applied to biological samples, Raman spectroscopy suffers quite often from fluorescence problems generated either from native components or from the preparation method itself. The proposed approach minimizes the impact of fluorescence from sample preparation. It has been tested on stomach content of three planktivorous species: the Atlantic herring *Clupea harengus*, the European pilchard *Sardina pilchardus*, and the European anchovy *Engraulis encrasicolus*. These species have been selected as they are some of the most captured fish species and are thus of economic importance. Moreover, they are directly subjected to MP pollution, because they are planktivorous and mainly filter-feeding. It is the first study that proposes an isolation method of AP in stomach contents of fish and based on sodium hypochlorite.

Materials and Methods

Reference Samples

The method was set up using several types of plastic and cotton. These families were chosen due to their high production and worldwide consumption. Five replicates of seven polymers families [polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS), polycarbonate (PC), and polyamide (PA)] and one piece of cotton were tested to ensure their resistance to the different treatments used for isolation. These MP were obtained by mashing with a sander or cutting common plastics found in supermarkets: plastic bottle (PET), pipe (PVC), packaging (PE), food storage (PP), plastic bowl (PS), or in the lab: transparent tube (PC) and net (PA). The particles sizes range between 0.5 and 4 mm. During the isolation method, all samples were weighed between each step to detect a possible degradation. At the end of the experiment, particles were briefly observed under a dissecting microscope and analyzed by Raman spectroscopy.

Fish Sampling

Three specimens of three planktivorous species (the Atlantic herring *Clupea harengus,* the sardine *Sardina pilchardus*, and the European anchovy *Engraulis encrasicolus*) were sampled in European seas. Herrings were sampled during the International Bottom Trawl Survey (IBTS) in January 2013 in the North Sea. Sardines were

sampled during the International Bottom Trawl Survey (IBTS) in January 2013 in the English Channel. Anchovies were sampled during the Pélagiques Méditerranée (PELMED) survey in July 2013 in the Gulf of Lions. Both surveys were organized by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). Their total lengths (TL) range between 11 and 28 cm. The mean lengths of herrings, sardines, and anchovies were 24.5, 23.7, and 12.0 cm, respectively.

Sample Preparation

The protocol developed is summarized in a flowchart showing each step of the method (Fig. 1). Fish were dissected on board and whole stomachs were stored in a 30-ml formaldehyde solution (CH_2O 37 %, AppliChem, ITW Company, Germany) diluted in seawater in a 1:6 v/v ratio. In the laboratory, the stomach content was first extracted under a stereo microscope using classical dissection tools (scissor and forceps) and put into the formaldehyde solution. This solution was then filtered with a cellulose acetate membrane filter (5 μm porosity, Sartorius Stedim-Biotech, Germany). The membrane was rinsed with a 9 % sodium hypochlorite solution (NaClO 28.4 g/l 8°Chl, La Croix, Colgate, Brussels, Belgium) diluted with distilled water in a 1:3 v/v ratio, in order to completely collect the stomach content. NaClO concentration has been chosen according to Stojicic et al. (2010). The NaClO volume was brought up to 30 ml, and the digestion process was allowed to proceed overnight. Once the stomach content is digested, the NaClO solution was filtered with another filter

membrane of the same type. This membrane was rinsed with a nitric acid solution (HNO_3 65 %, Merck, Germany) diluted with a NaClO solution (HNO_3:NaClO 1:10 v/v ratio). The NaClO/HNO_3 volume was brought up to 30 ml. After 5 min, the NaClO/HNO_3 solution was filtered. The membrane was put into a 15-ml methanol solution (CH_3OH 99 %, Sigma-Aldrich, Chromasolv, Germany) enclosed into a bottle. The bottle was then exposed to ultrasounds (50 Hz) by an ultrasonic bath during 5 min (Grant Instruments, United Kingdom). The small-size shocks caused by ultrasounds take off particles from the membrane. Afterwards, the membrane was manually removed and the solution was ready for centrifugation. Methanol was then centrifuged at 5000 rpm during 5 min at room temperature. After centrifugation, the bottom was collected with a pipette (maximum of 4 ml methanol) and deposited on a 26-cm^2 polished stainless-steel plate. The plate was left for 2 h at room temperature to evaporate the methanol prior to analysis.

Particles Images and Weights

Before Raman analyses, AP on stainless steel were photographed using a MOC-510 Mueller-Optronic 5 megapixel CMOS camera. This camera was set on a stereo microscope with a maximum magnification of ×45. After spectroscopic analyses, they were weighed with an analytical balance (AX105, Mettler-Toledo, Switzerland) with an accuracy of 0.01 mg. The software ImageJ (v1.48, National Institutes of Health, U.S.A.) was used to measure the maximum length of each AP.

Fig. 1 Summary diagram of the isolation method. *SC* stomach content; *AP* anthropogenic particles. *Italicized* steps are not mandatory
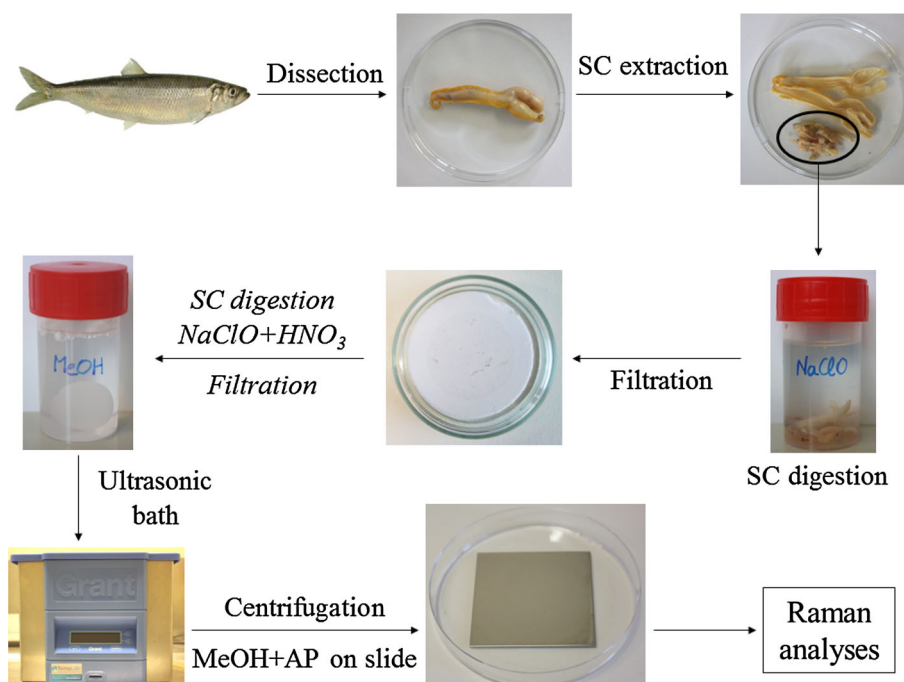
Raman Spectroscopy

Forty control AP (5 replicates of 7 MP and 1 piece of cotton) and particles found in fish stomach were analyzed using a LabRam 300 spectrometer (Jobin-Yvon) provided with an Olympus confocal microscope and an Andor BRDD Du401 CCD detector (Lepot 2011). Two lasers were used depending on the particle color: a Spectraphysics argon-ion laser (green laser, 514.5 nm) or a Torsana diode laser (red laser, 784.7 nm) and two objectives were used (magnification of ×50 and ×100) depending on the particle size. The maximum beam laser power on the sample was 5 mW (green laser) and 30 mW (red laser), but several neutral density filters were used most of the time to lower the power, thus avoiding degradation of the sample. The laser spot was focused on the target using a CCD camera. The integration times ranged from 5 to 50 s, depending on the sample. Where necessary, a baseline correction was applied to the recorded spectra using a polynomial regression model and homemade software. Matchings between recorded spectra and references from commercially available or homemade libraries were performed using the Thermo Specta 2.0 software.

Preventing Contamination

To avoid contamination, nitrile gloves were worn throughout manipulations, from dissection onboard to the end of the isolation method. All work surfaces and dissection materials were cleaned with ethanol 70 % (ethanol 99.8 %, Brenntag NV, Deerlijk, Belgium, diluted with distilled water). The cleaning was done using a white paper towel made of cellulose and lignin. Because it could contaminate samples in fibers (Foekema et al. 2013), white fibers presenting a cellulose/lignin Raman spectrum were removed from results. The isolation process was performed under an air flow hood except during the samples drying. Because particles might be very light and small (mainly fibers), they could be aspired by the air flow. Thus, to prevent airborne contamination, stainless-steel plates were placed under a metal sifter (36-µm mesh). Subsequently, all fibers with a length lower than 36 µm were not analyzed.

Results

Inertness of the Isolation Method Versus Commercial Plastics and Cotton Samples

The proposed isolation method was first tested on commercial plastics and cotton samples by following the mass variation after each step. All materials were unaffected by formaldehyde storing and NaClO treatment (Online

Resource 1). The final solvent used was methanol, which has caused a mass loss of 25 % on average for PVC particles only. The other plastic materials were not significantly degraded by methanol when comparing average values from five replicates.

Finally, Raman spectra were recorded for each sample before and after our chemical treatment. The spectra were found identical showing that our isolation method did not affect the chemical nature of the studied compounds. Examples of Raman spectra are shown in Fig. 2. In addition, the fluorescence level did not increase despite the chemical treatment.

Efficiency of Stomach Contents Degradation

Figure 3 shows the efficiency of the NaClO and NaClO/HNO_3 treatments. The first step (degradation by NaClO) is the most powerful; the difference between a filter membrane before and after NaClO degradation is obvious. Stomach contents are almost completely degraded by NaClO alone. Then, the solution of NaClO/HNO_3 enhances the degradation process. Finally, the filter membrane is free of organic matter except particles, including AP, which are not degraded by NaClO.
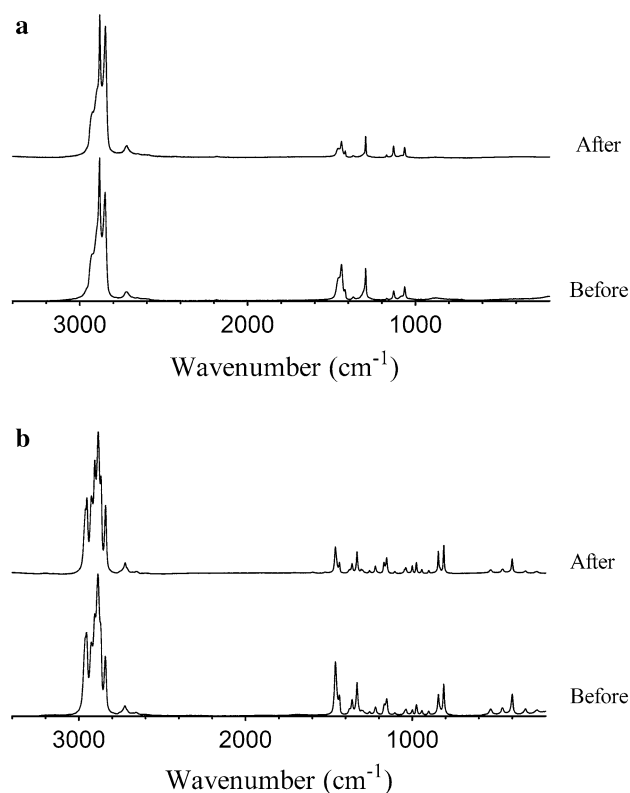
Fig. 2 **a** PE Raman spectra before and after treatment. **b** PP Raman spectra before and after treatment

Fig. 3 Stomach contents from anchovies on filter membranes before sodium hypochlorite treatment, after sodium hypochlorite treatment, and after NaClO/HNO$_3$ treatment

| Stomach contents before treatment | → | Stomach contents after NaClO treatment | → | Stomach contents after NaClO/HNO$_3$ treatment |
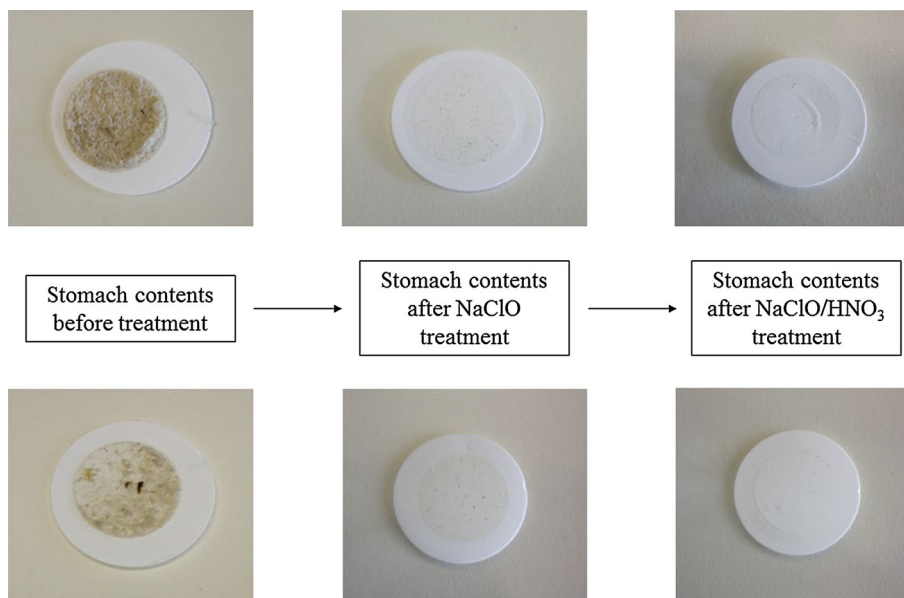
Anthropogenic Particles in Stomach Contents

Chemical Composition

The nine stomach contents had 35 non-degraded particles: 12, 10, and 13 in the stomach contents of herrings, sardines, and anchovies, respectively. Due to the very small laser irradiation spot size (approximately 1 μm^3 with our confocal microscope), the Raman spectra recorded concerns only a very small volume of the sample. In order to obtain representative Raman spectra of the sample, the technique was applied by scanning these particles with the laser on different areas. Examples of spectra are shown in Fig. 4.

Interestingly, only 11 of the 35 particles were made of plastic (examples are shown in Fig. 5). They were of different colors (transparent, white, blue, and green) and exhibiting different shapes. Only PE, PP, and PET were identified in these 11 particles.

The majority of "non-plastic" particles were made of vegetal materials, such as cellulose (Fig. 4b) or lignin. In some cases, Raman analyses showed a mixture of a dye and the material constituting the particle (Fig. 6).

The particles left are made of (1) collagen I, (2) dyes, and (3) sodium nitrate. Raman analyses of three particles showed only dye spectra. Four particles were unidentified by our software due to poor-quality spectra and/or a lack of spectra in our database.

Measurements and Weights

Particles length in fish stomachs ranged between 0.11 and 9.49 mm with a mean of 1.59 mm ($n = 34$, standard error
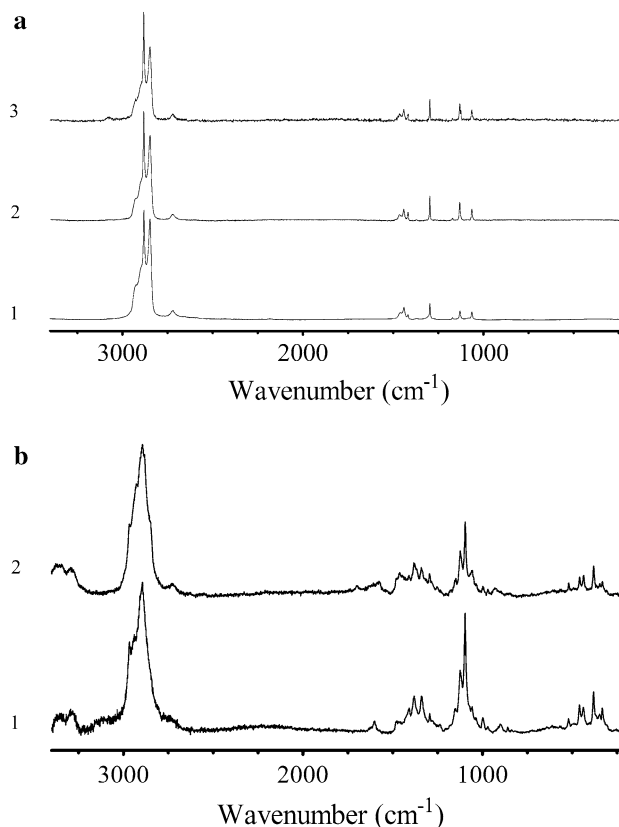
Fig. 4 a *1* PE Raman spectrum from our personal library, *2* PE Raman spectrum of a particle found in the anchovy's stomach, and *3* PE Raman spectrum of a particle found in the herring's stomach. **b** *1* Cellulose Raman spectrum from our database, and *2* cellulose Raman spectrum of a fiber found in the sardine's stomach

(SE) = ±0.41). Plastic particles had a mean length of 2.08 mm ($n = 11$, SE = ±0.83), with a minimal value of 0.22 mm and a maximal value of 9.49 mm. The longest
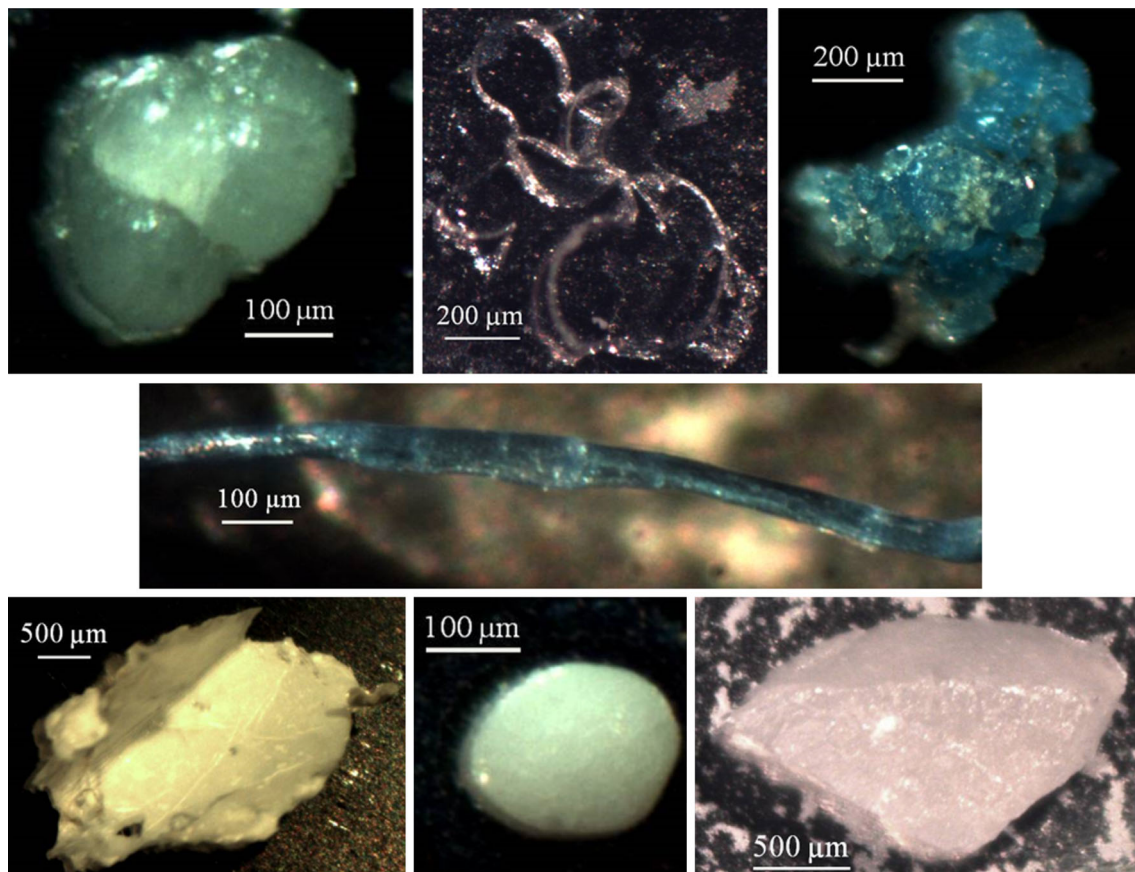
Fig. 5 Example of plastics isolated from stomach content by the isolation method. As identified by Raman spectroscopy, from *left to right*, *upper row* PE, PET, and PE; *middle row* PP; *bottom row* PET/PP, PP, and PE

particle was found in a sardine's stomach and was made of PP. The total weight of plastic particles, all three species included, is 1.89 mg.

Discussion

The purpose of this study was to develop a reliable analytic protocol allowing the identification of AP in stomach contents of fish. The nature of these AP appeared to be similar with previous studies (Foekema et al. 2013). Of 35 particles, 16 were confirmed as being from anthropogenic sources characterized by different compositions in terms of MP and dyes. The majority of the 19 other particles are fibers and are made of vegetal material, such as cellulose. As it is usually found in clothes, these fibers could have an anthropogenic origin. Unlike the study of Lusher et al. (2013), any rayon fiber was found. Rayon has a close but different spectrum than cellulose and lignin (Cho 2007). In the anchovy's stomach, two particles were made of $NaNO_3$, probably resulting from the mixing of the NaClO solution with HNO_3. Analyses on a larger number of

stomach contents are being performed, and the composition, number, and size of AP will be discussed in another publication.

Cellulose acetate fibers also were found in different stomach contents, but this result has to be interpreted with caution, because the filter membrane used in this method also is made of cellulose acetate. Therefore, we cannot exclude contamination from the membrane during the ultrasonic treatment. On the other hand, the ultrasonic bath did not damage AP, because no item was found in duplicate.

To perform AP isolation, several solutions were used according to several parameters. NaClO can provoke particles discoloration, which allows a direct analysis of the material by Raman spectroscopy. However, in the present study, several particles kept their original color, which allowed assigning them an anthropogenic origin. Methanol was chosen, because it is inexpensive, volatile, and has a density lower than almost any type of plastic or textile fiber (i.e., 0.79 g/cm^3). PE, PP, PET, PS, and PVC, which are the most common plastics (Plastics Europe 2013), have a density superior to 0.9 g/cm^3, whereas a cotton fiber has a
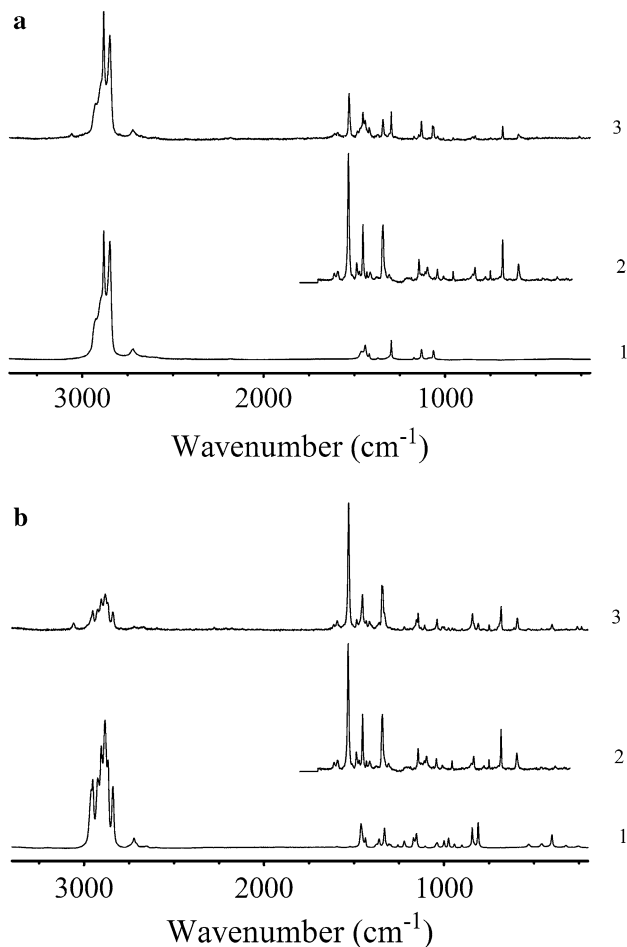
Fig. 6 Raman spectra. **a** *1* Polyethylene (PE), *2* Ingrain Blue (IB), spectrum from our database, and *3* PE spectrum of a particle found in the anchovy's stomach. **b** *1* Polypropylene (PP), *2* IB spectrum from our database, and *3* PP spectrum of a fiber found in the sardine's stomach

density of 1.55 g/cm^3 (Morton and Hearle 2008). The downside use of methanol is related to the observed mass loss for PVC particles. In this particular case, a more appropriate solvent should be used.

The selected species are ram-feeders (Van der Lingen et al. 2009), and their diet mainly consists in copepods (Plounevez and Champalbert 1999; Möllmann et al. 2004; Cunha et al. 2005), which are very fatty (Ohman 1997; Evjemo et al. 2003). Acidification with HNO$_3$ is required to enhance the digestion process. Indeed, NaClO acidification favors the HClO neutral form, which reacts with lipids (Spickett et al. 2000). Then, sodium hypochlorite also is a powerful muscle dissolvent depending on its concentration, pH, and exposure time (Christensen et al. 2008).

Depending on the number of particles obtained after treatment, one sample can be analyzed in 48 h (sampling and dissection excluded). The most time-consuming steps of the process are (1) digestion by NaClO (overnight) and (2) Raman analyses. The latter step can be very long if particles are numerous and made of several materials, such as cellulose, lignin, and dyes, requiring low laser power and high integration times. Plastic particles are however easily identified, whereas cellulose or other vegetal materials produced weak Raman spectra with some noise.

Other methodologies have been proposed but can suffer from several drawbacks. The rose Bengal staining appears to be a rapid method to bring out plastic particles (Davison and Asch 2011). This stain does not change microplastic color but can dye other organic materials. Consequently, red and rose plastic particles can be confused with organic matter and vegetal fibers, such as cellulose, cannot be isolated, because they have the same color as stomach content. Cole et al. (2014) compared several digestion protocols on zooplankton samples. Among the solutions tested to digest biological samples, the proteinase-K treatment was found to be the most efficient (efficacy superior to 97 %). Moreover, this protocol showed no visible impact on MP tested and fits to Fourier transform infrared spectroscopy (FT-IR) analyses. The only disadvantage of this method is the cost of a proteinase-K solution. An additional minor problem is that FT-IR analyses require a minimal surface for a particle to be analyzed.

Our protocol displays several advantages regarding time, cost, and efficiency. First, the process takes place faster than the KOH digestion process, which takes 2–3 weeks to complete (Foekema et al. 2013). Secondly, each type of polymer tested is resistant to all solutions used, except a loss in mass of 25 % for PVC particles. Polyamide can be recovered after the whole process, by contrast with the method of Claessens et al. (2013) using HNO$_3$ digestion at high temperatures and several durations.

The extraction and isolation method proposed in this paper, when coupled with Raman spectroscopy, offers an improvement in the field of marine microplastic pollution detection. This method also could be used for AP isolation for marine animals with carnivorous diets if volumes of solutions used and the NaClO/HNO$_3$ ratio are adapted following the fat contents of prey. The proposed methodology should contribute to evaluate MP impacts on marine organisms by a more accurate determination of the type and number of ingested MP.

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