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Morphology of the filtration apparatus of three planktivorous fishes and relation with ingested anthropogenic particles

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ABSTRACT

Anthropogenic particles (APs), including microplastics, are ingested by a wide variety of marine organisms. Exposure of *Clupeiformes* (e.g. herrings, anchovies, sardines) is poorly studied despite their economic and ecological importance. This study aims to describe the morphology of the filtration apparatus of three wild-caught *Clupeiformes* (*Sardina pilchardus*, *Clupea harengus* and *Engraulis encrasicolus*) and to relate the results to ingested APs. Consequently, the species with the more efficient filtration apparatus will be more likely to ingest APs. We hypothesized that sardines were the most exposed species. The filtration area and particle retention threshold were determined in the three species, with sardines displaying the highest filtration area and the closest gill rakers. Sardines ingested more fibers and smaller fragments, confirming that it is the most efficient filtering species. These two results lead to the conclusion that, among the three studied, the sardine is the species most exposed to APs.

1. Introduction

It is estimated that in 2010, up to 13 million tons of plastic ended up in oceans (Jambeck et al., 2015). This may cause negative impacts on wildlife (Laist, 1997; Wright et al., 2013) because the plastic can be ingested by many marine organisms (Cole et al., 2011), causing mechanical (Bugoni and Krause, 2001; Boren et al., 2006; Gregory, 2009) and/or toxicological harm (Browne et al., 2013; Rochman et al., 2013). Macroplastics (>5 mm) and microplastics (MPs; 0.1 μm to 5 mm; Klaine et al., 2012; Koelmans et al., 2015) are ingested by a wide range of organisms including marine birds (Brandão et al., 2011; Fife et al., 2015; Jiménez et al., 2015), marine mammals (Walker and Coe, 1989; Secchi and Zarzur, 1999; Jacobsen et al., 2010), marine turtles (Bjorndal et al., 1994; da Silva Mendes et al., 2015), fish (Collard et al., 2015; Romeo et al., 2015), zooplankton (Cole et al., 2013), and mollusks (Van Cauwenberghe and Janssen, 2014). In laboratory experiments, these plastics have been shown to be transferred from one trophic level to another (Farrell and Nelson, 2013; Setälä et al., 2014). Plastic material is considered an endocrine disruptor (Rochman et al., 2014). In addition, once ingested, anthropogenic particles (APs, which include MPs and other particles with certified anthropogenic origins such as artificially dyed fibers), can introduce several types of pollutants, including PCBs, triclosan, PAHs, and PBDEs within the organism (Besseling et al., 2013; Browne et al., 2013; Rochman et al., 2013), also causing, for example, endocrine disruption (Rochman et al., 2014) and hepatic stress (Rochman et al., 2013).

Teleosts have been reported to ingest APs (Foekema et al., 2013; Lusher et al., 2013; Collard et al., 2015; Neves et al., 2015), including MPs. However, they have different feeding mechanisms allowing the seizure of different kinds of prey, which means that the route of exposure might be different. In bony and cartilaginous fishes, gill rakers (GRs) are found at the level of the branchial basket (Gibson, 1988; Gerking, 1994). The primary function of these GRs is to protect the gill epithelium by retaining particles from the water flow during breathing (Lagler et al., 1962; Elsheikh, 2013). In some species, such as *Clupeiformes*, however, GRs have acquired a second function related to feeding (Elsheikh, 2013; Magnuson and Heitz, 1971). Filter-feeder fishes possess numerous and elongated rakers that are used as a net to extract food from the water flow and direct it toward the esophagus (Gibson, 1988). These rakers can be rod-like or fitted with small denticles, also called microspines (Iwata, 1976), microbranchiospines (Smith and Sanderson, 2007) or teeth (Gibson, 1988). These denticles have been reported in distantly teleost families such as *Clupeidae*

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(Gibson, 1988), *Cyprinidae* (Iwata, 1976), *Comephoridae* (Jakubowski, 1996), *Carangidae* (Sanderson et al., 1996), and also in some elasmobranchs (Misty Paig-Tran and Summers, 2014). Differences in mesh reflects the ability to catch different kinds of prey. In parallel, this should also support the fact that filtration efficiency would change species' capacity to consume APs.

In this study, we compare three planktivorous *Clupeiformes* that are all highly consumed fish products by humans: the Atlantic herring (*Clupea harengus*, Linnaeus 1758), the European pilchard (or sardine; *Sardina pilchardus*, Walbaum 1792) and the European anchovy (*Engraulis encrasicolus*, Linnaeus 1758). For each of these three species, we aim to determine the degree of exposure to AP pollution. To this end, two complementary approaches are used. Based on GRs and denticles morphometry, we define a new method that accurately evaluates the filtration areas and the minimum diameter of particles ingested. The degree of exposure is compared with APs found and characterized in sixty stomach contents from wild fish, providing a first picture of the impact on taxa.

2. Materials and methods

2.1. Sampling

Three planktivorous species (*C. harengus*, *S. pilchardus* and *E. encrasicolus*) were sampled. Fish were caught in three different zones (Fig. 1): the English Channel, the Northwestern Mediterranean Sea and the Northeastern Atlantic (Bay of Biscay), and at three different periods (Table 1). All sampling surveys were organized by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). Fig. 1 was made with Google Earth (Google, Mountain View, CA, U.S.A.).

Twenty individuals of each species were used for stomach contents analysis and five individuals of each species were used for morphological analysis. Individuals were dissected on-board. Gill baskets and stomachs were directly stored in a 5% formaldehyde solution. Total lengths (TLs) of fish were recorded. The first left gill arch was used for pictures and measurements (Alexandrino et al., 2006; Costalago and Palomera, 2014; Costalago et al., 2015).

2.2. Morphological study

2.2.1. Light microscopy

Gill arches were observed with a stereomicroscope (Zeiss Stemi 2000-C, Edmunds optics, Germany) and photographed with a 5 megapixels camera (Tucsen ISH500 v1.48, Xintu Photonics Co., China). Different measurements (Fig. 2) were carried out using ImageJ v1.48 software (National Institutes of Health, U.S.A.) on lengths of epibranchial, ceratobranchial, hypobranchial and GRs. Length of gill arches was calculated by summing up the epi-, cerato- and hypobranchial lengths.

2.2.2. Scanning electron microscopy

Other structures of the gill arches were observed in scanning electron microscopy (SEM), including the gap between GRs and denticles, the thickness of GRs and denticles, and the length of denticles (Fig. 2). Gill arches were dehydrated through a graded ethanol series then mounted on a glass slide and sputter-coated with a 20 nm Pt in a BALZERS SCD 030 unit. Two individuals from each species were used to measure denticles parameters. Pictures were taken with the Orion software (v 6.60.6) in a SEM Jeol JSM-840A (Japan) working at 20 kV of accelerating voltage.

2.2.3. Filtration area calculation and particle retention

To calculate filtration areas and particle retention, three different calculations were used. The first one, based on the method developed by Magnuson and Heitz (1971), consists of adding the area covered by GRs on the epibranchial (upper area) to the area covered by GRs on both cerato- and hypobranchial (lower area) of one gill arch (Fig. 3). The second calculation, which was developed by Gibson (1988), takes into account the space occupied by the GRs and adds the areas of open spaces between GRs where water flows. Finally, the third calculation, called “alpha” is a formula that we have developed with the aim of taking denticles into account.

The alpha formula uses Gibson's formula with additional parameters in order to include the space occupied by denticles in the calculation:

$$F = (\Sigma L - L_{max}) * (\overline{G} - 2x) \quad \text{where} \quad x = L_d{}^* \sin\alpha$$

Fig. 1. Map presenting all sampling points. Black symbols: sampling points for the contamination study; grey symbols: sampling points for both morphological and contamination studies; white symbols: sampling points for the morphological study.

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Table 1
Sampling parameters for each species.

Species	Study	Sampling period	Location	Survey
Engraulis encrasicolus (n = 20)	Contamination	July 2013	Mediterranean Sea	PELMED (Bigot, 2013)
	Morphological	October 2013	Bay of Biscay	EVHOE (Salaun et al., 2013)
Sardina pilchardus (n = 20)	Contamination	January 2013	English Channel	IBTS (Verin, 2013)
	Morphological	October 2013	Bay of Biscay	EVHOE (Salaun et al., 2013)
Clupea harengus (n = 20)	Contamination Morphological	January 2013	English Channel	IBTS (Verin, 2013)

F = filtration area, L = GR length, $\overline{G}$ = mean gap between GRs, α = angle between the denticle and the blade of GR, L_d = denticle length.

The calculated minimum diameter of particles trapped in GRs and denticles is represented in diagrams made with the software AutoCAD (AutoDesk, San Rafael, CA, U.S.A.).

2.3. Contamination study

The methodology used for AP isolation and analysis has been described previously (Collard et al., 2015) and is briefly detailed below.

2.3.1. Preventing contamination and procedural blanks

To minimize contamination, nitrile gloves were worn throughout the whole isolation process, from on-board dissection 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. Therefore, fibers presenting a cellulose/lignin Raman spectrum were removed from results. The isolation process was performed under an airflow hood, except while samples were drying to avoid any loss of fibers through aspiration process in the airflow hood. To prevent airborne contamination, stainless-steel plates were placed under a metal sifter (36-μm mesh). No fibers <36 μm in length were found.

From the three procedural blanks analyzed alongside the samples, no polymers were found.

2.3.2. Sample preparation

Sample preparation is described in Collard et al. (2015). Briefly, stomach contents were poured into a 9% NaClO solution overnight. The remaining solution was filtered with a cellulose acetate filter membrane (5 μm porosity) which was rinsed with a 99% methanol solution. It was then centrifuged at 5000 rpm for 5 min. The bottom was then collected and deposited on a stainless steel plate for Raman spectroscopy analysis.

2.3.3. Particle images and weights

Before Raman analysis, all particles on the stainless steel plate were photographed using a MOC-510 Mueller-Optronic 5 megapixel CMOS camera. This camera was set on a stereomicroscope with a maximum magnification of 50×. After spectroscopic analysis, they were weighed with an analytical balance (AX105, Mettler-Toledo, Switzerland) with an accuracy of 0.01 mg. The software ImageJ was used to measure the length (at the longest point) of each AP.

2.3.4. Raman spectroscopy analysis

A LabRam 300 spectrometer (Jobin-Yvon) equipped with an Olympus confocal microscope and Andor BRDD Du401 CCD detector was used to analyze particles. 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). 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

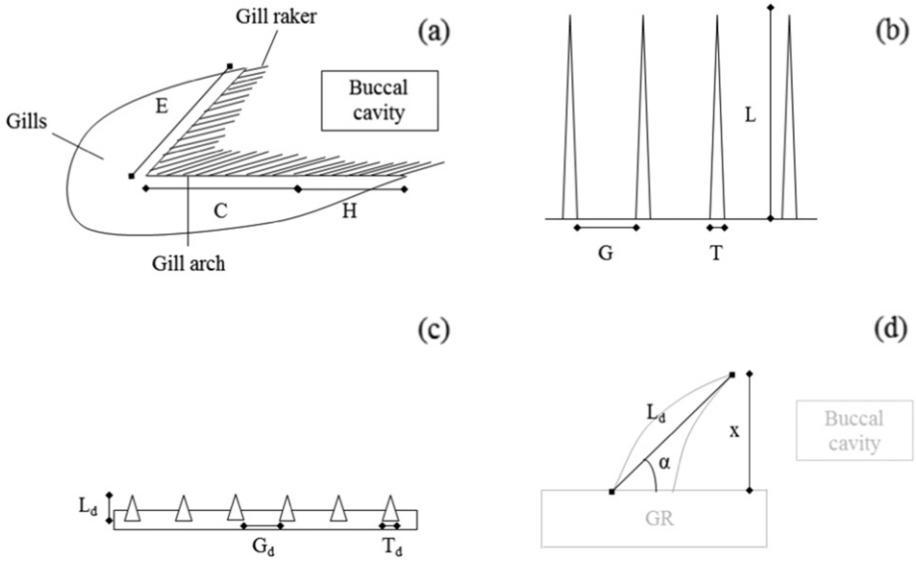

Fig. 2. Schematic representation of measured parameters of (a) gill arches, (b) GR, (c) denticles on GR and (d) the filtration area calculation. C: ceratobranchial length; E: epibranchial length; G: gap between GR; G_d: gap between denticles; H: hypobranchial length; L: GR length; L_d: denticle length; T: GR thickness; T_d: denticle thickness; X: denticle height using the inclination angle α.

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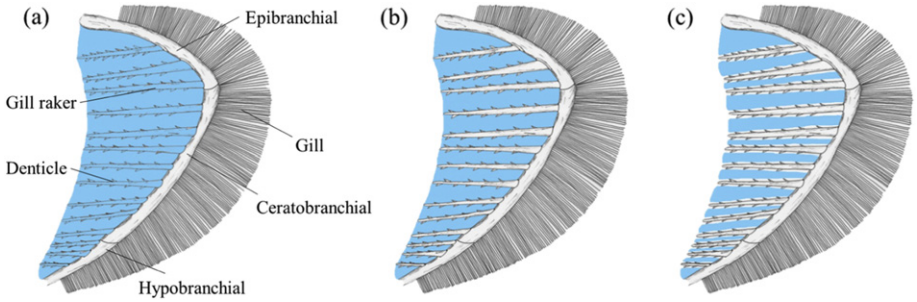

Fig. 3. Schematic representations of filtration areas calculated following the formula of (a) Magnuson and Heitz (1971), (b) Gibson (1988), and (c) our alpha formula. In each case, the area corresponds to the surface in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the time to lower the power, thus avoiding degradation of the sample. The integration times ranged from 5 s to 50 s, depending on the sample. Matching, between recorded spectra and references from commercially available or personal libraries, was performed using the Thermo Specta 2.0 software.

2.4. Statistics

Statistical analysis was performed with the GraphPad Prism software (v5.03, GraphPad software Inc., California, U.S.A.). Data were tested for normality using a Kolmogorov-Smirnov test. For the morphological study, normal distributions were analyzed with the ANOVA 1 test, followed by Tukey's multiple comparison test ($p < 0.05$). When data showed non-normality, they were analyzed with the non-parametric Kruskal-Wallis test. The Dunn's multiple comparison test was then used to compare all sample pairs ($p < 0.05$). Results are expressed in mean ± standard deviation. For the contamination study, comparisons between species were performed with a Fisher's test on a contingency table.

3. Results

3.1. Morphological study

3.1.1. Gill arches

Herrings have the longest gill arch (43.8 ± 2.6 mm), followed by sardines (42.7 ± 3.6 mm), and anchovies (29.1 ± 0.5 mm) (Table 2). Similarly, herrings have the longest ceratobranchial and hypobranchial, followed by sardines and anchovies.

3.1.2. Gill rakers

In each species, the GRs can be compared to a suite of superposed planks that lie on their large surface. This means that the large upper and lower areas correspond to the dorsal and ventral sides respectively, whereas the narrow sides correspond to the labial (or medial), and jugal (or lateral) sides.

Four morphologic parameters were measured and analyzed for the three species: the number, length, and thickness of GRs, and the gap between them (Table 2).

Herrings showed the thickest and the most spaced GRs (Dunn's multiple comparison, $p < 0.05$). Sardines had the most numerous (Tukey's multiple comparison, $p < 0.05$), the closest, the thinnest, and the longest GRs (Dunn's multiple comparison, $p < 0.05$). Anchovies had the smallest GRs across the whole gill arch (Dunn's multiple comparison, $p < 0.05$).

3.1.3. Denticles

All three species have denticles. Anchovies have four rows of denticles on each GR. Two rows are found on the edges of the labial side and are in line with the GR (Fig. 4), meaning they were not used in the calculation of the filtration area. The dorsal and ventral sides each possess a row of denticles that are alternating distichous. They are sickle-shaped, pointing toward the buccal cavity and forming an angle of 40° with the GRs.

Sardines and herrings show two rows of denticles that are on the dorsal and ventral side of the GRs. Sardines denticles are flattened and diabolo-shaped with a serrated distal end. These teeth form an angle of 25° with the GR. Denticles at the distal GR end are falciform. Herring denticles are conical, acute and straightened, forming an angle of 23° with the GR. Sometimes, denticles are found in pairs, as shown in the herring in Fig. 3.

When comparing measurements (Dunn's multiple comparison, $p < 0.05$), anchovies have the tallest denticles (0.071 ± 0.019 mm), followed by herrings (0.062 ± 0.016 mm), and sardines (0.054 ± 0.016 mm). Herrings have the thickest denticles (0.046 ± 0.014 mm) (Dunn's multiple comparison, $p < 0.05$), followed by anchovies (0.022 ± 0.007 mm), and sardines (0.019 ± 0.007 mm). The most spaced denticles are found in anchovies (with a gap of 0.173 ± 0.052 mm) while sardines have the closest denticles (with a gap of 0.098 ± 0.021 mm).

3.1.4. Filtration areas

Three different formulas were used (Fig. 5). For the three species, Magnuson's formula gave the highest value of the three formulas, but this was only significant for anchovies and herrings (Tukey's multiple comparison, $p < 0.05$). Calculations with our alpha formula always gave a smaller filtration area than Gibson's formula (31% in anchovies, 14% in herrings and 16% in sardines). When using our alpha formula, it has been shown that sardines have the highest filtration area (Tukey's multiple comparison, $p < 0.05$), followed by herrings and anchovies.

Table 2
Summary of measured parameters. Results are presented as mean ± standard deviation. Data are averaged on a *E. encrasicolus* individual of 150 ± 6 mm, a *C. harengus* individual of 290 ± 16 mm, and a *S. pilchardus* individual of 210 ± 27 mm. Number, length, gap and thickness of gill rakers are given for the whole gill arch.

Parameter	Data	*Engraulis encrasicolus* (n = 5)	*Clupea harengus* (n = 5)	*Sardina pilchardus* (n = 5)
Gill arch length	Raw (mm)	29.1 ± 0.5	43.8 ± 2.6	42.7 ± 3.6
Number of GRs	Raw	66.6 ± 2.3	63.8 ± 2.9	112 ± 11
GR length	Raw (mm)	4.3 ± 1.0	6.4 ± 1.0	7.9 ± 1.8
GR gap	Raw (μm)	323 ± 38	381 ± 37	265 ± 15
GR thickness	Raw (μm)	37 ± 12	49 ± 3	20 ± 6

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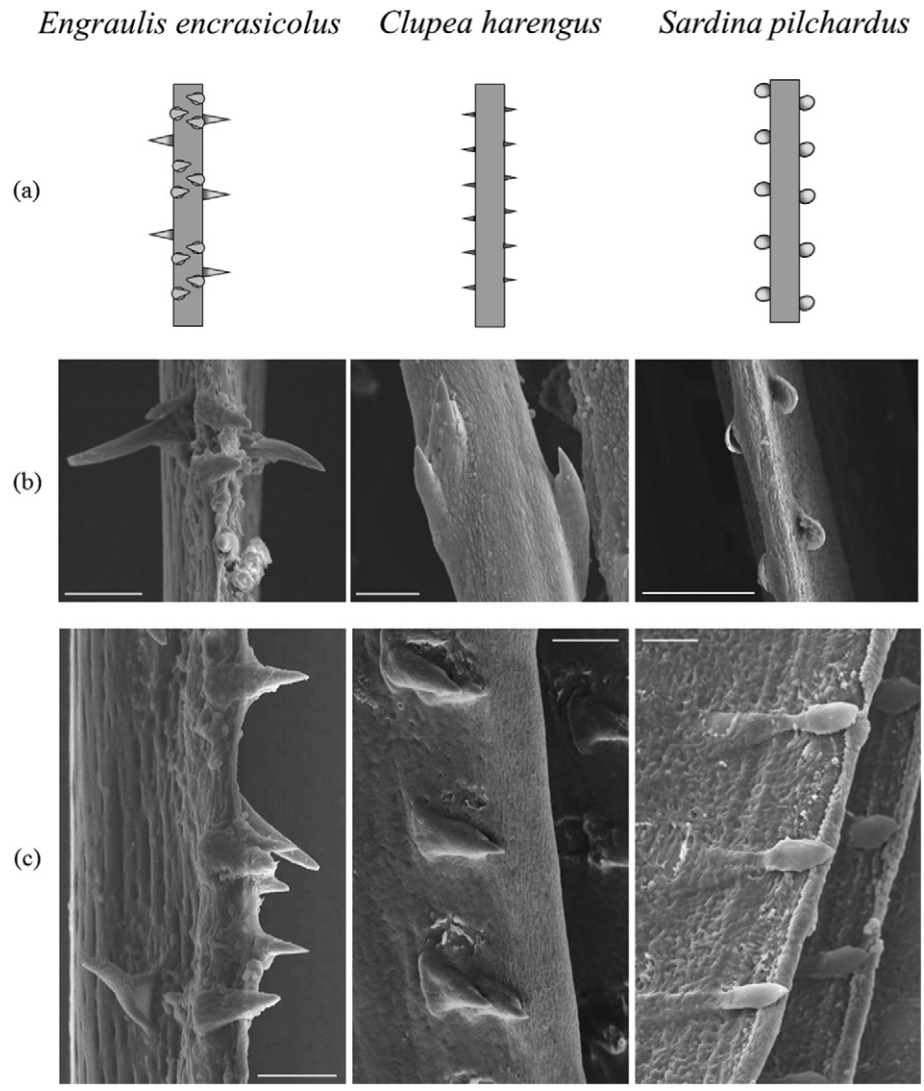

Fig. 4. Denticles illustrations in the three species: (a) schematic representation of the GRs in lingual view, (b) SEM pictures of the lingual side and (c) SEM pictures showing both the dorsal and lingual sides of the GRs. Scale bar: 50 μm.

Regardless of formulas, sardines have always the highest filtration area but this was significant only with Gibson's and our alpha formulas.

3.1.5. Particle retention

The minimum diameter of particles that may be trapped in GRs and denticles has been calculated in two ways: for particles retained by two denticles, or by four denticles (Fig. 6). The upper part of the diagram shows the mean raw data for an anchovy of 150 mm in length, a herring of 290 mm in length, and a sardine of 210 mm in length. While the sardine is much larger than the anchovy, the minimum diameters are similar.

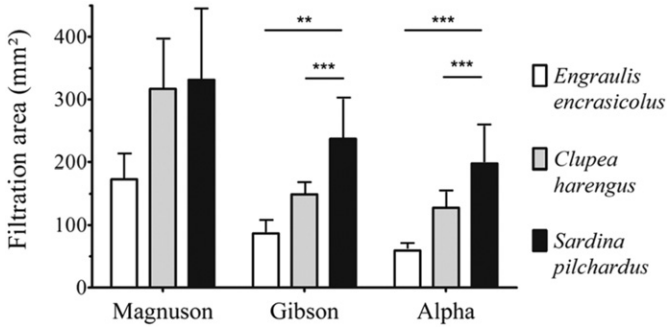

Fig. 5. Filtration areas calculated with the three formulas for an anchovy of 150 mm, a herring of 290 mm, and a sardine of 210 mm. Asterisks indicate a significant difference between fish species for the same formula used (Tukey test, $p < 0.05$).

3.2. Contamination study

We defined APs as plastic particles and other particles that are artificially dyed (textile fibers). With the help of Raman spectroscopy, 67 APs were found in the 60 individual stomachs: 25 APs in anchovies, 21 in herrings, and 21 in sardines (Table 3). Among these APs, 43 were made of plastic polymers: 17 plastic particles were ingested by anchovies, 11 by sardines and 15 by herrings. 40% of anchovies, 45% of sardines and 50% of herrings had plastic in their stomachs. Eight different polymers were recorded (Fig. 7): PE (37%), PP (26%), PET (16%), polyacrylonitrile (PAN, 7%), polystyrene (PS, 5%), polyamide (PA, 5%), polyethylene glycol (PEG, 2%), and poly(butyl methacrylate) (PBMA, 2%). Anthropogenic particle lengths ranged between 0.13 mm and 22.4 mm. Sardines ingested the smallest anthropogenic fragments (0.31 ± 0.08 mm), followed by anchovies (0.80 ± 0.73 mm), and herrings (0.87 ± 1.20 mm), but this could not be assessed statistically due to a number of fragments in the sardines that were too small. Different shapes were found (Fig. 8) and divided in two categories: fibers and

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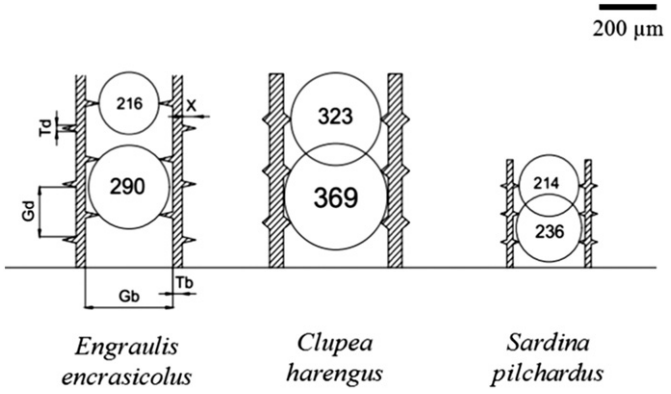

Fig. 6. Diagrams showing the minimum diameter of particles retained in the three species. Gb: gap between GRs; Gd: gap between denticles; Tb: GR thickness; Td: denticle thickness; X: denticle length.
Modified from Gibson (1988).

fragments. All anthropogenic fragments were made of plastic polymers. Sardines ingested more fibers than anchovies (Fisher's test, $p = 0.032$) and herrings ($p = 0.011$), while no difference was found between herrings and anchovies ($p = 0.561$). Fibers represented 38% and 48% of ingested APs in herrings and anchovies respectively, against 81% in sardines. Many APs were colored but the use of NaClO during the sample preparation may have discolored many particles. Sodium hypochlorite cleaves the azo-link of some dyes (Gregory and Stead, 1978; Robinson et al., 2001), leading to the bleaching of particles or fibers. Consequently, results about AP color are neither shown nor discussed.

4. Discussion

Long and numerous GRs determine what *Clupeiformes* will ingest during seawater filtration (Gibson, 1988; Alexandrino et al., 2006; Dibattista et al., 2012; Costalago et al., 2015). This also means that these structures will determine which APs will be ingested during feeding. Our results suggest that sardines would be the species most likely to ingest APs, compared to herring and anchovy, because sardines have the largest filtration area and the smallest gap between GRs. The minimum size of particles that will be retained by the filtration apparatus of sardines is smaller than particles retained by herrings and anchovies. Moreover, this result is corroborated with the size and the type of APs ingested: sardines ingested more fibers and smaller fragments than the two other species studied.

We are aware that fishes were sampled in different seas, potentially leading to different exposure in terms of shape and size of APs. Nevertheless, keeping these *Clupeiforme* species in an aquarium is very hard (if not impossible) and thus expensive, particularly if several replicates are needed for statistical analysis. Besides, fishing all three of these species in the same region is challenging; the only region that could accommodate this is the English Channel, and while this region has been sampled during IBTS 2013 and 2014 (Verin, 2013, 2014), the number of individuals caught of the three species was not satisfactory.

Plastic particle ingestion by fish causes several impacts due to polymer and associated pollutants (Cedervall et al., 2012; Rochman et al., 2013). Experimental studies have found that ingested microplastics cause liver stress (Rochman et al., 2013), alteration of the endocrine system (Rochman et al., 2014), and behavioral changes (Cedervall et al., 2012; Mattsson et al., 2015). When associated with pollutants such as pyrene, PAHs, PCBs or PBDEs the same impacts occur (Rochman et al., 2013, 2014) and others appear such as a significant inhibition of enzymatic activity in *Pomatoschistus microps* (Oliveira et al., 2013). Sometimes, impacts are more severe, such as pronounced alterations of the distal intestine in *Dicentrarchus labrax* (Pedà et al., 2016). If some species proportionally ingest more microplastics than others, these species will likely be more affected. Besides, translocation of nanoplastics in fish has been reported in laboratory (Kashiwada, 2006) while translocation of microplastics is more discussed (Avio et al., 2015) and seems to depend on particle size (Lu et al., 2016). Translocation of nanoplastics and small MPs leads to impacts such as inflammation and lipid accumulation in liver (Lu et al., 2016). If translocation of MPs occurs in wild fish, we could expect that species that are more likely to ingest MPs would be more affected by translocation and its impacts.

Planktivory ability in *Clupeiformes* is most probably enhanced by the different denticles that reduce the mesh and whose sharp extremities help in retaining food. Their shape and arrangement seem related to phylogenetic signals. Denticles of our *S. pilchardus* sardine have the same structure as the Pacific sardine (*Sardinops sagax*): they are arranged in a single row along each gill and their anterior tip is modified in a flattened nodule (Rykaczewski, 2009). Also, the European anchovy used here (*E. encrasicolus*) and the Northern anchovy (*E. mordax*) show morphologically similar denticles that form several rows (Rykaczewski, 2009). Sardines seem to have movable denticles which become erect from the GR blade when water flows in the buccal cavity (Rykaczewski, 2009), meaning that they could ingest smaller particles than the theoretical diameter calculated in this study. Moreover, sardines, herrings and anchovies produce sticky mucus that covers gill arches, GRs and denticles (Alsafy, 2013). This mucus could be involved in the filtering process by decreasing the mesh size of the branchial sieve (Northcott and Beveridge, 1988), by aggregating particles, and by facilitating their transport to the esophagus (Sanderson et al., 1996). Therefore, the calculated minimum particle size which can be ingested is certainly overestimated.

Table 3
Amount and lengths of APs and MPs according to their shape and the species in which they were found.

		Engraulis encrasicolus (n = 20)	*Clupea harengus* (n = 20)	*Sardina pilchardus* (n = 20)
APs (all)	Number	25	21	21
	Median length (mm)	0.99	0.60	1.48
	Min–max (mm)	0.22–22.4	0.13–6.6	0.25–9.5
APs (fibers)	Number	12	8	17
	Median length (mm)	1.25	2.12	2.21
	Min–max (mm)	0.42–22.4	0.22–6.6	0.63–9.5
APs (fragments)	Number	13	13	4
	Median length (mm)	0.68	0.47	0.29
	Min–max (mm)	0.22–2.9	0.13–3.7	0.25–0.42
MPs (all)	Number	17	15	11
	Median length (mm)	0.79	0.58	1.48
	Min–max (mm)	0.22–2.9	0.13–3.9	0.25–9.5
MPs (fibers)	Number	4	2	7
	Median length (mm)	1.75	2.99	2.21
	Min–max (mm)	1.2–2.7	2.1–3.9	0.97–9.5

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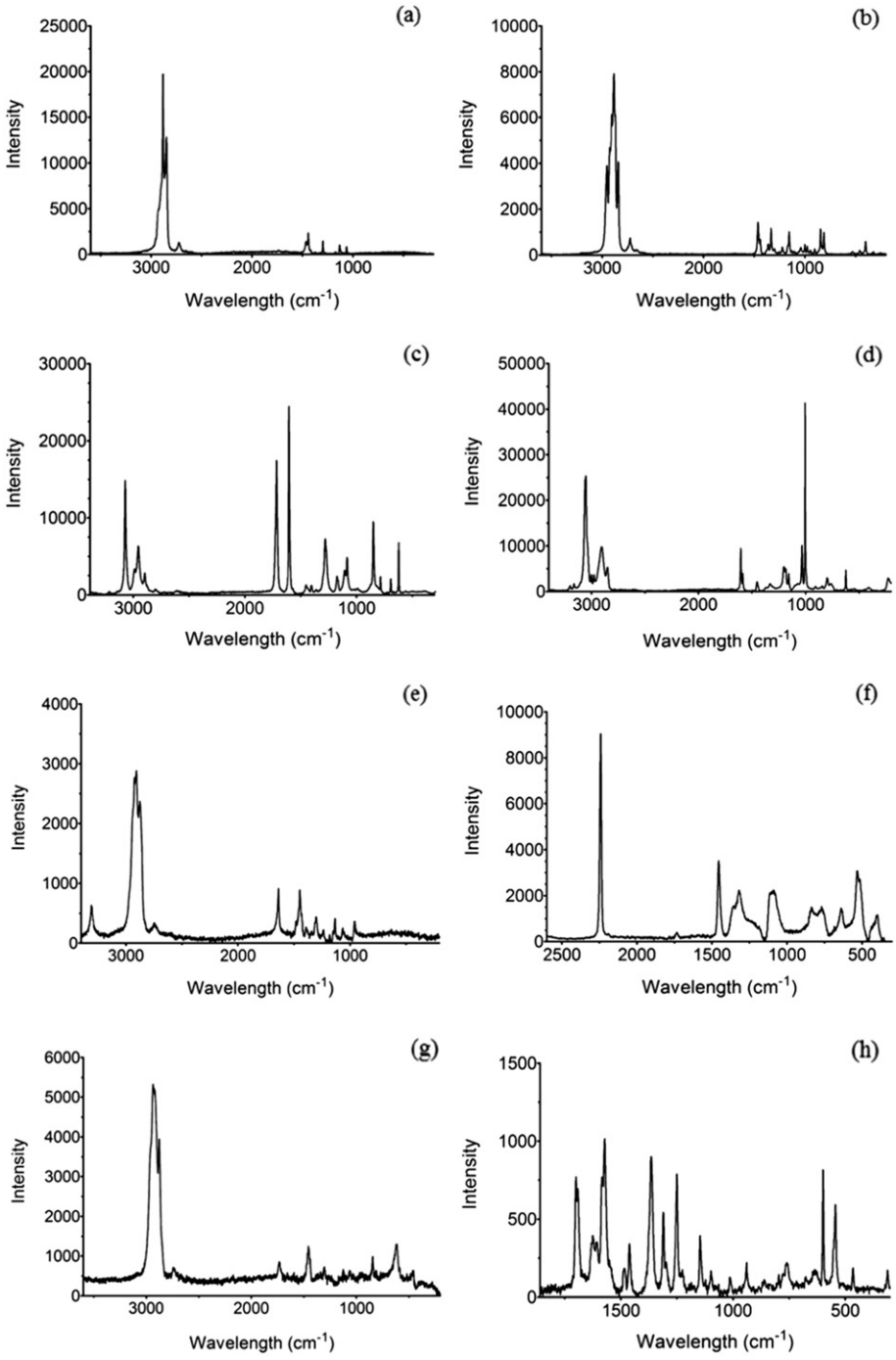

Fig. 7. Overview of spectra recorded: (a) PE fiber, (b) PP fragment, (c) PET fragment, (d) PS fragment, (e) PA fiber, (f) PAN fiber, (g) PBMA fragment, and (h) fiber artificially dyed with Vat Blue 1.

In addition to morphological features, the three *Clupeiformes* studied differ by their feeding ecology. *Clupeiformes* can switch from the filter-feeding (ram-feeding in particular) to the particulate-feeding mode. During ram feeding, fish open their mouth to surround prey while swimming. By contrast, particulate feeders visually detect prey before capturing them (Lazzaro, 1987). Herring and anchovy are known to regularly switch to particulate-feeding according to light intensity (Batty et al., 1990; Bulgakova, 1996) or prey abundance and size (Batty et al., 1986; Gerking, 1994; Tanaka et al., 2006). The particulate-feeding mode implies that the fish could choose the item to ingest, but we do not know whether they can be lured by visual cues such as color and shape. It appears that the dominant mode in sardines is filter-feeding (Garrido et al., 2007; Nikolioudakis et al., 2012; Garrido and Van der Lingen, 2014). This allows the ingestion of more planktonic organisms but also increases the probability of AP ingestion. Further investigation is needed to assess whether herrings, anchovies and sardines are able to differentiate food from APs.

In addition, sardines' apparently more efficient filtration apparatus supports the idea that they can ingest more fibers, which are easily caught in GRs and denticles. Moreover, the sardines ingested smaller microplastic fragments than both the anchovies and herrings. By contrast, the anchovies ingested more APs of all shapes than either the sardines or herrings, which could be explained by the difference in sampling area. Anchovies were sampled in the Mediterranean Sea, which is

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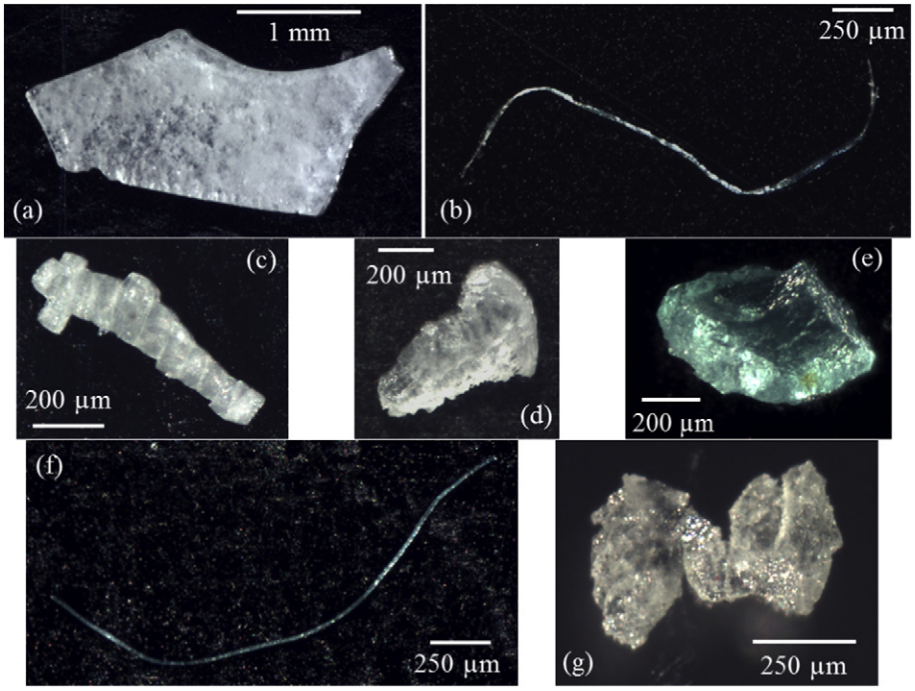

Fig. 8. Isolated particles from stomach contents observed with a stereomicroscope. (a) PE fragment, (b) dyed fiber (Vat Blue 1), (c) PP fragment, (d) PE fragment, (e) PP fragment, (f) PAN fiber, (g) PE fragment.

known to be one of the most plastic-polluted marine areas in the world (Cózar et al., 2015). Fibers made of cellulose that were not associated with artificial dyes have also been found in all species. These were not recognized as APs because cellulose is a natural material. Sodium hypochlorite, used during sample processing, is known to degrade artificial dyes (Robinson et al., 2001; Urano and Fukuzaki, 2011), therefore it cannot be excluded that previously dyed cellulose samples could be bleached during the processing, and consequently dyes were not detected by Raman spectroscopy.

Comparing our contamination results with other studies is not recommended because methodologies to extract and isolate MPs from stomach contents are not standardized within the scientific community (Hidalgo-Ruz et al., 2012). To isolate plastic particles, some studies have used visual criteria that could lead to erroneous interpretation (Remy et al., 2015), while others have used chemical methods. However, when looking at other studies dealing with AP ingestion by fish, our three species are among the most contaminated fish species (Table S1, supplementary material). This raises the question of the potential impacts on the human population, because two of the species (herrings and sardines) are among the 11 most fished species in the world (FAO, 2014).

Why do some species ingest more APs than others? The current body of knowledge does not provide a straightforward answer, and several parameters have to be taken into account. Habitat does not seem to have an influence, as demonstrated by Lusher et al. (2013) and Neves et al. (2015). It is not known whether MPs are deliberately ingested and if some characteristics (shape, color, texture) influence the organism's choice. The ingestion of MPs is the first step of processes leading to mechanical and toxicological harm, including translocation and transfer through food webs (Setälä et al., 2014). This highlights the need for further studies, and mainly experimental studies, to find which parameter(s) influence AP ingestion.

In conclusion, we have provided (1) a detailed description of morphometric and meristic characteristics of the filtration apparatus of three *Clupeiformes* species, (2) a new formula to calculate the filtration area in fish, (3) data about AP (and MP) ingestion in three commercial fish species and (4) evidence that the morphology of the buccal cavity is an important parameter to study AP ingestion by fish; sardines being the most exposed species in our study.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.12.067.

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