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Assessment of the health status of the European anchovy (*Engraulis encrasicolus*) in the NW Mediterranean Sea from an interdisciplinary approach and implications for food safety

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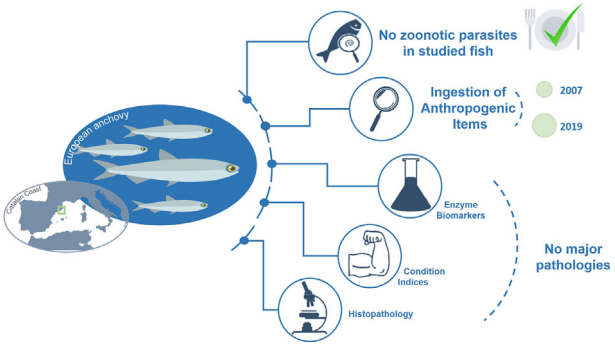
HIGHLIGHTS

- Sampled European anchovies did not show any relevant pathology.
- No zoonotic parasites were found in the studied fish.
- Half of fish contain anthropogenic items but with no impact on health descriptors.
- Higher pollution levels were found in 2019 with respect to 12 years earlier.

GRAPHICAL ABSTRACT



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ABSTRACT

The European anchovy (*Engraulis encrasicolus*) is a small pelagic fish with an outstanding commercial value supporting important fisheries and is a key component of pelagic ecosystems in the Mediterranean Sea. Progressive reductions in the population size of this species has been observed in the Mediterranean Sea during recent decades, accompanied by a decline in the body condition, as well as the size/age of maturation. Nonetheless, the health status has not been yet assessed using a holistic approach. Herein, we analyse the health status of the European anchovy, integrating distinct indicators from fish condition, enzymatic biomarkers, presence of tissue alterations, and parasite descriptors. In addition, we analyse the presence of anthropogenic items (AIs) in the digestive tract of fish and their potential impact on health status. Additionally, we assess the differences between current AIs values and those recorded over 12 years ago. None of the health indicators studied provided evidence of relevant pathologic conditions affecting this fish species in the studied area. However, changes in the pattern of liver parenchyma were found. Compared with anchovy populations from other distribution areas, no zoonotic parasites were recorded in this study, demonstrating a reduced risks associated with foodborne transmission to humans. AIs, such as fibres and plastic particles, were found in the digestive tract of half of the fish analysed. A significant increase was detected in AIs prevalence between 2007 (40 %) and 2019 (70 %), alongside differences in the abundance and typology of the AIs, though this does not seem to have impacted fish health yet. Therefore, our work underscores the importance of implementing a regular program to monitor the health status of this key species to better understand population dynamics and their drivers.

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1. Introduction

The European anchovy (*Engraulis encrasicolus*) is a well-known small pelagic fish species widely distributed in the NW Atlantic Ocean, mainly in near-European and African coastal areas, though also present in the Mediterranean and Black Sea (Whitehead et al., 1988). This species is highly valued in Mediterranean markets as it supports important local pelagic fisheries. Its popularity as a commercial fish species is partly due to the particularly rich nutritional profile it boasts, including a high content of essential fatty acids, including polyunsaturated fatty acids (PUFAs) — among which are the omega 3 fatty acids, such as docosahexaenoic acid (DHA) and ecosapentaeonic (EPA) — and omega-6 fatty acids, like arachidonic acid (ARA) (Zlatanos and Laskaridis, 2007). Due to its significant biomass at mid-trophic levels, this species is the main prey for numerous predators, thus playing a major role in energy transfer, connecting lower to higher trophic levels in marine ecosystems (Cury et al., 2000). For these reasons, the anchovy is considered an important resource not only for its great value for human nutrition, but also for its role in the food chains of pelagic ecosystems. Small pelagic fish are widely known for their rapid and significant population fluctuations, rendering their management especially difficult (Bakun, 1997). Progressive declines of commercially important small pelagic fish populations — including anchovies — have been observed in the Mediterranean Sea alongside changes in the population structure (Van Beveren et al., 2014). While the abundance of anchovy remains relatively high, both biomass and the mean size of individuals have diminished dramatically, accompanied by a decline in the body condition and size/age of maturation (Albo-Puigserver et al., 2021; Brosset et al., 2015; Saraux et al., 2019; Van Beveren et al., 2014). The prevailing explanatory hypothesis for this implicates various stressors, stemming from changes in the environment and food availability. Changes in the plankton community affect the diet of this species, resulting in a shift towards feeding on smaller zooplankton species (Queiros et al., 2019; Thoral et al., 2021; Van Beveren et al., 2014). However, other drivers, such as fishing pressure or increased levels of pollutants including microplastics might also be influencing the individual health of fish and therefore the population dynamics (Lefebvre et al., 2019; Saraux et al., 2019). Long-term survival, and maintenance of anchovy stocks is of vital importance for both commercial and ecological reasons; this can only be ensured by supporting the good health status of populations.

Due to these complex and multifactorial influences, evaluations of the condition and health status of wild fish populations necessitate the integration of multiple distinct indicators, in a holistic approach. This analysis should include general indices such as overall fish condition, to more specific indicators such as the evaluation of potential pathological features by histopathology, the presence of alterations in enzymatic biomarker patterns, and parasite descriptors, among others.

Fish body/condition indices are based on the relationship that exists between the mass of certain organs with respect to the general mass or fish size; they allow comparisons between individuals of the same population or between distinct populations. They are considered a simple way to infer overall health of fish and alterations can indicate the occurrence of diseases or other physiological events that may be compromising the species' fitness. For instance, the Le Cren relative index (Kn) is the main method used to estimate the body condition of an individual or population (Brosset et al., 2015), and fasting or feeding intensity can be assessed by the stomach fullness index (Hyslop, 1980). Hepatosomatic and gonadosomatic indices — both widely used in ecological studies and in stock evaluations in fisheries (Basilone et al., 2020; Brosset et al., 2017, Brosset et al., 2015; Stevenson and Woods, 2006) — give information about the physiological status of the individuals, in regard to the accumulation of short-term reserves and reproductive capacity, respectively (Wootton, 1989).

Moreover, environmental stressors can be reflected in alterations at different levels, from biochemical pathways, to cells, tissues, organs, systems, fish and/or fish stocks. Biochemical markers in fish are known to be particularly sensitive to environmental changes (Van der Oost et al., 2003). Thus, studies that combine different sets of biomarker enzymes — involved in biochemical and metabolic pathways that are influenced by the presence of xenobiotics — and the effects of this, e.g. alterations in levels of neurotoxicity, detoxification, and oxidative stress, are highly recommended (Mejdoub et al., 2017; Solé and Sanchez-Hernandez, 2018). Such markers respond to both natural and anthropogenic stressors, providing a broader perspective and a better understanding of the observed dynamics (Cajaraville et al., 2000; Galloway et al., 2002; Matozzo et al., 2018). Important knowledge gaps remain; for instance we know of only one study that has addressed the response of P450 monooxygenase 1A in anchovies from impacted areas (Basilone et al., 2018).

In many cases, pathologies associated with biological agents (parasites, virus, bacteria, fungi) or non-infectious diseases (neoplasia, pathological behavioural changes, genetic diseases) present in wild fish populations are also modulated by environmental impacts. These diseases or alterations in wild fish populations can be detected using different diagnostic methodologies and in particular, pathological and histopathological techniques are considered some of the most reliable and suitable tools for a general health assessment (Au, 2004; Costa, 2018; Feist et al., 2004; Stentiford et al., 2003). They allow for the identification of not only the early warning signs of disease and injury in cells, tissues, or organs, but also chronic exposure and the subsequent effects at the population or community level. The most common target organs used in histopathology are the liver (due to its role in transformation, storage, and detoxification) and gills — which are in direct contact to the environment. Additionally, the digestive tract, kidney, and gonads may be highly relevant for histopathological diagnostics (Costa, 2018) due to their role in fish metabolism and because of their sensitivity and direct exposure to environmental factors or pathogens. Apart from a few specific studies describing the histology of the visual organ sense (Heß, 2009; Kondrashev et al., 2012) in anchovies, neither the normal histology of adults nor the specific histopathological alterations have yet been described.

Fish parasite communities are widely used as health indicators of both organisms and ecosystems, since parasitic infestations affect not only fish health, but can also respond to environmental changes depending on the parasites' lifecycle and the nature of pollutants (Mackenzie et al., 1995; Marcogliese, 2005; Sures, 2001). Moreover, as a species for human consumption, the parasite fauna of European anchovy has been described and studied, especially with regard to zoonotic nematodes (Ferrer-Maza et al., 2016; Rello et al., 2009). These zoonotic parasites play a relevant role in human health and may trigger a food safety issue (Cipriani et al., 2018). Nematode larvae reach the fish when feeding, penetrate the intestinal wall and then encyst on the surface of the internal organs and/or migrate towards the musculature (Cipriani et al., 2018; Mattiucci and Nascetti, 2008). They can be transmitted to humans — who act as paratenic hosts — by ingesting infected raw or undercooked fish. Taking into account the wide range of culinary preparations in many countries of the Mediterranean basin, as well as the traditional method of processing and preserving (brined in salt and preserved in oil and salt mixture, or pickled in vinegar), the presence of these zoonotic parasites in anchovies is considered a risk for human health (Cipriani et al., 2018).

The Mediterranean Sea is a semi-enclosed, highly populated basin with a very ancient intensive use of marine and terrestrial resources that results in heavy anthropogenic pressure from land to the sea. During the last few decades, marine litter has increased exponentially (Ryan et al., 2009), becoming one of the greatest threats that marine ecosystems and their associated biota have ever faced, this is an ever-growing cause of concern worldwide. Between 4 and 12 million tonnes of plastic enters the world's oceans annually (Jambeck et al., 2015). Once in the environment, they are transported long distances thanks to their buoyant and persistent properties and current evidence suggests that plastics — and the products of their fragmentation — are now ubiquitous in oceans worldwide (Cózar et al., 2014). Microplastics (plastic fragments smaller than 5 mm; Frias and Nash, 2019) have been extensively described in both vertebrate and invertebrate organisms. The ingestion of particles of anthropogenic origin by small pelagic fish — including the anchovy — has been widely described in the NW Mediterranean (Compa et al., 2018; Lefebvre et al., 2019),

confirming the ingestion of microplastic fragments, plastic fibres, or cellulose fibres (Capone et al., 2020; Compa et al., 2018; Lefebvre et al., 2019). Although the possible hazardous effects have been addressed in laboratory experiments, studies inferring the effects in wild populations are scarce, though increasing (Rodríguez-Romeu et al., 2020).

Therefore, the assessment of the effects of active or accidental ingestion of anthropogenic items (AIs) — including microplastics but also other particles from anthropogenic origins such as cellulose fibres — on the health status of wild marine biota should be properly addressed, in order to better understand their potential impact at the population and ecosystem levels, as well as the potential risks regarding food safety and human health.

For these reasons, the main aim of the present study is to assess the health status of the European anchovy sampled from three different geographical areas of the NW Mediterranean Sea using a holistic approach, combining different evaluation methodologies: biological indices, enzymatic biomarkers, histological tissue alterations, and parasite descriptors — specifically focusing on zoonotic parasites. In addition, we analyse the presence of AIs in the digestive tract of fish and their potential impact on the fish condition and health status, as well as the differences between current AIs levels and those found in fish over 10 years previous.

2. Materials and methods

2.1. Study area and sample collection

A total of 150 European anchovies were captured from three distinct locations (Fig. 1, Table 1) off the Catalan coast (NW Mediterranean) within the framework of the BIOMARE (Spanish Ministry of Science and Innovation), SOMPESCA (Department of Agriculture, Livestock, Fisheries and Food, Catalonia, Spain) and PLASMAR (Spanish Ministry of Science, Innovation and Universities project) multidisciplinary projects. Fish were collected aboard commercial trawling vessels during 2007 (30 specimens) off Barcelona and with commercial purse seiner fishing vessels during 2019 at three different sites (30 specimens each; same location off Barcelona, and a northern and southern locations nearby Blanes and Tarragona, respectively) (Fig. 1, Table 1). Fish captured in 2007 were immediately fixed in 10 % buffered formalin and stored. In fish captured in 2019, before fixation, a portion of dorsal muscle (0.5 g, *w*/w) and a portion of liver (0.1 g, w/w) were dissected, weighed and stored in dry ice for subsequent enzymatic biomarker analysis. Thereafter, fish were immediately fixed in 10 % buffered formalin and stored in the laboratory. Thirty additional fish per location were frozen (−20 °C) on board to complement parasitological studies.

2.2. Laboratory procedures

Prior to dissection in the laboratory, each specimen was measured to the nearest mm (total length = TL and standard length = SL) and weighed to the nearest g (total weight = TW). To minimize airborne contamination, all procedures were performed in a laminar flow cabinet, which was previously cleaned, and the laboratory equipment and tools rinsed twice with deionized filtered (50 μm) water. Nitrile gloves and exclusive cotton lab coats were used at all times. The gastrointestinal tract was removed by dissection following procedures based on previous work (Lusher et al., 2013), from the top of the oesophagus to the anus. Stomach (SW), liver (LW), and gonads (GW) were weighed inside the laminar flow hood using a precision scale to the nearest mg. The spleen was also removed, and eviscerated weight (EW) was recorded to the nearest g. All organs including the dissected gastrointestinal tract (stomach, caeca, and intestine) were stored separately in filtered (50 μm) 70 % ethanol in individual glass vials previously rinsed with deionized filtered (50 μm) water for subsequent observations.

2.3. Condition and health assessment

Fish condition was assessed by the gonadosomatic index (GSI = (GW/EW) x 100), the hepatosomatic index (HSI = (LW/EW) × 100) and Le Cren's relative body condition index (Kn = EW / (α × TL^{β})), where Kn is the relative body condition, α and β are the slope and the intercept of the weight-length relationship representing the entire dataset of sampled fish

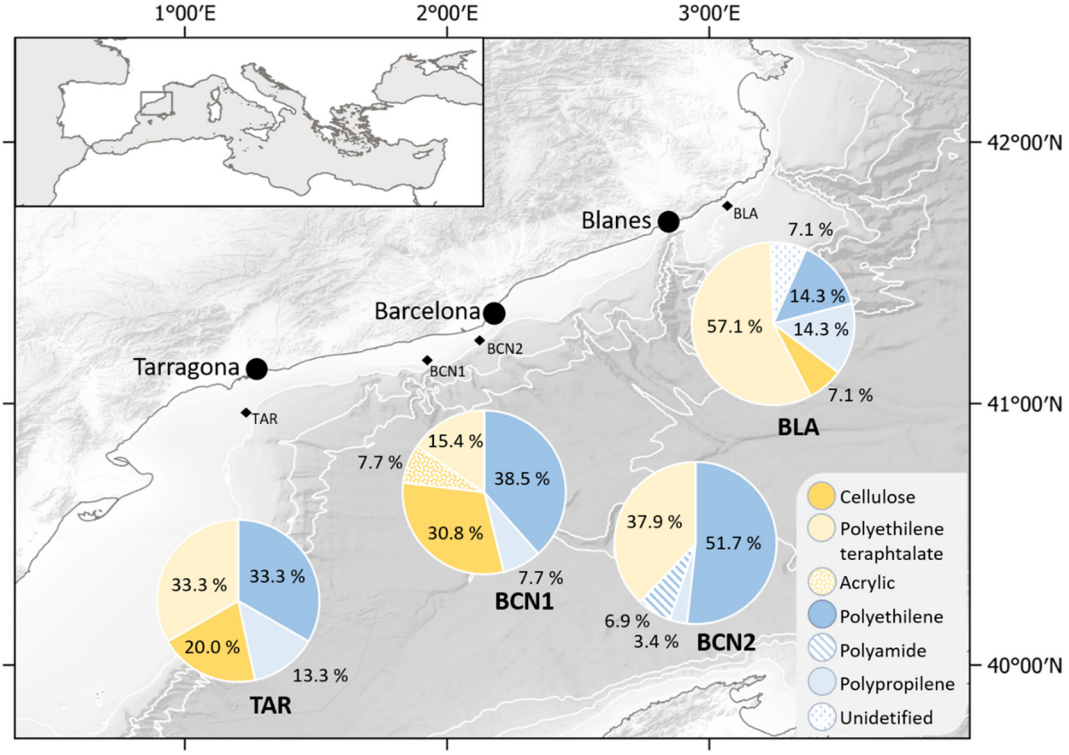

Fig. 1. Map of sampling area where sampling for European anchovy occurred. The diamond shape (♦) indicates sampling stations. From north to south, Blanes (BLA), Barcelona (in 2007, BCN1, and in 2019, BCN2), and Tarragona (TAR). Pie charts represent the percentage of each polymer type from the anthropogenic items identified by micro-FTIR in each location. Base map retrieved from the web map services of the EMODnet Bathymetry Consortium (2020).

Table 1
Cruise data (station, location, year, date, latitude, and longitude) for each sampling site. Mean and standard deviation (SD) of standard length (SL, cm), total weight (TW, g), gonadosomatic index (GSI), hepatosomatic index (HSI), Le Cren relative condition index (Kn), stomach fullness (FULL) and body perimeter in the anterior (P1), middle (P2) and posterior (P3) parts of the body. Mean and standard deviation (SD) of enzymatic activities of Acetylcholinesterase (AChE), Lactate dehydrogenase (LDH), Cytrate synthase (CS), Carboxyl esterase (CbE), Catalase (CAT), Glutatione-S-transferase (GST) and Ethoxyresorufin-O-deethylase (EROD). Significant differences between years (Barcelona 2007–2019) are represented by superscript letters (a and b), while the absence of superscript letter means no significant differences. Differences among localities (Barcelona–Blanes–Tarragona in 2019), are expressed by superscript numbers (1, 2 and 3), while the same superscript number or their absence means no significant differences.

Station	BCN1		BCN2		BLA		TAR	
Location	Barcelona		Barcelona		Blanes		Tarragona	
Year	2007		2019		2019		2019	
Date	20/07/2007		18/07/2019		22/07/2019		25/07/2019	
Lat.	41° 8′18.96”N		41°14′44.88” N		41°45′48.12”N		40°55′21.60”N	
Long.	1°45′41.54″E		2° 9′20.10″E		3° 3′47.40″E		1°16′43.20″E	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
SL	12.04 [a]	(0.37)	12.46 [b 1]	(0.71)	11.39 [2]	(0.52)	11.09 [3]	(0.46)
TW	16.97 [a]	(1.58)	18.53 [b 1]	(3.24)	13.40 [2]	(1.67)	14.05 [2]	(1.80)
GSI	4.53	(1.60)	4.56 [1]	(1.22)	4.57 [1]	(1.24)	3.72 [2]	(1.11)
HSI	1.75 [a]	(0.81)	2.25 [b 1]	(0.85)	1.53 [2]	(0.48)	2.25 [1]	(0.62)
Kn	1.04 [a]	(0.06)	0.97 [b 1]	(0.05)	0.97 [1]	(0.05)	1.01 [2]	(0.05)
FULL	1.48 [a]	(1.03)	0.48 [b]	(0.32)	0.40	(0.22)	0.58	(0.50)
P1	–	–	52.55 [1]	(4.62)	45.4 [2]	(5.08)	43.97 [2]	(6.44)
P2	–	–	47.07 [1]	(3.59)	41.75 [2]	(2.39)	41.35 [2]	(4.02)
P3	–	–	33.83 [1]	(2.69)	31.16 [2]	(1.76)	31.58 [2]	(2.29)
AChE [i]	–	–	21.3 [1]	(4.77)	33.18 [2]	(10.67)	28.37 [2]	(8.36)
LDH [i]	–	–	5915.18 [1]	(504.87)	5917.09 [1]	(440.40)	6840.94 [2]	(409.59)
CS [i]	–	–	77.09 [1 2]	(5.63)	81.43 [2]	(6.99)	75.74 [1]	(8.53)
CbE [i]	–	–	46.68 [1]	(13.81)	50.11 [1]	(13.75)	76.37 [2]	(35.40)
CAT [j]	–	–	311.5 [1]	(124.54)	456.82 [2]	(134.69)	451.7 [2]	(131.13)
GST [i]	–	–	163.81 [1]	(59.35)	236.95 [2]	(65.9)	229.8 [2]	(66.20)
EROD [k]	–	–	0.79 [1]	(0.33)	0.86 [1]	(0.18)	1.24 [2]	(0.38)

[i] $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$.
[j] $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$.
[k] $pmol \cdot min^{-1} \cdot mg\ protein^{-1}$.

(Le Cren, 1951). Feeding intensity was measured by the stomach fullness index (FULL = (CW/EW) × 100), which was calculated using the total stomach content weight (CW) recorded after screening for potential AIs.

To complement fish condition assessment, height, width, and perimeter were measured; this was performed in three different parts of the body — beginning of the dorsal fin (P1), beginning of the anal fin (P2), and midpoint between the end of the anal fin and the caudal peduncle (P3). In addition, a semi-quantitative analysis of the perivisceral fat was conducted from histological sections. For this purpose, a random subsample of 10 fish per location (2019 specimens) were used to determine a semi-quantitative indicator of size of adipocytes by screening the perivisceral fat tissue. The diameter of a minimum of 100 adipocytes was measured using images obtained at 20× magnification by a camera attached to the microscope (Leica microscope model: DM 5000 dB) and image-processing software (ProgRes® C3). According to adipocyte's size, four categories were obtained (0 = no fat tissue; 1 = small size adipocyte; 2 = medium size adipocyte and 3 = large size adipocyte). Using these criteria, all adipocytes from the rest of the samples were classified for subsequent analyses (Fig. 2).

For the enzymatic biomarker analyses, a muscle portion of around 0.3 g wet weight was used for acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and citrate synthase (CS) determinations. A portion of liver of around 0.07 g wet weight was used to analyse Glutathione-S-transferase (GST), catalase (CAT), carboxylesterase (CbE), and ethoxyresorufin-O-deethylase (EROD). Assays were performed following the procedures described in past studies in the area (Antó et al., 2009; Koenig et al., 2013; Solé and Sanchez-Hernandez, 2018).

A portion of gonad, liver, spleen, kidney, stomach, caeca, intestine, and gills were embedded in paraffin, sectioned at 5 μm and stained with Haematoxylin and Eosin for histopathological assessment. All histological sections were completely screened under the microscope. When alterations were detected, a morphological evaluation of each alteration was performed. The prevalence of the lesions (percentage of fish affected by a specific alteration) was calculated. When required, some of these sections were

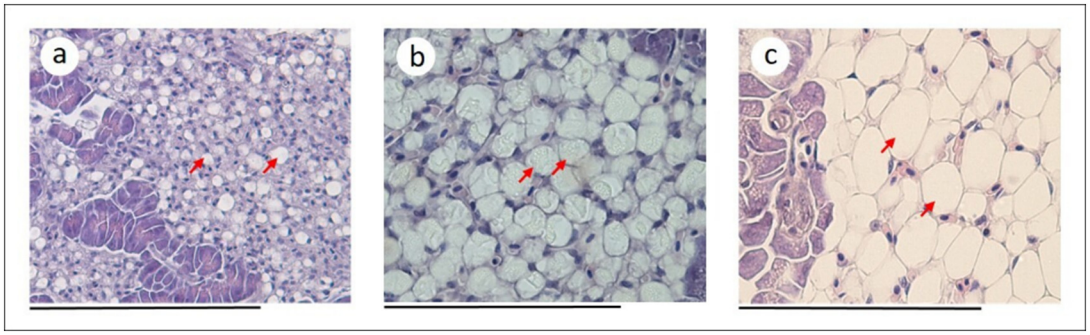

Fig. 2. Optical microscope images giving examples of fat tissue in European anchovy in which adipocytes (indicated by red arrows) of different size classes: a) small size, b) medium size, and c) large size, can be seen. Scale bars at the bottom left of each picture represent 0.1 mm.

additionally stained with Periodic Acid Schiff (PAS) and Sudan stains. The spleen was also chosen for a quantitative evaluation of the density of melanomacrophage centres (MMCs), due to the ease of dissection of the whole organ and the possibility to obtain complete radial sections (Fournie et al., 2001; Manera et al., 2000). For this purpose, three fields of view (0.23 mm^2/screen) were randomly selected from each section of the spleen at 200× and examined microscopically. Area and number of MMCs (mean area = MA.MMC and number = nMMC, respectively) of each field were measured using a MicroComp Integrated Image Analysis System, and a size discriminator was used to eliminate objects smaller than 100 μm^2.

External surfaces and gills were checked macroscopically for ectoparasites and the rest of the organs, including stomach, caeca, intestine, gonads, spleen, brain, body cavity, and muscle were carefully inspected for endoparasites under a stereomicroscope. The location of parasites within the fish was annotated. Digeneans and cestodes were stained with iron acetocarmine and permanently mounted in Canada balsam. Nematodes were temporally cleared and mounted in glycerine before identification. Parasites were identified under an optical microscope to the lowest possible taxonomic level. In addition, six digeneans, two nematodes and two monogeneans of thawed European anchovy were subjected to molecular analyses. DNA from all samples was extracted with Qiagen TM (Valencia, California) DNeasys Blood and Tissue Kit. Partial nuclear large subunit ribosomal DNA (28S rDNA) and partial fragments of mitochondrial cytochrome *c* oxidase 1 (cox1) gene were amplified (50 μl total volume) using ExcelTaqTM SMOBIO_ PCR Master Mix (Taiwan) containing: 5× concentrated master mix, that is, a mixture of recombinant Taq DNA polymerase, reaction buffer, MgCl2 (2 mM), dNTPs (0.2 mM), and enzyme stabilizer, 0.25 μM of each PCR primer and 2 μl of extracted gDNA. Primer pairs and amplification conditions were used as follows: partial fragments of the cox1 gene were amplified using the primers LCO1490 (forward, 5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (reverse, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) under the following thermocycling conditions: initial denaturation at 95 °C for 15 min followed by 35 cycles (denaturation for 5 min at 80 °C, followed by 1 min 30 s at 92 °C, annealing for 1 min at 42 °C, and extension for 2 min at 72 °C), and a final extension step at 72 °C for 10 min. Partial fragments of the 28S rDNA gene were amplified using the primers LSU5F (forward, 5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) and ECD2R (reverse, 5′-CTT GGT CCG TGT TTC AAG ACG GG-3′) (Littlewood et al., 2000) under the following thermocycling conditions: initial denaturation at 95 °C for 2 min followed by 30 cycles (denaturation for 50 s at 95 °C, annealing for 50 s at 52 °C, and extension for 50 s at 72 °C), and a final extension step at 72 °C for 7 min. In every PCR run, a negative and a positive control were used to detect any potential contamination, and to have a reliable sample to compare with, respectively.

PCR products were visualised on RedGel-stained 1 % agarose gels, purified, and sequenced by Macrogen (Amsterdam, Netherlands). Sequencing primers were the same as for the PCR. Valid sequences were aligned using BioEdit 7.0.1 (Hall, 1999), variable sites were checked visually for accuracy, and final sequences were submitted to GenBank (*Hysterothylacium aduncum* accession numbers ON514619 and ON514624; *Lecithaster* accession number ON524172). Parasite prevalence (P%) and mean abundance (MA) were calculated following previous studies (Bush et al., 1997), and richness and Brillouin diversity indices for each individual were also calculated.

2.4. Anthropogenic item (AI) extraction and characterisation

Stomachs were opened longitudinally — with a sagittal cut — and each half was carefully rinsed, before being stored separately. The area of the half-sectioned stomach of each fish was calculated. The content of the stomach, caeca, and intestine was carefully screened under a stereoscopic binocular at 5× to 40× magnification. To prevent airborne contamination, the stereomicroscope and working area was isolated from the surroundings using an isolation device adapted from the one proposed by Torre et al. (2016), and the interior was carefully washed before use to minimize the presence of airborne fibres. The laboratory dissection material was also rinsed with filtered deionized water twice before use. Uncovered Petri dishes containing filtered deionized water were placed inside and outside the isolation device to assess airborne contamination. Only fibre-shaped items were found in both controls, and the number and morphological characteristics of the airborne fibres were recorded before and after each sample examination. Contamination found in the inside controls (average values of 1.16 fibres per digestive sample screened) was 15.6 times lower than contamination in outside controls, thus indicating the efficiency of the isolation device in reducing potential contamination. Fibres found in the inside controls were clean and always appeared on the surface of the water (indicating that they were deposited from the air). Therefore, fibres from digestive tracts were only counted if they were clearly embedded in the digestive content and/or with detritus attached; these were clearly differentiated from those floating on the surface, which were excluded thereafter. Therefore, no correction factor was applied to the final values of the fibres reported. All manipulations were carried out under the stereomicroscope. Finally, the content of stomach, caeca and intestine was carefully separated and stored for a subsequent analysis.

The prevalence of fish with anthropogenic items (% AIs; percentage of fish with AIs within their digestive tract with respect to the total number of fish analysed) was calculated. AIs detected were collected and mounted between glass slides in filtrated deionized water and observed under the microscope. For the anthropogenic fibres, length, mean cross section (based on three random measures), and area were obtained. For anthropogenic particles, the maximum length and area were recorded. Images were obtained using a camera attached to the microscope and were subsequently measured using image-processing software (ProgRes® C3).

Total number of AIs were counted for each individual (nAI) and their locations within the digestive tract (stomach, caeca, and intestines) were annotated. Mean abundance (nAI = number of AIs/total number of individuals), and mean intensity (mean intensity = number of AIs/individuals with ingested AIs) were also calculated. AIs were divided into categories depending on their form (anthropogenic particles or anthropogenic fibres). Total length of AIs (TLAI) was calculated by adding the length of each AIs observed inside the digestive tract of each individual. The percentage of stomach occupation by AIs was calculated (proportion of stomach occupation = (sum of area of AIs in stomach/stomach area) x (100)). The fibres found in the digestive tract of fish were carefully observed under the microscope, characterized and classified visually into distinct typologies (cellulosic and plastic), according to their morphological features, following the criteria of Rodríguez-Romeu et al. (2020). Fourier-Transformed Infrared Spectrometry (FTIR) was performed on a randomly selected subsample of 72 anthropogenic items (35 anthropogenic fibres and 37 anthropogenic particles) corresponding to a total of 56 % of AIs found.

Spectra of fragments and films were recorded using a Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Germany) equipped with a diamond attenuated total reflectance (ATR) unit (16 scans cm^{-1}, 800–3600 cm^{-1}). Fibre spectra were recorded at Scientific and Technological Centres (CCitUB, University of Barcelona) using a micro-FTIR Thermo Scientific Nicolet iN10 MX, equipped with an Imaging Detector (4 scans cm^{-1}, 800–4000 cm^{-1}). Resulting spectra were treated (baseline corrections, peak normalization, and selection of characteristic band applied) with Spectragryph 1.2.11 and compared with reference spectra. Spectra from 11 common reference polymers were included in a custom library (cellulose, acrylic, nylon/polyamide, high-density and low-density polyethylene, polyethylene terephthalate, polypropylene, polyurethane, and polystyrene). Similarity correlation indices between sample and reference spectrum were calculated for characteristic bands (from 1800 to 670 cm^{-1} wavelengths) and values >70 % similarity were selected. Results were further checked by visual correlation of peaks and by using the KnowItAll® (Bio-Rad, USA) software, to compare spectra with a broader database.

2.5. Data analysis

In order to characterize the adipocyte diameter, measured adipocytes of the fat tissue were classified into four clusters by partitioning around

medoids (PAM). The k-medoids algorithm was applied in order to classify the dataset into different groups or clusters from a matrix of dissimilarity. Each cluster is represented by one object, which is located in the centre of the cluster. The k clusters are established by assigning each object of the dataset to the nearest representative object — thus objects that show a high level of similarity are grouped together, while objects that are dissimilar to each other belong to different clusters (Kaufman and Rousseeuw, 1990) — using the PAMK function implemented in the package fpc 2.2–3 in R Studio (version: 4.0.3). All variables were tested for normality and equality of variance using the Shapiro-Wilk and Levenne's test respectively.

To assess differences in explanatory and response variables among localities (Barcelona, Blanes, and Tarragona) and between years (2007 and 2019) when possible, general linear models (GLM, gaussian models) or generalized linear models (GZM, gamma models) were applied for biological indices (SL, TW, Kn, GSI, HSI and FULL), for enzymatic biomarkers, body perimeters, diversity parasite descriptors (richness and Brillouin diversity index), and percentage of AIs occupation. Differences among localities and years for the prevalence of AIs, and between localities for the adipocyte diameter and prevalence of histological alterations and parasites were tested using a GZM (binomial model, link logit). For mean parasite abundance (total parasites, endoparasites, and only parasites from digestive tract) a GZM based on negative binomial models (link log) was applied. Finally, mean intensity and abundances of AIs were tested for differences among localities and year using GZMs (poisson models, link log). When necessary, SL was considered as covariate, including stomach fullness for AI approximations.

Correlations between condition indices, biomarkers, parasites, and AIs variables were tested in order to detect any possible relationships among them. This was done using Pearson's correlation tests and non-parametric Spearman's correlation tests (when normality was not satisfied), and plotted with the corrplot R package (Wei and Simko, 2017). Parasites have been suggested as a possible factor affecting the retention of microplastics (Hernandez-Milian et al., 2019; Pennino et al., 2020). Thus, the total number of parasites, and those found in the digestive tract only, were considered as explanatory variables in subsequent analyses. These variables were included in models to test the association between parasite load and levels of ingestion of AIs.

Multiple factor analysis (MFA) was used to assess the possible effects of AIs on fish health condition indices (including biomarkers). This multivariate data analysis enables the evaluation and identification of individuals as characterized by sets of variables (both quantitative and qualitative) which are structured into groups on this basis. Thus, the differences within groups are minimized, whilst differences between groups are maximized. Distinct MFAs were conducted using the following as explanatory variables: nAI, TLAI, abundance of fibres (plastic/non-plastic), abundance of particles, and abundance of plastic and non-plastic anthropogenic items. Data analysis was performed using R Studio software (version: 4.0.3). For each statistical hypothesis test, significance was set at 0.05.

3. Results

3.1. Health status

3.1.1. Condition indices

Body condition indices of fish are shown in Table 1. For 2019, significantly higher values were found in Barcelona with respect to other locations in standard length (Barcelona–Blanes $t = -7.165$ $p < 0.01$; Barcelona–Tarragona $t = -10.150$, $p < 0.01$) and total weight (Barcelona–Blanes $t = -8.817$, $p < 0.01$; Barcelona–Tarragona $t = 8.221$, $p < 0.01$); therefore these results call for further analyses. In addition, the Barcelona fish demonstrate significantly higher values in body circumferences P1 (Barcelona–Blanes, $t = 6.905$, $p < 0.01$; Barcelona–Tarragona, $t = 7.140$, $p < 0.01$); P2 (Barcelona–Blanes, $t = 7.090$ $p < 0.01$; Barcelona–Tarragona, $t = 7.918$, $p < 0.01$); P3 (Barcelona–Blanes, $t = 4.673$, $p < 0.01$; Barcelona–Tarragona, $t = 4.338$, $p < 0.01$). Fish sampled in Tarragona showed significantly higher values of the Kn condition index ($t = 2.667$, $p < 0.01$). Whereas significantly lower values were found in Blanes for the HSI (Barcelona–Blanes, $t = -8.817$, $p < 0.01$; Tarragona-Blanes, $t = 8.221$, $p < 0.01$) and in Tarragona for GSI index (Barcelona-Tarragona, $t = -2.823$, $p < 0.01$; Blanes–Tarragona, $t = -2.458$, $p < 0.05$). No significant differences in stomach fullness among localities were found.

Histologically, differences in the size of the adipocytes in the perivisceral fat tissue were observed (Fig. 2). Cluster partitioning around medoids of adipocyte diameter gave three clusters. Small size: Ø <1.52 μm; medium size Ø >12.52 to <23.18 μm; and large size Ø >23.18 μm. Comparison of adipocyte clusters showed significant differences among localities, and fish from Barcelona had more small size adipocytes (cluster 1) compared to Blanes and Tarragona ($z = -2.772$, $p < 0.05$).

When comparing fish from 2007 and 2019 (Table 1), significantly higher values of standard length ($t = -2.907$, $p < 0.05$), TW ($t = -2.719$, $p < 0.05$) and HSI ($t = -2.021$, $p < 0.05$) were observed in anchovies sampled in 2019 when compared with fish obtained in 2007. Meanwhile significantly higher values of Kn condition index ($t = -9.069$, $p < 0.01$) and stomach fullness ($t = 5.450$, $p < 0.01$) were detected in 2007 when compared to 2019. No significant differences in GSI were found between years for Barcelona.

3.1.2. Enzymatic biomarkers

When compared to other locations, fish obtained in Barcelona showed significantly lower values for AChE (Barcelona–Blanes, $t = -3.568$, $p < 0.005$; Barcelona–Tarragona, $t = -2.181$, $p < 0.05$); CAT (Barcelona–Blanes, $t = -2.842$, $p < 0.05$; Barcelona–Tarragona, $t = -2.534$, $p < 0.05$) and GST (Barcelona–Blanes, $t = -2.92$, $p < 0.05$; Barcelona–Tarragona, $t = -2.468$, $p < 0.05$) (Table 1). Instead, Tarragona showed significantly higher values for LDH (Barcelona–Tarragona $t = -4.430$ $p < 0.001$; Blanes–Tarragona $t = -5.231$, $p < 0.001$); CbE (Barcelona–Tarragona $t = -3.160$, $p < 0.05$; Blanes–Tarragona $t = 2.070$, $p < 0.05$) and EROD (Barcelona–Tarragona $t = -3.081$, $p < 0.005$; Blanes–Tarragona $t = -3.081$, $p < 0.005$). Blanes showed significantly higher values for CS when compared to Tarragona (Blanes–Tarragona, $t = 2.070$, $p < 0.05$). None of these differences were related to SL.

3.1.3. Histological observation and alterations

Melanomacrophage centers (MMCs), when present, were mostly located in the spleen, but also in a smaller number and size in the kidneys and liver. Total prevalence of fish with splenic MMCs was 68 %; this was significantly higher in Barcelona when compared to Tarragona ($z = -2.061$, $p < 0.05$). The number of MMCs (nMMC) observed per fish ranged from 0 to 7, with a mean value of 1.23 MMC/ind. (SD = 1.58). The area of MMCs ranged from 246.40 μm^2 to 6046 μm^2, and mean area (MA.MMC) was 2253.92 μm^2/ind. (SD: 1442.74). Comparing nMMC among localities, fish sampled in Barcelona showed significantly higher values than Tarragona ($t = 2.417$, $p < 0.05$; Table 2). No significant differences in MA. MMC were found among localities.

No relevant histopathological alterations were found in the analysed samples, although some minor histological alterations were found in the livers of some fish. Vacuole-like structures were observed in the cytoplasm of hepatocytes in 65 % of the fish (Fig. 3a). Fish from Barcelona showed a higher prevalence of these structures when compared to Tarragona ($t = -2.212$, $p < 0.05$ (Table 2). These structures were not reactive to PAS, nor to Sudan staining. Other liver alterations consisted of the occasional presence of small patches of inflammatory foci, mainly composed of lymphocytes and sometimes a few macrophages (Fig. 3b). These foci were found in 15 % of individuals, and their prevalence was higher in Barcelona than in Blanes, though these differences were not significant (Table 2). No inflammatory foci were found in Tarragona (Table 2). In addition, gill epitheliocystis were observed in the gills of 15 % of fish sampled from Tarragona, though always in a very low intensity. No alterations or damage (epithelial erosion, haemorrhages, or inflammation) potentially associated to mechanical abrasion by AIs were detected in the intestinal or gastric structures. Other histological observations were explained by the parasite infestation — typically by nematodes and digenean larvae — detected in the parasitological study. These lesions were mainly observed as

Table 2
Parasite descriptors, and histopathological alterations found in European anchovy (*Engraulis encrasicolus*). Prevalence (P%) of parasites, epitheliocystis, inflammatory foci and vacuoles-like structures; mean abundance (MA) and standard deviation (SD) of parasites, and melanomacrophage centres (MMCs). Mean and standard deviation (SD) of the area (μm^2) of melanomacrophage centers (MA. MMC in) and number of melanomacrophage centers (nMMC). Different superscript numbers (1 and 2) show significant differences ($p < 0.05$) among localities, while the same superscript number or their absence means no significant differences.

Locality		Barcelona				Blanes				Tarragona		
Year		2019				2019				2019		
PARASITES		P%	MA	(SD)		P%	MA	(SD)		P%	MA	(SD)
METAZOA												
CNIDARIA												
Myxozoa		25	–	–		30	–	–		25	–	–
NEMATODA		70	1.70^{1}	(1.62)		63	1.07^{2}	(1.22)		63	0.97^{2}	(0.96)
PLATYHELMINTHES												
Trematoda		100	32.03^{1}	(23.94)		100	27.57^{1}	(21.67)		100	15.30^{2}	(12.89)
Digenea		97	30.83^{1}	(24.14)		100	27.23^{12}	(21.77)		100	14.57^{2}	(12.71)
Monogenea		50^{1}	0.87^{1}	(1.04)		13^{2}	0.13^{2}	(0.35)		40^{1}	0.70^{1}	(1.06)
Cestoda		27^{1}	0.33^{1}	(0.61)		10^{1}	0.20^{12}	(0.66)		3^{2}	0.03^{2}	(0.18)
PROTISTA												
APICOMPLEXA		40	–	–		15	–			20	–	–
CILIOPHORA		10	–	–		5	–			–	–	–
Parasites Species Richness			4.20^{1}	(1.16)			3.47^{2}	(1.2)			3.30^{2}	(0.92)
			MSR/MD	(SD)			MSR/MD	(SD)			MSR/MD	(SD)
Brillouin Diversity Index (H′)			0.78^{1}	(0.27)			0.71^{1}	(0.3)			0.61^{2}	(0.19)
HISTOPATHOLOGY		P%				P%				P%		
Epitheliocystis		–				–				15		
Inflammatory foci		25				20				–		
Vacuoles-like structures		80^{1}				70^{12}				45^{2}		
Melanomacrophage centers (MMCs)		80^{1}				76^{12}				47^{2}		
	nMMC	(SD)	MA.MMC	(SD)	nMMC	(SD)	MA.MMC	(SD)	nMMC	(SD)	MA.MMC	(SD)
	1.57^{1}	(1.71)	1865.04	(1663.53)	0.92^{1}	(0.80)	1496.04	(1284.66)	0.39^{2}	(0.60)	1206.07	(1761.04)

small granulomas or cysts in stomach and intestinal walls, pancreatic tissue, and the liver, and contained whole or degraded forms of the parasites.

3.1.4. Parasitological load

Every fish individual assessed ($n = 90$) showed at least one parasite. Globally, a total of 1360 parasites belonging to at least ten different taxa were identified: four digeneans (*Aphanurus virgula*, *Lecithaster* spp., unidentified metacercariae and an unidentified juvenile digenea), three nematodes (*Hysterothylacium aduncum*, *H. fabri* and unidentified encysted nematodes), two monogeneans (one unidentified Monopystochotylea and the Poliopystochotylea *Pseudacanthocotyloides heterocotyle*) and Tetraphyllidea fam. Gen. sp. larvae (Table 2). None of the unidentified nematodes matched the morphological characteristics of an Anisakidae species.

Regarding differences across locations, Brillouin Diversity index showed significantly higher values of parasites in fish sampled in Barcelona and Blanes when compared to values from Tarragona ($t = 2.897$, $p < 0.005$) (Table 2). Parasite Richness showed significantly higher values in Barcelona than other locations (Barcelona–Tarragona $t = 3.345$, $p < 0.005$; Barcelona–Blanes $t = 2.960$, $p < 0.005$) (Table 2). Nematodes were significantly more abundant in Barcelona ($z = -3.007$, $p < 0.005$) as compared to the other two locations, whereas digeneans and cestodes where higher only when compared to Tarragona ($z = -2.109$, $p < 0.05$ and $z = -2.062$, $p < 0.05$, respectively). Monogeneans were significantly less abundant in fish from Blanes (Blanes–Barcelona, $z = 2.380$, $p < 0.05$; Blanes–Tarragona z-value: 2.973, $p < 0.01$). Regarding the prevalence of parasite taxa, cestodes showed lower values in Tarragona compared to Barcelona, (Barcelona–Tarragona $z = -2.146$, $p < 0.05$), while Blanes showed significantly lower values for monogeneans compared to other locations ((Barcelona–Blanes, $z = -2.882$, $p < 0.005$; Tarragona–Blanes, $z = 2.243$ $p < 0.05$) (Table 2).

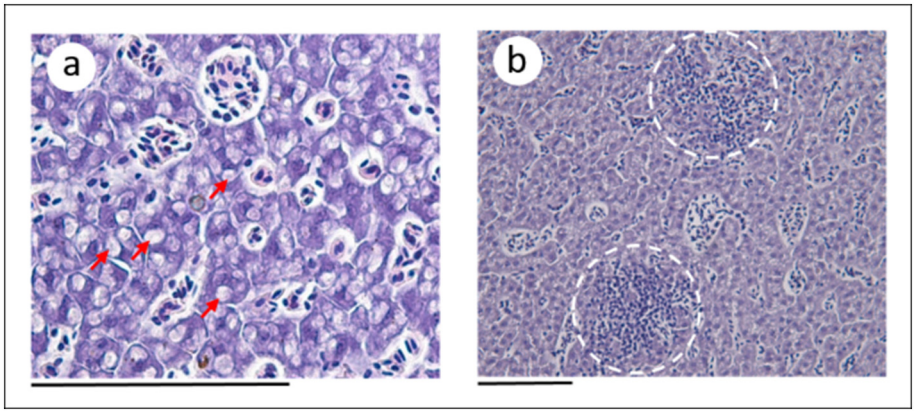

Fig. 3. Optical microscope images of the main histological alterations found in livers of European anchovy. Showing a) vacuole-like structures (indicated by red arrows) in the cytoplasm of hepatocytes, and b) inflammatory foci formed by patches of immune cells (surrounded by dashed white circles). Scale bars at the bottom left of each picture represent 0.1 mm.

Table 3
Prevalence (%) of fish ingesting anthropogenic items. Mean and standard deviation (SD) of mean intensity (number of items per fish ingesting anthropogenic items), mean abundance of anthropogenic Items (nAI), total plastic items, number of particles (n Particles), number of fibres (n Fibres), number of plastic fibres (n Plastic Fibres), number of cellulosic fibres (n Cellulose fibres, total length of anthropogenic items (TLAI) and percentage of stomach occupation by anthropogenic items (proportion of stomach occupation). Significant differences between years (Barcelona 2007–2019) are represented by superscript letters (a and b), while the absence of superscript letter means no significant differences. Differences among localities (Barcelona–Blanes–Tarragona in 2019), are expressed by superscript numbers (1 and 2), while the same superscript number or their absence means no significant differences.

Locality	Barcelona				Blanes		Tarragona	
Year	2007		2019		2019		2019	
ANTHROPOGENIC ITEMS								
Prevalence (%)	40^{a}		70^{b1}		53^{12}		33^{2}	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Mean Intensity	1.58	(0.67)	2.71	(1.76)	2.00	(1.41)	2.00	(1.33)
nAI	0.63^{a}	(0.89)	1.9^{b1}	(1.94)	1.07^{12}	(1.44)	0.67^{2}	(1.21)
Total plastic	0.43^{a}	(0.63)	1.23^{b1}	(1.68)	0.37^{2}	(0.72)	0.40^{2}	(0.81)
n Particles	0.23^{a}	(0.50)	1.17^{b1}	(1.68)	0.33^{2}	(0.66)	0.23^{2}	(0.68)
n Fibres	0.40	(0.72)	0.73	(1.17)	0.73	(1.28)	0.43	(0.73)
n Plastic fibres	0.20	(0.48)	0.07	(0.25)	0.03	(0.18)	0.17	(0.46)
n Cellulose fibres	0.20^{a}	(0.48)	0.67^{b}	(1.09)	0.70	(1.24)	0.27	(0.52)
TLAI	2.16	(5.73)	4.20^{1}	(3.07)	1.76^{2}	(2.53)	2.37^{2}	(3.89)
Proportion (%) of stomach occupation	0.31^{a}	(0.88)	1.05^{b1}	(1.66)	0.18^{2}	(0.53)	0.43^{2}	(1.18)

3.2. Ingestion of anthropogenic items by European anchovy and polymer characterisation

Half of the anchovies analysed (59 of 120; total prevalence 49.17 %) contained AIs in their digestive tract (Table 3), with a total mean intensity of ingested AIs of 2.17 (SD = 1.46). A total of 128 AIs were found in the digestive tract of analysed fish, 69 were fibres (53.4 %), including cellulosic and plastic fibres, and 59 (46.6 %) were particles, englobing films, and fragments (Fig. 4a). Most of the items (102) were found in the stomach, while nine were found in the pyloric caeca, and 17 in the intestine. Particles were located mainly in the stomach (55) and only four small particles were found in the intestine. Only fibres were found in the pyloric caeca. The proportion of the stomach occupied by AIs ranged from 0.004 to 4.95 % (Table 3).

The size of 95 % of the items found ranged between 0.25 mm to 4.82 mm, with a mean size of 1.99 mm (SD = 1.21); this fits within the definition of microplastic (Frias and Nash, 2019). Moreover, six items larger than 5 mm were also found, ranging from 5.23 mm to 6.88 mm, with a mean size of 6.31 mm (SD = 0.63) and one exceptional item of 13.82 mm. Due to their plastic basis, the items bigger than 5 mm were also considered in subsequent analyses. The TLAI was 2.37 mm/ind. (SD = 3.89). From the 59 particles (46.1 % of total AIs), 40 items (31.3 %) were fragments and 19 (14.8 %)

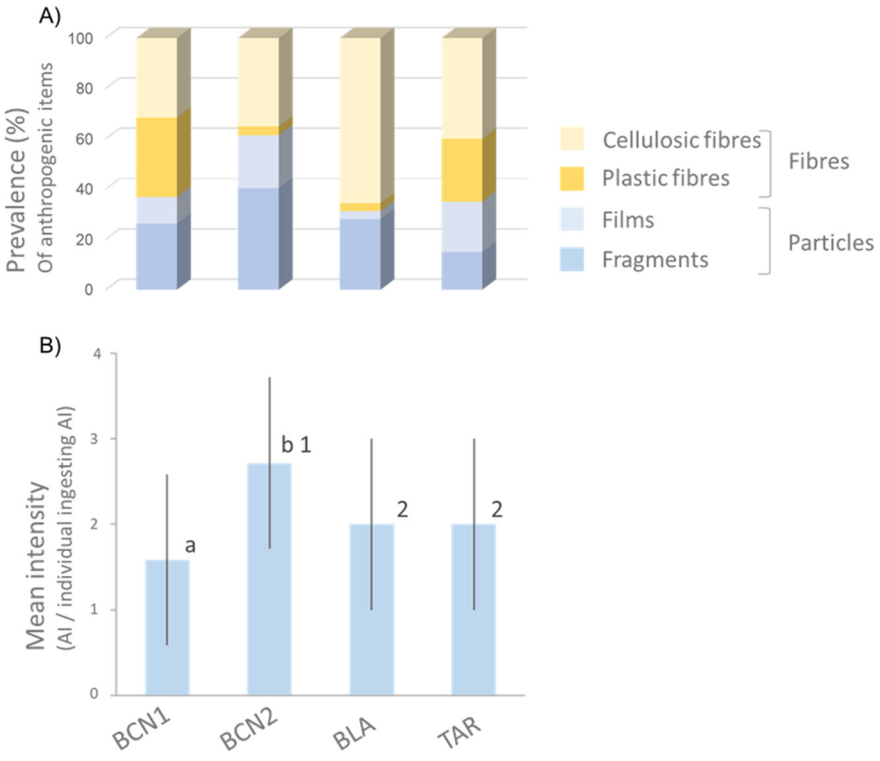

Fig. 4. Values of ingestion of anthropogenic items (AIs) by European anchovy showing A) Prevalence as a percentage of the AIs ingested by typology (fibres and particles), composition of fibres (plastic or cellulosic) in the sampling stations. B) mean intensity (number of AIs/number of fish ingesting AIs). Data are shown for Barcelona in 2007 (BCN1) and 2019 (BCN2), and in Blanes and Tarragona in 2019 (BLA and TAR, respectively). Different letters and numbers show significant differences between years (Barcelona 2007–2019) and localities (Barcelona–Blanes–Tarragona in 2019), respectively ($p < 0.05$).

were films. Moreover, 69 items (53.9 %) were fibres, 14 of these (10.9 %) were plastic-like fibres, while 55 (42.97 %) were cellulose-like fibres.

All fibres visually classified as plastic were correctly identified as synthetic polymers by micro-FTIR, while all those classified as cellulosic were identified as cellulose — which corresponded to 36.6 % of all polymers identified. Synthetic polymer identification showed polyethylene (38 %) as the most abundant polymer, followed by polyethylene terephthalate (11.3 %), polypropylene (8.5 %), polyamide (2.8 %), and acrylic (1.4 %). Polyethylene, polypropylene, and polyamide were identified only in particles, while polyethylene terephthalate and acrylic were in fibres.

When comparing the three sampled stations in 2019, Barcelona showed the higher prevalence ($z = -2.77$, $p < 0.005$) of fish ingesting AIs, and AIs were also more abundant ($z = -2.798$, $p < 0.05$); this was followed by Blanes and Tarragona, although significant differences were only found between Barcelona and Tarragona (Fig. 1, Table 3). Barcelona was also the location with a significantly higher abundance of particles (Blanes–Barcelona, $z = -2.657$, $p < 0.05$; Tarragona–Barcelona, $z = -2.832$, $p = 0.005$). Total length of AIs was higher in Barcelona (TLAI, Barcelona–Blanes, $t = 2.087$, $p < 0.05$; Barcelona–Tarragona, $t = 2.515$, $p < 0.05$), as was the proportion of stomach occupation by AIs (Barcelona–Blanes $t = 2.818$, $p < 0.005$; Tarragona–Barcelona, $t = -2.551$, $p < 0.05$). None of these differences were related to fish size or to stomach fullness. Mean intensity was also higher in Barcelona compared to the other two locations, but with no significant trend ($p > 0.05$) (Fig. 4b, Table 3).

3.3. Differences in anthropogenic items ingestion over a 12-year gap

When comparing the AIs ingestion in Barcelona between two distinct sampling occasions separated by 12 years, a significant increase was detected in AIs prevalence (2007: 40 %; 2019: 70 %) ($z = 2.296$, $p < 0.05$) (Fig. 1). Mean intensity and TLAI also showed higher values in 2019 than in 2007, but this difference was not significant (Fig. 4b, Table 3).

The abundance of AIs, and the proportion of stomach occupation by AIs were significantly higher in 2019 as compared to 2007 (nAI, $z = 2.473$, $p \leq 0.05$; $z = 2.337$, $p < 0.05$; respectively) (Table 3). Fibre abundances were not significantly different between years, while particles were more abundant in 2019 ($z = 2.515$, $p \leq 0.05$). Likewise, the percentage of particles from the total AIs was higher in 2019 (61.5 % respect to 37 % in 2007). If only particles were considered, fragments were the most abundant shape in both years. None of these differences were related to SL or stomach fullness. Regarding polymer identification, considering only particles, polyethylene was the most abundant polymer in both years. However, with fibres, polyethylene terephthalate was the most abundant polymer in 2007, whereas cellulose fibres were more abundant in 2019 ($z = 2.587$, $p < 0.005$).

3.4. Relationship between health status and potential stressors

MFA for the different locations explained 30.32 % of variability with the first two axes (Fig. 5). The quantitative variables that explained the variability in the first dimension concerned mainly anthropogenic items (AIs abundance, TLAI, and percentage of stomach occupation by AIs). Regarding the second dimension, abundance of parasites of the digestive tract — which were slightly associated with fish size — explained most of the variability, followed by enzymes (Fig. 5a).

A slight separation of data according to the sampling location was observed (Fig. 5b). In general, individuals of each locality were similar to each other, but some individuals were more differentiated in Barcelona. Dispersion along the first dimension means fish from Barcelona had higher values of descriptors of anthropogenic items (AIs abundance, AIs length and proportion of stomach occupation by AIs); dispersion along the second-dimension indicates bigger fish and higher parasite abundances. The correlation matrix showed that fish size was not correlated with parasite descriptors: parasite Richness (rho = 0.44, $p < 0.01$) and Brillouin Diversity Index (rho = 0.30, $p < 0.01$), nor with the abundance of the different taxonomic groups of parasites identified in the digestive tract, such as digeneans (rho = 0.32, $p < 0.01$); cestodes (rho = 0.27, $p < 0.05$); nematodes (rho = 0.34, $p < 0.01$) or with the total abundance of parasites in the digestive tract (rho = 0.35, $p < 0.01$).

No significant relationships were found among parasite descriptors and enzymatic biomarkers or histological alterations (Fig. S1). Likewise, no significant relationships were found between AIs and response variables, including health status descriptors, condition indices, enzymatic biomarkers, adipocyte diameters, total parasite abundance, abundance of parasites in the digestive tract, or histological alterations. Moreover, when parasites were considered as variables in models testing for their potential impact on fish health, no significant relationship with the response variables was found.

3.5. Temporal comparison

When considering data obtained of fish sampled in Barcelona in 2007 and 2019, the MFA performed explained 60.92 % of the variability (Fig. 6a

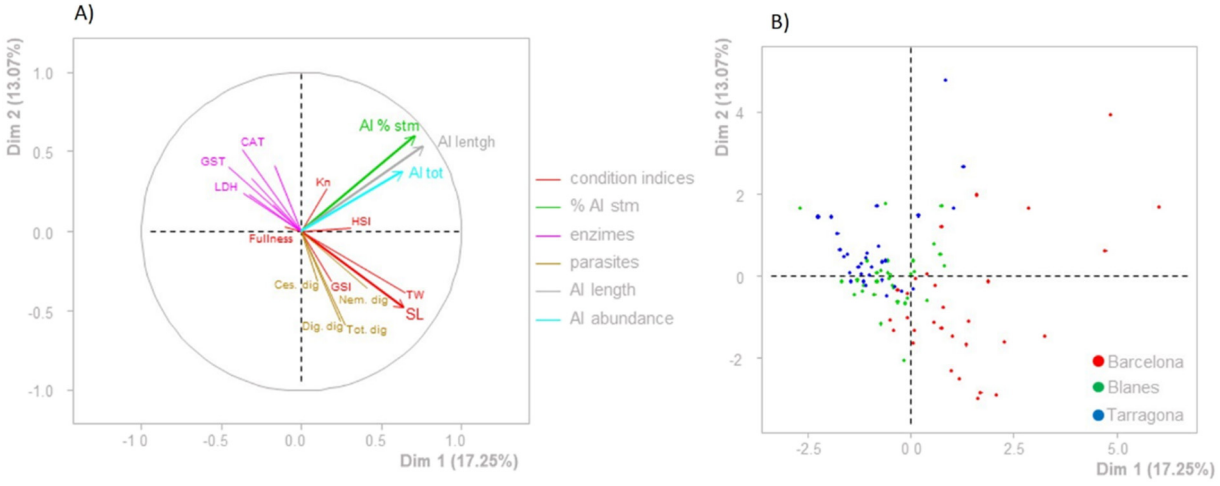

Fig. 5. Multiple factor analysis (MFA) among fish from locations in 2019. (A) MFA among body condition indices (standard length (SL); total weight (TW); Le Cren relative condition index (Kn); stomach fullness (Fullness); hepatosomatic index (HSI); and gonadosomatic index (GSI)), abundance of anthropogenic items (AI abundance, (total anthropogenic items (AI tot)), length of anthropogenic items (AI length), percentage of stomach occupation by anthropogenic items (% AI stm), enzymes (catalase (CAT), glutathione-S-transferase (GST), lactate dehydrogenase (LDH)) and parasites (cestodes in the digestive tract (Ces. dig), digeneans in the digestive tract (Dig. dig), nematodes in the digestive tract (Nem. dig) and total parasites in the digestive tract (Tot. dig)). (B) Factor map of the MFA, individuals are represented by dots and locations by colours.

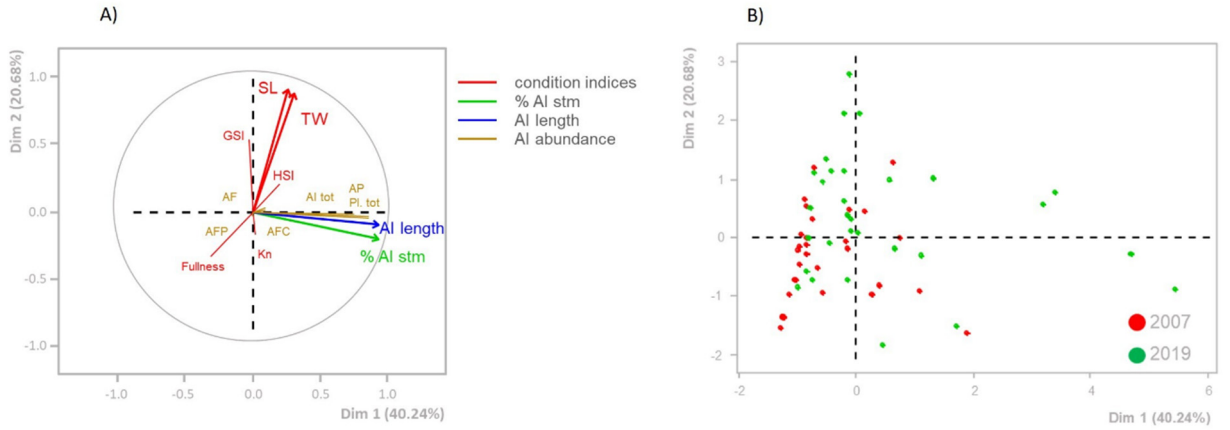

Fig. 6. Multiple factor analysis among fish from Barcelona between a 12-year interval (2007 vs 2019). (A) Multiple factor analysis between body condition indices (standard length (SL); total weight (TW); Le Cren relative condition index (Kn); stomach fullness (Fullness); hepatosomatic index (HSI); gonadosomatic index (GSI) and stomach fullness (Fullness)), abundance of anthropogenic items (AI abundance, total anthropogenic items (AI tot), anthropogenic particles (AI), anthropogenic fibres (AF), cellulosic anthropogenic fibres (AFC), plastic anthropogenic fibres (AFP), total plastic items (PL. tot)), length of anthropogenic items (AI length) and percentage of stomach occupation by anthropogenic items (proportion (%) AI stm). (B) Factor map of the MFA, individuals are represented by dots and years by colours.

and b). AIs length and the proportion of stomach occupation by AIs were the variables that explained most of the variability in the first dimension, while SL and TW were the variables with a higher contribution in the second dimension (Fig. 6a). Between years (Fig. 6b), all individuals were similar except some individuals in 2019 that were dispersed along the first dimension, meaning higher values of anthropogenic items (TLAI and proportion of stomach occupation).

4. Discussion

4.1. Current body condition and health status of European anchovy

The results of this study indicate that the European anchovy populations from the NW Mediterranean Sea are not affected by relevant pathologies. Moreover, the absence of zoonotic parasites in the analysed fish strongly suggests a low prevalence of these kind of parasites in the studied area, when compared with fish stocks in other geographical locations. However, substantial declines in the stock size, mean body size and/or condition have been observed in the NW Mediterranean Sea (Albo-Puigserver et al., 2019; Brosset et al., 2017, Brosset et al., 2016, Brosset et al., 2015; Ferrer-Maza et al., 2016; Saraux et al., 2019; Van Beveren et al., 2014) resulting in profound changes in the structure of the stocks and a major decline in landings and fishing activity (Brosset et al., 2017; Coll and Bellido, 2020; Saraux et al., 2019). Studies using condition indices, lipid content, and the fatty-acid profile of the muscle of European anchovy have revealed that the health status of this species is impaired due to malnourishment (Biton-Porsmoguer et al., 2020). This situation seems to be linked to changes in plankton abundance and the community composition over recent decades (Zarubin et al., 2014).

Differences in condition indices (HSI, GSI and Kn) among localities analysed by Multi-factorial analysis (MFA) and Spearman's correlation test clearly indicate that these are not related to any of the potential stressors analysed (parasites or AIs); and the variability observed may be due to the sampling design used to ensure sample size uniformity. The observed changes in body condition are probably driven by physiological conditions related to the reproductive stage, as well as environmental-related changes such as season, environmental fluctuations, or food availability (Brosset et al., 2015). Specimens of the present study were sampled during their reproductive period. This species is known to display daily spawning synchronicity (Basilone et al., 2015) which can affect daily variability in the population, such differences are evident in the somatic and reproductive states (Basilone et al., 2006; García Lafuente et al., 2002). Moreover, several authors have highlighted how small pelagic populations are able to spawn in a preferred geographical area and within a range of environmental conditions (Bakun, 1997; Motos et al., 1996). Among these variables, depth appears to play a key role, together with oceanic features and food availability (Giannoulaki et al., 2013; Motos et al., 1996; Somarakis et al., 2006).

Previous studies revealed that sardines (*Sardina pilchardus*) with more anthropogenic particles ingested had the lowest body condition in the Mediterranean Sea (Compa et al., 2018). We find no clear evidence of that in our study, in keeping with other work on anchovies in the Balearic Sea or the Gulf of Lion (Compa et al., 2018; Lefebvre et al., 2019); though in the Ligurian Sea, a positive correlation between body condition and number of ingested fragments was found (Capone et al., 2020). Given the contradictory nature of these results, the potential negative effects of AIs on body condition of European anchovy in the wild remain unclear.

Results from the histological analysis of fat tissue indicate differences in adipocyte size clusters among localities, but these differences were not related to condition indices nor to enzymatic biomarkers. The size of adipocytes has been widely used for analysis of fat indices in humans or mammals (Salans et al., 1973) and changes in size of adipocytes (hypertrophy) of the mesenteric fat tissue have been described in relation to fat content and composition of diets in farmed fish (Cruz-Garcia et al., 2011; Landgraf et al., 2017; Oka et al., 2010). It is known that adipose tissue undergoes dynamic remodelling in short periods of time, in response to changes in the nutritional status in farmed fish (Navarro and Gutiérrez, 1995). Visceral fat in particular may respond to the mobilization of lipid storage during periods of physiologically high-energy demand that necessitate catabolism of body reserves — such as during reproduction — or when high levels of lipids are included in the diet (Company et al., 1999). In wild fish, it is much more difficult to assess how environment, diet, and fish physiology can affect lipid storage dynamics in fat tissue. Therefore in this case, only specific differences in food availability among different sampling areas could be used to explain these differences, although the high variability in fish condition found within a population prevents any further conclusions.

Although parasitism has been identified as a further factor affecting the body condition (Kn) of several fish species in the Mediterranean (e.g. Ferrer-Maza et al., 2016, Ferrer-Maza et al., 2015), our results do not show a negative relationship between parasite infestation and condition indices, or with other health indicators such as biomarkers, clearly indicating that the effect on these fish populations is negligible. These findings also support previous observations from the same study area (Biton-Porsmoguer et al.,

2020; Dallarés et al., 2014; Pérez-i-García et al., 2017; Rodríguez-Romeu et al., 2020).

The different values of enzymatic biomarkers among localities could not be clearly correlated with any potential stressors (AIs, parasites) nor with fish size (SL), which suggests that this variability could be linked to a high levels of variation among individuals (Antó et al., 2009). The usage of biomarkers related to oxidative stress (CAT, GST), detoxification of xenobiotics (CbE), neurotoxicity (AChE,) tissue damage (EROD), aerobic- (CS) and anaerobic- (LDH) metabolism have been widely employed as biomarkers in aquatic organisms to evaluate the effects of contaminants like microplastics (Prokić et al., 2019). However, since most of the patterns observed in biomarkers response are limited to experimental exposures under controlled conditions, it is extremely difficult to draw a parallel in the field, where conditions are highly variable and usually not adequately monitored. In addition, concentrations used in these experimental studies are usually very high, as the experimental approach is purely toxicological, so the levels of plastics used are unrealistically outside the range of concentrations typically reported in the natural environment (Burns and Boxall, 2018). In natural conditions, many factors may be influencing response of fish concurrently, making it difficult to find strong correlations unless fish populations had been exposed to severe stress conditions during a certain period (e.g. an oil spill; Penela-Arenaz et al., 2009).

In the present study, the differences in the number of splenic MMCs identified in fish among the different locations do not show correlations with size or with the presence of potential stressors. The assessment of MMCs in liver of European anchovies has been previously proposed as a biomarker of environmental pollutant exposure in the Tyrrhenian Sea (Basilone et al., 2018). The increase in size and number of MMCs has been related to various histopathological and inflammatory conditions (Manrique et al., 2014), as well as to pollutant exposure, environmental degradation (Carrassón et al., 2008; Carreras-Colom et al., 2022b), and parasitological infections (Carrassón et al., 2008; Pérez-i-García et al., 2017). However, it should be noted that in addition to the response to anthropogenic stressors, MMCs can also respond to natural variability such as species, age (Stentiford et al., 2003), sex (Fournie et al., 2001), spawning phase (Kumar et al., 2016), diseases, or even unspecific environmental factors such as seasonality (Carreras-Colom et al., 2022b). Therefore, before using it as an indicator in environmental monitoring programs, it is necessary to thoroughly characterize and evaluate the natural variability of MMCs in the target species. In this sense, the present work may be foundational for future studies of anchovy-based MMC assessment.

The absence of relevant histological alterations in the fish examined in this study is particularly interesting, as this confirms the evident lack of a clear impact from diseases on the health of the anchovies sampled. Only very mild alterations were observed, and all of them affected a very limited area of the tissues or were minor metabolic changes. Although inflammatory foci have been described as a possible response to a polluted ecosystem (Bernet et al., 1999; Feist et al., 2004), in the present study, they are found with very low prevalence and intensity; this is similar to the observations of other wild fish in the same area, which also found no relationship between these foci and AIs or parasites (Rodríguez-Romeu et al., 2020). These changes are part of the natural variability of populations, and can be due to factors related to the biology or normal development of the species. The high percentage of fish with vacuole-like structures in the liver is noteworthy, but unfortunately their nature and cause are still unknown. The negative PAS and Sudan stains indicate that they are neither composed of lipids nor glycogen. However, as many lipids are usually removed during histological processing, the vacuole-like structures may correspond in fact, to lipidic droplets. As the liver plays a key role in lipid metabolism in many fish species, these changes in the hepatic parenchyma structure may be related to changes in the nutritional status of fish. For instance, potential transient accumulations of lipids that is associated with lipid absorption, or lipid mobilization in certain physiological conditions such as vitellogenesis. Thus, further studies on this aspect are needed to understand the significance of these changes.

4.2. Parasitological load

Our results indicate that the parasite community of European anchovies along the Catalan coast has a very low prevalence of zoonotic parasites, far smaller than in other Mediterranean areas and the Atlantic waters surrounding the Iberian Peninsula. Moreover, since all endoparasites found were located within the digestive tract, mesenteric tissue, or visceral cavity, and no parasites were found in the flesh of this species, these findings indicate little risk to humans and thus give an added value for the fishing and commercialization of anchovies from the Catalan coast. The parasite fauna of the European anchovy in the Mediterranean and Atlantic waters has been extensively described, particularly regarding nematode taxa, but also digeneans to some extent (Dessier et al., 2016). *Aphanurus virgula* — in addition to affecting anchovies — is considered a main parasite of *Boops boops*, but is also described in other sparids such as *Pagellus erytrhinus* or clupeids such as *Sardina pilchardus* (Kostadinova et al., 2004). *Lecithasater confusus* has been previously described in anchovies (Dessier et al., 2016), but our genetic and morphological analyses on unidentified *Lecithaster* spp. did not confirm the species, and indicates therefore a possible new species for the genus. Regardless, both digeneans found in our samples were adults, which indicates that they were in their definitive host and should not have a zoonotic potential. Regarding the nematodes species observed — *Hysterothylacium aduncum* and *H. fabri* — these have been cited in a wide number of fish species. The *Hysterothylacium* genera has been reported only once as non-invasive anisakidosis (Yagi et al., 1996) and may have been involved in some cases of food allergies (Valero et al., 2003). Contrary to other species such as *Anisakis* spp., *Hysterothylacium* species are not recognized as truly pathogenic for humans, possibly because their final host are not mammals but fish (Cipriani et al., 2018) — which do not have the ability to thermoregulate and maintain high body temperatures — and therefore have body temperatures that depend on the environment and are usually lower. Furthermore, considering that *Hysterothylacium* larvae does not migrate into the flesh of the fish host, nematodes of this genera cannot be considered as a concern for food safety (Levsen and Karl, 2014). No nematodes belonging to the *Anisakis* genera were found in our study, despite being previously described in the studied area — although in low numbers (Ferrer-Maza et al., 2016; Biton-Porsmoguer et al., 2020). The higher presence of *Anisakis* spp. in anchovies from the East Atlantic Ocean as compared to the West Mediterranean Sea (Rello et al., 2009) is well-known, as happens in other pelagic (Molina-Fernández et al., 2015) and demersal fish species (Gómez-Mateos et al., 2016, and references therein). Regarding the Mediterranean Sea, higher values of *Anisakis* spp. have been found in the Ligurian Sea compared to southern areas (Rello et al., 2009), and Roca-Geronès et al. (2020) reported a higher prevalence of *A. simplex* in the Adriatic Sea when comparing it with the Western Mediterranean. On the Catalan Coast, the absence of *Anisakis* spp. herein is in agreement with the low prevalence reported by Ferrer-Maza et al. (2016) in anchovies sampled off the northernmost coast of Catalonia, and Biton-Porsmoguer et al. (2020) reported a prevalence of 23 % infection rate of the fish studied. Results by Cipriani et al. (2018) on anchovies parasitized by *A. pegreffi* are also in concordance with our results, since this parasite was not reported in the Mediterranean Spanish coast (Alboran and Balearic Seas), while a prevalence of over 70 % was observed in the Adriatic Sea.

4.3. Anthropogenic items (AIs)

Concerning the detection of AIs in the sampled anchovy populations, 50 % of the analysed fish presented AIs — including particles and fibres — in their digestive tract. Despite the high variability observed between studies, our results are in the average range of values reported by similar analyses of anchovies in the Mediterranean Sea. This variability is noteworthy in the prevalence of ingestion, with values of >90 % in the Adriatic Sea or the Gulf of Lion, while in other areas — such as the Ligurian Sea — the prevalence is lower than 45 % (Capone et al., 2020; Lefebvre et al., 2019; Misic et al., 2022; Renzi et al., 2019). This wide range of reported values is found not only between different Mediterranean areas, but also in the

same geographical area as in the present study, where prevalence ranged from 6.6 to 60 % (Compa et al., 2018; Pennino et al., 2020). Moreover, our average ingestion values (1.07 AIs/ind.; SD = 1.49) are slightly higher than those obtained in previous studies, in which the average values reported are usually <1 item/ind. Those differences could be due to variation in environmental concentrations, but also to the different extraction and identification methodologies used, such as digestion and filtration, visual selection or AIs size selection; this unfortunately hinders the comparison of results (Simon-Sánchez et al., 2022). When considering differences among our sampled locations, the prevalence and mean intensity of plastic ingestion are higher in Barcelona, due to the higher prevalence of particles, which are also larger, with no differences in the number of ingested fibres among locations.

Particles found in our anchovies are made of PP and PE — polymers that abound on the surface due to their low density — which allows for a naturally buoyancy and makes them more available for organisms inhabiting the water column (like this pelagic fish species). The sinking process is determined by the weathering and biofouling, this allows AIs to sink and be available to sub-surface waters and organisms inhabiting deeper habitats (Andrady, 2011). Fibres of cellulose or PET are expected to have a faster sinking rate due to their higher density, reaching the bottom faster. Once there, they may be retained in the sediments where they have been seen dominating the polymer composition of the fibres present (Woodall et al., 2014), and are available to the benthonic organisms inhabiting these environments (Carreras-Colom et al., 2022a, Carreras-Colom et al., 2018; Rodríguez-Romeu et al., 2020).

We found that the stomach is the organ with the greatest accumulation of AIs, particularly of larger fragments that were only observed in this location; these findings are similar to those of Capone et al. (2020). Conversely, fibres were found throughout the entire digestive system, including the pyloric caeca. However, the greatest amount of AIs found in the stomach still does not represent >1 % occupation of this organ, suggesting that particles do not accumulate and are eventually egested (Grigorakis et al., 2017; Roch et al., 2021). The presence of microplastics in tissues is a controversial topic (Burns and Boxall, 2018). Microplastics are measured using extraction techniques that are based on organ disintegration such as powdering or digestion (Avio et al., 2015b). However, these techniques are prone to influence from airborne contamination during processing and do not allow the observation or assessment of the exact location of AIs in the tissues, and the possible interaction and effects. The presence of anthropogenic particles (323 μm) in livers of anchovies captured in the Mediterranean Sea was described by Collard et al. (2017). In this paper, authors argued that due to the technique used (cryosections of tissue and observation in a polarized light microscope), it was not possible to precisely locate microplastics within the tissue. Recent studies in intestinal cell cultures have demonstrated that nano-sized plastic particles (beads of 50 nm of diameter) can be internalized by intestinal human cells, but without any evident detrimental effect in the cells (Domenech et al., 2020). This is not an unexpected finding due to cells having mechanisms — such as pinocytosis — by which they can intake substances into their cytoplasm through vesicles of 100 to 200 nm in diameter (Guyton and Hall, 2001). However, for larger-sized particles, as is the case for the identified particles from the anchovies' digestive tracts, the mechanisms of absorption, transport, and distribution into the blood stream are much more difficult and thus likely happen more rarely. For these items, entry into the body and subsequent presence within tissues would be dealt with far more aggressively — as with any foreign bodies that generally cause tissue damage — and an associated inflammatory response would follow. In our study, no such signs were observed.

Some authors have hypothesized that parasite aggregations within the digestive tract could retain microplastics and cause their aggregation or accumulation. In the present study, no relationship between AIs abundance (including plastic or non-plastic items) and total parasite infestation was found. Not even when considering only parasites found in the digestive tract or when considering only parasites found in the stomach. Our results contrast with the results obtained by Pennino et al. (2020) in the same area one year previous, where a positive relationship between the abundance of parasites and the number of microplastics in stomachs of European anchovy was found. These authors suggested that the individuals distributed in more polluted areas — and thus feeding on more microplastics — also had a higher probability of being infected by parasites. However, previous studies using a similar approach in red mullet fish (*Mullus barbatus*) did not find a relationship between AIs/microplastics and parasite infestation (Rodríguez-Romeu et al., 2020). In large marine mammals, such as grey seals (*Halichoerus grypus*), the interacting aggregation of both parasites and microplastics (fragments and fibres) along the intestine was not conclusive, since the number of microplastics were not significantly related to parasites (Hernandez-Milian et al., 2019).

During the last decade, public discourse flagging fish as a significant route of exposure to plastics in humans has grown. However, considering the amounts of AIs found in fish (1.07 AIs/fish), and the fact that digestive tracts (containing the AIs) of fish are generally discarded before cooking, it should be stressed that fish consumption may be a far less significant route of exposure than previously thought. Moreover, the amounts of AIs ingested from consuming fish would be much lower than the amounts potentially ingested from other sources; Catarino et al. (2018) calculated that, within a year, humans ingest 13,700–68,400 microplastics from household dust, while ingesting a mean of 123 microplastics/year via mussels (equivalent to 190 AIs/year via fish). Furthermore, these values are also far lower than those found in bottled water (10–1000 micropastics/L).

4.3.1. Changes in AIs levels over a 12-year interval

The percentage of fish ingesting AIs in Barcelona is higher in 2019 than 2007, which could indicate an increasing trend during years. Not only is prevalence higher, but also the number and size of AIs also increased. The presence of AIs in the gastrointestinal tracts of the studied fish captured in 2007 demonstrates that ingestion of this type of debris has been occurring for at least twelve years. This trend was also described in previous works in the same area, both in fish and crustacean species (Carreras-Colom et al., 2018; Rodríguez-Romeu et al., 2020). Moreover, changes in the proportion of fibres and particles, and their polymer composition, could be revealing a change in this type of pollution. This could be due to various factors, especially since the study area is very close to a highly populated area (Andrady, 2011; Derraik, 2002; Jambeck et al., 2015), hence the characteristics of ingested AIs likely reflects changes in the debris accumulated in the marine environment over the last decade (Avio et al., 2015a). However, those changes could also be the result of oscillations of climatic and meteorological factors (Misic et al., 2022), among others, occurring in the same area at different times and resulting in a non-predictable increase in anthropogenic fibres. For instance, Carreras-Colom et al. (2020) reported a thirty-fold increase in the values of fibre ingestion in shrimp over a three-month period, inferring that this could be the result of increased arrival of terrestrial pollutants into the sea due to increased rainfall events. Therefore, it is not possible to clearly establish an upward trend over the years based on our data alone, and further investigations should be performed to establish a clear temporal trend.

5. Conclusions

Despite clear changes in anchovy populations observed during the last decade — including a steady decline in their abundance and size — our work indicates that in general terms, the fish studied do not show relevant pathologies that directly affect their health status. Moreover, the negligible presence of zoonotic parasites in the studied area confirms this species as suitable and safe for human consumption, thus giving added commercial value to anchovies caught in this Mediterranean area.

As with many other species, anchovies ingest anthropogenic plastic particles, as well as plastic- and cellulosic-fibres. This is not a new phenomenon; ingestion of plastics was also observed in individuals captured in 2007. The ingestion of this kind of debris appears to show geographical trends, with individuals from Barcelona showing higher levels of AIs when compared to other locations along the coast. Although several studies have

highlighted the potential negative effects of these kind of pollutants, this study demonstrates that no signs of health impacts were associated with AIs ingestion in wild fish at the present level. In conclusion, the problematic population dynamics of this species do not seem to be due to pathological reasons, but are instead likely multifactorial and may be linked to wider changes in anchovy community structure. In a broader sense, our results reinforce the utility of the European anchovy as an epipelagic species suitable for the monitoring of this type of marine pollutant, both spatially and temporally. This species could therefore be an important addition to future studies and monitoring programmes that aim to include fish with varied feeding behaviours and habitats.

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CRediT authorship contribution statement

Oriol Rodríguez-Romeu: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Anna Soler-Membrives:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision. **Francesc Padrós:** Formal analysis, Writing - review & editing. **Sara Dallarés:** Methodology, Formal analysis, Writing - review & editing. **Ester Carreras-Colom:** Methodology, Formal analysis, Writing - review & editing. **Maite Carrassón:** Writing - review & editing, Funding acquisition. **Maria Constenla:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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