Universitat Autònoma de Barcelona

Fishing among plastics: Micro-litter ingestion in demersal and pelagic fish species from the NW Mediterranean Sea and its potential impact on health condition

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A dissertation submitted by Oriol Rodríguez-Romeu in fulfilment of the requirements for the degree of Doctor of Philosophy granted by the International Doctorate in Aquaculture in September 2022.

Supervisor and tutor

Maria Constenla Matalobos

Universitat Autònoma de Barcelona

Supervisor

Anna Soler-Membrives

Universitat Autònoma de Barcelona

PhD candidate

Oriol Rodríguez-Romeu

This study was supported by:

"BIOMARE" project (CTM2006- 13508- C02- 01MAR) and the Complementary Action (CTM2006- 28145- E/MAR), funded by the Spanish Ministry of Science and Technology.

"SOMPESCA" project (ARP059/19/00003), funded by (European Maritime and Fisheries Fund (EMFF) and supported by the Catalan Department of Agriculture, Livestock, Fisheries and Food with the project

"PLASMAR" project (RTI2018-094806-B- 100), funded by the Spanish Ministry of Science, Innovation and Universities.

Oriol Rodríguez-Romeu benefits from a "Personal Investigador en Formació" (PIF) grant funded by Universitat Autònoma deBarcelona (UAB)

A la meva família

Croyez ceux qui cherchent la vérité,

doutez de ceux qui la trouvent.

[Creu en aquells que busquen la veritat,

dubta dels qui ja l'han trobat]

- André Gide –

Acknowledgements / Agraïments

A qualsevol que llegeixi aquesta tesi i que a més estigui llegint aquest apartat m'agradaria que sabés que la redacció del manuscrit de la tesi, en una nit qualsevol, va començar just per aquí, pels agraïments. Si no hagués començat per aquí, potser no hauria començat mai.

El camí que m'ha portat fins aquí no ha estat fàcil, després de viatjar per la recerca durant aquests anys he aprés moltes lliçons tant a nivell personal com professional i també he fet alguna troballa. Però haig de dir, sense cap dubte a equivocar-me, que la millor troballa que he fet han sigut les persones que m'han acompanyat durant aquesta etapa. Sols puc agrair de tot cor a totes les persones que han fet que aquesta tesi no naufragues i que finalment naveguem en aigües tranquil·les rumb a port.

En primer lloc, haig d'estar infinitament agraït a les meves directores de tesi, l'Anna i la Maria, de veritat no podria haver tingut unes de millors. Gràcies per guiar-me de forma magistral i incansable, per exigir-me esforç quan calia i sobretot per la vostra qualitat humana i la comprensió en els mals moments. Gràcies per fer-me sentir que en comptes de tenir dues *Jefas* he guanyat dues amigues.

Anna, gràcies per la teva infinita alegria i energia positiva, per la teva eterna paciència amb els meus excels infumables i per repassar l'estadística les vegades fes falta. Per la teva clarividència i per fer que quan jo ho veia negre al final fos (al menys) gris clar.

Maria, gràcies per estirar-me de les orelles quan calía o saber quan deixar-me al meu aire, per ser tant perspicaç i posar ordre quan sembla que tot se'n va en orris i alhora ser tant divertida. També per demostrar-me que voler es poder.

No puc deixar de donar les gràcies a la Maite, gràcies per confiar en mi i per donar-me la oportunitat d'emprendre una tesi i repescar-me cap a la recerca quan, segurament no ho sàpigues, estava a punt de tirar la tovallola.

Com no Ester, per a tu tinc un agraïment especial, perquè portem en això des del principi i no podria haver tingut una companya durant la tesi millor que tu. Gràcies, per tants bons moments, per escoltar les meves cabòries, per donar sempre bones idees i per estar sempre disposada a donar-me un cop de mà. Perquè simplement ets molt *crack* i perquè durant tots aquests anys has sigut una font constant d'inspiració i de motivació. Per cert!, dóna-li també les gràcies a la teva mare de part meva, per fer possible que tinguem unes campanes d'aïllament tant *pros* i que han aguantat tota la tesi.

Moltes gràcies a la resta de la família de Veterinària, perquè a aquestes altures per a mi ja sou més que simplement un equip. Sito, moltes gràcies per introduir-me en el món de la histologia i fer-me entrenar l'ull crític tant per a les mostres del microscopi com a nivell científic en general, per generar tantes bones idees i per donar sempre un punt de vista diferent i anar més enllà. També per tants bons moments repassant filmografia i bandes sonores mítiques i pel teu sentit de l'humor. A la Celia, per fer miracles amb el micròtom i aconseguir un tall perfecte encara que costés cent intents. Per tallar i preparar mostres histològiques com ningú, però també per tantes bones estones a l'hora de dinar. Sara, gràcies per estar sempre disposada a ajudar, pel teu compromís i per la gran tasca que has fet en la identificació de paràsits i en els anàlisis d'enzims. Encarna, gràcies pel teu particular optimisme i per aportar una mica de filosofia *Zen* als

moments d'estrès i bogeria. També, gràcies a la Laura i l'Andrea, que inicien el camí de la tesi amb la mateixa il·lusió amb la que vaig començar i han portat una glopada d'aire fresc durant aquest últim any de tesi, per haver pogut sortir-se'n amb els anàlisis químics i de passada enllestir algunes de les meves mostres que ja donava per perdudes. Finalment, també m'agradaria agrair els alumnes de pràctiques perquè sense la seva tasca avançar en la recerca seria impossible.

A en Francesc, l'Anna Garriga i l'Ana Filipa, per tantes bones estones a l'hora de dinar permetre un parèntesi i que no tot fos ciència i per acostar-se a fer una abraçada quan va fer falta. També a la resta de companys de la unitat, Fernando, Pilar, Òscar, Javier per donar resposta a tots els dubtes administratius, solucionar la logística de docència i agilitzar sempre tota la paperassa.

I would like to thank Claire Saraux to facilitate access to the IFREMER facilities and believe and help in the design of the experimental approach I proposed with sardines. Also special thanks to Quentin Schull who was the responsible researcher during my stay in Palavas-Les-Flots IFREMER station in France. Thanks for your confidence in me and, many thanks to be there all time to accomplish our objectives. Also thanks to the staff, Gilbert Dutto, Vincent Duchet, Emmanuel Roque and Lisanais Moreto for their invaluable help during my stay. It was some hard months but I felt happy there.

I would like also tank to the international evaluators of my thesis for their quick evaluation and for their positive feedback, I really apreciate it.

A tothom que d'alguna manera ha aportat el seu petit granet de sorra a aquesta tesi, o no tant petit, amb la identificació química de plàstics amb tècniques d'espectroscòpia. Mariano de l'Institut de Ciència de Materials de Barcelona, perquè sense la teva traça i el teu coneixement del RAMAN però sobretot per la teva paciència per aconseguir bons espectres el primer capítol de la tesi no existiria. També al Servei d'Anàlisi Química de la UAB i serveis cientificotècnics de la UB (CCitUB) per haver fet possible l'anàlisi de la resta de mostres.

A la gent de la mar, als pescadors de les diferents confraries (Blanes, Barcelona i Tarragona) per deixar-nos entrar a casa vostra i per ajudar-nos a aconseguir tot el material que calgués. Per donar-nos les vostres impressions, trametre les vostres inquietuds i preguntes i tractar-nos sempre tant bé.

Finalment, si miro enrere, no puc evitar recordar moltes persones de l'àmbit acadèmic que d'alguna manera tindrien una part de culpa de que jo sigui aquí. Malauradament no els puc anomenar tots, però no puc evitar agrair a les persones que vaig conèixer durant la meva estada com a estudiant a l'Institut de Ciències del Mar de Barcelona, especialment a en Joan Cartes que d'alguna manera va fer que definitivament em piqués el cuquet de la recerca.

Als meus amics Alfonso, Ángel, Carmen, Celia, Darío, Joana, Manuel, Natxo, Sergi B., Sergi G. i Víctor. Puntals i confidents, companys de fatigues i dolenteries, consellers i terapeutes, gràcies per ser-hi, per escoltar les meves malediccions, celebrar les meves fites i estar en els bons i mals moments quan ha calgut. Gràcies a tots i totes per apaivagar les meves preocupacions entre riures, muntar barbacoes sanadores, tardes de terrasses miraculoses i escapades de pesca terapèutiques.

Gràcies a la meva àvia i al meu avi, allà on estigui, tiets i tietes, *titas* i *titos*, perquè sempre n'esteu molt de mi i us alegreu per mi encara que no acabeu d'entendre això que faig,

Finalment, a la meva família perquè sense vosaltres no seria res i perquè passi el que passi, sempre he tingut el vostre suport i amor incondicional. Gràcies al meu pare, perquè d'alguna forma va ser el primer en veure que això d'empaitar bitxos i peixos *era lo mío* i tot i que li resultés estrany mai li va costar portar-me al camp, al riu o al mar quan li ho demanava. Avui hauríem pogut celebrar aquest triomf, i tot i que malauradament ja no podrà ser, només puc donar-te gràcies per tots els sacrificis que vas fer i que no et vaig poder agrair. A la meva mare, gràcies per ser sempre un puntal, per creure cegament en mi i per ensenyar-me que quan sembla que tot va en contra, sempre queden forces per tornar-se a aixecar i continuar endavant. Eloi, tot i que ets el petit, gràcies per fer-me de germà gran i servir-me d'exemple i cuidar-me quan m'ha fet falta.

Gràcies a TOTS i TOTES (disculpeu-me als que he oblidat mencionar) per recolzar-me en aquest viatge.

Oriol Rodríguez Romeu

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ABSTRACT

ABSTRACT:

The present thesis analyses the ingestion of micro-litter / anthropogenic items (AIs), including microplastics but also anthropogenic fibres (AFs) of different origin (synthetic and cellulose based), in the demersal red mullet (*Mullus barbatus*) and the foraging pelagic European anchovy (*Engraulis encrasicolus*) in different locations along the Catalan coast (Blanes, Barcelona and Tarragona). These commercial species are target species for monitoring this type of pollution in different habitats in the Mediterranean Sea.

We show how the ingestion of micro-litter occurs in both fish species in their natural environment, not only recently (in samples obtained from fishing vessels in 2018 and 2019, for red mullets and European anchovies respectively) but also 15 years ago (in samples obtained during 2007). AIs were present in 50% of the analysed digestive tracts in both fish species, with a general mean values of 1.48 AIs/ind (SD=1.98) for red mullets [mean intensity 2.95 (SD= 1.83)] and 1.07 (SD=1.50) for European anchovy [mean intensity 2.17 (SD=1.46)]. The percentage of fish ingesting AIs have been increased in 46% and 30% for red mullets and European anchovies, respectively, comparing individuals from Barcelona in recent years with those obtained during 2007. AIs were screened from digestive tract of fish and were morphological categorised by visual inspection. After their selection based on their morphological features, polymer identification was performed by using spectroscopic techniques (μ-RAMAN and FTIR and μ-FTIR). In both fish species, the most abundant shape were AFs, especially in red mullets in which fibres represent the 95 % of the total items. In this species, the fibres were mainly composed by cellulose (67%) and polyethylene terephthalate (31%), with a mean length of 2.50 mm (SD = 2.24). AFs in European anchovy (54 % of the total items found) were also composed by cellulose (42.97 %) and plastic (10.9 %), but particles (46 %) composed by fragments and films of synthetic polymers (such as polyethylene, polypropylene, and polyamide) were also found. Moreover, our results reveal geographical differences along the study area with higher levels of ingestion of AIs in fish collected in the surrounding area of Barcelona in both fish species.

Likewise, the potential effects of exposure to these pollutants have been analysed by evaluating the general health status of the target species using different indicators and at different levels, from more specific, such as enzyme biomarkers or histological alterations to a more general level, such as condition indices or parasitic infestation. Although some histological alterations have been found in both fish species, as well as differences in some bioindicators among the different locations, no relationship has been found between these and the presence and/or abundance of AIs.

On the other hand, in order to determine the importance of feeding behaviour and temperature in the ingestion of AFs and also its potential negative effects, specimens of European sardine (*Sardina pilchardus*) have been obtained from the natural environment and have been kept under laboratory conditions and exposed to this type of pollution. Our results demonstrated that when sardines were feeding by filtration (less selective feeding mode), they ingest more fibres (4.95 fibres/ind (SD=3.43)) compared to the number of fibres they ingest when feeding by catching food selectively (0.6 fibres/ind (SD=1.04)). Moreover, after ingestion, fibres travel throughout the digestive tract of sardines until their total egestion, with no evidence of retention or accumulation over time. The ingestion of these plastic fibres seems to not impoverish the body condition of sardines, but feeding behaviour by filtering. Finally, higher temperatures seem to affect the pattern of fibre expulsion, especially in filter-feeding sardines, thus, changes in the environment such us climate change might act as a synergistic factor and contribute negatively to the AIs ingestion.

1.INTRODUCTION

1. INTRODUCTION

During the last decades a significant decrease of biological diversity have been observed in worldwide ecosystems (Sax and Gaines, 2003). The cause of this deterioration is considered multifactorial, which means that it is associated with different factors, both natural and anthropogenic. The oceans are not exempt of these threats, which can occur in various ways, from more global to local levels, and include climate change, habitat loss by coastal and seabed alteration, overexploitation of natural resources, introduction of exotic species, pathogens or pollution (Sax and Gaines, 2003 and references therein). All of these factors can cause changes in the health of marine organisms, ultimately affecting the distribution and abundance of their populations (Wood et al., 2013). The progressive depletion of marine biological resources within coastal and continental shelf areas has led to increased interest in monitoring marine ecosystems (Danovaro et al., 2016; Melet et al., 2020; Zampoukas et al., 2014).

1.1. The Mediterranean Sea: From the cradle of western civilization to the decline of its natural resources.

The Mediterranean Sea, due to its geological history together with its climatic and hydrological variability, has been considered as one of the regions with richer ecological niches in the world, becoming a hotspot of biodiversity. Likewise, the exceptional natural conditions of the Mediterranean basin sustained and allowed the settlement and development of human activities throughout the history, playing a vital important role in the development of modern western civilization (Margalef, 1985). The exploitation of marine resources, especially fishing resources, is intrinsically linked to the history of the Mediterranean Sea, and has supported important fisheries over centuries that have contributed to the economic and social development of the region. Nowadays, fishing landings oscillate to around 800,000 tonnes annually, mostly concentrated in the western Mediterranean and Adriatic Sea, and with a total landing value estimated higher than 3 billion euros per year (FAO 2020). Small pelagics (mainly European anchovy *Engraulis encrasicolus* and European sardine *Sardina pilchardus*) and medium/small sized demersal fish (e.g., European hake *Merluccius merluccius*, red mullet *Mullus barbatus* or several Sparidae fish species) comprise the bulk of the total landings (FAO, 2018). However, after reporting a gradual increase in catches in the Mediterranean (peaked in mid-1990 at around 1.1 million tonnes) (FAO, 2020), landings have been followed by a continuous dramatic decline during the last decades (Pauly et al., 2014; Pauly and Zeller, 2016; Piroddi et

al., 2020). Even in some areas like the Black Sea, where especially pelagic fisheries suffer such great variations, the market has ceased to be profitable and has collapsed (FAO, 2020) (Fig. 1).

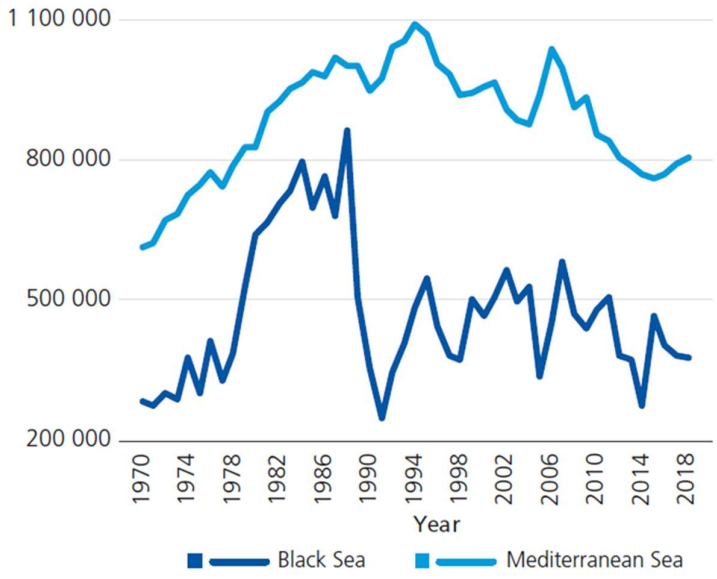

Fig. 1. Total landings in the Mediterranean and the Black Sea per year, 1970–2018. Source: The State of Mediterranean and Black Sea Fisheries 2020 (FAO 2021).

Natural variability may rule on food availability and environmental conditions, which will have a direct effect to natural marine populations and subsequently to fish landings. In addition, stressors from anthropogenic origin, such as fishing pressure, reduction of nutrient inputs due to the reduction of river discharges, alteration of habitats, eutrophication and increasing levels of pollutants, are also affecting these populations, placing them in a critical situation for sustainability. All these stressors are indeed a multifactorial-caused problem.

Mediterranean basin houses about 40% of the EU's population and approximately 10 % of world's coastal population, which means that receives water from densely populated areas resulting in a tremendous pressure of human activity from land to the sea. Moreover, it is a semi-closed basin with a low water renewal which promotes the accumulation of waste of anthropogenic origin (Durrieu de Madron et al., 2011). Therefore, the Mediterranean Sea, so-called the nest of biodiversity, is conversely also considered a hotspot of pollution (Costalago and Palomera, 2014). Among this pollution, marine litter, mostly composed of plastic, has been fervently considered as one of the types of pollution that has become widespread not only in

the Mediterranean but also all over the world, as it results from poor waste-prevention and management schemes, as well as improper behaviour by the consumer society.

1.2. Plastics and marine litter: An emerging problem

Waste product from human activities can potentially end up in the natural environment driving into possible negative effects. Technological development has great benefits; however, every technological leap is followed by the appearance of new potential / emerging pollutants that can lead to potentially negative consequences. In 1907, the chemical industry created the first completely synthetic polymeric material, and during the 20th century, the last great material developed in our technological history, plastics, were created. The emerging of this cheaper, easier to produce, and more accessible material was a completely revolution (Streit-bianchi et al., 2020). Plastics are key to the momentum of the industrial revolution, because of their remarkable utility and versatility. They are useful for an enormous variety of uses, such as building, industry (tools, machines, pipelines), textile, foodstuff (wrapping, water bottles) hygiene and pharmacy (cosmetics, medicine encapsulations, disposable sanitary material), among others (PEMRG, 2021) (Fig. 2). This material is currently essential and consequently its production and consumption has increased in recent decades. In 1990, the global production of plastic materials amounted to 100 million metric tons; this figure increased fourfold to almost 400 million metric tons in 2020 (Plastics Europe, 2021).

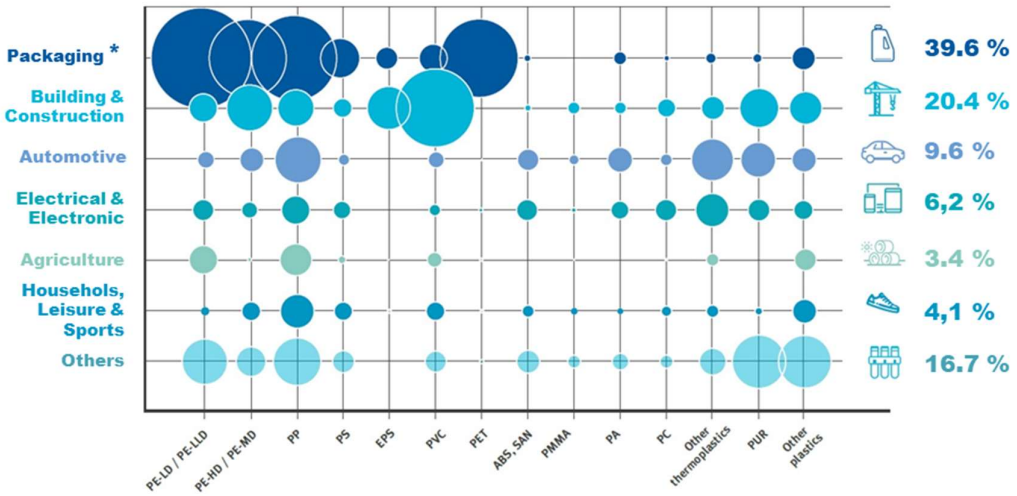

Fig. 2. Main usages and polymers of plastics. * Including commercial and industrial packaging. "Others" includes plastics for furniture, medical applications machinery and mechanical engineering, technical parts etc. (PE-HD: Polyethylene high density; PE-MD: Polyethylene, medium density; PE-LD: Polyethylene, low density PE-LLD: Polyethylene, linear low density; PP: Polypropylene: PS: Polystyrene; EPS: Expanded polystyrene; PVC; PET: Polyethylene terephthalate; ABS: Acrylonitrile butadiene styrene resin; ASA: Acrylonitrile styrene acrylate resin; PMMA: Polymethyl methacrylate; PA: Polyamides and PUR Polyurethane.) SOURCE: Plastics Europe Market Research Group (PEMRG) and Conversion Market & Strategy GmbH (2021).

However, the massive use of this material has also led to massive amounts of litter. Plastic litter generated on land is transported to the oceans as its final destination. Thus, the marine environment is considered the great sink for plastic pollution (Fig. 3). It is estimated that more than 150 million metric tons of plastics have accumulated in the world's oceans, while 4.6-12.7 million metric tons are added every year (J. R. Jambeck et al., 2015). According to recent estimations, the annual flow of plastic waste into the ocean could almost triple by 2040 to 29 million metric tons per year (UNEP, 2021). Nowadays, plastic litter is not only the most abundant anthropogenic waste, but also one of the most persistent in the environment. The properties that make this material interesting for our use are a disadvantage when they reach the marine environment. Composed by practically inert material, their chemical decomposition is practically negligible in natural conditions (Worm et al., 2017). However, physical weathering drives this material into fragmentation in smaller pieces and so far from disappearing, moves the problem into a different scale.

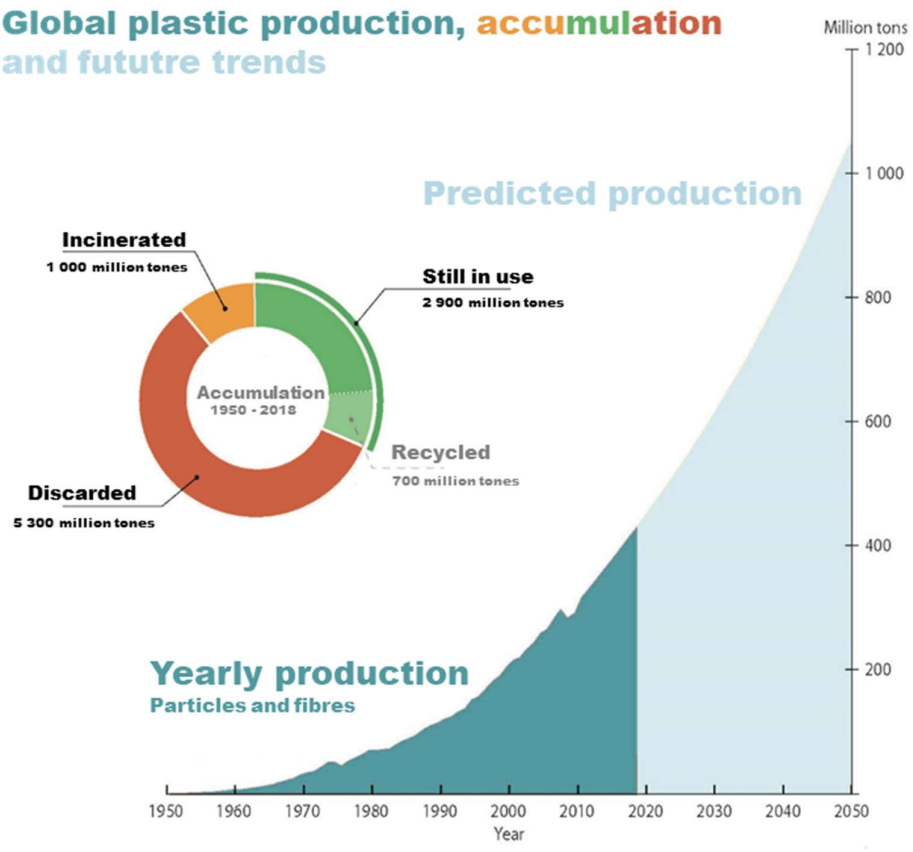

Fig. 3. Current production, accumulation of plastic and predicted production trend until 2050. Adapted from Illustrated by GRID-Arendal. Data from UNEP 2021, adapted from Jambeck et al. 2018.

Microplastics defined as synthetic solid particles of varied shapes and sizes, ranging between 1 µm and either 1 or 5 mm (Frias and Nash, 2019; Hartmann et al., 2019), have been described and reported in oceans worldwide. They can be classified according to their shape (which includes beads, fibres, films, and fragments (Hartmann et al., 2019)), colour, polymer composition or their origin. Small plastics are produced directly by the industry (primary microplastics) as raw material to be used in the manufacture of larger plastics or to be used as part of other products. However, as stated before, microplastics can also be produced by weathering (secondary microplastics) after bigger items reach marine environment (Fig. 4). Microplastics can be found along shores, a in open waters or deep seas, and both in the water column and in sediments (Avio et al., 2017). Amongst microliter, fibres are the most ubiquitous prevalent type of microplastic observed in the marine environment (Browne et al., 2011; Suaria et al., 2020), and have gained much attention in recent years. Anthropogenic fibres (AFs) (Lahens et al., 2018) refer not only to the plastic / synthetic fibres from petrochemical origin (i.e. polyester, polyamide, polypropylene, etc.), but also to non-synthetic fibres, which include artificial fibres from artificial cellulose or silk (i.e. viscose, rayon), and the natural fibres (i.e. cotton, wool); all of which are used in the textile and apparel industries. Non-synthetic fibres from the textile industry or urban wastewater treatment plants may also reach the aquatic environment throughout similar pathways. In contrast to plastic fibres, these non-synthetic fibres—despite being inherently unnatural— just started recently to receive attention from scientist and general public.

This set of small anthropogenic items (AIs) which englobes not only microplastics but also anthropogenic fibres are also usually referred by the synonymous term of micro-litter to be differentiated to the bigger litter as known as macro-litter.

1.3. Marine organisms facing micro-litter pollution: Up-taking and potential impact.

Before the first hypotheses of the potential negative effects of micro-litter emerged, attention was already focused in the 1980s on macro-litter (Laist, 1987). The first recordings of wildlife, mainly macroinvertebrates, entangled in plastic waste such as plastic bags, lost or discarded fishing nets and a wide variety of consumer goods, brought firstly all the attention. It was clear that entanglement had negative effects affecting movement capacity, breathing or feeding and eventually drive animals dying (Baulch and Perry, 2014), however, while marine megafauna received most off attention in this regard, it was evident that smaller organisms,

such as fish and invertebrates, would suffer similar impacts. For example, ghost fishing nets could be seen as a particular case of plastic entanglement for fish (Brown and Macfadyen, 2007).

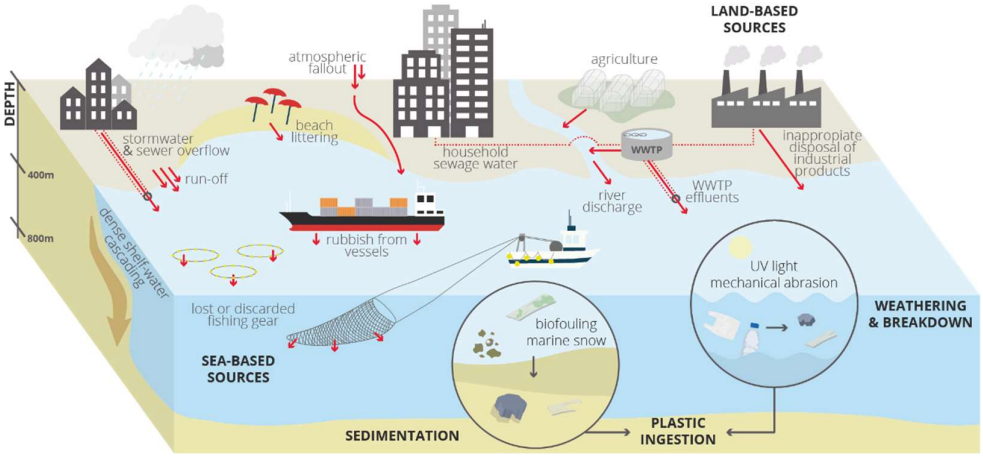

Fig. 4. Main sources and pathways of plastic and microplastic litter into the environment. Source: Original picture from Ester Carreras-Colom, 2021. Unravelling the (micro)plastic threat: the case study of plastic ingestion in *Aristeus antennatus* and *Nephrops norvegicus* from the NW Mediterranean Sea and its potential impact on health condition. PhD thesis. Universitat Autònoma de Barcelona.

The increasing abundance of micro-litter in the environment caused the first hypotheses of possible environmental implications (Moore et al., 2001; Thompson et al., 2004). Moreover, the fact that its ingestion has been demonstrated in living organisms of different taxonomic groups and the possibility that this type of pollutant may reach the food chain has drawn tremendous attention for scientists, popular media, and society since the first 2000's (Law, 2017).

The pathway of micro litter ingestion has been discussed and it has been considered that some organisms may actively ingest plastics due to their resemblance to natural prey (Ory et al., 2018a; Schuyler et al., 2012). However, passive ingestion has also been suggested as an important route, since micro-litter can be potentially ingested with prey (or in the prey itself, which could be considered trophic transfer) or from the sediment or while suspended in the seawater (Browne et al., 2007; Cole et al., 2011). Overall, the scarcity of studies simultaneously evaluating levels of micro-litter ingestion in organisms with different feeding strategies and environmental concentrations hinder the proper evaluation of feeding strategy or trophic transfer as significant factors for increased micro-litter ingestion.

Although the ingestion of micro-litter by biota is an undisputable fact, its mere presence in the digestive tracts is not enough to quantify the possible negative effects. Some authors pointed out that, when ingested, micro-litter items can interfere with the process of feeding, causing a false sensation of satiation, blocking or injuring the digestive tract, affecting digestion and absorption of food and even interact with the digestive microbiota (Foekema et al., 2013; Lusher et al., 2017; Murray and Cowie, 2011; Welden and Cowie, 2016). Thus, micro-litter can ultimately interfer with nutrient absorption and therefore might drive to reduced growth rates, diminished predator avoidance and reproductive failure (Wright et al., 2013), and finally negatively affect the body condition of individuals. However, these negative effects have not been clearly confirmed in wild organisms, since contradictory results have been observed, even in the same species. For instance, the body condition of the Mediterranean small pelagic fish species *Sardina pilchardus* and *Engraulis encrasicolus*, two species sharing the same habitat and food resources, showed a negative correlation with micro-litter ingestion in some studies, but is not feasible in others (Compa et al., 2018; Lefebvre et al., 2019; Pennino et al., 2020). The results from experimental studies under controlled conditions carried out in laboratories provided more evidences to this hypothesis. For instance, following controlled exposures, marine worms and sediment-dwelling bivalves displayed decreased energy reserves (Bour et al., 2018; Wright et al., 2013), green shore crabs showed a reduced scope for growth (Watts et al., 2014) and Norwegian lobster reduced its body condition (Welden and Cowie, 2016). In fish, however, this fact does not seem to be so evident (Alomar et al., 2021; Critchell and Hoogenboom, 2018).

On the other hand, micro-litter has also been stated able to adsorb on their surface other pollutants present in the environment, such as heavy metals (Avio et al., 2017; Brennecke et al., 2016) or organic compounds (polycyclic aromatic hydrocarbons (PAH) or polychlorinated biphenyls (PCBs)) (Heskett et al., 2012), thus acting as carriers of this substances and transfer it to biota (Rochman et al., 2013a; Teuten et al., 2009). Likewise, some experimental studies indicate that it can cause biochemical effects and induce toxic effects (Avio et al., 2017; Lu et al., 2016; Rochman et al., 2013a). Further studies have demonstrated that organic pollutants are present in micro-litter recovered from the field and can be successfully transferred to biota under experimental conditions (Bowley et al., 2021). However, a recent study provided evidence that the fraction of organic pollutants would be smaller compared to other media like prey (Koelmans et al., 2013).

Despite these experimental studies, currently, there are still doubts about the real impact in wild populations, where micro-litter might not be as pure as newly-synthetized polymers, concentrations are in a different order of magnitude, and other stressors (eg. other pollutant

sources, biological agents) might be interacting together, among others. Thus, in natural fish populations, these effects have been speculated from what is found in the individuals, without having appropriate health indicators.

1.4. Going beyond: Assessing health status of marine (fish) organisms

The evaluation of the health status of marine fish constitutes an important approach recognized internationally under descriptor 8 of the Marine Strategy Framework Directive (DMEM) to provide monitoring of Good Ecological Status (Lyons et al., 2010). However, this purpose can be very challenging in natural environments since organisms may be subject to multifactorial impacts. Indeed, there is no single indicator that may unequivocally measure environmental degradation. By this, in order to assess the overall quality of the aquatic environment, a holistic approach integrating various biological levels and combining different techniques to assess possible responses in the exposed organisms to pollutants has been shown as the most proficient (Dabrowska et al., 2017). So, to rise an overview of the population wellbeing, available indicators should be considered altogether. This means from an infra-individual level (enzyme biomarkers, cellular or tissue alteration) to more generalist indicators for populations, such as fish condition, or for communities, such as parasite infestation (Fig. 5).

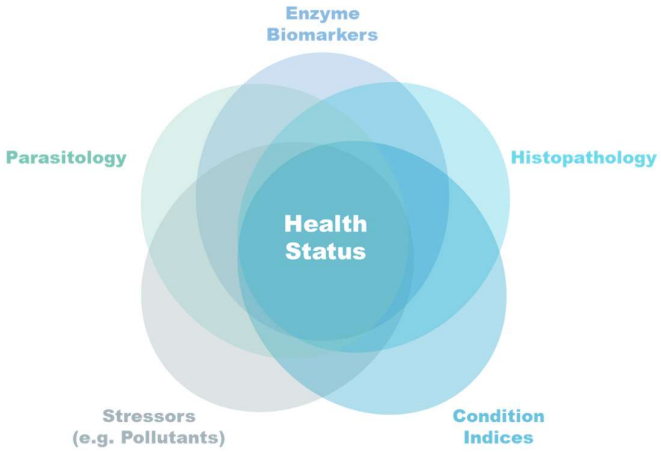

Fig. 5. Venn's diagram representing the main health indicators to assess the health status of fish from a holistic approach.

Enzymatic biomarkers in fish are widely accepted by providing a rapid response to contaminants (Van der Oost et al., 2003). The determination of multiple enzymatic activities makes it possible to integrate effects occurring at several physiological pathways (Crespo and Solé, 2016). For instance, cholinesterase activities are widely used as indicators of exposure to

chemical contaminants (Fulton and Key, 2001), whereas antioxidant enzymes such as catalase have been related to oxidative stress (Valavanidis et al., 2006; Winston and Di Giulio, 1991). In any case, there are several studies supporting their use in the evaluation of the exposure to environmental pollutants, including plastics, in a wide range of organisms, by this their use together with other biomarkers is strongly advised (Koenig et al., 2013; Solé et al., 2010b, 2010a; Suman et al., 2021).

Histological assessment has been proved a useful tool to evaluate the biological effects of pollutants in fish (Feist et al., 2015; Stentiford et al., 2003), since they reflect the true state of health of the organism (Costa et al., 2009). Common target organs for histological evaluation are those that are in direct contact with the surrounding environment (e.g. gills, digestive tract) and act as a barrier between the external / internal environment of fish. Moreover, other organs such as liver, driving metabolism by transformation, storage, and even elimination of xenobiotics, or kidney responsible of hematopoietic function and formation of cells of the immune system are considered one of the main target organs for histological diagnosis (Au, 2004; Bernet et al., 1999; Costa et al., 2010; Feist et al., 2004). The effectiveness of this approach has promoted the development of guidelines such as developed by ICES and the Program for Quality Assurance of Biological Effects in Monitoring (BEQUALM) (Feist et al., 2004; BEQUALM, 2005). The methodology proposed in this guideline allows to identify not only the early warning signs of disease and injury in cells, tissues, or organs but also the subsequent extrapolations to the level of population/community.

Body condition indices, including the body / relative condition factor and the hepatosomatic and gonadosomatic indices, are measures of body mass relative to size. They are widely used in ecological studies and are considered an estimate of the nutritional status or fitness, which may result from past foraging success, feeding intensity or exposure to environmental stress (Jakob et al., 1996; Jones and Obst, 2000). Hepatosomatic index is frequently related to the exposure to pollutants (Goede and Barton, 1990; Khan, 2010), generally increasing in hepatocytes' size, number, or both, in polluted areas (Goede and Barton, 1990). On the other side, body condition factor it is known to decrease in fish after exposure to stressors, such as heavy metals (Bervoets and Blust, 2003; Merciai et al., 2014). However, given the multiple factors (e.g., seasonal trends affecting food availability, reproduction events, changes in lipid content or individual genetic variability) affecting those indices, and mainly the gonadosomatic index, these should be carefully interpreted and not considered regardless of other fitness measures (Wilder et al., 2016).

The study of parasite communities of marine organisms, mainly in fish, have emerged during recent decades as an important tool for assessing environmental health at a community level. They are a good tool to be used as bioindicators of pollution effects, based on their responses to host related and environmental factors (Mackenzie et al., 1995; Marcogliese, 2005; Sasal et al., 2007; Sures, 2001). Parasite populations of organisms can change against environmental, either increasing or decreasing in abundances, depending on their life cycle and the nature of the change (Mackenzie et al., 1995; Marcogliese, 2005). Nevertheless, stressors such as pollution are usually associated with a reduction in the species richness of fish parasite communities (Carreras-Aubets et al., 2012; Marcogliese, 2005), denoting an impact on the ecosystem's health.

To sum up, all these indicators, when used together, will help to elucidate whether the potential negative effects of external factors such as pollutants are affecting only at individual level or contrary, they are moved into bigger scale affecting population level, which means that they could have some more relevant environmental implications.

1.5. Monitoring micro-litter: Key species as bioindicators of a new pollutant

By stated above, the potential negative influence of micro-litter on the marine environment and its organisms has been recognized as a priority descriptor in the Marine Strategy Framework Directive (MSFD). It includes marine litter ingestion by biota as one of the required descriptors (D10C3) for monitoring and assessing the good environmental status. Member States are obliged to establish their own protocols of monitoring, including the target species to be assessed. Moreover, member states are required to ensure that "The amount of litter and micro-litter ingested by marine animals is at levels that does not adversely affect the health of the species concerned" (EU 2017/848).

The use of organisms with relevant ecological importance to monitor the ingestion of micro-litter has been shown to be effective with different examples as the EcoQ0 indicator, such as northern fulmars (*Fulmarus glacialis*) (Van Franeker et al., 2011), used since 2007 in the North Sea (Convention for the Protection of the Marine Environment of the North-East Atlantic, OSPAR). However, the use of organisms as monitoring tool has been shown to be effective but it is not free of constraints. Some species, despite being potential candidates, do not have a wide enough distribution or are simply not found in certain areas. In other cases, although they are present and adequate, their scarcity or their degree of protection/conservation status as vulnerable species, make monitoring programs even more difficult. One example is the

loggerhead turtle (*Caretta caretta*) which has been proposed for the monitoring of macroplastic ingestion trends in the Mediterranean Sea (IMAP Candidate Indicator 24). The target species should therefore be abundant and wide distributed, be resident or highly philopatric, and have a relative short digestion period to ensure that the ingestion of the target pollutant (i.e. micro-litter) occurs in the vicinity of the investigated habitat (Fossi et al., 2018). In addition, the use of commercially important species could enable the estimation of the potential transfer of plastics, and their associated contaminants, from seafood to humans (Fossi et al., 2018). For instance, species such as mussels (*Mytilus edulis* and *M. galloprovincialis*) have been long and extensively used in monitoring programs because of their sessile lifestyle, wide distribution and filtering capacity that maximises the exposure and uptake of chemical pollutants in the environment. These species have been proved potential usefulness in monitoring microplastics (Li et al., 2016; Phuong et al., 2018). Nevertheless, the size range of particles they can ingest is limited, they can only reflect pollution levels of coastal areas, and sample processing is generally complex (Rochman et al., 2013a). Thus, there is a need in using a suitable variety of target species, to trace different size range of micro-litter pollution at each of the marine compartments (coast and oceanic, benthic and pelagic, shallow and deep waters).

Several species of fish have been reported ingesting micro-litter worldwide revealing fish as a very interesting group to monitor this kind of pollution (Wootton et al., 2021). Moreover, abundant and commonly caught species in fisheries allows obtaining comparable spatial and temporal information among different areas. Considering different descriptors such as species distribution, gut length, commercial value, vagility and occurrence, the most suitable target species to monitor micro-litter ingestion in the Mediterranean Sea have recently been proposed (Bray et al., 2019). Species selected are also mainly commercial target and key species of different habitats (Fig. 6). The European anchovy (*Engraulis encrasicolus*) has been proposed as the best monitor species for pelagic ecosystem, while the red mullet (*Mullus barbatus*) for continental shelf demersal bottoms.

1.6. Target species selected: The European anchovy and the red mullet.

The European anchovy (*Engraulis encrasicolus*) and European sardine (*Sardina pilchardus*) are the two main species of clupeids inhabiting the Mediterranean Sea and are key species for the epipelagic ecosystems. They also support more than the 40 % of landings of the Mediterranean fisheries (FAO, 2020). Due to its significant biomass at mid-trophic levels, these species are the main prey for numerous predators, thus playing a major role in energy transfer,

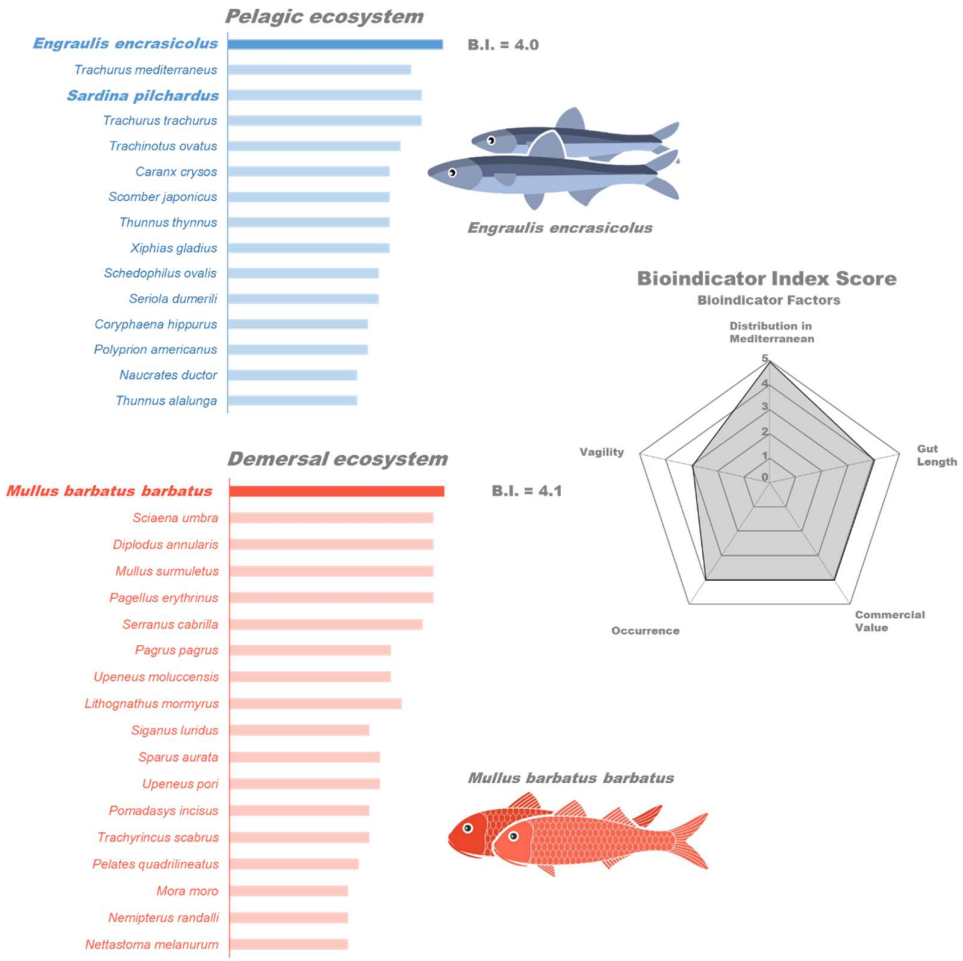

Fig. 6. Suitable species for monitoring microplastic ingestion by fish in the Mediterranean for demersal and pelagic ecosystem. B.I. : bioindicator index score. Adapted and data from Bray et al., 2019.

connecting lower to higher trophic levels in marine ecosystems (Cury et al., 2000). However, small pelagic fish are widely known for their rapid and significant population fluctuations (Bakun, 1997; Lloret et al., 2001).

During the last decades progressive declines of small pelagic fish populations have been recorded in the Mediterranean Sea, especially for Sardines, alongside changes in the population structure (Van Beveren et al., 2014). Moreover, while the abundance of anchovy remains relatively high, both biomass and the mean size of individuals have dramatically diminished, accompanied by a decline in the body condition and size/age of maturation (Albo-Puigserver et al., 2021; Biton-Porsmoguer et al., 2020; Brosset et al., 2015b; Saraux et al., 2019; Van Beveren et al., 2014).

The ingestion of micro-litter by these species has been recorded in whole Mediterranean Basin, from the Gulf of Lions (Collard et al., 2017; Lefebvre et al., 2019), Balearic Basin (Compa et al., 2018; Pennino et al., 2020), Ligurian Sea (Capone et al., 2020), Adriatic Sea (Renzi et al., 2019), and Tyrrhenian Sea (Savoca et al., 2020). By this, both species have been proposed as good candidates for monitoring micro-litter ingestion in pelagic ecosystems in the Mediterranean Sea (Bray et al., 2019). However, the greater abundance of anchovies compared to sardines makes the latter more suitable species for micro-litter monitoring.

On the other side, it should be considered that although these species have a great commercial value, they have always been studied in their natural environment, which have some limitations. For this reason, the need to understand in a more refined way parameters of their biology, such as the rate of growth, fecundity and larval development, has led to the development of protocols for obtaining and maintaining these species in captivity. Currently it is possible to obtain and maintain species from the wild, generally clupeids (Ohkubo et al., 2022; Queiros et al., 2019a; Savoca et al., 2017), which opens a new window to be used as a model organism, even to try to answer questions such those that micro-litter pollution has generated.

Red mullets, (*Mullus barbatus*, Linnaeus, 1758) is a benthic fish species widely spread in the Mediterranean Sea and the North-Eastern Atlantic, where they inhabit the continental shelf above gravel, sandy, and muddy bottoms up to 500 m in depth (Lloris, 2015). Despite it corresponds to the 1.9 % of the total landings from Mediterranean fisheries, it is considered one of the main target species from demersal ecosystems. Its diet and feeding behaviour are based on seeking and feeding on benthic invertebrates buried in the seafloor such as polychaetes, decapods and small crustaceans (Bautista-Vega et al., 2008; Labropoulou and Papadopoulou-Smith, 1999; Machias and Labropoulou, 2002). So, the species is in constant contact with sediment, and therefore, it is also exposed to the pollutants that are deposited in this area (Van Cauwenberghe et al., 2015). Thus, it has been widely used as a sentinel species for several pollutants including heavy metals, persistent organic pollutants (POP's) (Carreras-Aubets et al., 2012; Zorita et al., 2008) and micro-litter from demersal habitats (Bray et al., 2019).

Red mullet has been reported ingesting micro-litter in several areas of the Mediterranean Sea including along the Turkish shore, Adriatic, Ionian and Tyrrhenian Seas, and on the Mediterranean Spanish Coast (Avio et al., 2015a; Bellas et al., 2016; Capillo et al., 2020; Digka et al., 2018; Güven et al., 2017). However, the possible health effects of this ingestion have not been addressed and remains an important issue for a reliable risk assessment of these pollutants.

2.OBJECTIVES

2. OBJECTIVES

The main goal of this thesis is to determine the potential impact of micro-litter ingestion (microplastics and anthropogenic fibres) on wild commercial fish species off the Catalan Sea (North-Western Mediterranean Sea). Two wild species (the red mullet and the European anchovy) were selected as they are considered the most suitable target species for monitoring this type of pollution for benthic and pelagic habitats, respectively. Moreover, the European sardine (*Sardina pilchardus*), another key species of pelagic ecosystems, was selected as a model species to perform an experimental assay to determine the pattern of plastic fibre ingestion and egestion and their effects according to feeding behaviour.

To reach this main goal, five different specific objectives have been established:

1- To characterize the micro-litter ingestion by target species (prevalence, abundance, typology and composition).

2- To compare the ingestion of micro-litter according to location and temporality.

3- To evaluate the health status of the target species combining different methodologies: biological indices, enzymatic biomarkers, histological tissue alterations, and parasite descriptors.

4- To analyse a possible relationship between the ingestion of micro-litter and health indicators.

5- To determine a relationship between the amount of micro-litter ingested and the feeding behaviour of commercial fish species.

3.CHAPTER I

Are anthropogenic fibres a real problem for red mullets (*Mullus barbatus*) from the NW Mediterranean?

Oriol Rodríguez-Romeu, María Constenla, Maite Carrassón, Mariano Campoy-Quiles, Anna Soler-Membrives

Science of the Total Environment 733 (2020) 139336
DOI: https://doi.org/10.1016/j.scitotenv.2020.139336

ABSTRACT

Microfibres are among the most prevalent type of microplastics in marine environments. Man-made fibres derived from cellulose are distributed worldwide, but are often confused with synthetic plastic fibres and consequently neglected. All these fibres may adversely affect aquatic organisms, but their levels and potential effects in wild fish remain unknown. We analysed anthropogenic fibre (AF) ingestion in the red mullet (*Mullus barbatus*), at both temporal and geographical scales, to assess potential effects of these fibres on fish health condition. AFs were present in 50% of fish digestive tracts, with a mean of 1.48 AFs per individual (SD=1.98). In Barcelona, an increase of 46% in AF ingestion was observed in 2018 compared to 2007. AF ingestion also increases by 20% when Barcelona is compared to a less urban area (the town of Blanes). Visual characterization of fibres by typologies—corroborated by Raman spectroscopy—allowed classification and identification of 88% of AFs as cellulosic (57%), and synthetic polymers (PET) (31%). In all sampling stations, the only histopathological alterations were cysts of unknown etiology, and the most abundant parasites were nematodes. None of these alterations, parasite load, or other fish health indicators (condition indices) indicate an effect of AF ingestion.

HIGHLIGTHS

- Studies of marine micro-litter should consider plastic and non-plastic anthropogenic fibres (AFs).
- An accurate visual approach is useful to differentiate synthetic and cellulosic fibre types.
- Higher prevalence and abundance of AFs in demersal fish are detected near to polluted areas.
- PET and cellulosic fibres were already common in Mullus barbatus 10 years ago.
- No evidences of health effects attributable to AFs were detected for M. barbatus.

1. INTRODUCTION

Pollution by organic synthetic polymers, commonly known as plastics, in the ocean was first reported by scientists in the 1970s (Carpenter et al., 1972; Carpenter and Smith, 1972; Colton et al., 1972) and has since drawn tremendous attention in recent years in science, popular media, and society (Law, 2017). Currently, plastic pollution is considered a major threat to marine and terrestrial ecosystems globally (Derraik, 2002). Worldwide, it is estimated that between 4 and 12 million tonnes of plastic enters the world's oceans annually, mainly from coastal inputs (Jambeck et al., 2015); plastic has become ubiquitous in the ocean in only few decades. Small plastic debris—classified as microplastics (MPs) if their size is smaller than 5 mm (Hartmann et al., 2019)—are found globally (Cózar et al., 2014), either floating or deposited on the seafloor. Though they are particularly abundant in the coastal shallows around populated areas (Alomar et al., 2016), MPs are found even in the most remote locations, including the deep seafloor (Woodall et al., 2014), uninhabited islands (Lavers and Bond, 2017), the Arctic (Cózar et al., 2017), and the coastlines and surrounding waters of Antarctica (Waller et al., 2017). MPs can be classified according to their shape, which includes beads, fibres, films, and fragments (Hartmann et al., 2019). Fibres are among the most prevalent type of MPs observed in the marine environment (Browne et al., 2011). Fibre pollution has gained much attention in recent years. Anthropogenic fibres (AFs) (Lahens et al., 2018) refer not only to the plastic/ synthetic fibres from petrochemical origin (i.e. polyester, polyamide, polypropylene, etc.), but also to non-synthetic fibres, which include artificial fibres from artificial cellulose or silk (i.e. viscose, rayon), and the natural fibres (i.e. cotton, wool); all of which are used in the textile and apparel industries. Non-synthetic fibres from the textile industry or urban wastewater treatment plants also reach the aquatic environment. In contrast to plastic fibres, these non-synthetic fibres—despite being inherently unnatural— have received little attention from environmentalists (Stanton et al., 2019). They are also distributed worldwide (Gago et al., 2018), even in the gastrointestinal tract of organisms, and can be often confused with synthetic plastic fibres due to their similar morphological features (Remy et al., 2015; Savoca et al., 2019). All these anthropogenic fibres—with their additives or dyes, and their capability to bond other contaminants to their surfaces—are suspected of adversely affecting aquatic organisms (Burgos-Aceves et al., 2018a, 2018b; Faggio et al., 2018; Prokić et al., 2019). However, the levels of this pollution, and its potential effects in wild fish remain unknown.

The Mediterranean Sea is a semi-enclosed, highly populated basin, exposed to heavy coastal pressures, such as maritime traffic, waste discharges, and river inputs—these determine the densities of marine debris including marine plastics (Alomar et al., 2017; Barnes et al., 2009;

Deudero and Alomar, 2015; Jambeck et al., 2015). It is estimated that between 1000 and 3000 tons of plastic are floating on the Mediterranean Sea (Cózar et al., 2015). In the North West Mediterranean, AFs have been reported in several seafloor environments including estuarine, coastal areas (Alomar et al., 2016; Simon-Sánchez et al., 2019), and deep-sea zones (Sanchez-Vidal et al., 2018). Their ingestion by marine organisms has also been reported for both offshore and inshore fish (Bellas et al., 2016), as well as for deep-sea organisms such as fish and crustaceans (Carreras-Colom et al., 2018; Romeu et al., 2016). *Mullus barbatus* (Linnaeus, 1758) —commonly called the red mullet—is a benthic fish species widely spread in the Mediterranean Sea and the North Eastern Atlantic, where they inhabit the continental shelf above gravel, sandy, and muddy bottoms up to 500 m in depth (Lloris, 2015). Due to its diet and feeding behaviour (Bautista-Vega et al., 2008), this species is in constant contact with sediment, and therefore, it is also exposed to the pollutants that are deposited in this area (Van Cauwenberghe et al., 2015). Thus, it has been widely proposed as a sentinel species for a number of pollutants (Bray et al., 2019; Carreras- Aubets et al., 2012). AFs ingestion by the red mullet has been reported in several areas of the Mediterranean Sea including along the Turkish shore, Adriatic, Ionian and Tyrrhenian Seas, and on the Mediterranean Spanish Coast (Avio et al., 2015; Bellas et al., 2016; Capillo et al., 2020; Digka et al., 2018; Güven et al., 2017). Although these studies have shown red mullet do ingest AFs, the possible effects of this have not been addressed; this therefore remains an important issue for a reliable risk assessment of these pollutants.

Negative effects associated with AF and MP ingestion have been observed under laboratory experiments, where organisms were exposed to particular concentration levels of pollutants. However, the ranges studied were acutely high, and therefore mimicked environmentally unrealistic concentrations (Cunningham and Sigwart, 2019). The health status of wild fish populations is generally evaluated by integrating many indicators, from individual fish condition, to cellular or tissue alteration, and parasite infestation; together these give an overview of population wellbeing. Fish condition indices can alert us to the occurrence of diseases or other physiological features before mortality events. For instance, the hepatosomatic and gonadosomatic indices—which are widely used both in ecological studies and for evaluation of wild fish stocks in fisheries (Brosset et al., 2015; Stevenson and Woods, 2006)— give information about the physiological status related to the capacity/ accumulation of short-term reserves and the reproductive capacity, respectively (Wootton, 1989). Fulton's condition factor is the main indicator of the fattening of an individual/population (Nash et al., 2006), and fasting or feeding intensity can be obtained by the stomach fullness index (Hyslop, 1980). In the NW Mediterranean, a few studies have suggested that oxidative stress and other

effects in condition are related to ingested AFs in wild fish (Alomar et al., 2017; Compa et al., 2018). Moreover, sub-lethal environmental stress, e.g. that produced by pollutants, can be reflected in cellular or tissue alterations of different fish organs. The presence or changes in the intensity of these histopathological alterations caused by pollutants, such as heavy metals or organic compounds, can be assessed by the microscopic observation of target organs (Au, 2004; Costa, 2018; Stentiford et al., 2003). The most common target organs used in histopathology to assess the effects of aquatic pollutants are the liver and gills, but also the digestive tract, kidney, and gonads are highly relevant (Costa, 2018). Histopathological alterations caused by plastics (e.g. Inflammation, cell death, necrosis) are also described in fish after MP exposure in laboratory conditions (Ahrendt et al., 2020; Kögel et al., 2020), but studies in wild marine fish are scarce. Finally, fish parasite load is a widely used indicator of the health of both organisms and ecosystems, since parasite populations can either increase or decrease against environmental changes depending on their life cycle and the nature of pollutants (Mackenzie et al., 1995; Marcogliese, 2005; Sures, 2001). However, studies linking the presence of plastics with parasites are extremely rare. If high levels of MPs or AFs within the digestive tract affects fish condition, this could favour parasite infestation. Hernandez-Milian et al. (2019) hypothesized that parasite aggregations within intestines could retain microplastics and cause their aggregation. Therefore, besides being bioindicators of pollutants of marine ecosystems, parasites could increase the accumulation of microplastics within biota, and therefore increase the risk of damage to host health.

In the present study, we analysed the prevalence, abundance, size, and composition of AFs in the digestive tract of red mullet individuals by comparing two different years with a 10-year gap (2007 and 2018), and in two different locations along the Spanish NW Mediterranean Sea coast. The aims of the present study are: 1) To assess the presence of AFs in the digestive tract of *M. barbatus* from the NW Mediterranean Sea, and to: 2) infer differences between the prevalence, abundance, and typologies of ingested AFs in 2018, with those ingested over 10 years prior; 3) explore geographical variations in the prevalence, abundance, and typologies of the ingested AFs between two localities (a highly urban and a less-urban area); 4) assess the potential effect of AFs on fish health by applying health indicators such as biological and condition indices, tissue histological alterations, and parasite descriptors; and finally; 5) discuss the possible source or origin of the different kind of AFs within *M. barbatus*.

2. MATERIALS AND METHODS

2.1. Study area and sample collection

A total of 118 *Mullus barbatus* were captured at depths of between 60 and 130 m from the continental shelf off the Catalan coast (NW Mediterranean) within the framework of BIOMARE (Spanish Ministry of Science and Innovation) and SOMPESCA (Department of Agriculture, Livestock, Fisheries and Food, Catalonia, Spain) multidisciplinary projects (Table 1). Two areas were sampled, one around 5 miles off the coast of Barcelona, and the other offshore at the smaller nearby town of Blanes (Fig. 1). Barcelona city and the near metropolitan zone is an industrialized and densely populated coastal area when compared to Blanes. While Barcelona shore is under the influence of two main rivers (Llobregat and Besòs) which, in addition to typical seasonal abrupt discharges have a continuous flow regime throughout all year, the shore off Blanes is under the influence of a single smaller river (Tordera river) that has a mainly seasonal pattern of water discharge. Fish were collected aboard commercial and scientific fishing vessels during 2007 (48 specimens) from only Barcelona, and with commercial fishing vessels during 2018 (70 specimens) at two different sites (Barcelona—same site as in 2007—and in Blanes), in both cases fish were collected during two seasons (spring and summer) (Table 1). Two different fishing gears were used: a semi-balloon otter trawl, OTSB14 (Merrett and Marshall, 1980), and a commercial fishing trawl (BOU). Fish were immediately fixed in 10% buffered formalin and transported to the laboratory for analyses. An abdominal incision was made in order to improve the fixation process of internal organs.

Table 1 Cruise data (station, location, year, season, depth, latitude and longitude) for each sampling site, including the number of fish analysed (n). Mean and standard deviation (SD) of standard length (SL, cm), total weight (TW, g), gonadosomatic index (GSI), hepatosomatic index (HSI), condition factor (K), and fullness (FULL).

Station	Location	Year	Season	Depth	Lat.	Long.	n	SL	(SD)	TW	(SD)	GSI	(SD)	HSI	(SD)	K	(SD)	(SD)	Full
BCN1	Barcelona	2007	Spring	62 m	41°24′35.10″N	2°20′31.80″E	19	9.46	(0.79)	16.20	(3.91)	1.48	(0.51)	1.71	(0.46)	1.68	(0.22)	(0.12)	0.27
BCN2	Barcelona	2007	Summer	62 m	41°25′25.92″N	2°21′1.68″E	29	12.60	(1.90)	41.67	(22.55)	2.21	(1.60)	1.74	(1.48)	1.75	(0.37)	(0.20)	0.43
BCN3	Barcelona	2018	Spring	93 m	41°19′1.56″N	2°14′16.44″E	15	12.08	(1.31)	30.27	(10.15)	2.19	(1.44)	1.39	(0.47)	1.49	(0.25)	(0.13)	0.24
BCN4	Barcelona	2018	Summer	106 m	41°11′21.54″N	2° 4′23.16″E	20	13.99	(0.86)	51.17	(9.25)	1.72	(1.82)	1.78	(0.47)	1.67	(0.51)	(0.13)	0.69
BLN1	Blanes	2018	Spring	130 m	41°34′59.52″N	3°10′13.62″E	15	14.93	(1.23)	62.70	(17.14)	4.84	(2.76)	1.85	(0.77)	1.57	(0.24)	(0.12)	0.42
BLN2	Blanes	2018	Summer	110 m	41°32′51.36″N	2°44′34.80″E	20	17.26	(1.72	49.82)	(14.94)	3.41	(3.59)	1.90	(0.78)	1.65	(0.60)	(0.13)	0.70

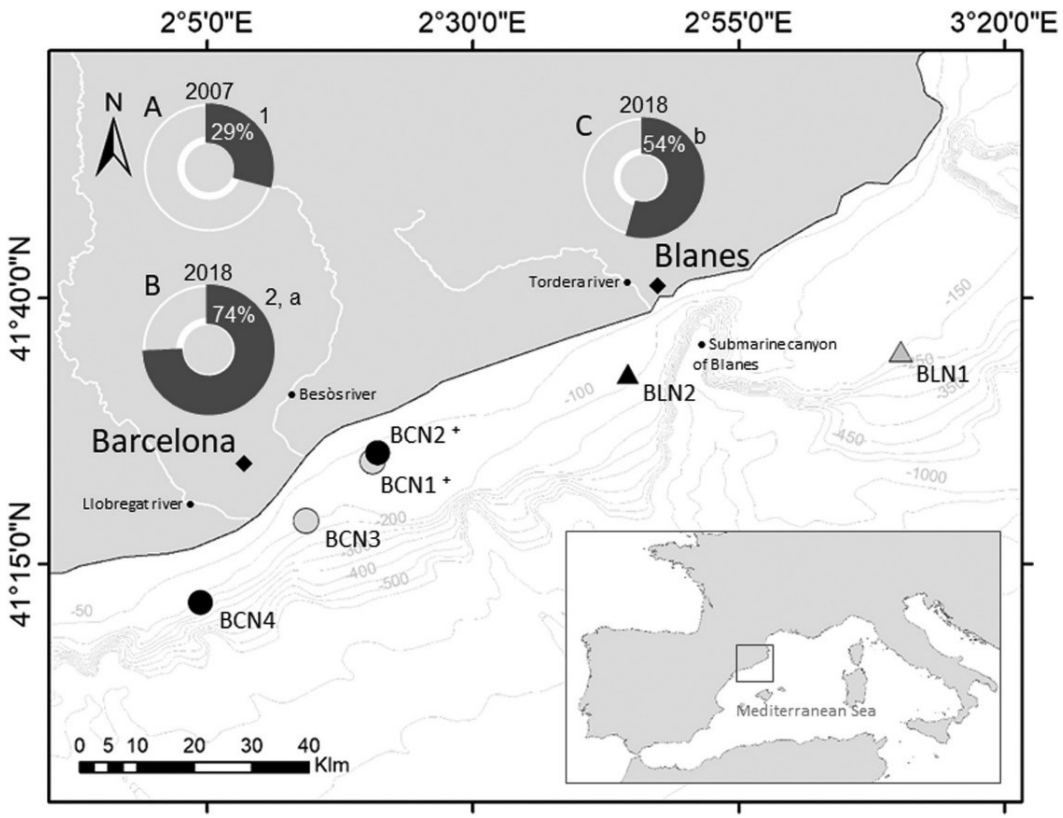

Fig. 1. Map of sampling area. Circles (○) indicate sampling stations near Barcelona and triangles (Δ) near Blanes. Black and grey represent summer and spring respectively, (+) indicates 2007 sample stations. Ring graphs indicate prevalence of fish containing anthropogenic fibres in Barcelona 2007 (A), Barcelona 2018 (B), and Blanes 2018 (C). Different numbers and letters show significant differences between years (Barcelona 2007–2018) and localities (Barcelona–Blanes in 2018), respectively ($p < 0.05$).

2.2. Laboratory procedures

Once at the laboratory, each specimen was measured to the nearest mm (total length = TL and standard length = SL), (Froese and Pauly, 2019), and weighed to the nearest g (total weight=TW) and dissected. To minimize airborne contamination, all procedures were performed in a laminar flow cabinet, which was previously cleaned; and all laboratory equipment and tools were rinsed with deionized (50 µm) water. Nitrile gloves and cotton lab coats exclusive to this work were used throughout. The gastrointestinal tract was removed by dissection following 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 then the individuals were weighed again to the nearest g (eviscerated weight = EW). All organs including the dissected gastrointestinal tract (stomach, caeca, and intestines) were stored separately in filtered 70% ethanol in

individual glass vials previously rinsed with deionized filtered (50 µm) water for subsequent observations.

2.3. Isolation and visual inspection of anthropogenic items (fibres and fragments)

The contents of the stomach, caeca, and intestine was carefully screened under a stereoscopic binocular at 10× to 45× of magnification. To prevent background contamination, the stereomicroscope and work area was isolated from the exterior by an isolation device adapted from the one proposed by Torre et al. (2016), and the interior was carefully washed before its use to minimize the presence of airborne anthropogenic items. The laboratory dissection material was also rinsed with filtered deionized water twice before use. Procedural controls, which consist of uncovered Petri dishes filled with filtered water, were placed inside and outside of the isolation device during digestive content screening, in order to assess potential airborne contamination levels. Only fibre-shaped items were found in both controls. Contamination found in the inside controls (average values of 0.22 fibres per digestive sample screened) was 3.6 times less abundant 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 contents 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.

To avoid misidentification of AFs with vegetal remains (e.g. seagrass or algae), a selection criterion adapted from that proposed by Hidalgo- Ruz et al. (2012) was used for fibres. AFs detected were collected and mounted between glass slides in filtrated deionized water and observed under the microscope. Those that presented vegetal morphological features such as cellular or organic structures were discarded. Length and mean cross section (based on three random measures) were obtained for fibres, and only cross-section for fragments. Images were obtained using a Leica camera model: CTR 5000, attached to the Leica microscope model: DM 5000 DB, and measured by image-processing software (ProgRes® C3). Anthropogenic items were counted for each individual, and their localization within the digestive tract (stomach, caeca, and intestines) was recorded.

The AFs found in the digestive tracts of fish were carefully observed under the microscope, characterized, and classified into distinct typologies according to their morphological features: general appearance (GA) and microscopic appearance (MA) (cross-

section shape, patterns of the fibre's body, shape and appearance of the ends, breakages and alterations of the fibre's body, birefringence, and colour) (Robertson et al., 2017). Prevalence of each typology was calculated as percentage of each fibre type with respect to the total amount of AFs.

2.4. Raman characterization of AFs

After visual classification, 25% of the fibres of each type (39 AFs in total) were randomly selected to be identified by Raman scattering.

Raman spectra were measured using a WITec Alpha300RA device. The experiments were performed under ambient conditions, employing low acquisition times (typically 100 ms) and moderate laser powers (488 nm excitation, typically 1.5 mW except for cotton-like fibres, for which it was increased) to minimize laser-induced degradation of the fibres. Fibres were imaged (several hundreds of spectra per image) through a 40× objective lens and using a motorized stage. Data were clustered using the Witec Project 5 software to account for the in/out positions of the fibres. All spectra within each fibre were then averaged after removing the cosmic ray hit pixels and background. The measured spectra were compared against a custom library, with known target polymers (see Supplementary material), and commercial Raman library BioRad KnowItAll® Informatics System–Raman ID Expert (2015) software. Hit Quality Index (HQI) was associated with each reference spectrum in order to allow polymer identification. HQI is a numerical measure of the closeness of fit between the unknown spectrum and each reference spectrum. The minimum match value between the obtained spectra and the library used for characterization was 70%.

2.5. Health assessment

Fish condition was assessed by the gonadosomatic index (GSI = (GW/TW) × 100), the hepatosomatic index (HSI = (HW/TW) × 100) and the Fulton's body condition factor (K = EW × 100/$(SL)^3$). Feeding intensity was measured by the stomach-fullness index (FULL = (CW/ EW) × 100), which was calculated using the total stomach content weight (CW).

A portion of gonad, liver, spleen, kidney, stomach (after isolation of anthropogenic items), and gills were embedded in paraffin and processed by routine histology. A section (5 µm) of each organ was stained with Haematoxylin and Eosin for histopathological assessment. All histological samples were screened completely in order to observe histological alterations under the microscope. The aim of this analysis was to detect the possible histological alterations (e.g. inflammation, cell death; see Kögel et al. 2020) which may be related to the ingestion of AFs or

associated toxic substances, but not to detect the fibres themselves. The spleen was chosen for the quantitative study of the melanomacrophage centres (MMC), due to the ease of ablation in this organ and the possibility of obtaining 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 spleen and examined microscopically at 200×. Area and number of MMCs (Mean area = MA.MMC, and number = nMMC) of each field were measured using a MicroComp Integrated Image Analysis System, and a size discriminator was used to eliminate objects smaller than 100 µm2.

External surfaces and gills were checked macroscopically for ectoparasites, and the rest of the organs, including stomach, caeca, intestine (after AF screening), and the internal body wash were carefully inspected for endoparasites under a stereomicroscope. Digeneans and cestodes were stained with iron acetocarmine and permanently mounted in Canada balsam. Nematodes were temporally cleared and mounted in glycerin before identification. Parasites were identified under an optic microscope to the lowest taxonomic level possible (see Supplementary material).

2.6. Data analysis

The prevalence of AFs was calculated as the proportion of fish containing AFs within their digestive tract with respect to the total number of fish. The number of AFs was determined for each individual (nAF). Total length of AFs (TLAF) was calculated by adding together the length of each AF observed inside the digestive tract of the individual, in order to give a more realistic value of the volume occupied by these fibres in the digestive.

Data were tested for normality using the Shapiro-Wilk test in order to assess differences in the number (nAF) and size (TLAF) of AF found in different parts of fish digestive tracts. When data did not satisfy the assumptions of normality, non-parametric Kruskal-Wallis tests were used.

In order to characterize the fibre size, each AF of the whole digestive tract was classified by size into four clusters by partitioning around medoids (PAM) (Kaufman and Rousseeuw, 1990), using the PAMK function implemented in the package fpc 2.2–3 in R Studio 3.5.0. A Chi-Squared test was used for testing changes in proportions of AFs (AF fish prevalence, size categories, and typology) or alternatively, the Fisher exact test was used when sample size was small and where expected values were <5.

As preliminary data analyses did not show significant differences in AFs (in neither number nor length) between spring and summer samples within the same year—neither in 2007 nor 2018 (data not shown)—seasonality was not considered a factor in the following analyses. The analyses between 2007 and 2018 was conducted using the subset of samples from Barcelona (2007–2018), and the geographical comparison was performed with the subset of samples from2018 (Barcelona and Blanes).

To assess differences in prevalence of AFs, nAF, and TLAF by year (2007 and 2018) and locality (Barcelona and Blanes) generalized Linear Models (GZM) (binary logistic model and negative binomial model) and general linear model (GLM) respectively, were used, with SL as a covariate.

Prevalence of the histological alterations (i.e. cysts of unknown etiology) was calculated as the proportion of fish containing the alteration with respect to the total number of fish. Parasite prevalence (P%) and mean abundance (MA) were calculated following previous studies (Bush et al., 1997), and parasite richness (R), and parasite diversity for each individual was also calculated using the Shannon Index (H'). Differences in biological indices were also tested comparing year and locality, with SL as a covariate: K and HSI using GLM; GSI, FULL, splenic melanomacrophage centres (nMMC andMA.MMC) and parasites (total number of parasites = nPAR) using GZM (gamma with log link and negative binomial model); and prevalence of histological alterations by GZM (binary logistic model).

To test possible correlations between anthropogenic fibres (nAF and TLAF) and histological alterations and parasite prevalence (for each taxonomic group), GZM (binary logistic model) were also used. To test correlations between anthropogenic fibres (nAF and TLAF) and parasite descriptors (R and H'), GZM (negative binomial model) and GLM, respectively, were used. In order to test the potential implication of the abundance of parasites (total abundance, and for each taxonomic group) on the retention of anthropogenic fibres within the digestive tract (nAF and TLAF), GZM (negative binomial model) and GLM, respectively, were used; in this case, tests were performed both by taking the digestive tract as a whole, and by comparing each organ (stomach, pyloric caeca, and intestine). To test possible effects of anthropogenic fibre abundance on values of parasite infection (total abundance and for each taxonomic group), non-parametric Spearman's correlation tests were performed. Since no relationship between anthropogenic fibres and parasite infection level was detected (see results below), and as parasite infection can also affect fish health indices, the number of parasites (nPAR) was considered as an explanatory variable to elucidate the relationships between AFs and biological

health indices. For this, a multivariate ordination method was used in Xlstat (version 2019.21.3.62256 http://www.xlstat.com). Redundancy analysis (RDA) is a multivariate method appropriate for testing or visualizing correlations or covariances between the response and explanatory variables; essentially modelling a cause-effect relationship. To identify the possibility of nAF, TLAF, and number of parasites (nPAR) as explanatory variables, for the fish health indices K, HSI, GSI and FULL as response variables, RDA was used with 500 Monte Carlo significance permutation tests. Finally, Pearson's correlation tests and non-parametric Spearman's correlation tests (when normality was not satisfied) were performed to assess the possible effect of AFs on the biological fish health indices K, HSI, GSI, FULL, and SL.

3. RESULTS

3.1. Ingestion of anthropogenic items by *M. barbatus*

A total of 167 AFs were found in the digestive tracts of *M. barbatus*. In addition, 7 fragments (size range = 0.12 – 0.72 mm, mean length = 0.32 mm (SD = 0.20)) were also found in the stomachs of five distinct fish: five fragments in Barcelona in 2018, and one each at Barcelona in 2007 and Blanes in 2018. Due to their small number, they were not considered in subsequent analyses. Mean AF length was 2.50 mm (SD = 2.24), ranging from 0.37mm to 14.80 mm. Partitioning around medoids based on each AF produced four clusters: small (S): ≤ 1.9 mm; medium (M): from 2 to 3.9 mm; large (L): from 4 to 7.3 mm and extra large (XL) ≥ 7.3 mm (Fig. 2). The most abundant size was small (53.45%) (X^2 = 14.129, p = 0.02), followed by medium (34.48%), while less abundant were the large and extra-large fibres (8.62% and 3.45%, respectively). Half of the red mullets analysed contained AFs in their digestive tract (59 out of 118): 28 and 95 from Barcelona (2007 and 2018 respectively), and 51 red mullets from Blanes in 2018. The mean number of AFs per individual (nAF) was 1.48 (SD = 1.98), and the mean TLAF was 3.55 mm/individual (SD = 6.17). No significant differences (p > 0.05) were found between the number of AFs in different parts of the digestive tract. The smallest TLAF were found in the caeca (K–W = 7.025, p = 0.027), followed by the intestine, and stomach (no significant differences). Comparing fish size and ingestion of AFs, a significant positive correlation between nAF and SL was found (X^2 = 8.689, p = 0.003) but not for TLAF.

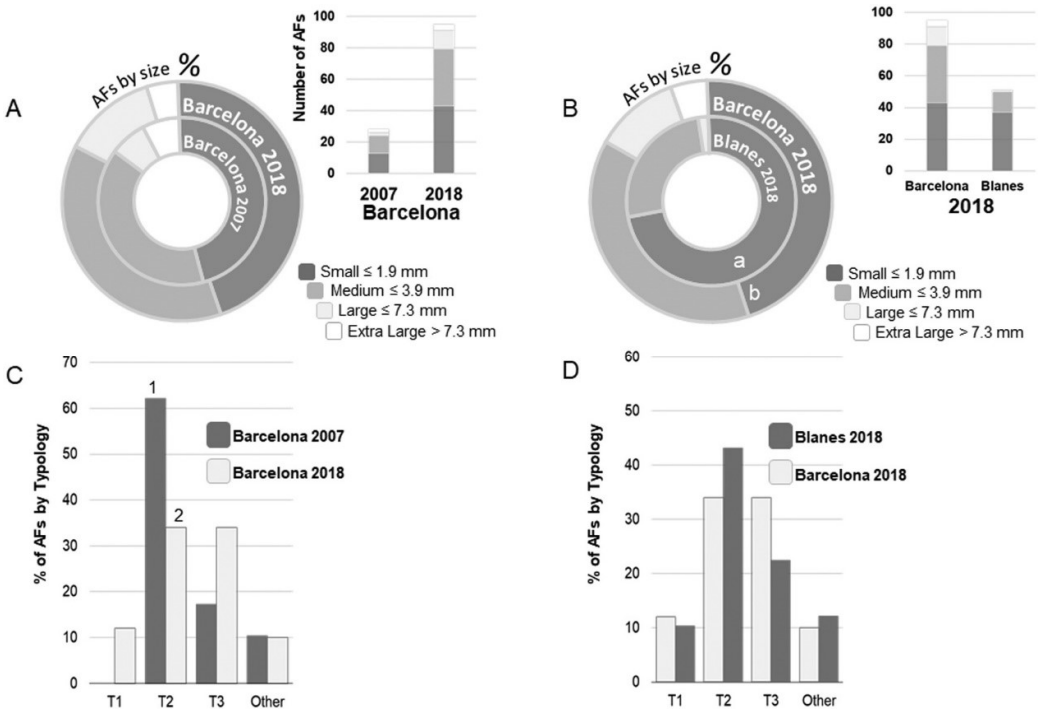

Fig. 2. Levels and typologies of anthropogenic fibres (AFs). Percentage (ring graph) and number (bar graph) of AFs by size between years A) and on a spatial scale B). Percentage of different anthropogenic fibres by typologies between years C) and on a spatial scale D). Different letters (a, b) show significant differences in AF size between localities (Barcelona–Blanes in 2018) ($p < 0.05$). Different numbers (1,2) in typologies are expressed when significant differences were found between years (Barcelona 2007–2018) ($p < 0.05$). Labels expressed in the X axis of figures C and D correspond to different fibre typologies described in the text: T1 (cellulose), T2 (cotton-shaped cellulose) T3 (PET), and others (unidentified).

3.2. AF characterization

The 167 AFs were classified into five distinct typologies according to GA and MA:

Typology 1 (T1): Prevalence: 10.69%, size range: 0.90–5 mm. GA: Cellulosic-like fibre, easily deformable; either with a circular section shape and a maximum thickness in the middle which narrows to pointed ends (Fig. 3, Typology 1, left), or long and smoothly striated and flat with angle-shaped folds (Fig. 3, Typology 1, right). Fibres are transparent, never dyed, slightly birefringent/iridescent. MA: From solid to clearly frayed edges; both crystalline and amorphous regions, without any pattern. A remarkably quick laser burnout was observed during Raman spectroscopy in samples of this typology. All samples shared similar spectra and were identified as cellulose (HQI 75.32%) (Fig. 4C).

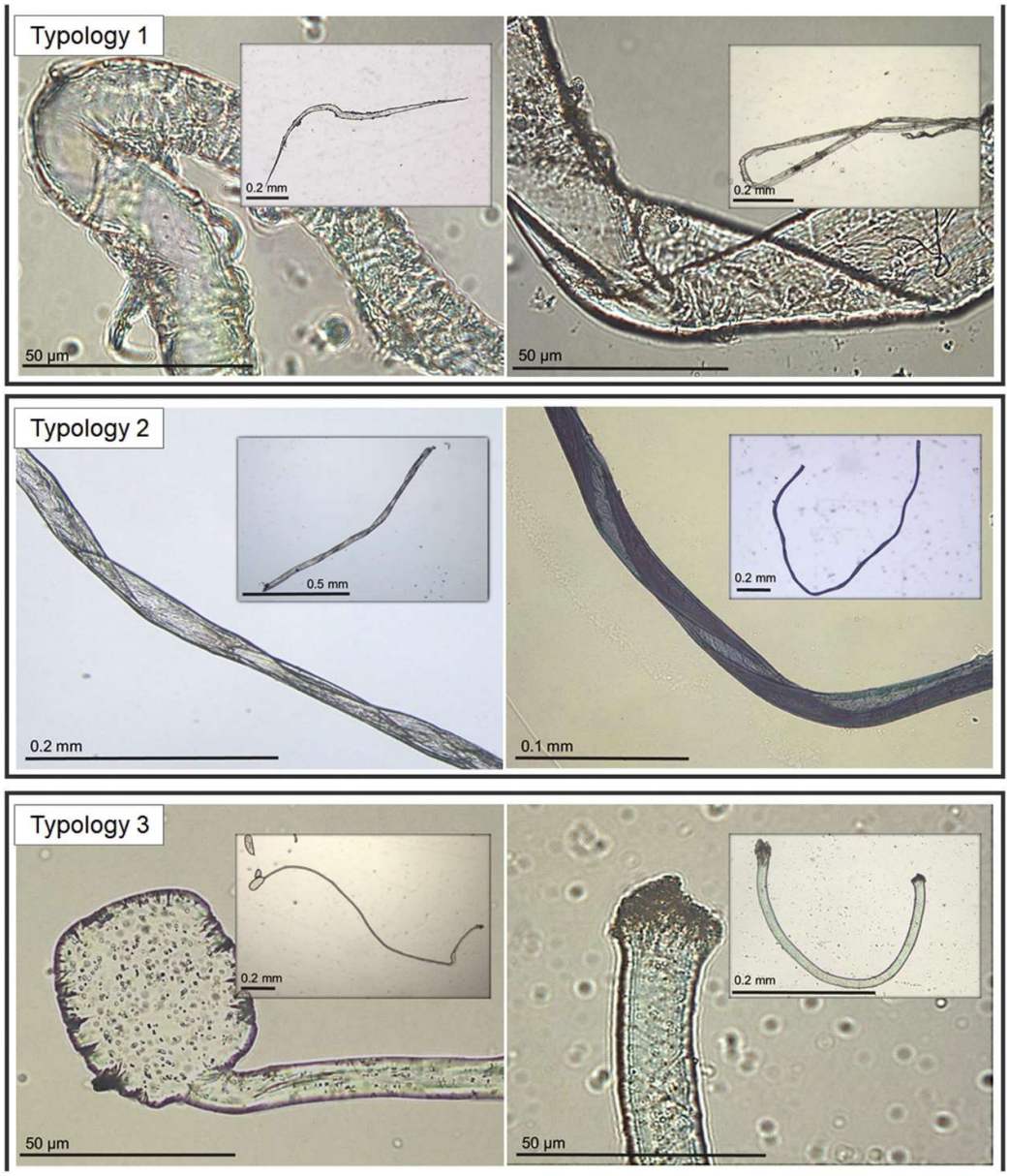

Fig. 3. Optical microscope images of anthropogenic fibres (AFs) found in the guts of *Mullus barbatus*. Distinctive features of each typology are shown with detail of the general appearance of AFs in the small box. Typology 1 identified as cellulose, typology 2 as cotton-shaped cellulose, and typology 3 as PET.

Typology 2 (T2): Prevalence: 46.10%, size range: 0.37–9.30 mm. GA: Curly cotton-shaped fibres with flat section, from clearly to slightly twisted; straight, frayed or with broken ends; sometimes angle folded; from translucent (Fig. 3, Typology 2, left) to deep blue dyed (Fig. 3, Typology 2, right). MA: non or slightly birefringent flat twisted fibre with solid edges. Spectra obtained in colourless samples of this category showed a similar pattern, and 80% were identified as cellulose (HQI > 70–80%) (Fig. 4C). When dyed, pigment was identified in 71.43% of samples which corresponded to Indigo dye spectra (HQI > 80%) (Fig. 4C–E).

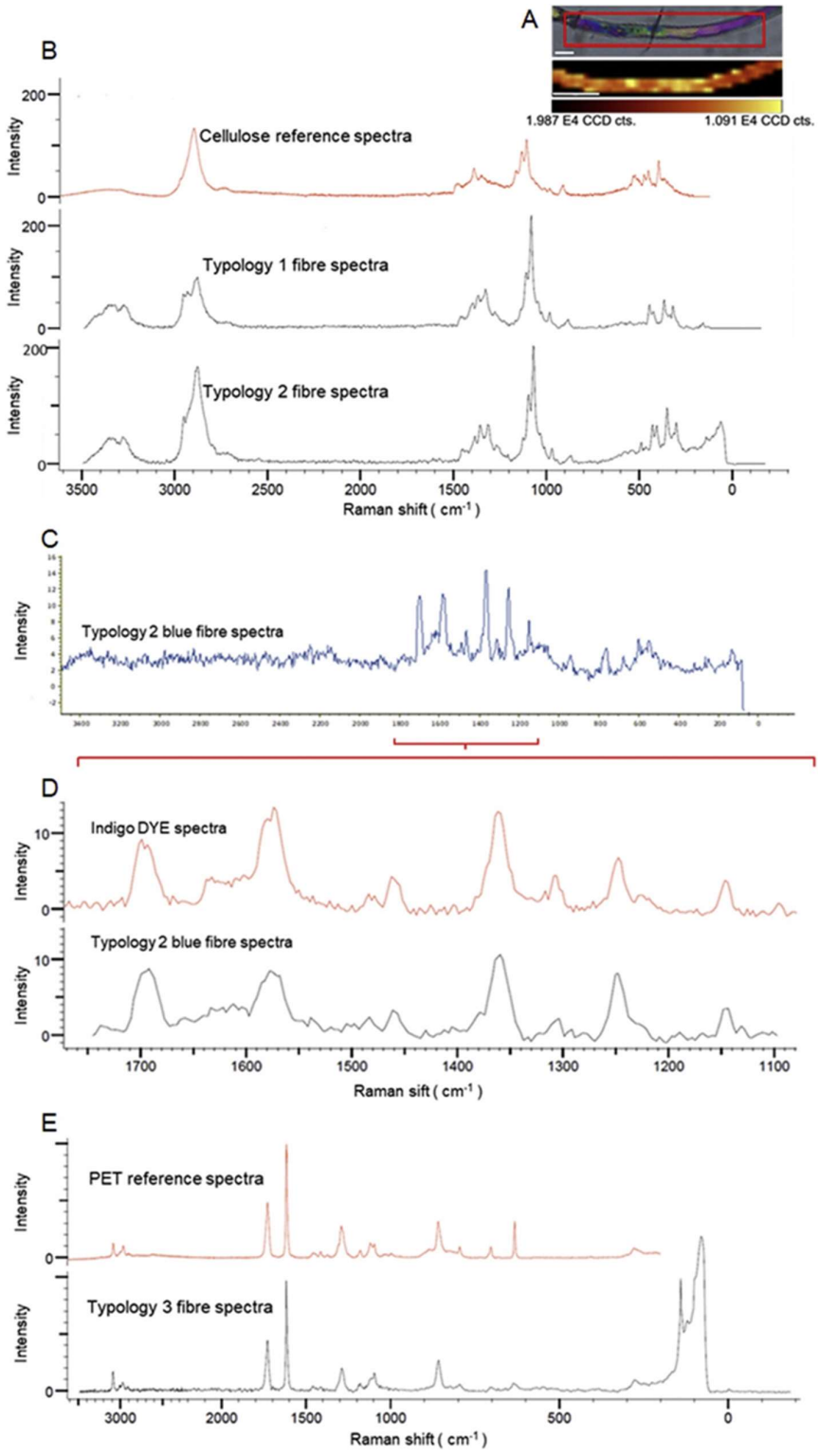

Fig. 4. Raman analyses. A) Example of fibre area analysed during Raman spectroscopy inwhich optical image (scale bar=100 µm) of fibre selection area (upper image) and Raman image (scale bar=50 µm) of the selected sampled area (inferior) are shown. The colour scale bar below represents intensity of the integrated spectral band. B–E) Raman scattering spectra of: Cellulose reference spectra compared to Typology 1 (HQI 75%) and 2 (HQI N 70–80%) comparedwith cellulose spectra (B), Typology 2 blue fibre spectra (C), section ofmaximum intensity of typology 2 blue fibre spectra compared to Indigo dye spectra (HQI N 80%) (D) and typology 3 (HQI ≥ 90) comparedwith PET reference spectra (E).

Typology 3 (T3): Prevalence: 31.14%, size range: 0.50–14.80 mm. GA: Rigid fibre with circular section and solid edges without fraying; if short usually hook shaped; frequently interrupted by molten flat areas of squashed appearance; ends with similar molten flat areas (Fig. 3, Typology 3, left) or club-shaped ends with a splintered breakage appearance (Fig. 3, Typology 3, right); colouration is from transparent to yellowish or slightly dyed (red, blue, yellow or green); crystalline without amorphous regions; MA: Discontinuities with a grainy appearance all along the fibre. Spectra of all samples (100%) analysed with Raman scattering from these typologies were identified as polyethylene terephthalate (PET) (HQI ≥ 90) (Fig. 4B).

Other fibres: Prevalence: 11.38%, size range: 1.10–6.30 mm. Other minority AFs found, these did not fit any of the previous descriptions and therefore were grouped into this category. No conclusive similarities were found in these spectra against reference spectra. Raman spectroscopy corroborated the correct visual classification by fibre morphology, since in all analysed cases, fibres from the same category share similar spectra.

Raman spectroscopy together with visual classification allowed identification of 56.79% of fibres as cellulose based (T1 and 2), and 31.14% of fibres as PET (T3), which corresponds to a total of 87.93% of the AFs characterized and identified.

3.3. Differences in AFs between a decade gap

A significant increase was detected in prevalence of AFs between 2007 (29%) and 2018 (74%) in Barcelona (X^2 = 15.157, $p < 0.001$) (Fig. 1, Table 2). The increase was also significant regarding the number of AFs per fish (nAF, X^2 = 26.286, $p < 0.001$), and the total AF length (TLAF, $F_{81,1}$=15.466, $p < 0.001$). None of these differences were associated with fish size (SL). No significant differences were found in the proportion (%) of AFs by size class between 2007 and 2018. Regarding typologies, T3 (PET) had higher values in 2018 (40%) with respect to 2007 (20%), although a X-squared test (X^2) was not significant due to the low sample size. In contrast, T2 (Cellulose) decreased significantly in 2018 (X2 = 6.847, p = 0.009).

3.4. Geographical variability of AFs

No significant differences in prevalence of AFs were detected between localities sampled in 2018 (Fig. 1, Table 2). However, AFs in Barcelona were both significantly more abundant (nAF, X^2 = 4.747, p = 0.029) and larger (TLAF, $F_{68,70}$ = 9.684, p = 0.003; large-sized AFs

in Barcelona, X^2 = 4.658, p = 0.033; small-sized AFs in Blanes: X^2 = 9.974, p = 0.002). None of these differences were related to fish size (SL). No significant differences were found among typologies between localities.

3.5. Effect of AFs on fish health

Indices of fish health status (HSI, GSI, K, and FULL) are shown in Table 1. Differences between years were found for K; this was significantly lower in 2018 ($F_{82,1}$ = 9.941, p < 0.005). FULL and GSI showed significant higher values in 2018, but this was related to fish size (X^2= 4.057, p= 0.044 and X^2=6.385, p < 0.05). Regarding the geographical comparison, only GSI was significantly lower (X^2 = 8.640, p = 0.003) in Barcelona (no association with fish size was detected). Spearman's test on nAF and TLAF did not show any correlation (p > 0.05) between AFs and biological health indices (HSI, GSI, K, and FULL).

Table 2. Anthropogenic fibres, parasite descriptors, and histopathological alterations found in *Mullus barbatus*. Mean and standard deviation (SD) of the number (nAFs) and total length (TLAFs) of the anthropogenic fibres found in the digestive system of *M. barbatus*. Prevalence (P%) of parasites and Cyst of Unknown Etiology (CUEs), mean abundance (MA) and standard deviation (SD) of parasites and Melanomacrophage centres (MMCs), and mean tissue area (A. Me., µm2) and standard deviation (SD) of MMC. Different numbers and letters show significant differences between years (Barcelona 2007–2018) and localities (Barcelona–Blanes in 2018), respectively (p < 0.05).

Locality	Barcelona								Blanes			
Year	2007				2018							
ANTHROPOGENIC FIBRES			Mean	(SD)			Mean	(SD)			Mean	(SD)
nAF			0.56[1]	(1.03)			2.77[2 a]	(2.43)			1.46[b]	(1.79)
TLAF			1.65[1]	(3.75)			7.64[2 a]	(9.18)			2.44[b]	(3.04)
PARASITES		P%	MA	(SD)		P%	MA	(SD)		P%	MA	(SD)
METAZOA												
CNIDARIA												
Myxozoa		16.67	-	-		8.57	-	-		17.14	-	-
NEMATODA		87.50	7.13 [1]	(6.97)		100	17.29 [2 a]	(11.35)		100	29 [b]	(32.81)
PLATYHELMINTHES												
Trematoda												
Digenea		83.33	3.06	(2.92)		77.14	3.54	(4.07)		82.86	5.51	(6.34)
Monogenea		2.08	0.02	(0.14)		5.71	0.06	(0.24)		-	-	
Cestoda		2.08	0.02	(0.14)		5.71	0.11	(0.32)		5.71	0.17	(0.86)
ARTHROPODA												
Copepoda		-	-	-		8.57	0.17	(0.62)		2.86	0.03	(0.17)
Isopoda		22.92	0.33	(0.72)		2.86	0.02	(0.17)		-	-	
PROTISTA												
AMOEBOZOA		8.33	-	-		-	-	-		-	-	-
APICOMPLEXA		-	-	-		-	-	-		5.71	-	-
CILIOPHORA		-	-	-		-	-	-		5.71	-	-
Shannon Diversity Index (H')	1.56				1.76				1.27			
Parasites Species Richness	4.10				5.26				3.86			
HISTOPATHOLOGY		P%				P%				P%		
Epitheliocystis		4.17				2.86				-		
Cysts of Unknown Etiology (CUEs)		25[1]				5.71[2 a]				34.29[b]		
	MA	(SD)	A. Me.	(SD)	MA	(SD)	A. Me.	(SD)	MA	(SD)	A. Me.	(SD)
Melanomacrophage centers (MMCs)	5.5[1]	(1.55)	1612.74	(606.28)	14.07 [2]	(8.99)	1388.64	(614.62)	7.39	(4.33)	1514.80	(792.58)

No relevant histopathological alteration was found in any organ of the analysed fish, except for cysts of unknown etiology (CUEs) observed in gills. CUEs consisted of cysts located mainly within gill filaments or lamellae, typically surrounded by cartilaginous tissue. Significant higher prevalence of CUEs was detected in Barcelona in 2007 (X2 = 5.369, p < 0.05) and in Blanes (X2 = 8.929, p < 0.05), with respect to Barcelona in 2018. No correlations between CUEs with fish size (SL) and anthropogenic fibres (nAF and TLAF) were found (GZM, p N 0.05). A significantly higher number of MMCs were detected in the spleens of fish from Barcelona in 2018 (X2 = 16.543, p < 0.001) than in 2007, but their size did not differ statistically (MA.MMC: p > 0.05). No significant differences were found for the size or number of MMCs between Barcelona and Blanes. No link between MMCs and SL was found (GZM; p > 0.05). Spearman's correlation test did not give any correlation of nMMC, nor MA.MMC when considering the anthropogenic fibres (nAF and TLAF).

A total of 2464 parasites were found in 118 *M. barbatus*, and only one individual was not parasitized. The most abundant group were nematodes, followed by digeneans, crustaceans, and cestodes (Table 2). Significantly higher values of parasite diversity and richness were found in Barcelona in 2018 (K–W = 5.293, p < 0.005 and K–W = 8.701, p < 0.005, respectively) compared to 2007. Significantly higher total abundance of parasites (X^2 = 6.6768, p = 0.009) was also found in 2018, particularly due to the higher abundance of nematodes (X^2 = 14.551, p < 0.001). Regarding the geographical comparison, higher values of parasite diversity and richness (K–W = 13.909, p < 0.005 and K–W = 11.130, p < 0.005, respectively) were found in Barcelona compared to Blanes. However, a significantly higher abundance of parasites was found in Blanes (X^2 = 4.200, p = 0.040), again due to the higher abundance of nematodes (X^2 = 4.478, p = 0.034). No significant differences by year or locality were found for the rest of the taxonomic groups of parasites (Table 2). A positive correlation was found between the number of parasites and the SL of fish (X^2 = 4.747, p = 0.029). No correlation (Spearman's correlation test, p > 0.05) was found between the total number of metazoan parasites (or each taxonomic group) and AFs (nAFs and TLAF), nor when considering only parasites within digestive tract (GZM, p > 0.05).

RDA explained 99.71% of variability by the first two axes. A positive relationship was observed between the number of parasites and fullness, as well as a weak negative relationship between the number of parasites, nAF, and TLAF with the condition indices K and HSI (Fig. 5), although none were significant.

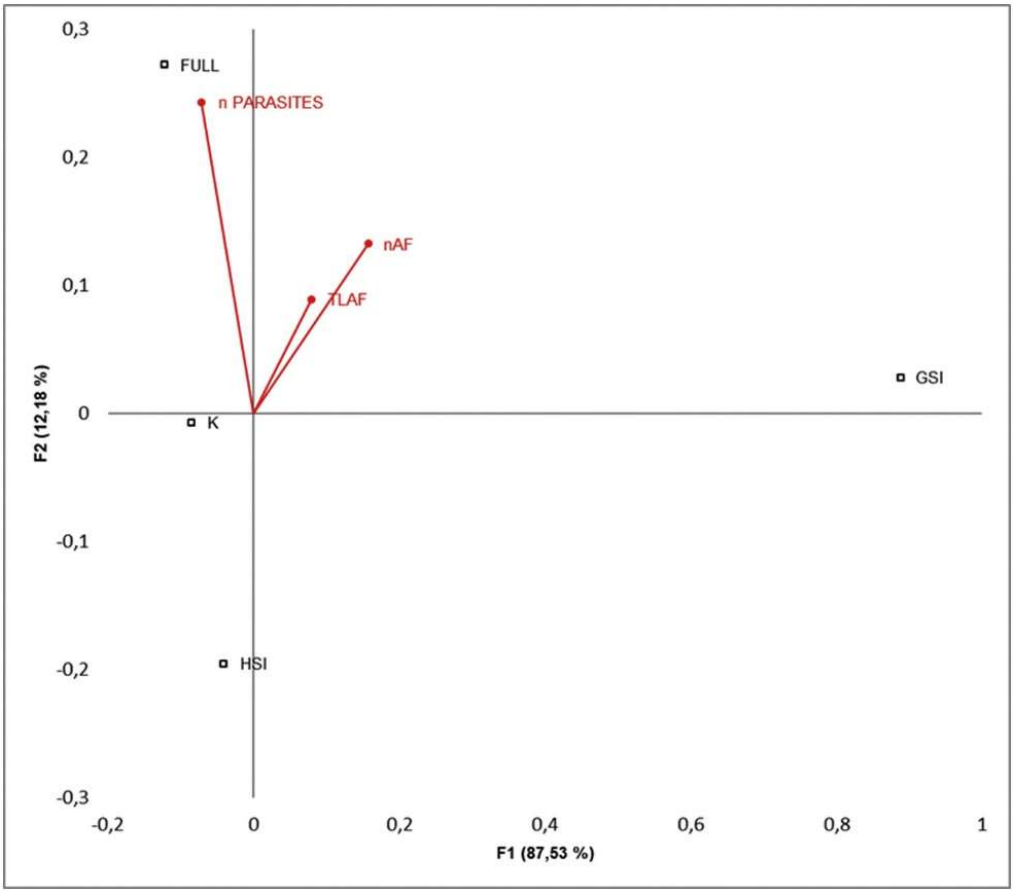

Fig. 5. Redundancy analysis (RDA) between anthropogenic fibres (AFs) and parasites with condition indices. Number (nAF) and total length (TLAF) of AFs and the number of parasites (nPARASITES) as explanatory variables with respect to the response variables of the fish health indices: condition factor (K), hepatosomatic index (HSI), gonadosomatic index (GSI), and fullness (FULL).

4. DISCUSSION

This study provides new information on the prevalence and abundance of AFs in wild organisms, and assesses the potential impact of this pollutant on the health status of fish. Half of the red mullet analysed in the present study presented AFs (plastic and non-plastic fibres) in their digestive tract, this is slightly higher than the prevalence reported by Bellas et al., (2016) in the same area (prevalence of 33% in Barcelona coast). However, similar values are found when comparing the average number of AFs per individual (1.48 AFs / ind.; SD = 1.98) in our study versus 1.75 (SD = 1.14) microplastics (MP)/ind. found by Bellas et al., (2016). This comparison must be considered with caution however, as Bellas et al., (2016) do not report on non-synthetic fibres. Differences in MPs among other areas in both basins of the Mediterranean Sea are usual: Spanish Mediterranean Coast (prevalence 18.8%, mean = 1.9 (SD = 1.29) MPs/ind., Bellas et al., 2016), Turkish shores (66% of fish ingested, with a mean of 2.12 (SD = 1.39)

MPs/ind., Güven et al., 2017), Adriatic Sea (64%, mean = 1.57 (SD = 0.78) MPs/ind.; Avio et al., 2015), Ionian Sea (32%, mean = 0.5 (SD = 0.20) MPs/ind.; Digka et al., 2018). Alomar et al. (2017) found slightly lower values in the sister species *M. surmuletus* in the nearby geographical area (Balearic Sea, 27.3%, with a mean number of 0.42MPs/ind.). Differences among these studies may greatly depend in the methodology used in each case. Most include only plastic items, and some of them also included other non-fibre items (e.g. microbeads or fragments), which increases the number of MPs per individual. Our results include mainly AFs; their detection is based on visual screening, and this includes plastic and non-plastic fibres, resulting in more than half of AFs being non-plastic fibres. Conversely, many of the previous studies have focused exclusively on plastic fibres, underestimating the number of AFs and making it difficult to compare these values among studies. Most of the studies based on MPs either purposefully discard non-plastic polymers, or generally use digestion and density separation methods to separate plastic material from organic matter (Hidalgo-Ruz et al., 2012). This digestion of organic matter with KOH, NaOH or H_2O_2, or by incubation at high temperatures, disintegrate cellulosic fibres in most cases (Dehaut et al., 2016) and may affect the artificial polymer integrity. As such, AFs are frequently neglected in studies.

4.1. AF characterization

Fibres are the major abundant shape found in *M. barbatus* in the present study (97% of debris were fibres), which is in agreement with other studies of the Mediterranean areas (Avio et al., 2015; Giani et al., 2019; Güven et al., 2017). There is only a single study of *M. barbatus* that reports an opposite trend in the Northern Ionian Sea (Digka et al., 2018), where fragments seemed to be the most important shape. Regarding size, the largest fibre found in our study is 14.8 mm, much larger than those reported in previous studies (sizes up to 3 mm or 5 mm) (Bellas et al., 2016; Digka et al., 2018). However, most of the fibres found in our fish were shorter than 4 mm (88%), which fits with the microplastic / microfibre size definition (MPs < 5 mm, Hartmann et al., 2019). The size of the fibres following this definition are based on their length, so fibres longer than 5 mm are not usually considered in studies of microplastics. Nonetheless, longer fibres (> 5 mm) found in guts usually appear entangled or folded on themselves or with other fibres (Carreras-Colomet al., 2018; Lusher et al., 2013), and may occupy a similar volume in the stomach to shorter fibres or other microplastics. Thus, this definition by size should be regarded with caution in ecological and health assessment studies of organisms. In order to give a more

realistic value of the volume occupied by these fibres in the digestive tract, we include the measure of the addition of each fibre length observed in each individual.

The results presented in this study demonstrate the importance of an accurate visual approach, that enables the discrimination between distinct fibre types (88% of AFs can be classified), and can infer their possible composition and origin, prior to analyses based on spectroscopic techniques. As Robertson et al., (2017) point out, AFs can be characterized visually by many morphological parameters which give them a high degree of variability, such as the colour, diameter, shape, particles included in the fibre, shape of the ends, cross-sectional shape, and size. This enables the identification of natural fibres (of animal, vegetable, or mineral origin), man-made fibres derived from natural polymers, such as cellulose (the regenerated fibres), and true synthetic fibres synthesized from simple organic chemicals found in petrochemicals (Robertson et al., 2017; Stanton et al., 2019). For example, colour is not useful to distinguish between natural (e.g., cotton) and artificial (e.g., viscose) fibres (Remy et al., 2015), but delustrants (such as paraffin wax or titanium dioxide) enable the discrimination of artificial fibres (Robertson et al., 2017). These substances are additives used during the manufacturing process to reduce the brightness of the resulting polymer, and are perceived under the microscope as inclusions of a grain like appearance (Robertson et al., 2017) such those observed in T3 fibres. Raman spectroscopy later confirms the non-natural composition (PET) in this type of fibre. The molten areas found in T3 fibres have been well described for synthetic textile fibres such as polyesters (De Wael et al., 2011; Lepot et al., 2008). These parts are formed by ironing, or during the industrial fabrication process (DeWael et al., 2011), and is another characteristic feature that enables their classification as a thermoplastic, to further confirm its PET composition.

Our results show that 57% of the fibres in the gastrointestinal tract of *M. barbatus* are cellulosic, versus 31% from PET, this is in agreement with the fact that cellulosic fibres are slightly dominant over synthetic polymers in some Mediterranean marine environments (Sanchez-Vidal et al., 2018). Cellulose-based fibres are one of the most used fibres in the textile industry (Textile World, 2015), these includes natural fibres such as cotton, and man–made regenerated fibres produced by dissolving a cellulose-based raw material, such as viscose or rayon. Cotton can be recognized by its flattened and twisted appearance (Robertson et al., 2017), as represented by the fibres grouped in T2 in the present study. This characteristic curly shape comes from the natural twist or convolution formed during natural fibre growth. Moreover, a high percentage of indigo blue dye is further detected by Raman spectroscopy analysis in T2 fibres, which is also the most used dye in blue-dyed cotton fibres, called denim fibres, that are characteristic of Jeanswear (De Wael et al., 2011; Grieve et al., 2006; Grieve and Biermann, 1997; Robertson et

al., 2017). Due to their morphology and colour, cellulosic fibres classified as T1 do not seem to have a textile origin.

The morphology of synthetic fibres, and the use of certain dyes in these fibres suggests that most anthropogenic fibres found in our study may have a textile origin (46% of T2—cotton-shaped fibres—plus 31% of T3—PET). How textile fibres from the waste effluent of washing machines travel via wastewater to sewage treatment plants is well described (Dris et al., 2015; Leslie et al., 2017), as is how they end up in the ocean (Browne et al., 2011; Napper and Thompson, 2016). Different habitats accumulate different types of marine debris (Anastasopoulou et al., 2018) due to their density. While denser polymers (e.g. PET and cellulose) tend to sink to the seabed, lighter particles are more commonly found floating in pelagic waters (e.g. low-density polyethylene LDPE) (Andrady, 2011). Although some exceptions have been described (Bottari et al., 2019), demersal fish species, such as *M. barbatus*, ingest more dense polymers when compared to species with shallower habitats (Alomar et al., 2017; Avio et al., 2015).

In the Mediterranean continental shelf and deep seafloors, specifically in the same geographical region of the present study, cellulosic fibres are the most abundant (80%), followed by PET (12.9%), acrylic (polymethyl methacrylate), polyamide, polyethylene, and polypropylene (Sanchez-Vidal et al., 2018); this is in agreement with the relative importance of the fibres found within the digestive tract of *M. barbatus* by the present study. PET is the most abundant synthetic polymer fibre found in *M. surmuletus* in Mediterranean Sea (Alomar et al., 2017). However, several studies in the Mediterranean Sea have also found non-plastic fibres—mainly cellulose-based—in different fish species (Compa et al., 2018; Savoca et al., 2019); such as the case in the present study. As previously explained, non-plastic fibres could be easily mistaken with plastic (Remy et al., 2015), so its importance in marine environments could be greater that is currently appreciated.

4.2. Differences between a decade gap

Not only the prevalence of AFs in Barcelona is higher in 2018 with respect to the same area in 2007, but also nAF and TLAF values were 5 and 4 times higher in 2018 than 2007, respectively.

The presence of AFs in the gastrointestinal tracts of the studied fish from 2007 demonstrates how the ingestion of this type of debris is not a new phenomenon in the NW

Mediterranean Sea. The sharp increase, not only in its prevalence but also in the number of AFs in 2018, could be the reflection of the accumulation of this debris in the marine environment (Avio et al., 2015) over the last decade. However, this interpretation should be taken with caution because differences between years could be also attributed to sporadic oscillations of different events in the same area at different times, resulting in a stochastic increase in AFs. For example, rain regime in the Barcelona area was especially abundant during 2018 (984.2 mm) compared to mean value (580.6 mm) over the past 10 years (Meteocat, 2019), which may result in an exceptional increase of river discharges in that area. Therefore, it is not possible to clearly establish an upward trend over the years based on our data.

The results seem to indicate a change between the years of the study in the levels of contamination of cellulosic fibres towards PET fibres, since significantly lower values are detected of T2 fibres (characterized as cellulose with a possible textile origin) in 2018. Changes in textile demands of recent decades may support this tendency because, currently, synthetic fibres such as polyesters like PET dominate the global fibre market, and have overtaken natural cotton, which has a declining production year-on-year (Carr, 2017).

4.3. Geographical variability

AF abundance is higher in Barcelona than in Blanes, and despite not being significant, the number of fish containing AFs are also higher in Barcelona. In addition, higher values of TLAF (three times higher in Barcelona with respect to Blanes) may be due to both an increase in the number of ingested fibres, and the larger size of these fibres. The high levels of AFs found in Barcelona as compared to Blanes may result from the specific level of anthropization of the surrounding land areas, the level of water discharge by rivers, and the specific features of the continental shelf. The main inputs of plastic to the marine environment come from industrialized and densely populated coastal areas (Andrady, 2011; Derraik, 2002; Jambeck et al., 2015), such the area surrounding Barcelona. Several pathways of dispersion of microfibres into the marine environment have been described, such as atmospheric fallout (Dris et al., 2017), waste water treatment plants, and storm-water runoff (Wagner and Lambert, 2018). The latter are considered as a critical input of synthetic fibres to the aquatic environment via river basins which finally discharge into the ocean (Horton et al., 2017b; Murphy et al., 2016). Both presently studied areas are under the influence of different rivers which drain water from urbanized areas, so the presence of AFs is expected (de Haan et al., 2019; Sanchez-Vidal et al., 2013). The continuous river regime, and the higher level of flow in Barcelona compared to Blanes may

explain the higher levels of AFs found in Barcelona. Moreover, the submarine canyon of Blanes—a great canyon that drives sediment transport into the deep-sea and minimizes sediment deposition in southern continental shelf—partially reduces sediment discharge and pollutants from this river. The northern shelf of Blanes is already a sediment-starved natural area, due the same effect of northern submarine canyons (Durán et al., 2014). In addition to river discharge, estuarine and river benthic sediments influence the microfiber accumulation (Horton et al., 2017a; Simon-Sánchez et al., 2019), that are eventually retained in the continental shelf (Sanchez-Vidal et al., 2018). Muddy and fine-grained sediments, like those of the Llobregat and Besòs rivers, may have a greater retention of pollutants (Van Cauwenberghe et al., 2013) in contrast to sandy sediments, like the sandy course immature sediments of the Tordera river (Durán et al., 2014). Hence, the continental shelf off Barcelona where red mullets inhabit may accumulate higher amounts of AFs when compared to the Blanes shelf.

The absence of significant differences in fibre composition between Barcelona and Blanes suggests that the two sites reflect a similar source or similar contribution in both areas, driven by human population density. However, a higher proportion of T2 fibres was found in Blanes, which may suggest a source of this cotton typology specific to this area.

4.4. Effect of AFs on fish health

In the present study, the levels of ingested AFs in *M. barbatus* do not seem to interfere with feeding activity, nutritional state, or reproductive capacity, since no relationship is found between the number or size of AFs and fish health condition indicators (HIS, GSI, K, FULL). Differences by year and locality observed in GSI, FULL, and K are most probably related to fish size and maturity, which can be easily related to fish biology (e.g. reproduction events). GSI is a consequence of gonad development that requires use of fish lipid reserves, therefore higher values of GSI result in a reduction of K. To restore this loss of energy, feeding intensity increases, which is reflected in higher values of FULL (Wootton, 1989). In wild pelagic species, such as *Sardina pilchardus*, lower values of K have been attributed to higher values of AF ingestion, although this relationship is not clearcut, since other variables such as latitude seem to be confounding the issue (Compa et al., 2018). In fact, effects of MP on body length or condition factors are ambiguous, as both reduced and increased levels are reported (Kögel et al., 2020).

It is known that the ingestion of fibres —both plastic and non-plastic —can result in agglomerations of fibres due to their shape; these may block and affect the physical performance of fish digestive tracts (Lusher et al., 2013). This is an effect demonstrated in some

wild crustaceans, and is probably enhanced by the characteristic anatomy of their digestive tracts (Carreras-Colom et al., 2018; Welden and Cowie, 2016). However, in wild fish, these effects are not usual. Entangled and folded fibres are observed in *M. barbatus* in the present study but without producing balls, probably due to the small number of AFs ingested, and the shape of the digestive tract in this species. The digestive tract of the red mullet does not present too many modifications, apart from the pyloric caeca which is present several digitations (Le Pommelet and Silan, 1998). Smaller fibres were found in this part of the intestine but—as other authors suggest (Grigorakis et al., 2017) — they seem to be normally egested without accumulation, since no differences in number of fibres are observed along the entire digestive tract.

Histopathological alterations attributable to AF ingestion are not found in present study. CUEs is the only histological alteration found in *M. barbatus*, with a similar prevalence described by previous studies in NW Mediterranean Sea (Carreras-Aubets et al., 2011). Although the presence of this alteration has been linked to environmental pollution (Carreras-Aubets et al., 2011; Munday and Brand, 1992), no relationship with AFs were found in our study. Splenic MMCs are known to increase in size and frequency, due to multiple factors such as: fish age, infectious processes, cell destruction, recycling or storage of endogenous and exogenous materials, and environmental chronic stress (Agius and Roberts, 2003). In this study, the change in the number of splenic MMCs between years is not related to AFs. However, under laboratory conditions, histopathological alteration due to the ingestion of anthropogenic particles are commonly reported following artificially exposures (Kögel et al., 2020; Limonta et al., 2019). It is important to note that the concentration of MP used in laboratory analysis is usually far higher than is found under natural conditions, and that concentration seems to be one of the most important factors regarding harm caused to fish (Kögel et al., 2020). In these studies, the most frequent alterations reported in fish linked to MP (>10 µm) were reduction in activity, increase in physiological stress, and hormonal dysregulation, together with intestinal damage (Kögel et al., 2020). Alterations in the digestive tract epithelium in direct contact with ingested AFs may be expected, but this appears not to be the case in *M. barbatus*. Our findings agree with those of other authors (Batel et al., 2020; Jovanović, 2017), which indicate that ingested MPs pass along the intestinal lumen without causing harm. Although some authors detect plastics of up to 0.6 mm in liver (Avio et al. 2015), it is highly unlikely that MPs or AFs as large as those in this study are able to pass through the intestinal barrier and reach other organs; only smaller AFs (usually < 100-150 µm) or their additives (often toxic) may do so. Some of the alterations described in the liver due to MP exposure (and associated toxins or pollutants) include glycogen

depletion, fatty vacuolation, inflammatory infiltration, or necrosis (Sures, 2001; Lu et al., 2016; Rochman et al., 2013). However, none of these signs are observed in fish from the present study, which again indicate that AFs ingested by *M. barbatus* are excreted without any adverse effects.

Parasites of the red mullet have been well described in the Mediterranean Sea, as has the variation of parasites in relation to environmental pollutants (Carreras-Aubets et al., 2011, 2012). In the present study, higher parasite abundance, richness, and diversity are observed in 2018 and in Blanes (when comparing locations), but also in relation to fish size; this is especially due to the high numbers of nematodes. This is a common phenomenon associated with host longevity and space available for especially long-lived larval nematodes (González and Acuña, 2000). Parasites are commonly used as early warning bioindicators for environmental and fish health assessment (e.g. Lafferty, 1997; Marcogliese, 2005). However, there are no studies relating variations in parasites communities with plastics. In the present study, the ingestion of AFs in *M. barbatus* does not seem to have effects on parasite descriptors, nor vice versa. Thus, the parasites within the digestive tracts of *M. barbatus* do not seem to interfere with the retention and accumulation of MPs, which goes against the hypothesis of Hernandez- Milian et al. (2019).

5. CONCLUSIONS

The ingestion of AFs by *Mullus barbatus* is not a new phenomenon in the Mediterranean Sea since it was already detected 10 years ago. The provided visual fibre characterization, corroborated by spectroscopic techniques, allow the identification of distinct fibres typologies in both synthetic polymers and cellulose. The most abundant fibres found herein were cellulosic-based fibres followed by PET fibres, with a possible textile origin, while other typologies were rare. *M. barbatus* appear to show geographical changes in the number of ingested AFs, and this also differed between years. However, in no cases did health status indicators (condition indices, histopathological, and parasitological analyses) reflect an effect of AF ingestion. Although several studies indicate the potential negative effect of these kind of pollutants, this study demonstrates that no damage to the health condition was detected at levels of AF ingestion for wild fish. Our results reinforce the use of the red mullet as a benthic species suitable for the monitoring of this type of marine debris, both spatially and temporally. Furthermore, fish with varied feeding behaviours and living habitats should be also monitored in future work, as AFs may affect them in different ways.

CRediT authorship contribution statement

Oriol Rodríguez-Romeu: Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. María Constenla: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. Maite Carrassón: Conceptualization, Conceptualization, Project administration, Writing - review & editing, Funding acquisition. Mariano Campoy-Quiles: Formal analysis, Resources, Writing - review & editing, Funding acquisition. Anna Soler-Membrives: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by Spanish Ministry of Science and Technology with the "BIOMARE" project (CTM2006- 13508- C02- 01MAR) and the Complementary Action (CTM2006- 28145- E/MAR), the Catalan Department of Agriculture, Livestock, Fisheries and Food (European Maritime and Fisheries Fund (EMFF) with the project "SOMPESCA" (ARP059/19/00003) and the Spanish Ministry of Science, Innovation and Universities with the "PLASMAR" project (RTI2018-094806-B- 100) and the "Severo Ochoa" excellence program of ICMAB (SEV- 2015-0496). MCQ thanks the Ministerio de Ciencia, Innovación y Universidades MICINN for the "Severo Ochoa" excellence program of ICMAB with reference SEV-2015-0496. Authors would like to thank Mrs. Maria José Ramos-Sosa for her invaluable research assistance. We also thank all staff of the oceanographic vessel "García del Cid", the fleet of the commercial fishing vessels of Barcelona and Blanes, and fisherman associations "Confraria de Pescadors de Barcelona" and "Confraria de Pescadors de Blanes" for their support during sampling cruises. We are very grateful to Amy MacLeod (EditingZoo) for the English editing.

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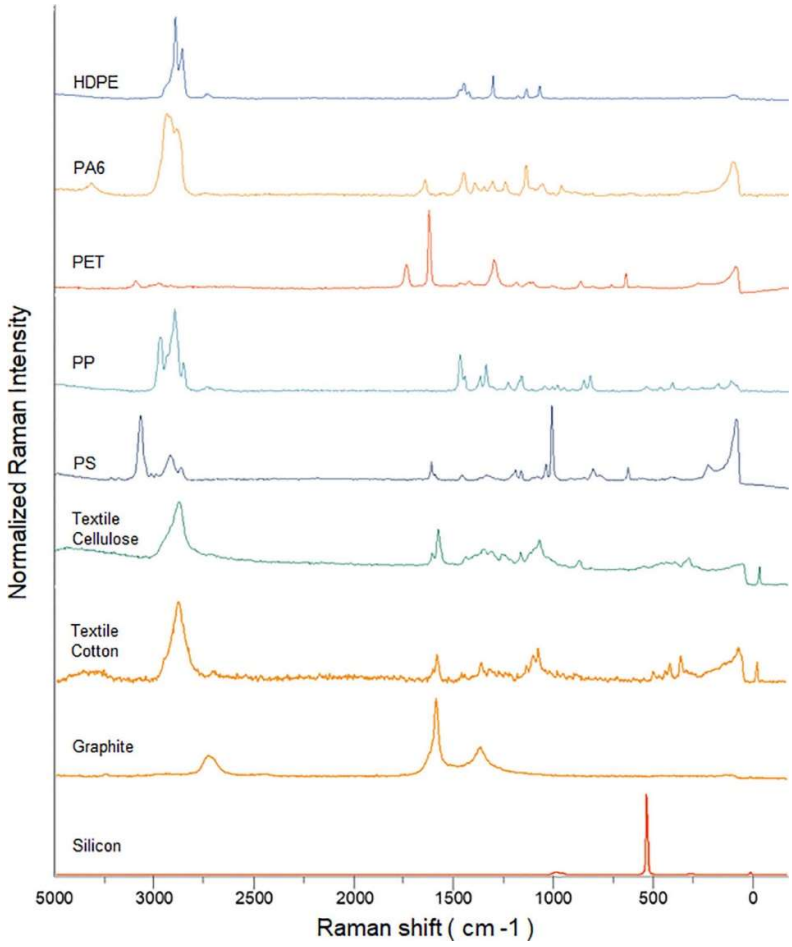
SUPLEMENTARY MATERIAL



Supplementary figure. Custom library spectra obtained by Raman spectroscopy of target materials. High density polyethylene (HDPE), nylon (PA6), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), textile cellulose (rayon), textile cotton, graphite, and silicon.

Supplementary table. Prevalence (P%), mean abundance (MA), and standard deviation (SD) of parasite species for each taxonomic group found in Mullus barbatus

Locality	Barcelona						Blanes		
Year	2007			2018					
PARASITES	P%	MA	SD	P%	MA	SD	P%	MA	SD
METAZOA									
CNIDARIA									
Myxozoa und.	16,7	-	-	8,6	-	-	17,1	-	-
NEMATODA									
Nematoda und.	56,3	1,98	(2,55)	85,7	3,51	(2,83)	57,1	2,69	(3,10)
Rhabditida									
Hysterothylacium aduncum larva	12,5	0,21	(0,68)	11,4	0,14	(0,43)	8,6	0,09	(0,28)
Hysterothylacium fabri larva	79,2	3,69	(4,25)	91,4	9,09	(8,25)	97,1	22,89	(29,02)
Hysterothylacium sp. larva	37,5	0,81	(1,51)	82,9	2,37	(2,84)	42,9	2,29	(4,02)
Ascarophis sp.	2,1	0,02	(0,14)	5,7	0,06	(0,24)	5,7	0,06	(0,24)
Contracaecum sp. larva	10,4	0,15	(0,55)	17,1	0,29	(0,71)	5,7	0,11	(0,53)
Cucullanus sp.	4,2	0,04	(0,20)	34,3	1,09	(2,25)	14,3	0,31	(1,08)
Dichelyne sp.	-	-	-	11,4	0,17	(0,57)	8,6	0,14	(0,55)
Raphidascaris sp.	-	-	-	2,9	0,03	(0,17)	2,9	0,03	(0,17)
Trichinellida									
Capillaria sp.	12,5	0,13	(0,33)	22,9	0,29	(0,57)	14,3	0,31	(0,90)
Capiloroides sp.	2,1	0,04	(0,29)	11,4	0,14	(0,43)	2,9	0,03	(0,17)
Paracapillaria sp.	4,2	0,06	(0,32)	11,4	0,11	(0,32)	5,7	0,06	(0,24)
PLATYHELMINTHES									
Trematoda									
Digenea und.	31,3	0,58	(1,01)	25,7	0,34	(0,68)	11,4	0,14	(0,43)
Aponurus mulli	10,4	0,19	(0,76)	8,6	0,11	(0,40)	2,9	0,11	(0,68)
Opecoloides furcatus	66,7	1,98	(2,35)	60,0	2,66	(3,79)	65,7	4,77	(6,01)
Paracanthium furcatum	8,3	0,13	(0,49)	11,4	0,11	(0,40)	11,4	0,11	(0,40)
Posornynchus crucibulum	-	-	-	2,9	0,03	(0,17)	-	-	-
Protoctrema bacilliobatum	4,2	0,13	(0,73)	5,7	0,14	(0,69)	14,3	0,34	(1,39)
Pseudopecoeloides sp.	6,3	0,06	(0,24)	2,9	0,09	(0,51)	-	-	-
Monogenea									
Monogenea und.	2,1	0,02	(0,14)	5,7	0,06	(0,24)	-	-	-
Cestoda									
Scolex pleuronectis larva	2,1	0,10	(0,72)	-	-	-	-	-	-
Nybelinia sp.	2,1	0,02	(0,14)	5,7	0,06	(0,24)	5,7	0,17	(0,86)
ARTHROPODA									
Copepoda									
Hatschekia mulli	-	-	-	8,6	0,17	(0,62)	2,9	0,03	(0,17)
Isopoda									
Gnathia sp. (praniza larva)	22.9	0.33	(0.72)	2.9	0.03	(0.17)	-	-	-
PROTISTA									
AMOEBOZOA									
Amoeba und.	8.3	-	-	-	-	-	-	-	-
APICOMPLEXA									
Coccidiasina und.	-	-	-	-	-	-	5.7	-	-
CILIOPHORA									
Trichodina sp.	-	-	-	-	-	-	-	-	-
Ciliophora und.	-	-	-	-	-	-	5.7	-	-

Supplementary data to this article can be also found online at https://doi.org/10.1016/j.scitotenv.2020.139336.

4.CHAPTER II:

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.

Oriol Rodríguez-Romeu, Anna Soler-Membrives, Francesc Padrós, Sara Dallarés, Ester Carreras-Colom, Maite Carrassón, Maria Constenla

Science of the Total Environment 841 (2022) 156539

DOI: http://dx.doi.org/10.1016/j.scitotenv.2022.156539

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 risk 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.

H I G H L I G H T S

- 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.

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. MATERIAL 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.

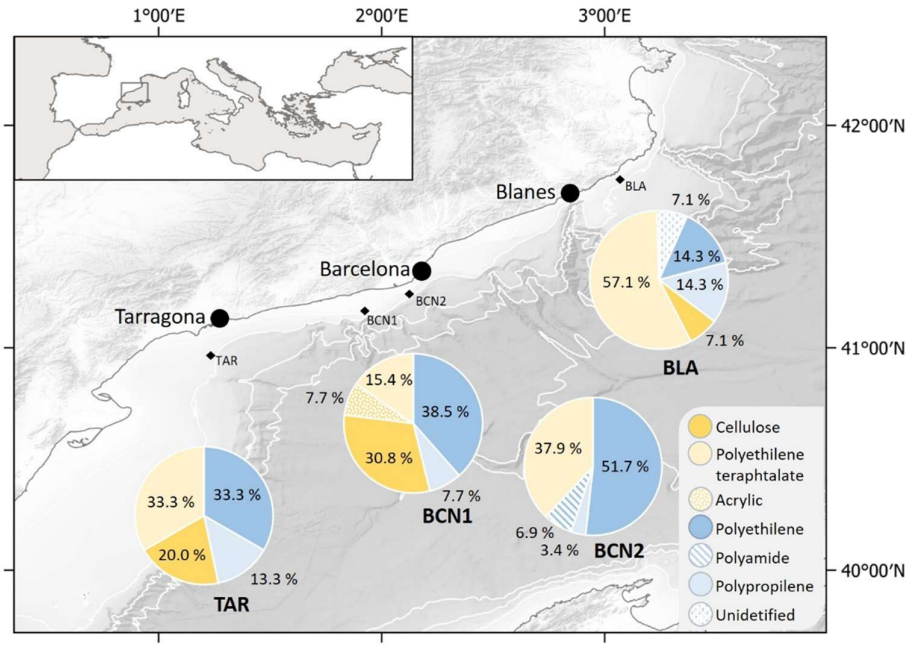


Fig. 1. Map of sampling area where sampling for European anchovy occurred. The diamond shape (♦) indicates sampling stations. From north to south, Blanes (BLA), Barcelona (in 2007, BCN1, and in 2019, BCN2), and Tarragona (TAR). Pie charts represent the percentage of each polymer type from the anthropogenic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

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 / ($\alpha \times TL^{\beta}$)), 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 (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 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 thermos cycling conditions: initial denaturation at 95 °C for 15 min followed by 35 cycles (denaturation for 5 min at 80 °C, followed by 1min 30 s at 92 °C, annealing for 1min 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 thermos cycling 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

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 · min-1 · mg protein-1 ; j µmol · min-1 · mg protein-1 ; k pmol · min-1 · mg protein-1

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.

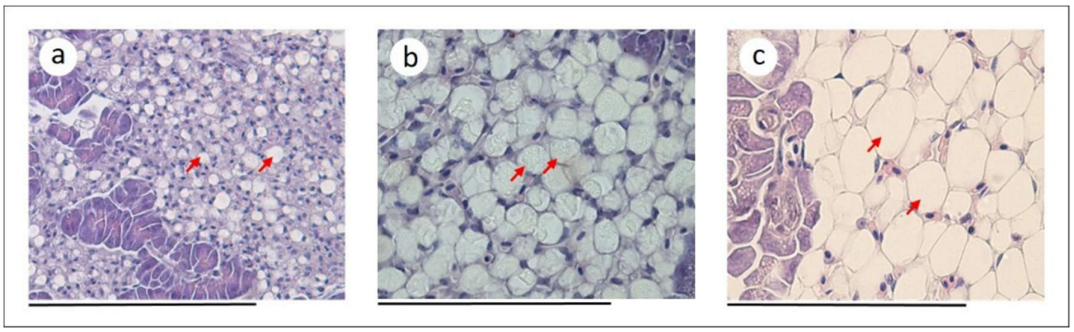


Figure 2. Optical microscope images giving examples of fat tissue in European anchovy in which adipocytes (indicated by red arrows) of different size classes: a) small size, b) medium size, and c) large size, can be seen. Scale bars at the bottom left of each picture represent 0.1 mm.

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).

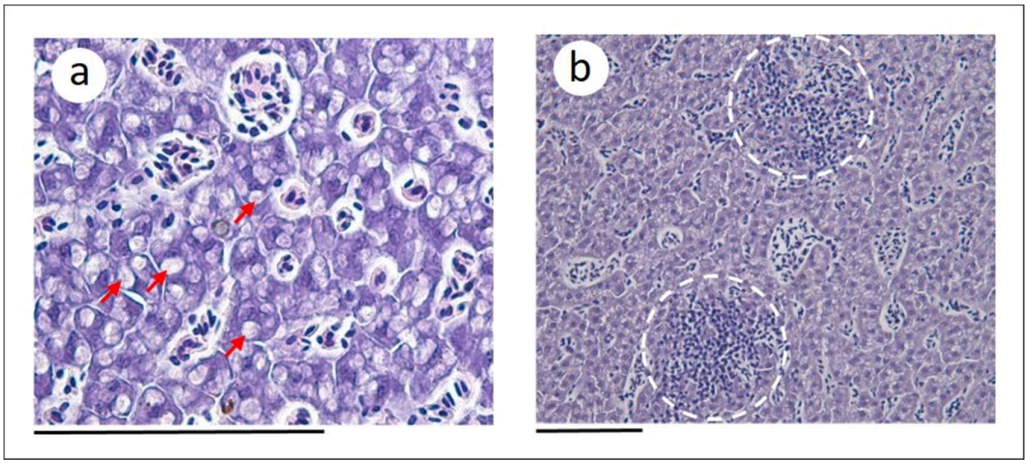


Fig. 3. Optical microscope images of the main histological alterations found in livers of European anchovy. Showing a) vacuole-like structures (indicated by red arrows) in the cytoplasm of hepatocytes, and b) inflammatory foci formed by patches of immune cells (surrounded by dashed white circles). Scale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

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 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).

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).

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 (µm2) 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 [1 2]	(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 [1 2]	(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 [1 2]				45 [2]		
Melanomacrophage centers (MMCs)		80 [1]				76 [1 2]				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)

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 $^{b\,1}$		53 $^{1\,2}$		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 $^{b\,1}$	(1.94)	1.07 $^{1\,2}$	(1.44)	0.67 ^{2}	(1.21)
Total plastic	0.43 ^{a}	(0.63)	1.23 $^{b\,1}$	(1.68)	0.37 ^{2}	(0.72)	0.40 ^{2}	(0.81)
n Particles	0.23 ^{a}	(0.50)	1.17 $^{b\,1}$	(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 $^{b\,1}$	(1.66)	0.18 ^{2}	(0.53)	0.43 ^{2}	(1.18)

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 %) 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).

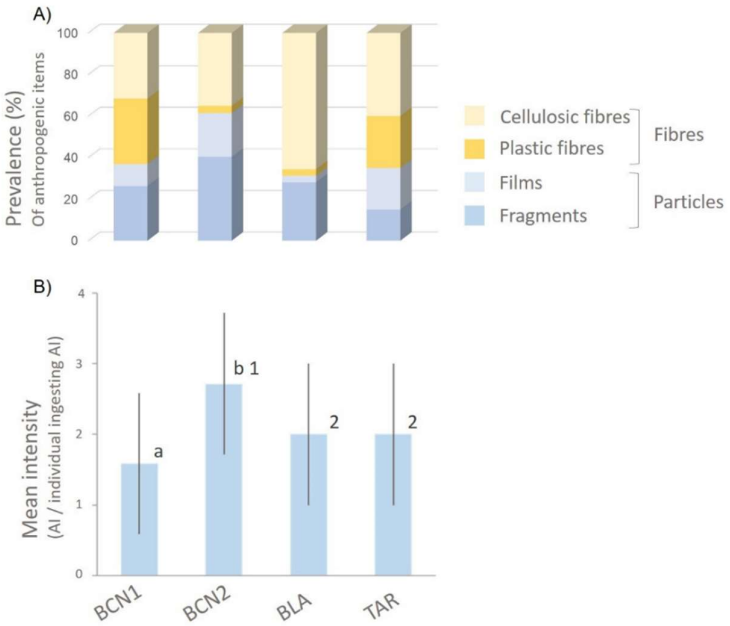


Figure 4. Values of ingestion of anthropogenic items (AIs) by European anchovy showing A) Prevalence as a percentage of the AIs ingested by typology (fibres and particles), composition of fibres (plastic or cellulosic) in the sampling stations. And B) mean intensity (number of AIs/number of fish ingesting 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 ($p < 0.05$).

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 ≤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≤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).

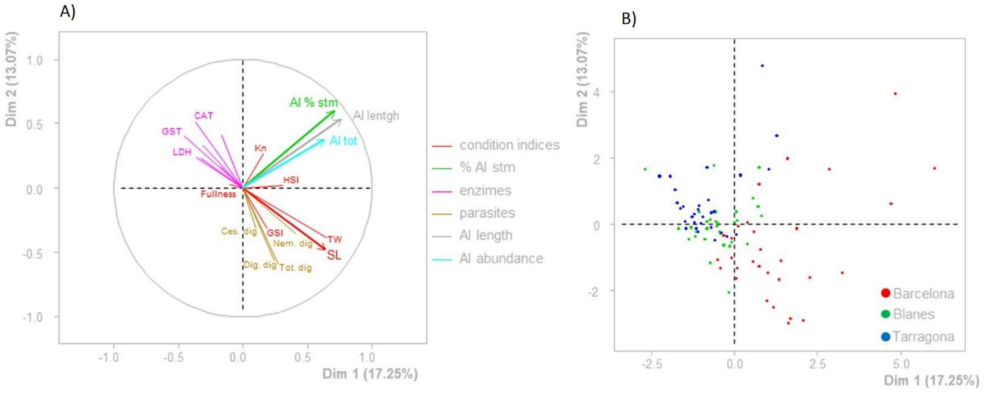


Figure 5. Multiple factor analysis (MFA) among fish from locations in 2019. (A) MFA among body condition indices (standard length (SL); total weight (TW); Le Cren relative condition index (Kn); stomach fullness (Fullness); hepatosomatic index (HSI); and gonadosomatic index (GSI)), abundance of anthropogenic 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.

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 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).

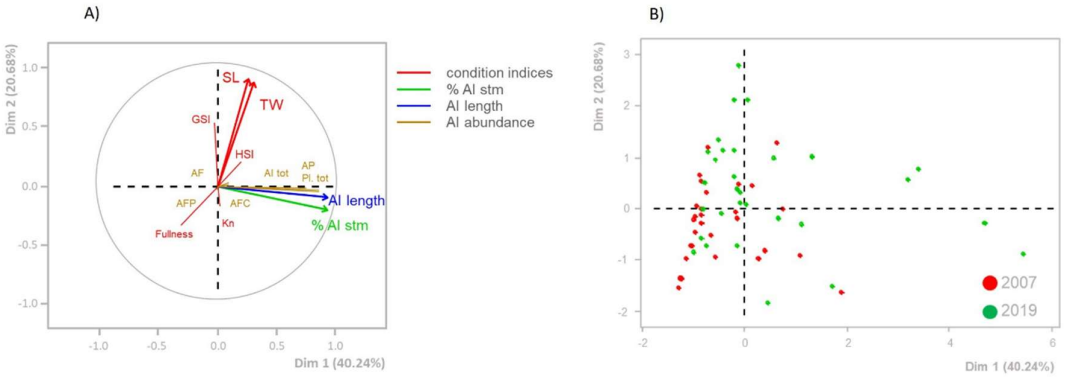


Figure 6. Multiple factor analysis among fish from Barcelona between a 12-year interval (2007 vs 2019). (A) Multiple factor analysis between body condition indices (standard length (SL); total weight (TW); Le Cren relative condition index (Kn); stomach fullness (Fullness); hepatosomatic index (HSI); 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.

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 nematode's 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.

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maite Carrasson reports financial support was provided by Autonomous University of Barcelona. Maria Constenla reports financial support was provided by Autonomous University of Barcelona.

Acknowledgements

This study was supported by the Spanish Ministry of Science and Technology "BIOMARE" project (CTM2006-13508-C02-01MAR) and the Complementary Action (CTM2006-28145-E/MAR); the project "SOMPESCA" (ARP059/19/00003) by the Catalan Department of agriculture, Livestock, Fisheries and Food and European Maritime and Fisheries Fund (EMFF)) and the Spanish Ministry of Science, Innovation and Universities project "PLASMAR" (RTI2018-094806-B-100). Authorswould like to thank Andrea Millán Sánchez for her invaluable research assistance. We also thank the fleet of commercial fishing vessels of Barcelona, Tarragona, and Blanes, and the fisherman associations "Confraria de Pescadors de Barcelona", "Confraria de Pescadors de Tarragona", and "Confraria de Pescadors de Blanes" for their support during sampling cruises. We are very grateful to Amy MacLeod (EditingZoo) for the English editing. Finally, authors would like to thank the reviewers for their valuable comments and suggestions to improve the quality of the manuscript.

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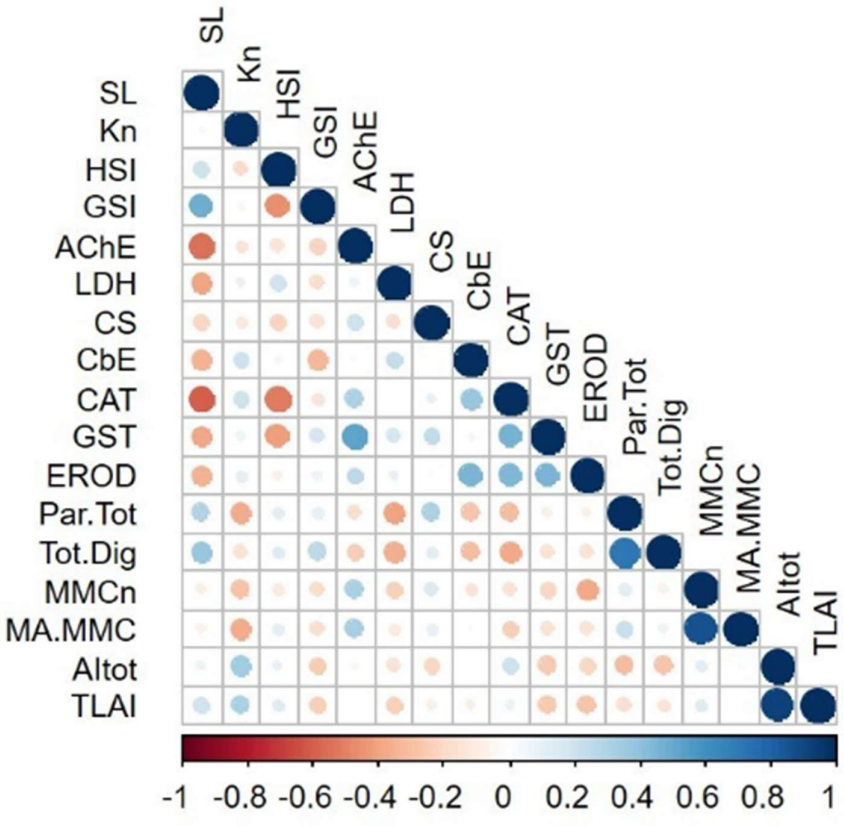

Figure S1. Spearman's correlations amongst individual's size (SL), condition indices (Kn = relative condition factor, HSI = hepatosomatic index, and GSI = gonadosomatic index. Enzymes (AChE = Acetylcholinesterase, LDH = Lactate dehydrogenase , CS = Cytrate synthase , CbE = Carboxyl esterase , CAT = Catalase , GST = Glutatione-S-transferase and EROD = Ethoxyresorufin-O-deethylase). Parasites (Par.Tot = Total number of parasites/individual and Tot.Dig = total number of parasites within the digestive tract/individual). Histological alterations (MMCn = number of melanomacrophage centres , MA.MMC = mean area of melanomacrophage centres) and anthropogenic items (Altot = total number of anthropogenic items/individual and TLAI = Total length of anthropogenic items/individual).

Supplementary data to this article can be also found online at

https://doi.org/10.1016/j.scitotenv.2022.156539.

5.CHAPTER III:

Plastic fibre ingestion by European sardine (*Sardina pilchardus*) in relation to feeding behaviour under experimental conditions.

ABSTRACT

European sardine (*Sardina pilchardus*) is a foraging fish species which has experimented important declines in their wild populations during the last decades. The decrease on its biomass have had a negative impact on its landings, driving their commercial fishing non-viable in some Mediterranean regions. The causes of this declines are not clear but seems to be mainly related to changes with planktonic communities. Moreover, this species has been described ingesting plastic fibres along its natural distribution pointing out this kind of pollution as a possible new stressful factor affecting their populations. In this study we develop a novel method to maintain wild fish under experimental conditions to test the possible factors affecting fibre ingestion in sardines. We demonstrate that sardines can ingest fibres from water, but the amount of fibres ingested is highly dependent on the feeding behaviour. Sardines feeding by filtration ingest less food but more plastic fibres (4.95 ± 3.43 fibres / ind), compared to sardines that feed by catching (0.6 ± 1.04 fibres / ind). Moreover, a decrease of sardine body condition factor was detected in sardines feeding by filtering, thus mainly related with the amount of food they ingest rather than with fibre ingestion. However, higher temperatures seem to affect the pattern of fibre expulsion in filter-feeding sardines which, together with reduction of zooplankton communities, might finally act synergistically distressing the fitness of this species, and therefore aggravating the problem in wild populations.

HIGHLIGTHS

- A novel set-up design for experimentation with fish and plastic fibres (89% of fibre recovery)
- Wild sardines reared in experimental conditions ingest plastic fibres in realistic concentration
- Fibre expulsion last until 50-60 hours after ingestion (almost twice the time to excrete the total amount of faeces in filter-feeding sardines).
- Sardines with filtering behaviour ingest less food, but more plastic fibres compared to the ones feeding by catching
- The higher temperature affects the pattern of fibre expulsion in filter-feeding sardines
- No relation is found between body condition with fibre ingestion, but with feeding behaviour

1. INTRODUCTION

The ubiquity of small plastic particles known as microplastics, < 5 mm (Frias and Nash, 2019; Hartmann et al., 2019), is a major concern throughout the world's oceans (UNEP, 2016). It is estimated that 4.8 to 15.11 million metric tons of plastic marine debris enter in the ocean every year (J. Jambeck et al., 2015; Lebreton et al., 2017). Fragmentation of larger plastics over time has also increased the presence of microplastic fragments and fibres from water surface layer down into deep ocean sediments (Browne et al., 2011; Lusher et al., 2015; Van Cauwenberghe et al., 2013). Fibres are reported as the most prevalent type of item found around the world (Gago et al., 2018a) and can constitute up to 91% of microplastics / anthropogenic pollution collected globally in water samples (Barrows et al., 2018). The concentrations of these fibres (both synthetic and cellulosic) vary a lot depending on locations from 0.02 to 25.8 fibres litter^{-1}, with a median concentration of 1.7 fibres litter^{-1} (Suaria et al., 2020).

Semi enclosed and highly populated areas such as the Mediterranean basin seems to be hotspots accumulation of anthropogenic fibres (4.6 fibres litter $^{-1}$; Suaria et al., 2020); and their small size and wide availability increase the chance for ingestion by marine organisms (Browne et al., 2008; Lusher et al., 2017). In fact, the ingestion of fibres has been well documented from a wide range of taxonomic groups, from zooplankton (Cole et al., 2013; Setälä et al., 2014), benthic invertebrates (Goldstein and Goodwin, 2013; Murray and Cowie, 2011; Watts et al., 2014; Wright et al., 2013) seabirds (Thiel et al., 2018), marine mammals (Hernandez-Milian et al., 2019), and fish of different trophic level (Neves et al., 2015) including small forage /planktivorous fish such as clupeiforms (Compa et al., 2018; Lefebvre et al., 2019; Savoca et al., 2020).

Microplastics are suggested to be potential carriers of other pollutants when ingested, such as metals, organic contaminants (Gauquie et al., 2015; Rochman et al., 2013b) or being vectors of pathogens (Bowley et al., 2021). The deleterious consequences of microplastics ingestion has been assessed in experimental conditions, and differ from one taxon to another: food activity modification (Besseling et al., 2013), food assimilation deficiency (Blarer and Burkhardt-Holm, 2016), growth retardation (Lo and Chan, 2018), reduced reproduction (Cole et al., 2015), neurotoxicity (Qiao et al., 2019), reduced survival and locomotion (Tosetto et al., 2016) and impaired cognitive abilities (Crump et al., 2020). These effects vary depending on the quantity of microplastics during the experiments (mostly acute experiments use high

concentrations of MP) and on the time of exposure. In order to assess how natural populations might be affected by microplastic contamination in wild fish, studies using realistic concentrations of MP exposition are needed in controlled conditions.

In this study, we used small pelagic fish, namely wild sardines (*Sardina pilchardus*) from the NW Mediterranean Sea, brought into captivity as a model to study microplastic dynamics after ingestion in natural population. Together with other small pelagic fish species, sardines are key components of marine ecosystems worldwide modulating population dynamics of both lower and upper trophic levels (Cury et al., 2000). Moreover, they support important fisheries and local economies, such as in the Mediterranean Sea, where small pelagic species represent almost 50% of the total fish landings (Lleonart and Maynou, 2003). However, the biomass of this species has decreased in last decades due to a sharp decline in individual size and mass (Saraux et al., 2019; Van Beveren et al., 2014). This decline seems to be primarily related to increased natural mortality of older individuals, and to changes in the environment and food availability rather than to overfishing, predation pressure or the presence of pathogens (Brosset et al., 2017, 2016a, 2015b; Queiros et al., 2018). A bottom-up control of the sardine population due to a shift in their planktonic prey towards smaller less nutritious species, has been proposed as a mechanism underlying lower growth and body condition (Brosset et al., 2016b; Saraux et al., 2019). Pollution like microplastics could be amplifying the problem, and, although the number of microplastics found in sardine guts in the wild was not that high (Lefebvre et al., 2019), it is still under investigation. Furthermore, sardines display different feeding strategies depending on prey size (Garrido et al., 2008, 2007) offering the possibility to investigate how feeding behaviour can affect microplastics ingestion. When food available consists in large sized prey (e.g. copepods) they preferably display particulate-feeding, i.e. targeting single prey. However, when only small size prey / food is available (e.g. phytoplankton) they can switch into a less selective feeding behaviour based on filtering (Costalago et al., 2015; Garrido et al., 2007). Interestingly, a strong evidence of systematic changes in plankton abundance and their community structure over recent decades has been found, not only in the Mediterranean Sea but also in many areas worldwide (Aberle et al., 2012; Feuilloley et al., 2022; Herrmann et al., 2014; Winder et al., 2012) often resulting into a smaller size (Daufresne et al., 2009). Climate change is of course associated with an increase in sea temperature (e.g. + 0.2°C per decade in the last 35 years in the Gulf of Lions; Feuilloley et al., 2022) and also should affect prey size for sardines, as plankton size is expected to continue decreasing with higher temperatures. This might trigger an increase in the less selective feeding behaviour, that is filtration, for sardines. An increase in temperature should also increase all fish physiological rates (Clarke et al., 2017;

Seebacher et al., 2014) such as digestion. Whether this will amplify microplastic ingestion by those fish remains to be investigated.

Thus, the main aim of the present study was to assess the ingestion of microfibers by sardines in relation with the different feeding behaviours (filtering and catching), and how this might be affected by climate change. To reach this goal, the specific aims of this study were: i) to study the ingestion of microfibers in realistic concentrations in a wild fish species (*Sardina pilchardus*); ii) to test whether sardine feeding behaviour and environmental temperature affect the fibre ingestion and the potential fibre retention in sardine digestive tract and finally iv) to determine whether the ingestion of plastic fibres can affect body condition, which is especially important in the context of the recent changes observed in the NW Mediterranean Sea sardine population as microplastics are known to cause detrimental effects on individuals ingesting them.

2. METHODS

2.1. Sardines capture and rearing conditions

Wild sardines were captured off Sète (South of France) in March 2020 and brought back to the Ifremer Palavas-les-Flots research station. The protocol for acclimation and weaning onto commercial pellets was the same as detailed in Queiros et al., (2019). Once the acclimation period was finished and sanitary conditions verified, sardines were anaesthetized with benzocaine at 140 ppm, and 80 specimens were selected and distributed in eight experimental tanks (95 L, 10 fish per tank). Measurements of length and weight were carried out, so as to ensure a similar weight and length in all tanks (28.00 g (SD = 2.97) and 150.10 mm (SD= 3.06) respectively). Tanks were kept in an open system by a constant flow of 160 L /h with filtered seawater. Temperature was controlled and photoperiod was gradually increased until achieve the equivalent of summertime, light: 14 h/day. Fish were in these tanks 15 days before the experiments started.

The daily food ration was settled at 1 % of fish total biomass, calculated from the mean of biomass of the eight tanks. Subsequently four tanks (1 to 4) were designated to be fed with small size pellet (0.1 mm) whereas the other four tanks (5 to 8) were fed with large size pellets (1.2 mm) (Fig. 1). The two food sizes were selected to elicit two distinct foraging modes, filter-feeding versus catching-feeding behaviour (Queiros et al., 2019b). During the feeding time (30 min) the water flow was interrupted to avoid losing food and to ensure that all food was

consumed by the sardines. Since sardines are suspension feeders, and do not catch remaining food that fell down to the bottom of the tanks: in tanks fed with small pellets (filter-feeding), food was sprinkled manually on water surface and an air bubble aerator was introduced to help keeping food in suspension; whereas in tanks fed with big pellets (catching), fish was fed manually little by little to ensure equally food spreading in all tanks and to prevent food reaching the bottom without being consumed. The distribution of food was carried out in three intakes throughout the day. The first ration consisted of 50% of the total amount of food at the beginning of the day and the rest was distributed in two more portions (25% each) at four and eight hours respectively after the first feeding event. To ensure removing any deposition of particles in the bottom of tanks and a correct sample collection during the subsequent experiments, entrance and exit of the water flow was designed to generate a vortex to obtain a constant cleaning effect. In this way all the contents of the tank were concentrated in the centre and came out through the drain pipe connected to a waste collection system. This system consisted of a 20 L decantation cylinder (120 cm height x 20 cm diameter). The water inlet was located in the centre of the cylinder allowing the heaviest particles to sink. At the base of the decanter there was an output valve that could be manually opened for sample collection. Moreover, decantation cylinder had a top overflow pipe through which water flowed constantly to collect free particles.

2.2. Sampling protocol, selection and detection of marked faeces, plastic fibres and image analysis

A standardized sampling protocol consisting in completely emptying each decantation cylinder of water and rinse it carefully to collect the content on filters (15 µm nylon mesh) was carried out in all tanks. In turn, the filter located in the overflow was replaced between each sampling and examined.

Food was marked with a fluorescent water-insoluble pigment (CC Moore Fluor Bait Dye). The amount of pigment used corresponded to a concentration of 1% in weight to a given amount of food. For its preparation, pigment was added directly to the fish pellets of the two granule sizes and shacked for 5 minutes to allow the pigment to mix uniformly. It allowed to differentiate coloured faeces, under UV light, from the remains of food or other faeces produced before or after treatment meal.

Plastic fibres consisted on fluorescent commercial (Flocking LDT) nylon flock fibres of 1mm length and 20 µm diameter, to mimic the most frequently described fibres described in

natural environments (Suaria et al., 2020) and in particular in the Gulf of Lions both in water column and in small pelagic fish (Lefebvre et al., 2019).

The use of fluorescent pigments allowed the easy detection of coloured faeces and fibres in the filters obtained at each sampling. For this purpose, the filters were illuminated using a 360 nm wavelength ultraviolet light bulb and photographed under standardized conditions by a Canon 60D camera using a 100mm macro lens. The pictures obtained were processed using image analysis piece of software Fiji ImageJ 2.1.2 (Schindelin et al., 2012). The presence or absence of fibres was checked and counted for every filter. In decanter filters, fibres were counted twice, before and after faeces disaggregation (mechanically by water jet).

2.3. Experimental design

All fish manipulations were performed under anaesthesia conditions and all procedures were in accordance with the French and the EU legislation regarding animal experimentation (APAFIS, Permissions No. 29810-2021021113024423 v2).

2.3.1. No fish experiment (set-up)

An experiment was performed without fish, before their transfer into the experimental tanks (Fig. 1a). The aim of this experiment was to test and validate the sampling protocol and to determine: (1) how plastic fibres passed through the tank; (2) the time needed to remove the fibres from the tanks; (3) and the percentage of recovered fibres.

This experiment was conducted once using eight tanks. After introducing fibres in tanks, an air bubble aerator was connected and kept during 5 min to allow homogeneous fibres dispersion. Aeration was connected again four and eight hours later in four tanks (i.e. tanks simulating the filtering condition). For the rest of the tanks (i.e. tanks simulating the catching condition) no aeration was added. Fibres concentration was settled at 5 fibres per L^{-1} (400 fibres / tank) to mimic the mean values reported for the Mediterranean Sea (Suaria et al., 2020). Fibres were introduced in the system at the beginning of the experiment, and sampling was performed every two hours for each tank.

2.3.2. Dyed food experiment: Tracing food transit

The aim of this second experiment was to determine the time of food transit, i.e. time that elapses between a food intake without plastics and its complete expulsion. This also allowed us to assess the timeframe of sampling for subsequent experiments.

This experiment was also conducted once using eight tanks and was performed with fish reared in the same conditions as explained in section 2.1 (Fig. 1b). Fish were maintained at 16°C. Fish were fed with dyed food in the first intake of the day (which correspond to 50% of the daily ration). The following daily rations (25% at four and eight hours later, respectively), consisted of normal non-dyed food. After feeding fish, sampling was performed every hour until coloured faeces disappeared. Note that filters were not sampled at night.

2.3.3. Plastic fibre experiment

The aim of this experiment was to compare the ingestion of plastic fibres by sardines as well as the fibre expulsion dynamics between both feeding behaviours at two different temperatures.

This experiment was conducted using eight tanks: four tanks designed for filtering feeding (three treatment tanks with fibres and one control tank without fibres) and four tanks designed for catching (three treatment tanks and one control). Control tanks were sampled in the same way as treatment way to monitor potential crossed contamination. The experiment (Fig. 1c) was performed exactly in the same way as the dyed food experiment, only with the addition of plastic fibres during the first meal of the day. Sardines were fed with dyed food (50% of the daily ration) at the beginning of the experiment and again with normal non-dyed food at four and eight hours later, 25% of the daily ration respectively. As for the no fish experiment (section 2.3.1.), 400 fibres (same concentration, same sizes, etc.) were added to the tank, at the same time as the dyed food. Sampling of faeces and fibres was then carried out every two hours. Two different experiments were performed successively at two different temperatures of 16 and 19 ºC respectively. Note that temperature was increased gradually over seven days between the two experiments, so as to ensure fish had time to acclimate to this new temperature before the second experiment started.

Biometric data (total weight and length) of all 80 individuals were recorded after each experiment, setting approximately every two weeks.

2.3.4. Plastic fibres within sardine digestive tract

Finally, to verify and quantify the ingestion of plastic fibres by sardines, a last experiment was carried out using the same design as the previous one. However, in this case, one fish from each tank was sacrificed every four hours (until 40 hours) by a lethal dose of benzocaine (1000 ppm) (four fish per feeding behaviour and time). As this procedure was terminal for the fish, this experiment was only performed at 19°C. Each fish was weighed, measured and dissected. The stomach content was weighed, and the gastrointestinal tract (stomach and intestine) was carefully screened for the presence of fibres under a stereoscopic binocular at 10× to 45× of magnification and UV light. All fibres were counted.

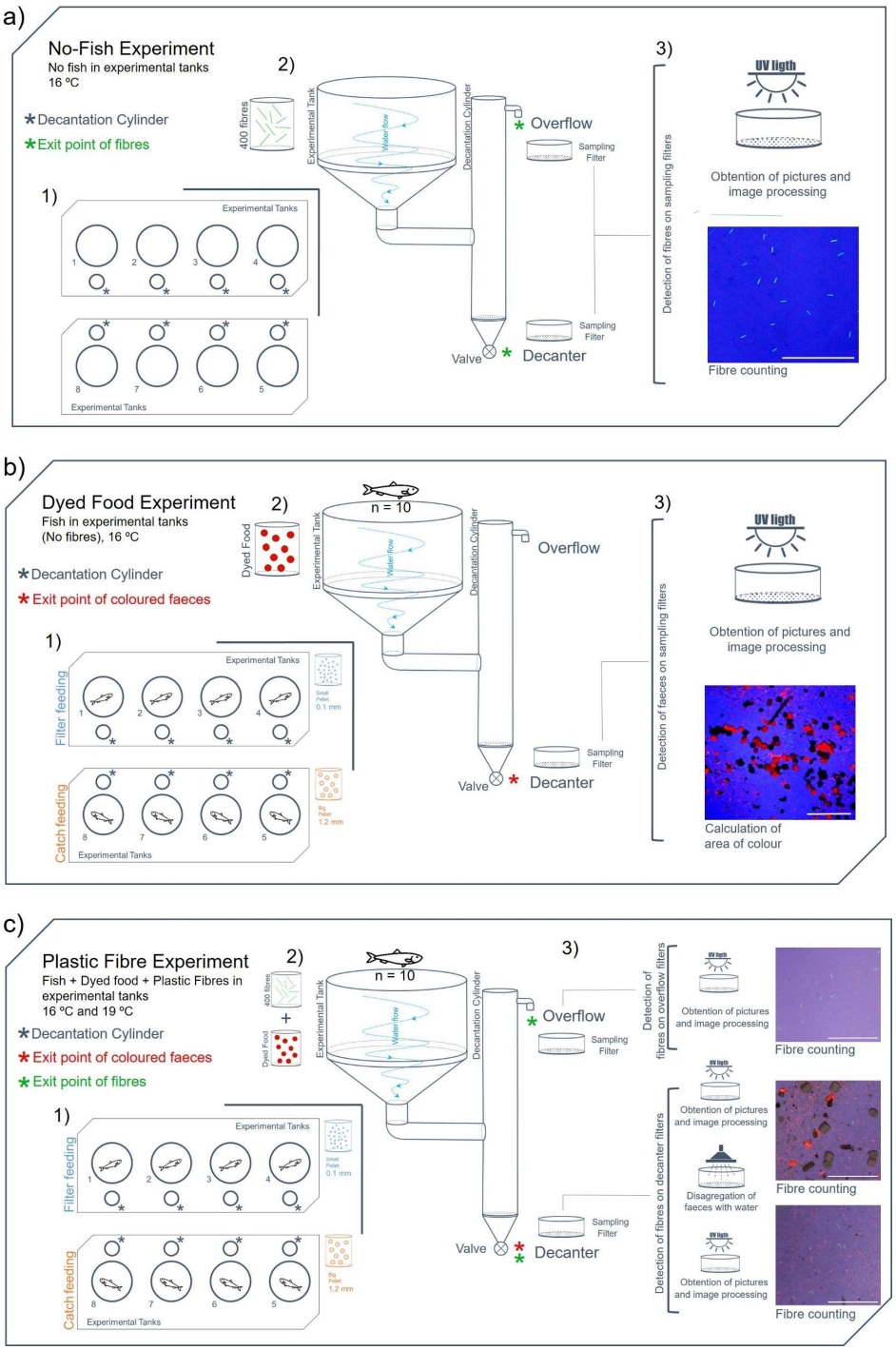

Fig. 1. Scheme of the performed experiments for the set-up experiment: (a) without fish; (b) with fish feeding with dyed-food but without plastic fibres; and (c) with fish feeding with dyed-food and plastic fibres. Number 1) indicates the experimentation room, disposition of experimental tanks, decantation cylinders and size of food used (when fish were present in tanks)**.** Number 2) show an example of an 80 L experimentation tank, decantation cylinder and fibre concentration or / and dyed food used in every experiment for every tank. Green asterisks indicate the exit points of the set up where fibres where sampled, while the red ones indicate the exit points where dyed faeces where sampled). Number 3) show examples of pictures obtained from sampling filters under UV light (360 nm wavelength) where fluorescent fibres and / or dyed faeces can be observed to be counted in each sampling time. Scale bars at the bottom right of each picture represent 15 mm.

2.4. Data analysis

2.4.1. Image analyses and fibre counting

Fibres recovered on overflow and decanter during set-up experiment were counted and kinetic curve was calculated for overflow, decanter and the sum of both, for each tank individually and grouping tanks depending on the feeding behaviour manipulation (filtering or catching)

The amount of coloured faeces (CF) during pre-experiment was calculated as area percentage, in number of pixels, from the obtained pictures in every sampling time as:

(CF = PFC / TPP) x 100))

Where PFC are pixels of fluorescent colour in the picture at a specific sampling time and TPP the total number of pixels of the picture

Standardized amount of coloured faeces (SCF) was calculated to ease comparisons iamongst tanks and treatments as:

(SFC = PFC / MFC) x 100)

where MFC is the maximum value of fluorescent colour in the experiment. Finally, the kinetic of food transit was assessed for the experiment.

In experiments where sardines were fed with plastic fibres, collected fibres in the overflow and in decanters as well as fibres embedded in faeces (FF) were calculated as:

FF = TF - NIF

where NIF are the non-ingested fibres found free in the filters (as counted before faeces disaggregation) and TF the total number of fibres (non-ingested fibres + fibres contained in faeces) after faeces disaggregation. The kinetic of fibres expulsion was monitored for experiments performed at the two distinct temperatures (16 and 19 ^{o}C).

To assess the plastic fibres within sardine digestive tract the prevalence (P%) was calculated as:

P% = (FF / TF) x 100

where FF is the number of fish displaying fibres within their digestive tract and TF the total of fish analysed. Fibres found in the different parts of the digestive tract were considered separately and together in regards to the feeding behaviour.

2.4.2. Fish condition indices

Based on the weight of the stomach content, stomach fullness index (Hyslop, 1980) was calculated as:

Fullness = (Stomach content weight / total fish weight) x 100

Body Condition Index (Kn) of each sardine was calculated with the Le Cren index Kn as estimated by Brosset et al., (2015a) using the data obtained during biometrics:

$Kn = TW /(0.00607 \times TL^{3.057})$

where TW the total weight in g and TL is the total length in cm.

4.2.3. Statistical analyses

To assess differences in explanatory and response variables amongst tanks individually and grouping it depending on feeding behaviour (filtering and catching), mixed models of repeated measures were applied to test differences in the number of fibres in filters (overflow and decanter) and total fibres in the no-fish experiment. The same models were used to test differences in the number of fibres over time in the experiments where sardines were fed with plastics. Residuals normality and homoscedasticity were tested using Shapiro-Wilk and Levenne's test respectively.

Differences in the amount of coloured faeces were addressed using Generalized Linear Models, (GM, gamma model). Differences in the prevalence, stomach fullness and abundance of plastic fibres found in the digestive tract of sardines between feeding behaviour, were tested using a GZM (binomial model, link logit), GZM (gamma model) and GZM (Poisson model, link log), respectively. The possible differences in the values of weight, length and condition factor index (Kn) obtained from the different biometrics along time were addressed using parametric ANOVA and 2-way-ANOVA of repeated measures and post-hoc pairwise comparison (Bonferroni correction) tests were used, otherwise non-parametric Friedman test was applied. 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 No fish experiment (set-up)

In the absence of fish, most fibres introduced in the tanks were recovered on our filters (89% on average [84-97.8%]), either in the overflow (62.65%) or in the decanter (37.5%). Values (mean and SD) of total fibres, and separately fibres recovered in overflow and decanter are shown in Fig. 2a. When looking at the dynamics of fibre collection over time, more than 70% of fibres came out of the system during the first hour and approximately 90 % of fibres came out of the system within 5 hours (Fig. 2b, c and d). After 26 hours, the system was empty of fibres. The number of fibres recovered from tanks over time was similar in the overflow and decanter, and did not show significant differences among tanks and between filtering and catching tanks ($p>0.05$).

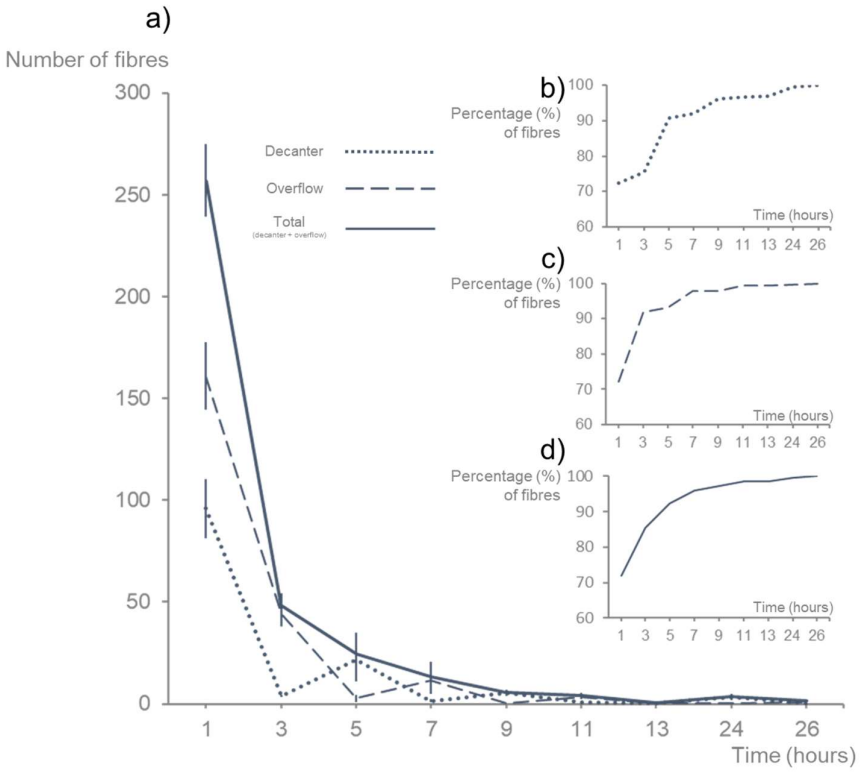

Fig. 2. Kinetics of plastic fibres during the no-fish experiment: (a) Number of recovered fibres (mean and SD) exiting from the tanks through the recovery system; (b) Accumulation curve in percentage (%) of the fibres recovered in the overflow; (c) Accumulation curve in percentage (%) of the fibres recovered in the decanter; and (d) Accumulation curve in percentage (%) of the total fibres recovered from the system.

3.2 Dyed-food experiment

Coloured faeces appeared after five hours post-ingestion of dyed food and 11 to 12 hours post-feeding were required for the fish to produce half of the total amount of faeces, for both feeding behaviours (Fig. 3). Most faeces were collected before the first night started (14h post-feeding) and the transit and production of coloured faeces could be considered finished during the second day-time period (28-38h post-feeding), despite a few occasional coloured faeces were detected in the third day. When comparing between feeding treatments, filter-feeding fish seemed to exhibit a faster transit compared to sardines fed with large pellets (90% after 14h vs. 70%). Further, sardines fed with large pellets (catching behaviour), produced significantly more coloured faeces (CF) overall (filtering = 0.06 ± 0.03 vs. catching = 1.02 ± 0.17, $t = 2.96$, $p < 0.005$) compared to the filter-feeding ones (Fig. 4a).

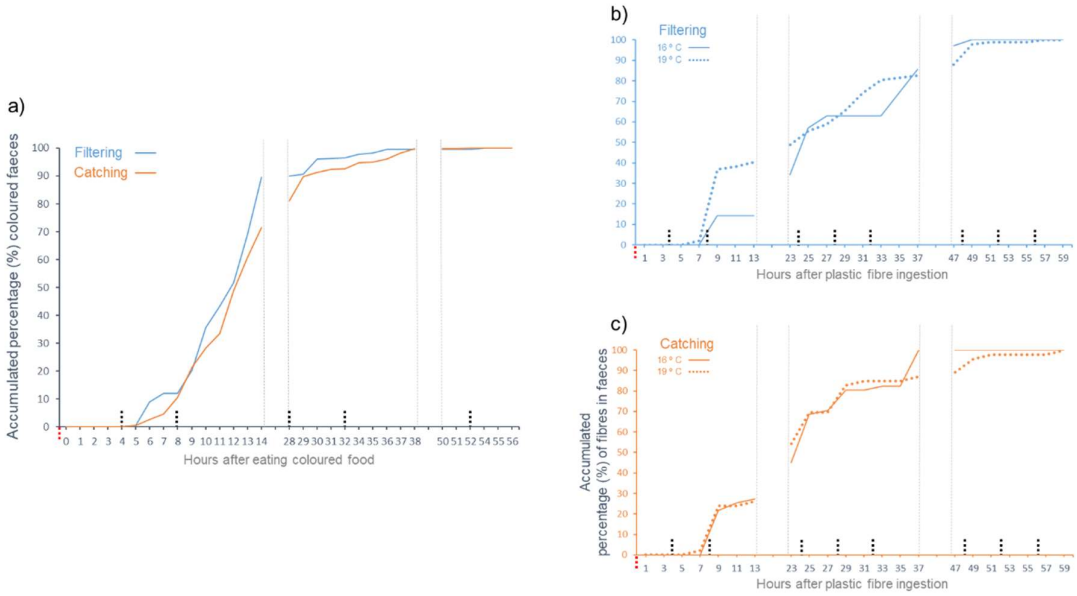

Fig. 3. Dynamics of the expulsion of coloured faeces and fibres (expressed in accumulated percentage %) for sardines feed on small pellets (Filtering) and big pellets (Catching): a) Dynamics of the expulsion of coloured faeces in both feeding behaviour; b) Dynamics of the expulsion of fibres contained in the faeces in sardines with filtering behaviour; and c) Dynamics of the expulsion of fibres in sardines with catching behaviour. Dashed red lines, coinciding with time "0" express the moment when fish were fed with stained food or plastic fibres. Black dashed lines express the subsequent feeding times with normal non-stained food. Spaces between solid bars, express a gap in the sampling during night-time.

3.3. Plastic fibres experiment

In the first five hours post fibre distribution, the total number of fibres recovered at 16 and 19 ºC were lower (16 ºC: 245.83 ± 38.7 fibres; 19ºC: 237 ± 34.1 fibres) compared to the no

fish experiment (no fish: 329.3 ± 19.7 fibres) with significant differences (16 ^{o}C, t = -6.26, p <0.0001 and 19 ^{o}C, t = -6.09, p <0.0001), suggesting that fish indeed ingested fibres. Further, the number of fibres expelled from the system within the first five hours was 20 % lower in filtering sardine tanks than in catching tanks. This decrease was significant, regardless of the temperature (z = 6.41, p <0.005, Fig. 4b).

At 16 ^{o}C, fibres were detected in faeces after 9 hours post ingestion (three hours after detecting the first coloured faeces) in both feeding behaviours. The 50 % of fibres were detected after 23 to 25 hours. No more fibres were found in faeces after 37 hours for catching and 52 hours for filtering (Fig. 3).

A similar pattern was observed at 19 ^{o}C especially for catching sardines, but faeces with fibres seem to appear sooner (one or two hours) at higher temperature in both feeding behaviours, and to disappear later especially with catching behaviour (after 56 for catching and 54 hours for filtering) (Fig. 3).

No fibres were found in any of the control tanks at any time, ensuring not crossed contamination of fibres during sampling.

3.4. Plastic fibres within sardine's digestive tract

Over all sampling hours (4 to 40 h post-fibre distribution), the proportion of fish that presented at least one fibre in their gastrointestinal tract (P%) was significantly higher (z = 3.71, p <0.001) in filter-feeding fish (93.3%) compared to those feeding by catching (40%). The number of fibres found in the digestive tract of dissected fish was also significantly higher (z = 7.87, p <0.001) for filter-feeding sardines (4.95 ± 3.43 fibres / ind vs 0.6 ± 1.04 fibres / ind) (Fig 4c). When considering stomach and intestine separately, most fibres were found in the stomach (86 %). Filter-feeding sardines also displayed higher numbers of fibres in both organs compared to the ones feeding by catching, although differences were significant only in the stomach (z = 7.35, p <0.001, Fig. 4d and 4e).

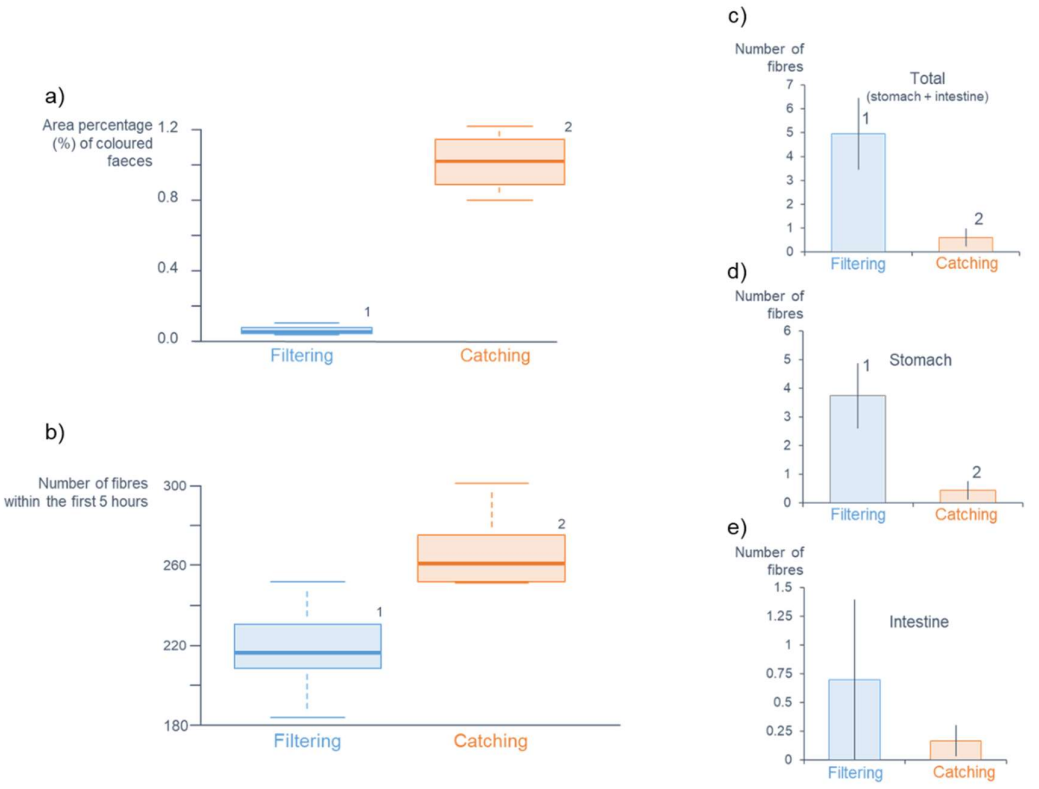

Fig. 4. Number of coloured faeces and fibres for sardines fed on small pellets (Filtering) and big pellets (Catching): a) Boxplot of the total amount of coloured faeces recovered at the end of dyed-food experiment from tanks of both feeding behaviour; b) Boxplot of the total number of plastic fibres recovered during the first five hours in the plastic fibre experiment from tanks of both feeding behaviour b); c-e). Number of fibres (mean and SD) found in the digestive tract of sardines after dissection (c) total stomach + intestine), (d) stomach and (e) intestine). Numbers express significant differences between feeding behaviours.

3.5. Biometrics

Fish exposed to plastic fibres did not show significant differences in biometrics (length, weight and condition index) compared to control fish, in none of the two feeding behaviours. A clear progressive decrease over time was observed in weight and condition index for filter-feeding fish, showing significant lower values at the end of the experimentation period (RM ANOVA: 4.96, $p < 0.005$ and RM ANOVA: 7.94, $p < 0.001$, weight and condition index respectively). Conversely, biometric data in sardines feeding by catching did not show significant changes over time.

Significant differences between both feeding behaviour were found for body condition index (RM Two-way ANOVA = 4.26, $p < 0.05$) in the two last samplings and for weight (RM Two-way ANOVA = 4.29, $p < 0.005$) only in the last one (Fig. 5). Regarding stomach fullness, fish with catching behaviour showed higher significant values compared to the ones fed with small pellets ($t = 3.863$, $p < 0.005$).

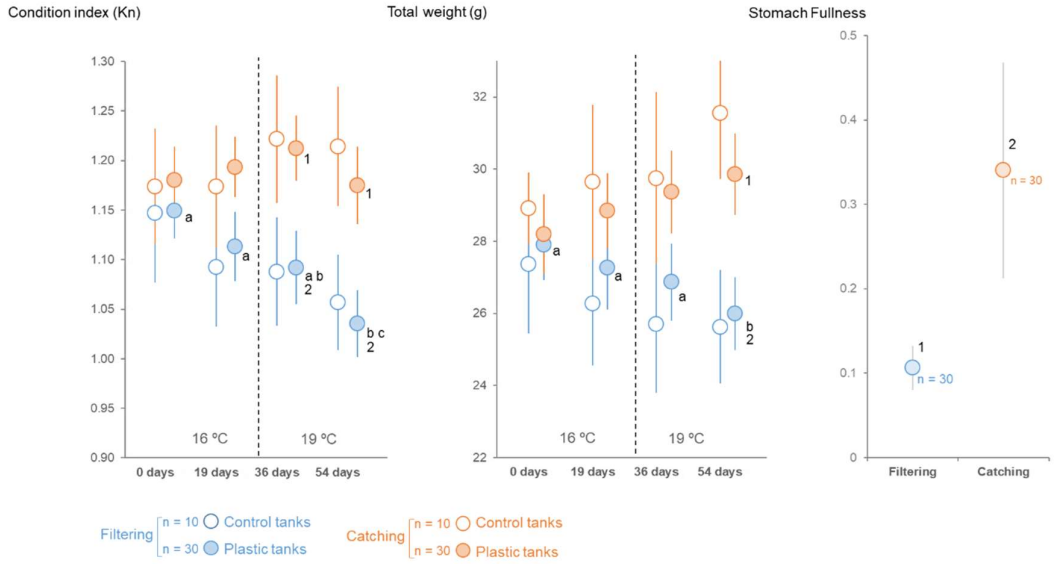

Fig. 5. Values of the biometrics (Biom. 1 to 4) according to the feeding behaviour (filtering vs. catching), for condition index (factor Kn) and total weight (g), performed throughout the experimentation period, and stomach fullness obtained by dissection after the last biometry. Letters express significant differences in the same feeding behaviour while numbers show significant differences between feeding behaviours.

4. DISCUSSION

To the best of our knowledge this is the first time that a study of plastic fibre ingestion in Mediterranean wild clupeids was performed under experimental conditions. In addition, this is the first study that demonstrates, in realistic concentrations, that sardines ingest plastic fibres from water by catching and also when the feeding mode is switched to filtering, and it is reported that the amount of ingested fibres depends on the feeding behaviour.

4.1. Set-up design and fibres detection

The "no fish" experiment shows that there is a good fibre recovery rate (near 90%), validating the set-up designed when challenging to track the fibre ingestion by organisms in realistic concentrations. In this system, during the first hour, most of the fibres are retreated from the tanks. The renewal speed of water tank seems adequate, as fibres are disposable during the feeding time for fish (30 minutes) but do not remain in the tanks along the experiment duration. Although it would be advisable to work with smaller volumes of water in tanks when experimenting with these small-sized fibres (i.e. nylon flock fibres of 1mm length), wild small pelagic fish, as a gregarious species, needs to live with other congeners and cannot be

maintained in captive facilities in small tanks, as they change their behaviour, even they stop feeding. However, the use of fluorescent fibres strikingly streamlines the detectability of these small fibres in the overflow and mainly the decanter filters, avoids the possible confusion of plastic fibres with airborne contamination, and seems not to interfere to the feeding behaviours of fish. In conclusion, this set-up experiment seems to be a compromising design to be faceable and to be as close as possible to the natural environment, i.e. water pumped from the sea, natural photoperiod to have natural behaviour of sardines, the number and density of fish per tank allowed for the formation of schools (Queiros et al. 2019); and fibres used mimics the most frequently found in the environment (Lefebvre et al., 2019; Suaria et al., 2020).

4.2. Feeding behaviour and ingestion of plastic fibres by sardines

Most of the studies performed for studying the dynamics of fecal pellets production and digestion in fish are conducted in captive fish that are common in aquaculture such as the gilthead seabream *Sparus aurata*, the Senegalese sole *Solea senegalensis* (Gilannejad et al., 2019) or the European seabass *Dicentrarchus labrax* (Adamidou et al., 2009). However, feeding time and frequency on gut transit is particularly species-specific due to the variance on the digestive system morphology and feeding behaviour of each species (Rønnestad et al., 2013). Although time of gastric evacuation has been calculated from wild samples or under laboratory conditions with some other clupeids (Tudela and Palomera, 1995; Van Der Lingen, 1998; Bulkagova, 1993) no literature has been yet produced for sardines, thus, this is the first study to infer the gut transit curve in this commercially important species.

Both feeding behaviours seem to have a similar food expulsion pattern, producing most of the amount of faeces during the first 14h post-feeding and ending around the 32h post-feeding. That is, in these experimental conditions, sardines digest a meal in half a day. The feeding biology of the sardine is well known in terms of behaviour and diet composition (Costalago and Palomera, 2014) but the feeding frequency has not yet been discussed in experimental studies. When comparing between feeding behaviours, fish fed with small pellets (filtering feeding) seemed to exhibit a faster transit compared to sardines fed with large pellets (catching), which agrees with typical digestibility of food within the gastrointestinal system. A similar pattern is described in other species such as *Sardinops sagax* in which individuals feeding on phytoplankton have faster gastric evacuation rates compared to the ones eating on bigger zooplanktonic items (Van Der Lingen, 1998). Further, sardines fed with large pellets produced significantly more coloured faeces overall, i.e. they eat more when catching. This is in agreement

to other cultured species such as the gilthead seabream, that prefer large-sized pellets and those induced a greater amount of feed waste (Busti et al. 2022).

The differences in the number of fibres recovered during the first hours of experiment comparing no fish and fish experiments demonstrates that sardines are ingesting fibres during feeding. In the same way, the lower number of fibres recovered in filtering tanks during the first hours indicate that filter-feeding sardines ingest more fibres than catching (Fig. 4b), fact that is then confirmed when dissecting fish (Fig. 4c-e).

The 50% of the fibres were excreted within 23-25 hours, which is twice the time required by sardines to excrete the half of the total amount of faeces, indicating fibres retention time is higher than the processing time of food items. However, if we take into account the total time needed to excrete all fibres, this higher retention is continued in sardines with filtering feeding (52 vs 38 hours), but is not seen in sardines feeding by catching. So, feeding behaviour seems to also influence in fibre retention. This time needed to excrete all fibres in sardines seems similar to what other pelagic species need to excrete MP (about 44 hours in *Engraulis japonicus*; (Ohkubo et al., 2022)) but higher if we compare with demersal species (about 25 hours *Pagrus major* or *Sparus auratus*; (Jovanović et al., 2018; Ohkubo et al., 2022)). In any case, as stated before, feeding time and frequency on gut transit is particularly species-specific, so it is important to know normal food excretion time in each species in order to properly evaluate the potential retention of AIs in digestive tract. The typology and size of AIs seems to also interfere with this retention as smaller items could potentially persist for long periods within digestive tract (Liu et al., 2021).

4.3. Impact of plastic ingestion in sardines

Different impacts on fish health (e.g. decreased survival and energy storages, alterations in the activity of biomarkers, alterations of metabolisms and in different tissues, increased feeding time, effects on body length…) are commonly reported following artificially exposures of microplastics (Kögel et al., 2020 and references therein). However, it is important to highlight that the concentration of MP used under laboratory conditions is usually far higher than what is found under natural conditions, and precisely such concentration seems to be one of the most important factors regarding harm caused to fish (Kögel et al., 2020). In our study, we tried to reproduce what happen in the wild using realistic concentrations of fibres found in the environment [from 0.02 to 25.8 fibres litter^{-1}, with a median concentration of 1.7 fibres litter^{-1}

(Suaria et al., 2020)]. With these concentrations (5 fibres litter^{-1}), no impact is observed in fish length, weight or body condition related to the ingestion of fibres.

Effects of microplastics on body length or condition factors are ambiguous, as both reduced and increased levels are reported (Kögel et al., 2020). For example, in wild pelagic species, lower values of condition index have been attributed to higher values of anthropogenic fibre ingestion in *Sardina pilchardus* but do not in *Engraulis encrasicolus* (Compa et al., 2018; de Vries et al., 2020), nor in *Gadus morhua* or *Pollachius virens* (de Vries et al., 2020). No significant effect of plastic exposure was observed in growth or body condition on the omnivorous fish *Diplodus sargus* (Müller et al., 2020) nor on the planktivorous fish *Acanthochromis polyacanthus* (Critchell and Hoogenboom, 2018) under laboratory conditions. However, in the later study, when food was totally replaced by plastic, there was a negative effect on the growth and body condition of the fish, which also emphasize the importance of concentration.

In our study, the low values of stomach fullness and fitness impoverishment seen in filter-feeding sardines compared to sardines that displayed a catching behaviour may be attributable to feeding behaviour rather than to plastic ingestion, as no differences can be seen comparing control and plastic tanks. The fitness loss when filtering perfectly fits with other experiments carried out on captive adult sardines. Queiros et al., (2019) show that sardines exposed to small food particles (mean of 0.1 mm ranging between 80 and 250 mm) display a filter-feeding behaviour while changing into catching when exposed to bigger pellets (mean of 1.2 mm, ranging between 900 and 1500 mm). The authors also demonstrate that body condition, growth and energetic reserves are significantly impacted by the feeding, as filtering sardines need to consume twice as much as those feeding on large items (by catching) to reach the same body condition and growth rate. Both facts (i.e. small food forces filter feeding; filtering drives to a drop in fitness), when combined, may have serious consequences in the natural environment, since the plankton downsize due to global warming (Aberle et al., 2012; Feuilloley et al., 2022; Herrmann et al., 2014; Winder et al., 2012) may trigger this phenomenon, affecting to the sardine wild populations and the fisheries based on this species. In addition, as if that were not enough, filtering sardines eat more plastic fibres than catching ones, thus adding an additional potential threat to the survival of this species.

CONCLUSIONS

In this study we developed a novel method suitable to perform experiments with plastic fibres and fish. This study demonstrates that studied fish, sardines, can ingest fibres from the

water. The number of fibres ingested is highly dependent on the feeding behaviour, since when they display a less selective feeding mode based on filtration they ingest more fibres compared to the most selective feeding mode based on catching. Body condition factor is not affected by the ingestion of fibres, but fish displaying filter-feeding behaviour present worst body condition factor because they ingest less amount of food compared to fish feeding by catching. As plankton size is expected to continue decrease in the context of climate change, this might trigger an increase in the less selective feeding behaviour. By this, further investigation needs to be addressed in the future to unravel the possible synergic effect of microplastic ingestion in relation to new scenarios derived from changes in the environment.

Acknowledgements

We thank to Quentin Schull and Claire Saraux for their confidence in this project and for opening the access to their facilities to address this study. Also thank to the staff of *Station de recherché en pisciculture de mediterranée* of *Institut Français de Recherche pour l'Exploitation de la Mer* (IFREMER) De Palavas-Les-Flots, specially to Gilbert Dutto, Vincent Duchet, Emmanuel Roque and Lisanais Moreto for their invaluable help during experimentation period.

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6.GENERAL DISCUSSION

6. GENERAL DISCUSSION

6.1. Lessons learned from the study of micro-litter / Anthropogenic items (AIs) ingestion

Since the presence of microplastics in the marine environment was detected, and especially when their ingestion by biota was demonstrated, scientists, popular media, and society, immediately took the attention to this major concern. In response to the environmental problems that this type of pollutant carries with, it was included in the Marine Strategy Framework Directive (MSFD) monitoring plans, which opened a window to a new field of research that has generated an overwhelming number of publications in recent years.

However, as it is a new discipline of study, the bases for its study were not well defined, especially with regard to the methodologies for the extraction, characterization and identification of microplastics. Thus, despite generating a large volume of new information, the reported data come from different methodologies and protocols, and thus are often difficult to compare. For this reason, before discussing the specific results derived from this thesis, we consider that it is important to highlight some aspects about de analysis of AIs.

6.1.1. Approaching the study of micro-litter / AIs with a visual inspection method

Visual inspection was selected and has been used throughout our studies (Chapters I & II) because it is a useful and very direct method to approach AIs ingestion and it is considered as the simplest way to address microplastic screening, since it does not require material or complex protocols. This point is important for two main reasons: it avoids the loss of AIs throughout the process, and it reduces notably the possible airborne contamination if it is performed under clean and controlled conditions. However, it is a technique with possible limitations derived from the observer that should be considered. For this approach, the observer needs training in the use of optical devices and the observation and manipulation of small materials under the stereomicroscope. In addition, experience in observation of stomach contents is highly recommended to avoid the misidentification of AIs by confusing them with items of the diet (Fanelli et al., 2014; Romeu et al., 2016). Once these skills are acquired, visual screening is a useful and very direct method to approach micro-litter ingestion.

Since the first studies of microplastics, the methodologies for their observation and quantification have been mainly focused on its isolation from the matrix in which they were embedded, with the aim to discriminate them from other items present in the samples that can be easily misleading. One example is the study of microplastics in environmental samples (water and sediments) where zooplanktonic organisms (e.g. copepods, gelatinous fauna), mineral material, and organic matter can be easily misleading with microplastics. The same problem is

found when trying to address the presence of microplastics or other AIs in the digestive tract of fish: both organic remains from the diet and mineral or sediment remains can be found in their stomachs and intestines as well, confounding in the same way the identification of AIs. To deal with this issue and to make microplastics more easily to find, several protocols have been proposed based on the disintegration of organic matter, especially by digestion with acidic, alkaline or enzymatic agents (Claessens et al., 2013; Enders et al., 2017; Foekema et al., 2013; Rochman et al., 2015) . These methods have been revealed effective, but they are not exempt of limitations. One limitation is the cost-effectiveness, depending on the type of the digestion agent. For example, although enzymatic digestion is presented as the most effective, its high cost makes it unfeasible to be used routinely for monitoring programs with large number or voluminous samples (Catarino et al., 2017; Cole et al., 2014). Other more affordable options of digestion such as those based on the use of acid or alkaline agents are all of them time consuming. This time of digestion can be reduced by increasing temperature during the process, however the combination of higher temperatures and aggressive corrosive agents have been demonstrated to impact microplastic particles, potentially leading to its complete degradation (Hurley et al., 2018) In fact, one of the main limitation to these types of digestion (either enzymatic or chemical) is that they cause an almost complete degradation of other interesting and abundant anthropogenic fibres, such as cellulose fibres (Dehaut et al., 2016, own unpublished results).

As shown in chapters I & II, the AIs found in the studied fish was composed by microplastic particles (films and fragments) and anthropogenic fibres, both of synthetic polymer and cellulose. Visual characterization has revealed effective in inspection and isolation of AIs ingested by studied organisms. However, in some cases, during the visual screening the identification of these items, especially when are particles, needs to consider other physical features such as the hardness, consistency, roughness, brittleness or flexibility, which are obtained by the manipulation (with precision pliers) under the stereomicroscope. Those features should be considered together with true visual characteristics such as colour, birefringence, gloss, and fine surface texture (proposed by Lusher et al., 2020) when analysing AIs.

On the other hand, contrary to particles, anthropogenic fibres are more easily to recognise, especially when they are clearly dyed with colours. However, due to their aspect sometimes is easily to confuse them with other organic / prey remains of the digestive tract, such as polychaete setae, animal hairs, threadlike antennae, appendages of crustaceans, vegetal debris, and even some nematodes. In these cases, morphology gives us the key to differentiate organic remains from true fibres. The presence of ornamentations, segmentations, scaled

appearance, or cellular structures are typical in items of organic origin, and allow us to discard them as possible AIs. Anthropogenic fibres present different appearance: plastic fibres can have a circular, bi-lobed or tri-lobed section and normally have a uniform appearance without external ornamentation, although they may have grain-like structures due to the additives included during their production; whereas cellulosic fibres that probably comes from textile origin, does not have a cellular structure, and have a characteristic appearance of a helical ribbon easily recognizable (Robertson et al., 2017).

Thanks to these morphological features, AIs, and especially for anthropogenic fibres, can be classified and grouped into different categories (as shown in chapters I & II), which give the first approach of their composition, discriminating between cellulose-like fibres and synthetic / plastic –like ones. However, and although a good-trained eye could differentiate most of the AIs, the chemical identification based on their visual characteristics could be somewhat risky and requires more specific and refined identification techniques.

6.1.2. Optimizing the proper polymer identification of AIs

To solve the chemical analysis of the AIs found throughout our studies, two techniques widely used in microplastic studies have been used: Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR). Although both techniques are useful, from our experience, the advantages of FTIR over Raman should be highlighted. Raman spectroscopy has a number of features that difficult polymer identification, for example, and probably the main experimental limitations, the fluorescence phenomenon generated by the chemical composition of many of the materials when excited by visible laser light (Araujo et al., 2018). This means that, several times, certain additives present in plastics, such as certain pigments, give an intense signal that can mask the true polymer spectra, making polymer identification impossible. Although identification of these additives gives valuable information to infer material composition, many of these pigments are nonspecific. Unfortunately, in those dyed items, the polymer identification is not possible with Raman and must be supported with additional information. For these reasons, the polymer identification in the study of red mullet (Chapter I), was a bit harder and more time-consuming. On the other side, the spectra obtained in FTIR-based techniques have usually enough quality to be analysed and properly define their composition. Probably, the most marked limitation of identifying polymers by FTIR is that the presence of organic matter adhered to the sample might generate noise affecting the quality of the spectra (Xu et al., 2019). In this case, this issue can be solved ensuring clean samples prior to FTIR analyses. Therefore, after the study of AIs in European anchovy (Chapter II), we strongly recommend the use of FTIR for the polymer identification better than Raman spectroscopy.

The confirmation of the AI chemical composition in the digestive tracts of fish is essential to avoid misidentifications, especially regarding anthropogenic fibres. Its synthetic nature needs to be confirmed to avoid overestimation of plastic fibres with respect to natural ones or those man-made fibres derived from cellulose. In contrast to plastic fibres, these non-synthetic fibres —despite being inherently unnatural— have received little attention (Stanton et al., 2019), even being one of the most important component of microfibres in worldwide oceans (Suaria et al., 2020) and biota (Chapters I & II). However, both plastic and cellulosic fibres—with their additives or dyes, and their capability to bond other contaminants to their surfaces—are suspected of adversely affecting aquatic organisms (Burgos-Aceves et al., 2018b, 2018a; Prokić et al., 2019). Thus, it is important to consider all of them when monitoring pollution in biota.

6.1.3. The importance of quality assurance and quality control (QA/QC) procedures

As shown along this thesis, anthropogenic fibres are the most abundant type of micro-litter ingested by organisms, but they are also abundant and ubiquitous worldwide (Gago et al., 2018b; Suaria et al., 2020) and especially in the marine environment. Unfortunately, in the first studies, they were usually discarded from the analyses due to their similarity with those from airborne contamination. But nowadays, they are commonly included in micro-litter studies, so which the measures to avoid or control contamination of the samples are paramount. There are two clear strategies to control contamination, based on monitoring and mitigation.

Monitoring focuses on sampling and quantifying the amount of fibres that exist in the working area. The most used method consists on placing Petri dishes with filtered water as control blanks to ensures there are no fibres in the working area (Lusher et al., 2017). However, by our observations and preliminary trials performed before examination of samples of this thesis, this method was discarded. Obtaining controls with no fibres and therefore consider samples free of contamination with no other additionally measures is neither viable nor realistic, due the huge ubiquity of fibres suspended in air, even inside a workspace. Another strategy used is the sampling of the contamination fibres and subsequently applying a correction factor to infer the total number of fibres ingested (Lusher et al., 2017). However, this approach has some limitations due the impossibility to know which items are part of the sample and which ones are contamination. This fact may drive into serious limitations to the further selection of the items to be analysed by spectroscopy techniques to determine chemical composition.

On the other hand, mitigation strategies addressing airborne contamination are based on the avoidance of this contamination entering the samples by as much as possible clean conditions in the working area. Clean conditions by filtrating air using EPA filters to ensure good quality results can be reached by processing samples in clean rooms or in laminar flow cabinets

(Lusher et al., 2017). However, if these devices are not available, another more affordable options based in smaller isolation devices, such as those proposed by (Torre et al., 2016), have been demonstrated very effective by reducing possible contamination by up to 90%. In our studies, the contamination found in the controls inside the isolation device ranged from 3.6 to 15.6 times lower than contamination in outside controls, thus indicating the efficiency of the isolation device when reducing potential contamination. Furthermore, 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), so different to those from digestive tract, and thus no correction factor was needed.

In all cases, regardless of the chosen method, cleaning of working surfaces and all material with filtered deionised water or ethanol is essential. Moreover, the use of glass or iron tools (to avoid plastic ones) and the use of gloves is highly recommended. Cotton lab coats have been extensively used to avoid contamination from synthetic fibres (Lusher et al., 2017), however, since cellulose fibres are included in the screening, this measure seems to be less important and makes more sense to use natural or synthetic but highly coloured lab coats (e.g. striking colours, fluorescent colours) to identify easily the fibres to can excluded them of the analyses.

6.2. Abundance and typology of AIs and their relationship with ingestion mechanisms and feeding behaviour

Once the analysis of the AIs ingested has been addressed, the values obtained from organisms in the natural environment, as well as those obtained under experimental conditions needs an interpretation. The scarcity of studies simultaneously evaluating levels of plastic ingestion in organisms with different feeding strategies and environmental concentrations hinder the proper evaluation of feeding strategy or trophic transfer as significant factors for increased plastic ingestion. Varying levels of plastic ingestion in terms of prevalence and abundance have been reported among marine organisms, and this variability has been mainly attributed to environmental differences and intrinsic factors such as the feeding behaviour (Avio et al., 2020). Far from thinking that organisms ingest this kind of pollution by the mere fact it is present in the environment, this phenomenon seems to be more complex and highly variable both at the inter and intraspecific level.

6.2.1. AIs values in target species: geographical and temporal comparisons

In general terms, half of red mullets and European anchovies of our study presented at least one ingested AI with differences in the typologies. European anchovy seems to ingest more voluminous and variety of micro-litter, including fibres and particles, compared to red mullet,

which only seems to ingest fibres. In fact, fibres are the most common type of AIs in both species, except for European anchovy from Barcelona where particles were up to almost 50 %. Regarding to polymer composition and considering only fibres, (i.e. excluding particles from European anchovy) cellulose was the most predominant polymer, especially in Barcelona and Blanes. This is in agreement with the fact that cellulosic fibres are slightly dominant over synthetic polymers in some Mediterranean marine environments (Sanchez-Vidal et al., 2018) and also worldwide (Suaria et al., 2020).

Our results also reveal geographical differences along the study area regarding the abundance of AIs, with higher levels of ingestion in fish collected in the surrounding area of Barcelona. This pattern has been previously found in crustaceans such as the deep-sea red shrimp *Aristeus antennatus* and the Norway lobster *Nephrops norvegicus* in the same study area, showing loads of anthropogenic fibres thirty times higher in samples collected near to Barcelona compared to the ones collected northern and southern (Carreras-Colom et al., 2022, 2018). Considering that the main inputs of plastic and other debris to the marine environment come from industrialized and densely populated coastal areas (Andrady, 2011; Derraik, 2002; J. R. Jambeck et al., 2015), these findings are consistent since Barcelona is the largest city on the Mediterranean coast and supports a high level of anthropization. From the several input pathways of litter into the marine environment that have been described, wastewater treatment plants and storm-water runoff (Wagner and Lambert, 2018), are considered as a critical inputs of litter into the aquatic environment via river basins which finally discharge into the ocean (Horton et al., 2017; Murphy et al., 2016).

Differences in the results of temporal comparison are also noticeable. Our results show higher AIs values in years 2018 and 2019 compared to those obtained in 2007. The data on the production and arrival of waste in the marine environment increases every year and would support the hypothesis that there is a trend to the rise in the ingestion of this type of pollutants by organisms. Unfortunately, we do not have continuous set of data over time to confirm this hypothesis. Thus, it should be considered also the possibility that the observed differences between years could be influenced by occasional increases in the amount of micro-litter in the environment due to sporadic events that favour the entry of micro-litter into the marine environment through rivers during seasonal flood events.

6.2.2. Comparing distinct feeding behaviours

Considering results of the ingestion of fibres by sardines, it is very interesting to see how both prevalence and abundance of ingested fibres is highly depending on the feeding behaviour they display. It has been hypothesised that some organisms, mainly visual predators, such as sea

turtles and certain fish may actively ingest plastics because of their resemblance with natural prey (Ory et al., 2018b; Schuyler et al., 2012). However, visual information is not the single stimuli driving plastics to be misidentified with food. Anthropogenic materials entering the marine environment can acquire an odour signature by the presence of certain substances such as dimethyl sulphide and its chemical precursor, dimethylsulfoniopropionate (DeBose et al., 2008; Dove, 2015) due to the biofouling process. These substances activate the same chemoreceptors responsible to detect food sources, especially for zooplanktivorous species, driving plastics to be perceived as food (Savoca et al., 2017, 2016). However, active up-take of AIs due to confounding them with food is not clear in our target species, at least in red mullet. Instead, they seem to ingest them inadvertently/accidentally. Red mullets detect potential preys buried in sediments with its highly sensitive barbels to subsequently dig it up with his mouth and separate from sediment before ingest it (Labropoulou and Eleftheriou, 1997; McCormick, 1995). This capacity to discriminate food items from the surrounding material (e.g. sand, mud, vegetal remains) may explain the low presence of particles compared to anthropogenic fibres in their stomach contents. Small fibres may not be detected or may be associated with ingested prey.

Feeding habits based on less selective feeding strategies such as filtration may explain higher values of AIs ingestion compared to a strategies based on direct food identification and selection (Karlsson et al., 2017). European anchovy has similar feeding habits than sardines, switching between filtering and direct food catching depending on food availability in the water column (Costalago et al., 2015). The fact that this species can display a less selective feeding mode, i.e. filtration, may explain higher amounts of ingestion of AIs but also more variety (fragments, films and fibres). Actually, in the experimental study of Chapter III we confirmed that filter feeding sardines ingest more plastic fibres than those feeding by catching.

6.2.3. Comparing different marine habitats (pelagic/benthic)

Passive ingestion has been suggested as an important route for AIs uptake in marine organisms, being potentially ingested with prey, from the sediment or while suspended in the seawater (Browne et al., 2007; Cole et al., 2011). As a consequence, those organisms whose food sources overlap with the distribution of higher concentrations of plastics, i.e., benthic habitats, have higher probabilities of ingesting AIs (Karlsson et al., 2017; Wright et al., 2013).

Organisms inhabiting benthic habitats, displaying different feeding behaviours (planktivorous, predator, deposit- and filter-feeders, both fish and invertebrates) have been confirmed to ingest plastics in natural settings (Avio et al., 2020; Li et al., 2016; Markic et al., 2020; Murray and Cowie, 2011; Taylor et al., 2016). However, a higher frequency of microplastic

ingestion was observed in benthopelagic species than pelagic and benthic ones, which was attributed to their higher possibilities to interact with microplastics from the two compartments (Avio et al., 2020).

Different habitats accumulate different types of marine debris (Anastasopoulou et al., 2018) due to their density. While denser polymers (e.g. PET and cellulose) tend to sink to the seabed, lighter particles are more commonly found floating in pelagic waters (e.g. low-density polyethylene LDPE or Polypropilene) (Andrady, 2011). The latter ones should be more accessible for species such as European anchovy, as happens in our study, in which stomach contents particles of these denser polymers were found. In the Mediterranean continental shelf and deep seafloors, specifically in the same geographical region of the present study, cellulosic fibres are the most abundant (80%), followed by PET (12.9%) (Sanchez-Vidal et al., 2018). By this, demersal fish species, such as red mullets, should ingest denser polymers (i.e. cellulose) when compared to species from shallower habitats such as European anchovy (Alomar et al., 2017; Avio et al., 2015b), in accordance with our results.

6.3. Impact of AIs to health and overall considerations

Due to the fact that AIs have a purely anthropic and inherently non-natural origin, its presence in the environment, but especially their ingestion by biota reaching the trophic chain, started the discussion on its potential impacts. These has been mainly focused on the possibility of bioaccumulation and especially toxicological effects.

During this thesis, the potential toxicological impact of AIs was also analysed and, as it is shown in the results throughout the different chapters, no relationship was detected between the ingested AIs and the health indicators studied, thus indicating no negative effects due to this ingestion. No relationship was found with the different enzyme biomarkers indicating metabolic alterations, oxidative stress or neurotoxicity in relation to studied stressors. The presence of histological alterations attributable to AIs ingestion such as injuries on the digestive tract (due to the direct contact with AIs) or alterations of organs such as glycogen depletion, fatty vacuolation, inflammatory infiltration, or necrosis in liver (especially due to the toxins or pollutants they content) has not been detected or has not been correlated to AIs. Other histological alterations observed (e.g. little and patched inflammatory foci, differences in the fat of liver parenchyma or cysts of unknown etiology) can be considered within normality and part of the natural variability of the studied populations. Finally, no relationship was found when considering more general indicators based on morphological indices / condition factor or parasitic communities. In addition, parasites do not seem to affect the retention of AIs in the

digestive tract of red mullet nor of the European anchovy, as other authors have suggested (Pennino et al., 2020).

The absence of impact on fish health together with the little amount of AIs found in the digestive tract of the studied fish suggest that there is no accumulation and its presence may be due to a constant turnover. As we observed in the experiments with sardines, the time of AIs residence inside the fish it is relatively short, although higher than the food transit time, since all fibres ingested are egested by the end of the second day. Thus, once AIs are ingested, they travel throughout the digestive tract to be finally egested (as confirmed in Chapter III).

Ingestion of AIs is depending on the size of the item ingested, which obviously must be small enough to be available for ingestion. However, once within digestive tract they are more or less easily expelled depending on their size, shape and the organism's morphology of the digestive. For example, fibres due to their shape (long and thin) can result in agglomerations, forming tangled balls, within certain morphological features of digestive tracts [e.g crustaceans; (Carreras-Colom et al., 2020, 2018; Murray and Cowie, 2011; Welden and Cowie, 2016)], however this retention does not seem to be extended over time and items retained are finally expelled by other physiological events such as molting. If AIs are big enough, they can cause perforations or internal abrasions or even lead to gut obstructions (Markic et al., 2020; Oehlmann et al., 2009; Wright et al., 2013). But, as stated before, none of these alterations were observed in red mullets or anchovies, probably due to the small size and the little amount of AIs found within their digestive tract.

Apart from the potential damage they can produce directly within digestive tract, AIs are suggested to produce secondary deleterial effects due to their additives such as phthalates, bisphenol-A or polybrominated diphenyl ethers (Browne et al., 2013; Koelmans et al., 2014) but also by acting as a potential carriers of other pollutants such as heavy metals, organic contaminants (Gauquie et al., 2015; Rochman et al., 2013b) or being vectors of pathogens (Bowley et al., 2021). The deleterious consequences of the ingestion of this kind of pollutants has been assessed in experimental conditions, differing from one taxon to another, and include: food activity modification (Besseling et al., 2013) food assimilation deficiency (Blarer and Burkhardt-Holm, 2016), growth retardation (Lo and Chan, 2018), reduced reproduction (Cole et al., 2015), neurotoxicity (Qiao et al., 2019), reduced survival or locomotion (Tosetto et al., 2016). These effects vary depending on the organisms that are exposed, but also on the time of exposure, the particle size and condition / shape or the polymer type (Kögel et al., 2020). But probably, one of the most relevant factor is the concentration, since most of the experimental studies are acute experiments using high and environmentally unrealistic concentrations.

Our experiments performed with sardines (Chapter III) were designed to mimic realistic fibre size and concentrations (Suaria et al., 2020) and, as a result, no changes in body condition factor (Kn) due to fibre ingestion were found. Nonetheless, changes found, i.e. impoverishment of fitness, was mainly related to feeding behaviour (in filter-feeding sardines) rather than to fibre ingestion.

This kind of approaches, performing experiments with wild animals that are naturally exposed to this kind of pollutants using realistic concentrations are very necessary to know what is really happen in the wild and may be the next step for further investigations.

The fact that currently we are not able to detect negative effects does not mean that these do not exist, or that they might not appear in the future. Furthermore, there are other factors inherent to the biology of organisms but also others affecting marine environment that should be considered in a holistic way. For example, the climate change affecting marine communities can play a synergic negative effect or a cascading of related effects. This is the case of the small pelagic sardine, that higher temperatures might induce a reduction of zooplankton communities, in turn, causing a swift in the feeding behaviour in favour to filtering, and may changes patterns of fibre expulsion (Chapter II), which at the end might affect their fitness.

Accounting for the precautionary principle in order to avoid a potential threshold effect and far from ignoring the problem, it is mandatory to continue developing tools to correctly assess this type of contamination. Therefore, further investigations as well as the continuous monitoring programs of this kind pollution are now more necessary than ever.

7.CONCLUSIONS

CONCLUSIONS

1. The ingestion of Anthropogenic items (AIs) is common throughout the Balearic Sea in the two fish species analysed, *Mullus barbatus* and *Engraulis encrasicolus*, as half of the studied fish presented at least one AI in their digestive tract. They both exhibit, in general, varied but overall low values of AIs'ingestion compared to those reported for other marine organisms in the area (i.e., decapod crustaceans). (Chapters I & II)

2. In both species, AIs are dominated by anthropogenic fibres (AF), which are mainly cellulose based while synthetic ones are composed by polyethylene terephthalate (PET) or acrylic. European anchovy also ingests other AIs as fragments and films composed of synthetic polymers such as polyethylene, polypropylene and polyamide. (Chapters I & II)

3. Higher abundances and prevalence of ingested AIs are observed in individuals sampled off Barcelona compared to the other locations along the Catalan coast. This reinforces the hypothesis that highly populated metropolitan areas such as Barcelona might be a significant source of this pollution, making it more available for biota. (Chapters I & II)

4. AIs are also found in samples obtained more than 10 years ago (2007) in both fish species, demonstrating that the ingestion of this kind of pollutant is not a new phenomenon in the studied area. (Chapters I & II)

5. Visual characterisation based on the observation of morphological features of AIs but especially for AFs, is an affordable and effective method to select them prior to spectroscopy analysis to confirm their polymer composition. (Chapters I & II)

6. Although some alterations in health bioindicators (enzymatic biomarkers, histological alterations, condition indices and parasite communities) are found in both fish species, none of them can be attributable to AIs. (Chapters I & II)

7. Cysts of unknown etiology in gills of red mullets and some vacuole-like structures and inflammatory foci in livers of European anchovies are found in samples among the Catalan Sea, but these alterations are commonly found in wild fish and could be considered part of the natural variability of the studied populations. (Chapters I & II)

8. The analysis of the parasite fauna of European anchovy showed a negligible presence of zoonotic parasites in the studied area compared to other areas of its natural distribution. (Chapter II)

9. The presence of parasites within digestive tract do not seem to cause retention of AIs in red mullets nor European anchovies. (Chapters I & II)

10. After ingestion, fibres travel throughout the digestive tract of sardines until their total egestion, with no evidence of retention or accumulation over time. (Chapter III)

11. Plastic fibres do not seem to impoverish the body condition of sardines, but feeding behaviour. Filter-feeding tends to diminish body condition in comparison to catching. (Chapter III)

12. The higher temperature affects the pattern of fibre expulsion in filter-feeding sardines, thus climate change might act as a synergistic factor and contribute negatively to the AIs ingestion. (Chapter III)

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