Universitat
de les Illes Balears

DOCTORAL THESIS

2020

PLASTIC LITTER IN SEAFLOOR HABITATS OF THE BALEARIC ISLANDS AND ITS IMPLICATIONS FOR MARINE SPECIES

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Universitat
de les Illes Balears

INSTITUTO ESPAÑOL DE OCEANOGRAFÍA 1914

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Doctoral Programme of Marine Ecology

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Doctor by the Universitat de les Illes Balears

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Doctoral thesis completed by Carme Alomar Mascaró fulfilling the requirements for the doctoral degree of the Marine Ecology Doctoral Programme of the *Universitat de les Illes Balears,* under the supervision of Dr. Salud Deudero Company and Dr. Beatriz Guijarro González.

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A mis padres, hermanos y hermana

Acknowledgements

En primer lugar me gustaría agradecer a mis directoras de tesis, Salud Deudero y Bea Guijarro, gracias por guiarme y ayudarme a lo largo de este crecimiento profesional.

Esta tesis ha sido posible gracias al apoyo incondicional de toda mi familia, tíos, tías, primos y primas, gracias, gracias y gracias por estar siempre conmigo, guiarme y por nunca dejarme sola en este camino.

Montse, Maite, Elvira, Salud, esto no tiene sentido sin vosotras, gracias por tanto!

Muchas gracias a todos mis compañeros y compañeras del COB-IEO ☺

Gracias a todas aquellas personas con las que he compartido despacho; sala multidisciplinar, Sandra, David, Anabel, Miquel, Matías, Pilar, Patricia muchas gracias por los buenos momentos!

Este largo recorrido no hubiese sido igual sin el apoyo de tantísima gente, y en especial al de vosotras y al equipo Queens… gracias por estar a mi lado, tanto en los momentos buenos, como en los no tan buenos; perquè no hi ha moments dolents, sinó menos bons!

Y a mis abuelas, que siempre me envían fuerzas de allí donde estén...

Una vez más ¡Gracias! a todos los que me habéis acompañado durante este proyecto

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List of publications

This thesis is a compendium of five research papers, of which four have been published in international scientific journals and one is under revision. A list of the research papers including the journal Impact Factor (IF) according to the Journal Citation Report (JCR), the SJR indicator and quartile of publication (Q) is given in this section.

Deudero, S. and Alomar, C. 2015. Mediterranean marine biodiversity under threat: reviewing influence of marine litter on species. Marine Pollution Bulletin, 98 (1–29): 58–68. https://doi.org/10.1016/j.marpolbul.2015.07.012 (Chapter 3). **IF =3.099, SJR=1.252, Q=Q1**

Alomar, C., Compa, M., Deudero, S., Guijarro, B. 2020. Spatial and temporal distribution of marine litter on the seafloor of the Balearic Islands (western Mediterranean Sea). Deep Sea Research Part I, 155, 103178. https://doi.org/10.1016/j.dsr.2019.103178 (Chapter 4). **IF=2.848, SJR=1.446, Q=Q1 (2018)**

Alomar, C., Deudero, S., Compa, M., Guijarro, B. Exploring the relation between plastic ingestion in species and its presence in seafloor bottoms, under review (Chapter 5).

Alomar, C and Deudero, S. 2017. Evidence of microplastic ingestion in the shark *Galeus melastomus* Rafinesque, 1810 in the continental shelf off the western Mediterranean Sea. Environmental Pollution, 223: 223-229. https://doi.org/10.1016/j.envpol.2017.01.015 (Chapter 6). **IF=4.358, SJR=1.615, Q=Q1**

Alomar, C., Sureda, A., Capó, X., Guijarro, B., Tejada, S., Deudero, S. 2017. Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress. Environmental Research, 159: 135–142. https://doi.org/10.1016/j.envres.2017.07.043 (Chapter 7). **IF=4.732, SJR=1.605, Q=Q1**

The following research papers are also in line with the present thesis:

Alomar, C., Estarellas, F., Deudero, S. 2016. Microplastics in the Mediterranean sea: Deposition in coastal shallow sediments, spatial variation and preferential grain size. Marine Environmental Research, 115: 1-10. https://doi.org/10.1016/j.marenvres.2016.01.005

Nadal, M.A., Alomar, C., Deudero, S. 2016. High levels of microplastic ingestion by the semipelagic fish bogue *Boops boops* (L.) around the Balearic Islands. Environmental Pollution, 214:517-532. http://dx.doi.org/10.1016/j.envpol.2016.04.054.

List of abbreviations and acronyms

AChE	Acetylcholinesterase
AICc	Akaike’s Information Criterion
ANCOVA	Analysis Of Covariance
ANOVA	Analysis Of Variance
BSA	Bovine Serum Albumin
CAT	Catalase
CBD	Convention on Biological Diversity
CDNB	1- Chloro-2,4-Dinitrobenzene
CO_2	Carbon dioxide
DDTs	Dichloro-Diphenyl-Trichloroethane
DE	Deviance
EC	European Commission
EMFF	European Maritime and Fisheries Fund
F	Female
FAO	Food and Agriculture Organization
FBCP	Federació Balear de Cofradies de Pescadors
FO	Frequency of Occurrence
FTIR	Fourier Transform Infrared spectroscopy
FW	Fresh Weight
GAMs	Generalized Additive Models (GAMs)
GES	Good Environmental Status
GESAMP	Joint Group of Experts on the Scientific Aspects of Marine
GFCM	General Fisheries Commission for the Mediterranean
GIS	Geographic Information System
GPS	Global Positioning System

GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSAs	Geographical Subareas
GSH	Glutathione
GST	Glutathione S-Transferase
GW	Gastrointestinal Weight
HCl	Hydrogen chloride
HD	High Definition
I	Immature
IDW	Inverse Distance Weighting
IUCN	International Union for Conservation of Nature
LDPE	Low-Density Polyethylene
M	Male
MDA	Malondialdehyde
MDS	Multidimensional Scaling
MEDITS	International bottom trawl survey in the Mediterranean
MPAs	Marine Protected Areas
MPs	Microplastics
MSFD	Marine Strategy Framework Directive
PAHs	Polycyclic Aromatic Hydrocarbons
PBDEs	Polybrominated Diphenyl Ethers
PBTs	Persistent Toxic Substances
PCBs	Polychlorinated Biphenyls
PERMANOVA	Permutational Multivariate Analysis of Variance
PERMDISP	Permutational Analyses of Multivariate Dispersions
PET	Polyethylene Terephthalate
pH	Potential Hydrogen

PhD	Philosophiae Doctor
Phe	Phenanthrene
POPs	Persistent Organic Pollutants
PRIMER	Plymouth Routines in Multivariate Ecological Research
PVC	Polyvinyl Chloride
R^2	R-squared
ROS	Reactive Oxygen Species
ROV	Remotely Operated Vehicle
SE	Standard Error
SEM	Standard Error of the Mean
SIMPER	Percentage Similarities
SOD	Superoxide Dismutases
TL	Total Length
TOC	Total Organic Carbon
U	Undefined
UK	United Kingdom
UNEP	United Nations Environment Program
UV	Ultraviolet
VI	Vacuity Index
W	Weight
We	Eviscerated Weight
Wg	Gastrointestinal Weight

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Summary

Over the last years, marine litter has become a global problem arising the awareness of the scientific community and highlighting the urgent need to understand the implications that this, especially the plastic fraction, might have on different sea compartments such as the seafloor and also on marine organisms. The Balearic Islands (western Mediterranean Sea) are exposed to human stressors such as an intense use of the coast and the marine environment associated to leisure activities, recreational and professional fishing, maritime transport and intense coastal urbanization, amongst others, all of which are contributors of marine litter into the sea, which can impact marine ecosystems and biodiversity.

This thesis aims to investigate seafloor litter, especially the plastic fraction, in areas around the Balearic Islands exposed to anthropogenic pressures such as fishing and to determine factors influencing its distribution. It also proposes to understand the exposure of these areas to plastic pollution by combining different indicators of plastic litter as well as assessing microplastic ingestion in seafloor species and physiological effects associated to this ingestion.

A bibliographic review at a Mediterranean basin scale exploring areas exposed to marine litter as well as analyzing the implications of these on species was performed in first place. This evidenced that the effects of plastics on biota depended on the taxonomic group and plastic size. Results also showed that first studies documented ingestion or entanglement of plastics mainly with marine mammals, whereas macrobenthic invertebrate species and algae were also identified in floating objects or observed to colonize litter items on the seabed and some fish species were seen to have plastic items amongst their stomach contents (Chapter 3).

In an approach to study marine litter from the continental shelf to the middle slope of fishing grounds around the Balearic Islands, data on different litter fractions (clinker, glass, metal, rubber, fishing material, paper, plastic and cloth) from 15 years of bottom trawl surveys was analyzed. Abundance data on marine litter showed an unequal distribution according to the different fractions analyzed and plastic was the second most common litter fraction (after glass) with a mean (± standard error) value of 2.73 ± 0.26 kg/km^2. A Generalized Additive Model (GAM) was applied to explore seafloor plastics patterns indicating that its distribution is influenced by sampling area, bathymetric strata (depth) and distance to the coastline with highest abundances along the northwestern coast of Mallorca (Chapter 4).

A twofold approach combining two indicators on Marine Litter (Descriptor 10) of the Marine Strategy Framework Directive (MSFD): litter deposited on the seafloor (indicator 10.1.2) and litter ingested by marine animals (indicator 10.2.1), was developed (Overlap Index) in an attempt to asses, simultaneously and at the same sampling point, exposure of seafloor areas to plastic pollution. This Overlap Index

increased with depth suggesting that plastic pollution is more dependent on it than spatial coverage and it also reflected that surrounding areas of an anthropogenized bay and Marine Protected Areas (MPAs) were more exposed to plastic pollution (Chapter 5).

In order to evaluate plastic ingestion in seafloor species, microplastic ingestion in two common and abundant demersal species but with different spatial distribution was investigated through visual sorting of stomachs and gastrointestinal tracts. Microplastic ingestion in *Mullus surmuletus* (0.42 ± 0.04 microplastics/individual) was higher than in *Galeus melastomus* (0.34 ± 0.07 microplastics/individual) probably related to differences in seafloor plastic abundance with depth (Chapter 6 and 7).

Finally, the physiological effects associated to microplastic ingestion in red mullet, *M. surmuletus*, a species of commercial interest in the study area, were assessed through biomarkers of oxidative stress and cellular damage related to the generation of Reactive Oxygen Species (ROS). Results indicated a slight increase of the enzyme Glutathione S-Transferase (GST), related to the detoxification system of fish as a response to microplastic ingestion in the marine environment (Chapter 7).

This thesis demonstrates that seafloor areas of the Balearic Islands are subjected to plastic pollution depending on geographical factors, such as sampling area and depth, and that some areas including the northwestern and southwestern of Mallorca seem to be more vulnerable to plastic pollution. Moreover, evidence of the ingestion of microplastics by several species is provided, reflecting that some species living or feeding close to the seafloor are exposed to plastic pollution. Specifically, it has been seen that *M. surmuletus* and *G. melastomus* could be valid indicator species of plastic pollution in these areas. Finally, the results from this thesis allow building on to the generation and transference of knowledge related to this field of research. All of this could be taken into consideration when developing management and conservation measures and plans for plastic pollution at a local and regional scale.

Resum

Durant els darrers anys, les deixalles marines s'han convertit en un problema a escala global, captant l'atenció de la comunitat científica i destacant la necessitat reial i urgent d'entendre les implicacions que aquestes poden tenir, especialment la fracció de plàstics als diferents compartiments del medi marí, com pot ser el fons marí, i sobre les espècies marines. Les Illes Balears (Mediterrani occidental) estan sotmeses a pressions humanes incloent, entre altres, l'ús intensiu de la seva zona costanera associat a activitats recreatives, transport marítim, pesca recreativa i professional i una elevada pressió urbanística, totes elles contribuïdores de la pol·lució per plàstics al medi marí que poden impactar sobre aquests ecosistemes i la seva biodiversitat.

L'objectiu principal d'aquesta tesi és investigar les deixalles al fons marí, especialment la fracció dels plàstics, en àrees del Mar Balear exposades a pressions antropogèniques com son les àrees de pesca, i determinar quins factors poden influir a la distribució dels plàstics al fons. També es proposa analitzar l'exposició d'aquestes àrees als plàstics mitjançant la combinació de diferents indicadors així com estudiar la ingesta de microplàstics a espècies de fons i els efectes fisiològics associats a aquesta ingesta.

En primer lloc es va realitzar una revisió bibliogràfica explorant les àrees del Mar Mediterrani més exposades a les deixalles marines i les implicacions que aquestes poden tenir sobre les espècies. Els resultats d'aquesta revisió bibliogràfica evidencien que els efectes depenen del grup taxonòmic i de la mida dels plàstics i assenyalen que els primers estudis tractaven la ingesta o asfíxia dels plàstics principalment a mamífers marins, mentre que els macroinvertebrats i algues s'identificaren colonitzant objectes flotant a la superfície de la mar o dipositats als fons marí i també s'observaren plàstics als continguts estomacals de peixos (Capítol 3).

Per l'estudi de deixalles marines a la plataforma continental i al talús d'envoltant les Illes Balears, es va analitzar l'abundància de les diferents fraccions de deixalles (clínquer, vidre, metall, goma, material de pesca, paper, plàstics i teles) a partir de 15 anys de dades obtingudes a campanyes científiques. L'abundància de les deixalles marines presenta una distribució desigual en funció de la fracció analitzada i els plàstics són la segona fracció més comuna (després del vidre) amb un valor mitjà (± error estàndard) de 2.73 ± 0.26 kg/km^2. Es va aplicar un Model Additiu Generalitzat (GAM) per l'estudi dels plàstics al fons marí i es va demostrar que l'àrea de mostreig, l'estrata batimètrica (fondària) i la distància a la costa, influeixen en la seva distribució amb una major acumulació al nord-oest de Mallorca (Capítol 4).

Per tal d'estudiar l'exposició del fons marí als plàstics d'una forma simultània i a la mateixa àrea de mostreig, es va desenvolupar un índex de solapament (*Overlap Index*) que combina dos indicadors de deixalles marines (Descriptor 10) integrats a la Directiva marc sobre l'estratègia marina: deixalles al fons marí (indicador 10.1.2) i deixalles ingerides pels organismes marins (indicador 10.2.1). Aquest índex de solapament

augmenta amb la fondària suggerint que la pol·lució pels plàstics és més dependent d'aquesta que de la cobertura espacial, al mateix temps que reflecteix que una badia antropogenitzada i algunes Àrees Marines Protegides es troben més exposades a la pol·lució pels plàstics en comparació amb altres àrees estudiades (Capítol 5).

Es va analitzar la ingesta de microplàstics a dues espècies demersals, comuns i abundants a la zona d'estudi però amb diferent distribució espacial mitjançant l'anàlisi visual d'estómacs i tractes gastrointestinals. La ingesta de microplàstics a *Mullus surmuletus* (0.42 ± 0.04 microplàstics/individu) va ser major que a *Galeus melastomus* (0.34 ± 0.07 microplàstics/individu), probablement relacionat amb les diferències de plàstics amb la fondària (Capítols 6 i 7).

Finalment, es va estudiar els efectes fisiològics associats a la ingesta de microplàstics al moll, *M. surmuletus*, una espècie d'interès comercial a l'àrea d'estudi, mitjançant l'aplicació de biomarcadors d'estrès oxidatiu i dany cel·lular associats amb la generació d'espècies reactives d'oxigen (ROS). Els resultats indiquen un lleuger augment en l'activitat de l'enzim glutatió-S-transferas (GST), relacionat amb el sistema de detoxificació de l’individu com a resposta a la ingesta de microplàstics al medi marí (Capítol 7)

Aquesta tesi dóna evidència de que els fons marins d'envoltant les Illes Balears estan sotmesos a la pol·lució per plàstics i que això depèn de factors geogràfics com l'àrea d'estudi i la fondària, i que algunes àrees, incloent la costa nord-occidental i sud-occidental de Mallorca semblen ser més vulnerables a aquest tipus de pol·lució. A més es fonamenta la ingesta de microplàstics per diverses espècies, assenyalant que algunes d’elles que viuen o s'alimenten a prop del fons es veuen exposades a la pol·lució per plàstics. Concretament, s’ha vist que *M. surmuletus* i *G. melastomus*, podrien ser vàlids indicadors de plàstics a l'àrea d'estudi. Finalment, els resultats d'aquesta tesi permeten enfortir la generació i transferència de coneixement relacionat amb aquest camp d'investigació. Tot això hauria de considerar-se a l'hora de desenvolupar mesures i plans de conservació i gestió per fer front a la pol·lució per plàstics tant a escala local com regional.

Resumen

Durante los últimos años, la basura marina se ha convertido en un problema global, captando la atención de la comunidad científica y destacando la necesidad real y urgente de comprender las implicaciones que esta, especialmente la fracción de plásticos, puede tener sobre los diferentes compartimentos del medio marino, como el fondo del mar y los organismos. Las Islas Baleares (Mar Mediterráneo occidental) están expuestas a presiones antropogénicas tales como, un uso intenso de su costa y el medio marino asociado a actividades recreativas, transporte marítimo, pesca recreativa y profesional, una intensa presión urbanística, todas ellas contribuidoras de plásticos al medio marino que pueden impactar sobre estos ecosistemas y su biodiversidad.

La presente tesis tiene como objetivo investigar la basura marina, especialmente la fracción de plásticos, en el fondo marino de áreas del Mar Balear expuestas a presiones antropogénicas como pueden ser la pesca, y determinar qué factores influyen en la distribución de estos. Mediante la combinación de distintos indicadores, se pretende determinar las áreas más expuestas a este tipo de polución, así como abordar el estudio de la ingesta de microplásticos en especies de fondo y los efectos fisiológicos asociados a la ingesta de estos.

En primer lugar se llevó a cabo una revisión bibliográfica sobre las áreas más expuestas a las basuras marinas en el Mar Mediterráneo y las implicaciones que estas pueden tener sobre las especies marinas. De este análisis se pudo observar que los efectos de los plásticos sobre la biota dependen del grupo taxonómico y del tamaño del plástico. Asimismo, los primeros estudios científicos trataban la ingesta o enredo de plásticos principalmente en mamíferos marinos, mientras que algunas especies de invertebrados y algas se observaron colonizando objetos flotando en la superficie o depositados en el fondo del mar, además de documentarse plásticos en los contenidos estomacales de ciertas especies ícticas (Capítulo 3).

Para el estudio de la basura marina sobre la plataforma continental y el talud de áreas expuestas a la pesca de alrededor de las Islas Baleares, se analizó la abundancia de distintas fracciones de basura (clínker, vidrio, metal, goma, material de pesca, papel, plásticos y telas) procedentes de 15 años de campañas científicas. Se observó una distribución desigual a lo largo del área prospectada en función de la fracción analizada y los plásticos constituían la segunda fracción más abundante (después del vidrio) en la zona de estudio con un valor medio (± error estándar) de 2.73 ± 0.26 kg/km^2. Para el análisis de plásticos en el fondo marino, se aplicó un Modelo Aditivo Generalizado (GAM) indicando que el área de estudio, el estrato batimétrico (profundidad) y la distancia a costa definían la distribución de estos con una mayor acumulación en la zona noroeste de Mallorca (Capítulo 4).

Con el propósito de analizar la exposición del fondo marino a los plásticos de una manera simultánea y en el mismo punto de muestreo, se desarrolló un índice de

solapamiento (*Overlap Index*) mediante la combinación de dos indicadores de basuras marinas (Descriptor 10) de la Directiva Marco sobre la Estrategia Marina: desechos depositados en los fondos marinos (indicador 10.1.2) y desechos ingeridos por los animales marinos (indicador 10.2.1). Este índice de solapamiento incrementaba con la profundidad sugiriendo que la afectación por plásticos es más dependiente de esta que de la cobertura espacial. Este índice también indicó que la zona de una bahía antropogenizada y algunas zonas de Áreas Marinas Protegidas estaban más expuestas a la polución por plásticos en comparación con otras zonas estudiadas (Capítulo 5).

Con el fin de evaluar la ingesta de plásticos en especies demersales, se investigó la ingesta de microplásticos en dos especies comunes y abundantes pero con distinta distribución espacial mediante el análisis visual de estómagos y tractos gastrointestinales. La ingesta de microplásticos en *Mullus surmuletu*s (0.42 ± 0.04 microplásticos/individuo) fue mayor que en *Galeus melastomus* (0.34 ± 0.07 microplásticos/individuo), probablemente asociado a las diferencias de plásticos con la profundidad (Capítulo 6 y 7).

Finalmente, se estudiaron los efectos fisiológicos asociados a la ingesta de microplasticos en el salmonete, *M. surmuletus*, una especie de interés comercial en el área de estudio, mediante la aplicación de biomarcadores de estrés oxidativo y daño celular relacionados con la generación de especies reactivas de oxígeno (ROS). Estos biomarcadores indicaron un ligero aumento en la actividad de la enzima glutatión-S-transferasa (GST), asociados al sistema de detoxificación del individuo como respuesta a la ingesta de microplásticos en el medio marino (Capítulo 7).

Esta tesis da evidencia de que el fondo marino está expuesto a la polución por plásticos dependiendo de factores geográficos como, el área de estudio y la profundidad, y que algunas áreas incluyendo el noroeste y suroeste de Mallorca son más vulnerables a los plásticos marinos. Además, se demuestra que hay una ingesta de microplásticos en varias especies, reflejando que algunas de estas que viven o se alimentan cerca del fondo del mar están expuestas a este tipo de polución. Concretamente, se ha visto que *M. surmuletus* y *G. melastomus* pueden ser validos indicadores de plásticos en el área de estudio. Finalmente, los resultados permiten construir sobre la generación y transferencia de conocimiento relacionados a este campo de investigación. Todo ello debería tenerse en consideración a la hora de desarrollar medidas y planes de conservación y gestión para hacer frente a la polución por plásticos tanto a escala local como regional.

Chapter 1: General Introduction

The Mediterranean Sea is a hotspot for biodiversity with unique geologic, biogeographic, physical and ecological features (Coll et al., 2010) but it is also very susceptible to human pressures given its geographical and political situation. This sea is highly anthropogenized receiving more than 25% of the global tourism, being home to 7% of the worlds' coastal human population and with 30% of the worldwide maritime transport navigating through its waters (UNEP, 2009). All of these lead to coastal development, contamination, acceleration of invasive species, desalination, habitat loss and degradation, overfishing, resource extraction and eutrophication, amongst others (Coll et al., 2010). However, in the last few decades marine litter has become a real threat for marine ecosystems in this basin and higher concentrations of marine litter have been detected here rather than elsewhere (Cózar et al., 2015; UNEP, 2015). The semi-enclosed nature of this basin, with a low water exchange rate with other seas and oceans and its oceanographic regimes favor these high concentrations (Cózar et al., 2015) and 62 million items of macrolitter are thought to be floating along the sea surface of the entire basin (Suaria and Aliani, 2014). Scientific research has documented marine litter in all sea compartments of the Mediterranean basin from the sea surface to the seafloor and from coastal to offshore areas (Martellini et al., 2018; Compa et al., 2019a; Ruiz-Orejón et al., 2019) as well as ingested in biota (Fossi et al., 2018). *Therefore, given this scenario and the exposure of marine ecosystems to plastic litter, this thesis begins by addressing marine litter, especially the plastic fraction, at a Mediterranean basin scale, reviewing most affected areas, as well as determining the implications that plastics might have on different taxonomic groups by categorizing effects into ingestion/entanglement and colonization/rafting (Chapter 3).*

According to the United Nations Environment Program (UNEP) marine litter is defined as ''any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment''. Given plastics' durable and flexible characteristics as well as massive use, plastic is the most common litter fraction found throughout the marine environment (Bergmann et al., 2015) and it is patchily distributed along coastal and oceanic grounds; continental shelves and slopes, submarine canyons, seamounts, banks and ocean ridges in a wide range of sizes from a micro to a macro size scale (Cauwenberghe et al., 2013; Pham et al., 2014; Tubau et al., 2015; Lopez-Lopez et al., 2017; Spedicato et al., 2019). Estimates of litter densities on the seafloor (0.4 to 48 items/ha^2; Pham et al., 2014) are higher than those for floating marine litter (0.021 items/ha^2) (UNEP, 2016) and size of plastic can determine the effects that these might have on marine organisms. Whereas smaller plastics will be available for a larger range of organisms to ingest and/or filter (Lusher et al., 2013), larger items will be also ingested/filtered by organisms but might also have an entangling effect upon species and can be colonized or used as shelter by marine organisms having the potential to alter seafloor habitats by adding new substrates or by overlaying sediment, inhibiting gas exchange and interfering with life (Galil, 2006). Not only size of plastics will determine ecological effects upon species but this parameter can be used as an indicator of plastic sources. According to plastics, these can be categorized into nano-, micro-, meso- and macro plastics (GESAMP, 2019); microplastics (MPs) particles with a diameter smaller than 5 mm, are classified into primary microplastics which are intentionally produced at a microscopic scale, either as precursors to other products (e.g. plastic pellets; Costa et al., 2010) or for direct use as abrasives in cleaning products or rotomilling (Browne, 2015) while secondary microplastics result from the degradation of larger plastics into small items due to mechanical, photolytic and/or chemical degradation processes in the marine environment and biodegradation under environmental conditions (Mathalon and Hill, 2014; Gewert et al., 2015).

In the northwestern Mediterranean Sea, first studies on the distribution and abundance of marine litter date back to the 90´s when up to 924 litter items were caught in one single trawl around Marseille (Galgani et al., 1995) and up to 100,000 items of marine litter/km² were quantified in the continental shelf of the Ligurian Sea (Galgani et al., 2000). A more recent study quantified marine litter in 90% of the sampled stations along the continental shelf and upper slope of the entire Mediterranean Sea and identified hotspot areas, mainly in the western and eastern parts of this sea (Spedicato et al., 2019). The continental shelf is exposed to human pressures including leisure activities (bathing, sailing, diving…), recreational and professional fishing, maritime transport and terrestrial waste inputs (direct and indirect) which are all sources of marine litter. However, even though the vast majority of marine litter sources are land-based (80%) in regrets to sea-based sources (20%) (Jambeck et al., 2015), hydrodynamic processes and marine currents play an important role in the transportation and diffusion of litter (Tubau et al., 2015) and consequently higher abundances might be detected in deeper areas such as submarine canyons (Galgani et al., 1995; Pham et al., 2014) which may act as dispersion vectors.

The Balearic Islands are located in the western region of the Mediterranean Sea and are exposed to human stressors including commercial and recreational fishing and all types of maritime traffic, as well as densely populated and urbanized areas along the coastline which increase during the summer season and directly or indirectly impact the marine environment. Moreover, in this area, highest abundance of floating anthropogenic and plastic litter has been quantified in coastal areas during August which coincide with the maximum peak of tourism (Compa et al., 2019a). Geographically, these islands constitute the Balearic Promontory which is elevated between 1000 to 2000 m above the surrounding marine Algerian and Balearic sub-basins (Acosta et al., 2002, 2004) and it is characterized by more oligotrophic waters than adjacent waters of the Iberian coast and the Gulf of Lions due to the absence of rivers (Estrada 1996). In contrast to the Catalan coast, where submarine canyons with high quantities of marine litter have been detected (Tubau et al., 2015), submarine canyons are scarce on the Balearic margin (Canals and Ballesteros, 1997). Given all these factors determining seafloor litter, abundance and distribution patterns as well as trends, especially of the plastic fraction, should be studied in relation to seafloor geomorphology, hydrodynamic characteristics, distance from the coast and exposure to anthropogenic activities of the sampling area. *For this reason, in this thesis marine litter, especially the plastic fraction, is studied in seafloor areas around the Balearic Islands exposed to human pressures, particularly fishing activities, and ranging from the continental shelf to the middle slope and up to 756 m depth and 21 nautical miles from the coast (Chapter 4).*

Additionally, these islands are influenced by two main currents: the Northern Current which circulates from the Gulf of Lion along the continental slope off the northwestern Mediterranean Sea into the Balearic sub-basin and by the Algerian Current which interacts with the Alboran Sea eddies and spreads fresh surface water from the Atlantic Ocean (Atlantic Waters) towards the Balearic Islands (Amores and Montserrat, 2014). Therefore, besides of the input of marine litter from local sources, these currents could be transporting marine litter from distant areas to these islands after having circulated along large metropolitan areas off the French and Spanish Mediterranean coasts as well as along the North African coast. Consequently, the Balearic Islands, which are separated from the Spanish mainland by maximum depths of 2000 m and a distance of 95 nautical miles (Guijarro, 2012), can be a source of marine litter resulting from high human activities ongoing on coastal and inland areas, especially during the summer season, but can also be receptors of transferred marine litter from adjacent and more distant areas. This could be the case in coastal areas of the Cabrera Archipelago Maritime-Terrestrial National Park (Balearic Islands), where microplastics were found with higher abundances in the integral zone of the Marine Protected Area (MPA), with up to 0.90 ± 0.10 MPs/g, than in coastal sediments of a nearby urbanized area in Mallorca (Balearic Islands) suggesting transferred contamination from adjacent anthropogenized areas to this MPA (Alomar et al., 2016). Moreover, microplastics identified in the urbanized area were filament type, reflecting a more urban related origin of plastic pollution (Browne et al., 2011; Remy et al., 2015) whereas those observed in the MPA had a fragmented typology linked to the fragmentation of larger plastics, in the marine environment (Wagner et al., 2014), and possibly reaching the MPA from close and/or distant anthropogenized areas.

Given the ubiquity and persistence of plastics from coastal to offshore areas due to dispersion processes that can transfer plastic pollution across MPAs boundaries, which are the primary tool for *in situ* habitat and biodiversity conservation (Coll et al., 2012), plastics are available for a wide range of organisms from protected to non-protected areas, with different ecological and biological traits and showing different sensitivity to marine plastics. Ingestion of plastics in wild species was first documented in the early 70's in coastal waters of southern New England in larval and juvenile stages of demersal and pelagic fish (Carpenter et al., 1972). Moreover, by the early 90's it was already thought that the ingestion of plastic particles might affect fish by blocking their digestive tract and reducing their feeding activity, as well as causing ulceration or other physical injury to the stomach lining (Hoss and Settle, 1990). Since then, reports on the number of species affected by plastic ingestion has been increasing and up to date, in the Mediterranean basin, most of the studies have focused on demersal (32.9%) and pelagic (27.7%), followed by benthic (14.7%), benthopelagic (16.5%), neritic (5.3%) and mesopelagic (2.9%) species (Fossi et al., 2018). Moreover, in this region of the world, species in coastal areas are at a higher risk of ingesting plastic than offshore species and in the Balearic Sea the overall predicted risk of plastic ingestion in marine species is higher than in other areas of the Mediterranean basin (Compa et al., 2019b). Ingested plastics in species might be dependent on several factors such as their biological and ecological characteristics, habitat and home range, feeding mechanisms and behavior as well as their spatial distribution (Fossi et al., 2018). Consequently, indicator species reflecting plastic ingestion from coastal to offshore areas and from benthic to pelagic environments should be considered when studying the effect of plastics upon species (Schwacke et al., 2013).

Moreover, when studying plastic ingestion, besides studying species with different trophic levels, multiple species occupying the same area and at the same time, should be considered as high species diversity and plastic overlap has been observed at a Mediterranean basin scale (Compa et al., 2019b). *Thus, a twofold approach addressing at the same time, seafloor plastics and microplastic ingestion in multiple species and from the same sampling area is explored in the frame of this thesis (Chapter 5).* This approach not only allows determining areas exposed to plastic pollution and plastic ingestion within species but relates two indicators under Descriptor 10 (Marine Litter) of the Marine Strategy Framework Directive (MSFD, 2008/56/EC): trends in the amount, composition, spatial distribution and sources of litter deposited on the seafloor (indicator 10.1.2) and trends in the amount and composition of litter ingested by marine animals (indicator 10.2.1). During the last years in the European Union, the MSFD has been building on the application of indicators related to marine litter, amongst others, in order to achieve and maintain a Good Environmental Status (GES) of marine waters. In this sense, generation of data related to the amounts and distribution of marine litter in sea surface, water column and seafloor as well as baseline data on plastic ingestion values in indicator species is a priority and a real need for conservation policies of marine ecosystems within this region.

Whereas the presence of plastics in the sea surface represents only a fraction of the total input and can be transitory (Gallo et al., 2018), the seafloor is seen as the ultimate fate for marine plastics (Courtene-Jones et al., 2017). This is because plastics with a density which exceeds that of seawater (1.02 g cm^3) sink and accumulate in sediments (Woodall et al., 2015) as well as that plastics can change their density due to biofouling (Barnes et al., 2009), by aggregation with denser objects or because of aging, in addition to getting entrained into turbulent flows (Tubau et al., 2015) or degrading into smaller items (Andrady, 2011) which finally sink to deeper areas. Consequently, demersal species, particulary those with a feeding behavior link to the seafloor, are at higher risk of ingesting plastics than pelagic species (Compa et al., 2019b). Some demersal species might be more vulnerable to marine litter pollution than others, as indicated by the red mullet fish, *Mullus barbatus* Linnaeus, 1758, which ingests twice as much microplastics than *Scyliorhinus canicula* (Linnaeus, 1758) and *Merluccius merluccius* (Linnaeus,

1758) (Bellas et al., 2016). These osteichthyes and elasmobranchs species are used for monitoring marine contamination within the Spanish Marine Pollution Monitoring Program (SMP) (Bellas et al., 2016), and in accordance with bioindicator criteria selection (Fossi et al., 2018) they or species from the same congener can be thought as suitable candidate species for monitoring spatial and temporal trends of ingested litter in seafloor habitats (Bellas et al., 2016). As ingestion rates should reflect both plastic abundances at the species habitat and ingestion occurrence in species, selection of species with an important and pivotal role in food webs of the study area is important, enhancing the comparison and transferability of results and monitoring strategies amongst taxonomic groups with similar feeding traits and analogous habitats from different geographical areas. *In this sense, microplastic ingestion in seafloor species is studied as a potential indicator of plastic pollution in the study area. Two abundant demersal species of the Balearic Islands, with different spatial distribution along the continental shelf and middle slope, such as Mullus surmuletus, which shows a more specialist diet, mainly feeding on polychaetes but also crustaceans (Bautista-Vega et al., 2008), and Galeus melastomus, with a more generalist and opportunistic diet preying on shrimps, cephalopods, fishes and euphausiids are investigated (Carbonell et al., 2003; Valls et al., 2011) (Chapter 6 and Chapter 7).*

Moreover, plastic impacts on biota have to be looked at further on than the ingestion of anthropogenic particles, as plastics may release toxic chemicals (Day et al., 1985) from the material itself, which are added during manufacturing processes (Teuten et al., 2009), or sorbed to their surface once in the marine environment (Rochman et al., 2013). Metals and Persistent Organic Pollutants (POPs), such as polychlorinated biphenyls (PCBs), dichloro-diphenyltrichloroethane (DDTs) and polycyclic aromatic hydrocarbons (PAHs) can be found amongst the components which are sorbed to plastics in the marine environment (Rios et al., 2007; Rochman et al., 2014; Gewert et al., 2015). In addition, chemicals such as bisphenol A, phthalates, nonylphenol and polybrominated diphenyl esters are added to plastic polymers during manufacturing processes (Rios et al., 2007; Rochman et al., 2014) to improve properties of the final product (Teuten et al., 2009) making them more stable, durable and resistant to degradation (Gewert et al., 2015). This means that as plastics are expected to persist in the environment for hundreds or even thousands of years (Barnes et al., 2009), hydrophobic monomers and plastic addictives can accumulate with time on their surface causing toxicity, carcinogenesis, endocrine disruption and physical harm (Laist, 1997; Wright et al., 2013; Jeong and Choi, 2019) as well as producing bioaccumulation of persistent toxic substances across organisms and the trophic web (Koelmans et al., 2013).

Physiological effects such as inflammation, lipid accumulation and oxidative stress in fish liver (Lu et al., 2016) and structural and functional deterioration of fish intestine (Pedà et al., 2016), as well as alterations in liver (Espinosa et al., 2019) have been detected due to microplastic ingestion under laboratory conditions. Recently alterations in wild fish tissues' have also been observed, suggesting lipid oxidative damage in gills and muscle and neurotoxicity related to plastic pollution (Barboza et al., 2019). In the marine environment, exposure of organisms to certain toxic compounds can induce the overproduction of Reactive Oxygen Species (ROS) leading to oxidative damage of macromolecules of tissues (Sureda et al., 2006). The application of biomarkers related to the generation of ROS involving enzymes such as Superoxide Dismutases (SOD), Catalase (CAT) and Glutatione-S-Transferase (GST) and the use of Malondialdehyde (MDA) as a biomarker of lipid peroxidation have been assessed in aquatic organisms as indicators of environmental stress (SOD, CAT) and detoxification (GST) (Karami et al., 2016) in response to marine pollution (Sureda et al., 2011). Consequently, oxidative damage in species due to plastic ingestion (and associated contaminants) can be assessed through the enzymatic response of macromolecules in selected organism tissues (Guzzetti et al., 2018; Barboza et al., 2019; Jeong and Choi, 2019). In this sense, scientific data on plastic ingestion in marine species as well as physiological effects upon species is needed to understand at a first stage, plastic effects at species level to move further on to investigate implications at a population level and lastly consider the whole ecosystem scale. Moreover, given the transferability of plastics (and associated contaminants) along the food web, which might have implications for human beings (Barboza et al., 2019), besides of the biological and ecological characteristics of the selected study species, the commercial interest of these should be considered when assessing plastic pollution and its implications across organisms. *In this sense, the physiological response to microplastic ingestion of red mullet fish, Mullus surmuletus, an important target species of the commercial fishing fleet in the study area (Moranta et al., 2008), is assessed through biomarkers of oxidative stress and cellular damage in fish liver at a final stage of this thesis (Chapter 7).*

Chapter 2: Aim of the study

The aim of this thesis is to study litter in seafloor areas from 50 m to 800 m depth at a mesoscale level around the Balearic Islands by analyzing factors determining plastic distribution in this area as well as investigating simultaneously seafloor plastics and microplastic ingestion in multiple species from the same sampling area. This thesis also aims at quantifying microplastics ingestion in two common demersal species, *Galeus melastomus* and *Mullus surmuletus,* and assessing the physiological response of *M. surmuletus* to microplastic ingestion in the marine environment. Consequently, the specific aims of this thesis are:

-To review the available quantitative data on marine litter, specifically the plastic fraction, from the seafloor and the sea surface in the Mediterranean Sea basin, as well as the existing literature on the interactions of marine litter with marine biota in this region (Chapter 3).

-To quantify marine litter from the continental shelf to the middle slope of fishing grounds in the Balearic Islands and investigate factors influencing seafloor plastic distribution in this area (Chapter 4).

-To determine seafloor areas exposed to plastic pollution by applying a twofold approach addressing, at the same time and in the same area, two indicators of marine litter: seafloor plastic abundance and microplastic ingestion in biota (Chapter 5).

-To quantify microplastic ingestion in two common demersal species (*Galeus melastomus* and *Mullus surmuletus*) with different spatial distribution along the continental shelf and slope of the study area (Chapter 6 and 7).

-To assess the physiological response of *Mullus surmuletus*, a species of commercial interest, to microplastic ingestion through biomarkes of oxidative stress and cellular damage related to the generation of Reactive Oxygen Species (Chapter 7).

Marine Pollution Bulletin 98 (2015) 58–68

Chapter 3: Mediterranean marine biodiversity under threat: Reviewing influence of marine litter on species

Salud Deudero and Carme Alomar

Abstract

The Mediterranean Sea is one of the most polluted seas worldwide, especially with regard to plastics. The presence of this emerging man made contaminant in marine environments precludes large effects and interactions with species exposed to massive litter quantities. In this review, available data of floating and seafloor litter around Mediterranean sub-basins are reported. A review of scientific literature on the interaction of plastic with marine biota resulted in the identification of 134 species, several taxa and feeding strategies affected from 1986 to 2014. Data from 17,334 individuals showed different levels of ingestion and effects on catalogued IUCN species (marine mammals and sea turtles) in addition to several pelagic fish and elasmobranchs. Biodiversity is certainly under threat, and knowledge of the extent of taxa affected is of concern considering the increasing plastic loads in the Mediterranean Sea and worldwide.

Keywords: Marine litter, Plastics, Biodiversity, Ingestion, Conservation

3.1 Introduction

Marine litter is increasing worldwide (Barnes et al., 2009) and is considered to be an emerging issue threatening marine biodiversity. The United Nations Environment Program (UNEP) defines Marine litter as ‘‘any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment’’. This organization has estimated that 6.4 million tonnes of litter enter the oceans every year (UNEP, 2009), with 62 million macrolitter items currently floating on the surface of the entire Mediterranean basin (Suaria and Aliani, 2014). The most abundant marine litter are polymers derived from plastics. Plastic has been produced on planet Earth for just over a century (Gorman, 1993), and several studies have revealed that plastic loads and presence are increasing in marine ecosystems worldwide, possibly provoking alterations at the species, community or ecosystem level. Concentrations of plastic (plastic islands) are present in the main subtropical gyres of the North and South Atlantic and Pacific (Leichter, 2011; Eriksen et al., 2014) and Indian Oceans (Barnes, 2004). In the Mediterranean Sea, Pham et al. (2014) have reported that plastics are the most prevalent litter items found on deep sea floors, while Suaria and Aliani (2014) have stated that plastic objects account for 82% of all man-made floating items. Consequences of the extent of this ‘plastic era’ can be observed on multiple scales, and many approaches to address these issues are just beginning to be developed. Moreover, other litter types are accumulating in marine environments, such as glass, paper, cardboard, metal, cloth, rubber, fishing-related waste, munitions, wood, cigarette filters and tips, sanitary and sewage-related litter, ropes, toys and strapping bands (UNEP, 2011).

Litter enters the marine environment and proliferates, migrates and accumulates in natural habitats worldwide. Whereas macrolitter washed up on shores is primarily assessed (Gabrielides et al., 1991; Galgani et al., 2000), floating and seafloor marine litter have been investigated less frequently (Galgani et al., 1995, 1996; Ramirez-Llodra et al., 2013; Suaria and Aliani, 2014). Recently, high macrolitter densities in submarine canyons have been reported (Pham et al., 2014), demonstrating evidence of transferred marine litter pollution. The plastic fraction of litter is highly persistent and resistant to biodegradation but finally fragments into small pieces that remain in the environment for many years (Klemchuk, 1990; Derraik, 2002; Barnes et al., 2009).

These features of marine plastics cause distress to marine organisms. Therefore, marine biota interact with plastics in several manners, resulting in digestion, entanglement, toxicity, carcinogenesis, endocrine disruption and physical harm, including internal abrasion and blockage, and they can also facilitate invasive species spread (Laist, 1997; Wright et al., 2013). Moreover, under ordinary environmental conditions, the availability of hydrophobic pollutants in seawater increases due to adsorption onto plastic litter (Thompson et al., 2009; Cole et al., 2011), which increases their environmental persistence, highlighting the importance of plastics as vectors of pollutant transfer across organisms (Teuten et al., 2007; Tanaka et al., 2013). These facts combined with the ubiquity and inherent persistence of plastic polymers highlight the need to assess the effects of the introduction of plastics to various marine habitats (epipelagic, pelagic, demersal and coastal or offshore habitats) on organisms. Moreover, there are many ‘red-listed’ species that are affected by direct effects of plastics, either by ingestion or entanglement and are in highly jeopardised situations (Baulch and Perry, 2014). Marine organisms can become entangled in loops or openings of floating or sunken marine litter, particularly discarded fishing gear, plastic packing rings and packing strapping bands (Katsanevakis, 2008), and benthic organisms may colonise these artificial structures as substrate settlement, refuge or reproduction sites (Katsanevakis et al., 2007). In addition, many species are voracious predators, visually chasing prey, and in many cases, confusion linked to the resemblance of prey to zooplanktonic organisms (jellyfish, siphonophores, euphausiids, fish larvae and juveniles, amongst others) can occur (Katsanevakis, 2008; Anastasopoulou et al., 2013). Therefore, the quantification of marine litter either as substrates or as particles that are ingested or entangled is essential, as well as the determination of the extent of taxa affected to facilitate future research.

In the Mediterranean, several taxa have been studied, mainly marine mammals and reptiles; however, few studies have revealed effects on fish or invertebrates, despite the fact that ingestion of plastics by fish was discovered many years ago (Carpenter et al., 1972; Hoss and Settle, 1990). In addition, few studies have evaluated plastics in the stomachs of epipelagic (Lusher et al., 2013) and mesopelagic fish (Davison and Asch,

2011) or the transference of litter across food webs to larger predators (Eriksson and Burton, 2003) and ultimately, to humans (Romeo et al., 2015).

Marine organisms have adapted to fluctuating environmental conditions (temperature, pH, CO_2, salinity, carbonates, etc.) and their physiological mechanisms have evolved to cope with changes that occur over geological time. However, marine litter, especially plastics, are brand new durable substances in nature that have only been present for less than 100 years. Therefore, the evolutionary development of adaptive responses of organisms to these materials has not yet occurred. Increased loads of plastics in oceans and litter exposure together with the limited number of studies addressing these emergent issues indicate the need to conduct further research.

Therefore, the aims of this study were threefold as follows: (1) to review available quantitative data on marine litter (either floating or seafloor) in the Mediterranean basin; (2) to revise existing literature on interactions of marine litter, mainly plastics, with marine biota in the Mediterranean Sea; and (3) to provide a species compendium of organisms that ingest marine litter at sea, particularly focusing on IUCN-listed species.

3.2 Materials and methods

3.2.1 Bibliographic research

A review of documents, including scientific papers, grey literature and reports of European projects, was conducted to obtain a representative number of documents on marine litter and litter interactions and effects with marine biota at the Mediterranean basin scale. A search was performed of 3 major scientific databases, including Scopus, ISI Web of Knowledge and Google Scholar, using a list of key research terms to identify relevant scientific papers. The research criteria were based on studies of the Mediterranean Sea, and no date limitation filter was applied. The terms used to search the databases were as follows: marine plastics Mediterranean, marine litter Mediterranean, marine debris Mediterranean, invertebrates and marine plastics, invertebrates and marine litter, invertebrates and marine debris, vertebrates and marine plastics, vertebrates and marine litter, marine mammals and marine debris, marine mammals and marine plastics, marine mammals and marine litter and marine mammals and marine debris. The bibliographic research was conducted for marine litter of all size classes (mega, macro, meso and micro) except for nanolitter (GESAMP, 2015). Marine litter at beaches, laboratory experiments and effects on seabirds were deliberately excluded to strictly focus on marine species and surface, pelagic and seafloor environments.

To study marine litter and specifically, plastic fraction around the Mediterranean basin, data on the percentages of plastic items on the seafloor and in the pelagic realm of the water column and floating litter were considered. Most of the data on plastic items (%) reported by the studies were obtained during offshore cruises, when samples were collected with trawls at different depths, and bongo nets and manta trawls were dragged through surface waters. For this part of the review, the information was divided according to the region, year, plastic items (%), seafloor and surface areas and source of data.

To evaluate the documents on the interaction and effects of litter with marine biota, data on litter were assessed, with an emphasis on plastics, to identify the main taxa affected (algae, seagrass, invertebrates, sea turtles, fish and marine mammals), the main types of interactions and effects, the feeding strategies and the catalogued species involved (following the IUCN classification). Information from the reviewed documents was classified according to the species, taxa, number of individuals in the study, quantified litter (%) and source of data. In addition, litter was classified as general litter or plastic litter, and when possible, litter items were specified (metal, wood, glass, styrofoam, ropes and monofilaments or fishing net materials). The types of interactions and effects were classified as direct effects, ingestion/entanglement or interactions, colonisation/rafting. Ingestion and entanglement are direct effects of plastics and were grouped together because most studies reported both of these impacts simultaneously. Feeding strategies (filter feeders, suspension feeders, detritivorous feeders and predators), and IUCN categories were assigned to species. Information on feeding strategies and IUCN categories was extracted from several databases (FishBase (www.fishbase.org), the Reptile Database (www.reptile-database.org), the World Cetacea Database (www.marinespecies.org/cetacea/) and The IUCN Red List of Threatened Species. Other information, such as the study area, habitat of the species in the study (offshore/coastal) and study date, were recorded from the reviewed documents.

3.2.2 Data analysis

Descriptive analysis of the plastic data around the Mediterranean Sea was conducted to provide a wide perspective of plastic items (%) in different areas of the basin from all available data between 1979 and 2014. For this purpose, a database was built, including the percentages of litter items and geographic positions determined from the bibliographic research, and it was integrated into a geographic information system (GIS) to display spatial litter patterns in the Mediterranean basin (ArcGIS 9.3).

To evaluate the effects and interactions of litter with marine biota, a comparison of taxa and feeding strategies was conducted for each of the considered subgroups (ingestion/entanglement and colonisation/rafting). Quantitative data on the effects and interactions of litter on marine biota were expressed as percentages obtained from the literature records when provided, and in other cases, the relative numbers of individuals exhibiting marine litter ingestion/entanglement and colonisation/rafting were calculated. The mean and standard error (se) values derived from all studies were calculated according to the faunal group (marine mammals, sea turtles, fish, invertebrates, algae and seagrass) and feeding strategies (filter feeders, suspension feeders, detritivorous feeders and predators), and species were assessed together using a compiled species list.

The evaluation of litter ingestion by the IUCN catalogued species was performed by determining the number of studies, total number of individuals studied, percentage of individuals ingesting litter items (litter ingestion %), plastic occurrence or general litter occurrence and coastal or offshore habitat of the species. Further, the litter ingested by all pelagic species was evaluated by calculating similarity matrices using normalised data for the un-transformed percentage of litter ingestion and by performing non-metric multidimensional scaling (MDS) (PRIMER; Anderson et al., 2008).

3.3 Results

3.3.1 Bibliographic research

A total of 79 documents were obtained from the bibliographic research, which included scientific papers, PhD theses and reports from research projects. The citation list was examined, and a total of 24 studies were found to satisfy the criteria for plastic data around the Mediterranean Sea (30%), and another 29 studies (37%) satisfied the criteria for the interaction of marine litter with Mediterranean biota. The papers in each category were analysed for the most relevant findings to improve the knowledge of plastic around the Mediterranean Sea and the interaction of litter with marine biota.

3.3.2 Marine litter in the Mediterranean sub-basins

Available data on marine litter differed amongst the studied areas (Fig. 3.1). The distribution of the examined studies revealed that plastic around the Mediterranean Sea showed highest reported values in the northwestern Mediterranean (76–96%), especially in the Tyrrhenian Sea (96%), and the lowest amount was found in the Lybian Sea (18%) (Fig. 3.1). With regard to the period of time studied, the studies conducted in 2013 reported the highest amounts of plastic (90–100%), and those conducted in 2009 found the lowest (6%). Although there was a wide variation in plastic items (%), which was probably due to the unequal number of studies, there was a shift from superficial to deep-sea studies. The first reports of floating plastic litter reported 65% plastic marine litter items in 1979, and this value increased to 83% in 1997. The first studies on seafloor plastic were conducted in 1993, reporting 77% plastic marine litter items (Fig. 3.2). Considering the high variability in examined studies, there was a trend of higher amounts of plastic items (%) reported in those studies conducted in areas close to populated urban zones (Fig. 3.1).

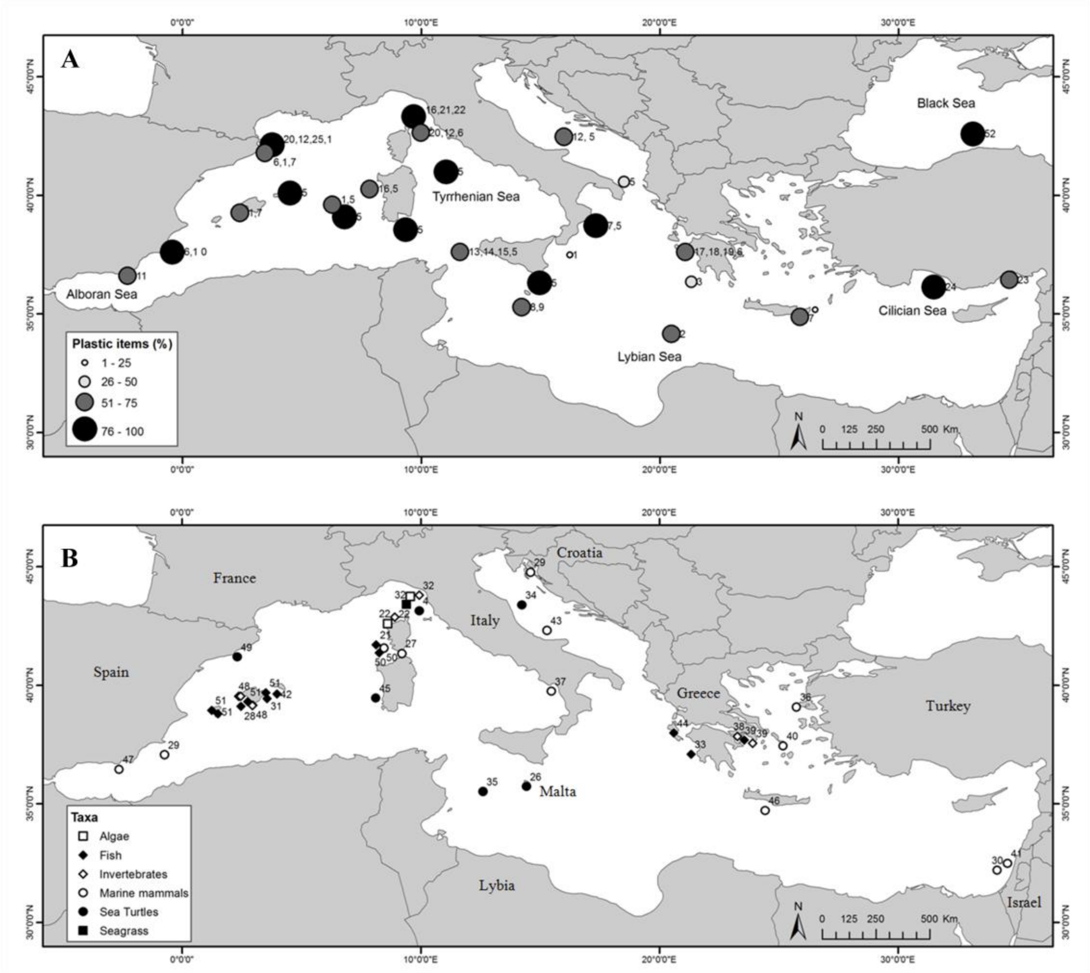

Fig. 3.1 Location of geographic regions in the Mediterranean and Black Sea where research has been conducted on (a) plastic (%) and (b) marine litter and biota. References: (1) Pham et al. (2014), (2) McCoy (1988), (3) Galil et al. (1995), (4) Campani et al. (2013), (5) Suaria and Aliani (2014), (6) Sánchez et al. (2013), (7) Ramirez-Llodra et al. (2013), (8) Morris (1980), (9) Mifsud et al. (2013), (10) Ecopuertos (2014), (11) Marino et al. (1989), (12) Galgani et al. (2000), (13) Ragonese et al. (1994), (14) Cannizarro et al. (1996), (15) Bianchini and Ragonese (1999), (16) Fossi et al. (2012), (17) Stefatos et al. (1999), (18) Katsanevakis and Katsarou (2004), (19) Koutsodendris et al. (2008), (20) Galgani et al. (1995), (21) Collignon et al. (2012), (22) Collignon et al. (2014), (23) Eryasar et al. (2014), (24) Güven et al. (2013), (25) Galgani et al. (1996), (26) Gramentz (1988), (27) Viale et al. (1992), (28) Massutí et al. (1998), (29) Baulch and Perry (2014), (30) Shoham-Frider et al. (2002), (31) Deudero (1998), (32) Aliani and Molcard (2003), (33) Madurell (2003), (34) Lazar and Gracan (2011), (35) Casale et al. (2008), (36) Tonay et al. (2007), (37) Pace et al. (2008), (38) Akoumianaki et al. (2008), (39) Katsanevakis et al. (2007), (40) Katsanevakis (2008), (41) Levy et al. (2009), (42) MEDITS project, (43) Mazzariol et al. (2011), (44) Anastasopoulou et al. (2013), (45) Camedda et al. (2014), (46) Roberts (2003), (47) de Stephanis et al. (2013), (48) Deudero et al. (2014), (49) Tomás et al. (2002), (50) Fossi et al. (2014), (51) Deudero and Alomar (2014), (52) Topçu and Öztürk (2010).

3.3.3 Biodiversity affected by marine litter

A total of 41% of the studies of litter and marine biota have reported interactions and effects on marine mammals, and invertebrates (14%) have been the least studied faunal group. The effects caused by marine litter have been classified mainly as ingestion/ entanglement (90%) and to a lesser extent interactions as colonisation/rafting have been reported (10%). The vast majority of revised documents (76%) have reported information on several types of plastic litter (plastic bags, plastic sheets, plastic monofilaments and ropes), while some (14%) have reported information only on non-plastic litter (wood, metal, glass, tar and non-plastic fishing gear). A small percentage of reviewed documents (10%) have classified items as general debris but have not specified litter types. The spatial coverage of the studies of marine litter and biota has included a wide range of depths (0–850 m) and a broad time period (from 1986 to 2014). These studies covered large areas, encompassing all of the Mediterranean basin, from Croatia (2 studies; 7%) to France (3 studies; 10%), Greece (5 studies; 17%), Israel (2 studies; 7%), Italy (7 studies; 24%), Malta (1 study; 3%), Spain (8 studies; 28%) and Turkey (1 study; 3%) (Fig. 3.1).

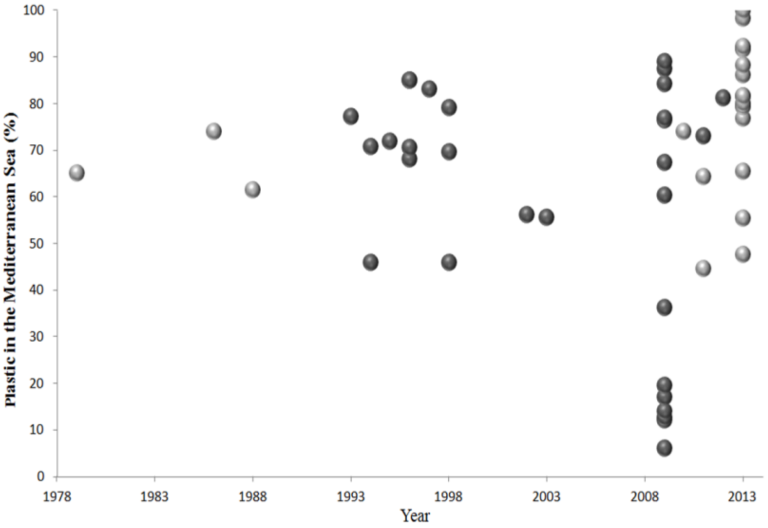

Fig. 3.2 Available data of plastic items (%) determined in seafloor (black) and surface (grey) marine areas from examined studies conducted in the Mediterranean Sea.

Several species (134) have been studied since 1986, although the number of species examined annually has been rather low and very variable, ranging from 1 to 42 species/year according to examined studies (Table 3.1). Over 28 years, most investigations have focused on sea turtles and marine mammals, while only 4 have examined invertebrates, and very few have assessed fishes. The sea turtle *Caretta caretta* was the first species examined in the Mediterranean, and a total of 6 studies have been performed on this species. Published data from 2005 to 2006 and 2010 are remarkable, including almost 30 species (Table 3.1).

Year	Species	N_t species	N_t studies	References
1986	*Caretta caretta*	1	1	Gramentz (1988)
1989	*Physeter macrocephalus*	1	1	Viale et al. (1992)
1990–1991	*Coryphaena hippurus*	1	1	Massutí et al. (1998)
1990–2012	*Tursiops truncatus, Delphinus delphis, Stenella coeruleoalba*	3	1	Aparicio pers.comm in Baulch and Perry (2014)
1990–2013	*Tursiops truncatus*	1	1	Duars pers.comm in Baulch and Perry (2014)
1992–2012	*Stenella coeruleoalba*	1	1	Duars pers.comm in Baulch and Perry (2014)
1993–1999	*Grampus griseus*	1	1	Shoham-Frider et al. (2002)
1994–1998	*Balistes carolinensis, Coryphaena hippurus, Naucrates ductor, Polyprion americanus, Schedophilus ovalis, Seriola dumerili, Trachurus mediterraneus, Trachurus picturatus*	8	1	Deudero (1998)
1997	*Algae, Arbacia lixula, Bowerbankia gracilis, Callopora lineata, Clytia hemisphaerica, Cymodocea nodosa, Cystoseira sp., Doto sp., Electra posidoniae, Eudendrium sp., Fiona pinnata, Fosliella farinosa, Gonothyraea loveni, Idotea metallica, Laomedea angulata, Lepas pectinata, Membranipora membranacea, Nereis falsa, Obelia dichotoma, Phtisica marina, Posidonia oceanica, Spirobranchus polytrema*	22	1	Aliani and Molcard (2003)
1999–2000	*Coelorhynchus coelorhynchus, Etmopterus spinax, Galeus melastomus, Helicolenus dactylopterus, Hoplostethus mediterraneus, Hymenocephalus italicus, Nezumia sclerorhynchus, Lepidorhombus boscii*	8	1	Madurell (2003)
2000–2002	*Balaenoptera physalus*	1	1	Duars pers.comm in Baulch and Perry (2014)
2001	*Physeter macrocephalus*	1	1	Roberts (2003)
2001–2004	*Caretta caretta*	1	1	Lazar and Gracan (2011)
2001–2005	*Caretta caretta*	1	1	Casale et al. (2008)
2001–2008	*Grampus griseus*	1	1	Duars pers.comm in Baulch and Perry (2014)
2002–2003	*Phocoena phocoena*	1	1	Tonay et al. (2007)
2004	*Physeter macrocephalus*	1	1	Pace et al. (2008)
2005–2006	*Alicia mirabilis, Amphitritides kuehlmanni, Aonides oxycephala, Apseudes latreilli, Aricidea capensis bansei, Aricidea catherinae, Aricidea cerrutii, Cestopagurus timidus, Chondrilla nucula, Chone duneri, Chthamalus stellatus, Ciona intestinalis, Eunice vittata, Gobius geniporus, Hexaplex trunculus, Loripes lacteus, Lumbrineris gracilis, Marphysa belli, Mastobranchus trinchesi, Microdeutopus gryllotalpa, Micronephthys maryae, Paradoneis lyra, Prionospio ehlersi, Protodorvillea kefersteini, Pseudoleiocapitella fauveli, Serranus hepatus, Sigambra tentaculata, Tellina compressa*	28	2	Katsanevakis et al. (2007) Akoumianaki et al. (2008)
2006	*Physeter macrocephalus*	1	1	Katsanevakis (2008)
2006–2013	*Balaenoptera physalus*	1	1	Fossi et al. (2014)
2007	*Tursiops truncatus*	1	1	Levy et al. (2009)
2007–2012	*Chauliodus sloani, Chelidonichthys cuculus, Chelidonichthys lastoviza, Coelorhynchus coelorhynchus, Conger conger, Dypturus oxyrhinchus, Etmopterus spinax, Galeus melastomus, Glossanodon leioglossus, Helicolenus dactylopterus, Lepidorhombus boscii, Lepidotrigla cavillone, Lepidotrigla dieuzeidei, Lophius budegassa, Lophius piscatorius, Merluccius merluccius, Micromesistius poutassou, Mullus barbatus, Mullus surmuletus, Myliobatis aquila, Nezumia aequalis, Pagellus acarne, Pagellus erythrinus, Phycis blennoides, Raja clavata, Raja miraletus, Raja montagui, Raja naevus, Raja polystigma, Raja radula, Scorpaena notata, Scorpaena scrofa, Scyliorhinus canicula, Serranus cabrilla, Spicara smaris, Synchiropus phaeton, Trachinus draco, Trigla lyra, Trisopterus minutus, Uranoscopus scaber, Zeus faber*	42	1	MEDITS survey
2007–2013	*Cetorhinus maximus*	1	1	Fossi et al. (2014)
2008–2012	*Caretta caretta*	1	1	Camedda et al. (2014)
2009	*Physeter macrocephalus*	1	1	Mazzariol et al. (2011)
2010	*Brama brama, Centrophorus granulosus, Conger conger, Epigonus telescopus, Etmopterus spinax, Galeus melastomus, Helicolenus dactylopterus, Lepidopus caudatus, Merluccius merluccius, Micromesistius poutassou, Molva macrophthalma, Mora moro, Myctophum punctatum, Nettastoma melanurum, Pagellus bogaraveo, Phycis blennoides, Polyprion americanus, Pteroplatytrygon violacea, Raja clavata, Raja oxyrinchus, Schedophilus ovalis, Scorpaena elongata, Scyliorhinus canicula, Squalus acanthias, Squalus blainville, Sudis hyalina, Xiphias gladius*	27	2	Collignon et al. (2012) Anastasopoulou et al. (2013)
2010–2011	*Caretta caretta*	1	1	Campani et al. (2013)
2011–2012	*Algae, Foraminifera*	2	1	Collignon et al. (2014)
2012	*Physeter macrocephalus*	1	1	de Stephanis et al. (2013)
2014	*Boops boops, Holothuria forskali*	2	2	Deudero and Alomar (2014), Deudero et al. (2014)
N/A	*Caretta caretta*	1	1	Tomás et al. (2002)
Total		134	29	

Table 3.1 Studies linking marine litter and marine biota in the Mediterranean Sea, from 1986 to 2014, with indication of number of species in each study (Nt species), number of studies (Nt studies) per period of time (year) and scientific reference.

The interaction and effects of marine litter with biota is classified into the following two subgroups: (a) ingestion/entanglement and (b) colonisation/rafting (Fig. 3.3). Fish were found to be influenced by both subgroups, with the greatest proportion of interactions (67%) related to the use of marine litter deployed on the seafloor or floating objects as shelter, however caution has to be taken when interpreting these results as they consider a low number of studies. Marine mammals and sea turtles are affected by plastic only through ingestion/entanglement, while invertebrates, algae and seagrass colonised marine litter objects.

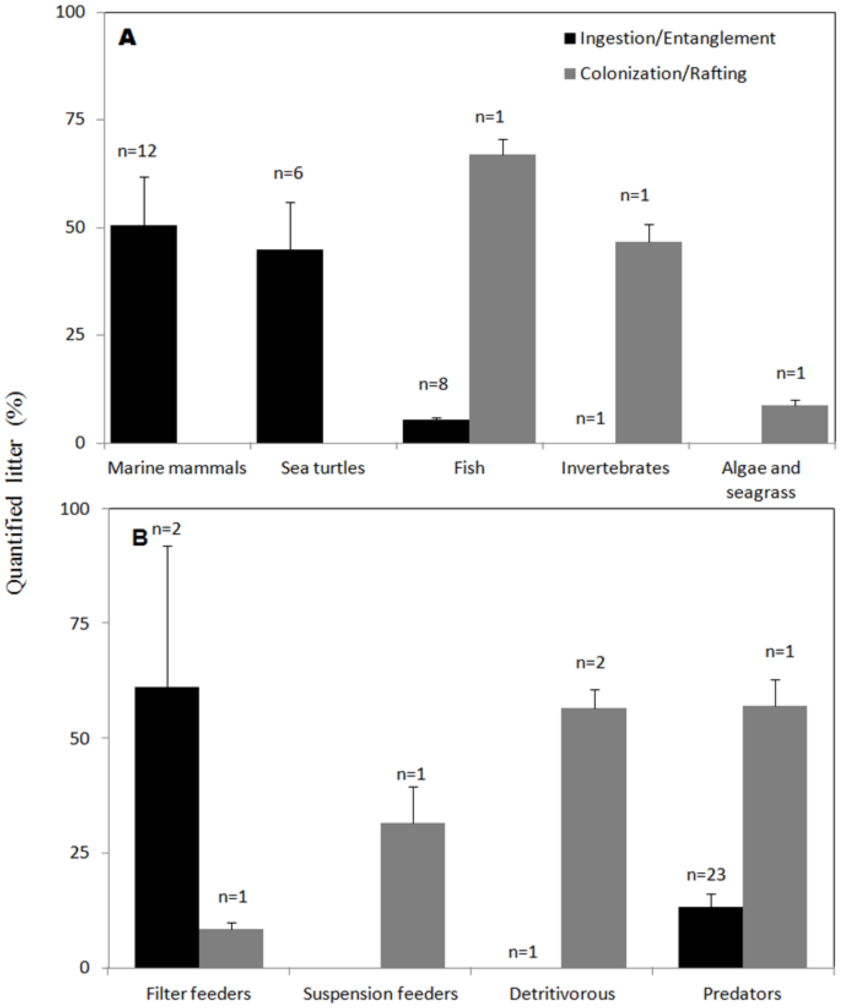

Fig. 3.3 Quantified litter (mean % and SE) from examined studies due to ingestion/ entanglement and colonisation/rafting for (A) different taxa and (B) according to different feeding strategies. Number of studies (n) shown above each bar.

IUCN category	**Taxa**	**Species** (N_t= **67 species)**	**Nº studies**	**Nº individuals**	**Litter ingestion (%)**	**Plastic Occurence**	**General Litter Occurence**	**Offshore/ Coastal**
Critically Endangered (CR)	Marine mammal	*Phocoena phocoena*	1	42	12	Yes	No	Coastal
Data Deficient (DD)	Fish	*Coelorhynchus coelorhynchus*	1	85	0	Yes	No	Offshore
		Myliobatis aquila	1	24	0	Yes	No	Offshore
		Polyprion americanus	1	11	55	Yes	Yes	Offshore
		Raja radula	1	46	0	Yes	No	Offshore
		Squalus blainville	1	75	1	Yes	No	Offshore
Endangered (EN)	Marine mammal	*Balaenoptera physalus*	2	7	71	No	Yes	Offshore
		Delphinus delphis	1	18	0	No	No	Offshore
	Sea turtle	*Caretta caretta*	6	438	37	Yes	Yes	Offshore
Least Concern (LC)	Fish	*Coryphaena hippurus*	2	551	7	Yes	Yes	Offshore
		Etmopterus spinax	3	323	6	Yes	No	Offshore
		Galeus melastomus	3	1320	3	Yes	Yes	Offshore
		Pagellus acarne	1	71	0	Yes	No	Offshore
		Pagellus erythrinus	1	276	0	Yes	No	Offshore
		Pteroplatytrygon violacea	1	2	50	Yes	No	Offshore
		Raja miraletus	1	41	0	Yes	No	Offshore
		Raja montagui	1	43	0	Yes	No	Offshore
		Raja naevus	1	40	0	Yes	No	Offshore
		Scyliorhinus canicula	1	1094	0	Yes	No	Offshore
		Spicara smaris	1	33	0	Yes	No	Offshore
		Trisopterus minutus	1	41	0	Yes	No	Offshore
		Xiphias gladius	1	1	0	No	Yes	Offshore
	Marine mammal	*Grampus griseus*	2	10	10	Yes	No	Offshore
		Stenella coeruleoalba	2	133	3	No	Yes	Offshore
		Tursiops truncatus	3	214	9	No	Yes	Coastal
Not Evaluated (NE)	Echinodermata	*Holothuria forskali*	1	30	7	Yes	No	Coastal
	Fish	*Balistes carolinensis*	1	22	14	No	Yes	Offshore
		Boops boops	1	117	29	Yes	Yes	Coastal
		Chauliodus sloani	1	70	0	Yes	No	Offshore
		Chelidonichthys cuculus	1	926	0	Yes	No	Offshore
		Chelidonichthys lastoviza	1	713	0	Yes	No	Offshore
		Conger conger	1	41	0	Yes	No	Offshore
		Dypturus oxyrhinchus	1	52	0	Yes	No	Offshore
		Glossanodon leioglossus	1	20	0	Yes	No	Offshore
		Helicolenus dactylopterus	2	628	1	Yes	No	Offshore
		Hoplostethus mediterraneus	1	212	1	No	No	Offshore
		Lepidorhombus boscii	1	630	0	Yes	No	Offshore
		Lepidotrigla cavillone	1	148	0	Yes	No	Offshore
		Lepidotrigla dieuzeidei	1	75	0	Yes	No	Offshore
		Lophius budegassa	1	169	0	Yes	No	Offshore
		Lophius piscatorius	1	192	0	Yes	No	Offshore
		Merluccius merluccius	1	1583	0	Yes	No	Offshore
		Micromesistius poutassou	1	374	0	Yes	No	Offshore
		Mullus barbatus	1	21	0	Yes	No	Coastal
		Mullus surmuletus	1	708	0	Yes	No	Coastal
		Myctophum punctatum	1	1	100	Yes	No	Offshore
		Naucrates ductor	1	319	18	Yes	Yes	Offshore
		Nezumia aequalis	1	30	0	Yes	No	Offshore
		Pagellus bogaraveo	1	60	2	No	Yes	Offshore
		Phycis blennoides	1	482	0	Yes	No	Offshore
		Schedophilus ovalis	1	12	50	Yes	No	Offshore
		Scorpaena notata	1	408	0	Yes	No	Offshore
		Scorpaena scrofa	1	352	0	Yes	No	Offshore
		Seriola dumerili	1	180	2	Yes	Yes	Offshore
		Serranus cabrilla	1	592	0	Yes	No	Offshore
		Synchiropus phaeton	1	31	0	Yes	No	Offshore
		Trachinus draco	1	1143	0	Yes	No	Offshore
		Trachurus mediterraneus	1	103	1	No	Yes	Coastal
		Trachurus picturatus	1	614	1	No	No	Offshore
		Trigla lyra	1	267	0	Yes	No	Offshore
		Uranoscopus scaber	1	40	0	Yes	No	Offshore
		Zeus faber	1	474	0	Yes	No	Offshore

IUCN category	Taxa	Species (N_t= 67 species)	Nº studies	Nº individuals	Litter ingestion (%)	Plastic Occurence	General Litter Occurence	Offshore/ Coastal
Vulnerable (VU)	Fish	*Cetorhinus maximus*	1	6	83	No	Yes	Offshore
		Squalus acanthias	1	10	0	No	No	Offshore
	Marine mammal	*Physeter macrocephalus*	6	23	83	Yes	Yes	Offshore
Near Threatened (NT)	Fish	*Raja clavata*	1	465	0	Yes	No	Offshore
		Raja polystigma	1	52	0	Yes	No	Offshore
Total number of individuals				17334				

Table 3.2 Litter ingestion in taxa according to protection status, based on IUCN category, number of studies (No. studies), number of individuals (No. individuals), litter effects (%), plastic occurrence, general litter occurrence and habitat (offshore/coastal).

Marine organisms using different feeding strategies interacted with litter items that were either accumulated on the seafloor or floating. Predators, followed by detritivorous feeders, suspension feeders and finally, filter feeders, were found to colonise marine litter items. Biota affected by ingestion/entanglement exhibited a different pattern and no studies reported the ingestion/entanglement of marine litter by detritivorous or suspension feeders (Fig. 3.3).

A total of 17,334 individuals were examined, and several species revealed no ingestion of marine litter, although a high number of individuals of these species were analysed (>133 individuals of the same species), including the fishes *Merluccius merluccius*, *Lepidorhombus boscii*, *Chelidonichthys cuculus*, *Chelidonichthys lastoviza*, *Trachinus draco*, *Mullus surmuletus*, *Scorpaena scrofa*, *S. notata*, *Phycis blennoides*, *Zeus faber*, *Serranus cabrilla* and *Pagellus erythrinus*, and the elasmobranchs *Scyliorhinus canicula* and *Raja clavata*. In addition, some species with high numbers of individuals sampled showed infrequent ingestion of marine litter, such as the fishes *Helicolenus dactylopterus* (1% ingestion) and *Trachurus picturatus* (1% ingestion), the elasmobranchs *Squalus blainville* (1% ingestion), *Galeus melastomus* (3% ingestion), and *Etmopterus spinax* (6% ingestion) and the marine mammal *Stenella coeruleoalba* (3% ingestion). Conversely, some species were highly affected, including the fishes *Polyprion americanus* and *Naucrates ductor*, the vulnerable elasmobranch *Cetorhinus maximus* and the elasmobranch *Pteroplatytrygon violacea*, the endangered sea turtle *C. caretta*, and marine mammals assigned to different IUCN categories, including the critically endangered *Phocoena phocoena*, the endangered *Balaenoptera physalus* and the vulnerable *Physeter macrocephalus* (Table 3.2). It must be pointed out that sampled group size varied amongst species ranging from 1 individual (*Myctophum punctatum*) to 1583 individuals (*M. merluccius*).

In particular, species with more than 1% ingestion were examined (Table 3.3). Species affected by ingestion were mainly large-sized organisms, such as the baleens *B. physalus* and *P. macrocephalus*, with ingestion rates of 100%, and the large elasmobranch *C. maximus* (83%), followed by the turtle *C. caretta*. The fish *M. punctatum* presented an ingestion rate of 100%, despite its small size, however only one individual was assessed. The invertebrate *Holothuria forskali* was determined to ingest plastic (monofilaments). General litter and plastics were the main litter types ingested by organisms. Plastic items and monofilaments were present in 60% of individuals showing more than 1% ingestion. The elasmobranch *G. melastomus* was found to ingest metal items.

Specie	Litter ingestion (%)	Litter Type	References
Balaenoptera physalus	100	General litter	Fossi et al (2014)
Balistes carolinensis	14	General litter	Deudero (1998)
Boops boops	29	Plastics, monofilaments	Deudero et al (2014)
Caretta caretta	6	General litter, Plastics, Metal	Gramentz (1988)
Caretta caretta	28	General litter and Plastics	Camedda et al (2013)
Caretta caretta	35	General litter, Plastics, Styrofoam, Ropes and monofilaments	Lazar and Gracan (2011)
Caretta caretta	48	Plastics	Lazar and Gracan (2011)
Caretta caretta	71	General litter, Plastics, Styrofoam, Ropes and monofilaments	Campani et al (2013)
Caretta caretta	80	General litter, Plastics, Styrofoam, Wood, Fishing net material	Tomás et al (2002)
Cetorhinus maximus	83	General litter	Fossi et al (2014)
Coryphaena hippurus	6	Plastics, Wood, Ropes and monofilaments	Deudero (1998)
Coryphaena hippurus	7	General litter, Wood, Ropes and monofilaments	Massutí et al (1998)
Etmopterus spinax	6	Plastics	Anastasopoulou et al (2013)
Etmopterus spinax	8	General litter	Madurell (2003)
Galeus melastomus	3	Plastics, Metal, Wood	Anastasopoulou et al (2013)
Galeus melastomus	13	General litter	Madurell (2003)
Grampus griseus	33	Plastics	Shoham-Frider et al (2002)
Helicolenus dactylopterus	2	General litter	Madurell (2003)
Holothuria forskali	7	Monofilaments	Deudero et al (2014)
Myctophum punctatum	100	General litter	Colligon et al (2012)
Naucrates ductor	18	Plastics, Wood	Deudero (1998)
Pagellus bogaraveo	2	General litter	Anastasopoulou et al (2013)
Phocoena phocoena	12	Plastics	Tonay et al (2007)
Physeter macrocephalus	50	General litter	Katsanevakis (2008)
Physeter macrocephalus	100	Plastics	Viale et al. (1992) Roberts (2003) de Stephanis et al (2013)
Physeter macrocephalus	100	Plastics, Ropes and monofilaments, Fishing net material	Mazzariol et al (2011)
Physeter macrocephalus	100	Fishing net material	Pace et al (2008)
Polyprion americanus	55	Plastics	Deudero (1998)
Pteroplatytrygon violacea	50	Plastics	Anastasopoulou et al (2013)
Schedophilus ovalis	50	Plastics	Deudero (1998)
Seriola dumerili	2	Plastics	Deudero (1998)
Squalus blainville	1	Plastics	Anastasopoulou et al (2013)
Stenella coeruleoalba	4	General litter	Baulch and Perry (2014)
Trachurus mediterraneus	1	General litter	Deudero (1998)
Trachurus picturatus	1	General litter	Deudero (1998)
Tursiops truncatus	10	General litter	Baulch and Perry (2014)
Tursiops truncatus	100	General litter, Ropes and monofilaments, Fishing net material	Levy et al (2009)

Table 3.3 Marine species presenting litter ingestion (for species accounting for more than 1% ingestion) according to litter type and references.

Pelagic species showed variable levels of litter ingestion, depending on the species (Fig. 3.4). Mesopelagic fishes from the Myctophydiae family were affected by litter, followed by medium-sized pelagic fishes, such as *Boops boops*, and epipelagic fishes, such as *Schedophilus ovalis*, the dolphin-fish *Coryphaena hippurus*, *Seriola dumerili* and *Balistes carolinensis*. Juveniles of *Trachurus* spp. were less affected by litter (Fig. 3.4).

According to marine mammals, interactions widely differed amongst misticeta and odontoceta and within families, and large effects were observed for *P. macrocephalus* (average mean ingestion value of 91%) followed by *B. physalus* (50%) and *P. phocoena* (12%) and *C. maximus* (83%) (Fig. 3.4). Conversely, the dolphins *Tursiops truncatus* and *S. coeruleoalba* did not ingest litter, while sea turtles were affected in almost 45%

of ingestion (Fig. 3.4). MDS revealed the grouping of the large filter feeders *C. maximus* and *B. physalus* together, while sea turtles were not grouped with any other species, nor was *C. hippurus*, which was one of the least affected pelagic species. Marine mammals, such as *Grampus griseus* and *P. phocoena*, which were sampled at the eastern most part of the Mediterranean basin, were found to be closely associated by MDS.

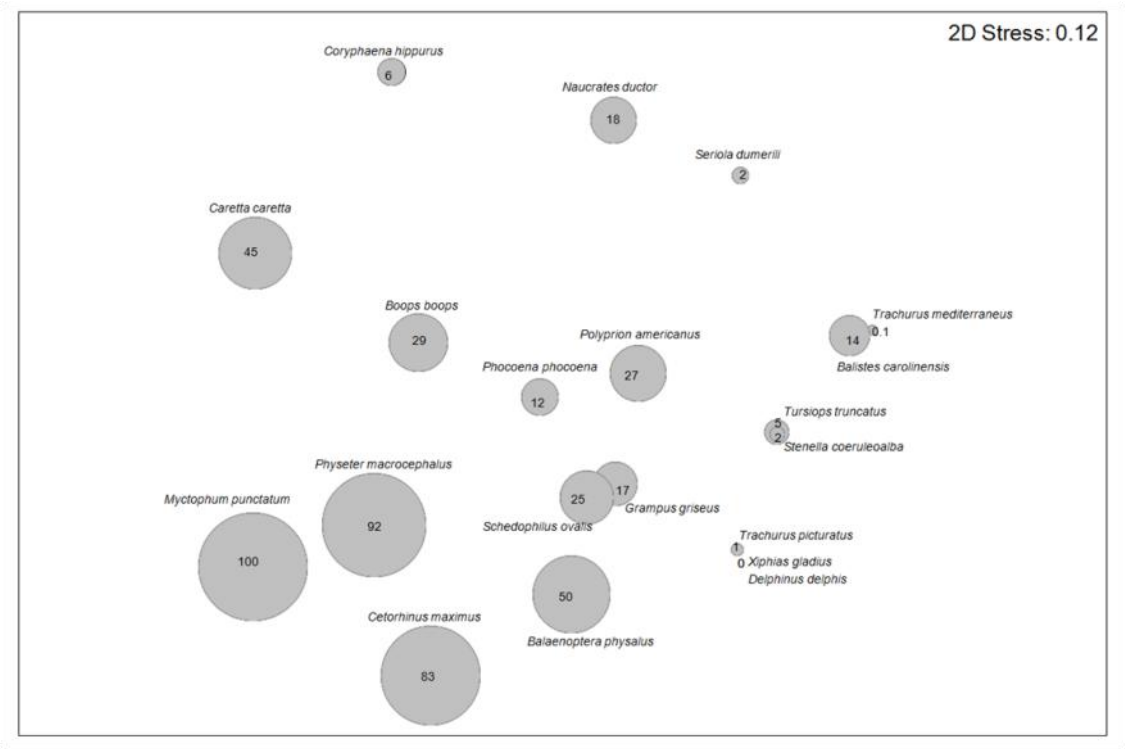

Fig. 3.4 Non-multidimensional scaling (MDS) calculated for pelagic species (marine mammals, fishes, sea turtles) for mean values of litter ingestion after normalisation and Euclidean distance. Bubble reflect mean litter ingestion % per species.

3.4 Discussion

This bibliographic review shows that marine biodiversity in the Mediterranean Sea is affected by marine litter, especially plastic pollution. Spatial and temporal differences of plastic litter reported in examined studies could be observed, however caution must be taken due to the unequal distribution of studies, biases in sampling methods and different sample sizes. Interactions and effects of litter with marine biota were classified as ingestion/entanglement and colonisation/rafting, respectively, and they were found to differentially affect marine organisms. Some key species, such as sea turtles and marine mammals, were particularly affected, with many vulnerable and endangered species threatened by litter, and many pelagic fish species were also affected. Some organisms, such as fish, were not very susceptible to litter exposure; however, ghost fishing due to discarded fishing materials must be considered. The high number of taxa influenced by marine litter highlights the magnitude of this emerging threat to biodiversity, indicating the urgent need to promote effective management through investigations of sinks and sources, strict legislation and enforced measures with regard to the deposition and reuse of plastics.

The Mediterranean Sea is highly rich in species and endemism (Coll et al., 2010) and is one of the most polluted and threatened semi-enclosed seas worldwide (Costello et al., 2010). In addition, it attracts 25% of international tourism and 30% of shipping traffic, affecting litter composition (Ramirez-Llodra et al., 2013). Marine litter is ubiquitous, and loads are increasing at coastal and offshore areas. Recent studies have revealed very high levels of marine litter in the Mediterranean (Pham et al., 2014; Suaria and Aliani, 2014), and impacts of coastal activities, especially with regard to litter distribution and biodiversity loss, are likely to be high. Severely declining numbers and losses of key species have been documented for species including fish and shark and especially predatory species due to human impact activities (Myers et al., 2007). Marine litter around the Mediterranean Sea is not evenly distributed, although the amount of plastic has increased over time (Barnes et al., 2009). The quantities of floating particles are within the order of those of main subtropical gyres of the North Atlantic and North Pacific (Leichter, 2011). Plastic litter has been studied since 1979 throughout the Mediterranean basin, from the Alboran Sea to the Cilician Sea, including the Black Sea. Hotspots of litter accumulation include shores close to populated areas and submarine canyons (Pham et al., 2014), where the degradation processes of plastics are much lower due to deterioration of ultraviolet (UV) light, haline environments and the cooling effect of the sea (Gregory, 1999). Similarities in litter item quantities have been found between plastic samples collected from the deep sea and those floating on the surface of the Mediterranean Sea. Ramirez-Llodra et al. (2013) have found the presence of plastics in 92.8% of deep-sea samples collected on a regional scale from the Mediterranean Sea, and Suaria and Aliani (2014) have observed plastic items in 82% of sea surface samples. In addition, Pham et al. (2014) have reported a higher density of litter on the seafloor (0.4– 48 items/ha) compared with that of floating litter (0.021 items/ha) throughout the Mediterranean Sea.

The latest research performed on a regional scale showed mean values of deep-sea macrolitter ranging from 0.6 ± 0.4 kg/ha (continental slope of the central Mediterranean) to 4.0 ± 1.8 kg/ha (continental slope of the western Mediterranean) (Pham et al., 2014). The levels observed throughout the Mediterranean Sea were within range, and even higher than seabed debris around the world (Kanehiro et al., 1996). Marine litter distribution is affected by natural factors (such as geomorphology, hydrography, water flows from incoming rivers, winds, storms and natural disasters) and human activities (urban areas, shipping and trading routes and fishing). Strong water flows from rivers can transport litter from coastal areas down to deep waters (Galgani et al., 1996). Sánchez et al. (2013) have observed that the variability of marine litter between sampling sites is higher than that between areas. Pham et al. (2014) have shown a general increase in litter density at locations closer to shores, in accordance with previous studies performed on the French Mediterranean coast (Galgani et al., 1996) and off of the California coast (Watters et al., 2010). In addition, most studies of the deposition of marine litter on the seafloor have been performed in trawling areas (Sánchez et al., 2013). Litter deployed on the seafloor can alter habitat characteristics by adding new substrates or by overlaying sediment, inhibiting gas exchange and interfering with life on the seabed. Katsanevakis et al. (2007) have demonstrated increases in the abundances of some species in littered areas along with a substantial change in the megafauna community structure, with the establishment of new relationships at the intraspecific and interspecific levels and novel predator– prey interactions.

This review establishes a comprehensive list of marine organisms that in some way interact and/or are affected by marine litter. As expected, marine litter, especially plastic items, affect many taxa, ranging from invertebrates (polychaetes, ascidians, bryozoans, sponges, etc.) to vertebrates, such as sea turtles, fishes and marine mammals (including the largest animal in the sea, the fin whale *B. physalus*). Effects reported by studies of marine litter have varied from entanglement, ingestion and suffocation to general debilitation. In 1997, Laist reviewed over 250 marine species affected by entanglement and ingestion. In our review, 134 species were found to be affected by litter, which is half of the amount reported worldwide by Laist (1997), although this author included seabirds, while our study intentionally excluded this faunal group. In the Mediterranean, studies on biodiversity have mainly focused on marine mammals and reptiles, and there have been few reports of plastic ingestion by invertebrates or fish. Nevertheless, some studies of the diets and stomach contents of Mediterranean fish have recorded marine litter ingestion (Deudero, 1998; Massutí et al., 1998; Madurell, 2003), although it was not the aim of these studies. For invertebrates, studies of macrobenthic fauna have focused on the examination of litter colonisation before and after the introduction of litter items (Katsanevakis et al., 2007; Akoumianaki et al., 2008), and macrobenthic species have also been identified in floating objects (Aliani and Molcard, 2003). However, research on litter, specifically on microplastics and its biological effects on invertebrates have been rather restricted to controlled laboratory experiments conducted in Europe, mainly in the UK (Browne et al., 2008; Farrell and Nelson, 2013). In addition, previous laboratory experiments studying microsphere transfer across food webs have demonstrated the potential of plastic microparticle transfer via planktonic organisms from one trophic level (mesozooplankton) to a higher level (macrozooplankton) (Farrell and Nelson, 2013; Setälä et al., 2014).

This review revealed that mictophidae lanternfishes were affected by litter ingestion. Previous studies conducted in the North Pacific Central Gyre have demonstrated 35% plastic ingestion by mesopelagic fishes (Boerger et al., 2010). The biomass of pelagic planktivorous fish plays a key role in pelagic food webs, representing a central link for larger predators. Moreover, the diel vertical migration of these planktivorous fishes may act as a biological pump, transferring plastic litter from surface waters to mesopelagic waters through faecal pellet excretion.

Offshore epipelagic fish species exhibited moderate levels of plastic ingestion, with several species ingesting marine litter, including *C. hippurus*, *S. dumerili*, *S. ovalis* and

N. ductor (Deudero, 2001). In the pelagic environment, optimised feeding is linked to visual and voracious prey capture behaviours; thus, particle selection may be linked to mouth biometry instead of nutritional quality. These observations are in agreement with data on the ingestion of macroplastics by large predatory fishes in subsurface waters (Choy and Drazen, 2013). Again, more focus should be placed on organisms inhabiting layers other than surface waters because the magnitude and extent of litter in these areas have been underestimated worldwide. Ultimately, marine litter sinks to deeper layers through weathering, smothering and the biological transfer of particles, leading to important ecological consequences at all levels of the food web within the depth gradient. Indeed, deep-sea fishes have been shown to exhibit high litter ingestion rates in the eastern Mediterranean (Anastasopoulou et al., 2013). These authors have suggested that the types of litter ingested are related to the feeding behaviours of fish species; for example, *G. melastomus*, which is a nektobenthic opportunistic feeder (e.g., Madurell, 2003), swallowed all types of debris, while the pelagic and bathypelagic feeders *P. violacea* and *S. blainville* ingested only plastic bags, and hard plastics were found in *E. spinax* and *Pagellus bogaraveo*, a finding that may be related to their bathybenthic feeding habits (Madurell, 2003).

Marine mammals are highly affected by ingestion on a global scale (Baulch and Perry, 2014). Although most studies rely on stranded individuals, large individuals, such as *P. macrocephalus* or the fin whale *B. physalus*, have been identified with large pieces of megaplastic in their stomachs, predominantly plastic sheets. Entanglement has been recorded in the sperm whale *P. macrocephalus*, with many individuals being exposed to driftnets (Pace et al., 2008). Most odontocetes exhibit low levels of plastic ingestion, with the exception of *Grampus griseus*, which could mistake plastics for squids (Shoham-Frider et al., 2002). Baulch and Perry (2014) have reported ingestion rates of debris as high as 31% in some marine mammal populations, and sub-lethal effects could result in impacts at the population level. However, extrapolating ingestion rates of stranded populations to those of wild populations must be performed with caution, and further examinations of the interactions and effects of plastic with large and wild marine mammals must be carried out.

Marine litter affected the sea turtle *C. caretta* in 45% of the studies evaluated, and these studies focused on the quantification of ingestion/entanglement. Studies have demonstrated the preferential ingestion of white plastics in contrast with blue and red plastics (Camedda et al., 2014), which is possibly linked to the visual confusion of white plastics with jellyfishes. Pelagic juveniles of all sea turtle species exhibit a high frequency of debris ingestion due to their indiscriminate pelagic feeding (Bjorndal, 1997).

According to feeding strategies, predator species exhibited a wide variety of trophic traits (piscivorous feeders, mesograzers, invertebrate feeders, etc.) and thus exhibited greater responses to marine litter in association with their ecological traits. In the pelagic environment, large filter feeders, including marine mammals and elasmobranchs, were exposed to ingestion/entanglement, such as the basking shark *C. maximus* (Fossi et al., 2014).

Ingestion and entanglement between marine litter and marine organisms have been reported for 663 species, and 15% of the species showing interactions are on the IUCN red list (CBD, 2012). In this review, several IUCN red-listed species were found to be affected by plastics, indicating the need to enforce conservation policies for protected species and the integration of marine litter into management plans. The outcomes of this review indicate that vulnerable and endangered catalogued species (mainly marine mammals) are influenced by litter accumulation in the pelagic environment. Thus, population decline may be attributed to marine litter together with several drivers, such as overfishing, acoustic pollution, prey shifts and climate change. In addition, Fossi et al. (2012) have detected leached plastic additives in Mediterranean fin whales, indicating chronic exposure to toxins as a result of microplastic ingestion. Main physical impacts of microplastics on marine organisms have been documented such as internal and/or external abrasions, ulcers and blockages of the digestive tract which can result in satiation, starvation and physical deterioration (Wright et al., 2013). Moreover, nano- and microplastics impose increasing threats to marine biota, although in the Mediterranean, no studies of the cross-effects of plastic size and marine biota at the ecosystem level have been conducted.

The findings of marine litter throughout the Mediterranean Sea and its interactions and effects with wildlife were possibly affected by bias due to the uses of several sampling procedures, spatio-temporal variation, the unequal number of studies performed and disparity in the number of species selected in each study. In addition, most of the examined studies refer to meso/macro litter ingested by marine species which must be leading to a subestimation of litter ingestion as smaller fractions (micro and nano) have been overlooked. However, considering the increase in global plastic production from 5 million tones in the 1960s to 280 million tones and recovery rates of only 13% (CBD, 2012), the issue of plastic ingestion represents a real challenge, and further investigations focusing on the impacts on marine biota must be established. In the Mediterranean Sea, in addition to the general lack of available data on organisms affected by marine litter, several factors, such as the mortality and morbidity rates due to plastic litter effects at the population level, the survival rate, the attachment of biofilm to plastics, the routes of dispersal, and the accumulation of plastic components along the food web must be elucidated in field conditions. This information will allow for the integration of studies at basin scale and overlapping information on currents regimes connecting distant areas (Pinardi and Masetti, 2000) in this semi-enclosed sea.

3.5 Acknowledgements

The authors are indebted to M. Valls for providing access to the MEDITS data, M. Vázquez-Luis for providing assistance with the GIS maps and E. Ólafsson for the critical revision.

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Deep Sea Research Part I 155 (2020) 103178

Chapter 4: Spatial and temporal distribution of marine litter on the seafloor of the Balearic Islands (western Mediterranean Sea)

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Abstract

Marine litter, and more specifically the plastic fraction, have been studied in the seafloor around the Balearic Islands. A total of 806 scientific bottom trawl hauls from 15 years of surveys (2001–2015) were analyzed covering a depth range from 38 to 800 m. Marine litter was detected in 88% of the hauls sampled, with a mean value of 1.39±0.13 kg/km^2. For the plastic fraction, the mean value obtained was 2.73 ±0.26 kg/km^2, with its presence in 66% of the sampled hauls. Amongst a series of analyzed factors, that may explain the distribution of seafloor plastics in the study area, sampling area, bathymetric strata and distance to the coastline were identified as significant. High quantities of seafloor plastics were observed along the northwestern coast of Mallorca, which could be related to oceanographic features, and in the continental shelf, close to the coastline, as well as in the upper slope. However, no increasing or decreasing temporal trend in abundance of seafloor plastics was seen throughout the 15 years of scientific surveys.

Keywords: Pollution, Plastic, Benthic litter, Seabed, Human pressure

4.1 Introduction

Over the course of the last years, marine litter has raised concern throughout the scientific community and general public (Goldstein et al., 2013). Marine litter is defined as any persistent, manufactured or processed solid material disposed of or abandoned in the marine and coastal environment (UNEP, 2005) and can be classified into different categories. This type of pollution has been quantified in all sea compartments from the sea surface (Compa et al., 2019) to the seafloor (García-Rivera et al., 2018). Once in the marine environment, floating litter, especially the plastic fraction, can degrade into smaller items (Andrady, 2011) as well as change its density due to biofouling (Barnes et al., 2009), by aggregation with denser objects or because of aging and can be entrained into turbulent flows (Tubau et al., 2015) finally sinking into deeper areas. Consequently, the seafloor area has been seen as the possible ultimate fate of marine litter (Courtene-Jones et al., 2017). Reported macro litter densities from continental shelves to the deep sea areas of European seabeds are higher than reported floating marine litter values for surface waters around the world (Pham et al., 2014). For example, in the Mediterranean Sea, up to 100,000 items of marine litter/km^2 have been quantified on the seafloor (Galgani et al., 2000) and litter densities in this benthic area (0.4–48 litter items/ha^2; Pham et al., 2014) are higher than estimates for floating marine litter (0.021 items/ha^2) (UNEP, 2016).

Sources of marine litter have been classified into land-based sources (80%) including tourism, industrial and recreational areas, terrestrial runoffs, river discharges, waste water effluents, sewage treatment plants and pipes and into sea-based sources (20%) which include commercial and recreational shipping, aquaculture and fishing (Ryan et al., 2009; Tubau et al., 2015; Jambeck et al., 2015). Given that the continental shelf and the upper slope are subjected to anthropogenic impacts such as fishing, maritime transport and terrestrial waste inputs, it is important to explore marine litter in these areas as they are exposed to most of the major contributors of litter pollution to the marine environment.

Studies on the distribution and abundance of marine litter in the northwestern Mediterranean Sea date back to the early 90 s. Galgani et al. (1995) identified pieces of plastic, glass and plastic bottles, metallic objects, glass and fishing gear on the continental shelf of the western region of this semi-enclosed sea which is not only a hotspot area for biodiversity (Bianchi and Morri, 2000) but also for marine litter (Deudero and Alomar, 2015). Furthermore, estimates of marine litter on the mentioned continental shelf, which did not exceed 200 m in depth, resulted in 175 million items of litter, 77% being plastics (Galgani et al., 1995). More recent studies along the western Mediterranean basin have also quantified marine litter on the continental shelf and upper slope showing mean plastic values of 8.4 ± 50.0 kg/km^2 in these areas exposed to human pressures and impacts (García-Rivera et al., 2018).

Submarine geomorphology, geographical settings, hydrodynamics and bathymetric stratification play an important role in seafloor plastic distribution (Galgani et al., 2000) and it is known that plastics can be transported by currents, tides and climate to distant areas from their sources (Galgani et al., 2013). As a matter of fact, litter of Italian origin has been observed along the French coast (Galgani et al., 1995) which could most likely be attributed to the key role of transportation processes along the coast due to upwellings, gyres, eddies, and other water movements (Millot, 1987; Brasseur, 1991).

Given that 70% of marine plastic items are hypothesized to sink to the seafloor (Engler, 2012) and the difficulty in determining sinking areas of plastic due to environmental characteristics, it is important to study seafloor plastics at a regional scale. The Mediterranean International scientific bottom-trawl surveys (MEDITS), which covers the entire Mediterranean and Black Sea, applying standardized protocols has been identified as an appropriate monitoring tool to obtain standardized information on marine litter composition and distribution in accordance with the Marine Directive Framework Directive (MSFD, 2008/56/EC) (García-Rivera et al., 2018). Data from the MEDITS scientific surveys is collected at the same sampling locations year after year, thus providing comparable data throughout the basin and over time.

Moreover, knowing that plastics accumulate in seafloor areas and that degradation processes are long (UNEP, 2009), quantification over time, and not only at specific times, should be attempted in order to provide a wider knowledge of distribution patterns and trends in the marine environment. Until now, few studies of marine litter include data with a temporal scale larger than 10 years (Compa et al., 2019; García-Rivera et al., 2018). Along the Spanish Mediterranean coast, seafloor litter was quantified for 11 years demonstrating that quantities in 2007 and 2011 were significantly higher than some other years (García-Rivera et al., 2018) but no evidence of an overall significative descending tendency was detected (García-Rivera et al., 2018) reflecting once again the need to increase the temporality of datasets.

A recent study in the Balearic Islands, Western Mediterranean Sea, documented that over 347 tones of floating litter were removed from coastal waters over the course of 11 years (2005–2015) of monitoring surveys during the summer months (Compa et al., 2019). The plastic fraction composed 54% of the floating litter and spatio-temporal patterns showed higher plastic concentrations in the northwestern and southeastern regions (Compa et al., 2019). Knowing that a large percentage of plastics are determined to sink into the seafloor and given the evidence of floating plastics in this area, it is significant to assess marine litter in seafloor areas of this part of the Mediterranean basin. This is especially important as the Balearic Islands are exposed to human stressors that impact coastal habitats (Deudero and Alomar, 2015). These activities include commercial and recreational maritime routes, and commercial and professional fishing, as well as densely populated and urbanized areas along the coastline which increase the amount of litter during the summer season (Compa et al., 2019).

Therefore, having evidence of high quantities of floating plastics in the Balearic Islands which can eventually sink into the seafloor and knowing that plastics have implications on marine ecosystems, this study aims at a) analyzing the spatial and temporal distribution of marine litter, with particular reference to the plastic fraction, on the seafloor from the continental shelf to the middle slope and b) determining which variables (sampling location, year, bathymetric strata, seafloor type, distance to the coastline) are more significant in defining seafloor plastic distribution patterns.

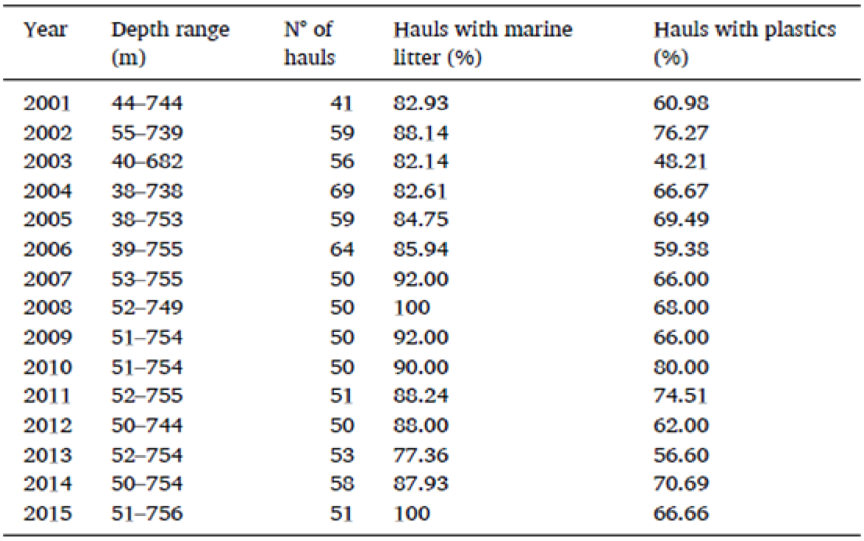
4.2 Material and methods

To assess spatial and temporal distribution of seafloor marine litter, with particular reference to the plastic fraction, data from scientific surveys conducted over a 15 year period (2001–2015) from the continental shelf to the middle slope in the surrounding grounds of the Balearic Islands was analyzed.

Data was collected during annual bottom trawl surveys, following an internationally agreed protocol (MEDITS, Mediterranean International scientific bottom-trawl; Barnes et al., 2009; Anonymous, 2017). These surveys aim at obtaining standardized basic information on the density, distribution and demographic structure of benthic and demersal species. Annual cruises were carried aboard R/V “Francisco de Paula Navarro” (2001–2006; length: 30 m; 178 gtr; nominal engine power: 759 hp) R/V “Cornide de Saavedra” (2007–2013; length: 67 m; 1113.13 gtr; nominal engine power: 1500 + 750 hp) and R/V “Miguel Oliver” (2014–2015; length: 70 m; 2495 gtr; nominal engine power: 2 x 1000 kw) during late spring and early summer (Table 4.1).

Bottom trawl hauls were conducted between depths of 38 and 800 m. Sampling was carried out during daytime hours using a bottom trawl gear designed for experimental fishing with scientific purpose (GOC 73; Dremière et al., 1999), with a cod end of 20 mm. The number of stations per survey varied between 41 and 69 (Table 4.1), with a towing speed of around 3 knots and a trawling time between 20 and 60 min, depending on the depth. Each haul was tracked using GPS and the opening of the net was monitored using a SCANMAR system. Horizontal and vertical net openings were estimated, on average to be of 16 m and 2.7–3.2 m, respectively.

Once aboard, marine litter was sorted out and classified into different categories: glass, plastics, metal, fishing material, clinker, cloth, rubber and paper. For all hauls, abundance of each litter fraction was calculated as the standardized weight (kg) of captured litter per surveyed area (kg/km^2).



Year	Depth range (m)	N° of hauls	Hauls with marine litter (%)	Hauls with plastics (%)
2001	44–744	41	82.93	60.98
2002	55–739	59	88.14	76.27
2003	40–682	56	82.14	48.21
2004	38–738	69	82.61	66.67
2005	38–753	59	84.75	69.49
2006	39–755	64	85.94	59.38
2007	53–755	50	92.00	66.00
2008	52–749	50	100	68.00
2009	51–754	50	92.00	66.00
2010	51–754	50	90.00	80.00
2011	52–755	51	88.24	74.51
2012	50–744	50	88.00	62.00
2013	52–754	53	77.36	56.60
2014	50–754	58	87.93	70.69
2015	51–756	51	100	66.66

Table 4.1 Summary of the scientific surveys considered for the study of the spatial and temporal distribution of seafloor plastics in the Balearic Islands with indication of sampling year, sampled depth range, number of bottom trawl hauls analyzed and percentage of hauls with marine litter and plastics.

4.2.1 Data analysis

The spatial distribution of seafloor plastic from the continental shelf to the middle slope off the Balearic Islands throughout 15 years of surveys was interpolated using the Inverse Distance Weight (IDW) method. The IDW was calculated on a 1° x 1° cell size using the interpolation plugin in QGIS (QGIS Development Team, 2018).

A Generalized Additive Model (GAM) was implemented to study the spatial and temporal distribution of plastics regarding: sampling area, year, bathymetric strata, seafloor type and sampling distance to the coastline (in nautical miles). In the model, to consider the spatial distribution of the sampled area, the latitude and longitude were considered as smoother in the base model. The bathymetric range was divided into the four bathymetric stratas defined in the MEDITS sampling protocol: stratum B (51–100 m, shallow shelf), stratum C (101–200 m, deep shelf), stratum D (201–500 m, upper slope) and stratum E (501–800 m, middle slope) while seafloor was categorized into sand, muddy-sandy, maërl, rhodophytes and crinoids. A full GAM model was assessed which considered the following variables: latitude and longitude for each sampling location, year, bathymetric strata, seafloor type and distance to the coastline. Year was treated as a categorical variable to calculate the effect of collected plastic in a given year and determine temporal variability. Finally, for significant parameters, a Wald test was performed to determine which explanatory variables significantly differed from each other. The residuals of the best-fit model were inspected to assure normality of data as well as to determine which bestfit model differed significantly from the null model. A backward stepwise approach was used to determine the most parsimonious model. One term was removed for each model and each model was compared based on the lowest Akaike's Information Criterion corrected (AICc) value. This was used as a measure of the goodness of fit as well as to determine the optimal number of model parameters, in which only the significant explanatory variables were retained. Data analysis was performed using the MGCV package (Wood, 2017) and all analyses were done with R version 3.4.3 (R Core Team, 2016).

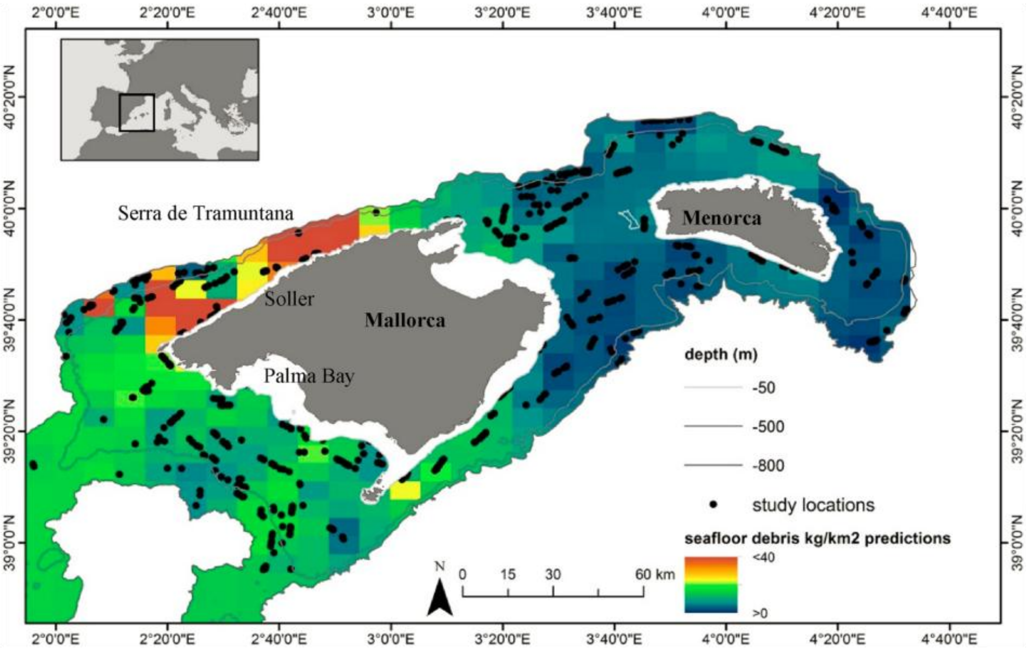
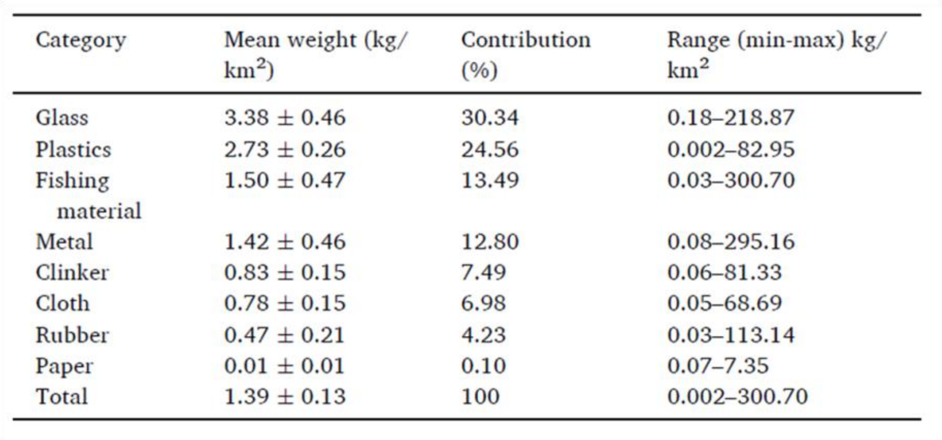


Fig. 4.1 Predictions of seafloor plastics (kg/km^2) in the study area based on the results from the Inverse Distance Weighting (IDW). The bathymetric isolines represent the 50, 500 and 800 m depths and the black points are the sampling locations during 15 years of surveys (2001–2015). The predictions for seafloor plastics have been masked to reflect the surveyed area between 50 – 800 m depth.

4.3 Results

A total of 806 bottom trawl hauls were conducted, from the continental shelf to the middle slope around the Balearic Islands, between 2001 and 2015 (Fig. 4.1). Bottom trawl hauls with marine litter accounted for 88% of the total hauls, with a mean value of 1.39 ± 0.13 kg of marine litter/km^2 during these 15 years of surveys. By type and weight of litter, glass was the most abundant with a mean value of 3.38 ± 0.46 kg/km^2 followed by the plastic fraction with a mean value of 2.73 ± 0.26 kg/km^2. On the other hand, rubber and paper were the least common fractions, in terms of weight, with mean values of 0.47 ± 0.21 kg/km^2 and 0.01 ± 0.01 kg/km^2, respectively (Table 4.2). When considering the presence of marine litter in the study area, glass, plastics, metal, clinker and cloth were the fractions with a broader presence whereas fishing material was less observed and rubber and paper were only occasionally observed throughout the 15-years of survey (Fig. 4.2).



Category	Mean weight (kg/km^2)	Contribution (%)	Range (min-max) kg/km^2
Glass	3.38 ± 0.46	30.34	0.18–218.87
Plastics	2.73 ± 0.26	24.56	0.002–82.95
Fishing material	1.50 ± 0.47	13.49	0.03–300.70
Metal	1.42 ± 0.46	12.80	0.08–295.16
Clinker	0.83 ± 0.15	7.49	0.06–81.33
Cloth	0.78 ± 0.15	6.98	0.05–68.69
Rubber	0.47 ± 0.21	4.23	0.03–113.14
Paper	0.01 ± 0.01	0.10	0.07–7.35
Total	1.39 ± 0.13	100	0.002–300.70

Table 4.2 Mean (± standard error (se)) weight for the eight categories of marine litter obtained in hauls of this study. Contribution of each category regarding the total amount of marine litter, by weight, is expressed in percentage. Minimum and maximum values for each litter category calculated without considering the 0 values.

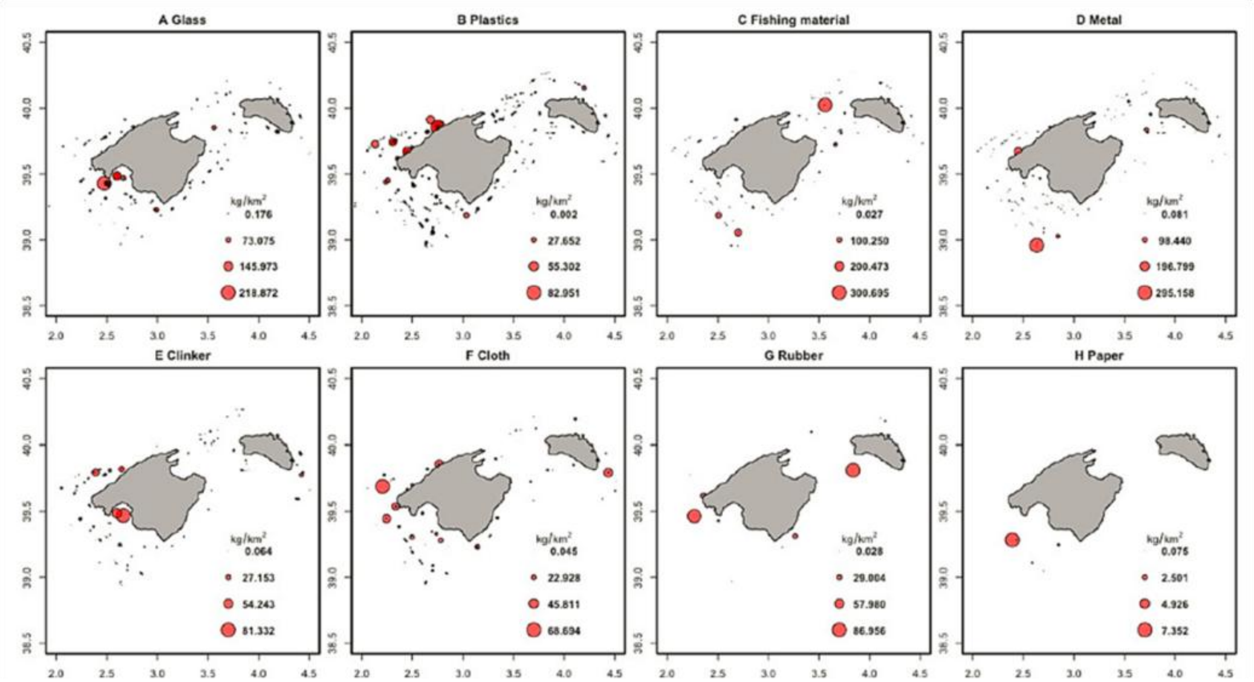


Fig. 4.2 Spatial distribution of the abundance (kg/km^2) of the different marine litter fractions: Glass (A), Plastics (B), Fishing material (C), Metal (D), Clinker (E), Cloth (F), Rubber (G) and Paper (H) obtained in the scientific bottom trawl hauls from 2001 to 2015. For best visualization of the abundance, hauls with 0 kg/km^2 of marine litter were not represented.

The plastic fraction was present in 66% of the hauls analyzed, with the highest quantities observed along the northwestern coast of Mallorca (max. 82.95 kg/km^2) and the lowest values of seafloor plastics in the eastern part of Mallorca and south of Menorca (Fig. 4.1). Regarding the temporal distribution of seafloor plastics, no temporal trend was observed within the 15 years of survey. Maximum seafloor mean plastic values were observed in 2007 (3.97 ± 1.93 kg/km^2) and in 2012 (4.40 ±1.93 kg/km^2) and the lowest abundance, in terms of weight, were observed in 2015 (1.32 ± 0.78 kg/km^2). Noticeably, variability (se) of mean values was very large throughout the sampling period (Fig. 4.3a), which indicates high variability among the samples analyzed.

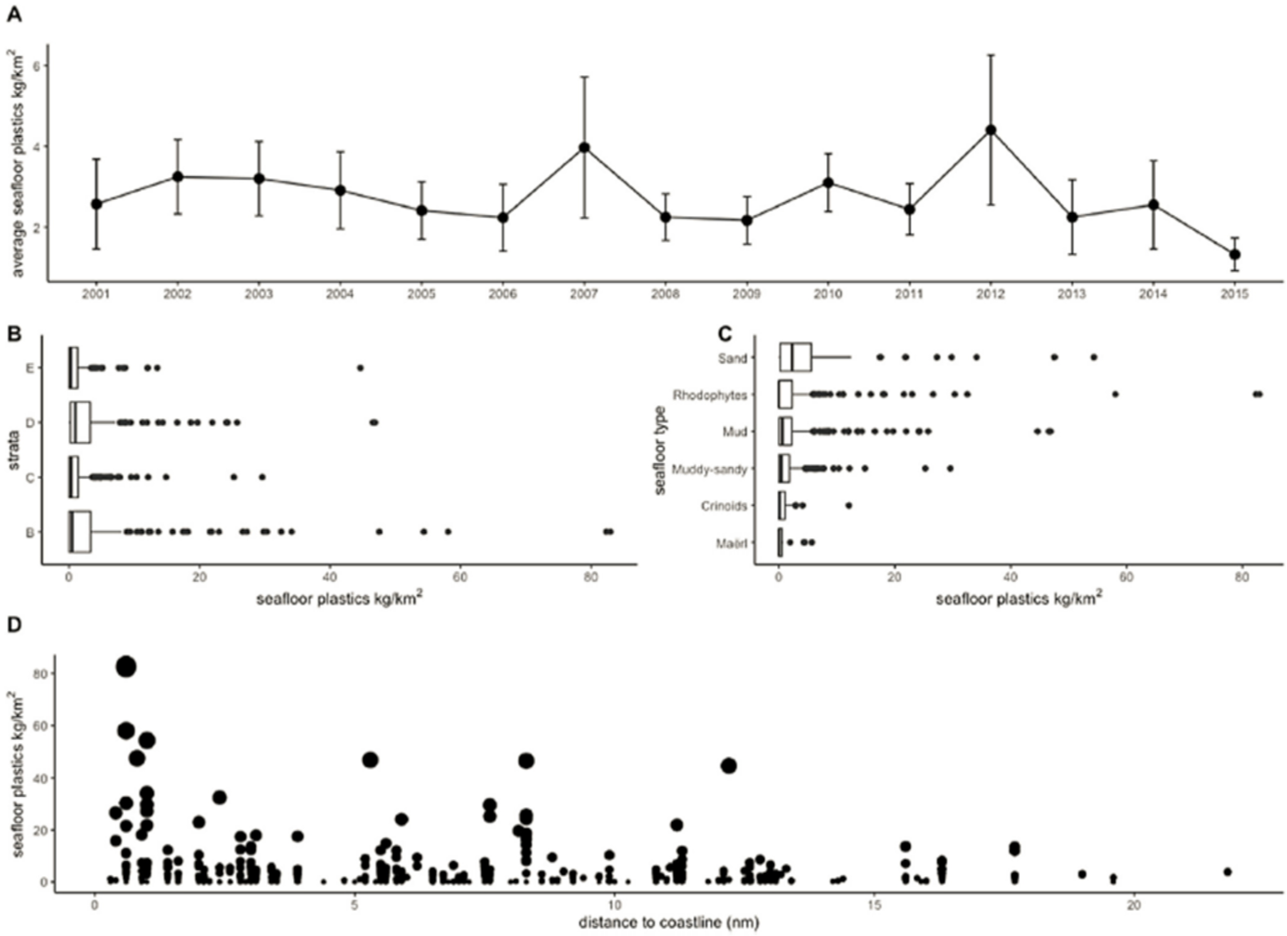
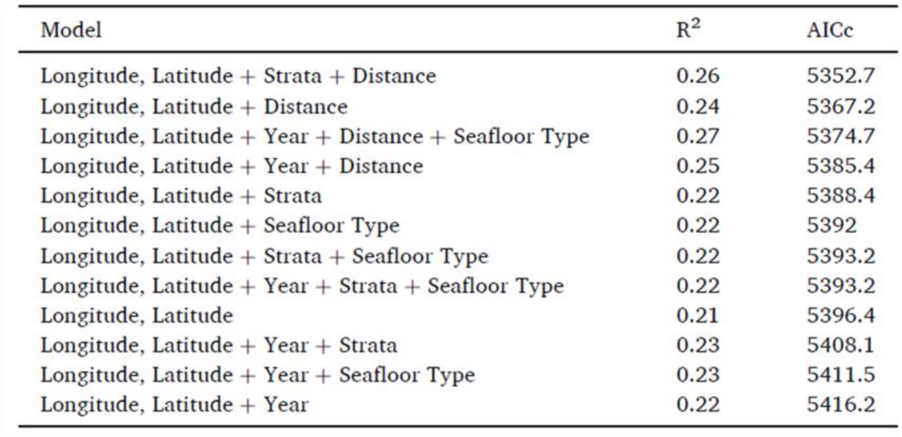


Fig. 4.3 Seafloor plastics (kg/km^2) for each of the parameters used to model their distribution around the Balearic Islands: A) sampling year (mean values, error bars indicate the standard error of the mean), B) bathymetric strata: B (51–100 m), C (101–200 m), D (201–500 m) and E (501–800 m), C) seafloor type (mäerl, crinoids, mud, muddy-sandy, rhodophytes and sand) and D) distance to the coastline (nm). In the box plots (B and C) the first, median and third quartiles are shown, the vertical line represents the median (50% quartile).

According to bathymetric strata (depth), the highest seafloor mean plastic values were found in the shallowest strata, the shallow shelf (B, 51–100 m) and in the upper slope (D, 201–500 m) with mean values of 3.76 ± 0.55 kg/km^2 and 3.71 ± 0.64 kg/km^2, respectively (Fig. 4.3b). On the other hand, the lowest abundance of plastics were given in the deep shelf (C, 101–200 m) and middle slope (E, 501–800 m) with mean values of 1.43 ± 0.22 kg/km^2 and 1.49 ± 0.36 kg/km^2, respectively.

When considering seafloor type, the highest quantities of mean plastic values were found in sandy areas, 5.95 ± 1.17 kg/km^2, followed by rodophytes (3.55 ± 0.79 kg/km^2) seafloors. On the other hand, the lowest values were given in mäerl (0.65 ± 0.23 kg/km^2) and crinoids (0.88 ± 0.28 kg/km^2) seafloors. Intermediate mean plastic values were observed for muddy (2.57 ± 0.37 kg/km^2) and muddy-sandy (1.74 ± 0.26 kg/km^2) seafloors (Fig. 4.3c).

Regarding distance from the coastline, a decrease of plastic abundance in the seafloor was seen with an increase of distance from the coastline (Fig. 4.3d). Most maximum values of seafloor plastics abundance, by weight, were given in seafloor areas within the first 5 nm from the coastline, which corresponds to coastal waters according to legislation (Fig. 4.3d).



Model	R^2	AICc
Longitude, Latitude + Strata + Distance	0.26	5352.7
Longitude, Latitude + Distance	0.24	5367.2
Longitude, Latitude + Year + Distance + Seafloor Type	0.27	5374.7
Longitude, Latitude + Year + Distance	0.25	5385.4
Longitude, Latitude + Strata	0.22	5388.4
Longitude, Latitude + Seafloor Type	0.22	5392
Longitude, Latitude + Strata + Seafloor Type	0.22	5393.2
Longitude, Latitude + Year + Strata + Seafloor Type	0.22	5393.2
Longitude, Latitude	0.21	5396.4
Longitude, Latitude + Year + Strata	0.23	5408.1
Longitude, Latitude + Year + Seafloor Type	0.23	5411.5
Longitude, Latitude + Year	0.22	5416.2

Table 4.3 Summary of the model selection approach for the Generalized Additive Models (GAMs) based on the R-squared (R^2) and Akaike's Information Criterion corrected value (AICc). All models included the spatially explicit terms for longitude and latitude as the base model and a backward stepwise approach was applied where the contribution of each covariate was considered from the initial full model. Relevant models for the GAM approach are described here and the first model was selected as the best model for describing the spatial and temporal distribution of seafloor plastics.

According to the Generalized Additive Model (GAM), the best model explaining seafloor plastics included latitude, longitude (sampling area), strata and distance from the coastline and over a quarter of the variation (26%) was explained by this model (Table 4.3). According to the results of this model, sampling area (p-value < 0.001), bathymetric strata (p-value < 0.01) and distance from the coastline (p-value < 0.001) were significantly explaining the spatio-temporal distribution of seafloor plastic from the continental shelf to the middle slope off the Balearic Islands (Table 4.4). GAM results highlighted the spatial distribution of plastic litter on the seafloor showing highest concentrations in the northwestern coast (Fig. 4.4a). When considering the explanatory variables given by the GAM model (sampling area, distance from the coastline and bathymetric strata), for bathymetric strata a significant effect was found between stratas B and depths of 201–500 m (strata D) (Wald, p-value < 0.01) and strata C with D (Wald, p-value < 0.001), highlighting the accumulation of seafloor plastics at depths of 201–500 m (Fig. 4.4b). Finally, regarding results of the GAM model, for distance from the coastline, two peaks of high seafloor plastic abundances were observed, one within closer distances from the coastline with variable abundances and another peak of seafloor plastic abundances further away from the coastline (Fig. 4.4c). Sampling year and seafloor type weren't significant explanatory variables in the model thus they weren't considered in the final model (Table 4.3).

Parametric coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	1.4542	0.8051	1.81E+00	0.07128	.
Strata C	0.5185	1.1855	0.437	0.66194	
Strata D	4.3412	1.434	3.027	0.00255	**
Strata E	2.3557	1.6913	1.393	0.16407	

Smooth terms:

	edf	Ref.df	F	p-value	
Latitude and Longitude	19.183	23.729	3.719	9.30E-09	***
Coastline distance (nm)	8.405	8.864	7.104	1.94E-09	***

Table 4.4 Summary of the results from the best-fit Generalized Additive Model (GAMs) after the backward stepwise model selection. The following contributing parameters explain the distribution of seafloor plastics from the continental shelf to the middle slope around the Balearic Islands. For each of the parameters and terms analyzed, the significance interval is expressed as the following: ***P < 0.001 and **P < 0.01.

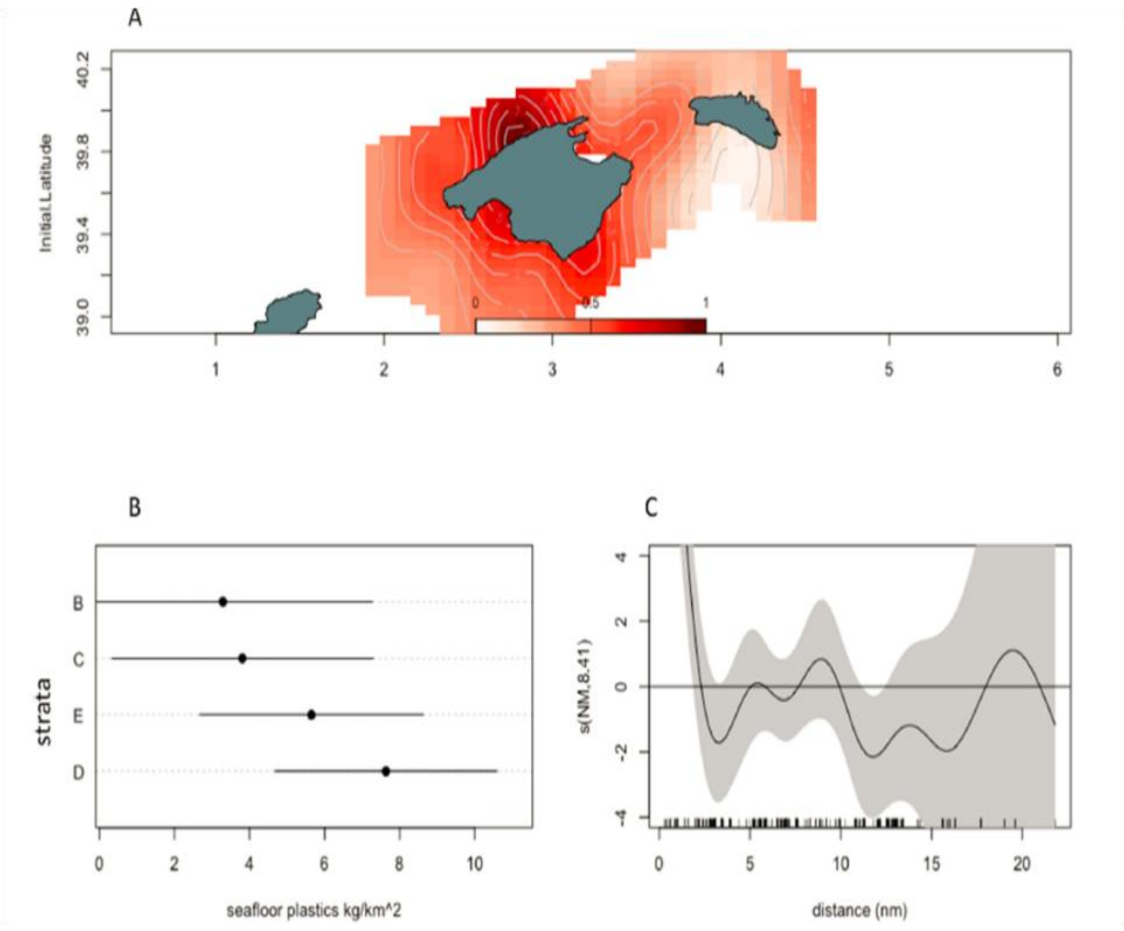


Fig. 4.4 Summary of the partial effects and fitted values of each coefficient from the best-fit GAM model: spatially explicit effect (latitude and longitude) to consider the sampling area, dark red area indicates high effect, (A), bathymetric strata (B) and distance from the coastline (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

4.4 Discussion

With this study we provide further evidence of marine litter in seafloor areas covering a depth range from 38 to 800 m around the Balearic Islands over a 15 year time period. Furthermore, results from this research, suggest that bathymetric strata (depth), distance from the coastline and sampling area are explaining the distribution of seafloor plastics between the continental shelf and the middle slope of the Balearic Islands. Plastics were present in 66% of the bottom trawl hauls and by mean weight, plastics were the second most common category of the study area after glass. This is consistent with other studies from the Mediterranean Sea where the most common categories are plastic, metal and glass (García-Rivera et al., 2017; Koutsodendris et al., 2008; Alvito et al., 2018).

However, marine litter values in seafloor areas of the continental shelf and middle slope from this study (1.39 ± 0.13 kg/km^2) are much lower than previously reported values from adjacent seafloor areas along the continental shelf and upper slope of the Spanish Mediterranean coast (9.8 ± 42.9 kg/km^2; García-Rivera et al., 2018) and from more distant areas in the Adriatic Sea (85 ± 26 kg/km^2; Strafella et al., 2015 and 82 ± 34 kg/km^2; Pasquini et al., 2016) and Greece (overall density of 13.4 kg/km^2; Koutsodendris et al., 2008). It is important to mention that the sampling in Greece was conducted in semi-enclosed gulfs with river run-offs (Koutsodendris et al., 2008) and that the Adriatic Sea is composed of an elongated basin, hosting a large number of rivers which drain through industrial centers of large cities and finally discharge their waters into this basin (Strafella et al., 2015). This enclosed nature, with a lower circulation and water exchange rate with open seas and multiple river run-offs in the Gulfs of Greece and Adriatic Sea, could explain the higher values of marine litter detected in these areas compared to areas from the Western Mediterranean basin (Balearic Islands and Spanish Mediterranean Coast).

Marine litter was quantified in a high percentage of hauls (88%) from the study area and according to the different litter fractions, it is interesting to note that clinker was observed in this study area and with highest quantities in the south of the bay of Palma, which hosts the main and major port of the Balearic Islands. Additionally, clinker was also present in bottom trawl hauls around Soller, which traditionally has been an important port for goods exchange with the Spanish mainland and France in addition to being a military port. Clinker is the residue of burnt charcoal of steam-powered vessels and was dumped overboard along shipping lanes about 150 years ago (Ramirez-Llodra et al., 2011) which could be explaining the higher abundances found in the vicinity of these two ports compared to other areas. Moreover, clinker has also been reported as a common type of litter in trawl samples, eventually becoming dominant in deep basins and continental slopes (Pham et al., 2014). Therefore, the presence of clinker could be considered as an indicator of the persistence of marine litter in marine environments for more than a hundred years. Besides, some studies have suggested that metal is a vessel-based source as it is not expected to travel long distances and it is likely to be found in maritime routes (Horsman, 1982; Moore and Allen, 2000; Stefatos et al., 1999; Koutsodendris et al., 2008). However, this wouldn’t be the case for metals in the study area, as higher quantities are not given in the main maritime routes but in isolated areas and its presence could be considered as sporadic.

As already mentioned, plastic was the second most abundant fraction and accounted for nearly one quarter (24.56%) of all of the marine litter, which is lower than previously reported percentages but higher than one study conducted with the same sampling methodology in the shelf of Antalya (17%; Olguner et al., 2018). However, in Sardinian waters, the percentage weight of plastic, also obtained within the MEDITS survey, was of 38% during three years of survey (Alvito et al., 2018) and in the Maltese Islands, plastic contribution, however based on number of items, was of 47% (Mifsud et al., 2013) whereas in Greece plastic accounted for 56% (Koutsodendris et al., 2008) and in the Central Adriatic Sea, contribution of plastic by weight was of 62% (Pasquini et al., 2016). Moreover, mean plastic values of our study (2.73 ± 0.26 kg/km^2) are much lower than values obtained, also during a MEDITS survey, along the Spanish Mediterranean seafloor (8.4 ± 50.0 kg/km^2; García-Rivera et al., 2018) and in Sardinian waters (7.35 ± 2.37 kg/km^2; Alvito et al., 2018) and even much lower than in the Central Adriatic Sea (49 ± 25 kg/km^2; Pasquini et al., 2016). It is remarkable that within the Spanish Mediterranean coast, significantly higher amounts of plastics were given in the Alboran Sea than in Valencia and Tramontana, which are closer to the Balearic Islands, and had seafloor plastic values mainly in the range of values from our study (0.1–5 kg/km^2). Compared to our study area, the Spanish Mediterranean continental coast is very extensive and sampled locations in García-Rivera et al. (2018) were spread out from the Strait of Gibraltar to the French border. This coast has a higher intensity of human pressures, for example, a higher level of marine traffic along the Gibraltar strait and also marine traffic entering and leaving Barcelona’s port and a higher intensity of fishing and merchant vessels (García-Rivera et al., 2018) which could be influencing the higher values of seafloor plastics in this coast compared to those from the Balearic Islands. The result of multiple factors such as human population density and fishing intensity has also been reflected in the surrounding grounds of the island of Sardinia as fishing related items on shallow coastal areas of the Strait of Sicily represented a smaller percentage of the total litter abundances quantified, suggesting a land based origin of plastics (Consoli et al., 2018b) than in deeper areas, where lost or abandoned fishing lines contributed to 98% of the overall litter density in seafloor areas of banks of the Strait of Sicily where fishing activities take place (Consoli et al., 2018a).

Contrary to our observation, Pham et al. (2014) documented higher seafloor litter values on the slope south of Mallorca than in other sampling areas of the Mediterranean Sea (0.7–1.8 kg/ha). However, we have to take into account that these authors prospected areas deeper than the ones analyzed in our study (up to 1500 m in the former study versus 800 m in our study). In fact, there is scientific evidence reporting much lower quantities of marine litter on continental slopes than on submarine canyons, seamounts, banks and ocean ridges (Tubau et al., 2015; Pierdomenico et al., 2019), which may indicate that higher quantities of marine litter are in general expected in areas that can act as vectors for litter transportation than those considered in our study area. Moreover, litter density in the same sampling area of Sardinia but at different depths, reported much higher densities in deeper depths of up to 220 m (2.13 ± 0.84 items/100 m^2) than in shallower depths of up to 30 m (0.11 ± 0.16 items/100 m^2) (Consoli et al., 2018a,b) and Olguner et al. (2018) observed a general trend in increasing litter accumulation with depth in the eastern Mediterranean Sea. According to the modeling approach of this study, when considering the explanatory variables for seafloor plastic distribution (bathymetric strata (depth), distance from the coastline and sampling area), highest abundances are reported at depths compromising between 201 – 500 m which would also reflect a trend of higher litter abundances in deeper areas. However, according to mean seafloor plastic values, higher abundances were also given in the shallowest bathymetric strata, the continental shelf (51–100 m) as well as in the upper slope (201–500 m). This could have implications for species living in these areas as bathymetric gradient has been seen to be the principal factor for species association (Massutí and Reñones, 2005), as seen for *Mullus surmuletus* and *Galeus melastomus*, two demersal species for which microplastic ingestion has already been quantified in this same area (Alomar and Deudero, 2017; Alomar et al., 2017). *Mullus surmuletus* shows a bathymetric distribution restricted to depths shallower than 200 m (Massutí and Reñones, 2005) with highest densities in the shallow shelf (Ordines et al., 2006). According to our results, high densities of plastic have been observed at these depths and for *M. surmuletus* plastic occurrence was documented in 27% of the sampled individuals with a mean value of 0.42 ± 0.04 microplastics/individual (Alomar et al., 2017). On the other hand, *G. melastomus* is distributed in the slope (Ramirez-Llodra et al., 2011), especially in the middle slope (Guijarro, 2012), in which low densities of plastic have been found according to results from our study. According to previous research in the same sampling area, almost 17% of the analyzed *G. melastomus* showed ingestion of microplastics, with a mean value of 0.34 ± 0.07 microplastics/ individual (Alomar et al., 2017) which are lower rates than those observed for *M. surmulletus* which is living in an area with higher observed abundances of plastics. Although these results are only for two species, future studies relating the presence of plastics in the seafloor with plastic ingestion in species of the same grounds as well as other possible implications (entanglement and colonization) should be considered for different taxonomic species in the study area.

In the study area, maximum abundances of seafloor plastics were found within the first 5 nm from the coastline, which is in accordance with other studies observing an overall increase of plastics in areas closer to the coast (García-Rivera et al., 2017). However, a peak in seafloor plastic abundance was also observed in areas further away from the coast when the modeling approach was applied giving further evidence of the patchiness in seafloor plastic distribution (Pierdomenico et al., 2019). Even though marine litter has its origin in sea based and land based sources (Jambeck et al., 2015), transport processes taking place within the marine environment seem equally important as external sources for the redistribution of marine litter on the seafloor (Ioakeimidis et al., 2014). Consequently, due to plastic properties and environmental characteristics, such as geomorphology and water circulation, plastics will travel long distances from their source point before sinking (Pham et al., 2014). Thus, higher values in a given area can be influenced by the regional circulation and not only be dependent on the anthropogenic characteristics of the sampling area. In this sense, transferred contamination, which could be the result of higher seafloor plastic abundances in areas far from the coastline of the study area, has already been seen in the Balearic Islands where coastal sediments from Marine Protected Areas (MPAs) showed higher microplastic quantities than those from urbanized areas (Alomar et al., 2016).

Nevertheless, in the study area, high coastal seafloor plastic abundances could also suggest an input of plastics from a local land-based origin and also from coastal sea-based source possibly linked to high human pressures, including, recreational activities and tourism, maritime transport and recreational and professional fishing in coastal areas. Indeed, higher quantities of litter in areas located close to the coastline have already been observed in another Mediterranean touristic island, Sardinia, giving notice of how tourism can influence detected patterns of benthic litter (Alvito et al., 2018). Moreover, a qualitative analysis, regarding maritime traffic, showed that merchant ships were the main source of marine litter in open waters while recreational and fishing vessels were the main source in coastal areas (García-Rivera et al., 2017). Nevertheless, in the study area, even though the MEDITS survey is directly conducted in fishing grounds, fishing material was not as spread out along the seafloor as other categories such as glass, plastics, cloth and rubber. Surprisingly, in the west of Mallorca (specifically Soller) high abundances of plastics were observed but few fishing material was seen, even though this is the zone with the highest fishing pressure of the study area, especially in the middle slope (600–750 m), targeting a highly valuable crustacean, *Aristeus antennatus* (Moranta et al., 2008) and fishing-related litter is suggested to belinked to high value commercial species (Consoli et al., 2018a).

Previous studies have identified Derelict Fishing Gear (DFG) as the most abundant type of litter (60–90%) in rocky environments (Melli et al., 2017) which may be linked to the highest probabilities of entanglement of gears in these type of bottoms, in comparison with the sandy-muddy bottoms in which the bottom trawl fleet usually operates, as it is in the west of Mallorca. However, given that mainly soft bottoms were analyzed due to rocky bottoms not being prospected by bottom trawl surveys, a direct comparison between habitats is not possible. Moreover, caution must be taken when comparing different habitats as sampling methodologies can be selective to litter quantification and characterization (Consoli et al., 2018a). Given that higher marine litter estimates have been observed over rocky bottoms than soft bottoms (Melli et al., 2017), it could be thought that rocky environments from the same sampling area would present higher abundances of marine litter and fishing related items. However, another possibility for the low abundance of fishing material in this sampled area (west of Mallorca), is that these are well known fishing grounds for local fishermen which could be reducing the probability of lost gear due to fishing practices in the prospected areas or even fishing items could have been removed by the continuous fishing activity in these grounds (García-Rivera et al., 2017).

Higher seafloor plastic abundances observed along the northwestern coast of Mallorca could also be related to the regional circulation in this area of the Mediterranean basin. Here, the circulation is dominated by the Northern Current which carries down Atlantic waters from the Gulf of Lions along the continental slope of the Iberian Peninsula and into the Balearic sub-basin (Monserrat et al., 2008). This could be influencing the distribution of seafloor plastics as the Northern Current, circulating along the continental slope of the Iberian Peninsula, could transport plastics from areas close to large Metropolitan cities and river discharges from the Gulf of Lions and along the Spanish coast towards the northwestern part of Mallorca. However, the regional oceanographic circulation in this area shows interannual variability, related to the climatic variability in the Gulf of Lions as the flow and intensity of the Northern Current reaching the study area can be modified depending on the conditions of the previous winter. That is, in the case of a preceding cold winter, the Northern Current may be blocked when reaching the Ibiza Channel and recirculates cyclonically joining the Balearic Current without significant transport of waters through the Ibiza Channel (Monserrat et al., 2008) and consequently a lower potential transportation rate of plastics into the Balearic Islands. These different water circulation interannual patterns may result in a high variability of plastic transportation to the study area that might be influencing the absence of a temporal trend throughout the 15 years of analyzed data.

Besides this high variability in the temporal trend of plastics, a high variability within sampling locations of the study area is also observed with plastic values ranging from 0.002 to 83 kg/km^2 This high variability is also given within the Mediterranean basin and in the eastern region, seafloor litter, also sampled according to MEDITS protocols, ranged from 0.02 and 559 kg/km^2 (Olguner et al., 2018). Even though the MEDITS survey takes place only once a year, the results from our study can provide us with a snapshot of marine litter quantities at a specific time of the year and within 15 consecutive years. Due to the fact that plastic degradation processes can be long, even lasting up to hundreds or thousands of years (Barnes et al., 2009), one could think that higher differences would be given within years and not within a same year. However, no temporal trend was seen during the 15 years of consecutive surveys even though maximum values were given in 2007 and 2012. The absence of a clear trend and the slow degradation processes of plastics in the marine environment which make it more difficult to detect decreasing tendencies due to human changes in plastic use, reflects the need to produce and analyze larger time series in order to detect temporal trends of seafloor plastics.

In summary, this study provides with further scientific evidence of marine litter in seafloor areas in the Mediterranean Sea, one of the most polluted seas worldwide (Galgani et al., 2014; Pierdomenico et al., 2019). Lower seafloor mean plastic values have been obtained in the Balearic Islands compared to other nearby and further areas in the Mediterranean basin, giving clear evidence of the variability of plastics within this semi-enclosed basin. In our study area, sampling area, depth and distance from the coastline are factors than seem to explain the distribution of plastics from the continental shelf to the middle slope. However, further progress is needed to include other factors such as oceanographic data, currents intensity or human pressures (i.e. fishing, maritime traffic, sewage discharges) in research dealing with seafloor plastic distribution. This would allow for a better understanding of the driving forces determining the spatial and temporal distribution of marine litter.

4.5 Acknowledgements

M. Compa is the recipient of an FPI Fellowship from the Conselleria d’Innovació, Recerca i Turisme of the regional Government of the Balearic Islands co-financed by the European Social Fund as part of the FSE 2014–2020 operational program. The BALAR surveys were funded by the Spanish Institute of Oceanography (IEO) and the MEDITS surveys has been co-funded by the IEO and the European Union through the European Maritime and Fisheries Fund (EMFF) within the National Program of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy. The authors wish to thank the crew and scientific team participating in the surveys. Acknowledgements to the European Commission DG Environment project: “Support Mediterranean Member States towards coherent and Coordinated Implementation of the second phase of the MSFD – MEDCIS” with Grant no. 11.0661/2016/748067/SUB/ENV.C2.

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Chapter 5: Exploring the relation between plastic ingestion in species and its presence in seafloor bottoms

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Abstract

In order to have a wider understanding of the impacts of plastics on marine ecosystems, studies should approach different environmental compartments, such as seafloor and biota, at the same time. The aim of this research is to study the relation between microplastic ingestion in species and the amount of seafloor plastics caught in the same bottom trawls hauls of the western Mediterranean Sea to describe a potential overlap between these two indicators of plastic pollution. According to results, 15% of the sampled individuals ingested microplastics with a mean value of 0.30 ± 0.40 microplastics/individuals. Regarding seafloor plastics, these were present in 58% of the hauls with a mean value of 1.31 ± 0.09 kg/km^2. The highest overlap between ingestion of microplastic in species and seafloor plastics was observed in the bay of Palma, the most urbanized area, but also in areas close to Marine Protected Areas (MPAs).

Keywords: Biota, Microplastics, Marine litter, Balearic Islands, Indicators

5.1 Introduction

The Mediterranean Sea, a semi-enclosed basin and a sensitive ecosystem, is affected by marine litter (Galil et al., 1995; Barnes et al., 2009; Galgani et al., 2010) and according to previous studies, 62 million pieces of litter are floating in this region of the world (Suaria and Aliani, 2014). Marine litter has its origin deriving from anthropogenic activities which can be land based and/or sea based including but not limited to: landfills, rivers and floodwaters, untreated municipal sewage, littering of beaches and coastal areas, disposal from ships, intentionally abandoned or accidentally lost fishing material, aquaculture and recreational activities in the sea (UNEP, 2009). It is estimated that 80% of litter found in the marine environment originates from land based sources (Jambeck et al., 2015) and a study carried out at a Mediterranean basin scale showed that the number of floating plastic particles was higher in the proximity of coastal areas than areas further away from the coast (Ruiz-Orejón et al., 2016). Moreover, high densities of anthropogenic items (more than 100,000 items/km^2) were found on the seafloor closer to metropolitan areas (Galgani et al., 2013). It is known that ocean circulation can transfer marine litter from source areas to distant areas and floating macro-litter on the sea surface will finally sink to the seafloor, be washed ashore, or fragmented into micro particles (Arcangeli et al., 2018). However, current knowledge on residence time of floating macro-litter, sinking and deposition rates is still unknown and due to this information gap, deposited litter in seafloor habitats can give indication of accumulation areas.

Marine topography will determine sinking areas and there is already scientific data reporting occurrence of plastic on seafloors with values ranging from 42.5% to 95% of the inorganic material found in hauls of professional trawlers (Ioakeimidis et al., 2014). Plastics and microplastics have already been quantified in soft, rocky, deep submarine canyons and coastal seafloors (Pham et al., 2014; Tubau et al., 2015; Alomar et al., 2016; Lopez-Lopez et al., 2017). In the Spanish southeastern Mediterranean seafloor, by weight, 75.9% of the litter obtained by commercial trawlers was plastic, metal and glass (García-Rivera et al., 2017). Marine litter in bottom trawl catches in the Mersin Bay, in the northeastern Mediterranean basin, also showed that plastics were the most abundant fraction accounting for 73% in terms of weight (Eryaşar, et al., 2014). In contrast, in three Italian regions of the Tyrrhenian Sea, plastics were only recorded occasionally with Remotely Operated Vehicle (ROV) but a direct impact on benthic organisms such as gorgonians, corals and sponges was observed (Angiolillo et al., 2015). Moreover, in seafloor areas of the banks of the Strait of Sicily, 30% of the litter detected caused entanglement/coverage to fauna (specifically 16 species) and 15% caused damage to sessile fauna (Consoli et al., 2018a).

Therefore, some marine species which live and feed on or close to seafloor areas are at high risk of ingesting or being entangled in discarded litter, highlighting the need to identify potential accumulation and sensitive zones of this type of pollution. Plastics have already been observed in species with different trophic guilds such as teleosts, elasmobranchs, cephalopods, bivalves and crustaceans (Deudero and Alomar, 2015). For example, 63.50% of benthic fish and 36.50% of pelagic fish studied from Portuguese waters ingested microplastics (Neves et al., 2015) and in the Mediterranean Sea, plastic ingestion has been quantified in deep-water fish (Anastasopoulou et al., 2013), demersal fish (Alomar and Deudero, 2017; Alomar et al., 2017), semipelagic fish (Nadal et al., 2017) and small and large pelagic fish (Romeo et al., 2015; Compa et al., 2018, Ríos-Fuster et al., 2019). Along the Mediterranean coast, *Mullus barbatus* ingest microplastics with a mean value of 1.90 ± 1.29 microplastics/individual and with an occurrence of 18.8% of the sampled individuals (Bellas et al., 2016). Authors suggested that, as this species is currently used as a biomonitor for marine pollution monitoring within the Spanish Marine Pollution Monitoring Programme (SMP), it might be also a suitable indicator for monitoring spatial and temporal trends of seafloor litter (Bellas et al., 2016).

Understanding the degree to which biota ingest plastics is essential to monitor and define levels that harm organisms, populations and ultimately, species' ecological functioning, and therefore the community structure (Fossi et al., 2018). Concern on marine litter has been increasingly attracting the attention of stakeholders and policy makers during the last years. In this sense, in the European Union (EU), the Marine Strategy Framework Directive (MSFD, 2008/56/EC) aims at achieving a Good Environmental Status (GES) of EU marine waters by 2020. This directive identifies the distribution of litter as highly variable and indicates that there is a needed to give response to the properties and quantities of marine litter in coastal and marine environments. There is an urge to further develop several indicators, notably those related to marine litter and biological impacts and in this sense, indicators 10.1.2 and 10.2.1 of descriptor 10 from the MSFD correspond respectively to characteristics of litter in the marine and coastal environment and to the impacts of litter on marine life.

In order to evaluate and locate sensitive zones of marine litter, environmental compartments (sea surface, water column and seafloor) and biota should be looked at simultaneously. For example, quantification of microplastics in environmental (water and sediment) and biota (fish) samples showed some indication that fish from places with a higher concentration of microplastics in the environment also ingested higher number of microplastics (Güven et al., 2017). Similarly, it is important to assess the relation between plastic accumulated in seafloor areas and ingestion in other taxonomic species such as cephalopods and crustaceans, as well as fish.

In the Mediterranean Sea, the Mediterranean International scientific bottom-trawl surveys (MEDITS) aim to obtain standardized information on benthic and demersal species and habitats, in terms of geographical distribution, abundance and biomass indices as well as demographic structure, from the continental shelf to the middle slope. All the catches obtained during the scientific experimental fishing trawls are recorded, including all types of litter, such as plastics. In parallel, the analysis of the stomach contents allows to estimate plastic ingestion rates of species captured in the same trawls. Consequently, this type of information can give simultaneous response to the two previously described indicators, 10.1.2 and 10.2.1, from the MSFD.

Therefore, the main aim of this research is to study the relation between microplastic ingestion in species and the amount of seafloor plastics collected simultaneously in the same bottom trawls in the western Mediterranean Sea to describe a potential overlap between these two indicators of plastic pollution within the MSFD. As a first step, the distribution of plastics was quantified both as ingested microplastics and seafloor plastics, followed by an overlapping analysis of both approaches. Secondly, the distribution of microplastic ingestion and the overlapping was explored according to several variables and finally a prediction model was developed for the spatial distribution of ingested microplastics, seafloor plastics and the Overlap Index.

5.2 Material and Methods

5.2.1 Field work

Sampling was carried out during the Mediterranean International scientific bottom-trawl surveys (MEDITS) in 2015. This scientific survey has been conducted annually since 2001 during late spring around the Balearic Islands, Western Mediterranean Sea, covering the soft bottoms of the continental shelf and upper and middle slopes between 50 and 800 m. In the 2015 expedition, a total of 55 hauls comprising depths between 46 and 756 m were carried out. To study the overlap between microplastic ingested in marine species and seafloor plastics, data from 43 hauls ranging from 46 to 756 m depth was analyzed. Bottom trawl hauls were performed during the daytime using an experimental net (GOC37) designed for scientific purposes with a vertical opening slightly superior to most common professional gears and a cod end mesh size of 20 mm (Bertrand et al., 2002). These hauls were conducted at a mean speed of 3 knots and during 20 - 60 minutes depending on the sampling depth.

Once aboard, the entire catch was sorted out, classified and individuals were counted and measured. Abundances of species in each haul were standardized to one kilometer square, using the horizontal opening of the net and the distance covered in each haul, obtained using the SCANMAR system (Catch Control Systems, Scanmar As, Åsgårdstrand, Norway) and Global Positioning System (GPS).

For the purpose of this study, microplastic ingestion was studied in 40 species, corresponding to species with more than 5 sampled individuals (Table 5.1). For a certain number of individuals, specimens were dissected and plastics in their gastrointestinal tracts were identified onboard following standardized protocols (Nadal et al., 2016). Regarding the other specimens, their gastrointestinal tracts were removed onboard and frozen for posterior plastic identification in the laboratory following the same standardized protocols (Nadal et al., 2016).

According to marine litter obtained in the bottom trawl hauls, this was classified into 8 different categories: coal, glass, metal, rubber, fishing material, paper, plastic and cloth. Each fraction was weighted separately and following the same protocol as species, litter abundances were standardized to one kilometer square. For this study, only the plastic fraction was considered.

Species	**n**	**Mean ± SE**	**Plastic ingestion (%)**
Argentina sphyraena	5	0	0
Aristaeomorpha foliacea	5	0	0
Aristeus antennatus	9	0.11 ± 0.11	11
Boops boops	24	0.33 ± 0.18	17
Capros aper	18	0	0
Centracanthus cirrus	10	0	0
Chelidonichthys cuculus	20	0.75± 0.37	35
Citharus linguatula	6	0	0
Engraulis encrasicolus	24	0	0
Gadiculus argenteus	12	0	0
Galeus melastomus	37	0.19 ± 0.08	16
Glossanodon leioglossus	5	0	0
Helicolenus dactylopterus	10	0.20 ± 0.20	10
Hymenocephalus italicus	5	0	0
Lepidotrigla cavillone	10	0	0
Lepidotrigla dieuzeidei	22	0	0
Merluccius merluccius	8	0	0
Nephrops norvegicus	8	0.63 ± 0.32	38
Nezumia aequalis	20	0.20 ± 0.16	10
Octopus vulgaris	6	0	0
Pagellus acarne	8	0.63 ± 0.26	50
Pasiphaea multidentata	11	0	0
Phycis blennoides	5	0	0
Plesionika martia	5	0	0
Raja clavata	11	1.73 ± 0.62	64
Sardina pilchardus	7	0.14 ± 0.14	14
Scyliorhinus canicula	13	0.08 ± 0.08	8
Serranus cabrilla	47	1.06 ± 0.17	57
Serranus hepatus	10	0	0
Spicara smaris	39	0.51 ± 0.21	23
Spondyliosoma cantharus	10	2.0 ± 1.09	50
Synchiropus phaeton	26	0.04 ± 0.04	4
Synodus saurus	5	0	0
Trachinus draco	15	0.07 ± 0.07	7
Trachurus mediterraneus	10	0.40 ± 0.22	30
Trachurus picturatus	5	0.20 ±0.20	20
Trachurus trachurus	40	0	0
Trigloporus lastoviza	5	0	0
Trisopterus minutus	5	0	0
Zeus faber	5	0	0
Total	546	0.30 ± 0.04	15

Table 5.1 Microplastic ingestion values in sampled species: number of sampled individuals for each species (n), Mean ± Standard Error (SE) ingestion values for each

species and percentage occurrence of individuals with microplastics (MPs) in their gastrointestinal tracts (MP ingestion (%)).

5.2.2 Laboratory work

Microplastic identification in individuals (Table 5.1) was done by visual sorting of the gastrointestinal tracts under a stereomicroscope (Euromex NZ 1903-S) with optical enhancement from 6.7x to 40.5x and with an attached CMEX 3.0 MP camera using a calibration software, Image Focus® 4.0 (Euromex software). The number of plastic items in each species' gastrointestinal tract was recorded. Measures to avoid (airborne) contamination were adopted while handling and processing samples in the laboratory (Woodall et al., 2015). During the gastrointestinal tract analyses, two glass Petri dishes were placed at each side of the stereomicroscope and checked for plastics before and after each sample. All material used was rinsed twice before use with distilled water and alcohol and all working surfaces were cleaned with alcohol. A 100% cotton laboratory coat was worn at all times during all analysis procedures.

5.2.3 Data analysis

To study microplastic ingestion in seafloor species, for each sampled individual, the number of microplastics in its gastrointestinal tracts was recorded. Occurrence of microplastic ingestion in each study species was calculated as the total number of individuals of the species ingesting microplastics divided by the total number of sampled individuals of that given species (full and empty gastrointestinal tracts) and multiplied by 100. The mean and the standard error values of microplastic ingestion per species were also calculated (microplastics/individual). In order to calculate the Overlap Index, the standardized microplastic ingestion per bottom trawl haul (standardized ingestion) was calculated as the mean microplastic ingestion of species as a whole in that haul multiplied by the total number of individuals (abundance) caught in the same bottom trawl haul and divided by the number of individuals analyzed for microplastic ingestion in the bottom trawl haul. This standardization was calculated because, in some cases due to sample size, only a subset of the individuals captured were analyzed for microplastic ingestion. To study seafloor plastic abundance, for each bottom trawl haul the abundance of plastics was calculated as the standardized weight of captured plastics (kilograms) per surveyed area in kilometers squared (kg/km^2). To study the relation between microplastic ingested in seafloor species and seafloor plastics, an adaptation of the spatial overlap index in Puerta et al. (2016) was calculated. This Overlap Index was calculated as the natural logarithm of the multiplied mean standardized ingestion in each bottom trawl haul and the abundance of seafloor plastics in the same bottom trawl haul plus 1.

$$\text{Overlap Index} = \log (X_{haulz} * Y_{haulz} + 1)$$

Where X= mean standardized microplastic ingestion of all species caught in haul z (standarized ingestion)

Y= seafloor plastics in haul z (kg/km^2)

5.2.4 Statistical analysis

Generalized Additive Models (GAMs) were used to explore microplastic ingestion per species (microplastics/individual), standardized ingestion and the Overlap Index. When studying microplastic ingestion per species (microplastics/individual), variables used in the GAM model were sampling point (haul latitude and longitude), depth, seafloor plastic abundance in the bottom trawl haul and species in the hauls. Additionally, GAMs were also constructed to study the response of the standardized ingestion according to sampling point (haul latitude and longitude), depth and seafloor plastic abundance in hauls. Finally, GAMs were also built to explore the Overlap Index for each haul according to sampling point (haul latitude and longitude) and depth.

A backward approach, in which only the significant explanatory variables were retained, was used to get the best model. Model selection was based on the Akaike's Information Criterion (AIC), which was used as a measure of the goodness of fit as well as the optimal number of model parameters, the best one having the smallest AIC value. Model performance was measured with the null deviance explained and the adjusted regression coefficient (R^2). According to the GAM models, a significant level of $p < 0.1$ was considered. All analyses were implemented with the mgcv package (Wood, 2017) using the R version 3.0.2 (www.R-project.org/).

Interpolation prediction maps for microplastic ingestion in species, seafloor plastics and the overlap index were estimated using the Kriging function from the Geospatial Toolbox ArcGIS. The interpolation method for each model was cross validated by comparing the predicted values to the observed values and the models with the lowest prediction mean and root-mean-square errors were identified (Krivoruchko, 2011). For each model, the average distance between all sampling stations was 60 nautical miles with a maximum and minimum number of nearest numbers assigned as 5 km. This approach allowed us to estimate values for all non-sampled locations within the Balearic Islands as well as the uncertainty of the predictions. All measured locations were included in the predictions.

5.3 Results

A total of 546 gastrointestinal tracts corresponding to 40 species were analyzed for microplastic ingestion (Table 5.1). Of all sampled individuals, 15% ingested microplastics with a mean value of 0.30 ± 0.04 microplastics/individual. According to all sampled species, 18 species (representing 45% of the analyzed species) showed ingestion of microplastics while 22 species (55%) showed no microplastic ingestion in their gastrointestinal tracts (5 - 40 individuals/species). Therefore, mean ingestion values for species ranged from 0 to 2.0 ± 1.09 microplastics/individual. This highest mean value corresponded to *Spondyliosoma cantharus* (10 individuals), followed by *Raja clavata* (11 individuals) and *Serranus cabrilla* (47 individuals), with 1.73 ± 0.62 microplastics/individual and 1.06 ± 0.17 microplastics/individual, respectively. The rest of the species that presented ingested microplastic in their gastrointestinal tracts showed mean values lower than 1 microplastic/individual (Table 5.1). Similarly, species with the highest percentage of occurrence of individuals with microplastics in their gastrointestinal tracts were again *R. clavata*, *S. cabrilla* and *S. cantharus*, as well as *P. acarne*, with values between 50% and 64%; the rest of the species showed percentage occurrence of individuals with microplastics lower than 50% (Table 5.1). On the other hand, *G. melastomus*, *N. aequalis*, *H. dactylopterus* and *T. picturatus* showed similar ingestion values amongst them (0.19 - 0.20 microplastics/individual) and also *T. draco* showed similar ingestion values to *S. canicula*, with 0.07 ± 0.07 and 0.08 ± 0.08 microplastics/individual, respectively (Table 5.1). When analyzing all species as a whole, highest mean ingestion values of microplastics where observed in the eastern part of Mallorca and around Menorca Island (Fig.5.1).

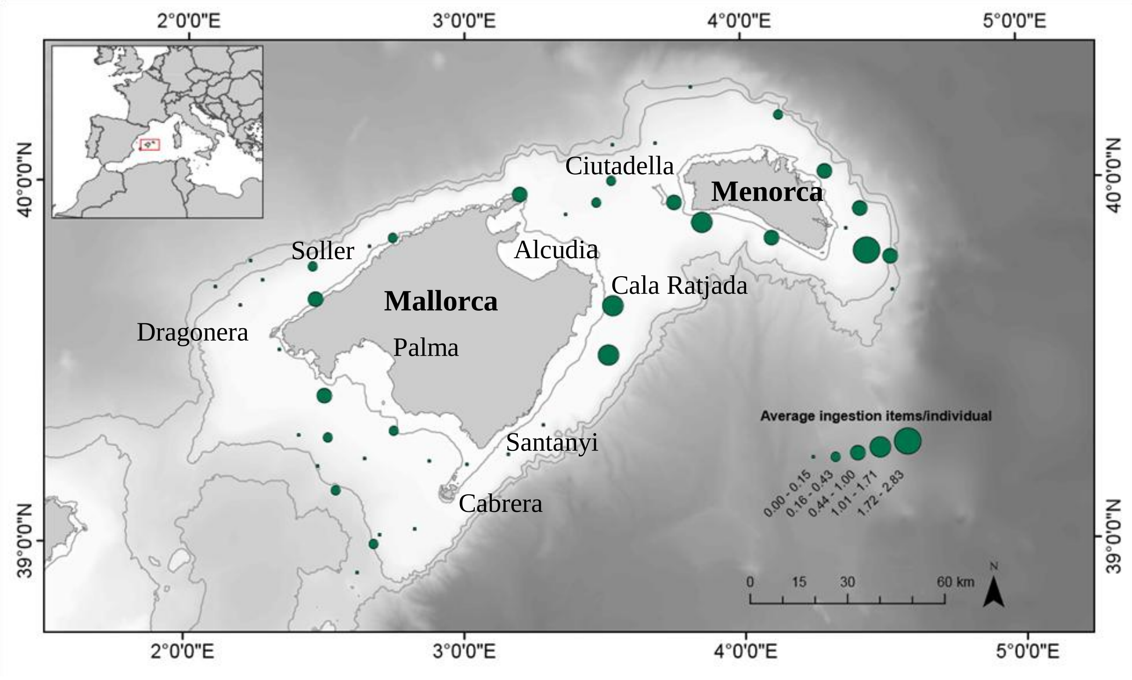

Fig. 5.1 Distribution map showing mean microplastic ingestion for all species analyzed at each bottom trawl haul during the 2015 MEDITS survey. Circles represent average microplastic ingestion classified into: 0 – 0.15; 0.16 – 0.43; 0.44 – 1.00; 1.01 – 1.71; 1.72 – 2.83 microplastics/individual and circle size increases with ingestion rates. Name of main islands of the study area are in bold.

Seafloor plastics were present in 58% of the hauls ranging from 46 to 756 m depth (Fig. 5.2). A mean abundance of 1.31 ± 0.09 kg/km^2 was obtained in the study area with values ranging from 0 to 18.65 kg/km^2 of seafloor plastics. The highest catches of seafloor plastics were found in the south and west of Mallorca (1.93 and 18.65 kg/km^2, respectively), including stations around the National Park of Cabrera, with values higher than 0.76 kg/km^2.

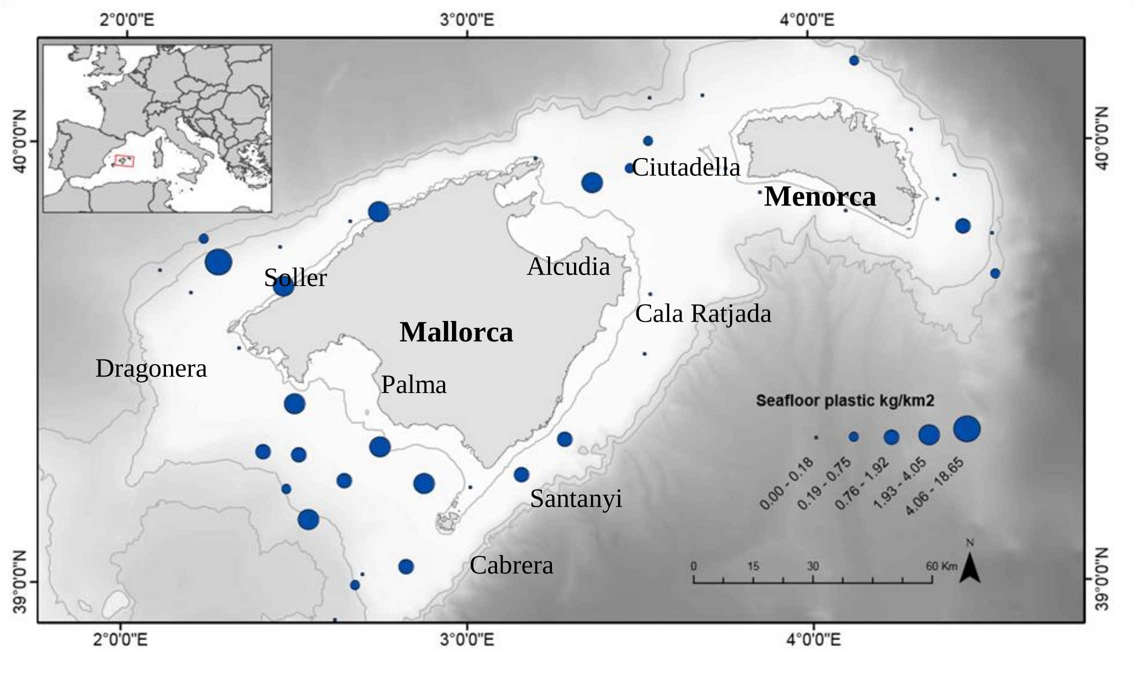

Fig. 5.2 Distribution map of seafloor plastic abundance quantified in the 44 sampled hauls around Mallorca and Menorca and between 46 and 756 meters depth during the 2015 MEDITS survey. Circles represent seafloor plastic abundance (kg/km^2) classified into: 0 – 0.18; 0.19 – 0.75; 0.76 – 1.92; 1.93 – 4.05; 4.06 – 18.65 kg/km^2 and circle size increases with seafloor plastic abundance.

From all bottom trawl samples analyzed, in 40% of them both microplastic ingestion in species and seafloor plastics were quantified. This Overlap Index, calculated for each haul, ranged from 0 to 5.93. A mean Overlap Index of 0.83 ± 0.24 was observed in the whole study area with lowest values in the eastern part of Mallorca and the highest in the south and west of Mallorca (Fig. 5.3).

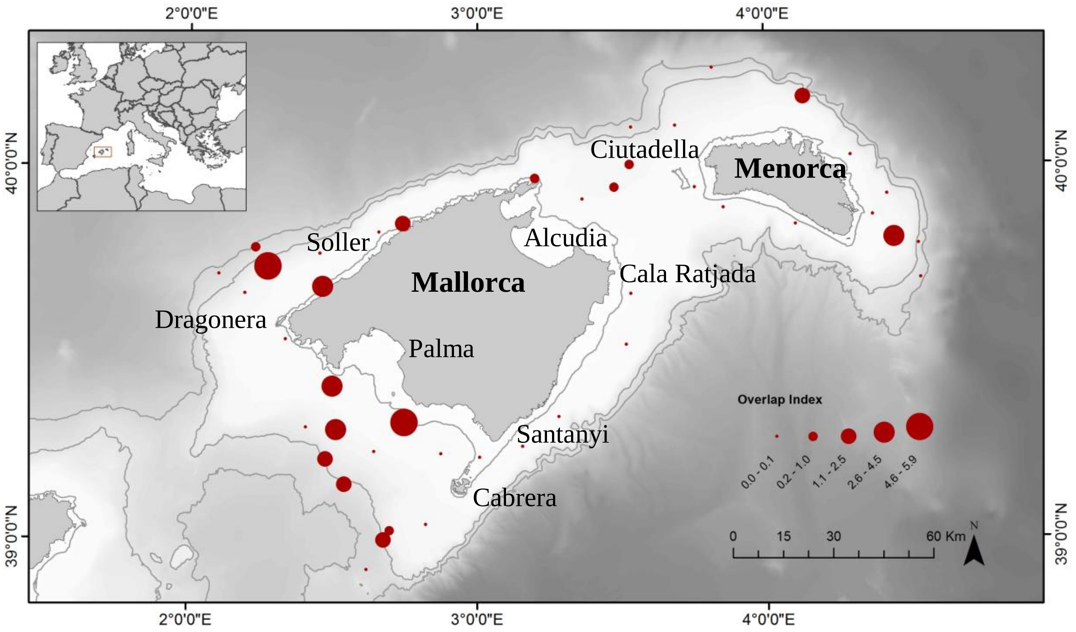

Fig. 5.3 Distribution map of the Overlap Index obtained as the natural logarithm of the multiplied mean microplastic ingestion in all species at each haul and the abundance of seafloor plastics in the same haul plus 1. Circles represent Overlap Index classified into: 0.00 – 0.10; 0.20 – 1.00; 1.10 – 2.50; 2.60 – 4.50; 4.60 – 5.90 and circle size increases with value of Overlap Index.

Regarding the Generalized Additive Model (GAM) used to explore microplastic ingestion per species (microplastics/individual), the deviance explained by the model accounting for sampling point, depth, seafloor plastic abundance and species was of 26.2% (Figure 5.4a). Results revealed that latitude (p-value = 0.00504), longitude (p-value = 0.00225), seafloor plastics (p-value = 0.05286) and species (p-value < 0.001) were significantly related with microplastic ingestion in individuals. Higher amounts of plastics in bottom trawl hauls meant higher ingestion values in sampled individuals (p-value = 0.05286). According to species, *Chelidonichthys cuculus* (p-value < 0.1), *Raja clavata* (p-value < 0.001), *Serranus cabrilla* (p-value < 0.05) and *Spondylosoma cantharus* (p-value < 0.001) showed higher significant ingestion values compared to the other analyzed species (Table 5.2).

When exploring the response of the standardized ingestion for all species as a whole in each haul according to sampling point, depth and seafloor plastics, a positive relation was seen between the standardized microplastic ingestion and seafloor plastics (R^2= 0.09, 19.2% of the deviation explained; Fig. 5.4b). However, when comparing microplastic ingestion with seafloor plastics in the same haul, those bottom trawl hauls with higher amounts of seafloor plastics also showed a slightly higher standardized ingestion value of microplastics for all species as a whole (p-value = 0.0734). Finally, when analyzing the response of the Overlap Index for each haul according to sampling point and depth, GAM results suggested a weak correlation between the Overlap Index and depth (R^2=0.11, 20.5% deviance explained, p-value = 0.0635; Fig 5.4c) (Table 5.2).

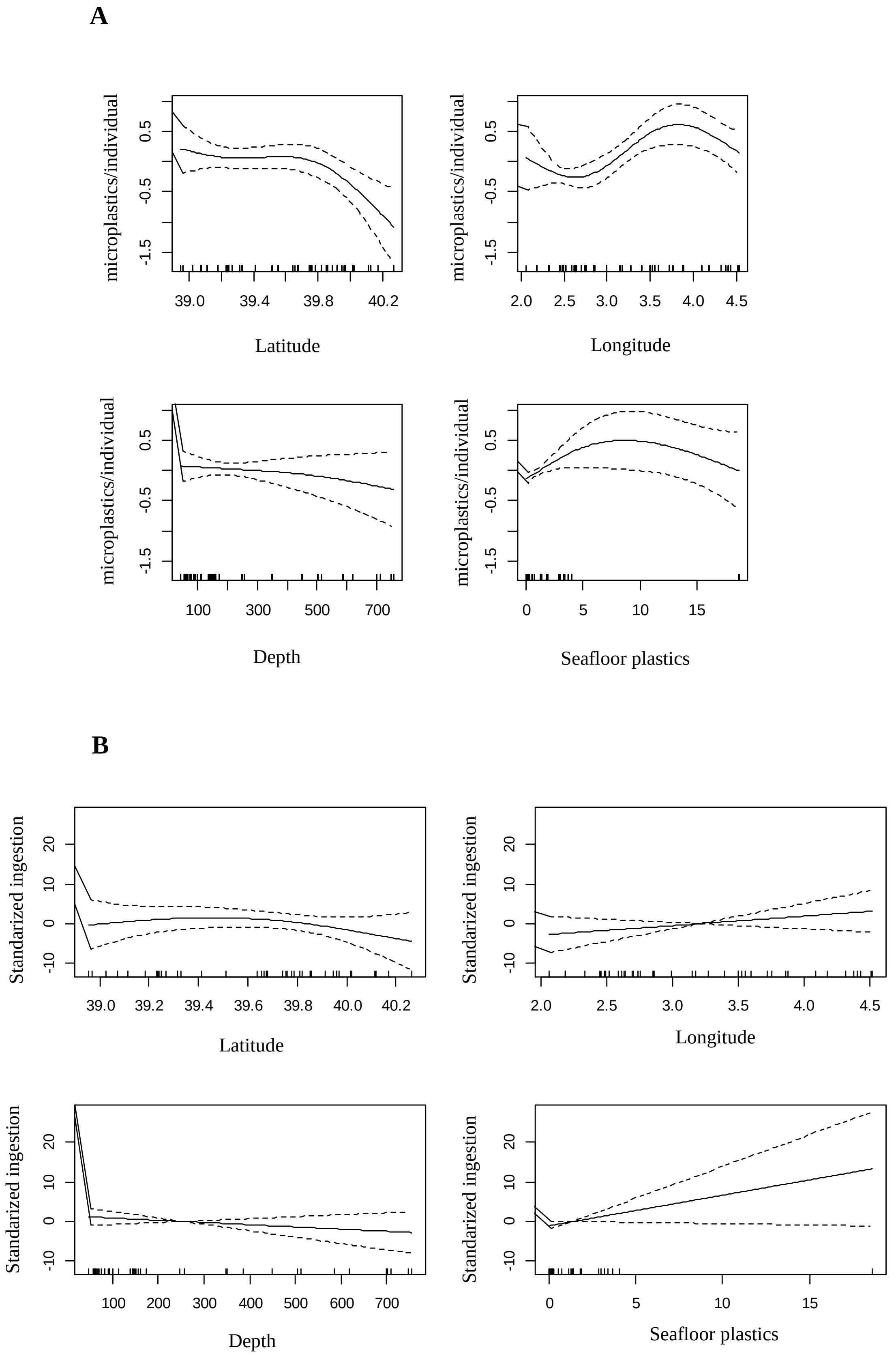

Fig. 5.4 Graphs for the best significant Generalized Additive Models (GAM) for the response of microplastic ingestion in species (microplastics/individuals) (A), standardized ingestion (B)...(*continues*)

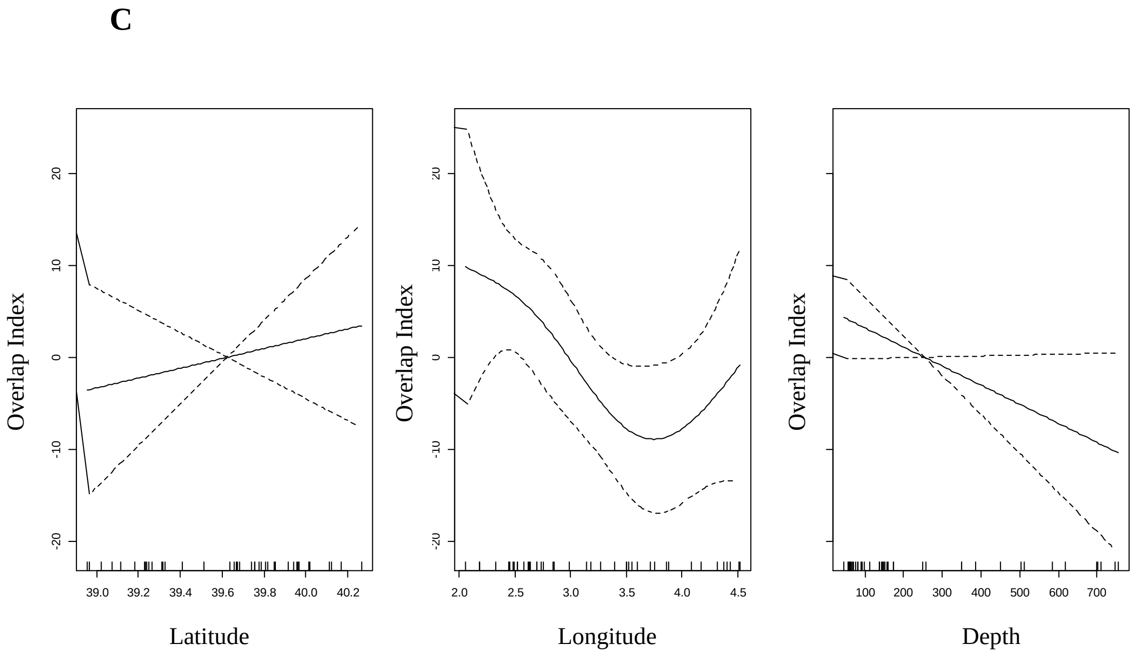

Fig. 5.4 *cont…* and the Overlap Index (C) according to different variables of study: latitude, longitude, species, seafloor plastics and depth.

To produce predictive maps for microplastic ingestion for all species as a whole, seafloor plastics and the Overlap Index, interpolation methods were applied. This permits to identify the uncertainty of the interpolation and ordinary Kriging was identified as the best modeling approach for each of the interpolations with the lowest mean predictions for mean ingestion (0.010), seafloor plastic (0.040) and the Overlap Index (0.074) and except for microplastic ingestion, with Root-Mean-Square Error value close to 1 (Table 5.3). For the ingestion interpolation model for each haul, predictions were highest between the south coast of Menorca and the east coast of Mallorca with prediction values ranging from 0.53 to 0.82 microplastics/individual (Fig. 5.5a) and with a prediction error ranging from 0.28 and 0.32 microplastics/individual (Fig. 5.5b).

The interpolative predictions for seafloor plastics identified higher concentrations of seafloor plastics along the northwestern coast and along the southwestern coast of Mallorca with predicted values ranging from 1.8-3.2 kg/km^2. Lower overall values of seafloor plastics were found surrounding Menorca (predicted values ranging 0.12-0.52 kg/km^2) (Fig. 5.5c).

According to the predictions for the Overlap Index, this was higher along the Mallorca Channel with values ranging from 1.80-2.80 kg/km^2 (Fig. 5.5e). The lowest Overlap Index predictions were found off the coast of Ciutadella, on the island of Menorca, with values ranging from 0-0.22 (Fig. 5.5e). The range of prediction errors for the Overlap Index was smaller than for all the other models (0.36–0.60) (Fig. 5.5f). Overall, prediction errors of the Overlap Index increase with increased distance from the coastline, which is also the case for the interpolation models for seafloor plastics (Fig. 5.5d,f).

	Latitude	Longitude	Depth	Seafloor plastics	Species	GCV	R^2	DE (%)
Microlastic ingestion in species	**	**	n.s	*	***	0.81125	0.19	26.2
Standarized plastic ingestion	n.s	n.s	n.s	+		66.345	0.09	19.2
Overlap Index	n.s	n.s	+	-	-	247.68	0.11	20.5

Table 5.2 Results of generalized additive models (GAMs) applied for the variables: microplastic ingestion in species (microplastics/individual), standardized ingestion of all species for each bottom trawl haul and the overlap index calculated for each bottom trawl haul. GCV generalized cross-validation score: R^2= R-squared, DE: deviance explained, *$P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, + $P< 0.1$ and n.s not significant ($P > 0.1$) Parameters not included in the analyses are marked as “-“.

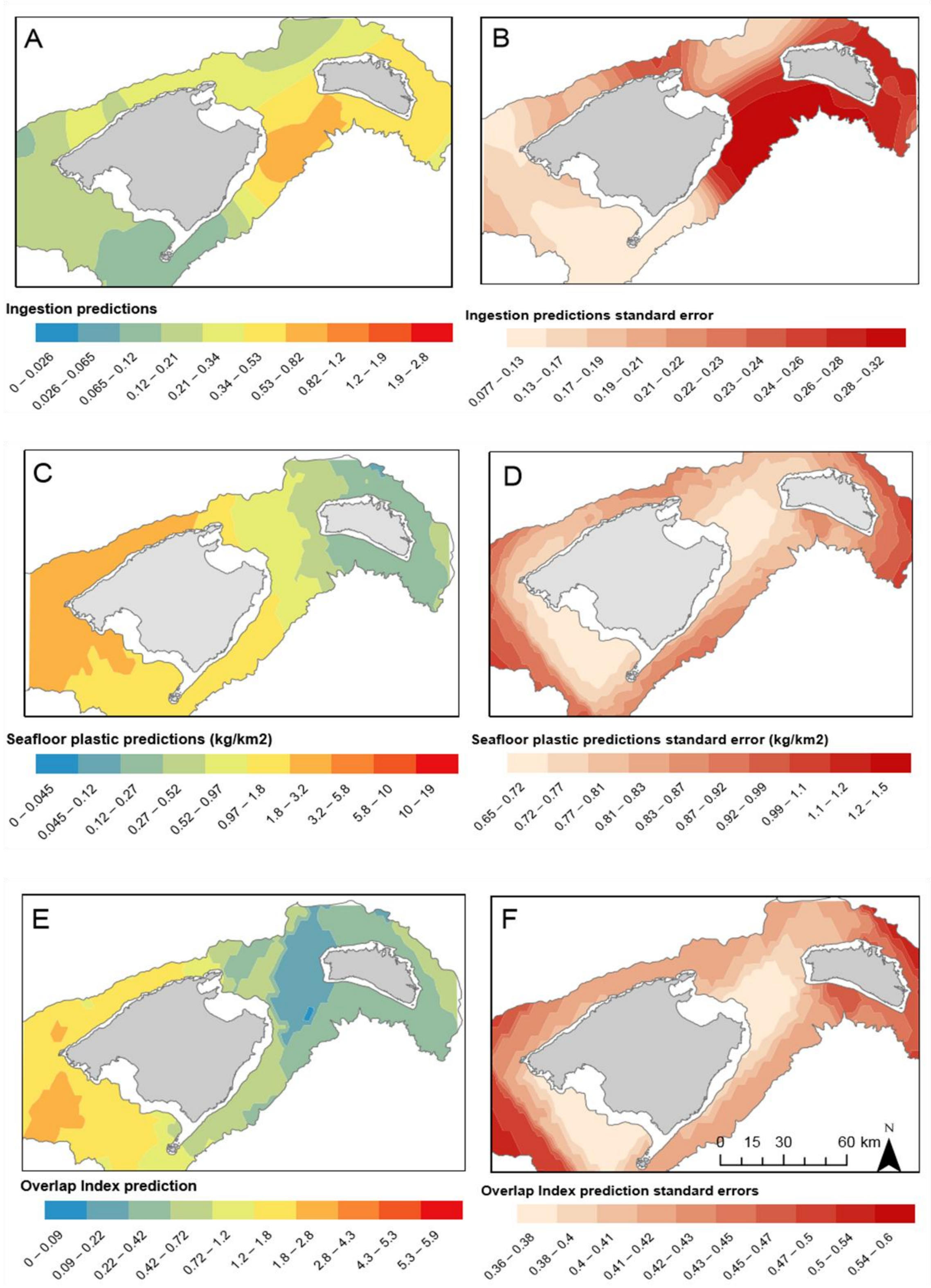

Fig. 5.5 Interpolative predictive maps created using the Kriging function for ingestion of microplastic in species (A) and the corresponding prediction errors (B), for seafloor plastics (C) and the corresponding prediction errors (D) and for the Overlap Index (E) and its prediction errors (F).

	Ordinary Kriging Prediction mean	Root-mean-square error	Inverse Distance Weighting Prediction mean	Root-mean-square error
Ingestion items/individual	0.010	0.566	0.011	0.546
Plastic kg/km^2	0.004	1.027	0.007	1.391
Overlap Index	0.074	1.556	0.120	1.771

Table 5.3 Results from the interpolation modeling approaches for ingestion, seafloor plastics and the Overlap Index: Ordinary Kriging and Inverse Distance Weighting.

5.4 Discussion

This scientific research has assessed microplastic ingestion in a wide range of species (40 species including osteichthyes, elasmobranchs, crustaceans and cephalopods) as well as quantifying seafloor plastics in the same sampling area. Microplastic ingestion in individuals was related to sampling point, seafloor plastics and species and highest ingestion values were observed on the eastern part of Mallorca and around Menorca Island. However, a higher Overlap Index between microplastic ingestion in species and seafloor plastics is predicted along the west coast of Mallorca and in the Mallorca Channel. Additionally, this Overlap Index increases with depth, possibly indicating that plastic pollution is more dependent on depth than spatial coverage as it has already been suggested by other authors (Consoli et al., 2018a,b; Olguner et al., 2018; Pierdomenico et al., 2019).

According to this study, 15% of the sampled individuals ingested microplastics with a mean value of 0.30 ± 0.40 microplastics/individuals. This corresponded to 45% of the sampled species (18 species) showing microplastic ingestion. Regarding the interpolation models, prediction of microplastic ingestion in species increases in the southern coast of the island of Menorca and the eastern coast of Mallorca. Whereas some of the studied species (*Boops boops* and *Galeus melastomus*) have already been documented to ingest microplastic items in the same study area (Nadal et al., 2016; Alomar and Deudero, 2017), up to our knowledge, this is the first time that microplastic ingestion has been quantified in *Chelidonichthys cuculus*, which shows the fourth highest mean ingestion value (0.75 ± 0.37 microplastics/individual) after *Spondyliosoma cantharus* (2.0 ± 1.09 microplastics/individual), *Raja clavata* (1.73 ± 0.62 microplastics/individual) and *Serranus cabrilla* (1.06 ± 0.17 microplastics/individual). A similar percentage of occurrence and mean ingestion values were observed in the small spotted catshark, *Scyliorhinus canicula* (0.08 ± 0.08 microplastics/individual) and the greater weever *Trachinus draco* (0.07 ± 0.07 microplastics/individual) sampled in the study area, regarding of having different feeding strategies. The small spotted catshark is a well known opportunistic active predator with a diverse prey range (Hunter et al. 2005; Sims et al. 2006) while *T. draco* has a foraging strategy where prey handling time and satiation level is important, waiting for prey encounter and not showing preference for larger prey items (Gill 2003).

The thornback ray (*Raja clavata*) is also an opportunistic predator with high mobility and has shown a higher percentage occurrence (64%) and mean ingestion values (1.73 ± 0.62 microplastics/individual) than *Scylorhinus canicula*. However, microplastic ingestion and occurrence in *S. canicula* from the study area (0.08 ± 0.08 microplastics/individual and 8% occurrence) is lower than in the Spanish Atlantic waters where 15 % of the sampled *S. canicula* ingested microplastics (Bellas et al., 2016). Highest reported ingestion values are observed in two demersal (*Raja clavata* and *Serranus cabrilla*) and a benthopelagic (*Spondyliosoma cantharus*) species, but as already stated by other authors (Markic et al., 2019) there is no relation between ingestion of plastic and habitats. For example, six pelagic species have been studied (*Engraulis encrasicolus*, *Gadiculus argenteus*, *Sardina pilchardus*, *Spicara smaris*, *Trachinus draco* and *Trachurus trachurus*) and half of them showed no ingestion of microplastic regardless that some studies report higher frequency of occurrence of plastic in pelagic species (Markic et al., 2019).

On the other hand, some of the analyzed species such as *Nephrops norvegicus*, *Citharus linguatula* and *Trachurus trachurus* which have also been sampled in other areas as the Clyde Sea, northeastern Ionian Sea and the South Adriatic Sea, respectively (Murray and Cowie 2011; Anastasopoulou et al., 2018), where higher amounts of seafloor plastic have been quantified than in the study area (Strafella et al., 2015; Pasquini et al., 2016), showed lower occurrence or absence of litter ingestion in the Balearic Islands (case of *C. linguatula*). Additionally, 9 % of sampled *Sardina pilchardus* of the north Adriatic Sea and 50 % of sampled sardines in the south Adriatic Sea ingested litter (Anastasopoulou et al., 2018) while in our study 14% of *Sardina pilchardus* ingested microplastic items with a mean value of 0.14 ± 0.14 microplastics/individual. This result is consistent with ingestion rates found in sardines along the Spanish Mediterranean Coast (Compa et al., 2018), where 15% of the sampled individuals ingested a mean value of 0.20 ± 0.23 anthropogenic particles/individual. Moreover, a study comparing ingestion rate in three pelagic species, including *Trachurus trachurus* and a semi-pelagic species (*Boops boops*) along the Spanish Mediterranean coast and Balearic Islands concluded that individuals from the peninsular coast showed higher ingestion occurrence (36%) than those from the Balearic Islands (12%) (Ríos-Fuster, 2019). Given that higher plastics along the peninsula coast have been observed than in the Balearic Islands (García-Rivera et al., 2017; Alomar et al., 2020), this fact could be influencing higher ingestion rates in species from the Spanish mainland coast than those from the Balearic Islands. Consequently, variability in microplastic ingestion within species could be attributed to environmental factors such as geography but also to dissimilarities in diet composition within the same species from different areas. For example, it is known that populations of *A. antennatus* from the Spanish mainland and insular coast (Balearic Islands) have different diets (Cartes, 1994; Cartes et al., 2008*)* and Carreras-Colom et al (2018) attributed natural feeding as the most likely route of microplastic intake in *A. antennatus* from Barcelona (Spanish mainland). In this case, individuals with a higher presence of microplastics had a higher presence of endobentic preys and prey relative diversity, whereas contribution of swimming benthos, hyperbenthic preys, was higher in shrimps without microplastics. The dissimilarities between our results (11% of the sampled individuals ingesting plastics) and those in Carreras-Colom et al (2018) (39% of the sampled individuals ingesting plastics) could be explained by the differences in diet found between mainland and insular populations of *A. antennatus* (Cartes, 1994; Cartes et al., 2008) as higher contribution of zooplankton (an no microplastic ingestion) in diet from shrimps close to the Balearic Islands has been reported (Cartes, 1994; Cartes et al., 2008) which could be linked to the lower occurrence of plastics in shrimps analyzed in our study area in comparison to other areas.

Up to date, only 7.5% of the 6,563 catalogued commercial marine and diadromous fish species have been studied for plastic ingestion (Markic et al., 2019) and here we analyzed microplastic ingestion in some commercially important species such as the European hake, *Merluccius merluccius* and the John Dory, *Zeus faber* but they do not show occurrence of microplastic ingestion. Contrary to our results, ingestion in the bentho-pelagic teleost *Zeus faber* and in the demersal teleost *Merluccius merluccius* has already been reported in the Thyrrenian Sea (mean values of 1.77 ± 2.35 plastics/individual and frequency of occurrence of 51.4%; Bottari et al., 2019) and in the Gulf of Cadiz, (frequency of occurrence of 16.7%; Bellas et al., 2016), respectively. However, in agreement with our results no plastic ingestion was observed in the gastrointestinal tracts of the silver hake, (*Merluccius bilinearis*) from Canadian waters even though an elevated number of individuals were analyzed (134) (Liboiron et al., 2018). When selecting indicator species, it is interesting to note that none of the *Phycis blennoides* (5 individuals) of our study ingest microplastic items and Cartes et al. (2016) examined the diet of large fish in the Balearic Basin and also did not find any type of antropogenic particles in *Phycis blennoides*. Moreover, out of a larger sample size (46 individuals) Anastopolou et al. (2013) did not observe litter in the guts of this species from deep-sea waters of the Eastern Ionian Sea. Thus, according to these studies, it seems that *Phycis blennoides* is less affected by plastic pollution as other species from the same sampling area have shown ingestion of plastics while *P. blennoides* has not.

Given the observed high variability in plastic ingestion rates within species and studies, notice should be taken regarding sample size of each studied species. For example, from our set of data, except for *Trachurus picturatus*, all species with a sample size of 5 individuals showed no ingestion of microplastic items (Table 5.1). In this sense, 7 individuals was the minimum samples size for detecting microplastic ingestion. Some protocols have already recommended a minimum sample size of 30 individuals (Anastasopolou and Mytilineou, 2015) while other authors establish this size in 10 specimens per assessment (Markic et al., 2019). In our study area, none of the 40 *Trachurus trachurus* analyzed ingested microplastics while ten specimens of *Helicolenus dactylopterus* showed an occurrence of 10% of ingestion with a mean value of 0.20 ± 0.20 microplastics/individual. Consequently, in our case, it could be thought that a higher sample size does not implicitly mean a higher microplastic ingestion rate nor occurrence but also sample size could be reducing the confidence interval when estimating the number of microplastic ingested in species thus caution must be taken when comparing and giving conclusions from results.

Species and their locations should not be considered in isolation, instead studies should approach multiple species occupying the same areas at the same time (Compa et al., 2019) implying that plastic should be quantified both inside species and in their living area. According to results from this study, seafloor plastics were present in 58% of the hauls with species catch showing a mean value of 1.31 ± 0.09 kg of plastics/km^2. Higher quantities of plastics were quantified in the west of Mallorca and more than 0.52 kg/km^2 of seafloor plastics are predicted around the Island of Mallorca whereas lower estimate values are predicted in Menorca (0.52 kg/km^2) which is a recognized as a Biosphere Reserve by the UNESCO (8th of October 1993), including the marine environment since June 2019. Moreover, highest predicted values of seafloor plastics are given along the Tramuntana coast of Mallorca (northwestern coast), which is influenced by the Northern Current, and within Palma Bay and Andratx. The Northern Current, which flows from the Gulf of Lion through large metropolitan areas as Marseille and Barcelona, to the Balearic Islands, can influence the amount of seafloor plastics reaching this area (Alomar et al., 2020). Plastics have mostly a terrestrial origin (Jembeck et al., 2015) and highest seafloor plastic quantities in Palma Bay could be related to the fact that this area hosts the mayor city and port of the Balearic Islands and recent back tracking modeling suggest that most plastic particles in this area come from anthropogenic activities within the bay and not from more distant areas (Compa et al., 2020). Thus, probably linked to the surrounding human pressures and water circulation, areas close to Marine Protected Areas (Dragonera and Cabrera) and marine Reserves (in Palma Bay) show predicted seafloor plastic values nearly twice as much as those surrounding seafloors of less anthropogenized areas as Menorca.

When looking at the twofold approach relating microplastic ingestion in species and seafloor plastics, results from this study suggest a positive correlation between these two indicators, 10.1.2 (amount of litter in the marine and coastal environments) and 10.2.1 (impacts of litter on marine life), of the MSFD. In this sense, species caught in hauls with a higher amount of plastics also showed higher ingestion values. It is known that biota represents a reservoir for microplastic mass estimates and the identification of plastics in stomach contents indirectly reflect the presence of this pollutant in the marine environment (Van Sebille et al., 2015). Moreover, studying plastic ingestion in species which feed on the seafloor or close to it is important as plastic loads in water samples may not increase over time due to loss through fragmentation or biofouling but trends in sediment samples increase (Markic et al., 2019). Therefore, results from this study are probably indicating that the species from the sampling area might be reflecting plastic pollution in the same area, being suitable plastic pollution bioindicators. In this study, species living far from the coast have been documented to ingest microplastics and a recent study identifies coastal species as being at a higher risk of ingesting plastic than those from open areas (Compa et al., 2019). Data from our study was obtained at a maximum distance of 18 nm from the coast and in 40% of the bottom trawl hauls both microplastic ingestion in species and seafloor plastics have been quantified indicating the ubiquity of plastic pollution and affectation in areas far away from urban development. According to this Overlap Index, the coast off Menorca (especially outside Ciutadella) and the eastern coast of Mallorca (which was the most affected in terms of species microplastic ingestion) are the lowest impacted areas by plastic pollution. Consequently, even though predictions of the Overlap Index do indicate areas more susceptible to plastic pollution, the combination of both indicators (seafloor plastics and microplastic ingestion in species) reduces the identified exposure areas to this type of pollution in contrast to when both indicators are analyzed independently. However, an increase of the Overlap Index is observed with sampling depth, suggesting that deeper areas are more exposed to plastic pollution which is not surprising as higher abundances of litter have been reported in deep areas (Tubau et al., 2015) but further research is needed. Moreover, this outcome is of ecological interest as in the marine environment, depth is the main factor structuring megafaunal assemblages (Ramón et al., 2014). Furthermore, overlap predicted values are higher along the Tramuntana coast and Palma Bay including surrounding areas of Marine Protected areas of Sa Dragonera and Cabrera which could be having further ecological and biological implications as the productivity and diversity of suprabenthos in this area is described as high (Cartes et al., 2011).

Up to our knowledge, this is the first assessment in studying the overlap between seafloor plastics and microplastic ingestion in species in the Mediterranean region but there are other studies investigating this overlap regarding cetaceans and marine reptiles in the Mediterranean pelagic environment. For example, Fossi et al. (2017) showed a clear overlap between pelagic areas with high densities of microplastics and the feeding grounds of fin whales in the Ligurian and Sardinia Sea, in the Mediterranean northwestern region, indicating that these marine mammals are subjected to a high level of microplastic intake while feeding in these areas. Given that mesoscale oceanic dynamics generating upwelling (divergence) and downwelling (convergence), are likely to drive both the concentration of floating particles and the presence of favorable feeding habitats for several species (D'Amico et al., 2003), it is not surprising that feeding habitats of some pelagic species overlap with accumulation areas of plastics when this overlap is assessed from simultaneous visual surveys for floating litter and cetaceans. Contrary to what it is observed in this pelagic zone, when assessing seafloor plastic and turtle density distribution, Carman et al. (2014) did not find a statistically significant association between seafloor plastics and turtle distribution and authors attributed this absence of association to limitations in the data set, as data on plastics and turtles were not obtained in the frame of the same sampling design highlighting the need to carry out evaluations with a spatial analysis approach (Carman et al., 2014), which is the case of our study.

Finally, from results of our research we can conclude that plastic is present from the continental shelf and upper slope of the western Mediterranean Sea as well as being available for marine species. Species with different biology and ecology are ingesting microplastics with a high variability within and amongst species and ingestion values in the study area are lower than in other geographical locations where higher amounts of environmental plastics have been quantified. Moreover, predicted values for seafloor plastics and the Overlap Index suggest that areas surrounding Mallorca are more susceptible to plastic pollution than areas surrounding Menorca, which is integrally a Biosphere Reserve by the UNESCO. With this study we provide quantitative data as well as predicted values which can help defining marine litter threshold values and indices for marine conservation in protected and non-protected areas which can be available for different stakeholders involved in the promotion and maintenance of good environmental status of marine ecosystems.

5.5 Acknowledgements

M. Compa is the recipient of an FPI Fellowship from Conselleria d'Innovació, Recerca I Turisme of the regional Government of the Balearic Islands co-financed by the European Social Fund as part of the FSE 2014–2020 operational program. The MEDITS surveys has been co-funded by the Spanish Institute of Oceanography (IEO) and the European Union through the European Maritime and Fisheries Fund (EMFF) within the National Program of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy. The authors wish to thank the crew and scientific team participating in the surveys. Acknowledgements to the EU-funded Interreg Med project: Plastic Busters MPAs: preserving biodiversity from plastics in Mediterranean Marine Protected Areas, co-financed by the European Regional Development Fund. Authors want to acknowledge the valuable contributions done by anonymous reviewers.

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Environmental Pollution 223 (2017) 223-229

Chapter 6: Evidence of microplastic ingestion in the shark *Galeus melastomus* Rafinesque, 1810 in the continental shelf off the western Mediterranean Sea

Carme Alomar, Salud Deudero

Abstract

Microplastic (<5 mm) ingestion has been recorded in *Galeus melastomus*, the blackmouth catshark, around the Balearic Islands. In total 125 individuals were analyzed for microplastic ingestion. Results have shown that 16.80% of the specimens had ingested a mean value of 0.34 ± 0.07 microplastics/individual. Stomach fullness index ranged from 0.86 to 38.89% and regression analyses showed that fuller stomachs contained more microplastics. A higher quantity of filament type microplastics were identified compared to granular or hard plastic type. No significant differences were given between ingestion values of two locations over the continental shelf providing further evidence of the ubiquitous distribution of microplastics. The findings in this study reflect the availability of this man made contaminant to marine species in seafloor habitats. Based on results from this study, data on microplastic ingestion could be used to study trends in the amount and composition of litter ingested by marine animals in accordance with descriptor 10 of the Marine Strategy Framework Directive.

Keywords: Elasmobranchs, Stomach fullness index, Marine litter, Seafloor, Balearic Islands

6.1 Introduction

Plastic pollution in the Mediterranean Sea has been documented (Pham et al., 2014) and recent studies have demonstrated that plastic is ingested by both pelagic and benthic fish species (Neves et al., 2015; Romeo et al., 2015; Lusher et al., 2013). The Balearic Islands, in the western Mediterranean Sea, are highly exposed to plastic pollution (Deudero and Alomar, 2015) and microplastic (<5 mm) concentrations have already been detected in coastal shallow waters (Alomar et al., 2016), deep sea areas (Woodall et al., 2014) and surface waters (Faure et al., 2015).

In addition, semipelagic fish from the Balearic Islands ingest microplastic filaments (Nadal et al., 2016) providing further evidence of the transfer of microplastics from the marine environment to biota. Microplastics have the potential to cause mortality, reduction of feeding activity, inhibited growth and development, endocrine disruption, energy disturbance, oxidative stress, immunity, neurotransmission dysfunction and even genotoxicity (Wright et al., 2013; Rochman et al., 2014; Avio et al., 2015). Physiological effects produced by microplastic ingestion, such as inflammation and lipid accumulations in liver as well as induction of oxidative stress have already been detected in zebrafish (Lu et al., 2016). Therefore, concerning scientific observations of physiological effects derived from microplastic ingestion, consequences for the marine food web are expected.

Amongst benthic fish species, the blackmouth catshark *Galeus melastomus* Rafinesque, 1810 has been recorded to ingest plastic debris in deep-waters from the Ionian Sea (Anastasopoulou et al., 2013). This species is considered a trophic generalist, preying on shrimps, cephalopods, fishes and euphausiids (Carrasson et al., 1992; Olaso et al., 1998; Fanelli et al., 2009; Valls et al., 2011). *Galeus melastomus* is an important bycatch species in demersal and longline fisheries and is usually discarded in the traditional deep trawl fishery for rose shrimp, *Aristeus antennatus* (Tursi et al., 1993; Torres et al., 2001). Research surveys indicate that it is an abundant demersal species (Valls et al., 2011) and the most abundant shark on the upper and middle slopes to depths of about 1400 m living in muddy bottoms (Baino et al., 2001; Rey et al., 2004). Therefore, assessing vulnerability of this elasmobranch to marine litter is essential and data on ingestion of microplastics could provide a snapshot of the availability of this contaminant in the marine environment at a wide spatial scale. Consequently, this data could be useful to assess "Trends in the amount and composition of litter ingested by marine animals" giving response to indicator 10.2.1 of descriptor 10 in the European Marine Strategy Framework Directive (MSFD Directive, 2008/56/EC).

Given that the Mediterranean Sea is highly exposed to plastic concentrations (Deudero and Alomar, 2015) and given the scarcity on data on microplastic ingestion in elasmobranchs but the pivotal role which chondrichthyans play in the structure of marine food webs worldwide (Albo-Puigserver et al., 2015) this research aims at: 1) Quantify microplastic ingested by the elasmobranch *Galeus melastomus* at two locations over the continental shelf with expected loads of microplastic inputs and 2) Explore trends of microplastic ingestion according to the percentage stomach fullness index of *Galeus melastomus*

6.2 Materials and methods

6.2.1 Sampling and visual sorting of stomach contents

A total of 125 *Galeus melastomus* were sampled in two locations around Mallorca Island to study microplastic ingestion by elasmobranchs (Fig.6.1). A total of 81 individuals were collected in Palma and 44 individuals in Soller over the continental shelf at approximately 600 m depth. Samples were provided from the bycatch of commercial trawling fishing vessels which had a 40 mm minimum mesh size net designed to capture demersal species (DOL, n1967, 21 December 2006). After ships arrived at seaports, samples were frozen and stored in bags at -18ºC until further laboratory procedures. In addition, individuals of other chondrichthyes species: *Scyliorhinus canicula* (5 individuals), *Chimaera monstrosa* (2 individuals) and *Etmopterus spinax* (8 individuals) were also sampled upon availability.

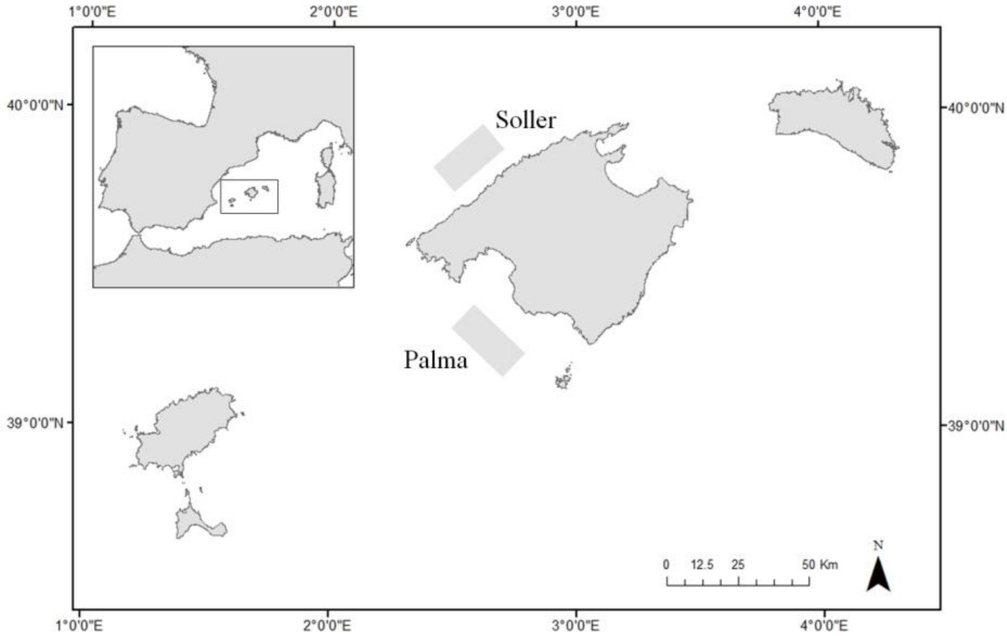

Fig. 6.1 Sampling locations (grey boxes: Soller and Palma) of elasmobranchs around Mallorca Island (Balearic Islands, Western Mediterranean) for assessment of microplastic ingestion.

Once at the laboratory, the samples were individually thawed at room temperature and biological parameters were recorded: total length (TL mm), fresh weight (FW), sex was recorded and classified into female, male and immature (i.e. individuals smaller than 140 mm with no defined sex). A percentage stomach fullness index was assigned to each individual from 0% (empty stomachs) to 100% (full stomachs). For microplastic identification, stomachs were removed and directly dissected following previous procedures (Nadal et al., 2016). Stomach contents were analysed in laboratory using a stereomicroscope (Euromex NZ 1903-S) with a CMEX 3.0 MP camera attached to it which included a special calibration software, ImageFocus® 4.0. (Euromex software). Optical enhancement from 6.7x to 40.5x was applied and microplastics were identified and stored in glass vials with deionized water. Glass vials were then send to the University of Eastern Finland (SIB Labs) for polymer identification. Particles in each glass vial were photographed with Leica EZ4 stereomicroscope with a HD camera and analyzed with Imaging Fourier Transform Infrared (FTIR) spectroscopy (30 scans, 4000-700 cm^{-1} PerkinElmer Spectrum Spotlight 300). Commercial and custom-made spectral databases were utilized for microplastic identification. Type of microplastics identified was classified into fragments, filaments and films following the Marine Strategy Framework Directive technical subgroup on marine litter (Galgani et al., 2013) and colour of microplastics was also recorded. For each studied specimen the number of microplastics ingested per gram of fresh weight of *Galeus melastomus* (MPs/g FW) considering all stomachs (full and empty) was provided. The approximate extraction and sorting time for microplastic identification was of twenty minutes per sample and depending on the fullness of the stomach it could increase to one hour per individual.

Measures to avoid (airborne) contamination were adopted while handling and processing samples (Alomar et al., 2016). During the sorting procedure at the laboratory, two glass Petri dishes were placed at both side of the stereomicroscope and checked for microplastics before and after each sample. If a filament, granular or film man made structure was detected, type and colour of these was recorded following the same criterion as for microplastic identification in elasmobranchs species.

6.2.2 Statistical analysis

A permutational multivariate analysis of variance (PERMANOVA) was applied to test for significant differences in microplastic ingestion per g FW of *Galeus melastomus* according to sampling locations and sex. The experimental design incorporated the two factors: ‘location’ (fixed) with two levels: Soller and Palma and ‘sex’ (random) with three levels: female, male and immature. The variable microplastic ingestion per g FW was transformed using the fourth root, and the resemblance matrix based on the Euclidean distance was calculated.

A non-parametric multidimensional scaling (MDS) was applied as the ordination method for exploring relationships between MPs/g FW and the percentage stomach fullness index of *Galeus melastomus*. The same transformed data and resemblance matrix (fourth root and Euclidean distance resemblance matrix) as in the PERMANOVA analyses were used. The factor percentage stomach fullness index was added to the two-dimensional space. All statistical analyses were performed using Primer V6 and the add-on package PERMANOVA+ (Anderson et al., 2008).

Regression analyses applying Pearson correlation were conducted to analyse the correlation between MPs/g FW and the percentage stomach fullness index and the number of microplastic ingested and total length (mm) of *Galeus melastomus* with the statistical package SPSS.

6.3 Results

In total 125 individuals of *Galeus melastomus* were analyzed for microplastic ingestion. Total elasmobranch length ranged from 115 to 560 mm, fresh weight from 5.07 to 399.78 g and sex ratio male: female:immature was 1:0.58:0.11. Percentage stomach fullness index ranged from 0.86 to 38.89%. Table 6.1 shows mean biological parameters recorded for *Galeus melastomus* in our study.

In Soller, 18.18% of the sampled *Galeus melastomus* (full and empty stomachs) had ingested microplastics with mean values of 0.32 ± 0.11 MPs/ind., whereas in Palma 16.05% of the individuals contained microplastics in their stomachs with mean values of 0.35 ± 0.10 MPs/ind. (Table 6.1). In both locations, maximum values of microplastics reported were 4 microplastics in one individual. No significant correlation was found between size (TL) and number of microplastics ingested per individual ($r = 0.001$ $p > 0.05$).

	n	TL (mm)	FW (g)	Sex ratio (M:F:I)	Stomach Fullness Index (%)	%MPs (n)	MPs/ind.
Palma	81	322.47 ± 9.33	104.47 ± 7.76	1 : 0.42 : 0	12.23 ± 0.46	16.05 (13)	0.35 ± 0.10
Soller	44	257 ± 13.44	65.47 ± 11.55	1 : 1.12 : 0.47	17.48 ± 2.64	18.18 (8)	0.32 ± 0.11
Total	125	299.42 ± 8.14	90.74 ± 6.65	1 : 0.58 : 0.11	14.08 ± 0.51	16.80 (21)	0.34 ± 0.07

Table 6.1 Biological data recorded for *Galeus melastomus* along with microplastic ingestion. n= number of individuals sampled per location and in total; mean total length TL (mm); mean fresh weight FW (g); sex ratio (M: males, F: females, I: immature); percentage stomach fullness index (%); percentage of individuals with ingestion of microplastics (%) and mean microplastics ingested per number of individuals (MPs/ind.) with indication of number of individuals showing ingestion in between brackets.

Ingested values of MPs/g FW for *Galeus melastomus* were 0.006 ± 0.002 MPs/g FW in Palma and 0.027 ± 0.013 MPs/g FW in Soller, and there were no significant differences between locations (Table 6.2, PERMANOVA $p > 0.05$, Fig. 6.2). However, significant differences were given in Soller according to sex: immature *Galeus melastomus* in Soller ingested significantly higher quantities of microplastics (0.14 ± 0.06 MPs/g FW) than females (0.002 ± 0.001 MPs/g FW) and males (0.001 ± 0.002 MPs/g FW) (Table 6.2, PERMANOVA $p < 0.001$). In Palma, only females and males were caught, showing ingestion values of 0.01 ± 0.006 MPs/g FW and 0.003 ± 0.002 MPs/g FW, respectively with no statistical significant differences (PERMANOVA $p > 0.05$). Excluding immature elasmobranchs, females showed higher mean values than males but with no significant differences (PERMANOVA $p > 0.05$).

Source of variation	MPs/FW (g)		
	df	MS	Pseudo-F
Location	1	0.021	$209.5^{n.s}$
Sex	2	0.35	12.49**
Location x Sex	1	9.9×10^{-5}	$0.0036^{n.s}$
Residual	120	0.028	
Total	124		

n.s = no significant differences

** $p < 0.001$

Table 6.2 Results of the two-factor multivariate PERMANOVA for microplastics ingestion/FW (g) in *Galeus melastomus*. Factors include location (Soller and Palma) and sex (female, male and immature).

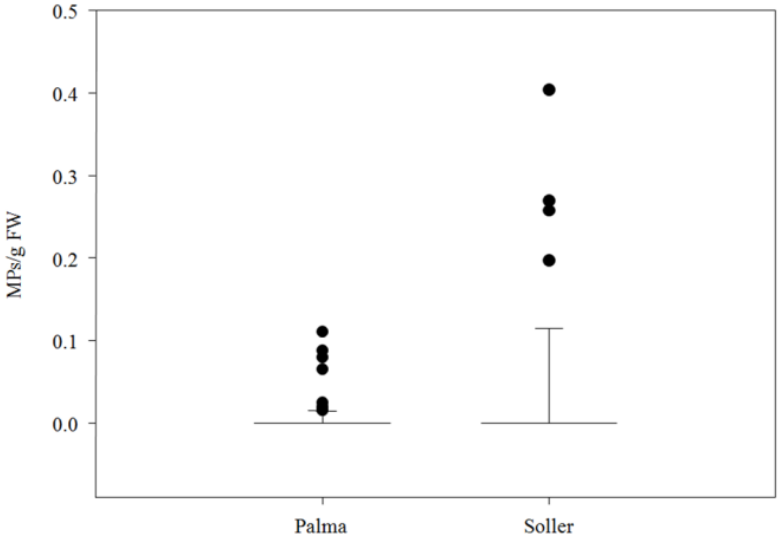

Fig. 6.2 Values of microplastics (MPs) ingested per gram of fresh weight of *Galeus melastomus* at both sampling locations. Horizontal lines represent the third quartile, whiskers maximum values and dots individual microplastic ingestion values.

Regarding microplastic type identified in stomachs of *Galeus melastomus*, 86.36% of the particles were filament type, while 12.12% were fragment and 1.51% film type. Filament microplastics were composed of a diversity of chemical polymers while films were only composed by polypropylene (Table 6.3). According to polymers in the identified microplastics, the most common polymers were cellophane (33.33%) and Polyethylene Terephthalate (PET) (27.27%) (Table 6.4). Microplastics were made up of five different colours being transparent the most abundant identified colour (Fig. 6.3).

	Black	Blue	Red	Transparent	White
Filament (86.36 %)					
Cellophane	22.73	31.82	18.18	27.27	-
Polyacrylonitrile (PAN)	-	100	-	-	-
Polyethylene terephthalate (PET)	11.11	16.67	5.56	66.67	-
Poly(Ethyl Acrylate)	-	-	-	100	-
Polyacrylate	12.50	37.50	12.50	37.50	-
Polyamide (PA)	-	-	50	50	-
Polypropylene (PP)	-	-	-	100	-
Film (1. 51 %)					
Polypropylene (PP)	-	-	-	100	-
Fragment (12.12 %)					25
Polyethylene (PE)	-	-	-	33.33	66.67
Polypropylene (PP)	-	100	-	-	-
Alkyd	-	100	-	-	-

Table 6.3 Characterization of microplastics identified in *Galeus melastomus* classified into type (Filament, Film and Fragment), colour and polymer. Values expressed as percentages.

Microplastic polymer	%
Cellophane	33.33
Polyacrylonitrile (PAN)	4.55
Polyethylene (PE)	4.55
Polyethylene terephthalate (PET)	27.27
Poly(Ethyl Acrylate)	1.52
Polyacrylate	12.12
Polyamide (PA)	3.03
Polypropylene (PP)	12.12
Alkyd	1.52

Table 6.4 Polymers identified with FTIR in microplastics ingested by *Galeus melastomus.*

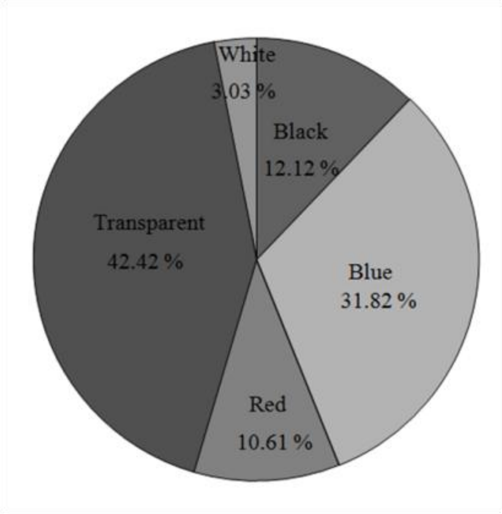

Fig. 6.3 Colour percentage breakdown of microplastics identified in *Galeus melastomus* stomachs.

According to the other three species sampled, ingestion values ranged from 0 MPs/g FW (*Scyliorhinus canicula* and *Chimaera monstrosa*) to 0.01 ± 0.005 MPs/g FW (*Etmopterus spinax*) showing that only half of the *Etmopterus spinax* had ingested microplastics. Only filament type microplastics were identified in *Etmopterus spinax* and no white colours were found in this case. According to polymers identified in the filaments these were as follows: cellophane (37.5%), PET (50%) and polyacrylate (12.5%).

Finally, a total of 10 fibres identified as cotton with FTIR spectroscopy were recorded as airborne contamination while handling and processing samples at the laboratory. These fibres were detected on separate days during 26% of the days that the visual sorting took place under the stereomicroscope.

In general terms, *Galeus melastomus* that exhibited higher percentage stomach fullness index (26-27%) had ingested more microplastics/g FW than individuals with more empty stomachs (Fig. 6.4, MDS (2D stress: 0.01)). The regression analyses showed that MPs/g FW was significantly and positively correlated with the percentage stomach fullness index (r = 0.331 p < 0.01).

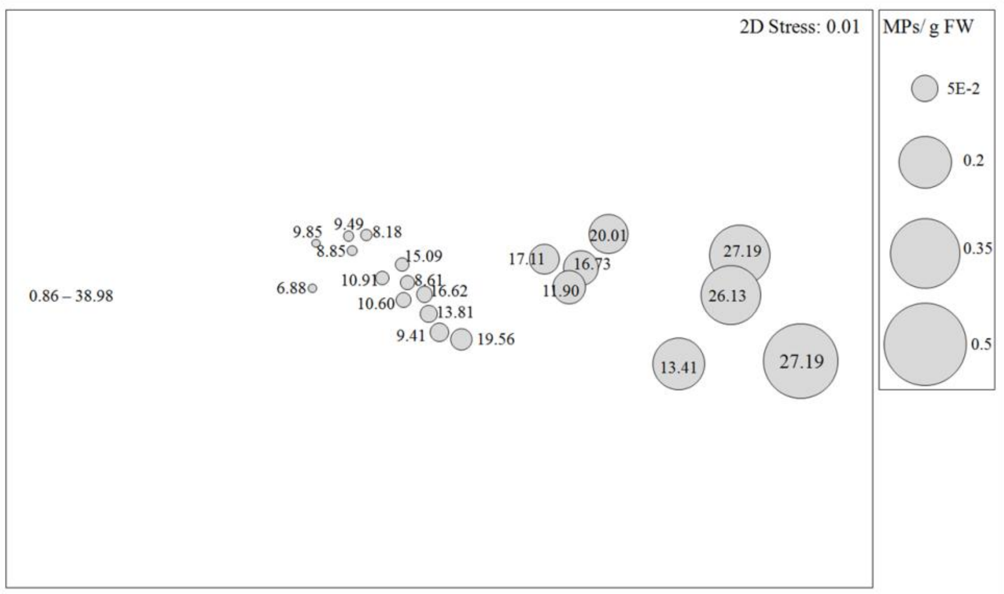

Fig. 6.4 Multidimensional scaling MDS calculated for MPs/g FW of *Galeus melastomus* after transformation with the fourth root function and the Euclidean distance resemblance. Bubbles reflect MPs/g FW and numbers inside them represent stomach fullness index (%) for *Galeus melastomus.*

6.4 Discussion

Microplastics have been identified in 21 of 125 individuals of the blackmouth catshark *Galeus melastomus* in the continental shelf around the Balearic Islands at about 600 m depth. This species is common and abundant in demersal habitats, therefore the relatively high percentage of specimens ingesting microplastics provides further indication that seafloor habitats are a sink for microplastic loads, as previously reported in other studies on marine litter distribution (Galgani et al., 1995; 1996; 2000).

Percentage of microplastic ingestion (16-18%) in our study is slightly higher than those previously reported also for *Galeus melastomus* in the Eastern Ionian Sea; 3.2% (Anastasopoulou et al., 2013) and 12.5% (Madurell, 2003), which included all debris categories. These results are in agreement with the general patterns of availability and large scale distribution of marine debris in the Mediterranean, since litter densities from trawl surveys in continental slopes have reflected higher amounts of litter in the western Mediterranean (4.0 ± 1.8 kg/ha) than those recorded from the central (0.6 ± 0.4 kg/ha) and eastern (1.1 ± 0.3 kg/ha) region (Pham et al., 2014). Also in deep basin regions higher values have been reported in western areas (1.8 ± 1.5 kg/ha) than in central (1.7

± 0.6 kg/ha) and eastern areas (1.2 ± 0.3 kg/ha) of the Mediterranean Sea (Pham et al., 2014).

When comparing to other demersal species, the proportion of *Galeus melastomus* ingesting microplastics in our study is higher than the percentage of *Etmopterus spinax* ingesting plastics in the eastern Mediterranean Sea (Madurell, 2003; 7.8%) and slightly lower than red mullets from Barcelona, western Mediterranean (Bellas et al., 2016; 33%). Preliminary results of microplastic ingestion in our study suggest that *Galeus melastomus* is feeding on higher quantities of microplastics than *Scyliorhinus canicula*, *Chimaera monstrosa* and *Etmopterus spinax* from the same sampling area. All four species were caught in the same fishing hauls suggesting that the blackmouth catshark is more vulnerable to microplastic ingestion although caution on sample size effects must be taken.

A possible explanation for the difference in microplastic ingestion in demersal species could be linked to feeding strategies and traits since previous studies have related them to microplastic ingestion (Anastasopoulou et al., 2013; Romeo et al., 2015). While small individuals of *Galeus melastomus* (150-350 mm TL) feed mainly on cephalopods, medium sized individuals (351-450 mm TL) feed on decapods and crustaceans and large specimens (>451 mm TL) seem to be more generalist-feeders (Fanelli et al., 2009). Elasmobranch size range in our study is rather large (115-560 mm TL) and no significant correlation was found between size and number of microplastics ingested per individual ($r = 0.001$ $p > 0.05$) but significant differences were observed in Soller for immature elasmobranchs which ingested higher quantities of microplastics suggesting a differences in microplastic ingestion with ontogenic changes. Nevertheless, for female and male mature individuals it could be suggested that microplastics are being ingested by *Galeus melastomus* independently of their preferential prey and it could be related to the more generalist feeding strategies of adults. As *Galeus melastomus* with fuller stomachs have ingested more microplastics that might come directly from their food preys (bioaccumulation), the retention time of microplastics in gastrointestinal tracts could be the same as the other ingested preys. Nevertheless, caution must be taken when assessing microplastic retention in gastrointestinal tracts as there is no clear trend of their mechanism once ingested. The uptake of microplastic particles and the transfer of potential harmful substances along with microplastics has been described in literature for a variety of organisms, especially invertebrates but the potential accumulation of very small microplastic particles along food webs has not been investigated so far (Batel et al., 2016).

Galeus melastomus are also ingesting microplastics which are directly available from the seafloor and water column, which are important sources of microplastics, and several authors refer to microplastic fibers as being more abundant in the marine environment than fragments (Claessens et al., 2011; Lusher et al., 2013; Rochman et al., 2015). In the present study a higher quantity of filament type microplastics were identified in the stomach contents of *Galeus melastomus* compared to granular or hard plastic type. This finding contrast previous feeding studies that found large pieces of chelae species such as *Pagurus alatus*, *Geryon longipes* and *Calocaris macandrae* in the stomach contents of *Galeus melastomus* as well as natantian decapods and eupausiids (Fanelli et al., 2009) which have a hard exoskeleton resembling more hard plastics rather than filament type microplastic. In addition, Anastasopoulou et al. (2013) also found hard plastic in *Galeus melastomus* which has been rarely found in our study. Consequently, further research is needed to set up the uptake procedures in microplastic ingestion by *Galeus melastomus* and explain the higher quantities of miscroplastic filaments in individuals from the western Mediterranean area.

According to chemical characterization of microplastics, cellophane followed by polyethylene terephthalate (PET) were the most predominant types of polymers. Cellophane is used in food packaging and cigarette wrappers as well as a release agent in the manufacture of fiberglass and rubber products and is often used as coatings combined with synthetic polymers (Yang et al., 2015). Cellophane packages can be discarded into the marine environment where they degraded into smaller particles being available for organisms. It is known that plastics with a density that exceeds that of seawater (1.02 g/cm^3) sink and accumulate in the sediment (Woodall et al., 2015). Thus cellophane and bottles composed of PET which have a high density (1.44-1.51 g/cm^3 and 1.38 g/cm^3 respectively) once in the environment can break down and microplastics can sink into sediments being available for species living in these areas such as *Galeus melastomus*. In addition, low density particles tend to float on the sea surface (Suaria and Aliani, 2014) or in suspension in the water column (Fossi et al., 2012) but through other processes such as density modification due to biofouling, role of currents, water circulation, plastics can be transported through long distances (Eriksen et al., 2014) being available to organisms inhabitating different environments and distant from the source of microplastic inputs.

In this study we have sampled *Galeus melastomus* of two locations over the continental shelf at 600 m depth showing no significant difference in ingestion rates. This study area is constrained to the southwest of Mallorca Island and both sampling locations have similar habitat type characteristics and anthropogenic pressures. By broadening the sampling area to a more regional scale including different regions of the Mediterranean Sea, it could be seen if *Galeus melastomus* still show similar ingestion rates around the Mediterranean basin or significant differences are observed for example between ingestion values of individuals from areas close to large metropolitan areas and specimens from areas less exposed to human pressures. In addition, variability of values is higher in Soller than Palma and it could be related to biological factors rather than abiotic factors since immature *Galeus melastomus* ingest higher quantities of microplastics (an order of magnitude higher than males and females) spreading the dispersion of results obtained in Soller. Higher ingestion values in immature specimens could have cross effects since maturity stage might have an effect on the ingestion rates of microplastics, as smaller individuals need more energy for growth which could indirectly mean a higher intake of microplastics along with other preys.

Elasmobranch samples in our study have been provided by commercial trawling boats that have been operating at the same traditional fishing grounds for species such as *Mullus surmuletus*, *Merluccius merluccius*, *Nephrops norvegicus* and *Aristeus antennatus* (Palmer et al., 2009). Therefore, it could be quite probable that *Galeus melastomus* from both locations are receiving a similar degree of anthropogenic pressure, thus explaining nearly the same mean ingested values per individual (Palma 0.35 ± 0.10 MPs/ind. and Soller 0.32 ± 0.11 MPs/ind.). However, this also means that these individuals are more exposed to microplastic loads and reported ingestion values could be higher than those expected in non-commercial fishing areas. Nevertheless, diffusive sources of microplastics and the vital role which fishing activities, oceanographic regimes and river inputs have in marine litter transportation and distribution must be considered when assessing microplastic distribution and effects in marine species (Galgani et al., 2000; Law et al., 2010; Woodall et al., 2014).

It is known that biota represents a reservoir for microplastic mass estimates (Van Sebille et al., 2015) and the identification of microplastics in stomach contents indirectly reflects the presence of this pollutant in the marine environment. Knowing that in the western Mediterranean, *Galeus melastomus* is the most abundant demersal shark found up to 1400 m depths (Moranta et al., 1998; Rey et al., 2004), it is a common by-catch species and usually discarded (Tursi et al., 1993; Torres et al., 2001) and has been documented to ingest microplastics it fosters knowledge to use this type of data in accordance with the Marine Strategy Framework Directive (MFSD). Data on microplastic ingestion by marine biota should be available for interoperable use in all MFSD marine regions of European Seas (Galgani et al., 2013) and therefore development of common indicators for marine litter assessment is a real need and urge. Future work should be addressed in determining appropriate key species, at least at a sea regional level, and establishing a minimum sample size enabling a proper quantification of marine litter distribution, patterns, its potential risks and ecological effects at species level without compromising selected species populations.

6.5 Acknowledgements

This study was partially funded by Ajuts per dur a terme Accions Especials de Recerca i Desenvolupament 2015-2016 del Govern de les Illes Balears, Ref: 17/2015. We thank A. Frank for assistance during the laboratory work and M. Compa for providing Fig. 6.1.

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Chapter 7: Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress

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Abstract

A total of 417 striped red mullet, *Mullus surmuletus*, were analyzed to study microplastic ingestion and livers of fish were assessed to study effects of microplastics. Nearly one third (27.30%) of the individuals were quantified to ingest microplastics although there was no evidence of oxidative stress or cellular damage in the liver of fish which had ingested microplastics. A small increase in the activity of glutathione S-transferase (GST) of *M. surmuletus* was detected which could be suggesting an induction of the detoxification systems but these findings should be tested in laboratory conditions under a controlled diet and known concentration of microplastics. Fish from trammel fisheries, operating closer to land and targeting larger individuals, showed higher mean ingestion values than fish from trawling fisheries, and were related to body size, as microplastics ingested increased with total fish length. Consequently, ingestion values of microplastics were not related to sampling distance from land giving further evidence of the ubiquity of microplastics in the marine environment. Finally, Fourier Transform Infrared Spectroscopy (FTIR) analysis showed that the vast majority of microplastics were filament type and polyethylene terephthalate (PET) was the main identified component.

Keywords: Biomarkers, Oxidative stress, Effects, Mediterranean Sea, FTIR

7.1 Introduction

Despite emerging research on macro- and micro- plastic ingestion by fish species (Deudero and Alomar, 2015; Battaglia et al., 2016; Bellas et al., 2016; Nadal et al., 2016; Rummel et al., 2016), knowledge on physiological effects caused by direct consumption of microplastics in wild fish is limited. Microplastics can be classified into primary microplastics which are intentionally produced at a microscopic scale and to be that size (Costa et al., 2010; Browne, 2015). This category of microplastics includes production pellets/powders and engineered plastic microbeads which are used in cosmetic formulations, cleaning products and industrial abrasives (GESAMP, 2016). On the other hand, secondary microplastics result from the degradation of larger plastics into smaller pieces mainly due to hydrolysis and biodegradation under environmental conditions (weathering, photo-oxidation) (Mathalon and Hill, 2014; Gewert et al., 2015).

Risks derived from microplastics come from the material itself as well as from the chemicals and pollutants sorbed to the surface (Rochman et al., 2013). Metals and persistant organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), dichloro-diphenyltrichloroethane (DDTs) and polycyclic aromatic hydrocarbons (PAHs) can be found amongst the components which are sorbed to microplastics in the environment (Rios et al., 2007; Rochman et al., 2014; Gewert et al., 2015). As plastics are expected to persist in the environment for hundreds or even thousands of years (Barnes et al., 2009), hydrophobic POPs can accumulate with time on the surface of plastics resulting in higher concentrations of these pollutants in plastic surfaces than in the environment (Gewert et al., 2015). In this sense, Mato et al. (2001) detected concentrations of polychlorinated biphenyls (PCBs) on polypropylene pellets 10^6 times higher than in the surrounding water column. Due to the higher surface area to volume ratio of microplastics, these sorb higher concentrations of pollutants than larger plastics (Cole et al., 2011). In addition, chemicals such as bisphenol A, phthalates, nonylphenol and polybrominated diphenyl esters are added to microplastics during manufacturing processes (Rios et al., 2007; Rochman et al., 2014). Additives are added to plastic polymers to improve properties of the final product (Teuten et al., 2009) making them more stable, durable and resistant to degradation (Gewert et al., 2015). Normally, these additives are not bound to polymers and they can leach from the plastic as it degrades once in the environment (Stringer and Johnston, 2001). These chemicals become available for organisms (Rios et al., 2007; Rochman et al., 2014) and can enter cells, chemically interacting with biological molecules and causing endocrine system disruptions (Teuten et al., 2009). Therefore biological consequences such as oestrogenic effects (Sonnenschein and Soto, 1998) and reduction of testosterone production (Foster, 2006) might occur.

Physiological effects have been detected after exposure of fish to polystyrene microplastics as induction of oxidative stress in Danio rerio (Hamilton, 1822) and inflammation and lipid accumulation in the liver were quantified (Lu et al., 2016). Furthermore, exposure experiments have shown that the combination of plastics and sorbed contaminants (PAHs, PCBs and polybrominated diphenyl ethers (PBDEs)) ingested by fish caused liver toxicity and pathology (Rochman et al., 2013). In addition, Pedà et al. (2016) gave evidence of structural and functional deterioration of the intestine of *Dicentrarchus labrax* (Linnaeus, 1758) exposed for 90 days to polyvinyl chloride (PVC) pellets.

At present there are few studies in wild conditions dealing with physiological effects derived from the ingestion of microplastics and associated chemicals. Despite of this, it is known that exposure of marine organisms to certain toxic compounds in the marine environment can induce the overproduction of reactive oxygen species (ROS) leading to oxidative damage to macromolecules of tissues (Sureda et al., 2006). The fish are characterized by a complex antioxidant system which includes enzymes such as superoxide dismutases (SOD) and catalase (CAT) but also the glutathione peroxidase (GPX) and the glutathione reductase (GR). In addition, the enzyme glutathione S-transferase (GST) which is involved in phase II detoxification process, produces a more hydrophilic glutathione conjugate of the toxic compound favouring their elimination

(Sureda et al., 2006). Malondialdehyde (MDA) is an end-product of the oxidative damage to lipids and it is used as a biomarker of the lipid peroxidation. Enzyme activity has been used to study the response of fish to exogenous substances and under several situations when the rate of ROS generation exceeds that of their removal, oxidative stress and increasing oxidative markers will occur (Sureda et al., 2006; Banaee et al., 2013). Thus, activation of enzymes and an increase in MDA levels in fish species exposed to certain toxic compounds is expected and enzymes can be used as biomarkers of environmental stress (SOD, CAT) and detoxification (GST) in aquatic organisms (Karami et al., 2016).

Given the evidence of microplastic ingestion in fish species (Lusher et al., 2013; Romeo et al., 2015, 2016; Bellas et al., 2016; Alomar and Deudero, 2017), physiological effects derived from the ingestion of an exogenous substance such as microplastic are a reality which needs to be investigated. A recent study has shown that the red mullet fish *Mullus barbatus* Linnaeus, 1758 ingested twice as much microplastics than other demersal species in the same study (*Scyliorhinus canicula* (Linnaeus, 1758) and *Merluccius merluccius* (Linnaeus, 1758) (Bellas et al., 2016). Consequently, the congeneric striped red mullet *Mullus surmuletus* Linnaeus, 1758, also a demersal species found in depths ranging from 5 to 400 m, is sensitive to marine debris contamination and microplastic ingestion. Adult habitat includes broken and rough grounds but also sand and soft bottoms (< 100 m), feeding on benthic organisms such as shrimps, amphipods, polychaetes, molluscs and benthic fishes.

The Mediterranean basin is a hotspot for the marine diversity and it is considered a sensitive ecosystem exposed to invasive species, fishing and touristic activities, maritime routes and urbanized coastal areas which can increase marine debris (Galgani et al., 1995). In the Balearic Islands, western Mediterranean, *M. surmuletus* is one of the main target species of the bottom trawling fishery along the continental shelf (50–80 m), where it is found in detrital bottoms of rhodophytic and corallinic algae and it is also an important commercial catch in the deep shelf (125–235 m) (Ordines et al., 2006; Palmer et al., 2009). In addition, it is also caught by the small-scale fishery at shallower waters with gillnet and trammel net (Reñones et al., 1995) where microplastic concentrations have already been quantified (Alomar et al., 2016). Therefore, high detected (Faure et al., 2015) and predicted concentrations (Van sebille et al., 2015) of microplastics in the Mediterranean basin warn of the potential physiological and ecological risks that these man-made particles might have on marine organisms in this region. Consequently, the aims of this research were i) to study the ingestion of microplastics in a highly valued commercial species: *Mullus surmuletus*, ii) to describe the characteristics of microplastics identified in *Mullus surmuletus* and iii) to investigate the enzyme response and cellular oxidative damage in this species associated to the ingestion of microplastics.

7.2 Material and methods

7.2.1 Field sampling

Sampling was conducted during the winter period from November 2014 to April 2015 to assess microplastic ingestion by *M. surmuletus*. In total, 417 individuals were obtained from bottom trawl and trammel fishing vessels operating from five different ports of Mallorca Island: Palma, Port d'Andratx, Port d'Alcúdia, Cala Ratjada and Santanyí, which operate in nearby fishing areas (Fig. 7.1). The only exception was Cala Ratjada, where only samples from bottom trawlers were available. Catches of *M. surmuletus* from the bottom trawl fleet mostly come from hauls carried out between 50 and 100 m depth and those from trammel fishing vessels come from hauls shallower than 50 m depth. Both bottom trawl and trammel fishing vessels spend one day at sea. Individuals which were alive when arrived onboard were selected for biochemical analysis. Each fish was identified; their liver was immediately extracted and frozen with liquid nitrogen and the individuals were preserved in ice until landing. These fish, together with others whose liver was not removed, were analyzed in a fresh condition at the laboratory on arrival at port.

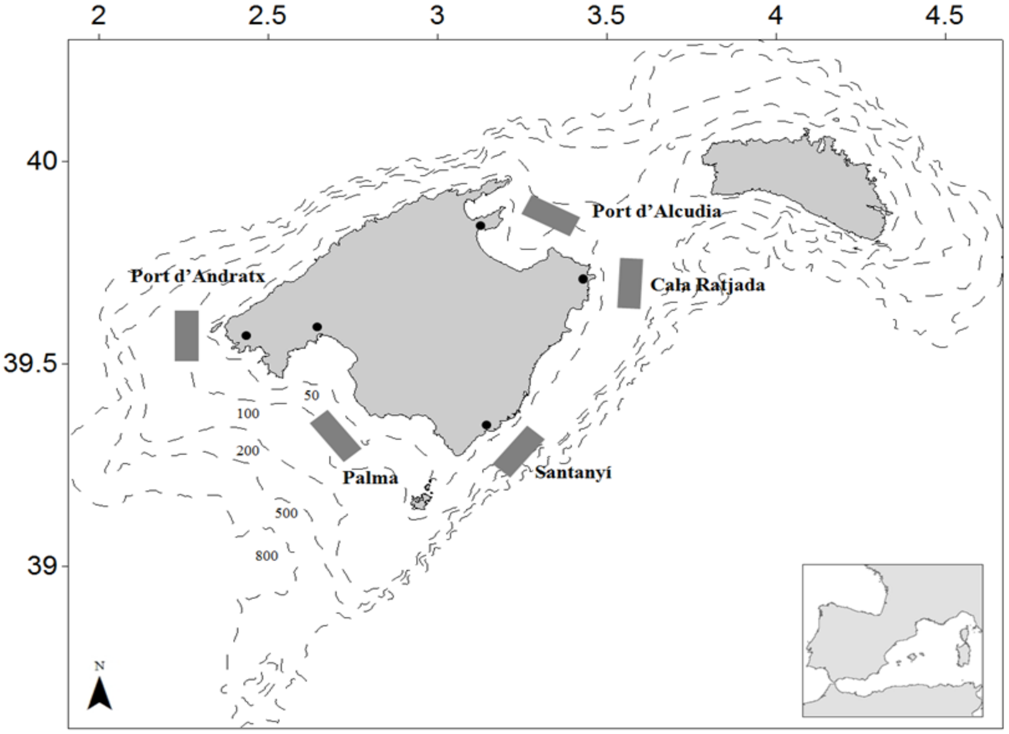

Fig. 7.1 Map of the Balearic Islands showing the sampling areas (shaded rectangles) of *Mullus surmuletus* to study microplastic ingestion and enzyme response and cellular oxidative damage in *Mullus surmuletus* liver regarding the ingestion of microplastics. Dots correspond to the closest port to each sampling area. Isobaths (dotted lines) are drawn for depths 50, 100, 200, 500 and 800 m.

7.2.2 Laboratory analysis

Once at the laboratory, biological parameters of *M. surmuletus* including total length (TL) to the nearest 0.1 cm, fresh weight (FW), gastrointestinal weight (GW) and sex (female (F), male (M), undefined (U)) were recorded (Table 7.1). Gastrointestinal tracts were removed and stored in glass vials with pure undenatured ethanol for further microplastic identification.

Fishing vessels	**TL (cm)**	**FW (g)**	**GW(g)**	**Sex ratio (M:F:U)**
Trawling (302)	18.32 ± 0.22	86.82 ± 3.12	3.55 ± 0.15	120:171:11
Trammel (115)	22.84 ± 0.28	171.15 ± 6.70	8.43 ± 0.47	26:89:0
All vessels (417)	19.57 ± 0.21	110.07 ± 3.50	4.90 ± 0.20	146:260:11

Table 7.1 Biological parameters measured in all individuals of *Mullus surmuletus* of this study. Data is displayed as mean ± standard error (SE) for total length (TL), fresh weight (FW), gastrointestinal weight (GW) and sex ratio. Number of individuals sampled with each type of fishing vessel is expressed in brackets.

7.2.3 Microplastic identification

Microplastic identification in *M. surmuletus* was done by visual sorting of stomach contents under a stereomicroscope (Euromex NZ 1903-S) with optical enhancement from 6.7x to 40.5x and with an attached CMEX 3.0 MP camera using a calibration software, Image Focus® 4.0 (Euromex software). Number, colour and shape of microplastics observed in each stomach was recorded and then stored in glass vials for further polymer verification and identification of a subset of particles at the University of Eastern Finland (SIB Labs). Particles in each glass vial were photographed with Leica EZ4 stereomicroscope with a HD camera and chemically identified with Imaging Fourier Transform Infrared (FTIR) spectroscopy (30 scans, 4000–700 cm^{-1} PerkinElmer Spectrum Spotlight 300). Commercial and custom-made spectral databases were utilized for microplastic identification. The level of certainty to accept polymers was established using a threshold for the spectra identification of 70% of similarity when comparing the spectrum from the sample to the commercial and custom-made spectral databases. Particles were classified into filament or fragment type according to the Marine Strategy Framework Directive technical subgroup on marine litter (Galgani et al., 2013).

Measures to avoid (airborne) contamination were adopted while handling and processing samples in the laboratory (Woodall et al., 2015). During the stomach content analyses procedure, two glass Petri dishes were placed at each side of the stereomicroscope and checked for microplastics before and after each sample. All material used was rinsed twice before use with distilled water and alcohol and all working surfaces were cleaned with alcohol. A 100% cotton laboratory coat was worn at all times during all analysis procedures. The approximate extraction and sorting time for microplastic identification in *M. surmuletus* stomach was of a maximum of 30 min depending on the quantity of digestive content.

7.2.4 Enzyme activities

To analyze microplastic ingestion effect on the antioxidant and detoxification system of *M. surmuletus*, a subset of specimens showing ingestion and not showing ingestion of microplastics were analyzed. Liver samples of 43 *M. surmuletus* with ingestion (n=19) and without ingestion (n = 24) of MPs were homogenized in ten volumes (w/v) of 100 mM Tris-HCL buffer pH 7.5 and homogenates were centrifugated at 9000×g during 10 min at 4 °C (Sureda et al., 2006). After centrifugation, supernatants were collected and immediately used for biochemical analysis. Antioxidant enzyme activities of superoxide dismutases (SOD) and catalase (CAT), detoxification enzyme activity of glutathione Stransferase (GST) and malondialdehyde (MDA) levels were determined on the supernatants. Total protein content was determined in all liver samples by a colorimetric method (Biorad Protein Assay), using bovine serum albumin (BSA) as a standard to normalize all biochemical results.

All enzymatic activities were determined with a Shimadzu UV-2100 spectrophotometer at 25 °C. SOD activity (pKat/mg protein) was determined by the degree of inhibition of the reduction of cytochrome C by the superoxide anion generated by the xanthine oxidase/ hypoxanthine system (McCord and Fridovich, 1969). Enzymatic activity was monitored at 550 nm and calculations were based on an absorption coefficient (ε) of 28.1 mM^{-1} cm^{-1}. Catalase activity (mK/mg protein; $K = s^{-1}$) was determined using the method described by Aebi (1984), based on the decomposition of H_2O_2 in 50 mM phosphate buffer, monitoring the decrease in absorbance at 240 nm. Detoxification enzyme activity of GST (nKat/mg protein) was determined with the method of Habig et al. (1974) using reduced glutathione (GSH) and 1- chloro-2,4-dinitrobenzene (CDNB) as substrates. The activity was recorded at 340 nm with $\varepsilon = mM^{-1}$ cm^{-1}.

7.2.5 MDA determination

MDA levels, as a marker of lipid peroxidation, were analyzed by a colorimetric assay kit for MDA determination based on the reaction of MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm, following the manufacturer's instructions (Sigma-Aldrich, Spain). Briefly, samples or standards were placed in glass tubes containing n-methyl-2-phenyl-indole (10.3 mM) in acetonitrile: methanol (3:1). Then, HCl (12 N) was added, and the samples were incubated for 1 h at 45 °C. Absorbance was measured at 586 nm and results expressed as nmol MDA/mg protein.

7.2.6 Data analysis

Bottom trawl and small-scale (trammel) fisheries operate at different depth ranges (see Section 7.2.1) and therefore samples obtained from both gears were analyzed separately. Due to potential differences in size composition of *Mullus surmuletus* of trammel and bottom trawling a Mann-Whitney test was applied to test for this significance. For each different gear, relation between fish size and microplastic ingestion was assessed applying Pearson correlation coefficient. In addition, an analysis of covariances (ANCOVA) was conducted to test if differences between microplastic ingestion in fish from both fishing gears was a result of fishing vessels operating at different depths and distance from the shoreline or a consequence of fish size as trammel nets target larger individuals (FAO-GFCM, 2015). In the ANCOVA analyses, fishing gear was assigned as the factor, microplastic ingestion as the variable and total length as the covariate.

For each fishing gear, a permutational multivariate ANOVA (PERMANOVA) was applied to test significant differences in microplastic ingestion in *M. surmuletus* according to area (fixed factor). The variable, number of microplastics, was transformed using the fourth root transformation and the resemblance matrix was built applying the D1 Euclidean distance. Total length was assigned as a covariable in the PERMANOVA analyses. In addition, a distance-based test for homogeneity of multivariate dispersion (PERMDISP) was conducted for the factor “Area” to test dispersion of microplastic ingestion values.

To assess antioxidant and detoxification mechanisms along with lipid peroxidation related to microplastic ingestion, significant differences between *M. surmuletus* showing ingestion and not showing ingestion of microplastics were tested for each biomarker (SOD, CAT, GST and MDA) applying a Kruskal-Wallis non-parametric test. In addition, significant differences between biomarkers and fish total length, sampling area and fishing gear were also analyzed (Kruskal-Wallis nonparametric test).

All statistical analyses were conducted with PRIMER v.6 and its addon package PERMANOVAþ (Anderson et al., 2008) and SPSS 11.5 software for windows.

7.3 Results

7.3.1 Microplastic ingestion in Mullus surmuletus

A total of 417 *Mullus surmuletus* were sampled, 27.30% (114 individuals) of which contained microplastics in their stomachs (full and empty stomachs). A mean number of 0.42 ± 0.04 MPs/individual was identified and the majority of the recovered microplastics (97%) were filament type.

In addition, mean ingestion values ranged from 0.04 ± 0.04 MPs/individual (Port d′Andratx; trawling gear) to 1.07 ± 0.26 MPs/individual (Santanyí; trammel net) (Table 7.2). As expected, significant differences ($p<0.05$) were detected in total length of *M. surmuletus* caught with both gears, with mean lower values from the trawling gear (18.32 ± 0.22 cm, mode = 20.40 cm) than from the trammel nets 22.84 ± 0.28, mode = 21.40 cm) (Table 7.1). A significant and positive correlation was found between fish size and microplastic ingestion for each independent type of gear (Pearson correlation, $r = 0.201$; $p<0.01$ for trawling and Pearson correlation, $r = 0.205$; $p<0.05$ for trammel).

	Trawling		Trammel	
Area	n	MPs/individual	n	MPs/individual
Palma	13	0.92 ± 0.29*	17	0.41 ± 0.21
Port d'Alcúdia	33	0.39 ± 0.14	20	0.40 ± 0.21
Cala Ratjada	121	0.34 ± 0.06	-	-
Santanyí	109	0.28 ± 0.06	28	1.07 ± 0.26
Port d'Andratx	26	0.04 ± 0.04	50	0.66 ± 0.14
All	302	0.32 ± 0.04	115	0.68 ± 0.10

* p < 0.05. Data is displayed as mean ± standard error (SE)

Table 7.2 Microplastic ingestion values for *Mullus surmuletus* (MPs/individual) around Mallorca Island according to fishing vessel (Trawling and Trammel) and number of individuals sampled at each area (n).

According to fishing gears, 23.50% of all fish sampled with trawling gear ingested microplastics with mean values of 0.32 ± 0.04 MPs/individual while 37.4% of all *M. surmuletus* caught with trammel nets ingested microplastics with mean values of 0.68 ± 0.10 MPs/individual. For trawling, ingestion mean values were significantly higher in Palma (Table 7.3; PERMANOVA; p<0.05) and showed higher dispersion in Palma and significantly lower dispersion in Port d′Andratx compared to the other areas (PERMDISP; Table 7.3; p<0.05). In addition to the significant differences observed amongst areas, significant differences were also observed in the total length of *M. surmulletus* (Table 7.3; PERMANOVA; p<0.01).

	Trawling			Trammel		
Source of variation						
	df	MS	Pseudo-F	df	MS	Pseudo-F
Area	4	0.61	3.13*	3	0.46	1.52
Total length	1	2.24	11.41**	1	0.32	1.04
Residual	296	0.20		110	0.31	
Total	301			114		
	PERMDISP mean ± SE for area					
Palma	0.56 ± 0.03			0.41 ± 0.07		
Port d'Alcúdia	0.41 ± 0.04			0.41 ± 0.06		
Cala Ratjada	0.41 ± 0.02			-		
Santanyí	0.35 ± 0.02			0.59 ± 0.02		
Port d'Andratx	0.07 ± 0.04*			0.53 ± 0.02		

* p < 0.05.
** p < 0.01.

Table 7.3 Results of permutational multivariate ANOVA (PERMANOVA) for microplastic ingestion in *Mullus surmuletus* and results of the multivariate dispersion PERMDISP analysis for microplastic ingestion/individual in each area independently for trawling and trammel vessels.

Instead, for fish caught with trammel nets, microplastic ingestion values were more homogenous and highest mean values were given in Santanyí but no significant differences were observed amongst areas (Table 7.3; PERMANOVA; p>0.05). Additionally, no significant differences were given for the total length of *M. surmulletus* (Table 7.3; PERMANOVA; p>0.05).

In addition, analysis of covariances (ANCOVA) results showed that there were no significant differences in microplastic ingestion in *M. surmuletus* from trammel and trawling fishing vessels when total body length of *M. surmuletus* was controlled (ANCOVA; p>0.05). Therefore, it seems that higher ingestion values in *M. surmuletus* from trammel nets are due to body size (ingestion rates increase with body size and trammel nets target larger individuals) and not to the fact that trammel fishing vessels are operating closer to land.

According to FTIR characterization of polymers, polyethylene terephthalate (PET; 36.36%) followed by cellophane (30.30%) and polyacrylate (15.15%) were the most common microplastics identified in *M. surmuletus* (Table 7.4 and Fig. 7.2). Nine different colours were identified with the most common being; blue (30.30%), black (27.27%) and transparent (21.21%) (Fig. 7.3).

Polymer type	Composition (%)
Polyethylene terephthalate (PET)	36.36
Cellophane	30.30
Polyacrylate	15.15
Polyacrylonitrile (PAN)	12.12
Alkyd	3.03
Polystyrene acrilonitrile methyl methacrylate	3.03

Table 7.4 Polymer type composition (%) identified with imaging Fourier Transform Infrared (FTIR) spectroscopy analysis in microplastic ingested by *Mullus surmuletus.*

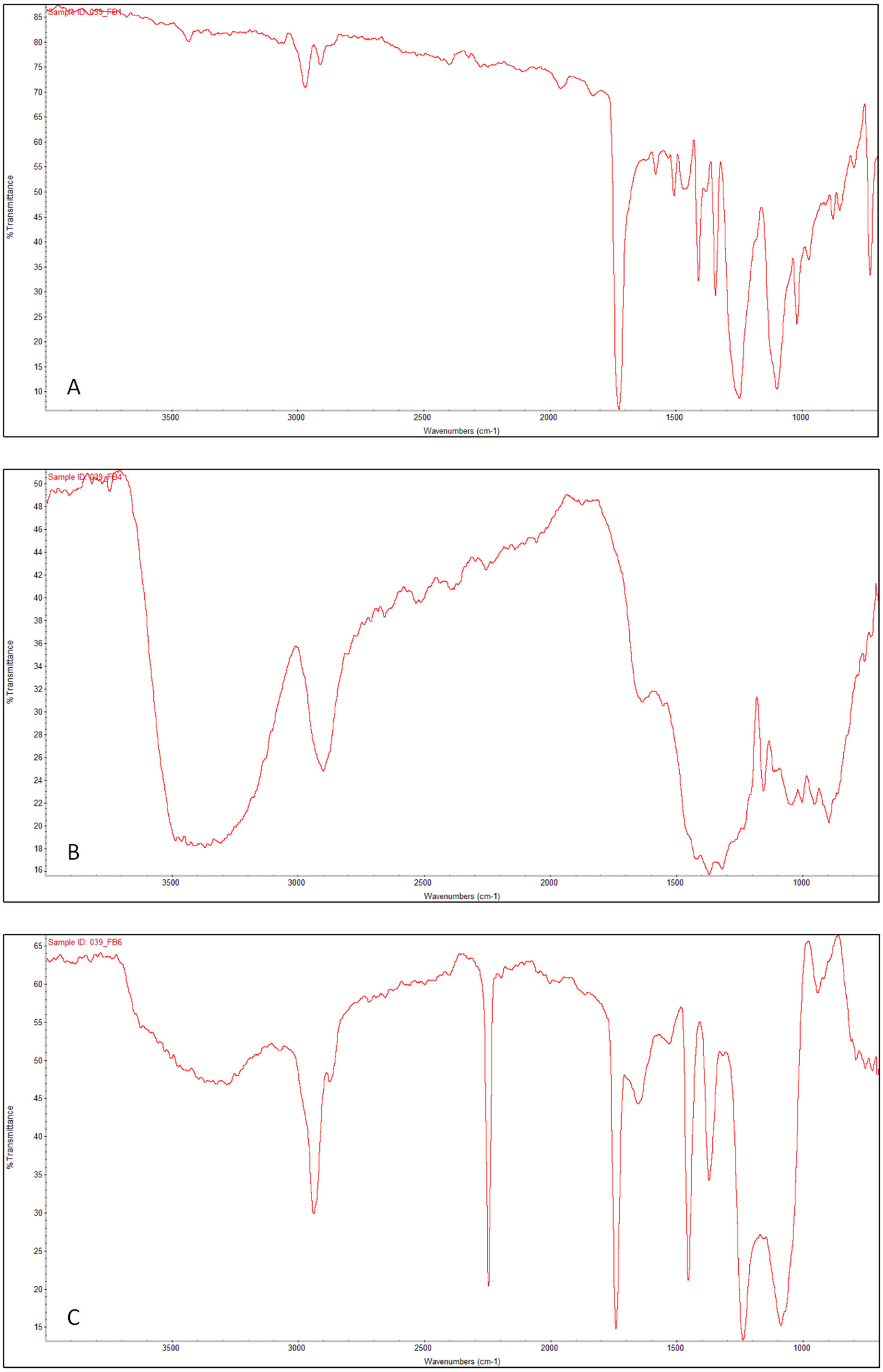

Fig. 7.2 Fourier Transform Infrared (FTIR) spectra (30 scans, 4000–700 cm^{-1} PerkinElmer Spectrum Spotlight 300) for the three most common polymers identified in stomachs of *Mullus surmuletus*: A) polyethylene terephthalate (PET) B) cellophane and C) polyacrylate.

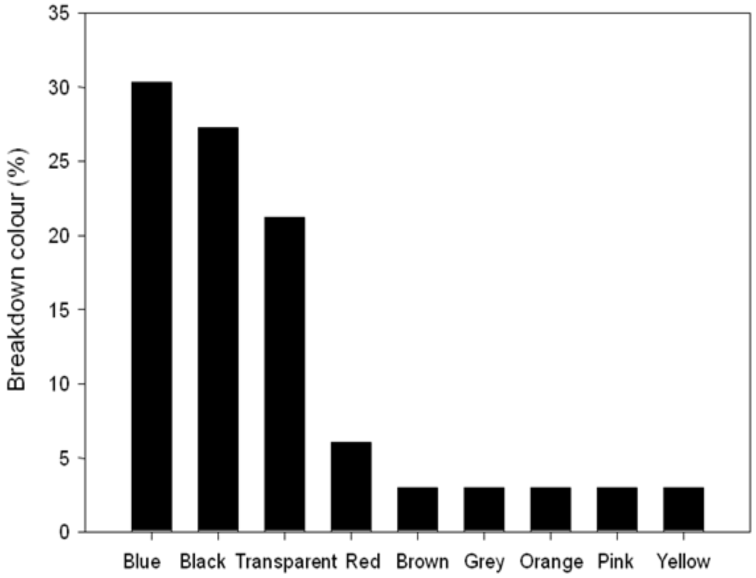

Fig. 7.3 Percentage colour breakdown of microplastics in *Mullus surmuletus´* stomach identified with imaging Fourier Transform Infrared (FTIR) spectroscopy analysis.

7.3.2 Biochemical biomarkers associated to microplastic ingestion

For all biomarkers assessed in *M. surmuletus*, mean values were higher in fish liver which had ingested microplastics (Table 7.5) but significant differences were only observed for the detoxification enzyme GST (Kruskal-Wallis; $p<0.05$). In addition, no significant differences between the enzymatic biomarkers -SOD, CAT, GST- and fish total length, fishing gear and sampling area were detected (Kruskal- Wallis; $p>0.05$). For lipid peroxidation, significant differences were evidenced in MDA concentrations for area (Kruskal-Wallis; $p<0.05$) with the highest values in Port d'Alcúdia and the lowest in Port d'Andratx.

Biomarker	with MPs (19)	No MPs (24)	χ^2	p
SOD (pKat/mg prot)	2.34 ± 0.49	1.34 ± 0.34	3.037	0.089
CAT (mK/mg prot)	30.87 ± 5.29	25.78 ± 3.57	0.678	0.415
GST (nKat/mg prot)*	9.47 ± 1.14	6.80 ± 0.72	4.266	0.045
MDA (nmol/mg prot)	13.18 ± 1.14	12.62 ± 0.72	0.635	0.430

* $p < 0.05$ (Kruskal-Wallis test).

Table 7.5 Enzymatic activities (SOD, CAT and GST) and MDA levels in the liver of *Mullus surmuletus* with ingestion of microplastics (with MPs) and with no ingestion of microplastics (No MPs). Data represent mean ± SEM, brackets numbers indicate the individuals analyzed.

7.4 Discussion

7.4.1 Microplastic ingestion by Mullus surmuletus

Results from this study provide further evidence of the ingestion of microplastics by a demersal species, *Mullus surmuletus,* in the western Mediterranean region. Ingestion of microplastics was evident as almost one third of *M. surmuletus* analyzed have ingested microplastics. These results are similar to previously reported ingestion values in other fish species from the Mediterranean Basin. For example, Bellas et al. (2016) reported that 18.18% of *Mullus barbatus* ingested a mean value of 1.90 ± 1.29 MPs/individual. Furthermore, 16.80% of the shark, *Galeus melastomus,* in the same area showed mean ingestion values of 0.34 ± 0.07 microplastics/individual (Alomar and Deudero, 2017). Also, Neves et al. (2015) reported mean values of 1.66 ± 0.57 MPs/ individuals in *M. surmuletus* from local markets and 2 MPs in one *M. surmuletus* obtained directly from a trawling fishing vessels. Anastasopoulou et al. (2013) studied debris ingestion in deep-water fish of the Eastern Mediterranean Sea giving mean values of 1.30 ± 0.20 debris items/individual with the highest value observed for the pelagic stingray *Pteroplatytrygon violacea*. In addition, it is interesting to note that ingestion of microplastics in the demersal fish *M. surmuletus* (27.30% and mean values of 0.42 ± 0.04 MPs/individual) are considerably lower that reported values for the semipelagic fish *Boops* in the same sampling area (Nadal et al., 2016; 58% and mean values 3.75±0.25 MPs/individuals). Seafloor habitats are considered the ultimate sink for plastics in the marine environment (Bellas et al., 2016) and ingested microplastics in marine species can indirectly provide information on microplastic loads in the environment (Van Sebille et al., 2015). Thus, higher loads of microplastics would be expected in sediments than in the water column and consequently in species feeding on the seafloor. Nevertheless when reviewing results from the literature it can be seen that ingestion values in pelagic species are similar or even higher than reported values for demersal species. For example, when comparing pelagic and demersal fish species from the English Channel, 38% of the pelagic species and 35% of the demersal species had ingested microplastics but there were no significant differences and mean values were of 1.90 ± 0.10 MPs/individual (Lusher et al., 2013). Furthermore, in the North Sea and Baltic Sea, 3.40% of the demersal (mean values of 0.03 ± 0.18 plastic items/individual) and 10.70% of the pelagic fish (mean values of 0.19 ± 0.61 plastic items/individual) ingested plastic with significantly higher frequency in pelagic feeders (Rummel et al., 2016). On the other hand, 63.50% of benthic fish and 36.50% of pelagic fish species ingested microplastics with mean values of 0.27 ± 0.63 MPs/individuals in Portuguese waters (Neves et al., 2015). In addition, ingestion values of plastic in large pelagic fish (bluefin tuna, albacore, swordfish) in the Central Mediterranean Sea ranged from 12.5% to 32.4% (Romeo et al., 2015) being these ingestion percentages comparable or even higher in the case of bluefin tuna (34.2%) than those provided for demersal species.

In fact, the proportion of ingested microplastics reported in this study is even higher when considering only individuals caught with trammel nets. This is likely to be the result of biological factors; *M. surmuletus* from trammel nets were significantly larger than those from trawling vessels and results of this investigation have demonstrated that ingestion of microplastics increases with fish size. It is known that, 80% of marine debris originates from land based sources (Jambeck et al., 2015) and trammel fishing vessels operate in shallower waters which are closer to land than trawling vessels. In this sense, it could be thought that trammel fishing areas are more exposed to microplastic inflow from urbanized areas and tourism related activities (Galgani et al., 2000) in comparison to trawling fishing areas. However, analysis of covariances suggested that there were no significant differences between fish of both fishing gears when body size was controlled, meaning that in this case, distance from land had no effect in ingestion patterns of microplastics. Thus, abiotic factors such as geomorphology and hydrodynamics may play a vital role in the transport, spread and diffusion of particles to more distant areas (Oliveira et al., 2015) and must be also accounted as explanatory parameters of microplastic ingestion in fish species.

7.4.2 Characterization of microplastics

In the scope of this research, the vast majority of identified microplastics were filament type which is more indicative of a sewage origin, being textiles an important source (Browne et al., 2011). Furthermore, a recent study from the same area concluded that filament type microplastics were found close to populated areas while fragment type were more common in a Marine Protected Area (MPA), free from waste waters inputs and human activities (Alomar et al., 2016). According to plastic polymers, polyethylene terephthalate, which is used in the manufacture of bottles, is the most common polymer identified in *M. surmuletus* stomachs of this study. Fishing gears are also composed of highly resistant plastics including polymers such as nylon, polyamide, polyester (Lusher et al., 2013) and polyethylene terephthalate. Litter from fishing activities has been seen to be particular common on seafloor habitats which are targeted by commercial fishing activities (Pham et al., 2014). Consequently, a potential source of microplastic ingestion by *M. surmulletus* could be this discarded fishing material in these grounds. Other polymers recorded, such as alkyd and polyacrylonitrile have also been identified in demersal species including *M. surmuletus* off the Portuguese coast (Neves et al., 2015). In pelagic and demersal fish from the North Sea and Baltic Sea the vast majority of polymers were made of polyethylene while only one particle of polyethylene terephthalate was recorded (Rummel et al., 2016). Common polymers found in the marine environment such as low and high density polyethylene, polypropylene, polyster, nylon and polystyrene (Andrady et al., 2011) and which have been identified in fish species of some areas including the Adriatic Sea (Avio et al., 2015) and north of Europe (Rummel et al., 2016) have not been detected in *M. surmuletu*s from the Balearic Islands. Consequently, nearly one third of the analyzed polymers were composed of cellophane; rarely identified in other marine fish species. However, cellophane which is included as tobacco packaging and wrappers (Conservancy, 2005) and as packaging debris (Simmonds, 2012) has been isolated and identified from seawater samples (Castillo et al., 2016) and in marine organisms such as turtles, the endangered Florida manatee (Katsanevakis, 2008) and dolphins (Simmonds, 2012). When assessing microplastic ingestion in fish species, caution has to be taken as the detected fibers are not always made of plastic (Remy el at, 2015). Lusher et al. (2013) found that over half of the polymers ingested by fish in the English Channel were made of rayon, an artificial textile material made of reconstituted cellulose compounds. In the present study, 52% of the particles were non-plastic, mainly cotton, linen, viscose and wool.

Colours of microplastics were similar to other studies with blue, black and transparent being the most abundant colours (Lusher et al., 2013; Bellas et al., 2016; Rummel et al., 2016). It is hypothesized that predatory fish ingest transparent plastic items similar to preys such as salps or siphonophores (Choy and Drazen, 2013) and white, clear and blue fibers similar to preys have been also identified in planktivores fish (Boerger et al., 2010) giving evidence of visual confusion between preys and microplastics. In the case of *M. surmuletus* they could be indirectly ingesting microplastics when detecting preys in the same way as red mullet with their barbells (Bellas et al., 2016).

7.4.3 Enzyme response and cellular damage in Mullus surmuletus's liver

Although this study provides with no evidence of oxidative stress or cellular damage in liver of fish which have ingested microplastics in field conditions, it suggests a slight increase of the activity of GST probably reflecting an induction of the detoxification system. In animals, the metabolism which inactivates exogenous molecules is mainly in the liver. These molecules, which could be toxic, should be rapidly metabolized and/or excreted in order to avoid its accumulation in the body (Sureda et al., 2006). In fact, there is evidence of severe glycogen depletion, fatty vacuolation, cellular necrosis, and lesions affecting liver in fish that were exposed to a mixture of polyethylene with chemical pollutants in the marine environment (Rochman et al., 2013). Most of the available literature about the potential effects of microplastics on oxidative stress or inflammatory makers in fish has been carried out only under laboratory conditions. In these studies results are variable since the species used, exposure time and particle size/type of microplastics are different in each case. Exposure to 5 μm diameter polystyrene microplastics in zebrafish (*Danio rerio*) during 7 days increased activities of SOD and CAT in the liver (Lu et al., 2016). In addition, polystyrene and polycarbonate nanoplastic particles also primed the neutrophils for phagocytosis, degranulation and oxidative burst after 2-h treatment in the fathead minnow (*Pimephales promelas*) (Greven et al., 2016). Microplastics were able to inhibit acetylcholinesterase activity whereas no effects were reported in GST activity or lipid peroxidation levels in the common goby (*Pomatoschistus microps*) (Oliveira et al., 2013). Bisphenol A, a plasticizer used widely in manufacturing polycarbonate plastics and epoxy resins, when supplied at 100 μM but not at lower concentrations increased the gene expression on stress genes such as heat shock protein 70 and interleukin-1 beta but not on SOD or reduced glutathione in Atlantic salmon kidney after 24-h exposure (Yazdani et al., 2016). Experimental work with the European sea bass fed on polluted and non-polluted microplastic pellets demonstrated that not only did microplastics induce histological problems but pollutants attached to their surface caused moderated structural alterations in fish within 30 days of exposure (Pedà et al., 2016). Thus, in the present study, the absence of cellular damage or evidence of oxidative stress related to the excess of ROS generated as by-products of biotransformation reactions could be indicating no apparent effect of microplastics in *M. surmuletus* liver or a short time exposure to microplastics which enables symptoms to show up and be diagnosed. Similarly to our study, experimental research has demonstrated microplastic ingestion and accumulation in organisms, *Artemia nauplii* and transference to *Danio rerio* but no observable physical harm in the intestinal tracts of the zebrafish (Batel et al., 2016). A recent study investigating the impacts associated with virgin and phenanthrene (Phe)-loaded low-density polyethylene (LDPE) fragments on a set of biomarker response in the juvenile freshwater African catfish, *Clarias gariepinus*, showed that liver and gills failed to exhibit any traces of microplastics or concentrations of the major classes of contaminants associated in the virgin microplastics within those organs (Karami et al., 2016). Instead, the release of ethylene monomers from LDPE under the activity of digestive enzymes and acids could result in the formation of ethylene oxide and ethylene glycol which are toxic for humans and animals (Grosse et al., 2007). In the light of these findings, more scientific research, combining both laboratory and field conditions, is needed to enlarge and build up the scientific knowledge related to the ingestion of microplastics and the derived ecological and biological risks and implications in species.

7.4.4 Final remarks

On the basis of this study, *M. surmuletus*, caught with two different types of fishing gears, exhibited no oxidative stress despite ingesting microplastics in the marine environment. *Mullus surmuletus* is an important commercial species in the Mediterranean region. Therefore, the lack of relevant oxidative stress response and cellular damage at this stage precludes no concern for human health. These findings reflect difficulties in studying microplastic effects in a wild population of fish species and should be corroborated with laboratory conditions. In this way, the real time of exposure to a defined pollutant and the exact concentration supplied would be known allowing for a direct and clear response of *M. surmuletus* towards the ingestion of microplastics. Finally, data on microplastic ingestion as the one provided in this study is important within the European Marine Strategy Framework Directive (MSFD Directive, 2008/56/EC) as it helps understanding and defining the trends in the amount and composition of litter ingested by marine animals which is a requirement within descriptor 10.2.1 of this directive.

7.5 Acknowledgements

This study was partially funded by the European Commission DG Environment project: “Support Mediterranean Member States towards coherent and Coordinated Implementation of the second phase of the MSFD – MEDCIS” with Grant no. 11.0661/2016/748067/SUB/ENV.C2. Sampling was carried out under PESCAMED CUATRO project, cofounded by the EU through the European Maritime and Fisheries Fund (EMFF) within the National Program of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries

Policy. Special thanks to V. Rubio and N. González for assistance throughout the biological sampling at the Spanish Institute of Oceanography (IEO), Balearic Centre of Oceanography (COB) and to A. Gascon, M. Compà and A. Garcia during sorting analyses. Acknowledgments to T. Gajst, T. Bizjak and A. Koistinen for the FTIR analyses at the University of Eastern Finland. A. Sureda was supported by the Programme of Promotion of Biomedical Research and Health Sciences CIBEROBN CB12/03/30038. The authors hereby acknowledge the Ph.D. grant provided by the University of the Balearic Islands.

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Chapter 8: General discussion

Marine litter is frequently addressed as an emergent topic (Veiga et al., 2016); however in the Mediterranean Sea, as seen from the intense bibliographic review (Chapter 3), this type of pollution has been documented since 1979 in the sea surface and since 1993 in the seafloor. From then onwards, marine litter has been a fast growing field of research and this is reflected through the increase of publications regarding litter effects on marine biodiversity from 29 documents reviewed in 2014 (Chapter 3) to 48 documents included in the last bibliographic review also at a Mediterranean basin scale (Fossi et al., 2018). At a first stage, litter effects on marine biota were mainly focused on ingestion/entanglement and marine mammals, being plastic the most studied fraction in comparison to other types of litter: wood, metal, glass, tar and non-plastic fishing material. However, since plastic large-scale production began around the 1950s (Geyer et al., 2017) these items have been susceptible to reach the marine environment and are available to all taxonomic groups, not only marine mammals. Moreover, ingestion was already observed in stomach contents of some pelagic and demersal fish in the Mediterranean Sea even though it was not the main aim of the investigation (Deudero, 1998; Massutí et al., 1998; Madurell, 2003). It has been during the last years that the scientific community has taken into consideration the ingestion of plastic in fish species and a clear example of this is that from 79 documents reviewed from worldwide fish in the last review by Markic et al. (2019), 29 studies were published in 2018, the same amount reviewed in the bibliographic research of the present thesis which covered data from 1986 to 2014 but considering only the Mediterranean Sea (Chapter 3).

From the bibliographic review at a Mediterranean basin scale (Chapter 3), it seems that the effects of plastic on marine biota depend on the taxonomic group and plastic size; whereas macrobenthic invertebrate species and algae were identified in floating objects or seen to colonize litter items on the seabed, marine mammals were mainly affected through entanglement in plastic objects or through ingestion of megaplastics such as plastic sheets. According to fish species, these might swallow plastic pieces misleading them for prey or can be ingesting plastic incidentally to normal feeding (Hoss and Settle, 1990). As revealed in the reviewed studies in Chapter 3, in the pelagic realm particle selection might be linked to mouth biometry instead of nutritional quality, while in the seafloor plastic ingestion could be linked to feeding behavior with opportunistic species ingesting a wider range of litter items which is also reinforced in Chapter 6 where it is seen that *Galeus melastomus*, with a non-selective feeding behavior (Carbonell et al., 2003), ingested filament, fragment and film type microplastics whereas only filament type microplastics have been observed in the gastrointestinal tracts of the semi-pelagic fish *Boops boops* from the same study area (Nadal et al., 2016). Moreover, in a recent investigation, *G. melastomus* was the only species, out of five analyzed species, to ingest fragment type microplastics (Capillo et al., 2019) giving further evidence of the higher diversity, in terms of plastic items, found in *G. melastomus*' gastrointestinal tract regarding the other investigated demersal species.

Consequently, as observed from the reviewed investigations conducted at a Mediterranean basin scale, plastic occurrence in biota and typology of ingested items is variable from one species to another as well as in the different marine compartments (seafloor and sea surface). When examining marine litter from the continental shelf to the middle slope of fishing grounds of the Balearic Islands (Chapter 4), this type of pollution is quantified in areas of up to 756 m depth and 21 nautical miles away from the coastline, being plastics the second most abundant litter fraction after glass (in terms of weight). These findings are consistent with other studies from the Mediterranean Sea where litter has also been quantified in fishing grounds of up to 800 m depth (Spedicato et al., 2019) and plastics, metals and glass are the most common litter fractions (Koutsodendris et al., 2008; García-Rivera et al., 2017; Alvito et al., 2018). However, when referring to the quantified abundance of seafloor litter around the Balearic Islands (1.39 ± 0.13 kg/km^2), this amount as well as the abundance of the plastic fraction (2.73 ± 0.26 kg/km^2) is lower than reported values around other regions of the Mediterranean Sea such as the Gulfs of Greece, Adriatic Sea and the Spanish Mediterranean mainland coast (Koutsodendris et al., 2008; Strafella et al., 2015; Pasquini et al., 2016; García-Rivera et al., 2018). These lower values of seafloor litter and plastics observed in the study area could be attributed to the lack of high industrialized areas in the Balearic Islands, the absence of river run-offs with a constant water flow into the sea and the scarceness of enclosed gulfs which might be the reason for higher amounts of seafloor litter and plastics quantified in the Gulfs of Greece and Adriatic Sea (Koutsodendris et al., 2008; Strafella et al., 2015; Pasquini et al., 2016). Additionally, given that macrolitter can be washed ahead from the continental shelves to submarine canyons and abyssal depths by bottom currents (Spedicato et al., 2019), an increase in seafloor litter and plastic abundance could be thought of in deeper seafloor habitats of the study area compared to the prospected fishing ground in this thesis.

Quantification of marine litter at the studied fishing grounds shows an unequal distribution according to the different fractions analysed: glass, plastics, metal, fishing material, clinker, cloth, rubber and paper (Chapter 4). Whereas plastics are present in more than half of the analysed hauls (66%) and glass, metal, clinker and cloth show a broad presence throughout the sampling area, fishing material is less observed and rubber and paper are only occasionally observed throughout the 15-years of survey giving further evidence of the patchy distribution of marine litter in the Mediterranean basin (Pierdominico et al., 2019). It is well known that marine currents can transport light litter items such as plastics from source areas to more distant areas while, heavier items such as clinker, metals and glass are not expected to travel large distance once in the marine environment but to sink close to their source point (Fortibuoni et al., 2019). Consequently, items such as metals are expected to be found close to the main maritime routes (Koutsodendris et al., 2008), which is not the case in the study area, as higher quantities are not observed in the main maritime routes but in isolated areas and the presence of metal could be considered as sporadic. On the other hand, clinker is observed in the study area close to one traditionally important military port (Soller) and the main port of the Balearic Islands (Palma), giving further evidence of the persistence of litter throughout many years in the marine environment as clinker is the residue of burnt charcoal of steam-powered vessels dumped overboard along shipping lanes about 150 years ago (Ramirez-Llodra et al., 2011).

Marine litter distribution is linked to both environmental and anthropogenic factors such as submarine geomorphology, geographical settings, hydrodynamics and bathymetric stratification and marine traffic, highly populated areas, rivers and ports (Galgani et al., 2000; Spedicato et al., 2019). When applying a modeling approach which considers different factors (sampling area, year, bathymetric strata, seafloor type and sampling distance to the coastline) to investigate seafloor plastic distribution from the continental shelf to the middle slope of fishing grounds (Chapter 4), we can observe that sampling area, bathymetric strata (depth) and distance to the coastline are defining seafloor plastic distribution around the Balearic Islands with accumulation of seafloor plastics along the northwestern coast of Mallorca and at depths compromising 201–500 m. These results are consistent with a recent modeling approach at a Mediterranean basin scale, where bottom depth and slope were seen to be important drivers of macrolitter distribution in similar seafloor habitats with four maximum peaks of litter abundance; one at shallower waters and the other three peaks around 200, 400 and 600 m depth (Spedicato et al., 2019). In the study area, a peak of seafloor plastic abundance in shallower coastal areas within the first 5 nm from the coast is also observed possibly linked to coastal human activities, which is also the reason for higher seafloor plastic abundances in nearby coastal areas of the Spanish Mediterranean coast (García-Rivera et al., 2017). On the other hand, the high seafloor plastic abundance at deeper areas, 201-500 m depth in the study area, is possibly linked to a cascading effect of litter transport to deeper depths because of water circulation (Galgani et al., 1996). This finding is also important since depth is known to be the main factor structuring species association in marine communities, including the study area (Massutí and Reñones, 2005), and the highest seafloor plastic abundances modelled at depths compromising from 201 to 500 m could be affecting marine species such as *Nephrops norvegicus*, *Helicolenus dactylopterus*, *Phycis blennoides* and *Octopus vulgaris* amongst others which live in this bathymetric strata (Massutí and Reñones, 2005; Guijarro, 2012). Moreover, two of these species, *H. dactylopterus* and *N. norvegicus* have been reported to ingest microplastics in the study area with an occurrence of 10% and 38%, respectively (Chapter 5).

The Overlap Index demonstrates that the availability of seafloor plastics for species living or feeding close to this area is a reality and evidence of this is that 45% of the investigated species from a total of 40 species including osteichthyes, elasmobranchs, cephalopods and crustaceans ingest microplastics with a mean value of 0.30 ± 0.04 microplastics/individual (Chapter 5). In the study area, this mean value is lower than reported ingestion values for some of the same species (*N. norvegicus*, *Citharus linguatula* and *Trachurus trachurus*) analyzed in other regions of the Mediterranean Sea such as the Clyde Sea, northeastern Ionian Sea and the South Adriatic Sea (Murray and Cowie, 2011; Anastasopoulou et al., 2018) where, as previously stated, also higher amounts of seafloor plastics have been quantified in comparison to those obtained in the study area possibly affecting plastic ingestion in species. Moreover, a high variability is observed amongst the 18 species ingesting microplastics with mean values ranging from 0 to 2.0 ± 1.09 microplastics/individual corresponding this highest value to *Spondyliosoma cantharus,* followed by *Raja clavata* and *Serranus cabrilla* while the rest of the species ingesting microplastics show mean values lower than 1 microplastic/individual. This high variability within species in the study area can be attributed to sampling location as for example higher seafloor litter variability can be given within nearby sampling areas than among faraway sites along the entire Mediterranean Sea (Spedicato et al., 2019). However, high variability within the same species can also be attributed to dissimilarities in diet composition of the same species in different areas, as it is the case for two populations of *Aristeus antennatus* in the Western Mediterranean Sea, which show different diets in the Spanish mainland coast and in the insular coast (Balearic Islands) (Cartes, 1994; Cartes et al., 2008) as well as different plastic ingestion values in these two areas (Carreras-Colom et al., 2018).

When assessing the Overlap Index which considers two indicators of the Marine Strategy Framework Directive (MSFD) simultaneously: seafloor plastic abundance and microplastic ingested in species from the same bottom trawl hauls (Chapter 5), those hauls with higher amounts of plastics also correspond to hauls capturing species with higher microplastic ingestion values. Moreover, the Overlap Index, increases with depth possibly indicating, once more, that plastic pollution is more dependent on depth than spatial coverage (Consoli et al., 2018a,b; Olguner et al., 2018; Pierdomenico et al., 2019). Additionally, the Overlap Index shows a decreasing trend from the east to the south-west of Mallorca and predictions for this index suggest a mayor exposure to plastic pollution along the northwestern coast of Mallorca (Serra de Tramuntana). The Northern Current, which flows from the Gulf of Lion (recently determined a hotspot area of seafloor litter (Spedicato et al., 2019)) through large metropolitan areas such as Marseille and Barcelona, to the Balearic Islands, could be influencing the higher amounts of plastics quantified in the seafloor of this northwestern area (Chapter 4). Moreover, given that the Northern Current increases primary production in this area (Estrada, 1996; Fernández de Puelles et al., 2004), this zone should be considered as a vulnerable area to plastic pollution exposure. A high overlap between seafloor plastics and microplastic ingestion in species is also given and predicted in the southwestern of Mallorca; in the bay of Palma (including the *Badia de Palma* Marine Reserve) and in areas surrounding four other marine reserves: *Migjorn de Mallorca*, *L'Illa del Toro, Illes Malgrats* and *Freu de Sa Dragonera* which are protected by the autonomous government of the Balearic Islands given that uses and the exploitation of the marine environment are regulated in them. Predicted values for the Overlap Index are also higher around the Cabrera Archipelago Maritime-Terrestrial National Park in comparission to seafloor areas of the east of Mallorca and around Menorca. All of this suggests that special ecological concern should be taken over these areas as production/biomass and diversity increases with depth in the south of Mallorca (Cartes et al., 2011) and the Overlap Index increases with depth and it is highest in this area of the the Balearic Islands. Even though predictions of the Overlap Index do indicate areas more susceptible to plastic pollution, the combination of both indicators (seafloor plastics and microplastic ingestion in species) reduces the identified exposure areas to this type of pollution. Consequently, a multiple-species approach, studying ingestion values in several species from different depths and with different feeding strategies should be also considered when studying plastic ingestion in biota and selecting adequate indicator species of this type of pollution.

In the study area, *Mullus surmuletus* which is restricted to depths shallower than 200 m (Massutí and Reñones, 2005) and *Galeus melastomus* which is distributed in the slope (Ramírez-Amaro et al., 2016), especially in the middle slope (Guijarro and Massutí, 2006) ingest microplastics in seafloor areas (Chapter 6 and 7). According to these two demersal species, almost 17% of the analysed *G. melastomus* showed ingestion of microplastics, with a mean value of 0.34 ± 0.07 microplastics/individual (Chapter 6) which is lower than ingestion values reported in Chapter 7 for *M. surmuletus* (27% of the sampled individuals with mean values of 0.42 ± 0.04 microplastics/individual). *Galeus melastomus* reach highest abundances in deeper areas (> 500m) (Moranta et al., 1998), where lower seafloor plastic abundances have been quantified in comparassion to the shallow shelf (Chapter 4), where *M. surmuletus* lives and shows higher microplastic ingestion values than *G. melastomus*. Considering that these two species are common and abundant in demersal habitats, microplastic ingestion and occurrence provides with further indication that seafloor habitats are a sink for microplastics which are available for marine organisms (Galgani et al., 1995; 1996; 2000). Moreover, these species could be valid indicators of plastic pollution in these grounds, as *M. surmuletus* and *G. melastomus* from different locations exposed to similar human pressures do not show significant differences according to microplastic ingestion and location (Chapter 6 and 7), probably reporting similar environmental microplastic loads in these seafloor areas.

As already mentioned in the introduction of this thesis, *Mullus barbatus* are used for monitoring marine pollution within the Spanish Marine Pollution Monitoring Program (SMP) (Bellas et al., 2016) and having seen that its congenic, *M. surmuletus*, is also susceptible to plastic pollution along the Mediterranean Sea, these species could be suitable candidate indicator species for this type of pollution. Moreover, coupling microplastic studies to the already ongoing contamination monitoring is feasible (Bellas et al., 2016; Capillo et al., 2019) and it should not compromise additional individuals for this assessment. Furthermore, the physiological response to microplastic ingestion of indicator species should be also considered when assessing marine plastics and its effects upon organisms. In this sense, when applying a set of biomarkers of oxidative stress and cellular damage including Superoxide Dismutases (SOD), Catalase (CAT), Glutation-S-Transferasa (GST) and Malondialdehyde (MDA) to study the physiological response of *Mullus surmuletus* to microplastic ingestion in the study area (Chapter 7), a slightly increase of the activity of the GST is detected. Glutation-S-Transferasa is one of the first biomarkers to activate in front of several toxins (Sureda et al., 2006) probably reflecting an induction of the detoxification system of wild *M. surmuletus* when ingesting microplastics. Similarly, in Portuguese waters, wild fish ingesting microplastics showed lipid oxidative damage in their gills and dorsal muscle as well as an increase of acetylcholinesterase (AChE) in brain (Barboza et al., 2019). As already stated, indicator species not only should attempt to reflect plastic abundances in the marine environment through plastic ingestion, but should also aim at giving evidence of the physiological damage caused by this ingestion and its associated contaminants. The use of biomarkers such as the ones applied in this thesis have been efficiently applied under controlled conditions in laboratory experiments to assess the physiological response of species to the uptake and accumulation of ingested microplastics (Oliveira et al., 2013; Lu et al., 2016). However, in the marine environment, controlling conditions such as exposure time to plastics and its concentration is difficult and consequently, the application of biomarkers in field conditions presents a greater challenge given that lesions affecting species will be variable and dependent on the study species, particle size/type, concentration and exposure time to this pollutant. This is evidenced when studying the physiological response of *M. surmuletus* to microplastic ingestion, as the absence of cellular damage or evidence of oxidative stress related to the excess of Reactive Oxygen Species (ROS) generated as a by-product of biotransformation reactions due to microplastic ingestion could be indicating no apparent effect of microplastics in fish liver or a short time exposure to microplastics which enables symptoms to show up and be diagnosed. Nevertheless, given that *M. surmuletus* is an important commercial species in the Mediterranean region, the lack of relevant oxidative stress and cellular damage could preclude no concern for human health at this stage.

Chapter 9: General conclusions

I) From the bibliographic review, scientific studies have reported floating plastics since 1979 and seafloor plastics since 1993 in the Mediterranean Sea. According to 17,334 individuals from 29 different studies, species are affected by marine litter through ingestion, entanglement, colonization and rafting and this depends on the taxonomic group and plastic size.

II) Marine litter is found from the continental shelf to the middle slope of fishing grounds of the Balearic Islands with an unequal distribution according to litter fractions analyzed. After glass, the plastic fraction (mean ± standard error (se) value of 2.73 ± 0.26 kg/km^2) is the most abundant fraction in terms of weight. Sampling area, bathymetric strata (depth) and distance to the coastline are influencing seafloor plastic distribution.

III) The Overlap Index increases with depth indicating that plastic pollution is more dependent on depth than spatial coverage and that deep-sea species might be more vulnerable to plastic ingestion. This twofold approach addressing simultaneously seafloor plastics and microplastic ingestion in species also suggests that the surrounding areas of a highly anthropogenized bay including Marine Protected Areas are more exposed to plastic pollution.

IV) Even though seafloor plastics are patchily distributed and variability of microplastic ingestion values within and amongst species is high, these values are lower than reported values in other areas of the Mediterranean Sea and according to marine litter indicators of the Marine Strategy Framework Directive (MSFD), the northwestern and southwestern of Mallorca seem to be the most exposed areas to plastic pollution in the Balearic Islands.

V) *Mullus surmuletus* and *Galeus melastomus,* two species with different spatial distribution, showed different abundances of ingested microplastics from the seafloor around the Balearic Islands. *Mullus surmuletus*, which is found in the continental shelf where high abundances of seafloor plastics are observed (51 to 100 m depth) shows higher microplastic ingestion values (mean ± se) (0.42 ± 0.04 microplastics/individual and 27% of occurrence) than *Galeus melastomus* (0.34 ± 0.07 microplastics/individual and 17% occurrence) which is found in the middle slope where lower abundances of seafloor plastics have been quantified. The evidence of the ingestion of microplastics by several species of osteichthyes, elasmobranchs, cephalopods and crustaceans sampled close to the seafloor coupled to this ingestion, suggests that seafloor species such as, *Mullus surmuletus* and *Galeus melastomus* could be valid indicatos of plastic pollution in this study area.

VI) *Mullus surmuletus* individuals with microplastics exhibit a slight increase in the enzymatic activity of Glutathione S-transferase (GST) compared to individuals with no microplastics in their stomachs. Consequently, GST seems to be the best biomarker of oxidative stress in fish liver for this species as a response to plastic pollution in the marine environment.

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