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Phthalates as Plastic Tracers in a Pelagic Food Web from an Open Oceanic Environment

DOCTORAL THESIS

Annalisa Sambolino
DOCTORATE IN BIOLOGICAL SCIENCES

UNIVERSIDADE da MADEIRA
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Resumo

A investigação científica nas ciências ambientais e biológicas preocupa-se cada vez mais dos efeitos adversos das atividades humanas. A poluição por plásticos tem recebido atenção global devido ao seu evidente impacto ambiental, especialmente nos ecossistemas marinhos. Os microplásticos (MPs), definidos como partículas de plástico com menos de 5 mm, são de particular preocupação devido à sua persistência e capacidade de se infiltrar nas cadeias alimentares. No entanto, o ambiente pelágico continua a ser um dos ambientes menos explorados e mais desafiantes para estudar. Este estudo teve como objetivo investigar a presença de microplásticos numa cadeia alimentar pelágica num ecossistema oceânico, examinando em simultâneo a ocorrência de aditivos plásticos, especificamente os ftalatos (PAEs), como potenciais "tracers". A amostragem ao longo de um ano das águas superficiais ao redor de uma ilha oceânica revelou uma presença consistente de MPs, especialmente microfibras, com flutuações sazonais significativas na razão entre MPs e zooplâncton. A análise do conteúdo estomacal das espécies de peixes pelágicos e lulas mais prevalentes que habitam estas águas confirmou que a maioria dos indivíduos ingeriu MPs, e foi observada uma correlação entre a abundância de MPs e a concentração de PAEs, particularmente DIBP (di-isobutyl phthalate), nos seus tecidos. A análise de PAEs em amostras de biópsia de cetáceos que se alimentam destas presas, revelou uma presença persistente e alarmantemente elevada destes aditivos plásticos. Isso sugere uma exposição crônica e duradoura, provavelmente por meio de transferência trófica. A utilização de indicadores indiretos, como os aditivos plásticos, revela-se de grande importância em ambientes de difícil acesso e investigação, especialmente ao estudar animais protegidos, como a megafauna marinha, na qual as amostras são limitadas e de difícil obtenção. Neste estudo, foram desenvolvidas e validadas metodologias inovadoras para a deteção e quantificação de PAEs em biota marinho. A deteção de MPs e PAEs em vários

níveis desta teia trófica oceanopelágica destaca a presença generalizada destes contaminantes antropogénicos mesmo em regiões oceânicas remotas.

Palavras-chave

Cadeia alimentar pelágica; Cetáceos; Ecossistema oceânico; Ftalatos; Ilha da Madeira; Microplásticos; Oceano Atlântico; Poluição por plástico.

Abstract

Scientific research in environmental and biological sciences is increasingly concerned with human activities' adverse effects. Plastic pollution has garnered global attention due to its evident environmental impact, particularly in marine ecosystems. Microplastics (MPs), defined as plastic particles smaller than 5 mm, are of particular concern for their persistence and ability to infiltrate food chains. Yet, the pelagic realm remains one of the least explored and most challenging environments to study. This study aimed to investigate the presence of MPs within a pelagic food web in an oceanic ecosystem while also examining the occurrence of plastic additives, specifically phthalates (PAEs), as potential tracers. Annual sampling of surface waters surrounding an oceanic island revealed a consistent presence of MPs, especially microfibres, with significant seasonal fluctuations in the ratio of MPs to zooplankton. Analysis of stomach contents in prevalent pelagic fish and squid species inhabiting these waters confirmed that most individuals had ingested MPs, and a correlation was observed between MPs abundance and PAEs concentration, particularly DIBP (di-isobutyl phthalate), in their tissues. The application of PAEs analysis in biopsy samples from free-ranging cetaceans, which feed on these prey species, revealed a persistent and alarmingly high presence of these plastic additives. This suggests chronic and enduring exposure, likely through trophic transfer. The use of indirect proxies, such as plastic additives, proves invaluable in environments that are challenging to access and investigate, especially when studying protected animals like marine megafauna, for which samples are limited and difficult to obtain. In this work, innovative methodologies were developed and validated for the detection and quantification of PAEs in marine biota. The detection of MPs and PAEs at various levels of this oceanic-pelagic trophic web underscores the widespread presence of these anthropogenic contaminants even in remote oceanic regions.

Keywords

Atlantic Ocean; Cetaceans; Madeira Island; Microplastics; Oceanic ecosystem; Pelagic food web; Phthalates; Plastic pollution.

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List of Abbreviations

AA	Arachidonic Acid
ACN	Acetonitrile
AIC	Akaike Information Criteria
BBP	Butyl benzyl phthalate
BHT	Butylated Hydroxytoluene
CAS	Chemical Abstracts Service
CE	Conformité Européenne
CH	Cyclohexane
Chla	Chlorophyll-*a* concentrationn
CI	Confidence Intervals
DBP	Di-n-Butyl phthalate
DCHP	Dicyclohexyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHA	Di(2-ethylhexyl) adipate
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DHA	Docosahexaenoic acid
DHP	Dihexyl phthalate
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate
DINP	Diisononyl phthalate
DIPP	Diisopentyl phthalate
DMP	Dimethyl phthalate
DNA	Deoxyribonucleic acid
DNHP	Di-n-hexyl phthalate
DNOP	Di-n-octyl phthalate
DNPP	Di-n-pentyl phthalate
DPHP	Di(2-propylheptyl) phthalate
DPP	Dipropyl phthalate
d.w.	Dry weight
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EU	European Union
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FL	Florida
FO	Frequency of Occurrence
FTIR	Fourier-transform Infrared Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GIT	Gastrointestinal Tract

GLMM	Generalized Linear Mixed Model
GPS	Global Positioning System
GSI	Gastrosomatic Index
HMW	High Molecular Weight
HOC	Hydrophobic Organic Chemicals
HSI	Hepatosomatic Index
IME	Island Mass Effect
IPMA	Portuguese Institute for Sea and Atmosphere
KOH	Potassium hydroxide
K_{ow}	Octanol-water partition coefficient
LCL	Lowest Calibration Level
LMW	Low Molecular Weight
LOQ	Limits of Quantification
MBP	Monobutyl phthalate
MBZP	Monobenzyl phthalate
MDL	Method Detection Limit
ME	Matrix Effect
MEHP	Mono(2-ethylhexyl) phthalate
MM	Molecular Mass
MP	Microplastic
MPA	Marine Protected Area
MS	Mass Spectrometry
MUFA	Monounsaturated Fatty Acid
NA	Not Available
NE	North East
NOAA	National Oceanic and Atmospheric Administration
nMDS	non-metric Multidimensional Scaling
NW	North West
PA	Polyamide
PAE	Phthalic Acid Esters - Phthalate Esters - Phthalates
PBDE	Polybrominated Diphenyl Ether
PC	Principal Component
PCA	Principal Component Analysis
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PE	Polyethylene
PERMANOVA	Permutational Multivariate Analysis of Variance
PERMDISP	Permutational Multivariate Analysis of Dispersion
PET	Polyethylene terephthalate
POP	Persistent Organic Pollutants
PP	Polypropylene
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PS	Polystyrene
PSA	Primary Secondary Amine

PUFA	Polyunsaturated Fatty Acid
PVA	Polyvinyl Alcohol
PVC	Polyvinyl Chloride
PVDF	Polyvinylidene Fluoride
QFASA	Quantitative Fatty Acid Signature Analysis
QQ	Quantile-Quantile
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RSD	Relative Standard Deviation
SANTE	Directorate-General for Health and Food Safety
SD	Standard Deviation
SFA	Saturated Fatty Acid
SIM	Single Ion Mode
SST	Sea Surface Temperature
SVHC	Substances of Very High Concern
TDI	Tolerable Daily Intake
UNEP	United Nations Environment Programme
USA	United States of America
UV	Ultraviolet
VIF	Variance Inflation Factor
WHO	World Health Organization
w.w.	Wet weight
WWTP	Waste Water Treatment Plant

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CHAPTER I

GENERAL INTRODUCTION

Chapter 1 - General Introduction

1. Marine plastic pollution

The term "plastic" encompasses a broad spectrum of synthetic or semi-synthetic materials with a high molecular weight and a polymeric structure. Polymers that primarily derive from fossil fuel-based petrochemicals like natural gas or petroleum, can soften upon heating and be molded, are generally categorised as “plastic” materials (Thompson et al. 2009; Plastic-Europe 2006). Since its civilian adoption became widespread in the 1950s (post-World War II), worldwide plastic manufacturing has rapidly increased (Geyer, Jambeck, and Law 2017). The polymers with the highest production and widespread distribution include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA), polyethylene terephthalate (PET), and polyvinyl alcohol (PVA) (Figure 1.1) (Andrady 2011). The low cost and high versatility of these materials have driven a continual rise in global production, with a robust and ongoing plastic demand (PlasticsEurope 2021). This surge has been intensified by a global transition from reusable to disposable items. Notably, PE and PP are predominantly employed in crafting single-use plastic products (comprising nearly 50% of plastic items), such as cutlery, shopping bags, and packaging (PlasticsEurope 2021).

Since then, the management and treatment of plastic items on land and at sea have been insufficient to prevent them from entering the ocean (Borrelle et al. 2020), where they quickly disperse and are highly persistent. As a consequence, a rise in ocean plastics has been witnessed in the last decades (Ostle et al. 2019), and concerning concentrations of plastic debris have been reported in all oceans, including remote areas such as the Arctic (Cozar et al. 2014; Bergmann et al. 2022). Plastics have thus been recognised as a major global environmental

issue due to their accumulation in oceans worldwide and the discovery of adverse health effects on living organisms (UNEP 2014).

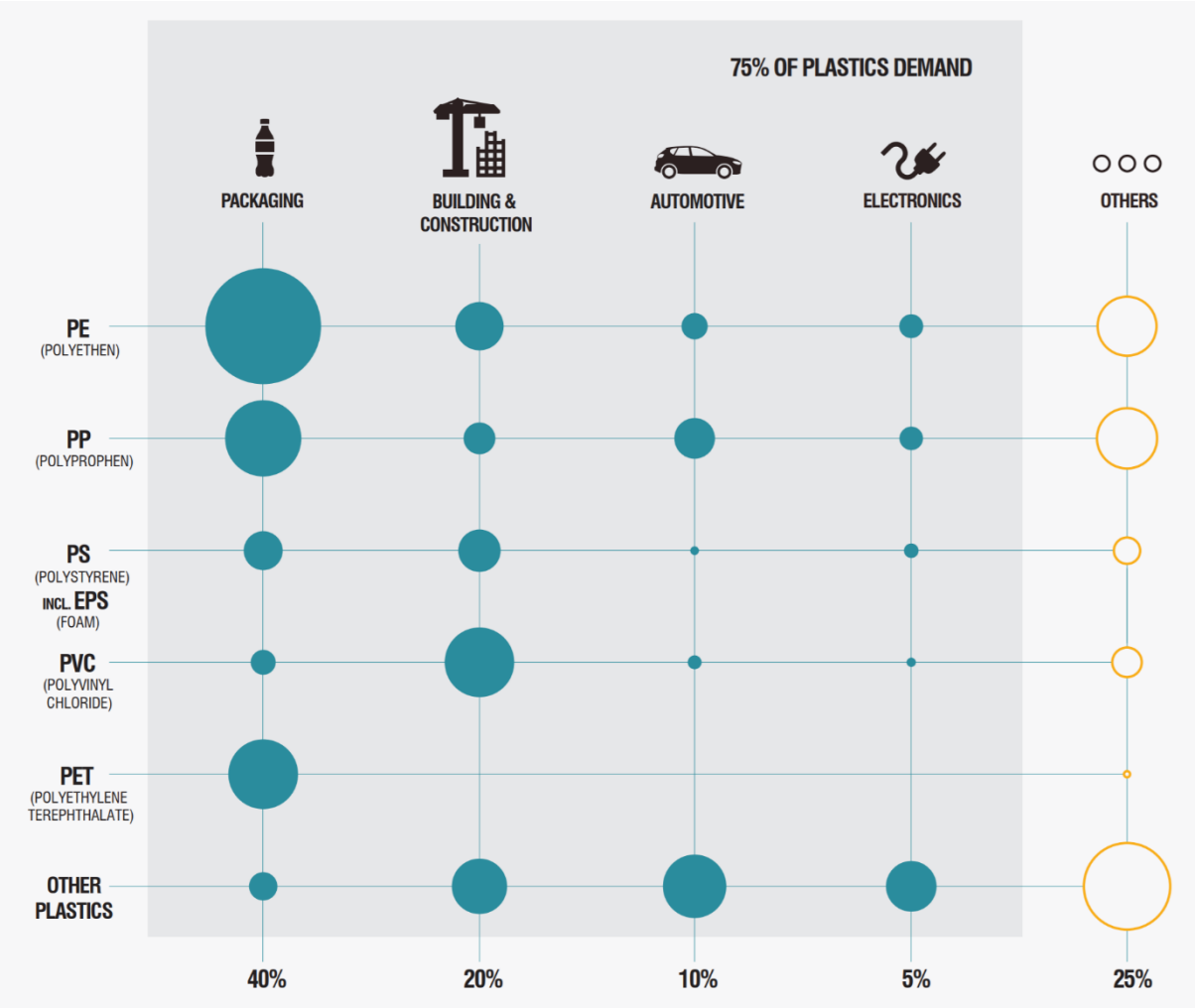


Figure 1.1 Share of European plastics demand in 2017 and the corresponding polymer composition. Image: Setting the Facts Straight on Plastics | World Economic Forum. Adapted from Materials Economic.

Research on plastic debris occurrence and its impact on the marine environment has quickly intensified in recent decades. Yet, this investigative drive has predominantly focused on coastal ecosystems. This preference is attributed to the relative ease of accessing and sampling these regions, coupled with the prevailing notion that coastal areas are more susceptible to contamination due to their proximity to anthropogenic sources of pollution. A search in Scopus using the words "plastic" AND "pollution" AND "coastal" vs" pelagic" clearly shows the wide disparity between the number of published research in the two areas (Figure 1.2).

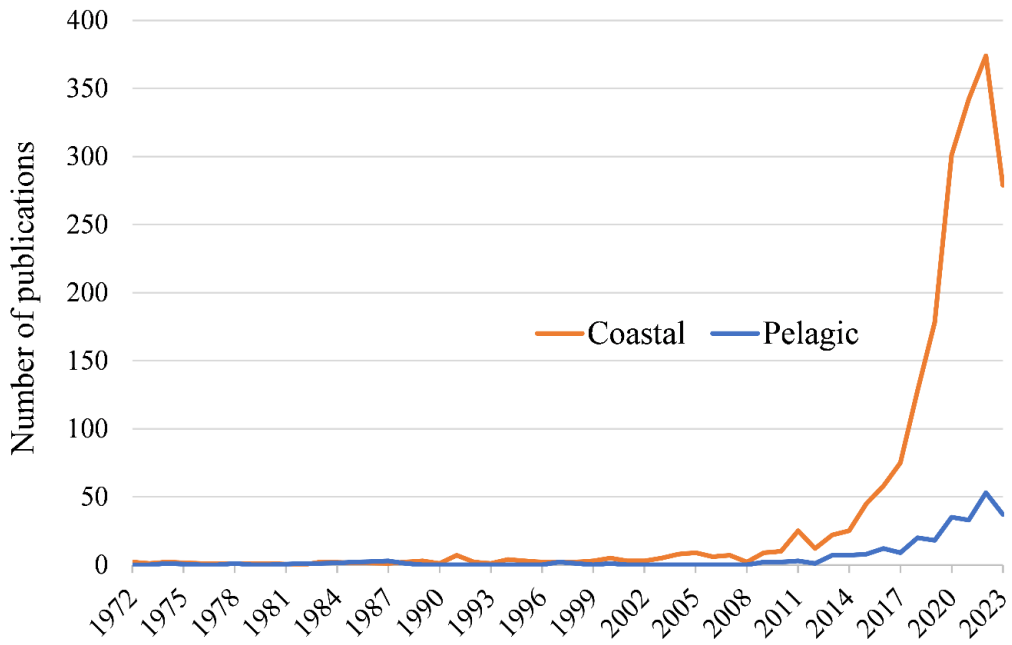


Figure 1.2 Number of publications found on Scopus using the keywords "plastic" AND "pollution" AND "coastal" (orange line) vs "plastic" AND "pollution" AND "pelagic" (blue line). Note that 2023 is an incomplete year (Jan - Jul).

While these efforts have significantly enhanced our comprehension of the plastic problem, the focus on coastal ecosystems has inherently left gaps in our knowledge regarding the extent of plastic debris in more remote and less accessible pelagic regions of the open ocean. Floating plastic debris can be transported and dispersed by oceanic physical processes, traveling long distances and impacting ecosystems far from where they originated (Van Sebille et al. 2020; Chenillat et al. 2021). Therefore, plastic debris are found to be widespread in all oceans, and they accumulate in specific areas due to oceanographic convergence processes, such as oceanic gyres and mesoscale eddies (Cozar et al. 2014; Brach et al. 2018; Van Sebille et al. 2020). Plastic debris have been found at the highest abundance in sub-tropical gyres (Cozar et al. 2014), which, however, have low levels of marine biodiversity due to low productive waters (Seki and Polovina 2019). Conversely, other converging processes, such as mesoscale eddies, can occur in areas with higher productivity and biodiversity hotspots, such as oceanic islands. The dispersion and accumulation of plastic debris in the open ocean constitute a worrying environmental hazard to islands, exposing them to increasing pollution

over which they have little control (Lavers and Bond 2017; Monteiro, Ivar do Sul, and Costa 2018; Cardoso and Caldeira 2021). Indeed, plastic debris can have dangerous repercussions on marine organisms, associated mainly with entanglement, ingestion and intestinal blockage, leaching, absorption and release of toxic pollutants, potential dispersion of invasive species and contamination by plastic particles in marine food webs.

2. Microplastic uptake in marine food webs

The formation and ubiquitous presence of microplastics (*i.e.,* plastic items < 5 mm - MPs) in the marine environment is a worrying consequence of plastic pollution due to its undisclosed effects on ecosystems' health (Barnes et al. 2009). This worrying environmental issue that has seen increasing scientific, public, and political interest in the last decade (Sedlak 2017; Hartmann et al. 2019). Dispersed in the ocean, plastic debris are exposed to physical and chemical processes, weathering and breaking down into smaller and smaller pieces, defined as MPs of secondary origin (Thompson et al. 2004). In addition, "primary" MPs are manufactured in specific industries such as cosmetics (e.g., microbeads) and textiles (e.g., synthetic microfibres) (Gregory 1996). Consequently, most plastic items in the ocean fall in the size range of 1-5 mm (Cozar et al. 2014), and the oceans' surface layer worldwide now contains more than 5×10^{12} pieces (Eriksen et al. 2014).

The term "microplastics" started to be employed in 2004, when Thompson et al. described microscopic plastic particles with diameters down to 20 μm widespread in the pelagic zone and sedimentary habitats of the North Sea, suggesting they resulted from the degradation of larger plastic items. Later in 2008, during a workshop hosted by NOAA, experts defined the limit size of 5 mm for plastic particles to be considered MPs (Arthur, Baker, and Bamford 2009). As research developed further, technologies allowed to identify smaller and smaller particles and submicron-size particles were also detected in the environment and defined as nanoplastics

(Koelmans, Besseling, and Shim 2015; Gigault et al. 2018). Because of their small size and synthetic origin, these particles can be easily ingested indirectly or directly (due to mistaking the particles for food) and introduced into food webs where they will persist, posing a concerning threat to marine life (Figure 1.3).

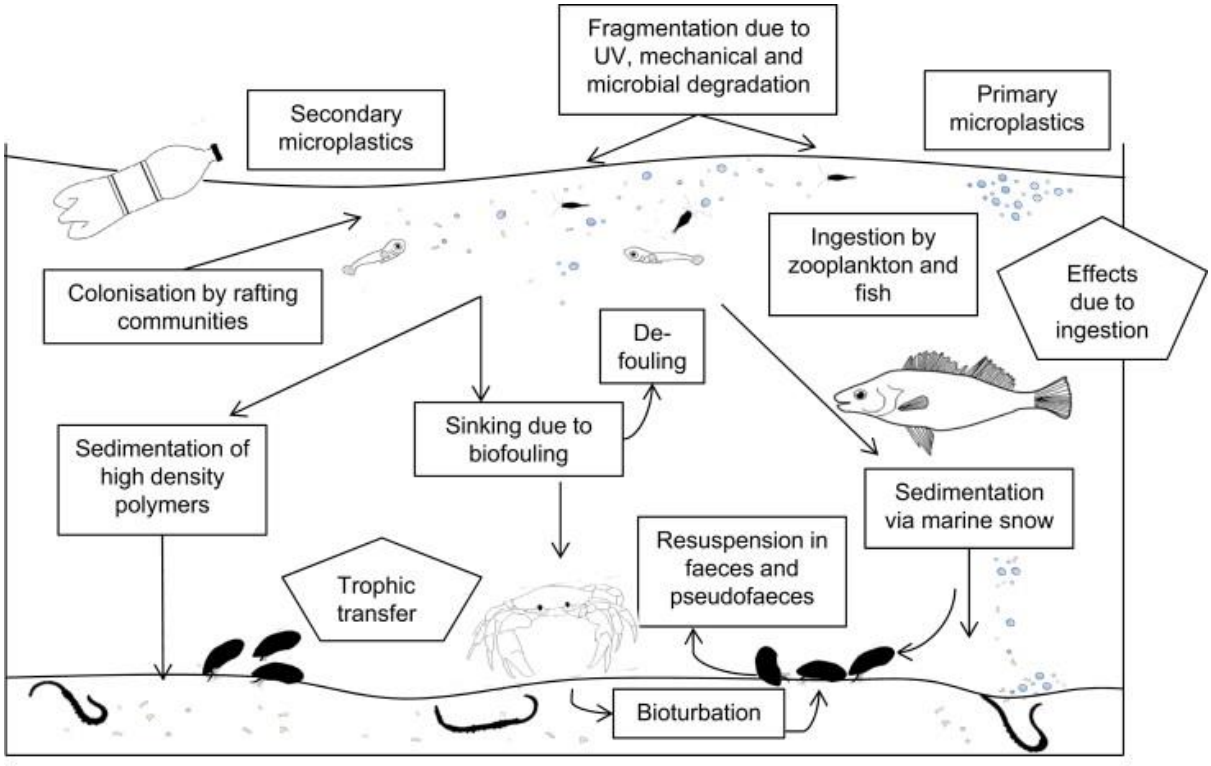


Figure 1.3 Potential pathways for the transport of microplastics and their biological interactions in the marine environment. Image: Wright et al., 2013.

MPs have been detected in a wide range of marine species, across all the trophic levels, from zooplankton to marine mammals and seabirds (Carney Almroth et al. 2018). MP ingestion by zooplankton has been experimentally demonstrated (Cole et al. 2013) and directly observed in the marine environment (Desforges, Galbraith, and Ross 2015). Trophic transfer to higher levels has also been documented (Farrell 2013; Setälä, Fleming-Lehtinen, and Lehtiniemi 2014). According to a recent study, 88% of the studied turtle specimens, 59.5% of marine mammals, 50.4% of sea birds, and 42% of fish ingested MPs, with a mean number of MPs per individual ranging from 121.7 (marine turtles) to 2.6 (fish) (Ugwu, Herrera, and Gómez 2021).

Notably, except for birds, the greatest proportion of MPs found in all marine species were fibres.

The high prevalence of microfibres in the natural environment is frequently observed in plastic pollution studies. Synthetic and semi-synthetic microfibres, originating from textile usage and laundering, have become prevalent due to the dominance of synthetic polymers, especially polyester, in the textile industry since the mid-1990s, surpassing cotton (Sillanpää and Sainio 2017). Their small size and light weight make them highly resistant to removal in conventional wastewater treatment processes (WWTPs) (McIlwraith et al. 2019; Ngo et al. 2019). Consequently, fibres are now the most commonly encountered type of human-made particles in global microplastic pollution surveys (Gago et al. 2018; Suaria et al. 2020) and they have been detected in a wide range of compartments, including drinking water, commercial seafood, marine organisms from zooplankton to mammals, and even in the air (O'Brien et al. 2020; Prata et al. 2020).

Marine organisms living in areas with higher concentrations of MPs are exposed to a higher risk of ingestion. Evidence suggests that coastal ecosystems are the most vulnerable to MP contamination, as the inputs from rivers and waste waters constitute the major pathway and source of MPs in the marine environment (Thompson et al. 2004; Browne et al. 2011). Nevertheless, the transport of MPs by oceanographic processes over long distances implies that pelagic organisms living in offshore waters can also be affected. However, our understanding of the extent of this impact remains limited. Due to the crucial role of zooplankton as the foundation of the pelagic food web, a prevalent method for assessing the risk of MP ingestion by pelagic organisms involves analysing the ratio of MP particles to zooplankton organisms (e.g., Collignon et al. 2012; Frias, Otero, and Sobral 2014; Kang, Kwon, and Shim 2015; Herrera et al. 2020). Open ocean waters are generally less productive and contain lower densities of food items. In areas such as the sub-tropical oceanic gyre, which are intensely

oligotrophic and accumulate high concentrations of plastic debris, this ratio has been found to exceed six plastic items per individual zooplankton (Moore et al. 2001). As such, the impact of MP pollution in pelagic ecosystems should not be underestimated.

MP consumption by marine biota can have a variety of deleterious consequences. Physical impacts include mechanical obstruction of the digestive tract or breathing apparatus, which can result in satiation, starvation and physical deterioration. In turn, this can lead to reduced reproductive fitness, drowning, diminished predator avoidance, impaired feeding ability and ultimately, death (Figure 1.4) (Wright, Thompson, and Galloway 2013). In addition, plastic particles may carry toxic substances into the organisms (Crawford and Quinn 2017), which have the potential to be transferred along the food web (Gall and Thompson 2015). Certain persistent organic pollutants (POPs) can be absorbed from the environment and carried by plastic particles, such as organochlorine compounds (e.g., Polychlorinated biphenyls – PCBs, dichlorodiphenyltrichloroethane - DDT) and heavy metals (Andrady 2011). Furthermore, plastic items often contain chemical additives such as plasticisers, thermal stabilisers, antioxidants, UV stabilisers, and colorants, that are incorporated during the manufacture of the polymers to improve their performance. These additives vary based on the polymer type and desired product. Many of these chemical compounds are frequently detected in field MP samples, environmental samples, and marine biota, and they can exhibit hazardous properties, such as endocrine disruption (Figure 1.4) (Hermabessiere et al. 2017).

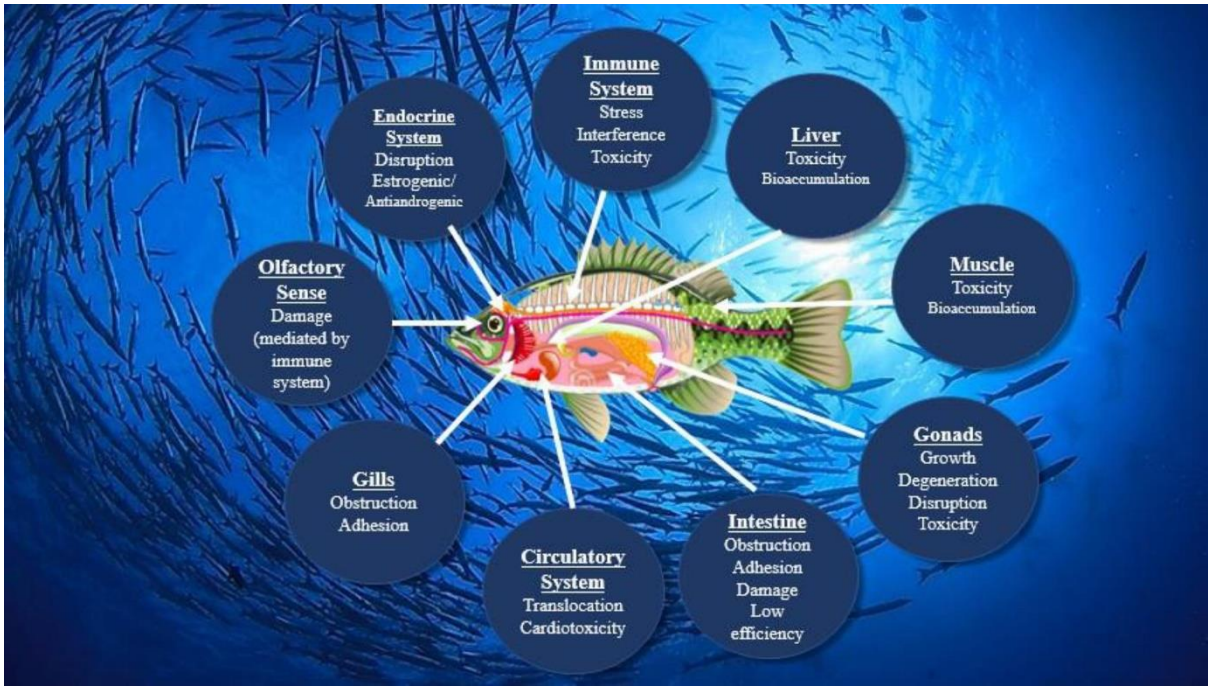


Figure 1.4 Possible toxic effects and impact of microplastics and its associated chemicals on the marine biota. Image: Gola et al., 2021.

3. Phthalates in the marine environment

Phthalates (Phthalate Esters or Phthalic Acid Esters – PAEs) are a class of plasticisers that received increasing attention for their ubiquitous presence in the environment (Net et al. 2015; Paluselli et al. 2019; Paluselli and Kim 2020; Hidalgo-Serrano et al. 2022). PAEs are manufactured chemicals introduced as plasticisers in the 1920s, mainly in the production of PVC, to increase the flexibility and softness of plastic polymers. PAEs are the most commonly used plasticiser worldwide, and they can account for up to 70% of the weight of the final plastic product (Hahladakis et al. 2018).

Phthalates-softened PVC plastic is extensively employed in a wide array of everyday items commonly found in households, extending to sectors like packaging (e.g., food packaging), construction (e.g., vinyl flooring and wall covering), furniture (e.g., sheets and coverings), automobiles, and telecommunications (e.g., wires and cables) (Figure 1.5).

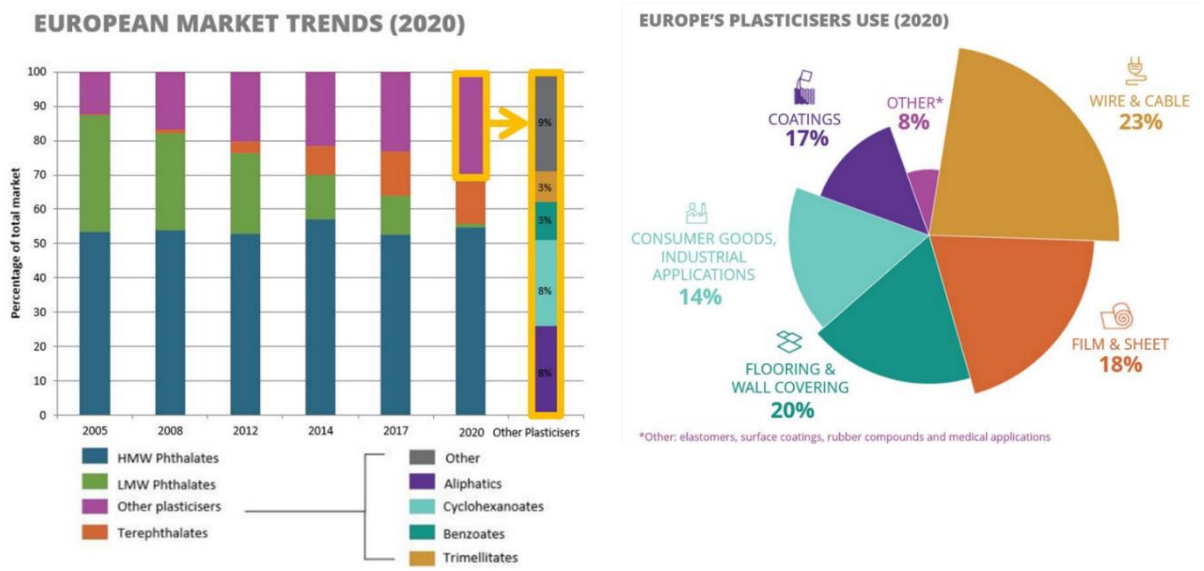
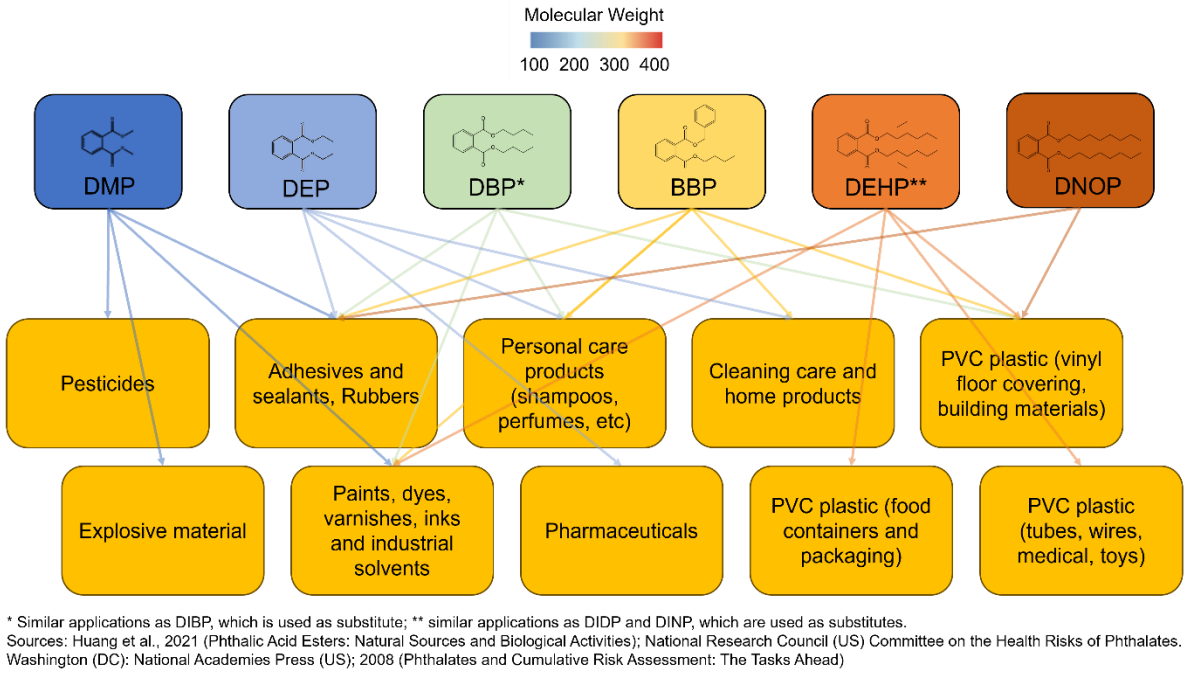


Figure 1.5 Plasticisers' production and use in Europe. HMW: High Molecular Weight; LMW: Low Molecular Weight. Graph taken from Plasticisers.org. Source: 2020 IHS and European Plasticisers estimates.

Different PAEs have a wide range of different properties (Table 1.1) and, thus, different applications (Figure 1.6). Other applications than plasticisers encompass adhesives, detergents, air fresheners, lubricating oils, and clothing. They are also found in personal-care products like soaps, shampoos, hair sprays, perfumes, and nail polishes. Certain PAEs serve as coatings for pharmaceuticals, herbal preparations, and dietary supplements (Teuten et al. 2009; Net et al. 2015; Heudorf, Mersch-Sundermann, and Angerer 2007; Giuliani et al. 2020).

Table 1.1 Chemical structure and properties of some of the most common PAEs. Data taken from SciFinder® and PubChem databases. MM: Molecular mass.

Analyte	Full name	Molecular formula	MM (g/mol)	Solubility in water (g/L, 25 ºC)	Vapor pressure (mmHg, 25 ºC)	Log K_{OW}	Melting point (ºC)	Boiling point (ºC)
DMP	Dimethyl phthalate	$C_{10}H_{10}O_4$	194.2	4.3	3.08·10-3	1.60	5.5	284
DEP	Diethyl phthalate	$C_{12}H_{14}O_4$	222.2	1.08	2.1·10-3	2.47	-3	295
DIBP	Di-isobutyl phthalate	$C_6H_{22}O_4$	278.3	0.0062	$4.76 \cdot 10^{-5}$	4.11	-37	320
DBP	Di-n-butyl-phthalate	$C_{16}H_{22}O_4$	278.2	0.0112	$2.01 \cdot 10^{-5}$	4.72	-35	340
BBP	Butyl-Benzyl-phthalate	$C_{19}H_{20}O_4$	312.1	2.69	$8.25 \cdot 10^{-6}$	4.73	-35	370
DCHP	Dicyclohexyl phthalate	$C_{20}H_{26}O_4$	330.2	4.0	$8.69 \cdot 10^{-7}$	6.20	66	225
DEHP	Di-(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.3	0.00027	$1.42 \cdot 10^{-7}$	7.60	-55	230
DNOP	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.6	0.000022	$1.0 \cdot 10^{-7}$	8.20	-25	385
DINP	Diisononyl phthalate	$C_{26}H_{42}O_4$	419.3	0.0002	$5.40 \cdot 10^{-7}$	9.37	-48	406
DIDP	Diisodecyl phthalate	$C_{28}H_{46}O_4$	446.3	0.00028	$5.28 \cdot 10^{-7}$	10.36	-50	423



* Similar applications as DIBP, which is used as substitute; ** similar applications as DIDP and DINP, which are used as substitutes.
Sources: Huang et al., 2021 (Phthalic Acid Esters: Natural Sources and Biological Activities); National Research Council (US) Committee on the Health Risks of Phthalates. Washington (DC): National Academies Press (US); 2008 (Phthalates and Cumulative Risk Assessment: The Tasks Ahead)

Figure 1.6 Applications of some of the most common PAEs.

Phthalates can be categorised into two groups: low molecular weight (LMW), comprising dialkyl phthalates with side chains ranging from C3 to C6 as the longest straight chain carbon backbone, and high molecular weight (HMW) phthalates, encompassing dialkyl phthalates with side chains ranging from C7 to C13 as the longest straight chain carbon backbone (ECHA 2013). These designations are in line with established structure-activity relationships: LMW phthalates (e.g., DEHP, DBP, BBP, and DIBP) are associated with adverse reproductive effects, while HMW phthalates (e.g., DINP, DIDP, DPHP) are not linked to such effects, and thus, are used to substitute hazardous phthalates (Figure 1.7) (Fabjan et al. 2006; Saillenfait et al. 2011; 2013).

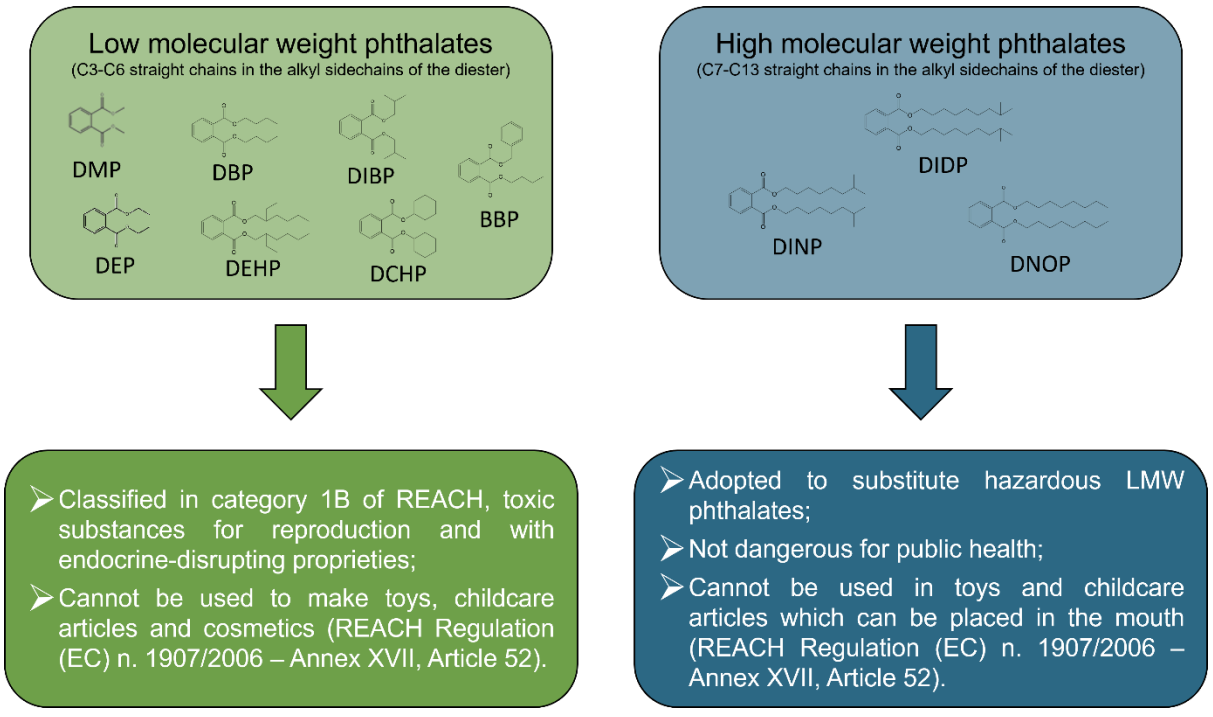


Figure 1.7 Categorization of the two main classes of PAEs, as defined by the European Chemicals Agency (ECHA).

LMW phthalates like DEHP, DBP, DIBP, and BBP are classified in the Substances of Very High Concern (SVHC) list as toxic to reproduction (Repr. 1B), signifying potential harm to fertility and fetal development. DBP and BBP are classified as very toxic to aquatic life (aquatic acute 1), with BBP also designated as having long-lasting effects on aquatic ecosystems (aquatic chronic 1). Ortho-phthalates designated as hazardous to reproduction

(Repr. 1B) are subject to restrictions both in their individual forms and when found in mixtures intended for consumer use. Since July 2020, DEHP, DBP, DIBP, and BBP have faced extensive restrictions in various products, including children's swimming aids, flooring, coated fabrics, recreational equipment, mattresses, footwear, office supplies, consumer clothing or related accessories as well as in other textiles that come into contact with the skin. Notably, DEHP, DBP, BBP, and DIBP, initially listed for reproductive toxicity, were expanded in November 2021 to encompass endocrine-disrupting effects, necessitating REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) authorisation for certain previously exempted uses. Notable examples encompass DEHP application in food contact materials, medical devices, immediate medicinal packaging due to environmental risks, and BBP and DBP usage in immediate medicinal packaging. Although certain phthalates are banned within Europe, their regulatory status differs outside the EU and products incorporating these phthalates might still be present in the EU market (Table 1.2).

Table 1.2. Restrictions of PAEs regulations for plastic food contact materials and drinking water (from Tran et al., 2022).

Phthalate (PAEs)	**Regulation of European Union (EU) for phthalate in plastic food contact materials**			
	Maximum content by weight (%)	Specific migration limit (mg kg^{-1})	Tolerable daily intake (μg kg^{-1} bodyweight day^{-1})	
BBP	≤ 0.1	≤ 30	50	
DBP	≤ 0.05	≤ 0.3	50	
DEHP	≤ 0.1	≤ 1.5	50	
DIDP	≤ 0.1	≤ 9	–	
DINP	≤ 0.1	≤ 9	50	
	Regulation in and outside EU for DEHP levels in drinking water (μg L^{-1})			
	WHO	EU	U.S.	Japan
DEHP	8 μg L^{-1}	8 μg L^{-1}	6 μg L^{-1}	100 μg L^{-1}

PAEs are mainly applied as external plasticisers: they are not covalently bonded to the plastic resin matrix but are held through intermolecular forces, such as hydrogen bonding and other van der Waals forces. Due to the weak attraction between the plasticiser molecule and the polymer chain, the plasticiser is easily lost through volatility, extraction, migration, chemical degradation, and biological degradation. As a result, PAEs can easily leach into both abiotic and biotic environmental components (Wadey 2003; Katsikantami et al. 2016; Hidalgo-Serrano et al. 2022). These plastic additives can thus be released into the marine environment by numerous pathways, including wastewater, atmospheric transport, and river runoff (Xie et al. 2007; Net et al. 2015; Hidalgo-Serrano et al. 2022) (Figure 1.8). The massive amount of plastic waste in the marine environment constitutes a major input of PAEs contamination in the ocean (Cao et al. 2022).

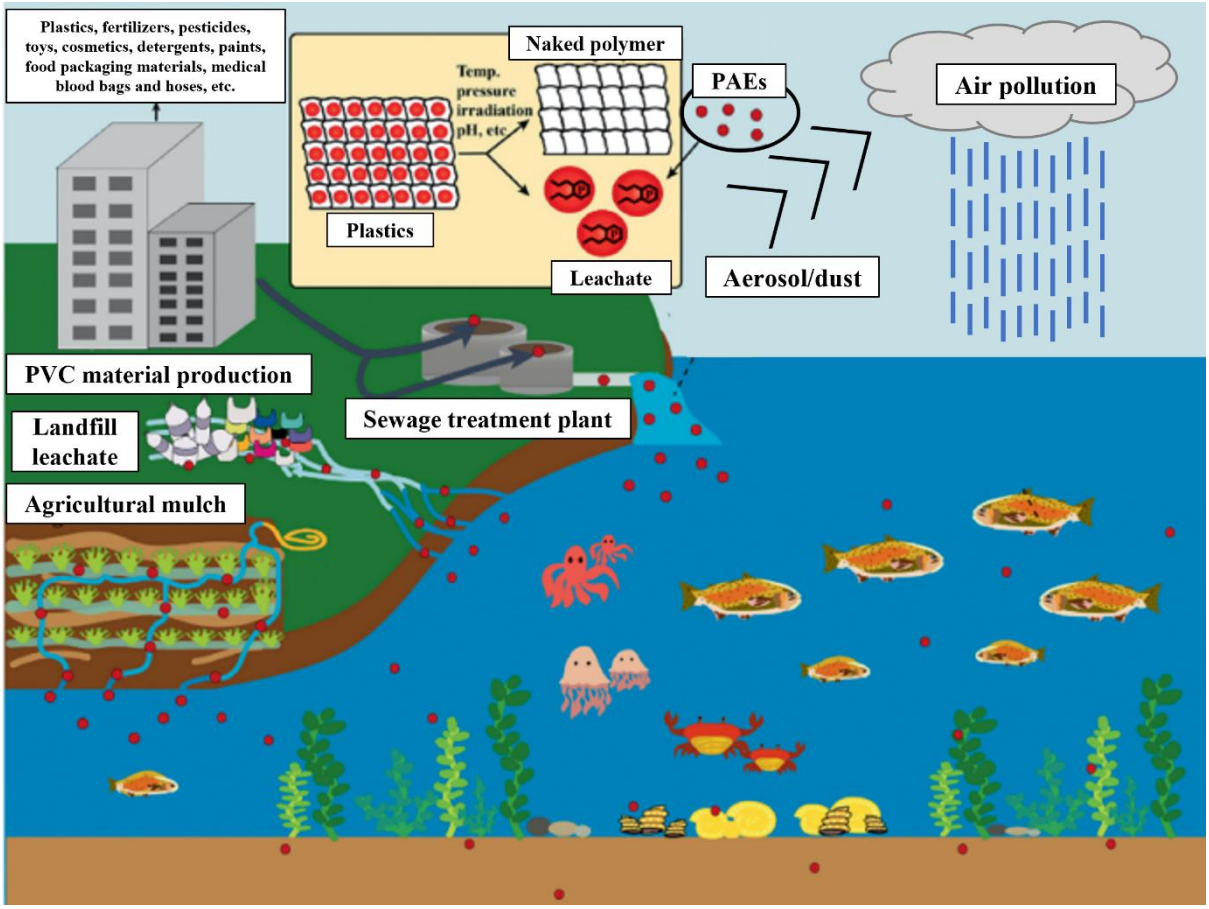


Figure 1.8 Sources, transport and pathways of PAEs into the marine environment. Representation modified from Karim et al., 2022.

Given their high octanol-water partition coefficient (K_{ow}), some phthalates are highly lipophilic. When (micro)plastics are ingested by aquatic organisms, gut surfactants, acidic conditions, and elevated temperatures (in warm-blooded animals) in the animal's gut increase the release of these lipophilic plastic-associated chemicals, which may accumulate in the organisms' tissues and subsequently transfer along the food chain (Bakir, Rowland, and Thompson 2014; Coffin et al. 2019). For this reason, phthalates are commonly detected in marine samples, including seawater, sediment, and biota (Net et al. 2015; Hidalgo-Serrano et al. 2022). However, PAEs are not persistent and are biodegraded in the environment (Net et al. 2015). They are quickly metabolised and excreted in mammals (Wittassek and Angerer 2008; Hart et al. 2018) and do not biomagnify in marine food webs (Gobas et al. 2003; Mackintosh et al. 2004). Nonetheless, their continuous environmental release might lead to high concentrations and chronic exposure in marine organisms (Pamplona-Silva et al. 2018; Gani, Tyagi, and Kazmi 2017; Warner and Flaws 2018).

4. Use of phthalates as plastic tracers

As previously mentioned, PAEs are the most common plasticisers worldwide and are easily leached into the environment. For this reason, some authors have proposed the use of PAEs as "plastic tracers" *i.e.,* a compound that, if detected in an organism's tissue, would indicate previous or co-occurrent exposure to plastic (usually through ingestion) (e.g., Fossi et al. 2012; Hardesty et al. 2015; Baini et al. 2017; Vered et al. 2019). Although logical, such a hypothesis is still controversial, since PAEs contamination in marine compartments can originate from other sources besides plastics, such as polluted effluents, industrial waste, wastewater discharges, and atmospheric deposition (Hidalgo-Serrano et al. 2022).

To define the state-of-the-art knowledge regarding the use of phthalates as plastic tracers in the marine environment, a systematic review was carried out following the Preferred

Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA; McIvor et al. 2022; Correia et al. 2023). Records were searched on Google Scholar and Scopus using the words "phthalate" OR "PAE" OR "phthalic acid ester" AND "plastic" OR "microplastic" OR "litter" OR "debris" AND "marine" OR "ocean" OR "sea" OR "basin", including only peer-reviewed research paper (excluding reviews, book chapters, and grey literature such as thesis, reports or conference communications) published between 1990 and June 2023. Other inclusion/exclusion criteria are listed in Table 1.3.

Table 1.3. Inclusion and exclusion criteria for the selection of studies in the systematic review.

Inclusion criteria	Exclusion criteria
Published between 1990 and June 2023	Published outside of the range specified
Peer-reviewed original research articles	Reviews, book chapters, grey literature such as thesis, reports, conference communications etc.
Field studies	Laboratory or modeling studies
Studies conducted in the marine environment	Studies conducted in freshwater
Studies investigating the relationship between MPs and PAEs	Studies that reported MPs and PAEs concentrations without analysing their relationship

Only the first ten pages of results from Google Scholar were considered, due to the negligible probability of finding further relevant publications past the 10th results page (McIvor et al. 2022; Correia et al. 2023). However, the following ten pages were checked, screening the papers' titles to ensure not to miss any important study, considering the inclusion/exclusion criteria. In addition, the most cited paper in Scopus, which could be included by the inclusion/exclusion criteria (Baini et al. 2017), was introduced in the "connecting papers platform" (https://www.connectedpapers.com) to identify other papers

which might have been neglected. A flowchart of the whole research and selection process is represented in Figure 1.9.

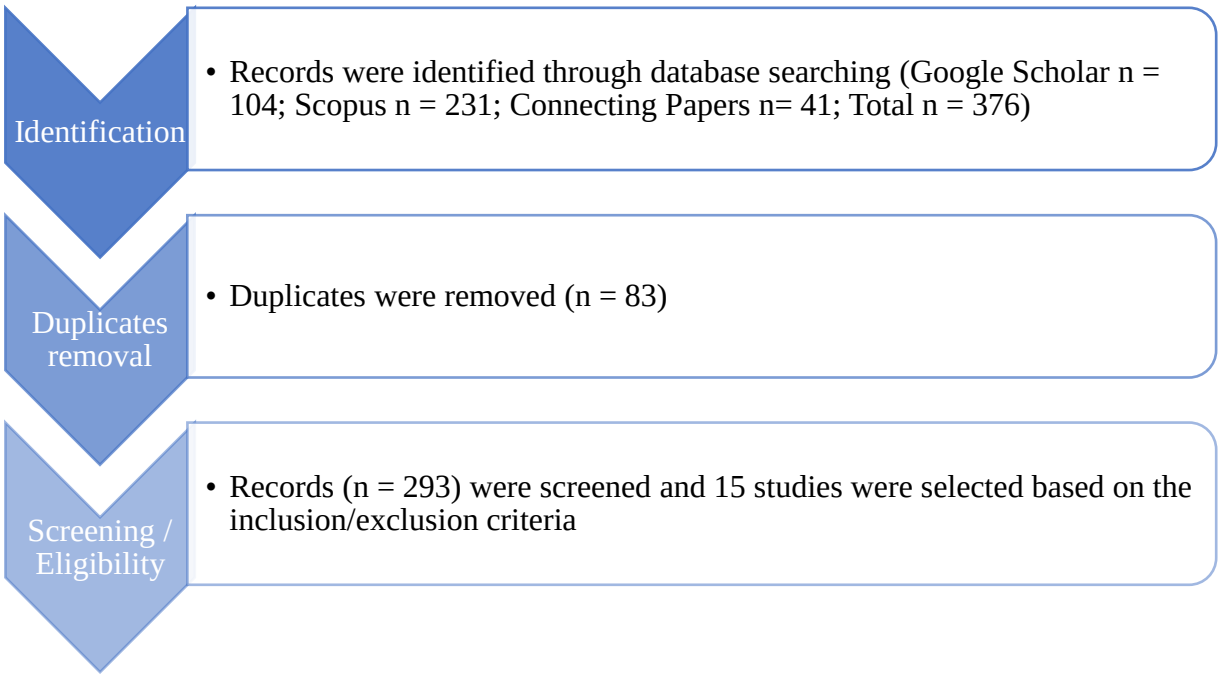


Figure 1.9 Flowchart of the literature search and selection process with the number (n) of studies in each step.

Among all the studies identified through the literature search, over 90% were published after 2013 (Figure 1.10). The highest number of publications (55) was reached in 2021.

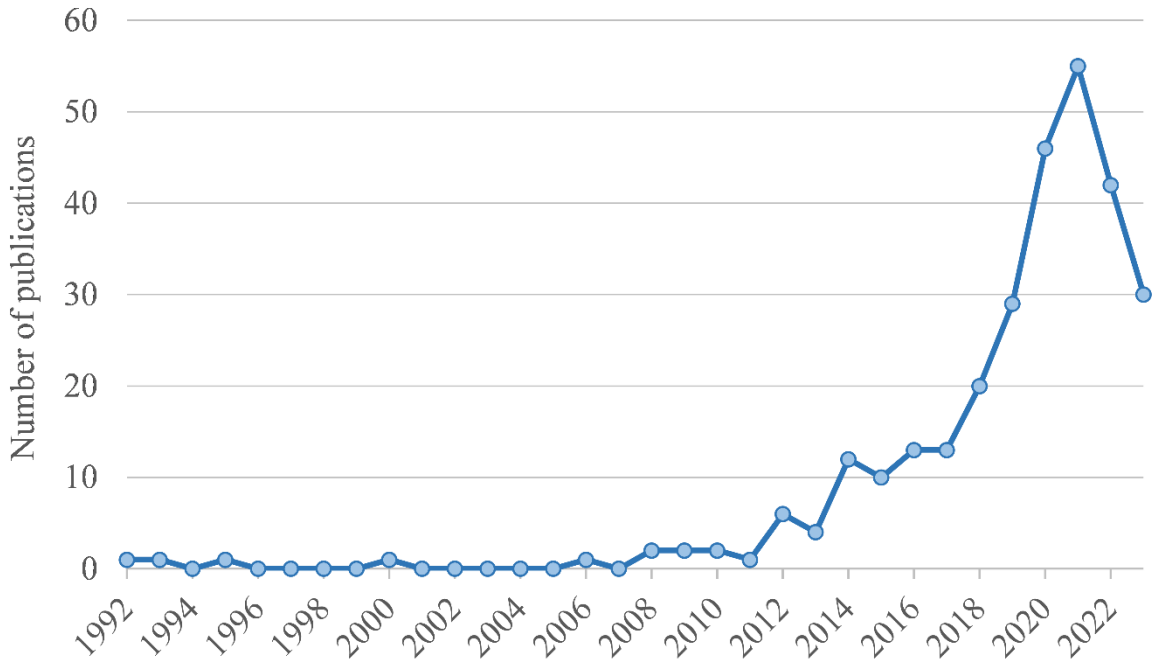


Figure 1.10 Temporal trend of publications found through literature search after removal of duplicates (n=293). Note that 2023 was an incomplete year (Jan-Jun).

Only 15 studies were selected after screening 293 non-duplicated records (listed in Table 1.4); however, among the excluded articles, many supported or used the "plastic tracers" hypothesis.

Several studies reported the leaching of PAEs from plastic particles in the aquatic environment in laboratory conditions (e.g., Paluselli et al. 2019; Henkel, Hüffer, and Hofmann 2022; Gulizia et al. 2023; Li and Tang 2023; Zhong et al. 2023), while others investigated and reported enhanced leaching in simulated gut conditions (e.g., Bakir, Rowland, and Thompson 2014; Coffin et al. 2019; Trujillo-Rodríguez et al. 2021). A consistent number of papers reported the detection of PAEs in plastic particles found in the marine environment *(*e.g., Llorca et al. 2021; Rani et al. 2021; Takdastan et al. 2021; Pal et al. 2023; Tun et al. 2023); others reported PAEs concentrations in different marine environmental compartments and assumed their presence as an indicator of plastic exposure, although plastic items were not investigated (e.g., Zhang et al. 2019; Montano et al. 2020; Andrea Paluselli and Kim 2020; F. Saliu et al. 2020; Savoca et al. 2018; 2021).

Furthermore, some studies reported both MPs and PAEs concentrations but did not examine their correlation (e.g., Fossi et al. 2016; Padula et al. 2020; Polidoro, Lewis, and Clement 2022; Talley et al. 2022; Squillante et al. 2023). Notably, a considerable amount of the screened papers reported new methodologies for PAEs extraction and analysis (e.g., He et al. 2015; Baini et al. 2017; ZHANG et al. 2017; Fauvelle et al. 2018; Wu et al. 2018; Akoueson et al. 2022), highlighting how the analysis of PAEs in environmental and biological matrices is a highly challenging task, primarily due to widespread background contamination in laboratory products and high matrix-effects of the target samples (Guo and Kannan 2012; Hidalgo-Serrano et al. 2022).

A summary of the chosen studies is provided in Table 1.4, and their respective geographical locations are shown in Figure 1.11. Among these, one study reported results for

three different taxa (bivalves, holothurians, fish; Rios-Fuster et al. 2022), which were considered separately, for a total of 17 examined MPs-PAEs relationships. Different types of samples were analysed in the considered studies: sediments (n = 3), surface seawaters (n = 3), plankton/neuston (n = 3), and marine biota (invertebrates n = 4; vertebrates n = 4). Out of the 17 analysed relationships, nine studies reported a positive correlation between MPs abundance and PAEs concentrations, while eight studies did not find any correlation.

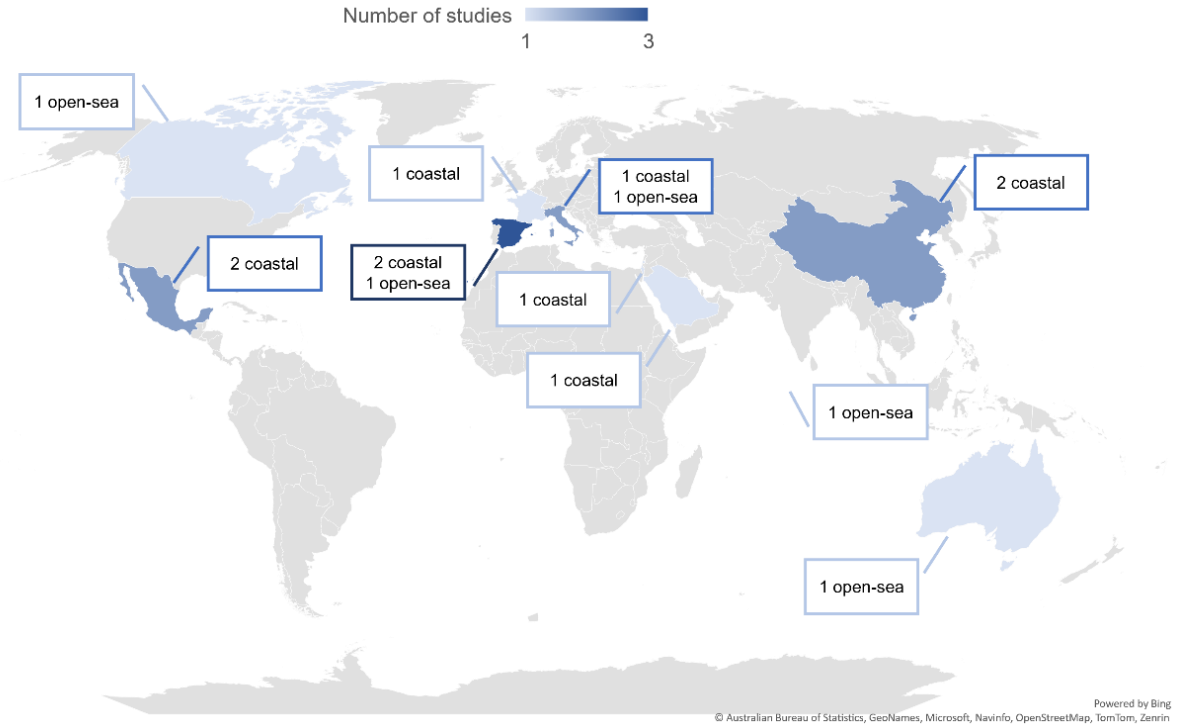


Figure 1.11 World map displaying the locations and the number of studies per country identified through the systematic literature review. Information in white boxes specifies whether the studies were conducted in coastal or open-sea waters.

These contradictory findings regarding the relation between PAE concentrations and MPs abundance, and hence the use of PAEs as plastic tracers, might be attributed to a significant input of PAEs from sources other than plastics. This phenomenon may be particularly relevant in coastal areas near wastewater and industrial effluents (Schmidt et al.

2021). In addition, the rapid metabolic degradation of PAEs in vertebrates might also play a crucial role.

The results of this literature analysis show the scarcity of data on the co-occurrence of MPs and PAEs in marine ecosystems, especially in open ocean waters, as well as the significant uncertainty surrounding the use of PAEs as plastic tracers, emphasising the need for further research.

Table 1.4. Summary table of the 15 studies selected according to the inclusion/exclusion criteria of the systematic review.

Location (country)	Environment	Type of sample	Species (if applicable)	MPs abundance (mean)	Type of PAEs	Individual PAEs concentrations (range)	Significant MPs-PAEs correlation	Correlated MPs - PAEs	Reference
North-west Mediterranean Sea (Italy)	open-sea waters	plankton/neuston	-	-	MBZP, MBP, MEHP, DNHP, BBP, DEHP, DIOP, DnDP	6 - 2709 ng/g dw	Yes (positive)	MPs < 0.5 mm with MEHP, MBzP, BBP + MPs 0.5-1 mm and 2.5-5 mm with BBP, MBP	Baini et al., 2017
Gulf of Mexico (Campeche, Mexico)	coastal waters	sediment	-	-	DMP, DEP, DBP, BBP, DEHP, DnOP, DEHA	65 - 6970 ng/g dw	Yes (positive)	Total MPs with ∑7PAEs	Borges Ramirez et al., 2019
Red Sea (Saudi Arabia)	coastal waters	surface seawater	-	0.04 ± 0.02 items/m3	DMP, DEP, DBP, BBP, DEHP, DnOP	0.8 - 1124 ng/L	Yes (positive)	Total MPs (as surface area, mm2/mm3) with ∑6PAEs	Dhavamani et al., 2022
La Paz Bay (Mexico)	coastal waters	plankton/neuston	-	-	MBZP, MBP, MEHP, DNHP, BBP, DEHP	0 - 3055.2 ng/g d.w.	No	-	Galli et al., 2023
North-east Queensland (Australia)	open-sea waters	digestive tract (MPs) and preen oil from uropygial gland (PAEs)	Short-tailed shearwaters (*Puffinus tenuirostris*), wedge-tailed shearwaters	-	DMP, DBP, DEHP	0 - 220 ng/mL	Yes (positive)	Total MPs with DBP and DEHP	Hardesty et al., 2015

			(*Ardenna pacifica*)						
Jiaozhou Bay (China)	estuarine/coastal waters (semi enclosed bay)	sediment	-	4527.5 ± 348.8 µg/kg	DEHP	0 - 591.2 ng/g	No	-	Li et al., 2021
Jiaozhou Bay (China)	estuarine/coastal waters (semi enclosed bay)	surface seawater	-	80.46 ± 44.82 items/m3	DMP, DEP, DBP, BBP, DEHP, DnOP	< MDL - 617.18 ng/L	Yes (positive)	Total MPs (items/m3) with ∑6PAEs (ng/L)	Liu et al., 2020
Macaronesia, North-East Atlantic (Spain)	open-sea waters	digestive tract (MPs) and muscle (PAEs)	Six odontocete species (*S. coeruleoalba, T. truncatus, G. griseus, G. macrorhynchus , K. berviceps, L. hosei*)	59.08 ± 40.52 fibres/individual	DEP, BBP, DEHP	0 - 1533 ng/g	No	-	Montoto-Martínez et al., 2021
Labrador Strait (Canada)	open-sea waters	digestive tract (MPs) and preen oil from uropygial gland (PAEs)	Northern fulmars (*Fulmarus glacialis*)	31.6 ± 32.3 items/individual	DMP, DEP, DBP, BBP, DEHP, DnOP	Not detected (high MDL)	No	-	Provencher et al., 2020
Sardinia, Western Mediterranean Sea (Italy)	coastal waters	digestive tract (MPs) and gonads (PAEs)	Sea urchins (*Paracentrotus lividus*)	1.0 ± 0.30 items/individual	DMP, DEP, DBP, BBP, DEHP	0 - 73 ng/g	Yes (positive)	Fibres with DEHP	Raguso et al., 2022
Cabrera MPA, Western Mediterranean Sea (Spain)	coastal waters	whole organism	Bivalves (*Arca noae*)	4.83 ± 5.35 items/individual	DEP, DBP, DEHP	-	No	-	Rios-Fuster et al., 2022
Cabrera MPA, Western Mediterranean Sea (Spain)	coastal waters	digestive tract (MPs) and muscle (PAEs)	Holoturians (*Holothuria forskalii, Holothuria poli, and Holothuria tubulosa*)	12.7 ± 7.3 items/individual	DEP, DBP, DEHP	-	Yes (positive)	Total MPs with DEP and DEHP	Rios-Fuster et al., 2022

Cabrera MPA, Western Mediterranean Sea (Spain)	coastal waters	digestive tract (MPs) and muscle (PAEs)	Fish (*Oblada melanura, Diplodus vulgaris, Serranus cabrilla, Serranus scriba*)	3 ± 4.4 items/individual	DEP, DBP, DEHP	-	No	-	Rios-Fuster et al., 2022
Barcelona continental shelf (Spain)	coastal waters	sediment	-	381.9 ± 348.2 items/Kg dw	DMP, DEP, DIBP, BBP, DHP, DEHA, DEHP, DnOP, DCHP, DiNP, DiDP	10 - 1040 ng/g dw	Yes (positive)	Fibres with DEHP, Total MPs with DEHP	Saliu et al., 2023
Faafu Atoll (Maldives)	open-sea waters	plankton/neuston	-	0.46 ± 0.15 items/m3	DMP, DEP, MEHP, DBP, BBP, DEHP	<MDL - 228 ng/g dw	Yes (only qualitative, no statistics)	Total MPs with ∑6PAEs	Saliu et al., 2019
NW Mediterranean Sea (France)	coastal waters	surface seawaters	-	0.051 ± 0.05 items/m3	DMP, DEP, DBP, DiBP, BBP, DEHP, DnOP	100 - 527 ng/L 454 ng/L	No	-	Schmidt et al., 2021
Eastern Mediterranean Sea, Red Sea (Israel)	coastal waters	whole organism	Ascidians (*Herdmania momus, Microcosmus exasperatus*)	1.37 ± 1.29 items/individual	DBP, DEHP, DnOP	0 - 13000 ng/g dw	No	-	Vered et al., 2019

5. Thesis objectives and outline

The present thesis aims to investigate the occurrence and interactions of PAEs and MPs in an open oceanic environment, focusing on the pelagic food web. The study focused on Madeira Island, a remote oceanic island characterised by a narrow shelf, a predominant deep-sea environment, and oligotrophic waters. The study aims to answer the following questions:

(i) Is the pelagic food web from an open oceanic environment affected by MPs contamination, and at which level?

(ii) Are PAEs found in co-occurrence with MPs in pelagic organisms, and could they be used as indicators of (micro)plastic exposure in an oceanic environment?

(iii) How are pelagic top predators, such as cetaceans, affected by PAEs contamination?

To do so, four steps were followed:

i) Planktonic samples were collected for one year to seasonally characterise the contamination by MPs in pelagic waters and relate this with the abundance and diversity of zooplankton (Chapter 2);

ii) An accurate, simple, and effective methodology for PAEs extraction and analysis from biological samples using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method together with GC-MS was developed and validated (Chapter 3);

iii) A modified version of the QuEChERS extraction method was adapted and validated for small quantities of cetacean blubber (Chapter 4);

iv) The most common species of small pelagic fish and mesopelagic squids were collected for one year; MPs and PAEs were analysed in their tissues and were correlated to confirm PAEs as valuable indicators of plastic exposure (Chapter 5);

v) Biopsy samples of free-ranging individuals of short-finned pilot whale (*Globicephala macrorhynchus*) and common bottlenose dolphin (*Tursiops truncatus*) were collected and analysed for PAEs concentrations to determine the burden of plasticiser pollution in pelagic top predators (Chapter 6);

6. Thesis publications

Chapter 2:

Sambolino, Annalisa, Inma Herrera, Soledad Álvarez, Alexandra Rosa, Filipe Alves, João Canning-Clode, Nereida Cordeiro, Ana Dinis, and Manfred Kaufmann. 2022. 'Seasonal Variation in Microplastics and Zooplankton Abundances and Characteristics: The Ecological Vulnerability of an Oceanic Island System'. *Marine Pollution Bulletin* 181: 113906. https://doi.org/10.1016/j.marpolbul.2022.113906.

The author of this thesis, as first author of the publication, contributed in conceiving the study, designing the methodologies, collecting samples and data, conducting laboratory and statistical analyses, organizing data, creating figures and tables, and composing the initial draft, as well as reviewing and editing the manuscript.

Chapter 3:

Sambolino, Annalisa, Cecilia Ortega-Zamora, Javier González-Sálamo, Ana Dinis, Nereida Cordeiro, Joao Canning-Clode, and Javier Hernández-Borges. 2022. 'Determination of Phthalic Acid Esters and Di (2-Ethylhexyl) Adipate in Fish and Squid Using the Ammonium Formate Version of the QuEChERS Method Combined with Gas Chromatography Mass Spectrometry'. *Food Chemistry* 380: 132174. https://doi.org/10.1016/j.foodchem.2022.132174

The author of this thesis, as first author of the publication, contributed in collecting samples and data, conducting laboratory and statistical analyses, organizing data, creating tables, and reviewing and editing the manuscript.

Chapter 4:

Sambolino, Annalisa, Marta Rodriguez, Jesus De la Fuente, Manuel Arbelo, Antonio Fernández, Manfred Kaufmann, Nereida Cordeiro, and Ana Dinis. 2024. 'Optimization and Validation of a Micro–QuEChERS Method for Phthalates Detection in Small Samples of Cetacean Blubber'. MethodsX 12 (June): 102502. https://doi.org/10.1016/j.mex.2023.102502.

The author of this thesis, as first author of the publication, contributed in conceiving the study, designing the methodologies, conducting statistical analyses, organizing data, creating tables, and composing the initial draft, as well as reviewing and editing the manuscript.

Chapter 5:

Sambolino, Annalisa, Eva Iniguez, Inma Herrera, Manfred Kaufmann, Ana Dinis, and Nereida Cordeiro. 2023. 'Microplastic Ingestion and Plastic Additive Detection in Pelagic Squid and Fish: Implications for Bioindicators and Plastic Tracers in Open Oceanic Food Webs'. *Science of The Total Environment*, 164952. https://doi.org/10.1016/j.scitotenv.2023.164952

The author of this thesis, as first author of the publication, contributed in conceiving the study, designing the methodologies, collecting samples and data, conducting laboratory and statistical analyses, organizing data, creating figures and tables, and composing the initial draft, as well as reviewing and editing the manuscript.

Chapter 6:

Sambolino, Annalisa, Filipe Alves, Marta Rodriguez, Mieke Weyn, Rita Ferreira, Ana M. Correia, Massimiliano Rosso, Manfred Kaufmann, Nereida Cordeiro and Ana Dinis.

(originally submitted September 2023; first revision submitted February 2024; second revision submitted April 2024). 'Phthalates and Fatty Acid Markers in Free-ranging Cetaceans from an Insular Oceanic Region: Ecological Niches as Drivers of Contamination'. *Environmental Pollution* (*ENVPOL-D-23-06966*).

The author of this thesis, as first author of the publication, contributed in conceiving the study, designing the methodologies, collecting samples and data, conducting statistical analyses, organizing data, creating figures and tables, and composing the initial draft, as well as reviewing and editing the manuscript.

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CHAPTER II

SEASONAL VARIATION IN MICROPLASTICS AND ZOOPLANKTON ABUNDANCES AND CHARACTERISTICS: THE ECOLOGICAL VULNERABILITY OF AN OCEANIC ISLAND SYSTEM

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Chapter 2 - Seasonal Variation in Microplastics and Zooplankton Abundances and Characteristics: The Ecological Vulnerability of an Oceanic Island System

Abstract

The ingestion of microplastics (MPs - plastic particles <5 mm) by planktivorous organisms represents a significant threat to marine food webs. To investigate how seasonality might affect plastic intake in oceanic islands' ecosystems, relative abundances and composition of MPs and mesozooplankton samples collected off Madeira Island (NE Atlantic) between February 2019 and January 2020 were analysed. MPs were found in all samples, with fibres accounting for 89 % of the particles. MPs and zooplankton mean abundance was 0.262 items/m^3 and 18.137 individuals/m^3, respectively. Their monthly variations follow the seasonal fluctuation of environmental parameters, such as currents, chlorophyll-*a* concentration, sea surface temperature and precipitation intensity. A higher MPs/zooplankton ratio was recorded in the warm season (May-Oct), reaching 0.068 items/individual when considering large-sized particles (1000–5000 μm). This is the first study to assess the seasonal variability of MPs in an oceanic island system providing essential information respecting its ecological impact in pelagic environments.

Keywords

Macaronesia, Marine trophic web, Marine litter, Oceanographic parameters, Pelagic environment, Plastic pollution.

1. Introduction

Plastic production is ever-increasing, with plastic litter accumulating in the environment worldwide and being found in high abundance in specific oceanic areas (Cozar et al., 2014). Exposed to weathering, plastics break down into smaller pieces, which are generally defined as microplastics (MPs) when they reach dimensions smaller than 5 mm (GESAMP, 2016; Frias and Nash, 2019). Furthermore, several industries directly manufacture plastics of such microscopic sizes (defined as "primary microplastics") (Cole et al., 2011). As studies concerning MPs are emerging, there is clear evidence of a widespread distribution of such contaminants in the marine environment and their adverse effects on biota (Botterell et al., 2019; Hale et al., 2020; Mallik et al., 2021). MPs are raising particular concern, mainly because of the high likelihood of entering marine trophic webs, occupying the same size fraction as zooplanktonic organisms (Hidalgo-Ruz et al., 2012; Wright et al., 2013).

Zooplankton represents one of the basic components of marine food webs. Zooplanktivorous predators are abundant in the ocean, and they can accidentally ingest plastic particles, mistaking them for food (Boerger et al., 2010; Lusher et al., 2013; Barboza et al., 2020). The feeding mechanisms of marine predators are still poorly understood. However, several studies suggested that planktivorous organisms apply a selective behaviour, preferring larger preys when available and choosing specific shapes and colours (Gardner, 1981; Hansen et al., 1997; Barton et al., 2013). Shaw and Day (1994) hypothesised that some marine organisms selectively ingest white and lighted colour plastic fragments, mistaking them for food. Ory et al. (2017) found a higher presence of blue MPs in the gastrointestinal tract of a visual predatory fish (*Decapterus muroadsi*), suggesting that these plastic particles were mistaken for blue copepod prey. Ingestion rates, however, also largely depend on the concentration at which potential prey is found (Wright et al., 2013; Kiørboe and Hirst, 2014). Thus, analysing sizes, characteristics, and relative abundances of MPs and zooplankton in

marine environments is crucial to understand the probability of plastic intake in the trophic web.

Plastic ingestion by planktivorous organisms has been reported in marine environments worldwide (Boerger et al., 2010; Lusher et al., 2013; Ory et al., 2017; Barboza et al., 2020). MPs are often associated with toxic pollutants (e.g., plastic additives such as phthalates and bisphenols, heavy metals, polybrominated diphenyl ether (PBDE), polychlorinated biphenyls (PCBs)), and they can negatively affect marine organisms both chemically *(*e.g., oxidative damage, endocrine disruption, immunity response) and physically (e.g., blockage of the digestive system) (Brennecke et al., 2016; Galloway et al., 2017; Cunha et al., 2020). Epipelagic fish can be particularly vulnerable to such a threat, as many synthetic particles are found at the sea surface, being made of low-density material and buoyant (Barnes et al., 2009; Cole et al., 2011). For this reason, most studies examined the presence of MPs in neustonic samples and reported the MPs/zooplankton ratio as an indicator to infer plastic ingestion by zooplankton feeders (e.g., Moore et al., 2001; Collignon et al., 2014; Kang et al., 2015).

However, such ratio might be overestimated in neustonic samples, as zooplanktonic organisms are more widespread in the water column than MPs, mainly found in high concentrations in the first centimetres of the sea surface (Vasilopoulou et al., 2021). Sub-surface samples collected by vertical hauls are representative of the whole water column, but the trawls are punctual and filter smaller volumes of water. In contrast, oblique or sub-surface horizontal transects could be the most representative of the real threat posed by MPs ingestion for epipelagic organisms, inferred by the MPs/zooplankton ratio.

Oceanic dynamics can greatly affect the occurrence and distribution of MPs and zooplankton in marine environments (van Sebille et al., 2020), especially when considering deep ocean island ecosystems. These pristine and remote ecosystems are often considered

important biodiversity hotspots (Gove et al., 2016). The productivity enhancement in the waters surrounding oceanic islands is usually related to island-induced physical processes, such as the formation of wakes, eddies, fronts, and upwelling cells (defined as the Island Mass Effect - IME) (e.g. Caldeira et al., 2002; Hasegawa, 2019). However, local events of island surface run-off, with discharges of freshwater, terrigenous sediments, suspended matter and nutrients, can also significantly contribute to IME (Rosa et al., 2022). Similarly, the transport and distribution of plastic particles have been described in relation to physical processes such as ocean currents and wind (Cardoso and Caldeira, 2021; Brach et al., 2018) or precipitation events (Lima et al., 2015), and their seasonal variations can determine the convergence, accumulation, or dispersion of marine litter.

Cyclic fluctuations of critical environmental variables such as sea surface temperature, chlorophyll-*a* concentration and nutrient availability determine the zooplankton abundance and composition in the area (Longhurst, 1995; Mackas et al., 2012). Consequently, seasonality also affects the occurrence and feeding habits of the upper trophic levels (marine predators) and their vulnerability to plastic ingestion.

Thus, it is crucial to investigate the annual or seasonal variation of MPs and zooplankton characteristics and abundances to understand the vulnerability of marine food webs to plastic ingestion. To the best of our knowledge, only a few studies have analysed such variation (Collignon et al., 2014; Lima et al., 2015; Kang et al., 2015), and none has focused on ecosystems with complex dynamics such as a remote oceanic island.

In this context, the present study aims to quantify and characterise MPs and mesozooplankton found in sub-surface water samples collected in a pelagic environment off the south coast of Madeira Island (NE Atlantic Ocean) to (i) identify co-occurrence in size ranges and other characteristics (colour and shape), (ii) describe the seasonal variation in their abundance and composition, (iii) relate such variation with environmental variables, and iv)

identify critical seasons and size range for plastic ingestion according to higher MPs/zooplankton ratio.

2. Material and methods

2.1. Study Area

Surrounded by the abyssal plain of Madeira to the west and the African Continent to the east, Madeira Island is characterised by a pelagic and oligotrophic environment, with a narrow continental shelf and deep submarine canyons (Longhurst, 1995; Geldmacher et al., 2000; Narciso et al., 2019). Madeira Island is located at the edge of the Atlantic subtropical gyre, affected mainly by the Azores Current (Caldeira et al., 2002). Such subtropical current circulation can mediate the transportation and accumulation of plastic particles (Cardoso and Caldeira, 2021). The study area is also characterised by a relatively steady wind regime, under a predominant northeasterly flow, corresponding to the NE Atlantic trade winds (Caldeira et al., 2002). That flow gives rise to local acceleration near the island flanks, especially in summer, where two tip-jets are often present (Alves et al., 2020). The opposite sign of vorticity produced at the two tip-jets leads to the production of anticyclonic eddies near the east flank and cyclonic eddies near the west flank (Alves et al., 2020, Alves et al., 2021; Miranda et al., 2021). Furthermore, a high mountain ridge (ca. 1800 m) in the island's interior obstructs the dominant northeast trade winds, leading to warmer and sheltered waters in the south of Madeira (Caldeira et al., 2002; Caldeira and Sangrà, 2012; Azevedo et al., 2021).

The sampling area (Figure 2.1), located off the south coast of Madeira, is thus characterised by calm and warm waters, and it is often in the convergence zone of two opposite eddies. The area was chosen in the proximity of a particularly productive area (named “Picos”), which is the main aim of the small pelagic purse-seine fishery in Madeira (Tejerina et al., 2019). The effluents of two preliminary wastewater treatment plants (WWTPs - Funchal and Câmara

de Lobos), which serve the major portion of the population of Madeira Island, are present within a few kilometres from the sampling site (Figure 2.1 and Figure S2.1). Nearby is also located the river outlet of one of the largest drainage basins of the island (Ribeira dos Socorridos) (Rosa et al., 2022).

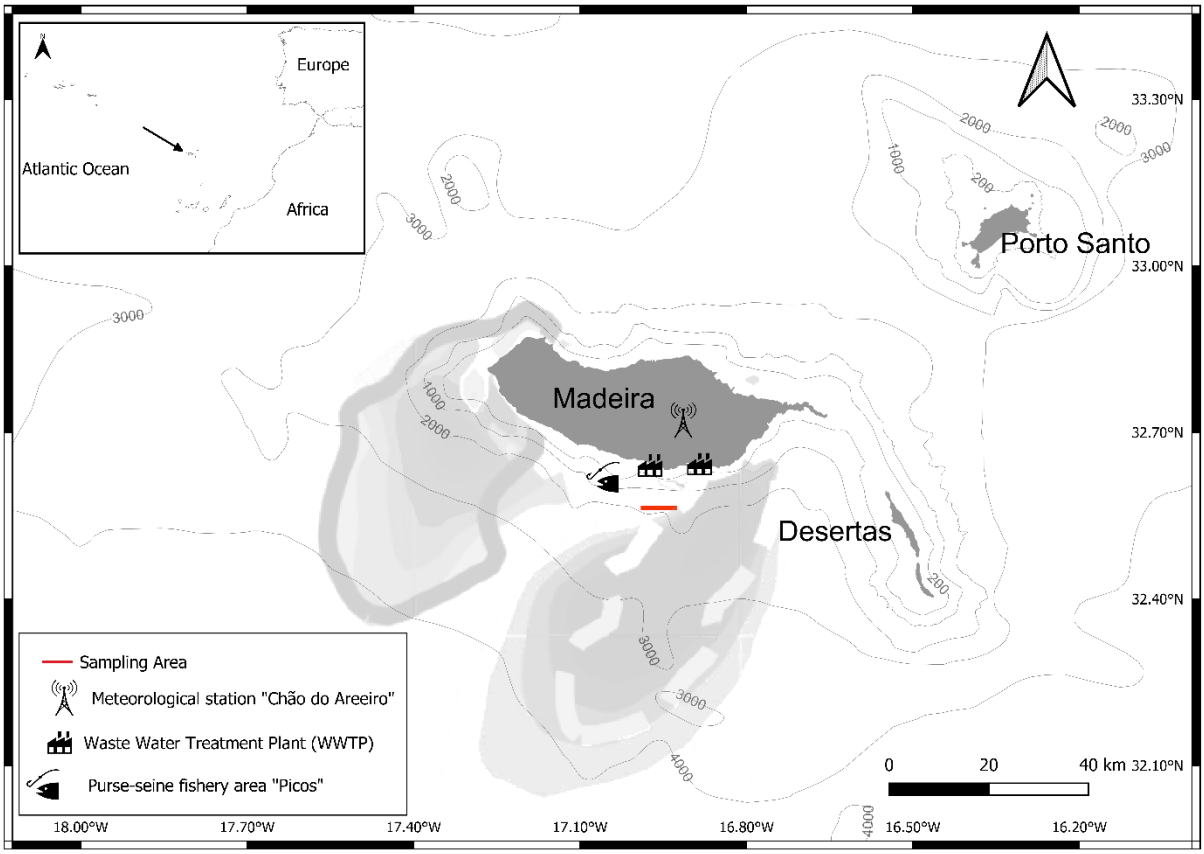

Figure 2.1 Location of the sampling area and points of interest. Thick lines and shades on the flanks of the island represent the oceanic eddies formations that take place mainly during summer, with cyclonic (solid dark line) and anticyclonic (dashed clear line) currents (adapted from Alves et al., 2021).

2.2. Sampling

Samples were collected using an Apstein plankton net (Hydro-Bios, Kiel, Germany) with a net mouth of 40 cm in diameter (0.125 m^2), mesh size 335 µm, and 100 cm net bag length. The Apstein net is a lightweighted version of the more common WP-2 net, serving for horizontal, vertical, or oblique tows. Horizontal tows were performed below the water surface (2–3 m), applying a light weight at the net mouth. The net was towed for 20 mins at 2–3 knots, approximately 25 m from the back of the boat, avoiding turbulence from the boat engine (Rigid

Inflatable Boat, <10 m length, with 150 cc engine). Samplings were performed only in good sea conditions (Beaufort scale ≤3) to maintain a steady linear course at a constant speed during the trawls. Trawled distance and water volume were measured with a mechanical flowmeter (General Oceanics Inc., USA) attached to the net. The net was rinsed thoroughly on board from the outside with seawater. The sample was then directly poured from the net collector into a 250 ml glass jar and preserved with formaldehyde (final concentration 4 %) until laboratory analysis. Samplings were performed during the daytime (9 am – 4 pm), with a monthly occurrence from February 2019 to January 2020, except in August due to logistical constraints.

2.3. Laboratory analysis

Each sample was filtered through 1 mm and 0.5 mm sieves in the laboratory, to obtain 3 sub-samples from 3 different size classes (335–500, 500–1000, 1000–5000 μm). For the two lower size classes, a 10–20 % aliquot of each subsample was used for taxonomic identification and quantification of zooplankton, while the rest was used for MPs analysis. The entire sub-samples from the larger size class (1000–5000 μm) were visually inspected for identification of planktonic organisms first and then for MPs, given the lower abundance of planktonic organisms found in this size class.

2.4. Zooplankton analysis

The zooplankton composition was determined by classification into the following 15 taxonomic groups: Amphipoda, Annelida, Chaetognatha, Cladocera, Copepoda, Crustaceans larvae, Decapoda, Echinodermata larvae, Eggs, Fish larvae, Mollusca, Ostracoda, Siphonophora, Thaliacea and Gelatinous (other). Only groups whose total abundance proportion was >1 % were considered in the analysis.

2.5. MPs analysis

To facilitate the visual identification of plastic particles, the organic matter in the sample was digested using H_2O_2 15 % heated at 40 °C for 24 h, following the methodology proposed by Frias et al. (2019). This method keeps a low temperature (40 °C), which does not affect the integrity of plastic particles (Alfonso et al., 2021). Previous studies observed no significant changes to microplastic particles following H_2O_2 digestion, including no evidence of microplastic bleaching, while the organic matter is either digested or decolourised (Avio et al., 2015; Hurley et al., 2018). Such protocol improves the chances of not including false positives in microplastic identification, especially microfibers. Furthermore, digestion protocols that make use of oxidising agents (as H_2O_2) usually yield high recovery rates (85–90 %) for the plastic particles in the samples (as reviewed in Way et al., 2022).

After digestion, samples were directly examined under the dissection microscope for the presence of MPs. Analysis was performed using a stereomicroscope (LEICA S9i) with an integrated camera (IC80 HD) to photograph each particle (Leica Software). Only particles smaller than 5 mm (defined as MPs) were considered for the analysis. We categorised particles according to colour (black, white, transparent, blue, yellow, red, green, other colours) and shape (fragments, fibres, lines, and films), while size classes were considered as those corresponding to the three sub-samples (335–500, 500–1000, 1000–5000 μm).

Particles were classified as plastics when showing homogenous colour, thickness, texture, and absence of cellular structures (Hidalgo-Ruz et al., 2012). When in doubt on suspected plastic particles, the hot needle test was used to observe the melting point of the material (Lusher et al., 2017). Moreover, staining with Nile Red was performed on the isolated particles to assess their fluorescence. Nile Red is a lipophilic fluorescent dye with a preferential adsorption to polymers compared to other inorganic interference (Maes et al., 2017). The co-staining of natural organic material can also occur (Shim et al., 2016), thus, the method should be used only after digestion of all organic contaminants through chemical oxidation (Lee and

Chae, 2021). The Nile Red solution was prepared in methanol at a concentration of 1 mg/ml. Few drops were placed over the sample to fully cover the particles and they were left to sit in the solution for 30 min at room temperature. Observations were performed using the microscopic I3 filter (excitation 450–490 nm, emission 515 nm) with a Leica DM2700P coupled with a CoolLED's pE-300lite LED fluorescent illumination system (Figure S2.2).

To avoid imaging bias, data acquisition was sequentially performed by the same operator and data discovery was only performed after finishing data gathering. Plastic particles were not further identified by polymer type.

2.6. Quality assurance and control

Special measures were considered to avoid contamination of samples, especially regarding airborne contamination. All lab-ware used for storing and processing the samples was made of non-plastic material and previously washed at least 3 times with MilliQ water. All the solutions used were previously filtered through a 20 μm mesh sieve. The processing time of samples was maintained to minimum, and samples were always covered while not processed or analysed. Samples were processed under a clean fume hood, a controlled and protected environment from the airborne deposit. Cotton lab coats and nitrile gloves were always used during the process and analyses. Contamination controls (clean Petri dishes) were placed during analysis every time the sample was open to register potential airborne particle deposition. A mean number of 3.1 (±2.7) fibres were found in the controls, comprising black, transparent, blue, red, and other colours and all the three size classes, corresponding to 8.7 % of the mean number of fibres found in samples. The results presented in this study were not corrected for contamination.

2.7. Environmental variables data

Precipitation data measured every 10 mins at the meteorological station of Chão do Areeiro (see Figure 2.1 for station location) were provided by the Portuguese Institute for Sea and Atmosphere (IPMA). Monthly averages of satellite-derived chlorophyll-*a* (Chla) concentrations and daily averages of Sea Surface Temperature (SST), calculated with values within the −2000 m isobath around the coast (as described in Rosa et al., 2022), were obtained from the Copernicus Marine Service (https://marine.copernicus.eu/).The satellite-derived Chla concentrations were based on a blended gridded product (Level 4; OCEANCOLOUR_ATL_CHL_L4_REP_OBSERVATIONS_009_091) at 1 km spatial resolution (Figure S2.3). SST were based on a Level 4 product (SST_ATL_SST_L4_REP_OBSERVATIONS_010_026) at ca. 5 km spatial resolution (Figure S2.4).

2.8. Statistical analysis

Shapiro-Wilk test and a visual inspection of the abundance data with histograms and QQ plots, analysing skewness, were used to assess normality. Given the non-normality of the data (Shapiro-Wilk normality test, p-value <0.05), the Mann-Whitney-Wilcoxon test was used to find significant differences in abundances and MP/zooplankton ratio in cold vs warm season (see Section 3.3). Spearman's correlation was used to test correlations between the abundances of MPs and zooplankton and environmental variables (precipitation, Chla and SST). All statistical analyses were performed with RStudio (version 1.4.1103) (RStudio Team, 2020) and GraphPad Prism (version 9.3.1 for Windows) (GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results

3.1. MPs and zooplankton characteristics

A total of 445 particles were detected and analysed. MPs were found in all the samples, where the fibres accounted for 89 % of the particles (Figure 2.2A). However, 82 % of the samples contained other types of plastic particles, i.e., fragments, films, and/or lines (Figure S2.2). Fibres were mainly black, while clear colours (transparent and white) were predominant in films and fragments (Figure 2.2B). Overall, black was the most common colour (40 %), followed by red (14 %), blue (13 %) and transparent (13 %) (Figure 2.2C). A mean number of 44 particles was detected per sample, with a mean abundance of 0.26 items/m^3. Small (335–500 μm) black fibres were the most common particles (20 %), and black fibres from all size categories represented 39 % of all particles. Also, large (1000–5000 μm) white fragments were commonly found, representing 2 % of the total number of particles. In general, fibres and films were primarily represented in the two smaller size classes (335–500, 500–1000 μm), while lines and fragments were more recurrent in the two bigger size classes (500–1000, 1000–5000 μm) (Figure 2.2D).

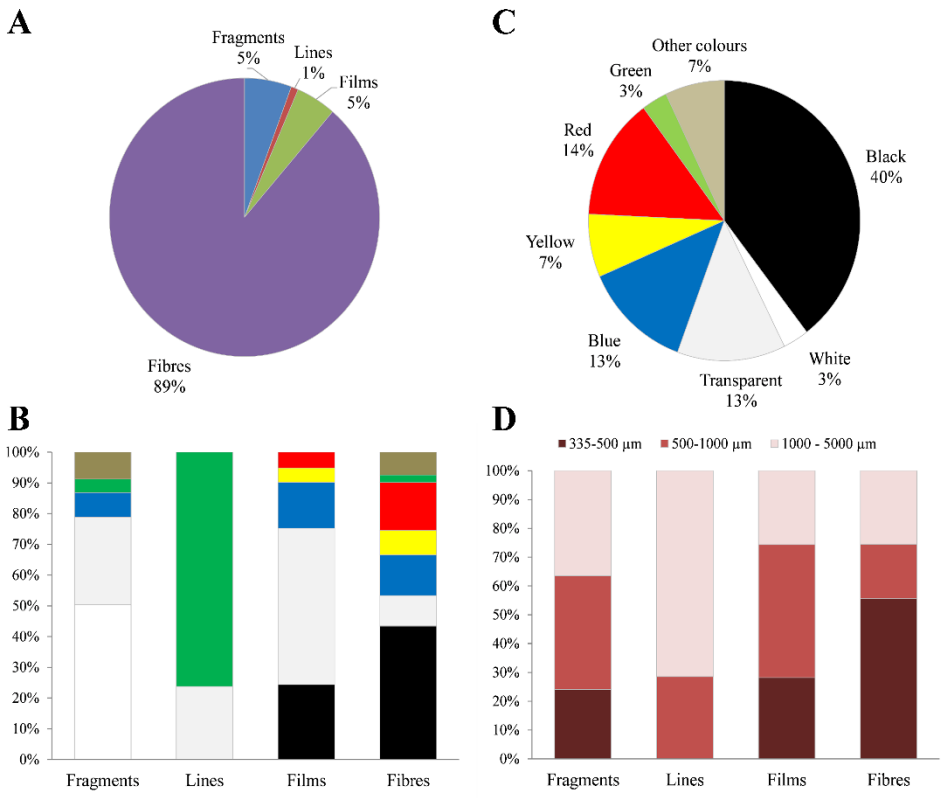

Figure 2.2 Characteristics of MPs found in the samples: proportion of MPs type categories (A), proportion of colours per MPs type (B), proportion of colour composition of MPs (C) and proportion of size categories for types of MPs (D).

In the zooplankton analysis, Thaliacea and Copepoda were the most common taxa encountered (representing 28.0 % and 24.7 % of the total, respectively), together with Cladocera (17.0 %) and eggs (14.1 %), they represented over 80 % of the community (Figure 2.3A). Chaetognatha was the most diverse taxa in terms of size classes, being abundant also in large size classes (1000–5000 µm), contrary to all the other taxa, which were mainly abundant in the smaller and medium size (Figure 2.3B). Other groups were recorded in low abundances (< 1 % of the total): Amphipoda, Annelida, Crustacean's larvae, Echinodermata larvae, Fish larvae, Gelatinous (other), Ostracoda.

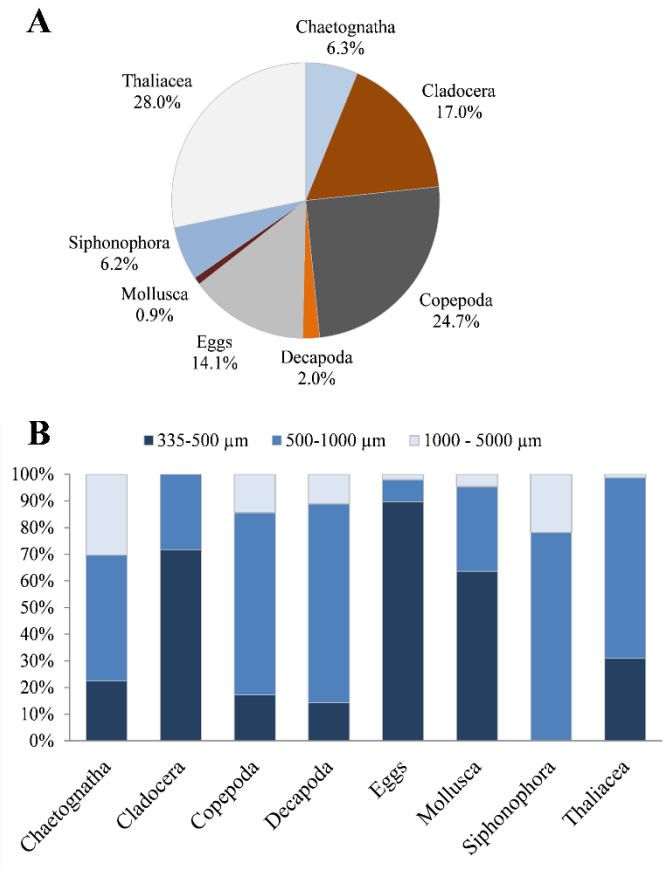

Figure 2.3 Characteristics of zooplankton found in the samples: taxonomic classification of zooplankton (A) and proportion of size categories for zooplanktonic taxa (B).

The sizes, colours, and shapes of the MPs were relatively homogeneous through all sampling occasions (Figure S2.5). Exceptions were the sample collected in April that presented higher quantities of small (335–500 µm) particles (mainly constituted by dark fibres), and the samples collected in July and September, that presented a larger diversity of shapes and sizes (i.e., higher numbers of fragments, lines and films when compared with other months where fibres dominated).

High diversity in taxa was recorded in the different samples (Figure S2.5). Thaliacea was extremely abundant (28.0 %) and was the dominant taxa in February and April samplings (>60 %) but scarce or almost absent on the other occasions. Copepods were numerous and represented the most abundant taxa (> 30 %) between July and January but were found in great numbers also in all the other samplings. March sampling was mainly characterised by large

amounts of Cladocera (ca. 30 %) and Eggs (ca. 70 %), differing from other months. Cladocera was also the dominant taxa (> 50 %) in the samples collected in May and June. In all the samples, the dominant size class of organisms was the medium one (500–1000 μm), besides February, March, May, and June, with organisms mainly in the small size class (335–500 μm) (Figure S2.5).

3.2. MPs and zooplankton relative abundances and environmental variables

The mean abundance of MPs and zooplankton found was 0.262 items/m^3 and 18.137 individuals/m^3 (n = 11), respectively (Table S2.1). Monthly abundances represented along with environmental variables are shown in Figure 2.4.

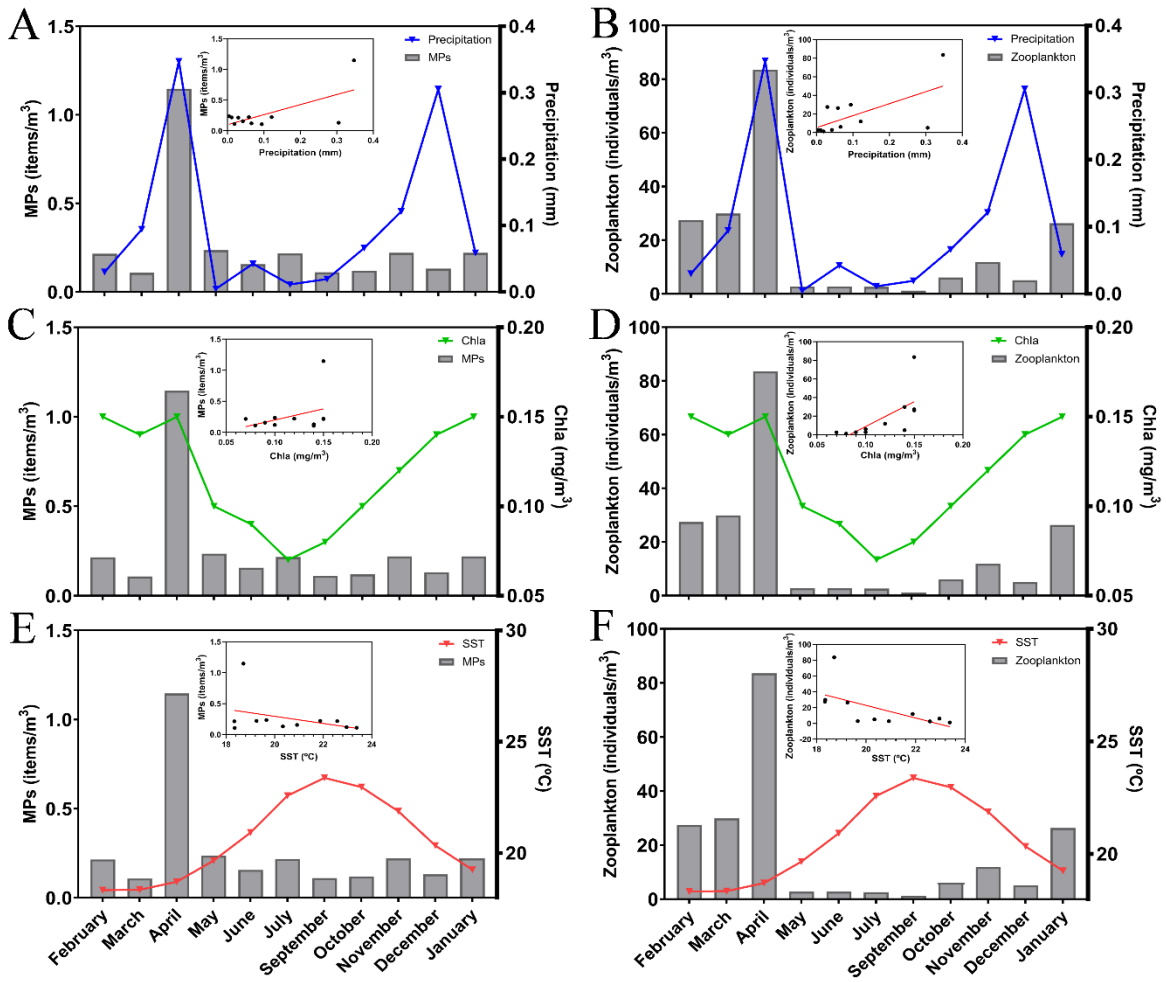

Figure 2.4 Monthly variability of MPs (A, C, E) and zooplankton abundances (B, D, F) represented along with monthly averages of precipitation intensity (A, B), chlorophyll-a concentration (Chla) (C, D) and sea surface temperature (SST) (E, F). Linear regression for each couple of variables is represented embedded in each graph.

The correlation matrix (Figure 2.5) shows, as expected, a significant correlation between zooplankton abundance, Chla and SST. No significant correlation was found between the abundance of MPs and zooplankton, although there was an abnormally similar high abundance of both MPs and zooplankton in April (Figure 2.4). The monthly average precipitation data shows a peak in April, however, MPs abundance was not correlated with monthly average precipitation, while a positive correlation was found between precipitation and zooplankton abundance.

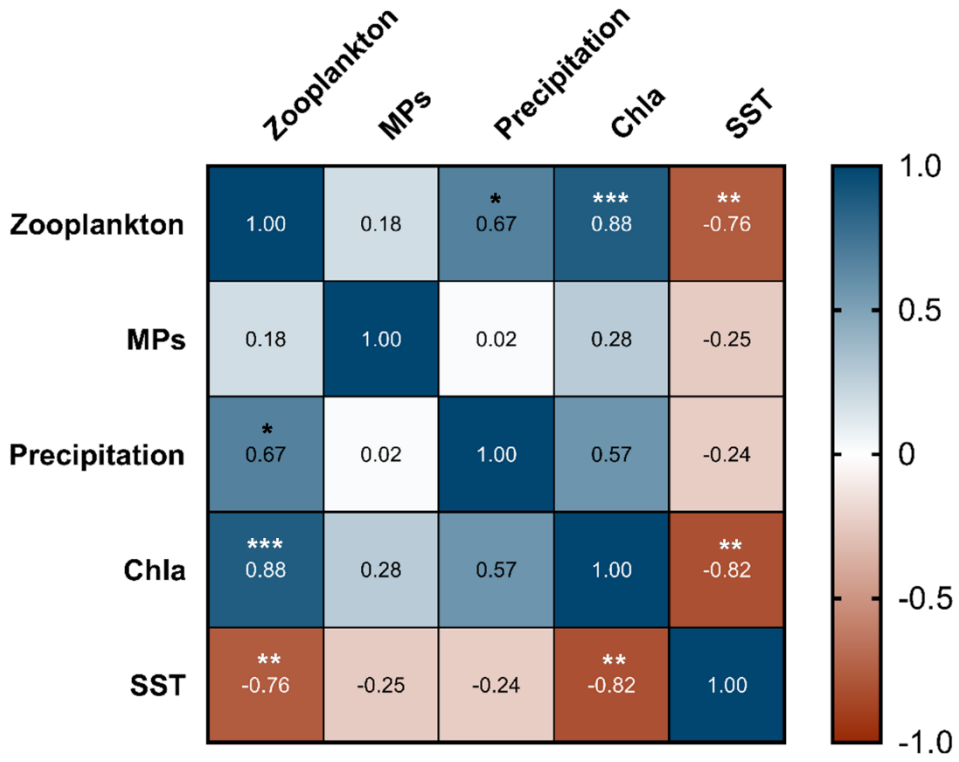

Figure 2.5 Correlation matrix (Spearman's correlation, rho index) for MPs-zooplankton abundances and environmental variables (precipitation, chlorophyll-a concentration (Chla), sea surface temperature (SST) – monthly averages). Significant correlations are indicated with: * *p*-value ≤0.05; ** *p*-value ≤0.01; *** *p*-value ≤0.001.

3.3. Abundances' seasonal variation and MPs/zooplankton ratio

Madeira Island is a subtropical region, with an intra-annual variation in SST of approximately 6 °C, recording maximum temperature in August and September (ca. 24 °C) and minimum temperature in February and March (ca. 18 °C) (Figure S2.4) (Caldeira et al.,

2002; Schäfer et al., 2019). As it is already reported in nearby subtropical regions, such as the Canary Islands (Landeira and Lozano-Soldevilla, 2018), also in Madeira, it is expected that plankton variation is mainly linked to one warm season that goes from May to October and one cold season from November to April. Chla concentrations derived from satellite observations support such seasonal differentiation: Chla concentrations around the island in 2019 were very low between May and October and higher between November and April (Figure S2.3). Therefore, we tested if the abundances of MPs and zooplankton from the samples collected were significantly different in these two main seasons. MPs abundances in cold and warm seasons were not significantly different ($W = 18$, p-value = 0.662) (Figure 2.6A), but, as expected, zooplankton abundance was significantly higher in cold season ($W = 29$, p-value = 0.009) (Figure 2.6B). Consequently, the MPs/zooplankton ratio was significantly higher in the warm season ($W = 1$, p-value = 0.008), given the lower abundance of zooplankton in these months (Figure 2.6C). The comparison within different size classes shows a significantly higher abundance of zooplankton in the cold season only for the middle size class (500–1000 μm) (Figure 2.6B). A higher amount of Copepoda and Thaliacea was the main responsible for such difference (Figure S2.5). However, the abundance of zooplankton was generally scarce within the largest class (1000–5000 μm), especially in the warm season. For this reason, the largest size class was the one where we could find the highest MPs/zooplankton ratio, with mean (± SD) values of 0.42 (± 0.52) (range 0.06–1.34) in the warm season and 0.67 (± 1.39) (range 0.01–3.5) in the cold season.

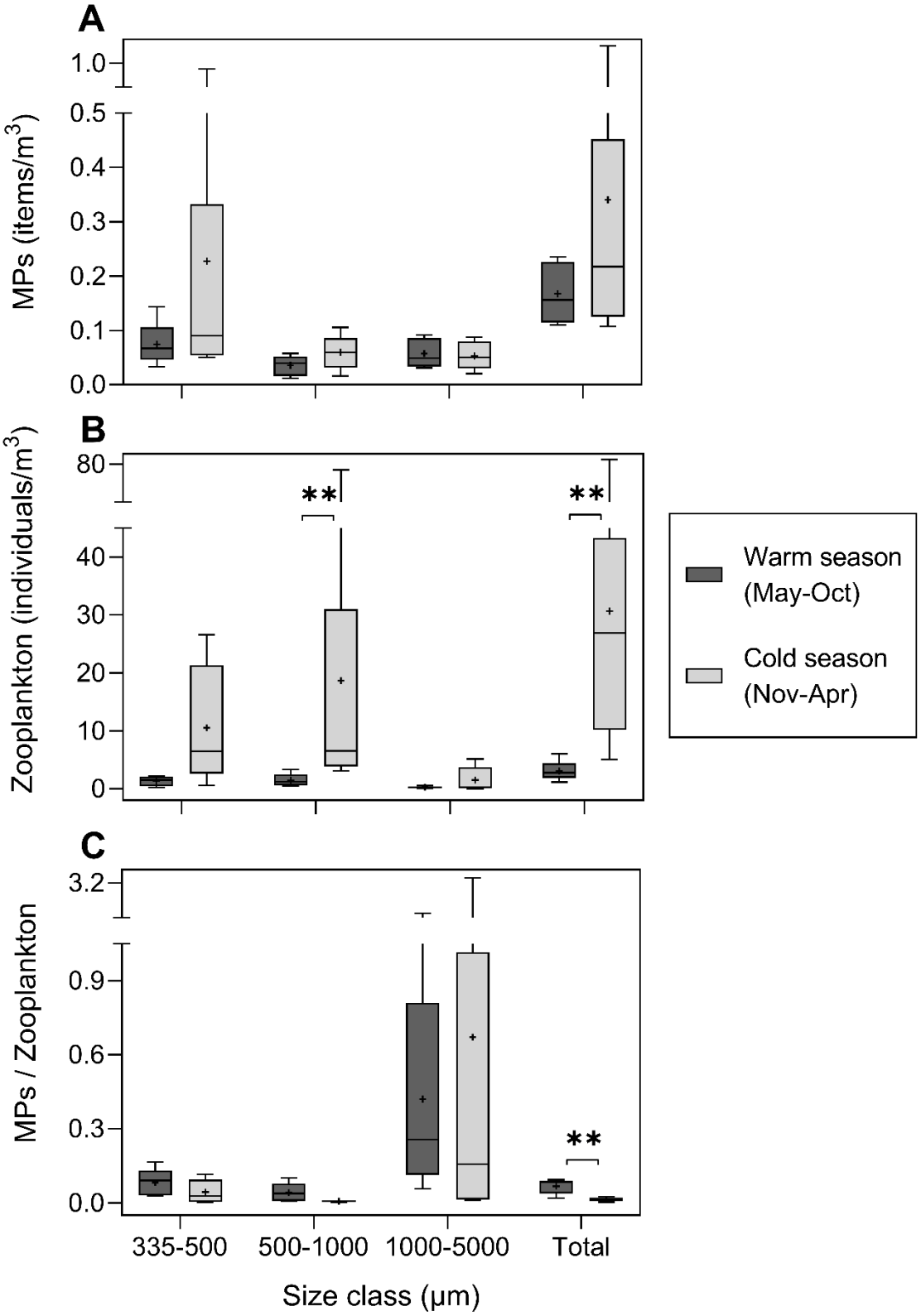

Figure 2.6 Abundance of microplastics (A), zooplankton (B) and MPs/zooplankton ratio (C) divided by size classes and compared by season (Warm – Cold). In each boxplot, the median (solid line) and the mean (plus symbol) are indicated in the centre of the box, and the edges of the box are the 25th and 75th percentiles; whiskers extend to the most extreme data points (min and max). Significant differences resulting from the Mann-Whitney-Wilcoxon Test are indicated with ** (*p*-value <0.01).

4. Discussion

The present study analysed the seasonal variation in abundances and characteristics of MPs and zooplankton collected in waters from a complex oceanic island system from the North-East Atlantic Ocean. Madeira Island is located on the external range of the North Atlantic Subtropical Gyre, an area of convergence where plastic debris is known to accumulate (Cozar et al., 2014). Through a modeling study, Cardoso and Caldeira (2021) found that Madeira Archipelago is significantly vulnerable to marine litter originating from distant sources. Litter particles, predominantly coming from the west coast of North America, are drifted by southward currents to Madeira (Gulf stream, Azores Current, Portugal, and Canary currents), mainly intercepting the north side of the island. Furthermore, Alves et al. (2021) found that north-east trade winds, which are especially strong and predominant in the summer season around Madeira Island, lead to a higher occurrence of anticyclonic eddies during that season in the southern waters of Madeira, where the samples were collected. Recent studies suggest that mesoscale anticyclonic eddies might trap, concentrate, and potentially transport microplastics (Brach et al., 2018). Thus, the higher diversity in particles encountered in the summer months (July and September) off the south coast of Madeira could be mainly associated with an aggregation of long-distance origin particles. Indeed, light coloured particles as fragments, lines and films are typically associated with the photo-degradation caused by prolonged exposure to the sunlight (Cole et al., 2011). In these months, a wider variety of taxa and size classes of zooplankton were also identified (Figure S2.5). Reasonably, oceanic physical processes can equally affect zooplankton distribution as much as microplastics, and eddies are also known to congregate nutrients and organisms (Zhang et al., 2014). This phenomenon should be considered regarding the potential impact of plastic pollution in open-ocean ecosystems, as such coincidence in the seasonal variation of MPs and zooplankton traits can increase the likelihood of MPs ingestion by planktivorous organisms. Furthermore, moderate

to high levels of water turbulence have been predicted to increase prey's ingestion rates due to higher frequency in particle contacts, further increasing such possibility (Botterell et al., 2019; Saiz et al., 2003).

In contrast, higher quantities of small (335–500 μm) particles, mainly constituted by dark fibres, were recorded in winter (especially in April). Dark coloured, small fibres are typically correlated with an influx of land-based contamination, as in the proximity of a wastewater treatment plant or a riverine source (Browne et al., 2011; Hale et al., 2020). A wastewater treatment effluent (Câmara de Lobos) and a river outlet (Ribeira dos Socorridos) that are in proximity of the sampling site might represent the primary sources of particles in this case (Figure 2.1). Despite the intermittent flow of the southern rivers (Prada et al., 2005), a recent numerical study demonstrated that the streams of Madeira might play an important role in the delivery of land-based material to coastal waters during precipitation events (Rosa et al., 2022). Higher precipitation intensity recorded in April and a peak in MPs abundance in this same month (Figure 2.4) support this theory.

Abundances of MPs showed little variation in different months, besides April. Indeed, both MPs and zooplankton abundances were exceptionally high in April (Figure 2.4; Table S2.1). Although no correlation was found in the abundances of microplastic and zooplankton, one can speculate that the high values observed for both in this sampling occasion might be somehow related. Collignon et al. (2014) also found higher values in the Mediterranean Sea in this same month. Vasilopoulou et al. (2021) found a significant correlation between the abundance of zooplankton and plastic particles, but they noticed that this correlation was even more significant when only fibres were considered in a specific coastal area. Lima et al. (2015) found a positive correlation between the abundance of plastic particles and rainfall in an estuarine environment.

A correlation between the monthly average precipitation and the microplastic abundance was not found in the present study, but a significant positive correlation was found between precipitation and zooplankton abundance. In comparison with estuarine or coastal environments, it can be expected that particles accumulation and dispersion in waters surrounding oceanic islands are more heavily subjected to physical oceanographic processes such as currents and winds, respect of surface run-off due to local meteorological events (Gove et al., 2016; Cardoso and Caldeira, 2021; Rosa et al., 2022). However, recent findings highlighted the underestimated impact of surface run-off episodes on suspended particulate matter and Chla concentration in waters surroundings Madeira Island (Rosa et al., 2022). Indeed, the high quantity of dark fibres recorded in April suggests that recent high-intensity precipitation events might be responsible for this particle's abundant presence in the island surroundings, which can even disperse into the pelagic environment.

A previous study in Macaronesia on neustonic samples also described microplastics and zooplankton, collected in August 2017, off the South coast of Madeira Island (Herrera et al., 2020). Overall, in those samples, higher percentages (47.5 %) of fragments and lower percentages (30 %) of fibres were found (Herrera et al., 2020). However, samplings were performed exclusively during summer, and such percentages are similar to those found in the samples collected in summer months (July and September) in the present study. Regarding the composition of the zooplankton community, Herrera et al. (2020) mainly found high percentages of fish eggs (60 %) and Copepoda (38.1 %). Copepoda is also the taxon found in major quantities in summer samples (July–September) from this study; however, only small percentages of fish eggs were recorded. A possible explanation is a misclassification by Herrera et al. of the dinoflagellate microalgae *Pyrocystis pseudonoctiluca* (found in very high quantities in summer samples from the present study, data not reported) which can easily be mistaken for fish eggs given the similar shape and size. Moreover, the average

MPs/zooplankton ratio found by Herrera et al. (2020) in the South of Madeira is similar but slightly lower than the one found in the present study for the warm season (Table 2.1).

MPs/zooplankton ratios found in other studies are summarised in Table 2.1. Such ratios can have significant spatial and temporal variations, given the seasonal influence of environmental variables on the zooplankton blooms and particle transport. However, most of those studies only investigated a short temporal window. Kang et al. (2015) studied the variation in MPs/zooplankton ratio before and after the rainy seasons in two bays of the Southern Sea of Korea. They found that the ratio decreased after the rainy season, mainly due to a higher abundance in the zooplankton and a dispersion of the MPs particles generated from coastal human activities. Vasilopoulou et al. (2021) analysed zooplankton and microplastics abundances off the coasts of Cyprus and did not find any significant difference in the MPs/zooplankton ratio among different seasons. However, samples were collected with vertical howls from 50 m depth, where the composition and abundance of both microplastics and zooplankton can be highly different from surface or sub-surface horizontal trawls. Collignon et al. (2014) analysed the annual variation of microplastics and zooplankton in the Bay of Calvi (Mediterranean Sea – Corsica) and investigated the relations within size classes. Coherently with the present results, the largest size class (2–5 mm) is the one where they found the highest MPs/zooplankton ratio, given the lower abundances of planktonic organisms in that size range.

Table 2.1 Summary of MPs and zooplankton abundances and MPs/zooplankton ratio found in other studies, with similar sampling techniques.

Site	Sampling type	Season	MPs[1] (items/m^3)	Zoo[1] (ind/m^3)	MPs / Zoo[1]	Reference
North Western Mediterranean Sea	Manta net (333 µm)	Summer (Jul – Aug)	-	-	0.5	Collignon et al. 2012
Bay of Calvi (Mediterranean – Corsica)	WP-2 net (200 µm)	All year	0.255	560.3	<0.002	Collignon et al. 2014
Goiana Estuary (Brazil)	Plankton net (300 µm)	All year	0.26	136.46	0.0018	Lima et al. 2014
Costa Vicentina (Portugal)	Neuston net (280 µm)	Winter (Jan)	0.036 ± 0.027	-	0.14	Frias et al. 2014
Aveiro (Portugal)	Neuston net (280 µm)	Spring (May)	0.002 ± 0.001	-	0.04	Frias et al. 2014
Lisboa (Portugal)	Neuston net (280 µm)	Winter (Jan)	0.033 ± 0.021	-	0.12	Frias et al. 2014
Algarve (Portugal)	Neuston net (280 µm)	Winter (Jan)	0.014 ± 0.012	-	0.05	Frias et al. 2014
Geoje Bay-Southern Sea of Korea	Manta net (330 µm)	Before rainy season	1.92 ± 1.84	22 ± 30	0.086 ± 0.061	Kang et al. 2015
Geoje Bay-Southern Sea of Korea	Manta net (330 µm)	After rainy season	5.51 ± 11.24	251 ± 173	0.022 ± 0.065	Kang et al. 2015
Jinhae Bay-Southern Sea of Korea	Manta net (330 µm)	Before rainy season	1.68 ± 0.81	102 ± 110	0.016 ± 0.007	Kang et al. 2015

[1] Data are expressed as Mean ± SD (when possible).

Jinhae Bay-Southern Sea of Korea	Manta net (330 µm)	After rainy season	1.07 ± 0.34	281 ± 297	0.004 ± 0.001	Kang et al. 2015
Pelagic Mediterranean Sea	Manta net (330 µm)	Summer (Aug – Sep)	1.73	-	0.5	Faure et al. 2015
North Atlantic Ocean (Azores)	Bongo net (200 µm)	Summer (July)	-	-	0.002	Herrera et al. 2020
North Atlantic Ocean (Madeira)	Manta net (200 µm)	Summer (August)	-	-	0.021	Herrera et al. 2020
North Atlantic Ocean (Canaries)	Manta net (200 µm)	All year	-	-	0.032	Herrera et al. 2020
Coasts of Cyprus (Eastern Mediterranean)	WP-2 (200 µm)	All year	41.31 ± 22.41	-	0.088 ± 0.130	Vasilopoulou et al. 2021
Cabrera MPA – coastal area (Italy)	Manta net (335 µm)	Summer (Jun – Aug)	3.52 ± 8.81	3.92 ± 2.36	0.14 ± 0.17	Fagiano et al. 2022
Madeira Island (Portugal)	Apstein net (335 µm)	Cold season (Nov – Apr)	0.34 ± 0.40	30.69 ± 27.69	0.013 ± 0.008	This study
Madeira Island (Portugal)	Apstein net (335 µm)	Warm season (May – Oct)	0.17 ± 0.06	3.07 ± 1.80	0.068 ± 0.030	This study

The predatory feeding behaviour of planktivorous fish, which visually encounter prey and feed raptorially, leads to selectivity for larger-sized prey in greater proportions than those available in the environment (Nilsson, 1972; Gardner, 1981). When plastic particles of comparable size to large planktonic organisms are abundant in the environment, with high MPs/zooplankton ratio, the probability of ingesting plastic might increase for marine predators with selective behaviour. The large size class (1000–5000 μm) of zooplankton in our samples was mainly constituted by Chaetognatha, Siphonophora, Copepoda and Decapoda (Figure 2.3B). Lines and fragments were the main shapes found in the large size class, but also films and fibres (measured on the length). These microplastics' colours and shapes often resemble those of meso-zooplanktonic organisms (Figure S2.6) and can easily be mistaken for food by planktivorous fish. However, fish can indirectly ingest any particles present in the water during the feeding events, with the likelihood of microplastic ingestion defined from the contaminants' environmental concentration (Wright et al., 2013). In this sense, smaller particles have higher chances to enter in the food chain, as they can be involuntary ingested by a higher variety of marine organisms, especially when considering filter-feeders.

The present study exposes the evidence that fibres can be abundantly found in a pelagic environment. Fibres are the most common synthetic particles found in most studies concerning MPs contamination in the marine environment, contributing to 35 % of the world ocean's MPs burden (Hale et al., 2020). They are also the dominant shape of MPs detected in the gastrointestinal tracts of marine fish worldwide (Wang et al., 2020). Even though these predators could actively feed on bigger plastics, which are more similar to zooplankton, they involuntarily ingest fibres present in high concentrations in the environment. A pelagic species of planktivorous fish (*Scomber scomber*) analysed in the nearby archipelago of Canary Islands showed a high rate of microplastic ingestion (Herrera et al., 2019). The main particles found in the gastrointestinal tracts were fibres (74 %), followed by fragments (12 %), paint chips

(12 %), lines (1 %) and films (1 %). Excluding the paint chips (that could be present as contamination from the commercial fishing boats), the shape's proportion described here reflects the one found in the present study.

Our samples were collected nearby an important area of purse-seine fishery of small pelagic fishes, being the Atlantic chub mackerel (*Scomber colias*) and the blue jack mackerel (*Trachurus picturatus*), the two most abundant catches (Tejerina et al., 2019; Romero et al., 2021*a*). Romero et al. (2021*a*) analysed the diet of these two fish species in Madeira and found that they can feed on a wide variety of prey, but mainly on zooplankton, with copepods being the most important group in their diet. They also identified a seasonal variation in the diet of these fish, mainly due to the different spatial and temporal distribution of prey. In spring and summer, copepods still represented one of the main taxa found in the stomach contents. Copepoda was also one of the most common taxa in our samples, together with Thaliacea (Figure 2.3A). These species occupy intermediate trophic levels and are key prey species in the pelagic food web for many predators, such as tunas (e.g., Romero et al., 2021*b*), seabirds (e.g., Alonso et al., 2014) and cetaceans (Burkhardt-Holm and N'Guyen, 2019). Furthermore, they have a substantial commercial value, are widely consumed by the local population, and are often used as bait in tuna fishing (Hermida and Delgado, 2016; Tejerina et al., 2019; Romero et al., 2021*b*). Plastic ingestion by these key species would lead to the trophic transfer of such contaminants to pelagic apex predators and ultimately to humans. Further studies which analyse the MPs ingestion by planktivorous fish in Madeira are needed.

The present results suggest a higher likelihood of plastic ingestion in the summer months in the southern waters of Madeira. Such a period coincides with the occurrence of some large pelagic predators, which feed mainly on epipelagic planktivorous fishes. For example, skipjack tuna (*Katsuwonus pelamis*) and the bigeye tuna (*Thunnus obesus*) constitute important commercial species in the region, and they occur seasonally in the warmer summer

and early autumn months, mainly between June and October (Romero et al., 2021*b*). The diet of these species is based mainly on epipelagic fishes, such as blue jack mackerel (*Trachurus picturatus*) and Atlantic chub mackerel (*Scomber colias*) (Romero et al., 2021*b*). Other large predators, such as delphinids and baleen whales, which feed mainly on small pelagic fishes, also occur in the area in high numbers during these months (Alves et al., 2018; Fernandez et al., 2021).

Although the consequences of microplastics ingestion on marine organisms are still far from being fully understood, their harmful effects, especially linked with the presence of plastic additives or absorbed toxic compounds, have been largely proved (Mallik et al., 2021). Chronic ingestion of microplastic by marine organisms or by humans, might lead to long-term negative effects, such as metabolic abnormalities (Sutton et al., 2016), endocrine disruption (Teuten et al., 2009) and liver toxicity (Rochman et al., 2013), that might finally lead to negative ecological consequences on the ecosystem diversity and stability (Ma et al., 2020).

5. Conclusion

In the present study, we highlighted the importance of the seasonal influence on the occurrence of MPs and zooplankton in a complex environment such as a deep ocean island. A variation in the composition of microplastics and the zooplankton community was found throughout the year in Madeira waters (NE Atlantic), suggesting they follow the seasonal fluctuation of oceanic parameters and, to some extent, precipitation events. We found that the warm season (May-Oct) could represent a period of greater concern, with a higher probability of plastic ingestion by marine organisms, given the higher MPs/zooplankton ratio and the co-occurrence of seasonal marine predators. Also, we identified a higher MPs/zooplankton ratio concerning large particles (size range 1000–5000 μm) and found that fibres are the most numerous particles present in these pelagic waters suggesting that they could be the most

ingested by planktivorous organisms. Furthermore, the results contributed to baseline knowledge on the zooplankton community inhabiting Madeiran waters and its seasonal variation.

Deep oceanic islands' ecosystems can be deeply affected both by oceanographic dynamics and island-related meteorological events. Considering the seasonal variations and the interaction among these environmental variables is crucial when investigating the zooplankton communities and the microplastic occurrence in these environments. In our case, results showed that long-distance origin particles (mainly present in the summer months) and fibres from WWTP discharges (predominants in the rainy months) constitute fundamental sources of contamination. Such results, suggesting the different provenance of the MPs found in the studied ecosystems, provide essential knowledge which allows informed environmental management decisions. Such information will also serve to assess the ecological impact of this contaminant on marine food webs in these vulnerable environments, which is still far from being fully understood.

Supplementary data

Supplementary data to this chapter can be found online at https://doi.org/10.1016/j.marpolbul.2022.113906 and in Appendix A of this document.

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CHAPTER III

DETERMINATION OF PHTHALIC ACID ESTERS AND DI(2-ETHYLHEXYL) ADIPATE IN FISH AND SQUID USING THE AMMONIUM FORMATE VERSION OF THE QUECHERS METHOD COMBINED WITH GAS CHROMATOGRAPHY MASS SPECTROMETRY

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Chapter 3 - Determination of Phthalic Acid Esters and Di (2-Ethylhexyl) Adipate in Fish and Squid Using the Ammonium Formate Version of the QuEChERS Method Combined with Gas Chromatography Mass Spectrometry

Abstract

In the present study, the ammonium formate version of the QuEChERS method, considered highly advantageous in relation to instrument maintenance and other issues, was applied for the first time to extract a group of twelve phthalic acid esters (PAEs, *i.e.* dipropyl phthalate, DPP; diisobutyl phthalate, DIBP; dibutyl phthalate, DBP; diisopentyl phthalate, DIPP; di-*n*-pentyl phthalate, DNPP; dihexyl phthalate, DHP; butyl benzyl phthalate, BBP; dicyclohexyl phthalate, DCHP; di(2-ethylhexyl) phthalate, DEHP; di-*n*-octyl phthalate, DNOP; diisononyl phthalate, DINP; and diisodecyl phthalate, DIDP) and one adipate (di(2-ethylhexyl) adipate, DEHA) from two species of fish (*Scomber colias* and *Katsuwonus pelamis*) and one of squid (*Loligo gahi*). The method was validated in terms of linearity, trueness and matrix effects. Determination coefficients (R^2) for matrix-matched calibration curves were higher than 0.99 in all cases, being the lowest calibration levels in the range 0.5–10 ng/g. Mean recovery values were between 70 and 117% with relative standard deviation values ≤20%. Matrix effects were soft (between −20 and +20%) for most analytes and matrices, except in squid samples, which was mostly medium with a moderate ion suppression. The analysis of 10 samples of each type showed the presence of DIBP, DBP and DEHP at concentrations up to 44.2 ± 2.1 ng/g of wet weight in some of the samples and species, still not representing concerning values when considering the daily intake of such species of seafood in the human diet (tolerable daily intake -TDI- values were not exceeded). Results demonstrated that the ammonium formate version of the QuEChERS method can be applied with success for the extraction and determination of the selected PAEs and DEHA in fish and squid samples.

Keywords

Fish, Squid, QuEChERS, Ammonium formate, Phthalic acid esters, Gas chromatography mass spectrometry

1. Introduction

Phthalic acid esters (PAEs) are manufactured chemicals which were first introduced in the 1920s. They are widely used as plasticizers in the plastic industry, mainly -but not limited to- in the production of polyvinyl chloride, to increase plastic plasticity by reducing intermolecular forces and, therefore, to facilitate its moulding. Some of them are also used as solvents and fragrances fixers, as well as additives in medical devices, household, cosmetics and personal care products (Katsikantami et al., 2016). Since they are not chemically bonded to the polymeric matrix, they easily migrate to their surrounding environment and, as a result, they are considered ubiquitous chemicals (Fasano et al., 2015, Katsikantami et al., 2016).

PAEs are the main type of plasticizers used nowadays; in fact, they accounted for 55% world production of plasticizers in 2020 (IHS Markit, 2021). As a result of their wide application and also of their ubiquitous presence in the environment, in the last years, important concern has arisen regarding the negative effects of PAEs and their metabolites on human health, which include their capacity to mimic the actions of natural hormones in the organism, producing several endocrine system disorders (Chang et al., 2021, Huang et al., 2021, Yang et al., 2015). Nevertheless, further research is still needed to effectively evaluate their toxic potential, especially the long-term effects. As a consequence, many public organizations/administrations have initiated actions to control/limit their use. This is the case of the EU, which banned di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) in all toys and childcare articles, and diisononyl

phthalate (DINP), diisodecyl phthalate (DIDP) and di-*n*-octyl phthalate (DNOP) in those articles that children could take to their mouth (The European Comission, 2006). More recently, the EU through its REACH regulation restricted the use of the four phthalates DEHP, DBP, diisobutyl phthalate (DIBP), and BBP coming into force in July 2020 (The European Comission, 2018), due to their demonstrated endocrine disrupting properties with effects on human health (Endocrine, 2019). According to it, the four PAEs are "restricted to a concentration equal to or below 0.1% by weight individually or in any combination in any plasticized material in articles used by consumers or in indoor areas". Earlier, in 2011 and 2012, these PAEs were identified as substances of very high concern (SVHCs) and added to the EU authorization list for being toxic and affecting reproduction mechanisms (European Chemicals Agency, 2021).

Regarding food contact materials, the use of DBP, DEHP, BBP, DINP and DIDP has been limited to certain situations also establishing specific migration limits (The European Commission, 2007). In this sense, on February 2019, EFSA panel on Food Contact Materials, Enzymes, and Processing Aids (CEP Panel) published their updated draft opinion on the risk assessment of such five PAEs, in which they established a group tolerable daily intake (TDI) of 50 μg/kg of body weight (b.w.) per day for DBP, BBP, DEHP and DINP, and for DIDP its own TDI of 150 μg/kg of b.w. per day (Silano & Baviera, 2019). The World Health Organization (WHO) has also established a TDI for DEHP of 25 μg/kg of b.w. and recommends not to exceed a concentration of 8 μg/L in drinking water (World Health Organization, 2003). In this regard, it should also be remarked that DEHP has also been included in the watch list given in Directive 2013/38/EU as a priority substance in the field of water policy (The European Commission, 2013).

In this context, it is more than evident that the determination of the presence of PAEs in food products is of high importance since the major route of exposure for the human beings

is food ingestion (Yang et al., 2015). Several studies have already reported the presence of PAEs in fishery products (Castro-Jiménez and Ratola, 2020, Hidalgo-Serrano et al., 2021, Xu et al., 2018). Such contamination can either be a result of the migration of PAEs from plastic packaging or of their absorption from the aquatic environment (Abdel daiem et al., 2012, Hahladakis et al., 2018). In fact, the ubiquitous presence of these compounds in marine waters and their lipophilic properties, might facilitate their accumulation in marine organisms (Hahladakis et al., 2018). Under this last respect, it should be indicated that PAEs have been recently proposed as plastic tracers in the marine environment, since despite that there are many restrictions on the manufacture and application of PAEs, these chemicals are still prevalent in the aquatic environments (Baini et al., 2017, Vered et al., 2019).

The complexity of fishery products requires the application of reliable and effective sample preparation methods for their analysis, which, following the current trends in the field, also demand simple and sustainable procedures with a minimum risk for humans. In this sense, the QuEChERS method (standing for *Quick, Easy, Cheap, Effective, Rugged and Safe*) is nowadays considered as a mega method, since it has demonstrated to be effective in the extraction of a wide variety of analytes and matrices after some adaptations (González-Curbelo et al., 2015, Socas-Rodríguez et al., 2017, Varela-Martínez et al., 2020). It is also considered a green method, as a result of the low amounts of solvents, reagents and energy required and their low toxicity (Varela-Martínez et al., 2020). Regarding the specific extraction of PAEs from fish and squid sample, the QuEChERS method has been applied in a very reduced number of occasions as shown in Table S2.1 of the Supplementary Data. Some of those works have also found some of the selected PAEs in the target samples by applying different versions of the QuEChERS method, though the most common has been the original/classical version in which acetonitrile (ACN) is used in the extraction step together with NaCl to promote partitioning as well as to produce a salting out effect, and $MgSO_4$ to also promote partitioning

and to heat the mixture around 40 °C as a result of the exothermic hydration process (Varela-Martínez et al., 2020). Despite the advantages of the use of both salts, trace amounts of them make necessary to intensify the periodic maintenance of the chromatographic systems like liners replacements in gas chromatography (GC) or the cleaning of the ion source in liquid chromatography-mass spectrometry (LC-MS), as well as in this last case contribute to the formation of sodium adducts. In 2014, González-Curbelo *et al.* proposed a modification of the method using ammonium formate instead of NaCl and $MgSO_4$ since it is also able to induce phases separation and it minimizes the disadvantages of the use of magnesium and sodium salts in MS analysis and also enhances the ionization of the analytes (González-Curbelo, Lehotay, Hernández-Borges, & Rodríguez-Delgado, 2014). Furthermore, the use of ammonium formate has also shown to have a similar performance as previous versions and to reduce the amount of co-extracted materials, leading to cleaner extracts and to a lower matrix effect (ME) (González-Curbelo et al., 2014, Han et al., 2016, Varela-Martínez et al., 2020). However, and despite its clear advantages, the ammonium formate version has not been fully explored as other versions of the QuEChERS method have been, probably as a result of the commercialization of a good number of QuEChERS kits under "classical" formulations to facilitate its application. Therefore, it is still necessary to study in depth this highly advantageous version and to extent its application to a wide variety of matrices and analytes.

In this context, the aim of this study was to apply for the first time the ammonium formate version of the QuEChERS method to the extraction of a group of 12 PAEs and one adipate (di(2-ethylhexyl) adipate, DEHA) from fish and squid samples (*Scomber colias*, *Katsuwonus pelamis* and *Loligo gahi*) in order to evaluate its performance as well as to study the ME. Ten samples of each type bought in local markets were analysed to check the possible presence of these compounds in the three selected species. This work represents the first application of the ammonium formate version of the QuEChERS method to these types of

samples and the first report of the presence of PAEs and DEHA in seafood species consumed in the Canary Islands.

2. Material and methods

2.1. Chemicals

The analytical standards that were used were dipropyl phthalate (DPP, CAS 131-16-8), DIBP (CAS 84-69-5), DBP (CAS 84-74-2), diisopentyl phthalate (DIPP, CAS 605-50-5), di-*n*-pentyl phthalate (DNPP, CAS 131-18-0), dihexyl phthalate (DHP, CAS 84-75-3), BBP (CAS 85-68-7), DEHA (CAS 103-23-1), dicyclohexyl phthalate (DCHP, CAS 84-61-7), DEHP (CAS 117-81-7), DNOP (CAS 117-84-0), DINP (CAS 20548-62-3) and DIDP (CAS 89-16-7). In addition, DBP-3,4,5,6-d_4 (DBP-d_4, CAS 93952-11-5), DNPP-3,4,5,6-d_4 (DNPP-d_4, CAS 358730–89-9), DHP-3,4,5,6-d_4 (DHP-d_4, CAS 1015854-55-3) and DEHP-3,4,5,6-d_4 (DEHP-d_4, CAS 93951–87-2) were used as internal standards (ISs). All of them had a purity greater than 97.0% and were acquired from Sigma-Aldrich (Madrid, Spain) and Dr. Ehrenstorfer (Augsburg, Germany). Table S3.2 of the Supplementary Data shows the chemical structures and properties of the studied PAEs and DEHA.

Individual stock solutions of each compound of interest and each IS were prepared at concentrations between 900 and 1100 mg/L in cyclohexane and stored in the darkness at −18 °C. Mix working solutions of all analytes and ISs were prepared at different concentrations in cyclohexane and stored at −18 °C in the darkness. All chemicals were used without further purification.

Tap water was purified with an Elix Essential water purification system, and then it was deionized using a Milli-Q gradient system A10 from Millipore (Burlington, MA). ACN of LC-MS grade and ammonium formate (purity 98.0%) were from VWR International Eurolab

(Barcelona, Spain). Primary secondary amine (PSA) and C_{18} were from Agilent Technologies (Santa Clara, CA, USA), and $MgSO_4$ monohydrate (purity 97%) was from Sigma-Aldrich (Madrid, Spain).

2.2. Apparatus and software

An 8860 GC system provided with an autosampler was used for analytes separation, which was coupled to a 5977B single quadrupole (Q) mass spectrometer for analytes detection, both from Agilent Technologies. The carrier gas was helium at a flow rate of 1.2 mL/min. Separation was performed in a HP-5 ms Ultra Inert column ((5%-phenyl)-methylpolysiloxane, 30 m × 250 μm × 0.25 μm) from Agilent Technologies. The temperature gradient program was as follows: temperature was increased from 60 to 170 °C at 40 °C/min, and finally increased to 310 °C at 10 °C/min and held for 3 min reaching a total run time of 20.75 min. Injection was carried out in the splitless mode (the split was opened after 0.75 min with a purge flow of 40 mL/min) at 280 °C and the injection volume was 2 μL. Other parameters that were established were the temperature of the ion source at 230 °C, the temperature of the transfer line at 280 °C and an ionization energy of −70 eV. In addition, single ion monitoring (SIM) mode was picked out. Enhanced MassHunter software from Agilent Technologies was used to control the GC–MS system.

A vortex was used to shake and mix the samples with the solvent or salts, and a Mega Star 3.0R centrifuge was used to separate the different layers from the homogeneous solution, both of them from VWR International. A 224i-1S analytical balance was also employed from Sartorius (Goettingen, Germany).

2.3. Samples

Patagonian squid (*Loligo gahi*, hereafter squid), Atlantic chub mackerel (*Scomber colias*, hereafter mackerel) and skipjack tuna (*Katsuwonus pelamis*, hereafter tuna) were

purchased from local markets in Tenerife (Canary Islands, Spain). When purchased, they were all covered in aluminium foil and immediately taken to the laboratory where they were cleaned and dissected. Dorsal muscles of fishes and mantles and tentacles of squids were extracted, triturated and frozen with liquid nitrogen. Afterwards, the frozen samples were grounded in a metal laboratory homogenizer to obtain a homogeneous powder. Once the sample reached room temperature, it was spiked with the analytes and/or ISs and allowed to stand for at least 20 min before the application of the ammonium formate version of the QuEChERS method.

For recovery studies, samples were spiked to yield 5, 10, 75 and 150 ng/g of wet weight (w.w.) depending on the analyte and matrix and 125 ng/g of w.w. for the ISs. Water content was determined by weighing 5 g of each sample in triplicate in porcelain capsules and letting it dry in an oven at 120 °C for 1.5 h, after which they were cooled in a desiccator and weighted until constant weight.

2.4. QuEChERS method

Five grams of homogenized tissue sample and 5 mL of ACN were introduced into a round bottom glass tube of 25 mL with a screw cap and was vigorously vortexed for 1 min. Then, 2.5 g of ammonium formate were added, and the sample was vortexed again for 1 min more and centrifugated for 5 min at 2500 rpm. Afterwards, 1 mL of the supernatant was transferred to a 15 mL round bottom glass tube containing 150 mg of $MgSO_4$ monohydrate, 50 mg of PSA and 50 mg of C_{18}. Then, the tube was vortexed for 1 min and centrifuged for 5 min at 2500 rpm. The resulting supernatant was filtered using a 0.2 µm polyvinylidene fluoride (PVDF) filter from Whatman™ (GE Healthcare, United States). Finally, 200 µL were transferred to a vial for injection in the GC–MS system.

2.5. Minimization and control of contamination

Volumetric glassware was cleaned with a sulphuric acid (95%, w/w, VWR International) solution of Nochromix® from Godax Laboratories (Maryland, USA) for 24 h. Non-volumetric glassware was cleaned by heating it up to 550 °C for 4–5 h (Muffle Carbolite CWF 11/13). High purity solvents were used in all cases as well as PAEs free pipette tips and gloves. Procedural blanks (analysis without sample) were carried out with every batch of samples.

3. Results and Discussion

3.1. GC-MS determination and application of the ammonium formate version of the QuEChERS method

In this work, GC–MS equipped with a single quadrupole analyser was used for the separation and detection of the 12 target PAEs and DEHA. The selected PAEs include those phthalates currently regulated by the EU in its different legislative actions, in particular, DBP, BBP, DEHP and DINP for which the EU and the WHO (only in the case of DEHP) have established TDIs. Table S3.2 of the Supplementary Data compiles the physicochemical properties of the selected analytes. In general, the length of the alkyl chains determines their different properties, like their hydrophobicity which increases with the increase of the chains (Yang et al., 2015). This also influences their chromatographic behaviour since long chain PAEs like DNOP, DINP and DIDP are eluted last in either GC or LC. Among the selected analytes, DEHA has also been included, since it is one of the most applied and studied alternative plasticisers to PAEs (Bui et al., 2016). As an example, it is among the plasticisers with the highest annual production in the EU, between 10,000 and 100,000 tonnes/year (Bui et al., 2016).

Though PAEs have also been determined by LC, they are more frequently determined by GC since they have enough volatility and thermal stability (González-Sálamo et al.,

2018, Martín-Pozo et al., 2021). In our case, the thermal gradient described in the Experimental Section was applied, obtaining a complete separation of the target analytes in less than 18 min. Concerning the ISs, isotopically labelled ISs were used. In particular, DBP-d_4 was used as IS of DPP, DBP and BBP, DNPP-d_4 of DIBP, DIPP and DNPP, DHP-d_4 of DHP, DEHA and DCHP, while DEHP-d_4 was used as IS of DEHP, DNOP, DINP and DIDP, the longer chain PAEs. The MS system was operated in the SIM mode. Table S3.3 of the Supplementary Data shows the quantifier and the two qualifier ions selected as well as the retention time of each analyte. Relative ion intensities with a ± 20% maximum permitted tolerance as well as the retention time were also considered as identification points (The European Commission, 2002). It should be remarked that the MS or tandem mass spectrometry (MS/MS) fragmentation pathways of most PAEs with alkyl side chains are similar, giving the *m*/*z* 149 as the most intensive parent ion, which corresponds to the protonated phthalic anhydride as the result of the fragmentation of the aliphatic side chains (Yin et al., 2014). As a consequence, the selection of *m*/*z* 149 for the quantification can make the determination of PAEs a very difficult task due to its low selectivity, being necessary a good resolution between peaks. To overcome this lack of selectivity, two qualifiers were monitored.

Apart from the previous consideration, it should also be taken into account that PAEs are ubiquitous in analytical laboratories, being necessary to minimize and control PAEs contamination. For this purpose, glassware should be used as much as possible, as well as PAEs free plastics (if employed) which should be carefully checked before being used. High purity solvents and reagents should also be selected since they contain less amounts of plasticizers and, what is more important, procedural blanks should be analysed on a daily basis. All these precautions have been taken into consideration, as indicated in the Experimental Section, in particular, the analysis of procedural blanks with each batch of samples.

As previously pointed out, the ammonium formate version of the QuEChERS method was studied by applying the experimental conditions indicated in Section 2.4, which is also summarized in Figure 3.1, in which ammonium formate was added instead of NaCl and $MgSO_4$. In this case, 5 g of homogenized fish (*Scomber colias* and *Katsuwonus pelamis*) or squid (*Loligo gahi*) were accurately weighted and 5 mL of ACN were added, followed by 1 min of vortex agitation. Then, 2.5 g of ammonium formate were added and 1 min more of vortex was applied. Phase separation was quickly achieved with relatively clean extracts after centrifugation, and 1 mL of the ACN layer was transferred to a glass tube containing 150 mg $MgSO_4$, 50 mg PSA (for polar matrix interferences removal, *i.e.* organic acids) and 50 mg C_{18} (for fat removal). Once vortex agitated and centrifuged, the supernatant was filtered and directly injected in the GC system avoiding any evaporation or further step that may yield to analyte loses. To facilitate sample comminution and to enhance analyte extraction, samples were frozen with liquid nitrogen and homogenized in a metal laboratory homogenizer. A fine powder was obtained in each case and, once at room temperature, 5 g of each sample were weighted and the QuEChERS method was applied. At the same time, 5 g of each sample were weighted in triplicate in porcelain capsules and their water content was determined in order to provide the final content of each PAE in dry weight (d.w.) (see Experimental Section for details).

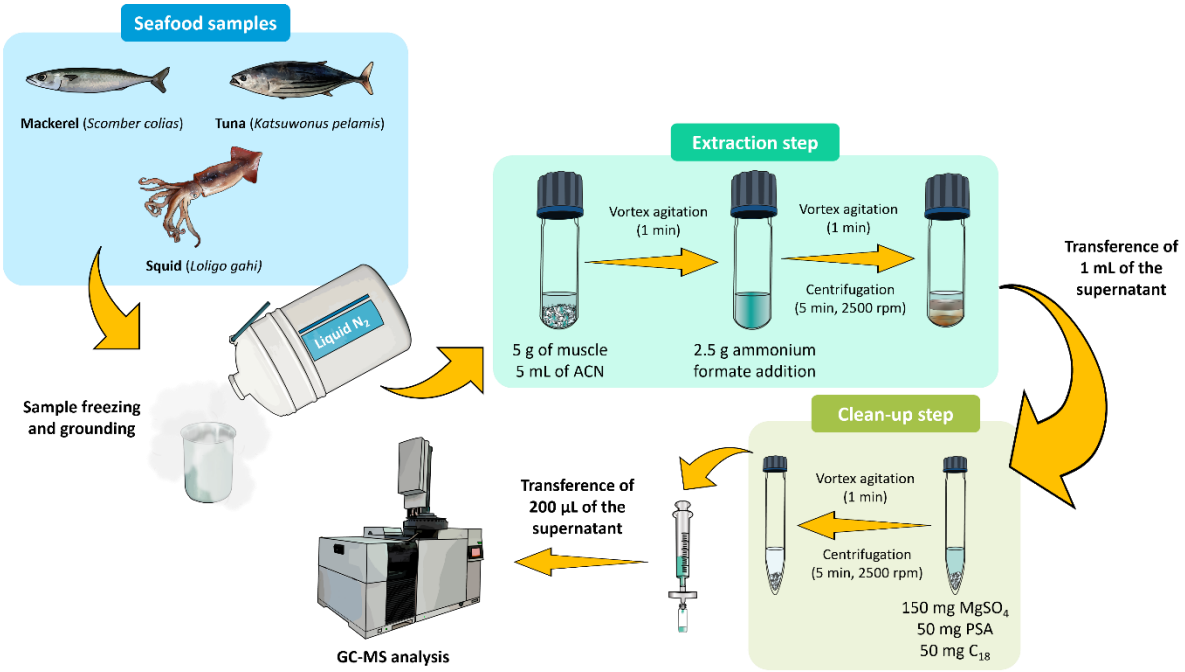

Figure 3.1 General scheme of the sample pre-treatment and QuEChERS extraction method applied in this work.

3.2. Matrix-matched calibration and matrix effect evaluation

In order to evaluate the existence of ME, which should be assessed at an initial method validation stage, matrix-matched calibration curves were obtained, by spiking the final extracts with the ISs (at 125 ng/g) and the target analytes (at eight concentration levels). Each matrix-matched standard was injected in triplicate and the GC liner was changed between the calibration of different matrices in order to correctly evaluate the ME. Non-spiked samples were also analysed and the ISs were added before the extraction in order to check/correct the possible presence of PAEs in the samples; in case a PAE or DEHA was found, the signal was subtracted for the calculations. Method performance acceptability criteria proposed by the SANTE Guidelines (SANTE/12682/2019, 2020) were also adopted in this study.

Table 3.1 shows the full calibration curves, including the studied linear range, the confidence intervals of the slope and intercept, as well as the determination coefficients (R^2) for all the target analytes, considering the IS previously indicated for each analyte. As can be seen in the table, R^2 values were higher than 0.99 in all cases. Regarding the lowest calibration

levels (LCLs), they ranged between 0.5 and 10 µg/L (equivalent to 0.5–10 ng/g, respectively) being the signal-to-noise (S/N) ratio in all cases equal or higher than 10. Regarding DIDP in tuna samples, an important interference precluded the correct quantification of the analyte and, therefore, matrix-matched calibration curves could not be obtained. LCLs were taken as the limits of quantification of the method, which, once proper calculation was made taken into account the solid nature of the samples, were less than 5 ng/g for all the analytes in the matrices analysed, except for DBP in tuna which was 10 ng/g.

Table 3.1 Matrix-matched calibration data of the selected PAEs and DEHA and matrix effect (ME) percentage in mackerel, squid and tuna (DBP-d_4 was used as IS of DPP, DBP and BBP, DNPP-d_4 was used as IS of DIBP, DIPP and DNPP, DHP-d_4 was used as IS of DHP, DEHA and DCHP, while DEHP-d_4 was used as IS of DEHP, DNOP, DINP and DIDP).

Analyte	Sample	Studied linear range (µg/L)*	Regression equation (n=8)		$s_{y/x}$	R^2	ME (%)**
			$b \pm s_b \cdot t_{(0.05;6)}$	$a \pm s_a \cdot t_{(0.05;6)}$			
DPP	Mackerel	1-150	$5.43 \cdot 10^{-3} \pm 4.66 \cdot 10^{-4}$	$4.82 \cdot 10^{-3} \pm 3.72 \cdot 10^{-2}$	$2.73 \cdot 10^{-2}$	0.9945	-35
	Squid	1-150	$2.10 \cdot 10^{-3} \pm 5.08 \cdot 10^{-5}$	$3.59 \cdot 10^{-4} \pm 4.05 \cdot 10^{-3}$	$2.97 \cdot 10^{-3}$	0.9996	-75
	Tuna	0.5-150	$5.02 \cdot 10^{-3} \pm 1.90 \cdot 10^{-4}$	$3.01 \cdot 10^{-3} \pm 1.42 \cdot 10^{-2}$	$1.24 \cdot 10^{-2}$	0.9986	-40
DIBP	Mackerel	5-150	$5.94 \cdot 10^{-3} \pm 2.06 \cdot 10^{-4}$	$-9.73 \cdot 10^{-3} \pm 1.78 \cdot 10^{-2}$	$1.03 \cdot 10^{-2}$	0.9994	-27
	Squid	1-150	$2.49 \cdot 10^{-3} \pm 1.93 \cdot 10^{-5}$	$1.39 \cdot 10^{-2} \pm 1.54 \cdot 10^{-3}$	$1.13 \cdot 10^{-3}$	1.0000	-69
	Tuna	1-150	$5.71 \cdot 10^{-3} \pm 2.17 \cdot 10^{-4}$	$2.76 \cdot 10^{-2} \pm 1.73 \cdot 10^{-2}$	$1.27 \cdot 10^{-2}$	0.9989	-29
DBP	Mackerel	1-150	$8.94 \cdot 10^{-3} \pm 9.65 \cdot 10^{-4}$	$3.35 \cdot 10^{-2} \pm 7.69 \cdot 10^{-2}$	$5.65 \cdot 10^{-2}$	0.9913	18
	Squid	5-150	$3.59 \cdot 10^{-3} \pm 5.47 \cdot 10^{-5}$	$-1.33 \cdot 10^{-2} \pm 4.71 \cdot 10^{-3}$	$2.72 \cdot 10^{-3}$	0.9999	-53
	Tuna	10-150	$8.15 \cdot 10^{-3} \pm 1.18 \cdot 10^{-3}$	$9.85 \cdot 10^{-2} \pm 1.11 \cdot 10^{-1}$	$4.52 \cdot 10^{-2}$	0.9938	7
DIPP	Mackerel	0.5-150	$5.27 \cdot 10^{-3} \pm 6.34 \cdot 10^{-5}$	$-8.13 \cdot 10^{-5} \pm 4.73 \cdot 10^{-3}$	$4.12 \cdot 10^{-3}$	0.9999	10
	Squid	1-150	$2.37 \cdot 10^{-3} \pm 2.89 \cdot 10^{-5}$	$2.12 \cdot 10^{-5} \pm 2.30 \cdot 10^{-3}$	$1.69 \cdot 10^{-3}$	0.9999	-51
	Tuna	0.5-150	$5.09 \cdot 10^{-3} \pm 7.79 \cdot 10^{-5}$	$4.98 \cdot 10^{-3} \pm 5.81 \cdot 10^{-3}$	$5.07 \cdot 10^{-3}$	0.9998	6
DNPP	Mackerel	0.5-150	$8.44 \cdot 10^{-3} \pm 8.10 \cdot 10^{-5}$	$-6.26 \cdot 10^{-4} \pm 6.04 \cdot 10^{-3}$	$5.27 \cdot 10^{-3}$	0.9999	11
	Squid	1-150	$3.75 \cdot 10^{-3} \pm 3.96 \cdot 10^{-5}$	$1.17 \cdot 10^{-3} \pm 3.16 \cdot 10^{-3}$	$2.32 \cdot 10^{-3}$	0.9999	-51
	Tuna	0.5-150	$8.01 \cdot 10^{-3} \pm 1.21 \cdot 10^{-4}$	$1.10 \cdot 10^{-3} \pm 9.05 \cdot 10^{-3}$	$7.89 \cdot 10^{-3}$	0.9998	5
DHP	Mackerel	5-150	$8.46 \cdot 10^{-3} \pm 1.47 \cdot 10^{-4}$	$1.95 \cdot 10^{-3} \pm 1.27 \cdot 10^{-2}$	$7.34 \cdot 10^{-3}$	0.9998	7
	Squid	5-150	$4.13 \cdot 10^{-3} \pm 6.00 \cdot 10^{-5}$	$3.45 \cdot 10^{-4} \pm 5.16 \cdot 10^{-3}$	$2.99 \cdot 10^{-3}$	0.9999	-48
	Tuna	5-150	$8.62 \cdot 10^{-3} \pm 2.04 \cdot 10^{-4}$	$4.91 \cdot 10^{-3} \pm 1.76 \cdot 10^{-2}$	$1.02 \cdot 10^{-2}$	0.9997	9
BBP	Mackerel	5-150	$3.17 \cdot 10^{-3} \pm 1.74 \cdot 10^{-4}$	$1.12 \cdot 10^{-3} \pm 1.50 \cdot 10^{-2}$	$8.67 \cdot 10^{-3}$	0.9984	68

	Squid	5-150	$1.72 \cdot 10^{-3} \pm 4.37 \cdot 10^{-5}$	$-2.01 \cdot 10^{-4} \pm 3.77 \cdot 10^{-3}$	$2.18 \cdot 10^{-3}$	0.9997	-9
	Tuna	5-150	$3.72 \cdot 10^{-3} \pm 1.64 \cdot 10^{-4}$	$-3.05 \cdot 10^{-3} \pm 1.41 \cdot 10^{-2}$	$8.16 \cdot 10^{-3}$	0.9990	97
	Mackerel	5-150	$2.97 \cdot 10^{-3} \pm 5.50 \cdot 10^{-5}$	$4.44 \cdot 10^{-3} \pm 4.74 \cdot 10^{-3}$	$2.74 \cdot 10^{-3}$	0.9998	15
DEHA	Squid	5-150	$1.42 \cdot 10^{-3} \pm 7.36 \cdot 10^{-5}$	$3.46 \cdot 10^{-3} \pm 6.34 \cdot 10^{-3}$	$3.67 \cdot 10^{-3}$	0.9986	-45
	Tuna	5-150	$2.88 \cdot 10^{-3} \pm 6.14 \cdot 10^{-5}$	$3.07 \cdot 10^{-3} \pm 5.29 \cdot 10^{-3}$	$3.06 \cdot 10^{-3}$	0.9998	11
	Mackerel	5-150	$6.02 \cdot 10^{-3} \pm 1.32 \cdot 10^{-4}$	$1.02 \cdot 10^{-3} \pm 1.14 \cdot 10^{-2}$	$6.60 \cdot 10^{-3}$	0.9997	17
DCHP	Squid	5-150	$3.08 \cdot 10^{-3} \pm 4.27 \cdot 10^{-5}$	$3.01 \cdot 10^{-5} \pm 3.67 \cdot 10^{-3}$	$2.13 \cdot 10^{-3}$	0.9999	-40
	Tuna	5-150	$6.26 \cdot 10^{-3} \pm 1.04 \cdot 10^{-4}$	$9.30 \cdot 10^{-4} \pm 8.93 \cdot 10^{-3}$	$5.17 \cdot 10^{-3}$	0.9999	22
	Mackerel	5-150	$7.57 \cdot 10^{-3} \pm 1.59 \cdot 10^{-4}$	$-1.46 \cdot 10^{-2} \pm 1.37 \cdot 10^{-2}$	$7.92 \cdot 10^{-3}$	0.9998	5
DEHP	Squid	5-150	$3.98 \cdot 10^{-3} \pm 4.33 \cdot 10^{-5}$	$-2.95 \cdot 10^{-2} \pm 3.73 \cdot 10^{-3}$	$2.16 \cdot 10^{-3}$	0.9999	-45
	Tuna	5-150	$7.69 \cdot 10^{-3} \pm 2.20 \cdot 10^{-4}$	$2.69 \cdot 10^{-2} \pm 1.90 \cdot 10^{-2}$	$1.10 \cdot 10^{-2}$	0.9996	6
	Mackerel	5-150	$1.23 \cdot 10^{-2} \pm 2.79 \cdot 10^{-4}$	$-1.82 \cdot 10^{-3} \pm 2.40 \cdot 10^{-2}$	$1.39 \cdot 10^{-2}$	0.9997	10
DNOP	Squid	5-150	$5.57 \cdot 10^{-3} \pm 4.05 \cdot 10^{-4}$	$-1.45 \cdot 10^{-2} \pm 3.49 \cdot 10^{-2}$	$2.02 \cdot 10^{-2}$	0.9973	-50
	Tuna	5-150	$1.29 \cdot 10^{-2} \pm 1.88 \cdot 10^{-4}$	$-5.10 \cdot 10^{-3} \pm 1.62 \cdot 10^{-2}$	$9.38 \cdot 10^{-3}$	0.9999	15
	Mackerel	5-150	$8.60 \cdot 10^{-3} \pm 2.86 \cdot 10^{-4}$	$-8.79 \cdot 10^{-3} \pm 2.47 \cdot 10^{-2}$	$1.43 \cdot 10^{-2}$	0.9994	3
DINP	Squid	5-150	$3.70 \cdot 10^{-3} \pm 2.88 \cdot 10^{-4}$	$-9.69 \cdot 10^{-3} \pm 2.48 \cdot 10^{-2}$	$1.43 \cdot 10^{-2}$	0.9969	-56
	Tuna	5-150	$9.51 \cdot 10^{-3} \pm 6.88 \cdot 10^{-5}$	$2.99 \cdot 10^{-2} \pm 5.92 \cdot 10^{-3}$	$3.43 \cdot 10^{-3}$	1.0000	14
	Mackerel	5-150	$7.28 \cdot 10^{-3} \pm 2.57 \cdot 10^{-4}$	$-8.11 \cdot 10^{-3} \pm 2.21 \cdot 10^{-2}$	$1.28 \cdot 10^{-2}$	0.9994	-16
DIDP	Squid	5-150	$3.18 \cdot 10^{-3} \pm 4.15 \cdot 10^{-4}$	$-1.03 \cdot 10^{-2} \pm 3.58 \cdot 10^{-2}$	$2.07 \cdot 10^{-2}$	0.9912	-63
	Tuna	-	-	-	-	-	-

b: slope; s_b: standard deviation of the slope; a: intercept; s_a: standard deviation of the intercept; R^2: determination coefficient; $s_{y/x}$: standard deviation of the estimate.
*Also equivalent to ng/g in real samples. **Calculated following the equation used by Kwon et al. (Kwon et al., 2012).

Table S3.4 of the Supplementary Data also shows the analogous data obtained for solvent calibration which was obtained in order to calculate the ME using the following equation: ME (%) = (slope of matrix-matched calibration curve − slope of pure solvent-based calibration curve)/(slope of pure solvent-based calibration curve) × 100 (Kwon, Lehotay, & Geis-Asteggiante, 2012). ME values are also shown in Table 3.1, though they have also been represented for each matrix in Figure 3.2 *vs* the retention time of each PAE. Negative ME values mean that a signal suppression is taking place, while positive values correspond to a signal enhancement. When the percentage ranges between −20 and 20%, a soft ME takes place and matrix-matched calibration is not required. However, ME in the ranges between −20 and −50% or between 20 and 50% correspond to a medium ME while values higher than 50 or lower than −50% correspond to a strong/significant ME. In both cases, matrix-matched calibration is necessary. From the figure, it is clear that for both mackerel and tuna samples a soft ME takes place for most of the selected PAEs, though for few of them ME is medium and mainly caused by signal suppression. On the contrary, for squid samples, an important signal suppression (strong/significant in most cases) can be observed which clearly indicates the need to develop matrix-matched calibration, though, in general, ME percentages are not extremely high.

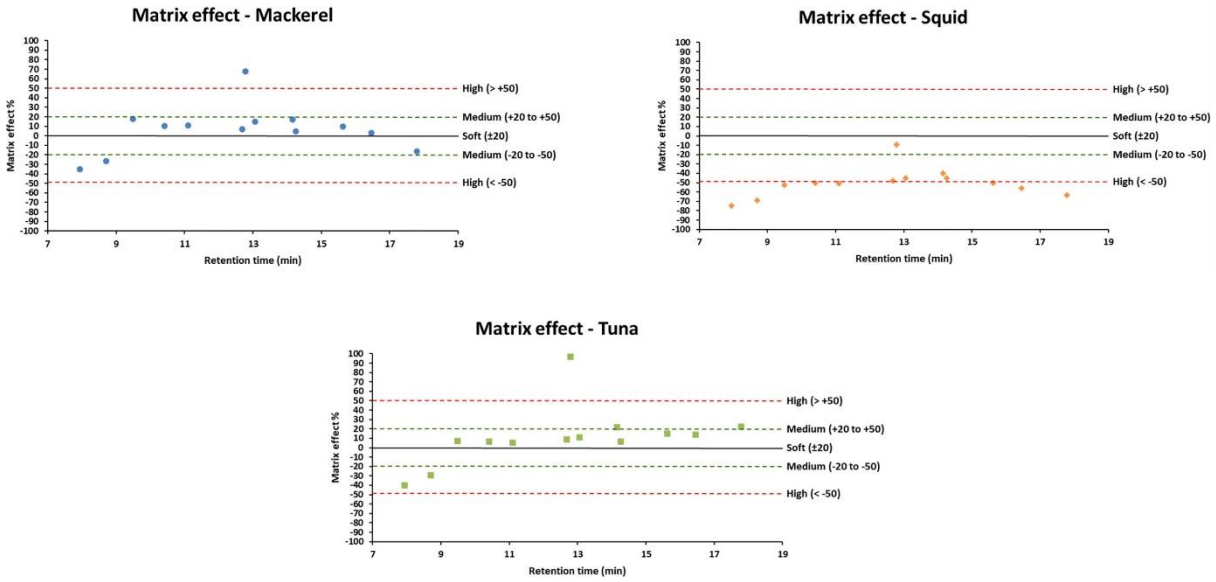

Figure 3.2 Distribution of the ME (%) vs the retention time (min) of each PAE and DEHA for mackerel, squid and tuna matrix after the application of the QuEChERS-GC–MS method.

3.3. Trueness

In order to evaluate the trueness of the method, a recovery study was carried out at three concentration levels by developing five consecutive extractions at each level. Samples were spiked with the analytes and ISs and let to stand for at least 20 min at room temperature before the application of the QuEChERS method. Concentration of level 1 was 5 ng/g (except for DBP in tuna which was 10 ng/g), 75 ng/g for level 2 and 150 ng/g for level 3 in the three types of samples. The three levels covered low, medium, and high concentrations of the linearity range of the target compounds. Table 3.2 shows the relative recovery values obtained at each level in which it can clearly be seen that acceptable recovery values, between 70 and 120% with relative standard deviation (RSD) values below 20% were obtained for most of the target PAEs and levels, similar values were also obtained for absolute recovery values, which clearly shows the high extraction efficiency and precision of the method. Though, as can be seen in Table 3.2, few of those values are outside this range (which have been marked in the table in bold), RSD values are also consistent, since they are lower than 20%. Moreover, if mean recovery values of the three levels are considered for each sample, it can be seen that they range between 70

and 117% with RSD values ≤ 20%, which are also acceptable criteria according to SANTE guidelines (SANTE/12682/2019, 2020). To better appreciate this issue, mean RSD values have been plotted versus mean recovery values as shown in Figure 3.3, and the range 0–20% for RSD values and 70–120% for recovery values have been marked.

Table 3.2 Relative recovery and RSD values of the target analytes in mackerel, squid, and tuna (n = 5 at each spiking level).

Analytes	Sample	Level 1	Level 2	Level 3	Mean
		Recovery % (RSD %)	**Recovery % (RSD %)**	**Recovery % (RSD %)**	**Recovery % (RSD %)**
DPP	Mackerel	106 (5)	110 (6)	103 (4)	107 (5)
	Squid	**131** (6)	105 (6)	**122** (2)	117 (11)
	Tuna	**136** (4)	106 (2)	110 (4)	114 (11)
DIBP	Mackerel	**69** (12)	106 (7)	107 (3)	94 (20)
	Squid	72 (14)	100 (6)	93 (1)	89 (15)
	Tuna	86 (5)	99 (3)	107 (5)	97 (10)
DBP	Mackerel	89 (6)	96 (2)	97 (4)	94 (6)
	Squid	79 (6)	101 (1)	85 (11)	90 (13)
	Tuna	**59** (13)	96 (3)	98 (0)	87 (20)
DIPP	Mackerel	99 (2)	98 (4)	97 (4)	98 (3)
	Squid	103 (5)	104 (2)	99 (1)	102 (3)
	Tuna	83 (3)	104 (1)	111 (1)	100 (13)
DNPP	Mackerel	96 (1)	96 (3)	95 (4)	96 (3)
	Squid	94 (3)	102 (1)	95 (1)	97 (4)
	Tuna	77 (2)	104 (1)	109 (1)	97 (15)
DHP	Mackerel	95 (1)	95 (4)	94 (4)	95 (3)
	Squid	93 (2)	103 (2)	100 (5)	99 (5)
	Tuna	81 (3)	103 (1)	108 (1)	97 (13)
BBP	Mackerel	97 (1)	91 (5)	96 (4)	95 (4)
	Squid	**68** (1)	96 (5)	86 (13)	86 (16)
	Tuna	**64** (1)	102 (3)	107 (3)	95 (19)
DEHA	Mackerel	90 (4)	79 (5)	81 (7)	83 (8)
	Squid	84 (10)	92 (2)	88 (1)	88 (6)
	Tuna	85 (6)	94 (2)	96 (2)	92 (6)
DCHP	Mackerel	97 (2)	95 (5)	97 (4)	96 (4)
	Squid	93 (3)	104 (1)	100 (5)	99 (6)
	Tuna	78 (3)	104 (2)	109 (2)	97 (15)
DEHP	Mackerel	114 (7)	101 (5)	97 (4)	104 (9)

	Squid	72 (6)	102 (2)	100 (6)	93 (16)
	Tuna	77 (10)	103 (2)	114 (8)	98 (18)
DNOP	Mackerel	92 (2)	90 (4)	92 (5)	91 (4)
	Squid	89 (3)	108 (2)	96 (1)	98 (9)
	Tuna	82 (4)	105 (3)	109 (2)	99 (13)
DINP	Mackerel	92 (6)	80 (5)	83 (6)	85 (8)
	Squid	77 (4)	99 (2)	87 (2)	88 (11)
	Tuna	78 (4)	93 (3)	98 (2)	90 (10)
DIDP	Mackerel	112 (5)	79 (6)	80 (6)	89 (18)
	Squid	**55** (11)	83 (4)	71 (2)	70 (18)
	Tuna	-	-	-	-

Level 1: 5 ng/g of w.w. except for DBP in tuna which was 10 ng/g of w.w.; level 2: 75 ng/g of w.w.; and level 3: 150 ng/g w.w. for all the analytes. Data outside the 70-120% range for recovery values and 0-20% for RSD values are in bold.

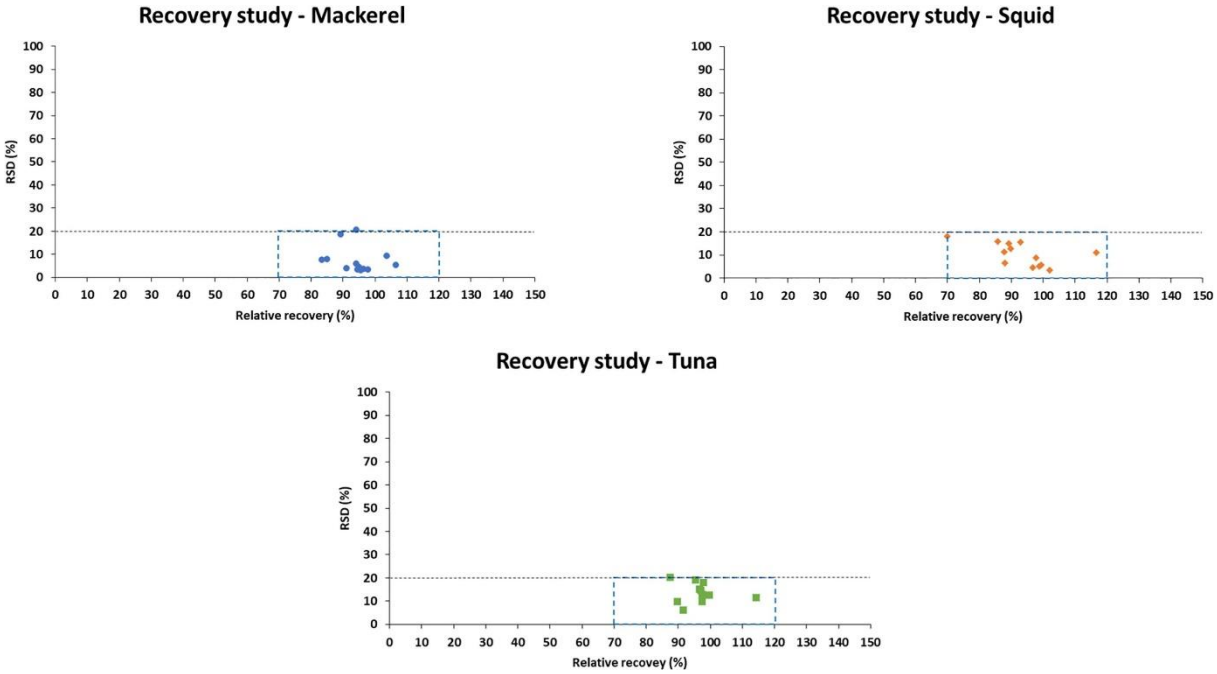

Figure 3.3 Overall RSD values (%) vs relative recovery (%) of each PAE and DEHA in each matrix after the application of the QuEChERS-GC–MS method. Compounds with RSD less than 20% and relative recovery values in the 70–120% range are in the indicated box.

Figure 3.4 shows a chromatogram of the separation of a squid sample spiked at the medium concentration level, while Figure 3.5 shows a GC–MS chromatogram of (Figure 3.5A) DIBP in a squid sample, (Figure 3.5B) DBP in a squid sample, and (Figure 3.5C) DEHP in a mackerel sample. As can be seen, in all cases, the analytes could be perfectly identified and quantified. Similar chromatograms were obtained for the rest of the samples except, as previously mentioned for DIDP in tuna.

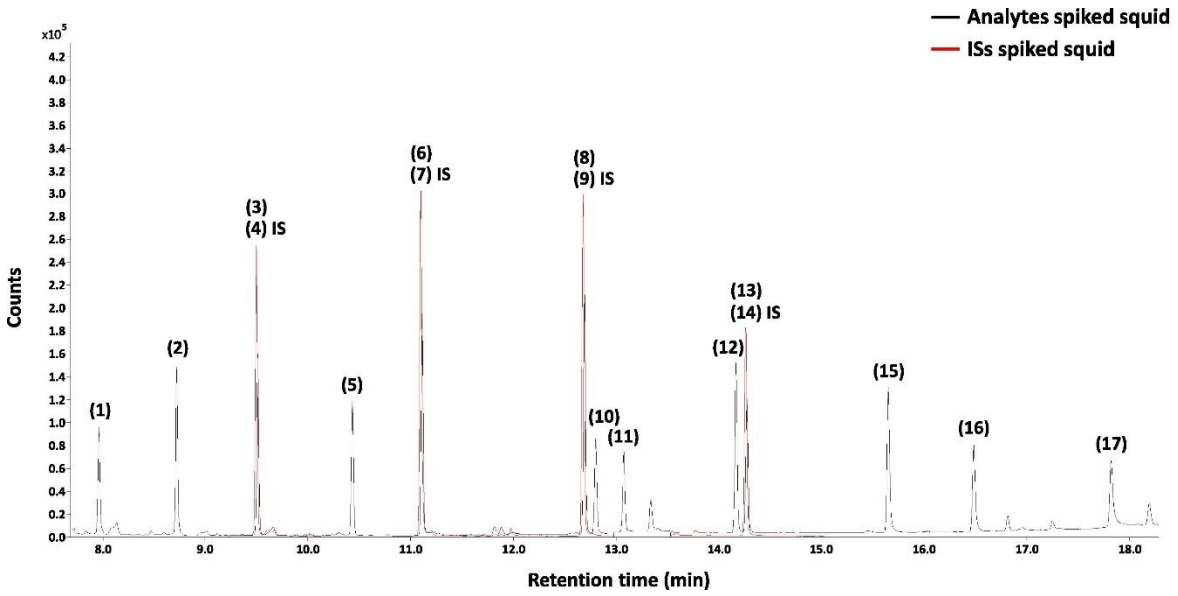

Figure 3.4 GC–MS chromatogram of a spiked squid sample at 75 ng/g level after the application of the ammonium formate version of the QuEChERS method. Peak identification: DPP (1), DIBP (2), DBP (3), DBP-d4 (4, IS), DIPP (5), DNPP (6), DNPP-d4 (7, IS), DHP (8), DHP-d4 (9, IS), BBP (10), DEHA (11), DCHP (12), DEHP (13), DEHP-d4 (14, IS), DNOP (15), DINP (16), DIDP (17).

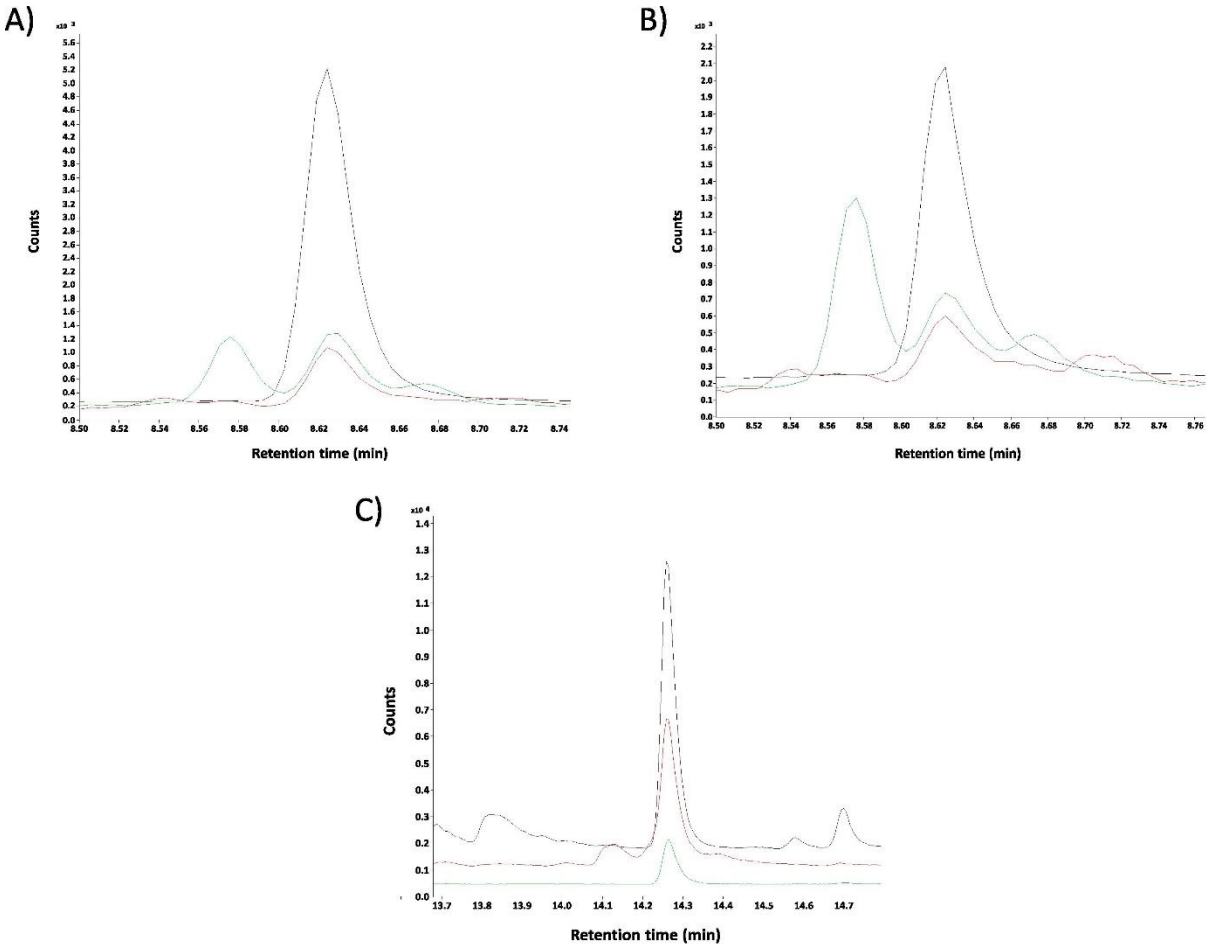

Figure 3.5 GC–MS chromatogram of A) DIBP in a squid sample, B) DBP in a squid sample, and C) DEHP in a mackerel sample after the application of the ammonium formate version of the QuEChERS method. All three PAEs have m/z 149 as the quantification ion (black line). For DIBP and DBP the qualifier ions are 205 (red line) and 223 (green line), while for DEHP they are 167 (red line) and 279 (green line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Real sample analysis

Once the method was validated, it was applied to the analysis of ten samples of each type. For this purpose, the complete muscle of each individual was cut, frozen with liquid nitrogen and homogenized until a fine powder was obtained. Afterwards, a portion of 5 g of each sample was analysed in duplicate. At the same time, the water content of each sample was determined, which ranged between 65.7 and 75.2% for mackerel, between 83.9 and 87.8% for squid and between 70.5 and 71.9% for tuna samples. Table 3.3 shows the results of the analysis of the 30 samples expressed as ng/g of w.w.

Table 3.3 Results of the analysis of mackerel, squid, and tuna samples after the application of the QuEChERS-GC–MS method.

	Sample	Sampling date	Analytes (ng/g) wet weight		
			DIBP	DBP	DEHP
Mackerel	1	June 21st, 2021	n.d.	n.d.	n.d.
	2	June 21st, 2021	n.d.	n.d.	n.d.
	3	June 22nd, 2021	n.d.	n.d.	n.d.
	4	June 22nd, 2021	10.2 ± 3.4	n.d.	n.d.
	5	June 23rd, 2021	7.24 ± 3.39	n.d.	44.2 ± 2.1
	6	June 23rd, 2021	5.82 ± 3.40	n.d.	43.2 ± 2.1
	7	June 24th, 2021	n.d.	n.d.	n.d.
	8	June 24th, 2021	n.d.	n.d.	n.d.
	9	June 24th, 2021	n.d.	n.d.	n.d.
	10	June 24th, 2021	n.d.	n.d.	n.d.
Squid	1	July 10th, 2021	2.95 ± 0.80	n.d.	< LCL
	2	July 10th, 2021	6.70 ± 0.80	< LCL	< LCL
	3	July 10th, 2021	2.92 ± 0.80	< LCL	< LCL
	4	July 10th, 2021	1.17 ± 0.80	n.d.	< LCL
	5	July 10th, 2021	n.d.	< LCL	< LCL
	6	July 10th, 2021	n.d.	n.d.	5.32 ± 1.06
	7	July 10th, 2021	n.d.	< LCL	n.d.
	8	July 10th, 2021	n.d.	10.9 ± 1.5	< LCL
	9	July 10th, 2021	n.d.	< LCL	< LCL
	10	July 10th, 2021	n.d.	< LCL	< LCL
Tuna	1	July 21st, 2021	< LCL	n.d.	24.5 ± 2.8
	2	July 23rd, 2021	< LCL	< LCL	n.d.
	3	July 23rd, 2021	< LCL	n.d.	n.d.
	4	July 23rd, 2021	< LCL	n.d.	n.d.
	5	July 23rd, 2021	< LCL	n.d.	n.d.
	6	July 23rd, 2021	n.d.	n.d.	n.d.
	7	July 23rd, 2021	n.d.	n.d.	< LCL
	8	July 23rd, 2021	< LCL	n.d.	n.d.
	9	July 23rd, 2021	n.d.	n.d.	< LCL
	10	July 23rd, 2021	n.d.	n.d.	n.d.

As can be seen in the table, only DIBP and DEHP were found above the LCL in some mackerel samples, as well as DIBP, DBP and DEHP in some squid samples and DEHP in tuna samples. Since some of the samples were collected in the same date as they were analysed, the concentration for some of them are quite similar, being DEHP the one with the highest variability. Among the PAEs found, TDIs have only been established for DEHP while no maximum residue limits have been stablished in Europe for these compounds and matrices. If an average consumption of 125–150 g of fish fillets or 200–250 g of whole fish with 2–4 servings/week is considered (advisable dietary intake in Spain (Guidelines, 2021)), the TDI of DBP and DEHP of 50 µg/kg of b.w. is not exceeded in any case, neither individually nor considering the group TDI established for DBP, BBP, DEHP and DINP. As an example, the ingestion of 150 g of the mackerel with the highest concentration of DEHP (44.2 ± 2.1 ng/g of w.w.) means a single ingestion of less than 7 µg per person.

Besides, in some of the squid samples, also the PAEs DBP and DEHP were found below the LCL while for some tuna samples DIBP, DBP, and DEHP were found below such levels too. Among them, TDI values have only been established for DBP, but since the concentration are below the LCLs, its TDI is not exceeded either.

Regarding previous works in which similar samples have been analysed, similar concentration ranges of PAEs were also found. Castro-Jiménez and Ratola (Castro-Jiménez & Ratola, 2020) found total PAEs concentrations of 19–83 ng/g for Atlantic bonito *Sarda sarda* and European hake *Merluccius merluccius*. Xu *et al.* (Xu et al., 2018) found in different fish samples (species were not indicated) similar PAEs to the ones of our work, in particular, DIBP, DBP and DEHP in the range 38.47–763.22 ng/g of w.w. Very recently, Hidalgo-Serrano *et al.* (Hidalgo-Serrano et al., 2021) analysed different samples of European squid (*Loligo vulgaris*) and fish (Atlantic salmon *Salmo salar*, Atlantic mackerel *Scomber scombrus* and sole *Solea solea*) finding concentrations up to 978 ng/g of d.w. In this case,

DEHP, DBP and BBP together with other PAEs not in common with this work were also found though in most cases below the limits of quantification of the method.

4. Conclusions

The application of the ammonium formate version of the QuEChERS method to extract 12 PAEs and the adipate DEHA from two species of fish (*Scomber colias* and *Katsuwonus pelamis*) and one of squid (*Loligo gahi*) resulted satisfactory in terms of linearity (matrix-matched calibration) and recovery values, except in the case of DIDP which could not be perfectly quantified in tuna samples as a result of an important chromatographic interference. ME was negligible for most analytes in the case of fish samples, though for squid a moderate/high signal suppression was found. The analysis of 10 samples of each species revealed the presence of DIBP, DBP and DEHP above the LCLs in some of the samples, as well as other PAEs below such level. Even though, the TDIs for those PAEs for which such limit has been established were not exceeded in any case. The application of this version of the method, as previously reported, is highly advantageous from an instrumental point of view, being still so simple and easy to apply as expected for the QuEChERS method.

Supplementary data

Supplementary data to this chapter can be found online at https://doi.org/10.1016/j.foodchem.2022.132174 and in Appendix B of this document.

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CHAPTER IV

OPTIMIZATION AND VALIDATION OF A MICRO–QUECHERS METHOD FOR PHTHALATES DETECTION IN SMALL SAMPLES OF CETACEAN BLUBBER

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Chapter 4 - Optimization and Validation of a Micro–QuEChERS Method for Phthalates Detection in Small Samples of Cetacean Blubber

Abstract

In this study, an innovative method was developed to detect and quantify phthalates in fresh cetacean blubber. An adaptation of the ammonium formate QuEChERS method was used and adapted as a micro-extraction for small quantities of samples. Significantly, this technique utilized minimal quantities of reagents and salts, with the additional implementation of rigorous Quality Assurance/Quality Control protocols to further reduce background contamination. To ensure the reliability of this method, comprehensive validation procedures were conducted, with a specific focus on two widely studied cetacean species: the common bottlenose dolphin (*Tursiops truncatus*) and the short-finned pilot whale (*Globicephala macrorhynchus*). Determination coefficients (R2) for matrix-matched calibration were >0.93 with limits of quantifications (LOQ) of the method in the range of 5–10 ng/g. Mean recovery values were between 40 and 100 %. This novel methodology holds particular relevance for environmental research studies, offering the capability to detect emerging contaminants with minimal sample requirements. This aspect is particularly valuable in investigations that involve free-ranging animals and rely on biopsy sampling. It allows for the assessment of contaminant levels in healthy individuals within wild populations, enhancing our understanding of ecological impacts and potential conservation measures.

• A micro-extraction adaptation of the ammonium formate QuEChERS method was developed and applied to a small quantity of fresh cetacean blubber to detect phthalates.

• Small quantities of reagents and salts were used, and additional Quality Assurance/ Quality Control procedures were taken to further minimize background contamination.

• Method validation was carried out for two cosmopolitan and extensively studied cetacean species: the common bottlenose dolphin (*Tursiops truncatus*) and the short-finned pilot whale (*Globicephala macrorhynchus*).

Keywords

Bottlenose dolphin; Pilot whale; Phthalic acid esters; Plastic additives; GC-MS; Gas chromatography-mass spectrometry

1. Method details

A modified version of the QuEChERS method described in Sambolino et al. [1] was applied to a small quantity of cetacean blubber for phthalates extraction and purification (named micro- QuEChERS). The present method, in order to be applied in 100x smaller quantity of tissue, sees a considerably reduced amount of extraction reagents, while maintaining the same amount of purification salts, given the higher fat concentrations of the target tissue. An extra step was introduced to concentrate the final extract, given the limited amount of analytes extractable from such small samples. For this reason, additional cleaning procedures were implemented to control background contamination, minimizing the risk of co-extraction of phthalates in reagents and glassware. The target analytes in this modified approach are seven phthalates which are the most commonly encountered in the marine environment [2], [3], [4].

Blubber tissue was collected from stranded cetaceans (common bottlenose dolphin - *Tursiops truncatus* and short-finned pilot whale - *Globicephala macrorhynchus*) and kept frozen at −20° C until the day of analysis. On the analysis day, a small portion of sample (50 mg, wet weight [w.w.]) was cut, added to a 15 mL glass tube, and mashed, still frozen, with a glass rod for at least 1 min. One ml of Acetonitrile (ACN) was then added to the glass tube

with the freeze-homogenized sample, vortexed for 1 min, then 0.5 g of ammonium formate was added, in order to induce phase separation [5]; the mixture was vortexed again for 1 min and then centrifuged for 5 min at 2500 rpm. For the purification step, all the supernatant was transferred to a glass tube containing 150 mg of MgSO4, 50 mg of Primary Secondary Amine (PSA), and 50 mg of C18. The ratio of ammonium formate to ACN and MgSO4 in PSA and C18 followed established methodologies [1,5], although quantities of reagents were considerably reduced in comparison to the original method [1]. The mixture was again vortexed for 1 min and centrifuged for 5 min at 2500 rpm. The resulting supernatant (200 µl) was transferred to a GC vial, evaporated overnight, and then reconstituted in 50 µl of Cyclohexane (CH).

To minimize contamination, all the glassware was carefully cleaned, immersed in an acid bath for 24 h, and then muffled at 550° C overnight [1,6]. No plastic material besides pipette tips (phthalate-free) was used during the procedure. To minimize background contamination from organic reagents, ACN and CH were double distilled before use [7] and salts were washed three times with methanol. Two procedural blanks were analyzed with each batch of samples, and an average of the two blank values (RSD < 10 %) was subtracted from the final results. High-purity solvents and reagents were used. Methanol, ACN and CH of LC-MS grade, ammonium formate (purity ≥ 97.0 %) and MgSO4 (purity ≥ 98.0 %) were from VWR International Eurolab (Barcelona, Spain). PSA and C18 were from Agilent Technologies (Santa Clara, CA, USA).

Seven phthalates (Phthalic Acid Ester - PAE) were the target analytes (chemical structures and proprieties in Table 4.1). High purity standards (>98 %) of each PAE and two isotopically labeled PAEs (DEP-D4 and DBP-D4, used as internal standards) were acquired from Sigma-Aldrich (Madrid, Spain) and Dr. Ehrenstorfer (Augsburg, Germany). The determination and quantification of the analytes was carried out with an Agilent 6890 GC

(Agilent Technologies, California, USA), equipped with an Ultra Inert HP-5 ms capillary column (30 m × 0.25 mm inner diameter, 0.25 µm film thickness), coupled with an Agilent 5973 Network MS (Agilent Technologies, California, USA), in the selected ion monitoring (SIM) mode. The oven temperature program started at 60 °C (1 min), then increased to 190 °C at 45 °C/min, and finally increased to 310 °C at 35 °C/min and held for 3 min. Injection was in splitless mode (purge time 0.75 min; purge flow 40 ml/min) at 280 °C, with an injection volume of 2 µl. The detection parameters follow those described in Sambolino et al. [1]; retention times, ion qualifiers and quantifiers of selected PAEs for this study are provided in Table 4.2.

Table 4.1 Chemical structure and properties of the studied phthalates.

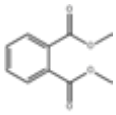
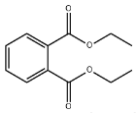
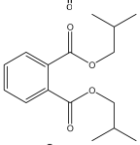
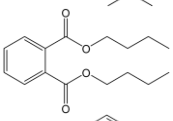
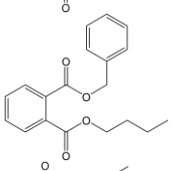
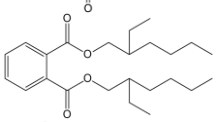
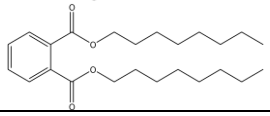
Analyte	Structure	Molecular formula	MM (g/mol)	Solubility in water (g/L, 25 ºC)	Vapor pressure (mmHg, 25 ºC)	Log Kow	Melting point (ºC)	Boiling point (ºC)
DMP		$C_{10}H_{10}O_4$	194.2	4.3	$3.08 \cdot 10^{-3}$	1.60	5.5	284
DEP		$C_{12}H_{14}O_4$	222.2	1.08	$2.1 \cdot 10^{-3}$	2.47	-3	295
DIBP		$C_{16}H_{22}O_4$	278.3	0.0062[a]	$4.76 \cdot 10^{-5}$	4.11	-37	320
DBP		$C_{16}H_{22}O_4$	278.2	0.0112	$2.01 \cdot 10^{-5}$	4.72	-35	340
BBP		$C_{19}H_{20}O_4$	312.1	0.00269	$8.25 \cdot 10^{-6}$	4.73	-35	370
DEHP		$C_{24}H_{38}O_4$	390.3	0.00027	$1.42 \cdot 10^{-7}$	7.60	-55	230
DNOP		$C_{24}H_{38}O_4$	390.6	0.000022	$1.0 \cdot 10^{-7}$	8.20	-25	385

Table 4.2 Retention times and m/z values of quantifier and qualifier ions in GC-MS analyses of the target analytes and internal standards (in bold).

Analyte	Retention time (min)	Quantifier (m/z)	Qualifier 1 (m/z)	Qualifier 2 (m/z)
DMP	4.678	163	77	194
DEP-d_4	5.090	153	181	80
DEP	5.096	149	177	76
DIBP	5.872	149	223	104
DBP-d_4	6.122	153	209	227
DBP	6.126	149	205	223
BBP	7.152	149	91	206
DEHP	7.559	149	167	279
DNOP	8.028	149	167	279

Ionization energy of 70 eV in all cases.

2. Method validation

The modified QuEChERS method (micro-QuEChERS) was validated through several steps. First, an instrument calibration using the internal standard approach was done in order to quantify the blank values and calculate the matrix effect (Table 4.3).

Recoveries studies at two spiking levels (25 and 150 ng/g w.w.) and matrix-matched calibrations with the internal standard method were then carried out for both matrices (blubber samples from the two species) (Table 4.4, 4.5). The equations coefficient obtained were used to quantify the compounds in the analyzed samples. The studied linear range, the matrix effect (ME) and the limits of quantifications (LOQ) of the method, considered as the lowest calibration level with signal to noise ratio > 10, are reported in Table 4.5 for the two different matrices analyzed.

Table 4.3 Internal instrumental calibration data of the target analytes.

Analyte	Studied linear range (µg/L)	Regression equation (n=8)		$s_{y/x}$	R^2
		$b \pm s_b \cdot t_{(0.05;7)}$	$a \pm s_a \cdot t_{(0.05;7)}$		
DMP	5 - 300	$(5.37 \pm 0.49) \cdot 10^{-3}$	$(0.54 \pm 6.7) \cdot 10^{-2}$	$4.67 \cdot 10^{-2}$	0.9938
DEP	5 - 300	$(7.68 \pm 0.72) \cdot 10^{-3}$	$(7.64 \pm 9.84) \cdot 10^{-2}$	$6.86 \cdot 10^{-2}$	0.9934
DIBP	5 - 300	$(10.19 \pm 1.24) \cdot 10^{-3}$	$(4.06 \pm 17.57) \cdot 10^{-2}$	$10.92 \cdot 10^{-2}$	0.9924
DBP	5 - 300	$(11.68 \pm 1.05) \cdot 10^{-3}$	$(1.31 \pm 14.43) \cdot 10^{-2}$	$10.06 \cdot 10^{-2}$	0.9939
BBP	5 - 300	$(4.19 \pm 0.42) \cdot 10^{-3}$	$(5.88 \pm 6.57) \cdot 10^{-2}$	$3.11 \cdot 10^{-2}$	0.9970
DEHP	5 - 300	$(6.44 \pm 0.73) \cdot 10^{-3}$	$(3.22 \pm 10.65) \cdot 10^{-2}$	$5.39 \cdot 10^{-2}$	0.9962
DNOP	5 - 300	$(9.42 \pm 1.04) \cdot 10^{-3}$	$(1.52 \pm 16.36) \cdot 10^{-2}$	$7.74 \cdot 10^{-2}$	0.9964

b: slope; S_b: standard error of the slope; $t_{(0.05;7)}$: t-multiplier for 95% confidence interval calculation; a: intercept; S_a: standard error of the intercept; $s_{y/x}$: standard error of the estimate; R^2: determination coefficient.

The method yielded recoveries with relatively high variation. For *T. truncatus* samples, mean relative recoveries were between 85 – 100 % for five of the seven PAEs; DMP and DNOP held lower recoveries (40% and 75%, respectively). For *G. macrorhynchus*, mean recoveries ranged between 53% and 100 % (Table 4.4). Matrix-matched calibrations with the internal standard method were calculated for each matrix, obtaining linear regression with fitting $R^2 >$ 0.93. Limits of quantifications (LOQ) of the method ranged between 5-10 ng/g w.w. (Table 4.5). The matrix effect varied depending on the matrix and the analyte, staying within the ± 20% limits, except for DBP and BBP in *G. macrorhynchus* (-33% and -27%, respectively) and DMP, BBP, and DNOP in *T. truncatus* (+42%, +37% and +48% respectively).

Real samples analysis was conducted on portions of biopsies from the two species (*G. macrorhynchus*, n=15; *T. truncatus*, n = 9), longitudinally cut to encompass all the blubber layers. Results on the PAEs concentrations are reported and further discussed in the related research article (Chapter 6).

Table 4.4 Relative recovery (%) and RSD values (in brackets) of the target analytes from recovery studies with two spiking levels on blubber

Matrix (Species)	Analyte	Level 1	Level 2	Mean
Short-finned pilot whale (*Globicephala macrorhynchus*)	DMP	88 (37)	108 (43)	100 (38)
	DEP	61 (30)	121 (41)	97 (51)
	DIBP	72 (5)	103 (4)	88 (20)
	DBP	59 (6)	74 (3)	67 (13)
	BBP	64 (5)	41 (9)	53 (24)
	DEHP	70 (9)	37 (10)	53 (35)
	DNOP	162 (13)	23 (14)	93 (83)
Bottlenose dolphin (*Tursiops truncatus*)	DMP	45 (21)	36 (20)	40 (22)
	DEP	82 (22)	92 (6)	87 (15)
	DIBP	83 (28)	88 (8)	85 (18)
	DBP	92 (20)	107 (11)	100 (16)
	BBP	114 (31)	78 (7)	92 (28)
	DEHP	110 (20)	83 (3)	97 (21)
	DNOP	75 (4)	57 (11)	65 (17)

Level 1: 25 ng/g of w.w; level 2: 150 ng/g of w.w. Data outside the 70-120% range for recovery values and 0-20% for RSD values are in bold.

Table 4.5 Matrix-matched calibration data of the selected PAEs, with limits of quantification (LOQ) and matrix effect (ME) percentage in cetacean blubber samples.

Matrix (species)	Analyte	Studied linear range (ng/g)	Regression equation (n=8)		$s_{y/x}$	R^2	LOQ (ng/g)*	ME (%)**
			$b \pm s_b \cdot t_{(0.05;7)}$	$a \pm s_a \cdot t_{(0.05;7)}$				
Short-finned pilot whale (*Globicephala macrorhynchus*)	DMP	5 – 300	$(4.39 \pm 1.58)\cdot10^{-3}$	$(0.81 \pm 11.34)\cdot10^{-2}$	$0.59\cdot10^{-1}$	0.9631	10	-18
	DEP	5 – 300	$(8.27 \pm 2.14)\cdot10^{-3}$	$(0.61 \pm 31.68)\cdot10^{-2}$	$1.72\cdot10^{-1}$	0.9664	10	8
	DIBP	5 – 300	$(10.9 \pm 1.29)\cdot10^{-3}$	$(0.37 \pm 17.56)\cdot10^{-2}$	$1.29\cdot10^{-1}$	0.9894	5	7
	DBP	5 – 300	$(7.84 \pm 3.94)\cdot10^{-3}$	$(0.55 \pm 57.51)\cdot10^{-2}$	$2.91\cdot10^{-1}$	0.9302	5	-33
	BBP	5 – 300	$(3.07 \pm 0.24)\cdot10^{-3}$	$(0.32 \pm 1.94)\cdot10^{-2}$	$0.14\cdot10^{-1}$	0.9953	10	-27
	DEHP	5 – 300	$(5.8 \pm 3.98)\cdot10^{-3}$	$(0.73 \pm 64.76)\cdot10^{-2}$	$2.03\cdot10^{-1}$	0.9515	5	-10
	DNOP	5 – 300	$(9.3 \pm 0.4)\cdot10^{-3}$	$(0.71 \pm 4.97)\cdot10^{-2}$	$0.39\cdot10^{-1}$	0.9986	10	-1

Bottlenose dolphin (*Tursiops truncatus*)	DMP	5 – 300	$(7.62 \pm 1.11)\cdot10^{-3}$	$(0.14 \pm 13.86)\cdot10^{-2}$	$1.09\cdot10^{-1}$	0.9842	5	42
	DEP	5 – 300	$(8.27 \pm 5.02)\cdot10^{-3}$	$(0.35 \pm 86.33)\cdot10^{-2}$	$2.52\cdot10^{-1}$	0.9617	5	8
	DIBP	5 – 300	$(12.02 \pm 2.04)\cdot10^{-3}$	$(0.3 \pm 31.41)\cdot10^{-2}$	$1.57\cdot10^{-1}$	0.9915	5	18
	DBP	5 – 300	$(11.38 \pm 9.4)\cdot10^{-3}$	$(0.28 \pm 145.43)\cdot10^{-2}$	$5.25\cdot10^{-1}$	0.9314	5	-3
	BBP	5 – 300	$(5.73 \pm 0.52)\cdot10^{-3}$	$(0.19 \pm 6.7)\cdot10^{-2}$	$0.55\cdot10^{-1}$	0.9917	10	37
	DEHP	5 – 300	$(6.9 \pm 1.34)\cdot10^{-3}$	$(0.42 \pm 20.7)\cdot10^{-2}$	$1.01\cdot10^{-1}$	0.9889	5	7
	DNOP	5 – 300	$(13.96 \pm 1.37)\cdot10^{-3}$	$(0.18 \pm 17.57)\cdot10^{-2}$	$1.46\cdot10^{-1}$	0.9904	10	48

b: slope; Sb: standard deviation of the slope; t(0.05;7): t-multiplier for 95% confidence interval calculation; a: intercept; Sa: standard deviation of the intercept; sy/x: standard deviation of the estimate; R2: determination coefficient. *Calculated as the lowest calibration level with S/N>10; **Calculated following the equation used by Kwon et al. (Kwon, Lehotay, & Geis-Asteggiante, 2012). [https://doi.org/10.1016/j.chroma.2012.10.059]

3. Additional information

The present manuscript proposes a new rapid, cost-effective methodology to analyze phthalates in small blubber samples from two odontocete species. Bottlenose dolphins and pilot whales, extensively studied among delphinid species due to their widespread abundance and distribution in temperate and tropical waters [8,9], face significant anthropogenic impacts, sharing habitats and resources with human activities [1,10]. Conservation efforts necessitate monitoring their contaminant status. The use of biopsy sampling in free-ranging individuals offers a superior assessment of wild population health compared to stranded animals [11,12]. However, these samples are extremely limited in quantity, demanding innovative methodologies for their effective use. To the best of our knowledge, only one other method [13] has been developed for working with such samples. Our refined approach, aligning with QuEChERS extraction principles, proves notably more efficient and faster, utilizing only half the sample quantity (50 mg vs. 100 mg).

Previous studies [14,15] indicate that pollutant concentrations may vary based on blubber layer stratification and sample body location. Therefore, analyzed biopsy samples

should encompass all layers, from beneath the skin to above the muscle, with due consideration to the sample's body location.

The proposed method is anticipated to produce comparable results for other odontocete species, however, blubber tissue in different cetacean species exhibits variability in thickness and triglycerides/wax esters composition [16]. Thus, when applying the method to new species, validation is strongly recommended.

Analyzing blubber is challenging due to its high lipid content, posing interference with chromatographic analysis. While employing ACN facilitates lipid removal from fat tissues, an additional cleanup step is essential [17]. Dispersive solid-phase extraction (d-SPE) with a mixture of MgSO4, PSA and C18 was found effective for coextractives removal from acetonitrile extract [18]. However, some coeluates extracted during the procedure may interfere with the final analysis, causing signal suppression [19], which was observed in the matrix effect (Table 4.5). Recovery rates for most analytes ranged from 80-100%. However, analytes like DMP exhibited a significant loss, potentially attributed to the drying step, resulting in a lower rate of 40%.

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CHAPTER V

MICROPLASTIC INGESTION AND PLASTIC ADDITIVE DETECTION IN PELAGIC SQUID AND FISH: IMPLICATIONS FOR BIOINDICATORS AND PLASTIC TRACERS IN OPEN OCEANIC FOOD WEBS

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Chapter 5 - Microplastic Ingestion and Plastic Additive Detection in Pelagic Squid and Fish: Implications for Bioindicators and Plastic Tracers in Open Oceanic Food Webs

Abstract

The ubiquitous presence of microplastics (MPs) in the ocean represents a potential threat to marine organisms, with poorly understood long-term adverse effects, including exposure to plastic additives. The present study investigated the ingestion of MPs in two epipelagic fish species (*Trachurus picturatus* and *Scomber colias*) and three pelagic squid species (*Loligo vulgaris*, *Ommastrephes caroli* and *Sthenoteuthis pteropus*) from an open oceanic region of the Northeast Atlantic. Seven phthalate esters (PAEs) were also analysed in the organisms' tissue, and the potential correlation between PAEs concentrations and ingested MPs was investigated. Seventy-two fish and 20 squid specimens were collected and analysed. MPs were found in the digestive tract of all species and in the squid species' gills and ink sacs. The highest occurrence of MPs was in the stomach of *S. colias* (85 %) and the lowest in the stomach and ink sac of *O. caroli* and *L. vulgaris* (12 %). Most of the particles identified (>90 %) were fibres. Among all the ecological and biological factors considered (dietary preferences, season, body size, total weight, liver weight, hepatosomatic index and gastrosomatic index), only gastrosomatic index (GSI) and season were significant predictors of MPs ingestion in fish species, with a greater likelihood of ingestion in the cold season and in specimens with higher GSI values (*i.e.* higher feeding intensity). Four PAEs (DEP, DIBP, BBP, DEHP) were detected in all the species analysed, with average ∑PAEs concentrations ranging between 10.31 and 30.86 ng/g (wet weight). DIBP was positively correlated with ingested MPs, suggesting this compound might represent a “plastic tracer”. This study looks into the problem of MPs ingestion for pelagic species in an open oceanic region, highlighting

the most suitable bioindicators and providing essential insights into the factors that may influence ingestion rates. Additionally, the detection of PAEs in all species indicates the need for further research on the contamination sources, the effects of these chemicals on marine organisms, and the potential risks to human health through seafood consumption.

Keywords

MPs, Ocean contamination, Plastic additives, Ingestion of microplastics, Pelagic fish species, Phthalate esters.

1. Introduction

The widespread contamination by microplastics (MPs; plastic particles <5 mm) represents a global threat to open ocean ecosystems in many different aspects (Cózar et al., 2014; Gestoso et al., 2019; Herrera et al., 2020; McIvor et al., 2023). MPs primarily originate from land-based sources, such as laundry discharges and breakage of improperly disposed waste, and enter the ocean through streams, rivers, and sewage from populated areas, especially when wastewater treatment is poor or deficient (Li et al., 2020; Vassilenko et al., 2021; Sambolino et al., 2022a). Winds and ocean currents facilitate the transport of MPs across long distances, resulting in their ubiquity in water bodies (Van Sebille et al., 2020; Ross et al., 2021). The Atlantic Ocean is no exception to this global phenomenon, even in areas that once were considered pristine (Cózar et al., 2014).

Low-density buoyant MPs are found in higher quantities in the first few meters of the water surface and, subjected to atmospheric and oceanographic processes, get incorporated into the current circulation system and accumulate in specific convergent zones (Law et al., 2010;

Brach et al., 2018). The Northeast Atlantic, for instance, harbors one of the subtropical gyres, an anticyclonic ocean circulation responsible for transporting, collecting and aggregating plastic particles (Law et al., 2010; Silvestrova and Stepanova, 2021). These currents also carry plastic litter from the American continent through the Macaronesian archipelagos (Pham et al., 2020; Cardoso and Caldeira, 2021).

Epipelagic planktivorous fish have recently gained attention for their high susceptibility of ingesting these low-density, small-sized synthetic particles (MPs) that are predominantly in the surface layers of the water column (Herrera et al., 2019; Lopes et al., 2020; Pereira et al., 2020). While fish species have been extensively studied, there is a lack of knowledge regarding other taxa that feed in the same zone, such as squids. Cephalopods are important prey for various marine predators, such as demersal and pelagic fish and marine mammals (e.g. dos Santos and Haimovici, 1998; Yamamura and Inada, 2001; Bearzi et al., 2011). They exhibit a wide-ranging diet, consuming fish, crustaceans, cephalopods, and polychaetes (Pierce et al., 1994; Ivanovic and Brunetti, 2004; Valls et al., 2015; Merten et al., 2017), making them key species with a significant impact on the trophodynamics of marine ecosystems and a crucial role in pelagic food webs (Gasalla et al., 2010; Navarro et al., 2013; Coll et al., 2013; Merten et al., 2017). Mesopelagic species typically perform diel vertical migration, feeding at night within depth ranges of 0–200 m, in the epipelagic layer.

The high bioavailability of MPs in the epipelagic zone significantly increases the likelihood of MPs being ingested by these marine organisms (Thompson et al., 2004; Jovanović, 2017; Wang et al., 2020). Other factors, such as species-specific feeding strategies and selectivity, may also play a pivotal role. For example, some studies suggested that carnivorous predators might be more exposed to MPs through trophic transfer (Sequeira et al., 2020), while others have shown evidence that filter-feeding organisms exhibit the highest ingestion rates (Kahane-Rapport et al., 2022). MPs bear resemblance to plankton in shape,

colour and size, leading to direct ingestion when mistaken for food or indirect ingestion through contaminated prey during feeding events (Jovanović, 2017). Passive pathways of microplastic uptakes, such as accidental ingestion while feeding or drinking (a typical behaviour in marine fish species), are also possible (Roch et al., 2020).

Regardless of the uptake mechanism, ingesting MPs can have harmful consequences for marine organisms (as reviewed by Wang et al., 2020 and Koelmans et al., 2022). In fish species, the presence of MPs in the gastrointestinal tracts can lead to false satiation, impaired reproduction, slowed growth rate, and oxidative stress (Jovanović, 2017; Hossain and Olden, 2022). However, MPs do not seem to accumulate in the gastrointestinal tract of fish. Instead, they are likely excreted at a similar rate to ingestion and without observed biomagnification along the trophic chain (Jovanović, 2017; Miller et al., 2020). For example, a recent controlled experiment observed that most individuals from two marine fish species (Indian medaka and clown anemonefish) excreted all the previously ingested polyethylene particles within 24 h (Okamoto et al., 2022). Conversely, other studies have observed the accumulation of fine plastic particles (< 100 μm) in organs, such as gills, intestines, and liver of fish (ex. Wang et al., 2019; Prata et al., 2022; Lee et al., 2023). In fact, small (< 100 μm) MPs can migrate to different tissues through the vascular system and cellular passages, posing concerning health repercussions for the organisms (Jovanović, 2017; Wang et al., 2020).

Another concerning consequence of MP uptake in marine organisms is the exposure to hazardous chemical compounds associated with plastics (Do et al., 2022). Plastics can adsorb hydrophobic organic chemicals (HOCs), heavy metals, and other pervasive compounds, which can then be transferred to organisms (Brennecke et al., 2016; Hartmann et al., 2017; Koelmans et al., 2016). Of particular concern are the toxic chemical compounds, known as plastic additives, which are incorporated into plastics during manufacturing and can subsequently be

released from MPs into the environment (Hermabessiere et al., 2017; Hahladakis et al., 2018; Do et al., 2022).

Plastic polymers can be used for a wide range of applications beyond consumer products, including textiles (synthetic fibres), foams, coatings, adhesives, and sealants. During manufacturing, various organic additives, such as plasticisers, flame retardants, photo-stabilisers, antioxidants, and pigments, are intentionally mixed with polymers to improve their performance, functionality, and aging properties (Stevens, 1990; Hahladakis et al., 2018). Plasticisers, in particular, can be added in concentrations up to 70 % of the wet weight to improve the flexibility, durability, and stretchability of the material (Hahladakis et al., 2018). Many of these organic additives are hazardous to aquatic life, exhibiting carcinogenic, mutagenic, or endocrine-disrupting properties in marine invertebrates and fish (Hermabessiere et al., 2017). In nearly all cases, additives are not covalently bonded to the polymer matrix and can leach into the surrounding media, especially in lipophilic matrices like sediment and biota (Teuten et al., 2009; Andrade et al., 2021). Recent studies suggest that the hydrophobic properties of plastic additives limit the leaching of these compounds from MPs into the aquatic environment. However, if marine organisms ingest the MPs, the stomach and fish oils present in their gastric environment may accelerate the leaching process, resulting in higher concentrations of the chemicals being absorbed by biological tissues (Andrade et al., 2021; Sun et al., 2021).

Due to their ubiquity, one class of plasticisers that is receiving increasing attention is phthalates, or phthalic acid esters (PAEs). The solubility of certain types of phthalates in water and their extensive use in a wide range of products emphasise the need to assess their potential impact on the environment and human health (Heudorf et al., 2007; Katsikantami et al., 2016; Paluselli et al., 2018a; Chen et al., 2022). Phthalates with smaller molecular structures and lower molecular weights, such as diethyl phthalate (DEP) and dibutyl phthalate (DBP) are more

soluble in water and are often used in personal care products, pharmaceuticals, dyes, pesticides, and varnishes (Giuliani et al., 2020). However, phthalates are primarily used as plasticisers to add flexibility to plastic materials. Bis (2–ethylhexyl) phthalate (DEHP) is, historically, the one produced in the largest quantities as the most common plasticiser for the production of PVC (Heudorf et al., 2007). Di-n-propyl phthalate (DPP), diethyl phthalate (DEP), diisobutylphthalate (DIBP), and dibutyl phthalate (DBP) are added in polyethylene terephthalate (PET) (Hahladakis et al., 2018). DBP is also used for the production of cellulose acetate plastics. Other polymers, such as polyethylene (PE) and polystyrene (PS), also can contain and release phthalates (Fasano et al., 2012; Hahladakis et al., 2018; Paluselli et al., 2018a). Therefore, the substantial amount of plastic waste in the marine environment might represent a significant source of PAEs pollution (Cao et al., 2022).

In the past few years, PAEs have been widely detected in the marine environment, particularly in marine biota (Net et al., 2015; Baini et al., 2017; Schmidt et al., 2021; Sambolino et al., 2022b; Squillante et al., 2023), with DEHP, DBP, DIBP, and DEP as the most frequently detected ones (Hidalgo-Serrano et al., 2022). These compounds, known for their endocrine-disrupting properties, have been extensively studied due to their harmful effects on animal and human health (Oehlmann et al., 2009; Katsikantami et al., 2016). PAEs can negatively impact the immune system, endocrine system, metabolism, development, and behaviour of aquatic animals such as fish and invertebrates, leading to fertility issues, reduced hatchability, impaired embryonic development, and altered sex ratios (Zhang et al., 2021). Exposure to phthalates has also been associated with fertility problems, respiratory diseases, childhood obesity, and neuropsychological disorders in humans (Katsikantami et al., 2016). Due to their endocrine-disrupting properties and reproductive toxicity, DEHP, BBP, DBP, and DIBP have been listed as Substances of Very High Concern (SVHC) under the REACH (Registration, Evaluation,

Authorisation and Restriction of Chemicals) regulation, resulting in restrictions or bans on their production and use in the manufacturing (Reg. 1907 CE/2006).

Although the potential absorption of PAEs through the ingestion of MPs in marine organisms and the use of phthalates as "plastic tracers" has been proposed by several authors (Fossi et al., 2014; Baini et al., 2017), the correlation between MPs ingestion and PAEs concentrations is not always clear, and results are sometimes contradictory (Schmidt et al., 2021). Therefore, this paper aims to investigate the relationship between MPs uptake and phthalates concentration, as well as their associations with biological and ecological variables in two epipelagic fish species (Atlantic chub mackerel *Scomber colias* and blue jack mackerel *Trachurus picturatus*) and three mesopelagic squid species (neon flying squid *Ommastrephes caroli*, previously known as *Ommastrephes bartramii* (Fernández-Álvarez et al., 2020), orange-back flying squid *Sthenoteuthis pteropus*, and European squid *Loligo vulgaris*) from the North East Atlantic Ocean.

The Atlantic chub mackerel and the blue jack mackerel are fish with a pelagic-neritic distribution that can be found at the surface down to ~300 m deep and feed on small zooplankton and fish (Romero et al., 2021). The neon flying squid and the orange-back flying squid are mesopelagic squid species that inhabit waters down to 1500 m depth during the day but perform diel vertical migration for feeding at night in the epipelagic layers (0–200 m depth). The European squid is a benthopelagic species commonly found near the continental shelf and slope, occurring at depths ranging from a few meters to several hundred meters. These squids are fast-growing carnivorous predators with short lifespans. Their diet is opportunistic and includes a wide variety of prey, shifting from zooplanktonic crustaceans and micronektonic fish in the early stages to squids and fish (mainly myctophids) in the adult stages (Pierce et al., 1994; Ivanovic and Brunetti, 2004; Merten et al., 2017).

The present study provides novel data on MPs and plastic additives (PAEs) in squids from the NE Atlantic, which are under-represented species in the field of MPs research. The study aims to determine whether i) squid species could represent equal or better bioindicators of MPs contamination compared to small epipelagic fish in an open ocean environment, ii) MPs accumulate in the gastrointestinal tracts (stomach or intestine) of the studied fish species, iii) dietary preferences, feeding intensity, biological parameters (size, weight, liver weight, hepatosomatic index) and season significantly affect MPs ingestion in the studied fish species and iv) PAEs concentrations are correlated with the number of ingested MPs, and thus, validating the potentiality of PAEs as "plastic tracers" in pelagic food webs.

2. Material and methods

2.1. Sampling and sample preparation

Madeira Island is located in the NE Atlantic, at the edge of the subtropical Atlantic gyre. With a narrow continental shelf and deep submarine canyons, the island is characterised by a pelagic and oligotrophic environment (Canning-Clode et al., 2008; Narciso et al., 2019) and by a predominant north-easterly current that mediates the transportation and aggregation of plastic particles to the island (Cardoso and Caldeira, 2021). Madeira island is situated ~650 km from the West African Coast and ~850 km from the southern tip of Portugal, surrounded by oceanic waters, and thus, represents a privileged location for studying pelagic ecosystems from the open ocean with a low anthropic impact (relative to coastal waters and semi-enclosed basins).

Fish and squid specimens were bought from the main local market in Funchal, Madeira Island, from February 2019 to January 2020, wrapped in aluminum foil, and stored at −20 °C until further analysis. Both fish and squid specimens were caught using light attraction purse seine from local fishermen and transported to the market within 24 h (small-pelagic fishery, as described in Tejerina et al., 2019). Small pelagic fish are found all year round, while squids are

only caught between August and December, as it is reflected in the study's sampling period (Table 5.1). Although plastic material is used during fishery operations, and possible contamination might occur, such related items (such as paint chips from the boat or rope pieces from the fishing nets) were not detected in the gastrointestinal tracts of the organisms or, if encountered, were excluded from the analysis (as was the case of one paint chip found in one specimen). Furthermore, the short time frame between the catch, collection, and storage of the specimens should prevent significant absorption of plastic additives. However, some contamination deriving from the catching and handling of the organisms by the fishermen cannot be excluded. Hence, the most external layer of the dissected fish muscles and squid mantles was discarded, and only the internal parts were used for PAEs analysis.

Table 5.1 Sampling data, body size, MPs occurrence and mean number of MPs per individual (per each body compartment analysed), and total concentrations of phthalates per each species (analysed in the muscle and in the mantle for fish and squid, respectively). Body compartments analysed differ for fish and squid species (stomach and intestine in fish, stomach, gills and ink sac in squids).

Species	Sampling period (mm/yyyy)	n	Body size range (Mean) (cm)	MPs occurrence (%)				MPs / individual (Mean ± SD)**				∑ PAEs (ng/g) w.w. (Mean ± SD)
				Stomach	Intestine	Gills	Ink sac	Stomach	Intestine	Gills	Ink sac	
Scomber colias	02/2019 – 01/1020	37	14.3 – 31.6 (22.5)	85	61	-	-	5.18 ± 5.66	1.54 ± 2.01	-	-	19.43 ± 11.99
Trachurus picturatus	02/2019 – 01/2020	38	14.7 – 27 (18.7)	74	45	-	-	3.29 ± 3.49	1.00 ± 1.52	-	-	10.31 ± 5.94
Sthenoteuthis pteropus	08/2020	4	21.9 – 25.5 (23.9)*	75	-	75	25	8.75 ± 12.34	-	1.00 ± 0.82	0.25 ± 0.50	16.61 ± 12.2
Loligo vulgaris	09/2019 – 12/2019	8	12 – 34.2 (19.3)*	12.5	-	37.5	37.5	0.25 ± 0.71	-	0.50 ± 0.76	2.75 ± 4.80	20.69 ± 19.35
Ommastrephes caroli	08/2020	8	12.5 – 18.2 (14.5)*	12.5	-	62.5	12.5	0.13 ± 0.35	-	1.88 ± 2.36	0.13 ± 0.35	30.86 ± 8.95

* Mantle Length (ML) **calculated including all individuals (with and without MPs)

On the day of analysis, individuals were dissected after being defrosted. Total length (nearest 0.1 cm), total and gutted weight (fish weight minus viscera, ± 0.1 g), gastrointestinal tract (GIT) and liver weight (± 0.001 g) of each fish were recorded with a caliper and digital balance, respectively. Gastrosomatic (GSI) and hepatosomatic indexes (HSI) were calculated as follows:

$$GSI = \frac{\text{GIT weight(g)}}{\text{Total weight (g)}} \times 100 \quad (1)$$

$$HSI = \frac{\text{Liver weight(g)}}{\text{Total weight (g)}} \times 100 \quad (2)$$

The GSI measures the proportion of the gut mass to the total body mass and is an indicator of fish feeding activity (feeding intensity) (Mohammadizadeh et al., 2010; Renzi et al., 2019). The HSI is commonly used to indicate a fish's health and nutritional status, as the liver plays a key role in energy metabolism and nutrient storage (Chaves et al., 2017; Leão et al., 2021). An elevated HSI can indicate the presence of liver diseases or exposure to toxins, while a low HSI can indicate poor nutritional status or a reduction in energy reserves (Facey et al., 2005; Al-Ghais, 2013). Stomach and intestine contents were collected for MPs analysis. An aliquot (10–20 %) of the stomach contents (only for fish) was also analysed for taxonomic identification and quantification of prey. Zooplankton composition was determined by classification into the following 14 taxonomic groups: Copepoda, Decapoda (Malacostraca), Chaetognatha, Appendicularia, Cladocera, Amphipoda, Ostracoda, Annelida (Polychaeta), Siphonophora, Thaliacea (Salps), Mollusca (pteropods and other gastropods), Fish, Eggs, Platyhelminthes (flatworms and other worms). Only groups for which the total abundance proportion was >1 % were considered in the analysis.

Mantle length and total weight were recorded for squid specimens, and stomach, gills, and ink sacs were extracted for MPs analysis. The muscle of ten fishes of each species and the

mantle and tentacles of all squid specimens were removed and stored at −20 °C in aluminum foil for further analysis of PAEs.

2.2. Microplastic analysis and quality control/assurance

Tissues from the different body compartments (fish: stomach and intestine, squid: stomach, gills, ink sac) were digested with KOH 10 % at 40 °C for 24 h and then with H_2O_2 15 % at 40 °C for 24 h, following the recommendation of Frias et al. (2018), keeping low temperatures to avoid the risk of plastic polymers degradation (Alfonso et al., 2021). The digested contents were filtered through a 50 µm mesh and visually examined under a stereomicroscope (LEICA S9i) using an integrated camera (IC80 HD) to photograph and measure all the suspected plastic particles (Leica Software). The particles were classified, depending on texture and shape, into fragments, fibres, lines, paint sheets, and films and, depending on size, into five size classes: < 0.5, 0.5–1, 1–2.5, 2.5–5 mm. They were also classified based on the colours (black, white, transparent, blue, yellow, red, green, and other colours). Particles were classified as plastics when showing homogenous colour, thickness, texture, and absence of cellular structures (Hidalgo-Ruz et al., 2012). When in doubt, the hot needle test was used to observe the material's melting point (Lusher et al., 2017). However, no polymer analysis was available for this study, and the visual determination error, especially in small (< 500 µm) fibres, can reach 70 % (Lusher et al., 2017), giving some uncertainty whether they are synthetic or natural (e.g. cotton, linen, manila, kenaf, sisal rope, silk, wool, cellulose). Conversely, the use of an oxidising agent (H_2O_2) in the digestion process helps prevent false positives when identifying microplastics, as it digests or discolours organic materials such as cotton, linen, manila, kenaf, sisal rope, silk, wool, and cellulose (Avio et al., 2015; Hurley et al., 2018). Obtaining mostly dark fibres in the results (see results section), a consistent inclusion of non-treated natural fibres seems very unlikely.

Procedures to minimise contamination followed recommendations from Prata et al. (2021). All the lab ware and dissection tools used were made of non-plastic material and were always rinsed three times with MilliQ water before use. Samples were processed under a clean fume hood with the air pump off, a controlled and protected environment from the airborne deposit. Cotton lab coats and nitrile gloves were always used. All solutions used were previously filtered through a 20 μm stainless-steel mesh sieve. The processing time of the samples was kept to a minimum, samples were always covered while not processed or analysed, and a clean Petri dish was placed next to the work area any time the sample was open as airborne contamination control (both during sample processing and analysis). A mean number of 1.57 (± 0.84 SD) fibres in black, blue, and red colours, belonging to the three lower size classes, were found in the controls, and the fibres count in each sample was corrected accordingly, subtracting fibres with correspondent characteristics.

2.3. Phthalates analysis and quality control/assurance

Seven PAEs (dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-n-butyl phthalate (DBP), benzyl-butyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DNOP)) were investigated in this study (for chemical structures and properties, see Table S5.1 of Supplementary Data). High purity standards (> 98 %) of each PAE and two isotopically labeled PAEs (DEP-D_4 and DBP-D_4, used as internal standards) were acquired from Sigma-Aldrich (Madrid, Spain) and Dr. Ehrenstorfer (Augsburg, Germany).

Phthalates extraction and purification were performed according to the QuEChERS method described in Sambolino et al., 2022b. Briefly, 5 g of freeze-homogenised sample (wet weight) was added to a round-bottom glass tube with 5 mL of acetonitrile (ACN) and vortexed for 1 min. Then, 2.5 g of ammonium formate was added, the mixture was vortexed again for 1

min and then centrifuged for 5 min at 2500 rpm. One mL of supernatant was transferred to a 15 mL round-bottom glass tube containing 150 mg of $MgSO_4$, 50 mg of primary secondary amine (PSA), and 50 mg of octadecane (C18). The mixture was again vortexed for 1 min and centrifuged for 5 min at 2500 rpm. The resulting supernatant was transferred to another vial, and 2 µL was directly injected into the GC–MS system.

To minimise contamination, all the glassware was previously incinerated at 550 °C overnight, and any plastic material (screw caps and pipette tips) was cleaned three times with methanol in an ultrasonic bath for 15 min. Procedural blanks were analysed with each batch of samples, and blank values were subtracted from the final results. High-purity solvents and reagents were used. The ACN (LC-MS grade), ammonium formate (purity ≥97.0 %), and MgSO4 (purity ≥98.0 %) were from VWR International Eurolab (Barcelona, Spain). The PSA and C18 were from Agilent Technologies (Santa Clara, CA, USA).

The PAEs determination was performed on an Agilent 6890 GC, coupled with an Agilent 5973 Network MS (Agilent Technologies, USA) in the selected ion monitoring (SIM) mode. The detailed detection methods also follow those described in Sambolino et al., 2022b; retention times, ion qualifiers, and quantifiers of selected PAEs for this study are provided in Table S5.2 of Supplementary Data. Matrix-matched calibrations with the internal standard method were calculated for each matrix, obtaining linear regression with fitting $R2 > 0.99$ (Table S5.4 of Supplementary Data). The equations coefficient obtained were used to quantify the compounds in the analysed samples. RSD values accepted were below 20 %. The limits of quantifications (LOQ) of the method, considered as the lowest calibration level with $S/N > 10$, are described in Table S5.4 for all the different matrices analysed and ranged between 5 and 20 ng/g.

2.4. Statistical analysis

All statistical analyses were performed with R version 4.1.2 (R Core Team, 2021) with a significance level of 0.05. Response variables (MPs abundance and PAEs concentrations) were not normally distributed (Shapiro-Wilk normality test); thus, non-parametric tests (Mann-Whitney test, Kruskal-Wallis test followed by Dunn pairwise test, with p-values adjusted with the Bonferroni method) were performed to find significant differences between body compartments within the same species and among different species, using the R packages "stats" (R Core Team, 2021) and "FSA" (Ogle et al., 2022), and plotted using "ggplot2" (Wickham, 2016).

PCA (Principal Component Analysis) and PERMANOVA (Permutation test for adonis under reduced model) were performed on non-transformed data of prey composition, MPs characteristics, and PAEs concentrations using the "vegan" R package (Oksanen et al., 2013). MPs abundance was transformed into a categorical factor "MPs contamination level" with two levels: high (> 2 MPs per individual) and low (≤ 2 MPs per individual), being 2 the median value of MPs per individual. Then, PCA and PERMANOVA were performed on PAEs concentration profiles to explore correlations among PAEs and separation among samples from these two groups. When detected, outliers were excluded from the PCA analysis. PCA is an exploratory analysis that extracts Principal Components (PCs) from the combination of different inter-correlated variables, which can summarise and better explain the variation in the dataset. The loadings of different variables in each PC also indicate the most correlated variables. The first two PCs in terms of the amount of variation explained (%), were used for the visualisation of the multidimensional data.

Generalised Linear Mixed-Effects Models fit by maximum likelihood - Laplace Approximation (GLMMs) were fitted on fish data, using the "lme4" R package (Bates et al., 2015) to assess the effects of season, species, and biological parameters such as body size, total weight, gastrointestinal tract (GIT) weight, liver weight, gastrosomatic index (GSI) and

hepatosomatic index (HSI) on the abundance of ingested MPs (MPs per individual, from stomach only). Only two main seasons were considered, as suggested by Sambolino et al. (2022a): one warm season from May to October and one cold season from November to April. Squid data were excluded from this analysis due to the scarce sample size and the limiting seasonal sampling. The model for negative binomial distribution was applied since data showed overdispersion. All models included month as a random effect and were validated with residual analysis ("DHARMa" package; Hartig, 2022). Correlation matrices and Variance Inflation Factor (VIF) were calculated to study collinearity between predictor variables and exclude from the same model highly correlated ones (VIF > 5). Model selections were based on the information-theoretic approach (Lukacs et al., 2007)) by comparing models AICs (Akaike's Information Criterion; Akaike, 1974). The correlation between ingested MPs abundance and PAEs concentrations was tested with the Spearman correlation test and plotted in a correlation matrix.

3. Results and discussion

3.1. MPs abundance and characteristics in fish and squid species

A summary of the sampling data, fish and squid characteristics, and the MPs abundance is presented in Table 5.1. The sampled fish species exhibited similar body size ranges. However, the squid species displayed greater variations, with one species (*S. pteropus*) considerably larger than the others and one (*L. vulgaris*) showing a rapid increase in body size during the reproductive season.

In both fish species, MPs were found in higher occurrence and number in the stomach than in the intestine (Mann-Whitney test p-values <0.001, Figure 5.1A, B), suggesting that MPs do not accumulate in the latter. Indeed, previous studies have also suggested that MPs have a relatively short residence time in fish GIT, with excretion rates equal to or greater

than ingestion rates, thereby preventing bioaccumulation in this compartment (Jovanović, 2017; Jovanović et al., 2018). The higher numbers of MPs in the stomach may be attributed to its bigger size and a potentially longer retention time of the ingested items than in the intestine (Figure S5.1). In squid species, only *O. caroli* presented a significantly higher number of MPs in gills compared to the other compartments (Figure 5.1C, D, E). Gills are one of the largest organs in squid species (Figure S5.2), are in constant contact with the surrounding environment, and are directly involved in water filtration, thus, it is expected they intercept a substantial amount of particles, including microplastics. Gong et al. (2021) also reported high amounts of MPs in the gills of jumbo squid (*Dosidicus gigas*), comparable with those found in the stomach. Other studies analysing both gastrointestinal tracts and gills of marine species also found that the adherence of MPs to gills represents a primary pathway of MPs uptake, in addition to ingestion (Kolandhasamy et al., 2018; Zhang et al., 2021). Notably, relatively high levels of MPs were detected in the ink sac of two individuals of L. *vulgaris* (7 and 13 particles, respectively), all of which were blue and black fibres. To the best of our knowledge, this is the first study to analyse the presence of MPs in the ink sac of a cephalopod species. The origin of these fibres remains unclear; however, their relatively long size (0.5–2.5 mm) and the connection of this organ to the external environment through the anus, suggest that they might have directly entered from the surrounding environment, rather than migrated through the vascular system.

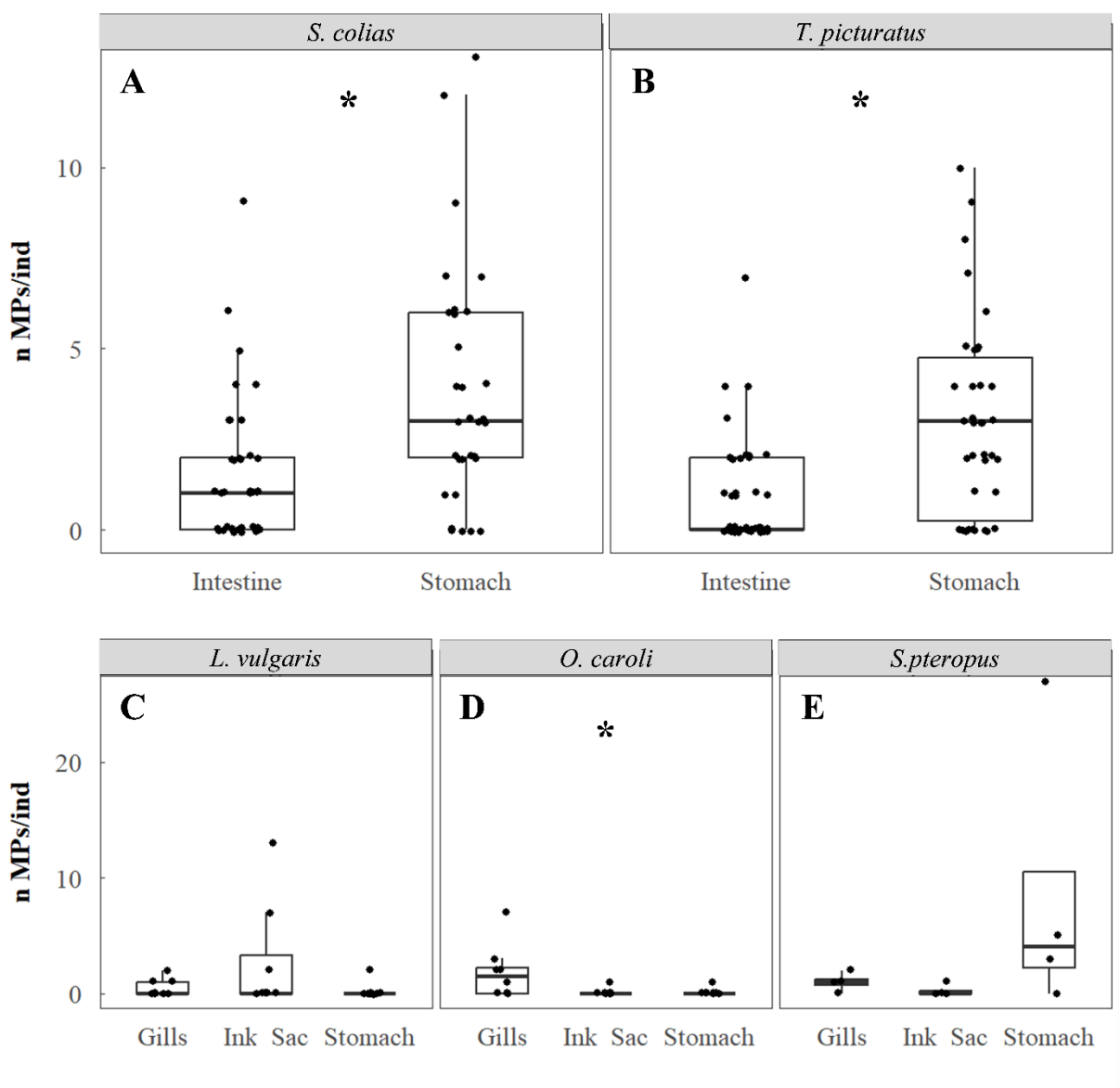

Figure 5.1 Boxplots of the number of microplastics found in different body compartments per each individual of *S. colias* (A), *T. picturatus* (B), *L. vulgaris* (C), *O. caroli* (D), *S. pteropus* (E). Significant differences (*p*-value <0.05) tested with Mann-Whytney (A and B) and Kruskal-Wallis test (C, D, and E) are indicated with an asterisk.

The occurrence and average number of MPs per individual were higher in the fish species (*S. colias* and *T. picturatus*) and *S. pteropus* compared to the two smaller squid species (*O. caroli* and L. *vulgaris*) (Table 5.1). Overall, *S. pteropus* exhibited the highest number of ingested MPs per individual, primarily due to one individual found with 27 MPs in its stomach. *S. pteropus* is a mesopelagic carnivore squid with an opportunistic and highly variable diet, particularly in the adult stage, feeding on several species of nektonic fish and squids and increasing its trophic level alongside size (Merten et al., 2017). The trophic transfer

of MPs and the relation between MPs ingestion and trophic level are still under discussion (Miller et al., 2020). However, the larger size and higher trophic level of *S. pteropus* may explain the high number of ingested MPs. Nevertheless, low sample size ($n = 4$) and high variation among samples (RSD = 141 %) prevented the identification of a significant difference in the abundance of ingested MPs compared to other squid species. Significant differences in ingested MPs were observed between the two fish species and the two smaller squid species (Dunn test post-hoc comparison) (Figure 5.2). *S. colias* exhibited the highest values and most significant difference (p-values <0.01). In contrast, *O. caroli* and *L. vulgaris* presented a low occurrence of MPs (12.5 % in both) and low numbers of ingested MPs per individual (0.13 and 0.25, respectively). The difference in MPs ingestion could be attributed to different feeding behaviours, dietary habits, or the water depth at which they feed. Mesopelagic squid usually feed in surface waters at night. However, depending on the abundance of prey items, life stage, and specific dietary habits, they may also feed in deeper waters (Pierce et al., 1994; Ivanovic and Brunetti, 2004). The vertical distribution of MPs in the water column highly varies along the depth profile, and organisms feeding at different depths are exposed differently to MPs (Choy et al., 2019).

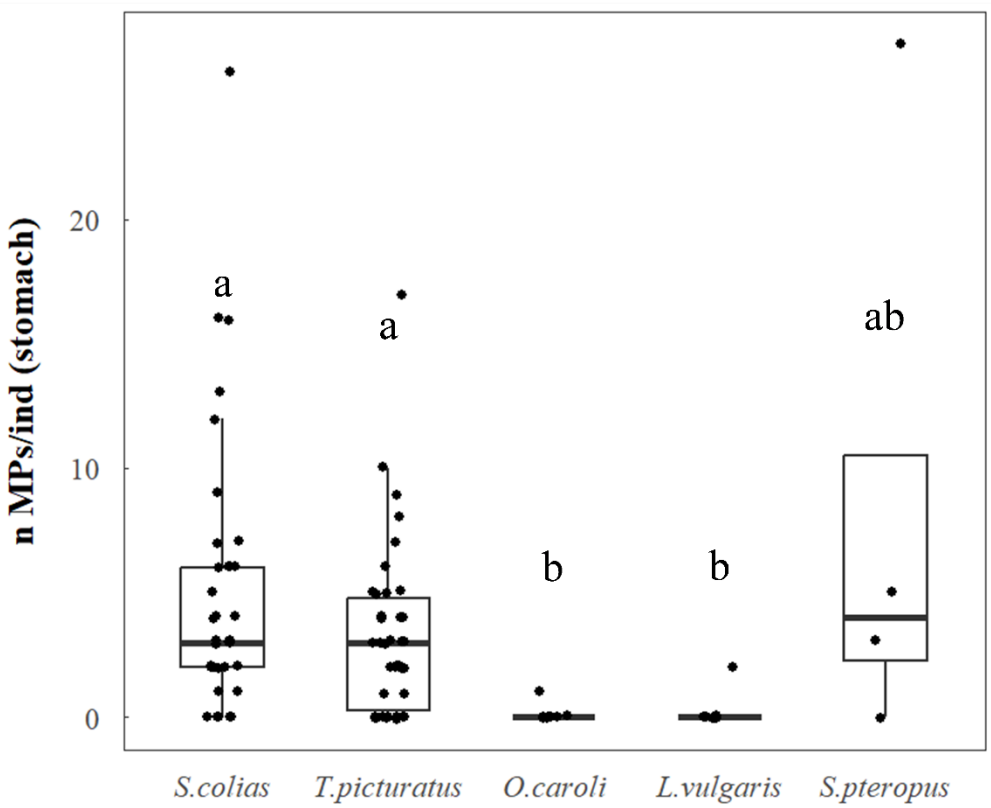

Figure 5.2 Boxplot of the number of microplastics per individual found in the stomach of the five studied species. Significant differences (p-value <0.05) in the number of microplastics per individual in different species are represented with different letters (Dunn - Kruskal-Wallis multiple comparisons with Bonferroni correction).

Previous studies comparing MPs ingestion in *S. colias* with other species also found this species to have a higher occurrence (%) and typically higher numbers of ingested MPs (Herrera et al., 2019; Barboza et al., 2020; Lopes et al., 2020; Pereira et al., 2020). Accordingly, *S. colias* has been suggested as an indicator species for assessing environmental status concerning MPs contamination (Lopes et al., 2020). The occurrence of MPs found in *S. colias* in the present study (85 %) is similar to that found by Herrera et al. (2019) (78.3 %), Lopes et al. (2020) (64 %) and Barboza et al. (2020) (62 %). In contrast, Pereira et al. (2020) found a considerably lower proportion of MPs in pelagic fish species in the Azores (about 16 % for *S. colias*) because they excluded all the suspected cellulosic fibres from their counting.

Fibres were the dominant type of MPs found in all species and body compartments, accounting for over 80 % of the total (Figure 5.3). Blue and black fibres were the most common

particles overall, comprising over 80 % of the total number of fibres. However, exceptions were observed in the stomachs of L. *vulgaris* and *O. caroli*, where only three fibres of green, transparent, and yellow colours were found. Fragments were the second most common item, exclusively found in the stomachs of *S. colias*. Films were found in only one specimen of *S. pteropus,* and one line was found in the stomach of *S. colias*. Paint chips were excluded from the analysis as they were found only in one individual and might be due to contamination during fishing operations. These results align with what was found in most studies, with black and blue fibres representing the most considerable proportion of particles detected in pelagic organisms (*e.g.* Herrera et al., 2019; Koongolla et al., 2020; Lopes et al., 2020; Gong et al., 2021; Valente et al., 2022; Trani et al., 2023) and sea surface waters (Suaria et al., 2020; Silvestrova and Stepanova, 2021; Sambolino et al., 2022a). Marked inter-specific differences in MPs found in squid and fish might be associated with different dietary habits and inhabited depth. However, the fibres found in *S. pteropus* were more similar to those found in the fish species, suggesting that they might primarily result from trophic transfer through the ingestion of contaminated fish, which is consistent with the bigger size and thus higher trophic level of *S. pteropus*.

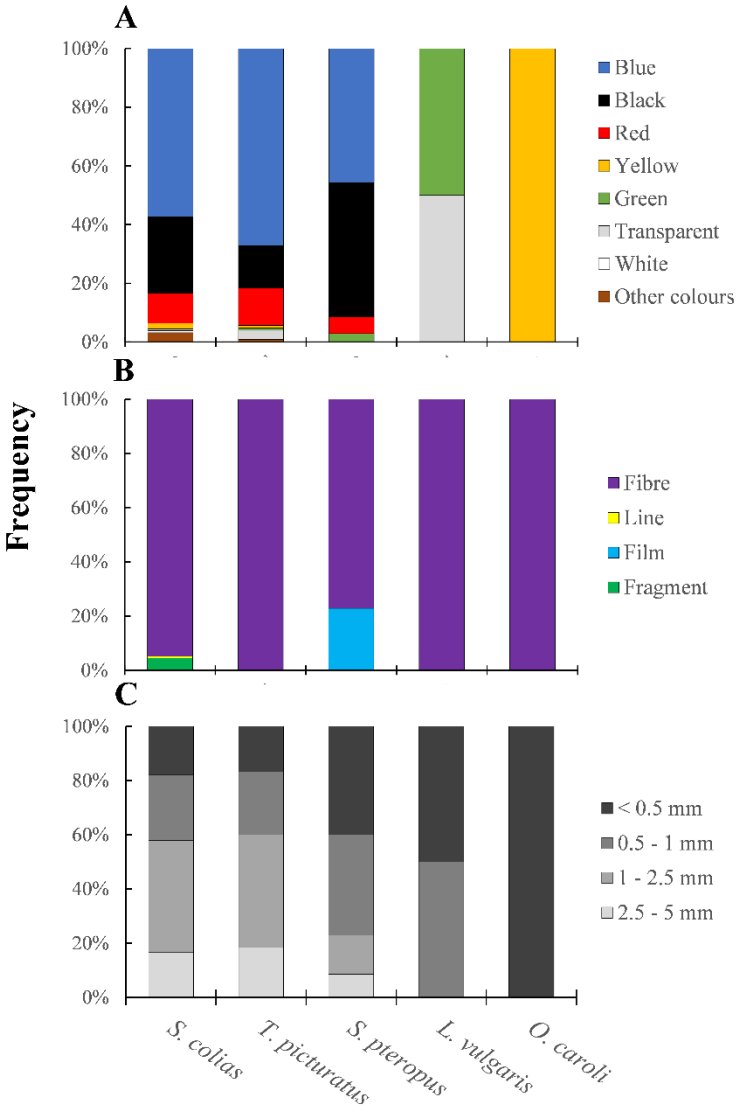

Figure 5.3 Proportion of microplastics colour (A), shape (B) and size class (C) found in the stomachs of the five studied species.

3.2. Relations between ingested MPs and ecological parameter in fish

Copepods dominated prey composition in the two fish species, followed by salps and eggs in *S. colias* and Decapoda (Crustacea: Malacostraca) and Mollusca (Gastropoda mainly pteropods) in *T. picturatus* (Figure S5.3 in Supplementary Data). A higher percentage of *T. picturatus* stomachs (55 %) were found empty, compared to *S. colias* (0 %). To investigate possible differences in feeding strategy and MPs selectivity, a principal component analysis (PCA) was performed on MPs characteristics (colour, shape, and size) and prey composition

obtained from the stomach analysis of the fish species to find qualitative differences among the samples, based on species and season (Figure 5.4). The results showed that *S. colias* and *T. picturatus* did not ingest different microplastics (PERMANOVA, $p = 0.239$, Figure 5.4A) even though their prey composition was significantly different (PERMANOVA, $p = 0.001$, Figure 5.4B). Fish species with MPs with more diverse characteristics were found in the warm season (in terms of colours and shapes), while in the cold season, dark fibres were more represented, even though the difference was not significant (PERMANOVA, $p = 0.116$, Figure 5.4C). Season did not significantly influence prey composition (PERMANOVA, $p = 0.641$, Figure 5.4D).

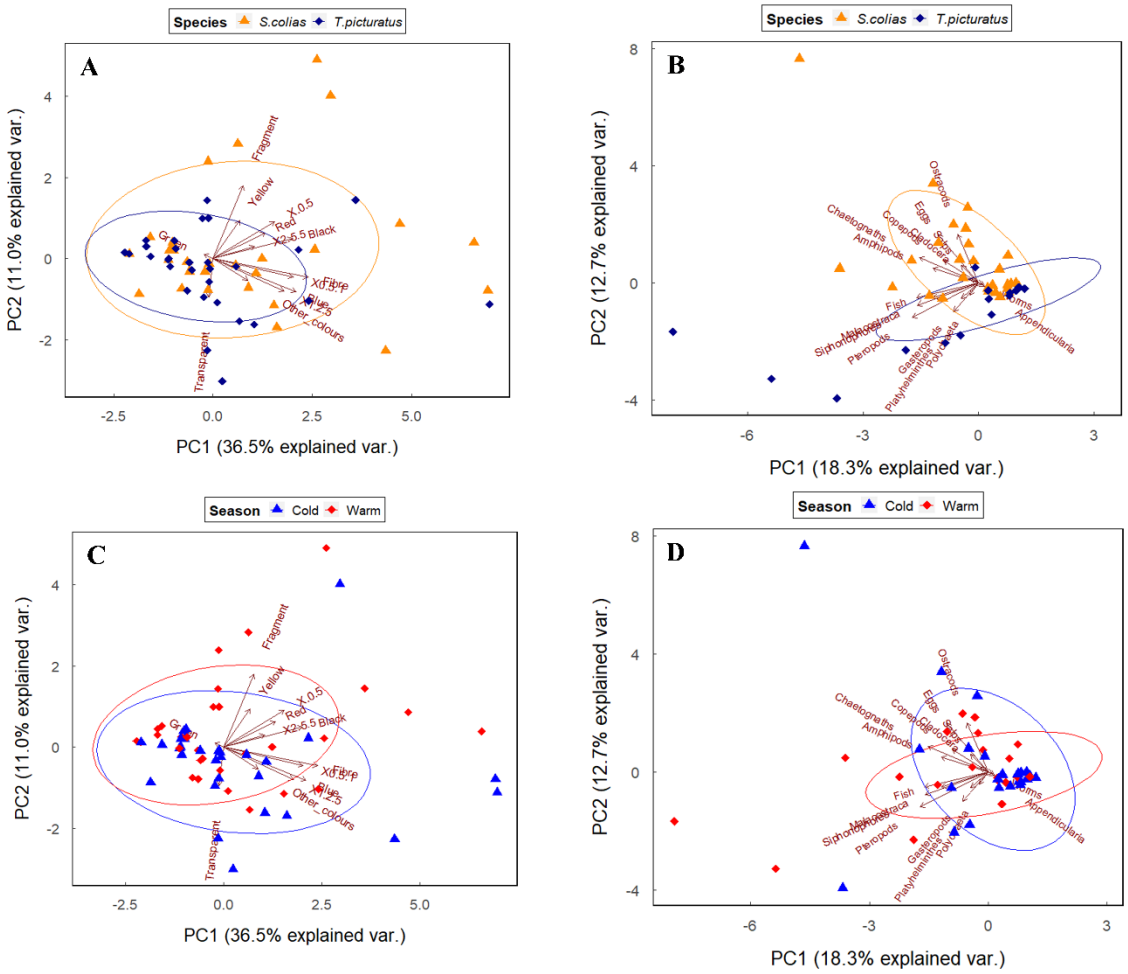

Figure 5.4 Biplots of Principal Component Analysis (PCA) of MPs characteristics by species (A) and by season (C), and prey composition by species (B) and by season (D), performed on data from fish species (*S. colias* and *T. picturatus*). Each point represents an individual sample; different colours and ellipses represent different factors groupings. Significance difference between groups (PERMANOVA, *p*-value <0.05) was only detected for prey composition by species (B).

Romero et al. (2021) also recorded different prey compositions for *S. colias* and *T. picturatus* from Madeira Archipelago. Here, seasonal variation was observed in the diets of the two species, mainly due to fluctuations in the presence of fish as prey, which were not detected in the samples from the current study. A previous study on MPs characterisation in sub-surface seawater samples from Madeira Island revealed a greater diversification in the types of MPs during the warm season (Sambolino et al., 2022a). This could be explained by the strong and constant north-easterly winds that occur in the summer months, facilitating the transport of MPs and creating convergence areas (eddies) in the south of the island, where plastic particles mix (Cardoso and Caldeira, 2021). However, this phenomenon occurs occasionally and may not significantly impact the overall composition of MPs. Nevertheless, it emphasizes the importance of seasonal or monthly sampling to get an accurate perspective on the composition and primary sources of MPs in a location, as sporadic sampling can yield misleading results. The absence of difference in the characteristics of ingested MPs between the two fish species, despite differences in prey composition, suggests that they may employ -with different feeding strategies, but do not selectively ingest different types of MPs. Instead, they may unintentionally ingest whatever is available in the surrounding environment during the feeding events.

To understand the relationship between MPs ingestion, biological parameters and environmental variables (season), generalised linear mixed models (GLMM) were fitted to the data (Table S5.5). The best-fitting model based on the lowest AIC value, included the predictor variables of season, hepatosomatic index (HSI), and gastrosomatic index (GSI). Body size, body weight, and species did not show any effect on MPs ingestion. Moreover, body weight and body size did not significantly differ by season (Mann-Whitney test, p-value >0.05). The model indicated that season and GSI were significant predictors of the abundance of ingested MPs (Table 5.2), with a higher GSI and cold season predicting a higher number of ingested

MPs per individual (Figure 5.5 – GSI and season). Although the inclusion of HSI improved the model's fitting, it was not found to be a significant factor.

Table 5.2 Summary of the best-fitting model (GLMM) results with the predictor variables Season, GSI and HSI on the abundance of ingested microplastics (MPs per individual stomach), including "month" as random factor, for the two fish species *S. colias* and *T. picturatus*. Bold font indicates significant factors (*p*-value < 0.05).

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.60385	0.36443	1.657	0.0975
SeasonWarm	**-0.58573**	**0.25948**	**-2.257**	**0.024**
HSI	0.29511	0.44065	0.67	0.503
GSI	**0.14082**	**0.06278**	**2.243**	**0.0249**

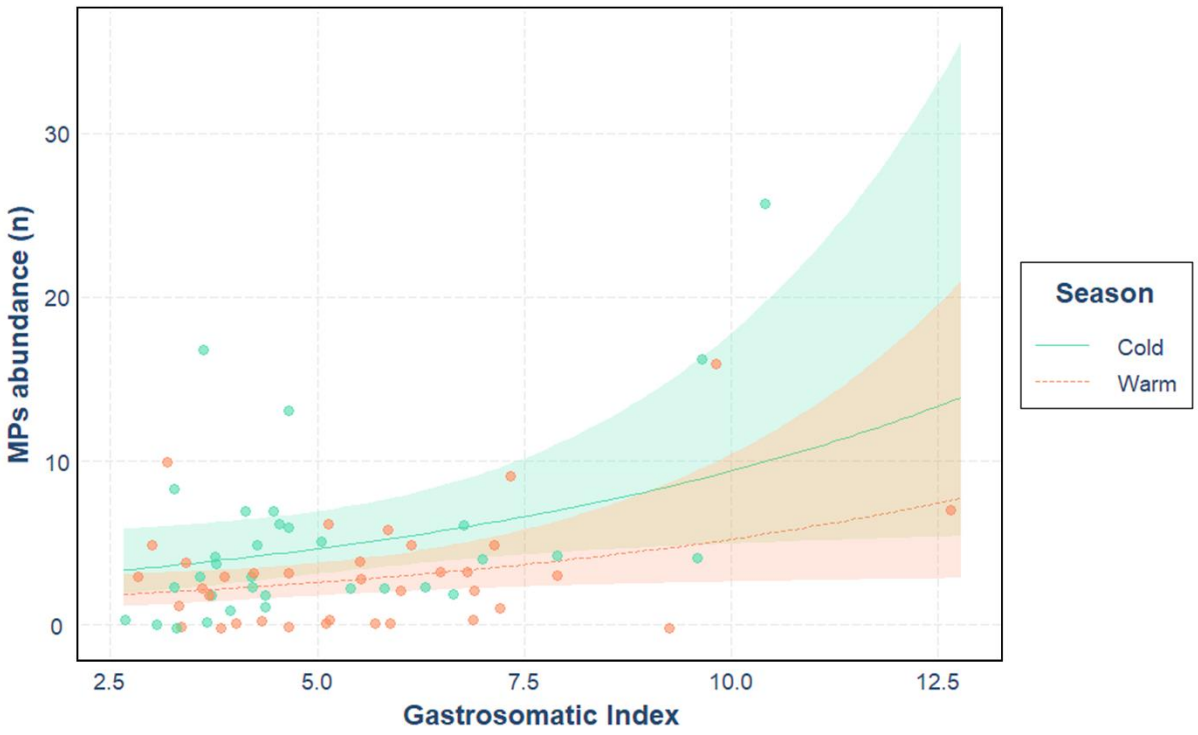

Figure 5.5 Plot of predicting model (GLMM) with fitting lines and 95 % Confidence Intervals (CI) of the abundance of ingested MPs (n per individual stomach) based on gastrosomatic index (GSI) values and season (Cold – Warm) for the two fish species (*S. colias* and *T. picturatus*).

The higher probability of fish ingesting microplastic as the GSI increases indicates a direct relationship between plastic ingestion and feeding intensity. This finding supports previous hypotheses formulated in MPs research: *i)* MPs are not retained in the stomach nor the intestine for a longer time than food items and do not accumulate; *ii)* MPs might be in aggregation or higher quantities in "feeding areas", adhering to or inside zooplankton, *iii)* MPs ingestion occurs mainly during feeding events, rather than outside (as could be the case of passive ingestion during water drinking). Previous studies have found a significant correlation between GIT fullness and the number of ingested MPs (Alomar and Deudero, 2017) or between GIT weight and the frequency of MPs occurrence (Valente et al., 2019; Valente et al., 2022). These findings also suggest that the retention time of MPs and food items may be similar, and the authors hypothesised that MPs ingestion prevalently occurs through ingestion of contaminated prey. The present study's results cannot confirm whether the MPs ingestion happens directly or indirectly (through contaminated prey). Despite feeding on different prey, *S. colias* and *T. picturatus* ingested similar types of MPs suggesting that MPs ingestion is likely accidental and direct, dependent on their bioavailability in the environment; however, it is also possible that different prey contained the same type of MPs, which were then transferred to the pelagic fish.

Trani et al. (2023) also found that feeding intensity (inferred by a body condition factor K) predicted a higher abundance of ingested MPs in three fish species, while body size and weight were not significant factors. Sbrana et al. (2020) investigated the relationship between MPs ingestion and biological parameters in a small pelagic fish (*Boops boops*) and found no significant relationship between stomach fullness and the abundance of ingested MPs. Nevertheless, they found a relationship with a body index (Kn), suggesting that fish with lower body conditions are more prone to ingesting MPs. In the present study, HSI was not a significant predictor, but its inclusion in the model improved the model's ability to explain the

response variable, as indicated by a lower AIC value. It is worth mentioning that a slightly increased HSI, which may indicate liver diseases or exposure to toxins, was associated (though not significantly) with increased MPs ingestion (Figure S5.4 in Supplementary Data). The season was also a significant predictor variable, which can be related to variations in the abundance and distribution of MPs in the water column or changes in fish feeding behaviour in response to seasonal fluctuations in prey availability. However, no significant difference was found when comparing GSI values between seasons (Mann-Whitney, p-value >0.05). Therefore, the higher abundance of ingested MPs in the cold season is likely due to the higher availability of MPs. This could be explained by a larger amount of fibres entering from the island's wastewater treatments during rainy months, as suggested by Sambolino et al. (2022a). Indeed, laundry discharge and river effluents are a leading source of microfibres pollution in the marine environment (Vassilenko et al., 2021). The impact of effluents on the input of sediment and related debris into the ocean was found to be especially relevant in Madeira Island (Rosa et al., 2022).

3.3. PAEs concentration and correlation with ingested MPs

All seven PAEs studied were detected in the samples (Table S5.6 in Supplementary Data). Among them, DMP and DNOP were detected in only one specimen, while DIBP and DEHP were the most frequent and abundant. DBP was detected in all three species where it was analysed, however, its results had to be discarded in *T. picturatus* and *O. caroli* due to high blank contamination values in the respective batches. Therefore, only DEP, DIBP, BBP and DEHP were considered for statistical analysis and are visually displayed (Figure 5.6, Figure 5.7). The concentrations of these four PAEs differed significantly among species, with DIBP found in higher abundance in *S. colias,* and *S. pteropus* and BBP and DEHP found in higher abundance in small squid species *O. caroli* and L. *vulgaris*. DEP was only found in *S. colias* and *T. picturatus*. The total concentration of PAEs (∑ PAEs - calculated including these

4 PAEs only) showed no significant difference due to high variance (*p*-value = 0.08736, data reported in Table 5.1). Different concentrations of PAEs in different species might be related to their varied feeding habits, differences in PAEs absorption and metabolism, and/or different concentrations of PAEs in their respective environments. In fact, Paluselli et al. (2018b) found variations in PAEs concentration profiles at distinct depths in the Mediterranean Sea. The concentrations of PAEs detected in this study were relatively low compared to those observed in marine organisms from the Mediterranean Sea or China, where the concentrations were one or two orders of magnitude higher (Hidalgo-Serrano et al., 2022). For instance, a crab species from Hangzhou Bay in China reported a concentration of DIBP of 5313 ng/g (Hu et al., 2020), while shrimp samples bought in Spanish markets were found with 3393 ng/g of DEP (Hidalgo-Serrano et al., 2020). However, the concentrations of PAEs found in fish and squid species from the Canary Islands were like those found in the present study (Sambolino et al., 2022b).

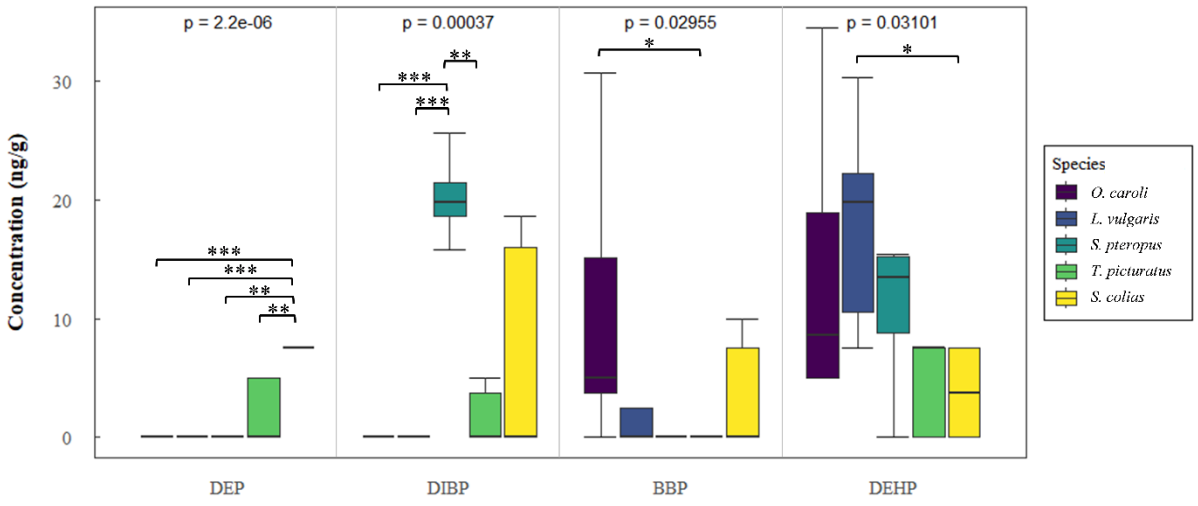

Figure 5.6 Concentrations (ng/g, wet weight) of the four main PAEs detected in the five studied species (*O. caroli* n = 8, *L. vulgaris* n = 8, *S. pteropus* n = 4, *T. picturatus* n = 10, *S. colias* n = 10). P-values reported on the top of each boxplot were calculated with Kruskal-Wallis test, and bars with asterisks represent significant differences between species from pairwise comparison (Dunn's test with Bonferroni correction). Significance codes: '***' *p*-value <0.001 '**' $p < 0.01$ '*' $p < 0.05$.

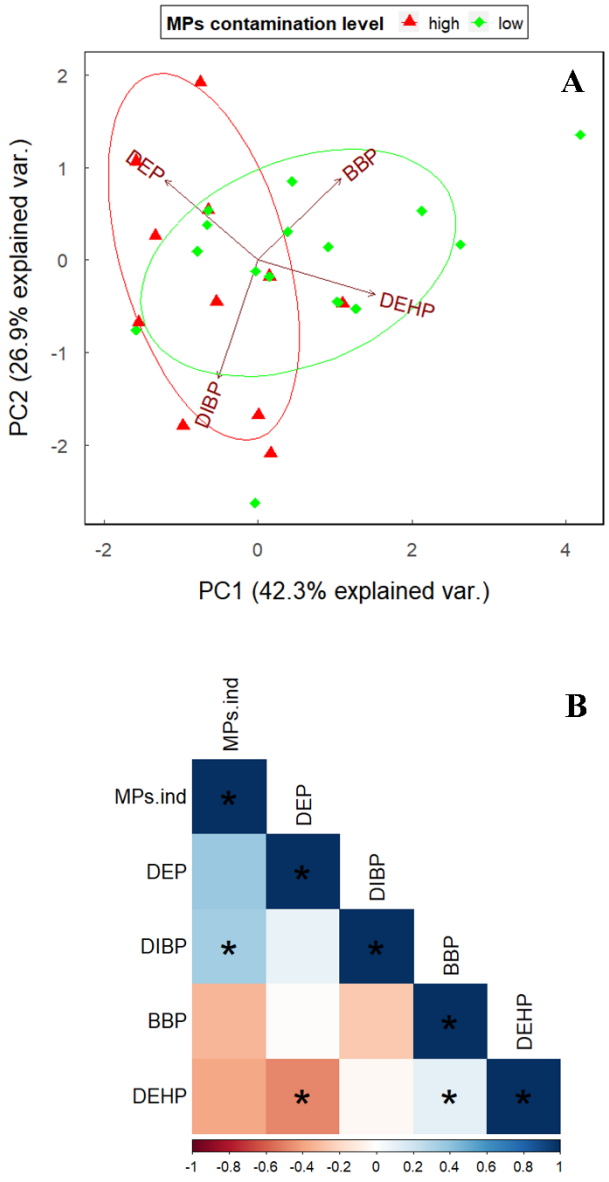

Figure 5.7 Biplot of Principal Component Analysis (PCA) of four PAEs concentrations (ng/g, wet weight) detected in the five studied species (*O. caroli* n = 8, *L. vulgaris* n = 8, *S. pteropus* n = 4, *T. picturatus* n = 10, *S. colias* n = 10), grouped by different MPs contamination level (low ≤ median value of MPs/individual stomach < high) (A) and correlation matrix of the four PAEs concentrations with the abundance of ingested MPs (MPs.ind = MPs/individual stomach) (B). Asterisks indicate significant correlation (Spearman's correlation test, *p*-value <0.05).

As one source of PAEs contamination in the environment is attributed to plastic pollution (Cao et al., 2022), the relationship between PAEs concentrations and the abundance of ingested MPs was explored. The first two Principal Components of the PCA explained 42.3 and 26.9 % of the variance, respectively (both returning Eigenvalues >1) (Figure 5.7A). PC1 had negative loadings for DEP and DIBP and positive loadings for BBP and DEHP, indicating

inter-correlation between these compound pairs; however, none of the loading values was above 0.7. Grouping samples by MPs contamination level showed that high MPs contamination is related to higher DEP and DIBP concentrations (Figure 5.7A). PERMANOVA confirmed significant differences in PAEs distribution depending on MPs contamination level (*p*-value = 0.011). Spearman's correlation tests revealed that, among all PAEs, DIBP is the only one correlated with the abundance of ingested MPs (MPs per individual stomach). Additionally, DEHP showed a significant correlation with BBP and DEP. These results should be interpreted with caution given the relatively low sample size (total $n = 40$) and the different metabolic responses of different species to PAEs contamination, which are not considered here. However, previous evidence suggested that MPs could be a source of PAE contamination in marine biota. For instance, Saliu et al. (2019) found that corals living in areas most affected by MPs contamination had higher PAEs concentrations. Baini et al. (2017) found a positive correlation between MPs and PAEs concentrations in planktonic samples. However, Schmidt et al. (2021) did not find any correlation and suggested that in coastal systems, MPs abundances cannot be taken as a proxy of contamination by organic plastic additives and *vice versa*, given the larger contribution deriving from other sources, such as household greywater or industrial inputs.

Many PAEs have been detected in microplastics, including in fibres. MP particles identified as PE, PS, polypropylene, polyamide, chlorinated PE, and chlorosulfonated PE were associated with DEHP, DBP, DIBP, DEP, DMP, and 2,4-di-tert-butylphenol (2,4-DTBP), which are used as antioxidant additives (Fries et al., 2013). DIBP has been identified as one of the primary PAEs released from PE bags (Paluselli et al., 2018b). However, the microplastics found in this study, which correlated with DIBP concentrations, were primarily fibres. In a separate study, Sørensen et al. (2021) detected DEHP, DEP, and BBP leaching into seawater from PET,

PA, and wool fibres. Additionally, other researchers have reported significant concentrations of phthalates in synthetic clothing (Tang et al., 2020; Chen et al., 2022).

A complex paradox in MPs research is the wide presence of cellulosic fibres, which can constitute up to 80 % of the sample, in the marine environment (Suaria et al., 2020). The high occurrence of these fibres suggested that they might not biodegrade in the environment (Suaria et al., 2020). One possible reason is the common use of plasticisers during the manufacturing of natural fibres, as is the case of cellulosic fibres (Lo Nostro et al., 2002; Phuong and Lazzeri, 2012). Wool was found to have higher levels of bisphenols and benzophenones than synthetic fibres (Sait et al., 2021). Rayon and cotton are frequently processed, finished, dyed, and coated with additives such as resins, softeners, and flame retardants, which may drastically slow their degradation (Li et al., 2010). As such, the hazard posed by treated natural fibres, which are widely available and persistent in the marine environment, should not be underestimated in future studies, and immediate action is crucial to address this issue.

3.4. Limitations and future perspectives

The present investigation has shed light upon the intricate interaction between MPs and marine biota. Nevertheless, it opens multiple avenues for future exploration and refinement. Although restrained by a modest sample size, it has succeeded in elucidating noteworthy insights on MPs ingestion in *S. colias* and *T. picturatus*. Serving as a springboard, these initial results provide a foundation for broader investigations incorporating larger sample sizes and a wider array of species.

This investigation has also unveiled a correlation between concentrations of PAEs and the abundance of ingested MPs, despite not considering the differential metabolic responses to PAEs contamination among species. This information opens the path for future investigations to delve deeper into the species-specific metabolic responses to PAEs contamination, which

would contribute to a more nuanced understanding of the interaction of PAEs with different marine organisms.

Within the MPs spectrum, this investigation predominantly identified fibres, contributing with valuable insights into the nature of MPs ingested by marine biota. However, the polymers could not be identified. To construct a more comprehensive picture of MPs in marine environments, future investigations could extend their focus to the polymer composition of MPs and the associated plastic additives.

Thus, this investigation sets the stage for a more comprehensive exploration of the interactions between MPs, PAEs, and marine biota. The constraints of the current study should be viewed not as deficiencies, but as opportunities for future refinement. It is anticipated that ensuing research will build upon this foundation, further enhancing our understanding of the extent and implications of plastic pollution in marine environments.

4. Conclusions

In this study, microplastic (MPs) ingestion was scrutinized in NE Atlantic squid and small epipelagic fish, highlighting their potential as bioindicators in pelagic food webs. Investigation into the mechanisms of MPs ingestion indicated non-selective consumption through active feeding, without evidence of accumulation in the gastrointestinal tracts or direct size-dependent contamination. The influence of seasonal variations on MPs bioavailability and ingestion rates was confirmed. Furthermore, a thorough examination was conducted on the relationship between MPs ingestion and the presence of plastic additives (PAEs) in these species. A correlation was identified between the abundance of ingested MPs and the levels of DIBP, validating the supposition of PAEs, particularly DIBP, as potential ‘plastic tracers’ in pelagic food webs. This correlation appears to hold true in low anthropized areas, such as the present study area, where other pathways of PAEs contamination are limited. Therefore, the

present study emphasizes the presence and potential risks of MPs and PAEs in the open oceanic food webs and provides compelling evidence for the role of PAEs as 'plastic tracers', pointing to the importance of further research on the sources of contamination, the impacts of these contaminants on marine organisms, and the potential risks to human health through seafood consumption.

Supplementary data

Supplementary data to this chapter can be found online at https://doi.org/10.1016/j.scitotenv.2023.164952 and in Appendix C of this document.

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CHAPTER VI

PHTHALATES AND FATTY ACID MARKERS IN FREE-RANGING CETACEANS FROM AN INSULAR OCEANIC REGION: ECOLOGICAL NICHES AS DRIVERS OF CONTAMINATION

This chapter is submitted as:

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Chapter 6 - Phthalates and Fatty Acid Markers in Free-ranging Cetaceans from an Insular Oceanic Region: Ecological Niches as Drivers of Contamination

Abstract

Plastic additives such as phthalates are ubiquitous contaminants in the marine environment, with concerning impacts on marine organisms and overall ecosystems' health. Valuable information about the status and resilience of marine ecosystems can be obtained through the monitoring of key indicator species, such as cetaceans. In this study, fatty acid profiles and phthalates were examined in blubber biopsies of free-ranging individuals from two delphinid species (short-finned pilot whale – *Globicephala macrorhynchus*; common bottlenose dolphin – *Tursiops truncatus*) in a remote island system. This investigation aimed to explore the relations between trophic niches (epipelagic vs. mesopelagic), contamination levels, and the health status of individuals within different ecological and biological groups (defined by species, residency patterns and sex). Multivariate analysis of selected dietary fatty acids revealed a clear niche segregation between the two species. Di-n-butylphthalate (DBP), diethyl phthalate (DEP), and bis(2-ethylhexyl) phthalate (DEHP) were the most prevalent among the seven studied phthalates, with the highest concentration reached by DEHP in a bottlenose dolphin (4697.34 ± 113.45 ng/g). Phthalates concentrations were different between the two species (Mean ∑ PAEs: 947.56 ± 1558.34 in bottlenose dolphin, 229.98 ± 158.86 ng/g in pilot whale), with bottlenose dolphins mainly affected by higher concentrations of DEHP and pilot whales by DEP and DBP. Health markers suggested pilot whales might suffer poorer physiological conditions, although fatty acid profiles also showed high metabolic differences between the two species. Phthalate levels showed no differences by ecological or biological groups, seasons, or years. This study is the first to assess the extent of plastic additive

contamination in free-ranging cetaceans off Madeira Island, an isolated region within the Atlantic Ocean, underscoring the intricate relationship between ecological niches and contaminant exposure. Monitoring these chemicals and their potential impacts is vital to assess wild population health, inform conservation strategies, and protect critical species and habitats.

Keywords

Atlantic Ocean; Biopsy blubber; Free-ranging cetaceans; Madeira Island; Odontoceti; Phthalic acid esters; Plastic additives.

1. Introduction

Plastic debris, microplastics, and their associated chemical additives are emerging contaminants of great concern due to their extensive and escalating presence in the ocean (Chiba et al., 2018; Cozar et al., 2014; Lusher et al., 2015b; Xie et al., 2007) and their long-term impacts on marine ecosystems, especially once they enter the food web (Guzzetti et al., 2018; Hermabessiere et al., 2017; Koelmans et al., 2022). Phthalates (Phthalate Esters or Phthalic Acid Esters – PAEs), a common class of plastic additives, are pervasive environmental contaminants known to have detrimental health effects as endocrine disruptors (Hidalgo-Serrano et al., 2022; Katsikantami et al., 2016; Net et al., 2015; Zhang et al., 2021). Initially introduced in the 1920s as plasticizers to enhance flexibility and softness in polyvinyl chloride (PVC) production, PAEs are now widely used in most plastic materials, comprising up to 70% of the final product (Hahladakis et al., 2018). Short-chained phthalates, which are more water-soluble, also find applications in personal care products, adhesives, varnishes, paints, cosmetics, and pharmaceuticals (Net et al., 2015). Given their non-chemically bonded nature, PAEs readily leach into the environment and enter the ocean through various pathways, including wastewater, atmospheric transport, river runoff, and plastic waste (Cao et al., 2022; Katsikantami et al., 2016; Xie et al., 2007). Consequently, PAEs are commonly detected in

marine environments, encompassing seawater, sediment, and biota (Hidalgo-Serrano et al., 2022; Net et al., 2015).

Phthalates with a high octanol-water partition coefficient (K_{ow}) are highly lipophilic and can accumulate in sediments and organisms' tissues; however, they are quickly metabolized and excreted in mammals (Hart et al., 2020; Wittassek and Angerer, 2008). Although they are not believed to biomagnify in marine food webs (Gobas et al., 2003; Mackintosh et al., 2004), continuous environmental release and chronic exposure to PAEs can have serious adverse effects on marine organisms, including immune system disruption, metabolic alterations, developmental abnormalities, and behavioral changes (Zhang et al., 2021). Phthalate exposure, particularly DEHP, in rats, has been linked to adverse effects on testis development, steroid hormone synthesis, and androgen-dependent tissues, leading to reduced fertility, reproductive tract malformations, decreased testosterone and sperm production, as well as cardiovascular toxicity (Dobrzynska et al., 2012; Mariana et al., 2016). Chronic exposure to phthalates in mice and rats can also activate inflammatory processes and cause oxidative damage (Brassea-Pérez et al., 2022). Furthermore, human exposure to phthalates has been linked to fertility issues, respiratory conditions, childhood obesity, and cognitive abnormalities (Katsikantami et al., 2016). Consequently, certain PAEs have been restricted or prohibited in their production and use in the EU and the USA (Regulations CE 1907/2006 and 2021/2045, 16 CFR Part 1307).

Monitoring the occurrence of these hazardous chemicals in marine ecosystems is crucial for devising strategies to mitigate their environmental and human health impacts. Cetaceans serve as valuable indicators of pelagic ecosystem health (Bossart, 2011; Fossi et al., 2020; Lemos et al., 2013), occupying a unique trophic position as top predators that consume a multitude of species across a wide depth range in the water column. Changes in cetacean body condition and health parameters reflect alterations in prey abundance, availability, and exposure to environmental contaminants (Cossaboon et al., 2019; Jepson et al., 2016). Due to

their distinctive physiology, feeding behavior, and long lifespan, cetaceans are particularly susceptible to pollutants exposure (Bossart, 2011), which can cause reproductive failure, immune system suppression, and even mortality (Jepson et al., 2005; Murphy et al., 2018). In addition, as a result of the overlap between maritime human activities and ecologically vital areas, marine mammals are increasingly subjected to other anthropogenic threats such as fisheries, marine traffic, and climate change (Halpern et al., 2008; McIvor et al., 2022). Considering their critical ecological and commercial importance (Mazzoldi et al., 2019; Pimiento et al., 2020), it is imperative to monitor the health status of these populations and implement protective measures for their conservation. A common procedure for studying cetacean populations is analyzing tissues from stranded animals. However, evaluating health conditions based on strandings may result in a biased assessment of the population status (*e.g.,* over-representation of diseased individuals) (Fossi et al., 2020). Biopsy sampling from free-ranging cetaceans removes concerns over tissue quality and post-mortem changes (Hobbs et al., 2003); additionally, it allows for the study of metabolic and biomarkers responses alongside contaminant burden, elucidating physiological differences, body condition, and contaminants' adverse effects on the studied individuals (Fossi et al., 2018).

In marine ecology, fatty acids (FAs) have emerged as important biomarkers that can provide insights into dietary sources, trophic interactions, and physiological conditions of marine organisms (Budge et al., 2006; Dalsgaard et al., 2003). Certain FAs are employed as "trophic markers" since they pass up in the food chain mostly unmodified, serving as indicators of predator-prey relationships (Budge et al., 2006; Parrish et al., 2000). In marine mammals, lipids (dominant in the form of triglyceride, i.e., a glycerol with three fatty acids) are mainly stored in the blubber and mobilized in times of energetic needs (Iverson, 2009). Therefore, the blubber lipid content can indicate the overall energy reserves and body condition of the animal (Gómez-Campos et al., 2011; Stirling et al., 2008). Blubber is also an accumulation site for

lipophilic pollutants and can undergo changes in FA composition due to high contaminant levels, including organic pollutants like polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and heavy metals (Guitart et al., 1996; Ruiz-Hernández et al., 2022). Also phthalates, such as DEHP, can disrupt lipid metabolism in cetaceans by activating peroxisome proliferator-activated receptors (Routti et al., 2021; Xie et al., 2023b). Furthermore, long-term exposure to PAEs has been associated with FA alterations and metabolic disorders in humans (Harley et al., 2017; Li et al., 2020). Despite these concerning health implications, the relationship between these pollutants and lipid metabolism in cetacean species remains largely underexplored (Routti et al., 2021; Xie et al., 2023b).

In this study, the blubber of free-ranging individuals of short-finned pilot whale (*Globicephala macrorhynchus*, hereafter "pilot whale") and common bottlenose dolphin (*Tursiops truncatus,* hereafter "bottlenose dolphin"), inhabiting a remote oceanic insular environment, was examined to assess PAE contamination levels and FA profiles. Variations in habitat use, feeding ecology, and physiological adaptations among individuals may influence pollutant absorption and response (Brown et al., 2015; Righetti et al., 2023); however, this has scarcely been explored in oceanic species. As such, this study aims to assess whether (i) the two species exhibited separate trophic niches, (ii) ecological and biological factors influenced their susceptibility to PAEs contamination, and (iii) health conditions differed across groups with distinct levels of contamination.

2. Material and methods

2.1. Sampling

Blubber samples were collected from free-ranging pilot whales (n=45) and bottlenose dolphins (n=39) in the southern waters of Madeira Island (eastern North Atlantic, Figure 1), between 2017 and 2022 (Table S6.1). Three dedicated campaigns were carried out in 2017-

2018 (as reported in Alves et al., 2020), while samples from 2019 to 2022 were collected opportunistically.

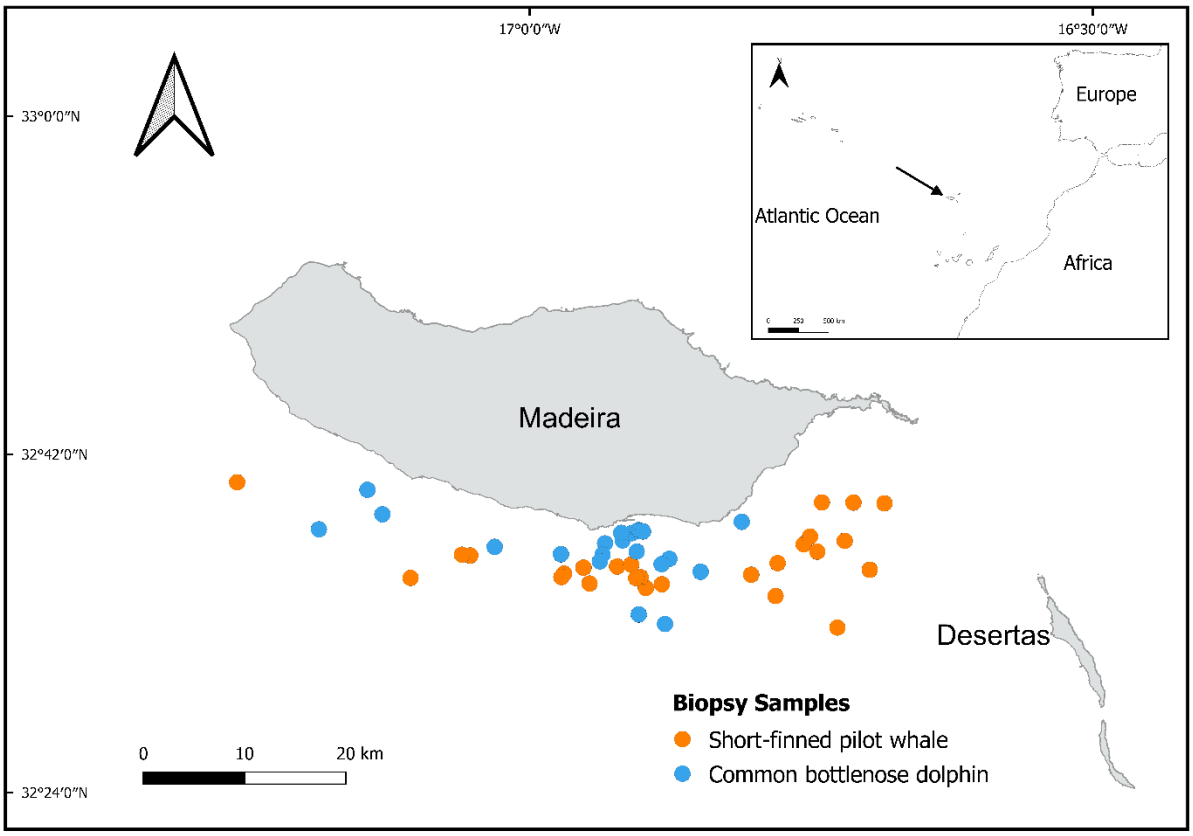

Figure 6.1 Map of Madeira showing the locations where biopsy samples of short-finned pilot whales (*Globicephala macrorhynchus*) and common bottlenose dolphins (*Tursiops truncatus*) were collected.

Madeira is part of the Macaronesia biogeographical region (Spalding et al., 2007), and it is remotely situated ~650 km from the West African coast and ~850 km from the southern tip of Portugal (Figure 6.1). The island is characterized by a narrow shelf and deep submarine canyons and is surrounded by oceanic, oligotrophic waters (Caldeira et al., 2002; Canning-Clode et al., 2008). Despite its remote location, Madeira Island is affected by marine pollution in its surrounding waters due to consistent discharges from local streams and its position at the edge of the North-Atlantic subtropical gyre (Álvarez et al., 2020; Rosa et al., 2022; Sambolino et al., 2022a)

A darting biopsy system operated by experienced researchers with legal permits (see ethical approval) was used to obtain samples, as described in Alves et al. (2020). The biopsies were collected from the animals' flanks, just below the dorsal fin, and the same type of sampling tip (darts specially designed for small cetaceans by Finn Larsen, Ceta-Dart40) was used for the two sampled species. Only adult-sized individuals with no sign of emaciation and not accompanied by calves were targeted. Sighting data (GPS coordinates) and individual identification photographs of the biopsied animals were taken whenever possible, using digital cameras with zoom lenses (*e.g.,* Würsig and Jefferson, 1990).

Biopsy samples were stored in phthalate-free polypropylene cryovials, immersed in liquid nitrogen in the field, and placed at -80 ºC within the same day of collection until further treatment and analysis. On the day of the analysis, the blubber content of the biopsies was sectioned longitudinally (to comprise all the possible layers), and 30-50 mg (wet weight) of tissue per sample was used for each analysis (Table S6.1).

2.2. Sex determination

Determination of the sex of all individuals was done genetically, using the skin from the biopsies. Genomic DNA was extracted using a standard high-salt protocol outlined in Sambrook et al. (1989). Details of the PCR and amplification conditions are described in Alves et al. (2020).

2.3. Residency patterns

Residency patterns of pilot whales were determined from photographic-identification of the biopsied animals, as described by Alves et al. (2020). It was based on the data set of individual-specific encounter histories (from 2003), where only individuals that exhibited multiyear and year-round (i.e., in the four seasons) site fidelity were termed residents. Only four individuals (from 2019-2021) could not be photographed and were not attributed any

residency pattern (Table S6.1). On most occasions, bottlenose dolphins were not adequately photographed or had insufficient marking to allow correct individual identification; thus, residency patterns were not determined for this species.

2.4. Temporal analysis

Given that FA composition can be affected by changes in diet and environmental conditions across seasons (Gonçalves et al., 2012; Hartwich et al., 2013; Sushchik et al., 2017), the seasonal variation was examined for fatty acid analysis, considering samples collected in different periods: Autumn 2017 - Spring 2018 - Autumn 2018 for pilot whales and Autumn 2017 - Spring 2018 - Summer 2018 for bottlenose dolphins (aggregation based on the sampling campaigns - Table S6.1). For phthalates, only inter-annual temporal variation was considered (due to the scarcity of samples from different seasons).

2.5. Fatty acid analysis

About 30 mg of each sample was freeze-dried and shredded prior to analysis. Total lipids were not extracted due to the small size of the samples. The method for fatty acid methyl esters (FAMEs) preparation and detection is the same as described by Fernandes et al. (2019). Briefly, a mixture of ethyl acetate–methanol (1:19 v/v), with the antioxidant Butylated Hydroxytoluene (BHT, 0.01%), was added in a glass tube with the freeze-dried sample, then kept in a hot bath at 80 °C for one hour. FAMEs were analyzed by gas chromatography (Agilent HP 6890 - California, USA) equipped with a mass selective detector (Agilent 5973N - California, USA) and a fused silica capillary column DB-5ms (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) from Agilent J&W. The GC oven was programmed as follows: initial temperature 40 °C for 5 min; temperature gradient 2 °C/min; final temperature 250 °C, held for 5 min; injector temperature, 260 °C; transfer-line temperature, 260 °C; split ratio, 1:100. Helium was used as the carrier gas with a flow of 1.0 mL/min. The FAMEs were identified

through comparison of retention times and mass spectra obtained from "bacterial acid methyl esters CP mix" and "Supelco 37 component FAME mix" standards from Supelco (Missouri, USA). To quantify the FAs, the internal standard method was used, adding heneicosanoic acid (C21:0) from Sigma-Aldrich (Missouri, USA) as an internal standard at the initial step of the extraction. The results were expressed in g/g dry weight (d.w.) and percentage of each individual FA in relation to the total FA, with the quantification made according to the response factor determined for each FA present in the standards (or the most similar one), in comparison with C21:0 (internal standard). Methanol, BHT, and heptane (used for the standard solutions) were acquired by Sigma Aldrich (St. Louis, MO), while ethyl acetate was supplied by Merck (Darmstadt, Germany).

2.6. Phthalates analysis

Dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-n-butyl phthalate (DBP), benzyl-butyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DNOP) were the target analytes in this study.

A modified version of the QuEChERS method described in Chapter 3 (Sambolino et al., 2022b) was applied and validated for the extraction and purification of PAEs from a small quantity of cetacean blubber ("Micro – QuEChERS"). The QuEChERS method (standing for Quick, Easy, Cheap, Effective, Rugged, and Safe) offers several advantages over traditional extraction methods, involving lower quantity of reagents, reduced costs, and shorter operational time (Varela-Martínez et al., 2020). This method has proven effective for a broad range of analytes and matrices (González-Curbelo et al., 2015), although only recently its application to marine mammal blubber has been explored (Pedersen et al., 2023). Method details and validation procedure are provided in Chapter 4 (Sambolino et al., 2024). Briefly, a small quantity of frozen sample (50 mg, wet weight) was homogenized, then one ml of

acetonitrile and 0.5 g of ammonium formate were added; the mixture was vortexed and centrifuged. For the clean-up, all the supernatant was transferred to a glass tube containing the purifying salts ($MgSO_4$, PSA and C18). The mixture was again vortexed, centrifuged, and the resulting supernatant was transferred to a GC vial, evaporated overnight, and then reconstituted in 50 µl of Cyclohexane.

Stringent QA/QC measures were taken to ensure analysis accuracy and precision, which are described in Chapter 4 (Sambolino et al., 2024), where the detection method, equipment (GC-MS), and reagents are also reported. To summarize some: two procedural blanks were analyzed with each batch of samples, and an average of the two blank values (RSD < 10 %) was subtracted from the final results; matrix-matched calibration curves (8 points) with internal standard method were used to quantify the concentrations of the analytes, and limits of detection and quantification were determined. All the PAEs concentrations reported here are in wet weight (w.w.).

2.7. Data analysis

Only FAs found in percentages > 0.1% (in the average value per species in at least one of the two species) were considered for statistical analysis, to minimize analytical variation associated with low level FAs (Remili et al., 2022). Dietary FAs were selected to infer niche segregation (Budge et al., 2006) based on Iverson et al. (2004). These fatty acids exclusively originate from the diet and serve as excellent indicators of dietary variations in marine mammals. FAs used as health markers were selected based on an extended literature search (*e.g.,* Balk et al., 2011; Filimonova et al., 2016; Hook et al., 2014; Tocher, 2010) and were resumed on two ratios which are markers of inflammatory condition / oxidative stress (the ratio of arachidonic acid on eicosapentaenoic acid (AA/EPA) and the ratio of omega-6 on omega-3

polyunsaturated fatty acids (n6-PUFAs/n3-PUFAs) and a nutritional condition marker (total fatty acids content g/g d..w.).

Given the non-normal distribution of data (tested with the Shapiro-Wilk test), non-parametric tests were used. Pairwise comparison of FAs and PAEs concentrations between groups (species, sex, residency pattern) was performed with the Mann-Whitney test. Non-metric Multidimensional Analysis (nMDS, metaMDS R function) based on Bray-Curtis dissimilarities among samples and clustered heatmaps (pheatmap function with clustering method ward.D2) were used to visualize data and identify clusters for dietary FAs and PAEs profiles. PERMANOVA (Permutation Analysis of Variance, adonis2 R function) was used to test differences among groups for FAs and PAEs profiles. The homogeneity of multivariate dispersion among groups was tested by PERMDISP (betadisp R function) (Anderson, 2006). When different PERMANOVA models with different combinations of factors were tested, model selections were based on the information-theoretic approach (Anderson and Burnham, 2004) by comparing models AIC (Akaike's Information Criterion; Akaike, 1974). All statistical analyses were performed with R version 4.1.2 (R Core Team, 2022), using the packages "vegan", "pheatmap", "ggord" and "ggplot2", and a significance level of 0.05 was considered.

3. Results

3.1. Fatty acid profiles

Twenty FAs with a relative proportion > 0.1% were identified in the blubber samples of pilot whales and bottlenose dolphins, with significantly different overall profiles (PERMANOVA, $p < 0.001$). Univariate pairwise tests (Mann-Whitney) revealed that almost all FAs' proportions significantly differed between species, as well as the total proportions of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids (Table 6.1). Very few FAs significantly differed between subgroups (Table 6.1), and none of the

ecological or biological variables (sex, residency pattern, season) significantly affected the overall FA composition (PERMANOVA, $p > 0.05$).

3.2. Trophic niches

The nMDS analysis on nine selected dietary FAs (C18:2n6, C20:1n9, C20:4n6, C20:5n3, C22:1n11, C22:4n6, C22:5n6, C22:6n3, C24:1n9) reveals that the two species have distinct trophic niches (Figure 6.2A), as also supported by the PERMANOVA ($p < 0.001$) and PERMDISP analysis (TukeyHSD $p<0.05$). The same analysis performed on two groups of pilot whales, differentiated by residency pattern, did not show any clear separation (PERMANOVA, $p = 0.521$); however, visual inspection of the nMDS and analysis of dispersion (PERMDISP) suggest that resident individuals have a less variate diet compared to non-resident ones (average distance to centroid: Resident 0.1310, Non-Resident 0.1904), although only a nearly significant difference in dispersion was found (TukeyHSD test, $p = 0.09$). The PERMANOVA analysis of dietary FA datasets looking at temporal variation (applied separately for pilot whales and bottlenose dolphins) showed no significant seasonal or inter-annual differences. Similarly, the composition of dietary FA did not differ between sexes.

Table 6.1 Fatty acid (FA) profiles (% of individual FA on total FA) in blubber of short-finned pilot whales (*Globicephala macrorhynchus*, n=30) and common bottlenose dolphins (*Tursiops truncatus*, n=29). Data expressed as mean ± standard deviation. Only FA > 0.1% (average per species) were considered. Values in bold show significant differences between species, residency pattern, or sex (Mann-Whitney test, p-value < 0.05).

	Short-finned pilot whale					**Common bottlenose dolphin**		
	Residency Pattern		**Sex**			**Sex**		
	Resident (*n=11*)	**Non-Resident (*n=19*)**	**Females (*n=17*)**	**Males (*n=13*)**	**Total (*n=30*)**	**Females (*n=16*)**	**Males (*n=13*)**	**Total (*n=29*)**
C14:0	2.33 ± 0.34	2.59 ± 0.45	**2.63 ± 0.44**	**2.31 ± 0.34**	2.49 ± 0.43	2.96 ± 0.93	2.61 ± 0.4	2.8 ± 0.75
C15:0	0.3 ± 0.07	0.32 ± 0.07	0.33 ± 0.08	0.28 ± 0.05	0.31 ± 0.07	0.33 ± 0.06	0.32 ± 0.04	0.32 ± 0.05
C16:0	10.45 ± 3.25	12.33 ± 2.72	12.16 ± 2.74	10.96 ± 3.33	**11.64 ± 3.02**	5.15 ± 1.97	4.32 ± 1.28	**4.78 ± 1.72**
C17:0	0.33 ± 0.11	0.36 ± 0.1	**0.39 ± 0.1**	**0.29 ± 0.09**	**0.35 ± 0.1**	0.13 ± 0.06	0.13 ± 0.07	**0.13 ± 0.06**
C18:0	2.32 ± 0.46	2.51 ± 0.35	2.5 ± 0.38	2.36 ± 0.42	**2.44 ± 0.4**	1.24 ± 0.34	1.02 ± 0.42	**1.14 ± 0.39**
∑ SFA	16.19 ± 4.08	18.52 ± 3.3	18.51 ± 3.39	16.56 ± 3.96	**17.66 ± 3.72**	10.62 ± 2.88	9.37 ± 1.76	**10.05 ± 2.48**
C14:1n5	**0.37 ± 0.14**	**0.23 ± 0.17**	0.25 ± 0.14	0.32 ± 0.2	**0.28 ± 0.17**	1.2 ± 0.52	1.49 ± 0.84	**1.33 ± 0.68**
C16:1n7	11.34 ± 1.91	9.81 ± 2.55	**9.48 ± 2.33**	**11.53 ± 2.08**	**10.37 ± 2.42**	15.5 ± 5.69	15.17 ± 5.2	**15.35 ± 5.38**
C16:1n9	1.39 ± 0.41	1.45 ± 0.59	1.58 ± 0.58	1.22 ± 0.36	1.43 ± 0.52	1.49 ± 0.55	1.84 ± 0.92	1.65 ± 0.75
C17:1nx	0.82 ± 0.1	0.7 ± 0.24	0.74 ± 0.2	0.75 ± 0.22	0.74 ± 0.2	0.62 ± 0.22	0.7 ± 0.17	0.65 ± 0.2
C18:1n9	46.68 ± 5.43	48.3 ± 6.32	46.04 ± 5.61	49.88 ± 5.92	**47.7 ± 5.97**	28.68 ± 6.2	27.51 ± 4.83	**28.16 ± 5.56**
C20:1n9	4.82 ± 0.68	4.93 ± 1.41	5.18 ± 1.2	4.5 ± 1.09	**4.89 ± 1.18**	1.28 ± 0.56	1.4 ± 0.6	**1.33 ± 0.57**
C22:1n11	0.65 ± 0.17	0.69 ± 0.4	0.72 ± 0.35	0.62 ± 0.3	**0.68 ± 0.33**	0.17 ± 0.12	0.22 ± 0.14	**0.19 ± 0.13**
C24:1n9	0.38 ± 0.12	0.34 ± 0.21	0.37 ± 0.2	0.34 ± 0.15	**0.36 ± 0.18**	0.15 ± 0.09	0.12 ± 0.03	**0.13 ± 0.07**

∑ MUFA	67.41 ± 4.38	67.37 ± 5.74	**65.47 ± 5.29**	**69.89 ± 4.02**	**67.39 ± 5.2**	50.15 ± 11.73	49.94 ± 11.34	**50.05 ± 11.35**
C18:2n6	0.75 ± 0.12	0.71 ± 0.21	0.74 ± 0.19	0.7 ± 0.18	**0.72 ± 0.18**	1.61 ± 0.27	1.66 ± 0.21	**1.63 ± 0.24**
C20:4n6 (AA)	2.07 ± 0.51	1.93 ± 0.81	1.92 ± 0.56	2.06 ± 0.89	**1.98 ± 0.71**	1.48 ± 0.63	1.57 ± 0.42	**1.52 ± 0.54**
C20:5n3 (EPA)	2.38 ± 1.25	2.13 ± 1.37	2.47 ± 1.21	1.9 ± 1.42	**2.22 ± 1.31**	4.23 ± 1.5	5 ± 0.84	**4.57 ± 1.29**
C22:5n6	0.07 ± 0.11	0.09 ± 0.15	0.09 ± 0.15	0.06 ± 0.12	**0.08 ± 0.13**	0.74 ± 0.62	0.69 ± 0.59	**0.72 ± 0.6**
C22:6n3 (DHA)	8.2 ± 3.53	6.71 ± 3.66	7.82 ± 3.57	6.52 ± 3.69	**7.26 ± 3.62**	25.08 ± 9.23	25.2 ± 7.97	**25.14 ± 8.53**
C22:4n6	0.17 ± 0.14	0.29 ± 0.39	**0.34 ± 0.39**	**0.12 ± 0.14**	**0.24 ± 0.33**	0.36 ± 0.28	0.4 ± 0.28	**0.38 ± 0.28**
C22:5n3	1.71 ± 0.79	1.33 ± 1.14	1.53 ± 1.12	1.39 ± 0.93	**1.47 ± 1.03**	4.4 ± 2.09	4.59 ± 1.68	**4.49 ± 1.89**
∑ PUFA	16.28 ± 6.21	14 ± 6.95	15.87 ± 6.59	13.48 ± 6.8	**14.83 ± 6.67**	39.23 ± 13.3	40.69 ± 10.57	**39.89 ± 11.96**

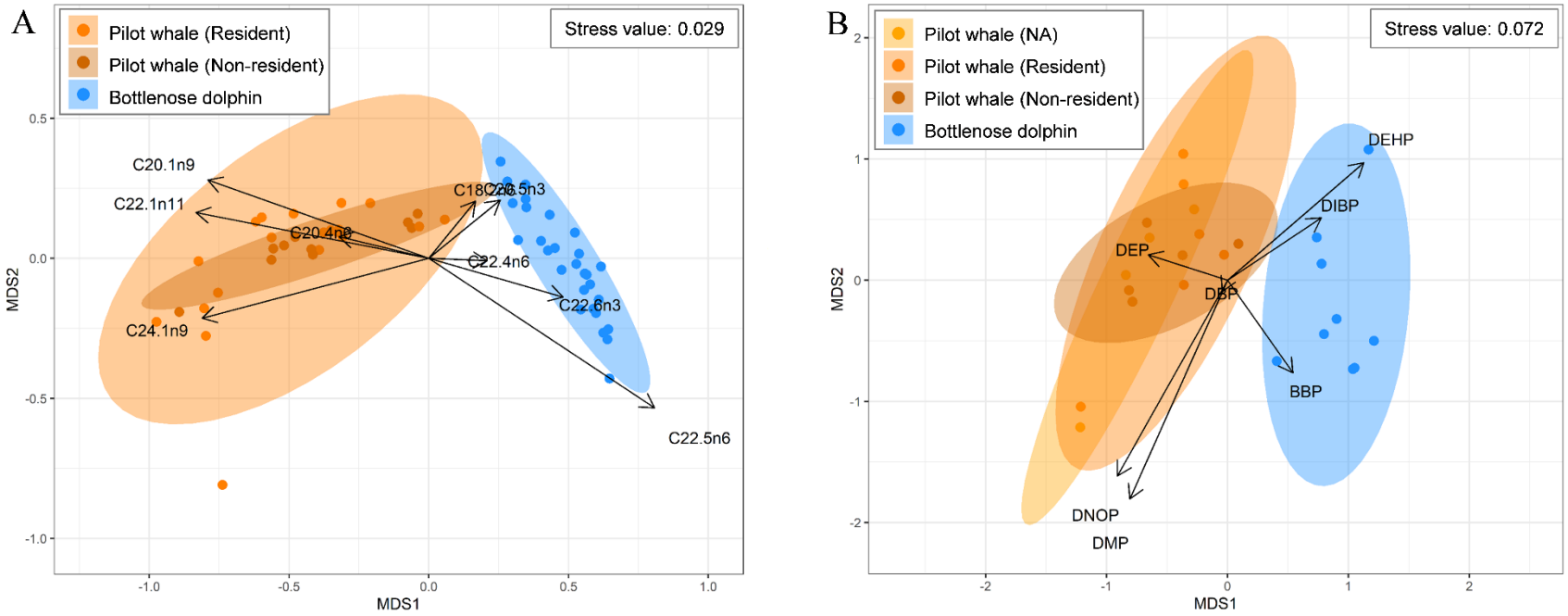

Figure 6.2 Non-metric Multidimensional Scaling (nMDS) of dietary fatty acids (A) and phthalates (B) detected in blubber samples of short-finned pilot whales (*Globicephala macrorhynchus* – FAs, n=30; PAEs, n=15) and common bottlenose dolphins (*Tursiops truncatus* – FAs, n=30; PAEs, n=9). Individuals from the two species and groups of short-finned pilot whales with different residency patterns are shown with different colors. NA = residency pattern not available.

3.3. Phthalates concentrations

All seven PAEs were detected in variable concentrations, and all samples analyzed contained PAEs (Table 6.2 and Table S6.2).

The nMDS applied to PAEs concentrations allowed a great representation of the entire dataset in reduced dimensions (stress value <0.1) (Figure 6.2B). Also in this case, samples showed clearly distinct PAEs profiles for the two species, which are mainly separated by DEP (more abundant in pilot whales), DEHP, DIBP, and BBP (more abundant in bottlenose dolphins).

Table 6.2 Descriptive statistics (frequency of occurrence [FO], mean, standard deviation [SD], minimum [Min] and maximum [Max] values) of phthalates (PAEs) concentrations (ng/g, wet weight) found in the blubber of biopsy samples of short-finned pilot whales (*Globicephala macrorhynchus*, n=15) and common bottlenose dolphins (*Tursiops truncatus*, n=9).

	Short-finned pilot whale					**Common bottlenose dolphin**				
	FO (%)	**Mean**	**SD**	**Min**	**Max**	**FO (%)**	**Mean**	**SD**	**Min**	**Max**
DMP	73	8.63	8.18	0.00	25.98	0	0.00	0.00	0.00	0.00
DEP	100	62.21	98.49	5.00	404.30	33	8.46	14.63	0.00	38.94
DIBP	13	1.79	5.23	0.00	19.41	33	19.84	33.87	0.00	92.70
DBP	80	133.40	101.19	0.00	284.11	56	123.85	231.67	0.00	717.59
BBP	80	4.36	2.65	0.00	10.41	100	17.29	16.24	5.00	57.11
DEHP	40	18.37	29.70	0.00	93.63	100	778.12	1475.35	127.30	4697.34
DNOP	13	1.22	3.60	0.00	13.37	0	0.00	0.00	0.00	0.00
∑ PAEs	100	229.98	158.86	10.00	581.78	100	947.56	1558.34	168.49	4987.16

DMP and DNOP, grouped by hierarchical clustering (Figure 6.3), were detected solely in pilot whales and were the least abundant. DNOP was found in only one sample of pilot whale, and DMP was detected in concentrations < 26 ng/g in 11 pilot whale samples. On the contrary, DBP and DEHP (also clustered) were the most abundant PAEs, detected in

concentrations up to 718 and 517 ng/g, respectively (both found in samples of bottlenose dolphins). One bottlenose dolphin sample was found to have a 10x higher concentration of DEHP, corresponding to 4697 ng/g; this outlier was excluded from multivariate analysis. Hierarchical cluster analysis (Figure 6.3) revealed a clear separation in PAEs concentration profiles between the two species. One bottlenose dolphin (Tt52) also constituted a separate cluster due to the atypical high value of DBP (which was generally lower in bottlenose dolphin samples compared to pilot whales).

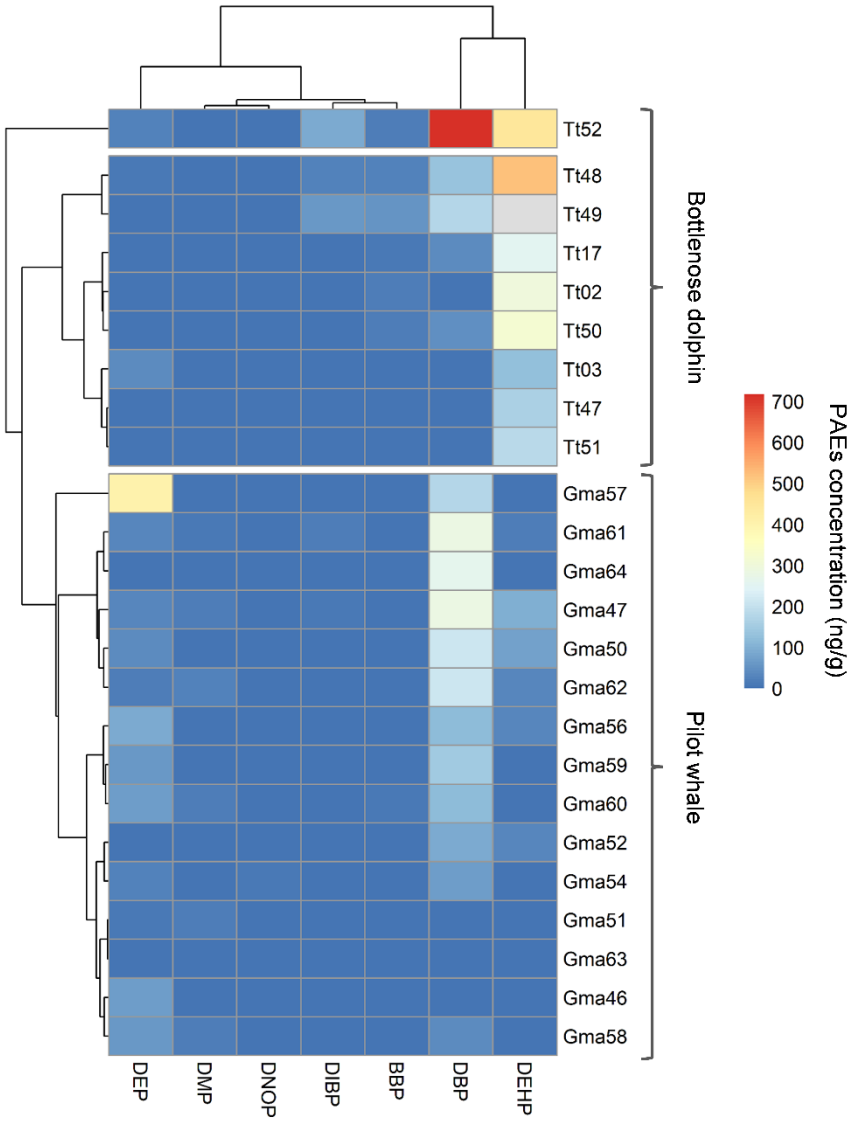

Figure 6.3 Heatmap of phthalates (PAEs) concentrations detected in blubber samples of short-finned pilot whales (*Globicephala macrorhynchus* – Gma, n=15) and common bottlenose dolphins (*Tursiops truncatus* – Tt, n=9), with hierarchical clusters. Information on individual codes is reported in Table S6.1 (Supplementary Data). DEHP from Tt49 (outlier value) is not reported here for better visual representation in the color scale.

DMP, DEP, BBP and DEHP concentrations significantly differed between the two species (Figure 6.4A). However, no differences were found between individuals from different sex and residency patterns (Figure 6.4B,C,D). PERMANOVA models were applied to three datasets: all individuals together (n = 24); only pilot whales with known residency patterns (n = 11); only bottlenose dolphins (n = 9). For pilot whales, the factor "year" was considered a two-level factor, joining 2019 to 2020 and 2021 to 2022, to balance the test design. Models ranked by the lowest AIC value are reported in Table 6.3. Confirming the results from the univariate tests, species was the only factor that significantly affected PAEs concentrations, while neither year, sex, nor residency pattern had any significant effect.

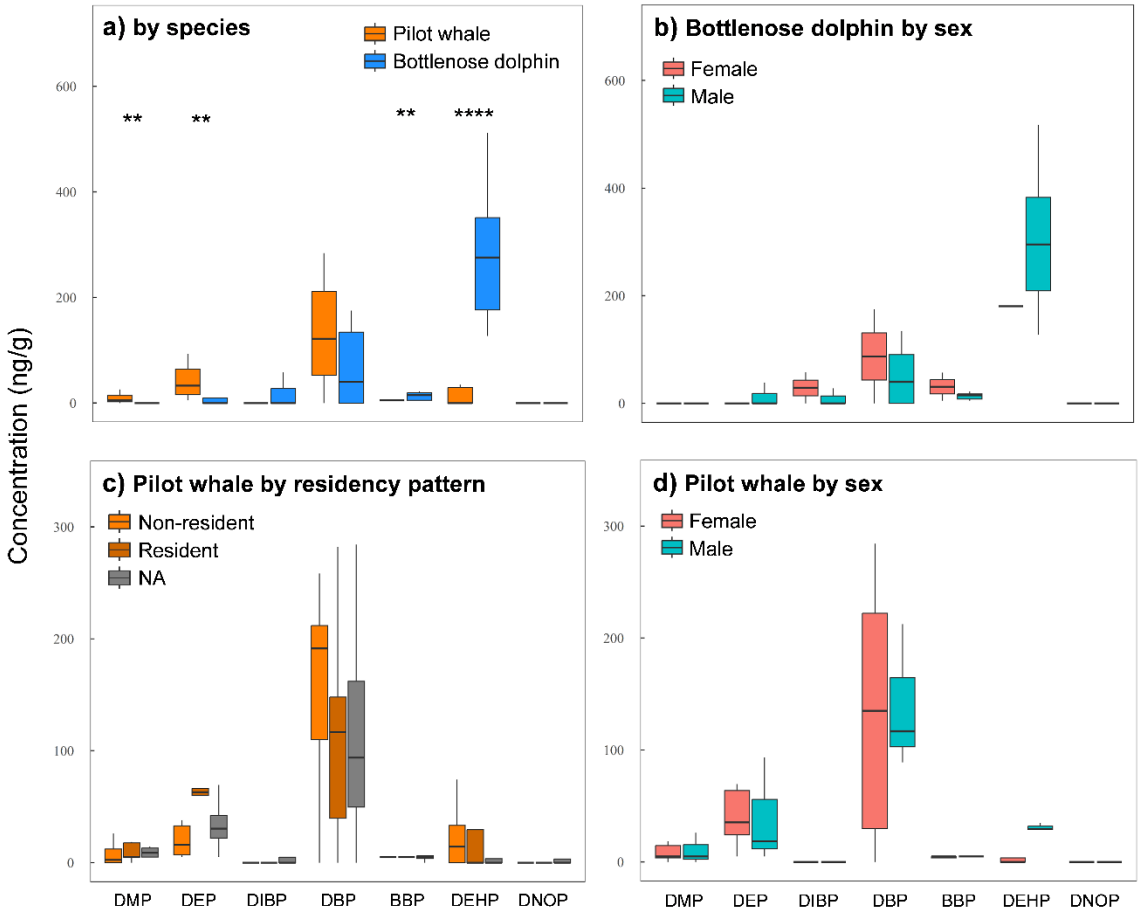

Figure 6.4 Boxplots of phthalates concentrations found in blubber tissue of short-finned pilot whales (*Globicephala macrorhynchus*, n=15) and common bottlenose dolphins (*Tursiops truncatus*, n=9) for comparison between the two studied species (a), between female and male individual bottlenose dolphins (b), between resident and non-resident individual pilot whales (c) and between female and male individual pilot whales (d). Significant differences (Mann-Whitney test) are indicated with ** ($p < 0.01$) and **** ($p < 0.0001$).

Table 6.3 Results of PERMANOVA applied on individual phthalates (PAEs) concentrations (DMP, DEP, DBP, DIBP, BBP, DEHP, DNOP), using three datasets: all short-finned pilot whales (*Globicephala macrorhynchus*) and common bottlenose dolphins (*Tursiops truncatus*) ("mod.GmaTt", n = 24), only pilot whales with known residency pattern ("mod.Gma", n = 11), and only bottlenose dolphins ("mod.Tt", n = 9). The best fitting model per each dataset is ranked by the lowest Akaike Information Criteria (AIC). Only "Species" (in bold) was a significant factor. Df = degrees of freedom.

	Model	Variance explained (%)	Df	AIC
mod.Gma.Tt1	**Species**	33.921	22	-39.42749
mod.Gma2	Residency pattern	14.018	9	-17.19726
mod.Gma1	Sex	11.47	9	-16.87611
mod.Gma4	Sex + Residency pattern	24.513	8	-16.6293
mod.Gma3	Year	4.275	9	-16.01654
mod.Gma6	Residency pattern + Year	18.671	8	-15.80925
mod.Gma5	Sex + Year	15.96	8	-15.4486
mod.Gma7	Sex + Residency Pattern + Year	29.317	7	-15.35252
mod.Gma9	Sex * Residency pattern	24.67	7	-14.65217
mod.Gma10	Year * Residency pattern	24.049	7	-14.56182
mod.Gma8	Sex * Year	21.465	7	-14.19377
mod.Gma12	Sex * Residency pattern * Year	28.873	6	-13.28366
mod.Gma11	Sex * Residency pattern * Year	40.349	4	-11.2192
mod.Tt2	Year	14.603	7	-15.51924
mod.Tt1	Sex	5.108	7	-14.57037
mod.Tt3	Sex + Year	15.356	6	-13.59895
mod.Tt4	Sex * Year	15.356	6	-13.59895

3.4. Health markers

The comparison of health markers showed significant differences between the two species, with a higher ratio of arachidonic acid on eicosapentaenoic acid (AA/EPA) and PUFAs-n6 on PUFAs-n3 (∑n6/∑n3) in pilot whales, and higher concentrations of total FAs concentrations (g/g d.w.) in bottlenose dolphins. The marker AA/EPA was also significantly higher in male individual pilot whales compared to females (Figure 6.5).

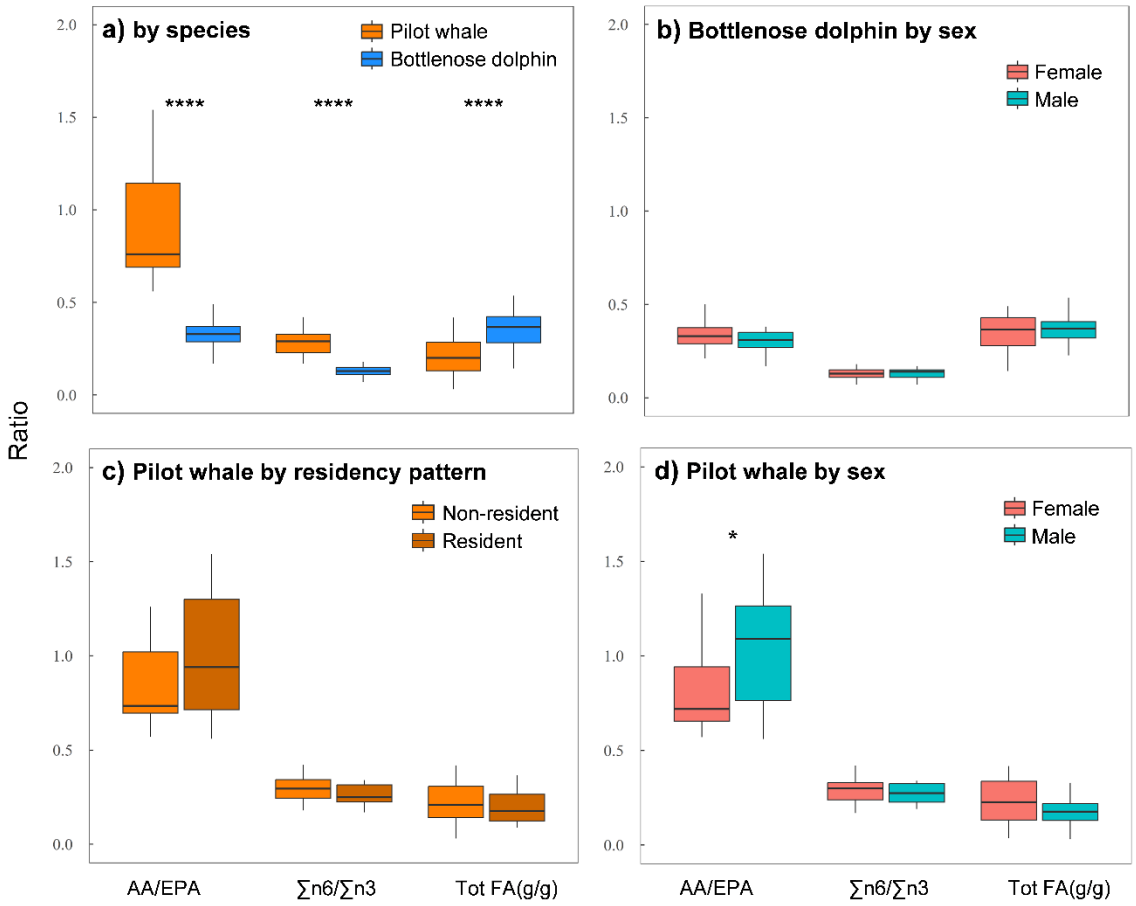

Figure 6.5 Boxplots of health biomarkers derived from fatty acids analysis in the blubber tissue of short-finned pilot whales (*Globicephala macrorhynchus*, n=30) and common bottlenose dolphins (*Tursiops truncatus*, n=30) for comparison between the two studied species (a), between female and male individual bottlenose dolphins (b), between resident and non-resident individual pilot whales (c) and between female and male individual pilot whales (d). Significant differences (Mann-Whitney test) are indicated with * ($p < 0.05$) and **** ($p < 0.0001$).

4. Discussion

In the face of increasing threats and degradation to marine ecosystems, understanding the fundamental exposure patterns and impacts of emerging contaminants on marine organisms is of paramount importance. Cetaceans play a crucial role as environmental sentinel species, making this study significantly valuable for uncovering how plastic additives affect delphinid species from an oceanic region. The two species examined in this research are common in the study region (Alves et al., 2018) and have an essential role in maintaining the structure and function of the environment they inhabit (Pearson et al., 2023; Roman et al., 2014). Short-

finned pilot whales thrive in tropical to warm-temperate regions, predominantly feeding on squids during vertical dives in deep waters (Aguilar Soto et al., 2008; Alves et al., 2013a; Fernandez et al., 2009; Quick et al., 2017). On the other hand, common bottlenose dolphins, a cosmopolitan species, mainly reside in coastal waters, relying on various bottom-dwellers and pelagic fish and squid for sustenance (Dias et al., 2023; Dinis et al., 2016a; Wells and Scott, 2018). Both species exhibit site fidelity in the studied region (Alves et al., 2013b; Dinis et al., 2016b), although some individuals venture widely between neighboring archipelagos (Canaries and Azores) (Alves et al., 2019; Dinis et al., 2021).

4.1. Fatty acid profiles and trophic niches

Blubber FA profiles determined in the two species were largely consistent with those reported in previous studies conducted in nearby archipelagos. In pilot whales from the Canary Islands, Iniguez (2020) found similar concentrations of MUFA (67.15%), SFA (23.33%), and PUFA (7.19%). In bottlenose dolphins from the Azores, Walton et al. (2007) also identified the same most abundant FAs, but they found lower amounts of PUFA (18.3% vs. 39.9%) and higher amounts of SFA and MUFA (16.7% and 63.5% vs. 10.1% and 50.1%, respectively). Similarly, percentages of FAs comparable to those found in bottlenose dolphins were observed in two other epipelagic delphinid species in Madeira (Atlantic spotted dolphin - *Stenella frontalis* and common dolphin - *Delphinus delphis*) (Quérouil et al., 2013).

Trophic interactions in cetaceans are typically investigated using MUFAs and PUFAs of exclusive dietary origin (Budge et al., 2006; Iverson et al., 2004), given the high FAs endogenous metabolism in marine mammals, which results in FA modification and de novo synthesis (Cooper et al., 2005). The nMDS analysis on dietary FAs in this study confirmed the expected feeding resource partitioning between the two cetacean species. The fatty acids primarily responsible for this segregation are well-known trophic markers from pelagic marine

ecosystems (Dalsgaard et al., 2003; Parrish et al., 2015). For example, C20:1n9 and C22:1n11, found in higher percentages in pilot whales, are typically associated to copepod-consuming mesopelagic fish and squid (Parrish et al., 2015; Pethybridge et al., 2011). On the other hand, essential long-chained PUFAs, such as DHA and EPA, found in higher levels in bottlenose dolphins, are exclusively synthesized from autotrophic organisms and primarily linked to diets based on dinoflagellates and diatoms (Parrish et al., 2015), which could suggest a prevalent feeding on epipelagic fish. Indeed, as widely acknowledged and affirmed in prior studies, pilot whales are specialized deep feeders, primarily hunting mesopelagic squids, while bottlenose dolphins are opportunistic epipelagic feeders that may occasionally prey on benthic species (Bode et al., 2022; Dias et al., 2023). Despite frequently being observed in association (Alves et al., 2018; Dinis et al., 2016b), their distinct physiology, diving behavior, social structures, and preferred habitats allow them to occupy different ecological niches.

4.2. PAEs contamination

The nMDS analysis of PAE concentrations also revealed a clear species-level separation, suggesting that the ecological roles of the two species influence their exposure to these pollutants. Trophic transfer is a common contamination pathway, and their distinct diets may explain the variations in PAE contamination. Xie et al. (2023a) for example found that continuous trophic transfer of PAE metabolites through chronically contaminated prey led to high concentrations in their predators, challenging the concept of biodilution of PAEs and PAEs-metabolites in marine food chains. A previous study (Sambolino et al., 2023) detected PAEs in common cetacean prey (fish and squids) in Madeira; however, uncertainty exists about their exact contribution to the diet of the studied species. Feeding depth may also impact PAE exposure differences in the two species. Paluselli et al. (2018) found PAE concentrations increasing with depth in the Mediterranean Sea, indicating accumulation in sediments and resuspension near the bottom. DBP concentrations were highest in deeper waters, while DEHP

dominated in the first 250 meters. Our results support these findings, with bottlenose dolphins inhabiting shallower waters showing higher DEHP concentrations, and deep-diving pilot whales having relatively higher DBP concentrations.

No significant differences in PAEs contamination levels related to biological/ecological factors (sex and residency pattern) nor temporal trends (inter-annual) were observed. This finding is consistent with previous studies on fin whales (*Balaenoptera physalus*) and bottlenose dolphins, where no significant effects of biological or temporal factors on PAEs concentrations were found (Garcia-Garin et al., 2022; Hart et al., 2018). It is worth noting that PAEs studies often exhibit high inter-individual variability in the type and amount of PAEs (Baini et al., 2017; Fossi et al., 2014; Hart et al., 2018). Unlike other contaminants like PCBs (Remili et al., 2021; Ross et al., 2000), phthalate concentrations seem not to be influenced by sex-specific pathways such as gestation and lactation (Hart et al., 2018). While none of these factors significantly affected PAEs concentrations, the residency pattern emerged as the best explanatory model for pilot whales, with variations in PAEs concentration dispersion observed in comparison to the non-resident individuals. The resident population of pilot whales from Madeira, with restricted feeding grounds and site fidelity to an anthropized area, may be more vulnerable to anthropogenic stressors (Alves et al., 2020, 2013b; Sambolino et al., 2022c). Therefore, continuous monitoring of pollutant hazards is crucial for this population's conservation.

The concentrations of PAEs found in blubber tissue samples of pilot whales and bottlenose dolphins collected in Madeira were consistent with those found in other studies conducted in the North Atlantic (Garcia-Garin et al., 2022; Montoto-Martínez et al., 2021; Routti et al., 2021), which also found DEP, DBP and DEHP as the most abundant PAEs. Montoto-Martínez et al. (2021) investigated muscle samples from stranded dolphins in Madeira, detecting individual PAE concentrations of up to 1533 ng/g. Although these values

are in the same range as our findings, the highest concentration observed in our study is four times greater. Notably, analyses performed on stranded animals might yield lower concentration values, possibly due to reduced intake in the hours prior to stranding (i.e., lack of feeding), followed by subsequent metabolization of most PAEs. However, other factors such as environmental conditions, the type of tissue analyzed (muscle vs. blubber), or the analytical methodologies used may have also contributed to the observed differences.

The presence of PAEs in marine animals can also serve as an indicator of potential plastic ingestion, as supported by numerous studies (Baini et al., 2017; Hardesty et al., 2015; Paluselli et al., 2018; Sambolino et al., 2023; Schmidt et al., 2021). This study revealed a higher PAEs burden in bottlenose dolphins when compared to pilot whales. Deep-diving cetaceans, that solely rely on echolocation for hunting, are particularly impacted by the ingestion of plastic litter resembling deep-sea cephalopods, such as plastic bags (Coram et al., 2021; Lusher et al., 2018, 2015a). While plastic debris has been found in the gastrointestinal tracts of stranded deep-diving species like sperm whales (*Physeter macrocephalus*) and beaked whales (Ziphiidae) (*e.g.,* Alstrup et al., 2021; Lusher et al., 2015a; O'Connell and Berrow, 2010; Podestà et al., 2015; Unger et al., 2016), studies have reported very few cases of plastic ingestion in stranded pilot whales (*Globicephala sp.*) (Lusher et al., 2018; Puig-Lozano et al., 2018; Walker and Coe, 1989), seemingly indicating that pilot whales are less susceptible to plastic ingestion than other deep-diving species. In contrast, bottlenose dolphins primarily feed in the epipelagic zone, where plastic debris, particularly microplastics, are highly concentrated (Pabortsava and Lampitt, 2020). Additionally, macroplastic has been found in the gastrointestinal tracts of several bottlenose dolphin specimens (Jerbi et al., 2021; Lusher et al., 2018; Solomando et al., 2022).

4.3. Health status and risk assessment

The findings here presented suggest that bottlenose dolphins may exhibit a more favorable health status compared to pilot whales, given their lower AA/EPA and ∑n6/∑n3 ratios. The physiological role of omega-3 PUFAs, such as EPA, is still not well understood in dolphins. However, these FAs have an important role in mammals' health, including promoting cardiovascular health and protecting against neurological and inflammatory diseases (Harris & Schmitt, 2014). In cells, n3-PUFAs act as protective chemical mediators that balance the inflammatory effect of n6-PUFAs (Weaver et al., 2008). The AA/EPA ratio, which focuses on the molecules competing for conversion to bioactive eicosanoids, is potentially a more relevant form of the ∑n6/∑n3 ratio (Harris et al., 2006). Although primarily studied in humans, these health markers are also significant in marine species, as exemplified by Dhurmeea et al. (2018) in female albacore tunas (*Thunnus alalunga*). Higher FA content (g/g d..w.) in bottlenose dolphins, compared to pilot whales, also could indicate superior energy storage and nutritional condition (Gómez-Campos et al., 2011; Stirling et al., 2008). However, inter-species comparisons of such markers should be interpreted cautiously due to potential metabolic and physiological differences. Nevertheless, our results are in accordance with a recent study (Alves et al., 2020) that also suggested better ecophysiological conditions in bottlenose dolphins compared to pilot whales in Madeira. PAEs burden, which was lower in pilot whales, is unlikely the leading cause of their lower health condition scores. Instead, other environmental variables and anthropogenic pressures (or a combination of them) may be responsible. For example, Sambolino et al. (2022c) highlighted the higher exposure of resident individual pilot whales to whale-watching activities, warranting further investigation into the impacts of such human pressures. Likewise, a significantly higher AA/EPA ratio was observed in male pilot whales in comparison to females, potentially suggesting a heightened inflammatory state within this group. Additional factors, including varying levels of exposure

to environmental factors or physiological distinctions between genders, may also contribute to this discrepancy.

The exposure of these marine mammals to DEP, DBP, and DEHP raises concern due to the well-established adverse health effects of these chemicals observed in laboratory and human studies. These effects encompass altered hormone synthesis and transport, male genital developmental abnormalities, reproductive impairment and liver toxicoses, including liver cancer (Hart et al., 2020, 2018). The updated risk assessment by the European Food Safety Authority (EFSA, 2019) established a group tolerable daily intake (TDI) of 50 µg/kg (50 ng/g) body weight (bw) for DBP, BBP, and DEHP, based on reproductive toxic effects (specifically, fetal testosterone reduction). While these limits are formulated for human health, it is reasonable to consider that similar toxicological mechanisms can occur in marine mammals. This study revealed remarkably high levels of these compounds in the blubber of the studied species when compared to the TDI value. The average values surpassed the TDI for DBP in both pilot whales and bottlenose dolphins, as well as for DEHP in bottlenose dolphins. Notably, one case of DEHP in a bottlenose dolphin nearly reached 100 times the TDI. The detection of such elevated PAEs concentrations, particularly DEHP, underscores the urgent necessity for further investigation into the exposure and impacts of these chemicals on marine mammal populations, especially those potentially exposed to heightened plastic debris and associated pollutants, such as bottlenose dolphins.

5. Conclusions

The present study investigated the fatty acid (FA) composition and phthalates (PAEs) concentrations in the blubber of two oceanic cetacean species to assess differences in their ecological niches, potential exposure to harmful plastic additives and health status. The current findings also provide the first insights and baseline information into the levels of plastic

additives in free-ranging cetaceans that inhabit the waters around Madeira Island. Significant differences in FA composition and PAE levels were found between the two delphinid species, suggesting distinct metabolisms, dietary preferences and pathways of contaminants exposures. Bottlenose dolphins exhibited elevated PAE levels, potentially linked to their habitat and dietary preferences. Further research is needed to understand the health implications.

Despite the challenges of studying PAEs in field biota samples, investigations on wild populations, particularly those involving free-ranging animals, are crucial for marine ecosystem conservation and threat mitigation. These results highlight the pervasive nature of plastic pollution and its associated chemicals, even in remote areas like oceanic islands. Additionally, they emphasize the vulnerability of cetaceans to the absorption of hazardous chemical additives with reproductive toxic properties, potentially leading to significant long-term adverse impacts on populations. Further research linking the presence of these chemicals to health impacts, using metabolic biomarkers or other health indicators, is necessary for a comprehensive understanding of the risks posed by plastic pollution in marine environments.

Ethical approval. Biopsies were obtained in accordance following guidelines and regulations of Instituto de Florestas e Conservação da Natureza (Instituto Português - Região Autónoma da Madeira), under sampling permits 1.856/2017, 508/2018, 08/IFCN/2018, 02/IFCN/2019, 02/IFCN/2020, 01/IFCN/2021, and 01/IFCN/2022 - FAU MAD.

Supplementary data

Supplementary data to this chapter can be found in Appendix D of this document.

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CHAPTER VII

GENERAL DISCUSSION, FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

Chapter 7 – General Discussion, Final Conclusions and Future Recommendations

1. General Discussion

(Micro)plastics and their associated additives represent some of the most pervasive and hazardous emergent contaminants in our modern world. The marine environment, in particular, faces significant impacts due to the indiscriminate disposal of waste into the ocean, making it a final destination for these pollutants (Chen et al. 2022; Fauser, Vorkamp, and Strand 2022; Koelmans et al. 2022). Since their initial detection in 1970s (Napper and Thompson 2020), numerous studies have diligently investigated the presence and potential effects of microplastics in marine biota, sediments, and water samples (Hossain and Olden 2022; Gola et al. 2021; Ugwu, Herrera, and Gómez 2021; Wang, Ge, and Yu 2020). These contaminants have been found even in the most remote areas, such as the Arctict (Barnes 2005; Bergmann et al. 2022), and with the continuous and escalating production of plastics their environmental prevalence is expected to escalate further (Jambeck et al. 2015; Europe 2021).

Despite their widespread occurrence, the ecological impact of these emerging contaminants in oceanic regions remains relatively unexplored, particularly concerning pelagic organisms, mainly due to the limited accessibility of these ecosystems (Hylland and Vethaak 2011; Fossi et al. 2014; Fossi and Depledge 2014). Oceanic islands offer unique and privileged locations for investigating the open oceanic environment, particularly when studying pelagic organisms (Alves et al. 2020). Madeira Island, in particular, is remotely situated about 500 km from the closest continental coast (West Africa) and is mainly surrounded by deep ocean, providing researchers with relatively easy access to the pelagic realm away from continental influences.

Cetaceans are regarded as exceptional sentinels of the pelagic ecosystem health due to their high trophic level, long lifespan, and subsequent heightened susceptibility to environmental alterations and contaminants (Bossart, 2011; Cossaboon et al., 2019; Fossi et al., 2020). When monitoring protected marine megafauna species like cetaceans in the wild, direct measurements of MP exposure are often unfeasible, and the use of indirect indicators is particularly relevant (Hermabessiere et al. 2017). One such indicator is the level of accumulated plastic additives in the environment or within organism tissues, which has been considered a proxy for plastic exposure in the oceans due to the release of these additives from dispersed plastic debris (Chen et al. 2022). Phthalates (PAEs) have been proposed as tracers to evaluate microplastic exposure in marine organisms due to their ubiquity and their wide use as plasticizers (Fossi et al. 2012; Hardesty et al. 2015; Baini et al. 2017). Furthermore, PAEs are pervasive and hazardous toxic substances (EFSA, 2019), and their concentrations in the environment and among wild populations should be regularly monitored.

Effective environmental management necessitates a comprehensive understanding of the ecological implications stemming from anthropogenic pollution. With this thorough knowledge, successful strategies can be developed and implemented to safeguard and preserve oceanic ecosystems. Therefore, addressing these knowledge gaps and continuing in-depth investigations into the presence, uptake mechanisms, and effects of MPs and their associated chemicals on marine organisms and the broader pelagic ecosystem is vital for informed conservation efforts and sustainable management of marine environments.

With this purpose, the occurrence and interactions of MPs and PAEs in the pelagic environment of an oceanic region (Madeira Island) were investigated in this thesis, encompassing organisms across various trophic levels, using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method coupled with Gas Chromatography - Mass

Spectrometry to detect PAEs in the target species. In addition, potential effects in lipid metabolism (through fatty acid profiles analysis) were investigated in cetacean populations.

In Chapter 2, the occurrence of MPs, together with zooplankton, was investigated in the surface waters of a pelagic area along one year of sampling to assess the baseline concentrations (in comparison to coastal areas) and to determine the influence of environmental factors such as rain and oceanographic processes on its seasonal abundance. During summer (July - September), the presence of anticyclonic eddies contributed to the aggregation of long-distance-origin particles, particularly photo-degraded light-coloured fragments, lines, and films. In contrast, in the rainy months (especially April), an increase in dark-coloured small fibres was recorded, likely resulting from land-based contamination which entered through wastewater treatment and river outlets. Zooplankton abundance was also significantly higher in the cold season. The ratio of MPs to zooplankton significantly varied due to environmental variables and seasonal influences, with higher values in the summer months. The potential risk of microfibres ingestion by planktivorous fish was highlighted in this study, and it was then confirmed by the findings of Chapter 5.

Chapter 3 and 4 of this thesis report the development and validation of *ad-hoc* methodologies for the detection and quantification of PAEs in tissue of pelagic fish and squid species (Chapter 3) and in small biopsy-blubber samples of cetacean species (Chapter 4).

In Chapter 5, the occurrence of MP and PAEs in planktivorous fish and higher trophic level organisms, such as squids, was investigated, considering their role as bioindicators in pelagic food webs. MPs, mainly in the form of fibres, were detected in all species, and findings indicated that MPs were consumed non-selectively through active feeding, with no evidence of accumulation in gastrointestinal tracts or size-dependent ingestion rate. Also, a seasonal variation in MPs ingestion rates was observed, with a major MPs uptake in the cold (rainy) season, suggesting a direct relation with a higher bioavailability of microfibres discharged after

the rain events. The correlation between MPs ingestion and plastic additives (PAEs) was also examined, and a significant relationship was found between ingested MPs and DIBP levels, validating the use of PAEs, especially DIBP, as plastic tracers in pelagic food webs.

In Chapter 6, the phthalate concentrations in blubber samples of free-ranging individuals from two important cetacean species (the short-finned pilot whale – *Globicephala macrorhynchus* and the common bottlenose dolphin – *Tursiops truncatus*) were examined, thus assessing their exposure to plastics. Blubber fatty acid (FA) profiles of pilot whales and bottlenose dolphins were also analyzed, revealing differences in metabolism, trophic adaptations and physiological status. The results confirmed distinct diets for the two species, which may be the leading cause of variations in the concentration of phthalate additives (PAEs) in their blubber tissues. Bottlenose dolphins showed a higher PAEs burden, likely due to their feeding habits in plastic-rich areas, such as the epipelagic zone. There was no clear correlation between PAEs burden and metabolic or physiological lipid alterations. Although the potential adverse health effects of PAEs were not evident in this case, further investigation is required.

The research addressed three main questions: (i) the extent of MPs contamination in the pelagic food web, (ii) the co-occurrence of PAEs with MPs in pelagic organisms as potential indicators of plastic exposure, and (iii) the occurrence and potential impact of PAEs on pelagic top predators, specifically cetaceans.

The occurrence of MPs in surface seawater and marine organisms from a pelagic area was unveiled in Chapter 2 and 5. The concentrations of MPs found in the surface seawater were found to be comparable to some coastal regions (e.g., Bay of Calvi - Collignon et al. 2014; Goiana Estuary – Lima, Costa, and Barletta 2014) or even higher (e.g., Portugal coast – Frias, Otero, and Sobral 2014), but much lower than some highly impacted areas (e.g., Southern Sea

of Korea – Kang, Kwon, and Shim 2015; Pelagic Mediterranean Sea – Faure et al. 2015; Cabrera MPA – Fagiano et al. 2022). It must be stressed that no clear standardization of sampling and analysis techniques in MPs research makes comparisons between different studies very difficult. Oceanic island pelagic ecosystems can face significant impacts from plastic debris transported by ocean currents. Surprisingly, the most concerning input comes from fibres, aligning with global marine environment results (Andrady 2011; Suaria et al. 2020). A rise in fibre concentrations during the rainy (cold) season was indicated by both studies, suggesting substantial MPs input from riverine discharges. These results underscore how populated islands like Madeira can significantly affect marine ecosystems, becoming an additional source of pollution. Moreover, plastic-carrying oceanographic processes such as ocean gyres, eddies, or coastal currents can lead to oceanic islands functioning as plastic retention zones (Monteiro, Ivar do Sul, and Costa 2018). These islands are ecologically unique, with exceptional biodiversity and endemism, rendering them highly sensitive to the impacts of plastic pollution (Ivar do Sul et al. 2013; Ivar do Sul, Costa, and Fillmann 2014; Monteiro, Ivar do Sul, and Costa 2018).

The correlation between MPs and PAEs was studied in Chapter 5, offering validation of the use of PAEs as plastic tracers. In particular, the abundance of MPs (mainly constituted by fibres) found in the gastrointestinal tracts of fish and squids showed a positive correlation with the concentration of DIBP found in the animals' muscle and mantle, respectively. The use of PAEs as plastic tracers has been proposed in many studies in the last decade (Fossi et al. 2012; Fossi et al. 2014; Hardesty et al. 2015; Baini et al. 2017); however, a direct correlation between the abundance of MPs and the concentration of PAEs detected in the same organism has been rarely explored (Table 1.5), neglecting the possibility that the concentrations of PAEs detected might derive from different sources than plastics. While this can be especially a problem in coastal areas where other anthropogenic sources of pollution are consistent, in

pelagic waters, the input of PAEs can be expected to derive mainly from the drifting plastic debris, which keeps leaching additives (Do, Ha, and Kwon 2022; Schmidt et al. 2021). Furthermore, the direct relation with absorption from the plastic present in the environment can easily be shaded by the relatively quick metabolism of PAEs in the organisms if these two are detected at different times (Hart et al. 2020; Goutte et al. 2020; Hu et al. 2016; Wittassek and Angerer 2008). As such, it must be stressed that the detection of PAEs mainly indicates a short-term exposure to plastic, and that PAEs have not been previously found accumulating in marine food webs (Mackintosh et al. 2004; Gobas et al. 2003).

PAEs, although not persistent pollutants with bioaccumulation potential, are a cause for concern as they are ubiquitously and pervasively found in the marine environment, can act as endocrine disruptors, and have the potential to transfer along the food chain, particularly affecting pelagic top predators like cetaceans. Chapter 6 examined the occurrence of PAEs in two top-predator cetacean species. Notably, higher concentrations of PAEs were found in cetaceans compared to their prey, which suggests potential short-term bioaccumulation in high trophic level species that consume numerous contaminated preys. This phenomenon may also be related to the different lipid concentrations present in the species and tissues analyzed. Blubber tissue of cetaceans, known for accumulating lipophilic contaminants like PAEs, holds much higher lipid levels than fish and squid tissues.

The study did not characterize food web relationships nor the biomagnification potential of PAEs due to the challenges of using fatty acid profiles of prey and predators to construct diet estimations. Although a quantitative estimation analysis has previously been proposed (QFASA – Iverson et al., 2004), its use requires conversion factors derived from experimental feeding studies, which are scarce for wild cetacean species. Moreover, the wide and variable range of marine organisms that pelagic cetaceans feed on, many of which are

rarely observed and live in deep waters, poses significant difficulties in constructing a complete food web. Combining various trophic techniques, such as fatty acids, stable isotopes, and stomach contents, would be necessary to determine the trophic relationships effectively. Stable isotope analysis, for example, can define trophic levels and aid in determining a biomagnification factor, as demonstrated by Xie et al. (2023). To fully understand the trophic transfer mechanisms of MPs and PAEs in marine food webs, it is crucial to understand trophic relationships and PAEs metabolism, and trophic transfer experiments should also be carried out in laboratory conditions.

Most animals rapidly metabolize PAEs into their monomeric metabolites (mono-phthalates - mPAEs). Therefore, future studies should not overlook these metabolites in their detection analysis. Detecting mPAEs requires additional procedures, as these compounds are not volatile and need derivatization for being analyzed with GC-MS or the use of Liquid Chromatography (LC). Including mPAEs in the analysis can provide valuable information on past, "longer-term" exposure to plastics, and studying the presence of PAEs and mPAEs in multiple samples collected from the same animal at different times can offer insights into the metabolism mechanism and rate of the target species.

Evidence of adverse effects of PAEs in studied species was not disclosed in the present thesis; however, the broader understanding of the occurrence, distribution, and intake patterns of these emerging contaminants in the marine ecosystem lays a crucial foundation for future research and management strategies to address their potential ecological impacts. Chronic exposure to plastics in environmental conditions, even in low MP concentrations, leads to subsequent chronic exposure to endocrine-disruptor plasticizers such as PAEs, whose long-term adverse effects are still not fully understood. Future studies should focus on uncovering long-term impacts associated with chronic exposure to MPs and PAEs in marine organisms,

monitoring the levels of the contaminants, understanding trends in exposure, and using metabolic or cellular biomarkers that can provide insights into the physiological effects.

One of the main challenges encountered in this research field relates to the analytical aspect of detecting PAEs in biological tissues. Therefore, Chapters 3 and 4 focused on the development, adaptation, and validation of the analysis methodology. As ubiquitous contaminants, PAEs can be found in everyday life objects, including laboratory and field equipment, leading to background contamination issues that require careful attention during sampling, processing, and analysis. Addressing this aspect is crucial to ensure confident results regarding the actual occurrence of PAEs in the marine environment. For instance, Chapter 5 was unable to present results on one of the main PAEs typically detected in the environment (DBP) due to unexpected and abnormal concentrations in the procedural blank samples. In subsequent studies (Chapter 6), additional measures were taken to tackle this problem, such as doubling the number of procedural blanks, incorporating more de-contamination procedures for laboratory materials, and performing the extraction process under a laminar flow cabinet. Another challenge in the analysis of PAEs in biological samples is the interference of lipids, especially in lipid-rich samples like blubber tissue, with the chromatographic detection signal of PAEs. This challenge was addressed by introducing purifying salts in the clean-up phase of the extraction. Nevertheless, further research is necessary to improve methodologies for PAEs determination in blubber tissues.

2. Final Conclusions and Future Recommendations

This thesis contributes to a deeper understanding of the occurrence and relationships of MPs and PAEs in a pelagic ecosystem. It was shown how they are pervasive emergent contaminants in the studied oceanic region, posing significant risks to the ecosystem. Some main conclusions can be evinced from this thesis:

1. Oceanic islands, such as Madeira Island, have been demonstrated as valuable sites for studying pelagic food webs. However, the land-based anthropogenic impact derived from the island was superior to what was expected. The vulnerability of the island system to plastic-carrying meteo-oceanographic processes, which can contribute to the accumulation of MPs (and, subsequently, PAEs), was also shown.

2. Using PAEs as tracers to evaluate MP exposure in marine organisms is worthwhile when direct measurements are unavailable. This thesis further validated their use, uncovering the positive correlation between MPs abundance and one of the PAEs (DIBP) concentrations found in marine species, although more studies are necessary to understand their direct relationships better.

3. The potential short-term accumulation of PAEs in high trophic level species, such as cetaceans, emphasizes the importance of studying the impacts of MPs and associated additives on pelagic food webs, up to top predators. Investigating the occurrence, interactions, and potential effects of emerging contaminants in marine food webs is essential for a comprehensive understanding of their potential long-term implications for marine ecosystems.

4. The QuEChERS method, coupled with GC-MS, both in its “macro” and “micro” versions, allowed for the analysis of PAEs in lipid-rich biological tissues, providing a valuable tool to detect their presence and potential impact on marine organisms.

Along with the conclusions, a summary of future recommendations is also presented:

1. Standardize sampling and analysis techniques in MPs research internationally to enable meaningful comparisons between studies.

2. Identify biomarkers of chronic exposure to MPs and PAEs to understand their long-term impacts on marine ecosystems. Further investigate the potential health effects of PAEs in

marine organisms, particularly in top predators like cetaceans, to understand the broader implications of plastic contamination in marine ecosystems.

3. Consider metabolites (mPAEs) in PAEs detection analysis to provide insights into "longer" term exposure to plastics and to better understand the metabolism rate and mechanisms of PAEs in marine organisms.

4. Explore the potential bioaccumulation of PAEs along pelagic food webs, gaining a deeper understanding of trophic relationships using multiple techniques such as fatty acids, stable isotopes, and stomach contents. Also, PAEs metabolism and trophic transfer mechanisms should be further explored through controlled laboratory studies in organisms from different trophic levels.

5. Improve analytical methodologies for PAEs determination in blubber and other lipid-rich biological tissues (such as liver) of marine organisms to get better analytical accuracy and sensitivity. At the same time, it is essential to address background contamination risk during field sampling and in laboratories to ensure accurate assessments of the occurrence of PAEs in the marine environment.

6. Focus on long-term monitoring programs to assess the ecological impacts of MPs and PAEs chronic exposure on marine organisms over extended periods. Ecological models can be developed to evaluate cumulative effects, as well as their long-term consequences, considering interactions with other stressors. These efforts are crucial to develop and enforce effective environmental management strategies and conservation efforts.

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APPENDIXES

APPENDIX A - Supplementary Data to Chapter 2

Appendix A consists of the Supplementary Data from the following published manuscript:

Sambolino, Annalisa, Inma Herrera, Soledad Álvarez, Alexandra Rosa, Filipe Alves, João Canning-Clode, Nereida Cordeiro, Ana Dinis, and Manfred Kaufmann. 2022. 'Seasonal Variation in Microplastics and Zooplankton Abundances and Characteristics: The Ecological Vulnerability of an Oceanic Island System'. *Marine Pollution Bulletin* 181: 113906. https://doi.org/10.1016/j.marpolbul.2022.113906.

APPENDIX A - Supplementary Data to Chapter 2

Table S2.1 Microplastics (MPs) and zooplankton (Zoo; ind = individuals) abundances and MPs/zooplankton ratio, per each size class and in total, per each sample collected.

				Size class 335 - 500 µm			Size class 500 - 1000 µm			Size class 1000 - 5000 µm			Total		
Sample	**Date**	**Distance (m)**	**Volume (m^3)**	**MPs (items/m^3)**	**Zoo (ind/m^3)**	**MPs / Zoo ratio**	**MPs (items/m^3)**	**Zoo (ind/m^3)**	**MPs / Zoo ratio**	**MPs (items/m^3)**	**Zoo (ind/m^3)**	**MPs / Zoo ratio**	**MPs (items/m^3)**	**Zoo (ind/m^3)**	**MPs / Zoo ratio**
PPl1	19/02/2019	1200	151	0.095	19.596	0.005	0.080	7.626	0.010	0.040	0.212	0.188	0.215	27.434	0.008
PPl2	20/03/2019	1564	197	0.050	26.607	0.002	0.037	3.129	0.012	0.020	0.153	0.133	0.107	29.889	0.004
PPl3	15/04/2019	634	80	0.953	8.164	0.117	0.106	75.357	0.001	0.088	0.025	3.500	1.147	83.546	0.014
PPl4	10/05/2019	1589	200	0.143	1.502	0.095	0.012	1.202	0.010	0.080	0.060	1.333	0.235	2.764	0.085
PPl5	28/06/2019	1620	204	0.059	2.113	0.028	0.047	0.467	0.101	0.049	0.192	0.256	0.156	2.771	0.056
PPl6	29/07/2019	1822	229	0.067	0.743	0.091	0.058	1.529	0.038	0.092	0.319	0.288	0.217	2.590	0.084
PPl8	10/09/2019	2379	299	0.033	0.201	0.165	0.040	0.753	0.053	0.037	0.214	0.172	0.110	1.168	0.094
PPl9	14/10/2019	2326	292	0.068	2.156	0.031	0.021	3.370	0.006	0.031	0.544	0.057	0.119	6.070	0.020
PPl10	06/11/2019	1880	236	0.126	3.259	0.039	0.061	5.438	0.011	0.034	3.195	0.011	0.220	11.892	0.019
PPl11	02/12/2019	2389	300	0.055	0.633	0.087	0.016	4.113	0.004	0.060	0.330	0.182	0.131	5.076	0.026
PPl12	09/01/2020	1961	246	0.085	4.869	0.017	0.058	16.272	0.004	0.077	5.166	0.015	0.220	26.307	0.008
Mean ± SD:		1760 ± 532	221 ± 67	0.158 ± 0.266	6.349 ± 8.728	0.062 ± 0.053	0.049 ± 0.028	10.841 ± 21.865	0.023 ± 0.031	0.055 ± 0.025	0.946 ± 1.664	0.558 ± 1.043	0.262 ± 0.298	18.137 ± 24.345	0.038 ± 0.035

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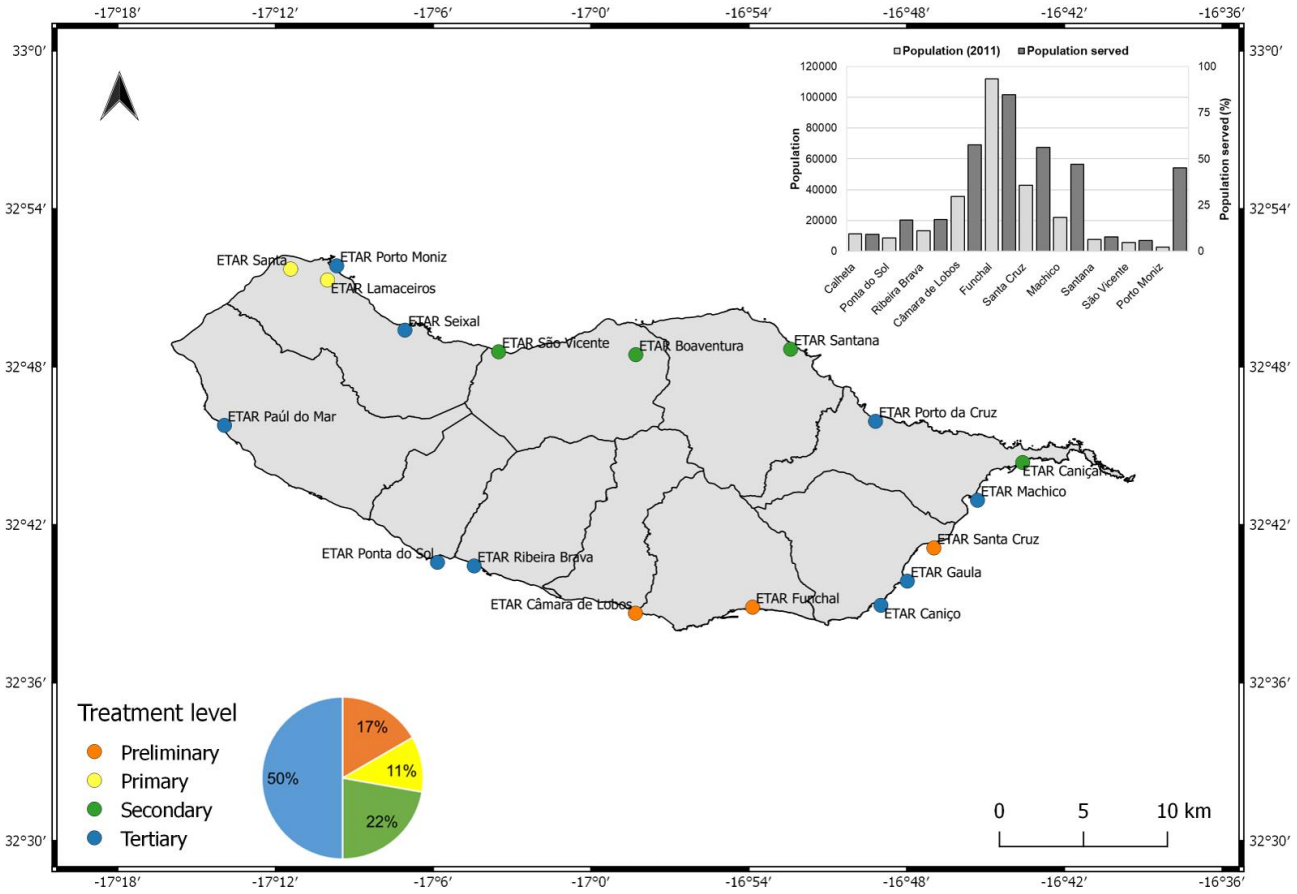

Figure S2.1 Location of wastewater treatment plants (WWTP = ETAR) in Madeira Island, with corresponding treatment levels. A histogram of the population served by each installation is also shown on the top right.

APPENDIX A - Supplementary Data to Chapter 2

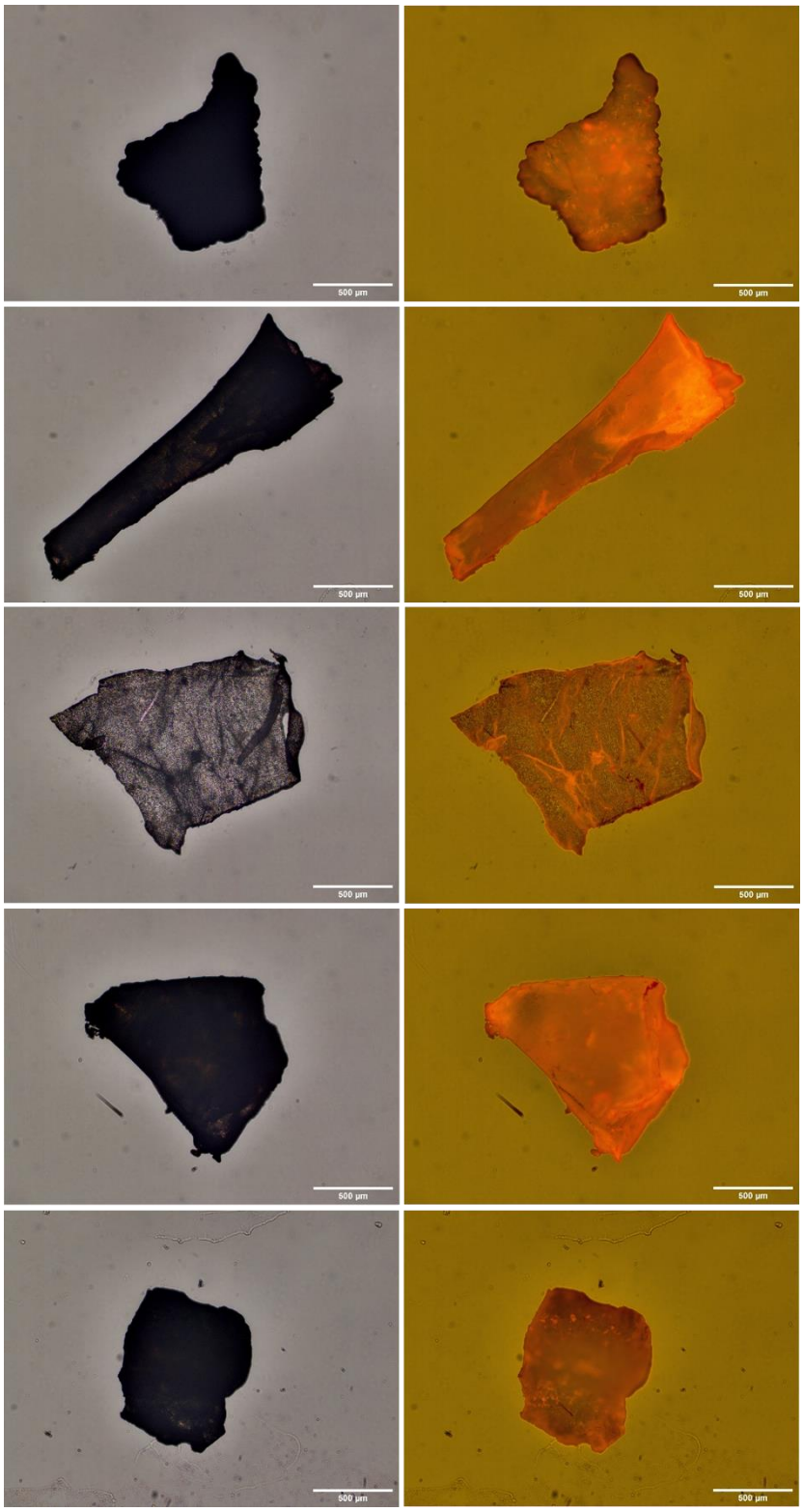

Figure S2.2 Examples of microplastics stained with Nile Red, magnified and photographed under a white-light source (left) and a blue LED light (Leica I3 filter - excitation 450-490 nm, emission 515).

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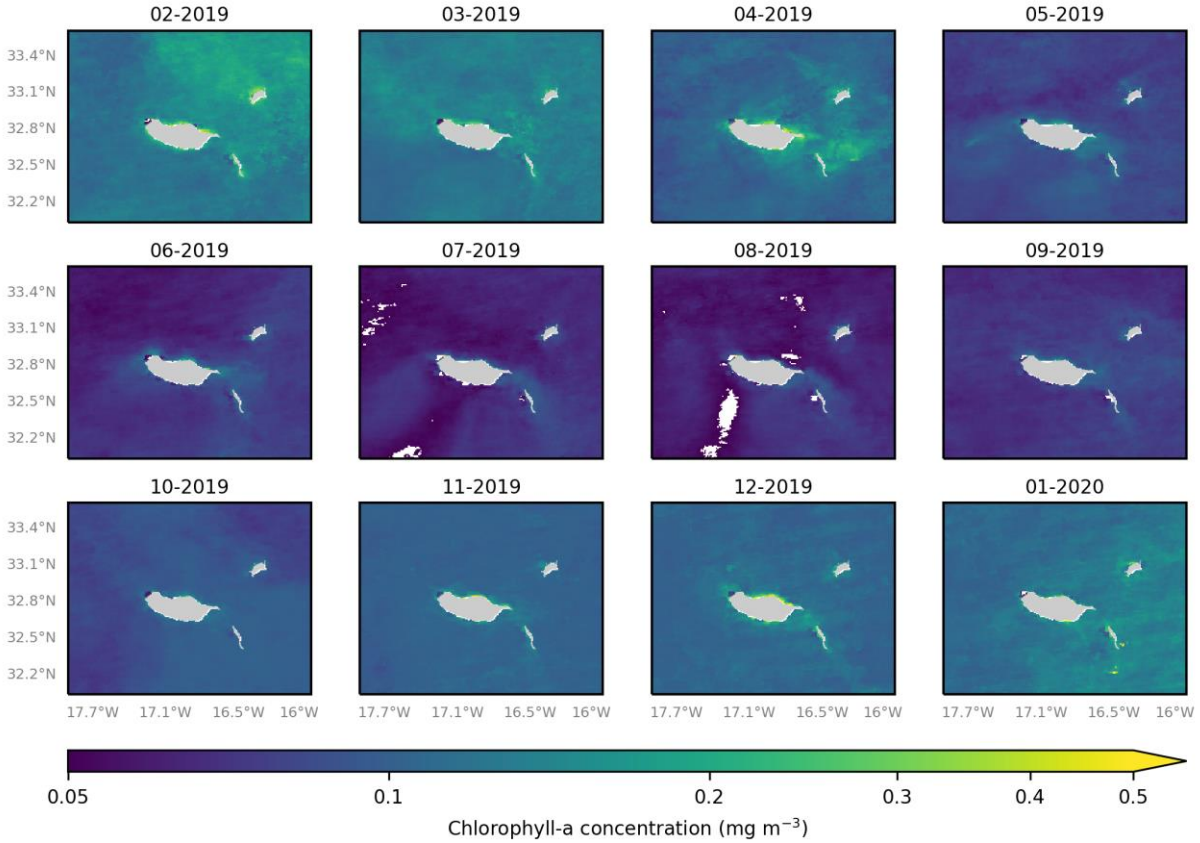

Figure S2.3 Monthly average of Chlorophyll-*a* concentrations (mg/m3) from February 2019 to January 2020 around Madeira Island. Data were obtained from the Copernicus Marine Service datalog.

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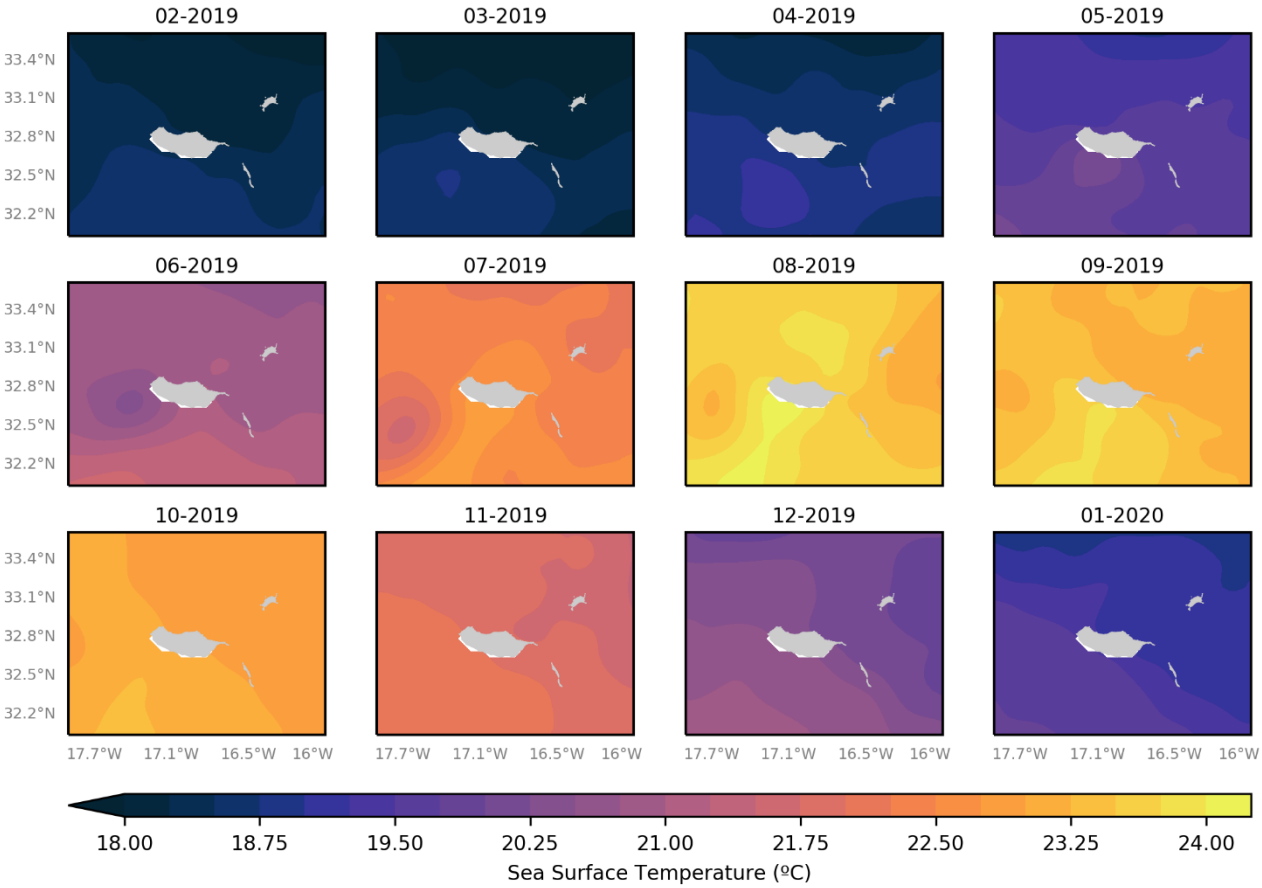

Figure S2.4 Monthly average of Sea Surface Temperature (ºC) from February 2019 to January 2020 around Madeira Island. Data were obtained from the Copernicus Marine Service datalog.

APPENDIX A - Supplementary Data to Chapter 2

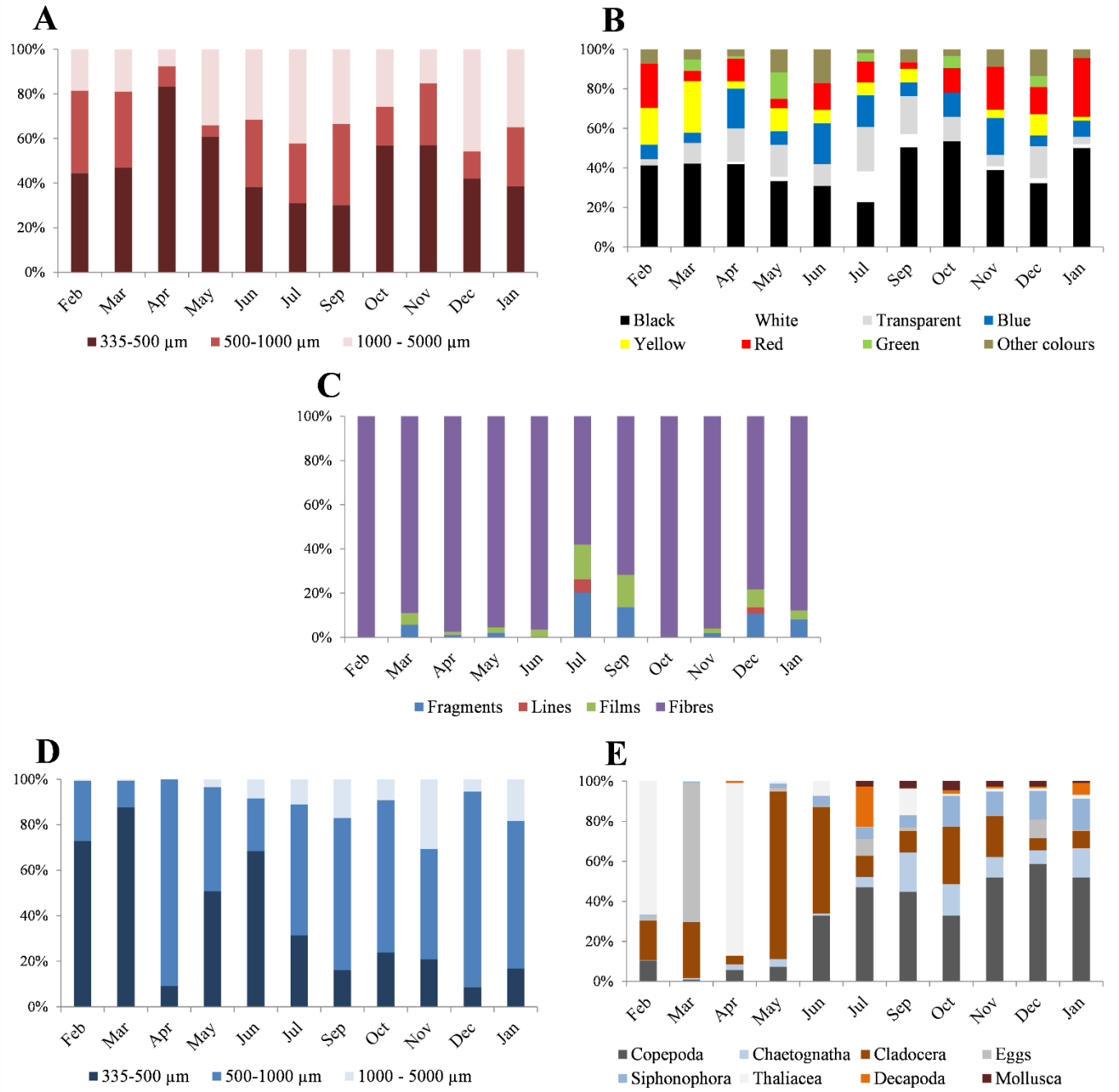

Figure S2.5 Proportion of size categories (A), colours (B) and types (C) of microplastics and size categories (D) and taxonomic groups (E) of zooplankton, per each sample collected in the sampling area between February 2019 and January 2020 (see Table S2.1 for all sampling data, e.g. total abundance of each sample).

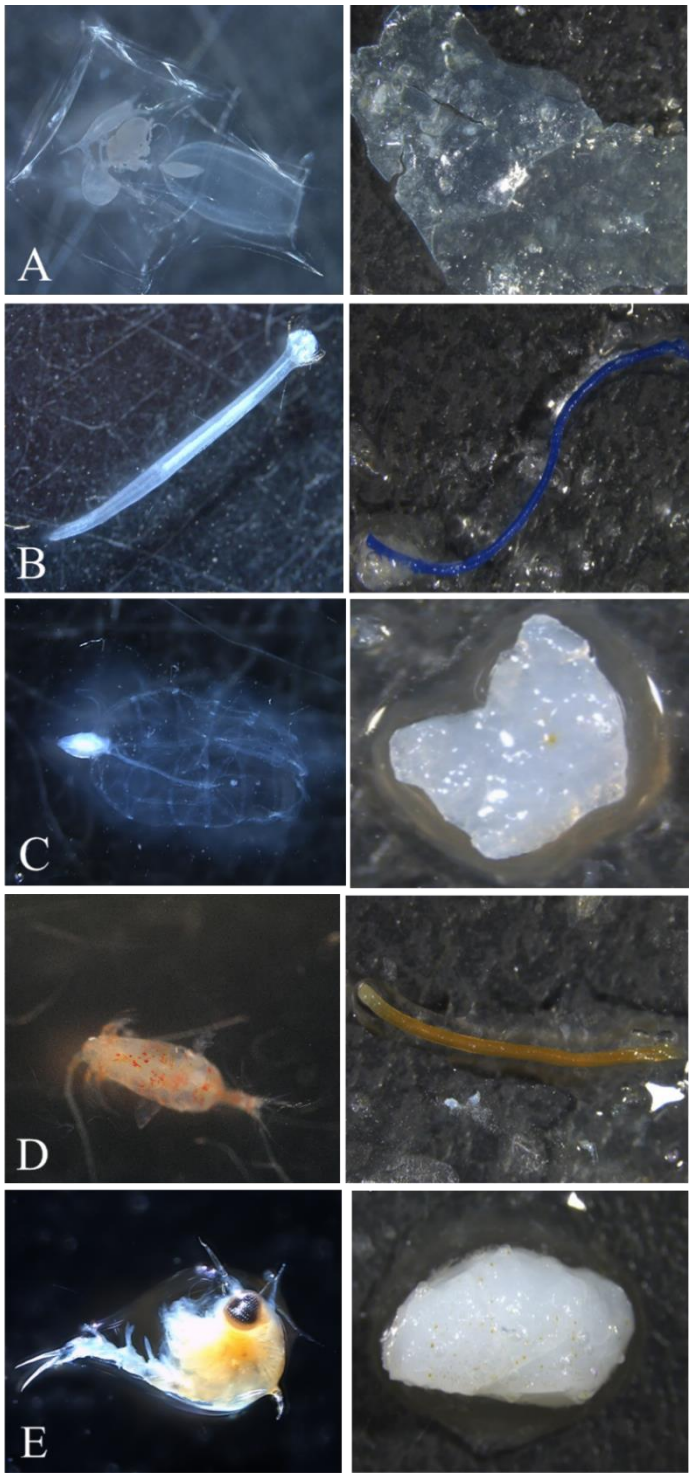

Figure S2.6 Representation of similarities among meso-zooplanktonic organisms and microplastic particles. Examples of zooplanktonic taxa that could be more easily mistaken for plastic particles (i.e. belonging to the largest size classes) are represented next to plastic particles with physical resemblance: A) Siphonophora B) Chaetognatha C) Thaliacea, D) Copepoda and E) Decapoda (Crustacean larvae). Both zooplankton and microplastics represented are of a size range between 1 and 5 mm. Pictures were not obtained from samples from the present study, but they were obtained from the same study area and serve as exemplification. Zooplankton pictures were taken by Inma Herrera. Microplastics pictures were taken by Annalisa Sambolino.

APPENDIX B - Supplementary Data to Chapter 3

Appendix B consists of the Supplementary Data from the following published manuscript:

Sambolino, Annalisa, Cecilia Ortega-Zamora, Javier González-Sálamo, Ana Dinis, Nereida Cordeiro, João Canning-Clode, and Javier Hernández-Borges. 2022. 'Determination of Phthalic Acid Esters and Di (2-Ethylhexyl) Adipate in Fish and Squid Using the Ammonium Formate Version of the QuEChERS Method Combined with Gas Chromatography Mass Spectrometry'. *Food Chemistry* 380: 132174. https://doi.org/10.1016/j.foodchem.2022.132174

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Table S3.1 Previous works in which the QuEChERS method has been applied to the analysis of fish or squid samples and comparative with this study.

Number of analysed PAEs	Matrix (species and amount)	Partitioning step	dSPE	Determination technique	Recovery % (RSD %)	LOQ_{method}	Concentrations found	Comments	Reference
5	Fish (-) and shrimp (-) (2 g)	10 mL EtOH/H_2O (80:20 v/v) + 0.5 g $MgSO_4$	2 mL of supernatant were transferred + 20 mg PSA	GC-MS	80-91% (1-5%)	8,430-32,030 ng/L	No PAEs were found	-	(Wang et al., 2013)
7	Fish (Atlantic bonito, *Sarda sarda* and European hake *Merluccius merluccius*) (0.5 g)	10 mL DCM/EtOAc (1/1, v/v) + 1 g $MgSO_4$ + 0.75 g PSA + 0.38 g C_{18}	All the supernatant was transferred + 1.2 g Florisil + 0.8 g alumina	GC-MS	49-77% (11-12%)	0.002-0.02 ng/g	19-83 ng/g of d.w. (total amount)	Samples were freeze-dried	(Castro-Jiménez and Ratola, 2020)
19	Fish (-) (5 g)	5 mL of ACN (1 % acetic acid) + 2 g NaCl	3 mL of supernatant were transferred + 150 mg of PSA + 450 mg of anhydrous $MgSO_4$	GC-MS/MS	71-116% (4-16%)	0.05-20 ng/g of w.w.	38.47-763.22 ng/g w.w. of DIBP, DBP and DEHP	-	(Xu et al., 2018)
12	Squid (*Loligo vulgaris*), fish (salmon *Salmo salar*, mackerel *Scomber scombrus* and sole Solea solea) (1 g)	10 mL water at pH 2 + 10 mL of ACN + 4 g of anhydrous $MgSO_4$ + 1 g NaCl + 0.5 g of sodium citrate dibasic sesquihydrate + 1 g of	5 mL of supernatant were transferred + 1 Lipifiltr® push-through cartridge (for PAEs) or 200 mg of bulk C_{18} sorbent	UHPLC-MS/MS	13-79% (4-17%)	5-250 ng/g of d.w.	All PAEs were found at concentrations in the range 10-978 ng/g d.w.	Samples were freeze-dried	(Hidalgo-Serrano et al., 2021)

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		sodium citrate tribasic dihydrate	(for phthalate monoesters)						
12+DEHA	Squid (*Loligo gahi*) and fish (tuna *Katsuwonus pelamis* and mackerel *Scomber colias*) (5 g)	5 mL ACN + 2.5 g ammonium formate	1 mL of supernatant was transferred + 150 mg $MgSO_4$ + 50 mg PSA + 50 mg C_{18}	GC-MS	70-117% (< 20%)	0.5-5 ng/g of w.w.	DIBP, DBP and DEHP were found in the range 1.17-44.2 of ng/g w.w. in some cases	Samples were frozen with liquid nitrogen	This method

ACN: acetonitrile; BBP: benzyl butyl phthalate; BMPP: bis(4-methyl-2-pentyl) phthalate; DAP: diallyl phthalate; DBEP: di(2-butoxyethyl) phthalate; DBP: dibutyl phthalate; DCHP: dicyclohexyl phthalate; DCM: dichloromethane; DEEP: di(2-ethoxyethyl) phthalate; DEHA: di(2-ethylhexyl) adipate; DEHP: di(2-ethylhexyl) phthalate; DEP: diethyl phthalate; DHP: dihexyl phthalate; DIBP: diisobutyl phthalate; DIDP: diisodecyl phthalate; DINP: diisononyl phthalate; DIPP: diisopentyl phthalate; DIPrP: diisopropyl phthalate; DMEP: di(2-methoxyethyl) phthalate; DMP: dimethyl phthalate; DNOP: di-*n*-octyl phthalate; DNP: dinonyl phthalate; DNPP: di-*n*-pentyl phthalate; DPhP: diphenyl phthalate; DPP: dipropyl phthalate; dSPE: dispersive solid-phase extraction; d.w.: dry weight; EtOAc: ethyl acetate; EtOH: ethanol; GC: gas chromatography; LOQ: limit of quantification; MBP: monobutyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEP: monoethyl phthalate; MMP: monomethyl phthalate; MOP: monooctyl phthalate; MS/MS: tandem mass spectrometry; MS: mass spectrometry; PAE: phthalic acid ester; PSA: primary secondary amine; RSD: relative standard deviation; UHPLC: ultra-high-performance liquid chromatography; w.w.: wet weight.

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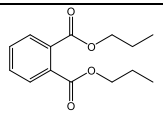
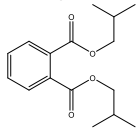
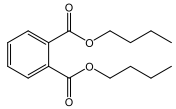
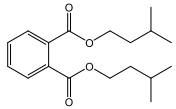
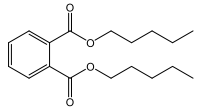
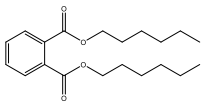
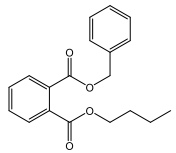
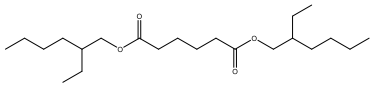
Table S3.2 Chemical structure and properties of the studied PAEs and DEHA.

Analyte	Structure	Molecular formula	MM (g/mol)	Solubility in water (g/L, 25 ºC)	Vapor pressure (mmHg, 25 ºC)	Log Kow	Melting point (ºC)	Boiling point (ºC)
DPP		$C_{14}H_{18}O_4$	250.1	37.9	$3.89 \cdot 10^{-4}$[a]	10.47	-	318
DIBP		$C_6H_{22}O_4$	278.3	0.0062[d]	$4.76 \cdot 10^{-5}$	4.11	-37	320
DBP		$C_{16}H_{22}O_4$	278.2	0.0112	$2.01 \cdot 10^{-5}$	4.72	-35	340
DIPP		$C_{18}H_{26}O_4$	306.2	0.20	$3.54 \cdot 10^{-4}$[b]	5.50[c]	-	336
DNPP		$C_{18}H_{26}O_4$	306.2	0.0008	$2.8 \cdot 10^{-5}$[c]	5.62	-55	342
DHP		$C_{20}H_{30}O_4$	334.4	0.00005	$1.40 \cdot 10^{-5}$	6.82	-58	302
BBP		$C_{19}H_{20}O_4$	312.1	2.69	$8.25 \cdot 10^{-6}$	4.73	-35	370
DEHA		$C_{22}H_{42}O_4$	370.6	0.00078[b]	$8.5 \cdot 10^{-7}$	6.11	-68	335

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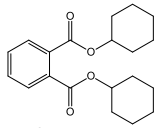
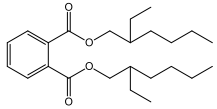
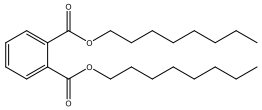
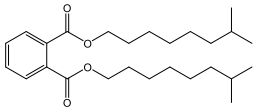
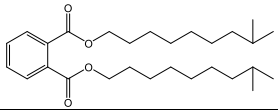
DCHP		$C_{20}H_{26}O_4$	330.2	4.0[d]	$8.69 \cdot 10^{-7}$	6.20	66	225
DEHP		$C_{24}H_{38}O_4$	390.3	0.00027	$1.42 \cdot 10^{-7}$	7.60	-55	230
DNOP		$C_{24}H_{38}O_4$	390.6	0.000022	$1.0 \cdot 10^{-7}$	8.20	-25	385
DINP		$C_{26}H_{42}O_4$	419.3	0.0002[e]	$5.40 \cdot 10^{-7}$	9.37	-48	406
DIDP		$C_{28}H_{46}O_4$	446.3	0.00028	$5.28 \cdot 10^{-7}$	10.36	-50	423

[a)] Calculated. [b)] 22 ºC. [c)] Predicted value. [d)] 24 ºC. [e)] 20 ºC. Data taken from SciFinder® and PubChem databases. MM: Molecular mass.

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Table S3.3 Retention times, quantifier, and qualifier m/z values in GC-MS analyses of the selected PAEs, DEHA and ISs. Ionization energy of -70 eV in all cases.

Analyte	Retention time (min)	RSD values % (n=40 injections)	Quantifier (m/z)	Qualifier 1 (m/z)	Qualifier 2 (m/z)
DPP	7.943	0.01	149	191	209
DIBP	8.702	0.03	149	205	223
DBP-d4	9.494	0.03	153	209	227
DBP	9.494	0.01	149	205	223
DIPP	10.413	0.01	149	237	219
DNPP-d4	11.096	0.01	153	223	241
DNPP	11.096	0.02	149	219	237
DHP-d4	12.679	0.02	153	255	237
DHP	12.679	0.02	149	251	233
BBP	12.784	0.03	149	91	206
DEHA	13.054	0.02	129	112	147
DCHP	14.152	0.01	149	167	249
DEHP-d4	14.257	0.01	153	283	171
DEHP	14.257	0.01	149	167	279
DNOP	15.630	0.01	149	167	279
DINP	16.459	0.01	149	167	293
DIDP	17.786	0.01	149	167	307

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Table S3.4 Internal instrumental calibration data of the target analytes (DBP-d4 was used as IS of DPP, DBP and BBP, DNPP-d4 was used as IS of DIBP, DIPP and DNPP, DHP-d4 was used as IS of DHP, DEHA and DCHP, while DEHP-d4 was used as IS of DEHP, DNOP, DINP and DIDP).

Analyte	Studied linear range (µg/L)	Regression equation (n=8)		$s_{y/x}$	R^2	LCL (µg/L)*
		$b \pm s_b \cdot t_{(0.05;6)}$	$a \pm s_a \cdot t_{(0.05;6)}$			
DPP	0.5-150	$8.34 \cdot 10^{-3} \pm 4.40 \cdot 10^{-4}$	$-2.05 \cdot 10^{-2} \pm 3.28 \cdot 10^{-2}$	$2.86 \cdot 10^{-2}$	0.9972	0.5
DIBP	0.5-150	$8.10 \cdot 10^{-3} \pm 4.68 \cdot 10^{-4}$	$-1.99 \cdot 10^{-2} \pm 3.49 \cdot 10^{-2}$	$3.04 \cdot 10^{-2}$	0.9967	0.5
DBP	0.5-150	$7.59 \cdot 10^{-3} \pm 2.97 \cdot 10^{-4}$	$-1.57 \cdot 10^{-2} \pm 2.21 \cdot 10^{-2}$	$1.93 \cdot 10^{-2}$	0.9985	0.5
DIPP	0.5-150	$4.78 \cdot 10^{-3} \pm 1.52 \cdot 10^{-4}$	$-9.09 \cdot 10^{-3} \pm 1.14 \cdot 10^{-2}$	$9.92 \cdot 10^{-3}$	0.9990	0.5
DNPP	0.5-150	$7.60 \cdot 10^{-3} \pm 3.26 \cdot 10^{-4}$	$1.69 \cdot 10^{-2} \pm 2.43 \cdot 10^{-2}$	$2.12 \cdot 10^{-2}$	0.9982	0.5
DHP	5-150	$7.90 \cdot 10^{-3} \pm 2.84 \cdot 10^{-4}$	$-2.27 \cdot 10^{-2} \pm 2.44 \cdot 10^{-2}$	$1.41 \cdot 10^{-2}$	0.9993	5
BBP	5-150	$1.89 \cdot 10^{-3} \pm 6.54 \cdot 10^{-5}$	$-2.80 \cdot 10^{-3} \pm 5.63 \cdot 10^{-3}$	$3.26 \cdot 10^{-3}$	0.9994	5
DEHA	5-150	$2.59 \cdot 10^{-3} \pm 9.66 \cdot 10^{-5}$	$-5.29 \cdot 10^{-3} \pm 8.32 \cdot 10^{-3}$	$4.82 \cdot 10^{-3}$	0.9993	5
DCHP	5-150	$5.15 \cdot 10^{-3} \pm 1.93 \cdot 10^{-4}$	$-1.62 \cdot 10^{-2} \pm 1.66 \cdot 10^{-2}$	$9.61 \cdot 10^{-3}$	0.9993	5
DEHP	5-150	$7.23 \cdot 10^{-3} \pm 2.41 \cdot 10^{-4}$	$-1.46 \cdot 10^{-2} \pm 2.08 \cdot 10^{-2}$	$1.20 \cdot 10^{-2}$	0.9994	5
DNOP	5-150	$1.12 \cdot 10^{-2} \pm 4.78 \cdot 10^{-4}$	$-2.39 \cdot 10^{-2} \pm 4.11 \cdot 10^{-2}$	$2.38 \cdot 10^{-2}$	0.9991	5
DINP	5-150	$8.34 \cdot 10^{-3} \pm 3.60 \cdot 10^{-4}$	$-1.83 \cdot 10^{-2} \pm 3.10 \cdot 10^{-2}$	$1.79 \cdot 10^{-2}$	0.9990	5
DIDP	5-150	$8.67 \cdot 10^{-3} \pm 3.71 \cdot 10^{-4}$	$-2.09 \cdot 10^{-2} \pm 3.19 \cdot 10^{-2}$	$1.85 \cdot 10^{-2}$	0.9991	5

b: slope; s_b: standard deviation of the slope; a: intercept; s_a: standard deviation of the intercept; R^2: determination coefficient; $s_{y/x}$: standard deviation of the estimate.
*Equivalent to 0.5 and 5 ng/g.

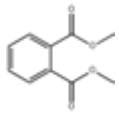
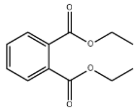
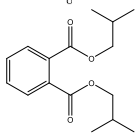
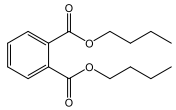
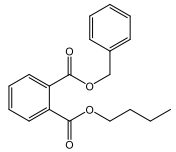
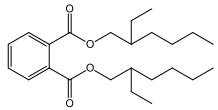
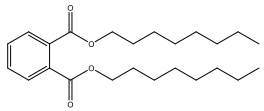
APPENDIX C - Supplementary Data to Chapter 5

Appendix C consists of the Supplementary Data from the following published manuscript:

Sambolino, Annalisa, Eva Iniguez, Inma Herrera, Manfred Kaufmann, Ana Dinis, and Nereida Cordeiro. 2023. 'Microplastic Ingestion and Plastic Additive Detection in Pelagic Squid and Fish: Implications for Bioindicators and Plastic Tracers in Open Oceanic Food Webs'. *Science of The Total Environment*, 164952. https://doi.org/10.1016/j.scitotenv.2023.164952

APPENDIX C - Supplementary Data to Chapter 5

Table S5.1 Chemical structure and properties of the studied PAEs.

Analyte	Structure	Molecular formula	MM (g/mol)	Solubility in water (g/L, 25 ºC)	Vapor pressure (mmHg, 25 ºC)	Log K_{OW}	Melting point (ºC)	Boiling point (ºC)
DMP		$C_{10}H_{10}O_4$	194.2	4.3	$3.08 \cdot 10^{-3}$	1.60	5.5	284
DEP		$C_{12}H_{14}O_4$	222.2	1.08	$2.1 \cdot 10^{-3}$	2.47	-3	295
DIBP		$C_{16}H_{22}O_4$	278.3	0.0062[a]	$4.76 \cdot 10^{-5}$	4.11	-37	320
DBP		$C_{16}H_{22}O_4$	278.2	0.0112	$2.01 \cdot 10^{-5}$	4.72	-35	340
BBP		$C_{19}H_{20}O_4$	312.1	0.00269	$8.25 \cdot 10^{-6}$	4.73	-35	370
DEHP		$C_{24}H_{38}O_4$	390.3	0.00027	$1.42 \cdot 10^{-7}$	7.60	-55	230
DNOP		$C_{24}H_{38}O_4$	390.6	0.000022	$1.0 \cdot 10^{-7}$	8.20	-25	385

[a] 24 ºC. Data taken from SciFinder® and PubChem databases. MM: Molecular mass.

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Table S5.2 Retention times and m/z values of quantifier and qualifier ions in GC-MS analyses of the selected PAEs and ISs. Ionization energy of 70 eV in all cases.

Analyte	Retention time (min)	Quantifier (m/z)	Qualifier 1 (m/z)	Qualifier 2 (m/z)
DMP	4.757	163	194	77
DEP-d_4	5.149	153	181	80
DEP	5.153	149	177	76
DIBP	5.925	149	223	104
DBP-d_4	6.181	153	209	227
DBP	6.184	149	205	223
BBP	7.210	149	91	206
DEHP	7.610	149	167	279
DNOP	8.089	149	167	279

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Table S5.3 Internal instrumental calibration data of the target analytes.

Analyte	Studied linear range (µg/L)	Regression equation (n=8)		$s_{y/x}$	R^2
		$b \pm s_b \cdot t_{(0.05;6)}$**	$a \pm s_a \cdot t_{(0.05;6)}$		
DMP	0.5-150	$(3.44 \pm 0.43) \cdot 10^{-3}$	$(0.57 \pm 2.85) \cdot 10^{-2}$	$1.38 \cdot 10^{-2}$	0.9954
DEP	0.5-150	$(5.84 \pm 0.1) \cdot 10^{-3}$	$(1.51 \pm 0.78) \cdot 10^{-2}$	$0.68 \cdot 10^{-2}$	0.9997
DIBP	0.5-150	$(5.82 \pm 0.41) \cdot 10^{-3}$	$(4.51 \pm 3.02) \cdot 10^{-2}$	$2.64 \cdot 10^{-2}$	0.9952
DBP	0.5-150	$(7.25 \pm 1.04) \cdot 10^{-3}$	$(-0.52 \pm 7.12) \cdot 10^{-2}$	$4.14 \cdot 10^{-2}$	0.9939
BBP	0.5-150	$(4.33 \pm 0.41) \cdot 10^{-3}$	$(1.55 \pm 3.24) \cdot 10^{-2}$	$2.38 \cdot 10^{-2}$	0.9934
DEHP	0.5-150	$(6.82 \pm 1.2) \cdot 10^{-3}$	$(3.8 \pm 8.16) \cdot 10^{-2}$	$4.75 \cdot 10^{-2}$	0.9910
DNOP	0.5-150	$(8.67 \pm 0.76) \cdot 10^{-3}$	$(4 \pm 6.08) \cdot 10^{-2}$	$4.47 \cdot 10^{-2}$	0.9942

b: slope; S_b: standard deviation of the slope; a: intercept; S_a: standard deviation of the intercept; R^2: determination coefficient; $s_{y/x}$: standard deviation of the estimate.

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Table S5.4 Matrix-matched calibration data of the selected PAEs, with method limits of quantification (LOQ) and matrix effect (ME) percentage in mackerel and squid.

Matrix (species)	Analyte	Studied linear range (ng g^{-1})	Regression equation (n=8)		$s_{y/x}$	R^2	LOQ (ng g^{-1})*	ME (%)**
			$b \pm s_b \cdot t_{(0.05;6)}$**	$a \pm s_a \cdot t_{(0.05;6)}$				
Atlantic chub mackerel (*Scomber colias*)	DMP	-	-	-	-	-	-	-
	DEP	1-150	$(7.47 \pm 0.7) \cdot 10^{-3}$	$(0.34 \pm 4.89) \cdot 10^{-2}$	$3.32 \cdot 10^{-2}$	0.9954	15	28
	DIBP	0.5-150	$(6.88 \pm 0.58) \cdot 10^{-3}$	$(-1.87 \pm 4.36) \cdot 10^{-2}$	$3.81 \cdot 10^{-2}$	0.9928	10	18
	DBP	0.5-150	$(8.15 \pm 0.8) \cdot 10^{-3}$	$(-1.61 \pm 5.98) \cdot 10^{-2}$	$5.21 \cdot 10^{-2}$	0.9904	15	12
	BBP	1-150	$(3.07 \pm 0.24) \cdot 10^{-3}$	$(0.32 \pm 1.94) \cdot 10^{-2}$	$1.43 \cdot 10^{-2}$	0.9953	20	-29
	DEHP	0.5-150	$(4.76 \pm 0.33) \cdot 10^{-3}$	$(0.49 \pm 2.63) \cdot 10^{-2}$	$1.93 \cdot 10^{-2}$	0.9964	15	-30
	DNOP	0.5-150	$(7.35 \pm 0.67) \cdot 10^{-3}$	$(-0.76 \pm 4.99) \cdot 10^{-2}$	$4.35 \cdot 10^{-2}$	0.9918	15	-15
Blue jack mackerel (*Trachurus picturatus*)	DMP	0.5-150	$(5.17 \pm 0.5) \cdot 10^{-3}$	$(1.03 \pm 3.72) \cdot 10^{-2}$	$3.04 \cdot 10^{-2}$	0.9930	10	50
	DEP	0.5-150	$(5.45 \pm 0.2) \cdot 10^{-3}$	$(-1.1 \pm 1.52) \cdot 10^{-2}$	$1.32 \cdot 10^{-2}$	0.9986	10	-7
	DIBP	0.5-150	$(6.66 \pm 0.37) \cdot 10^{-3}$	$(2.78 \pm 2.73) \cdot 10^{-2}$	$2.38 \cdot 10^{-2}$	0.9970	10	14
	DBP	-	-	-	-	-	-	-
	BBP	0.5-150	$(2.9 \pm 0.13) \cdot 10^{-3}$	$(0.15 \pm 0.95) \cdot 10^{-2}$	$0.83 \cdot 10^{-2}$	0.9981	20	-33
	DEHP	0.5-150	$(5.02 \pm 0.73) \cdot 10^{-3}$	$(0.97 \pm 6.86) \cdot 10^{-2}$	$3.13 \cdot 10^{-2}$	0.9937	15	-26
	DNOP	0.5-150	$(6.5 \pm 0.6) \cdot 10^{-3}$	$(1.24 \pm 4.46) \cdot 10^{-2}$	$3.89 \cdot 10^{-2}$	0.9916	10	-25
European squid or common squid (*Loligo vulgaris*)	DMP	0.5-150	$(1.8 \pm 0.13) \cdot 10^{-3}$	$(0.3 \pm 0.91) \cdot 10^{-2}$	$0.65 \cdot 10^{-2}$	0.9972	10	-48
	DEP	0.5-150	$(5.06 \pm 0.34) \cdot 10^{-3}$	$(0.18 \pm 2.56) \cdot 10^{-2}$	$2.23 \cdot 10^{-2}$	0.9954	10	-13
	DIBP	0.5-150	$(7.05 \pm 0.46) \cdot 10^{-3}$	$(0.99 \pm 3.43) \cdot 10^{-2}$	$2.81 \cdot 10^{-2}$	0.9968	5	21
	DBP	0.5-150	$(7.75 \pm 0.51) \cdot 10^{-3}$	$(2.39 \pm 3.81) \cdot 10^{-2}$	$3.33 \cdot 10^{-2}$	0.9957	15	7
	BBP	0.5-150	$(2.56 \pm 0.14) \cdot 10^{-3}$	$(1.15 \pm 0.91) \cdot 10^{-2}$	$0.75 \cdot 10^{-2}$	0.9977	20	-41
	DEHP	0.5-150	$(5.56 \pm 0.38) \cdot 10^{-3}$	$(1.27 \pm 3) \cdot 10^{-2}$	$2.21 \cdot 10^{-2}$	0.9965	15	-19
	DNOP	0.5-150	$(5.53 \pm 0.33) \cdot 10^{-3}$	$(1.94 \pm 2.1) \cdot 10^{-2}$	$1.74 \cdot 10^{-2}$	0.9974	5	-36

Neon flying squid (*Ommastrephes caroli*)	DMP	0.5-150	$(2.09 \pm 0.34) \cdot 10^{-3}$	$(1.13 \pm 2.32) \cdot 10^{-2}$	$1.36 \cdot 10^{-2}$	0.9922	10	-39
	DEP	0.5-150	$(5.27 \pm 0.19) \cdot 10^{-3}$	$(1.02 \pm 1.55) \cdot 10^{-2}$	$1.15 \cdot 10^{-2}$	0.9990	5	-10
	DIBP	0.5-150	$(6.28 \pm 0.49) \cdot 10^{-3}$	$(2.88 \pm 3.91) \cdot 10^{-2}$	$2.9 \cdot 10^{-2}$	0.9954	5	8
	DBP	-	-	-	-	-	-	-
	BBP	0.5-150	$(2.58 \pm 0.27) \cdot 10^{-3}$	$(0.57 \pm 2.18) \cdot 10^{-2}$	$1.47 \cdot 10^{-2}$	0.9943	10	-40
	DEHP	0.5-150	$(5.02 \pm 0.74) \cdot 10^{-3}$	$(0.97 \pm 6.51) \cdot 10^{-2}$	$3.24 \cdot 10^{-2}$	0.9936	10	-26
	DNOP	0.5-150	$(5.57 \pm 0.56) \cdot 10^{-3}$	$(-0.47 \pm 4.48) \cdot 10^{-2}$	$3.32 \cdot 10^{-2}$	0.9924	5	-36
Orangeback flying squid (*Sthenoteuthis pteropus*)	DMP	0.5-150	$(3.49 \pm 0.27) \cdot 10^{-3}$	$(2.39 \pm 1.98) \cdot 10^{-2}$	$1.73 \cdot 10^{-2}$	0.9942	10	2
	DEP	0.5-150	$(4.37 \pm 0.31) \cdot 10^{-3}$	$(1.24 \pm 2) \cdot 10^{-2}$	$1.66 \cdot 10^{-2}$	0.9962	5	-25
	DIBP	0.5-150	$(5.08 \pm 0.43) \cdot 10^{-3}$	$(-2.17 \pm 2.95) \cdot 10^{-2}$	$2.11 \cdot 10^{-2}$	0.9963	10	-13
	DBP	5-150	$(8.1 \pm 1.44) \cdot 10^{-3}$	$(-1.71 \pm 9.53) \cdot 10^{-2}$	$4.62 \cdot 10^{-2}$	0.9908	10	12
	BBP	0.5-150	$(3.36 \pm 0.32) \cdot 10^{-3}$	$(1.66 \pm 2.39) \cdot 10^{-2}$	$2.09 \cdot 10^{-2}$	0.9909	10	-22
	DEHP	0.5-150	$(4.41 \pm 0.43) \cdot 10^{-3}$	$(2.5 \pm 3) \cdot 10^{-2}$	$2.04 \cdot 10^{-2}$	0.9950	10	-35
	DNOP	0.5-150	$(7.65 \pm 0.64) \cdot 10^{-3}$	$(1.19 \pm 4.75) \cdot 10^{-2}$	$4.14 \cdot 10^{-2}$	0.9931	5	-12

b: slope; S_b: standard deviation of the slope; a: intercept; S_a: standard deviation of the intercept; R^2: determination coefficient; $s_{y/x}$: standard deviation of the estimate. *Calculated as the lowest calibration level with S/N>10. **Calculated following the equation used by Kwon et al. (Kwon, Lehotay, & Geis-Asteggiante, 2012). [https://doi.org/10.1016/j.chroma.2012.10.059]

Figure S5.1 Stomach and intestine of *S. colias* after a dissection (illustrated as example, no plastic containers were used in the dissections of specimens in this study).

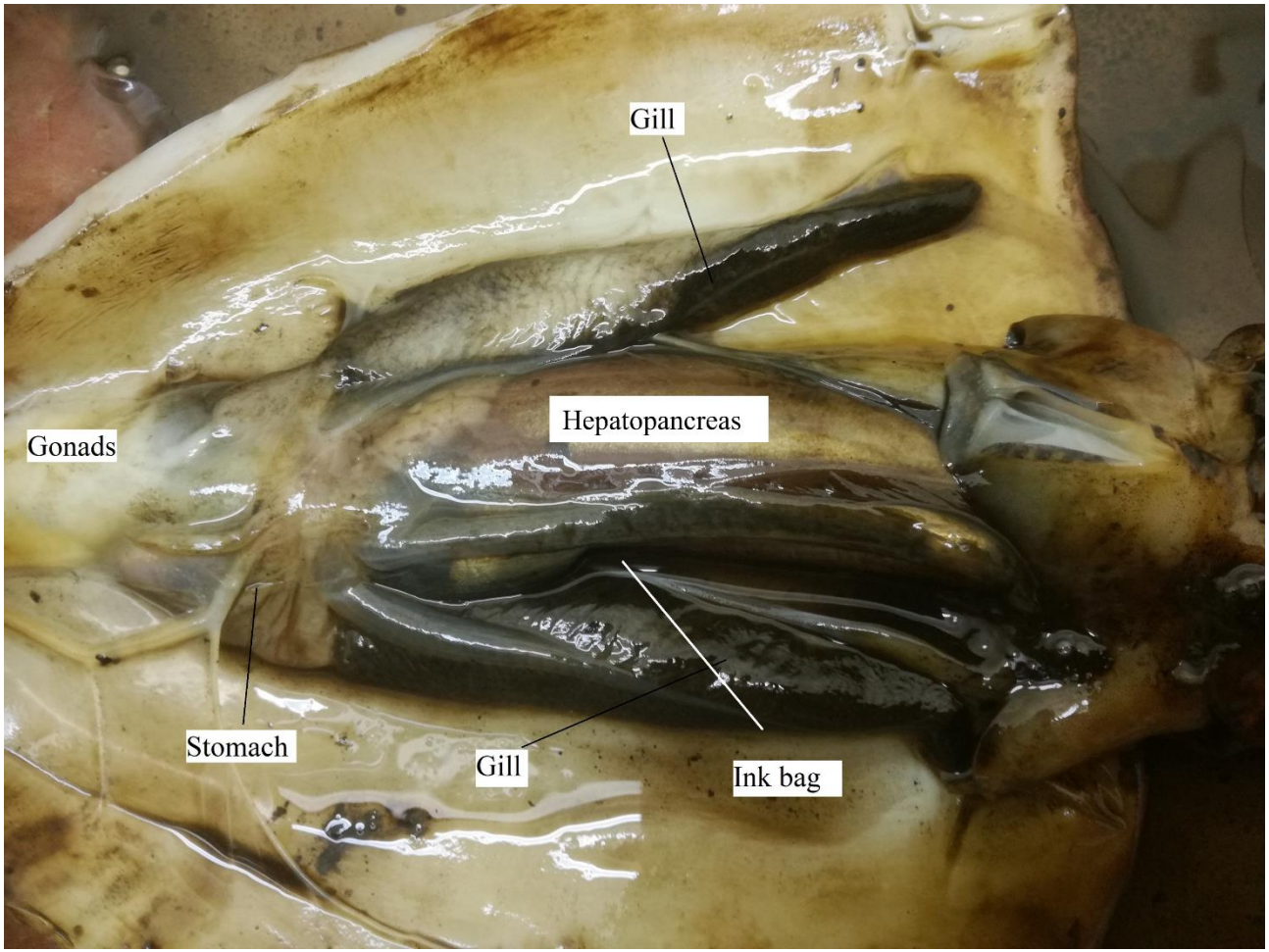

Figure S5.2 Dissection diagram of *O. caroli*, showing the position of stomach, gills and ink sac.

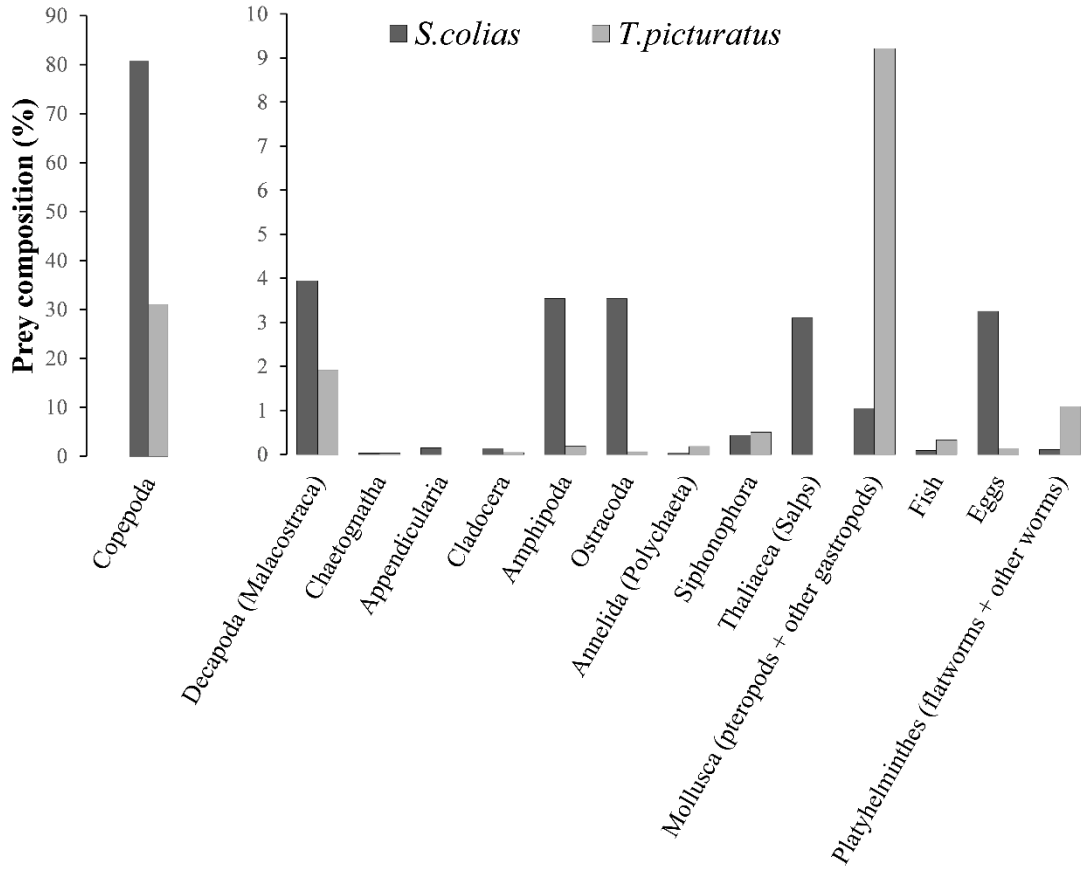

Figure S5.3 Prey composition found in the stomach of *S. colias* and *T. picturatus*. A different scale was used for copepods for visualization purposes.

Table S5.5 Results of Generalized Linear Mixed Models ranked by lowest Akaike Information Criterion (AIC) for the response variable abundance of ingested microplastic (MPs/ind, found in the stomach). Month was included in all models as random factor. Predictor variables were combined based on ecological coherence and non-collinearity of biological parameters (VIF < 5). The best-fitting model, chosen based on the lowest AIC is shown in bold. HSI = Hepatosomatic Index; GSI = Gastrosomatic Index; GIT = Gastrointestinal Tract.

Model predictor variables (Fixed factors)	df	AIC
Season + HSI + GSI	6	344.137
Season + Liver weight + GSI	6	344.54
Season + Species + HSI + GSI	7	346.1166
Species + Season + GSI + Liver weight	7	346.4492
Season + Body Size + Liver weight + GSI	7	346.5339
Season + GSI	5	346.7603
Species + GSI + Liver weight	6	347.3897
Season + Species + Body Size + HSI + GSI	8	348.0713
GSI	4	348.2115
Species + Season + GSI	6	348.6468
Season + Body Size + GSI	6	348.7254
HSI	4	349.7228
Species + GSI	5	350.1686
Species + Body Size + HSI	6	350.6517
Liver weight + GIT weight	5	350.8277
Liver weight	4	351.3228
Body Size + Liver weight	5	352.7512
Season + GIT weight	5	352.7956

Season + Species + GIT weight	6	354.2953
Season	4	370.0124
Season + Species + Total weight	6	371.1081
Total weight	4	371.3752
Body Size	4	371.4039

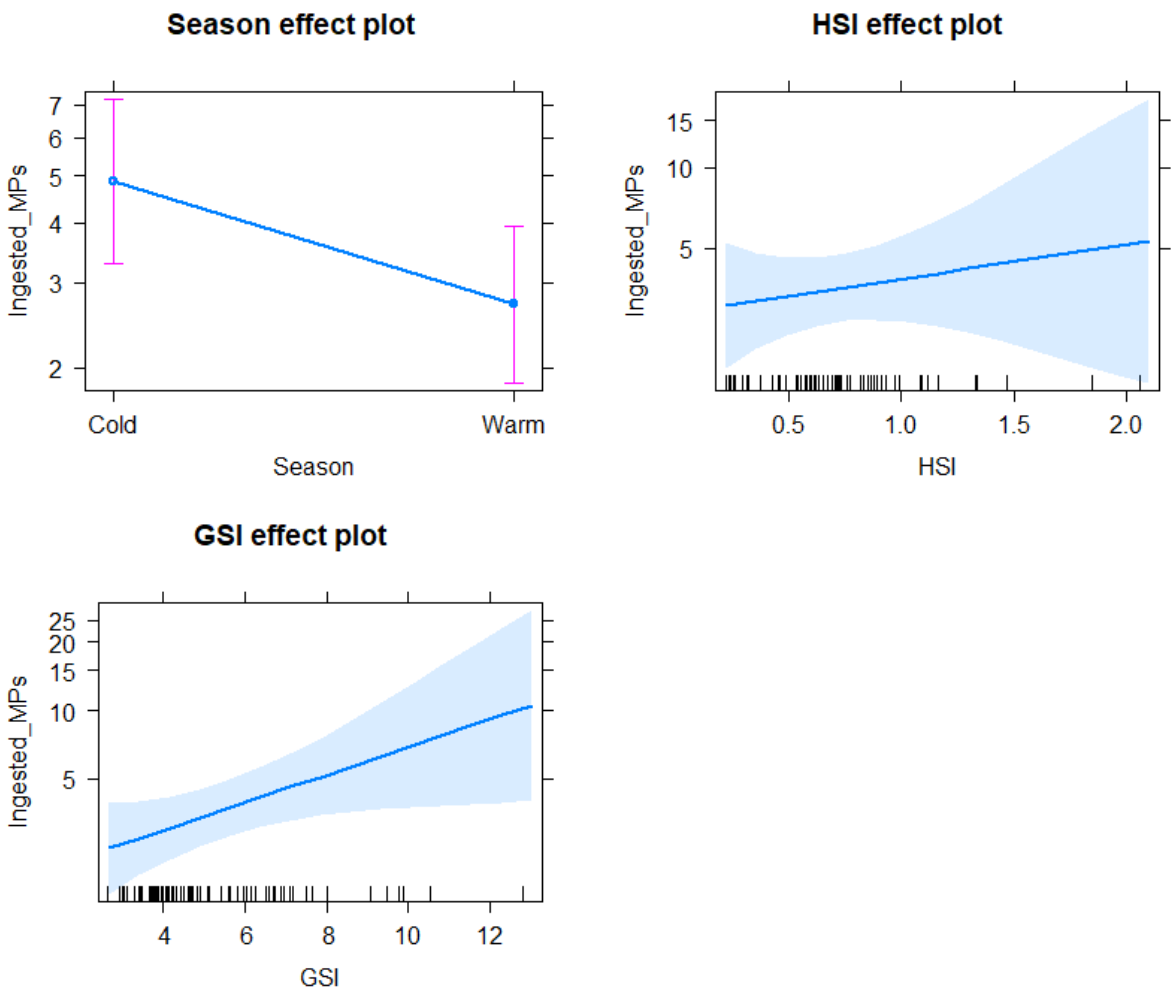

Figure S5.4 Effect plots (with CI 95%) of the predictor factors Season, Gastrosomatic Index (GSI) and Hepatosomatic index (HSI) of the best-fitting Generalized Linear Mixed Model, on the response variable abundance of ingested microplastic (MPs/ind, found in the stomach). Note that only Season and GSI were significant factors.

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Table S5.6 Concentrations of phthalates in the mackerel and squid samples analysed.

Matrix (species)	Sample	Analytes (ng g^{-1}) wet weight							Analytes (ng g^{-1}) dry weight						
		DMP	DEP	DIBP	DBP	BBP	DEHP	DNOP	DMP	DEP	DIBP	DBP	BBP	DEHP	DNOP
Atlantic chub mackerel (*Scomber colias*)	Sc 05	-	< LOQ	18.65 ± 0.19	15.52 ± 1.00	n.d.	< LOQ	n.d.	-	< LOQ	60.47 ± 0.62	50.32 ± 3.26	n.d.	< LOQ	n.d.
	Sc 18	-	< LOQ	17.69 ± 2.39	16.40 ± 0.20	n.d.	< LOQ	n.d.	-	< LOQ	57.34 ± 7.75	53.17 ± 0.66	n.d.	< LOQ	n.d.
	Sc 31	-	< LOQ	**21.33 ± 6.43**	**20.20 ± 5.93**	n.d.	< LOQ	n.d.	-	< LOQ	**69.16 ± 20.83**	**65.48 ± 19.23**	n.d.	< LOQ	n.d.
	Sc 34	-	< LOQ	**73.11 ± 42.83**	**62.71 ± 40.10**	n.d.	< LOQ	< LOQ	-	< LOQ	**237. 02 ± 138.85**	**203.32 ± 130.01**	n.d.	< LOQ	< LOQ
	Sc 37	-	< LOQ	15.45 ± 2.28	**16.86 ± 9.09**	< LOQ	< LOQ	n.d.	-	< LOQ	50.08 ± 7.38	**54.66 ± 29.46**	< LOQ	< LOQ	n.d.
	Sc 12	-	< LOQ	n.d.	28.15 ± 0.92	< LOQ	n.d.	n.d.	-	< LOQ	n.d.	81.54 ± 2.66	< LOQ	n.d.	n.d.
	Sc 13	-	< LOQ	n.d.	17.06 ± 1.65	n.d.	n.d.	n.d.	-	< LOQ	n.d.	49.42 ± 4.77	n.d.	n.d.	n.d.
	Sc 22	-	< LOQ	n.d.	20.23 ± 0.86	n.d.	n.d.	n.d.	-	< LOQ	n.d.	58.59 ± 2.49	n.d.	n.d.	n.d.
	Sc 25	-	< LOQ	n.d.	**20.19 ± 5.09**	< LOQ	n.d.	n.d.	-	< LOQ	n.d.	**58.47 ± 14.75**	< LOQ	n.d.	n.d.
	Sc 09	-	< LOQ	n.d.	16.75 ± 2.03	n.d.	n.d.	n.d.	-	< LOQ	n.d.	48.52 ± 5.88	n.d.	n.d.	n.d.

Blue jack mackerel (*Trachurus picturatus*)	Tp 9	n.d.	< LOQ	< LOQ	-	n.d.	< LOQ	n.d.	n.d.	< LOQ	< LOQ	-	n.d.	< LOQ	n.d.
	Tp 10	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.
	Tp 18	n.d.	n.d.	n.d.	-	n.d.	**23.95 ± 29.12**	n.d.	n.d.	n.d.	n.d.	-	n.d.	**83.66 ± 101.70**	n.d.
	Tp 20	n.d.	< LOQ	n.d.	-	n.d.	< LOQ	n.d.	n.d.	< LOQ	n.d.	-	n.d.	< LOQ	n.d.
	Tp 22	n.d.	n.d.	n.d.	-	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	-	< LOQ	n.d.	n.d.
	Tp 26	n.d.	n.d.	< LOQ	-	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	-	n.d.	n.d.	n.d.
	Tp 27	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.
	Tp 28	n.d.	< LOQ	n.d.	-	n.d.	< LOQ	n.d.	n.d.	< LOQ	n.d.	-	n.d.	< LOQ	n.d.
	Tp 33	n.d.	< LOQ	< LOQ	-	n.d.	n.d.	n.d.	n.d.	< LOQ	< LOQ	-	n.d.	n.d.	n.d.
	Tp 36	n.d.	n.d.	n.d.	-	n.d.	20.61 ± 3.08	n.d.	n.d.	n.d.	n.d.	-	n.d.	73.23 ± 10.94	n.d.
European squid or common squid (*Loligo vulgaris*)	Lv 37	n.d.	n.d.	n.d.	n.d.	n.d.	19.97 ± 1.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	78.22 ± 4.62	n.d.
	Lv 38	n.d.	n.d.	n.d.	< LOQ	< LOQ	**25.57 ± 12.11**	n.d.	n.d.	n.d.	n.d.	< LOQ	< LOQ	**86.57 ± 41.01**	n.d.
	Lv 41	n.d.	< LOQ	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	< LOQ	n.d.

	Lv 43	n.d.	n.d.	n.d.	< LOQ	< LOQ	30.31 ± 2.05	n.d.	n.d.	n.d.	n.d.	< LOQ	< LOQ	107.96 ± 7.29	n.d.
	Lv 46	n.d.	n.d.	n.d.	< LOQ	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	< LOQ	n.d.
	Lv 49	n.d.	n.d.	n.d.	n.d.	n.d.	19.59 ± 2.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	65.28 ± 9.44	n.d.
	Lv 51	n.d.	n.d.	n.d.	n.d.	n.d.	**19.44 ± 4.27**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**63.91 ± 14.04**	n.d.
	Lv 52	n.d.	n.d.	n.d.	n.d.	n.d.	23.01± 0.71	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	106.12 ± 3.28	n.d.
	Oc 17	< LOQ	n.d.	n.d.	-	30.74 ± 4.34	**37.74 ± 17.30**	n.d.	< LOQ	n.d.	n.d.	-	117.22 ± 16.55	**143.89 ± 65.97**	n.d.
	Oc 21	n.d.	n.d.	n.d.	-	11.85 ± 0.13	21.20 ± 6.27	n.d.	n.d.	n.d.	n.d.	-	57.51 ± 0.62	102.90 ± 30.43	n.d.
	Oc 23	n.d.	n.d.	n.d.	-	< LOQ	< LOQ	n.d.	n.d.	n.d.	n.d.	-	< LOQ	< LOQ	n.d.
Neon flying squid (*Ommastrephes caroli*)	Oc 24	n.d.	n.d.	n.d.	-	< LOQ	12.28 ± 0.87	n.d.	n.d.	n.d.	n.d.	-	< LOQ	36.85 ± 2.62	n.d.
	Oc 27	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.
	Oc 28	n.d.	n.d.	n.d.	-	< LOQ	**39.74 ± 12.34**	n.d.	n.d.	n.d.	n.d.	-	< LOQ	**149.20 ± 46.31**	n.d.
	Oc 31	n.d.	n.d.	n.d.	-	24.95 ± 0.84	34.49 ± 5.39	n.d.	n.d.	n.d.	n.d.	-	119.09 ± 4.02	164.65 ± 25.75	n.d.
	Oc 32	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	-	n.d.	< LOQ	n.d.

	Sp 15	n.d.	n.d.	20.12 ± 3.69	**12.12 ± 6.33**	n.d.	n.d.	n.d.	n.d.	n.d.	70.78 ± 12.96	**42.62 ± 22.26**	n.d.	n.d.	n.d.
Orangeback flying squid (*Sthenoteuthis pteropus*)	Sp 16	n.d.	n.d.	19.53 ± 0.56	< LOQ	n.d.	15.47 ± 0.40	n.d.	n.d.	n.d.	80.74 ± 2.32	< LOQ	n.d.	63.97 ± 1.66	n.d.
	Sp 34	n.d.	n.d.	25.63 ± 3.52	16.98 ± 0.46	n.d.	15.09 ± 0.46	n.d.	n.d.	n.d.	106.20± 14.60	70.32 ± 12.86	n.d.	62.51 ± 1.90	n.d.
	Sp 35	n.d.	n.d.	15.80 ± 1.20	n.d.	n.d.	11.78 ± 0.55	n.d.	n.d.	n.d.	69.36 ± 5.27	n.d.	n.d.	51.70 ± 2.41	n.d.

< LOQ : below the limits of quantification, considered for statistical analysis as ½ of the LOQ value
n.d.: not detected (no visible peak)
Values in bold have RSD > 20% and were not considered for statistical analysis

APPENDIX D - Supplementary Data to Chapter 6

Appendix D consists of the Supplementary Data from the following submitted manuscript:

Sambolino, Annalisa, Filipe Alves, Marta Rodriguez, Mieke Weyn, Rita Ferreira, Ana M. Correia, Massimiliano Rosso, Manfred Kaufmann, Nereida Cordeiro and Ana Dinis. (submitted September 2023). 'Phthalates and Fatty Acid Markers in Free-ranging Cetaceans from an Insular Oceanic Region: Ecological Niches as Drivers of Contamination'. *Environmental Pollution* (*ENVPOL-D-23-06966).*

APPENDIX D - Supplementary Data to Chapter 6

Table S6.1 Collected samples and ecological information of the short-finned pilot whales (*Globicephala macrorhynchus*, n=45) and common bottlenose dolphins (*Tursiops truncatus*, n=39) used for fatty acid analysis (first section) and PAEs (phthalate esters) analysis (second section). See Materials and Methods of Chapter 6 for the criteria used in the 'Residency pattern'.

	Pilot whales					Bottlenose dolphins			
	ID	**Date of collection**	**Season**	**Sex**	**Residency pattern**	**ID**	**Date of collection**	**Season**	**Sex**
Fatty acids analysis	Gma02	03/11/2017	Autumn 2017	F	Non-resident	Tt04	15/11/2017	Autumn 2017	F
	Gma04	03/11/2017	Autumn 2017	F	Non-resident	Tt05	15/11/2017	Autumn 2017	F
	Gma06	06/11/2017	Autumn 2017	F	Non-resident	Tt06	15/11/2017	Autumn 2017	F
	Gma07	06/11/2017	Autumn 2017	M	Resident	Tt07	15/11/2017	Autumn 2017	M
	Gma08	13/11/2017	Autumn 2017	F	Non-resident	Tt08	15/11/2017	Autumn 2017	F
	Gma09	13/11/2017	Autumn 2017	M	Non-resident	Tt09	15/11/2017	Autumn 2017	M
	Gma12	17/11/2017	Autumn 2017	F	Non-resident	Tt10	15/11/2017	Autumn 2017	F
	Gma13	17/11/2017	Autumn 2017	M	Non-resident	Tt12	17/11/2017	Autumn 2017	F
	Gma14	18/11/2017	Autumn 2017	M	Resident	Tt13	17/11/2017	Autumn 2017	M
	Gma17	18/11/2017	Autumn 2017	F	Resident	Tt14	17/11/2017	Autumn 2017	F
	Gma18	20/03/2018	Spring 2018	F	Non-resident	Tt15	19/11/2017	Autumn 2017	M
	Gma19	20/03/2018	Spring 2018	M	Non-resident	Tt16	20/11/2017	Autumn 2017	F
	Gma20	20/03/2018	Spring 2018	F	Non-resident	Tt18	16/03/2018	Spring 2018	F
	Gma21	20/03/2018	Spring 2018	F	Non-resident	Tt19	22/03/2018	Spring 2018	M
	Gma23	23/03/2018	Spring 2018	M	Non-resident	Tt20	22/03/2018	Spring 2018	M
	Gma25	18/04/2018	Spring 2018	M	Resident	Tt21	22/03/2018	Spring 2018	M
	Gma27	21/05/2018	Spring 2018	M	Resident	Tt22	22/03/2018	Spring 2018	F

	Gma28	21/05/2018	Spring 2018	F	Resident	Tt23	22/03/2018	Spring 2018	M
	Gma29	21/05/2018	Spring 2018	M	Resident	Tt25	22/03/2018	Spring 2018	F
	Gma31	28/09/2018	Autumn 2018	F	Non-resident	Tt27	16/04/2018	Spring 2018	F
	Gma32	28/09/2018	Autumn 2018	F	Non-resident	Tt28	16/04/2018	Spring 2018	F
	Gma33	28/09/2018	Autumn 2018	F	Non-resident	Tt29	16/04/2018	Spring 2018	F
	Gma35	28/09/2018	Autumn 2018	F	Non-resident	Tt31	16/04/2018	Spring 2018	M
	Gma36	29/09/2018	Autumn 2018	F	Resident	Tt33	17/04/2018	Spring 2018	F
	Gma37	29/09/2018	Autumn 2018	M	Non-resident	Tt34	17/04/2018	Spring 2018	M
	Gma39	02/10/2018	Autumn 2018	M	Non-resident	Tt36	17/04/2018	Spring 2018	M
	Gma40	04/10/2018	Autumn 2018	F	Resident	Tt37	26/04/2018	Spring 2018	M
	Gma41	04/10/2018	Autumn 2018	M	Resident	Tt39	01/06/2018	Summer 2018	F
	Gma43	05/10/2018	Autumn 2018	F	Non-resident	Tt42	31/07/2018	Summer 2018	F
	Gma44	05/10/2018	Autumn 2018	M	Resident	Tt43	31/07/2018	Summer 2018	M
	Gma46	10/01/2019	-	F	Resident	Tt02	14/11/2017	-	M
	Gma47	07/02/2019	-	F	Resident	Tt03	15/11/2017	-	M
	Gma50	11/10/2019	-	F	Non-resident	Tt17	20/11/2017	-	M
	Gma51	16/10/2019	-	F	Non-resident	Tt47	25/07/2019	-	M
PAEs analysis	Gma52	16/10/2019	-	M	Non-resident	Tt48	25/07/2019	-	M
	Gma54	19/11/2019	-	F	NA	Tt49	18/09/2019	-	F
	Gma56	28/10/2020	-	M	Resident	Tt50	18/09/2019	-	M
	Gma57	09/11/2020	-	F	Non-resident	Tt51	16/10/2019	-	F
	Gma58	12/02/2021	-	F	Resident	Tt52	16/10/2019	-	M

Gma59	21/04/2021	-	F	Resident
Gma60	21/06/2021	-	F	NA
Gma61	25/08/2021	-	F	NA
Gma62	02/09/2021	-	M	Non-resident
Gma63	19/10/2021	-	F	NA
Gma64	03/11/2022	-	F	Non-resident

APPENDIX D - Supplementary Data to Chapter 6

Table S6.2 Results of the phthalates (PAEs) analysis of cetacean blubber samples of short-finned pilot whales (*Globicephala macrorhynchus*, n=15) and common bottlenose dolphins (*Tursiops truncatus*, n=9).

Matrix (species)	Sample	Analyte (ng g^{-1}) wet weight						
		DMP	**DEP**	**DIBP**	**DBP**	**BBP**	**DEHP**	**DNOP**
Pilot whales	Gma46	n.d.	66.53 ± 27.47	n.d.	n.d.	<LOQ	n.d.	n.d.
	Gma47	18.56 ± 1.53	30.18 ± 3.55	7.38 ± 1.88	281.95 ± 9.45	<LOQ	93.63 ± 15.96	<LOQ
	Gma50	<LOQ	37.82 ± 0.58	n.d.	210.27 ± 8.15	<LOQ	74.25 ± 6.67	n.d.
	Gma51	14.94 ± 2.1	13.48 ± 4.24	n.d.	n.d.	n.d.	n.d.	n.d.
	Gma52	n.d.	<LOQ	n.d.	88.98 ± 8.58	<LOQ	28.76 ± 13.98	n.d.
	Gma54	<LOQ	27.89 ± 3.74	n.d.	66.28 ± 7.64	<LOQ	n.d.	13.37 ± 2.83
	Gma56	<LOQ	93.24 ± 4.6	n.d.	116.76 ± 8.01	<LOQ	29.5 ± 3.91	n.d.
	Gma57	n.d.	404.3 ± 7.23	n.d.	172.48 ± 5.91	<LOQ	n.d.	n.d.
	Gma58	17.73 ± 0.58	63.12 ± 2.14	n.d.	39.76 ± 2.56	<LOQ	n.d.	n.d.
	Gma59	<LOQ	60.25 ± 4.31	n.d.	148.1 ± 5.45	n.d.	n.d.	n.d.
	Gma60	14.47 ± 0.52	69.56 ± 7.04	n.d.	121.58 ± 2.39	10.41 ± 0.87	n.d.	n.d.
	Gma61	12.82 ± 0.84	33.11 ± 6.17	19.41 ± 0.44	284.11 ± 1.77	<LOQ	14.46 ± 10.59	n.d.
	Gma62	25.98 ± 0.86	18.6 ± 1.63	n.d.	212.4 ± 10.68	<LOQ	34.95 ± 9.42	n.d.
	Gma63	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
	Gma64	n.d.	<LOQ	n.d.	258.38 ± 3.8	<LOQ	n.d.	n.d.

Bottlenose dolphins	Tt02	n.d.	n.d.	n.d.	n.d.	15.46 ± 2.38	295.56 ± 8.28	n.d.
	Tt03	n.d.	38.94 ± 3.08	n.d.	n.d.	<LOQ	127.3 ± 23.71	n.d.
	Tt17	n.d.	n.d.	n.d.	40.1 ± 3.13	11.32 ± 3.12	254.99 ± 19.13	n.d.
	Tt47	n.d.	n.d.	n.d.	n.d.	<LOQ	163.49 ± 9.99	n.d.
	Tt48	n.d.	9.99 ± 4.7	28.01 ± 13.18	134.21 ± 11.89	21.85 ± 2.57	517.77 ± 5.78	n.d.
	Tt49	n.d.	n.d.	57.82 ± 3.72	174.89 ± 1.51	57.11 ± 3.02	4697.34 ± 113.45	n.d.
	Tt50	n.d.	n.d.	n.d.	47.9 ± 17.88	14.99 ± 1.93	318.91 ± 3.72	n.d.
	Tt51	n.d.	n.d.	n.d.	n.d.	<LOQ	180.79 ± 14.25	n.d.
	Tt 52	n.d.	27.19 ± 8.48	92.7 ± 22.21	717.59 ± 36.51	19.85 ± 1.82	446.97 ± 19.82	n.d.

< LOQ: below the limits of quantification, ½ of the LOQ value was considered for statistical analysis; n.d.: analyte not detected (below limit of detection); values in bold have RSD > 20%