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Drivers and effects of plastic contamination in aquatic ecosystems

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Table of contents

Summary

Marine litter indicates any solid material manufactured or processed by man, which is discarded or disposed in the marine environment after its use. Marine litter is documented across all oceans, with apparently no bathymetric or geographical boundary. The most prevalent sort of marine litter is plastic, which is found in all aquatic ecosystems, even in remote areas far from human activities. Anthropogenic litter enters the marine environment from various sources such as: sea-based (e.g., vessel-traffic and fisheries), or from land-based (e.g., coastal tourism or river run-off), in which rivers play a major role in its transport and transfer in other water bodies such as lakes and oceans. The seafloor has been recognized as the major sink for marine debris and the study of litter accumulation dynamics represents a fundamental tool to evaluate possible removal actions. Plastic already dispersed in the marine environment undergoes through environmental degradation and is further fragmented into smaller particles which called MicroPlastics (MPs), with a dimension comprised between 1 μm and 5 mm, that currently represent one of the most ubiquitous, persistent, yet poorly understood contaminant in terms of exposure and contamination to *biota*, as well as possible effects to organisms.

To cope with the paucity of information on a very wide and complex topic that is marine litter and MP contamination, the present thesis encompasses different contaminants and environments: from seafloor macro-litter (Chapter 2) to MPs (Chapter 3 and 4), and from the marine benthic environment and relative biota (Chapter 2 and 3) to freshwater environment and biota (Chapter 4).

More in detail, this thesis aims to analyse the spatial and temporal variability of seafloor macro-litter in the Food and Agriculture Organization (FAO) Geographical Sub-Area 11 (Sardinian seas, western Mediterranean) and to test whether different litter categories may result

in geographically segregated seafloor litter hotspots. Analyses are based on data on different litter categories (plastic, rubber, metal, glass, cloth, wood, paper, other) collected over a 7 years period, in the framework of the MEDiterranean International Trawl Survey. Results show the absence of any temporal trend in seafloor macro-litter density and weight, but rather indicate a spatial and bathymetric segregation of different litter categories. The data shows how different sources and physical features of macro-litter items (*i.e.*, plastic and fishing gear, rubber, glass, metals, and cloth) led to spatially segregated accumulation hotspots. The study points out how the identification of seafloor macro-litter hotspots using aggregated data that include plastic items could obscure the identification of other segregated but yet relevant hotspots of other macro-litter categories accumulated in the marine environment (Chapter 2).

Accumulation hotspots, moreover, can be source of MPs that can eventually contaminate benthic fauna. In Chapter 3, the exposure to MPs was evaluated using economically and ecologically key crustaceans *Palinurus elephas* (Fabricius, 1787) and *Nephrops norvegicus* (Linnaeus, 1758) collected from sites located on the western coast of Sardinia, at depths comprised between 25 and 660 m was investigated. All specimens contained MPs (100% occurrence), with *P. elephas* being significantly more contaminated (9.1 ± 1.75 vs. 3.2 ± 0.45 MPs individual^{-1}), ingesting larger MPs with different polymeric composition. The results suggest that both *P. elephas and N. norvegicus* could be valuable bioindicators and charismatic species that could represent excellent flagship species for raising awareness toward the global issue of plastic in the marine environment (Chapter 3).

Finally, moving to freshwater environments, the last chapter is a study looking at the effects of microplastic pollution on fish behaviour. This part of the thesis was developed and conducted during my stay abroad in the Institute of Hydrobiology, Biology Centre CAS, České Budějovice, Czech Republic. The topic is extremely underexplored and if plastic ingestion is documented in a multitude of organisms, potential effects and even less effects on organisms' behaviour are very far from being understood. The target species were the native crucian carp (*Carassius carassius*) and invasive gibel carp (*Carassius gibelio*), that were exposed to MPs via contaminated food pellets (using polyethylene or tire wear particles) and compared to a control group for 60 days. Then, differences in behavioural changes and swimming capacity were tested. The results show that exposure to MPs affected fish behaviour, and in particular the native species changed its behaviour toward bolder levels, potentially exposing them to higher predation rates in the wild. (Chapter 4).

The thesis further emphasizes that plastic pollution, an ever-arising challenging issue to all environmentalists needs to be taken care of. The study highlights the smaller yet dense hotspots of the accumulated plastic litter along the Sardinian coasts due to various factors such as oceanic currents and geomorphology. Contributing to the diverse scientific literature for the ingestion of the MPs by several species, here in our study the decapod crustaceans such as *P. elephas* and *N.norvegicus* share a similar feeding habitat that is scavenging activity and digestion. This common feeding habitat of the species suggests that they could be potential bioindicators for the MP contamination. Lastly, the pollutants such as polyethylene and tire wear particles in their sub-lethal doses of exposure to the fish exhibited behavioural changes after two-month treatment eventually impact the species interactions. All these results contribute to the present literature and set an example for the toxicity experiments wherein the higher does could be tested for interactions with other organisms especially with predators. Further, the mitigation actions could be the foundation for implementing more skilled and advanced management of the macro litter, especially plastic.

Chapter 1: General Introduction

1.1 Overview of Marine litter

Marine litter is increasing worldwide and is considered to be an emerging issue threatening marine biodiversity (Barnes et al., 2009). The United Nations Environment Program (UNEP) defines Marine litter as ‘‘any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment’’. Its major component is plastic, in all its forms, as it has become an integral part of daily life in the decades because of its profoundly adaptable nature (Ryan, 2015; Spedicato et al., 2019). Once in the marine environment, plastic causes a wide variety of negative impacts: from mere aesthetic degradation of beaches and marine habitats to the interaction with *biota*, which is documented to cause severe impacts at many trophic levels (Derraik, 2002; Laist, 1987). According to UNEP by 2050, there will be more plastic in the oceans than fish (by weight) (Xu et al., 2020). Consequences of the extent of this ‘plastic era’ can be observed on multiple scales, and many approaches to address these issues are just beginning to be developed (Deudero and Alomar, 2015). Despite marine litter and plastic litter are used almost as synonyms, marine litter encompasses a much wider range of categories: glass, paper, cardboard, metal, cloth, rubber, fishing-related waste, wood and sewage-related litter (Deudero and Alomar, 2015). Despite the widespread recognition of the problem, evidence suggests that plastic pollution in the marine environment is ever increasing (Moore, 2008; Ryan et al., 2009).

1.2 Origin of plastic waste in water bodies

The sources of these contaminants are divided into two major categories: sea-based and land-based sources. (Eryaşar et al., 2014). Land-based debris comprises all different kinds of anthropic items derived from touristic, agricultural, industrial and domestic activities (Galgani et al., 2000; Moore and Allen, 2000). It is broadly accepted that land-based sources account for 78 % of marine litter in the world's oceans, while marine-based debris, accounts for 22 % (ship traffic recreational, commercial and occupational, aquaculture facilities and energy production) (Alvito et al., 2018; Ruiz et al., 2022). Rivers plays an major role in transport of plastic litter from land-to-ocean. (Castro-jiménez et al., 2019). Direct dumping at riverbanks, runoff from urban and rural areas, and effluents from sewage facilities are some potential sources of riverine litter (Rech et al., 2014). At this stage, atmospheric and natural climate factors including storms, flooding events, wind, wave motion, and currents lead to their growth, further transportation, and eventually deposition on the seabed (Ryan et al., 2009).

1.3 Mediterranean Sea polluted by Macro litter

The Mediterranean Sea has unique geology, biogeographic, physical, and biological traits that make it a hotspot for biodiversity (Coll et al., 2010). The Mediterranean Sea is considered as a predominately complex ecosystem because of its higher biodiversity which is being impacted by anthropogenic pressure. Mediterranean is highly anthropized along its coasts, receiving more than 25% of the global tourism, being home to 7% of the worlds' coastal human population and with 30% of the worldwide maritime transport navigating through its waters (UNEP, 2009). All this contributes to eutrophication, habitat loss, accelerated invasive species, contamination, overfishing among other things (Coll et al., 2010). In this basin marine litter have become a real threat for marine ecosystem over the years and higher concentrations of marine litter have been detected here as compared to other oceans (Cózar et al., 2015). The semi-enclosed nature of the basin, its low water exchange rate with adjacent seas and oceans, and its oceanographic regimes all favours these high concentrations of marine litter (Cózar et al., 2015). Approximately 6.4 million tonnes of litter enters the ocean of which 62 million macrolitter items currently floating on the surface of the entire Mediterranean basin (Deudero and Alomar, 2015; Suaria and Aliani, 2014). According to Pham et al. (2014) plastics are the most prevalent litter items found on deep sea floors in the Mediterranean Sea, out of which 82% of all man-made floating items are of plastics (Suaria and Aliani, 2014).

The first studies on the distribution and abundance of marine litter in the northwest Mediterranean Sea date back to the 1990s, about 924 pieces of litter were collected around Marseille (Galgani et al., 1995), and up to 100,000 items of marine litter/km^{-2} were measured in the continental shelf of the Ligurian Sea (Galgani et al., 2000). The marine litter in the deep Mediterranean Sea ranges from the 97 items of marine litter/km^{-2} from Maltese islands (Mifsud et al., 2013). The central Mediterranean Sea, has an abundance of 1.2 items/10 m to 13.2 items/10 m at a depth of around 300 m to 520 m of sea level. (Pierdomenico et al., 2019). The presence of marine litter within the catch is usually high in the northern areas of the Mediterranean Sea while decreasing in the southern side, while at the industrial section it was found to be on the opposite trend where the northern location faced the least amount of marketable products (Galimany et al., 2019). Hotspots of the marine litter were identified mainly in the western and eastern parts of the sea (Spedicato et al., 2019). With respect to Sardinia, available literature reports how a total of 918 items were collected in three years survey in the Mediterranean basin such as Sardinia in which plastic was the dominant component of litter 35.15 ± 3.89 items km^{-2} which confirms the ubiquity of anthropic benthic litter found in the marine environment even within a relatively low-populated zone (Alvito et al., 2018). Cau et al. (2018) confirmed that anthropogenic waste had reached the deep Mediterranean Sea the density ranged more than 1300 litter items per km^{-2}, and plastic items are consistently the most abundant category of marine litter found in the deep sea.

Given that the Mediterranean Sea harbours between 4-25% of the world's marine species (Coll et al., 2010), plastic pollution in conjunction with all the anthropogenic impacts present in the area (i.e., fishing, habitat loss, climate change) must be regarded as a particularly concerning threat to the marine wildlife of the area.

1.4 Plastic pollution- A brief history

Plastic has changed the way we live. Due to its durability, multi-function and affordability, plastic had become extremely popular for everyday use (Andrady and Neal, 2009). The question of, why plastic? It makes the people to think about the need for plastic, whether it is needed or to be eradicated. Plastic can be produced at a low cost, and there are countless applications, including construction materials, apparel, household, personal goods, and packaging etc. Plastic production worldwide resulted in the generation of 6300 million metric tons of waste as of 2015, out of which 9% is recycled, 12% incinerated and 79% is dispersed in the natural environment (Geyer et al., 2017; Robin et al., 2020). Due to the increasing global production and use have led the accumulation of plastics in the environment. The majority of plastics produced are used in single use applications such as packaging (40% of the demand for plastic in Europe; Plastics Europe, 2015). It includes plastic bags, microbeads, cutlery, straws, polystyrene such as cups, food containers, and sachet water wrappers (Xanthos and Walker, 2017). Nearly 25% of single use plastic is estimated to be reduced to safeguard pollution in upcoming years. This is to be eradicated by producing recycled plastics and eliminating single use plastic. In marine environment millions of tonnes of plastic waste ends up and due to their low density, they are readily dispersed by currents and wind, sometimes travelling thousands of kilometres (Jambeck et al., 2015; Ryan et al., 2009).

1.5 Plastic degradation in the environment

As a rule, widely used plastics do not naturally degrade to a large degree when released into the environment (Marqués-Calvo et al., 2006; Zheng et al., 2005). This may not come as a surprise given that many polymers are incredibly stable and durable, which is one of the main reasons for their popularity and extensive use (Yamada-Onodera et al., 2001). Plastics deteriorate in the environment via four processes: photodegradation, thermooxidative degradation, hydrolytic degradation and biodegradation by microorganisms (Andrady, 2011). Generally, the natural degradation of plastic begins with photodegradation, which leads to thermos oxidative degradation (Webb et al., 2013). Ultraviolet radiation from the sun provides the activation energy necessary to start the incorporation of oxygen atoms into the polymer (Raquez et al., 2011). This causes the plastic to become brittle and to break into smaller pieces until the polymer chains reach sufficiently low molecular weight to be metabolized by microorganisms (Zheng et al., 2005). These microbes either convert the carbon in the polymer chains to carbon dioxide or incorporate it into biomolecules (Andrady, 2011). While the mechanisms of weathering and degradation are the same in the marine environment as on land, the rate at which they proceed in the former can be significantly slower than in the latter (Müller et al., 2001) which leads to their accumulation in the environment for a longer time (Liu et al., 2021).

1.6 Introduction to microplastics

1.6.1 Definition of microplastics

A heterogeneous range of plastics with sizes ranging from 1 μm and 5 mm are referred as microplastics (MPs) (Frias and Nash, 2019).They are divided into four categories such macroplastics (>25 mm), mesoplastics (5–25 mm), microplastics (MPs) (<5 mm) and nanoplastics (<100 nm) (Kim et al., 2015; Lee et al., 2013; Robin et al., 2020). Around the world, MPs have been found in diverse ecosystems (lakes, rivers, seas, etc.), from the bottoms of the oceans to the sediment of land areas that border the water bodies, like beaches and coasts (Galloway and Lewis, 2017). MPs have a heterogeneous array of shapes and sizes, such as spheres, pellets, irregular fragments, filaments, film, foamed plastic, granules and fibres in the marine environment (Hidalgo-Ruz et al., 2012; Wright et al., 2013).

1.6.2. Types and sources of MPs

Several terms have been used to describe and categorize sources of MPs namely, “primary” and “secondary” (Arthur et al., 2009; Lehtiniemi et al., 2018). The primary sources are those in which MPs are produced through extrusion or grinding, either as precursors to other products (Costa et al., 2010) or for direct use (e.g., abrasives in cleaning products or roto-milling). The common everyday practices which emit MPs into domestic sewage are the use of personal care products containing so called microbeads and the washing of synthetic clothing (Browne et al., 2011; Zitko and Hanlon, 1991). The range of primary MPs particle types includes fragments (Lusher et al., 2015), fibres (Rummel et al., 2016), pellets (Nobre et al., 2015), film (Kang et al., 2015) and spheres (Li et al., 2016). Pharmaceutical and cosmetics industries contribute most of the spheres present in the marine ecosystem (Zitko and Hanlon, 1991), whereas “secondary sources” of MPs are those which are formed in the environment from the fragmentation of larger plastic material into smaller pieces (Cashman et al., 2020). Road runoff is described as a “direct source” (sewage or stormwater), whereas fragmentation of existing plastic debris was described as an “indirect source” of MPs to the environment (Browne, 2015). Sewage treatment plants (STP) receive high amounts of MPs originating from urban runoff (sewage system) and domestic sewage in their influent. Because of their small size these MPs are difficult for the STP processes to remove or retain. Sewage treatment facilities are therefore considered a significant source of MPs in freshwater environments (Van Cauwenberghe, 2016). The most common type of MPs is called as synthetic polymers. A few synthetic polymers are polypropylene, acrylic, polyethylene, polyamide and polystyrene (Andrady and Neal, 2009; Boyle and Örmeci, 2020). Out of which polypropylene and polyethylene are comprising 45–50% of the global production (Bobori et al., 2022; Geyer, 2020). Hence polyethylene is the most prevalent polymer found in aquatic habitats (Enders et al., 2015; Limonta et al., 2019; Plastic-Europe, 2006; Rezania et al., 2018).

1.7 Ecological consequences of MPs pollution

Factors affecting the availability of MPs to organisms are manifold (Van Cauwenberghe, 2016). Due to their resemblance to natural foods or considering it as prey items, MPs have been reported to be consumed by variety of organisms (Crawford and Quinn, 2017; Liu et al., 2021; Wang et al., 2019). The uptake of MPs by the organisms depends upon the position of these particles present in the water column which is determined by the plastic's density (Van Cauwenberghe, 2016). Planktivorous and filter feeders found on the upper water column, are more likely to come into contact with positively buoyant MPs. In contrast, as negatively buoyant MPs descend to the ocean floor, they will become available to benthic suspension and deposit feeders.

1.7.1 Microplastics in biota

Microplastics are a multifaceted concoction of polymers that contain chemical additives and that could interact with abiotic and biotic components of the marine environments (Mariano et al., 2021). Numerous laboratory based investigations and field studies report that a wide range of aquatic organisms are susceptible to MPs ingestion, mostly due to accidental ingestion driven by MPs mistaken for preys due to similar morphology and size (Bobori et al., 2022). Early in the 1990s, it was already believed that ingesting plastic particles could harm fish by obstructing their digestive tracts, decreasing their feeding activity (Hoss and Settle, 1990). More findings about the species affected by plastic ingestion have been published, and as of now, the majority of studies in the Mediterranean basin have focused on demersal (32.9%) and pelagic (27.7%) species, followed by benthic (14.7%), benthopelagic (16.5%), neritic (5.3%), and mesopelagic (2.9%) species (Fossi et al., 2018).

Till date there is little information about MPs being consumed by wild decapods in European marine waters. The published data regard the Norway lobster (Cau et al., 2019; Devriese et al., 2017; Martinelli et al., 2021), the brown shrimp (Devriese et al., 2015), the Symi shrimp (Bordbar et al., 2018) and the blue and red shrimp (Carreras-Colom et al., 2018; Cau et al., 2019), the green crab (Acar and Ates, 2018; Piarulli et al., 2019), *Maja squinado* (Welden et al., 2018) and European spiny lobster (Kampouris et al., 2023). The majority of decapods, especially prawns, shrimps, crabs, lobsters, and crayfish, are caught or cultured for food, and as a result, they represent an essential part of the diets of humans living in coastal regions (D'Costa, 2022). However, Decapods caught from areas of high anthropogenic stress can accumulate pollutants to concentrations much higher than those found in the environment (Batvari et al., 2016) thus, posing a severe threat to the humans that consume them. Therefore decapods are suitable for use as model organisms to test the toxicity of various environmental pollutants (Anderson and Phillips, 2016).

Microplastics can be considered as "pervasive environmental pollutants" that are ingested by many organisms (Doyle et al., 2022). It is one of the emerging "global issues" as it directly concerns human consumption (Bhuyan, 2022; Thiele et al., 2021). It was observed that long-term exposure to MPs can also influence ecological dynamics of aquatic communities. Another study by Grøsvik et al. (2022) reviewed MPs present in benthic and pelagic invertebrates from the Arctic. It was suggested that indicator species is needed, which should be used for monitoring MPs at the coastal regions. The meta-analysis comparing the impact of MPs on various taxonomic groups covering crustaceans, bivalves, annelids, gastropods, and insects in terms of growth, reproduction and survival showed that even though there were adverse impacts due to MPs, however, there was no particular pattern to be found (Doyle et al., 2022). In a separate study, Barboza et al. (2020) documented the toxicological aspects of MP ingestion in fish the findings of the study revealed a higher level of oxidative damage in the gills and muscles, along with neurotoxicity in the fish that had higher exposure to MPs.

The toxic effects are also seen in chemicals present in MPs, so that MPs uptake by fish may causes severe damage to changes in immune related gene expression, damage to tissues, and oxidative stress. Fish suffer highly from neurotoxicity, behavioural abnormalities, and growth retention (Au et al., 2015; Besseling et al., 2014; Chen et al., 2020; de Sá et al., 2015; Della Torre et al., 2014; Espinosa et al., 2019; Paul-Pont et al., 2016; Rist et al., 2016; Tang et al., 2018; Xia et al., 2020; Yu et al., 2018).

1.7.2 Effects of MPs on fish behaviour

The exposure of MPs to fish have shown to impact their behaviour Chen et al. (2022) studied the changes in fish behaviour in terms of boldness when zebrafish were exposed to MPs. Due to the aggressive behaviour of the bold zebrafish, these accumulated more MPs compared to the shy ones. In another laboratory-based study, the feeding behaviour of the hybrid group juveniles varied with the types of MPs (Xu and Li, 2021). These fish were able to differentiate MPs as inedible, but it was suggested that dense stocking of the fish can still make them ingest these pollutants. Direct evidence of MP accumulation was reported by Hasegawa and Nakaoka (2021), where the MP exposure route of mysids, *Neomysis* with benthic fish, *Myoxocephalus brandti* in a model of prey and predator was examined. It was found from the results of the trophic transfer experiment that the mass and amount of the MPs ingested is dependent on the source and concentration of plastics. Moreover, the trophic transfer was higher from the prey to the predator than from direct ingestion.

1.8 Tire Wear Particles: The sheath pollutant that's becoming a major threat to the environment

The ongoing discussion about the consequences of plastic waste and MPs in the aquatic environment has brought to light the fact that organic pollutants can enter the water in both dissolved and particulate forms. Research on MPs has mostly focused on thermoplastic material like polyethylene or polystyrene and did not consider elastomers such as rubber (Wagner et al., 2018). According to ISO (ISO, 2013), rubber is not considered a plastic whereas others suggests using a broader definition of MPs that would include all man-made macromolecular materials, including rubber (Verschoor, 2015). Tire wear particles (TWPs) are generated on the road surface, the particles are emitted to the air or instantly intermixed with road wear particles (Thorpe and Harrison, 2008). TWPs have been estimated and identified as potential environmental contaminants, but there is a dearth of information on their occurrence, toxicity, risk, and transit pathways (Wagner et al., 2018). Many ecotoxicological studies on zooplankton, mussels and fish have shown significant effects of TWPs and the leachates on various physiological and behavioural characteristics although only at concentrations exceeding environmentally relevant conditions (Capolupo et al., 2020; Halle et al., 2021; Halsband et al., 2020; Järlskog, 2022; Koski et al., 2021; Marwood et al., 2011). However more monitoring studies and analyses of environmental concentrations in various matrices using comparable and reliable analytical methods are therefore required. According to the precautionary principle, TWPs emissions should be seen as an environmental risk until a thorough assessment has been properly evaluated.

1.8.1 Formation and composition of TWPs

TWPs are formed by the mechanical abrasion of tires on the road, and research shows that these particles account for 5–10% of the microplastic emissions that enter the world's oceans (Hann et al., 2018; Kole et al., 2017). During the lifetime of a tire, approximately 10–20 % of the total mass is worn off resulting in an annual global release of 6000000 tons (Kukutschová et al., 2011; Sugiura et al., 2021; Ziajahromi et al., 2020). It is estimated about 500,000 tonnes TWPs are generated annually in the EU alone (Hann et al., 2018). Around 50–140 thousand tonnes of these TWPs are released into surface waters in the EU each year (Page et al., 2022). Tire tread mainly consists of 40–60% rubber (elastomers), both natural rubber (polyisoprene) and synthetic rubber (mainly polybutadiene and styrene-butadiene-rubber) together with various fillers (e.g., silicon and/or carbon black to increase lifespan, rolling resistance and hardness), it also include variety of tire additives as well, including preservatives (halogenated cyanoalkanes), antioxidants (amines, phenols), desiccants (calcium oxide), plasticizers (aromatic and aliphatic esters) and processing aids (mineral oil, peptone) (Sommer et al., 2018; Sundt et al., 2014; Wagner et al., 2018).

1.8.2 Effects of TWPs

The majority of research has been focused on the toxicity of the leachate component deriving from TWPs. Exposure to TWPs' leachates can cause mortality in freshwater organisms (Gualtieri et al., 2005). One of the most commonly identified components of tire debris leachate is high levels of zinc (Zn), a heavy metal known to be toxic to aquatic organisms (Attar and Maly, 1982; Gualtieri et al., 2005). In fact, it is estimated that up to 36% of Zn released into the atmosphere comes from Tire particles (Councell, 2004). The compounds leaching from tires into water include benzothiazole, aluminium, 1-indanone, zinc, N,N′-diphenylurea, N,N′-diphenylguanidine and many other chemicals (Chibwe et al., 2022; Halle et al., 2021). The tire particle leachate has a potential toxic effect on fish behaviour and performance. Leachate from pristine tires showed an acute and long-term toxic effect than suspension from worn tires in freshwater *Hyalella azteca* (Halle et al., 2021). In another study, tire particle leachate, N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6-PPD-quinone), a transformed product of the tire antiozonant chemical 6-PPD, showed species-specific toxicity. At a concentration of 1.3–1.8 μg/L, 6PPD-quinone was specifically toxic, namely, causing acute mortality syndrome in only juvenile and adult coho salmon (*Oncorhynchus kisutch*) and not in closely related chum salmon (*Oncorhynchus keta*) (McIntyre et al., 2021).

Overall, there is much work done focusing on the leachates but there is dearth information regarding the toxicity of TWPs themselves, and it has not yet been investigated that or demonstrated that TWPs are hazardous to freshwater model species. Micro and nanoplastic research have primarily focused on marine environments (Blettler et al., 2018); however, because freshwater environments are known to accumulate and convey tire wear particles (Cunningham et al., 2022; Wagner et al., 2018), it is important to consider how toxicity may differ for freshwater organisms.

1.8.3 Pollution Resistance Hypothesis

It has been well established that industrialization has introduced pollutants to the global environment (El Haj et al., 2019). However, exotic or invasive species tend to tolerate the environmental stressors in a better way than the native species (El Haj et al., 2019; Sánchez et al., 2016; Varó et al., 2015). This is in contradiction to the notion that the native species get more adapted to the pollution acquired through chemical evolution compared to the invasive species (Ruggeri et al., 2019). In a biodiversity and climate change study from Spain, the role of Arsenic pollution for the invasion of same exotic shrimps, over the same native shrimp was compared

(Sánchez et al., 2016). The findings of the study showed that with increasing temperatures from 25˚ to 29˚C, the survival, growth and resistance of the exotic species from polluted sites was drastically better compared than the native species. Similarly, resistance levels of the freshwater bivalves, *Anodontites trapesialis* (native species) was compared with *Limnoperna fortunei* (exotic species), both equally exposed to copper and herbicide contamination (El Haj et al., 2019). The results showed that the native species was more sensitive to the pollutants than the exotic species, implying higher resistance of the exotic species. In contrast, Pais-Costa et al.(2019) in their study focusing on shrimps observed that the native species, *A. parthenogenetica* was better physiologically and reproductively adapted to zinc contamination in comparison to *A. franciscana*, the exotic species. From all the studies, it can be implied that the understanding the complex interactions occurring due to biological invasions, contaminants, and the change in the climate can be contradictory and therefore, requires more focus in research.

1.9 Research gap

- Benthic marine litter has a patchy documentation in terms of distribution, abundance, and composition in the Mediterranean Sea. Therefore, a standardized protocol needs to be recognized by which evaluation of multiple years can be carried out, and the standardized data series which could help to identify seafloor macro-litter hotspots. The effective reduction of marine litter contamination should begin with a cap in the production of hazardous materials, with plastic as main target (Bergmann et al., 2022). However, for that fraction of macro-litter already dispersed in sea bottoms worldwide, mitigation initiatives (i.e., clean-up actions and restoration of highly contaminated sites) represent the most realistic and feasible strategy to pursue, still considering the enormous limitations that arise to work in the marine environment, at high depths (Canals et al., 2021). *Therefore, the need* *for guidelines aimed at identifying best practices to plan mitigation actions, brings the objective of our first chapter (Chapter2) of the thesis, where we tested as to whether different litter categories may result in geographically segregated and eventually more accessible hotspots, where mitigation actions could be prioritized.*

- Quantification of the MPs in the environment is extremely complex and the need to assess proper bioindicators and surrogate descriptors of such threat is compelling. Focusing more specifically in the benthic *dominium*, decapod crustaceans with scavenging feeding behaviour are among the most reliable and effective organisms. *With this background, Chapter 3 focusses on the aim to assess and compare the eventual significant differences in microplastic contamination for two ecologically relevant crustaceans dwelling in European Waters: The European spiny lobster Palinurus elephas and the Norwegian lobster Nephrops norvegicus.*
- The MPs & TWPs are polluting the aquatic world and consequently affecting the health of the species, had been stated in the study Franzellitti et al. (2019). The TWPs which is the major source of microplastic. The studies have been focused on the leachates and more evidence for acute poisoning from road run-off in case of TWPs. But, till date little information is available regarding the occurrence, course, risk, transport pathways, and toxicity of TWPs in the environment. *Chapter 4 deals with the adverse environmental effects of MPs and TWPs pollution and sublethal, but nevertheless important changes in species interactions which may promote cascading effects throughout biological hierarchy. This may have huge unforeseen implications from individual effects towards population and community levels. Further, if native species are more impacted that their invasive counterparts, it is likely fostering biological invasions in some. Therefore, our study*

focusses to compare the behaviour and performance of two carp species exposed to MPs of PE and TWPs and to test as to whether invasive gibel carp can show different response to contamination and get better adapted to polluted environment than the native crucian carp.

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Chapter 2: Scattered accumulation hotspots of macro-litter on the seafloor: Insights for mitigation actions

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1. Introduction

Poorly managed litter is contaminating the world's oceans and human-made marine litter is continuously entering the marine environment, with significant impacts on marine ecosystems (Canals et al., 2021; Chiba et al., 2018). It is estimated that plastic alone account for ca. 8 million tons of the litter that enters into the marine environment and constitutes numerically ca. 60–80% of the whole amount of marine litter (Bergmann et al., 2017; https://litterbase.awi.de/litter_graph ; Jambeck et al., 2015) so that the terms plastic and marine litter are used almost as synonyms. Litter entering the marine environment can cause various negative interactions with *biota*, including the well-documented accidental ingestion, which will represent a severe challenge for the next decades since litter and plastic particularly undergoes environmental and mechanical deterioration/fragmentation, thus becoming more accessible to *biota* (Jâms et al., 2020).

Beach clean-ups are efficient initiatives to raise awareness of the extent of litter pollution (Arroyo Schnell et al., 2017; Frantzi et al., 2021); however, the ocean seafloor is out of sight to most of society, and, as such, its vulnerability remains largely unknown to the general public (Canals et al., 2021). This perception does contrast with evidence from recent scientific reports devoted to seafloor litter, which strongly indicate that the seafloor represents the ultimate sink for the great majority of items that enter the marine environment (Cau et al., 2018; Peng et al., 2020;

Woodall et al., 2014), especially plastic items, including the well-known microplastics (1 μm–5 mm in size) (Frias and Nash, 2019).

Within the European Union, marine litter has been recognized as a major issue in European seas since implementing the Marine Strategy Framework Directive (MSFD; 2008/56/EC), within which marine litter quantification is the 10th of 11 descriptors proposed for evaluating the environmental status of European seas (Galgani et al., 2013; Hanke et al., 2013). The Mediterranean is among the worlds' oceans exhibiting the most considerable accumulation of marine litter (Eriksen et al., 2014; Pham et al., 2014; Ramirez-Llodra et al., 2011) and, indeed, thousands of items (up to 20,000 km^{-2}; Pierdomenico et al., 2019) have been estimated to sit on its seafloor. However, this could be even more prominent in other areas, for which no data exist. The assessment of the abundance, distribution, and effects of marine litter on the ocean floor is primarily challenged by our limited knowledge of this environment. Indeed, studies carried out so far have investigated benthic litter abundance, composition, and distribution mainly on continental shelves and slope, whereas data from deeper habitats such as adjacent bathyal plains (Bergmann and Klages, 2012; Galgani et al., 2000; Pham et al., 2014; Ramirez-Llodra et al., 2013) are far less abundant, primarily due to the difficulties and associated costs of deep-sea sampling (Barnes et al., 2009). While the concept of litter-free seas has proved to be utopian, more realistic targets set the reduction of plastic contamination in the environment through multiple mitigation measures that should act synergically to meet ambitious goals (Borrelle et al., 2020; Lau et al., 2020). One of these measures is the removal of marine litter already accumulated in the ocean (Rochman, 2016). Recently, researchers aimed at identifying areas of the sea where floating plastic removal could be more effective (Sherman and Van Sebille, 2016). However, this sort of effort doesn't have a ‘benthic’ counterpart, despite the availability of numerous studies dealing with the identification of seafloor litter hotspots (*e.g.*, Franceschini et al., 2019; Garofalo et al., 2020).

The distribution, abundance, and composition of benthic litter in the Mediterranean Sea have patchy documentation, further complicated by the use of different sampling techniques. Over the last decade, much effort has been put into assessing baselines of seafloor litter’s abundance and standardizing data acquisition protocols, which was recognized as vital to implement litter reduction policies and adequate monitoring schemes (Galgani et al., 2013; Hanke et al., 2013; Kershaw et al., 2019).

What could be considered the ‘second step’ of the process is the evaluation of multiple years, standardized data series, which are rare (Canals et al., 2021; Galgani et al., 2021; Martínez et al., 2020). Indeed, it has been recently documented how difficult it is to provide a solid answer to whether seafloor macro-litter amounts in marine environments are increasing or not, due to the heterogeneous distribution of data and the lack of continuous standardized surveys (Galgani et al., 2021). These kinds of data would allow to identify seafloor macro-litter ’hotspots’, which are defined as those areas where macro-litter accumulates (Tubau et al., 2015) and, pragmatically, where eventual mitigation actions should be prioritized.

This study aims at analyzing the spatial and temporal variability of seafloor litter in the Food and Agriculture Organization (FAO) Geographical Sub-Area 11 (Sardinian seas, western Mediterranean), taking advantage of the MEDiterranean International Trawl Survey (MEDITS) (Spedicato et al., 2019a) standardized protocol of surveys conducted for 7 years (from 2013 to 2019). The present work also aims to test whether different litter categories (according to the MEDITS protocol) may result in geographically segregated seafloor litter hotspots, thus providing useful guidelines for prioritizing eventual action foreseen in the framework of plastic reduction policies.

2. Materials and Methods

2.1. Study area

The island of Sardinia is located within the FAO Geographic Sub Area 11 (http://www.fao.org/gfcm/data/maps/gsas/en/); here, we are grouping the two FAO GSA11.1 and 11.2 in a single unit. Sardinian seas represent an interesting area within the Mediterranean' basin due to its geographical location and geomorphology (Figs. 1 and 2). The sea bottoms along the coasts are not homogenous in terms of extension, geomorphology and associated biodiversity (Cau et al., 2017a, 2017b). The eastern Sardinian continental margin represents the passive margin of the Tyrrhenian basin, delimited to the north by the Etruschi seamount and to the south by the Ichnusa Seamount (Moccia et al., 2021). The eastern sea bottoms are characterized by a narrow continental shelf that terminates at about 60–100 m depth in the south and central eastern coasts, while around 200m depth in the northeastern coast. Along the entire eastern side of the island, the continental shelf and slope are connected to inland orographic structures and river basins; narrow inlets, separated by high and steep mountains, are connected through a very narrow continental shelf with irregular bottoms to the slope incised by profound canyons (Harris and Whiteway, 2011; Mascle et al., 2001; Sulli, 2000). Hydrologically, the eastern part of Sardinia is characterized by the Levantine Intermediate Water (LIW; Fig. 2) (Millot, 2013), that flows along the entire eastern coast of the island. A part of the LIW, in addition, circulates cyclonically in the southern part of the Tyrrhenian basin and flows along the northern slope of the Sardinia Channel (SC). According to Astraldi et al. (2002) the intermediate and deep layers of the SC are filled by the LIW and the Tyrrhenian Deep Water that outflow into the Algero-Provençal Basin and the Western

Mediterranean Deep Water with an eastward flow (Fig. 2). The study area also includes the SC that, together with the Strait of Sicily and the Sardinia–Sicily passage, is considered one of the crucial areas in the Mediterranean Sea in the control of water mass exchange between the Eastern and the Western Mediterranean (Astraldi et al., 2002).

The western coast of Sardinia shows the presence of a wider continental shelf that can extend for hundreds of kilometres from the coast, especially on the southwestern side of the island. With respect to hydrological features, the Western Sardinian Current reaches its maximum intensity in the south-west corner of the island due to topographic constraints and the synergic action of mesoscale features such as anticyclonic eddies. In addition, a coastal upwelling of deep waters along these coasts which is triggered by strong mistral winds, has been recently discovered in this region (Olita et al., 2013).

2.2. Data collection

Data used in the present study were collected in the framework of the MEDITS survey, conducted from 2013 to 2019. Spatial patterns of data collected between 2013 and 2015 have been described in Alvito et al. (2018), while the present study focuses on the entire temporal range between 2013 and 2019, including the analysis of accumulation hotspots.

A total of 707 hauls, approximately 100 hauls year $^{-1}$, were positioned following a depth stratified sampling scheme with random drawing of hauls positions within each bathymetric *stratum*, namely A (0–50m); B (51–100m); C (101–200); D (201–500) and E (501-800). The number of hauls per each *stratum* (~20 hauls year^{-1}) was performed within each of seven sub-zones of GSA11 (see Table 1 and Fig. 1; AAVV, 2017).

Once onboard, seafloor macro-litter was sorted and classified according to nine major categories (i.e., L1: Plastic; L2: Rubber; L3: Metal; L4: Glass/Concrete; L5: Cloth; L6: Processed wood; L7: Paper and cardboard; L8: Other; L9: Unspecified), and corresponding sub-categories (Supplementary Table 1). To obtain standardized weight and density indices, the total weight and the number of items collected per each sub-category were standardized according to the swept area, expressed as number of items and weight (in Kilograms) per km^2.

2.3. Data processing and statistical analysis

To test for significant spatio-temporal differences in seafloor litter density (n. of items km^{-2}) and abundance (kg km^{-2}), a non-parametric permutational analysis of variance (PERMANOVA; software PRIMER 7+) was performed. The statistical routine was based on Euclidean distance-based resemblance matrixes, built used untransformed data on density and weight. The PERMANOVA was ran in an univariate environment using the total density and weight of macro-litter, separately, as response variables. The multivariate analyses used the n. of items km^{-2} or Kg km^{-2} of the nine major seafloor macro-litter, as response variable. Both routines were conducted using a design based on three fixed orthogonal factors and their interactions; in detail: (i) Year (7 levels); (ii) Zone (7 levels); (iii) Stratum (5 levels), with a variable number of replicates. Differences in seafloor macro-litter abundance across years were also tested singularly using the five most recurrent macro-litter categories in terms of density and weight (i. e., plastic, metal, glass, rubber and cloth).

2.4. Seafloor macro-litter hotspots

In order to highlight seabed hotspots of litter accumulation, abundance data from the MEDITS survey were interpolated, and then areas of high densities were identified. For each category, interpolated values were evaluated by 2D interpolating via Inverse Distance Weighting (IDW) (Falivene Aldea et al., 2010) on a grid of 1 km^2, thereby obtaining an extended map of the potential zones where plastic litter tends to sink and accumulate according to the historical series, which has already been applied in Franceschini et al. (2021). The method consists in fitting a thin plate spline surface to irregularly spaced data. The assumed method is an additive model where longitude, latitude, and depth were considered as dimensional surfaces for litter density estimation. The smoothing parameter is chosen by generalized cross-validation (CV) (Wise, 2011). Model development was carried out using *fields* functions in R environment (R Core Team, 2022).

Hotspots of recurrent litter accumulation were evidenced by selecting the 90th percentile of the obtained interpolated distribution. In this case, we wanted to highlight areas where major quantities of litter were likely to occur. This was not obviously aimed at predicting local quantities of seabed litter, but rather highlight areas that by conformation of the seabed, sea currents or other environmental/anthropogenic factors appear to be stable accumulation points in the area of interest from a qualitative point of view. For the production of the hotspots maps, the plastic macro-category was further divided in 'Fishing Related Items' (FRI) and 'Other Plastic'. FRI included the sub-categories 'fishing nets', 'fishing lines' and 'other fishing related items', while all other sub-categories were included in 'plastic' (Supplementary Table 1).

3. Results

The total swept area covered by the survey was ~44 km^2. Among the 707 hauls conducted between 2013 and 2019, 461 showed the presence of seafloor macro-litter (65%). In more detail, the highest number of hauls showing the presence of macro-litter (83) was recorded in 2015, corresponding to 82% of the hauls conducted that year; on the contrary, the lowest (55) was recorded in 2016 (55% of the year' hauls). Overall, a total of 1908 items were collected from 2013 to 2019, corresponding to a total of 531 kg of seafloor litter (some examples of collected items are reported in Fig. 3).

3.1. Spatio-temporal trends

During the study period, the average number of collected items was 29.8 ± 1.22 items km^{-2} (average ± standard error), with a maximum of 37.2 ± 3 items km^{-2} in 2014 and a minimum of 25.6 ± 2 items km^{-2} recorded in 2018. With respect to the weight of seafloor macro-litter, the average weight recorded in the study period was 9.1 ± 1.96 kg km^{-2}, with a maximum of 14.2 ± 5.2 kg km^{-2} in 2019 and a minimum of 4.8 ± 1.2 kg km^{-2} in 2018. The highest macro-litter density was recorded in zones 6 and 4, showing an average density of 33.9 ± 1.9 and 31 ± 2.9 items km^{-2}, respectively, while the lowest density was in zone 1 (22.9 ± 1.9 items km^{-2}).

According to the PERMANOVA routine, there was a lack of significant differences in seafloor macro-litter density and weight across years (Table 2). The absence of significance was also confirmed when each of the five major categories of macro-litter was considered singularly, with the only exception of glass, which showed significant differences in weight across time, with a maximum average value of 10.5 ± 0.9 kg km^{-2} observed in 2016 and a minimum value of 4.6 ± 0.3 kg km^{-2} observed in 2017 (Supplementary Table 2).

While the interaction of the three factors considered simultaneously (*i.e*. ‘year’, ‘*strata*’ and ‘zone’) was not significant, the interaction of the factors’ ‘*strata*’ and ‘zone’ was statistically significant ($p < 0.001$; Table 2), emphasizing a significant variability in the spatial and bathymetric distribution and composition of seafloor macro-litter.

3.2. Seafloor macro-litter composition

The PERMANOVA routine showed the absence of any significant difference in seafloor macro-litter composition across years, but rather a significant difference in composition among different bathymetric *strata* and geographical zones (Table 2). With respect to relative density of seafloor macro-litter items, plastic was the dominant macro-category across investigated years

(2013–2019), with an average of ~67% of the total number of items. The highest record was observed in 2019 (78%), followed by 2018 and 2017 (74% and 73% respectively) (Fig. 4). The spatial analysis confirmed how the plastic macro-category was dominant in all geographical sub-areas (67% on average), with a maximum value of 86% recorded for zone 5 (Supplementary Fig. 1). In all the five bathymetric *strata* (A-E) plastic constituted 67% of collected material, followed by metal and glass (8% each) and cloth (7%). *Stratum* E showed the highest values of plastic macro-litter (74%), followed by *strata* B and A, 73% and 67% respectively, whereas *stratum* C recorded the lowest value (60%; Supplementary Fig. 2). After plastic, the most representative categories of macro-litter were metal and glass, ranging between 5% and 10% and between 2% and 20%, respectively (Supplementary Fig. 2).

More in detail, plastic consisted mostly of bags and food wrappers (45% of all plastic items) and 44% of the total n. of plastic items found along the investigated bathymetric profile (Supplementary Figs. 4 and 5), while fishing related items accounted cumulatively for ca. 15%. Metals macro-category was dominated by beverage cans (Supplementary Figs. 6–8) while glass was constituted almost exclusively by glass bottles (see Supplementary Figs. 9–11). Further details of spatial, temporal, and bathymetric distribution of cloth and rubber sub-categories are reported in Supplementary Figs. 12–17.

In terms of weight, plastic remained the most abundant macro-category, with an average of 62% during the study period, with a maximum of 81% in 2019 (Fig. 4; Supplementary Figs. 1 and 2). Plastic was followed by rubber, glass, and cloth (all ~8%). Contrarily to the analysis of density, the major component of the plastic macro-category in terms of weight were fishing related items (cumulatively 83%), followed by smaller and lighter objects (Supplementary Fig. 3). Fishing nets were most abundant in the deepest *stratum* E (79% of the total weight; Supplementary Fig.

5). Rubber (tyres 63%, 'others' 37%) and cloth, composed by large pieces (*e.g.* carpets, mattresses, etc; 44%), clothing (39%) and natural ropes (17%) were the second and third heavier categories (Supplementary Figs. 15–17). Metals were mostly composed by large metallic objects (53%) and middle size container (20%), and constituted cumulatively 6% of the total weight of macro-litter (Supplementary Figs. 6 and 7) and about 81% of the macro-litter found in the deepest *stratum* E (Supplementary Fig. 8). Glass (bottles 98% and ceramic jars 2%) constituted 6% of the total weight collected during the study period and was dominated by glass bottles across all bathymetric and spatial boundaries considered in this study (Supplementary Figs. 9–11).

3.3. Seafloor macro-litter hotspot

Results showed that for the plastic macro-category, which was split in fishing-related items (see FRI in Fig. 5) (*e.g.*, fishing nets, lines, etc.) and all other plastic items (see 'Plastic' in Fig. 5), major concentrations of items occur in the south-west area of the island. Hotspots for rubber items showed a reasonably homogeneous distribution around the Sardinia coast, with the highest peaks in the south-eastern area. Similar values were found for the metal debris. The glass category showed a hotspot in the north-western area of the island, while the highest values of fiber and cloth are more likely to occur in the south-eastern area (Fig. 5). Considering the additive contribution of all the above mentioned categories, the "overall" hotspot area is mainly concentrated in the southern part of the island.

4. Discussion

4.1. Spatio- temporal patterns and accumulation hotspots

Based on regular surveys conducted over a 7 years' time series, our results confirm the absence of a significant temporal trend in seafloor macro-litter quantities, regardless of the typology, as already observed and commented in a recent reflection proposed by (Galgani et al. (2021).

The absence of any temporal trend could suggest that the standing stock in surveyed fishing grounds reached an equilibrium status between input and output. The ‘output’ likely involves remobilization of macro-litter (*i.e.*, secondary dispersion) due to human activities such as dredging and trawling and also the progressive burial due to sediment flows and resettling sedimentary particles (Tubau et al., 2015). On this aspect, very recent and interesting results have been reported by Brandon et al. (2019), documenting a doubled contamination of (micro) plastic in sediments over a multidecadal time scale, which indirectly already accumulated on the seafloor.

The observed variability among years is likely related to variations in the abundance of plastic macro-litter, which represents the most conspicuous fraction of collected items, regardless of the geographical area or the bathymetric *stratum* considered. The numerically dominant sub-categories are characterized by light weight (*e.g.*, plastic bags, sheets and bottles) and can thus be transported over long distances following marine currents, and accumulate in proximity of peculiar geomorphologies on the seafloor (Galgani et al., 2000; Spedicato et al., 2019b). The distribution of plastic hotspots resulted in few delimited areas, mainly located on the northern, western, and southern coasts, while they were absent on the eastern shore. The western coast is one of the most dynamic areas of the Mediterranean Sea (Olita et al., 2013). It is characterized by strong northwest winds (Mistral and Tramontane) and by the presence of a southeastward current coming from the Balearic Island, that around 40° N separates into two branches flowing northward and southward when approaching the coast (Hernandez-Lasheras and Mourre, 2018). The northward flow branch may be one of the factors that enhanced the accumulation of plastic and rubber categories in the gulf of Alguer (north-western corner), together with high tourism activities to which this area is subject especially during summer. The southward branch, also known as the Western Sardinian Current, might be one of the leading transport pathways contributing to the accumulation of macro-litter objects in the south-western corner of the island. Moreover, the presence of two minor islands (Carloforte and Sant'Antioco), represents a physical barrier to the Western Sardinian Current at its maximum intensity (Olita et al., 2013), possibly favoring the formation of macro-litter hotspots washed up from the western continental shelf. Additionally, the presence of mesoscale anticyclonic eddies observed in this area (Knoll et al., 2017) could be another hydrological contributor to the accumulation of seafloor macro-litter as they could facilitate the collection and sinking of items to the seabed (Spedicato et al., 2019b).

Coupled with this highly dynamic hydrological scenario, the geomorphology of the seafloor is another critical feature that acts synergically to the spatial distribution of seafloor macro-litter hotspots (Galgani et al., 2000). Along the western Sardinian coast, the presence of a large continental shelf, characterized by extensive areas of outcropping and sub-outcropping rocky volcanic substrata (Conforti et al., 2016; Deiana et al., 2021), could possibly facilitate the entangling and thus the accumulation of drifting objects on the seafloor. On the contrary, the eastern coast' short continental shelf is incised by numerous submarine canyons, with their heads positioned at short distances from the shoreline (Harris and Whiteway, 2011); this setting likely favours the transport of lighter objects (i.e., single-use plastic and metal objects) towards deeper sea bottoms (Cau et al., 2017a, 2017b; Dominguez-Carrió et al., 2020; Zhong and Peng, 2021), possibly explaining the absence of major hotspots along the central and southeastern continental shelf.

Land-sourced macro-litter can be a major fraction of the total litter retrieved, which may flow from distant inland sources (Meijer et al., 2021; Pierdomenico et al., 2019). Moreover, such input can be exacerbated by heavy rainfalls, river floods, sewage overflow, or can carry large amounts of debris to beaches and coastal waters in a matter of a few hours or days, part of which subsequently spreads seawards and settles to the seafloor (Canals et al., 2021). Four of the largest Sardinian's rivers (i.e., Flumini Mannu, Rio Mannu, Coghinas and Tirso; (Alvito et al., 2018) discharge their water and at least part of the land-based litter accumulated along their path (Tramoy et al., 2019), in proximity of highlighted hotspots in the southern (Gulf of Cagliari), northern (Gulf of Asinara) and western (Gulf of Oristano) Sardinian coasts. All these rivers pass nearby all most densely populated Sardinian cities (i.e., Cagliari, Oristano, and Sassari; Fig. 1), and they flow into distinct gulfs, which, because of hydrological dynamics, tend to accumulate litter debris instead of washing them away by currents (Katsanevakis and Katsarou, 2004). A fifth big Sardinian river, the Flumendosa river, discharges its water on the island's south-eastern coast. However, the presence of the San Lorenzo canyon in front of the Flumendosa rivers mouth might wash litter objects from the continental shelf down to the submarine canyons, as already documented by Tubau et al. (2015) in the northwestern Mediterranean.

Heavier objects hotspots such as those belonging to the rubber or glass macro-category were generally closer to the coastline, mostly in the proximity of the larger ports (Cagliari, Oristano and Porto Torres) or far from the coast but close to the most trafficked maritime routes such as the one those across the Gulf of Bonifacio, the port of Olbia or the Sardinian channel (i.e., the southernmost portion of the island), which suggests that these objects can possibly result from illegal dumping since this and other types of heavy litter (*e.g.*, metal objects) originate from direct sea-based point sources. According to available information extrapolated by Maritime State Property Concessions (Italian Ministry of Infrastructure and Transports), the glass hotspot could be related to both intense tourism and shipping activity that occurs most intensely along the northeastern coastline (Scotti et al., 2021). Also, hydrodynamic drivers such as the Northern Tyrrhenian Cyclone (east of the Strait of Bonifacio), could be invoked as a driver of local accumulation pattern by predictive model studies (Mansui et al., 2020).

Plastic litter hotspots are of particular concern due to the well documented detrimental effects that its persistence in the environment can possibly trigger, such as: (i) the introduction of artificial substrata that can locally enhance benthic biodiversity (the so-called ‘plastic benefits paradox’; Carugati et al., 2021; Song et al., 2021), (ii) the possible fragmentation into smaller particles, possibly mediated by *biota* that can ideally become more and more bioavailable to multiple levels of the trophic web (Cau et al., 2020, 2019; Fossi et al., 2018; Franceschini et al., 2021), (iii) compromise the biogeochemistry and alter microbial communities in sediments (Seeley et al., 2020), and (iv) provide physical damage to structuring fauna, in case of lost fishing gears (Angiolillo and Fortibuoni, 2020; Cau et al., 2017a, 2017b; Galgani et al., 2018). Nonetheless, beside the enormous attention of the scientific community for the plastic problem, other undocumented yet detrimental effects of other litter categories to the ecosystem could likely be discovered. As examples, glass is generally perceived as the least harmful material despite its durability; however, as per any hard substratum entering the marine environment, it can be colonized by sessile biota (*e.g.,* Fig. 3C) and thereby alter local benthic communities (Martínez et al., 2020); moreover, fragmentation processes occurring in the marine environment are still poorly understood. Another example are car tyres (the major component occurring in rubber hotspots, according to our results, see Fig. 3E) whose deterioration and eventual fragmentation once dispersed in the marine environment is poorly understood either, while knowledge on the increasing presence of tyre wear particles generated from roads into the environment is building up (Wagner et al., 2018).

4.2. Insights for mitigation actions

The effective reduction of plastic contamination in the future can be achieved through three broad management strategies that can be summarized in: (i) plastic litter reduction, (ii) better waste management, and (iii) environmental recovery (*i.e.*, clean-up actions and restoration of highly contaminated sites). These mitigation strategies should act efficiently and synergically to achieve ambitious goals, since one strategy alone would have little to no hope of reducing plastic contamination in the environment, oceans included (Borrelle et al., 2020).

The removal of litter from the environment could be desirable under particular circumstances, but in many cases lacks a detailed consideration of the possible effectiveness, costs and benefits (Falk-Andersson et al., 2020). This argument is of particular relevance for the marine environment, for which very limited knowledge is available and for which many physical constraints exist, such as the rough geomorphology of the seabed and well-known technical difficulties in operating in the deep-sea. Fishing For Litter (FFL) initiatives have been noted as effective and low-cost strategies for removing seafloor macro-litter from oceans, at least from fishing grounds, even though several difficulties arise in their execution and implementation (Cho, 2009; Ronchi et al., 2019). This is due to the wide spatial coverage of trawling activities, often performed in areas where fishermen are the only operators capable of providing this type of social service, nonetheless during their regular working routine.

Our results pointed out how identifying seafloor macro-litter hotspots as a single cumulative category could be misleading if the aim is to identify areas where mitigation strategies could be prioritized and where, as example, fishing for litter activities could be performed. The accumulation hotspot analysis, performed separately per each category identified in the MEDITS protocol, showed segregated distribution for the five categories investigated (Fig. 5), with some of them being closer to shore and at shallower depth compared to others. This segregation pattern is ascribable to the macro-litter's intrinsic features, which, depending on material and dimensions, can float on the sea surface before sinking due to changes in specific weight enhanced by biofouling, or reach the seafloor directly once discarded. While the former case is the one that mostly relates to plastic items (Ryan, 2015), the latter relates the most with heavier objects such as rubber, glass, and metal.

A portion of fishing-related items, which constituted the majority of macro-litter in terms of weight, could be removed by fishing for litter activities in case of smaller objects such as fishing lines and ropes, pieces of nets, and other smaller items. On the contrary it would be very demanding in terms of tools and skills required to remove larger and heavier lost fishing gears. On this aspects, bottom-trawling vessels using hooks and ropes are the cheapest way for removing DFGs, but it strongly impacts the seabed mechanically (Cho, 2011). Additionally, it could also be ineffective since nets and ropes are often lost due to their entanglement in small rocky outcrops of the seabed or structuring communities (Angiolillo et al., 2015), that would be likely ripped out. To tackle this issue, a synergic, sustainable action that might involve diverse parties and technologies (such as professional divers, where feasible, and remote sensing) should be adopted (Cho, 2011; Madricardo et al., 2020; Morishige and McElwee, 2012).

5. Conclusion

Despite the almost exponential increase in the number of studies dealing with seafloor macro-litter over the past decade, our knowledge on the topic remains limited when coping with the necessity to remove that fraction of macro-litter already accumulated in the marine environment. Numerous surveys have shown that litter can accumulate more likely in some locations rather than in others, and underwater video footage often documented severe contamination that can be visually compared to landfills, with thousands of items per km^{-2} (Canals et al., 2021; Pierdomenico et al., 2019).

Our results are the first to document a scattered distribution of different macro-litter categories, which likely depend on their sources and mobility through the marine environment. Based on our observations, we stress how the identification of macro-litter hotspots using aggregated density data (i.e., including the dominant category of plastic) might lead to the misidentification of smaller yet considerable macro-litter hotspots. Indeed, our analyses highlighted how in GSA11, several hotspots of different macro-litter categories are located close to shorelines and at shallower depths, compared to other litter categories. Considering this, and considering how removal activities are still in their infancy, prioritizing such more accessible fishing grounds could represent a solid test bed for developing more challenging remediation activities. At present, envisioning macro-litter removal without taking advantage of FFL initiatives appears unlike due to the enormous costs and technical difficulties. Nonetheless, the environmental effects of macro-litter burial in marine sediments and the effects that might arise from the removal from the seabed still have to be determined and compared to assess the lowest-impact strategy. Moreover, the necessity to increase studies on the quantification of marginal benefits and costs associated with the removal of seafloor macro-litter can inform socially efficient future management and policy. The combination of targeted removals, developing policies that work to reduce contributing factors and incentivize plastic reduction and prevention would likely be most effective.

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7. Figures and Tables

Tables

			n. items km^{-2}		Kg km^{-2}	
Years	Hauls	Swept Area (km^2)	Average	St. Error	Average	St. Error
2013	101	6.1	32.6	3.4	5.8	1.3
2014	102	5.7	37.2	3.0	8.2	1.5
2015	101	6.4	26.6	1.5	10.4	3.2
2016	100	6.2	27.2	2.6	5.9	1.5
2017	100	5.8	30.6	2.3	11.0	3.4
2018	102	6.4	25.6	2.0	4.8	1.2
2019	101	7.2	28.8	2.9	14.2	5.2
TOTAL	707	44				

		n. items km^{-2}		Kg km^{-2}	
Zones	Hauls	Average	St. Error	Average	St. Error
1	63	22.9	1.9	8.6	3.1
2	126	29.8	3.5	6.2	2.0
3	70	26.5	1.4	10.4	3.8
4	68	31.0	2.9	25.9	14.5
5	80	27.9	2.8	11.8	4.7
6	184	33.9	1.9	6.3	1.2
7	116	28.1	2.0	6.4	1.7

		n. items km^{-2}		Kg km^{-2}	
Strata	Hauls	Average	St. Error	Average	St. Error
A	142	42.4	2.7	14.1	4.2
B	131	44.2	3.0	12.1	2.3
C	168	34.9	2.3	16.7	6.3
D	148	17.8	0.8	3.2	0.8
E	118	18.2	1.1	2.4	0.8

Table 1. Summary of the MEDITS survey conducted in Sardinian Sea, reporting the number of hauls and the swept area per year, geographical sub-area within GSA11, and bathymetric *stratum*. Also, the average density and weight of items per km^2 (±st. error) are reported per category.

UNIVARIATE density (n. items km^{-1})				
Source	df	MS	Pseudo-F	P(MC)
Year	6	1153	0.6910	0.762
Zone	6	4094	2.4541	0.003
Stratum	4	4136	2.4791	0.009
Year*Zone	36	1485	0.8899	0.730
Year*Stratum	24	1275	0.7639	0.885
Zone*Stratum	24	2550	1.5282	0.013
Year*Zone*Stratum	116	1267	0.7596	0.996
Res	247	1668		
Total	463			

MULTIVARIATE density (n. items km^{-1})				
Source	df	MS	Pseudo-F	P(MC)
Year	6	3462.8	1.328	0.098
Zone	6	5967.1	2.288	0.001
Stratum	4	5883.2	2.256	0.004
Year*Zone	36	2608	1.000	0.485
Year*Stratum	24	2732.5	1.048	0.335
Zone*Stratum	24	3925.8	1.505	0.002
Year*Zone*Stratum	116	2464	0.945	0.796
Res	247	2607.3		
Total	463			

UNIVARIATE abundance (Kg km^{-1})				
Source	df	MS	Pseudo-F	P(MC)
Year	6	2251.8	0.8915	0.601
Zone	6	3914.2	1.5497	0.05
Stratum	4	7820.5	3.0963	0.001
Year*Zone	36	2082.7	0.8246	0.898
Year*Stratum	24	2293.3	0.9079	0.694

Zone*Stratum	24	4085.7	1.6176	0.004
Year*Zone*Stratum	116	2348.7	0.9299	0.791
Res	247	2525.7		
Total	463			
MULTIVARIATE abundance (Kg km^{-1})				
Source	df	MS	Pseudo-F	P(MC)
Year	6	4124.7	1.225	0.138
Zone	6	5848.7	1.738	0.007
Stratum	4	8317.9	2.471	0.001
Year*Zone	36	3563.3	1.058	0.256
Year*Stratum	24	3533.4	1.049	0.322
Zone*Stratum	24	4937.5	1.467	0.001
Year*Zone*Stratum	116	3520.8	1.046	0.189
Res	247	3365.8		
Total	463			

Table 2. Output from the PERMANOVA analysis (main test). Significant Monte Carlo procedure p-values [P (MC)] are reported in bold.

Figures

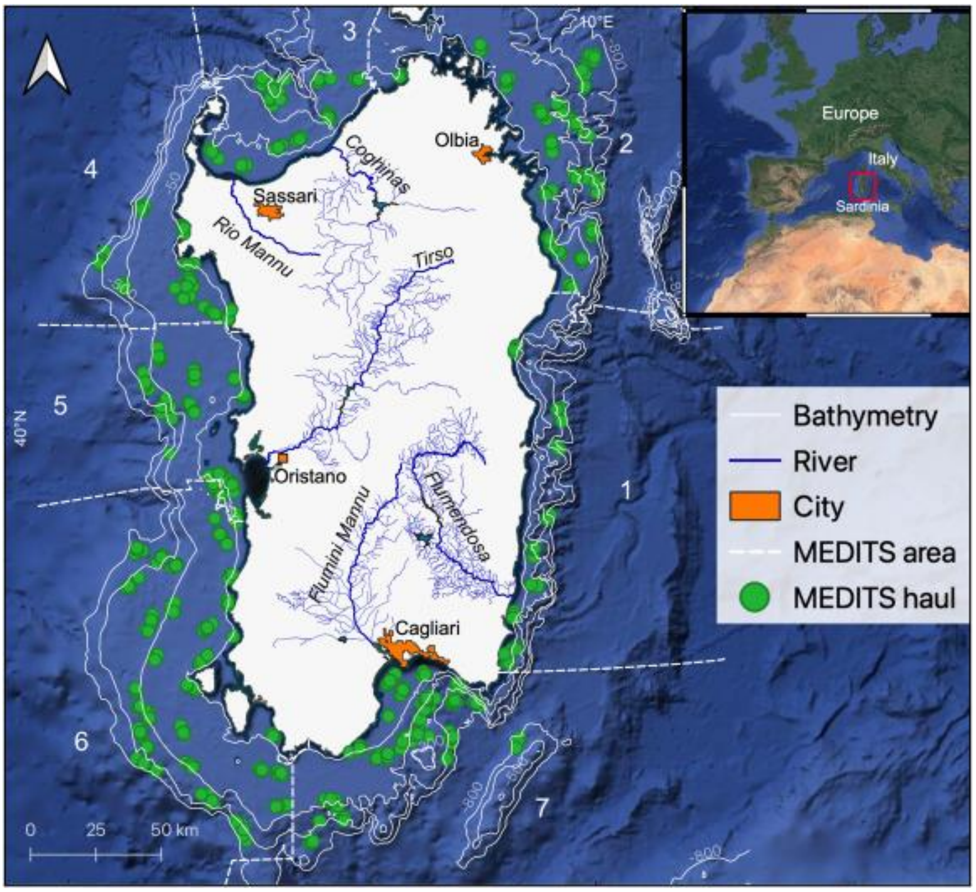


Figure 1. Map of the study area. Green dots represent hauls performed in the study period (2013-2019). The map also includes major cities and rivers of the island of Sardinia. White dashed lines represent the delimitations of the 7 geographical sub-areas comprised within GSA 11.

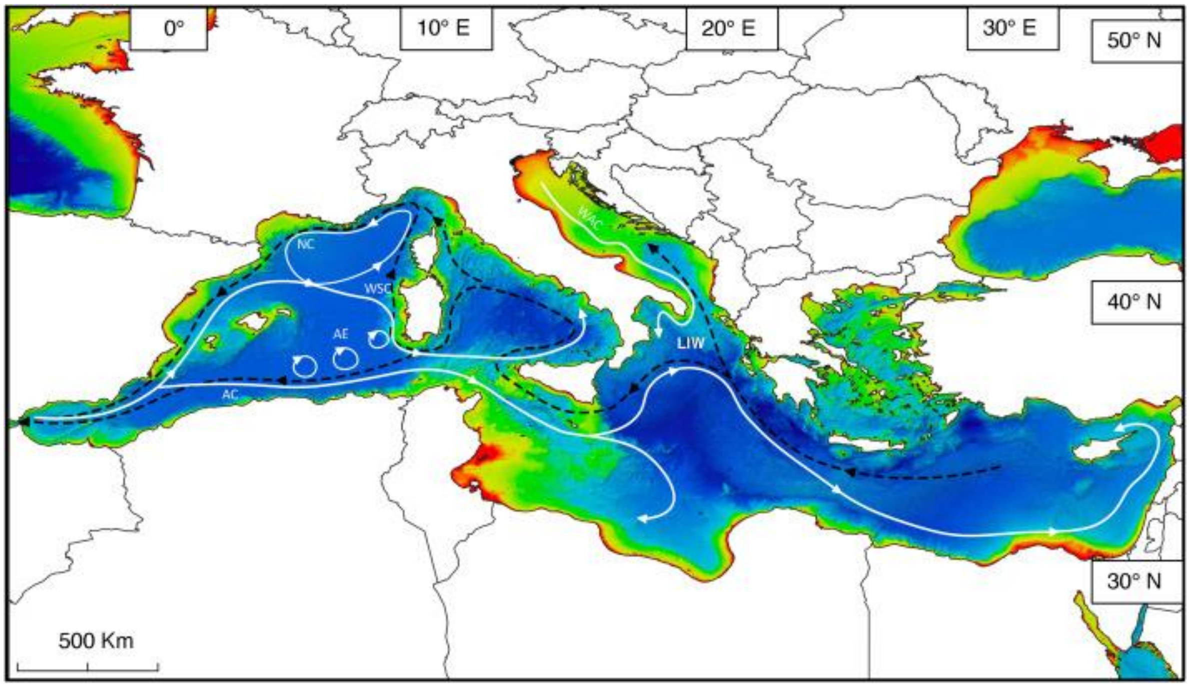


Figure 2. The hydrological context of the Mediterranean Sea showing the paths of the main surface circulation currents (white arrows) and the Levantine Intermediate Water (LIW) (dashed black arrow). Along the surface circulation, the Western Sardinian Current (WSC), Northern Current (NC), Algerian current (AC), Algerian Eddies (AE), and Western Adriatic Current (WAC) are highlighted. Bathymetry has been downloaded from http://www.emodnet-bathymetry.eu. The right panel is modified from Millot, (2013); Olita et al. (2013); Poulain et al. (2012).

Figure 3. Multipanel showing different macro-litter categories collected through the MEDITS trawl survey in the study period: A) Large metal objects found at ca. 160 m depth; B) deteriorated plastic and aluminium cans; C) Glass bottles showing the presence of encrusting fauna on it; D) Fishing pot covered with eggs of Loligo sp.; E) Tyres recovered from ca. 160m depth and F) Plastic films and bags.

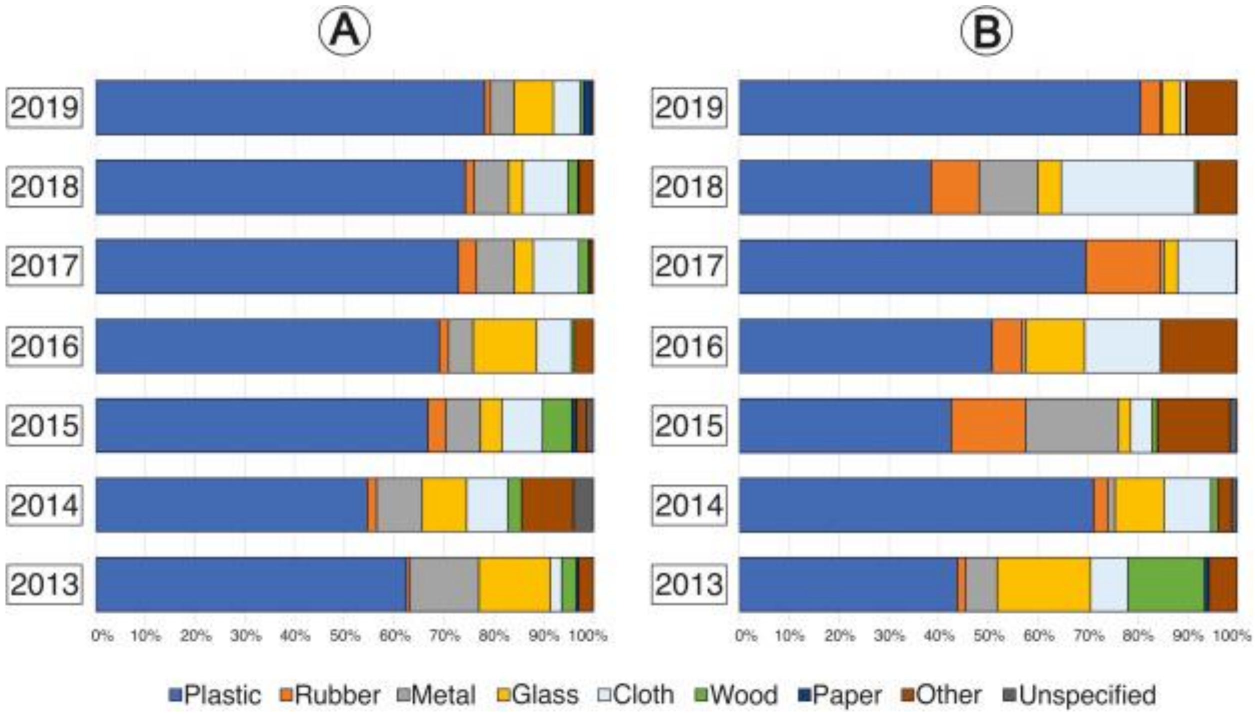


Figure 4. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of seafloor macro-litter during the study period, comprised between 2013 and 2019.

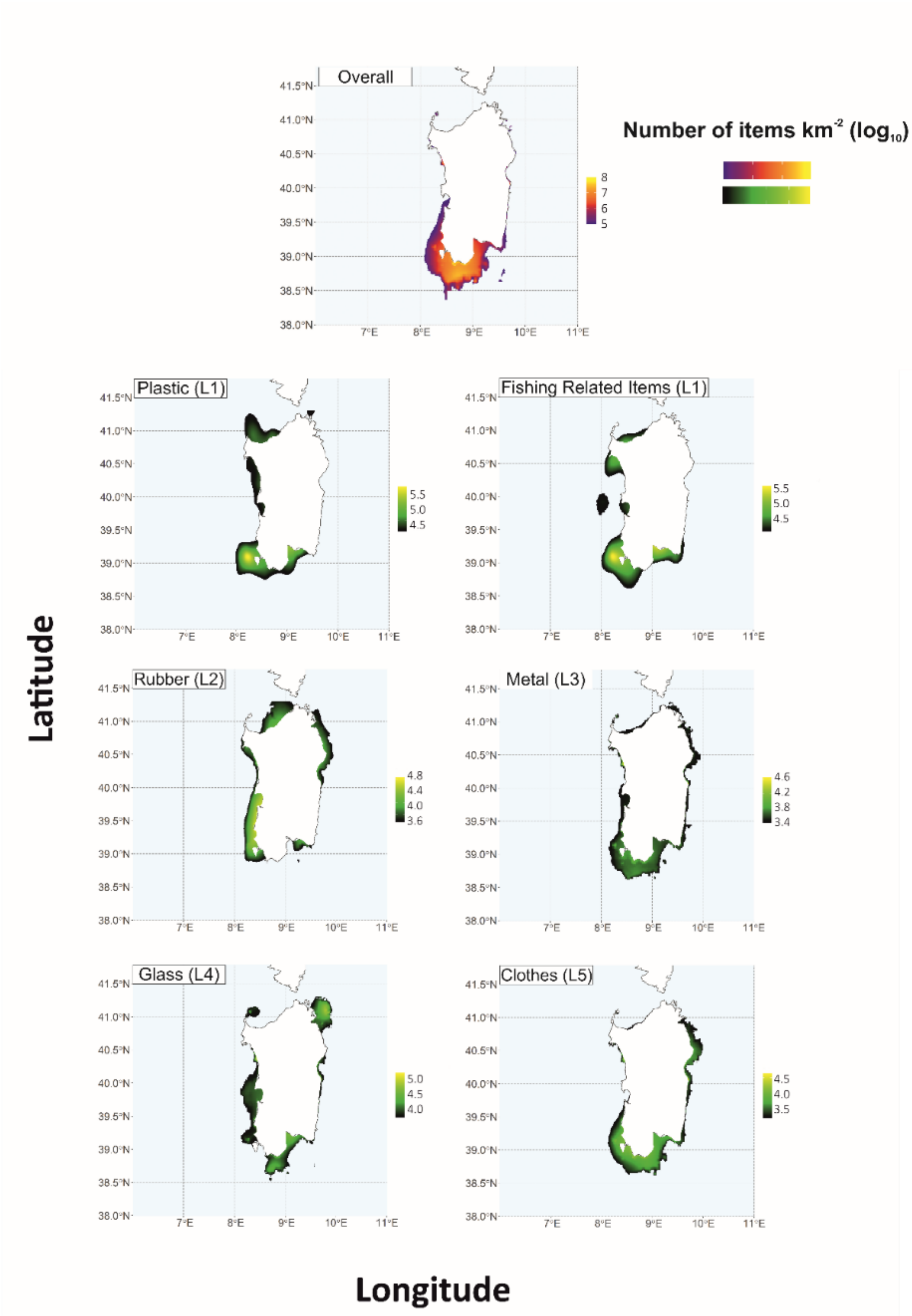


Figure 5. Map of seafloor macro-litter hotspot identified in GSA11, represented both as cumulative results of the five most recurrent litter categories (Overall) and divided according to each of the most representative seafloor macro-litter categories. The scale at the right of the figures

is reported as logarithmic weighted n. of items. * = the 'Plastic' macro-category was split in 'Fishing Related Items', which included sub-categories 'fishing nets', 'fishing lines' and 'other fishing related items'; while all other sub-categories were included in 'plastic' (i.e., plastic bags, sheets, etc; Supplementary Table 1).

SUPPORTING INFORMATION FOR:

Scattered accumulation hotspots of macro-litter on the seafloor: Insights for mitigation actions

Alessandro Cau, Simone Franceschini*, Davide Moccia, **Pankaj A. Gorule**, Blondine Agus, Andrea Bellodi, Rita Cannas, Laura Carugati, Danila Cuccu, Claudia Dessì, , Martina F. Marongiu, Riccardo Melis, Antonello Mulas, Riccardo Porceddu, Cristina Porcu, Tommaso Russo & Maria Cristina Follesa

Number of pages: 11

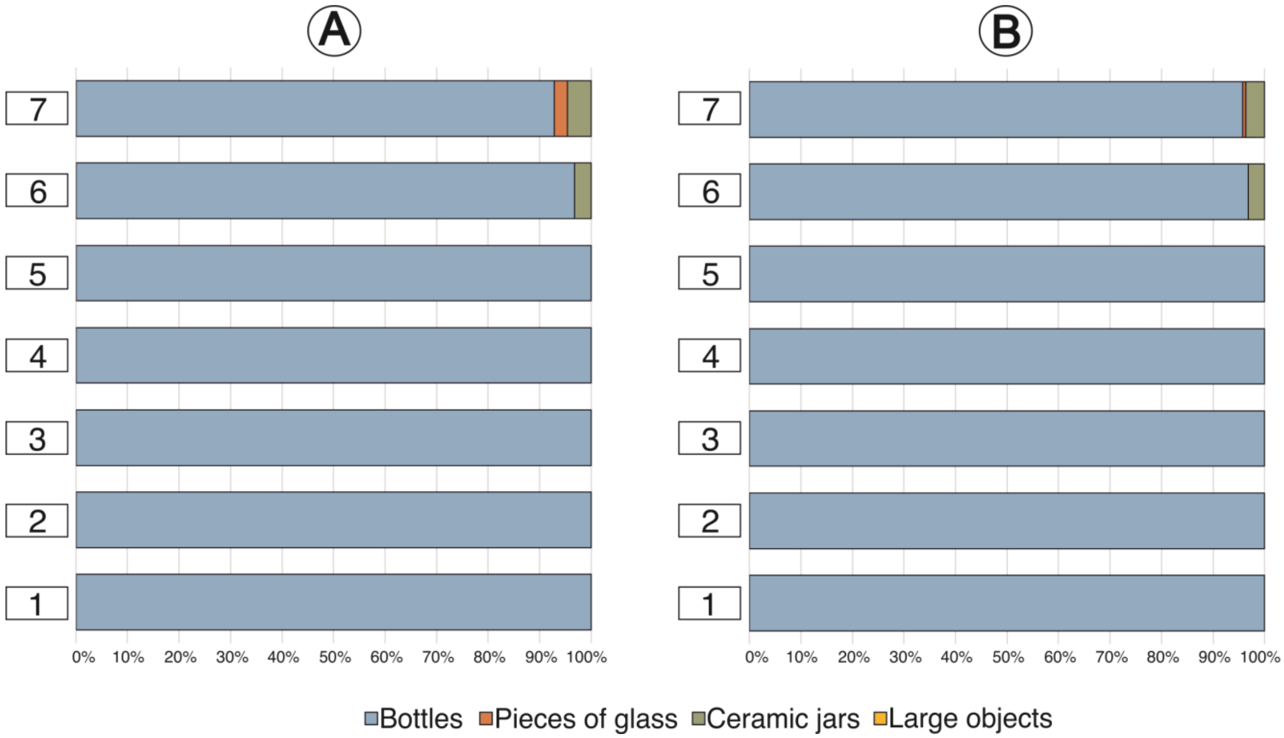
Number of Tables: 1

Number of figures: 17

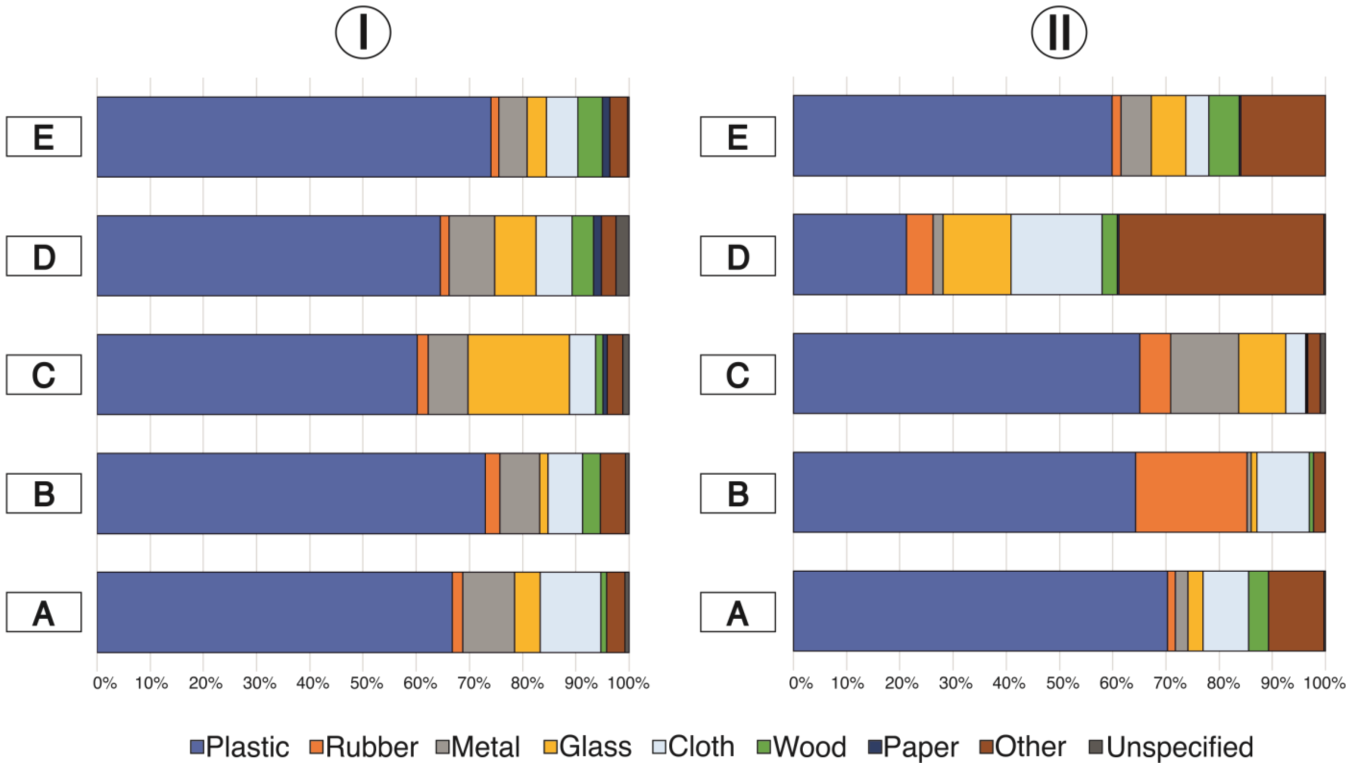
L0 No litter in the net
L1 Plastic (including PVC, polypropylene, polyethylene)
L1a. Bags L1b. Bottles L1c. Food wrappers L1d. Sheets (table-cover, etc.) L1e. Hard plastic objects (crates, containers, tubes, ashtrays, lids, etc.) L1f. Fishing nets L1g. Fishing lines L1h. Other fishing related (pots, floats, etc.) L1i. Synthetic ropes/strapping bands L1j. others
L2 Rubber
L2a. Tyres L2b. Other (gloves, floats, boots/shoes, oilskins, sanitaria)
L3 Metal
L3a. Beverage cans L3b. Other food cans/wrappers L3c. Middle size containers (of paint, oil, chemicals) L3d. Large metallic objects (barrels, pieces of machinery, electric appliances) L3e. Cables

L3f. Fishing related (hooks, spears, etc.) L3g. remnant from the war
L4 Glass / Ceramic/Concrete
L4a. Bottles L4b. Pieces of glass L4c. Ceramic jars L4d. Large objects (ceramic basins, etc.)
L5 Cloth (textile) **/ Natural fibres**
L5a. Clothing (clothes, shoes, etc.) L5b. Large pieces (carpets, mattresses, etc.) L5c. Natural ropes L5d. Sanitaria (diapers, cotton buds, etc.)
L6 Wood processed (palettes, crates, etc.)
L7 Paper and cardboard
L8 Other
L9 Unspecified

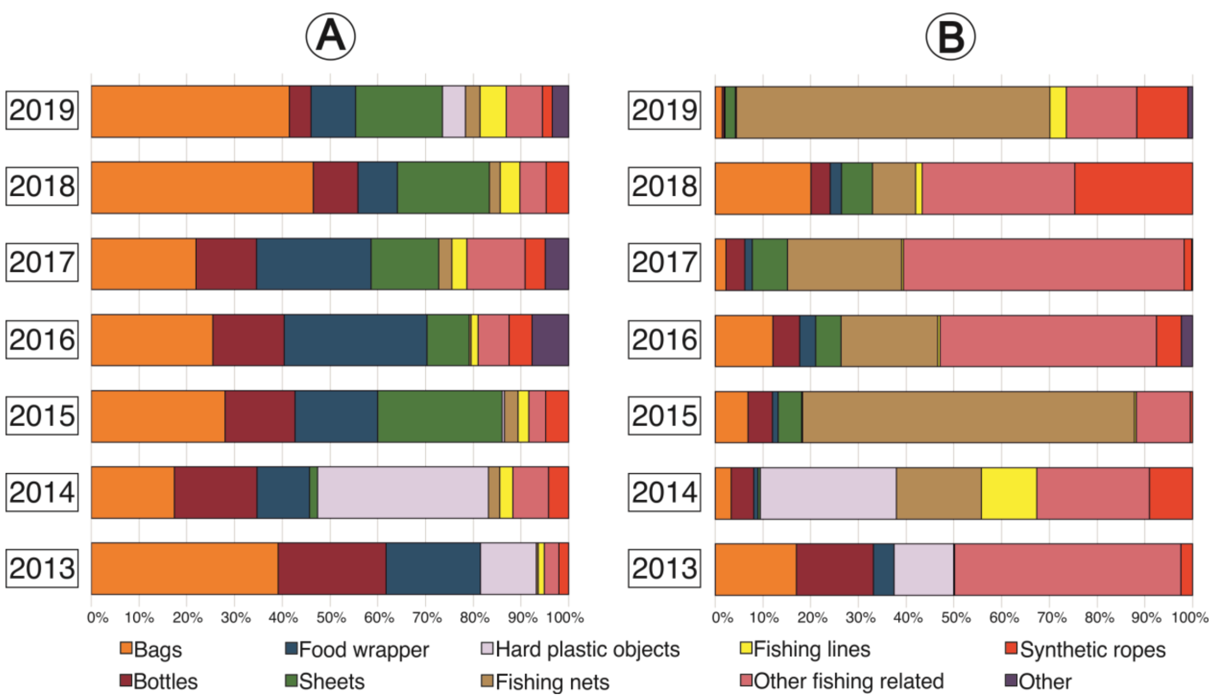
Supplementary Table 1. Summary of the seafloor macro-litter categories comprised within the MEDITS protocol (*i.e.*, from L1 to L9), and relative sub-categories.



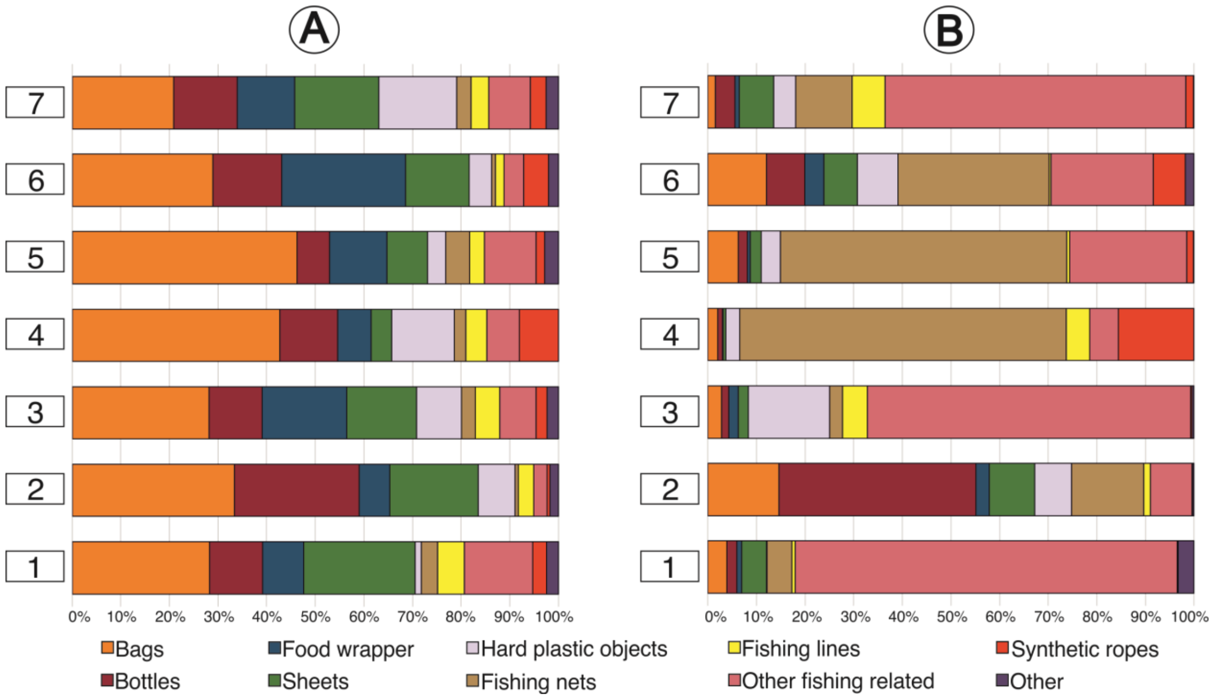
Supplementary Figure 1. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of each litter category, per each of the geographical sub-areas within GSA 11.



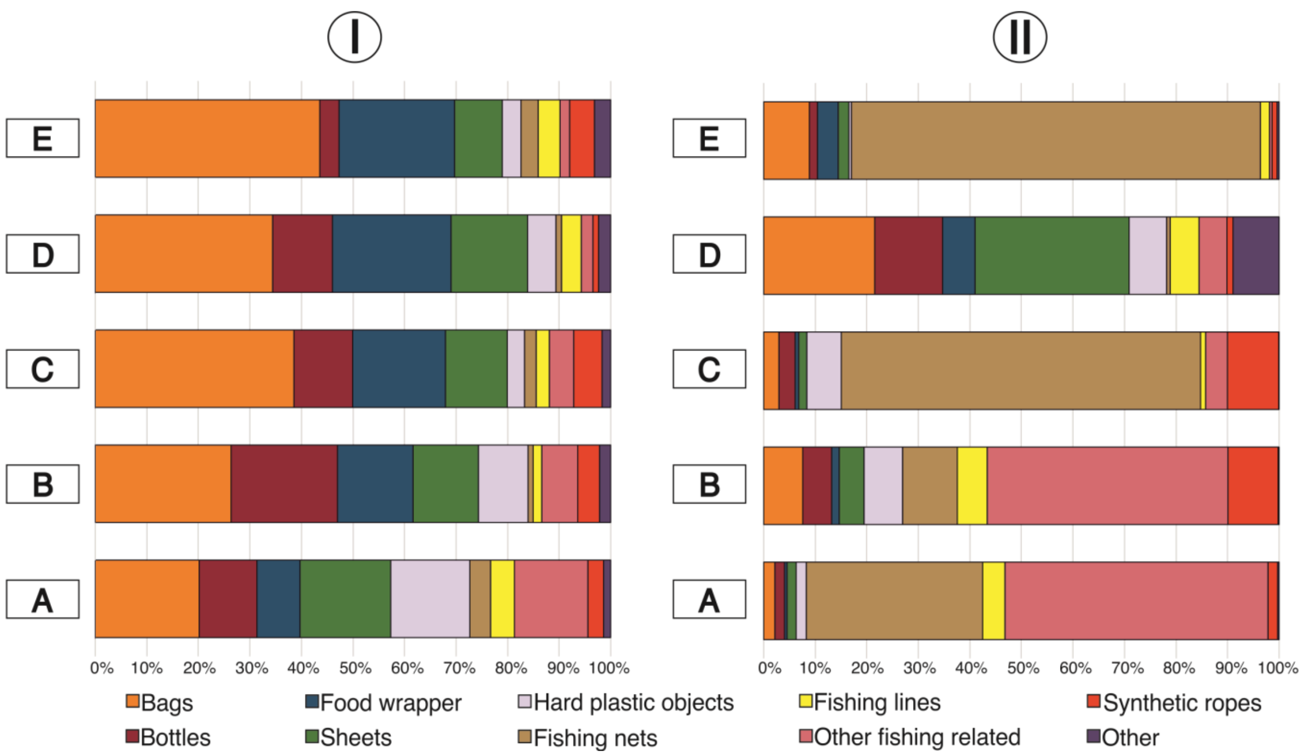
Supplementary Figure 2. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of each litter category, per each of the bathymetric strata of the MEDITS protocol.



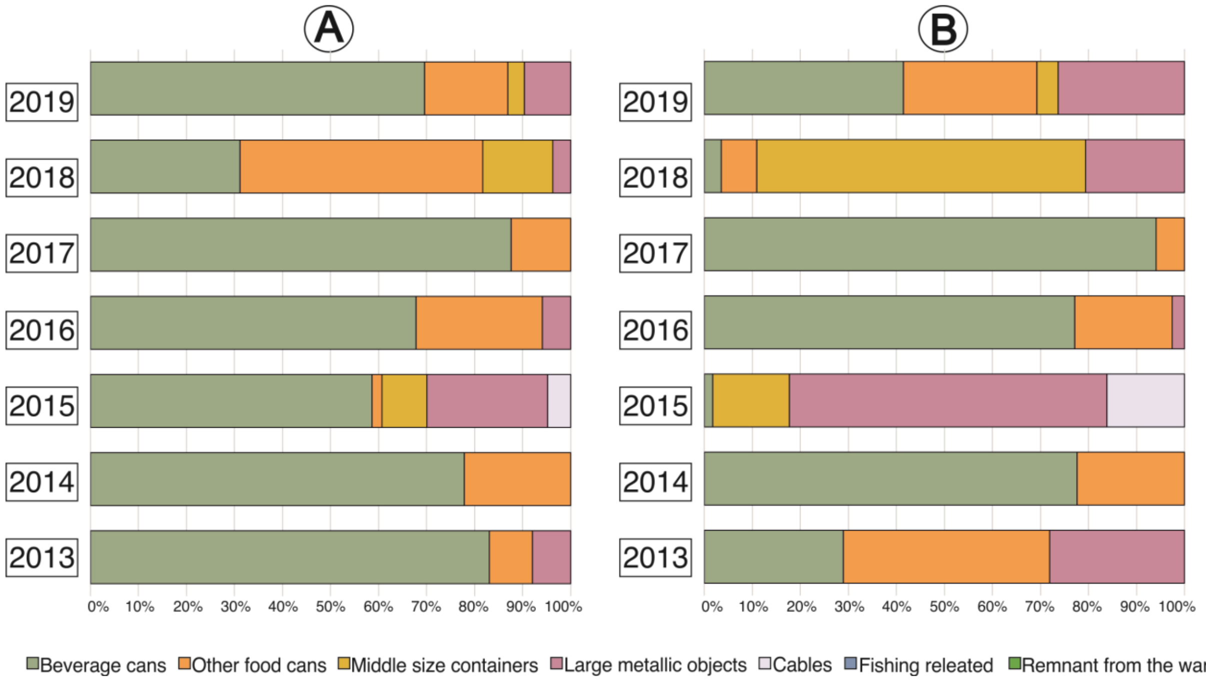
Supplementary Figure 3. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of plastic's subcategories during the study period, comprised between 2013 and 2019.



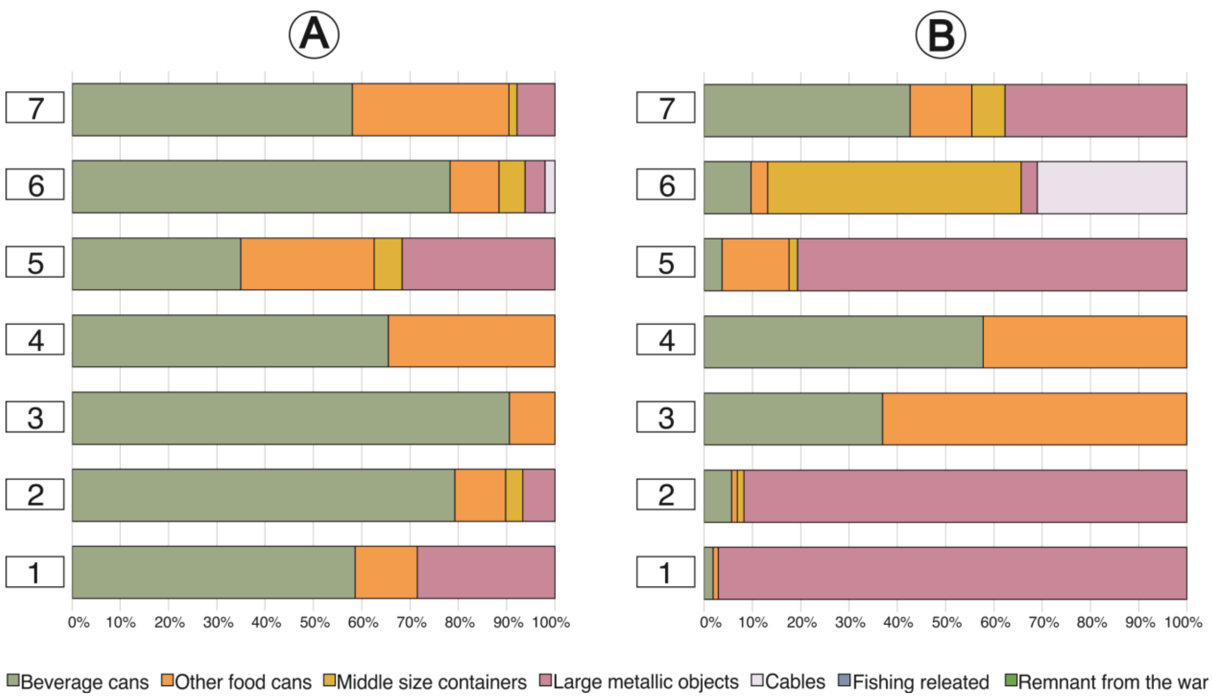
Supplementary Figure 4. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of plastic's subcategories, per each of the geographical sub-areas within GSA 11.



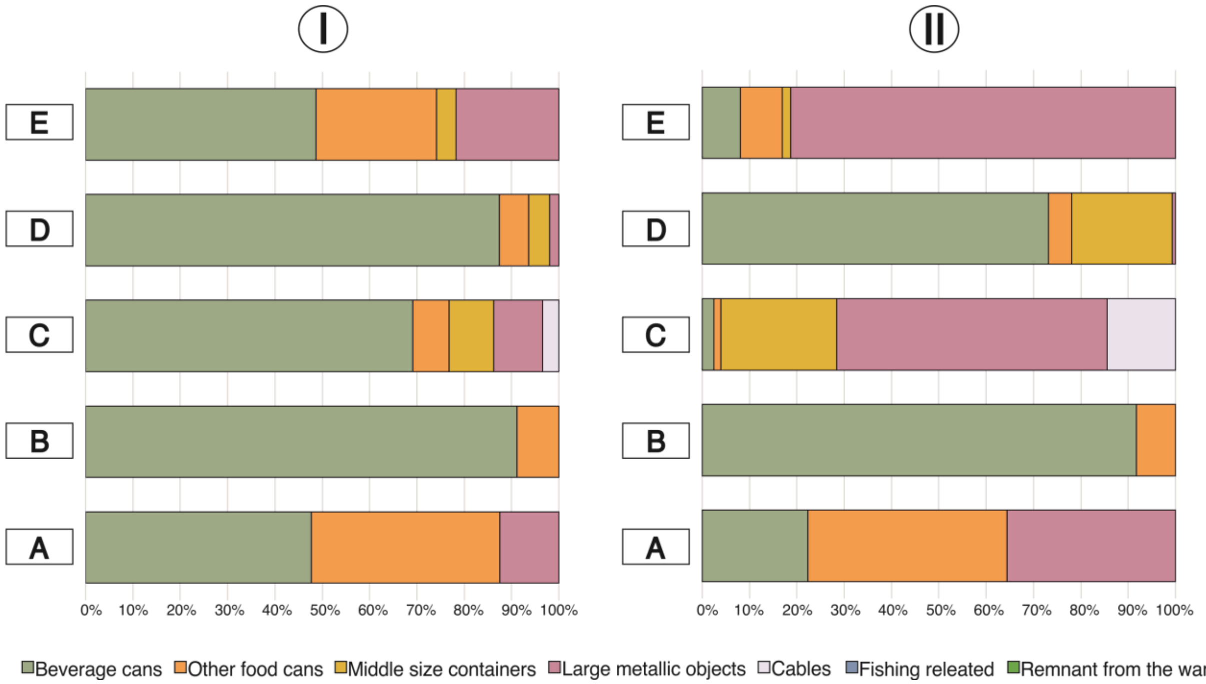
Supplementary Figure 5. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of plastic's subcategories, per each of the bathymetric strata of the MEDITS protocol.



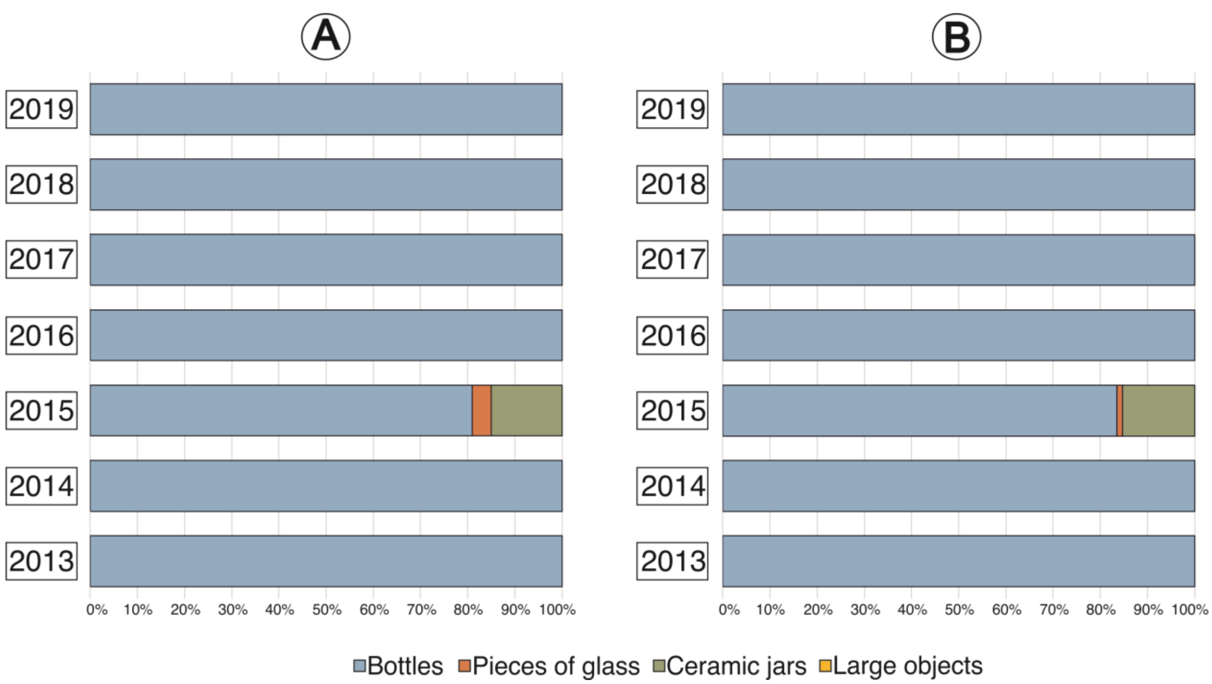
Supplementary Figure 6. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of metal's subcategories during the study period, comprised between 2013 and 2019.



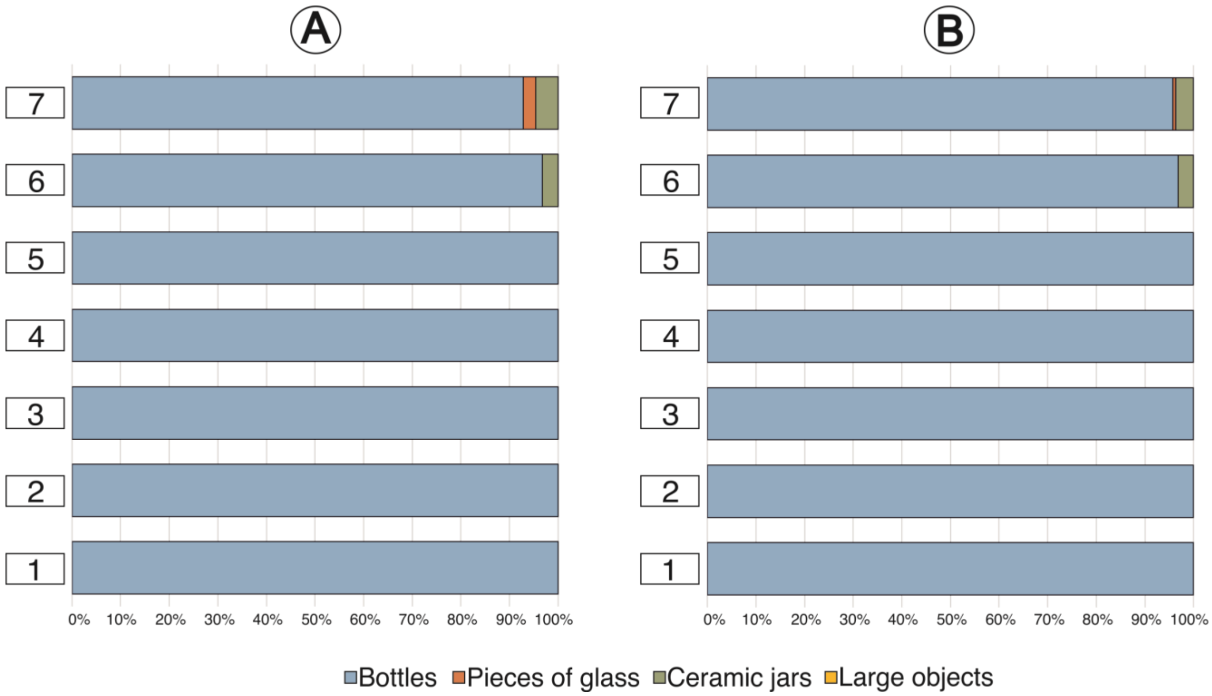
Supplementary Figure 7. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of metal's subcategories, per each of the geographical sub-areas within GSA 11.



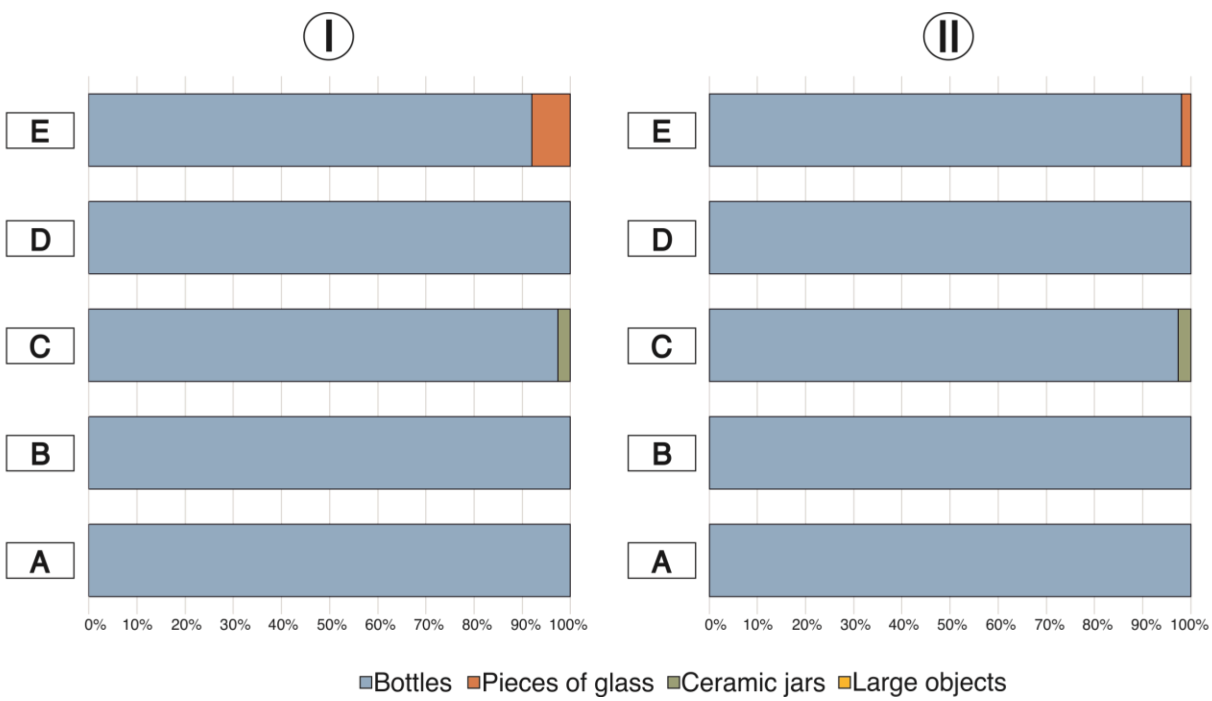
Supplementary Figure 8. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of metal's subcategories, per each of the bathymetric strata of the MEDITS protocol.



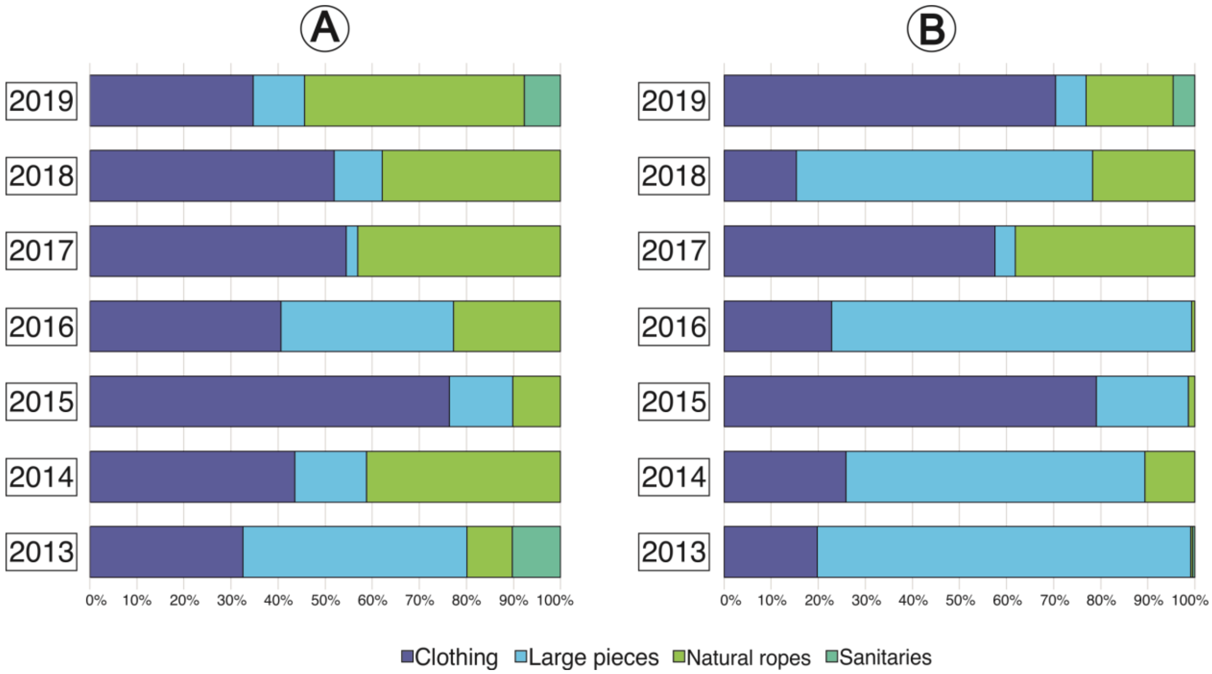
Supplementary Figure 9. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of glass subcategories during the study period, comprised between 2013 and 2019.



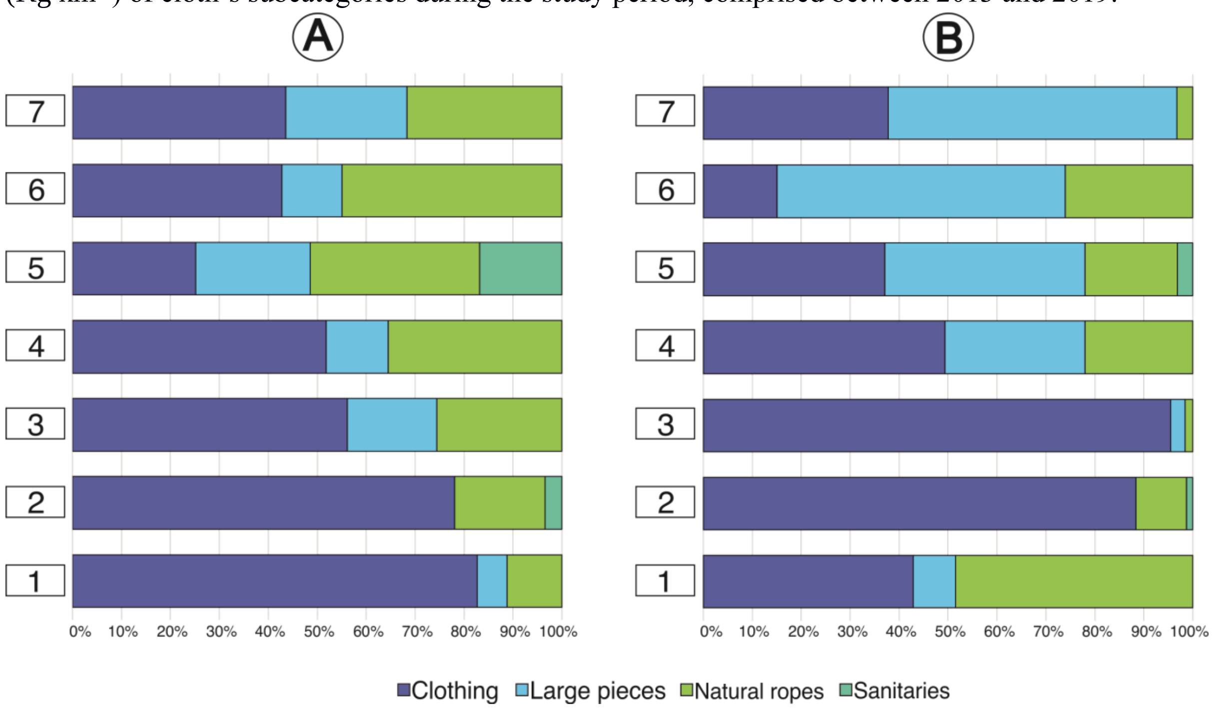
Supplementary Figure 10. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of glass subcategories, per each of the geographical sub-areas within GSA 11.



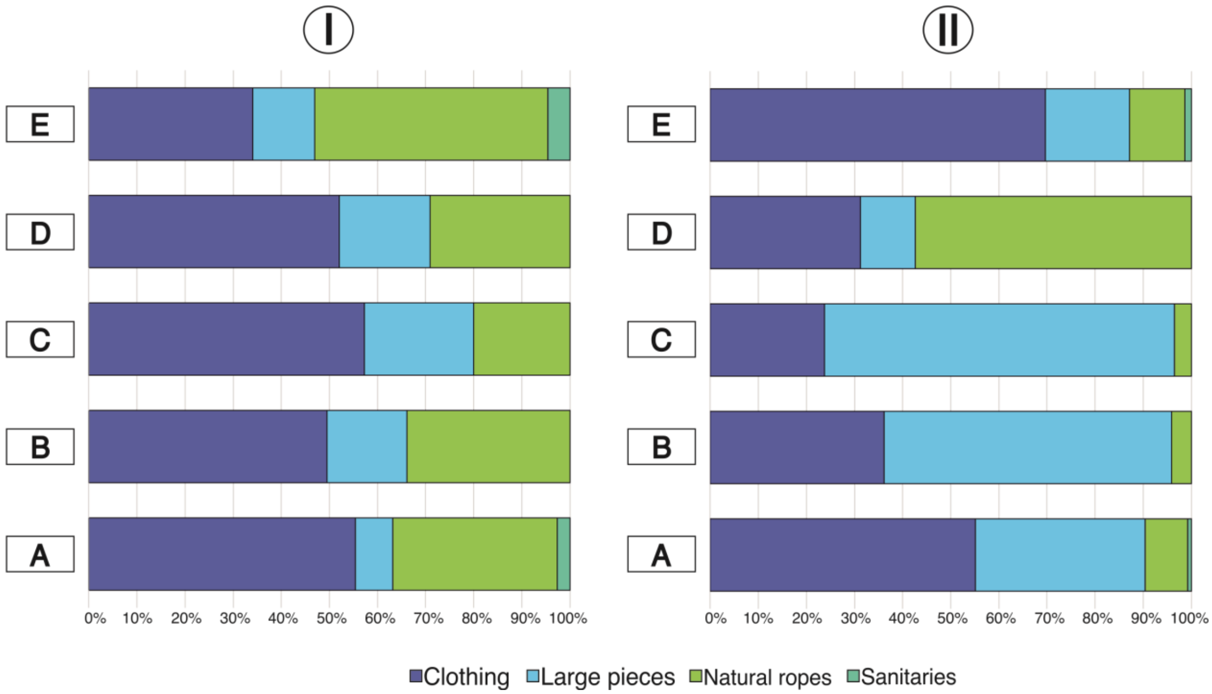
Supplementary Figure 11. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of glass subcategories, per each of the bathymetric strata of the MEDITS protocol.



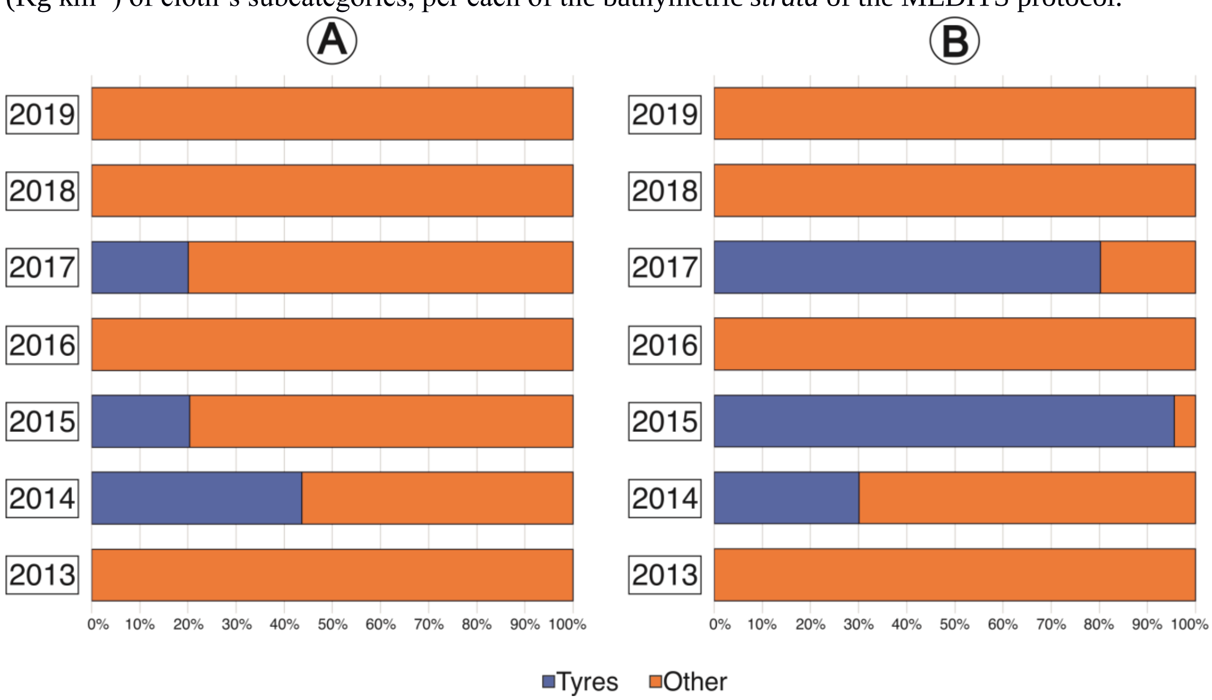
Supplementary Figure 12. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of cloth's subcategories during the study period, comprised between 2013 and 2019.



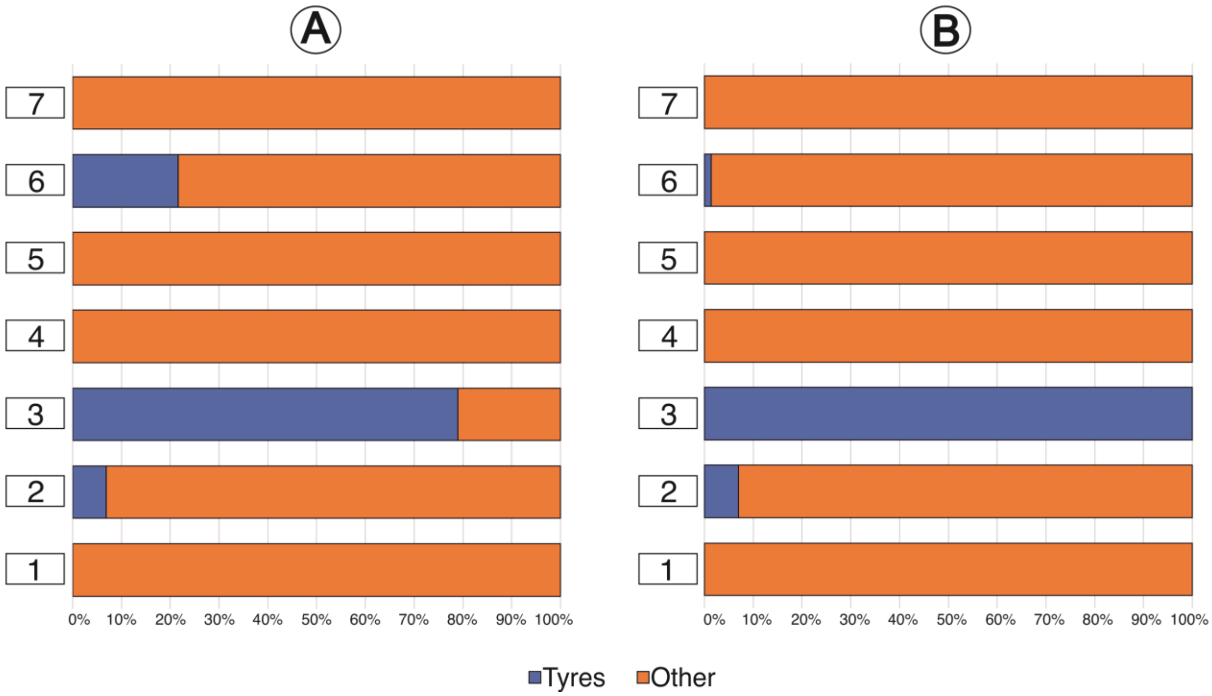
Supplementary Figure 13. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of cloth's subcategories, per each of the geographical sub-areas within GSA 11.



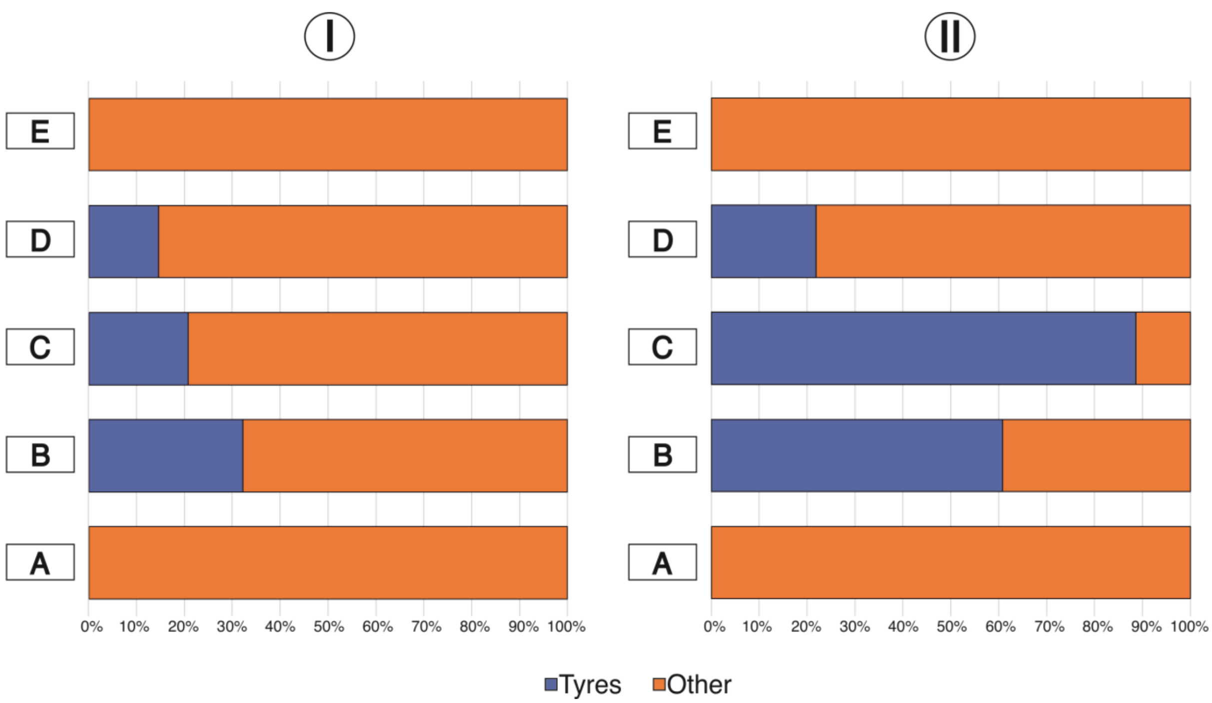
Supplementary Figure 14. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of cloth's subcategories, per each of the bathymetric *strata* of the MEDITS protocol.



Supplementary Figure 15. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of rubber subcategories during the study period, comprised between 2013 and 2019.



Supplementary Figure 16. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of rubber subcategories, per each of the geographical sub-areas within GSA 11.



Supplementary Figure 17. Percentage composition in terms of (A) density (n. items km^{-2}) and (B) weight (Kg km^{-2}) of rubber subcategories, per each of the bathymetric *strata* of the MEDITS protocol.

Chapter 3: Comparative microplastic load in two decapod crustaceans *Palinurus elephas* (Fabricius, 1787) and *Nephrops norvegicus* (Linnaeus, 1758)

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1. Introduction

Since 1950s, plastic production generated ca. 5 billion tons of waste, currently dispersed in the environment (Geyer et al., 2017). It has been estimated that 5-8 Mt of plastic move from land to oceans on a yearly basis, with trillions of plastic items currently floating at sea (Eriksen et al., 2014). However, the abundance of floating litter on oceans' surface was measured to be lower than what has been forecasted by the most conservative models (Cozar et al., 2014; Eriksen et al., 2014). While recent studies proposed that riverine input might have been overestimated (Weiss et al., 2021), it is widely recognized that the sink of objects represent the major explanation for such discrepancy. The existence of an initial floating stage, followed by sink and deposition on the seafloor after a more or less long period of time, renders plastic items capable of reaching also secluded environments such as polar regions and the deep oceans' floor (Jambeck et al., 2015; Peeken et al., 2018; Peng et al., 2020). The most emblematic sign of this is a plastic bag documented at ca. 10,900 m depth in the Mariana Trench (Chiba et al., 2018).

Processes like biofouling or physical weathering can change specific weight of plastic (Kowalski et al., 2016; Zettler et al., 2013), triggering their sink into the water columns and thus, as anticipated, ocean floors represent the final sink for plastic particles (Woodall et al., 2014), as demonstrated by the exponential increase in deposition occurred over the last decades (Brandon et al., 2019). Plastic can also slowly degrade through biological, mechanical and physical processes that cause its fragmentation into smaller particles that are called MicroPlastics (MPs), if their dimension is comprised between 1 μm and 5 mm (Frias and Nash, 2019).

Such dimensional range renders this contaminant particularly suitable for accidental ingestion by marine *biota*, with vagile benthic fauna being particularly exposed compared to other organisms (Bour et al., 2018; Carreras-Colom et al., 2018; Cau et al., 2019a; Murray and Cowie, 2011). The size of MPs particles influences their ingestion and egestion rates and their isolation from tissues of marine organisms, by itself, does not represent a reliable proxy for particle retention (Cau et al., 2020), nor for their accumulation but rather a snapshot of the exposure that organisms experience in the specific environment.

Within the European Union, the Marine Strategy Framework Directive evaluates the environmental status of European seas (MSFD; 2008/56/EC) through 11 descriptors, within which marine litter quantification (and MPs therein) is one of those (Descriptor 10) (Galgani et al., 2013); thus, the necessity and the research for efficient bioindicators is building up constantly across scientific literature (Bonanno and Orlando-Bonaca, 2018; Fossi et al., 2018). This is particularly relevant for the Mediterranean, which is among the most contaminated (or at least most investigated) basins worldwide (Canals et al., 2021). The Mediterranean Sea is estimated to retain 5-10% of the global plastic mass dispersed in oceans (Suaria et al., 2016; Van Sebille et al., 2015), and the resident associated *biota* showed to diffusely ingest MPs, both in the pelagic and benthic *dominium* (Cau et al., 2019a).

Recent scientific literature emphasized how some decapod crustaceans that show a tight association with the seabed are particularly exposed to MPs: this is the case of Norwegian langoustine *Nephrops norvegicus* (L. 1758) and European spiny lobster *Palinurus elephas* (F. 1787). While the former is widely acknowledged as flagship species for MPs contamination across EU waters (Carreras-Colom et al., 2022a; Cau et al., 2019a; Hara et al., 2020; Joyce et al., 2022a), the latter has only very recently been identified as exposed to MPs (and nanoparticles) through a study conducted in the Aegean sea, Greece, where authors stressed the urgent need to provide additional data over a broader geographical scale (Kampouris et al., 2023).

Crustaceans belonging to the family *Palinuridae* are among the most highly priced seafood in the world, and their fishery often represent the backbone of export economy in some regions (e.g., Caribbean countries; Higgs et al. 2016). European spiny lobster *P. elephas* is distributed across the Mediterranean Sea, but also across the eastern part of the Atlantic Ocean, from North Africa to Scotland. Its fishery was first recorded in the 15th century BC, and the popularity of spiny lobsters as *gourmet food* took off in the 19th century and consistently increased till present days, where living specimens of *P. elephas* can be sold at retail prices comprised between 40 and 120€ Kg^{-1} (Cau et al., 2019b; Groeneveld et al., 2013). With these premises, it is not surprising that European spiny lobster is currently classified as “Vulnerable”, by the International Union for Conservation of Nature (IUCN), mostly due to its continuous overfishing (Follesa et al., 2014; Goñi and Latrouite, 2005).

N. norvegicus (fam. *Nephropidae*) is a benthic decapod inhabiting European temperate and cold waters. Similarly to European spiny lobster, langoustine is a millions of Euros worth fishery resource in Europe, since it is highly appreciated as *gourmet* seafood either, with a retail price comparable to that of other crustaceans such as lobsters, spiny lobsters or deep sea shrimps (Cau et al., 2019b; Ungfors et al., 2013). Langoustine is a key element in muddy bottoms trophic webs, and it shows a wide bathymetric distribution (up to 800 m depth), mostly restricted to deep waters in the Mediterranean area (>200 m depth). The continuous scavenging behaviour on the seabed allows langoustines to interact with other benthic species, but also with sediment-water fluxes and resuspended sediment (Cristo et al., 1998); that could potentially expose *N. norvegicus* to accidental ingestion of MPs. It has thus been pointed as a reliable bioindicator of MPs contamination of the deep seabed (Carreras-Colom et al., 2022b, 2022a; Cau et al., 2019a; Franceschini et al., 2021; Murray and Cowie, 2011).

Contrarily to *N. norvegicus*, within the Mediterranean basin, *P. elephas* dwells in shallow waters up to 200 m depth (Goñi and Latrouite, 2005; Groeneveld et al., 2013), while both species share similar trophic habits since *P. elephas* is considered as well as an omnivorous and scavenging species, that renders European spiny lobster at least as exposed as *N. norvegicus*. Moreover, with very few exceptions (Cau et al., 2020; Joyce et al., 2022b), all available information on MPs occurrence in these species reflects the isolation of these particles from their stomach contents or through the digestion of the entire digestive apparatus (Avio et al., 2020; Hara et al., 2020; Joyce et al., 2022a, 2022b; Murray and Cowie, 2011; Welden and Cowie, 2016a). This prevents to establish if and how benthic crustaceans can play a role in modulating the environmental fate and bioavailability of MPs through the ingestion, mechanical fragmentation, and egestion process, as recently documented by two studies performed in controlled and wild conditions (Cau et al., 2020; Dawson et al., 2018).

The present study aims to investigate and compare MPs ingestion in the European spiny lobster *P. elephas* and the Norwegian lobster *Nephrops norvegicus* sampled from coastal and deeper waters from Sardinian waters, in Italy. As anticipated, these species are commonly and extensively fished for human consumption and are greatly exposed to MPs ingestion, while exhibiting similar feeding strategies in two segregated bathymetric distribution range. Thus, obtained results were expected to provide novel insights as to whether ecologically similar organisms might suffer from different exposure and consequent ingestion of MPs.

2. Materials and methods

2.1. Specimen collection and treatment

For *N. norvegicus*, samples were collected from 2 sites around the Sardinia island in 2019 (Fig. 1), in the framework of the MEDiterranean International Trawl Survey (MEDITS), at depths comprised between 412 and 627 m. A total of 15 stomachs and intestines were extracted. For *P. elephas* samples were collected from 3 sites from the western coast of Sardinia in 2020 (Fig. 1), from both artisanal and professional fisheries operating using trammel nets and trawlers, at depths comprised between 25 and 90 m. A total of 14 stomachs and intestines were extracted. Ranges of biometric data (and sex ratio) of analysed specimens were Carapace Length (CL) 25.2 – 41.2 mm for *N. norvegicus* (9 males and 6 females), and CL 73 –117.9 mm for *P. elephas* (6 males and 8 females). For *P. elephas*, stomach and intestine weight were recorded, separately and individual weight, which ranged from 350 gr to 202 Kg.

2.2. MPs extraction and characterization

Specimens were collected and transported in the laboratory using an ice box and placed in cold storage (-20 °C) to avoid the risk of contamination from sampling activities. Samples were thawed at room temperature and each specimen was dissected following the standard dissection method to remove the stomachs and intestines, which were then placed separately in aluminium foils and stored at -20 °C, until analysis. To assure contamination free samples, necessary precautions were taken when handling and processing the samples to prevent aerial and solvent contamination with MPs. Digestion of the digestive tract was carried out using a 10% potassium hydroxide (KOH) at 40 °C for 48 h, for both species as recommended by Hara et al. (2020). The MPs extraction procedure was based on a density-separation step through a NaCl hypersaline solution (density 1.2 gr cm^{-3}), where supernatant solution was collected through glass beaker. For *P. elephas*, since stomachs contained inorganic and organic detritus, supernatant solution was subjected to mechanical stirring process and a preliminary density separation step through a NaCl hypersaline solution, after the digestion process performed with KOH. The supernatant collected from both the species was filtered using a vacuum pump (VCP130) through 47 mm Sartorius® cellulose nitrate membrane filters (pore size 8 μm). The filter was then transferred onto a labelled petri dish followed by, partial digestion in diluted hydrogen peroxide (15%), sorting and chemical characterization. The method has been validated and standardized on samples spiked with MPs of different types and sizes by (Avio et al., 2015) and used for MPs extraction in the same species targeted by the present study (Avio et al., 2020; Cau et al., 2020, 2019a; Martinelli et al., 2021). When compared with other available methodologies, it showed a recovery yield higher than 90% for particles smaller than 100 μm and 95% for greater ones, with no effects on particle characteristics such as shape or colour.

During sorting, all retrieved particles were observed under a microscope, photographed and categorized according to shape in: i) fragments (small, irregular shaped particles, crystals, rigid, thick); ii) film (irregular shapes, thin and flexible, transparent particles); iii) pellet (cylindrical particles); iv) fiber (elongated, thin, straight particles, frayed ends); v) sphere- like (cubical, sphere); vi) foam (lightweight particles with spongy texture). Once isolated, MPs were measured at their largest cross section under a stereomicroscope. When possible, the length of particles was estimated, image analysis CPCe (Kohler and Gill, 2006). All extracted particles were characterized using a µFT-IR microscope (Spotlight i200, Perkin Elmer) coupled to a spectrometer (Spectrum Two, Perkin Elmer). The measurements were made using the µATR mode. Following back-ground scans, 32 scans were performed for each particle, with a resolution of 4 cm^{-1}. Spectrum 10 software was used for the output spectra and the identification of polymers was performed by comparison with libraries of standard spectra. Polymers matching for more than 70% with the reference spectra were validated, while polymers with a match comprised between 60% and 70% underwent into a more critical interpretation of the spectra (Bour et al., 2018).

2.3. Quality Assurance and Quality Control

The operator was wearing acid green cotton lab coat to identify possible fibres coming from it. Before starting the extractions, and between each process step, benches were cleaned with milli-Q water and all the solutions used were pre-filtered through a nitrate acetate membrane with pore size of 0.45 µm. Glass and metal materials were used whenever possible and rinsed thrice with prefiltered milli-Q water before use and wrapped in aluminium foil when not in use. After rinsing, all containers were covered with aluminium foils, which were also kept during digestion, stirring, decantation and filtration steps. After filtration, membranes were kept in glass petri dishes, previously rinsed with prefiltered milli-Q water. To reduce background contamination cotton lab coats were used all the time, and special attention was paid to limit the wearing of synthetic clothes. NaCl solution was prepared in distilled water and further filtered on cellulose membranes (0.45 µm pore size). Contamination controls were also included (one control for each batch of samples was treated in parallel to samples), consisting of prefiltered hypersaline solution that undertook all the steps of the protocol. Despite such precautions, it was not possible to fully avoid airborne contamination and some textile fibres were found in the control membranes. We then applied total subtraction of items as correction method, based on a spectral similarity and visual characteristics (Kroon et al., 2018). In brief, fibres were checked with the actual samples and compared, both visually and spectrally. Such fibres were considered as external contamination and they were not included in the dataset. These potential extraneous contaminants form a visual and spectral contaminant library against which all sample items and when particle matches a contaminant or control library item with > 80 % spectral similarity and visual similarity (i.e., same colour, shape, texture), the particle is removed from the dataset. This correction method provides a count of total sample particles minus items confirmed to be contaminant particles.

2.4. Statistical analysis

PERMutational ANalysis Of VAriance (‘PERMANOVA’; Anderson et al. 2008) based on Euclidean distance resemblance matrixes (untransformed data) was used to test for significant differences in MPs polymeric composition among the two investigated species, which was used a single factor (2 levels, fixed) as unique source of variation. The n. of particles ind^{-1} of each polymer type isolated as response variable. Differences in the number and size of particles among the two species were tested using the Mann-Whitney test. Moreover, within each species, using the same statistical routine, we tested for different contamination (both in terms of number of particles and size) between stomach and intestine. The PERMANOVA was used to test for differences in the polymeric composition among the two compartments, in this case using the different compartment (i.e., stomach or intestine: 2 levels, fixed) as unique source of variation. Due to the limited number of samples, it was not possible to test for geographical differences within the sub-region object of the study, nor for bathymetric trends.

3. Results

3.1. MPs in Palinurus elephas

More than 2,000 particles were extracted from the 2 species (>1,300 for *P. elephas* and >700 for *N. norvegicus*) and sorted for the chemical characterization through µFT-IR (Fig. 2). All the 14 specimens of *P. elephas* had MPs their digestive tract (100% of occurrence); more in detail, 13 stomachs and 12 intestines, out of 14 (Fig.3). After data correction, out of the total number of particles isolated, 127 of them were made of plastic, 87 for stomachs and 40 for intestines. The weight of stomachs ranged from 4.2 to 24.9 gr while intestines' weight ranged from a minimum of 0.4 gr to a maximum of 10.4 gr.

The average number of MPs was 9.1 ± 1.75 MPs ind. $^{-1}$, ranging from 3 to a maximum of 25 MPs (Fig. 4) with no significant correlation to the weight of stomachs or intestines and with sex or dimensions of organisms (in terms of weight and/or LC). More in detail, considering only positive individuals, the average number of particles was 6.7 ± 1.3 in stomachs and 3.4 ± 0.9 in intestines. Overall, MPs were detected in all individuals (100% occurrence; Fig. 3), corresponding to a frequency of ~92% in stomachs and 85% in intestine.

There was no significant difference in the size of particles isolated from stomachs and intestines of *P. elephas*, which had overall an average size of 1.63 ± 0.22 mm, with those isolated from intestine (avg. 1.82 ± 0.22 mm) being slightly bigger than those found in stomachs (avg. 1.82 ± 0.22 mm; Fig 5). The smallest particles isolated from stomach were 41 µm and 64 µm in intestine while the largest were 9.7 mm and 6.39 mm, both of which clearly outsize the definition of MPs and fall in the definition of meso-plastics (>5 mm). Out of the total of plastic particles extracted from the 14 individuals, 6 particles were larger than 5 mm, 4 in stomachs and 2 in intestines.

Overall, the size-frequency distribution (Fig. 6) showed how ~50% of the particles isolated were smaller than 1 mm in length, while the most representative size class was the same for both stomach and intestine, which was the range comprised between 1 and 2 mm, accounting for >20% of the total. With respect to the shape, overall, plastic fibers represented the dominant category (62% of the total particles), followed by films (27%), followed by foams and spheres (both 4%) and fragments and pellets cumulatively accounting for the remaining 3% (Fig. 7). Considering stomach and intestine separately, while the general pattern was similar in both compartments, films were more abundant in the stomach, where the relative abundance raised to 35%.

The µFT-IR analysis revealed the presence of 8 polymers, within which the dominant was polyester (PEST, 67%), polyamide (PA, 15%), polyethylene (PE, 6%), followed by polypropylene (PP, 3%), polystyrene (PS, 3%) and other polymers (ethylene vinyl acetate, polyacrylate, thermoplastic elastomer) cumulatively accounting for the remaining 10% (Fig.8). Polymeric composition of particles isolated from stomach and intestine did not show any significant difference.

The analysis of particles' colours showed a wide heterogeneity, with transparent MPs being the dominant category (29% of the total), followed by blue (23%), red (14%) and black (10%) while remaining colours (green, yellow, purple, brown and others) accounted for the remaining 24% (Fig. 9).

3.2. MPs in Nephrops norvegicus

The chemical µFT-IR characterization of extracted particles revealed a total of 48 MPs isolated cumulatively from both stomach and intestine of *N. norvegicus*. Overall, MPs were detected in all individuals (100% occurrence), corresponding to a frequency of ~87% in stomachs and 80% in intestines (Fig.10). The average number of particles (within positive individual) was 3.2 ± 0.45 MPs individual^{-1}, ranging from 1 up to a maximum of 6 MPs individual^{-1} (Fig. 11) without significant differences between stomachs and intestines, which showed an average of 2 ± 0.26 and 1.83 ± 0.24 MPs for stomachs and intestines, respectively

The size classes of MPs ranged from a minimum of 0.10 to a maximum of 1.20 mm. Considering isolated particles cumulatively, the average size was 0.44 ± 0.3 mm. Stomach and intestine showed a significant difference in particles size (Mann-Whitney test, $p<0.001$), with the ones isolated from the intestine being significantly smaller (0.28 ± 0.03 mm) than those found in the stomach of the organisms (0.58 ± 0.05 mm) (Fig. 12). More in detail, 91% of the particles isolated from the intestine were smaller than 0.5 mm, while the same specific range of size comprised only 46% of those isolated from the stomach (Fig. 13). The overall pattern of shapes showed the dominance of fragments (56%), followed by fibers (36%) and films (8%) (Fig. 14), with no significant difference among shapes observed within stomachs and intestines.

Overall, 6 typologies of polymers were identified (Figure 15): PE and PEST were the more represented (24% and 39% respectively), followed by PP (12%), PS (9%), PA (5%), while PU, acrylic polymers, Ethylene-vinyl acetate (EVA), silicon and copolymers cumulatively accounted for ca. 9% of total polymers. There was no significant difference in polymeric composition among stomach and intestine. The most dominant colour was transparent (59% of total particles), followed by black (23%) and white (6%); green, yellow, and red particles cumulatively accounted for the remaining 6% (Fig. 16). The comparison of the two species confirmed a significantly higher number of MPs in *P. elephas*, compared to *N. norvegicus* (M-W, $p<0.001$), and also a different polymeric composition (PERMANOVA, $p<0.001$, Table 1).

4. Discussion

The ingestion of MPs by marine organisms is widely documented and patterns of potential transfer through trophic webs are increasingly being documented. Because of the ubiquity of plastic contamination (and MPs in particular) that encompasses all compartments of the marine environment from sea surface to the sea bottom, it is complicated to identify efficient and reliable bioindicators. This is also confirmed by the consistent increase of scientific literature aimed at documenting contaminated organisms that potentially could be adopted as surrogate descriptors of MPs contamination: sharks, jellyfish, crustaceans, mammals and fishes (Bray et al., 2019; Carreras-Colom et al., 2022a; Fossi et al., 2018; Macali et al., 2018; Sbrana et al., 2022). Similarly, to fishermen using different gear to target different species according to their peculiar features, different bioindicators are representative of specific compartments of the marine environment, according to their biology and ecology. In our case, we focussed our attention on *P. elephas* and *N. norvegicus*, which are typical inhabitants of Mediterranean benthic environments across a very wide bathymetric range that goes from few meters to ca. 200 m depth in case of *P. elephas* and from ca. 200 m up to 800 m depth in the case of *N. norvegicus*. While showing different movement patterns, with langoustines being more static compared to spiny lobsters (Follesa et al., 2015; Mulas et al., 2022; Sbrana et al., 2019), the two species share the same scavenging behaviour, which has been highlighted as the trophic strategy that most likely expose benthic organisms to the accidental ingestion of MPs (Andrades et al., 2019).

Our results confirmed these species as highly exposed to MPs ingestion, with an occurrence of particles in 100% of analysed specimens. Nonetheless, the number of MPs observed in *P. elephas* was much lower, up to one order of magnitude, compared to those reported in the only available study that documented ca. 250 MPs ind^{-1} in samples of this charismatic species from NW

Aegean sea (Kampouris et al., 2023). The two studies showed similar polymeric composition, with different abundance of PA and PVC as principal difference, that could be likely representative of different local contamination in investigated areas and sites. Scavenging crustaceans are known for being representative of local contamination and the different polymeric composition of isolated particles compared to Aegean samples, might suggest that different quantities and qualities of polymers characterize Sardinian benthic habitats. With respect to the extraction protocol, both the present study and Kampouris et al. (2023) used a pre-digestion and density separation based approach, which has been used on several organisms, including decapod crustaceans (Avio et al., 2020; Cau et al., 2019a): the slight adaptations to the peculiar necessities of MPs extraction in *P. elephas* (e.g, a further density separation step for full stomachs with lot of detritus), would hardly justify such discrepancies.

The spiny lobster *P. elephas* lacks a significant body of literature on MPs contamination, since less than 100 specimens have been processed so far in the whole Mediterranean, making difficult to establish if MPs contamination of this species in the Mediterranean area can be as heterogeneous as per other crustaceans such as *Aristeus antennatus*, *Aristaeomorpha foliacea* or *N. norvegicus*, with very different levels of MPs ingestion according to the geographic areas and sites (Carreras-Colom et al., 2022a, 2018; D’Iglio et al., 2022; Hara et al., 2020; Joyce et al., 2022a).

As previously observed in the Greek study (Kampouris et al., 2023), our results confirmed that the number of MPs retrieved in *P. elephas* is not influenced by how empty or full are the stomach or intestine, weight or dimensions of individuals, nor the total weight of the specimen. Interestingly, we also observed large pieces piece of fishing nets (i.e., up to 6 cm) in the stomach of a specimen collected by means of trammel nets. That specific individual (sample id=2; Fig. 3) was the one showing the highest n. of particles ind^{-1} (n=25), with red particles of polyamide being dominants (likely fragmented from the ingested net), supporting the intuition that fishing gears can easily become a source of plastic particles ingestion (Fig. 17), beside their documented destructive activity in case of lost fishing gears (i.e., ghost fishing, Cau et al., 2017; Dominguez-Carrió et al., 2020).

Contrarily to spiny lobster, scientific literature has documented *N. norvegicus* contamination across different areas and bathymetries, both in the Mediterranean (Avio et al., 2020; Carreras-Colom et al., 2022a; Cau et al., 2019a; Martinelli et al., 2021) and in the Atlantic (Hara et al., 2020; Joyce et al., 2022a; Murray and Cowie, 2011), with Mediterranean samples being generally more contaminated than their Atlantic counterpart. Even within Mediterranean basin, langoustines can show variability in MPs abundance (i.e., n. part ind^{-1}), according to different study areas, different anatomical parts from which MPs are isolated (e.g., Martinelli et al., 2021), different extraction protocols and/or focus of the study (e.g., microfibers; Carreras-Colom et al., 2022a). Results here presented (average 3.2 ± 0.45 part ind^{-1}) are lower in abundance, when compared with other studies from Sardinian sea: 5.5 ± 0.8 MPs (Cau et al., 2019a) and 4.8 ± 0.76 (Cau et al., 2020), showing however similar or higher occurrence rates (>80%). It is worth of mention, however, that above mentioned studies reported average values that were representative of different study areas and were based over a larger sample number. Langoustines from Catalan sea showed a larger number of particles (i.e., fibres) in their stomachs (7.60 ± 12.01 st.dev, ind^{-1}; Carreras-Colom et al., 2022a), while the two studies conducted in the Adriatic region showed similar or higher occurrence but contrasting results in terms of abundance, with one study reporting lower contamination (1 ± 0 part. ind^{-1}; Avio et al., 2020) and the other higher contamination (4.9 ± 2.4 part. stomach^{-1}; Martinelli et al., 2021).

The two crustacean species of this study have similar feeding strategies and prey preferences, but different trophic behaviour: *P. elephas* is capable of moving for long distances (Follesa et al., 2015; Mulas et al., 2022) and is representative of both rocky and muddy habitats due to its migrations and opportunistic feeding strategy, while *N. norvegicus* feeds mostly within a small bottom area around its burrows (Sbrana et al., 2019). Because of this, in case both species are collected within the area of their partially overlapping bathymetric distribution, they would essentially provide different information in terms of spatial representativeness for MPs contamination. However, it is relevant to emphasize how the huge discrepancy in available information for these species renders *N. norvegicus* a more informative and reliable bioindicator, since little is currently known on factors (e.g., retention rates of both fragments and fibers) that might influence *P. elephas* contamination. Nonetheless, *N. norvegicus* can be a reliable bioindicator for microfibers contamination, even without extraction protocol (Carreras-Colom et al., 2022a), while this or other potentials for *P. elephas* still need to be determined. Last but not least, technical difficulties arise when processing large sized stomachs like those of *P. elephas*, where often considerable amount of detritus might be encountered. We here stress the need to both extend investigations on *P. elephas* to better understand its peculiarities as bioindicator, as well as for developing effective extraction protocols that can be applied for monitoring purposes over a large number of samples.With respect to the polymeric composition, most of MPs extracted from both *N. norvegicus* and *P. elephas* were composed by PE, PES and PP confirming previous observations that highlighted packaging materials and textile products as the major source of exposure for benthic organisms.

The peculiar gastrointestinal tract of *N. norvegicus* can act as a bottleneck for ingested MPs (Welden and Cowie, 2016b), with larger ones being retained and accumulated in the stomachs that are not designed for cutting flexible and resistant filamentous materials such as fibers (Carreras-Colom et al. 2022a). However, smaller particles can be easily egested, even microfibers, where a size-dependent egestion can occur (Joyce et al., 2022b). Recent evidence also documented that the action of the gastric mill of langoustine can be responsible for the fragmentation and re-distribution of smaller 'secondary' MPs in the environment, thus modulating and extending their environmental path (Cau et al., 2020). Since the gastric mill is a common feature of these species, we tested if also *P. elephas* could eventually modulate the environmental fate of MPs in benthic environments.

Our results do not support this hypothesis for spiny lobster since particles were significantly larger than those found in *N. norvegicus* but did not show any significant difference among stomach and intestine, with the latter being slightly larger than those in the stomachs. Results here presented are the first available on the extraction of MPs from the two parts of the digestive trait of *P. elephas* and, despite being based over a limited number of samples, suggest that biologically mediated fragmentation of MPs particles might not occur in *P. elephas*. On the contrary, the significant differences in particles size between stomach and intestine of *N. norvegicus* corroborated the hypothesis described in Cau et al. (2020).

5. Conclusion

` In conclusion, we confirm and further extend the awareness of the high exposure of these crustaceans to MPs, rendering spiny lobsters and langoustines either valuable bioindicators that belong to the most important stocks in the FAO Major Fishing Areas of European competence, but also species with socio-cultural relevance within Mediterranean and EU communities. Being regarded as gourmet food and being also amongst the most charismatic, flagship species for citizens, they could trigger and enhance environmental awareness and consciousness of the vastity of the impact derived from plastic contamination (Cau et al., 2019a; Kampouris et al., 2023).

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7. Figures and Tables

Tables

POLYMERIC COMPOSITION				
P. elephas vs. N. norvegicus				
Source	df	MS	Pseudo-F	P(MC)
Species	1	79.78	8.82	0.001
Residual	50	9.042		
Total	51			

Table 1 Output of the PERMANOVA routine, testing for differences in the polymeric composition of the particles retrieved from the specimens of *N. norvegicus* and *P. elephas.*

Figures

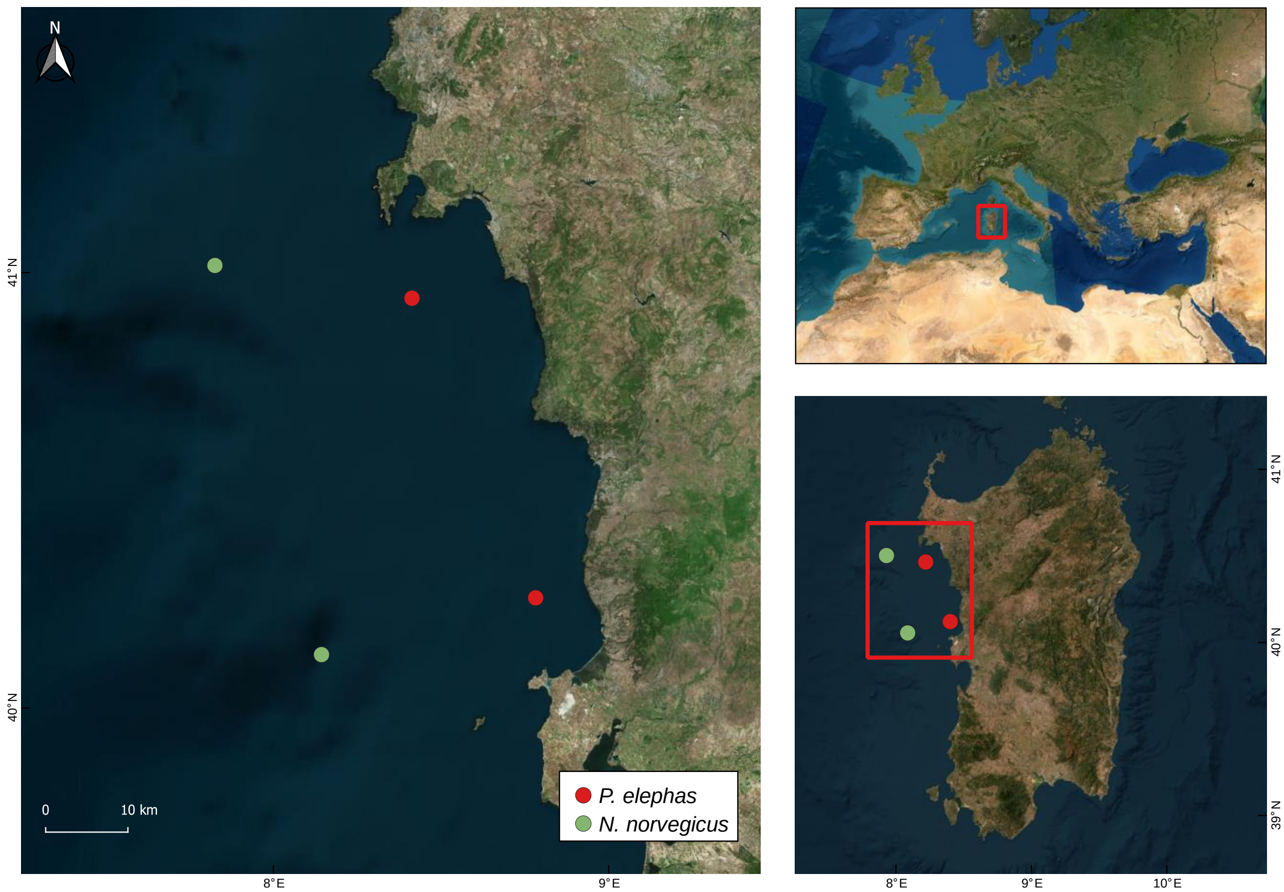

Figure 1. Map of the study area where specimens of *N. norvegicus* (green dots) and *P. elephas* (red dots) have been collected. Bottom-left white bar represents a scale of 10Km.

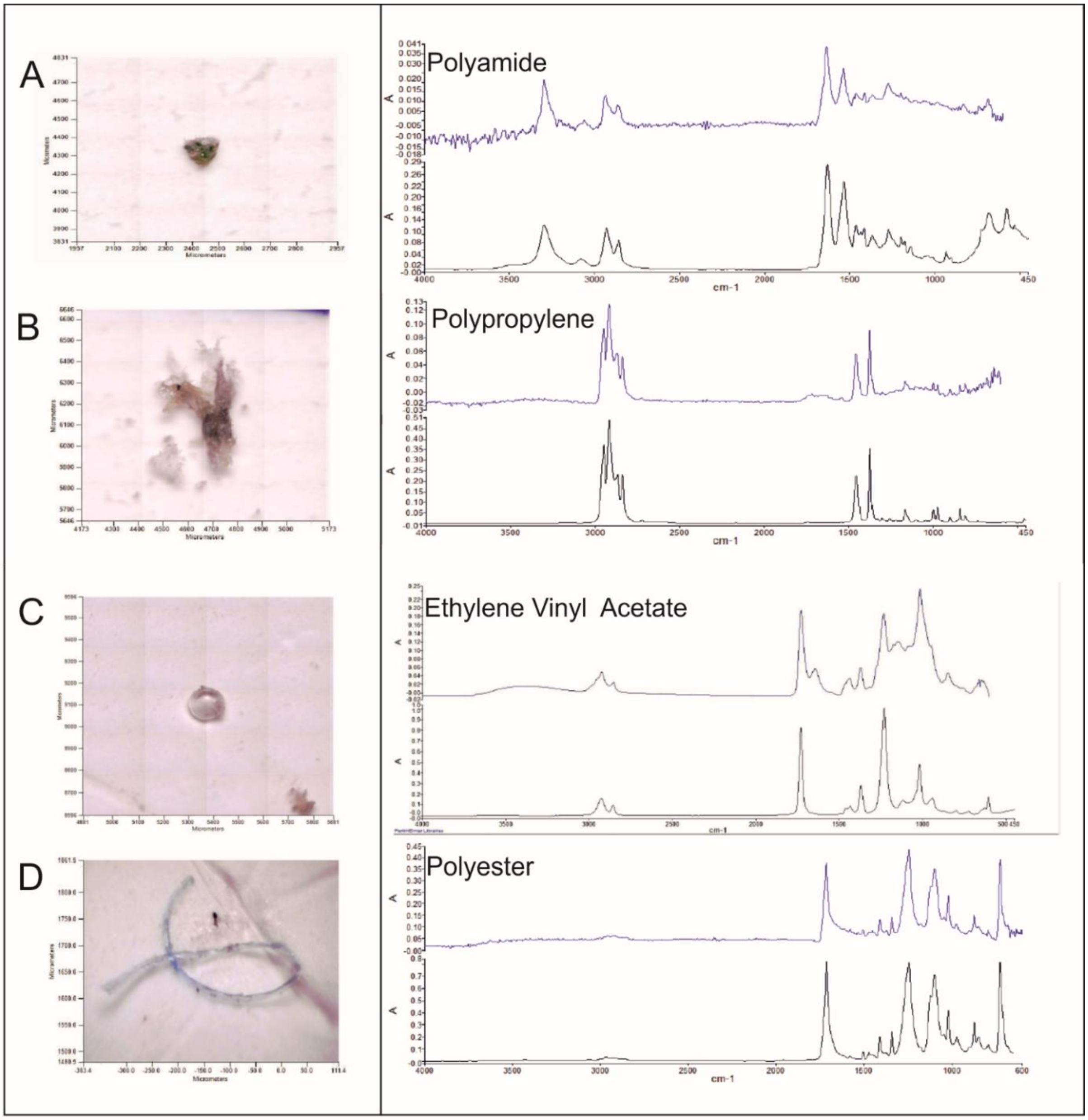

Figure 2. Examples of MPs extracted from *P. elephas* and *N. norvegicus* and corresponding µFT-IR spectra. (A) polyamide fragment; (B) polypropylene particle, (C) ethylene vinyl acetate sphere, (D) polyester fiber. The blue lines represent the characterized particles, while dark lines correspond to the reference spectra.

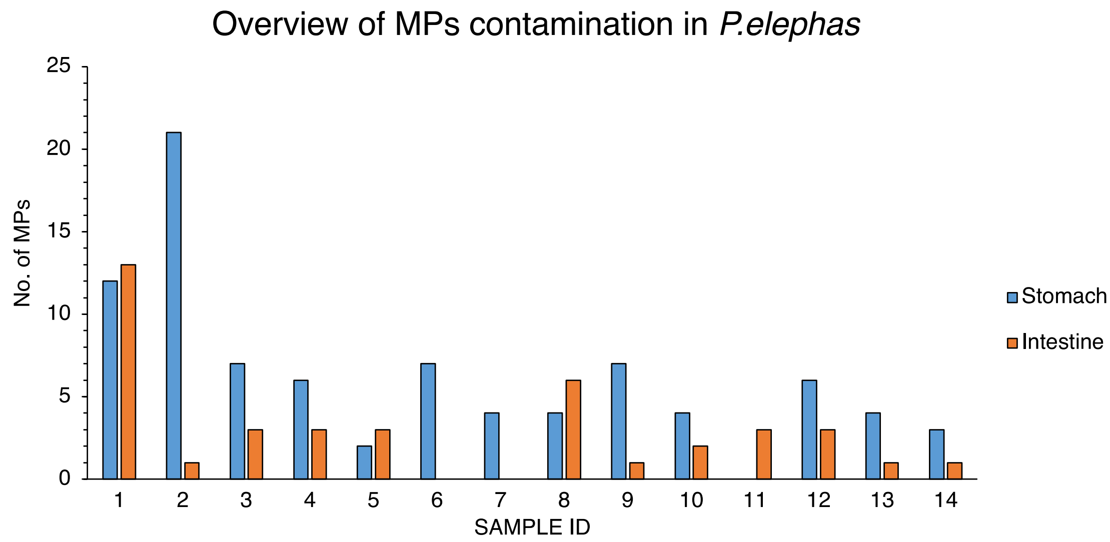

Figure 3. Histogram showing the number of MPs isolated from stomach and intestine of *P. elephas* considered in the present study.

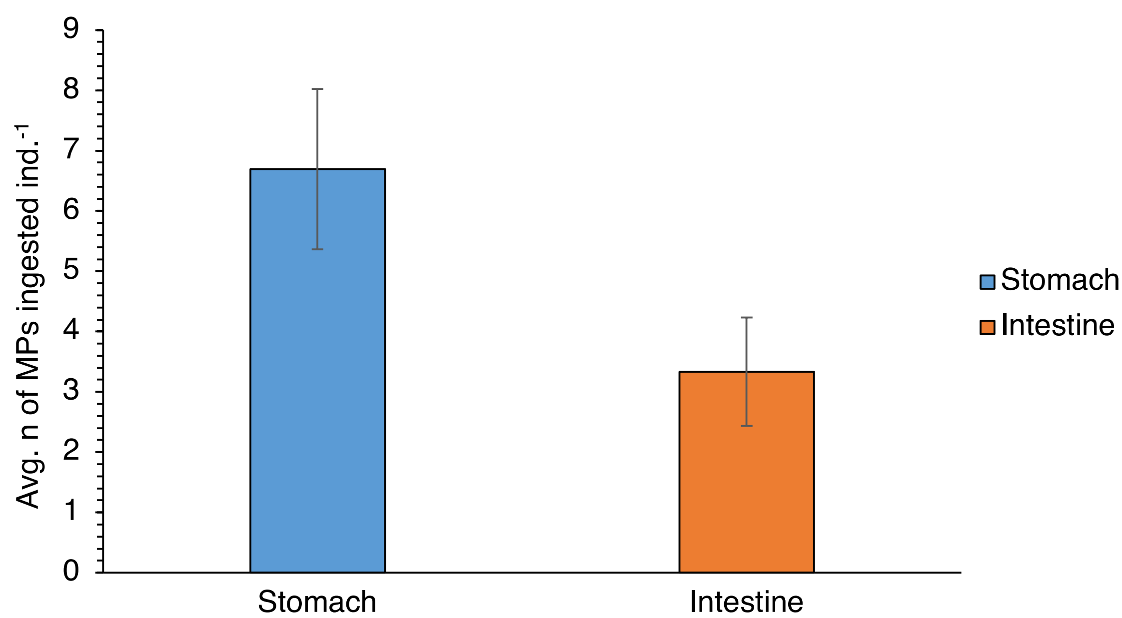

Figure 4. Histogram showing the average number of particles of MPs (± st. err) isolated from stomachs and intestines of *P. elephas*.

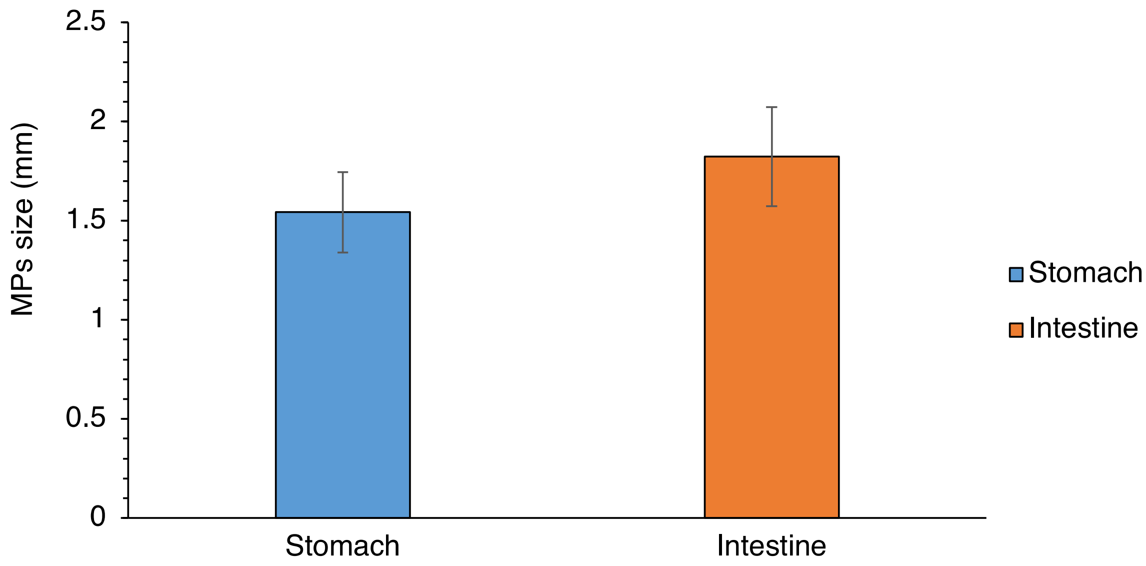

Figure 5. Histogram showing the average size (in mm) of MPs isolated from stomachs and intestines of *P. elephas*

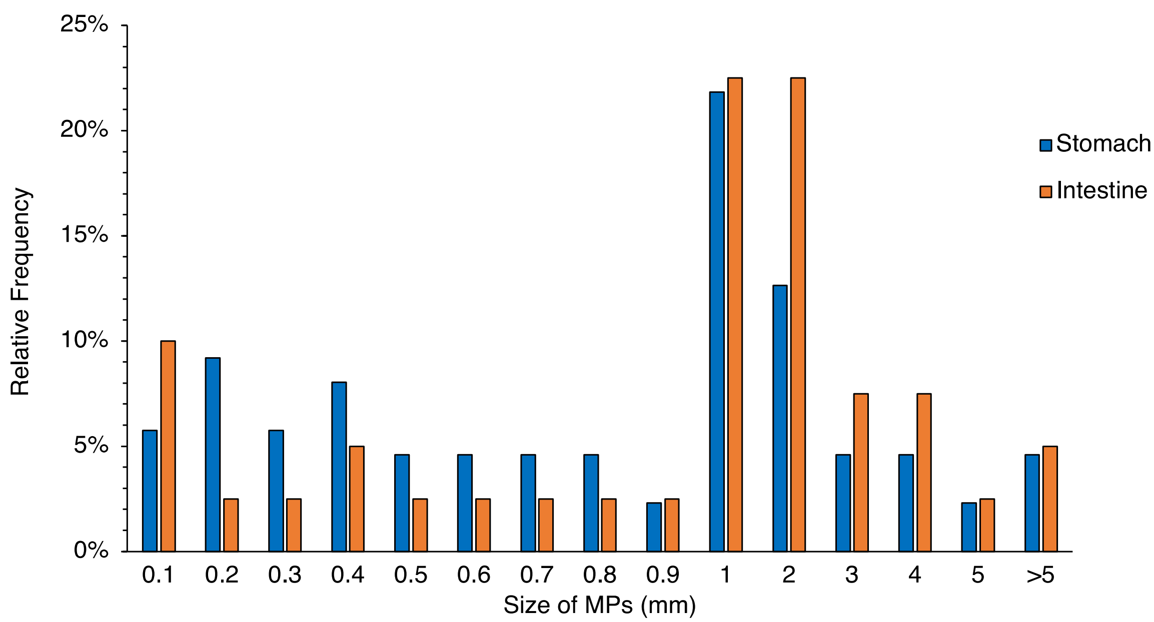

Figure 6. Histogram showing the size (in mm) frequency distribution of MPs isolated from stomachs (blue) and intestines (orange) of *P. elephas*.

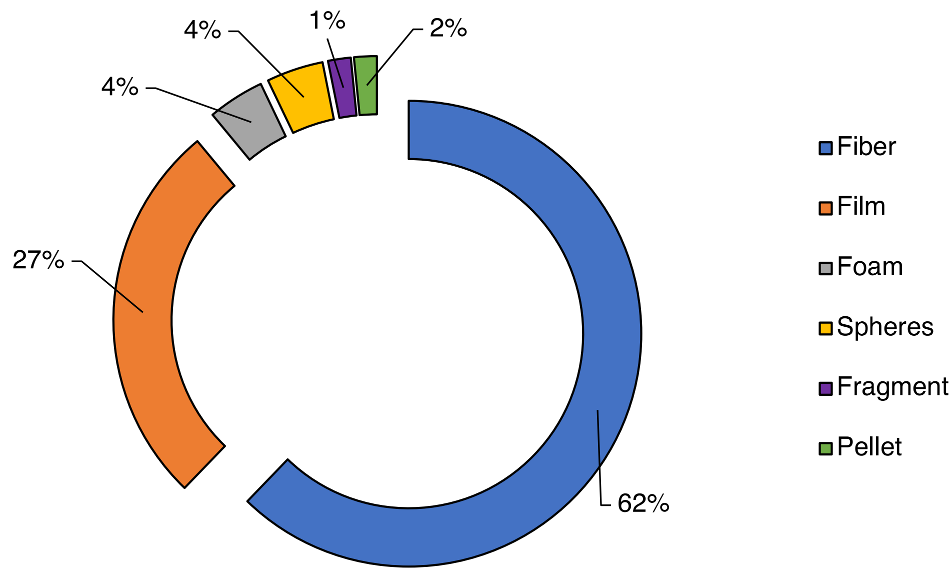

Figure 7. Relative shape composition (%) of MPs retrieved in the gastrointestinal tract of *P. elephas*.

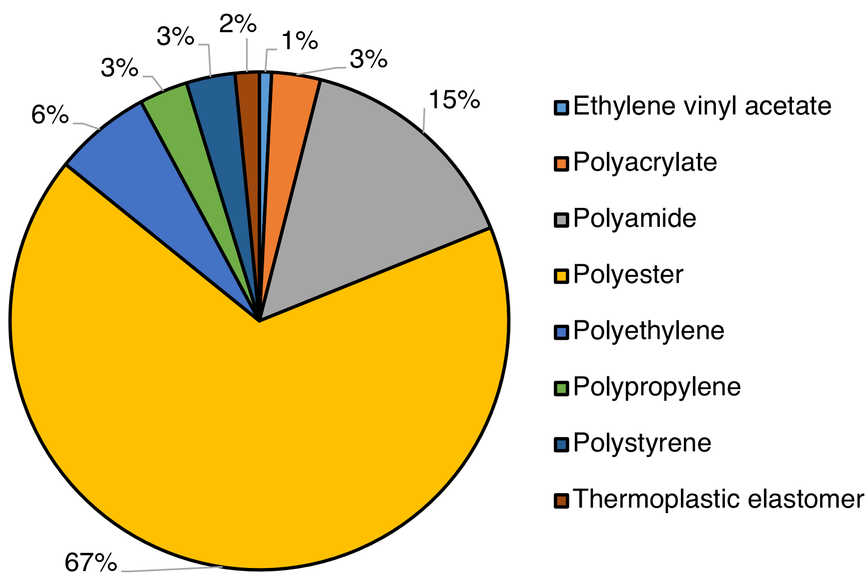

Figure 8. Relative polymeric composition (%) of MPs retrieved in the gastrointestinal tract of *P. elephas*.

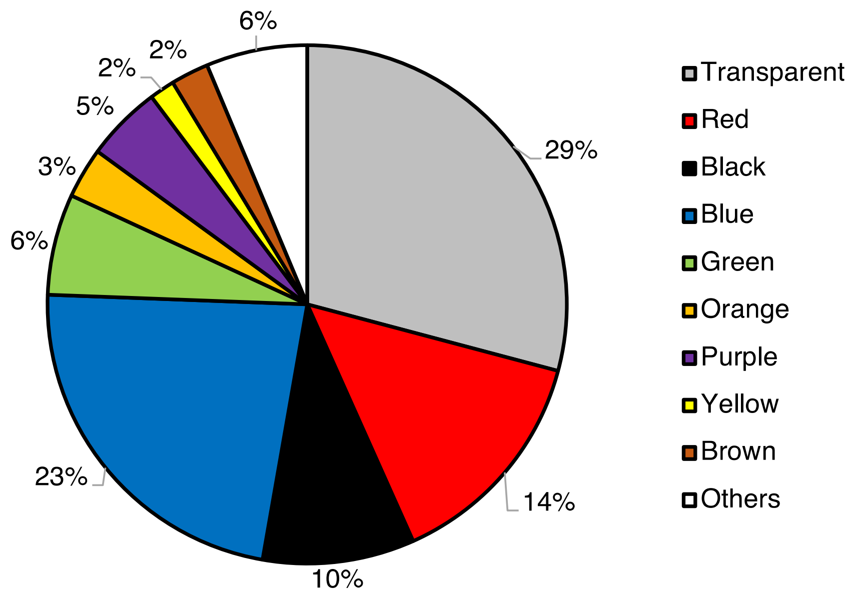

Figure 9. Colour composition (%) of MPs retrieved in the gastrointestinal tract of *P. elephas*.

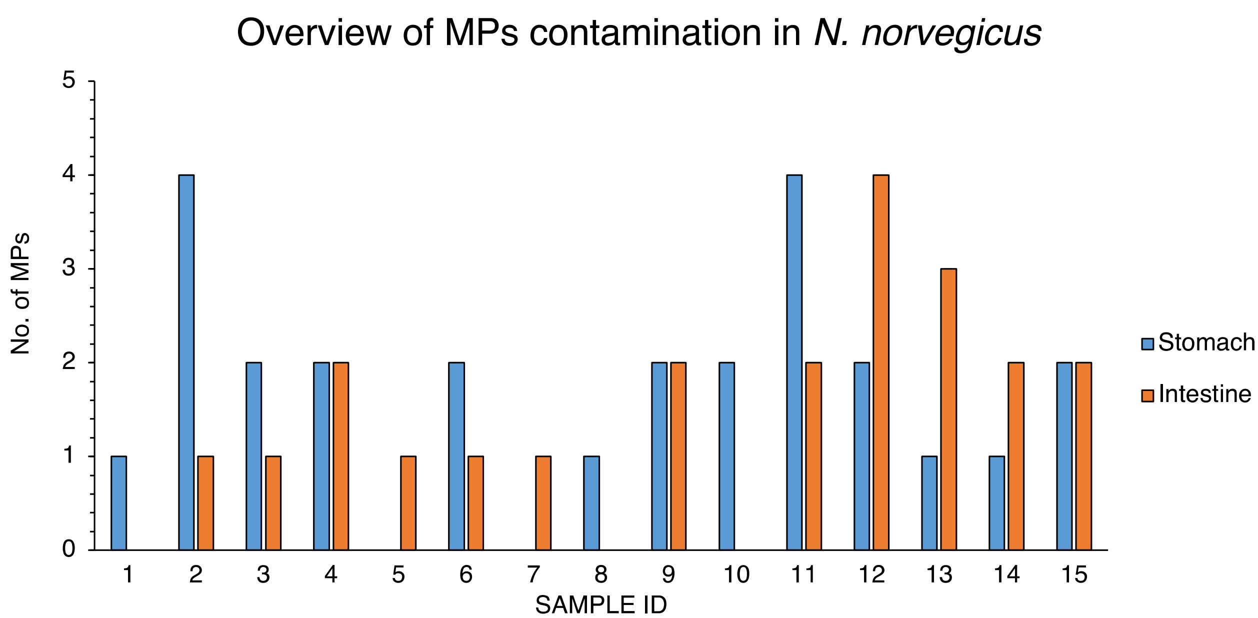

Figure 10. Histogram showing the number of MPs isolated from stomach and intestine in each sample of *N.norvegicus* considered in the present study.

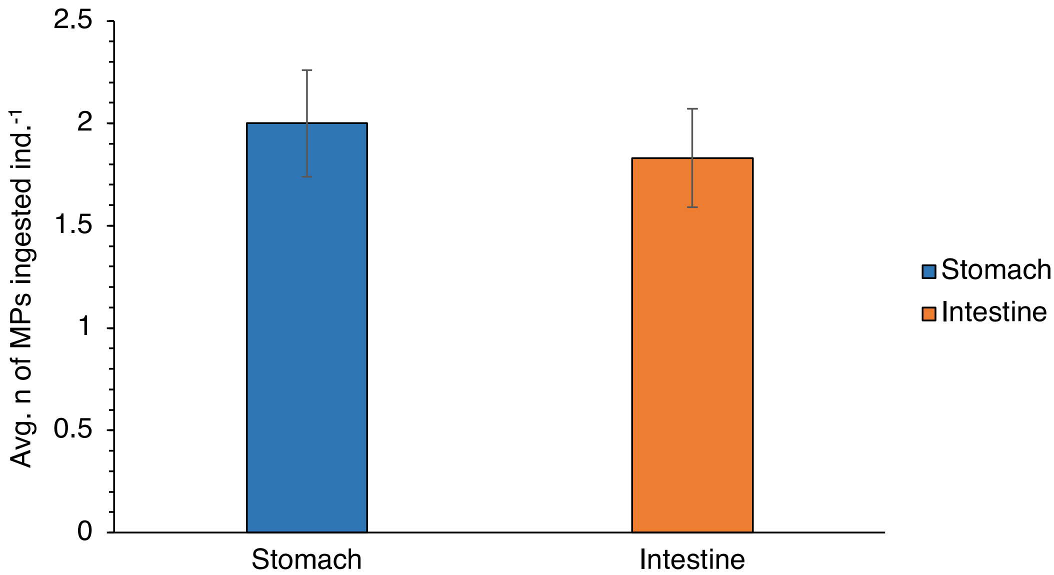

Figure 11. Histogram showing the average number of particles of MPs (± st. err) isolated from stomachs and intestines of *N. norvegicus*.

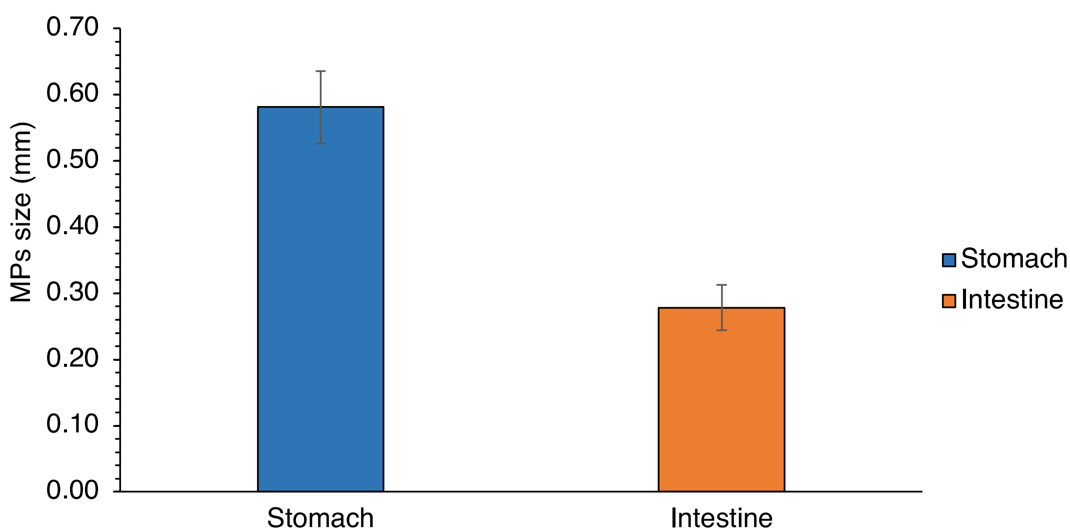

Figure 12. Histogram showing the average size (in mm) of MPs particles extracted from stomachs and intestines of *N. norvegicus*.

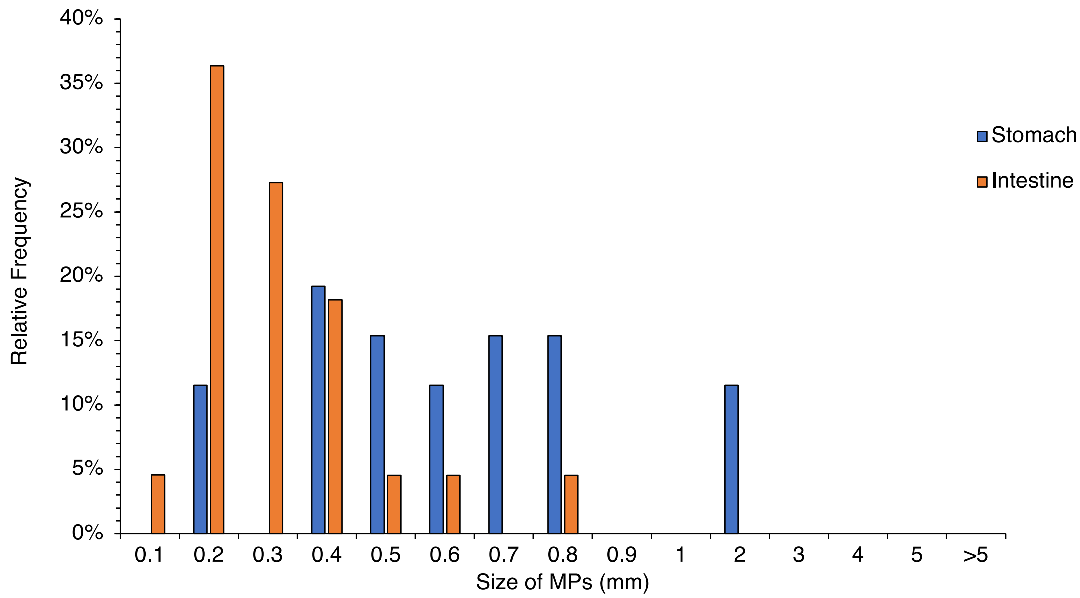

Figure 13. Histogram showing the size (in mm) frequency distribution of MPs isolated from stomachs (blue) and intestines (orange) of *N. norvegicus.*

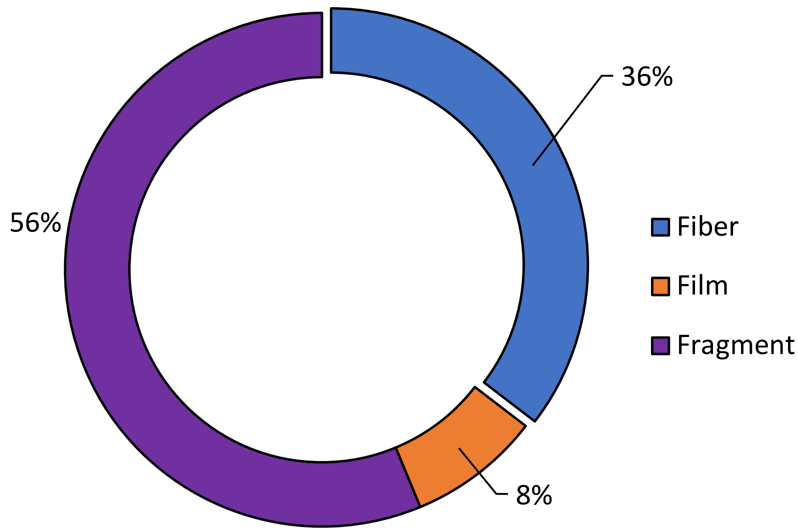

Figure 14. Relative shape composition (%) of MPs retrieved in the gastrointestinal tract of *N. norvegicus*.

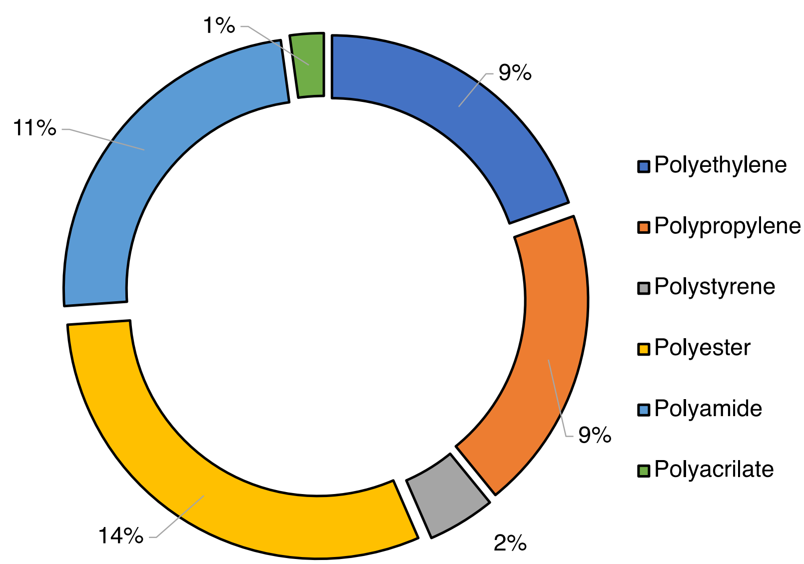

Figure 15. Relative polymeric composition (%) of MPs retrieved in the gastrointestinal tract of *N. norvegicus*.

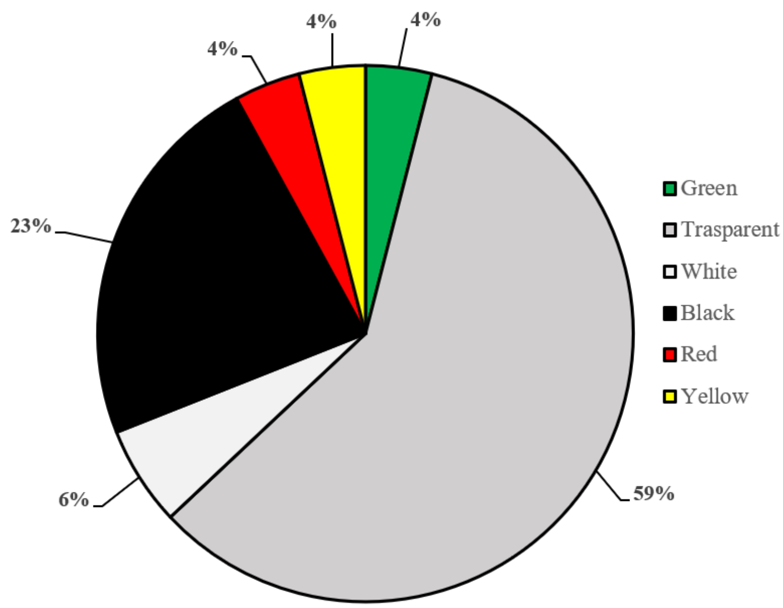

Figure 16. Colour composition (%) of MPs retrieved in the gastrointestinal tract of *N. norvegicus*.

Figure 17. Piece of trammel net in stomach content of *P.elephas*

Chapter 4: Long-term exposure to polyethylene and tire wear particles: Effects on risk-taking behaviour in invasive and native fish

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(Manuscript submitted)

1. Introduction

Plastic production worldwide resulted in the generation of billions of tons of unmanageable waste, the majority of which is currently dispersed across all environmental matrixes (Geyer et al., 2017; Robin et al., 2020). Plastic waste mostly ends up in water bodies such as lakes and ocean, with rivers playing a major role in its transport and transfer across different habitats (McNeish et al., 2018). Scientific community paid particular attention on those plastics that deteriorate and fragment into smaller particles, generating microplastics (MPs) with a dimension comprised between 1 μm and 5 mm (Frias and Nash, 2019). Generally, MPs in rivers originate from land-based sources, i.e., anthropogenic sources due to industrial activities and population densities (Birch et al., 2020). MPs can be found in soil, atmosphere, marine and freshwater sediments, surface or ground water and *biota* (Boyle and Örmeci, 2020; Hidalgo-Ruz et al., 2012; Kim et al., 2015; Lee et al., 2013; Liu et al., 2021; Robin et al., 2020). With respect to the latter category, MPs can be accidentally ingested by many aquatic organisms, causing mechanical damage of tissues but also, depending on polymers, physiological and behavioural responses like immune system disturbances, oxidative stress, developmental defects and changes including growth suppression or abnormal feeding selectivity (Au et al., 2015; Besseling et al., 2014; Caccamo et al., 2016; de Sá et al., 2015; e Silva et al., 2016; Espinosa et al., 2019; Rist et al., 2016; Zhang et al., 2022).

Among conventional plastics that dominate the world market, polyethylene (PE) has a major role, PE has several characteristics that made it attractive in trade: low cost, ease of manufacture, chemical resistance, processability, flexibility, versatility, toughness, and excellent electrical insulation (Bardají et al., 2020). PE reached a widespread use since 1950s in packaging products with usually short shelf life like plastic bags, bottles, cups, containers. Thus, it is not surprising that represent one of the most recurrent type of MPs retrieved in aquatic environments, regardless of the matrix of dominium explored (Kumar et al., 2021).

Tire wear particles (TWPs) are generated from the mechanical abrasion of tire material during use on roads and gained considerable attention as part of MPs that contaminate aquatic environments (Wagner et al., 2018). TWPs get dispersed in aquatic environments through various paths such as wastewater, road water runoff, and atmospheric deposition (Kukutschová et al., 2011; Sugiura et al., 2021; Ziajahromi et al., 2020). It is estimated about 500,000 tonnes TWPs are generated annually in the EU alone and 50–140,000 tonnes are released annually in EU surface waters (Hann et al., 2018; Page et al., 2022). TWPs are made of a complex mixture of rubber (e.g., styrene butadiene), embedded asphalt, pavement minerals and other minerals (copper and zinc) (Eisentraut et al., 2018; Panko et al., 2013). Leachates of TWPs have been the main focus for ecotoxicity studies, while studies based on real TWPs that involve both the physical and the chemical component of the contaminant are almost inexistent and should be prioritized (Wagner et al., 2018). TWPs can also carry metals (e.g., copper, among others) that can affect both biological (i.e., reduced growth) and ecological traits (i.e., prey-predator interactions, behavioural patterns or swimming performance (Siddiqui et al., 2022; Gosavi et al., 2020).

While a great effort has been made over the last years to document the contamination from MPs in aquatic environment, knowledge on the effects that exposure to specific polymers might have on aquatic organisms is still scant (Bucci et al., 2020). Behavioural changes in response to exposure to pollutants is becoming an increasingly important topic in ecotoxicology (Tosetto et al., 2017) and since both PE and TWPs are more prone to be collected in and transferred through freshwater environments (Wagner et al., 2018), freshwater species represent a perfect target to investigate these neglected consequences (Cunningham et al., 2022). As a general pattern, small amounts of pollutants can induce behavioural changes (Bae and Park, 2014) and despite that fact that changes in animal health may not be noticeable, behavioural change may have severe consequences on animal interactions within the community (Scott and Sloman, 2004), thus allowing to identify and infer about sub-individual effects that eventually can alter population or even communities dynamics along the biological hierarchy.

As a potential cumulative effect of MPs contamination in aquatic environment, "Pollution Resistance Hypothesis postulates that invasive species may have a competitive advantage in environments that are polluted or contaminated with toxic substances”. The hypothesis proposes that invasive species have evolved traits that enable them to tolerate or detoxify pollutants, giving them a competitive edge over native species that may be more susceptible to pollution (Crooks et al., 2011; El Haj et al., 2019; Varó et al., 2015). To address this hypothesis with declining native species and corresponding invasive species, we used native crucian carp (*Carassius carassius*, L. 1758) sharply declining in European waters (Kottelat and Freyhof, 2007; Tapkir et al., 2022) due to invasion of gibel carp (*Carassius gibelio*, K. 1782) (Jeffries et al., 2016; Kottelat and Freyhof, 2007). The invasive gibel carp was imported into Romania in the mid-20th century (Szalay, 1954; Tóth, 1976) and has since spread across the European continent (Kottelat and Freyhof, 2007;

Ribeiro et al., 2015). During the gibel carp invasion, most of crucian carp populations went extirpated due to the higher competitive ability of the invasive carp.

With these premises, the present study compares behavioural and performance responses of two *Carassius* species exposed to MPs of PE and TWPs, to test as to whether invasive gibel carp can show different response to contamination and get better adapted to polluted environment than the native crucian carp. To do so, we exposed model fish species to food pellets contaminated with 0.1% mass of TWPs or PE. We hypothesised that exposure to TWPs and PE would: i) alter fish behaviour towards bolder and risk-taking behaviour, and ii) reduce fish maximum swimming capacity, thus potentially interacting with predator-prey relationship. Finally, we iii) expected that native species will be more affected by TWPs and PE than invasive species.

2. Materials and Methods

2.1. Preparation of microplastics

To be as representative as possible of realistic environmental scenarios, manipulative experiments described in the present work were conducted using particled produced from dismissed commercial Tires and Polyethylene produced from dismissed commercial plastic bottles, both retrieved from aquatic environments during activities of clean-up of rivers from macro-litter. This choice was to guarantee that tyres and plastics underwent through usual usage but also environmental weathering (i.e., photo-, thermo- and mechanical-deterioration). Effects on biota can be caused by physical interactions between particle and organisms, according to particles' size and shape, and by associated compounds released from the particles (Skjolding et al., 2016). Thus, it is crucial to create experimental designs that allow scientists to properly discriminate physical factors (size and shape of particles) from chemicals (i.e., polymeric composition), within the same level of the biological hierarchy (Bucci et al., 2020). To cope with this need, particles of both polymers were manually milled to create irregularly shaped particles, and then sieved to a defined size range, comprised between 70 and 210 µm. This size range was considered relevant for the purpose of the experiment since it encompasses at the same time the most common shape and size range of runoff and/or shredded tires (Charters et al., 2015).

2.2. Pyrolysis GC-IRMS

Tire wear particles were analysed with a micro-furnace pyrolyzer frontier (EGA/PY-3030D) coupled to a GC-MS (ISQ QD, Thermo Scientific, Bremen, Germany) following the method of (Rødland et al., 2022), wherein the pure samples of the TWPs were taken in the quantities of 50 µg ,150 µg, 500 µg for the three injections of the sample, and the optimal amount was 500 µg. The pyrolysis for the sample was conducted in single-shot mode at 650°at a flow rate of 1ml /min. Injections were made using a 20:1 split and with a pyrolyzer interface temperature at 310° C. The selected markers used and the compounds detected are mentioned in Table 1.

2.3. Diet

The feeds were manufactured in a lab at the Institute of Aquaculture and Protection of Waters, University of South Bohemia. A hammer mill grinder (Mistral 50 L, River System SRL, Italy), with an 18-mm mesh, was used to grind commercial feed (C-3 Carpe F, Skretting, Stavanger, Norway). A portion of the ground feed (1000 g) and PE (1 g) or TWPs (1 g) were thoroughly mixed in a container to achieve homogeneity and experimental Groups TWPs 0.1 and PE 0.1. Subsequently, 0.2 L of warm water (40 °C) was gradually added and thoroughly mixed into the feed. Water was added until the mixture did not break apart when handled with the hand. Then the mixture was passed through the mixer, mini pelleting machine (2-mm diameter plate, Bottene, Bottene Fratelli Snc, Italy) and cutter (Cutter LT3, FAC srl, Italy) to form pellets. The pellets were then placed in paper-lined trays and placed in an air oven for 24-105 hours at 55 °C.

The feeds were dried, then the oven was shut off and they were left there until it cooled to room temperature (20 °C). Feeds were stored at – 20 °C in plastic bags until fed to the fish. The control diet contain 0% plastic and it was prepared using the same procedure to have similar surface textures, and to avoid any unforeseen interferences.

2.4. Study animal collection and maintenance

Live crucian carps (n=60) ranging in size from 81-108 mm and averaging 93.53mm ± 6.49 standard deviation (SD) were collected from Pavlov u Herálce (49.5039411N, 15.4285000E) and live invasive gibel carp (n=60) ranging in size from 81-105 mm and averaging 90.95 mm ± 5.7 SD were collected from the same site. To prevent the mortality the fish were carried to the lab in the plastic barrels filled with dechlorinated oxygenated water. Fish were kept in glass aquariums (64 x 60 x 45 cm) filled with 130 L of clean, oxygenated water with 20 °C in the laboratory in groups of ten fish. The photoperiod was 12 h light (9:00 a.m. light on) and 12 h dark (9:00 p.m. light off). The aquariums were fitted with aerators for continuous oxygen and water from all the aquariums was changed after every fourteen days. All fish were allowed to acclimatize for 30 days prior to the experimentation.

2.5. Experimental design

A total of 120 fish according to the similar body weight were distributed in 12 experimental tanks (n=10/tank). The native and invasive species were kept separately into three treatments: i) control treatment, ii) PE treatment, and iii) TWPs treatment with 40 fish for each treatment. The fish were given anaesthesia with MS-222 before tagging, and their standard length (in mm) and weight (in g) were recorded. During tagging, 3-4 scales were removed and a 2-3 mm vertical incision 3 cm posterior to the pelvic fin (Šmejkal et al., 2019), then a passive integrated transponder tag (PIT tag, Oregon RFID, Oregon, USA; half-duplex; length: 12 mm; diameter: 2.12 mm;

weight: 0.1 g; ISO 11784/11785 compatible) was inserted into the body cavity. The fish were given healing time of 30 days before starting the experiment. The fish were exposed for 14 and 60 days to three different treatment diets: control, PE, TWPs and were fed daily at 1% of body weight supplied once a day. Fish were monitored throughout the experiment for any potential indications of poor health status (i.e., feeding behaviour, swimming activity and condition of fins). Physical and chemical parameters of the water: pH and dissolved oxygen (mg/L) were measured weakly basis with a with a CALSENS 1.4 PC software (Aqualabo, France) and manage DIGISENS (OPTOD, Ponsel, France) water quality sensors range through a PC.

2.6. Fish Behaviour Assays

2.6.1 Boldness – emergence time

The scoring behaviour was performed in a rectangular arena (57 cm long × 57 cm wide) filled with dechlorinated tap water. A start box was placed at one end of the arena and air stone was placed for aeration. The start box had a sliding closing on one side, and it was operated by a pulley. Body weight, standard length and total length of each fish were measured a day prior to the experiment. The fish were fed 24h before the experiment. The test fish was removed from the fish tanks using a hand net and placed into a start box using a pulley, the adjustable doors were maintained closed to keep the fish out of the test arena. The fish was allowed to acclimatise for 10 min and later with the help of pulley the doors were gently opened, allowing the fish to come out in the test arena. The videos were recorded for 20 min prior opening the entry doors. To minimize the effects of human observers on fish behaviour a webcam (Logitech C270) was positioned directly above the apparatus and utilized to record videos. Videos were recorded for fish emergence in the test arena and the emergence time was noted when the complete fish (i.e., fish fully visible in the arena) came out of the start box (Tosetto et al., 2017). Highest scores i.e., 1200 seconds were given to the fish which did not appear in the test arena.

2.6.2 Open field test

The open field was represented by a rectangular arena (57 cm long × 57cm wide) and was filled with oxygenated water. The bottom of the apparatus was divided into the centre, which made up 60% of the whole arena, and the peripheral, which made up 40% respectively (Araújo and Malafaia, 2020). Body measurements (weight, standard length and total length) of each fish were measured a day prior of the experiment. The fish were fed 24hrs before the experiments. The fish was individually introduced in the centre of the arena and allowed to acclimatize for 10 min and later recording them using Webcam (Logitech C270) connected to the computer for 20 min using a iSPY software (www.ispyconnect.com). The distance travelled by fish (measured in cm) was calculated as well as the ratio of fish locomotion in the arena's centre to total locomotion during the test period. Video records were analysed in the LoliTrack 5 (Loligo Systems, Tjele, Denmark).

2.6.3. Swimming performance

Evaluation of swimming performance and oxygen consumption was performed in a 5 L (testing section 28 x 7.5 x 7.5 cm) Steffensen-type swimming tunnel respirometer (Loligo systems, Tjele, Denmark) submerged in a buffer tank that was connected to an aerated temperature-controlled 50 L reservoir tank allowing continuous water exchange. The swimming chamber was linked to a buffer tank through a flush pump (5 L_/min, Eheim GmbH, Deizisau, Germany) to ensure adequate dissolved oxygen concentration for swimming performance testing. Flow calibrations were performed using a handheld flowmeter (Flowtherm, Höntzsch GmbH, Waiblingen, Germany). The level of dissolved oxygen in the test chamber was kept above 70% throughout the experiment (Tran et al., 2021). The temperature and dissolved oxygen in the swimming chamber were continuously monitored using a fibreoptic oxygen probe and a temperature probe coupled to the Witrox 1 (Loligo Systems, Tjele, Denmark). Water temperature was maintained at 20 ± 0.1 ◦C. The tank was covered with paper to prevent the disturbance from the outside. The water flow and dissolved oxygen in the swimming chamber were monitored and controlled by the system via the AutoResp software (Loligo Systems, Tjele, Denmark).

2.7. Experiment protocol

One hundred and twenty fish (40 fish/diet group) were used in swimming tests following 24 h without feeding. Standard length (in mm), and weight (in g) were recorded prior to the experiments. Fish were randomly and rotationally selected from the species and treatment groups (single fish was measured at a time) and defined by PIT tag number. Individual fish were transferred in the swimming tunnel and allowed to acclimatize to the test conditions for 10 min with water flow velocity of 10 cm×s^{-1}. The tunnel was closed to avoid the water exchange in the surrounding tank. The initial velocity was set at 10 cm×s^{-1} and stepwise to 5 cm×s^{-1} every two min in stepwise increments with terminating at a maximum of 150 cm×s^{-1} or until the fish is exhausted (stopped swimming). The small increments of velocity and time in our protocol were set to minimize stress of tested fish, the protocol follows Tran et al. (2021). The swimming test was terminated when the test fish remained at the rear grid for more than 10s without any activity. Critical swimming speed was calculated using the equation previously described (Brett, 1964):

$U_{crit} = U_{max} + (T_{max}/T_{interval} * U_{interval})$, where U_{max} is highest velocity recorded at fatigue (cm×s^{-1}); $U_{interval}$ is velocity interval (5 cm×s^{-1}); T_{max} is spent time at fatigued velocity; and $T_{interval}$ is the time interval (2 min).

2.8. Data analysis

Possible effects of species, treatment, initial SL and time of exposure on the behavioural parameters (emergence time, activity and open field test) were analysed using linear mixed effect models (LMMs). Individual fish identity was used as a random intercept in the models to account for the among-individual differences in behaviour. Model selection based on the corrected Akaike information criterion (AICc) was used to identify the most parsimonious model with the lowest AICc and other plausible models (ΔAICc ≤ 2) for the data (Burnham and Anderson, 2002). Four models for each behavioural variable were fit, including the full model with species, treatment, initial SL and the statistical interaction between treatment and time and possible sub-models (behaviour parameter ~ species; species + treatment; species + treatment +SL) were among the candidate models of individual fish behaviour in the experiment. The analyses have been conducted in R (R Core Team, 2022) using packages nlme and jtools (Long, 2022; Pinheiro et al., 2021). To gain better insight in the changing behaviour across different treatments and time of exposure, forest plots obtained using Log-transformed ratios of mean response variables (i.e., boldness, open-field test and swimming activity) in fish fed with contaminated pellet (impact: PE and TWPs respectively) and controls. Forest plots were performed using R Studio (R Development Core Team, 2016), through the meta-analysis packages “metafor” (Quintana, 2015; Viechtbauer, 2010) and “robumeta”; the script has been created by Quintana (2015) and modified using the Log-transformed ratios of means as effect size.

To test whether exposure to TWPs or PE reduced the fish swimming capacity using cm$\times$s^{-1} and body lengths $\times$s^{-1}, a general linear model was constructed with swimming performance as response variables and explanatory variables species, treatment and fish weight.

3. Results

No mortality of fish in experimental conditions was observed. Comparison of LMMs of fish emergence time showed that the most parsimonious model was the most complex with explanatory variables species, initial SL, treatment, interaction of treatment and time and random intercept of fish ID with R^2 of 0.27. Effect of species and SL was not significant in the model, as well as the treatment itself, but emergence time significantly decreased in interaction with time at 60 days being lowest in both PE and TWPs treatments (Table 2, Fig. 1). Similarly, fish behaviour in open field test was best explained by the most complex model with R^2 of 0.36. Species identity was significant in the model and further significant change was observed between time at 60 days and PE with higher utilization of open field in crucian carp than in other groups and time of experiment (Table 3, Fig. 2). In the case of fish activity, the most parsimonious model was simple model with the only explanatory variable species, however, AIC criterion was very similar for all models. The most complex model showed significant effect of time at 14 and 60 for TWPs (Table 4, Fig. 3).

The most complex GLM with fish swimming speed was chosen based on AIC criterion and showed high significance of species on maximum swimming speed ($t = 9.34$, $p < 0.001$; Figure 4) and positive effect of TWPs treatment on swimming speed in gibel carp ($t = 2.22$, $p = 0.03$; Fig. 4). The best model with response variable swimming speed expressed as body lengths$\times s^{-1}$ was GLM with species as explanatory variable ($t = 9.63$, $p < 0.001$).

Forest plots analysis show how, compared to controls, PE contamination led to a significantly negative effect (i.e., decrease) for crucian carp in open field test, after 14 days of exposure (LnR = -0.49 ± 0.28; 95% CI; 95% CI) and after 60 days for boldness (LnR = -0.58 ± 0.28; 95% CI). On the contrary PE led to a positive (i.e., increase) effect in boldness for gibel carp after 60 days (LnR = 0.43 ± 0.46; 95% CI; Fig. 5). TWPs exerted a significantly positive effect for gibel carp in open field test after 14 days of exposure (LnR = 0.20 ± 0.19; 95% CI), and a significant negative effect in boldness after 60 days of exposure (LnR = -0.47 ± 0.46; 95% CI; Fig. 5).

4. Discussion

Our results suggests that, within laboratory settings, sublethal dose of PE and TWPs may cause fish to change their behaviour in direction to active and bold behaviour especially in native crucian carp. In the present study we also obtained highly significant results in case of species on the maximum swimming speed indicating a positive shift caused by TWPs exposure. The swimming speed of the invasive species increased significantly, which indicate that there is eventually a threat to the species interactions since locomotion is being affected by experimental treatments, especially in TWPs. Such induced behavioural changes may lead to more risk-taking behaviour in the wild, having potential repercussions in predator-induced mortality rates (Houston et al., 1993; Hulthén et al., 2017; McCormick et al., 2018). The ecotoxic effect of PE MPs on different fish species has been addressed in a study from laboratory settings, trophic transfer of PE from *Poecilia reticulata* (primary consumer) to *Danio rerio* adults (secondary consumer) resulted in behavioural changes such as deficit in anti-predatory defensive response in organisms of upper trophic level. The study suggested ecological consequences of potential transfer of harmful pollutants through the food chain between vertebrates (da Costa Araújo et al., 2020). The study (Araújo and Malafaia, 2020) suggested that PE MPs accumulation in the tadpole *Physalaemus cuvieri* affects the locomotion ability, anxiogenic behaviour, and antipredator response deficit in anurans exposed to potential predators.

Evidence of Pollution Resistance Hypothesis

Our results also provided support to the "Pollution Resistance Hypothesis", showing that if any species reacted to toxicity of PE and TWPs used in our study, it was native species and invasive gibel carp induced weaker response. Pollution Resistance Hypothesis is likely valid only in selected scenarios: The opposite example can be native *Artemia* species which are extremely resistant to Hg and this prevents invasion of non-native *Artemia franciscana* (Pais-Costa et al., 2020).

Most of available knowledge highlights gibel carp low sensitivity to environmental contaminants and pollution (Gkelis et al., 2006; Kagalou et al., 2008; Perdikaris et al., 2012), while comparative information of these two focal species in relation to pollutants is still missing. Results here presented further corroborate the higher resistance of invasive species to contaminants and provide insights on the potential effects that, synergically with other disturbances, could strengthen biological invasion and replacement in European freshwater ecosystems. While replacement of native fish by invasive species have likely many reasons including competitive abilities of the two species (Tapkir et al., 2022), mostly pronounced decline of crucian carp was observed in degraded and suboptimal habitat such as oxbows of large river systems (Kottelat and Freyhof, 2007; Lusk et al., 2010), which collaborates with Pollution Resistance Hypothesis.

Recorded effects of pollutants on behaviour

Pharmaceutical products and/or complex mixtures of pollutants abetted by abiotic factors such as temperature or pH also affect behaviour and cognitive performance of aquatic ecosystems (Jacquin et al., 2020). Exposure to drugs dissolved in wastewater or agricultural effluents have consequences on behaviour of fish species. Contrary to the belief that oxazepam, an anxiolytic or antidepressant drug can induce reduction in anti-predator behaviour of fish species, Fahlman et al.

(2021) showed no impact of high concentration oxazepam on the anti-predator behaviour of European perch (*Perca fluviatilis*) suggesting development of genetic resistance to the chemical environment. Yet, in another study, European perch cultured in a semi-natural ecosystem did not show any strong behavioural changes such as anti-predation behaviour. The author posits that the difference in fish behaviour in laboratory and natural setting could be masked by abiotic factors such as temperature (Lagesson et al., 2018). Besides, the exposure to oxazepam increased the boldness and activity of European perch under laboratory settings suggesting that ecotoxicological behavioural assays performed under laboratory settings could be used to predict the fish behaviour in their natural lake ecosystem (Klaminder et al., 2016). Similarly, Brodin et al. (2013) reported increased activity, reduced sociality and higher feeding rate in European perch exposed to oxazepam.

Toxicity of PE and TWPs: what is currently known

The compounds leaching from tires into water include a multitude of chemicals (Chibwe et al., 2022; Halle et al., 2021), which has potential toxic effect on fish behaviour and performance. Toxicity studies documented differential effects of leachate originated from worn and new tires, with the latter showing long-term acute toxic effect in freshwater *Hyalella azteca* (Halle et al., 2021). The toxicity of tire leachate has also been addressed in the early life stages of fish such as zebrafish embryos, which displayed reduced swimming performance (i.e., velocity, locomotive behaviour, total distance travelled) in a dose-dependent manner upon exposure to 6PPD and 6PPD-quinone (Varshney et al., 2022). Studies also indicated toxic effect of TWPs leachate on marine phytoplankton, the base of marine food webs, which showed reduced growth rate in three phytoplankton species, namely, the cryptophyte *Rhodomonas salina*, the diatom *Thalassiosira weissflogii* and the dinoflagellate *Heterocapsa steinii* (Page et al., 2022).

In adult zebrafish (*Danio rerio*) ingestion of PE of size 10-600 µm at a concentration of 2mg/L resulted in abnormal behaviours including erratic movement, seizures and downward tail bent (Mak et al., 2019). The ingestion of weathered polyethylene elicited stress defensive response in whiteleg shrimp (*Penaeus vannamei*) (Hariharan et al., 2022). The short term trophic transfer of MPs from beach hoppers to fish did not affect fish behaviour however there was a shift in boldness with fish becoming shyer due to change in diet (Tosetto et al., 2017). The study (Bobori et al., 2022) exposed two freshwater fish species, the zebrafish (*Danio rerio*) and perch (*Perca fluviatilis*) for 21 days to polyethylene microplastics (PE-MPs) sized 10–45 μm and 106–125 μm and the results showed that the smaller in size 10 PE-MPs were more potent toxicants they induced higher oxidative stress, in the liver and gills of both fish.

Besides plastic, other contaminants such as pesticides, pharmaceutical products and/or complex mixtures of pollutants abetted by abiotic factors such as temperature or pH also affect behaviour and cognitive performance of aquatic ecosystems (Jacquin et al., 2020). For instance, influence in swimming behaviour, acceleration and social behaviour due to combined toxicity of PE and glyphosate, a pesticide, was observed in the common carp (*Cyprinus carpio*) (Chen et al., 2022). An adverse effect on the free-swimming behaviour or inhibition of swimming behaviour was reported by Chen et al. (2022). The cefalexin, an antibiotic, alone or in combination with MPs at high temperature (>20 °C) reduced predatory performance in juveniles of the common goby (*Pomatoschistus microps*) (Fonte et al., 2016). Further, polyethylene sorbed with organic contaminants such as benzo(a)pyrene (BaP) resulted in swimming hyperactivity while PE sorbed with benzophenone-3 (BP3) resulted in swimming hypoactivity in larvae of marine medaka (*Oryzias melastigma)* (Le Bihanic et al., 2020).

The research done so far indicates that the effect of TWPs, PE and other pollutants on the behaviour may vary depending on the fish species that are tested. These studies are of ecological importance; the longer exposure to pollutants can disturb the fitness, cognitive, physiology and behaviour pattern of fish from an early stage of life (Jacquin et al., 2020). Accumulation of these pollutants can result in interpopulation divergence and affect ecosystems disproportionately by selectively removing sensitive species. Further, these pollutants can also be transferred via the food chain to higher strata of organisms or predators leading to local adaptation.

Role of behaviour in predator-prey interactions

In the comparison of invasive gibel and native crucian carp, the study showed that although gibel carp had in experiments overall tendency to have higher scores in activity, maximum swimming performance or open field test (i.e., more active and exploratory behaviour), gibel carp also took much longer to emerge from box, suggesting more timid behaviour. During the experiment, there was a tendency to habituation in fish, e.g., reduced time of emergence and increased activity, which could also diminish the visibility of impacts of PE and TWPs on the behaviour. To avoid habituation effects, more natural conditions, such as outdoor mesocosms and telemetry testing can be used to address behavioural changes (Lennox et al., 2021; Šmejkal et al., 2022). Especially crucian carp was found to be susceptible to habituation, and its behaviour was changing in expected direction also in control treatments, and with overall trend of crucian carps being substantially more relaxed in laboratory compared to gibel carps.

Native crucian carps are usually not strong in avoiding predation (Holopainen et al., 1997), and their main adaptation represent inducible morphological change of body depth (Brönmark and Miner, 1992; Domenici et al., 2008; Hulthén et al., 2014), or eventual switch from nocturnal to aperiodic activity (Pettersson et al., 2001). In comparison with crucian carp, invasive gibel carp thrive even in predator-rich environment, such as main channel and oxbows of Elbe River, Czech Republic (Daněk et al., 2012; Lusk et al., 2010). The observed change in the behavioural trends in relation to predation risks in crucian carp needs to be tested before drawing any conclusions, but it may have a potential to further weaken its antipredation skill.

Relevance for species and environment

Despite crucian carp extreme resistance to abiotic factors (Blažka, 1958), native crucian carp is facing the decline due to introduction and competitive abilities of invasive gibel (Jeffries et al., 2016; Kottelat and Freyhof, 2007; Tapkir et al., 2022). Both native crucian carp and invasive gibel carp are well adapted to non-favourable conditions of aging pools of floodplains; e.g. they are extremely hypoxia and heat tolerant species (Antonova, 2010; Bundgaard et al., 2020; Jackson, 2000; Karvonen et al., 2005; Piironen and Holopainen, 1986). Furthermore, gibel carp was found to be surviving ammonia concentrations (12.5 mg L^{-1}; pH 8.6) (Nathanailides et al., 2003) and toxic cyanobacterial blooms (Perdikaris et al., 2012) by storing toxins in the liver and other tissues (i.e. ovaries, brain, intestine, muscle and kidneys) (Gkelis et al., 2006; Kagalou et al., 2008). Such high environmental tolerance to abiotic environmental factors is hypothesized to made gibel carp strong enough to outcompete native crucian carp in its suboptimal and degraded habitat (Kottelat and Freyhof, 2007), rendering gibel carp one of Europe’s most effective invaders (Perdikaris et al., 2012). While crucian carp is known to prefer floodplain channels with rooted floating aquatic vegetation (Sayer et al., 2011; Tonn et al., 1992; Wheeler, 2000, 1981), gibel carp can withstand man-made as well as natural waterways such as streams, rivers, canals, dams, reservoirs, estuaries and ponds (Tarkan et al., 2012; Vetemaa et al., 2005), which are exposed to high level of contamination, and where crucian carp populations dropped in recent decades (Lusk et al., 2010; Lusková et al., 2010).

In the present work, we used a concentration of MPs 0.1% incorporated in food pellets. Published field data on MPs concentrations water or sediment concentrations in freshwater environments provided limited useful information since the vast majority reports concentrations on particle count rather than weight basis (Bellasi et al., 2020; Kumar et al., 2021), which make the comparison with our pellet-based concentrations impractical. Nonetheless it is impossible to estimate the actual exposure to fauna starting from the environmental concentration, despite some insights are available on the fact that bio fouled particles show enhanced chances of misfeeding (Yagi et al., 2022). However, the very few weight-based concentration of MPs, regardless of the matrix, reported concentrations higher or similar to the one used in the present study and we thus believe our exposure concentrations are relevant, particularly for foundational studies like the present one. In relation to studied species, it may be expected that native crucian carp receives slightly higher dose of contaminants than gibel carp, since crucian trophic position is higher (feeding solely on zooplankton and benthic invertebrate prey), while part of gibel carp diet consists of plant material (de Meo et al., 2022; Özdilek and Jones, 2014; Tapkir et al., 2023).

5. Conclusion

Our study focused on long-term effects of sublethal concentrations of PE and TWPs, while at the moment there is more evidence for acute poisoning from road run-off in case of TWPs and negative effects annexed with the accidental ingestion of PE particles. We observed a tendency of crucian carp to reduce its scores related to cautious behaviour (reduced emergence time and increased use of open space in PE treatment). It remains to be tested if higher doses of longer exposure to pollutants would lead to more pronounced changes and whether such changes would be relevant for interaction with other organisms, especially with predators. Our results, based on relevant sub-lethal concentrations of TWPs, put emphasis on the need to further identify potential effects of the multitude of polymers included in the macro-category MPs.

Focussing specifically on TWPs could represent a urgency since plastic production, use and management will likely undergo through a deep remodulation that will hopefully result in reduced plastic input in the environment (e.g. global plastic treaty; Bergmann et al., 2022). This, however, can’t be true for TWPs simply because, at present, there is no other options that could replace tires for large scale road transportation. Thus there will likely be a consistent input of TWPs over the next decade, through its numerous pathways that span from atmospheric deposition to runoff waters (Wagner et al., 2018). So, the important goal is to evaluate potential negative impacts on the environment and sublethal but yet important changes in species interactions that, in specific cases, can eventually foster biological invasions in some ecosystems.

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7. Figures and Tables

Tables

Compound	Formula	m/z marker
2-Aminopyridine	$C_5H_6N_2$	94
Benzyl ether	$C_{14}H_{14}O$	198
p-Xylene	C_8H_{10}	106
Styrene	C_8H_8	104
Bicyclo [5.1.0] octane, 8-methylene	C_9H_{14}	122
Pentacyclo [5.2.1.0(1,5).0(5,9).0(6,8)] decane	$C_{10}H_{12}$	132
Benzene, isothiocyanato	C_7H_5NS	135
15-Isobutyl-(13αH)-isocopalane	$C_{24}H_{44}$	332
Cyclopropylphenylmethane	$C_{10}H_{12}$	132
Pseudoephedrine	$C_{10}H_{15}NO$	165

Table 1. Summary of the compounds detected in the 500 µg of the TWPs sample at the mentioned m/z ratios achieved by the Pyrolysis GC-MS.

FIXED EFFECTS:

	Est	S.E.	t val.	d.f	p
Intercept	18.96	203.77	0.09	174.77	0.93
Species Gibel carp	47.69	28.24	1.69	116.88	0.09
SL	1.90	2.15	0.88	171.48	0.38
Treatment PE	-23.12	49.36	-0.47	319.08	0.64
Treatment TWPs	18.30	49.36	0.37	319.07	0.71
Treatment control Time 14	-33.41	43.84	-0.76	234.08	0.45
Treatment PE Time 14	-35.23	43.91	0.80	235.26	0.42
Treatment TWPs Time 14	-73.85	43.92	-1.68	235.37	0.09
Treatment control Time 60	-133.43	44.21	-3.02	240.41	0.00
Treatment PE Time 60	-106.37	44.39	-2.40	243.44	0.02
Treatment TWPs Time 60	-182.14	44.23	-4.12	240.78	0.00

Table 2. The linear mixed effects regression (lmer) model for the Boldness- Emergence time test of both the species GC and CC for the observations recorded for a group of 120 fishes, 40 fishes each for control, 14 day as well as 60 day exposure period. The estimated parametric coefficients and their significance (coefficients for Species, SL, Treatment and the relation between treatment with respect to time, respectively) in the model. The adjusted R^2 of the lmer model with the random intercept of the fish ID dependent variable Emergence time was 0.27 and deviance explained by the ICC values was up to 21%. Significant p values of the explanatory variables were calculated using Satterthwaite d.f.

FIXED EFFECTS:

	Est	S.E.	t val.	d.f	p
Intercept	-0.12	0.13	-0.89	184.93	0.37
Species Gibel carp	0.08	0.02	4.45	117.10	0.00
SL	0.00	0.00	1.96	182.51	0.05
Treatment PE	-0.03	0.03	-0.85	300.65	0.40
Treatment TWPs	-0.00	0.03	-0.12	300.65	0.91
Treatment control Time 14	0.06	0.03	2.12	234.07	0.04
Treatment PE Time 14	0.05	0.03	1.82	235.39	0.07
Treatment TWPs Time 14	0.06	0.03	2.15	235.51	0.03
Treatment control Time 60	0.03	0.03	1.08	241.09	0.28
Treatment PE Time 60	0.06	0.03	2.28	244.44	0.02
Treatment TWPs Time 60	0.01	0.03	0.27	241.50	0.79

Table 3. The linear mixed effects regression (lmer) model for the Open Field test of both the species GC and CC for the observations recorded for a group of 120 fishes, 40 fishes each for control, 14 day as well as 60 day exposure period. The estimated parametric coefficients and their significance (coefficients for Species, SL, Treatment and the relation between treatment with respect to time, respectively) in the linear mixed effects regression model (lmer). The adjusted R^2 of the lmer model with the random intercept of the fish ID dependent on variable RatioN was 0.36 and deviance explained by the ICC values was up to 28%. Significant p values of the explanatory variables such as the fish identity were calculated using Satterthwaite d.f. The most appropriate parsimonious model was selected based on AIC criterion with the most complex variables.

FIXED EFFECTS:

	Est	S.E.	t val.	d.f	p
Intercept	6087.21	2783.74	2.19	165.27	0.03
Species Gibel carp	588.99	378.98	1.55	116.40	0.12
SL	-1.27	29.40	-0.04	161.02	0.97
Treatment PE	-737.33	698.99	-1.05	335.18	0.29
Treatment TWPs	-760.97	699.00	-1.09	335.17	0.28
Treatment control Time 14	95.57	648.98	0.15	233.83	0.88
Treatment PE Time 14	-35.10	649.85	-0.05	234.87	0.96
Treatment TWPs Time 14	1684.65	649.93	2.59	234.97	0.01
Treatment control Time 60	253.28	653.64	0.39	239.42	0.70
Treatment PE Time 60	1043.68	655.89	1.59	242.10	0.11
Treatment TWPs Time 60	1546.91	653.91	2.37	239.74	0.02

Table 4. The linear mixed effects regression (lmer) model for the total activity of the fishes in the Open Field test of both the species GC and CC for the observations recorded for a group of 120 fishes, 40 fishes each for control, 14 day as well as 60 day exposure period. The estimated parametric coefficients and their significance (coefficients for Species, SL, Treatment and the relation between treatment with respect to time, respectively) in model (lmer). The adjusted R^2 of the model with the random intercept of the fish ID dependent on variable TotalN was 0.18 and deviance explained by the ICC values was up to 14%. Significant p values of the explanatory variables such as the fish identity with respect to time were calculated using Satterthwaite d.f.

Figures

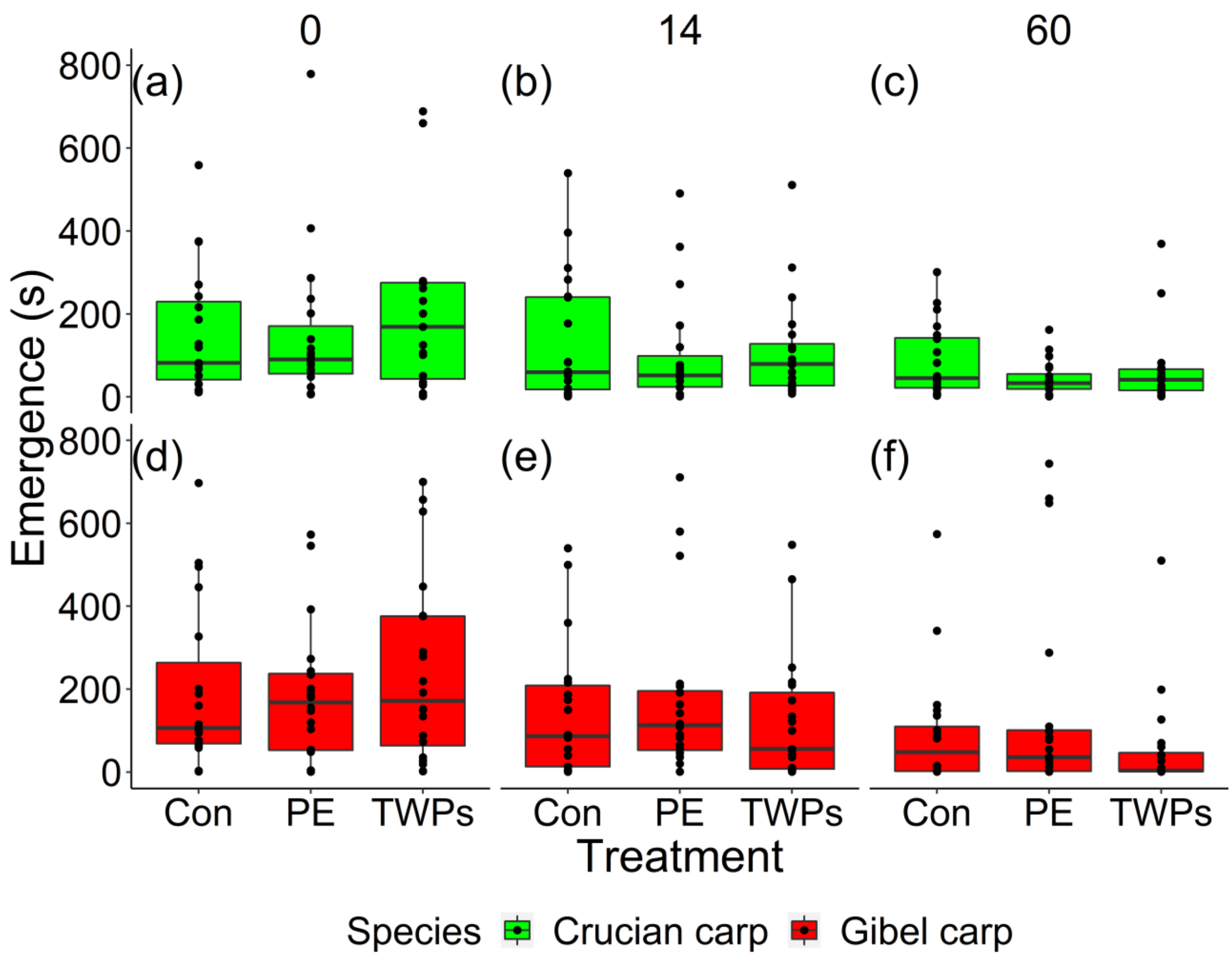

Figure 1. Emergence time (a proxy of fish boldness) differed between native crucian carp (*Carassius carassius*) and invasive gibel carp (*C. gibelio*) and declined with experimental duration (evaluated at 0, 14 and 60 day). PE treatment - polyethylene microplastics 0.1 % food content, TWPs – tire wear particles 0.1 % food content, Control – no addition in the food pellets. Points = individual data; boxplots: thick line = median, box = 50% of interquartile range, whiskers = outer 25% of interquartile range excluding outliers.

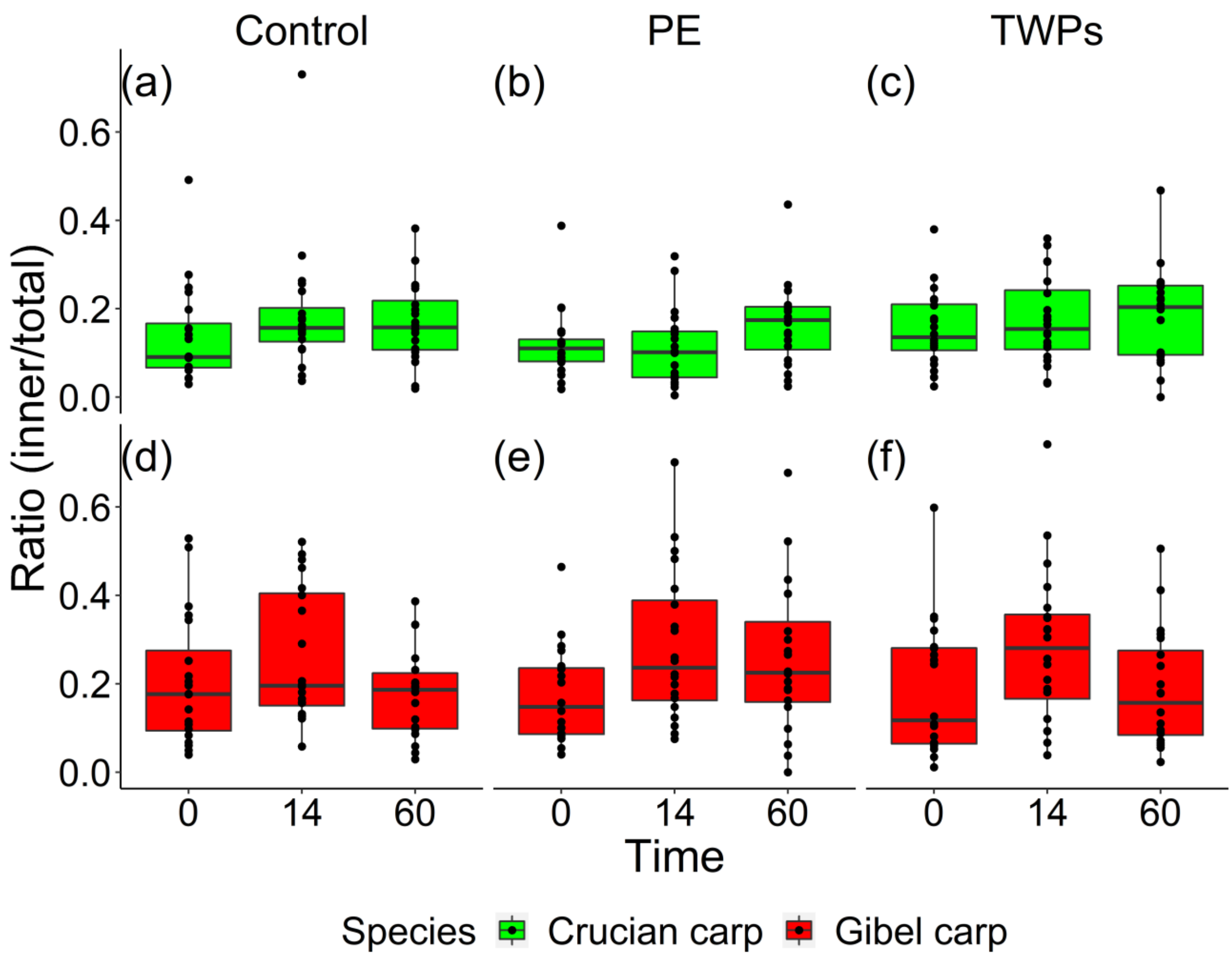

Figure 2. The ratio of the inner to the total amount of time spent by the native crucian carp (*Carassius carassius*) and the invasive gibel carp (*C. gibelio*) during the exposure to the Control, PE and TWPs treatment. The native species was more prone to risk at the end of PE treatment. PE treatment - polyethylene microplastics 0.1 % food content, TWPs – tire wear particles 0.1 % food content, Control – no addition in the food pellets. Points = individual data; boxplots: thick line = median, box = 50% of interquartile range, whiskers = outer 25% of interquartile range excluding outliers.

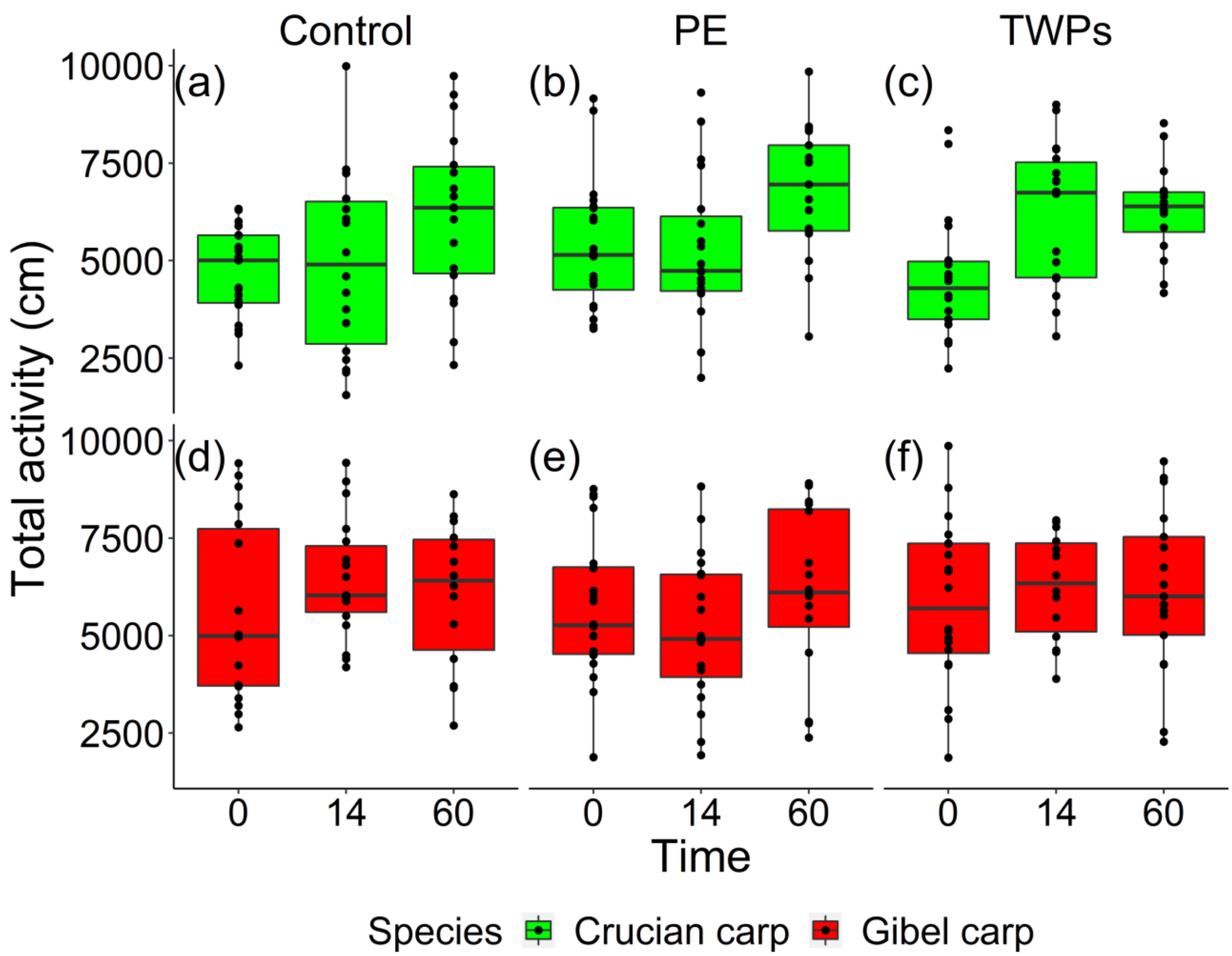

Figure 3. Total activity of crucian carp (*Carassius carassius*) and invasive species (*C. gibelio*) represented as the total distance swam in cm during the behavioural trials. Native species wherein the invasive species have been more locomotive than the native species in all the treatments. PE treatment - polyethylene microplastics 0.1 % food content, TWPs – tire wear particles 0.1 % food content, Control – no addition in the food pellets. Points = individual data; boxplots: thick line = median, box = 50% of interquartile range, whiskers = outer 25% of interquartile range excluding outliers.

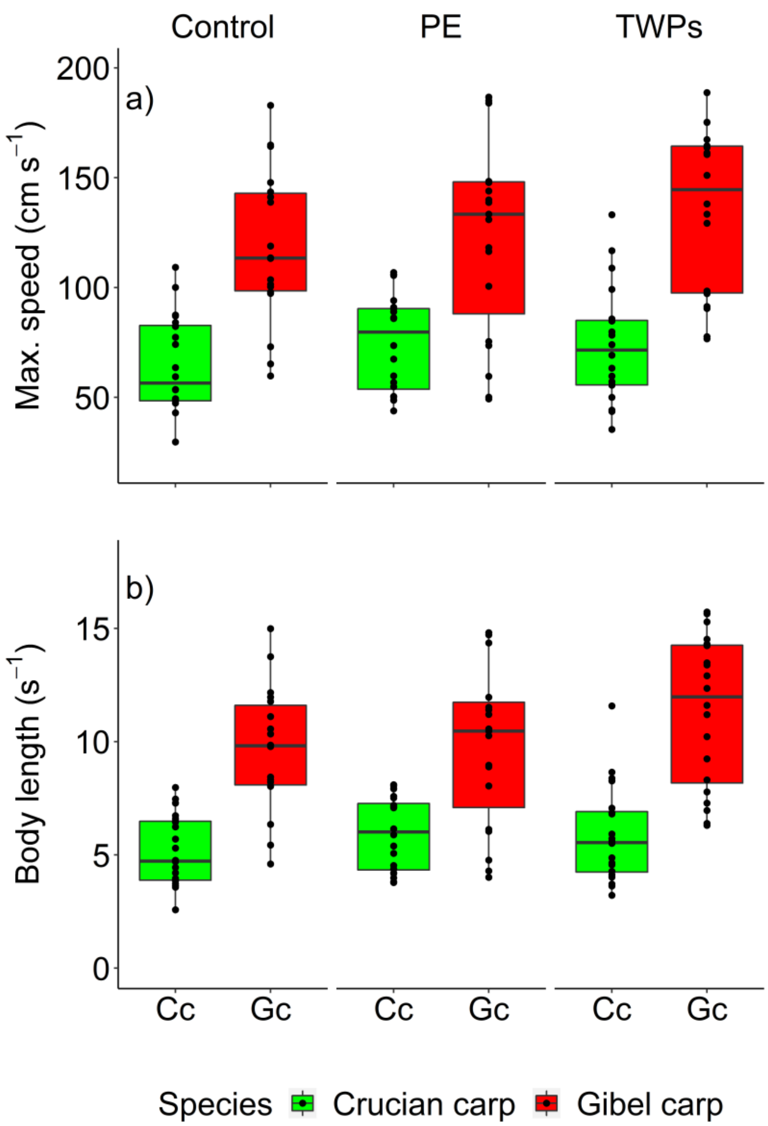

Figure 4. Swimming activity of the crucian carp (*Carassius carassius*) and invasive species (*C. gibelio*) represented as the total distance swam in cm s^{-1} at the end of 60-day experimental period. Invasive species had better swimming performance than the native species in all the treatments. PE treatment - polyethylene microplastics 0.1 % food content, TWPs – tire wear particles 0.1 % food content, Control – no addition in the food pellets. Points = individual data; boxplots: thick line = median, box = 50% of interquartile range, whiskers = outer 25% of interquartile range excluding outliers.

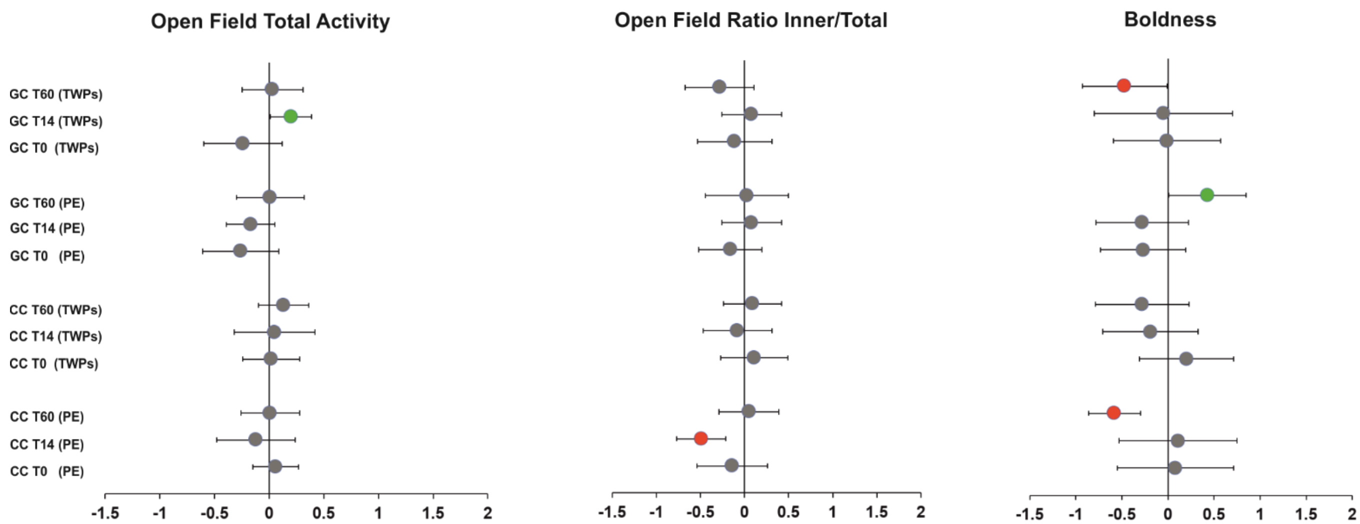

Figure 5. Forest Plot showing cumulative impacts on fish of the native *Carassius Carassius* (CC) and the invasive *C. gibelio* (GC) due to different treatments of PE and TWPs (ln–response ratio; mean ± 95% confidence intervals). Red circles: negative ratios; green circles: positive ratios; grey circles: non-significant ratio, across time 0, 14 and 60 days. Cumulative effects are significant if confidence intervals do not overlap zero.

Chapter 5: General discussion

Marine litter is frequently addressed as an emergent topic; however, this type of pollution has been identified in the Mediterranean Sea since 1993 on the seafloor and since 1979 on the sea surface (Deudero and Alomar, 2015; Veiga et al., 2016). Since then, research on marine litter has rapidly expanded but, when it comes to the consequences of litter on marine life, plastic has been the most thoroughly studied substance when compared to other types of litter, such as wood, metal, glass, tyres, and non-plastic fishing line. However, since the 1950s, when large-scale plastic production started, macro litter has entered the marine environment and is accessible to all taxonomic groups, not just marine organisms.

Chapter 2 of the thesis was dedicated to study the marine litter from the fishing grounds of the Sardinia Islands, quantified in areas of up to 800 m depth. The study revealed in terms of density and weight, plastic was the most dominant macro-category across investigated years (2013-2019), the five bathymetric *strata* (A-E), and five zones (1-7) followed by metal, glass, clothes, rubber. However, when referring to the average weight of litter recorded in the study period around the Sardinia islands (9.1 ± 1.96 kg km^{-2}), while the average number of collected items was (29.8 ± 1.22 items km^{-2}), which is lower than reported values around other regions of the Mediterranean Sea such as the Gulfs of Greece, Adriatic Sea, Gulf of Echinadhes, Gulf of Patras, South Western Portugal and the Spanish Mediterranean mainland coast (Koutsodendris et al., 2008; Mifsud et al., 2013; Neves et al., 2015; Pasquini et al., 2016; Stefatos et al., 1999; Strafella et al., 2015). These lower values of seafloor litter observed in the study area could be attributed to the lack of high industrialized areas in the Sardinia islands, the absence of river run-offs with a constant water flow into the sea and the corroborate hypothesis that marine litter occurrence increases beside population density. In this regard, the island of Sardinia represent an interesting case study, where anthropic/demographic factors (i.e., highly populated areas, rivers and big commercial ports) and riverine input are very low; it is thus a case where to better assess maritime traffic impact (i.e., glass and large metal objects that can't be dislocated by marine currents) and drifting floating plastics that was been dumped elsewhere and then transported during the "floating life" of plastic, likely from other areas of the basin. In our study we suggest that mitigation actions for the clearance of the fraction of macro-litter already accumulated in the marine environment should be based on solid (i.e., temporally and spatially) scientific evidence and then further implemented to be more effective in terms of costs and benefits. Our work on the hotspot distribution provides a far more holistic approach in order to recognise the smaller yet significantly densely accumulated hotspots of the macro-litter and thereby, help the designated local authorities to design the management and reduction strategies for the plastic macro-litter.

In chapter 3, considering the results emerged from the first study and in light of the urgent need to identify reliable surrogate descriptors of plastic contamination in the marine environment, we decided to further extend the investigation to assess and compare the eventual significant differences in microplastic contamination for two ecologically relevant crustaceans dwelling in European Waters: The European spiny lobster *Palinurus elephas* and the Norwegian lobster *Nephrops norvegicus,* which has already been identified as reliable and efficient bioindicator. Both species are regarded as major fishery resources and *gourmet* food, thus potentially representing flagship species for plastic contamination, with the former dwelling in shallower areas (up to 200 m) compared to the latter, that dwell from 200 to 800 m depth. Indeed, the two crustaceans object of our study have a huge discrepancy in scientific effort that document MPs contamination, with *P.elephas* showing the least amount of literature as compared to the *N .norvegicus*, for which there is a diversity of study based on the Mediterranean waters (Avio et al., 2020; Carreras-Colom et al., 2022; Cau et al., 2019; Martinelli et al., 2021) as well as the Atlantic waters (Hara et al., 2020; Joyce et al., 2022; Murray and Cowie, 2011). Our results point out 100% occurrence of MP particles in all the gastrointestinal tracts of the samples of both the species, with significant differences in the number and size of particles isolated from the two species. Moreover, we further confirm the fragmentation of MPs mediated by *N. norvegicus* while it was not possible to document this peculiar feature in *P. elephas*. The number of MPs reported in our study for *P. elephas* is much less as compared to Greek waters, the only available study on the species (Kampouris et al., 2023). Further investigations are necessary to provide a wider picture of MPs contamination across Mediterranean regions. The similar scavenging habitat of both the species along with the greater mobility feature of the *P.elephas*, provides the results which could be useful to declare these species as bio-indicators of the wide bathymetric and geographical range for the EU and because of their high economical value as a gourmet delicacy it could be an excellent flagship species for plastic contamination.

In chapter 4, the focus of the study was laid on the behavioural changes due to sub- lethal doses of PE and TWPs on the native and invasive species of freshwater fish. The statistical analysis such as the LMMs, GLMs and forest plots for different experiments such as the boldness, open field and the total swimming activity tests of the native crucian carp and the invasive gibel carp showed risk taking behaviour in native and invasive fish. The increase or decrease in the fish activity differed significantly on the 14 days and the 60 days of the treatment with PE and TWPs. The forest plot analysis summarized the results in general shows that the PE treatment had a decrease in the fish activity period and emergence time of the native species for the open field test on the 14-day exposure and 60-day exposure for the boldness test, respectively. Whereas in the case of TWPs treatment, it was observed that the invasive carp had an increase in the fish activity period on the 14-day exposure in case of open field test and a significant decrease in the emergence time in case of boldness on the 60-day exposure. Our results could be compared with the work on the long term acute toxic effects on the freshwater *Hyalella azteca* due to leachates from the worn and new tires (Halle et al., 2021). Prey- predator interactions in different freshwater fishes have already been studied wherein, the PE contamination resulted in the abnormal movements, increase in oxidative stress, trophic transfer of MPs in the food chain has also been studied in the available literature. With the available scientific literature on the ingestion of MPs, other contaminants such as pesticides, pharmaceutical products and/or complex mixtures of pollutants abetted by abiotic factors such as temperature or pH and trophic transfer of these pollutants, it is evident that the behaviour may vary depending on the fish species that are tested. In our case, the results show that although the concentrations used were not lethal to the fish, they affected their behaviour, and in particular the native species changed their behaviour toward bolder levels, potentially exposing them to higher predation rates in the wild, especially in case of TWPs. These results support the Pollution Resistance Hypothesis and provide information on the sublethal effects of tire wear particles in freshwaters. Very few studies report the results based on the weight-based concentration of the MPs regardless of the matrix, couldn't be of much help for us to select the concentration of the food pellets that were used for our study. Therefore, we can clearly believe that the concentration of the food pellet with the contaminant used in our study could be a foundational study for the exposure concentration studies in the future. Also, with the fact that not much work has been conducted on the pollutant TWPs, our results are quite significant and reliable and thereby, justifies our selection of the key pollutants for this study. Our findings demonstrate that invasive species are more tolerant of pollutants and give light on potential repercussions that, when combined with other disturbances, could result in biological invasion and replacement in European freshwater ecosystems.

Chapter 6: Final conclusions

The key general conclusions that can be drawn from this study are as follows:

- The present study in Chapter 2, deals about the analyzed results regarding the hotspot distribution of the scattered accumulated macro-litter of different categories (depending on different sources and mobility) along the Sardinian coasts, needs to be taken into immediate consideration for the waste management and efficient removal. Since, there is a high tendency of missing out the smaller yet considerable macro-litter hotspots on the basis of the density data of the plastic debris as well as other heavy debris categories like rubber and metals.
- Out of the different categories of the macro-litter, plastic contributed to the highest percentages followed by rubber. Both, plastic and rubber are the source for the rise to the numerous toxic pollutants such as microplastics, nano-plastics, meso-plastics and tire wear particles to name a few (Chapter 2).
- The surveyed data could be a source of help for the decision-making bodies for setting up new norms for bringing the level of plastic pollution to a lower value and thereby save the marine ecosystem, from the eco- toxicological effects of these plastic debris breakdown over ages. Therefore, the need to prioritize the efficient future waste management and reduction strategies such as clean-up actions and restoration of highly contaminated sites needs to be implemented at an urgent note keeping in mind the cost as well as the possible effectiveness of the mitigation strategy on the environment (Chapter 2).
- Plastic ingestion in the ecologically relevant decapod crustaceans, i.e., *Nephrops norvegicus* and *Palinurus elephas* is a matter of serious concern since, both the

crustaceans are also a part of the gourmet delicacies. Therefore, they possess a threat to the marine trophic food chain and eventually human health. (Chapter 3).

- Fragmentation of MPs mediated by benthic crustacean digestion was confirmed for *N. norvegicus* and not for *P. elephas*, suggesting that the size of individuals could play a crucial role in determining if and how plastic particles can be further fragmented during the egestion processes where gastric mill act. Our study reveals not only fragmented small particles in the stomachs and intestines of the crustaceans but, also pieces of fishing nets of large dimensions (i.e., up to 6 cm) in stomachs, exclusively in those specimens that were collected by means of trammel nets, corroborating the idea that fishing gears can easily become a source of plastic particles ingestion.
- It can be said that both species showed the incidence of MPs of different polymers, size ranges and colours from different primary sources likely from laundry, cosmetics, industrial and domestic discharges. Thereby, budget to clean up the beach coasts and even the seafloors, contribute to the upgradation of existing mitigation plans such as the Reduce, Reuse and Recycle plans and most importantly, assist local authorities in processing plastic waste with better positive effectiveness to the environment.
- The macro category of the pollutant that is TWPs needs to be taken into consideration for better remodulation for reducing the plastic pollution and thereby the associated risks to the aquatic ecosystems. While the long-term goal in the case of MPs is to reduce the load on the environment and there are alternatives to do so, there are currently no other options that could replace tires for road transport on a large scale.
- Our study demonstrates how PE and TWPs exposure may lead to significant changes in fish behaviour, that can potentially influence higher levels of the biological hierarchy:

altered individual behaviour can alter prey-predator interactions at population level and community level, in the case of a more resistant invasive species, as per the case study here investigated.

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Appendix

Publications And Presentations (topics related to the present Ph.D. thesis)

(* Co-first authors)

• Cau, A., Franceschini, S., Moccia, D., **Gorule, P. A.,** Agus, B., Bellodi, A., Cannas, R., Carugati, L., Cuccu, D., Dessì, C., Marongiu, M.F., Melis, R., Mulas, A., Porceddu, R., Porcu, C., Russo, & Follesa, M. C. (2022). Scattered accumulation hotspots of macro-litter on the seafloor: Insights for mitigation actions. *Environmental Pollution*, *292*, 118338. https://doi.org/10.1016/j.envpol.2021.118338

•Cau, A. *****, **Gorule, P. A. *,** Bellodi, A., Carreras-Colom, E., Moccia, D., Pittura, L., Regoli, F., & Follesa, M. C. Comparative microplastic load in the two decapod crustaceans *Palinurus elephas* and *Nephrops norvegicus.* (*Accepted, Marine Pollution Bulletin*)

Communicated

• **Gorule, P. A.,** Šmejkal, M., Tapkir, S., Stepanyshyna, Y., Stejskal, V., Follesa, M.C., & Cau A. Long-term exposure to polyethylene and tire wear particles: Effects on risk-taking behaviour in invasive and native fish

Poster Presentations

• **Gorule, P.A.,** Pittura, L., Follesa, M.C., Cau, Al. (2021). Microplastic contamination in two benthic decapod crustaceans from Sardinian seas. XXV Congress of Italian Society of Oceanography and Limnology (AIOL), Italy 30th June- 2nd July 2021.

• **Gorule, P.A.,** Pittura, L., Follesa, M.C., Cau, Al. (2021). Microplastic contamination in two benthic decapod crustaceans from Sardinian seas. XV Meeting of doctoral students and young researchers in ecology and aquatic systems sciences Italian Society of Oceanography and Limnology (AIOL) and Italian Society of Ecology (SItE), Italy 13- 15th April 2021.

Publications (publications on topics not related to the present Ph.D. thesis.)

•Pise, M., Gosavi, S. M., **Gorule, P. A.,** Verma, C. R., Kharat, S. S., Kalous, L., & Kumkar, P. (2022). Osteological description of Indian lepidophagous catfish *Pachypterus khavalchor*

(Siluriformes: Horabagridae) from the Western Ghats of India. *Journal of Vertebrate Biology*, 71(22021), 22021-1. https://doi.org/10.25225/jvb.22021

• Kumkar, P., Pise, M., **Gorule, P.A.,** Verma, C.R., Kalous, L. (2021). Two new species of the hillstream loach genus *Indoreonectes* from the northern Western Ghats of India (Teleostei: Nemacheilidae). *Vertebrate Zoology* 71: 517-533. https://doi.org/10.3897/vz.71.e62814

• Verma, C.R.*, **Gorule, P.A.*,** Kumkar, P., & Gosavi, S.M. (2020). Morpho-histochemical adaptations of the digestive tract in Gangetic mud-eel *Ophichthys cuchia* (Hamilton 1822) support utilization of mud-dwelling prey. *Acta Histochemica*, 122(7), 151602. https://doi.org/10.1016/j.acthis.2020.151602

• **Gorule, P.A.,** Gosavi, S.M., Kharat, S.S., & Verma, C.R. (2020). Osteological description of Indian Skipper Frog *Euphlyctis cyanophlyctis* (Anura: Dicroglossidae) from the Western Ghats of India. *Journal of Threatened*, 35(9), 16136-16142. https://doi.org/10.11609/jott.6031.12.9.16136-16142

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