UNIVERSITÀ DEGLI STUDI DI MESSINA

TESI DI DOTTORATO DI RICERCA IN BIOLOGIA APPLICATA E MEDICINA SPERIMENTALE

CURRICULUM IN SCIENZE BIOLOGICHE ED AMBIENTALI

XXXIV CICLO

SSD BIO/07

Multitrophic and Experimental Studies on Plastic Abundance and their Potential Effects on Marine Organisms from Mediterranean Sea

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Anno Accademico 2020/2021

Abstract:

The environmental pollution is one of the main issues of last decades, despite the many kinds of pollutants arising from many sources, one of the main concerns for scientific community is the Plastic Pollution. The plastic contaminants, especially those of micro-size, can follow the entire cycle of ecosystems contaminating many different environments including both vegetables and animals' organisms in which the last trophic step are humans. The plastic pollution is already persistent worldwide and, in all environments, from air to soil and water masses. Among these last, the Mediterranean Sea is one of the main polluted marine environments, reaching the sadly known name of "The Plastic Soup". Considering both the high amount of plastic daily enters seas and oceans and the ecological importance of the Mediterranean Sea, the aim of this study was to survey the plastic contamination status of some marine species that inhabit the basin. The study was divided in two phases: first environmental survey, and second in house experiments to assess microplastic effects on biota. During the environmental survey, different marine animal species were analysed, both caught by trawling fishing and sampled, and the plastic contents analysed and characterized. In total were counted 599 plastic items in 527 specimens (mean 1.3 plastic/specimen) belonging to 15 species in which 45.16% organisms were positive to plastic ingestion. The particles analysed by spectroscopies analysis were cellulose (CL), Kraton G, Polyamide (PA), Polyethylene (PE), Polypropylene (PP), Polytetrafluoroethylene (PTFE), Rayon and Nylon. During the second phase of the study was valuated the influence of microsphere of PE (10µm diameter) on feeding behaviour and life cycle of the brine shrimps *Artemia salina* used as experimental zooplankton model. The plastics microspheres were administered at different concentrations 0,1,10,102,103,104 MPs/ml in two different groups: Group A (without food source) and Group B (with food source). The ingestion degree was observed in treatment A4, A5 and from B1 to B5 and the main variations between the two groups was observed at exposure times T0, T6, T12, T24 vs. T48 in the Group A and at T0, T6, T12, T24 vs. T96 in Group

B. The trial highlighted the influence of MPs on growth in *A. salina* as well: the main variance in Group A was between A0 and all other treatment while in Group B the MPs less influenced the body development than Group A. The comparison between the multitrophic and experimental approaches to plastic ingestion increased the knowledge regarding the dynamics that regulate the plastic distribution in the environment using organisms of different taxa as propagation vector. Moreover, the influence of plastic particles used during experimental phases, allows to understand the potential effect of MPs to the development stages of zooplankton.

DECLARATION

I hereby declare that the results presented are to the best of my knowledge correct, and that this thesis represents my own original work, carried out during the designated research project period, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work. I therefore contributed in the study design and execution of the experiments, sampling, data acquisition, their analysis and interpretation. The thesis project (2018-2021) was a joint effort of several research teams from different institutes, mainly: The Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences, the Department of Veterinary Science and the Department of Mathematical and Computational Sciences, Physical Science and Earth Science, University of Messina. I am responsible for any eventual plagiarism. This thesis was verified using the software Plagiarism Checker X 2019, showing a percentage lower than (18%).

ACKNOWLEDGEMENT

To begin with, I would like to express my sincere gratitude to my supervisor Professor Nunziacarla Spanò that support me during all my student career since bachelor's degree with her teaching, support, and advice. Thanks to co-supervisor Professor Serena Savoca (University of Messina) for her teachings, support, guidance and valuable advice on all aspects of the thesis and for his support during the PhD period.

A special thanks to Professor Gioele Capillo, for his extreme patience and valuable advice. Thanks to all my colleagues of University of Messina, in particular Dr. Marco Albano for helping whenever needed. Dr. Sergio Famulari and Dr. Claudio d'Iglio, Dr. Sabrina Natale for help and advice on the field and in the laboratory activities and sampling essential to the success of the thesis; Dr. Fabiano Capparucci, Dr. Claudio Gervasi and Dr. Giovanni De Benedetto for help in many kinds of laboratory activities, Dr. Dario Di Fresco and Dr. Davide di Paola for their support during analysing process in laboratory.

I would like to thank Professor Fabio Marino for his advice about work field, laboratory procedures and for making available the Centro di Ittiopatologia Sperimentale della Sicilia (CISS) during experimental tests.

I Sincerely thank to Dr. Teresa Bottari and Dr. Monique Mancuso, for helping, support, patience, and collaborations given in research activities during last few years.

Thank you to my family and best friends that have endured my mood swings. Its certain that wouldn't be where I am today without their help and support.

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1. Introduction

The Mediterranean Sea is considered a hotspot of biodiversity and abundance in species. Many factors contribute together or individually to these features, such as: geomorphology, geography, oceanography, paleo geography and ecology (Bianchi et al., 2012; Bianchi and Morri, 2000; Coll et al., 2010; Spanò and Domenico, 2017).

Since ancient times, the Mediterranean basin, has attracted the entire scientific community, including geologists, biogeographers, zoologists and ecologists (Bianchi et al., 2012).

This, leaded to an organic and continuously updated knowledge about richness and abundance of species in this basin. Already from the 4th century BC, the first historical naturalist, Aristotle, described the life and the environment of Aegean Sea (Voultsiadou and Vafidis, 2007). Plinius, in the 1st century AD, in his naturalistic treaty *Historia naturalis liber IX*, summarized the existing knowledge about the fauna in the Mediterranean Sea called "*mare nostrum*" (our sea) by Romans (Bianchi et al., 2012). Study and research on the Mediterranean Sea continued thanks to several Universities founded starting from 11th century AD, in Italy and in other Mediterranean countries and many marine biological institutions since 1862, with the foundation of the "Stazione Zoologica Anton Dohrn" (Bianchi et al., 2012; Riedl, 1980).

Concerning the geographic data, the Mediterranean basin connects through the Black Sea in the east, and to the Atlantic Ocean in the west through the Strait of Gibraltar (Coll et al., 2010). In the southeast, the basin is linked by the Suez Canal with the Indian Ocean and the Red Sea (Fig.1). The Mediterranean Sea is separated by Sicily and the coast of Tunisia in two main subregions; the western and the eastern, having an extension of 0.85 and 1.65 million Km^2 respectively (Coll et al., 2010).

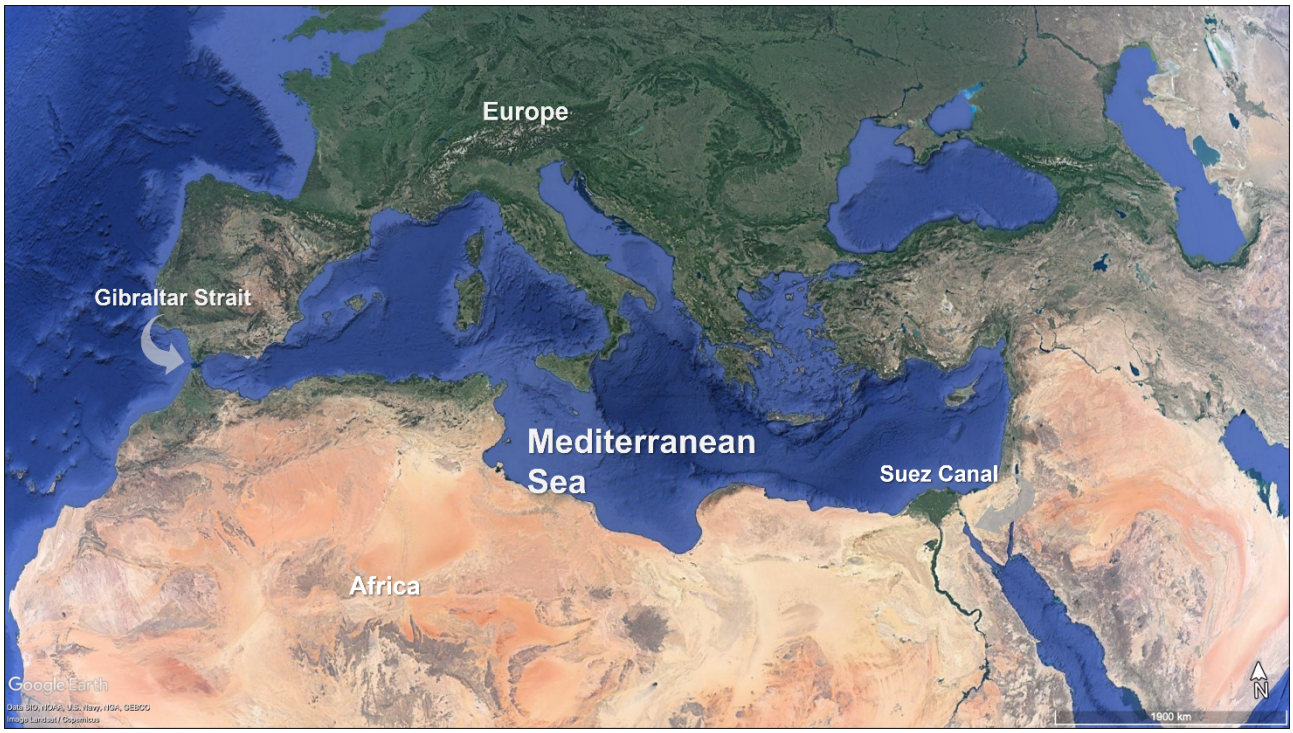

Fig.1: Mediterranean Sea and the connection with Atlantic Ocean (Strait of Gibraltar) and Indian Ocean (Suez Canal)

The Mediterranean Sea is a semi-closed basin in which the evaporation degree is higher than the precipitation one (Spanò and Domenico, 2017). This causes the average salinity of the Mediterranean Sea to oscillate between 37.5 and 39.5 ppt (Emig and Geistdoerfer, 2004).

Based on the high evaporation degree in the Mediterranean basin compared to scarce intake by rivers and rains, the biggest mass of water come from the Atlantic Ocean. The Atlantic waters, colder and with lower salinity degree than Mediterranean one, enter the basin passing through the Strait of Gibraltar (Coll et al., 2010). However, the Strait of Gibraltar has low depth (±280m), this allows only the passage of the Atlantic superficial water, that goes to mix with those in the basin. This, together with the characteristic climate of the region with hot and dry summers and cool and humid winters, contributes to make the Mediterranean Sea temperate during all the year with an average surface temperature of 13°C (Coll et al., 2010; Rohling and Abu-Zied, 2009; Roveri et al., 2008).

298 The evaporation degree is higher in the eastern half of the basin causing the decrease of water
299 level and the consequent increase of salinity from west to east (Coll et al., 2010).
300 The generated pressure gradient pushes the Atlantic waters into Mediterranean ones where they
301 sink in the Levantine Sea becoming Levantine Intermediate Waters (LIW). LIW passes through
302 the whole Mediterranean Sea and finally exit the Strait of Gibraltar (Coll et al., 2010; Garcia-
303 Castellanos et al., 2009).
304 Geomorphological features, currents, river and municipal discharges, and the different climate
305 factors contributes to make the Mediterranean basin a high diversified trophic basin (Bosc et
306 al., 2004; Coll et al., 2010; Zavatarelli et al., 1998). In general, the basin is considered
307 oligotrophic due to the regional features, however, the strong environmental gradients cause
308 the eastern part to be more oligotrophic than the western (Danovaro et al., 1999). On the other
309 hand, from northern to southern and from western to eastern the trophic production decreases
310 and it is inversely related to the increase in temperature and salinity. Moreover, these variation
311 factors define many environments and ecosystems into the same Mediterranean basin, in
312 addition to the environmental conditions, the fauna and flora can change. This is one of the
313 main reasons for the high biodiversity degree detectable in the Mediterranean Sea. For instance,
314 close to Gibraltar (in the east) the Alboran Sea shows many affinities with the Atlantic Ocean
315 both for flora and fauna due to the incoming flow of ocean waters (Fig.2) (Bianchi et al., 2012;
316 Harmelin and D'Hondt, 1993). On the other hand, the Tyrrhenian Sea is more isolated from the
317 western part of the basin, it is contoured by mountains that can cover the action of
318 meteorological events that usually are conditions of the western Mediterranean basin (Astraldi
319 et al., 1995). These features influence the average surface water temperature that increases
320 compared to other areas. Thus, the biota of the Tyrrhenian Sea is featured by species with
321 subtropical affinity (Bianchi et al., 2012).
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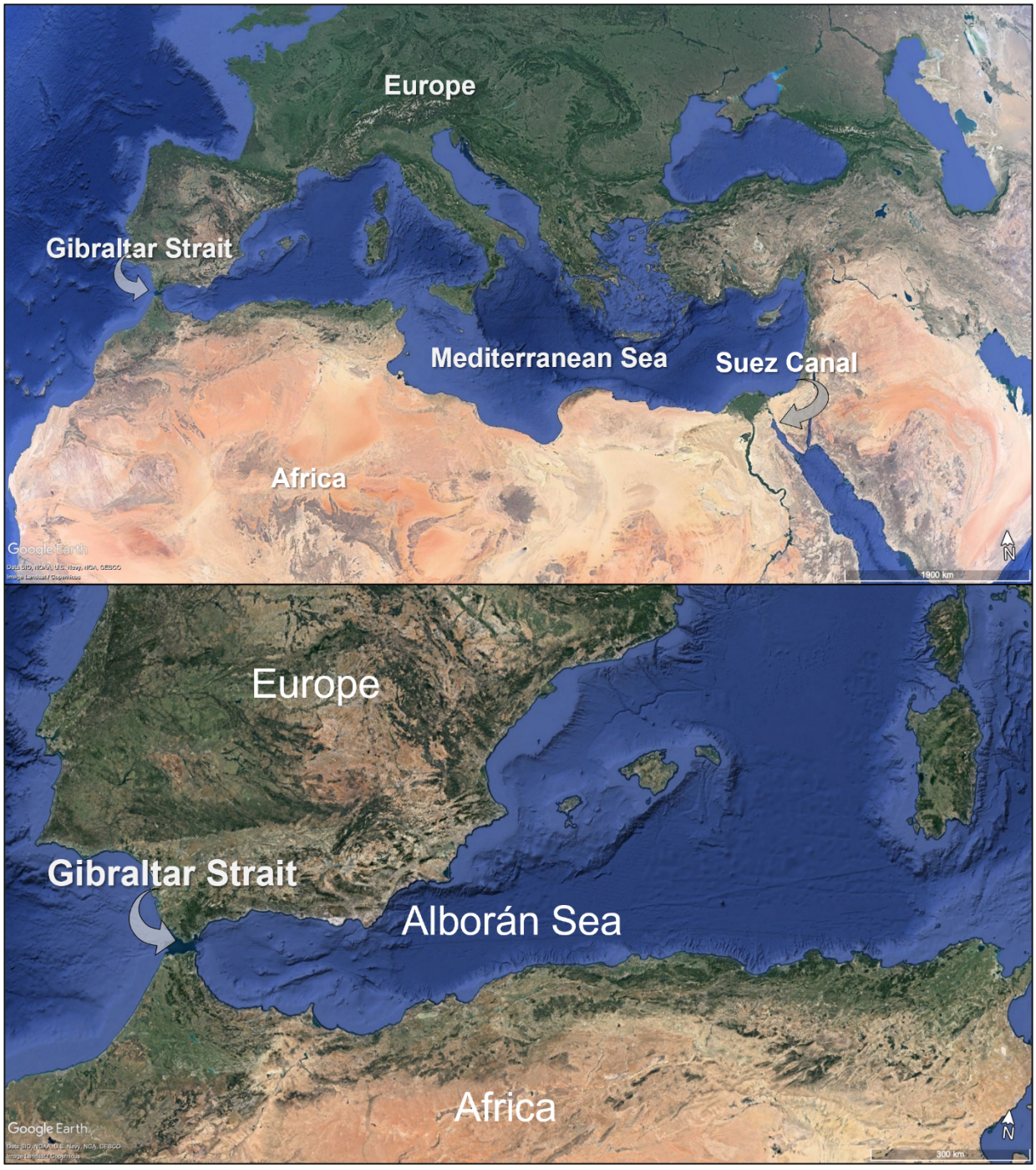

Fig.2: The Alboran Sea that is featured by many affinities with Atlantic Ocean due to their proximity that central Mediterranean basin (Astraldi et al., 1995)

Another isolated area of the Mediterranean basin is the Adriatic Sea that can be divided in three areas: north, central, and south (Fig.3). The northern part is very peculiar; it is influenced by cool winters, many river inputs that cause the decrease of salinity degree and the tidal range that makes it more like the northern Atlantic than the Mediterranean basin. Consequently, this influences the biota characterized by disjunct Atlantic-Adriatic species. This contributes to the reason why this area is called the northern-Adriatic "sub-Atlanticism". In the same way, the Mediterranean endemic species are scarce and the diversity in this Sea is the lowest compared to the entire basin (Bianchi, 2004; Bianchi et al., 2012). The central Adriatic Sea is still scarce in endemic species; however, it is richer than the northern part (Bianchi, 2004; Bianchi et al., 2012). On the other hand, the southern Adriatic Sea, has a reduced affinity to the western part; it is considered as a transition spot between the northern and central Adriatic Sea and the rest of the Mediterranean basin (Bianchi, 2004; Bianchi et al., 2012).

Fig. 3: the division of the Adriatic is in three sub-areas, North Adriatic Sea, Central Adriatic Sea, South Adriatic Sea (Bianchi et al., 2012)

Thus, these features contribute to making the Mediterranean Sea a place of different features with many varieties of species.

Moreover, another factor acting on the richness and diversity in species in this basin is due to the horizontal and vertical currents (Astraldi et al., 1995; Lambshead et al., 2000). Indeed, the big water mass moves up and down the thermocline allowing both the distribution of nutrients along the water column and oxygen supply to the bottom. The deep currents, rich in nutrients, are going out the Strait of Gibraltar, decreasing the trophic level. Thus, the poor abundance of nutrient together with the flow of currents and the temperature homogeneity increase the water transparency.

As currents in the Mediterranean Sea distribute nutrients, they play a role in contamination by nano and micro of the whole water column (Pedrotti et al., 2016). The energetic Northern Current having strong density gradient, can influence the plastic distribution close the shore (Pedrotti et al., 2016). However, in the Mediterranean basin, the structures of high plastic

accumulation, are instable structure usually (Mansui et al., 2015). This is due to the periodic strong wind that mixing and spreads seawards both plastic by land-based source and riverine waters. This, together with the complex circulation patterns may be the cause of the lack of stable plastic accumulation structures in open sea (Mansui et al., 2015; Pedrotti et al., 2016). However, as showed in the results of Pedrotti et al., (2016), the hydrodynamic features of the central part of the Mediterranean Sea could temporarily retain high plastic accumulation towards offshore regions.

In the basin, there are many important ecological habitats featured by rocky, sandy, and muddy bottom; characteristic intertidal zones such as beachrock (Capillo et al., 2018; S. Savoca et al., 2020), the seagrass meadows of *Posidonia oceanica*, and both deep-sea and pelagic environments with unique species and ecosystem (Goren and Galil, 2001; Green et al., 2004). The entire area of Mediterranean Sea is a spot for species inhabiting temperate and subtropical environments (Sara, 1985; Bianchi and Morri, 2000; Coll et al., 2010). Several species that live in this sea have a relevant conservation concern, as many cetacean's species, sea turtle and the Mediterranean monk seal are classified as "endangered" on the Red List of International Union for Conservation of Nature (IUCN). The basin is the main spawning area of a relevant economic pelagic species as the bluefin tuna *Thunnus thynnus* (Delaugerre, 1987; MacKenzie et al., 2009).

All the cited features, as well as many other, make the Mediterranean Sea a unique environment that deserves to be preserved. Preservation and protection of such natural environment, as Mediterranean Sea, need at the bases the continuously updated knowledge.

1.1 Human influence in Mediterranean Sea

The Mediterranean Sea, for millennia, since Roman times to date, both for strategic geographical location, climatic condition, and for availability of different sources, supported human civilization and its development. The entire Mediterranean area is characterized by heavy demographic, industrial and urban pressures, and, of course, climate change (Bianchi et al., 2012; Coll et al., 2010; Lotze et al., 2006; Voultsiadou et al., 2010). These are the reasons why the basin became overexploited for many biological resources and suffers high level of marine pollution and habitat modification (Coll et al., 2009). Based on the high demographic degree of the region, the demand for biological marine resources is high, and it is constantly increasing (Bianchi et al., 2012; Papaconstantinou and Farrugio, 2000). The decrease of fish stocks as a food source is of considerable concern in society, being strictly linked to overfishing events. The inadequate and scarce management of fishery activities cause alteration of the entire ecosystem in all the aquatic environments, Mediterranean basin included. The high demand of marine food sourced together with the high fish effort and development of fishing technique and technologies is burdening on stocks and in general on marine ecosystems (Papaconstantinou and Farrugio, 2000). Moreover, at depth ranging between 50 and 700m, the trawling commercial fishing has a double influence on ground bottoms of the Mediterranean Sea; the alteration/destruction of the benthic ecosystem and both from this and fishing itself, impact on ecological equilibria of stock in terms of species abundance and diversity. In general, the assessments highlight that the main economic species, both demersal and pelagic, are fully or overexploited (Coll et al., 2009; Farrugio et al., 1993; Palomera et al., 2007; Papaconstantinou and Farrugio, 2000). The catches by trawl include in the net an elevated and diversified mix of species being part of different taxa such as molluscs (cephalopods), crustaceans (stomatopods) and fish (both teleosts and elasmobranchs). As suggested by (Colloca et al., 2003; De Juan et al., 2007) these trawl techniques lead to ecological community shift, indeed, reducing the biodiversity of demersal ecosystems, the pelagic food chain is changing from fish to jellyfish (Purcell et al., 2007).

1.2 Marine pollution

As above mentioned, the overexploitation of marine resources is a relevant issue in the Mediterranean Sea. However, another main issue is related to the marine pollution of the area.

Pollution can be defined as a form of contamination, with anthropic origin, influencing and impacting ecosystems and the organisms in it, potentionally burdening the growth, reproduction, and the life cycle of both animal and vegetable species, including humans (Cremean and Techera, 2012). Consequently, the marine pollution as suggested by the Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP), part of the UN Convention on the Law of the Sea (UNCLOS) 1982 (Article 1.4) (Cremean and Techera, 2012), could be defined as:

"*The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water, and reduction of amenities."*

The marine pollution can have four main origins: the first is attributed to the land through discharges (44%) or the atmosphere (33%), while 12% due to maritime activity or shipping accidents, finally from dumping of sewage and garbage (11%) (Cremean and Techera, 2012). Moreover, pollutants can be classified in many ways as follow: *i*) basing to their physicochemical composition; inorganic like atmospheric pollutant (NO_2, NO_3 and SO_2) or metal ions, or organic like wastewater that come from petroleum derivatives and agricultural land, *ii*) basing on their physical state; solid like plastic debris, gases like organic compounds and solutes as remains of antibiotics, medication, and agricultural run-off and fertilizers (N_2), *iii*) basing on their persistence in the environment; biodegradable pollutants like cooking waste, manure or sewage; pollutants that spontaneously dissipate and conservative or persistent pollutants are not subject to bacterial attack and strongly resistant to degradation such as heavy metals (copper, lead, mercury etc.), radioactive wastes, dioxins and pesticides, *iiii*) finally, the

pollution can be point source or nonpoint source; the first when the pollution source can be traceable to an identifiable and single point, while can be considered the pollution source has a diffuse source that is not traceable to a specific location in the last case (Cremean and Techera, 2012).

Thus, there are many pollutants and kinds of pollution that could impact on marine environments, however, to date the more given attention concern is the plastic pollution and its influence on marine ecosystems.

1.3 Plastic pollution in marine environment

Plastic materials have many uses for daily societal benefits due to their properties, they are practical and versatile for many fields, thus, their uses became indispensable for modern civilization (Browne et al., 2010; Monteiro et al., 2018; Zhang et al., 2017). This is the reason why each year more than 320 million tons of plastics are produced worldwide. It is estimated that this trend will double in the next 20 years (Andrady, 2011; Capillo et al., 2020; Wright and Kelly, 2017). The main issue of this huge production is related to the scarce or inadequate management of the plastic waste. Indeed, only a small part of this plastic produced is correctly recycled, about 6-26% (Barnes et al., 2009; Dris et al., 2015b). The remaining part of the plastic products is conferred in landfills, or worse, ends up in environments directly (Barnes et al., 2009; Capillo et al., 2020; Dris et al., 2015a). Thus, the plastic wastes that enter the ocean and sea can come from different pathways; for instance, it was estimated that a range between 4.8 and 12.7 million metric tons of plastic waste come from land-based sources each year enter the oceans. As well cruise or private ships, maritime activities in general as fishing (both professional and recreational) and illegal dumping cause the increase of marine plastic pollution (Bottari et al., 2019; Cooper and Corcoran, 2010; Ryan et al., 2009) (Fig.4). Based on the high pollution degree, apart from marine environment, the plastic pollution was defined by UN environment, 2018 (Sidhu and Desai, 2018) as “one of the biggest environmental challenges of this

456 lifetime". The first report about a worrying plastic accumulation in sea dates back in 1972 (Carpenter
457 and Smith, 1972), from this date, the study of this topic increased for understanding the distribution
458 and accumulation ways of plastic polymers and the potential impact on ecosystems and the organisms
459 that are part of it.
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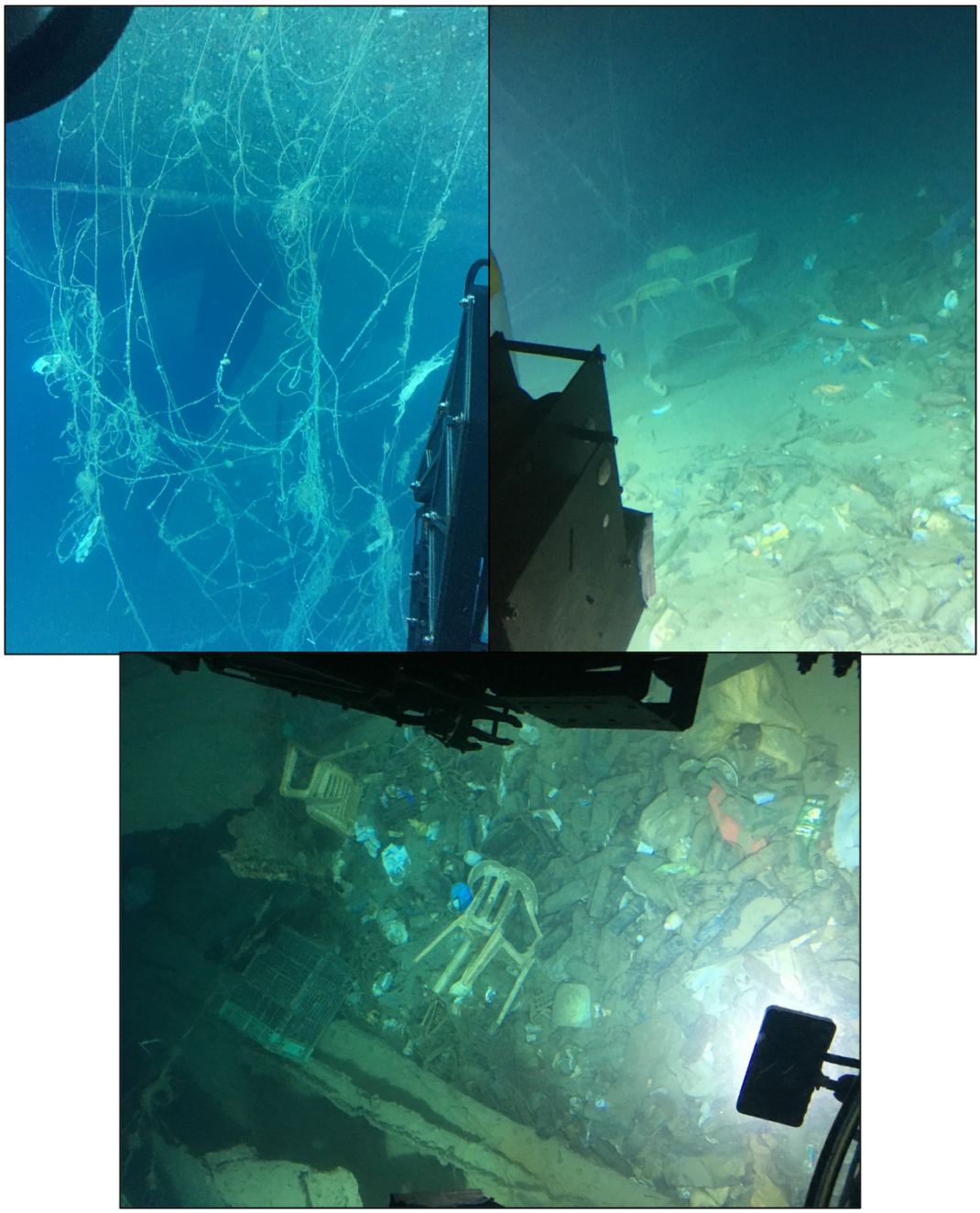

Fig. 4: Evidence of plastic garbage from different sources

Once in the sea, the distribution of plastic polymers can occupy different water strata, from the superficial ones to the bottom. Plastic litter distribution depends on many features both by plastic characteristics and the environmental factors. The dynamics that act on the distribution of these materials can be influenced both by the plastic polymer features as size, shape, density and by

environmental factors as river flow and marine currents, tidal, coastal winds and colonization of plastic surface by some marine organisms (Albano et al., 2021a; Capillo et al., 2020).

The plastic litter can be classified based on its size as follows: megaplastic>50cm, 50cm<microplastic<25cm, 25cm<mesoplastic<5mm and microplastic<5mm and nanoplastic<100nm (Capillo et al., 2020; Galgani et al., 2013; Hartmann et al., 2015)). Along the water column of oceans and seas, all these particle classes can be recognized. Concerning microplastics (MPs), they can be classified as primary or secondary (Costa et al., 2010; Mathalon and Hill, 2014). In the first case, are those MPs that have been originally manufactured at those sizes for a precious use as cosmetics preparation, facial detergents, toothpastes, textile fibres, and many other (Zhang et al., 2017). The secondary MPs are those that originate from the fragmentation of larger pieces (Costa et al., 2010; Mathalon and Hill, 2014). The fragmentation process of the materials is driven by mechanical, chemical, and physical factors as waves, sand impacts, and photodegradation (Albano et al., 2021a; Bottari et al., 2019; Capillo et al., 2020; Da Costa et al., 2016). On the other hand, based on particle density, these materials can float to the surface or at different depths of the water column, or sink to the bottom. More specifically, the particles with lower density than sea water, such as PE (Polyethylene) and PP (Polypropylene), tend to float along the water column than the plastics denser, such as PVC (Polyvinyl Chloride), will tend to sink (Bottari et al., 2019). Some biological factor can also influence the distribution of MPs along the water column, as the biofouling and in general the colonization by organisms on the plastic surface, increasing the density of these materials that sink to the bottom faster (Albano et al., 2021a; Lobelle and Cunliffe, 2011; Quero and Luna, 2017; Ye and Andrady, 1991). The degradation rate of plastic debris depends on the plastic polymer and the presence of chemical additives in it, environmental temperature, oxygen concentration, UV rays, depth, and pressures.

The most MPs present in marine environments are secondary (Duis and Coors, 2016), and are considered worldwide ubiquitous in the oceans and seas.

The most common MPs polymer detected in marine environment are Polyvinyl Chloride (PVC), Polyethylene (PE), Polypropylene (PP), Polystyrene (PS) and Polyethylene Terephthalate (PET) (Avio et al., 2017).

Moreover, recently, have also been considered as pollutants the semi-synthetic or natural fibers that come from urban centres wastewaters or textile industry (Capillo et al., 2020; Savoca et al., 2019a). These anthropogenic fibers, also named "man-made fibers", include textile materials of natural plant or animal origin and by cellulosic sources such as viscose/rayon (Henry et al., 2019; Savoca et al., 2019b). Despite the researchers focusing mainly on plastic and especially microplastic pollution in the marine environment, man-made fibers, to date, become very common and widespread pollutants. Maybe, in the past, the scientific community did not pay much attention to these fibers cause, often, they did not differentiate from plastic fibers with petroleum-based origin; on the other hand, recently many studies highlighted and identified the differences between the fibers of cellulosic polymers and synthetic textile fibers (Barrows et al., 2018; Carney Almroth et al., 2018; Remy et al., 2015). The wide use of these fibers in many activities such as agriculture and fisheries cause the increasing production per year (2%) adding to those of synthetic plastic, promoting the already known marine pollution (Dris et al., 2018; Savoca et al., 2019b).

As already said, the plastic pollution is an issue that burdens in all marine environments, from polar regions to equator, making up 60-80% of all marine litter (Cable et al., 2017; Jambeck et al., 2015; Lusher et al., 2015b; Savoca et al., 2019a).

1.3.1 Plastic pollution in Mediterranean Sea

The Mediterranean Sea was described as one of the main regions affected by microplastic pollution, hence, the name "plastic soup" (Mancuso et al., 2019; Suaria et al., 2016). Basing on this high pollution degree, regional and local plans to survey, monitoring and management the impact of litter to Mediterranean marine environment have been developed. Both the Regional Plan on Marine Litter

519 Management in the Mediterranean (UNEP/MAP, 2015) and the Marine Strategy Framework
520 Directive (MSFD) are the main tools for the protection by marine litter pollution in the Mediterranean
521 basin (Galgani et al., 2013). The application of these directives during environmental surveys,
522 promote the implementation of data about the abundance and distribution of marine litter and their
523 potential presence in the different species along the trophic web.

524 1.3.2 The influence of Plastic Pollution in the Trophic Web

525 Plastic and above all microplastics are ubiquitous in each environment, from air to land, from lake
526 and rivers to sea and oceans (Cable et al., 2017; Chae and An, 2018; Rillig, 2012). Because of their
527 characteristic resistance to corrosion and degradation in general, these materials persist for many
528 years in the environment accumulating to different ecosystems (Bottari et al., 2019).
529 In marine environments, these particles can induce both toxics and mechanical impact in the aquatic
530 species (Neves et al., 2015; Savoca et al., 2020). Indeed, for what concern the mechanical impact, the
531 debris ingestion can be considered neutral, if the plastic items are simply ingested and pass the
532 gastrointestinal tract to be excreted with feces later, while sub-lethal to lethal effects can be found
533 when the plastic debris pierce or block the Gastrointestinal tract of the animals (Wilcox et al., 2018).
534 Another mechanical damage comes from the fishing activities such as the abandoned nets which
535 represent a death trap for many species that get stuck, go to meet skin abrasion or death in the case,
536 for instance, in which these animals such as reptiles and marine mammals cannot rise to the surface
537 to breathe, or remain entangled for long time slowly dying. Apart from the specific case, in general,
538 the mechanical impact of plastic ingestion can influence the animal welfare; indeed, the obstruction
539 of food ways or the swim leads as energetic deficiency caused by feeding reduction to death in some
540 cases (Cole et al., 2015; Guzzetti et al., 2018; Savoca et al., 2019a; Strungaru et al., 2019).
541 Furthermore, to date, it is hard to establish an exact quantitative relationship between plastic ingestion
542 rate and its consequences in the organisms (Wilcox et al., 2018).

543 On the other hand, toxic hazard come from the endogenous chemical additives into plastic materials,
544 such as carcinogenic substances, flame retardants, dyes, phthalates, and bisphenol A incorporated
545 during the manufacture process; these additives give more durability to plastic materials (Bottari et
546 al., 2019; Teuten et al., 2009). Furthermore, in addition to already present additives, the plastic debris
547 can adsorb organic pollutants, and hydrophobic compounds such as pesticides, Polychlorinated
548 Biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbons (PAHs) (Bakir et al., 2014). Again, heavy
549 metal ions, such as copper, zinc etc., can adhere to plastic surfaces. Also, microorganisms (both
550 pathogenic and non) can adhere to MPs surface becoming part of the so-named Plastisphere (Amaral-
551 Zettler et al., 2020). Microorganisms covered particles can distribute through the aquatic
552 environments following the above explained processes. Thus, plastic wastes can be considered as
553 vectors for the mobility and distribution of many kinds of pollutants and microorganisms in marine
554 ecosystems (Brennecke et al., 2016; Koelmans et al., 2016; Gassel and Rochman, 2019). The
555 ingestion of plastic debris, with their adhered compounds, pollutants, and microorganisms encumber
556 many species along the food web. Indeed, highlighting the toxic effects, the ingestion of MPs can
557 induce disturbances and alterations to endocrine system, physiological, genetic, morphological, and
558 neural damages verifiable in the behavioural changes and alterations of the organisms as well.
559 The ingestion of plastic materials can be classified as primary (direct) and secondary (indirect)
560 (Bottari et al., 2019; Romeo et al., 2015). In the first case, the animal feeds on plastic directly. This
561 can happen based on the feeding behaviour of the species. For instance, the whales such as
562 *Balaenoptera bonaerensis* (Burmeister, 1867), that during the predation are usual to swim slow to
563 gobble water with many preys, can directly ingest plastic present in the water column together with
564 preys (Friedlaender et al., 2014; Goldbogen et al., 2011).
565 On the other hand, secondary ingestion consists in the ingestion of a prey already contaminated by
566 the plastic (Bottari et al., 2019; Capillo et al., 2020; Romeo et al., 2015). Primary and secondary
567 ingestion creates a process that led to the distribution of above-mentioned particles along the food

web reaching all the biotic compartments. Thus, the plastic ingested can be moved and accumulated in many species at different trophic steps causing bioaccumulation and biomagnification (Albano et al., 2021a, 2021b; Capillo et al., 2020; Savoca et al., 2020, 2019).

Basing on the already argued plastic pollution dynamics, in this study were chosen target species to survey their polymers contamination degree.

The target species, both bony fish, cartilaginous fish and cnidaria, and their ecological features are listed below.

1.1.1.1 Bony fish

***Boops boops* (Linnaeus, 1758)**

The bogue *Boops boops* (Linnaeus, 1758) (Fig.9) is a sparid distributed in the Eastern Atlantic along the shores from Norway to Angola including the Canary Islands and Cape Verde, and in the Mediterranean and Black Sea. The bogue is a gregarious species inhabits shelf or coastal pelagic environments having different bottom, both sandy, muddy, and rocky (Arechavala-Lopez et al., 2011; Arechavala-López et al., 2008; Monteiro et al., 2006). It is a demersal to semi pelagic species that lives in a depth range between 0 and 350 m (Monteiro et al., 2006). The diet is variable, it is considered an omnivore species feeding planktonic animals (especially jellyfish) and vegetables (Arechavala-Lopez et al., 2011; Arechavala-López et al., 2008; Costa, 1991; Milisenda et al., 2014)). It is commonly fished in the Mediterranean basin and the region with the highest catches are Spain (13.4 t), Algeria (6.4 t) and Italy (3.6 t) (Nadal et al., 2016).

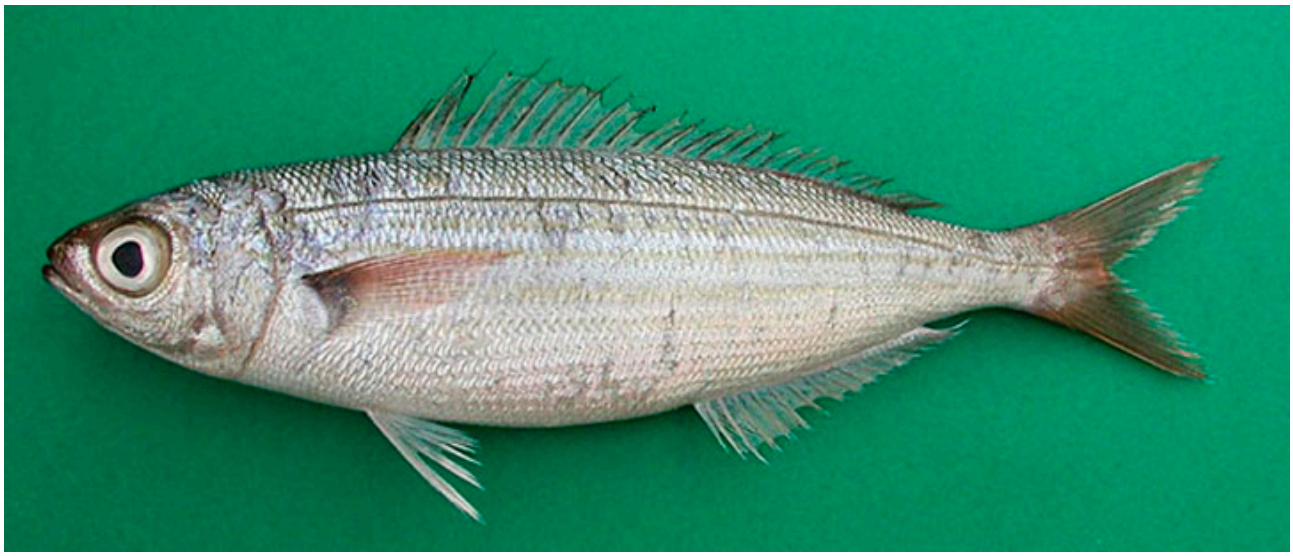

Fig. 5: The bogue Boops boops (Linnaeus, 1758)

Chlorophthalmus agassizi (Bonaparte, 1840)

The Shortnose greeneye, *Chlorophthalmus agassizi* (Bonaparte, 1840) (Fig.6), generally, live in a depth range between 50 and 1000m (Anastasopoulou et al., 2006; Wheeler et al., 1986). The species inhabits the Eastern Atlantic Sea, from Spain to Senegal and especially in the Mediterranean Sea, it is very abundant in the Ionian Sea where distributes from 300 to 600m depth (D'Onghia et al., 2003; Politou et al., 2003). As showed by Anastasopoulou et al., (2006) and Anastasopoulou and Kapiris, (2008), *C. agassizi* can be divided into three different size group based on their total length (TL); large (TL>150mm), medium (100mm<TL<150mm) and small (TL<100mm). The shortnose greeneye is a demersal fish that lives along clay and sandy bottom, feeding mainly small crustaceans as copepods, decapods and euphausiids (Anastasopoulou and Kapiris, 2008). However, was showed as the diet composition of *C. agassizi* undergoes seasonal changes both for diversity and number of preys ingested, indeed, in winter the stocks show the lowest degree ingestion both for diversity and number of preys (Amundsen et al., 1996), while during spring crustaceans, especially copepods, are the mainly preys (Anastasopoulou and Kapiris, 2008). Summer is the season with the high number of preys in which mainly were fishes (Anastasopoulou and Kapiris, 2008). Autumn is the season with

608 the highest diversity degree, indeed, as showed by Amundsen et al., (1996) and Anastasopoulou and
609 Kapiris, (2008), the shortnose greeneye have a mixed feeding strategy adding polychaetes, hydrozoa
610 and plants detritus to classic diet composition in crustacean. *C. agassizi*, along the central and eastern
611 part of Mediterranean Sea is an abundant bycatch species (Anastasopoulou and Kapiris, 2008).
612

613
614 *Fig. 6: The Shortnose greeneye, Chlorophthalmus agassizi (Bonaparte, 1840)*
615

616 ***Engraulis encrasicolus* (Linnaeus, 1758)**

617 The European anchovy *Engraulis encrasicolus* inhabits Northeast Atlantic, Mediterranean Sea, and
618 the adjacent sea areas (Palomera, 1992; Reid, 1967) (Fig.7). The species are distributed in a wide
619 range of environment such as bays, estuaries, and lagoons. Moreover, *E. encrasicolus* inhabits
620 environments with different features, from extreme conditions areas with low trophic productivity,
621 like Black Sea, to area with high trophic productivity with warm waters. During spawning phase,
622 usually between April and November of the specimens, they move inshore (Frimodt, 1995; Palomera,
623 1992). *E. encrasicolus* tend to move in surface during summer period while goes down to -400m in
624 winter (Frimodt, 1995). The European anchovy is one of the main commercial species along
625 Mediterranean Sea, indeed, are fished up to 270000 tons every year (FAO, 2018).

Fig. 7: The European anchovy Engraulis encrasicolus (Linnaeus, 1758)

***Sardina pilchardus* (Walbaum, 1792)**

Sardina pilchardus (Walbaum, 1792) (Fig.8) is a species distributed in some areas of northeast Atlantic, and in the Mediterranean Sea is very abundant in the western part and Adriatic Sea while rare in the eastern part (Olivar et al., 2001; Santos et al., 2006; Tsikliras and Koutrakis, 2013). The European pilchard inhabits both offshore and close to the shore in a depth range between 10 to 100m depending to their life stage (Santos et al., 2006). About the spawning period, the European pilchard, such as most clupeids, is a batch spawner with several reproduction time during the season (Amenzoui et al., 2006; Tsikliras and Koutrakis, 2013). *S. pilchardus* is an important commercial species representing the 15-20% of the fished species in the Mediterranean basin (Tsikliras and Koutrakis, 2013).

Fig. 8: The European pilchard Sardina pilchardus (Walbaum, 1792)

642 ***Lepidopus caudatus* (Euphrasen, 1788)**

643 The silver scabbardfish, *Lepidopus caudatus* (Euphrasen, 1788) (Fig.9) is a species inhabits warm
644 waters of the world (Demestre et al., 1993). It is a mesopelagic teleost living on the edge of continental
645 shelf over sandy and muddy bottom, down to 620 m depth however, the vertical distribution of the
646 specimen change in relation to season, indeed, during winter the silver scabbardfish is abundant on
647 the continental shelf while moves to deeper environment during other seasons (Demestre et al., 1993;
648 Falsone et al., 2021; Mytilineou et al., 2005). The silver scabbardfish is a predator with a polyphagous
649 diet, the mainly preys are teleosts like Clupeidae and Myctophidae, crustacean such as Euphausiacea
650 and Decapoda, and cephalopods. The reproduction period for males goes from March to April while
651 from February to October with the maximum activity starting from April (Demestre et al., 1993). The
652 silver scabbardfish is a species with a moderate commercial value in many countries such as Morocco,
653 Portugal, and New Zealand while in Italy, Spain, Albania and Tunisia the large specimens have an
654 high economic value, indeed, the smaller ones are discarded (Demestre et al., 1993; Falsone et al.,
655 2021; FAO, 2018; Figueiredo et al., 2015).

Fig. 9: The silver scabbardfish, Lepidopus caudatus (Euphrasen, 1788)

***Merluccius merluccius* (Linnaeus, 1758)**

The European hake *Merluccius merluccius* (Fig.10) (Linnaeus, 1758) is a nektobenthic fish species (Carpentieri et al., 2005). The European hake is distributed in a depth range from 20 to 1000m in an area that goes from North Norway to the Gulf of Guinea in the northeast Atlantic and both in Mediterranean and Black Sea (Casey and Pereiro, 1995; Fischer et al., 1987; Murua, 2010). Both juveniles and small hakes, usually, tend to live on muddy bottoms on the continental shelf while adults live on the slope of the shelf featured by canyons and cliffs (Mancuso et al., 2019; Murua, 2010). In general, the European hake lives close to the bottom during the day, moving usually between 70 and 370m depth at night (Cohen et al., 1990). The feeding habits of European hake shift based on its ontogenetic development, indeed, *M. merluccius* is a top predator that in juvenile stage (<16cm TL) tends to preys on euphausiids, changing the diet after one year age (>16cm TL) eating both

benthic and nektonic fishes (Gobiidae, Clupeidae)(Ardizzone and Corsi, 1997; Colloca et al., 2003). The last stage corresponding to the achievement of sexual maturity (36cm TL) with another change in diet, indeed, the growing of the hake is reflected by the increase of prey's weight and the decreasing of their abundance (Ardizzone and Corsi, 1997; Carpentieri et al., 2005; Colloca et al., 2003; Velasco and Olaso, 1998). The European hake is an ecological and economic significant species that recently reported in the Mediterranean Sea as overfished status and near threatened (NT) in the red list of International Union for Conservation of Nature (IUCN) (Busalacchi et al., 2010; Colloca et al., 2013).

Fig. 10: The European hake Merluccius merluccius (Linnaeus, 1758)

***Mullus barbatus barbatus* (Linnaues 1758)**

The red mullet *Mullus barbatus barbatus* (Linnaues 1758) (Fig.11) is a demersal fish that inhabits both sandy and muddy bottoms with a bathymetric preference between 5 and 250m in shelf bottoms (Relini et al., 1999; Sieli et al., 2011). The species is distributed along the eastern Atlantic Sea and the entire Mediterranean Sea (Lombarte et al., 2000; Maravelias et al., 2007). The red mullet usually fed both benthic crustaceans, polychaeta and molluscs (Sieli et al., 2011). The spawning period of the species in the Mediterranean Sea change basing on the specific place of the basin but in general is

687 during spring and summer, for instance, for the species caught in the Tyrrhenian Sea and analysed in
688 this study, the spawning period of the specimens was from April to July (Follesa and Carbonara,
689 2019). The red mullet is a demersal species having a high commercial value in the Mediterranean
690 basin (Caddy, 1993; Christos D. Maravelias et al., 2007; Reñones et al., 1995).

691

692 *Fig.11: The red mullet Mullus barbatus barbatus (Linnaues 1758)*

693

694 ***Pagellus bogaraveo* (Brünnich, 1768)**

695 The blackspot seabream *Pagellus bogaraveo* (Brünnich, 1768) (Fig.12) is a benthopelagic species
696 generally inhabits the Mediterranean Sea in which the young individuals are found near the shore
697 while adults down to 400m depth (Chilari et al., 2006; Wheeler et al., 1986). In Atlantic Ocean, the
698 blackspot seabream adult, are found on the slope of the shelf down to 700m depth, both for fish that
699 inhabit Mediterranean and Atlantic waters, they live along rocky, sandy, and muddy bottoms (Chilari
700 et al., 2006; Wheeler et al., 1986). *P. bogaraveo*, as other sparids, exhibits protandry hermaphroditism

in which the female sexual maturity is around 28cm in size and 32cm for male in Mediterranean Sea, while in Atlantic Ocean the sexual maturity size is 26cm and 29cm for male and female respectively (Chilari et al., 2006; Estácio et al., 2001; Krug, 1989). The diet composition of blackspot seabream is wide, the dominant preys are fishes followed by crustaceans, thaliaceans, ophiuroids and gastropods (Morato et al., 2001). Cause the slow-growing and long-life, *P. bogaraveo* is vulnerable to fishing pressure, indeed, the General Fisheries Commission for the Mediterranean (CGPM) adopted Recommendation GFCM/41/2017/2 about the blackspot seabream fishery that aims to improving the exploitation of the stock (Hermida et al., 2013; FAO, 2018).

Fig.12: The blackspot seabream Pagellus bogaraveo (Brünnich, 1768)

***Pagellus erythrinus* (Linnaeus 1758)**

The common pandora *Pagellus erythrinus* (Linnaeus 1758) (Fig.13), is a demersal species inhabits muddy-sandy and rocky bottom at depth range between 20 and 300m, it is a gregarious species (Busalacchi et al., 2014; Miguel Neves Santos et al., 1995; Wheeler et al., 1986). The common pandora live along the Atlantic Ocean, from Europe (Norway) to Africa (Angola) including Mediterranean and Black Sea (Busalacchi et al., 2014; Pajuelo and Lorenzo, 1998). The species can reach a Total Length (TL) about 60cm but commonly the range size is between 10 and 30cm, *P.*

719 *erythrinus* shows protogynous hermaphroditism, indeed, in the second- or third-year age the females
720 became males, in a range size about 18cm TL (Girardin and Qunignard, 1985; Pajuelo and Lorenzo,
721 1998; Papaconstantinou et al., 1988; Relini et al., 1999). In Mediterranean Sea, the spawning season
722 ranges from March to November, while in the Atlantic Ocean from May to August (Coelho et al.,
723 2010; Girardin and Qunignard, 1985; Hoşsucu et al., 2003; Pajuelo and Lorenzo, 1998;
724 Papaconstantinou et al., 1988; Tsikliras et al., 2010). Around the second or third age year, the species
725 reach sexual maturity (Girardin and Qunignard, 1985; Pajuelo and Lorenzo, 1998). The diet
726 composition consists of: Decapoda, Polychaeta, Cephalopoda, Bivalvia, Euphausiacea, Mysidacea
727 and teleost's (Ardizzone and Messina, 1983; Šantić et al., 2011). Legislation on fisheries, along
728 Mediterranean Sea, sets the minimum size caught for the common pandora at 15cm TL, in Italy, the
729 stock of this species, represent one of the most common seabreams landed (IREPA 2011) (EU
730 Regulation 1967/2006) (Busalacchi et al., 2014). The common pandora is one of the mainly captured
731 species in central Mediterranean basin (FAO, 2018).

732

733 *Fig.13: The common pandora Pagellus erythrinus (Linnaeus 1758)*

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736

***Trigla lyra* (Linnaeus, 1758)**

The piper gurnards *Trigla lyra* (Linnaeus, 1758) (Fig.14) is a teleost inhabits sandy and gravelly bottom (Costa, 1991). It is a demersal species lives to 400m depth and distributed along Italian shores above all to Sicilian coasts (Costa, 1991). The piper gurnard is a voracious species fed on molluscs, crustaceans and teleost's like callionymidae (Costa, 1991). Even if it has good meat, is not a high commercial species, indeed, the specimens are not valued in all Italian regions (Costa, 1991).

Fig.14: The piper gurnards Trigla lyra (Linnaeus, 1758)

***Zeus faber* (Linnaeus, 1758)**

The john dory, *Zeus faber* (Linnaeus, 1758) (Fig.15), is a species worldwide distributed and abundant in the Mediterranean Sea, usually along sandy bottom from 5 to 200m depth (Briguglio et al., 2017; Busalacchi et al., 2010; Costa, 1991; Münzing et al., 1970). It is a voracious bentho-pelagic teleost that feeds mainly on fishes and molluscs that are rapidly ingested by the suction that is the feeding habit of the species (Costa, 1991; Silva, 1999; Stergiou and Fourtouni, 1991). Despite the high commercial interest of the species, the knowledge about ecology habits is quite scarce (Costa, 1991; C. D. Maravelias et al., 2007). In the Mediterranean basin, usually, the john dory is fished by trawl or with fisheries artisanal methods (C. D. Maravelias et al., 2007; Stergiou et al., 1997). Moreover, *Z. faber* has a high commercial value, above all in the Adriatic Sea (Bottari et al., 2019; Briguglio et al., 2017; C. D. Maravelias et al., 2007).

Fig.15: The john dory Zeus faber (Linnaeus, 1758)

1.1.1.2 Cartilaginous fish

***Galeus melastomus* (Rafinesque, 1810)**

The blackmounth catshark *Galeus melastomus* (Rafinesque, 1810) (Fig.16) is a benthopelagic elasmobranch that prefers muddy bottoms in a depth range between 200 and 500 even if it can habit in a wide range between 50 and 1400m (Moranta et al., 1998; Rey et al., 2005). *Galeus melastomus* is distributed in the eastern Atlantic Ocean and along Mediterranean Sea where is the main species of the genus than *Galeus atlanticus* more abundant in eastern part of the basin, in the Alboran Sea (Castilho et al., 2007; Moranta et al., 1998; Rey et al., 2005). It is an oviparous species in which the females can release from 1 to 4 egg cases along muddy bottoms (Capapé et al., 2008). In Mediterranean Sea, as reported by Capapé et al., (2008), the species spawning during all year even if the peak period is between spring and summer. It is a generalist forager feeds on worms (polychaetes) molluscs (cephalopods), crustacean (shrimps, crabs) and bony fish, moreover, there is little shift in

772 diet composition according to variation in depth and size of shark specimens (Carrassón et al., 1992;
773 Cortés, 1999; Macpherson, 1979; Olaso and Rodriguez-Marin, 1995).
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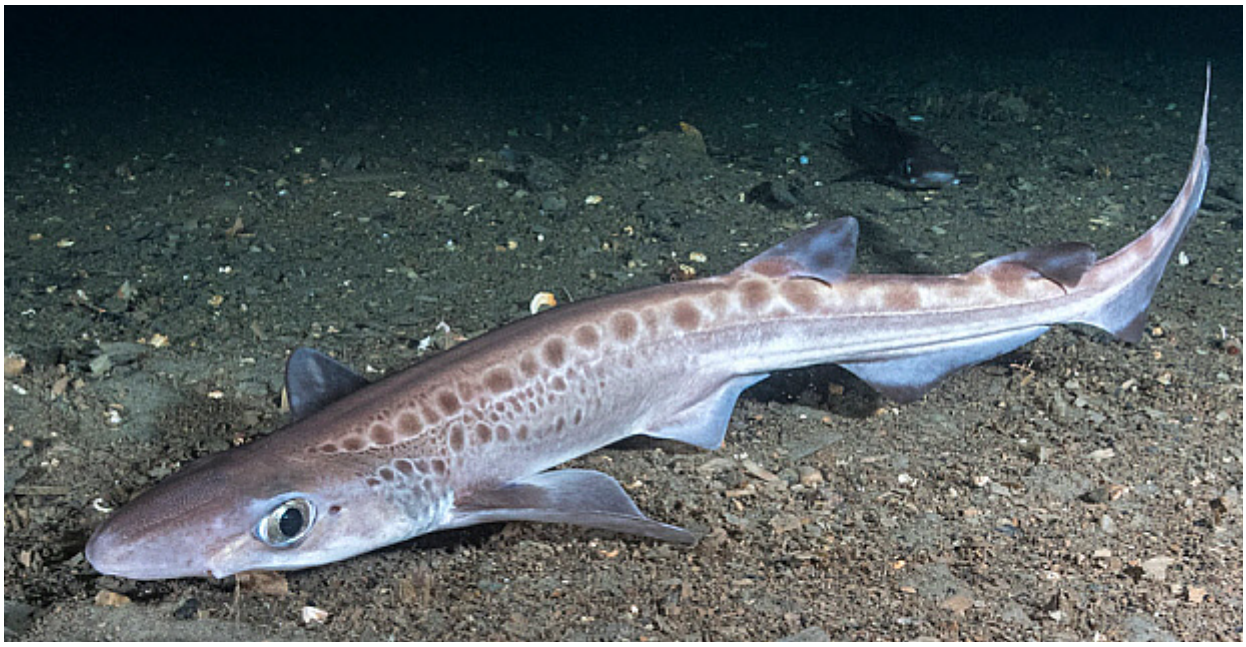
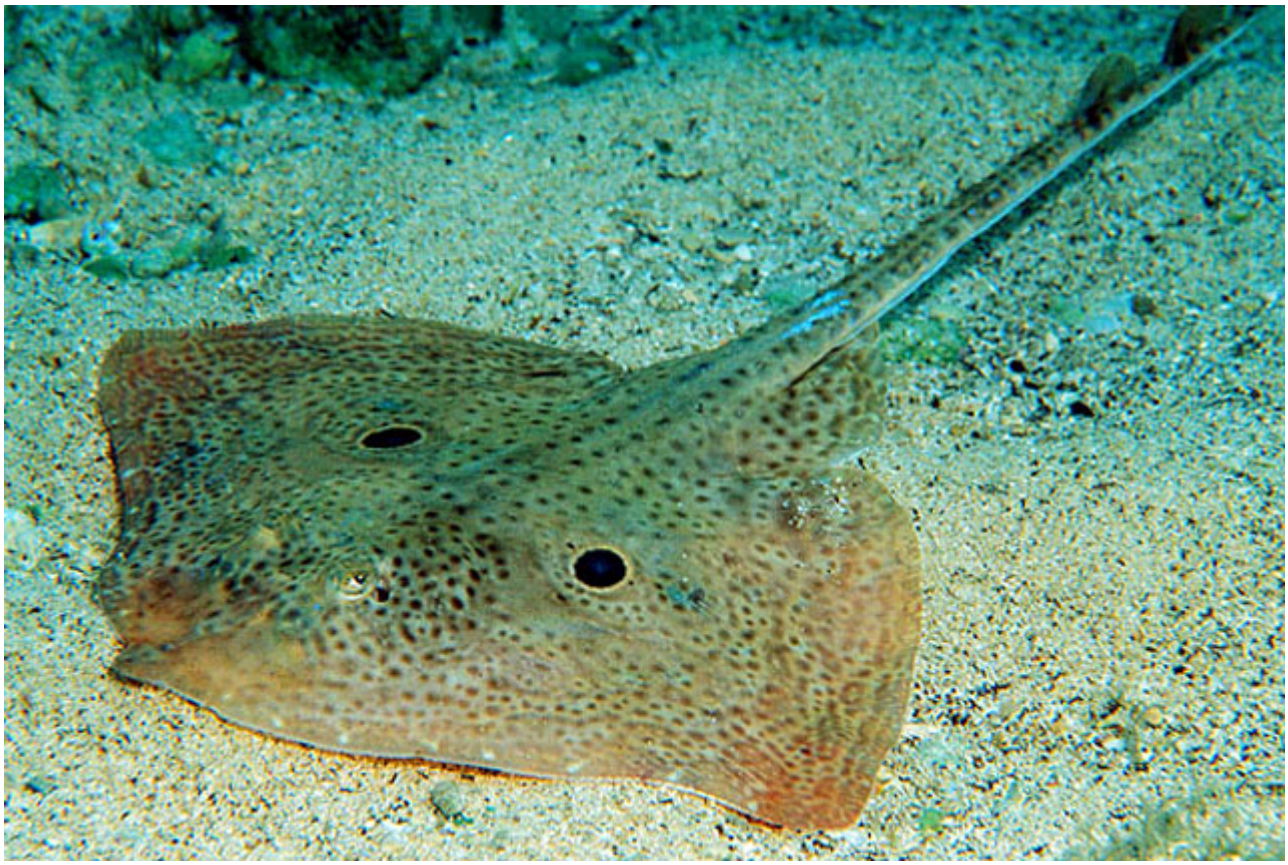


775
776 *Fig.16: The blackmounth catshark Galeus melastomus (Rafinesque, 1810)*
777

778 ***Raja miraletus* (Linnaeus, 1758)**

779 The brown ray *Raja miraletus* (Linnaeus, 1758) (Fig.17) is a species distributed in the Northeast
780 Atlantic and common in Mediterranean basin, especially in Adriatic Sea (Capapé et al., 2007; Capapé
781 and Azouz, 1976; Costa, 1991). The specimens inhabit along sandy and muddy bottoms to 400m
782 depth, however, during summer it can approach close to the shore to 30m depth (Costa, 1991;
783 Mytilineou et al., 2005).
784 The diet composition include mainly molluscs and crustaceans (Costa, 1991; Šantić et al., 2013).
785 The brown ray is an oviparous species, the egg cases are featured by oblong capsule and stiff horns,
786 they are released in muddy or sandy bottoms (Bor, 2002). The eggs have a length between 4.2 and
787 4.6cm and each female specimen can release to 40-70 egg cases in a year. The spawning period goes
788 from spring to summer (Bor, 2002).

789 The species has not a high commercial value due to its meat that are not considered very delicious,
790 for this reason, the specimens, usually caught by trawl, are discarded at sea directly (Capapé et al.,
791 2007; Costa, 1991).



792

793 *Fig.17: The brown ray Raja miraletus (Linnaeus, 1758)*

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795 ***Scyliorhinus canicula* (Linnaeus, 1758)**

796 The Lesser spotted dogfish *Scyliorhinus canicula* (Linnaeus, 1758) (Fig.18) is a shark distributed in
797 north sea, north-eastern Atlantic ocean, Norwegian Sea and Mediterranean basin especially close to
798 Italian shores where is very common (Costa, 1991; Soares and De Carvalho, 2019). The specimens
799 inhabits many ecosystem including gravel, muddy, sandy bottoms and coralline and algal
800 environments (Muus and Nielsen, 1999). In the Mediterranean Sea usually inhabits muddy bottom
801 from 20 to 400m depth (Costa, 1991). It is a nocturnal species in which the males remain on the
802 bottom while females from low depth hiding caves wait for prey opportunity (Costa, 1991; Sims et

al., 2001). It is a generalist foragers, indeed, the diet composition is wide and diversified including both invertebrate like polychaetes, molluscs, crustaceans and vertebrate like small teleost fishes (Compagno, 1984; Costa, 1991; Thollot, 1992). It is an oviparous species releasing egg cases during all year, however, the peak is in June and July (Ellis and Shackley, 1997). The egg cases are released by females on biological structures both vegetable like macroalgae and sea grass and animals like poriferans and corals (Costa, 1991; Ellis and Shackley, 1997; Wheeler, 1978). The eggs are anchored to biological structures via tendrils positioned on each corner of the capsule (Compagno, 1984; Costa, 1991; Ellis and Shackley, 1997). The shape of the eggs is rectangular and they has a length range between 5 and 7cm, and 1.5 to 7cm in width (Bor, 2002; Costa, 1991). Despite the specimens are capture by trawl it is a by-catch species, indeed, the Lesser spotted dogfish has not an high economic value (Costa, 1991; Revill et al., 2005).

Fig.18: The Lesser spotted dogfish Scyliorhinus canicula (Linnaeus, 1758)

1.1.1.3 Cnidaria

***Pelagia noctiluca* (Forsskal, 1775)**

In the Mediterranean basin there are about 12 species of Scyphomedusae mainly represented by the mauve stinger *Pelagia noctiluca* (Canepa et al., 2014; Gili and Pagès, 2005) (Fig.19). The mauve stinger is considered a holoplanktonic species due to the lack of the benthic phase during its life cycle (Canepa et al., 2014). This feature permit to *P. noctiluca* to inhabits different environments with

different water parameters, indeed, the populations of this species are widely distributed from temperate waters of the North Sea to the subtropical like Mediterranean Sea (Canepa et al., 2014; Licandro et al., 2010). Along the water column, the mauve stinger is distributed usually close to the surface in a bathymetric range between 10 and 30m that is in line with the depth of the higher level of halocline/pycnocline and especially with the nocturnal vertical migration of the zooplankton that represent the main prey of *P. noctiluca* (Graham et al., 2003; Mariottini et al., 2008). The species is a generalist planktonic predator feeding both on ichthyoplankton and holoplankton (Canepa et al., 2014; Giorgi et al., 1991; Malej et al., 1993).

Fig.19: The mauve stinger Pelagia noctiluca (Forsskal, 1775)

1.4 Plastic pollution: experimental survey

As already argued, the plastic materials, from mega to nano sizes, are ubiquitous in the marine environment. In the last decade, many studies focused on the mechanical and toxic effects of the plastic on marine organisms and as these materials can go through the food web through bioaccumulation and biomagnification phenomena. However, recent studies surveyed the influence of plastic ingestion in marine organisms adopting experimental approaches. The controlled environment and its fixed parameters together with the known plastic administration utilized during experimental surveys, can increase the knowledge about the interaction between the plastic materials and the animal models. Indeed, this approach can lead to an increase in knowledge about the plastic feeding behaviour and the influence of these materials in the life cycle of the species. In accordance, in this study, was used a biological model for the experimental approach.

1.4.1 *Artemia salina* (Linnaeus, 1758)

Artemia salina (Linnaeus, 1758) (Fig.20) is a crustacean belongs to the order Anostraca, subclass Branchiopoda, that lives in extreme environment as lakes and pools featured by high salinity degree (Browne and MacDonald, 1982; Savoca et al., 2020; Ward-Booth and Reiss, 1988).

Fig. 20: the brine shrimps Artemia salina (Linnaeus, 1758)

The order Anostraca, without carapace, is featured by a trunk with 20 or more segments. Both exopodite and endopodite are featured by a single flattened lobe with dense setae along the border. The name of the class Branchiopoda "gill feet", came from the epipodite into the coxa that serves as a gill, moreover, these appendages are adapted for locomotion and filtration feeding, mainly on microalgae (Ward-Booth and Reiss, 1988). During swimming, the brine shrimp produce a water current generated by the beating of the thoracic limbs. These movements allow, as well as the locomotion, the gathering of the food (Abatzopoulos et al., 2002). The eggs of *A. salina* hatch in a stage of free-living larvae (nauplii) that reach the adult stage through a series of gradual changes (Instars) (Criel and Macrae, 2002). During these changes, before the complete adult's formation, somites and appendages are added to the specimens. Before the spawning, males and females swim

865 closely together, one on top of the other, until the females begin to develop two egg sacs. The
866 produced eggs are remarkably strong, they can resist adverse conditions, thus, based on the
867 environmental conditions that can be favourable, the laid eggs may hatch within hours or can lie
868 quiescent for months as well (Criel and Macrae, 2002).
869 The genus *Artemia* is considered an optimal model organism for environmental toxicology studies
870 thanks to their easy management features (Rajabi et al., 2015). Moreover, the brine shrimps due to
871 the small size range, life cycle and easy breeding, have a crucial role in the aquaculture facilities
872 where it's used as food source for many aquatic species (Abatzopoulos et al., 2002; Clegg and
873 Trotman, 2002).
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2. Aim of thesis

As already argued in the sections before, plastic pollution is a worldwide issue. The Mediterranean Sea, the "plastic soup", resulted in one of the major plastic polluted areas. Based on the ecological importance of the Mediterranean Sea, characterized by high abundance and diversity of species, it became of fundamental importance to broad the knowledge about the plastic pollution level of the area and its influence/presence in the organisms inhabit in. Furthermore, many species of the basin have high commercial value for human consumption, thus, they become potential plastic particles vectors to human creating risks for the health of the final consumer. For these reasons, the aim of this study was to investigate the distribution and abundance of plastic materials and their influence on marine species.

The research question has been approached by using two different ways:

- **Multitrophic approach (Environmental Survey)**; the survey about the presence and abundance of plastic materials, especially microplastics, in marine species was conducted with environmental sampling. The target species were chosen basing on their trophic habits; indeed, the species were both pelagic, nektonic, and benthic, on the other hand, also the feeding habits of the chosen species were different to better show both the different ingestion degree in terms of abundance and the way in which the plastic particles could be ingested such as primary or secondary ingestion. The aim of this kind of sampling was to collect a high amount of data for distribution and abundance of plastics both from all the water column and at different trophic levels from consumers to predators.
- **Experimental approach (Trial)**: an animal model was used for the administration of plastic particles to investigate the influence of microparticles on the feeding behaviour, growth, and development of the selected species. The chosen animal model was a zooplanktonic species

both for the easily management during the different trial phases and to better understand the dynamics about the plastic ingestion on the primary consumers.

3. Materials and methods

3.1 Environmental Survey

3.1.1 Study Area

The study included sampling along three different areas, thus, in June 2017 was performed experimental trawl surveys on board of the Scientific Research Vessel “Dallaporta” in the central Tyrrhenian Sea. The specific area of trawl was the Gulf of Patti (Fig.21); it is a zone between cape Milazzo (eastern limit) and cape Calavà (western limit) (Busalacchi et al., 2014; Mangano et al., 2015). In this area, in which there exists since 1990 the regional law N. 25/90 about Fishery Exclusion Zone, the artisanal methods through set gillnets and trammel nets are the only allowed fishing activities (Mangano et al., 2014).

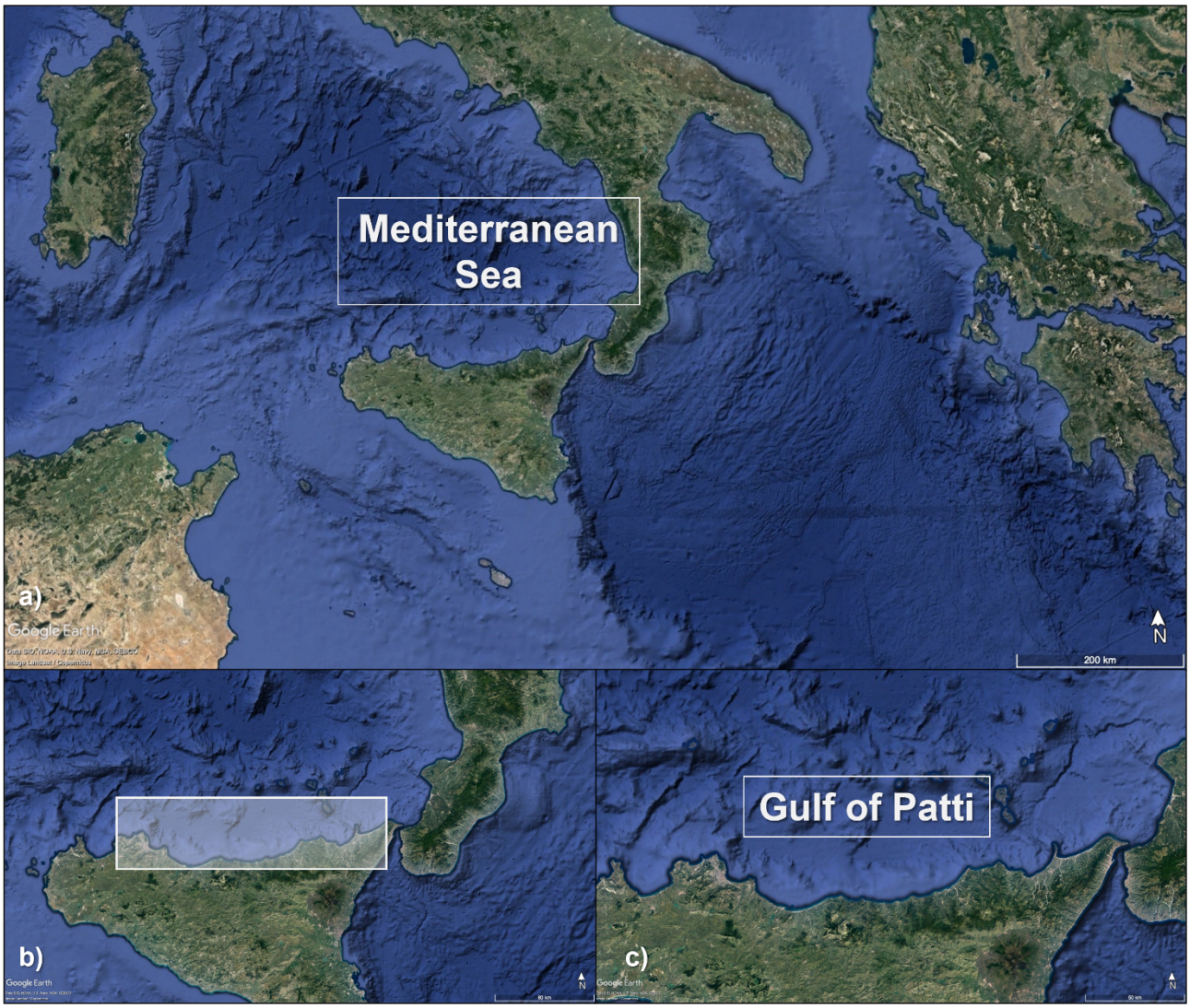


Fig. 21:a)the entire area of GSA10, b)c) Fishery Exclusion Zone of Gulf of Patti

In October 2019, on board the fishing boat "Pegaso", the experimental trawl surveys (MEDITS) in the south of Sicily, more precisely in the Sicily Channel (GSA16-Fig. 22) was performed. The trawling hauls were performed in a depth range between 50 and 500m.

Fig. 22: the entire area of Sicily channel (GSA16)

The Strait of Messina (Fig.23) represented the third sampling area of the study in which, in 2019, was surveyed the cnidarian species *Pelagia noctiluca*. The Messina's Strait is one of the most peculiar environments in the Mediterranean Sea, featured by high hydrodynamics due to strong and continuous tidal and stationary currents. Indeed, this place is a geographical point of union between the Ionian and Tyrrhenian waters, thus, the meeting between water masses with different chemical-

physical parameters and the geomorphological features generates peculiar phenomena as deep waters rose up and turbulence that can evolve in cut and sea stairs (Spanò and Domenico, 2017).

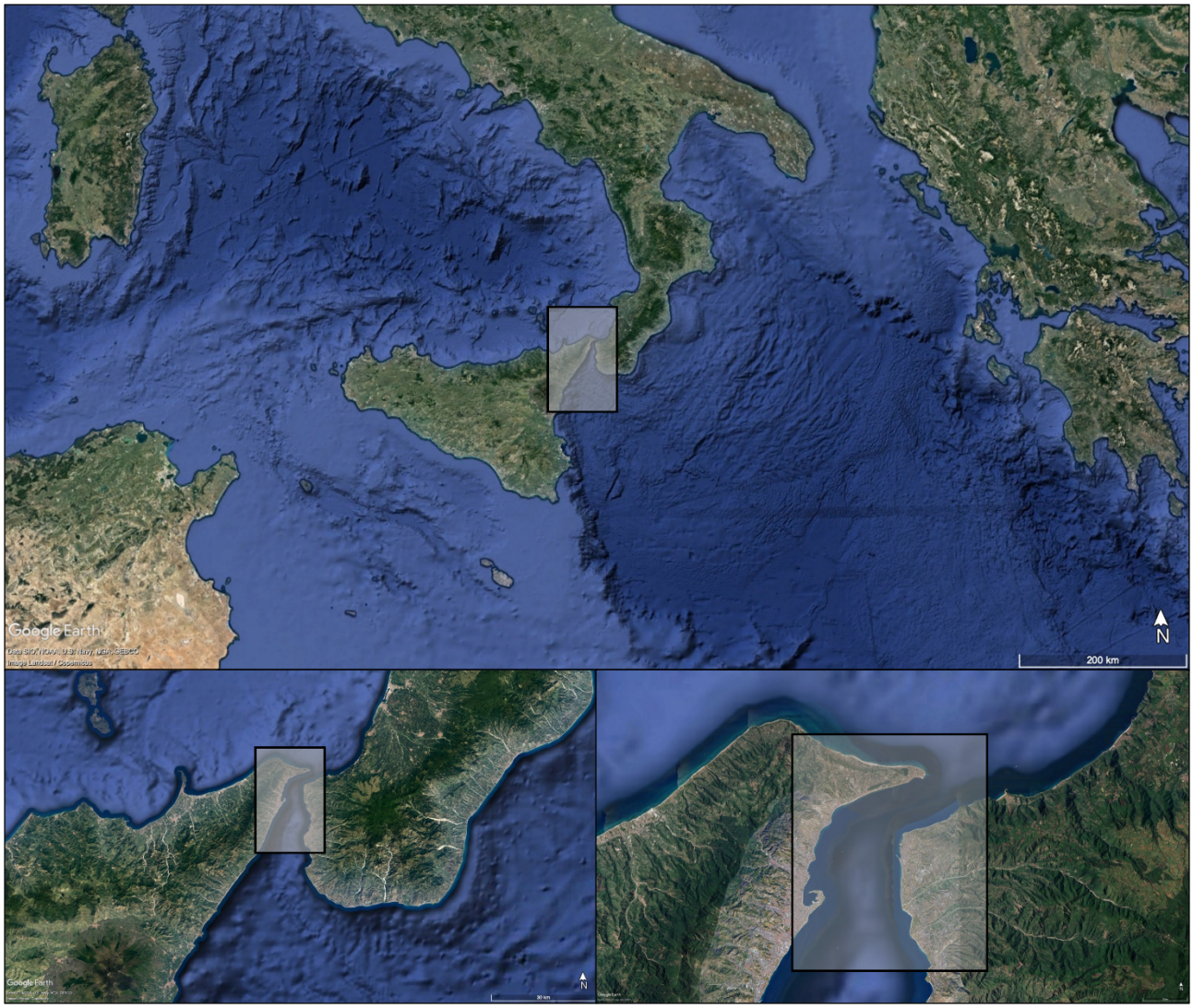


Fig. 23: Strait of Messina in which the Tyrrhenian and Ionic water mass meet

More precisely, the jellyfish specimens were sampled in a particular habitat of the Strait of Messina named beachrock (Capillo et al., 2018; S. Savoca et al., 2020) (Fig.24).

This intertidal zone, featured by sedimentary formation represented by an extensive stretch of shore, is located in the north-eastern part of Messina between the villages of Faro (38◦15'43" N, 15◦38'13" E) and Ganzirri (38°25'69" N, 15°61'24" E). Furthermore, beachrock is an area included in the Oriented Natural Reserve of Capo Peloro (Messina, Italy) SCI (ITA030008).

Fig. 24: b)c)The sampling Area of Pelagia noctiluca, d)one of the characteristic tidal ponds of Beachrock in which the specimens were sampled

3.1.2 Sampling design

All the specimens were collected by trawl except for the cnidaria *Pelagia noctiluca* (Forsskål, 1775) and samples of juvenile of clupeid fishes, mainly *Engraulis encrasicolus* (Linnaeus, 1758) and *Sardina pilchardus* (Walbaum, 1792).

Jellyfishes were sampled during the spring 2019 directly from the shore of the Strait of Messina, where the specimens were collected using free-plastic equipment as sterile glass containers equipped with metal screw caps. The fresh samples were directly transported to the laboratory.

The clupeid fishes were obtained in February 2019 thanks to the local authorities coming from illegal fishing in the Southern Tyrrhenian Sea (Gulf of Patti). The fresh specimens were transported to the laboratory in a refrigerated and plastic free container. Once in laboratory, the specimens were

963 immediately classified and divided in three subsamples: the first (S1) consisted of identified species
964 in *E. encrasicolus* and *S. pilchardus*; second (S2) composed by mixed-species, mainly European
965 anchovy, and pilchard; and the last (S3) was classified basing on observed skin-adhered contaminants
966 and consisted of mixed-species.
967 For what concern the species caught by trawl in the Gulf of Patti and in the Sicily Channel, the
968 morphological measurements of all samples were done on board directly. The collected data included
969 total body length (TL, cm), body weight (W, g) and when possible, sex differences (M/F) of the
970 specimens. Once recorded morphometric data, all the specimens were frozen at -20°C directly on
971 board to be, at the end of the survey transferred to the laboratory. The caught species were both bony
972 and cartilaginous fishes. In general, all the specimens captured by trawl were demersal/bentho-pelagic
973 species, thus, the various haul depths allowed the capture of many demersal fishes with different
974 depth distribution range.
975

976 ### 3.1.3 Microplastics extraction methods

977 The plastic occurrence in selected organisms were surveyed using two main approaches: *i)* Visual
978 Sorting, and *ii)* Chemical Digestion protocol.
979

980 #### *3.1.3.1 Visual Sorting Method*

981 Stomachs and guts were removed from fish samples and analyzed. Each gastrointestinal tract was
982 put in a Petri plate and observed directly under stereoscope to detect any plastic particles. Once
983 extracted, the MPs were washed in distilled water, centrifuged twice for 30 s to remove the organic
984 residues and then stored in vials for further polymer identification by spectroscopy analysis.
985 Number, lenght, color and shape of MPs observed in each stomach was recorded.
986 Except for the samples of *S. canicula* from Sicily channel, all the specimens caught by trawl were
987 analysed using the visual sorting method.
988

3.1.3.2 Chemical Digestion Method

For samples not checked by visual sorting, a chemical digestion protocol was applied. In general, the sample was placed in a flask with 10% KOH (minimum ratio 1:5 weight/volume), covered with aluminium foil and placed in an oscillator incubator for 72h to remove organic matter (Avio et al., 2015). The digested samples were then decanted in graduated glass cylinder with hypersaline NaCl solution that allows the separation of two phases by density in which we expected the distribution of plastic particles close to the surface of the solution (Albano et al., 2021b; Avio et al., 2015; Savoca et al., 2020). Regarding fish samples, the NaCl solution for phase separation was 15%, while 10% for cnidaria specimens due to the highest salt and collagen concentration of tissue of the last one. The supernatant of the solution was decanted in a glass beaker to be filtered. The filtration process was carried out using a vacuum system (Millipore) and glass fiber membrane 0.7µm pore size and 47mm diameter (Whatman GF/F, UK). Filter membranes placed in the Petri dishes were exposed to the laboratory air and used as control (blank) during the entire laboratory procedure in parallel to the sample's treatment.

The filters were then put in sterile petri glass dishes for subsequent analysis.

The method was used for *S. canicula* caught in the Sicily Channel, the clupeids *E. encrasicolus* and *S. pilchardus*, and *P. noctiluca*.

3.1.3.3 Samples treatment and plastic contamination prevention procedures

Samples treatment in the laboratory was different based on their own characteristic, as described below.

Pelagia noctiluca

All the 49 specimens of mauve stinger were divided in 4 pools based on their range size (Table1). Umbrella (U) and Oral Arms (O.A.) of each specimen were divided and every pool was variable in

number both for U and O.A. according to their range size. All the samples, before analysis, were washed twice in deionized water to remove any external particles and to check any plastic inside the specimens.

Engraulis encrasicolus* and *Sardina pilchardus

The total of 264 specimens were divided in three subsamples (S1, S2, S3). In S1, 46 individuals were identified, 19 as *Engraulis encrasicolus* and 27 as *Sardina pilchardus*. The morphometric measurements were recorded (Total Length, total weight). Each specimen was checked for any skin contamination by microplastics and then gently washed twice using deionized water to remove the potential occurrence of plastic. The sample S1 was subsequently divided in two pools for chemical digestion. In S2, the total sample weighed 30g and was composed of 188 mixed specimens of *E. encrasicolus* and *S. pilchardus*. In this case, the specimens did not rinse to survey the natural condition of juvenile's fishes that usually are eaten fresh without any previous washing by the consumer. S2 was fully digested through chemical methods. The third subsample S3, that contained 30 mixed juvenile specimens, was observed using stereoscope microscopy before, then specimens with skin-adhered particles were selected for SEM observation due to the strength of the particles that could not be removed by the skin mucus after an intensive rinse.

Specimens caught by trawl

The total of specimens caught by trawl (432) were dissected to check for plastic presence in the body surface of specimens and the GIT of each sample was gently removed. The organs were rinsed with pre-filtered water, to prevent plastic contamination, and put into petri glass where they were cut longitudinally through scissors and the contents were removed by laboratory spoon to be observed using the stereoscope (Leica M205C) to individuate any particles. The particles detected were classified based on their size, colour and shape. The same methods, except for the organ rinsed, were used for *T. lyra* and *M. barbatus barbatus* in which the gill lamellae were removed and checked under stereoscope as well. Based on the little size and low specific weight of the MPs (Hale et al., 2020),

these particles tend to easily spread in air and indoor environments (Dris et al., 2017, 2015a; Zhang et al., 2020). For these reasons, in this study, during all the steps of samples manipulation, precautions have been takes against plastic contamination as reported by Bessa et al., (2019). The same contamination prevention procedures were adopted for all samples; first, the processed sample were carried out in a closed room with restricted access to scientific staff both during necroscopy and analysis. The staff acceded to the workspace dressed only with clothing that were not polymer nature or artificial textiles too, thus, the 100% cotton laboratory coat and cotton gloves were always worn. The workspace was cleaned both before and after each daywork, similarly, tools and equipment such as scalpels, tweezers, petri dishes etc. were washed with ultrapure Milli-Q water filtered (0.22 μm) before and rinsed with ethanol after (Bessa et al., 2019). During analysis, manipulation and dissection of samples, glass fiber membrane 0.7μm pore size and 47mm diameter (Whatman GF/F, UK) were leave in an open petri dish and exposed to the laboratory air as control blanks (Giani et al., 2019). When possible, all the procedures were carried out under the laboratory flow cabinet.

3.1.4 Microscopy and plastic classification

Regardless of the used method for plastic occurrence (sorting/chemical), the particles were observed under stereoscope (Leica M205C) and photographed to define size, shape and colour to obtain a univocal classification. The Scanning Electron Microscope (SEM) (Zeiss Supra 40) was used to investigate the surface morphology of the samples. According to *Marine Strategy Framework Directive* (MSFD) (Galgani et al., 2013) plastic debris were classified based on their size as: macroplastic>25mm, 25mm<mesoplastic<5mm and microplastic<5mm. Plastic particles were divided based on different colours and shapes as fibres or fragments. The ImageJ Software was used to analyse the MPs images captured by Stereoscope.

3.1.5 Plastic characterization by spectroscopy: Raman, ATR-FTIR and SEM-EDX

The particles found in the samples, both from visual sorting and chemical digestion methods, were stored in a glass Petri disk and spectroscopic techniques were applied to characterize the composition of the particles. Furthermore, these techniques have been employed for the reliability of the observation through stereoscope about shape, colour, and size of plastic debris. The microscopes used for spectroscopy techniques were micro-Raman (Witech Alpha 300 RS), Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) (Bruker Vertex 80 V FTIR) and the combined Scanning Electron Microscopy- Energy Dispersive X-ray (SEM-EDX). The three techniques were used both separately and together as complementary. Both micro-Raman and micro-FTIR allow a fast and non-destructive identification and characterization of plastic polymers by the vibrational recognition of their molecular structure (Capillo et al., 2020; Costa et al., 2010; Güven et al., 2017). The used micro-Raman was coupled with a microscope by 50X objective and allowed the focus on the surface fibers. The exciting source used came from an air-cooled Coherent Compass Sapphire Laser with an excitation of 532 nm. In some cases, both to obtain a sufficiently intense Raman signal and to reduce the autofluorescence of the polymers, a near infrared 785nm wavelength was used. The power of the laser was fixed at about 0.5 mW to avoid the heating of the particles, the laser beam was focused to the sample surface with a light spot about 0.7 μm in diameter. The spectra were integrated from 1 to 50s depending on the sample, to enhance the quality of the spectra and reduce the noise, with an accumulation number of 10.

Using both spectroscopy techniques, it can happen that the IR has strong intensities bands in which Raman are weak and vice-versa. The Raman has a higher spatial resolution (1μm) than FTIR (10-20μm), thus, the Raman spectroscopy is more suitable about microparticles having very small size e.g. <20μm. Furthermore, in Raman the presence of a confocal microscope helps in the focusing of

the laser beam on the sample. For these reasons, was always preferred the Raman than FTIR in the analysis about microfibers, however, in the case that the samples have strong fluorescent signals was preferred the use of FTIR in which the infrared spectra were acquired in in Attenuated Total Reflection (ATR) configuration. On the other hand, the application of Raman spectroscopy allows a better identification of organic matter woven in the plastic than FTIR.

The characterization of the surface microfibers morphology and their atomic composition was investigated with the use of the SEM (Zeiss Supra40) that was equipped with an Energy Dispersive X-Ray probe (EDX).

3.1.6 Statistical analysis

In the study were applied univariate analysis, both parametric and nonparametric tests. The Chi-squared test ($\chi 2$) was performed to survey potential significative differences between abundance of plastics in the species, including sexual differences, or between the different colours of plastic ingested. The Spearman's rank correlation was used to check any potential relation between the morphometric features of the specimens and the number and size of plastic. The significance level of Spearman test was set up at $p<0.005$. The analysis of variance was applied through ANOVA as well. To determine any potential differences between the abundance of MPs and their features (size and colour), was performed by Kruskal-Wallis one- way ANOVA ($p < 0.05$). Moreover, the Chi-squared test was performed as well to check potential differences between the presence of plastic materials in the two sexes.

The statistical analysis was processed using PAST software.

Regarding the samples of *S. canicula* come from GSA16, data were grouped (pools) into three groups basing on the total length class (LC) (LC<35cm, 35cm<LC<40cm and LC>40.5cm) and depth range (50-100m, 101-200m, 210-500m) of the specimens. Were calculated, both for each LC and depth

range, the frequency of occurrence (FO%) and the average number of plastic debris extracted by the GIT on the total number of individuals (N. plastic debris/N. all examined individuals).

Finally, cluster analysis and non-parametric multi-dimensional scaling (nMDS) were used to highlight microparticle feature similarities between the fish groups analysed. After the data square root transformation, the Bray-Curtis similarities were calculated. Statistical analyses were performed using PRIMER6-E.

3.2 Experimental Trial

According to the aim of the study, microcosms experiments were performed to highlight the influence of certain abundance of polymer microparticles on the feeding behaviour and the development of the brine shrimp *Artemia salina* (Linnaeus, 1758). The brine shrimp was used as a model organism to study the distribution of microplastics along the web chain based on the features of the specimens that are the primary living food source of many farmed species. Before the start of the trial, all the equipment was double washed and sterilized. The solution in which the brine shrimps have been immersed, was sterilized artificial marine water made with Caledonia Reef Salt (ReeFlowers Aquarium Solutions, Istanbul, Turkey) and ultrapure water came from Milli-Q; Merck, Darmstadt, Germany. The water parameters, during all the trial time, had a determined and pre-established degree as follows: Salinity 37 ppt; pH 8.2 ± 0.1; Dissolved Oxygen (DO) 6.6 ml/L.

3.2.1 Microalgae *Dunaliella salina* as food source for *A. salina*

Dunaliella salina (Teodoresco,1905) (Fig.25) is a halophilic unicellular green alga. The high versatility of the photosynthetic apparatus of this alga permit to growth in different light areas, thanks to the ability to accumulate high amount of β-carotene (Abu-Ghosh et al., 2015; Fu et al., 2013;

Lamers et al., 2010; Ye et al., 2008). Many studies highlighted the properties of this alga species as a biotechnological model system for the β-carotene extraction (Abu-Ghosh et al., 2015; Borowitzka et al., 1984; Lamers et al., 2012). In this study, both for average diameter (± 10µm) and the easily growth management of the *D. salina*, it was used as the only food source for the brine shrimps during the experimental trial.

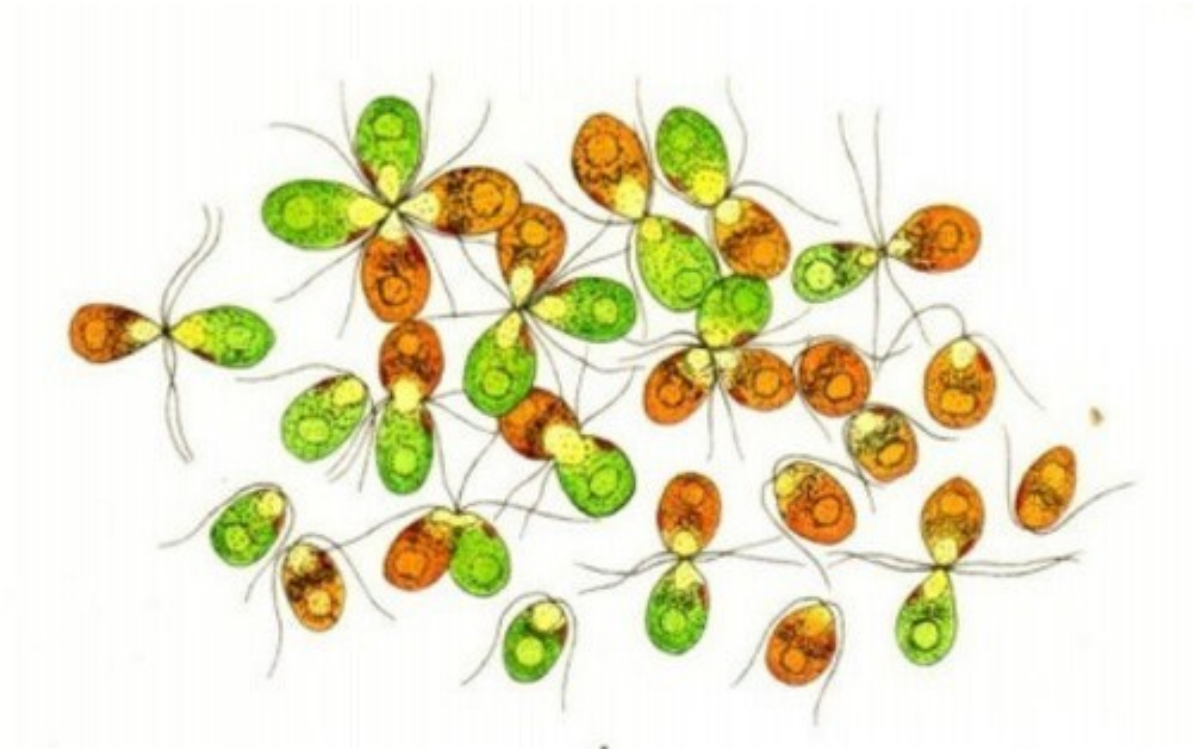


Fig. 25:The microalgae Dunaliella salina (Teodoresco 1905)

3.2.2 Plastic Microspheres and experimental solutions

The used plastic microspheres during the trial were 10 µm (diameter) of Polybead® Blue Dyed Microspheres purchased by Polysciences in a 2 ml package in a 2.5% aqueous suspension with a concentration of 4.55 x 10^7 particles/ml. The choice of this precise kind and size of plastics came from the feature of the brine shrimps and its feeding habits. Indeed, the size of the microparticles is close to those of the alga *D. salina* chosen as food source for the *A. salina* specimens. The colour blue

of the purchased plastics was chosen to better contrast against the specimens, being different from any other organic matter potentially present during the experimental activities. During the trial, solutions were used with five different concentrations of MPs as follows: 1, 10, 100, 1000 and 10000 MPs/ml. The solutions came from a MPs stock solution diluted that was prepared in sterilized artificial marine water. The different diluted solutions were used both at start of the experiment and during the same (for solution replacement) to contrast potential problems related to the distribution of the MPs in water solution as aggregation, sedimentation, and adhesion both to surface and beaker wall. All solutions were diluted in triplicate before the administration of the trial phases, observing a Bürker Türk counting chamber (Marienfeld, Lauda-Königshofen, Germany) under a microscope (Leica DM6B) at 40x magnification.

3.2.3 Management of Brine Shrimps and Algae

Since the start of the trial the larval stage of *Artemia salina* that was obtained from the shop Blue Line Artemia Cyst (Acquariomania, Macerata, Italy) were used. The brine shrimps' specimens were hatched in a beaker containing 1L saline solution with determined and pre-established parameters as shown before. During the hatching phase of the brine shrimps, strong aeration and a temperature of 26°C (±0.5°C) have been maintained. Pure strain of microalgae *D. salina*, used as food source for *A. salina*, was cultured at 20°C (±1°C), 4500 lux 24-h light photoperiod in f/2 medium and with a salinity degree of 30 ppt both after the hatching and during all the trial time of the brine shrimps. To ensure and check the feeding rate of the specimens during the different phases of the experiment, before the administration, the concentration per ml of *D. salina* was counted in triplicate for all solutions in the Bürker Türk counting chamber through microscope at 40x magnification. The main water parameters as temperature, salinity, pH and DO were measured through a multiparameter probe (Sanfilippo et al., 2016).

3.2.4 Experimental design

The trial activities were carried out in the Center of Experimental Fishpathology of Sicily (CISS), Messina, Italy (Marino et al., 2016b; Salvaggio et al., 2016). According to parameters and conditions showed before, after 24h of incubation, the instar I of *A. salina* nauplii were obtained, they were collected in a S50 Sadgewick Rafter Counting Chamber (Marienfeld, Lauda- Königshofen, Germany) and counted under a stereoscope Leica M205 C (Leica Microsystems, Milan, Italy). In total, twelve experimental groups in triplicate were set up. The experimental groups were transferred, through an automatic pipette, in 10ml glass test tubes in which there were 6 different MPs concentrations as follows: 0, 1, 10, 100, 1000 and 10000 MPs/ml. The experimental groups were managed in two different ways, in half of them were added *D. salina* as food source (Group A) while in the other half only MPs were present (Group B). The reference of the treatments is summarized in Table1.

197 *Table 1: The treatments used during the trial with Artemia salina*

Experimental Groups	Concentration N°MP/mL
A0	0
A1	1
A2	10
A3	100
A4	1000
A5	10000
B0	0
B1	1
B2	10
B3	100
B4	1000
B5	10000

198

199 The total solution in each test tube was 6ml, the experimental containers were kept in a rack at
200 laboratory for 168h with a temperature of 24°C (±1°C), with a photoperiod of 12D:12N. Sixty
201 specimens of *A. salina* nauplii (10 nauplii/ml) were transferred in each replicate. It was decided to set
202 up seven time points for sampling as follows: 0, 6, 12, 24, 48, 96, 168h. In each sampling, during

every time point, five brine shrimps per each replicate were kept, transferred, and clarified in 4% buffered formalin, observed, and photographed under the microscope Leica DM6B equipped with Imaging system (Leica DFC7000T, Microsystems, Milan, Italy) and LAS X software for further analysis. In each treatment and for each time point, the total length of brine shrimps was recorded, using the size from naupliar eye to the anus of the specimens as landmark. Both the experimental groups (A and B) have been checked every day to record the potential mortality of the specimens, in the case of it, the brine shrimps were collected with a glass pipette and transferred on a slide to be analysed and photographed under microscope. To avoid the sedimentation of the MPs in the test tubes and to facilitate the feeding of the brine shrimps, every day, each treatment of the experimental solutions was replaced by 90% (Wang et al., 2019a). The microscope Leica DM6B was used to check and quantify the MPs in the intestinal tract of *A. salina*, this counting and identification have been performed thanks to the recognizable features of the used plastics (Dahms et al., 2020; Windsor et al., 2019). Moreover, both from each group and time point of the experimental phases, several images of the specimens were observed under microscope at different magnification and captured with the imaging system (Leica DFC7000T, LAS X software) for further analysis through ImageJ software (Abràmoff et al., 2007; Aljaibachi and Callaghan, 2018). Sometimes, to obtain a better image and quantification of the MPs observed, the cover slide was gently pressed to specimens for a better spread of the MPs on the slide.

3.2.4.1 Pre-experimental Tests

The pre-experimental tests were performed to survey the distribution of the plastic in the saline solution, the potential adhesion to the wall of test tubes and aggregation with microalgae, finally, the feeding rate of the brine shrimps. The preliminary tests allowed a better management of MPs during

the experimental phases. The test results showed that after 48h, the MPs started sedimentation despite the constant motion activity of the brine shrimp nauplii. Using the already shown method as proposed by Bergami et al. (2016), before the trial, we surveyed the 24-h ingestion/egestion rate of *A. salina*. The results showed that after 3h post-ingestion, the nauplii of brine shrimps ejected 90% of MPs administered. Thus, basing on the shown data, we decided to replace the solution every day to avoid the decreasing of the MPs concentration during the experimental time phases.

3.2.5 Feeding rate and algal growth

To evaluate both the feeding rate and behaviour of the brine shrimps, a parallel test was performed adopting the same set up of the main experiment, except for the quantification of *A. salina* in each test tube. Indeed, only a specimen was added in each 10ml test tube, but like the main experiment, with the 6 different concentrations (0, 1, 10, 100, 1000, 10000 MPs/ml) and in triplicate. As in the main experiment, the specimens were sampled at different time points (24, 48, 96 and 168h) and counted under microscope at 40x magnification in a Bürker Türk counting chamber. As shown by Frost, (1972), the cells/larvae/h method was used to estimate the feeding rate of the brine shrimps on algae. Before each daily replacement of the algal food source, the pure culture of *D. salina* was counted to ensure the exact concentration. Moreover, a control test, without *A. salina* feeding, was performed to evaluate the algal growth. Using the ImageJ software, through the image acquisition, at each time point was applied an estimation of the MPs occupied the intestinal tract of the brine shrimps, thus, these were subtracted from the ingestion of *D. salina* (Abràmoff et al., 2007).

3.2.6 Data Elaboration and Statistical analysis

The total length of intestinal tract and the entire body of the brine shrimps were measured using ImageJ software, thus, as reported by Wang et al., 2019b, was valued at the developing stages of the

specimens of the control group. Data are expressed as means ± standard deviation (SD). Evaluating the total length and mortality rate in the different experimental groups, and significant differences in microplastic abundance, the parametric One-way Analysis of Variance (ANOVA) or nonparametric Kruskal–Wallis tests, followed by Fisher's Least Significant Differences (LSD) post-hoc test were used. Differences were considered significant at $p<0.05$.

4. Results

4.1 Environmental survey

The results obtained in this study are reported in the sections below. Data are described and divided according to the plastic extraction protocol applied to each sample (Visual sorting/Chemical Digestion).

4.1.1 Plastic occurrence investigated by Visual Sorting

The specimens analysed by visual sorting method were those caught by trawl in the Gulf of Patti. In a total of 371 specimens, belonging to 12 species, were analyzed. The morphometric measurements of the species were reported in Table2.

Table 2: the morphometric measurement of all the species caught by trawl and their sex

		Lenght (cm)			Weight (g)			Sex (n°)	
	N° specimens	Range	Mean	SD	Range	Mean	SD	M	F
Boops boops	30	12.5÷25.5	20,3	3,2	54÷155	86,9	35,3	13	17
Chlorophthalmus agassizi	43	12÷16.5	14,3	1,2	13.16÷39.84	25,7	6,7	I	
Galeus melastomus	75	32÷52.5	43	4,3	104÷483	248,6	74,8	32	43
Lepidopus caudatus	32	70÷109	95	10,3	219÷1053	587	188,3	I	
Merluccius merluccius	67	18÷42	25,9	6,3	39.8÷664	159,4	132	29	38
Mullus barbatus barbatus	21	11÷22.5	17,2	4,1	15÷134	66,8	40,7	9	12
Pagellus bogaraveo	24	14÷21	16,3	1,6	41÷122	62,7	18,4	6	18
Pagellus erythrinus	15	16÷25	18,7	2,2	53÷187	86,6	32	I	
Raja miraletus	1	45	-	-	41	-	-	1	0
Scyliorhinus canicula	12	19.8÷51.5	40,7	9	100÷497	275,3	140	8	4
Trigla lyra	16	10.0÷16	13,9	2,4	13÷36	27	9,48	I	
Zeus faber	35	7÷50	18,4	12,3	6.0÷2080	244	492	I	

One hundred twenty-three specimens were positive to plastic contamination (33.15%), and a total of 369 plastic particles were detected. The ingested particles were distributed through the species as follow: 80 in *Boops boops* (63.3%), 9 in *Chlorophthalmus agassizi* (14%), 6 in *Galeus melastomus* (8%), 152 in *Lepidopus caudatus* (78.1%), 31 in *Merluccius merluccius* (46.3%), 6 in *Mullus barbatus barbatus* (14.3%), 3 in *Pagellus bogaraveo* (12.5%), 1 in *Pagellus erythrinus* (6.6%), 2 in *Raja miraletus* (100%), 13 in *Scyliorhinus canicula* (33.3%), 4 in *Trigla lyra* (25%) and 62 in *Zeus faber* (51.4%) (Tab.3). Except for *T. lyra* in which there were isolated fibers between gill lamellae, all particles were found in the GIT of the specimens.

Table 3: Results to plastic ingestion degrees in the specimens caught by trawl in the Gulf of Patti

Species	Item/Specimen	MPs positive %
Boops boops	2.7	63.3
Chlorophthalmus agassizi	0.2	14
Galeus melastomus	0.1	8
Lepidopus caudatus	4.8	78.1
Merluccius merluccius	0.4	46.3
Mullus barbatus barbatus	0.3	14.3
Pagellus bogaraveo	0.1	12.5
Pagellus erythrinus	0.1	6.6
Raja miraletus	1.0	100
Scyliorhinus canicula	1.1	33.3
Trigla lyra	0.3	25
Zeus faber	1.8	51.4

4.1.1.1 Bony fish

Boops boops

A total of 30 *B. boops* individuals, 80 plastic fibers were found in 19 specimens (63.3%) with an average of 2.7 items/specimen without significant differences ($\chi^2 = 0.03$, $P > 0.05$) in relation to sexes (61.5% males, 64.7% females). All plastic items were found in the GIT (37.5%). Selected optical images of particles found in the bogue specimens are shown in Figure 26. The Spearman test ($P > 0.05$) did not show a significant correlation between fish length and fibers number. The length range of fibers was from 0.5 to 30 mm, including plastic items from micro to macro size in which the most abundant were microplastics (74% <5mm). Concerning fibers colour, most were black (95%) with a small percentage in red color (5%) (Fig.27).

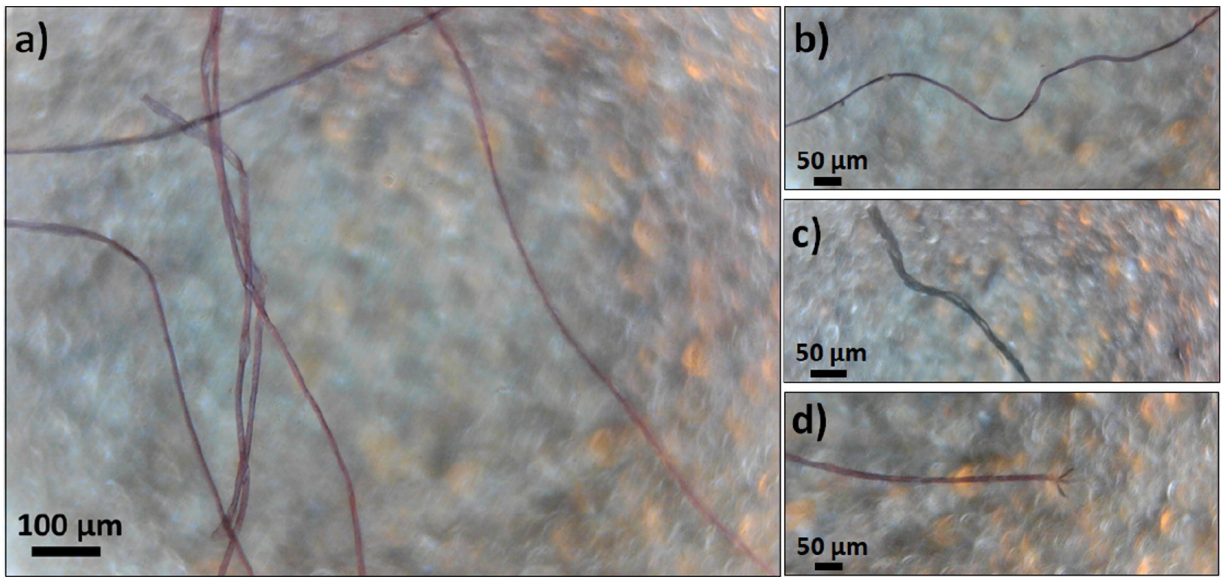
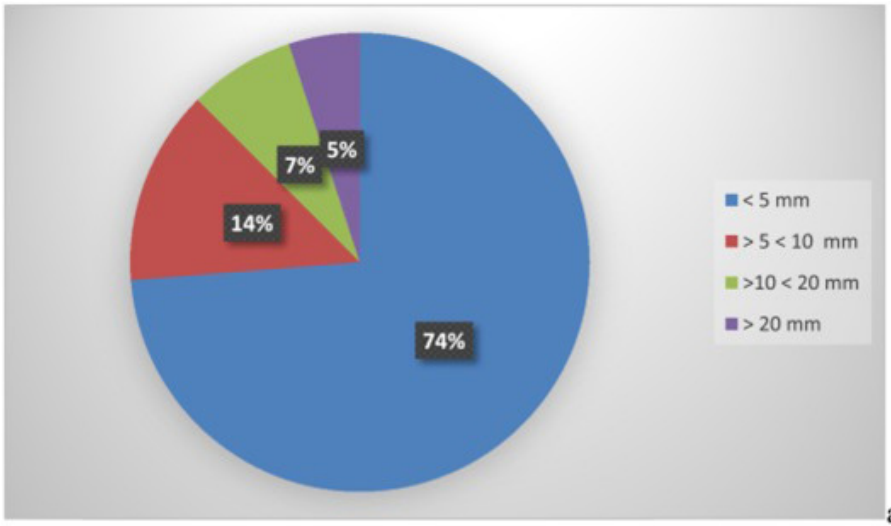
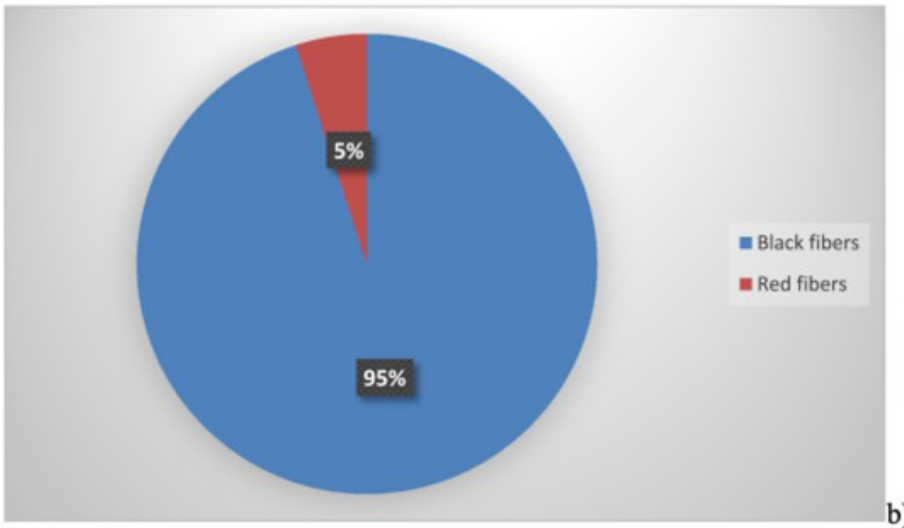


Fig. 26: Optical image of the fibers found in the specimens of Boops boops



a)



b)

Figure 27: pencentage of fibers classified by size (a) and colour (b) extracted from Boops boops

Chlorophthalmus agassizi

A total of 43 *Chlorophthalmus agassizi* were analysed. Nine plastic fibers were found in six specimens (14%) with an average of 0.2 items/specimen. The plastic contamination was observed only in the GIT (21%) of shortnoose greeneye. All the particles detected were microplastics with a length range between 1 and 4.5 mm. The colours detected were blue and black in which the first was more abundant (77.7%) than black (22.2%) (Table4). Figure28 shows one representative black fiber found in the GIT of *C. agassizi*.

Table 4: The features of the found MPs in Chlorophtalmus agassizi; Colour, N°, Length range (mm)

Colour	N° MPs	Lenght Range
Black	2	1-2.78
Blue	7	1.36-4.55

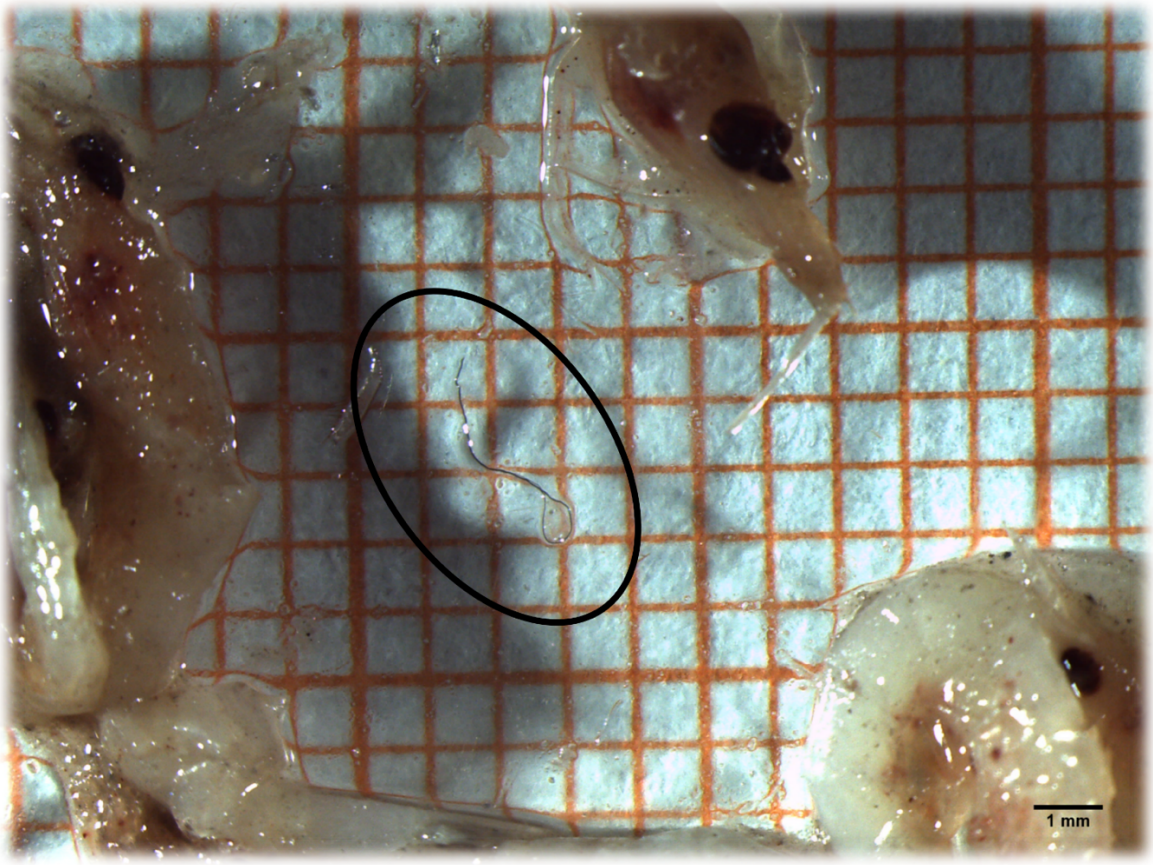


Fig. 28: representative black fiber found in the GIT of C. agassizi

Lepidopus caudatus

Thirty-two specimens of *L. caudatus* were analyzed. The plastic particles (152 items) were observed in the GITs of 25 specimens, with a plastic relevance of 78.1% and an average of 4.8 items/specimen (from 1 to 23 items). The plastic debris were only in fiber shape with a length range between 0.5 and

15mm in which the main particles were categorized as microplastics (94%) than the remaining 6% as mesoplastics. However, the prevalent size class in length of observed microplastics was between 1 and 2.5mm.

The black fibers were the most abundant (95.4%) followed by a minor percentage of red fibers (4%) and light blue fibers (0.6%) (Table5).

Merluccius merluccius

A total of 67 specimens of *M. merluccius* were analysed. The specimens, basing on the gonadal developmental stage, were both adults and sub-adults. A total of 31 plastic particles were found only in the GIT of 46.3% European hakes without significant differences ($\chi 2 = 0.3$, $P > 0.05$) between females (44.7%) and males (48.3%). The microplastic length range was between 0.7-2.95mm and all of these were in black colour. A representative image of particle found in the GIT is reported in Figure 29.

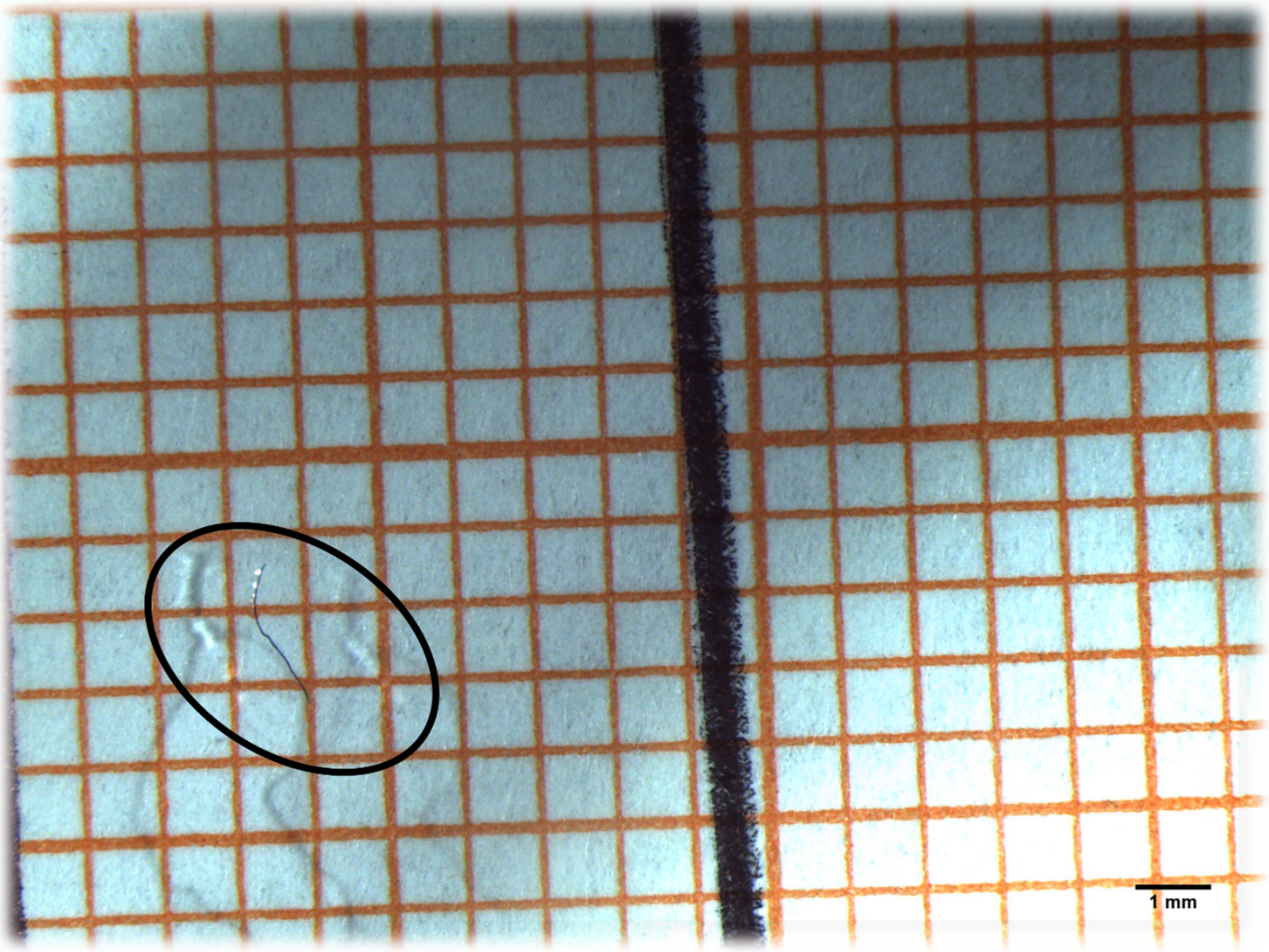


Fig. 29: One of the 38 black fibres found in the GIT of Merluccius merluccius

Mullus barbatus barbatus

The total *M. barbatus barbatus* checked were 21 in which 12 were females (57.1%) and 9 males (42.8%). The 14.3% of the specimens resulted positive to plastic contamination in the GIT with an amount of 6 debris and 0.3 items/specimen. All the plastics detected were fibers and the dominant colour was black. One of the fibres found in the specimens is shown in Figure 30.

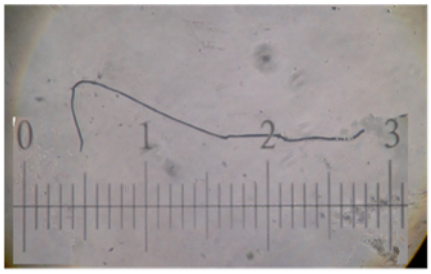


Fig. 30: Teflon fibre found in the red mullet M. barbatus barbatus

Pagellus bogaraveo* and *Pagellus erythrinus

A total of 39 species of seabreams were analysed in which 24 (61.5%) were *P. bogaraveo* and 15 (38.5%) *P. erythrinus*. The plastic particles were found in the GIT of 4 specimens (9.1%), 3 *P. bogaraveo* and 1 *P. erythrinus* respectively. For both species was calculated 0.1 items/specimens. All the items were fibers in shape and only the black was observed as a colour feature.

Particles detected in two species of seabreams are shown in Figure 31 (a, b, and c).

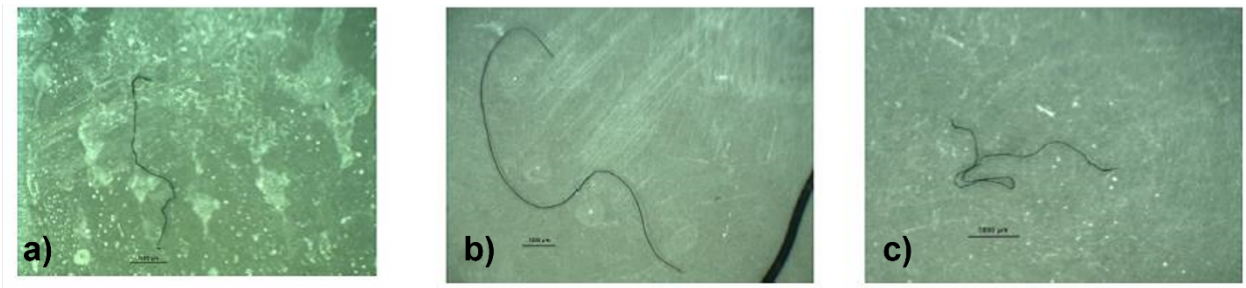


Fig.31:MPs found in a)Pagellus erythrinus and b)c) P. bogaraveo

Trigla lyra

In the 16 specimens of *T. lyra* analysed were found 4 positive individuals to plastic ingestion, showing a positivity of 25%. The colours of observed fibers were black (85%) and red (15%).

Zeus faber

A total of 35 specimens of John Dory *Zeus faber* were analysed. The 51.4% (18) of specimens resulted positive to plastic contamination along the GIT with an average of 1.8 items/specimen and a total of 62 plastic items were found. The length range of the particles were from 0.5 to 5.3 mm, including from micro to meso size class. The most common plastics were black fibers 96.7% than light blue fragments 3.2%. The features of found plastics in *Z. faber* are reported in Table6.

Table 5: The features of the found MPs in Zeus faber; Colour, N° MPs, Length (mm)and shape

Colour	N° MPs	Length (mm)			Shape
		Range	Mean	SD	
Black	60	0.5-5.3	2.84	1.30	fibers
Light blue	2	1	1	-	fragment

4.1.1.2 Cartilaginous fish

Galeus melastomus

A total of 75 Blackmouth catshark were analysed, 32 males (42.6%) and 43 females (57.3%). In 6 specimens, plastic particles were found (8%) with an average of 0.1items/specimen. The predominant shape of plastic was as fibers (83.3%), while in some specimens were found fragments (16.6%). Moreover, in a blackmouth catshark individual was found a macroplastic debris (Fig. 32).
The plastic colours recorded during the sorting observation were black and white, more in detail, about 80% of plastics were black fibers than the remaining 20% represented by white fragments.

Fig.32: The microplastic fragment of PE found in the GIT of the blackmouth catshark Galeus melastomus

Raja miraletus

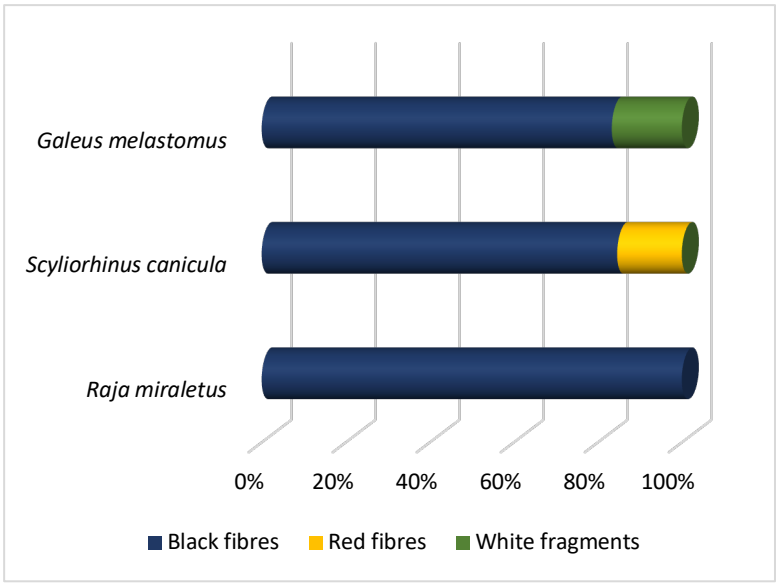
A single specimen of *R. miraletus* was analysed, a female of 45cm length and 41g weight. The specimens showed the occurrence of 2 black fibers in the GIT.

***Scyliorhinus canicula* (Gulf of Patti)**

The specimens of *S. canicula* analysed were 12 in which 8 (66.6%) were males and 4 (33.3%) females. A total of 13 plastic debris were found in 4 specimens (33.3%) with an average of 1.1 items/specimen. All the particles were in fiber shape and the highest abundance of size class was micro (92.3%) than meso (7.6%), however, a macrofilament was found as well (Fig. 33). The plastic particles found in the GIT were both in black (84.7%) and red (15.3%) colour.

Fig. 33:Macrofilament found in the GIT of Scyliorhinus canicula

The results of plastic occurrence and features of the recorded plastics in cartilaginous fish, except for *S. canicula* from Sicily channel, are reported in Figure34 and 35 .



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403 *Fig. 34:Percentage of plastics recorded in cartilagineous fish categorized based on their shape and colour*

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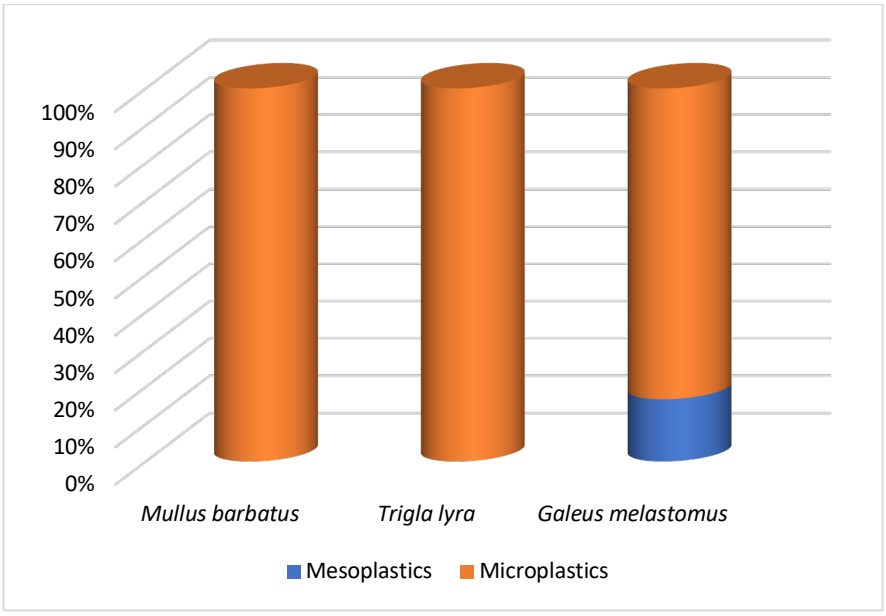

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407 *Fig. 35: Percentage of plastics recorded in cartilagineous fish categorized based on their*

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4.1.2 Plastic extraction by chemical digestion

4.1.2.1 Jellyfish

The 49 specimens of *Pelagia noctiluca* collected were divided and classified in relation to their umbrella diameter range size in four different pools: pool-1 (3-5cm), pool-2 (5-8 cm), pool-3 (8-10 cm) and pool-4 (>10 cm). The length and diameter of Oral Arms (OA) and Umbrella (U) respectively, and the total weight of *P. noctiluca* are reported in Table6. The umbrella and oral arms were processed separately during the digestion procedures, but the potential plastic presence and abundance were recorded as part of the same pool. A total of 55 plastic particles were isolated in which 53 (96.3%) microplastics and 2 (3.6%) mesoplastics, ranging in size between 0.09 and 9.4mm. The plastic colours observed were various, despite the black was the most abundant (80%). Analysing in detail the abundance and the features of plastics in each pool, was observed as follow:

- The abundance of plastics in pool-1 was 14.5%, it was the only group with all black particles, ranging in size between 0.09 and 2mm.
- Pool-2 was the only one featured by the presence of several colours (black, blue, red and white). It showed 7.27% of microplastics and with the bigger size of particles than other groups, ranging between 0.1 and 9.4mm length, including both micro and mesoplastics.
- Pool-3 showed 38.10% microplastics, blue and black, with a length range between 0.1 and 3.3mm.
- Finally, Pool-4 showed the highest percentage of plastics (40%) than other pools with a length between 0.1 and 2.9mm, it was featured by plastics in black, blue, and light blue colours.

Table 6: Morphometric measurements of Pelagia noctiluca

	Diameter (cm)			Length (cm)			Weight (g)			Examined Specimens (N°)
	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	
Pool 1	3.8÷5.0	4,6	0,6	0÷4.5	2,7	2,4	2.2÷12.0	6,1	5,2	3
Pool 2	5.3÷7.9	6,9	0,8	3.2÷13.7	8,1	2,7	2.4÷56.6	15,1	12,6	23
Pool 3	8.1÷9.9	9	0,5	7.7÷13.8	10,6	1,7	17.0÷81.4	37,1	14,6	20
Pool 4	10.0÷10.8	10,3	0,4	9.6÷13.5	10,9	2,2	41.2÷47.1	43,2	3,3	3

The statistical analysis did not highlight significant differences in size and number of microplastics between the considered pools ($p > 0.05$), except for the colour ($p < 0.05$). Considering umbrella and oral arms separately, these showed a percentage of plastic abundance corresponding to 62% and 38% respectively. In this case, as well, no significant statistically differences were highlighted, except in the Pool-3 regarding the plastic size from umbrella and oral arms ($p < 0.05$). A weak correlation ($p < 0.05$ and $p = 0.31$ respectively) was calculated between the particle size and the weight of specimens.

4.1.2.2 Clupeid fish

A total of 46 specimens were exanimated. The morphometric measurements of specimens analysed are reported in Table 7.

Table 7: the morphometric measurements of Sardina pilchardus and Engraulis encrasicolus

	Length (cm)			Weight (g)			Examined Specimens (N°)
	Range	Mean	SD	Range	Mean	SD	
Sardina pilchardus	2.99÷4.04	3,26	0,25	0.16÷0.49	0,23	0,07	19
Engraulis encrasicolus	2.20÷3.15	2,68	0,27	0.05÷0.12	0,08	0,01	27
Total							46

In the sample S1 (27 *E. encrasicolus,* 19 *S. pilchardus*) were found 17 microplastics both adhered to external surface and in the GIT of investigated specimens. More precisely, were found 7 microplastics in *E. encrasicolus* specimens and 10 in *S. pilchardus* specimens. The European pilchard was the subsample in which was recorded the highest average of plastic debris (0.53 items/specimen) than the European anchovy with an average of abundance of 0.26 items/specimen. Regarding the shape of particles isolated, the observations revealed the occurrence of only fibers in *S. pilchardus* while in *E. encrasicolus* was observed the presence of 1 fragment. ANOVA analysis did not highlight significant differences ($p>0.05$) in MPs mean length, 0.79 mm in *E. encrasicolus* and 1.90 mm in *S. pilchardus*, between the two species. Not even the Spearman Test ($p>0.05$) highlighted correlation between MP size and length of specimens. The most common fibers colour observed was black in both species (42.8% and 30% in *E. encrasicolus* and *S. pilchardus* respectively) followed by other colours as blue, red etc. as showed in Figure 36.

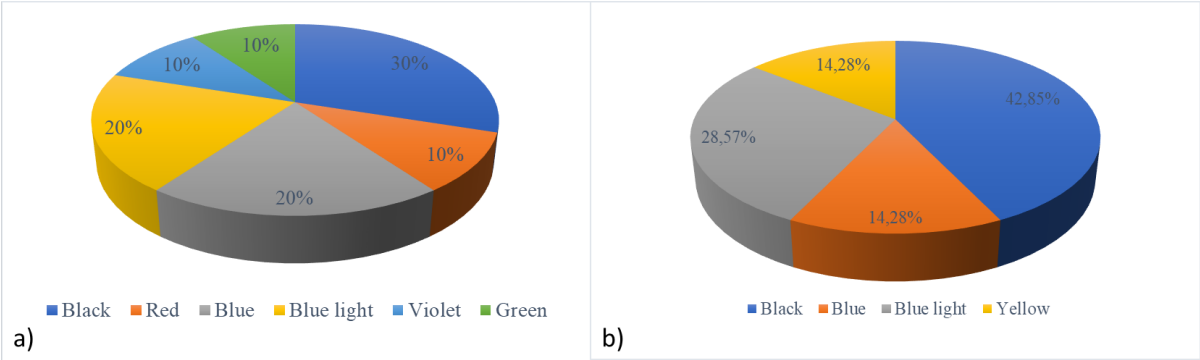


Fig. 36: the percentage of plastic found in a) S. pilchardus and b) E. encrasicolus basing on different colours

The likelihood ratio Chi-square test did not highlight significant differences ($p>0.05$) between the colours of MPs and the two species. In the sample S2, composed by 30g of mixed species, were found 30 MPs in which the most were in fibers shape (93.3%) than fragments (6.7%). The length range of particles was between 0.2 and 4mm and the prevalent colour was black (63.3%) followed by blue (13.3%), red (10%), yellow (6.6%) and light blue fragments (6.6%) (Table8). The representative sequence of optical images of plastic particles are showed in Figure 37.

Table 8: The features of the found MPs in the clupeids fishes; Colour, N°, Length (mm) and shape

Colour	N° MPs	Length (mm)			Shape
		Range	Mean	SD	
Black	19	0.2-4	1,12	0,99	Fiber
Red	3	0.5-1	0,83	0,28	Fiber
Blue	4	0.4-4	1,47	1,7	Fiber
Light blue	2	0.5-1	0,75	0,35	Fragment
Yellow	2	1÷2	1,5	0,7	Fiber

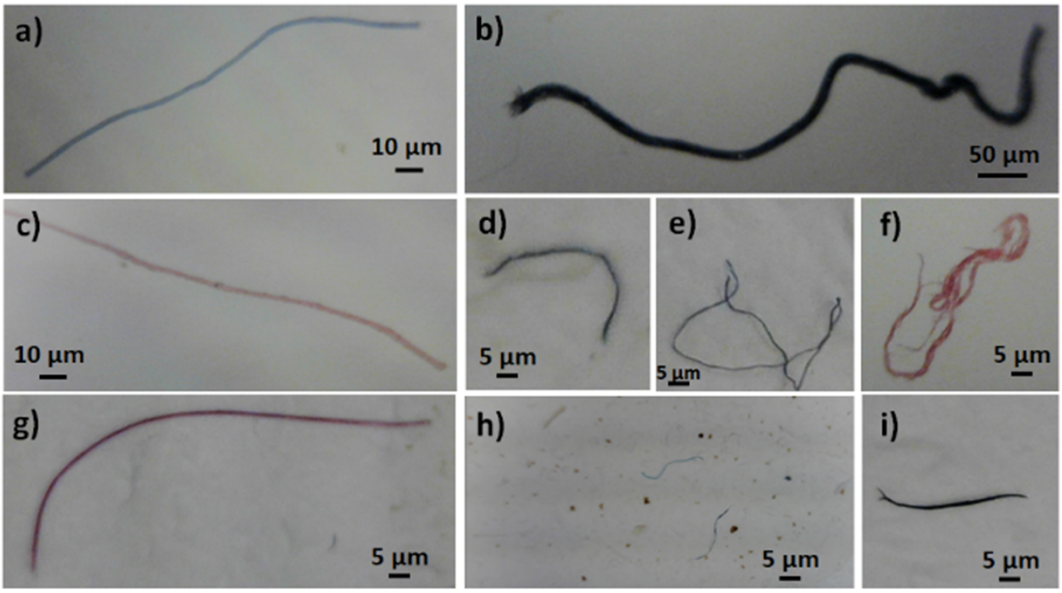


Fig. 37: representative optical image of MPs extracted by the juveniles clupeid fishes and having different shape features

Starting from these images' differences about diameters and colours of MPs were observed; linear fibers (until to 100µm) with a diameter of 2-3µm (a, b, g). In images d), e), and f) the fibers were tangle and filamentous appearing as a very weakened tissue. In images h) and i), the observed fibers were featured by the edges with smooth surface and cracked rounded shapes, after, through spectroscopy techniques, it was observed that this was due to the mechanical breaking of the polymer/cellulose chains.

4.1.2.3 *Scyliorhinus canicula* (Sicily Channel)

Plastic Presence in GITs

Coming from experimental trawling survey in the GSA16, were analysed a total of 61 *S. canicula* ranging from 24.5 to 55cm in length and a weight between 40 and 330 g. From the 61 examined specimens the presence of plastic was observed in forty-nine individuals (FO:80.3%). In the 61

positive specimens were found a total of 185 plastic debris (2.6 items/specimens) in the GITs belonging to different size: macroplastics (1%), mesoplastics (4%), large microplastics (46%) and microplastics (49%) (Fig. 38).

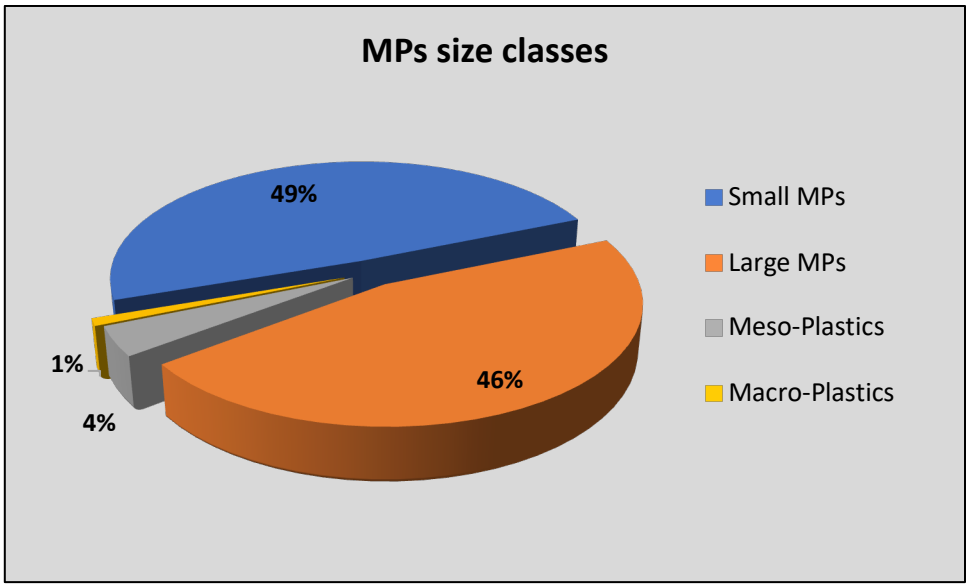


Fig.38: The different size class of plastic found in the GIT of Scyliorhinus canicula:

The observed length range of plastic particles was between 0.03 and 42.3mm in which fibers were the most abundant shape (85%) followed by fragment shape (15%- Fig.39).

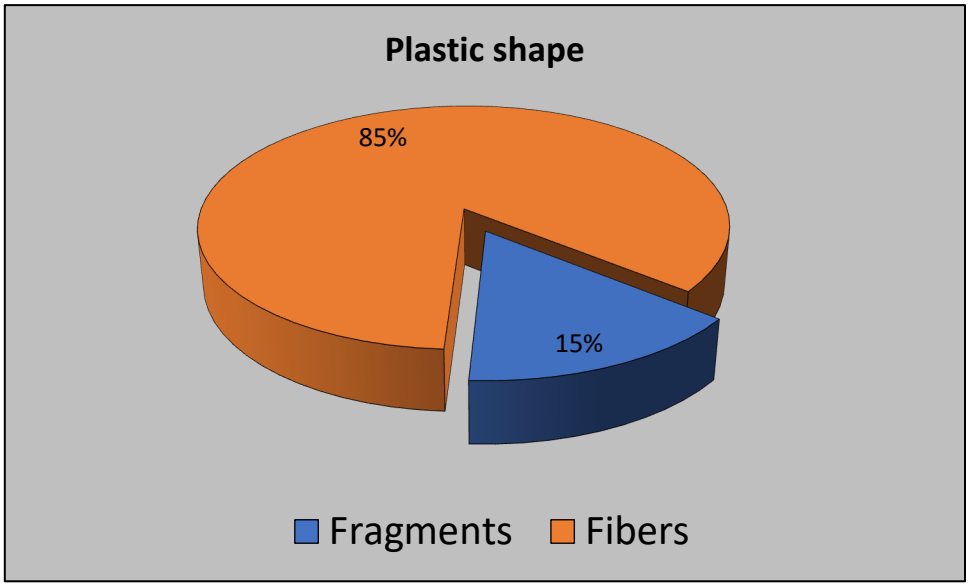


Fig.39: The classification of found plastics basing on their shape

For what concerning the observed colours the predominant was black (21%) followed by blue (18%), transparent (15%) and red (11%), however, were observed as well other colours in minor percentage such as brown, white, light blue and other shades (Fig. 40).

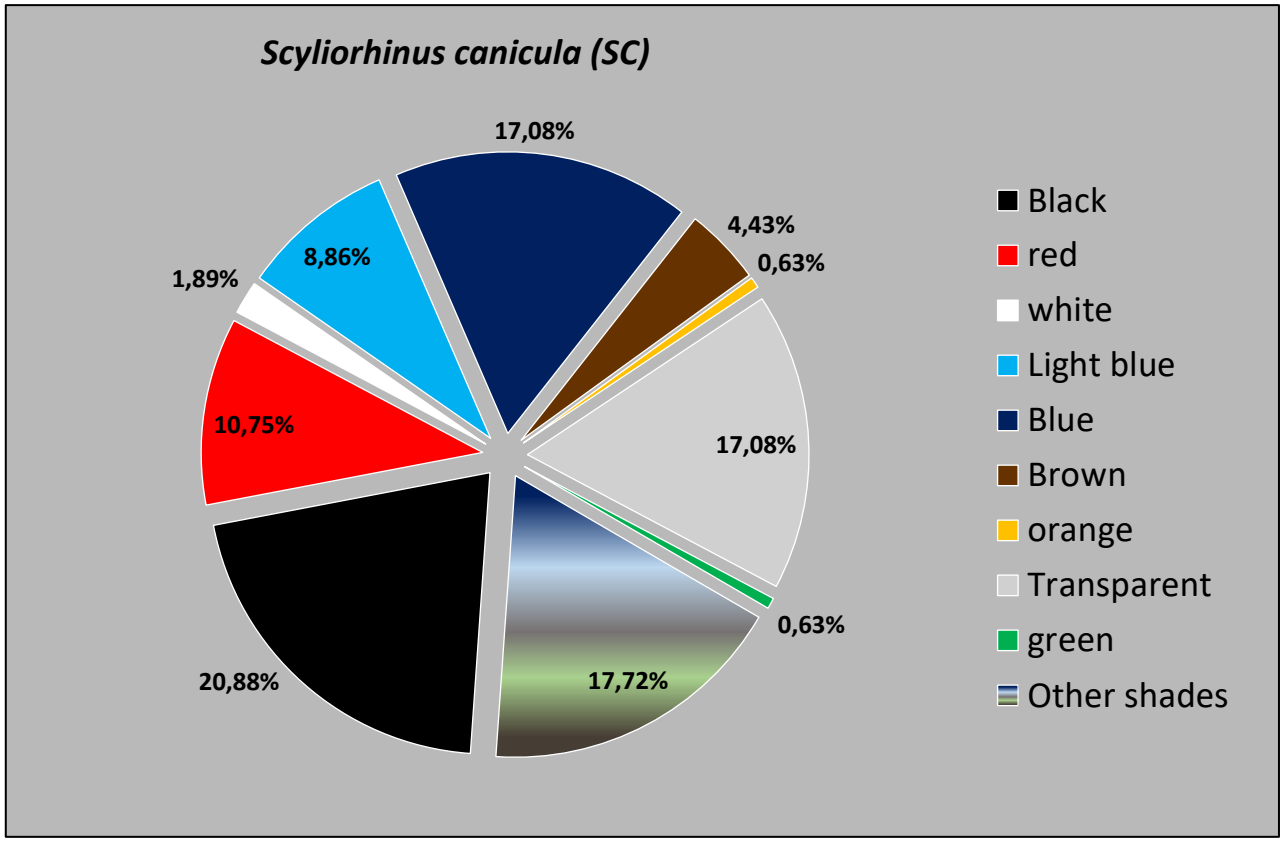


Fig.40: classification of found plastics basing on their colours

Plastic ingestion in relation to sex, length class, depth range and sampling site

In the 61 specimens examined 32 were females and 29 males, results highlighted the higher frequency of plastic occurrence in males (86.2%) than females (75%) and no significant differences were highlighted ($\chi 2$: 1.1; $p>0.05$). On the other hand, the abundance of plastic materials was higher in the females' specimens, with a range between 1 and 12 items (3.0 items/specimens), than in males with a number range between 1 and 20 (2.1 items/specimens).

The plastic presence was observed in all three-length class, showing the increasing of the plastic abundance per specimen in accordance with length class form 1.1 (LC<35) to 3.2 (LC-45), and a frequency of plastic occurrence from 57.1 in LC<35 to 85% in LC-45.

For what concerns the relation of plastic ingested with depth range, it showed the highest number of items/specimens (4.7) between 50 and 100m while the lowest was 2.2 between 201 and 500m deep.

Comparison of plastic contamination level among the species analysed

The Cluster analysis and MDS (Fig. 41-43) grouped the analysed species in two main clusters carachetrized by a 40% of similarity, on the basis of microplastics features. The first cluster showed high similarity of microplastics abundance, size and color distribution between *B. boops*, *Pagellus* spp., *M. mercluccius*, *M. barbatus* and *R. miraletus*. Within the same cluster 80% similarity was found between *Pagellus spp*., *M. merluccius*, *M. barbatus* and *R. miraletus*. The second cluster showed high similarity of microplastics abundance, size and color distribution between *C. agassizi*, *Z. faber*, *L. caudatus*, *T. lyra*, *S. canicula* and *G. melastomus*. Within the same cluster 60% similarity was found between *Z. faber*, *L. caudatus*, *T. lyra*, *S. canicula* and *G. melastomus.*

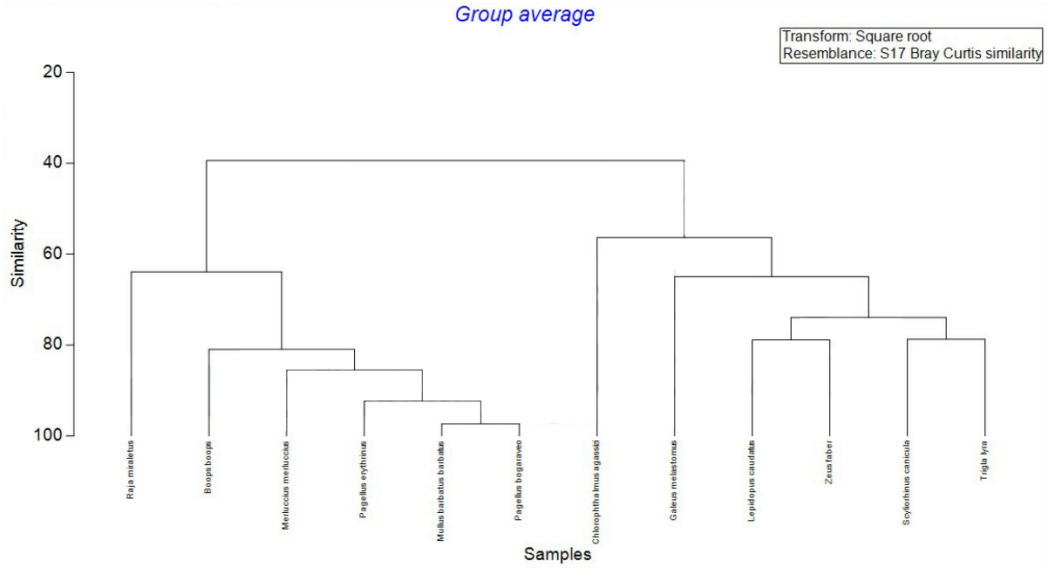
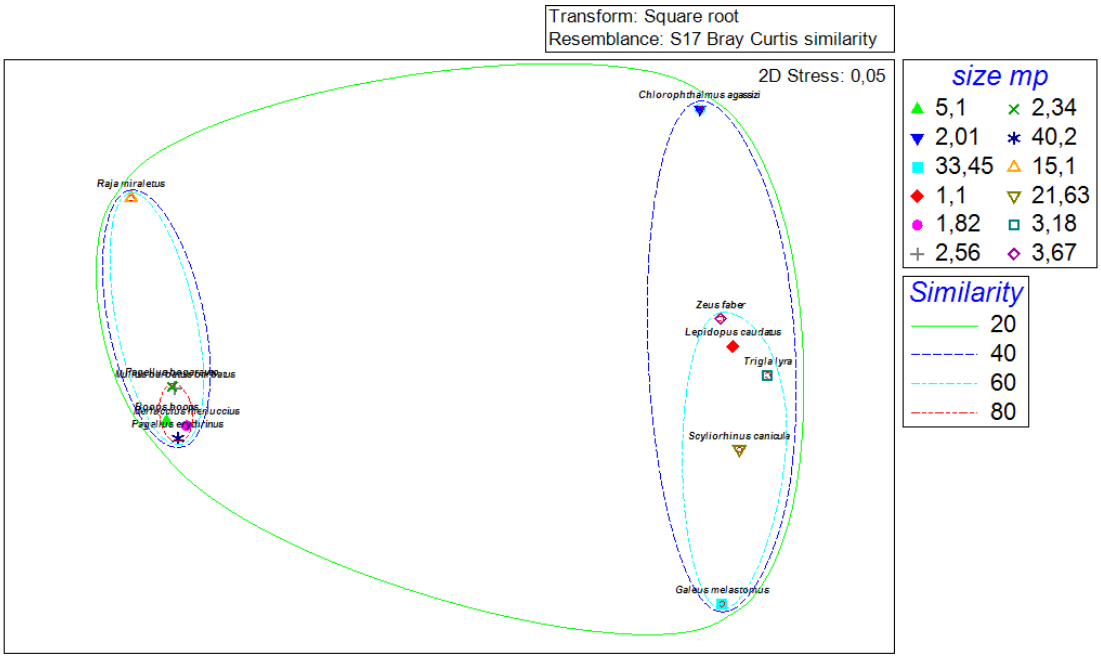


Fig. 41 Similarities in the microparticle abundance between analysed species



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537 *Figure 42 Similarities in the microparticle size between analysed species*

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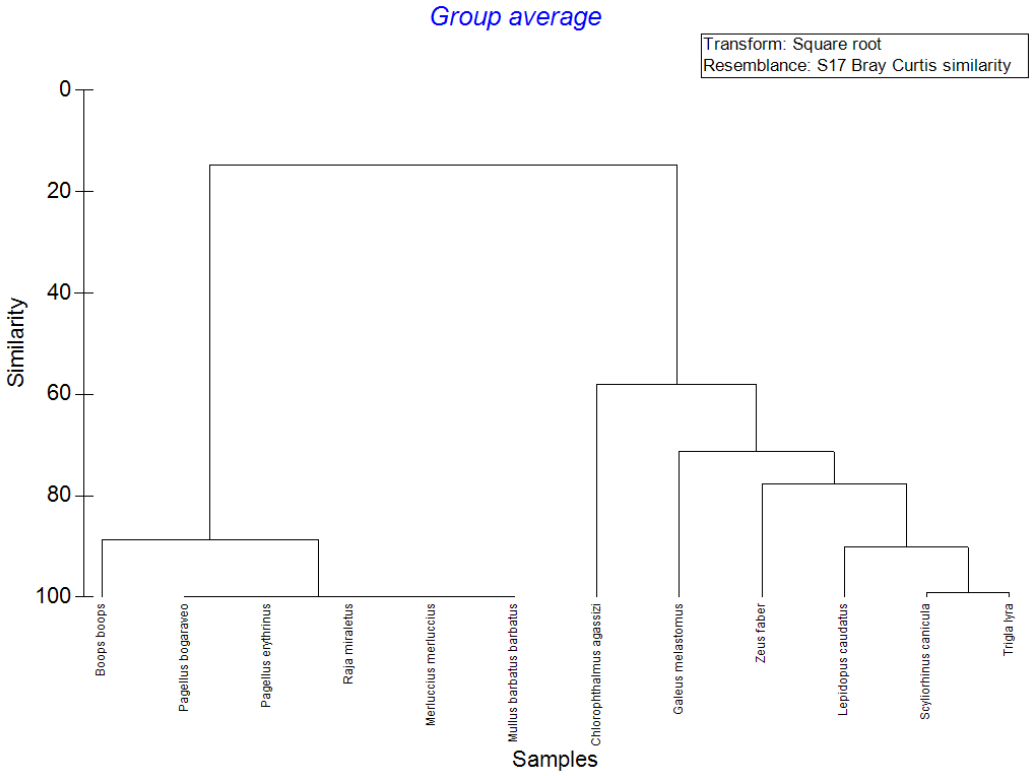

Figure 43 Similarities in the microparticle colours between analysed species.

Concernig *S. canicula* specimens collected from Gulf of Patti and Sicilian Channel interesting differences were observed between the two population in terms of microplastics abundance, size and color ($p<0.05$) (Fig. 44-46).

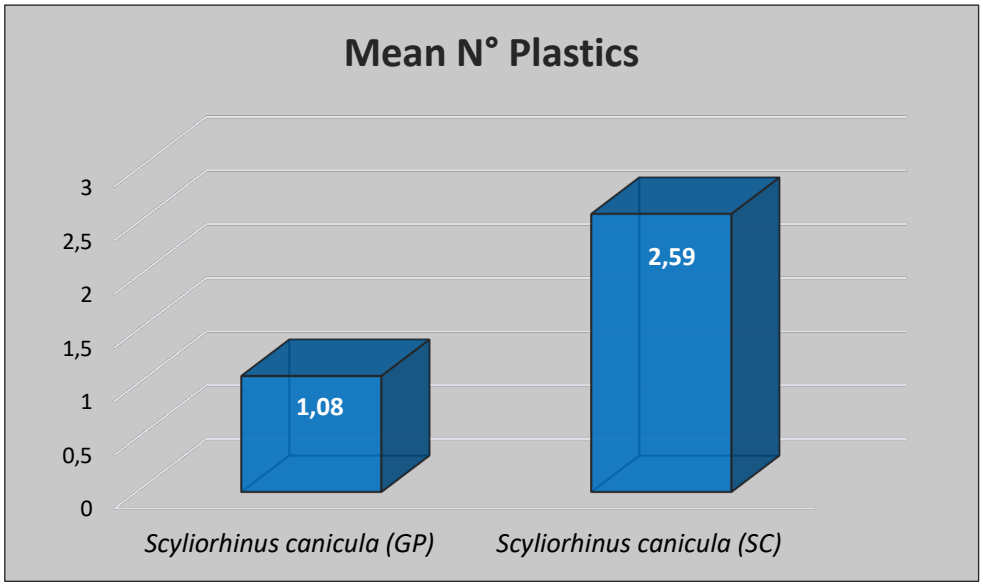

Figure 44 Abundance of particles found in the S. canicula specimens collected from Gulf of Patti (GP) and Sicilian Channel (SC)

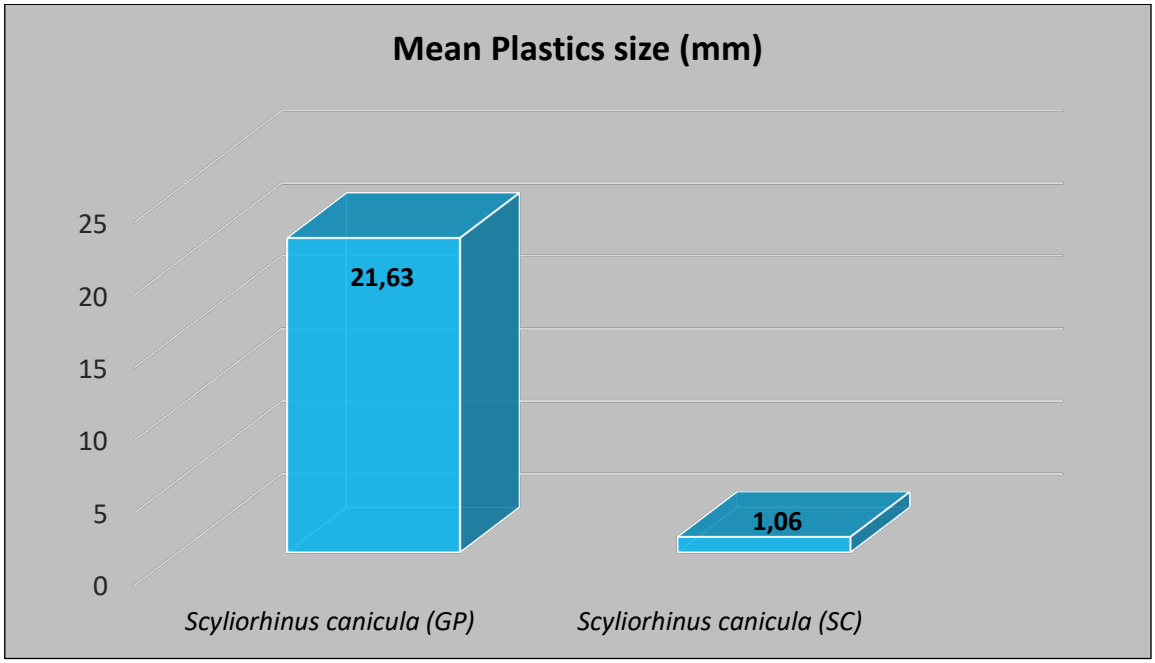

Figure 45 Size of particles found in the S. canicula specimens collected from Gulf of Patti (GP) and Sicilian Channel (SC)

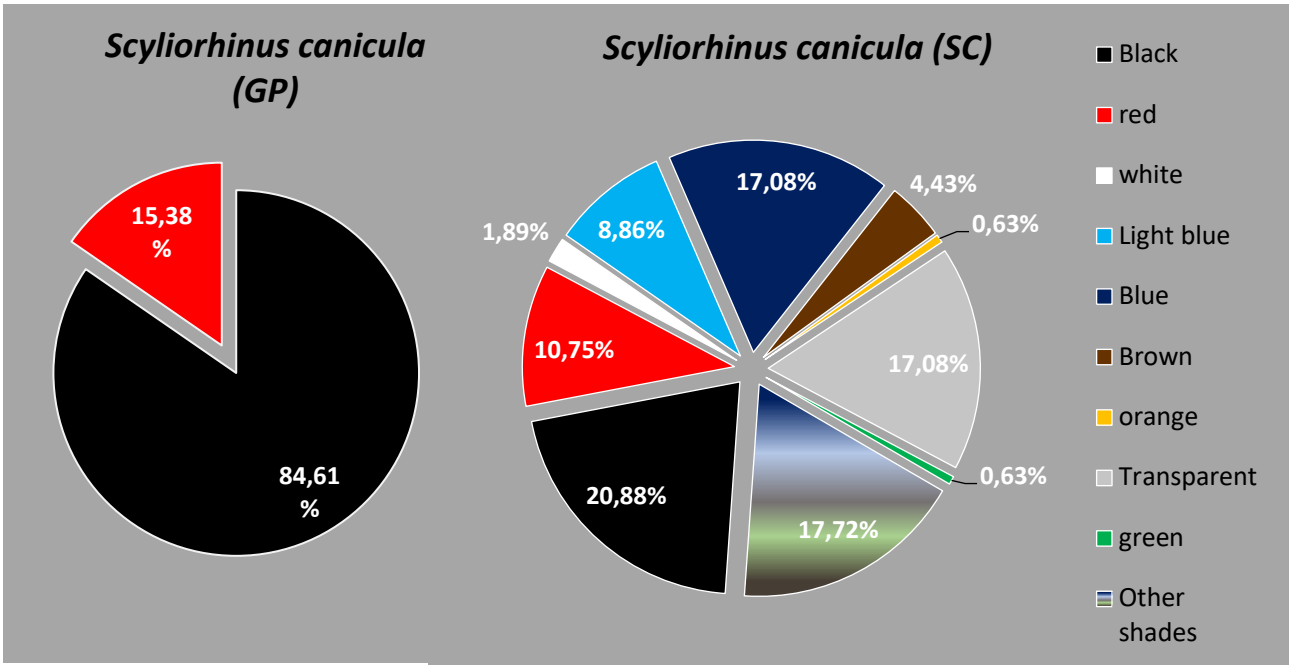

Figure 46 Colors composition of particles found in the S. canicula specimens collected from Gulf of Patti (GP) and Sicilian Channel (SC)

4.1.3 Scanning Electron Microscope (SEM)

The Scanning Electron Microscope technique was applied to juvenile clupeid fish (*Engraulis encrasicolus*, *Sardina pilchardus*), more precisely in the sub-sample S3. The SEM observation on the sample, highlighted in two specimens of European pilchard (*S. pilchardus*), the presence of entangled microfibers in the skin mucus (Fig. 47). The observation exhibited a compact and tight structure of the microfibers polymeric surface, however, in some parts, the polymers had an open and loose structure which resulted in a partial degradation of them.

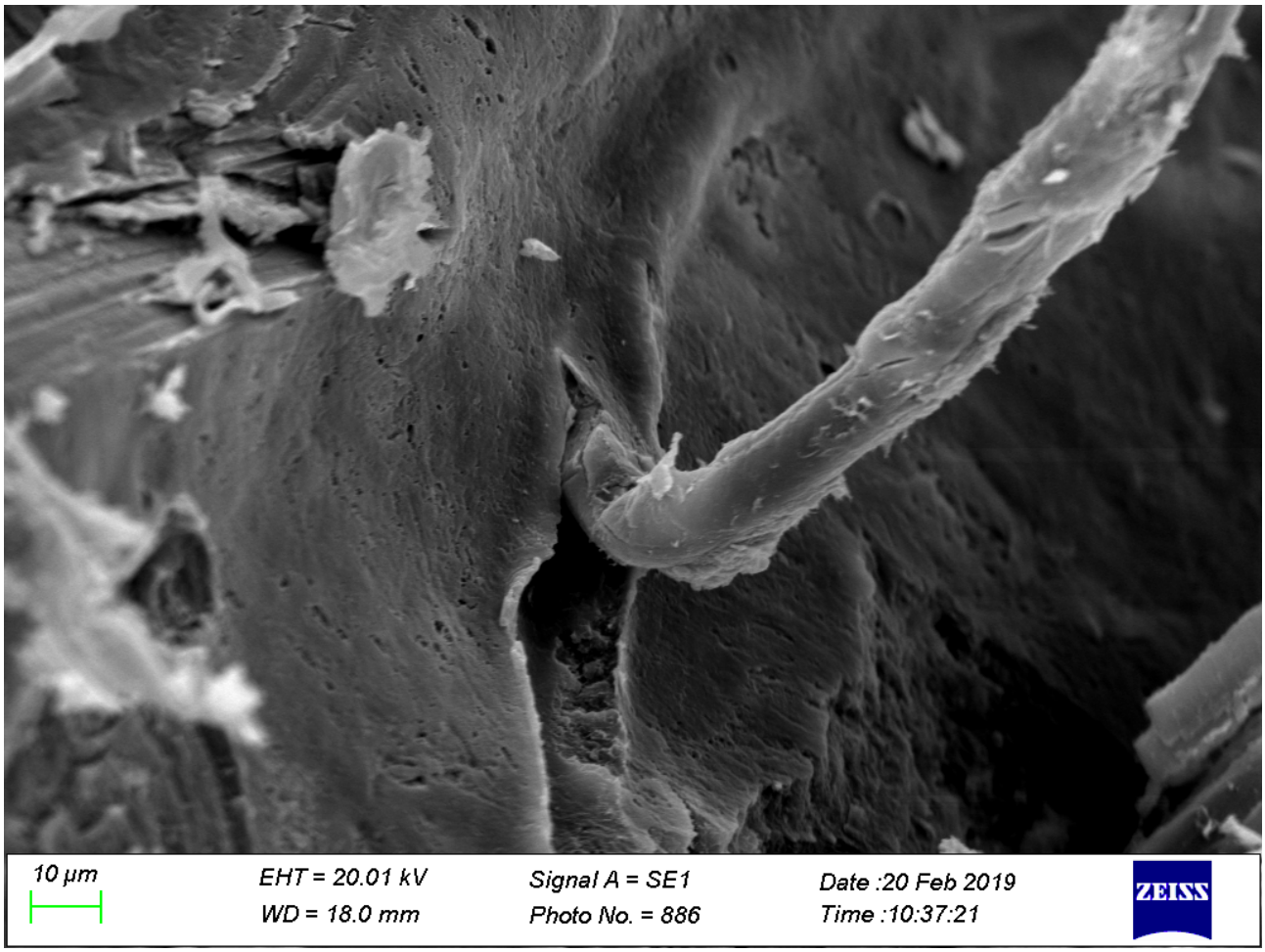

Fig.47: SEM image of microfiber trapped in the skin mucous in the external side os Sardina pilchardus

4.1.4 Characterization by spectroscopy technique

4.1.5 Raman

Analysing the fibers found in *B. boops* through Raman Spectroscopy, no changes in the feature peaks of the spectra were observed. The acquired spectra in two points of the fibers are shown in Figure 48. The main Raman peaks were in three regions as follows: 1095–1250 cm^{-1}, 1280–1450 cm^{-1}, and 1530–1670 cm^{-1}.

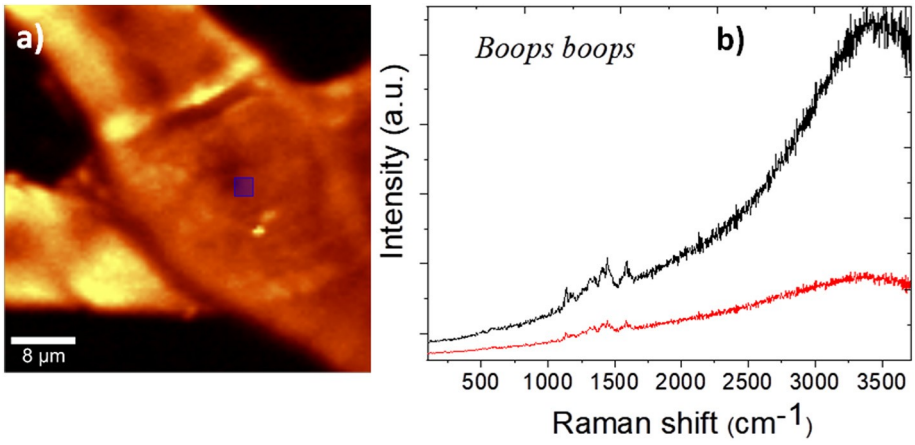

Fig.48: a) The false colour image of the microfibers by the fluorescence signal at ~ 3500 cm−1 and b) corresponding Raman spectra

Based on the chemical features of the analysed particles, the Raman spectroscopy highlighted the cellulose fibers constituted by linear polymers of glucose monomers. In Figure 49 are shown the structures of cellulose, viscose rayon, a man-made fiber composed of 100% regenerated cellulose, and cellulosic fibers obtained through viscose regeneration process.

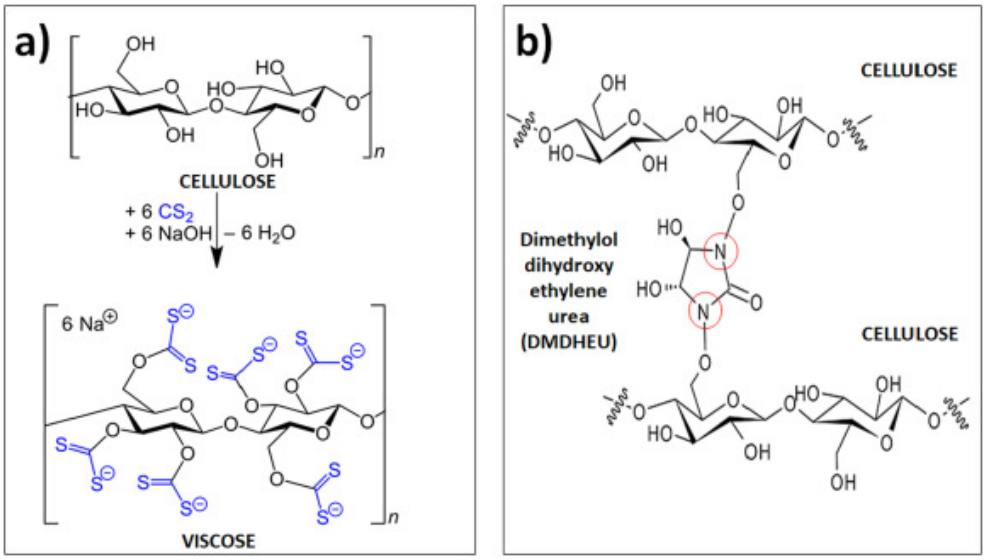

Figure 49:Reactions occurring during the preparation of a)viscose rayon and b) wrinkle-free finish crosslinking with two cellulose chains.

Starting from the particles detected in the GIT of *L. caudatus* and *Z. faber*, in Figure 50 is showed a false colour image of plastics due to the fluorescence signal that sometimes masks Raman signals.

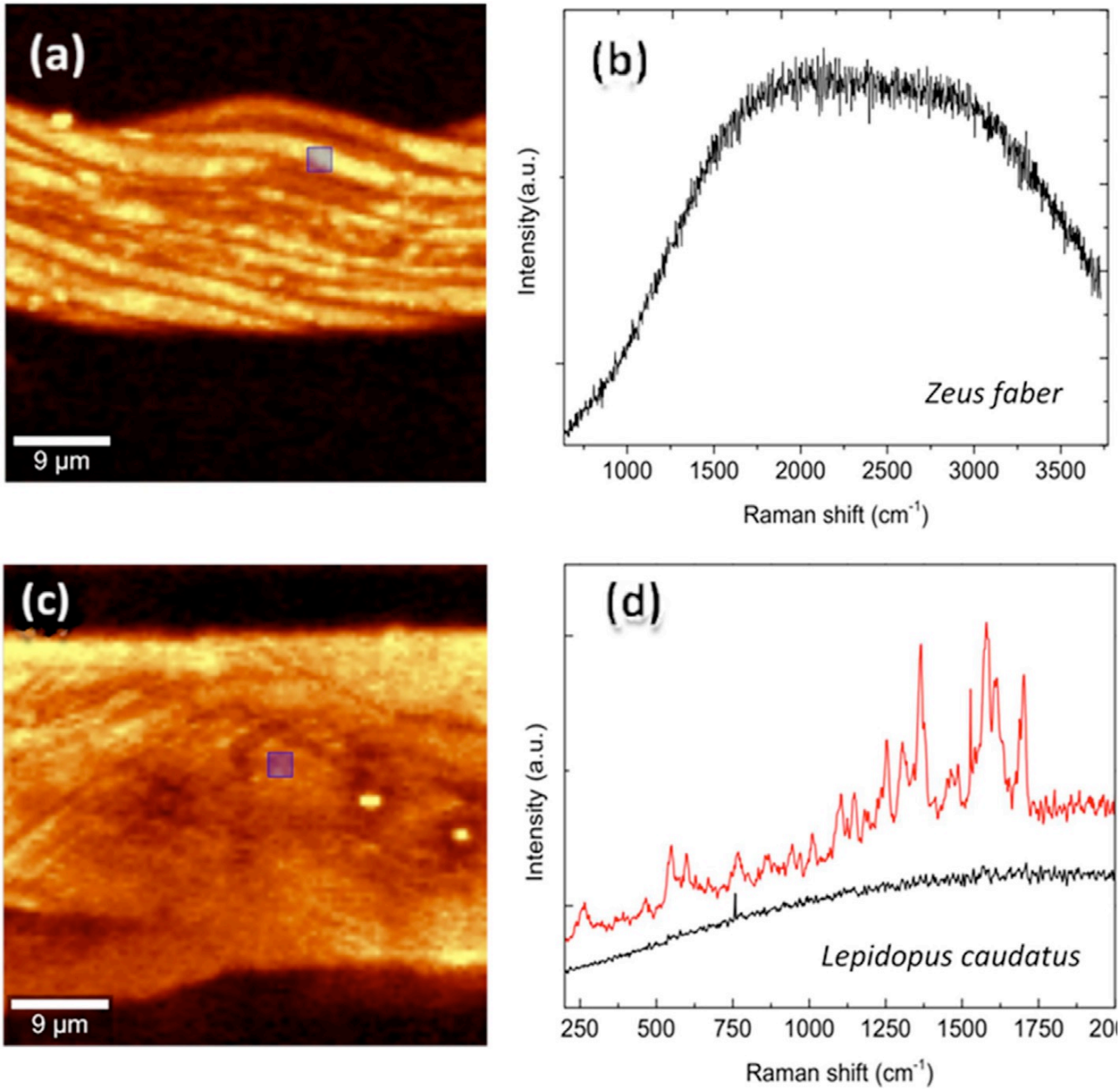

Fig. 50: a),c) the fluorescence signal with false colour image and b),d) their corresponding Raman spectra

Thus, the fluorescence images highlighted the differences in terms of porosity, roughness, and presence of protuberances in the MPs extracted by the two species. The protuberances were more pronounced in the polymers came from the GIT of John Dory. The spectra showed different vibrational modes as an index of different chemical nature of the plastics (Fig.50 b,d). Indeed, the identified polymers were: polyamide (PA), polypropylene (PP), nylon and polyethylene (PE). More

in detail, the Raman analysis allowed to detect the plastics found in the GIT of *L. caudatus* as a mixture of common PP, acrylic polymers, and PP.

4.1.6 Raman and FTIR analyses

Isolated particles from cartilaginous species (only the *S. canicula* caught in the Gulf of Patti), *M. barbatus barbatus, T. lyra* and both the clupeid fish *E. encrasicolus* and *S. pilchardus* were analysed using both Raman and FTIR. The microfibers analysed came from *T. lyra*, where those were extracted by the gill lamellae. All these microfibers showed the same spectra by Raman analysis with typical bands of cellulose (CL)(Fig.51).

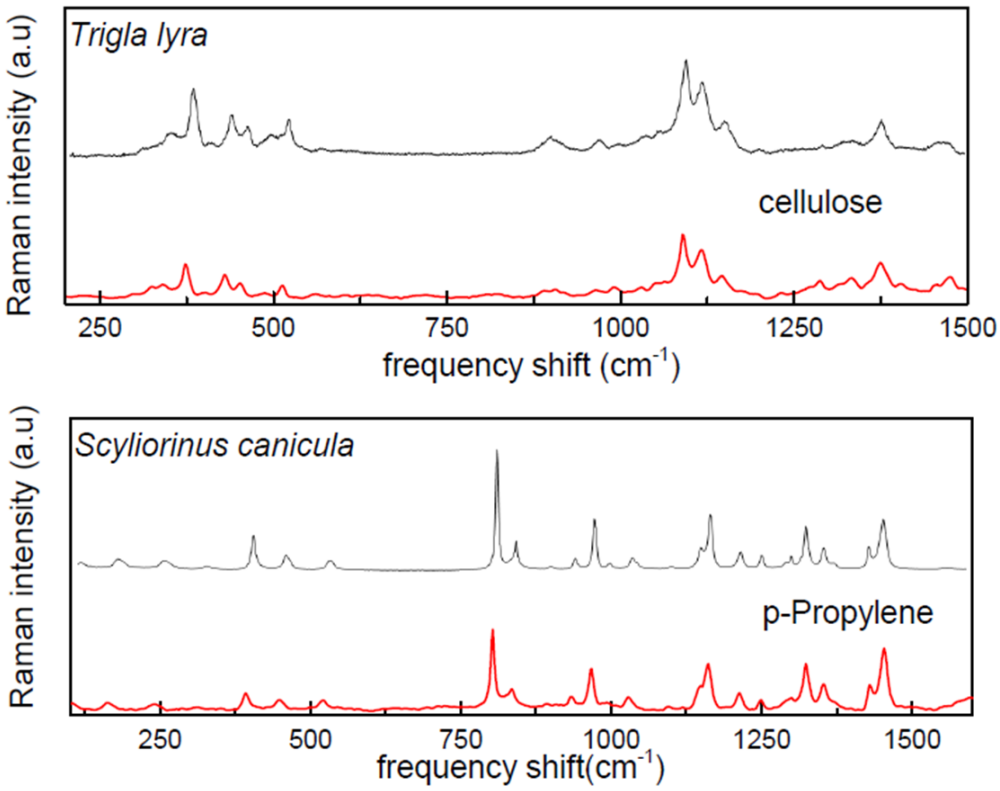

Fig.51: The comparison between the fibres (red) found in the specimens with the related reference spectra of Raman (grey)

In the specimens of *M. barbatus barbatus* the type polymers were PTFE (75%) and PA (25%). The cartilaginous fish showed a higher abundance of plastics compared to *T. lyra* and *M. barbatus barbatus*. More in detail, in *G. melastomus* the polymeric compositions were both 50% of PA and

PE. In the *S. canicula* the plastic contamination was represented by PP, CL and PTFE. In the last specimen of elasmobranch, *R. miraletus*, the only analysed fiber was identified as Kraton G for 85%. In Figure 41are reported the comparison between the Raman spectra of analysed fibers and those obtained from libraries. In the case that the fibers showed a strong fluorescence background, may be traceable to dyes or additives, they were analysed by ATR-FTIR. In Figure 52 are reported the infrared spectra of some samples.

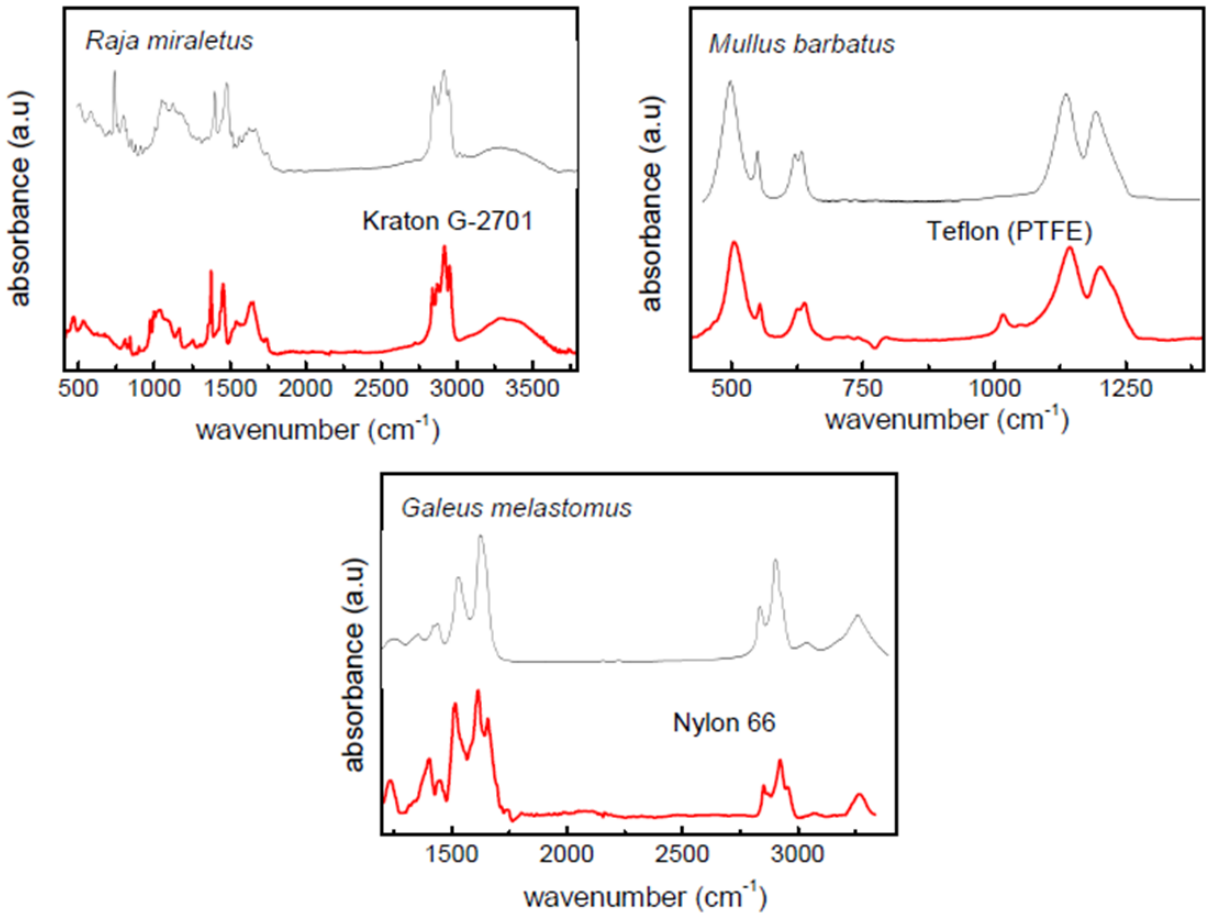

Fig. 52: Infrared spectra of selected fibers found in fishes (red) compared with related reference spectra (grey).

However, due to the poor quality of spectra that showed no identifiable or very few peaks, two of the fibers analysed were not unequivocally identified. Finally, regarding the large fragment and

microfilament shown in (Figs. 31,32), found in the GIT of *G. melastomus* and *S. canicula* respectively, were classified by Raman analysis as Polyethylene in the first species and Polypropylene in the second.

In the clupeids fish sample, the S1 and S2 were investigated using Raman and FTIR spectroscopy. The identified Raman peaks were associated to: Synthetic cellulose, polyamide (PA), polypropylene (PP) and Polyethylene (PE). The peaks recorded during the FTIR analysis revealed the assignments of different kinds of polymers as follows: Nylon, Rayon, polyester, polypropylene, polyacrylonitrile, polyethylene and polyamide. Furthermore, dyes and other additives, such as antioxidants or UV stabilizer, were recorded by the absorbance peaks.

For what concerning the *Pagellus spp*. specimens, the comparison of Raman and FT-IR spectroscopies highlighted that the only polymer composition was by Nylon 66 (polyamide).

4.1.7 SEM-EDX

The SEM microscope equipped with EDX was used for analysis in *B. boops, L. caudatus* and *Zeus faber*. For the first species, the analysis confirmed that the chemical bonding configurations are compatible with the cellulose detected by the Raman spectroscopy in the particles extracted by bogues. Similarly, also for *L. caudatus* and *Z. faber*, the SEM-EDX measurements confirmed the analysis through Raman spectroscopies.

4.2 Experimental Trial

Results highlighted significant differences in plastic ingestion, mortality, and growth rate ($p<0.05$) based on different experimental groups and their features as MPs concentrations and time exposure.

4.2.1 Evaluation of MPs Ingestion and Mortality

Both experimental groups A and B showed plastic ingestion in the specimens of brine shrimps (Fig.53,54).

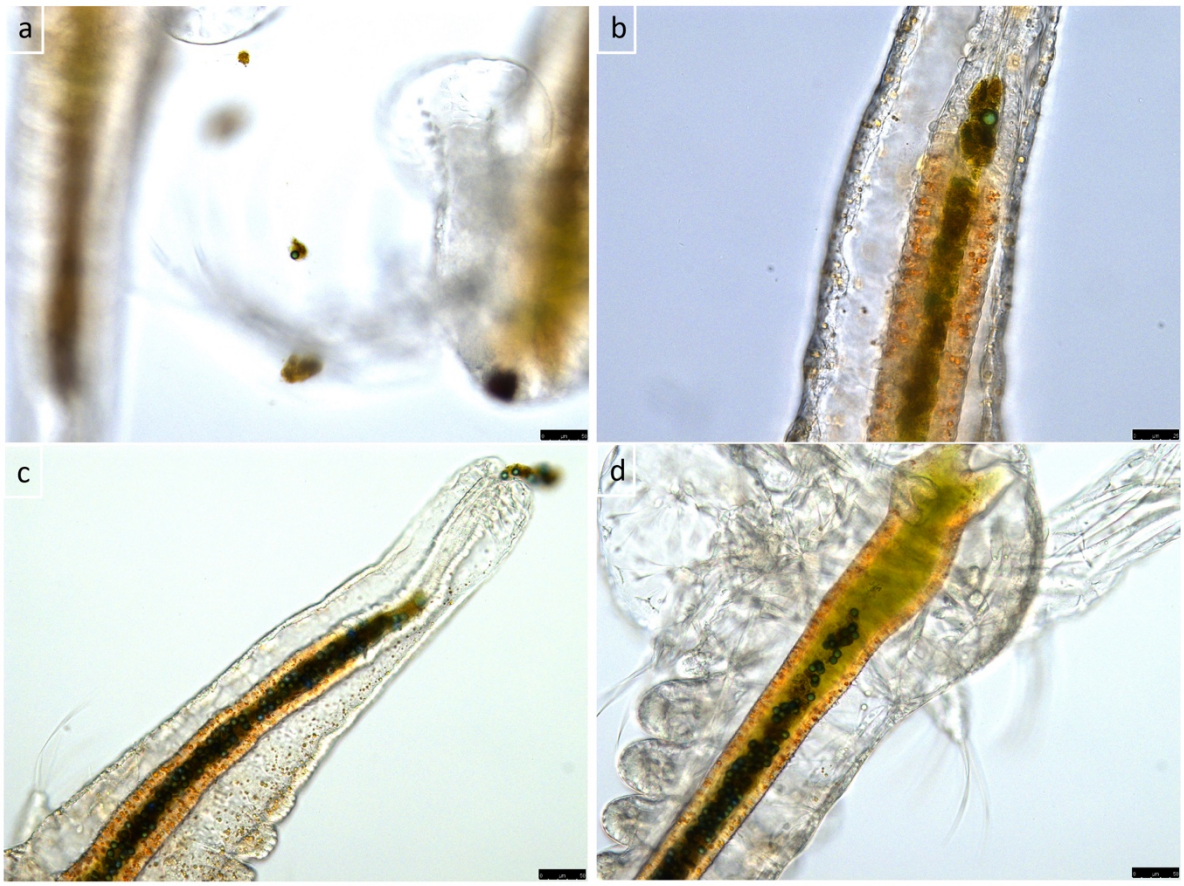

Fig.53:evidence of the Blue Dyed Microspheres detected in the specimens of GroupA at T48, a) A3(10^2 MPs/ml) b) A4 (10^3 MPs/ml); c-d) A5 (10^4 MPs/ml). Scale bars: a) 25µm; b),d)50µm

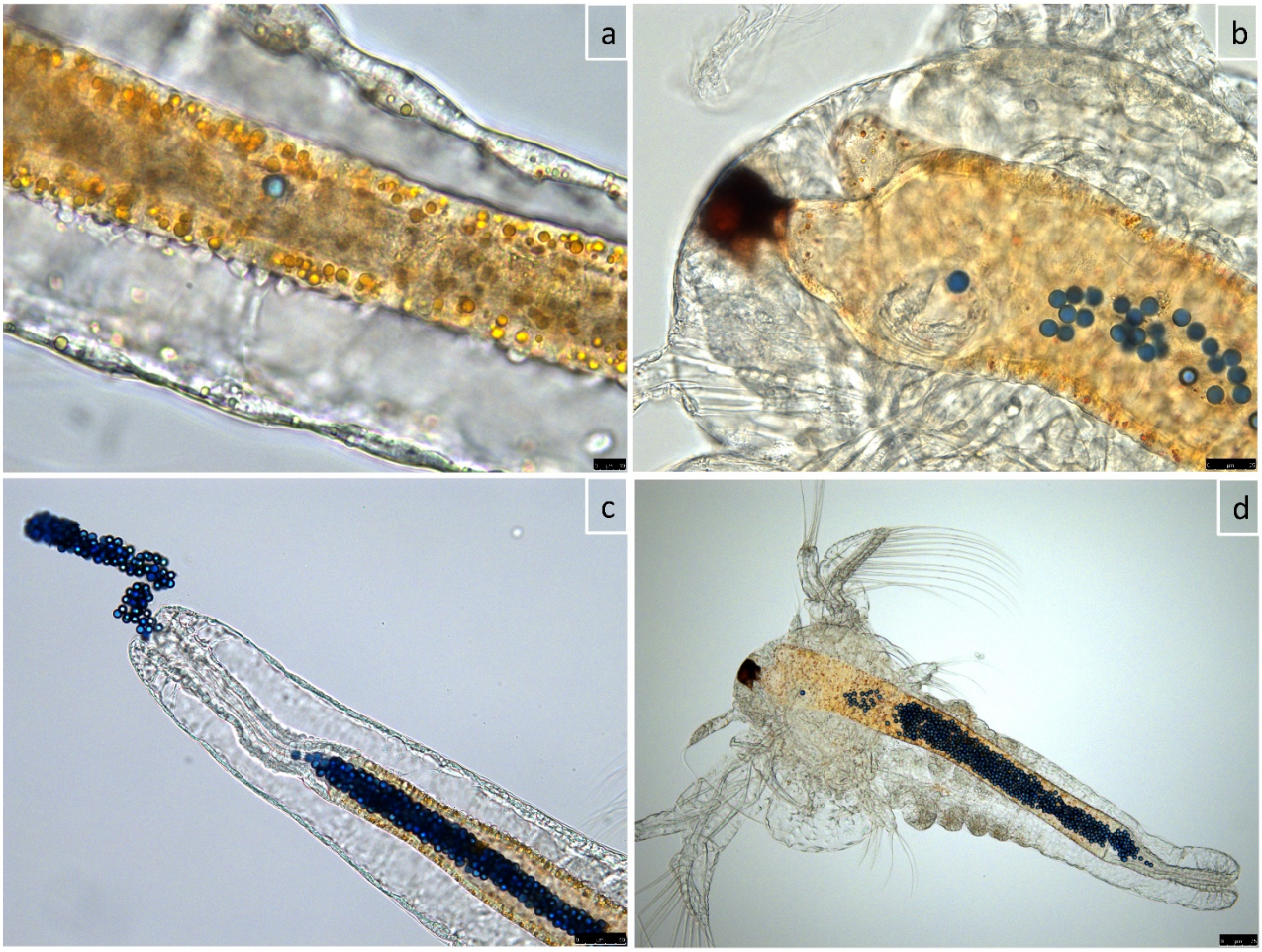

Fig.54: evidence of the Blue Dyed Microspheres detected in the specimens of GroupB at T48: a) B1 (1 MPs/ml); b)B4 (10^3 MPs/ml); c),d):B5 (10^4 MPs/ml). Scale bars: a)10 µm; b)25 µm; c)50 µm;d)75 µm .

The blue dyed microspheres were found in treatments A4, A5 and from B1 to B5 (Table 9). Regarding the MPs ingestion, the concentration of 10^4 MPs/ml was that with significant differences ($p = 0.003$) compared to other concentrations. The main variations in MPs ingestion between treatment A and B were observed between the exposure time T0, T6, T12 and T24 vs. T48 in the Group A and between T0, T6, T12 and T24 vs. T96 in the Group B. More precisely, the Group-A, based on exposure concentration, highlighted significant variation for MPs ingestion ($p<0.001$). The main differences for mortality rates were observed between T168 vs. T0, T168 vs. T12 ($p = 0.014$) according to exposure time ($p < 0.001$). In Group-B were observed similar results, indeed, also in this case the

MPs abundance was dependent by exposure concentration ($p = 0.001$). As for the Group-A, the mortality rate showed a time-dependent trend ($p < 0.001$) by exposure time, with mortality peaks at T96 and T168.

Table 9: The ingested MPs for each experimental group (Goup A and B) and their exposure time. Data are exposed as means±SD (n=3)

	Exposure time						
Experimental Groups	**T0**	**T6**	**T12**	**T24**	**T48**	**T96**	**T168**
A0	0	0	0	0	0	0	0
A1	0	0	0	0	0	0	0
A2	0	0	0	0	0	0	0
A3	0	0	0	0	0	0	0
A4	0	0	0.4 ± 0.38	2.2 ± 1.57	2.4 ± 1.46	4.4 ± 1.44	4.4 ± 1.32
A5	0	1.4 ± 1.16	13.2 ± 12.4	43.6 ± 28.7	122 ± 51.8	108.2 ± 38.3	110.2 ± 31.4
B0	0	0	0	0	0	0	
B1	0	0	0	0.2 ± 0.44	0.6 ± 0.54	0.8 ± 0.83	
B2	0	0	1±1	1.4 ± 0.54	2.8 ± 1.92	2.2 ± 1.48	
B3	0	1.4 ± 1.94	7.4 ± 7.66	6.4 ± 4.39	8 ± 9.46	5.4 ± 4.15	
B4	0	22.4 ± 29.3	23.8 ± 14.9	72.8 ± 28.1	85.6 ± 9.81	75 ± 49.4	
B5	0	37.6 ± 51.5	157.4 ± 68.6	169.4 ± 65.6	300.4 ± 42	306.2 ± 49.3	

4.2.2 Effect on growth and development by MPs ingestion

The measurements of the total body of the specimens showed significant differences between Group-A and Group-B ($p = 0.028$) (Fig.55), these results were highlighted by the analysis of variance as well, which confirmed a significant variance between both experimental Groups.

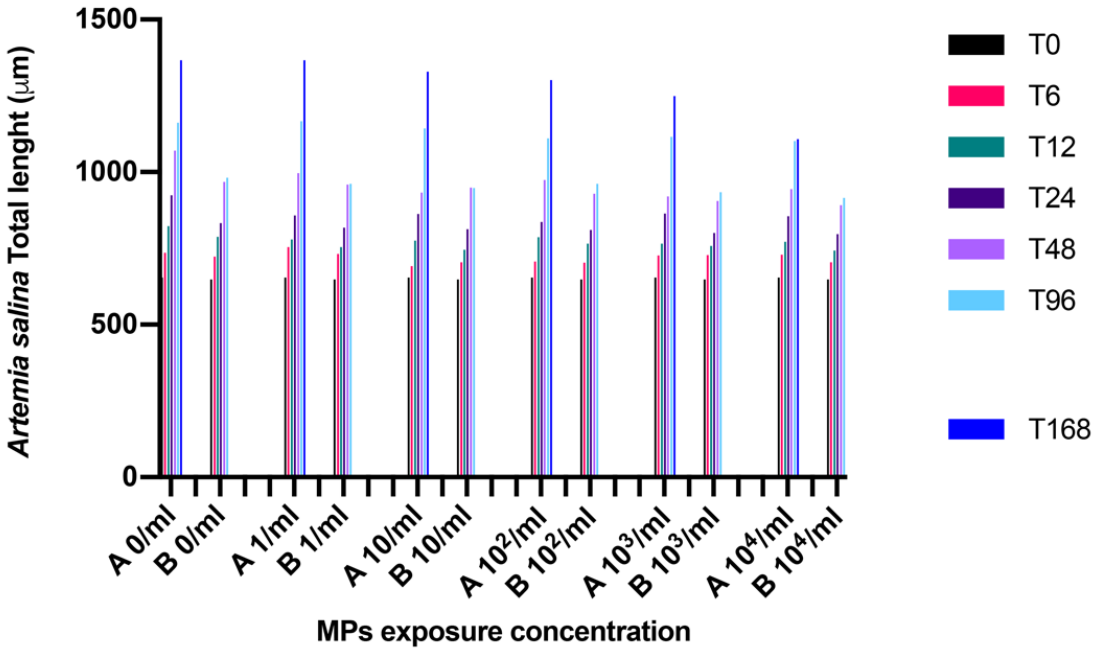

Fig.55: the body length of Artemia salina during each concentration and experimental time

More precisely, in Group-A the significant variance was observed between A0 and all other treatments ($p < 0.001$), except in A1 ($p > 0.05$). The highest differences for total body length (258µm) were at T168 between A0 (1366.824 µm) and A5 (1108.746 µm) and of 116µm between A0 (1366.824 µm) and A4 (1249.606 µm). Between A0 (1162.024µm) and A5 (1101.746µm) at T96, 61µm of difference was detected. Regarding Group-B was observed that the concentration of microspheres less influenced the total body length of *A. salina* compared to Group-A, indeed, it showed a difference of 66µm comparing B0 (981.26µm) with B5 (915.164µm) at T96. Significant differences between the two Groups were observed regarding the anatomic features that mark the development stage (Instars) of *A. salina*. These differences were highlighted by the images of analysed specimens of brine shrimps. Comparing both Groups, it is possible to observe a significant delay of the Group-B in the development stage than Group-A specimens. In the Group-B (B0) the maximum development stage reached, before the total mortality due to starvation, ranged between instars II and III, in which the only active appendages were the antennae due to the total absence of thoracopods and the naupliar eye was simple. Instead, Group-A (A0) reached at the same time point

the Instar IV, in which an evident primordial thoracopods and a differentiation of the naupliar eye were present. Furthermore, in Group-A at T168, instar V, there were a complete differentiation of eye in compounds and median and a prominent formation of thoracopods that become swimming appendages (Fig.46). More precisely, in the Group-A, only A0 at T168 reached instar V, while A4 reached instar IV and A5 instar III (Fig.56).

Fig. 56: Development stages of Artemia salina nauplii in GroupA during six treatments (From A0 to A5) and time (From 0 to 168h). Scale bars:75µm for T0-t12; 250 µm for T24-T168

On the other hand, in Group-B was not detected evident anatomical differences basing on different treatments (Fig.57)

Fig.57: Development stages of Artemia salina nauplii in GroupB during six treatments (From B0 to B5) in time (0 to 96h). Scale bars: 75 µm for T0-T48; 250 µm forT96

4.2.3 Effect on Feeding Behaviour by MPs ingestion

Results of the parallel experiment shown in section 3.2.5 (Feeding rate and algal growth) are summarized in Figure 58. The maximum microalgal ingestion rate reduction at T48 was 50.58% when

exposed to 10^4 MPs/ml, while at T96 was 24.08% with an exposition of 10^3 MPs/ml, finally, at T24 when exposed to 10^2 MPs/ml was 18.70%.

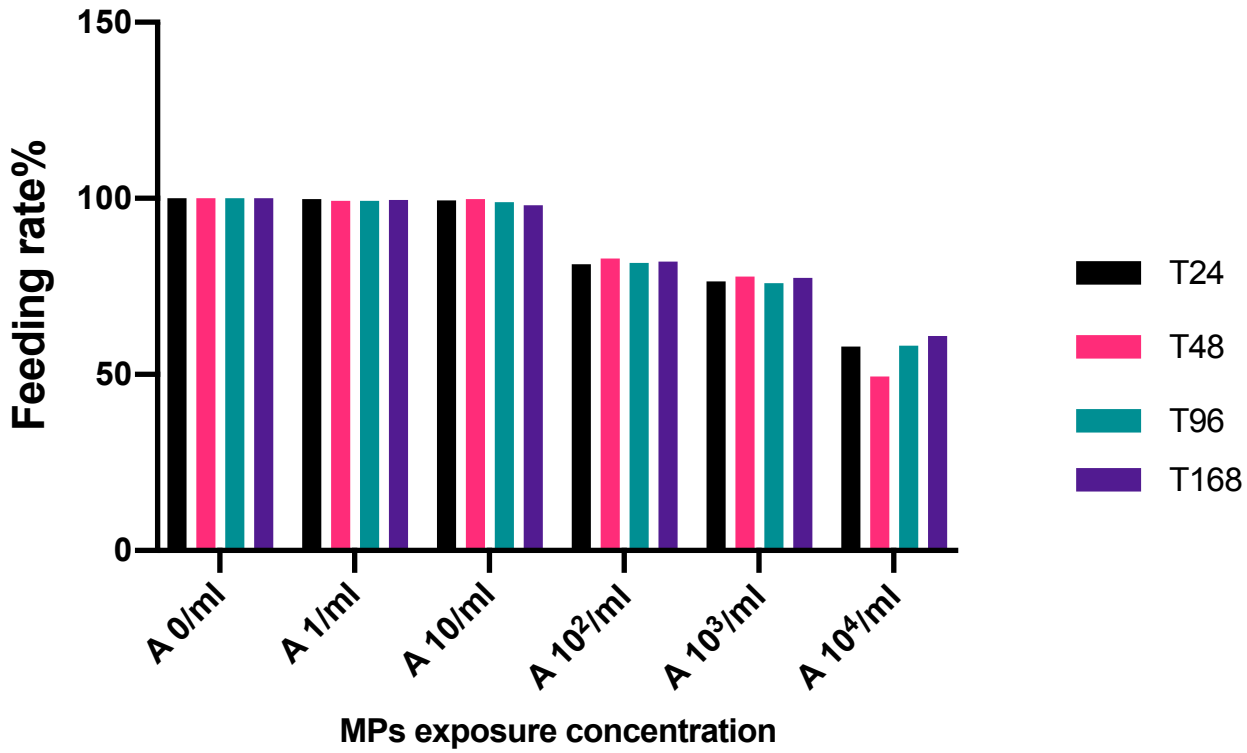

Fig.58: Artemia salina feeding rate% during each concentration and experimental time

In the case of 10^4 MPs/ml concentration at T168 was recorded a time-dependent trend (39.09%) for the ingestion rate of the microalgae. During the trial, it was not considered that the influence of concentration came from the algal growth for the ingestion rate data because the results obtained from the control of algae, highlighted that in absence of *A. salina* the growth of algal concentration cannot be considered neglectable. As shown in Figure 48, the results about the area measurement of ingestion highlighted a heavy reduction of area unoccupied by MPs in A5, B5 and B4. In the treatments A5 and B5 the maximum degree of MPs filling was 44.43% and 90.82% respectively. Moreover, the

730 percentage of microalgae ingestion rate revealed a negative correlation both with MPs ingestion ($r = -0.7$; $p < 0.001$) and with the MPs-filled area ($r = -0.9$; $p < 0.001$).

732

5. Discussion

The widespread presence of microplastics in aquatic environments, has attracted the attention of the scientific community. Microplastics may severely impact biotic and abiotic compartments of aquatic ecosystems. In the present thesis the ingestion of microplastics have been investigated in several feral and edible fish species, including different life stages of some species. The results of this study highlighted the ubiquitary presence of plastic particles in the considered species caught in different areas of the central Mediterranean Sea. Indeed, the sampling of the species showed how the presence of plastic contaminants can change in terms of abundance and distribution along the entire set of samples considered. Moreover, the experimental survey, as well as giving more knowledge about the plastic feeding behaviour, showed as the plastic accumulation could influence the body development of the model organism.

Based on the global issue of marine plastic pollution, it became fundamental to understand the distribution and abundance of the plastic in biodiversity hotspots such as the Mediterranean Sea and how the plastic ingestion by animals can influence the growth and life cycle of these.

5.1 Environmental Survey

5.1.1 Visual sorting

Except for the specimens of spotted catshark caught in the Sicily Channel, in all the trawled species caught in the Gulf of Patti the plastic presence and abundance were checked by visual sorting.

The features of the plastic materials, such as chemical composition, shape and colour found in the samples, can deepen knowledge about *i)* potential toxic effects on organisms *ii)* source of these materials *iii)* possible preference of the specimens related to shape and colour of MPs that can be exchange as food source and directly ingested. For instance, in the marine invertebrate, the plastic fibers seems to be more toxic than plastic in spheres or fragments shape (Gray and Weinstein, 2017)

as showed in some trials in which different plastic particles shape were administered to shrimps (Au et al., 2015; Gray and Weinstein, 2017).

In general, this study permitted an increase and, in some cases, to give new data about plastic contamination along areas in which no adequate sampling was done yet to survey the ecological impact of plastic polymers. Thus, the obtained results can increase the knowledge about the plastic pollution in the Mediterranean Sea and in many ecological and economic important species.

5.1.1.1 Teleost fish

Boops boops

Basing on the trophic habits of the bogue, it has been proposed as target species and indicator for microplastic pollution at small scales in the Mediterranean Pelagic domain (Battaglia et al., 2017) https://plasticbustersmpas.interreg-med.eu/. Indeed, the bogue is an omnivorous mesopelagic species, thus, feeding both on benthic and pelagic species, can carries the plastics ingested along the water column. Furthermore, the bogues, according to energy transfer from low to high trophic level to the top predator of the food chain could transfer the plastic ingested as well through biomagnification (Savoca et al., 2020; Savoca et al., 2019b).

Despite the presence of fibers in the GIT (63.3%) of the specimens, the low number of these suggest the small retention time of the particles in the guts of bogue. As suggested by several authors (Bessa et al., 2018; McGoran et al., 2017; Nelms et al., 2018; Rochman et al., 2015), usually fish tend to ingest dark coloured fibers, mostly black. In line with literature and with the most spread fibers colour in the Gulf of Patti (Mancuso et al., 2019; Savoca et al., 2019b), in the bogue specimens the dominant colour of observed fibers was black. As shown by results, in the specimens of bogue were found man-made cellulosic fibers. There are few studies about the pollution of this kind of fibers, however, Woodall et al., (2014) highlighted the contribution of these materials to a percentage of 56.9% to total

microfibers from the Atlantic Ocean bottom, giving more importance to the man-made cellulose fibers pollution. The man-made fibers are not considered an environmental issue, thus, the real impact of these fibers is related to their associations with additives and dyes that could be potentially harmful to marine species. Indeed, additives such as flame retardants or dyes as direct blue 22 and direct red 28 are carcinogenic substances (Henry et al., 2019; Remy et al., 2015). Coming from the characterization of the fiber samples, the presence of metal or metal oxide nanoparticles as Cu oxides were due to the features of these components that are usually anchored to the cotton textile clothing, in this case the man-made fibers in general. Moreover, a great indication that the fibers extracted from GIT were man-made cellulose materials, was the presence of sodium ions.

Chlorophtalmus agassizi

Despite many studies about plastic contamination in deep environments, there was no evidence of plastic ingestion by the shortnose greeneye. Thus, this represents the first report of plastic ingestion in *C. agassizi*. The specimens mainly fed on sandy and clay bottoms that are the areas with the major impact by fishing trawling and where the plastics tend to sink as reported by Fabri and colleagues (2014).

Like other sampled and analysed species, *C. agassizi* showed ingestion only of dark colour plastics (blue and black) that is probably due to the contamination degree of the surveyed area. Furthermore, the shortnoose greeneye is a prey included in the diet composition of many predators already reported in this study for plastic presence in the GIT. Thus, the capture of *C. agassizi* by predators such as *L. caudatus* and *G. melastomus* represent a secondary plastic ingestion which can imply bioaccumulation and biomagnification phenomena.

Direct data about the plastic ingestion in this demersal species are scarce or completely absent, thus, it would be appropriate to begin a survey to increase in knowledge about the plastic ingestion in this and other under looked demersal species.

Lepidopus caudatus

The silver scabbard fish is an important species both for its economic and ecologic value, in this study was highlighted for the first time the evidence of plastic presence in GIT of the specimens caught in the southern Tyrrhenian Sea than Anastasopoulou et al., (2013) that showed the presence of the plastic materials in eastern Mediterranean Sea. Moreover, in the study of Anastasopoulou and colleagues any plastics were found in the guts of the *L. caudatus* probably to the low sample size analysed than in this study where in 32 specimens of silver scabbard fish were found both micro and meso plastics. Thus, the results revealed the high susceptibility of this species to plastic ingestion (78.1%). Apart from the main concern about the consumption of this species as a human food source, it's important to consider the ecological impact of the plastic contamination in these organisms. The silver scabbard fish is an important mesopelagic species preyed on by important top predators such as bluefin tuna and swordfish. Thus, it is logical to think that *L. caudatus* can constitute a vector for plastics to higher trophic levels becoming one of those species that could increase the biomagnification and bioaccumulation events of plastic polymers along the trophic web (Battaglia et al., 2013; Romeo et al., 2009).

Merluccius merluccius

As already argued before and as reported in literature, fish tends to ingest mostly dark-colored particles. In line with this, during the observation of the European Hake's GIT, only fibers in black colour. The possible origin of these colour fibers could be related to different sources such as cosmetics and hygiene products in the case of primary microplastics or by textiles and fishing industries in the case of secondary microplastics (Mancuso et al., 2019; Veiga et al., 2016).

Mullus barbatus barbatus

The red mullet is a species with benthic behaviour, for its trophic behaviour is considered a bio-indicator species (Bottari et al., 2016; Carreras-Aubets et al., 2012; Mangano et al., 2017). Comparing the results of plastic ingestion in *M. barbatus barbatus* with other studies, in this was reported that the percentage of microparticles ingested was lower (14.28%) than in the cases of Adriatic Sea (64%), Turkish (42%), Greek (32%) and Spanish (19%) (Avio et al., 2015; Bellas et al., 2016; Digka et al., 2018; Giani et al., 2019; Güven et al., 2017). Also for the characterized plastics, the results were different from other studies. In the western Mediterranean Sea, Alomar and Deudero, (2017) highlighted the major plastic contamination was featured by Polyethylene terephthalate (PET, 36.36%, Alomar and Deudero, 2017) than in this study the characterized plastic most abundant were composed of polytetrafluoroethylene (PTFE, 75%).

Pagellus bogaraveo* and *Pagellus erythrinus

In general, the data about plastic ingestion of important commercial value species with demersal behaviour are scarce above all about the *Pagellus* (Anastasopoulou et al., 2013; Bellas et al., 2016; Güven et al., 2017; Neves et al., 2015). Thus, this study represents the first report about the plastic ingestion by *Pagellus spp.* in the Tyrrhenian coasts. Comparing with what reported by Anastasopoulou et al., (2013) the percentage of *P. bogaraveo* specimens positive to plastic ingestion (1.7%) along the Ionian Sea, in this study was highlighted a higher percentage of MPs ingestion (12.5%) in the same species. On the other hand, the fibres shapes were in accord with previous observations. For what concerns the *P. erythrinus* instead the plastic ingestion percentage highlighted in this study was 6.7% that is lower if compared with results reported for coastal Turkish waters (22%) (Güven et al., 2017).

Studying the literature about the percentages of plastic ingestion by demersal and pelagic fishes, the results are in contrast. Indeed, Rummel et al., (2016) reported higher plastic ingestion in pelagic

857 species (10.7%) than demersal ones (3.4%). On the other hand, many studies highlighted the absence
858 of differences in the percentage of plastic ingestion between species with the two different trophic
859 behaviours.
860 Based on the only type of polymer present in the GIT of *Pagellus* specimens (Nylon 66), we can
861 hypothesize that high levels of this particles could have come from the fragmentation of fishing nets
862 and ropes and residues of textile fibers as well. Moreover, basing on the high-density degree of this
863 polymer (1.14g/cm^3), this kind of fibers can easily sink to the sea bottom where can be ingested by
864 demersal species as in this case for the two congeners of seabreams.
865
866 ***Trigla lyra***
867 The piper gurnard is a species with benthic behaviour, thus, feeding on the bottom and due to its
868 limited displacements, like red mullet *M. barbatus barbatus*, could be considered a bio-indicator
869 species. This trophic behaviour could be the reason for the presence of man-made fibers in the gill
870 rakers.
871 The identified microfibers both came from gills and GIT of the piper gurnard were all composed by
872 cellulose (100%). Even if the exact impact of these materials on the gill tissue is not clear, it is easily
873 conceivable that these fibers can cause mechanical damage with alteration of the respiratory system.
874
875 ***Zeus faber***
876 According to what has already been said about the high abundance of black fibers in the study area,
877 also in the case of *Zeus faber*, the dominant colour of observed fibers was the black. In contrast with
878 the Atlantic sampled species in which the specimens were contaminated mainly by rayon and
879 polyamide (Lusher et al., 2015a), in this study the fibers were characterized by nylon and
880 polyethylene composition. Moreover, the presence of zinc in the particles is significant, indeed, this
881 indicates the fibers capacity to adsorb the metals acting as vectors for the marine pollution along the

food web (Brennecke et al., 2016; Gassel and Rochman, 2019). Zinc is a metal that can induce several effects to marine species such as morphological alterations and oxidative stress (Huang et al., 2018; Montalbano et al., 2018). Thus, the presence of this metal in the john dory is worrying not just for its potential bioaccumulation and biomagnification along the trophic web, but also for the human consumption of a species of high commercial value like *Z. faber* (Barboza et al., 2018a; Bottari et al., 2019).

5.1.1.2 Cartilaginous fishes

The cartilaginous fishes, especially sharks, are ecological important species having a crucial role in the marine food webs as top predators, unfortunately, many recent studies showed the high levels of plastic ingested by these species revealing their high sensibility to plastic pollution (Capillo et al., 2020; Smith et al., 2018; Valente et al., 2019).

Galeus melastomus

The blackmouth catshark *G. melastomus* is a benthopelagic species that prey mainly on demersal invertebrates, such as cephalopods and shrimps, and on mesopelagic fishes (Bottari et al., 2017; Fischer et al., 1987; Giacopello et al., 2013; Rinelli et al., 2005). However, the blackmouth catshark in its diet include myctophidae and lanternfishes as well, that are species in which (Romeo et al., 2016) highlighted the presence of plastic items along the gastrointestinal tract. Comparing the results of plastic ingestion in *G. melastomus* (8%) with other studies this trend changes basing on the studied area. Indeed, along the eastern Ionian Sea were showed plastic ingestion percentage that goes from 3.2% to 12.5% (Anastasopoulou et al., 2013; Madurell and Cartes, 2003; respectively) while a degree between 3.2-4.8% and 16-18% in the western Mediterranean Sea (Cartes et al., 2016; Alomar and

907 Deudero, 2017; respectively). Regarding the composition of ingested plastics by *G. melastomus*, the
908 spectroscopy analysis revealed the presence of nylon for microfibers and polyethylene for the
909 microplastic fragment. These data differ from what reported by Alomar and Deudero, (2017) in the
910 western Mediterranean Sea in which the analysed plastics were in cellophane (33.3%) and
911 polyethylene terephthalate (27.3%) composition. These differences probably are attributable both to
912 the different pollution level and surveyed area.
913
914 ***Raja miraletus***
915 In the only specimens of *Raja miraletus*, the present polymer was Kraton G. With the name Kraton
916 G means several high-performance elastomers that are styrenic block copolymers (SBC) made by
917 polystyrene and rubber blocks. These materials are widely used as personal care products and based
918 on my knowledge from studies in literature, this is the first report about this kind of contamination in
919 the marine environment.
920 The ingestion of Kraton G and in general of plastic polymers in *R. miraletus* is traceable to the trophic
921 and food habits of the species that being a benthic fish feeds mainly on the bottom in which are
922 reported high levels of plastic particles and fibers (Capapé and Azouz, 1976; Fastelli et al., 2016;
923 Sanchez-Vidal et al., 2018; Woodall et al., 2014).
924
925 ***Scyliorhynus canicula* (Gulf of Patti)**
926 The small-spotted catshark is a demersal predator that feeds both on benthic and demersal preys
927 including fish, crustaceans, cephalopods, and polychaetes (Bottari et al., 2014; Busalacchi et al.,
928 2010; Lauriano et al., 2019). Comparing the results about the percentage of plastic ingestion (33%)
929 with those in Spanish Atlantic and Mediterranean coasts (15.3%) is evident the higher degree of MPs
930 ingestion in the present study, however, due to absence of characterization of plastic polymers in
931 (Bellas et al., 2016) is not possible to comparing data. On the other hand, comparing the results of the

sampled sharks in this study is evident that the plastic ingestion degree in *S. canicula* is higher (33%) than in *G. melastomus* (8%) and the most frequent polymer occurred in the GIT was polypropylene (71.4%). However, these results are in contrast with the hypothesis of Alomar and Deudero, (2017) that the blackmouth catshark is the more vulnerable species to plastic ingestion compared with other cartilaginous fish.

5.1.2 Chemical Digestion

Pelagia noctiluca

The sampling area of the Strait of Messina plays a crucial role for the survey of plastic ingestion in *P. noctiluca*. The interested area is featured by a particular geomorphology and bathymetry that makes the place interested strong currents and hydrodynamics (Albano et al., 2021b), these phenomena promote the distribution and concentration both of planktonic species and plastic particles that can be potentially ingested by my case study. For these reasons, this study can be considered the first evidence of plastic occurrence along different body parts of mauve stinger. Indeed, poor knowledge is present in literature reporting the contamination by plastic in *P. noctiluca* along Mediterranean coasts, as error ingestion of fragments, for instance, the specimens has ingested garbage trying to find food (Macali et al., 2018).

P. noctiluca, through appropriate predatory structures, actively preying (Morand et al., 1987). More in detail, oral arms are the predatory structures, and the umbrella is the seat of the gastrovascular cavity, this could be the reason why the plastics were found in both predatory structures and umbrella. Basing on literature, the feeding behaviour of *P. noctiluca* is described as a non-selective predation, thus, having a vary diet based more on food availability than a real preference, in which the more frequent species are different types of zooplankton and ichthyoplankton (Hall et al., 2015; Rosa et al., 2013). Results supported the hypothesis of the shown feeding behaviour; indeed, no linear correlation was shown between the size of the specimens and the percentage of microplastics

occurring. However, between the size of plastic particles and the weight of the specimens was found a negative correlation. This data could be related to two main factors: *i)* the non-selective predation way of the mauve stingers, *ii)* the fragmentation process of the particles (Dawson et al., 2018). Moreover, the common prey of the analysed specimens come into contact with plastic materials, thus, these phenomena could result in a biomagnification process (Sun et al., 2017).

Based on what was assessed, it is conceivable that the microplastics observed came from both biomagnification and accidental ingestion; this hypothesis is corroborated as well by the length classification of the particles ranging between 90µm and 9.4mm.

Concerning the particle colours found, another time was confirmed the non-selective predation of *P. noctiluca*, the most abundance of black particles is due to the interested area.

Jellyfish is a prey of many species that inhabit the Mediterranean Sea that consume them as an integral part of the diet or occasionally (Milisenda et al., 2014), as *Boops boops*. This can lead to secondary ingestion of plastic particles in marine predators that fed on jellyfish, creating the bases of biomagnification along the food web (Romeo et al., 2015, 2009; Wilcox et al., 2018). Moreover, after death, jellyfish tend to precipitate to the deep environment promoting the contamination of the marine bottom (Courtene-Jones et al., 2017).

Clupeid fish (*Sardina pilchardus, Engraulis encrasicolus*)

Studies in literature about the features of plastics ingested in marine juveniles' species are very few and anyway they are unexplored (Rizzi et al., 2019; Steer et al., 2017). Thus, this can be considered the first study about the ingestion of microplastics in the white juvenile and late-larval stage in the Mediterranean Sea.

The MPs occurrence was observed in all the subsamples considered (S1, S2, S3). Moreover, analysing the features of the plastics in terms of size, colour, and composition, it is clear that the study of plastic contamination in the fish larval stages needs more attention by the scientific community. The

surveyed features in this study are very important to increase in knowledge both for the influence of polymers on juveniles' stages of species and for the comparison of these stages with adults. Interesting is compare results showed by Lefebvre et al., (2019) with mine. In the specimens of sardines were recorded 0.53 items/specimen and 0.26 items/specimen in European anchovies showing the same range in the adult specimens of the same species analysed by Lefebvre and collaborators in which were recorded from 0.20 to 2.14 items/specimens in sardines and from 0.11 to 0.85 items/specimens for European anchovies.

The spectroscopy methods highlighted the presence of already reported contaminant polymers such as PE, PA, PU, PPA and man-made cellulose. Moreover, it showed the presence of Nylon as well. Few studies reported the presence of Nylon as marine contaminant, however, recently the ingestion of this polymer in the same area was reported as great presence inducing this kind of polymer pollution (Bottari et al., 2019; Capillo et al., 2020; Mancuso et al., 2019; Romeo et al., 2016, 2015; Savoca et al., 2019a, 2019b). Except for a fragment, the only shape recorded in the clupeid fish was in fibers like shown in the papers of (Compa et al., 2018) and (Lefebvre et al., 2019) for adult specimens. The main issue of microplastic contamination is related to the impact on fish health, indeed, the plastic particles can act as a vehicle for the distribution of biological “contaminants”, many chemical compounds, and heavy metals. There are many studies in literature that were demonstrated the capacity of MPs to adsorb compounds such as styrene, phthalates, PAHs, toxic metals, PBA and PCB (Barboza et al., 2018b). These compounds could come by the uptake of MPs in the environment directly or by production processes and the main influence on fish species include endocrine disruption, carcinogenesis, and neurotoxicity (Gallowaya and Lewisa, 2016; Hahladakis et al., 2018; Rochman et al., 2013; Thompson et al., 2009; Wright and Kelly, 2017). In addition to physiological and toxicological damage of the plastics to marine organisms, already explored in literature, there is another kind of damage that comes from the biological “contaminants” adhered to the surface of plastic materials. Indeed, basing on the bonds of some plastic polymers, it can favour

2006 the interaction with bacteria and fungi. For instance, polyurethanes are a kind of polymer with
2007 versatile features used in many applications, they are formed by a reaction of isocyanate, that have a
2008 functional group -N=C=O, with a functional group -OH of an alcohol such as polypropylene and
2009 polyethylene oxides (Urban, 1993). Thus, the presence on plastic surfaces by the biological
2010 "contaminants" such as microorganisms can be considered an issue in terms of pathogens spread in
2011 which the impact on marine species could lead to infection coming from MPs ingestion (Keswani et
2012 al., 2016). About that, the presence on plastic surfaces of *Aeromonas salmonicida*, *Vibrio spp.*,
2013 *Escherichia coli*, *Bacillus cereus* and *Stenotrophomonas maltophila* was reported (Barboza et al.,
2014 2018b).
2015 Moreover, we have to consider the mechanical damage of skin-adhered and ingested microplastics as
2016 recorded in my analysis as well. The recorded plastics of considerable dimensions isolated from
2017 clupeids, thus, can induce both the occlusion of GIT and/or the reduction of exchange surface in gills
2018 (Wright et al., 2013).
2019 However, the contamination degree of the analysed specimens could be related to two main factors:
2020 the sampling net used for fishing the species, and the storage method of the specimens after catch.
2021 More in detail, the sampling net was composed of nylon having a mesh size between 1 and 2mm.
2022 Moreover, the storage methods in fisheries place the fish in a polystyrene box with a different colour
2023 nylon net sheet, to keep the good "quality" of fish to consumers.
2024 The subsample S2 was digested without pre-washed to observe the status of the specimens similar to
2025 what they sell, indeed, in line with traditional Italian cooking these kinds of clupeid fish are eaten raw
2026 without previous washing to maintain the taste. These species are served fried as well, in this case
2027 another issue come, indeed, the frying process is about 170-190°C, consequently these are the
2028 temperatures in which the plastic compounds are transformed into mutagenic and carcinogenic
2029 compounds as dioxins (Smith et al., 2018). Thus, the exposure of plastics and this case of MPs cause

2030 the release of toxic compounds, on the other hand, the MPs goes to another one fragmentation and
2031 degradation process when exposed to high temperature (Renzi et al., 2019; Shibamoto et al., 2007).
2032 As already argued in this study and literature in general, the plastic contamination causes behavioural
2033 and physiological effects on fish, furthermore, these effects can change based on the life stages of the
2034 examined species (Pannetier et al., 2020). The larval stage is considered that with the most
2035 vulnerability, however, to date there aren't studies about the physiological influence of plastic
2036 ingestion in wild larval stage fish (Köster et al., 2003). However, there are many experimental studies
2037 that showed the negative influence of plastic ingestion in juvenile fish in which were observed
2038 neurotoxicity, toxicant accumulation, reduction of the mobility, malnutrition, the perforation and
2039 blockage of the gut and decreasing of predator capacity (Barboza et al., 2018a; de Sá et al., 2015).
2040 Probably, the negative effects in the early life stages of fish contaminated by plastics are more harmful
2041 than in adults. Indeed, the plastic ingestion causing the underdevelopment of organs both in larval
2042 and juvenile stages, can reduce the ability to eliminate the chemical compound of the MPs (Collicutt
2043 et al., 2019; Gove et al., 2019; Kühn et al., 2018; Ory et al., 2018).
2044 Obviously, the fish health, and consequently the growth, reproduction and mortality, influence
2045 directly the stock productivity and abundance (Galgani, 2015; Lloret et al., 2012; Marino et al.,
2046 2016a). Thus, plastic pollution is an issue that needs to be under control because of the direct and
2047 indirect effects on fish, stocks and consequently to fisheries.
2048

2049 ***Scyliorhynus canicula* (Sicily Channel)**

2050 In line with the aim of this thesis, besides to increase in knowledge about the marine pollution degree
2051 in the Mediterranean Sea, the survey of specimens of *S. canicula* report for the first time the
2052 relationship between plastic occurrence and feeding habits (nutritional profile) of a shark species in
2053 wild environment, despite other studies performed in laboratory conditions. Indeed, basing on my

2054 literature study, only (Barboza et al., 2020) reported data from the Northeast Atlantic Ocean in wild
2055 specimens of *Dicentrachus labrax*, *Scomber colias* and *Trachurus trachurus*.
2056 In accordance with (Neves et al., 2015) and (Bellas et al., 2016), the most represented plastic shape
2057 was fibers followed by fragments. These data are supported by other studies as well, for instance, in
2058 (Mancia et al., 2020) the 83.3% of observed plastics were in fiber shape, this degree is very similar if
2059 compared with results of this study (85%). Also, in Capillo et al., (2020) was shown that most
2060 observed plastics were fibers (33%), however, this degree is lower than mine. On the other hand, in
2061 (Pedà et al., 2020) only filaments were observed.
2062 For what concerning the most common colours, black was the more abundant (21%) followed by blue
2063 (18%), transparent and red and in minor percentage different colour shades like Bellas et al., (2016)
2064 and Capillo et al., (2020) that reported black as the predominant colour. On the other hand, Pedà et
2065 al., (2020) showed a prevalence of transparent and white.
2066 Like what already reported in the survey along the Gulf of Patti, the differences to plastic colours are
2067 probably related to the contamination of the study area in which the *S. canicula* can ingest plastic
2068 through direct or indirect way (Barboza et al., 2020). Thus, the predominance of the black and dark
2069 colours plastics in the GIT of the small spotted catshark may be related to the confusion of the predator
2070 that swaps the MPs with prey (de Sá et al., 2015). Additionally, the difference observed in the
2071 microplastic abundance between the samples of small-spotted catsharks collected from the Gulf of
2072 Patti and Sicily channel analysed in this study, can be related to the different plastic contamination
2073 degree as well as the hydro dynamism that characterize each investigated area.
2074
2075
2076

5.2 Experimental trial

Despite the many studies in literature about the impacts of the microparticles in marine organisms, the real and exact dynamics of the influence of these materials to biological and immunological function still need to be better understood (Albano et al., 2021a; Granek et al., 2020). The results showed by the experiments on *A. salina* should be considered useful to increase the poor knowledge about the fate of MPs in wild environments. Thus, the administration of 10µm microspheres to the first life cycle of the brine shrimps highlighted that these particles could influence the biology of these crustaceans. First, the experiments highlighted the easily assimilation capacity of brine shrimps to the ingestion of particles through filtration that is the method used to feed by zooplankton in general. Consequently, the use of *A. salina* for the administration trial showed that this organism is an excellent model for the study of the influence of MPs to the development stage of zooplankton and for the consequently biomagnification and bioaccumulation phenomena along the food web (Alava, 2020; Ivorra et al., 2019; Motta et al., 2019; Provencher et al., 2019).

Like other experimental studies about MPs ingestion by zooplankton crustaceans (Rosenkranz et al., 2009; Wang et al., 2019a), in the trial were observed the first ingestion of MPs 6h post hatching in a dose-dependent manner and a maximum ingestible value after 48h of exposure. In table 7 was reported the differences in the replicas with and without algae administration as well as MPs. Results highlight the lower ingestion of MPs in the replicas with *D. salina*, probably this is due to rare encounters with plastic debris aggregation than microalgae (Wang et al., 2019a). In literature there are many studies in which were showed the environmental concentration of MPs about 1 MPs/ml (Barboza and Gimenez, 2015; Dubaish and Liebezeit, 2013; Kunz et al., 2016; Lozano and Mouat, 2008), this is interesting if compare with my results regarding the experimental replicas, more precisely at 1, 10, and 100 MPs/ml, with microalgae in which into the brine shrimps no MPs were found. However, at 10 and 100 MPs/ml concentration were found MPs egested in faecal pellets aggregation. Comparing results of this study with those of Wang et al., (2019a) and (2019b), the

detection problem is due to disproportion between microalgal and MPs debris in water. This suggested that even if no particles were detected in the *A. salina* a minimal percentage of plastics was ingested.

On the other hand, in the replicas without *D. salina*, the presence of MPs inside the brine shrimps was detected starting from 24h exposure (T24). This data highlight that with no other suspended matter, *A. salina* was actively filtering plastic microspheres holding in the intestinal tract these materials for a long time as shown for other cases for zooplanktonic organisms (Cole et al., 2013; Wu et al., 2020). Moreover, in accordance with Wang et al., (2019a) and Bergami et al., (2016) with *A. parthenogenetica* and *A. franciscana* respectively, results demonstrated the no selective feeding behaviour of *A. salina*.

For mortality, this parameter was chosen based on previously toxicity studies about *A. salina* (Zhu et al., 2017a, 2017b). In the Group B, between 96 and 120 h, was observed an event of massive mortality, it due to starvation obviously. Thus, this is the reason of total absence of specimens at T168 in the same experimental Group that made all the results of the Group A and B comparable until T96. Consequently, in the Group B, the mortality degree was strong time-dependent while comparing the study results by Group A with mortality trend of *A. parthenogenetica* showed by Wang et al., (2019a,2019b) was highlighted low differences with an absence of an acute toxic influence by MPs exposure. However, the development delay due to high concentration particles administered, from 10^2 to 10^4 MPs/ml, showed a low influence on mortality in the different time exposure of both Group A and B. These results are coherent with the higher development delay highlighted from A3 to A5 if compared to control A0 that obviously caused a higher mortality (Zhang et al., 2019). As demonstrated by Cole et al., (2013) for copepods, and in my case highlighted in Figure 459, in most cases, the dead specimens observed were contaminated by MPs corroborating the ingestion and retention capacities of brine shrimps until the end of vital activity.

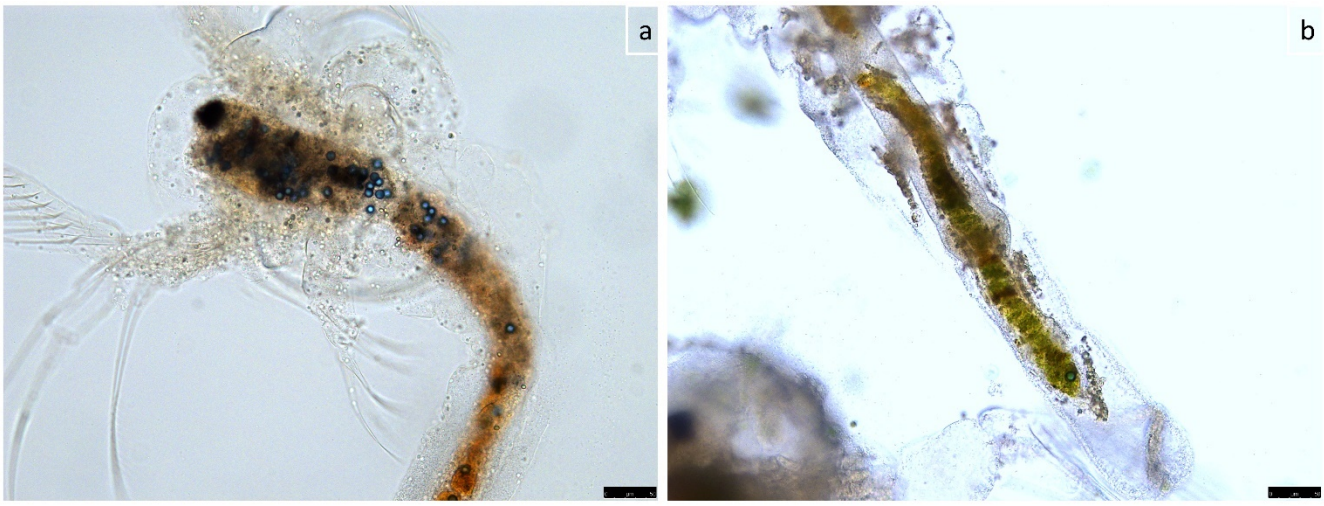

Fig. 59: Microspheres detected in the died specimens, more in detail a) GroupB, b)GroupA. Scale bars: 50 µm

As reported in literature, the features like the total body length variations are used to evaluate the toxicity degree (Nunes et al., 2006; Stara et al., 2020). The results of the study highlighted that the decreasing trend of average total body length of brine shrimps was inversely proportional to the increase of MPs concentration in the solutions for Group A. Results are in contrast with those reported by Wang et al., (2019a) in which no differences were showed about the growth rate depending on MPs concentration in *A. parthenogenetica*, on the other hand, the study of Kokalj et al., (2018) for *A. franciscana* supported my data. However, in this study the results highlighted biggest differences at T96 between A4, A5, B4, B5 and control, more in detail, the higher degree was in Group A showing a more significant influence of microspheres ingestion in the replicas with food source. Precisely, in Group A this influence was more highlighted at T168 showing a correlation of growth with the exposure time as well, this was as expected by a normal developing rate. Thus, basing on these data, during experiment was observed an evaluable trend between the two experimental Groups with the same negative influence on growth due to increase in MPs ingestion. However, of course as expected, a significant difference in total body length was observed comparing Group A and B due to the absence of *D. salina* as food source.

For those concerning the development stages of *A. salina*, data showed the major differences between Groups A and B as expected. At the end of exposure T96 of the control of Group B was reached the maximum instars comprised between II and III, indeed, a mass mortality event was recorded after this 96h due obviously to the absence of microalgae as food source.

On the other hand, the Group A in which the *D. salina* as food source was added, reached at T96 the instar IV, showing normal anatomical development of the species like shown by (Anderson, 1966; Benesch, 1969). At the end of exposure time (168h) was reached the instar V with a complete differentiation of the eye and an evident formation of thoracopods that become the swimming appendages. Thus, it's clear that the presence of the food source in Group A allowed a linear and normal anatomical development of brine shrimps. If compared the replicates A4 and A5, that reached respectively instars IV and III, to control in which the instar V was reached, was highlighted a different average development. However, these data are in contrast compared to these showed by (Wang et al., 2019b) in which no differences were highlighted in the developmental trend of *A. parthenogenetica* based on the different MPs concentration administration.

In the toxicology studies, the filtration rate, as in the case of *A. salina*, is one of the main focused targets and as found in literature for *A. phartenogenetica* (Wang et al., 2019a), *A. franciscana* (Bergami et al., 2016), *D. magna* (Kokalj et al., 2018) and *Calanus helgolandicus* (Cole et al., 2015), the ingestion by plastic materials leads a decreasing in feeding rate in a dose-dependent manner (Germanov et al., 2019; Oliveira et al., 2018; Rosenkranz et al., 2009; Stara et al., 2020). Consequently, we assess that the delay of development trend in the specimens of this study is due to the presence of the MPs that filling the intestinal tracts reduce the ingestion of *D. salina*. The results, in accordance with those found in literature about toxicology, showed in the Group A an high negative correlation between the ingestion rate of microalgae and the one from MPs in the brine shrimps. During the trial, it was observed at T48 for the replica B5 that the average maximum reduction of ingestion rate was 50.58%. This data is related both to maximum reduction of intestinal area for MPs

presence and maximum MPs ingestion degree, furthermore, both values were detected at the same time point. More in detail, at T168 the ingestion rate of microalgae was increased to 39.09% and MPs ingestion rate with a similar value, thus, demonstrated the feeding behaviour of *A. salina* that feds on plastic filtering in a dose-dependent and non-selective manner.

Moreover, the growth of microalgal concentration did not influence the ingestion rate during the trial. More precisely, it was observed that in absence of *A. salina* no significant variation was observed in the concentration growth of *D. salina*. This is due to the feature conditions of the algal cultivation that does not reach concentration in the pure culture also in the case of 24D photoperiod. However, the used photoperiod (12D:12N) during the trial steps did not help the algal growth in concentration. In the studies about the toxicity in zooplankton, the intestinal tract is a significant target (Bergami et al., 2016; Kokalj et al., 2018; Wang et al., 2019a). This is the reason why we focused on the feeding behaviour of brine shrimps. The results about the intestinal area occupied by the microspheres highlighted a strong negative correlation between the area occupied by the MPs and microalgae ingestion rate. Indeed, the absence of microalgae as a food source showed a maximum filling degree of MPs of 90.82% at T96 in B5, while in Group A the filling degree was 44.43% at T96 in A5. Comparing these results, it is clear that in the cases of the presence of a similar food source, the availability of the intestinal area for the filling by MPs was reduced and vice versa.

6. Conclusion

The multispecies and experimental approaches used in this study allowed to increase the knowledge about the plastic distribution and potential influences on organisms of different taxa inhabit many domains. In the bogue *Boops boops* was observed the plastic and man-made cellulose presence even if the abundance of these particles were quite low.

In the present study was highlighted for the first time, the presence of plastic particles in the shortnose greeneyes *Chlorophthalmus agassizi*. This evidence suggests the presence of plastic items to the bottoms of high depths, environments in which *Chlorophthalmus agassizi* usually inhabits, furthermore, the presented data about this species can provide an update for the *Marine Strategy Framework Directive*.

The presence with high percentage of PA, PP, PE, and nylon in the high commercial value species as *Lepidopus caudatus* and *Zeus faber* is a proof of the distribution of the plastic items to higher trophic levels. Furthermore, the characterization of the polymers gave knowledge about the source of these materials like textiles and packaging industries.

The predominant presence in *Mullus barbatus barbatus* of PTFE and PA can be traced back to textile and clothes industries in line with the sampling place (Gulf of Patti) situated close to populated area. Nylon was the only kind of fibres found in the two species *Pagellus bogaraveo* and *Pagellus erythrinus*. These fibers are usually used in fishing industries suggesting that the presence of the polymers in the cited species is due probably to the fragmentation of nets and ropes.

Similar to *Mullus barbatus barbatus*, *Trigla lyra* is a demersal species that feds mainly on bottom swallowing sediment and preys together then expels the sediment, this feeding behaviour can explain the presence of cellulose fibers in the gill lamellae of the analysed specimens.

The ingestion of nylon and PE by *Galeus melastomus* and the different abundance of observed particles compared with other specimens sampled in other area highlight the different contamination level of the environments and as the vulnerabilities of this species depends by the area that's inhabits. In contrast to *Galeus melastomus, Scyliorhinus canicula* sampled in the Gulf of Patti showed an higher degree of plastic ingestion due to their feeding habits close to the bottom. However, in this case as well, the sampling area played a crucial role in the plastic contamination degree, indeed, the specimens of *Scyliorhinus canicula* came from GSA16 showed an higher plastic ingestion degree that those from GSA10.

The presence of plastic items in the elasmobranch *Raja miraletus* is due both to ecological habits of the species that feeds on bottom and to the high degree of contaminated bottoms.

Basing on the ecological habits of the planktonic feeders *Pelagia noctiluca*, it can be considered a bioindicators along the water column. Furthermore, future studies are needed to increase the role of this species as vector for microplastics in marine environments to better understand the dynamics along the trophic web.

The sampling of juvenile clupeid fishes for plastic occurrence, highlighted the needs to survey more juvenile species that are the food source of many species to higher trophic levels. These kinds of surveys could help in the understand of the biomagnification and accumulation of plastic litter along the food web.

The trial about the plastic administration in *Artemia salina* showed as this experimental model is strongly influenced by the plastic concentrations and presence/absence of food source (*Dunaliella salina*) both in their feeding rate and stage development.

In conclusion the environmental survey allowed to better understand the distribution and abundance of plastic materials in the marine environments and the species that inhabits in. Moreover, the experimental survey with *Artemia salina* permitted to increase the knowledge about the plastic

ingestion dynamics of zooplankton, as this can change their usually development stage (Instar) and how the plastic particles can move along the levels of trophic web.

These results represent an important baseline in assessing the abundance and distribution of microplastics in terms of ingestion and trophic transfer in the marine environment. However, further studies on nanoplastics, both in natural and experimental conditions are essential, as there is concern that nanoplastics may have a high biological impact at all trophic levels.

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8. Glossary

ATR-FTIR = Attenuated Total Reflectance Fourier transform infrared

CGPM = General Fisheries Commission for the Mediterranean

EDX = Energy Dispersive X-ray

Fry = white late-larval and juvenile stages of clupeid fishes

GIT= Gastrointestinal Tract

MSFD= Marine Strategy Framework Directive

MPs = Microplastics

OA = Oral Arms

SEM= Scanning electron microscope

U = Umbrella

List of original papers

1. **The mauve stinger *Pelagia noctiluca* (Cnidaria, Scyphozoa) plastics contamination, the Strait of Messina case.** Albano M., Panarello G., Di Paola D., D'Angelo G., Granata A., Savoca S., Capillo G.
2. **The influence of polystyrene microspheres abundance on development and feeding behavior of *Artemia salina* (Linnaeus, 1758).** Albano M., Panarello G., Di Paola D., Capparucci F., Crupi R., Gugliandolo E., Spanò N. Capillo G., Savoca S.
3. **Quali-quantitative analysis of plastics and synthetic microfibers found in demersal species from Southern Tyrrhenian Sea (Central Mediterranean).** Capillo, G., Savoca, S., Panarello, G., Mancuso, M., Branca, C., Romano, V., D'Angelo, G., Bottari, T., Spanò, N.
4. **Plastics occurrence in juveniles of *Engraulis encrasicolus* and *Sardina pilchardus* in the Southern Tyrrhenian Sea.** Savoca, S., Bottari, T., Fazio, E., Bonsignore, M., Mancuso, M., Luna, G., Romeo, T., D'Urso, L., Capillo, G., Panarello, G., Greco, S., Compagnini, G., Lanteri, G., Crupi, R., Neri, F., Spanò, N.
5. **Detection of artificial cellulose microfibers in *Boops boops* from the northern coasts of Sicily (Central Mediterranean)**. Savoca, S., Capillo, G., Mancuso, M., Faggio, C., Panarello, G., Crupi, R., Bonsignore, M., D'Urso, L., Compagnini, G., Neri, F., Fazio, E., Romeo, T., Bottari, T., Spanò, N.
6. **Plastics occurrence in the gastrointestinal tract of *Zeus faber* and *Lepidopus caudatus* from the Tyrrhenian Sea**. Bottari, T., Savoca, S., Mancuso, M., Capillo, G., Panarello, G., Bonsignore, M., Crupi, R., Sanfilippo, M., D'Urso, L., Compagnini, G., Neri, F., Romeo, T., Luna, G.M., Spanò, N., Fazio, E.