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Microbiological and spectroscopic analysis of microplastics in commercial fishes

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ABSTRACT

The aim of this thesis is the evaluation of the plastic impact on ecosystems and especially on human health. First, the attention will be focused on the identification of plastic pollutants, extracted by aquatic organisms, and their physico-chemical characterization. Microplastics characterization has been carried out by using some spectroscopic techniques (IR optical absorption, micro-Raman and X-ray photoelectron spectroscopies) and scanning electron microscopy (SEM/EDX and STEM). A screening for susceptibility to antibiotics of bacteria isolated from plastics extracted by gills and gastrointestinal tract will be presented and discussed. Plastics washing processes of synthetic clothings have been lately identified as responsible for about 35% of primary microplastic release in aquatic environment. The released fibres and debris behave as potential vectors for spread of pathogenic bacteria through aquatic organisms, contributing to the diffusion of multiple antibiotic resistance in marine environments. Ultimately, microplastics play a potential and crucial role in conveying antibiotic resistance by humans food ingestion.

In *Chapter 1*, an overview of microplastics pollution in aquatic environments and their classification are reported.

In *Chapter 2*, results about the effect of water temperature, salinity and pH on the immune response of mussels from Faro Lake and Tyrrehenian sea by flow cytometry and Raman spectroscopy are presented and discussed.

In *Chapter 3*, the methodologies generally adopted for sampling, identification and characterization of plastics extracted by fishes are described, focusing the attention on the mechanisms underlying the techniques used to investigate the microplastics composition and their morphological properties.

In *Chapter 4*, the occurrence of microplastics in *Zeus faber* and *Lepidopus caudatus* from the Tyrrhenian Sea, juveniles of *Engraulis encrasicolus* and *Sardina*

pilchardus from Southern Tyrrhenian Sea, *Boops boops* from the northern coasts of Sicily (Central Mediterranean) was evaluated in order to quantify the amount and the typology of plastics within the gastrointestinal tract of the samples and, in turn, to evidence that Mediterranean sea is affected by plastic pollution.

In *Chapter 5*, the potential effects of plastics on humans' health are remarked. To this purpose, an in-depth study of the plastics extracted by the *Pagellus erithrynus*, going from the northernmost portion of the Strait of Messina, was carried out. Particularly, microbiological assays showed the presence of Enterobacteriaceae, Aeromonadaceae, Vibrionaceae, and Pseudomonaceae families both in gills and gastrointestinal tract. Bacterial isolates were screened for susceptibility to antibiotics using the *Kirby-Bauer* test, choosing the molecules most used in human therapy.

Finally, the main contents of the thesis are summarized in the last chapter, remarking challenges and future perspectives. Still, there are significant disconnects in the integration of knowledge derived from laboratory and field studies. It is well known and also emerged by the results reported in this thesis that microplastic transport over a range of spatial scales and with different residence times will be influenced by particle characteristics, external forces (e.g. flow regimes), physical site characteristics (e.g. bottom topography), the degree of biofouling, and anthropogenic activity (e.g. dam release), however there is a lack of data on this. Nevertheless, it is predicted that impacts on biota will mirror that of the marine environment. Thus, some efforts in this direction will be still made to safeguard the health of the system and of the future generations.

Acknowledgements

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Chapter 1

Microplastics in the marine environment

Plastic contamination of marine environments has become significant worldwide due to the increasing use of polymeric materials for commonly objects, mostly disposable. The sources, the points of accumulation and the effects on aquatic environment are still unclear today. It is well known that the evaluation of the plastic impact on ecosystems requires an accurate chemical-physical characterization of these contaminants and, secondly, an assessment of their impact on the aquatic environment where organisms at the top of the food chain live.

In this chapter, an overview about microplastics sources, classification, potential remediation and future path of research and development to be carried out to mitigate the problem are presented.

1.1 An overview of microplastics pollution in aquatic environments: an emerging issue of international concern

The increasing demands of plastics have led to a drastic increase in their production (see Fig.1.1). For this, plastics should remain in the top of the political agenda in Europe and across the world not only to minimise plastic leakage and pollution but also to promote sustainable growth and to stimulate both green and blue-economies. Several marine ecology studies show the threat posed by plastic waste which ending up in aquatic environments every year (millions of tons) (Plastics Europe, 2015). The United Nations-Oceans organization defined this

situation as "a planetary crisis" since plastic production is expected to increase globally (Andrady, 2011); (Galgani et al., 2010).

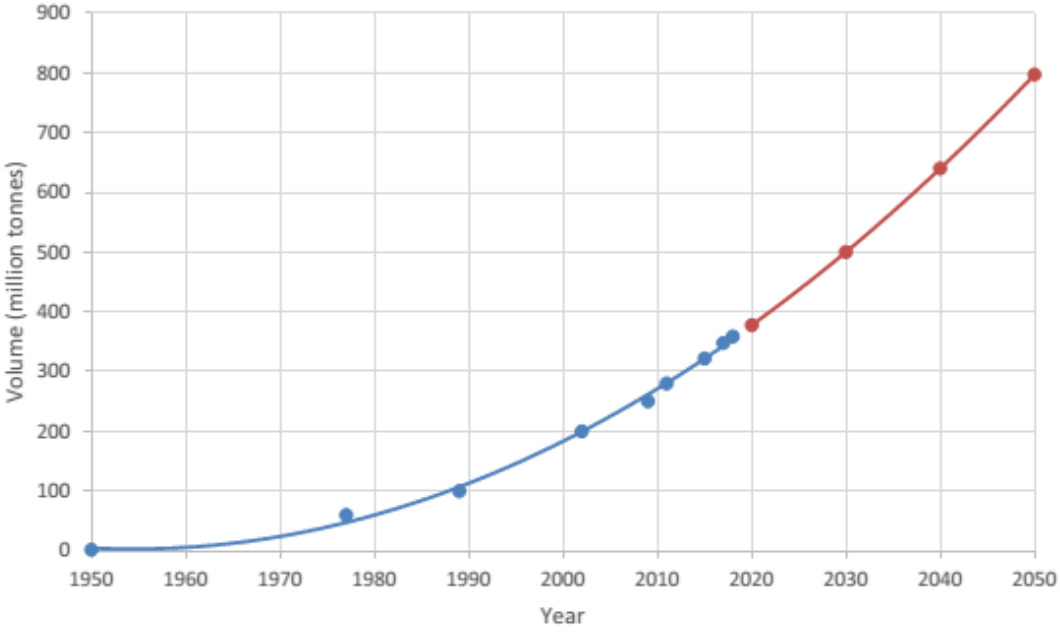

Figure 1.1: Global production volume of plastics. Blue line, production 1950 to 2018; red line, forecast production 2020 to 2050. Data; 1950 to 2016 (Plastics Europe, 2016); 2017-2018 (Plastics Europe, 2019). Forecast 2020 to 2050 (Rouch, 2021).

Currently, the biological fragmentation of big plastics into microplastics is the predominant wake-up call. Recent studies show that microplastics pose a significant risk in the ocean for animals at the top of the food chain such as whales, sharks and manta rays, which inadvertently ingest them through Antarctic krill (Besseling et al, 2015); (Lusher et al, 2015). Antarctic krill, in turn, incorporates microplastics and transforms them into nanoplastics through digestive processes and is capable of fragmenting polyethylene (PE) microspheres (less than 5 millimeters in size) until to nanoplastics of different sizes (below 0.1 micrometers) (Dawson et al, 2018). Krill is ecologically important because it is the staple food of many marine animals through the food chain. During fragmentation, plastic takes on new physical and chemical characteristics that are not yet well defined, increasing the possibility of being harmful for organisms (Fig. 1.2). Chemical effects are particularly problematic during the decomposition phase since toxic elements, such as phthalates and

bisphenol A, are released, and subsequently interfer with the hormonal systems of vertebrates (including humans) and invertebrates (Avio et al, 2016); (Ng et al, 2018). Consequences have been reported on both male and female reproductive organs and also are linked with childhood obesity (Andersson, 2014).

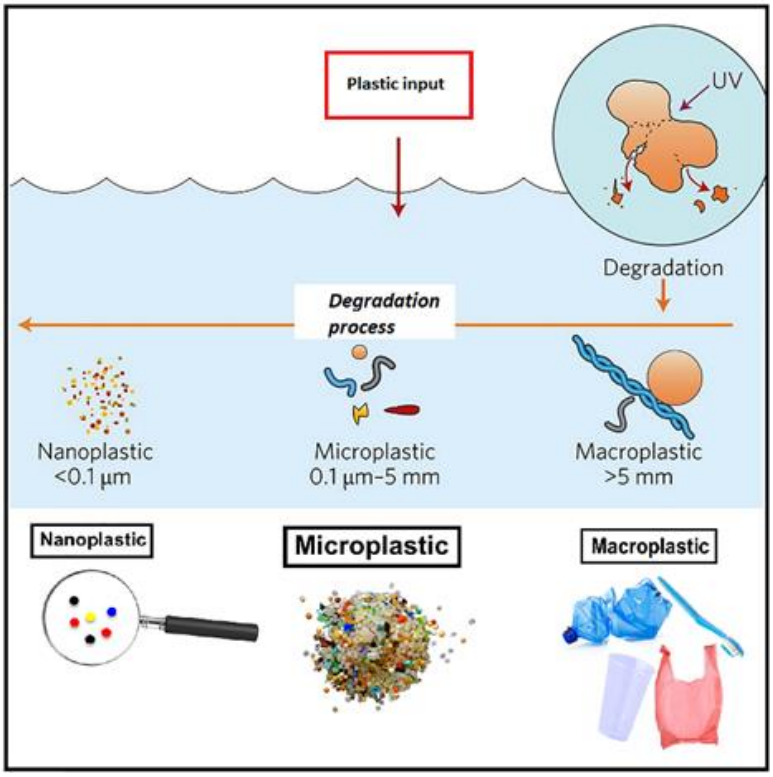

Figure 1.2: Representative scheme of chemical-physical degradation plastics process.

The impact of plastics on the environment occurs on different levels: from the spread of bacteria to structural changes in the soil to damage the entire species. The plastic fragments can "transport" pathogens on the surface (bacterial adhesions) and thus cause antibiotic resistance in humans. A study on the journal Marine Environmental Research reports the presence of the *Vibrio parahaemolyticus* bacterium on floating microplastics from Baltic Sea (same situation could happen on land) (Kirstein et al, 2016). In addition, microplastics ingested by aquatic organisms can be unabsorbed in the gastro-intestinal tract-and they are eliminated by defecation. As a consequence, they are accumulated in the sediments of the ocean floor (Lusher et al, 2017); (Gonçalves et al, 2019). Fig. 1.3 shows a representative diagram of how

microplastics enter the food chain. An example is given by the mussels which tend to accumulate microplastics by filtering large quantities of water. So, mussels represent a risk related to the health of their consumers (aquatic organisms or humans) (Van Cauwenberghe et al, 2014). This will be discussed more specifically in Chapter 2, where immunological and structural properties of hemocyte populations in *Mytilus galloprovincialis* coming from Mediterranean Sea were investigated. Mediterranean Sea is considered a unique ecosystem. Despite its small size (less 1% of the total ocean area), it hosts a disproportionately large amount of biodiversity (up to 18% of all marine species). These species are coming increasingly under threat due to marine litter pollution. Thus, we need to leave the plastics age behind and rethink the future of plastics towards a plastics-free.

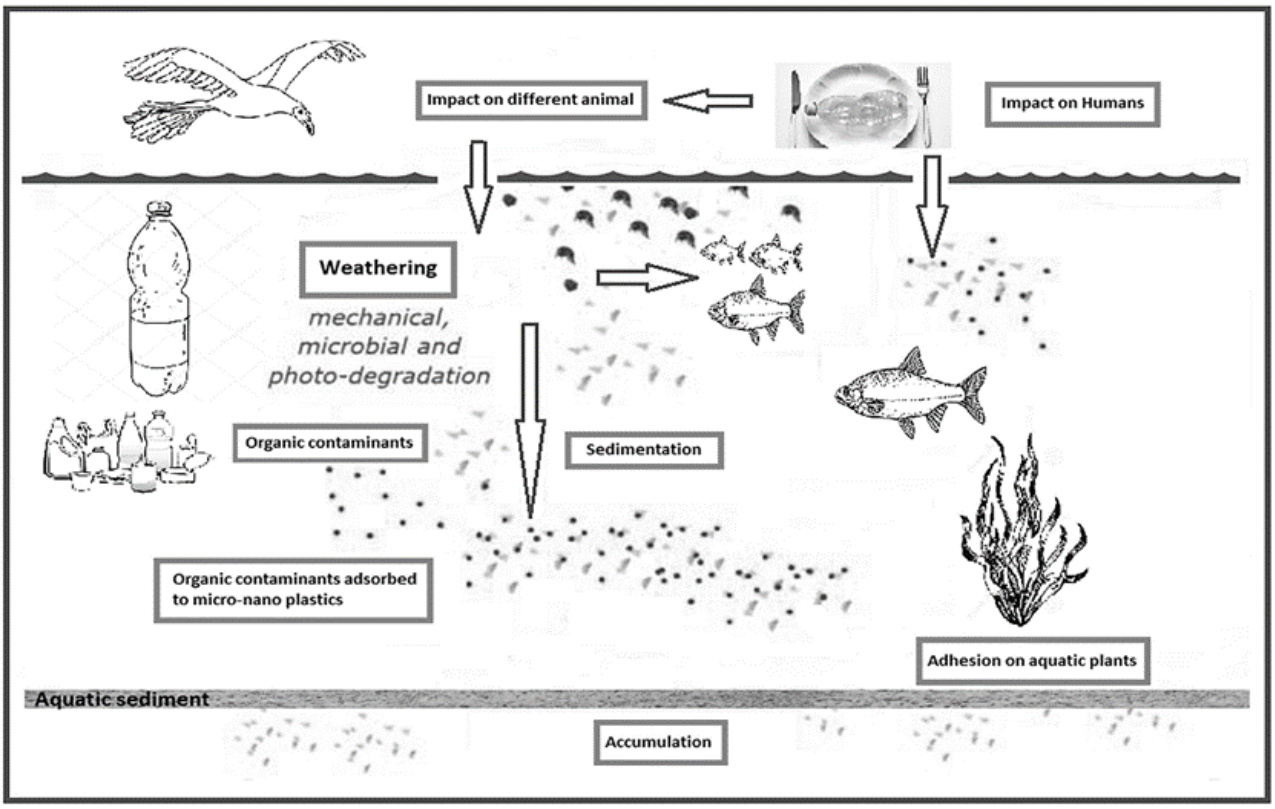

Figure 1.3: Microplastics and the food chain.

Unfortunately, today Mediterranean Sea is a marine area considered one of the great accumulation for marine plastic pollution: 83% of the total number of collected items are microplastics (Cózar et al, 2015). Particularly, the most abundant polymer

floating in the Mediterranean Sea was polyethylene (52%), followed by polypropylene (16%), synthetic paints (7.7%), polyamides (6.6) and epoxy resins (5%) (Suaria et al, 2016).

As is well known, microplastics are able to move from one ecosystem to another (aquatic-terrestrial-airborne), exploiting atmospheric phenomena or biological organisms such as insects as vectors (Majer et al, 2012); (Goldstein et al, 2012). Particularly, the smallest plastics are able to enter in water cycle following transport in the clouds (condensing, precipitate to the ground). This ultimate step represents the precursor to reach humans (via drinking, tap or bottled water and so on) (Pivokonsky et al, 2018). When plastics waste end up in the aquatic environment, they will interact with surroundings organisms (Schwarzet et al, 2019); (Wright et al, 2013). This is demonstrated by the presence of plastics, toxin and chemical compounds in the muscle and fat tissue of many fishes; hence, once in the large fish and bird trophic levels, humans may be at risk from ingestion of highly contaminated animals. As a top predator, man is particularly susceptible because of his wide variety of prey (as omnivore), including many species that regularly ingest microplastics, such as crustaceans, fish, bivalves, and some fowl. Ultimately, this implies that the transfer of plastics is "guided" by aquatic organisms to man, through the interaction between predator and prey (Egbeocha et al, 2018); (Lusher, 2015).

For the above reasons, World Health Organization (WHO) makes an urgent call for the assessment of environmental pollution due to microplastics and its effect on human health. In particular, there is a paucity of information on the toxicity mechanisms of microplastics in animal studies, and despite their documented presence in food products, no policy has been in place so far, to monitor and regulates microplastics in commercial foods meant for human consumption. Although there are policies and regulations with respect to plastics, these are only in a few countries and in most instances are not fully implemented due to socioeconomic reasons, so they do not address the problem across the entire life cycle of plastics from production to disposal.

1.2 Classification of plastics

1.2.1 Classification according to size, shape and colour

Plastics are generally classified by following morphological characteristics: size, shape and colour. A scientifically rigorous definition of plastics pieces provides for the subdivision into: (Rios Mendoza et al, 2018)

i. macro *ii.* meso *iii.* micro and *iv.* nano-size ranges.

Plastics fragments in aquatic environment with dimension larger than 2 cm are defined as ***macroplastics***. In turn, macroplastics can be fragmented into smaller particles after UV thermal oxidation, wind action and sea waves and/or mechanical processes forming plastics debris in the micrometer- nanometer range (Winton et al, 2020); (Andrady, 2011); (Wagner et al, 2014). Debrites characterized by dimension between 2.5 cm to 5 mm are named ***mesoplastics*** (GESAMP, 2015). Finally, plastics pollutants that have dimensions ranging from a few μm to 5 mm, commonly present in sea water, are defined as ***microplastic*** (MPs), while plastics with a size ≤0.1 μm are defined ***nanoplastics*** (NPs) (Law et al, 2014); (Barnes et al, 2009); (Boyle et al, 2020).

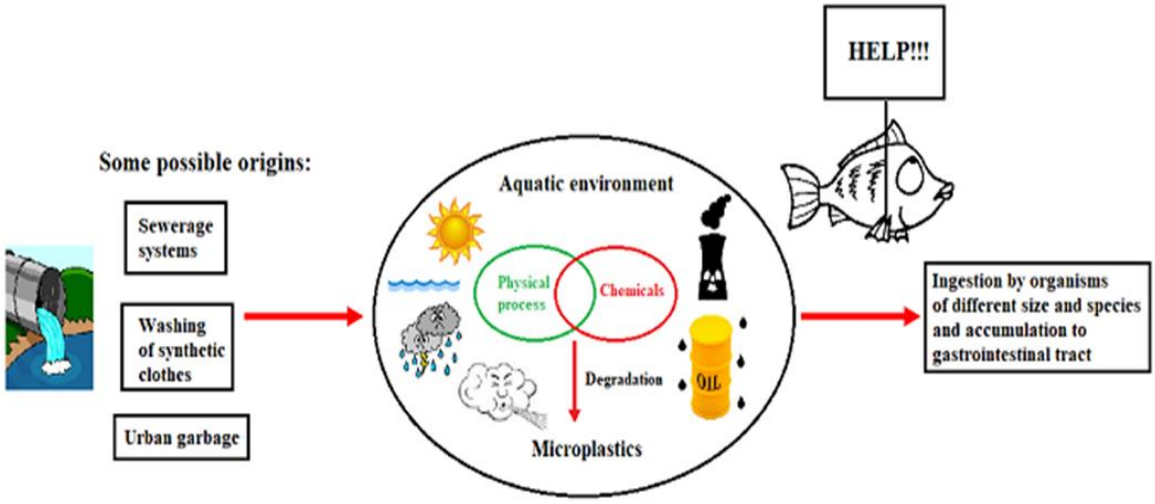

Figure 1.4: Representative scheme of the potential routes of entry of plastic contaminants in aquatic environment (left panel), fragmentation and/or alteration of the morphology-dimension and interaction with further pollutants (central panel), contact with organisms aquatic (right panel).

It is important to outline that micro-nanoplastics contribute to increase plastic bioavailability, once are ingested by marine organisms. To represent these processes, in Fig.1.4 is reported a representative scheme of the potential routes of entry of plastic contaminants in aquatic environment, their fragmentation and so the alteration of their morphology and interactions with further pollutants until the contact with aquatic organisms. Furthermore, regarding microplastic classification, researchers also refer to plastic's shape for their physical characterization (Lusher et al, 2017). Analytical results show that eight shape types namely fragments, flake, fibers, foams, pellets, films, beads and sponges, were present in marine environment (see Fig. 1.5 and Table 1.1).

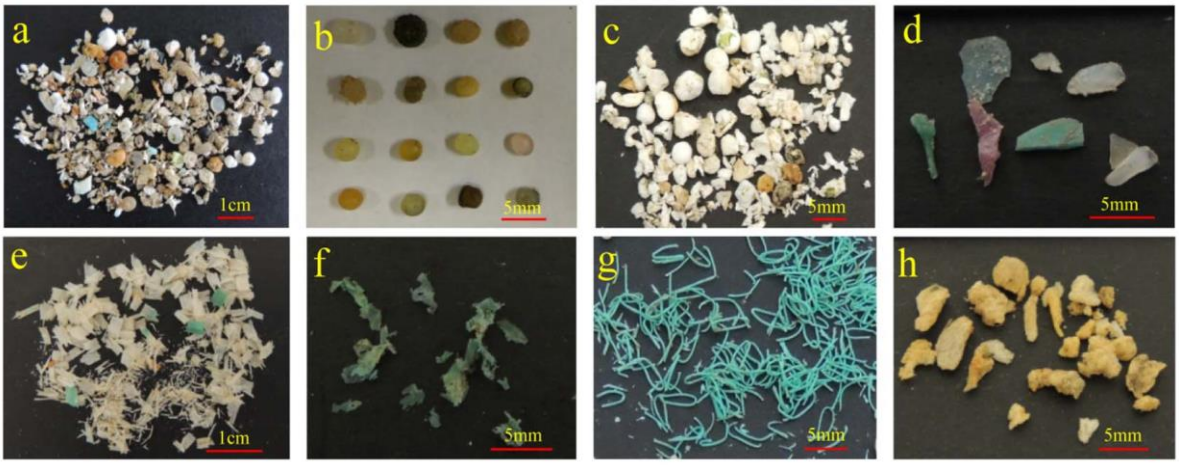

Figure 1.5: Different shapes types of microplastics; a, mixed microplastics; b, pellets; c, foams; d, fragments; e, flakes; f, films; g, fibers (fishing lines); h, sponges (Zhou et al, 2018).

Table 1.1: Shape Classification for microplastics (Lusher et al, 2017).

Shape	Definitions
Fragments	Irregular shaped particles, hard, jagged, crystals, fluff, powder, granules.
Flakes	Flat sheets of plastic
Fibers	Filaments, microfibres, strands, threads
Foams	Lightweight, polystyrene, expanded polystyrene
Pellets	Resin Pellets, nurdles, nibs
Films	Flat, flexible particle with smooth or angular edges
Beads	Microbeads, microspheres
Sponges	Lightweight and porous.

Finally, to further complete the plastics classification, the colour is another important parameter (Rocha-Santosa et al, 2015). This choice is explained taking into account that the different colours of plastics are able to attract predators who mistake them for their prey (Kühn et al, 2015). Colours change are due to ultraviolet radiations or to bacterial adhesion. In addition, the fragmentation, bleaching, or the microbiota fouling induce chromatic change and could bring to an incorrected identification (Eriksson et al, 2003); (McGivney et al, 2020).

1.2.2 Classification according to origin and degradation effects

Generally, the microplastics are divided into "*primary*" and "*secondary*" (see Fig. 1.6). The "primary plastics" are those intentionally produced, either for direct use or as precursors of other products. So, they result from the direct input of man-made emissions (GESAMP, 2015).

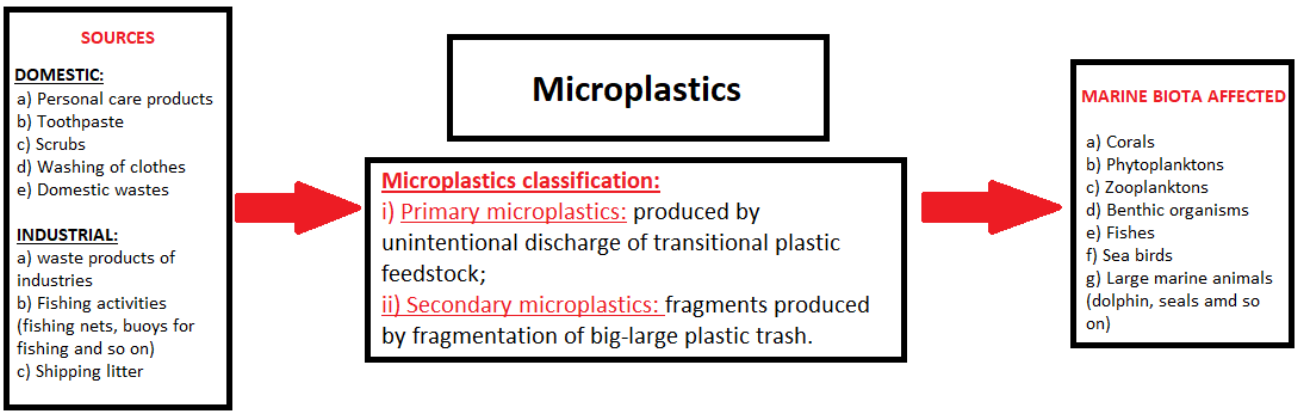

Figure 1.6: Schematic representation of plastics (primary and secondary) in relation to sources and marine biota connection.

The major primary plastics are (Hantoro et al, 2019):

(i) man-made polymers. Personal care consumer products, as facial scrubs and common toothpastes, industrial or commercial products belong to this group;

(ii) inherent collateral products of other industrial activities (i.e industrial abrasives, drilling fluids, raw materials (nurdles= raw materials for the plastics industry) and so on);

(iii) plastic sourced as accidental or deliberate spillage i.e., pellets loss from plastic factories and transport.

The main source of primary microplastics in the aquatic environment is represented by plastic pollution originated by washing processes of synthetic textiles (Boucher et al, 2017)]. Browne et al. discovered that the polyester and acrylic fibres used in clothing were the same found in habitats with sewage-discharges (Browne et al, 2011). The release of microplastics from synthetic clothes is due to thermic and mechanic stresses that laundry machine causes during washing process (see Fig.1.7). Fibers can detach from the yarns that constitute the textile. Since their dimensions, the microfibers might pass wastewater treatment plants (WWTPs) and reach directly the aquatic environment. Since synthetic fibres (polyester, polypropylene, nylon, etc.) represent almost the 60% of the annual global consumption, they make a strong contribution to pollution from microplastics (De Falco et al, 2017).

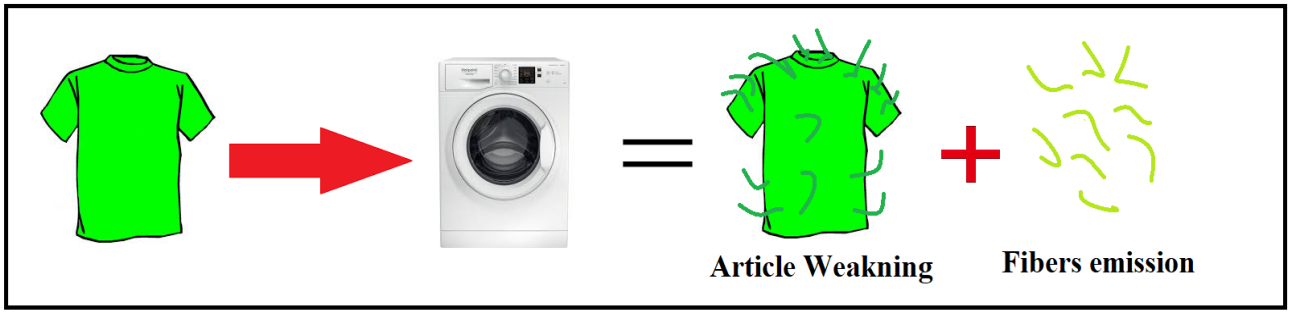

Figure 1.7: Scheme of the release of microplastics from synthetic clothes due to machine during washing process.

Otherwise, "secondary plastics" are indicated as secondary pollution sources in which larger plastic debris after photo-degradation, biodegradation, photo-oxidative and thermo-oxidative degradation leads to the formation of smaller plastic

pieces (GESAMP, 2015); (Lambert et al, 2018). As said before, plastics are typically made up by organic polymers of high molecular mass (meaning each molecule can have a lot of atoms bound together) with the addition of several dangerous substances (fillers, plasticizers, colorants, stabilizers and processing aids (Jansen, 2016). Most plastics are made from synthetic resins (polymers) obtained through the polymerization industrial process (Derraik, 2002); (Wright et al, 2013). Into polymer's categories, both virgin plastic resin pellets (easily transported prior to manufacture of plastic objects) as well as the resins mixed (or blended) with several additives to enhance the performance of the materials are included (Hahladakisa et al, 2018).

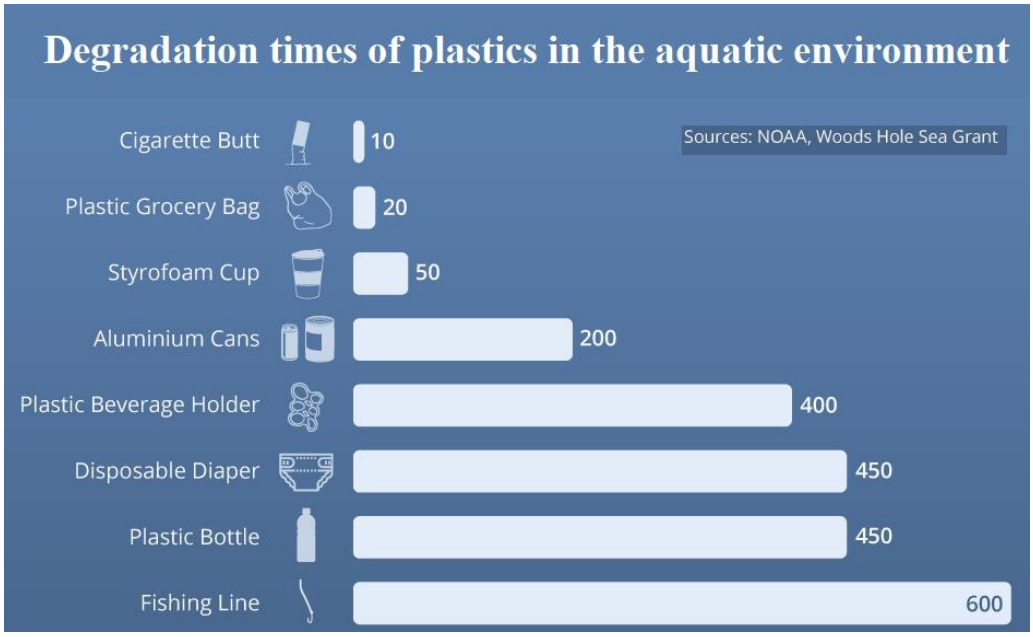

Figure 1.8: Degradation times of plastics in acquatic environment.

In Fig. 1.8 a schematic representation of plastics degradation time in the acquatic environment is shown. Due to lightweight and durable nature, plastics has become a default elements of marine environment (Moore, 2008). Plastics does not biodegrade in nature but only shatters into smaller pieces even more difficult to eliminate (Moore, 2008); (Thompson et al, 2009). For example, some items take a long time to degrade (fishing line, bottle and so on), others less (cigarette, plastics bag and so

on). Moreover, it is relevant remember that the smaller plastics particles are easier to ingest, thereby increasing the susceptibility, enhanced leaching, desorption and adsorption potentials of the microplastics (Velzeboer et al, 2014); (Auta et al, 2017). As said before, global market produces several types of plastic but typically 6 classes of plastics are identified: (Nerland et al, 2014); (Ritchie, 2018)

- polyethylene (**PE**, high and low density),
- polypropylene **(PP)**,
- polyvinyl chloride **(PVC)**,
- polystyrene (**PS**, including expanded EPS),
- polyurethane (**PUR**) and
- polyethylene terephthalate (**PET**)

Additional informations are reported in Table 1.2.

Table 1.2: Main types of plastic and their characteristics (Nerland et al, 2014); (Ritchie, 2018).

Plastics	Characteristics
PE	**PE** products include containers for milk shampoos and conditioners, soap bottles, detergents. Moreover, **HDPE (high density)** products are commonly recycled. **LDPE (low dencity)** is so metimes recycled. It is both durable and flexible. Is very know as cling-film, sandwich bags, and plastic grocery bags and so on.
PP	**PP** is strong plastic and can able to higher temperatures. It is used to make yogurt pots, syrup bottles and is used for plastic bottle caps.
PVC	**PVC** is used for all kinds of pipes and tiles. Is a very strong material by can use only for material without food contact.
PS	**PS** is commonly recycled, and is ever present for example for disposable coffee cups, plastic cutlery and so on.
PUR	**PUR** or **PU** is composed by organic units joined by carbamate (urethane) links and are thermosetting (not melt when heated). Are used for production of durable elastomeric wheels and tires (such as escalator, shopping cart, elevator), synthetic fibers (e.g., Spandex), hard-plastic parts (e.g., for electronic instruments) and so on.
PET	**PET** plastic is used to make many common items like beverage bottles, medicine jars, clothing and carpet fibre. Items made from this plastic are commonly recycled.

Moreover, there is the category **"others"** among which are counted amidic and polyamide synthetic materials like Nylon (PA), Acrylonitrile butadiene styrene

(ABS), Polycarbonate (PC) Layered or multi-material mixed polymers (GESAMP, 2015).

The fate, the distribution and the dissemination of microplastics in the aquatic environment is different between beach and bottom sediments and this situation is still not fully understood. Certainly, the presence of them is however closely linked to the intrinsic densities of the plastic. In fact, the buoyancy of plastic polymers is different; for instance, PE and PP are float on the water surface because have lower density than the water while PVC and PET have higher densities than water, so they go down the water surface (Kärrman et al, 2016). So, an important property influencing the behaviour of plastics in the marine environment is the density with respect to the density of seawater. Further, also the development of biofilms on the plastics surface sufficiently contributes to alter the density to float it (Tu et al, 2020).

Recently, a question that creates a lot of interest and concern is the ability of some pathogenic microorganisms to bind to the plastics present in the aquatic environment and be carried over a long range. In turn, the plastics with the pathogenic bacteria, ingested by an aquatic organism, can reach humans and also create antibiotic resistance (Murphy et al, 2020). These plastic surfaces can give natural habitats both for microbial colonization and biofilm formation, allowing opportunistic microorganisms and invasive species the migration (Zettler et al, 2013). In normal conditions, bacteria are not supposed to survive for very long in coastal waters, but the presence of microplastics changes things. They can attach to the plastic and form a biofilm on the surface. It creates a more welcoming environment for them, raising their chances of survival. They can live longer, travel further away, and even reproduce. What is more, within these biofilms, proximity between the bacteria enables the exchange of DNA, including antibiotic-resistant genes. This process is called horizontal gene transfer. This means that, in these environments, extracellular DNA (free DNA) carrying antibiotic-resistant genes is potentially present, representing a high risk of contagion for non-resistant bacteria.

Antibiotic resistance is considered by the World Health Organization (WHO) as one of the biggest threats to global health, food security and development today. It occurs when, as a result of exposure to antibiotics, bacteria progressively develop the ability to combat their action. Although this is a naturally-occurring phenomenon, the misuse and/or overuse of antibiotics have dramatically accelerated the process, imperiling the incommensurable progress achieved in the fight against infectious diseases globally since the invention of penicillin in the early 20th century. To limit the propagation of this scourge, WHO recommends intensive monitoring for the identification and surveillance of critical hot spots. Point sources of pollution, such as wastewater treatment plants or agricultural facilities, are suspected of playing a major role in the dissemination of antibiotic-resistant bacteria and genes in the natural environment.

Finally, Persistent Organic Pollutants (POPs) are hazardous human-made chemicals that are resistant to biodegradation in the aquatic environment. Polychlorinated biphenyls (PCBs), different organochlorine pesticides (e.g. DDTs and HCHs) and brominated flame-retardants are all POPs. These chemicals have a high affinity for oils and fats (tecnically are lipophilic), are able to accumulate in fatty tissues organisms and, since plastic pellets are also lipophilic, have a good affinity for them, too. It was demonstrated that plastics are able to sorb POPs from the aquatic environment but should be remember insecticides, pesticides and chemicals from industries spilled in sea water via waste- and run-off waters, too (Teuten et al, 2007); (Lee et al, 2014); (Sci. Environment Pol., 2017). The interaction between POPs and plastics depends on their physical and chemical properties. Usually, the absorption of chemicals occurs on the amorphous portions of the polymers rather than the crystalline ones (Nerland et al, 2014). In aquatic environments, usually the highest concentration of POPs is found in the surface water that is the highest point in which plastics accumulate too (Cole et al, 2011). Ultimately, POPs associated with MPs are considered dangerous pollutants since they tend to accumulate in the environment, are toxic, possible carcinogen and able

to influence the immune, reproductive and endocrine systems of animals (Fries et al, 2011).

1.3 Detection of antibiotic resistance in bacterial strains isolated on microplastics

Plastics wastes (macro-meso and micro-nano plastics) spilled in aquatic environment represent a good surface for the attachment of microbial populations and biofilms. Therefore, these surfaces become a possible growth site for viruses and several pathogen bacteria in relation to hydrophobicity, properties and floating ability of plastics (Zettler et al, 2013). So, the microbial colonisation of plastics can also impact particle buoyancy and transport (Naik et al, 2019). The buoyant and persistent nature of plastic could contribute significantly to the survival and transport, across large distances, of microorganisms that associate with plastics surfaces (see Fig. 1.9). Plastic debris, in all its dimensional and shape variations, have a low biodegradability making them persistent in the environment and potential vectors for spreading pathogens.

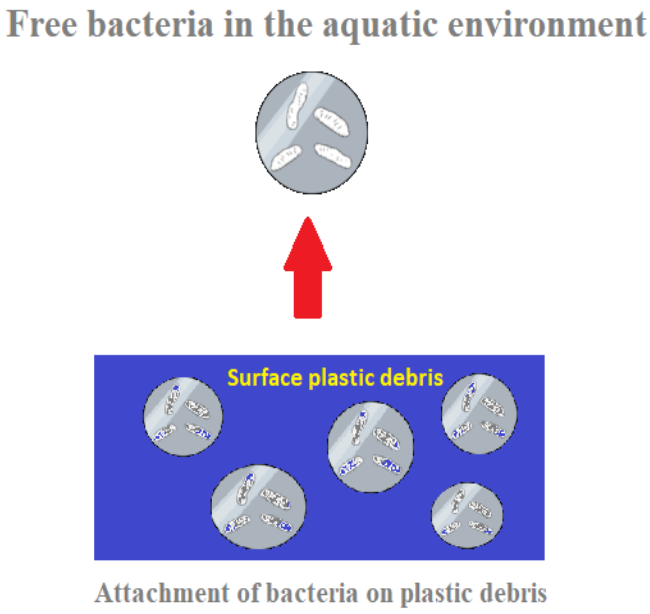

Figure 1.9: Representation of interactions between generic aquatic bacteria and plastics debris. Initially bacteria live free in aquatic environment, after the microorganisms are able to attach on plastic surface.

The microscopic community able to attach plastics surface consists of several types of microorganisms that include a mix of eukaryotic and prokaryotic cells, such as diatoms, coccolitophores, dinoflagellates, fungi and bacteria (Reisser et al, 2014) (Zettler et al, 2013). Obviously, size, shape, type and surface roughness of plastic debris influence the diversity and selectivity of the taxa that will grow on it (Carson et al, 2013). For example, bacteria belonging to the genus of *Vibrio* spp. can populate floating plastics debris in aquatic environment. *Vibrio* spp. is a well-known genus of bacteria containing strains pathogenic for humans (e.g. cholera) and animals. *Vibrio* spp. bacteria are the most numerous species compared to all microorganisms detected on plastics surface (Gregory, 2009); (Oberbeckmann et al, 2017). On the overall, plastics debris could acting as a potential vector for the wide-scale dissemination of pathogens around the globe via marine currents, and so they may vector pathogens into the gut. Microbial attachment to plastic particles can enhance both microbial dispersal and survival, as biofilms offer protection from environmental stress and enhanced opportunities for the sharing of beneficial traits via horizontal gene transfer (Wagner et al, 2014); (Zettler et al, 2013); (Lear et al, 2021). Moreover, during weathering of microplastics, many chemical additives (metal, metalloid, aromatic compounds, plasticizers, antioxidants, heat stabilizers, slip agents) are released/leached and this provides a perfect environment for chemical mixture mediated co-selection of antibiotic resistance in bacterial pathogens (Hahladakis et al., 2018). Some studies have demonstrated that the surfaces of different types of plastics, for e.g. polyethylene (PE) and polyethylene terephthalate (PET), submerged in seawater, are able to become rapidly colonised by heterotrophic bacteria, and that these organisms persist for longer periods respect to those free in the surrounding seawater (Lobelle et al, 2011). During the bacterial adhesion phase, changes in bacterial colonisation of plastics occur. In fact, initially, the first phase of colonisation is characterized by a higher species variability (Jones et al, 2006). Alphaproteobacteria and Gammaproteobacteria resulted the most dominant group of bacteria colonising plastics surface (Zettler et al, 2013); (Jones et

al, 2006). Specifically, Gammaproteobacteria contain several pathogens harmful to human health, including *Salmonella* spp. and *Vibrio cholerae* (Zettler et al, 2013).

The emerging plastic contaminant has the potential to further enhance antibiotic resistance by providing porous micro ecosystems named “plastisphere". In these plastisphere antibiotic resistance is promoted by: (Baker-Austin et al., 2006); (Seiler et al, 2012)

(i) cross-resistance in which resistance mechanisms to heavy metals and antibiotics are physiologically coupled (for example, efflux pumps)

(ii) co-resistance in which antibiotic resistance genes and metal resistance genes are present on the same mobile genetic element and thus genetically coupled.

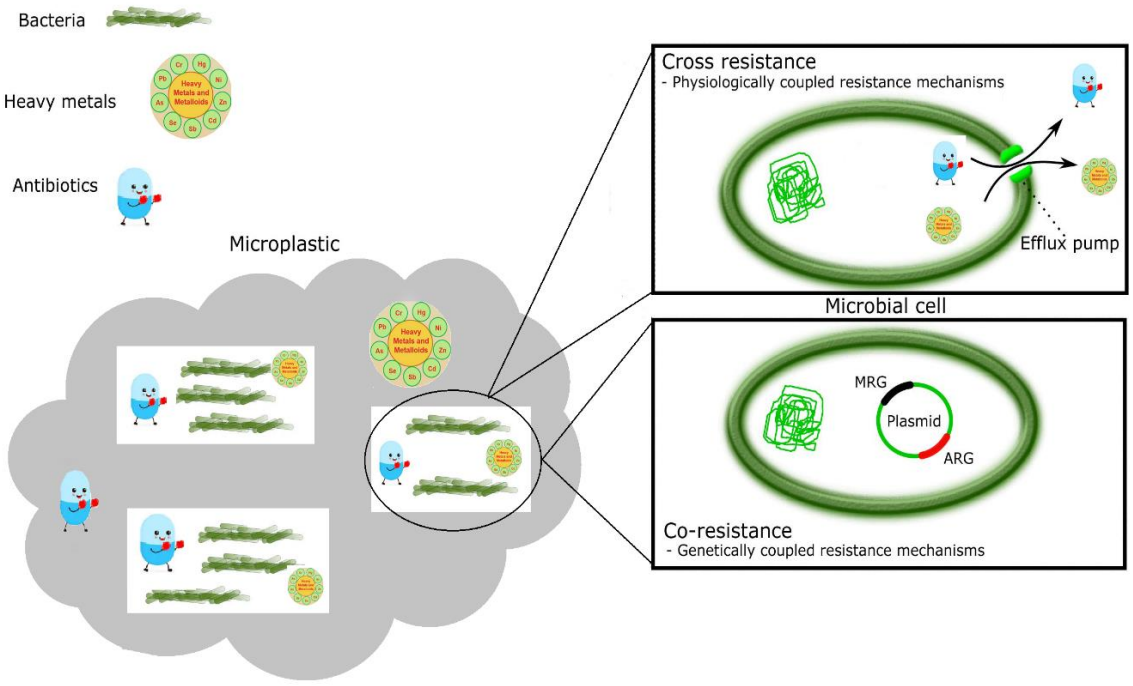

Figure 1.10: Representation of an example of plastic miniature ecosystem, where antibiotic resistance is promoted by (i) cross-resistance in which resistance mechanisms to heavy metals and antibiotics are physiologically coupled (for example, efflux pumps) and (ii) co-resistance in which antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) are present on the same mobile genetic element and thus genetically coupled.

Therefore, a rigorous selection of metal resistance, for example in animal gut, anthropogenically contaminated soils and water bodies, leads to an automatic

selection of antibiotic resistance genes (Baker-Austin et al., 2006); (Seiler et al, 2012); (Li et al., 2017) (see Fig. 1.10). Probably, these resistant bacteria come from human and animal populations treated with antibiotics and then travel through wastewater into aquatic environment (Berkner et al, 2014).

It was found that some bacteria (potential human pathogens e.g. *Pseudomonas* spp., *Acinetobacter* spp., *Aeromonas* spp., *Proteus* spp., and *Listeria* spp.) isolated from 22 seafood samples from 16 different fish and seafood species (purchased at supermarkets and fish market in the province of Jaen -Spain- during the 2013 and 2014 years) resist to multiple antibiotics, biocides, preservatives and metals through co-selection mechanism (Romero et al., 2017). Nowadays, multi-drug resistant bacteria (particularly those producing extended-spectrum β-lactamases) have become a major public health concern. Romero et al, 2017 determined a 75.86% of the 87 isolates studied were resistant to at least one antibiotic or one biocide, and 6.90% were multiply resistant to at least three biocides and at least three antibiotics. The correlations between the different antimicrobials tested for the 87 bacterial isolates are shown in Fig. 1.11.

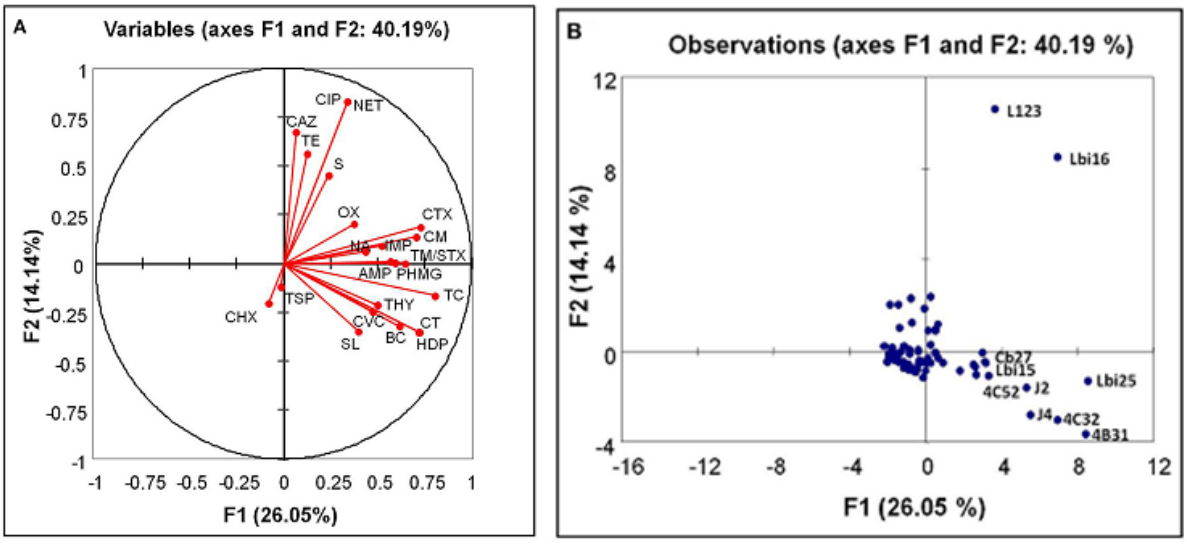

Figure 1.11: Biplot for biocide tolerance and antimicrobial resistance (scores) in the 87 bacterial isolates (variables) from seafoods. Antimicrobials (A), red dots, and isolates (B), blue dots are indicated. In (B), the letters indicate the bacterial isolates with an outstanding high number of antimicrobial resistance traits. BC, benzalkonium chloride; CT, cetrimide; HDP, hexadecylpyridinium chloride; TC, triclosan; CF, hexachlorophene; PHMG, poly-(hexamethylen guanidinium) hydrochloride; OX, P3 oxonia; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; CM, chloramphenicol; S, streptomycin; TE, tetracycline; NA, nalidixic acid; TM/STX, trimethoprim-sulfamethoxazole; CVC, carvacrol; THY, thymol; SL, sodium lactate; TSP, trisodium phosphate (Romero et al, 2017).

A sub-set of 30 isolates, selected according to antimicrobial resistance profile and food type, were identified by 16S rDNA sequencing; then, the genetic determinants for biocide and metal tolerance and antibiotic resistance were investigated. The selected isolates were identified as *Pseudomonas* (63.33%), *Acinetobacter* (13.33%), *Aeromonas* (13.33%), *Shewanella*, *Proteus* and *Listeria* (one isolate each) (Romero et al, 2017). These results suggest that exposure to metals could contribute the co-selection for antibiotic resistance as well as the potential of bacteria present on seafoods to be involved in the transmission of antimicrobial resistance genes. Similar evidences are reported regarding the isolation of *Vibrio parahaemolyticus*, resistant to antibiotics and heavy metals, from shrimps going from Shanghai fish market (He et al., 2016). Finally, potential human pathogens *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* associated with floating microplastics (polyethylene, polypropylene and polystyrene) was reported from North and Baltic sea (Kirstein et al., 2016) while, microplastic-associated bacterial assemblages were analysed through high-through put sequencing in the intertidal zone of the Yangtze estuary. The presence of bacterial communities (e.g. Rhodobacterales, Sphingomonadales and Rhizobiales), responsible for pathogenesis in human, fish and corals has been evidenced (Jiang et al., 2018).

Hence, microplastics in marine environment behaves as a potential vector for spread of pathogenic bacterium (as the *Aeromonas salmonicida*) through aquatic organisms. Its presence is relevant since the *Aeromonas salmonicida* causes human infection after consumption of infected fish (Virsek et al., 2017). Recently, Yang et al, 2019 have estimated the abundance of ARGs (in the 7.07×10^{-4}-1.21×10^{-2} range) and MRGs (5.51×10^{-3}– 4.82×10^{-2} copies per 16S rRNA) in microbial communities found on plastic particles going from the North Pacific Gyre. In Fig. 1.12 is reported the relative abundance of antibiotic resistance genes in seawater, macroplastics microbiota and microplastics microbiota at different sampling sites. ARGs and MRGs in plastics microbiota are significantly greater than those in seawater microbiota in the North Pacifi Gyre. Multidrug resistance genes and multi-metal

resistance genes are resulted the main classes of genes detected in plastic microbiota, while no significant differences in the abundance or diversity of ARGs and MRGs between macroplastics biota and microplastics biota emerged, indicating that particle size had no effect on resistance genes (Yang et al, 2019). Furthermore, some ARGs and MRGs had a higher incidence of non-random co-occurrence, suggesting that the co-effects of selection for antibiotic or metal resistance are important factors influencing the resistome of the microbiota on the plastic particles. These results indicated that ARGs on plastics in the marine environment were more abundant and more diverse than in only seawater (Yang et al, 2019).

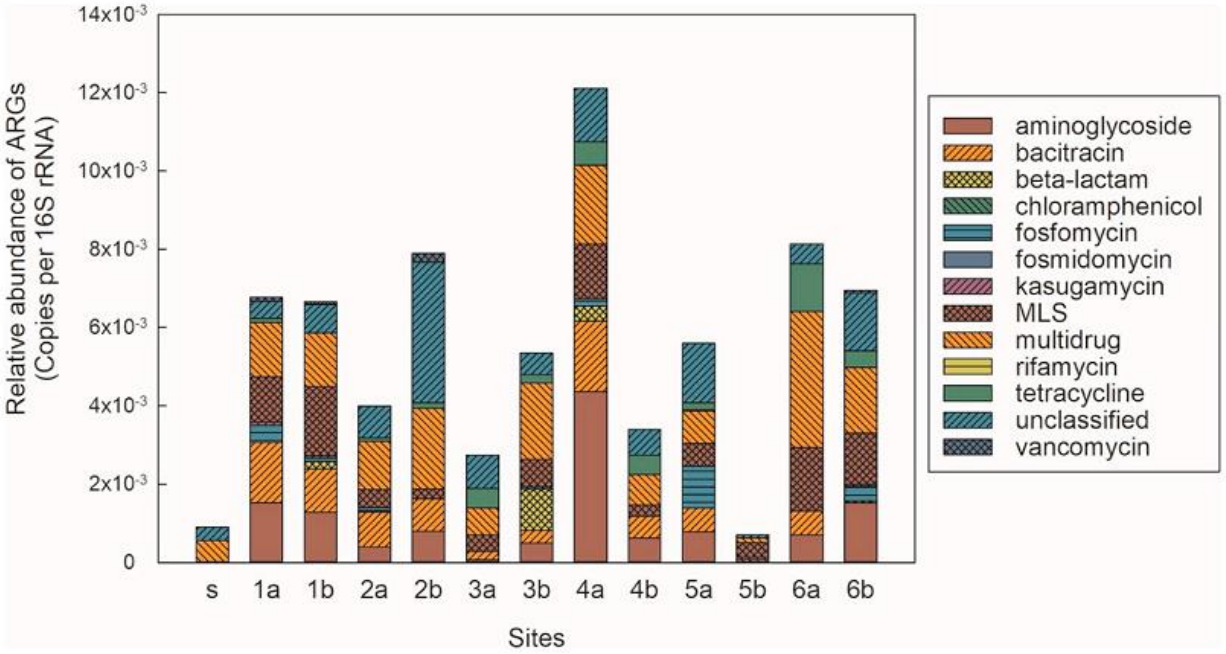

Figure 1.12: The relative abundance of antibiotic resistance genes in seawater, (a) macroplastics microbiota and (b) microplastics microbiota at different sampling sites (MLS: macrolide–lincosamide–streptogramin) (Yang et al 2019).

In this contest, it is important to outline that multiple drug resistant human pathogens enter the marine ecosystem through faecal discharge, directly without treatment. This explains the increase incidence of antibiotic resistant pathogens (*Vibrio* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Shigella* spp., *Enterococcus* spp., *Escherichia coli*, *Clostridium perfringens*, *Staphylococcus* spp. and *Campylobacter* spp.) in the last decade mainly along west coast of India (Poharkar

et al., 2017). In addition, the microplastics absorption of antibiotics (sulfadiazine, ciprofloxacin, amoxicillin, trimethoprim and tetracycline) result in their long-range dispersion, so entering into food chain (Li et al., 2018). Specifically, Sun et al., 2018 demonstrated that sophorolipid can stimulate bacteria/phage mediated antibiotic resistant gene dispersion in microplastic-tetracycline co-contaminated soil (Sun et al., 2018). Another interesting evidence is related to the increased rate of plasmid DNA transfer in phylogenetically-diverse bacteria associated with microplastics as compared to free-living bacteria in aquatic environment (Arias-Andres et al., 2018). Despite these evidences, still today there is a gap in knowledge about co-selection of antibiotic resistance when environment is co-contaminated with mixture of chemicals. It is still difficult to determine which chemicals are involve in co-selection mechanisms, and also to define the indirect effects due to bacterial adhesion and antibiotic resistance.

UniME researchers on this issue wanted to contribute analysing the antibiotic resistance of bacterial strains detected on plastics extracted by *Pagellus erythrinus*, chosen because it represents an example of a "good experimental model" easy to manipulate and available at low cost. *Pagellus erythrinus* is frequent in the seas of Sicily as well as in Atlantic Ocean, and in the North Sea. This fish is much appreciated for its meats that are low in fat, rich in potassium and high biological value proteins and, therefore, its consumption is particularly suitable for feeding children and the elderly.

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Chapter 2

Effects of water temperature, salinity and pH on the immune response of mussel *Mytilus galloprovincialis*

Many environmental factors including temperature, salinity or pH, affect the structure and functioning of aquatic communities, which are simultaneously exposed to the fluctuations of these multiple factors. For the entire aquatic biocoenosis, mussels are considered as suitable sentinel organisms. In fact, hemocytes represent the main mediators of immunity in invertebrates and their morpho-functional properties have been widely investigated as biomarkers in environmental monitoring.

The hemocytes count provides information on the physical and chemical parameters of water in which the mussel live, allowing to assess the relationship between these factors and the susceptibility of organism changes in environmental conditions. An increase of total haemocytes can result from proliferation or movement of cells from tissues into hemolymph, whereas a haemocyte reduction can rely on cell lysis or recruitment from hemolymph to tissues. These two compartments can be considered in a dynamic balance and various factors are involved in bilateral shifts of hemocytes, as well as presence of pathogens, body accumulation of contaminants, nutrients availability, genetic characteristics, or more generally stress-correlated situations.

In this first part of this chapter, a global assessment of environmental stressors in aquatic environment are reported from literature while, in the second section, data collected to investigate immunological and structural characteristics of hemocyte populations in *Mytilus galloprovincialis* (Bivalvia: Mytilidae) coming from two

different Sicilian habitats (Faro Lake and Tyrrhenian sea) are presented and discussed.

2.1 Source and effects of environmental stressors in aquatic environment: a global assessment

Stress is a condition in which intrinsic or extrinsic factors modify the homeostasis of an organism. Exposure to a stressor typically results in a stress response, which is the answer behavioral and physiological used by the organism to compensate for the change induced. Many studies examining the organism stress response are focused on a single environmental stressor (for example salinity or temperature) (Ern et al, 2014). However, globally 97.7% of the aquatic environment is affected by multiple stressors in aquatic environment which acts simultaneously (Segner et al, 2014); (Halpern et al, 2015).

Continuous growth of human populations and use of natural systems (oceans, seas, river and lakes) have enhanced anthropogenic environmental stressors (Halpernet al, 2019). Specifically, antropogenic activities (agriculture, fishing, and so on) together with global climate change due to humanity (e.g. global warming) are determining profound and irreversible changes in aquatic ecosystems (Hewittet al, 2016). In turn, stressors can create synergistic and/or antagonistic interactions (Maheret al, 2019). Synergistic interactions occur when the combined effect of more stressors are greater than the sum of the individual stressors (i.e. additive effect), commonly considered as the no effect model. On the other hand, when the combined effect of multiple stressors leads to a smaller response than that predicted by the null model, the interaction is considered antagonistic and responses to stress happen (Maheret al, 2019).

In addition, the environmental factors can interact and alter the physiological state to bioma in several way, as the bioavailability of toxicants is influenced by changing environmental conditions (pH, salinity, temperature and so on) (Gordon et

al, 2003); (Hooper et al, 2013). Temperature stress in organisms might occur due to climatic extremes or discharges of heated waste water by power plants. In general, the uptake of toxic substances in relation to high temperatures determines alteration of metabolic processes (Rosen et al, 2015). In relation to pH variation, some fishes living in acidic water, normally due to pollution, experience increased number and turnover rate of ionocytes, increased mucus production possibly leading to suffocation, and break down of gill tissue (Laurent et al, 1991). On the other hand, some species have evolved by adapting to live in low pH environments. These fishes regulate body pH by production and excretion of H^+ ions and by manipulating Na^+ and Cl^- influx through Na^+/H^+ and HCO_3^-/Cl exchange mechanisms in cell membranes (Bell, 2013). At last, other fishes have evolved adaptations and withstands to low pH environments, altering Na^+ and Cl^- efflux rates without changing influx rates to maintain ionic homeostasis (Hwang et al, 2007). On the overall, multiple stressors change an organism's sensitivity: for example, the exposure to a toxic substance narrows the organism's tolerance to environmental changes, enhancing negative effects (Sundrum et al, 2015). Generally, stressors-exposed organisms invest more energy in costly detoxification processes at the expense of immunity with potential consequences for parasite resistance (Du et al, 2018); (Dunier, et al, 1993); (Teixeira Alves et al, 2020).

For all that, living organisms (specifically acquatic organisms) are increasingly used as bioindicators of marine pollution (such as plastics residue) since they adsorb and accumulate matter from their habitat. Mussels especially are valuable bioindicators because of their wide geographical distribution, sessile nature, and easy sampling. In Figure 2.1 is shown a general mulluscan hemolymph composition. As a sedentary, filter-feeding animal, the mussel *Mytilus galloprovincialis* is a sentinel species, known as a good bioaccumulator of trace elements.

Hemocytes represent an important constituent of mussel hemolymph and play fundamental roles in the clearance of nitrogenous catabolites, the detoxification of toxic compounds, the storage of nutrients, and immunosurveillance, assuring the

animal survival by coping with foreign, potentially pathogenic microbes entering the organism. In most cases, however, their high concentration may be induced by environmental contamination to capture stressors such as handling, transportation or exposure to air (Jussila et al, 1999); (Renwrantz et al, 2011); (Malham et al, 2002).

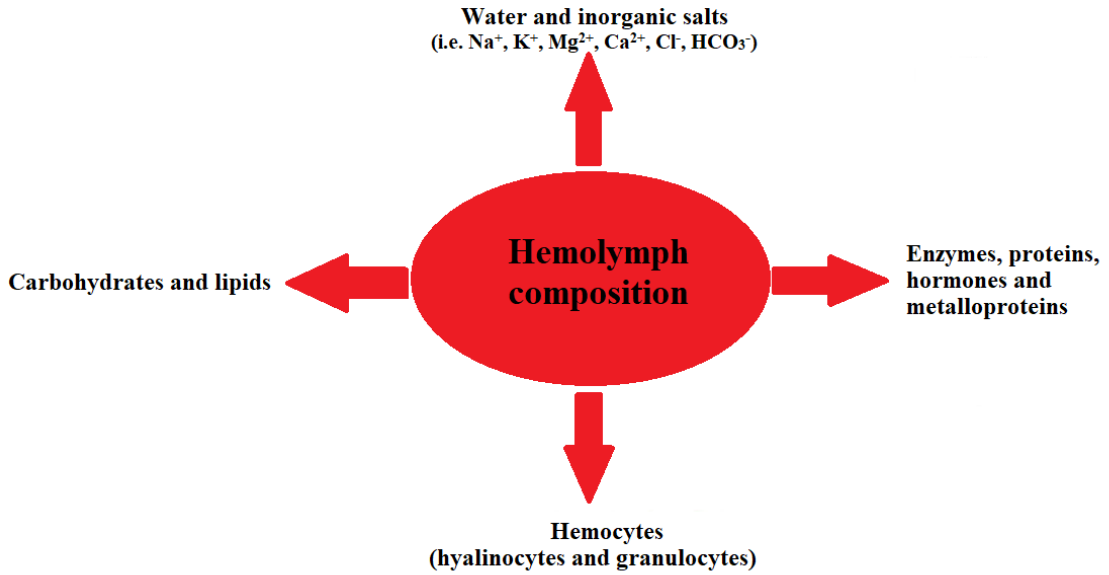


Figure 2.1: General mulluscan hemolymph composition.

The roles hemocytes in bivalves are: wound healing, transport and digestion of nutrients and immune defense (Rebelo et al, 2013). Moreover, bivalves are characterized by having two major types of hemocytes: agranular hemocytes, (without granules) and granular hemocytes, (which contain granules) (Rebelo et al, 2013); (Donaghy et al, 2009). More in details, it is possible identifyed in *Mytilus galloprovincialis*: 1) agranular hemocytes (called hyalinocytes), 2) three types of granular cells (called basophilic granulocytes), 3) acidophilic granulocytes, 4) intermediate cells which contain both acidophilic and basophilic granules (Tame et al, 2015).

2.2 Identification of hemocyte populations in the mussel *Mytilus galloprovincialis* from Faro Lake and Tyrrhenian Sea by flow cytometry and micro-Raman spectroscopy

In this section, immunological and structural characteristics of hemocyte populations in the mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae), going from two different Sicilian habitats (Faro Lake and Tyrrhenian sea), investigated by flow cytometric and micro-Raman spectroscopy analyses was presented and discussed. Samples's morphology and their immune-related activities were investigated by means of flow cytometry while cells structural organization was analyzed using the micro-Raman spectroscopy technique. The changes of specific Raman biomolecular signatures were followed to discriminate hemocytes going from Lake Faro and Tyrrhenian Sea. On the overall, we investigated about the effects of water temperature, salinity and pH on the immune response and also on the lipids and proteins conformational structures of mussels which live in two different habitat. These information could be useful to understand the variations of the hemocyte population in mussels in relation to anthropogenic loads, contributing to the identification of new “biomarkers" (see also Ref. Parrino et al, 2019 (Parrino et al, 2019).

To this aim, it is important to outline that Faro lake is a typical example of a natural confined environment, where eutrophication can trigger stress with negative repercussions on the aquatic ecosystem. The lake is characterized by euryhaline waters, as there is an injection of fresh water from aquifers, which mixes with salt water through the channels communicating with the sea. It represents an example of a meromictic basin and, therefore, the sediments are not involved in a mixing process. The Tyrrhenian Sea, in general in the Strait of Messina, degrades slowly reaching 500 m of depth between the two shores of Sicily and Calabria. The Tyrrhenian waters are strongly influenced by tidal exchange regime, typical of the

Strait of Messina (De Domenico 1987); (Mosetti, 1988); (Fasulo et al, 2008). In the area of sampling, the nature of the seabed is rocky. In the next sub-sections, details about mussels and water sampling procedure adopted as well as information about the characterization procedures are illustrated.

2.2.1 Animals sampling and study area

Three hundred and sixty mussels *Mytilus galloprovincialis* were investigated during the study carried out in November 2017. They were divided into two equal groups on the basis of the site of collection. Sixty *Mytilus galloprovincialis* were caught in Faro Lake (group A), and sixty *Mytilus galloprovincialis* were caught in Tyrrhenian Sea (group B), and all mussels were sampled in triplicate for each site. For both sites of collection (Fig. 2.2), water physico-chemical parameters were measured.

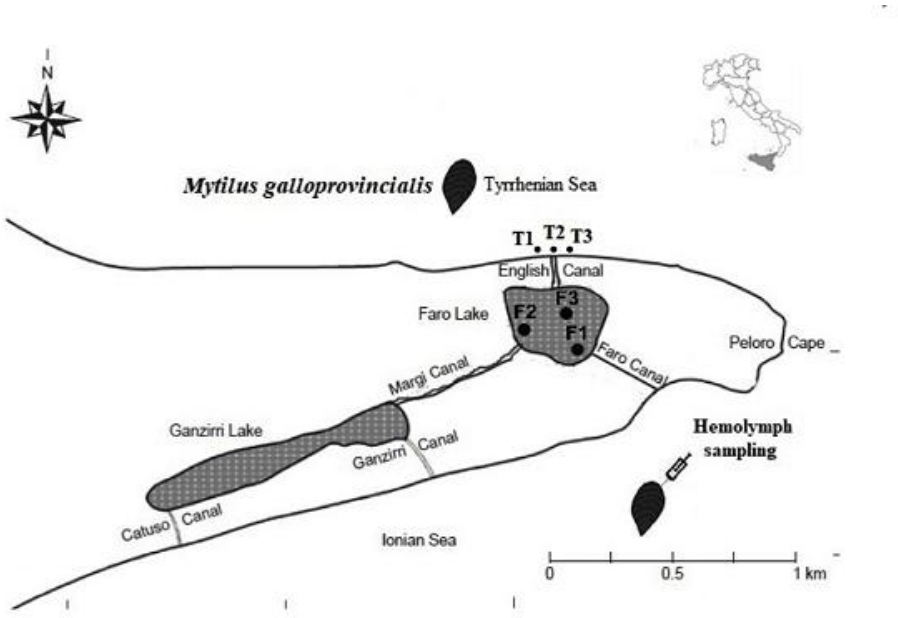


Figure 2.2: Map depicting locations of the sampling sites, in the Tyrrhenian sea (T1, T2 and T3) and Faro Lake (F1, F2 and F3).

Water sampling was carried out in the same date of mussel sampling, in three stations of Faro Lake (F1, F2 and F3) and Tyrrhenian Sea (T1, T2 and T3), in triplicate. The three stations on each location were selected randomly and the

distances among them were about 3 m (see Fig. 2.2). A multiparameter probe Multi 340i/SET – WTW was used to monitor the temperature (Table 2.1).

Table 2.1: Physico-chemical parameters of the Faro Lake and Tyrrhenian Sea during the autumn season.

Parameters	Faro Lake	Tyrrhenian Sea
Temperature (°C)	14.5 ± 0.1	17.8 ± 0.2*
Conducibility (mS/cm)	52.6 ± 0.1	56.5 ± 0.2*
Salinity (PSU)	37°/oo ± 0.1	36°/oo ± 0.2*
Dissolved Oxygen (mg/l)	7.6 ± 0.1	5.6 ± 0.2*
pH	8.13 ± 0.1	7.16 ± 0.3*
Ammonium 10 (mg/l)	0.2 ± 0.1	0.3 ± 0.2*
Free chlorine (mg/l)	0.07 ± 0.1	0.04 ± 0.2*
Total chlorine (mg/l)	0.18 ± 0.1	0.08 ± 0.3*
Total phosphate (mg/l)	1.6 ± 0.1	1.2 ± 0.2*
Orthophosphate (mg/l)	0.4 ± 0.1	0.3 ± 0.3*

∗ Multi 340i/SET – WTW (*$P < 0.05$).

2.2.2 Water sampling and analysis

A portable Multi 340i/SET (WTW Wissenschaftlich, Weilheim, Germany) was used to estimate temperature, pH, conductivity and dissolved oxygen (DO), directly in the sampling sites. Hence, surface water samples were collected in polyethylene bottles and transferred, under refrigeration at temperature of 4 °C, to the laboratory. The physico-chemical properties of the water samples were analyzed following standard methods (Apha, 1995). Especially, nutrients (e.g. ammonia, phosphate, ortophosphate, nitrites) of samples, previously filtered through a 0.45- μm Millipore membrane filter paper, were estimated following the standard APHA (1995) colorimetric approach and using a Filterphotometer (PF-11 MN, Macherey-Nagel GmbH and Co. – D ren, Germany) spectrometer

2.2.3 Collection of hemolymph and total hemocyte counts

Hemolymph was withdrawn from the posterior adductor muscle of each mussel using a 5 mL syringe and a 25-gauge needle. All sample were stored at temperature of 4 °C for 1 day before the analysis. The estimated number of circulating hemocytes or total hemocyte counts (THC) is (J: 18.5±3.1°–106 cells mL^{-1}), and then decreased drastically in sexually mature animals (M: 11.8±1.1°–106 cells mL^{-1}). After spawning, scallop THC returned to standard levels (S: 25.8±2.8°–106 cells mL^{-1})

(Table 2.2). No significant differences were observed between animals. Flow cytometric analyses were performed as previously described (Ashton-Alcox et al, 1998) within 5 h of drawing on whole hemocyte samples. Hemolymph (2–10 mL) was transferred to a plastic tube (whose volume is 2.0 or 15 mL) and kept on ice for less than 5 min until further use. So, the samples were collected in EDTA tubes and also by a pDC instrument to acquire cell membrane patches. All the so prepared samples were analyzed by a multispectral flow cytometer (ImageStreamx (Amnis, Seattle, WA), combining standard microscopy with flow cytometry. This technique allows to analyse up to 100 cells/s, acquiring simultaneously six images for each cell, including bright field, scatter, and multiple fluorescent images. To this scope, the integrated software INSPIRE was used to run the ImageStreamx. For each experiment, cells (pDCs, LCL, and mDCs) were stained with respective markers or PHK-26 and suspended in 50 μl buffer (cold PBS with 1% FCS and 0.05% sodium azide) in 0.6-ml microcentrifuge tubes. Samples were acquired in the following order: unlabelled, single-color fluorescence controls, and finally, the experimental samples. Samples were always left on ice. At least 10,000 cells/experimental sample and 2000 cells/single-color control were acquired for each sample. After each sample was injected into the flow cell and before the acquisition, we have waited for the formation of a single core stream. Then, the acquired images were analyzed using the IDEAS image-analysis software (Amnis). Two hemocyte populations were found in the hemolymph of *Mytilus galloprovincialis*: hyaline hemocytes (HH) and granular hemocytes (GH). The GH are spherical to fusiform cells (6–12 μm), containing abundant cytoplasmic granules (≤ 1 μm) that appeared eosinophilic or basophilic on iemsa staining. Their nucleus (3-6 μm) was characteristically polymorphic, assuming a spherical or bi-to multilobulated shape (Figs. 2.5 and 2.6).

2.2.4 Flow cytometry and micro-Raman spectroscopy

When a monochromatic laser light interacts with a sample, most of the photons are scattered: 1) without any change in energy (the so called elastic or Rayleigh

scattering) which when the electrons in a molecule oscillate in resonance with the applied electric field of the incident light; 2) undergoing a change in energy (the so called inelastic scattering-i.e. Raman shift). The change of the incident photon energy indicates molecular polarizability (dipole moment induced by electric field) changes. A plot of the intensity of the inelastically scattered light as a function of the energy change is called Raman spectrum which is a distinct chemical fi ngerprint for each investigate molecule/material. For several years, the evolution in terms of Raman mapping has allowed to generate images based on the sample's Raman spectra, so showing the distribution of individual chemical components, polymorphs and phases, and variation in crystallinity (Bumbrah, et al, 2016). Today they are available on the market either dispersive Raman spectrophotometer equipped with prisms or gratings and non-dispersive Raman spectrophotometer which uses a Michelson interferometer placed into a Fourier Transform Raman spectrophotometer (Lewandowska 2010).

Figure 2.3: Raman setup instrument.

In our case, to carry out micro-Raman measurements, the 20–25 μL hemolymph aliquot was deposited on a CaF_2 slide and left by air-drying in sterile

conditions for one day. Then, each slide was directly observed and analyzed. All tests were performed in duplicate. In Fig. 2.3 is shown a photo of the microRaman setup adopted. Raman spectra were acquired using the 532 nm excitation wavelength coming from a solid state semiconductor laser. The optics of an Olympus BX40 confocal microscope allow to focalize the 1-mW laser beam onto an area of about 2 μm^2 on the sample surface. The elastically scattered radiation was rejected by an edge filter while the backscattered radiation was collected by the same optics of the microscope and dispersed by a monochromator.

This latter is equipped with a 600 line/mm grating which allows investigation in the 200–2000 cm^{-1} spectral range, with a spectral resolution of about 2.0 cm^{-1}. Finally, a Peltier-cooled CCD sensor was used to record the spectra, usually averaged for a period of 10 s. We outline that, after the acquisition, no cell showed evidence of damage in the proximity of irradiated area. Further, the random collecting of Raman spectra in arbitrary cell location preserve data reproducibility of tested samples.

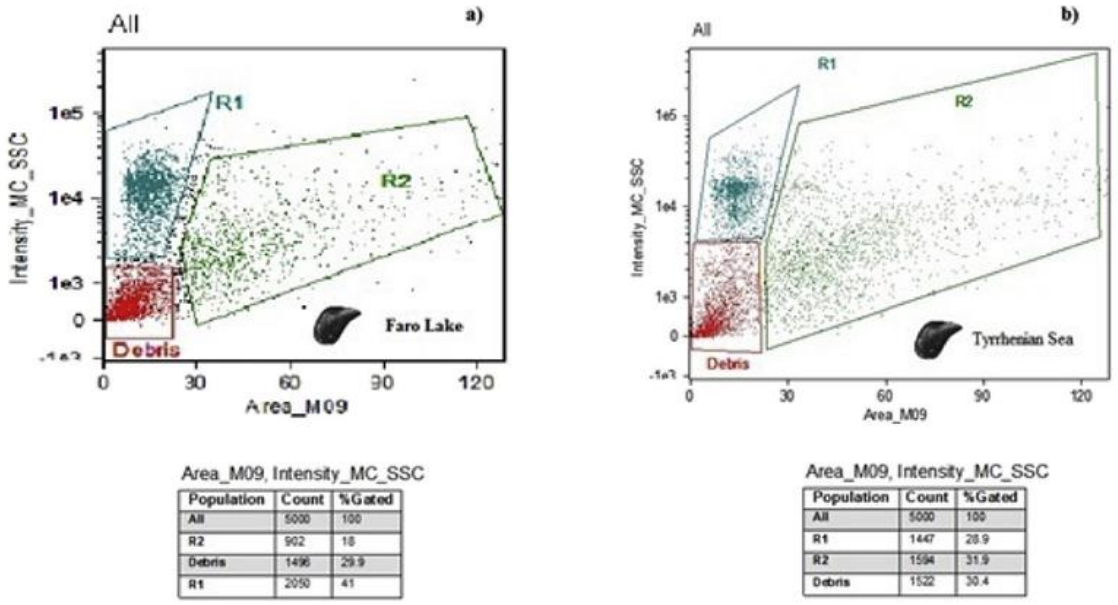
2.2.5 Statistical analysis

Data obtained for hemocyte populations were tested for normality using Kolmogorov-Smirnov test. Unpaired t-test was used to determine signifi cant differences in chemical and physical parameters of two sampling sites and halinocytes (R1) and granulocytes (R2) measured in group A and group B. $P < 0.05$ was considered statistically signifi cant for all analysis. Data were analyzed at 95% confi dence level and all calculations were carried out. Data were analyzed using statistical software prism v. 5.00 (Graphpad Software Ldt., USA, 2003).

2.2.6 Immunological and structural characterization

The physico-chemical parameters and the statistical results of hemocyte sub-populations from *Mytilus galloprovincialis* (Bivalvia: Mytilidae) captured in two different habitats, Faro Lake and Tyrrhenian Sea (Sicily, Italy) are shown in Table 2.1. As shown in Fig. 2.4, different hemocyte populations (R1: halinocytes and R2: granulocytes) with cells population count in mussel *Mytilus galloprovincialis* from

Lake Faro (a) and Tyrrhenian Sea (b) - (Sicily, Italy) have been evidenced by flow cytometric analysis. Significant differences were found in halinocytes (R1) and granulocytes (R2) between the two groups of *Mytilus galloprovincialis* (Table 2.2). Furthermore, unpaired T-test showed that halinocytes (R1) values were significantly higher ($P < 0.001$) in group A with respect to group B. An opposite behaviour is shown by granulocytes (R2) (Ballarin et al, 2014); (Ladha r-Chaabouni et al, 2016).



Area_M09, Intensity_MC_SSC

Population	Count	%Gated
All	5000	100
R2	902	18
Debris	1496	29.9
R1	2050	41

Area_M09, Intensity_MC_SSC

Population	Count	%Gated
All	5000	100
R1	1447	28.9
R2	1594	31.9
Debris	1522	30.4

Figure 2.4: Flow cytometric analysis on hemocyte populations (R1: halinocytes and R2: granulocytes) with cells population count in mussel Mytilus galloprovincialis from Lake Faro (a) and Tyrrhenian Sea.

Table 2.2: Statistical results on hemocyte sub-populations from *Mytilus galloprovincialis* (Bivalvia: Mytilidae) captured in two different habitats, Faro Lake and Tyrrhenian Sea (Sicily, Italy).

Mytilus galloprovincialis (n = 120)	Hemocyte	Mean ± SD	95% C·I	25th-75th P
Faro Lake	Halinocytes (R1)	2055 ± 138[a]	1990 –2119	2023 –2090
(n = 60)	Granulocytes (R2)	909 ± 47 [a]	887 –931	890 –930
	Total (R1+R2)	2964		
Tyrrhenian Sea	Halinocytes (R1)	1453 ± 69[b]	1421 –1485	1400 –1500
(n = 60)	Granulocytes (R2)	1600 ± 125[b]	1542 –1658	1500 –1675
	Total (R1+R2)	3053		

C.I. = Confidence interval of mean; P = percentile.

The mean values denoted with different letters within the same hemocyte subpopulation are statistically significant ($P < 0.05$).

In Fig. 2.5 are shown some optical microscopic pictures (a-f, g-h) of the Tyrrhenian Sea and Faro Lake haemocytes samples and the corresponding representative Raman spectra (Fig. 2.5i). Fig. 2.5b –c, g show that the spherical cells are very different in size (1–3 μm in size). In particular, the Faro Lake sample consists of few nanoparticles smaller than 5 μm in diameter. Some of them are isolated while others are agglomerated or overlapped (Fig. 2.5 d –f, h). Moreover, as can be seen from Fig. 2.5i, the Raman spectrum of Faro Lake mussels look very different from that of Tyrrhenian Sea, reflecting different local structural arrangements. On the entire Tyrrhenian Sea cells, there is no remarkable evidence of the presence of the Amide II (α-helix) Raman scattering feature at 1458 cm^{-1} while the contribution at 1074 cm^{-1}, due to the lipid deformation, dominates. In details, Table 2.3 reports the main identified spectral bands, correlated to the presence of specific biomolecules, here used as markers for nucleic acids, lipids and proteins.

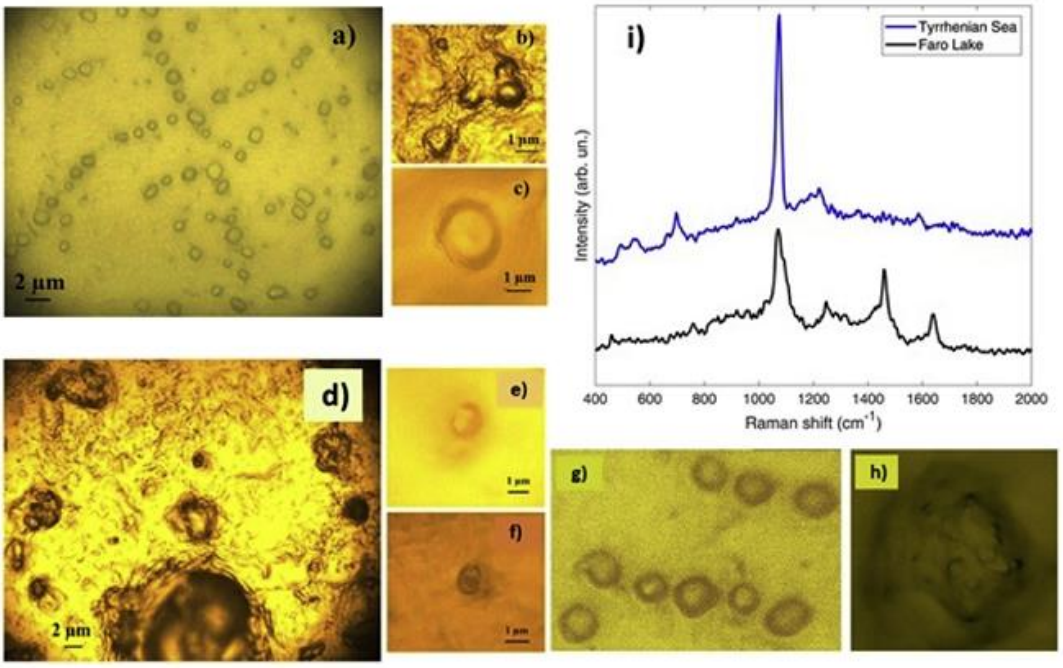


Figure 2.5: Optical microscope images of the Tyrrhenian sea (a, b, c, g) and Faro Lake (d, e, f, h) samples and the corresponding representative Raman spectra (i).

Specifically, the contributions in the 650–1100 cm^{-1} region are attributed to DNA, triglycerides, glucose and nucleic acids, while those in the 1200–1800 cm^{-1} region

are mainly ascribed to amides and lipids and partly to nucleic acids (i.e. adenine, guanine) (Fazio et al, 2018); (Franco et al, 2017); (Lentini et al, 2016); (Fazio et al, 2016) (Movasaghi et al, 2007). Micro-Raman spectroscopy provided an excellent tool, in giving local structural information by selectively probing a microscopic scattering volume. Hence for a more in-depth analysis, we analyse the micro-Raman maps obtained by the integration of Amide II and lipid characteristic Raman bands.

Table 2.3: Main Raman marker bands and their assignments (Fazio et al, 2018) (Movasaghi et al, 2007).

Raman band (cm^{-1})	Vibrational modes	Assignments
480 –965	C-C twisting, δ (=CH) wagging	Protein, Lipid/Protein
990 –995	C-O, C-C stretching	RNA (Ribose), benzene ring
1070	ν (C –C) or ν (C –O)	Lipids, Triglycerides, glucose
1074	ν (C –C) or ν (C –O)	Lipid (Phospholipids)
1090 –1100	PO_2^- and O –P –O backbone stretching	DNA (Nucleic acids)
1140	ν (C –C)	Lipids
1180 –1330	Amide III (β-sheet and random coil)	Protein (secondary structure)
1458	Amide II (α-helix)	Protein nucleic acid modes
1462	$\delta(CH_2)$ or CH_2/CH_3 wagging	Deformation of lipids, disaccharides
1490	C-N stretching, C-H bending	DNA (Guanine, Adenine), amino radical cations
1640	Amide I band	Protein band

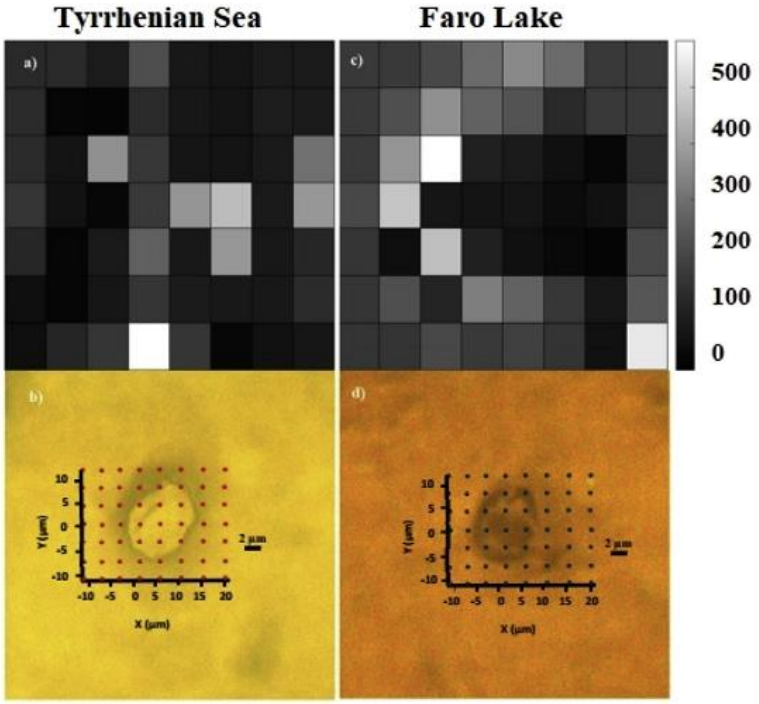


Figure 2.6: Optical microscopy pictures and micro-Raman false color map (20 μm ×30 μm) obtained following the ratio of the (Amide II/Lipid) integrated area of the Tyrrhenian Sea (a,b) and Faro Lake (c,d) haemocytes samples.

In Fig. 2.6 is report the maps obtained by the ratio between the Amide II and lipid integrated area of the Tyrrhenian Sea and Faro Lake haemocytes samples. The obtained values are represented on a 255 Gy scale (Fig. 2.6 a, c). Brighter spots correspond to higher integrated area values. As can be seen from the images, in Tyrrhenian Sea mussel the contribution of the lipids dominates on the full cell while those of Amide II is nearly zero. On the contrary, the almost uniform intensity distribution of the Amide II/lipid ratio indicates that Amide II and lipid are homogeneously distributed inside the Faro lake cell even if, as expected, proteins features are localized mainly in the nucleus region while lipid ones are in the cytoplasmatic region (Fazio et al, 2018); (Movasaghi et al, 2007).

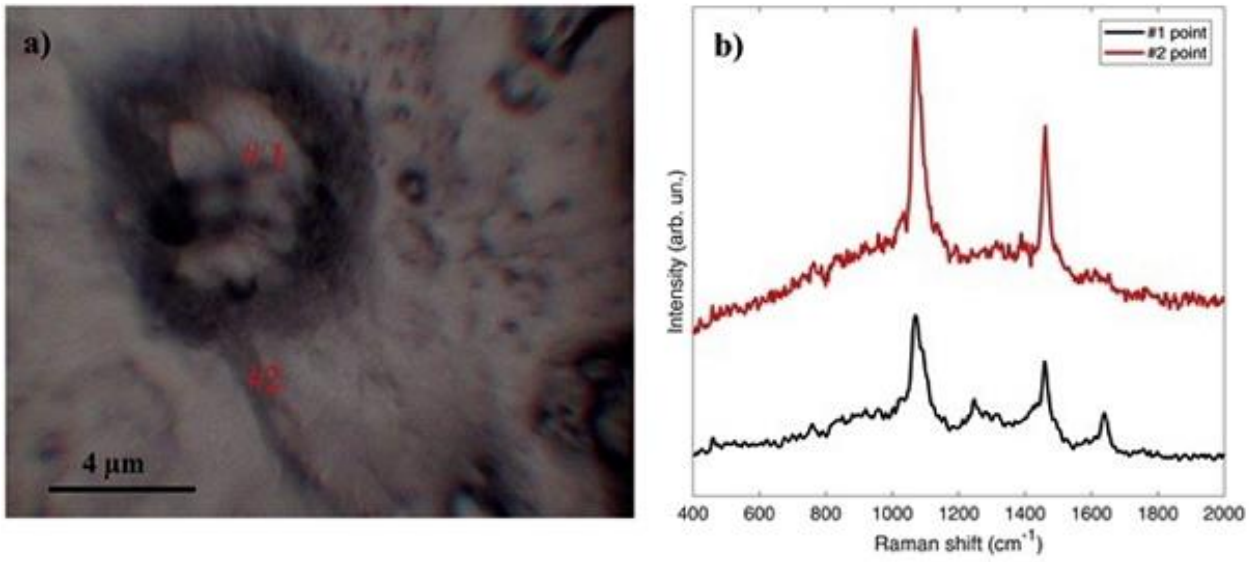


Figure 2.7: Faro Lake optical microscope image characterized by a protrusion, called "pseudopodia" (a), and Raman spectra in two different cells points.

Furthermore, we have observed that few Faro lake cells exhibiting an irregularly-shape, presumably associated to a spontaneous apoptotic process (Fig. 2.7). This modification of status is correlated with the projection of protrusions called "pseudopodia" (point #2 in Fig. 2.7 a). We outline that these morphological features have been observed only in the Faro Lake sample.

Mussels are considered as suitable sentinel organisms for the entire aquatic biocoenosis. Hemocytes represent the main mediators of immunity in invertebrates

and their morpho-functional properties have been widely investigated as biomarkers in environmental monitoring (Perez et al, 2011). The hemocytes count provides information on the physical and chemical parameters of water in which the mussel live, allowing to assess the relationship between these factors and the susceptibility of organism changes in environmental conditions. An increase of total haemocytes can result from proliferation or movement of cells from tissues into hemolymph, whereas a haemocyte reduction can rely on cell lysis or recruitment from hemolymph to tissues (Pipe et al, 1995). These two compartments can be considered in a dynamic balance and various factors are involved in bilateral shifts of hemocytes, as well as presence of pathogens, body accumulation of contaminants, nutrients availability, genetic characteristics, or more generally stress-correlated situations (Renwrantz et al, 2011); (Comesaña et al, 2012); (Allam et al, 2000); (Amachree et al, 2013).

In the present study, the recorded values of pH, ammonium 10, free chlorine, total chlorine, floride 2, total phosphate, potassium, although different between the two habitats, are within the permitted values (*European Commission, EEA, Primary Environmental Indicators, 2001)* and not visible between the two sites monitored. However, an increase of total haemocytes (3053 cells) with a significant increase of granulocytes was observed in *Mytilus galloprovincialis* caught in Tyrrhenian sea with respect to Faro lake. The Tyrrhenian Sea water temperature of 17.8 °C was higher than those of 15.5 °C in Faro lake. Thus, it seems that total hemocytes value could be directly influenced by water temperature, as already observed in clams and mussels (Soudant et al 2011).

However, there are controversial data in the literature. Indeed, in marine bivalves it was observed that the number of circulating hemocytes increases upon reaching the temperature up to 28–32 °C (Monari et al, 2007) while other research reported that hyperthermia causes a significant decrease of total hemocytes in a temperature and time-dependent manner (Yao et al, 2013). At this point, we analyzed the effects of dissolved oxygen (DO) and salinity on total hemocytes. As shown in Table 2.1, DO

and salinity values are lower in Tyrrhenian Sea with respect to Faro Lake. Dissolved oxygen and salinity showed an inverse relationship with the total hemocytes count, probably depending on osmotic adjustment of the hemolymph compartment (Fisher 1986). Moreover, the amount of circulating hemocytes increases under hypoxic conditions. In this case, the low DO on the stimulation of heart rate acts as compensatory mechanism to maintain the oxygen tissue perfusion (Sussarellu et al, 2012).

We have observed that total hemocytes count varies when exposed to specific environmental stressors such as temperature and salinity (Matozzo et al, 2003). Generally, hydrogen peroxide can produce an excess of hydroxyl radical via a Fenton mediated mechanism. This may induce acute oxidative injury if not scavenged or removed effectively by antioxidants. For example, acute oxidative stress induced changes in nicotinamide adenine dinucleotides in mouse skeletal muscles. There are several biochemical assay methods to estimate oxidative injury in cells. However, they do not provide information on the biochemical changes as the cells get damaged progressively under oxidative stress. Otherwise, micro-Raman spectroscopy combined to optical microscopy offers the possibility to monitor, point by point, the chemical composition of live cells undergoing oxidative stress (Zoladeket A. al, 2010); (Zoladek A.B. et al, 2010).

In our case, we have observed that Faro Lake Raman peaks related to nucleic acids, lipids and proteins in the 700–1800 cm^{-1} showed several changes with respect to Tyrrhenian Sea (Table 2.3), indicating the breakdown of the phosphodiester backbone, a change of the protein conformational structure as well as of the nuclear bases (Chaabouni et al, 2016). The hemocyte degradation against the not-self (i.e. the real activation of the hemocyte) occurs through the production of oxygen free radicals (ROS) (Panara et al, 1996); (Mosca et al 2013). In some circumstances of ROS overproduction, the protection afforded by antioxidant defence mechanisms might be overcome, thereby leading to oxidative damage to tissue macromolecules including DNA, proteins and lipids. Xenobiotic-enhanced oxyradical generation can

be a possible mechanism of pollution toxicity (Livingstone et al, 1990). In addition, the observed spectral variations of the 1074 cm^{-1} and 1458 cm^{-1} peaks, ascribed to the lipid and Amide II (α-helix) contributions, support this hypothesis. In addition, the whole structure external to the hemocyte of the Faro Lake mussel was altered compared to those of Tyrrhenian Sea, not only for the extroflexion of the pseudopodia (point #2 in Fig. 2.7a) for which the disappearance of the Raman feature at about 1610 cm^{-1} was observed but also in terms of an increase in protein intake and specifically in actin, indicating a cytoskeleton reorganization (red spectrum in Fig. 2.7b).

On the overall, the observed biomolecular changes and the statistically significant increase in halinocytes (R1) in mussels sampled in Faro Lake with respect to those from the Tyrrhenian sea, are closely linked to the disruption of immune parameters, in turn, induced by the different values of some qualitative water parameters (temperature, salinity, dissolved oxygen, pH, ammonium 10, free chlorine, total chlorine, total phosphate, orthophosphate) in the two habitat. All these bio-chemical changes could be induced by the "glucose content" in the cells, probably used by lysosomes to activate enzymatic species involved in this process (Chaabouni et al, 2016). An intense metabolic cellular activity occurred in the hemolymph of Faro lake as indicated by the modifications of the C=C stretching vibrations of the Amide I α-helix structure and of the Amide III components linked to tubulin, heterodimeric globular protein containing subunits α and β and cytoskeleton element (Diptima et al, 2013).

Summarizing, the combined effects of the environmental stressors have a great impact on bivalves, increasing the susceptibility to pathogens and also favouring natural mortality events. Finally, the aforementioned linkage between hemocyte counts and environmental parameters in *Mytilus galloprovincialis* can be considered as a useful tool to evaluate the effects of environmental stressors on the immune response, revealing in advance the development of potential critical situations for mussel survival. Nevertheless, further studies that compare the total

hemocytes population of *Mytilus galloprovincialis*, collected in different sites, with several levels of pollution are necessary to clarify and fortify the knowledge of the linkage between hemocytes count population and habitat, to identify useful relationships on physiological adaptations to different environmental conditions.

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Chapter 3

Sampling and identification of microplastics ingested by fishes from the Central Mediterranean sea

The *United Nations Environment Programme* (UNEP) has identified plastic pollution as a critical problem, the scale and degree of this environmental issue is comparable to that of climate change. There is currently much public and political debate surrounding the issue of microplastics as additives to household and industrial products, and the methods by which impacts of said microplastics on the environment are to be measured. Determining the degree to which biota consume microplastics is essential to determine and monitor 'good environmental status' for plastic pollution.

It is imperative to be able to accurately isolate, identify and enumerate microplastic debris consumed by or entangled with biota.

Here, methods employed in the extraction, identification and quantification of microplastic particles ingested by biota are described, considering the effectiveness and limitations of a range of field sampling, laboratory exposure, extraction, analytical techniques, and mainly steps for mitigating contamination. In the second part of the chapter, an overview of conventional analytical techniques used to discriminate the nature of microplastics in terms of morphology and composition is reported.

3.1 Conventional methods for isolating microplastic in fishes

Several techniques, whose suitability can be determined by assessing the degradation impact on microplastics and their recoverability from dosed environmental samples, have been developed to extract microplastics ingested by aquatic organisms. The common methods for extracting microplastics from aquatic organism are:

1. dissection
2. depuration
3. digestion (different approaches)

3.1.1 ***Dissection:***

Dissection procedures are carried out to study target specific tissues, mostly digestive tract (including the stomach and intestine), also to quantify the amount of microplastics by invertebrates and vertebrates including pelagic and demersal species (Dey et al, 2021). In brief, the dissection consists in cutting the body section of interest to extract the tissue. The main simple steps are: 1) cut the skin from the anal fin along the belly of the fish to the operculum; 2) removal of the operculum and the pectoral fin, including the pectoral girdle; 3) cut the skin, beginning from above, along the side of the fish, down to the anal fin; 4) identify and carefully remove the gastrointestinal tract containing the microplastics. This standard method allows to collect tissues that can be used to assess whether plastic-based materials are the cause of a fish contamination.

3.1.2 ***Depuration:***

This procedure allows to recover any externally adhered plastics prior to their specific treatment. Generally, this procedure is achieved by washing the analyzed organism with water, saline water or using forceps (Desforges et al, 2015). A depuration step can be used to eliminate transient microplastics present in the

intestinal tract. Depuration is facilitated containing the specimens under study in media microplastics-free (e.g. freshwater, seawater, sediment), with or without food, and leaving sufficient time for complete gut evacuation (Van Cauwenberghe et al, 2014); (Watts et al, 2015).

3.1.3 ***Digestion:***

It is a process used to remove organic material that would interfere with the plastics characterization analyses in the environmental organisms samples which contain dense amounts of naturally occurring organic materials, such as zooplankton, phytoplankton, remnants of aquatic organisms or biofims (e.g. brown algae or bacterial fim) attached to the surface of the plastic particles (Su et al, 2018); (Felsing et al, 2018). Digestion is conducted using strong oxidizing agents that anyway can degraded the synthetic polymers until a total damage when plastics previously exposed abrasion and photodegradation are treated. In fact, these polymers are structurally more fragile and less resistant to chemicals utilized for digestion treatments (Pfeiffer et al, 2020). A multi-step treatment is often adopted to ensure complete removal of biofims and organic materials attached on plastics surface, which ultimately limit a good visual observation and/or spectral identification of plastics. Four major classes of digesting agents have been employed to eliminate organic materials:

a) Chemical digestion with simple and/or mixtures of strong acids

Chemical digestion with strong acids, such as nitric acid (HNO_3), hydrochloric acid (HCl) and perchloric acid ($HClO_4$) is a useful treatment to remove biological residues, thus facilitating isolation of plastic particles (Catarino et al, 2018); (Naidoo et al, 2019). In table 3.1 some detail about chemical digestion using acids are reported.

Some plastics (e.g. nylon, PET) have low resistance to acids and may be degraded. Nitric acid (HNO_3) is a strong oxidizing mineral acid, capable of molecular cleavage and rapid dissolution of biogenic material. When tested against

hydrochloric acid (HCl), hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH), HNO_3 resulted in the highest digestion efficacies, with >98% weight loss of biological tissue. Researchers have found that polymeric particles, including polyethylene (PE) and polystyrene (PS), dissolved following overnight exposure and 30 minutes boiling with 22.5 M HNO_3. Otherwise, polyamide (PA, Nylon), polyester (PET) and polycarbonate have low resistance to acids, even at low concentrations.

Table 3.1: Summary of acid digestion methods of plastics.

Acid digestion	Plastic degradation	Advantages	Disadvantages
$HClO_4$	Unknown	➢ Easy method ➢ Overnight	➢ Recovery rates showed a loss of product after treatment ➢ Expensive ➢ Ability to use FTIR/Raman spectroscopy following separation unknown ➢ Applicability to all sample types unknown ➢ High chemical hazards – corrosive acid ➢ Often needs to be heated/boiled to digest biologically rich samples, which could result in loss of plastics
HCl	Alteration to PET and PVC following treatment	➢ Easy method ➢ 12 h	➢ Recovery rates showed a weight change after treatment ➢ Expensive ➢ Ability to use FTIR/Raman spectroscopy following separation unknown ➢ Applicability to all sample types unknown
HNO_3	Alteration to PS and PA following treatment	➢ Easy method ➢ Overnight ➢ Inexpensive	➢ High chemical hazards – corrosive acid ➢ Often needs to be heated/boiled to digest biologically rich samples, which could result in loss of plastics ➢ Ability to use FTIR/Raman spectroscopy following separation unknown ➢ Applicability to all sample types unknown

HCl seems to be the least effective in treating large quantities of biological material (Maes et al, 2017). Karami et al. (Karami et al, 2017b) found that HCl (37%) at 25°C induces a digestion efficiency > 95%, although it caused PET to melt. On the overall, it's clear that acid digestion should be used with caution, as it may lead to underestimation of plastics in organisms samples.

b) <u>Bases or alkaline digestion</u>

Alkalis method, involving the use of strong bases (sodium hydroxide (NaOH) or potassium hydroxide (KOH), can be used to remove biological material by hydrolysing chemical bonds and denaturing (Cole et al, 2014); (Foekema et al,

2013). However, alkaline digestion, being deposited on plastic surfaces, may damage or discolour plastics, not allowing spectroscopic characterization (Qiu et al, 2016); (Wagner et al, 2014). In Table 3.2 are summarized the main advantages/disadvantages using alcaline digestion.

Table 3.2: Summary of alcaline digestion methods of plastics.

Alcaline digestion	Plastic degradation (verifid for)	Advantages	Disadvantages
NaOH	Unknown	➢ Easy method ➢ Low chemical hazards and relatively inexpensive ➢ Ability to use FTIR/Raman spectroscopy following separation	➢ Digestion time of 3 weeks ➢ May be necessary to heat sample, which may cause loss of plastics ➢ Applicability to all sample types unknown
KOH	Loss of PET; yellowing of PA; Degradation of LDPE, CA and PA	➢ Digestion 24h	➢ Recovery rates reported only by weight, not abundance ➢ Applicability to all sample unknown ➢ Known to leave behind reaction residue on plastics, may hinder FTIR if not cleaned ➢ Some chemical hazards

c) <u>Oxidative digestion</u>

Oxidative digestion methods using hydrogen peroxide (H_2O_2) at different concentrations (15–35%), and operating temperatures (from room temperature up to 70°C), and reaction times (from a few hours to a week) are largely used to treat plastic based materials extracted by fish (Nuelle et al, 2014). Like other types of digestion, oxidative digestion is both easy and relatively inexpensive and can be applied to all samples type. In addition the digestion time required is short (< 1 hour) and recovery rates are relatively high (> 85%). However, H_2O_2 oxidation has been shown to negatively impact extraction effiiencies of PS MPs < 100 µm in size from soil and biosolids (Wang et al, 2018). H_2O_2 is more efficient than NaOH and HCl in dissolving organic matter, inducing only colour fading (Nuelle et al, 2014); (Zhao et al, 2017). $FeSO_4$ (Fenton's reagent) can also be used to remove organic matter such as MPs from organic-rich wastewater samples, although it has been shown to work best with H_2O_2 (Tagg et al, 2016). $FeSO_4$ destroys organic components, such as highly chlorinated aromatic compounds or inorganic compounds, that are typically

resistant to H_2O_2, and therefore may prove more effective in removing all organic components from complex environmental substrates (Pignatello et al, 2006). In table 3.3 some detail about chemical digestion using acids are reported.

Table 3.3: Summary of oxidative digestion methods of plastics.

Oxidative digestion	Plastic degradation (verifid for)	Advantages	Disadvantages
$FeSO_4$ (catalyst)	Unknown	➢ Easy method ➢ Relatively inexpensive ➢ Digestion times (< 1 h) ➢ Recovery rates > 85% ➢ Low chemical hazards	➢ Ability to use FTIR/Raman spectroscopy ➢ following separation unknown ➢ Applicability to all sample types unknown ➢ Often needs to be heated/boiled to digest ➢ biologically rich samples, which could result in loss of plastics
H_2O_2	Degradation of PA; colour change of PET	➢ Easy methodology ➢ Relatively inexpensive ➢ Digestion times (< 1 hour) ➢ Recovery rates > 85% ➢ Ability to use FTIR/Raman ➢ spectroscopy following separation ➢ Can be applied to all sample types	

d) Enzymatic digestion

In contrast to chemical digestion techniques, enzymes method used for hydrolyzing proteins and breaking down tissues, ensures no loss, degradation or surface change of plastics. Moreover, this procedure is less hazardous to human health (Cole et al, 2014); (Courtene-Jones et al, 2017).

Table 3.4: Summary of enzimatic digestion methods of plastics.

Enzimatic digestion	Plastic degradation (verifid for)	Advantages	Disadvantages
Proteinase K	Unknown	➢ Digestion time of ≈ 3 h ➢ Low chemical hazards ➢ Ability to use FTIR/Raman following separation	➢ Recovery rates unknown ➢ Applicability to all sample types unknown ➢ Relatively very expensive ➢ Methodology more complex than simple acid digestion ➢ Not common in laboratories
Corolase 7089	PET, HDPE and PA	➢ Fast method ≈ 1 h ➢ Recovery rates of 93% ➢ Ability to apply FTIR/Raman following separation	➢ Need to heat sample to 60°C – may result in loss of plastic ➢ Applicability to all sample types unknown ➢ Not common in laboratories
Trypsin	PET, HDPE, PVC, PP, PS and PA	➢ Digestion time of 30 minutes ➢ Low chemical hazards	➢ Recovery rates unknown ➢ Ability to use FTIR/Raman spectroscopy following separation unknown ➢ Applicability to all sample types unknown ➢ Effect on plastic types unknown ➢ Very expensive ➢ Methodology more complex than simple acid ➢ Not common in laboratories

Enzyme efficiency will vary with the type of organic material present in the sample (see Table 3.4). For example, trypsin can be used to extract microplastics from biological samples, providing a rapid, cost-efficient and effective method of separation, while the use of Corolase 7089 (AB Enzymes GmbH, Darmstadt, Germany) is associated with high recovery rates (93% ± 10%) when separating MPs from soft tissue, such as muscle tissue (Catarino et al, 2018). The best performance is achieved with proteinase K, with the highest recovery rate (97%) and no plastic degradation (Karlsson et al, 2017).

Finally, several other methods of extracting plastics from organic matter have been reported in the literature. For example the use of microwaves and ultrasonication, which are useful in combination with other digestion of sludge using NaOH (Jin et al, 2018). The use of sodium hypochlorite (NaClO; 24.8 g/L) was reported as an efficient method in the digestion of fish stomach contents without affecting plastics, but potentially causing discoloration (Collard et al, 2018). Other studies have also suggested that a combination of methods, for example, acid and alkalin digestion used sequentially (e.g. NaOH and HNO_3), can provide good digestion of biological material and high recovery rates (95%), with few changes of the morphological and chemical characteristics of microplastics (Roch et al, 2017). NaCl filtration followed by H_2O_2 digestion resulted in successful extraction of microplastics from fish guts, with good recovery rates (80–90% dependent on particle size and class) (Avio et al, 2015). Ultimately, the most common technique to preconcentrate or isolate microplastics is the density separation approach. Density separation exploits the buoyancy of plastic particles in solutions with a higherdensity than that of plastics (ρ=0.9–1.6 g cm^{-3}), while the mineral fraction (for instance silica, ρ >2.0 g cm^{-3}) remains at the bottom (Enders et al, 2020). To date, there is no standardized density separation procedure for microplastic extraction from fishes. In principle, the plastics sample is mixed with a density solution, and floating plastic particles are collected after a certain amount of time. However, studies vary greatly in terms of sample amounts, applied density solutions, and the technical setup.

3.2 Procedures adopted for processing our plastics in view of physical and chemical characterizations

As described in the previous paragraph, several methods are used for sampling and microplastic samples preparation (e.g., organic digestion and density separation) which influence the interpretation of results and hamper their comparability. Really, the essential basis for the interpretation of the results of microplastic monitoring is the consistency of a sampling strategy and of pre-treatment of the microplastics to favour their identification both by electronic and spectroscopic techniques.

The procedures adopted for the sampling and microplastics preparation of our samples were the most accredited and widely reported in the literature (Stock et al, 2019); (Lusher et al, 2017). Here, they are described in detail. First, fish samples were processed in a closed room with restricted access. Workspaces and tools were cleaned from any particle according to the protocol proposed by Bessa et al. (Bessa et al, 2018). All clothing worn during laboratory work were of non-polymer nature and no artificial textiles too.

The gastrointestinal tract of *Zeus Faber, Lepidopus caudatus* and *Boops boops* was exposed to air for the minimum time possible and rinsed with pre-filtered deionized water to avoid any contamination of the sample; after that it was cut open longitudinally with a scissors within a Petri dish. The content was gently removed and examined under optical microscope. Furthermore, filter papers in Petri dishes exposed to the laboratory air were used as control blanks during analysis of each sample (Giani et al, 2019). Then, isolated plastics debris were recorded and categorized based on their size, colour and shape (fiber and fragment). The microplastics were categorized in three class sizes (0.5 –1 mm; 1 –2.5 mm; 2.5 –5 mm) according to Marine Strategy Framework Directive (MSFD) protocol (Galgani et al, 2013). In addition, a subset (20%) of fibers were washed in distilled water, centrifuged twice for 30 s to remove the organic residues. All that to carry out the spectroscopic measurements in order to identify their polymer chemical nature.

To this aim, plastics extraced were placed on corning slides and covered with a cover slip to avoid their dispersion.

Wih respect to *Zeus Faber*, *Lepidopus caudatus* and *Boops boops*, a different sampling procedure was applied for the set of *Sardina pilchardus* and *Engraulis encrasicolus*, given the small size of these species. The specimens were gently washed twice using deionized water to remove foreign particles. Subsequently, they were grouped in two pools and subjected to chemical digestion, following the protocol designed by Avio et al. (2015) in Ref. (Avio et al, 2015). Samples were placed in a 250 ml conical glass flask with addition of calculated quantity of 10% KOH solution (minimum ratio 1:5 w/v), the flask was covered with aluminum foil. In order to remove the organic matter, the flasks were placed in an oscillation incubator to be continuously stirred at room temperature for 72 h. Each sample was then put into a graduated glass cylinder and hypersaline NaCl solution (15%) was added in order to obtain a separation of the two phases by density. The supernatant was collected in a glass beaker, and filtered through a glass fiber membrane having 0.7 mm pore size and 47 mm diameter (Whatman GF/F, UK) using a vacuum system (Millipore). The procedure was carried out twice in order to prevent any loss of sample. Neat filters were used as blank, following the same procedure of the samples. All steps were carried out at room temperature. The membranes were placed in sterile Petri glass dishes for subsequent observations under the stereomicroscope in order to isolate plastic debris. The isolated samples were recorded and categorized based on their shape (fibers and fragment), size and colour.

In addition, randomly selected subset (20%) of plastic samples retrieved from *S. pilchardus* and *E. encrasicolus* was used for the chemical/structural characterization of the isolated polymers. The random selection was done considering only a part (20%) of analyzed surfaces (filters). Selected MPs samples were washed in distilled water, centrifuged twice for 30 s to remove the organic residues and then stored in glass vials.

3.3 Plastic polymers characterization

It is well known from literature that studies based on visual identification of microplastics and non-plastic interferences, even with the aid of optical microscopy, are vulnerable to false negatives and false positives (Song et al, 2015); (Shim et al, 2017). In the last few years, sophisticated analytical techniques are adopted to more accurately determine microplastic morphology and composition, including complementary combinations of manual or automated Fourier transform infrared (FTIR) micro-spectroscopy, scanning electron microscopy plus energy-dispersive X-ray spectroscopy (SEM/EDS), or Raman micro-spectroscopy. In Fig. 3.1 is shown a scheme of the main physical and chemical characterization techniques adopted for microplastic identification.

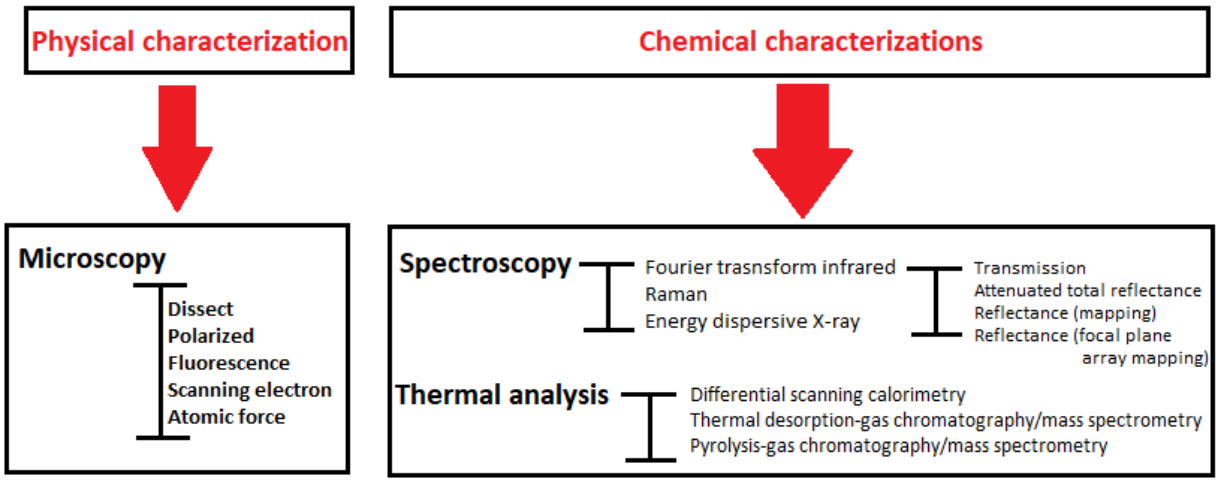

Figure 3.1: Summary of currently used physical and chemical characterization methods for microplastic analysis.

Below, a brief description of the diagnostic techniques widely used for the characterization of microplastics (also adopted in our experiments) is reported, underlining their specific peculiarities.

3.3.1 Visual sorting and size/colour classification by optical microscope

Visual inspection and the counting of microparticles were commonly performed using a conventional optical microscope. This simple and fast approach allows the identification of microplastics with a size within 2–5 mm range (Song et al, 2015). Specifically, a stereo microscope was used to identify microplastics based on physical appearance: samples different from tissues (git) (i.e. more rounded, pitted, fibre-like, coloured or transparent) were identified and counted, and pieces in sizes ranging < 5 mm microplastics and 5 –25 mm mesoplastics (Cole et al, 2011); (Shim et al, 2015). Microplastic visualization and classification in terms of size and macroscopic structure remains an essential step in classifying micro-plastics, and is perfectly acceptable when supported by subsequent polymer analysis of sub-samples, generally carried out by spectroscopic and electronic techniques, described below.

3.3.2 Scanning Electron Microscopy

For physical characterisation of microplastics, optical microscopy can be used as an easy and fast method of pre-screening of the samples. Subsequently, selected samples can be moved to scanning electron microscope (SEM) combined with energy dispersive X-ray spectrometer (EDX) to obtain high-magnified, high-resolution images of the investigated plastics, and also to collect qualitative information about their chemical composition (Gniadek et al, 2019). A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample.

For the characterization of the microplastic analyzed in this thesis, the Zeiss Supra 40 field ion microscope equipped with an Energy Dispersive X-ray (EDX)

probe was used. Images were acquired at an accelerating voltage of 20 kV and at a working distance of 4 mm. SEM apparatus is coupled with an EDX spectrometer to carry out energy dispersive X-ray (EDX) mapping analysis in order to define the species constituting the plastics. In this case, when primary electron beam hits the sample surface, different interactions (among others–X-ray) are generated. So, EDX can provide qualitative information about elements and their spatial distribution within the sample. Particularly, the combined use of electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX) gives detailed information on the elemental composition of microplastics with additional information on the in-organic additives they contain. Ultimately, SEM-EDX aids in differentiating natural materials forms microplastics due to simultaneously collected images and elemental mapping.

3.3.3 Raman and Fourier transform infrared spectroscopies

The most promising and frequently used techniques for microplastics detection and characterisation are the Fourier transform infrared (FTIR) and Raman spectroscopies (Käppler et al, 2016). IR and Raman spectroscopies are considered complementary approaches. Raman spectroscopy depends on a change in polarizability of a molecule, whereas IR spectroscopy depends on a change in the dipole moment. Raman spectroscopy measures relative frequencies at which a sample scatters radiation, unlike IR spectroscopy which measures absolute frequencies at which a sample absorbs radiation. Hence, FTIR spectroscopy is sensitive to hetero-nuclear functional group vibrations and polar bonds, especially OH stretching in water. During FTIR measurements, light is absorbed in different amounts by the investigated sample and at distinct frequencies which correspond to the vibrational frequencies of the bonds in the sample.

Recently, FTIR spectrometer are coupled to an optical microscope that can work both in reflection and in transmission mode. In our work, infrared absorption measurements were carried out in the 3500–500 cm^{-1} range using a Fourier

transform infrared (FTIR) Perkin-Elmer spectrophotometer, working both in Attenuated Total Reflection (ATR) and transmission configuration. In Fig. 3.2 is shown a photo of the instrumentation used.

Figure 3.2: photo of FTIR setup.

FTIR and Raman gives complementary information. In fact, Raman is sensitive to homo-nuclear molecular bonds; for example, it can distinguish between C-C, C=C and C≡C bonds. Collecting the light diffused from a sample, Raman spectroscopy give information regarding vibration modes of the molecules that form the sample. Ultimately, it gives a molecular fingerprint of materials. Raman measurements consists in the illumination of a sample with monochromatic light of frequency w_0 that lead to energetic transitions (vibrational or electronical). After this transition, the system will emit a photon in order to release the energy absorbed. An huge part of this light will have the same frequency w_0 of the incident light (Scattering Rayleigh), but there is a small fraction that will have a frequency *wi* such that $\Delta E = h\ (w_0 - wi)$.

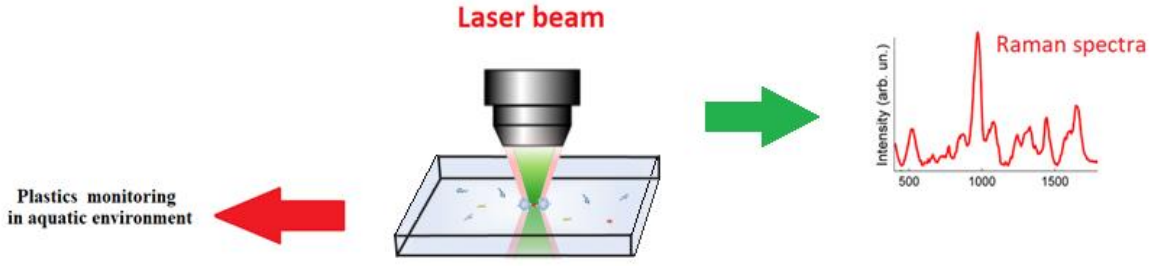

Figure 3.3: Schematic representation laser beam of Raman spectroscopy directed to microplastics extracted by *Pagellus erythrinus.*

In this thesis, Raman spectra of selected plastics were recorded by a Witech Alpha 300 RS Raman spectrometer, exciting with the 532 nm line of a Coherent Compass Sapphire Laser (Coherent). In some cases a near infrared excitation source (785-nm) of a High Power Single Frequency Diode Laser (Toptica Photonics) was also employed in order to eliminate sample fluorescence from polymers, but, at the same time, obtaining sufficiently intense Raman signals to be detected by the Charge Couple Devices (CCD). The 532 nm laser was focused on the sample surface through the 50 X long working distance objective of the microscope, at a low laser power (about 0.5 mW) to avoid laser-induced heating of the specimens. Integration times were varied, depending on the signal-to-noise ratio, between 1 and 10 s, with an accumulation number of 10. For a complete characterization of the samples, epi-fluorescence images were acquired using the optics of an Olympus BX40 microscope coupled with a mercury arc light source and equipped with an ultraviolet excitation filter centered in the blue region (450 nm).

3.3.4 X-ray Photoelectron Spectroscopy

Along with conventional Raman and IR techniques, X-ray Photoelectron Spectroscopy (XPS) technique is increasingly being used. XPS allows to have quantitative information about the chemical composition of the plastics surface. XPS measure the energy distribution of electron that are emitted from atoms and molecules core levels. In particular, when a material is irradiated with X-Ray photoelectron are emitted with an energy equal to the difference between the one of the incident photon $h\nu$ and the ionization energy of the atom E_{ion}: $KE = h\nu - E_{ion}$. Moreover, the binding energy measured in this way changes by few eV in relation with the atoms bonds (this effect is known as chemical shift). In this way, it is possible to evaluate both the elemental atom percentage in the samples and their bonding configuration. XPS data, shown in this thesis, are collected using a UHV system equipped with the MgKα radiation of a conventional twin-anode Al/Mg Kα source. The photoelectron spectra were collected by a means of a hemispherical

sector analyser CLAM 100 from VC Instruments. The XPS core levels measured were referred to the Ag 3d5/2 level (368.0 eV), measured on a small silver paint droplet deposited on the sample surface, in order to take into account some small charging effects.

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Chapter 4

Microplastics occurrence in edible fish species collected in different geographical sub-areas of the Mediterranean Sea

Mediterranean sea represents a semi-closed sea with highest land pollution (80% of the total) due to population density, intensive fishing, shipping, touristic and industrial activities concentrated in its coasts. Global models predict some of the highest concentrations of floating plastics in the world to occur in the Mediterranean Sea (more than one million tonnes of plastic). Microplastics have been detected not only in surface water and water columns but also in sediments, deep seafloor, and biota including fish and seafood for human consumption. Thus, Mediterranean Sea is defined as great accumulation zone for marine litter (Cincinelli et al, 2019); (Lebreton et al, 2012). Plastic pollution in the Mediterranean Sea poses considerable risk to ecosystems and human health, also causing adverse economic impacts on coastal communities, taking into account that Mediterranean Sea is characterized by biodiversity hotspot and a critically polluted area. All the organisms living in this environment, such as birds and fishes, are not immune to plastics, rather they come into contact and accumulate them (Llorca et al, 2020). In 2016, the *UN Environment Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria* (IMAP) adopted the Candidate Indicator 24 "*Trends in the amount of litter ingested by or entangling marine organisms focusing on selected mammals, marine birds, and marine turtles*" under Ecological Objective 10 (EO10) i.e. Marine Litter. Work is underway to define the most representative species (such as *Merluccius merluccius*, *Mullus barbatus*, *Seriola dumerili*, *Thunnus thynnus*, *Xyphias gladius* samples) to be used for this indicator. It emerges that

further research including multiple fish species is needed to create a database to clear identify indicators species for marine plastic litter ingestion.

During the PhD activities, for a more complete evaluation of the plastics presence across the Mediterranean Sea food web, microplastics were isolated by *Zeus faber* and *Lepidopus caudatus* from the Tyrrhenian Sea, juveniles of *Engraulis encrasicolus* and *Sardina pilchardus* from Southern Tyrrhenian Sea, *Boops boops* from the northern coasts of Sicily (Central Mediterranean) and subsequently analyzed by chemical and physical techniques. Then, the main problems and shortcomings associated to microplastics analyses such as their identification and quantification or the necessity of standardised protocols have been outlined. Specifically, an in-depth study of the plastics extracted by the *Pagellus erithrynus* going from the northernmost portion of the Strait of Messina, coastal waters of the Ionic Sea was carried out mainly looking to bacteria adhesion on plastics and their susceptibility to tested antibiotics.

4.1 Plastics occurrence in the gastrointestinal tract of *Zeus faber* and *Lepidopus caudatus* from the Tyrrhenian Sea

Here, results reported in Ref. (Bottari et al, 2019) about the plastics occurence in two commercially important marine teleosts (*Zeus faber* and *Lepidopus caudatus*) from the northern coasts of Sicily (Tyrrhenian Sea) are presented and discussed.

Zeus faber (Linnaeus, 1758) is a saltwater bentho-pelagic teleost fish, member of the Zeidae family which generally lives in all temperate and tropical waters, including the Mediterranean and Black Seas. It is found on sandy bottoms in a depth range between 5 and 400 m, usually between 50 and 150 m. The body is ellipsoidal and is flattened on the sides. The mouth is large and can be extended forward. *Zeus faber* eyes are large and the head has numerous thorns and roughness. It feeds mainly bony fishes and occasionally on cephalopods and crustaceans (Briguglio et al, 2017).

The second type of fish from which the microplastics were extracted is the *Lepidopus caudatus* (Euphrasen, 1788). It is a mesopelagic species widely distributed in warm waters of the world, is a saltwater fish belonging to the Trichiuridae family. In the Southern Tyrrhenian Sea, *Lepidopus caudatus* represent an important typifying the upper slope demersal fish community. It is a very active carnivorous fish that feeds on squid, crustaceans and fish (Busalacchi et al 2010). In the Central Mediterranean Sea, these are species of high economic value as their meat is very valuable.

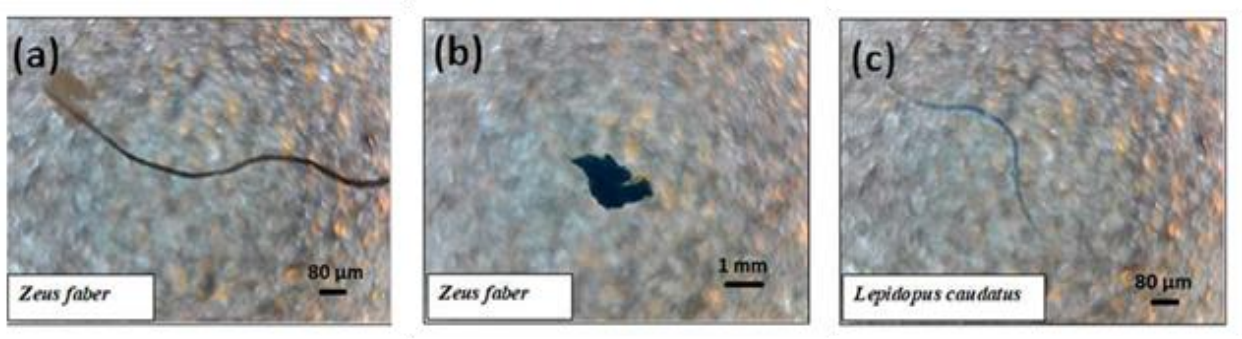

Figure 4.1: Optical microscopy images of plastics extracted from sampled fish (a, b, c).

A total of 43 individuals belonging to the two species (18 *Zeus faber* and 25 *Lepidopus caudatus*) had ingested plastic items. A total of 213 plastic particles were found from the gastrointestinal tract of all fish specimens. Plastics occurrence was higher in *Lepidopus caudatus* (78.1%) than *Zeus faber* (51.4%) as counted by optical microscope observation. In *Zeus Faber* and *Lepidopus caudatus* samples, the plastics particles extracted from the gastrointestinal tract show a mostly uniform distribution in terms of color. Black fibers are shown as the most common (95.4 to 96.8%), followed by red fibers (4.0%) and celestial debris (3.2%). They also have an average diameter of about 25 μm and an irregular shape mainly in the terminal portion (Fig. 4.1).

Plastics characterization was carried out by micro-Raman spectroscopy allowing to identify the main type of plastics. Fig. 4.2 (left panel) shows false color images of the plastics obtained following the fluorescence signal which, in some cases, masks the Raman signals (signals obtained using a laser excitation source at a wavelength of 532 nm).

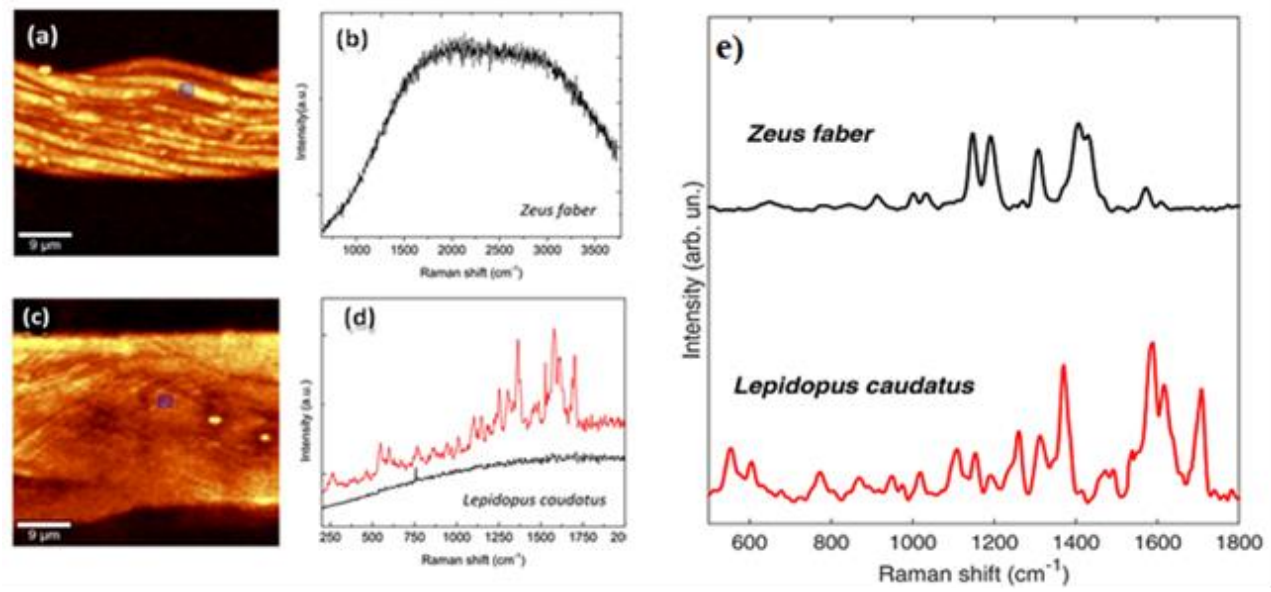

Figure 4.2: In the left panel, false color images of the plastics obtained as result of the fluorescence signal (a, c) and respective Raman spectra in different points of the samples (b, d). In the right panel (e), Raman spectra of the plastics extracted from the sampled fish.

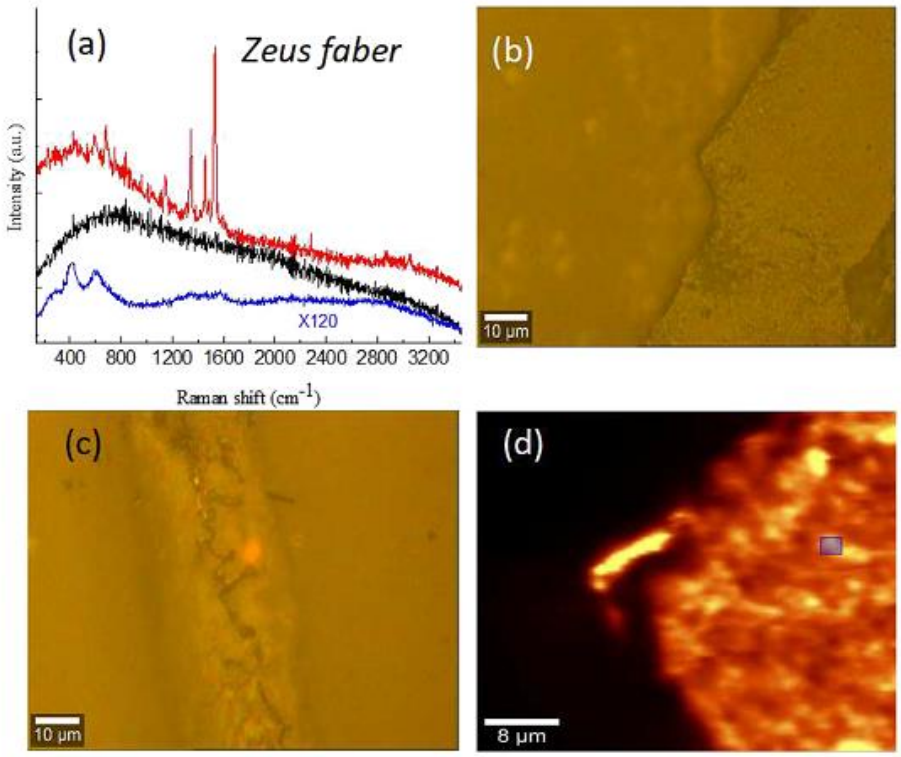

Figure 4.3: Raman spectra (a), optical (b, c) and false colour (d) images of the microplastics extracted by the gastrointestinal tract of Zeus faber that show the zinc oxide Raman bands. Microplastics extracted by *Zeus faber* were a mixture of PE and nylon.

The fluorescence images (Fig. 4.2 a, c) show that the microplastics extracted from the two fish differ in terms of roughness, porosity, presence of veins and protuberances. Furthermore, the nature of microplastics was determined by the Raman signals and mainly attributed to polypropylene (PP), polyamide (PA), nylon and, to a lesser extent, polyethylene (PE). More specifically, the Raman data revealed that the plastic extracted from *Lepidopus caudatus* is made up of a mixture of common polymers of PP, PA and acrylics. As can be seen in Figure 4.2 e, Raman spectrum of *Lepidopus caudatus* shows intense signals in the spectral region between 1200-1700 cm^{-1}, characteristic of the contributions of the [-C (= O) -NH-] amide groups (typically the polyamides generally called nylon).

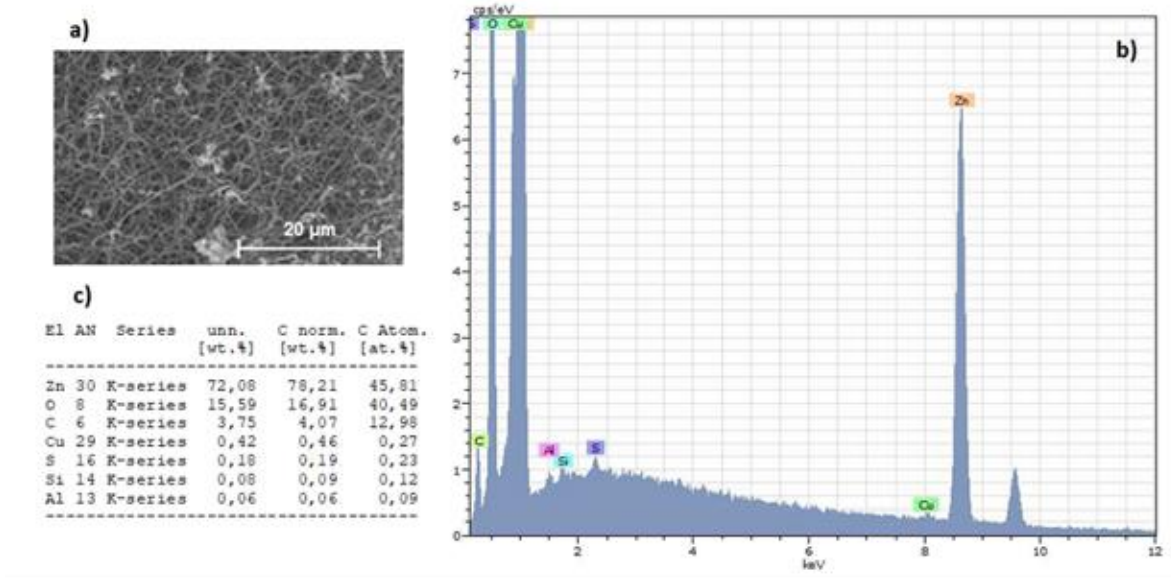

Figure 4.4: SEM-EDX data relating to the microfibers extracted from the examined fish. At the top left in inset (a) the SEM image of the fibers is shown, in insets (b and c) the results obtained by the EDX probe that detected a significant Zn signal are shown.

Otherwise, in relation to the plastics found in the *Zeus faber*, in addition to the Raman signals typical of PE and nylon, the vibrational contributions related to zinc oxide in the spectral region between 300-1000 cm^{-1} have been identified (Fig. 4.3). The presence of zinc was further ascertained by SEM-EDX analysis, increasingly highlighting the ability of microplastics to absorb metals and act as a vector for their dispersion (Fig. 4.4).

On the overall, a high percentage of plastics debris, mainly composed by fibers made of PP, PE, PA, and nylon, were systematically observed *Zeus Faber* and *Lepidopus caudatus*. The plastic polymers found in this study likely originate from industrial sources, including packaging and textiles.

4.2 Plastics occurrence in juveniles of *Engraulis encrasicolus* and *Sardina pilchardus* in the Southern Tyrrhenian Sea

The occurrence of microplastics in the gastrointestinal tract of adult specimens of *Zeus Faber and Lepidopus caudatus* was followed by the same analyses on late-larval and juvenile stages of clupeid fishes. This choice since this type of fishes plays an important role within the pelagic food web in Mediterranean sea (Savoca et al, 2020). In fact, the juvenile stages of clupeid fishes, mainly European pilchard *Sardina pilchardus* (Walbaum, 1792), and European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) represent an ecological-economical high-value ecosystem fraction. *Sardina pilchardus* and *Engraulis encrasicolus* represent an appreciated example of fish intended for human consumption (especially in Mediterranean countries) and ingested, given its very small size, without evisceration.

During PhD activities, for the first time to our knowledge, some pieces of microplastics isolated from *Sardina pilchardus and Engraulis encrasicolus* were analyzed. They are highly appreciated as a delicacy for human consumption and also they considered as sentinel species for microplastics pollution (Fossi et al, 2017). Ten fibers, ranging in size between 0.5 and 5 mm, were isolated from *S. pilchardus* and 6 fibers and 1 fragment in the *E. encrasicolus* specimens were observed. The colour distribution of ingested plastics was mostly uniform across both fish species analyzed and black fibers the most common (30% and 42.8% in *S. pilchardus* and in *E. encrasicolus*, respectively), followed by light blue and blue debris. Representative optical images of plastics are shown in Fig. 4.5. Differences in terms of diameters and colours were observed: many of them were very long (until to 100 μm) linear fibers with a diameter of 2–3 μm (a–c, g). Fibers showed a strong green fluorescence when irradiated with blue light (see Fig. 4.6 a), indicating the presence of fluorescent disperse dyes into the plastics.

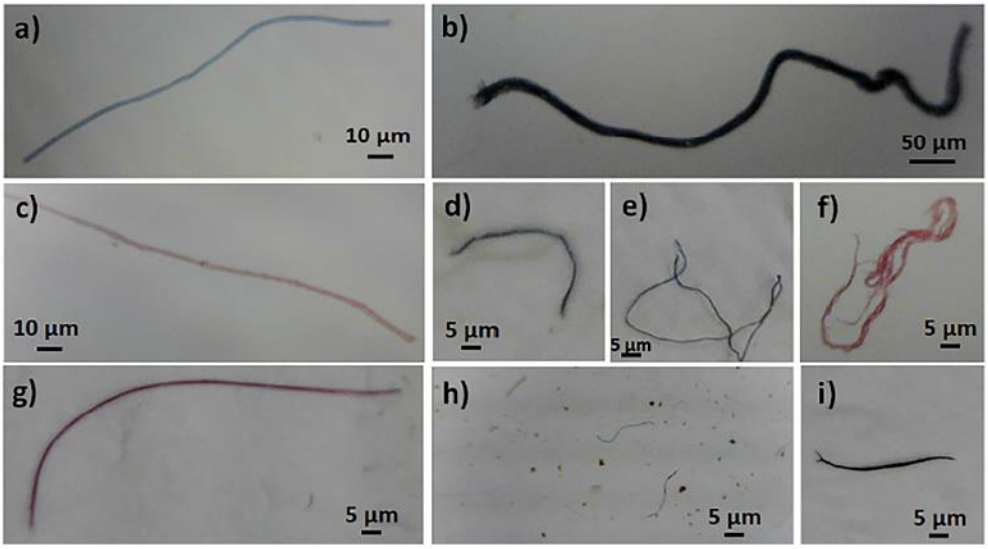

Figure 4.5: Representative optical microscope images of microplastics from *S. pilchardus* and *E. encrasicolus*.

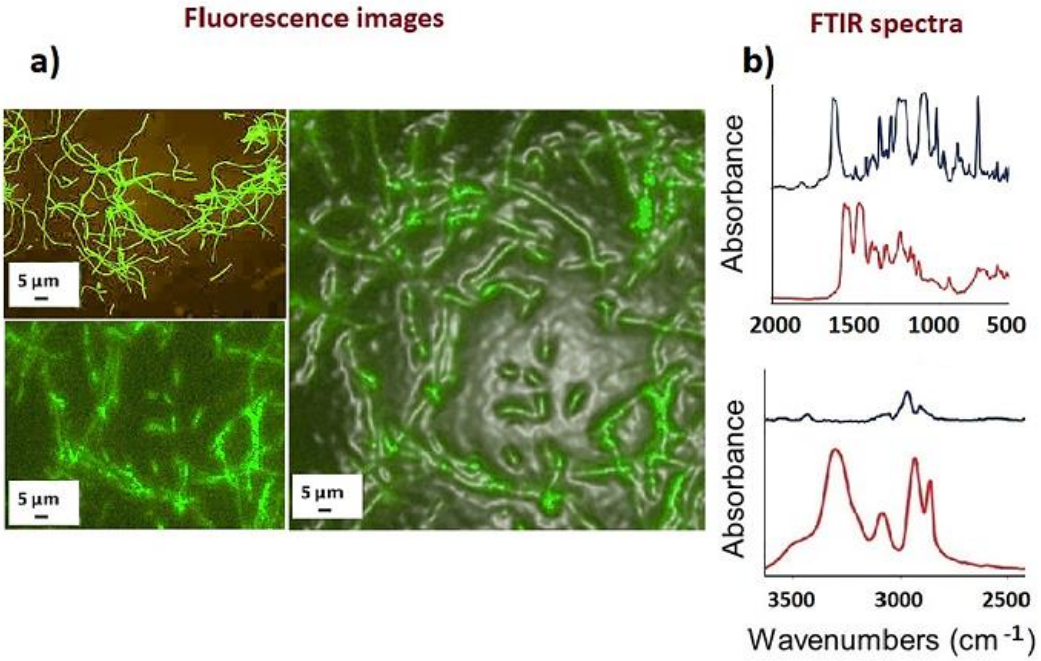

Figure 4.6: Epi- fluorescence images (panel a) and FTIR data (panel b) of plastics extracted by *Sardina pilchardus*. The extracted plastics were characterized by black (plotted FTIR red line) and blue (plotted FTIR black line) colours. Epi-fluorescence was detected using the optics of a 604 microscope coupled with a mercury arc light source and equipped with an ultraviolet excitation 605 filter centred in the blue region (450 nm).

To exclude unwanted auto fluorescence signals, fluorescence images on the filters (without samples) and on filters covered with only aquatic environment were also collected. In both cases, no significant fluorescence was acquired; in any case,

the detected low signal was subtracted to that of all the investigated samples (i.e. those going from microplastics), previously placed on the filters after a centrifugation process. The autofluorescence of microalgae or cyanobacteria, potentially present as contaminant together the fish tissue were ruled out from the analysis. In Fig. 4.6b are shown representative FTIR spectra acquired on the investigated plastics. The detected FTIR peaks can be associated to the characteristic spectral assignments of different types of polymers: polyester, polypropylene, polyacrylonitrile, polyethylene, polyamide, nylon and rayon. In addition, the absorbance peaks in the 1000 –1800 and 2500–3500 cm^{-1} indicate the presence of a very small amount of an organic component, dyes and other additives (i.e. UV stabilizer, antioxidant, antiblock to prevent electrostatic charges).

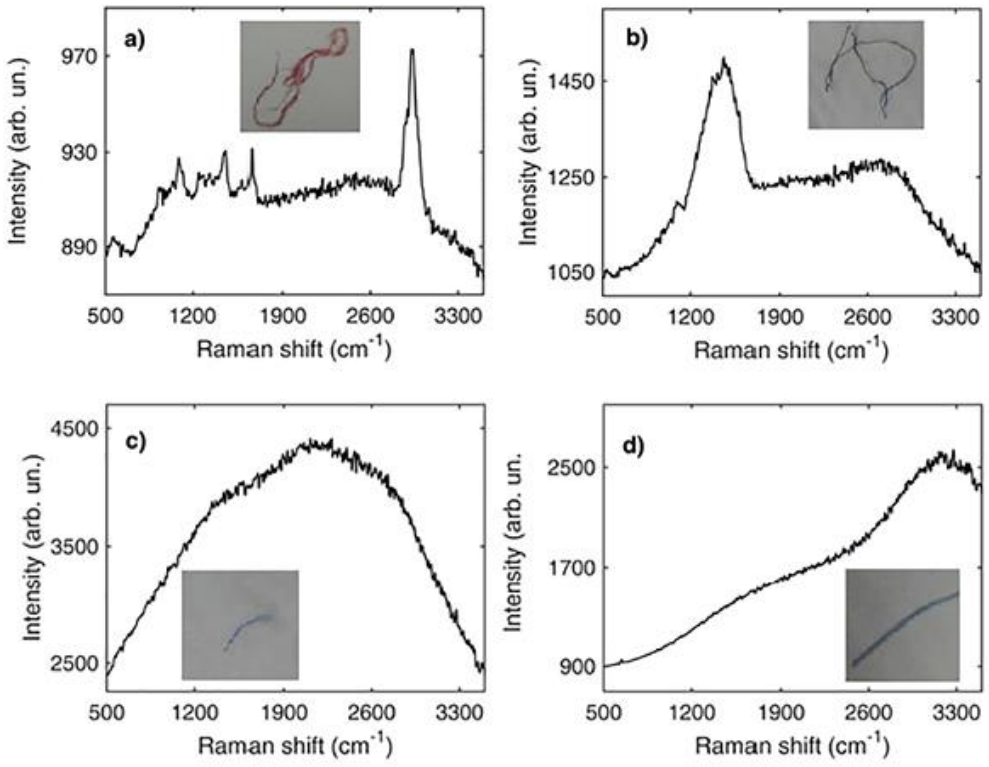

Figure 4.7: Raman spectra collected on: tangled microplastics characterized by a red and black (panels a, b) colour; by black microplastic fiber (panels c, d).

In Fig. 4.7 are shown representative Raman spectra collected on microfibers characterized by different colours. Raman spectra of the samples associated with

residual biological materials or dyed loaded polymers were accompanied by a broad auto- fluorescence background, which complicate the identification process of plastics, and by a broad band assigned to the C-C vibrational mode in the 1200–1600 cm^{-1} range.

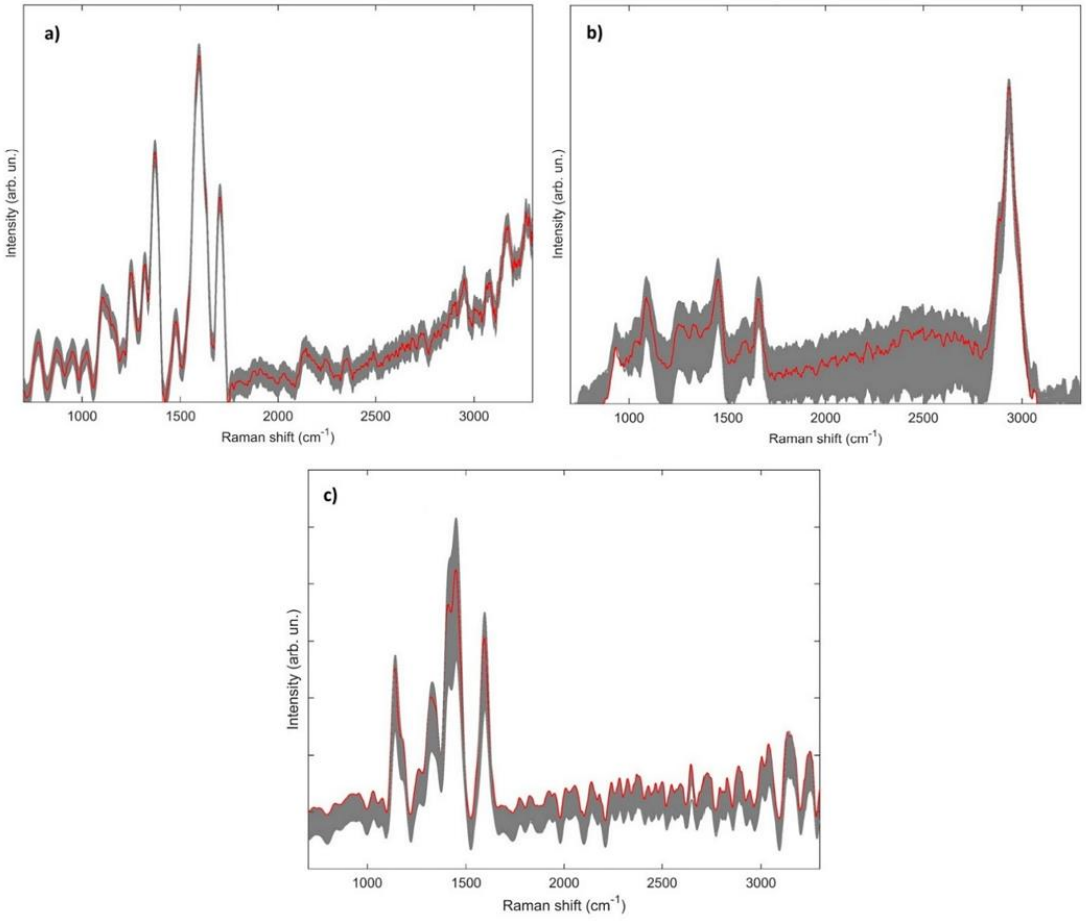

Figure 4.8: Three mean Raman spectra, averaged over all the Raman spectra acquired, referred to three different type of microplastics extracted by fishes. The relative standard deviation values are indicated by the shaded area. Obtained Raman spectra were mainly associated to a mixture of nylon, polyethylene, polyurethane (a), a mixture of polyamide (PA) and polyurethane (b), a mixture of polypropylene and man-made cellulose based materials (c).

To better define the chemical nature, a more detailed analysis was performed. In Fig. 4.8a–c are shown three representative mean Raman spectra (red line), averaged over all the Raman spectra acquired, and the standard deviation values limit.

The Raman peaks identified were associated to polypropylene (PP), polyamide

(PA), nylon, polyethylene (PE) and synthetic cellulose based materials. It's probably that tha broad band at the higher wavenumber also refers to the residual biological components (proteins, lipids, nucleic acid) of the fish from which the microplastic was extracted or ascribed to antioxidant signals, such as phenolic and amine type antioxidant, generally added to plastics manufacture to improve their stability. Thus, the organic/antioxidant components have been also identified, even if not uniquely, by Raman analysis.

On the overall, combining Raman and FTIR evidences, a good identification of plastic fibers chemical structure in digestive tract and/or on the external mucosal surface was obtained. In conclusion, this further set of results demonstrated that *Sardina pilchardus* and *Engraulis encrasicolus*, very small fishes, are accumulators of microplastics. Clearly, this study represents only a data baseline for a future in-depth study about MPs pollution in several life stages of Clupeid in the Mediterranean Sea.

4.3 Detection of artificial cellulose microfibers in *Boops boops* from the northern coasts of Sicily (Central Mediterranean)

In this paragraph, results about some specimens of *Boops boops*, namely bogue, from the northern coasts of Sicily (Central Mediterranean) were presented and discussed (Savoca et al, 2019). Bogue was chosen as target species as indicated by the *European Marine Strategy Framework Directive (MSFD 2008/56/EC)* in order to estimate the "microplastics status" in the gastointestinal tract contents (Hanke et al, 2013).

Boops boops (Linnaeus, 1758) is a marine bony fish belonging to the Sparidae family. Boga has a large range distribution from the eastern Atlantic, Norway to Angola, to the Mediterranean Sea (Busalacchi et al, 2010). It is a gregarious species, goes up in the surfaces purely during the darkness of the night (Bottari et al, 2013).

Bogue is omnivorous, demersal and semi-pelagic feeder, it can generally be found down to 100 m, it feeds crustaceans, porifera, molluscs benthic and pelagic prey like copepoda. In our analysis, a total of 19 (63.3%) individuals had ingested fibers, without significant differences (χ^2 = 0.03, P > 0.05) between males and females (61.5% and 64.7%, respectively). A total of 80 fibers in the GIT's bogues were found. In the specimens of *Boops boops*, most of the fibers found are black (95%) and a small percentage of red (5%). The optical images of the microfibers extracted from bogues' GIT are shown in Fig. 4.9. They have characterized by an average diameter of about 20 μm and an irregular shape mostly in the fibers terminal. More protrusions are clearly visible in the points where the fibers are overlapped. This weaving determines a crossing of the colours (red and violet).

Further information on plastics morphology and their composition was obtained carrying out SEM-EDX analyses. In Figure 4.10 are shown SEM-EDX results collected on the investigated microplastics. SEM images show the structure of the fibers made up of bundles of filaments whose surface is smooth and flat (a-c). On the right, the EDX probe reveals the presence of C, O and N and other elements, such as traces of K, Na, Cu and S and Cl (d).

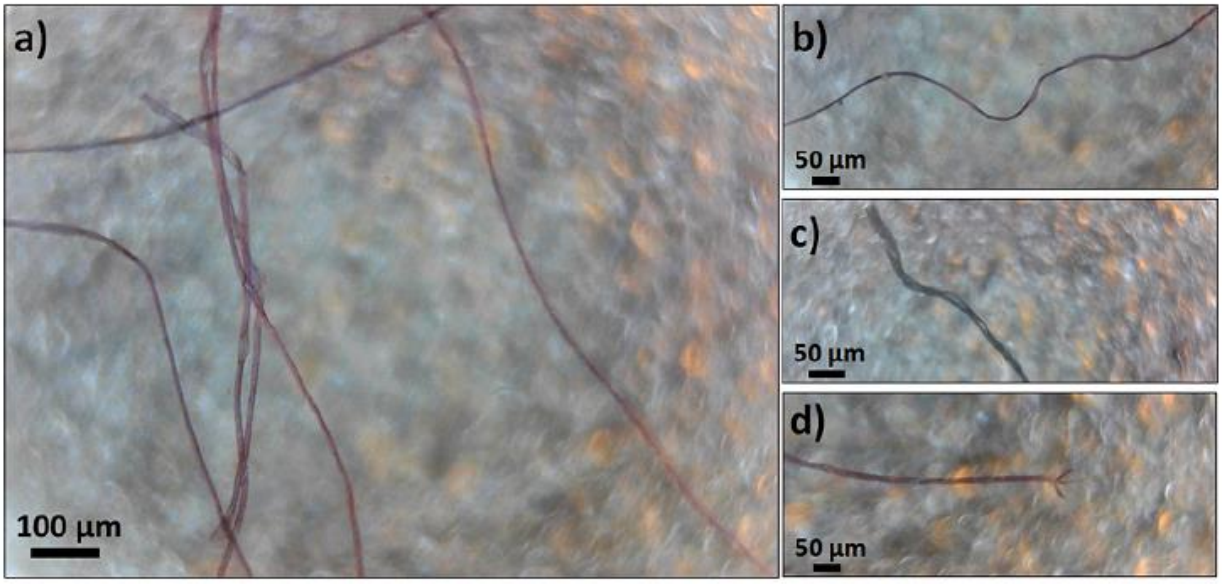

Figure 4.9: Optical images of the fibers ingested by *Boobs boops* specimens.

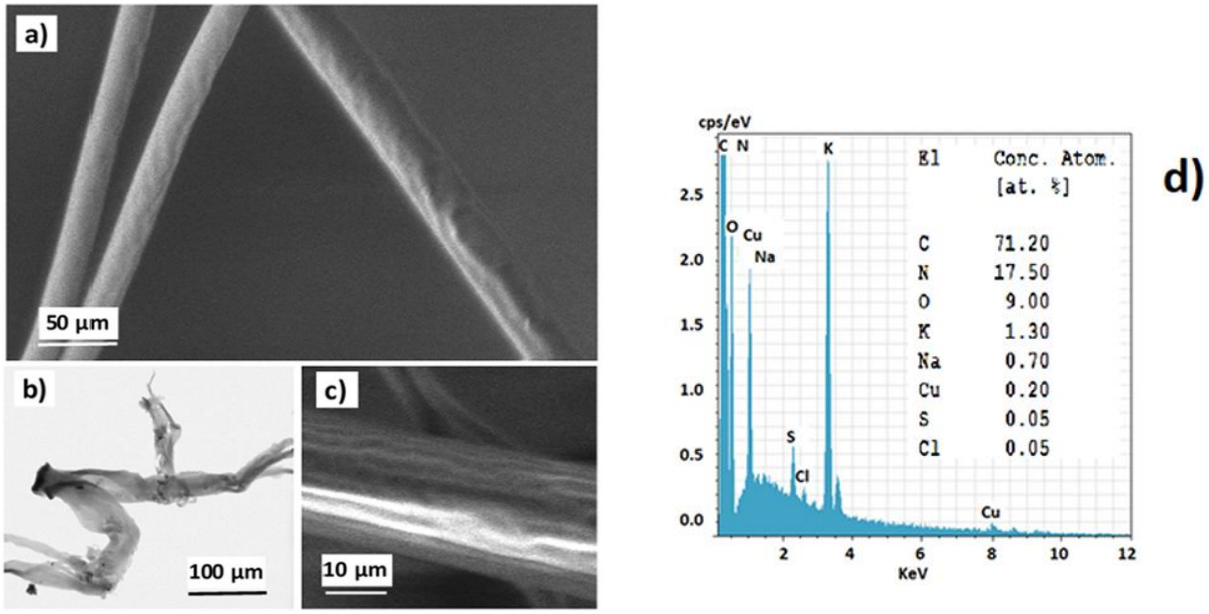

Figure 4.10: On the left, SEM images of the fibers (a-c). On the right, EDX data (d).

The identification of the nature of the plastics was determined analyzing Raman and XPS data. In Fig. 4.11b is shown a representative Raman spectrum of the investigated samples. The most significant Raman peaks are summarized in Table 1 and they found in the following three spectral regions:1095–1250 cm^{-1}, 1280–1450 cm^{-1}, and 1530–1670 cm^{-1}.

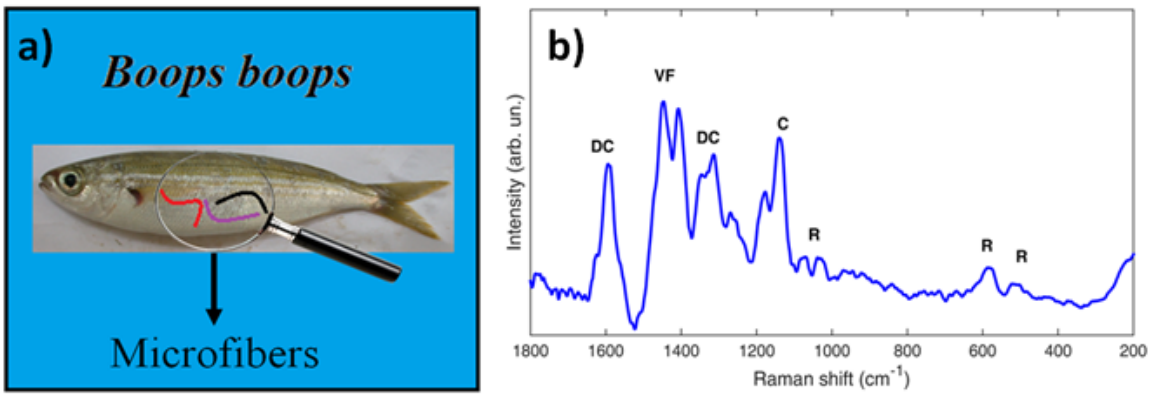

Figure 4.11: Scheme of the fibers extracted from *Boop boops* (a) and Raman spectrum (b). The signs refer to the dye added to cotton (DC), viscose fiber (VF), pure cotton (C) and rayon (R).

Table 1: Raman peaks and their vibrational modes and assignments.

Raman peaks (cm^{-1})	Vibrational modes	Assignments[a]
1620	C-C stretching	Dyecotton
1595	C=C, C=N stretching	Viscose fiber/dye cellulose fibers
1445	CH_2deformation modes	Viscose fiber
1408	C-H_2plane bending	Dyecotton
1347	C-H_2wagging, H-C-C, H-C-O, H-O-C stretching and CH_3 stretching	Dyecotton/acetate cellulose
1316	H-C-C bending	Cellulose fibers
1260 –1280	CH_2twisting/C-N stretching, N-H bending	Cotton/DMDHEU treated cellulose/nylon
1000 –1250	C=S stretching/C-O-C asymmetric vibrations	Viscose fibers/ester cellulose or acetatef iber from wood pulp treated with acetic acid in presence of sulfuric acid/glucose rings of cellulose and hemicellulose/glycoside bonds of only hemicellulose. Synthetic cellulose fibers/Dye cotton
1140	C-C asymmetric ring stretching, C-N stretching	Rayon/glucopyranose ring Rayon
1060 –1130	C-O-C asymmetric stretching	Rayon/Incomplete regeneration of xanthate derivative
1038	C-O stretching	back into the form of cellulose during the rayon
550 –650	C-S-C stretch	process/arti ficial cellulose acetate (triacetate) as a result of processing of natural raw materials Rayon
515	Skeletal C-O-C, C-C-C, O-C-C and O-C-O bending	

Synthetic fibers: result of chemical synthesis. Cellulose acetate is a natural plastic, which is manufactured from purified natural cellulose. Thus, in primary form cellulose acetate cannot be processed as a thermoplastic. It can only be processed by dissolving in a solvent and spinning or casting. For example, the basis of acetate and triacetate fibers is not pure cellulose, as in the case of viscose, but cellulose acetate. The fabric obtained from them is often called "rayon". In fact, it is very similar to natural silk, has the same shiny surface but, unlike silk, it will simply dissolve in acetone. The ester cellulose fibers are terms used to describe fibers made from cellulose acetate. Acetate is derived from cellulose by reacting purified cellulose from wood pulp with acetic acid and acetic anhydride in the presence of sulfuric acid.

[a] Artificial fibers: result of special processing of natural raw materials.

The vibrational modes in the 1095–1250 cm^{-1} region was mainly related to skeletal, symmetric, and asymmetric glycosidic ring breathing (C=O stretching, C-O-C antisymmetric stretching, C-C and O-H bending modes), while the Raman features observed in the 1280–1450 cm^{-1}, are due to the H-C-C, H-C-O, H-O-C bending modes and to the -CH_3 deformation stretching mode. Moreover, the contributions in the 1530–1670 cm^{-1} are ascribed to the H-C-H and H-O-C bending modes (Kavkler et al, 2011). All these features are characteristics of treated and bonded cellulose fibers, constituted by linear polymers of glucose monomers and linked by –glycosidic linkages and hydrogen bond rearrangements (Chiriu et al, 2018).

Raman analysis evidenced that some of the vibrational modes of cellulose and cellulose acetate are located in the same spectral regions (650–1600 cm^{-1}) since, as expected, they are originated by the same carbon oxygen vibrational modes (see Ref. Sánchez-Márquez et al, 2015) and Fig. 4.12).

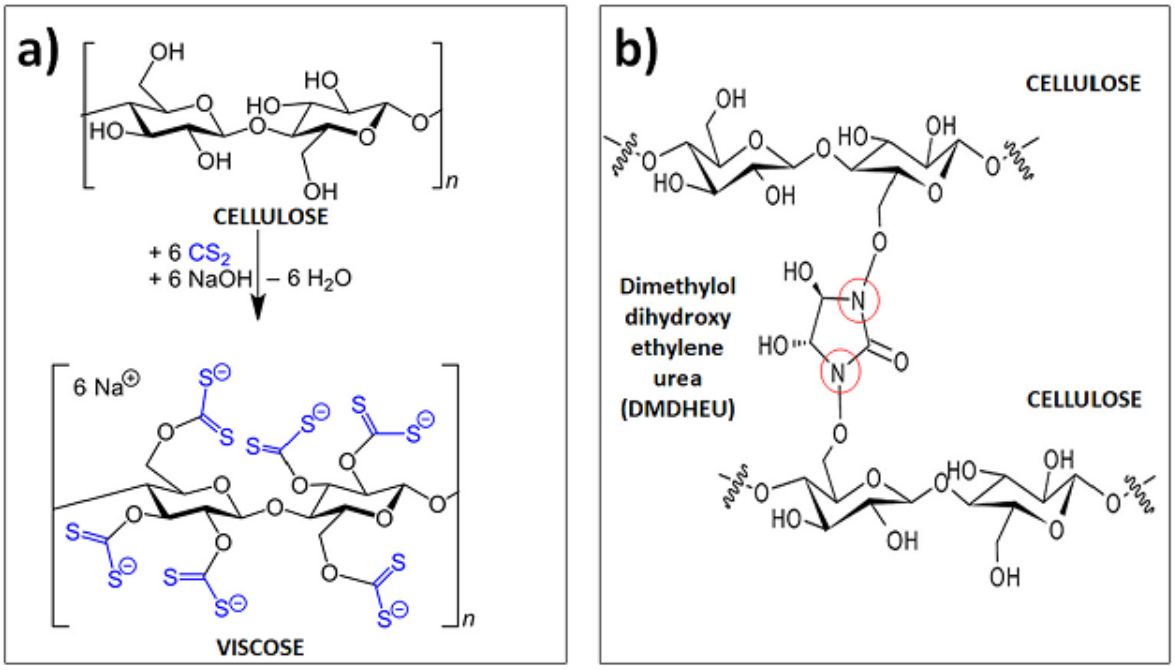

Figure 4.12: Reactions occurring during the preparation of (a) viscose rayon, a man-made fiber composed of 100% regenerated cellulose, and (b) wrinkle-free finish crosslinking with two cellulose chains.

However, in the 800–940 cm^{-1} range, the peaks due to CH_3 rocking, twisting and wagging modes (markers of cellulose with two acetyl groups per glucose module, as shown in the chemical structure of acetate cellulose shown in Fig. 4.13) are totally absent in our spectra. Hence, Raman signals seem to come mainly from chemical treated cellulose used for clothes or other commercial textile products and, in part, from nylon (contained in fishing line and tents, rope, carpet and women's stockings whose chemical structure is shown in Fig. 4.13b), which is a synthetic product constituted by linear polyamides characterized by (CO-NH) groups.

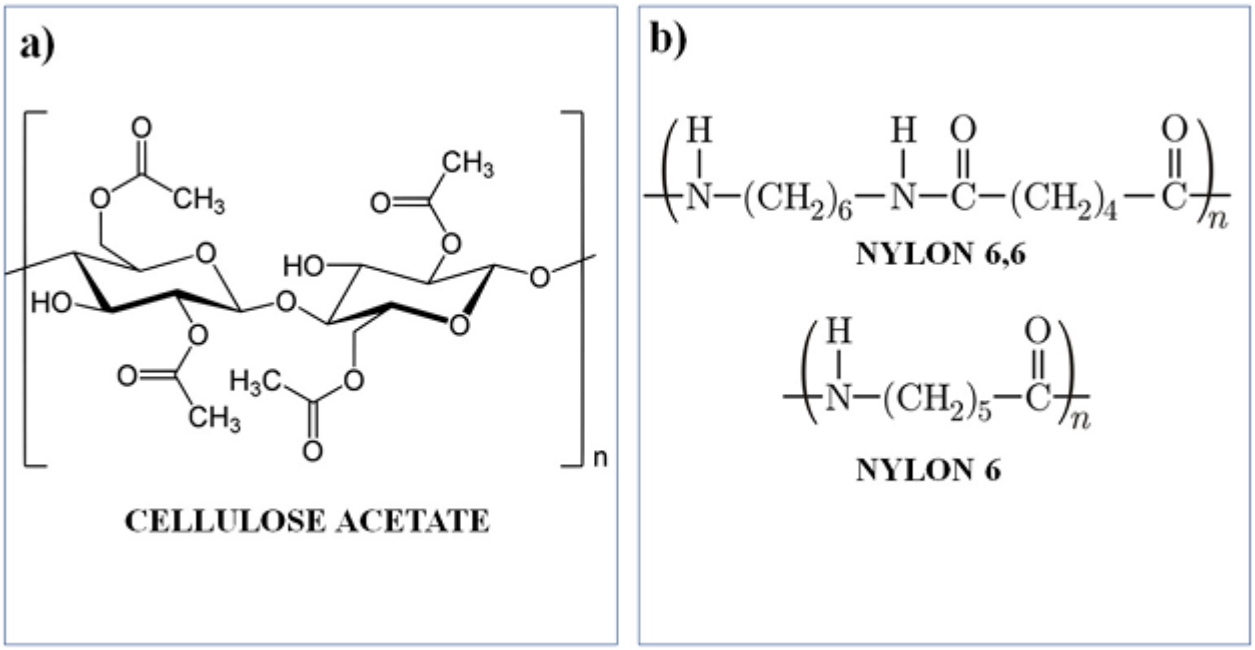

Figure 4.13: Chemical structure of acetate cellulose (a) and nylon (b).

It emerged that the nature of the microplastics extracted from *Boops boops* is attributable to materials whose main component is the synthetic cellulose of which the products used in the clothing sector are generally made. In particular, Raman spectra shown the presence of contributions related to dyes that are generally added to the cellulosic fibers. Since the Raman spectra of rayon/viscose (artificial silk from cellulose) and cellulose fibers are quite similar, further information about the compositional nature of the fibers was obtained by analyzing the chemical bonds at the surface level of the samples performing XPS analysis.

Specifically, XPS measurements gives qualitative and quantitative information about the fibers surface chemical composition and the relative bonding configurations, mainly taking into account the EDX elemental analysis results indicating the presence of nitrogen, sodium and sulfur atomic elements. First, XPS data indicate that few of the fibers analyzed exhibits high amounts of C (82.85%) and O (13.15%) as well as a lower N (1.95%) and S (2.05) content with respect to the high amount of the investigated fibers for which the following composition has been found: 0.00% N, 0.00% S, 13.2% O, and 86.8% C.

Regarding the surface bonding configurations, a deconvolution of the main XPS profiles was made. In Fig. 4.14 are shown the C 1s, N 1s and S 2p XPS high-resolution lineshapes acquired on different fibers extracted by *Boops boops*.

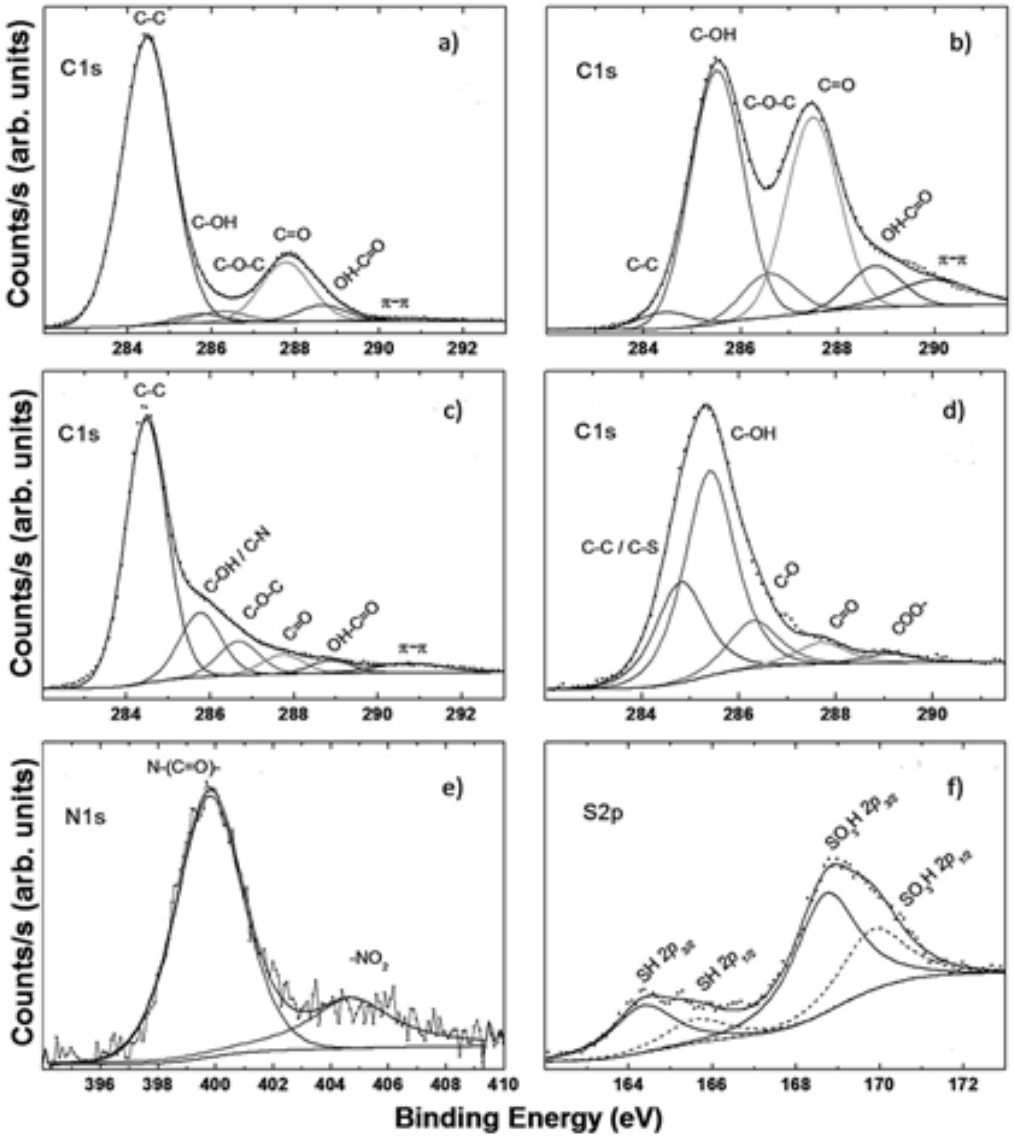

Figure 4.14: High resolution XPS spectra and relative deconvolution. Some fibers analyzed have high amounts of C (82.85%), O (13.15%), and lower content of N (1.95%) and S (2.05).

Relevant modifications can be observed looking to C 1s profiles shown in Fig. 4.14 a-d. The C1s main contribution at 284.5 eV is attributed to C-C in the aromatic ring while the other features at the higher binding energies are ascribed to oxygen in different surface functionalities (C-OH, C-O-C, C=O, OH-C=O). However, the sub-

bandsat about 286 eV and 285 eV are assigned to C-N and C-S bonds when the signals due to nitrogen and sulfur species are collected. In correspondence, the N 1 s high-resolution profile shows two peaks at about 400 and 407 eV, representative of the formation of the N-(C=O) functionality and of nitro groups ($-NO_2$), respectively. Moreover, some fibers are characterized by the presence of C-S/S-S/S-H bonds at 164.0 eV and by the oxidized/hydroxide sulfur bonds at about 168.5 eV.

Ultimately, the combination of EDX, Raman and XPS analyses indicates that the fibers composition and the chemical bonding configurations are strongly compatible with that of regenerated cellulose (Kumar et al, 2019). On the overall, these data further underline the presence of man-made cellulose fibers in the marine environment that needs the problem to be monitored to avoid negative conseguences in the future. Then, this study represents an interesting start point to better understand how this new pollution phenomenon is wide but, once again, more studies are needed for a broad vision of the problem.

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Chapter 5

Microplastics: biological impact and potential effects on human population

The World Health Organization (WHO) emphasized the ubiquitous microplastics presence in the environment and aroused great concern regarding the exposition and effects of microplastics on human health (Campanale et al, 2020). Fishes have been chosen as sensitive indicators and organisms model for evaluation-quantization of plastics in aquatic environment (Schartl 2014); (Van Der Oost et al, 2003); (Azevedo-Santos et al, 2019). About 0.3% of microplastics (with size less than 150 μm) is uptaked in humans, while a lower fraction (0.1%), containing particles that are greater than 10 μm, should be able of reaching both organs and cellular membranes and passing through the blood–brain barrier and placenta (Barboza et al, 2018).

Microplastic surfaces in aquatic environments easily resemble the size and structure of naturally occurring particles to which bacteria adhere, becoming 'vehicles' for bacteria strains (Guo et al, 2020). Therefore, microplastics host microorganisms posing threats in three ways: i) through long distance transport to fragile areas e.g. by marine currents-warming waters increasing disease risk; ii) longer retention times in animals and plants (both wild and farmed), a problem that involves food chains; iii) and the possibility that the antimicrobial resistance genes could transfer among bacterial species (Lear et al, 2021). These considerations suggest that plastic pollution could have ramifications on disease transmission and treatment in addition to environmental consequences and human exposure to contaminated air, water, and food. Furthermore, bacterial biofilms found on microplastics free in aquatic ecosystems have been shown to include bacteria with

antibiotic-resistant genes (Guo et al, 2020). Many bacteria can participate in horizontal gene transfer (HGT), facilitating the sharing of antibiotic resistance genes among bacteria of the same or different species (Alanis 2005). These resistant bacteria originate especially in human and animal populations treated with antibiotics and then they travel through wastewater into river, lake and marine ecosystems (Berkner et al, 2014). The possibility that plastic pollution can facilitate resistance to antibiotics has critical implications for the spread of diseases and their management (Zhu et al, 2020). Although researchers have made important strides in understanding the direct effects of microplastics on animal and plant life, the consequences of plastic pollution, including the bacteria adhesion and their antibiotic resistance, remain unclear (Kukkola et al, 2021).

To better understand and evaluate whether aquatic organisms of Sicily may contain microplastics which, in turn, are capable of hosting bacteria resistant to some antibiotics, fishes samples were analyzed. Specifically, *Pagellus erythrinus* (Linnaeus, 1758) samples as model of study for plastics detection characterization and for bacterial adhesion-antibiotic resistances evaluations were selected.

5.1 Samples collection of *Pagellus erythrinus* and study of area

Pagellus erythrinus belongs to the order of Percomorphi, family of marine Teleostei fishes, genus *Pagellus*. The name *P. erythrinus* derives from ancient Greek language "ἐρυθρός" that means "*red color*" with obvious reference to its dominant color (see Fig. 5.1).

Table 5.1: *Pagellus* scientific classifications.

	SCIENTIFIC NAME: P. ERYTHRINUS (LINNEO, 1758)
CLASS	Actinopterygii
ORDER	Perciformes
FAMILY	Sparidae
GENUS	*Pagellus*

Figure 5.1: *P. erythrinus*'s sample.

P. erythrinus is a demersal fish belonging to the Sparid family, whose geographical distribution extends from the Mediterranean Sea, the Black Sea, along the west coast of Europe and Africa, to Norway and Angola, so it has a relatively wide distribution (Fisher et al, 1987). *P. erythrinus* shows protogynous hermaphroditism (Pajuelo et al, 1998), where females become males at sizes between 12.8 and 20.3 cm (Metin et al, 2011). However, the pattern is not consistent as small males and, even large females, are frequently identified (Larrañeta 1964). Bathymetric range extends from the shallow subtidal down to about 300 m, but they are most common at depths from 20-100 m (Fisher et al, 1987). Generally, *Pagellus* can be found at depths ranging between 20 and 100 m even reaching 320 m in various regions (Bauchot et al, 1987).

Usual length in catches is 10-30 cm and can grow up to 60 cm (Bauchot et al, 1987). In Mediterranean Sea, these species are mainly caught by bottom trawl and they are considered as an appreciated fishery resource in Black and Mediterranean seas as well as in the Atlantic Ocean. Particularly, the catch of *P. erythrinus* represents an important resource for fishermen in Sicily.

Figure 5.2: sampling area FAO 37.2.2 (Ionian Division).

To conduct the research, samples of *P. erythrinus* were purchased in a popular supermarket chain of Messina (Sicily, Italy) in January-February and September-December 2020, and in February-March 2021. Geographical origin and capture methods of samples were provided directly by the fishmonger at the same time of the purchase (sampling notes of the vessel), and reported below:

"Pagellus's samples were caught in Sicily, in the most northern portion of the Strait of Messina, FAO 37.2.2 (Ionian Division), in the Ionian Sea aboard of a fishing vessel by bottom trawls" (Fig. 5.2). The samples selected for the research were intended for sale and common human consumption.

5.2 Laboratory procedures: sampling and laboratory transport of *Pagellus erythrinus* samples

Forty fishes for a total of 13 samplings were bought and wrapped in food transporting containers with transparent film closed (as shown in the Fig. 5.3). Then, samples were transferred to the laboratory and stored at 4°C before to carry out analyses.

Figure 5.3: *Pagellus* purchased packed in polystyrene tray.

Fresh and not frozen fish samples were used in order to obtain information on the bacterial flora, which would have been in part lost if the samples had been frozen. Fishes were treated in laboratory with restricted access to prevent any contamination of tissue fibers or microplastics. Laboratory procedures related to manipulation of fishes and tissues, bacteriological assays and processing of samples for spectroscopic analysis at University Hospital "G. Martino", Department BIOMORF, University of Messina (Italy) were carried out.

Workspaces (laboratory table and surfaces) were previously cleaned with alcohol solution and tools (scissors, extraction forceps and scalpels) were autoclaved before starting each operation. During the visual procedure, the specimens were exposed to the air for the minimum possible time. All the materials used for fishes

manipulation (dissection-extraction) were rigorously cleaned with alcohol and then with sterilized distilled water. The scheme for the treatment was summarized in Fig. 5.4.

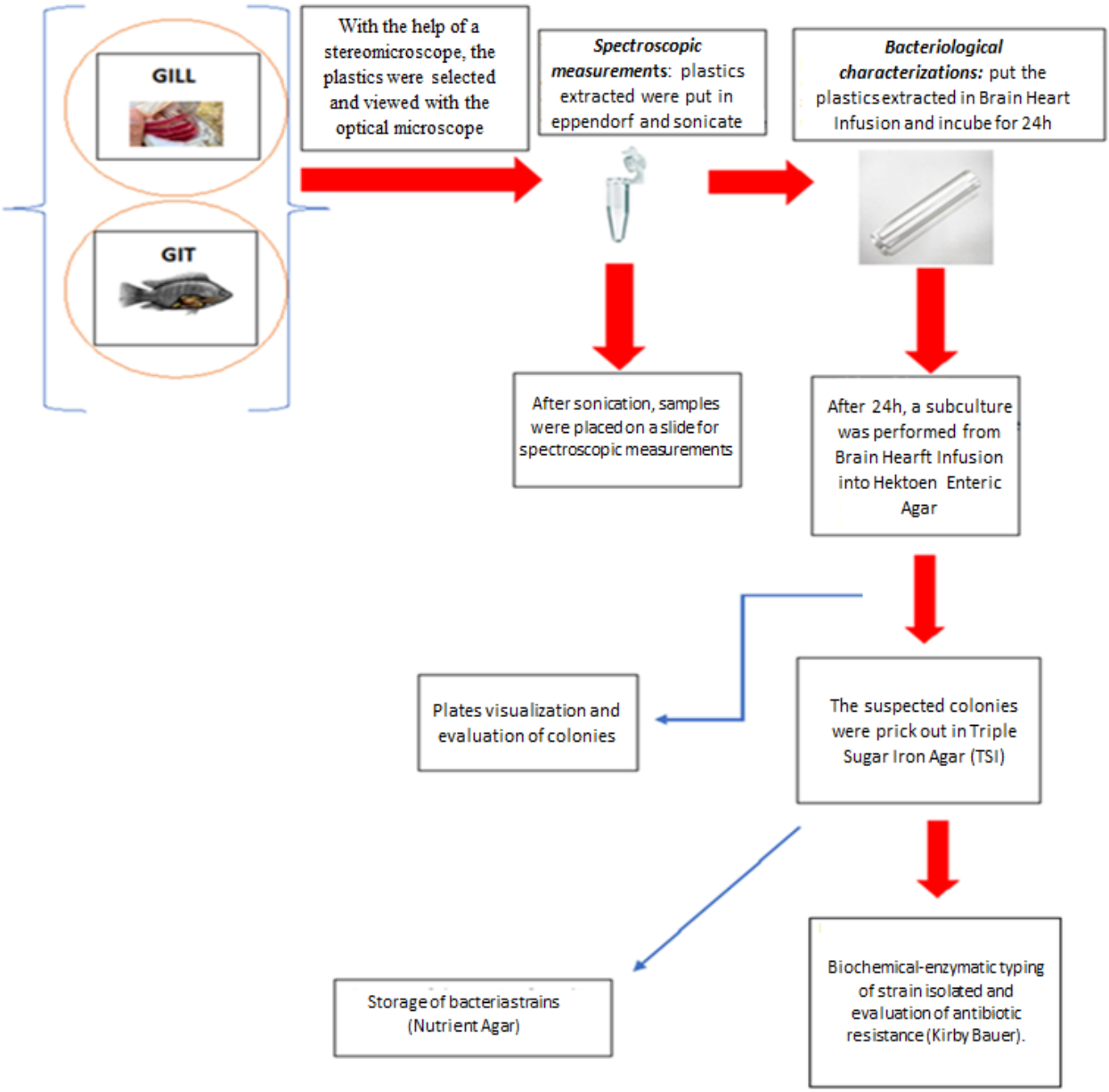


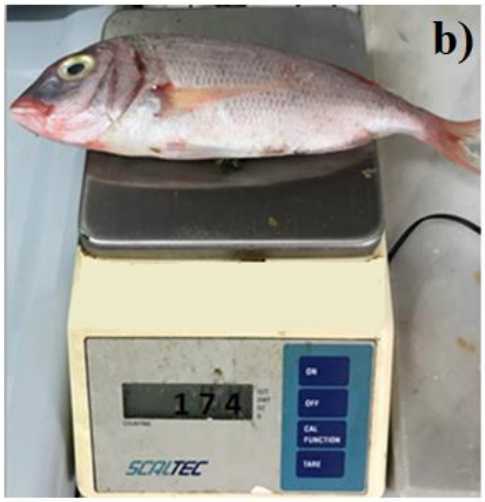
Figure 5.4: scheme for the treatment of plastics in *Pagellus.*

Pagellus's samples were dissected and longitudinally opened, within a sterile glass Petri dish. The components considered exogenous to the normal structure of gills and gastrointestinal tract (GIT) were collected. In order to elucidate the origin of an

eventual presence of microplastics, samples were treated differentially. Initially, for each fish a part of the microplastics were put into vials for bacterial cultivation in liquid enrichment medium (*Brain Heart Infusion Broth*). After an overnight incubation at 37±1°C, a subculture was performed in a *Hektoen Enteric Agar* (HEA) plate for the isolation of any enterobacteria presence. The remaining 50% of plastics in Eppendorf were sonicated and deposited on glass slides to carry out Raman spectroscopic diagnosis.

5.3 *Pagellus erythrinus*: biometric parameters (length and weight)

Before dissection process, *Pagellus*'s samples were carefully measured in term of the length (mm) and weight (g), as shown in Fig. 5.5 **a)** and **b)**.



c)

Lenght and weight	
Length (cm)	range 19,5-28
Weight (g)	range 106-400

Figure 5.5: a) length method (mm), **b)** weight (g) and **c)** data (length and weight) of *Pagellus.*

Biometric parameters measurements were performed with suitable measuring boards, tapes or calipers. Overall, length measurements were made using scales and calipers with the fish lying on its right side, with the snout facing left, the mouth closed, the fish body and tail straightened along the midline. Generally, fishes should

be measured while it is fresh and wet, near to the relaxed live condition as possible; fish will shrink rapidly on drying. When the fishes are measured on fishing craft or in markets at the time of unloading, this problem will not arise. If samples are taken away for later observation, a means of conversion of measurements to fresh, wet condition may have to be employed. Fish in rigor mortis (stiffness after death) should be flexed gently before they are measured. In our case, each specimen was measured in terms of total length from the buccal to the caudal end. More in detail, the definition of "total length" was given by *Bagenal (1978)* (Bagenal et al, 1978) and here cited: "*Total length is the greatest length of the fish from its anterior extremity to the end of its tail fin. In fishes having a forked tail, for example, the two lobes are moved to the position which gives the maximum length measurement (whichever may be the longer lobe is used)*". In addition, the weight of samples was determined using a conventional analytical balance (see Fig. 5.5 **b)**). Morphological measurements including total body length (TL, cm) and body weight (W, g) were performed for each specimen (Fig. 5.5 **c)**). The total length TL varies between 19,5 cm and 28 cm, while the total weight W changes between 106 g and 400 g (see Fig.5.5 **c)**).

5.4 *Pagellus* dissection and tissues extraction. Collections, visual identification and classification of plastics

Among the tissue, for our studies, gills and GIT were chosen being structures closely in contact with pollutants present in aquatic environment (i.e. plastics). The gills lie behind and to the side of the mouth cavity and are characterized by filaments supported by the gill arches and filled with blood vessels, which give gills a typical red color. Water taken continuously through the mouth passes backward between the gill bars and over the gill filaments, where the exchange of gases takes place. These structures represent the first filter structure that comes into contact with the pollutants present in the environment. The blood capillaries in the gill filaments are

close to the gill surface to absorb oxygen from the water and to release excess carbon dioxide to the water (Rodriguez et al, 2019). Gill's structure of *P. erythrinus* are shown in Fig. 5.6.

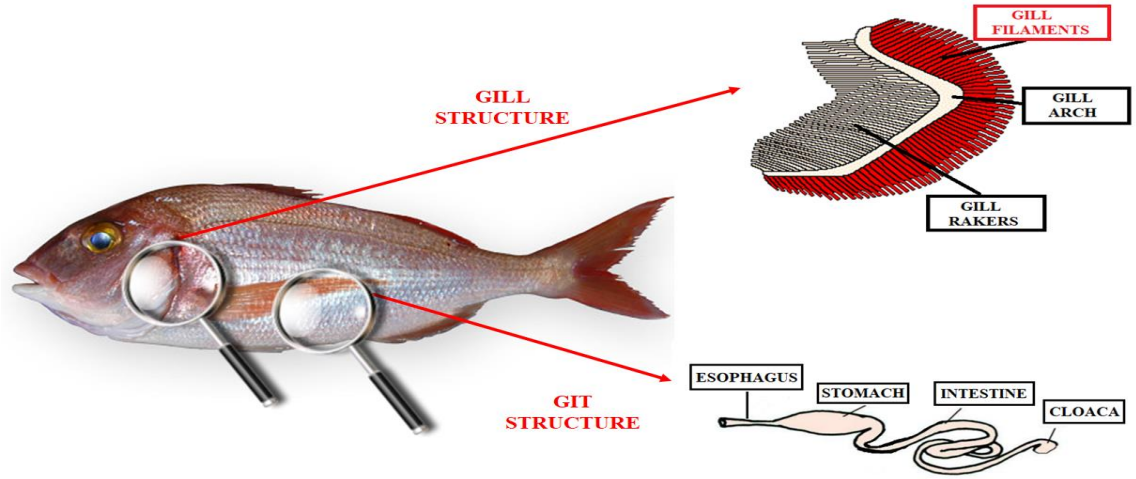


Figure 5.6: Representation of internal structure of gill with arc, filaments and rakers and GIT with esophagus, stomach, intestine and cloaca of *Pagellus erythrinus*.

Then, GIT was chosen as second structure of study. GIT tract is basically a tube that courses through the body. The GIT tract in *P. erythrinus* is divided into the following characteristic regions: oesophagus, stomach, mid intestine, distal intestine and cloaca (see Fig. 5.6). From the opening of the mouth, the buccopharynx is linked with the anterior intestine through a short oesophagus with a rather strict lumen. Two several regions can be distinguished in the oesophagus, based on the histological features of the mucosa: an anterior region lined by a pseudostratified, ciliated epithelium, and a dilated posterior region lined by a simple, cuboidal epithelium, from which the stomach is originate and connected (Olsen et al, 1977). Stomach is a portion of the digestive tract with distinctive cell lining, where acid is secreted, with some digestive enzymes like pepsin (Olsen et al, 1977); (Caruso et al, 2001). Food ingested through the mouth then moves first through the esophagus and then through the stomach. As mentioned before, enzymes from the liver and pancreas also intervene in digestion. Then, the nutrients are absorbed through the intestinal villi and the feces excreted through the anus.

The instruments and materials used for all *P. erythrinus* dissection procedure were:

- Microscope;
- Magnifying Glass with Light;
-Dissection sterile set (scissors, scalpel and forceps);
-Lab coat, gloves;
-Security goggles;
-Sterile Glass Petri dishes.

Figure 5.8 a) gills extraction and **b)** incision with sterile scalpel of *P. erythrinus.*

As shown in Fig. 5.8 **a)**, the first step of dissection was the extractions of gills; subsequently, a shallow longitude incision along the ventral left-hand side, which extends from the anterior of the anus to below the gill arches, was performed (Fig. 5.8 **b)**. It is important to cut deep enough to gain access to the GIT without damaging the organs (Monit. and Sampl. Manual, 2018). Then, with the help of sterile tweezers, the external structure was gently lifted and the anterior and posterior ends of the GIT were cut transversely. At the end, the removed GIT was placed in a sterile Petri dish. Immediately after the extraction of the chosen body portions (gill and GIT), the components considered exogenous to the normal structure of the gills and

GIT were collected (see Fig. 5.9). In order to conduct bacteriological analyzes and subsequent spectroscopic characterizations, plastics were treated differentially as described in the previous section "*Laboratory procedures: samplings and transport in laboratory of Pagellus erythrinus samples*".

First, the extracted plastic samples were first examined with the stereomicroscope. All plastic fragments/fibers were carefully hand-picked with sterile tweezers, partly transferred to tubes for the pre-treatment of sonication in order to carry out spectroscopic measurements and partly placed in glass tubes with liquid medium for the first step of bacteriological treatment.

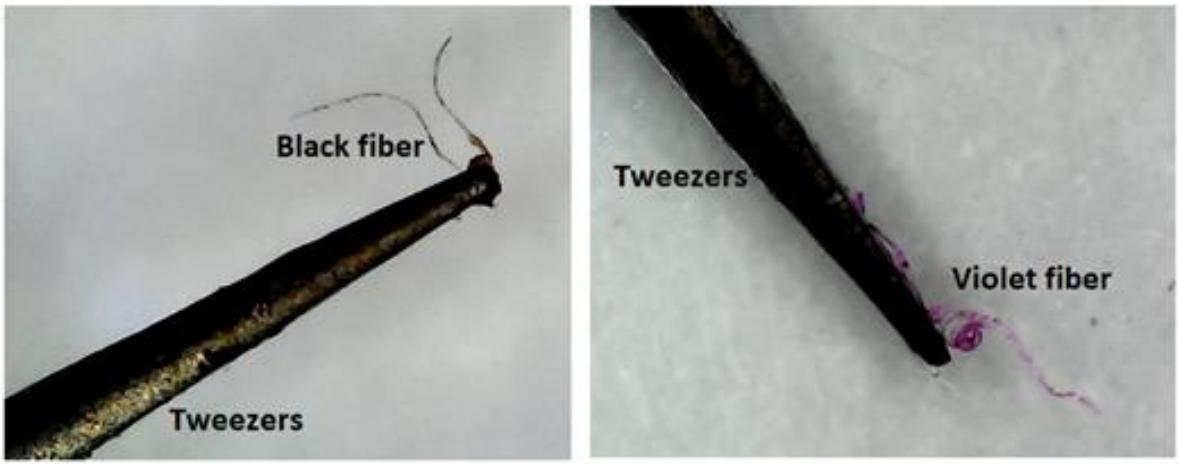


Figure 5.9: sterile tweezers and fibers found in samples of *P. erythrinus.*

The extracted fibers/fragments were classified according to the ***type:***

I) filaments (elongated fibers);

II) fragments; and

III) color:

a) black;
b) red;
c) red-orange;
d) white/transparent;
e) white/yellow;
f) purple;
g) yellow-black;
h) blue-yellow and red complex.

Overall, 40 samples of *P. erythrinus* were analyzed, of which 33 had ingested plastic items (82,5%). A total of 109 plastic particles were found, of which 47 (43%) from the gills and 62 (57%) from GIT. In *P. erythrinus* samples, the particles extracted from the gill showed a variety in the distribution of colors. Black plastics were the most common (43%), followed by white-transparent (40%), red (11%), blue (2%), violet (2%) and yellow-trasparent (2%) (Fig. 5.10).

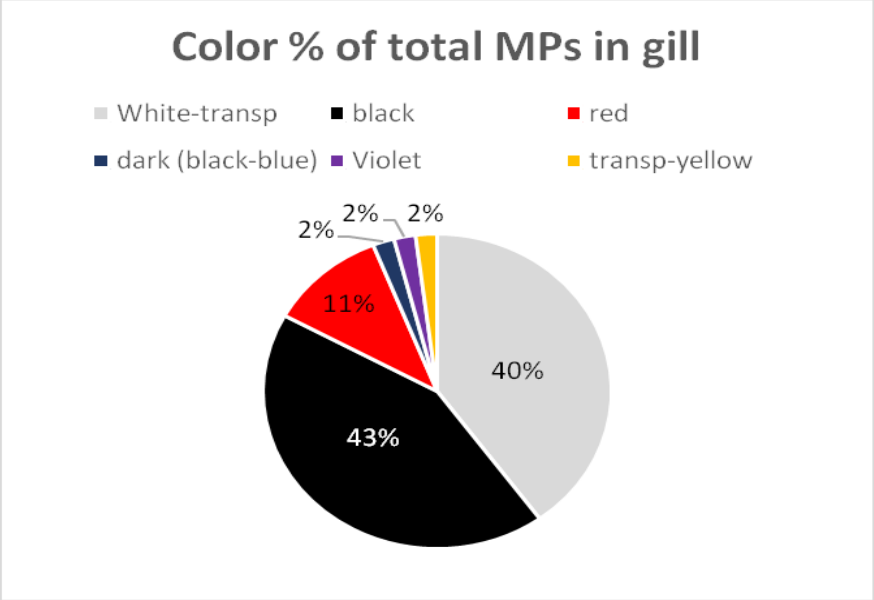


Figure 5.10: Percentage (%) of plastics, isolated from gills of *P. erythrinus* categorized by colour.

A further subdivision has been made considering that 19 fibers (40%) and 28 fragments (60%) have been extracted by the gills (Fig. 5.11).

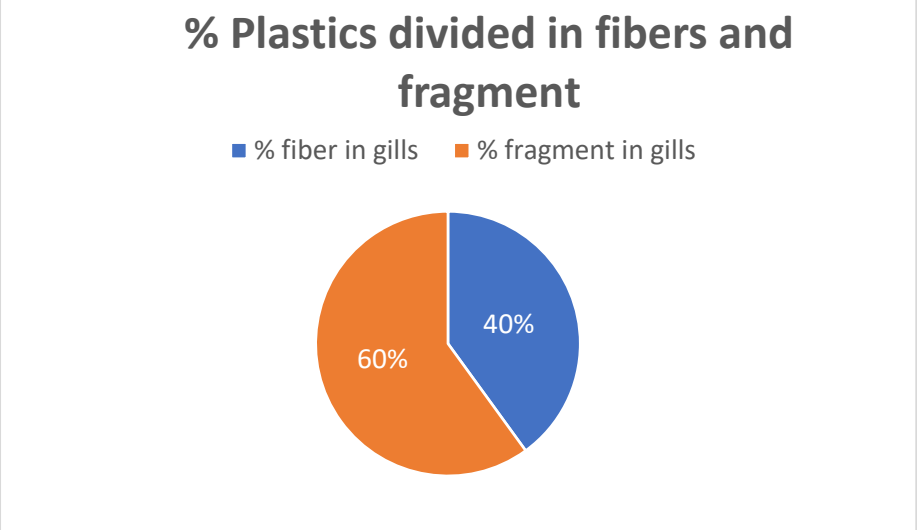


Figure 5.11: Percentage (%) of plastics from gills of *P. erythrinus* classified in fibers and fragments.

Moreover, the plastics found in the GIT showed a variety of colors too. In fact, white-transparent plastics are the most frequent (50%), followed by black (24%), red (18%) a mix of blue-yellow-red (2%), violet (2%) and yellow-transparent (4%) (Fig. 5.12). Furthermore, for the plastics extracted by GIT, a further subdivision has been made: 30 (48%) of fibers and 32 (52%) of fragments (see Fig. 5.13).

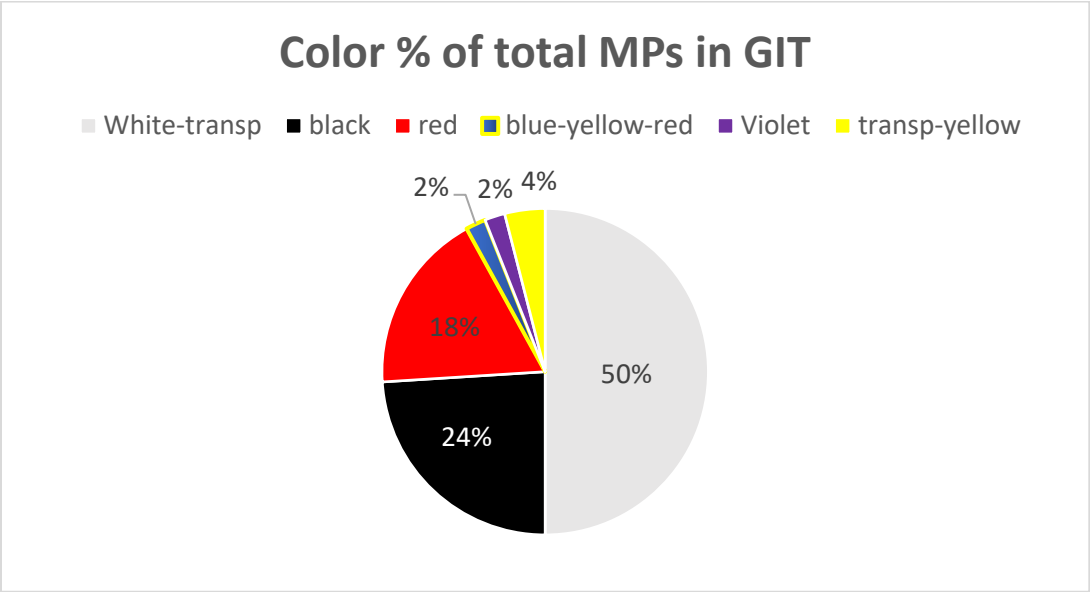


Figure 5.12: Percentage (%) of plastics, isolated from GIT of *P. erythrinus* categorized by colour.

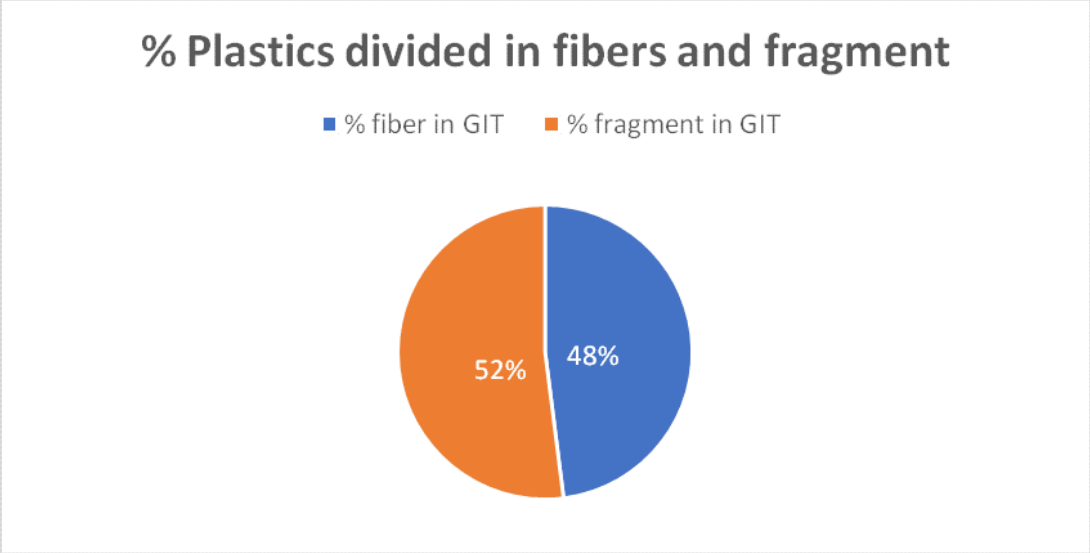


Figure 5.13: Percentage (%) of plastics isolated from GIT of *P. erythrinus* classified in fibers and fragments.

Representative optical images of the microplastics extracted from gills and GIT are shown in Figs 5.14 and 5.15. These microplastics were characterized by an average diameter of about 25 μm and an irregular shape, especially in the fibers terminal. Some fibers showed a very thick diameter and many fragments wrap around themselves and overlap, as shown in Fig. 5.15b.

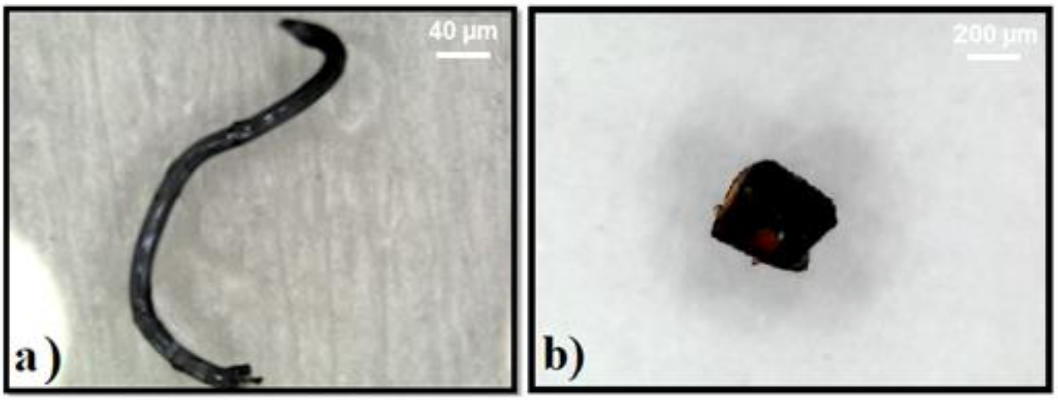


Figure 5.14: Representative optical microscope images of the different type of microplastics extracted from gills of *Pagellus*. Elongated microfiber of dark colour identified in panel a) and a fragment plastics particle of red colour in the right panel b) are shown.

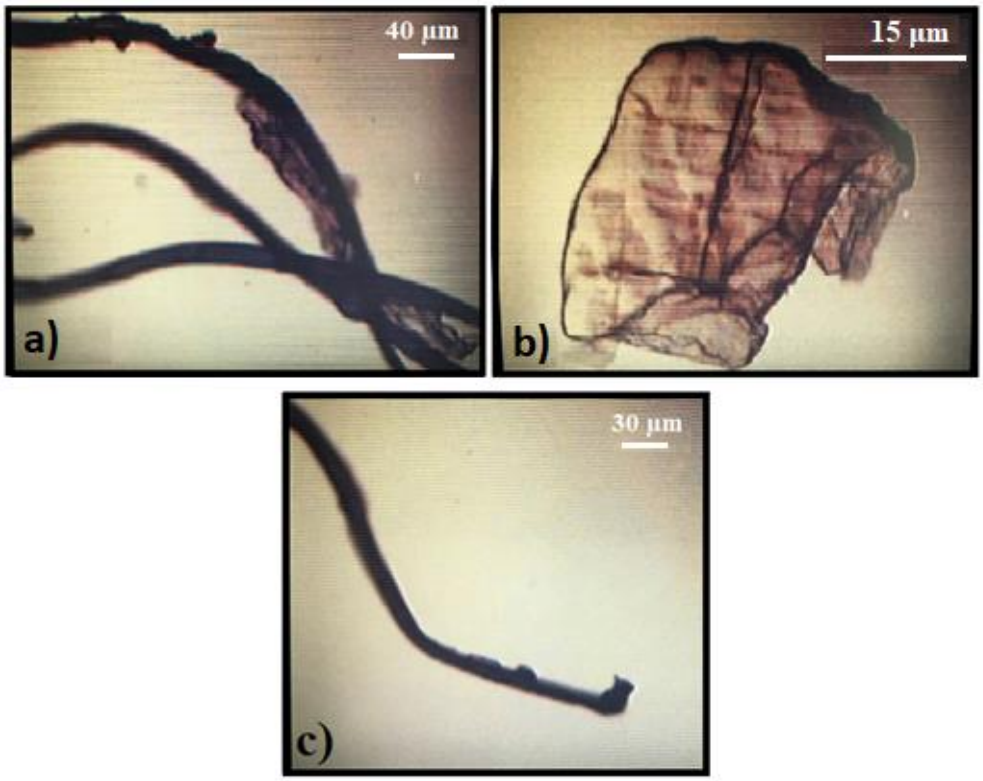


Figure 5.15: Representative optical microscope images of the different type of microplastics extracted from GIT of *Pagellus*. Elongated microfibers in panel a) and c), and another plastics type with a fragment shape in panel b) are shown.

5.5 Raman spectroscopy measurements and microplastics characterization

Some pieces of plastics extracted from the gills and gastrointestinal tract of *Pagellus erytrhinus* were collected to carry out chemical-physical measurements. Then, the plastics were put in distilled water and sonicated for 20 minutes. Subsequently, the fish organic component have been removed and the plastics samples were placed and covered with corning slides to avoid their dispersion, as shown in Fig.5.16. Raman measurements were carried out in the Optical Spectroscopy Laboratory in Mathematical and Computational Sciences, Physics Sciences and Earth Sciences (MIFT) Department of University of Messina (Italy).

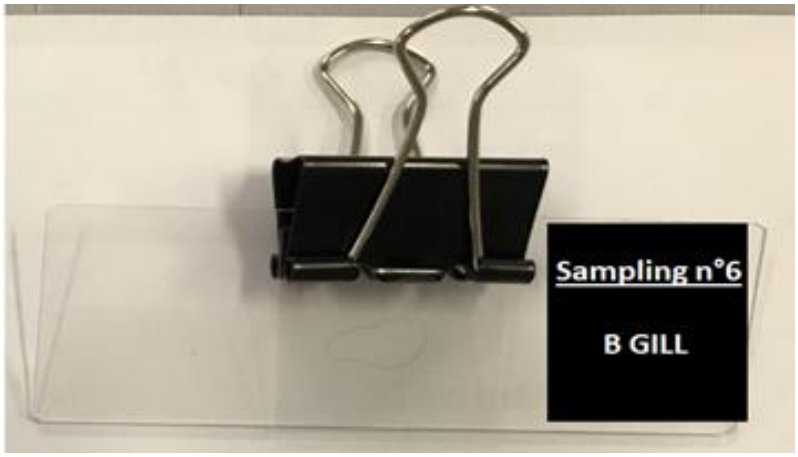


Figure 5.16: Example of black plastic fiber extracted by gill of *P. erythrinus* and ready to Raman measurements.

Raman spectra of plastics were recorded by the XploRA Horiba spectrometer. The 532 nm laser was focused on the sample surface through the 50 X long working distance objective of the microscope, at a low laser power (about 0.5 mW) to avoid laser-induced heating of the specimens. For single acquisition, integration times were varied, depending on the signal-to-noise ratio, between 1 and 50 s, with an accumulation number of 5. µ-Raman mapping took 4h to scan each sample surface using a point distance of 10 µm and an integration time of 0.5 s per point.

Zeiss Supra 40 field ion microscope equipped with an Energy Dispersive X-ray (EDX) probe was used to characterize the microfibers morphology and their atomic composition. Microplastics are found in various shapes, sizes, colors, and polymer types. Plastic particles can be difficult to distinguish visually under a light microscope since microplastic materials appear similar in shape and size to sand particles. For example, a dark blue plastic fiber appears next to undigested pieces of fish tissue. Although some microparticles are obviously plastic (blue fragments), other particles could be white sand or gelatin.

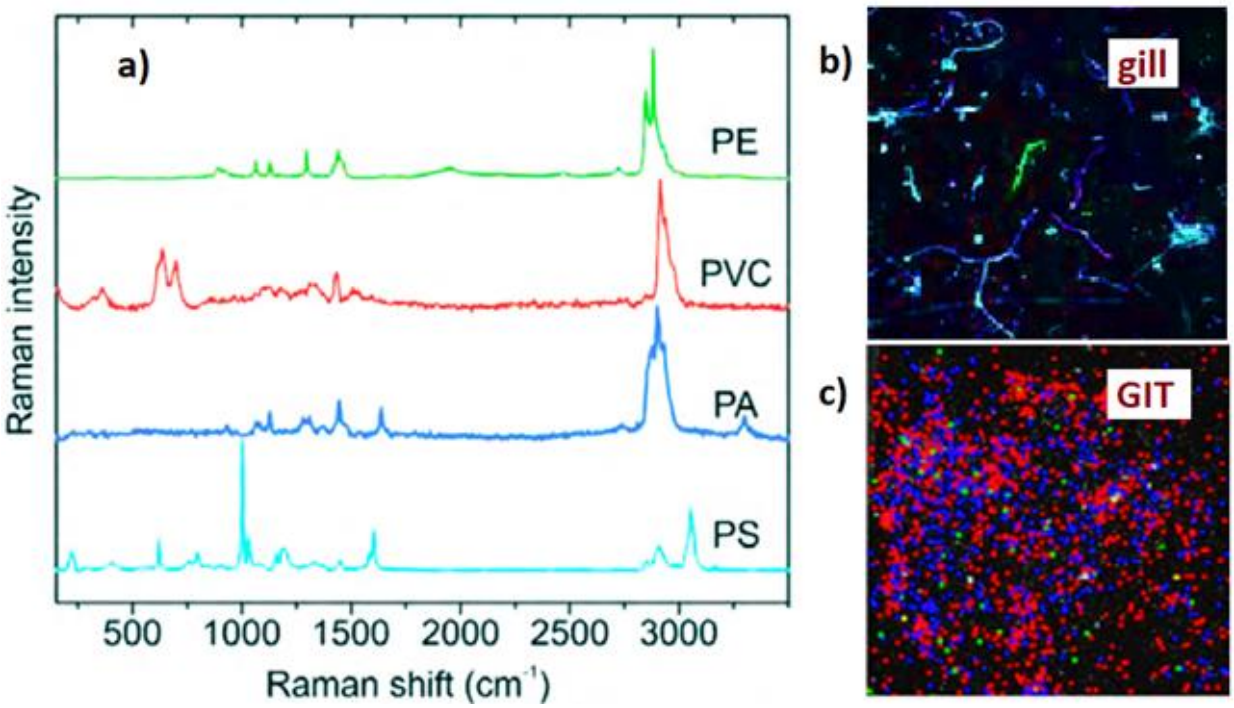


Figure 5.17: Raman spectra on MPs extracted from gills and GIT **a)** and false-colouring mapping denoting the spectral intensity in the 2780-3050 cm^{-1} range **(b, c).** Scale bar 100 µm for Raman mapping.

Visual detection relies upon morphological criteria to decide whether a given pieces is made of plastic; this approach gives good results for bigger MPs (generally greater than 100 mm) but progressively fails for smaller MPs size and when morphological characteristics become less distinguishable. In these cases, a peculiar identification by spectroscopic techniques is largely used to define the composition of the material, distinguishing between inorganic and organic one.

In our case, µ-Raman mapping analyses were carried out on MPs extracted from gills and GIT. Their representative Raman spectra are shown in Fig. 5.17a. Some Raman features observed are characteristics of each plastic; for example, the peak centered at 1000 cm^{-1} is characteristic of PS, those at 1059 cm^{-1} of PE, and that at 695 cm^{-1} of PVC (Xu et al, 2020); (Dąbrowska, 2021). Furthermore, by mapping images via the spectral intensity of the characteristic and fingerprint peaks in the 2780-3050 cm^{-1} range, the microplastics extracted by gill and GIT, including polyethylene (PE), polyvinyl chloride (PVC), polyamide (PA) and polystyrene (PS), have been individually identified and visualized (see Fig. 5.17b, c). The false-color mapping has allowed to further evidence the shape of the microplastics (fragments, fibers and agglomerates).

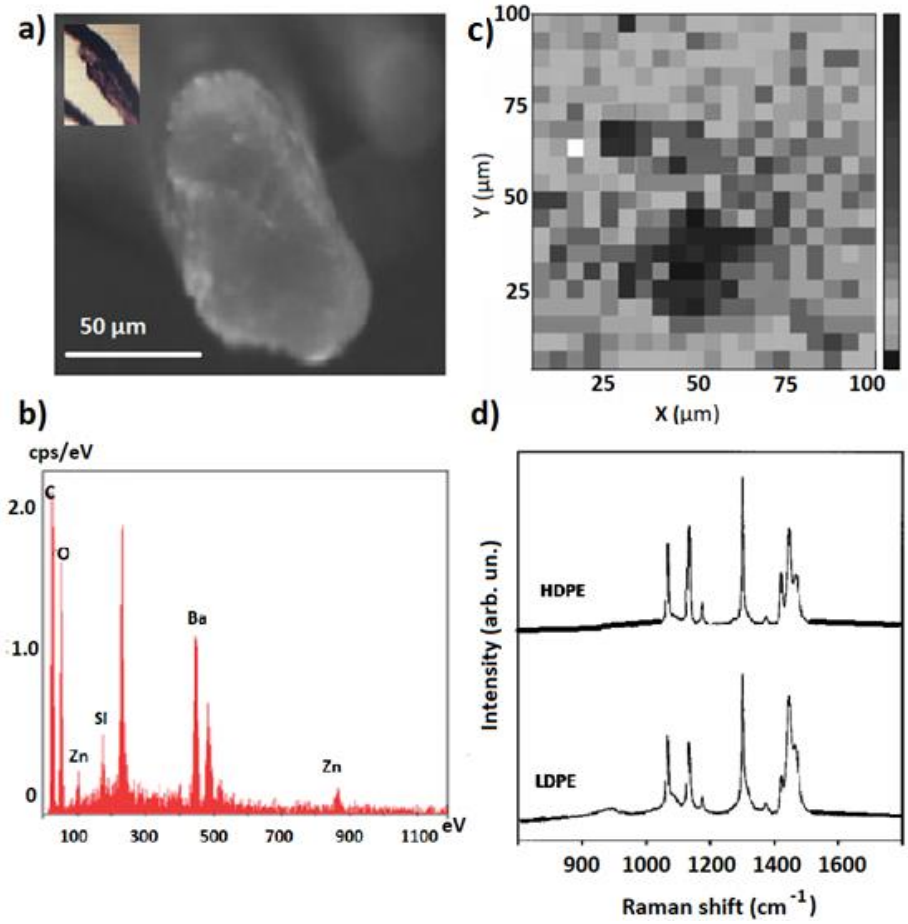


Figure 5.18: SEM image **a)**, EDX spectrum **b)**, Raman mapping and representative Raman spectra (**c, d**) of a plastic extracted by GIT. Scale bar 100 µm for Raman mapping. Raman features are those typical of *Low and High-Density Polyethylene (LHDPE-HDPE).*

In Fig. 5.18a is shown a SEM image in which the feature, already observed by the optical microscope (inset), represents a plastic fragment. The same region was analyzed mapping the 1460 cm^{-1} Raman intensity, a characteristic feature of both *Low and High-Density Polyethylene (LHDPE-HDPE)* (Fig 18d). Raman spectra of LDPE and HDPE are very similar (Allen et al, 1999), but it was observed that the maximum intensity of the Raman band at 1460 cm^{-1} increases with the reduction of LDPE in the polymer blend.

Finally, EDX probe allowed to detect trace of Zn, Si and Ba indicating the presence of metal into plastics.

5.6 Microbiological analysis for identification of bacteria on microplastics

Microplastics, like every surface in the world, can easily colonized by environmental bacteria. Several bacterial species are ubiquitous in aquatic environment and are able to adhere over all surfaces, including plastics (Zettler et al, 2013). As already said, plastics are abundant, stable and inert, so they could represent a good surface-substratum for bacterial colonization in aquatic ecosystems (McCormick et al, 2014); (Zettler et al, 2013). Planktonic bacteria are able to attach plastics in aquatic environment and also to create biofilms; so bacterical communities are diversified among different plastic types (Oberbeckmann et al, 2015): relatively smooth and inert plastics have a surface more hydrophobic and less colonized by bacteria, while rougher and charged surfaces are more hydrophilic and allow better colonization (Thompson et al, 2009). More in detail, how to study bacteria adherent on plastics and what media are used to identification?

5.6.1 Bacterial cultures: liquid and solid media

Bacterial strains, adherent to the plastics in gills and GIT of *P. erytrinus* specimens, were cultured initially in ***Brain Heart Infusion*** broth (BHI) (Difco

Laboratory, Detroit, MI), a liquid non-specific culture medium rich in nutrients, suitable for the cultivation of several Gram positive and negative bacterial strains, such as streptococci, meningococci and pneumococci, but also for fungi and yeasts (Zimbro et al, 2009). More information about medium composition can be found in the Appendix at the end of this chapter (Table A1). Each plastic was incubated overnight into BHI at 37°C. After the incubating time, solid medium ***Hektoen Enteric Agar*** (HEA) for bacterial colonies isolations was chosen. HEA is a moderately selective medium used for the isolation and cultivation of Gram-negative enteric microorganisms, for example *Escherichia coli* or *Shigella* spp, from faeces, foodstuffs and other materials of sanitary importance. Also in this case, more information about medium composition can be found in the Appendix at the end of this chapter (Table A2).

5.6.2 Triple Sugar Iron (TSI) and Mackenzie test

Colonies isolated into HEA were then cultured in identification medium as ***Triple sugar iron agar*** (TSI). TSI agar is a differential medium used to determine carbohydrate fermentation and H_2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fementatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and on the production of hydrogen sulfide. TSI is used for *Enterobacteriaceae* identification, although it can be useful also for other gram-negative bacteria (Hajna, 1945). The presence of sucrose in TSI agar allows the earlier detection of coliform bacteria that ferment sucrose more rapidly than lactose. More information about medium composition can be found in the appendix at the end of the chapter (Table A3).

Mackenzie test **(**Mackenzie et al, 1948) is a biochemical assay carried out to determine the ability of bacteria to convert tryptophan into indole (see Fig.5.19). Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria expressing tryptophanase enzyme. Indole is generated by reductive

deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan is removed.

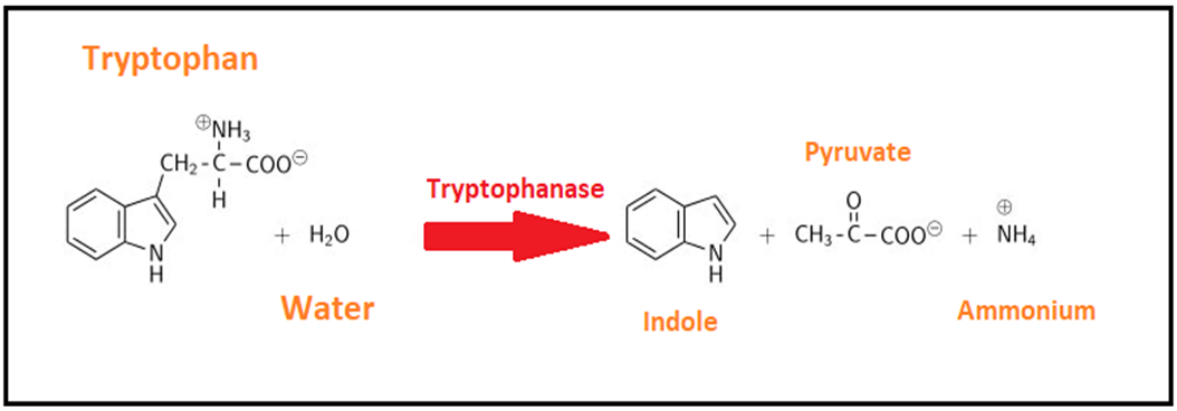


Figure 5.19: Representation of chemical reaction.

Final products of the reaction are indole, pyruvic acid, ammonium (NH_4^+) and energy. Pyridoxal phosphate is a necessary coenzyme. When indole is combined with *Kovac's Reagent* (which contains hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol), the solution turns from yellow to cherry red. Because amyl alcohol is not soluble in water, the red coloration will form in an oily layer at the top of the medium. More information about medium composition can be found in the Appendix at the end of this chapter (Table A4).

5.6.3 Bacteria identification

Plastics extracted from *Pagellus'* tissues (gills and GIT) and treated with liquid and solid media as described in section 5.2 "Laboratory procedures" paragraph 5.2, showed adhesion of bacteria strains. A total of 40 bacterial strains (Table 5.7) were isolated; of these, 22 (55%) from the gills and 18 (45%) from the GIT, respectively. Moreover, Table 5.7 shows that the most frequent strains detected in gills belonged to the *Vibrio* spp and *Enterobacter* spp while *Vibrio* spp, *Enterobacter* spp and *Aeromonas hydrophila* were the most frequent detected in GIT. This finding was

predictable because of the large presence of these strains in marine environments. The rest of the detected strains is made up by species normally present in the intestinal tract as commensal flora. However, it is possible to highlight the presence of bacteria, such as *Klebsiella pneumoniae*, *Pseudomonas* spp and *Proteus mirabilis,* in which recently important antibiotic resistance have been found (Ahmed et al, 2019) and with a crucial role in human pathology.

Table 5.7: bacterial strains, numbers and percentage of microorganism isolated from the gills and GIT of *Pagellus* sample.

BACTERIA STRANS (n°) (%)	Provenance of the tissue
Vibrio spp (4) (10)	GILL
Enterobacter spp (4) (10)	GILL
E. coli (2) (5)	GILL
Proteus mirabilis (2) (5)	GILL
Pseudomonas spp (2) (5)	GILL
Vibrio/Aeromonas i- (2)(5)	GILL
Aeromonas hydrophila (2)(5)	GILL
Klebsiella pneumoniae (1) (2,5)	GILL
Serratia spp (1) (2,5)	GILL
Citrobacter spp (1) (2,5)	GILL
Enterobacter spp/ Klebsiella (1) (2,5)	GILL

BACTERIA STRANS (n°) (%)	Provenance of the tissue
Vibrio spp (4) (10)	GIT
Enterobacter spp (3) (7,5)	GIT
Aeromonas hydrophila (3) (7,5)	GIT
Proteus mirabilis (2) (5)	GIT
Klebsiella pneumoniae (2) (5)	GIT
Citrobacter spp (1) (2,5)	GIT
Serratia spp (1) (2,5)	GIT
Pseudomonas spp (1) (2,5)	GIT
Enterobacter spp/ Klebsiella (1) (2,5)	GIT

5.6.4 *Kirby-Bauer* test: Screening for antibiotics susceptibility

The bacterial isolates were screened for antibiotic susceptibility by the ***Kirby-Bauer test*** with three replicates performed for each isolate (see Fig. 5.20) (Bauer et al, 1966). The isolates were harvested and then suspended in sterile water adjusted

to a 0.5 McFarland turbidity standard (bioMérieux), corresponding to 1.5×10^8 CFU ml^{-1}. The inoculum was placed onto Mueller-Hinton (MH) agar plates. The produced diameters of inhibition were measured after 24 h of incubation at 37°C. Commercially available antibacterial disks (Oxoid) to determine the resistance patterns of the isolates against 32 different antibiotics were used.

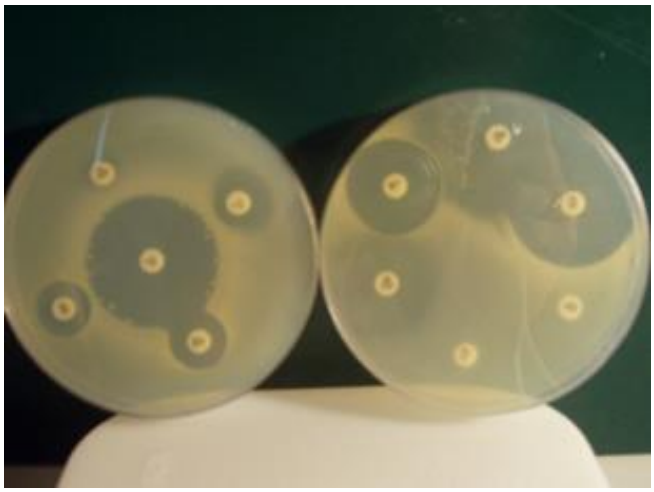


Figure 5.20: Müller Hinton Agar plates used for antibiogram (*Kirby Bauer* test).

For Gram negative bacteria, the following antibiotics were used: Nalidixic Acid (NA); Pipemidic Acid (PI); Ampicillin (AMP); Aztreonam (ATM); Azithromycin (AZM); Amoxicillin and Clavulanic Acid (AZT); Carbenicillin (CAR); Cefazolin (KZ); Ceftazidime (CAZ); Cefotaxime (CTX); Cefoxitin (FOX); Ceftazidime (CAZ); Ceftriaxone (CRO); Cefuroxime (CXM); Ciproflxacin (CIP); Chloramphenicol (C); Colistin Sulphate (CS); Ceftriaxone (CRO); Fosfomycin (FOS); Gentamycin (CN); Imipenem (IPM); Levoflxacin (LEV); Mezlocillin (MEZ); Netilmicin (NET); Nitrofurantoin (F); Norflxacin (NOR); Oflxacin (OFX); Piperacillin (PRL); Rifampicin (RD); Tetracycline (TE); Tigecycline (TGC); Tobramycin (TOB).

The diameter of the inhibition zone around each disk was measured with a precision caliper (Mitutoyo, Andover, UK). Each bacterial species was classified as resistant (R), intermediately resistant (I) or sensitive (S) according to the breakpoints established by the EUCAST (2017).

The results of *Kirby-Bauer test* are shown in Fig. 5.21. All the strains were resistant to fusidic acid. High resistance level against the antibiotic ampicillin (81%) was found.

A widespread resistance to the antibiotics cefazolin (68%), carbenicillin (57%), cefozitin (53%), amoxicillin-clavulanic acid (augmentin) (41%) while a moderate sensitivity to azitrhomicin (35%), fosfomycin (30%), rifampicin (28%), tetracycline (23%), nitrofurantoin (25%) were detected. Conversely, a high sensitivity to nalidixic acid (15%), pipemidic acid (6%), aztreonam (13%), cefotaxime (5%), ceftazidime (15%), cefuroxime (15%), tigecycline (15%), chloramphenicol (10%), gentamicin (3%), mezlocillin (8%), norfloxacin (5), ofloxacin, piperacillin, tobramycin and neomycin (3%) was observed (see Fig. 5.21).

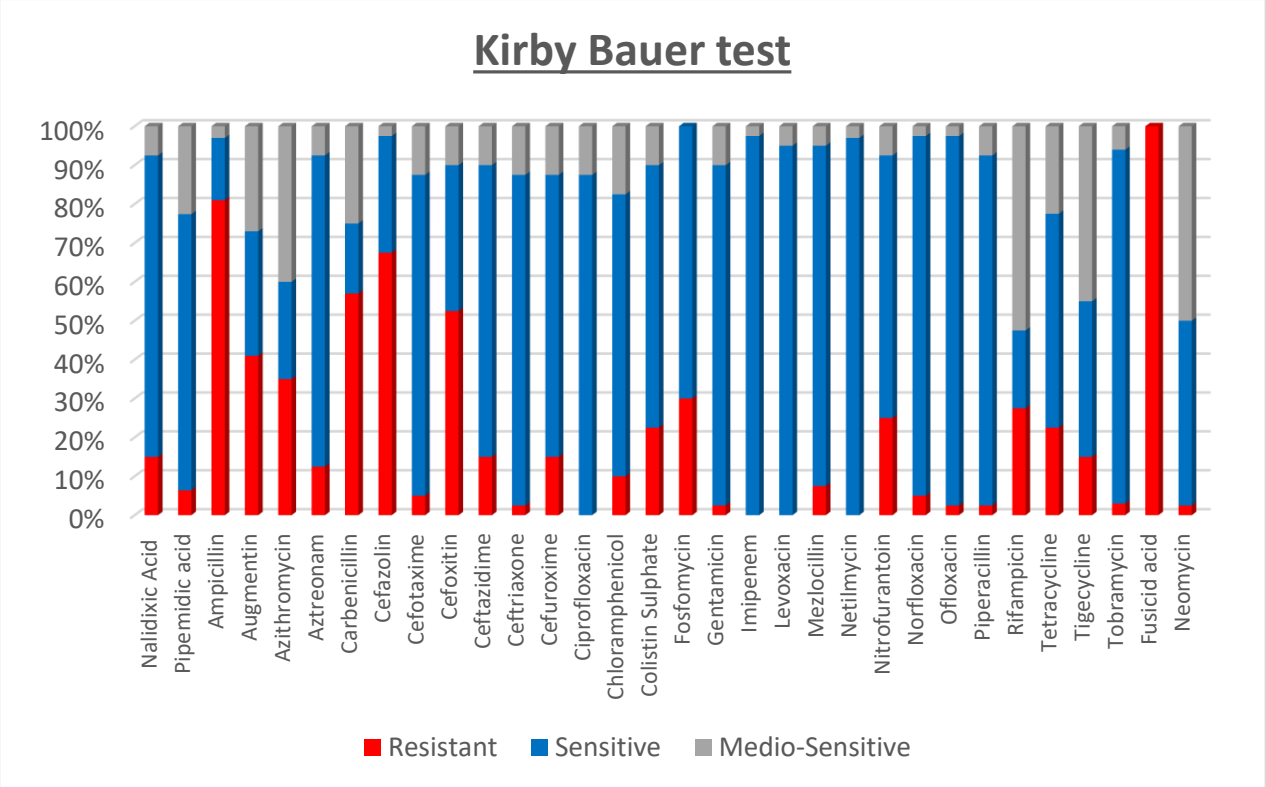


Figure 5.21: Here is reported the percentage of resistant, medio-sensitive or sensitive bacterial strains compared to the total of the isolates, as obtained from *Kirby- Bauer test* performed against eachas antibiotic.

Moreover, the susceptibility of bacterial isolates to aminoglycosides, cephalosporins and quinolones was evaluated, due to their very common use for the treatment of bacterial infections.

Resistance to cephalosporins in high percentage of strains (50-60% of the total) to cefazolin and cefoxitin, sensibility to ceftadizime and cefuroxime (15%) and high sensibility against cefotaxime and ceftriazone (from 2.5% to 5%) was detected (see Fig. 5.22).

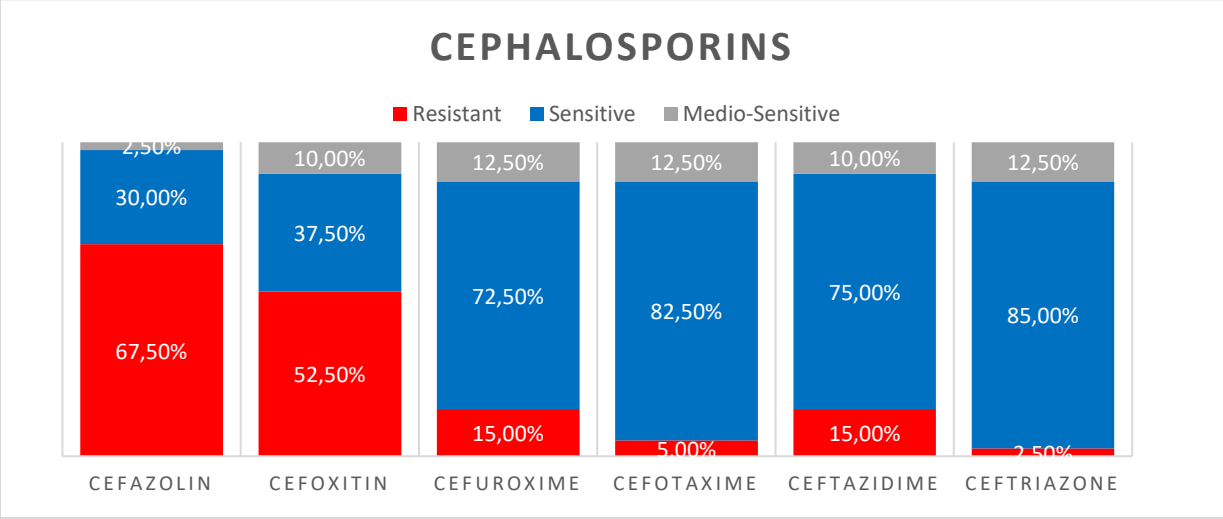


Figure 5.22: Antibiotic susceptibility profiles of the bacterial isolates against cephalosporins. First-generation cephalosporins include cefoxitin; second-generation subgroups include cefuroxime and finally, third-generation cephalosporins comprise cefotaxime, ceftazidime and ceftriaxone.

This very large level of resistance among cephalosporins is surely secondary to the different therapeutic usage time of the chosen molecules. Indeed, cefazolin and cefoxitin, both belonging to the first generation of cephalosporins, have been used for much longer than the other ones and this can determine a higher rate of resistance. Conversely, resistance to cefuroxime, ceftazidime and ceftriaxone, all belonging to the third generation of cephalosporins and for this reason used for less time than the first ones, were much less common.

Finally, resistance to aminoglycosides (gentamicin, tobramycin, neomycin and netilmycin) was detected in a low percentage of strains (2.5-3% of the total) (see Fig. 5.23).

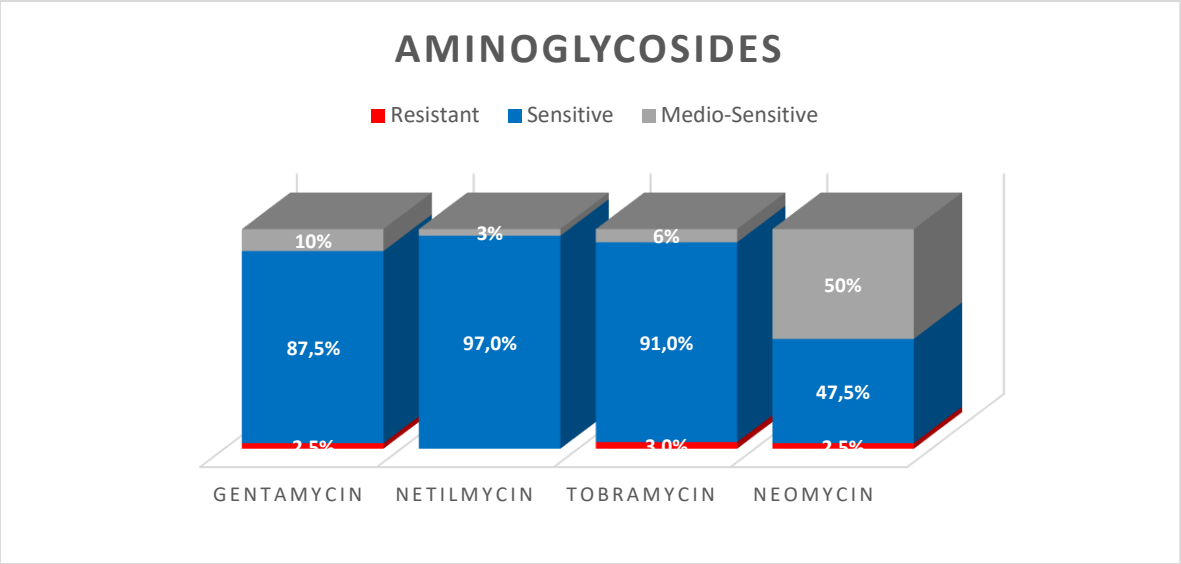


Figure 5.23: Antibiotic susceptibility profiles of the bacterial isolates against aminoglycosides.

Resistance to quinolones (nalidixin acid, ciprofloxacin, levofloxacin, ofloxacin, norfloxacin) was observed only in a discrete number of strains (from 2.5 to 15% of the total) **(**see Fig. 5.24) as well as for the cephalosporins, the highest rate of resistance (15%) was found towards nalidixic acid, the first quinolone antibiotic used, while very low resistance rates were found for the most recent quinolones drugs.

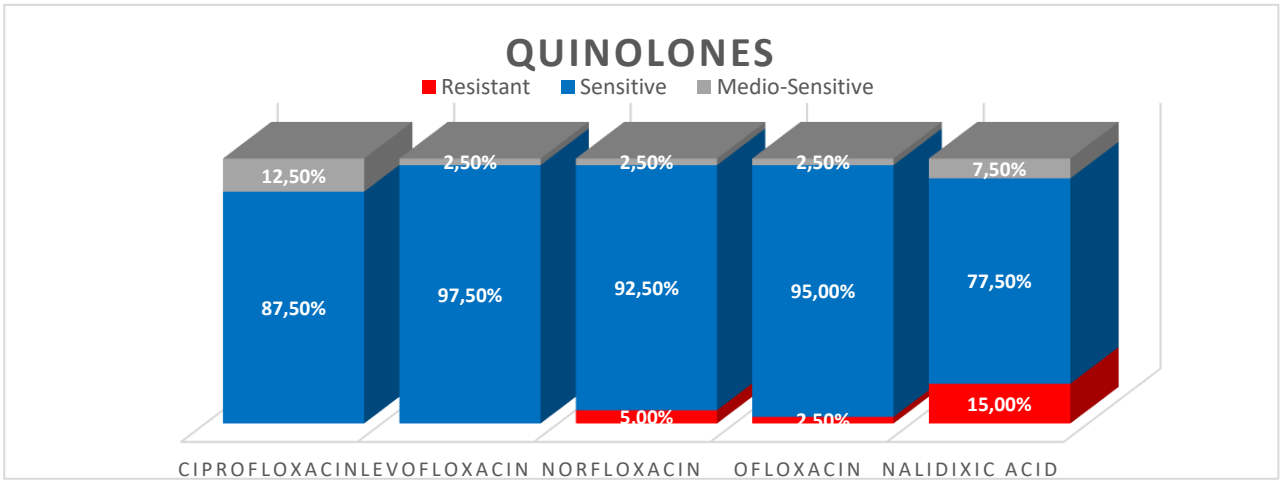


Figure 5.24: Antibiotic susceptibility profiles of the bacterial isolates against quinolones

Therefore, plastics pollutants have a proven ability to affect the growth of bacteria and composition of bacterial communities, which may further influence the spread of antibiotic resistance genes (ARG) in the environment. So, to understand the interactions between these emerging contaminants, we investigated

the variations of the ARG levels due to resistance-sensibility to antibiotics in plastics found in gills and GIT of *P. erytrinus* samples. The results showed that ARGs were enriched in the plastics-attached groups, especially in gills strains of *Vibrio* spp and *Enterobacter* spp were found, while *Vibrio* spp, *Enterobacter* spp and *Aeromonas hydrophila* were the most frequent detected ARGs in GIT.

On the overall, the current study evidenced that the presence of aquatic plastics into tissues of *P. erytrinus* promoted the propagation of ARGs. These findings have important implications for understanding the environmental risks of the combined pollution of plastics and ARGs. In addition, the *Kirby-Bauer* disk diffusion susceptibility test to 32 antibiotics indicates a widespread resistance to the antibiotics cefazolin (68%), carbenicillin (57%), cefozitin (53%), amoxicillin-clavulanic acid (augmentin) (41%), while a moderate sensitivity to azitrhomicin (35%), fosfomycin (30%), rifampicin (28%), tetracycline (23%), nitrofurantoin (25%) were observed. Here, we outline that, after entering aquatic environments, antibiotics usually undergo natural attenuation due to physico-chemical processes, like hydrolysis, photolysis, oxidation and reduction, and/or microbial biodegradation (Sun et al., 2018). On the other hand, marine bacteria are sentinels of environmental impacts due to their genome plasticity making them able to respond to xenobiotics and chemical contaminants (Nogales et al., 2011); (Caruso et al., 2016). Moreover, the presence of antibiotic residues in highly anthropogenically impacted areas, or in areas close to hospitals, is known to act as a selective pressure for antibiotics resistance (Davies et al, 2010). So, the potential for pathogenicity of bacteria strains isolates from marine plastics into tissues *P. erytrinus*, which include genes encoding ARGs represent a serious problem not only for aquatic environment but also for human health. In this contest, our study strengthens the notion that plastic pollutants may serve as vectors for transport offish pathogen as well as other opportunistic human pathogens in the marine environment. Our results are a further portion of this unexplored puzzle even if in-

depth follow-up studies are still needed for better understanding the role of plastic in the spread of antibiotic resistant pathogens in the marine environment.

In fact, in recent years the phenomenon of antibiotic resistance has also involved the food world, becoming one of the main problems of public health. The phenomenon is constantly increasing due to the widespread use of antibiotics, not only in human therapy, but also in animal husbandry and veterinary with the consequence of a rapid selection of resistant strains. The dramatic consequence of the selection of these bacteria is the loss of therapeutic efficacy of antibiotics with serious risks for the health of both humans and animals. Resistance to antibiotics can be transmitted to humans through food with various mechanisms:

1) direct transmission (raw materials contaminated with resistant strains that can colonize or infect humans after ingestion); 2) resistance transfer (food contaminated with resistant bacteria during the preparation and handling phases); 3) ingestion of resistant bacteria (through ingestion of contaminated fresh products, such as products in aquaculture and horticulture). Indeed, recent estimates based on data from EARSNet (European Surveillance of Antimicrobial Resistance Network) show that every year, more than 670 000 infections occur in the EU / EEA due to antibiotic-resistant bacteria and that around 33 000 people die as a direct result of these infections. The related cost to the health systems of the EU / EEA countries is approximately € 1.1 billion.

The phenomenon is also a Food Safety problem: the often indiscriminate use of antibiotics in farm animals for therapeutic treatment, prevention or growth promotion (the latter practices now prohibited in Europe), allows resistant bacteria and resistance genes to reach man through the food chain. The foods we consume, especially if raw, can be contaminated with bacteria and therefore represent a potential way of transmission of resistant strains. Throughout the world, various authorities are responsible for food safety. In Europe, the EFSA (European Food Safety Authority) assesses, among other things, the risks related to feed safety and EFSA and ECDC (European Center for Disease Control) supervise antibiotic

resistance in animals and humans, using the data reported by the Member States. EFSA and ECDC agencies collaborate with the EMA (European Medicines Agency) to analyze the relationship between the use of antibiotics and resistance in both food-producing animals and humans.

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5.7 Appendix 1: media composition and information

Brain Heart Infusion (HB)

Enzymatic digest of animal tissues and brain-heart infusion provide amino acids, nitrogen, carbon, vitamins and minerals for bacterial growth. Glucose is the carbohydrate source. Sodium chloride maintains the osmotic balance of the medium. Disodium phosphate is the buffering agent. The protocol plans to suspend 37 g of medium powder in 1 liter of distilled or deionized water. Heat shaking frequently until completely dissolved. Distribute into tubes and sterilize in autoclave at 121°C for 15 minutes.

Table A1: Brain Heart Infusion (HB) composition.

Typical Formula	(g/l)
Enzymatic Digest of Animal Tissues	10.0
Dehydrated Calf Brain Infusion	12.5
Dehydrated Beef Heart Infusion	5.0
Glucose	2.0
Sodium Chloride	5.0
Disodium Hydrogen Phosphate, Anhydrous	2.5
Final pH 7.4 ± 0.2 at 25°C	

Hektoen Enteric Agar (HEA)

Enzymatic digest of meat provides amino acids, nitrogen, carbon, vitamins and minerals for organisms growth. Yeast extract is a source of vitamins. Lactose, saccharose and salicin are fermentable carbohydrates. The presence of bile salts and acid fuchsin inhibits Gram-positive organisms. Sodium chloride maintains the osmotic balance of the medium. Ammonium ferric citrate and sodium thiosulfate enable the detection of hydrogen sulfide production. Bromothymol blue together with acid fuchsin are the pH indicator system. Agar is the solidifying agent. The protocol plans provides to suspend 76 g of the powder in 1 liter of distilled water.
Heat to boil shaking frequently until completely dissolved. This type of medium doesn't have to be autoclaved. So, melt the content of the bottle in water bath until completely dissolved. Then cool about at 45-50°C, mix well and aseptically distribute into Petri dishes.

Table A2: Hektoen Enteric Agar composition

Typical Formula	(g/l)
Enzymatic Digest of Meat	12.0
Yeast Extract	3.0
Lactose	12.0
Saccharose	12.0
Salicin	2.0
Bile Salts No.3	9.5
Sodium Chloride	5.0
Sodium Thiosulfate	5.0
Ammonium Ferric Citrate	1.5
Acid Fuchsin	0.1
Bromothymol Blue	0.065
Agar	15.0
Final pH 7.5 ± 0.2 at 25°C	

Triple Sugar Iron Agar (TSI)

Triple Sugar Iron Agar is a medium used for the identification of Enterobacteriaceae, grown on soils selective or moderately selective, based on the fermentation of lactose, glucose and sucrose, on the production of hydrogen sulphide and carbon dioxide.

Table A3: Triple Sugar Iron (TSI) composition

Typical Formula	(g/l)
Beef extract	3.0
Yeast extract	3.0
Peptone	20.0
Glucose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous sulfate or ferrous ammonium sulfate	0.2
NaCl	5.0
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	13.0
Distilled water	1,000

The protocol provides to suspend 64,6 g of the powder in 1 liter of distilled water. Heat shaking frequently until completely dissolved. TSI medium must be sterilized at 121°C for 15 minutes in autoclave.

A. With a sterilized straight inoculation needle touch the top of a well-isolated colony;
B. Inoculate TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant;
C. Incubate the tube at 37°C for 24 hours.

Interpretation of Triple Sugar Iron Agar

1. If lactose (or sucrose) is fermented, a large amount of acid catabolites are produced, which turns yellow the phenol red indicator in the slant. Some organisms generate gases, which produces bubbles and/or cracks on the medium;
2. If lactose is not fermented but the amount of glucose is, the oxygen-deficient butt will be yellow, but on the slant the produced will be oxidized to carbon dioxide and water by the organism and the slant will be red (alkaline or neutral pH);
3. If H_2S is produced, the black color of ferrous sulfide is seen;

4. If any sugar is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of the production of ammonia from the oxidative deamination of amino acids.

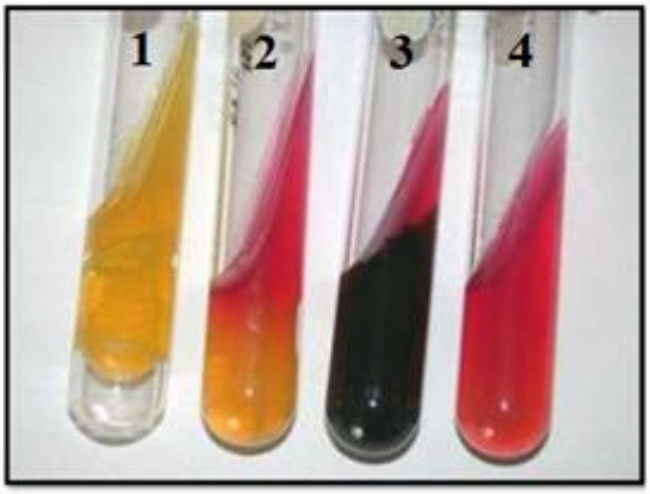

Figure 5.25: TSI in relation to the numerations (1,2,3 and 4) in the above presented list-text.

Mackenzie test

Peptone-Tryptone Water, given the high tryptophan content of tryptone, is particularly suitable as a substrate for determining indole production. The ability to metabolize tryptophan with indole formation is a distinguishing feature of some bacterial species; the test is therefore useful for the purpose the identification and classification of microorganisms. Incubation time and temperature vary to depending on the bacterial species under examination; the presence of indole can be detected with Kovac's reagent.

Table A4: Kovac's Reagent.

Kovac's Reagent	(g/ml)
p-Dimethylaminobenzaldehyde	50.0 g
Hydrochloric Acid, 37%	250.0 ml
Amyl Alcohol	750.0 ml

1. Take a sterilized tube containing Peptone Water;
2. Inoculate the tube aseptically with a colony grown for 24 h;
3. Incubate the tube at 37°C for 24 h;
4. After the incubation, add 2-3 drops of Kovac's Reagent into the medium;
5. Observe for the presence or absence of red ring.

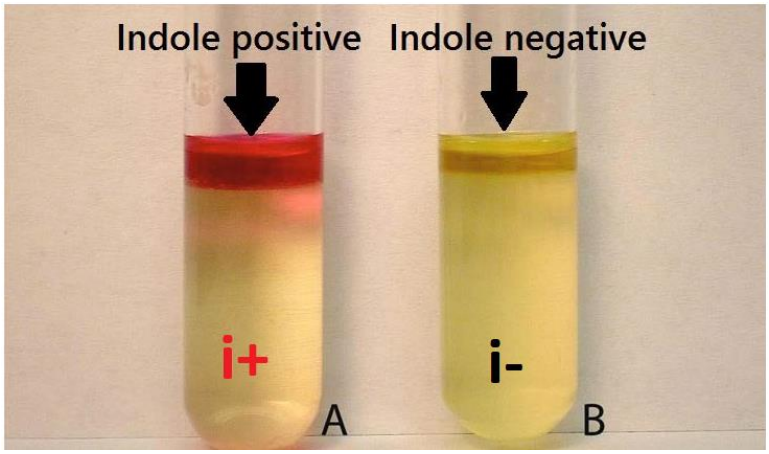

Figure 5.26: Visible reaction and formation of "cherry-red ring"

Chapter 6

Conclusions, challenges and future perspectives

In this thesis we have tried to give an assessment to identify the main sources, fate and effects of microplastics in the Italian sea to provide an improved understanding of the scale of the problem posed by the presence of microplastics, and of the link between larger plastic items (macro- and mega-plastics) and the generation of 'secondary' microplastics. From these studies it can be concluded that it is advisable to integrate science and social science efforts early on in order to make sure the public is informed of the issue and potential solutions. Certainly, many initiatives can contribute to the goal of increasing the awareness on the impacts of microplastics.

Today, there is no one perfect solution to the issue, but there are numerous approaches, such as changing the legislation, improving plastic waste facilities and management, changing plastic use and consumption, and education and public engagement should be part of these. A combination of these factors will help address the issue of microplastics in the aquatic environment. Overall, understanding risk (and benefit) perception is important, as these have been found to influence behaviour and acceptability of regulatory approaches. There has been recent interest in a potential technological solution of mechanical cleaning of the open ocean. Unfortunately, ascribing responsibility and capabilities to technological solutions could encourage a negative spill-over effect, promoting an unintended behaviour of people not managing their waste responsibly. Such a spill-over effect would be particularly concerning if the technological fix was actually ineffective.

In addition to reviewing the limited research on microplastics and making inferences from related research, this thesis has highlighted where literature

data dedicated to microplastics explicitly is missing. For instance, very little is known about individuals' knowledge and understanding, perceived risks, and the associated consequences of microplastics specifically on humans.

Future research needs to consider methodologically these aspects. For instance, studies on individuals' knowledge and understanding of microplastics should consider regional, demographic, and individual differences. Also, surveys should include a wider geographical coverage, since to date only a select number of countries is represented (e.g. US and Europe). Finally, new studies should address the economic consequences of microplastics. At the same time, attention should be focused on potential solutions and their acceptability, including social 'solutions' such as education, public engagement and behaviour change campaigns.

Thus, comprehensive improvement to waste generation and management practices is essential in order to reduce the entry of plastics and microplastics into the marine environment. This requires an adequate understanding of the relative importance of different types of materials and sources at global, regional and sub-regional scales, and the socio-economic sectors involved. The input of macro-plastics and microplastics is highly variable and poorly quantified on a regional basis, presenting great difficulties in designing and implementing cost-effective mitigation strategies. Moreover, reducing the input of macro-plastics represents the most effective way of minimizing the increase in the abundance of microplastics in the sea.

On the overall, the main future challenges and prospective are summarized:

1) identify and create a database with the main sources and categories of plastics and microplastics entering Mediterranean and Tyrrenia sea. To reach this aim, identify probable 'hotspots' of land- and sea-based sources for plastic and microplastics, using a combination of targeted modelling, knowledge of actual and potential sources (e.g. coastal tourism, aquaculture, fisheries, riverine inputs, urban inputs), environmental and societal data. This will allow

mitigation measures to be better targeted, and used to predict and verify their effectiveness. Among the 'hot spots' identified by EU, Mediterranean Sea is included.

2) Utilize end-of-life plastic as a valuable resource rather than a waste product. This will be aided by the development of innovative and effective solutions as an intrinsic part of the circular economy. In this way 'unwanted' plastic can be seen as a useful resource, with commercial value, rather than a waste problem requiring the allocation of scarce public and private sector resources. Such action reduces our reliance on non-renewable reserves of oil and gas to produce plastics and reduces the need for waste management, for example via landfill. This is a rapidly developing field that is being embraced by business and institutions, and it needs to be encouraged at a global level. However, adequate controls have to be in place to ensure that plastic waste treams are separated appropriately, to reduce the potential for unnecessary and unwanted cross-contamination, especially of consumer products made with recycled plastic. Commercially available 'biodegradable' plastics do not offer a viable alternative, and in most cases will not lead to a reduction in microplastic formation.

3) Promote greater awareness of the impacts of plastics and microplastics in the marine environment. For this scope, facilitate the transfer of complex and uncertain scientific findings into a language that can be understood by target stakeholder groups (e.g. industrial production, manufacturing, retail, fisheries, aquaculture, coastal tourism, shipping). Take proper account of regional, cultural, gender, economic, educational and other demographic differences, in assessing perceptions and behaviours.

4) Include particles in the nanosize range in future assessments of the impact of plastics. The word 'microplastics' refers particles ranging in size over several orders of magnitude, from particles several mm in diameter to those in the

nano size range (<1 μm). Particles in these size ranges may be introduced directly or may gradually form by fragmentation. The sampling methods normally used to collect microplastics tend to exclude material <330 μm. This means there is very limited information about the occurrence of finer plastic particles, including nano-plastics, in our sea and particularly the degree to which nano-plastics interact with biota internally. The available evidence from the medical, pharmaceutical and toxicology literature suggests that nano-sized particles are much more likely to cross cell membranes and induce a response that may adversely affect marine life. Therefore they have the potential to pose a greater risk to the organism than micro-sized plastics. Unfortunately, at present it is not possible to provide a credible assessment of the extent to which nanoplastics do present a risk.

5) Evaluate the potential significance of plastics and microplastics as a vector for organisms in future assessments. There is emerging evidence that some organic compounds present as additives, in relatively high concentrations in some categories of plastic, are capable of transferring into the body tissue of fish-eating sea birds. This has been demonstrated for PBDE23 flameretardants using a chemical fingerprinting technique, which can distinguish the composition of PBDEs in the plastic particles and the tissue of prey species. The extent to which other chemicals are bioavailable to species of interest is unknown. The role of digestive fluids in influencing transfer rates (e.g. utilizing fish oil for digestion in birds) is also unclear. The extent to which this poses a risk to individual organisms, or to the population as a whole, and to predators of the affected species, including humans, remains untested.

6) Future assessments should address the chemical risk posed by ingested microplastics in greater depth. To reach this aim, compare information from laboratory-based experiments of the bioavailability of the target chemicals with field-based observations of their distribution in the tissue of marine

organisms. Moreover, take account of gut retention times and gut environment when assessing risk of damage, also including considerations of microplastic particle size and shape.

Ultimately, it will important to encourage the inclusion of expertise on pharmacology, mammalian toxicology, nano-polymer sciences and nano-engineering in future assessments to critically review laboratory-based experiments. All that to examine the behaviour and potential effects of micro/nano-plastics in order to evaluate their relevance to the natural environment and to assess current sampling and detection methods for nano-sized plastic particles, particularly in biota.