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Corso di laurea magistrale

Biologia marina

Prime prove della presenza di microplastiche nel sangue e nelle gonadi del tonno rosso atlantico (*Thunnus thynnus*): implicazioni sulla riproduzione

First evidence of presence of microplastics in blood and gonads of Atlantic bluefin tuna (*Thunnus thynnus*): implication in reproduction

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RIASSUNTO

La produzione su larga scala di materiale plastico, iniziata a partire dagli anni ’40, ha portato, negli ultimi 70 anni, ad un aumento dell’inquinamento dato da plastiche e microplastiche a livello globale, interessando anche aree incontaminate come i Poli. Attualmente la massiccia presenza di microplastiche nell’ambiente marino ha sollevato molte preoccupazioni, poiché le microplastiche, ormai considerate inquinanti ubiquitari, possono rappresentare un rischio per gli organismi marini a causa della loro elevata biodisponibilità. Infatti, le microplastiche possono essere ingerite, poiché scambiate per prede, da organismi ai più bassi livelli trofici e poi con il trasferimento lungo la catena trofica raggiungono anche i livelli più elevati. Inoltre, negli ultimi anni molti studi si sono focalizzati anche sulla presenza di altri contaminanti adsorbiti sulla superficie delle microplastiche oppure aggiunti durante il processo di produzione delle materie plastiche.

Il presente lavoro ha lo scopo di studiare la presenza ed il possibile impatto negativo delle microplastiche nelle gonadi di una specie molto importante dal punto di vista commerciale, come il tonno rosso Atlantico (*Thunnus thynnus*). Il tonno rosso è considerata una specie top predator, quindi considerando le

sue abitudini alimentari, può entrare in contatto con le microplastiche sia tramite ingestione diretta che indirettamente attraverso il trasferimento trofico da parte di prede contaminate.

Durante questo progetto è stato messo a punto un protocollo ottimale per l'estrazione delle microplastiche a partire da matrici biotiche. È stata utilizzata una soluzione basica (10% KOH) per rimuovere l'eccesso di materiale organico e quindi facilitare l'analisi delle particelle sintetiche trovate. Il tipo di polimero trovato è stato confermato grazie alle successive analisi spettroscopiche al Raman.

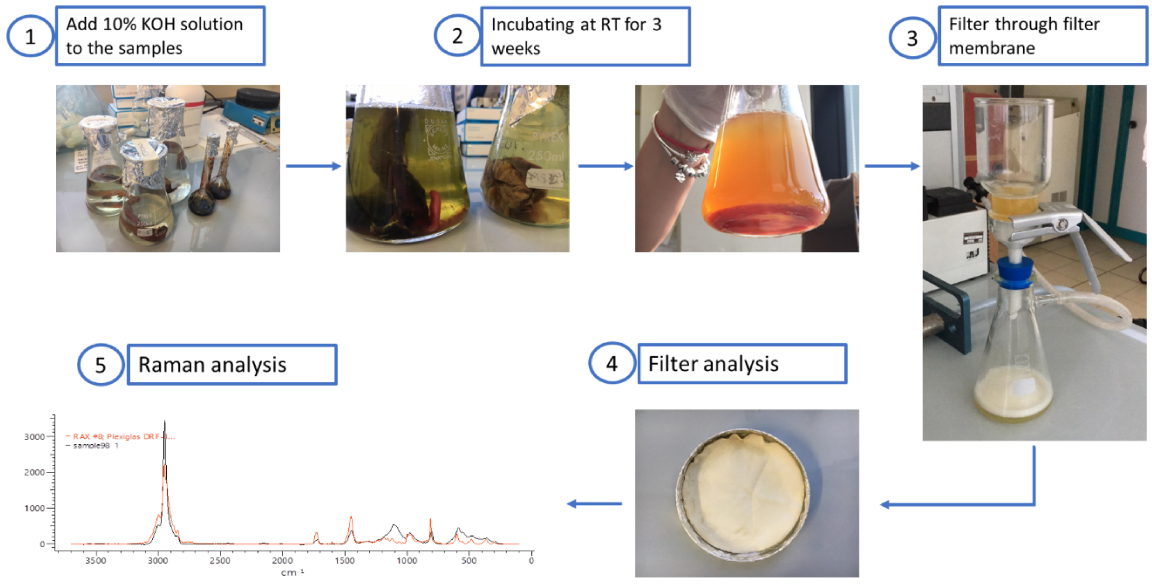

Figure 1. Rappresentazione grafica del protocollo utilizzato

I risultati ottenuti attestano la presenza di microplastiche in gonadi, intestino e sangue di tonno rosso Atlantico. Inoltre, mostrano una differenza tra i livelli ritrovati in organismi maschili e femminili, poiché la più alta incidenza sia di materiale esogeno che di centri di melanomacrofagi è stata trovata in individui femminili nel gruppo non-riproduttivo. In aggiunta molti frammenti trovati erano ricoperti da pigmenti inorganici, la cui presenza non ha permesso l'identificazione spettroscopica del materiale sottostante.

I risultati di questo studio rappresentano una prima prova della presenza di contaminazione da microplastiche nel tonno rosso Atlantico. Inoltre, la presenza di pigmenti inorganici pone le basi per future ricerche focalizzate sulla loro possibile tossicità per gli organismi marini.

1 INTRODUCTION

1.1 MICROPLASTICS

1.1.1 Origin, sources and types of plastics

Coastal and marine areas are under constant and increasing pressure from human activities. Pollutants such as pesticides, persistent organic pollutants (POPs), hydrocarbons, heavy metals, plastics and microplastics have a huge impact on the marine ecosystem. Marine litter has become a global environmental problem that affects all parts of our oceans and causes biological, economic and environmental issues that occur due to poor waste management practices (Auta et al. 2017).

One of the most important components of marine litter is plastic which makes up about 80 to 85% of it (Dehaut et al. 2016). Thanks to its economic, light and durable properties, plastic occupies a very important place in our society with vast commercial, industrial, medicinal and municipal applications. However, plastic waste has contaminated the environment through its disposable applications, such as plastic bags with low recovery value and degradation problems (Wang et al. 2016). World annual production of plastics

has been constantly increasing since 1950, being estimated at 0.5 million tonnes in 1960, 311 million tonnes in 2014 and 348 million tonnes in 2017, of which 64.4 million tonnes in Europe alone (PlasticsEurope 2018). Asia is the largest producer of plastic materials, followed by Europe, North America, Middle East Africa and Latin America (Prokić et al. 2019). It is reported that the level of microplastics pollution is higher in undeveloped areas due to the lack of proper waste management leading to a transfer of pollution from the ground to the ocean (Rezania et al. 2018). It is estimated that at least 8 million tonnes of plastic waste ends in the oceans each year (Wang et al. 2019). The World Economic Forum in 2016 estimated that by 2050 there will be more plastics by weight in our oceans than fish (Farady 2019).

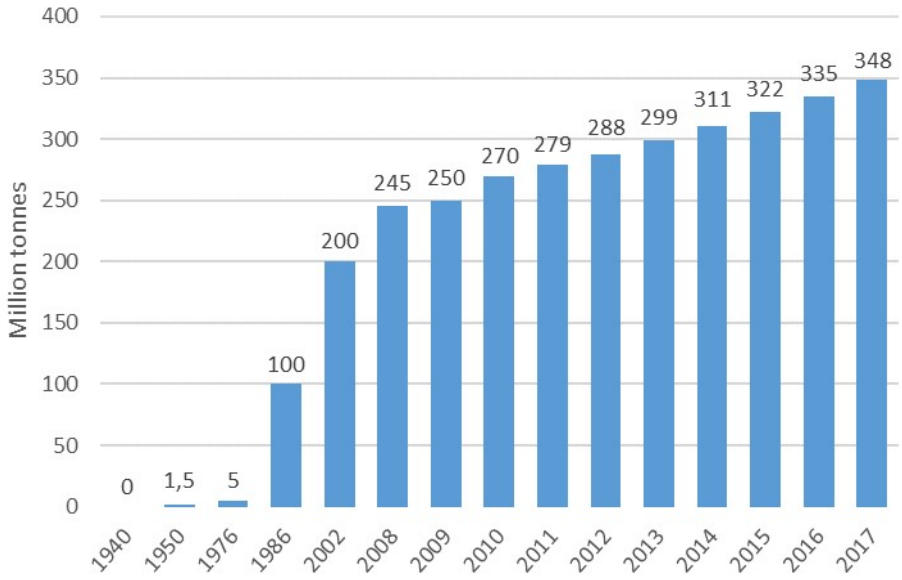

Figure 2. Increase in plastic production worldwide

Plastic is a term derived from the Latin "*plasticus*", which derives from the Greek "*plastikos*" and was used to describe something that could be molded or suitable for molding (PlasticsEurope 2018). Generally, plastics are based on polymer structures with complex mixtures of mainly synthetic organic compounds that are linked together during polymerization. Plastic was first invented in 1839 by Eduard Simon as polystyrene (a strong plastic created from ethylene and benzene); following its utility and versatility in the production of other materials such as beverage cups and peanut packaging. In 1862, Alexander Parkers invented the first artificial plastic called Parkesine (an organic material derived from cellulose that, once heated, could be shaped and maintain its shape when cooled). By 1897, the efforts to produce chalkboards led to the synthesis of casein plastic from milk proteins mixed with formaldehyde. In 1899, Arthur Smith discovered phenol-formaldehyde resins that were used as a substitute for ebonite in electrical insulation. In 1907 Leo Hendrik Baekeland improved the phenol-formaldehyde reaction techniques to develop the first synthetic resin (first modern plastic) under the brand name Bakelite (Alimba and Faggio 2019). The invention of Bakelite increased the knowledge of low-cost techniques that have improved the production of modern plastics: a large family of different materials with various characteristics, properties, and uses, designed to meet the very different needs of thousands of end products. The plastic discovery was

subsequently followed by the mass production of plastic related products starting from the 1940s (Alimba and Faggio 2019; PlasticsEurope 2018).

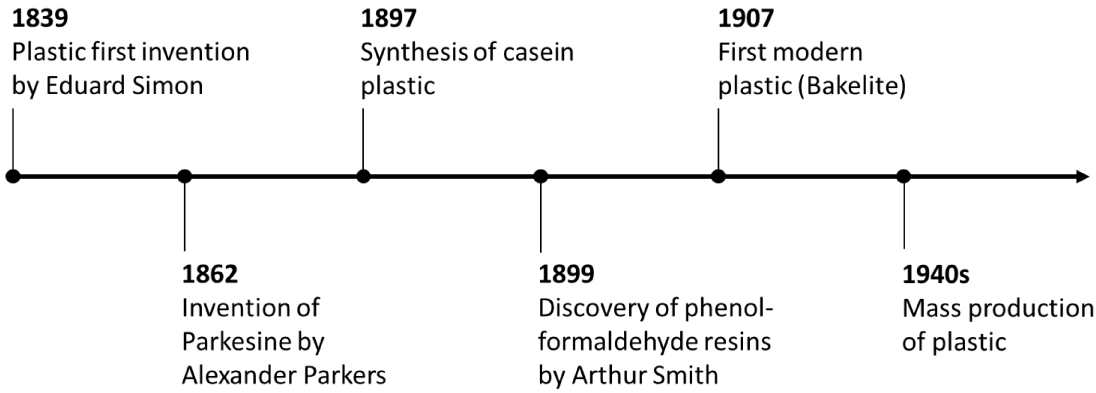

Figure 3. Plastic evolution timeline

Today's plastic can be fossil-based or biologically-based and, in both cases, they can also be biodegradable. Plastics can be divided into two major categories: thermosets and thermoplastics. Thermoset plastics are a family of plastics that cannot be re-melted and reformed if heated, as they will undergo chemical modification, leading to degradation of their respective characteristics. This category includes polyurethane (PUR), epoxy resins, silicone, vinyl ester, epoxy resins, acrylic resins and unsaturated polyester, and represents plastics that are used in insulation, coating, adhesive, composite, pneumatic and balloon applications. On the contrary, thermoplastics melt when heated and hardened when cooled, in a reversible way: therefore, they can be heated, remodelled and frozen repeatedly. Polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC),

polyethylene terephthalate (PET), polystyrene (PS), expanded polystyrene (EPS), polyamide (PA), polycarbonate (PC) belong to this category, and represent plastic materials used for bottle, food container, pipe, textile, fishing gear, milk jug, film, bag, cigarette butt, just to mention a few (PlasticsEurope 2018).

Nowadays, plastics include more than twenty families of polymers among which six are known as the "big six": polypropylene (PP), high and low density polyethylene (HDPE and LDPE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET) and polystyrene (PS), which are mainly produced from fossil fuels such as oil, natural gas or coal, and are designed to meet the very different needs of the end products. The "big six" represent 80% of plastic production in Europe.

Table 1. Different plastics for different needs (Wang et al. 2016)

Classifications	Abbreviation	Density (g L^{-1})	Products	Recycling symbols
Polyester	PET	1.37	Soft drink, water, juice, beer bottles	
High-density polyethylene	HDPE	0.94	Milk jugs, juice bottles, bleach, detergent and household cleaner bottles, butter and yogurt containers	
Polyvinyl chloride	PVC	1.38	Window cleaner and detergent bottles, shampoo bottles, cooking oil bottles, clear food packaging, medical equipment, boots	
Low-density polyethylene	LDPE	0.91-0.93	Plastic bags, six-pack rings, netting, drinking straws, wire cables	
Polypropylene	PP	0.85-0.93	Rope, bottle caps, netting, car bumpers, flowerpot, folders	
Polystyrene	PS	1.05	Disposable plates and cups, meat trays, egg cartons, carry-out containers, aspirin bottles	
Others			DVDs, sunglasses, phone and computer cases, signs and displays, nylon, “bullet-proof” materials	

Plastics are mostly non-biodegradable, and because of their nature as durable substances, their presence and impacts on the marine environment will continue for decade to come (Fackelmann and Sommer 2019; Farady 2019). Since a very small amount of plastic is recycled, it undergoes fragmentation or degrades at a very slow rate, being accumulated in all environments. Released plastics are commonly subjected to progressive fragmentation under the action of physical-chemical and biotic environmental factors, such as mechanical abrasion, ultraviolet radiation and biological degradation by microorganisms (Wang et al. 2019). Given this continual fragmentation of plastic debris into smaller particles, their concentrations are likely to increase with decreasing size over time (Wright et al., 2013).

The first observation of plastic pollution in the marine ecosystem was recorded in 1972 by Carpenter and Smith, while they were sampling the pelagic Sargassum community of the Western Sargasso Sea (Carpenter and Smith 1972). They attributed their observation to the dumping of waste from cities or cargo and passenger ships and suggested that as the rate of plastic production increases, combined with bad waste disposal practices, plastic litter will accumulate at sea over time. Forty years after their report, plastic pollution in the marine environment has become a growing issue of global environmental pollution and public health (Alimba and Faggio 2019; Dehaut et al. 2016).

Plastics found in the aquatic environment are different in size, colour, specific density, chemical composition and shape. According to the size they can be divided into four different categories: macroplastics (> 25 mm), mesoplastics (5-25 mm), microplastics (< 5 mm) and nanoplastics (< 1 μm) (Gigault et al. 2018).

The term "microplastics" (MP) was introduced in 2004 to describe microscopic particles of plastic debris in the marine environment. In the first international microplastics workshop held by the National Oceanographic and Atmospheric Agency (NOAAA) in Washington, United States, it was concluded that MPs should include all plastic debris with a diameter of less than 5 mm. Likewise, the European Union with the Marine Strategy Framework Directive (MSFD) adopted the NOAAA definition and set the 5 mm diameter of plastic litter as the upper limit for the classification of microplastics (Alimba and Faggio 2019).

Researchers have divided microplastics according to their shape into five groups: microbeads (used in personal care products), nurdles (used in manufacturing), fibers (generated from clothing), foam (derived from food containers and drink cups), fragments (derived from larger plastic products degradation) and films. Moreover, MPs have been further classified into large microplastics (1-5 mm) and small microplastics (0.3-1 mm), based on the characteristic of continuous breakdown (Wu et al. 2019).

MPs are further classified into two different categories, primary and secondary microplastics according to their origin and source in the marine environment. Primary microplastics are mainly released from terrestrial sources directly into the marine environment and are purposely produced for specific industrial or domestic applications, such as resin pellets in the plastics industry, or as precursors for the production of consumer products such as cosmetics, exfoliating facial scrubs, toothpaste, abrasives present in cleaning agents, insect repellents, sunscreen and synthetic clothing fibers. These tiny particles enter directly into the marine environment through wastewater and sewage or industrial discharges. Secondary microplastics present in the marine environment result from *in situ* fragmentation of larger plastics into small particles through photothermal degradation, oxidation and mechanical abrasion (Alimba and Faggio 2019; Auta et al. 2017). Recent studies have shown that most microplastics, either produced as primary and secondary, are supposed to continue to fragment until they reach the nano-size scale (< 1μm), or degrade continuously until the polymer is entirely mineralized into carbon dioxide (CO_2), water and biomass (Alimba and Faggio 2019).

MPs reach the marine environment through different pathways, both from terrestrial and marine activities. A large amount of this plastic debris comes from continental sources that enter the marine environment mostly through

rivers, industrial and urban effluents and the runoff of beach sediments. The other part comes from direct inputs, like offshore industrial activities (e.g. aquaculture, oil and gas extraction), loss of fishing nets and waste released during maritime activities, including tourism (Barboza et al. 2018). Microplastics also get into the marine environment via storm, sewers, wind and currents and some are transported out to sea via runoff (Alomaret al. 2016; Auta et al. 2017).

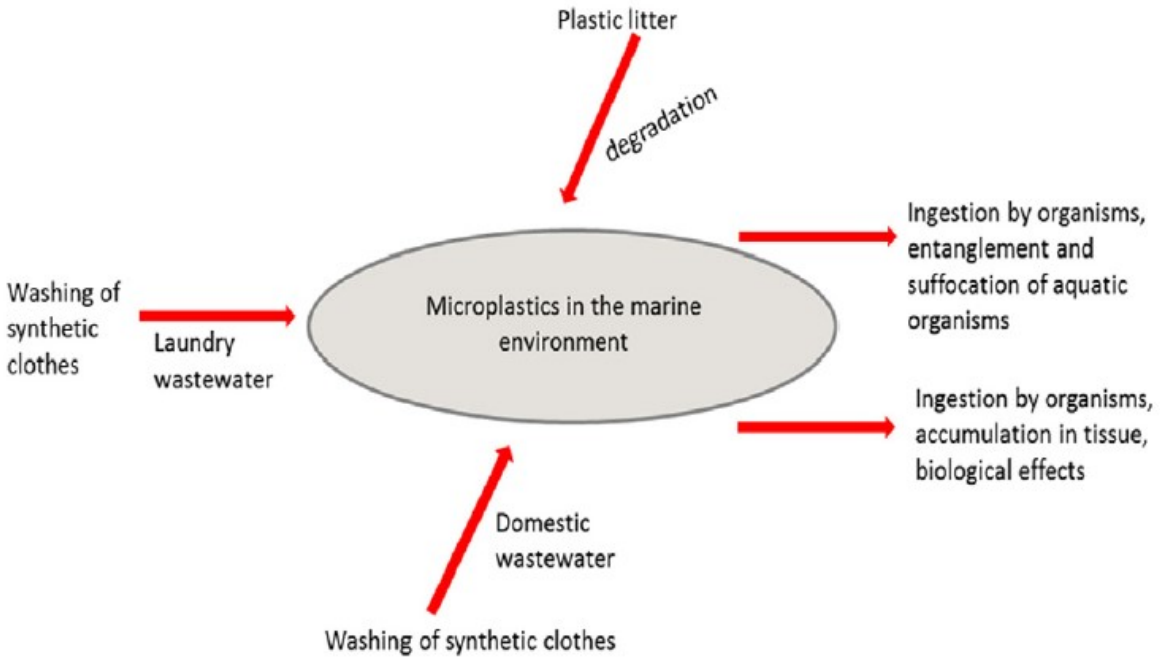

Figure 4. Sources of microplastics in the marine environment (Auta et al. 2017)

The size of the microplastics and their low density contributes to the widespread transport and distribution over long distances. These small marine plastics debris are abundant and present in all aquatic habitats in the world

and also found in the Arctic and Antarctic seas, transported by ocean currents and wind. As a result they are now considered "ubiquitous pollutants".

Because of their low density, plastic debris float on the sea surface, and therefore tends to accumulate in large subtropical ocean gyres. Floating microplastics have been found in five large offshore accumulation zones, such as the North Pacific subtropical gyre, the North Atlantic subtropical gyre, the South Pacific subtropical gyre, the South Atlantic subtropical gyre and the Indian Ocean gyre (Wu et al. 2019).

Another way through which microplastics could enter the oceans is through zooplankton feces, as demonstrated by Cole et al. (2016). In this study, they exposed zooplankton (*Calanus helgolandicus* and *Calanus typicus*) to polystyrene microplastics. The organisms easily fed on the microplastics that passed through the intestine, were encapsulated in feces and were egested. After egestion, the feces sank and were subsequently ingested by the larger copepod. The study showed that MPs can be indirectly ingested through the consumption of faecal pellets, demonstrating that faecal pellets are a source of microplastics in the marine environment.

1.1.2 Evidence in marine organisms

It has been observed that 690 species of aquatic fauna, including 86% of all sea turtle species, 44% of all seabird species and 43% of all marine mammal

species have been exposed to plastic debris. Plastic debris is found in the intestinal content of fish worldwide, including estuaries, pelagic and demersal habitats (Wang et al. 2016).

Microplastics can be uptaken by marine organisms by different processes, among these, ingestion is believed to be the main microplastic exposure route for several marine species (Barboza et al. 2018). Evidence of microplastics ingestion has been observed in a long list of aquatic fauna ranging from small invertebrates to large predatory mammals (Wang et al. 2019). Ingestion of microplastics by marine organisms in most cases is accidental because the particle is often mistaken for food, although some can be specifically targeted by some organisms. There are many factors that influence the possibility of ingestion of microplastics. Compared with predators, filter and deposit feeders are more susceptible to microplastics uptake, because of their unselective feeding strategy. The microplastics size determines the aquatic taxa that ingest them, since organisms are more likely to consume particles with a similar size range as their natural preys. The varying polymer densities of microplastics influence their vertical distribution in the aquatic environment from surface water to benthic sediment, thus influencing the bioavailability to low-density floating plastics such as PE and PP, while benthic taxa are more likely to encounter high-density plastics, such as PVC, PS and PET. The buoyancy of plastics is also influenced by biofouling,

because the formation of biofilms on the surface of microplastics can affect their vertical transport. Other factors such as the colour, shape and abundance of microplastics can also affect the bioavailability of microplastics in the aquatic environment (Wang et al. 2019). This poses a great risk to the organisms as the ingestion of these tiny plastic particles have been reported to reduce the feeding rate, the body mass, the metabolic rate, the allocation of energy for growth and the swimming performance, to decrease predatory performance and fertilization, to change behavioural responses, to cause larval abnormalities, neurotoxicity, oxidative stress (Barboza et al. 2018; Wang et al. 2019), intestinal damage (Cole et al. 2013) and several other adverse effects. Many studies showed that the ingested plastic blocked the flow of food materials in the alimentary canal leading to false satiation and to increase starvation, compromised feeding potential and digestion; therefore, increase in morbidity and mortality (Alimba and Faggio 2019; Auta et al. 2017; Lusher et al. 2013; Wang et al. 2019).

Several studies have been carried out on microplastic ingestion by different fish species. Romeo et al. (2015) investigated the presence of plastic debris in stomach of large pelagic fish in the Mediterranean Sea, including swordfish (*Xiphias gladius*), bluefin tuna (*Thunnus thynnus*) and albacore (*Thunnus alalonga*), and observed that 18.2% of the stomach contents contained micro-, meso- and macro-plastic debris. Lusher et al. (2013) found microplastics in

36.5% of the gastrointestinal tracts of pelagic and demersal fish. Neves et al. (2015) reported the presence of microplastics in the stomach in 63.5% of benthic fish and 36.5% pelagic fish species. Xiong et al. (2018) found microplastics in all intestinal tracts of East Asian finless porpoises (*Neophocaena asiaeorientalis sunameri*) analysed in the study. Abbasi et al. (2018) confirmed the presence of microplastics in almost all the analysed tissues of fish and prawn from the Musa Estuary in the Persian Gulf. Karbalaei et al. (2019) reported the presence of plastic debris in organs and gills of 9 of 11 commercial marine fish species. Bernardini et al. (2018) found plastic litter in 25% of blue sharks (*Prionace glauca*) from the Ligurian Sea and reported that juvenile are more likely to ingest marine litter than adults. Collard et al. (2017) found microplastics in livers of European anchovies (*Engraulis encrasicolus*) and highlight the translocation issue from intestinal tracts to other tissues. Baalkhuyur et al. (2018) reported the presence of microplastics fragments in the gastrointestinal tracts of 14.6% analysed fish from the Saudi Arabian Red Sea coast. The presence of plastic debris has also been detected in seafood sold for human consumption, as well as in fish and shellfish purchased from markets (Barboza et al. 2018).

1.1.2.1 Fate of microplastic ingested by marine organism

Different studies demonstrate that microplastics can be taken up by different marine organism and once ingested:

- Microplastics can be eliminated from the organism through excretion or production of pseudofaeces, thereby having no long-lasting effect on the organism (Browne et al. 2008).
- Microplastic can remain within the organism and translocate among tissues, with unknown precise mechanisms.
- Microplastics can be retained and have negative effects on the organism that ingested them.
- Microplastics can be transferred to the progeny and have negative effects either on the adults and the offspring and their development.
- Lastly, organisms that retain microplastics may subsequently be fed upon by other higher animals in the food web and thereby transferring the microplastics to other animals in the trophic level.

1.1.2.2 Effect on marine organisms

Microplastics are thought to accumulate in aquatic organisms only in tissue in direct contact with water, such as gastrointestinal and respiratory tissues. However, as scientific studies are increasing, it is becoming clear that other tissues may be impacted by particle accumulation, arguing the onset of

translocation processes, and broadening potential impacts of microplastics on a variety of physiological functions.

In bivalve molluscs, gills are the first site of particles uptake, while a second route occurred via ciliary movement in the stomach, intestine and digestive tubules, followed by microplastic translocation towards haemolymph (Browne et al. 2008; Franzellitti et al. 2019).

Browne et al. (2008) utilized mussel, *Mytilus edulis*, as a bioindicator to investigate the mechanisms of accumulation and toxicity of microspheres of polystyrene (diameter of 3 and 9,6 μm) following its ingestion. They observed that initially the polystyrene microspheres accumulated in the gut cavity and digestives tubes of the exposed mussels; within 3 days the polystyrene translocated from the gut to the circulatory system and was tracked in the haemolymph persisting for over 48 days; furthermore, smaller particles were more abundant than larger particles. However, the translocated polystyrene did not cause significant reduction in the oxidative status, viability and phagocytic activity of haemolymph, or filter-feeding activity. It is possible that the polystyrene microspheres particles permeated the cell membrane of the epithelial lining of the mussel gut via endocytosis-like mechanisms.

This has been confirmed by Lei et al. (2018) who compared the toxicity of five common types of microplastics: polyamides, polyethylene, polypropylene, polyvinyl chloride and polystyrene particles using zebrafish

(*Danio rerio*) and fluorescently labelled transgenic nematodes, *Caenorhabditis elegans*. They exposed zebrafish and nematode to polyamides, polyethylene, polypropylene, polyvinyl chloride and polystyrene particles with a mean diameter of ca. 70 μm for 10 days. Histopathological analysis of the fish gut showed that they caused intestinal damage including cracking of villi and splitting of enterocytes. Furthermore, the microplastics reduced calcium level but increased glutathione S-transferase 4 expressions in the nematode intestine (an indication of intestinal damage via oxidative stress). Moreover, following the 2 days exposure, the microplastics significantly inhibited survival rates, body length and reproduction of *C. elegans*, but with no or low lethality in *D. rerio*. Lu et al. (2016) treated *D. rerio* with microplastics and the results showed that the activities of superoxide dismutase (SOD) and catalase (CAT) in liver significantly increased, suggesting the onset of oxidative stress. Karami et al. (2017) found that microplastics had no effects on antioxidant-related biomarkers in zebrafish. The reports that polystyrene is capable of eliciting oxidative stress in *D. rerio* (Lu et al. 2016) and *C. elegans* (Lei et al. 2018) suggested that microplastics-induced toxicity is based on free radical formation. The overproduction of radicals may alter physiological homeostasis of cellular components via supressing the activity of antioxidant systems (oxidative stress). The overwhelming production of ROS is usually accompanied by damage to cellular macromolecules including DNA, carbohydrate, lipid and

protein structures. This damage may be associated with genome instability, biochemical and pathophysiological alterations and carcinogenesis (Alimba and Faggio 2019).

Cole et al. (2016) demonstrated the effect of polystyrene microbeads on the feeding, function and fertility of the marine copepod *Calanus helgolandicus*. It was observed that copepods exposed to microplastics ingested fewer algal cells, resulting in a significant reduction in carbon biomass. Prolonged exposure resulted in death of some of the copepods, fewer egg productions and decreased reproduction output, which affected hatching.

Gardon et al. (2018) exposed oyster, *Pinctada margaritifera*, to polystyrene microbeads (diameter of 6 and 10 μm) for 2 months and monitored the impacts of polystyrene on the overall physiology of the animal via ingestion and respiration rate, assimilation efficiency and reproductive potentials. Polystyrene caused significant decrease in the assimilation efficiency; furthermore, the gonads from exposed oysters were starved of energy balance required to maintain the animals' metabolism through the production of metabolites derived from germ cells phagocytosis. This report was consistent with Tallec et al. (2018) wherein polystyrene particles of varying sizes (50 nm, 500 nm and 2μm) affected fertilization, embryo-larval development and metamorphosis of Pacific oysters, *Crassotea gigas*. The microplastics significantly decreased fertilization success and embryo-larval development

with numerous malformations, which climax with total development arrest in the exposed oysters.

It is described that microplastic could downregulate the transcription of gonadotropin-releasing hormone (GnRH) in hypothalamus of African catfish (*Clarias gariepinus*) (Karami et al. 2016), and vitellogenin (Vtg) and choriogenin (Chg) in the liver of Japanese medaka (*Oryzias latipes*) (Rochman et al. 2014). In the hypothalamus-pituitary-gonadal (HPG) axis, GnRH is a physiologic regulator of the release of gonadotrophins (GtHs), which regulate gonadal steroidogenesis and gametogenesis in vertebrates. The downregulation of GnRH, Vgt and Chg genes suggests that microplastics might cause reproductive disruption (Wang et al. 2019). In the study by Wang et al. (2019) marine medaka were exposed to environmentally relevant concentrations of polystyrene microspheres with a diameter of 10 μm for 60 days. This study found that long-term exposure to microplastics not only caused oxidative stress and tissue damage, but also disrupted the reproductive endocrine system in a sex-dependent manner. After 60 days of exposure, polystyrene microplastics accumulated in the gill, gut and liver. These results revealed that microplastics could enter fish bodies through the processes of ingestion and respiration, and most of them were excreted by the digestive system, but a small amount of microplastics could be transported into liver via the circulatory system. Microplastic caused a significant increase in MDA

(malondialdehyde) levels in the gill, intestine, liver and gonad. As an end-product of the oxidative damage to lipids, the increase of MDA indicated that microplastic exposure caused the production of reactive oxygen species (ROS) and occurrence of lipid peroxidation. Moreover, the activity of CAT was decreased after microplastic exposure, indicating that the normal ROS-mediated oxidative process might be inhibited. In addition, changes in the activity of SOD, GST, GSH and GSH-PX enzymes also confirmed the disturbance of microplastics on the oxidation process. Correspondingly, obvious morphological abnormalities were found in these tissues. This study also demonstrated that microplastic exposure decreased the levels of sex hormones in female plasma by inhibiting their synthesis. In male fish the transcription of genes involved in the HPG axis and steroidogenesis pathway were upregulated, and the levels of plasma E_2 and testosterone were slightly increased, which was contrary to the results of the female. These findings demonstrate that polystyrene microplastics exhibited sex-specific endocrine disruption.

A first evidence on maternal transfer of nanoplastics in fish is provided by a laboratory study with zebrafish carried out by Pitt et al. (2018), who exposed adult zebrafish to dietary labelled polystyrene (mean diameter of 42 nm) and observed the transfer of nanoplastics from parents to offspring. Polystyrene significantly reduced glutathione reductase activity in the brain, muscle and

testes of parents. In addition, polystyrene was detected in the yolk sac, gastrointestinal tract, liver and pancreas of the F1 embryos. These data suggested that polystyrene is maternally transferred to the offspring via accumulation in the eggs of exposed females, probably due to nanoplastic interaction with plasma proteins in oocytes. Bradycardia was also observed in embryos in addition to reduction in the activity of glutathione reductase and levels of thiols. It was concluded from the outcome of the study that polystyrene modified the antioxidant system in both adult tissue and F1 larvae.

1.1.3 Microplastic and other pollutants

Microplastics contain organic pollutants, either added during plastic production (Diethylhexyl phthalate, DEHP) or adsorbed from sea water. Thereby serving as scavengers and transporters of organic contaminants such as persistent organic pollutants (POPs), metals and endocrine disrupting chemicals. In addition microplastics can also serve as vehicles for pathogens (Bakir, Rowland, and Thompson 2014). These chemical are found in high concentration in the sea surface microlayer, where low density microplastics also exist in large numbers (Teuten et al. 2009; Wang et al. 2016). The sorption capacity of microplastic is influenced by the type of polymer and its state (glassy or rubbery) (Auta et al. 2017). Additionally, different types of

plastic may have different impacts on organisms, since the type of resin used in plastic manufacture can affect the propensity of the plastic to adsorb toxins (Farady 2019).

Microplastics that have been covered with POPs, heavy metals or other pollutants may be carried across the oceans and easily contaminate other ecosystems. During plastic fragmentation process, organic chemicals, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dichloro-diphenyl-trichloroethane and its metabolites (DDTs), polybrominated diphenyl ethers (PBDEs), alkylphenols and bisphenol A are released along with its fragments into the environment. Furthermore, the material may be ingested by marine organisms, which is transported along the food chain. These toxic chemical contaminants have a wide range of harmful effects such as causing cancer and endocrine disruption, birth defects, immune system problems and child development issues (Alimba and Faggio 2019; Auta et al. 2017; Teuten et al. 2009).

Besseling et al. (2013) provided the first evidence of a correlation between polystyrene microplastic concentration in sediment and a significant internal bioaccumulation of PCBs in *Arenicola marina* in a controlled experiment. This caused weight loss and reduction in feeding activity and fitness of the exposed *A. marina*. Rainieri et al. (2018) exposed zebrafish to feed supplement with microplastics and sorbed with mixtures of PCBs,

perfluorinated compounds and methylmercury. They observed that combined microplastics and sorbed contaminants significantly altered homeostasis in the liver, brain, intestine and muscle than microplastics and contaminants alone.

The assimilation of polybrominated diphenyl ethers (PBDEs) from microplastic by *Allorchestes compresa* was reported by Chua et al. (2014), and the assimilation by fish was reported by Wardrop et al. (2016).

Batel et al. (2016) investigated the transfer of microplastics and potentially harmful substances between different trophic levels in the marine environment. In the study, *Artemia spp.* nauplii were subjected to high concentrations of microplastics and found to have ingested and accumulated microplastic particles. In high concentration these were subsequently transferred to zebrafish which fed on the nauplii. Although some of the accumulated microplastic particles were excreted out of the organisms, some got retained within the epithelial cells and the intestinal villi. The microplastic particles acted as a vector for the transfer of associated persistent organic pollutant benzo[α]pyrene (BaP) from the nauplii to the zebrafish, and the substance was retained in the intestinal tract. However, no physical harm was observed in both nauplii and zebrafish. The study clearly proved that microplastics and associated harmful substances can be transferred along food chains across various trophic levels.

Kim et al. (2017) investigated the toxicity of variable and fixed combinations of two types of polystyrene microplastics with the nickel (Ni) on *Daphnia magna*. They observed that the toxicity of Ni in combination with either of the two microplastics differ from that of Ni alone and concluded that the toxic effects of microplastics and pollutants may vary depending on the specific properties of the pollutant and microplastic functional group.

Barboza et al. (2018) exposed the European seabass (*Dicentratus labrax*) to microplastics, mercury and microplastics-mercury mixtures. Fish exposed to the highest mercury concentration had significantly higher mean concentrations of the metal in the brain and in the muscle than animals exposed to the lowest mercury concentration, this indicated that fish take up mercury from water and that it reaches the brain and muscles. Moreover, the analyses of mercury concentrations in the brain and in the muscle in relation to microplastics concentration in the water indicated that microplastics influence the mercury concentration in brain and muscle of fish. Microplastics alone and mercury alone caused neurotoxicity, lipid peroxidation in brain and muscle, and changed the activity of energy-related enzymes (LDH and IDH). Overall, microplastic-mercury mixtures caused effects on the same biomarkers but evidence of toxicological interactions between microplastic and mercury was found.

1.1.3.1 Plastisphere

In addition to chemicals, microbes and other organisms have been found on plastic debris. The term “Plastisphere” refers to the environment and community associated to floating plastic debris in the sea. In addition to meiofauna, plastics offer attractive shelter and create new ecological niches for bacterial communities. These communities are mainly composed by keystone species in biofilm formation, other species degrading microplastics and some hitchhikers potentially pathogens. Main bacterial colonies seem to be generally attracted by microplastics as a support rather than by the type of the polymer component itself. Biofilms are highly heterogeneous environments and offer several ecological advantages for multitudes of associated bacteria, can accumulate nutrients, offers a protective barrier and associated bacteria can organize the degradation of complex substrates. All these features may promote the establishment and growth of different types of bacterial communities, including potential pathogens, such as *Vibrio spp.*, *Escherichia coli, Stenotrophomonas maltophilia, Bacillus cereus* and *Aeromonas salmonicida* (Barboza et al. 2018; Franzellitti et al. 2019). In the North Adriatic Sea, the fish pathogenic bacteria *Aeromonas salmonicida* was detected onto microplastic fragments. This pathogen species, native from temperate waters and higher latitudes, is usually not present in the Mediterranean Sea, and its presence on microplastic can represent a new source for contamination to fish and humans. The genus *Vibrio*, which

includes many species that are pathogens for humans and marine organisms, has been encountered in several parts of the world as microplastic-associated communities (Franzellitti et al. 2019).

This phenomenon favours the transfer of species from different environments. Therefore, it has been suggested that plastic debris may increase the global risk of animal and human diseases via new contaminations and infection routes, introduction of pathogens and their vectors into new areas through the environmental spread of microplastics. Additionally, the “plastisphere” may also include exotic invasive species (pathogens or not) that may contribute to loss of biodiversity and other negative ecological and economic impacts (Barboza et al. 2018).

1.2 MELANOMACROPHAGE CENTERS (MMCs)

1.2.1 MMCs morphology

Melanomacrophage centers (MMCs), also known as macrophage aggregates, are nodular accumulation of closely packed melanomacrophages (or melanin-macrophages, MMs), which are pigments-containing cells that are generally found inside the reticulo-endothelial matrix of hematopoietic tissue in teleost fish, although they also exist in gills, brain and gonads (Manrique et al. 2014). They may also develop in association with chronic inflammatory lesions elsewhere in the body and during ovarian atresia (Agius and Roberts 2003). In fish with morphologically distinct aggregations (Osteichthyes), the centers are usually rounded, although they may have some irregularity in outlines and are separated from the surrounding tissue by an argyrophilic capsule and are most often found in close relationship with blood vessel (Kalita et al. 2019). It has been observed that MMCs quickly remove injected foreign material from the circulation (Steinel and Bolnick 2017). Macrophages are normally packed to form large aggregates and are bigger after phagocytic activity on heterogeneous material, such as cell debris, melanin pigments, haemosiderin granules, residues of lipofuscin, protein aggregates and neutral mucopolysaccharides (Manrique et al. 2014). MMs are darkly pigmented due

to high lipofuscin, melanin and hemosiderin content which make them histologically distinguishable *via* light microscopy (Steinel and Bolnick 2017). The morphological appearance of MMCs may vary in different species, different organs and different physiological conditions within the same species, such as age, gender, nutritional status and degree of tissue catabolism, tissue type, thermal stress, iron and haemoglobin metabolism, pathological and inflammatory conditions and immunological processes (Borucinska et al. 2009; Manrique et al. 2014).

1.2.2 MMCs functions

Melanomacrophage are an important component of the immune system of fishes (Kalita et al. 2019) and MMCs are thought to play dual roles, participating both in immune defences and normal, non-immunological, physiological processes. Like other macrophages, MMs' primary function is phagocytosis (Steinel and Bolnick 2017). Several studies have suggested that the general function of MMCs is to destroy, detoxify and recycle endogenous and exogenous substances, such as discarded material originating from erythrocytic and cell metabolic activity (Manrique et al. 2014). Primarily, melanomacrophage move freely from blood vessels by mechanisms of margination, diapedesis and migration towards lymphoreticular parenchyma giving rise to aggregates, the melanomacrophage centers (Passantino et al.

2014). In addition, MMCs perform an important role in relation to the response to foreign bodies, including infectious agents (Manrique et al. 2014). These findings highlight the importance of MMCs in debris clearance and long-term storage of highly indigestible and/or toxic materials (Steinel and Bolnick 2017). As might be expected in a structure with these functions, it has been observed that MMCs increase in size and number as fish grow older and tissues degenerate (Agius and Roberts 2003).

1.2.3 MMCs as indicators of environmental stress

Given that the stress increases the susceptibility of fish to diseases, fish inhabiting a polluted environment can reflect degraded environmental conditions through altered activity of the immune system or the non-specific defences. Changes in size of the melanomacrophage centers can occur physiologically, in association with aging, but there are also numerous instances of their increasing in size or frequency in relation to pollutants (Agius and Roberts 2003).

MMCs provide a widely applicable tool for immunology, as they are near-ubiquitous across vertebrates: MMCs are reported in over 130 fish species and are also present in amphibians and most reptile; are accessible and readily measured and can be statistically compared using imaging techniques. (Steinel and Bolnick 2017). MMCs increase in size or frequency is commonly

used as a reliable biomarker for the state of the fish health and for the water quality, in terms of both deoxygenation and chemical pollution, in the aquatic environment (Agius and Roberts 2003; Stosik et al. 2019).

The increase of MMCs have been used as a stress biomarker in numerous environmental studies in fish, including exposure to polychlorinated biphenyls (PCBs) (Anderson et al. 2003; Hinck et al. 2007), immunosuppressive toxins like polyaromatic hydrocarbons (PAHs) (Haaparanta et al. 1996; Payne and Fancey 1989), silver nanoparticles (Sayed and Younes 2017) and generally polluted environments (Facey et al. 2005; Macchi et al. 1992; Mikaelian et al. 1998; Spazier et al. 1992; Wolke 1992; Wolke et al. 1985). Passantino et al. (2014) investigated the presence of MMCs in the liver of Atlantic bluefin tuna (*Thunnus thynnus*). They found out that there is a MMCs stronger proliferation in fish caught in the Northern Adriatic Sea compared with the ones caught in the central Adriatic Sea. The authors hypothesized that this difference is a consequence of the higher degree of environmental pollution in the Northern Adriatic Sea.

1.2.4 Evidence of MMCs in gonads

In ovaries and testes the presence of melanomacrophage has been related to degradation and resorption of unspawned oocytes and sperm during gonadal regression. Similarly to splenic and hepatic MMCs, gonadal MMCs have

been suggested as putative tissue biomarkers of environmental stress, based upon observation of increased melanomacrophage abundance in testes of fish collected from polluted areas (Micale et al. 2019). It is possible that certain stimuli can induce the migration of macrophage into gonadal tissue and after phagocytosis of necrotic or cell debris may accumulate into melanomacrophage aggregates (Blazer 2002).

Ravaglia and Maggese (1995) studied the MMCs in swamp eel *Synbranchus marmoratus* and suggested they may act as "atretic cell cleaners", parasitic encystations, sperm degeneration and non-specific tissue resorption. Louiz et al. (2009) reported an increase of melanomacrophage centers in testes of *Gobius niger* from Bizerta lagoon (Tunisia), a highly impacted site. Pieterse et al. (2010) assessed the presence of MMCs in ovaries and testes of the sharptooth catfish *Clarias gariepinus* from an urban nature reserve in South Africa, which shows high levels of metals (Cd, As, Pb and Hg) and organic pollutants, such as dichlorodiphenyltrichloroethane (DDT) and its metabolites. Kaptaner (2015) found several types of histological alterations, including melanomacrophage centers, and show evidence of elevate oxidative stress in the ovaries of *Alburnus tarichi* in Lake Van, Turkey. The author demonstrates that the abnormalities are related to increased oxidative stress as a result of elevated levels of pollution in the study area.

1.3 THUNNUS THYNNUS

The Atlantic Bluefin Tuna (ABFT, *Thunnus thynnus* Linnaeus 1758) is a fascinating species: one of the fastest, largest and most wide-ranging teleost fish in the oceans. It can grow to a length >300 cm and attain a mass of 680 kg (Teo et al. 2007), the largest fish 679 kg was caught in Nova Scotia in 1979 (Api et al. 2018). Atlantic bluefin tuna represent an important fishing resource at a global scale and because of its commercial importance it is intensely fished and considered overexploited since the 1980s (Corriero et al. 2005; Santamaria et al. 2009).

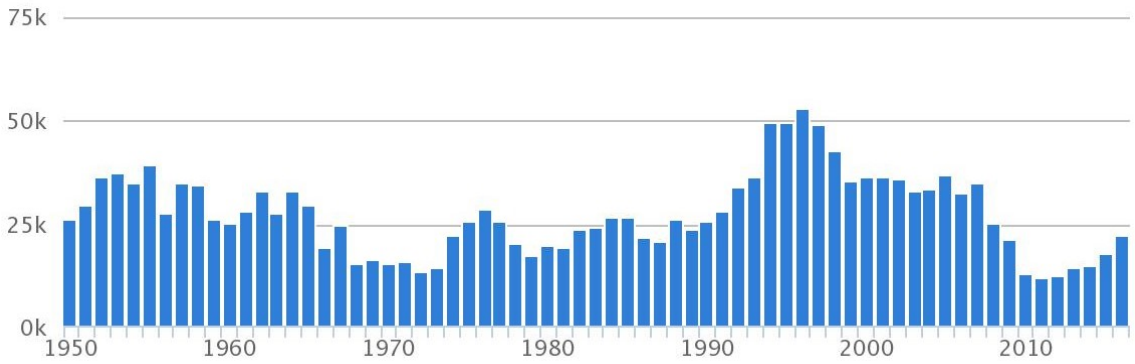

Figure 5. ABFT global capture production in tonnes (FAO fishStat)

1.3.1 Distribution

Atlantic bluefin tuna (ABFT) have a wide geographical distribution, but mainly live in the temperate pelagic ecosystem of the entire North Atlantic and its adjacent waters, for example the Gulf of Mexico, Gulf of St. Lawrence and the Mediterranean Sea. Among the tuna, ABFT has the widest geographical distribution and is the only large pelagic fish living permanently in temperate Atlantic waters (Powers and Fromentin 2005). Bluefin tuna are highly migratory species that seems to display a homing behaviour and spawning site fidelity to primary spawning areas in both the Mediterranean Sea and Gulf of Mexico. Bluefin tuna preferentially occupy the surface and subsurface waters of the coastal and open-sea areas, but they frequently dive to depths of more than 1,000 m (ICCAT 2017).

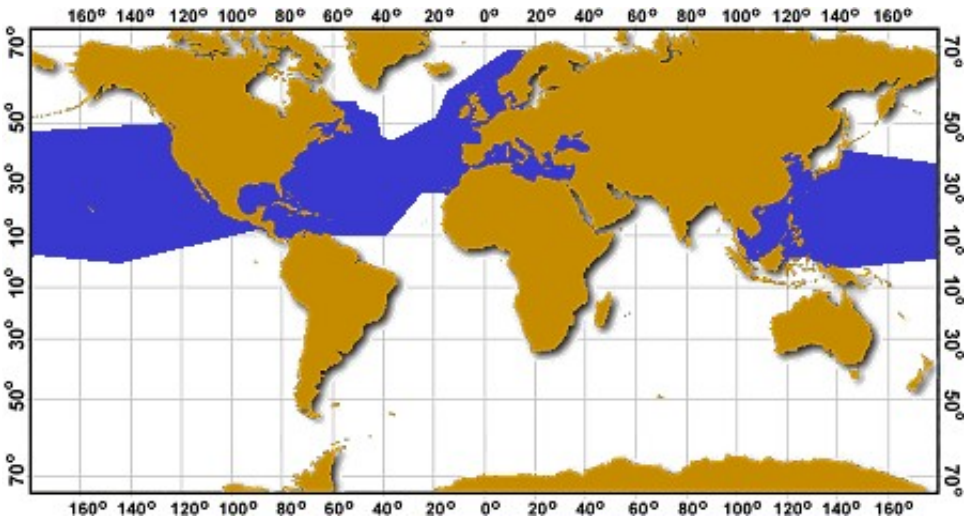

Figure 6. Tuna global distribution map (FAO)

The International Commission for the Conservation of Atlantic Tunas (ICCAT) regulates the bluefin tuna fishery since 1970 and currently recognizes two different stocks, the west and the east Atlantic stock separated by the 45°W meridian, the latter including the Mediterranean Sea (Corriero et al. 2005). Based on electronic tagging data these two stocks have different spawning area (Mediterranean Sea and Gulf of Mexico) but they have an overlapping distribution in North Atlantic feeding grounds (Powers and Fromentin 2005).

1.3.2 Physiological characteristics

Atlantic Bluefin Tuna is a pelagic fish that swim constantly in order to maintain hydrostatic equilibrium and are ram ventilators. Tunas are also capable of rapid burst and acceleration in pursuit of pray and during rapid depth changes. In addition, tuna can tolerate cold as well as warm water, cause its able to generate high metabolic heat within the red muscle, which is used to elevate and maintain muscle, eye, brain and visceral temperatures above water temperatures (Addis et al. 2009; Powers and Fromentin 2005). Atlantic bluefin tuna make rapid, ocean basin-scale migration, ranging from cold subpolar foraging grounds to discrete breeding grounds in warm, subtropical waters during the spawning season (Teo et al. 2007). *T. thynnus* has the ability to conserve metabolic heat through counter current vascular

heat exchangers (*retia mirabilia*) and can maintain a steady-state body temperature that is greater than environmental water temperature. This physiological specialization, known as regional endothermy, improves tuna performance regardless of environmental temperature and allows bluefin tuna to expand their temperature range both vertically and latitudinally (Addis et al. 2009). The elevated body temperature of bluefin tuna increase their capacity for rapid migrations by enhancing the power output of their muscle (Block et al. 2001).

1.3.3 Feeding ecology

The feeding habits have been described for bluefin tuna populations in the western North Atlantic (Estrada et al. 2005), eastern North Atlantic (Ortiz de Zarate and Cort 1986), Pacific (Pinkas et al. 1971), western Mediterranean Sea (Sarà and Sarà 2007) and eastern Mediterranean Sea (Karakulak et al. 2009). From these investigations Atlantic bluefin tuna has been identify as an opportunistic species, able to exploit a great variety of resources. Moreover, the presence in the gastric content of pelagic, epipelagic, mesopelagic and benthonic organisms, including bony fish, squid and crustaceans, pointed out that *T. thynnus* performed diurnal vertical migration. The dominant prey can vary according to the abundance of the different prey species presence where tuna live, depending on their distribution. *T. thynnus* diets can also include

jellyfish and salps, as well as demersal and sessile species such as octopus, crabs and sponges. In general, juveniles feed more on crustaceans, fish and cephalopods, while adults feed primarily on fish, frequently Atlantic herring (*Clupea harengus*), anchovy, sand lance (*Ammodytes americanus*), sardine, sprat, bluefish and Atlantic mackerel (*Scomber scombrus*) (Estrada et al. 2005; Karakulak et al. 2009). Typically, ABFT stomach contents are dominated by one or two prey species, such as Atlantic herring and sand lance in the West Atlantic or anchovy in the East Atlantic and Mediterranean. No clear relationship has been demonstrated between prey length and size of ABFT: both small and large ABFT display similar prey-size spectra (Powers and Fromentin 2005).

1.3.4 Reproduction

Bluefin tuna is oviparous and iteroparous like all tuna species, and a batch spawner. In females ABFT the ovary is characterized by an asynchronous oocyte development, in which oocytes at different stages can be simultaneously found in reproductively active ovaries. Ovaries are classified in four stages: resting (R), active non-spawning (ANS), active spawning (AS) and inactive mature (IM). Males ABFT have an unrestricted spermatogonial testicular type, where spermatogonia occur along the greater part of the

testicular tubules. Testis maturity is classified as early spermatogenesis stage (ES), late spermatogenesis stage (LS) and spent (S) (Carnevali et al. 2019).

Like most fish, egg production appears to be age or size dependent: a 5 years old female produces an average of 5 million eggs, while a 15-20 years female can carry up to 45 million eggs (Powers and Fromentin 2005).

The ABFT spawning grounds differ from its trophic areas, the spawning takes place in warmer waters (between 23 and 25°C) of specific and restricted locations (around the Balearic Islands, Sicily and the Gulf of Mexico) and occurs only once a year, from June for the western Atlantic population and from May for the eastern Atlantic population (Powers and Fromentin 2005; Sarà and Sarà 2007). Karakulak et al. (2004) identified a new spawning area in the Northern Levantine Sea and the reproductive period is around mid-late May, almost one month earlier than the spawning period in the other Mediterranean grounds.

Knowledge of first sexual maturity has important implications for stock management and regulation of the fishery. Western Atlantic bluefin tuna mature at the age of 6 and are considered fully mature by the age of 8, at a weight of 135kg. On the other hand eastern Atlantic bluefin tuna mature at the age of 3, at a weight of 15 kg and are fully mature by the age of 5 (Corriero et al. 2005).

2 AIM OF THE STUDY

The main objective of this study is to provide initial evidence of the presence of microplastics in a commercially important specie, such as the Atlantic bluefin tuna (*Thunnus thynnus)*. Moreover, by carrying out the analyses in different compartments, such as intestine, blood, and gonads, it is also possible to establish a connection between the ingestion of microplastics through the diet and their possible translocation to other body compartments, such gonads.

In addition, the present study aims to evaluate the possible negative effects of microplastics on reproduction through histological analyses of the gonads.

3 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Samples were collected in the western Mediterranean Sea from February to July of 2019. A total of 64 (34 female and 30 male) Atlantic bluefin tuna (*Thunnus thynnus*) were caught from the fishermen from a wild population in three different areas: 25 in the Adriatic Sea and 7 in Sicily by longline and 32 in Sardinia in two sampling sites, Carloforte and Cala Vinagra, by tuna trap.

The longline is a commercial fishing technique that uses a long line, called the main line, with baited hooks attached at intervals by means of branch lines called snoods. The Mediterranean tuna trap is a passive capture method that relies upon the natural swimming behaviour of the tuna on their reproductive migration routes along the Mediterranean coast.

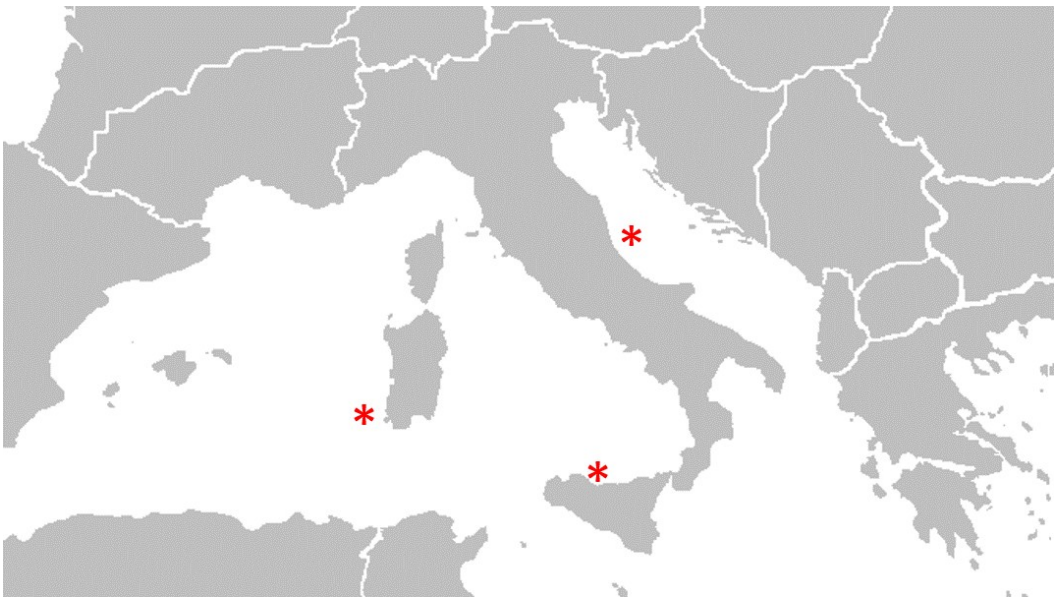

Figure 7. Map of the study area and localization of sampling sites in Mediterranean Sea: Adriatic Sea, Sicily and Sardinia

All sampled fish were measured, weighted and gutted right after the capture. In addition, they were subjected to macroscopic and biometric analysis, to evaluate: total length (straight fork length, SFL and curved fork length, CFL); head length (HD); first predorsal length (LD); total weight; gutted weight; gonad weight and sex (table 2).

Table 2. Size of sampled Atlantic bluefin tuna from different locations

Sampling site	Month	Sex	n°	Mean fork length (cm)	Mean total weight (kg)
Adriatic Sea	March/April	F	13	130.36±9.4	42.15±5.8
		M	9	132.40±10	48.11±8.9
Sardinia	May	F	16	163.29±43.6	102.64±67.7
		M	16	194.40±53.3	153.01±108
Sicily	June	F	4	143.75±28	58.75±32.9
		M	3	121.33±4.0	37.17±9.1
Adriatic Sea	July	F	1	124.00	27.00
		M	2	133.00±8.5	31.00±5.6

For this study samples of gonads, blood and intestines were taken. Due to the commercial importance of tuna, especially for the ovary, it was not possible to sample all individuals, only 15 from the Adriatic Sea are complete with gonads, blood and intestines. From those from Sicily and Sardinia, it was possible to collect only the blood.

Figure 8. Photograph of a sampled tuna

3.2 MICROPLASTIC ANALYSIS

3.2.1 Microplastic Extraction

Plastic isolation from intestine and gonad of the sampled fish was performed according to the method of Foekema et al. (2013). The organs were weighed, and pieces of ca. 20 g were placed in a clean glass container. A 10% potassium hydroxide (KOH) solution was prepared using 8μm-filtered deionized water and KOH tablets (Sigma-Aldrich). This solution was added to each jar in a ratio with the sample of 1:10 (w/v). The containers were then sealed and incubated at room temperature for 3 weeks. Plastic isolation from blood samples was performed according to the method of Foekema et al. (2013) after modification of the protocol, reducing the 3 weeks at room temperature step to a 24 hours incubation step at room temperature, according to Dehaut et al. (2016). The previous steps were the same as for intestine and gonad samples.

Digestates were then filtered through 8 μm filter membrane (Whatman 540) using a vacuum pump connected to a filter funnel. The filter papers were dried at room temperature and stored in Petri dishes carefully covered with aluminium foil for the following visual identification of particles.

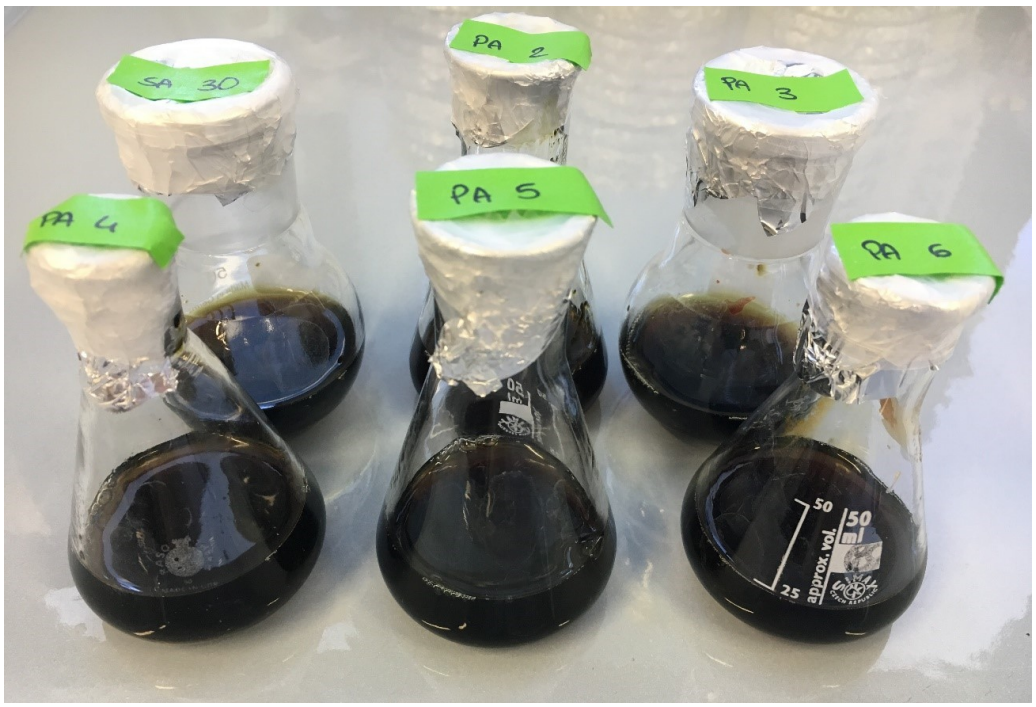

Figure 9. Preparation of blood samples

3.2.1 Contamination Prevention

To avoid plastic contamination, all the work surfaces and the used supplies were washed with 70% ethanol; single-use latex gloves and cotton laboratory coat were worn during experiments and sample handling. The sampling was performed on a clean metallic bench. All the tissue sampled were mantained in metallic packaging, such as aluminium foil. The collection of blood samples was performed with a glass pipette and the blood is then preserved in polypropylene tubes, appropriately not considered during the following microplastic particles characterization analyses. All liquids (deionized water for cleaning and for preparation of KOH solution) were filtered through 1.6 µm filter membrane (Whatman GF/A). Glassware and instruments, including,

scissors, tweezers and scalpels, were washed using dishwashing liquid, rinsed with deionized water and finally rinsed with 1.6 μm-filtered deionized water. Since the experiments were conducted without the use of the laminar flow hood, the plastic fibres found in the samples were not taken into account in the results.

Throughout the analysis process blank filters have been used to evaluate possible contamination due to the presence of microplastics in the air. Blank filters have been prepared following all the steps used for sample processing.

3.2.2 Visual Identification

Visual inspection of 8 μm filter membranes was conducted using a Stereomicroscope OPTECH GZ 808, equipped with a digital photocamera DELTA PIX INVENIO II 10SIII. All particles resembling MPs were measured, photographed and sampled in small glass tubes containing deionized filtered water. The content of these tubes was then placed on the same 8μm filters for Raman Spectroscopy analysis.

3.2.3 Raman Spectroscopy Analyses

Particles were analyzed directly on the filter over a range of 150 to 3000 cm^{-1} using a Raman spectrometer (Horiba XploRA Nano) equipped with a 785 nm laser diode, coupled with a charge-coupled device detector (Horiba Synapse).

Before the library search, raw spectra were submitted to a baseline correction and normalization procedure, in order to reduce noise and enhance the spectrum quality (Labspec 6, Horiba Scientific). Pre-processed spectra were then evaluated and compared to the spectral library of the KnowItAll software from Bio-Rad. The Correlation algorithm (KnowItAll, Bio-Rad) was used to evaluate each query spectrum to the spectra of the databases.

3.3 HISTOLOGICAL ANALYSIS

3.3.1 Fixation and storage

After sampling has been completed, samples were placed in tubes containing a fixative solution for histological processing. The fixative solution used was a Formaldehyde and Glutaraldehyde solution ($NaH_2PO_4{\cdot}H_2O$ + NaOH + Formaldehyde 36.5% + Glutaraldehyde 25% + H_2O). the samples were stored directly in the fixative solution at 4°C.

3.3.2 Inclusion

The inclusion process consisted of a first dehydration step of samples through serial baths of increasing EtOH concentration, then samples were cleared in Xylene and finally embedded in paraffin. The whole protocol includes: 45' EtOH 50%, 45' EtOH 70%, 45' EtOH 80%, 45' EtOH 95%, 60' EtOH 100%, 60' EtOH 100%, 15' Xylene (I), 30' Xylene (II), 90' Paraffin 46–48° C, 120' Paraffin 58–60° C.

3.3.3 Sectioning

After 12 hours the paraffin was hardened, and paraffin blocks were excised out of the metallic support. In order to perform sectioning, a microtome Leica

model RM2125 RTS (Leica Biosystems Wetzlar, Germany) was used. The sections were taken at a distance of 15 cuts. The chosen standard thickness was 5 μm.

3.3.4 Staining and closure

The slides were used for Mayer's Haematoxylin – Eosin staining. The protocol applied to perform the staining was the following: 10' Xylene (I), 10' Xylene (II), 5' EtOH 100% (I), 5' EtOH 100% (II), 5' EtOH 95%, 5' EtOH 80%, 5' EtOH 70%, 10' H_2O, 2.15' Mayer's Haematoxylin, 5' H_2O, 50'' Eosin, 2' H_2O, serial steps of 3'' in increasing EtOH (70, 80, 95), 1' EtOH 100% and eventually 15' Xylene.

Once the staining step was concluded the slides were closed with the mounting media SafeMount (Bio Optica, Milano, Italy) and finally closed with coverslips.

3.3.5 Area covered with MMCs in the gonads

Each gonad sample was observed under a Zeiss Axioskop optical microscope (Oberkochen, Germany) connected to a high-resolution digital camera (Canon EOS 6D). In order to establish the microscopic stage of maturity and estimate the melanomacrophage centers (MMCs) area, each individual microphotograph were captured.

In order to investigate the melanomacrophage center area in gonads the analysis of the histological section from male and female gonads was done according to the methodology of Papadakis et al. (2013). For each gonad, five microphotographs (surface area = 4915200 μm^2) were randomly taken at x20 magnification from sections obtained from different areas of the gonad. A total number of 280 photographs were analysed, and the total area covered with MMCs was calculated using an image analysis software (Image J). The results are expressed as the percentage with respect to the total area of the gonad tissue.

4 RESULTS

4.1 CHARACTERIZATION OF MICROPLASTICS

Microplastic polymers were identified using Raman spectroscopy and compared with reference spectra found in the database. As a result, it has been possible to confirm with certainty the presence of microplastics in the intestine, blood and gonads of Atlantic Bluefin Tuna (*Thunnus thynnus*) (Figure 10). Moreover, from the analyses carried out on several fragments it was possible to identify only the spectrum of the pigment, as the presence of the pigment completely obscured the spectrum of the underlying fragment (Figure 11, 12, 13).

In particular, the spectral analysis showed that the spectrum of the semi-transparent fragment of about 40 μm present in the intestine was almost completely superimposable to the spectrum of the synthetic material "Plexiglas DRF-100" (polymethylmethacrylate, PMMA) (Figure 10A). While the spherical fragment of about 15 μm of yellow colour present in the blood has been identified as polymethylmethacrylate (Figure 10B). And lastly, the

spectral analysis of the spherical fragment of about 20µm of black/purple color present in the ovary showed that the spectrum of the fragment was partly superimposable to the vinyl chloride copolymer and partly to the Heliogen superimposable to the vinyl chloride copolymer and partly to the Heliogen turquoise dye. (Figure 10C).

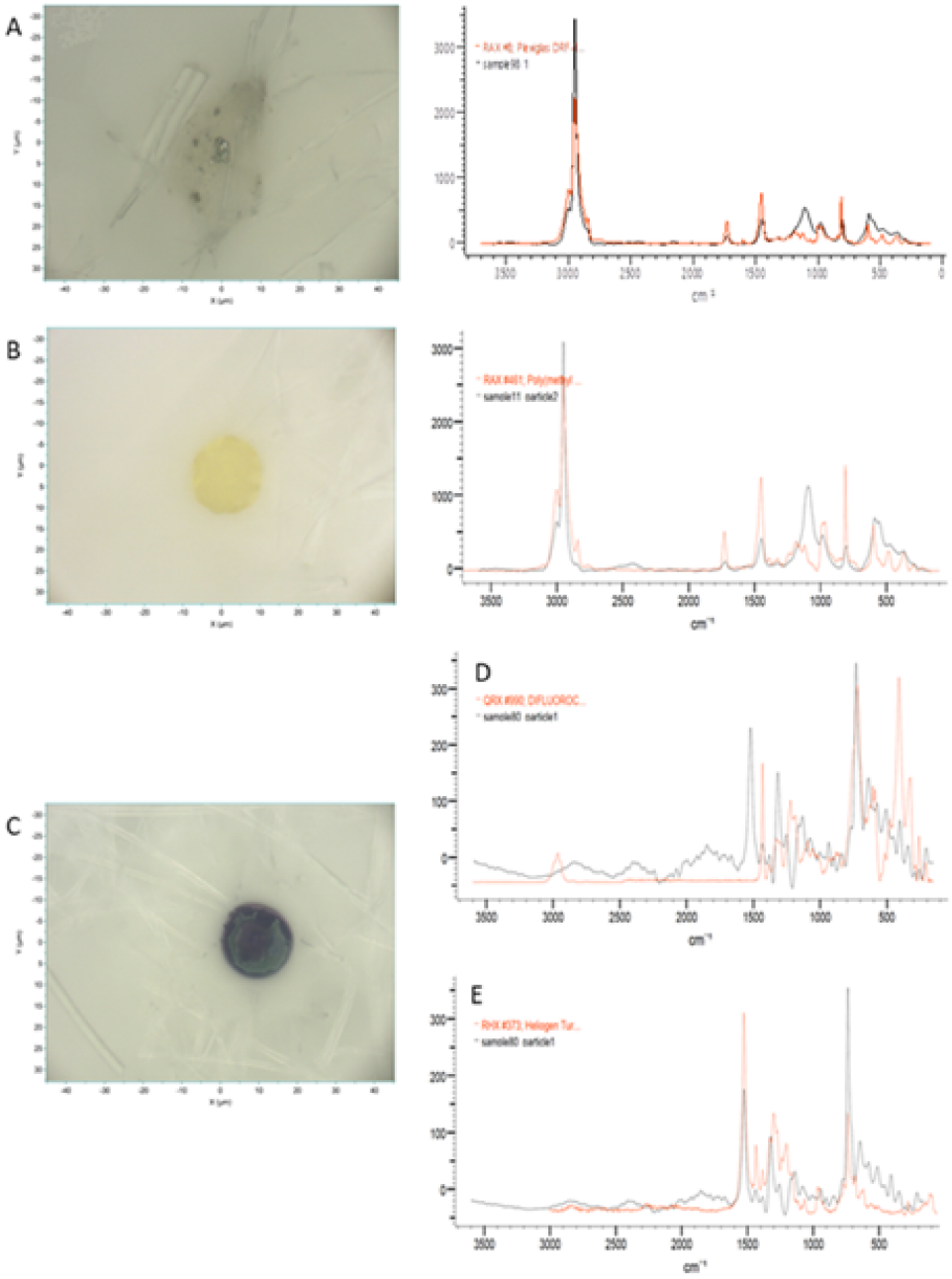

Figure 10. Microplastics found in blood, intestine and gonad of Atlantic Bluefin Tuna and their relative spectra

Among the pigments found, copper phthalocyanine and Red Ochra have the highest incidence. In particular, four fragments were found showing the spectrum of copper phthalocyanine (Pigment Blue 15) in female gonad samples (Figures 11 A and B) and both male and female intestine samples (Figures 11 C and D). The particles found are fragments and spheres with sizes ranging from 5 to 30µm.

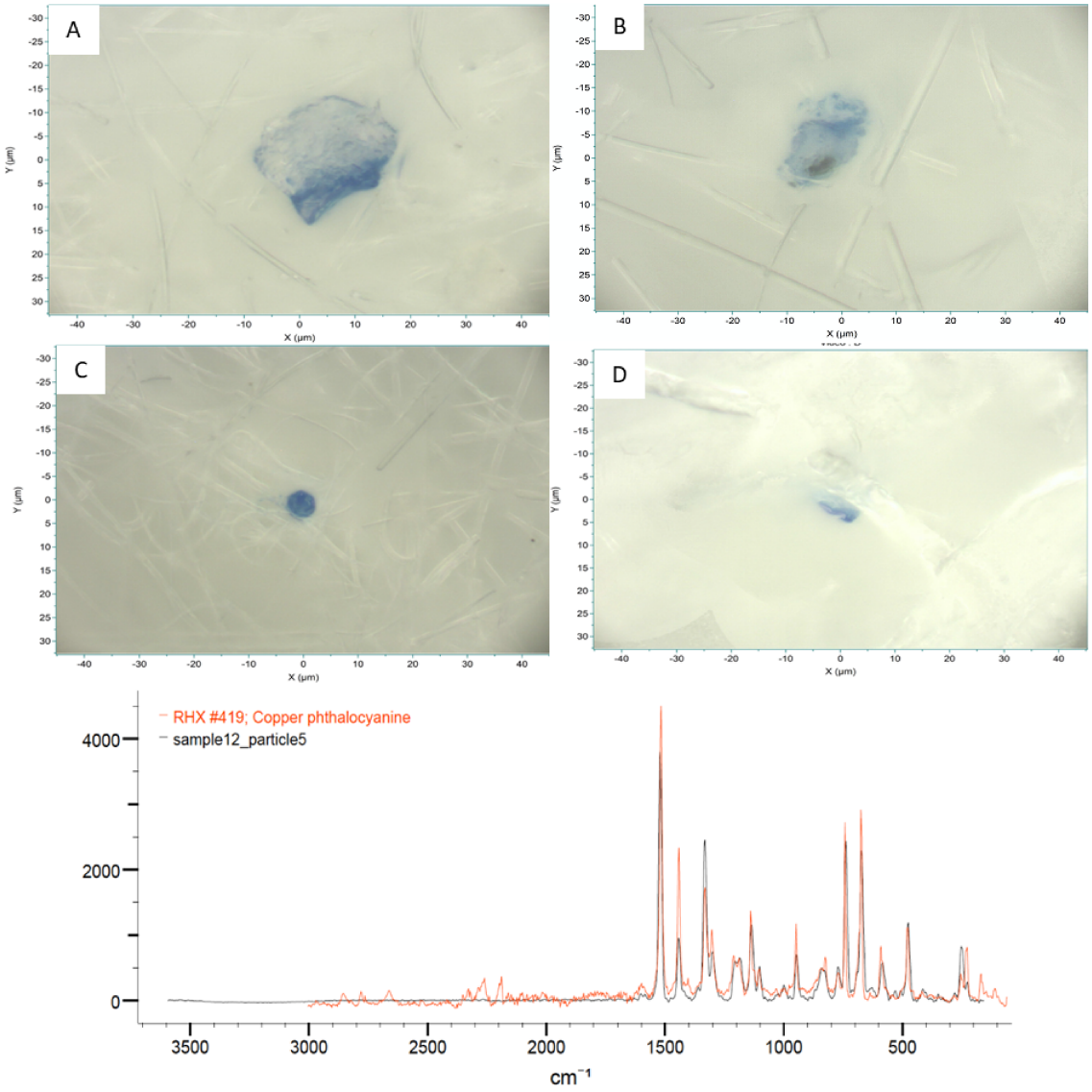

Figure 11. Copper Phthtalocyanine pigment fragments and its spectrum

The fragments identified with the Red Ochre spectrum (Red 102 pigment) are shown in figure 12, with the relative spectrum. Four fragments were found, two of them in the ovary (Figures 12 A and B) and the other two in the female (Figures 12 C) and male (Figures 12 D) intestine. The fragments show a size of about 15 µm, while the spectral analysis of the larger fragment, about 100 µm (Figures 12 D), allowed to identify the dark part as titanium dioxide, while the brownish portion of the particle was identified as Red Ochre inorganic pigment.

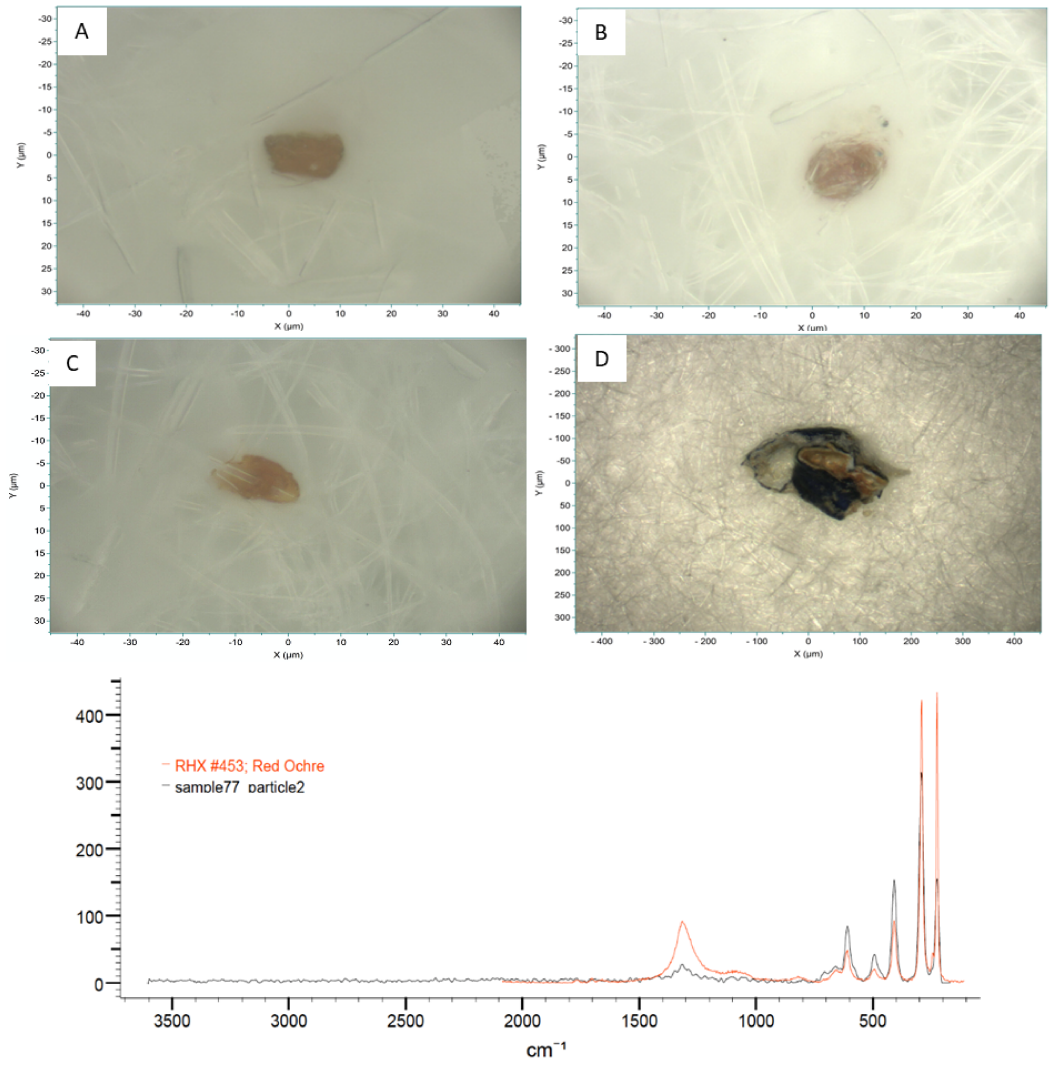

Figure 12. Red Ochre pigment fragments and its spectrum

The other analysed fragments are shown in Figure 13 with their spectra. In more detail, a fragment of about 10 μm was identified with the pigment Levafix Blue E-RA (Bayer) present in the blood of a female sample (Figure 13 A); two fragments of 15 and 10 μm identified with the pigment Sicotrans red (Red Pigment 101) found in female intestine samples (Figure 13 B); a fragment of about 15 μm identified as Umber pigment (Pigment brown 7) found in the ovary (Figure 13 C); a 5 μm fragment identified with the Neozapon blue FLE pigment (Solvent Blue 70) found in the ovary (Figure 13 D); and finally two fragments of 10 and 20 μm identified with the Heliogen turquoise pigment (Pigment Blue 70) found in the ovary (Figure 13 E).

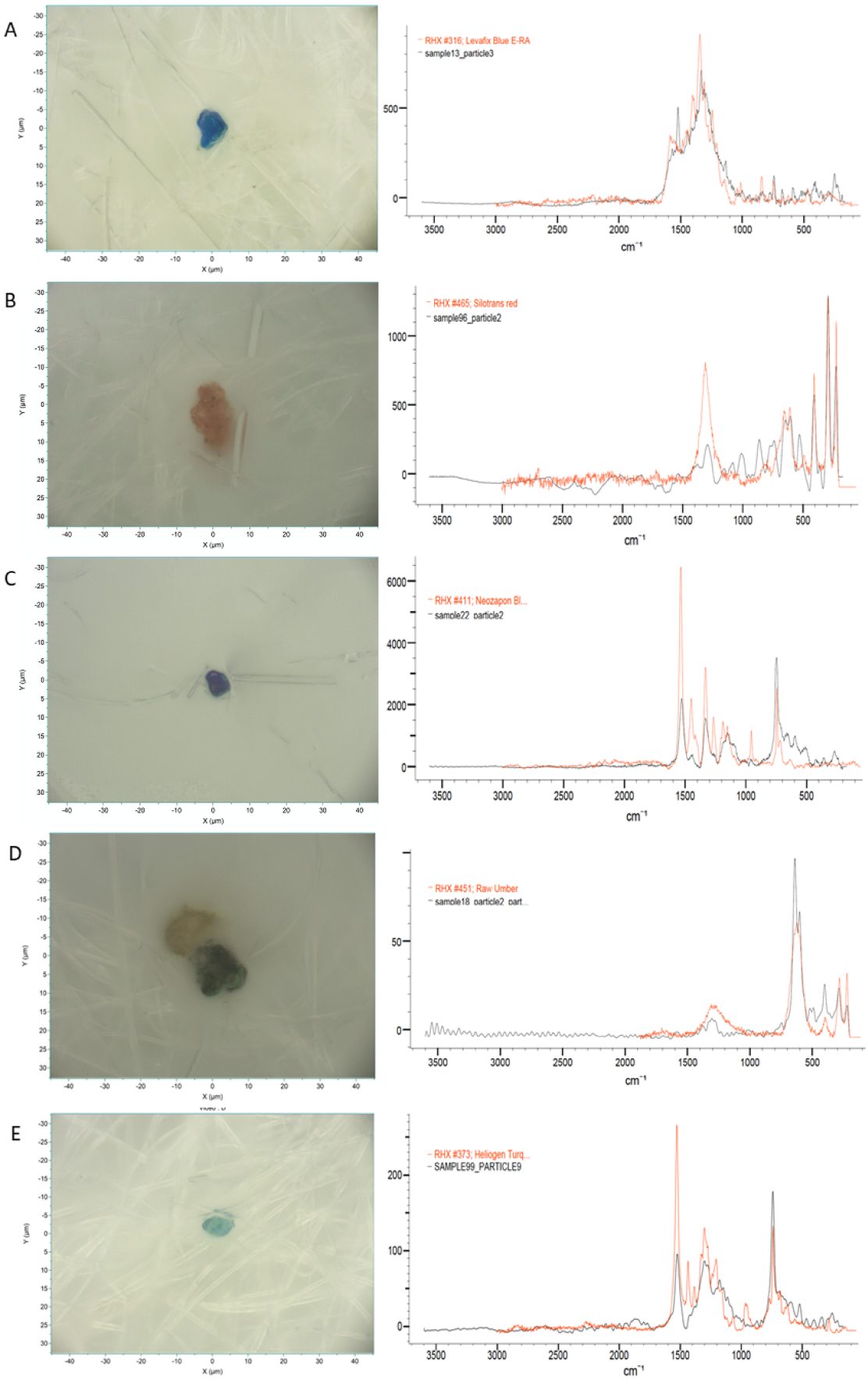

Figure 13. Various pigments found in gonads, intestine and blood and their spectra

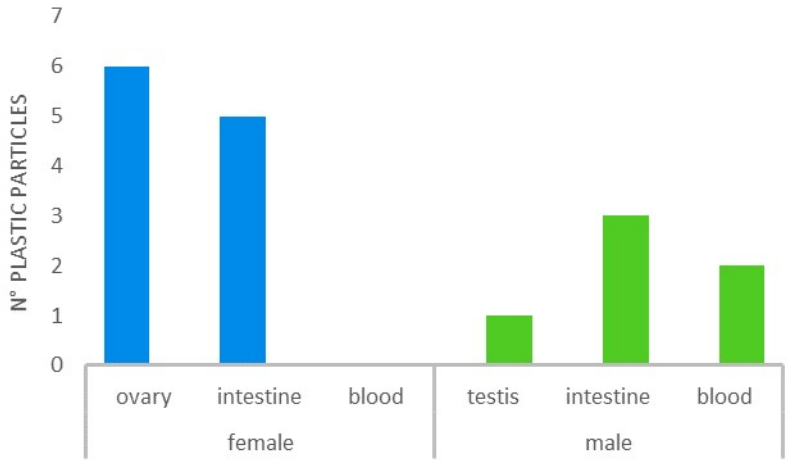

Figure 14. Number of plastic particles found in female and male samples

4.2 GONADAL HISTOLOGY

The histological appearance of tuna gonads is shown in Figures 15 and 16. Particularly in the non-reproductive group the ovaries are classified as resting (R), and the testis are in early spermatogenesis (ES) or spent (S). While in reproductive group the testis are in late spermatogenesis (LS) or spent (S) and ovaries are classified as active non-spawning (ANS) and active spawning (AS). In the analysed sections yellow-brownish aggregates of cells and pigments that constituted the melanomacrophage centers are visible.

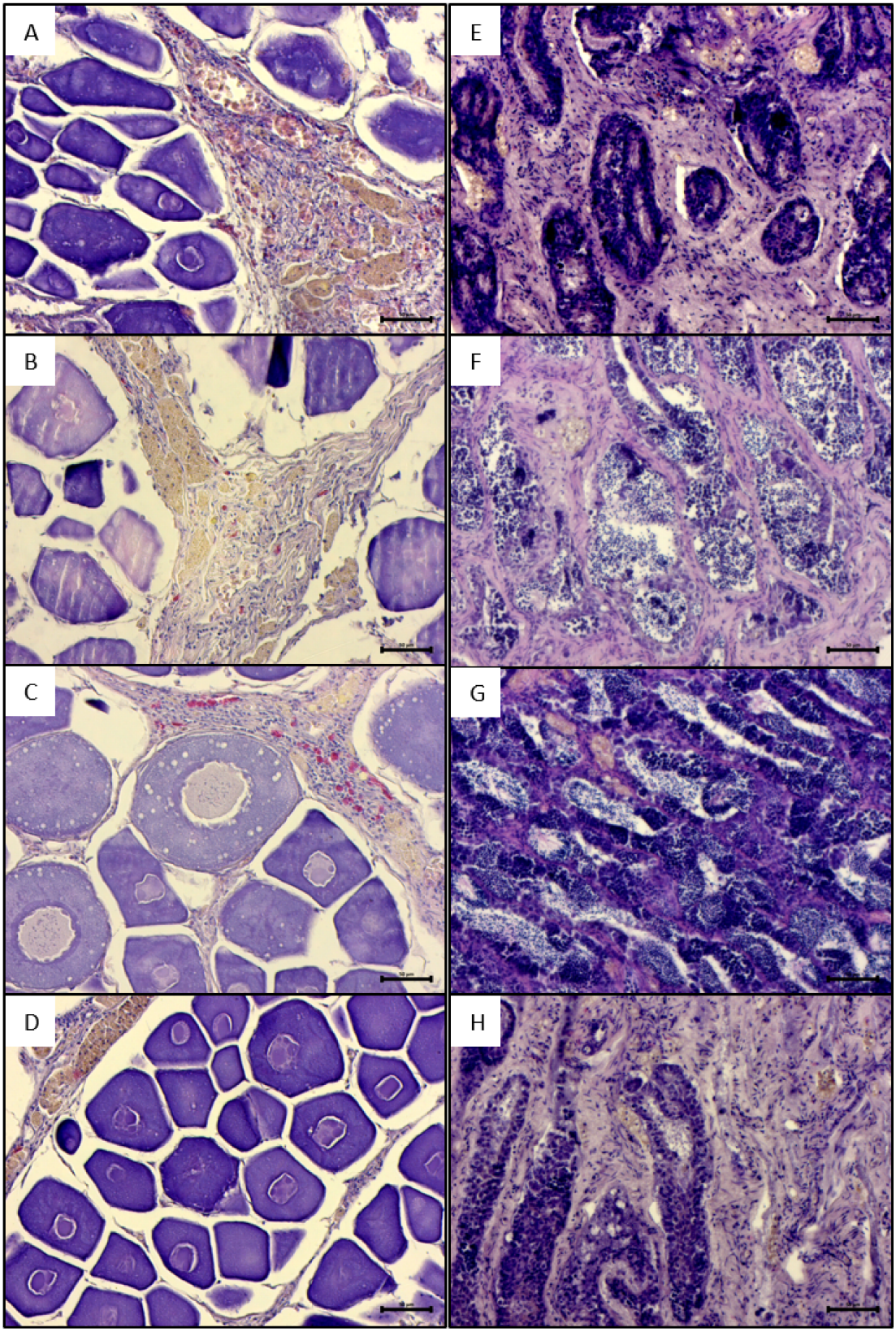

Figure 15. Tissue sections of non-reproductive tuna ovaries (A, B, C, and D) and testis (E, F, G and H). Scale bar is 50 µm and magnification 20x

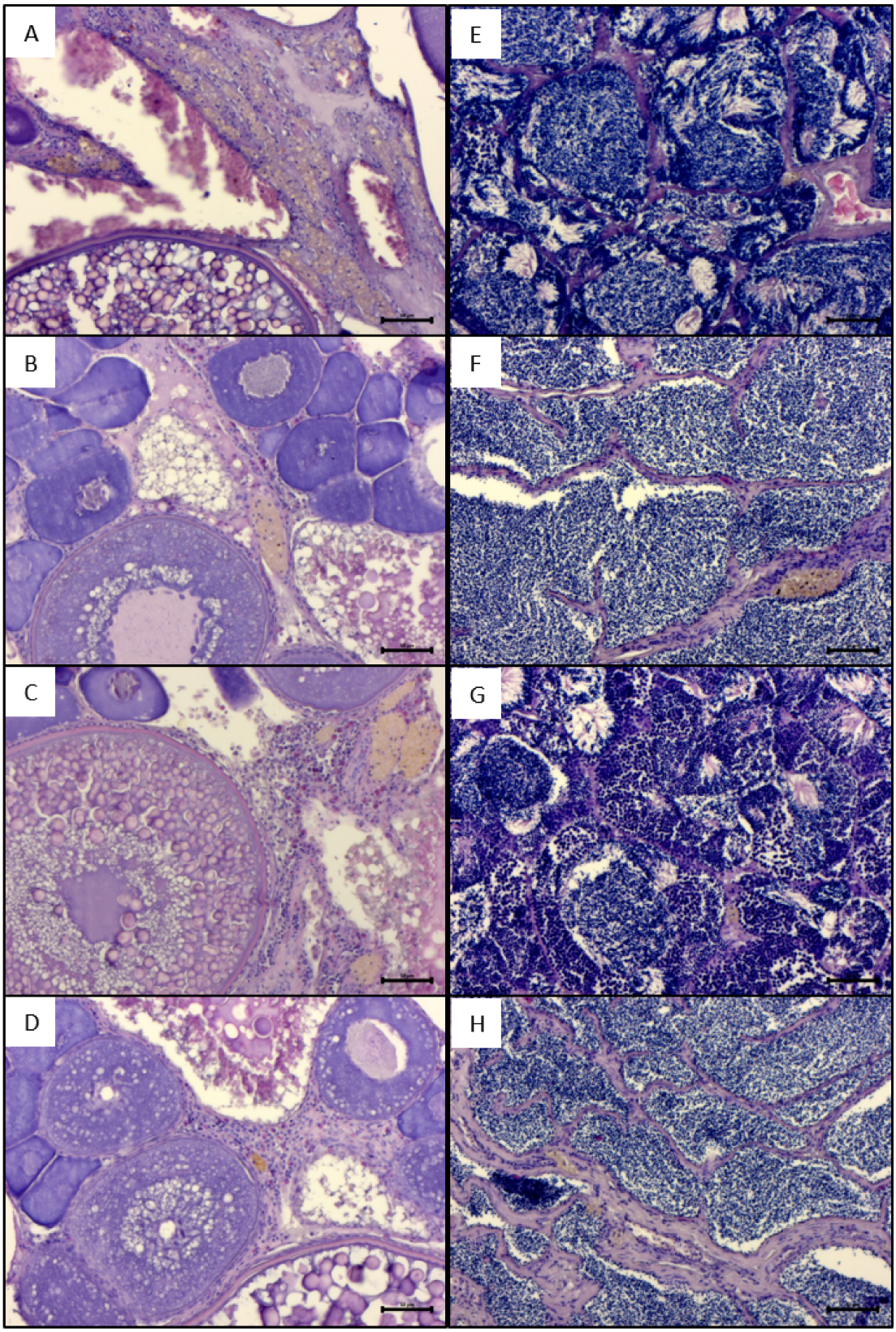

Figure 16. Tissue sections of reproductive tuna ovaries (A, B, C and D) and testis (E, F, G and H). Scale bar is 50µm and magnification 20x

The results of the quantitative analysis of the melanomacrophage centers are shown in Table 3. The histological sections of ovaries and testis in both reproductive and non-reproductive groups revealed the presence of melanomacrophage centers. The results show that females have a higher incidence of MMCs compared to males, with the highest value in the non reproductive group. While the lowest value of MMCs incidence is in reproductive males.

Table 3. Percentage of Melanomacrophage centers in non-reproductive and reproductive groups

Stage	n°	Sex	Length (cm)	Total weight (kg)	Gonadal weight (kg)	MMCs area (%)
Non reproductive	13	F	137.7±9.9	40.6±7.0	0.31±0.1	0.89±0.7
	10	M	143.4±11.3	44.3±10.1	0.16±0.0	0.31±0.3
Reproductive	14	F	167.83±42.2	93.7±58.4	2.75±2.2	0.51±0.6
	17	M	174.81±53.7	120.25±106.4	4.47±4.1	0.03±0.1

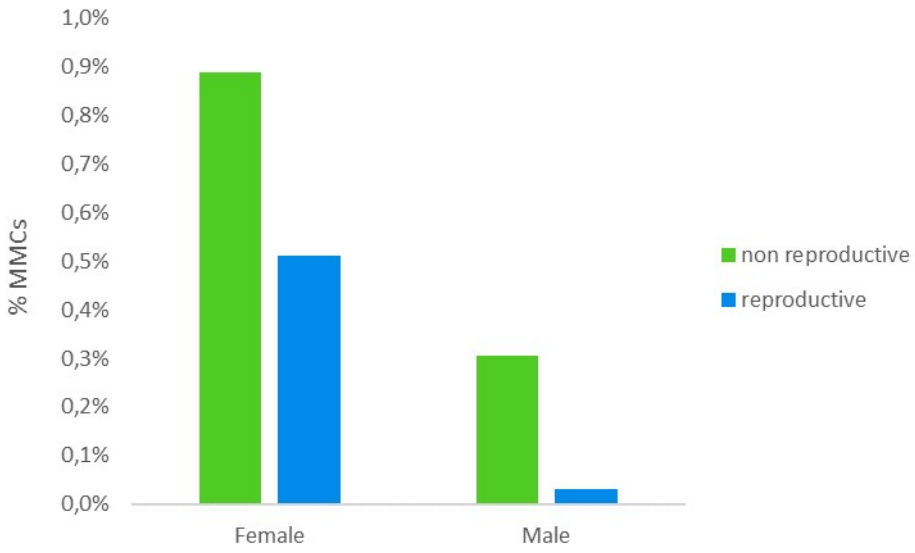

Figure 17. MMCs incidence in female and male

Table 4. Comparative results for female non-reproductive group

CFL (cm)	Total weight (kg)	Gonadal weight (kg)	Gonads	Blood	Intestine	MMCs (%)
129	**35**	**0.3**	**Pigment Blue 70 (20 µm)**	**-**	**-**	**0.891**
119	**30**	**0.2**	**Pigment Red 102 (15 µm)**	**-**	**-**	**1.572**
144	46	0.2	-	-	-	0.450
140	44	0.3	-	-	Pigment Red 101 (15µm)	0.057
138	38	0.3	-	-	Pigment Red 102 (15µm)	0.655
141	**43**	**0.3**	**Solvent Blue 70 (5µm)**	**-**	**Pigment Blue 15 (5µm)**	**1.620**
151	51	0.4	-	-	-	0.345
146	46	0.4	-	-	-	0.318
142	47	0.3	-	-	-	0.111
124	**27**	**0.4**	**Pigment Red 102 (15µm)**	**-**	**-**	**0.844**

Table 5. Comparative results for male non-reproductive group

CFL (cm)	Total weight (kg)	Gonadal weight (kg)	Gonads	Blood	Intestine	MMCs (%)
140	39	0.2	-	Levafix Blue E-RA (Bayer) (10 µm)	Pigment Ble 15 (10 µm) Pigment Red 102 (100 µm)	0.162
135	41	0.2	-	-	-	0.172
133	37	0.1	-	Polymethyl methacrylate (15 µm)	Plexiglas DRF-100 (40 µm)	0.011
142	41	0.1	-	-	-	0.299
152	55	0.2	-	-	-	0.600
159	57	0.2	-	-	-	0.299
162	60	0.2	-	-	-	0.222
145	**47**	**0.1**	**Pigment Brown 7 (15 µm)**	**-**	**-**	**0.910**
127	29	1.3	-	-	-	0.000
139	37	0.4	-	-	-	0.386

5 DISCUSSION

The Atlantic Bluefin Tuna (*Thunnus thynnus*) represent an important fishing resource and currently is considered overexploited (Corriero et al. 2005; Santamaria et al. 2009). Due to its commercial importance there are many studies focusing on its distribution (Corriero et al. 2005; Powers and Fromentin 2005; ICCAT 2017), reproductive biology (Carnevali et al. 2019; Corriero et al. 2005), behaviour (Addis et al. 2009; Teo et al. 2007) and feeding ecology (Estrada et al. 2005; Karakulak et al. 2009; Ortiz de Zarate and Cort 1986; Pinkas et al. 1971; Sarà and Sarà 2007). However, there is a lack of studies regarding the presence of contaminants in this key species.

Given the limited data in the literature regarding the presence and the negative impact of microplastics in Atlantic Bluefin Tuna it is difficult to compare our results with other previous studies. The presence of plastic debris in the gastrointestinal tract of ABFT has been confirmed by Romeo et al. (2015). However, this is the first study to directly identify microplastics in the gonads and blood of Atlantic Bluefin Tuna. Therefore, in the first place in this project, an effective protocol for the extraction of microplastics from different tissues, such as gonads, intestines and blood, has been developed. In addition,

the data obtained from the microplastics analysis were compared with the histological data for the presence of melanomacrophage centers in the gonads.

Several protocols have been developed for the extraction of microplastics from biotic matrices. These use different solutions, such as KOH (Foekema et al. 2013), HCl (Karl et al. 2014), HNO_3 (Van Cauwenberghe et al. 2015), HNO_3 and $HClO_4$ (De Witte et al. 2014), NaOH (Cole et al. 2014) and $K_2S_2O_4$ and NaOH (Maher et al. 2002). Every protocol has a different yield. In particular, the Foekema et al. (2013) protocol was used for this study, which was later optimized for our needs. Unlike other protocols present in the literature, the use of a 10% KOH solution allows the extraction of microplastics from the tissue without damaging them with the basic solution, and consequently allows a better spectroscopic analysis of the particles.

The Raman spectroscopy results show the presence of microplastics of different types, shapes, colour and sizes in gonads, blood and intestines of sexually mature Atlantic Bluefin Tuna samples. Since microplastics are now considered as ubiquitous pollutants in the marine environment, it is relatively easy to expect their presence in aquatic organisms, particularly in the digestive tract of a top predator, as shown by many studies carried out on several species. The interesting result of this study is the presence of microplastics in the gonads of tuna. Therefore, after ingestion through food, the microplastics are then transferred from the intestine into the blood and

finally transported through the circulatory system to other organs. The translocation of microplastics from the digestive tract was first demonstrated by Browne et al. (2008) in *Mytilus edulis*. They observed that the MPs translocated from the gut to the circulatory system and were then tracked in the haemolymph. The transfer of microplastics at membrane level has also been studied by Rossi et al. (2014), who utilized coarse-grained molecular simulations to demonstrated that polystyrene particles, with a diameter of up to 7 nm, permeated easily into the lipid membranes via alterations of the membrane bilayer structure.

During our analysis only two fragments were found in the blood, but it should be considered that blood samples of about 1 ml are probably not representative for an animal with a blood volume in the order of litres. In contrast to the gonad where the samples taken were about 20 g compared to an average total gonad weight of ca. 230 g. Therefore, for future analysis, in order to have data on the presence of microplastics also in the blood, larger samples will have to be taken.

The presence of microplastics in gonads can raise the problem of bioaccumulation and biomagnification of these foreign bodies in organs where excretion processes are basically non-existent. Therefore, it can be expected that organisms at higher trophic levels, such as tuna, can ingest microplastics either from water or through their prey (Hollman et al. 2013).

As a result, it can be assumed that older organisms show greater contamination by microplastics than juveniles. Although in this study it was seen that the individuals who showed a greater presence of foreign bodies in the gonad did not match the older individuals. This may be due to several factors, such as different eating habits between juveniles and adults or varying environmental contamination between different areas, because bluefin tuna is a migratory species and therefore does not always occupy the same area.

Moreover, the results obtained from Raman spectroscopy show the presence of many fragments in which the spectrum does not correspond to that of plastic materials as it is completely obscured by the spectrum of different pigments, such as copper phthalocyanine, Levafix blue E-RA (Bayer), Heliogen turquoise, Neozapon Blue FLE, Sicotrans red, Red Ochre, Umber and titanium dioxide. In particular, copper phthalocyanine, Levafix blue E-RA, Heliogen turquoise and Neozapon Blue FLE are highly useful in developing blue and green hues for plastic materials. For instance, copper phthalocyanine is the most commonly used phthalocyanine blue in the plastic industry, is used in almost all pigment applications, especially for paints, in rubber, linoleum, PVC and plastics (O'Neil, 2006). While Sicotrans red, Red Ochre are classified as red pigment and widely used paints and coatings, plastics, rubbers, leather industries, papers and pottery. Umber is classified as red/brownish pigment and typical used in coatings, decorative paints and

wood stains. Titanium dioxide is used as a white pigment in paints, paper, rubber, plastics, opacifying agent, cosmetics, inks and in water paints (O'Neil, 2006; Lewis, 2007). Levafix blue E-RA is mostly used in textile industry and classified as a toxic compound. Kalpana et al. (2012) demonstrate the efficiency of using the white rod fungi to decolorize and degrade the sulphonated azoic reactive Levafix Blue E-RA dye granulates, with no production of toxic metabolites during the process. This study is important to improve the methods in order to remove the dyes from industrial effluents, before they reach the marine environment.

Therefore, it was not possible to classify all the fragments found as microplastics due to the presence of the pigment, but the searches carried out show that very often these pigments are used in the colouring of plastic materials. Thus, we could expect that some of the fragments found in gonads, intestines and blood can be considered microplastics.

The second part of this work focuses on histological analysis in order to allow the correlation of data on the presence of microplastics in the gonads and the possible histological alterations found, probably caused by the presence of exogenous material. Melanomacrophage centers, which are normally present in the organism, develop in association with chronic inflammatory lesions in the body and during ovarian atresia (Agius and Roberts 2003). However, in certain conditions, there is an higher incidence of MMCs due to the presence

of foreign bodies (Manrique et al. 2014; Sayed and Younes 2017), immunosopressive toxins (Anderson et al. 2003; Haaparanta et al. 1996; Payne and Fancey 1989) or in polluted environments (Facey et al. 2005; Micale et al. 2019; Passantino et al. 2014; Wolke 1992). Comparing the percentages of melanomacrophage centers and the presence of foreign bodies in the female gonad during the non-reproductive period, there is a positive correlation. In fact, foreign bodies have been found only in organisms where the incidence of melanomacrophage centers is higher than 0.84%. It is clear that there is a link between a higher incidence of MMCs and a higher level of contamination by exogenous material. Therefore the results are in accordance with several other studies where the MMCs are used as a biomarker for the environmental quality (Agius and Roberts 2003; Mikaelian et al. 1998; Spazier et al. 1992; Stosik et al. 2019). In addition, the foreign bodies found in the gonad are smaller than 30 μm, so they are particles that can pass through blood vessels and consequently reach the gonad.

The analysis of microplastics in the female gonad of organisms in the reproductive period was not possible, so only the histological data are available for these samples. These data show that the percentage of melanomacrophage centers is on average lower than in the non-reproductive group, but among the 14 female samples two of them show a percentage of

MMCs higher than 0.84%. Therefore, we could expect the presence of microplastics in the gonad of these two samples.

Lastly, analysing the male gonads, it can be seen first of all that the percentage of melanomacrophage centers both in the non-reproductive and reproductive group is much lower than that of the females, but also the presence of microplastics is almost null. Only one sample of male gonad shows the presence of a foreign body, and even in this case, as in females, the percentage of MMCs is higher than 0.84%.

Therefore, in both ovaries and testis it has been observed that the percentage of melanomacrophage centers and the presence of microplastics or fragments of exogenous material are positively correlated. In particular, foreign bodies have been observed only in those gonads that show a percentage of melanomacrophage centers greater than 0.84%. So even if only histological data are available, the presence of microplastics could be deduced in samples where there is a higher incidence of MMCs.

Clearly, more information and further studies are needed to validate this hypothesis, especially on how microplastics are transported in the circulatory system. Plastics are generally lipophilic substances, so we could expect them to be linked to lipids in the blood. This could also explain why the microplastics are less represented in the testis than in the ovary, where there is a greater mobilization of lipids with the incorporation of vitellogenin and

neutral fatty acids in the oocytes. The interesting fact is that the highest incidence of the presence of microplastics occurs in the non-reproductive group, where vitellogenin is not yet present. Therefore, it could mean that the microplastics found in the ovary arrived during the previous reproductive season and then accumulated. If the microplastics are actually transported with the vitellogenin it is also possible that the transfer from the mother to the offspring takes place, as already demonstrated with the nanoplastic on the zebrafish by Pitt et al. (2018).

6 CONCLUSION

The results of this study represent a first evidence that microplastic pollution represents an emerging threat even in a top predator specie, such as the tuna (*Thunnus thynnus*). For the analysis, a valuable protocol for the microplastics extraction from biotic matrix with a 10% KOH solution was identified and optimized. Microplastics contamination in the gonads as well as fragments of exogenus material covered by pigments were found in intestine, gonads and blood of the Atlantic Bluefin Tuna. However, the presence of the pigments did not allow the identification of the underlying material.

Furthermore, the occurrence of foreign bodies differs between male and female gonads, with an higher concentration in the ovary. In particular, a positive correlation was seen between the presence of fragments covered by inorganic pigments and a higher incidence of melanomacrophage centers (MMCs) in the ovary of the non-reproductive group. Therefore, a negative effect in the gonad caused by the presence of these fragments can be hypothesized. This could then lead to reproductive dysfunction in animals more contaminated and more exposed to microplastics.

In conclusion, the results are essential to enrich the already very negative picture of the microplastics contamination in the marine environment of a very important commercial fish. Moreover, the presence of pigments lays the basis for future research focusing on their possible toxicity to marine organisms.

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