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CICLO XXXVII

ENVIRONMENTALMONITORING SYSTEMS AIMED AT DEVELOPING PROTOCOLS FOR HUMAN HEALTH

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ABSTRACT

Plastic pollution is one of the main global environmental problems of the 21st century. Through the course of the rivers, most plastic waste ends up in the sea, where a slow degradation determines its fragmentation into smaller particles, commonly known as microplastics (MPs) and nanoplastics (NPs). The main problem of the permanence of microplastics in the seas is their ability to interact with aquatic organisms, thus entering the food chain and reaching humans through the consumption of contaminated fish. The toxicity of microplastics is mainly linked to the fact that, during their permanence in the ecosystem, they can absorb and become a vehicle of harmful substances to humans, including pathogenic microorganisms, drugs, antibiotics, and heavy metals. As for heavy metals, some of them are indispensable for normal human biochemical processes, but others, such as lead and mercury, are extremely toxic.

SoLute Carriers (SLCs) represent the main target of heavy metals due to their localization on the cell surface. In particular, relevant importance are the organic cation transporters that form a subfamily of the larger SLC22 family because, in addition to the physiological role of transporting endogenous organic cations, are crucial in the disposition of drugs and the interaction with xenobiotics. Among these transporters, OCTN1 appeared in vertebrates during evolution and has homologs in some fishes. Interestingly, OCTN1 harbours seven cysteine residues, already known as a target of a prototype heavy metal, namely mercury chloride.

The following study aims to analyze microplastics sampled on the surface of the Mediterranean Sea in six particularly polluted areas of the Calabrian coasts. The size, shape, color, and polymeric composition of the samples have been investigated. Most of the microplastics were very small, with shape and color critical for

interactions with biota. In addition, the presence of heavy metals potentially harmful to humans was detected. Subsequently, one of the most important freshwater fish species in the human diet (*Oncorhynchus mykiss*) has been used as a model for studying the release of these metals from microplastics by the simulation of its digestion process. Interestingly, the release from microplastics of chromium, lead, cadmium, and zinc was significant, especially during the gastric phase of the digestive process, due to the very acidic pH. Lead was also detected by SP-ICP-MS, probably due to solid deposits on the surface of the plastics. Furthermore, the powder obtained from some reference plastic materials showed that the amount of metals released depends on the size of the microplastics and, consequently, on the surface area exposed to the digestive fluids. Finally, the effect of these heavy metals on the human organism was evaluated through interaction studies with the organic cation transporter OCTN1. The potency of heavy metals on OCTN1 was evaluated through IC_{50} analysis, using the experimental system of the proteoliposome, and the values were in the micromolar range. In addition, the mechanism of interaction of these xenobiotics with the protein was elucidated through site-directed mutagenesis and computational analyses. To suggest some potential scavengers, the effects of some reducing agents were studied. Some of these can reverse the negative effects of heavy metals on OCTN1.

ABBREVIATIONS

1PW4 Crystal Structure of the Glycerol-3-Phosphate

4J05 Crystal Structure of a Eukaryotic Phosphate Transporter

ABC ATP Binding Cassette

Ach Acetylcholine

ADME Gene encoding for a protein conventionally considered central to the absorption, distribution, metabolism, and elimination of drugs

Arg Arginine

ARPA Regional Agency for Environmental Protection

ATP Adenosine Triphosphate

ATR Attenuated Total Reflectance

ATSDR Agency for Toxic Substances and Disease Registry

BSA Bovine serum albumin

$C_{12}E_8$ Octaethylene Glycol Monododecyl Ether

$CaCl_2$ Calcium Chloride

cAMP Cyclic adenosine monophosphate

$CdCl_2$ Cadmium chloride

CH_4 Methane

CO_2 Carbon Dioxide

$CrCl_2$ Chromium (II) chloride

CRSM Marine Strategy Regional Center

Cryo-EM Cryogenic Electron Microscopy

Cys Cysteine

Cys-less Without Cysteines

DDM n-dodecyl D-β maltoside

DNA Deoxyribonucleic Acid

DTE 1,4 Dithioerythritol

EDS Energy Dispersive Spectrometer

EDTA Ethylenediaminetetraacetic acid

EU European Union

EVA Ethylene-vinyl Acetate

FT-IR Fourier Transform Infrared Spectroscopy

GABA Gamma-Aminobutyric Acid

Glu Glutamic Acid

GLUT Human Glucose Transporter

H_2O Water

H_2O_2 Hydrogen Peroxide

HCl Hydrochloric Acid

HDPE Hig-density Polyethylene

$HgCl_2$ Mercury (II) chloride

HgS Mercuric Sulfide

HNO_3 Nitric Acid

IARC International Agency for Research on Cancer

IBD Inflammatory Bowel Disease

IBS Irritable Bowel Syndrome

IC_{50} Half-maximal Inhibitory Concentration

ICP-MS Plasma Mass Spectrometry

IPA Aromatic Hydrocarbon

IUPAC International Union and Applied Chemistry

KCl Potassium Chloride

LDPE Low-density Polyethylene

Leu Leucine

LLDPE Linear-density Polyethylene

LQ Limit of Quantification

MeHg Methylmercury

$MgSO_4$ Magnesium Sulphate

MP Microplastic

mRNA Messenger Ribonucleic acid

MTSET 2-(Trimethylammonium)ethyl methanethiosulfonate, Bromide

nAChR Nicotinic Receptor

NaCl Sodium Chloride

NaHCO3 Sodium Hydrogen Carbonate

NaOH Sodium Hydroxide

NDB Nucleotide-binding Domain

NNCS Non-Neuronal Cholinergic System

NP Nanoplastic

OAT Organic Anion Transporters

OCT Organic Cation Transporters

OCTN Organic Cation Transporters Novel

OCTN1 Organic Cation Transporter Novel 1

PA Polyamide

$Pb(NO_3)_2$ Lead nitrate

Pb_3O_4 Lead Tetroxide

PBC Polychlorinated Biphenyl

$PbCrO_4$ Lead Chromate

PDB Protein Data Bank

PDC Primary Carnitine Deficiency

PES Polyester

PET Polyethylene Terephthalate

pK Dissociation Constant

PLP Pyridoxal 5'-phosphate

POP Persistent organic pollutant

PP Polypropylene

PS Polystyrene

PTFE Politetrafluoroetilene

PTM Post-translational Modification

PVC Polyvinyl Chloride

ROS reactive oxygen species

SDS–PAGE Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

SEM Scanning Electron Microscopy

SGF Simulated Gastric Fluid

SH Thiol Group

SIF Simulated Intestinal Fluid

SLC SoLute Carriers

SP-ICP-MS Single Particle Plasma Mass Spectrometry

TEA Tetraethylammonium

TFM Tetrafluormethaxyl

TMD Transmembrane Domain

Tyr Tyrosine

UC Ulcerative Colitis

URAT Uric Acid Transporter

UV Ultraviolet Radiation

VachT Vesicular acetylcholine transporter

WHO World Health Organization

WT Wild Type

xCT Cystine/Glutamate Antiporter

$ZnCl_2$ Zinc chloride

ΔG activation energy

CHAPTER 1:

Introduction

1.1 PLASTIC

“The substance that nature forgot to create,” is how Paul John Flory, winner of the Nobel Prize in Chemistry in 1974, defined plastic. The IUPAC (International Union of Pure and Applied Chemistry) defines it as “polymeric materials that may contain other substances to improve their properties and reduce costs.”

Plastics are synthetic organic polymers made by linking hundreds or thousands of organic subunits, i.e. monomers, together via strong covalent chemical bonds and are made from non-renewable petrochemical products, so-called because they are derived from fossil oil, natural gas, and coal. They are cheap, durable, versatile, and heterogeneous, so they are present in every aspect of our lives [1]. Their low cost and excellent moisture barrier properties make them ideal for packaging materials, sterile medical uses, and construction, among others; for this reason, classic materials such as glass, metal, and paper have been replaced by more economically advantageous plastic packaging [2]. The first synthetic polymer is "Bakelite" (made by a condensation reaction of phenol with formaldehyde) and dates to the beginning of the 20th century, more precisely in 1907. Subsequently, fairly cheap techniques have been optimized to allow the production of a large variety of plastics [3]. The real mass production of plastic products began in the 1950s and has grown exponentially since then, reaching 380 Mt per year in 2015 [3], representing 8% of the world's oil production. The largest shares of the plastic market belong to low-cost basic thermoplastic polymers, namely polyethylene terephthalate (PET), high-, low- and linear-low-density polyethylene (HDPE, LDPE,

and LLDPE), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS), polyester (PES), polyamide (PA) **(Figure 1.)**[1, 2, 4].

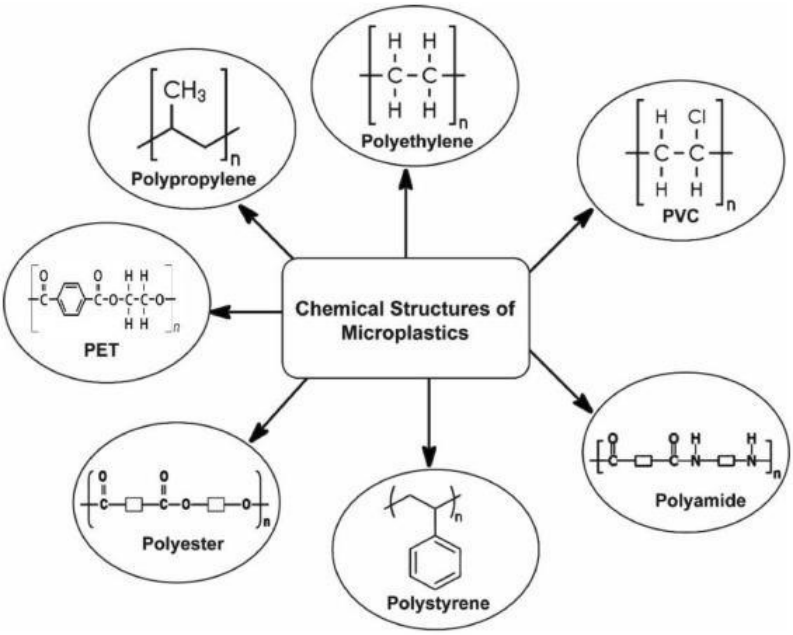

Figure 1*. Chemical structures of some common MPs polymers[4].*

Although these plastics are inexpensive, each is a highly engineered material with precise physical properties. They can be molded into virtually any desired shape by rotation, injection, extrusion, compression, blow molding, or thermoforming. Their material properties are adjusted during and after synthesis to achieve the desired strength, permeability, porosity, opacity, and color. For example, polyolefins are particularly durable, a property due to their high molecular weight and hydrophobicity (chemical and biological inertness), and the absence of functional groups that are susceptible to attack by microbial enzymes, light, water, etc [5]. Plastic objects can be divided into two main categories: those defined as “disposable,” and those that must last a long time, maintaining their physical and mechanical properties. For this reason, for the second category, different production protocols have been developed to increase their durability over time: by building new molecules; creating mixtures of

several different polymers (blends); by adding other components, called additives, which can be organic fillers, colorants, oxidant stabilizers, UV stabilizers, plasticizers, lubricants [6]. All these fascinating characteristics of plastic products also make them particularly long-lived when they are discarded. Various antioxidants and stabilizers, which are used to extend the useful life of plastics, dramatically slow down their environmental degradation. Consequently, despite the social benefits associated with plastics being far-reaching, this material is the subject of ever-increasing environmental concerns [7]. Globally, only 18% of plastic waste is recycled and 24% is incinerated. The remaining 58% is disposed of in landfills or released into the natural environment, where plastic accumulates and persists for a long period, we are talking about hundreds of years. [8-10].

1.1.1 Marine Litter

Plastic waste that ends up in the marine ecosystem is defined as "marine litter", which means "any persistent solid material manufactured, processed, discarded or abandoned in the marine environment" [11]. Marine litter is one of the major issues of the 21st century [12]. The sources of this material are highly correlated with the lack of effective waste management infrastructure. It is estimated that almost 90% of the plastic entering the ocean comes from just 10 rivers, all located in Asia or Africa [13]. Plastic debris in the oceans is associated with persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), pesticides, and polycyclic aromatic hydrocarbons (IPAs), due to the higher affinity that these hydrophobic molecules have for plastics compared to their affinity for sediments or water [14]. About 80% of the plastic present in the oceans comes from

terrestrial activities (urban landfills, malfunctioning sewage systems, industries) that are transported to the seas thanks to rivers; while the remaining 20% comes from maritime activities (cruises, recreational and commercial fishing, aquaculture) [12] [15, 16].

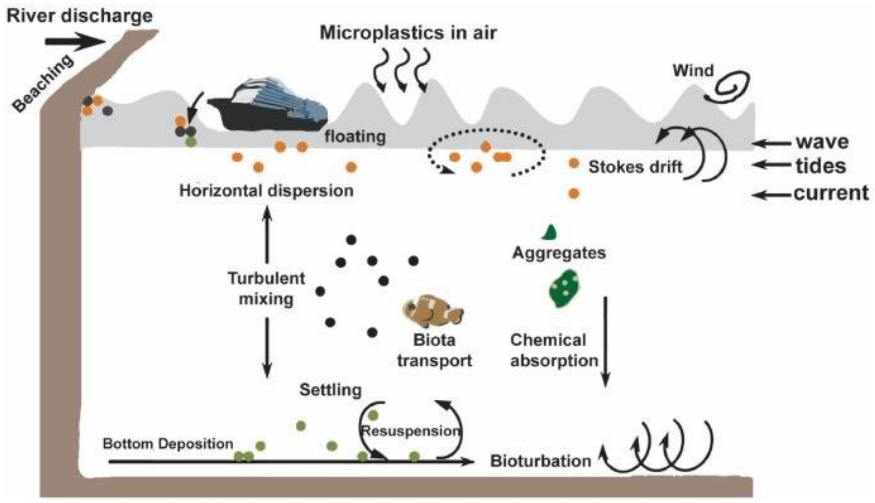

Figure 2. *Diagram of the MPs transport pathways in the ocean[17]*

Once in the sea, plastics undergo a degradation process over time determined by chemical, physical, and biological actions and this leads to the formation of very small fragments, measuring millimeters and/or micrometers [18]. As regards chemical-physical actions, the main protagonist is the oxygen present in the air. It can interact with polymers creating free radicals that cause breaks in the polymer chains, with exothermic reactions. Immediately after, UV solar radiation can cause the formation of free radicals and the consequent breaking of the chain; the presence of water also induces hydrolysis reactions. The breaking of the chains causes the phenomenon of chemo-crystallization, that is, the shorter molecules reorganize themselves into crystalline spherules with a volumetric contraction that causes the formation of surface cracks that propagate throughout the body producing increasingly smaller fragments [6]. Biological degradation involves mechanisms

involving extracellular enzymes produced by environmental bacteria and other microorganisms to break the chemical bonds that bind plastic molecules. The colonization of the surface of plastics by the microbial community, described as the plastisphere, results in the degradation of the polymer into its constituent monomers, which are then broken down into carbon dioxide (CO_2), methane (CH_4), and water (H_2O) [19, 20]. Different types of plastic polymers have been reported to have variable sensitivity to the action of enzymes and UV radiation [21]. Some characteristics of plastic products can influence the rate of degradation processes, both biotic and abiotic, including the complex polymer structure and the material components.

1.1.2 Microplastics

The term "microplastics" (MPs) was used for the first time [22] to indicate the accumulation of microscopic plastic fragments observed both in marine sediments and in water columns. Based on their size, plastic fragments are classified as macroplastics (>25 mm), mesoplastics (between 5 and 25 mm), microplastics (between 300 μm and 5 mm), and nanoplastics (<300 μm). [23]. However, within the scientific community, there is still not much consensus on this type of classification. Some authors define MPs as particles with dimensions in a range between 1 μm and 5 mm, while the dimensions of NPs would fall in a range between 1 nm and 1 μm; others have defined NPs as "particles smaller than 100 nm", based on the European Commission's definition of nanomaterial size which is 1-100 nm [24]. To date, microplastics have been observed virtually everywhere, from the poles to the equator; they have even been reported in considerable concentrations even in remote sites such as Antarctica and on the

bottom of the Mariana Trench [9, 25, 26]. This is because, once introduced into the sea, they can be transported by currents and winds and return to the coasts again, or they can become part of gyres. A gyre is a large system of circulating ocean currents formed by global wind patterns and forces created by the Earth's rotation. These natural phenomena act in a similar way to vortices, dragging debris (including plastic) towards their center. Thanks to these movements, waste tends to accumulate in five "ocean garbage patches" located in the North Atlantic, South Atlantic, North Pacific, South Pacific, and Indian Oceans. The largest of these areas is the Great Pacific Garbage Patch, located between Hawaii and California. The largest percentage of plastic present in this region would mainly derive from sources in Asia and would arrive there through the Kuroshio, also known as the "black current", as well as from intensive fishing activities in the Pacific Ocean [27]. The Mediterranean Sea is indicated as the sixth largest accumulation area of floating marine plastic waste [28].

Based on their origin, microplastics can be divided into primary and secondary. Primary microplastics are particles intentionally manufactured as such and used as raw material for the production of larger objects such as cosmetic products (e.g. scrubs, detergents, toothpaste) and industrial abrasives. The microspheres present in cosmetics and detergents end up directly in water through sewage discharges, it is unthinkable that these are effectively removed with wastewater treatment. Secondary microplastics instead derive from the fragmentation and/or degradation of larger plastic waste, this category also includes fibers released from synthetic fabrics. In addition to this classification, MPs can be further classified based on their shape into fragments, sheets, granules, fibers, foams, and pellets [29].

1.1.3 Health risks

In addition to being an environmental pollution problem, microplastics represent a major problem for human health. It has been highlighted that these substances have been found in various foods such as beer, honey, sea salt, canned sardines, and tap water [30, 31], as well as in many commercially important fish species [32]. When microplastics enter the seas, they can be mistaken for food due to their small size and be ingested by fish, thus entering the food chain until they reach our tables through the consumption of contaminated fish products. Generally, fish species consumed whole (such as mollusks and crustaceans, anchovies, and sardines) represent a greater threat than gutted fish or shelled shrimp. However, as reported in [33] the concentration of MPs present in two species of dried fish (*Chelon subviridis* and *Johnius belangerii*) is significantly higher than that present in the removed organs (viscera and gills), this leads us to believe that evisceration does not necessarily eliminate the risk of microplastic intake. Furthermore, the presence of microplastics has recently been detected in the muscle of some species of fish such as *Ephinephelus coiodes, Platycephalus indicus, Sphyraena jello, Saurida tumbil, Sillago sihama,* and in crustaceans such as *Peneaus Semisulcatus* [34, 35]. These observations raise concerns about what the potential effects on humans could be.

It should be noted that humans are exposed to microplastics through three routes: 1) ingestion of foods containing them, 2) inhalation of microplastics in the air, and 3) skin contact with particles contained in products, fabrics, or dust **(Figure 3).**

Figure 3. *Pathways of human exposure to microplastics[36].*

Inhalation and dermal contact with microplastics are considered less significant exposure routes, while ingestion of contaminated substances, especially fish products, represents the main exposure route and it is assumed that on average 39,000 to 52,000 particles are ingested per year. After ingestion, microplastics could be absorbed at the intestinal level and Peyer's patches are the main site of absorption and translocation. In particular, M cells, specialized epithelial cells lining Peyer's patches, are responsible for the absorption of particles by endocytosis and also for their transport from the intestinal lumen to the lymphoid tissues of the mucosa. Translocation can then occur through systemic circulation, reaching secondary target organs, including the liver, kidney, spleen, heart, and brain. Size, shape, solubility, and surface charge influence the cytotoxicity of plastic particles in cells and tissues *in vivo*. In particular, following exposure to high concentrations, the cell may undergo necrosis or apoptosis, since the surfactant molecules present on the surface of plastic particles appear to be able

to break the lipid bilayer of the plasma membrane. Lower levels of surfactants would instead be sufficient to alter important membrane structures such as proteoglycans and other extracellular matrix structures, as well as interfere with signaling processes based on receptor-ligand interaction. At the same time, nanoplastics could be absorbed by endocytosis. In this way, nanoplastics released into the cytosol would interfere with the functioning of various organelles, such as mitochondria and the nucleus, as well as with cellular processes such as the formation of mitotic spindles and chromosome migration [37, 38].

1.1.4 Microplastics and environmental contaminants

The potential impact of micro- and nanoplastics on aquatic ecosystems may go beyond the simple effect due to the ingestion of particles, due to their ability to absorb and transport different types of contaminants (pharmaceutical compounds, agrochemicals, organic, inorganic, and heavy metals) [39]. Without considering all the harmful substances used in the production of the plastic substances themselves [40]. It has been established that plastic waste dispersed in the environment can absorb, and therefore release, some molecules originating from drugs, such as phenanthrene, ciprofloxacin, and antibiotics [41]. What is of greatest concern are heavy metals, of which microplastics are the main carriers, these can be absorbed from the surrounding environment or used as additives in production processes. Plastic fragments that remain in the marine environment for a long time have a higher metal content than "virgin" plastics, this is because absorption increases over time, also due to the larger surface area that is gradually created, due to continuous fragmentation [42].

1.2 HEAVY METALS

Heavy metals are chemical elements that have a density at least 5 times higher than that of water, therefore at least 5 g/cm^{-3}, have an atomic mass higher than 23 or an atomic number higher than 20. Consequently, this category includes rare earth elements, transition elements, Bi, elements that form amphoteric oxides such as Al, Ga, In, Tl, Sn, Pb, Sb, Po, and some metalloids such as Ge, As, Te [38]. Heavy metals are naturally present in our environment and can derive from natural processes such as volcanic eruptions, however, their environmental contamination derives mainly from human activities and one of the main causes is their use as additives during the production process of plastics [38]. Although European regulations prohibit the use of dangerous additives for the production of plastics, it is still possible to find them in some consumer goods available in the EU [40]. Some metals, such as Al and Zn, are used as flame inhibitors and retardants; Zn, Pb, Cr, Co, and Cd are used as inorganic pigments; antimony and tin compounds are used to prevent the degradation of plastics due to UV radiation, the presence of oxygen, and other atmospheric agents. As, Sb, and Sn are also used as biocides to make polymers resistant to microbial attack [38]. However, it is important to point out that microplastics are vehicle of heavy metals because these are used in the polymer production processes, and they can be adsorbed from the surrounding environment when plastic products are dispersed as waste. The adsorption processes are various and can be influenced by different factors. Adsorption probably occurs through interactions between divalent cations (e.g. Cd^{2+}, $Pb^{2+,}$ etc.) with charged or polar regions of the plastic surface and through non-specific interactions between neutral organometallic complexes and the hydrophobic surface of the

bulk plastic medium. These bonds are influenced by factors such as pH, salinity variations, photo-oxidative erosion, biofilm formation, increased polymer polarity, and plastic porosity [43-46]. It has been demonstrated that microplastics dispersed and aged in the sea have a greater capacity for adsorbing heavy metals, due to the modifications that the polymers undergo over time, the increase in surface area induced by continuous fragmentation, and the presence of biofilms and chemical precipitates. In summary, microplastics dispersed in the environment, loaded with intrinsic (additive) and extrinsic (environmental) heavy metals, can be transported into the food chain to reach aquatic organisms and, consequently, humans [47].

1.2.1 Heavy metals and aquatic environment

Once plastic particles are ingested by fish, during digestion they can release the heavy metals they carry through leaching processes, which are then absorbed by aquatic organisms through several steps: first, the metals bind to the mucus of the intestinal lumen, then they are transferred to the mucosal epithelium via the apical membranes of enterocytes and finally they are exported, through the apical membranes of enterocytes, into the blood or extracellular fluid [48]. At high concentrations, metals can accumulate, causing toxicity. Their toxicity is mainly linked to carcinogenesis through mechanisms that act at different molecular levels, including inhibition of DNA repair and modulation. It is known that they can bind to sulfhydryl groups of cysteine residues of proteins such as metallothioneins causing their depletion [49].

1.2.2 Heavy Metals and Human Health

The consumption of contaminated fish triggers humans to come into contact with heavy metals. About their effects on the human organism, high concentrations of heavy metals have been shown to cause cellular and tissue damage, leading to a variety of adverse effects and human diseases. Al, Sb, As, Ba, Cd, Cr(II), Co, Cu, Pb, Hg, Ni, Se, Sn, and V are defined as metalloestrogens, i.e., they show a high affinity for estrogen receptors because they can mimic activation by estrogen; for this reason, they are potentially linked to breast cancer [38, 50-56]. According to the IARC (International Agency for Research on Cancer) guidelines, arsenic, cadmium, chromium, lead, and mercury are classified as human carcinogens. Cadmium has been suggested to participate in promoting cell apoptosis and DNA methylation, providing oxidative stress, causing DNA damage, increasing bone fractures in postmenopausal women, and lipid peroxidation. Lead is responsible for damage to DNA repair systems, production of ROS (reactive oxygen species), deregulation of oncogenic genes, and various central nervous system effects, including impairment of motor and cognitive functions, convulsions, coma, and death. Arsenic contamination could cause cancer of the urinary bladder, lungs, liver, and kidneys. As for mercury, it affects two target organs: the central nervous system and the kidney [38].

1.3 MARINE POLLUTION

Heavy metals are the object of study of this research even if they are not the sole environmental pollutants. Human activities, such as urbanization and increasing industrial development, lead to the development and release into the marine environment of numerous contaminants including oils, persistent organic pollutants (POPs) and new Emerging Pollutants (EPs) which include pharmaceutical compounds (triclosan, triclosan, losartan, Ffluoxetine) cosmetics, endocrine disruptors, biocides (chlorothalonil, DCOIT) and illegal drugs (in particular cocaine and crack). Like heavy metals, all these substances can be absorbed and transported by microplastics and, once dissolved in the marine environment, can be absorbed by organisms becoming bioavailable for other organisms, bioaccumulative and biomagnifiable along the entire food chain, until reaching humans in very high concentrations [57]. Among POPs, PAHs are of particular concern. These hydrocarbons can cause genotoxic, mutagenic and carcinogenic damage [58]. In the ecotoxicological field, these substances are detected with some tests such as: comet test, micronucleus test and neutral red test. The comet test is a very sensitive test because it detects DNA breaks caused by these substances at very low concentrations. The micronucleus test, on the other hand, is aimed at the identification of pollutants in a broader context, i.e. by measuring the frequency of micronuclei it is possible to evaluate the effects of chronic exposure at the population level. Finally, the neutral red test is used to evaluate the stability of the lysosomal membrane by determining the retention time of the neutral red dye (NRRT) [57]. As for emerging contaminants, due to their recent detection in the environment, most EPs are not regulated and their impact on aquatic

organisms is still poorly known. However, some general responses are prevalent, such as those related to the metabolism of xenobiotics (i.e., substances foreign to the organism or ecosystem, including EPs) [59]. Since the biotransformation of xenobiotics induces an increase in metabolism, augment of ROS occurs with consequent activation of the antioxidant system. Indeed, some pollutants can induce oxidative stress with consequent increase in antioxidant enzyme activity, such as glutathione peroxidase (GPx). Furthermore, oxidative stress can induce DNA damage and lipid peroxidation (LPO) due to the oxidation of these biomolecules. Also in the case of emerging contaminants, comet, micronucleus and neutral red tests can be used [57].

1.4 MEMBRANE TRANSPORTERS

Membrane transporters are considered excellent targets in environmental toxicology, as in this case, because these proteins are exposed on the cell surface. Therefore, they represent a forefront target for xenobiotics including drugs and pollutants such as heavy metals [60]. The biochemical function of membrane transporters is to allow the passage of nutrients and catabolites through cell membranes, which are otherwise impermeable to any molecules [61]. In biological systems, there are two main routes for transport: channels and transporters. Channels are mainly responsible for the traffic of ions, which pass from one side of the membrane to the other downhill their concentration gradient and thanks to precise interactions with amino acid residues of the channels. While channels are specific for ions, transporters catalyze the traffic of a larger variety of molecules such as nutrients, metabolites, catabolites, cofactors, and ions. The transmembrane passage step of a substance represents a highly energetic stage, comparable to the transition state of an enzyme-catalyzed chemical reaction. In both cases, an activation barrier must be overcome to reach the intermediate stage. The activation energy (ΔG) required for the translocation of a polar solute across the lipid bilayer is so large that lipid bilayers are virtually impermeable to polar species and charged species. Membrane proteins lower the activation energy barrier thus greatly increasing the transport rate of the flux across the membrane. However, transporters are not enzymes, since their substrates are not chemically modified but are transported from one compartment to another. However, like enzymes, transporters bind their substrates with high specificity through noncovalent interactions. Transporters traverse the lipid bilayer of the membrane, forming a transmembrane pore lined with hydrophilic

amino acid side chains, which further lowers the ΔG for transmembrane passage. The result of this process is an increase of several orders of magnitude in the rate of passage of the substrate through the membrane, with respect to diffusion (**Figure 5**) [62].

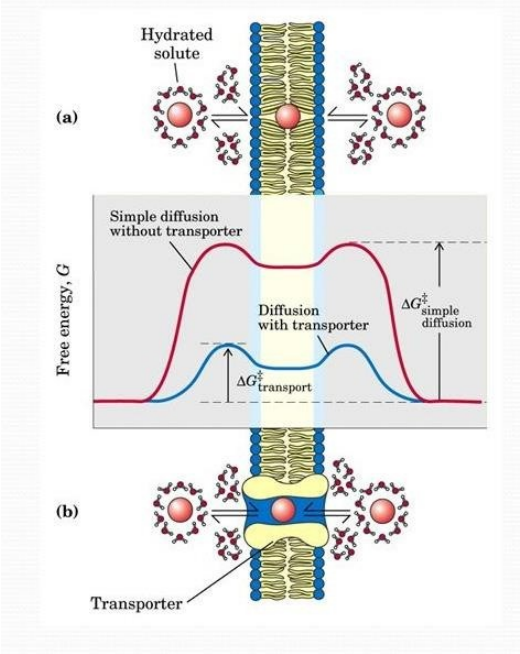

Figure 5. *Energy changes accompanying the passage of a solute across the lipid bilayer of a biological membrane. (a) transport without a membrane protein; (b) a carrier protein reduces the value of ΔG.*

Transporters are classified into primary and secondary active transporters. In the first case, the transport ΔG derives from the hydrolysis of ATP; in the second case, the flow of a solute provides the ΔG for the transport of another solute against the electrochemical gradient. The primary active transporters are called ABC (ATP binding cassette) transporters and are grouped into 7 families. ABC transporters have domains capable of binding ATP (NDB) at the cytosolic level, that have the task of hydrolyzing ATP from which the driving force for transport reactions comes. These proteins are found in both prokaryotes and eukaryotes, in the former they serve exclusively for the absorption of nutrients, while in the latter, they work both in uptake and efflux. The

secondary active transporters are classified into: 1) Uniporters, which catalyze the transport of a single species independently of other species. Transport occurs according to the concentration gradient of the substance, which creates the driving force necessary for passage through the cell membrane, from a more concentrated area to a less concentrated area; 2) Symporters, which catalyze the transport of two or more molecular species in the same direction. In this case, the transport of a substance along its own concentration gradient creates the driving force necessary for the transport of a second substance against its own concentration gradient (from a less concentrated area to a more concentrated one) in the same direction as the first; 3) Antiporters, which catalyze the exchange of one or more molecular species with another in opposite directions. The transport of a substance along its concentration gradient creates the driving force necessary for the transport of another substance against its concentration gradient but in the opposite direction to the first **(Figure 6)** [62].

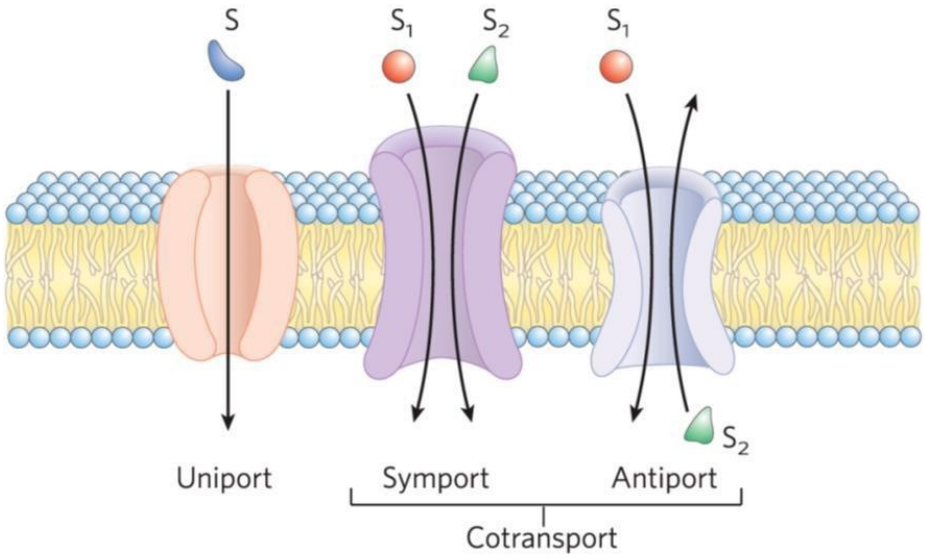

Figure 6. *Secondary active transporters [62]*

1.4.1 Biological membrane composition

Biological membranes are made up of a lipid bilayer that constitutes a barrier for the passage of ions and virtually all molecules. The main lipids that constitute eukaryotic membranes are glycerophospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatic acid), sphingolipids and sterols including cholesterol (the most abundant in mammals). Lipid rafts are domains of the plasma membrane particularly rich in cholesterol that binds sphingolipids, this makes the bilayer thicker and more ordered than other areas that contain more phospholipids and are more fluid. Proteins are incorporated into this lipid bilayer, they are positioned so that their hydrophobic domains are in contact with the fatty acyl chains of the lipids. The orientation of lipids and proteins in the bilayer is asymmetric and forms the so-called "fluid mosaic model" [62].

1.4.2 SLC Transporters

The secondary active transporters are called also SLC (SoLute Carriers). The SLC group includes 52 families of secondary active transporters for a total of 400 genes that, thanks to alternative splicing processes, give rise to more than 800 proteins [63]. As stated in the previous paragraph, SLCs derive energy from the concentration gradients of the solutes or ions involved that are co-transported with the solutes in the same or opposite directions. Such gradients are always generated by the hydrolysis of ATP (secondary active transporters) [63]. SLC proteins transport a wide range of molecules, including sugars, amino acids, vitamins, nucleotides, metals, inorganic ions, organic anions, oligopeptides, and drugs. Many of these proteins are

polyspecific, others transport only one biomolecule, and still, others are considered "orphans", that is, without a known substrate. On average, SLC families contain seven members (sharing at least 20-25% of sequence), but 8 families contain only one (SLC32, SLC40, SLC48, SLC50, SLC53, SLC61, SLC62, SLC64), while the largest, SLC25, contains 53 members. Structurally, by analyzing hydropathy profiles, SLC transporters contain 1 to 16 transmembrane domains, although approximately 83% contain 7 to 12 transmembrane domains. SLC transporters are abundant in the body and are essential for several vital processes: the import of nutrients into cells, the generation of electrical and chemical signals, the regulation of cell volume, and the absorption and delivery of drugs. [61].

1.5 SLC22

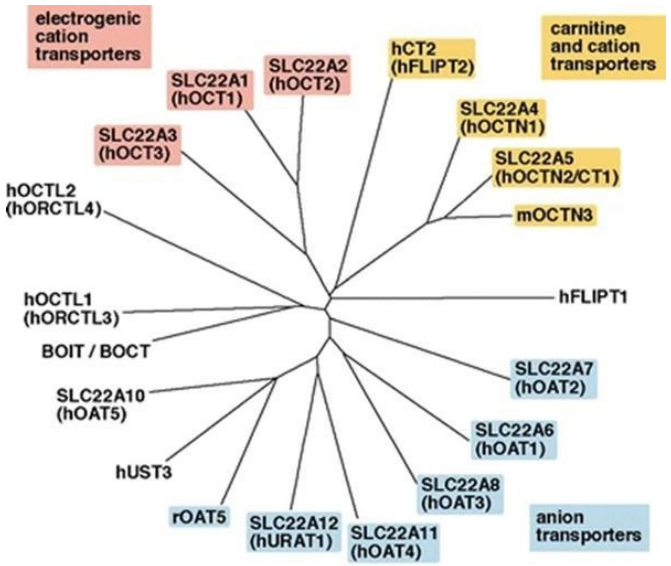

Figure 7. *Phylogenetic tree of the human transporters of the SLC22 family[64].*

The SLC22 family is one of the largest SLC families with 30 members, 13 of which are located on the plasma membrane. SLC22 proteins have been grouped into two main subfamilies based on their phylogenetic history, which are further divided into other subgroups: Organic Anion Transporters (OAT) and Organic Cation Transporters (OCT). OATs are divided into Oat, Oat-like, and Oat-related subgroups; while OCTs are divided into Oct, Octn, and Oct-related. From a structural point of view, all SLC22 are made up of 12 α-helical transmembrane segments (TMDs), a large extracellular loop located between TMDs 1 and 2 that is glycosylated and in some cases mediates homo-oligomerization, and a large intracellular loop located between TMDs 6 and 7 that is involved in post-translational regulation [65]. From a functional point of view, most SLC22 transporters are

polyspecific, that is, they transport structurally diverse substrates and many other compounds can act as high- or low-affinity inhibitors [66]. Transporters within the same subgroup show similarities about transport mechanism and substrate selectivity, furthermore, different substrates can be transported or inhibited by transporters of different subgroups. OCT1-3 translocate organic cations, weak bases, and some neutral compounds in both directions of the plasma membrane, and the electrochemical force is provided by the electrochemical gradients of the compounds being transported. Among the OCTNs, human OCTN1 mediates the uniport of organic cations, including ergothioneine, and zwitterions, such as carnitine; human OCTN2 and human OCT6 mediate the uniport of organic cations and the cotransport of Na^+/Carnitine. OAT1-6 and 10 and URAT1 mediate the transport of organic anions in both directions. [65]. They also play an important role in the transport of drugs and metals across cell membranes [61].

1.5.1 SLC22A4: expression and evolutionary aspects

OCTN1 (SLC22A4) is an organic cation transporter identified more than twenty years ago; it belongs to the OCTN (Organic Transporters Cation Novel) subfamily together with OCTN2 and OCTN3 [67].

The *Octn1* gene is located at 5q31.1, consists of 49,823 nucleotides with 11 exons, and encodes a protein of 551 aa, with a molecular mass of 62,155 Da [68]. From an evolutionary point of view, OCTNs appear in vertebrates and, therefore later in evolution. OCTN3, in particular, has been observed in rodents and zebrafish but not in humans, in which only OCTN1 and OCTN2 are present with a sequence identity of 76% [68, 69]. In addition to terrestrial animals closer to

humans, OCTN1 homologs have been reported in aquatic species such as *Danio rerio* (zebrafish) [70] and rainbow trout, as above-stated. In the latter species, the presence of an ergothioneine transporter has been tested, although no information is available on the possible physiological function of ergothioneine in salmonids [71]. The expression of OCTN1 in humans has been widely studied revealing that OCTN1 is a ubiquitous transporter, with mRNA and protein, being quantified in 56 different mammalian cell lines and 59 tissues. OCTN1 was found highly expressed in the renal epithelium, trachea, bone marrow, liver, skeletal muscle, prostate, lung, pancreas, intestine, placenta, heart, uterus, spleen, spinal cord and even neurons. Finally, OCTN1 was also found expressed in many cancer cell lines [60].

Like all SLC22s, OCTN1 is constituted by 12 transmembrane domains, with an α-helix secondary structure, connected by hydrophilic portions (loops). In particular, the first extracellular loop, connecting the first and the second transmembrane domains, is very large and harbors potential PTM sites, such as N-glycosylation site. Moreover, a large intracellular loop is present between the sixth and the seventh transmembrane domains, with potential regulatory functions [72]. There are structural similarities between the three members of the OCTN group, with the greatest similarity found among the 12 transmembrane segments [73]. Indeed, this portion of the protein shows an average degree of identity of 68.9%, while the C-terminal end and the intracellular hydrophilic loop are very different from each other. These observations suggest that the specificity of the different transporters maybe linked to the intracellular loops and the C-terminal portion [72, 73].

1.5.2 SLC22A4: functional aspects and involvement in human diseases

OCTN1 is considered a polyspecific transporter since the list of substrates recognized by OCTN1 is very long as reported in Table 1.

Substrate	Description
Acetylcholine	Human metabolite
Choline	Human metabolite
Carnitine	Human metabolite
2-Deoxycytidine	Human metabolite
Ergothioneine	Fungal metabolite
Homostacidrin	Plant metabolite
Spermine	Antioxidant, immunosuppressive agent, and human metabolite
Stachydrine	Plant metabolite

Table1. *Major endogenous substrates of OCTN1. Dice adapted from* [60].

The best substrates are some organic cations and zwitterions. Regarding organic cations, the first molecule identified as a substrate is tetraethylammonium (TEA) which, however, is only a prototype of organic cations. The first physiological substrate discovered was carnitine, then ergothioneine, a mushroom metabolite, and immediately

after acetylcholine [60]. Various studies agree on the fact that the transporter mediates a unidirectional transport of substrates with a different mechanism depending on the cationic or zwitterionic nature of the substrate; indeed, cationic substrates are transported in a Na^+-independent fashion and zwitterions are transported with a Na^+-dependent mode of transport. Recently, it has been demonstrated that the transport of carnitine is dependent on sodium, while the transport of acetylcholine is inhibited by sodium [60, 74].

An intriguing aspect of OCTN1-catalyzed transport, is the positive regulation by internal ATP. Indeed, despite the differences existing among transport of Ach, TEA, and Carnitine in terms of Na^+-dependence, when looking at the allosteric regulation by intracellular ATP, the transport reactions of the different OCTN1 substrates share this common stimulation by ATP. It is important to highlight that this effect is not due to nucleotide hydrolysis, since the same effects have been using ANTP, the non-hydrolyzable analogue of ATP. Furthermore, activation by intracellular ATP is quite specific since internal AMP and cAMP do not exert the same effect. The lateral specificity of the ATP effect may depend on the orientation of the transporter in the cell membrane: sequence analysis of hOCTN1 reveals that the nucleotide-binding motif is localized intracellularly, as also deduced from the hydropathic profile of the protein. [75]. In good agreement, experiments conducted in intact cells showed that ATP depletion may affect OCTN1 transport function [67].

-Acetylcholine transport by OCTN1

Besides being a neurotransmitter, Ach is the key player in the non-neuronal cholinergic system present in the airways, alimentary tract, skin, placenta, heart, skeletal muscle, urogenital tract, and other

tissues where it is implicated in many cellular pathways. In line with the key role played by acetylcholine, at the level of these tissues, the expression of choline acetyltransferase, cholinesterase, and acetylcholine receptors has been described. However, in some of these tissues, the vesicular acetylcholine transporter VAchT is not expressed, which could justify the involvement of OCTN1. This pathway is involved in the regulation of inflammation: indeed, acetylcholine plays an important role in inducing anti-inflammatory effects through high-affinity binding to the α7-nAChR receptor, expressed in many non-neuronal tissues. For this reason, OCTN1 is expected to play a crucial role in immune cells [75]. OCTN1 modulates both the uptake and efflux of acetylcholine[75]. The similar K_m on the outer and inner membrane side of hOCTN1 indicated that the outer and inner binding sites can be assembled from common amino acid residues, alternatively exposed to the outside or the inside, depending on the conformational state (outward or inward) of the transporter. The V_{max}, however, appears to be lower in the outward transport direction and this could be explained by the different activation energies associated with the conformational changes required for the two opposing transport processes or, alternatively, the two processes could be modulated differently by effectors, such as ATP [75]. Exploring the kinetic effects of other OCTN1 substrates on this transport pathway, carnitine, betaine, ergothioneine, glucose, creatinine, creatine, and GABA were found to not affect acetylcholine transport when used at concentrations as low as 1 mM. In contrast, acetylcholine transport was inhibited by spermine and spermidine via mixed inhibition at concentrations as low as 0.5 mM and was also significantly suppressed by choline, acetylcarnitine, γ-butyrobetaine, tetraethylammonium, and tetramethylammonium via a competitive inhibition mechanism. Similarly, $HgCl_2$ and MTSET

compounds known to interact with SH residues, as well as PLP, which interacts with NH_2 residues, inhibited the transporter to varying degrees [75]. Acetylcholine efflux appears to be slightly stimulated by intracellular K^+, while it is not affected by the presence of extracellular Na^+, which, on the contrary, strongly impairs acetylcholine uptake. These ionic effects are quite specific, since Mg^{2+} or the neutral osmolytic sucrose, do not affect the transporter activity in either the external or internal compartments. The specific lateral regulation by Na^+ and K^+ is well correlated with the asymmetric structure of the transport protein hOCTN1 [76]. The mechanism by which Na^+ ions asymmetrically regulate acetylcholine transport is based on a competition between Na^+ and acetylcholine for the same binding site with very similar half-saturation constants, i.e. 1.0 mM for acetylcholine and 1.2 mM for Na^+ [75]. The competitive mechanism also explains the lack of inhibition of acetylcholine efflux by external Na^+. Furthermore, according to the asymmetric structure of hOCTN1 and the different molecular mechanisms of efflux versus uptake, it is plausible that the internal site for acetylcholine does not interact with inorganic cations as efficiently as the external one, thus internal ions do not compete with acetylcholine efflux. The slight stimulatory effect of internal K^+ on uptake and efflux could be due to interaction with a site other than that of acetylcholine, as is also the case for ATP. Considering that under physiological conditions, the extracellular Na^+ concentration is normally above 50 mM, hOCTN1 should be mainly involved in catalyzing the efflux of acetylcholine into the extracellular environment rather than the uptake [76].

-Carnitine transport by OCTN1

Unlike acetylcholine, carnitine transport is stimulated by the presence of external Na^+. This has been highlighted by a recent work by Pochini et al. in which the transport of TEA and carnitine was studied through a combined approach of in vitro assay and molecular docking simulations. TEA and Carnitine are respectively a cation and a zwitterion, both recognized and transported by OCTN1 but, while TEA is a prototype substrate, carnitine is a physiological substrate that must be distributed between different tissues. Experimentally it has been seen that there is no reciprocal influence between these two substrates due, most likely, to different interactions with the transporter and this also explains an opposite dependence for sodium. The kinetics for the transport of these two substrates is cooperative with a Hill coefficient of 1.25±0.29. TEA transport, unlike carnitine transport and similar to Acetylcholine transport, is strongly inhibited by the presence of sodium. In silico analyses have revealed a probable binding site for sodium to which TEA can also bind, namely the glutamate residue 381. A homologue of this amino acid residue is found in the three-dimensional structure of OCT3, solved experimentally by Cryo-EM and used as a template for the homology modelling of OCTN1, where it is responsible for binding to cations. This has been demonstrated by experimental data showing a competitive inhibition by Na^+ towards TEA. In computational analyses, carnitine revealed able to bind to the same glutamate residue to which TEA and sodium bind; intriguingly, in the presence of Na^+ which occupies the site of E381 residue, carnitine becomes able to interact with OCTN1 at the level of another amino acid residue, namely Arginine R469 [74]. These results on OCTN1 confirm the previously hypothesized existence of more than one binding site for some SLC22 members, such as OCT1, providing an explanation for the

abilities of these cation transporters to interact not only with cations but also with substrates of opposite or more complex chemical nature, such as anions or zwitterions; these complex features are related, in the case of OCTN1, with the still undefined physiological role[77].

1.5.2.a Acetylcholine and Crohn's Disease

As stated above, one of the main functions of OCTN1 in the non-neuronal cholinergic system is the efflux transport of acetylcholine from cells. It has been shown that the ability of OCTN1 to modulate acetylcholine efflux is strongly altered in the L503F mutant, an isoform associated with inflammatory bowel disease or Crohn's disease. In this mutant, Leu-503 is replaced with a phenylalanine and it has been seen that this replacement reduces the Vmax of the transporter, without however altering the K_m [75, 78]. Furthermore, it has been shown that the absorption of carnitine in the L503F variant is also reduced it is 2.3 times lower than the WT protein with a reduction of the V_{max}. The reduction of carnitine determines an increase in intestinal inflammation, due to the incorrect elimination of bacteria and to a compromise of the β-oxidation of fatty acids and, if all this is added the fact that it increases the absorption of toxins such as putrescine, deriving from bacterial catabolism, the fundamental role of carnitine as a response to oxidative stress is clear [78]. Given the importance of acetylcholine and the non-neuronal cholinergic system, this mutation is linked to the inflammation of the intestinal wall in Crohn's disease [75]. Indeed, the gene encoding for OCTN1 maps in the IBD 5 (Inflammatory bowel disease) locus on chromosome 5, which has been linked to susceptibility to Crohn's disease, ulcerative colitis, and rheumatoid arthritis[75]. Crohn's disease and ulcerative colitis are the most common forms of idiopathic IBD.

Despite differences in their clinical and histological features, both forms of IBD, but especially UC, are associated with an increased risk of developing colorectal cancer [79].

1.5.2.b Carnitine and PDC

OCTN2 is the main carnitine transporter in cells due to its high affinity for this substance, and the relationship of this transporter with primary carnitine deficiency (PDC) has been well described and acknowledged in literature [80]. In this frame, the ability of OCTN1 to transport carnitine includes this transporter in the list of those responsible for compensating the lack of OCTN2 in PCD. The presence of OCTN1 in cell membranes allows the treatment of PCD with high doses of carnitine making this genetic disease compatible with life [74].

1.5.2.c Cancer

Intriguingly enough, the same OCTN1 variant linked to Crohn's disease has also been linked to colorectal cancer further confirming that chronic inflammation is a pro-oncogenic condition [102]. More recently, in a case report of irritable bowel syndrome (IBS) characterized by a dysfunctional microbiota-gut-brain axis, the involvement of the same L503F variant was found [79].

Since this transporter is part of the non-neuronal cholinergic system, it is also implicated in the physiopathology of lung cancer. In this case, acetylcholine, transported by OCTN1, through nicotinic and muscarinic receptors can modulate the proliferation and induction of epithelial-mesenchymal transcription of cancer. In this case, the function of OCTN1 is therefore linked to that of other receptors, and it

is important to underline that nAChR receptors are not only located at the level of the external plasma membrane but also at the level of the external mitochondrial membrane, which suggests a potential localization of OCTN1 also at the mitochondrial level [60].

1.5.2.d Ergothioneine as an Antioxidant

Ergothioneine is produced by fungi and cyanobacteria and absorbed by humans through diet. It has antioxidant properties but cannot be considered a vitamin as it is not essential for human metabolism. Its deficiency is not associated with any disease, although in OCTN1 knockout mice a greater sensitivity to oxidative stress has been observed compared to wild-type mice. The role of this metabolite has yet to be understood [81].

1.5.3 SLC22A4: structural aspects

As for most transporters of higher eukaryotes, the three-dimensional structure of OCTN1 is not yet available. This is due to several difficulties typical of the handling of membrane proteins including, but not limited to, the co-existence of large hydrophobic and hydrophilic portions that hamper the possibility of obtaining stable crystals for X-ray or enough pure proteins for CryoEM technologies. Then, even if CryoEM revolutionized the field of structural biology, not every protein has been solved yet. Therefore, computational analysis has been employed for obtaining structural information thanks to *in silico* technologies and several attempts at homology modeling have been made. The first model of OCTN1 obtained through homology studies dates back to 2012, but the first 142 amino acid residues including the first transmembrane domain and the largest extracellular

loop are missing [82]. In 2013, another attempt was made using the Modeller 9v9 software, and the glycerol-3-phosphate transporter (1PW4) was used as a template. In this case, the amino acid residues 43-141 were missing [83]. Another server used was Phyre2 which identified the eukaryotic phosphate transporter from *Piriformospora indica* (4J05) as the best template. In this case, the hydrophilic loop was present but did not have a well-defined structure [84, 85]. Another attempt was made using the automatic server I-TASSER and the human glucose transporter GLUT3 as a model [86]. Thanks to the experimental resolution of the structure of OCT3 [87], in 2024 the last homology structure of OCTN1 was created using the human organic cation transporter 3 as a template, with which it shares more than 30% identity. Of the three available structures of OCT3, PDB:7ZH6 was used, that is, the structure of the transporter complexed with its inhibitor, i.e. corticosterone. The modeling was done using Prime. The most critical part of this homology was the extracellular loop (residues 41-142) since the two sequences show very low homology (**Figure 8**)[74].

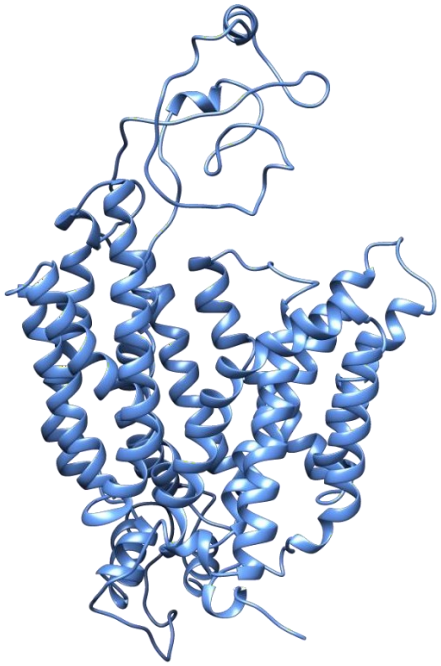

Figure 8. *OCTN1 homology model built with MIB2 software using the hOCT3 structure as a template.*

Once the structure was obtained, it was possible to perform molecular dynamics simulations that allowed the identification of the key interaction sites for most of the ligands: Tyr211, Glu381, and Arg469 are the significant residues engaged in the formation of multiple hydrogen bonds and disulfide bridges with the substrates. The role of Na^+ in solute transport has also been studied. It had already been shown that the co-transport of this ion increased the transport of ergothioneine by OCTN1 while other substrates, such as TEA and Ach, show a reduced transport rate when sodium is present at higher concentrations [74]. Simulations allowed the identification of a putative sodium binding site at the level of Glu308 [88].

1.6 INTERACTION BETWEEN OCTN1 AND DRUGS/XENOBIOTICS

Plasma membrane transporters are of interest as drugs and xenobiotics targets. Due to their location, they can be considered "frontier proteins", that is, they are proteins on the front line between the external environment and the intracellular body districts. Indeed, transporters located in the apical membrane of the intestinal epithelium are accessible to administered drugs, while internal tissues are accessible by the bloodstream. OCTN1 is a favorable target due to its ubiquitous localization in almost all tissues [68]. Many drugs have been proposed as substrates or interactors of OCTN1, indeed, OCTN1 be classified as an ADME gene, i.e. a gene encoding for a protein conventionally considered central to the absorption, distribution, metabolism, and elimination of drugs [60]. One of the xenobiotics that is of greatest concern today is heavy metals. The key aspect of this protein, and the main reason for its involvement in the present study, is its ability to interact with these polluting compounds. The interaction between OCTN1 and heavy metals occurs thanks to the presence of 7 cysteine residues in its primary structure. Already in [84] it was demonstrated that OCTN1 can interact with mercury compounds such as MeHg because these substances react with the SH groups of cysteines. To demonstrate this, a homology structural model of OCTN1 based on (PiPT, PDB 4J05) was used and, through site-directed mutagenesis, several mutants were created in which, in turn, each cysteine residue was replaced by an alanine residue. The choice of Ala depends on the size and hydrophobicity of the side chain of this amino acid, which are similar to those of Cys but inability to react with SH residues. This has demonstrated not only that interactions between environmental contaminants and OCTN1 occur at the level of cysteines,

but also that the cysteines responsible for the interaction are C50A and C136A. Furthermore, computational analysis revealed that four residues of OCTN1 are located in the extracellular loop and, therefore, easily reachable by external reagents [84].

1.7 CYSTEINE: A TARGET AMINO ACID

Cysteine is considered a fundamental amino acid for the correct performance of cellular functions. It can be absorbed from the diet or derived from the endogenous degradation of proteins. Considering the oxidizing conditions of extracellular environment, cysteine is present in its oxidized form, namely cystine, that is converted into cysteine in the cytosolic reducing environment. The cellular absorption of cystine occurs thanks to a specific membrane transporter belonging to the SLC family, more specifically it is SLC7A11 (xCT) which, through an antiport mechanism, allows the entry of cystine by exchanging it with glutamate [89, 90]. The cysteine/cystine ratio is fundamental for maintaining redox homeostasis in the intra and extracellular environment because it can interact with macromolecules containing thiols. This work is performed together with the reduced/oxidized tripeptide glutamyl-cysteinyl-glycine (glutathione). It should be noted, however, that these two systems are independent of each other [91]. Alterations of xCT function may cause ferroptosis, a type of cell death linked to lipid peroxidation further highlighting the importance of the transporter in maintaining redox homeostasis through the regulation of cysteine transport [90]. From an evolutionary point of view, cysteine appeared much later than other amino acids, together with glycine, proline, and tryptophan. However, over time it was proven to be a crucial amino acid for cell life, so since its appearance, its frequency has increased more and more up to higher organisms; even if it is currently one of the least present amino acids, with a frequency of 2.3% in the entire human proteome [92]. The great importance of cysteine is linked to the presence of a thiol group at the level of its side chain (-SH) which is the player of a sizable number of different reactions in

cells. First of all, cysteine is involved in the formation of disulfide bridges (-SS-) responsible also for the modification of the protein structure; further, cysteine is involved in the regulation of the redox potential; it is subject to oxidation by reactive oxygen species (ROS); it leads to interactions with gaseous signaling molecules such as NO, H_2S and with amines and amides. In agreement with the overall aim of the present thesis work, cysteine residues present in the plasma membrane transporter OCTN1 are considered for their ability to coordinate metals and metalloids linked to microplastics sampled in Mediterranean Sea, which makes this amino acid fascinating in the physiological and, above all, toxicological field [63]. The fact that cysteine is involved in all these reactions and post-translational modifications makes its classification from the hydrophobicity point of view very difficult and, consequently, it is not possible to univocally define an amino acid with which to replace it in mutagenesis experiments, as happened in this thesis work. Indeed, the choice of mutants depends on the position that cysteine occupies in the protein structure taken into consideration: in some cases, the hydrophilic serine could be preferred while in others, as in the present study, the hydrophobic alanine [93]. In all human transporters belonging to the SLC family, at least one cysteine residue is present (except the zinc transporter SLC39A11) with an average of 12.

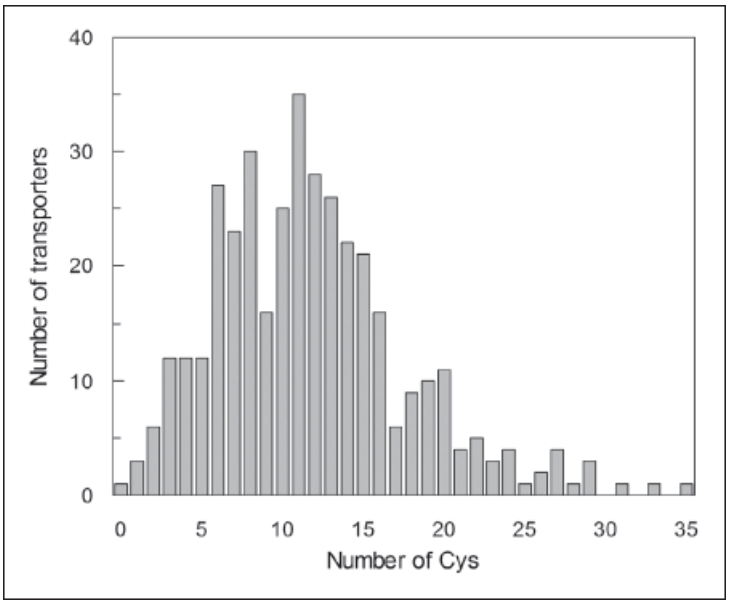

Figure 9. *Distribution of SLC transporters versus the number of cysteine residues* [63].

If these residues are sufficiently close, they can be in a thiol or disulfide state and, in the first case, they are an excellent target for drugs and xenobiotics. In this regard, S-metallic bonds are of significant importance, as occur at the level of metallothioneins, which are fundamental from a physiological point of view. Furthermore, at the level of cysteine, important post-translational modifications occur which make the human proteome even more complex[63].In this study, we focus on the ability of cysteines to bind heavy metals, important environmental pollutants, that and harmful to human health.

1.8 Oncorhynchus mykiss

Most studies on the effect of microplastics on marine ecosystems consider different species, including mussels (*Mytilus galloprovincialis*), sardines (Sardina *pilchardus*), common red mullets (*Pagellus erythrinus*) and red mullets (*Mullus barbatus*). These species are among the proposed indicators for microplastics in the seas due to their wide spatial distribution, commercial importance, habitat and feeding strategies, as well as documented ingestion of microplastics[94]. However, although in this thesis work we focused on monitoring the Mediterranean Sea for the presence of plastic pollutants, most of the waste reaches the seas through the rivers. Therefore, for this reason it was interesting to study the impact of these harmful substances also in a freshwater environment, taking as an example a particular species, i.e the rainbow trout (*Oncorhynchus mykiss*), for several reasons. At first, *Oncorhynchus mykiss* is a fish of great commercial importance since is one of the main products consumed by humans. Then, it is one of the most common freshwater fish species in aquaculture and the seventeenth most widely cultivated commercially important finned fish in the world. In the EU, the second world producer of trout, Italy is in first place for its consumption [95]. Furthermore, it is considered a sentinel species of pollution, because it can provide information on the types, quantities, availability, and effects of environmental contaminants. However, what makes this species the most suitable for this work is the fact that recently, a characteristic has been highlighted that brings this species closer to humans, regarding the effects of heavy metals. The presence of an ergothioneine transporter has been discovered in this fish that has a 53% homology with the human ergothioneine transporter, namely OCTN1 (**Figure 4**). This

protein is one of the main ones responsible for interactions with heavy metals in humans, due to the presence of 7 cysteine residues in its primary structure; it is, therefore, possible that also in the trout the interactions with these very dangerous contaminants are modulated in the same way, since by carrying out an alignment study, it emerged that the cysteines responsible for the interactions between OCTN1 and metals are conserved [71]. In this study, we focused on the leaching process of heavy metals from microplastics during the digestion process of trout.

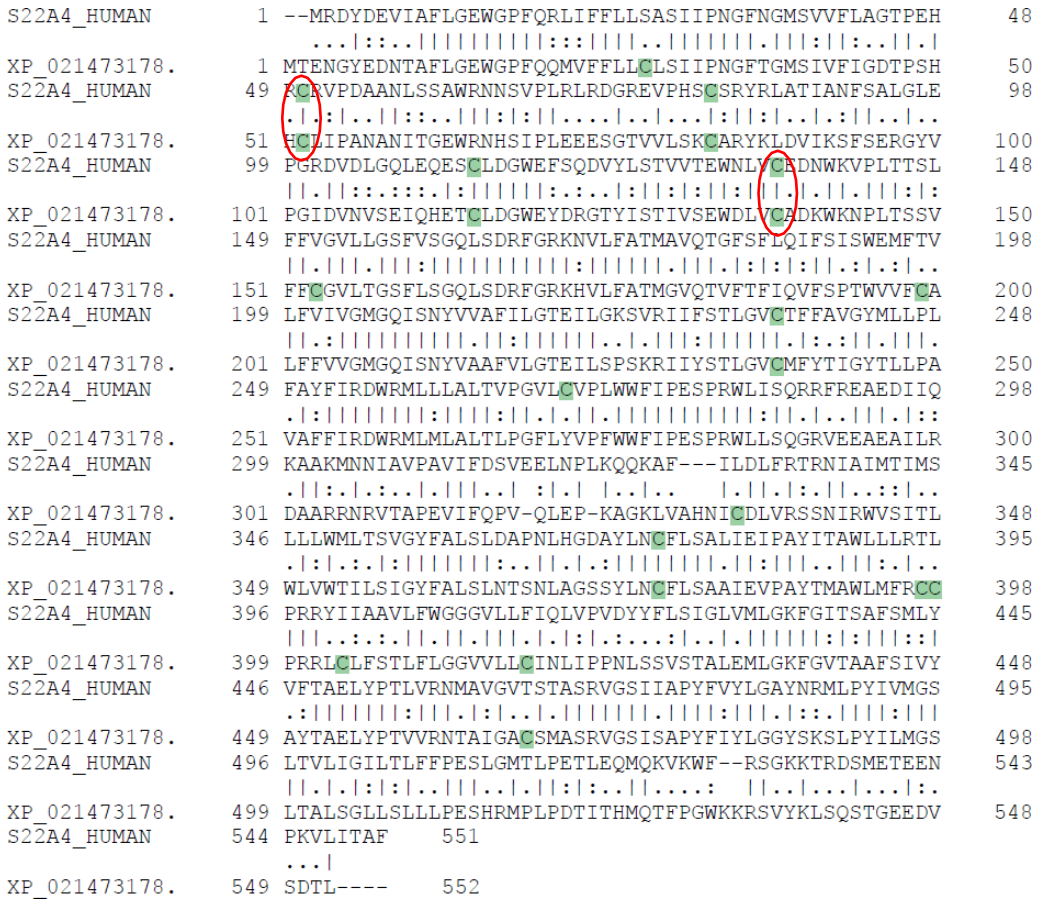

Figure 4. *Sequence alignment between human OCTN1 and a possible ergothioneine transporter found in Onchorynchus mikiss. The cysteines C50 and C136 of the human isoform are highlighted and are conserved in the sequences of the salmonid transporters.*

The above-mentioned marine species have already been used for monitoring the Mediterranean Sea at the level of the Northern Ionian Sea, that is the area between the Adriatic Sea and the Ionian Sea, considered problematic for waste accumulation, taking into account coastal tourism and recreational activities, including poor waste management practices, fishing, aquaculture and maritime transport [94]. However, it would be interesting to use these same biomarkers to study the effects of microplastics also in the areas involved in this thesis work, since they were found to be highly contaminated especially due to the presence of important commercial ports.

CHAPTER 2:

Results

2.1 OBJECTIVE OF THE RESEARCH PROJECT

The overall aim of this thesis work was to evaluate the pollution rate of the environment in which we live and the effect it has on the human organism. To achieve these objectives, we started by monitoring the Calabrian coasts of the Mediterranean Sea and we reached the microscopic levels by studying pollutants present in the microplastics, i.e. some heavy metals on the functionality of a benchmark membrane protein that is OCTN1. It is important to highlight that almost all waste materials that are dispersed in the environment, through rivers, reach the sea, which turns into a large garbage dump. Among these waste materials, those that cause the most concern nowadays are plastic waste because, taking a very long time to degrade, they remain in our seas, reducing themselves to increasingly smaller fragments. Through sampling and studying microplastics dispersed in the sea, we were able to analyze how much the *Mare Nostrum* is polluted, making a comparison between the two coasts and between different periods of the year. The main problem with microplastics is that, since they reach very small dimensions and are also generally colored, they can be mistaken for food and ingested by aquatic organisms. Many of the aquatic organisms that ingest microplastics end up on our tables, and consequently, through the consumption of contaminated organisms, also humans ingest these substances. It must be considered that microplastics are not only dangerous as such, but also because they are carriers of many harmful substances that can be absorbed during their stay in the ecosystem, or can be used directly in the production processes of the plastics themselves. Heavy metals are one of the main toxic compounds carried by plastics. At this point, our goal was to

understand whether the heavy metals transported by plastics could be released by them after being ingested by fish. The reason is that in this way aquatic organisms absorb these substances and release them to humans through the food chain. For this reason, the research project moved on to study the digestion of fish to understand whether the release of heavy metals from plastics actually occurs, and in what quantities. A particular freshwater species, *Oncorhyncus mykiss* (rainbow trout), was examined because, while it is true that our seas are highly polluted, it i salso true that most of this pollution is transported by rivers and, furthermore, it is one of the most commercially important species; therefore, it is very present in the human diet. After having demonstrated the presence of plastic pollutants in the Mediterranean Sea and how these transport heavy metals that, through the consumption of contaminated fish, reach humans, the last part of the thesis was focused on the study of the effect of these toxic substances on a human target. In particular, we identified a member of the SoLute (SLC) carriers which represent a benchmark of first-level targets for heavy metals due to their localization on the cell surface. Among SLCs, the main targets of heavy metals are probably organic cation transporters belonging to a subgroup of the SLC22 family because, in addition to the physiological capacity to convey the absorption and release of organic cations, they are also important for interactions with drugs and xenobiotics. The protein OCTN1 (SLC22A4) was chosen due to its ubiquitous expression, the conservation in sea fishes, the already documented ability to interact with mercury and mercury-derived compounds and, above all, due to the presence of 4 cysteine residues well exposed to the extracellular environment, as above- mentioned.

The first part of the project is explained by the first article submitted reported below. Microplastics were sampled by operators of

the Regional Environmental Protection Agency of the Calabria Region (Arpacal) at three sites on the Ionian coast and three sites on the Tyrrhenian coast. The samplings were done in duplicate during two periods of 2021: March and June. The plastics were classified one by one based on size, shape and colour using a stereomicroscope and based on their polymeric composition through non-destructive Fourier transform infrared spectroscopy (FT-IR) analyses. Subsequently, other non-destructive analyses were performed using scanning electron microscopy (SEM) in order to understand whether the samples being analysed were carriers of heavy metals and, if so, which metals they were; therefore, qualitative but not quantitative analyses were performed (Brunetti et al., 2024). – **Article 1.**

The intermediate part of the project is reported in the second article submitted. Qualitative analyses performed on microplastics during the first phase revealed the presence of numerous heavy metals. Eight of these were chosen based on their dangerousness for the environment and for humans: Mercury, Chromium, Cadmium, Arsenic, Tin, Antimony, Zinc and Lead. The percentage in which these metals are released from microplastics during the digestion of *Oncorhyncus mykiss* was studied through *in vitro* assays, carried out with solutions that mimic the composition of the digestive fluids of the fish species, namely gastric fluid and intestinal fluid. After simulating the digestive process, the fluids were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to measure the concentration of metals that pass from the plastics to the fish. Furthermore, through size reduction analyses, it was possible to demonstrate how the exposed surface of the plastics is fundamental for the quantity of metals released (Bolea et al., 2024) – **Article 2 under submission.**

The last part of this thesis work is included in the last submitted

article. The heavy metals found in microplastics first, and then tested in the in vitro digestion of fish species, were finally tested on hOCNT1 using the tool of reconstitution in proteoliposomes in wich the recombinant OCTN1, produced in *E.coli* and purified by affinity chromatograhy, was inserted with the same orientation as in native membrane. This experimental setup allowed us to study a single protein in an isolated environment to precisely define the molecular mechanisms of inhibition combining approaches in vitro and *in silico*, in the absence of interferences deriving from other cell systems. Importantly, this *in vitro* technique allows us to avoid animal testing (Brunetti et al., 2024) – **Article 3.**

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Article

Examining Microplastics Along the Calabrian Coastline: Analysis of Key Characteristics and Metal Contamination

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Abstract: Plastic pollution is a major concern today. Microplastics (MPs), due to their small size, can enter the food chain and cause serious harm to living organisms. The Mediterranean Sea is the sixth largest accumulation area for plastic waste, including MPs, worldwide. In this study, we analyzed the distribution, shape, color, size, and polymer composition of MPs (having dimensions between 330 μm and 5 mm), collected from the water surface in six areas along the Calabrian coast, Italy. A prevalence of polyethylene was detected, with higher concentrations of MPs found in the Gioia Tauro and Cetraro areas. Additionally, heavy metals were identified within the MPs, suggesting that these particles could act as environmental carriers of such elements into the food chain.

Keywords: plastic pollution; microplastic polymeric composition; Mediterranean Sea; Calabrian coast; heavy metals

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1. Introduction

Today, plastic pollution represents one of the main environmental concerns worldwide due to its distribution (there is no area of the planet immune to this problem) and permanence in ecosystems; and it is estimated that at least 5.3 trillion plastic particles are currently floating in the seas [1]. The Mediterranean Sea is the sixth largest accumulation area of floating marine plastic waste, and this is due to its hydrodynamics. It is in fact a semi-closed convective basin and this structure determines not only the maintenance of local plastic pollution, but also the entry of floating waste from the Atlantic Ocean [2–4]. The main origin of these marine contaminants is recognized to be litter on beaches and coasts, fishing activities, and, most importantly, the contribution of rivers carrying municipal wastewater discharges [5–10]. The main problem is that the biodegradation of plastics in marine waters is extremely slow; this causes them to be transported over long distances, ensuring that plastic pollution reaches even the most remote areas of the planet. Furthermore, the salinity of the water, solar radiation, and mechanical degradation determine the reduction of plastic waste into increasingly smaller fragments, favoring interactions with the biota [11–14]. The continuous process of the fragmentation of plastic leads to the formation of very small particles called microplastics (MPs). This term defines particles with dimensions between 300 μm and 5 mm [15,16], although, currently, the range 1 μm–5 mm is accepted [17,18].

Precisely as a result of their small size and their resistance, these substances, together with all the toxic substances they contain, are easily ingested by aquatic and terrestrial organisms, thus entering the food chain, and reaching humans [19–22]. It must be taken into consideration that MPs are distributed at the level of surface waters, the water column, coastal sediments, and deep waters, and this compartmentalization depends on their polymeric composition, and, therefore, on their density. Consequently, they will have different interactions with aquatic organisms [23]. Many studies have highlighted how MPs have been found in various foods such as beer [24], honey [25], sea salt [26], canned sardines [27], mineral water [28], and tap water [29]. This obviously poses a risk to human health, both as a vehicle for accumulated toxic substances and for intrinsic additives.

Many studies have shown how plastics are able to absorb molecules derived from drugs and antibiotics [30] and heavy metals [31]. Plastics are carriers of metals, both those that are adsorbed from the surrounding environment and those that are used in the plastic-manufacturing process itself as plasticizers, stabilizers, color pigments, fillers and extenders, flame retardants, blowing agents, antioxidants, impact modifiers, lubricants, and antimicrobial agents [32,33]. Heavy metals are elements with a high density compared to that of water, and, since toxicity and density are related, they are capable of inducing toxicity at low levels of exposure. In recent years, the public health concern associated with environmental contamination by these metals has grown [34]. Humans are exposed to toxic metals that come from various industrial, agricultural, domestic, and technological processes. It has been observed that natural phenomena such as weathering and volcanic eruptions are sources of pollution, while metal processing in refineries, coal burning in power plants, oil combustion, nuclear power plants, microelectronics, the conservation of wood, and paper processing are some of the important industrial sources [35]. Some of these metals such as cobalt (Co), copper (Cu), chromium (as Cr(III)), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) are essential for the biological functions of plants and animals; however, at high levels, they interfere with metabolic reactions in organism systems and are toxic. While other heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), chromium as Cr(IV), uranium (U), or arsenic (As) are not useful for living organisms and they are extremely toxic and are capable of reducing plant growth due to the reduced photosynthetic activity, reduced mineral nutrition, and reduced activity of essential enzymes. Furthermore, they could lead to cancer in humans; in fact, these toxic metals can accumulate in the body if consumed with contaminated foods through the food chain and become risky to health [36].

This study aimed to monitor the Calabrian (Italy) coastline to assess the quantity and characteristics of MPs, their composition, and heavy metals they may carry.

For this purpose, MPs (small and large microplastics) has been sampled in six areas on the Calabrian, more specifically, three stations on the Ionian coast and three stations on the Tyrrhenian coast. Microscopic observations made it possible to make a count of particles in each collected sample, as well as to identify the color, the shape and the size of each microplastic. Infrared spectroscopy allowed us to identify the polymeric composition, while, by means of electronic microscopy coupled with energy-dispersive spectrometry and inductively coupled plasma-mass spectrometry, it was possible to identify the presence of heavy metals carried by MPs.

2. Materials and Methods

2.1. Study Area

The microplastic samples analyzed were taken from the coasts of the Calabria Region, south of the Italian peninsula, and the sampling areas of the Operational Plan of the Marine Strategy of Calabria of the Regional Agency for Environmental Protection (ARPA) were

used [37]. Sampling sites were chosen both on the Ionian Coast and on the Tyrrhenian Coast, for a total of 6 sites. The choice of areas was made based on certain factors, such as the distance from direct input sources, such as river mouths, port facilities, significant urban settlements, or accumulation areas for local hydrodynamic conditions. For each area, samples were taken from three distances from the coast, 0.5, 1.5, and 6 nautical miles (M). From north to south, the sampling areas are the following: Mouth of the Crati River (Cosenza), Mouth of the Neto River (Crotone), and Mouth of the Corace River (Catanzaro) for the Ionian Coast; and Cetraro (Cosenza), Vibo Marina (Vibo Valentia), and Gioia Tauro (Reggio Calabria) for the Tyrrhenian Coast (Figure 1). The sampled areas are part of the monitoring activities conducted since 2015 under the framework of the Community Directive 2008/56/EC Marine Strategy, carried out by the Marine Strategy Regional Center (CRSM) of ARPA Calabria.

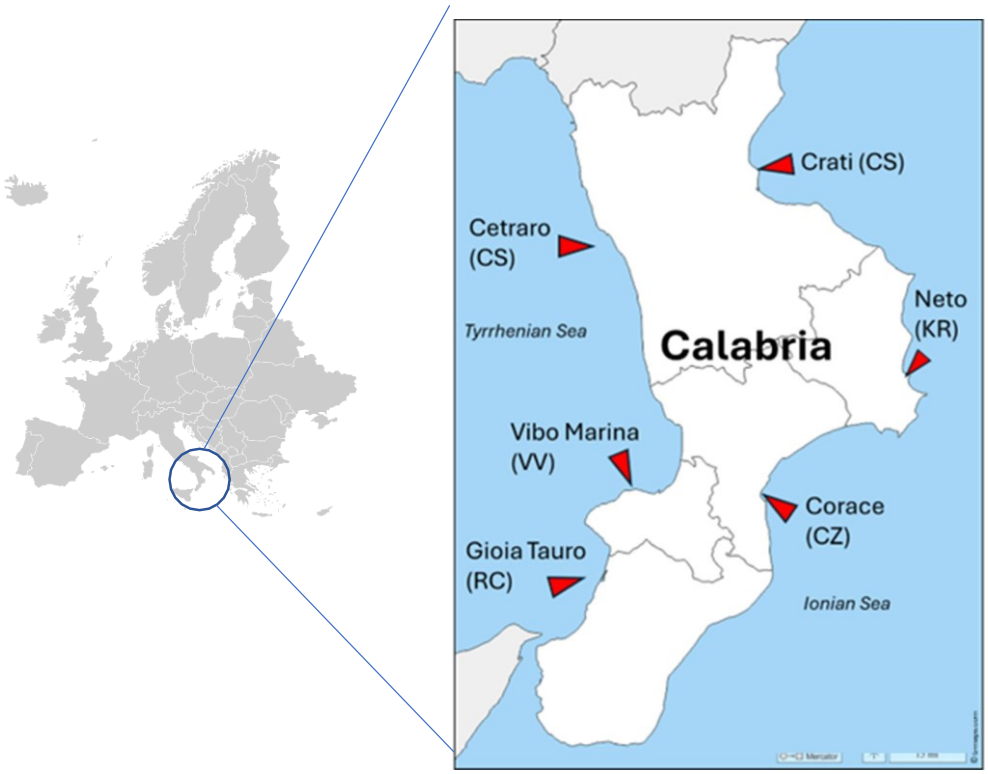

Figure 1. Sampling areas on the Ionian Coast (Crati, Neto, and Corace) and on the Tyrrhenian Coast (Cetraro, Vibo Marina, and Gioia Tauro).

2.2. Sampling

The MP sampling was carried out in March 2022 and June 2022 as described in [37]. MPs were sampled using a 2.5 m-long manta trawl with a mesh size of 333 µm with a rectangular frame opening of 25 × 50 cm. The manta ray was towed to the surface, against the current, for about 20 min from the ship's starboard side at an average speed of 2.5 knots. To avoid wake turbulence, all sampling was taken from the starboard side of the vessel, beyond the bow wave. After each haul, the net was rinsed with sea water, and, subsequently, the collected material was screened through two stacked stainless-steel sieves with a mesh void of 5 mm and the underlying one of 300 µm. The accumulated residues were transferred to a glass vial with 70% alcohol and stored at room temperature. For each area, 3 samples have been collected (one at 0.5 M, one at 1.5 M, and one at 6 M from the coast). The number of particles collected from each sampling has been divided by the surface area (distance towed x horizontal dimension of the frame) to obtain the abundance per square meter, and divided by the volume (distance towed × surface of the frame) to obtain the abundance per volume.

2.3. Characterization

The samples were visually inspected under a stereomicroscope (Zeiss Axiolab microscope equipped with a digital camera to acquire images), and, using laboratory tweezers, suspected MP particles were carefully collected, placed in a Petri dish, and washed with double-distilled water to separate them from other organic residues. The criteria taken into consideration to classify a potential MP particle are the following: (1) absence of cellular or organic structures; (2) a homogeneous thickness across the particles; and (3) homogeneous colors. Once isolated, potential MPs were counted and photographed, and their maximum length (mm) was recorded, considering the largest diameter, shape, and color. All samples were examined and double-checked by two different researchers to confirm that MP counts were consistent and conservative. In order to confirm the polymeric nature of the samples (suspected MPs) and to allow specific identification of the different types of plastic, samples were analyzed by FTIR investigations; for this purpose, a PerkinElmer Spectrum 100 spectrophotometer equipped with an attenuated total reflectance (ATR) accessory has been used. Infrared spectra were recorded in ATR mode, in the range of 500–4000 cm^{-1} with a resolution of 4 cm^{-1}. After background scans, 16 scans per particle were performed and CO_2 interference was removed for clarity. The obtained spectra were then compared with a library of standard polymer spectra and accepted with a similarity threshold greater than 70%. All MPs collected in the first sampling were analyzed by FTIR, while, regarding MPs from the second sampling, only 15 were analyzed for each transect (five for each of the three sampling distances from the coast). After confirming the polymeric nature of the samples, to detect the potential presence of metals on their surface, they were analyzed by scanning electron microscopy (SEM), using a Zeiss Crossbeam 350 microscope, equipped with an energy-dispersive spectrometer (EDS). We analyzed 60 MPs collected in March (10 for each transect), and 6 MPs collected in June (1 for each transect) by SEM-EDS.

All samples were grouped in Ionian and Tyrrhenian coast and analyzed by ICP-MS. The samples were subjected to acid digestion before analysis. Microwave-assisted digestion was performed using a One-Touch MARS6 (CEM Corporation, Charlotte, NC USA) with tetrafluormethaxyl (TFM) vessels. The operating conditions are reported in Table S1. For digestion, 50 mg of each sample was placed in the vessels with 2.3 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30%). After the digestion program, the samples were diluted with ultrapure water obtained from a Milli-Q system (Millipore, Bellerica, MA, USA), then analyzed by ICP-MS. A Perkin Elmer Elan DRC-e mass spectrometer (Toronto, ON, Canada) was used. Instrumental parameters are described in Table S2. For statistical tests, IBM SPSS software has been used. The Shapiro–Wilk test was performed to evaluate the normality of the abundance data (H_0, null hypothesis: population is normally distributed). Since not all data were normally distributed, the Kruskal–Wallis non-parametric test was employed to assess the significance of variations among particle abundances (H_0, null hypothesis: particle abundances of each sample come from the same population). Additionally, results from the size, shape, color, and composition analysis were analyzed using the chi-square test (H_0, null hypothesis: size/shape/color/composition classes ratios are independent from the sampling site and/or sampling period).

3. Results and Discussions

3.1. Distribution of Microplastics

In Table 1, the results about the counting of the sampled MPs are summarized. Taking into account all samples, the average particle abundance is equal to 0.06 particles/m^2 (0.24 particles/m^2); it is worth noting that there is a great variability in this parameter ranging from 0.01 to 0.3 m^2. However, this value is lower than that obtained after the sampling campaign in 2021 in the same areas (0.13 particles/m^2) [38]. The abundances,

calculated as an average of all results from each coast (Figure 2), suggest that there is not a significant difference in the number of MPs among the Tyrrhenian and Ionian coast in the two sampling periods; this is confirmed by a statistical test (Table S3). Looking at the abundance values in Table 1, it is quite evident that samples collected at Corace and Neto (Ionian side) showed a lower abundance in June with respect to March. Looking at the single values calculated for each area, abundances rarely exceed 0.10 particles/m^2, except for Cetraro (up to 0.16 particles m^2) and Gioia Tauro (up to 0.30 particles m^2). This could be explained by the fact that two important commercial ports are located in Cetraro and Gioia Tauro; it is a bit surprising that Vibo Valentia has a lower abundance, despite being located near another commercial port. This indicates that the presence of ports is not the only element that can justify the high presence of plastic; in fact, the surface circulation of the Mediterranean Sea must also be taken into consideration [39–44]. The Mediterranean Sea is a semi-enclosed basin, connected to the Atlantic Ocean through the Strait of Gibraltar and this hydrodynamic model determines the entry of plastic pollutants from the ocean [43,45]. This is a less saline current, which, once it enters the basin, is diverted to the right by the Coriolis Force, skirting all the coasts in an anti-clockwise direction. Added to this phenomenon are also the surface winds that blow towards the Tyrrhenian coast, contributing to the accumulation of material. Precisely for this reason, the largest accumulation areas are the Tyrrhenian coast, and, particularly, the Strait of Messina, which acts as a funnel for marine waste. On the Ionian coast, the opposite phenomenon is observed; that is, the winds and currents tend to move the waste towards the open sea. It is, therefore, normal to attribute the difference between its coasts to the marine and atmospheric circulation, which makes the Tyrrhenian Sea an area of accumulation of materials, both local and coming from distant places [46,47].

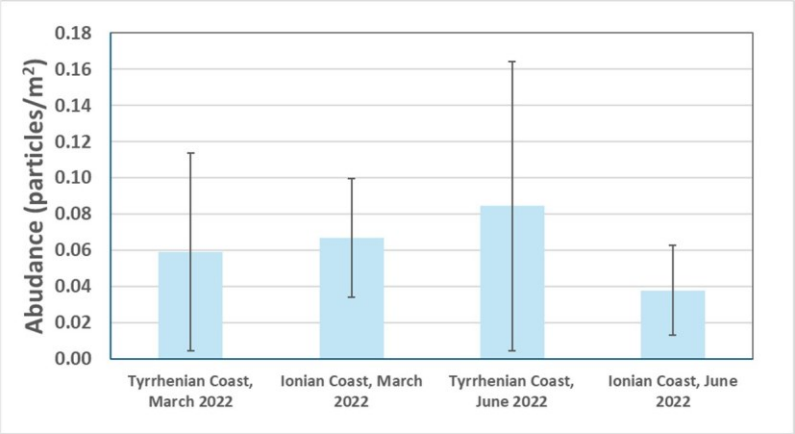

Figure 2. Differences among coasts and sampling periods.

The concentration of MPs on the Calabrian coasts, detected in the present study, is comparable with what was measured in other studies carried out in the Mediterranean Sea (Table 2). However, there appears to be a big difference regarding studies conducted in other areas of the world, in particular, the Atlantic Ocean and the Asian coasts. This difference, however, is obvious, because, globally, waste tends to accumulate in five "ocean garbage patches" located at the North Atlantic, South Atlantic, North Pacific, South Pacific, and Indian levels. The largest of these areas is the Great Pacific Garbage Patch, located between Hawaii and California. The highest percentage of plastic present in this region would derive from sources present in Asia and would reach there through the Kuroshio, also known as the "black current" [48].

Table 1. Quantity and origin of collected MPs.

Sampling Site	Location	Starting Coordinates		Distance from Seashore (Nautical Miles–M)	March 2022		June 2022	
		Latitude	Longitude		N° of Particles	Abundance (particles/m^2)	N° of Particles	Abundance (particles/m^2)
Cetraro	Tyrrhenian Coast	39.50683	15.934	0.5	42	0.04	17	0.02
		39.50083	15.9076	1.5	167	0.16	25	0.02
		39.45016	15.7566	6	29	0.03	40	0.04
Vibo Marina	Tyrrhenian Coast	38.733595	16.089434	0.5	12	0.01	40	0.03
		38.719219	16.099541	1.5	47	0.04	21	0.02
		38.797072	16.038261	6	79	0.07	17	0.01
Gioia Tauro	Tyrrhenian Coast	38.4344	15.87206	0.5	16	0.02	219	0.26
		38.44173	15.85291	1.5	26	0.02	319	0.30
		38.46905	15.76335	6	176	0.14	70	0.06
Mean values of Tyrrhenian Coast						0.06 ± 0.05		0.08 ± 0.07
Crati River Mouth	Ionian Coast	39.727313	16.541585	0.5	31	0.04	40	0.05
		39.732025	16.561949	1.5	49	0.05	25	0.03
		39.756677	16.652299	6	64	0.10	30	0.04
Neto River Mouth	Ionian Coast	39.200117	17.157133	0.5	32	0.10	15	0.04
		39.202667	17.17865	1.5	41	0.10	18	0.05
		39.198202	17.273467	6	36	0.10	34	0.09
Corace River Mouth	Ionian Coast	38.811868	16.617133	0.5	22	0.02	21	0.02
		38.798977	16.637915	1.5	37	0.04	13	0.01
		38.76624	16.700495	6	51	0.05	12	0.01
Mean values of Ionian Coast						0.07 ± 0.03		0.04 ± 0.02

Table 2. Microplastic concentrations in sea.

Study Area	MPs Range (mm)	Average Abundance (particles/m^2)	Ref.
Calabrian Coasts	0.33–5	0.06 particles/m^2	This study
Calabrian Coasts	0.33–5	0.13 particles/m^2	[38]
North Western Mediterranean Sea	0.30–5	0.12 particles/m^2	[49]
Western Mediterranean Sea	0.33–5	0.13 particles/m^2	[50]
Western Mediterranean Sea—Adriatic	0.20–20	0.40 particles/m^2	[51]
Mediterranean Sea—Corsica	0.20–2	0.06 particles/m^2	[52]
Central–Western Mediterranean Sea	0.33–5	0.15 particles/m^2	[53]
	MPs Range (mm)	**Main Abundance (particles/m^3)**	
Calabrian Coasts	0.33–5	0.24 particles/m^3	This study
Sardinian Sea	0.33–5	0.17 particles/m^3	[54]
Ligurian Sea	0.20–5	0.49 particles/m^3	[55]
Mediterranean Sea	0.20–5	0.24 particles/m^2	[56]
North Atlantic	0.33–4.75	1.70 particles/m^3	[57]
North-East Atlantic	0.25–5	2.46 particles/m^3	[58]
East Asian Sea	0.35–5	3.70 particles/m^3	[59]
Seto Inland Sea	0.30–5	0.39 particles/m^3	[60]
Arctic Polar Waters	0.30–5	0.34 particles/m^3	[61]
Bohai Sea	0.50–5	0.33 particles/m^3	[62]

3.2. Physical Characterization

The size of MPs is a determining factor for their interaction with marine organisms and for their ingestion. At sea, plastic waste is degraded into increasingly smaller fragments, increasing in number quantity as the size decreases [63].

The size of MPs is a determining factor for their interaction with marine organisms and for their ingestion. [63]. In Figure 3, distribution of the sizes of the identified MPs it is shown. It is clear that the dimension class 1–2 mm is the most abundant in all sampled areas. Such distributions are quite similar with that found by elaborating the 2021 data in the same areas, with the same sampling method [38], but also with other studies in other areas [59,63]. However, due to the sampling methods with a cutoff at 330 microns, there is no chance at all to detect particles smaller than that size. Regarding the upper limit of the distribution, for the sampling of MPs, filters with pores smaller than 5 mm are used; however, samples such as filaments, which have an elongated shape, still manage to pass through the pores even if they are larger in size and this is the reason why plastics were found larger in size. The presence of such sized particles is an alarming factor for ingestion by marine organisms, because, the smaller they are, the higher the probability that they enter the food chain; in fact, the number of particles in the various organisms increases as the dimensions decrease [64,65]. The distributions in Figure 3 have also been tested with the chi-square statistical tool, and the results are reported in Table S4. According to the result, the size classes' abundance depends on the sampling sites and on the period as well.

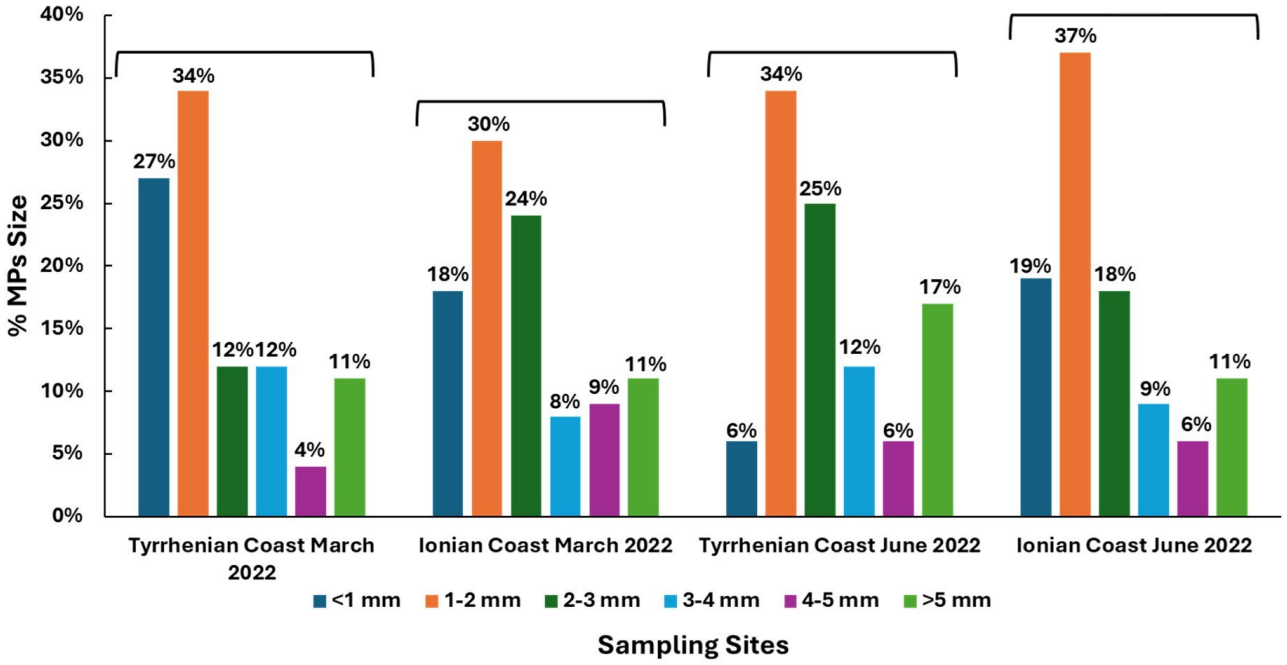

Figure 3. Summary of results of size analysis of MPs.

Another important factor for the classification of MPs is the shape, also implicated in the potentially harmful effects that these substances have. Based on their appearance they are classified into six different forms: fragment, sheet, filament, granule, foam, and pellet [38]. In Figure 4, a summary of the percentages of the shapes detected has been reported.

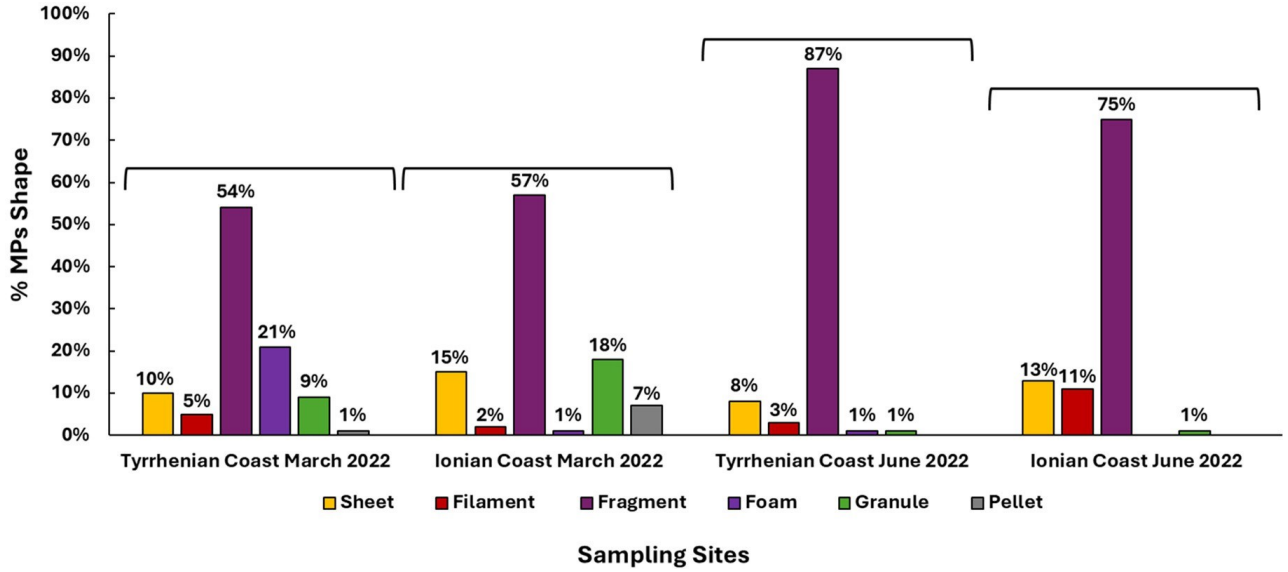

Figure 4. Summary of results of shape analysis of MPs.

The fragment shape is the most abundant in all sampled areas, ranging from 54 to 87%, whereas the sheet shape has an abundance between 8 and 15%. Despite the fragment prevalence, the Chi-square test revealed that such distribution profiles are dependent on the sampling site and on the period (Table S5). The filament shape reaches a maximum of 11% on the Tyrrhenian coast in March, while foam has been significantly found on the Ionian coast in March. Pellets and granules are considered primary MPs, while all the others are defined as secondary as they originate over time through photochemical, mechanical, and biological processes [66]. It could be hypothesized that the fragments derive from hard and packaging plastics, the sheets from plastic bags, and the filaments from fishing lines or textiles [67,68]. Filaments are rather insidious forms (or fragments), because, due to their structure, if they are ingested by aquatic organisms, they can seriously damage both the intestine and the gills with lethal consequences [65,69].

Although the percentage of fibers found is low, their presence could be much higher because they originate mainly from the fragmentation of fishing lines and textiles which, once they end up in the sea, tend to sink, while only a small part remains on the surface. Moreover, fibers also come from wastewater treatment plants effluent as textile residues; then, this source seems to not be the main contributor to the pollution in the area studied.

In Figure 5, the results about the color assessment have been reported. The white and transparent color are the most abundant. Red, blue, green, and other colors are found in relatively low percentages. A statistical test (Table S6) revealed that the color pattern depends on the sampling site and on the sampling period; then, the differences among the distribution are not only ascribable to statistical fluctuations. Colors have been observed that interfere with the ability of marine organisms to distinguish between plastic and natural food [70]. White and transparent MPs are most dangerous ones, because they are more easily mistaken for food by marine organisms and enter the food chain in greater quantities [71]. These latter types of MPs could derive from plastic bags that are used daily, but also from colored particles which, upon entering water and being subjected to various processes, lose their color. Indeed, many samples found had acquired a pale-yellow color and showed rounded corners, due to long environmental exposure. Black, on the other hand, represents one of the most used colors to produce plastics which, precisely because of their color, are very difficult to recycle. This pigment is usually made with carbon black and specific techniques are required for the disposal of plastic waste containing it [72].

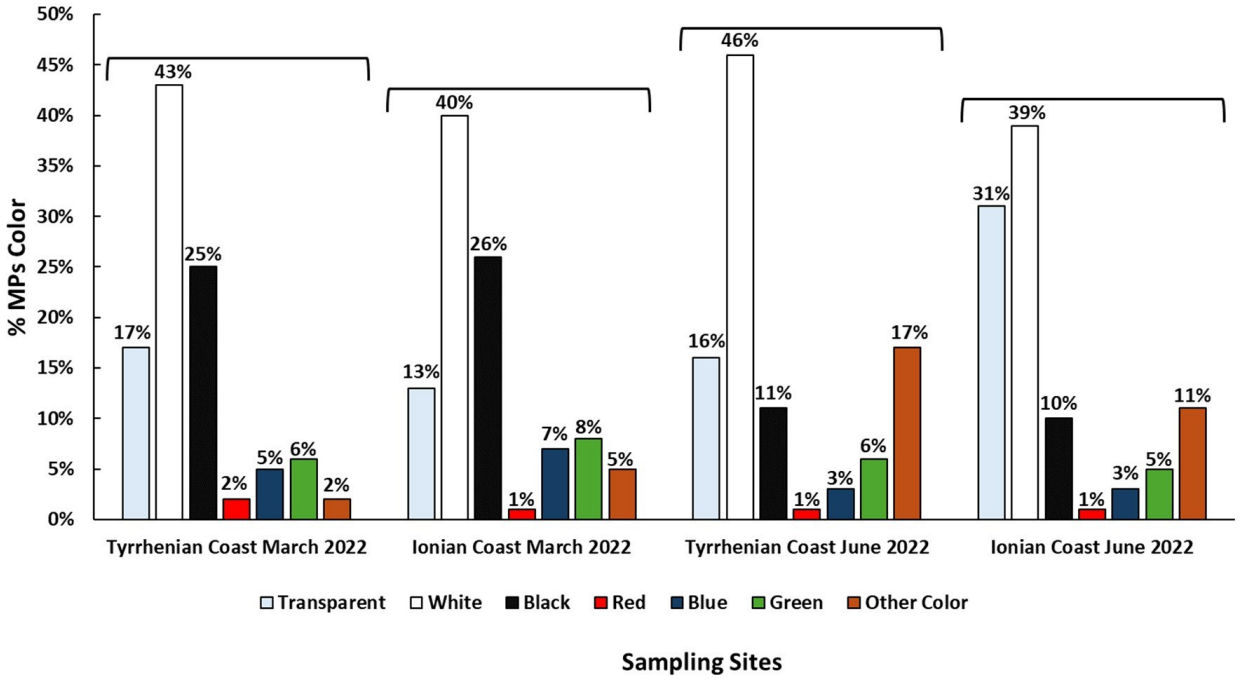

Figure 5. Summary of results of color analysis of MPs.

3.3. Compositional Features

The MP samples were analyzed by ATR FTIR to determine their polymeric composition. The results are summarized in Figure 6, and shows that polyethylene (PE) is the most abundant polymer (67–89%), followed by polypropylene (PP) (11–27%). Moreover, in this case, the chi-square test revealed a dependence of the pattern of polymers on the sampling site/period (Table S7). PE and PP are part of the polyolefin family, thermoplastic compounds produced through the polymerization process [73].

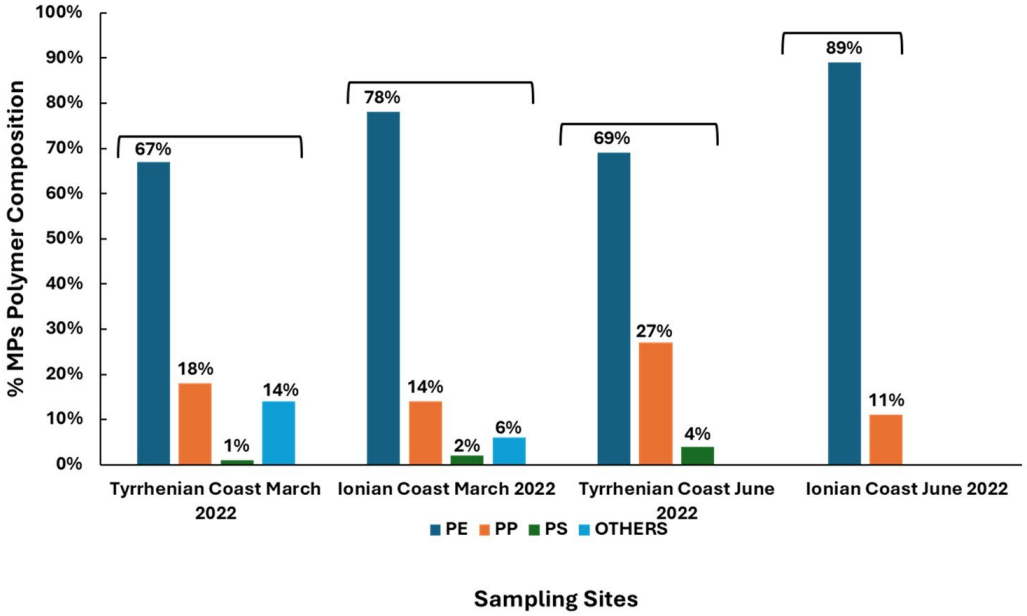

Figure 6. Summary of results of composition analysis of MPs. The term "others" includes the following: polyvinyl chloride, polyethylene terephthalate, polyurethane, acrylonitrile butadiene styrene, and polyamide.

Based on worldwide plastic production data starting from the 1990s [74], an estimation of the production percentages of each polymer has been carried out (Table 3).

Table 3. Estimation of the worldwide production of each polymer, expressed in percentage.

Polymer	%	% of Polymer Particles in This Research
Polyethylene (PE)	33	75
Polypropylene (PP)	20	17
Polyvinyl chloride (PVC)	17	8
Polystyrene (PS)	10	
Polyethylene terephthalate (PET)	7	
Others (i.e., polyurethane, acrylonitrile butadiene styrene, polyamide, and polycarbonate)	13	
Total	100	100

There is an accordance between the worldwide polymer production and the results of this research: in both cases, PE is the most diffused polymers. However, if we consider the percentages, the data do not fit each other, since, in our samples, PE seems to be overestimated with respect to the all-other polymers. To explain this, it must be considered that PE and PP have a lower density than that of water, so they can float on the surface of the water. PS, PET, and other substances have a higher density than that of water, so they tend to sink [75]; however, in this study, polymers with a density greater than that of water were also detected. This is due to the fact that the distribution of MPs in the sea is not determined only by the density, but also by other factors such as wind, temperature, salinity, and hydrodynamic conditions; these variables ensure that even polymers with a high density are able to rise to the surface [76]. Similarly, even a part of low-density polymers, such as PE and PP, can sink and, consequently, not be picked up during sampling. and, therefore, they are the most abundant in the samplings that are carried out [73–80]. In addition, looking at the percentages of PE and PP in Table 3, the ratios seem to be not comparable (PE/PP: 1.65 worldwide production; 4.4 in collected samples). The reason of this discordance is not clear; however, some aspects can be taken into account. PP is less resistant than PE to UV rays and oxidation processes; therefore, once it ends up in the sea, it ages very quickly and breaks down into very small particles, which form nanoplastics; consequently, the concentrations of PP could be underestimated compared to those of PE which is the most abundant substance. Another explanation can be related to the fact that PE is more used as packaging, which are the objects that can more easily be dispersed into environment [74–79].

3.4. Analysis of Metals

The microplastic samples were also analyzed by using a scanning electron microscope (SEM) coupled with an energy-dispersive spectrometer (EDS) to obtain information on micromorphological features and elemental composition. SEM observations highlighted the presence of brighter spots, which indicate heavier elements with respect to the bulk, in which C of the polymer is predominant (Figure 7). All bright spots highlighted on the analyzed side of MP have been analyzed by EDS analysis, and the results are summarized in Table S8; it reports the frequency of detection of each element (element detection limit = s/n > 10).

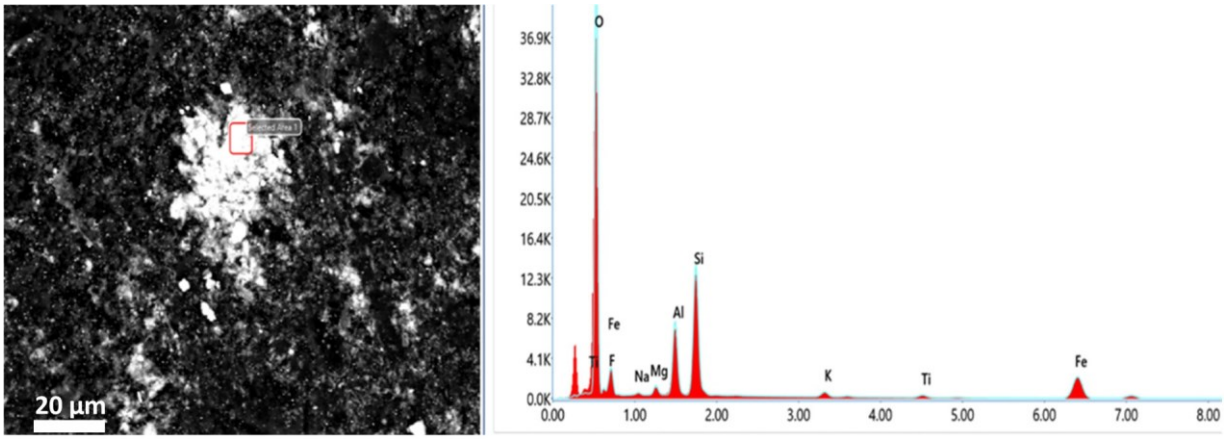

Figure 7. Example of image and related spectrum representing the elements detected during the SEM-EDS analysis.

The most frequent elements detected in all spots (Si, Na, K, Ca, Al, and Mg) suggest that most of the inorganic fraction detected on MPs is ascribable to natural sediments. However, the frequency of some heavier elements like Pb and Ba can also suggest the contribution of a certain level of pollution. From this type of analysis, it is hard to understand whether these elements are endogenous, i.e., used in the production processes of the plastic itself, or exogenous, i.e., if they were absorbed from the surrounding environment during the fragmentation process in the marine environment. However, some evidence can be collected; an element which has been found on bright spots, and was not present, or present with a significant lower EDS signal in the surrounding area, suggests that the element itself may be exogenous.

To confirm that point, some "virgin" polyethylene and polypropylene polymers, coming from common objects and not dispersed into the environment, were observed by the SEM microscope. In these plastics, the light spots (heavier elements with respect to elements of the bulk) are distributed uniformly over the entire surface (Figure S1a,b), while, in plastics sampled in the sea (Figure S1c,d), the light spots are not uniformly distributed and have very different sizes between them. All analyzed samples show a morphology attributable to Figure S1c,d; then, it is reasonable that the detected elements are mainly exogenous.

In order to obtain quantitative information about the amount of some metals in the sampled MPs, an ICP-MS analysis was performed on samples grouped in the Ionian and Tyrrhenian coasts. Among the elements determined (with the results shown in Table 4), lead is the most abundant, followed by chromium and zinc. It is worth noting that the concentration of lead is higher in MPs on the Ionian coast with respect to the Tyrrhenian coast (two to four times higher). Moreover, in the case of chromium, there is a higher amount of this metal in MPs from the Ionian coast. These results suggest that MPs in the Ionian Sea are more impacted by pollutants. In order to compare these metal contents with other results both from the MP analysis and also from marine sediments, some results are also reported in Table 4.

Comparing our results with those obtained from MPs of Indian coral reefs [81], MPs from the Calabrian coast are less contaminated by all metals considered in this research. In addition, there is a coherence between the lead content measured in sediments from nearby Mediterranean areas [82,83] and the lead measured in MPs in this research.

Table 4. Results of ICP-MS analysis performed on MPs sampled on Ionian and Tyrrhenian coast in March and June 2022. Some results of previous studies are also reported.

	Pb	Cd	Cr	As	Sb	Sn	Zn
	Concentration $\mu g \cdot g^{-1}$						
Ionian Coast, 03/2022	147 ± 3	10.3 ± 0.1	140 ± 1.1	0.53 ± 0.01	0.16 ± 0.05	0.31 ± 0.2	61.1 ± 0.1
Ionian Coast, 06/2022	205 ± 5	1.3 ± 0.03	153 ± 1.3	0.63 ± 0.02	0.016 ± 0.001	0.028 ± 0.016	47.9 ± 0.6
Tyrrhenian Coast, 03/2022	72 ± 1.4	1.3 ± 0.06	104 ± 3	0.446 ± 0.03	1.06 ± 0.15	0.464 ± 0.07	26.3 ± 0.7
Tyrrhenian Coast, 06/2022	53.3 ± 0.3	17.1 ± 0.3	98 ± 1.2	0.431 ± 0.03	0.079 ± 0.05	0.57 ± 0.05	46 ± 1
UK, PE pellets [84]	1.72	-	-	-	-	-	0.25
Indian coral reef, MPs [85]	218–3765	10–212	27 -191	0.03–1	11–653	1–2	2187–3076
Gulf of Lions and the Ligurian Sea, sediments, Tyrrhenian sea [86]	19–86	-	-	-	-	-	-
Mar Piccolo Taranto (Ionian sea), sediments [87]	44–111	0.6	-	-	-	1.9–5.6	165–241

These results confirm that MPs carry heavy metals, which are harmful even at low concentrations and persist in the aquatic environment. Commonly, "heavy metals" are defined as elements with a density of at least 5 g/cm^{-3}, which have an atomic mass greater than 23 or an atomic number greater than 20 [84–86]. Heavy metals present in the environment, both of natural and anthropogenic origin, can be affected by sorption processes on plastic waste, which depend on several factors [87–93]. However, the presence of heavy metals on MPs can derive from their production processes (endogenous); in fact, metals can be used as additives to improve the quality of the plastic product: dyes (zinc, lead, chromium, cobalt, and cadmium), heat- and flame-retardants (bromine and chlorine), or stabilizers (cadmium and tin) [94]. In our case, it is difficult to quantitatively distinguish the fraction of each exogenous and endogenous metal and this point has to be investigated in further research. Table 5 summarized the different uses of metals as polymer additives together with the potential effects on human health.

Table 5. Main heavy metals used as additives and their effects on human health.

Element	Polymer	Additives	Effects on Human Health	Ref.
Al	PET, PE, PVC	Stabilizer, inorganic pigments, and flame retardants	Metal-estrogen, breast cancer	[95–97]
Ti	PVC	Inorganic pigments, UV stabilizers	Cytotoxicity on human epithelial lung and colon cells	[95,98,99]
Cu	-	Biocides	Inducing DNA strand breaks and oxidation; formation of reactive oxygen species (ROS)	[86,95,96, 100]
Cr	PE, PP, PVC	Inorganic pigments	Severe cardiovascular, respiratory, hematological, gastrointestinal, renal, hepatic, and neurological effects; allergic reactions to the body; nasal septum ulcer; possibly death	[86,101]
Mn	-	Inorganic pigments	Neurodegenerative disorder	[95,102]
Ba	PVC	Inorganic pigments and UV stabilizers	Metal-estrogen, breast cancer; cardiovascular and kidney diseases; metabolic, neurological, and mental disorders	[95–97,103]

Table 5. *Cont.*

Element	Polymer	Additives	Effects on Human Health	Ref.
Pb	All types of plastics where red pigments are used	UV stabilizers; heat stabilizers and inorganic pigments	Anemia; hypertension; miscarriages; disruption of nervous systems; brain damage; infertility; oxidative stress and cell damage	[86,95–97,101,104]
Zn	PE, PP, PVC	Stabilizers, inorganic pigments, and flame retardants	-	[95,97]
Sn	PVC, Foam, PU	UV stabilizers and biocides	Metal-estrogen; breast cancer; skin rashes; stomach complaints; nausea; vomiting, diarrhea; abdominal pain; headache and palpitations; potential clastogen	[95–97,104]
Co	PET	Inorganic pigments	Formation of reactive oxygen species (ROS); neurological (e.g., hearing and visual impairment); cardiovascular and endocrine deficits	[86,96,105]
Cd	PVC	UV stabilizers, inorganic pigments, heat stabilizers	Changes in metabolism of calcium, phosphorus and bone; osteomalacia and bone fractures in postmenopausal women; lipid peroxidation and in the promotion of carcinogenesis; cellular apoptosis; DNA methylation	[86,96,106]
Hg	PU	Biocides	Mutagen or carcinogen; induction of the disruption of DNA molecular structure and brain damage	[86,96,107,108]
As	LDPE, PVC, Polyesters	Biocides	Congenital disabilities; carcinogen: lung, skin, liver, bladder, kidneys; gastrointestinal damage; death	[95,96,101]
Ba	PVC	Inorganic pigments and UV stabilizers	Metal–estrogen, breast cancer; cardiovascular and kidney diseases; metabolic, neurological, and mental disorders	[95–97,102]
Sb	Various plastics	Biocides and flame retardants	Metal–estrogen; breast cancer	[95–97]

In biological systems, heavy metals have been reported to exert an action on cellular components such as the cell membrane and cytoplasmic organelles, as well as on some enzymes involved in metabolism, detoxification, and damage repair [36,109].

The most frequent metals identified in MPs in this research and reported in Table 5 are as follows: Al, Cr, Cu, and Pb. Copper is an essential element for human survival, but, at high concentrations, it is harmful because it is responsible for DNA damage and the formation of ROS, as well as causing serious pathologies such as Wilson's Syndrome. Lead is considered an extremely toxic and carcinogenic element even at low concentrations.

4. Conclusions

In this study, we collected and analyzed microplastics (MPs) from six marine areas along the Calabrian coast. An average MP abundance of 0.06 particles/m^2 has been calculated. The results suggest that there is not a prevalence of MPs between the Tyrrhenian and Ionian Sea; however, in the areas of Cetraro and Gioa Tauro, greater abundance values have been highlighted, likely due to the presence of two important commercial ports. White/transparent fragments of polyethylene having sizes between 1 and 2 mm are the prevalent physical and chemical features of the samples analyzed. The variability in the size, color, shape, and polymeric composition of the MPs was found to depend on the sampling site and time period, suggesting that these characteristics are not consistent across time and space. The presence of exogenous materials' sorption on the MPs' surface has been observed by electron microscopy of the fragments collected. The analysis of such materials revealed the presence of several elements, including heavy metals such as Pb, which come from the surrounding environment, proving that MPs can act as carriers of

heavy metals in the food chain. Further studies are needed to understand the ability of a living organism to absorb such elements.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments12010004/s1. Table S1. Temperature program for microwave-assisted digestion of polymers. Table S2. Instrumental parameters for ICP-MS measurements. Table S3. Abundance data used for Shapiro-Wilk test and Kruskal-Wallis tests, and test results. Table S4. χ^2 tests performed on results of size analysis, H_0, null hypothesis: size classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected. Table S5. χ^2 tests performed on results of shape analysis. H_0, null hypothesis: shape classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected. Table S6. χ^2 tests performed on results of color analysis. H_0, null hypothesis: color classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected. Table S7. χ^2 tests performed on results of composition analysis. H_0, null hypothesis: composition classes ratios are independent from the sampling site and/or sampling period. H0 null hypothesis is rejected. Table S8. Elements detected in samples of Microplastics, Ionian Coast and Tyrrhenian Coast, March and June sampling. Analyzed by Scanning Electron Microscopy (SEM-EDS). Figure S1. Elements present on (a) "virgin" polyethylene sample (magnification 399X); (b) "virgin" polypropylene sample (magnification 963×); (c) sample of environmental polyethylene (magnification 516×); (d) sample of environmental polypropylene (magnification 532×). Analyzed by backscattered electrons (BSE).

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Supplementary Information

Table S1. Temperature program for microwave-assisted digestion of polymers.

Step	Power (W)	Temperature (°C)	Ramp (min)	Hold (min)
1	1000	120	4	0
2	1000	180	3	0
3	1000	220	4	0
4	1000	220	0	20

Table S2. Instrumental parameters for ICP-MS measurements.

Parameter	Setting
RF Power	1600 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1.2 L min^{-1}
Nebulizer gas flow rate	1.14 L min^{-1}
Sample flow rate	1.0 mL min^{-1}
Isotopes monitored	^{53}Cr; ^{64}Zn; ^{75}As; ^{110}Cd; ^{120}Sn; ^{121}Sb; ^{200}Hg; ^{208}Pb

Table S3. Abundance data used for Shapiro-Wilk test and Kruskal-Wallis tests, and test results.

MPs abundace (particles/m²)			
Tyrrhenian Coast March 2022	**Ionian Coast March 2022**	**Tyrrhenian Coast June 2022**	**Ionian Coast June 2022**
0.04	0.04	0.02	0.05
0.16	0.05	0.02	0.03
0.03	0.10	0.04	0.04
0.01	0.10	0.03	0.04
0.04	0.10	0.02	0.05
0.07	0.10	0.01	0.09
0.02	0.02	0.26	0.02
0.02	0.04	0.30	0.01
0.14	0.05	0.06	0.01
Shapiro-Wilk test p-value (H0, null hypothesis: population is normally distributed)			
0.019392	0.023178	0.000512	0.281041
Kruskal-Wallis test p-value (H0, null hypothesis: particle abundances of each sample come from the same population)			
0.352			

Table S4. χ^2 test performed on results of size analysis, H_0, null hypothesis: size classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected.

Size (mm)	Observed results – number of MPs				
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022	TOT
<1	143	66	143	11	**363**
1-2	169	111	283	61	**624**
2-3	71	87	137	61	**356**
3-4	70	30	70	29	**199**
4-5	25	29	45	12	**111**
>5	66	40	90	33	**229**
TOT	**544**	**363**	**768**	**207**	**1882**

Size (mm)	Expected results – number of MPs			
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022
<1	104.87	69.98	148.05	40.10
1-2	180.27	120.29	254.50	68.93
2-3	102.85	68.63	145.20	39.32
3-4	57.49	38.36	81.16	21.98
4-5	32.07	21.40	45.27	12.26
>5	66.45	44.34	93.81	25.41

$\chi^2 =$ 83.531 $\chi^2_{.005}\, df(15) =$ 24.996 p-value<0.05

Table S5. χ^2 test performed on results of shape analysis. H_0, null hypothesis: shape classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected.

Shape	Observed results – number of MPs				
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022	TOT
Fragment	323	201	668	170	**1362**
Sheet	56	55	60	30	**201**
filament	30	15	30	5	**80**
Foam	0	1	1	0	**2**
Granule	129	67	9	3	**208**
Pellet	5	24	0	0	**29**
TOT	**543**	**363**	**768**	**208**	**1882**

Shape	Expected results – number of MPs			
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022
Fragment	392.97	262.70	555.80	150.53
Sheet	57.99	38.77	82.02	22.21
filament	23.08	15.43	32.65	8.84
Foam	0.58	0.39	0.82	0.22
Granule	60.01	40.12	84.88	22.99
Pellet	8.37	5.59	11.83	3.21

$\chi^2 =$ 332.904 $\chi^2_{.005}\, df(15) =$ 24.996 p-value<0.05

Table S6. χ^2 test performed on results of color analysis. H_0, null hypothesis: color classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected.

Color	Observed results – number of MPs				
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022	TOT
Transparent	57	46	129	55	**287**
White	261	148	352	82	**843**
Black	150	95	82	23	**350**
Red	11	4	7	2	**24**
Blue	31	25	27	5	**88**
Green	34	28	44	10	**116**
Other Color	0	17	129	28	**174**
TOT	**544**	**363**	**770**	**205**	**1882**

Color	Expected results – number of MPs			
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022
Transparent	83.05	55.42	117.55	32.98
White	242.83	162.04	343.71	96.42
Black	101.16	67.50	143.18	40.16
Red	7.18	4.79	10.17	2.85
Blue	25.86	17.26	36.61	10.27
Green	33.62	22.44	47.59	13.35
Other Color	50.29	33.56	71.18	19.97

$\chi^2 =$ 223.060 $\chi^2_{.005}\,df(18) =$ 28.869 p-value<0.05

Table S7. χ^2 test performed on results of composition analysis. H_0, null hypothesis: composition classes ratios are independent from the sampling site and/or sampling period. H_0 null hypothesis is rejected.

Polymer	Observed results – number of MPs				
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022	TOT
PE	212	188	29	35	**464**
PP	59	35	10	7	**111**
PS	2	4	0	0	**6**
Others	50	18	0	0	**68**
TOT	**323**	**245**	**39**	**42**	**649**

Polymer	Expected results – number of MPs			
	Tyrrhenian Coast March 2022	Ionian Coast March 2022	Tyrrhenian Coast June 2022	Ionian Coast June 2022
PE	230.93	175.16	27.88	30.03
PP	55.24	41.90	6.67	7.18
PS	2.99	2.27	0.36	0.39
Others	33.84	25.67	4.09	4.40

$\chi^2 =$ 27.316 $\chi^2_{.005}\,df(9) =$ 16.919 p-value<0.05

Table S8. Elements detected in samples of Microplastics, Ionian Coast and Tyrrhenian Coast, March and June sampling. Analyzed by Scanning Electron Microscopy (SEM-EDS).

Detected Elements	Detection Frequency (%)			
	Ionian Coast, 03/2022 (60 particles analyzed)	**Ionian Coast, 06/2022** (6 particles analyzed)	**Tirrhenyan Coast, 03/2022** (60 particles analyzed)	**Tirrhenyan Coast, 06/2022** (6 particles analyzed)
Si	100	100	100	100
Na	94	45	84	75
K	93	36	80	50
Ca	84	36	81	50
Al	81	82	84	100
Fe	70	45	86	87
Mg	50	54	67	75
Ti	49	9	41	37
Cr	37	27	21	25
Mn	35	0	24	0
Ni	25	9	16	25
S	25	54	29	12
Ag	17	0	2	0
Cu	14	0	19	0
Mo	12	9	5	0
Ba	10	27	9	12
Pb	10	27	2	0
Zn	6	0	5	0
F	6	9	13	25
Sn	5	9	2	0
P	5	27	15	12

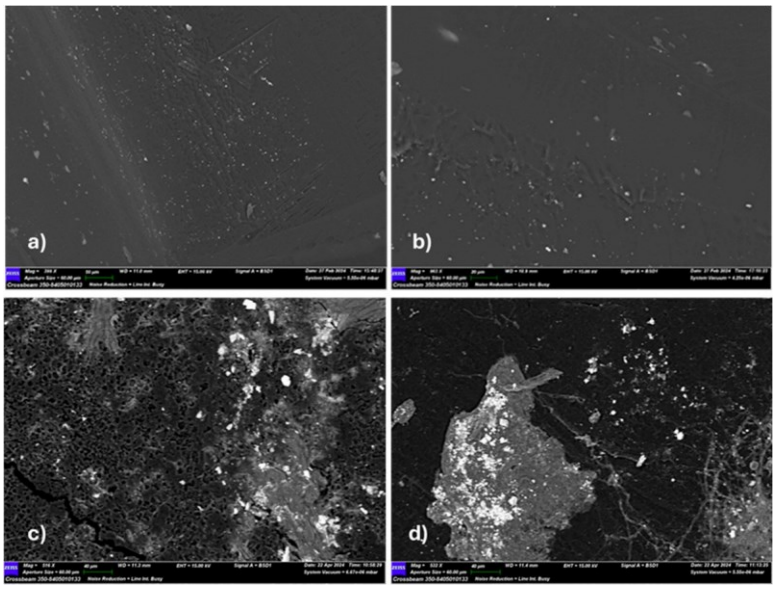

Figure S1. Elements present on a) "virgin" polyethylene sample (magnification 399X); b) "virgin" polypropylene sample (magnification 963X); c) sample of environmental polyethylene (magnification 516X); d) sample of environmental polypropylene (magnification 532X). Analyzed by backscattered electrons (BSE).

Release of heavy metals during *in vitro* fish gastrointestinal digestion from microplastics collected at Calabrian coasts

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Abstract: Migration of different environmentally relevant elements (Pb, Cd, Cr, As, Sb, Sn, Zn, and Hg) from microplastics collected at different points on the Calabrian coast (areas of both Tyrrhenian and Ionian seas) during simulated fish digestion processes has been studied. The effect of particle size and polymer composition on migration processes has been studied using three different polymers (low density polyethylene (LDPE), polypropylene (PP), and polyvinyl chloride (PVC)) as models. *In vitro* fish digestion simulation consists of two different phases: gastric (simulated gastric fluid (SGF)) and intestinal (simulated intestinal fluid (SIF)). In general, larger percentages of released metal were found during the gastric phase with respect to the intestinal, likely due to the more acidic conditions along the gastric phase. The total amount of migrated metals after the whole process (SGF+SIF) was also measured, being lower than the initially migrated during the gastric step. In comparison, the amounts of metals migrated during the intestinal phase were not significant for most of the metals studied, diluting consequently the concentration of the metals at the end of the process. Reduction of the polymeric material size (from diameters of several mm (pellets) to 300-500 µm in average (milled)) leads to higher concentrations released during both digestion phases for most of the metals studied. Plastics collected on the Calabrian coast also show metal migration during digestion simulations, being significant for chromium, lead, cadmium and zinc. Particulates containing lead were also detected by single particle ICP-MS, which may correspond to solid deposits on plastic surfaces released during digestion simulations.

Keywords: heavy metals, microplastics, ICP-MS, Mediterranean Sea

1.- Introduction

The issue of plastics in the seas represents one of the main problems of the 21st century [1]. About 80% of the plastic present in the oceans derives from terrestrial activities (urban landfills, malfunctioning sewage systems, industries) that are transported to the seas thanks to rivers; while the remaining 20% derives from maritime activities (cruises, recreational and commercial fishing, aquaculture) [2, 3]. To date, microplastics have been observed everywhere, from the poles to the equator; in fact, they have been reported in considerable concentrations even in remote sites such as Antarctica and on the seabed of the Mariana Trench [4, 5]. This happens because, once introduced into the sea, they can become part of the gyres. These natural phenomena act in a similar way to vortices, dragging debris (including plastic) towards their center. Due to these movements, waste tends to accumulate in five ''ocean garbage patches'' located in the North Atlantic, South Atlantic, North Pacific, South Pacific, and Indian Oceans. The largest of these areas is the Great Pacific Garbage Patch, located between Hawaii and California [6]. The Mediterranean

Sea is indicated as the sixth-largest accumulation area of floating marine plastic waste, due to its hydrodynamics. This Sea is a semi-closed convective basin and this structure determines, not only the maintenance of local plastic pollution but also the entry of floating waste from the Atlantic Ocean [7, 8]. Once in the sea, plastics undergo a degradation process over time determined by chemical, physical, and biological actions that lead to the formation of very small fragments, of millimeters and/or micrometers sizes, defined as Microplastics (MPs) [9]. As regards chemical- physical actions, oxygen present in the air is one of the most important, interacting with polymers and creating free radicals that cause breaks in the polymer chains. UV solar radiation also contributes to the formation of these free radicals inducing hydrolysis reactions. The breaking of the chains causes the phenomenon of chemo-crystallization, creating crystalline spherules with a volumetric contraction, producing increasingly smaller fragments [10]. Biological degradation is also produced and includes mechanisms that involve extracellular enzymes produced by environmental bacteria and other microorganisms that break the chemical bonds that bind the plastic molecules[11, 12]. It has been reported that different types of plastic polymers have variable sensitivity to the action of enzymes and UV rays [13]. Some characteristics of plastic products can influence the speed of degradation processes, both biotic and abiotic, including the complex polymer structure and the components of the materials. It has been highlighted that MPs have been found in many commercially important fish species [14]. Essentially, when microplastics enter the seas, they can be mistaken for food due to their small size and be ingested by fish, thus entering the food chain. Generally, fish species consumed whole (such as mollusks and crustaceans, anchovies, and sardines) pose a greater threat than gutted fish or peeled shrimp. However, the presence of microplastics has recently been detected in the muscle of some fish species such as Ephinephelus coiodes, Platycephalus indicus, Sphyraena jello, Saurida tumbil, Sillago sihama, and in crustaceans such as Peneaus Semisulcatus [15, 16]. These observations raise concerns about what could be the potential effects on humans, considering the presence of metals that the sea microplastics could contain. In fact, the bioaccessibility of heavy metals through the ingestion of tire particles has been already demonstrated [17, 18]. Metals may remain in plastics as catalytic or reaction residues, although their principal primary source is functional additives. Some metal-based additives are insoluble inorganic compounds, partially soluble organic compounds, or organometallic liquids or salts [19]. These metal-based additives have a wide range of functions in plastics, mainly as fillers or pigments for color, but they can also act as flame retardants, biocides, antimicrobial agents, or lubricants. Due to concerns about the environmental impact of those inorganic and organometallic compounds, because of their non-biodegradable nature, leading to bioaccumulation in the food chain [20], there has been a gradual shift towards organic compounds or non-metal-based alternatives. Although hazardous metal-based additives are no longer intentionally incorporated into contemporary plastics, at least in Europe, it is still possible that such additives would be still employed in certain consumer goods available in the EU [19]. In any case, all these plastics containing metal-based additives still persist in soils, sediments, and waters.

Therefore, this study aims to clarify the role of microplastics in the bioaccessibility of metals in gastric and intestinal fluids of a model fish species by highlighting the potential for solubilization of polymer-bound elements during intestinal transit.

2.- Materials and Methods

2.1. Materials and samples

Three different plastic materials: Low Density Polyethylene (LDPE), Polypropylene (PP) and Polyvinyl chloride (PVC) were used as models for digestion migration assays. Certified reference material ERM-EC681m (JRC, Geel, Belgium) was used as model for LDPE. This material is

commercialized as pellets of 3x3 mm. Fragments of plastics collected on beaches of the French Atlantic coast were analyzed and fragments of PP were isolated and subsequently used as model for PP. Finally, PVC in powder (Sigma, St. Louis, MO) was used as model for PVC.

Plastics from four environmental (marine water) samples were collected and studied. Samples from the Calabrian Ionian Coast (Southern Italy) were collected in Corace, Neto, and Crati (river mouths), whereas those from the Calabrian Tyrrhenian Coast were collected in Cetraro, Vibo Marina, and Gioia Tauro (port and tourist areas). All of them were collected during two campaigns, along the first (I) and second (II) half of 2022. Samples from each coast were combined in the experimental procedure, giving four different samples: Ionian (I and II) and Tyrrhenian (I and II). The characterization of such samples is reported elsewhere [21].

Plastic composition varied from one coast to another, being 77% PE and 14% PP on average for the Ionian coast samples and 66% PE and 18% PP for the Tyrrhenian coast ones. Ethylene- vinyl acetate (EVA), polystyrene (PS) and polyamide were also detected in different proportions in both types of samples.

Sodium Chloride (NaCl), Potassium Chloride (KCl), Magnesium Sulphate ($MgSO_4$), Calcium Chloride ($CaCl_2$), Pepsin, Porcine Bile Extract, Pancreatin, Sodium Hydrogen Carbonate ($NaHCO_3$), HEPES were purchased from Sigma (St. Louis, MO). All the reagents used were of analytical grade. Ultrapure water (18 MU cm of resistivity) was obtained from a Milli-Q purification device (Millipore Co., Bedford, MA, USA).

2.2. Methods

2.2.1. Size reduction of raw plastic materials used as models.

A ball mill MM400 (Retsch, Haan, Germany) was used to reduce the size of LDPE, PP, and PVC materials described in 2.1. Two different procedures were compared: a wet milling based on the use of methanol inside the jars together with the polymer, and a cryogenic milling based on the use of a liquid nitrogen bath. As for the first protocol, to reduce 1 g of LDPE, 7 milling cycles lasting 5 minutes each at a frequency of 25 Hz were required, adding 2 mL of methanol in every cycle. As for the second protocol, to reduce 5 g (2.5 g in each container) of LDPE, 12 milling cycles of 2 minutes each at 30 Hz were required. Before each milling, containers with the samples were placed in the liquid nitrogen bath for 5 minutes. To reduce 5 g of PP and PVC with cryogenic milling, two cycles of 2 minutes each at 30 Hz were required. Frequency and times were adjusted following the recommendation of the mill manufacturer, whereas the number of cycles was optimized by visual inspection after each cycle, in terms of homogeneity and particle size.

2.2.2. Determination of size distributions by laser diffraction

A Mastersizer 3000E (Malvern Panalytical, UK) was used to characterize the size distribution of each material after milling, using both protocols described in 2.2.1. Less than 1 g was used for measurements, introduced as solid dispersion by aspiration. Five replicates were made for each sample and average sizes were used along the study.

2.2.3. Characterization of plastics collected at Calabrian coasts and plastic models.

2.2.3.1. Determination of metal concentration by ICP-MS.

The samples were subjected to acid digestion to determine the total amount of heavy metals. The protocols used along this work were adapted from [22]. A microwave-assisted digestion was performed using a One Touch MARS6 (CEM Corporation, Charlotte, NC USA) with TFM™ (modified PTFE) vessels. The operating conditions are reported in Table S1. For digestion, 50 mg

of each sample were placed in Teflon vessels with 2.3 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30%). After the digestion program, the samples were diluted with ultrapure water, then analyzed by ICP-MS. A Perkin Elmer Elan DRC-e mass spectrometer (Toronto, Canada) was used for ICP-MS measurements. Data acquisition was performed using a dwell time of 50 ms, 20 sweeps, and 10 replicates per measurement. Instrumental parameters are described in Table S2. Instrumental limit of quantification (LQ) was calculated as 10 times of blank standard deviation divided by linear regression slope.

2.2.4. In vitro digestion

In vitro digestion protocol was adapted from [18]. Basically, it consists of 3 hours of digestion in a Simulated Gastric Fluid (SGF), to simulate the transit time in the fish stomach, followed by 23 hours of digestion in a Simulated Intestinal Fluid (SIF), to simulate the transit time in the fish intestine. The composition of both simulated fluids and protocol are described in detail in SI.

2.2.5. Analysis of digestion media after in vitro assays for metal released determination

After *in vitro* digestion, the digestion media (SGF, SIF, and SGF+SIF) were filtered at 0.45 µm with PTFE filters (Omnipore, Merk), diluted with ultrapure water (1:20) and analyzed by ICP- MS, using the same conditions as described in Table S2. Matrix recovery assays were conducted in all media (SGF, SIF, SGF+SIF) for all metals monitored by addition of metal concentration standards and measured under the same conditions as samples. Recovery values between 85% and 130% were obtained for all the elements, except for As in the samples collected at sea, which were lower than 20%, which justifies the values obtained, below the limit of quantification.

Concentration results are expressed as the difference between metals concentration levels found in simulated fluids after digestion conditions in the presence of the corresponding plastic and in absence. Differences statistically not significant are expressed as below the limit of quantification, which corresponds to the limit calculated from the concentration of the metal in the different simulated fluids.

Samples from Ionian and Tyrrhenian Coast subjected to the *in vitro* assays were also measured in single particle mode (SP-ICP-MS). For this purpose, a Perkin Elmer NexION 2000B mass spectrometer was used. Following the *in vitro* digestion process, the SGF+SIF digestion media were filtered at 10 µm with PTFE filters (Omnipore, Merck) and subsequently diluted 10 times with ultrapure water prior to analysis by SP-ICP-MS. The sample introduction system consisted of an Asperon^{TM} linear pass spray chamber (Perkin Elmer, Toronto, Canada), equipped with a flow focusing nebulizer (Ingeniatrics, Sevilla, Spain). A µDx Single Cell Autosampler (Elemental Scientific, Omaha, NE, USA) equipped with a syringe pump operating at 10 µL min^{-1} was used for sample introduction. ^{208}Pb was monitored at a dwell time of 100 µs for a total acquisition time of 60 s. Default instrumental parameters are listed in Table S2.

3.- Results and discussion

3.1. Effect of particle size on metal released.

Firstly, the effect of the size of microparticles on the amount of metal released was studied. The certified reference material ERM-EC681m (trace elements in polyethylene) was used for this assay. This material is commercialized as pellets of 3x3 mm made of pure LDPE and pigments. This material was reduced in size by the milling process described in section 2.2.1. Although similar size distributions were obtained following the two different milling procedures (cryogenic and wet) (see Figure S1), both milling conditions were used throughout this study. Size distribution obtained by laser diffraction shows a maximum at 272 µm, with 10% of volume

population below 100 μm. Raw and milled materials were conducted under the same *in vitro* digestion procedure described in section 2.2.4. and results are compared in Table 1.

Table 1. Effect of particle size and milling conditions on metal released during in vitro digestion for LDPE material. Hg was also measured, but all values were below the limit of quantification of the method (0.1 μg L^{-1}), referred to the blank level in the corresponding simulated fluids (see section 2.2.5). Mean ± standard deviation (n=3).

LDPE form	Digestion phase	Pb	Cr	Cd	Sb	Sn	Zn
		Concentration (μg L^{-1})					
Pellet	Gastric	0.5±0.2	163±1	0.5±0.2	0.05±0.1	0.6±0.1	15±2
Wet milled		50±2	227±1	16±0.6	10.1±0.5	0.5±0.1	10±2
Cryo milled		24.5±0.3	131±2	6.0±0.1	7.0±0.2	6.0±0.1	<0.4
Pellet	Intestinal	0.9±0.3	151±1	0.7±0.2	0.6±0.2	1.2±0.1	47±3
Wet milled		4.2±0.4	243±1	2.05±0.2	6.5±0.3	1.1±0.1	<5
Cryo milled		1.8±0.9	44±3	0.5±0. 1	<0.6	2.0±0.1	<5

In general, higher concentrations were obtained for most elements determined for the milled material (cryogenic and wet) in both media, gastric and intestinal. However, the ratio between the concentrations observed varies to a large extent from one metal to another and between milling conditions. For instance, lead, antimony and cadmium show the largest difference, with ratios around 1:10 or even of 1:100, whereas chromium showed small differences, being even the opposite (smaller the concentration released from the milled material) in the case of Sn and Zn (exception made for Sn with the cryogenic milling). This last behavior is opposite to that expected since the surface of contact is much lower in the case of the raw material (pellets). The high concentration values of zinc obtained in the blank (probably due to the impurities in the different reactants used during the intestinal phase) and the large uncertainty associated with the values for the rest of the samples analyzed, make these results not significant. In any case, other variables different from particle size should be considered to explain all these results. Given that intestinal phase lasts much longer (23 hours) than the gastric one (3 hours), it is possible that chromium, tin or zinc releases following a different kinetic than the rest of metals, similarly to that observed in [18] for zinc.

If milling conditions are compared, higher concentrations were observed with wet milling for most metals. The slight differences observed in the size distributions between these two procedures (Figure S1) do not seem to justify such differences, although they may contribute to some extent. It is possible that the use of methanol during the milling process could modify the composition on the surface of the material, facilitating the release of metals, assuming that they are homogeneously distributed. For this reason, and considering that cryogenic milling is less aggressive to the milled material, this procedure was selected for the rest of the experiments. In any case, the trend discussed above about the effect of the total surface remains the same, with larger amounts of metals released as the particle size is reduced (larger surface area).

If the composition of the additives is considered, which is the main source of these released elements, Zn and Sn were present as sulfides, same as cadmium and mercury. Sulfides of Zn, Sn, and Cd are insoluble in water, although they can be solubilized in a small extent in acid media (pKs values around 25), whereas HgS is quite insoluble, which would explain the low values

(below limit of quantification in all cases) obtained. It is possible that some adsorption processes onto the materials would play a significant role after the release of these metals, reducing the total concentration remaining in the medium in the case of the milled materials.

Precisely, the acidity of the gastric medium could also explain the higher concentrations of some metals released during this phase compared to the intestinal phase, in which pH is increased up to values around 7. This effect was also observed by other authors using the same simulated digestion [18].

3.2. Metals released from microplastics used as models during simulation of fish digestion phases.

Three common polymers, LDPE, PP, and PVC were used as models for metal release in *in vitro* fish digestion studies. Table 2 shows the results obtained for these three materials under different conditions. Along this study, "gastric and intestinal" phase is referred to the total bio- accessible fraction of metals after digestion, whereas individual phases were also analyzed, so percentages of metals released on each phase could be compared. As previously discussed, larger concentrations of released metal were found during the gastric phase for the three materials studied.

Table 2. Concentrations of metals in digestion media after in vitro assays. Mean ± standard deviation (n=3). Concentration values expressed as the difference with respect to blank values. All Hg concentrations were lower than the limit of quantification (0.1 μg L^{-1}).

Material	Digestion phase	Pb	Cr	Cd	As	Sb	Sn	Zn
		Concentration (μg L^{-1})						
PVC	Gastric	<1	<20	<1	<2	<0.6	<2	<0.4
PP		75±1	27.9±0.1	6.8±0.1	6.0±0.1	1.01±0.01	<2	<0.4
LDPE		24±1	54.1±0.2	6.4±0.4	7±1	5.7±0.4	<2	<0.4
PVC	Intestinal	<1	<20	<0.3	<2	<1	<1	<5
PP		2.32±0.02	<20	1.0±0.1	<2	<1	<1	<5
LDPE		1.84±0.04	<20	0.4±0.1	<2	1.96±0.01	<1	<5
PVC	Gastric and Intestinal	<1	<20	<1	<2	<0.6	<1	<5
PP		22.2±0.5	24±1	6.0±0.4	<2	1.5±0.4	<1	<5
LDPE		10.8±0.5	56.3±0.1	4.0±0.1	<2	6.7±0.1	<1	<5

The lower concentrations observed at the end of the process seem to correspond to the lower concentrations released and the dilution required during the intestinal phase. Comparing the different polymer materials, the lowest concentrations of metals released correspond, in general, to the PVC. However, this comparison should be made in relative terms, considering the total metal concentrations in the bulk materials (see Table S3), so results can be expressed as percentages of metal released with respect to the total metal content in the solid, as shown in Figure 1.

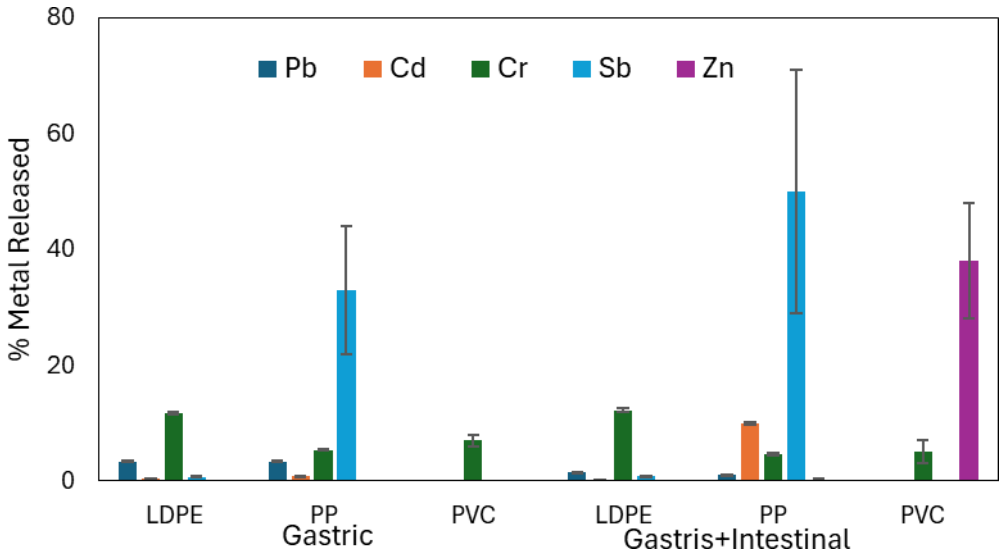

Figure 1. Percentage of metal released during gastric phase and at the end of the digestion (after both gastric and intestinal phases).

Percentages were calculated after the gastric phase and at the end of the digestive process for the three microplastics considered. In general, percentages lower than 15% were obtained for most of the metals determined, except for Sb in the case of PP. In these cases, the high uncertainty values observed, due to the low concentration found in the PP for Sb (see Table S3), make these results less significant. In general, PVC shows no significant migration in comparison to the other two materials (LDPE is a reference material that contains several pigments, whereas PP is a fraction of microplastics collected in the environment). Despite these differences, chromium is released in a significant percentage in the rest of cases, especially from LDPE. This material contains chromium as $CaCrO_4$, which may explain its solubility in acidic media, remaining in the final medium after the intestinal phase. Lead and cadmium show a similar behavior, with larger percentages during the gastric phase.

Therefore, it is not possible to establish a general pattern to explain the differences observed between the different materials and metals studied. Some elements, such as Sb, seems to be released significantly during the intestinal phase from PP, which suggest the presence of metal forms accessible by complexation with the proteins added during this phase, although the large uncertainty associated to these data should also considered. C. Catrouillet et al [20] found that Zn, together with some other elements, such as Al or Mn, progressively released from the plastic surface to the external environment by the analysis of acidic leaching and acid digestion with plastics collected in the environment. By contrast, metals such as Cu, Pb, Cr, and Cd, were adsorbed in the altered plastic surface, being released afterwards during the acidic leaching assay. As previously discussed, Masset et al [18] also observed an increment on the percentage of total Zn solubilized during the intestinal phase, whereas no significant desorption occurred in this phase for other metals such as Fe, Pb or Co, although the total concentration of these metals in the original material was also lower. Considering all these potential effects, it may be justified by the different behavior observed between the three different plastics models used. In the case of PP, environmentally aged plastic, adsorption processes suffered during this time on its altered surface can make some metals more accessible respect to those present in the LDPE, which corresponds to an unaltered plastic, so released metals during the digestion simulation come from the known additives it contains. Even though, dynamic sorption processes may also occur during the digestion, modifying the amount of metal finally bio-accessible.

3.3. Metals released from samples collected at Calabrian Coasts. Comparison with microplastic models. Effect of particle size.

Total metal concentrations in these samples were determined following the procedure described in section 2.2.3. and results shown in Table S3. These concentrations are in good agreement with those found by other authors in environmentally aged ocean plastic fragments [23-25]. In general, metals in environmental plastics can be found as adsorbed species, which depends on the surface plastic composition and may increase its reactivity towards the metals present in the environment, or as additives [24] as previously discussed. Some strategies have been developed to distinguish between these two forms based on the metal concentration profiles in microplastics surface. This study [24] revealed that there was a large variety of profiles depending on the origin of these microplastics, although some elements, such as As and Zn, showed larger concentrations at the subsurface layers, whereas other elements, such as Pb or Sb present constant distributions, which suggests they were additives in these plastics. It is evident that metal release processes during digestion will be affected by the forms (sorbed vs. additives) these metals are present in plastics, as observed with the models already studied.

Plastics collected from different areas of Calabrian coast (see details in section 2.1), were subjected to the same digestion procedure as plastics models and results are shown in table 3.

Table 3. Concentrations of metals in digestion media after in vitro assays. Mean ± standard deviation (n=3). Concentration values expressed as the difference with respect to blank values.

Sample	Digestion phase	Pb	Cr	Cd	As	Sb	Sn	Zn	Hg
		Concentration (μg L^{-1})							
Ionian I	Gastric	6.4±0.1	<20	1.0±0.1	/	<0.01	<2	103±9	<0.1
Tyrrhenian I		5±1	<20	0.20±0.02	/	<0.01	<2	<0.4	<0.1
Ionian II		23±8	<20	0.8±0.1	/	<0.01	<2	51.4±0.1	<0.1
Tyrrhenian II		<1	<20	0.8±0.1	/	0.5±0.3	<2	102±44	<0.1
Ionian I	Gastric and intestinal	1.3±0.4	<20	1.0±0.1	<2	<0.01	<1	88±30	<0.1
Tyrrhenian I		<1	28±8	<0.5	<2	0.3±0.2	<1	<5	0.40±0.04
Ionian II		4±2	<20	0.6±0.1	<2	<0.01	<1	<5	0.2±0.1
Tyrrhenian II		<1	<20	<0.5	<2	0.6±0.3	<1	63±25	0.2±0.1

Since microplastics used along this study were not classified by color or composition, the results correspond to the bulk material, trying to simulate a real scenario of microplastics ingested by fishes. Pb and Zn are the metals released to a larger extent, although concentrations are lower than those observed with the microplastics models. If samples from the two coasts are compared, no significant differences are observed. Similar discussion as in section 3.2 about the influence of chemical forms of these metals in plastics on the release process can be done here. Plastics collected were observed by SEM and elemental composition analyzed by EDS, as described in [21]. Results showed that, while in the case of raw plastics used in that work, metals were distributed uniformly all over the surface analyzed, the plastics collected at sea showed some spots containing metals (Si, Na, K or Al were the most frequent, but Cr, Ti, Pb or Zn were also detected), which suggests that these metals may be present on the plastic surfaces as deposits sorbed onto their surface. When these samples were analyzed by single particle inductively coupled plasma mass spectrometry (SP-ICP-MS), which allows the simultaneous discrimination between dissolved species and particles [26], some signals corresponding to particles containing Pb were detected. Figure 2 shows the time scans obtained for the four different samples from Calabrian coast when monitoring Pb. As in the case of Table 3, these samples had previously been subjected to the whole digestion process (gastric and intestinal phase). Time scans for ultrapure

water and digestion medium blank are also shown in Figure 2 for comparative purposes. As can be observed, micro/nanoparticles containing Pb were detected for all Tyrrhenian and Ionian Sea samples, being this value higher in the case of Ionian II sample, which is in agreement with lead concentrations released to media shown in Table 3. These particles containing Pb could be related to lead released from the microplastics themselves as insoluble forms (Pb_3O_4, $PbCrO_4$), although the formation of insoluble hydroxides in the medium itself once the Pb^{2+} is released cannot be discarded. Therefore, it is possible that metals deposit on microplastic surfaces in the environment, facilitating their release during digestion processes as insoluble forms (particulates) afterwards. In any case, further experiments should be carried out with more samples and monitoring more elements to confirm these results.

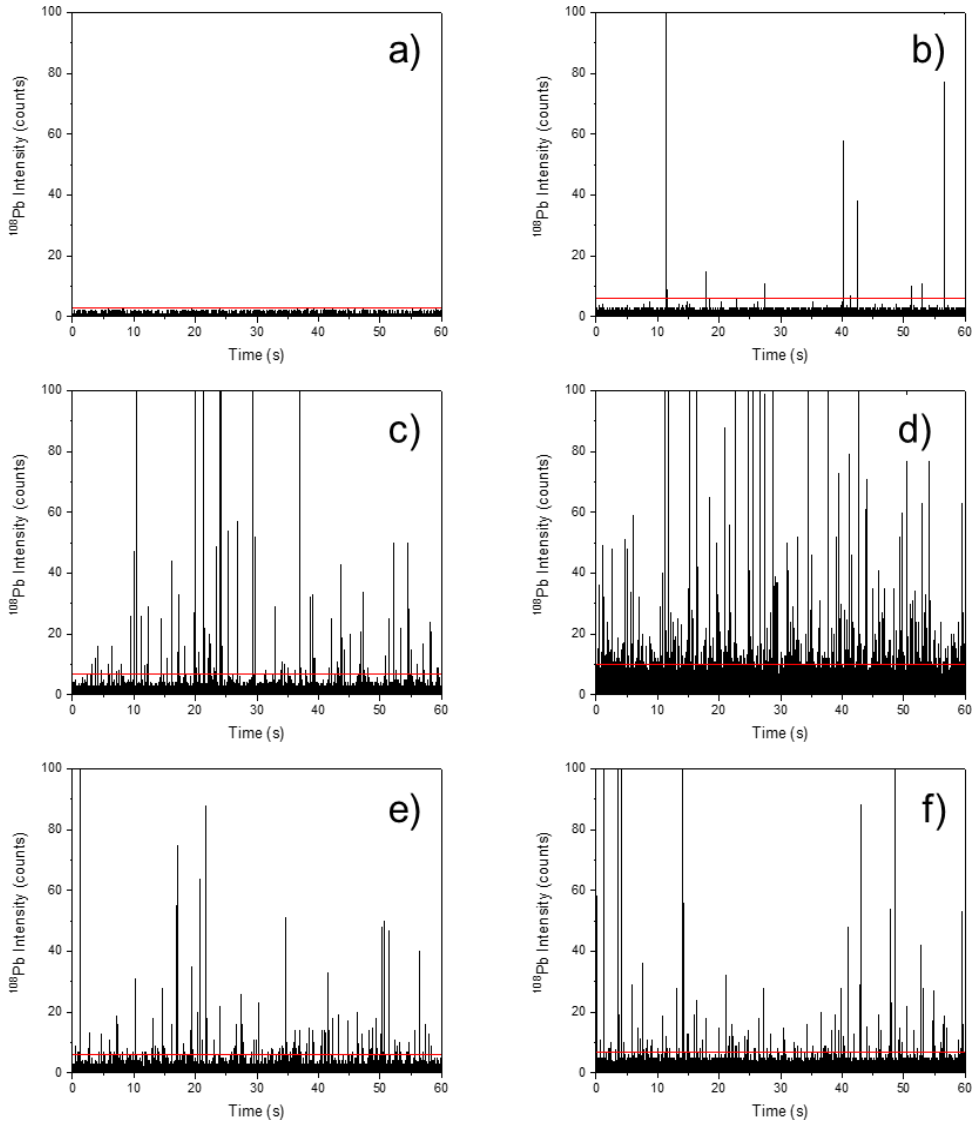

Figure 2. ^{108}Pb SP-ICP-MS time scans for a) ultrapure water b) Blank (SGF+SFI); c) Ionian I; d) Ionian II; e) Tyrrhenian I; f) Tyrrhenian II. Red line: critical value for identification of particle events.

If percentages of metal released with respect to the amount of metal present in the samples are directly compared (see figure S2), it makes evident the differences between the environmental samples and those selected as models, being much larger in the latter case (except for Zn, although large uncertainties were observed).

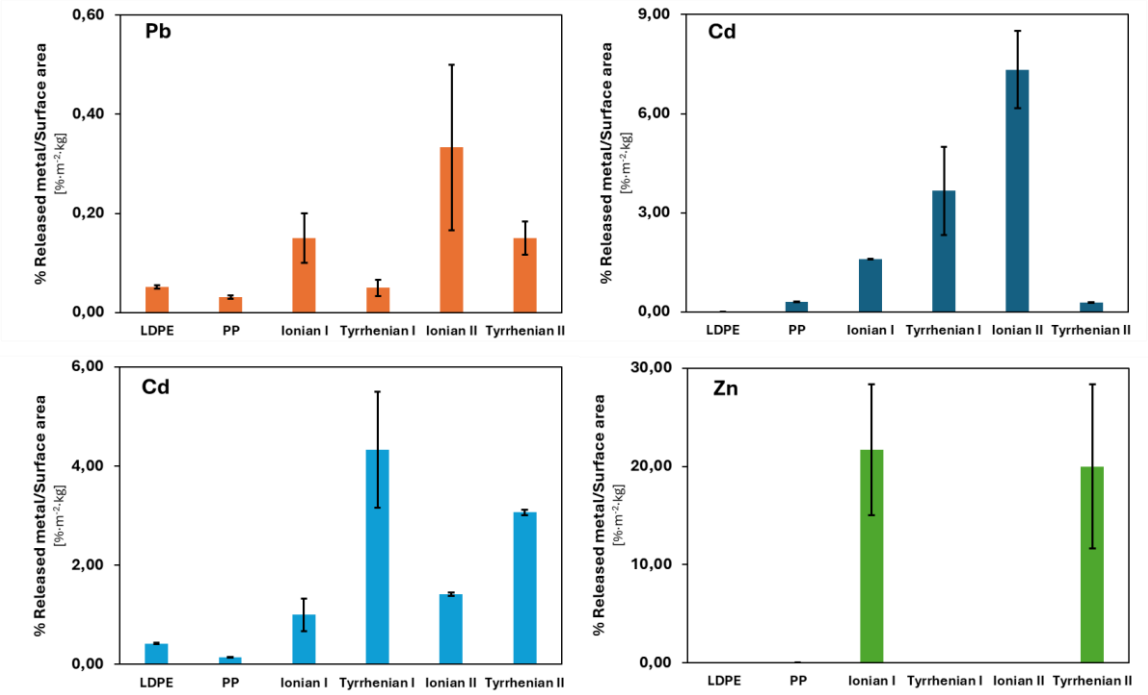

Figure 3. Percentage of metal released at the end of the digestion (after both gastric and intestinal phases) normalized to the surface area.

That would lead to the wrong impression that the amount of each metal released from these samples is less significant than those observed for those plastics used as models. However, the effect of the surface area should be considered, given its effect on this kind of processes, as previously discussed in section 3.1. Figure 3 shows the percentage of metal released per surface area unit (the total surface area of the mass of microplastic added to the synthetic digestion media was estimated considering the average size and density for each sample). In general, for most metals, the percentages in environmental samples are comparable to those obtained with the microplastics models, which suggests that surface area plays a relevant role on the release of metals during digestion process. In fact, predictions of the complete release of metals or metallic compounds from very small particles of nanometer to micrometer dimensions estimate that it could take hours to days, being nanoplastics exposed to the environment rapidly depleted of metal additives [19].

4.- Conclusions

Microplastics collected in areas of the Calabrian coasts carry heavy metals which, through the digestion process of marine organisms, can reach humans via the food chain. Procedures based on gastric simulation in fishes represent a valuable tool for understanding these processes. Release of Pb, Cd, Cr or Zn during simulation digestion in fish has been demonstrated. Percentages of metal released may become relevant as microplastic sizes reduce, as suggested by the results obtained when the surface area is also considered. However, the chemical forms of these metals are also relevant to explain the release processes involved during digestive simulations. As expected, the use of additives based on metals as insoluble forms make them less bio-accessible, but the acidic medium during gastric phase or the presence of complexing agents during intestinal phase may favour these processes. In addition, the potential adsorption of metals on the microplastic surface once they are incorporated into the environmental systems, may increase the bio-accessibility of these metals, acting as vectors for these contaminants (having a Trojan horse effect). The release of metals present either as additives or deposited on the

microplastics surface, as particles, has been detected in the case of lead by SP-ICP-MS. The nature of these particles detected containing lead could not be determined, and they could correspond to lead released in its original form as additive, or even as part of a small fragment of microplastic released, although the formation of insoluble forms during digestion cannot be discarded. Obviously, the chemical form of the released metal will have a different impact on its bioavailability and potential toxicity. Further speciation studies are required to determine the true nature of these species and their actual impact on environmental systems and its bioaccessibility.

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Supplementary information

1.- In vitro digestion protocol

A total of 0.1 gg of sample is placed in a 15 mL falcon to which 10 mL of simulated gastric fluid (SGF) is added at t=0, obtaining a solid/liquid ratio of 0.01. The flasks are immediately wrapped in aluminum foil to simulate a dark environment and placed on an orbiting oscillator at 130 rpm, for 3 hours at room temperature (20 °C). After three hours, the flasks are centrifuged at 2400 rpm, for 10 min at 20 °C and, by pipetting, SGF is collected and replaced with 10 mL of simulated intestinal fluid (SIF) to maintain the solid/liquid ratio of 0.01. The falcons are again wrapped in aluminium foil and placed on an orbiting oscillator at 130 rpm, for 23 hours at room temperature (20 °C). The collected SGF is stored in polypropylene tubes at 4 °C. After this time, the falcons are centrifuged again and, by pipetting, the liquid part SIF) is separated from the solid part and stored at 4 °C. This protocol was also performed with a variation: at the end of the 3-hour gastric phase, the SGF is not removed and replaced by SIF, but 180 µL of concentrated SIF is added to it. This variation was made in order to give continuity to the protocol and avoid losing plastic samples during the replacement process and it is referred along the text as SGF+SIF.
Gastric fluid and intestinal fluid are prepared starting from a buffer consisting of 8819.6 mg/L NaCl, 224.4 mg/L KCl, 108.8 mg/L MgSO4, 148.2 mg/L CaCl2. Gastric fluid is prepared by acidifying the buffer to pH=2 with 32% HCl. Subsequently, 10 mg of pepsin are dissolved in 10 mL of ultrapure water and added to the buffer in an amount of 20 µL/mL. The gastric fluid is instead prepared by adding to the starting buffer 4 mg/mL of porcine bile extract, 2 mg/mL of pancreatin, 0.84 mg/mL of NaHCO3 and 20 µL/mL of 1 M HEPES. In the case of the continuous protocol (180 µL of SIF are added to the SGF) the intestinal fluid is prepared in a way that, by adding a small volume in 10 mL of gastric fluid, the concentration of its components does not change compared to the initial protocol. Consequently, the concentrated SIF, in this case, will be composed of 200 g/L of porcine bile extract, 200 g/L of Pancreatin, 53 g/L of NaHCO3, 238.5 g/L of HEPES, dissolved in ultrapure water. After adding 180 µL of concentrated SIF for the second phase of digestion, the pH is adjusted to 7 with NaOH. The composition of the gastric fluid and intestinal fluid was adapted from [18, 27].

2.- Tables

Table S1. Temperature program for microwave-assisted digestion of polymers.

Sample	Step	Power (W)	Temperature (°C)	Ramp (min)	Hold (min)
PE+PP	1	1000	120	4	0
	2	1000	180	3	0
	3	1000	220	4	0
	4	1000	220	0	20
PVC	1	1000	220	10	0
	2	1000	220	0	20

Table S2. Instrumental parameters for ICP-MS measurements.

ICP-MS	Perkin Elmer Elan DRC-e	Perkin Elmer NexION 2000B
Instrumental parameters		
RF power	1200 W	1600 W
Argon gas flow rate Plasma	15 L min^{-1}	15 L min^{-1}
Auxiliary	1.2 L min^{-1}	1.2 L min^{-1}
Nebulizer	1.14 L min^{-1}	1 L min^{-1}
Make-up	-	0.2 L min^{-1}
Sample flow rate	1.0 mL min^{-1}	0.01 mL min^{-1}
Isotopes monitored	^{53}Cr; ^{64}Zn; ^{75}As; ^{110}Cd; ^{120}Sn; ^{121}Sb; ^{200}Hg; ^{208}Pb	^{208}Pb

Table S3. Total content of metals in plastic models and samples. (n=3).

	Pb	Cd	Cr	As	Sb	Sn	Zn
	concentration μg·g^{-1}						
PVC	2.4±0.1	0.01±0.005	3.0±0.1	0.10±0.01	0.04±0.01	0.20±0.01	6.0±0.1
PP	219±4	87±1	52±1	0.20±0.03	0.30±0.1	0.20±0.03	40±1
LDPE	71±2	136±1	46±1	14.0±0.1	86±7	99±6	1002±18
Ionian I	147±3	10.3±0.1	140±1	0.53±0.01	0.16±0.05	0.57±0.01	61.1±0.1
Tyrrhenian I	72±1	1.30±0.06	104±3	0.45±0.03	1.1±0.1	0.46±0.07	26.3±0.7
Ionian II	205±5	1.30±0.03	153±1	0.63±0.02	0.048±0.02	0.23±0.04	47.9±0.6
Tyrrhenian II	53.3±0.3	17.1±0.3	98±1	0.43±0.03	0.17±0.03	0.72±0.04	46±1

3.- Figures

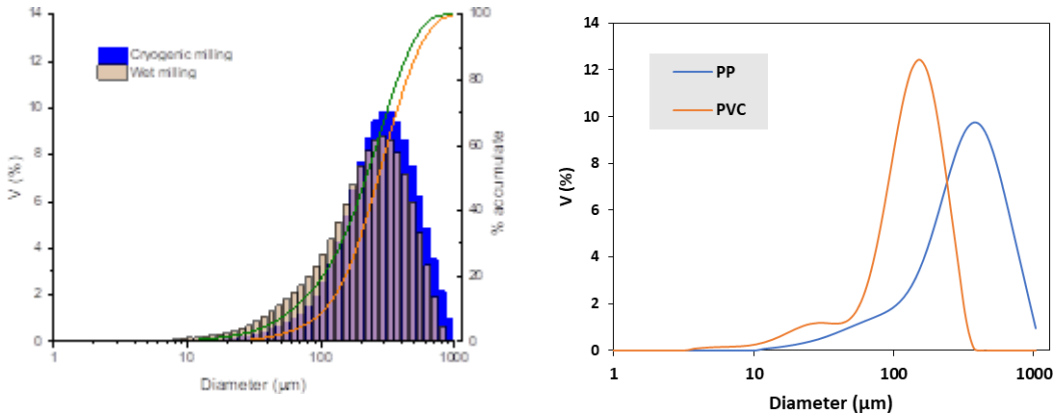

Figure S1. Left Size distribution of ERM-EC681m material after milling under two different conditions: wet milling with methanol, and cryogenic milling using a bath of liquid nitrogen. Right Size distribution of PP and PVC samples.

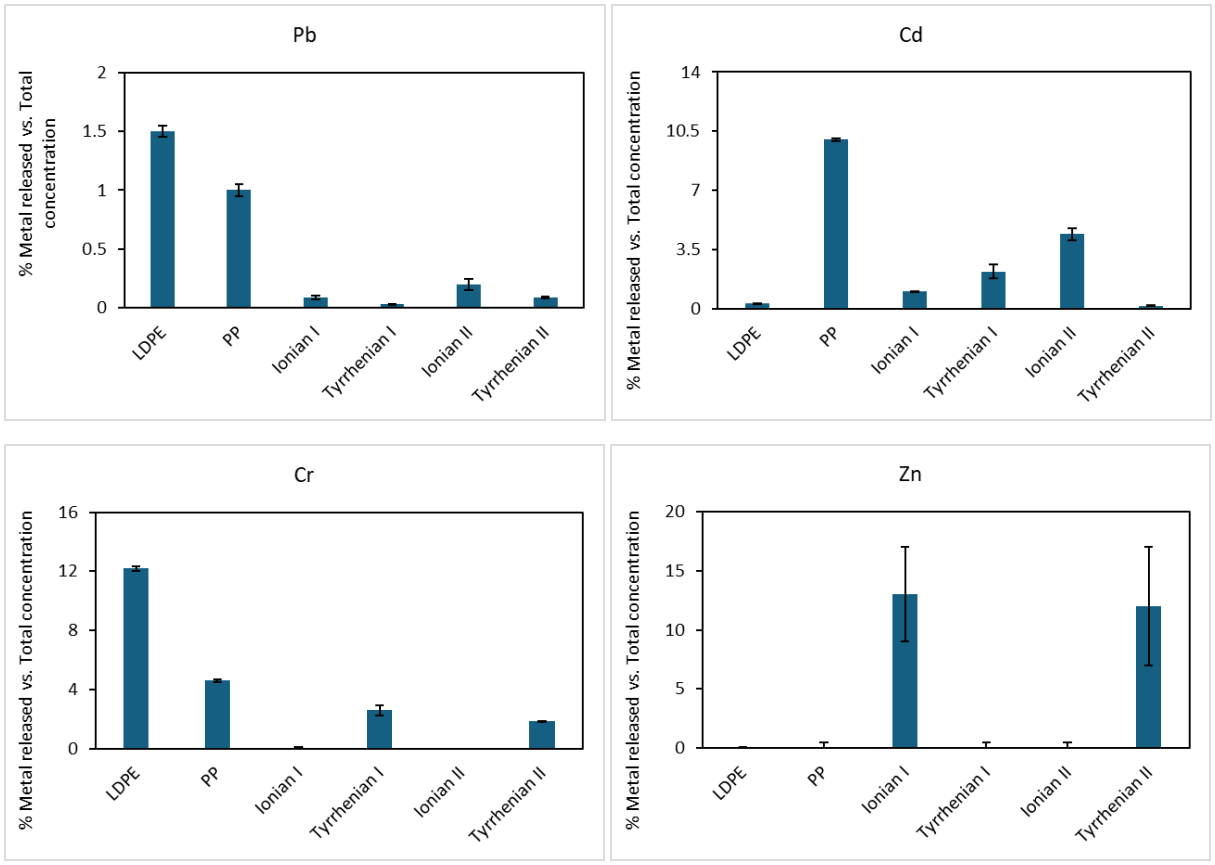

Figure S2. Percentage of metal released at the end of the digestion (after both gastric and intestinal phases) with respect to the total amount of metal in the microplastics.

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OCTN1 (SLC22A4) as a Target of Heavy Metals: Its Possible Role in Microplastic Threats

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Abstract: Microplastics represent a threat due to their ability to enter the food chain, with harmful consequences for living organisms. The riskiness of these particles is also linked to the release of other contaminants, such as heavy metals. Solute Carriers (SLCs) represent eminent examples of first-level targets of heavy metals due to their localization on the cell surface. Putative targets of heavy metals are the organic cation transporters that form a sub-clade of the SLC22 family. Besides the physiological role in the absorption/release of endogenous organic cations, these transporters are crucial in drug disposition and their interaction with xenobiotics. In this work, the human SLC22A4, commonly known as OCTN1, was used as a benchmark to test interactions with heavy metals released by microplastics, exploiting the proteoliposome tool. The potency of metals to interfere with the OCTN1 function has been evaluated by measuring IC50 values calculated in the micromolar range. The molecular mechanism of interaction has been defined using site-directed mutagenesis and computational analyses. Finally, some chemical and physiological thiol-reacting compounds show the capacity to rescue the metal-inhibited OCTN1 function. The conclusions drawn on OCTN1 can be extended to other members of the SLC22 family and orthologous transporters in fish.

Keywords: SLC22A4; heavy metals; liposomes; acetylcholine; pollution

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1. Introduction

Plastic pollution represents one of the global threats to living organisms, with both seas and oceans being ideal basins for accumulating great amounts of these contaminants. Indeed, islands of plastic waste have grown over the years in marine environments, reaching more than 250 K tons of total plastic [1]. The danger of plastic waste in the environment is related to several factors, including their physical, mechanical, and chemical features. In this respect, increasing efforts are underway to accomplish a systematic classification of plastic particles according to their size and composition, and currently, three main categories have been defined (Table 1) [2].

Table 1. The classification of microplastics according to their size.

Particles Size (mm)	Classification
>5 mm	Macroplastics
Between 5 mm and 0.3 mm	Microplastics
<0.3 mm	Nanoplastics

When looking at living organisms as a target, microplastics are those raising major concerns because their small size facilitates ingestion and accumulation in the entire food chain, including humans [3,4]. Furthermore, microplastics are vehicles of other contaminants with unpredictable global effects on metabolism, cell proliferation, and even more intricate and complex interactions with entire organisms [5]. Therefore, great efforts are needed to fully characterize the collection of potential threats linked to the worldwide spread of microplastics. In this respect, appropriate biomarkers for monitoring the harmful effects of plastic pollution are needed in living organisms, including humans.

1.1. Microplastics as Carriers of Heavy Metals

In this very large and open field of investigation, several studies highlighted the possibility for microplastics to accumulate and release heavy metals [6–9], which are absorbed during the production process or by their interaction with other pollution sources [10]. Generally, the release of metals follows kinetics linked to the diffusion of the metals themselves inside the plastic particles and depends, besides other parameters, on microplastic diameters and porosity, pH, the salinity of the medium in which they are found, and solar radiation. Moreover, gastrointestinal fluids of aquatic animals can digest microplastics and, hence, release heavy metals [11,12]; it has been demonstrated that ingested microplastics can release heavy metals in human tissues as well [3,13]. Then, microplastics represent another source of heavy metals, which are well known as extremely dangerous for all living organisms. Indeed, these toxicants can accumulate in tissues with mechanisms known as biomagnification [14,15]. Once in cells, some heavy metals can form metal–thiol bonds with cysteine residues of proteins, altering their stability/mobility and functionality in cells. The wide range of negative effects of heavy metal contamination is related to the sizable number of their molecular targets and, hence, cell pathway alterations. Among the biological targets of heavy metals, there are membrane proteins belonging to the SLC superfamily, which contain an average of 11 cysteine residues in their structure and at least 1 cysteine residue in each protein, with only one exception [16].

1.2. OCTN1 (SLC22A4): A Benchmark for Studying Interactions with Heavy Metals

In line with previous data, the fourth member of the SLC22 family, namely OCTN1, is an acknowledged target of mercury-derived compounds [17]. OCTN1 can also recognize several drugs and, hence, contribute to their delivery to human tissues [18]. For these reasons, SLC22 proteins are included in the FDA guidelines as major players in pharmacodynamics and drug disposition [19]. OCTN1 has been described as a poly-specific transporter in ex vivo [20] and in vitro [21] models, given its ability to recognize both cations or zwitterions as substrates with different mechanisms. Indeed, this transporter mediates the uptake and the efflux of physiological cations such as acetylcholine and choline in a sodium-independent fashion [21]; at the same time, OCTN1 can recognize the fungi metabolite ergothioneine and the vitamin-like cofactor carnitine with a sodium-dependent mechanism [22–24]. Recently, the two independent pathways for cation/zwitterion binding and translocation were investigated by in silico and in vitro strategies [23].

1.3. OCTN1 in the Non-Neuronal Cholinergic System

This broad substrate specificity highlights various roles in cell physiology, from the absorption of the antioxidant ergothioneine and carnitine to the release of acetylcholine with anti-inflammatory action in the non-neuronal cholinergic system (NNCS) [19]. Furthermore, the low-affinity carnitine transport can compensate for the lack or misfunction of the high-affinity carnitine transporter OCTN2 [20,23]. In addition, OCTN1 is involved in the disposition of carnitine or its derivative acetyl-carnitine when pharmacologically administered as neuroprotectants [25,26]. This evidence links the OCTN1 expression and function to the RSST (Remote Sensing and Signalling Theory) [27,28] as a player of the responsive/adaptive system allowing inter/intra-organ (gut–brain and gut–kidney axis) and inter-organismal communication. In good agreement, NNCS is active in all kingdoms

of life due to the role of acetylcholine in the control of basic cell functions [29,30]. Finally, the involvement of OCTN1 in chronic inflammatory diseases and metabolic disorders suggests its role in cell senescence and ageing processes characterized by the so-called "inflammaging" phenotype [31,32]. Moving from the above-described premises, in this work, we aim to employ the human OCTN1 as a benchmark to study the effect of heavy metals potentially released by microplastics after ingestion by fishes and, therefore, by humans. In this research, OCTN1 could represent one of the food chain rings affected by microplastic pollution; indeed, OCTN1, which evolved with vertebrates, has orthologs in species other than humans, among which fishes also enter the human food chain [33,34]. Therefore, unravelling the molecular mechanism of heavy metal–OCTN1 interactions could add important information for explaining and, hence, preventing or treating the effects of heavy metal pollution in humans.

2. Results and Discussion

The heavy metals reported in Table 2 were identified in microplastics sampled in six stations of the Calabria Region coast, using qualitative and quantitative approaches. In brief, Ionian and Tyrrhenian sites were sampled, and microplastics were analyzed in terms of their size, shape, colour and metal content with the following relative abundance: Lead > Chromium > Zinc > Cadmium. These findings are in good agreement with an already published study on the release of heavy metals from microplastics in the marine environment [12].

Table 2. Toxicity level of metals tested according to ATSDR. High toxicity (+++), Low toxicity (+).

Heavy Metals	Level of Toxicity	Identifier
Mercury	+++	https://www.atsdr.cdc.gov/toxprofiles/tp46.pdf (accessed on 21 October 2024)
Cadmium	+++	https://www.atsdr.cdc.gov/toxprofiles/tp5.pdf (accessed on 21 October 2024).
Zinc	+	https://www.atsdr.cdc.gov/toxprofiles/tp60.pdf (accessed on 21 October 2024).
Lead	+++	https://www.atsdr.cdc.gov/toxprofiles/tp13.pdf (accessed on 21 October 2024).
Chromium	+++	https://www.atsdr.cdc.gov/toxprofiles/tp7.pdf (accessed on 21 October 2024).

As mentioned above, OCTN1 can be considered an ideal biological candidate in the context of environmental toxicology studies due to several important features: (i) it is a cell membrane protein being considered a first-level target for xenobiotics [35]; (ii) it harbours in its structure seven cysteine residues, which can be targets of mercury [17]; (iii) it plays multiple functions in humans, mediating the absorption/release of physiologically relevant molecules [19]; (iv) it has orthologues in different vertebrates including fishes, such as *Danio rerio* [33] and *Oncorhynchus mykiss* [34], which is one of the most common cold-water fish species in aquaculture and the seventeenth widely cultivated commercially important finfish in the world and, hence, in the human diet [36]; and (v) it is also a drug transporter, hence, the impairment of its function may also affect drug distribution and response in humans and other animals. It is noteworthy that even if exposure to heavy metals is a well-known human threat [17] (https://www.who.int/publications/i/item/WHO-FWC-PHE-EPE-16.01-eng (accessed on 21 October 2024)), it acquires more relevance for global health since the concept of the exposome emerged [37] (WHO). Indeed, considering an "exposome" referring to the totality of exposures to internal and external factors, the widespread distribution of heavy metals, together with their strong ability to interact with macromolecules, can be considered an emblem in such a continuously evolving research area.

2.1. Reconstitution of Human OCTN1 in Proteoliposomes and Effects of Heavy Metals

Moving from these premises, the effect of Hg^{2+}, Cd^{2+}, Cr^{2+}, Pb^{2+}, and Zn^{2+} found to be released by microplastics was evaluated on the human OCTN1 using the well-characterized experimental model of proteoliposomes (Figure 1) in which the effect of Hg^{2+} was previously established [17].

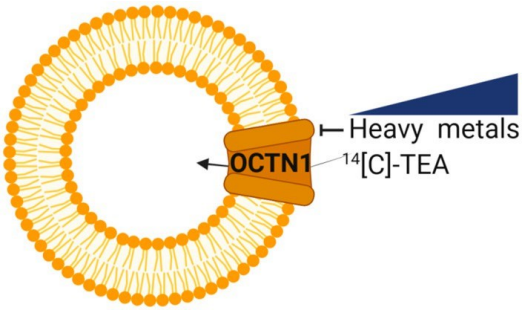

Figure 1. Inhibition of [^{14}C]-TEA transport mediated by OCTN1 in the presence of heavy metals. The experimental model is depicted as a sketch. In orange the protein OCTN1; the arrow indicates the direction in which OCTN1-mediated [^{14}C]-TEA transport occurs. The crossed-out line indicates inhibition of [^{14}C]-TEA transport by heavy metals (blue).

This experimental system successfully mimics the cell conditions since OCTN1 is inserted in the artificial membrane with the same orientation as in the native membrane, i.e., with the internal and external compartments of liposomes corresponding to the internal and external environment of cells. This allows reliable information on the specific effects of the toxicants on the single protein included in the membrane to be obtained (Figure 1). In this system, the effect of heavy metals was tested on the uptake of radiolabelled TEA (Tetraethylammonium), i.e., the well-known and widely used prototype of a cationic substrate of OCTN1 [38]. The addition of divalent metals strongly inhibited the uptake of [^{14}C] TEA in proteoliposomes (Figure 2). It has to be stressed here that our experimental model, which is exquisitely in vitro, is intended as a proof-of-concept approach devoted to the identification of a molecular target of environmental pollutants, such as heavy metals; therefore, the used concentration in these experiments was intentionally high, as an initial screening, assuming the condition of a high-polluted environment or a biomagnification phenomenon in tissue, which is a common feature for heavy metals [14,39–41].

To evaluate the potency of inhibition, dose–response analyses were performed (Figure 3).

As usual for potent inhibitors, the measured IC_{50} values were in the micromolar range, i.e., from 70 to 200 µM (Table 3). It is not trivial to consider that these concentrations are lower than those locally reached in the organism upon microplastic ingestion due to the release mechanism, which depends on several mechanisms, some of which are still not completely explained [42].

Table 3. IC_{50} values (µM) calculated from dose–response analysis of OCTN1 WT in the presence of $HgCl_2$, $CdCl_2$, $ZnCl_2$, $Pb(NO_3)_2$, and $CrCl_2$ on [^{14}C]-TEA uptake.

Heavy Metals	IC_{50} (µM)
$HgCl_2$	121 ± 7
$CdCl_2$	56 ± 4
$ZnCl_2$	57 ± 17
$Pb(NO_3)_2$	90 ± 19
$CrCl_2$	170 ± 15

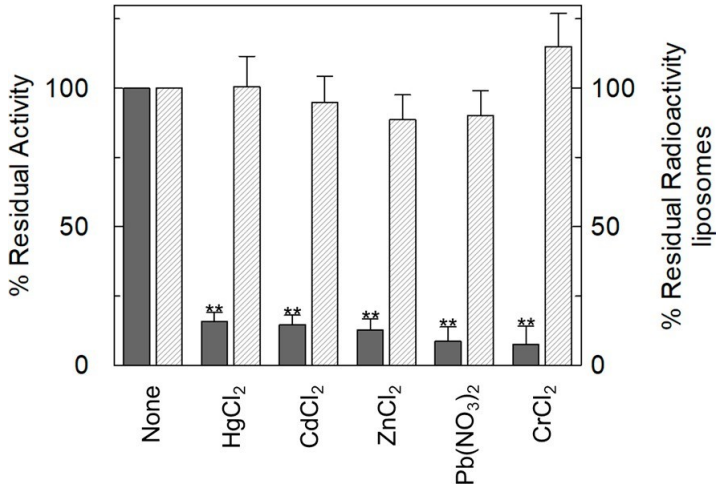

Figure 2. The inhibition of [^{14}C]-TEA transport mediated by OCTN1 in heavy metals. The protein reconstitution procedure is described in the Section 3. The uptake started with the addition of 0.1 mM [^{14}C]-TEA to proteoliposomes in the presence of 250 μM of indicated metals. The transport was stopped after 60 min according to the stop inhibitor method. Percent residual activity with respect to the control (without heavy metals addition) is reported (dark grey bars). To the right of the y-axis, data collected on liposomes without incorporated proteins are reported as the percentage of residual radioactivity associated with liposomes (white bars) due to diffusion/association with the vesicles with respect to the control (liposomes without heavy metals addition). The amount of radioactivity detected in liposome controls was no higher than 15% with respect to that which entered proteoliposomes harbouring OCTN1 in the membrane. The values are the means ± SD from three independent experiments. Data in the presence of the heavy metals were significantly different from the control (without heavy metals addition), as estimated by Student's *t*-test (** $p < 0.01$).

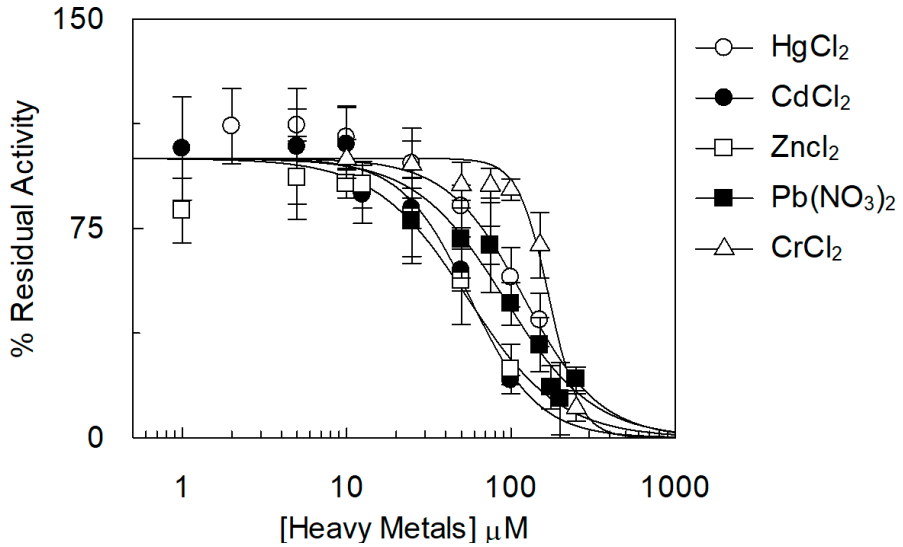

Figure 3. Dose–response analysis of the inhibition of the [^{14}C]-TEA transport by heavy metals. The reconstitution was performed as described in the Section 3. The transport was started by adding 0.1 mM [^{14}C]-TEA to proteoliposomes together with the indicated concentrations of $HgCl_2$ (empty circle), $CdCl_2$ (filled circle), $ZnCl_2$ (empty square), $Pb(NO_3)_2$ (filled square), and $CrCl_2$ (empty triangle), and ended with the stop-inhibitor method after 60 min. The figure shows the residual activity as a percentage compared to the control (without heavy metals addition). The values are means ± SD from three independent experiments.

2.2. Role of OCTN1-Cys Residues in the Interaction with Heavy Metals

As stated in the introduction, heavy metals are known to interact with cysteine residues of the target proteins via the formation of metal–thiol bonds. Some canonical binding sites for metals have been described, such as the CXXC, CXXXC, and MxCXXC motifs [16]. These are found in different proteins, i.e., intracellular targets and membrane proteins, including those responsible for the direct transport of heavy metals in cells [43–45]. Interestingly enough, non-canonical binding motifs are also expected to exist based on the spatial positioning of close thiol residues according to the 3D structure of protein microdomains [46]. This property, which cannot be identified in the primary structure of proteins, further explains the wide toxicity exerted by heavy metals [46]. It has to be stressed that a specificity issue arises from the available data in the literature, considering that the same signature may recognize different metals [47]. OCTN1 contains vicinal thiol residues, which are not adjacent in the protein sequence, forming microdomains previously identified as targets of Hg and Hg-derivatives by site-directed mutagenesis and in vitro transport assays [17]. Therefore, we hypothesized that the effects described in Figure 3 and Table 3 are ascribable to the interactions of the tested heavy metals with the cysteine residues of OCTN1 via the formation of metal–thiol bonds. To support this hypothesis, the effect of physiological and not physiological-reducing reagents was evaluated on the inhibition exerted by heavy metals (Figure 4).

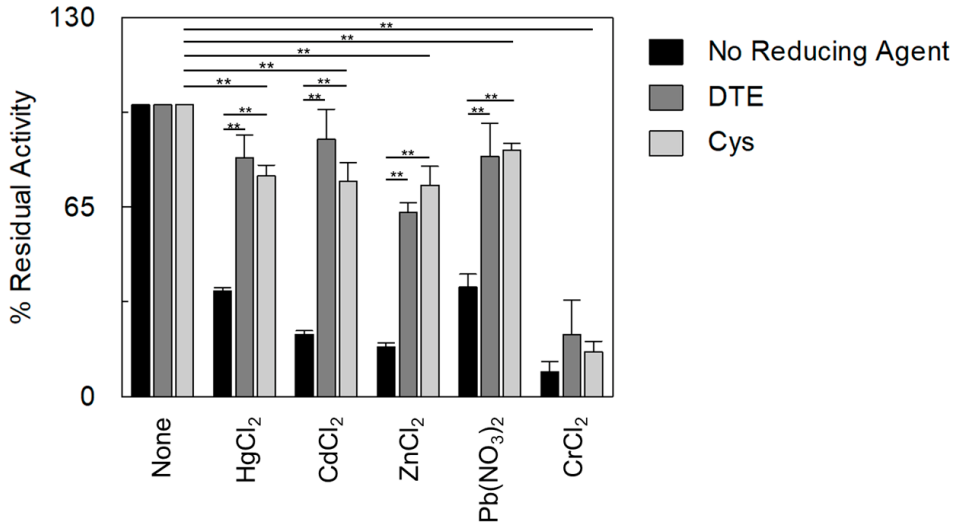

Figure 4. Effects of DTE and Cys on the inhibition of [^{14}C]-TEA transport mediated by OCTN1 with heavy metals. The protein reconstitution procedure is described in the Section 3. The uptake started with the addition of 0.1 mM [^{14}C]-TEA to proteoliposomes together with 100 μM $HgCl_2$ or $CdCl_2$ or $ZnCl_2$ or $Pb(NO_3)_2$, or 250 μM $CrCl_2$ in the absence of reducing agents (black bars) or in the presence of 5 mM reducing agents; DTE or Cys are indicated by dark grey or light grey bars, respectively. The uptake ended with the stop-inhibitor method after 60 min. The percentage of residual activity with respect to the control (without heavy metals addition) was reported. The values are means ± SD from three independent experiments. They were significantly different from samples without reducing agents and from the control (without heavy metals addition), as estimated by Student's *t*-test (** $p < 0.01$).

Indeed, these reagents can cleave, in most cases, the metal–thiol bond [17]. In good agreement with the proposed role of OCTN1-Cys residues, the addition of DTE and cysteine prevented the inhibitory effects of all the tested divalent cations with the only exception of Cr^{2+}, the ability of which to interact with residues other than Cys has been described [48]. It has to be stressed that four out of the seven Cys residues of OCTN1 are located in the large extracellular loop between TM1 and TM2, making them an ideal target(s) for xenobiotics.

2.3. Computational Analyses on hOCTN1 Homology Model

Then, computational analyses were performed to support experimental data: the homology model of human OCTN1 was built based on the CryoEM structure of human OCT3 [23] and analyzed using the MIB2 tool for predicting the binding template of Cd^{2+}, i.e., the most effective among the tested metals (Table 3). The MIB2 tool constructs metal ion-binding templates that consist of a protein spatial region of 3.5 Å around the ion. These regions are selected from 3D structures containing ions deposed in the Protein Data Bank (PDB) or predicted by AlphaFold. Then, the structure to be analyzed (such as OCTN1) is locally screened against the ion-binding templates, as previously described [49]. As shown in Figure 5, the best-scored Cd^{2+} binding site on the outward-facing part of OCTN1 includes Cys50, Cys81, Cys113, and Leu114 residues.

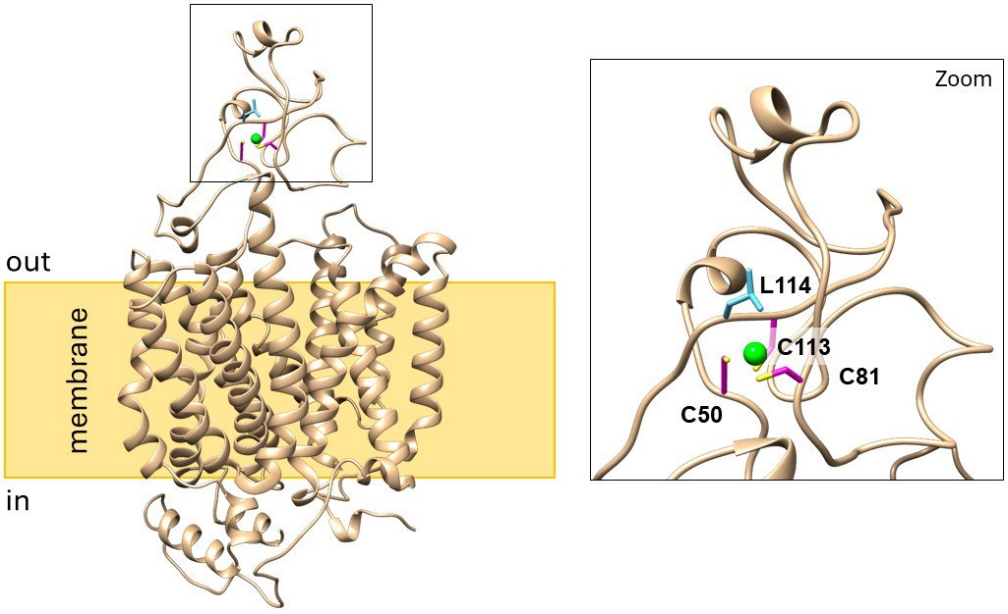

Figure 5. Lateral view of the homology model of human OCTN1 showing the predicted Cadmium binding site. The 3D structure of OCTN1 is shown with ribbon representation (sand). Cadmium is in green; the amino acids involved in the binding are depicted in magenta/yellow (C50, C80, C113) and Cyan (L114). Molecular graphics and the visualization of MIB2 results were performed with the UCSF Chimera v.1.14 software (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, CA, USA).

2.4. Analyses of Cys-Less Mutant of the Human OCTN1 Reconstituted in Proteoliposomes

To definitively prove the involvement of OCTN1 Cys residues in interactions with metals, another strategy was adopted using an OCTN1 mutant in which all cysteine residues were mutated to alanine (Cys-less OCTN1).

The data reported in Figure 6 show that the Cys-less OCTN1 was virtually not affected by heavy metals; the residual-measured inhibition can be ascribed to the presence of other residues, such as His, whose ability to coordinate heavy metals is well acknowledged [50]. The only exception was again Cr^{2+}, confirming that the inhibitory effect on OCTN1 may be due to a molecular mechanism involving residues other than cysteine [48]. To better evaluate the effect of heavy metals on the physiological transport properties of OCTN1, the physiological substrate acetylcholine was also tested on WT and Cys-less OCTN1 (Figure 7).

Overlapping results were obtained with respect to TEA, further demonstrating that OCTN1's functionality is altered when assaying a physiological substrate.

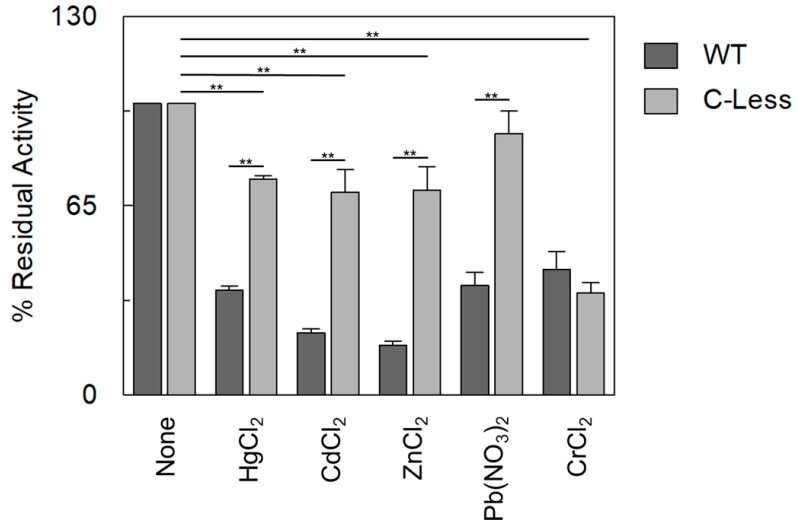

Figure 6. The inhibition of [^{14}C]-TEA transport mediated by OCTN1 WT and C-less in the presence of heavy metals. The reconstitution procedure of WT (dark grey bars) and C-less (light grey bars) is described in the Section 3. The uptake started with the addition of 0.1 mM [^{14}C]-TEA to proteoliposomes in the presence of 100 μM $HgCl_2$ or $CdCl_2$ or $ZnCl_2$ or $Pb(NO_3)_2$, or 250 μM $CrCl_2$. The transport was stopped after 60 min according to the stop-inhibitor method. The percentage of residual activity of OCTN1 WT and OCTN1 C-less with respect to the control was reported. The values are means ± SD from three independent experiments. Significant differences in C-less were obtained with respect to the control (without heavy metals addition) and C-less with respect to OCTN1-WT, as estimated by Student's *t*-test (** $p < 0.01$).

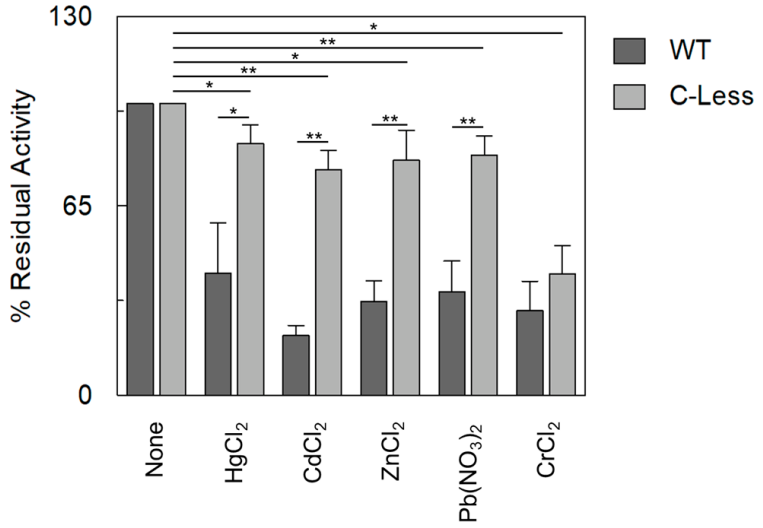

Figure 7. The inhibition of [^{3}H]-Ach transport mediated by OCTN1 WT and C-less in the presence of heavy metals. The WT (dark grey bars) and C-less (light grey bars) proteins were reconstituted as described in the Section 3. The transport was started by adding 0.1 mM [^{3}H]-Ach to proteoliposomes in the presence of 100 μM $HgCl_2$ or $CdCl_2$ or $ZnCl_2$ or $Pb(NO_3)_2$, or 250 μM $CrCl_2$. The transport was stopped after 60 min according to the stop-inhibitor method. The percentage of residual activity of OCTN1 WT and OCTN1 C-less with respect to the control was reported. The values are means ± SD from three independent experiments. Significant differences in the C-less were obtained with respect to the control (without heavy metals addition) and of C-less with respect to OCTN1-WT, as estimated by Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

2.5. Acetylcholine-Mediated Transport of OCTN1: Inhibition and Link with NNCS

It has to be highlighted that, under physiological conditions, OCTN1 is involved in the non-quantal release of acetylcholine from cells in the so-called non-neuronal cholinergic system [19,51–53]. Therefore, a different transport assay was used in which the efflux of radiolabelled acetylcholine was measured from proteoliposomes, mimicking the cell release [21] (Figure 8A).

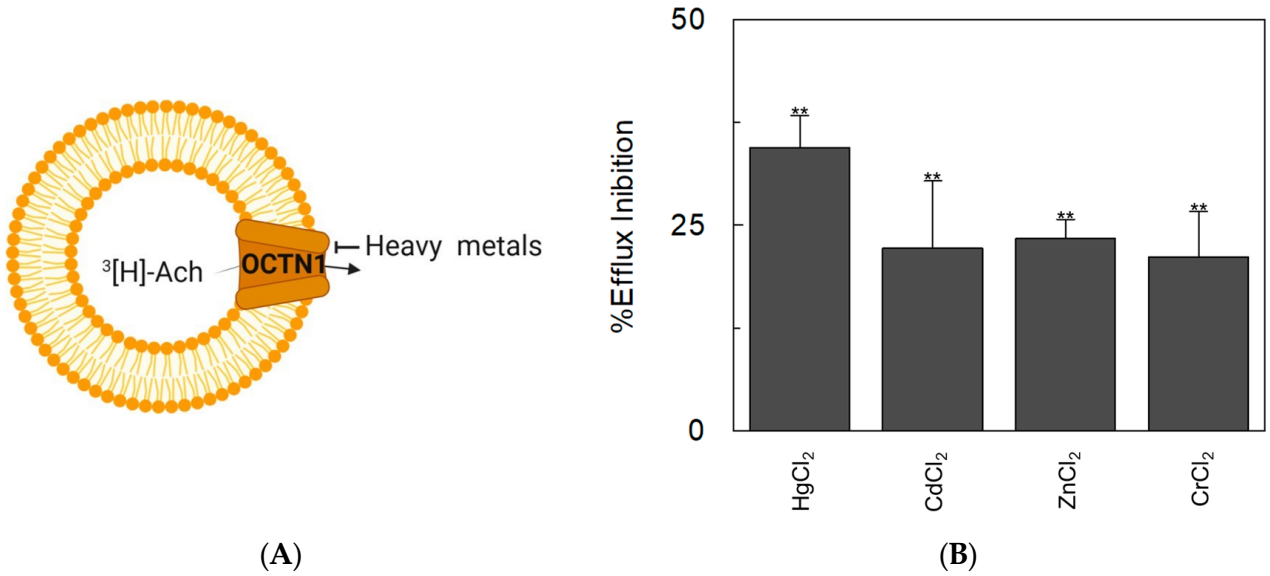

Figure 8. The inhibition of [^{3}H]-Ach efflux mediated by OCTN1 WT in the presence of heavy metals. (**A**) The experimental model is depicted as a sketch. In orange, the protein OCTN1; the arrow indicates the direction in which OCTN1-mediated [^{3}H]-Ach transport occurs. The crossed-out line indicates inhibition of [^{3}H]-Ach transport by heavy metals. (**B**) The protein reconstitution procedure is described in the Section 3. The uptake of 0.1 mM [^{3}H]-Ach was performed in 120 min. Then, the efflux of [^{3}H]-Ach was measured in the presence of 100 μM indicated heavy metals. The efflux was stopped after 60 min according to the stop inhibitor method. Data were calculated as the percentage of efflux inhibition with respect to efflux without externally added heavy metals. Results are the mean ± SD of three independent experiments. Data in the presence of the heavy metals were significantly different from the control (without heavy metals addition), as estimated by Student's *t*-test (** $p < 0.01$).

Interestingly, the OCTN1 efflux of radiolabelled acetylcholine was inhibited by the addition of heavy metals in the external environment (trans-inhibition) (Figure 8B). These results indicate that the presence of extracellular metals may also impair, besides the uptake of physiological compounds or cationic drugs, the non-quantal release of acetylcholine, thus impairing its anti-inflammatory action.

Indeed, besides its well-known role as a neurotransmitter, acetylcholine plays pleiotropic functions in non-neuronal tissues, including airways, the intestine, skin, heart, skeletal muscle, placenta, urogenital tract, pancreas islets, and others [29,53,54]. In these districts, acetylcholine is involved in modulating cell proliferation and differentiation, cell–cell communication, and inflammation. In good agreement, the link between OCTN1 and inflammatory diseases has been demonstrated for a long time [55,56]. In good agreement, the gene encoding for OCTN1 maps in the IBD 5 (Inflammatory Bowel Disease) locus on chromosome 5, which has been linked to susceptibility to Crohn's disease, ulcerative colitis, and rheumatoid arthritis; moreover, a natural mutation of OCTN1, namely L503F, is found in Crohn's disease patients. The mutant protein shows an impaired ability to release acetylcholine [57], the impaired uptake of carnitine [24] and ergothioneine, and a fungi metabolite with an antioxidant role [58,59]. It is noteworthy that the presence of chronic inflammation is nowadays considered one of the main drivers for cell senescence and ageing,

further highlighting the importance of the molecules and macromolecules responsible for handling oxidative stress in cells, which could also be induced by heavy metals.

2.6. OCTN1 as a Meeting Point Between Humans and Fish

Finally, in a broader environmental context, it has to be highlighted that the orthologue *Oncorhynchus mykiss* has 6 Cys residues conserved with OCTN1 (Figure 9).

```
S22A4_HUMAN         1 --MRDYDEVIAFLGEWGPFQRLIFFLLSASIIPNGFNGMSVVFLAGTPEH     48
                        ...|::..||||||||||:::||||..|||||||.|||:||:..||.|
XP_021473178.       1 MTENGYEDNTAFLGEWGPFQQMVFFLLCLSIIPNGFTGMSIVFIGDTPSH     50
S22A4_HUMAN        49 RCRVPDAANLSSAWRNNSVPLRLRDGREVPHSCSRYRLATIANFSALGLE     98
                      .|.:|..||::..|||:|:||....|..|...|:||:|..|.:||..|..
XP_021473178.      51 HCLIPANANITGEWRNHSIPLEEESGTVVLSKCARYKLDVIKSFSERGYV    100
S22A4_HUMAN        99 PGRDVDLGQLEQESCLDGWEFSQDVYLSTVVTEWNLVCEDNWKVPLTTSL    148
                      ||.||::.:::.|:||||||:.:..|:||:|:||:|||.|.||.|||:|:
XP_021473178.     101 PGIDVNVSEIQHETCLDGWEYDRGTYISTIVSEWDLVCADKWKNPLTSSV    150
S22A4_HUMAN       149 FFVGVLLGSFVSGQLSDRFGRKNVLFATMAVQTGFSFLQIFSISWEMFTV    198
                      ||.|||.|||:||||||||||:||||||.|||.|:|:|:||.:|.:|..
XP_021473178.     151 FFCGVLTGSFLSGQLSDRFGRKHVLFATMGVQTVFTFIQVFSPTWVVFCA    200
S22A4_HUMAN       199 LFVIVGMGQISNYVVAFILGTEILGKSVRIIFSTLGVCTFFAVGYMLLPL    248
                      ||.:||||||||||.||:||||||..|.|||:||||||.|:.:||.|||.
XP_021473178.     201 LFFVVGMGQISNYVAAFVLGTEILSPSKRIIYSTLGVCMFYTIGYTLLPA    250
S22A4_HUMAN       249 FAYFIRDWRMLLLALTVPGVLCVPLWWFIPESPRWLISQRRFREAEDIIQ    298
                      .|:||||||||:||||:||.|.||.||||||||||||:||.|..|||.|::
XP_021473178.     251 VAFFIRDWRMLMLALTLPGFLYVPFWWFIPESPRWLLSQGRVEEAEAILR    300
S22A4_HUMAN       299 KAAKMNNIAVPAVIFDSVEELNPLKQQKAF---ILDLFRTRNIAIMTIMS    345
                      .||:.|.:..|.|||..| :|.| |..|..   |.||.|:.||..::|..
XP_021473178.     301 DAARRNRVTAPEVIFQPV-QLEP-KAGKLVAHNICDLVRSSNIRWVSITL    348
S22A4_HUMAN       346 LLLWMLTSVGYFALSLDAPNLHGDAYLNCFLSALIEIPAYITAWLLLRTL    395
                      .|:|.:.|:|||||||:..||.|.:|||||||.||:|||..|||:.|..
XP_021473178.     349 WLVWTILSIGYFALSLNTSNLAGSSYLNCFLSAAIEVPAYTMAWLMFRCC    398
S22A4_HUMAN       396 PRRYIIAAVLFWGGGVLLFIQLVPVDYYFLSIGLVMLGKFGITSAFSMLY    445
                      |||..:.:.||.||.|||.|.|:|.:...:|..|.|||||:|:||||::|
XP_021473178.     399 PRRLCLFSTLFLGGVVLLCINLIPPNLSSVSTALEMLGKFGVTAAFSIVY    448
S22A4_HUMAN       446 VFTAELYPTLVRNMAVGVTSTASRVGSIIAPYFVYLGAYNRMLPYIVMGS    495
                      .:|||||||:|||.|:|..|.|||||||.||||:|||.|::.||||:|||
XP_021473178.     449 AYTAELYPTVVRNTAIGACSMASRVGSISAPYFIYLGGYSKSLPYILMGS    498
S22A4_HUMAN       496 LTVLIGILTLFFPESLGMTLPETLEQMQKVKWF--RSGKKTRDSMETEEN    543
                      ||.|.|:|:|..|||..|.||:|:..||....:  ||..|...|...|:.
XP_021473178.     499 LTALSGLLSLLLPESHRMPLPDTITHMQTFPGWKKRSVYKLSQSTGEEDV    548
S22A4_HUMAN       544 PKVLITAF    551
                      ...|
XP_021473178.     549 SDTL----    552
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Figure 9. Sequence alignment between human OCTN1 and a possible ergothioneine transporter found in *Oncorhynchus mykiss*. The conserved cysteine residues are highlighted in green. The sequences were taken from UniProt, and the alignment was performed with EMBOSS Needle.

This suggests a common inhibitory mechanism among fish and human isoforms of OCTN1, further demonstrating the wide effects of heavy metals in different species by interacting with a similar target.

3. Materials and Methods

3.1. Materials

1,4 Dithioerythritol (DTE), n-dodecyl D-β maltoside (DDM), N-Lauroylsarkosine sodium salt (Sarkosyl), Amberlite XAD-4, Octaethylene glycol monododecyl ether ($C_{12}E_8$), BIS-Tris, Sephadex G-75, L-α-Phosphatidylcholine, Cholesterol, Imidazole, Tetramethylammonium chloride (TEA), Acetylcholine chloride (Ach), Chromium (II) chloride ($CrCl_2$), Zinc chloride ($ZnCl_2$), L-Cystein, Mercury (II) chloride ($HgCl_2$), Lead nitrate ($Pb(NO_3)_2$), Cadmium chloride ($CdCl_2$), Hemicholinium-3, Adenosine-5′-Triphosphate disodium salt, and His-select resin were purchased from Merck Life Science (Milan, Italy); Acetylcholine iodide [acetyl-^{3}H]-Acetylcholine was obtained from Perkin-Elmer; [ethyl-1-^{14}C]-TEA was obtained from American Radiolabeled Chemicals; and PD-10 Desalting Column was obtained from GE Healthcare UK LTD. All the other reagents were of analytical grade.

3.2. Over-Expression of OCTN1 WT and C-Less Mutant

The over-expression of OCTN1 wild type (WT) and C-less mutant proteins, as previously described in [17], has been performed in *E. coli* Rosetta(DE3)pLysS, as described in [60,61].

3.3. Purification of WT and Mutant OCTN1 Transporter

The WT (Figure 10A) and C-less (Figure 10B) OCTN1 proteins were purified as previously described [23] with some modifications. In brief, the insoluble fraction of 4 mL (OCTN1-WT) or 20 mL (OCTN1-C-less) of cell lysate was washed with 2 mL of Tris/HCl 0.1 M (pH 8.0) and centrifuged at 12,000× g for 5 min at 4 °C. The pellet was solubilized with 100 µL of 0.1 M DTE, 54 µL of 10% sarkosyl, and 400 µL of 8 M Urea. Then, 546 µL of a buffer containing 10% glycerol, 200 mM NaCl, 20 mM Tris/HCl (pH 8.0), and 0.1% sarkosyl were added. The resulting mixture was centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant was applied onto a column filled with His-select Ni^{2+}-chelating affinity gel (0.7 cm diameter, 5.2 cm height) pre-conditioned with 8 mL of a buffer containing 10% glycerol, 0.1% sarkosyl, 200 mM NaCl, and 20 mM Tris/HCl (pH 8.0). After 1 h of incubation at 4 °C, the elution was performed with 5 mL of a buffer containing 10% glycerol, 200 mM NaCl, 20 mM Tris/HCl (pH 8.0), 0.05% DDM, and 1 mM DTE, and then 3 mL of the same buffer containing 10 mM imidazole. OCTN1 was eluted with 4 mL of 200 mM imidazole added with 0.05% DDM, 1 mM DTE, 10% glycerol, and 20 mM Tris/HCl (pH 8.0). The eluate (2 mL) containing OCTN1 was applied onto a Desalting Column (PD-10), previously equilibrated with 25 mL of a solution composed of 0.05% DDM, 1 mM DTE, and 10% glycerol, 20 mM Tris/HCl (pH 8.0). The elution of OCTN1 was obtained by applying 3.5 mL of the same solution described above. The first 1.5 mL was discarded, and the following 2 mL, containing OCTN1, was collected and used for reconstitution in proteoliposomes.

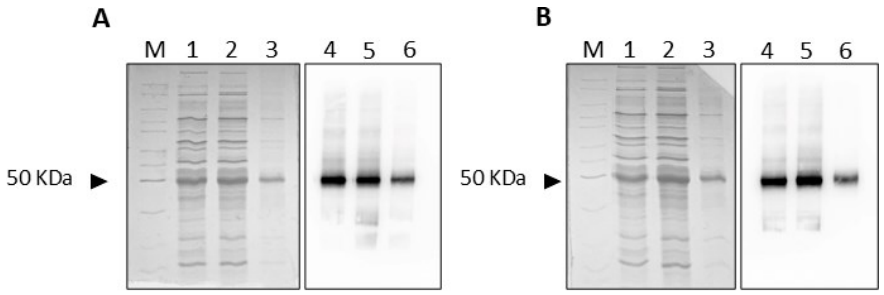

Figure 10. SDS-PAGE and Western blot analysis. In (**A**), the purified WT-OCTN1 is shown, and in (**B**), the purified C-less OCTN1 is shown. Lanes 1 and 4: cell lysates of the over-expressed proteins. Lanes 2 and 5: supernatant of solubilized proteins before purification. Lanes 3 and 6: purified proteins, as described in Section 3. Lane M: molecular mass marker (Thermo Fisher Scientific, Milan Italy—product code: 26614). Samples were separated by SDS–PAGE and stained using Coomassie Blue (**Left**) or detected by using Anti-His antibody in the Western blotting procedure (**Right**).

3.4. Liposome Preparation

To remove calcium phosphate from phospholipids, 3 mM EDTA was added to 10% egg yolk phospholipids and incubated for 15 min at room temperature. Then, chloroform was added in a 1:1 ratio with phospholipids, and the solution was centrifuged for 15 min at 12,000× *g*, at 4° C, using a fixed angle rotor. The supernatant containing clean phospholipids in chloroform was evaporated by a rotavapor at 40° C. Then, 25 mg of cholesterol was added to 100 mg phospholipids and dissolved with chloroform. After incubation, under rotatory stirring (30 °C 15 min 750 rpm), the solution was dried using a rotavapor. The lipid film was resuspended in water (10% final concentration), and single-bilayer liposomes were prepared by two sonication cycles of 1 min (1 pulse ON and 1 pulse OFF, 40 W) with a Vibracell VCX-130 sonifier (VWR, Milan, Italy) [62].

3.5. Reconstitution of OCTN1 Transporter into Liposomes

After desalting, the protein was reconstituted in liposomes. The composition of the initial mixture used for reconstitution was as follows: 50 µg of protein (or desalt buffer in liposomes without the incorporated protein), 80 µL of 10% $C_{12}E_8$, 120 µL of 10% egg yolk phospholipids with 25% cholesterol in the form of sonicated liposomes, 16 mM ATP disodium salt, and 5 mM BisTris/HCl (pH 7.5) in a final volume of 700 µL. The purified OCTN1 (WT or C-less mutant) was inserted in the liposomal membrane using the batch-wise technique. This procedure used 0.5 g of Amberlite XAD-4 to remove the detergent from mixed micelles consisting of proteins, phospholipids and detergent at 23 °C for 40 min at 1200 rpm, as previously described [63].

3.6. Transport Measurements

Proteoliposomes and liposomes (600 µL) were applied onto a Sephadex G-75 (Merck Life Science, Milan, Italy) column (0.7 cm diameter × 15 cm height) pre-equilibrated with 5 mM BIS-Tris/HCl (pH 8.0) and eluted with 600 µL of the same buffer. Samples of 100 µL each were prepared from the 600 µL pool. The transport was initiated by [^{14}C]-TEA or [^{3}H]-Ach at the concentration of 0.1 mM to the proteoliposome samples and stopped after 60 min according to the stop-inhibitor method [57]. In the blank samples, the inhibitor (1 mM Hemicholinium-3) was added at time zero. After the transport time, each sample was applied onto a Sephadex G-75 column (0.6 cm diameter × 8 cm height) and pre-equilibrated with 50 mM NaCl to remove the radioactivity not taken up. Proteoliposomes were eluted with 50 mM NaCl (1 mL), added to 3 mL Pico-Fluor Plus, vortexed, and counted. The radioactivity in the blank samples was used to subtract background radioactivity from the protein-associated one.

For efflux measurements, proteoliposomes (600 µL) were preloaded with radioactive 0.1 mM [^{3}H]-Ach for 120 min. External compounds were removed by another passage of the proteoliposomes through Sephadex G-75. The efflux measurement was started by adding 100 µM of the indicated heavy metals. The transport was stopped after 60 min by using the stop-inhibitor method, as previously reported. The percentage of residual activity with respect to the control was reported.

3.7. Other Methods

The amount of purified protein was estimated from stain-free 12% SDS–PAGE gels using the Chemidoc imaging system equipped with Image lab software (Bio-Rad Laboratories srl, Milan, Italy) in absolute quantification using BSA as a standard. Recombinant OCTN1 WT and C-less proteins were immuno-detected using the Monoclonal Anti-polyhistidine–Peroxidase antibody 1:10,000 after 1 h incubation at room temperature. The reaction was detected by Electro Chemi Luminescence (ECL). The human OCTN1 homology model was obtained using the 3D structure of OCT3 (PDB ID: 7ZH0) by Modeller 10.2 [23,64]. This structure was analyzed by the MIB2 server to predict a potential Cd^{2+} binding site [65].

Sequence alignment was performed with EMBOSS Needle and Protein sequences, which were taken from the GenBank database [66,67].

Illustration sketches were prepared using https://www.biorender.com/ (accessed on 22 October 2024).

3.8. Data Analysis

All experimental data were derived from the mean of three independent experiments, and results were expressed as means ± SD. IC_{50} values were derived from data fitting the IC_{50} equation using Grafit v 5.0.13 software (Erithacus Software (v 5.0.13), East Grinstead, UK).

Comparisons between the two groups were performed with the two-tailed Student's unpaired *t*-test for * $p < 0.05$ and ** $p < 0.01$.

4. Conclusions

In this work, the human transporter OCTN1 was exploited as a benchmark for deciphering the molecular mechanism of interactions with heavy metals potentially released by aquatic microplastics. It has to be stressed that the collected results may have great relevance in the context of environmental health because OCTN1 emerged with vertebrates and, hence, orthologues are present in humans and fishes. Due to the biomagnification phenomena, the effects of heavy metals could not appear in a short time as acute effects and could be only appreciable after a long period of chronic exposure. Therefore, it is of paramount importance to understand the action and mechanisms of heavy metals to prevent or treat the consequences of long-term exposure. It is noteworthy that the mechanisms described for OCTN1 may be common to other biological targets in humans and in other living organisms, further enlarging the collection of negative effects ascribed to heavy metals exposure. As an example, within the SLC22 family, OCT members have conserved cysteine residues in the same position of the metal-binding site of OCTN1 [68]. In this scenario, it can be hypothesized as a beneficial effect to use some antioxidants, such as cysteine or acetyl-cysteine, in individuals subjected to heavy metal exposure. Intriguingly, the negative effects of heavy metals can be worsened by interactions with microplastics and nanoplastics, whose risks for biota are already well known [4]. Taken together, the described data highlight two key and complementary needs in this research field: (i) experimental tools devoted to studying the effects of pollutants related to microplastic spread and (ii) improvements in the identification of novel targets across different species to reduce the risks and face future challenges.

Author Contributions: Conceptualization, L.P., M.S. and C.I.; methodology, L.S.B., M.G., L.P. and L.C.; software, L.C., R.S. and L.S.B.; validation, L.S.B., L.P., M.S., M.F.L.R. and C.I.; formal analysis, L.S.B., R.S., L.C. and C.I.; investigation, L.S.B., R.S., L.P., M.S., L.C. and M.G.; resources, M.F.L.R. and C.I.; data curation, L.S.B. and R.S.; writing—original draft preparation, L.S.B., M.S. and C.I.; writing—review and editing, L.S.B., L.P., R.S., M.S., L.C. and C.I.; visualization, L.S.B., R.S. and L.C.; supervision, C.I.; project administration, C.I.; funding acquisition, L.P. and M.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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CHAPTER 3:

Conclusions

Plastic has made a significant contribution to the growth of the global economy as well as major social benefits, in particular the commercial boom of plastic occurred after the end of the Second World War. Since then, plastic has been spread everywhere, in household appliances, toys, clothes, cars, food containers, machinery and means for agriculture and commerce, home furnishings and kitchen utensils. It is so fundamental today in our daily lives that we take it for granted without realizing its pervasiveness. The wide use of plastic is due not only to its extreme versatility, but also to its durability and low cost in terms of processing compared to other materials, such as glass and metal. The global production of plastic materials in the last seventy years has increased significantly, going from 1.5 million tons in 1950 to 359 million tons in 2020. The big problem with plastic, however, is its disposal, due to the very slow degradation that characterizes this material. Before 1980, 100% of plastic waste was landfilled or dispersed in the environment. Its persistence in ecosystems and worldwide distribution makes plastic pollution one of the major global environmental issues of the 21st century. Once in the environment, depending on exposure to solar radiation, mechanical forces or interactions with organisms, plastic can slowly degrade; then, fragmentation into smaller particles commonly known as microplastics (MP) and nanoplastics (NP) can occur. Microplastics have been observed virtually everywhere, from the poles to the equator; in fact, they have been reported in considerable concentrations even in remote sites such as Antarctica. Most of the plastics produced and released into the environment, through the course of rivers, reach the seas and oceans where, thanks to the action of the winds, 5 large accumulation zones of plastic waste have formed. The Mediterranean Sea has been proposed as the sixth largest accumulation zone of marine litter. The

Mediterranean basin, which by extension represents 1% of the planet's seas, contains 7% of all microplastics that pollute the oceans. This immense collection of floating plastic is probably due to the hydrodynamics of the Mediterranean Sea, a semi-closed convective basin and it has been demonstrated that this characteristic hydrodynamic pattern allows to retain plastic waste and at the same time allows the entry of other plastic waste from the Atlantic Ocean. The polymeric composition of the fragments influences their separation and distribution on the water surface, in the water column, on the coasts and on the seabed. In fact, based on the density, the polymers remain for more or less time on the water surface, therefore they will have different interactions with the biota. In this study we collected and analyzed microplastics (MP) from 6 marine areas along the Calabrian coast. The results suggest that there is no clear prevalence of MP between the Tyrrhenian and Ionian Seas, however in the areas of Cetraro and Gioia Tauro higher density values were highlighted, probably due to the presence of two important commercial ports. White/transparent polyethylene fragments with a size between 1 and 2 mm are the main physical and chemical characteristics of the analyzed samples. This leads us to think that most of these wastes come from common plastic bags that we use daily. In addition, we also observed the presence of exogenous materials adsorption on the surface of the MPs by electron microscopy. The analysis of these materials revealed the presence of several elements, including heavy metals such as Pb, which come from the surrounding environment, demonstrating that the MPs can act as heavy metal carriers in the food chain. In fact, it must be considered that plastic fragments present in the sea can be ingested by marine organisms and, through the food chain, reach humans. The harmful effects of microplastics on living organisms can be classified

into two main categories: a) Physical effects, these concern size, shape and concentration; b) Chemical effects, related to the hazardous chemicals associated with micro- and nanoplastics. Indeed, it has been demonstrated that, during their stay in the ecosystem, microplastics can absorb and become vectors of various substances harmful to humans, including pathogenic microorganisms and parasites, drugs and antibiotics and heavy metals. Heavy metals, in particular, can be both absorbed from the external environment (extrinsic) and used in the production processes of the plastics themselves (intrinsic) in the form of colorants, stabilizers, plasticizers or adjuvants, in order to improve the qualities of plastic polymers. Although hazardous metal-based additives are no longer intentionally incorporated into contemporary plastics, at least in Europe, it is still possible that such additives are still used in some consumer goods available in the EU. Some of these metals such as copper (Cu), magnesium (Mg), and zinc (Zn) are essential for humans however, at high levels they interfere with metabolic reactions in organism systems and are toxic. While other heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), uranium (U) or arsenic (As) are not useful for living organisms and they are extremely toxic. Those just mentioned, in particular, are considered priority metals of great importance for public health. In this work we have demonstrated that Microplastics collected in areas of the Calabrian coasts carry heavy metals which, through the digestion process of marine organisms, can potentially reach humans via the food chain, thus representing a risk for human health. Procedures based on gastric simulation in fishes represent a valuable tool for understanding these processes. Release of Pb, Cd, Cr or Zn during simulation digestion in fish has been demonstrated. Percentages of metal released may become relevant as microplastic sizes reduce. In fact, the sampled plastics had

dimensions between 1 and 2 mm, however, we were also able to demonstrate that, as the size of the plastics decreases, the exposed surface increases and, consequently, the quantity of metals released. At the biological level, we have demonstrated how these toxic substances can interact with the human organism, and we have done so by demonstrating their ability to inhibit the cellular membrane transporter SLC22A4. The effects of metals on this particular protein occur thanks to binding interactions between xenobiotics and some cysteine residues present in the structure of OCTN1. This mechanism has been demonstrated in this thesis work through the study of the activity of the OCTN1 mutant for all cysteines: the protein, devoid of cysteines, is not inhibited by heavy metals. From this perspective, the negative effects on the human organism could be much broader if we consider the fact that other membrane proteins retain the same cysteine residues in the same binding positions of OCTN1; some examples are the OCT transporters, which are part of the same SLC22 superfamily as OCTN1. Furthermore, if we consider the phenomenon of biomagnification, the effects of metals could not manifest themselves in a short time, but after a long period of chronic exposure. Precisely for this reason it is extremely important to study these substances in depth and understand how to intervene in adverse circumstances. In this work, we have demonstrated the beneficial effect of some antioxidants such as cysteine or reducing agents such as DTE, capable of preventing the bond between heavy metals and OCTN1.

From an experimental point of view, with this work we have demonstrated how the use of combined techniques, such as mass spectrometry, bioinformatics and the experimental system of proteoliposomes, has proven extremely effective in understanding the amount of heavy metals that, from microplastics, can reach humans,

and the extent of damages induced inthe organism; finally, we were also able to identify some detoxifying substances in vitro. Even if the described approaches are widely used in different disciplines, this thesis work is characterized by a great interdisciplinarity that allowed describing an entire phenomenon from inert plastics, to environment, to organisms, to molecules, further highliting the need to carry out cross-sectional studies for improving human life and global health.

Despite the work carried out, the study of heavy metals, their effects on the environment and on humans is still in its infancy and requires greater efforts to fully understand the chemical, molecular and pharmaceutical mechanisms. This thesis work aims to be a starting point to demonstrate that humans are part of the ecosystem and that what damages the environment in which we live inevitably affects us. It is a starting point to understand how to cure the damage done because, if it is true that we cannot go back, it is also true that we can and must find a way to move forward, and research is the only possible way.

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