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Mussel watch program for microplastics in the Mediterranean sea: Identification of biomarkers of exposure using *Mytilus galloprovincialis*

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ABSTRACT

Microplastics (MPs) are ubiquitous pollutants that have also been detected in the aquatic ecosystems at high concentrations. The use of shellfish as bioindicators is widespread for assessing and monitoring the environmental quality in both freshwater and marine environments. On this path, biomarkers represent an effective tool in monitoring programs. This minireview would broaden the existing knowledge on biomarkers of MPs in the Mediterranean mussel *Mytilus galloprovincialis*. This species was selected as it is widely distributed across the Mediterranean Sea and used as a bioindicator to monitor the presence of MPs in the marine environment. The literature search returned only 11 studies, mainly related to oxidative stress biomarkers. Although certain biomarkers were explored to estimate the effects of MPs on *M. galloprovincialis*, a battery of standardized and validated biomarkers as well as the inclusion of new ones are needed in future studies to obtain more comparable and robust findings across the Mediterranean Sea.

1. Introduction

Plastic material is composed of long polymer chains and is used in many aspects of human life. According to its extremely favorable properties and worldwide use, its production has been continuously increasing since the 1950s (Zhang et al., 2021). Plastic is a general term for a material that can be formed into many different shapes and is mainly composed of carbon, hydrogen, oxygen, chloride, and silicon. In addition, there are many additives such as plasticizers, stabilizers, antioxidants, pigments, and flame retardants (Zhang et al., 2021) that change the properties of plastics and make them more durable, lightweight, and resistant to degradation (He et al., 2022; Manzoor et al., 2022). Plastic debris can be classified by size into mega- (>100 mm), macro- (100–20 mm), meso- (20–5 mm), micro- (5000–10 μm), and nano- (<100 nm) plastics (Renzi et al., 2018; Harris, 2020; Pignattelli et al., 2021a).

Microplastics (MPs) are considered new-generation pollutants with adverse effects on various environmental compartments such as the atmosphere, aquatic ecosystems and biological resources and soil ecosystems (Ma et al., 2020; Renzi et al., 2019; Renzi et al., 2020; Pignattelli et al., 2021b). Microplastics can be divided into primary and secondary, according to different sources (Liu et al., 2021). Primary MPs refer to plastics manufactured as such and found in cosmetics, detergents, beauty products, plastic beads, etc. Secondary MPs are plastics that originate from larger plastic parts and become MPs through various degradation processes (Liu et al., 2021). The degradation of secondary MPs can occur through different types of processes that can occur either independently or in collaboration. These processes are photooxidative degradation, thermal degradation, ozone degradation, catalytic degradation, mechanochemical degradation, and biodegradation (Manzoor et al., 2022). The toxicity of MPs is not only related to the toxics they release into the environment, but also to their size and shape; in

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particular, smaller MPs and nanoplastic particles are more toxic to organisms (Llorca et al., 2020). Higher toxicity in terms of size and shape is also attributed to MPs, due to their longer residence time in organisms (Ma et al., 2020).

Microplastics in aquatic environment are the result of improper waste disposal (Zhang et al., 2021); they account for 93 % of plastic waste in the oceans (Gedik and Eryaşar, 2020), entering the aquatic environment through a variety of pathways, primarily surface runoff, fishing, aquaculture, and other human activities (Pastorino et al., 2021). The fate of MPs in these ecosystems is influenced by ocean currents and hydrodynamic processes which potentially facilitate their transport to long-range distances and accumulation in high-mountain ecosystems, making them a global pollutant (He et al., 2022; Pastorino et al., 2022). In addition, the ubiquity of MPs can have adverse effects on the environment and biota, such as light scattering impairment, chemical alteration due to oxygen deprivation, accumulation, and biomagnification across different trophic levels (Manzoor et al., 2022). Microplastics are capable of absorbing and accumulating pollutants from air, soil, and seawater, such as persistent organic pollutants, or attaching to other biological surfaces and forming biofilms (Kinjo et al., 2019; He et al., 2022). The attraction of microorganisms and the formation of biofilms inevitably change the properties of MPs and add new variables, which in turn can lead to more toxic MPs for aquatic organisms and environment. Biofilms are believed to have good adsorption properties and accumulate heavy metals, antibiotics, and other toxic substances that can easily spread through the aquatic food web (Llorca et al., 2020; Piccardo et al., 2021; He et al., 2022). Many aquatic organisms are used as promising tools for biomonitoring programs and assessing the environmental quality. Throughout time and evolution, organisms have had to adapt to environmental changes and develop defense strategies and mechanisms to survive; when the organism's threshold is crossed, it ceases to exist (Gadzała-Kopciuch et al., 2004). These adaptive mechanisms are used to fathom the amount, type, and effects of pollutants and/or contaminants on organisms, environment, and the impact on human society (Box et al., 2007). Bioindicators and biomonitoring are closely related, as it is possible to obtain quantitative information about the state of health of the ecosystem and the environmental quality by using bioindicators (Parmar et al., 2016). For aquatic environments, bivalves as *Mytilus galloprovincialis* have been found to be the most suitable organisms for biomonitoring, becoming good bioindicators for their natural habitat (Li et al., 2019). Indeed, *M. galloprovincialis* has a high ecological and commercial relevance in the Mediterranean Sea, where MPs contamination is also of particular concern (Grbin et al., 2019). Moreover, *M. galloprovincialis* was chosen for this minireview because it has been proposed by the International Council for the Exploration of the Sea to monitor MP pollution in the marine environment (Bråte et al., 2018).

Biomarkers are a useful tool for assessing and identifying stress and toxic effects in organisms, as they are normally produced during the life cycle of any organism. Their use in risk assessments is currently recommended to characterize the sub-lethal alterations and toxicological pathways at the root of major environmental impacts in aquatic ecosystems. They can be divided into specific and non-specific biomarkers related to the stress or pollutant/contaminant typology (Sarkar et al., 2006). A good biomarker must be measurable, have significant differentiation between noise and signa, and be responsive to the changes in the organism and its conditions (Aronson and Ferner, 2017). Mussel biomarkers can be a range of compounds depending on tissue, time of exposure and many other factors. They are considered sensitive and could indicate early warning signals of environmental quality (González-Fernández et al., 2015). Biological responses are defined as changes that occur in different domains, from cellular to physiological and can be studied in different species such as body fluids, tissues, or organs (González-Fernández et al., 2015; Aronson and Ferner, 2017). This minireview provides a general overview of the status and effects of MPs on aquatic ecosystems, illustrating the usefulness of *M. galloprovincialis* as a good bioindicator for the marine ecosystems. In particular, the main aim is to highlight and relate to each other the biomarkers used to assess the impact of MPs on the bivalve *Mytilus galloprovincialis*. To do this, a literature search was performed in the Scopus (https://www.scopus.com/search/) and Web of Science (https://clarivate.com/) databases using the keywords: "*Mytilus galloprovincialis*" OR "bivalve" AND 'biomarkers' AND "microplastics" across all publication years until 5 June 2022.

2. Plastics

In the context of sedimentology, to better understand plastic pollution, its effects, and its migration or transport through different media, numerous studies are being conducted that attempt to combine MP particles with normal sediment particles such as clay and silt. Once settled in the environment, all plastic particles eventually settle to the seafloor, either through degradation, density differences, or a process known as biofouling, even via animal excretions (Harris, 2020; Llorca et al., 2020). Numerous considerations should be made before sampling an area for MPs in organisms or sediments. The sampled area must be identified as a net sink for sediment and MPs or net erosion for sediment and MPs, or it may be in equilibrium (Harris, 2020). This information could provide insight into the occurrence of MPs in the sediment and water column and provide insight into the extent of pollution to organisms, from sediment feeders up the trophic chain. If the above ideas are implemented over time (monitoring), this could provide clues to the improvement or degradation of the organism/area being studied for the past and future. Due to varying locations, pollution levels, and anthropogenic activities, sediment is saturated with MPs to varying degrees, ranging from 3 particles/kg to 11,600 particles/kg (Harris, 2020). Studies near Salina Island in the Aeolian Archipelago found values of 99–431 particles/kg dry weight (Renzi et al., 2018). Studies in the Barents Sea indicate that bivalves can accumulate significantly greater amounts of MPs than their environment; it is reported that bivalves had 3.7×10^4 elements/kg dry weight compared to sediment and seawater, which had 48 elements/kg dry weight and 27 elements/L, respectively (Li et al., 2019). One thing that is agreed upon by almost all researchers is that the predominant form of MPs particles is fibers (Li et al., 2019; Harris, 2020).

2.1. *Plastic degradation*

As mentioned earlier, MPs can be deformed or degraded by a variety of principles, from biodegradation to hydrolysis (Sharma and Chatterjee, 2017; Bhatt et al., 2021). Although plastic is resistant to degradation and long-lived in the environment, it degrades slowly over the years, during which time the plastic particles can change, release toxic substances, and alter the structure of the polymers (Zhang et al., 2021).

2.2. *Photodegradation of plastic*

The driving force behind the decomposition of plastics is light: UV light and visible light initiate the processes to reform various end groups of the polymer (Manzoor et al., 2022). Depending on the purity of the plastic, different degradation rates occur; typical for UV degradation is the reaction of chromophores with oxygen to form radicals (Zhang et al., 2021).

2.3. *Thermal degradation of plastic*

Like photodegradation, thermal degradation of plastics begins with the help of photodegradation, with it and oxygen it begins to weather, and radicals are released. Thermal degradation lasts only if the necessary energy is available for the reactions. This degradation depends on pressure, temperature, or energy differences and the substrate or degradation area, as it varies in water sediment or on the beach (Zhang et al., 202; Manzoor et al., 2022).

2.4. Biodegradation of plastic

This form of degradation occurs through the presence of microorganisms, either at the molecular, microscopic, or macroscopic level. It is assumed that the by-products produced by this degradation are not toxic (Manzoor et al., 2022). The process of biodegradation can also be considered biofouling, which can have negative effects on the ecosystem and organisms, as mentioned earlier (He et al., 2022). Many other processes are involved in plastic degradation that can interact and have positive or negative consequences for MP degradation or the environment and its biota (Fastelli et al., 2016; Ma et al., 2020; Zhang et al., 2021). Once plastic is discarded, degradation processes begin in different ways that can increase the number of particles in the medium. Studies show that heavily populated areas and areas with high industrial production correlate with more MP particles in the environment and in organisms. Fig. 1 shows different mechanisms of plastic degradation and their partitioning (Sharma and Chatterjee, 2017).

A holistic understanding of these processes can shed light on future MP pollution problems and ways to address them. Depending on the type of degradation and the process it undergoes, different methods can be used to determine various parameters and increase knowledge about the fate of MPs in the environment and organisms. These different methods are listed in Table 1.

2.5. Biofilm or biofouling

Biofouling is a process in which microorganisms attach to the substrate and/or MP particles and form communities. The most common biofilms are bacteria, algae, and fungi (He et al., 2022). Biofilm formation usually occurs according to the following processes: formation, reversible or irreversible adhesion, depending on the MP and the type of "glue" used by the microorganisms, which can be either covalent, ionic, hydrogen bonded, or formed by proteins, and production of biofilm formation and spreading of the organisms, as shown in Fig. 2 (He et al., 2022). Due to the different density of plastics, MP particles can migrate vertically through the water column with the help of the biofilm and eventually accumulate in the sediment (Zhang et al., 2021).

2.6. Plastic toxicity

Microplastics enter the marine environment primarily through human activities (i.e., aquaculture, fishing, tourism, industrial and domestic wastewater systems) and their distribution is highly variable (Duis and Coors, 2016). A positive relationship has been established between the increase in human population density and the abundance of MPs, which could lead to an increase in plastic debris accumulating in the marine environment (Shahul Hamid et al., 2018). Physicochemical properties of MPs (i.e., size, chemical composition), hydrodynamic factors, and environmental characteristics (i.e., water currents, turbidity, temperature, wind) can influence their transport dynamics and, as a result, their distribution and accumulation in various marine areas (Ding et al., 2021).

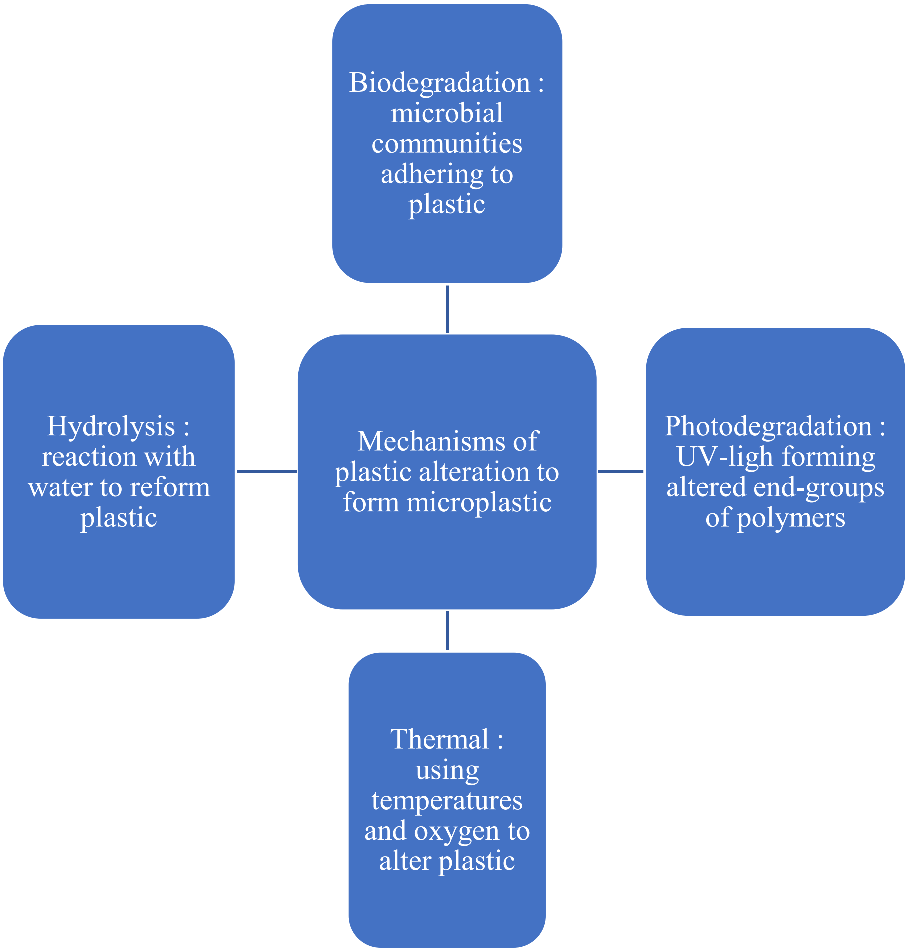

Fig. 1. Degradation of plastic particles by different mechanisms (Sharma and Chatterjee, 2017, modified).

Table 1
Methods for identification of plastic features. FTIR: Fourier transform infrared spectroscopy; XRF: X-ray fluorescence spectrometry; GC/MS: gas chromatography coupled with mass spectrometry; XRD: X-ray diffraction; DSC: differential scanning calorimetry; TGA: thermogravimetric analysis; TOC: total organic carbon; LSC: liquid scintillation counting; GC: gas chromatography.

Type	Technique	Method	Reference
Chemical composition	FTIR	FTIR measures the absolute value of IR-radiation absorbed by the microplastic particle	Gedik and Eryaşar, 2020
	Raman	Sample reflects radiation at different frequencies which Raman spectroscopy can measure	Vandermeersch et al., 2015
	XRF	Samples when exposed to outside energy their individual atoms emit X-ray photons which XRF can quantify	Zhang et al., 2021
	GC/MS	Degrading microplastic samples release additives which can be analysed quantitatively and qualitatively by comparing them with standard concentrations	Li et al., 2019; Liu et al., 2021
Shape and texture	Spectrophotometer	Spectrophotometer and Colorimeter are used to measure colour and Haze amount of scatter light that is reflected from the plastic particle	Sharma and Chatterjee, 2017
	Colorimeter		Zhang et al., 2021
	Microscope	Microscope use visible light and lenses to magnify the sample and to identify its shape, texture and colour but has limited range	Harris, 2020
Physicochemical properties	XRD	Analysing crystallinity in a sample XRD is used to measure order between molecules	Zhang et al., 2021
	DSC	DSC can analyse crystallinity by measuring heat required to melt the plastic particle	Zhang et al., 2021
	Potentiometric titration	Plastic can have different charges when exposed to time and weathering, with Potentiometric titration we can measure surface charge	Zhang et al., 2021
	TGA	TGA measures thermal stability of a plastic particle, it is used to monitor weight in relation of time and temperature changes	Liu et al., 2021
Mechanical properties	Instron universal materials testing machine	This technique follows standard methods by ASTM and ISO and is used to determine tensile properties, sheer strength, and other mechanical properties	Zhang et al., 2021
Mineralization	TOC analyser	TOC analyser can analyse carbon concentration by combusting the sample and measuring CO_2 released	Zhang et al., 2021
	LSC	LSC measures radioactive changes in sample when it is degrading	Zhang et al., 2021
	GC	GC can be used to measure CO_2 and CH_4 with and IR detector released from the sample	Liu et al., 2021
Microplastic identification	Pyrolysis	Pyrolysis is used to degrade microplastic particles with high temperature in inert atmosphere	Rebelein et al., 2021

There is little agreement on the biological consequences of MP pollution. Indeed, while large plastic particles (i.e., *meso* and macroplastics) can cause easily visible effects at the organism level, such as suffocation, entanglement, or intestinal blockage (O'Donovan et al., 2018), the direct and indirect physiological effects of small size particles (micro- and nano-plastics) on aquatic animals mainly remain unknown. Several studies have shown that once ingested, MPs can accumulate within the aquatic organisms and be translocated between body tissues, or that they can be eliminated for excretion or egestion via pseudofaeces (Graham et al., 2019). The accumulated MPs can have a variety of negative effects on the organism that ingests them. Internal and/or external injuries, mechanical damages, blockages of the gut tract resulting in pseudo-satiety sensation and physiological stress, alteration of feeding and retardation of growth, reduction in fertility, fecundity, and progeny survival rate are some of these (Graham et al., 2019). Furthermore, MPs can be used to introduce toxic compounds into marine organisms (Cole et al., 2011). Dark-colored, or black MP particles are reported to tend to adsorb PCBs (polychlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons) much more strongly (Ma et al., 2020). Chemical additives (i.e., flame retardants, nanoparticles) added to plastic products during manufacturing processes to improve their final properties, as well as environmental contaminants (i.e., persistent organic pollutants, hydrocarbons and heavy metals) adsorbing on their surface by the marine environment can cause changes in metabolic and reproductive activity, a decrease in immune response, oxidative stress, cellular or sub-cellular toxicity and inflammation in marine biota (Padervand et al., 2020). Several additives are not covalently bonded to plastic polymers; therefore, they can migrate to the surface of the material and release chemicals into the environment (Do et al., 2022). In effluents from wastewater treatment plants, surface waters, and marine waters, additives have been detected (Do et al., 2022). Among them, bisphenol A and phthalates are two commonly plastic additives that are recognized as probable endocrine disruptors

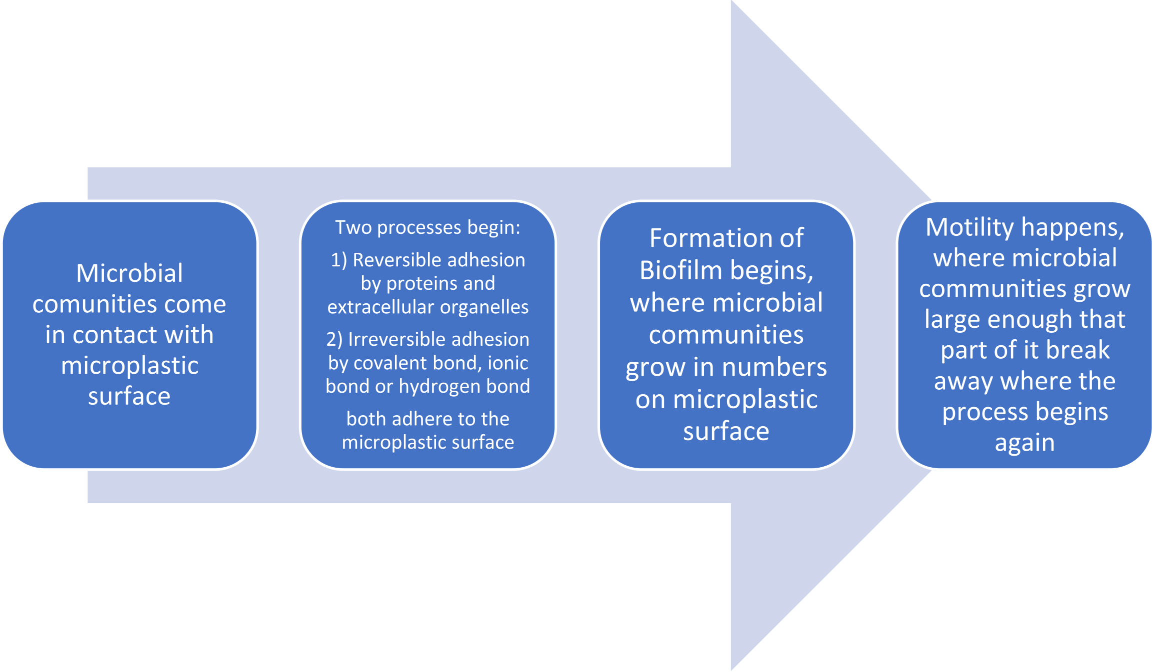

Fig. 2. Mechanisms of biofilm formation.

due to their capacity to interfere with hormone regulation in both human and wildlife (Hermabessiere et al., 2017). Finally, through bioaccumulation and biomagnification processes, MPs can be transferred to the food chain alone or in combination with other pollutants, with potential risks for human health (Lehel and Murphy, 2021). The accumulation of these particles could have effects on chromosomes and lead to infertility, obesity, and cancer (Sharma and Chatterjee, 2017).

Because MP particles are similar in size and shape to the natural prey of predators and filter feeders, mussels are thought to be most affected by the toxicity of MP pollution (Kinjo et al., 2019). Because these organisms constantly filtering a large volume of seawater (about 50 mL of seawater is pumped per minute [Li et al., 2019]), they can also consume MPs that are similar in size to their natural prey. For example, Paul-Pont et al. (2016) showed an increase in hemocyte mortality in *Mytilus* spp. Exposed to polystyrene MPs. Cole et al. (2020) reported that MP particles can penetrate fluids and tissues of *Mytilus* spp., which in turn translates into an immune response and the induction of cytotoxicity. Disorders in osmoregulation, energy and protein metabolism, and oxidative stress were found in *M. galloprovincialis* exposed to polystyrene MPs.

3. *Mytilus galloprovincialis* as bioindicator of pollution

Aquatic mollusks represent optimal and common model organisms for ecotoxicological risk assessment and ecosystem monitoring because they meet all the characteristics normally required, such as abundance, limited mobility, and longest (>1 year) possible life span (Jong et al., 2022). Bivalves are among the largest taxa in the animal kingdom and are cosmopolitan in temperate intertidal zones (Cappello et al., 2018). The marine bivalve *Mytilus galloprovincialis* is a popular shellfish and a good bioindicator for a variety of toxicological and environmental studies. Properties as sensitivity to pollutants, wide geographic distribution and sedentary lifestyle, ease of sampling make organisms efficient tools for assessing the environmental quality. Mussels are sessile organisms and static filter-feeder (Cappello et al., 2018) and represent a biological resource of high economic importance, but they are continuously exposed to many pollutants, due to their feeding activity. Intake of pollutants, through transfer and accumulation in tissues (Box et al., 2007), pose a health risk to them, resulting in an imbalance at the whole food chain, including humans (Li et al., 2015). Additionally, their hard shell and ease of handling in the laboratory minimize the risk of contamination during experiments (Li et al., 2019), making them excellent bioindicators. Furthermore, their low metabolic detoxification (Bolognesi et al., 2004) and close correlation with their habitat make mussels suitable for biomonitoring studies related to many toxicants, as well as MPs (Li et al., 2019). Ward et al. (2019) indicate that bivalves are selective particle eater, and that particle size, shape and surface characteristics affect rejection or ingestion of food and MPs. *Mytilus galloprovincialis* can select particles at the palps (Ward et al., 2019), which is important when laboratory experiments are performing with specific shapes and sizes of MPs, as they are very different and more complex from natural habitat, in terms of type, shape, size and composition, thought to be related to the degree of toxicity they cause (Zhang et al., 2021). An important observation that relates to the natural environment and the laboratory is the saturation of gills and labial palps, as this is likely to influence the uptake or rejection of particles such as MPs (Ward et al., 2019). Another selection of MP particles that should be considered is the width of the mouth of bivalves, which can be quite wide but still has an upper limit reported to be in the range of 600–900 μm (Ward et al., 2019).

4. Biomarkers of microplastics in *mytilus galloprovincialis*

Biomarkers are a variety of biological indicators that are measurable at the quantitative level and indicate changes at the cellular, biochemical, molecular, and physiological levels (Lionetto et al., 2019). These indicators can be measured at the cellular level, body fluid level, tissue level or organ level. They can also provide us with information about the exposure or effect of xenobiotics (Gonzalez-Fernandez et al., 2015). In particular, the use of mussel gills for biochemical and biomonitoring studies could be useful because they are more sensitive to toxic pollutants and MP particles and increase the concentration of antioxidants to prevent oxidative damage (Bolognesi et al., 2004; Capó et al., 2021b).

When an organism is exposed to stress or external pressures some responses are triggered, such as the production of reactive oxygen species (ROS), which in turn trigger the production of antioxidant compounds, both enzymatic and non-enzymatic, to prevent oxidative damage (Lam and Gray 2003; Pastorino et al., 2020). Many pollutants as MPs can disrupt redox homeostasis, forcing the antioxidant system to work overtime to restore balance (Trestrail et al., 2020). Conceptually, the interaction between pollutants and the antioxidant system can be divided into three stages. An oxidative challenge (stage I) can be posed by a pollutant, necessitating an energetically demanding response from the antioxidant system (stage II). If the organism's response is insufficient to deal with the oxidative challenge, it will suffer from oxidative stress (stage III) (Trestrail et al., 2020).

The first stage of pollutant-induced redox homeostasis disruption occurs when the pollutant creates an oxidative challenge in the tissues of the exposed animal by rapidly increasing ROS concentrations (Pastorino et al., 2020). This increase in ROS concentration can occur when a pollutant initiates a physiological pathway that generates ROS (Magara et al., 2021), or when it increases the organism's aerobic respiration rate and, as a result, the levels of by-product ROS (Elia et al., 2020). ROS concentrations can act as a biomarker in this early stage, indicating whether a pollutant posed an oxidative challenge. The response of the antioxidant system is involved in the second stage of redox homeostasis disruption. If the homeostatic antioxidant capacity is sufficient, the first-stage ROS concentration spike will be immediately neutralized, and redox homeostasis will not be disrupted. If the homeostatic antioxidant capacity is insufficient to counteract the excess ROS, energy will be expended to synthesize antioxidant enzymes and molecules, increasing the organism's antioxidant capacity (Lushchak, 2011). Redox homeostasis is restored when the antioxidant capacity is sufficient to neutralize the excess ROS. The second stage antioxidant response is associated with a plethora of redox biomarkers. Since antioxidant capacity is influenced by a wide range of enzymes and molecules (Halliwell, 2007), and new antioxidant molecules are constantly being discovered, measuring all the antioxidant system's molecules and enzymes is quite challenging. Thus, only antioxidant biomarkers involved in critical pathways are commonly assessed.

4.1. Results from literature search

Results from literature search in Scopus (https://www.scopus.com/search/) and Web of Science (https://clarivate.com/) databases retrieved only 11 studies on biomarkers of MPs in *M. galloprovincialis* (Table 2).

In Table 3 are listed the biomarkers commonly used to detect toxicity in *M. galloprovincialis* as well as their locations and functions.

Generally, almost all studies were performed in laboratory under

Table 2
Results from literature search (Scopus and Web of Science databases) on biomarkers of microplastics in *Mytilus galloprovincialis.* It is also indicated the type of study (E = experimental; F = field) and biomarker.

Reference	Study type	Biomarker
Abidli et al., 2021	E	Oxidative stress
Capó et al., 2021b	F	Oxidative stress
Capolupo et al., 2021a	E	Oxidative stress; histopathology
Capolupo et al., 2021b	E	Oxidative stress; histopathology
Capolupo et al., 2021c	E	Oxidative stress; histopathology; immunological parametres
Cappello et al., 2021	E	Metabolomics
Choi et al., 2022	E	Reproductive; neurotoxic
González-Soto et al., 2019	E	Oxidative stress; histopathology
Pittura et al., 2018	E	Oxidative stress; histopathology; immunological parametres
Romdhani et al., 2022	E	Cytotoxic and genotoxic
Trestrail et al., 2021	E	Enzymatic digestive activity

Table 3
Biomarkers commonly used to detect toxicity in *Mytilus galloprovincialis* as well as their location and function.

Biomarker	Location	Function	Reference
Activity of carbohydrases	Digestive gland	Catalyzes the breakdown of carbohydrates into simple sugar	Trestrail et al., 2021
Acetylcholinesterase inhibition, AChE	Gills	Servers as neurotransmitter	Pittura et al., 2018; Capolupo et al., 2021a, 2021b, 2021c Choi et al., 2022
Catalase (CAT)	Digestive gland and gills	Involved in removal of hydrogen peroxide	Pittura et al., 2018; González-Soto et al., 2019; Abidli et al., 2021; Capolupo et al., 2021a, 2021b,2021c; Choi et al., 2022
DNA fragmentation rate	Haemolymph	Separation or breaking of DNA strands into pieces due to contaminant exposure	Pittura et al., 2018; Romdhani et al., 2022
Glutathione (GHS)	Digestive gland and gills	Serves in metabolism and detoxification processes	Pittura et al., 2018; Capó et al., 2021b
Glutathione peroxidase (GPx)	Gills	Catalyses the reduction of hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxide radicals to alcohols and oxygen	Pittura et al., 2018; Capó et al., 2021b
Glutathione reductase (GR)	Gills	Maintaining the supply of reduced glutathione	Pittura et al., 2018; Capó et al., 2021b
Glutathione S-transferase (GST)	Digestive gland	Allows the conjugation of the reduced form of glutathione (GSH) to pollutant compounds	Pittura et al., 2018; Abidli et al., 2021; Capó et al., 2021b Capolupo et al., 2021a, 2021b, 2021c
Intra-lysosomal content of lipofuscin	Digestive gland	Oxidative alteration of macromolecules by oxygen-derived free radicals generated in reactions catalyzed by redox-active iron of low molecular weight	Capolupo et al., 2021a, 2021b, 2021c
Lysosomal β-hexosaminidase activity	Digestive gland	Breaking down toxic substances and act as recycling centre	González-Soto et al., 2019
Lysosomal Membrane Stability (LMS)	Haemolymph	Controls the passage of material into and out of lysosomes	Pittura et al., 2018; Capolupo et al., 2021a, 2021b,2021c; Romdhani et al., 2022
Lysosome to cytoplasm volume ratio	Digestive gland	Advanced physio pathological condition in mussel hepatopancreas	Capolupo et al., 2021b
Lysozyme-specific activity	Haemolymph	Catalyzes the cleavage of β-1,4-glycosidic bonds	Capolupo et al., 2021c

(*continued on next page*)

Table 3 (*continued*)

Biomarker	Location	Function	Reference
		between *N*-acetyl muramic acid and *N*-acetyl glucosamine in peptidoglycan	
Malondialdehyde	Digestive gland	Product of lipid peroxidation	Pittura et al., 2018; Abidli et al., 2021; Cole et al., 2020; Capò et al., 2021b; Capolupo et al., 2021b, 2021c
Metallothionein	Digestive gland, gills	Cysteine (Cys)-rich protein that acts as a crucial antioxidant	Capolupo et al., 2021b
Micronuclei frequency	Haemolymph	Biomarker of chromosomal damage	Pittura et al., 2018; Romdhani et al., 2022
Necrosis of digestive tubule epithelium	Digestive gland	Damage to epithelial cells useful to adsorb substances into the body and restricting the entry of harmful substances	González-Soto et al., 2019;
Phagocytic activity	Haemolymph	Represents a vital facet of the innate immune response to pathogens, and plays an essential role in initiating the adaptive immune response	Capolupo et al., 2021c
Presence of brown cells in connective tissue and brown aggregates in epithelium	Digestive gland	Non-specific indicator of environmental pollution	González-Soto et al., 2019
Protease activity	Digestive gland	Catalyses hydrolysis of the dietary proteins	Trestrail et al., 2021
Protein carbonyls derivates	Gills	Marker of oxidative damage	Capò et al., 2021b
Thiobarbituric Acid	Digestive gland, gills	By-product of lipid peroxidation	Cole et al., 2020
Transcriptional parameters	Mussel embryos	Transcripts encoding lysosomal enzymes	Capolupo et al., 2018
Steroid hormones	Haemolymph	Endogenous regulators of gametogenesis	Choi et al., 2022
Superoxide dismutase (SOD)	Digestive gland and gills	Antioxidant enzyme that serves as protection from toxic effect of reactive oxygen species and free radicals	Cole et al., 2020; Choi et al., 2022

controlled conditions, except the work of Capò et al. (2021b) in which *M. galloprovincialis* were distributed in three areas with different anthropogenic impacts. This is due to the difficulty to set an experiment in the field controlling the number and the type of MPs. Indeed, in an open environment like marine ecosystem, it can be difficult put a fixed number of MPs and obtain certainly the contact between particles and organisms. Obviously, tests set in laboratory follow the indication of environmental dose of contaminant and ty to reproduce real environmental conditions. The biggest difference between experiments in laboratory and in field is the absence of dilution in laboratory and the absent or reduced recycle of the water. Also, the number of the organisms than can be exposed in laboratory is different by the number of organisms than can be exposed in a natural scenario.

Even though, results obtained from laboratory experiments are adaptable to natural ecosystem because the doses used in laboratory are usually realistic respect environmental level of contamination. Moreover, the quantity of organisms, the volume of water and the control of parameters (i.e., dissolved oxygen, salinity, pH, etc.) are proportioned to natural environmental.

It can be interesting and important try to create some experiment in natural field or in same similar location, also for simulate wave movement, change of temperature, and light condition during the day.

As regard the type of biomarker, most of published papers focused the attention on biomarkers of oxidative stress. Indeed, the measurement of antioxidant compounds involved in detoxification of ROS, such as glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione (GSH) and lipid peroxidation (LPO) has been shown to be an appropriate assessment tool in this framework for two reasons (Lam, 2009): a) the mechanisms that counteract ROS are well known and extensively studied; b) they can act as sentinel compounds to prevent ROS due to their immediate production.

Glutathione S-transferase plays a key role in the phase II of detoxification involved in the conjugation of GSH with phase I enzymes; its activity has been found to be increased in the gills of mussels when exposed to MPs (Capó et al., 2021a) and PP leachates (Capolupo et al., 2021b). Contrary, polystyrene MPs did not induce any GST changes in *M. galloprovincialis* gills (Capolupo et al., 2021c) nor in the digestive gland of *M. galloprovincialis* exposed to chrysene-sorbed polystyrene MPs (Capolupo et al., 2021a). Superoxide dismutase is the main actor in the dismutation process to obtain hydrogen superoxide (H_2O_2) from the superoxide anion (O_2^-); its suitability as a biomarker is due to its rapid response to stress; normally, its activity is increased under stress conditions, especially in the gills, since they are the first to be exposed to toxins (Box et al., 2007). The study on oxidative stress biomarkers by Box et al. (2007) highlight that the levels of GPx and SOD are significantly higher in the digestive glands than in the gills for the polluted area, further supporting these biomarkers as indicators of pollutants. In an experiment conducted under controlled conditions with *M. galloprovincialis*, SOD activity was found to be significantly increased in the first 24 h after exposure to 500 ng/mL of polystyrene microspheres (PS-MP) and polyamide microfibers (PA-MF), especially in the gills and digestive glands, but after 7 days of exposure, activity decreased and was similar to that of control organisms (Cole et al., 2020). On the other hand, other studies using larger plastics (100–1000 PE-MP/mL) for 4 days on *Mytilus* spp. showed no significant SOD activity after 24 h (Wang et al., 2020), confirming that SOD activity is related and inversely proportional to plastic size. CAT is only involved in the reduction of H_2O_2 to H_2O at high H_2O_2 levels, while at low levels it is actively involved in the detoxification of other compounds, with H_2O_2 reduction always occurring (Gonzalez-Fernandez et al., 2015). In *in situ* experiments (aquaculture cages), a higher activity of CAT was found (Capó et al., 2021b) in gills of *M. galloprovincialis* exposed to MPs. Abidliet al. (2020) showed how polyethylene microplastics (PE-MPs) can induce alteration in CAT activity. Moreover, *M. galloprovincialis* exposed to leachates from different polymers showed higher CAT activity in digestive gland than gills since peroxidation processes are frequently modulated in response to toxicant exposure (Capolupo et al., 2021b). Like the enzymes mentioned above, GPx is also involved in the removal of H_2O_2 (Capó et al., 2021b); although GR is not directly involved in the detoxification of ROS, its function is equally important as it is involved in maintaining the ratio of GSSG (oxidized glutathione)/GSH, which in turn plays a key role in cellular homeostasis (Gonzalez-Fernandez et al., 2015); GSH, in turn, is actively involved in the detoxification of ROS (Box et al., 2007). The activity of GR was found to be higher in field experiments compared to the control group (Capó et al., 2021b). Glutathione was detected in greater amounts in the digestive gland of *M. galloprovincialis*, which is a known organ for detoxification of pollutants, and higher than normal levels may indicate stress (Cappello et al., 2021).

Lipid peroxidation is considered a toxicity biomarker because it is used to assess lipid peroxidation of membranes, i.e., it reflects the damage caused by ROS (Gonzalez-Fernandez et al., 2015; Box et al., 2007). It is already expressed both *in situ* and under controlled environmental conditions, and in the latter, it was found particularly higher in the gills after 24 h of exposure (Cole et al., 2020; Capó et al., 2021b).

Malondialdehyde (MDA) levels are commonly used to assess oxidative stress as a product of lipid peroxidation. For example, exposure of *M. galloprovincialis* to polystyrene microplastics (PS-MP, 3 μm) and nanoplastics (PS-NP, 50 nm) resulted in the up-regulation of MDA in the digestive gland, indicating that lipid peroxidation phenomena might be induced by PS particles (Capolupo et al., 2021c).

Proton nuclear magnetic resonance (NMR) metabolomics has been also used in aquatic ecotoxicology since it allows simultaneous identification of changes in metabolic pathways after contaminants exposure (Cappello, 2020). However, only few studies have used NMR-based metabolomics to investigate the effects of MPs on aquatic biota (i.e., Lu et al., 2016, Qiao et al., 2019). The research performed on metabolites in *M. galloprovincialis* and their expression under stress conditions in relation to polystyrene MP particles by Cappello et al. (2021) could provide a basis for studying toxic responses to MPs and the results could be compared globally, at spatial and temporal level, providing a consistent protocol for monitoring baselines. Cappello et al. (2021) highlight some biomarkers that could provide clear signs of decreasing or increasing levels. On this path, glycine was a compound that steadily decreased while GHS dramatically increased in the first 24 h after exposure to MPs. Lactate, glycogen, glucose, isoleucine, leucine, alanine, tyrosine, and valine increased in *M. galloprovincialis* exposed to MPs compared to control groups and could be used to determine ecotoxicity tests and MPs poisoning and responses (Cappello et al., 2021).

Genotoxicity biomarkers investigated in published literature mainly include DNA fragmentation rate and micronucleus frequency which provide information about the pollution taking place (Cole et al. 2020; Romdhani et al., 2022). Micronucleus test is used as a function of time and could be an indicator for monitoring purposes, while DNA strand breakage could be used for recent exposure to environmental stressors and contaminants in the organism (Bolognesi et al., 2004). Romdhani et al. (2022) assessed the impact of ecologically relevant MPs concentrations alone or combined with benzo[*a*]pyrene (MP-B[*a*]P) by means of micronucleus frequency and DNA fragmentation rate, highlighting an increase in groups exposed to MPs. The same findings were also shared by Cole et al. (2020) who exposed mussels to polystyrene MPs. Another important aspect that should not be underestimated is the difference between wild and caged mussels. Wild mussels have been shown to contain higher levels of accumulated heavy metals and persistent organic pollutants and therefore have higher micronuclei levels than caged mussels (Bolognesi et al., 2004). On the other hand, caged mussels have higher levels of DNA strand breaks, likely due to recent exposure to contaminants (Bolognesi et al., 2004).

Histological and cytological biomarkers are also a powerful tool for detecting and characterizing the biological endpoints of toxicants, and histopathology has been shown to be a sensitive indicator of health in aquatic organisms (Rašković and Berillis, 2022). For examples, neutral lipid (NL) accumulation, as well as the lysosome to cytoplasm volume ratio (LYS/CYT) significantly enhanced in *M. galloprovincialis* exposed for 7 days to leachates from car tire rubber, polypropylene, polyethylene terephthalate, polystyrene, and polyvinyl chloride (Capolupo et al., 2021b). Increase in LYS/CYT is an advanced physio-pathological condition in mussels, and it is thought to precede highly hazardous processes to the viability of digestive cells and digestive gland functions (Capolupo et al., 2021b). Similarly, neutral lipid accumulation is a lipidosis biomarker that is thought to be caused by either an increase in cytosolic lipid content or a decrease in fatty acid processing (Capolupo et al., 2021b).

Several authors have already measured immune system impairment in marine organisms exposed to MPs (i.e., Paul-Pont et al., 2016). On this path, immunological effects on lysosomal membrane stability and phagocytosis were also observed in *M. galloprovincialis* exposed to MPs (Capolupo et al., 2021a, 2021b,2021c; Romdhani et al., 2022).

Microplastics can also interfere with the activities of digestive enzymes, which convert carbohydrates, proteins, and fats into subunits that animals can absorb and use for energetic purpose (Karasov and Douglas, 2013). Because MPs were found in the digestive systems of aquatic invertebrates and can be retained for long periods of time (Fernández and Albentosa, 2019), there is ample opportunity for MPs to interfere with digestive enzyme functions. For such reason, Trestrail et al. (2021) determined the activities of seven key digestive enzymes in the digestive gland of *M. galloprovincialis*, highlighting how polymer type significantly affected the activities of carbohydrase enzymes.

Neurotoxicity biomarkers (i.e., acetylcholinesterase inhibition, AChE) were assessed by Capolupo et al. (2021a, 2021b, 2021c) and Choi et al. (2022). For example, Capolupo et al. (2021a) and Capolupo et al., (2021b) showed a decrease in acetylcholinesterase activity in *M. galloprovincialis* exposed to polystyrene MPs and PVC leachates, respectively.

In bivalves the steroid hormones estradiol and testosterone act as endogenous regulators of gametogenesis (Gauthier-Clerc et al., 2006). Estradiol is thought to regulate several reproductive processes in bivalves, including increasing oocyte diameter and ovarian protein content in female oysters and promoting vitellogenin protein accumulation (Li et al., 1998). Thus, the levels of estradiol and testosterone in bivalves influence the reproductive cycle and can be used as biomarkers of reproductive toxicity. Choi et al. (2022) found a decrease of both estradiol and testosterone in *M. galloprovincialis* after polyethylene terephthalate microfiber exposure, suggesting a disruption of the reproductive cycles of mussels.

Another important study, not illustrated in the Table 3, is represented by Cappello et al. (2021) which used for the first time a protonic Nuclear Magnetic Resonance (H NMR)-based metabolomic approach to examine the effects of short-term exposure of *M. galloprovincialis* to polystyrene MPs. Although several metabolites were found, amino acids and osmolytes metabolites involved in the energy metabolism represented the major classes of compounds. Alanine serves as osmolyte and is involved in nitrogen metabolism, and it is present in gills, digestive gland, and posterior adductor muscle. Acetoacetate presents in posterior adductor muscle and digestive gland, servers as energy supply for cell activity and as signaling molecule. Betaine serves as osmolyte, but it is present only in digestive gland; the same thing for dimethylglycine and homarine. Instead, hypotaurine and taurine is present also in gills and posterior adductor muscle. Glutamate is a precursor for synthesis of glutamine and later glutathione which serves as protection against oxidative damage, and it is present in digestive gland and posterior adductor muscle. Glycine serves as osmolyte, carbon energy source for generation of ATP in digestive gland, gills, and posterior adductor muscle. Glycogen, useful for reproductive cycle, can be found in gills and digestive gland. Leucine and isoleucine, in digestive gland and posterior adductor muscle, serves as metabolite in immune system, energy reserves and is precursor for formation of lymphocytes. Finally, valine serves as proteinogenic amino acid as precursor for formation of lymphocytes and essential in immune system and we can find it in digestive gland, gills, and posterior adductor muscle. Thus, an integrated description of the perturbations induced by MPs in the metabolome of *M. galloprovincialis* was successfully reported by Cappello et al. (2021). However, there is a lack of information about metabolic pattern in *M. galloprovincialis* that need to be filled in order to consider metabolites as useful biomarkers to reveal MPs toxicity.

5. Conclusions and future perspectives

The amount of information and knowledge about the serious but underappreciated problem of the toxic effects of MPs on organisms is growing. Although MPs affect the normal physiology and causing toxic effects on aquatic organisms, we know very little about biomarkers, which are indicator of normal and abnormal biological processes or responses at lower levels of biological organization (i.e., biochemical, cellular, or physiological levels). This minireview summarized the present situation in *M. galloprovincialis*, one of the most important bioindicators for the marine environment. The bibliometric analysis showed that several biomarkers were assessed. However, a battery of standardized and validated biomarkers is needed in future studies to obtain more comparable findings. Another challenge is the incorporation of new biomarkers (i.e., genetic, metabolomic, transcriptomic, histological, reproductive) at different levels of biological organization, as well as a multidisciplinary approach (adequate integration of multiple indicators) to perform a risk assessment, encouraging the use of chemometrics. Indeed, chemometric tools (correlation and factor analyses) and several numerical models should be used to assess ecological and human (non-carcinogenic and carcinogenic) health risk due to MPs exposure.

Another aspect is that organism usually answer with a stress or an increment of some enzymes after an exposition to MPs, but it is not clear if organisms answer to plastic or to some components of this or to some substances related to plastic, as phthalates. Related to this, the acidification of the oceans and, in consequence, the change of pH, and the increasing of the temperature can change the relation between plastic and contaminant, but also the availability for organism and the possibility of absorbing it.

Furthermore, other interesting aspects should be represented by microbiota studies associated to MPs exposure. Finally, one of the major challenges ahead of us include determining the exact mechanisms of MPs toxicity, either alone or in combination with other environmental contaminants, as well as determining the exact mechanisms of MPs uptake by gill and gut cells and translocation of the ingested MP into the circulation and target tissues.

CRediT authorship contribution statement

Francesca Provenza: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Darian Rampih:** Conceptualization, Writing – original draft, Writing – review & editing. **Sara Pignattelli:** Conceptualization, Writing – original draft, Writing – review & editing. **Paolo Pastorino:** Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Damià Barceló:** Conceptualization, Data curation, Writing – review & editing. **Marino Prearo:** Conceptualization, Writing – review & editing. **Antonietta Specchiulli:** Conceptualization, Writing – original draft, Writing – review & editing. **Monia Renzi:** Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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