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Review

Pollution Biomarkers in the Framework of Marine Biodiversity Conservation: State of Art and Perspectives

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Abstract: Marine biodiversity is threatened by several anthropogenic pressures. Pollution deriving from the discharge of chemical contaminants in the sea represents one of the main threats to the marine environment, influencing the health of organisms, their ability to recover their homeostatic status, and in turn endangering biodiversity. Molecular and cellular responses to chemical pollutants, known as biomarkers, are effect-based methodologies useful for detecting exposure and for assessing the effects of pollutants on biota in environmental monitoring. The present review analyzes and discusses the recent literature on the use of biomarkers in the framework of biodiversity conservation. The study shows that pollution biomarkers can be useful tools for monitoring and assessment of pollution threat to marine biodiversity, both in the environmental quality monitoring of protected areas and the assessment of the health status of species at risk. Moreover, key areas of the research that need further development are suggested, such as the development of omics-based biomarkers specifically addressed to conservation purposes and their validation in the field, the extension of the biomarker study to a wider number of endangered species, and the development of organic guidelines for the application of the biomarker approach in support to conservation policies and management.

Keywords: biomarkers; marine protected area; endangered species; biodiversity; biomonitoring; pollution

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1. Introduction

Chemical pollution derived from the discharge of chemical contaminants in the sea, from both point and non-point pollution sources, represents one of the main threats to the marine environments and their resources and services and remains a great environmental challenge [1,2]. Both sea-based and land-based anthropogenic activities result in the release of contaminants into the marine environment. Shipping is a source of pollutants through accidental spillages, operational discharges, and antifouling paint leaching; mariculture accounts for medicinal product, biocide, and food additive release; offshore activities produce drill cuttings and hydrocarbon release; dredging of sediment and dumping at sea contribute to water column contaminant level increase [3]. Moreover, the release of chemical contaminants into the sea from land-based activities, such as urban wastewater discharge, industrial and agricultural activities, mining, and runoff from coastal areas, contributes dramatically to the contamination of the seas. Chemical contaminants such as oil-based products, fertilizers, pesticides, trace metals, antifouling compounds, plastic materials, pharmaceutical, and veterinary products represent a worldwide threat to the health of the marine environment [4], influencing the health of marine organisms, their ability to recover their homeostatic status, and in turn endangering biodiversity. Several of them are contaminants of emerging concern (CECs), which include a wide array of anthropogenic chemicals that have no regulatory standards yet [5,6].

Chemical pollution is recognized as one of the major pressures driving biodiversity loss worldwide [7], in particular, in coastal areas because of the high anthropogenic use of the coastal habitats. Coastal benthic ecosystems are characterized by a high diversity of biotopes and high biodiversity, but unfortunately, most of the pollution is concentrated in these areas. Several cases have been described in the literature about the serious reduction of fish and invertebrate populations nearby the sites of effluent discharge [8,9]. Chemicals absorbed by the organisms through the gills, the gastrointestinal tract, and the tegument can interact with biological macromolecules, producing several toxicological effects at the cellular and molecular levels. This includes enzyme inhibition, alterations of transport properties, alteration of the functioning of membrane and intracellular receptors, alterations of intracellular signaling pathways, oxidative stress, and DNA damage [10–14]. These primary effects at the molecular and cellular levels can produce integrated toxicity effects over time, including impairment of organ and systems functioning such as neurotoxic effects, immunological responses, hepatotoxicity, behavioral changes, reproductive and developmental alterations, endocrine disruption, and genotoxicity [2]. These integrated toxicological effects resulting in adverse outcomes at the organism level can endanger species survival at a wider time scale. Moreover, the toxicological effects that chemical contaminants can exert on living organisms are made even more complex and multifaceted by the simultaneous presence of multiple contaminants in the environment which can exert additive or synergistic effects [15]. For example, some persistent marine pollutants can exacerbate the adverse effects of certain pesticides as well as other persistent organic pollutants (POPs) in marine organisms [16]. In addition, the harmful effects of pollutants are aggravated by the concomitant pressures of climate change and ocean acidification [17].

Healthy oceans are among the main objectives of the EU by 2030. To reach these goals, water quality monitoring and assessment assume a fundamental role. The development of effect-based methodologies, which provide the tools for the early detection of the biological effects of chemical contaminants in organisms, can give a useful contribution in this field. The study of the molecular, cellular, and physiological alterations in the organism in relation to the exposure to chemical pollutants has contributed to developing several markers (biomarkers) of exposure and toxicological responses to chemical pollutants [18,19]. The application of the biomarker approach in marine environment monitoring and assessment, integrated into the physicochemical analysis of the environmental matrices, has greatly increased in recent years. This is mainly due to the fact that the assessment of the entity of the organism exposure to pollutants in a certain environment and the extent of the suffered toxicological effects is of fundamental importance for decision making related to habitat and species protection, ecosystem services provision, adoption of remediation procedures, or impacted area monitoring [20].

Recently, the application of the biomarker approach in biomonitoring is considered with great interest in the field of biodiversity conservations. Considering that chemical pollution is recognized as one of the major pressures driving biodiversity reduction loss worldwide [7], the study of the responses of the organisms to the anthropogenic alterations of the environment that may cause or contribute to population decline can support biodiversity conservation strategies. Biomarkers have been recently applied to several research areas of the biodiversity conservation field, including environmental quality monitoring of protected areas and the assessment of the health status of species at risk.

The present review analyzes and discusses the recent literature in the field suggesting key areas of further research and, at the same time, identifies some perspectives for the development of novel biomarkers useful for biodiversity conservation.

2. Pollution Biomarkers

Pollution biomarkers are defined as pollutant-induced alterations in molecular and cellular components of the organisms as a consequence of exposure of the organisms to pollutants in their environment [21]. The harmful effects of pollutants are generally first exerted at lower levels of the biological organization before disturbances are manifested at the population, community, or ecosystem levels on a larger time scale. Therefore, biomarkers measured at the molecular or cellular level have been proposed as sensitive "early warning" tools for biological effect measurement in environmental quality assessment [22]. In particular, exposure biomarkers are early reversible cellular changes in the organism, which provide early detection of the exposure of the organisms to pollutants. Effect biomarkers assess the toxicological effects exerted on the organisms by the exposure to pollutants and are directly related to the risk of adverse health effects. Biomarkers of susceptibility are intrinsic characteristics of the organism that account for increased sensitivity to the effects of an environmental pollutant.

Examples of exposure biomarkers are provided by detoxification responses early activated by the organism to protect itself from the toxicity of pollutants. Metallothioneins are highly conserved low-molecular-weight, cysteine-rich metal-binding proteins showing a high affinity for metal ions belonging to the IB and IIB groups. These proteins account for metal homeostasis in the cell and are over-expressed when organisms are exposed to high metal concentrations in their environment [23]. The analysis of metallothionein concentration in bivalve mollusk hepatopancreas and fish liver has proved to be a valuable specific biomarker of exposure to trace metals (in particular Zn, Cu, Cd, Hg, Ag) in coastal marine environmental biomonitoring [24–26], allowing to discriminate between different levels of exposure of the organisms and, in turn, allowing to discriminate between different levels of contamination. Enzymes involved in the biotransformation of xenobiotics, such as cytochrome P450s in the liver of vertebrates, are also employed as exposure biomarkers due to their substrate inducibility and specificity. Increased activity and expression of cytochrome P450-dependent monooxygenases (CYP1A) in fish liver is a specific biomarker of exposure to polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). They are commonly used in the monitoring of coastal and marine areas polluted by hydrocarbons, industrial discharge, and urban sewerage systems [27]. A widely utilized exposure biomarker is also represented by the alterations in the blood concentration of the estrogen-inducible protein vitellogenin. It is a precursor to egg yolk which is physiologically synthesized by the liver of female oviparous vertebrates during oocyte maturation. The detection of vitellogenin in the blood of male fish is successfully used as a specific biomarker of exposure to xenoestrogen compounds [28].

Examples of biomarkers of effect are represented by genotoxicity biomarkers since DNA alterations are particularly important for the development of potential risk and adverse health effects [13]. Other examples are represented by oxidative stress biomarkers, such as alteration in the activity of antioxidant enzymes, depletion of intracellular antioxidant defenses, and induction of lipid membrane peroxidation, which are important biomarkers of effect due to the central role played by oxidative stress in several pathological conditions [29,30]. Alterations in the lysosomal system, such as lysosomal membrane destabilization, have been used as a biomarker of effect related to liver damage and tumor progression in the liver of various fish species [31], a prognostic indicator for pathologies in animals, and widely utilized in both aquatic and terrestrial organisms [32,33]. Moreover, cholinesterase inhibition is directly linked with the mechanisms of toxic action of organophosphorus and carbamate insecticides, whose use has increased in recent decades around the world, and also their impact on non-target organisms in aquatic environments. It is widely used as a specific biomarker of neurotoxic effects [34].

An enzymatic biomarker that has been receiving attention in recent years is represented by alteration in the activity and expression of carbonic anhydrase, a ubiquitous metalloenzyme involved in several physiological functions, such as respiratory gas exchange, pH homeostasis, transepithelial transports, metabolic processes, and calcification. It has been proved to be sensitive to many pollutants and in several species [35–40] and has been proposed as a candidate for the assessment of global and local impacts in biomonitoring programs [41].

Examples of biomarkers of susceptibility are represented by the presence of specific polymorphic genes involved, for example, in the metabolism of the pollutant or in the repair mechanisms that can increase the susceptibility of an organism to the toxicity of a substance or class of substances [42].

The specificity of biomarkers ranges from specific biomarkers, which allow identifying the specific pollutant or the specific chemical class of pollutants responsible for the responses observed in the organisms, to general biomarkers, which respond to a variety of environmental chemical stressors such as DNA damage or oxidative stress. Given the complexity of environmental pollution, with multiple chemicals contaminating the environmental matrices and the variety of biological responses that can be induced in the organisms, batteries of biomarkers with different specificity are generally used to properly assess the impacts of pollution on the organisms in a certain environment.

Biological responses to pollutant exposure can be chosen as biomarkers and, in turn, can provide an accurate assessment of exposure and toxicological effects if they meet specific requirements, such as their dose-response behavior to pollutant exposure over a range of environmentally realistic concentrations, their link to important biological processes, and the knowledge of their natural variability, which is fundamental for discriminating the pollutant-induced response from the physiological basal level [20]. In the natural environment, a number of confounding factors, including age, sex, nutritional status, spawning period, season, temperature, salinity, etc., may influence the biomarker responses to pollutant exposure. Therefore, a direct significant relationship between exposure to a certain substance and biomarker responses sometimes is hardly seen. The presence of confounding factors is an issue that needs to be carefully considered in the use of biomarkers in monitoring programs since the applicability of biomarkers is related to the capacity of discriminating between pollutant-induced responses from the natural-variability-related responses. The in-field validation of the use of biomarkers requires extensive knowledge of the effects of confounding factors on the studied biomarker responses. In this context, knowledge of the physiological status of the bioindicator organisms and the measurement of physico-chemical variables of the studied environment are essential for a correct interpretation of biomarker responses.

3. Pollution Biomarkers in Biomonitoring of Marine Protected Areas

Marine protected areas (MPAs) represent important tools in marine biodiversity conservation. They are increasingly being instituted worldwide to reduce the decline of biodiversity and conserve ecosystem function [43,44]. MPAs perform three key functions in modern conservation: conservation of marine biodiversity, preservation of productivity, and contribution to economic and social welfare. They involve the protective management of natural areas of seas, oceans, and estuaries according to specific protection objectives, such as habitats, biodiversity, and ecological processes conservation, species protection, and resources preservation.

Pollution has been recognized as one of the main menaces to MPAs [45,46]. As recently reviewed by Abessa et al. [47], a great number of MPAs show some signs of chemical pollution. Several MPAs are located near sources of pollution, such as industrial activities, harbors, agricultural farms, urban areas, and sewage outfalls. Contaminants may be introduced from adjacent areas [48], and marine currents can transport pollutants over long distances from pollution sources. Pollution should be considered a critical aspect in the creation and management of an MPA, which should require the identification and the assessment of the extent of the pollution pressure on the native species and ecosystems [49]. Pollution conditions are unknown in most MPAs worldwide, and even when some information is available, it is often inadequate to assess the threats to biodiversity or to address further actions [47]. In this context, there is an urgent need for adequate diagnostic tools useful for monitoring and assessment of the health status of the organisms in MPAs. The biomarker approach can meet this need since it allows to detect exposure and biological effects that are occurring in the organisms due to the presence of chemical substances in the environmental matrices.

The review of the literature produced in the past 20 years on the biomarker approach application in MPA biomonitoring all over the world is summarized in Table 1. The criterion for the inclusion of a paper in this review was represented by the fact that the work concerned the study of at least an MPA and included the experimental collection of specimens and the analysis of molecular and cellular biomarkers. The research was carried out on Scopus and Web of Science employing "marine protected areas*", or "marine reserve*" or "marine sanctuary*" or "marine park*" and "biomarker*" as search terms. A total of 22 studies were included in the analysis.

Table 1. Literature produced in the past 20 years on the application of pollution biomarkers in marine protected area biomonitoring all over the world. The criterion for the inclusion of a paper was represented by the fact that the work concerned the study of at least a marine protected area and included the experimental collection of specimens and the analysis of molecular and cellular biomarkers. The research was carried out on Scopus and Web of Science employing "marine protected areas*", or "marine reserve*" or "marine sanctuary*" or "marine park*" and "biomarker*" as search terms.

Protected Areas	Bioindicator Species	Bioindicator Class	Endpoint	Biomarkers Analyzed	Ref.
Europe					
Egadi Islands Marine Protected Area (Italy)	*Coris julis*, *Patella caerulea*, *Paracentrotus lividus*	Osteichthyes Gastropoda Echinoidea	Detoxification of organic pollutants	EthoxyresorufinO-deethylase, BaPMO, NADH ferry red, and NADH cyt c	[50]
Tremiti Islands Marine Protected Area (Italy)	*Paracentrotus lividus*	Echinoidea	Coelomocytes alterations	Coelomocytes subpopulations ratio, heat-shock protein 70	[51]
National Park of La Maddalena Arcipelago (Italy)	*Mytilus galloprovincialis*	Bivalvia	Lysosomal alterations	Lysosomal membrane stability, lipofuscin content, neutral lipid contents, lysosomal structural changes	[52]
Capo Peloro Natural Reserve (Italy)	*Atherina boyeri*	Osteichthyes	Detoxification of organic pollutants, neurotoxicity, genotoxicity	Acetylcholinesterase, benzo(a)pyrene-monooxygenase, polycyclic aromatic hydrocarbons metabolites in bile, erythrocytic nuclear abnormalities assay	[53]
The Pelagos Sanctuary (International Sanctuary for the Protection of Mediterranean Marine Mammals) (Italy, France)	*Meganyctiphanes norvegica*	Malacostraca	Detoxification of organic pollutants, neurotoxicity, response to xenoestrogens	Cytochrome P450, BaPMO activity, NADPH cytochromec reductase, NADH-ferricyanide reductase, esterases, porphyrins, vitellogenin, zona radiata proteins, acetylcholinesterase	[54]
	Stenella coeruleoalba	Mammalia	Detoxification of organic pollutants, oxidative stress	Cytochrome P4501A, cytochrome P4502B, catalase	[55]
	Balaenoptera physalus	Mammalia	Detoxification of organic chemical pollutants, oxidative stress	Cytochrome P4501A, cytochrome P4502B, lipoperoxidation	[56]
	Balaenoptera physalus, *Physeter macrocephalus*	Mammalia	Metal excretion	Metals in the fecal material	[57]
North America					
Florida Keys National Marine Sanctuary (U.S.A.)	*Montastraea annularis*	Anthozoa	Oxidative stress, stress protein multidrug resistance induction	Superoxide dismutase, glutathione peroxidase, glutathione-s-transferase, heat-shock proteins, metabolic condition, multixenobiotic resistance proteins	[58]

Table 1. *Cont.*

Protected Areas	Bioindicator Species	Bioindicator Class	Endpoint	Biomarkers Analyzed	Ref.
Veracruz Coral Reef System National Park (Mexico)	*Haemulon Aurolineatum, Ocyurus chrysurus*	Osteichthyes	Detoxification of organic chemical pollutants, response to xenoestrogens	Cytochrome P4501A, vitellogenin, glutathione-S-transferase, PAH metabolites in fish bile	[59]
Natural protected area of Laguna Madre in the Gulf of Mexico (Mexico)	*Chione elevata*	Bivalvia	Neurotoxic effects, oxidative stress, metabolic alterations	Acetylcholinesterase, butyrylcholinesterase, carboxylesterase, alkaline phosphatase, glutathione s-transferase, oxygen radical absorbance capacity	[60]
South America					
Morrocoy National Park (Venezuela)	*Siderastrea sidereal*	Anthozoa	Detoxification of organic chemical pollutants, oxidative stress	Cytochrome P450 I, cytochrome P450 II, NADPH reductase, glutathione *S*-transferase, catalase, superoxide dismutase	[61]
Parque Nacional Archipielago Los Roques (Venezuela)	*Siderastrea sidereal*	Anthozoa	Detoxification of organic chemical pollutants, oxidative stress	Cytochrome P450 I, cytochrome P450 II, NADPH reductase, glutathione *S*-transferase, catalase, superoxide dismutase	[61]
Fernando de Noronha Archipelago protected area (Brazil)	*Amphistegina lessonii*	Foraminifera	Oxidative stress, metal detoxification	Antioxidant capacity against peroxyl radicals, lipid peroxidation, protein carbonylation, metallothionein-like proteins	[62]
Paranaguá Bay protected areas (Brazil)	*Atherinella brasiliensis*	Osteichthyes	Neurotoxicity, detoxification of organic chemical pollutants, oxidative stress	Cholinesterase, ethoxyresorufinO-deethylase, glutathione S-transferase, catalase	[63]
Cananéia–Iguape–Peruíbe Environmental Protected Area (Brazil)	*Cathorops spixii*	Osteichthyes	Detoxification of organic pollutants, oxidative stress, genotoxicity, metal detoxification	Glutathione S-transferase, glutathione peroxidase, GSH levels, lipid peroxidation, DNA strand breaks, metallothonein	[64]
	Cathorops spixii	Osteichthyes	Genotoxicity	Comet assay, micronucleus test (MN), and nuclear abnormalities test (NA) in peripheral blood	[65]
Natural Protected Area San Antonio Bay (Argentina)	*Neohelice granulata*	Malacostraca	Detoxification of organic pollutants, oxidative stress, metal detoxification	Catalase, lipid radical content, lipid peroxidation, α-tocopherol, catalase, glutathione-S-transferases, metallothioneins	[66]
Cananéia–Iguape–Peruíbe Protected Area (Brazil)	*Callinectes danae*	Malacostraca	Genotoxicity, detoxification of organic pollutants, oxidative stress, metal detoxification, neurotoxicity	Glutathione S-transferase, glutathione peroxidase, intracellular glutathione, acetylcholinesterase, lipid peroxidation, metallothionein, DNA strand breaks	[67]

Table 1. *Cont.*

Protected Areas	Bioindicator Species	Bioindicator Class	Endpoint	Biomarkers Analyzed	Ref.
Estuarine Lagoon Complex of Iguape–Cananéia (Brazil)	*Gobioides broussonnetii*	Osteichthyes	Oxidative stress, genotoxicity, metal detoxification, histopathological alterations	Superoxide dismutase, catalase, glutathione peroxidase activity, glutathione S-transferase, glutathione, metallothionein, lipoperoxidation, micronuclei, histological alterations	[68]
Australia					
Great Barrier Reef (Australia)	*Plectropomus leopardus*	Osteichthyes	Detoxification of organic chemical pollutants, neurotoxicity	EROD, cholinesterase	[69]
	Acropora millepora	Anthozoa	Oxidative stress	Genetic loci involved in environmental stress tolerance and antioxidant capacity	[70]

The scientific interest in the use of pollution biomarkers in MPA biomonitoring has grown in the past decade, as indicated by the recent increment in the number of publications produced in this field: two papers were found in the period 2001–2005, three papers in the period 2006–2010, five papers in the period 2011–2015, and nine papers in the period 2016–2020.

As shown in Figure 1, the most investigated responses are represented by biomarkers of organic chemical pollutant detoxification (including cytochrome P540 family) and antioxidant and oxidative-stress-related biomarkers (such as antioxidant enzyme activity, lipid peroxidation, antioxidants depletion), followed by neurotoxicity (inhibition of the activity of acetylcholinesterase), genotoxicity (DNA damage assessed by comet assay, micronuclei, and erythrocyte nuclear abnormalities), metal detoxification biomarkers (induction of metallothionein), cytological/histological alterations, xenoestrogen exposure biomarker (vitellogenin), lysosomal alterations, heat-shock proteins, multidrug resistance induction, porphyrins, and biomarkers of pollutant excretion.

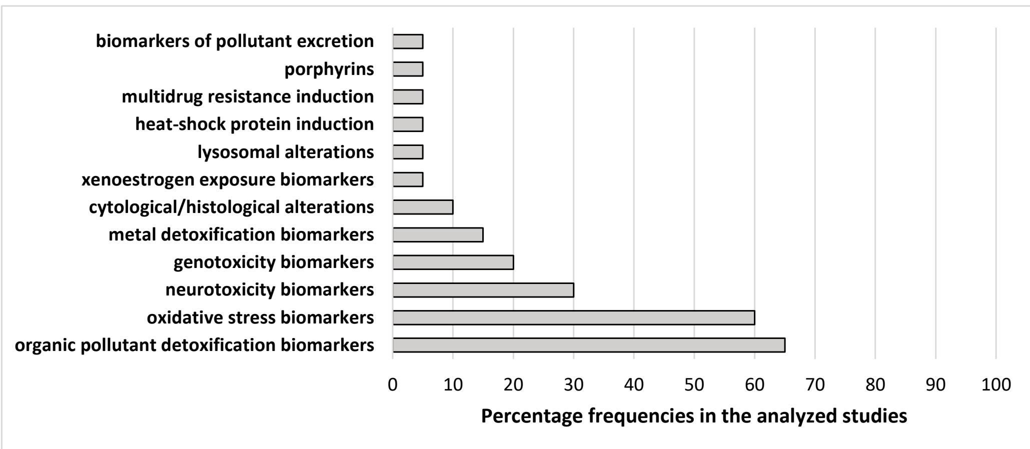

Figure 1. Percentage frequencies of different biomarkers in the studies on MPA pollution biomonitoring selected for this review, as reported in Table 1.

As observed in Table 1, fish are widely utilized as bioindicator species for biomarker analysis in MPAs, thanks to their high sensitivity to chemical pollutant exposure which triggers their antioxidant and biotransformation systems [34,71,72]. They were used for

MPA biomonitoring in 40% of the analyzed studies. Other bioindicator organisms investigated for biomarker responses in MPAs are represented by bivalve mollusks and crabs. Bivalve mollusks are extensively used as bioindicator organisms thanks to their wide distribution, sessile and filter-feeding nature, and tolerance to a wide range of pollutants. Their biological responses to pollutant exposure are extensively used as biomarkers in coastal and marine environmental monitoring and assessment [24,26,34,39,73]. Crabs are important components of coastal ecosystems and represent good bioindicator organisms for the biomarker approach application since they can accumulate contaminants absorbed from water and food in their tissues developing detectable molecular and cellular responses to pollution exposure and effects [74,75]. Corals have been employed for biomarker application in water quality assessment in coral reef environments [58,61,70]. In one study foraminifera were also successfully utilized for reef water quality assessment [62].

In the case of the only pelagic MPA in the Mediterranean Sea, the Pelagos Sanctuary (International Sanctuary for the Protection of Mediterranean Marine Mammals, Corsican-Ligurian Provencal Basin, Northern-Western Mediterranean Sea), biomarkers have been successfully applied to marine mammals, utilizing a nondestructive approach on animal biopsies and fecal material analysis [55–57]. In addition, Fossi et al. [54] explored the possibility to measure biomarker responses in zooplanktonic euphausiids for the assessment of the environmental quality of the Pelagos Sanctuary.

All the studies analyzed underline the usefulness of the biomarker approach responsiveness to environmental pollutants exposure as a tool for monitoring the environmental quality of MPAs, detecting eventual threats from anthropogenic pressures, and assessing the effectiveness of the adopted measures in MPAs to preserve the quality of the marine senvironment.

For example, in the Estuarine Lagoon Complex of Iguape–Cananéia (Brazil), the authors complemented the chemical analysis of sediments (metals, polycyclic aromatic hydrocarbons, pharmaceuticals, and personal care products were determined) with the analysis of alterations in antioxidant, biotransformation, histopathological, and genotoxic biomarkers in the fish *Gobioides broussonnetii* allowing to detect the contribution of anthropogenic activities to contaminant inputs and compromising of the conditions of fish in the area [68].

In the Cananéia–Iguape–Peruíbe Environmental Protected Area (Brazil), the use of a multimarker approach (including glutathione S-transferase, glutathione peroxidase, GSH levels, lipid peroxidation, metallothionein, and genotoxicity biomarkers, such as DNA strand breaks, comet assay, micronucleus test, and nuclear abnormalities) in the fish *Cathorops spixii*, paralleled by the analysis of metal body burden and polycyclic aromatic hydrocarbons in bile, allowed for the identification of both seasonal and spatial variations in pollution sources [64,65].

Moreover, Caliani et al. [53], who studied biomarkers in the key fish species *Atherina boyeri* of Capo Peloro lakes in Sicily (Italy), confirmed that a biomarker-based approach (including acetylcholinesterase, benzo(a)pyrene-monooxygenase, polycyclic aromatic hydrocarbons metabolites in bile, and erythrocytic nuclear abnormalities) can be useful for monitoring seasonal and spatial variations in pollution sources impacting MPAs according to variations in anthropogenic activities in the surrounding areas [53].

In the Paranaguá Bay (Brazil), the histopathological analysis of tissues of the fish *Atherinella brasiliensis* revealed a significant presence of severe pathological conditions in the liver and gills of the animals paralleled by biochemical alterations and DNA damage assessing the pollution threat to aquatic organisms coming from anthropogenic activities (urban, industrial, agricultural, and harbor activities) of the surrounding areas [63].

In the MPA of La Maddalena Archipelago (Italy), the biomarker approach using lysosomal biomarkers (lysosomal membrane stability, lipofuscin content, neutral lipid contents, lysosomal structural changes) in transplanted *Mytilus galloprovincialis* was coupled to the measure of trace metals in mussel tissues, the analysis of the presence of endocrine disruptors in the water column, and the in vitro cellular toxicity of POCIS (Polar Organic

Chemical Integrative Sampler) extracts on mussel hemocytes measured by lysosomal membrane stability assay [52]. This integrated approach allowed assessment of the effects of anthropic stressors in the MPA and to evaluate the effectiveness of the adopted measures to preserve the quality of the marine environment.

In the Tamaulipas Laguna Madre, the Gulf of Mexico, the use of a wide-ranging battery of biochemical biomarkers (acetylcholinesterase, butyrylcholinesterase, carboxylesterase, alkaline phosphatase, glutathione s-transferase, oxygen radical absorbance capacity) analyzed on the clam *Chione elevata* as a sentinel organism was integrated into a stress index, the Integrated Biomarker Response (IBR), and complemented by the chemical analysis of metals, organochlorine pesticides, and hydrocarbons in the sediments [60]. The authors underlined the potential of the approach to be a useful tool for monitoring the health status of the sentinel organisms and, in turn, the environmental quality of the MPA.

In the Pelagos Sanctuary (Italy-French), Fossi et al. [55] for the first time provided the first complete evidence of the toxicological stress in cetaceans living in the Pelagos Sanctuary by applying an integrated approach based on the analysis of persistent chemicals combined with biochemical markers of exposure to planar halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (such as cytochrome P4501A, cytochrome P4502B) and the antioxidant enzyme catalase to anthropogenic contaminants in striped dolphin (*Stenella coeruleoalba*) skin biopsies. Moreover, through the biomarker approach, Fossi et al. [56] investigated the potential toxicological effects of microplastics and their related contaminants on free-ranging fin whale populations.

The biomarker approach has been applied to reef environments, typical of many shallow coastal areas in tropical regions, which are at increased risk due to several threats, including pollution. Antioxidant defenses and metal detoxification analysis in foraminifers [62], biotransformation and neurotoxicity biomarker in reef fish [69], and detoxification proteins, antioxidant defense, metabolic profile, and stress proteins in corals [58,70] have been applied for gaining a measurable impact on selected reef environments caused by anthropogenic contaminants.

These studies underline the importance and usefulness of the biomarker approach for assessing the quality of the habitat in MPAs, in the detection of any space and time variations in anthropogenic pressures on the area, and the effectiveness of the conservation policies. Some studies utilized an integrated approach complementing the biomarker analysis with the measurement of the pollutant residues in the tissues of the organisms. The importance of this approach in the continuous monitoring of the health status of the organisms living in protected areas appears evident from these works; however, considerable research efforts still need to be made to fill some gaps such as the lack of specific guidelines for the use of these tools in MPA monitoring and assessment and for their useful application to MPA management. Guidelines should address the criteria for the employment of key species as sentinel organisms for the multi-biomarker approach application in MPA biomonitoring and the choice of suitable biomarker responses in relation to the specific protection objectives of MPAs. Moreover, guidelines should indicate protocols for the validation of the biological responses used and even the standardization of a biomarker-based index for specifically assessing the ecotoxic risk.

4. Biomarker Analysis in Endangered Species

In recent years, the biomarker approach has been also applied as a tool for detecting the health status of endangered species and their ecotoxicological risk in relation to exposure to pollutants.

In this field, most of the studies available on marine organisms refer to vertebrates, in particular turtles and marine mammals. Among turtles, the most investigated species are represented by *Caretta caretta* and *Coelonia mydas*, as recently reviewed by Finlayson et al. [76], which are included in the UCN Red List of Threatened Species. Most of the works on biomarkers in turtles are related to the study of protein expression and enzyme activity in relation to pollutant exposure with potential use as a biomarker. In particular, Richardson et al. [77] and Labrada-Martagón et al. [78] correlated oxidative stress biomarkers (catalase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase, lipid peroxidation) with contaminant levels, such as trace metals and organochlorine pesticides. Keller [79] correlated vitellogenin levels in blood with organic contaminant concentration in water. Moreover, in *C. caretta*, the application of the comet assay for assessing DNA damage has been investigated [80], and in both *C. mydas* and *C. caretta*, the induction of metallothionein has been related to the levels of metals in the tissues of the animals [81]. More recently, Casini et al. [82] provided the first ecotoxicological assessment of *C. caretta* in the Mediterranean Sea through a nondestructive integrated biomarker approach on blood, skin, and carapace biopsies. CYP1A expression, vitellogenin, lipoperoxidation, DNA damage, butyrylcholinesterase activity, cholinesterase activity, metallothionein, and gamma-glutamyl transferase were analyzed in parallel to the contaminant (OCs, PAHs, Pb, Cd, Hg) tissue concentration. A statistically significant positive correlation was found between DNA fragmentation and PAHs in blood and between gamma-glutamyl transferase in plasma and Cd in the carapace. The youngest animals showed higher levels of DNA fragmentations, butyrylcholinesterase inhibition, and gamma-glutamyl transferase induction, while older specimens showed the highest levels of erythrocyte nuclear abnormalities, presumably due to long-term exposure.

As regards marine mammals, CYP1A1 induction was detected in blubber biopsies of endangered false killer whales (*Pseudorca crassidens*) and nine other odontocete species from Hawaii [83] in parallel to contaminant chemical analysis. Significantly higher levels of CYP1A1 were observed confirming a biological response to contaminant exposure. The authors outlined them as contaminant burdens, and in turn, CYP1A1 expression levels were influenced by the different trophic positions of the studied species. More recently, Baini et al. [84] assessed levels of polychlorinated biphenyl (PCBs), polybrominated diphenyl ethers (PBDEs), and induction of cytochrome's P450 in skin biopsies of Cuvier's beaked whale, whose Mediterranean subpopulation has been categorized as vulnerable on the IUCN (International Union for Conservation of Nature) Red List of Threatened Species. Recently, Mancia et al. [85] explored the application of epigenetics to the study of the stress response related to xenobiotics exposure in the Mediterranean fin whale (*Balaenoptera physalus*).

Moreover, as recently reviewed by Consales and Marsili [86], the biomarker approach has been utilized for assessing the conservation status of Chondrichthyans, including near-threatened, vulnerable, and endangered species. The biomarkers utilized included biomarkers of organic chemical pollutant detoxification, antioxidant- and oxidative-stress-related responses, neurotoxicity, vitellogenin, and zona radiata protein, a sensitive biomarker for environmental estrogen exposure [87–93].

In comparison with vertebrates, the studies on endangered invertebrate species are far fewer. Sureda et al. [94] successfully applied biomarkers on the endangered species *Pinna nobilis*, the largest endemic Mediterranean bivalve subject to strict protection. The specimens were collected in the east and southeast of the islands of Ibiza and Formentera, affected by the oil spill from the sinking of the Don Pedro merchant ship in 2007. The measurement of antioxidant enzymes and lipid peroxidation, in parallel to the analysis of polycyclic aromatic hydrocarbons in the tissues of the animals, allowed the assessment of the oxidative stress induction in the organism and the recovery along time of the oxidative damage in lipids due to the activation of the enzymatic antioxidant response.

In all the above-reported studies, the analysis of the molecular and cellular responses to pollutant exposure in endangered species has contributed to the detection of exposure and the assessment of the impact of pollution on the conservation status of these species. These results encourage the application of the biomarker approach to other endangered species. In particular, the information on the health status and the threat of pollution on endangered invertebrate species is very scarce and could represent a research area in which pollution biomarkers can find application and development. These studies suggest how the biomarker approach has much to offer to policymakers in terms of risk assessment of endangered species, particularly in the identification of hazard, detection of the exposure, and assessment of the association of the response with the probability of a pathological outcome. However, in order to make the biomarker approach an effective and valuable tool for the protection of endangered species novel nondestructive approaches need to be developed, able to encompass the limitation of traditional methods which require manipulation and often sacrifice of the animal. Recently, Chaousis et al. [95] highlighted the fact that research on nondestructive biomarkers in wildlife is scarce and should be developed. On the basis of their analysis, the authors indicated in vitro methods, based on the use of tissue explants or cultured cells, combined with nondestructive sampling, as the most promising approach for nondestructive biomarker research development in wildlife. Moreover, the fruitful application of this tool for conservation purposes requires further research for the development and validation of more biomarker responses linked to population-level processes, such as reproduction and mortality, to be included in the contest of a multimarker approach based on an array of responses, to more strictly address fundamental issues in conservation. The development of novel biomarkers requires also a great research effort to acquire an extensive knowledge of natural variability of the responses considered in order to discriminate the alteration due to pollutant exposure from basal levels.

5. Perspectives for Future Research in the Biomarker Development in the Framework of Biodiversity Conservation

In the framework of biodiversity conservation, the research on pollution biomarkers can benefit from the recent development of "omics" technologies, such as genomics, proteomics, metabolomics, and the bioinformatics tools required to analyze the massive omics outputs. These methodologies show great potentiality for acquiring important knowledge regarding the mechanistic mode of action of pollutants, single or in a mixture, and their impact on the organisms' health status [96]. They allow one to deepen the knowledge on the toxicological mechanism of pollutants and to define the adverse outcome pathways (AOPs) associated with the exposure to pollutants through the analysis of the complex of responses that an organism can exert. Following exposure to pollutants, the organism exerts multiple responses including alterations in the expression of genes, changes in the levels of proteins, and variations in the concentrations of metabolites. The specific pattern of responses is related to the specific mode of action of pollutants, allowing the identification of pathways of responses and, in turn, the development of arrays of related biomarkers that can represent a sort of "signature" of exposure [97]. This allows extending the biomarker concept beyond the classic biomarker concept linked to the single biological endpoint.

However, a constraint to the application of the genomics principle to wildlife and endangered species conservation is represented by reduced or absent genomic information on non-model organisms. On the other hand, transcriptomics, which addresses changes in gene expression by focusing on mRNAs, as well as proteomics, which considers the entire set of proteins that are produced or modified by an organism, and metabolomics, which studies the whole metabolic profile of an organism, are widely employed in ecotoxicology [98–100].

Massive molecular information can be quickly acquired through RNAseq or proteomics, even when working with organisms for which genome sequences are not currently available. This aspect is of particular relevance for wildlife ecotoxicological analysis. The acquired information can be used to clarify the molecular modes of action of contaminants, to develop sensitive methods for biomarker quantification [101], and to assess and predict the vulnerability of organisms and species to pollutant exposure [102]. The reduced costs of sequencing technologies in recent years make possible the exploration of inter-population, intra-species, and inter-species variability in the response to pollutant exposure and also to assess variability in the sensitivity to pollutants during different stages of the life cycle of an organism through the analysis of alternative splicing, polypeptide cleavage, and post-translational modification [102]. This would help to develop species-specific biomarkers that would be of particular relevance in the field of the assessment of the health status of endangered species. It must be emphasized that great effort also has to be devoted not only to the development of new biomarkers but also to their validation in the field, including the assessment of the effect of environmental conditions (temperature, salinity, pH, etc.) and species-related factors (age, gender, reproduction cycle, etc.) on the biomarker responses analyzed to avoid non-correct interpretation. Moreover, another important aspect on which research efforts will need to focus is represented by the link between biological responses at the molecular level and the fitness of the species. This last aspect is to date almost neglected but is of fundamental importance for the employment of the multi-biomarker approach in the field of biodiversity conservation and could improve the environmental prognostic value of this tool.

6. Conclusions

In conclusion, the analyses and discussion of the recent literature suggest that pollution biomarkers have proved to be useful tools for monitoring and assessment of pollution threat to marine biodiversity, both in the environmental quality monitoring of protected areas and the assessment of the health status of species at risk.

However, great efforts should be still devoted to developing the research in this field. In particular, important issues that require further development concern (1) the development of new biomarkers specifically addressed to conservation purposes, also thanks to the development of omics technologies, (2) the extension of the study to a wider number of endangered species, and (3) their validation in the field and inclusion into organic guidelines that could support conservation policies and management.

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