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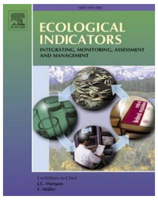

Review

# Biomarkers based tools to assess environmental and chemical stressors in aquatic systems

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## ABSTRACT

The quality of aquatic systems is threated by the huge amount of chemicals dispersed in the environment. Anthropogenic activities are one of the main causes of pollution by metals, water sewage, pesticides, pharmaceutical drugs and other contaminants. These substances have consequences on the environment bringing abiotic and biotic deterioration. Global Climate change (GCC) also affects the dynamics of aquatic communities affecting the toxicity of pollutants by altering their physiochemical properties and the adaptive capacity of organisms. Damages in ecosystems and thus in aquatic communities have consequences in functional and physiological roles, with impacts along the trophic food web and thus, in the food quality. The degradation and loss of the goods and services provided by theses ecosystems affect human population. To safeguard the environmental status, the European Union has implemented the Water Framework Directive (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC) legislations, which allow the use of biological tools to detect the quality of aquatic systems. The following review highlights the use of these biological tools to achieve the objective of both legislations that assures the good quality of the aquatic environment. The biological groups most used for the assessment of aquatic systems are: phytoplankton, macrophytes, benthic macroinvertebrates and fish, with several authors also consider zooplankton due to the sensitiveness of this community to stressors, although this group is not identified as a biological quality element (BQE) of WFD. Relying on bioindicators and biomarkers as supporting on chemical analyses, researchers should have a clear overview of environmental conditions. To ensure that biological tools are valid, they must deal with some criteria: they must be cheap and easy to perform; be sensitive to pollutant exposure; be reliable, with a short life span and easy to collect.

Although bioindicators and biomarkers may be used as early-warning indicators of the presence of stressors, they have limitations in their applications. Thus, should be assessed and identified the most accurate and suitable biomarker to be used as endpoint in ecotoxicological studies and the assessment of the environmental status.

## 1. Introduction

Water covers ¾ of the Earth surface. Marine water represents about the 97% of the water that covers the Earth and it is considered one of the most abundant resource on our planet (Karima and Shafiul Islam, 2019).

Marine waters and resources provide benefits to humans and are classified as ecosystem services, useful to conduce a better lifestyle (Barbier, 2017; Bryhn et al., 2020). An unsustainable use of marine waters and resources by human-beings has strongly altered the structure and function of many marine ecosystems worldwide (Österblom et al., 2017; Rocha et al., 2015; Selim et al., 2016). The anthropogenic impacts are manifested in different ways: loss of biodiversity (Worm et al., 2006), eutrophication (Nixon, 1995; Wurtsbaugh et al., 2019), chemicals and pollutants release (Amato et al., 2006; Fliedner et al., 2020; Tornero and Hanke, 2016), marine litter (Bergmann et al., 2015; Consoli et al., 2020; Deudero and Alomar, 2015). These stressors change the balance of aquatic ecosystems and lead to the reduction of wellness of animal and plant species with the consequent reduction of ecosystem services.

Chemical pollutants release in the environment can have worst effect

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when they pass from the environment from animal organisms because of the phenomenon of biomagnification (Falfushynska et al., 2019; Kelly et al., 2007), with consequent increase of toxicity. In the ecosystem is released a wide amount of emerging and persistent organic contaminants like butyltins (BTs), polycyclic aromatic hydrocarbons (PAHs), pesticides including pyrethroids, pharmaceuticals and personal care products (PCPs) and flame retardants (Rocha et al., 2018).

To protect and monitoring the marine seas the European Union has formulated two legislations: the Water Framework (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC). These legislations are based on the protection, maintenance and restoration of marine environments.

The detection of environmental changes from aquatic organisms is related to their structure and functionality which allow them to be really sensitive to environmental stressors (Littlefield-Wyer et al., 2008). Due to the various impacts of these chemicals, the environmental degradation cannot be determined by single biomarkers but it is necessary to use a battery of biomarkers based on measurements of complementary parameters to describe accurately the effects of contamination on organisms (Kim and Jung, 2016; Samanta et al., 2018; Sanchez and Porcher, 2009).

Nevertheless, the use of bioindicators and biomarkers has limits, therefore they are not completely reliable instruments but they are tools that if implemented based on chemical analysis, they can give a more precise and accurate response to the environmental status.

This review aims to report the main biomarkers used for the detection of stressors caused by chemical pollutants in aquatic system, mainly marine and estuarine environments. The classes taken into account are phytoplankton, zooplankton, benthos and fish.

## 2. Biological tools in ecological risk assessment

The European Union and other countries have adopted legislation during the years to guide and monitoring the water quality. The Water Framework Directive (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC) are the adopted legislations that contributed to achieve a “Good Ecological Status” and “Good Environmental Status” for estuarine, coastal and offshore waters (from 2015 to the WFD and from 2020 to the MSFD).

The main goal of the WFD is the protection, maintenance and recovery on inland surface, transitional and coastal waters alongside the promotion of the sustainable use of water and the reduction and/or elimination of pollutants, whereas MSFD defines 11 descriptors for the achievement of good environmental status including human-induced eutrophication, biological diversity and concentrations of contaminants.

Progressively the European Union included an integrative analysis of water quality going through the use of several biological quality elements: phytoplankton, macroalgae, phanerogams, benthic invertebrate fauna and, fishes (CIS-WFD, 2003). Scientists have developed indexes calculation methods based on richness, abundance and biomass of the community (Desrosiers et al., 2013). They are multimetric indexes, it means they include different parameters to have accurate results and those indexes are also combined with physico-chemical parameters (transparency, dissolved oxygen, nutrients and pollutants) of water because they cannot be neglected (CIS-WFD, 2003) to have an overview about the water quality status.

Combined approach is preferable even because of the constant movement of the water due to the action of winds, tides and currents, which makes difficult the measurement of water chemistry. For instance, to measure the nutrient level for oligotrophic waters – a parameter that is relied on the biological condition in an ecosystem – bioindicators used may be a more representative approach since organisms will integrate spatial and temporal changes in the water quality (Desrosiers et al., 2013).

The use of bioindicators helps to detect the presence of stressors, can be used as early warning of pollution or degradation (Linton and Warner, 2003) and they may compensate the constraints of chemical analysis alone. The approach given by biological tools has the advantage to provide information on the exposure and the effects of chemicals (even short-lived chemicals) on living organisms, while chemical analyses provide information about the presence and/or concentrations of the substances; it means that only chemical analysis does not reflect the response of aquatic organisms to harmful effects of pollutants (Martinez-Haro et al., 2015; Wan et al., 2018).

Discussions about bioindicators and biomarkers are often confusing. Even though these concepts are related, the fundamental difference is that biomarkers concentrate on measurement attributes, while bioindicators require validation in addition to measurement (McCarty et al., 2002).

The first biomarker approach was originally developed in pharmacology/medical toxicology, then it has become useful in the field of environmental assessment and monitoring (McCarty et al., 2002). As the definition by NAS/NRC (1989), and modified by Shugart et al. (1992), is referred “a biomarker is a xenobiotically-induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample”.

In 1996, McCarty and Munkittrick gave the following definition: “a biomarker is an anthropogenically-induced variation in biochemical, physiological, or ecological components or processes, structures, or functions that are measurable in a biological sample or system.”

Aquatic organisms inhabiting polluted ecosystems may be also considered as integrative tools for the detection of the impacts of chemical compounds in the environment and the health condition (Hagger et al., 2008). Hence, it is demonstrated that biomarkers are valid tools in the monitoring programs of the WFD, as part of the adaptation of the Directive to scientific and technical progress in accordance with the provisions of Article 20 (Sanchez and Porcher, 2009)

A biomarker may be considered a good tool for environmental assessment if it reflects the following criteria: it should be reliable, with a short life span and easy to sample; relatively cheap and easy to perform; should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter. The development of non-destructive techniques to evaluate biomarker responses is recommended for conservation of endangered species (Fossi and Marsili, 1997).

Furthermore, it is necessary to well define the baseline data of biomarkers to have a correspondent distinction between “noise” (natural variability) and “signal” (stress caused by contaminant). The relationship and the mechanism between biomarker response and pollutant exposure should be defined a priori. Knowing the dosage and time of laboratory experiments, the expectation and prediction on what happens in the field will be more realistic. The response also depend on the type of biomarker used; if it is sessile it could also reflect conditions at one site so it is more specific to identify a particular impact on the ecosystem (Stegeman and Lech, 1991; Van der Oost et al., 2003; Desrosiers et al., 2013).

## 3. The multi-level biomarkers approach in different biological groups

There are several pollutants introduced in the aquatic environment. In order to understand the toxicity of these pollutants we rely on biomonitoring activities, using bioindicators and biomarkers sensitive to changes in the environment. Toxicity can be easily identified and quantified measuring the appropriate biological responses (e.g., egg shell thickness, calcium metabolism) in environmentally or laboratory exposed organisms (Martinez-Haro et al., 2015).

Through literature, we found several bioindicators belonging to different biological groups: phytoplankton, zooplankton, benthos, fish.

We have integrated biomarker response (IBR) that employs biochemical, morphological and physiological features (Bignell et al., 2011; Brooks et al., 2009; De los Ríos et al., 2013; Lam, 2009). Still, based on the results obtained, it is expected to identify the best biomarker to be used as endpoint in ecotoxicological studies to detect the presence of specific pollutants.

McCarty and Munkittrick (1996) defined four recognizable classes of biomarkers: (1) biochemical (enzymes, hormones, metallothioneins); (2) physiological (growth, reproduction, energetics); (3) toxicological (behaviour, lethality, teratogenicity, mutagenitic, carcinogenitic) and (4) ecological/community (additions, deletions, alterations in ecosystem/community structures and relationships). These classes correspond to different biological functions and different levels of biological organization. As different studies reflect, there are strong responses from biological biomarkers than from higher level-organization biomarkers, and they are also favourite because of the tendency to be very cost-effective even if they require more commitment to be related on ecological significance (McCarty and Munkittrick, 1996).

Changes at the genetic/molecular level tend to occur first, followed by responses at cellular, tissue, organ and whole-body levels. Thus, by monitoring molecular and biochemical changes (e.g. gene-expression patterns, hormonal levels, activities of specific enzymes, structure and function of organelles), and physiological parameters (e.g. reproductive performance and growth rates), the potential harm of an agent can be assessed before more severe disturbances/consequences occur (Lam, 2009). The multi-level biomarker approach is widely used from researchers to obtain a more complete and integrative overview of what affect marine organisms health and, consequentially, the environment (Samanta et al., 2018).

Biomarkers at low organization level are identified for instance in molecules, pigments, fatty acids, lipid and phenolic biomarkers, because are good indicators to assess the health of an ecosystem and the degree of anthropogenic impact. Folch et al. (1956) made a simplified method to extract marine lipids in which the sample is ground in chloroform and methanol (2:1). Nature of lipids is heterogeneous, an extract contains as many as 16 different subclasses of both biogenic and anthropogenic origin (Parrish, 1988), it means it can give us much information and lipid can be separated from non-lipid contaminants in order to discover the type of chemicals from each environment (Parrish et al., 2005).

Also biomarkers of oxidative stress, even if they are not specific biomarkers, are good indicators of stress exerted by a wide range of pollutants in aquatic ecosystems (Van der Oost et al., 2003)

The WHO (World Health Organization) defined different categories of biomarkers: (1) a biomarker of exposure is “an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism”; (2) a biomarker of effect is “a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease” (World Health Orgnization, 1993).

Biomarkers of exposure are identified in the induction of proteins as metallothioneins, which respond following exposure to certain metal

**Table 1**
Biomarkers based tools to assess environmental and chemical stressors in aquatic system.

| Biological group | Genus/Species name | Type of stressor | Biomarker tool | Response | Reference |
|---|---|---|---|---|---|
| Phytoplankton | *Phaeocystis* spp. | Pollutants | Chlorophilla *a* (Chl-*a*) | Eutrophication; excess of N or P. | Tett et al. (2007) |
| | *Cyclotella meneghiniana*<br>*Melosira varians*<br>*Bacillaria paradoxa*<br>*Campylodiscus bicostatus*<br>*Campylodiscus echeneis*<br>*Cocconeis placentula*<br>*Cymbella affinis*<br>*Cymbella cistula*<br>*Navicula salinarum*<br>*Nitzschia hybrid*<br>*Nitzschia obtuse*<br>*Nitzschia scalaris*<br>*Nitzschia sigmoidea*<br>*Pleurosigma salinarum*<br>*Surirella ovalis* | Increase of temperature and turbity due to the discharge of water sewage | Abundance of organisms | Increase of the abundance of diatoms respect their normal status | Saad and Antoine (1983) |
| Zooplankton | *Acartia margalefi*<br>*Acartia latisetosa*<br>*Siriella clausi*<br>*Diamysis bahirensis*<br>*Siriella armata*<br>*Mysidopsis gibbosa*<br>*Euphausia crystallorophias*<br>*Euphausia superba*<br>*Streetsia challengeri*<br>*Meganycthiphanes norvegica*<br>*Eriphia verrucosa*<br>*Pachygrapsus marmoratus* | Mixture of contaminants (PAHs) | Acetylcholinesterase activity (AChE) | Increase in enzyme activity with increasing concentration of contaminants | Minutoli et al. (2002) |
| | *Cassiopea* sp. | Heavy metal cadmium (Cd) | Glycolytic enzymes (PK and LDH), malondialdehyde content (MDA) and chlorophyll *a* (Chl-*a*) | Low protein amount due to an excessive energy consume needed for maintenance | Aljbour et al. (2018) |
| | *Bosmina* spp.<br>*Daphnia* spp. | MeHg concentration | Fatty acid (FA) | Detection of water contaminated | Kainz et al. (2002) |

species, or cytochrome P450 monooxygenase system induced following exposure to organic pollutants such as aromatic hydrocarbons, polychlorinated biphenylsor dioxins.

Biomarkers of effect are for example: the enzyme delta-aminolevulinic acid dehydratase (ALAD), inhibited even at small levels of lead; the cholinesterase enzymes (ChE), inhibited following exposure to organophosphates and carbamates pesticides and also to some non-essential metals; the comet assay or micronucleus assay to evaluate DNA or chromosomal damage, respectively, due to genotoxins; or imposex phenomenon (imposition of male secondary sexual characteristics on gastropods females) due to organotin compounds (Martinez-Haro et al., 2015).

In Table 1 is listed the main biomarkers-based tools to assess environmental and chemical stressors in aquatic systems divided by biological group.

### 3.1. Phytoplankton

Phytoplankton is in the base of the productivity in aquatic environments and it is an important biological tool to define ecological status. The most used markers from this group are pigments (mainly chlorophyll a) and fatty acids, thought we know the nutritional quality of seston for herbivorous zooplankton (Taipale et al., 2016). These parameters cannot give real information about a specific type of pollution, but calculating an integrated index that includes phytoplankton biomass and abundance could help to discover the water quality condition (Revilla et al., 2009), since biomass is associated with the visible symptoms of eutrophication, and it is usually the cause of the practical problems resulting from eutrophication.

Moreover, phytoplankton species are well-defined as indicators for eutrophication. Phytoplankton species' sensitiveness to eutrophication that are specific to a particular polluted area, should be monitored to determine their abundance in normal condition and during blooms; if phytoplankton abundance increases, the toxicity may increases as well (Heslenfeld and Enserink, 2008).

Chl-a concentration changes depend on algal species; this is a weakness of the use of algae. A great variability is expected based on seasonality, light conditions and nutrient availability (Boyer et al., 2009). Tett in his study discovered *Phaeocystis* spp. as an indicator to detect water eutrophication and might indicate an excess of available N (or P) in relation to dissolved silica (Tett et al., 2007).

The response of aquatic species to pollution is reliable for the increase in abundance of phytoplankton. Among the causes of the increase of phytoplankton there are the discharge of sewage, an increase of chlorisity in the water, less oxygen dissolved, high temperatures and turbidity of waters. Among diatoms identified as indicator of brackish waters there are: *Cyclotella meneghiniana, Melosira varians, Bacillaria paradoxa, Campylodiscus bicostatus, Campylodiscus echeneis, Cocconeis placentula, Cymbella affinis, Cymbella cistula, Navicula salinarum, Nitzschia hybrid, Nitzschia obtuse, Nitzschia scalaris, Nitzschia sigmoidea, Pleurosigma salinarum, Surirella ovalis* especially to the increase of water temperature (Saad and Antoine, 1983).

### 3.2. Zooplankton

Modern biomarkers approach is applied on zooplankton community of marine and brackish environments to evaluate ecotoxicological risk and to give a warning signal of ecosystem health (Minutoli et al., 2002).

Several zooplankton organisms were used to estimate their potential application in biomarker approach. Studies were carried out with: the copepods *Acartia margalefi* and *Acartia latisetosa* collected in Ganzirri Lake (Messina); the mysid *Siriella clausi* collected in Faro Lake (Messina); the mysids *Diamysis bahirensis*, *Siriella armata* and *Mysidopsis gibbosa* collected in Stagnone di Marsala (Palermo); the Antarctic euphausiids *Euphausia crystallorophias* and *Euphausia superba*; the amphipod *Streetsia challengeri* and the euphausiid *Meganycthiphanes norvegica* collected after a shore-stranding along Messina's Ionian coast. Moreover, experiments were carried out with the benthic decapods *Eriphia verrucosa* and *Pachygrapsus marmoratus* from a rocky shore of Messina's Ionian coast (Minutoli et al., 2002).

An increase in acetylcholinesterase activity (AChE) with increasing concentration of pollutant was detected and in all the marine and brackish species for which it was possible to carry out the experiment. We could not neglect the effect of species sizes: the AChE activity is inversely proportional to size (Fossi et al., 1996); in fact *E. crystallorophias* esterase activity is higher than *E. superba* activity. Another difference is about the level occupied by the species in the trophic level; mysids *S. clausi, D. bahirensis, S. armata, M. gibbosa* showed a higher activity then copepods and euphausiids. AChE is a good biomarker to detect even other contaminants in different species, for instance for indicating organophosphate/carbamate in the cyprinid *Leuciscus cephalus* (Frenzilli et al., 2008; Matozzo et al., 2005), and pharmaceutical pollutants in *Carassius auratus* (Liu et al., 2014).

Metallothionein or metallothionein-like proteins (MTLP) are widely used as biomarkers of trace metal pollution (Hamza-Chaffai et al., 1997; Hauser-Davis et al., 2019; Le et al., 2016; Matin et al., 2019; Netzahualcoyotzi et al., 2019).

Jellyfish could be also defined as good bioindicators for polluted environments. Two main glycolytic enzymes (PK and LDH) malondialdehyde content (MDA a proxy to asses LPO) and both protein and chlorophyll a (Chl-a) content were measured in *Cassiopea* sp. (Aljbour et al., 2018). Jellyfish reacts to coastal pollution of the metal cadmium with no signs of oxidative stress damage. It means jellyfish has a good defence system but a low protein response likely due to an excessive energy consumption needed for maintenance

Fatty acids are one of the most important and used molecules, so they were took into consideration as good biomarkers of ecosystem health and of environmental stress (Filimonova et al., 2016). In zooplankton *Bosmina* spp. and *Daphnia* spp. were analysed fatty acid (FA) biomarkers to investigate the effect of organic matter ingestion on MeHg concentration (Kainz et al., 2002). Researches showed contamination water because of the presence of MeHg in ingested food.

### 3.3. Benthos

Macroinvertebrates are ubiquitous, abundant and visible without microscope. They include many insects, crustaceans, mites, molluscs and worms (Chessman, 2003).

Among the different organisms, benthic invertebrate fauna are the most frequent used in bioassays protocols (Martinez-Haro et al., 2015) to test acute and chronic toxicity from specific contaminants in water or sediments for a long time because of their long life cycle (Armon and Hänninen, 2015).

*Mytilus edulis* is the benthic organisms mainly used for biomonitoring (Falfushynska et al., 2019; Liang et al., 2020; Strubbia et al., 2019; Yuan et al., 2017). A good set of biomarkers was used from the higher to the lower organism level: heart rate and feeding/clearance rate to detect physiological responses of general condition; ferric reducing ability of plasma (FRAP) and haemolymph protein concentrations to detect and measure antioxidant activity; neutral red retention assay to carried out as a measure of lysosomal damage; phagocytosis activity to detect immunocompetence; micronucleus assay to detect DNA damage; acetylcholinesterase activity to detect exposure to organophosphate pesticides and carbamates; the length, width and tissue weight to detect metallothionein and exposure to metals (Hagger et al., 2008). Biomarkers showed severe reduction in esterase activity and increase in metallothionein concentrations due to the exposure to high concentrations of pesticides (organophosphate and/or carbamates) and metals. However, the selected biomarkers reflected early molecular mechanisms of action of contaminants whereas for biological effects monitoring, to be ecologically meaningful, it is generally agreed that a set of biomarkers at different levels of biological organisation should be chosen.

Other biomarkers used at lower levels were identified in the mollusc *Meretrix meretrix*: the lysosomal membrane stability (LMS); micronucleus frequency (MF); glutathione (GSH); acetylcholinesterase (AChE); metallothionein (MTs); glutathione reductase (GR); glutathione peroxidase (GPx); malondialdehyde (MDA). They were used to assess the water quality through general health of the animals. As results, biomarkers were discovered as good tools for assessing the effects of anthropogenic contaminants, particularly effluent-seawater mixtures in aquatic ecosystems (Wan et al., 2018).

Food sources of macroinvertebrates are a good signal to understand the level of pollutant in the environment, indeed reserves stored in the form of lipids are used from bivalves' food sources as biomarkers (Bergé and Barnathan, 2005). Also the amount of fatty tissue still it also depends on environmental conditions, season, sex, life stage, and gametogenic cycle (Larsson et al., 2018), which means that is necessary make further analysis.

Biomarker studies conducted at molecular or subcellular levels tend to be more repeatable and predictable, but the same biomarkers could give different responses depending on the organism. Glutathione S-transferase (GST) specific activity was determined in the gill and digestive gland tissues of *M. edulis* from a number of sites of varying pollution loads used to detect general oxidative stress. As results reveal, the elevated GST activity observed could be a result of organotin exposure. However, although the evidence for the presence of a GST inducer is good, the identification of the causative agent requires further clarification (Fitzpatrick et al., 1997). Increases in GST specific activity have been documented in laboratory exposures of bivalves to various organo-chemicals (Livingstone, 1991) such as dieldrin and lindane (Boryslawskyj et al., 1988). In contrast, there is no evidence to suggest that exposure of mussels to metals (e.g. Cu, Mn, Fe, and Pb) results in elevated GST levels (Regoli and Principato, 1995). Therefore, the potential exists for its use as a possible specific index of organic chemical pollution in the environment (Fitzpatrick et al., 1997).

Also, studies conducted on the barnacle *Balanus balanoides* showed elevated levels for GST activity as a result of the response to direct effect of environmental xenobiotics in the polluted site. For *Balanus balanoides*, biomarkers used are antioxidant enzymes such as catalase, SOD and GST, microsomal lipid peroxidation in digestive tissues in barnacles: their levels are sensitive to organisms' response to the exposure to the xenobiotics in the environment and are not influenced by changes in physical–chemical parameters (Niyogi et al., 2001).

A multi-integrated biomarker index approach was applied to assess marine PAHs pollution, metal toxicity and to evaluate marine environmental quality and health status of the clams of *Ruditapes philippinarum* (Aouini et al., 2018; Ji et al., 2019).

As the values of IBR showed, DNA damages the expression of superoxidase dismutase (SOD), 7-ethoxyresorufin-O-deethylase (EROD) and glutathione S-transferase (GST) activity, and lipid peroxidation (LPO) could be served as biomarkers to monitor the PAHs pollution in Laizhou Bay (Ji et al., 2019). Among biomarkers related to the Pb toxicity, the enzymatic activity of δ-aminolevulinic acid dehydratase (δ-ALAD) has been adopted as a specific tool. Metallothionein (MT), LPO and antioxidant enzymes activities, such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), GST and SOD have also been employed to assess the response of the clams to the metals (Aouini et al., 2018).

Biomarkers help also to identify the presence of pharmaceutical drugs. The response of two species of bivalves – *Venerupis decussata* and *Venerupis philippinarum* – to acute exposure of acetaminophen was determined. Results showed a significant increase in all oxidative stress biomarkers, evidencing the bioactivation of acetaminophen into a deleterious prooxidant, noticeable for low concentration (Antunes et al., 2013).

*Perna perna* was used as test to evaluate the cytotoxicity of Amoxicilin and Potassium Clavulanate, through the NRRT assay, which assesses the stability of lysosomal membrane (Souza et al., 2016). Lysosome is a good biomarker because it is the responsible organelle for encapsulation of xenobiotics; in the presence of a toxic compound, the lysosomal membrane permeability becomes brittle and all the absorbed content is extravasated to the cytosol of the cells (Lowe and Pipe, 1994). *Perna perna* mussels cytotoxic response to the antibiotics Amoxicillin and Potassium Clavulanate was observed in the cytotoxicity assay by NRRT method. The drugs presented about four times more toxicity in association than when tested isolated (Souza et al., 2016).

Metallothionein (MT) and lipid peroxidation (LPO) biomarkers were used to detect Cu effects and the toxicity in the amphipod *Gammarus locusta,* respectively. In fact, the maximum levels of MT and Cu body content were observed at day 6 and it coincided with the decrease in LPO estimated by the formation of thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA). Levels of MDA increased after one and two days of exposure and subsequently peaked at day 4 achieving the highest level, then returned to control values at both days 6 and 10 (Correia et al., 2002).

Metallothionein as controller for Cu cannot be used for all type of amphipods; *Dikerogammarus villosus* has great detoxification mechanisms that allow the accumulation of metals but also the survival of the organism (Maazouzi et al., 2008). For that reason, it cannot be used to detect metal pollution. A similar study-case is given by the talitrid amphipod *Orchestia gammarellus* already used to investigate copper and zinc concentration in a polluted site. After exposure of copper, zinc and cadmium in the laboratory MTLP concentration did not increase (Mouneyrac et al., 2002). This proves that the same biomarkers cannot work for all the species belonging to the same group.

### 3.4. Fish

Fish are considered among the most significant bioindicators in fresh (Authman et al., 2005; Rashed, 2001), and marine waters (Rodrigues et al., 2019; Teles et al., 2016; Willett et al., 1997) to estimate metal pollution level in contaminated environment. Studies on them could help to assess temporal changes in aquatic habitats due to the long-time exposure of fish to toxicants. First responses could be highlighted from the accumulation in their organs and tissues, then in subcellular alterations due to their continuous deleterious action (Hedayati, 2018). From the surrounding environment fish have the ability to accumulate pollutants in their fatty tissues. Monitoring pollution in fish is also important for human health because they are located at the end of the aquatic food chain and may accumulate metals; bioremediation has its climax before going to human beings through food causing chronic or acute diseases (Al-Yousuf et al., 1999).

*Lethrinus lentjan* fish was studied along the western coast of the United Arab Emirates on the Arabian Gulf, and used as means of evaluating metal pollution in coastal waters, like zinc, copper, manganese and cadmium. The levels of contaminants in liver tissues were higher compared to skin and muscle tissues, but the study reports that the fish does not constitute a risk factor for human health because levels appear to be below the permissible limits for human consumption (Al-Yousuf et al., 1999).

Liver is the main vulnerable organ because of the tendency to bioaccumulate chemicals through both bioconcentration (uptake via the water), and biomagnification (uptake via the food web (Kraybill, 1976)) and a consequently high induction rate of metallothioneins (MTs). Detoxification of chemicals is activated through enzyme-catalysed reactions (Dural et al., 2007; Holdway et al., 1995; Narra, 2016; Pait and Nelson, 2003; Yilmaz et al., 2010). One of the most used enzyme is cytochrome P4501A1 (CYP1A1), (Fatima and Ahmad, 2006; Goksøyr and Förlin, 1992; Livingstone, 1993; Payne et al., 1987; Spies et al., 1984; Stegeman and Lech, 1991) used as a biomarker of exposure to PAHs.

CYP1A is an enzyme belonging to mixed function oxygenase (MFO) that is involved in the biotransformation of PAHs, but its induction could produce damaging side effects through the formation of intermediates that are highly reactive, mutagenic and carcinogenic (Buhler and

Williams, 1988; Gelboin, 1980; Stegeman and Lech, 1991).

*Oreochromis mossambicus*, a widely distributed tropical euryhaline fish, was exposed in the laboratory to a pure PAH and a mixture of PAHs compounds. Fish was analysed after three days of exposure and after two weeks, with increasing concentration of pollutants. Hepatic concentrations of phenanthrene (pure PAH) for both experiment and results showed significantly higher phenanthrene levels in the liver for both the treatment groups. Thus, tilapia *Oreochromis mossambicus* showed to be good sentinel organisms for monitoring PAH pollution in tropical waters. Exposure to low concentrations of PAH leads to sublethal hepatic toxicity in fish as shown by the increased activity of serum sorbitol dehydrogenase, an indicator of liver damage. The use of multiple enzyme biomarkers such as CYP1A- and SSDH-activities in combination with bile fluorescent aromatic compounds (FACs) is especially recommended for biomonitoring the fish response to PAH, considering the ease of analyses of the parameters (Shailaja and D'Silva, 2003). The response given by the CYP1A system in fish could be influenced by external factors such as temperature, season or sexual hormones. Thus, it is not always possible to have a linear dose–response relationship between biomarker content and concentration chemicals (Sarkar et al., 2006).

The immunoassay of CYP1A should be conducted with catalytic assays to ensure a better quality of analysis (Collier et al., 1995).

Detection of PAHs could be also identified by using different integrated biomarkers. *Cynoscion guatucupa*, a wild fish from the Bahia Blanca Estuary (BBE), Argentina, was studied to assess the levels of 17 PAHs in sediments and muscle of the fish. The integrated biomarkers taken into account to detect PAH pollution involved metabolic enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), the lipid peroxidation in liver and muscle tissues and the condition factor (CF) (Recabarren-Villalón et al., 2019). Moreover, levels of certain biomolecules in muscle and liver tissues and physiological conditions of fish could be ascribed to PAH concentrations in muscle, proposing them as early integrated biomarkers of PAHs in *C. guatucupa* from the BBE. Specifically, LDH and AST/ALT activities, TBARS, and the CF appeared to be valid early integrated biomarkers for PAHs' concentrations in *C. guatucupa* that warn about environmental contamination levels (Recabarren-Villalón et al., 2019).

Researches about the presence of metals in fish conducted along the Red Sea coast of Hodeida, Yemen Republic, attested the presence of metals (Fe, Cu, Zn, Cd and Pb) in two species: *Pomadasys hasta* and *Lutjanus russellii*. IBRs were constituted by biochemical parameters of liver functions, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and kidney functions (urea and creatinine) as well as histopathological changes in gills, liver and kidney. Liver of both fish species proved to be the target organ for Fe and Cu while the kidney was considered as the target organ for Zn but also for Pb (Omar et al., 2014); this could be based on specific metabolic processes and coenzyme catalysed reactions.

Monitoring growth parameters and analysis of tissues is even possible to detect the presence of insecticides released in the environment. Studies discovered changes in enzymatic activities due to the presence in environment of two insecticides, chlorpyrifos (CPF) and monocrotophos (MCP), which caused decrease in growth rate and other biological processes of fish species (Sweilum, 2006). For example, the exposure of *Clarias batrachus* to MCP and CPF caused a significant decrease in red blood cells, haemoglobin and haematocrit in liver, muscle and gill tissues (Narra et al., 2017).

Organophosphates (OPs) are widely used as insecticides and studies demonstrate their effects on the rapid irreversible inhibition of the enzyme acetylcholinesterase (AChE).

AChE is envolved into the degradation of the transmitter acetylcholine in the synaptic gap of cholinergic synapses and neuromuscular junctions. OPs inhibits this enzyme and toxic effects range from sublethal and lethal conditions. Sturm (1999) reported characteristics of acetylcholinesterases from brain and muscle tissue of three marine teleosts, *Limanda limanda, Platichthys flesus* and *Serranus cabrilla*, to provide basal information for environmental monitoring in coastal and marine areas (Sturm et al., 1999).

## 4. Limits of bioindicators and biomarkers

Scientists are divided in using biomarkers at molecular, biochemical and subcellular levels and physiological levels; this doubt is due to the different level of prediction: while at low level biomarkers tend to be more repeatable and predictable, and they give excellent warning about contaminants, their ability to predict significant biological effects is limited. At high level biomarkers are usually more ecologically relevant, but slower to respond and more difficult to detect (Armon and Hänninen, 2015; Lam, 2009; Wu et al., 2005).

Most of the used biomarkers are less specific and respond to environmental stress in general. To establish the right biomarker is fundamental to know the detailed dose–response relationship between the biomarker and the contaminant considered. Not all the biomarkers can be used for the same issue or to allow an identification of the precise stressors, and it is also important to make a validation and/or site-specific confirmation, since it is not possible to make assumptions concerning the cause-effect linkages at the population, community, or ecosystem levels of organization based on a primary toxicology test data (McCarty et al., 2002). The numbers of ecotoxicological tools are not the same for different stressors: there are more ecotoxicological tools developed to screen the effect of priority pollutants such as metals, PAHs, PCBs, than emerging pollutants (Martinez-Haro et al., 2015). Further research is needed to develop and validate ecotoxicological tools as early-warning systems of emerging pollutants in order to not confuse the tools responses by factors relied on the environment such as food availability, water temperature (Niyogi et al., 2001) and reproductive activity (Canesi et al., 1991).

Another constrain about the use of biological indicators is the bioremediation and adaptability of species. Some organisms have the ability to repair damage induced by chemicals and if it occurs could be an underestimated analysis of the level of contaminant or the appearance of false negatives (Lam, 2009; Wu et al., 2005). An example is given by the B[a]P concentration in the hepatic DNA adduct levels of the green-lipped mussel *Perna viridis*; it increased to a maximum after exposure and after almost three weeks under continuous exposure to chemicals, with the level return to control level (Ching et al., 2001). Another case is demonstrated by the estuarine fish *Fundulus heteroclitus* (Atlantic Killifish), and its adaptation to Polychlorinated Biphenyls (PCBs) (Nacci et al., 2010) that shows clearly that even if the site is polluted the organism cannot be good detector to stressors in the environment.

## 5. Conclusion

From the literature review it emerges that investigations on biological tools such as bioindicators and biomarkers is a topic widely discussed over the years by scientists.

Among the most used biomarkers there are organisms belonging to the benthos and fish biological groups, which give more specific indications on the stressors present in the environment.

Literature research shows that the best way to have an accurate control of the environment and about chemicals dissolved in the ecosystem is by using a set of biomarkers to assess the best biomarker to be used as endpoint in ecotoxicological studies and to detect the presence of specific contaminant.

Scientists should take a detailed attention in research to highlight the limits of biomarkers and the right way to use organisms in monitoring approaches, because not all the biological tools can be used for the same purpose. Further research will reveal new biological tools for the assessment of aquatic systems which, combined with chemical analyses, will show the state of health of an ecosystem.

The application of biomarkers tools in ecological and ecotoxicological studies may allow early detecting the presence of chemicals stressors and early respond to those stressors, that may be used in monitoring environmental systems and thus in the maintenance of the health status of the ecosystems, which is the objective of the MFD and MSFD.

## 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.

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