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Are Mytilus species suitable bioindicators for assessing aquatic pollution along the Black Sea Coast? A review

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Abstract. This review aims to summarize the possibility of using mussels (*Mytilus* spp.) as bioindicators to assess aquatic pollution in the Black Sea in Bulgaria. In addition, the main responsive biomarkers that could be applied to study the negative effects of different toxicants on these species in terms of using the Marine Strategy Framework Directive and implementation of environmental quality standards (EQS) in marine biota are also discussed. A specific reference is made to plastic pollution, transplant mussel caging, and mussel watch programs - their application, challenges, and future perspectives in Bulgaria.

Key words: *Mytilus* spp., aquatic pollution, biomarkers, Black Sea, Bulgaria.

Introduction

Human activities produce various persistent organic and inorganic pollutants, such as polyromantic hydrocarbons (PAHs), polychlorinated bypehnils (PCBs), polybrominated diphenyl ethers (PBDEs), short-chain chlorinated paraffins (SCCPs), dioxins and trace metals, as well as plastic litter, which eventually end up in the marine environments and ocean (Guendouzi et al., 2020; Kouali et al., 2022). Hence, the marine environments, which are subjected to such diverse and toxic pollutants, are threatened – the integrity of habitats and their associated biota, from coastal to pelagic environments, and from benthonic to surface ecosystems (Anbuselvan et al., 2018; Bonanno & Orlando-Bonaca, 2018; Bonsignore et al., 2018; Urban-Malinga et al., 2018).

Environmental problems in Black Sea

The Black Sea is the world's largest land-locked inland sea between southeastern Europe and western Asia, and is surrounded by six countries - Romania, Bulgaria, Ukraine, Russia, Georgia, and Turkey (Fig. 1). It is 1210 km long from east to west and up to 560 km wide, has a maximum depth of 2212 m and a volume of 534000 km^3, and covers an area of 432000 km^2 (Özsoy &

Ünlüata, 1997; Sari et al., 2018). The Black Sea communicates with the Mediterranean Sea to the south and the Azov Sea to the north (Topping & Mee, 1998; Boran & Altınok, 2010).

However, the Black Sea suffers from serious and much challenging environmental problems. The sea's shallow, mixed surface waters receive river discharges, heavily loaded with nitrogen and phosphorus nutrients, and polluted with industrial and mining wastes. In addition, coastal industries appear to discharge wastes directly into the sea with little or no treatment. Thus, the life-supporting surface layer's water quality has seriously deteriorated (Fabry et al., 1993). Moreover, The Black Sea coastal zone is densely populated. The total population in the catchment area of the Black Sea is about 160 million, almost half of which is from non-coastal countries in the catchment area of the Danube (Sari et al., 2018). It has a permanent population of about 6 million, with another 4 million tourists during summer. Another important pollutant source is the Mediterranean inflow that transports municipal and industrial pollutants from the mega metropolitan Istanbul city with a population of 16 million (Sari et al., 2018).

Fig. 1. Map of the Black Sea in Europe.

The Black Sea's primary pollution sources are the rivers flowing into it. The major rivers flowing into the Black Sea and their discharges are the Danube (203 km^3 yr^{-1}), Dnieper (Dnepr) (54 km^3 yr^{-1}), Dniester (Dnestr) (9.3 km^3 yr^{-1}), Don (28 km^3 yr^{-1}), and Kuban (13 km^3 yr^{-1}) (Bakan & Özkoç, 2007). Furthermore, many smaller rivers along the Turkish and Bulgarian coasts contribute another 28 km^3 yr^{-1} to the water budget of the sea (Bakan & Özkoç, 2007). Moreover, the Danube is the major river delivering 58% of the total freshwater and sediment inputs to the Black Sea (Mee, 1992; Müftüoğlu, 2013). It is 2850 km long, with a drainage area of 817000 km^2 (Sari et al., 2018). The Danube and the other major rivers flow through the central and eastern European industrial towns and agricultural areas, and transport significant pollutants and natural inputs from mineralized and high-background rock-bearing areas. It drains into the Black Sea, passing through the lands of Germany, Austria, Slovakia, Hungary, Croatia, Serbia, Romania, Bulgaria, and Ukraine. 81 million people living in the drainage area influence its hydrological system (Sommerwerk et al., 2010). Hence, the Black Sea's sediments and water have been adversely affected by the transport of riverine anthropogenic pollutants over the last few centuries (Guieu et al., 1998; Secrieru & Secrieru, 2002; Yigiterhan & Murray, 2008; Mülayim & Balkıs, 2015) and sadly, this problem persists.

Plastic pollution is an environmental issue in many seas worldwide (Lebreton et al., 2017; Ryberg et al., 2019). The Black Sea is no exception, and it suffers from land-based pollution, including cities and sewage systems (Lechner et al., 2014; Berov & Klayn, 2020; D'Hont et al., 2021; González-Fernández et al., 2021; Pojar et al., 2021). The environmental problems are getting worse because the sea is semi-enclosed; thus, plastics tend to accumulate over time; the drainage area of the sea is approximately 2.5 million km^2 and is divided into 107 sub-basins, which drain through more than 20 countries located in the European and Asian continents; the sea also receives plastics from the three large transboundary rivers: Danube, Don, and Dnieper (Strokal et al., 2022).

There are already several surveys of marine litter on beaches, floating litter in the waters, and plastic pollution of the sediments that have been carried out in Turkey and Romania (Topçu et al., 2013; Suaria et al., 2015; Aytan et al., 2016; Kilinc, 2017; Oztekin & Bat, 2017; Săvucă et al., 2017; Terzi & Seyhan, 2017; Pojar & Stock, 2019; Aytan et al., 2020; Terzi et al., 2020), including a survey on the Western and Eastern Black Sea waters along the coasts of Ukraine, Russia, and Georgia (Slobodnik et al., 2018).

There are also some studies from Bulgaria (Moncheva et al., 2016; Simeonova et al., 2017; Simeonova & Chuturkova, 2019; Stanev & Ricker, 2019; Berov & Klayn, 2020; Miladinova et al., 2020); however, there is insufficient data on the negative effects of marine litter in the Bulgarian Black Sea, and how (micro)plastics could affect different biological indices in the aquatic organisms. The microplastic pollution in the Black Sea in Bulgaria as an environmental problem and its potential toxic threats for aquatic animals in Bulgaria has been recently reviewed by Todorova et al. (2023).

Marine Strategy Framework Directive

Within the Marine Strategy Framework Directive scope, the Member States of one marine region and neighboring countries, which share the same marine waters, must cooperate to protect the marine environment more effectively (Coatu et al., 2016). The Marine Strategy Framework Directive (MSFD, 2008/56/EC) requires all the EU Member States to achieve Good Environmental Status (GES) of the marine environment by 2020, considering 11 key descriptors of environmental status (Orlando-Bonaca et al., 2022). The presence of hazardous substances in biota represents relevant criteria and indicators for assessing the status of the Black Sea environment under Descriptor 8 ("Concentrations of contaminants are at levels not giving rise to pollution effects") and 9 ("Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards") (Coatu et al., 2016). Descriptor 10 (Properties and quantities of marine litter do not cause harm to the coastal and marine environment) focuses on the emerging problem of marine litter and its effects on the marine environment and biota. Specifically, the secondary criterion D10C3 defines that "The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned," "Litter and micro-litter classified in the categories 'artificial polymer materials' and 'other,' assessed in any species from the following groups: birds, mammals, reptiles, fish and invertebrates," and that "Member States shall establish that list of species to be assessed through regional or subregional cooperation." Indicators for this criterion should be developed to assess the GES in the current implementation cycle of MSFD. At the moment, this criterion has not been fully evaluated, as the threshold values for levels that may have lethal or sublethal effects on marine organisms have not yet been identified, while they should still be defined through regional or subregional cooperation. In addition, the secondary criterion D10C4 outlines "The number of individuals of each species, which are adversely affected due to litter, such as by entanglement, other types of injury or mortality, or health effects." Although this criterion appears ready to be used, monitoring campaigns for the census of marine organisms affected by litter are relatively occasional. A magnitude ranking of injuries due to litter (and explicitly plastic) ingestion has still to be developed. Eventually, the Commission Decision 2010/477/EU identified the indicator "Trends in amount and composition of litter ingested by marine animals (e.g., stomach analysis)" (10.2.1) (Galgani et al., 2013; Orlando-Bonaca et al., 2022).

According to the Barcelona Convention (2016), "Marine pollution knows no border, pollution in one country affects all other 21 countries." The awareness of the need for a regional approach has resulted in many formal and informal initiatives at global and regional levels (e.g., UNEP Regional Seas Program, 1974; OSPAR, 2009). Moreover, the "Common Indicator 18" of the Barcelona Convention proposed loggerheads as indicators of marine debris levels ashore or at sea for monitoring, achieving, or maintaining the GES, as defined by D10 of the MSFD (Barcelona Convention, 2016). In addition, the Integrated Monitoring and Assessment Program (IMAP) adopted in 2016 includes Ecological Objective 10 on Marine Litter (IMAP, 2016). Within this framework, the proposal for marine litter monitoring also includes Candidate Indicator 24: "Trends in the amount of litter ingested by or entangling marine organisms focusing on selected mammals, marine birds, and marine turtles (EO10)". So far, the most suitable species for this indicator have not been identified yet (Orlando-Bonaca et al., 2022).

Mussels as an important food source in aquaculture

Mytilus species (Fig. 2) are common in temperate seas all around the globe. They are widely used both as seafood, not just as sentinel organisms in monitoring anthropogenic pollution trends in marine waters (Goldberg, 1975, 1980; Farrington et al., 2016). Blue mussels have been an important food for humans for thousands of years, and mussel farming dates back to the Ancient Romans (Beyer et al., 2017). Mussels are economically important food species, accounting for more than a third (roughly 470 thousand tons) of production by weight of the aquaculture industry in the European Union (Eurostat, 2016). Therefore, ingestion of mussel seafood contaminated with various pollutants is a key source of potential health risks, such as neurotoxic, carcinogenic, and cardiovascular diseases for a man (Ersoy & Çelik, 2009). This is of primary concern in the case of top marine predators, as some metals and persistent organic pollutants (dichlorodiphenyltrichloroethane, DDT) accumulation tends to magnify along the food web, resulting in a higher potential risk to human health when high trophic level predators are consumed (Barone et al., 2018).

In recent years marine aquaculture along the Bulgarian Black Sea coast has been mainly related to the construction of mussel farms, which number has exceeded 30 installations. The mussels from the family Mytilidae, are important and prospective species traditionally consumed in Bulgaria and Europe (Cammilleri et al., 2020).

Mediterranean (black) mussels (*M. galloprovincialis*) are a valuable protein source, and the favorable conditions of the Black Sea (temperature, salinity, and food availability) stimulated mussel farming in this region. In recent years, the growing market interest in this species is based on the proven high nutritional quality and *M. galloprovincialis* have a future as a promising source of high-quality protein, polyunsaturated fatty acids, and essential macro and microelements (Peycheva et al., 2021a). Mussels are actually the only bivalves cultured on the Bulgarian coast of the Black Sea (Ministry of Agriculture and Food, 2016). They are mainly cultured on ropes suspended in the water column and attached to rafts. The aquacultured mussels are usually suspended 3-4 m above the sea bottom (Executive agency for fish and aquacultures - IARA, 2017). A few studies characterized this species as a beneficial food that could provide a well-balanced chemical composition and, through their consumption, could prevent various nonchronic diseases (Özden et al., 2010; Petrova-Pavlova et al., 2014; Merdzhanova et al., 2019; Peycheva et al., 2021b).

Blue mussels (*M. edulis*) are bivalve mollusks widely consumed as seafood, with a high content of proteins, omega-3 fatty acids, and vitamins (Akre et al., 2019; Gomez-Delgado et al., 2023). Moreover, Black Sea mussels are one of the most perspective novel food sources with a protein content equal to cattle meat. The mussels are also important natural biofilters – at 17°C temperature of the water, one mussel can filter almost 3 liters of seawater per hour. In recent years in Bulgaria, an increasing interest in this animal as profitable breeding culture has been increasing. However, today due to the industrial pollution of the Black Sea, there are serious obstacles in mussel farms (Ganchev et al., 2012). In this regard, the spatial extent of mussel beds in the Black Sea and Sea of Marmara was reported to have been declining drastically over the past years

(Zaitsev & Mamaev, 1997). The total biomass of mussels in the Black Sea was estimated at 25×106 tons in the 1960s, dropping to 7×106 tons in the 1980s, with juvenile specimens predominating (Zaitsev, 1992). Mass mortalities of *M. galloprovincialis* were observed in the region due to siltation and hypoxia (Gomoiu, 1992; Ozturk & Ozturk, 1996). Moreover, eutrophication, coastal constructions, sea dumping, and ship accidents (such as Rabunion-18, sinking with 20000 live sheep in the Bosphorus) were reported to be the leading cause of hypoxia in the region (Yurdun et al., 1995; Topçu et al., 2019).

Mussels as important sentinel organisms in aquatic toxicology

Using bioindicators as sentinels of the environment's health is a well-established biotechnology that provides qualitative and quantitative information on the impact of numerous pollutants and stressors (Siddig et al., 2016). The selection of appropriate bioindicator species follows general criteria that can also be applied to marine plastic pollution and should allow different marine habitats to be monitored. Given the ubiquitous nature of plastics in the oceans, cosmopolitan marine species should be considered the primary sentinels of environmental impacts. Larger ecological niches allow organisms to detect the same disturbances or stressors in different habitats (Bartell, 2006; Urban et al., 2012).

Any good bioindicator, in particular, should have some basic characteristics, including natural occurrence, abundance, ease of identification and sampling, moderate tolerance to disturbances and stresses, and broad geographic distribution corresponding to a range of exposures to a certain pollutant or stressor (Carignan & Villard, 2002; Caro, 2010).

The high number of taxa impacted by different marine plastics underscores the magnitude of this threat to biodiversity. Some key taxa, such as sea turtles and marine mammals, and various pelagic fish species were particularly affected. Furthermore, to date, numerous species of different taxonomic groups have been used as bioindicators of various marine pollutants, such as mollusks (Naimo, 1995; Guerlet et al., 2007; Dirrigl et al., 2018; Yancheva et al., 2018; Yancheva et al., 2022), among them - mussels, in particular, the blue mussel (*Mytilus edulis* Linnaeus, 1758) and the Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) (Cappello et al., 2013; Beyer et al., 2017), as well as various fish species (Yancheva et al., 2015).

Mytilus spp. as bioindicators for aquatic pollution

The genus *Mytilus* includes several closely related (congeneric) species (or subspecies) that can interbreed with each other and make fertile hybrids. It is often called the *Mytilus edulis* complex. Although the exact taxonomy within the *Mytilus* genus is not yet fully clarified, recent research indicates there are five species occurring in the Northern Hemisphere (*M. edulis, M. galloprovincialis, M. trossulus, M. californianus*, and *M. coruscus*) and two in the Southern Hemisphere (*M. galloprovincialis* and *M. platensis*). In contrast, the former *M. chilensis*, is currently considered a variant of *M. platensis* (Gaitan-Espitia et al., 2016). About 3.5 million years ago, *M. trossulus* and M. *edulis* diverged genetically, then around 2.5 million years ago, *M. edulis* and *M. galloprovincialis* diverged too (Fraïsse et al., 2014).

The blue mussel (*M. edulis*) and the Mediterranean mussel (*M. galloprovincialis*) are two well-known “early warning” bioindicators of marine pollution that are increasingly used to monitor the presence of microplastics (Avio et al., 2017; Phuong et al., 2017). Mussels are sessile suspension feeders that ingest microplastics to 500-fold higher concentrations (Van Cauwenberghe & Janssen, 2014; Van Cauwenberghe et al., 2015). Although short-term exposure to microplastics may not have significant biological effects (Browne et al., 2008), ingestion of microplastics by mussels has been shown to result in disruptive effects, such as a reduction in filtering activity (Wegner et al., 2012), tissue-dependent changes at the transcriptome level (Détrée & Gallardo-Escárate, 2017), histological changes, and severe inflammatory responses (von Moos et al., 2012).

M. edulis is boreo-temperate in its distribution on both coasts of the Atlantic Ocean. It is found in abundance, intertidally and subtidally, in both sheltered and exposed sites, attached to hard substrates or forming biogenic reefs. As summarized by Lynch et al. (2014), in the western Atlantic, *M. edulis* is historically found from the Arctic Sea, Canada (Dall, 1889) to North Carolina, United States (Stimpson, 1860; McDougall, 1943) and in the eastern Atlantic occurs from Norway (Christiansen, 1965) to the border of France and Spain (Sanjuan et al., 1994). In favorable conditions, *M. edulis* can grow to a shell length of more than 10 cm and have a lifespan of more than 20 years (Powell & Cummins, 1985; Sukhotin et al., 2007), although specimens larger than 8 cm and older than 10 years are uncommon.

M. edulis is a keystone coastal species with essential roles in ecosystem functioning, including habitat formation for diverse benthic communities (Joint Nature Conservation Committee, 2008) and nutrient recycling. They play an important role in benthic-pelagic coupling by removing large quantities of suspended organic matter from the water by filter-feeding, and through the production of faces and pseudofaces (Ward & Shumway, 2004) and process large volumes of water; for example, under optimal algal conditions, a 21.5 mm sized mussel will filter an average of 15 mL min^{-1} (Riisgård et al., 2014). Coupled with their wide geographical range and low metabolic transformation rates, these traits make blue mussels useful in monitoring programs for many potential and dissolved chemical pollutants (Scott et al., 2019).

M. galloprovincialis is endemic to the Mediterranean, Black, and Adriatic Seas and has expanded its range to the British Isles (Gosling, 1992). As explained by Livingstone (1992) and Uluturhan et al. (2019), the sessile nature of Mediterranean mussels renders them ideal candidates for molecular to physiological and ecological studies, and biomonitoring purposes of the water column.

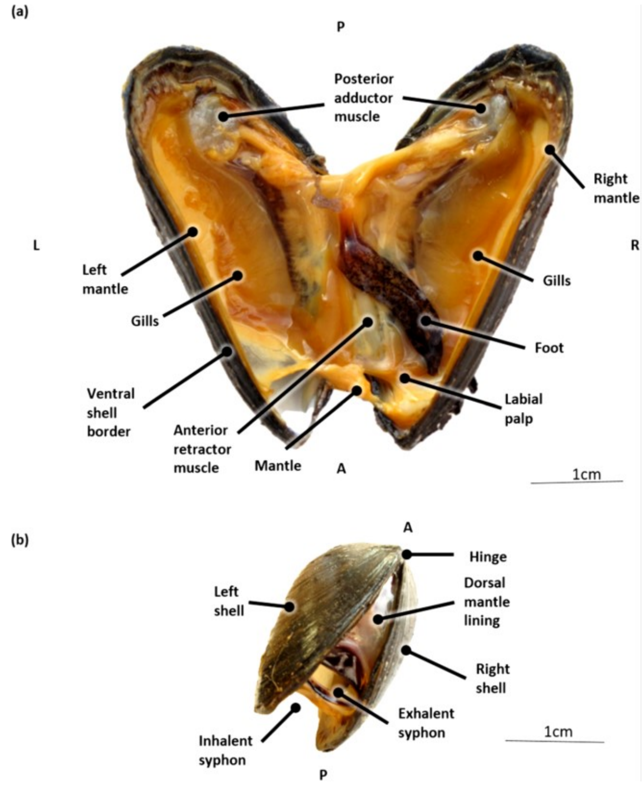

Fig. 2. *Mytilus edulis* - general anatomy and morphology of as presented by Eggermont et al. (2020).

Biomarkers in *Mytilus* species

The concept of biomarkers was set for the first time in 1987 by the US National Research Council (NRC) and defined as "xenobiotically-induced variations in cellular or biochemical components or processes, structures or functions that are measurable in a biological system or sample". In addition, due to their quick response to stress and potentially high toxicological relevance, biomarkers are often considered early warning indicators in detecting molecular, biochemical, or ecological effects (Dellali et al., 2021).

To assess the impact of ingested debris on marine organisms, Fossi et al. (2020) proposed a threefold monitoring approach that can combine an accurate measurement of debris and microplastic levels in animals, the assessment of plastic additives and persistent organic pollutants levels in tissues, and associated toxicological effects. According to Fossi et al. (2018a) and Fossi et al. (2018b), such a monitoring approach should be based on three types of collected data, and the three evaluations can be applied independently or simultaneously for different kinds of bioindicators (e.g., commercial species, protected species, stranded and/or hospitalized organisms, etc.): 1) the analysis of gastrointestinal contents to assess the debris (especially plastics) ingested by the animals, focusing on the occurrence (%), abundance (number), weight (g), color, size, and polymer topologies of the ingested macrodebris and microplastics; 2) the qualitative -quantitative analysis of plastic additives (various phthalates and polybrominated diphenyl ethers) and persistent, bioaccumulating, and toxic substances used as "potential" plastic tracers in the bioindicators tissues; 3) the analysis of the effects of debris ingestion through biomarker responses at different levels of biological organization (from variations in gene/protein expression to histological changes).

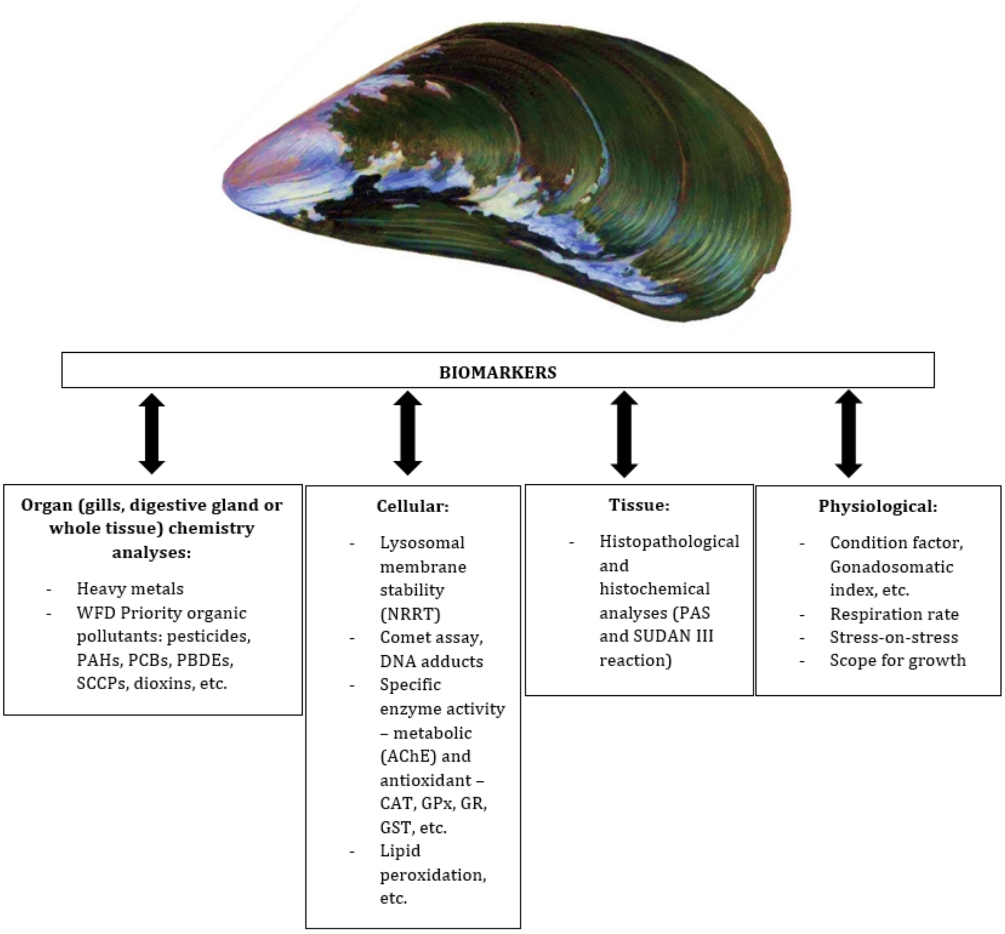

Fig. 3. Biomarkers in *Mytilus* species for assessing the effects of water pollution.

Here we present some of the most common and reliable biomarkers, which can be applied in *Mytilus* species when assessing aquatic pollution (Figure 3):

Neutral red retention time in lysosomes

Unlike fish, mussels do not have the same ability to metabolize organic compounds, but detoxification processes exist through other complex mechanisms that occur partly in the lysosomal compartment. Hence, the lysosomes play an important role as a natural immunological defense system in invertebrates (Lowe & Pipe, 1994; Lowe et al., 1995; Moore & Willows, 1998; Akcha et al., 2004; Mamaca et al., 2005). At the subcellular level, the lysosomal system has been identified as a particular target for the toxic effects of contaminants, and pathological alterations in lysosomes have been especially useful in the identification of adverse environmental impacts on marine organisms (Moore et al., 1996; Giamberini & Pihan, 1997; Moore et al., 2009).

When marine mollusks, such as mussels, are exposed to xenobiotics, one of the characteristic pathological alterations is decreased integrity of the lysosomal membrane (Moore, 1988). Lysosomal membrane integrity has also been reduced with increasing nonspecific stress (i.e., biotic and abiotic) (Moore, 1985). The mechanisms causing this alteration in membrane stability needs to be better understood. Still, it may involve the direct effects of chemicals on the membrane or the increased frequency of secondary lysosomes in toxicant-stressed cells (Mayer et al., 1992).

Lysosomes are important subcellular organelles that contain many hydrolytic enzymes, perform protein degradation and detoxify some foreign compounds. At the cellular level, lysosomal digestion pathways include phago-cytosis, endocytosis, and autophagy. The lysosomal membrane protects the cytosol and the rest of the cell from leakage of degradative enzymes. However, malfunctioning of lysosomes and their accumulation of toxic pollutants have been linked to lysosomal storage diseases and result in lysosomal injury and oxidative damage, in some cases leading to cell death (Moore et al., 2007). In this regard, the neutral red retention time (NRRT) assay takes advantage of this phenomenon by measuring the decreased retention time of neutral red dye within phagocytic haemocytes of a range of aquatic organisms, including mussels (Regoli, 1992; Tedesco et al., 2008). In the popular sentinel species, *M. edulis*, the haemocytes (from the adductor muscles or digestive gland) are essential immune system components (Hu et al., 2015). Therefore, NRTT has been reported as a useful indicator of the organism's overall health status because animals exposed to pollutants often have compromised lysosomal stability (Borenfreund & Puerner, 1985; Moore et al., 2009) and the loss of red dye in the cytosol (reduction of NRRT) indicates destabilization of the lysosomal membrane.

Oxidative stress

Biomarker responses in bivalves include the induction of antioxidant enzymes. They have been widely used to assess organic xenobiotics' impact in marine environments (Doyotte et al., 1997; Lau & Wong, 2003). During detoxification, enzymes, such as carboxylesterases (CEs), convert toxic compounds into more hydrophilic and reactive molecules to facilitate their elimination. In the second step, glutathione transferases (GSTs) conjugate xenobiotics metabolites with glutathione to convert them into more hydrophilic and less reactive molecules (Falfushynska et al., 2019). Accompanying the detoxifying process, reactive oxygen species (ROS) can be produced.

In front of this, organisms have developed a complex antioxidant system to avoid oxidative stress damage (Livingstone, 2001; Rios-Fuster et al., 2022). The antioxidant system, which is composed of enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRd), and glutathione-S-transferase (GST) (Capó et al., 2015; Capó et al., 2021) serves as a shield, which crucial role is to protect the cells against the ROS harmful effects and to reduce the possible damage due to their high reactivity (Vidal-Liñán et al., 2010; Kourdali et al., 2022).

However, if the ROS production is beyond the organisms' elimination capabilities, ROS can lead to the production of several biomolecules, such as malondialdehyde (MDA), which are lipids generating oxidative products and thus, can be used as biomarkers of oxidative damage (lipid peroxidation) (Bartoskova et al., 2013).

DNA damage

Many harmful substances sporadically present in the water can bioaccumulate in living organisms, induce DNA and/or cell damage, and even enter the trophic chain and affect distant ecosystems if some components of such chains are migratory. Increasing attention is being paid to using micronuclei (MN) as an index of cytogenetic damage in aquatic organisms.

Mussels are potentially suitable biological indicators of genotoxic pollution (Venier & Canova, 1996; Dixon et al., 2002). The induction of MN in different mussel species exposed to genotoxic compounds has been reported in several studies (Parolini et al., 2010; Dallas et al., 2013; Liu et al., 2014). Therefore, *Mytilus* spp. can be considered suitable for routine in situ surveys of genotoxic pollutants employing the micronucleus test as a biomarker, supporting Viarengo & Canesi (1991).

According to Bolognesi & Hayash (2011), the MN assay validation process in the genus *Mytilus* started more than 20 years ago (i.e., Heddle et al., 1983; Wrisberg & van der Gaag, 1992). Dose-related induction of MN by different pollutants or polluted water containing various mixtures of contaminants have been reported in gill cells and haemocytes - mitomycin C (Majone et al., 1987, 1990), ethyl methanesulfonate (EMS) (Wrisberg et al., 1992; Jha et al., 2005), dimethylbenz[a]anthracene (Bolognesi et al., 1996), benzo[a]pyrene (Venier et al., 1997), bisphenol A (Barsiene et al., 2006), phenanthrene (Koukouzika & Dimitriadis, 2008), and heavy metals (Bolognesi et al., 1999; Duroudier et al., 2021).

Histopathological and histochemical analyses

Histopathology is a sensitive tool for diagnosing direct and indirect toxic effects that affect the tissues (Kent et al., 2013); therefore, it is considered an excellent method for assessing environmental quality (Bignell et al., 2011). In addition, according to Hinton & Lauren (1990), histopathology is often the easiest method of assessing both short and long-term toxic effects for field assessments. On the other hand, Wester & Canton (1991) state that the histopathological methods are relatively labor-intensive and require good experience, but after all, they have the considerable advantage that pathological alterations in different tissues (e.g., gills, liver) can be observed individually, creating a direct link with physiological functions, such as growth, reproduction, respiration, and nutrition.

The histochemical techniques help to analyze the localization of lipids and glycogen at the cellular level. Furthermore, histochemistry's main advantage lies in analyzing biological phenomena in "particular cells". In this regard, the intensity of staining can be used for comparing the lipid and glycogen contents present in the (gills) cells of normal mussels compared to treated ones with different toxic compounds (Pathan et al., 2009).

Based on the studied literature on histochemical changes triggered by various toxicants, we can state that the histochemical methods, such as PAS (glycogen) and Sudan III (lipids) reactions mainly concern vertebrates, such as fish (and the liver), and to some smaller extent - invertebrates, such as mussels (and the gills and digestive gland) (Drastichová et al., 2005; Wolf & Wolfe, 2005, Figueiredo-Fernandes et al., 2006; El-Serafy, 2009; Singh, 2014).

Furthermore, glycogen synthesis and degradation mechanisms are studied primarily in mammal tissues, such as the liver (Smythe & Cohen, 1991; Bollen et al., 1998). These mechanisms seem similar in the gills, which are energy-consuming organs. Moreover, the mussel gills are attractive models for ecotoxicological studies because the gills are the first uptake site for many toxicants present in the aquatic environment and are often affected by exposure to pollutants (Gómez-Mendikute et al., 2005). In addition, the histochemical methods are relatively inexpensive compared to biochemical analyses; therefore, we encourage applying these tissue methods in monitoring programs and multi-biomarker approaches.

Biometric measurements

Biometric measurements are the easiest among all biomarkers to study. There are different approaches; some use wet weight, and some use dry weight; however, all are associated with the length and weight of treated and controlled mussels. Here we present the formulas of some of the most common condition indexes, which can provide information about impacted physiological processes:

CI total – (soft tissue weight/total weight) x 100;
CI 2 - soft tissue weight/shell weight;
CI 3 (state index) - soft tissue weight/shell length;
CI 4 (shell component index) - shell weight/shell weight + soft tissue weight;
CI 5 (condition factor) - soft tissue weight/shell lenght^3
(Lucas & Beninger, 1985; Rios-Fuster et al., 2022).

In addition, reproduction can be inhibited by sublethal environmental stress because animals reallocate energy away from gamete production and toward defense, and repair mechanisms (Michalek-Wagner & Willis, 2001).

The gonadosomatic index (GSI) is calculated according to the formula (Roff, 1992):
GSI = gonadal tissue weight / (gonadal + somatic tissue weight).

Stress-on-stress

According to Viarengo et al. (1995), contaminant exposure can decrease mussel tolerance to anoxia. The stress-on-stress (SOS) response represents the survival of mussels in the air (time to kill 50% of sample: LT_{50}; Thomas et al., 1999; Hellou & Law, 2003). It appeared as a sensitive and straightforward indicator of mussels' health, and therefore has been since applied in monitoring programs and laboratory studies with mussels. Holwerda et al. (1985) reviewed the general survival of invertebrates in air and listed blue mussels as surviving more than 30 days, the second highest of 22 species tested (Hammen, 1976). SOS response can also significantly reveal mussel exposure to a mixture of pollutants at very low concentrations, resembling field conditions. Moreover, the methodology utilized to evaluate the stress on stress response is simple, rapid, and low in cost and does not require sophisticated equipment. The SOS response was therefore proposed as an index of general stress at the organismal level, and has been since applied as a monitoring tool for assessing polluted aquatic ecosystems (Hellou & Law, 2003).

Scope for growth

Recent advances in environmental toxicology involving the close coupling of the sensitive stress response (scope for growth - SFG) and pollutant levels in the tissues of mussels have provided a powerful and cost-effective method of assessing environmental pollution (Widdows & Donkin, 1992; Widdows, 1998; Widdows et al., 2002). SFG itself results from various vital functions (filtration, ingestion, absorption, and respiration), a technique involving calculating the energy available for growth (Albentosa et al., 2012). This approach complements the established chemical monitoring programs by assessing whether the recorded contaminant levels are causing deleterious effects and whether all relevant toxicants are being measured (Widdows et al., 2002). In addition, SFG is a biomarker at the individual/whole organism level of biological complexity with high ecological relevance. This is very applicable to biomonitoring programs.

Hence, the SFG concept and method are sensitive indicators of environmental pollution in European waters (Widdows et al., 2002; Halldórsson et al., 2005). As explained by Albentosa et al. (2012), in 2007, the Spanish Marine Pollution monitoring program (SMP) conducted by the Spanish Institute of Oceanography added SFG as an environmental assessment technique to be integrated with the chemical parameters. Determining growth in organisms is one of the most sensitive methods available for detecting, quantifying, and identifying changes over time and space to the water quality of marine ecosystems since growth results from a combination of different physiological processes involved in energy acquisition and consumption. In short, it consists of evaluating the energy acquired by an organism after absorbing the food it has ingested. The difference in the organism's energy available for production (growth and reproduction) is lost in the respiratory and excretory processes.

The calculations of SFG are based upon the following equation:

$$SFG=(I-F)-R=(I \times AE)-R,$$

where I is the consumption of the energy available in the diet, F is the energy lost in the feces, AE is the absorption efficiency, and R is the energy consumed by respiration (Bayne & Newell, 1983).

The clearance rate (CR, expressed in L ind^{-1} h^{-1}) is calculated from the difference between inlet and outlet concentrations for the experimental system according to the equation (Riisgård, 2001):

$$CR=f \times (Ci-Co)/Ci$$

where f is the flow of water expressed in L/h, Ci is the inlet concentration, and Co is the outlet concentration, both expressed in particulate volume units, mm^3/L.

The organic ingestion rate (OIR, mg POM h^{-1}) was obtained by multiplying the clearance rate by the diet concentration (expressed in mg POM l^{-1}).

The absorption efficiency (AE) is calculated from the percentage of organic matter in the food and the feces according to Conover's ratio (1966);

$$AE=[(F-E)/((1-E) \times F)] \times 100,$$

where F is the percentage of organic matter (ash-free dry weight) in the food and E is the percentage of organic matter in the feces.

The absorption rate (AR, mg POM h^{-1}) was obtained by multiplying the ingestion rate by the absorption efficiency (AR=OIR x AE).

Respiration intensity rate

The rate of respiration reflects the metabolic activities of animals and the responses due to changes in the surrounding environment are a good indicator of the adjustment capacity of the organism (Kumar et al., 2012). Bivalve mollusks reflect immediate responses to toxic substances present in the surrounding water through changes in their physiological responses (Basha et al., 1988) and histological arrangement (Kumar et al., 2012). It is known that without time for acclimation, mussels typically reduce their clearance rate (volume of water passing through gills per unit time), thus potentially lowering their intake of oxygen (Aldridge et al., 1987). However, most bivalve mollusks reflect immediate responses to toxic substances present in the surrounding water by changes in physiological responses (Basha et al., 1988). In most cases, the respiration rate increases with the increase of the pollutant concentration and level of toxicity (Kumar et al., 2012). This is because the organism tries to deliver more oxygen to all tissues and organs triggered by the stress caused by toxic exposure.

Respiration intensity is calculated following Tsekov (1989);

$$I = Q_2/G,$$

where I – respiration rate index; G – weight of the mussels, in grams, Q_2 – oxygen consumed by the mussels between the two measurements (the difference between the oxygen levels before and after 1 h, $Q_2 = Q-Q_1$ hour).

Q is calculated following the formula:

$$Q = V \times q,$$

where: Q – total oxygen level; V – water volume, in liters; q –dissolved oxygen levels in 1 liter of water (mg L^{-1}).

Mussel caging and mussel watch programs

Environmental monitoring with mussels is often termed as Mussel Watch Program. Data from such monitoring is available from more than 50 nations, sometimes with data going back to the 1960s (Goldberg, 1986; Cantillo, 1998; Beliaeff et al., 1998). The relationship between the level of waterborne pollutants and bivalve tissue concentrations is well established within the Mussel Watch Program, which monitors over 150 organic and inorganic pollutants, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), the pesticide dichlorodiphenyltrichloroethane (DDT), etc. (National Oceanic and Atmospheric Administration, 2018). Since 1983, the Ontario Ministry of Environment (MOE) has used caged mussels (*Elliptio complanata* Lightfoot, 1786) to monitor contaminants in the Niagara River (Richman et al., 2011). Several studies have suggested that transplanted mussels can also be useful biomonitoring tools for evaluating environmental microplastic pollution (Brate et al., 2018). Mussel caging, transplants, and similar mussel watch programs have yet to be applied in Bulgaria. Gecheva et al. (2020) and Georgieva et al. (2022) are the first to report results from such field experiments in Bulgaria.

The current situation in Bulgaria – challenges and future perspectives

Data on mussels and their various biological disturbances due to pollution in the Black Sea in Bulgaria is extremely scarce. There is some research on the levels of heavy metals and various organic pollutants in the sea and mussels (Stoichev et al., 2007; Rizov & Georgieva, 2010; Georgieva et al., 2016a,b; Peteva et al., 2018a,b; Zhelyazkov et al., 2018; Manev et al., 2020; Bachvarova et al., 2022). At present, there are no studies regarding the spread and bioaccumulation of microplastics or their effects on the marine biota of the Bulgarian part of the Black Sea. Still, Alexandrova et al. (2022) recently provided the first preliminary data on the distribution and accumulation of microplastics in wedge clams (*Donax trunculus* Linnaeus, 1758) from selected localities along the Bulgarian Black Sea coast.

In sum, most of the results concern the qualities of mussels as a source of protein and therefore, the risk to humans (Peteva et al., 2020; Peycheva et al., 2022) or practices for growing and catching them or their importance for aquaculture (Petrova & Stoykov, 2009; Klisarova et al., 2020). Aquaculture is one of the pillars of the European Union's blue economy strategy. It is the subject of increasing interest on the Bulgarian Black Sea coast, but so far, mainly in establishing farms to cultivate *M. galloprovincialis* (Zahariev, 2021). Furthermore, during the last years, there has been an increased interest in cultivating Mediterranean mussels in Bulgaria, and several farms have been created in Sozopol, Kavarna, and Balchik (Nikolov et al., 2010). As mussels are considered a healthy food because of their high nutritional value, including high levels of polyunsaturated fatty acids, especially omega-3 fatty acids, recent research on the nutritional characteristics of shellfish from the Bulgarian coast showed high values of unsaturated fatty acids, high protein, and high fat-soluble vitamin content and how shellfish may provide health benefits for local populations (Merdzhanova et al., 2017; Stancheva et al., 2017; Merdzhanova et al., 2021; Panayotova et al., 2021).

Data on changes in different biological tools due to aquatic pollution is limited. Gorinstein et al. (2003) studied antioxidants in *M. galloprovincialis* as an indicator of Black Sea coastal pollution. Ganchev et al. (2012) tested in laboratory conditions how some spirohydantoins and their derivatives affected the mortality rate of *M. galloprovincialis*. Yakimov et al. (2018) estimated the pro/antioxidant status of M. *galloprovincialis* from different Bulgarian Black Sea coastal area sites and studied the oxidative stress levels to indicate stressful environmental conditions. In their recent study, Yakimov et al. (2020) further investigated the oxidative stress in different Bulgarian Black Sea bivalves - *Chamelea gallina, D. trunculus, M. galloprovincialis*, and their bioindicator potential. So did Nikolova et al. (2018) and Tsvetanova et al. (2022), but they investigated oxidative stress regarding seasonal changes. Nechev et al. (2006) and Nechev et al. (2007) followed the lipid and sterol changes due to the effect of cobalt ions on *M. galloprovincialis*. Peteva et al. (2018b) analyzed the marine toxin levels along the Black Sea food chain (phytoplankton and mussels). They discussed the metabolic changes they could undergo as they moved to higher trophic levels and assessed the potential human risk. In 2017 Vasileva et al. applied the Comet assay as a sensitive tool for genotoxicity assessment of environmental stress in *M. galloprovincialis* from the Bulgarian Black Sea coast.

Other species as potential bioindicators for aquatic pollution in the Black Sea in Bulgaria

Even though most of the results suggest that *Mytilus* spp. are probably the most suitable mollusk bioindicators, including for the assessment of microplastic pollution, not only in Bulgaria, but worldwide (Jamil et al., 1999; Beyer et al., 2017; Li et al., 2018, 2019; Monteiro et al., 2019; Gunaalan et al., 2020; Li et al., 2021; Abelouah et al., 2023; Xu et al., 2023) there are other species, which might need to be studied too.

Petrova-Pavlova (2014) describes that the sand mussel (*Mya arenaria* Linnaeus, 1758) is invasive species for Black Sea, which originates from the northern part of the Atlantic Ocean. In the Black Sea it was transported, probably in larval stage in 1960s. Along the Bulgarian Black Sea coast this species is distributed everywhere, but the largest aggregations are observed in the south coastal area in front of estuaries and bay aquatories. *Anadaraina equivalvis* (Bruguiere, 1789) is an invasive bivalve of Indo-Pacific fauna, which was first found in the Black Sea in 1968 and it has spread into the whole basin. The habitat of this clam is sandy-muddy bottoms between 3 to 15 m depth. *Chamelea gallina* (Linnaeus, 1758) is widely distributed in the Black Sea up to 25 meters, forming aggregations in the sublittoral zone of sandy ground. This species live buried in the sandy sediment at a depth of 15–20 cm and its life cycle is three years (Petrova-Pavlova, 2014).

The veined rapa whelk (*Rapana venosa* Valenciennes, 1846) is a predatory invasive species from Asia. It was first detected in the Black Sea in 1947 and fed mainly on mussels (*M. galloprovincialis*). The veined rapa whelks are suitable for human consumption and are gathered by divers. Data suggests this species could also be used for environmental pollution biomonitoring (Bat et al., 2000; Bat & Öztekin, 2015; Mülayim & Balkıs, 2015; Zhelyazkov et al., 2018).

The wedge clam (*D. trunculus*) inhabits fine sandy habitats of the upper littoral subzone and feeds by filtration on phytoplankton and suspended particulate matter. In the Bulgarian Black Sea coastal zone, *D. trunculus* usually dominates between 1.0 and 6.5 m depth, and is exposed to intense wave action and fluctuations of abiotic environmental factors (Gumus et al., 2020). Although local people do not traditionally consume wedge clams, *D. trunculus* are increasingly being collected with dredges for export due to their high price at foreign markets (Gumus et al., 2020). In this regard, Georgieva et al. (2021) presented results on PAH accumulation in *D. trunculus* from the Bulgarian Black Sea Coast. Georgieva et al. (2021) assessed the state of the marine environment along the Bulgarian Black Sea Coast by analyzing the acetylcholinesterase activity in wedge clam too. Olivieri et al. (2022) reported the first results on the uptake of microplastics in wedge clams from the Mediterranean Sea.

Conclusions

More scientists, among them, biologists and chemists from Bulgaria, are starting to study the negative consequences of Black Sea pollution on various marine species - not only mollusks, but also different economically important fish species. Therefore, we strongly recommend the implementation of a multi-biomarker approach that combines successfully most of the indicated in this review biomarkers for better results, the application of mussel caging, as well as an exchange of collective experience.

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