Multi-biomarker Approach as a Response of Oxidative Stress in *Mytilus galloprovincialis* (Lamarck, 1819) Obtained from the Algerian west coast

Rahma Belmokhtar[1,2*], Fayza Belmokhtar[1,3], Ahmed Kerfouf[4], Mohammed Bey Baba Hamed[1,5]

[1]Laboratory of Aquaculture and Bioremediation, University Ahmed Ben Bella Oran1, 31000 Oran, Algeria
[2]Department of Natural and Life Sciences, University Ibn-Khaldoun, 14000 Tiaret, Algeria
[3]Department of Medicine, University Abdelhamid Ibn Badis, 27000 Mostaganem, Algeria
[4]Laboratory of Eco-Development of Space, University Djillali Liabes, 22000 Sidi Bel Abbes, Algeria
[5]Higher School of Biological Sciences of Oran, BP 1042 Saim Mohamed (X-IAP), 31003 Oran, Algeria
Email: rahma.belmokhtar@univ-tiaret.dz

Abstract

The present study aimed to assess the oxidative stress potential of Mytilus galloprovincialis across four sites along the Algerian west coast from March 2022 to June 2022. Among these sites, three were previously identified as contaminated, while the Barbadjanie site was considered isolated and distant from pollution sources. We measured glutathione (GSH) and malondialdehyde (MDA) levels, as well as the specific activities of glutathione S-transferase (GST) and catalase (CAT) in the digestive glands of the mussels. Our findings revealed a significant decrease in GSH levels in mussels from the contaminated sites, indicating reduced antioxidant capacity. In contrast, MDA levels, GST, and CAT activities exhibited significant increases (P< 0.05) in the same mussels, suggesting heightened oxidative stress and increased cellular damage. To further evaluate the impact of pollution, we applied the Integrated Biomarker Response (IBR) index to the four biomarkers measured. The IBR analysis showed the lowest score for mussels from Barbadjanie, confirming its relatively low pollution impact, and the highest score for mussels from Beni Saf Port, indicating severe pollution effects. This multi-biomarker approach was effective in demonstrating oxidative stress responses in mussels, highlighting its reliability in assessing the impacts of environmental pollution on aquatic organisms. The study underscores the importance of using comprehensive biomonitoring tools to evaluate ecological health and pollution effects in marine environments.

Keywords: *Mytilus galloprovincialis, pollution, oxidative stress, biochemical parameters, Algeria*

Introduction

Mussels have been commonly used as monitoring organisms in several environmental studies. A battery of pollution-stress biomarkers has been developed, including biochemical, cytological, and gene expression-based assays (Saavedra and Bachere, 2006; Venier *et al*., 2006; Tanguy *et al*., 2008). The mussels *Mytilus galloprovincialis* are widely distributed in meso- and eutrophic waters of the Mediterranean Sea. They are common in harbors and also in open waters, where they may be characterized by their high capacity to tolerate and accumulate various pollutants, sedentary lifestyle, wide geographical distribution (Viarengo *et al*., 2007; Banni *et al*., 2014; Faggio *et al*., 2018; Uluturhan *et al*., 2019; Leite *et al*., 2022; Cunha *et al*., 2023).

The assessment of the state of the marine environment requires not only measuring concentrations of pollutants but also the level of cellular responses of marine bivalves to xenobiotics, overexploitation, and climatic changes (Gürkan, 2020). Reactive oxygen species are produced as a response of organisms to exposure to a number of pollutants. Many biomarkers of oxidative stress are used as indicators of pollution status (Capó *et al*., 2021a). Antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRd), glutathione peroxidase (GPx), glutathione S-transferase (GST) and the oxidation products of biomolecules, such as malondialdehyde (MDA) or proteins can be applied to assess the response of organisms to marine pollutants such as microplastics, hydrocarbons, and metals (Oliveira *et al*., 2018; Capó *et al*., 2021b; Provenza *et al*., 2022).

The biomarker responses in mussels can be depending on type of tissue, time of exposure and many other factors (González-Fernández *et al*., 2015). The contamination by heavy metal can have adverse effects on aquatic organisms after assimilation and accumulation. Cadmium (Cd) indirectly induces reactive oxygen species (ROS) production and lipid peroxidation through interference with antioxidant systems (Kamel *et al*.,

*) Corresponding author

https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.29.3.309-320
Received : 13-06-2024
Accepted : 15-08-2024

2014). At the sub lethal level, it can cause physiological effects and growth inhibitions (Chiffoleau *et al*., 2001). The toxic effects of lead (Pb) in the mussel *Mytilus edulis* can cause competition with divalent essential metals and perturb their metabolism especially calcium, magnesium and copper (Belabed, 2010). Copper (Cu) is an important and essential micro-element that acts as a respiratory pigment in marine invertebrates. Its accumulation in the cell is the cause of cytotoxicity, which is manifested by enzymatic inhibition of the glutathione reductase (Kamel *et al*., 2012). High levels of this metal can cause oxidative damage to lipids and proteins. It can also cause DNA deformation.

The main objective of the present study was to evaluate the oxidative stress potential of *M. galloprovincialis* population using four biomarkers: Reduced glutathione (GSH), malondialdehyde (MDA), as well as the specific activity of glutathione S-transferase (GST) and catalase (CAT), to estimating the environmental health of coastal ecosystems in the Algerian west coast.

Materials and Methods

The study area extends along the Algerian west coast, from the extreme west to the coasts of the town of Mostaganem. The choice of the sites was based on the quality of the site according to the distance and the proximity of sources of pollution. Our study was carried out at four sites (Figure 1.). Mussels *Mytilus galloprovincialis* were collected during the period of March 2022 to June 2022 on the Algerian west coast. Port of Ghazaouet, Port of Beni-Saf and Port of Salamandre were selected as contaminated sites, while Barbadjani site was selected as isolated area and very far from sources of pollution. Mussel collection was carried out by hand or by diving between 0 and 2 m below the seawater surface. Mussels were kept alive at 4°C. In the laboratory, the shells were measured and tissues weighted. The selected mussels were between 50mm and 60 mm (Gulf of Maine Council, 1992), which correspond to the size class of metabolically stable adults (Andral *et al*., 2004).

Condition index (CI)

The conditions indices were measured to determine the physiological condition of mussels. The CI was calculated using the ratio of the weight of soft tissue to the total weight (weight of soft tissue (g)/shell + soft tissues + palleal liquid (g)) of the mussel, multiplied by 100 (Amiard *et al*., 1998).

Biomarker analyses

Four biomarkers were analyzed in the mussels (GST: glutathione S-transferase, CAT: catalase, GSH: reduced glutathione, MDA: malondialdehyde). The digestive glands of five mussels were pooled in 1 tube , with 10 tubes from each station. Then, 1g from each tube was ground in phosphate buffer ((100 mM, pH 6.5 for protein, GSH and GST) (100 mM pH7.5 for CAT) and chlorure of potassium buffer 1.15% for MDA, using an Ultra-Turrax homogenizer (1:3w/v). The homogenate obtained was centrifuged at 9000 × g for 20min. The supernatant designated post-mitochondrial fraction (S9), which contained the cytosol, endoplasmic reticulum, Golgi apparatus and cytosolic proteins, was recovered for the biochemical assay.

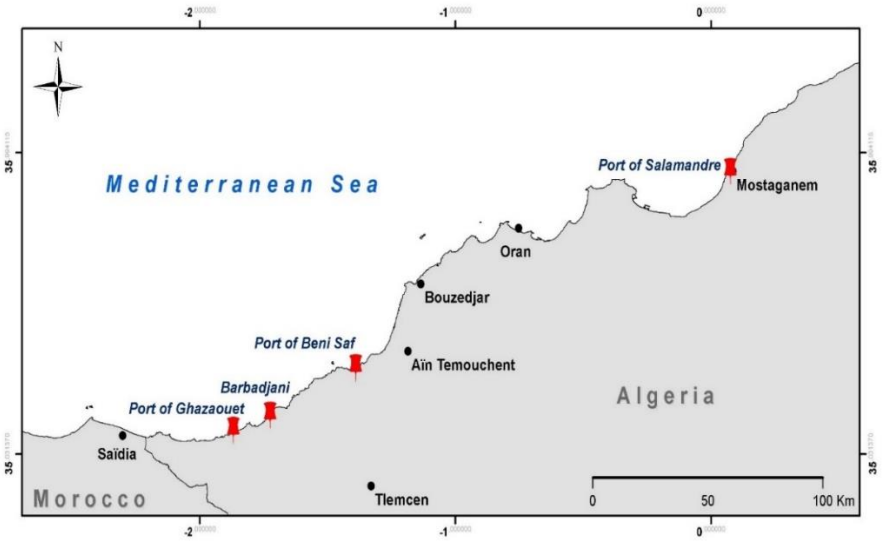

Figure 1. Geographic map of sampling stations for studied *Mytilis galloprovincialis* along the Algerian West Coast.
: Sampling stations

GST activity

The GST activity was performed using a modified method based on Habig *et al*. (1974). 20 mM 1-chloro-2,4-dinitro-benzene (CDNB; Sigma) was used as the substrate and 20 mM glutathione (GSH) as the co-substrate and10µl of the supernatant fraction S9. GST activity was estimated by measuring the formation rate of conjugated substrate (CDNB–GSH). This reaction was determined at 340 nm every minute for 05 min. The results were calculated as nmol of substrate hydrolyzed min^{-1} mg^{-1} relative to the protein in the sample.

CAT activity

CAT activity is determined following the method described by Saint-Denis *et al*. (1998), which measures the rate of enzymatic decomposition of H2O2 at 240 nm every 30s for 01 min. In this measurement, 990 µL of phosphate buffer (100 mM, pH 7.5) was added to 500 ml of H2O2 (500 mM) in a spectrophotometer tank. The enzymatic reaction starts by adding the fraction S9. Enzyme activity was expressed in µ**mole** min^{-1} mg^{-1} protein.

GSH

The GSH level was estimated according to the method of Weckbecker and Cory (1988), based on the absorbance of 2-nitro-5-mercapturic acid resulting from the reduction of 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) by the thiol (SH) group of the glutathione. The yellow precipitate formed was measured with a spectrophotometer at 412 nm. The glutathione concentration was expressed in nmol mg-1 protein.

MDA

The MDA assay was carried out according to the method of Uchiyama and Mihara (1978). This measure was based on the properties that some compounds, such as MDA react with thiobarbituric acid (TBA) to regenerate colored product (pink) which absorbs at 532 nm. MDA contents were determined using 1,1,3,3-tetra ethoxypropane as a standard with the results expressed in nmol mg-1 protein.

Protein

The protein content was determined at 595 nm according to the Bradford colorimetric method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

IBR Calculation

To assess the pollution status of each location, an integrated biomarker response (IBR) index was calculated using all the 4 biomarkers studied. A simple star plot graphic tool (Microsoft Excel) was applied to the data to visualize the results. The calculation procedure of IBR method of Beliaeff and Burgeot (2002) modified by Devin *et al*. (2014) was described below:

The mean value for a site (X) was standardized using the mean value for all sites (m) and the standard deviation for all sites (s) to produce a value we call

$$Y: Y=(X-m)/s$$

For each biomarker, we compute the value

$$Z = Y \text{ or } Z = -Y$$

according to the expected biological effect, respectively activation or inhibition.

The value S was computed, with

$$S = Z+|Min|$$

where Min is the minimal value observed for all sites for each biomarker.

Finally, all the Site values were plotted on a radar diagram. The IBR is calculated as the total area displayed by the radar diagram. All the Si values were shown on a radar diagram and IBR was computed as the sum of the triangles defined by k-standardized biomarkers.

$$IBR= \sum_{i=1}^{k} Ai, \text{ where } Ai=Si\times Si+1\times \sin(2\pi/k)/2.$$

Statistical analyses

Statistical analyses were conducted using STATISTICA 12.0 for Windows. Single biomarker variability between the stations or different heavy metal concentrations was investigated using a standard ANOVA. The significance level was set at $P<0.05$.

Condition index

The results obtained revealed that the maximum value (49.22±6.919) of the CI was found in mussels sampled from Barbadjani, while the minimum value was noted in the port of Ghazaouet (19.04±4.788). A significant difference for the CI was observed between the mussels sampled from the different sites ($P<0.01$) (Figure 2).

It was found that this index changes with the availability of food and the quality of diet (Mougraud *et al*., 2002; Romeo *et al*., 2003). Besides, De los Ríos *et al*. (2013) and Tsangaris *et al*. (2011) added that the highest CI is often observed in contaminated areas near the coast or within a plume of the river, where eutrophic water offers better food conditions for mussels. Akcha *et al*. (2022) indicated that the decrease in the muscle dry weight may be due to the decrease in the lipid content available for storage. Another possible explanation is related to the adaptive response for mussel survival, either through reducing the filtration rate or by the closure of the shell (Akcha *et al*., 2022).

Additionally, mussels can expend more energy on metal detoxification. Sustaining these detoxification mechanisms might demand significant energy consumption. Furthermore, biological factors such as genetic background (Knapen *et al*., 2007), size (Wallace *et al*., 2003), physiological conditions, and absorption routes (Ng *et al*., 2015) can enhance the capacity for metal detoxification (Rouane-Hacene *et al*., 2015).

The change in the CI of bivalves is used as a biomarker that provides integrated measures on the well-being of an organism, and on its physiological status (Lobel *et al*., 1991) and its growth (Lobel *et al*., 1991; Benali *et al*., 2015). This physiological biomarker can provide a good evaluation of the quality of water, particularly regarding the availability of nutrients. It may be influenced by normal parameters, such as the changes in temperature, oxygenation, salinity, availability of food, life cycle, etc. (Paul *et al*., 2003).

Glutathion reduced (GSH)

In GSH analysis, a significant (*P*=0.049) gradient was be observed with the highest value in Barbadjani site (57.8±15.4 nmol.mg^{-1} protein) compared with the other sites. In the port of Ghazaouet, we noted a lowest value (23.61±4.77 nmol.mg^{-1} protein) (Figure 3).

The relationship between the decrease in the GSH rate and the level of environmental contamination has been elucidated in several studies (Gwoździński *et al*., 2010; Sifi *et al*., 2013; Bouzahouane *et al*., 2018). GSH is key in metal scavenging in the organism due to the high affinity of metals to its (-SH) group (Jozefczak *et al*., 2012). It has been proposed to complex and detoxifies metal actions as soon as they enter the cells, representing a first line of defense against metal cytotoxicity.

According to the literature, metal accumulation in biological tissues can result in the reduction of GSH availability (Lavradas *et al*., 2016). Verlecar *et al*. (2007) reported a decrease in GSH level in *P. viridis* exposed to trace metal elements (TME). This reduction was observed in the digestive glands of *Crassostrea virginica*, Ringwood *et al*. (2004) in P. perna exposed to Cd. Also, Santovito *et al*. (2021) showed a decrease in GSH level in *M. galloprovincialis* exposed to Cd for up to 96 h.

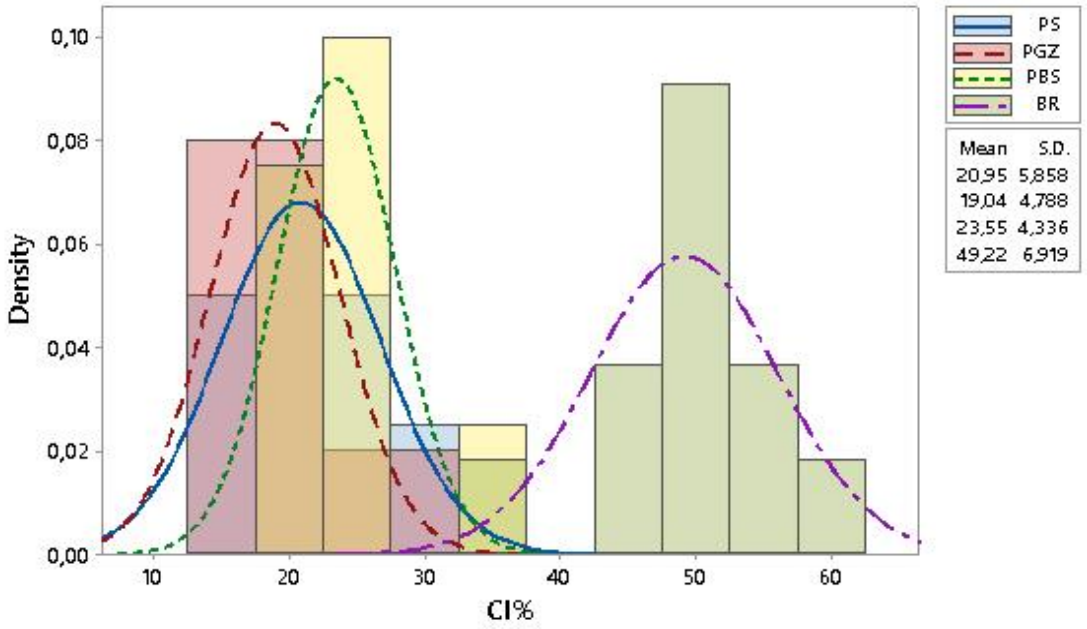

Figure 2. Variations of condition index in *Mytilus galloprovincialis* of different sites of study

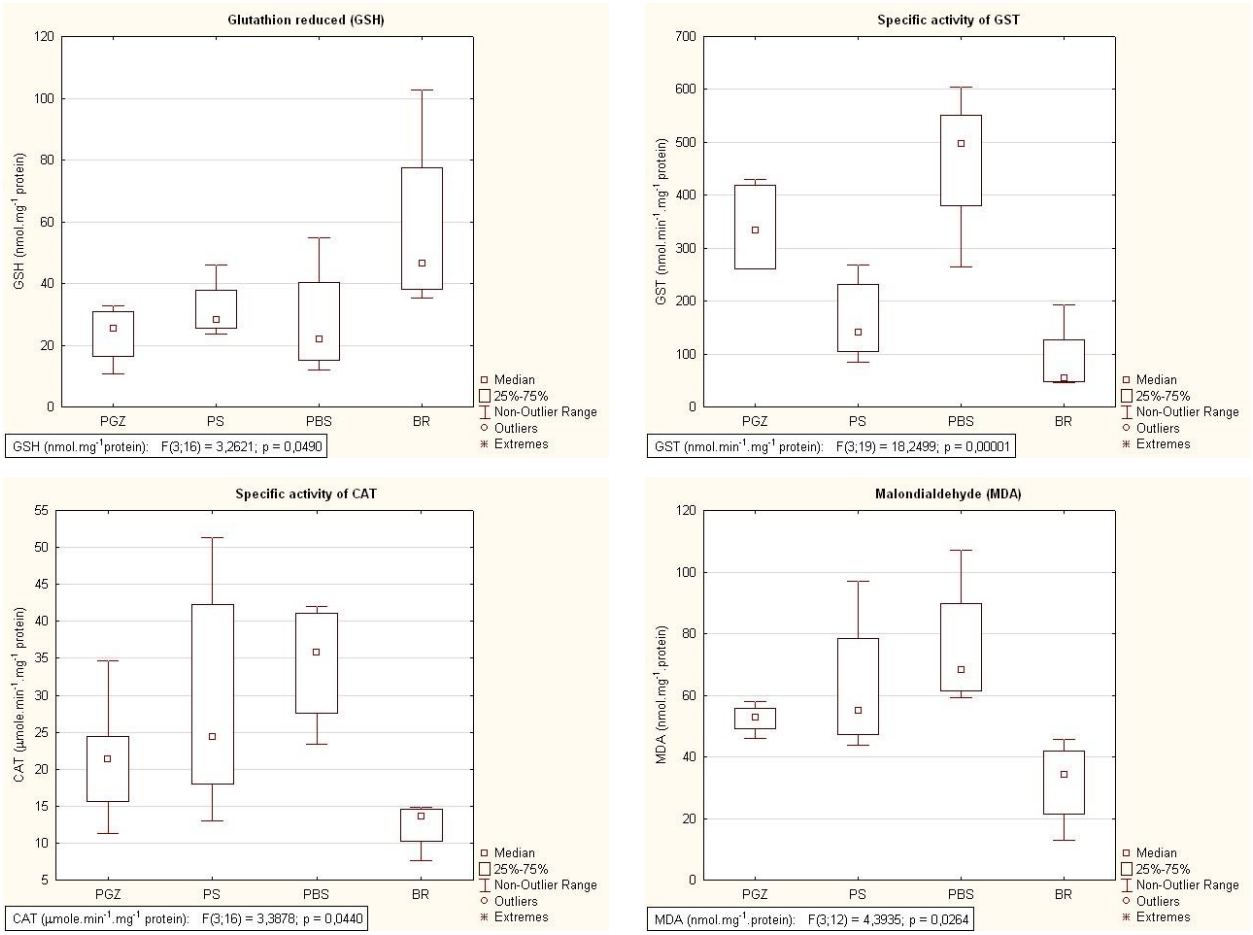

Figure 3. Box plots of biomarkers (GSH, GST, CAT and MDA) in digestive gland of *Mytilus galloprovincialis* at the study stations. Station codes as in Table 1. Each plot shows median, interquartile range, data range for each station shown. PGZ: Port of Ghazaouet, PBS: Port of Beni-Saf, PS: Port of Slamandre, BR: Barbadjani

Glutathion S-transferase (GST)

The highest values for GST activity were recorded at the port of Beni-Saf (465.5±71.6 nmol $min^{-1}.mg^{-1}$ protein). The lowest activity was observed close to the site of Barbadjani (87.1±35.4 $nmol.min^{-1}.mg^{-1}$ protein). A significant difference was been detected between the sites (*P*= 0.00001) (Figure 3).

Our results were similar to their of Van der *et al*. (2003), who showed similar activity between a reference site and a polluted site. Abbassi *et al*. (2015) have described a relationship between environmental pollution and GST activity in *M. galloprovincialis*. Measurement of GST activity in Mohammedia mussels of Morocco showed the response of these lasts to the stress generated by the ready contaminants (El Haimeur *et al*., 2017).

Barhoumi *et al*. (2014) showed that GST was induced in *M. galloprovincialis* by organochlorine pesticides such as Dichlorodiphenyltrichloroethane (DDT). The variation of GST activity may also be related to the age and the reproductive cycle (Giarratano *et al*., 2011). Although some field studies have reported a good response of GST in mussels exposed to organic compounds (Moreira and Guilhermino, 2005), others have detected little or no GST response (De Luca-Abbott *et al*., 2005). However, significant correlations were detected between GST and metal (Hg, Cd and Pb) concentrations in several studies reported for *M. galloprovincialis* (Canesi *et al*., 1999; Khessiba *et al*., 2001). Also, its activity has been found to be increased in the gills of mussels when exposed to Microplastics (Capo´ *et al*., 2021b) and polypropylene leachates (Capolupo *et al*., 2021b).

Catalase (CAT)

CAT activities were relatively different over the sites, and significant difference was noted (*P*=0.044). It ranged from 12.41±1.65 to

34.29±4.27µmol.min^{-1}.mg^{-1} protein for the Barbadjani and port of Beni-Saf respectively (Figure 3.). The significant increase in the CAT activity at polluted sites may suggest that mussels were subjected to oxidative stress. The induction of CAT activity can be attributed to higher levels of exogenous hydrogen peroxide, which is the major cellular precursor of the toxic hydroxyl radical (OH-) (Mejdoub *et al*., 2017). Our results are supported by the work of Benali *et al*. (2015), which focused on the strong CAT activity in *M. galloprovincialis*, on the Algerian west coast. Vlahogianni *et al*. (2007) also observed an increase of the CAT activity at a polluted site.

Guemouda *et al*. (2014) and Amri *et al*. (2017) demonstrated the rise of CAT activity in spring *in P. cultrifera* and *P. lividus*, respectively. The induction of this activity was observed in *M. galloprovincialis* due to TME (Vlahogianni *et al*., 2007); in *R. decussatus* collected from several sites on the Tunisian coast according to pollution gradients (Jebali *et al*., 2007); in *P. viridis* in response to organic contaminants (PAHs) and organochlorine pesticides (Richardson *et al*., 2008); and in *D. trunculus* (Bensouda and Soltani-Mazouni, 2014). This activity was high in *P. perna* in polluted sites compared to reference sites (El Jourmi *et al*., 2015). However, *in vitro* exposure of *M. galloprovincialis* to Cd (5 nm; 10^{-4}–10^{2} mg.Cd.L^{-1}; 24h), Cd^{2+} also caused higher CAT activity in the gills (Katsumiti *et al*., 2014), Also, a higher activity of CAT was found in gills of *M. galloprovincialis* exposed to microplastics (Capo´ *et al*., 2021b). Abidli *et al*. (2021) recorded that polyethylene microplastics can induce alteration in CAT activity.

Malondialdehyde (MDA)

In the port of Beni-Saf a higher MDA levels were recorded (75.6±10.9 nmol.mg^{-1}protein) compared with the others sites sampled, the difference was statistically significant (P=0.0264) (Figure 3). Malondialdehyde (MDA) levels are commonly used to assess oxidative stress as a product of lipid peroxidation of membranes. This reflects damage caused by ROS (González-Fernández *et al*., 2015). The significant increase in MDA formation in mussels from polluted sites was noted in the present study. Our results are similar to the results obtained by Vlahogianni *et al*. (2007), Kamal *et al*. (2014) and Abbassi *et al*. (2015) in *M. galloprovincialis*; by El Jourmi *et al*. (2015) in *P. perna*; and in the same species exposed to Cu and Cd by Khati *et al*. (2012). Machreki-Ajmi *et al*. (2008) showed an increase in MDA concentrations at a site with high Cd levels compared to a reference site. Soltani *et al*. (2012) and Sifi *et al*. (2013) found a high MDA level in *D. trunculus* at a site exposed to industrial pollution. Viarengo *et al*. (1990), who observed the increased lipid peroxidation in the digestive gland of *M. galloprovincialis* exposed to Cu (40 µg l^{-1} ind^{-1}) for six days. The exposure of *M. galloprovincialis* to polystyrene microplastics and nanoplastics resulted in an increase in MDA in the digestive gland, indicating that polystyrene particles may induce lipid peroxidation (Capolupo *et al*., 2021b).

Integrated biomarker response

In order to diagnose and compare the overall physiological state of mussels studied at the different sites, the IBR index was applied to the four biomarkers measured (Beliaeff and Burgeot, 2002). IBR values showed the lowest score for Barbadjani mussels and the highest score for Beni Saf Port mussels (Figure 4).

The highest values in radar graph were recorded at the three most stressful sites for mussel life (Port of Ghazaouet, Port of Beni-Saf and Port of Salamandre). It should be noted that these mussels coming from polluted sites are exposed to continual osmotic stress as a result of the domestic and industrial wastes discharged by the cities of Beni-Saf, Ghazaouet and Mostaganem. This corresponds perfectly with the significantly higher GST, CAT activities and MDA value.

Bocquené *et al*. (2004) combined AChE, GST, CAT activities and MDA concentrations to calculate the IBR index, on the common mussel *M. edulis* in the coast of Brittany (France) and they found that IBR index was strongly connected to MDA levels.

The application of the IBR for the five biomarkers (AChE, CAT, GST, MDA, MTs) by Abbassi *et al*. (2018), made it possible to assess the state of health of mussels on the coast of Sidi Ifni (Morocco) and thus to classify the study sites according to their levels of contamination. Perić and Burić (2019) show that exposing mussels *M. galloprovincialis* to 5 mg.L^{-1} of organo phosphorous pesticide chlorpyrifos resulted in a low IBR value, whereas 15 mg.L^{-1} of metal copper resulted in a high IBR value.

Accordingly, IBR has been proposed as a potentially beneficial method for quantitative monitoring of toxicity-induced stress in mussels. Nonetheless, the multi-biomarker method may not adequately respond to pollutants. Other suitable biomarkers should be combined in determining IBR values in the future with those already taken into account (Yuan *et al*., 2017).

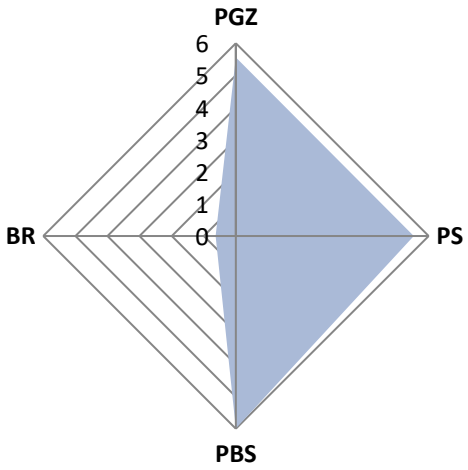

Figure 4. Starplot graphics on integrated biomarker response (IBR) index calculated from 4 biomarkers (GSH, GST, CAT and MDA) at the study stations. PGZ: Port of Ghazaouet, PBS: Port of Beni-Saf, PS: Port of Slamandre, BR: Barbadjani

Conclusion

The results obtained demonstrate the usefulness of the multi-biomarker approach, including the integrated biomarker response index. A global analysis of *M. galloprovincialis* responses of a parameter's battery reflects a discrimination of sites. It could be concluded that there were direct or indirect effects of climatic conditions and/or other exogenous and endogenous factors on the variation in the measured parameters, in particular the pollution level of the site. The simultaneous evaluation of MDA levels in parallel with the antioxidant defenses seems to be a suitable biomarker of toxicity in marine bivalves. This study confirms that variations of antioxidant parameters could be used as prospective biomarkers of toxicity in environmental monitoring programs; and demonstrates that *M. galloprovincialis* is a useful tool in biomonitoring of aquatic pollution and can be employed as a sentinel species.

References

Abbassi, M., Banaoui, A., Kaaya, A., Elkhou, A., Nadir, M. & Lefrere, L. 2015. Biomarker approach to the assessment of the health status of Moroccan marine ecosystems: preliminary study in Sidi Ifni coast (South of Morocco). *J. Materials Environ. Sci*., 6(11): 3086-3093.

Abbassi, M., Charioui, I., Banaoui, A., Nadir, M., Agnaou, M., Bah, M.M.H. & Kaaya, A. 2018. Use of the multimarker approach for the assessment of the state of health of the coastline of Sidi Ifni (Morocco): Index of the Integrated Response of Biomarkers (IBR). *Smetox J.,* 1(1):18- 25.

Abidli, S., Pinheiro, M., Lahbib, Y., Neuparth, T., Santos, M.M. & Trigui El Menif, N. 2021. Effects of environmentally relevant levels of polyethylene microplastic on *Mytilus galloprovincialis* (Mollusca: Bivalvia): filtration rate and oxidative stress. *Environ. Sci. Poll. Res.,* 28: 26643-26652.

Akcha, F., Coquillé, N., Sussarellu, R., Rouxel, J., Chouvelon, T., Gonzalez, P., Legeay, A., Bruzac, S., Sireau, T., Gonzalez, J.L. & Gourves, P.Y. 2022. Trophic transfer of copper decreases the condition index in *Crassostrea gigas* spat in concomitance with a change in the microalgal fatty acid profile and enhanced oyster energy demand. *Sci. Total Environ.,* 824: 153841.

Almeida, A.C., Gomes, T., Langford, K., Thomas, K.V. & Tollefsen, K.E. 2017. Oxidative stress in the algae *Chlamydomonas reinhardtii* exposed to biocides. *Aquatictoxicology,* 189: 50-59.

Amiard, J.C., Geffard, A., & Amiard-Triquet, C. 1998. La métallothionéine chez la moule *Mytilus edulis* comme biomarqueurs de pollution métallique: variabilité´ entre sites, saisons et organes. *J. Rech. Oceanogr.,* 23: 25–30.

Amri, S., Samar, M. F., Sellem, F. & Ouali, K. 2017.Seasonal antioxidant responses in the sea urchin *Paracentrotus lividus* (Lamarck 1816) used as a bioindicator of the environmental contamination in the South-East Mediterranean. *Mar. Poll. Bull.,* 122(1-2): 392-402.

Andral, B., Stanisiere, J.Y., Sauzade, D., Damier, E., Thebault, H, Galgani, F., & Boissery, P. 2004. Monitoring chemical contamination levels in

the Mediterranean based on the use of mussel caging. *Mar. Poll. Bull.,* 49(9-10): 704-712. https://doi.org/10.1016/j.marpolbul.2004.05.008

Banni M., Hajer A., Sforzini S., Oliveri C., Boussetta H., & Viarengo A. 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress Comp. *Biochem. Physiol. PartC: Toxicol. Pharmacol*., 160: 23-29, https://doi.org/10.1016/J.CBPC.2013.11.005

Barhoumi, B., Clérandeau, C., Gourves, P.Y., Le Menach, K., El Megdiche, Y., Peluhet, L., Budzinski, H., Baudrimont, M., Driss, M.R., Cachot, J., 2014. Pollution biomonitoring in the Bizerte lagoon (Tunisia), using combined chemical and biomarker analyses in grass goby, *Zosterisessor ophiocephalus* (Teleostei, Gobiidae). *Mar. Environ. Res.,* 101: 184-195. https://doi.org/10.1016/j.marenvres.2014.07.002.

Beliaeff, B. & Burgeot, T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. *Environ. Toxicol*. *Chem. Int. J.,* 21(6): 1316-1322.

Benali, I., Boutiba, Z., Merabet, A. & Chèvre, N. 2015.Integrated use of biomarkers and condition indices in mussels (*Mytilus galloprovincialis*) for monitoring pollution and development of biomarker index to assess the potential toxic of coastal sites. *Mar. Poll. Bull.,* 95(1): 385-394.

Bensouda, L. & Soltani-Mazouni, N. 2014. Measure of Oxidative Stress and Neurotoxicity Biomarkers in *Donax trunculus* from the Gulf of Annaba (Algeria): Case of the Year 2012. *Annual Res., Rev. Biol*., 4(12): 1902-1914. https://doi.org/10.9734/ARRB/2014/7318

Benzaoui, M.Y., Bentaallah, M.E.A., Ben Naoum, B.E., Kerfouf, A. & Boutiba Z. 2015. Heavy, metals (Hg, Cd, Pb) concentrations on *Mytilus galloprovincialis* (Lamarck, 1819) in Oran coastal waters (Western Algeria): new evidences. *J. Appl. Environ. Biol. Sci.,* 5(6): 1-9.

Bocquené, G., Chantereau, S., Clérendeau, C., Beausir, E., Ménard, D., Raffin, B., Minier, C., Burgeot, T., Leszkowicz, A.P. & Narbonne, J.F 2004. Biological effects of the "Erika" oil spill on the common mussel (*Mytilus edulis*). *Aquat. Living Res.*, 17(3): 309-316.

Bouzahouane, H., Barour, C., Sleimi, N. & Ouali, K. 2018. Multi-biomarkers approach to the assessment of the southeastern Mediterranean Sea health status: Preliminary study on *Stramonita haemastoma* used as a bioindicator for metal contamination. *Chemosphere*, 207: 725-741.

Bradford, M. 1976. A rapid and sensitive assay of protein utilizing the principle of dye binding. *Anal.Biochem.,*772: 242– 264.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M. & Gallo, G. 1999.Heavy metals and glutathione metabolism in mussel tissues. *Aquat. Toxicol.,* 46(1): 67-76.

Capó, X., Company, J.J., Alomar, C., Compa, M., Sureda, A., Grau, A. & Deudero, S. 2021b. Long-term exposure to virgin and seawater exposed microplastic enriched-diet causes liver oxidative stress and inflammation in gilthead sea bream *Sparus aurata*, Linnaeus, 1758. *Sci. Total Environ.,* 767: p.144976. https://doi.org/10. 1016/j.scitotenv.2021.144976

Capó, X., Rubio, M., Solomando, A., Alomar, C., Compa, M., Sureda, A. & Deudero, S., 2021a. Microplastic intake and enzymatic responses in *Mytilus galloprovincialis* reared at the vicinities of an aquaculture station. *Chemosphere,* 280: 130575. https://doi.org/10.1016/j.chemosphere.2021.130575

Capolupo, M., Gunaalan, K., Booth, A. M., Sørensen, L., Valbonesi, P. & Fabbri, E. 2021a.The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean mussel *Mytilus galloprovincialis*. *Environ. Poll.,* 283: 117081. https://doi.org/10.1016/j.envpol.2021.117081

Capolupo, M., Valbonesi, P. & Fabbri, E. 2021b. A Comparative Assessment of the Chronic Effects of Micro- and Nano-Plastics on the Physiology of the Mediterranean Mussel *Mytilus galloprovincialis. Nanomaterials*. 11(3): 649. https://doi.org/10.3390/nano11030649

Cunha, M., Silva, M.G., De Marchi, L., Morgado, R.G., Esteves, V.I., Meucci, V., Battaglia, F., Soares, A.M., Pretti, C. & Freitas, R., 2023. Toxic effects of a mixture of pharmaceuticals in *Mytilus galloprovincialis:* The case of 17α-ethinyl estradiol and salicylic acid. *Environ. Poll.,* 324: 121070. https://doi.org/10.1016/j.envpol.2023.121070

De los Ríos, A., Pérez, L., Ortiz-Zarragoitia, M., Serrano, T., Barbero, M.C., Echavarri-Erasun, B. & Cajaraville, M.P. 2013. Assessing the effects

of treated and untreated urban discharges to estuarine and coastal waters applying selected biomarkers on caged mussels. *Mar. Poll. Bull.,* 77(1-2): 251-265. https://doi.org/10.1016/j.marpolbul.2013.09.050

De Luca-Abbott, S.B., Richardson, B.J., McClellan, K.E., Zheng, G.J., Martin, M. & Lam, P.K. 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters. *Mar. Poll. Bull.,* 51(8-12): 694-707. https://doi.org/10.1016/j.marpolbul. 2005.01.010

De Marchi, L., Coppola, F., Soares, A. M., Pretti, C., Monserrat, J.M., Della Torre, C. & Freitas, R. 2019. Engineered nanomaterials: From their properties and applications, to their toxicity towards marine bivalves in a changing environment. *Environ. Res.,* 178: 108683. https://doi.org/10.1016/j.envres.2019.108683

Devin, S., Burgeot, T., Giambérini, L., Minguez, L. & Pain-Devin, S. 2014. The integrated biomarker response revisited: optimization to avoid misuse. *Environ. Sci. and Poll. Res.,* 21: 2448-2454. https://doi.org/10.1007/s11356-013-2169-9

El Haimeur, B., Zbiry, M., Bouhallaoui, M., Elkhiati, N., & Benhra, A. 2017.Multimarker approach to assess seasonal variation of pollutant effects in *Mytilus galloprovincialis* in the Casablanca region (Moroccan Atlantic coast). *Bull. Soc. Zool. Fr.,* 142(4): 203–223

El Jourmi, L., Amine, A., Boutaleb, N., Abouakil, N., Lazar, S. & El Antri, S. 2015. The use of biomarkers (catalase and malondialdehyde) in marine pollution monitoring: Spatial variability. *J. Mater. Environ. Sci.,* 6: 1592-1595.

Faggio, C., Tsarpali, V. & Dailianis, S. 2018. Mussel digestive gland as a model tissue for assessing xenobiotics: an overview. Sci. of the total environment, 636: 220-229. https://doi.org/10.1016/j.scitotenv.2018.04.264

Giarratano, E., Gil, M.N. & Malanga, G. 2011. Seasonal and pollution-induced variations in biomarkers of transplanted mussels within the Beagle Channel. *Mar. Poll. Bull.,* 62(6): 1337-1344. https://doi.org/10.1016/j.marpolbul.2011.03.037.

Giarratano, E., Gil, M.N. & Malanga, G. 2013. Assessment of antioxidant responses and trace metal accumulation by digestive gland of ribbed mussel *Aulacomyaatra atra* from Northern Patagonia. *Ecotoxic. Environ. Safety,* 92: 39-50. https://doi.org/10.1016/j.ecoenv.2013.02.007

González-Fernández, C., Albentosa, M., Campillo, J.A., José Fumega, L.V., Franco, A., Besada, V., González-quijano, A. & Bellas, J. 2015. Influence of mussel biological variability on pollution biomarkers. *Environ. Res*., 137: 14-31. https://doi.org/10.1016/j.envres.2014.11.015

Guemouda, M., Meghlaoui, Z., Daas, T., Daas-Maamcha, O. & Scaps, P. 2014. Monitoring pollution in East Algerian coasts using biochemical markers in the polychaete annelid *Perinereis cultrifera*. *Ann. Biol. Res*., 5(2):31-40.

Guendouzi, Y., Soualili, D.L., Fowler, S.W. & Boulahdid, M. 2020. Environmental and human health risk assessment of trace metals in the mussel ecosystem from the Southwestern Mediterranean. *Mar. Poll. Bull.,* 151: p110820. https://doi.org/10.1016/j.marpolbul.2019.110820

Gulf of Maine Project 1996. Keystone National Policy Dialogue on Ecosystem Management: Final Report, The Keystone Center (Colorado), B-13.

Gürkan, S.F. 2020. Assessments of antioxidant capacities of Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) from the different stations of Dardenelles Strait (Turkey). *Biharean Biologist*, 14(1): 10-15.

Gwoździński, K., Gonciarz, M., Kilańczyk, E., Kowalczyk, A., Pieniążek, A. & Brichon, G. 2010. Antioxidant enzyme activities and lipid peroxidation in from the French Mediterranean coast. *Oceanol. Hydrobiol. Stud*., 39(4): 33-43. https://doi.org/10.2478/v10009- 010-0059-8.

Jarque, S., Prats, E., Olivares, A., Casado, M., Ramon, M. & Pina, B. 2014. Seasonal variations of gene expression biomarkers in *Mytilus galloprovincialis* cultured populations: Temperature, oxidative stress and reproductive cycle as major modulators. *Sci. Total Environ.,* 499: 363-372. https://doi.org/10.1016/j.scitotenv.2014.08.064.

Jebali, J., Banni, M., Almeida, E. A. D. & Boussetta, H. 2007. Oxidative DNA damage levels and catalase activity in the clam *Ruditapes decussatus* as pollution biomarkers of Tunisian marine environment. *Environ. Monit. Assess.,* 124: 195-200. https://doi.org/10.1007/s10661-006-9217-6.

Jozefczak, M., Remans, T., Vangronsveld, J. & Cuypers, A. 2012. Glutathione is a key player in metal-induced oxidative stress defenses. *Int. J. Mol. Sci.,* 13(3):3145-3175. https://doi.org/ 10.3390/ ijms13033145.

Kamel, N., Attig, H., Dagnino, A., Boussetta, H. & Banni, M. 2012. Increased temperatures affect oxidative stress markers and detoxification response to benzo [a] pyrene exposure in mussel *Mytilus galloprovincialis*. *Arch. Environ. Contam. Toxicol.,* 63: 534-543. https://doi.org/ 10.1007/s00244-012-97903.

Kamel, N., Burgeot, T., Banni, M., Chalghaf, M., Devin, S., Minier, C. & Boussetta, H. 2014. Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from Bizerte lagoon. *Environ. Sci. Poll. Res.,* 21: 6108-6123. https:// doi.org/10.1007/s11356-014-2540-5

Katsumiti, A., Gilliland, D., Arostegui, I. & Cajaraville, M.P. 2014. Cytotoxicity and cellular mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the mussel *Mytilus galloprovincialis*. *Aquatic Toxicol.,* 153: 39-52. https://doi.org/10.1016/ j.aquatox.2014.02.003.

Khati, W., Ouali, K., Mouneyrac, C. & Banaoui, A. 2012. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use in biomonitoring. *Energy Procedia,* 18: 784-794. https://doi.org/10.1016/j.egypro.2012.05.094

Khessiba, A., Hoarau, P., Gnassia-Barelli, M., Aissa, P. & Roméo, M. 2001. Biochemical response of the mussel *Mytilus galloprovincialis* from Bizerta (Tunisia) to chemical pollutant exposure. *Arch. Environ. Contam. Toxicol.,* 40: 222-229. https://doi.org/10.1007/s0024400 10166

Knapen, D., Reynders, H., Bervoets, L., Verheyen, E. & Blust, R. 2007. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquatic. Toxicol*., 82(3): 163-172. https://doi. org/10.1016/j.aquatox.2007.02.008.

Lavradas, R.T., Rocha, R.C., Bordon, I.C., Saint'Pierre, T.D., Godoy, J.M. & Hauser-Davis, R. A. 2016. Differential metallothionein, reduced glutathione and metal levels in *Perna perna* mussels in two environmentally impacted tropical bays in southeastern Brazil. *Ecotox. Environ. Safety*, 129: 75-84. https://doi. org/10.1016/j.ecoenv.2016.03.011

Leite, D.P., Menegotto, I., da Cunha Lana, A.P. & Júnior, L.L.M. 2022. A new look at the potential role of marine plastic debris as a global vector of toxic benthic algae. *Sci. Total Environ.,* 838: p156262. https://doi.org/10.1016/j.scitotenv. 2022.156262

Lobel, P.B., Bajdik, C.D., Belkhode, S.P., Jackson, S.E. & Longerich, H.P. 1991. Improved protocol for collecting mussel watch specimens taking into account sex, size, condition, shell shape, and chronological age. *Arch. Environ. Contam. Toxicol.,* 21: 409-414. https://doi.org/10.10 07/BF01060364

Machreki-Ajmi, M., Ketata, I., Ladhar-Chaabouni, R. & Hamza-Chaffai, A. 2008.The effect of in situ cadmium contamination on some biomarkers in *Cerastoderma glaucum*. *Ecotoxicol.,* 17: 1-11 https://doi.org/10.1007/s10646-007-0166-9

Mejdoub, Z., Fahde, A., Loutfi, M. & Kabine, M. 2017.Oxidative stress responses of the mussel *Mytilus galloprovincialis* exposed to emissary's pollution in coastal areas of Casablanca. *Ocean Coast. Manag*., 136: 95-103. https://doi.org/ 10.1016/j.ocecoaman.2016. 11.018

Monteiro, H.P., Rodrigues, E.G., Reis, A.K.A., Longo Jr, L.S., Ogata, F.T., Moretti, A.I. & Stern, A. 2019. Nitric oxide and interactions with reactive oxygen species in the development of melanoma, breast, and colon cancer: A redox signaling perspective. *Nitric Oxide,* 89: 1-13. https://doi.org/10.1016/j.niox.2019.04.009

Moreira, S. M. & Guilhermino, L. 2005. The use of *Mytilus galloprovincialis* acetylcholinesterase and glutathione S-transferases activities as biomarkers of environmental contamination along the northwest Portuguese coast. *Environ. Monit. Assess.,* 105: 309-325. https://doi.org/ 10.1007/s10661-005-3854-z

Mougraud, Y., Martinez, E., Geffard, A., Andral, B., Stanisiere, J.Y., Amiard, J.C. 2002. Metallothionein concentration in the mussel *Mytilus galloprovincialis*as a biomarker of response to metal contamination: validation in the field. *Biomarkers,* 7: 479–490. https://doi. org/10.1080/1354750021000034528

Ng, J.C., Juhasz, A., Smith, E. & Naidu, R. 2015.Assessing the bioavailability and bioaccessibility of metals and metalloids. *Environ. Sci. Poll. Res.,* 22: 8802-8825. https://doi.org/10.1007/s11356-013-1820-9

Oliveira, P., Barboza, L.G.A., Branco, V., Figueiredo, N., Carvalho, C. & Guilhermino, L. 2018. Effects of microplastics and mercury in the freshwater bivalve *Corbicula fluminea* (Müller, 1774): filtration rate, biochemical biomarkers and mercury bioconcentration. *Ecotoxicol. Environ. Safety*, 164: 155-163. https://doi.org/10.1016/j.ecoenv.2018.07.062

Paul, K.S., Lam, W.U. & Rudolf, S. 2003.The use of bioindicators, biomarkers and analytical methods for the analysis of POPs in developing countries. STAP Workshop. University of Hong Kong, 79 pp.

Perić, L. & Burić, P. 2019. The effect of copper and chlorpyrifos co-exposure on biomarkers in the marine mussel *Mytilus galloprovincialis*. *Chemosphere,* 225: 126-134. https://doi.org/10.1016/j.chemosphere.2019.03.003

Piscopo, M., Ricciardiello, M., Palumbo, G. & Troisi, J. 2016. Selectivity of metal bioaccumulation and its relationship with glutathione S-transferase levels in gonadal and gill tissues of *Mytilus galloprovincialis* exposed to Ni (II), Cu (II) and Cd (II). *Rendiconti Lincei*., 27: 737-748. https://doi.org/10.1007/s12210-016-0564-0

Provenza, F., Rampih, D., Pignattelli, S., Pastorino, P., Barceló, D., Prearo, M., Specchiulli, A. & Renzi, M. 2022. Mussel watch program for microplastics in the Mediterranean Sea: Identification of biomarkers of exposure using *Mytilus galloprovincialis. Ecol. Indic.,* 142: 109-212 https://doi.org/10.1016/j.ecolind.2022.109212

Richardson, B.J., Mak, E., De Luca-Abbott, S.B., Martin, M., McClellan, K. & Lam, P.K. 2008. Antioxidant responses to polycyclic aromatic hydrocarbons and organochlorine pesticides in green-lipped mussels (*Perna viridis*): do mussels "integrate" biomarker responses? *Mar. Poll. Bull*., 57(6-12): 503-514. https://doi.org/10.1016/j.marpolbul.2008.02.032

Ringwood, A.H., Hoguet, J., Keppler, C. & Gielazyn, M. 2004. Linkages between cellular biomarker responses and reproductive success in oysters–*Crassostrea virginica*. *Mar. Environ. Res*., 58(2-5): 151-155. https://doi.org/10.1016/j.maren vres.2004.03.010

Romeo, M., Hoarau, P., Garello, G., Gnassia-Barelli, M., Girard, J.P. 2003. Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. *Environ. Pollut*., 122: 369–378. https://doi.org/10.1016/S0269-7491(02)003 03-2

Rouane-Hacene, O., Boutiba, Z., Belhaouari, B., Guibbolini-Sabatier, M. E., Francour, P. & Risso-de Faverney, C. 2015. Seasonal assessment of biological indices, bioaccumulation and bioavailability of heavy metals in mussels *Mytilus galloprovincialis* from Algerian west coast, applied to environmental monitoring. *Oceanologia,* 57(4): 362-374. https://doi.org/10.1016/j.oceano.2015.07.004

Saavedra C., Bachere E. 2006. Bivalve genomics. *Aquacul.*, 256(1-4): 1-14. https://doi.org/10.1016/j.aquaculture.2006.02.023

Santovito G, Trentin E, Gobbi I, Bisaccia P, Tallandini L, Irato P. 2021. Non-enzymatic antioxidant responses of *Mytilus galloprovincialis*: Insights into the physiological role against metal-induced oxidative stress. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.,* 240: p.108909. https://doi.org/10.1016/j.cbpc.2020.108909.

Sifi, K., Amira, A. & Soltani, N. 2013. Oxidative stress and biochemical composition in *Donax trunculus* (Mollusca, Bivalvia) from the gulf of Annaba (Algeria). *Adv. Environ. Biol.,* 7(4): 595-605.

Soltani, N., Amira, A., Sifi, K. & Beldi, H. 2012. Environmental monitoring of the Annaba gulf (Algeria): measurement of biomarkers in *Donax trunculus* and metallic pollution. *Bull. Soc. Zool. Fr*., 137(1-4): 51-60.

Tanguy A., Bierne N., Saavedra C., *et al*. 2008. Increasing genomic information in bivalves through new EST collections in four species: development of new genetic markers for environmental studies and genome evolution. *Gene,* 408(1-2): 27-36. https://doi.org/10.1016/j.gene.2007.10.021.

Tsangaris, C., Hatzianestis, I., Catsiki, V.A., Kormas, K.A., Strogyloudi, E., Neofitou, C., Andral, B. and Galgani, F., 2011. Active biomonitoring in Greek coastal waters: application of the integrated biomarker response index in relation to contaminant levels in caged mussels. *Sci. Total Environ.,* 412: 359-365. https://doi.org/10.1016/j.scitotenv.2011.10.028

Uluturhan, E., Darılmaz, E., Kontas, A., Bilgin, M., Alyuruk, H., Altay, O. & Sevgi, S. 2019. Seasonal variations of multi-biomarker responses to metals and pesticides pollution in *M. galloprovincialis* and *T. decussatus* from Homa Lagoon, Eastern Aegean Sea. *Mar. Poll. Bull.,*

141: 176-186. https://doi.org/10.1016/j.marpolbul.2019.02.035

Van der Oost, R., Beyer, J. & Vermeulen, N.P. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol*., 13(2): 57-149. https://doi.org/10.1016/S1382-6689(02)00126-6

Venier, L.A. and Pearce, J.L. 2006. The Use of Ground Beetles (Coleoptera: Carabidae) and Spiders (Araneae) as Bioindicators of Sustainable Forest Management: A Review. *Ecolog. Indic.,* 6: 780-793. http://doi.org/10.1016/j.ecolind.2005.0 3.005

Verlecar, X.N., Jena, K.B. & Chainy, G.B.N. 2007. Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. *Chem. Biol. Interact.,* 167(3): 219-226. https://doi.org/10.1016/j.cbi.2007.01.018

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M. N. & Orunesu, M. 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. C. Comp. Pharma*., 97(1): 37-42. https://doi.org/10.1016/0742-8413(90)90168-9

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E. & Koehler, A. 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comp. Biochem. Physiol. C. Toxicol. Pharma*., 146(3): 281-300. https://doi.org/10.1016/j.cbpc.2007.04.011

Vlahogianni, T., Dassenakis, M., Scoullos, M. J. & Valavanidis, A. 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Mar. Poll. Bull.,* 54(9): 1361-1371. https://doi.org/10.1016/j.marpolbul.2007.05.018

Wallace, W.G., Lee, B.G. & Luoma, S.N. 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.,* 249: 183-197.

Yuan, M., Wang, Y., Zhou, B., Jian, X., Dong, W. & Tang, X. 2017. An integrated biomarker response index for the mussel *Mytilus edulis* based on laboratory exposure to anthracene and field transplantation experiments. *Chin. J. Oceanol. Limnol.,* 35: 1165-1178. https://doi.org/10.10 07/s00343-017-60927