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The Use of Biomarkers and Biochemical Composition in Tissues of *Mullus surmuletus* to Assess the Pollution in Coastal Environment of Mediterranean Sea: A Comparative Study

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Abstract: *Environmental pollution is a serious threat for marine organisms. The aim of the present study is to assess and compare between East and West of Mediterranean coast by using antioxidant enzymes (bioindicators) as recommended by the International Council for the Exploration of the Sea (ICES) and MED-POL for monitoring the marine environment at sites particularly exposed to different pollutants e.g. anthropogenic and industrial waste. The activities of the antioxidant enzymes (glutathione-GSH, glutathione transferase-GST and Acetylcholine esterase-AchE) were studied in M. surmuletus. Significant differences are observed in the activity of oxidative stress at investigated regions. There are generally lower activities at West in different organs except AchE. There is highly significant difference of GSH and GST activities in liver and gills at East region. The inhibition of AchE activity explains the reduction of the enzymatic activity in industrial and agricultural region (East). Results showed an increased in GSH content and GST activity in M. surmuletus from East region compared to those West. It can be concluded that the useful of using antioxidant enzymes as environmental monitoring for detecting the variation between pollutants that changes the activity and the organization of antioxidative defense system from East region compared to those from West region.*

Keywords*: Acetylcholinesterase, Glutathione, Glutathione transferase, M.surmuletus, Mediterranean Sea*

I. Introduction

The marine environment is exposed to different anthropogenic pollutants generated by industrial, domestic and agricultural activities. Many pollutants are susceptible to interact with the physiological processes such as growth and reproduction lead to serious disruptions such as reduction of the animal populations, changes of the reproductive functions. The Mediterranean Sea, semiclosed basin surrounded by densely populated and industrialized countries has a low capacity of water interchange with the Atlantic Ocean and other surrounding seas. Alexandria city lies on the Mediterranean coast and it is one of the most important industrial centers, comprised 100 large factories and about 260 smaller ones [1]. It is also the main summer resort in Egypt; about 4 million citizen and two million summer visitors [2]. Human development in the Mediterranean region has extensively influenced the coastal areas and has led to a constant rate of pollution with toxic compounds.

The water in Eastern region (Damietta and Port Said) are exposed to agricultural drains contaminated with hazardous industrial wastes, domestic sewage, organic matter, fertilizers and pesticides, in addition to oil pollution from ships and oil terminal as in Port Said and Damietta [3, 4], while sources of water pollution in Western region (Salloum to El Alamein) are sewage from residential areas and resort areas. Previous studies recorded that heavy metals under certain environmental conditions might accumulate up to toxic concentrations and cause ecological damage [5,6].The use of biomarkers is important in environmental monitoring programs, and also as early warning signals of environmental distress in coastal and marine areas[7,8].

Many studies of antioxidant defense enzyme activities in aquatic organisms, particularly in fish, were designed to provide data for comparative studies or to examine the effects of environmental influences. The activity of antioxidant defense enzymes, glutathione content (GSH), glutathione-S-transferase (GST) and Acethycholinesterase (AchE) were measured in *Mullus surmuletus* collected from both East and West regions of Alexandria city along the Mediterranean Sea coast. Fish have been used as aquatic contamination indicators for many years. Red mullet are among the most valuable and highly priced fish species in Egypt, though widely distributed along the entire coast of Mediterranean, their major fisheries are located in the area from Alexandria to Port Said [9]. In present study the striped red mullet (*Mullus surmuletus)* is chosen as a bioindicator because it is considered a very important economic marine fish in Egypt. It is one of territorial fish of commercial interest in the region, which has been used in several studies of coastal pollution monitoring [10-13].

The major oxidative stress biomarkers in marine fish are SOD, CAT, and GSH-Px, as well as the biotransformation phase II enzyme GST[14]. Glutathione transferase enzyme is one of the phase II biotransformation enzyme systems; this enzyme is also the most sensitive biomarker for the influence of environmental pollution on the organism. It has been used to indicating aquatic environment pollution with

wastewater of municipal, industrial, agricultural or mining origin (organic industrial effluents) [15]. Fish liver is the main organ of xenobiotic metabolism [16], GST may represent up to l0% of total liver cytosolic proteins [17]. GST activity has been studied in various tissues of different fish species and the induction of antioxidant GST enzymes were measured in *Mullus barbatus* liver tissue at 5 stations along the French and Spanish coasts to demonstrate alterations due to the presence of free radicals from pollutant metabolism[18]. Mussels, such as *Mytilus edulis* and other marine bivalves, as well as fish (e.g. *Mullus sp., Platichthys flesus* L., *Zoarces viviparus, Perca sp.*) are widely used in monitoring programs as sensitive indicators, so-called sentinel organisms, of the exposure to, and the biological effects of metals and organic pollutants [19].

Some biomarkers are highly specific for individual chemicals; such biomarkers include inhibition of cholinesterase by organophosphate or carbamate [20]. Acetylcholinesterase (AchE) is an enzyme essential to the correct transmission of nerve impulse. Extensive studies on inhibition of cholinesterase activity by neurotoxic agents have clearly indicated that it can be used as a tool to diagnose organophosphorus pesticide poisoning in fish [21, 22&23] citied by Sarkar *et al*., 2006. Also Many stressors (including pesticides, drugs or metals) exert their activity by acting on the central nervous system; causing neurotoxicity it is caused by accumulation of excess of acetylcholine, cholinesterasic inhibition is a suitable tool [24].

The aim of this study is to assess and compare the activity of antioxidant enzymes in liver, gills and muscles of *M. surmuletus* collected from East and West regions of Alexandria for detecting the exposure and effect induced by chemical pollutants, industrial wastewater and domestic sewage along the Mediterranean coast.

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II. Materials And Methods

1. Sample collection and preparation

Forty five samples of *Mullus surmuletus* were obtained from East and West Mediterranean coast by local fishermen during November and December (2015) (Fig.1). Fifteen of *M. surmuletus* collected from East region with average length and weight (22.0cm, 122.9g). East region affected with intensive industrial wastewater and domestic sewage, fertilizers and pesticides from agricultural activities. Twenty eight of *M. surmuletus* collected from West region with average length and weight (14.1cm, 38.9g). West region affected with intensive anthropogenic sewage from residential areas and resort areas pollution. Fish samples were kept immediately in ice on polyethylene bags and transported to the physiology Lab of NIOF, Alexandria to sustain freshness. Fish liver, gills and muscle tissues were rapidly excised and kept frozen in cold storage at -20^{o}c until biochemical analysis. Samples from each organ were homogenized in Tris-buffer 0.1M, pH 7.5, therefore; centrifugation was carried out at 3000 rpm for 20 min.The supernatant was collected and stored at -20^{o}c for enzymatic determination.

Fig.1: Egyptian Mediterranean coast.

2. Biochemical analysis

2.1. Protein determination

Total protein concentration in the supernatant was determined according to the method of Lowry *et al.*[25] and expressed in mg/g wet mass.

2.2. Lipid determination

Total lipid concentration in the supernatant was determined according to the method of Folch *et al.* [26] and expressed in mg/g wet mass.

2.3. Determination of Reduced glutathione (GSH) content

GSH content was determined by using the method described by Beutler *et al.*[27].The method based on the reduction of 5, 5` dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration. The intensity of the color was measured at 412 nm. Glutathione content was expressed as mg/g tissue wet weight.

2.4. Determination of Glutathione transferase Activity (GST)

The GST activity was determined in liver, gills and muscles according to the method of Habig *et al.* [28] by using 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample using a (Spekoll 11) Spectrophotometer. The enzymes activity was expressed as U/min /gm tissue.

2.5. Determination of Acetylcholinesterase Activity (AchE)

AChE activity was spectrophotometrically determined in (liver, gills and muscles) according to the methodology described by Ellman *et al.* [29]. AchE present in samples degrades acetylthiocholine (substrate); forming acetate and thiocholine.This degradation makes the consequent conjugation of thiocholine with DTNB.This allows quantifying cholinesterase activity by assessing the absorbance increase at wave length of 412 nm. Acetylcholinesterase activity was expressed as U /min /gm tissue.

3. Statistical analyses

Statistical Analysis of data was carried out using SPSS Inc. version (16) statistical package. One-way analysis of variance (ANOVA) and Duncan multiple ranges were used to assess whether the significant difference between liver, gills and muscles among two regions. Correlation coefficient was calculated to analyze the relationship between different values of enzyme activity in investigated organs in East and West two regions. Data were expressed as means and standard deviation (M±SD) and the significance level was set at $P<0.05$.

III. Results

The studied regions of East and West Alexandria along the Mediterranean coast was selected because one receives industrial waste, domestic sewage and pesticides from agricultural activities and the other one receives sewage from residential areas and resort areas. Total protein content in the liver, gills and muscles of striped red mullet (*M. surmuletus*) at both East and West regions is shown in Figure (2).The presented results show that protein content was significantly higher at West than at East in three organs ($p<0.05$).The highest protein content was in muscles at both regions compared with its level in liver and gills, however, the lower value was in gills.

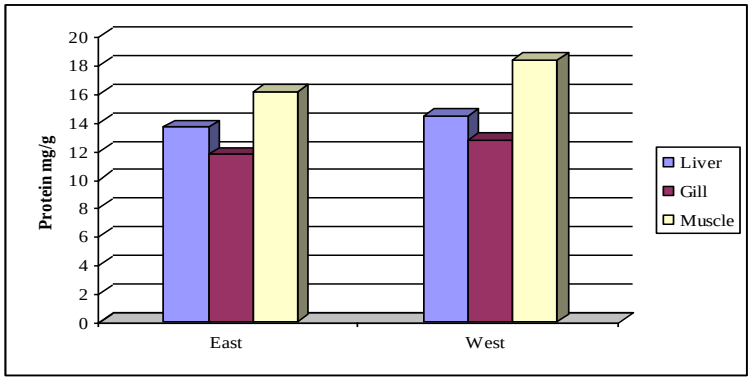

Fig. 2: Total protein concentration (mg/g wet mass) in the liver, gills and muscles of (*Mullus surmuletus*) from East and West of the Mediterranean Sea.

Figure (3) showed significantly increase in the total lipid levels in *Mullus surmuletus* that obtained from West more than East in investigated organs ($p<0.05$). The lipid content in liver was higher than in gills and muscles at West while lower lipid content was in gills at East region.

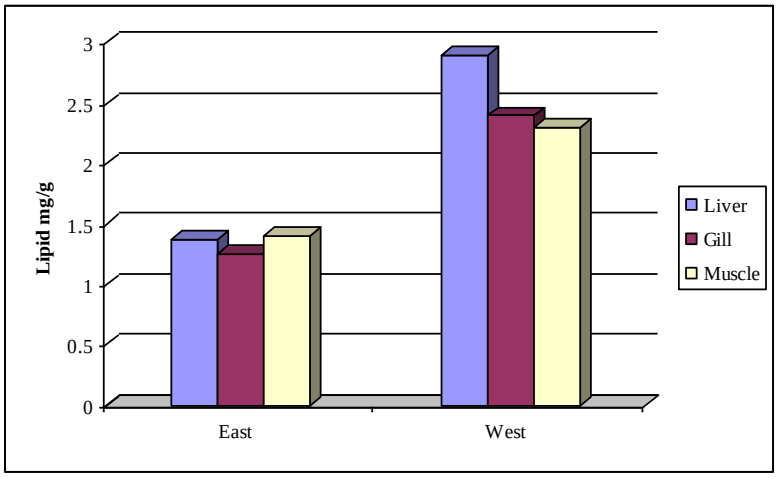

Fig.3: Total lipid concentration (mg/g wet mass) in the liver, gills and muscles of (*Mullus surmuletus*) from East and West of the Mediterranean Sea.

Figure (4) showed glutathione content (GSH) was lower in liver, gills and muscles in West than in East. In addition, liver GSH content was markedly higher than in gills and muscles at East. While it was higher in muscles than in gills and liver at West.

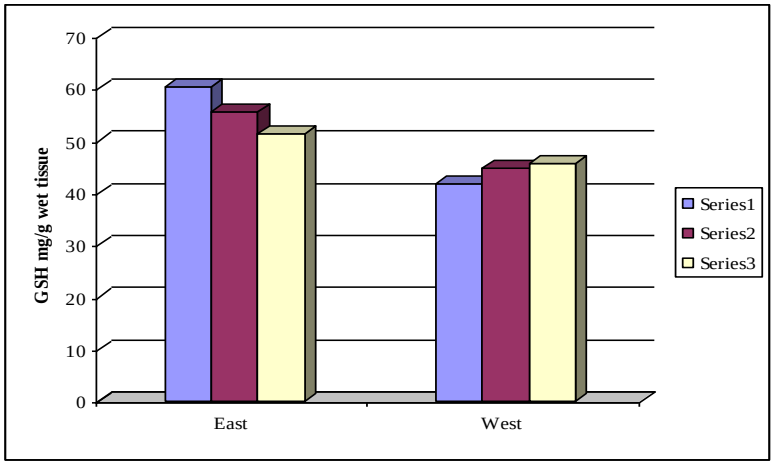

Fig.4: The GSH (mg/g protein) in the liver, gills and muscles of (*Mullus surmuletus*) from East and West of the Mediterranean Sea.

The activity of glutathione transferase (GST) enzyme was considerably lower in liver, gills and muscles at West region compared to East region. The higher GST activity was in gills and muscles at East region. The lowest GST activity was in muscles at West. (Figure 5).

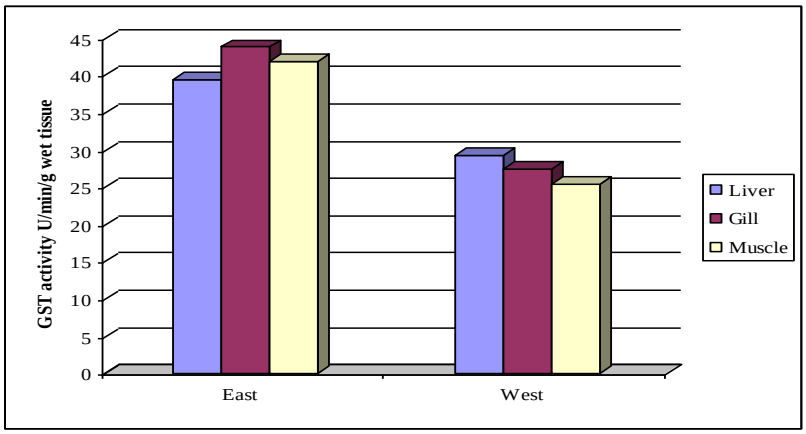

Fig.5: The activity of GST (U/mg protein) in the liver, gills and muscles of (*Mullus surmuletus*) from East and West of the Mediterranean Sea.

As presented in figure (6) the AchE activity in gills and muscles was significantly lower in East than in West while the highest values found in liver of both regions. Acetylcholine activity was higher in investigated organs in West more than in East.

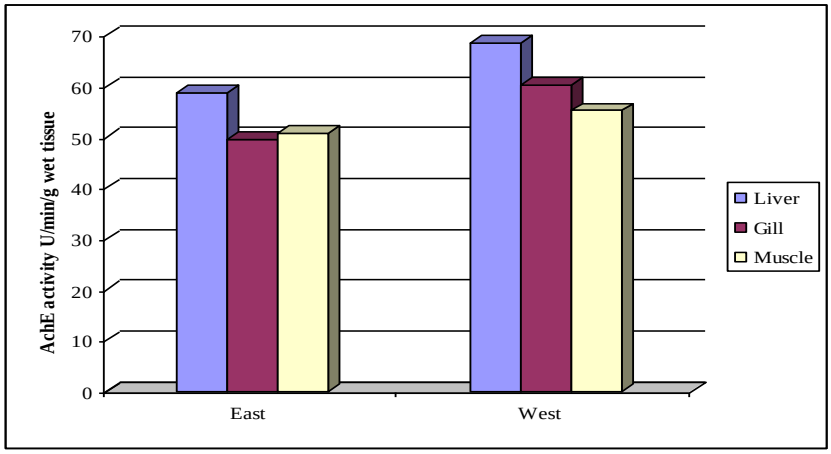

Fig.6: The activity of AchE (U/mg protein) in the liver, gills and muscles of (*Mullus surmuletus*) from East and West of the Mediterranean Sea

The results in table (1) observed the metabolic activity differences between the investigated tissues depending on tissue types and pollutants of region. The minimum values were observed in muscles except protein value recorded (17.51) mg/g while the maximum values was recorded in liver and gills of *M. surmuletus* for both regions. No statistical significant was found between protein content and AchE activity of the investigated organs from both East and West. Moreover, a significant differences ($p<0.05$) were observed for lipid-GST-GSH and AchE.

Table (1): The relation between means of protein, lipid and antioxidant enzymes activity in investigated organs of *Mullus surmuletus* from East and West of the Mediterranean Sea.

	Protein	Lipid	GSH	GST	AchE
Liver	14.17^{b}	2.38^{b}	48.23^{a}	32.88^{a}	65.16^{b}
Gill	12.38^{a}	2.03^{a}	48.60^{a}	33.18^{a}	56.53^{a}
Muscle	17.51^{c}	2.02^{a}	47.72^{a}	31.16^{a}	53.62^{a}

Means with different superscripts in the same column are significantly different at ($P<0.05$).

The abundance correlations between protein, lipid and the activity of GST, GSH, AchE enzymes in *M. surmuletus* are presented in tables (2-4).From the tables, one can see that the relations are highly significant with a $p<0.05$ (marked with'**') while others are less significant with a $p<0.01$ (marked with'*').The results from table (2) indicated a positive significant correlation between (protien and lipid) at $P<0.05$-(GST and GSH)at $P<0.01$, while a negative significant was between (lipid and GST, GSH) - (GST and AchE) - (GSH and AchE) at $P<0.01$ in liver fish from East and West.

Table (2): Correlation coefficients between protein, lipid and antioxidant enzymes in liver of *Mullus surmuletus* from East and West of the Mediterranean Sea.

	Protein	Lipid	GST	GSH	AchE
Protein	1	0.370*	-0.192	0.018	0.026
Lipid		1	-0.598**	-0.532**	0.410
GST			1	0.776**	-0.483**
GSH				1	-0.634**
AchE					1

*.

Correlation is significant at $P< 0.05$ **. Correlation is significant at $P<0.01$

Table (3) showed a negative significant correlation in gills of *M. surmuletus* between protein and GSH ($P<0.01$), lipid-GST, GSH and AchE-GST, GSH ($P<0.01$) and a positive correlation were noticed between protein-AchE ($P<0.05$), lipid- AchE and GST-GSH ($P<0.01$).

Table (3): Correlation coefficients between protein, lipid and antioxidant enzymes in gills of *Mullus surmuletus* from East and West of the Mediterranean Sea.

	Protein	Lipid	GST	GSH	AchE
Protein	1	0.275	-0.078	-0.433**	0.330*
Lipid		1	-0.683**	-0.656**	0.508**
GST			1	0.815**	-0.560**
GSH				1	-0.423**
AchE					1

*. Correlation is significant at P< 0.05 **. Correlation is significant at P<0.01

Table 4 represented the correlation coefficients of protein, lipid and GST,GSH and AchE in muscles of *M. surmuletus* from East and West. The results indicated that there was a positive correlation between protein, lipid and AchE (P<0.05), (P<0.01) respectively and between GSH and AchE (P<0.01). While a negative significant relationship were detected between protein and GST -lipid and GST, GSH - GSH and AchE (P<0.01). There was no significant difference for the protein values between East and West regions in investegated organs while the significant difference was clear for the antoxidant enzymes.

Table (4): Correlation coefficients between protein, lipid and antioxidant enzymes in muscles of *Mullus surmuletus* from East and West of the Mediterranean Sea.

	Protein	Lipid	GST	GSH	AchE
Protein	1	0.316*	-0.460**	0.217	0.440**
Lipid		1	-0.508**	-0.560**	-0.032
GST			1	0.109	-0.442**
GSH				1	0.438**
AchE					1

*. Correlation is significant at P< 0.05 **. Correlation is significant at P<0.01

IV. Discussion

The use of biomarkers stands for a fundamental approach in the assessment of ecosystem health.It allows the early detection of biological changes due to exposure to chemical pollutants, which may result in long-term physiological disturbances. They are sensitive indicators demonstrating the penetration of a toxic substance into the organism and its distribution among tissues; therefore they are the decisive indicators of the toxic effects [14].The United Nations Environment Programme has estimated that 650 million tons of sewage, 129,000 tons of mineral oil, 60,000 tons of mercury, 3,800 tons of lead and 36,000 tons of phosphates are dumped into the Mediterranean each year. Meanwhile, 70 per cent of the wastewater dumped into the Mediterranean is untreated.

The two investigated regions were chosen to compare the activity of antioxidant enzymes at East polluted with industrial wastewater, domestic and agricultural sewage and West region polluted with intensive anthropogenic sewage. In many biomonitoring studies, the liver is the main target organ for investigation because of its fast answer to environmental influences, high metabolic activity, and essential function in the organism while muscles have a lower metabolic rate.This investigation is important and have great significance to humans because of nutritional importance, especially in the case of commercially important fish species such as red mullet.

The present results indicated higher protein and lipid levels in West region of Mediterranean coast in liver and muscles of *M. surmuletus* while lower levels showed in gills from East region. In some fish species proteins are importance as bio-catalysts and hormones for control growth so variation of fish protein could be used as bioindicator for monitoring physiological status of tested fish. In red mullet species, maximum protein values has been obtained in spring, whether it is not observed considerable differences in seasons [30]. These differences related with; food intake, energy spending, migration, sexual changes during the spawning period, water temperature and salinity, seasons, environments and age [31].

In the present study GST activity was high sensitive to environmental changes in gills and muscles than in the liver of striped red mullet in both regions on contrary Pavlović *et al.* [32] showed less GST activity sensitive to environmental changes in the white muscle than in the liver of red mullet. Many laboratory experiments have demonstrated increased level of GST activity following exposure of various fish species to organic substances commonly occurring in the environment however results of laboratory tests do not always coincide with results obtained under field conditions. The differences may be caused by the fact that fish under natural conditions are exposed to a constantly changing composition of chemical substance. Moreover GST activity was significantly elevated in response of pollution; it could be related to adaptation to a continuous exposure to pollutants [33].

It is important that besides physiological and environmental factors the intrinsic biological variations such as size, tissue specificity, and natural variations of the biochemical responses, such as food availability become key factors to be considered [34]. Reduced glutathione (GSH) is a physiologically useful scavenger if the reaction with superoxide is prevented [35].The present results indicated that the induction of GSH and GST in liver and muscles of *M. surmuletus* at East was in agreement with that recorded in liver and kidney of *O. niloticus* captured from sewage polluted sites[36] and in liver of *O.niloticus* exposed to malathion for three weeks [37].One contrary, some studies of aquatic animals demonstrated that seasonal variations in different biomarkers were attributable to environmental and biological factors, mainly temperature and metabolic status of the animals, rather than to the site[38].

Acetylcholinesterase (AChE) enzyme is responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid. The inhibition of AChE is linked directly with the mechanism of toxic action of organophosphorus and carbamate insecticides, viz. irreversible or reversible binding to the catalytic site of the enzyme and potentiation of cholinergic effects [39].The present results indicated the inhibition of AchE in liver, gills and muscles of *M. surmuletus* from East region compared to West region. The inhibition and the lower activity of AchE could be explained by the leaching of pesticides into the sea from agriculture and metals moreover the reduction of AchE activity could be observed also in an industrialized region (East).These results are agreement with Lionetto *et al.* [40] who reported that heavy metals such as Zinc and Mercury can be considered as environmental inhibitors for AchE activity in fish. The quantification of this enzyme has been applied to laboratory and field studies with both vertebrates and invertebrates to assess exposure to organophosphorus and carbamate insecticides [41, 42]. On contrary the use of bioindicators, such as enzyme activities, in biomonitoring studies is often complicated, because levels of chemical pollutants in the environment often display wide seasonal variations in response to climate and other factors [43].

Cellular antioxidant defence represents a physiological system, and then changes in the activity of an individual antioxidant component should be accompanied by subsequent changes in the activity of others [44]. This can be explored statistically by correlation analysis, which determinates probability if the activity of one component is correlated with another. Statistically significant differences were detected between the three investigated organs in both regions, indicating a different degree of pollution. Our finding of a positive correlation between GST and GSH activities in both liver and gills tissues, suggesting a similar pattern for hydrogen peroxide elimination. At the same time, there was significant positive correlation between GSH and AchE activities.

Several positive correlations between measured antioxidant components in fish were agreement with [45] who suggested coordinated regulation of antioxidant enzymes to respond different developmental, seasonal and environmental impact during life cycle. Comparative analyses of correlations between antioxidant enzymes in different species, seasons and the environment have been performed in our previous studies [46, 47].Furthermore; the set of biomarkers used indicated different levels of stress in *M.surrmuletus* paralleling a coastal pollution degree. It is not obvious if this is a sign of impaired health or an adaptation response to a polluted environment. A part from natural biological cycles, the annual cycles of climatic conditions my also induce stress in the organisms thereby triggering antioxidant defences [43]. The results are in accordance with similar monitoring studies and represent a further support in the assessing the health of coastal areas and how it was affected on GSH, GST and AchE activities.

V. Conclusion

In conclusion the study provides further support for the use of biomarkers in assessing the health of coastal areas, and also the suitability of *Mullus surmuletus* as a guide species in the Mediterranean. The integrated use of AchE and antioxidant enzymes (glutathione or glutathione transferase) in *M. surrmuletus*, living in different compartment of marine coastal ecosystem, can find a useful application within the framework of marine coastal environment monitoring for detecting the possible exposure or effect induced by chemical pollutants, including pesticides, domestic sewage and industrial wastes, on living marine organism.

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