EVALUATION OF THE IMPACT OF POLLUTION IN THE GULF OF ANNABA (ALGERIA) BY MEASUREMENT OF ENVIRONMENTAL STRESS BIOMARKERS IN AN EDIBLE MOLLUSK BIVALVE *DONAX TRUNCULUS*

Fatima Amamra[1], Karima Sifi[2,*], Nouha Kaouachi[1], Noureddine Soltani[2]

[1]Laboratory of Aquatic and Terrestrial Ecosystems, Faculty of Natural and Live Sciences, Mohamed Cherif Messaadia Souk-Ahras University, Souk-Ahras 41000, Algeria
[2]Laboratory of Applied Animal Biology, Department of Biology, Faculty of Sciences, Badji Mokhtar Annaba University, BP 12, Annaba 23000, Algeria

ABSTRACT

Our study was aimed to assess the responses of three biomarkers, Acetylcholinesterase (AChE), Glutathione S-transferase (GST) and Malondialdehyde (MDA) in various tissues (gonad, mantle, digestive gland) of *Donax trunculus* (Bivalvia, Donacidae), a Mollusk Bivalve bioindicator of pollution in the Gulf of Annaba (Algeria). The samples were collected during the four seasons (winter, spring, summer, autumn) of 2016 year in three sites of Annaba gulf; Sidi Salem, site exposed to various sources of industrial and harbor pollution; Echatt, site subject to urban and agricultural wastes; and El Battah site distant from any source of pollution. The results showed a significant inhibition of AChE and induction of GST and MDA in individuals of Sidi Salem and Echatt as compared to El Battah with significant effects of both site and season. Indeed, the season effect was showed an inhibition of AChE and an induction of GST and MDA more pronounced during summer and spring compared to the other seasons. In addition, the comparison between tissues revealed a more marked response in gonad than mantle and digestive gland.

KEYWORDS:
Donax trunculus, Gulf of Annaba, Biomonitoring, Biomarkers, Pollution.

INTRODUCTION

Marine environment and especially coastal zones receive a large amount of contaminants from urban, agricultural, harbor and industrial activities [1]. These contaminants cause increased pollution of marine ecosystems and have a significant toxic impact on the health of living organisms [2]. Thus, the environmental monitoring or biomonitoring, is an important tool to determine the link between the current levels of pollution and the effects observed in the field [3]. To monitor the health of coastal systems, sentinel organisms such as mussels (bivalves) have been identified as suitable candidates to indicate levels of contaminants in the coastal environment and were proposed to be bioindicator species of pollution [4]. Among those mussels, *Donax trunculus*, is a sentinel organism that is widely distributed in Mediterranean Sea and used in ecotoxicology studies for the assessment of marine Mediterranean environment [5, 6]. Their characteristics such as bioaccumulation capacity, adequate body size, continuous availability throughout the year, ease of sampling and high longevity, make *D. trunculus* particularly useful as bioindicator of contamination changes [7, 5]. In addition, *D. trunculus* is an economically important species that is consumed by local population of Annaba (Algeria) [8]. Annaba city, coastal zone located in the extreme Northeast of Algeria has been known these last years a development of industry, advances in agriculture, and important demography leading to an increase a sea water and littoral contaminations, which are coming from industrial, harbor, agricultural and domestic activities [8, 9].

The use of biological markers or biomarkers measured at different levels of the biological organization is an important tool to understand the possible biological adverse effects of pollutants on organisms [10]. Biomarkers contributed to the development of the effective early warning systems of environmental pollution [11]. While some biomarkers are believed to demonstrate exposure to a specific group of contaminants (e.g. Acetylcholinesterase) [12], others can be used to indicate the cumulative effects of exposure to complex mixtures of contaminants such as a Glutathione S-transferase [13], and also a biomarker of oxidative stress like Malondialdehyde [14].

The aim of this study was to evaluate the seasonal responses of three biomarkers of environmental stress, the biomarker of neurotoxicity, Acetylcholinesterase; the Phase II detoxification enzyme, the Glutathione S-transferase; and the Malondialdehyde, a biomarker of lipid peroxidation of cell membranes during an oxidative stress. The biomarkers were determined in gonad, mantle and digestive gland of *D. trunculus*, collected from three sites of Annaba gulf (Algeria); El Battah, site considered to be a relatively clean, Echatt, site subjected to urban and agricultural wastes, and Sidi Salem, site located near several sources of pollution.

MATERIALS AND METHODS

Presentation of sampling sites. The gulf of Annaba is located in the Northeast of Algeria. It is limited by the Cap Rosa (8° 15' E and 36° 58' N) in the East and by the Cap Garde (7° 16' E and 36° 58' N) in the West. El Battah site (7° 56' E and 36°50' N), is located about 30 km to the East of Annaba far away from major human and industrial activities, and is considered to be a relatively clean site. Echatt (7° 52' E and 36° 49' N), is a site subjected to urban and agricultural pollution. However, Sidi Salem site (7° 47' E and 36° 50' N), which is located about 1 km to the East of Annaba city, is considered as a polluted area because it receives urban, harbor and industrial wastes (Fig. 1).

Biological material. Specimens of *D. trunculus* of standardized shell size (length 27 ± 1 mm) were collected during four seasons (winter, spring, summer, autumn) in 2016, from the three selected sites in the Annaba gulf. After sampling, *D. trunculus* were transported alive to the laboratory, and each species was quickly dissected and the tissues were removed (gonad, mantle, digestive gland) for biomarkers analysis.

Acetylcholinesterase analysis. The specific activity of AChE was determined according to the method described by [15]. The method is based on a coupled enzyme reaction involving acetylthiocholine as the specific substrate for AChE and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as an indicator for the enzyme reaction at 412 nm. Results are expressed as millimoles of thiocholine produced per minute per milligram of protein ($mmol.min^{-1}.mg^{-1}$ protein).

Glutathione S-transferase analysis. The GST activity was determined using the method described by [16] based on the GST catalyzed conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in CDNB conjugate was monitored at 340 nm and the enzyme activity was expressed in millimoles CDNB conjugate per minute per milligram of protein ($mmol.min^{-1}.mg^{-1}$ protein).

Malondialdehyde analysis. The lipid peroxidation was estimated by quantification of MDA rates using method of [17]. The principle of the method was based on a measurement of the color produced during the reaction of thiobarbituric acid (TBA) with MDA. The rate of MDA was measured at 532 nm and expressed as $mmol.mg^{-1}$ protein.

Protein quantification. Protein concentrations in the supernatants were measured according to [18] by using bovine serum albumin as standard. Absorbances were measured at 595 nm wavelength.

Statistical analysis. The results were expressed as mean ± standard deviation (SD). Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests, respectively. The variation of each parameter among sites and between seasons and tissues was tested by a two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. All statistical analysis was performed using GraphPad.Prism.v6. The significant difference was defined at $p< 0.05$.

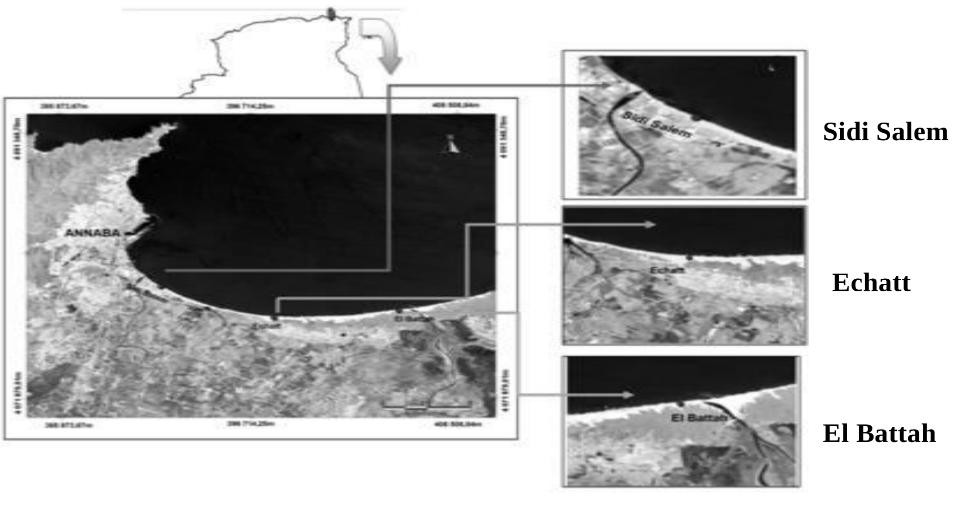

FIGURE 1
Location of sampling sites in the gulf of Annaba (Northeast of Algeria)

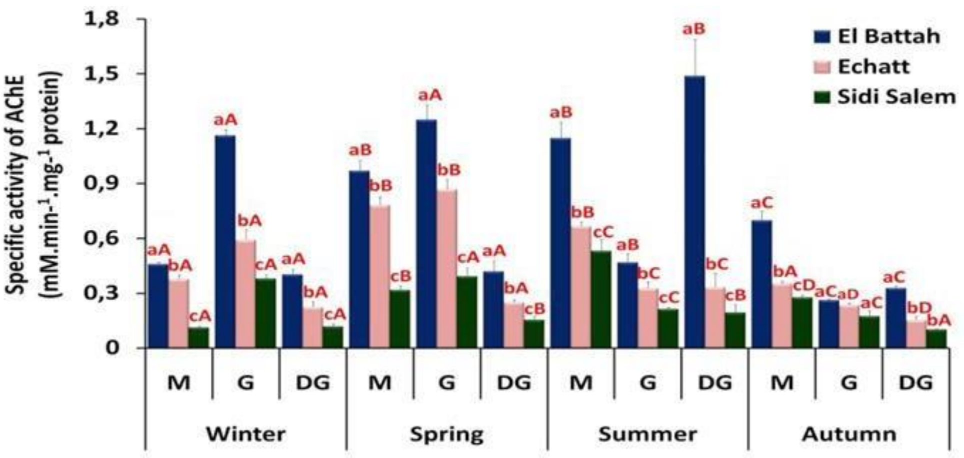

FIGURE 2
Seasonal specific activity of Acetylcholinesterase (mmol.min^{-1}.mg^{-1} protein) in mantle (M), gonad (G) and digestive gland (DG) of *D. trunculus,* sampled in three sites of Annaba gulf during 2016 (mean ± SD; n= 5). Means followed by same small case letters are not significantly different at p > 0.05 between sites in each tissue and season; while means followed by same capital letters are not significantly different at p > 0.05 across seasons within each tissue and site (Tukey's post hoc test, p < 0.05).

RESULTS AND DISCUSSION

Acetylcholinesterase activity. The results of seasonal variations of AChE in different tissues of *D. trunculus* are represented in Figure 2. The comparison between the three sites revealed a significant (p < 0.001) inhibition of AChE activities in individuals of Sidi Salem, then Echatt as compared to El Battah for the three tissues. The significant effect (p < 0.001) of season was observed with an inhibition of AChE more marked in summer and spring than autumn and winter seasons. The comparison between the tissues with ANOVA and Tukey's post-hoc test showed a significant (p < 0.001) inhibition of AChE at the gonad as compared to mantle and digestive gland (Table 1).

Acetylcholinesterase (AChE) is essential for the normal functioning of the central and peripheral nervous system. It is well known as a modulator to regulation of acetylcholine release from synaptic system. The AChE activity is used as biomarker of neurotoxic compounds in aquatic organisms. It is a target site of inhibition by organophosphate and carbamate pesticides [12] and other chemical compounds like heavy metals and hydrocarbons [19]. In our results, we reported the inhibition of AChE in *D. trunculus* of polluted sites compared to reference site. The inhibition of AChE was reported in the same species collected in polluted site in Tunis gulf as compared to reference site [5]. [20] have reported an inhibition of AChE in bivalve *Scrobicularia plana*, in polluted site as compared to reference site in France. In the other studies, AChE was reported to be inhibited in bivalve exposed to acute and subacute environmental contaminants including metals [1]; PAHs [13] and pesticides [21].

Glutathione S-transferase activity. The activities of GST (Fig. 3) recorded in *D. trunculus*, were increased significantly (p < 0.001) in organisms collected from polluted site of Sidi Salem, followed by Echatt as compared with El Battah site, this in all tissues. The season effect revealed a significant increase (p < 0.001) of GST activities more marked in summer and spring as compared with autumn and winter. Indeed, significant effects (p < 0.001) of both site and season were determined by ANOVA test. The activity of GST in tissues revealed a significant (p < 0.01) higher activities in gonad as compared with mantle and digestive gland tissues (Table 1).

The glutathione S-transferase (GST), a phase II detoxifying enzymes, represents one of the most basic mechanisms for detoxification system in marine organisms against a broad range of xenobiotics found in their environment [22]. Besides GST presents peroxidase activity, it is considered an indirect antioxidant, since it can eliminate a products of reactive oxygen species (ROS) generated during an oxidative stress [13]. Our results are agreeing with [23] and [7] who have reported an induction of GST in *D. trunculus* collected in polluted sites as compared to reference site. [13] have showed a significant induction of GST in mussels transplanted in harbor areas. An induction of GST has been reported in *Mytillus galloprovincialis* caged in impacted polluted areas in Sicily (Italy) as compared to reference site [24]. [25] have observed an induction of GST in gills of zebra mussels collected in sites along the Seine river (France) compared to reference site and a clear relationship between GST activities and amounts of bioaccumulated metals and PAHs was established. Induction of GST has been reported by recent studies in bivalves exposed to chemicals compounds like *M. galloprovincialis*, exposed to nickel [1]; *Venerupis decussata*, affected by permethrin and

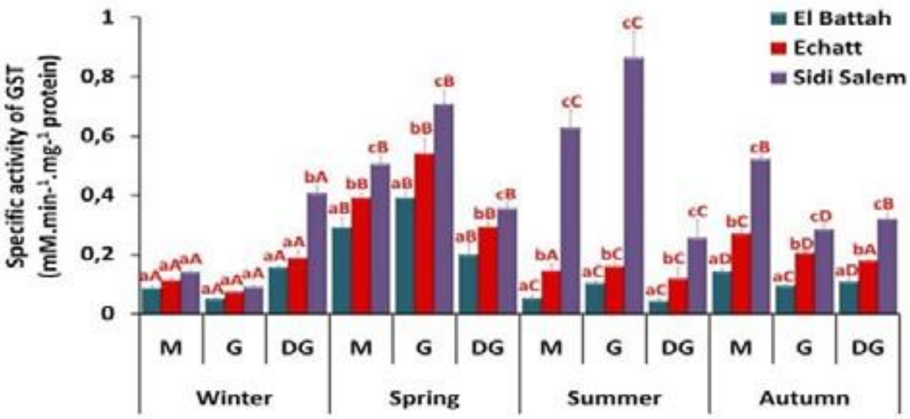

FIGURE 3
Seasonal specific activity of Glutathione S-transferase (mmol.min^{-1}.mg^{-1} protein) in mantle (M), gonad (G) and digestive gland (DG) of *D. trunculus*, sampled in three sites of Annaba gulf during 2016 (mean ± SD; n= 5). Means followed by same small case letters are not significantly different at p > 0.05 between sites in each tissue and season; while means followed by same capital letters are not significantly different at p > 0.05 across seasons within each tissue and site (Tukey's post hoc test, p < 0.05).

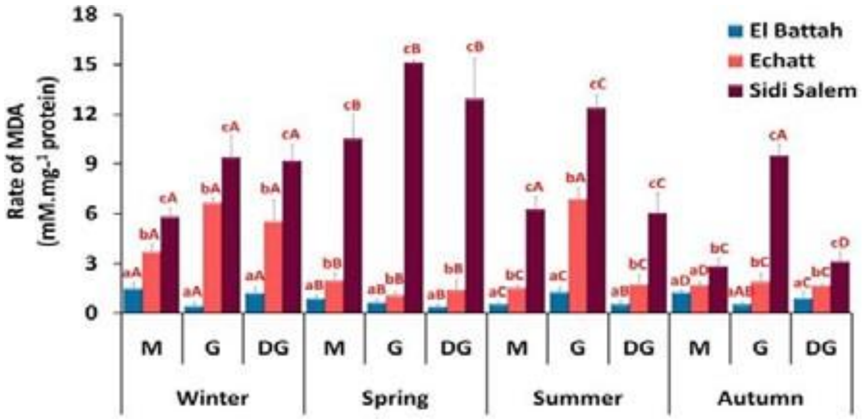

FIGURE 4
Seasonal rates of Malondialdehyde (mmol.mg^{-1} protein) in mantle (M), gonad (G) and digestive gland (DG) of *D. trunculus*, sampled in three sites of Annaba gulf during 2016 (mean ± SD; n= 5). Means followed by same small case letters are not significantly different at p > 0.05 between sites in each tissue and season; while means followed by same capital letters are not significantly different at p > 0.05 across seasons within each tissue and site (Tukey's post hoc test, p < 0.05).

anthracene [26]; *Ruditapes philippinarum* exposed to benzo-a-pyrene [27] and *Dreissena polymorpha* treated by the cocaine metabolite benzoylecgonime [28].

Malondialdehyde rates. The site effect showed that rates of MDA were significantly increased ($p < 0.001$) in *D. trunculus*, from polluted sites of Sidi Salem and Echatt compared to El Battah site (Fig. 4). The results of MDA revealed a significant effect ($p < 0.001$) of season with higher rates in spring and summer than in winter and autumn (Fig. 4). Furthermore, the tissues effect showed a significant ($p < 0.001$) higher values in gonad followed by digestive gland, then mantle (Table 1).

Oxidative stress is one of the most significant effects caused by xenobiotics and can be estimated by measuring the concentration of biomarkers of antioxidant activity or a products of damages like malondialdehyde (MDA) a biomarker of lipid peroxidation of polyunsaturated fatty acids of cell membranes [29]. Oxidative stress reflects an imbalance between pro-oxidants and antioxidants who favor a production of reactive oxygen species (ROS) responsible of cellular and/or molecular damages like deterioration of cell membranes (lipid peroxidation), DNAdamage and enzyme inactivation [30]. The present study showed an increase of MDA levels in *D. trunculus* from the polluted sites of Sidi Salem and Echatt as compared to El Battah. Increased in MDA concentrations were also detected in bivalve *Unio tumidus* exposed to polycyclic aromatic hydrocarbons [31], *Unio gibbus* exposed to insecticide cypermethrin [32] and *Corbicula fluminea* submitted to herbicides Atrazine and Roundup [33]. Other studies have reported an increase of MDA in mussels subjected to environmental stress such as *M. galloprovincialis* exposed to nickel and thermal stress [1]; *Elliptio complanata* exposed to zinc oxide nanoparticles [34]; *Aulacomya atra atra* under the effect of several metals [35] and *Lasmigona costata* submitted to wastewater effluents [14].

Season and tissues effects. Seasonal cyclic changes are well known to influence mussel's physiology [36]. Due to their intertidal region habitat, mollusks bivalve can be sensitive to seasonal variations of temperature. Thus, a change in seasonal abiotic factors can affect the bioavailability of pollutants to living organisms [37]. The differences between seasons revealed in our study were probably triggered by the seasonal variation of environmental factors in seawater, such as, elevated temperature [38]. This may explain our results as we noted a strong inhibition of AChE and a significant induction of GST and MDA in the summer and spring seasons where temperatures are high and may contribute to the concentration of pollutants. [39] were reported that higher reproductive activity and seasonal temperature can modulate and cause an increase in the antioxidant defenses in the digestive gland of *Perna perna*, with higher levels of GST. The same result has been reported in *Mytilus edulis* exposed to seasonal effect of temperature [37].

TABLE 1
Tissues effect of biomarkers response (AChE, GST, MDA): two-way analysis of variance (significantly difference at $p < 0.05$)

Biomarkers	Tissues	P
AChE	Gonad Mantle Digestive Gland	< 0.001
GST	Gonad Mantle Digestive Gland	< 0.001
MDA	Gonad Digestive Gland Mantle	< 0.001

In most cases, the chemical substances enter organism tissues and subsequently induce the toxic effects. Stress syndrome in bivalve mollusks can lead to shell defects, recession of the mantle and deterioration of the epithelium in the digestive gland and gonad [40]. Some studies have reported that contaminants in polluted water enter mollusks mainly through the epidermal cells of the gill and mantle, then transported by circulatory system to other organs within the mollusk, such as digestive gland, adductor muscle and gonad [41]. These tissues have been used as a target for studying the biomarker responses to several environmental stressors [36]. We have been found in our study a tissues effect with an AChE inhibition and an induction of GST and MDA more marked in gonad then mantle and digestive gland. The gonad appear more sensitive because it ensures the reproduction of the species and it is more sensitive during the spring and summer, seasons of sexual activity of *D. trunculus* [42].

CONCLUSION

The results of the present investigation suggest that *D. trunculus* is a suitable organism for use as bioindicator species. In addition, it is a sensitive non-target species that could be used in biomonitoring of Annaba gulf in Algeria based on biomarkers assays. The difference recorded between the sites is related to their level of exposition to pollution in the gulf of Annaba. Indeed, Echatt site receives waste of urban and agricultural origin while the Sidi Salem site is located near a factory that produces pesticides. Furthermore, this site receives heavy metals and other pollutants from harbor activities and urban discharges.

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CORRESPONDING AUTHOR

Karima Sifi
Laboratory of Applied Animal Biology,
Department of Biology,
Faculty of Sciences,
Badji Mokhtar Annaba University, BP 12,
Annaba 23000 – Algeria

e-mail: sifi_k23@yahoo.fr
karima.sifi@univ-annaba.dz