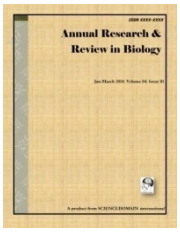

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Measure of Oxidative Stress and Neurotoxicity Biomarkers in *Donax trunculus* from the Gulf of Annaba (Algeria): Case of the Year 2012

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Authors' contributions

This work was carried out in collaboration between both authors. Author NSM conceived and designed the experiments. Authors NSM and LB performed the experiment, analyzed the data and wrote the manuscript. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The purpose of our study was to evaluate three biomarkers in an edible species, *Donax trunculus* (Mollusca, Bivalvia) associated with the environmental pollution in the Annaba gulf. The biomarkers selected were the antioxidant enzyme catalase (CAT), the detoxifying enzyme glutathione S-transferase (GST) and the neurotoxicity marker acetylcholinesterase (AChE).
Place and Duration of Study: Samples were collected monthly in 2012 at two sites in the Annaba gulf (Northeast Algeria): El Battah a relatively clean site and Sidi Salem, a polluted site.
Methodology: Mollusc bivalves (*D. trunculus*) with the same shell length (25±1mm) were collected monthly from the two sampling sites, transferred to the laboratory and dissected the same day. The mantle was dissected and samples were prepared for biomarker analyses. The enzymes activities were determined by using standard methods.
Results: Biochemical data showed a significant inhibition of AChE activity and a significant increase in both CAT and GST activities in *D. trunculus* at the site of Sidi Salem compared to El Battah. These changes are discussed in relation to environmental factors and suggested an influence of site quality on the health of *D. trunculus*.
Conclusion: The measurements of biomarkers in *D. trunculus* reveals differences between the two sites in relation to their pollution state confirming previous reports. The

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site of Sidi Salem is more contaminated due to their proximity to different pollution sources. Moreover, the current study gives additional data on the pollution in the gulf of Annaba.

Keywords: Donax trunculus; Gulf of Annaba; Biomarkers; CAT; AChE; GST.

1. INTRODUCTION

Pollution of the marine environment generates a significant stress in aquatic organisms [1]. Scientists and environmental managers have used biomonitoring program to assess the impact of pollution on various ecosystems; this control is based on the use of biological indicators of pollution. Bioindicators are organisms used to determine the presence, abundance and bioavailability of environmental contaminants through measures in these organisms or in several organs and/or tissues [2]. Various molluscs are used as bioindicators of physiological and cellular alterations caused by pollution [3-6] for their high bioaccumulation capacities. The environmental risk assessment involves the use of biomarkers [7]. A biomarker is defined as an observable and/or measurable change at the molecular, biochemical, cellular, organism, population or ecosystem which can be connected to an exhibition or the toxic effects of environmental chemical pollutants [7-9]. Biomarkers were classified following their specific response to certain pollutants or a particular effect. Thus, the authors typically distinguish biomarkers of exposure of effect and of susceptibility [8,10-12].

Biomarkers of exposure are induced by a type of pollutants and these biomarkers are described as specific, such as enzyme biomarkers of neurotoxicity as Acetylcholinesterase (AChE), the detoxifying enzymes such as Glutathione S-transferase (GST) and Cytochrome P450 (CYP) and also the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [13].

Acetylcholinesterase is an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetate at synapses [14]. AChE is a target site of inhibition by organophosphates and carbamates, which act by inhibiting its catalytic activity by binding to the active site of the enzyme instead of acetylcholine [14,15]. CAT (EC 1.11.1.6) is widely used as a marker involved in the primary defense against oxidative damage [16,17]. Oxidative stress results in the formation of many Reactive oxygen species (ROS) [18] causing cell and tissue damage [19]. The CAT activity tells us about the degree of alteration of the cell [20]. Thus, the CAT activity was reported in fish and bivalves exposed to organic pollutants [21,22]. GST is an important phase II enzyme that catalyzes the conjugation of reduced glutathione (GSH) to cellular components damaged by ROS attack, leading to their detoxication [23]. The GST activity can be triggered by some pollutants [24-27]. GST response to toxic chemicals follows a similar bell-shaped trend as CAT [28], while GST inhibition has been indicated as more a specific response to chemical challenge [29].

The aim of this study was to evaluate of the water quality of the Annaba Gulf (Northeast Algeria) by measuring the response of three biomarkers, a biomarker of neurotoxicity, AChE, a biomarker of oxidative stress, CAT and the phase II detoxifying enzyme GST in the edible mollusc, *Donax trunculus* (Mollusca, Bivalvia), associated with environmental pollution. This assessment was conducted monthly in 2012 at two sites, El Battah a relatively clean site and

Sidi Salem, a polluted site. The present study, in continuation to previous works, gives additional information on the water quality.

2. MATERIALS AND METHODS

2.1 Presentation of Sampling Sites

The gulf of Annaba is located in the east of Algeria. It is limited by the Rosa Cap (8°15 ' E and 36°38' N) in the East and by the Garde Cap (7°16' E and 36°68' N) in the West. El Battah site (36°50' N and 7°50' E) is located about 20km to the East of Annaba far from any human activities and is considered as a relatively clean site. Sidi Salem site (36°50' N and 7°47' E), located about 1km to the East of Annaba city, receiving industrial and domestic wastewater and considered as a polluted area (Fig. 1).

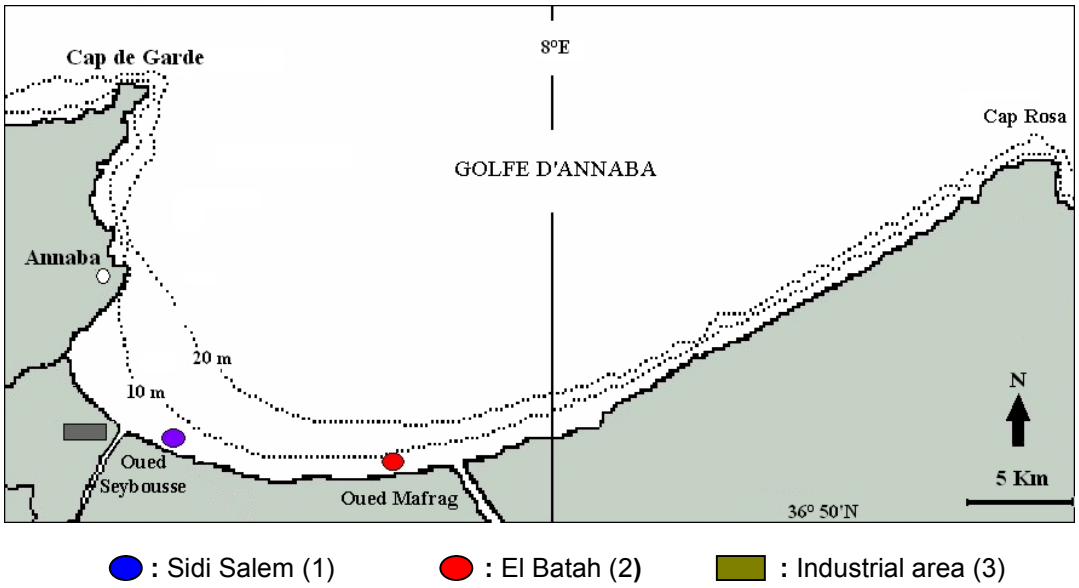

Fig. 1. Geographic location of the two sampling sites in the Annaba gulf (El Battah and Sidi Salem)

2.2 Samples Collection

Mollusc bivalve (*D. trunculus*) with the same shell length (25±1mm) was collected monthly in 2012, from two sampling sites (El Battah and Sidi Salem). The collected samples were transferred the same day to the laboratory in plastic trays containing sea water. The mantle of each individual was dissected the same day on ice, weighted and stored at -20ºC until required. For sample preparation, all procedures were carried out on crushed ice.

2.3 Biochemical Procedure

The analysis was made individually on each sample under laboratory conditions with a room temperature of above 20ºC in winter and 22ºC in summer.

Determinations of AChE activity were performed [30] with the use of Acetylthiocholine (ASCh) as substrate, 100 µl of supernatant are mixed in an incubation medium consisting of Tris buffer pH 7.0 (0.1M) and DTNB (0.01M). After incubation for 3 minutes, 100 µl iodide acétylthiocoline (AcSCh) are added to obtain a final concentration of 100 mM. The activity rate was measured as change in optical density (OD/min) at 412 nm.

CAT activities were assayed as described by [31], 50 µl of supernatant was mixed with an incubation medium composed of phosphate buffer pH 6.0 (0.1M) and hydrogen peroxide. The spectrophotometric reading was performed every 30 seconds at a wavelength of 240nm. The activity rate was measured as change in optical density (OD/min) at 240 nm.

GST activity was by [32], 200 µl of the supernatant are mixed in an incubation medium consisting of a mixture of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) diluted in a sodium phosphate buffer pH 6.0 (0.1M). The activity rate was measured as change in optical density (OD/min) at 340 nm. The final activity of AChE, CAT and GST was expressed as µMol/min/mg protein. Samples were dissected on ice and stored at -20ºC until required.

Total proteins were extracted following the procedure of Shibko et al. [33]. In brief, each sample consisting of one pair of ovaries was homogenized in 1 ml of trichloroacetic acid (20%) and then centrifuged (5,000g for 10 min). The resulting pellet was dissolved in 1 ml NaOH (0.1N for 24 h). Then, an aliquot (100 µl) was taken for the determination of total proteins according to Bradford [34] with blue brilliant of coomassie (G 250, Merck) as reagent and bovine serum albumin (Sigma) as standard.

All analyses were performed with 5 repeats each consisting of one piece (0.4-0.5g fresh weight) dissected from the median part of mantle from 5 different individuals.

2.4 Statistical Analysis

Results are presented as mean ± standard deviation (SD). Comparison of mean values between sites was estimated by Student's t-test. The effects of time, sampling sites were made using two-way analysis of variance (ANOVA). All statistical analyses were performed using MINITAB Software (Version 16, PA State College, USA). A significant difference was assumed when $P< 0.05$.

3. RESULTS

3.1 Change in Acetylcholinesterase Activity

Measurements of monthly AChE activity in mantle of mussels (*D. trunculus*) from two sampling locations (El Battah=EB and Sidi Salem=SS) are presented in Fig. 2. AChE activities ranged from 2.42 µMol/min/mg of proteins at Sidi Salem to 51.9 µMol/min/mg of proteins at El Battah and were generally higher at EB comparatively to SS; the differences between the two sites were significant in March, August and November ($P< 0.05$), highly significant difference was observed in October ($P< 0.01$) and very highly significant difference was revealed in September ($P< 0.001$). The results were confirmed by the two-way ANOVA since a significant ($P< 0.001$) effects of both site ($F= 50.66$; $df= 1, 92$) and month ($F= 189.98$; $df= 11, 92$) were noted (Table 1). It is well known that the temperature plays a major role in the influencing of the AChE activity. Thus, the temperature was

measured monthly in the two sampling sites (Table 2). Globally, there was no significant difference between the two sites.

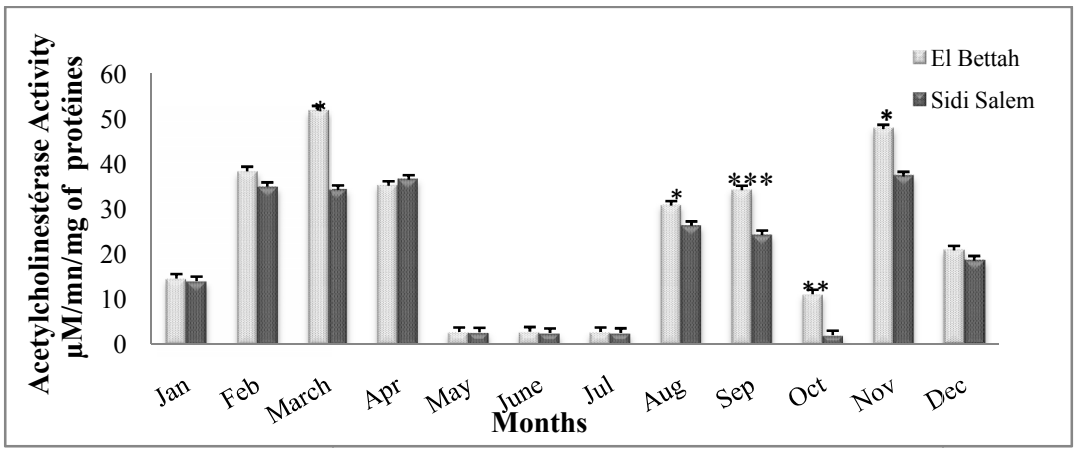

Fig. 2. Monthly variation of specific activity of Acetylcholinesterase (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf

*mean ± SD; n= 5; *P<0.05; **P<0.01; ***P<0.001*

Table 1. Monthly variation of specific activity of AChE (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf: two-way ANOVA

Sources of variation	DF	SS	MS	Fobs	P
Site	1	608.1	645.6	50.66	<0.001
Month	11	27002.6	2454.8	189.98	<0.001
Interaction Site/Month	11	890.3	81.2	6.29	<0.001
Residual error	92	1188.8	12.9	-	-
Total	115	29692.9	-	-	-

Table 2. Monthly variation of temperature in the two sampling sites in 2012

Sites / Months	El Battah	Sidi Salem
January	14.1	15.2
February	14.6	15.2
March	14.6	15.6
April	15.4	16.1
May	18.4	19.6
June	20.8	20.8
July	24.2	25.3
august	26.4	27.4
September	25.3	25.9
October	23.6	24.3
November	18.9	19.1
December	16.5	16.4
Mean ± SD	19.40±4.25	20.09±4.57

3.2 Change in Catalase Activity

CAT activities in *D. trunculus* collected monthly from two different sites are presented in Fig. 3. CAT activities ranged from 0.231 µM/min/mg of proteins at El Battah to 4.39 µM/min/mg of proteins at Sidi Salem. Significant differences were recorded in January (P< 0.05) and July (P< 0.01). Two-way ANOVA revealed no significant (P > 0.05) effect of site (F= 2.70; df= 1, 96) and a significant (P<0.001) effect of month (F= 59.28; df = 11, 96) (Table 3).

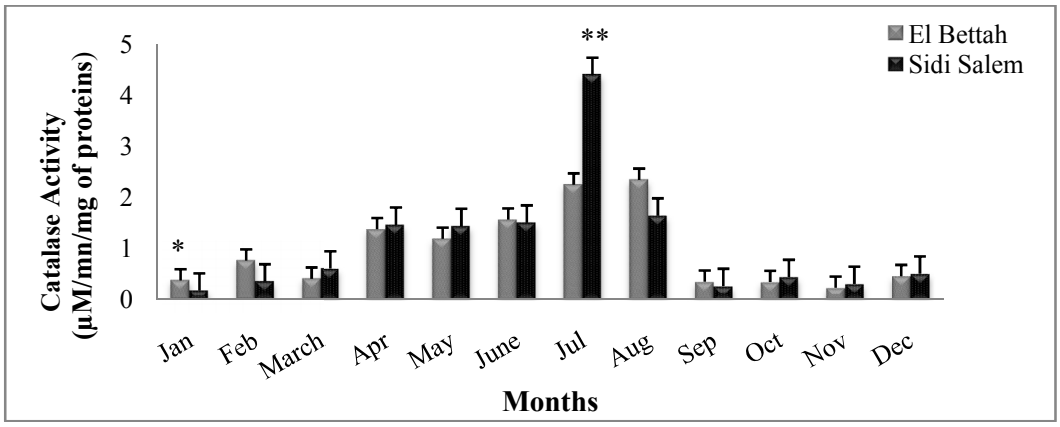

Fig. 3. Monthly variation of specific activity of catalase (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf

*mean ± SD; n= 5; *P<0.05; **P<0.01; ***P<0.001*

Table 3. Monthly variation of specific activity of CAT (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf: two-way ANOVA

Sources of variation	DF	SS	MS	Fobs	P
Site	1	0.3964	0.3964	2.70	0.104
Month	11	95.7063	8.70065	9.28	<0.001
Interaction Site/Month	11	13.0202	1.1837	8.06	<0.001
Residual error	96	14.0902	0.1468	-	-
Total	119	123.2130	-	-	-

3.3 Change in Glutathione S-transferase Activity

The results of monthly GST activities in mantle of *D. trunculus* are presented in Fig. 4. GST activity ranged between 3.31 µM/min/mg of proteins at El Battah to 34.45 µM/min/mg of proteins at Sidi Salem and overall higher values were found at the polluted site than those from the reference site with significant differences in March (P< 0.05) and February, May, July, October and December (P< 0.01). Significant effects *(P*< 0.001) of site (F=183.49; df= 1, 88) and months (F= 325.05; df=10, 88) were determined by two-way ANOVA test (Table 4).

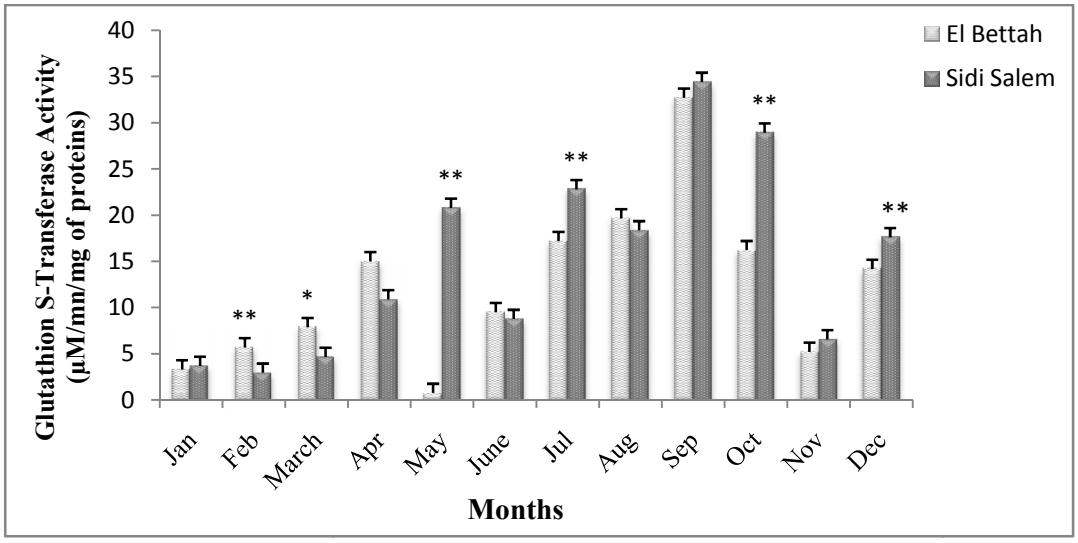

Fig. 4. Monthly variation of specific activity of glutathione S-transferase (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf

*mean ± SD; n= 5; *P<0.05; **P<0.01; ***P<0.001*

Table 4. Monthly variation of specific activity of GST (µM/min/mg of proteins) in *D. trunculus* from two sites in the Annaba gulf: two-way ANOVA.

Sources of variation	DF	SS	MS	Fobs	P
Site	1	2457.5	2457.5	183.49	<0.001
Month	10	43534.4	4353.4	325.05	<0.001
Interaction Site/Month	10	12917.7	1291.8	96.45	<0.001
Residual error	88	1178.6	13.4	-	-
Total	115	60088.2	-	-	-

4. DISCUSSION

The development of human factors in coastal areas causes an increasing deterioration of the quality of the marine environment, including increased intake of toxic elements through domestic and industrial wastes. This anthropogenic pollution affects adaptation and maintenance of aquatic populations such as bivalve mollusks [35]. The Gulf of Annaba is a recipient of a large amount of contaminants from urban, agricultural, harbour and industrial activities [36,37]. Pollution of the marine environment generates significant environmental stress in aquatic organisms. Among the biological tools recommended for marine pollution monitoring, biomarkers have been successfully incorporated in the assessment of the quality of the coastal environment during last year [38-39]. Aquatic organisms, especially marine bivalves, exhibit a variety of changes in enzymatic antioxidant defences after exposure to pollutants with oxidative potential [40,41]. In the present study, we have measured three biomarkers of environmental stress to evaluate their utility as biological tools for pollution monitoring in the Gulf of Annaba.

The neurological and behavioral activities of animals can be extremely sensitive to environmental contamination [42]. The inhibition of AChE activity was frequently used in

ecotoxicology to diagnose exposure to anticholinesterase chemical products such as organophosphates (OP) and carbamates [43-44]. Therefore, these disorders can affect locomotion and balance of exposed animals [45]. The acetylcholinesterase is a key enzyme in the central nervous system; it is responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetate at synapses [46]. If the action of this enzyme is blocked, the postsynaptic membrane is continually excited which leads to the accumulation of acetylcholine in the synaptic region causing hyper excitation which caused the death of the organism [47].

Monthly determination of AChE activity in individuals of *D. trunculus* in El Battah and Sidi Salem sampled during 2012 revealed a significant inhibition of the specific activity of AChE in individuals from Sidi Salem compared to those of El Battah. However, no significant difference was recorded between the two sites during the months of January, February, April, May, June, July and December. This can be explained by a diminution of chemical stress particularly in winter and spring. The site of Sidi Salem receives domestic and industrial wastes mainly from FERTIAL complex, specialized in the production of fertilizers and pesticides. This agrees with previous reports [48-51]. In a previous report, heavy metal levels were determined in *Donax trunculus* sampled from the same studied sites [50]. These authors showed a significant effect of seasons for all metals measured and there was a significant difference on Cd concentrations between sites.

It has been reported a change in the activity of AChE in *M. galloprovincialis* transplanted for one month at four sites in the Bay of Cannes (France), where the strongest inhibition AChE was recorded at the old port of Canne, the most polluted sites by heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls [52]. The AChE activity can be modulated by trace metals (Cd, Cu, Hg and Zn) or natural factors (seawater temperature, biotoxins or cyanobacteria in mussel tissues) [53,54].

A study performed on the clam, *R. decussatus*, in the lagoon of Bizerte (Tunisia) showed that the inhibition of AChE observed can be influenced by various biotic, abiotic parameters and the presence of pesticides from agricultural activity [55]. This inhibition was also reported in other bivalves, such as oysters *C. gigas* treated by carbofuron and malathion [56] molds *Ambuema plicata* [57,58] and *Scrobicularia plana* [59].

A significant increase in CAT activity was observed during the months of January and July in *D. trunculus* in the contaminated site (Sidi Salem) by comparison with the reference site (El Battah). In addition, a higher CAT activity is observed during the summer period compared to other periods in the site of Sidi Salem. These changes were probably related to the pollution level of this site [36,49]. Our results can be explained by the presence of anthropogenic activity at the site of Sidi Salem related to exposure to different sources of pollution. This exhibition has probably resulted in production of reactive oxygen due to oxidative stress in individuals of species *D. trunculus*. Among these reactive oxygen species, hydrogen peroxide (H_2O_2) enters the cells by diffusion, where it is normally converted into CAT by the oxygen (O_2) and water (H_2O), however, with the excess pollutants into the site of Sidi Salem, H_2O_2 can generate hydroxyl radicals and caused significant lipid peroxidation of cell membranes [60]. On the other hand, the values of CAT showed temporal changes in the two sampled sites. These biological responses can be modulated also by seasonal changes of both environmental and biological factors, potentially affecting responsiveness and sensitivity to pollutants [55]. Fluctuations obtained in our study are reported by several authors in different marine species, including, in *R. decussatus* taken from a polluted environment in Bizerte in Tunisia [55] and *Chamaelea gallina* exposed to PCB [60]. Other

authors found an increase in CAT activity, in *Bathymodiolus azoricus* exposed to thermal stress [59].

Monthly determination of GST activity in individuals of *D. trunculus* in El Battah and Sidi Salem sampled during 2012 revealed a significant higher activity of this enzyme in individuals from the multi-contaminated Sidi Salem site in comparison with the reference site (El Battah) [48-51] during the months of May, July, October and December. An increase in the GST activity can be generated by several factors such as temperature, pH, oxygen and the level of the pollution as it may be related to the reproductive cycle and the individual growth of *D. trunculus* [51]. In our case, there were no significant differences in temperature between the two sites in 2012.

Our results showed differences in the biomarkers responses between the two sites in relation to their pollution state confirming previous reports made in *D. trunculus* [48,51]. However, comparing the current data with a recent published paper [48], it is possible to notice some additional data. Indeed, the differences concern the duration (number of months per year) and the intensity (values of biomarker activity) of significant responses. The environmental stress was observed during a relatively short period (AChE 5 months in 2012 vs 9 months in 2010). This can be explained by differences in environmental factors between the two years affecting the pollution level in the gulf such as marine current and water bringing by the rivers during winter and spring. This contributes to dilution and homogenization of pollutants in the gulf. In contrast, although a shorter duration of stress, its intensity is more marked in 2012 (increase in CAT and GST activity and decrease in AChE activity) suggesting a higher level of pollution.

5. CONCLUSION

The measurements of biomarkers of environmental stress show *D. trunculus* from Sidi Salem a higher inhibition of AChE activity indicates the presence of neurotoxic pollutants such as organophosphates and carbamates, while a higher induction of CAT activity suggesting a significant oxidative stress in relation to increased level of pollution in this site as compared with values from El Battah samples. An increased GST activity reflects the induction of detoxification system after exposure to different xenobiotics resulting from urban and industrial activities, particularly in Sidi Salem. The overall results obtained reveals that *D. trunculus* from Sidi Salem are exposed to higher levels of pollution in relation to their proximity to different pollution sources. Moreover, the current study gives additional data on the pollution in the gulf of Annaba, which was comparable with previous studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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