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Metallothioneins as a biomarker of metallic pollution in *Donax trunculus* Linnaeus, 1758 (Mollusca Bivalvia) from the Gulf of Annaba (Algeria)

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ABSTRACT In recent years, the Gulf of Annaba has observed an increase in the pollution level, in particular by heavy metals. The present experiment aimed to assess pollution by measuring the rate of metallothioneins (MTs), a biomarker of metallic contamination, measured in the digestive gland of *Donax trunculus* Linnaeus, 1758 (Mollusca Bivalvia) an edible mollusk widely used in biomonitoring of water quality. This sentinel species was sampled during two seasons (winter and summer 2019) at two sites in the Gulf of Annaba (Northeast Algeria): El Battah site far from any source of pollution and Sidi Salem site exposed to various sources of pollution. The biochemical analyzes reveal spatio-temporal changes. The levels of proteins recorded in the two studied sites were higher in summer and values from El Battah individuals were significantly higher compared to those from Sidi Salem. In addition, they show an induction in MT rates in individuals from the two sites (Sidi Salem and El Battah) in summer with higher values recorded in individuals from Sidi Salem. This difference is related to the proximity of sources of contamination at Sidi Salem compared to El Battah. The rates of MTs vary also according to abiotic factors such as hydrodynamics or temperature which can explain the significant seasonal difference with a more marked induction in summer. Conclusively, our study shows the sensitivity of *D. trunculus* to chemical stress induced by heavy metal contamination as evidenced by an induction of MTs with significant effects of both sites and seasons.

KEY WORDS Pollution; Gulf of Annaba; *Donax trunculus*; Biomarker; Proteins; Metallothioneins.

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INTRODUCTION

The development of human activities is responsible for the chronic pollution of the environment by organic and inorganic contaminants (Larba & Soltani, 2014; Breitwieser et al., 2020; Douafer et al., 2020). The marine environment, and in particular coastal areas, receives a large amount of contaminants from urban, agricultural, port and industrial activities. These contaminants cause increased pollution of marine ecosystems and have a significant toxic impact on the health of living organisms (Amamra et al., 2019; Hamdani et al., 2020). However, the increase in ocean pollution caused by toxic metals has resulted in bioaccumulation of toxic metals in marine animals, particularly bivalves such as clams, and the consequent toxicity associated with heavy metals in these animals (Mengxu et al., 2020). Thus, biomonitoring is an important tool to determine the link between current pollution levels and observed effects in the field (Asker et al., 2016). Bivalves belong to the first-choice species as bioindicators for environmental and chemical stress. They are sentinel benthic organisms living as filter feeders and exposed to different environmental compartments (Laitano et al., 2016; Dos Santos et al., 2022).

Biomarkers are an important tool for understanding the possible harmful effects of pollutants on organisms (Boshoff et al., 2015). They are inhibition biomarkers such as metallothioneins (MTs) which are widely used as a biomarker of metal contamination by binding and removing toxic metals (Pedrini-Martha et al., 2017). A defense biomarker like MTs was considered in this study to investigate metal accumulation. MTs are low molecular weight, cysteine-rich proteins induced in response to various environmental stressors, including metal ions, cytokines and free radicals (Coyle et al., 2002). MTs are involved in metal uptake, storage and excretion (Breitwieser et al., 2020). Their concentrations in whole soft tissues or in a particular bivalve tissue have been widely used in ecotoxicological studies and biomonitoring programs (Yen Lea et al., 2016; Merad & Soltani, 2017; Pedrini-Martha et al., 2017).

In this context, the main objective of this work was to study the in situ seasonal responses (winter and summer) of a biochemical biomarker MTs in *D. trunculus*, a locally prevalent edible mollusk sampled at two sites in the Gulf of Annaba: a polluted site (Sidi Salem) and a relatively clean site (El Battah).

MATERIAL AND METHODS

Study area

The Gulf of Annaba is located in the Northeast of Algeria. It is limited by the Rosa Cap (8°14'13" E, 36° 56'46″ N) to the east and by the Gard Cap (7°16'00" E, 36°58'02"N) to the west.

The El Battah site (36°5' 49" N, 7°56'57" E), is located about 30 km to the east of Annaba far from any human activities and is considered as a relatively clean site because of its remoteness from anthropogenic activity, its important hydrodynamic exposure, and the species' abundance (Rabei et al., 2018; Boukari et al., 2021). Sidi Salem site (36°51'19" N, 7°47'48" E), located about 1 km to the east of Annaba city, receives industrial and domestic wastewater, and is considered as the polluted area (Fig. 1).

Samples collection

Mollusk bivalves *(D. trunculus*) with the same shell length (25 ± 1 mm) (Hamdani et al., 2020) were collected seasonally in the winter and summer of 2019, from two sampling sites (El Battah and Sidi Salem). Animals were transported in cold boxes to the laboratory: the dissection and the sam-

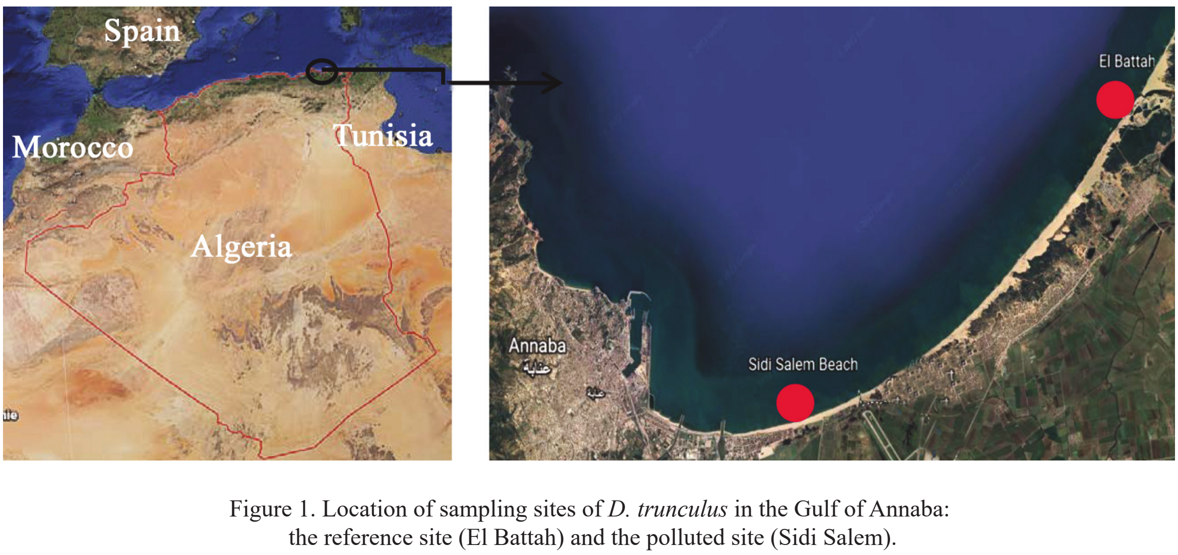

Figure 1. Location of sampling sites of *D. trunculus* in the Gulf of Annaba: the reference site (El Battah) and the polluted site (Sidi Salem).

pling of the digestive glands are carried out the same day of the fishing.

Physico-chemical parameters

The physico-chemical parameters of seawater were recorded using a multiparameter (Multi 340 i, Germany) during the study period at the two sites, El Battah and Sidi Salem. The following parameters were considered: temperature (°C), pH, salinity (PSU) and dissolved oxygen (%) (Table 1).

Protein quantification

The proteins were extracted following the procedure of Shibko et al. (1966) recently described (Boukari et al., 2021) and quantified according to the method of Bradford (1976). This method consists in measuring protein concentrations in the supernatants by using bovine serum albumin (Sigma) as a standard and Coomassie Brilliant Blue (BBC) as a reagent. The absorbance was read at a wavelength of 595 nm. The assay was conducted with 5 repeats.

Determination of metallothionein rate

The determination of metallothioneins in digestive glands was performed following the procedure of Viarengo et al. (1997) previously described (Amira et al., 2018). This method is based on the quantification of SH-residue contents by a spectrophotometric method using Ellman's reagent. Clams were collected (5 specimens from each site). Each digestive gland was dissected and analyzed individually. Absorbance was read at 412 nm and MT levels quantified by use glutathione (Sigma) as a standard. The results are expressed as μg/mg proteins.

Statistical Analysis

Data are represented as mean ± standard deviation (SD). The statistical analyses were performed using prism version 7 for Windows (GraphPad software, La Jolla California, USA, www.Graphpad.com). The comparison of the mean values between sites was made by Student's t-test. The combined effects of seasons and sites were investigated using two-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test.

RESULTS

The physico-chemical parameters of the sea water show average temperature values of 15.12±0.60 °C at El Battah and 14.54±0.31 °C at Sidi Salem. The value of pH is 7.83±0.20 at the site of El Battah and 7.87±0.12 at the site Sidi Salem. Concerning salinity a value of 22.50±0.45 PSU was recorded at the site El Battah and 24.20±0.60 PSU at Sidi Salem. The dissolved oxygen shows a value of 5.51±0.31 % for the site of El Battah and for the site of Sidi Salem 5.37±0.27 %. Statistical analysis by Student's t-test shows no significant difference between the two studied sites ($p>0.05$) for all parameters considered (Table 1).

Protein amounts

The results of the MTs assay obtained are expressed in terms of a quantity of protein. For this purpose, a regression line expressing the absorbance as a function of the quantity of the standard, albumin, was determined (Fig. 2).

Site / Parameters	El Battah	Sidi Salem
Temperature (°C)	15.12±0.60 a	14.54±0.31 a
pH	7.83±0.20 a	7.87±0.12 a
Salinity (PSU)	22.50±0.45 a	24.20±0.60 a
Dissolved oxygen (%)	5.51±0.31 a	5.37±0.27 a

Table 1. Physic-chemical parameters of water at the two sampling sites. For each parameter, mean values followed by the same letter are not significantly different ($p> 0.05$).

Site / Season	El Battah	Sidi Salem
Winter	79.61 ± 0.90 a A	71.68 ± 1.60 b A
Summer	96.10 ± 1.7 a B	80.72 ± 0.70 b B

Table 2. Total protein amounts (μg/mg tissue) in the digestive gland of *D. trunculus* during winter and summer 2019 (m ± SD; n= 5; For the same season, means followed by the same lowercase letter are not significantly different, while for the same site, means followed by the same uppercase letter are not significantly different at $p > 0.05$).

Biochemical analyses reveal a significant ($p<0.001$) increase in proteins levels at Sidi Salem compared to El Battah during the two seasons (Table 2). Maximum values were recorded in El Battah site with an average of 96.10 ± 1.7 (µg/mg tissue) in summer against minimum values in Sidi Salem site with an average rate 80.72 ± 0.70 (µg/mg tissue). A two-way ANOVA revealed significant ($p < 0.001$) effects site ($F_{1, 12}$= 14.92; $P < 0.0023$), season ($F_{(1, 12)}$= 12.42; $P < 0.0042$) and interaction site season was no significant ($F_{1, 12}$= 1.271; $P < 0.2817$).

Metallothionein rates

The results obtained concerning the levels of protein. For this purpose, a regression line expressing the absorbance as a function of the quantity of the standard, albumin, was determined (Fig. 2). MTs are summarized in Table 3. They reveal a significant increase ($p<0.001$) in the levels of MTs observed in Sidi Salem compared to El Battah during both two seasons. The maximum values were recorded at the Sidi Salem site with a mean of 4.10 ± 0.67 (µg/mg proteins) in summer, compared to the minimum values at the El Battah site with a mean level of 3.03 ± 0.38 (µg/mg protein). A two-way ANOVA revealed significant ($p < 0.001$) effect of site ($F_{1, 16}$ = 24.47; $P < 0.0001$) and season ($F_{1, 16}$ = 12.11; $P < 0.0031$). The site-season interaction was not significant ($F_{1, 16}$ = 1.527; $P < 0.2344$).

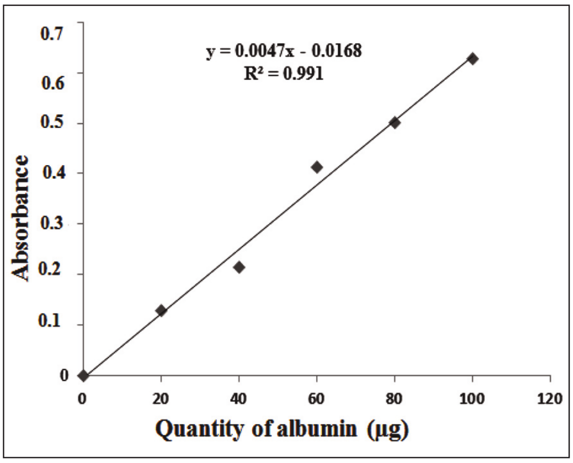

Figure 2. Protein assay: regression line expressing absorbance as a function of the amount of albumin (µg) (R^2 coefficient of determination).

Site / Season	El Battah	Sidi Salem
Winter	2.19 ± 0.53 a A	2.69 ± 0.37 a A
Summer	3.03 ± 0.38 b B	4.10 ± 0.67 b B

Table 3. Metallothionein rates (µg/mg proteins) in the digestive gland of *D. trunculus* during winter and summer 2019 (m ± SD; n= 5; For the same season, means followed by the same lowercase letter are not significantly different, while for the same site, means followed by the same uppercase letter are not significantly different at $p > 0.05$).

DISCUSSION

Changes in protein amounts

Proteins play an important role in repairing and protecting organisms from environmental damage (Umminger, 1977; Ansell et al., 1980; Maharajan et al., 2012). Protein production and expression reflect physiological responses to changing or stressful environmental conditions (Gotelli et al., 2012; Pedrosa et al., 2017). Under stressful conditions, proteins provide energy in metabolic pathways and biochemical reactions to counter this aggression (Maharajan et al., 2012; Do Nascimento et al., 2016).

Our study conducted in *D. trunculus* shows significant decreases in protein levels over the two seasons in the polluted site Sidi Salem compared to El Battah site. Similar results were reported by Yusseppone et al. (2020) where protein analysis in the digestive gland of *Diplodon chilensis* Gray 1828 (Bivalvia Hyriidae) transplanted into a polluted site showed a significant decrease. Since bivalves have a limited amount of carbohydrates and lipids, the next alternative source of energy to meet the increased demand is protein (Le Gal et al., 1997; Xuan et al., 2011; Yeung et al., 2017). Many bivalve species have shown a reduction in proteins to overcome Cd-induced stress: *Mytilopsis. sallei* (Recluz, 1849) (Bivalvia Dreissenidae) exposed to the concentration of 0.71 mg/L (Uma-Devi *et al.,* 1996), *Anodonta anatina* Linnaeus, 1758 (Bivalvia Unionidae) with algae containing the dose of

0.0059 µg/g dry weight (Ngo et al., 2012) or *Mytilus edulis* Linnaeus, 1758 (Bivalvia Mytilidae) treated at the concentration of 0.2 mg/L (Géret et al., 2002). The depletion of total protein levels suggests increased proteolysis and possible use of their degradation products for metabolic purposes, and may be due to increased catabolism and decreased anabolism of proteins, as reported in studies on the freshwater bivalve *Parreysia corrugata* (O.F. Müller, 1774) (Bivalvia Unionidae) (Deshmukh & Lomte, 1998).

Changes in metallothionein rates

Metallothionein levels were determined in the digestive gland of *D. trunculus* in individuals sampled at the El Battah site as well as at Sidi Salem in winter and summer of 2019; our data showed highest values recorded in summer. The statistical analysis reveals that there are significant effects of season and site, confirming previous observations made by Rabei et al. (2018) and Amira et al. (2018) in the same species at the same sites in the Gulf of Annaba. This response is related to the higher metal pollution levels in Sidi Salem compared to El Battah (Belabed et al., 2017; Amira et al., 2018; Rabei et al., 2018; Drif et al., 2019). In fact , the Gulf of Annaba has a strong industrial activity which contributes to the discharge of multiple pollutants such as heavy metals (Abdenour et al., 2000; Beldi et al., 2006; Larba & Soltani, 2014; Belabed et al., 2017, Amira et al., 2018; Drif et al., 2019) and the site of Sidi Salem is exposed to various pollutants from domestic, agricultural and industrial sources (Ounissi et al., 2016; Rabei et al., 2018; Kheroufi et al., 2021, Kroini et al., 2021). The relationship between the concentrations of heavy metals and MTs depends on the concentration and chemical speciation of the accumulated metals (Amira et al., 2018).

A recent report conducted in the same species also showed that MTs are induced by Cd with a concentration effect in several tissues (adductor muscle, gill, digestive gland) after 96 hours of exposure to various concentrations (Moncaleano-Niño et al., 2017). In the same biological model, Tlili et al. (2013) noted an increase in digestive gland MT levels of *D. trunculus* at a site close to the chemical and metallurgical industries in Tunisia. Sand grain size also affects metal availability (Amira et al., 2018; Manju et al., 2020). This explains the increase in rates at the Sidi Salem site, which is made up of fine sand, in contrast to the El Battah site, which is made up of medium sand (Amira et al., 2018). A similar induction has been recorded in Morocco where the proximity of the Mohammedia site (Morocco) to the discharge of a petrochemical company using metals (mainly mercury) among mussels *Mytilus galloprovincialis* (El Haimeur et al., 2017). The study by Mdelgi-Lasram et al. (2007) on clams *Rudipates decussatus* Linnaeus, 1758) (Bivalvia Veneridae) from the Bizerte lagoon (Tunis) at three sites shows that the levels of MTs in the digestive gland are different at the three sites, with a positive correlation with the degree of metal pollution of sites.

Moreover, the comparison of MTs rates between the two seasons (winter and summer) at the same sites (Sidi Salem and El Battah) reveals an increase in MTs rates in summer compared to winter for both sites. This difference may be linked to the fact that in winter, sea currents are stronger than in summer, thus contributing to the dilution of pollutants (Khelifi et al., 2007; Ounissi et al., 2016). However, a change in abiotic factors can affect the bioavailability of pollutants to living organisms and their penetration into tissues (Nahrgang et al., 2013). Indeed, increasing water temperature leads to an increase in the solubility of heavy metals (Hertika et al., 2018). Similarly, increasing salinity will have the same impact on the concentration of these elements (Kavun et al., 2002) and the decrease in dissolved oxygen concentration increases their toxicity (Rotchell et al., 2001). Furthermore, this difference may be related to increased wastewater and air emissions (Qaderi et al., 2018). In addition, other authors have suggested that MT levels may increase during the reproductive period and throughout cell proliferation and differentiation (Coyle et al., 2002; Boulajfene et al., 2019; Hamdani et al., 2020).

CONCLUSIONS

This paper reports the results of a biochemical biomarker MTs measured in the digestive gland of *D. trunculus*, a useful sentinel for environmental monitoring. The results will contribute to the development of an environmental monitoring program based on relevant bioindicators. The level of metallic pollution was found relatively high at Sidi Salem compared to El Battah.

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