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Shell alterations in *Hexaplex trunculus* collected in the vicinity of an impacted zone by industrial marine discharges (Gabès, Southern Mediterranean)

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ABSTRACT

Using the gastropod *Hexaplex trunculus,* a comparative study of the shell characteristics (morphometrics, microstructure and elemental composition) was carried out in the vicinity of Gabès city (Chatt Essalem, CE) which is known as seriously affected by industrial pollution, and two control zones (Ghanouche, GH and Teboulbou, TB). Gastropods from CE had larger, thicker and heavier shells, associated with bigger apertures and slighter soft bodies. Scanning electron microscope (SEM) revealed the presence of three shell layers having a crossed lamellar structure. The inner layer was thinner, while the median layer was thicker in snails from CE, suggestive of the ability of *H. trunculus* to remodel the shell microstructure accordingly. Moreover, the median and inner layers were marked by the presence of fibrous interbeds that were numerous and thicker in shells from CE. In this site, shell elemental alteration was also observed with a decrease in Ca, P, S, K and O contents against an increase in C, Cl and Mg compared to control sites. This investigation was in agreement with previous studies and further suggests the deleterious effect of coastal pollution generated by various industrial units implemented around Gabès city on marine organisms.

1. Introduction

The Gulf of Gabès is the most important fishing zone in Tunisia thanks to its shallow waters and its *Posidonia* seagrass, which makes it a major nursery ground in the southern Mediterranean (Hattab et al., 2013). Since the 1970s, the industrialization of this zone has led to a permanent degradation of the local ecosystems and it is now considered among the most affected areas in the Mediterranean (El Zrelli et al., 2018; Rabaoui et al., 2017). The major source of pollution is the industrial complex of Ghannouche-Gabès, which discharges untreated wastes represented by phosphogypsum dumped by the Tunisian Chemical Group as well as other discharges from the Fluorine Chemical Industry, having a north-south dispersion with a high accumulation in the area between Gabès and Ghannouch Ports (Fig. 1) (Béjaoui et al., 2004; El Zrelli et al., 2015; Rabaoui et al., 2017).In this zone, the water pollution index was reported to be seriously affected following the record of acidity, higher temperatures, and significant inputs of toxic metals like Cd and Cu (El Zrelli et al., 2018; Rabaoui et al., 2017). The harmful effects of industrial waste on the marine environment in the studied area are well documented (Béjaoui et al., 2004; El Zrelli et al., 2015; El Zrelli et al., 2018; Rabaoui et al., 2017). The main consequences are the appearance of red tides and affecting benthic communities with high mortality that resulted in a decrease in fishery production (El Kateb et al., 2018; El Zrelli et al., 2018).

The shell of molluscs is considered as an informative marker, as it exhibits changes according to several ecological and environmental alterations, like chemical contamination, temperature, desiccation, ocean acidification, wave exposure, food availability, depth, and predators (Abdelhady et al., 2018; Duquette et al., 2017; Gouveia et al., 2019; Harayashiki et al., 2020; Landro et al., 2021; Lopes-Lima et al., 2012; Nuñez et al., 2012; Oliveira et al., 2020). Considering environmental pollution, tributyltin (TBT) contamination is known to induce variations

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in oyster shell shape and thickening (Almeida et al., 1998; Higuera-Ruiz and Elorza, 2009). In the freshwater mussel *Anodonta cygnea* and the oyster *Crassostrea gigas*, alterations of the shell inner layer and shell composition have also been reported to occur following exposure to toxic metals (Almeida et al., 1998; Lopes-Lima et al., 2012). In the false-limpet *Siphonaria lessoni* organic malformations affecting the inner layer were observed and considered as a response to organochlorine pesticides and TBT exposures (Nuñez et al., 2012). In the limpet *Bostrycapulus odites*, thicker shells with affected microstructure and changed elemental composition were recorded in the polluted harbours of Argentina (Laitano et al., 2013). A fragile shell, reduced prismatic layer thickness, and enlargement of the periostracum layer in the mussel *Mytilus galloprovincialis*, as well as a significant decrease in organic fraction amounts, were linked to carbamazepine exposure (Oliveira et al., 2020). In a most general view, changes in shell biometric and morphometric parameters, as well as traditional biomarkers of exposure, were most likely caused by exposure to contaminated environments by toxic metals, organochlorines, polyaromatic hydrocarbons, TBT, and polychlorobiphenyls (Begliomini et al., 2017; Gouveia et al., 2019; Márquez et al., 2017; Primost et al., 2016). However, the use of mollusc shell as a biomarker of environmental contamination should be interpreted taking into account the natural conditions to avoid biases that may arise from such assessment studies (Gouveia et al., 2019; Harayashiki et al., 2020).

The banded murex, *Hexaplex trunculus* is a marine edible gastropod largely distributed throughout the Mediterranean Sea, occurring from the mid-littoral to infra-littoral zones and inhabiting both soft and hard substrates (Poppe Goto, 1993). In Tunisia, *H. trunculus* is particularly abundant in the Gulf of Gabès, where it occurs up to 90 m depth (Elhasni et al., 2010). It constitutes a target species collected by hand harvesters and by catch using various fishing gears, with maximal daily landings of 500 kg reached in the spring season (Elhasni et al., 2018). Based on previous investigations into the effect of industrial pollution on aquatic organisms, mainly in the Gulf of Gabès, we hypothesize that such contamination may have an impact on the *H. trunculus* shell. Thus, we here describe the shell characteristics in terms of shell width, shell aperture width, shell thickness, shell weight, microstructure, and elemental composition near the industrial complex of Ghanouche-Gabès, known as the most polluted area in the southern Mediterranean. The observed changes were compared with shells from reference areas.

2. Materials and methods

2.1. Study area and sampling

H. trunculus specimens ($n = 50$/station) with a shell length ranging between 40 and 76 mm, were obtained from local fishermen's gear in July 2016 (resting period of reproduction activity) at three sampling stations around the city of Gabès (Fig. 1). The first station is Chatt Essalem (CE), which is located in the inter-harbour area receiving a mixture of industrial (phosphogypsum and fluoridated wastewaters) and urban marine discharges. It is known as the most polluted site in Gabès with low coastal fishing activity (El Zrelli et al., 2018; Rabaoui et al., 2017). The other two stations are northern Ghanouche (GH) and Teboulbou (TB), which are characterized by the lack of anthropogenic sources of pollution, average to intensive coastal fishing activity, and are presumed to be non-polluted sites (El Zrelli et al., 2018; Rabaoui et al., 2017).In the sampled area, water salinity was reported to be 37.8 in CE, 38.0 in TB, and 40.7 in GH (Ayadi et al., 2015), with pH values ranging from 4.4 to 8.22 in CE, 6.9 to 8.19 in GH, and 7.2 to 8.18 in TB (Ayadi et al., 2015; Ben Amor and Gueddari, 2016). The substratum was sandy-mud (El Lakhrach et al., 2019), with similar hydrodynamics (Zayen et al., 2020).

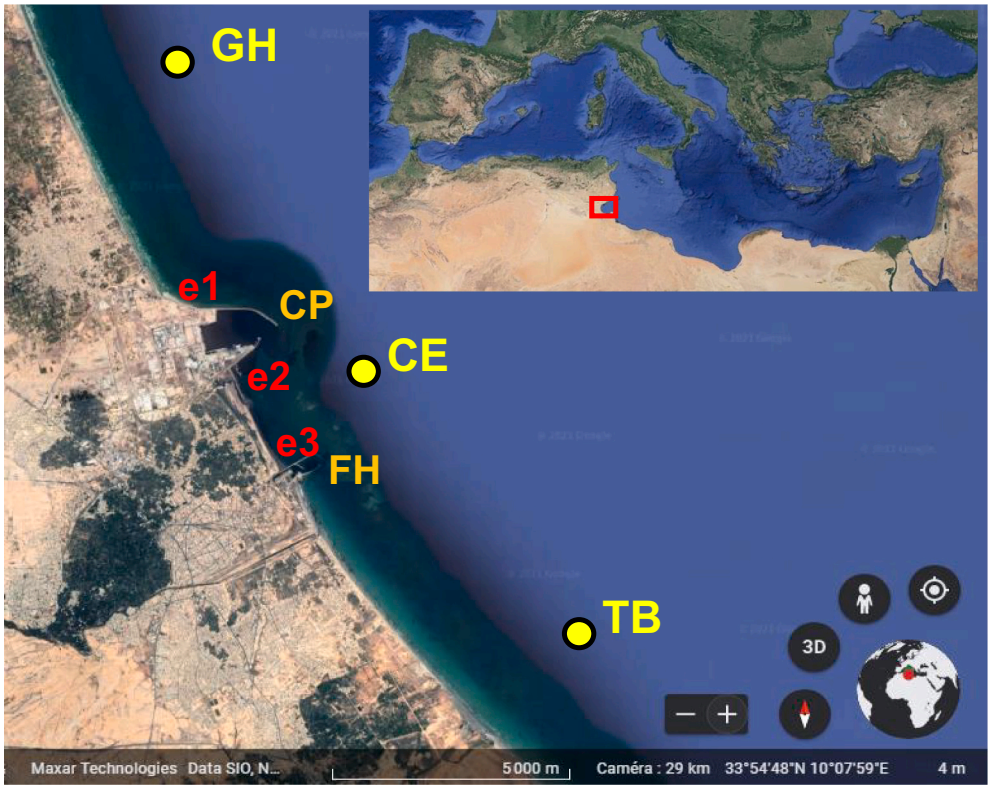


Fig. 1. Sampling sites of *Hexaplex trunculus* at the Gabès coasts. GH: Ghanouche, CE: Chatt Essalem, TB: Teboulbou. CP: Commercial port, FH: Fishing harbour. e1: Fluorine Chemical Industry emissary, e2: Tunisian Chemical Group phosphogypsum waste emissary, e3: urban wastewater marine emissary.

2.2. Lipid peroxidation level (LPO)

The digestive gland tissues (n = 10/station, not pooled) were homogenized in phosphate buffer saline pH 7.4 with an ultra-thurax T25 homogenizator, centrifuged (15 min at 10000*g*, 4 °C), and the supernatant used for protein determination (in triplicate) as described by Ohnishi and Barr (1978). Malondialdehyde (MDA) measurements were used to assess lipoperoxidation (Draper and Hadley, 1990). An aliquot of each homogenate was mixed with a BHT–TCA solution containing 1% (*w/v*) butylated hydroxytoluene (BHT) dissolved in 20% TCA (w/v) and centrifuged at 4000*g* for 15 min at 4 °C. Supernatant was blended with 0.6 N HCl and 120 mM thiobarbituric acid (TBA) in 26 mM Tris buffer. The mixture was heated at 80 °C for 10 min, cooled and absorbance measured at 532 nm with a spectrophotometer (Synergy HT, Biotek, USA) at 25 °C. Concentrations expressed in nmol MDA/mg protein were calculated using the absorbance coefficient of MDA-TBA complex 1.56 × 105 cm^{-1} M^{-1} (Draper and Hadley, 1990).

2.3. Biometric analyses

In the laboratory, gastropods (n = 150) were washed with deionized water to eliminate sediment and surface contaminants, and then stored at −20 °C until analysis. After thawing the gastropod samples, shell length (SL), shell width (SW) and shell aperture width (SA) were measured classically with a vernier caliper to the nearest 0.01 mm (Marzouk et al., 2016; Elhasni et al., 2018). Shell thickness (STH) was measured on a fresh cut taken at the last whorl directly under a binocular microscope using a micrograduated scale (Fig. 2). The shell and soft body were weighed (SWT and SBW) using a digital analytical balance (precision 0.001 g).

2.4. Shell microstructure and elemental composition

The mineral nature of the shell was investigated on fresh shell fractures (n = 10/station) for 15 min using Feighl reagent. The fractured surfaces were then examined under a stereomicroscope to reveal the grey metallic colour that characterizes the aragonite mineral reaction (Dauphin et al., 2012). A detailed examination of the shell layers was performed on ten individuals of similar size from each sampling site (45-50 mm in SL). This was accomplished by polishing sections, attacking them with formic acid, and metalizing them with Ag/Pd in a Denton Vacuum Desk II (Denton Vacuum, Inc. NJ, USA) sputter coater(Dauphin et al., 2012). The analyses were performed using a Phenom ProX scanning electron microscope (SEM) (Phenom World, Netherlands) outfitted with a CEB6 tip and a backscattered electron detector (BSE), which allowed the thickness of each layer composing the shell to be measured. The microscope was used with a 15 KeV acceleration voltage and a charge reduction system to avoid having to carbonize / metallize the area under analysis (Dauphin et al., 2018). At the same time, a semi-quantitative determination of Ca, C, O, P, S, Cl, K and Mg in the shells was performed by an Energy Dispersive Spectroscopy (EDS) analysis using the EID software (Dauphin et al., 2018; Laitano et al., 2013; Nuñez et al., 2012).

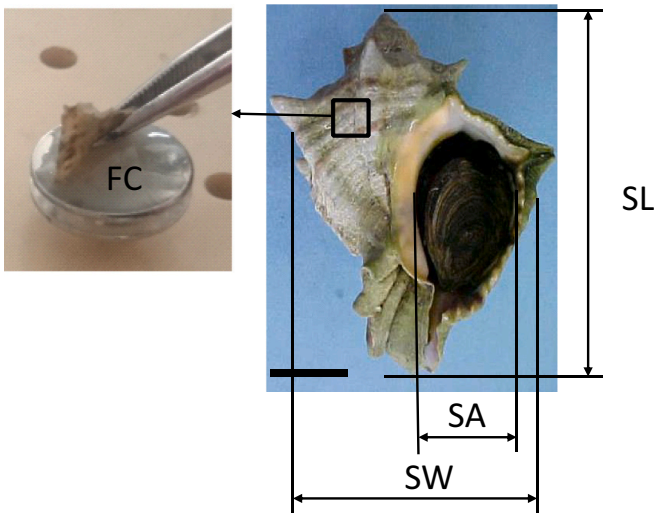


Fig. 2. Localization of the shell fresh cut used for thickness measurement and for making SEM observations. SL: Shell length, SA: Shell Aperture width, SW: Shell width, FC: Fresh cut fixed to the SEM plot. Scale bar = 10 mm.

2.5. Statistical analysis

The averages of shell biometrics, elemental content, shell thickness (SEM measurements), and MDA level among the three stations were subjected to one-way ANOVA (assumptions of normality of data and homogeneity of variances were achieved) and pairwise comparisons (whenever significant differences among groups were detected) using the Tukey test with sigmastat 3.5. The level of significance used is $p < 0.05$.

Morphometric relationships between SL (independent variable), SW, SA, STH, SBW and SWT were established for each population through regression analysis (least squares method for log transformed data), by fitting the power function ($Y = aX^b$) to raw data and assessing the degree of association between variables by the correlation coefficient (r). Being highly significant ($p < 0.001$) and displayed invariably high correlation coefficients (r = 0.951 to 0.973), morphometric characters were used to calculate three metric (SW/SL, SA/SL and STH/SL) and two weight (SBW/SL and SWT/SL) ratios for each individual. The calculated values of the morphological ratios were subjected to a multivariate analysis of variance (MANOVA) using PAST. A Wilk's Lambda test was used to determine whether there were significant differences between stations, and post hoc hotelling pairwise comparisons (Bonferroni corrected and uncorrected) were used to show what are those differences. The scatter from Canonical Variables Analysis (CVA) was then obtained for the three gastropod populations based on the calculated values of morphological ratios.

3. Results

3.1. Oxidative damage

The LPO values (mean ± SD) were 0.16 ± 0.01 nmol MDA/mg protein in GH, 0.32 ± 0.06 in CE and 0.23 ± 0.04 in TB, which were statistically different (ANOVA, F = 25.56, $p < 0.001$) (Fig. 3). Tukey Pairwise comparisons show that all values were different; snails collected in CE (the most contaminated site) had higher values than snails collected in GH ($p < 0.001$) and TB ($p < 0.01$). LPO levels were significantly higher in TB than in GH (p = 0.03).

3.2. Morphometric analysis

Biometric data of collected gastropods are reported in Table 1. The three populations showed similar shell lengths (ANOVA, p = 0.6). The population from CE presented larger shells (p-values <0.001), while those from GH and TB were similar (p = 0.27). SA values were also the highest in CE (p-values <0.001), being greater in TB than in GH (p = 0.01). STH values were similar between CE and GH (p = 0.06), and between GH and TB (p = 0.27), being higher in CE than in TB ($p < 0.001$). With regard to weight variables, the GH population presented a higher soft-body weight as compared to CE (p = 0.02), and was similar to TB (p = 0.33). CE and TB did not show a significant difference in their body weight (p = 0.41). However, shells from CE were heavier than those from GH and TB (p-values <0.001), which both had similar weights (p = 0.99).

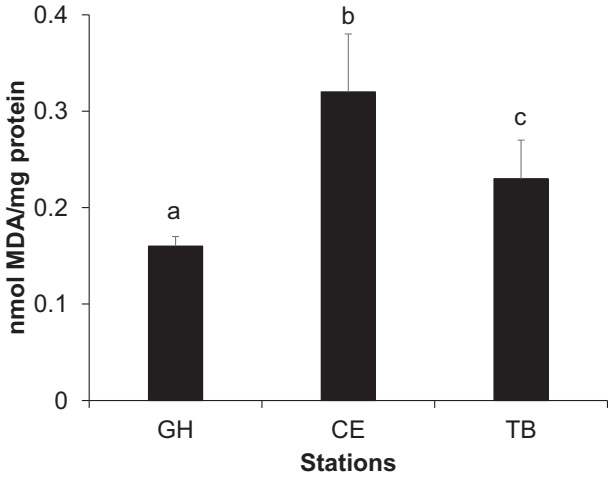


Fig. 3. Lipid peroxidation level (LPO) in *Hexaplex trunculus*. GH (Ghannouche), CE (Chatt Essalem), TB (Teboulbou). Different letters denote statistically significant differences (Tukey test).

Table 1
Biometric data and morphometric ratios of *Hexaplex trunculus* (mean ± SD) at the study sites (n = 50/station). GH (Ghanouche), CE (Chatt Essalem), TB (Teboulbou). Different letters denote statistically significant differences (Tukey test).

	CE	GH	TB
Biometric variables			
SL (mm)	54.48 ± 6.96^{a}	53.05 ± 7.25^{a}	54.19 ± 8.11^{a}
SL range	40.4–73.7	40.0–73.0	40.0–76.0
SW (mm)	34.54 ± 4.38^{a}	29.80 ± 4.46^{b}	31.14 ± 4.13^{b}
SA (mm)	15.65 ± 1.67^{a}	12.87 ± 1.45^{b}	13.82 ± 1.78^{c}
STH (mm)	0.65 ± 0.07^{a}	0.62 ± 0.06^{ab}	0.60 ± 0.08^{b}
SBW (g)	6.96 ± 2.53^{a}	8.74 ± 3.85^{b}	7.80 ± 3.43^{ab}
SWT (g)	15.40 ± 5.76^{a}	9.51 ± 3.63^{b}	9.46 ± 5.72^{b}
Biometric ratios			
SW/SL	0.63 ± 0.01^{a}	0.56 ± 0.00^{b}	0.57 ± 0.04^{b}
SA/SL	0.29 ± 0.00^{a}	0.24 ± 0.00^{b}	0.26 ± 0.00^{c}
STH/SL	11.87E-03 ± 0.00^{a}	11.68E-03 ± 0.00^{a}	11.12E-03 ± 0.00^{b}
SBW/SL	0.13 ± 0.03^{a}	0.16 ± 0.05^{b}	0.14 ± 0.05^{c}
SWT/SL	0.32 ± 0.04^{a}	0.18 ± 0.05^{b}	0.17 ± 0.04^{b}

Metric ratios were all higher in CE as compared to GH and TB ($0.02 \leq p < 0.001$). SW/SL was greater in CE ($p = 0.001$) and comparable in GH and TB ($p = 0.08$). SA/SL was also greater in CE, followed by TB and GH (all p-values <0.001). STH/SL is similar between CE and GH ($p = 0.16$), being greater than TB (p-values<0.001). Considering weight ratios, the population from CE presented significant differences, with SBW/SL being lower than GH ($p = 0.002$) and TB ($p < 0.001$), while SWT/SL was higher (p-values <0.001). A comparison between GH and TB revealed a significant difference for SBW/SL ($p < 0.001$) and similar values for SWT/SL ($p = 0.94$).

The MANOVA revealed that the linear morphometric ratios (SW/SL, SA/SL and STH/SL) differed between the three sites (Table 2). A subsequent pairwise test also revealed significant differences between the three populations (Table 2). The CVA analysis scatter plot showed clear segregation of the three gastropod populations according to the variations observed in CV1 (90.11%) than in CV2 (9.87%) (Fig. 4a). Based on CVA loadings on the first canonical variate, separation was due to variation in SA/SL. For the second canonical variate, loadings were mostly for STH/SL and, from afar, SW/SL. Subsequently, gastropods inhabiting GH and TB are grouped in the negative values of CV1 and gastropods of CE in the positive values (Fig. 4a). Considering CV2, gastropods inhabiting GH and CE are mostly distributed in the positive values, while those of TB are in the negative values. The distribution of individuals showed more regularity in CE, followed by GH and TB. Overall, this means that the CE population has a bigger shell aperture with more regular variability in shell width and thickness as compared to TB and GH (Fig. 4a). Organisms from TB have bigger apertures with thinner shells as compared to GH. When the weight ratios (SWT/SL and SBW/SL) were considered, MANOVA also revealed a significant difference between groups (Table 2), with pairwise comparisons being significant for CE as compared to GH and TB. While these later stations did not show significant variations ($p = 0.23$). The CVA analysis scatter plot showed the segregation of the CE population according to CV1 (80.06%), with loadings being for SBW/SL ratio, which means that snails from CE have lighter soft-bodies (Fig. 4b). The CV2 (19.93%) loadings were for SWT/SL with gastropods from GH and TB having high variability in the weight of their shells as compared to CE (Fig. 4b).

Table 2
Multivariate analysis of variance (MANOVA) of calculated morphological ratios at the the study sites. GH (Ghanouche), CE (Chatt Essalem), TB (Teboulbou).

Metric ratios (SW/SL, SA/L, STH/SL)			
MANOVA	Value	F	p-value
Wilk's Lambda test	0.015	334.3	1.4E-126
Pillai trace	1.256	81.6	2.28E-59
Pairwise comparison	GH vs. CE	GH vs. TB	CE vs. TB
Bonferroni uncorrected (p-value)	5.73E-87	3.38E-44	4.47E-70
Bonferroni corrected (p-value)	1.72E-86	1.01E-43	1.33E-69
Weight ratios (SBw/SL, SWT/SL)			
MANOVA	Value	F	p-value
Wilk's Lambda test	0.158	113.6	3.92E-58
Pillai trace	0.859	54.97	1.62E-34
Pairwise comparison	GH vs. CE	GH vs. TB	CE vs. TB
Bonferroni uncorrected (p-value)	6.0E-43	0.238	5.03E-41
Bonferroni corrected (p-value)	1.8E-42	0.714	1.51E-40

3.3. Microstructure and elemental composition

Feighl coloration of the fresh fractures showed the shell of *H. trunculus* is of an aragonitic nature. SEM analysis in the three populations revealed that three layers (external L1, median L2 and inner L3) with a crossed lamellar structure compose the shell (Fig. 5). However, the L2 and L3 layers were marked by numerous and thicker fibrous interbeds in CE as compared to the control sites (GH and TB)(Table 3, Fig. 6). When considering the SEM analysis, the total thickness of all the shell layers (L1 + L2+ L3) was similar among the studied populations (Table 3, F = 1.88, p = 0.23). The thickness of the external (L1) shell layer was also similar among the three gastropod populations (F = 2.38, $p = 0.17$). However, when comparing the median (L2) and inner (L3) layers, significant differences were recorded (Table 3, F = 12.38; $p = 0.007$, F = 9.93, $p = 0.012$, respectively). Indeed, L2 was significantly thicker in CE as compared to GH (Tukey test, $p < 0.001$) and TB (Tukey test, $p < 0.001$). No difference was recorded among control sites (Tukey test, $p = 0.79$). While L3 was significantly thicker in TB and GH compared to the impacted site (Tukey test, p-values <0.001), again, no differences were recorded between these two sites (Tukey test, $p = 0.86$).

Shell elemental composition also varied among the three sites (ANOVA, $24.1 < F < 2400$, $p < 0.001$; Table 4); except O concentration, which was similar ($F = 2.9$, $p = 0.07$). Ca, P, S and K contents were higher in GHand TB (Tukey test, $p < 0.001$), while C, Cl and Mg were higher in CE (Tukey test, $p < 0.001$).

4. Discussion

LPO has been widely used as a biomarker of exposure to a variety of hazardous substances (Begliomini et al., 2017; Belcheva et al., 2015; Gouveia et al., 2019; Lin et al., 2016). The higher LPO levels found in CE

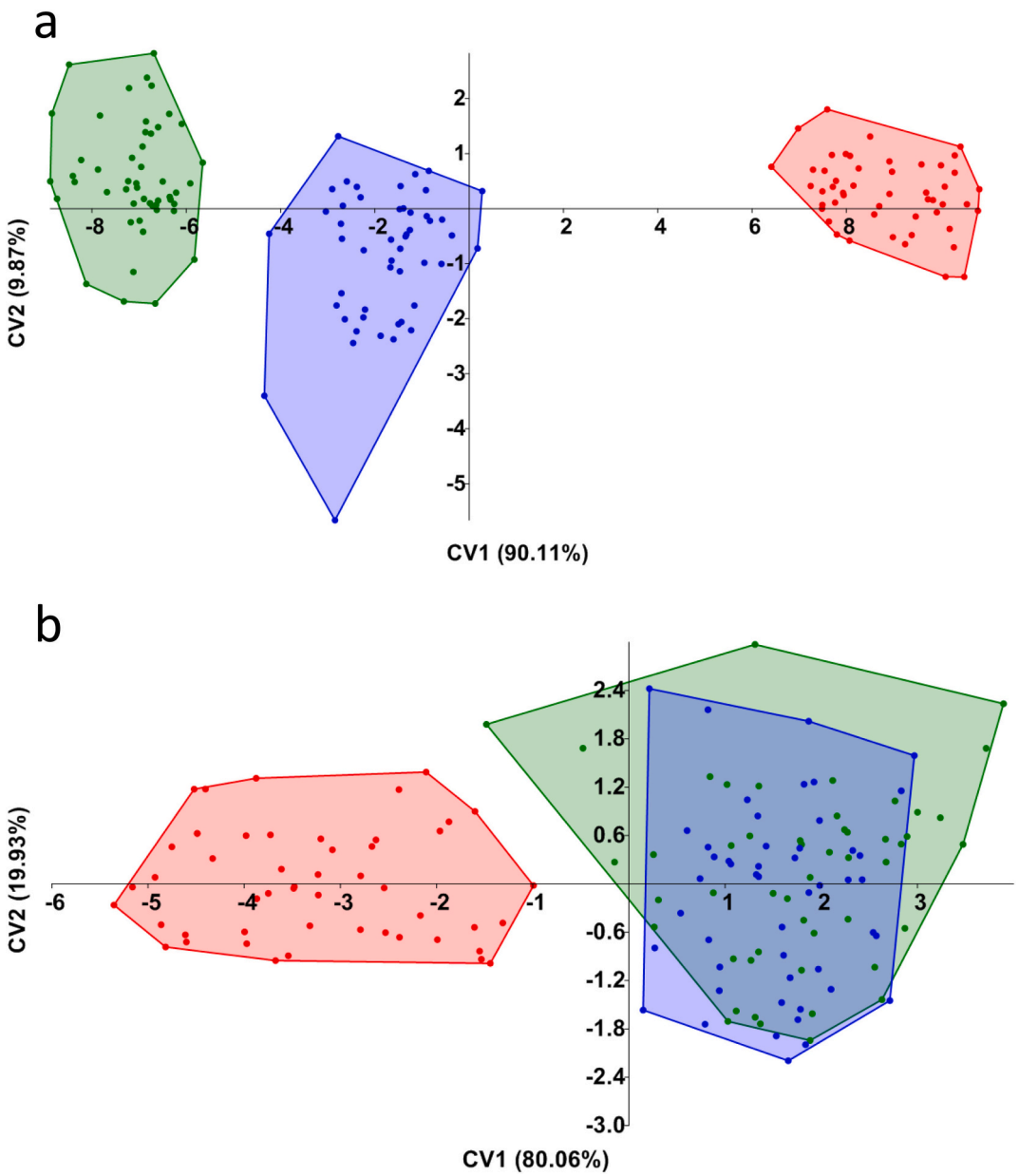


Fig. 4. Results of Canonical Variates Analysis (CVA) of three morphometric linear ratios (a) and two weight ratios (b) of *H. trunculus* from the studied sites. Green (Ghannouche, GH), Red (Chatt Essalem, CE), Blue (Teboulbou, TB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirm that gastropods are being affected by water contaminants. These chemicals can cause the formation of reactive oxygen species (ROS), which can cause toxic effects due to the generation of free radicals. The polyunsaturated fatty acids in cellular membranes are the primary targets of ROS (Belcheva et al., 2015; Oliveira et al., 2020). Similar findings were reported in gastropods from south-eastern Brazil, with LPO levels increasing in response to a pollution gradient of various contaminants in surface sediments (Begliomini et al., 2017; Gouveia et al., 2019).

The present investigation demonstrates that industrial (phosphogypsum and fluoridated wastewaters) contamination is most likely the cause of shell changes in *H. trunculus*. Indeed, the three populations had identical shell lengths, implying that the changes observed were not driven by differences in the size of the organisms in the sample. Shells were larger, thicker and heavier while soft tissues were lighter in the snails collected at the impacted site (CE). Similar effects on the soft tissue weight were reported in the gastropod *Nerita atramentosa* exposed to oil spills, with obvious effects at gonadal level, i.e. lower gonad weight coupled with changes in fertility indices (Battershill and Bergquist, 1982). In the limpets *Siphonaria lessoni* and *B. odites* decreased soft tissue weights were also reported in highly polluted areas (harbours) with shells being thicker (Laitano et al., 2013; Nuñez et al., 2012). Contrary to our findings, these studies reported that shell weight decreased when animals were exposed to environmental pollution and that shell hardness in polluted areas decreased. Indeed, thickening within lighter shells is a known reported pollution effect in bivalves (mainly oysters) in which shell thickening has been reported because of shell chambering due to exposure to TBT (Almeida et al., 1998; Alzieu et al., 1986). In the case of gastropods, no shell chambering was reported within the few available studies, although shell thickening was reported in harbours (polluted with TBT and hydrocarbons) and areas affected by mine residues (i.e. heavy metals) (Laitano et al., 2013; Nuñez et al., 2012). In our case, TBT cannot explain our results because the study area did not show significant TBT contamination and because the use of TBT

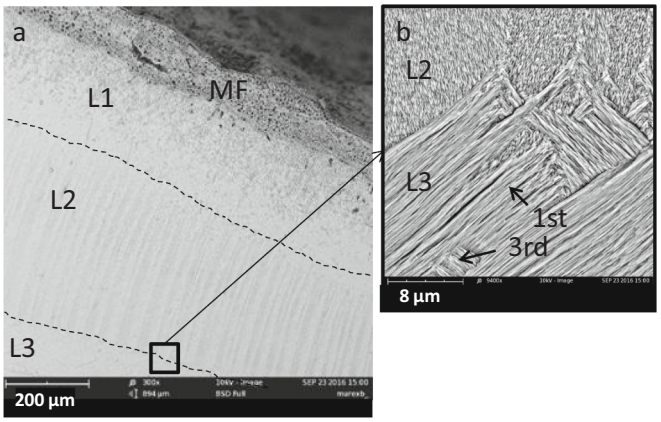


Fig. 5. SEM image of a fresh shell break in *H. trunculus* from Ghannouche (GH) showing (a) three aragonitic cross lamellar layers (L1: outer, L2: median, L3: inner) and (b) a detailed view of the transitional zone between L3 and L2. MF: micro-fouling, 1st: first order lamella, 3rd: third order lamella.

Table 3
Shell layers thickness (mean ± SD, µm) and number of interbeds (mean ± SD) variations for each of the study sites (n = 10/station). Different letters denote statistically significant differences (Tukey test).

	Impacted site	Control sites	
Shell layers	CE	GH	TB
External (L1)	235.33 ± 31,98^{a}	260.54 ± 16.11^{a}	220.33 ± 16.66^{a}
Median (L2)	450.74 ± 25.01^{a}	357.71 ± 26.24^{b}	371.17 ± 22.88^{b}
Inner (L3)	271.67 ± 27.14^{a}	348.00 ± 27.71^{b}	359.29 ± 23.53^{b}
Total layers	1019.74 ± 68.87^{a}	955.32 ± 41.26^{a}	946.50 ± 34.74^{a}
Number of interbeds	8.30 ± 1.25^{a}	3.30 ± 0.82^{b}	5.10 ± 0.74^{c}
(min-max)	(7–10)	(2–4)	(4–6)

has been prohibited in Tunisia since 2011 (Lahbib et al., 2018). Besides pollution, other factors that are known to affect shell thickness in gastropods are predation intensity (Avery and Etter, 2006; Charifson, 2019), coastal energy (Giraldo-López and Gómez-Schouben, 1999) and food availability (Charifson, 2019).Predators are present all along the study area, mainly the invasive crabs *Callinectes sapidus* and *Portunus segnis* but they are not likely to differ among the studied stations that are very close (14 km from GH to TB). *P. segnis*, in particular, was abundant, being captured in 60% of fishermen's fishnets (25 kg per 50 m of nets) and can reach more than 150 kg of crabs per 24 h in some extreme cases (Khamassi et al., 2019). Coastal energy and food availability are also similar among the three stations (Zayen et al., 2020), further supporting the role of environmental pollution in determining our results. However, the water currents directed north-south that allow the transfer of pollutants toward the southern areas might explain the changes reported in TB as compared to GH (El Zrelli et al., 2015, 2018; Zayen et al., 2020). In this context, to find the direct link between coastal contamination and shell modifications, in-vitro studies are suggested under controlled conditions.

To better describe the changes in shell thickening and weight, shell microstructure and elemental composition were investigated. SEM analyses, allowed measuring shell thickness with precision. Statistically, results were similar among the three sites, and this may be explained by the fact that shell microfouling could have bias in making precise shell thickness measurements using the stereomicroscope (Fig. 7).If this were the case, the contamination of the CE site may not have had a clear effect on shell thickening in *H. trunculus*. Shell microfouling could also have been a bias on elemental composition like Si and Mg that were present abundantly (Fig. 7). For that reason, fouled shells should be discarded from elemental analysis. The identification and thickness measurement of the three layers composing the shell of *H. trunculus* revealed that the

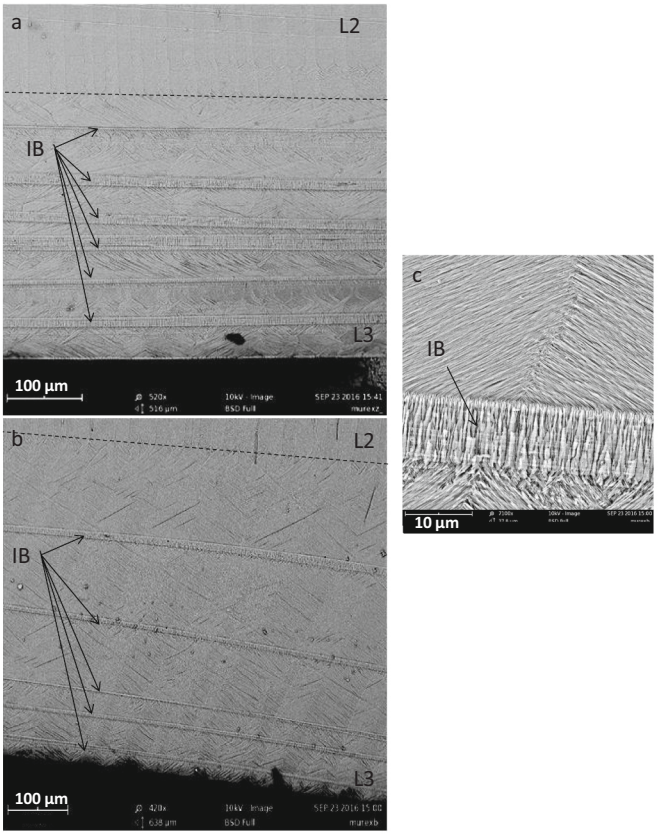


Fig. 6. SEM micrographs on fresh cuts in the shell of *H. trunculus* showing interbeds in CE (a) and TB (b). (c) Detail of an interbed (IB) from Chatt Essalem (CE). L2: median layer, L3: inner layer.

Table 4
Semi-quantitative elemental composition (average weight percentage ± SD, *n* = 10) of shells from high polluted site CE and control sites (GH and TB). Different letters denote statistically significant differences (Tukey test).

	Impacted site	Control sites	
Element %	CE	GH	TB
Ca	15.2 ± 1.6^{a}	46.4 ± 3.8^{b}	34.9 ± 2.4^{c}
C	34.1 ± 4.3^{a}	27.6 ± 5.4^{b}	20.0 ± 3.8^{c}
O	15.4 ± 2.8^{a}	16.0 ± 3.4^{a}	18.9 ± 4.1^{a}
P	0.0 ± 0.0^{a}	0.8 ± 0.04^{b}	0.4 ± 0.06^{c}
S	0.4 ± 0.2^{a}	2.5 ± 0.7^{b}	2.0 ± 0.5^{b}
Cl	0.8 ± 0.5^{a}	0.0 ± 0.0^{b}	0.3 ± 0.1^{b}
k	0.0 ± 0.0^{a}	1.6 ± 0.8^{b}	0.9 ± 0.3^{c}
Mg	0.5 ± 0.05^{a}	0.0 ± 0.0^{b}	0.2 ± 0.07^{c}

inner and median layers exhibit changes among the three sites. The median layer was thicker at the polluted site (CE) while the inner layer was thinner. They both exhibited more interbeds than control sites, which may suggest that the shells are continually reinforced, maybe because of environmental stress (Avery and Etter, 2006; Charifson, 2019). In that logic, exposure to contaminants does not induce an overall increase in shell thickness but rather shell layer remodeling. Such results were also reported in the snails *Nucella lapillus* and *Littorina littorea* exposed to high predation and food decrease (Avery and Etter, 2006; Charifson, 2019). Shell reinforcements have been considered to be investigated with energetically cheaper materials (homogenous aragonitic structure) which would allow fast growth, ensuring refuge for minimizing the risk of predation (Avery and Etter, 2006). In that sense, the preference of *H. trunculus* for thickening the median shell layer and growing numerous interbeds (maybe energetically cheaper structure)

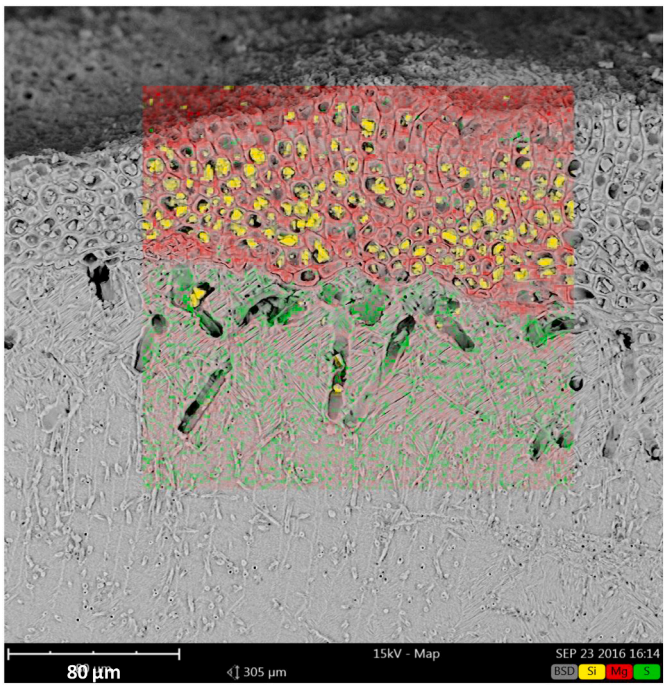


Fig. 7. Elemental composition mapping of a siliceous alga fouling on the external shell surface of *Hexaplex trunculus*. Yellow, red and green regions correspond to Si-, Mg-, and S-rich areas, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

could be a behaviour against chemical exposure. Additionally, while the L1 and L3 shell layers have the same (longitudinal) crossed lamellar structure, the median layer has a different (transversal) orientation. Further investigations focusing on how these modifications of the median layer affect shell strength and the associated energy cost needed for such changes are necessary to make any further conclusions.

To verify the changes recorded in the shell of *H. trunculus* in the contaminated site, elemental composition was investigated, namely targeting the elements known as indicative of shell alteration (Faubel et al., 2008; Gouveia et al., 2019; Laitano et al., 2013; Nuñez et al., 2012). The shell elemental composition varied among stations. The Ca concentration decreased significantly between the contaminated site and the two control sites, falling from 34.9–46.4% in TB and GH to 15.2% in CE. Similar studies investigated in *S. lessoni*, *B odites*, *Lottia subrugosa,* and *Siphonaria pectinata* have found that a decrease in calcium content indicates a change in shell composition (Gouveia et al., 2019; Laitano et al., 2013; Nuñez et al., 2012; Slama et al., 2021). In fact, the calcium shell content of the false limpet *S. lessoni* has decreased from 27% in unpolluted stations to only 7% in port stations (Nuñez et al., 2012). In *B. odites*, from 48% to 18% (Laitano et al., 2013). In *L. subrugosa*, from 38% to 15–31% (Begliomini et al., 2017; Gouveia et al., 2019). In *S. pectinata*, from 42.3% to 8% (Slama et al., 2021). Similarly, phosphorus and sulfur content have decreased in the contaminated site (from 0.4–0.8% to 0.0% for P and from 2 to 2.5% to 0.4% for S), which is in agreement with previous studies. Indeed, P and S content in L. *subrugosa* decreased from 2.29 to 0.31% and from 1.04 to 0.17%, respectively (Gouveia et al., 2019). In *S. pectinata*, P content decreased from 1.33 to 0.24% and S content from 1.23 to 0.29% (Slama et al., 2021). Considering that P and S are among the main elements composing proteins, we hypothesize that pollution is inducing a loss of the shell organic matrix. This is further supported by C and Cl content, whose levels increased in the contaminated station, despite that in this study, no organic malformations were observed on the inner shell surface like reported in other gastropods (Laitano et al., 2013; Nuñez et al., 2012). Presently, concentrations increased in CE from 20 to 27.6 to 34.1% for C and from 0 to 0.36 to 0.8% for Cl, being in agreement with those reported in *S. lessoni* (15.8–52.9% for C and 0–0.31% for Cl) and *B. odites* (34.1–73.5% for C and 0–0.42% for Cl) (Laitano et al., 2013; Nuñez et al., 2012).The registered decrease in Ca concentrations in shells collected in the polluted station (CE) may be due to the potential effect of the phosphogypsum-associated contaminants on the metabolic pathways implicated in shell biomineralization. Moreover, the shells from this station also displayed structural alterations, such as numerous and thick interbeds in the median layer. These findings corroborate experiments and field studies that registered a decrease in Ca levels in bivalve and gastropod shells exposed to contaminants like toxic metals (Faubel et al., 2008; Gouveia et al., 2019; Laitano et al., 2013; Nuñez et al., 2012). The mobilization of such elements and their partitioning to the shell is a known detoxification strategy in bivalves and gastropods (Nicholson and Lam, 2005). In the case of CE, toxic metals that were found in phosphogypsum discharges at significant concentrations (El Zrelli et al., 2018; Rabaoui et al., 2017), support the idea that they could play a leading role in shell alterations. Further investigations focusing on laboratory exposure of gastropods to selected heavy metals are necessary to verify their effects on shell biomineralization. In bivalves, Cd, Cu and Cr have been reported to cause alterations in shell biomineralization within the inner layer (Faubel et al., 2008). These elements are known to produce changes in the shell crystallization process and are indicative of an ion disturbance in shell calcification. The incorporation of such elements into the shells is most likely attributed to the similarity of their atomic radius to that of Ca, which allows them to reach the intracellular environment by competition (Matsuo et al., 2005).

5. Conclusions

Shell changes in *H. trunculus* reported in the contaminated site of Chatt Essalem compared to the two reference sites are suggestive of industrial wastewater (phosphogypsum) being the main cause. This investigation also revealed the ability of *H. trunculus* to adapt to environmental pollution by remodeling the median shell layer accordingly.

Credit authorship contribution statement

Youssef Lahbib: Conceptualization, Validation, Supervision, Writing - Review & Editing. Tasnime Slama: Conceptualization, Methodology, Investigation, Writing - Original Draft, Validation. Sami Abidli: Conceptualization, Investigation. Najoua Trigui El Menif: Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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