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Assessment of Environmental Quality in the Tamaulipas Laguna Madre, Gulf of Mexico, by Integrated Biomarker Response Using the Cross-Barred Venus Clam *Chione elevata*

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Abstract The entire Laguna Madre of Tamaulipas is a natural protected area and a Priority Marine Region of Mexico. However, its important biodiversity and high levels of endemism are threatened by the discharge of different pollutants and activities related to the ocean oil and gas industry. Therefore, the assessment of these effects on this marine ecosystem is of paramount importance. At present, the joint approach of monitoring chemical contaminant levels, alongside the use of pollution biomarkers as surrogate measures of biological impact within the environment, provides the better evaluation of the environmental hazard. Within this context, a biomonitoring study using native *Chione elevata* mussels sampled from four locations along the Mexican Laguna Madre coasts evaluated whether a battery of select biomarkers was suitable for identifying and quantifying pollution-induced stress in mussels. The levels of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CaE), alkaline phosphatase (ALP), glutathione s-transferase (GST), and the oxygen radical absorbance capacity (ORAC) were measured in soft tissues samples. Different metals (Cd, Pb, Cu, Zn, and Fe) as well as total heavy hydrocarbons were also determined in sediments. Higher concentrations of metals were observed in sampling localities with marine influence possibly related to the presence of marine grass. The concentration of total heavy hydrocarbons, as expected, was higher in sites with intensive fishing activity. The integrated biomarker response (IBR) and the condition index of mussels allowed discriminating between localities of continental and marine influence, revealing that the sampling stations with continental influence were subjected to a greater stress as a result of anthropogenic effects.

Keywords Clam · Biomarkers · IBR · Pollution · Laguna Madre

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1 Introduction

The daily mixing of fresh and salt water in estuaries leads to the existence of variable and dynamic physicochemical conditions. This makes it difficult to determine the effects of pollution caused by anthropogenic activities on aquatic organisms, despite the increasing level of these activities in these ecosystems (Sarkar 2006; Watson et al. 2011). Among the diverse types of marine ecosystems, the Laguna Madre in the Gulf of Mexico is the only coastal, hypersaline lagoon system on the North American continent and has been recognized as the largest of the seven known hypersaline ecosystems of the world. The Laguna Madre is classified as a UNESCO Biosphere Reserve, a RAMSAR site, and is considered a Priority Marine Region of Mexico (Arriaga-Cabrera et al. 1998). Specifically, this region has a diverse variety of subtropical and coastal ecosystems that provide habitat to many

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species including endemic turtles, shorebirds, and a wide diversity of invertebrates and aquatic vertebrates of economic importance for commercial and sport fisheries, such as the shrimp fishery which outstands by the number of fishermen depending on this activity (Rendón-von Osten and García-Guzmán 1996). In 2005, Mexico declared the entire Laguna Madre of Tamaulipas as a natural protected area, which is a milestone in the conservation of such a productive and diverse estuary. This designation, by itself, will not safeguard the lagoon from all the problems affecting it; however, it represents the commitment from the Mexican government towards more environmentally sound management practices. At present, this great biodiversity is especially threatened in the middle part of the Laguna Madre, in Mexico, by the discharge of different pollutants drained into the mouths of the Bravo and Soto la Marina rivers, as well as by diverse activities related to the ocean oil and gas industry (Botello et al. 2015).

A crucial step during the assessment of environmental quality is the monitoring and evaluation of the biological effects of pollutants using the right tools on adequate samples. The chemical monitoring of these affected environments is difficult and is ultimately restricted to the identification of a limited number of substances, moreover, the toxicity of the mixtures may be very different from that of the single compounds (Carvalho et al. 2014), and does not provide information on their biological significance, i.e., on their effects on biological communities (Serafim et al. 2012). In relation to this, sediments are important in aquatic ecosystems because they accumulate pollutants and mediate the exchange between particulate, dissolved, and biological phases (Andreotti and Gagneten 2006; Caeiro et al. 2009). It is for this reason that those organisms found in the sediments are considered among the most useful sentinels in the biomonitoring of environmental pollutants, especially mollusks as they are slow-moving or sessile, have a broad distribution, and are filter feeders with high bioaccumulation and low biotransformation rates (Vázquez-Silva et al. 2006). These organisms accumulate a wide range of contaminants and reflect changes in contaminant status of the environment in aquatic ecosystems (Damiens et al. 2007). Different approaches ranging from population-community level to sublethal responses of sentinel species are the current basis for most biomonitoring programs (Seabra Pereira et al. 2014). Regarding this, different marine bivalves of the genus *Mytilus*, *Ruditapes*, and *Crassostrea* have been widely used as sentinels for pollution monitoring programs in coastal waters (Moreira and Guilhermino 2005; Cotou et al. 2013). However, in most of the cases, the selection of the species relies in their abundance and distribution in the area of study, and not always considers the sensitivity of the species to certain pollutants (Leiniö and Lehtonen 2005). Among the bivalve species with potential to be used as bioindicators in the Laguna Madre stand out, *Crassostrea virginica*, *Anadara transversa*, *Isognomon alatus*, and *Chione cancellata* (*C. elevata*, *nomen novum*; Roopnarine and Vermeij 2000). The latter is the species with the wider distribution and higher abundance in this region (Torres-Cerón et al. 2014). *Chione* species have proven to be good sentinels for the evaluation of the impact of pollutants. In this way *C. elevata* was used to evaluate the effects of wastewater in Florida (McNulty 1961), while *C. californiensis*, *C. stutchburyi*, and *C. subrugosa* were used for the monitoring of DDT (Nuñez 1975), chlorinated hydrocarbons (Gutierrez-Galindo et al. 1988), and heavy metals (Green-Ruiz et al. 2005; Páez-Osuna et al. 1993) in the Gulf of California and New Zealand (Purchase and Fergusson 1986). However, these studies with *Chione* species were only focused on the residual determination of pollutants, and until the present day, a baseline on biochemical biomarkers has not been proven to evaluate the eventual physiological alterations provoked by pollutants in these mollusks. Because of the ubiquity of various chemicals in the aquatic environment, several biochemical endpoints (biomarkers) of pollutants can provide valuable information regarding the working mechanism of toxic compounds and thus have been tried as early warning signals relative to endpoints at higher levels of biological organization, with the potential to identify the presence of specific classes of toxicants, and account for deleterious effects (van Der Oost et al. 2003; Kim et al. 2010). Currently, several advances have been accomplished in the selection of biomarkers suited for marine bivalves aimed at evaluating the quality of the surrounding aquatic environment and an important consensus has been achieved concerning the use of a multi-biomarker biochemical approach as no single biomarker can unequivocally measure environmental degradation and usually more than one biomarker response is observed by exposure to pollutants (Cotou et al. 2013). Under this context, *C. elevata* could be a suitable candidate as a sentinel of environmental pollutants in the Laguna Madre, as well as in other regions of the Gulf of Mexico and the American Atlantic (Roopnarine and Vermeij 2000).

Considering the scarcity of data on the effect of pollutants in bivalves of the Laguna Madre in Mexico, and taking into account the wide distribution, high abundance, and earlier use of other species of the genus *Chione* in pollution studies, the present work was aimed at assessing the eventual impacts of pollutants on *C. elevata*, sampled at different locations, by means of a selected suite of biomarkers, whose response was combined and incorporated into a stress index, the Integrated Biomarker Response (IBR) proposed by Beliaeff and Burgeot (2002), to achieve a comprehensive evaluation of the health status of these mollusks.

2 Material and Methods

2.1 Study Area

Four sampling sites were selected to cover areas of continental and marine influence in the interior of the Laguna Madre. These sites were identified as follows (Fig. 1): *Crassostrea virginica* oyster bank (S1), coordinates 24° 28′ 58.1″ N and 97° 41′ 26″ W, which is a shallow area with patches of seagrass located in the lee of the barrier island and close to the mouth of the lagoon where shrimp enter and exit to the open sea. Zone of seagrass (S2), coordinates 24° 29′ 2.4″ N and 97° 41′ 52.6″ W, an area inside the deep lagoon covered by *Halodule wrightii* and *Syringodium filiforme*, characterized by an important abundance of penaeid shrimp. The ravine (S3), coordinates 24° 29′ 15.5″ N and 97° 46′ 02.5″ W, a shallow area located in the Catan lagoon close to the largest fishing village in the zone. The molar (S4), coordinates 24° 26′ 29.43″ N and 97° 46′ 7.71″ W, an area also located near the continent where shrimp concentrate before returning to the ocean.

2.2 Physicochemical and Contaminant Analyses

Sampling was performed during April 2010 and temperature, pH, and salinity were measured in situ at the collection sites using a digital pH meter (Combo Waterproof HI 98130) and a portable refractometer (Vista Model A366ATC). At each collection site, a quadrant of 1 m^2 was used and five sediment cores were obtained to locate the organisms. A sample of sediments from each sampling site was sent to the Laboratory of the College of Agronomy of the UANL, where the concentrations of Cd, Pb, Cu, Zn, and Fe were determined by Flame Atomic Absorption Spectroscopy (FAAS) according to the AS-14 method described in the NOM-021-SEMARNAT-2000 (Rodríguez-Fuentes and Rodríguez-Absi 2011). A 0.5-g sample was digested with 10 ml of nitric acid during 10 min at 175 °C and centrifuged at 3000 rpm during 10 min. Metals in the solution were determined by FAAS, based on a 0.1 to 4 ppm calibration curve for Fe and Cu; from 0.1 to 1 ppm for Zn; and from 0.5 to 2.5 ppm for Pb and Cd.

Another sample of sediments was sent to the College of Chemical Sciences of the UANL where organochlorine pesticides were evaluated by gas chromatography according to the 8070A method of the US Environmental Protection Agency (USEPA 2007). Extracts of sediments were prepared with hexane-acetone (1:1). After a cleanup step, the extracts were analyzed by injecting a measured aliquot into a gas chromatograph.

To determine the total heavy hydrocarbon fraction, the hydrocarbons were extracted from sediments by the Soxhlet extraction 3540C method (USEPA 1996; Fernández-Linares et al. 2006). Briefly, 5 g of air-dried soil were placed inside an extraction thimble and mixed with sodium sulfate, in a 1:1 relationship. Dichloromethane was used as the solvent for the extraction. The extraction was carried at 45 °C during 8 h, with 6–8 cycles per hour. The total heavy hydrocarbon fraction that remained at the bottom of the flask was quantified gravimetrically.

2.3 *C. elevata* Characteristics

The collection of clams was authorized by the Regional Direction of the Gulf of Mexico and Coastal Plain Region of the National Commission of Natural Protected Areas (CONANP) that administrates the Laguna Madre/Rio Bravo Natural Protected Area (Authorization number FOO7.DRPCGM/1160/18). *C. elevata* specimens obtained from each sampling site were transported at 4 °C to the Ecophysiology Laboratory of the College of Biological Sciences of the UANL and stored at − 70 °C until processed. The specimens were identified based on the description of Roopnarine and Vermeij (2000), and data on length, height, and shell thickness were registered, as well as the total weight and wet weight of soft tissues. The condition index [IC = (soft tissue wet weight/total weight) × 100] was individually determined.

2.4 Biomarkers

Biomarkers were analyzed using 10 adult specimens per sampling site (Moore and Lopez 1969). The whole soft

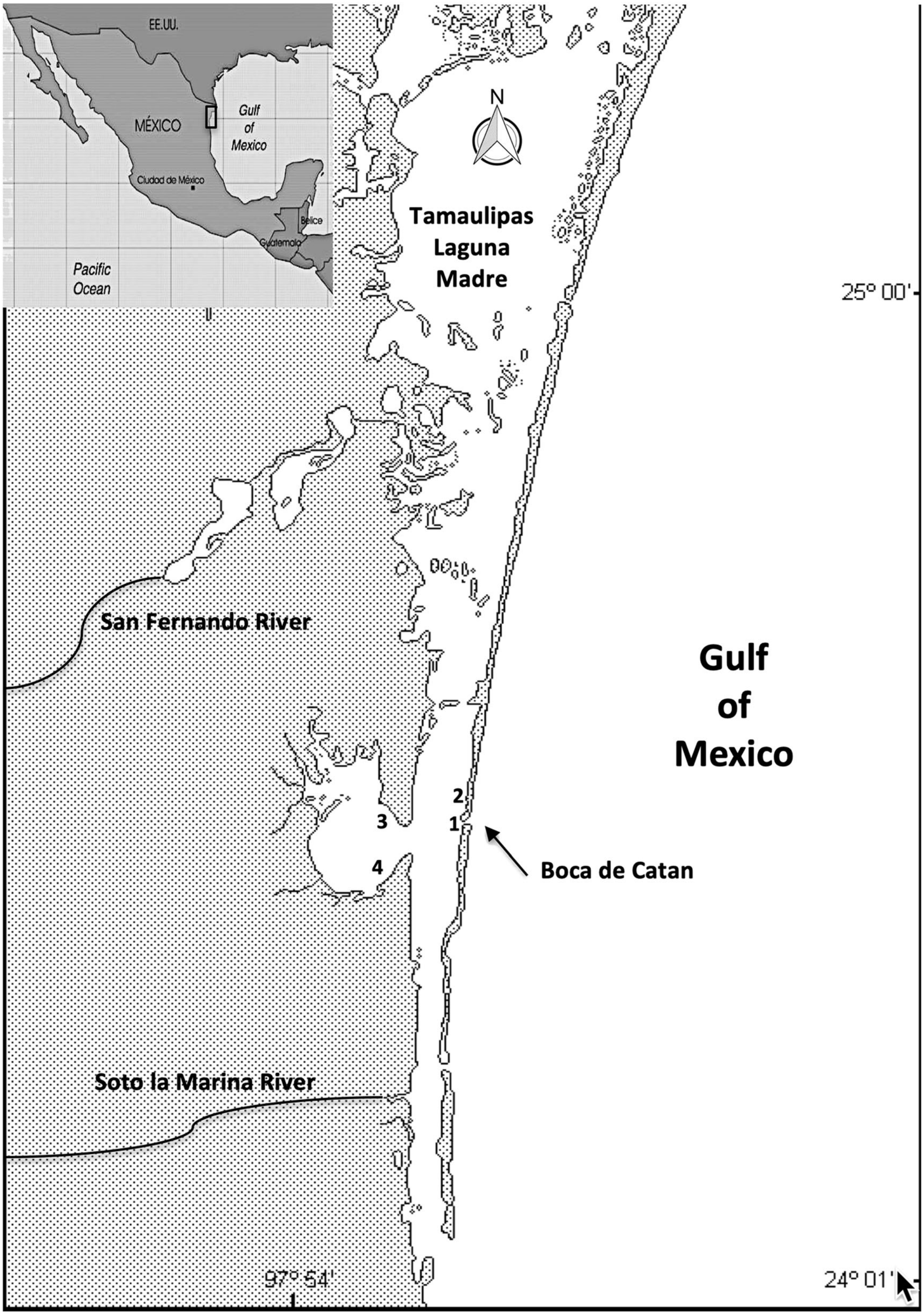

Fig. 1 Study area and location of *C. elevata* and sediment sampling sites in Laguna Madre Tamaulipas, Mexico. The numbers correspond to the sampling sites (S) indicated in the study

tissues of each individual were homogenized at 4 °C using a Wheaton-glass homogenizer (Glas-ColTM) at 333 rpm during 4 min in a Tris-HCl 50 mM (pH 7.1) buffer to a proportion of 1:10 (*W*/*V*). The homogenized material was centrifuged at 15,300 *g* at 4 °C for 30 min. The supernatant was separated and stored in 0.1 mL aliquots at − 70 °C to be used hereinafter as the enzymatic extract. Total soluble protein concentrations in the extracts were determined by the Bradford (1976) method using bovine albumin serum as a standard.

Esterases [acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CaE)], alkaline phosphatases(ALP), and glutathione S-transferase (GST) were determined by previously reported assays (Aguilera et al. 2015). In the case of esterases, the reaction mixture for AChE and BChE consisted of 280 μL of 5,5′-Dithiobis 2-nitrobenzoic acid in a PBS buffer 0.1 M (pH 7.8) and 10 μL of enzymatic extract. The reaction was initiated adding 10 μL of acetylthiocholine chloride (0.015 M) for the AChE activity or butyrylthiocholine chloride (0.015 M) for the BChE activity. To determine the activity of CaE 200 μL of buffer Tris-HCl 50 mM (pH 7.1), 10 μL of enzymatic extract and 100 μL of p-nitrophenyl acetate (2 mM) like substrate were added to initiate the reaction. Absorbance was immediately registered at 405 nm in intervals of 120 s up to 10 min.

ALP activity was measured using p-nitrophenyl phosphate as substrate. The reaction was performed using 200 μL of diethanolamine buffer (1.0 M) with 50 mM MgCl2 (pH 9.8), then 10 μL of the enzymatic extract and 10 μL of the substrate were added at a final concentration of 0.4 mM. Absorbance was immediately registered at 405 nm in the same conditions as the esterases.

GST activity was analyzed using L-glutathione as substrate. The reaction mixture consisted of 300 μL of Dulbecco' s buffer (pH 7.2) containing 1-chlorine-2,4-dinitrobenzene (CDNB) 100 mM and reduced L-glutathione (200 mM) with 10 μL of the enzymatic extract. Absorbance was immediately registered at 340 nm every 60 s for 10 min. For each sample, three analytical replicates were conducted in a microplate reader (ELx800TM, BioTek). The sample was replaced with buffer in the case of the control. The linearity of the reaction was verified with the Gen5 Sofware (BioTek). Enzymatic activity for esterases and ALP was expressed as the increase of absorbance per minute by milligram of protein in the extracts (ΔAbs min^{-1} mg $protein^{-1}$) and reported as mili-units (mU). GST activity was expressed as μmol mL^{-1} min^{-1} mg $protein^{-1}$ using for CDNB a molar extinction coefficient of 9.6 $Mmol^{-1}$ cm^{-1}.

2.5 Cellular Antioxidant Capacity

Cellular antioxidant capacity was determined by the previously reported assay of fluorescence-based Oxygen Radical Absorbance Capacity (ORAC-FL) (Barriga-Vallejo et al. 2017), which is an indirect method that monitors the antioxidant's ability to protect the fluorescent probe from free radical-mediated damage, and an azo-radical initiator, AAPH (2,2-azobis (2-amidinopropane) dihydrochloride). Protein was eliminated from the extracts by precipitation with perchloric acid (0.5 M) in a 1:1 (*v*:*v*) ratio. The reaction was carried out in a 75 mM phosphate buffer (pH 7.4). The reaction mixture consisted of 150 μL fluorescein (0.004 mM) and 25 μL of antioxidant (Trolox calibration solutions 0–100 μM), test samples or buffer in the case of blanks. The mixture was preincubated for 30 min at 37 °C in a microplate reader (Synergy 2 Biotek). AAPH solution (25 μL; 153 mM) was added rapidly to every well to start the reaction. Fluorescence (485-nm excitation and 528-nm emission) was recorded every minute for 60 min. Standard curve and samples calculations were performed with the Software Gen5 (Biotek) incorporated in the microplate reader. ORAC-FL values were expressed as Trolox equivalents in μmol^{-1} mg $prot^{-1}$.

2.6 Statistics

Data normality was evaluated using the Kolmogorov-Smirnoff test. Differences of morphological data among sampling sites were determined by the non-parametric test of Kruskal-Wallis and a Mann-Whitney test was performed to identify paired sampling sites that were statistically different ($p < 0.05$). Differences on biomarkers activities among sampling sites were analyzed by a one-way ANOVA and the Tukey multiple range test was used to identify differences among mean values at the 0.05 level. Statistical analyses were performed using the Statistica 9.0 software.

2.7 Integrated Biomarker Response

Integrated biomarker response (IBR) was calculated for each sampling site and for each biomarker following the method described by Beliaeff and Burgeot (2002).

Briefly, the data were standardized considering an inhibitory response for AchE, BchE, CaE, and ORAC and an activation response for the rest of the biomarkers. Data were represented in star plots for each sampling site following the sequence AchE, BchE, CaE, ALP, GST, and ORAC. The polygon area was calculated according to the formula modified by Devin et al. (2014) to obtain the IBR.

3 Results

3.1 Physicochemical and Contaminant Analyses

Physicochemical characteristics of the water, organic matter, conductivity, concentration of metals (mg kg^{-1}) and total heavy hydrocarbons (THH) of the sediments in the different sampling sites are shown in Table 1. Sediments of the sampling localities were classified from sand-silt to silt-sand, and all but sampling site S3, were strongly alkaline. Sediments of sampling localities with continental influence (S3 and S4) showed a lower concentration of organic matter (0.63 and 0.87%) and were highly saline (conductivity 32.1 and 30 dS/m), whereas the water in these stations showed a higher temperature and a higher salinity (40 °C and 38 ppt). In contrast, sediments of sampling localities with marine influence (S1 and S2) showed a higher concentration of organic matter and were highly saline (conductivity 47.6 and 41.7 dS/m) despite a lower water salinity. Most of the metals analyzed showed higher concentrations in sampling localities with marine influence (S2 and S1) compared to those of continental influence. The concentration of total heavy hydrocarbons was higher in S1 and S4 sampling stations (200 mg/kg) compared to S2 and S3 (100 mg/kg). Organochlorine pesticides showed levels below the limit of detection (< 2 mg/kg) in the sediments of all sampling sites.

3.2 Morphological Characteristics of *C. elevata*

Morphological characteristics of *C. elevata* were statistically different (*P* 0.05) among the sampling sites (Table 2). These differences could be attributed to a significantly higher shell length, shell height, shell width, and soft tissue weight of organisms from the sampling stations with continental influence (S3 and S4) compared to those with marine influence (S1 and S2). However, the condition index was significantly higher in specimens from the latter sampling sites.

3.3 Biomarkers

Results concerning the battery of biomarkers determined in *C. cancellata* populations are shown in Table 3.

Table 1 Physicochemical characteristics of water and concentrations of metals (mg kg^{-1}) and total heavy hydrocarbons (THH) of the sediments in the sampling locations

	Sampling station			
	S1	S2	S3	S4
Water				
Temperature (°C)	26	25	28	27
Salinity ($^{0}/_{00}$)	36	35	40	38
pH	10.5	10.7	6.03	8.29
Sediments				
Organic matter (%)	1.2507	1.2507	0.6253	0.8754
Conductivity (dS/m a 25 °C)	47.6	41.7	32.1	30.0
Cd (mg kg^{-1})	ND	ND	ND	ND
Pb (mg kg^{-1})	3.66	4.02	2.66	1.30
Cu (mg kg^{-1})	1.86	2.83	2.01	1.64
Zn (mg kg^{-1})	26.74	26.15	11.78	10.59
Fe (mg kg^{-1})	8369.08	8184.0	3425.25	3153.92
Total heavy hydrocarbons (mg kg^{-1})	200	100	100	200

Table 2 Means ± standard deviation of the morphological characteristics analyzed in different populations of *C. elevata* in the Tamaulipas Laguna Madre; the different letters indicate significant differences ($P < 0.05$) among localities

	Sampling station			
	S1	S2	S3	S4
Height (mm)	17.54 ± 10.91ab	13.61 ± 4.02 b	18.47 ± 7.04 ab	21.67 ± 3.21 a
Length (mm)	19.66 ± 12.36 ab	15.95 ± 4.93 b	22.35 ± 9.83 ab	24.34 ± 3.44 a
Width (mm)	11.12 ± 7.28 ab	8.46 ± 2.65 b	12.03 ± 5.53 ab	14.84 ± 2.87 a
Total weight (gr)	5.13 ± 4.94 ab	1.37 ± 0.88 b	3.54 ± 2.60 ab	5.20 ± 2.03 a
Wet weight (gr)	0.719 ± 0.687 ab	0.172 ± 0.121 b	0.523 ± 0.393 ab	0.521 ± 0.244 a
Condition index (%)	21.06 ± 2.33 a	20.09 ± 3.67 ab	17.65 ± 2.63 b	18.02 ± 2.59 b

The soluble protein of the extracts was statistically lower in organisms of the S3 and S4 sampling sites, while the highest values were observed in animals collected in sampling stations of marine influence. In the same sense, AChE, BChE, and GST enzymatic activities were higher in organisms from the sampling sites of continental influence (S3 and S4) compared to those of sampling sites with marine influence (S1 and S2). Clams from the S3 sampling site showed the significantly highest CaE and ALP activities compared to organisms from the rest of the sampling locations. Finally, the highest cellular antioxidant capacity (ORAC) was observed in organisms from the S1 sampling site compared to individuals from the rest of the sampling sites.

3.4 IBR

The greatest response to the different biomarkers (1.147) was observed in the S3 sampling site, followed by S2 and S4, with IBR values of 0.9106 and 0.7816, respectively, while the lowest response was observed in S1, with an IBR of only 0.6283 (Fig. 2a). ORAC was the biomarker that showed the greatest response in most populations S3, S4, and S2 (Figs. 2c–e), while a greater AChE response was observed at the S1 sampling site (Fig. 2b).

In the polygons obtained to represent the response to the biomarkers, ORAC showed the highest IBR values (IBR = 2.392) (Fig. 3a). On the other hand, in the ORAC polygon (Fig. 3g), the S3 sampling site stands out with the highest values displaying a rather similar shape to that of the site IBR polygon (Fig. 2a). GST, ALP, and CaE, with IBRs of 0.6049, 0.0285, and 1.168, respectively, also showed a higher response for the S3 sampling site (Figs. 3d–f), and the ALP response was exclusive for this site (Fig. 3e). The higher AChE and BChE IBR values (0.370 and 0.220), were observed in S1 and S2, respectively (Fig. 3b, c).

Table 3 Means ± standard deviation of the biomarkers analyzed in different populations of *C. elevata* in the Tamaulipas Laguna Madre; the different letters indicate significant differences ($P < 0.05$) between localities. AChE, BChE, CaE, ALP, GST, and ORAC

	Sampling station			
	S1	S2	S3	S4
Soluble protein (μg/ml)	1.917 ± 1.05 b	2.549 ± 1.57 c	1.205 ± 0.85 a	1.272 ± 0.46 a
AChE (mU)	9.664 ± 5.394 a	15.410 ± 9.94 a	26.208 ± 14.61 b	25.887 ± 13.106 b
BChE (mU)	151.03 ± 63.04 ab	138.98 ± 58.36 a	188.39 ± 60.84 b	180.26 ± 84.14 ab
CaE (mU)	11.715 ± 3.975 a	13.604 ± 8.916 a	24.172 ± 14.0 b	16.363 ± 4.138 a
ALP (mU)	17.416 ± 7.79 a	15.962 ± 5.545 a	23.794 ± 12.39 b	16.532 ± 4.356 a
GST (U)	70.5 ± 29.8 a	64.1 ± 37.8 a	105.5 ± 42.3 b	102.4 ± 26.8 b
ORAC (Trolox equivalents)	80.3 ± 13.0 b	57.8 ± 12.3 a	50.3 ± 18.4 a	51.7 ± 7.8 a

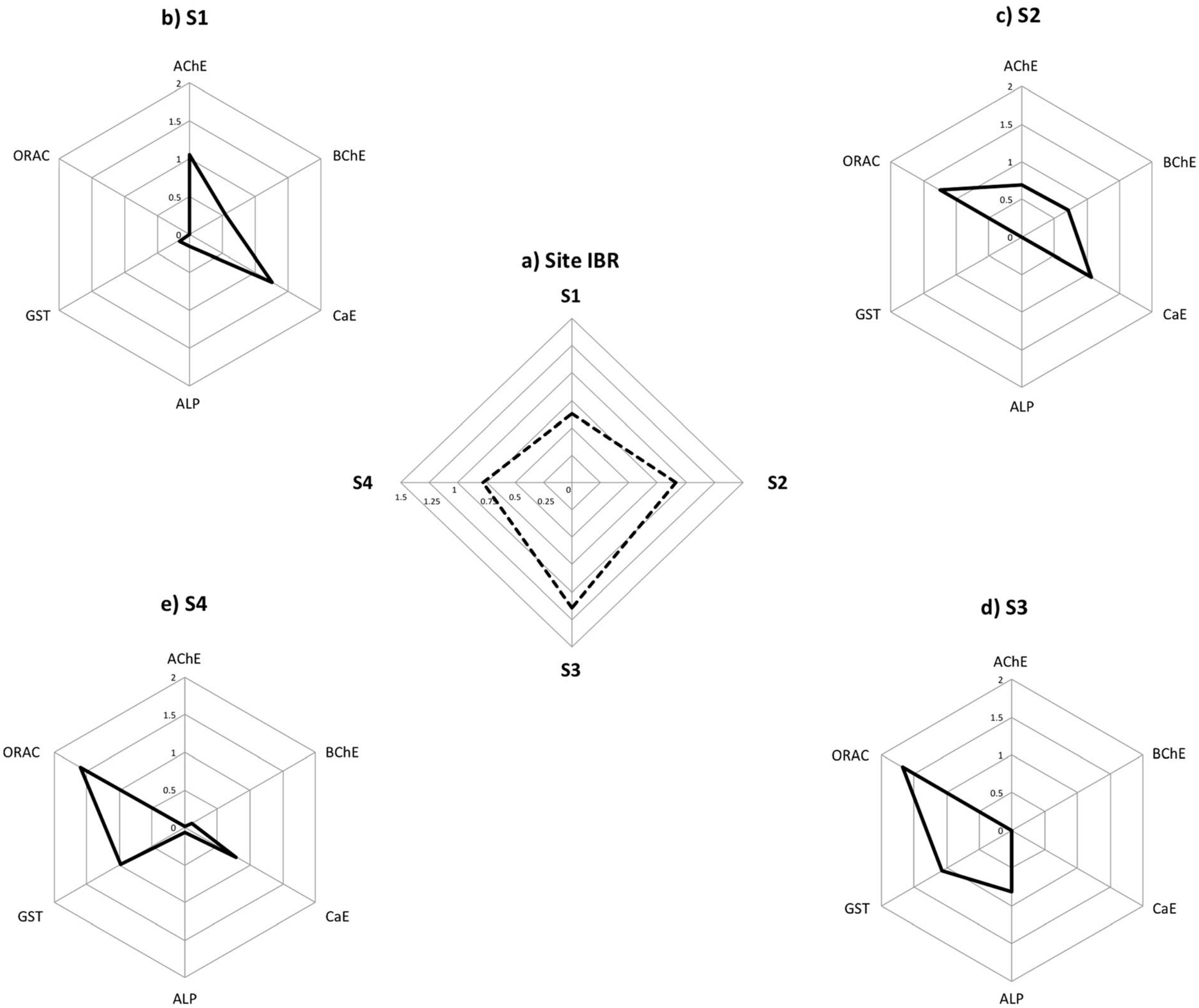

Fig. 2 Star plot of the Integrated Biomarker Response (IBR) for all *C. elevata* sampling sites (**a**) and star plots of IBRs for each sampling site (**b**- **e**) in the Tamaulipas Laguna Madre, Mexico. The size and form of the area polygons are indicative of the enzymatic response. The IBR values are presented in the text

4 Discussion

The main origin of sediments in the Laguna Madre are the basins of the Rio Grande (Bravo), the San Fernando River, and the Soto la Marina River; however, their distribution is determined by the inflow of marine water, the internal wave energy, and wind action (Yañez and Schlaepfer 1968). To have a complete scenario of these influences, the sampling sites were located between the San Fernando and Soto la Marina rivers (Fig. 1). The S3 and S4 sampling sites are located in the continent, whereas the S1 and S2 sites are in the proximity of Boca de Catan, with a marked marine influence. The complex interaction between the environmental factors that determine the dispersion and deposition of pollutants in this ecosystem, as well as their nature, makes it difficult to establish a clear relationship with the concentrations of the pollutants observed in the different sampling sites. In this way, the highest concentration of total oil hydrocarbons observed at the S1 and S4 sampling sites may be related to the important fishing activity because shrimp concentrate in these locations. The highest values of heavy metals observed at S2 and S1 may be the result of metal retention capacity of seagrass beds, which could also interfere in the dynamics of the pollutants (Riosmena-Rodríguez et al. 2010; de Araujo et al. 2014). Likewise, it is difficult to compare these results with previous reports of pollutants, considering the scarcity of information on this aspect for the Laguna Madre,

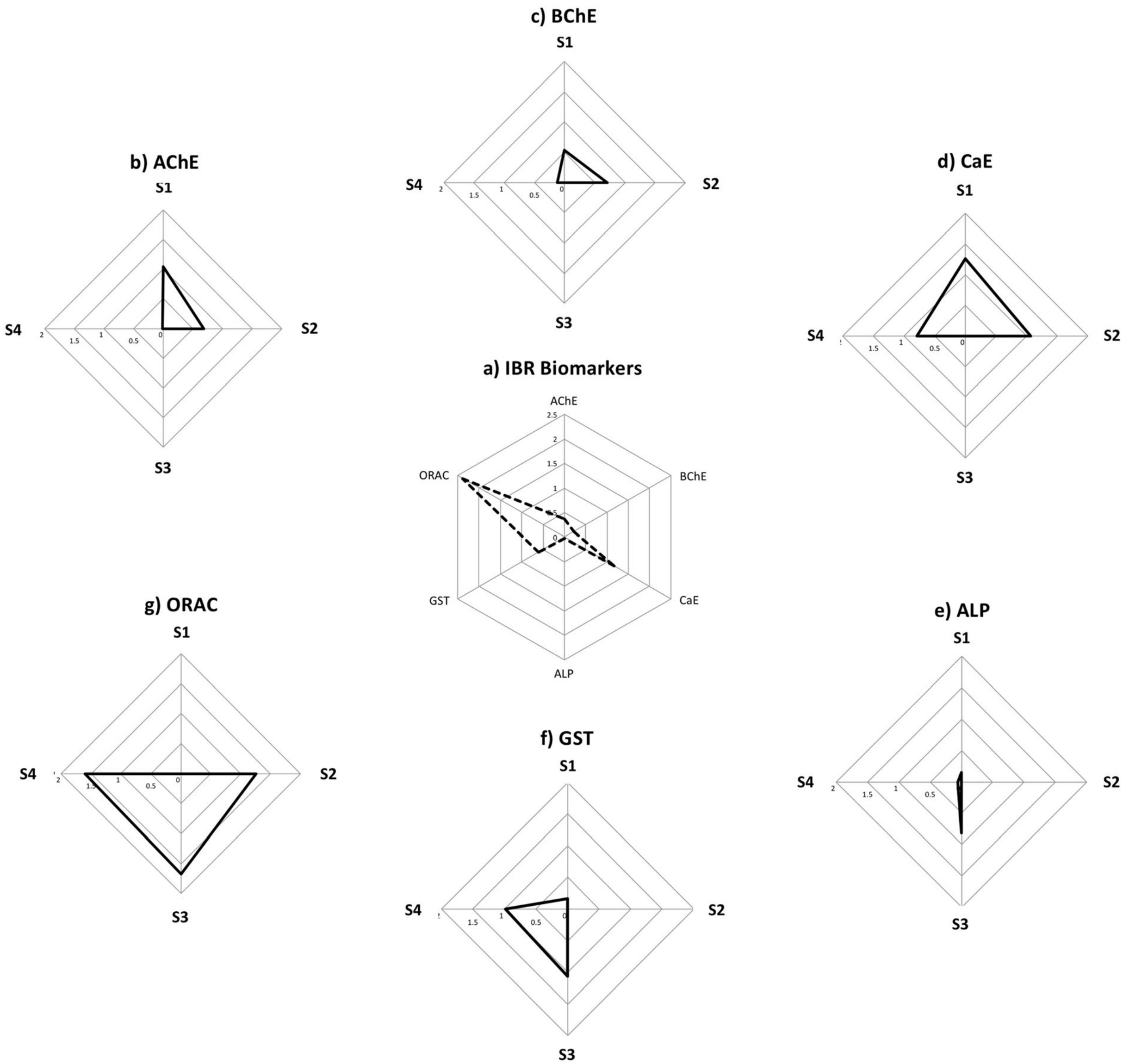

Fig. 3 Star plot of the Integrated Biomarker Response (IBR) for all the biomarkers evaluated in *C. elevata* (**a**) and star plots of IBRs for each biomarker in sampling sites S1 to S4 (**b**-**g**) of Laguna Madre Tamaulipas, Mexico. The size and form of the area polygons are indicative of the enzymatic response. The IBR values are presented in the text

particularly for the Tamaulipas area (Botello et al. 2015) where the present study was carried out. The current knowledge on the concentration of oil hydrocarbons and metals in the NW Gulf is restricted to the continental shelf off Tamaulipas, México (Ponce-Vélez et al. 2006) associated with the expanding activity of the local oil industry. Only Pulich (1980) reports metal concentrations in sediments in the Tamaulipas Laguna Madre, which may be a consequence of the proximity of the Burgos Basin, which is considered the third most important province in the world for its production and oil resources (Eguiluz de Antuñano 2011).

The higher salinity in sampling sites 3 and 4 are related to the evaporation rate caused by higher temperatures in these sites, while the lower pH in S3 may be attributed to nutrient runoff from fertilizer, human and animal waste, and other sources (Sunda and Cai 2012).

Regarding the morphological characteristics of *C. elevata*, it could be observed that the larger individuals were those of populations found in localities of continental influence, compared to localities of marine influence. However, the highest values of the condition index (CI) correspond to the clam populations inhabiting localities of marine influence, which could reflect the effect of salinity on the activity-related cost of osmoregulation in mussels of locations of continental influence. The CI is an ecophysiological measure of the health status of the animals that summarizes their physiological activity (growth, reproduction, secretion, etc.) under given environmental conditions and during the period of sexual dormancy; this quotient is considered a good indicator of mollusk growth (Tsangaris et al. 2010). Even though the CI has been related to gonadal development in *C. elevata* (Moore and Lopez 1969), this would not be the case in the present study as the sampling was carried out in April, when the organisms were out of their reproductive season, and thus the C.I. most likely would be reflecting a better physiological condition. Additionally, it has been stated that the CI decreases or remains unchanged in pollutant-exposed bivalves (Luna-Acosta et al. 2017).

Results of AChE, BChE, and GST activities show a clear disjunction of localities of greater marine influence (S1 and S2) with those located in the continent (S3 and S4). The lower AChE, BChE, and CaE activity values registered in the S1 and S2 localities would be indicative of general physiological stress (Lehtonen et al. 2006), and particularly of neurotoxic effects. Indeed, the inhibition of the activity of these enzymes has often been associated with different types of contaminants, mainly organophosphorus pesticides and carbamates, as well as with the presence of heavy metals, surfactants, and PAHs (Solé and Sanchez-Hernandez 2018). The lower activities of these enzymes concur with the higher concentration of metals found in S2 and S1. In fact, the results of IBR by biomarkers show the higher relative weight of AChE, as well as the BChE activity values in these two localities, whereas CaE also showed a relevant relative weight in S4 (Fig. 3d). These results are consistent because both biomarkers generally show similar responses to contaminants, although with different sensitivity, depending mainly on the species and/or tissue analyzed (Solé and Sanchez-Hernandez 2018). However, the AChE, BChE, and CaE IBR values are among the lowest compared to other biomarkers, which would indicate that neurotoxic effects by contaminants do not represent the main cause of stress in the *C. elevata* populations. This is also supported by the results of IBR by location, where S1 shows the lowest values of the battery of biomarkers, meaning that these are the populations showing the lowest stress attributable to contaminants.

In contrast, the higher GST activity values and higher relative weight (Figs. 2e, d and 3f) for the sites of continental influence (S3 and S4) would be indicative of the activation of detoxification processes provoked by organic contaminants as part of the phase II biotransformation pathway (Sheehan and Power 1999). The origin and type of these pollutants could not be determined in this work, but they might be complex mixtures of pollutants carried by the continental drainages towards the lagoon, particularly at the S3 sampling locality as a consequence of its proximity to the largest fishing community in the study area. Numerous studies in bivalve mollusks report the variation of GST activity by exposure to various xenobiotics (Cotou et al. 2013). The above statement can also be supported by the activity values of ALP, whose activity also increases in response to processes of detoxification of various pollutants (Mazorra et al. 2002). The values of both biomarkers were higher in sites of continental influence, but significantly higher for the S3 locality. This is consistent with the IBR results that showed that the relative weight of GST was similar for S3 and S4, whereas the relative weight of ALP was only important at the S3 sampling location (Fig. 2d, e). The IBR value for GST was intermediate with respect to other biomarkers, and the ALP was the lowest, which would indicate that *C. elevata* in these localities were partially responding to xenobiotics through detoxification processes.

Finally, a trend could be perceived concerning the higher values of ORAC in the localities of marine influence compared to those of continental influence, although the values were significantly higher only for the S1 sampling location. This biomarker quantifies the non-enzymatic antioxidant molecules present in organisms that can inhibit the action of free radicals (Barriga-Vallejo et al. 2017), therefore, lower values would represent a greater exposure to free radicals that may arise from the exposure to xenobiotics. This suggests that clams from the S3 sampling location would be more exposed to oxidative stress, followed by those from S4 and S2 localities, whereas individuals from the populations of the S1 locality would be the least exposed. These results which are clearly reflected in the ORAC IBR polygon (Fig. 3b) agree with the CI and the worst physicochemical conditions in the locations of continental influence. This statement is supported by other reports that emphasize that the toxicity of different pollutants is strongly dependent on the chemical characteristics of water and sediments (Ardestani et al. 2014). And also adds value to the use of sublethal responses to complement biological indices based on benthic macroinvertebrate taxa, particularly in moderately polluted sites where stressors are already affecting communities but not too strongly to be detected by biotic indices (Damásio et al. 2011). Moreover, the CI is dependent on food availability and if a poor index is obtained in areas characterized by good conditions, as reflected by the larger size of animals, as in S3 and S4, some other stressors must be present (Damiens et al. 2007). The lower CI in these sampling stations concur with reports that state that the CI may also indicate pollution stress because mussels that are exposed to a complex mixture of pollutants are forced to spend a great part of their energy budget in detoxification processes and maintenance of homeostasis to the detriment of CI (Pampanin et al. 2005; Tsangaris et al. 2011).

Additionally, ORAC showed the highest IBR value among the biomarkers analyzed (Fig. 3g), consequently when the IBR of the localities are plotted the influence of ORAC can be perceived in the shape of the IBR location polygon, indicating the most impacted areas (Fig. 2a). The antioxidant activity of non-enzymatic molecules evaluated by the ORAC technique is still underused in ecotoxicology studies. Nevertheless, because of its sensitivity and utility in the areas of food quality and physiology, it has gained relevance in ecotoxicology (Wiklund et al. 2014; Barriga-Vallejo et al. 2017). From these analyses, it could be considered that oxidative stress represents the greatest risk for the studied populations of *C. elevata*. Furthermore, heavy metals could be discarded as the main cause of oxidative stress in the S3 and S4 sampling localities, considering that a higher content of heavy metals was found in the localities of marine influence (S1 and S2).

As expected, the general results point to the localities with continental influence as those subjected to greater stress as a result of anthropogenic effects, as continental runoffs represent the main route of entry of agricultural, industrial, and urban pollutants to this area of the Laguna Madre. Oil spills from barges, discharge from the Mexican side of the Rio Grande, and hydrocarbon extraction are some of the threats posed by the high volume of commercial activities taking place on the Laguna Madre (Mendoza et al. 2011). The localities of greater marine influence normally have a lower contribution of pollutants and these could be associated with events such as oil spills during transport or extraction in the sea. Although oil spills from exploration and production occur only occasionally, no major oil spills have occurred in the Laguna Madre in Texas or Tamaulipas until now. The results of the presence of pollutants in the sediments analyzed in this work do not allow establishing a straightforward relationship with the battery of biomarkers employed in the present study. Often, a straightforward relationship between biomarker response to contaminants and further biological consequences at the individual or higher biological levels is difficult to establish, mainly due to the individual's ability to adapt and to the influence of natural variability (Serafim et al. 2012). On the other hand, it could be pointed out that the quantification of pollutants only took place in sediments and not in water, mainly due to technical limitations for an adequate qualitative and/or quantitative evaluation of pollutants, a situation that is relatively common in developing countries (Ruiz-Picos et al. 2017). This drawback is also clear in previous research on contaminants in sediments, water, or organisms for this region of the Laguna Madre, which also prevented us from establishing sampling sites where known levels of contaminants could have been considered. This scarcity of information led us to focus on the hypothesis of a higher impact of pollutants in sites with greater continental influence. In this sense, Botello et al. (2015) found the highest concentrations of PAHs and metals in water and sediments of NW Gulf of Mexico in sites just off the Laguna Madre where the sedimentary conditions on the shelf were profoundly influenced by the river runoff of three main rivers, namely, Bravo, Soto La Marina, and Panuco, and by the exporting capacity of two major coastal lagoons: Madre and Tamiahua.

The use of ecological or physiological indices to evaluate the quality of the environment or the impact of pollutants is becoming a worthwhile alternative, especially in developing countries, because it requires neither expensive nor very sophisticated equipment, thus reducing the operational, maintenance, and training costs invested in physicochemical laboratory analyses (Ruiz-Picos et al. 2017). However, the use of biomarkers in field studies can be problematic as there are often complex mixtures of pollutants in the environment, as well as species-specific variations in the biomarkers response and other variations attributable to seasonal fluctuations (Dellali et al. 2004). Therefore, there is a consensus that it is more useful to employ a wide battery of biomarkers considering that not all of them respond efficiently to the nature and magnitude of the pollutants, as well as the fact that certain biomarkers respond differently depending on the species (Cotou et al. 2013). Despite this, much progress has been made in the identification of biochemical biomarkers in marine bivalve mollusks that can be used for assessing environmental water quality and health status of the organisms (Cotou et al. 2013). In this regard, the biomarkers selected for this study correspond to the objectives of an ecotoxicological approach as early warning signals of the presence of environmental stressors. The choice of these biomarkers intended to include early responses to major types of contaminants such as metals, PAHs, PCBs, pesticides, and general stress. In the present study, the position of biochemical biomarkers on the star plot was based on their relevance to identify different biological endpoints, i.e., the inhibition of AchE, BChE, and CaE activities is associated with pollutant-induced neurotoxic effects, the activities ALP and GST are related to xenobiotics detoxification processes and ORAC is commonly used as an oxidative stress indicator.

Biomarker responses are indicative of xenobiotics inducing physiological stress, which may lead to changes of the microbenthic structures and deterioration on the ecological status over time. The stress indexes observed can be regarded as a toxicity indication, which may increase the susceptibility of benthic organisms to pathologies and mortality (Seabra Pereira et al. 2014). On the other hand, a new problem has emerged as a result of using many biomarkers: the difficulty in analyzing and integrating the response of different biomarkers, both by specialists and by environmental managers, decision makers, and others non-specialists. In response to this, different indexes have been proposed with the purpose of scoring and summarizing in a single value or graph the set of responses of different biomarkers. Among these indexes, the "Integrated Biomarker Responses" (IBR) described by Beliaeff and Burgeot (2002) was considered for this study as it provides an intuitive interpretation of the health status of the organisms (Kim et al. 2010) and is at the present one of the most used in field and laboratory studies. The number of biomarkers used in this study was appropriate to reveal differences in the physiological condition of mussels in the study area because, due to its mathematical basis, the IBR becomes more robust when the number of biomarkers increases. Moreover, when the set of biomarkers is relatively large (6–8), as it is the case in this study, the weight of one factor is markedly reduced, revealing well the relative weight of each biomarker, compared with cases when a small number (3–4) of biomarkers are used (Broeg and Lehtonen 2006). Additionally, in agreement with the results of the present study, a number of studies using different biomarker combinations have shown visual accordance of IBR with contaminant levels suggesting that the integrated biomarker responses may serve as a useful tool for quantitative monitoring of the toxicological effects of toxic compounds towards mollusks, even in the presence of mixtures of chemicals present at concentrations below or nearby their detection limits (Beliaeff and Burgeot 2002; Kim et al. 2010; Tsangaris et al. 2011; Luna-Acosta et al. 2017).

The clear differences in the values of biomarkers between stations with dissimilar characteristics showed that *C. elevata* is an adequate species for the biomonitoring of contaminants. Furthermore, the wide range extension of the species in the Gulf of Mexico makes it suitable for monitoring other locations. In this sense, Nigro et al. (2006) reported that alterations resulting from cumulative events have a better discriminating capacity when applied to chronically exposed native specimens, in contrast with transplanted exotic mussels used for the same purpose (Damiens et al. 2007).

In conclusion, the results of this study suggest that the use of a wide-ranging battery of biochemical biomarkers, analyzed by the IBR, on *C. elevata* as a sentinel organism has the potential to monitor the environmental quality in the Laguna Madre. However, it is deemed necessary to increase the amount of data to analyze and incorporate seasonal or climatic variations. It is also indispensable to match these studies with further physicochemical analysis, in laboratory and field, to set up a clearer relationship between the type and amount of pollutants with the activities of different biomarkers.

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