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RESEARCH REPORT

# Tissue biomarkers as vulnerability indicators in the clam *Polymesoda caroliniana*

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## Abstract

Vulnerability depends on the exposure and sensitivity levels of a system to a specific pressure, together with the capacity to cope, recover, or adapt to this pressure. We propose the use of well-known tissular techniques to measure the components of vulnerability. Immunohistochemistry and histopathology indicate the health status of living organisms and the environment. Therefore, these techniques should provide the necessary information to determine the vulnerability of an organism. Immunohistochemical analysis uses biomarkers to determine the presence of toxic compounds, reflecting the exposure level of an organism. Histopathological analysis reveals the environmental impact of a given toxin, reflecting the sensitivity level of the organism to said toxin. Here, we propose a strategy to use these techniques to assess the vulnerability of clams from Tecolutla, Veracruz. We developed categories for each vulnerability component using semi-quantitative scales. Briefly, we calculated the exposure level based on the average number of positive immunohistochemical biomarkers among several organs of clams. Then, we compared the prevalence of histological alterations with the exposure level to determine the sensitivity level. Finally, to estimate the recovery capacity, we placed the control group in a clean environment for 40 days. These led us to observe the capacity of the clams to reverse the effects of environmental stress. Clams showed a moderate level of exposure, a low sensitivity level, and an effective recovery capacity. In conclusion, these results indicate that clams have a low level of vulnerability. This proposal has the potential to guide future works assessing the vulnerability of organisms and later include them in the estimation of vulnerability from aquatic bodies.

**Key Words**: *Polymesoda caroliniana*; histopathological index; vulnerability; immunohistochemistry; environmental stress

## Introduction

Coastal areas are considered one of the most vulnerable environments affected by pollutants. Some studies have already proposed some vulnerability parameters for coastal areas (Ramírez and Torres, 2011). However, none of these assessments included animal physiological traits that describe the health status of living organisms. Environmental stress can cause measurable effects over the physiological state of different animals, compromising their health (Bayne *et al.*, 1976; Baum *et al.*, 1984; Häder *et al.*, 2020). Diverse methodological approaches measure these effects at different biological levels. Histopathological and

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immunohistochemical analyses evaluate the effects of environmental stress at the tissue level (Costa *et al.*, 2013; Cuevas *et al.*, 2015). On one hand, histopathological analysis determines the physiological consequences of pollutants over organisms, through a characterization of tissue injuries (Usheva *et al.*, 2006; Boscolo Papo *et al.*, 2014b). These toxic responses are semi-quantified through the histopathological index (Ih), detailing the health status of an organism (Costa *et al.*, 2013; Cuevas *et al.*, 2015). On the other hand, immunohistochemical analysis determines cytological responses related to the presence of some pollutants. Many biomarkers are used to this end, indicating several types of toxic compounds that affect living organisms (Moraga *et al.*, 2005; Wang *et al.*, 2010; Zhang *et al.*, 2012; Boscolo Papo *et al.*, 2014a). Consequently, the integration of histopathological and immunohistochemical approaches constitutes a valuable strategy to investigate the vulnerability of an organism to a specific environment (Boscolo Papo *et al.*, 2014a).

Tecolutla is located in Veracruz state, along the eastern coast of Mexico (Fig 1). The town of Tecolutla surrounds a river that has associated estuaries, canals, and mangroves, giving this locale huge biodiversity. The most important and growing economic activity in the zone is tourism, hence the river receives many discharges of wastewater, agrochemicals, and hydrocarbons, which impact on the natural environment (López-Portillo *et al.*, 2009; Arriaga-Gaona, 2009; Temino-Boes *et al.*, 2020). Water samples taken from the Tecolutla river exceed the limits of total and fecal coliform bacteria allowed according to Mexican standards (Arriaga-Gaona, 2009). Moreover, Veracruz state shows a high vulnerability, according to impact studies conducted on fishery production and damage to the mangrove (Ramírez and Torres, 2011). Nevertheless, none of these studies included physiological indicators of the health of living organisms. Clams are bioindicators of the health status of an environment (Markert *et al.*, 2003; Park *et al.*, 2007; Wu *et al.*, 2013; Boscolo Papo *et al.*, 2014a; Zhang *et al.*, 2014; Carneiro *et al.*, 2015; Cuevas *et al.*, 2015; Santovito *et al.*, 2015; Vodopivez *et al.*, 2015; Delgado-Alvarez *et al.*, 2019). Among the diversity of Tecolutla, we found the clam *Polymesoda caroliniana*, commonly named black clam. Therefore, these organisms are potential sources of vulnerability data.

Vulnerability depends on the exposure and sensitivity levels of a system to a specific pressure, together with the capacity to cope, recover, or adapt to this pressure (Villa and McLeod, 2002; Stein *et al.*, 2014; Carantoña and Hernández, 2017; Ocaña and Pech, 2018). These components are measured through indicators at different biological levels. Here, we created an approach to measuring the three vulnerability components by assessing the health status of the clams. The proposed approach includes the use of different tissular techniques to

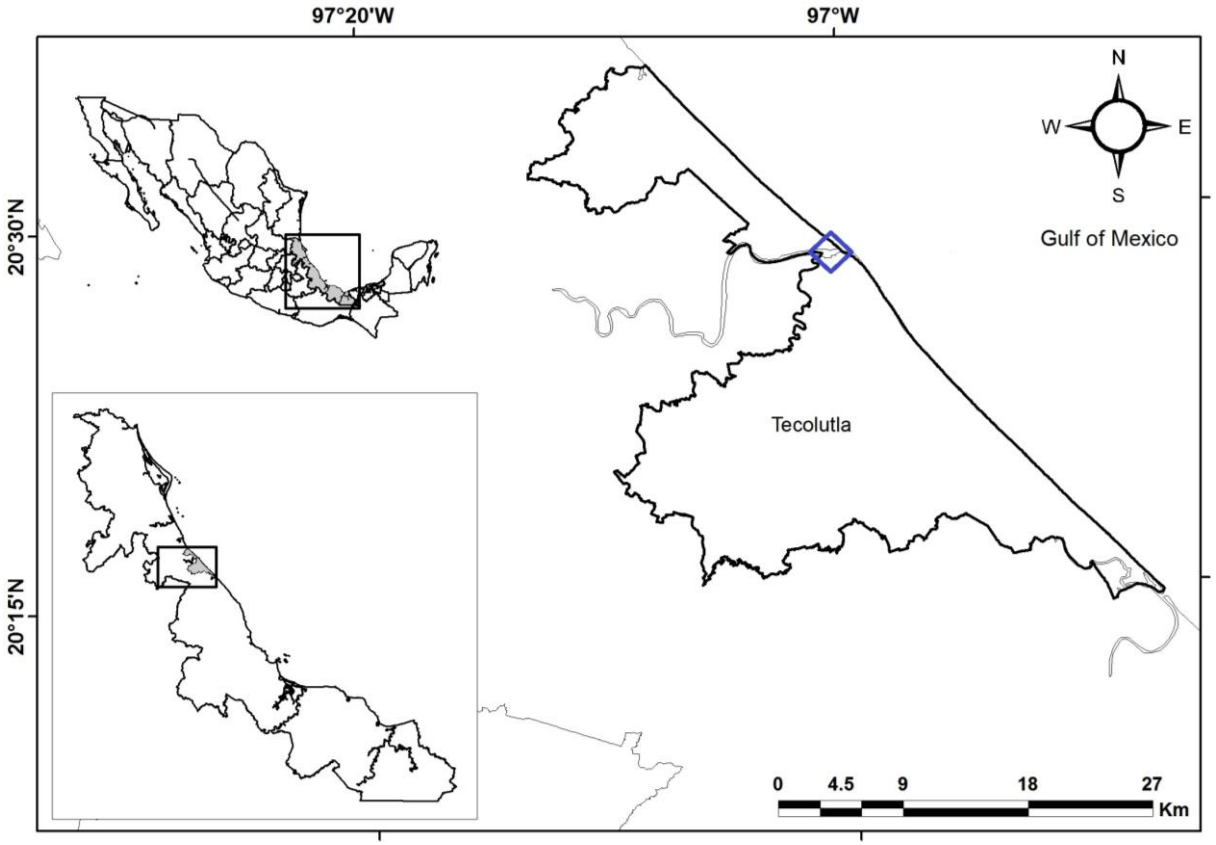


**Fig. 1** Map indicating the location of Tecolutla in Veracruz State, Mexico

this end. To measure the exposure level, we used immunohistochemical analysis. Immunohistochemical analysis reflects the level of exposure, indicating cytological responses to the presence of different pollutants. Then, to measure the sensitivity level, we correlated the prevalence of histopathological injuries with the level of exposure. Finally, we estimated the recovery capacity through a group of clams that depurated pollutants for 40 days in a clean environment. These organisms were shown to be sensitive enough to reflect that Tecolutla is polluted but also resilient enough to resist and survive environmental stress. Moreover, they showed an effective recovery capacity when introduced to a clean environment. Here we proposed the integration of histopathological and immunohistochemical analyses through semi-quantitative scales to assess the vulnerability of clams from Tecolutla. We created categories for each component, proposing approaches to their measurement. In conclusion, we determined that the clam *P. caroliniana* has a low vulnerability, based on the fact that clams could recover fully in a clean environment. This first proposal is a guide for future investigations to determine the vulnerability of specific organisms. The vulnerability of several organisms based on physiological traits should be included in the determination of the health status and vulnerability of an environment.

## Material and methods

*Sampling area and sample collection*

The Tecolutla River is located in the state of Veracruz between parallels 96°59′849″ W and 20°27′628″N, on the eastern coast, facing the Gulf of Mexico (Fig 1). Prior to collection, we obtained physicochemical water parameters (temperature, salinity, dissolved oxygen, and pH) with a Hach® model DR / 2000 direct reading spectrophotometer. The physicochemical parameters of Tecolutla, Veracruz characterize a typical estuarine environment: 22 °C, 2 ‰ salinity, 6.6 mg/l dissolved oxygen, and pH 7.7. These were later used to maintain the same conditions in the control group.

Local fishermen collected 100 adult *Polymesoda caroliniana* clams (4 to 5.5 cm in length). The clams were divided into a control and an experimental group, each containing 50 individuals. Transportation to the laboratory was in thermal boxes at 4 °C. Once they arrived, all the clams were cleaned with 70% alcohol. Then, all the clams were subjected to a macroscopic evaluation, and the following parameters were recorded: shell perforations and the presence of vermes or ectoparasites. Macroscopic analysis of *Polymesoda caroliniana* clams showed healthy organisms without shell distortion or perforations, but some of them had parasitic worms.

Immediately, the visceral mass of the experimental group was dissected and processed for histopathological and immunohistochemical analysis. The clams in the control group were maintained in seawater under the original physicochemical parameters (22 °C, 2‰ salinity, 6.6 mg/L dissolved oxygen, and pH 7.7) for 40 days. During the 40 days, the seawater was continuously aerated with partial water replacement every 72 h. The clams were fed with a suspension of 909,935 cells/ml microalgae culture of *Chlorella sp* every day. After 40 days of maintenance, these clams were subjected to histopathological and immunohistochemical analysis.

**Table 1** Percentage of dissemination degree assigned to each *a* value

| Dissemination degree | *a value* |
|---|---|
| 0 – 9% | 0 |
| 10 – 40% | 2 |
| 50 – 70% | 4 |
| 70 – 100% | 6 |

*Tissue preparation*

The visceral mass of all clams was fixed in 10% neutral buffered formalin for 48 h, dehydrated through a graded series of ethanol in a tissue processor (Leica, model TP1020), and embedded in paraffin using a paraffin embedding station (Leica, model EG1140-H). Serial 5-µ-thick sections were cut in a microtome (Microm, model HM3156).

*Histopathological analysis*

Histopathological slides were prepared by a standard technique, stained with hematoxylin-eosin (Cuevas *et al.*, 2015), and examined under light microscopy (Zeiss, PrimoStar) to observe the general morphology, presence of parasites, and histological alterations. For each clam, three slides were analyzed. For each slide, we observed six different optical fields, each one at three magnifications, 10X, 40X, and 100X. Three different people analyzed the slides separately following the same criteria. Each person observed and quantified the alterations to report the number of clams presenting each alteration in a prevalence matrix.

The histopathological analysis included: *i.* observation and description of tissue alterations, *ii.* construction of an alteration prevalence matrix, and *iii.* pathological importance factor (*w*) assignment and the degree of dissemination value (*a*). These values created a prevalence matrix, used to calculate the histopathological index.

*Histopathological index*

The histopathological index ($I_h$) is part of a protocol for the evaluation of aquatic pollution, and it was calculated according to previous studies (Bernet *et al.*, 1999; Costa *et al.*, 2013; Cuevas *et al.*, 2015). First, each type of alteration was categorized with respect to reaction patterns. A reaction pattern is the set of alterations in the functional unit of the targeted organ. The reaction patterns were tubular alterations and intertubular alterations. Then, we calculated the pathological importance factor (*w*) and the degree of dissemination (*a*). The pathological importance factor (*w*) is defined as the degree of damage or

compromise of an organ after an alteration on a scale of 0 to 3 (0 = absence, 1 = negligible, 2 = moderate, and 3 = severe pathological importance), according to the original work (Cuevas *et al.*, 2015; Costa *et al.*, 2013). Parasites were weighted as $w$ = 3, hemocytic infiltrations and atrophies as $w$ = 2, and brown cells and lipofuscin aggregates were assigned $w$ = 1, according to the original proposal for clams (Costa *et al.*, 2013; Cuevas *et al.*, 2015). The degree of dissemination ($a$) was the level of spread of a particular alteration in a functional unit or organ, in the range of zero to six. Therefore, to assign $a$ value, we used a prevalence matrix (Table 1).

Each $a$ value correspond to (0) unchanged, (2) mild occurrence, (4) moderate occurrence, and (6) severe occurrence, according to previous reports (Bernet *et al.*, 1999; Cuevas *et al.*, 2015). Later, we calculated the $I_h$ using the following equation (Costa *et al.*, 2013)

$$I_h = \frac{\sum_1^j w_j * a_{jh}}{\sum_1^j M_j}$$

Where $w_j$ is the pathological importance factor of each alteration, $a_{jh}$ is the degree of dissemination of an individual alteration, and $M_j$ is the maximum attributable value for the alteration, estimated from the maximum value of $w$ times the maximum value of $a$. This denominator normalizes $I_h$ to a value between 0 and 1, thus permitting comparisons between distinct organs.

*Immunohistochemistry*

For immunohistochemical staining, tissue slides were deparaffinized in xylene before hydration via a graded series of ethanol. To retrieve the heat-induced antigen, we used a recuperator (Diva Decloaker 20x DV2005, Biocare Medical) diluted 1:20 with distilled water in a digital electric pressure cooker (Decloaking Chamber, model DC2002, Biocare Medical) at 25 PSI and 125 °C for 5 min. Slides were washed with TBS (Auto Wash Buffer, 40X, Biocare Medical) and then cooled down progressively at room temperature for 20 min. Endogenous peroxidases were neutralized by incubating the slides with the blocker (endogenous peroxidase blocker PX968G, Biocare Medical) for 5 min at room temperature in an incubator chamber (model RMIQ105, Biocare Medical), to avoid natural temperature fluctuations. Excess blocking solution was removed by washing in TBS. Thereafter, all incubations were performed at room temperature. Serial sections were incubated for 45 min with 100 µL of the primary antibodies inside the incubator chamber. For metallothioneins, we used mouse monoclonal metallothionein antibody [UC1MT] (Genentex Catalog Number: GTX12228), dilution 1:30. For cytochrome P450, we used mouse monoclonal antibody CYP1A2 [15E2] (Genentex Catalog Number: GTX84638), dilution 1:50. Finally, for HSP70, we used a mouse monoclonal Hsp70 antibody [3A3] (Genentex® Catalog Number: GTX25439), dilution 1:50. TBS solution without primary antibody served as the control. After washing in TBS, sections were incubated with a polymer (EnVision+ System-HRP, Labelled Polymer (Mouse) K4000, Agilent Dako) that contained secondary antibody and streptavidin for 45 min. Then, we stained the slides with diaminobenzidine (DAB), using 1 ml of substrate buffer and 50 µL of chromogen (Dako Liquid DAB+ Substrate Chromogen System K3465, Agilent Dako) for 10 min. The slides were counterstained with hematoxylin (Tacha's Automated Hematoxylin, Biocare Medical) for 10 min and later dehydrated. Samples were mounted with a 50% synthetic xylene resin solution. Staining was performed in triplicate for each tissue of three clams. All slides were observed under a light microscope (Zeiss®, PrimoStar 176045) coupled to a digital camera (CANON, Powershot G10). Immunolabeling was considered positive when the staining intensity was greater than the background observed in the negative control. The specificity of the immunostaining was verified by incubating the sections with PBS instead of the specific primary antibody. This validation was performed for all antibodies in each the tissues. All preparations were kept in a buffer solution to avoid drying.

**Table 2** Relations between the prevalence of histopathological alterations and level of exposure. The sensitivity value was calculated by subtracting the prevalence minus exposure level. These relations resulted in a categorization of sensitivity (Very low = -2, Low = -1, Normal = 0, High = 1, Very high = 2)

| Exposure level | Value | Prevalence of histopathological alterations | Value | Sensitivity value | Sensitivity category |
|---|---|---|---|---|---|
| Minimal | 1 | Low prevalence | 1 | 0 | Normal |
| Moderate | 2 | | 1 | 1 | High |
| High | 3 | | 1 | 2 | Very high |
| Minimal | 1 | Moderate prevalence | 2 | -1 | Low |
| Moderate | 2 | | 2 | 0 | Normal |
| High | 3 | | 2 | 1 | High |
| Minimal | 1 | High prevalence | 3 | -2 | Very low |
| Moderate | 2 | | 3 | -1 | Very low |
| High | 3 | | 3 | 0 | Low |

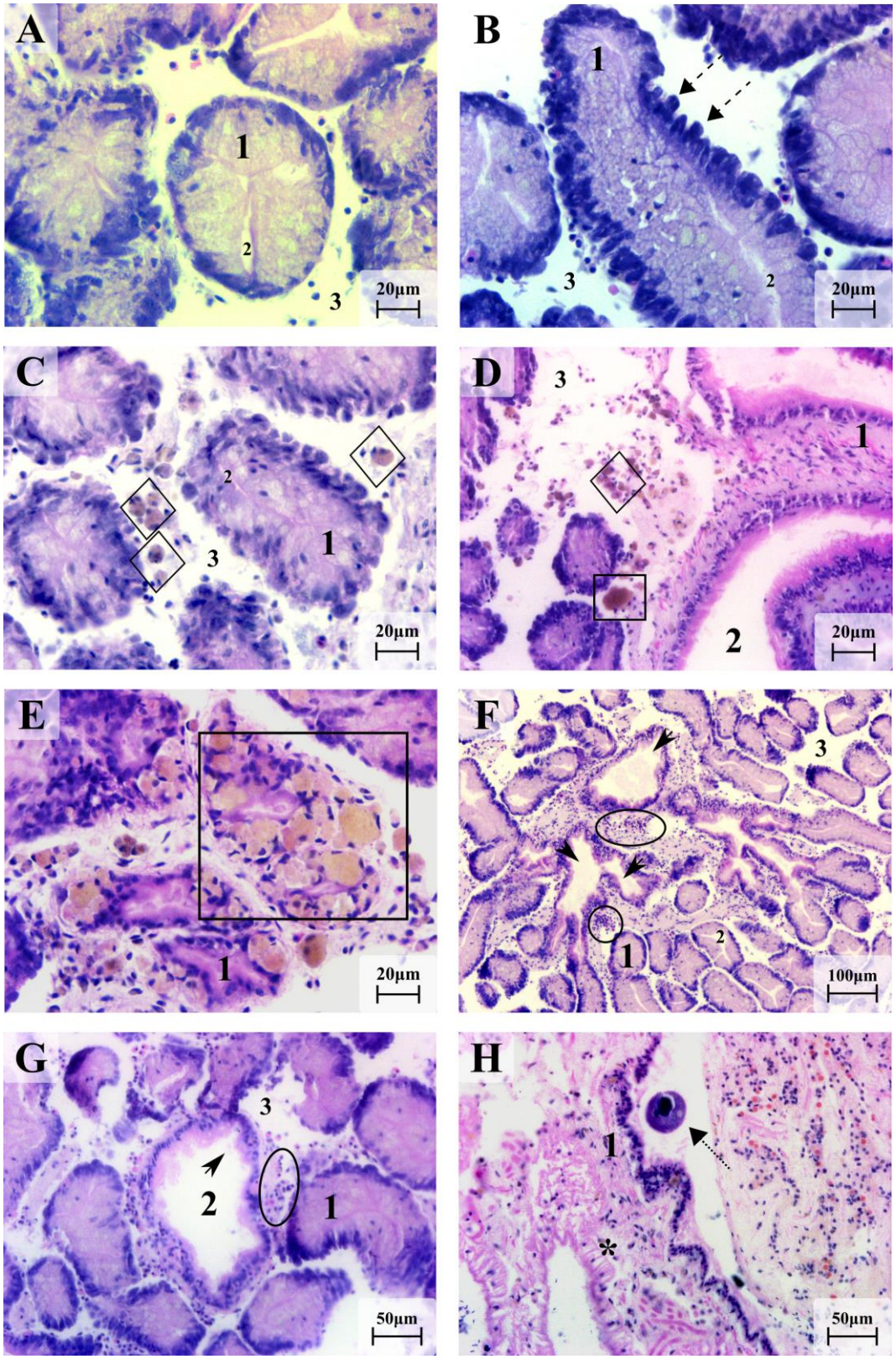


**Fig. 2** Digestive gland histological description of several organisms showing different dissemination degrees (a). A. Digestive tubules showing semi-circular normal aspects. Basophilic cells lining the lumen of digestive tubules. B. Secretory vesicles (dashed arrows) derived from epithelium. C. Digestive gland tubules showing few brown cells (a = 2, diamonds). D. Digestive gland tissue showing an increase of brown cells (a = 4), and some lipofuscin aggregates (a = 2, square). E. Tubules showing lipofuscin aggregates (square) with the highest dissemination degree (a = 6). F-G. Digestive gland showing atrophy (solid arrows) of the tubule epithelium (a = 2), and hemocyte infiltration (a = 6, circles). H. Digestive gland tissue showing inclusions of possible parasites (a = 2). 1. Epithelium 2. Lumen 3. Connective tissue. Hematoxylin-eosin staining

*Vulnerability parameters*

Vulnerability is composed of the levels of exposure, sensitivity, and recovery capacity (Ocaña and Pech, 2018). Exposure was measured through the number of immunohistochemical biomarkers identified. The immunohistochemical index ($I_I$) is the average of biomarkers found in different organs of the organisms. This average was categorized into four groups: no exposure ($I_I = 0$), minimal exposure ($I_I$ between 0.1 and 1), moderate exposure ($I_I$ between 1.1 and 2), and high exposure ($I_I$ between 2.1 and 3). Moreover, the sensitivity is the level of response according to the level of exposure to certain stresses (Ocaña and Pech, 2018). Therefore, the sensitivity relates the prevalence of histopathological alterations to the level of exposure, hence we assigned values to each category. The prevalence of histopathological alterations is a classification derived from the histopathological index, where 0 – 0.25 is low, 0.25 – 0.50 moderate, 0.50 – 0.75 high, and 0.75 – 1, very high (Costa *et al.*, 2013). We considered high and very high prevalence as the same category. All the organisms are sensitive to their environment to survive, thus we made four categories of sensitivity from very low to very high. These categories were assigned according to the following criteria. If an organism was exposed to a low level of exposure and had a low level of prevalence of alterations, then it had a normal sensitivity. However, if the organism was exposed to a low level of exposure but had a moderate or high prevalence of alterations, then the organism was highly sensitive (Table 2).

**Results**

*Clams from Tecolutla had a low prevalence of histopathological alterations*

The digestive glands have been used to assess pollution effects on many organisms, as epithelial cells lining the digestive glands are very sensitive to environmental stress (Usheva *et al.*, 2006). On one hand, the digestive gland of *P. caroliniana* clams in the control group showed tubular structures with an epithelial lining and a central lumen (Fig 2A, 2B). Moreover, on this epithelium we observed secretory vesicles (Fig 2B), which aid in the detoxifying processes of the clam (Usheva and Frolova, 2006). Around the tubules we observed connective tissue, which provides support to the digestive gland and a medium for oxygen and nutrients to diffuse to cells. Moreover, we found few hemocytes, brown cells and lipofuscin aggregates in the connective tissue, as expected. Most of this histological morphology agrees with the description of digestive glands in other clam species (Usheva and Frolova, 2006; Usheva *et al.*, 2006; Sıkdokur *et al.*, 2020; Bejaoui *et al.*, 2020; Costa *et al.*, 2013). On the other hand, as compared with the control groups, digestive gland sections from the experimental group underwent a change in their structure. Brown cells and lipofuscin aggregates in the slides from the experimental group increased (Fig 2C, 2D, 2E), as did hemocytic infiltrations, atrophy, and parasites (Fig 2F, 2G, 2H, and Table 3). These responses were classified into tubular and intertubular alterations. We used this classification to associate a pathological importance factor (*w*) and degree of dissemination (*a*) (Costa *et al.*, 2013), to further determine the general health status of clams from Tecolutla, Veracruz. The clams from the experimental group presented alterations with diverse pathological importance factors (*w*); however, the most disseminated pathologies had a low *w* (Table 4). These results suggest that the most prevalent pathologies are the least harmful for the organisms.

To integrate both *w* and *a*, we calculated the histopathological indices for these clams. The average histopathological index ($I_h$) for the control group was 0.01, while the average $I_h$ for the experimental group was 0.18. Based on these results, we concluded that the clams from Tecolutla, Veracruz had a low prevalence of histopathological alterations, according to previous reports classifying the $I_h$ (Costa *et al.*, 2013; Cuevas *et al.*, 2015).

*Clams from Tecolutla are exposed to several pollutants*

Besides histopathological analysis, other biomarkers are used to corroborate the impact of environmental stress. Some of these biomarkers include P450 cytochromes (CYPs), metallothionein proteins (MTs), and heat-shock proteins (HSP) (Moraga *et al.*, 2005; Boscolo Papo *et al.*, 2014a; b). We used these biomarkers to assess environmental

**Table 3** Pathological alterations and dissemination degree observed in clams from the experimental group. A total of 50 clams belonged to this group. Clams from the control group lacked any of these alterations. Dissemination degree (*a*) is the percentage of clams presenting each alteration

| Alteration | Number of clams with the alteration | Dissemination degree (*a*) |
|---|---|---|
| Brown cells | 47 | 95 |
| Lipofuscin aggregates | 30 | 60 |
| Hemocytic infiltrations | 20 | 40 |
| Atrophy | 10 | 25 |
| Parasites | 1 | 0.05 |

**Table 4** Values of pathological importance factor (*w*) and degree of dissemination (*a*) of clams from the experimental group. At the end is the $I_h$ for this group

| Reaction pattern | Alteration | *w* | *a* |
|---|---|---|---|
| Tubular alterations | Brown cells | 1 | 2 |
| | Lipofuscin aggregates | 1 | 4 |
| | Hemocytic Infiltrations | 2 | 0 |
| | Atrophy | 2 | 0 |
| | Parasites | 3 | 0 |
| Intertubular alterations | Browns cells | 1 | 6 |
| | Lipofuscin aggregates | 1 | 2 |
| | Hemocytic Infiltrations | 2 | 6 |
| | Atrophy | 2 | 6 |
| | Parasites | 3 | 2 |
| **Histopathological index ($I_h$)** | | 0.18 | |

stress effects on the clam of Tecolutla (Table 5). Digestive gland slides of the control group lacked any immunoreactivity. However, digestive gland slides of the experimental group showed positive immunoreactivity to P450 cytochrome (CYP) and heat-shock protein 70 (HSP70), but not to MTs. Both CYP and HSP70 were observed on the cytoplasm of the epithelial tissue lining of the digestive tubules (Fig 3A, 3B, 3C). These results suggest that injuries caused to the digestive gland by environmental stress are reversible, as the control group did not show immunoreactivity to these markers. Nevertheless, the digestive system of clams is composed of other organs, such as a short esophagus, a stomach, and a gut. Hence, we tested the same biomarkers on the rest of them. Stomach and gut tissues showed immunoreactivity to HSP70 and CYP but not to MTs (Fig 3D). The immunohistochemical responses indicated that the Tecolutla environment has many pollutants, but apparently the concentration of heavy metals is low, as the digestive system did not show any immunoreactivity.

Nevertheless, to obtain an overview about the overall health of the clams, we tested the same antibodies in other target organs (Table 5). The gills, the gonads and the foot are widely used to assess the health of many mollusks through histopathological and immunohistochemical approaches (Moraga *et al.*, 2005; Usheva *et al.*, 2006; Costa *et al.*, 2013; Boscolo Papo *et al.*, 2014a; Cuevas *et al.*, 2015; Sıkdokur *et al.*, 2020). In our clams, the gills showed immunoreactivity to HSP70 and CYP, but not to MTs. Meanwhile, the gonads showed immunoreactivity only to HSP70. Finally, the foot showed immunoreactivity to all the biomarkers, HSP70, CYP, and MTs (Fig 3E, 3F). To observe the accuracy of the immunohistochemical test, we showed that in the absence of the antibody, the color of the slides changes dramatically (Fig 3G, 3H). These organs have different levels of exposure to the environment, due to their diverse physiological functions. Accordingly, the gills are highly related to water quality, while the foot is more related to sediment quality. However, the general response to immunohistochemical biomarkers indicated that the environment is affecting all the organs. Furthermore, as the control group lacked immunoreactivity to any biomarker, we concluded that the health of our clams from Tecolutla could be restored if the quality of the environment is improved.

*Proposed approach to assess vulnerability using tissular analysis*

Vulnerability is the degree to which a system is susceptible to adverse effects caused by environmental stress (Villa and McLeod, 2002; Stein *et al.*, 2014; Gauthier *et al.*, 2014; Carantoña and Hernández, 2017; Ocaña and Pech, 2018). The strategies to estimate environmental vulnerability

**Table 5** Immunoreactivity responses in different organs of clams from the experimental group

| Organ | CYP | HSP70 | MTs | Total |
|---|---|---|---|---|
| **Digestive gland** | 1 | 1 | 0 | 2 |
| **Stomach** | 1 | 1 | 0 | 2 |
| **Gut** | 1 | 1 | 0 | 2 |
| **Gills** | 1 | 1 | 0 | 2 |
| **Gonad** | 0 | 1 | 0 | 1 |
| **Foot** | 1 | 1 | 1 | 3 |
| **Immunohistochemical index ($I_I$) = 2** | | | | |

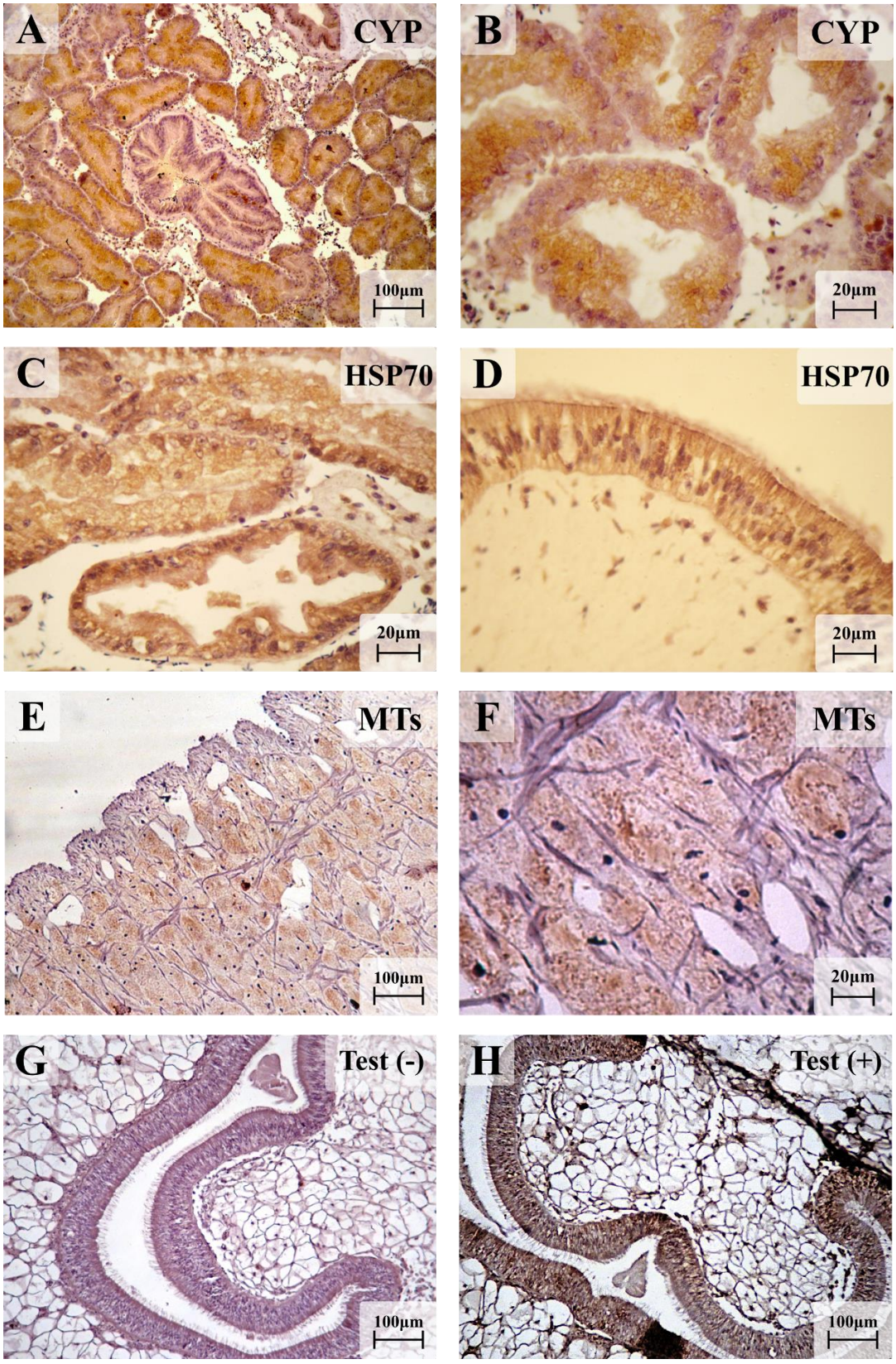


**Fig. 3** Immunoreactivity of CYP, HSP70, and MTs in different tissues of clam from Tecolutla. All sections are counterstained with Tacha Hematoxylin. A-B. Digestive gland showing an immunoreactivity to CYP. C. Digestive gland showing an immunoreactivity to HSP70. D. Intestine showing immunoreactivity to HSP70. E-F. Foot showing immunoreactivity to MTs. G-H Immunoreactivity controls showing difference in the brown staining, both from foot tissue. Negative control (G) processed with PBS instead of the HSP70 antibody and positive control (H) processed with the HSP70 antibody

include indicators categorized in anthropogenic, biological, geological, and meteorological components (Villa and McLeod, 2002; Skondras *et al.*, 2011; Karmaoui, 2015; Sahoo *et al.*, 2016; Harik *et al.*, 2017). However, these indicators do not include any physiological trait of the living organisms of each system. Therefore, we propose the integration of the histopathological index and immunohistochemical approaches as valuable tools to assess the vulnerability of clams from Tecolutla. The assessment of vulnerability includes the measure of exposure, sensitivity, and recovery capacity (Villa and McLeod, 2002; Ocaña and Pech, 2018). To begin with, exposure was measured through the immunoreactivity of different biomarkers. We created a range based on the immunohistochemical data previously presented. The average number of biomarkers found in the organs was classified into no, low, medium, and high exposure, called the Immunohistochemical Index ($I_I$) (Table 5). The digestive glands of clams from Tecolutla showed medium exposure, but very close to the high category (Table 6). We recommend the use of at least three different immunochemistry biomarkers in three or more target organs to assess exposure. The gills, digestive gland, and foot constitute the preferred organs to evaluate this (Moraga *et al.*, 2005; Usheva *et al.*, 2006; Boscolo Papo *et al.*, 2014a; Cuevas *et al.*, 2015). Afterwards, the sensitivity of clams was assessed through a comparison between the histopathological index ($I_h$) classification and the exposure level (Table 2). We determined that an organism is sensitive if the level of exposure is lower than the category of the $I_h$. In other words, an organism that shows a low prevalence of histopathological alterations in an environment with moderate exposure level has low sensitivity (Table 2). Therefore, clams from Tecolutla had low sensitivity to the environment. Finally, to assess recovery capacity, we observed both the $I_I$ and the $I_h$ of the control group. These clams depurated toxic molecules during 40 days in a clean environment, hence they constituted a good parameter to assess recovery capacity. Clams from the control group showed an exposure level of zero, according to $I_I$. Furthermore, these clams had an $I_h$ of 0.01, meaning that the prevalence of histopathological injuries was reversed almost completely. In other words, these organisms have a good recovery capacity (Table 6). However, another study is necessary to propose an approach that classifies the levels of recovery capacity in these organisms.

For the integration of the three vulnerability components in a mathematical model, we needed a value of recovery capacity. However, recovery capacity regulates vulnerability through the modulation of exposure and sensitivity (Adger *et al.*, 2007; Engle, 2011). Therefore, we concluded that clams from Tecolutla had a low vulnerability, as they reversed all the effects of environmental stress.

In summary, these physiological traits and their semi-quantification should be included in the vulnerability measurements of aquatic bodies. These traits represented directly the health status of Tecolutla, Veracruz. Briefly, these results indicate that the effects of pollutants in these organisms are still reversible. However, it is important to stop

**Table 6** Proposed semiquantification of parameters considered as indicators of vulnerability

| Vulnerability factor | Indicator | Scale | | Clams vulnerability |
|---|---|---|---|---|
| **Exposure** | Immunoreactivity index ($I_I$) | No exposure | 0 | Moderate exposure |
| | | Minimal | 0.1 - 1 | |
| | | Moderate | 1.1 - 2 | |
| | | High | 2.1 - 3 | |
| **Sensitivity** | Exposure vs prevalence of histopathological alterations ($I_I$ vs $I_h$) | Very low | -2 | Low sensitivity |
| | | Low | -1 | |
| | | Normal | 0 | |
| | | High | 1 | |
| | | Very high | 2 | |
| **Recovery capacity** | Control group assessment | Low | ? | Efficient recovery capacity |
| | | Moderate | ? | |
| | | High | ? | |

pollution on Tecolutla so that organisms can improve their health. This work sets a health record for future monitoring references. The full quantification of histopathological indexes for the rest of the organs and the integration with the immunohistochemical part is a perspective of this work that contains valuable information about the vulnerability of these organisms. Furthermore, it is important to develop an approach to classify the recovery capacity properly. Additionally, the perspective of this work includes the application of this strategy in other indicator organisms to fully estimate the vulnerability of Tecolutla.

## Discussion

Vulnerability is the degree to which a system is susceptible to adverse effects caused by environmental stress (Villa and McLeod, 2002; Gauthier *et al.*, 2014; Stein *et al.*, 2014; Carantoña and Hernández, 2017; Ocaña and Pech, 2018). Environmental vulnerability indexes created along the last years, through many approaches, take into account indicators categorized into anthropogenic, biological, geological, and meteorological components (Villa and McLeod, 2002; Skondras *et al.*, 2011; Gauthier *et al.*, 2014; Karmaoui, 2015; Sahoo *et al.*, 2016; Harik *et al.*, 2017). However, these indicators do not include any physiological traits of the living organisms of each system. Some approaches include water quality and plant distribution, while others include plant physiological traits (Esperón-Rodríguez and Barradas, 2015; Trevisan *et al.*, 2020). The biological responses of organisms reflect the health status of a particular environment. Recently, some vulnerability indexes analyzed responses based on specific biomarkers (Gauthier *et al.*, 2014; Chalghmi *et al.*, 2020). Vulnerability depends on the levels of exposure and sensitivity of a system to a specific pressure, altogether with the capacity to cope, recover, or adapt to this pressure (Villa and McLeod, 2002; Stein *et al.*, 2014; Carantoña and Hernández, 2017; Ocaña and Pech, 2018). These components can be assessed at different levels. Here, we created an approach to measure the three vulnerability components through the assessment of the health status of the clams (Table 6). The proposed approach includes the use of different tissular techniques to this end.

*Exposure*

Several human activities generate pressure on aquatic bodies. Vulnerability cannot be assessed without exposure to a pressure. Therefore, the first step is to determine the sources of pressure (Ocaña and Pech, 2018). Environmental exposure is related to the presence and absence of immunohistochemical biomarkers (Moraga *et al.*, 2005; Wang *et al.*, 2010; Boscolo Papo *et al.*, 2014a; Santovito *et al.*, 2015). These responses reflect the environmental stress that affects the organisms. The immunohistochemical approach determines the exposure to pollutants quickly, without the need for specific chemical procedures. The biomarkers used cover a broad range of pollutants. Among them, we used P450 cytochromes (CYPs), metallothionein proteins (MTs), and heat-shock proteins (HSP) that are expressed after exposure to pesticides, hydrocarbons, metals, heat shock, and other toxic compounds (Moraga *et al.*, 2005; Boscolo Papo *et al.*, 2014a; b). These biomarkers are widely used in different organisms to show exposure to environmental stress (Moraga *et al.*, 2005; Wang *et al.*, 2010; Boscolo Papo *et al.*, 2014a; Santovito *et al.*, 2015). Different target organs are used to this aim, such as the digestive gland, gills, foot, and gonads. To obtain an overview of the general exposure of the clams, we evaluated several target organs. These responses were compiled and averaged to create a range of different levels of exposure. Although the immunochemical approach lacks quantification of pollutants, averaging the immune reactivity to different biomarkers in different organs provides an overview of the level of exposure. The target organs differed in their reactivity to the biomarkers, because they have different physiological functions. The physiological function of each organ and its anatomical position determine its interaction with the environment. For instance, the foot and the gills are in direct contact with sediment and water, making them more exposed, while the gonads are less exposed because they do not interact directly with the environment. Our proposal weights equally the biomarkers exposed in different organs; however, it is necessary to determine if the organs should be weighted differently. Nevertheless, our proposal, using an immunochemical approach, includes two types of exposure information. On one side, it evaluates a broad range of pollutants to which organisms are exposed. On the other side, it includes different target organs with distinct metabolic pathways that cope with the toxic compounds.

*Sensitivity*

Sensitivity is the degree to which a system or species is affected by environmental stress (Stein *et al.*, 2014). A description of what makes a system sensitive is necessary, keeping in mind that each sensitivity level is specific to each pressure (Ocaña and Pech, 2018). All living organisms are sensitive to their environment to survive; however, the level of responses should be related to an exposure level to determine if the system is sensitive. Hence, to evaluate the organism's sensitivity, we related two aspects. On one hand, we measured the types and dissemination of the responses to a stress, called the prevalence of histopathological alterations. This prevalence categorizes the values of the histopathological index ($I_h$), for comparison among several organisms (Costa *et al.*, 2013; Cuevas *et al.*, 2015). Besides, the prevalence correlates with the health status of a specific environment. On the other hand, we correlated the prevalences with the exposure level to determine the clam's sensitivity to the pollutants present in Tecolutla. Histopathology is widely used to recognize the effects of environmental stress on the organisms (Bernet *et al.*, 1999; Usheva *et al.*, 2006; Costa *et al.*, 2013;

Boscolo Papo *et al.*, 2014a; b; Cuevas *et al.*, 2015; Sıkdokur *et al.*, 2020). It has been used in several organisms to determine the health status of aquatic bodies (Usheva *et al.*, 2006; Chalghmi *et al.*, 2020; Costa *et al.*, 2013; Boscolo Papo *et al.*, 2014a). Therefore, in our proposal, sensitivity is the measure of responses (prevalence of histopathological alterations) related to the exposure level. If the exposure level is equal to the prevalence of histopathological alterations, then the sensitivity is normal. In case the exposure level is lower than the prevalence, then the sensitivity is low. In contrast, if the exposure level is higher than the prevalence then the sensitivity is high. As far as we know, this is the first approach giving a semiquantitative method to assess sensitivity through the evaluation of physiological responses to pollutants.

*Recovery capacity*

The third component of vulnerability is adaptive capacity. Adaptive capacity refers to the ability of a species or system to cope with environmental impact with minimal disruption (Stein *et al.*, 2014). However, adaptive refers to the evolution of a system in ecology, and it is usually assessed through evolutionary potentials, such as plasticity, dispersal ability and evolutionary potential (Ocaña and Pech, 2018). Tecolutla lacks monitoring programs that assess health status through time, hence we required another strategy to evaluate this vulnerability component. We determined that the recovery capacity attribute could be used instead. Recovery capacity is usually assessed through both extrinsic factors and intrinsic traits. To evaluate this capacity, we develop an approach to evaluate the ability of the organism to cope with the consequences of environmental stress. A control group of clams lived in a clean environment for 40 days. Then, they were subjected to histopathological and immunohistochemical analysis to observe differences with the experimental group. We observed a full recovery of the control group. The control group did not show immunoreactivity to any exposure biomarker (immunochemical analysis). Moreover, it had a lower histopathological index ($I_h$ = 0.01) compared with the experimental group ($I_h$ = 0.18). Nonetheless, we lacked a categorization for this component. Another essay is necessary to evaluate different levels of recovery capacity related to different levels of exposure. Nevertheless, we concluded that, having the current level of exposure, the clams from Tecolutla have sufficient recovery capacity to reverse the consequences of environmental stress.

Briefly, we employed different indicators to measure the three components of vulnerability. However, to integrate them in a mathematical model, standardization, response scaling, weighting, and aggregation are necessary. Although we had semi-quantitative values for exposure and sensitivity levels, we lacked values for the recovery capacity component. Nonetheless, knowing that the clams were able to recover with a moderate exposure level and a low sensitivity, we can estimate the vulnerability. Recovery capacity was taken as a measure of adaptive capacity, hence both regulate vulnerability through modulation of exposure and sensitivity (Adger *et al.*, 2007; Engle, 2011). This agreed with the method we used to assess recovery capacity, evaluating exposure and sensitivity levels through the same methods and then compare the results of the control versus the experimental group. As we observed that the clams reverted almost all the effects of environmental stress, we determined that the recovery capacity exceeded the exposure and sensitivity. Therefore, these results indicated that clams from Tecolutla showed a low vulnerability. Higher recovery or adaptive capacity aids in reducing the effects of exposure and sensitivity and, in consequence, reduces the vulnerability of the system (ART Vulnerability & Risk Assessment Report, 2012; Stein *et al.*, 2014; Thomas *et al.*, 2019). The basic role of recovery or adaptive capacity is accepted as a positive attribute to reduce vulnerability (Engle, 2011; Thomas *et al.*, 2019). As previously mentioned, it is still necessary to develop an assay to identify the limits of the recovery capacity of clams at different exposures levels.

In conclusion, adding the vulnerability components, exposure, sensitivity, and recovery capacity, we determined that the clams of Tecolutla have a low vulnerability. These clams had a moderate exposure level, low sensitivity, and a high recovery capacity. Assets with a higher adaptive capacity, or recovery capacity, and low sensitivity better tolerate impacts, and therefore have a lower vulnerability (Engle, 2011; ART Vulnerability & Risk Assessment Report, 2012). As far as we know, this is the first approach to semi-quantifying the vulnerability of an organism. Besides, this proposal constitutes an appealing approach for organisms in aquatic bodies that lack monitoring programs. The perspective of this work is to create categories to divide the recovery capacities of several organisms and compare them. Also, we propose to determine the vulnerability from several organisms, in order to estimate the general vulnerability of an environment. Finally, along with other approaches we aim to determine in the future the vulnerability of Tecolutla, Veracruz.

**Declaration of conflict of interest**

The authors declare no conflict of interests.

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