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## Evaluation of marine biotoxins in *Crassostrea virginica* in Tampamachoco Lagoon and Tamiahua Lagoon, Veracruz

Evaluación de biotoxinas marinas en *Crassostrea virginica* en la Laguna de Tampamachoco y Laguna de Tamiahua, Veracruz



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### ABSTRACT

Marine biotoxins were evaluated in the oyster *Crassostrea virginica* collected from the Tampamachoco and Tamiahua lagoons in Veracruz, Mexico, through monthly samplings conducted from July 2018 to February 2019. Using optical microscopy, three genera of toxin-producing dinoflagellates were identified: *Alexandrium*, *Prorocentrum*, and *Gymnodinium*. The detection of biotoxins in oyster muscle was performed using mouse bioassays, rapid tests for paralytic shellfish poisoning (PSP), and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The results showed that paralytic shellfish toxins were not detected by any of the techniques used. However, chromatographic analysis allowed the identification of other marine biotoxins. In samples from Tampamachoco lagoon, spirolides were detected with maximum values of 10.09 mg/g in July, 11.39 mg/g in August, and 10.77 mg/g in September; gymnodimines reached 14.56 mg/g in December and 12.4 mg/g in February; and pectenotoxins were detected at 0.38 mg/g in December. In Tamiahua lagoon, spirolides were recorded at 1.76 mg/g in September and 4.33 mg/g in October; gymnodimines at 0.18 mg/g in August and 0.21 mg/g in November; pectenotoxins at 0.17 mg/g in December; and domoic acid at 13.76 mg/g in August and 14.45 mg/g in November. The detection of domoic acid, the toxin responsible for amnesic shellfish poisoning, represents the first report of this compound in these lagoons. Furthermore, the presence of spirolides, gymnodimines, and pectenotoxins highlights the need for additional studies to evaluate their potential toxicological risk to human health and to strengthen biotoxin monitoring programs in shellfish-producing areas.

**Keywords:** *Crassostrea virginica*, biotoxins, domoic acid.

### RESUMEN

Se evaluó la presencia de biotoxinas marinas en el ostión *Crassostrea virginica* proveniente de las lagunas de Tampamachoco y Tamiahua, Veracruz, México, mediante muestreos mensuales realizados de julio de 2018 a febrero de 2019. Mediante microscopía óptica se identificaron, tres géneros de dinoflagelados productores de toxinas: *Alexandrium*, *Prorocentrum* y *Gymnodinium*. La detección de biotoxinas en músculo del ostión se realizó con bioensayos en ratón, pruebas rápidas para toxina paralizante de moluscos (PSP) y cromatografía líquida acoplada a espectrometría de masas en tándem. Los resultados mostraron



que la toxina paralizante de moluscos no fue detectada con ninguna de las técnicas empleadas. Sin embargo, el análisis cromatográfico permitió identificar otras biotoxinas. En las muestras de Tampamachoco se detectaron espirolidos (10.09 mg/g en julio, 11.39 mg/g en agosto y 10.77 mg/g en septiembre), gymnodiminas (14.56 mg/g en diciembre y 12.4 mg/g en febrero) y pectenotoxinas (0.38 mg/g en diciembre). En Tamiahua se registraron espirolidos (1.76 mg/g en septiembre y 4.33 mg/g en octubre), gymnodiminas (0.18 mg/g en agosto y 0.21 mg/g en noviembre), pectenotoxinas (0.17 mg/g en diciembre) y ácido domoico, con 13.76 mg/g en agosto y 14.45 mg/g en noviembre. La detección de ácido domoico, responsable de la intoxicación amnésica por consumo de moluscos, representa el primer reporte de esta toxina en estas lagunas. Asimismo, la presencia de espirolidos, gymnodiminas y pectenotoxinas resalta la necesidad de realizar estudios adicionales para evaluar su posible riesgo toxicológico para la salud humana y fortalecer los programas de monitoreo de biotoxinas en zonas productoras de moluscos.

**Palabras Clave:** *Crassostrea virginica*, biotoxinas, ácido domoico.

## INTRODUCTION

Mexico is a producer of the American oyster *Crassostrea virginica*, which comprises a significant part of national oyster production and 90% of production in the Gulf of Mexico (Cáceres-Martínez & Vázquez-Yeomans, 2013). Moreover, Tampamachoco Lagoon and Tamiahua Lagoon provide 89% of oyster production in the state of Veracruz (Arias, 2014). The oyster is a significant commercial resource and a source of income for the regional communities participating in its extraction (Vidal-Briseño, 2015). Bivalve mollusks feed by filtering particles suspended in the water, thus accumulating toxins in their tissues that can potentially cause poisoning in humans when consumed. Mollusks are a source of protein for humans and are a valuable resource for both the local and regional economies of coastal populations (Braga et al., 2018). Paralytic shellfish poisons (PSPs) are a group of potent marine neurotoxins produced naturally in both fresh and marine water (Abi-Khalil et al., 2017). Among the components of PSP, saxitoxin, described as a potent water-soluble neuromuscular toxin, presents the highest level of toxicity (Marín et al., 2013). Amnesic (domoic acid) shellfish poisoning causes short-term-to-permanent memory loss, while it can also present serious gastrointestinal symptoms and, in acute cases, may cause death (Wright et al., 1989). Marine biotoxins, classified as paralyzing, neurotoxic, amnesic, diarrhoeal, ciguatoxic, and azaspiracidic marine phycotoxins, are produced by microalgae species and are classified according to the principal signs and symptoms they produce in humans (Sánchez-Bravo et al., 2016).

They are also classified, according to their chemical composition and physical properties, as either hydrophilic and hydrophobic (or lipophilic). The hydrophilic group includes amnesic shellfish poisons (ASPs) and PSPs, while lipophilic toxins include diarrhetic shellfish poison (DSPs), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs), gymnodimines (GYMs), spirolides (SPXs), and brevetoxins (PbTXs) (Botana, 2014). PSP is produced mainly by dinoflagellates of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, which accumulate in species which feed via filtration (Shin et al., 2017).



These toxins comprise more than 57 tetrahydropurine derivatives that can be divided into four categories, the toxins N-sulfocarbamoyl, decarboxyl, deoxycarbamoyl, and carbamate, to the last of which pertains saxitoxin (STX) (Wiese *et al.*, 2010).

In the Gulf of Mexico, Barón-Campis *et al.*, (2014) found 17 dinoflagellate species, of which six are potentially toxic and may pose a risk to mariculture and the region's main fisheries due to their ichthyotoxic and among which said authors highlight *Karenia brevis*, *Karenia cf. mikimotoi*, *Gambierdiscus* spp., *Ostreopsis* sp., and *Prorocentrum minimum*.

Figueroa & Weiss (1999) reported, for the northern region of the state of Veracruz, research on the taxonomic composition of the dinoflagellates of the coastal Tamiahua Lagoon, describing their role as toxic organisms involved in the formation of red tides. Of the 35 species they found, 23 had never been registered for that study area, 24 had been reported as red tide forming members, and seven corresponded to toxic species (*Amphidinium carterae*, *Gymnodinium breve*, *Prorocentrum lima*, *Prorocentrum micans*, and *Prorocentrum minimum*). A study conducted on the annual distribution and abundance patterns of harmful phytoplankton in the coastal area of Tuxpan Veracruz found a total of 265 phytoplankton species, 86 of which pertain to the dinoflagellates and among which were identified the three genera that cause PSP—*Gymnodinium*, *Alexandrium*, and *Pyrodinium* (Orduña-Medrano, 2012). On the other the hands, Pérez-Olmedo (2014) reported 130 species, of which 18 pertain to potentially toxic species that include species of the genus *Pyrodinium*. Said author also conducted an evaluation of the composition and abundance of toxic and harmful dinoflagellate species that form red tides in the coastal region of Tuxpan, finding 17 dinoflagellate species that are reported as toxic and are associated with both the formation of red tides and the three genera that produce PSP (Pérez-Olmedo, 2017).

## MATERIAL AND METHODS

The present study was carried out in Tampamachoco Lagoon and Tamiahua Lagoon from July 2018 to February 2019, in three oyster banks per lagoon, from which samples were collected for the detection of saxitoxin and the evaluation of the physicochemical parameters at each capture site. Moreover, a water sample was taken from each oyster bank at a depth of no greater than 30 cm and then fixed with Lugol's solution at 100% (Vicente *et al.*, 2005), with one drop applied per 100 ml (Méndez-Torres, 2016). The PSP reference solution (1 µg de saxitoxin/ml) was prepared using 1 ml standard solution in a 100-ml volumetric flask and brought to the desired volume with distilled water that had been acidified using HCL (pH=3). The resulting solution was kept stable under refrigeration at 3 to 4°C and with a pH of 2.0 to 4.0, based on Official Mexican Standard 13NOM-242-SSA-2009 (D.O.F., 2009). Said procedure used 40 male albino mice, who were healthy and weighed 19 to 21 gr.



Approximately 150 gr of oyster meat was collected for the analysis and then ground until homogenization was achieved. Solely 100 gr of the homogenized meat was used, to which 100 ml hydrochloric acid (HCl) (0.18 N) was added and then kept at a pH of between two and four. With HCL 0.001 M added to the solution, the supernatant was centrifuged for five minutes at 3000 rpm and the amount of liquid required for the bioassay then filtered. The bioassay used three mice, each of which were injected intraperitoneally with 1 ml of centrifuged extract. Each mouse was observed for 60 minutes and the moment of their death then recorded. The average period of time that elapsed until death after the application of undiluted extract was more than seven minutes, a finding used to determine the toxicity of the sample. The guidelines established in NOM-242-SSA-2009 for the extraction and handling of bivalve mollusks were applied during the bioassays conducted on the samples collected by the present study. On the same day that the bioassay was conducted, the conversion factor (CF) was determined, with five mice then inoculated with an appropriate dilution of the standard solution.

Mouse units (MUs) were obtained using Sommer's table, based on the time elapsed until death for each mouse. The MU for the mice that survived for more than 60 minutes was <0.875. The corrected mouse units (CMUs) were then calculated by multiplying the MUs by the weight correction factor and arranged in order from lowest to highest to obtain the median corrected mouse unit (MCMU). The concentration of saxitoxin equivalents was calculated via Equation 1:

$$\mu\text{g PSP}/100\text{gr mollusk meat} = \text{MCMU}/\text{ml} \times \text{CF} \times \text{DF} \times 200$$

where:

MCMU = median corrected mouse unit

CF = conversion factor

DF = dilution factor

200 = corresponds to the 100g of sample plus the 100g of hydrochloric acid

Any value of over 80  $\mu\text{g}/100\text{gr}$  was considered a risk value and meant that the individual from which it was obtained should be prohibited from human consumption.

If all the mice survived more than 60 minutes after the injection, this sample was reported as presenting a concentration lower than (<) the value obtained from the toxin concentration calculations.

The Scotia Rapid Testing procedures undertaken used individual vials with 400  $\mu\text{l}$  buffer, to which 100  $\mu\text{l}$  mollusk extract was added until reaching the black line marked on the dispenser. One hundred  $\mu\text{l}$  of the extract/buffer mixture was taken and placed in the cassette well identified as S, with the data then recorded an hour later and, finally, interpreted using the guide provided with the test kit, thus obtaining the test results (Figure 1).



**Figure 1. Guide for interpreting the rapid tests**

If the *T-line* is darker than the *C-line* given in the POSITIVE result set out in Figure 1, the result is NEGATIVE. The result is POSITIVE if the *T-line* is the same as or fainter than the *C-line* given in the POSITIVE result set out in Figure 1.

In the event of ambiguity in the interpretation of the test data, the result is considered POSITIVE. If the *C-line* is the same as or fainter than the *T-line* given in the INVALID result set out in Figure 1, the test is INVALID. In such a case, the assay should be repeated after first ensuring a pH of three for the extract. Should the same result continue to present, the manufacturer states that this would indicate that the sample contains an unknown agent that is inhibiting the antigen/antibody reaction and, thus, an alternative method of analysis should be sought. The toxin analysis involved placing  $2.00 \pm 0.05$  g homogenized tissue in a 50 ml centrifuge tube, to which 9.0 ml of 100% methanol was added and homogenized using a vortex for 3 min at maximum speed. The mixture was then centrifuged at 2000 RPM for 10 min at 20°C and the supernatant transferred to a 20 ml. volumetric flask, with the extraction then repeated over the pellet and another 9.0 ml methanol added, after which the sample was homogenized for one minute in an Ultra Turrax™ homogenizer. Moreover, the mixture was centrifuged again for 10 min at 2000 and 20°C. The supernatant was then transferred and combined with the first extract until the volume reached 20 ml with 100% methanol. Finally, an aliquot was filtered from said extract, using a 0.45-to-0.2- $\mu$ m methanol-compatible filter and then subject to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Once the results had been obtained, they were entered into an Excel database, separated by lagoon, and then graphed.



## RESULTS

The 30 water samples collected from Tampamachoco Lagoon and Tamiahua Lagoon were examined in a microscope, wherein, as with the 15 preserved oyster samples, the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* could not be observed.

During the process of standardizing the control test, the dilution of the stock solution revealed a time elapsed until death of five to seven minutes, wherein mouse M1 presented 6:21 min and mouse M5 the longest period of 7:38 min (Table 1).

**Table 1. Calculation of the conversion factor for standard dilution**

Mouse number	Weight	Innoculation	Last exhalation	Death
M2	20.24 g	0:00 s	7:23 min	7:23 min
M5	19.88 g	0:50 s	8:28 min	7:38 min
M9	20.94 g	1:45 min	8:35 min	6:50 min
M8	19.71 g	2:18 min	9:28 min	7:10 min
M1	20.72 g	4:07 min	10:28 min	6:21 min

A median time elapsed until death of 1.390 was then obtained, with all the mice subject to the bioassay presenting clinical signs such as extensive muscle paralysis, dyspnea, and death due to respiratory paralysis (Table 2).

**Table 2. Calculation of the conversion factor for dilution**

Mouse unit (MU)	Weight correction	Corrected mouse unit (CMU)	Ordering of the CMU data	Median CMU value	Clinical signs
1.328	1.006	1.336	1		Extensive muscle paralysis, dyspnea, death due to respiratory paralysis
1.294	0.997	1.390	3	1.390	Extensive muscle paralysis, dyspnea, death due to respiratory paralysis
1.417	1.027	1.455	4		Extensive muscle paralysis, dyspnea, death due to respiratory paralysis
1.363	0.991	1.351	2		Extensive muscle paralysis, dyspnea, death due to respiratory paralysis
1.516	1.021	1.548	5		Extensive muscle paralysis, dyspnea, death due to respiratory paralysis



The bioassays conducted on the samples taken from Tampamachoco Lagoon from July to October did not obtain maximum values of 80  $\mu\text{g}/100\text{gr}$ , as established by NOM 242, with all mice surviving the 1-ml oyster muscle extract injection and presenting a time elapsed until death of over an hour. The concentration presented by the mice was below 39.65  $\mu\text{g STX eq Kg}^{-1}$  tissue (Table 3). The test strip (Scotia Rapid Testing) used to determine the presence of PSP in the 30 oyster muscle samples obtained in Tampamachoco Lagoon and Tamiahua Lagoon from July-November revealed negative results (Figure 2).

The CF value was used to calculate the limit of quantification, which was 39.65  $\mu\text{g STX}/100\text{g}$  for the control test (Table 3).

**Table 3. Final CF calculation and limit of quantification**

AVERAGE CF	LIMIT OF QUANTIFICATION (LQ)
$\text{CF} = \frac{\text{diluted STX}}{\text{MCMU}} = \frac{0.333}{1.390} = 0.2395$	$\text{LQ} = \text{CMU} \times \text{CF} \times 200$
	$\text{LQ} = (0.8278)(0.2395)(200) = 39.65 \mu\text{g STX eq Kg}^{-1} \text{ tissue}$

Chromatograms were undertaken on the homogenized samples from Tampamachoco Lagoon and Tamiahua Lagoon in search of other toxins, revealing, in the Tampamachoco samples, spirolides that presented maximum values in July, August, and September. The highest gymnodimine values were observed in December, January, and February, while the highest pectenotoxin values presented in December (Figure 3).

The LC-MS/MS conducted on the Tamiahua Lagoon oyster muscle samples found high levels of domoic acid in August and October, gymnodimines in August and November, spirolides in September-October, and pectenotoxins in December (Figure 4).

The PSP values obtained, 39.65  $\mu\text{g STX eq Kg}^{-1}$  tissue, were lower than those permissible for human consumption under NOM 242, while the ASP levels (domoic acid) obtained were also lower than those permitted under the standard. Although spirolide, gymnodimine, and pectenotoxin levels remain unregulated with no official standard stipulating levels permissible for human consumption, acid domoic does generate symptoms that are harmful to public health (Table 4).



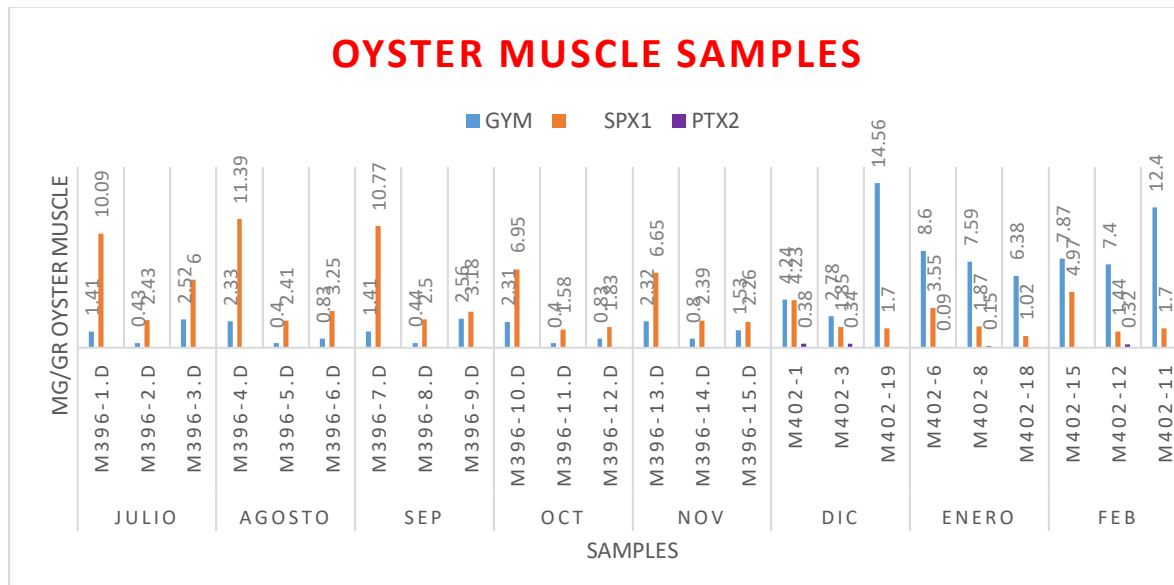


Figure 3. Marine biotoxin values presenting in the oyster muscle samples taken from Tampamachoco, corresponding to GYM (gymnodimines), SPX1 (spirolides), and PTX2 (pectenotoxins)

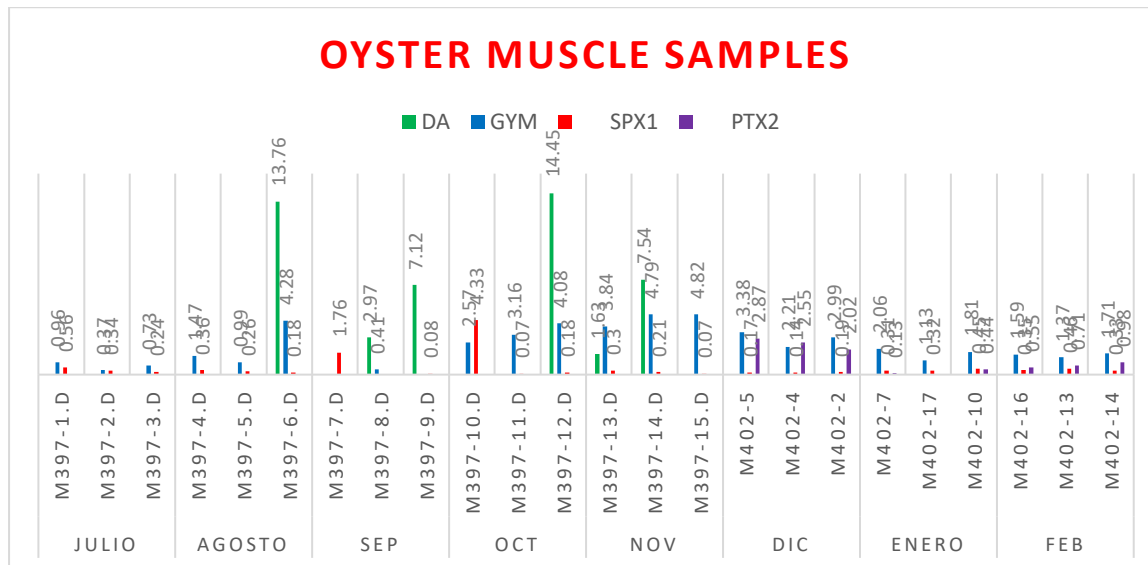


Figure 4. Marine biotoxins values presenting in the oyster muscle samples taken in Tamiahua Lagoon, corresponding to DA (domoic acid), GYM (gymnodimines), SPX1 (spirolides), and PTX2 (pectenotoxins)



**Table 4. Values obtained for marine biotoxins**

Marine biotoxins	Values observed in the muscle samples	Reference values stipulated by NOM 242
PSP	<39.65 µg/100gr	80 µg/100gr
ASP	<14.45 mg/gr	20 g/gr

## DISCUSSION

When the marine biotoxin concentrations in the *C. virginica* oyster muscle were evaluated by the present study, PSP was not found, while spirolides, gymnodimines, and pectenotoxins were identified in the samples obtained in both Tampamachoco Lagoon and Tamiahua Lagoon. Domoic acid was only observed in the oyster muscle samples taken in Tamiahua Lagoon. That the present study did not find *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, genera which produce marine biotoxins in Tampamachoco Lagoon, distinguishes it from research reported by [Orduña-Medrano \(2012\)](#), who observed the presence of *Alexandrium* in March and April and *Gymnodinium* in February and May. [Pérez-Olmedo \(2017\)](#) obtained *Gymnodinium* in samples taken in July and *Alexandrium* in June, July, August, September, and December samples, coinciding with the months in which sampling was undertaken by the present study. Dinoflagellate producers of toxins were not found in either Tampamachoco Lagoon or Tamiahua Lagoon. Toxin absorption and elimination rates can be affected by the temperature and pH of seawater, variables which influence phytoplankton growth and the toxin-accumulation capacity of the mollusk ([Webb et al., 2013](#)). Toxicity levels vary among bivalve species due to the differences in the toxin components retained and the purification/purge/filtering rate, given that some species rapidly toxins, while others take longer to detoxify themselves ([Deeds et al., 2008](#)). The bioassays conducted on the muscle of the oyster *Crassostrea virginica* did not obtain positive results, nor did they exceed the legally permissible limits of 80 µg STX, given that the times elapsed since death were longer than an hour. This contrasts with that described by [Ortiz-Castro \(2013\)](#), who report elevated concentrations of said toxin that range from 163 to 292 µg STX eq/100 g in *Choromytilus chorus* tissue obtained from natural banks. [Seguel & Sfeir \(2010\)](#) report low concentrations of 30 µg STX eq 100 g<sup>-1</sup> and a highest limit of 96 µg STX eq. 100 g<sup>-1</sup> via the use of the same mouse bioassay technique, observing elevated PSP concentrations due to the absence of algae bloom in the study area and an insufficient presence of the species of interest to find high levels of the toxin. While the mouse bioassay revealed little information on the toxin profile, it did obtain the total toxicity of the oyster muscle sample used. In light of the ethical pressures over the



use of live animals in scientific research, the present study details findings obtained via alternative analytical methods ([Hartung, 2010](#)).

Spirolides, gymnodimines, and pectenotoxins were the lipophilic toxins detected via LC-MS/MS in the oyster *C. virginica* for Tampamachoco Lagoon, while, for Tamihua Lagoon, spiroolides, gymnodimines, pectenotoxins, and domoic acid were observed via the same method. In general, low concentrations of the toxins spiroolides, gymnodimines, and domoic acid were detected, levels which did not reach the concentrations stipulated by NOM 059, with the presence of the species that produce them not found throughout the sampling period. To date, the present study constitutes the first report on the detection of the presence of marine biotoxins in the muscle of the oyster *Crassostrea virginica* in the Gulf of Mexico. Although the levels found in the muscle are relatively low, the possibility of finding higher concentrations in future studies cannot be excluded.

## CONCLUSIONS

The present study found the absence of paralyzing shellfish poison (PSP) in mollusks, while finding, at low levels, the presence of other marine biotoxins, such as domoic acid, spiroolides, gymnodimines, and pectenotoxins. The optical microscope techniques applied by the present research on the seawater samples studied were not able to identify the genera that produce the marine biotoxins *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. The mouse bioassays conducted on the oyster muscles sampled were not able to identify the presence of PSP. The absence of PSP was proven via the use of Scotia Rapid Testing, while the application of liquid chromatography coupled with tandem mass spectrometry revealed the presence of domoic acid, spiroolides, gymnodimines, and pectenotoxins in each lagoon and oyster bank studied. The values obtained for the marine biotoxins of interest were lower than the maximum permissible values established in NOM-242-SSA1. The presence of these compounds in Tampamachoco Lagoon and Tamiahua Lagoon, as indicated by the presence of amnesic toxin (domoic acid) in the mollusk samples obtained at the two sites, is a notable finding and is registered as the first report of the presence of this substance at these latitudes. The present study also identified the presence of pectenotoxins, spiroolides, and gymnodimines, with, to date, no studies having been undertaken to establish their toxicity in humans. However, this finding requires careful examination by future studies directly focusing on the availability of the corresponding toxin reference standards.



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