

Research Article

***Perkinsus marinus* in the pleasure oyster *Crassostrea corteziensis* cultivated on the southeast coast of the Gulf of California, Mexico**

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ABSTRACT. The protozoan *Perkinsus marinus* has been associated with high mortality episodes of the eastern oyster *Crassostrea virginica* in the USA. The presence of *P. marinus* on the pleasure oyster *Crassostrea corteziensis* cultivated in two estuaries on the southeast coast of the Gulf of California was evaluated. Oysters were collected monthly (September 2016 to September 2017) and analyzed using Ray's fluid thioglycollate medium (RFTM) and polymerase chain reaction (PCR). Water parameters and oyster biometrics were also recorded. Pathogen prevalence increased over time from 0 to 100% in oysters from La Pitahaya, and from 0 to 83.33% in those from Bacorehuis. At both oyster farms, infection intensity was light ($<1 \times 10^4$ parasites g⁻¹ wet tissue), pathogen prevalence and infection intensity were correlated with oyster size and weight, and there was a strong correlation between *P. marinus* prevalence and intensity (La Pitahaya $r = 0.91$; Bacorehuis $r = 0.82$). The oysters that resulted positive for *P. marinus* by RFTM also were assayed using PCR. *P. marinus* presence was confirmed in 98.27% (114/116) of the oysters from La Pitahaya, and 95.83% (46/48) of those from Bacorehuis. The detection of *P. marinus* confirms that this pathogen is well established in the area with high expression during the warmer season. Despite the light infection intensity of this parasite at both sites, health surveillance of this bivalve in the region is highly advisable.

Keywords: *Perkinsus marinus*; *Crassostrea corteziensis*; pathogen; diseases; prevalence; infection intensity; Gulf of California

INTRODUCTION

Diseases of farmed mollusks cost billions of dollars each year, and the movement of exotic pathogens to new culture areas is one of the most important factors for disease dispersion (Lafferty *et al.*, 2015). As in many countries (Soletchnick *et al.*, 2002; Langdon *et al.*, 2003; Taris *et al.*, 2007), oyster production in northwestern Mexico is based on the Japanese oyster (*Crassostrea gigas*) (Chávez-Villalba, 2014). This bi-

valve was first introduced in the 1970s on the Pacific coast of the Baja California Peninsula (Isla-Olivares, 1975), and later in the Gulf of California. Unusual episodes of high mortality in *C. gigas* associated with environmental factors (Cáceres-Martínez *et al.*, 2018), poor culture management (Cáceres-Martínez & Vásquez-Yeomans, 2013), and pathogens (Cáceres-Martínez, 2000) have been reported since 1997 (Vásquez-Yeomans *et al.*, 2004a). Therefore, the culture of the pleasure oyster *Crassostrea corteziensis*,

a native species from the Gulf of California, was suggested as an alternative to compensate for *C. gigas* losses (Chávez-Villalba *et al.*, 2005). However, concerns about *C. gigas* mortality related, for example, to the presence of parasites have recently extended to other bivalve mollusk species in the region, including *C. corteziensis* (Cáceres-Martínez *et al.*, 2016).

Different oyster species are affected by a variety of symbionts and pathogens that may cause some external and internal sings, including shell perforations (Gallo-García *et al.*, 2004) and damage to internal tissues (Vásquez-Yeomans, 2006; Aguirre-Macedo *et al.*, 2007; Cáceres-Martínez *et al.*, 2010). In other cases, high mortality at different oyster ages has been detected (Chávez-Romero *et al.*, 2011). Among oyster parasites reported in Mexico, Vásquez-Yeomans *et al.* (2004b) isolated bacteria in gill tissues from oysters collected during a mortality event in Baja California; Cáceres-Martínez & Vásquez-Yeomans (2013) reported the presence of a herpesvirus in *C. gigas* in Baja California, and Grijalva-Chon *et al.* (2013) detected a new type of ostreid herpes virus in *C. gigas* from the Gulf of California. Cáceres-Martínez *et al.* (2010) revealed the presence of Rickettsiales-like prokaryotes, turbellarians, encysted crustaceans, and ciliated protozoans in different tissues from *C. corteziensis* on the north-central coast of the Mexican Pacific. They concluded that the protozoan *Perkinsus marinus* represented the most significant risk to oyster health. The parasite presence in the Mexican Pacific derives from *C. virginica* mobilizations from the USA and the Gulf of Mexico. Cáceres-Martínez & Vásquez-Yeomans (2013) documented three records of *P. marinus* introductions in the Pacific coast: one from the Washington coast to the estuary Punta Banda, Baja California, in the 1970s; another from the transfer of oysters for commercial purposes from the Tamiahua Lagoon, Veracruz to Nayarit one decade later; and the last one by mobilization of the American oyster from Louisiana, USA, to San Jorge Bay, Sonora, to protect the oyster stock and production of the impact of Katrina hurricane, in 2005 (Cáceres-Martínez & Vásquez-Yeomans, 2013).

Different *Perkinsus*-like parasites have been reported in wild and cultivated bivalve species in and around the Gulf of California using different techniques given their presence, detection, confirmation, and infection degree. For instance, *P. marinus* was found in natural populations of *C. corteziensis* on the central coast of the Mexican Pacific (Cáceres-Martínez *et al.*, 2008) and the mangrove oyster *Saccostrea palmula* along the Sinaloa coast (Cáceres-Martínez *et al.*, 2012), as well as in the wild population of the black clam *Chione fluctifraga* in Kino Bay, Sonora (Enríquez-

Espinoza *et al.*, 2015). *Perkinsus* sp. has been diagnosed in a natural bank of the pen shell *Atrina maura* in Sinaloa (Góngora-Gómez *et al.*, 2016) and cultivated Japanese oysters along the coast of Sonora in the Gulf of California (Enríquez-Espinoza *et al.*, 2010) and on the north-central coast of Sinaloa (Villanueva-Fonseca & Escobedo-Bonilla, 2013). Cáceres-Martínez *et al.* (2016) determined the prevalence and intensity of *P. marinus* in two cultured populations of *C. corteziensis* in the state of Nayarit, Mexico, from 2007 to 2014 and pointed out that no unusual mortalities were related with the presence of the parasite.

The presence of *P. marinus* in pleasure oysters farmed off the north coast of Sinaloa, Mexico, remains unknown. Assessing the possible impact of this pathogen on *C. corteziensis* cultivated in this state is of paramount importance. Thus, this study's objective was to evaluate *P. marinus* in *C. corteziensis* at two oyster farms on the southeast coast of the Gulf of California.

MATERIALS AND METHODS

Oysters

Crassostrea corteziensis specimens were sampled at two oyster farms located in the Bacorehuis ($26^{\circ}06' - 26^{\circ}32'N$, $109^{\circ}01' - 109^{\circ}20'W$) and La Pitahaya ($25^{\circ}21' - 25^{\circ}24'N$, $108^{\circ}30' - 108^{\circ}45'W$) estuaries in northern Sinaloa, Mexico. According to the farmers' information, the oyster seed was produced at the Centro de Reproducción de Especies Marinas del Estado de Sonora (CREMES) of the Instituto de Acuacultura del Estado de Sonora, O.P.D., which were certified as parasites-free. The small oysters (4.5 mm size range) were transported to the cultural sites and acclimated following standard procedures (Gallo-García *et al.*, 2001). As traditionally performed in the region, oysters were cultured in a suspended long-line system, according to Villanueva-Fonseca *et al.* (2017). Thirty oysters were sampled monthly in both sites throughout a single culture cycle from September 2016 to September 2017 (13 months). The mean initial shell height (SH, major axis) and total weight (TW) were 4.35 ± 0.78 mm and 0.01 ± 0.004 g. The specimens were randomly collected from several oyster trays each month and transported live in 20 L plastic tanks for further analysis at the Centro Interdisciplinario de Investigaciones para el Desarrollo Integral Regional (IPN-CIIDIR) Unidad Sinaloa, Guasave, Sinaloa, Mexico. At each sampling, the oyster biometrics (SH and TW) and water parameters (temperature, salinity, dissolved oxygen, pH, transparency, depth, chlorophyll-a concentration, total suspended solids, and particulate organic and inorganic matters) were recorded in order to assess any possible correlation with parasite preva-

lence. The technical staff of each farm recorded survival according to their work routine.

Perkinsus marinus diagnosis

Once at the lab, oysters ($N = 30$ ind month $^{-1}$ site $^{-1}$) were immediately processed for *Perkinsus marinus* diagnosis. Fouling organisms and mud were removed from each oyster using a soft brush and a stream of freshwater. Next, specimens were opened, and the soft tissue divided into two subsamples. One subsample from each oyster was incubated in Ray's Fluid Thioglycollate Medium, RFTM (Ray, 1966; OIE, 2012) with the addition of antibiotics (penicillin and streptomycin) and an antifungal (nystatin) and incubated in the dark for 7 d at room temperature (23–25°C) to induce hypnospore formation; the other subsample was placed at -70°C for confirmatory PCR analysis. Subsequently, the tissues were digested (NaOH 2M, 60°C for 2–6 h) and rinsed with deionized water; next, they were placed on a slide, stained with 3% Lugol's solution, and observed under light microscopy (100x) for the presence of spherical, blue, or bluish-black *P. marinus* hypnospores. Samples with a high number of hypnospores were serially diluted to reduce the total cell number to a manageable number.

The number of hypnospores per gram of oyster tissue was calculated. The effect of *P. marinus* in the sampled oysters was evaluated based on the infection intensity and prevalence. The infection intensity was classified as negative (0 hypnospores), light ($<1 \times 10^4$ hypnospores g $^{-1}$), moderate (1×10^4 to 5×10^5 hypnospores g $^{-1}$), or heavy ($>5 \times 10^5$ hypnospores g $^{-1}$) (Bushek *et al.*, 1994). The pathogen prevalence was defined as the number of infected oysters in the sample and expressed as a percentage of the total number of oysters sampled (Thrusfield, 1995).

Specimens with a positive RFTM result were subjected to PCR. Total deoxyribonucleic acid (DNA) extraction was achieved using a DNAzol® kit following the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH). At the same time, PCR was performed using the primers PmarITS-70F (5'-CTT-TTG-YTW-GAG-WGT-TGC-GAG-ATG-3') and PmarITS-600R (5'-CGA-GTT-TGC-GAG-TAC-CTC-KAG-AG-3') that amplify a 509 of the ITS region of the *P. marinus* rRNA gene complex. The PCR mix was prepared as follows: 100 ng of DNA template; 1×PCR Buffer (10 mM Tris-HCl, pH 8.5, KCl 50 mM); MgCl₂ 1.5 mM; 0.2 mM of a mixture of dATP, dCTP, dGTP, and dTTP; 0.01 mM of each primer; and 1.5 U of proofreading *Taq* DNA polymerase (Bioline, Boston, MA, USA) in a final volume of 25 µL. Amplification conditions were: an initial denaturation step of 95°C for 5 min followed by 35 cycles of 94°C

for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final elongation of 72°C for 5 min (Audemard *et al.*, 2004). Also included in the reactions were a positive control (total genomic DNA of *P. marinus* infected tissue from *C. corteziensis*) and negative control in which sterile water was added instead of the template. All PCR analyses were performed in triplicate. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide (1 µg mL $^{-1}$), and visualized using ultraviolet light.

Statistical analysis

All data were tested for normality (Lilliefors test) and heteroscedasticity (Bartlett's test) to select appropriate statistical analyses. Prevalence values were arcsine transformed before analysis. The differences in the number of hypnospores per gram and the parasite prevalence in the oysters were compared between the two sites every month using a Mann-Whitney U test and a paired-sample t-test, respectively. Spearman's rank correlation was used to relate the infection intensity and prevalence data with the oyster biometrics and environmental parameters at the two oyster farms. All statistical analyses were performed using Statistica (Statsoft Inc., Tulsa, OK, USA). The significance level was set to $\alpha = 0.05$ for all tests.

RESULTS

Growth and mortality of oysters and environmental data

After one year of culture, the SH and TW of *Crassostrea corteziensis* from the La Pitahaya and Bacorehuis oyster farms were 62.74 ± 3.74 and 68.82 ± 6.77 mm and 35.09 ± 4.94 and 38.75 ± 11.27 g, respectively. The daily length and weight growth rate were 0.14 mm d^{-1} and 0.08 g d^{-1} in La Pitahaya and 0.18 mm d^{-1} and 0.09 g d^{-1} in Bacorehuis. The mortality was recorded during the autumn and winter seasons, from October 2016 to February 2017 at La Pitahaya and from November 2016 to January 2017 at Bacorehuis. Final survival was 64.43 and 75.56% for La Pitahaya and Bacorehuis farms, respectively. The mean water parameters for the La Pitahaya and Bacorehuis estuaries are shown in Table 1.

Perkinsus marinus diagnosis

The RFTM test shows that out of 390 oysters analyzed 116 (29.74%) and 48 (12.31%) from the La Pitahaya and Bacorehuis estuaries, respectively, tested positive for presumptive *Perkinsus marinus* hypnospores by RFTM, which showed spherical cells (5–70 µm diameter) stained black or bluish-black with Lugol's solution (Fig. 1).

Table 1. Mean water parameters in the La Pitahaya and Bacorehuis estuaries during 13 cultivation months (September 2016-September 2017).

	La Pitahaya	Bacorehuis
Temperature (°C)	27.64 ± 4.97	27.90 ± 4.18
Salinity	33.61 ± 2.18	32.38 ± 4.55
Dissolved oxygen (mg L ⁻¹)	5.84 ± 0.93	5.49 ± 1.98
pH	7.03 ± 0.84	7.10 ± 0.89
Transparency (m)	0.64 ± 0.29	0.53 ± 0.32
Depth (m)	1.45 ± 0.45	0.86 ± 0.30
Chlorophyll-a (mg m ⁻³)	6.70 ± 4.63	4.41 ± 3.35
Total suspended solids (mg L ⁻¹)	40.71 ± 12.68	37.15 ± 30.78
Particulate organic matter (mg L ⁻¹)	19.63 ± 2.38	19.68 ± 4.65
Particulate inorganic matter (mg L ⁻¹)	29.78 ± 10.55	27.46 ± 26.75

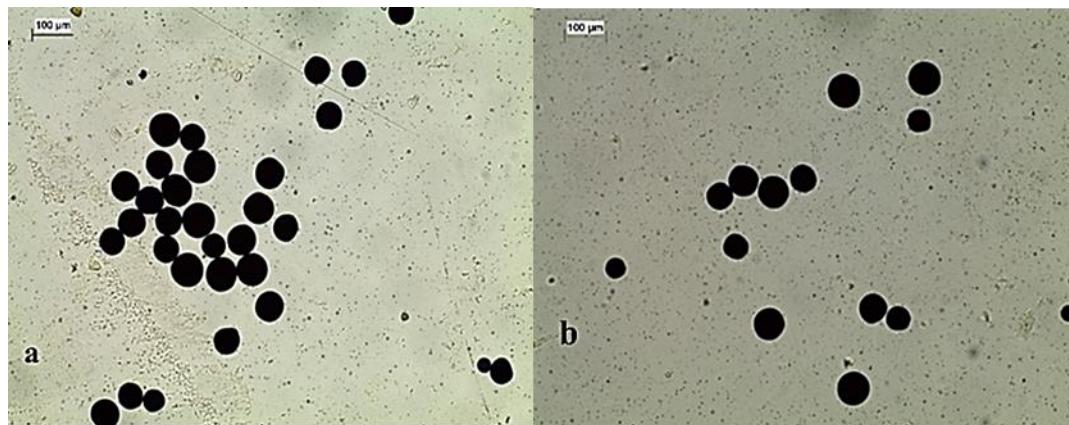


Figure 1. Detection of presumptive *Perkinsus marinus* hypnospores in *Crassostrea corteziensis* from a) La Pitahaya and b) Bacorehuis estuaries with Ray's fluid thioglycolate medium (RFTM) (100x).

Both the prevalence and the infection intensity of *P. marinus* in *C. corteziensis* at both oyster farms increased over time (Fig. 2).

For La Pitahaya, *P. marinus* was not detected (September-November 2016 and February 2017) or occurred with a low prevalence (December 2016, March-May 2017). Prevalence increased from June to September 2017, reaching 100% in the last two months. For Bacorehuis, the parasite was not detected from October to December 2016 and February 2017 or occurred with a low prevalence (January, March-July 2017). Prevalence increased in August, reaching 83.33% in the last month. At the end of the culture cycle, the number of hypnospores per g of wet tissue (wt) of the oysters cultivated at the La Pitahaya farm reached 9,242 hypnospores g⁻¹ wt, approximately three times that found in the oysters from the Bacorehuis farm (3,226 hypnospores g⁻¹ wt). The infection intensity was light at both oyster farms (<1×10⁴ parasites g⁻¹). Between sites, the prevalence was similar

($t = 1.37$, $P = 0.18$) but the parasite load was significantly different ($W = 8.33$, $P = 0.0008$). Table 2 shows the correlations between the prevalence and intensity of infection with oyster measurements and physicochemical parameters. The infection prevalence was strongly correlated with the infection intensity (La Pitahaya $r = 0.91$; Bacorehuis $r = 0.82$).

The specimens testing positive for *Perkinsus* by RFTM were subjected to *P. marinus* species identification by PCR analysis. At La Pitahaya and Bacorehuis, 114/116 (98.3%) and 46/48 (95.8%) cases were confirmed, respectively (Fig. 3).

DISCUSSION

Cáceres-Martínez *et al.* (2008, 2010, 2012) and Martínez-García *et al.* (2017) reported the occurrence of *Perkinsus marinus* in cultivated and wild bivalves along the north-central coast of the Mexican Pacific, suggesting a possible horizontal transmission of the pro-

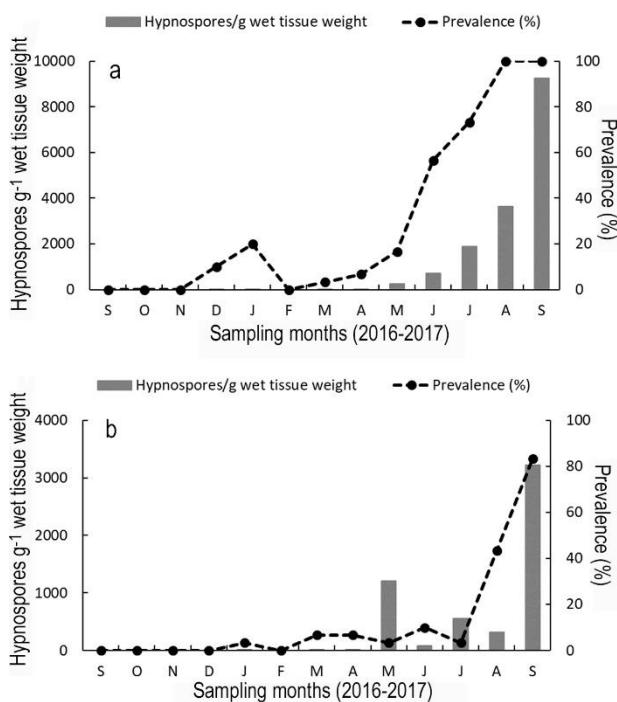


Figure 2. Intensity (hypnospores g^{-1} wet tissue weight) and prevalence of the infection of *Perkinsus marinus* in *C. corteziensis* in a) La Pitahaya and b) Bacorehuis estuaries.

protozoan in the zone. In the present study, the protozoan *P. marinus* was detected in *Crassostrea corteziensis* from both oyster farms by the presence of characteristic round bluish-black hypnospores by RFTM assay (OIE, 2003). Specimens with positive RFTM assay results were subsequently subjected to confirmatory PCR analysis. Oyster mortality at both sites was not consistent with the prevalence throughout the culture cycle. Mortality from October 2016 to February 2017 was recorded at low or null infection; meanwhile, the infection intensity increased from May 2017 onwards, coinciding with the increase in water temperature. The present study coincided with the dynamics of *Perkinsus* described for Cáceres-Martínez *et al.* (2016), that reported an established pattern of the prevalence and intensity of *Perkinsus* infection in the Nayarit coast, showing low values from late autumn to early spring, while the highest values reported were in late summer and early autumn values; these fluctuations are related to the temperature and salinity of the area. However, no correlation was found between the environmental variables with prevalence and infection intensity of *P. marinus* in both localities. The absence of a direct relationship with the temperature may reflect the time required by the parasite to multiply in the host in response to changing environmental conditions (Burreson & Ragone-Calvo, 1996). Therefore, oyster mortality cannot be attributed to the presence of presump-

Table 2. Spearman's rank-order correlations between prevalence and intensity of *Perkinsus marinus* infection, with oyster measurements and environmental parameters for *Crassostrea corteziensis* cultivated in the La Pitahaya and Bacorehuis farms, Sinaloa, Mexico. *r*: correlation, *P*: p-value, SL: shell length, SH: shell height, SW: shell weight, BW: body weight, DO: dissolved oxygen. Only significant correlations ($P < 0.05$) are shown.

	La Pitahaya		Bacorehuis	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Prevalence vs. SL	0.744	0.0099	0.794	0.0059
Prevalence vs. SH	0.845	0.0034	0.828	0.0041
Prevalence vs. SW	0.806	0.0052	0.856	0.0030
Prevalence vs. BW	0.867	0.0027	0.822	0.0044
Intensity vs. SL	0.810	0.0050	0.909	0.0016
Intensity vs. SH	0.908	0.0017	0.842	0.0035
Intensity vs. SW	0.885	0.0021	0.893	0.0020
Intensity vs. BW	0.919	0.0014	0.881	0.0023
Prevalence vs. DO			0.725	0.0119
Intensity vs. DO			0.638	0.0269

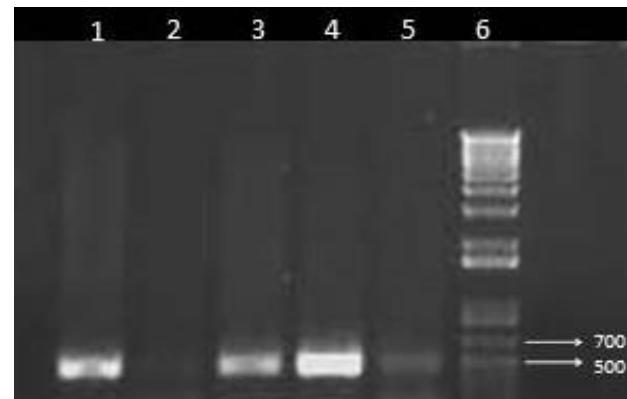


Figure 3. Agarose gel electrophoresis of PCR products. Lanes: 1) *Perkinsus marinus* (positive control), 2) sterile water (negative control), 3-4) fresh tissue (La Pitahaya), 5) fresh tissue (Bacorehuis), 6) molecular weight markers (1 Kb ladder).

tive hypnospores of *P. marinus*; instead, it could partially be explained by other aspects such as management and genetics.

Oyster growth and environmental parameters were similar at both sites. Only the *C. corteziensis* shell height and body weight were correlated with prevalence and intensity. At both locations, the infection prevalence and intensity increased with culture time, when oysters reached the highest growth values. Nevertheless, oysters were exposed to the parasite for only one year that can be a short time to favor the development of severe infections and mortalities, compared to the high mortalities of the eastern oyster *C. virginica* in the second year of cultivation registered on USA farms, attributed to *P.*

marinus (Cáceres-Martínez & Vásquez-Yeomans, 2013; Cáceres-Martínez *et al.*, 2016). According to Soniat (1996), the low levels of prevalence and intensity of *P. marinus* in young oysters can be explained because their cell division rate is similar to *Perkinsus*. While they are adults, their growth rate decreases, and the parasite can reach higher infection intensity, which is consistent with this work, because bigger oysters (>50 mm shell height) presented the highest prevalence and infection intensity of *P. marinus*. Contrary findings for other cultivated oyster species are reported by Enríquez-Espinoza *et al.* (2015) and da Silva *et al.* (2016), who found that infection rates were independent of oysters' size. According to Paynter *et al.* (2010) and Ehrich & Harris (2015), larger oysters have a larger gill area that consequently leads to a higher filtration rate favoring infection through the increased ingestion of parasite cells, which could explain our results. It is also possible that larger oysters may have been infected at earlier stages, and the parasite had more time to proliferate within the host, as well as the fact that the older the oysters are, their growth rate decrease favoring the parasite proliferation; questions that deserve further research. The mean prevalence of *P. marinus* infecting *C. corteziensis* ranged from moderate (29.74% La Pitahaya) to low (19.30% Bacorehuis) by RFTM; the infection prevalence exceeded 80% at the end of the culture cycle. Although the MFTR staining technique does not discriminate between species of the genus *Perkinsus* spp., it is cheap, simple (OIE, 2012), more sensitive when compared with histology (McLaughlin & Faisal, 1999) and the infection data collected may be counted (Auderman *et al.*, 2008) and categorized on a scale (Mackin, 1962; Bushek *et al.*, 1994). Also, RFTM is considered a reliable preliminary method for carrying out subsequent molecular assays (such as PCR) in processed tissues in helping to confirm parasite's species and, as in this case, to explain better the prevalence of *P. marinus* infection in *C. corteziensis*.

The results of the molecular analysis (PCR) for the positive RFTM specimens indicated parasite incidence exceeding 95%; this is comparable with the PCR results reported by da Silva *et al.* (2016) in three month old *Crassostrea gasar* in polyculture with shrimp but higher than that reported for cultivated *C. gigas* (Villanueva-Fonseca & Escobedo-Bonilla, 2013) and wild pen shell *Atrina maura* (Góngora-Gómez *et al.*, 2016) from locations near the La Pitahaya Estuary. These differences may be due to several factors, such as the low prevalence by RFTM and the light intensity levels obtained in the present assay and the susceptibility of other species to *P. marinus* (Calvo *et al.*, 1999). Moreover, the sample size used for parasite

detection combined with the low infection intensities (Reece *et al.*, 2008; Sabry *et al.*, 2009; Dantas-Neto *et al.*, 2015) may have affected the output results. Finally, the different environmental conditions at each place also contribute to determining the host-parasite interaction (Villalba *et al.*, 2004).

Of particular interest is the low infection level obtained for this oyster species on the north-central coast of Sinaloa, which coincides with the conclusions obtained by Cáceres-Martínez *et al.* (2012), Villanueva-Fonseca & Escobedo-Bonilla (2013), and Góngora-Gómez *et al.* (2016), who reported low infection by the same protozoan in other bivalve species in the region. While the production of commercially important bivalve species has not been affected significantly by *Perkinsus* sp. due to their low susceptibility to this pathogen, it nonetheless represents a potential disease vector for other mollusk species in the region (Villalba *et al.*, 2004). Therefore, sanitary measures to control the movement of bivalves from one culture site to another, and continuous monitoring of the protozoan in both cultivated and wild populations of bivalve mollusks should be implemented.

CONCLUSIONS

The infection intensity observed in this study was low. The presence of *Perkinsus marinus* in the north-central coast of Sinaloa confirms that this pathogen is well established in the area with high expression during the warmer season, as concluded by Cáceres-Martínez *et al.* (2016) for the same oyster species in natural populations of the state of Nayarit. Although no *Perkinsus*-related mortality has been reported for the north of the Sinaloa state, health surveillance of bivalve populations in the region is highly advisable, particularly for the native oyster *Crassostrea corteziensis*, which is an important component of the oyster industry.

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