UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA
FACULTAD DE CIENCIAS MARINAS
INSTITUTO DE INVESTIGACIONES OCEANOLÓGICAS
POSGRADO EN ECOLOGÍA MOLECULAR Y BIOTECNOLOGÍA

EVALUACIÓN DE *Candidatus* Xenohaliotis californiensis (*C*Xc) Y SU FAGO ASOCIADO p*C*Xc EN ABULÓN NEGRO DE BAJA CALIFORNIA

T E S I S

QUE PARA CUBRIR PARCIALMENTE LOS REQUISITOS NECESARIOS PARA OBTENER EL GRADO DE

MAESTRO EN CIENCIAS EN ECOLOGÍA MOLECULAR Y BIOTECNOLOGÍA

PRESENTA

CASANDRA DELGADILLO ANGUIANO

ENSENADA, BAJA CALIFORNIA A NOVIEMBRE DE 2021

PRÓLOGO

El presente escrito es la tesis desarrollada, en formato de borrador para artículo científico, de la estudiante **Casandra Delgadillo Anguiano**, durante el período de agosto de 2018 a noviembre de 2021, para cubrir parcialmente los requisitos de la **Maestría en Ciencias** del **Posgrado en Ecología Molecular y Biotecnología** de la **Universidad Autónoma de Baja California**.

La intención final del presente trabajo es someterlo a la revista de investigación: **Diseases of Aquatic Organisms**, cuyo factor de impacto es de **1.802** y, que sea revisado por pares para que eventualmente sea publicado.

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Resumen aprobado por: _____

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Las poblaciones naturales de abulón negro, *Haliotis cracherodii*, de California, EUA y Baja California, México, han enfrentado descensos masivos por más de treinta años. Los principales factores que se han considerado son sobrepesca, contaminación, cambio climático y enfermedades letales, como el síndrome de deshidratación (SD), causado por la bacteria intracelular *Candidatus* Xenohaliotis californiensis (*C*Xc). En años recientes, la presencia de un bacteriófago asociado, pCXc, infectando CXc fue detectado y reportado en algunas de las especies de abulón de California y Baja California. En el presente estudio, se analizaron 199 muestras de heces de abulón negro de 14 sitios a lo largo de la costa de Baja California, para determinar la presencia de *C*Xc y su posible infección con p*C*Xc. Encontramos que la prevalencia de la bacteria y de la bacteria infectada con el fago fue de 44% y 38% respectivamente. Encontramos también diferencias significativas en la relación entre la prevalencia y el promedio de longitudes de los abulones a lo largo de las zonas de estudio. Este resultado probablemente se deba a diferencias en la distribución de frecuentas de longitudes entre zonas. El único sitio que encontramos que la relación entre prevalencia y la longitud del abulón fue significativa fue en la zona 2, Isla Todos Santos, probablemente debido a que en esta zona se encontraron los abulones con mayores longitudes. También, detectamos que la prevalencia de la bacteria y el fago, tanto individualmente como en conjunto, no está relacionada con la densidad poblacional de los abulones presentes en el área de estudio. Adicionalmente, encontramos una correlación positiva en la prevalencia entre *C*Xc y p*C*Xc, sin embargo, no fue significativa. Finalmente, este estudio es el primero en detectar la presencia de *C*Xc infectado por p*C*Xc, en las poblaciones silvestres de abulón negro presente en Baja California.

Palabras clave: *Haliotis cracherodii*, abulón negro, *Candidatus* Xenohaliotis californiensis, *C*Xc, bacteriófago, p*C*Xc, Síndrome de Deshidratación

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Evaluation of *Candidatus* Xenohaliotis californiensis (*C*Xc) and its associated phage p*C*Xc in black abalone of Baja California

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Key words: *Haliotis cracherodii*, Black abalone, *Candidatus* Xenohaliotis californiensis, *C*Xc, bacteriophage, p*C*Xc, Withering Syndrome

Abstract

Natural populations of black abalone, *Haliotis cracherodii*, from California, USA and Baja California, Mexico, have faced massive declines for more than thirty years. The main factors that have been considered are overfishing, pollution, climate change and lethal diseases, such as withering syndrome (WS), caused by the intracellular bacterium *Candidatus* Xenohaliotis californiensis (*C*Xc). In recent years, the presence of an associated bacteriophage, p*C*Xc, infecting *C*Xc was detected and reported in some of the abalone species from California and Baja California. In the present study, 199 black abalone fecal samples from 14 sites along the Baja California coast were analyzed for the presence of *C*Xc and its possible infection with p*C*Xc. We found that the prevalence of bacteria and phage-infected bacteria was 44% and 38% respectively. We also found significant differences in the relationship between the prevalence and the mean lengths of abalones throughout the study areas. This result is probably due to differences in the frequency distribution of lengths between zones. The only site where we found that the relationship between prevalence and length of abalone was significant was in zone 2, Isla Todos Santos, probably due to the fact that abalones with the longest lengths were found in this zone. Also, we detected that the prevalence of the bacteria and the phage, both individually and together, is not related to the population density of the abalones present in the study area. Additionally, we found a positive correlation in the prevalence between *C*Xc and p*C*Xc, however, it was not significant. Finally, this study is the first to detect the presence of *C*Xc infected by p*C*Xc, in the wild populations of black abalone present in Baja California.

1. INTRODUCTION

Over the past three decades, the exposure to stressors such as pollution, overfishing, habitat degradation and infectious diseases have caused massive declines in wild abalone (*Haliotis* spp.) populations of California, USA and Baja California, Mexico (Hamm & Burton, 2000). However, current mass mortality events related to extreme environmental events, are expected to increase due climate change, temperature pikes or prolonged hypoxic conditions (Lonhart et al. 2019). Historically, these massive decreases first occurred in the fished species such as red, *H. rufescens*, green, *H. fulgens*, and pink, *H. corrugata*, abalone (González-Avilés & Ortiz-Quintanilla 1986). Then, the development of a commercial fishery for species considered less desirable such as the black abalone, *H. cracherodii*, followed (Neumann et al. 2010, Altstatt et al. 1996). However, massive declines beginning in 1986 were observed, and 98% of the once-abundant black abalone populations disappeared in many sites across California (Lafferty & Kuris 1993, Altstatt et al. 1996). These declines were initially attributed to starvation and high temperatures caused by the 1982-1983 El Niño-Southern Oscillation (ENSO) (Burge et al. 2016), but over time it was suggested an infectious disease was the cause (Burge et al. 2016, Valles-Rios 2000) as originally suggested by Lafferty and Kuris (1993). As a result of these declines, in 1999 the black abalone was included as Candidate Species for protection by the National Marine Fisheries Service and listed as Endangered in the US Endangered Species Act in 2009 (Neumann et al. 2010, VanBlaricom et al. 2009).

In Mexico, abalone commercial fishing began around 1860 and became one of the most important activities of the peninsula for over 50 years (León-Carballo & Muciño-

Díaz 1996, Sierra-Rodríguez et al. 2006). The first abalone fishing cooperative was established in 1940. Although there are sporadic records of black abalone catches around 1922, commercialization in Baja California began around the 1950s and reached a peak during the 1970s (Sierra-Rodríguez et al. 2006, VanBlaricom et al. 2009). Around the 1980s, high mortalities that affected farms and fisheries were reported after temperature rises resulting from the ENSO event of 1983-84 (Cáceres-Martínez 2002). Currently, black abalone in Mexico has practically vanished from areas where they were once abundant, such as Isla de Cedros and Islas San Benito, and is no longer considered for commercial fishing (Cáceres-Martínez 2002). In 1996 an agreement to stop black abalone catches in Baja California was stablished, however, a small fishery continued in some areas, such is the case of Isla Guadalupe, where black abalone represented 0.2% of the total catches in 2006 (Sierra-Rodríguez et al. 2006).

The actual cause of these massive mortalities remains unknown and could be attributed to overexploitation, climatic changes, diseases, or a combination of factors (Cáceres-Martínez 2002). One disease affecting abalone is the withering syndrome (WS), caused by the bacterium *Candidatus* Xenohaliotis californiensis (*C*Xc) (Crosson et al. 2014). This bacterium was first detected in 1985 in black abalone of Santa Cruz Island, California, after the 1983-84 ENSO event and was considered the etiological agent responsible for the chronical and lethal disease (VanBlaricom et al. 2009, Cruz-Flores et al. 2016). This pathogen is an obligated intracellular bacterium that infects the epithelial cells of the gastrointestinal track and posterior esophagus of abalone, although can spread to the stomach, digestive diverticula and rarely to the intestines (Brokordt et al. 2016, Cruz-Flores et al. 2016). Also, *C*Xc

causes morphological abnormalities in the digestive gland leading the organism to starvation, anorexia, lethargy, and death (Neumann et al. 2010, Crosson et al 2014). Apparently, *C*Xc is capable of survival outside its host indeterminately, however, it cannot be cultured under laboratory conditions, due its obligated intracellular nature. Therefore, detection and diagnosis depend mainly on histological analysis and/or molecular techniques (Valles-Ríos 2000, OIE 2009, Friedman et al. 2014, Muñoz-Flores 2014).

Bacterium *C*Xc is considered an endemic pathogen of Southern California and Baja California (Cruz-Flores et al. 2016). Its presence has been reported in red, pink, green, black, white, *H. sorenseni*, and flat, *H. walallensis*, abalone (Valles-Ríos 1998, Cruz-Flores 2017). Moreover, *C*Xc has also been introduced to other countries, such as China, Japan, Thailand, Taipei, Chile, Iceland, Spain, and Ireland, by transport of infected red abalone (*H. rufescens*) to culture centers (OIE 2009, Brokordt et al. 2016). Epidemic events of *C*Xc have been associated with high-water temperatures, which increase abalone mortality rates (Friedman et al. 2014, Ben-Horin et al. 2013). However, some abalone species seem less affected than others (Brokordt et al. 2017). Particularly, black abalone is very susceptible to WS, but the main reason remains unknown (Ben-Horin et al. 2013).

In addition, recent studies have detected the presence of a bacteriophage associated to *C*Xc, named p*C*Xc, in red and black abalone from California (Cruz-Flores et al. 2018, Moore et al. 2019), as well as red, blue, and pink abalone from Baja California, Mexico (Cruz-Flores et al. 2016). This suggests that both *C*Xc and p*C*Xc are well established in the area and could be considered endemic pathogens of the region (Cruz-Flores et al. 2016, Cáceres-Martínez et al. 2020). It also appears

that, when p*C*Xc is present, there is a decrease in the pathogenicity of *C*Xc (Moore et al. 2009, Cruz-Flores et al. 2016, Cruz-Flores 2017).

Previous studies (Table 1) on different abalone species from California and Baja California, have detected the prevalence of both *C*Xc and p*C*Xc through histological and genomic techniques. In Baja California, *C*Xc has been detected in black abalone only using histological methods (Valles-Rios 2000). Even though there are not official records of population sizes and health status of black abalone in Baja California, recent monitoring in some localities of the peninsula show a clear sign of population recovery and no signs of the disease (Cepeda-Ochoa 2019). Therefore, the aim of this study was to determine the prevalence of both *C*Xc and its associated phage hyperparasite, p*C*Xc, in wild populations of black abalone of northern Baja California, Mexico using molecular methods. We also evaluated differences on prevalence between zones, abalone length and abalone abundances.

Table 1

Previous studies using different methods to detect the prevalence of *C*Xc infected by p*C*Xc in abalone species present in California, Baja California and introduced in Chile**.**

Method	Abalone species	Reference
• Histology • PCR amplification with 16S rDNA gene (160 bp) • In situ hybridization	12 farmed red abalones (*H. rufescens*) from California. First observation of the infection of *C*Xc with p*C*Xc in abalone.	Friedman & Crosson 2012
• Histology • PCR amplification with 16S rDNA gene (160 bp)	159 farmed green abalones (*H. fulgens*) and 160 farmed pink abalones (*H. corrugata*) from Baja California. Prevalence of *C*Xc in green was 95.4% and 65.5% in pink abalone. Prevalence of p*C*Xc infecting *C*Xc was 96% in green and 100% in pink abalone.	Cruz-Flores 2013
• Histology • qPCR amplification with 16S rDNA gene Gene copy numbers: - per gram of sample with fecal and tissue samples - per ng of DNA extracted from filtered water	215 wild black abalones (*H. cracherodii*) from different sites along the shore of California and 90 farmed red abalones (30 infected with *C*Xc and 60 non-infected). Mortalities of abalone with *C*XC infected with p*C*Xc were lower than those that presented only *C*Xc. San Nicholas Island organisms showed an increased survivorship to the infection with *C*Xc.	Friedman et al. 2014
• Histology	125 farmed red abalones (50 infected with *C*Xc and 75 non-infected), 75 non-infected and farmed japanese abalone (*H. discus hannai*), and 75 farmed hybrids between red and Japanese species non-infected in Chile. Prevalence of *C*Xc was 33% and 20% in red and hybrids, respectively. Japanese abalones did not present the bacteria. Prevalence of p*C*Xc phage was 90% in red and 65% in hybrids.	González et al. 2014
• Histology • PCR amplification with 16S rRNA gene (160 bp)	182 wild green and 170 wild pink abalones from 7 localities in Baja California. Prevalence of *C*Xc was 80% in green and 62% in pink abalone. The prevalence of p*C*Xc was 22% in green and 31% in pink abalone. Both bacteria and phage were present in the 7 localities where the abalones were captured.	Cruz-Flores & Cáceres-Martínez 2016
• Histology	3 farmed red abalones and 2 wild pink abalones from Baja California. Prevalence of both *C*Xc and p*C*Xc was found in both abalone species.	Cruz-Flores et al. 2016
• Histology	473 farmed red abalones from 3 different farms from Chile. 65% of the organisms presented both *C*Xc and p*C*Xc and it was found that some families were less susceptive to the infection from both pathogens.	Brokordt et al. 2017
• Histology • qPCR amplification with 16S rRNA gene	192 farmed red and 192 farmed white abalones (*H. sorenseni*) from California. Prevalence of *C*Xc and p*C*Xc in both abalone species, with mortalities of 10% in red abalone and 100% in white.	Vater et al. 2017
• PCR amplification with thymidylate kinase gene and ORF 24 (412 bp)	1 farmed red abalone and 1 wild pink abalone from Baja California. Primer design specific to detect p*C*Xc and is the first genome sequence reported for this phage with a size of 35,728 bp in red and 35,736 bp in pink abalone.	Cruz-Flores 2018
• Histology	251 farmed red abalone from an aquaculture facility in Baja California, presumably exclusively with *C*Xc. Prevalence of only *C*Xc was 81% and infected by p*C*Xc was 16%.	Cáceres-Martínez et al. 2020

2. MATERIALS & METHODS

2.1 Study site and sample collection

Between spring 2018 and summer 2019, a total of 236 wild black abalone from 14 sites were sampled throughout the coast of northern Baja California, Mexico (Fig. 1). The sampling sites were divided into six zones by proximity and sample size: Zone 1 (n= 78): PBA, FOX, BJM, SAL; Zone 2 (n= 24): ITS; Zone 3 (n= 31): BUF, CKN; Zone 4 (n= 32): BCN, BOC, ERE, PBJ; Zone 5 (n=30): CUC, LOL, and Zone 6 (n= 4): ISG (Table 2). We excluded zone 6 (Isla Guadalupe) from further analysis due to the small sample size.

A random sampling was performed during low tide conditions in the rocky intertidal zone. Fecal samples were obtained using a semi-intrusive non-lethal sampling method consisting in scraping near the anus with a sterile nylon swab (Copan Diagnostics Nylon Flocked Dry Swabs). The swab was then preserved in 95% ethanol at -20°C until DNA extraction. Also, the total length in centimeters of all abalone was recorded (Table 2).

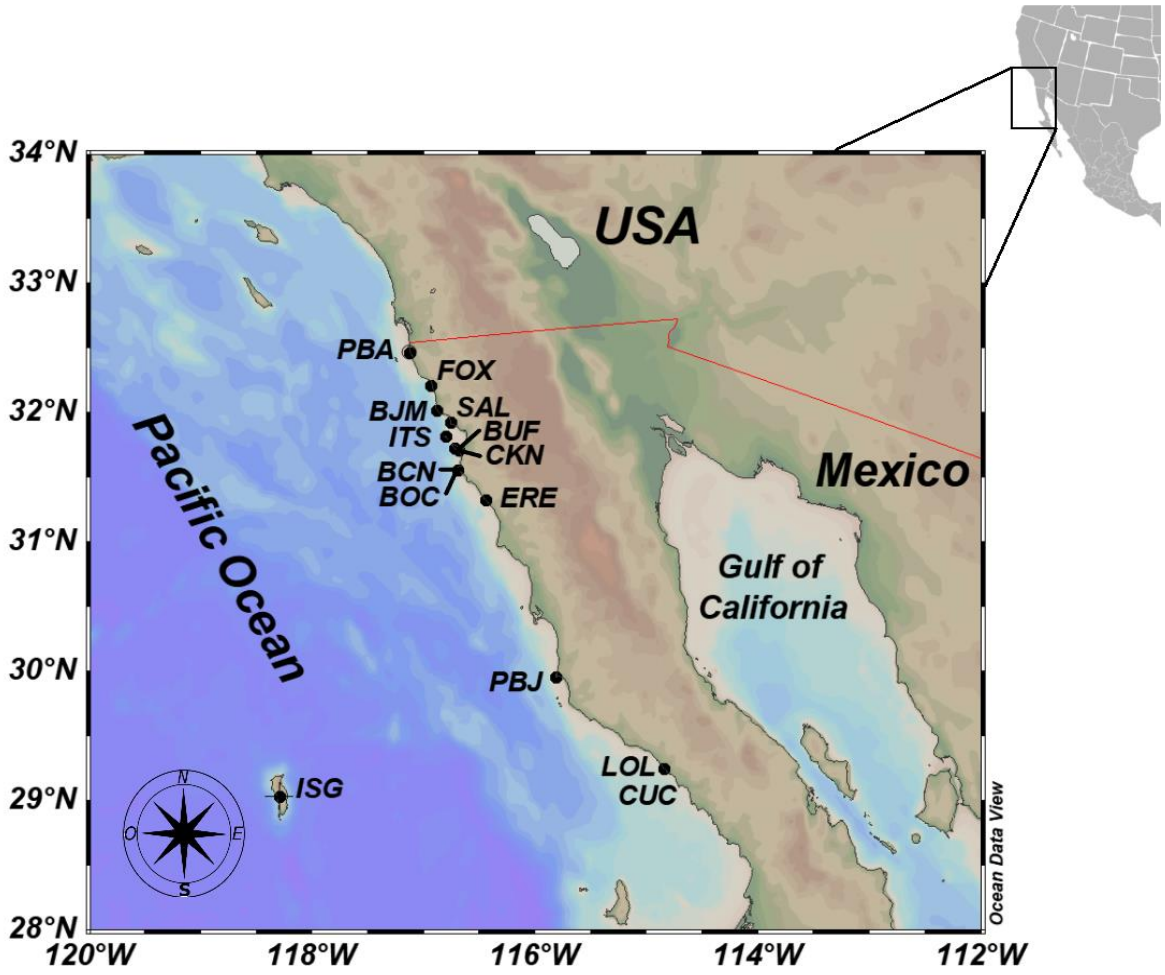

Fig. 1. Sampling sites for black abalone along the coast of Baja California, Mexico: Punta Bandera (PBA); Estudios FOX (FOX); Bajamar (BJM); Salsipuedes (SAL); Isla Todos Santos (ITS); Campo Kennedy (CKN); La Bufadora (BUF); Santo Tomás, La Bocana Norte (BCN); Santo Tomás, La Bocana (BOC); Eréndira (ERE); Punta Baja (PBJ); Faro San José, La Lola (LOL); Faro San José, El Cuchillo (CUC); Isla Guadalupe (ISG).

Table 2

Sampling sites grouped by zones, coordinates, site identification, total number of samples collected per locality, and population density.

Zone	Sampling site	Site ID	Latitude (N)	Longitude (W)	No. samples	Mean length (cm)	Density ind m^{-2}*
1	Punta Bandera	PBA	32.46	-117.118	2	8.5	0.0016
	Estudios FOX	FOX	32.20	-117.030	1	N/D	0.0009
	Bajamar	BJM	32.01	-116.876	8	3.9	0.2270
	Saldamando	SAL	31.92	-116.756	67	7.8	0.0842
2	Isla Todos Santos	ITS	31.81	-116.802	23	14.0	0.0220

3	La Bufadora	BUF	31.72	-116.715	30	6.4	0.0043
	Campo Kennedy	CKN	31.70	-116.680	1	N/D	0.0039
4	Santo Tomás, La Bocana Norte	BCN	31.55	-116.689	3	5.6	0.0007
	Santo Tomás, La Bocana	BOC	31.55	-116.689	9	6.5	0.0007
	Eréndira	ERE	31.32	-116.436	19	10.3	0.0100
	Punta Baja	PBJ	29.95	-115.807	1	11	0.0000
5	Faro San José, La Lola	LOL	29.24	-114.846	13	9,4	0.1136
	Faro San José, El Cuchillo	CUC	29.24	-114.842	17	11.6	0.1136
6	Isla Guadalupe	ISG	29.03	-118.290	4	12.5	N/D

*****Note: Density data from Cepeda-Ochoa 2019. Unavailable data marked as No Data (N/D).

2.2 Genomic DNA extraction and PCR detection of CXc and pCXc

Total DNA was extracted using the DNeasy® Blood and Tissue kit (QIAGEN) following the manufacturer's protocol, with slight modifications in: 1) step 6 with centrifugation at 13,000 rpm during 5 min and 2) step 7 with 100µl Buffer AE pipeted directly into the membrane of the spin tube and incubated at room temperature during 5 min. Then, we repeated this last step and eluted in a final volume of 200µl with Buffer AE. All DNA concentrations were quantified using UV spectrophotometer NanoDrop™ 2000 (ThermoFisher Scientific) and the products stored at -20°C until use. Genomic DNA integrity was evaluated in a 1.5% agarose gel electrophoresis with SuperBuffer 1X as described by Zhang et al. (2011).

To evaluate the prevalence of *C*Xc in the abalone feces, primers ss16S F (5' GCC TCA GTT TGG CTG GGT TCT TCA 3') and ss16S R (5' GAA TTG CCA CTT TAA AGT ATG GAC GG 3') were used to amplify a 426bp fragment of 16S rRNA gene according to PCR conditions described in Cicala et al. (2017). Also, the prevalence of p*C*Xc was evaluated using primers pCXcR4-F (5' CCA ACA CGA CTA GCT CCC TC 3') and pCXcR4-R (5' TCT GCA AGC TGT TTC TGG CT 3') to amplify a 412bp fragment that covers over the thymidylate kinase viral gene and ORF 24, according to PCR conditions described in Cruz- Flores et al. (2018). PCR was conducted in 12.5µL final volume containing 1µL of DNA, 10X PCR Buffer (Kapa Biosystems®),

0.2mM dNTPs (New England Biolabs), 0.3µM (*C*Xc) and 0.2µM (p*C*Xc) of each primer, and 0.5U of Taq polymerase (Kapa Biosystems®). Thermal cycling conditions for *C*Xc primers consisted of 4 min at 94°C, 40 cycles of 1 min at 94°C, 30s at 66°C and 10s at 72°C, followed by 8 min at 72°C (Cicala et al. 2017). For p*C*Xc detection, thermal cycling conditions consisted of 3 min at 95°C, 40 cycles of 10s at 95°C, 10s at 62°C and 10s at 72°C, followed by 3 min at 72°C (Cruz-Flores et al. 2018). PCR products were verified with a 1.5% agarose gel electrophoresis during 35 min at 80V using 5µL of the PCR product with 1µL of GelRed solution (Biotium) and the molecular marker DirectLoad™ of 100pb DNA Step Ladder (D3812, SIGMA-ALDRICH®). A histological evaluation was performed in one black abalone with clear signs of WS to be used as positive control for the detection of *C*Xc.

2.3 Data analysis

We tested for differences on abalone mean length between zones using a General Linear Model (GLM) and a *post hoc* Tukey-Kramer honestly significant difference test (HSD). We also calculated the lengths standard deviation and plot the length frequency distributions for each zone in order to characterize the abalone lengths among zones.

We recorded the presence of *C*Xc and p*C*Xc in each individual and estimated the prevalence in each zone. Also, we obtained the pathogens' prevalence distribution to evaluate if there was a certain length at which the pathogens were more abundant. We did this evaluation for three scenarios: total abalone positive for *C*Xc (T*C*Xc), total positive for p*C*Xc (Tp*C*Xc), and both pathogens present (*C*Xc-p*C*Xc). In order to evaluate the relationship between the pathogens' prevalence and abalone length

in each zone, we did three logistic regression models (LRM) and *post hoc* analyses when necessary. Prevalence was the dependent variable and abalone length, zone, and the interaction between length and zone were the independent variables.

We also explore the relationship between prevalence and abalone densities published in Cepeda-Ochoa (2019). We used linear regression analyses where abalone density was the independent variable and the pathogens' prevalence was the dependent variable. Finally, we determined if there is a correlation between the prevalence of Tp*C*Xc and the prevalence of T*C*Xc using a linear regression analysis. All statistical analyses were conducted with JMP 15.2.1 (SAS).

3. RESULTS

We found significant differences on abalone mean length between zones ($r^2 = 0.53$, $F = 39.82$, $p < 0.0001$). Zone 2 had the largest abalone mean length (13.99 $\pm$ 3.54 cm, Tukey-Kramer HSD $p < 0.05$, $Q = 2.76$), and a maximum of 22.5 cm, the biggest abalone recorded in this study. The mean length in zone 2 was 25% larger than zone 5 (10.59 $\pm$ 3.53 cm) and twice as big than zones 1 (6.89 $\pm$ 1.99 cm), 3 (6.35 $\pm$ 1.98 cm), and 4 (6.73 $\pm$ 2.22 cm). Zone 5 was also statistically different from zones 1, 3, and 4, which had similar mean lengths (Tukey-Kramer HSD $p < 0.05$, $Q = 2.76$) (Fig. 2a). Zones 2 and 5 were not only the zones with the largest mean lengths but also with a wider distribution of sizes (Fig. 2b).

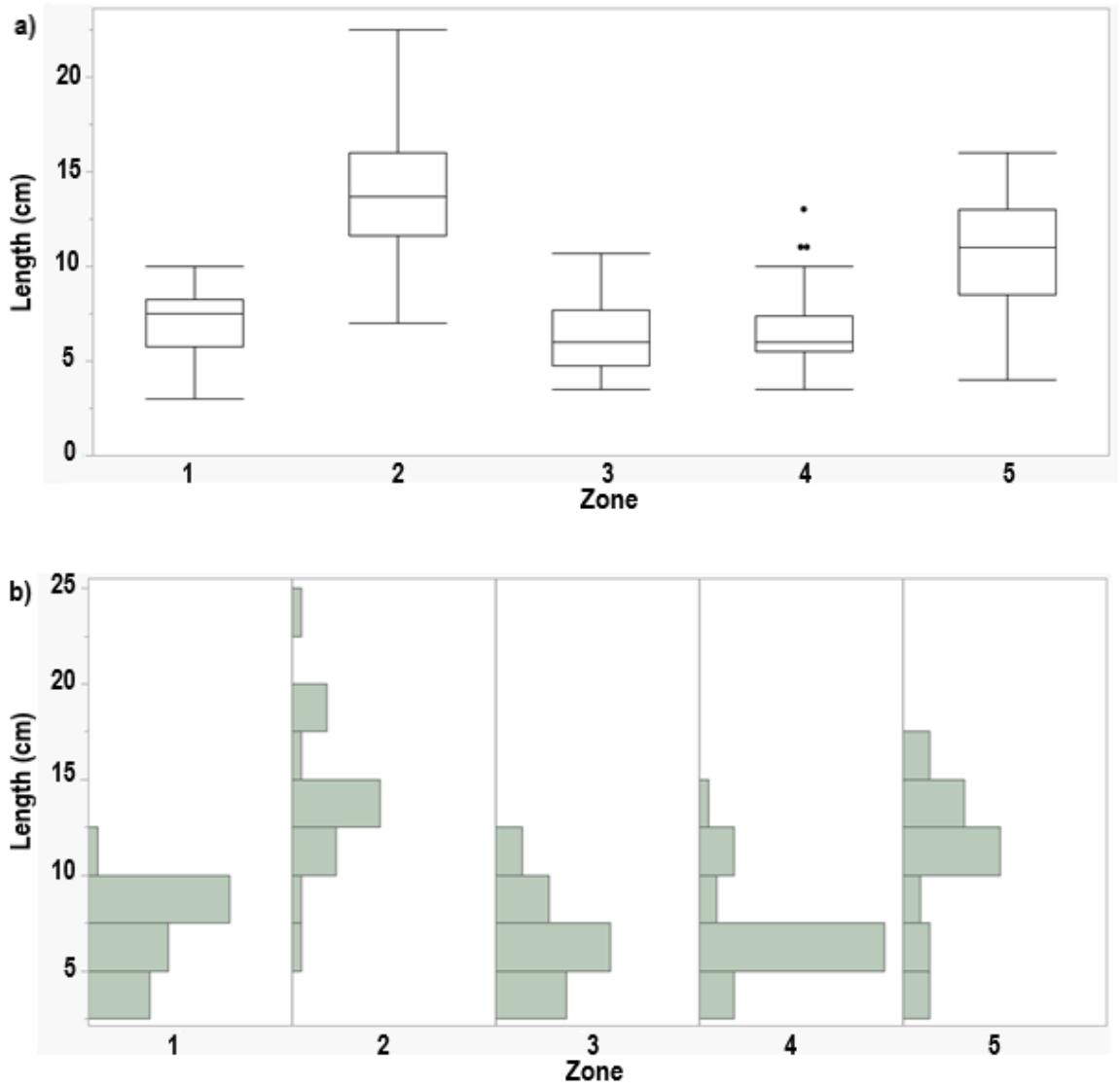

Fig 2. a) Variation in abalone mean length by zone; b) Length frequency distribution by zones.

Good quality genomic DNA and successful PCR amplification products were obtained for 199 individuals. We found that 11 individuals (6%) were positive for only *C*Xc, 68 (34%) were positive for only p*C*Xc, 76 (38%) presented both *C*Xc and p*C*Xc, and 44 (22%) were negative. We observed that the prevalence of *C*Xc and p*C*Xc fluctuated throughout the study area, but no geographic pattern was detected. For example, the northern zones 1 and 2 presented the highest prevalence of abalone

infected with both *C*Xc and p*C*Xc, with 47.4% and 70.8% respectively, while zone 4 showed the lowest (9.4%). Also, all four individuals from zone 6 (Isla Guadalupe) had both *C*Xc and p*C*Xc but were excluded from all other analyses given the small samples size. On the other hand, we did not detect any of the two pathogens in 59.4% of the individuals in zone 4 and 46.7% in zone 5, being the least infected zones (Fig. 3).

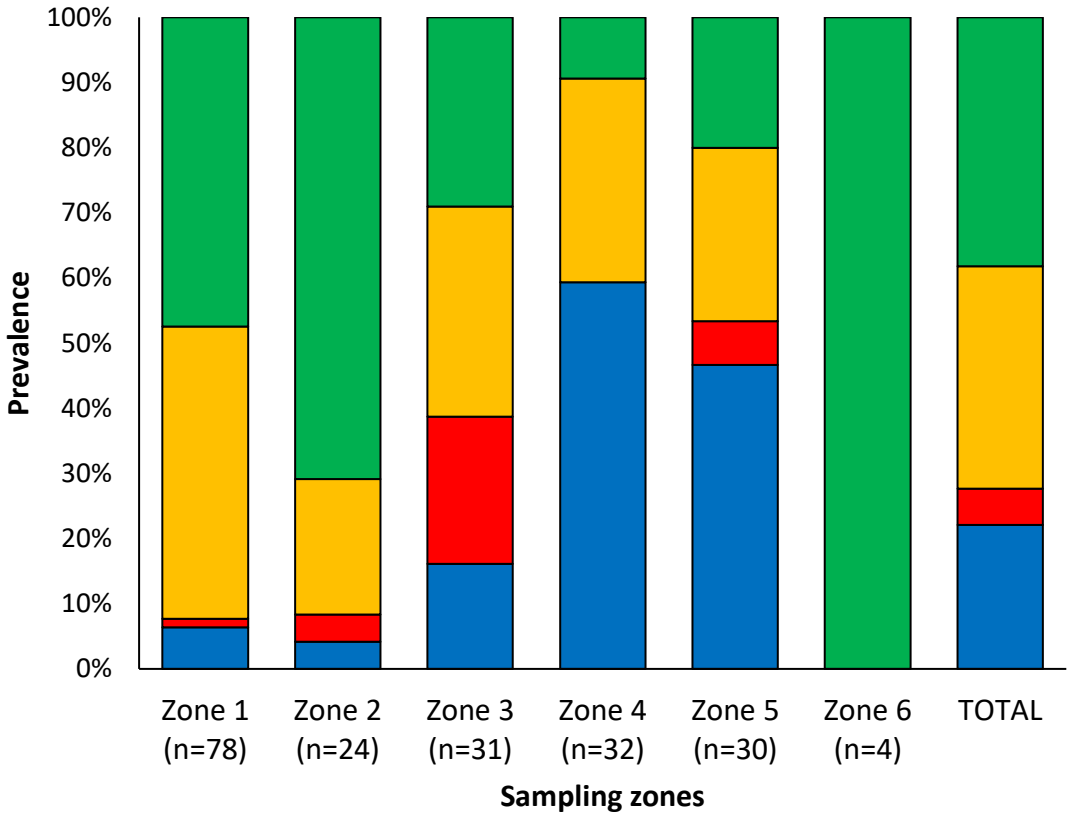

Fig. 3. Prevalence (%) of the bacteria *C*Xc and its associated phage p*C*Xc. Individuals were grouped as follow: no pathogens detected (blue), only *C*Xc (red), only p*C*Xc (yellow), and both *C*Xc and p*C*Xc detected (green) by zones: Zone 1 (PBA, FOX, BJM, SAL), Zone 2 (ITS), Zone 3 (BUF, CKN), Zone 4 (BCN, BOC, ERE, PBJ), Zone 5 (CUC, LOL) and Zone 6 (ISG).

The prevalence distribution vs length distribution comparison through a distribution analysis for T*C*Xc showed that zone 2 has the largest abalones with *C*Xc present (mean = 15.2 cm, max = 22.5 cm, min = 11.2 cm) from all zones, and are larger than

abalones with non-present *C*Xc in this zone (mean = 10.4 cm, max = 13.5 cm, min = 7.0 cm). Zone 5 had the second largest abalones with *C*Xc present (mean = 10.3 cm, max = 15.0 cm, min = 4.0 cm) however, non-significant difference with non-present was observed (mean = 10.7 cm, max = 16.0 cm, min = 4.0 cm). Zones 1, 3, and 4 had similar mean lengths between them and no differences between present and non-present were found (table 3, Fig. 4a). The prevalence distribution vs length distribution comparison for Tp*C*Xc shows that abalones with the p*C*Xc present are larger than those without p*C*Xc across all zones (table 3, Fig. 4b). Finally, the results of both pathogens (*C*Xc-p*C*Xc) were similar to that observed for T*C*Xc. Zone 2 presented the largest abalones for both *C*Xc-p*C*Xc present and non-present (mean length = 22.5 cm and 18.5 cm respectively) from all zones, followed by zone 5. The rest of the zones had similar length values among them, and no differences were found between present and non-present (Table 3, Fig. 4c).

Table 3

Mean, minimum and maximum length (cm) values of the sampled abalones for each pathogens' prevalence scenario, with the total of both infected and non-infected organisms, across the study zones. P: present; A: absent.

		Zone									
		1		2		3		4		5	
		P	A	P	A	P	A	P	A	P	A
T*C*Xc	n	38	40	18	6	16	15	3	29	8	22
	Mean (cm)	6.5	7.1	15.2	10.4	6.2	6.5	7.5	6.7	10.3	10.7
	Min (cm)	3.5	3.0	11.2	7.0	4.0	3.5	5.5	3.5	4.0	4.0
	Max (cm)	9.5	10.0	22.5	13.5	10.0	10.7	11.0	13.0	15.0	16.0
Tp*C*Xc	n	72	6	22	2	19	12	13	19	14	16
	Mean (cm)	6.8	7.5	13.9	14.4	6.9	5.5	6.5	6.9	11.8	9.6
	Min (cm)	3.0	7.0	7.0	10.3	4.0	3.5	3.5	4.0	6.0	4.0
	Max (cm)	10.0	8.0	22.5	18.5	10.5	10.7	11.0	13.0	16.0	15.0
***C*Xc - p*C*Xc**	n	37	41	17	7	9	22	3	29	6	24
	Mean (cm)	6.5	7.1	15.0	11.6	6.9	6.1	7.5	6.6	11.0	10.5
	Min (cm)	3.5	3.0	11.2	7.0	4.0	3.5	5.5	3.5	6.0	4.0
	Max (cm)	9.5	10.0	22.5	18.5	10.0	10.7	11.0	13.0	15.0	16.0

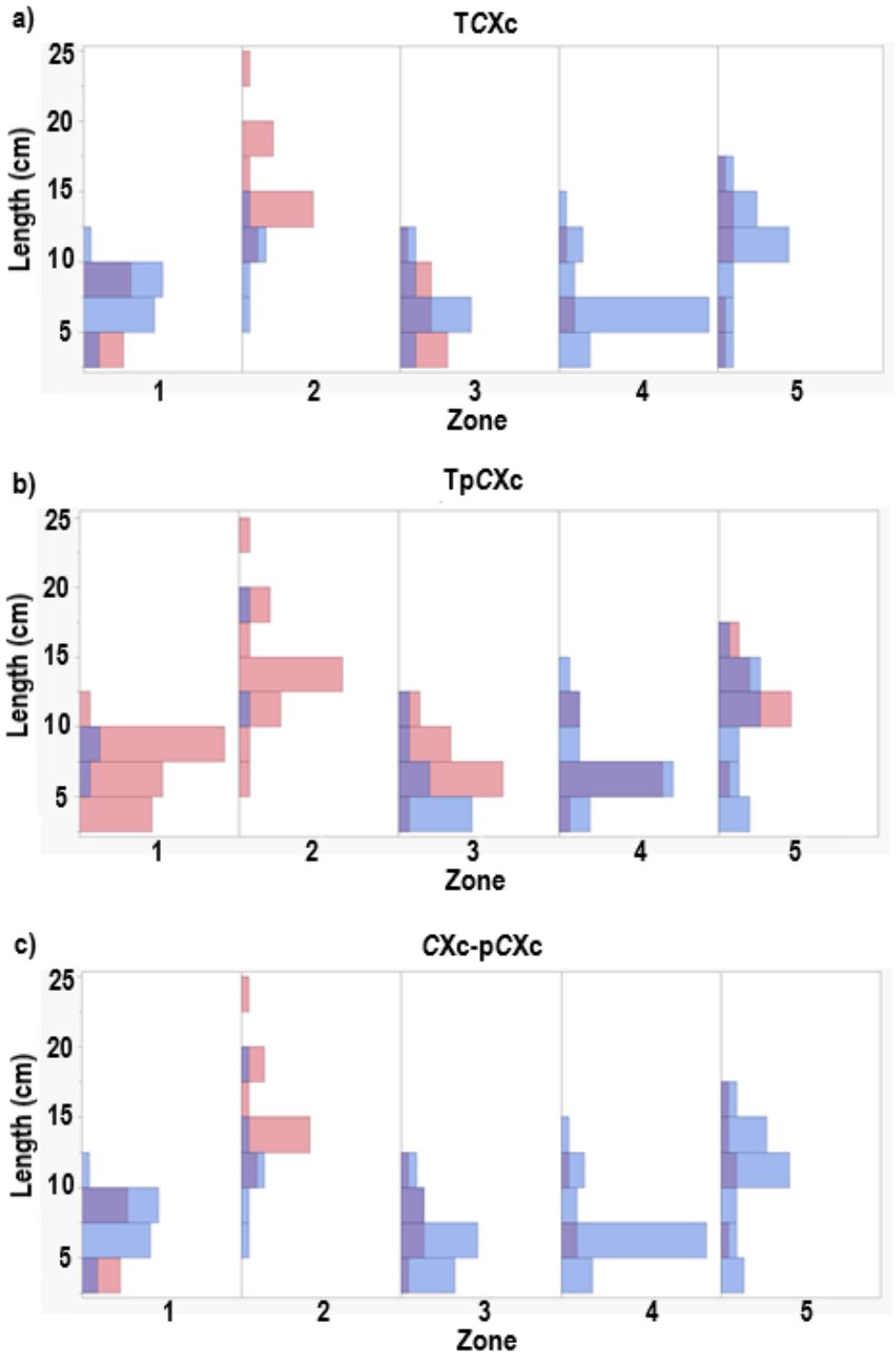

Fig. 4. Distribution analysis of the prevalence of: a) total *C*Xc (T*C*Xc), b) total p*C*Xc (Tp*C*Xc) and c) both pathogens (*C*Xc-p*C*Xc), within the individual abalone lengths recorded among the zones. Red is for pathogen(s) present and blue for no pathogens found.

We found no effect of zone or abalone length on prevalence of T*C*Xc, Tp*C*Xc, and both of the pathogens, *C*Xc-p*C*Xc. However, the interaction between zone and abalone length had an effect on the prevalence of T*C*Xc (Table 4). For that reason, we tested the effect of length for each zone separately using LRMs. Also, we tested for differences on prevalence across zones using a Nominal Logistic Model. We found that length had a significant relationship with T*C*Xc but only in zone 2 (r^2 = 0.53, χ^2 = 13.95, p = 0.0002). This relationship was positive with larger abalone more likely to be infected by *C*Xc (Fig. 5).

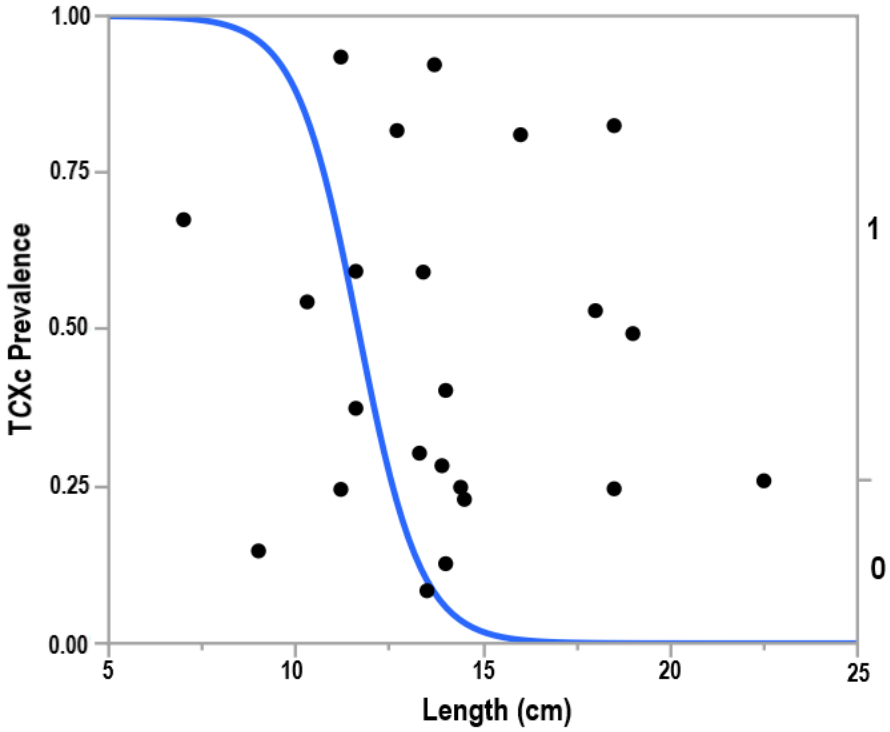

Fig. 5. Nominal logistic model showing the positive correlation found between the T*C*Xc prevalence and abalone length (cm) in zone 2 (ITS).

We also tested the effect of zone on T*C*Xc, Tp*C*Xc, and *C*Xc-p*C*Xc without the effect of length using a Contingency Analysis (Table 5). We found that zone 2 had the highest prevalence of *C*Xc (75%), meanwhile, zone 4 had the lowest with 9.4% and

were statistically different from zones 1 (48.7%), 3 (51.6%) and 5 (26.7%) that were close to total average 42.6%. The same pattern was observed for *C*Xc-p*C*Xc, where zone 2 had the highest prevalence (70.8%) and zone 4 has the lowest with 9.4%. They were also statistically different from zones 1 (47.4%), 3 (29.0%), and 5 (20.0%), that were close to the total average 36.9%. On the other hand, zone 1 presented the highest prevalence of p*C*Xc (92.3%), followed by zone 2 (91.7%). The total average was 71.8%, which was statistically similar to zone 3 (61.3%). Finally, zone 5 showed a 46.7% of prevalence and zone 4 presented the lowest with a 40.6%.

Table 4

Generalized linear model to examine the relationship among the pathogens' prevalence with the study zone, the abalones length and the interaction between the zone and the length. Significant values are bolded.

	Factor	r^2	df	χ^2	p - value
T*C*Xc	Zone	0.24	4	6.74	0.1504
	Length		1	3.02	0.0821
	Zone * Length		4	14.43	**0.0060**
Tp*C*Xc	Zone	0.22	4	22.30	**0.0002**
	Length		1	0.46	0.4982
	Zone * Length		4	5.55	0.2357
***C*Xc-p*C*Xc**	Zone	0.19	4	3.67	0.4523
	Length		1	2.23	0.1349
	Zone * Length		4	5.62	0.2289

Table 5

Contingency analysis to examine the relationship between the pathogens' prevalence with the study zone. Significant values are bolded.

Prevalence	r^2	df	χ^2	p - value
T*C*Xc	0.13	4	33.28	**< 0.0001**
Tp*C*Xc	0.22	4	49.86	**< 0.0001**
***C*Xc-p*C*Xc**	0.13	4	32.65	**< 0.0001**

We did not find any correlation between abalone densities and any of the pathogens' prevalence.

Finally, we observed a positive correlation (r^2 = 0.73) between the prevalence of T*C*Xc and the prevalence of Tp*C*Xc by zone, however, it was not statistically significant (p = 0.064) (Fig. 6).

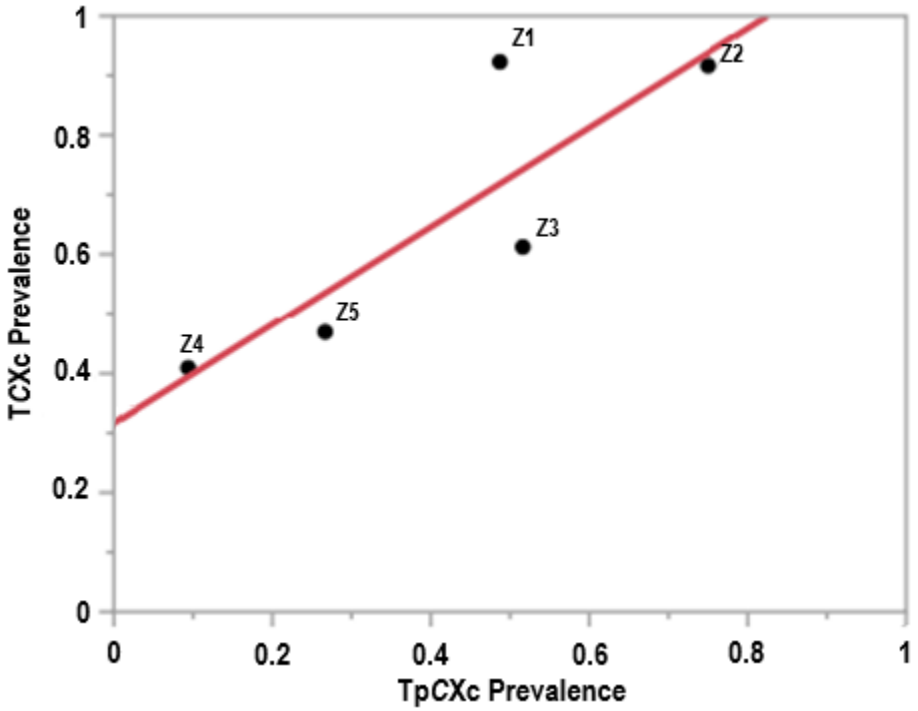

Fig. 6. Correlation between prevalence of T*C*Xc and prevalence of Tp*C*Xc by zone.

4. DISCUSSION

Black abalone has a crucial ecological role in coastal communities and once served as an important commercial and recreational fishery, however its populations are critically endangered due to massive declines attributed to over-exploitation and the withering syndrome (Raimondi et al. 2002, Harley & Rogers-Bennet 2004), which is known to be the most lethal disease affecting black abalone (Ockert 2005, VanBlaricom et al. 2009). Abalones infected by with *Candidatus* Xenohaliotis

californiensis not always develop the expression of the disease and subsequently death of the abalone which enables transmission before the host population declines (Ben-Horin et al. 2013). For example, some abalones may not present the external signs of WS despite a high load of *C*Xc, and on the contrary, abalones with a low load, could show external signs of the disease (Cáceres et al. 2020).

Recently, both *CXc* and its associated bacteriophage, p*C*Xc have been observed in multiple abalone species in California (Friedman et al. 2014, Vater et al. 2017) and Baja California (Cruz-Flores & Cáceres-Martínez 2016, Cruz-Flores et al. 2016). However, our findings are the first to show that both pathogens are widely distributed in black abalone populations of Baja California.

In the present study, we detected *C*Xc in 43.7% of the black abalones samples. This is a low prevalence compared to that recorded in green (80%) and pink (63%) wild abalone from Baja California through histology, and then corroborating the prevalence of the bacterium using PCR (Cruz-Flores et al. 2016). It was an even smaller prevalence compared to the found in black abalone from California (74-98%) also detected through histological analyses (OIE 2021). Moreover, Valles-Ríos (2000) also detected *C*Xc in 100% of black abalone from one population in Baja California Sur, through histology analysis. This variable prevalence between species could be related to genetic diversity, an important key factor in parasitic infection defense that determines the probability of an encounter between the pathogen and the host through habitat use and behavior or the response and development of a disease due to physiological compatibility (González et al. 2014). Although, black abalone probably possess a limited capability to develop resistance to new pathogens (such as *C*Xc), over time, its resistance to different pathogens is likely to

be enhanced due disease pressure (Friedman et al. 2014). Another reason that could explain black abalone susceptibility to WS is their unique occurrence in rocky intertidal habitats, where temperature changes can be extreme, allowing *C*Xc transmission and expression of WS (Ben-Horin et al. 2013). It has been reported that *C*Xc infection is slower in colder water, with an infection ratio of about 1% in temperatures below 13°C and around 72-94% in temperatures above 18°C (OIE 2021, Vater et al. 2017, Braid et al. 2005).

On the other hand, phages are known to play an important role in controlling bacterial activity and proliferation, with a 10-50% bacterial mortality (Crosson et al. 2020), and a significant reduction in symptoms of WS disease in black abalone (Brokordt et al. 2017). Also, improvement of black abalone survival has been observed when p*C*Xc is present even when environmental conditions are not favorable (Vater et al. 2017), however, it is still not known how temperature affects the bacteriophage and the possible genetic resistance to *CXc* of the different abalone species (NOAA 2020). In this study, we found a high prevalence of p*C*Xc when *C*Xc was present (38%), which might also play a role on the low prevalence of CXc we found in this study. Detection of p*C*Xc in Baja California has been reported only in blue and pink abalone with 22% and 31% prevalence respectively (Cruz-Flores et al. 2016). These results are similar to the 34.2% that we recorded.

It has been previously reported that bacterial load is lightly correlated with size, that age had an effect in the level of *C*Xc load when p*C*Xc was not present (Brokordt et al. 2017), and that the probability of infection increases with abalone size (OIE 2021). These could be explained due to older (and hence larger) organisms have a longer exposure to the environment. We only observed a positive correlation between *C*Xc

prevalence and length in one zone, even though we found variability in abalone mean length and the length frequency between zones. It is important to note that it has been reported that the probability of detection of the pathogens in organisms smaller than 10 mm through histology is reduced yet equal if the detection is using the PCR method (OIE 2021), however, our results showed that the prevalence of both pathogens was highest in the smallest organisms in some zones.

We also found that the pathogens prevalence in Baja California populations was not related to abalone densities. Since black abalones display a stacking dynamic, this allows an easy and rapidly spread of the disease within the population (Crosson et al. 2020), however the pathogens transmission capability can be influenced by host behavior, such as enhanced resistance to WS (Chambers et al. 2006), or a possible genetic variation for susceptibility (Brokordt et al. 2017), as well as pathogens reservoirs and vectors (Crosson et al. 2020). Also, WS had a major impact in aquaculture due to high organisms densities and stressful changing environments, which favors the disease transmission and spread (Brokordt et al. 2017). Nonetheless, it has been suggested that a directed fishery to infected individuals could control the parasites load in natural populations (Ben-Horin et al. 2016).

In recent intense monitoring of black abalone in both California and Baja California indicates a slow recovery of natural populations (NOAA 2020, Cepeda-Ochoa 2019). Our result might indicate that the recently recovered black abalone natural populations have not reached an abundance level in which *C*Xc, and thus, the phage, transmit more. Due to this presumed low abundance, we could assume that it might be a reason as to why we did not find an effective correlation between the pathogens prevalence and abalone densities.

On the other hand, Cepeda-Ochoa found no genetic structure among black abalone populations in Baja California (except for the subspecies from Isla Guadalupe), which suggest that the collected abalones could be descendants of the survivors of the mass mortality occurred in the 1990s and are more resistant to infection (Cepeda-Ochoa 2019). Additionally, a possible genetic variability loss that occurred around the mid 80's in California (USA) and the early 90's in Baja California (Mexico) when *C*Xc first appeared, could have resulted from a founder effect (Cicala et al. 2018).

In conclusion, this first study where *C*Xc infected by p*C*Xc in black abalone from Mexico has been detected, is an initial approach to determine and evaluate the prevalences of both the phage (p*C*Xc) and the bacterium (*C*Xc) in the wild populations of black abalone found along the Mexican coast. Although we did find that prevalence of the pathogens has a certain correlation with abalone lengths but none with abalone densities, we consider that a more robust sampling throughout the study area and advanced molecular analysis, such as qPCR, could provide more accurate results.

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6. CONCLUSIONES

Este trabajo es el primer estudio en el cual se reporta la prevalencia de la bacteria *C*Xc infectada por el bacteriófago p*C*Xc en las poblaciones silvestres de abulón negro de Baja California.

Se detectó que tanto la bacteria como el bacteriófago se encuentran ampliamente distribuidos en la zona de estudio, así como en las diferentes longitudes de abulón negro.

Asimismo, se encontró que hay una aparente correlación entre las prevalencias de la bacteria y el bacteriófago con la longitud de los organismos.

7. RECOMENDACIONES

Debido a que en general no se encontró una relación entre las prevalencias de los tres escenarios (*C*Xc, p*C*Xc y *C*Xc infectado con p*C*Xc) con la zona y la densidad poblacional de los abulones negros silvestres, es preferible realizar un muestreo más robusto tanto en toma de muestra como a lo largo de la zona de estudio, así como el uso de técnicas moleculares con una mayor resolución (por ejemplo, la qPCR) que nos permitan conocer la intensidad de las bacterias y los bacteriófagos presentes en las muestras.

8. ANEXOS

Se realizó una evaluación histológica en un abulón negro con claros signos de SD que fue utilizado como control positivo para detectar *C*Xc y, posteriormente p*C*Xc. El tejido gastrointestinal fue extirpado y fijado es solución de fijación Davidson durante 24 horas a -4°C y procesado para histología con parafina. Los portaobjetos desparafinados de 0.5µM fueron teñidos con hematoxilina y eosina y revisado para el análisis (Cáceres-Martínez et al. 2011, Andree et al. 2000).