Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Doctorado en Ciencias
Ciencias de la Vida
con orientación en Biotecnología Marina

Effects of Paralytic Shellfish Toxins on marine mammals, seabirds and geoduck fisheries in the Northern Gulf of California during 2015-2019

Tesis
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Presenta:

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Efectos de las toxinas paralizantes en mamíferos y aves marinas, y en las pesquerías de almeja generosa del Norte del Golfo de California durante 2015-2019

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Las toxinas paralizantes (PST) son un grupo de neurotoxinas producidas por algunas especies de dinoflagelados marinos. Durante los Florecimientos Algales Nocivos (FAN) de estas especies, las PST se acumulan en bivalvos, que son el principal vector de las toxinas al hombre. La intoxicación paralizante por consumo de mariscos (PSP) es el síndrome asociado a toxinas en moluscos producidas por FAN más importante en México, y el único relacionado con muertes humanas. Al menos cuatro FANs de *Gymnodinium catenatum* ocurrieron en el norte del Golfo de California (NGC) entre 2015 y 2019. En 2015, el FAN provocó la mortalidad de más de 1,000 aves y 240 mamíferos marinos de distintas especies. Además, al menos 5 personas presentaron síntomas de intoxicación PSP. *G. catenatum* se registró de enero a mayo. La sardina "bocona" (*Cetengraulis mysticetus*) fue el principal vector de las PST hacia los animales. Las actividades económicas también se han visto afectadas por los FAN en el NGC debido a la implementación de vedas a la pesca y exportación de la almeja generosa *Panopea globosa*. En este trabajo, se caracterizó el metabolismo de las PST en el sifón y la masa visceral de la almeja, esta información es necesaria para la elaboración de planes de manejo para esta importante pesquería. La toxicidad máxima detectada fue 16,740 µg STXeq Kg^{-1} en la masa visceral, con una tasa de depuración de 4.3% $día^{-1}$. C1&2 fueron los análogos más abundantes en muestras recolectadas al inicio del FAN, su concentración disminuyó en las primeras semanas y los análogos más tóxicos GTX5, dcGTX3 y dcSTX fueron detectados. Cinco y siete meses después de la aparición del FAN, se detectaron análogos de tipo M y STX. Esta es la primera descripción de la biotransformación de PSTs en *P. globlosa*. El monitoreo de las toxinas en bivalvos, particularmente durante un FAN, requiere la generación de resultados confiables en poco tiempo. Para satisfacer las demandas analíticas, las autoridades mexicanas implementaron el uso de Scotia Rapid Testing (SRT). En este trabajo, se compararon SRT y HPLC-PCOX, la metodología de referencia en EE.UU. y Canadá, con el bioensayo en ratón (MBA), la metodología de referencia en México. La buena correlación (R^2 = 0.91) entre HPLC-PCOX y MBA indica que HPLC-PCOX es adecuado para monitorear PSTs en *P. globosa*, mientras que STR no es una metodología precisa ya que se registraron más de 67% de falsos positivos. Los FAN de *G. catenatum* se han producido en el NGC entre enero y mayo cada año desde 2015. La abundancia máxima de células en la superficie alcanzó $311x10^3$ cél L^{-1}, lo que resultó en la acumulación de PSTs en bivalvos de importancia económica. La extracción de almeja y otros bivalvos estuvo prohibida hasta por 294 días en 2017, impactando a las comunidades locales. Los FAN de *G. catenatum* y los efectos de la acumulación de PST, son un fenómeno recurrente que debe ser considerado al implementar planes de manejo que promuevan el desarrollo socioeconómico mientras protegen la vida silvestre y la salud pública en el NGC.

Palabras clave: Florecimientos algales nocivos, *Gymnodinium catenatum, Panopea globosa*, HPLC-PCOX, Bioensayo en ratón

Abstract of the thesis presented by **Jennifer Medina Elizalde** as a partial requirement to obtain the Doctor in Life Sciences with orientation in marine biotechnology degree.

Effects of Paralytic Shellfish Toxins on marine mammals, seabirds and geoduck fisheries in the Northern Gulf of California during 2015-2019

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Paralytic Shellfish Toxins (PSTs) are a group of neurotoxins produced by some marine dinoflagellates species. During the harmful algal blooms (HABs) of these species, PSTs accumulate in bivalves which become the main vector of the toxins to humans. Paralytic Shellfish Poisoning (PSP) is the most important shellfish toxin syndrome in Mexico related to HABs and the only one associated with human fatalities. At least four HABs of *Gymnodinium catenatum* occurred in the Northern Gulf of California (NGC) between 2015 and 2019. In 2015, a *G. catenatum* HAB caused multispecies mass mortalities of sea mammals and seabirds. More than 1,000 seabirds and 240 marine mammals were affected. Also, the intoxication of at least five people were associated with this HAB. The presence of *G. catenatum* was registered from January to May. The sardine "bocona" (*Cetengraulis mysticetus*) was the main vector of the PSTs to animals. Economic activities have been also impacted by HABs in the NGC. Multiple prohibitions (sanitary bans) to the harvesting and exportation of the geoduck clam *Panopea globosa* have been implemented in the region. The metabolism of the PSTs in the siphon and visceral mass was characterized, this information is necessary when creating management plans for this important fishery. A maximum toxicity of 16,740 µgSTX eq Kg^{-1} was detected on the visceral mass, with a depuration rate of 4.3% day^{-1}. C1&2 were the most abundant analogues detected in geoduck samples collected during the HAB appearance. The concentration of these analogues decreased in the first weeks, and the more toxic analogues GTX5, dcGTX3 and dcSTX were detected. Five and seven months after the occurrence of the HAB, M-type analogues and STX were detected also in the visceral mass of the clams. This is the first description of the biotransformation of PST in *P. globlosa*. Monitoring of PSTs levels in bivalves, and particularly during HABs requires reliable results in a short period of time. To meet analytic demands, Mexican Health Authorities implemented the Scotia Rapid Testing (SRT). SRT and HPLC-PCOX, the reference methodology in the US and Canada, were compared with the mouse bioassay (MBA), the reference methodology in Mexico. The good correlation (R^2=0.91) between HPLC-PCOX and MBA indicates that HPLC-PCOX is suitable for monitoring PSTs in *P. globosa*. However, it was found that STR was not an accurate methodology as more than 67% of false positives were registered. *G. catenatum* HABs have occurred between January and May in the NGC every year since 2015. Surface maximum cell abundances reached 311 x 10^3 cell L^{-1}, resulting in the accumulation of PSTs bivalves of economic importance for the region. The extraction of geoduck and other bivalves was prohibited up to 294 days in 2017, causing an important socioeconomic impact to local communities. HABs of *G. catenatum* and the subsequent impacts due to the accumulation of PSTs are a recurrent phenomenon that must be considered when implementing management plans to promote socioeconomic development, while protecting the wildlife and the public health in the NGC.

Keywords: Harmful Algae Bloom, *Gymnodinium catenatum, Panopea globosa*, HPLC-PCOX, Mousse Bioassay

Dedication

A mi familia, José, Irene, Karen, Alan, Nestor, Camila

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Chapter 1. Introduction

1.1 Introduction

Paralytic Shellfish toxins (PSTs) are a group of more than 50 neurotoxic alkaloids of saxitoxin, and are produced by marine dinoflagellates, cyanobacteria, and bacteria (Wiese *et al.*, 2010). PSTs accumulate mainly in filter-feeding organisms, the main vectors of these toxins to humans being bivalve mollusks. PSTs bind reversibly to Na^+ channels (Cestèle and Catterall 2000), which prevents transmission of the nerve impulse in nerve and muscle fibers, leading to neuromuscular paralysis (Bricelj *et al.* 2005). Ingestion of contaminated shellfish mollusks can cause paralytic shellfish poisoning (PSP) resulting in headache, dizziness, vomiting, diarrhea, numbness, paralysis and, in high concentrations, death from respiratory distress within hours after ingestion (Botana, 2000; Llewellyn, 2006). Due to the risk of human intoxication, the concentration of PSTs in bivalve mollusks is regulated by international health authorities to a regulatory limit (LR) of 800 µg STXeq Kg^{-1} of tissue (Codex Alimentarius Committee, 2008).

In the Mexican Pacific and Gulf of California, harmful algal blooms (HABs) of PST-producing algal species have impacted on fauna (Núñez-Vázquez *et al.*, 2011) and human health since 1979 (Mee *et al.*, 1986; Band-Schmidt *et al.,* 2010; Lewitus *et al.*, 2012; Bustillos-Guzmán *et al.,* 2015; Bustillos-Guzmán *et al.,* 2016; Santiago-Morales, 2016). The dinoflagellate *Gymnodinium catenatum* is the main species responsible to produce PSTs in the Gulf of California (Bustillos-Guzmán *et al.*, 2016) and has been recorded on the coasts of Sonora, Baja California Sur (Gárate-Lizárraga, *et al.*, 2004) and Baja California (Murillo-Martínez, 2011). In the Northern Gulf of California (NGC) at least four HABs of *G. catenatum* have been recorded between 2015-2019 affecting the ecology, economy, and public health of the region. In this work, we describe the effect on marine mammals, seabirds, and geoduck fisheries of the presence of PSTs after those HABs.

In January 2015, a HAB of this dinoflagellate occurred in the NGC, resulting in the death of thousands of birds and hundreds of marine mammals due to PSP (García-Mendoza *et al.*, In preparation). In addition, the first human intoxications related to phycotoxins in Baja California occurred. In Bahía de los Ángeles, B.C., at least eight people presented signs of PSP after consuming contaminated wild clams (Tapia, 2015). Five of these cases were recognized by national health authorities as PSP (COFEPRIS, 2015). After the 2015 HAB, the Mexican health authorities (COFEPRIS) detected toxin levels up to 33 times higher than the RL, which caused the implementation of sanitary closures in Baja California and Sonora for almost all

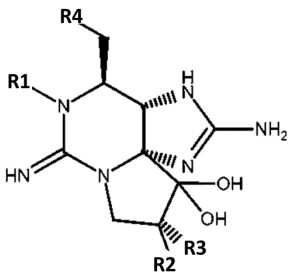
of 2015 (COFEPRIS, 2015). It should be noted that in the NGC, the extraction of geoduck clam *Panopea globosa* is one of the main economic activities, since it has a high commercial value in the international market, especially in China and USA. As a result, the ban had a negative impact on activities in the extraction areas and in the processing and export plants, which was reflected in the economy of the region.

In January 2017, 2018, and 2019, new blooms of *G. catenatum* occurred in the NGC. In 2017, PST concentrations in geoduck tissues up to 61 times higher than the RL were quantified (COFEPRIS, 2017). Fishing activities of other shellfish, such as the pen shell *Atrina maura* were also prohibited due to the detection of PSTs in their tissues. In these years the sanitary closures implemented after the HABs have lasted several months, negatively impacting the economy of the region.

Even when *P. globosa* is an important fisheries resource for the region, the processes of assimilation, transformation, and depuration of PST have not been described. In this study, we compared three methodologies for the detection of PSTs in geoduck tissues. Also, we described the transformation and depuration of PSTs in naturally contaminated clams. This information is essential to establish contingency plans and resource management, as well as to recognize the impact of these toxins on economic activities. On the other hand, a fundamental aspect related to mitigating the effects of HABs is to establish an adequate monitoring program for these events and to have robust methods for detecting phycotoxins that support adequate decision-making. This will protect public health and reduce losses in coastal economic activities.

1.1.1 Paralytic Shellfish Toxins

Saxitoxin (STX) is a hydrophilic molecule isolated in 1957 from the Alaskan butter clam *Saxidomus giganteus* (Schantz *et al.*, 1957). This molecule contains two guanidine groups, it is a tricyclic 3,4-propinoperhydropurine with formula $C_{10}H_{17}N_7O_4$, a molecular weight of 299 (Berlinck and Kossuga, 2005), and it is one of the most potent natural neurotoxins known (Figure 1; Llewellyn, 2006; Wiese *et al.*, 2010). The LD50 of STX, when ingested by humans, is 5.7 µg Kg^{-1}, and 0.6 µg Kg^{-1} when delivered by injection (Nguyen *et al.,* 2021).



R1	R2	R3	Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins	Others analogues M toxins		
H	H	H	STX	B1 (GTX5)	dcSTX			
OH	H	H	NEO	B2 (GTX6)	dcNEO			
OH	H	OSO_3^-	GTX1	C3	dcGTX1			
H	H	OSO_3^-	GTX2	C1	dcGTX2			
H	OSO_3^-	H	GTX3	C2	dcGTX3			
OH	OSO_3^-	H	GTX4	C4	dcGTX4			
H	OH	H				M1	M2	
H	OH	OH				M3	M4	
Not structurally characterized								M5
			R4 $OCONH_2$	R4 $OCONHCH_3$	R4 OH	R4 $OCONH_2$	R4 $OCONHCH_3$	

Figure 1. Structure of the main saxitoxin analogues grouped according to substituents of the side chains.

Since the discovery of STX, at least 57 analogues have been described (Wiese *et al.*, 2010). Saxitoxin and its analogues conform the group of the Paralytic Shellfish Toxins (PST), which are produced in freshwater environments by cyanobacteria of the genera *Anabaena, Cylindrospermopsis, Aphanizomenon, Planktothrix* and *Lyngbya*, and in marine environments by dinoflagellates of the genera *Gymnodinium, Alexandrium* and *Pyrodinium* (Jaime *et al.,* 2007).

Most PSTs present hydrophilic characteristics and differ from each other by the substituents on their R_4 side chain, which may be carbamate, sulfate or hydroxyl. Analogues vary in their potency or biological activity: the carbamate-type analogues (STX, neosaxitoxin NEO and gonyaulotoxins GTX1-4) are the most potent toxins since they have a higher affinity for the sodium channel; the N- sulfocarbamoyl toxins (B1&2 or GTX5&6, and C1-4) are the less powerful, while 3lobose3oyl toxins (dcGTX1-4, dcSTX and dcNEO) exhibit intermediate toxicity (Figure 1). In recent years, hydrophobic analogues isolated from the cyanobacteria *Lyngbya wollei* (Onodera *et al.,* 1997) and the dinoflagellate *G. catenatum* have been characterized (Negri *et al.*, 2003; Bustillos-Guzman *et al.*, 2011). The analogues from the cyanobacteria contain acetate as substituents on their side chains (Onodera *et al.,* 1997), while the substituents in the analogues from the dinoflagellate are hydroxybenzoate (Negri *et al.*, 2003; Bustillos-Guzman *et al.*, 2011).

In addition to the analogues discovered in the producing organisms, other toxins have been identified that may be metabolites or degradation products formed in bivalves during the natural purification process. In 2008, during a HAB of *Alexandrium tamarense* some of these toxins were isolated in blue mussels (*Mytilus edulis* and *M. trossalus*) from eastern Canada, named M toxins (Figure 1) (Aversano *et al.*, 2008). Later, during a HAB of *G. catenatum* in Portugal coasts Vale *et al.,* (2010) identified

some of these toxins in mussels (*M. galloprovinciallis*), cockles (*Cerastoderma edule*), and in both estuarine (*Ruditapes decussatus*) and marine clams (*Donax trunculus* and *Ensis spp*.). However, M toxins are not fully characterized due to the small amount that has been isolated from the containing mollusks (Aversano *et al.*, 2008; Vale, 2010).

During HABs of PST-producers, PSTs are incorporated into the food chain through vector organisms such as bivalves and fish, which accumulate the toxins by feeding on the producing algae (Botana, 2000; Wiese, *et al.,* 2010). Other non-filtering species such as crustaceans, squids and gastropods may incorporate toxins into their tissues by feeding on the vectors. Many of these organisms are part of the human diet and have caused poisonings and deaths (Jaime *et al.*, 2007). Paralytic Shellfish Poisoning (PSP) is a severe syndrome that can cause death from cardio-respiratory arrest (Llewellyn, 2006; Wiese *et al.,* 2010). Because of the risk of intoxications, the concentration of PSTs is regulated by health authorities to a limit of 800 μg STXeq kg^{-1} of tissue (Codex Alimentarius Committee, 2008). The presence and accumulation of PSTs is not only a public health problem but also an ecological and economic problem since they can cause the death of marine fauna and the prohibition (sanitary closure) of fishing activities in the affected areas (Wiese *et al.,* 2010).

HABs of PST-producing algae are often associated with mass mortalities of marine wildlife and have been implicated in acute intoxications of marine mammals (Geraci *et al.,* 1989; Reyero *et al.,* 1999; Landsberg, 2002; Starr, 2017). For example, between November 1987 and January 1988, fourteen humpback whales died in Cape Cod Massachusetts. PST-related activity was detected by mouse bioassay in liver, kidney, and stomach content of dead animals. Toxins were also detected in the viscera of mackerels captured from the region of the incidence (Geraci *et al.* 1998). From May to June 1997 over 100 monk seals died in the coast of Mauritania, and PST were detected in organs of some specimens (Hernandez *et al.,* 1998; Reyero *et al.,* 1999). From February to April 2015, the dead of 343 whales stranded on the southern coast of Chile was also associated with PST (Haussermann *et al.,* 2017). The species responsible for producing toxins was not detected during these events, and there was no conclusive evidence that PST caused the mortalities.

The massive mortality of other marine organisms has also been associated to PST poisoning. PSTs have affected numerous bird species, after the mortalities, the toxin content was evaluated, or intoxicating behavior was documented and linked to a HAB of the PSTs producer species. In Washington US, a die-off of approximately 225 birds of nine species occurred in May 1942, bird deaths suspected due to consumption of contaminated small fish and crustaceans (McKernan and Scheffer 1942). In Fame Islands

UK, in 1968 a total of 1999 shags (*Phalacrocorax aristotelis*) died during a HAB of *A. tamarensis*, and 156 shags and other species in 1975 (Coulson *et al.,* 1968; Armstrong *et al.,* 1978). In New Hampshire coast, in mid-September 1972 approximately 1600 duck (*Anas rubripes*) deaths occurred after feeding on toxic shellfish (e.g., *Mytilus spp., Siliqua spp., Ensis spp.*) contaminated by *A. tamarensis*. Maximum toxicity of 400 µg STXeq Kg^{-1} was quantified (Bicknell and Walsh, 1975). In 1974, 12,000 double-crested cormorant (*Phalacrocorax 5lobose*) and red-breasted merganser (*Mergus merganser*) and 20,000 *Lesser scaup* (*Aythya affinis*) died during a *Gymnodinium breve* HAB (Quick and Henderson, 1975; Forrester *et al.,* 1977). Identifying the cause of the death during sea bird mass mortalities is complex, and therefore the number of cases is probably underestimated or are often anecdotal (Shumway *et al.* 2003). Massive mortalities of sea turtles, such as green turtles (*Chelonia mydas*) and olive ridley turtles (*Lepidochelys olivacea*), have also been documented in El Salvador during 2005 and 2010-2017, and have been linked to PST intoxication (Licea *et al.,* 2008; Licea *et al.,* 2013, Amaya *et al.* 2018).

Although PSTs have been detected in affected animals during the occurrence of outbreaks, blooms of the toxin-producing organisms are not detected in the majority of incidents. Therefore, the association of mass mortalities with a causative organism is most often based on indirect evidence. Because of its unpredictability, the confirmation that a HAB is the cause of mass mortalities of marine wildlife is difficult, especially in marine mammals. The most documented case of PST-intoxicated animals is the multi-species mass mortality that occurred in August 2008 in the St. Lawrence Estuary, Canada (Starr *et al.,* 2017). During this event, a bloom of the dinoflagellate *A. tamarense* caused the deaths of ten beluga whales, seven harbor porpoises and 85 seals, fish, birds, and invertebrates (Starr *et al.,* 2017).

1.1.2 Paralytic Shellfish Toxins in the Mexican Pacific

A HAB is a proliferation of phytoplankton cells that can consume oxygen or nutrients in the water, essential for other organisms, affecting the aquatic biota. It can also cause physical damage to other organisms (gill obstruction) and/or cause intoxications through the production of chemical substances (toxins), altering the physiology of the affected organisms. In Mexico, HABs are common events, both on the Pacific coast and on the Gulf of Mexico and the Caribbean Sea. The HABs have increased substantially in the last 20 years, partly due to the greater number of researchers working on the subject, expanding the number of records and study areas, but also due to changes in ecosystems, either by natural or anthropogenic origin

(eutrophication, global warming, transport of organisms, deterioration of the coastal environment, increase in aquaculture activities, use of fertilizers for agriculture, etc.) (Band-Schmidt *et al.*, 2011).

In Mexico, PSP is the most important toxic syndrome related to HABs. It is the only syndrome associated with human deaths (Lewitus *et al.,* 2012), affecting to date 460 individuals and causing 32 deaths (Band-Schmidt *et al.,* 2010; Bustillos-Guzmán *et al.,* 2016; Santiago-Morales, 2016). *Gymnodinium catenatum,* is a PST-producer dinoflagellate that is distributed throughout the Mexican Pacific. It was first described in the Gulf of California (Graham, 1943), where blooms of the species have been observed since 1939. The first relationship between this dinoflagellate and PST was made in 1979, during a HAB which occurred in the Mexican states ranging from Sonora to Jalisco (Band-Schmidt *et al.,* 2010). During this event, cell concentrations of up to $6.6x10^6$ cells L^{-1} and toxicities of 76,400 μg STXeq Kg^{-1} in the clam *Donax sp.* And 17,200 μg STX eq kg^{-1} in the rock oyster *Crassostrea iridiscens* were reported. In addition, 19 intoxicated people, three human deaths, and massive fish mortalities were recorded (De la Garza-Aguilar, 1983; Mee *et al.,* 1986). Since then, other events associated with PSTs have occurred throughout the Mexican Pacific (Bustillos-Guzmán *et al.,* 2016). In November 1989, three people died and 99 were poisoned in the Gulf of Tehuantepec (Oaxaca), an accumulation of 8,110 μg STXeq kg^{-1} in oysters was documented (Saldate-Castañeda *et al.,* 1991). Between August 2001 and February 2002, another HAB caused the accumulation of up to 14,560 μg STXeq kg^{-1} in mussels and the intoxication of 17 people on the Oaxaca coast (Ramírez-Camarena *et al.,* 2004; Santiago- Morales, 2016; Band-Schmidt *et al.*, 2010).

G. catenatum is the species responsible for the accumulation of PSTs during PSP intoxications in the northeastern Mexican Pacific coast (Table 1), while *Pyrodinium bahamense* var. *compressum* is in the south (Lewitus *et al.*, 2012; Bustillos-Guzman *et al.*, 2016; Santiago-Morales, 2016). Most of the blooms recorded of these species have occurred between February and May when the sea water temperature ranges between 17 and 25°C (Gárate-Lizárraga *et al.,* 2004, 2006; Lewitus *et al.,* 2012).

Table 1. Harmful Algal Blooms caused by *Gymnodinium catenatum* recorded in the Gulf of California from 1943 to 2019.

Location	Date	Abundance (cells $L^{1)}$ of *G. catenatum*	Toxicity (µg STXeq Kg^{-1}) and vector species	References
29 °N Gulf of California	1939	~ 1 x 10^{6}	Nd	Graham, 1943
Puerto Libertad, Son.	1981	190 x10^{3}	Nd	Cortés-Altamirano *et al.* 1999
Bahía Concepción, BCS	1990	1.8x10^{2} – 3 x10^{3}	Nd	Gárate-Lizárraga *et al.* 2001
Bahía de La Paz, BCS	1997-1998	1.6X10^{2} – 2.6x10^{2}	4.0-5.6 in *Megapitaria squalida*	Gárate-Lizárraga *et al.* 2004b
Bahía Concepción, BCS	1999	5.7x10^{5}	638 – 2,980 in *Agropecten ventricosus*	Gárate-Lizárraga *et al.* 2004
Bahía Concepción, BCS	2000	500 – 4 x10^{6}	630 in *A. ventricosus*	Morquecho *et al.* 2003 Gárate-Lizárraga *et al.* 2004
Bahía de La Paz, BCS	2001	Nd	20-670 in *A. ventricosus* and PST in phytoplankton net samples	Band-Schmidt *et al.* 2005 Gárate-Lizárraga *et al.* 2006
Bahia Kun Kaak, Son	2003	Nd	Nd	García-Hernández *et al.* 2005
Bahía de La Paz, BCS	2003	1 – 1.2x10^{3}	Nd	Gárate-Lizárraga *et al.* 2006 López-Cortés *et al.* 2006
Bahía de los Ángeles, BC	2006	Nd	30-540 in *Nodipecten subnodosus*	Gárate-Lizárraga *et al.* 2007
Bahía de La Paz, BCS	2006	1 – 3.6x10^{3}	30-45 in *M. squalida* 40-90 in *Dosinia ponderosa*	López-Cortés *et al.* 2007 Gárate-Lizárraga *et al.* 2007b
Bahía de La Paz, BCS	2007	6 – 2.39x10^{6}	4.0–377.4 in *M. squalida, M. aurantiaca, D. ponderosa. Modiolus capax, Pinna rugosa, P. multicostata*	Hernández-Sandoval *et al.* 2009
Bahía de La Paz, BCS	2008	8 – 79x10^{3}	Nd	Gárate-Lizárraga *et al.* 2009
Northern Gulf of California	2015	152 x 10^{3}	16,740 in *Panopea globosa*	Medina-Elizalde *et al.* 2018
Northern Gulf of California	2017	283 x 10^{3}	49,166 in *Panopea globosa*	Medina-Elizalde *et al.* 2018
Northern Gulf of California	2018	15 x 10^{3}	10,500 in *Panopea 7lobose*	This work
Northern Gulf of California	2019	23 x 10^{3}	7,800 in *Panopea globosa*	This work

1.1.3 Mechanism of action of Paralytic Shellfish Toxins

Na^+ channels are voltage-dependent transmembrane proteins responsible for increasing sodium permeability that initiates an action potential (Cestele & Catterall, 2000). The voltage gated Na^+ channel of the mammalian brain is a complex of 3 subunits: α(260 kDa), β1 (36 kDa), and β2 (33 kDa). The α subunit consists of four homologous domains (I to IV), each one containing six transmembrane segments α helix (S1-6) and one reentrant segment (SS1/SS2), the channel pore is formed by *loops* reentrant (SS1-SS2) between the S5 and S6 segments of each one of the domains (Figure 2; Caterall, 2000; Cestèle and Catterall, 2000). The PSTs bind reversibly to these channels, the interaction occurs between guanidine groups charged positively in the toxins and the site 1 of the channel, formed by two amino acids located in SS2 of the N-terminal of the transmembrane segments S6 in each of the four domains (ISS2-S6: E387 and D384; IISS2-S6: E945 and E942; IIISS2-S6: D1426 and K1422; IVSS2-S6: D1717 and A1714) which causes the blockage of the pore (Cestèle and Catterall, 2000). Blocking the Na^+ channel prevents transmission of the nerve impulse in nerve and muscle fibers, leading to neuromuscular paralysis (Bricelj *et al.,* 2005). There is no treatment for PSP; only artificial respiration, fluid therapy, and activated charcoal are provided to help eliminate toxins not yet assimilated from the digestive tract) and wait for reversible symptoms to reduce (Llewellyn, 2006).

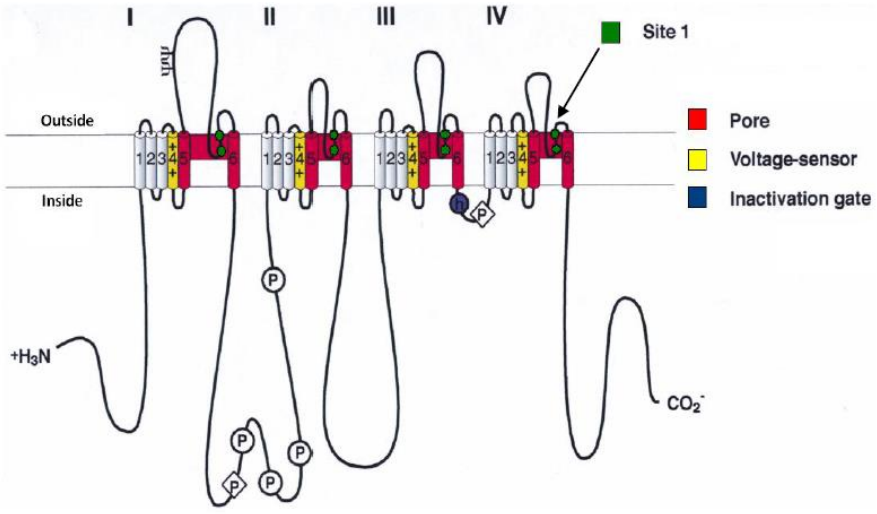


Figure 2. Proposed transmembrane arrangement of the α-subunit of Na^+ channels. Paralytic Shellfish Poisoning is caused by the interaction and blocking of channel site 1 by Paralytic Shellfish Toxins (Modified from Cestèle and Catterall, 2000).

1.1.4 Accumulation, transformation and depuration of Paralytic Shellfish Toxins in bivalves

Bivalves have significant interspecies differences in their ability to accumulate and depurate PSTs. Several feeding experiments have been developed in bivalve mollusks to investigate the rates of accumulation and elimination of PSTs in different tissues. For example, Bricelj *et al.* (2014) studied the accumulation and depuration of PSTs in juveniles of the clam *Spisula solidissima.* In the experiments, clams were fed for 14 days with *Alexandrium fundyense* and PSTs depuration was measured in viscera and siphon for 2.4 months at 5°, 12° and, 21°C. The experiment showed that juveniles accumulated the toxin in the whole organism at approximately $2.2x10^2$ μg STXeq Kg^{-1} per day. After feeding time, the whole organism showed an average concentration of PSTs 31,600 μg STXeq kg^{-1}, 173,500 μg STXeq kg^{-1} in visceral mass, and 9.500 μg STXeq kg^{-1} in siphon, indicating differential accumulation by tissues. Regarding the depuration process, depuration was observed at all three temperatures in the viscera, with a higher depuration rate at higher temperatures. In contrast, in the siphon, toxicity remained constant or increased during the experiment, regardless of temperature, indicating differential depuration.

Curtis *et al.* (2000) described the distribution of PSTs in the siphon, mantle and viscera of *P. generosa* (= *P. abrupta)* naturally contaminated from two localities and two depths on the coast of Washington, USA. During the study, each clam was analyzed separately to describe the intra and inter-population variation in toxin accumulation observed during monitoring programs. Although PST was detected in some mantle and siphon samples, the toxicity in these tissues never reached the regulatory level. Furthermore, up to 19,370 μg of STXeq kg^{-1} were detected in the viscera. The highest toxicity was detected in the shallowest area of one of the locations. Differences were observed between individuals from the two depths and two locations. The authors attributed these differences to factors such as differences in feeding rates, food availability due to vertical and horizontal depth gradients, reproductive status, individual sensitivity to PSTs, variation in body mass and the “shown factor”, which refers to the fact that at a given moment only 70% of the clam population will have their siphon sticking out of the sand, although they are not necessarily feeding.

Several studies (e.g., Cembella *et al.*, 1993, 1994; Shumway *et al.*, 1994) conducted on the accumulation of PSTs have concluded that in most bivalves the visceral mass contains between 80% and 98% of the total toxicity during the maximum point of intoxication. Tissues involved in the locomotion as foot, abductor and pallial muscles reach two to three times lower toxicities than viscera and contain less than 1% of total toxicity despite their high relative mass in some species (Bricelj & Shumway, 1998).

Depuration rates are also different among bivalve species, with no clear relationship between the amount of toxin incorporated and depuration rates (Bricelj & Shumway, 1998). Bricelj & Shumway (1998b) developed a classification of the species regarding their detoxification kinetics, which is based on detoxification rates or percentage of toxin loss per day. The detoxification rate is calculated by fitting the toxin concentration values over time to an exponential decay model with formula $y = ae^{-bx,}$ where *y* is the toxicity, *x* the detoxification time in days, *a* the initial toxicity (maximum detected), and *b* the amount of toxin removed per day. According to this scheme, species that detoxify quickly or moderately have depuration rates of between 6 and 17% of toxin loss per day and reach the regulatory limit between 1 and 10 weeks after the maximum intoxication point and depending on the amount of toxin assimilated. While slow detoxifiers take between three months and two years to reach the regulatory limit with rates in the range of 0.3-4% loss of toxin per day. This model may underestimate the time required to reach the RL in some clam species, such as *Saxidomus giganteus* or *Spisula solidissima*. These species show a process of detoxification of two phases, a rapid initial phase followed by a more slowly phase or exponential. It has been suggested that the initial phase represents the elimination in the stomach of non-assimilated toxins. In contrast, the second phase represents the release of assimilated toxins and the toxins incorporated into the tissues (Bricelj & Shumway, 1998b).

As previously mentioned, PSTs are all analogous of saxitoxin. The toxin profile of PST-producer dinoflagellates contains some of these analogues depending on the species, the strain, and the environmental conditions in which the organism grows (Band-Schmidt *et al.* 2014). For example, *Gymnodinium catenatum* is a chain-forming dinoflagellate distributed in tropical waters in many world regions (Hallegraeff *et al.,* 2012). The species produces at least 21 saxitoxin analogues (Negri *et al.,* 2007). Band-Schmidt *et al.* (2014) analyzed the effect of the temperature on growth, cellular toxicity, and toxic profile in isolates of this species from different locations in the Mexican Pacific. The authors found that the culture temperature modifies the growth rate, cell density, and toxic profile. Regarding the toxic profile, at 16°C, 80% of the molar content of PSTs was due to toxins C1&2, while at 33°C these toxins only contribute 20% of the molar total.

The toxin profile on dinoflagellates is sometimes different from that in bivalves, which indicates an active metabolism of mollusks (Cembella *et al.,* 1994). The transformation of PSTs *in vitro* has been documented. Sullivan *et al.* (1983) observed the transformation, from N- sulfocarbamoyl toxins (B1, C1, and C2) to toxins designated as STX-M, GTX-IIIM, and GTX-IIIM (because they had longer retention times than the carbamate type) in the viscera of the clam *Protothaca sraminea*. In this tissue, conversions occurred in approximately 4 hours, while in mantle, muscle, and siphon transformation rates were lower.

The new compounds were up to 25 times more toxic than the original toxins. The authors concluded that the transformation involves enzymatic hydrolysis of the carbamate group (Figure 3).

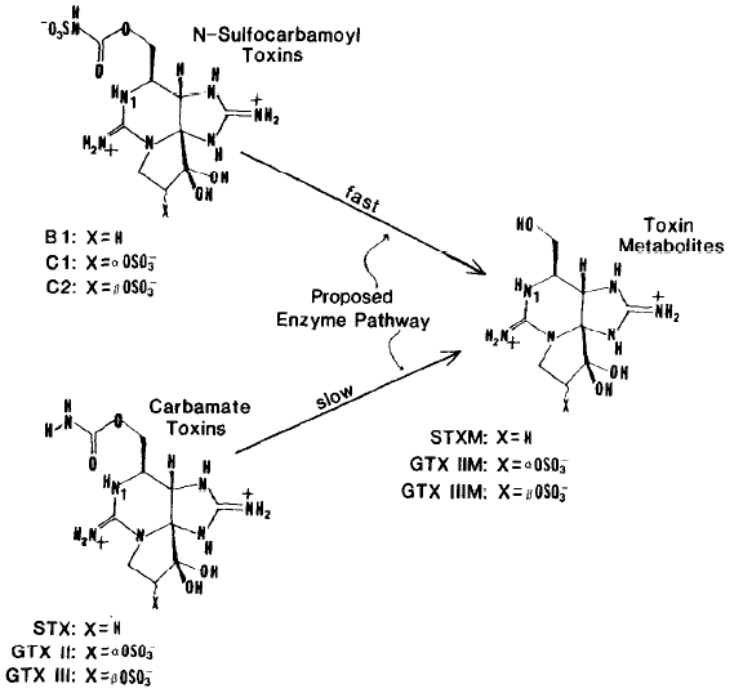


Figure 3. Metabolic transformations in the *Protothaca sraminea* clam involving hydrolysis of the carbamate group (Taken from Sullivan *et al.,* 1983).

Jaime *et al.* (2007) studied PSTs transformations in 10 species of mollusks: eight filter feeders, one carnivore, and one herbivore considering that the latter two do not obtain the PSTs directly from the producing microalgae. They found that the transformations into toxins by reduction, epimerization, and hydrolysis can be attributed to bacterial activity or to molluscan enzymes. In addition, they observed the highest conversion rates in digestive tissue. Depending on the species, there was a decrease in toxin concentration between 20% and 73% within 48 hours. In conclusion, they mention that some species, such as the herbivorous snail *Littorina littorea* and the clam *Cardium edul*, have efficient depuration mechanisms, converting PST into less toxic or more easily excreted metabolites. While other species like the oyster *Crassostrea gigas* and the clam *Mia edulis* have limited transformation and depuration capacity. However, Sullivan *et al.* (1983), Choi *et al.* (2003), Jaime *et al.* (2007) and Turner *et al.* (2013) have observed that in some bivalve species, especially clams, there is PST transformation into more toxic analogues, which increases the toxicity of edible parts and therefore the risk to human health.

Changes in the toxic profile in bivalve tissues have been suggested to be due to selective retention or elimination of individual toxins, epimerization or biotransformation processes such as reduction in the presence of natural reductants, hydrolysis at low pH, or enzymatic conversion (Bricelj & Shumway, 1998b, Figure 4).

Although there is information on the accumulation, transformation, and depuration of PST in some bivalves, the metabolism of these toxins in the geoduck clam *P. 12lobose,* an important resource in the NGC, is unknown.

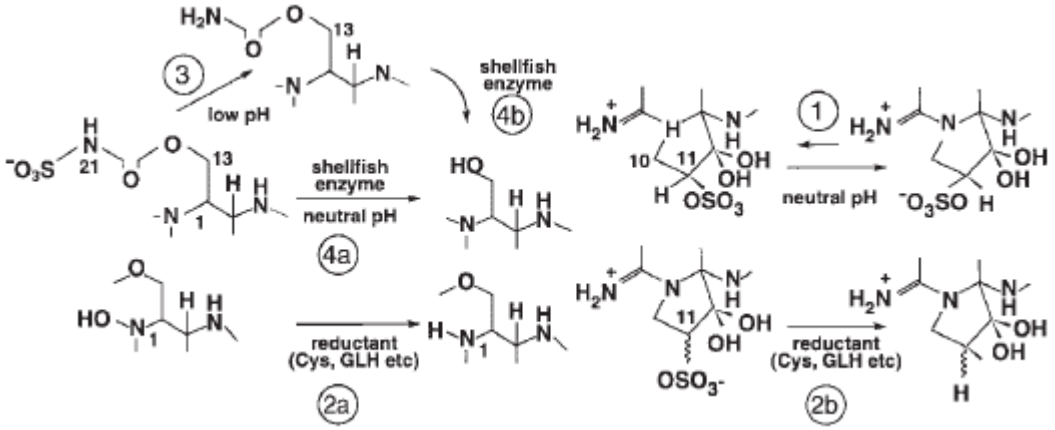


Figure 4. Scheme of the transformations of Paralytic Shellfish Toxins that occur *in vivo* in bivalves. 1. Epimerization of β to α Epimers. 2. Reduction (a) of GTX1 & 4 and NEO to GTX2 & 3 and STX, (b) of GTX1 & 4 and 2 & 3 to STX and NEO in the presence of sulfhydryl reducers such as cysteine and glutathione. 3. Acid hydrolysis. 4. Enzymatic hydrolysis (decarbamoylation): conversion of N- sulfocarbamoil (4a) or carbamate (4b) toxins to decarbamoyl toxin derivatives (Bricelj & Shumway, 1998 b).

1.1.5 Geoduck clam *Panopea globosa* (Dall, 1898)

Two species of clams of the *Panopea* genus are found on the Pacific coast of Mexico: *P. generosa* (Gould 1850) distributed from Alaska to Baja California (Goodwin and Pease 1987) and *P. 12lobose* (Dall 1898) endemic to the Gulf of California and distributed as far as Bahía Magdalena in Baja California Sur Pacific (Aragon-Noriega *et al.,* 2012; Hendrickx *et al.,* 2005). The genus *Panopea* is commonly known as "geoduck", "chiluda", or "siphon clam" (Ramírez-Félix *et al.,* 2012).

P. 13lobose is a large size species reaches a gross weight of up to 2 kg and 1 m siphon length, 197 mm in shell (right valve), and between 158 mm and 52 mm in height (Figure 5; Pérez Valencia, 2009). During the first 10 to 15 years (Strom *et al.,* 2004) its growth is fast, but growth rates vary considerably with temperature, substrate, and the depth to which it is exposed (Campbell *et al.,* 2004).

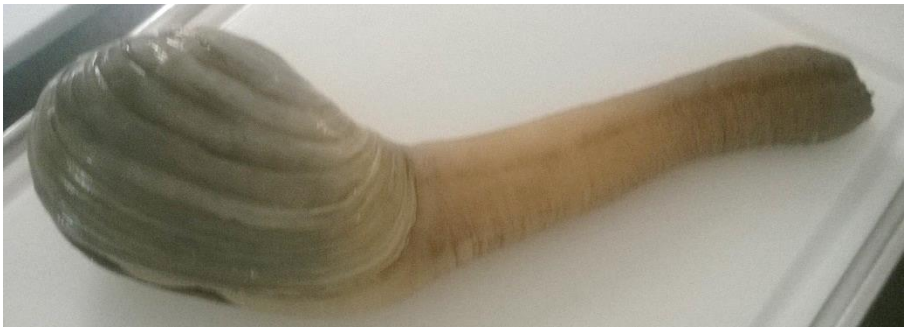


Figure 5. Geoduck clam *Panopea globosa*.

Geoduck clams live buried up to one meter deep in sandy and muddy substrates from the low intertidal zone down to depths of 110 m. It is considered one of the longest-lived species, reaching up to 146 years (Sloan and Robinson, 1984). The average age varies from one area to another, from 26.6 to 60.4 years (Bureau *et al.,* 2002).

1.1.6 Economic importance of the geoduck clam *Panopea globosa*

The geoduck clam has a high commercial value in the Asian market, as well as a high demand (DOF, 2012). In recent years, their fishing and export have generated profits of more than US$30 million (Rocha Olivares *et al.,* 2010), which makes it very attractive for local producers and is an important source of employment (Ramírez Félix *et al.,* 2012). The *P. 13lobose* clam has a lower price (40%) than *P. generosa.* The beachfront price ranges from two to four dollars per kilogram, depending on the access to fishing areas. The cost is established according to the distance to the harvesting areas and the productivity of the divers (Ramírez Félix *et al.,* 2008).

In the late 1990s, this clam was first detected in the Gulf of California. In 2002, the first fishing permits were granted in Sonora and later in Baja California for prospective purposes. In 2004 were allowed commercial fishing activities, and the first statistical records about the extraction of the species were made in 2005, and in 2007 its Fisheries Management Plan (PM) was published (DOF, 2012).

Geoduck represents one of the most important fisheries in Baja California. From 2008-2015, the average production was 1,460 tonnes per year, where *P. 14lobose* extraction represented 78% of the total (SEPESCABC, 2016). Of the total production, San Felipe has prominent relative importance, since it contributes between 42% and 59% of Baja California's production (Ramírez Félix *et al.,* 2012). The main marketing channel is the sale of the whole live product packaged in boxes of six to 12 clams in each. Most of the product is transported to Los Angeles, California, USA and from there to Asia (DOF, 2012).

1.1.7 Harmful algal blooms in the Northern Gulf of California

On January 13, 2015, national newspapers reported the presence of approximately 500 birds and four sea lions died on beaches near the town of San Felipe, BC. Reports of epizootics continued for several weeks. Days after the first epizootic, COFEPRIS detected the accumulation of high concentrations of PSTs in the geoduck clam (13,873 µg STXeq Kg^{-1} COFEPRIS, 2015), a species on which it maintains continuous monitoring as part of the Programa Mexicano de Sanidad de Moluscos Bivalvos (PMSMB) and due to the extraction activities that occur in the area. As a result, a sanitary ban was implemented on all bivalve species harvested in the region, and the recently extracted organisms were destroyed to prevent their commercialization and avoid human intoxications. Following the guidelines of the PMSMB, from the detection of the HAB, COFEPRIS intensified the monitoring of phytoplankton and phycotoxins in all the bivalve mollusks extraction zones in the NGC.

In January 2017, a new HAB of *G. catenatum* occurred in the area. The health authority detected toxin concentrations up to 178 times higher than the RL in geoduck clam (142,117 µg STXeq Kg^{-1}), this event was called "the most toxic red tide to date". Consequently, a sanitary closure was implemented for six months in all harvesting areas and intermittently the rest of the year, even in November 2017 some areas certified for the extraction of generous clam were closed (COFEPRIS, 2017).

In January 2018 and 2019, HABs of *G. catenatum* were also recorded in the NGC, although without the notorious effects that in previous events, in these years the closures were implemented for a few weeks in the different extraction areas.

1.1.8 Methods of detection of Paralytic Shellfish Toxins

For more than 60 years, the mouse bioassay (Mouse bioassay, MBA) was the method for determining toxicity in marine bivalves throughout the world. This has been the reference methodology in Mexico since 1993 when the regulation of these toxins was implemented (NOM-031-SSA1-1993). However, the expansion of the distribution limits of toxic algae, the cost of this method and its limitations, as well as the tendency to eliminate the use of live animals for routine analysis, have led to the development of alternative detection methods (Layock *et al.,* 2010, Turner *et al.,* 2011).

Bioassays are a direct measure of the toxicity of a sample regardless of its composition (the analogues present), while alternative methods usually comprise the quantitation of toxin concentrations, from which the total toxicity is calculated or inferred using Toxicity Equivalence Factors (TEFs). One of the alternative methods to MBA involves the use of high-performance liquid chromatography (HPLC) with Florescence Detection (FLD). To reduce the use of experimental animals and to have accurate methodologies for the routine monitoring of phycotoxins, the European EC 2019/627 established in January 2009 the AOAC Official Method of Analysis (OMA) 2005.06 precolumn oxidation HPLC-FLD (Pre-COX-LC-FLD or *Lawrence* method) as the official methodology in European Union (Turner *et al.,* 2019) to monitor PSTs toxins in bivalves. Countries such as the United Kingdom, the Republic of Ireland, Portugal, and Germany have implemented the methodology, as well as countries exporting bivalve mollusks to Europe, such as New Zealand and Chile (Turner *et al.,* 2019). In the USA, the AOAC 2011.02, post-column oxidation (HPLC-PCOX) was incorporated into the National Sanitation Shellfish Program (NSSP) in 2011 as an official methodology together with MBA (ISSC, 2012). Both of these HPLC-FLD methods were extensively validated before their implementation in the sanitary legislation of these countries (AOAC 2005.06: Turner *et al.,* 2011a, b; Turner *et al.,* 2014; Ben-Gigirey *et al.,* 2012; AOAC 2011.02: Van de Riet *et al.*, 2009; 2011). Other countries such as Korea (Song *et al.,* 2013) and China (Qiu *et al.,* 2020; Yue *et al.,* 2020), have also increased efforts to develop and validate these methodologies.

The disadvantage of the HPLC methods is that they require complex and expensive instrumentation, for example, the FLD or mass spectrometry (MS), as well as certified reference standards for preparation of working instrumental calibrants and TEF conversion factors to transform concentration data into toxicity values (Layock *et al.,* 2010).

Other alternatives are antibody assays, such as ELISA (Enzyme Linked Immuno Sorbant Assay) and immunochromatographic lateral flow (LFIA), which indirectly measure toxicity. A major disadvantage of these methods is that different cross-reactivities exist between different toxin analogues (Layock *et al.,* 2010). Consequently, an accurate measurement can only be performed if the toxin profile of the sample is known, and the appropriate mixture of analogues is used. If only one analog is used for calibration, the determinations will not be correct in samples containing other analogues, with significantly different cross-reactivities, which normally occurs in naturally contaminated tissues. Antibodies may be useful in bivalve monitoring only as general detection methods since the quantification of total toxicity is imprecise (Layock *et al.,* 2010).

An alternative to ELISA is lateral flow immunochromatography, from which the National Research Council (NRC) of Canada and the company Jellett Rapid Testing Ltd. (currently Scotia Rapid Testing) developed rapid tests for the detection of PSTs. In immunochromatography, the PSTs present in a sample competitively bind with saxitoxin in a test strip against anti saxitoxin (Turner *et al.,* 2012). With these tests, the analyzes can be performed in the field, outside the laboratory, with the minimum of material and infrastructure, and generating results a few minutes after extraction. This makes these tests a simple, economic, and fast method. The disadvantages are that they are not quantitative results, they only indicate if the concentration of toxins is below the detection limit (LOD). Another significant disadvantage is that due to cross-reactivity and specific toxicity, the detection limit is variable since it depends on the mixture of toxins present in the sample (Layock *et al.,* 2010).

Scotia Rapid Testing was developed as a screening method for regulatory labs to eliminate negative samples (Laycock *et al.,* 2001). However, in performance studies worldwide (Costa *et al.*, 2009; Laycock *et al.*, 2010; Turner *et al.,* 2015b; Dorantes-Aranda *et al.,* 2017), the kit showed false positives in between 50 and 98%. Due to the high percentage of false positives, the authors concluded that the methodology is not suitable for official control testing in direct replacement of the mouse bioassay.

In 2011, the USA NSSP included the SRT to the list of Approved Limited Use Methods for Marine Biotoxin Testing. As a result, the test could be used a) to determine when to perform a mouse bioassay in

a previously closed extraction area, b) as a substitute for a mouse bioassay to maintain an area in the open status after a negative result, and c) to establish a precautionary closure after a positive result (ISSC, 2012).

In 2015, Mexican health authorities incorporated Scotia Rapid Testing into the national shellfish sanitation program (Programa Mexicano de Sanidad de Moluscos Bivalvos, PMSMB) to monitor PSTs in shellfish. The use of STR was implemented to homologate the PMSMB with the USA NSSP. USA and Mexico are the only countries that use STR in their official biotoxins monitoring programs. The performance of STR has been studied on different shellfish samples from Alaska (Costa *et al.*, 2009), the cost of Canada (British Columbia) and USA (Alaska and Maine) (Laycock *et al.*, 2010), UK (Turner *et al.,* 2015b; Harrison et al., 2016) and New Zealand (Dorantes-Aranda *et al.,* 2017). These studies concluded that the high number of false positives (between 50 and 98%) make this methodology unsuitable for official control testing in direct replacement of the mouse bioassay.

In Mexico, analytical methods are not used in the regulatory monitoring program of PSTs in bivalve mollusks, and the SRT is routinely used. Still, its performance was not evaluated before its implementation. SRT closed its Canadian operations in February 2021, according to its website, “Production and sales of the SRT tests is being passed to AquaBC which is run by Dr David Cassis”. To this day, the use of SRT remains the screening methodology in the Mexican regulation, Programa Mexicano de Sanidad de Moluscos Bivalvos (https://www.gob.mx/cofepris/documentos/manual-de-laboratorios-del-pmsmsb).

1.2 Justification

HABs have become more frequent and intense in recent decades (Wiese *et al.,* 2010; Band-Schmidt *et al.,* 2011), becoming a challenge for health authorities who must protect public health and commercial interests at the same time (Laycock *et al.,* 2010).

In particular, HABs of the PST producer dinoflagellate *G. catenatum* have become recurrent in the north Gulf of California. The first sanitary ban in the region was implemented due to the accumulation of toxins in *P. globosa* just after COFEPRIS began monitoring PSTs in 2010. HABs in the NGC have occurred every year after 2015 causing epizootics, PSP cases, and affecting coastal socioeconomic activities due to implemented sanitary bans that have caused important losses to the geoduck fishery.

Management of the occurrence of PSTs in the NGC requires the generation of knowledge in different aspects of HAB phenomenology. For example, the accumulation, depuration, and transformation of PSTs in wild and commercial important species of the region must be evaluated. Also, the ecological impact of PSTs in the NGC region must be documented. It is necessary to generate information on the temporal appearance of toxins in the environment their causal agent, and the susceptibility of species to saxitoxin and its analogues. Also, the attention of problem requires robust toxin detection methods that generate reliable results to support monitoring programs. Overall, this information is necessary to establish schemes to mitigate the negative impact of the recurrent presence of PSTs in the NGC.

This work aimed to describe the effects of the Paralytic Shellfish Toxins on marine mammals, seabirds, and geoduck fisheries in the NGC during 2015-2019. The origin and impact of PSTs on wildlife and commercially important species were discussed. Also, the performance of different PSTs detection methods was evaluated, and the depuration and transformation of these toxins in the geoduck clam were characterized. The information generated is expected to develop toxin management schemes in this region to protect public health and reduce losses in coastal economic activities.

1.3 Objectives

1.3.1 Main Objective

Describe the effects of Paralytic Shellfish Toxins on marine mammals, seabirds and geoduck fishery in the Northern Gulf of California during 2015-2019.

1.3.2 Specific objectives

1. Describe the effect on wildlife of the presence of Paralytic Shellfish Toxins in the Northern Gulf of California during a Harmful Algal Bloom that occurred in January 2015

2. Characterize the transformation and depuration of Paralytic Shellfish Toxins in naturally contaminated geoduck clams *Panopea globosa*

3. Compare HPLC-PCOX and Scotia Rapid Testing methods with mouse bioassay for the detection and quantification of Paralytic Shellfish Toxins in geoduck clam tissues

4. Evaluate the impact of the presence of PST in the Northern Gulf of California during the period 2015-2019.

Chapter 2. Material and Methods

2.1 Effect of Paralytic Shellfish Toxins on wildlife in the Northern Gulf of California during a Harmful Algae Bloom that occurred in January 2015

In January 2015, a sampling campaign was carried out in the NGC after detecting a *G. catenatum* HAB. This campaign aimed to estimate the number of affected organisms and identify the cause of their death. In six sampling transects, established along 80 km of beach from San Felipe to Puertecitos, BC, all dead seabirds and mammals were counted and their geographical position marked (Figure 6). One dead pelican *Pelecanus occidentalis* and two blue-footed boobies *Sula nebouxii* were collected and necropsies were performed to obtain the stomach content, crop content and the organs of the digestive system. In addition, fish close to the one of the collected boobies that were probably regurgitated by it were collected. Organs, stomach, and crop contents were frozen at -20 °C until analysis by high performance liquid chromatography with post -column oxidation (HPLC-PCOX) according to Van de Riet *et al.* (2011).

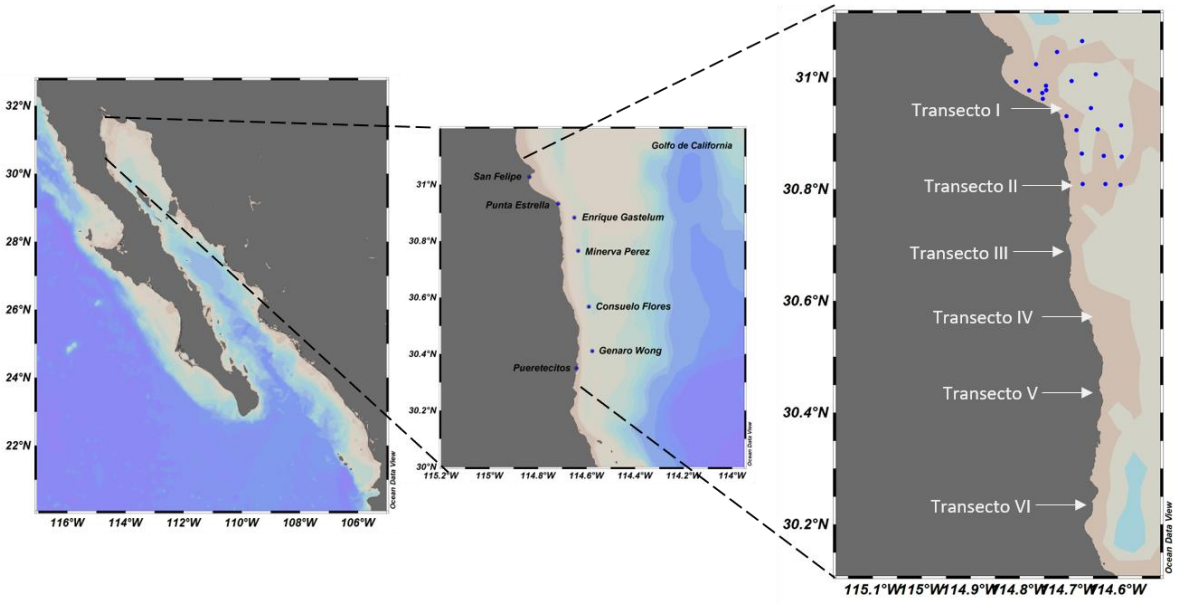


Figure 6. Location of the study area in the Northern Gulf of California. B. Location of four geoduck extraction areas, the sampling campaign at sea included the Minerva Pérez and Enrique Gastelum areas, the coastal sampling campaign occurred in front of four extraction areas. C. Transects implemented in the San Felipe-Puertecitos coast during January 2015 Harmful Algae Bloom.

Days after the sampling campaign, PROFEPA recovered and donated the fresh carcass of a common dolphin (*Delphinus capensis*) in which a necropsy was performed to recover all the organs. Organ samples from other dolphins were obtained by donation from different groups studying the HAB at the same time. Organs were frozen at -20 °C until analysis by high performance liquid chromatography with post -column oxidation (HPLC-PCOX) according to Van de Riet *et al.* (2011).

To extract the toxins, tissues were defrosted and homogenized in a blender. Five grams of each tissue homogenate were mixed with 5 ml of 0.1 M HCl and boiled for 5 minutes. After cooling to room temperature, samples were centrifuged at 3000 g for 10 min and an aliquot of 500 µl of the supernatant was recovered and deproteinized with 35 µl of a trichloroacetic acid (TCA) 30% solution. The pH was adjusted to 3 before and after the boiling step with 5 M NaOH or 5 M HCl. The pH was also adjusted after the addition of the TCA with 25 µl of 0.1 M NaOH. The aliquot was subsequently filtered through a 45 µm pore size nylon membrane filter before injection onto the HPLC-PCOX system.

Quantification of PSTs by HPLC–PCOX

The AOAC 2011.02 method was implemented to extract and quantify PSTs in shellfish tissue samples. The analysis was performed using an Agilent Technologies 1100 model HPLC (Agilent, USA) calibrated with dilutions prepared from eight certified reference standards obtained from the Institute of Biotoxin Metrology, National Research Council Canada (NRC, Halifax, Nova Scotia, Canada). For C toxins (C1&2) 5µl of each sample was injected onto an Agilent Zorbax SB-C8 (250 x 4.6 mm, 5 µm) HPLC column. For GTX toxins (GTX1&4, GTX2&3, dcGTX2&3, GTX5) and STX toxins (NeoSTX, STX, dcSTX) 10 µl of each sample were injected onto an Agilent Zorbax Bonus-RP (150 x 4.6 mm, 3.5 µm) column. After the chromatographic separation, samples were oxidized on-line in a Vector PCX derivatization instrument (Pickering Labs Inc., USA) and detected by fluorescence. Excitation was at 330 nm and emission detection at 395 nm. When the concentration of PSTs in samples was higher than the calibration curve concentration range, extracts were diluted with a 0.1 M HCl (pH 3) solution. The dilution factor was considered in the calculation of PSTs concentration.

The total toxicity of each sample was calculated by multiplying the concentration of each toxin, the molecular weight of STX diHCl and the toxicity equivalent factor (TEF) of each analog according to Oshima (1995; Table 2).

Table 2. Toxin equivalent factors (TEFs; Oshima, 1995) applied for quantitation and semi quantitation of PST analogues.

Analog	TEF	Analog	TEF	Analog	TEF
C1	0.01	**GTX2**	0.4	**dcNEO**	0.4
C2	0.1	**GTX3**	0.6	**STX**	1.0
C3	0.02	**GTX1**	0.99	**NEO**	0.9
C4	0.1	**GTX4**	0.7	**M1**	0.1[c]
dcGTX2	0.15	**GTX5**	0.06	**M2**	0.3
dcGTX3	0.4	**GTX6**	0.1	**M3**	0.1[c]
dcGTX1	0.5[a]	**doSTX**	0.05[b]	**M4**	0.3[d]
dcGTX4	0.5[a]	**dcSTX**	0.5	**M5**	0.1[c]

[a]dcGTX1 and dcGTX4 based on assumed toxicity equivalency factors (Sullivan, 1983)

[b]doSTX toxicity equivalency factor (Harwood *et al.*, 2014)

[c]Toxicity factors derived from EFSA GTX5 and GTX6.

[d]Toxicity factors derived from EFSA 11OH-STX.

2.2 Transformation and depuration of Paralytic Shellfish Toxins in naturally contaminated geoduck clams *Panopea globosa*

There are approximately 20 *Panopea* harvesting areas in the NGC (Figure 6). The laboratory FICOTOX has been monitoring the phytoplankton community intermittently since 2011 in surface water samples collected from a harvesting area located south from San Felipe, Baja California, Mexico (30°39'30" – 30°50'00" N and 114°34'00" – 114°41'15" W; Figure 7). This area is a federal concession Minerva Perez for geoduck extraction. After the detection of the bloom, water samples and geoduck clams were collected every week from January to June of 2015.

The phytoplankton community was evaluated in water samples collected at surface and from 10 m depth vertical net hauls. 250 ml of water were placed in dark plastic bottles (Nalgene type) and fixed with 1 to 2 mL of a concentrated lugol-acetate solution. This solution is recommended for routine evaluation of the phytoplankton community with the Utermöhl technique (Sournia, 1978). Lugol-acetate increases the settling velocity of the microalgae, can be used for the majority of phytoplankton species without damaging the cells and has low toxicity to humans compared with other fixatives (Andersen & Throndsen, 2004). The Utermöhl technique (Sournia, 1978) was used to identify and to enumerate phytoplankton cell abundance. 10 to 25 ml of the sample were sedimented for at least 24 h and the complete surface of the sedimentation chamber was analyzed with a LEICA DMI3000B (Leica Microsystems, Germany) inverted microscope. The abundance of *G. catenatum* (in cells L^{-1}) was calculated

according to the sedimented volume. A minimum of 500 cells were counted to estimate the relative abundance of *G. catenatum* to total phytoplankton cells (in %) present in samples collected from net hauls.

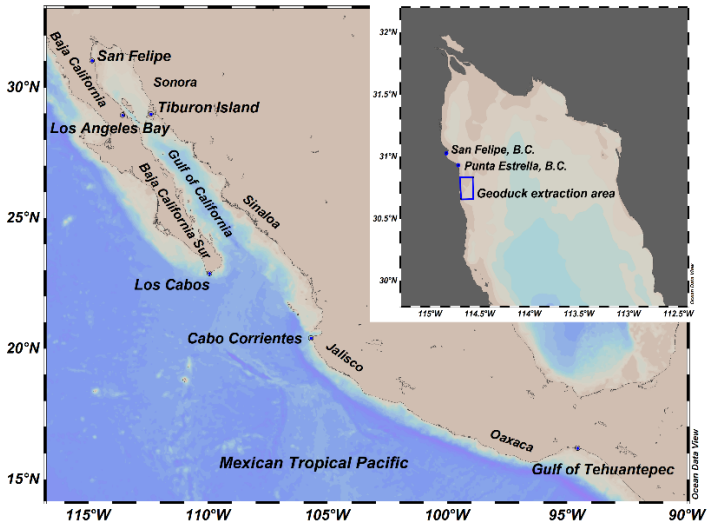


Figure 7. Gulf of California and Mexican Tropical Pacific where outbreaks of Paralytic Shellfish Toxins have been documented. The northern part of the Gulf of California and the location of federal approved (Minerva Perez) area for harvesting of *Panopea globosa* in San Felipe, BC, are presented in the inset.

Geoduck clams, collected by scuba diving at 12 to 20 m depth, were maintained in cool boxes and kept at 12° to 16 °C until delivery to the laboratory. Samples were transported to the FICOTOX laboratory less than 24 h after the collection. Due to internal company paperwork problems, some organisms could not be delivered to the laboratory after field sampling. Organisms collected on June 7 and 29, July 2 and 25, and August 7 and 8 were kept alive in the company's tanks unfed at 18°C prior to delivery of samples to the laboratory. These samples arrived at the laboratory no more than eight days after collection in the field.

Once in the laboratory, the organisms were dissected. Toxin content was evaluated both in the visceral mass containing all the organs of the geoduck and in the siphon, the muscular part of the organism. To obtain a representative sample and in the accordance with the recommendations of the national sanitary regulation for PSTs monitoring in geoduck (Secretaría de Salud, 2016), each sample consisted of three individuals. The tissues of the three dissected organisms were pooled, homogenized in a blender and kept frozen at -20°C until analysis by high performance liquid chromatography with post column oxidation (HPLC-PCOX) according to Van Riet *et al.* (2011, as previously described).

Quantification of PSTs by HILIC – MS/MS

Ten selected samples collected at different dates were also analyzed by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry (HILIC-MS/MS). After the centrifugation step the sample was lyophilized and sent for analysis to the Centre for Environment, Fisheries and Aquaculture Science (Cefas). There, the samples were subjected to semiautomated solid phase (SPE) clean-up using Supelclean ENVI-Carb 250 mg/3 ml SPE cartridges (Sigma-Aldrich, St. Louis, MO). Sample extracts were loaded onto individual carridges, desalted by flushing with deionized water and the toxins eluted and collected through the addition of 2 ml 20% acetonitrile (MeCN) + 0.25% acetic acid at 3 ml min^{-1}. SPE eluents were then vortex-mixed prior to dilution of 100 µl aliquots in 700 µl Verex polypropylene autosampler vials (Phenomenex, Manchester, UK) with 300 µl LC-MS grade MeCN.

HILIC-MS/MS was conducted according to Boundy *et al.* (2015) using a 1.7 µm, 2.1 × 150 mm Waters (Manchester, UK) Acquity BEH Amide UPLC column in conjunction with a Waters VanGuard BEH Amide guard cartridge. 2.0 µl were injected into a Waters Acquity UPLC I-Class coupled to a Waters Xevo TQ-S tandem quadrupole mass spectrometer (MS/MS). The HILIC-MS/MS PST method was calibrated with nine STX analogues with certified reference standards obtained from NRC (C1&2, dcGTX2&3, GTX2&3, GTX1&4, GTX5, dcSTX, dcNEO, NEO, STX) plus four reference materials (C3&4, GTX6, doSTX and tetrodotoxin TTX) from Cawthron Natural Compounds (CNC, New Zealand) and CIFGA laboratories (Lugo, Spain). Levels of toxins with no reference standards, such as dcGTX1&4 and M-toxins, were semi-quantified using the response factor of the nearest structural analog with a certified standard and applying a relative response factor (RRF, Boundy *et al.*, 2015) (1.8 for dcGTX1, 1.93 for dcGTX4 and 1 for M toxins, Turner *et al.*, 2015). In the absence of relative toxicity data for M-toxins, TEFs were taken from structurally similar PSTs analogues (Table 2).

Data analysis

An exponential decay function was fitted to the change of PSTs in time. Also, a lineal model and an exponential rise to a maximal regression model were fitted to the PSTs present in the siphon in relation to the concentration of these toxin in the visceral mass. The analysis was done with SigmaPlot 11.0 (Systat Software Inc.).

2.3 Comparation of HPLC-PCOX and Scotia Rapid Testing methods with mouse bioassay for the detection and quantification of Paralytic Shellfish Toxins in geoduck clam tissues

35 of the viscera homogenates from the previous experiment were used to perform this experiment. In addition to the HPLC-PCOX analysis performed above, the 35 samples were analyzed by Mouse Bioassay according to AOAC 958.08, and Scotia Rapid Testing according to manufacturer's instructions following the AOAC extraction method.

Quantification of PSTs by MBA

To extract the toxins, 100 grams of pooled viscera homogenate were mixed 1:1 with 0.18 M HCl and boiled for 5 minutes. After cooling to room temperature, the samples were centrifuged at 3000 g for 10 minutes. The supernatant was recovered and 1 ml injected intraperitoneally to each of 3 male mice Hsd:ICR weighing 18-20 g. Mice were observed for one hour with death time recorded and used to quantify the total sample toxicity.

Detection of PSTs by Scotia Rapid Test

The supernatant recovered for the mouse bioassay was also used to perform the Scotia Rapid Test. 100 µl of the supernatant were mixed with 400 µl of the loading buffer of the Scotia Rapid Test kit. The mixture was loaded into the cassette and interpreted after 35 minutes according to the manufacturer's instructions. For interpretation, the color of the T (test) and C (control) bands on the resulting strip was compared with the color of the bands on the test card provided with the kit. The sample is negative if the T-line is darker than the positive result in the card. The sample is positive if the T-line is equal to or fainter than the Positive result on the card.

According to the results of the analysis by HPLC-PCOX, 16 samples were selected to perform detection by Scotia Rapid test. Scotia Rapid Test claims sensitivity of 200 to 700 µg STXeq Kg^{-1} of shellfish tissue. After the initial analysis by Rapid Test, each sample was diluted with HCl 0.003 M pH 3 to get concentrations of 800, 400, 200 and 100 µg STXeq Kg^{-1} and loaded on the cassette following previous described instructions. Each test was interpreted by two analysts in a blind analysis. Considering the regulatory limit of 800 µg STXeq Kg^{-1}, a false positive was indicated when the analyst pointed the sample

as a positive and the toxin concentration calculated by HPLC-PCOX was below to the regulatory limit. A false negative was indicated when the analyst marked the sample as a negative and the toxin concentration calculated by HPLC-PCOX was above to the regulatory limit.

Data analysis

A linear model was fitted to the PSTs concentration calculated by HPLC-PCOX and MBA using SigmaPlot 12.0 (Systat Software Inc.).

2.4 Impact of the presence of Paralytic Shellfish Toxins in the Northern Gulf of California during 2015-2019

In 2010 COFEPRIS began a monthly monitoring of the PSTs in geoduck clam harvested in the certified extraction areas of the NGC (Figure 8). According to the Programa Mexicano de Sanidad de Moluscos Bivalvos, COFEPRIS obtains samples from each approved area and quantifies PSTs by MBA. After the HABs from 2015 to 2019, COFEPRIS increased the frequency of the monitoring and collected samples weekly. The data from this monitoring were obtained and normalized to the highest toxin concentration per year. Then, the normalized values were plotted with the ODV v 5.2.1 software to observe the distribution of toxicity over time. The DIVA function included in the software (Data-Interpolating Variational Analysis) was used to interpolate the data.

Phytoplankton relative and absolute abundances reported by Laboratory FICOTOX since 2011, PSTs concentration in clams reported by COFEPRIS since 2015, and PSTs concentration in clams calculated by HPLC-PCOX in this work were plotted to link the presence of the dinoflagellate and the toxin accumulation in clams. Plots were made using the software SigmaPlot v. 12.

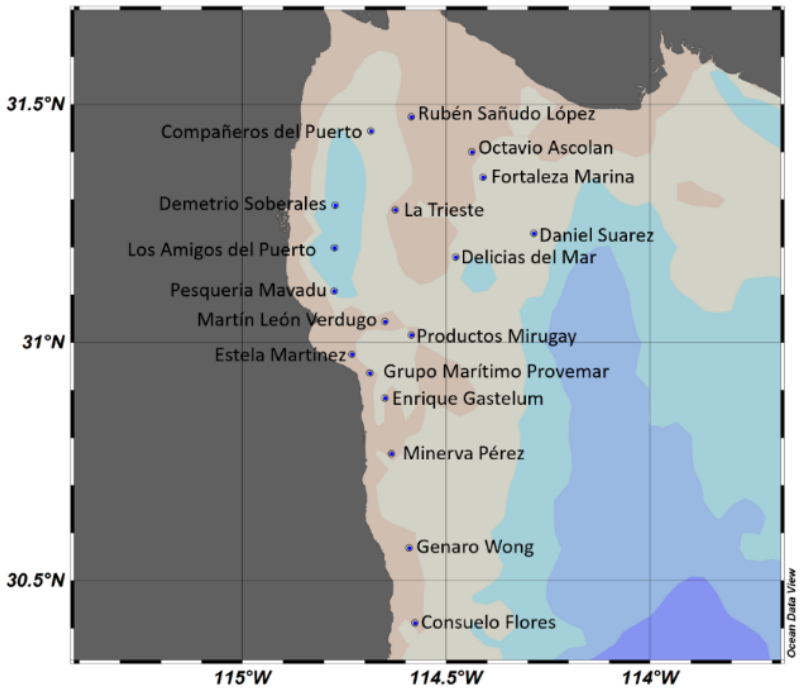


Figure 8. Location of the certified areas for geoduck clam extraction in the northern Gulf of California (the areas are polygons of different dimensions. The point and coordinates indicates the approximate center of each polygon).

Chapter 3. Results

3.1 Effect of Paralytic Shellfish Toxins on wildlife in the Northern Gulf of California during a Harmful Algae Bloom that occurred in January 2015

After the HAB detected in January 2015 in the NGC, the number of stranded animals in six transects in the coastline from San Felipe port to Puertecitos was documented. 467 seabirds and 25 dead marine mammals were detected (Table 3; Figure 9). Loons (*Gavia immer, Gavia sp*.) represented more than 80% of dead seabirds, followed by pelicans (*Pelecanus occidentalis*) and boobies (*Sula sp., Sula nebouxii*). A scoter (*Melanitta sp*.) and an unidentified molluscivorous duck were also found. The majority of the marine mammals affected were common dolphins (*Delphinus capensis*), although a Risso's dolphin (*Grampus griseus*) was also identified. In general, dolphin's carcasses were found in advanced states of decomposition. In the case of birds, fresh carcasses were observed and some still floating in the sea (they were not considered in the count of affected animals). In a bird resting area, were observed at least 5 birds that behave abnormally of the approximately 30 specimens that were on the site. Among the observed behaviors, one of the pelicans in that group was unable to support its head and coordinate its movements to have a controlled flight. A boobie was lying on the ground with minimum response to stimulus. In general, the affected organisms presented dyspnea, mydriasis and paralysis, and died approximately 30 min later. The estimate of the affected organisms after the monitoring campaign in January 2015 in approximately 80 km of coastline, assuming a heterogeneous distribution, is 11,648 (SD = 6,718) seabirds and 186 (SD = 90) marine mammals, which represents 146 birds km $^{-1}$ (SD = 84 km $^{-1}$) and 2.3 dolphins km $^{-1}$ (SD = 1.1 km $^{-1}$).

After this sampling campaign, reports of affected organisms continued during January with no records during February. However, a second period of mass mortalities occurred in March. From March 8 to 10, the research vessel Martin Sheen of the Sea Shepherd Conservation Society registered 33 dead dolphins from the Vaquita's refuge to San Luis Island. On March 11, PROFEPA found another 55 dolphins and four California sea lions stranded south from San Felipe to El Caracol fishing camp located 108 km south from this port. The FICOTOX lab conducted a new sampling campaign on March 13, registering 91 dolphins and a whale (*Balaenoptera sp.*) in 18.5 km of beach line south from San Felipe. The total number of specimens reported by different entities from January to March was 1,017 seabirds and 244 mammals (Table 3; Figure 9).

Table 3. Affected seabirds and marine mammals reported between January and March 2015 in the Northern Gulf of California.

Date	Organisms	Number of organisms	Location	Source and Comments
01/01/15	Dolphins	one	San Felipe	INECC. Recently stranded organisms
01/13/15	Seabirds California sea lion	550 4	Beaches south of San Felipe (Transect I)	PROFEPA. The remains of seabirds were removed from the beach by PROFEPA
01/17/15	Sea birds Dolphins	(?) 9 (3.4 km $^{-1}$)	Transect I 30°57‘26.03” N 114°45‘49.92” W	This work. The remains of seabirds were removed from the beach by PROFEPA
01/17/15	Sea birds Dolphins	189 (261 km $^{-1}$) 1 (1.4 km $^{-1}$)	Transect II 30°56‘24.26” N 114°43‘39.77” W	This work. Necropsies were performed on 3 loons and one boobie
01/17/15	Sea birds Dolphins	154 (153 km $^{-1}$) 4 (4 km $^{-1}$)	Transect III 30°56‘12.60” N 114°43‘14.52” W	This work. Necropsy of a pelican was performed
01/18/15	Sea birds Dolphins	15 (150 km $^{-1}$) 3 (2.2 km $^{-1}$)	Transect IV 30°22‘11.78” N 114°38‘33.71” W	This work. Dead marine count was performed on 100 m of beach
01/18/15	Sea birds Dolphins	14 (140 km $^{-1}$) 3 (1.7 km $^{-1}$)	Transect V 30°58‘17.96” N 114°41‘55.40” W	This work. Dead seabird count was performed on 100 m of beach
01/18/15	Sea birds Dolphins	95 (24 km $^{-1}$) 5 (1.24 km $^{-1}$)	Transect VI 30°49‘0.26” N 114°42‘0.28” W	Transect of 4 km traveled by sandpit vehicle
01/19/15	*Delphinus capensis*	one	PROFEPA San Felipe	PROFEPA. Recently stranded organism donated for necropsy
01/19/15	*Balaenoptera* sp.	One	In front of the Port of San Felipe	PROFEPA. Found floating
01/20/15	Dolphins	4	From the south of San Felipe to Punta Estrella	INECC and PROFEPA.
01/24/15	Dolphins	12	San Felipe	PROFEPA. PROFEPA and UABC performed necropsies of 8 organisms and obtained urine samples
01/24/15	California sea lion	4	San Felipe	PROFEPA
03/08/15 to the 03/10/15	Dolphins	33	Floating in different locations, from the vaquita marina refuge to the island of San Luis	Sea Shepherd Conservation Society
03/12/15	Dolphins California sea lion	55 4	South of San Felipe	PROFEPA
03/13/15	Dolphins *Balaenoptera* sp.	67 one	San Felipe to Punta Estrella (11 km) 6.09 km^{-1}	INECC and PROFEPA
03/14/15	Dolphins	24	7.5 km south of Punta Estrella, transect of 3.2 km^{-1}. A dolphin was floating in the vaquita marina shelter	INECC, CICESE and PROFEPA Stool samples were obtained from 2 organisms
03/17/15	Dolphin *Balaenoptera sp.*	8 one	South of the Gulf of Santa Clara, Sonora.	Information provided to INECC by José Haro Rodríguez. RB Upper Gulf of California and Colorado River Delta

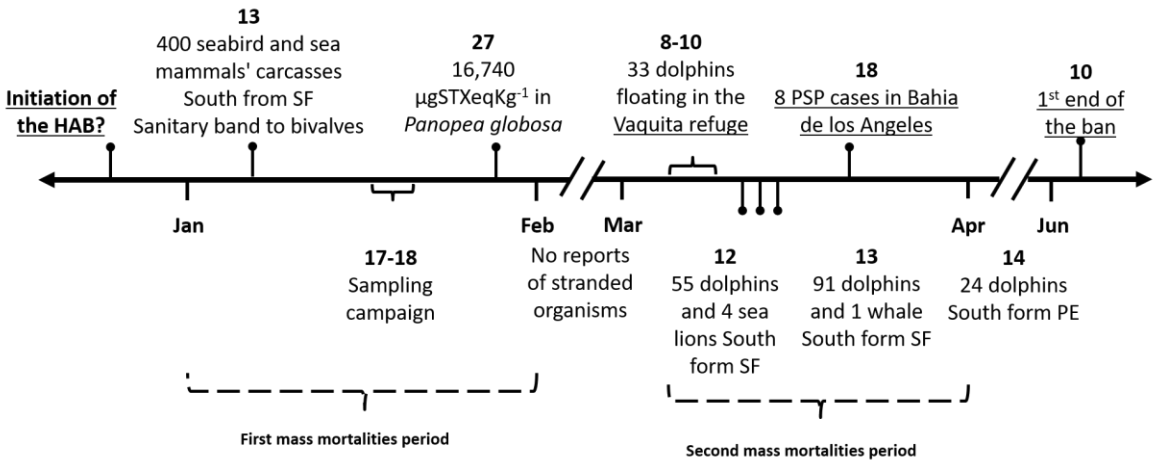


Figure 9. Events related to the 2015 *Gymnodinium catenatum* Harmful Algae Bloom that occurred in the Northern Gulf of California.

Biological material was collected during the January 2015 campaign, and necropsies were practiced in one dolphin and three seabirds when the degree of decomposition of the carcasses permitted this approximation. The presence of different phycotoxins was evaluated in these samples. The PST concentration calculated by HPLC-PCOX in tissues of dolphins, birds, and undigested fish from the crop of the birds or regurgitated by the boobies is detailed in table 4.

It was not possible to sample different organs in all the dolphins analyzed due to the degree of decomposition of the carcasses. In seven dolphins' kidneys collected in January (six males and one female) and intestine from two males, PSTs were detected in low concentrations. GTX4, Neo, GTX2 and STX were the main analogues identified in these samples (Table 4). In faeces of three dolphins collected in March, up to 3,500 µg STXeq Kg^{-1} were quantified, the main analogues identified in the samples were STX, GTX1&4 and Neo. A complete necropsy of one young female dolphin collected in January was performed, where PSTs were detected in 6 organs. The highest PST concentrations were found in the stomach (1,675 µg STXeq Kg^{-1}) and its content (2,168 µg STXeq Kg^{-1}), the analogues presented in the samples were STX, dcSTX and GTX2 (Figure 10).

Table 4.Concentration of Paralytic Shellfish Toxins in biological material collected during the 2015 Harmful Algal Bloom in the Northern Gulf of California.

Sample	µg STXeq Kg^{-1}	Main PST analogues
Seabirds – January 17, 2015		
Seabird 1 (loon, *Gavia immer*) – crop content	3,081	STX > dcSTX
Seabird 1 - stomach	354	dcSTX > STX > GTX4
Seabird 1 - liver	166	Neo > GTX2
Seabird 1 - intestine	97	dcSTX > GTX2
Seabird 2 (boobie, *Sula nebouxii*) – stomach	309	Neo > GTX2
Seabird 2- gut content	2,875	STX > dcSTX
Seabird 2 - liver	69	GTX2
Seabird 2 – intestine	596	STX > GTX4
Seabird 2 – gonad	Not detected	
Seabird 2 – kidney	98	dcSTX > GTX2
Seabird 3 (pelican, *Pelecanus occidentalis*) – gut content	2,143	STX > GTX1&4 > GTX2
Regurgitated fish	6,624	GTX5 >Neo > dcSTX
Dolphin (*Delphinus capensis*), female – January 19, 2015		
Content of the stomach	2,168	STX > dcSTX
Stomach	1,675	STX > GTX2
Intestine	75.1	GTX2 > GTX5 > dcSXT
Liver	70.5	STX > dcSXT >GTX2
Lungs	11.5	STX
Kidney	2.8	STX
Brain	Not detected	
Heart	Not detected	
Dolphins (*Delphinus capensis*) - January 24, 2015		
DH1 (Dolphin, female) – kidney	7.48	GTX4 > Neo
DM1 (Dolphin mal) – intestine	5.96	GTX4 > STX
DM1 (Dolphin male)- kidney	2.08	Neo > GTX2
DM2 (Dolphin male) – kidney	6.72	GTX4 > Neo
DM3 (Dolphin male) – intestine	6.14	GTX4 > STX
DM3 (Dolphin male) – kidney	1.98	Neo > GTX2
DM4 (Dolphin male) – kidney	5.26	GTX4 > Neo
DM5 (Dolphin male) – kidney	6.57	GTX4 > Neo
DM8 (Dolphin male) – kidney	6.21	GTX1 > Neo
Dolphin, March 14, 2015		
Dolphin 1 feces	2,536	STX > GTX1 > GTX2
Dolphin 2 feces	3,480	STX > GTX1 > GTX4
Dolphin 3 feces	2,976	SXT > NEO > GTX1

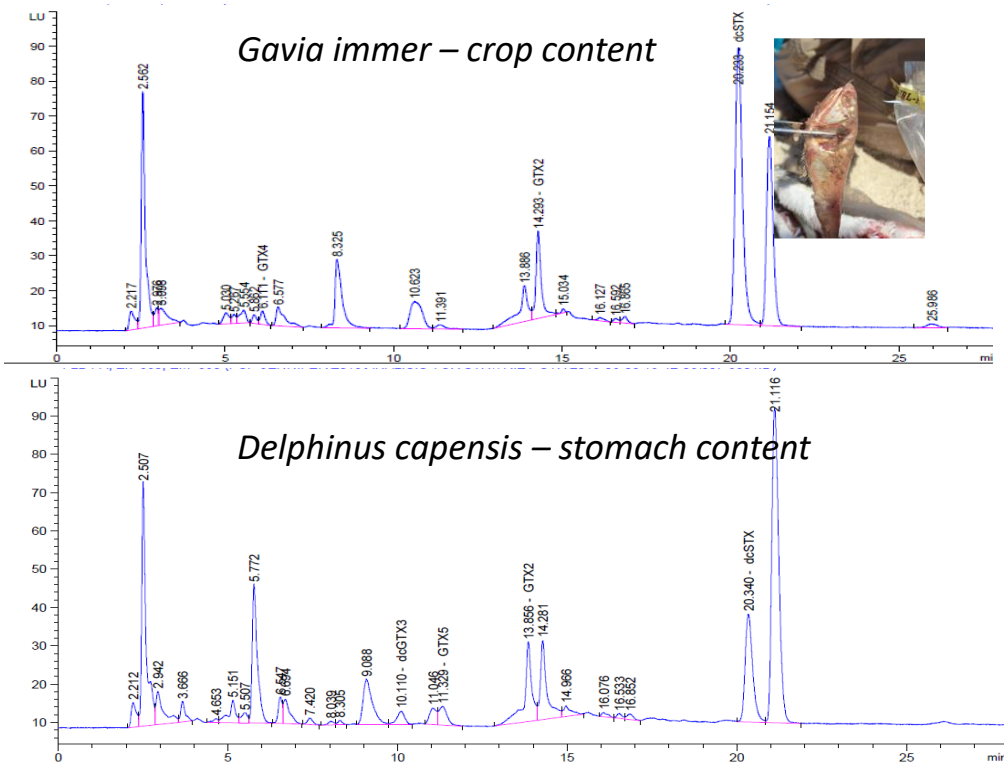


Figure 10. Paralytic Shellfish Toxin profile of the loon (*Gavia immer*) gut content (undigested anchovies) and of the stomach content of the dolphin (*Delphinus capensis*). The most abundant peaks after minute 20 are dcSTX and STX.

Necropsies were also conducted in a loon, a pelican and a boobie found severely affected in a bird-resting site. The highest PST concentrations were detected in the crop content of the loon with 3,081 µg STXeq Kg^{-1}, boobie with 2,875 µg STXeq Kg^{-1}, and stomach content of the pelican 2,143 µg STXeq Kg^{-1}. The main analogues present on these samples were STX and dcSTX in the loon and boobie, and STX, GTX1&4 and GTX2 in the pelican (Figure 10). The toxicity was also high in regurgitated fish collected in the boobies resting area, GTX5, Neo and dcSTX were the main analogues presented and the total toxicity was 6,624 µg STXeq Kg^{-1}.

In all the seabirds analyzed, the crop was full of undigested fish (sardine "bocona", *Cetengraulis mysticetus*). The comparison of the toxin profile of the fish samples collected from the gut indicates that this species were the principal vectors of the PST found in loons and dolphins (Figure 10). Saxitoxin and decarbamoylsaxitoxin (dcSTX) constituted more that 90 % of the PST detected in fish samples collected from the crop of loons and boobies, and stomach of dolphins.

G. catenatum produced the PST detected in dead animals and fish. In January 17 and 18, *G. catenatum* was patchily distributed in the sampling area from surface to 20 m depth (Figure 11). The highest abundance was 266 x 10^3 cell L^{-1} detected at surface close to the coastline in front of Punta Estrella area (Figure 11B). Also, a high abundance was detected sub superficially northwest and southwest form this site (Figure 11). *G. catenatum* was present between 5 to 15 m in these areas with abundances from 15 x 10^3 to 150 x 10^3 cell L^{-1}. The high accumulation areas of *G. catenatum* seemed to extend and be more disperse northwesterly from Punta Estrella towards the center of the Gulf (Figure 11). In contrast, the accumulation of phytoplankton biomass was more concentrated and located closer to the coast with an extension of approximately 80 km south from Punta Estrella. The extent of bloom was inferred according to the January 17 Modis-Aqua image assuming that the satellite derived Chlorophyll a (Chlsat) concentration was mainly associated with the presence of *G. cateatum* (Figure 11).

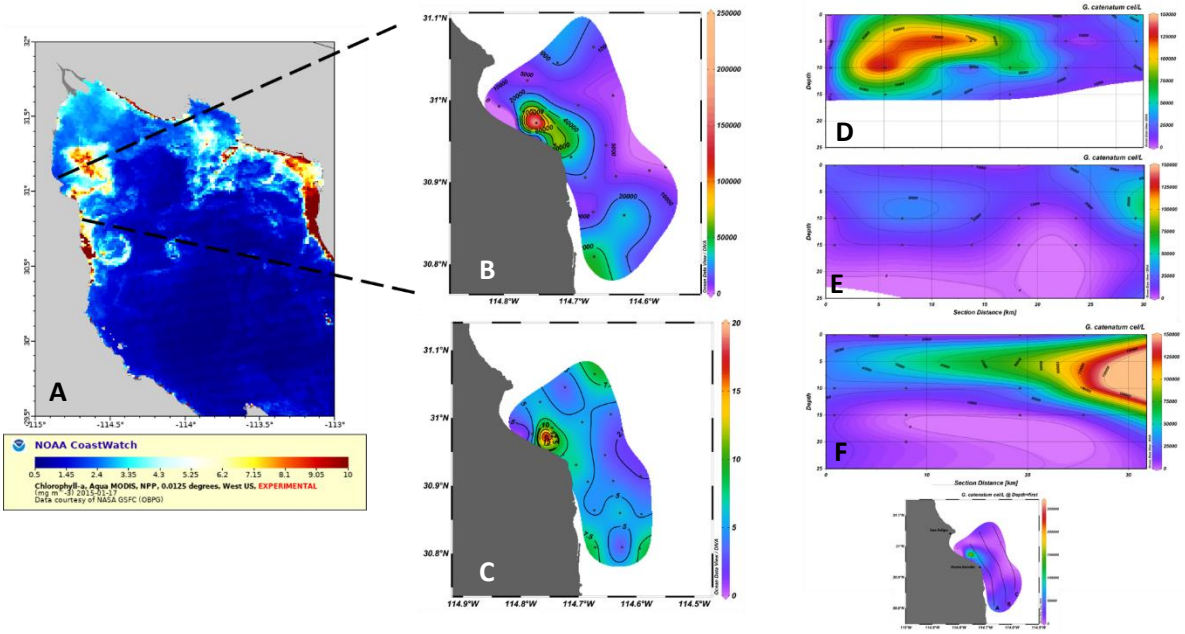


Figure 11. Distribution of *Gymnodinium catenatum* in January 17, 2015 in 18 sampling points. A. Modis-Aqua image. B. Distribution of *G. catenatum* at the Surface. C. Distribution of *G. catenatum* sub superficially. D-F Vertical distribution of *G. catenatum* according to three transects parallel to the coastal line.

The distribution of PST in particulate matter followed the distribution of *G. catenatum*. Maximum PST in particulate matter was 36 µg PST L^{-1} and was associated with the maximum *G. catenatum* cell abundance registered in front of Punta Estrella (Figure 12). High concentrations of PST were also present subsurfacely (Figure 12). The average cellular PST content was 50 pg $cell^{-1}$ equivalent to 3.06 pg STXeq $cell^{-}$

[1]. C toxins represented the major proportion of PST (95% of total molar contribution) and STX and dcSTX concentrations were low or undetectable in phytoplankton samples.

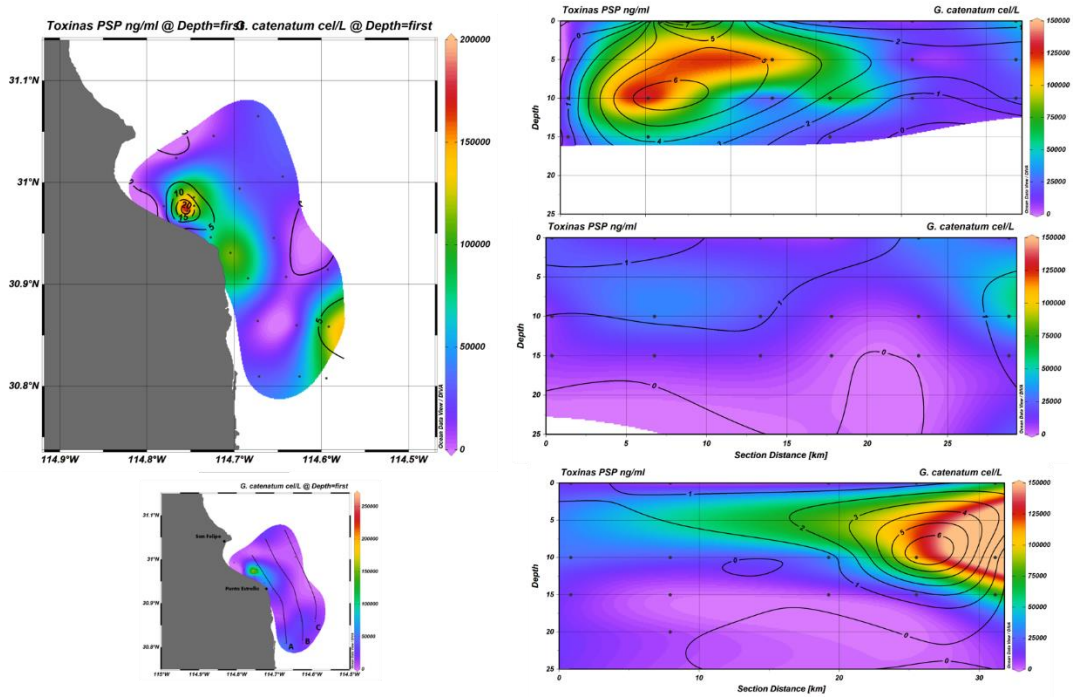


Figure 12. Distribution of PST in particulate matter in January 17, 2015 in 18 sampling points. A. Distribution of PST in particulate matter at the Surface. B-D Vertical distribution of PST in particulate matter according to three transects parallel to the coastal line.

3.2 Transformation and depuration of Paralytic Shellfish Toxins in naturally contaminated geoduck clams *Panopea globosa*

Gymnodinium catenatum abundance

The phytoplankton community was monitored intermittently from 2011 in surface water samples collected every two weeks or every month in the *P. globosa* extraction area located south of San Felipe. In general, *G. catenatum* was present from December to April, and surface abundances were no higher than $10x10^3$ cells L^{-1} before 2015. On January 14, 2015, the abundance of this dinoflagellate reached $152x10^3$ cells L^{-1}. From this date, surface and vertical net tow water samples were analyzed weekly. One week after the maximum abundance was detected, the presence of *G. catenatum* decreased to $9.3x10^3$ cells L^{-1}. Detection of this species at the water surface continued in the following weeks, and a second abundance

peak was registered on February 24 ($18.5x10^3$ cells L^{-1}). After this date, this species disappeared and was detected again in April and May at abundances lower than $3.8x10^3$ cells L^{-1} (Figure 13A).

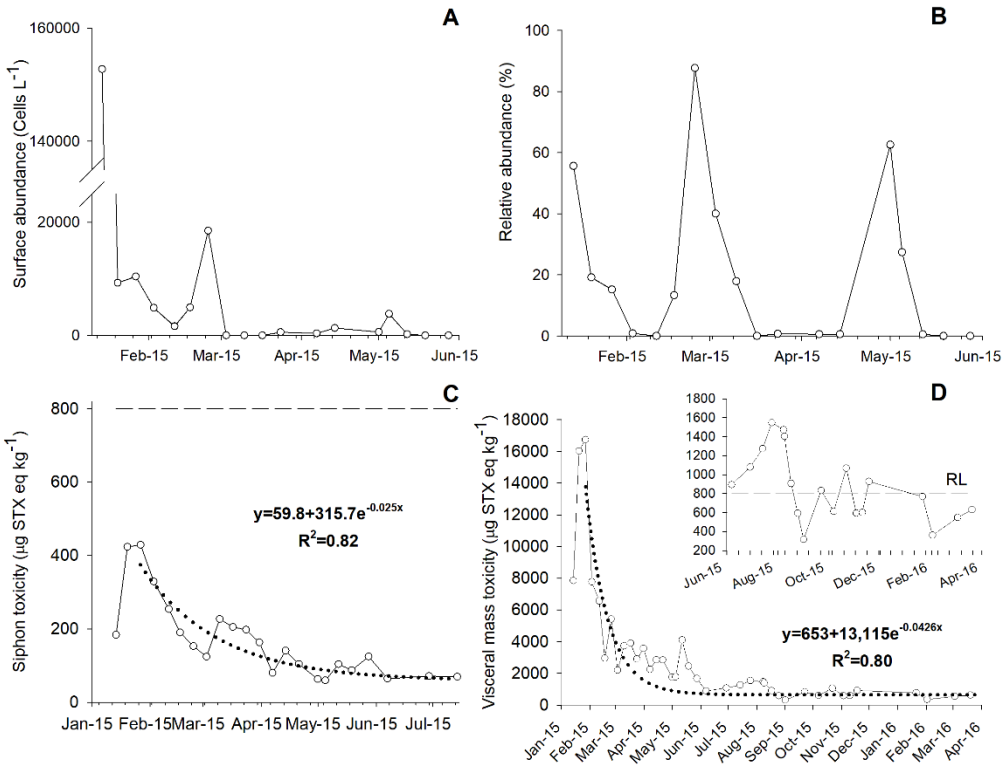


Figure 13. Surface (A) and relative abundance (B) of *Gymnodinium catenatum* during 2015 in the northern region of the Gulf of California. Toxicity associated with paralytic shellfish toxins in the siphon (C) and visceral mass (D) of geoduck samples collected from January 2015 to April of 2016. Dotted line shows the fitted of exponential decay function to the data and the dashed line is the regulatory limit (RL) of 800 µg STX eq kg^{-1}.

The abundance of *G. catenatum* assessed in the vertical net tow samples clearly showed the presence of the bloom in the area (Figure 13B). The relative abundance of this species (abundance of *G. catenatum* to total phytoplankton cells in the net sample) was 55% on January 14 and decreased in the following weeks. Relative abundance increased in February with a maximum of 87%. *G. catenatum* was detected in the net tow samples until March 10, disappearing after that. It was detected again on May 1 with a relative abundance of 62%. *G. catenatum* appeared in net tow samples on May 12 for the last time with a relative abundance of 27% (Figure 13B).

Toxicity in the siphon and visceral mass of *P. globosa*

The visceral mass contains the gut, the digestive gland, gonads, heart, kidney, and gills. Although this part of the clams is rarely consumed, the viscera mass is the target tissue for assessing PSTs concentration in geoduck clams. The muscle (siphon) is the part of the organism that is preferentially consumed. In both tissues, PST concentrations, as well as their transformation and depuration were evaluated. Toxin concentrations were determined in 23 siphon and 40 viscera samples collected in 2015. Also, toxin concentration in nine samples collected in 2017 were considered to analyze the relationship between siphon and viscera toxicity (see below).

PSTs were present in the siphon, although concentrations in all samples were below the regulatory limit of 800 µg STXeq kg^{-1} (Figure 9C). The highest concentration of PSTs was 429 µg STXeq kg^{-1} detected on January 27 and subsequently decreased. From the quantification of the maximum PSTs accumulation, the lowest concentration was detected after five months with 60 µg STXeq Kg^{-1} (Figure 13C). Therefore, no further siphon samples were processed after July. An exponential decay model was fitted to the data describing the reduction in toxicity level over time. The coefficient of determination (R^2) was 0.82 and the calculated depurating rate of PSTs in the siphon was 2.5% of toxin loss per day (Figure 13C).

P. globosa accumulated PSTs mainly in the visceral mass, with a maximum concentration of 16,740 µg STXeq kg^{-1} in January. This concentration represented 21 times the regulatory limit (RL; Figure 13D)., maintaining toxicity above the RL for approximately eight months. 210 days after the detection of the maximum concentration (August 25) only one sample was detected with a concentration below 800 µg STXeq kg^{-1}. However, toxicity increased again in the following weeks and fluctuated around the RL for the remainder of 2015. The last sample processed for the study was collected in March 2016 with a concentration of 629 µg STXeq kg^{-1}.

The change in total PSTs in viscera over time was also fitted to an exponential decay model (R^2=0.80). The maximum PSTs concentration detected on January 27 was the first data used in the analysis (Figure 13D). The calculated depuration rate was 4.3% of toxin loss per day. According to this, the PSTs concentration should have reached the RL 106 days after the maximum concentration was detected.

PSTs in the siphon was related to the concentration of these toxins in the visceral mass (Figure 14). PSTs concentrations detected in viscera and siphon after another HAB that occurred in January 2107 were also considered in this analysis. In January 24 of 2017, *G. catenatum* surface abundance reached 283 x 103

cells L^{-1} in the *P. globosa* extraction area. The relative abundance of *G. catenatum* in net tow samples was 87% on this date.

Following the 2017 HAB, concentrations above 800 µg STXeq kg^{-1} were detected in three siphon samples (Figure 14). An exponential rise to a maximal value function explains 78% (R^2 = 0.78) of the variance between PSTs present in the siphon in relation to the presence of these toxin in the viscera. Regression analyses were performed considering different models (lineal, exponential, polynomial). However, the sample with a toxicity in the visceral mass of 49,166 µg STXeq kg^{-1} and 1,063 µg STXeq kg^{-1} in the siphon was discarded because it was considered an outlier. PSTs in the siphon were linearly related (best fit) to the concentration detected in viscera below 20,000 µg STXeq kg^{-1} (R^2=0.72; Figure 14). According to this model, toxicity in viscera was 23.8 times higher than detected in siphon. Importantly, the toxicity in the siphon will exceed the RL when toxicity in visceral mass reaches 18,424 µg STXeq kg^{-1}.

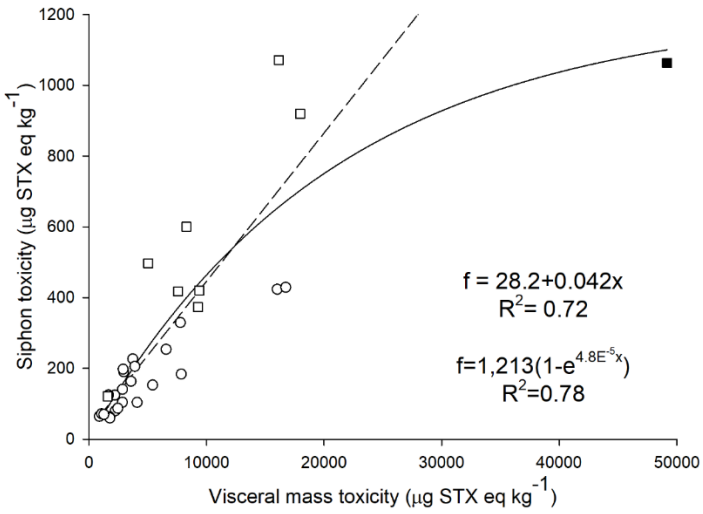


Figure 14.Relation between calculated toxicity of the siphon and visceral mass in geoduck samples collected during 2015 (circles) and 2017 (squares). The filled square shows the highest paralytic shellfish toxin concentration detected in viscera in 2017. This point was omitted (outlier) when a linear regression analysis was applied (R^2 = 0.72, dashed line; see text). An exponential rise to a maximal function was used for the regression analysis considering all data (R^2 = 0.78, solid line).

Paralytic shellfish toxins in the siphon and visceral mass of *P. globosa*

To characterize the accumulation and biotransformation process of PSTs it is important to evaluate the analogues present in the samples. Therefore, we evaluated the concentrations of twelve PSTs

analogues in both the siphon and visceral mass samples (Figure 15). Both tissues contained the major analogues C1&2, GTX2&3, GTX5, dcGTX2&3 and dcSTX. We detected trace amounts of GTX4, Neo and STX in some viscera samples, while STX and Neo were not detected in any of the siphon samples.

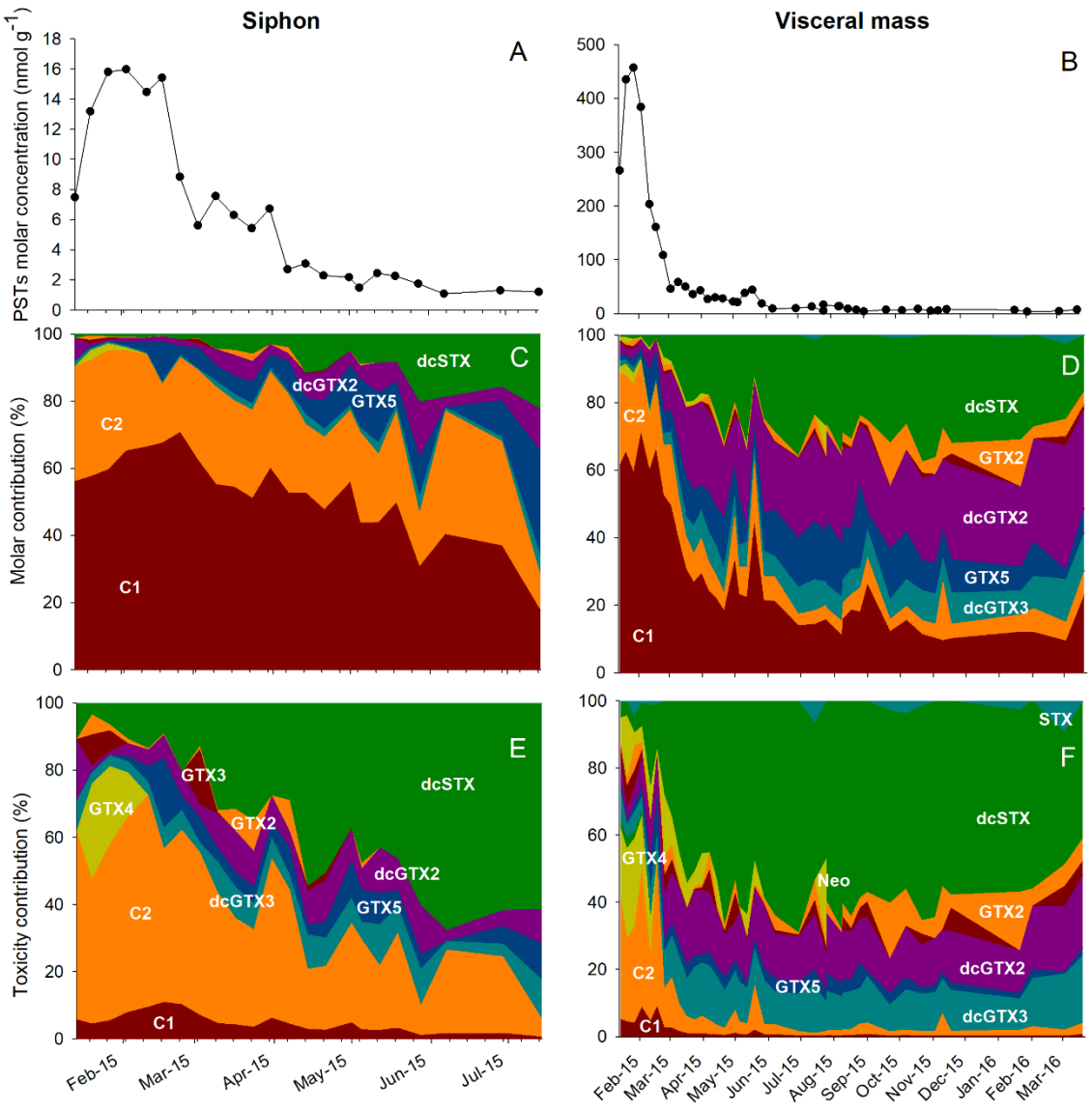


Figure 15. Paralytic toxins in the siphon (A,C,E) and in the visceral mass (B,D,F) of geoduck samples collected from January 2015 to April of 2016. The paralytic toxins molar concentration (nmol g^{-1}) is presented in A and B. The molar contribution (in %, C,D) and toxicity contribution (in %, E,F) of different paralytic toxins analogues detected during the sampling period is also shown. The toxicity of each analog was calculated according to the toxin equivalent factors shown in Table 2.

The molar content in the siphon was 16 nmol g^{-1} in February and decreased to 1 nmol g^{-1} in June 2015 (Figure 15A). C1&2 were the prevalent toxins in all the samples throughout the monitoring period. These analogues represented 95% of the total PSTs content in the siphon samples during February and decreased to 28% at the end of the sampling period (Figure 15C). The reduction of C1&2 was accompanied with an increase of GTX5, dcGTX2 and dcSTX to a maximum combined relative concentration of 60% (0.6 nmol g^{-1}) (Figure 15C). Type C toxins contributed up to 73% to the toxicity in January, with the reduction of C1&2 in siphon, dcSTX contribution to total toxicity increased significantly at the end of the sampling period (68% in June, Figure 15E).

Toxin content in the visceral mass was 457 nmol g^{-1} in January 2015 and decreased to 3.6 nmol g^{-1} in February 2016 (Figure 15B). Similar to the siphon samples, C1&2 were the dominant analogues (Figure 15D) in February 2015 and represented 90% of the molar content (384 nmol g^{-1}). In contrast with the siphon, there was a sharp reduction of the concentration of these toxins with time in viscera (Figure 15D). However, an increase of C toxins with respect to previous concentrations were detected two times in May. PSTs molar concentration of C toxins increased from 18% in April 24 to 33% on May 1, and from 22% in May 12 to 44% in May 19 (Figure 15D). The reduction of C toxins was accompanied with the appearance of GTX5 and the more potent toxins, dcSTX and dcGTX2 (Figure 15F). After February, dcSTX was the analogue that contributed the most to total toxicity. The highest dcSTX concentration of 338 nmol g^{-1} was detected in May 12, representing 63% of the 2,610 µg STX eq kg^{-1} calculated on this date. At the end of the monitoring period in 2016, low STX concentrations of 1.2 nmol g^{-1} were detected, which represent the 9% of the toxicity calculated in March 2016 (50 µg STX eq kg^{-1}).

There is a limitation on the detection and quantification of PSTs analogues by HPLC-PCOX. The detection of PSTs by this method was only directed to the commercially available toxin certified reference materials when the method was developed. Therefore, to evaluate the presence of other analogues ten selected viscera samples were also analyzed by HILIC-MS/MS. This method allowed the detection of 12 other analogues not detected by HPLC-PCOX, including type M toxins. The five M analogues described so far were all detected in the tissue samples. M5 was the most abundant M-toxin, followed by M1 and M3. M-toxins represented between 30% (230 nmol g^{-1}, January 20) and 75% (28 nmol g^{-1}, June 29) of total PSTs detected by HILIC-MS/MS (Table 5). There was not a clear pattern in time of the contribution of M-toxins to total PSTs. Considering the TEFs shown in Table 2, the concentration of these toxins represented 9,718 µg STXeq Kg^{-1} in January 20 (33% of total toxicity) and 1,838 µg STXeq Kg^{-1} in June 29 (48% of total toxicity, Table 5).

Table 5. Contribution of M-toxins to molar concentration (nmol g^{-1}) of Paralytic Shellfish Toxins and total toxicity (µg STXeq kg^{-1}) calculated by HILIC-MS/MS analysis in geoduck visceral mass samples.

Sample date	nmol g^{-1}		Toxicity (µg STXeq Kg^{-1})	
	Total	M-toxins	Total	M-toxins
1/14/2015	497.7	131.6 (52.9%)	1,8633.7	10,970.8 (58.9%)
1/20/2015	768.9	118 (30.7%)	29,448.9	9,842.4 (33.4%)
1/27/2015	1,086.9	241.4 (44.4%)	41,027	20,119 (49%)
1/17/2015	154.6	34.6 (44.8%)	6,026.6	2,884.1 (47.9%)
3/17/2015	58.1	15.3 (52.8%)	3236.5	1279.6 (39.5%)
5/1/2015	45.9	13.2 (57.7%)	3844.7	1103.6 (28.7%)
6/29/2015	37.8	14.1 (74.8%)	2450.2	1,179 (48.1%)
8/17/2015	51.6	13.3 (52.1%)	2813.1	1109.6 (39.4%)
8/25/2015	11.2	3.3 (58.5%)	885.9	274.7 (31%)
11/10/2015	12.0	2.8 (45.9%)	1023.7	230.5 (22.5%)

3.3 Comparation of HPLC-PCOX and Scotia Rapid Testing methods with mouse bioassay for the detection and quantification of Paralytic Shellfish Toxins in geoduck clam tissues

Comparation of the results obtained with HPLC-PCOX and MBA

Toxicity was calculated with HPLC-COX and MBA in 35 samples of viscera of *P. globosa*. Samples were collected during and after the HAB detected in January 2015 and 2017 in the NGC, near to San Felipe, BC.

Using MBA, the toxicity in samples ranged from the detection limit (320 µg STXeq Kg^{-1}) to 42,300 µg STXeq Kg^{-1}, with 75% of the samples having toxicity above the regulatory limit (800 µg STXeq Kg^{-1}). With HPLC-PCOX, toxicity ranged from 477 and 20,700 µg STXeq Kg^{-1}, and 70% of the samples showed a toxicity level higher than the regulatory limit. Following the 2017 HAB, a maximum toxicity of 70,642 µg STXeq Kg^{-1} was quantified by MBA, whereas sample analysis with HPLC-PCOX resulted in a maximum of 29,632 µg STXeq Kg^{-1} (Figure 16A).

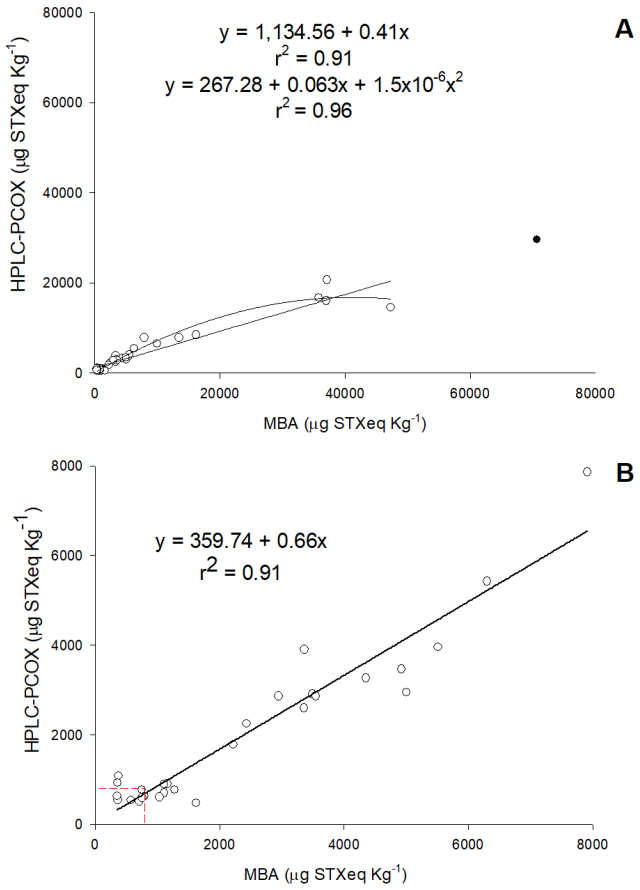


Figure 16. Quantification of Paralytic Shellfish Toxin in viscera of geoduck using HPLC-PCOX compared with MBA. A. Quantification and linear regression of 35 samples. The black circle indicates an outlier excluded from the linear regression. B. Quantification and linear regression of 28 samples which values were up to 10 times the RL. RL: red dashed line

The concentration of 12 PSTs analogues analyzed with HPLC-PCOX showed that 23% of the samples had a toxicity between 7,000 and 20,700 µg STXeq Kg^{-1} (Figure 16). In these samples, the analogues contributing most to the total molar concentration were C1&C2, which represented between 70-85%. Toxicity in 34% of the samples was between 1,700 and 5,500 µg STXeq Kg^{-1}, and C1&C2 represented between 30-40% of the total molar concentration while dcGTX2&3, GTX5 and dcSTX accounted for up to 30%, 10% and 12% respectively. In 43% of the samples the toxicity calculated was between 350 and 1,500 µg STXeq Kg^{-1}. In these samples, dcGTX2&3 contributed to 30% of the total molar contribution, while dcSTX and C1&2 contributed up to 35% and 20%, respectively.

During the analysis, interferences due to the naturally fluorescent compounds present in the viscera of the clams analyzed were observed (to confirm this, tissue samples were analyzed without

oxidant). At least four matrix peaks were detected between five and seven minutes, and it is here that GTX4 and GTX1 are eluted (Figure 17). Differences in matrix peaks were observed between samples collected in different times of the year. The chromatograms of samples from January and July showed that the matrix peak in July is lower than in January (Figure 17).

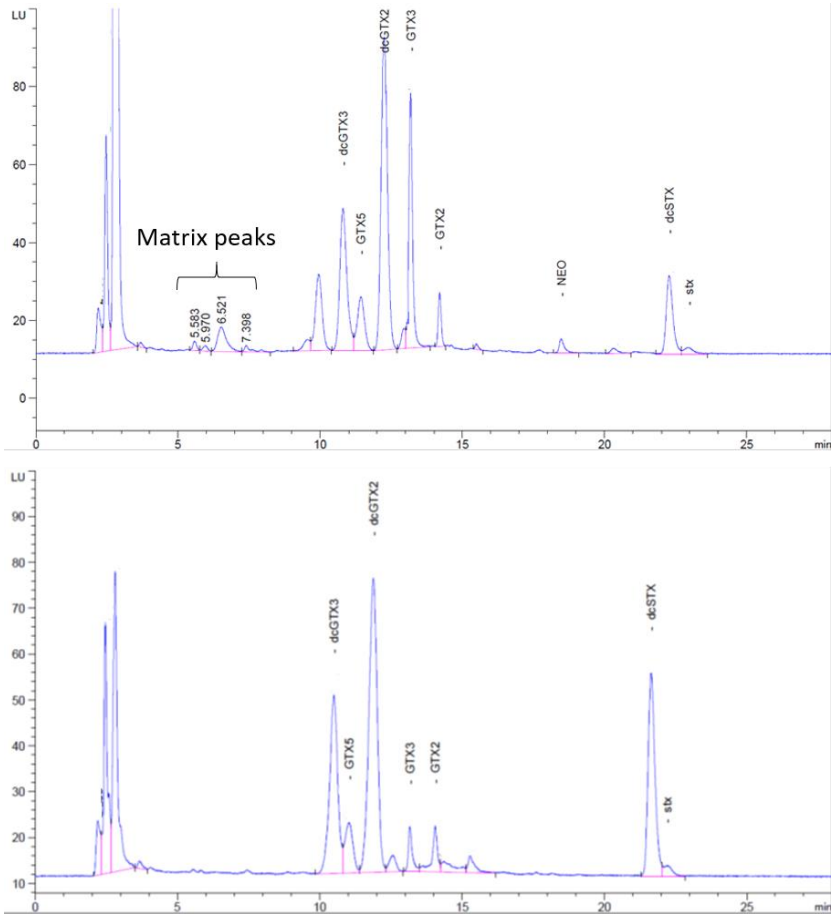


Figure 17. Chromatograms showing temporal variations of the matrix peaks. A. Samples collected in January 2015. B. Samples collected in July 2015

The correlation between the HPLC-PCOX and MBA was calculated using a quadratic and a linear function. The quadratic function showed a r^2 = 0.96 while in the linear function r^2 = 0.91. At high PSTs concentrations, the toxicity calculated with HPCL-PCOX is lower than the one obtained with MBA (a = 0.41x in a linear function).

Levels of PSTs higher than the regulatory limit (RL=800 µg STXeq kg^{-1}) were calculated in 22 of the 35 samples analyzed, and only seven samples showed lower concentrations than the RL. However, the methods showed inconsistent results in six samples. In four 4 of those, HPLC-PCOX calculated values lower than the RL, while MBA calculated values higher than the limit (Table 6). In other two samples, HPLC-PCOX calculated values higher than the regulatory limit when MBA showed values lower than the limit (Table 6).

Table 6. Geoduck samples in which the toxicity calculated with HPLC-PCOX and MBA compared to the regulatory limit was inconsistent. Columns a and b show the samples in which the toxicity with HPLC-PCOX was greater than the RL. Columns c and d show the samples in which the toxicity with HPLC-PCOX was lower than the RL

Toxicity (µg STXeq Kg^{-1}) with HPLC-PCOX ≥ RL		Toxicity (µg STXeq Kg^{-1}) with HPLC-PCOX ≤ RL	
a. MBA	b. HPLC-PCOX	c. MBA	d. HPLC-PCOX
372	1,079	1,108	703
356	927	1,276	768
		1,033	612
		1,621	477

Detection of PSTs by Scotia Rapid Testing

HPLC-PCOX analysis evaluated the concentration of 12 PSTs analogues in the visceral samples. The major analogues identified in the samples were C1&2, dcGTX2&3 and dcSTX (Figure 18). Considering the molar contribution of each of the 12 individual toxins and the total toxicity of the sample, 17 samples were selected for the analysis by Scotia Rapid Testing. Three profiles were included in the analysis (Figure 18, Table 7): profile 1) high proportion of type C toxins and high toxicities, as occurred at the beginning of the experimental period, profile 2) high proportion of decarbamoyl toxins (dcGTX2&3 and dcSTX) and intermediate toxicities, and profile 3) higher proportion of decarbamoyl toxins (dcGTX2 and dcSTX) and toxicities close to the RL in samples collected at the end of the experimental period.

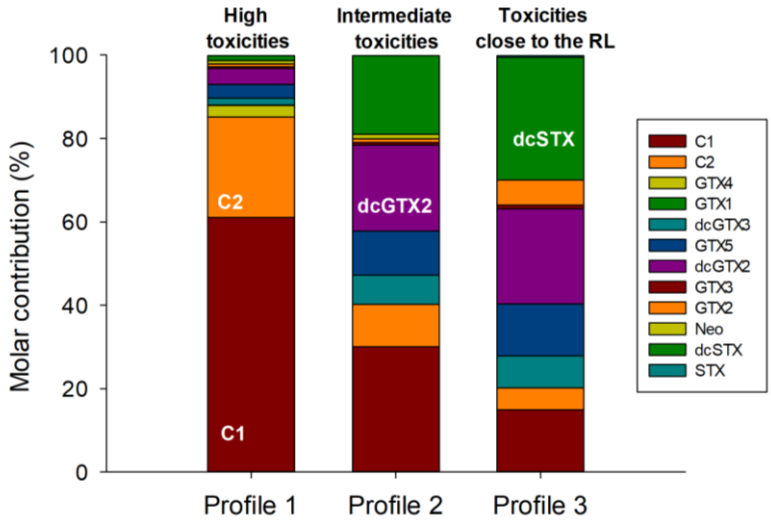


Figure 18. Average molar contribution (%) of each toxin to the total molar content in the different profiles analyzed. The molar contribution and total toxicities were quantified by HPLC-PCOX, samples from these profiles were analyzed by Scotia Rapid Testing.

Table 7. Molar contribution of the main toxins (%) to the total molar content and total toxicities in the different profiles analyzed by Scotia Rapid Testing (molar contribution and total toxicities were determined by HPLC-PCOX).

Profile	Number of samples	% of contribution to the total Molar concentration			Total toxicity (µg STXeq kg^{-1})
		C1&2	dcGTX2&3	dcSTX	
1	4	88-89	3-5	0.6-0.9	7,000-16,000
2	6	28-52	22-33	13-24	2,200-5,400
3	6	15-25	23-35	22-35	600-1,500

As described above, samples were diluted to concentrations of 800, 400, 200 and 100 µg STXeq Kg^{-1} to be analyzed by Scotia Rapid Testing PSP LFA by two technicians. Including diluted and undiluted, a total of 65 samples were analyzed (Table 8). Analyst 1 reported a total of 48% false positives and 0% false negatives. Analyst 2 reported a total of 31% false positives and 6% false negatives. Most of the false positives were recorded in profile 3, were the samples contained a higher proportion of decarbamoyl toxins (dcGTX2 and dcSTX) and the toxicities were close to the RL. In samples of the profile 3, both analysts reported 67% false positives. The false negatives were reported by the analyst 2 in the profile 1 dilution at 800 µg STXeq kg^{-1}, were the samples contained high proportion of type C toxins and high toxicities (Figure 19). The table 9 shows the dilutions where the false positives were calculated, except for one sample in the dilution 400 µg STXeq kg^{-1}, the 100% of the samples diluted to 400 and 200 µg STXeq kg^{-1} were false positives.

Table 8 Results of the Paralytic Shellfish Toxins analysis in geoduck samples by 2 analysts using the Scotia Rapid Testing method.

Profile	Analyst 1		Analyst 2		Total of samples per analyst	Total of false positives (both analysts)
	False positive	False negative	False positive	False negative		
1	9 / 45%	0	0	4 / 20%*	20	22.5%
2	12 / 40%	0	11 / 37%	0	30	38%
3	10 / 67%	0	10 / 67%	0	15	67%
Total	31 / 48%	0	21 / 32%	4 / 6%	65	

*All false negatives were reported in the dilution to 800 µg STXeq kg^{-1}

Figure 19. Scotia Rapid Testing strips obtained after dilutions. A. Profile 1. B. profile 2. C. Profile 3. The arrow points a false negative observed by analyst 2 in the dilution to 800 µg STXeq kg^{-1}.

Table 9 . Number of false positives of the total samples per dilution calculated in geoduck samples by 2 analysts per profile

Dilution (µg STXeq kg^{-1})	Analyst 1			Analyst 2		
	Profile 1	Profile 2	Profile 3	Profile 1	Profile 2	Profile 3
Real value	0	0	3*/6	0	0	3*/6
800	0	0	0	0	0	0
400	4/4	6/6	3/3	0	5/6	3/3
200	4/4	6/6	3/3	0	6/6	3/3
100	1/4	0	1/3	0	0	1/3

*Real value of the samples calculated by HPLC-PCOX 717, 629 and 461 µg STXeq kg^{-1}

3.4 Impact of the presence of Paralytic Shellfish Toxins in the Northern Gulf of California during 2015-2019

FICOTOX Lab has monitored the phytoplankton community in surface water samples and the PSTs in bivalves in the NGC intermittently since 2011. *G. catenatum* has been present in the NGC every year (2013 was not monitored). PSTs were detected for the first time by the regulatory authorities from April

to May 2010, when four *P. globosa* extraction areas were included in the Mexican sanitary program (PMSMB, COFEPRIS 2014). After this year no further sanitary bans were implemented until 2015.

COFEPRIS has monitored the presence of PSTs in geoduck clams harvested in the NGC on a monthly basis since 2010. After detection of high PSTs concentrations and as long as concentrations remain above the RL, COFEPRIS increases the frequency of monitoring and quantifies the PSTs in bivalves on a weekly basis. The concentrations obtained by this agency using MBA in 18 geoduck harvesting areas (Figure 8) were plotted to evaluate the spatial distribution of the PSTs in the NGC during the 2015-2019 HABs.

COFEPRIS detected PSTs in geoduck collected from January to June (Figure 20). The highest PSTs concentration of 18,800 µg STXeq Kg^{-1} was detected at the end of January (Figure 20b) in the harvesting area of Minerva Perez, located south of San Felipe (Figure 8). Throughout the monitoring period, a high concentration of toxins was detected in that area. PSTs were also detected in samples from adjacent areas such as Enrique Gastelum, Genaro Wong and Consuelo Flores, but in lower concentrations. In northern San Felipe, between February 10 and March 3, up to 3,840 µg STXeq Kg^{-1} were quantified in Los Amigos del Puerto area. From end of April to end of June, toxins were detected in the harvesting area closest to the coast, Estela Martinez, in concentrations of up to 2,400 µg STXeq Kg^{-1}.

As in 2015, in 2017 the maximum toxin concentration was detected in an area south of San Felipe (Figure 21). The highest toxin content reported in a mollusk in Mexico was detected in Enrique Gastelum area, where 152,852 µgSTXeqKg^{-1} was recorded on January 17. On that date high PSTs concentrations were also detected in other areas, in Consuelo Flores area a 109,000 µg STXeq Kg^{-1} were quantified located further south, and 68,000 µg STXeq Kg^{-1} were detected in Estela Martinez, the area closest to the coast. PSTs concentrations were also detected north of San Felipe, in Demetrio Soberales area with 23,000 µg STXeq Kg^{-1}.

Unlike the previous years, in 2018, the maximum toxin concentrations were detected in the area in front of San Felipe. In the Estela Martinez area, 53,000 µg STXeq Kg^{-1} were detected on January 23. In this year, high concentrations of PSTs were also registered in clams extracted from the Upper Gulf of California. After February, the toxins were detected in the areas Amigos del Puerto and Demetrio Soberales that are located north of San Felipe (Figure 22).

In 2019, toxin accumulation in clams was lower than reported. As in 2018, the highest toxicities were detected in the northernmost areas located in the upper Gulf of California (Figure 23). The areas with

the highest concentration were Pesquera Mar Profundo in January with 6,800 µg STXeq Kg^{-1}, and Martin Leon Verdugo in March 14 with 7,800 µg STXeq Kg^{-1}.

Since 2011, FICOTOX Lab has intermittently monitored the phytoplankton community in the NGC in 2 extraction areas (except in 2013). From 2011 to 2017, the water samples were obtained from Enrique Gastelum, starting in 2018, samples come from a sampling point located close to Estela Martinez (figure 8). In figure 24, these data were compared with the PSTs concentrations in geoduck's visceral samples calculated by COFEPRIS using MBA and in this work using HPLC-PCOX.

In 2011, *G. catenatum* was detected in January-February, May-June and October. Maximum concentrations were detected on February 1 with 3,100 cell L^{-1} and a relative abundance of 21%, and in June 1 of 5,200 cell L^{-1} and 82% of relative abundance. In 2012, the highest abundances were detected on April 16th with 17,200 cell L^{-1}. April was also the month with the highest abundance of *G. catenatum* (5,300 cell L^{-1}) in 2014. Except from 2016 when the maximum abundance was registered in April (April 19th, 9,160 cell L^{-1}), starting in 2015, the highest concentration of the species occurred in January: 2015 with 152,750 cell L^{-1}, 2017 with 311,000 cell L^{-1}, 2018 with 2,556 cell L^{-1} and 2019 with 18,918 cell L^{-1}.

From 2015-2019, both the dinoflagellate and the accumulation of toxins in geoduck were detected, starting with the first month of the year (Table 10). In 2017, COFEPRIS detected toxins from January to April, from January to March in 2018, and from January to May in 2019. There was a discrepancy on the data from 2015 between months of occurrence reported by COFEPRIS and this work. In 2015, we detected toxins in concentrations higher than the regulatory level during the whole year and even in January 2016, while COFEPRIS detected toxins only from January to June 2015.

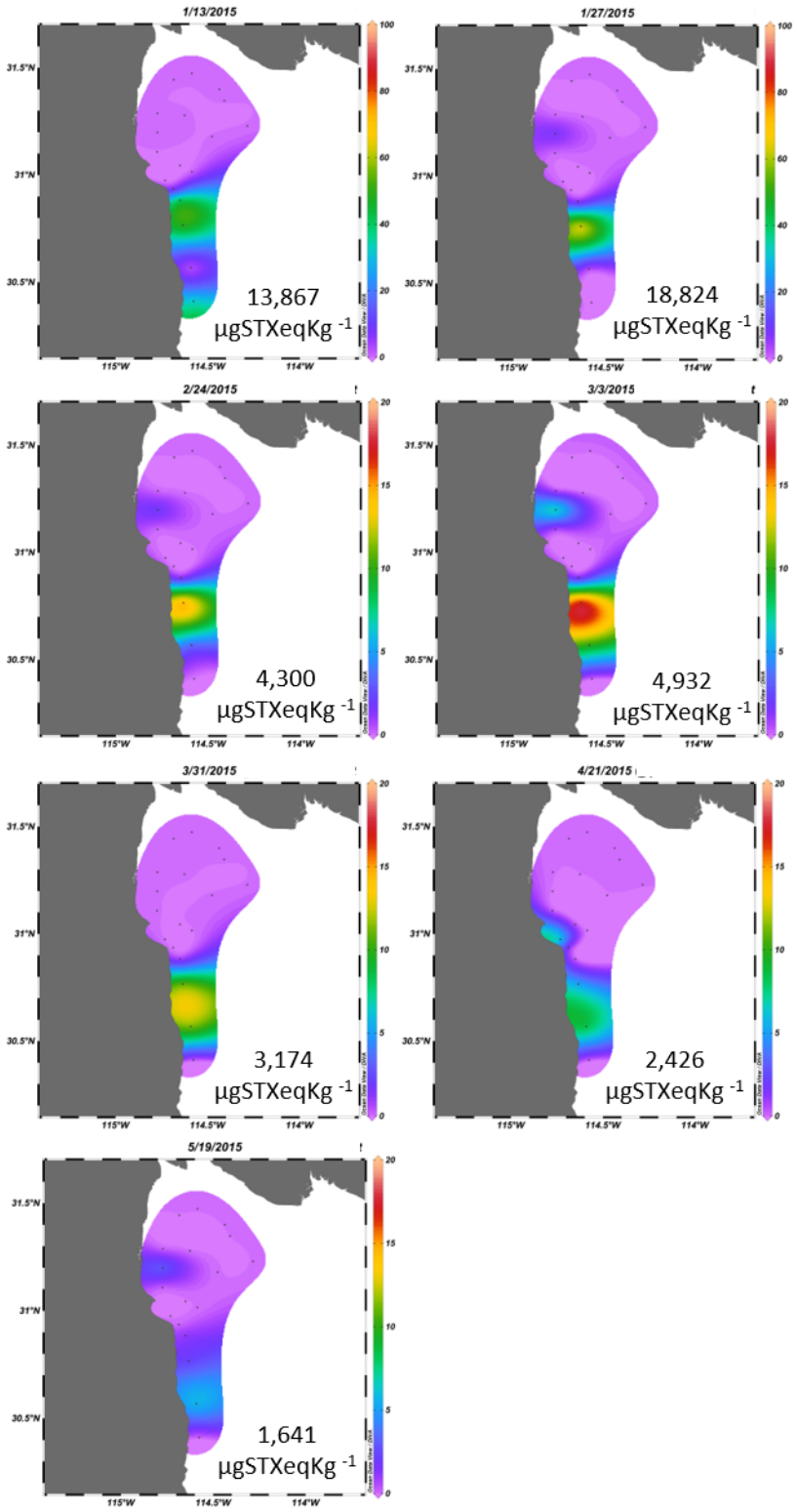


Figure 20. Spatial distribution of Paralytic Shellfish Toxins in 18 certified areas for the extraction of geoduck clam in the northern Gulf of California in 2015. Data obtained from COFEPRIS, 2015. The highest PSTs concentration per date is shown in each image.

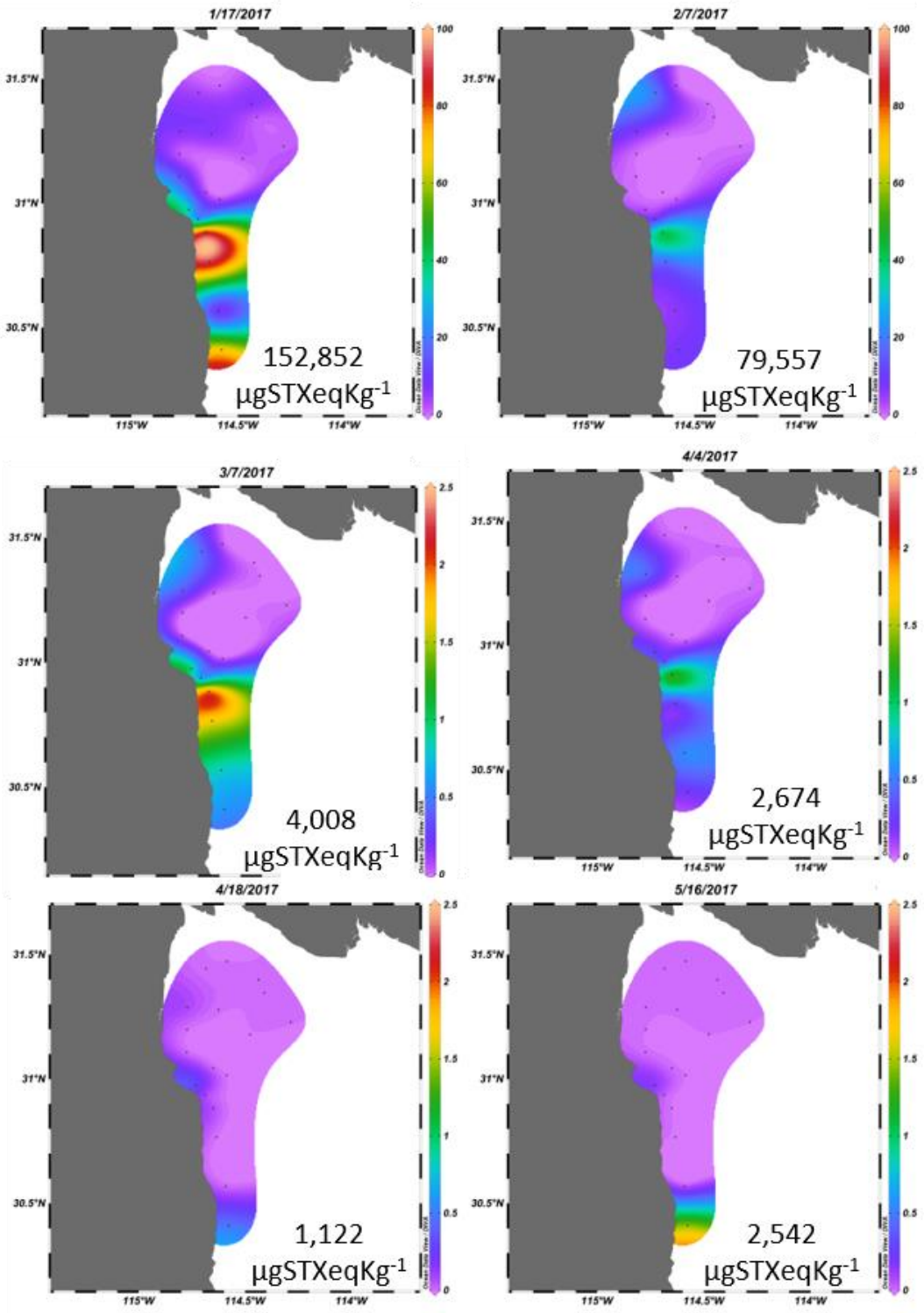


Figure 21. Spatial distribution of PSTs in 18 certified areas for the extraction of geoduck clam in the northern Gulf of California in January 2017. Data obtained from COFEPRIS, 2017. The highest PSTs concentration per date is shown in each image.

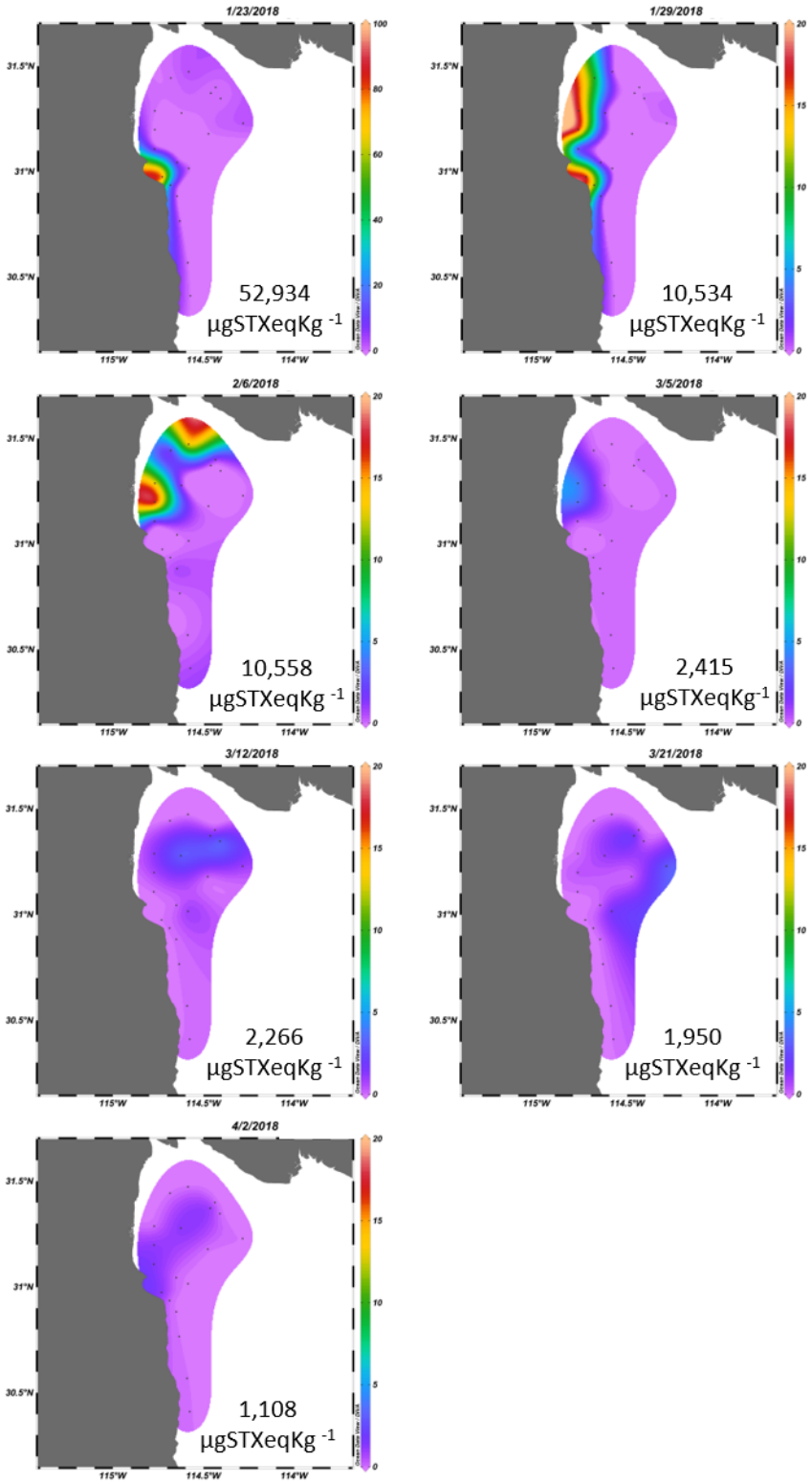


Figure 22. Spatial distribution of PSTs in 18 certified areas for the extraction of geoduck clam in the northern Gulf of California in February 2018. Data obtained from COFEPRIS, 2018. The highest PSTs concentration per date is shown in each image.

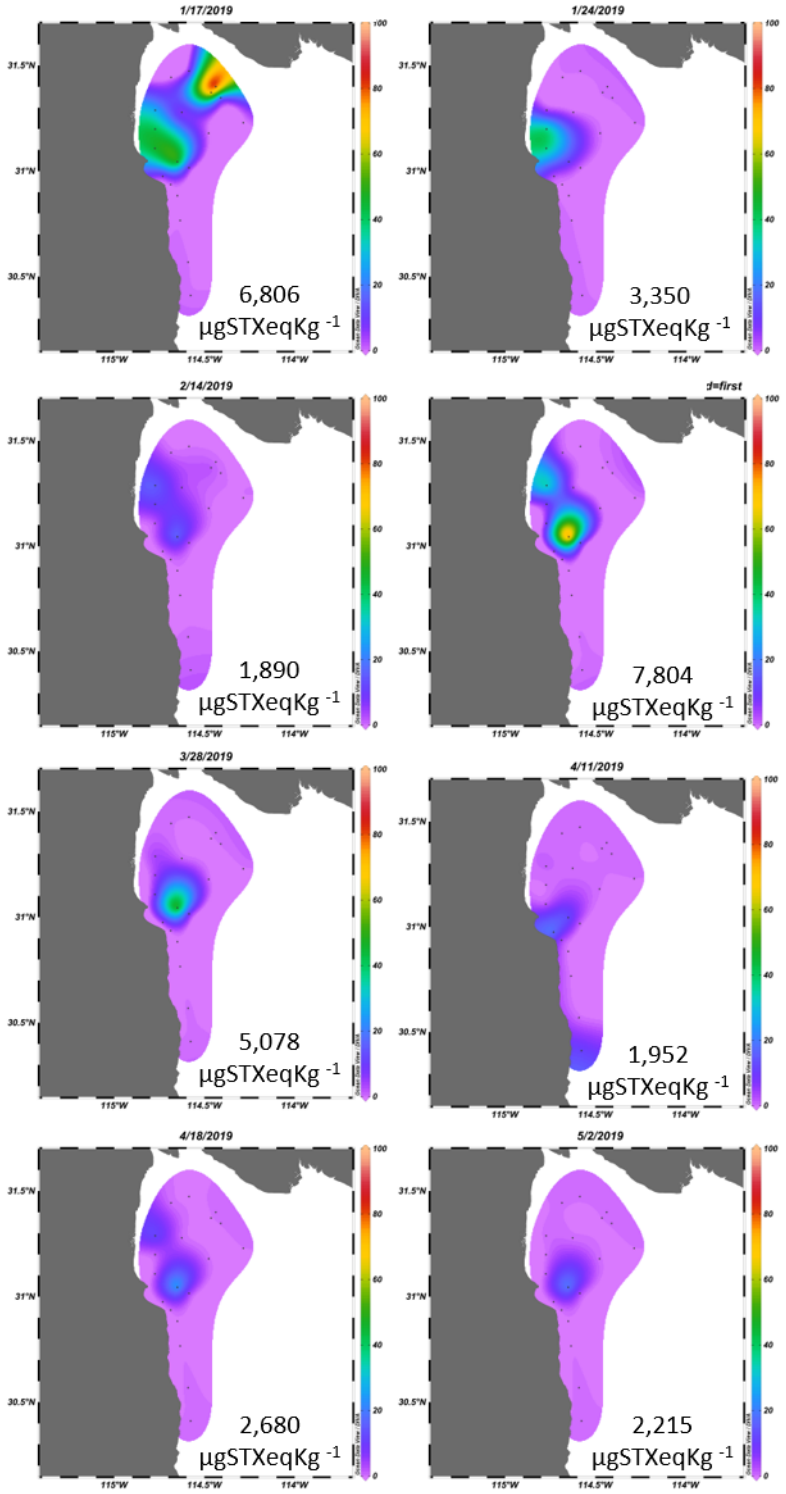


Figure 23 Spatial distribution of PSTs in 18 certified areas for the extraction of geoduck clam in the Northern Gulf of California in 2019. Data obtained from COFEPRIS, 2019. The highest PSTs concentration per date is shown in each image.

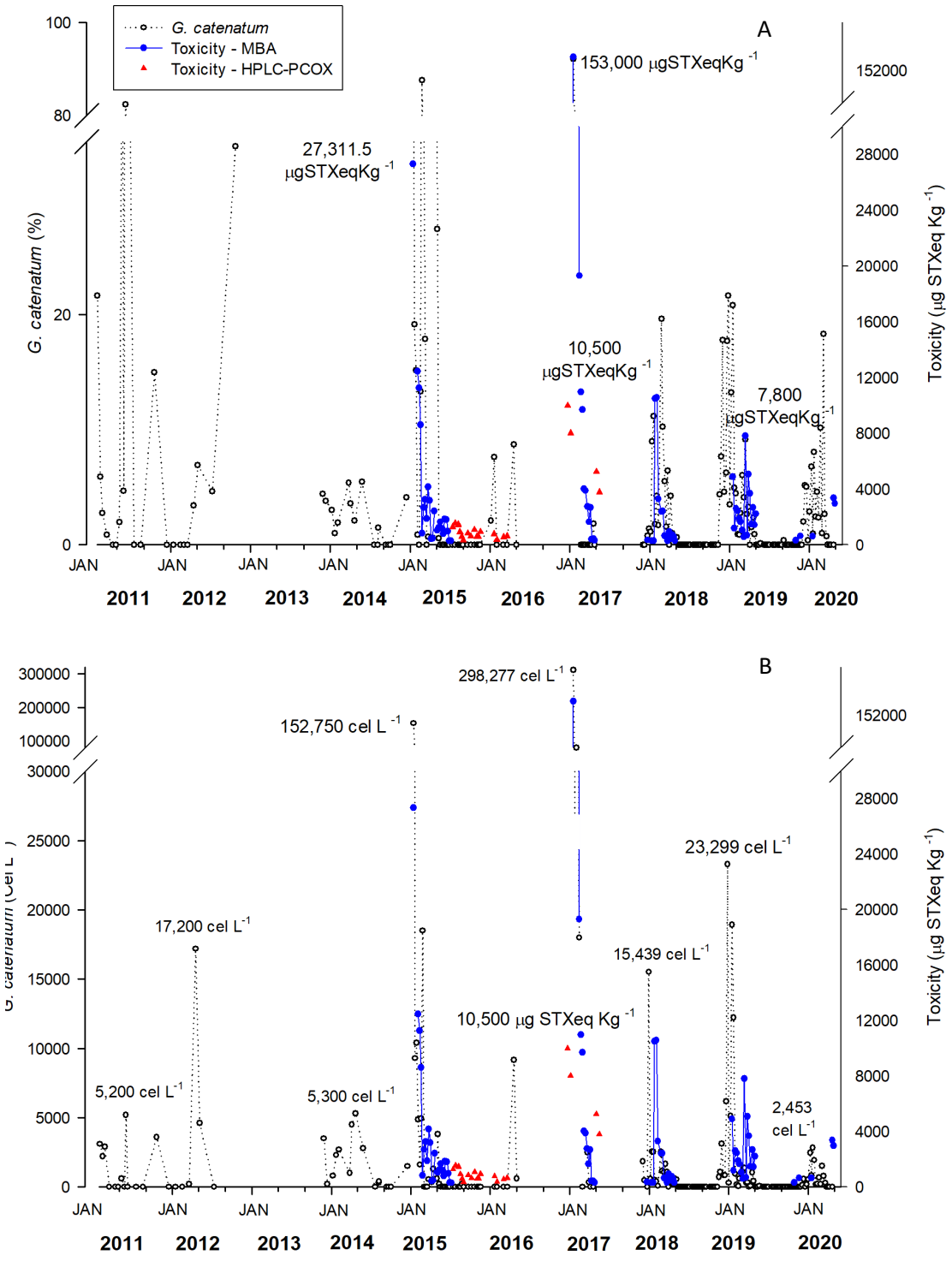


Figure 24. Relative (A) and absolute (B) abundance of *Gymnodinium catenatum* (dotted line) in the North of the Gulf of California from January 2011 to May 2020 and PSTs in viscera of clam (continuous line) from 2015 to 2020. PSTs were calculated by MBA (blue line, data obtained from COFEPRIS) and by HPLC-PCOX (red line, this work). In the upper panel, PSTs concentration calculated by MBA is shown, in the lower panel, the abundance of *G. catenatum* is presented.

Table 10. Months of occurrence and maximum abundances of *Gymnodinium catenatum* (data generated in this work, except for 2011 that was obtained from Murillo-Martinez, 2011) and months of occurrence of PSTs on geoduck's visceral samples (data from COFEPRIS 2015, 2016, 2017, 2018, 2019) in the NGC in the period 2011-2019.

	G catenatum			PSTs
	Months of occurrence	Maximum cell L^{-1}	Maximum %	Months detected
2011	Jan-Feb, May-Jun	5.2×10^3		Jan-Feb, Jun*
2012	Mar-Apr	5.3×10^3		
2014	Jan-Apr	5.3×10^3		
2015	Jan – May	153×10^3	87	Jan – Dec
2016	Jan & Apr	9×10^3	8	Jan
2017	Jan – Feb	311×10^3	92	Jan - Apr
2018	Jan- Mar	15×10^3	20	Jan - Mar
2019	Dec - Mar	23×10^3	20	Jan - May

Chapter 4. Discussions

4.1 Effect of Paralytic Shellfish Toxins on wildlife in the Northern Gulf of California during a Harmful Algae Bloom that occurred in January 2015

In this work, we documented multispecies mass mortalities caused by a *G. catenatum* HAB that occurred between January and March 2015 in the Northern Gulf of California (NGC). The total number of organisms reported by different entities from January to March was 1017 dead seabirds and 244 marine mammals. The east coast of Baja California is a remote area with low population density, so it was likely not all stranded animals were reported to the authorities. Therefore, the number of dead organisms registered could be an underrepresentation of the effect of this large-scale phenomenon. For example, in January the calculated abundance of dead seabirds was 146 organisms km^{-1} (SD =84 organisms km^{-1}, n=5 transects) and of 2.3 dolphin carcasses km^{-1} (SD=1.1 carcasses km^{-1}, n=5). Therefore, the estimation of negatively impacted organisms in the period of the survey (January 17 and 18) in approximately 80 km of coastline (Fig. 1) is 11,648 (SD = 6,718) seabirds and 186 (SD = 90) marine mammals. This estimation assumes a homogeneous distribution of the organisms in the prospected area and that double counting of dead organisms did not occur. This last assumption holds since only stranded animals above the mark of the highest sea level for the period of the sampling were counted. On the other hand, the stranding of dead animals depends on the coastline's local circulation and geomorphological characteristics. Therefore, a homogeneous distribution of stranded animals in 80 km of coastline is not realistic, and this was represented in the large variation of registered corpses per Km found at each transect. However, this rough estimation demonstrates the impact of a large-scale phenomenon with such duration as the one registered in the region.

Even when the implementation of several sanitary bans associated with the accumulation of PST in different mollusks species have been also associated with *G. catenatum* blooms in Mexico, Venezuela, and Ecuador (Band-Schmidt *et al.*, 2019), the west coast of Spain, Tasmania, and Taiwan (Hallegraef, 2012), there is no clear evidence of *G. catenatum* negatively impacting wildlife, or the information is anecdotal. To our knowledge, this is the only mass mortality event of multiple species of marine mammals and seabirds that have been related to PST produced by *G. catenatum*, and it is the only one with such important registered affectations to coastal activities. The HAB of *G. catenatum* affected at least six seabird and five marine mammal species. The only unconfirmed report of the affection of marine wildlife caused by *G. catenatum* is the dead of approximately 50 marine turtles in El Salvador. In 2013 fishermen reported

dozens of dead sea turtles floating offshore and 40 stranded corpses found on La Paz and La Libertad beaches during September and October 2013 (Amaya *et al.* 2018). *G. catenatum* was detected (5,400 cells L^{-1}) in phytoplankton samples taken in a northern location (Amaya *et al.* 2018), but no toxin confirmation was obtained from the dead animals.

To date, morbidity of wildlife including mass mortalities of marine mammals and seabirds related to PST has been related mainly to species of the genus *Alexandrium* and *Pyrodinium bahamenese* (Geraci *et al.,* 1998; Reyero *et al.,* 1999; Landsberg, 2002; Goya & Maldonado, 2014; Starr, 2017; Amaya *et al.,* 2018). Mass mortalities events related to *Alexandrium* and *Pyrodinium bahamenese* blooms are probably associated with the toxic potential of these species since they present a higher toxic potential than *G. catenatum*. Specifically, analogues with high toxicity such as GTX5, GTX6, STX and Neo are produced by *Pyrodinium bahamenese* (Montojo *et al.,* 2006). Toxin profile is different in the different species of *Alexandrium* in the world. In North America's strands, the dominant analogues are Neo, GTX4 and C2 (Bricelj *et al.,* 2014); *Alexandrium minimum* in Spain produces GTX1 and GTX4 as the principal toxins (Franco *et al.,* 1995), whereas GTX2 and/or GTX3 are more dominant in strains from Ireland, southwest UK and France (Lewis *et al*., 2018), while *Alexandrium catenella* from Scotland contains STX, Neo, GTX1&4 and C1&2, with other analogues such as GTX2&3 and GTX5 (Brown *et al.*, 2010). In contrast, N-sulfocarbamoyl C1 and C2 have one of the lowest TEF, representing the major proportion of PST analogues in *G. catenatum* (Bustillos *et al.,* 2016; Costa *et al.,* 2015). Therefore, the toxic potential of a microalgae species depends on its toxin profile and cell abundance. Toxin profiles in phytoplankton samples analyzed in this study were comparable to the ones reported previously for *G. catenatum* maintained in culture conditions and in natural environmental samples (Hallegaref *et al.,* 2012; Bustillos-Guzman *et al.,* 2016) since C1 and C2 toxins represented the major proportion (95%) of total molar content while dcSTX concentrations were low or undetectable. Also, the average cellular PST content (50 pg $cell^{-1}$ equivalent to 3.06 pg STXeq $cell^{-1}$) was comparable to the one reported for other blooms (Quijano-Scheggia, 2012).

The impact of HABs is related to the toxic potential of the species. Still, most importantly, it is associated with the cells abundance and duration of the phenomenon. The highest abundance registered during 2015-HAB (266 x 10^3 cell L^{-1}) was much lower than the maximum abundance registered in events documented in different regions worldwide. Abundances as high as 1.02×10^7 cell L^{-1} have been reported in Manzanillo Bay in the Mexican tropical pacific (reviewed in Band-Schmidt *et al.,* 2018). However, it must be taken with precaution since it was reported in a conference meeting and not in a peer-reviewed publication (reviewed in Band 2018). In the same area, a bloom with a maximum abundance of 3.65×10^6 cells L^{-1} was registered in 2012 (Quijano-Scheggia *et al.,* 2012). Maximum cells abundance in HABs reported

on another region was $1.2x10^6$ cell L^{-1} in Taiwan (Liu *et al.,* 2020) and Spain. However, no impact on wildlife has been documented in HABs with such high cell abundances.

As another factor considered to explain the multispecies die-offs registered due to the HAB of the NGC, we propose the fish sardine "bocona" (Cetengraulis mysticetus) as vector for the transference of the toxins to marine mammals and seabirds and as the principal cause for the magnification of *G. catenatum* toxic potential. Due to the full gut content in deceased birds containing undigested and semi-digested fish sampled in the first massive mass morality period, there was evidence that the organisms died in a short time after feeding. Fish sampled from the gut content and those found next to the dying bird (booby *Sula nebouxii*) showed the highest toxin content of all the biological material analyzed (2,875 µg STXeq Kg^{-1}). PST produced by the microalga was therefore transferred to birds and marine mammals most probably through these planktivorous fish. Low toxicity STX analogues constituted the major proportion of PST within the microalgal source. However, a high proportion of STX and dcSTX indicates that PST acquired by fish was transformed into more toxic analogues. A high content of STX and dcSTX was also observed in *Diplodus sargus* and *Dicentrorchus punctatus* during the die-off of monk seals registered in 1997 (Reyero *et al.,* 1999).

There are few studies related to the biotransformation of PST in fish. Costa *et al.* (2010) evaluated the accumulation of PST in sardines (*Sardina pilchardus*) exposed to blooms of *G. catenatum*. Fish exposed to maximum cell densities of $25x10^3$ cells L^{-1} accumulated a maximum of 531 µg STXeq Kg^{-1} in viscera (Costa *et al.* 2010). Contrary to our results, they reported that the PST profile in sardines was like that found in *G. catenatum* and therefore, no significant biotransformation occurred in fish tissues. However, there is evidence that PST biotransformation occurs in other species or that the route of exposure of fish favors the accumulation of more toxic analogues. For example, dcSTX and GTX5 constituted nearly 90% of toxins found in the Atlantic horse mackerel (*Trachurus trachurus*) related to *G. catenatum* maximum abundance of 5.0×10^3 cells L^{-1} (Lange and Costa, 2011). Likewise, changes in the toxin profile of this alga were also detected in the white seabream (*D. sargus*), in which only dcSTX was detected in the liver after two and six days of the exposure to different PST analogues extracted from *G. catenatum* (Costa *et al.*, 2012). N-sulfocarbamoyl and decarbamoyl toxins were eliminated or enzymatically transformed in *D. sargus* liver (Costa *et al.,* 2012). This is a clear evidence that PST are enzymatically transformed in fish (Costa, 2016). Toxin content and analogues detected in the anchovy *Cetengraulis mysticetus* during the HAB of the NGC evidenced that low toxic analogues were converted into more potent metabolites since dcSTX was not detected in phytoplankton samples and in fish was the main PST congener. Alternatively, analogues in the anchovy tissues were acquired through a zooplankton dietary intermediate. This was proposed by Lange

and Costa (2011) to explain the high levels of toxins detected in the Atlantic horse mackerel as *G. catenatum* abundance was relatively low (5.0 x 10^3 cells L^{-1}). Toxin content in the mackerel viscera was comparable (4,800 µgSTXeq Kg^{-1}) to the one detected in anchovy during the NGC HAB. Accumulation, metabolism and the ecological role of the accumulation of these metabolites in fish must be investigated. Most importantly, risk evaluation of human intoxication should be considered.

Other multispecies massive mortalities have been documented in the Upper Gulf of California. During January and mid-February of 1995 at least 425 marine mammals and 200 sea birds died also in the San-Felipe region (Vidal and Gallo, 1995). According to an official communication, this die-off was caused by cyanide poisoning associated with the dye used for drug smuggling activities in the region (PROFEPA 1995). However, this explanation was not plausible and other factors might have been the cause of this mass mortality (Vidal and Gallo 1995). No analysis of phycotoxins nor the evaluation of the presence of potentially harmful phytoplankton species were performed during this die-off. In January 1997, 766 birds (*Gavia immer*), 168 dolphins, nine sea lions, and four whales (*Balaenoptera physalus*) died also in the Upper Gulf of California. This die-off was associated with domoic acid intoxication (PROFEPA, 1997). Therefore, phyocotoxins represents a threat to marine mammals in this region. These compounds have an acute effect but also a chronic effect on marine species. It has been documented that chronical exposure to phycotoxins affect the reproductive success of sea lions and might compromise the health and reproduction of other marine mammals (Doucette et al 2012).

4.2 Transformation and depuration of Paralytic Shellfish Toxins in naturally contaminated geoduck clams *Panopea globosa*

Here, we report PSTs profiles in the viscera and the siphon tissues of *P. globosa*. This is the first characterization of the transformation and depuration of different analogues in a geoduck clam. The accumulation of PSTs in *P. globosa* was associated with a bloom of *G. catenatum* that was present in the NGC during the first months of 2015. *G. catenatum* have been reported in different areas of the Gulf of California (Band-Schmidt *et al.*, 2010), but they have not been registered in the NGC since there was not a continuous monitoring program in the region. Monitoring of PSTs in organisms extracted from areas located south of San Felipe initiated in 2010 and the first sanitary ban for the extraction of *P. globosa* was implemented the same year. As a consequence, FICOTOX Research Laboratory has been monitoring the

phytoplankton community in the area since 2011. They have documented that *G. catenatum* is a conspicuous species of the phytoplankton community from December to April with abundances lower than 10 x 10^3 cells L^{-1} (Murillo-Martinez, 2011). However, in 2015 the abundance reached 152 x 10^3 cells L^{-1} in samples collected at the surface of the water column. The presence of *G. catenatum* was related to a maximum toxicity of 16,740 µg STXeq Kg^{-1} (27 the RL) in viscera of *P. globosa*. In 2017, another bloom was registered in the region and maximum registered abundance of the dinoflagellate (283 x 10^3 cells L^{-1} in surface water samples) and associated accumulation of PSTs (49,166 µg STXeq kg^{-1} in viscera samples) in *P. globosa* increased importantly.

Although the relationship between the two variables is complex, the abundance of *G. catenatum* seems not particularly high when considering the PSTs concentrations and total sample toxicity quantified in the clams. Higher abundances of this species have been reported during HABs in other regions. In the Gulf of California, for example, *G. catenatum* blooms have been associated with wild and cultured animals die-offs, economical loses (Núñez-Vázquez *et al.*, 2011) and caused human intoxications and fatalities (De la Garza, 1983; Mee *et al.*, 1985; Morey-Gaines, 1982; Band-Schmidt *et al.*, 2010). Cell densities measured during these HABs were in the order of 1 x 10^6 cells L^{-1} (Morey-Gaines, 1982; De la Garza, 1983; Mee *et al.*, 1985; Band-Schmidt *et al.*, 2010). *G. catenatum* has also been reported in high abundances in other parts of the world. In Spain, abundances of 2 x 10^6 cells L^{-1} were reported in 1986 causing the accumulation of up to 26,400 µg STX eq kg^{-1} in mussels (Anderson *et al.*, 1989). In Portugal, the dinoflagellate has occurred since 1986, with blooms in autumn of 1986, 1990, 1992, 1994, 1995 and annually from 2005 to 2012 (Vale *et al.*, 2008; Silva *et al.*, 2015) with abundances as high as 22 x 10^4 cells L^{-1} in 2007 (Rodrigues *et al.*, 2012). Intermittent and multiannual scale blooms of *G. catenatum* are devastating to mussel aquaculture production in Galicia and to the harvesting of natural shellfish banks on the Galician and Portuguese coasts. Toxin levels of up to 60,000 µg STX eq. kg^{-1} in mussel meat were reported in 2005 (Pazos *et al.*, 2006; Vale *et al.*, 2008) and 2010, leading to prolonged harvesting closures lasting until the following spring (Trainer *et al.*, 2010). *G. catenatum* surface abundance did not reflect the magnitude of the HAB event in the NGC since this species can be distributed subsuperficially. The relative abundances detected in net tow samples of the NGC in this study confirmed this subsurface distribution.

The characterization of PST concentration changes in terms of STX eq is essential for regulation purposes. The high levels of toxin accumulation and the time required for toxin depuration in the clams was related to the long duration of the HAB. The presence of *G. catenatum*, in January, part of February and from March to May resulted in PSTs remaining in viscera above the RL for almost all 2015. The calculated depuration rate of *P. globosa* was 4.3% of STX eq loss per day. Therefore, according to the model

after the accumulation of 16,740 µg STX eq kg^{-1} in viscera, total PSTs should have reached the RL 106 days after this maximum level of toxicity. However, the RL was reached 104 days later than the date estimated by the exponential decay model. PST concentrations were found to decrease in time but there were periods when the toxicity increased compared to previous measurements (Fig. 9C and D). This increase in toxicity was associated with the increase in the abundance of *G. catenatum* in the water column that represented a continuous source of PSTs for geoduck clams. This consequently represents a bias in the calculation of the depuration rate in *P. globosa*. However, even with an under-estimation of the rate, it was clearly found that this species exhibits a low depuration rate. The organisms accumulated toxins for several months in their visceral tissues. Bricelj and Shumway (1998) classified bivalves that need several months to years to detoxify below the RL and present detoxification rates between 0.1 and 4% of STX eq loss per day, as a low depuration species. In contrast, a fast depurator bivalve has an elimination rate between 6% and 17% of toxin loss per day, and reach the RL between 1 and 10 weeks, depending on the amount of toxin assimilated (Bricelj & Shumway, 1998). *Panopea* species have low depurating physiology since *P. generosa* was also found to retain toxicity for long periods of time (Curtis *et al.*, 2000). Another low depuration clam species is *Saxidomus giganteus* with an average of 0.7% of STX eq loss per day in the whole tissues (Quayle, 1969; Madenwald, 1985; Price *et al.*, 1991), *Spisula solidissima* depurates at 1.9%, measured in whole animals (Shumway *et al.*, 1988), *Placopecten magellanicus* at 0.6% when pools of digestive tissues, gills and mantle were measured (Shumway *et al.*, 1988) and *Patinopecten yessoensis* with an average of 2.6% in digestive tissues (Nishihama, 1980; Ogata *et al.*, 1982; Tazawa *et al.*, 1988).

C1 and C2 were the most abundant analogues in the siphon and viscera in samples collected close to the HAB occurrence. These are the main analogues present in *G. catenatum* and represented 95% of PSTs detected in phytoplankton samples during the bloom, with dcGTX3, dcNeo and GTX2&3 comprising the remaining 5% (Bustillos-Guzmán *et al.*, 2016; García-Mendoza *et al.*, in preparation). Following the incorporation of toxins into the clam tissues through feeding activities, toxins transformation occurred principally in the viscera, where the low potency toxins C1&2 were converted into more toxic analogues such as dcGTX2 and dcSTX. The presence of higher toxicity analogues and the increase in their relative contribution to the total burden in viscera resulted in toxicities remaining above the RL for several months. Since there is a high number of analogues and different reactions involved in their transformation (Oshima, 1995; Cembella, 1994; Gueguen *et al.*, 2011; Turner *et al.*, 2012a, 2013), it is difficult to describe the different biotransformation steps and conversion rates that occurred in *P. globosa* after toxin bioaccumulation. However, we identified the principal biotransformation reactions in this species (Figure 25). Epimerization occurred between the epimeric pairs C1&2 and dcGTX2&3. C1 was then converted into GTX5 by desulfuration in R3 position or in dcGTX2 by desulfo carboxylation in R4. C1 can be also

transformed into dcSTX if both reactions occur in parallel. However, according to the time of appearance of PSTs analogues in *P. globosa* it is more likely that formation of GTX5 is an intermediate step for the formation of dcSTX by desulfo-carboxylation in the R4 position. These biotransformation reactions explain the observation of the reduction of C1&2, which was accompanied with an increase in GTX5, dcGTX2 and dcSTX to a maximum combined relative concentration of 59%. Even when enzymatic conversion of PSP toxin to decarbamoyl (dc) derivatives is uncommon among bivalves (Bricelj & Shumway, 1998) and has been only demonstrated in the Pacific clam *Protothaca staminea* (Sullivan *et al.*, 1983), the surf clam *Spisula solida* (Turner *et al.*, 2013), and in the Japanese clams *Mactra chinensis* and *Peronida venulosa* (Oshima, 1995), we identified in this study the production of dcGTX2&3 and dcSTX by N-sulfocarbamoyl toxins hydrolysis.

The characterization of the PSTs conversion steps and conversion rates become more complicated if the M-toxins are considered. Five M analogues were present in *P. globosa* tissues and represented a significant proportion between 30 to 75% of the PSTs detected in the samples. This is the first time that M- toxins are reported in geoduck clams. M-toxins were described in 2008 from blue mussels (*Mytilus edulis* and *M. trossalus*) from eastern Canada during a HAB of *Alexandrium tamarense* (Dell´Aversano *et al.*, 2008). M-toxins are most probably metabolites and or degradation products formed in shellfish since they have not been detected in microalgae (Dell´Aversano *et al.*, 2008). Five M-toxins analogues have been identified and Vale (2010) found that M1 is the most abundant in species that retain PSTs toxins for longer periods. To date, M1 has been reported in mussels (*M. galloprovinciallis*), cockles (*Cerastoderma edule*) and clams from estuarine (*Ruditapes decussatus*) and oceanic habitat (*Donax trunculus* and *Ensis* spp.) collected in Portugal during a bloom of *G. catenatum* (Vale, 2010). M1 could contribute to an important fraction of the PSTs profile (up to 70% of total GTX5) and has been proposed that it is originated from the metabolism of GTX5 (Vale 2010). In the case of *P. globosa*, M5 was the most abundant M-toxin. Probably, there is a rapid transformation of some other M-toxins into M5 in this species. Metabolism and biotransformation of this type of toxins has not yet been elucidated.

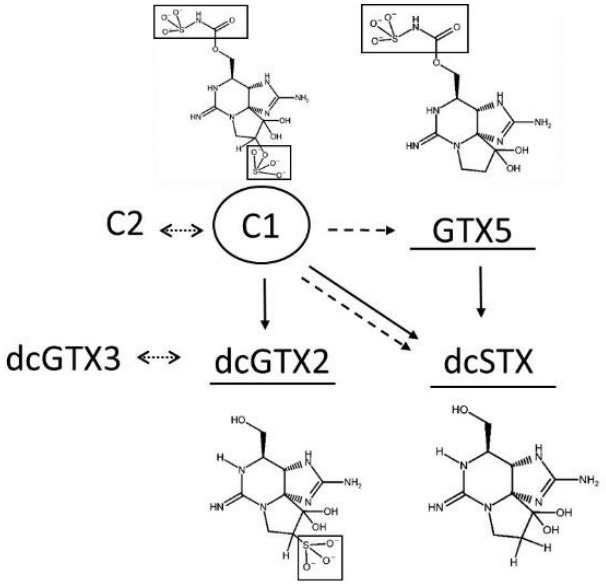


Figure 25. Diagram of assumed biotransformations that occurred in viscera of geoduck *Panopea globosa* contaminated with *Gymnodinium catenatum* paralytic shellfish toxins. The part of the molecule that is modified to become in another analog is highlighted in a square. Solid arrow: desulfo-carboxylation, dashed arrow: desulfurization, double arrow: epimerization.

The results of the present work have important implications for both regulation and management. Evaluation of the causative HAB species within the phytoplankton community should be performed more intensively during winter in the NGC to monitor the appearance of harmful species. Assessment for the presence of *G. catenatum* should be conducted not only on surface samples, but also on water samples throughout appropriate depths within the water column, such as vertical net hauls, tube integrated or hose integrated samples. Risk abundance indexes should also be developed to assess potential PSTs accumulation in geoduck clams above the RL.

In this study, *P. globosa* accumulated approximately 24 times more toxins in the viscera than in the siphon, which can be compared with Curtis *et al.* (2000) who reported that accumulation of PSTs in viscera of *P. generosa* was 30 times higher than in the siphon. At the beginning of the fishery in the 1970s, PSTs were not considered by the Washington State Department of Health as a public health risk because the geoduck viscera were presumed to be discarded. However, it was later discovered that some members of a Native American tribe and of Asian immigrant communities in USA consumed the viscera in soup (Curtis *et al.*, 2000). Therefore, the sanitary programs of USA included the viscera as the target tissue for PSTs analyzes. This approach was adopted recently in Mexico to be in accordance with the USA regulation (Secretaría de Salud, 2016). The visceral ball is easily separated from siphon and mantle without risk of contamination. Consequently, it is considered that consumption of the siphon is safe since muscle tissue does not accumulate high concentration of PSTs (Curtis *et al.*, 2000). Actual Geoduck Clam Biotoxin

Monitoring Plan of Alaska (Alaska Department of Environmental Conservation, 2017) considers evisceration and use of the siphon in some cases when unacceptable levels of PSTs are detected in viscera. We found that accumulation of toxins in the muscular tissue was proportional to the concentration of these toxins in the viscera. This indicates that a proportion of the toxins ingested by the organism or present in the viscera are translocated to the muscle (f = 28.2+0.042x). The siphon will exceed the RL when visceral mass accumulates 18,424 µg STX eq kg^{-1}. More data with high PSTs concentrations in siphon are needed to describe a more robust statistical relationship, but it is clear that siphon can exhibit toxicity above the regulatory limit, as actually occurred in the 2017 HAB. In this year, PSTs concentration in the geoduck viscera was nearly three times higher than in 2015 and the PSTs concentration in the siphon exceeded the RL on three dates. These results have important regulatory implications, since the siphon could have a concentration above the regulatory limit if there is a high accumulation of PSTs in viscera.

The mouse bioassay (MBA; Anon, 2005) was historically the approved method for PSTs detection and quantitation in shellfish for regulatory purposes in the world. Recently, European Community and USA sanitary legislations included HPLC-based methodologies as alternative methods to monitor these. The presence of M-type toxins in *P. globosa* represents a monitoring challenge since the MBA will be eliminated from sanitary monitoring programs. There are no certified reference standards of these toxins, qne quantification and estimated toxicity have been assumed based on structure–activity relationship (SAR) studies with other saxitoxin analogues (Dell´Aversano *et al.*, 2008). In the case of *P. globosa,* we estimated that M-toxins represented up 49% of calculated total toxicity in some samples, based on assumed TEFs. Furthermore, the contribution of M-toxins to total toxicity was not dependent on the depuration process in the clam. The relative concentration of these analogues did not vary with time after the clams were exposed to *G. catenatum*. Importantly, *P. globosa* represents a potential source of these toxins for isolation due to the high concentrations found in viscera. The results obtained in the present work are important to elucidate biotransformation pathways in the metabolism of PSTs in an economically important bivalve. In addition, they are important to develop management plans for the *P. globosa* fishery since PST producing blooms have become recurrent (last documented bloom occurred in January 2018) in the last years in the NGC.

4.3 Comparation of HPLC-PCOX and Scotia Rapid Testing methods with mouse bioassay for the detection and quantification of Paralytic Shellfish Toxins in geoduck clam tissues

G. catenatum blooms have become recurrent in the NGC. PSTs concentrations in geoduck harvested in the area reach high levels, resulting in high risks to human food safety (Comisión Federal para la Protección contra Riesgos Sanitarios, 2015), impacts on wildlife (Garcia-Mendoza *et al.*, in preparation) and negative socio-economic effects (Medina-Elizalde *et al.,* 2018). Precautionary shellfish closures increase impacts to the region's economy that is supported by the geoduck fishery. It is therefore important to establish a routine and effective monitoring program for the presence of potentially toxic microalgae and toxins in shellfish to manage impacts and protect both public health and economical activities. The MBA is the reference method to determinate the PSTs concentration in shellfish within Mexico, but the laboratories authorized to perform the analysis are too far from the harvesting areas. Besides, international laws are changing and regions wishing to export shellfish to Europe and other regions must utilize non-animal bioassays for official control testing. The applicability of an internationally validated method must be locally tested with the shellfish species of relevance. Here, we report the performance of two methodologies for the quantification of PST in the local matrix *P. globosa*, the AOAC 2011.2 HPLC-PCOX method and the Scotia Rapid Test immune chromatographic kit.

Comparation of HPLC-PCOX and MBA

In this study, 35 *P. globosa* viscera samples were analyzed by HPLC-PCOX and MBA methodologies. The results showed a good correlation between HPLC-PCOX and MBA (r^2=0.91) where the values calculated with HPLC-PCOX were lower than the ones calculated with MBA. This result contrasts with previous reports, Turner *et al.* (2011) compared the performance of MBA with other non-bioassay methodologies in four shellfish species of United Kingdom. When analyzed mussels and cockles, the authors reported a correlation r^2 of 0.875 between MBA and HPLC-PCOX, but the values of the total toxicity of the samplers calculated with the non-bioassay methodologies were higher than the ones calculated using MBA. Turner *et al.* (2011b) concluded that MBA was underestimating values for some shellfish speciesand further analysis will be necessary to determinate the causes. However, they also concluded that a precautionary approach to public health protection would imply the use of analytical methods in addition to or in

replacement of the MBA for official control monitoring of PSP in oysters would be appropriate. Nevertheless, in a study conducted with samples of different species of shellfish from four countries in Latin America, Turner *et al.* (2020) obtained the same results as the present work, values calculated with HPLC-PCOX were lower than the ones calculated with MBA ($r^2 = 0.68$).

The HPLC-PCOX method in the present study was calibrated with 12 analogues of PST, the total toxicity reported represents the sum of the individual toxicities of those analogues. In a previous work, Medina-Elizalde *et al.* (2018) reported the presence of M-toxins in visceral clam samples collected after the 2015 HAB, with the M-toxins representing between 33% and 48% of the total toxin content. Due to the characteristics of these analogues, they can be detected by liquid chromatography with tandem mass spectrometry detection (LC-MS-MS) methods but not by fluorescence detection (LC-FLD). The MBA procedure involves the replicate injection of mice with filtered, hydrochloric acid extracts of shellfish with subsequent death times being used to calculate sample toxicity (Turner *et al.* 2012). The acidic extract that is injected includes all the hydrophilic toxins presents in the shellfish tissue, including the M-toxins. The presence of these toxins in the samples may explain the differences between the values calculated with both methods. Turner *et al.* (2020) also analyzed geoduck samples from Mexico (*P. generosa*), found a high relative proportion of M-toxins in the samples and lower toxicities calculated with HPLC-PCOX compared with MBA.

The PST HPLC-PCOX method has been validated by Van de Riet *et al.* (2009, 2011) and incorporated to the Canadian and USA shellfish regulation (ICCS, 2012). The performance of the method has been compared with other approved methods, such as PreCOX (AOAC 2005.06) and MBA, showing its potential effective use for the characterization of candidate reference materials (Turner *et al.* 2014) and the determination of the total PST concentration in different shellfish matrices worldwide (Burrell *et al.* 2016; Rey *et al* 2015).

Rey *et al.* (2016) and Biré *et al.* (2003) described that the advantage of the HPLC-PCOX method over other AOAC methodologies, for the PSTs determination, is that it enables chromatographic separation and quantification of each epimer individually and shows a good correlation with the mouse bioassay. In contrast, it requires additional time to run sample batches as two analytical columns are required, and the column lifetime is shortened due to the use of ion-pairing reagents in mobile phases. It also shows interferences from naturally fluorescent compounds present in matrices. In the present study, the lifetime of the columns used was found to range between 10 and 350 samples, after which there was no further separation of analytes due to high pressures and blockages due to salt accumulation.

Interferences due to the naturally fluorescent compounds present in the tissues of the clams extracts analyzed were also observed. At least four matrix peaks between five and seven minutes were observed, and this is where GTX4 and GTX1 elute (Figure 5). To determine whether such peaks are related to toxin presence or matrix components, standards were spiked into the naturally contaminated samples and reanalyzed. Differences in the matrix peaks between samples collected in different times of the year were also noticed. Figure 18 shows the chromatograms of samples from January and July where the matrix peak in July is lower than in January. This temporal variation was previously described by Rey *et al.* (2015) in scallops, whose work concluded that the differences in matrix peaks depend on the species, date of collection and geographical area. However, the performance of the methodologies must be validated in each of the local matrices and chromatographic parameters as well as column lifetimes and other practicalities considered in routine monitoring.

In Mexico, the Programa Mexicano de Sanidad de Moluscos Bivalvos (PMSMB) implemented by the Health Regulatory Agency COFEPRIS is the current system for monitoring toxin concentrations in shellfish. The PMSMB is a program equivalent to the NSSP. Due to this equivalence and considering that the NSSP had incorporated the HPLC-PCOX methodology and the good correlation between MBA and HPLC-PCOX showed in this study, Mexican Shellfish Regulation could adopt HPLC-PCOX as an official methodology for the detection of PSTs in the geoduck *P. globosa*. This will represent an advantage for toxin monitoring in the country, and especially in the NGC and for laboratories located near harvest areas where animals for experimentation are not always available. Also, after a HAB where a high volume of analysis is necessary to protect public health and commercial interest.

Detection of PSTs by Scotia Rapid Testing

In the present study, 16 *P. globosa* viscera samples with different PST profile were selected to be analyzed by Scotia Rapid Test, the samples were diluted to test PST concentrations around the RL and also 1/2, 1/4 and 1/8 times the RL, a total of 65 samples were analyzed by two analysts in a blind assay. The results showed a high percentage of false positives, with values between 37% and 67%.

Scotia Rapid Test has been tested as a potential methodology for the detection of PSTs in shellfish in different countries (Costa *et al.*, 2009; Laycock *et al.*, 2010; Turner *et al.* 2015; Dorantes-Aranda *et al.,* 2017; Turner *et al.* 2020). During these studies, one of the main issues for the applicability of the method

was the low cross reactivity of the test for some toxins, especially when the PSTs profile on the shellfish was dominated for low reactivity toxins.

The PSTs profile of the shellfish species tested in UK by Turner *et al.* (2015) and in Tasmania by Dorantes-Aranda *et al.* (2017) was dominated by GTX1&4, a toxin with low cross reactivity (Table 11). Both studies reported false positives of up to 98% and 55% respectively, considering the RL of 800 µg STXeq kg^{-1}.

During and after the 2015 HAB in the NGC, the major analogues identified in the viscera of *P. globosa* were C1&2, dcGTX2&3 and dcSTX. These analogues have a low cross reactivity of 7%, 5% and 40%, respectively (Table 4), which explains the high number of false positives obtained in this study. Using the values of the individual toxins calculated by HPLC-PCOX, samples were categorized in three profiles. Profile one samples were dominated by C1&2 toxins, with a cross reactivity of seven, and the total percentage of false positives in this profile was 22.4%, considering both analysts. Profile two was dominated by dcGTX2&3 toxins with cross reactivity of five, with a total of 38% false positives. Finally, profile three was dominated by dcSTX with a cross reactivity of 40 and 60% false positives. Turner *et al.* (2020) also reported a high proportion of false positives on geoduck samples from Mexico (66%). The highest percentage of false positives occurred in the profile three, even when the cross-reactivity of the major analogue in that profile is not as low as the other two profiles. The false positives can be explained due to the toxicity of samples close to the RL in profile three. This has been previously observed by Costa *et al.* (2009), Laycock *et al.* (2010) and Turner *et al.* (2014), who reported that SRT produce positive results for shellfish samples containing low levels of PSP. Furthermore, most of the false positives were reported for samples diluted to 400 and 200 µg STXeq kg^{-1}, the sensitivity of SRT is 250 µgSTX kg^{-1}.

Table 11. Cross-reactivity (mole % relative to STX) of Scotia Rapid Test as specified by the manufacturer (Laycock *et al.*, 2010)

PST analogue	Cross-reactivity	PST analogue	Cross-reactivity
STX	100	C1&2	7
Neo	21	GTX5	40
GTX2&3	93	dcSTX	40
GTX1&4	3	dcGTX2&3	5

In this study, one of the analysts reported four false negatives from the 20 samples analyzed with the profile one. All false negatives were indicated after sample dilution to 800 µg STXeq kg^{-1} and around

the RL. This could be associated with the subjectivity of the method rather than the assay. Mackintosh *et al.* (2002) reported this subjectivity as a disadvantage of the method.

Mexican Health Authorities have incorporated the use of Scotia Rapid Testing to the PMSMB as a screening method. According to this method, samples are analyzed in an approved laboratory for SRT located near to the harvesting areas. After a negative result, the shellfish can be commercialized, but after positive results a precautionary closure is implemented, and the PSTs extract is sent to Mexico City (3,000 km from the area) for confirmation by the MBA reference method. The high proportion of false positives obtained with this methodology (22%-60%) consequently has a negative impact on the fishing activities in the area.

The use of toxin monitoring kits could be advantageous to the shellfish industry by providing valuable data on the levels of regulated shellfish toxins in fishery products. However, due to significant variability in the PST toxin profiles of different PSTs-producer species, as well as the widely different in the potency of PST analogues, the applicability of different commercial test kits for local product testing requires careful consideration (Dorantes-Aranda, *et al.* 2017). To be useful, the tests would have to accurately reflect the toxins levels determined in the samples using the reference method (Turner *et al.,* 2011b). Considering the high percentage of false positives calculated in this study, SRT is not an accurate methodology for determining PST concentration in the local matrix *P. globosa*.

This comparative study has demonstrated that the use of alternative methods to monitor PSTs must be evaluated in each region, focusing on commercially important species, to ensure that the method is adequate to protect both public health and economic interests.

4.4 Impact of the presence of Paralytic Shellfish Toxins in the Northern Gulf of California during 2015-2019

The distribution of the PSTs in shellfish obtained from the NGC during the HABs that occurred in the period 2015-2019 was calculated from the toxicity detected by COFEPRIS using MBA in a particular area. The distribution of the toxicity in the NGC can be associated to multiple factors, such as food availability due to the origin, geographic extent, duration and intensity (cell L^{-1}) of the HAB, ocean circulation and waves. Also, the physiology of the clams must be considered to explain the high spatial

variability of PST accumulation. This will be affected by feeding rates, toxin-metabolism and depuration process, reproductive status, individual sensitivity to PSTs, variation in body mass, and the "shown factor" which refers to the fact that at a given moment only 70% of the clam population will have their siphon out of the sand, although they are not necessarily feeding (Curtis *et al.,* 2000). Toxicity may also be related to the location of the HAB, as the clams filter-feed on nearby phytoplankton. In 2015 and 2017, the HAB occurred south of San Felipe, while in 2018 and 2019 it appeared near and north of that port.

Accumulation of toxins in clams was first reported by the authorities in April-May 2010 and after 2015. FICOTOX Lab started the monitoring of the phytoplankton community and PST in geoduck in 2011. Murillo-Martinez (2011) reported the presence of PSTs in viscera of geoduck in concentrations higher than the RL in January (4,530 µg STXeq Kg^{-1}), February (849 µg STXeq Kg^{-1}) and in June (949 µg STXeq Kg^{-1}). In 2012, 17,200 cell L^{-1} of the dinoflagellate were registered, this is a higher concentration than the one reported in 2018; however, PSTs in clams were not reported by COFEPRIS. A similar case occurred in 2011 and 2014, when 5,000 cell L^{-1} of the dinoflagellate were quantified but the authority did not report the accumulation of toxins in geoduck. On the contrary, 2,400 cell L^{-1} were quantified in 2020 and PSTs were detected. In 2018, the concentration of *G. catenatum* was not as high as reported in the previous year, however the PSTs concentration in viscera exxceeded the RL 13 times in one of the sampling areas and the sanitary bans ended in March. The highest concentration of *G. catenatum* in the NGC occurred in 2017, however, the sanitary ban was ended in April in most of the harvesting areas. This contrasts with the 2015-HAB, when the dinoflagellate concentration was not as high, but the PSTs were present the whole year. The difference between these years is the duration of the HAB. In 2015, the species was present from January to May, while in 2017, from January to mid-February.

The non-detection of PSTs in bivalves, or detection at concentrations below the regulatory level, from 2011 to 2014, even when the PST-producer was present, may be due to the monitoring activities. As mentioned above, monitoring is performed monthly, which allows obtaining the samples after the depuration process. The methodology used to analyze toxins may also affect these results. After analyzing samples with different methodologies, Turner *et al.* (2020) obtained false negatives from samples analyzed by MBA, due to the method performance issues or issues relating to toxin stability due to the storage/transportation issues. The geoduck clam biology and food availability are also factors that should be considered to explain the absence of PSTs in clams from 2011 to 2014. Curtis *et al.* (2000) found differences in toxins accumulation in two populations due to feeding rates, food availability due to vertical and horizontal depth gradients, reproductive status, individual sensitivity to PSTs, body mass variation, and the "shown factor".

The presence of *G. catenatum* and the accumulation of toxins in geoduck clams affects the region's economy due to the implementation of sanitary bans that prohibits the extraction of clams in order to prevent human intoxications. COFEPRIS establishes closures per area, according to the results of the monitoring performed in samples obtained from a particular harvest area. It is common to have some areas closed and others open. Once the ban is implemented by the authorities, weekly samples are analyzed using the official methodologies (MBA) until three continuous negative sample are obtained. The most impacted areas during 2015-HAB were Enrique Gastelum and Minerva Perez (Figure 8 and 20), which remained closed for 184 days throughout the year. If all the areas are considered (with no overlapping days), the fishery of the geoduck in the NGC was prohibited for 294 days. In 2017, fishing in the NGC was also banned for most part of the year (244 days) and the Enrique Gastelum and Minerva Perez areas were closed for 156 days. In 2018 and 2019, activities were prohibited for 151 and 112 days, respectively (Table 12).

The geoduck clam fishery in the NGC does not have a seasonal pattern, but from May to September 40% to 50% of the total annual quota is harvested (DOF 2012). December and January are also important months for harvesting geoduck for shipment to China. China celebrates New Year in February and there is a high demand for this product for such as celebration. When the 2015-HAB was detected, at least six tonnes of geoduck had been caught and were in tanks at the processing plants in preparation for shipment to Asia. All of that product was destroyed to prevent human intoxications. As fishing was banned until June, the Mexican product did not reach international markets that year. As consequence, the companies involved in clam harvesting suffered important financial losses, ceased operations, laid off employees or declared bankruptcy (Minerva Perez, Com. pers.).

There are no formal studies of the socioeconomic impact of HABs in the NGC, only news published in regional newspapers with interviews conducted with company CEOs, the population and authorities of San Felipe, the main port of entry for the harvested product. The news published on February 28, 2017 in Ensenada.net described the economic impact of the HABs that occurred in 2015 and 2017. According to this, 1,200 layoffs occurred, and 18 tonnes of geoduck were destroyed which represents a loss of USD 150,000 due to inventory destruction plus USD 2,000,000 lost due to unrealized sales.

Other fisheries have also been affected due to the presence of PSTs in the NGC. In 2017, 205 kg of the pectinide *Atrina maura* were destroyed after COFEPRIS detected toxins concentrations higher than the regulatory level. There are 19 pectinide extraction areas in the NGC that were banned due to the presence of PSTs (COFEPRIS 2017).

Table 12. Maximum toxicity detected by HPLC-PCOX in geoduck and total days of prohibition of the fishery each year in the period 2015-2019 in the Northern Gulf of California. Total days of the year considering all harvest areas, the days in parentheses indicate the days when fishing was banned in the area with the most closures.

Year	Days closed	Max toxicity (µg STXeq Kg^{-1})
2015	294 (184)	16,700
2016	12	-
2017	244 (156)	153,000
2018	151	15,500
2019	112	7,800

HABs of *G. catenatum* seems to become recurrent in the NGC. This study describes the impacts of the HABs on wildlife, for the economy due to closures the shellfish fishing activities, and for public health since the HAB caused the first intoxication case by PST in Baja California (and the latest reported for Mexico since 2002; Ramírez-Camarena *et al.*, 2004) recognized by Mexican health authorities. The Upper Gulf of California is a unique ecosystem that has received worldwide attention due to efforts to protect the Vaquita (*Phocoena sinus*), the most endangered cetacean in the word (Jaramillo-Legorreta *et al.,* 2017). The vaquita is subject to unsustainable bycatch in gillnets set by small-boat fishers targeting shrimp and finfish (Thomas *et al,* 2017), especially totoaba *(Totoaba macdonaldi*), an endemic fish with a lucrative illegal trade to China for swim bladders (Thomas, 2017). Recently, unprecedented efforts have been undertaken to protect the vaquita. Totoaba aquaculture is now a strategy activity, among others, to prevent illegal capture of this fish and reduce bycatch pressure to the remaining vaquita population. The conservation problems of endemic and commercially important species, together with the need for socio-economic development in the region, represent a management challenge for authorities at all levels of government. HABs add another degree of complexity and must be considered when designing the management programs.

Chapter 5 Conclusions

- *Gymnodinium catenatum* is responsible for the presence of paralytic shellfish toxins (PSTs) in the Northern Gulf of California (NGC) and its accumulation in bivalves.

- *G. catenatum* impacted the wildlife, the economy, and the public health of the Northern Gulf of California. The harmful algae bloom (HAB) which occurred in 2015 is the only confirmed case where several marine mammals and seabird species were affected by *G. catenatum*. The toxic potential was exacerbated by planktivorous fish that was the vector of transference of PST to affected organisms.

- The intoxications (paralytic shellfish poison, PSP) that occurred during the 2015-HAB are the first cases recognized in Baja California by health authorities.

- The PSTs profile was characterized for the first time in different tissues of *Panopea globsa*. and PSTs accumulated mainly in the visceral mass. Toxicity in viscera was 23.8 times higher than detected in siphon.

- After exposure of geoduck to *G. catenatum,* C1&2 were the dominant toxins in both tissues. More potent STX-analogues such as GTX5, dcGTX2 and dcSTX appeared in time after the accumulation of PSTs. The change in the toxin profile was related to biotransformation associated to the metabolism of the geoduck.

- The depuration rate in visceral mass of geoduck (the target tissue for regulatory monitoring) was 4.3% of reduction per day.

- M toxins were detected for the first time in visceral mass of *Panopea globosa*. The toxicity of these STX-analogues contributed up to 75% of the total toxicity in some samples using assumed toxicity equivalency factors.

- Constant sanitary closures associated with HABs affecting the industry have negatively impacted the economy of the region. HPLC-PCOX is a useful methodology to quantify PSTs toxins in geoduck in the NGC, that can be implemented to provide fast and accurate results. However, the use of Scotia Rapid Testing is not adequate due to the highly number of false positives that are obtained.

- HABs of the PST-producer, *G. catenatum* are a reoccurring problem in the NGC that must be adequately attended through the development of a knowledge-based management plan.

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