

Marine guanidinium neurotoxins: Biogenic origins and interactions, biosynthesis and pharmacology

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1. Introduction

The marine environment is a vast repository of biogenic toxins, as beautifully illustrated and described by Halstead (1965) in his classic treatise *Poisonous and venomous marine animals of the world*. Anecdotal references to naturally occurring biotoxins from marine organisms and their relationship to coastal settlements date back to before written history. In the mythologies of indigenous cultures, such poisons have been associated with magic, religion, and medicine, sometimes in an indistinguishable way. Ancient understanding of marine toxins and toxigenic source organisms recognized the inherent risks of human exposure, and in some cases, led to their creative exploitation in concoctions for prey capture and against human enemies.

Among marine biotoxins, the guanidinium neurotoxins have played a unique and intriguing historical role in the development of human civilization throughout the world. The association of guanidinium toxins with harmful algal blooms (HABs) and toxic fish (e.g., pufferfish) has been part of every society linked to the sea. Despite lack of knowledge on the ultimate biogenic sources, for centuries the effects of these potent toxins have been recognized as a risk to seafood consumption and exploited for their rapid neurotoxic properties. The characteristic symptoms (rapid paralyzing effect) and etiology of guanidinium toxin exposure make it possible to retroactively ascribe poisoning events to these toxins with some degree of confidence, even from anecdotal or early written descriptions and in the absence of archived source material. Marine guanidinium neurotoxins figure most prominently in seafood poisoning incidents, but occasionally they have also been used in hunting and as chemical warfare agents against humans. More recently, their exquisitely targeted ion-channel interactions have been exploited as in neuropharmacology and as therapeutants.

The known chemical structures and nomenclature of their biological origin of the guanidinium neurotoxins divide them into two major sub-groups, comprising the tetrodotoxins (TTXs), and the saxitoxins (STXs). The latter group is also known as paralytic shellfish toxins (PSTs) due to the frequent accumulation in shellfish and consequent poisoning of human consumers. The guanidinium neurotoxins are synthesized by and derived from diverse source organisms from marine, freshwater aquatic, and terrestrial environments, but herein they are considered only from the marine perspective. The TTXs and STXs share a key structural feature—the presence of a least one free positively charged guanidinium group. This moiety determines

the mode of action as a voltage-gated sodium channel (Na_v) blocker in vertebrates and thus accounts for the common paralyzing effect.

The toxicological aspects of guanidinium toxins have been extensively reviewed (Durán-Riveroll and Cembella, 2017; Wang, 2008; Wiese et al., 2010) as well as the technological approaches to monitoring these toxins in seafood (Botana et al., 2017; Stauffer et al., 2019; Vilarinho et al., 2018), and analytical and diagnostic methods for their detection (Bragg et al., 2018; Harju et al., 2015; Rodríguez et al., 2018; Wharton et al., 2017). Accordingly, this review focuses primarily on guanidinium toxins in the context of socioeconomic history of human poisoning events and exploitation as neurotoxic agents, their biogenic origin and biosynthetic pathways, pharmacological mechanisms of ion-channel binding, and their functional role in species interactions in marine systems.



2. Perspective on global socioeconomic and health consequences

2.1 Toxicological history of guanidinium neurotoxins and poisoning events

2.1.1 Links of harmful algal blooms to seafood poisoning

Circumstantial associations between harmful algal blooms (HABs) and marine neurotoxins causing seafood poisoning and marine faunal mortalities have been noted throughout recorded history, long before the biogenic origin and toxicity mechanisms were understood. Associations between HABs and toxicity events can be traced back to ancient Greeks, who referred to the “red sea” for the water discoloration caused by high magnitude blooms and their consequent suffering from noxious algal blooms. Virgil (70–19 BCJ) noted: *Nihil ilior alga* (there is nothing fouler than an alga), presumably with reference to similar bloom events in the Mediterranean. An early reference to a toxic HAB is found in the biblical book of Exodus (7:20–21; King James Version): “...and all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river; and there was blood throughout all the land of Egypt.” The misnomer, “red tides” is still a colloquial reference widely used to describe high biomass blooms causing water discoloration, and in some cases to refer to shellfish toxicity or bloom-linked faunal mortalities. In fact, most such “red tides” and their effects are not caused by bloom species known to produce guanidinium neurotoxins nor to cause shellfish poisoning.

Indigenous populations in coastal regions of North and South America, Europe, and east- and southeast Asia, and including islands and archipelagoes recognized a link to manifestations (“blooms”) in coastal waters and toxic seafood, particularly with wild-harvested shellfish. Throughout the world, folkloric traditions and anecdotes have described incidents of recurrent human poisoning after consumption of shellfish, with symptomology characteristic of “paralytic shellfish poisoning” (PSP).

In the pre-Columbian Americas, PSP was long recognized according to the characteristic symptomology. In his report to the Spanish Crown, Alvar Núñez Cabeza de Vaca, from a failed Spanish expedition to the Americas from 1478 to 1528, states that indigenous Mexicans knew the risk of consuming shellfish during the first months of the year. During this standard time for “red tides,” shellfish consumption was forbidden (Núñez-Cabeza de Vaca, 1542).

In eastern Canada, the potential hazard of PSP from shellfish consumption was noted by the French expeditionary Lescarbot in his *Histoire de la Nouvelle France* (1609, re-published in Lescarbot (1907)). Therein he describes that the natives of Nova Scotia refused to eat mussels and preferred to eat their dogs and even tree bark when starving.

Traditional knowledge of the native populations of the northeast Pacific of the Americas extending to Alaska indicates that PSP toxicity from shellfish consumption was already well-known prior to European and Russian exploration and settlement. Notably, the Tsimshian and Haida tribes from the Pacific Northwest traditionally avoided shellfish consumption during periods of “shiny water” (Durán-Riveroll et al., 2018). This refers to bioluminescence produced by high cell densities of often toxigenic dinoflagellates now known to cause PSP. During his expedition to the northwest Pacific coast of North America near the end of the 18th century, Captain George Vancouver recorded in the ship’s log a PSP outbreak (and one death) among several crew members who consumed toxic shellfish (Vancouver, 1798). Multiple cases of shellfish poisoning consistent with PSP were recorded later from the California coast, most notably a toxic episode in San Francisco, California, in 1927, where more than 100 victims were poisoned, and several died (Sommer and Meyer, 1935).

Historical cases of PSP are not well documented from Central America until the last few decades. The phenomenon was first officially recognized in Guatemala in 1987 following a massive PSP outbreak with 187 people poisoned, of which 26 died.

In South America, the first registered cases of PSP date from 1886. Classic PSP symptoms and several deaths of indigenous inhabitants after consumption of mussels from the Beagle Channel region (Ushuaia, Argentina) were described (Segers, 1908). Sporadic anecdotal reports mostly from afflicted native populations in southern Chile and Argentina persisted throughout the early 20th C. Since 1972, well-documented PSP outbreaks in the Magallanes Strait region (Guzmán et al., 1975) have been an ongoing concern to public health and the shellfish industry in southern Chile (Benavides et al., 1993). The PSP cases and death of two crew members of the ship *Constanza* after consuming mussels while sailing by the Valdés Peninsula in 1980 brought scientific attention to PSP along the Argentine coast. Subsequent identification of the culprit dinoflagellate and mouse bioassay toxicity testing revealed one of the highest toxicity levels ever detected in mussels ($50,000 \mu\text{g STX}_{\text{eq}} 100 \text{g}^{-1}$ mussel tissue) (Carreto et al., 1981). Since then, the southern region of South America is noted as a global hot spot for PSP toxicity in shellfish.

On the other side of the Atlantic, in medieval Europe shellfish poisoning incidents with symptomology consistent with PSP were believed to be related to mystical causes, perhaps the act of an angry God or evil spirits. The first written reference to putative PSP in Europe appeared in 1689 in a scientific article about poisonous mussels in *Ephémérides des curieux de la nature*, a French scientific periodical with views on medical issues (Halstead, 1965). Much later, a renowned Prussian physician and pathologist (Virchow, 1885) reported apparent PSP toxicity with 4 deaths and 10 non-lethal illnesses from mussels harvested from Wilhelmshaven on the Wadden Sea coast of Germany. In 1938, an apparent PSP event occurred in Belgium whereby 4 people died after consuming cultivated mussels harvested from a canal near Brugge (Woloszynska and Conrad, 1939). Mussel poisoning consistent with PSP has been reported from Norway, first from 1901 and extending to the early 1980s, and is particularly well known from Trondheimfjorden and Oslofjorden (Karlson et al., 2021).

2.1.2 Historical link of tetrodotoxicity with pufferfish

Toxicity events caused by fish consumption are known throughout recorded human history (Davis, 1983; Haque et al., 2008), for which the association of tetrodotoxicity with pufferfish is particularly well documented. The French archeologist Claude Gaillard noted in *Recherches sur les poissons représentés dans quelques tombeaux Égyptiens de l'ancien empire* that the pufferfish is represented by hieroglyphics of ancient tombs from the

Fifth Dynasty (ca. 2700 BCE); the species depicted *Tetraodon stellatus* is since recognized as poisonous (Gaillard et al., 1923). The biblical book Deuteronomy (14:9-10, cited King James Version) from the late 7th C. BCE, states: “*These ye shall eat of all that are in the waters: all that have fins and scales shall ye eat: And whatsoever hath not fins and scales ye may not eat; it is unclean unto you.*” Pufferfish are virtually scaleless, and their fins are so minuscule that most species appear finless.

In the Americas, the ancient Mayans also knew of the poisonous nature of pufferfish from the Caribbean. In *Relación de las cosas de Yucatán* from 1566, the Spanish explorer Diego de Landa Calderón wrote about this deadly fish: “There is a tiny fish so poisonous that nobody escapes from dying when eaten, and this happens extremely fast. This fish is recognized because it swells very much” (de Landa, 1566). A Jesuit cleric Francisco Javier Clavijero also referred to a poisonous pufferfish called “botete” from Baja California in *Historia de la Antigua o Baja California* (Clavijero, 1852).

In Asia, stories about pufferfish poisoning events and knowledge of these species has been recorded by the Chinese since the 16th C. Engelbert Kaempfer, a German physician and naturalist, documented in *The History of Japan* (The Japan Foundation Information Center Library, 2021) existing knowledge and predilection for the three different kinds of “furuhe”. These pufferfish preparations were considered a cuisine delicacy and sometimes used to commit suicide.

There are detailed anecdotes of tetrodotoxicity from several expeditions that left written records. Captain James Cook, in *A Voyage Towards the South Pole* describes how he and some members of his crew got severely sick after eating a small portion of fish soup. He describes an “extraordinary weakness and numbness all over our limbs” that lasted for several days (Chau et al., 2011; Halstead, 1965).

2.2 Symptomology and sequelae of poisoning by guanidinium toxins

Members of the major guanidinium toxin groups affiliated with saxitoxin (STX) and tetrodotoxin (TTX), albeit rather different in structure, produce similar pharmacological effects in mammals due to their similar interactions on a common site of voltage-gated sodium channels of the neuromuscular system. Symptoms of guanidinium toxin poisoning are primarily neurological and typically appear between several minutes to a few hours after consuming contaminated seafood. At high oral dosages, death can occur in the first 6 h (Underman and Leedom, 1993). Symptoms usually initiate with lips, tongue, face, and fingertips tingling, numbness, and burning sensation,

leading to limb paralysis. Other common symptoms are ataxia, giddiness, staggering, lethargy, dry throat and skin, incoherence, aphasia, rash, and sometimes fever. Intestinal distress, nausea, and vomiting have also been reported. In severe cases, progressively decreasing respiratory muscle movements reduces ventilatory efficiency and eventually causing respiratory paralysis (Ahmed, 1991; Al-Sabi et al., 2006). The time-course and severity of the poisoning depend heavily upon multiple factors, such as toxin dosage ingested, body weight, age, health status, and simultaneous alcohol consumption with seafood ingested by the victim.

No specific antidote is known for reversing the paralysis and other neurological effects associated with guanidinium toxin poisoning; the only effective treatment consists of maintaining breathing support for severe cases showing respiratory distress. Counteractive neuropharmacological agents (e.g., atropine) have been administered in severe PSP cases but were not found very effective. Stomach and gut evacuation in early stages and administration of activated charcoal can assist in binding and removing the remaining food-borne toxins from the victim's gut. Victims of PSP surviving past 12–24 h frequently recover within a short time (Llewellyn, 2006).

Based upon follow-up studies on human victims, there are few apparent long-term consequences of guanidinium toxin exposure; the toxins are reversibly bound from Na_V channels without noticeable neuropathology and rapidly eliminated from the body. Nevertheless, guanidinium neurotoxins are capable of crossing the blood-brain barrier in high doses (O'Neill et al., 2016), leading to concerns regarding long-term sequelae. Post mortem analysis of PSP victims has shown widespread distribution of STXs in brain, cerebrospinal fluid, liver, spleen, heart, adrenal and thyroid glands, kidneys, pancreas, and lungs (García et al., 2004). Clinical studies on rat cortical neurons showed that chronic exposure to TTX causes dendrite retraction, loss of dendritic spines, and degeneration of the neurons within 1–2 weeks; apoptotic processes are also triggered by miniature excitatory postsynaptic currents (Fishbein and Segal, 2007). Chronic exposure of neurites to low doses of guanidinium toxins could affect neurogenesis during CNS development (Brackenbury et al., 2010).

Chronic exposure to guanidinium toxins does not exclusively affect the nervous system of vertebrates. Reduction in metabolic enzyme activity has been observed in different animal models, with the potential that enzyme polymorphisms could yield individual differences in response to STX (O'Neill et al., 2016). Such genetic differences may affect susceptibility to effects of STX exposure among human populations to some extent.

Anecdotal claims of acquired toxin resistance among individuals or populations more frequently exposed to chronic sub-lethal doses have not been substantiated.

In vivo models have shown both significant changes in antioxidant mechanisms and DNA damage in response to these guanidinium toxins in both fish and mammals (da Silva et al., 2011; Hong et al., 2003). Drinking water spiked with STX and provided to laboratory rats for 30 days induced changes in the antioxidant mechanisms in brain and liver (Ramos et al., 2014). The consequences on antioxidant mechanisms of low-dose exposure to guanidinium toxins have not been evaluated in humans but may warrant critical consideration of chronic pathologies, and not only within the nervous system.

2.2.1 Exposure routes for guanidinium toxins

Most cases of human exposure to guanidinium toxins derive from inadvertent ingestion of these toxins from contaminated seafood. Guanidinium toxins can be accumulated and transferred throughout marine food webs in complex patterns and compartments (Fig. 1), not all of which are considered seafood. Common routes of human exposure to STXs versus TTXs are, in fact, usually distinct.

2.2.1.1 Paralytic shellfish poisoning

Despite the high PSP risk from consumption of seafood, such species represent a globally significant nutritional source and are particularly vital for indigenous populations. Cases of PSP typically arise from the consumption of bivalve mollusks, such as clams, mussels, scallops, oysters, or cockles. Bivalve mollusks are the most frequent proximal cause of PSP because they suspension-feed on toxic plankton and rapidly accumulate toxins, whereas toxin release rates are relatively slow—days to years to become non-toxic in some cases. Toxins can remain in mollusk tissues for weeks or even months (Bricelj and Shumway, 1998).

In some instances, PSP can also occur after eating marine gastropods (whelks, snails) and crustaceans (e.g., lobsters, crabs), particularly after consuming hepatopancreas. Carnivorous gastropods, cephalopods, and crustaceans can become highly toxic primarily through feeding on bivalve mollusks (Shumway, 1995) and thus pose a severe PSP risk as a secondary vector. Several species of xanthid crabs and the mangrove horseshoe crab, *Carcinoscorpius rotundicauda*, have been demonstrated to bear different kinds of guanidinium toxins (Inoue et al., 1968; Noguchi et al., 2011), including

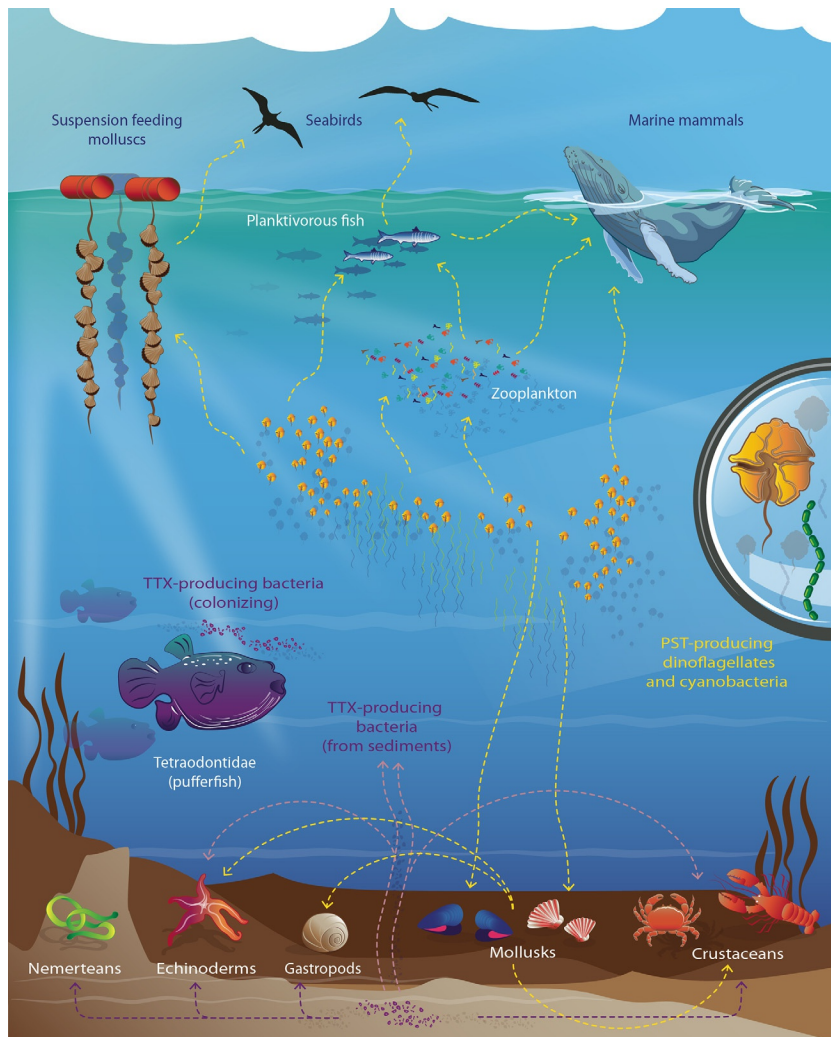


Fig. 1 Origin and fate of saxitoxins (STXs) and tetrodotoxins (TTXs) in the marine environment indicating food web transfer vectors and distribution among species.

both STXs and TTXs. Numerous PSP cases from crab consumption have been reported in the Japanese archipelago, but great individual and geographical toxicity variations occur (Noguchi et al., 1984). For some time, crabs themselves were believed to generate endogenous paralyzing toxins, but it is now known that these crustaceans acquire these toxins by carnivorous feeding on toxin-contaminated bivalve mollusks (Noguchi et al., 2011).

The sequestration of STXs varies dramatically among compartment organs of bivalve mollusks, such that if selected tissues are removed, the risk of PSP is significantly reduced (Cembella et al., 1993). In fact, in mature bivalve mollusks, STXs are retained primarily in the digestive system and the siphon and gonads. Little PSP toxicity is typically retained in the adductor muscle of shellfish or the skeletal muscle of finfish, but this is highly case- and species-specific.

The STXs accumulate in many marine species via the food web, particularly in bivalve shellfish by suspension-feeding on toxic dinoflagellates, but also in crustaceans and gastropods (Fig. 1). The toxins may be transferred to marine mammals and seabirds that feed by diverse mechanisms on zooplankton, mollusks, ichthyoplankton, and fish (Durán-Riveroll and Cembella, 2017). Marine mammals such as sea otters and seabirds which consume STX-contaminated shellfish suffer similar PSP symptoms, morbidity, and mortalities to human victims. Baleen whales can ingest high levels of STXs via filtering of krill and other zooplankton, whereas carnivorous (toothed) whales may acquire high toxin body burden by feeding on STX-contaminated fish (Durbin et al., 2002).

Outbreaks of PSP and numerous fatalities continue to be reported worldwide (Gestal-Otero, 2014) from Europe, Africa, the Americas, Asia and Oceania (Fig. 2). Most cases are linked to seafood consumption from the tropics with lesser frequency from temperate latitudes and few incidents from sub-arctic and polar latitudes. There are no known case reports from the high Arctic or Antarctica. Close to 2000 PSP cases are reported yearly around the world, with a mean fatality rate of about 15% (Hallegraeff, 1993). Children are more vulnerable and are subject to an elevated fatality rate (de Carvalho et al., 1998).

Successful toxin monitoring programs implemented in many affected countries have greatly reduced health risks and fatalities due to PSP (Etheridge, 2010). In Europe, the accepted lowest adverse effect level (LOAEL) in humans is $1.5 \mu\text{g STXeq kg}^{-1}$ body weight (b.w.) with an acute reference dose (ARfD) of $0.5 \mu\text{g STXeq kg}^{-1}$ b.w.; this yields a regulatory level of $800 \mu\text{g STXeq kg}^{-1}$ shellfish tissue for seafood safety (Boente-Juncal et al., 2020; EFSA, 2009). Such internationally recognized regulatory limits for STXs in seafood are frequently albeit not universally enforced for PSTs in seafood for human consumption.

Natural biotransformation into more toxic analogs can even enhance toxicity, and standard cooking procedures do little to reduce the total

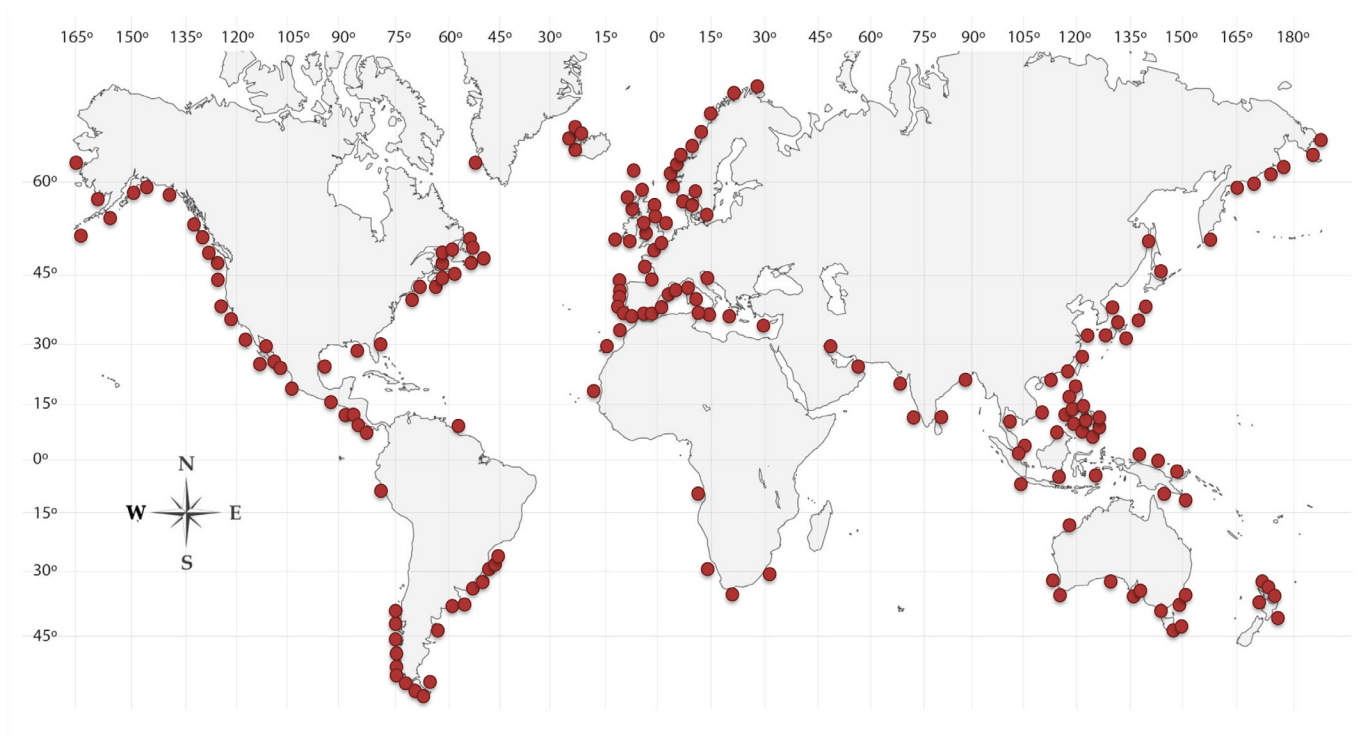


Fig. 2 Worldwide occurrence of paralytic shellfish toxin (PST) events reported up to 2021, representing human cases (including illnesses and fatalities), linked marine faunal mortalities, and toxin levels above regulatory limits in seafood. *Compiled and revised from Durán-Riveroll, L.M., Cembella, A.D., Correa-Basurto, J., 2018. Guanidinium toxins: natural biogenic origin, chemistry, biosynthesis, and biotechnological applications. In: La Barre, S., Bates, S.S. (Eds.), Blue Biotechnology: Production and Use of Marine Molecules. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 323–370. and HAEDAT, I.-I.-P., 2021. Harmful Algae Event Database. [http://haedat.iode.org/eventSearch.php?searchtext\[syndromeID\]=6](http://haedat.iode.org/eventSearch.php?searchtext[syndromeID]=6) [Online Available: Accessed 21 January 2021].*

toxin body burden in seafood (Costa et al., 2012). The PSTs are highly thermostable, although they can decompose under strong alkaline conditions (Durán-Riveroll and Cembella, 2017).

2.2.1.2 Tetrodotoxicity

Tetrodotoxicity in humans is most often due to consumption of members of the ray-finned fish order Tetraodontiformes, more specifically from within the family Tetraodontidae, commonly known as pufferfish. In fact, the guanidinium toxin group associated with tetrodotoxicity is named tetrodotoxin (TTX), and the syndrome is often referred to as “pufferfish poisoning” (PFP) (Field, 1998). The symptomology of tetrodotoxicity is rather characteristic, but it is not always possible to distinguish between fish poisoning caused by poor sanitary handling, preparation and storage, and cases of endogenous biotoxins. Many cases of fish toxicity are related to pathogenic bacteria or viruses, whereas endogenous biotoxins arise from the fish themselves or from symbiotic or commensal bacteria.

In practice, the designation PFP to describe tetrodotoxicity caused by TTXs is a misnomer—not all cases of tetrodotoxicity are due to ingestion of pufferfish and guanidinium toxins in pufferfish are not limited to TTX analogs. Rare cases of tetrodotoxicity in Japan have been documented due to ingestion of the trumpet shell, *Charonia*, and in Thailand from the eggs of the horseshoe crab, *Carcinoscorpius rotundicauda* (Kungsuwan et al., 1987). Cases of PFP in the eastern United States were attributed to several pufferfish species from Florida rich in STX analogs (but not TTXs), leading to the proposal to characterize this poisoning syndrome as saxitoxin pufferfish poisoning (SPFP) (Landsberg et al., 2006).

The highest recorded rates of tetrodotoxicity occur in east- and southeast Asia where pufferfish are traditionally and most commonly consumed. In Japan, the pufferfish delicacy known as “fugu” accounts for most cases. More than 100 fatalities per year were registered in Japan until 1960; from 1967 to 1976, 1105 cases and 372 deaths were recorded (Underman and Leedom, 1993). Since 1983, deaths from PFP have fallen dramatically to less than 10 per year, and most of these occur due to consumption of homemade dishes containing puffer fish liver. In Japan, where there is a regulatory limit of 2 mg TTXeq kg⁻¹ fish flesh, TTXs are responsible for 30–50 poisoning cases per year (Durán-Riveroll et al., 2018). In Europe, TTX is considered

an emerging toxin but is currently regulated only in the Netherlands, where it is included in the shellfish monitoring program at a regulatory limit of 44 g TTX kg⁻¹ shellfish tissue (Gerssen et al., 2018). Fish from the Tetraodontidae family may not be sold in European markets (Lago et al., 2015).

Cases of tetrodotoxicity are mainly linked to pufferfish harvested from tropical and sub-tropical waters, including from Taiwan, Hong Kong, China, Thailand, Singapore, Malaysia, Kiribati, Fiji, Australia, Papua New Guinea, Bangladesh, and even a few cases from the southern Pacific coast of North America (Durán-Riveroll et al., 2018; Noguchi and Ebesu, 2001). The sequestration of TTXs within seafood species leads to higher concentrations in particular organs, although the compartmentalization of toxins in fish is somewhat different from the STXs. In pufferfish, the highest tissue burdens of TTX are typically associated with the skin and internal organs, such as the gonads and liver, with much less toxicity in the skeletal muscle. In Japan, the Ministry of Health and Welfare has published a list of edible pufferfish species and prohibits the serving of the liver. Increasing local knowledge of the health risks, better health advisories on seafood safety and advances in the medical care of tetrodotoxicity victims, particularly in advanced economies, have reduced case and mortality rates in recent years.

Tetrodotoxicity among individual fish is highly variable and notoriously difficult to monitor. Despite general public awareness of the toxic potential of pufferfish, poisoning risk through pufferfish consumption remains high among indigenous populations in developing economies. Underreporting and poor case documentation yields statistical gaps and may account for the apparently infrequent occurrence of PFP in certain tropical and subtropical regions (Islam et al., 2011).

Tetrodotoxicity is an emerging threat in regions previously considered safe outside the sub-tropics and tropics and has penetrated even into temperate waters. This has been plausibly attributed to increasing water temperatures worldwide (Bane et al., 2014), causing a range expansion of toxigenic pufferfish. Ocean warming may also lead to distributional shifts in TTX-producing bacteria that do not colonize pufferfish. In recent years, there have been a number of reports of TTX occurring in benthic marine fauna from the United Kingdom, the Netherlands, and Iberia in Europe (Gerssen et al., 2018; Turner et al., 2015a,b), including in some cases bivalve shellfish species harvested as seafood.

2.3 Biogenesis of paralytic shellfish poisoning (PSP)

The historical biogeography of PSP events is described by cases of human poisonings, high toxin levels measured in seafood species, records of high biomass toxicogenic blooms, and associated marine faunal mortalities. The distribution of such events reveals a truly global distribution pattern (Fig. 2). Yet despite the long circumstantial associations to microalgal blooms, causative attribution of such paralyzing symptoms of shellfish toxicity to certain marine dinoflagellates did not occur until the early 20th Century.

Current knowledge indicates that major PSP events are exclusively caused by toxigenic members of several genera of marine dinoflagellates: the gonyaulacoids *Alexandrium*, *Centrodinium* and *Pyrodinium*, and the naked gymnodinoid *Gymnodinium* (Fig. 3). More than a dozen species of *Alexandrium* are known to produce STXs (Anderson et al., 2012), whereas in the heavily armored genus *Pyrodinium* only *P. bahamense* var. *compressum* is typically toxigenic (Cembella, 1998). Recently, *Centrodinium punctatum*, with a controversial taxonomic relationship to *Alexandrium*, was also found to

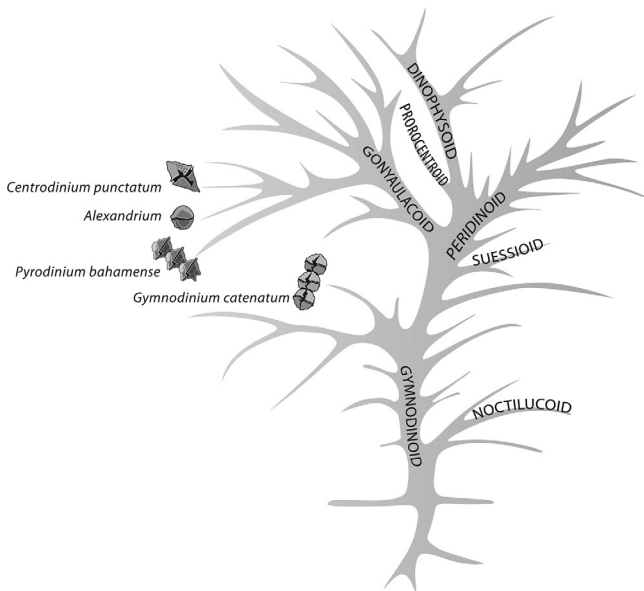


Fig. 3 Phylogenetic distribution of STX-biosynthetic capacity among extant marine dinoflagellates upon an evolutionary scheme proposed by Taylor et al. (2008) based on morphological, ultrastructural and molecular criteria.

produce multiple STX analogs (Shin et al., 2020). Only a single gymnodinoid species, the chain-forming *Gymnodinium catenatum* is confirmed to produce STXs (Hallegraeff et al., 2012).

Historically, the first confirmed association of causative dinoflagellate species with PSP was from poisoning events in California, attributed to a newly described chain-forming dinoflagellate *Gonyaulax catenella* (Sommer and Meyer, 1935; Whedon and Kofoed, 1936). Decades later, a morphologically similar but non-chain-forming dinoflagellate first described from southern England as *Gonyaulax tamarensis* was identified as a cause of PSP toxicity in shellfish from the Pacific Northwest of America and British Columbia and the primary source in Atlantic Canada (Riegel et al., 1949).

In the Indo-Pacific and Southeast Asia, the dinoflagellate *Pyrodinium bahamense* var. *compressum* has been associated with PSP since the 1970s. In Papua New Guinea, the first confirmed toxic bloom of this species occurred in 1972 (McLean, 1976), and subsequent bloom-related incidents were registered in Malaysia and Brunei (1976) and later in the Philippines (1983) (Maclean, 1989; Roy, 1977; Wiadnyana et al., 1996). Since then, *P. bahamense* is known as the dominant cause of PSP events in southeast Asia (Usup et al., 2012), with significant health risks and socioeconomic implications for seafood consumption.

In Central America, a major PSP outbreak in Guatemala in 1987 was the first attribution to toxic *P. bahamense* blooms in Latin America (Rodríguez et al., 1990). Subsequently, toxigenic blooms of this species have been associated with PSP events along the Pacific coast of Mexico (Alonso-Rodríguez et al., 2015) and in Costa Rica from the Gulf of Nicoya (Vargas Montero et al., 2008). *Pyrodinium bahamense* is also implicated as a source of STXs and contributor to a hazardous toxin reservoir in pufferfish associated with poisoning incidents in Florida (Landsberg et al., 2006).

The naked chain-forming dinoflagellate *Gymnodinium catenatum* was first described from the Gulf of California (Graham, 1943) but rarely reported from the plankton until the late 1970s. The first PSP events related to *G. catenatum* blooms date from 1976 from the Atlantic coast of Spain (Estrada et al., 1984). On the Pacific coast of Mexico in 1979, a massive toxigenic *G. catenatum* bloom was linked to a major fish kill, 19 cases of PSP, and 3 deaths (de la Garza-Aguilar, 1983; Mee et al., 1986). To date, in Latin America, 241 PSP cases with 14 fatalities in Mexico and Venezuela have been attributed to toxins from this species. Recently, mass mortalities of fish, marine mammals, seabirds, and cultured shrimp have occurred in

association with *G. catenatum* blooms, circumstantially due to STX toxigenicity. Prolonged closures of shellfish aquaculture facilities or harvesting from natural shellfish banks have caused important economic losses, mainly in the northern part of the Gulf of California (Band-Schmidt et al., 2019; Medina-Elizalde et al., 2018). Blooms of toxigenic *G. catenatum* associated with PSP events are reported with increasing frequency from at least 23 countries, including Australia, Japan, Portugal, Venezuela, Cuba, Uruguay, Morocco, Korea, New Zealand, the Philippines, Thailand, Singapore, Palau, and Malaysia (Hallegraeff et al., 2012).

Subsequent to the original linkages of PSP events to marine dinoflagellates, the culprit species have been frequently redescribed (occasionally in error), redefined, and subjected to almost continuous taxonomic and nomenclatural revisions. Currently revised taxonomic lists of marine dinoflagellates confirmed to produce STXs are freely available (IOC-Taxonomic List of Harmful Microalgae, Moestrup et al. (2021); AlgaeBase, Guiry and Guiry (2021)). Development of gene sequencing technology to supplement morphological descriptions has assisted in clarifying relationships among toxigenic dinoflagellate species but has also created further controversies regarding species assignment. Suffice to note here that earlier attempts to link species morphological descriptions with toxigenicity have not been generally convincing; within *Alexandrium*, obvious species descriptors such as chain-formation (“catenella”-type) versus single-cell (“tamarensis”-type) have not been supported in taxonomic revisions. Further details are beyond the scope of this review. Here only a few examples of proposed retroactive species assignments are noted for comparison. For example, the reference to chain-forming *Gonyaulax catenella* as the primary cause of PSP in the northeast Pacific would be reassigned to *Alexandrium catenella*. Most cases of PSP attributed to *Gonyaulax tamarensis* from Atlantic Canada, New England, and most of northern Europe, including early events from Norway (cited in Karlson et al., 2021) would shift to non-chain-forming *A. catenella* (ribotype Group 1) in the current nomenclature (John et al., 2014; Litaker et al., 2018). The apparent PSP incident in Belgium in 1938, associated with a gonyaulacoid (*Gonyaulax*-like) dinoflagellate identified as *Pyrodinium phoneus* (Woloszynska and Conrad, 1939), is most plausibly referable to *A. ostenfeldii*, originally described from Iceland.

Although all known cases of PSP can be referred to marine dinoflagellates, several species of cyanobacteria are also capable of STX biosynthesis. Among cyanobacteria, production of certain STX analogs has been

confirmed in at least nine genera comprising more than a dozen species, including *Dolichospermum circinale*, *Aphanizomenon* spp., *Lyngbya wollei*, and *Cylindrospermopsis raciborskii*, found primarily in fresh- and brackish waters (Christensen and Khan, 2020). A few cyanobacterial genotypes found in marine environments, particularly in the tropics, may also contain these toxins, but PSP events associated with such marine cyanobacterial blooms are unknown. The presence of STXs in free-living Eubacteria (“true bacteria”) or from isolated endosymbiotic bacteria from dinoflagellates has been reported in the early literature but remains unconfirmed by advanced genomic and toxin analytical methods.

Biosynthetic capacity for guanidinium toxins of the STX group is highly variable among species, genetic strain, and natural biogeographical populations of both dinoflagellates and cyanobacteria (Anderson et al., 2012; Cembella et al., 1993). Cell toxin content is also highly dependent upon growth rate and environmental conditions, leading to the conclusion that simple quantitative generalizations regarding toxigenicity of strain or species are largely inaccurate.

2.4 Guanidinium toxins in traditional medicine, ceremony and chemical warfare

Guanidinium neurotoxins have been associated historically with both hunting and warfare due to their paralyzing activity. Their neurological properties have been recognized in folklore and sometimes exploited in traditional medicine by the application of crude concoctions extracted from toxic species. Anecdotal reports from the Pacific Northwest of North America indicate that native tribes were well aware of the lethal paralyzing effect of seasonally harvested shellfish and may have deliberately served toxic cooked portions containing STXs to unwanted guests—an early example of chemical warfare against tribal enemies. Extracts of these shellfish toxins are not known to have been employed in tribal medicine and were rather avoided.

There are constraints against development of STXs and TTXs as chemical weapons—lack of sufficient supply of toxins and an appropriate administration route (oral, injected, or inhaled). The supply limitation can be alleviated through extraction and purification from high biomass natural sources, chemical synthesis, or biosynthesis. All procedures have drawbacks for the preparation of high quantities of these guanidinium neurotoxins, either due to the low concentrations in natural sources, the challenges of

chemical syntheses with multiple reaction steps and low final yields, and lack of knowledge on the toxin genetics for microbial bioproduction.

Consideration of STXs for military warfare was initiated in the USA during the early phases of the cold war with Eastern Europe. In fact, the impetus for extraction and purification of unknown STXs from Alaskan butter clams and dinoflagellates (Schantz *et al.*, 1975) was initiated and financed under contract with the U.S. Army Biological Warfare Laboratories at Fort Detrick, Maryland. This decade-long initiative yielded gram-quantities of purified STX retained by the U.S. military for chemical warfare research and later for potential deployment as anti-personnel agents by the CIA. In the 1960s, congressional investigations verified that part of the STX stockpile was intended for potential assassination attempts on foreign enemies (reputed to include Cuba's Fidel Castro). Also disclosed was the replacement by STX as an alternative to suicide agents such as cyanide or strychnine for captured spies and military personnel; the captured American U-2 spy plane pilot Francis Gary Powers was issued STX if caught by the Russians but failed to use it as directed (Powers and Gentry, 1970).

Subsequent civilian research since the 1970s has continued to discover aspects with potential military application. Aerosolized STX hydrochloride has been proven much more lethal than through other routes of administration. Though no human intoxication cases by inhalation have been reported, animal experiments suggest that death may occur within minutes (Sierra and Martínez-Álvarez, 2020). The chemical synthesis of STX (Tanino *et al.*, 1977) has raised the possibility of producing this toxin in sufficient quantities for mass application to populations.

Military interest in STX as a chemical warfare agent waned after the discovery that it was difficult to extract and purify in large quantities from natural sources, complicated to synthesize with high yield, and could not be readily aerosolized in an effective format for mass gas attacks. As far as is known, active research on chemical warfare application of STXs has been suspended globally, but the possibility of transferring or modifying the biosynthetic genes in alternative microorganisms could revive interest in mass production of these toxins for military purposes (Tucker, 2002). In any case, the stockpile of purified STXs from the CIA facilities has been distributed for toxicological and pharmacological research purposes and as calibration standards for bioassays and instrumental analysis of these toxins (Tester *et al.*, 2018).

Throughout human history, extracts of pufferfish have been exploited for their neurotoxic potency in hunting, medicinal and religious ceremonial preparation, and occasionally used against human enemies. Furthermore,

pufferfish extracts have been revealed as important ingredients in zombification concoctions for ceremonial and religious rituals throughout the Caribbean region (Davis, 1983), demonstrating relevant traditional knowledge on its toxicity.

Japanese and Chinese traditional medicine register the use of TTX preparations to alleviate several neurophysiological disorders (Newman and Cragg, 2016). One of the earliest deliberate lethal uses of TTX in Japan was for committing suicide and perhaps also to murder (Gage, 1971). Murder attempts with STX are also recorded from Sierra Leone and the United States (Llewellyn, 2015; Ohno, 2006).

In 18th Century Japan, pufferfish toxicity was tested by feeding war prisoners with the flesh and liver of the fish (study cited by Suehiro (1947)). It is debatable to consider such cases of forced human assays as within the jurisdiction of chemical warfare conventions (Haque et al., 2008; Llewellyn, 2015). Mystique and traditional preparation of “fugu” in Japan continue to favor the deliberate inclusion of some TTX to invoke symptoms of mild tetrodotoxicity (light-headedness, mild tingling, euphoria) without causing an extreme effect—a delicate balance based on unscientific techniques (Llewellyn, 2015).

Saxitoxin is the first and only marine molecule declared a Schedule 1 chemical weapon. Unlike STX, TTX is not considered a weapon under the Chemical Weapons Convention. Any facility producing more than 100 g STX per year must be declared, production facilities must be open to routine on-site inspection, and every transfer must be reported (Tucker, 2010). For decades, a permit from the Organization for the Prohibition of Chemical Weapons and the United Nations was needed to dispatch or receive even small quantities of STX of no consequence to human health or military application. This restriction on trans-shipment of STX negatively affected chemical, toxinological and neurological research. This problem has been partially alleviated (Llewellyn, 2015) by distribution of analogs and alternative formulations, e.g., STX-diHCl or STX-diacetate in solution, which are not covered under Schedule 1 inclusion of STX alone.



3. Evolutionary origins and functional biosynthesis

3.1 Biosynthesis of saxitoxin

Current hypotheses suggest that the biosynthetic genes for STX arose first in bacteria, but there is no conclusive evidence that extant bacteria are capable of synthesizing these toxins. In fact, half of the known dinoflagellate STX

biosynthetic gene cluster (*sxt*) homologs are linked to probable proteobacterial origin (Orr et al., 2013), although no strains are known to synthesize these toxins. The first putative STX biosynthetic gene cluster was identified in the cyanobacterium *Cylindrospermopsis raciborskii* T3 (Kellmann et al., 2008b). Minor gene cluster variants were later confirmed in other cyanobacteria, including *Anabaena circinalis* AWQC131C and *Aphanizomenon* sp. HN-5 (Kellmann et al., 2008a; Mihali et al., 2009). A core set of 14 genes (including *sxtA-sxtI*, *sxtP-sxtR*, *sxtS* and *sxtU*) were originally found among STX-producing cyanobacteria. About a dozen more variable elements of the *sxt* gene cluster have been subsequently identified. Presence or absence of individual elements of the gene cluster can account for the toxin composition profile differences among cyanobacterial strains and species (Soto-Liebe et al., 2010).

Comparative phylogenomic analysis of draft genomic assemblies of putative *sxt* genes in various filamentous cyanobacteria (Moustafa et al., 2009) suggests the biosynthetic genes were derived via multiple horizontal gene transfer (HGT) events. Alternative origins from multiple precursor bacteria were accompanied by subsequent coordination within and among multiple cyanobacterial lineages. Under this scenario, the establishment of ancestral toxigenic strains within filamentous cyanobacteria was succeeded by widespread and common loss of the relevant genes. This would account for the patchy distribution of toxigenicity (mostly absent) among extant cyanobacterial lineages.

Phylogenomic evidence from toxigenic cyanobacteria leaves open questions as to whether or not the development of STX gene homologs in dinoflagellates arose via single or multiple HGT events directly from STX-producing cyanobacteria or independently, perhaps from bacterial precursors. The hypothesis that convergent evolution of biosynthetic genes may account for their occurrence in phylogenetically unrelated dinoflagellates and cyanobacteria remain open because of the shared domain structures of the *sxtA* (Stüken et al., 2011) and *sxtG* (Orr et al., 2013) core genes. Nevertheless, the occurrence of multiple *sxt* homologs with high sequence identity (Orr et al., 2013; Stüken et al., 2011) renders this convergent origin hypothesis unlikely in such phylogenetically unrelated groups that lack a common ancestor. Close homologs of the cyanobacterial genes have been identified as nuclear-encoded genes in the STX-producing dinoflagellates *Alexandrium fundyense* (now renamed *A. catenella*) and *A. minutum* (Stüken et al., 2011). This similarity led to the hypothesis that the biosynthetic genes arose via horizontal gene transfer (HGT) between ancestral STX-producing

bacteria and dinoflagellate nuclei. Nevertheless, comparative analysis of the structure and assembly of respective STX gene clusters in cyanobacteria and dinoflagellates indicates enough dissimilarity to suggest substantial independent evolution. The current model (Wang et al., 2016) proposes a core cluster of about a dozen *sxt* genes shared between toxigenic dinoflagellates and cyanobacteria. These are linked to tailoring, regulatory and transporter *sxt* genes that are subject to independent evolution and modification to yield different STX-biosynthetic analogs among lineages. This could include secondary loss of key gene cluster elements necessary to complete biosynthesis.

The fact that among dinoflagellates STX is produced by three genera within a single gonyaulacoid family (*Alexandrium*, *Centrodinium* and *Pyrodinium*) and by a single species (*Gymnodinium catenatum*) from a distantly related gymnodinoid dinoflagellate order (Fig. 3) does tend to indicate that such bacteria-to-dinoflagellate horizontal gene transfer would likely have taken place prior to the separation of the gonyaulacoid genera *Alexandrium* and *Pyrodinium*. Under this scenario, this event was followed by a dinoflagellate-to-dinoflagellate horizontal gene transfer into *G. catenatum* (Stüken et al., 2011).

Whether or not the evolution of Na_V ion channels reflects the functional role of guanidinium toxins and the evolution and maintenance of the corresponding biosynthetic genes in cyanobacteria and dinoflagellates remains unknown. The proposed adaptive advantage of STX analogs in mediating Na^+ ion regulation and transport in extant toxigenic cyanobacteria from fresh and brackish waters (Pomati et al., 2004a,b) is consistent with this functional role. The osmoregulatory effect on Na^+/K^+ -ATPases and proton pump H^+ -ATPases for regulation of salinity/cell volume and pH equilibrium, respectively, could have been crucial to the co-evolution of biosynthetic genes for STX and susceptible ion-channels in cyanobacterial lineages.

In contrast, in marine dinoflagellates this osmoregulatory function for STXs may have been supplanted by their role in chemical defense against grazers or competitors (Cembella, 2003). If chemical defense, particularly against metazoan grazers, is indeed the primary function of these compounds in dinoflagellates, this implies that the evolutionary function and selective pressure on the biosynthetic genes may already have diverged from the cyanobacteria (Durán-Riveroll and Cembella, 2017). In that case, the potent effects of guanidinium neurotoxins on the Na_V channels of the highly evolved neuromuscular systems of marine mammals, seabirds, teleost fish,

and human consumers of toxic seafood must be viewed as “collateral damage” in the chemical arms race—but not of evolutionary adaptive significance.

3.2 Biogenesis of tetrodotoxin

The biogenic origin of TTXs remains controversial, although there is consensus that pufferfish are the major vector in marine environments. Originally, it was thought that TTXs were found only in fish of the family Tetraodontidae, presumably as a predatory defense mechanism (Matsumura, 1995). The TTXs are now known to occur in a wide diversity of phylogenetically unrelated organisms (Fuhrman, 1986), comprising 6 phyla of marine and terrestrial organisms (Chau et al., 2011). In the marine environment, TTXs have been found in pufferfish species of the genera *Arothron*, *Fugu*, *Lagocephalus*, *Sphoeroides*, *Takifugu*, and *Tetraodon*; in gastropods from the genera *Charonia*, *Nassarius*, *Oliva*, *Rapana*, and *Steromphala*; in the opisthobranch *Pleurobranchaea maculata*, and in starfish of the genus *Astropecten* (Pratheepa and Vasconcelos (2013) and references therein).

Evidence from early studies that non-toxic species could acquire tetrodotoxigenicity by feeding on toxic prey and becoming TTX bearers (Noguchi et al., 1982) led to alternative hypotheses on the biogenic origin of TTXs and transfer of toxigenicity: (i) endogenous synthesis by the members of genetically unrelated species, e.g., of fish or marine invertebrates; (ii) bioaccumulation through the food web; and (iii) synthesis by endosymbiont bacteria. Production of TTX by the isolates of the bacterial genera *Vibrio* and *Pseudomonas* confirmed the third hypothesis (Noguchi et al., 1986; Yasumoto et al., 1986), but of course does not rule out the alternatives. To date, at least 23 bacterial species have been found to produce TTX (Pratheepa and Vasconcelos, 2013). Pufferfish, gastropods, and other marine organisms acquire the toxin through the food web and/or from toxigenic bacteria while serving as bacterial hosts or vectors for the toxins (Bane et al., 2014; Kono et al., 2008; Matsui et al., 1990).



4. Discovery and properties of guanidinium neurotoxins

Guanidinium toxins are small molecules (low molecular weight, 200–600 Da) and mostly water-soluble. The guanidinium moiety is the main chemical characteristic shared by both STX and TTX groups, with

important implications for their specific potency. Each guanidinium moiety confers a positive charge to the molecule at physiological pH and accounts for the potent Na_V channel blocking activity. The guanidinium neurotoxins are among the most potent known natural toxins, with oral lethal dosages for humans in the low milligram range for the most toxic analogs of TTX (Bane et al., 2014) and STX (Wiese et al., 2010). In humans, the minimal lethal oral dose required to kill half of the subjects (MLD_{50}) is around 2 mg TTX, and an amount near this value will trigger poisoning symptoms (Noguchi and Arakawa, 2008). The lethal oral dose of STX in humans is between 1 and 4 mg, depending upon the age and physical condition of the victim.

4.1 Discovery and toxicological characteristics of STXs

The association of PSP with shellfish toxicity was well known but poorly understood by the beginning of the last century. In the late 1920s, PSP toxicity was referred to as “mytilism” after the vector mussel *Mytilus edulis*. The relationship with toxigenic dinoflagellates (*Gonyaulax catenella*) and mytilism was established after the 1927 PSP outbreak in California by feeding non-toxic mussels with cultures of *G. catenella*, demonstrating that the mussels became poisonous (Riegel et al., 1949; Sommer and Meyer, 1935). Discovery of the PSTs was guided and facilitated by the development of a standardized mouse bioassay, by defining a mouse unit (MU) as the minimum amount of toxin that would kill a 20-g male white mouse in 15 min after injected intraperitoneally with 1 mL of shellfish extract (Schantz, 1984).

Large amounts of toxic material were extracted and purified from the Alaska butter clam *Saxidomus giganteus* supplemented by small quantities from cultured dinoflagellates (Schantz, 1984). After a decade of work extracting and by mouse bioassay-guided fractionation, they obtained a highly purified potent substance ($5500 \pm 500 \text{ MU mg}^{-1}$) dubbed saxitoxin (STX) after the primary biological source (Mold et al., 1957). The structure of STX was confirmed by spectroscopy and specifically by X-ray diffraction of a single crystal (Schantz et al., 1975).

In the decades following the publication of the STX structure, the group was supplemented by several guanidinium analogs: (i) N-1 hydroxylated STX called neosaxitoxin (NEO) purified from scallops (Shimizu et al., 1978); (ii) carbamoyl C-11 sulfated STX epimers gonyautoxin 2

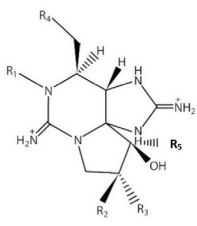
and gonyautoxin 3 (GTX2/3) (Boyer et al., 1978) and corresponding sulfated NEO epimers GTX1/4; and (iii) 21-N-sulfocarbamoyl derivatives GTX5/6 (or B1/B2) and C1–C4 of reduced potency (Schantz, 1984). Much later, a group of benzoyl (-GC) derivatives was characterized from the dinoflagellate *Gymnodinium catenatum* (Negri et al., 2003). To date, more than 60 naturally occurring STX analogs have been characterized from marine dinoflagellates, cyanobacteria, or from vector species (Fig. 4), including biotransformation products and biosynthetic precursors.

4.2 Discovery and toxicological characteristics of TTX

By the late 19th C, statistics of more than 100 Japanese dying every year from fugu consumption created an intense scientific interest in the nature of the chemistry and pharmacology of this unknown fish poison. In his comprehensive treatise, Halstead (1965) provides detailed historical references to the discovery of the toxicological properties and distribution of tetrodotoxicity. Serious research was initiated in the early 1880s, and studies soon determined that death was caused by asphyxia due to respiratory muscle paralysis. In 1883, visiting French and Russian doctors in Japan reported in *Comptes rendus des séances et mémoires de la Société de Biologie* the toxicological symptoms and autopsy findings in dogs exposed to multiple species of pufferfish. In 1889 an illustrated guide and atlas on poisonous fishes was published for the safety of naval personnel.

Independent attempts to isolate and characterize pufferfish poison in the 1890s yielded “tetrodonin” and “tetrodotoxin,” the latter considered to be useful in pharmacology. According to Suehiro (1947), the exact chemical nature of these preparations was never known, but they likely included the components now described as tetrodotoxins (TTX). Toxicity was fully understood only after the isolation of crude crystalline toxin (then named spheroidine) from the ovaries of pufferfish (*Fugu rubripes*) (Yokoo, 1950).

Structural elucidation of the key analog TTX was not confirmed until 1964 after the independent findings by three research groups were presented at the IUPAC Symposium of Chemistry of Natural Products (Goto et al., 1965; Tsuda et al., 1964; Woodward, 1964). The base compound TTX consists of six hydroxyl groups and a positively charged guanidinium group containing three nitrogen atoms and a pyrimidine ring. More than a dozen naturally occurring analogs have been discovered in marine species (Fig. 5), along with new analogs from terrestrial sources (mainly bacteria and amphibians) and novel synthetic derivatives.















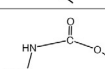
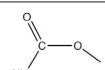




Analog	R ₁	R ₂	R ₃	R ₄	R ₅
STX	H	H	H		OH
NEO	OH	H	H		OH
GTX1	OH	H	OSO ₃ ⁻		OH
GTX2	H	H	OSO ₃ ⁻		OH
GTX3	H	OSO ₃ ⁻	H		OH
GTX4	OH	OSO ₃ ⁻	H		OH
B1	H	H	H		OH
B2	OH	H	H		OH
C1	H	H	OSO ₃ ⁻		OH
C2	H	OSO ₃ ⁻	H		OH
C3	OH	H	OSO ₃ ⁻		OH
C4	OH	OSO ₃ ⁻	H		OH
dcSTX	H	H	H		OH
dcNEO	OH	H	H		OH
dcGTX1	OH	H	OSO ₃ ⁻		OH
dcGTX2	H	H	OSO ₃ ⁻		OH
dcGTX3	H	OSO ₃ ⁻	H		OH
dcGTX4	OH	OSO ₃ ⁻	H		OH
GC1	H	H	OSO ₃ ⁻		OH
GC2	H	OSO ₃ ⁻	H		OH
GC3	H	H	H		OH
GC4*	OH	H	OSO ₃ ⁻		OH
GC5*	OH	OSO ₃ ⁻	H		OH
GC6*	OH	H	H		OH
GC1a*	H	H	OSO ₃ ⁻		OH
GC2a*	H	OSO ₃ ⁻	H		OH
GC3a*	H	H	H		OH
GC4a*	OH	H	OSO ₃ ⁻		OH
GC5a*	OH	OSO ₃ ⁻	H		OH
GC6a*	OH	H	H		OH
GC1b*	H	H	OSO ₃ ⁻		OH
GC2b*	H	OSO ₃ ⁻	H		OH
GC3b*	H	H	H		OH
GC4b*	OH	H	OSO ₃ ⁻		OH
GC5b*	OH	OSO ₃ ⁻	H		OH
GC6b*	OH	H	H		OH
LWTX1	H	H	OSO ₃ ⁻		H
LWTX2	H	H	OSO ₃ ⁻		OH
LWTX3	H	OSO ₃ ⁻	H		OH
LWTX5	H	H	H		OH
LWTX6	H	H	H		H
LWTX4	H	H	H		H
M1	H	OH	H		OH
M3	H	OH	OH		OH
M2	H	OH	H		OH
M4	H	OH	OH		OH

Fig. 4 Saxitoxin and major naturally occurring analogs in marine dinoflagellates and cyanobacteria. Left: 3,4,6-trialkyltetrahydropurine skeleton, common to all STX analogs. STX, saxitoxin; NEO, neosaxitoxin; GTX1–4, gonyautoxins 1–4; B1, B2, toxins B1 and B2; C1–C4, toxins C1–C4; dcSTX, decarbamoyl saxitoxin; dcNEO, decarbamoyl neosaxitoxin; dcGTX1–4, decarbamoyl gonyautoxins 1–4; LWTX1–6, lyngbytoxins 1–6; M1–M4, *Mytilus* toxins 1–4. Asterisks* for the benzoyl (GC)-toxins refer to putative structures determined by mass spectrometry with NMR support in some cases, but which remain to be confirmed.

4.3 Toxin potency of saxitoxin and analogs

Among the more than 60 known STX analogs, at least 18 are considered of toxicological significance (Fig. 6). Specific toxin potency in mammals ranges from the most potent analog groups as follows: carbamoyl >

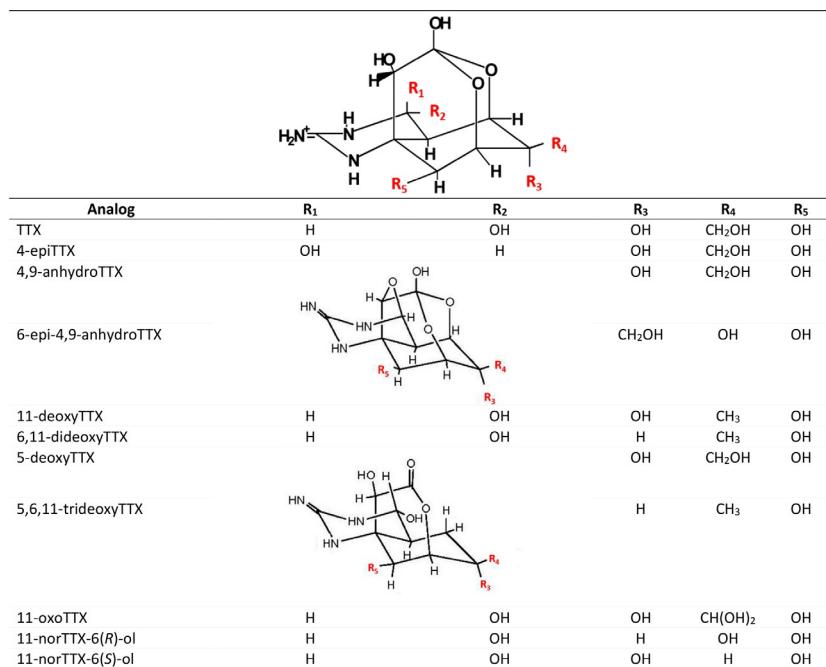


Fig. 5 Tetrodotoxin and main marine analogs found in pufferfishes and other marine fauna. Modified from Durán-Riveroll, L.M., Cembella, A.D., 2017. Guanidinium toxins and their interactions with voltage-gated sodium ion channels. *Mar. Drugs*, 15, 303; Yotsu-Yamashita, M., Abe, Y., Kudo, Y., Ritson-Williams, R., Paul, V.J., Konoki, K., Cho, Y., Adachi, M., Imazu, T., Nishikawa, T., 2013. First identification of 5, 11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. *Mar. Drugs*, 11, 2799–2813.

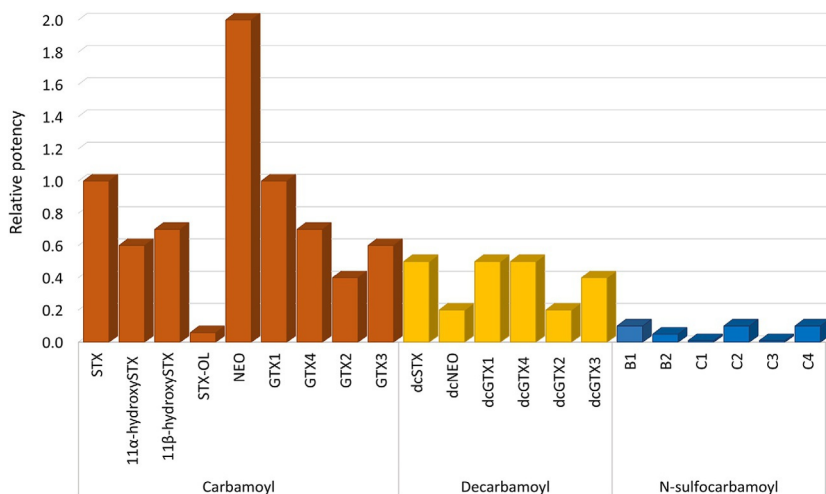


Fig. 6 Relative potency of saxitoxin (STX = 1) and major carbamoyl, decarbamoyl and N-sulfocarbamoyl analogs based on combined results of all existing toxicity studies and re-evaluated by electrophysiology as recommended values from FAO/WHO (2016).

decarbamoyl > *N*-sulfocarbamoyl; potencies of benzoyl (*p*-hydroxybenzoyl, di-hydroxybenzoyl, and sulfobenzoyl) analogs have not been specifically evaluated but are likely to be relatively low potency (Durán-Riveroll et al., 2016).

Variability in specific potency among STX analogs determined from the administration of purified toxins in the intraperitoneal (i.p.) mouse bioassay (MBA) is well defined and calibrated. However, this assay assumes that the dose–death time curves for all analogs are identical, leading to questionable validity of the assay for potency comparison among analogs (Munday et al., 2013). Previous reports on analogs toxic potency were inaccurate and had critical errors, as noted by the FAO/WHO (2016). Specifically, for the N-1 hydroxylated and some sulfated analogs (NEO, GTX1/4, GTX2/3), there is no correlation between the relative specific activity and relative toxicity by i.p. injection into mice. Recent reevaluation of the toxin potencies with certified materials and by adding oral administration testing (feeding and gavage), found significant differences from previously reported toxin equivalency factors (TEFs) (Munday et al., 2013). Furthermore, *in vitro* methods have established a more accurate TEF for the STX group by testing their binding to the Na_v channel in neuronal cultures (Vale et al., 2008). The European Food Safety Authority combined the results of all existing studies in a TEF list that was later re-evaluated with an electrophysiological (patch clamp) approach (Pérez et al., 2011). Different methodologies rendered mostly comparable results, except for neosaxitoxin (NEO), which showed a wide range of relative toxicity values, from 1 to 2.54 (EFSA, 2009; Munday et al., 2008). Finally, the FAO and WHO Expert Group, after contemplating all the previous TEFs reported (FAO/WHO, 2016), recommended the relative potency values of STXs as shown in Fig. 6.

4.4 Toxin potency of tetrodotoxin and analogs

Toxicity equivalency factors (TEFs) for TTXs are essential for the evaluation of relative risk but unlike for STXs, information on relative potencies of TTXs is limited. Even the definition of some terms like the minimum lethal dose (MLD) is highly variable and inconsistent among studies (FAO/WHO, 2016). The toxin potency of TTX and analogs has been explored rather differently from the techniques applied to the STX group. One approach has been to determine toxin potency within the TTX family by relative affinity binding of analogs to different Na_v subtypes. The use of various cell types, such as rat brain synaptosomes and neuroblastoma cell lines (Kudo et al., 2014; Yotsu-Yamashita et al., 1999, 2003) has been questioned because these cells express different Na_v subtypes. It is recognized that TTX and its analogs exhibit differential subtype selectivity (Tsukamoto et al., 2017).

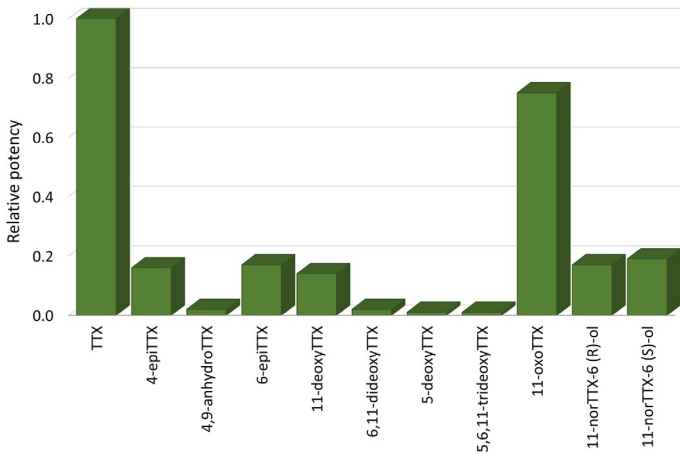


Fig. 7 Relative potency of tetrodotoxin (TTX = 1) and analogs determined by alternative methods. 6-epiTTX, 11-deoxyTTX, 5-deoxyTTX, and 11-norTTX-6 (S)-ol were tested by i.p. LD₅₀ ($\mu\text{g kg}^{-1}$ b.w.); 11-oxoTTX, and 11-norTTX-6 (R)-ol by i.p. LD_{100/99} ($\mu\text{g kg}^{-1}$ b.w.); 4-epiTTX, and 4,9-anhydroTTX by i.p. lethal potency (MU mg^{-1}); 5,6,11-trideoxyTTX by i.p. MLD ($\mu\text{g kg}^{-1}$ b.w.); and 6,11-dideoxyTTX by i.v. MLD ($\mu\text{g kg}^{-1}$ b.w.). Values compiled from FAO/WHO, 2016. *Technical Paper on Toxicity Equivalency Factors for Marine Biotoxins Associated With Bivalve Molluscs, Rome, pp. 108.*

Applying the patch-clamp technique, [Tsukamoto et al. \(2017\)](#) evaluated the ability of TTX analogs to block different channel subtypes by expressing different subtypes of human Na_v in HEK293T cells. Their findings showed that TTX is the most potent analog of the group, but as previously reported ([Satin et al., 1992](#)), some channel isoforms like Na_v1.5 are insensitive. Most analogs showed fairly low relative toxin potency (RTP) values, except for TTX (RTP = 1), and 11-oxoTTX (RTP = 0.75). The RTPs presented in [Fig. 7](#) represent an amalgam of different studies for the potency of TTX analogs expressed in different ways. In any case, with appropriate caveats, it is evident that most analogs are much less toxic than TTX.

4.5 Metabolism and biotransformation of STXs

Toxin compositional profiles of STXs occurring in shellfish and other marine fauna often differ substantially from that of the putative source dinoflagellate or cyanobacterium because of metabolic transformation processes in the vector species ([Bricelj et al., 1990](#); [Cembella et al., 1993](#); [Oshima, 1990](#)) ([Fig. 8](#)). Toxin compositional changes in shellfish may result from enzymatic activity, hydrolysis at low pH, or reductive conversions by natural

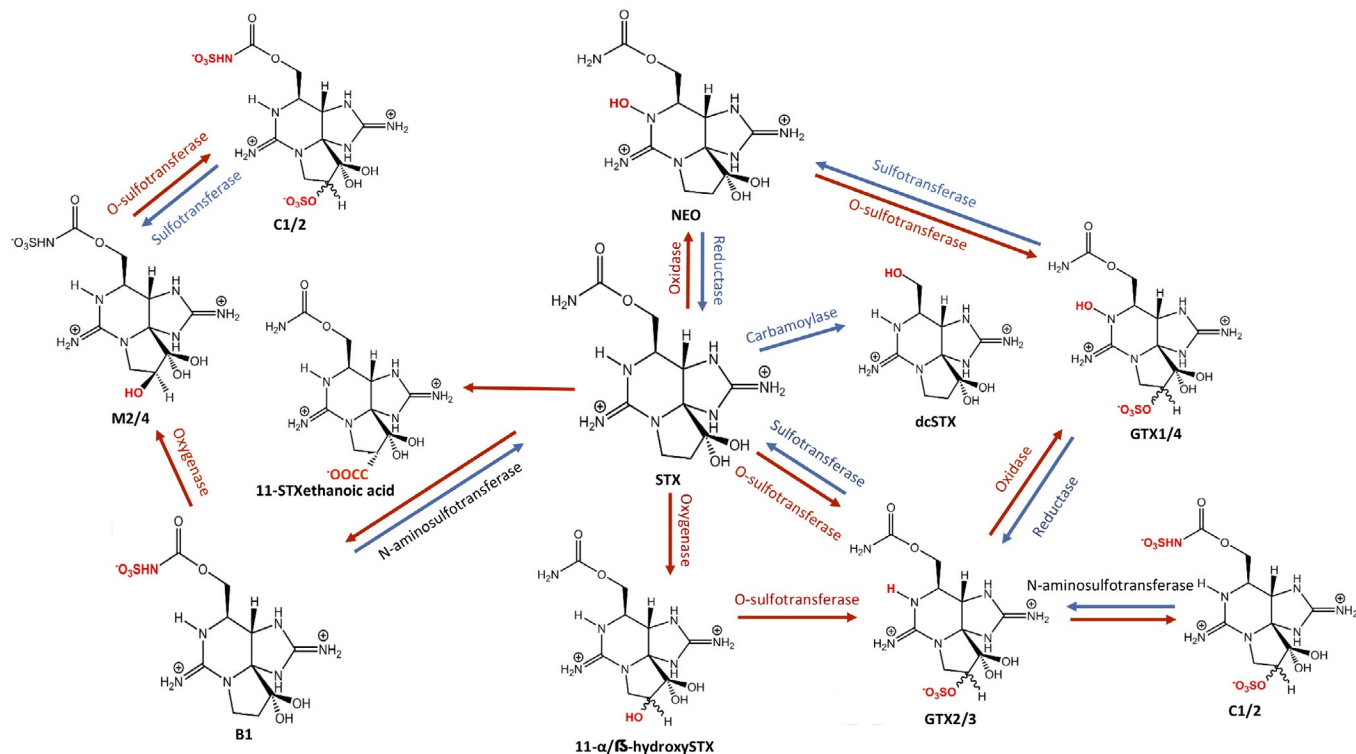


Fig. 8 Enzyme-mediated biotransformation pathways for STXs in dinoflagellates, cyanobacteria and vector species. Known biosynthetic reactions are shown with red arrows and catabolic reactions with blue arrows. Only the major reactions are shown; other conversions such as NEO to dcNEO and GTX2/3 to dcGTX2/3 or transformations of the benzoyl (GC-) toxins can occur but are not illustrated here. *Compiled from information from Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs, 8, 2185–211; Raposo, M.I., Gomes, M.T.S., Botelho, M.J., Rudnitskaya, A., 2020. Paralytic shellfish toxins (PST)-transforming enzymes: a review. Toxins, 12, 344.*

reductants in various tissues (Bricelj and Shumway, 1998). Enzymatic processes are capable of reducing the O-sulfate and N1 hydroxyl groups, as demonstrated with homogenate of the scallop *Placopecten magellanicus* (Shimizu and Yoshioka, 1981). Conversion of carbamoyl and N-sulfocarbamoyl into decarbamoyl analogs, particularly in certain clam species such as *Protothaca (Leukoma) staminea* (Sullivan et al., 1983), can be effected by endogenous carbamoylases. Similarly, the lateral chain of the benzoyl analogs (GC-toxins) can be enzymatically converted into their corresponding decarbamoyl analogs (Vale, 2008). Particularly common are sulfotransferase conversions of the labile N-sulfocarbamoyl analogs (B- and C-toxins), which are often highly enriched in dinoflagellates, to their respective gonyautoxin derivatives (GTX1–GTX4) (Oshima et al., 1993). On a molar basis this yields increased toxicity and hence enhanced PSP risk because of the higher specific potency of the carbamoyl GTXs.

Where the same STX analogs are found in toxigenic dinoflagellates or cyanobacteria and vector species may reflect metabolic conversions mediated by the same class of enzymes operating in biosynthetic versus degradative pathways. The sulfated carbamoyl derivatives GTX2/3 can be converted enzymatically into GTX1/4 analogs after incubation with *Alexandrium tamarense* cell homogenate. Similarly, a sulfate group can be added to GTX analogs at the 21-N position, transforming them into N-sulfocarbamoyl (C1 and C2) analogs after incubation with *G. catenatum* homogenate (Oshima, 1990; Oshima et al., 1993), presumably containing N-aminosulfotransferases.

Other unusual STX analogs are not found at all in the biogenic species but occur only as metabolites in vector species. For example, the M-toxins (Fig. 4), mollusk metabolites first detected in the mussel *Mytilus*, and later in cockles and clams, are not present in the toxigenic dinoflagellates (Dell'Aversano et al., 2008). Some STX analogs, such as saxitoxinethanoic acid (SEA) from a xanthid crab, and a novel carbamoyl-N-methylsaxitoxin (STX-uk) from a freshwater pufferfish, are most likely due to biotransformation within the toxic organism or by its associated microbiome (Arakawa et al., 1995; Fast et al., 2006; Zaman et al., 1998).



5. Pharmacology of guanidinium toxins

5.1 Structure of voltage-gated sodium channels

Voltage-gated sodium channels (Na_V) consist of a single protein complex formed by a pore-forming α -subunit of around 220–260 kDa and one to three auxiliary β -subunits of 33–36 kDa that do not have an active

relationship with the ion influx. The α -subunit contains four homologous domains (DI-DIV), and each domain is formed by six transmembrane segments (S1–S6) (Catterall et al., 2007; Cestèle and Catterall, 2000). Segments 5 and 6 of each domain shape the internal part of the pore. Between these segments, a reentrant amino acid chain loop called the P-loop, lines the ion permeation pathway (Guy and Conti, 1990) and forms the outer vestibule of the channel, where Site 1 for channel-blocking toxins interaction is located (Choudhary et al., 2002, 2007) (Fig. 9). All cells containing Na_V channels express multiple isoforms; nine Na_V isoforms (Na_V 1.1–1.9) have been characterized thus far (Goldin et al., 2000).

5.2 Interactions of guanidinium toxins with Na_V channels

The basic neurophysiology and mode of interactions of guanidinium toxins with Na_V ion channels have been explored for many decades and are now rather well understood (Durán-Riveroll and Cembella, 2017). In fact, the pursuit of understanding the mode of action of guanidinium neurotoxins *in vitro* and in animal model systems led to the discovery of the ion channel families (Thottumkara et al., 2014). Before ion channels were known, Hodgkin and Huxley (1952) investigated the mode of action of unknown guanidinium toxins based upon partial purification of STX and TTX. They noticed that these toxin preparations interfered with the creation of action potentials in nerves and voluntary muscles. The specific action of TTX in blocking the concentration increase of Na^+ ions into isolated lobster giant axons notably affected the so-called “sodium-carrying mechanism” (Narahashi et al., 1964). With the elucidation of the toxin structures, it became apparent that the positively charged guanidinium groups of both toxin groups were key for this activity.

Guanidinium toxins are highly specific Na_V blockers by binding to these proteins located in the cell membrane of excitable nerve, muscle, and endocrine cells. By allowing Na^+ ion influx, Na_V channels are responsible for triggering the action potential required to exert cell actions (Catterall et al., 2007). The toxin molecule fits the external orifice of the Na_V channel but is too large to penetrate the cell. Due to strong charge interactions with the protein residues, clogging of the ion passage occurs (Fig. 10), inhibiting action potential and thus causing neuro-muscular paralysis (Narahashi et al., 1964). STX and TTX block the Na_V channels with a 1:1 affinity (Noda et al., 1989); it has been proposed that their analogs bind to the same site but with different affinities, conferring different potencies (Tubaro et al., 2012).

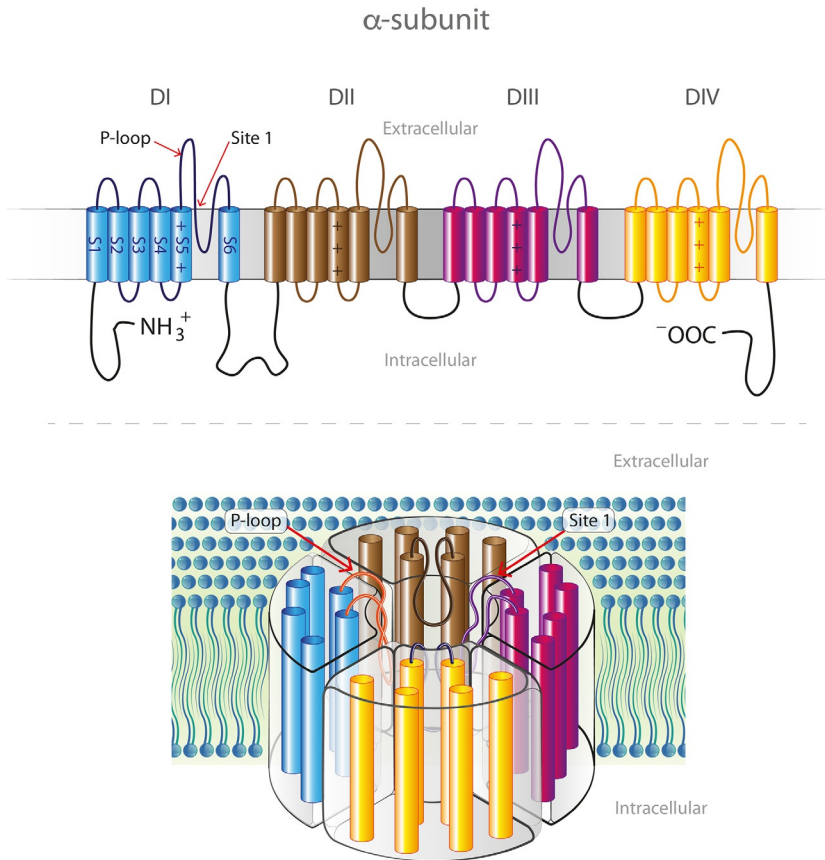


Fig. 9 Representation of the α -subunit of a voltage gated (Na_v) channel showing two-dimensional view of the unfolded pore-forming protein. DI-DIV, domains I-IV; S1-S6, transmembrane segments; Domain I P-loop and the binding site (Site 1) for STX and TTX and analogs are indicated. Three-dimensional view of the Na_v channel shows orientation of the P-loop and Site 1 in the cell membrane. Adapted from Durán-Riveroll, L.M., Cembella, A.D., 2017. Guanidinium toxins and their interactions with voltage-gated sodium ion channels. *Mar. Drugs*, 15, 303; after model of Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie*, 82, 883–892.

The ion selectivity filter of Na_v channels allows the entry of Na^+ ions exclusively. This filter is formed by one amino acid residue from each domain (Asp (D), Glu (E), Lys (K), and Ala (A), known as the DEKA motif) (Durán-Riveroll et al., 2016). Close to the DEKA motif, negatively charged amino acids create an electrostatic cloud to attract the Na^+ ions into the pore

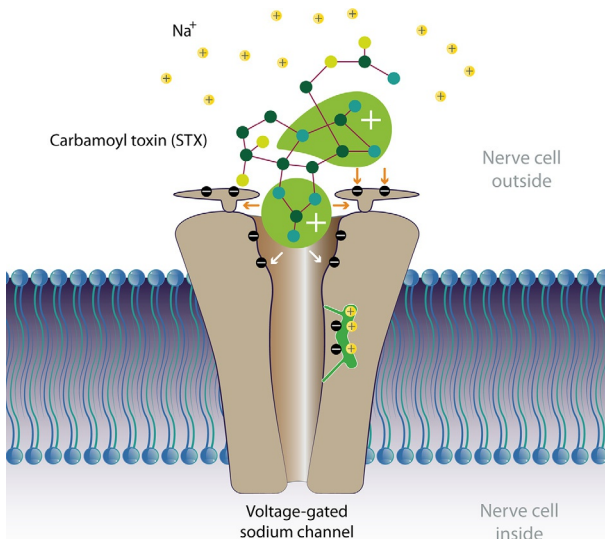


Fig. 10 Schematic representation of a voltage-gated sodium channel (Na_v) in the cell membrane and the mechanism of action of guanidinium toxins; in this case the positively charged guanidinium groups (marked in green) of the carbamoyl STX are blocking passage of sodium ions (Na^+).

(Mahdavi and Kuyucak, 2015). Na^+ ion conductance into the cell depends on these moieties, and any alteration in their residues will affect the channel performance (Toledo et al., 2016) and the sensitivity to the toxins.

There are several hypotheses for potency differences among STX and TTX analogs. One is that analogs bearing large functional moieties such as sulfate or benzoyl groups could affect the toxin binding due to hindrance effects; a second possibility is that the positive charges of the guanidinium groups are compensated with the negative charges of the sulfate groups of highly sulfated toxins, particularly the low potency N-sulfocarbamoyl analogs (Durán-Riveroll et al., 2016).

STX has been described to interact with other ion channels but by different mechanisms and with low affinity. Wang et al. (2003) reported that this toxin modifies the channel gating instead of blocking the pore of the human *ether-á-go-go* (hERG) K^+ channel. Likewise, some effect on the Ca_v channel, through reducing the Ca^{2+} ion current in ventricular myocytes has been found (Su et al., 2004). These alternative channel binding have not been observed for TTX.

5.3 *In silico* modeling of guanidinium toxin interactions with Na_v channels

More profound research is required to fully understand the interactions between guanidinium toxins and Na_v ion channels and binding to specific amino acid residues. (Durán-Riveroll and Cembella, 2017). Computational methods can facilitate visualizing the binding patterns even at atomic levels and calculate the binding free energies. Unfortunately, mammalian Na_v channels have not yet been crystallized or modeled. Instead, homologs have been modeled based on crystal structures of bacterial or red cockroach Na_v channels (Fig. 11) (Durán-Riveroll et al., 2016; Lipkind and Fozzard, 2000; Mahdavi and Kuyucak, 2014; Shen et al., 2017).

Three bioinformatic approaches to test ligand binding properties are feasible once the appropriate homology model of the target protein is selected: molecular docking, molecular dynamics, and quantitative structure-activity relationships (QSAR). Molecular docking is an *in silico* approach to calculate optimal free energy (ΔG) values through scoring samples and functions of interactions between the ligand (toxin) and the target (Na_v channel) (Bello et al., 2013). This approach has been used to identify essential amino acids involved in the recognition of the μ -conotoxins (Li et al., 2001), benzoyl STX analogs (GC-toxins) (Durán-Riveroll

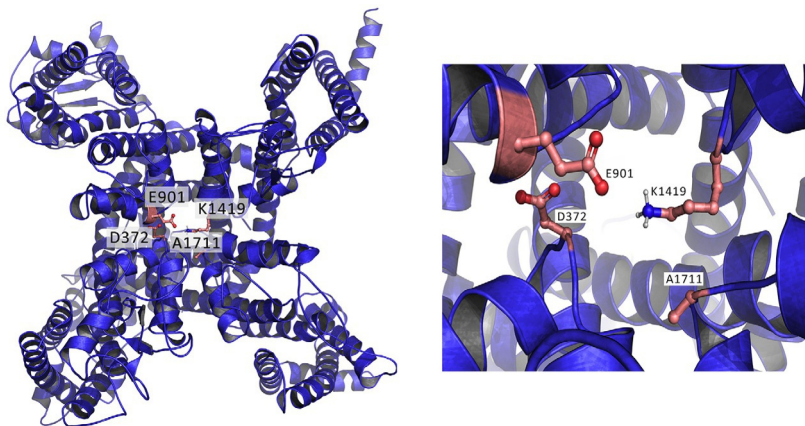


Fig. 11 Top view and pore close-up of a voltage-gated sodium channel (Na_v) constructed based on the crystal structure model of red cockroach *Periplaneta americana* reported by Shen et al. (2017). Residues conforming the DEKA selectivity filter are indicated.

et al., 2016), and TTX (Tikhonov and Zhorov, 2005). Toxin binding recognition relationships and essential recognition properties on the Na_v channel outer vestibules were revealed in great detail through molecular docking simulations with STX (Choudhary et al., 2002). Molecular docking has been used with TTX on Na_v1.4 channel isoform to predict the optimal toxin orientation. Computational results were consistent with experimental results (Tikhonov and Zhorov, 2005). These simulations can provide a valid approximation for toxicity prediction and could be a future alternative to animal subjects for toxicological studies (Durán-Riveroll and Cembella, 2017).

Molecular dynamics (MD) simulates the dynamic behavior of molecular systems, considering the membrane-associated biological parameters, and protein and ligand as flexible entities (Salmaso and Moro, 2018). As such, this approach compensates for some of the deficiencies of molecular docking simulations, which lack attention to several biological parameters (Rosales-Hernández and Correa-Basurto, 2015) and consider the protein as a rigid object and the ligand as a flexible molecule (Bello et al., 2013). Despite much higher computational cost, MD may yield more realistic results by simulating several protein conformations. Combining both simulation approaches could be an effective strategy to model targets and ligands behavior, but MD has not been widely used to analyze guanidinium toxin interactions.

The quantitative structure-activity relationship (QSAR) approach generates regression models that consider a wide variety of known interactions among molecules and converts them to numerical values. The predictors include the physical-chemical properties and molecular descriptors to yield an optimum relationship between ligand (toxin) parameters and biological responses (toxicity). Some early molecular descriptors were used to predict the potency of novel STX analogs (Llewellyn, 2007), but an important shortcoming for QSAR studies of new toxin analogs is the lack of biological data (Durán-Riveroll et al., 2018). To our knowledge, the QSAR approach has not been applied to guanidinium toxins.

None of the computational approaches are expected to fully describe Na_v ion channels and their interactions with various guanidinium pharmacophores. Nevertheless, the application of advanced computational tools, preferably in combination to generate different simulations, would undoubtedly contribute to a better understanding of Na_v channel structure, function, and binding mechanisms for guanidinium neurotoxins.



6. Pharmacological and therapeutic applications of guanidinium neurotoxins

Most pharmaceutical research on guanidinium toxins is focused on the treatment of pain, which is related to $\text{Na}_V1.7$ and 1.8 isoform function. These guanidinium alkaloids have favorable attributes due to their potent Na_V blocking properties while they do not bind significantly to the $\text{Na}_V1.5$ isoform, mainly expressed in heart tissues (Berde et al., 2011; Kohane et al., 1998). Therefore, the search for isoform-specific Na_V channel blockers is a promising opportunity for better pain treatments (Hameed, 2019). There is no evidence of tolerance factors that could lead to the need for higher doses and invoke addiction issues (Berde et al., 2011; Durán-Riveroll and Cembella, 2017). Nevertheless, due to their high acute toxicity and unknown chronic effects, research on guanidinium toxins as potential therapeutics has been rather limited (Lago et al., 2015).

6.1 Saxitoxin analogs in pharmacological applications

Neosaxitoxin (NEO) (Fig. 4) has been successfully used as a long-acting pain blocker by local infiltration into the bladder submucosa for bladder pain syndrome therapy. The analgesic effects lasted for the 90-day follow-up without further infiltrations and no adverse reactions to the toxin (Manriquez et al., 2015). The same analog has shown exciting results in the treatment of achalasia, a rare motility disorder of the esophagus that causes aperistalsis and results in an increased risk of esophageal carcinoma (Boeckxstaens et al., 2014). Sphincter relaxation was observed within 5 min, and the complete disappearance of the symptoms was reached after 4 h of the local application. The symptoms returned 8 days after the treatment, but this preliminary result revealed the pharmacological potential of this analog in the treatment of achalasia (Rodríguez-Navarro et al., 2006).

Gonyautoxins (Fig. 4) have also shown remarkable therapeutic potential and significant success in medical trials. The application of a mixture of GTX2/3 resulted in reduced healing times in chronic and acute anal fissures by paralyzing the anal muscle (Garrido et al., 2007). The same analogs have been similarly employed on chronic tension-type headache treatment and for knee arthroplasty. Chronic tension headache was treated by local muscle infiltration, with results within minutes in decreasing acute pain scores. Their mode of action is associated with activity eradication of the trapezius muscle, providing immediate pain relief, and preventing further pain episodes for more than 8 weeks (Lattes et al., 2009). Knee pain blockage for

the surgical procedure was achieved by local toxin infiltration of GTX2/3 without side effects or adverse reactions (Hinzpeter et al., 2016).

6.2 Tetrodotoxin in pharmacological applications

Even prior to the structural elucidation of TTXs, tetrodotoxic concoctions in Japan were applied to diverse maladies, including pain treatment due to cancer and leprosy, reduction of tetanus muscle spasms, and as an analgesic for patients with rheumatoid arthritis. A tetrodotoxic extract is believed to have contained around 0.2% TTX (Suehiro, 1993).

First successful studies on TTX as an anesthetic were performed on rabbits, where its application produced prolonged local anesthesia for corneal surgery (Schwartz et al., 1998). In humans, prolonged local anesthesia has been achieved with a mixture of TTX and bupivacaine combined with epinephrine to reduce the systemic toxicity of TTX and bupivacaine. The “high bupivacaine-low TTX-concentration” combination keeps the systemic dose within a safe range while providing the desired effects (Berde et al., 2011), aiding postoperative recovery after abdominal and orthopedic surgery (Dahl and Moiniche, 2009). Clinical phase III trials have been completed in Canada, the USA, and New Zealand for Halneuron[®] (<https://wexpharma.com/>) with TTX as the active ingredient for the treatment of cancer-related pain. Further development for chemotherapy-induced neuropathic pain treatment is anticipated because this preparation does not elicit opioid side effects. The safety profile is acceptable and there is no evidence of addiction or build-up tolerance, providing pain relief with reduced opioid consumption.



7. Conclusion

Enhanced knowledge on the biogenic origins and biosynthetic pathways can serve to interpret links to toxin heterogeneity and biogeographical and phylogenetic distribution of these respective guanidinium neurotoxin groups. Genetic mechanisms for toxin biosynthesis should be integrated with the modeling of receptor-binding interactions and the structural-functional affinities of Na_v ion channels. This will provide a template for determining the functional eco-evolutionary role of guanidinium neurotoxins in marine ecosystems. Finally, biotechnological exploitation in pharmaceutical research and therapeutic applications will ensure a bright future for productive use of these fascinating and mysterious neurotoxins that have played such a notorious role in sagas of human history.

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