UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF PHARMACY- SCHOOL OF MEDICINE AND SURGERY

Ph.D. program in

"Scienza del Farmaco"

XXXIVCYCLE

Ciguatoxins and Ciguatera Poisoning: forecasting risk, method development, and applied outbreak response

Christopher R. Loeffler

Tutor

Dott.ssa Luciana Tartaglione

Dott.ssa Dorina Bodi

Coordinator

Prof.ssa

Maria Valeria D'Auria

Table of contents

Contents

List of Tables

List of Figures

List of published articles

1. **Loeffler, C.R.**; Tartaglione, L.; Friedemann, M.; Spielmeyer, A.; Kappenstein, O.; Bodi, D. (**2021**) Ciguatera Mini Review: 21st Century Environmental Challenges and the Interdisciplinary Research Efforts Rising to Meet Them. Int. J. Environ. Res. Public Health, 18, 3027. https://doi.org/10.3390/ijerph18063027

   International Journal of Environmental Research and Public Health (ISSN: 1660-4601) Impact Factor (IF): 3.390, Scimago Journal Rank: Quartile (Q)1 Public, Environmental & Occupational Health, Q2 Public Health, Environmental and Occupational Health.

2. **Loeffler, C. R.**; Bodi, D.; Tartaglione, L.; Dell'Aversano, C.; & Preiss-Weigert, A. (**2021**). Improving *in vitro* ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). Harmful Algae, 103, 101994. https://doi.org/10.1016/j.hal.2021.101994

   Harmful Algae (ISSN: 1568-9883) IF: 4.273, Q1 Marine and Freshwater Biology (10/110) Aquatic Science (Rank 3/219), Q1 Plant Science (Rank 16/431).

3. **Loeffler, C.R.**, Abraham, A., Stopa, J.E., Flores Quintana, H.A., Jester, E.L.E., La Pinta, J., Deeds, J., Benner Jr., R.A., Adolf, J., (**2021**). Ciguatoxin in Hawai'i: Fisheries forecasting using geospatial and environmental analyses for the invasive *Cephalopholis argus* (Epinephelidae). Environmental Research, doi: https://doi.org/10.1016/j.envres.2021.112164

   Environmental Research (ISSN: 0013-9351) IF: 6.498, Q1 Public, Environmental & Occupational Health (rank 16/203), Q1 Environmental Science (31/340), Q1 Biochemistry (90/438).

4. Spielmeyer, A.; **Loeffler, C.R.**; Bodi, D. (**2021**) Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. *Toxins*, *13*, 630. https://doi.org/10.3390/toxins13090630

   Toxins (ISSN 2072-6651) IF: 4.546. Q1 Toxicology (14/93), Q1 Food Science & Technology (19/135).

5. ***Loeffler, C.R.**; Spielmeyer, A.; Friedemann, M.; Kapp, K.; Schwank U.; Kappenstein, O.; Bodi, D (submitted) Food safety risk in Germany from mislabeled imported fish: ciguatera outbreak trace-back, toxin elucidation, and public health implications. Food and chemical Toxicology.

   *Submitted December 2021

   Food and Chemical Toxicology https://www.journals.elsevier.com/food-and-chemical-toxicology (ISSN: 0278-6915) IF: 6.023, Q1 Toxicology (9/93), Food Science Q1 (27/299), Medicine (miscellaneous) Q2.

Presentations at National and International Symposia (~oral, *poster, +invited)

1. ~+**Loeffler C.R.**, Spielmeyer A., Bodi B., Kapp K., Blaschke V., Kappenstein O. Aufklärung eines Ciguatera-Ausbruchs in Deutschland 2017. Workshop of the German National Reference Laboratory for the detection of marine biotoxins. BfR-Seminar. 10-11th November 2021 Berlin, Germany.

2. ~**Loeffler** C.R., Spielmeyer A., Bodi B., Kapp K., Blaschke V., Kappenstein O. Ciguatera poisoning in Germany: Outbreak trace-back and ciguatoxin-like compounds in Lutjanus Bohar. 19th International Conference on Harmful Algae. October 10-15th **2021**. La Paz, Mexico (Online).

3. ~+**Loeffler C.R**., Neuro-2a assay introduction and collaboration discussion. Webex meeting with the University of Konstanz (online). 11th January 2021, Berlin, Germany.

4. ~**Loeffler** C.R. Marine toxins affecting neuronal function: implementation of an in vitro bio-assay and analytical methods. University of Naples Federico II, Cycle 34 second year presentation. November 25th **2020**, Naples, Italy (Online).

5. ~*#**Loeffler** C.R., Luciana T., Bodi D., Spielmeyer A., Kappenstein O., Borrelli F., Dell'Aversano C. 2020. Ciguatera Fish Poisoning: A problem for public health officials, medical professionals, clinicians, patients, fishers, and researchers. Giornate scientifiche della scuola di medicina E chirurgia, Farmacia E biotecnologie. March 2-3rd **2020** Naples, Italy. #accepted but not presented due to SARS-CoV-2 coronavirus restrictions.

6. ~+**Loeffler** C.R. The N2a in vitro cell based assay is an important detection method for marine biotoxins. Moving away from animal-based methods and towards a validated method. BfR-Seminar 2020 Zellkultursysteme, Martin-Lerche-Hörsaal. June 18th **2020** Berlin, Germany.

7. ~*+**Loeffler** C. R., Bodi D., Tartaglione L., Dell'Aversano C., Preiß-Weigert A. Improved detection of marine neurotoxins through the creation of an ouabain and veratridine resistant (OV-R) neuroblastoma (neuro-2a) cell line. SITOX 2nd meeting on 'Natural Toxins'. September 18-19th **2019** Parma, Italy.

8. ~**Loeffler** C.R., Tartaglione L., Bodi D., Dell'Aversano C, Preiß-Weigert A. Improving an in vitro method for detecting marine biotoxins. University of Naples Federico II, Cycle 34 first year presentation. November **2019**, Naples, Italy.

9. Bodi D., **Loeffler C.R.,** Preiß-Weigert A**.** The German National Reference Laboratory for Marine Biotoxins: Current Activities for Ciguatoxin Analysis. Meeting of the Japanese Food Reference Laboratory and BfR, July-10-2019, Berlin, Germany.

10. ~*+**Loeffler** C.R., Bodi D., Tartaglione L., Dell'Aversano C., Preiß-Weigert A. Neuroblastoma (neuro-2a) cell-based assay: Investigating factors affecting performance for detecting marine neurotoxins. Conférence nationale sur les algues toxiques. May 15-16th **2019**, Brest, France.

11. ~+**Loeffler** C.R., Dell'Aversano C., Tartaglione L., Bodi D., Preiß-Weigert A. A walkthrough the importance of interdisciplinary cooperation among toxicology, chemistry, and predictive

models in marine environments. International Conference THEMES. November 28-30th **2018**, Venice, Italy.

12. ~***Loeffler** C. R., Are ciguatoxins a risk in new and emerging areas of Europe? Development and validation of methods for the detection, quantification, and confirmation of ciguatoxins as crucial steps towards outbreak response. PreDoc Symposium. November 22nd **2018** Berlin, Germany.

13. ~***Loeffler** C.R., Bodi D., Preiß-Weigert A. Neuroblastoma cell-based assay optimization for harmonized marine biotoxin detection: reducing ouabain and veratridine oversensitivity. 18th International conference on harmful algae. October 21-26th **2018** Nantes, France.

14. **~+Loeffler C.R.**, Ciguatera Fish Poisoning: a beautiful problem 18th. BfR working group 8 meeting, 3rd July 2018. Berlin, Germany.

Thesis summary

Ciguatera poisoning (CP) is a global human health problem that is in need of further investigation. Currently, it is not possible to predict CP and detecting the presence of the responsible compounds, ciguatoxins (CTXs), is complex. Working within the current approaches to investigating CP, the research presented herein strives to enhance the understanding for three general points of focus for CP: (1) prediction, (2) detection, and (3) outbreak response. Chapter 1 provides the background for the ciguatoxins (CTXs) responsible for causing CP, their production, vectors, and relevance for human health. Chapter 2 describes an approach for a novel environmental CTX forecasting method. Wherein geospatial and environmental information are used for forecasting CTXs in an invasive fish species around the island of Hawai'i. Remotely observable environmental conditions that can successfully anticipate levels of CTX accumulation in a fish species is useful for communities dependent on fisheries which are impacted by CP. The research presented in Chapter 3 describes the potential for a significant improvement in detection sensitivity for CTX3C, CTX1B, and PbTx-3 using the *in vitro* OV-LS N2a-assay. Chapter 4 describes a traceback investigation into a cluster of cases of CP that occurred in Germany in 2017. The responsible lot consisted of fish that were identified as "mislabeled" according to the commercially declared species and all samples tested contained CTX-like toxicity; food fraud and a human health risk. Chapter 5 introduces a novel extraction method with improvements to speed and waste stream reduction, which are applicable to sample preparation efforts for routine toxin analysis. Together the research presented in this thesis provides several significant advancements for the topic of ciguatera and creates a pathway for further improving our prediction, detection, and prevention efforts moving forward for researching and addressing this human health problem in the 21st century.

Chapter 1. Ciguatera poisoning: the responsible toxin, vectors, and management efforts

Significance Statement

Globally, the livelihoods of over a billion people are reliant on marine ecosystems. The resources and ecosystem services that are provided by the marine environment contribute nutrition, income, and health benefits for global communities. Fish provide nearly 20% of the animal protein intake for more than 3.3 billion people worldwide [1]. The seafood trade sector involves vast portions of the global economy, annually, nearly 40% of all fisheries and aquaculture production is traded internationally [1]. One threat to the seafood trade, nutrition, and economic benefits is a global health problem called CP; worldwide, it is the most reported non-bacterial seafood-related illness. The compounds involved in CP are structurally complex, biologically active in small amounts, broadly produced, and their metabolites are found in many marine organisms, complicating control efforts. Many fundamental studies, e.g., identifying the toxin production, distribution, quantification, and qualification are lacking. Herein Chapter 1 is the background information regarding the history, impacts, occurrence, toxin source, vectors, detection methods, and management efforts for controlling CP.

1.1. Ciguatera history, background, and discovery of ciguatoxin

Marine biotoxins in the CTX-group, when contained in seafood, can result in severe intoxications in humans, which is described as CP. The word 'Ciguatera' was derived from the name used for the Turban shell (*Turbo pica*, since re-classified as *Cittarium pica*) called '*cigua'* by the Taíno (indigenous people of the Caribbean). When this sea snail (marine gastropod) was consumed, it was understood by the indigenous people that it can cause disturbances to the human digestive and nervous system (i.e., how certain fishes are known to cause CP) which they called ciguatera [2]. Therefore, the name ciguatera was given to describe a human seafood intoxication, which was known to occur after the consumption of tropical and sub-tropical fishes, or less frequently invertebrates, in Cuba. While in Cuba, a Portuguese naturalist by the name of Don Antonio Parra Callado suffered from ciguatera, along with his family. In 1787, he was seemingly the first person to describe CP in considerable detail, concerning the symptoms and empirical remedies

used at the time. In general, the consequences of consuming a fish containing ciguatoxins (CTXs) are felt directly by the individual, with symptoms presenting, often immediately, after the consumption of the fish. The disease is characterized by a variety of symptoms which are broadly clustered into three categories such as digestive, cardiovascular, and neurological. These symptoms are inherently variable due to factors such as, chronic exposure, amount of contaminated product consumed, body weight, susceptibility, region of seafood origin, etc.

The earliest reports of poisoning by tropical coral reef-associated fish occurred in the Caribbean in the 1550s, in the Pacific in 1606, and infamously with a mention in Captain James Cook's journal of a fish-poisoning incident that occurred in 1774 and was interpreted as an early documented case of CP [3-5]. Throughout the records of history, ciguatera has been written about and described as having been affecting people for at least the last 500 years, but because many of these stories were first encounters when exploring new regions, it is likely to have been a persistent, but undocumented, seafood intoxication problem pre-dating the first recorded illness reports [6,7]. There is speculation that Alexander the Great refused to let his troops eat fish due to concerns from Ciguatera [8], and one particularly astounding hypothesis concerning CP's impact on society was whether CP was a determining factor in the events leading up to the late Holocene Polynesian voyages of discovery. This hypothesis of CP induced exploration, was based on archaeological records of abrupt changes in fishing practices believed to be due to the concern that certain species, once relied upon for food, became hazardous to eat (which is a known description of fish in CP endemic regions). The loss of a staple food source was believed to be the event that caused the search for new islands with seafood that was safe for consumption in the Pacific [9].

These historical anecdotes are important for understanding CP in a wider context. That CP proceeds the impacts and activities of modern humans (i.e., before anthropogenic impacts) and that the causative organism, its toxins, and the process of CP is not a new phenomenon but has remained an unsolved enigma for resource and human health managers with a global occurrence pre-dating modern record keeping (Figure 1).

Through trial and error, historic civilizations recognized that certain fish could be hazardous to eat, however, at that time the reasons for 'why and how' they became toxic was never elucidated. To understand the conditions and situations that can result in the accumulation of toxins in fish, to the point of becoming hazardous to the consumer, Randall in 1958 postulated that algae of a benthic nature were the source from which the toxin originates and that it is transmitted through the food web [10]. He described the phenomena of ciguatera as 'an illness of occasional occurrence following the ingestion of various tropical reef and inshore fishes and possibly echinoids and gastropods'. He proposed (based on evidence working with others) that the toxin is cumulative based on the

observation that large piscivorous fish (e.g., barracuda) are the most toxic, that plankton-feeding fish appear non-toxic, whereas herbivores, detritivores, and invertivores can be poisonous.

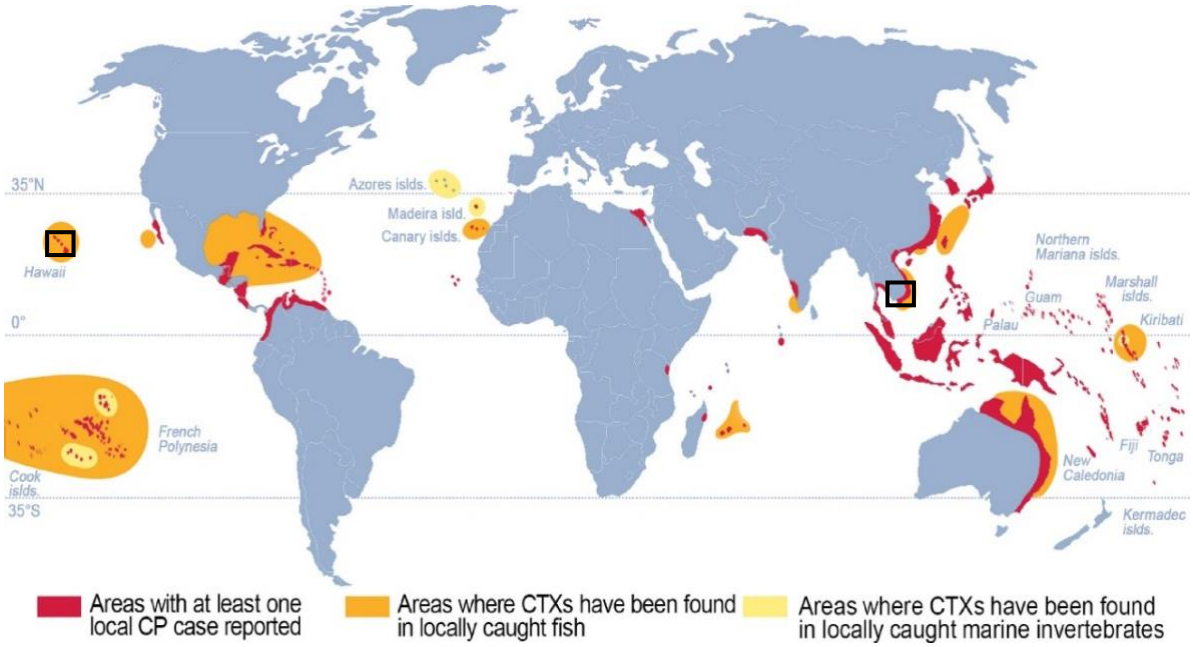


Figure 1. Global map of areas locally impacted by CP. Areas with at least one local CP case reported (red), CTXs confirmation in locally caught fish (orange) and marine invertebrates (yellow). Black squares are regions investigated in this thesis (Chapter 2 and 4). Map from Chinain, *et al.* [11].

Furthermore, the occurrence of toxic fish are spatially and temporally variable. Randall in 1958 suggested that a benthic organism was the source of the toxin [10], which would explain the observed site attachment differences for toxicity in certain species (i.e., species that tend to stay within a small home range). This was the first description of the situation leading up to a fish acquiring CTXs that remains accurate today. Following this hypothesis, the causative toxin was first isolated, named, and characterized by Scheuer et al. in 1967, via isolation from *Gymnothorax javanicus* (moray eel) from the Pacific Ocean [12]. With an example of the compound in hand, as a reference to compare, the search for the toxins sourced from the environment was greatly facilitated. Through a collaborative project between Japan and France, to find more examples of the compound in other fish (surgeonfishes, *Ctenochaetus striatus,* and *Acanthurus lineatus*, from Tahiti), two toxins were identified; a fat-soluble toxin and a water-soluble toxin [13]. The water-soluble toxin was named maitotoxin and it was purified according to standard procedures for polar lipids at the time, and the minimum lethal dose to mice by intraperitoneal injection (ip) was estimated at 15-20 mg kg^{-1} [14]. The fat-soluble toxin was compared to the reference CTX (from 1967) and found to be chromatographically identical. Furthermore, this compound was also found in the stomach contents of the fish, suggesting that CTXs were acquired through the diet. The identification of these toxins in the gut contents and liver of a Parrotfish (*Scaridae*) provided further evidence that a precursor toxin may exist in the diets of herbivores;

'presumably, a benthic organism containing little, or no chlorophyll may be responsible for producing the toxin' as postulated by Yasumoto et al. in 1977 [15]. Working in a region known to produce CTX-positive fish, the collaborative research group identified a toxic sample of detritus (based on the knowledge that parrotfish and surgeonfish consume that type of material, macroalgae and detritus) and separated different fractions using size exclusion filtration for testing, where one portion contained dinoflagellates [15,16]. They isolated the toxin from the detritus and used a bioassay to prove that the toxicity of the samples was attributable to (and varied with) the number of algal cells. This finding led them to conduct and refine the extraction procedures to identify the toxin compound contained in the sample. Knowing that the responsible organism was likely a dinoflagellate, the identification of a benthic dinoflagellate of potential implication for CP was finally accomplished and named *Gambierdiscus toxicus* by Adachi et Fukuyo [16-19] (Figure 2).

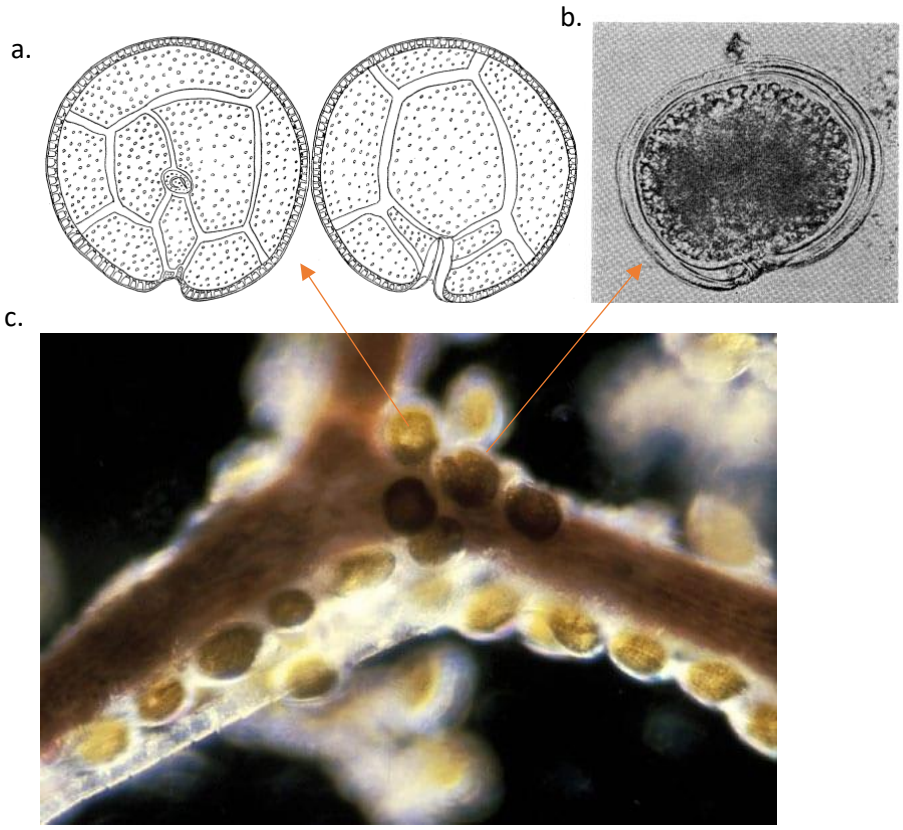


Figure 2. Thecal structure of the marine toxic dinoflagellate *Gambierdiscus* toxicus gen. et sp. nov. collected in a ciguatera endemic area. a) Plate pattern of epitheca (top down view) and hypotheca (bottom up view), b) live cell image, c) *Gambierdiscus* on macro algae. Adachi R. & Fukuyo Y. 1979 [16]. Bull. Jap. Soc. Sci. Fish. 45: 67-71, an open-access CC-BY article. C. Photo from Woods Hole Oceanographic Institute web page [20] © 2021 Woods Hole Oceanographic Institution. All Rights Reserved.

1.2. Impacts of ciguatoxins on human health

The biological activities of CTXs are extensive, producing more than 175 distinct symptoms in humans, likely due to the 30 different CTXs known (see section 1.4 for CTXs). CTXs are considered the most potent activators of voltage-gated sodium channels and induce calcium fluxes in the cytoplasm of various cells [21]. Fatalities attributed to CP have been documented, and descriptions of the overall mortality rate have been between less than 0.1% of cases to up to 10% [22-28]. Severe cases of CP can involve hypotension with bradycardia, respiratory difficulties, coma, and paralysis some of which can occasionally become fatal [29-32]. Modern (>1950's) reported mortality rates are low (<1%), mostly due to improvements in the quality of healthcare and symptom-managing medications now available. In rare cases of CP the symptoms experienced can persist for years which can be a highly incapacitating disease. Fish in general rarely accumulate sufficient levels of CTXs to be lethal [33] but the natural biological response to intoxication, includes self-evacuation (vomiting) of the remaining undigested product (incomplete absorption), and natural elimination routes for ingested toxins (i.e., via feces and urine). Several factors are involved with the severity of CP and include the type of fish consumed (higher predators are correlated with more severe symptomology), the animal part consumed (viscera, liver, head), the toxin content of the meal, previous underlying conditions of the consumer, age, and access to adequate medical care. Soups made from the head, liver, intestine, or roe (eggs) of fish can be particularly toxic as these parts of the fish can contain higher concentrations of CTXs and have been shown to influence the severity of a CP event [8,34-36]. Generally, severe cases of CP can be avoided when toxin intake is reduced through smaller portion sizes for a meal.

Less severe but common symptoms of CP include gastrointestinal disturbances with symptoms that can include diarrhea, vomiting, and abdominal pain. Neurological symptoms are also commonly reported and can include loss of energy, burning sensation of the skin (dysaesthesia), pruritus, joint pain, paraesthesia of the hands, mouth, and feet, headache, vertigo, and chills (Figure 3). Currently, there is no effective antidote or medicine to relieve the suffering associated with CP, rather the standard provisions include treatment and supportive care with fluids and electrolytes. Because no serologic test is yet available, cases of ciguatera or a diagnosis of ciguatera are based on an assessment of the patient's signs and symptoms (clinical presentation) in addition to having recently (within the last 1-48 hours) consumed a seafood product from a known ciguatera-prone (endemic) region. When available, analytical testing of a meal remnant, or if a portion is unavailable, a related lot can be analyzed for CTXs [23]. In the United States, the Food and Drug Administration (FDA) has established consumer and industry guidance levels for ciguatoxins in seafood products originating in the Pacific Ocean region (P-CTXs, currently described as CTX1B [37]) at 0.01 ng g^{-1} CTX1B eq., and in the tropical Atlantic, Gulf of Mexico, and the Caribbean at 0.1 ng g^{-1} C-CTX-1 eq. [38,39] (See section 1.4 for more

information on CTXs). While the levels are based on CTX content related to illnesses, they are not action levels as they would be established through regulations; however, the FDA retains the right to act, as deemed appropriate. For CTX1B it was suggested that human illness commonly occurred at values of 0.1 and 5 ng g^{-1}, or 1 ng consumed per kg body weight [32,40-46]. While the FDA-recommended guidance levels are based on CTX content related to illnesses, they do include a 10x safety factor to account for uncertainties such as measurement error and human consumption inconsistencies.

Food preparation methods do not destroy CTXs, therefore CP is not associated with, nor due to, the mishandling of fish during processing or storage and toxic fish. Fish with and without CTXs are broadly indistinguishable. In India, approximately 200 workers of a fish processing factory were poisoned after consuming meals prepared with the heads of snapper fish. The fish heads were discarded parts, a fisheries processing byproduct from the filets which were packaged for export (it is unknown what became of the exported product) [47].

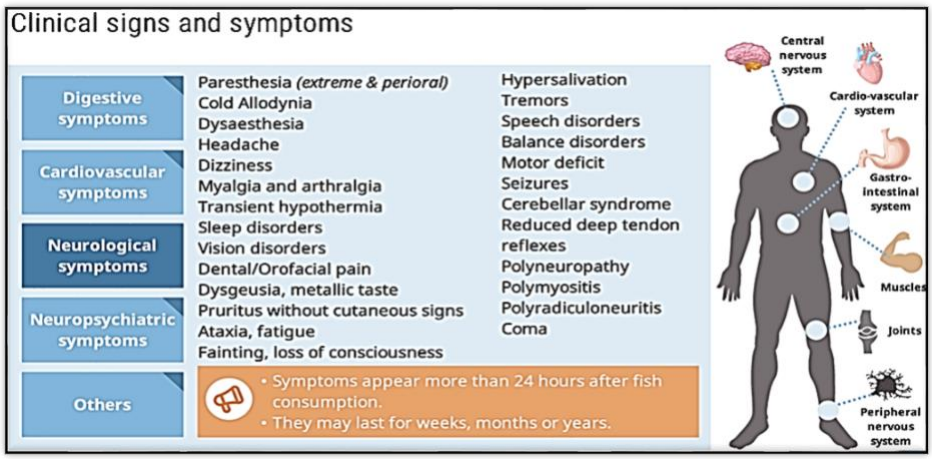


Figure 3. Clinical signs and symptoms with a specific focus on the neurological list of symptoms associated with CP. Figure modified from FAO and WHO ciguatera certified training course FAO E-learning academy. FAO WHO Ciguatera training slides. Slide 5 of 19. Monitoring and preventing ciguatera poisoning learning module – Lesson 3: Public health Ciguatera illness monitoring and reporting. Pg. 5 https://elearning.fao.org/course/view.php?id=648 [Date accessed August 2021]. © FAO 2020 reuse terms and conditions - http://www.fao.org/contact-us/terms/en/ [37].

The impacts due to CP are also felt on a societal level, in 1981 the South Pacific Epidemiological and Health Information Service reported a mean CP incidence of 109/100,000, which is a rate comparable to other disruptive notifiable diseases with serious impacts; including dengue 86/100,000, pulmonary tuberculosis 30/100,000 and gonorrhea 212/100,000; indicating that ciguatera is a

significant public health problem in the Pacific [48]. In the years between 1998-2008 seventeen Pacific Island Countries and Territories reported an annual incidence rate of 194 cases per 100,000 [37]. It is through these high rates of incidence that CP can create economic hardships beyond the physical illness, but also through protein source restrictions (fear of intoxication after fish consumption), loss of employment (reduced fishing efforts), and reduced self-sufficiency (dependence on imported food). In particular, these restrictions on fishing, jobs, and food can all have major ramifications on the local economy, culture, and outlook of small markets; such as small island nations (which have a restricted labor and industry market), as well as other coastal regions that are reliant, culturally or economically, upon locally caught seafood. Furthermore, any reduction in the supply of seafood products will harm the livelihood of over one billion people around the world who are dependent upon seafood products for income and animal protein [1].

1.3. Occurrence of CP

Global estimates of the number of people who suffer from CP range between 10,000-500,000 annually [23,33,49,50] (Figure 1). However, due to under-reporting, an accurate accounting of the impact remains unknown [23]. Challenges to fully understanding the CP impacts on humans are numerous and include (i) difficulties in identification and misdiagnosis due to diverse unspecific symptomology (e.g., gastrointestinal, neurologic, and cardiac) [51], (ii) severity related to consumption habits (what parts of the animal were eaten and how much) [26], (iii) patient pre-existing conditions or existing CTX body burden, (iv) willingness to seek medical treatment [52,53], (v) whether the healthcare system considers ciguatera to be a reportable illness [23,54], (vi) globalized intertwined food networks [55,56], and (vii) international trade and travel where the source of illness (traceback) can be difficult to ascertain [57,58], as reviewed in [59].

Before reporting a case of CP, a diagnosis must be made, and this is dependent upon the physician's recognition or awareness of CP, leading to the question and identification of a recent history of eating fish, clinical signs, symptoms, and presentation as well as confirmation of analytical testing results of the meal remnant by the person who suffered from CP. To unify the description of a case of CP, Friedman et al. (2017) created a possible universal case definition Table 1 [23]. In addition to cases, clusters are often described, which are defined and labeled with the geographic locations where the fish was purchased by the victims, indicating a common source. This method of labeling outbreaks is also based on the systematic ordering of clinical and laboratory data as well as the corresponding fish consumption data, and clusters are furthermore linked by a common batch, lot, or consignment of the product (i.e., fish) which then defines the geographic dimension of the CP outbreak [58]. In the European Union and European Economic Area countries, the incidence rates of CP are extremely low (0.0054 per 100,000 inhabitants per year, excluding travel-related cases). However, it is

believed that only 10-20% of cases are reported, based on available literature ([60,61]. The opinion that illness reporting rates in the EU, a non-endemic continental region so far, are potentially low is further supported by the results presented in Chapter 4 regarding a traceback investigation following a major outbreak of CP that occurred in Germany.

Due to environmental, health, religious, philosophical, and ethical reasons, some consumers have shifted their diets in the early 21st century. Ethical, health-conscious, and sustainable diets tend to be based on alternatives to animal-based protein (e.g., vegetarian, vegan, plant-based) [62-65]. This alternative protein source consumption is relevant for the accuracy of CP incidence rates; as consumers who abstain from fish consumption (and fish-based products), either entirely or based on region or species, should be excluded from CP risk assessments, to have an accurate representation of the potential risk group [50,53]. An example of a refined risk group study, the Florida Department of Health, United States, reported an unadjusted annual incidence rate of 0.2 reports of CP per 100,000 people, while an adjusted rate was projected to be 2,000 times higher at 400 per 100,000 (adjusted for underreporting and a focus on groups of high CP risk) [66].

Table 1. Possible universal case definition of ciguatera poisoning (CP), modified from Friedman et al. 2017 [23].

A case definition is a set of uniform criteria for identifying a disease, which is used for research purposes, clinical diagnosis, or public health surveillance. Concerning CP, a universal case definition, designed to account for the variability in symptom presentation for fish obtained from different geographic regions (e.g., Caribbean Sea, Indian Ocean, and the Pacific Ocean), is desirable to help identify cases consistently. Following is a possible case definition. This proposed definition is a refinement or modification of other CP clinical descriptions or case definitions (e.g., Centers for Disease Control and Prevention's Yellow Book, US Food and Drug Administration's Bad Bug Book [67], European Food Safety Authority's Framework Agreement), for global application:

Clinical Criteria	Patient who consumed saltwater (marine) fish that has been previously associated with CP, and, reports neurologic symptoms which may include any combination and sequence of paresthesia, dysesthesia, pruritus, allodynia, myalgia, arthralgia, and dizziness with onset up to approximately 48 h after eating the fish. Gastrointestinal (GI) symptoms (e.g., nausea, vomiting, and diarrhea) often precede or accompany the neurological symptoms, with GI symptom onset usually within minutes to 12 h after fish consumption. Cardiovascular symptoms and signs (hypotension and bradycardia) may also be present.
Laboratory Criteria	Confirmation of ciguatoxin(s) in implicated raw or cooked fish meal remnant.
Epidemiological Criteria	Exposure to the same fish source as a confirmed CP case.
Case Classification	-Confirmed case: Any patient meeting the clinical and laboratory criteria. -Probable case: Any patient meeting the clinical and epidemiological criteria -Possible case: Any patient meeting the clinical criteria after consuming a saltwater (marine) fish that is either NOT previously associated with ciguatera fish poisoning (CFP) or of unknown species; or any patient with an illness presentation that differs slightly from the clinical criteria or is an unusual presentation that, in the professional judgment of the healthcare provider, merits consideration for a CFP diagnosis; or any patient who meets the clinical criteria but where other etiologies have not been ruled out.
Outbreak definition	Two or more cases that are epidemiologically related.

*Source- Friedman et al. 2017 (and references therein). This article is an open-access article with distribution permitted under the terms and conditions of the Creative Commons Attribution CC BY license (http://creativecommons.org/licenses/by/4.0/) [23].

Underreporting remains a major problem and the reasons for an inaccurate accounting of the number of cases are numerous. In most CP incidents, symptoms are mild or self-resolving, which does not require the consultation of a health care professional, calling a poison control center, or seeking medical help. Without self-reporting these cases will not be included in any accounting of incidences of CP and mild cases that self-resolve without the need for seeing medical treatment or advice are a frequently cited reason for underreporting issues related to ciguatera. Several studies have been conducted using various survey methods including interviews by phone, mail, door to door, and email,

to try to achieve a better accounting of the incidence of CP in endemic areas. When attempting to account for incidences and demographic risk factors for CP Radke et al. (2015) identified three key levels of underreporting for their study [66]. These included i) people who do not seek medical attention, ii) people who seek medical attention for their illness but are not appropriately diagnosed, and iii) people who are diagnosed but not reported to the wider regional data collection and consolidation agency. Awareness of the CP problem is low in regions or areas where ciguatera cases are rare or unheard-of; this awareness can be low even among healthcare workers potentially leading to misdiagnosis [68]. Clinical registration with the International Classification of Diseases (ICD-10) under code T61.0: 'Ciguatera fish poisoning' or reporting CP cases with emergency department databases, public health, or data curation authorities is not always mandatory.

In addition to underreporting, misdiagnosis can be a problem for outbreak identification, the diagnosis of CP can be complicated by individual experiences or presentations that can be variable, even when multiple individuals consumed the same fish. Several different fish-related illnesses fall into the category of 'oral marine intoxications originating from fish'. These intoxications can present similar conditions to CP and therefore must be ruled out as a possible explanation of illness, these include, clupeotoxin poisoning, scombrotoxic fish poisoning, hallucinogenic fish poisoning (ichthyoallyeinotoxism), chimaera poisoning, cyclostome poisoning, elasmobranch poisoning, gempylidae-fish poisoning. Table 2 presents some examples of illnesses with clinical presentations similar to CP [23]. This is particularly important because products responsible for CP can have variable symptomology based on harvest locations, for example fish were harvested in the Atlantic and Caribbean are predominately linked with gastrointestinal type symptoms and those suffering CP with a product sourced from the Pacific tend to describe neurological symptoms as the predominant/commonly associated with CP events (i.e., each considered the hallmark of CP in the respective region). The international shipping of fish can complicate this regional CP recognition when fish from one ocean are sold in another (e.g., Pacific ciguatoxin contaminated fish intoxicating a person in the Caribbean CTX region). Sensitivity to CTXs affects consumers both in endemic and non-endemic regions alike, no immunity or tolerance to CTXs have been described. Because many seafood related illnesses can present gastrointestinal distress, having a clear identification of the causative fish can make a diagnosis more accurate. There have been recent advances in detection methods using biological samples (blood, urine) and if these techniques can be validated, they would constitute a clear path for clinical diagnosis which could be coupled with additional independent laboratory confirmation of CTX presence in the meal remnant [69,70].

Table 2. Examples of a selection of illnesses with clinical presentations similar to Ciguatera Poisoning (CP) modified from Friedeman et al. 2017 [23]*.

Illness	Cause	Symptom Presentation
Ciguatera Poisoning	Caused by ingestion of marine (saltwater) fish of invertebrates contaminated with ciguatoxins	Reports of neurologic symptoms that may include any combination and sequence of paresthesia, dysesthesia, pruritus, allodynia, myalgia, arthralgia, and dizziness with onset up to approximately 48 h after eating the marine animal. Gastrointestinal (GI) symptoms (e.g., nausea, vomiting, and diarrhea) often precede or accompany the neurological symptoms, with GI symptom onset usually within minutes to 12 h after fish consumption. Cardiovascular symptoms and signs (hypotension and bradycardia) may also be present.
Paralytic Shellfish Poisoning	Caused by ingestion of marine bivalve mollusks such as mussels, clams, and oysters, contaminated with saxitoxins	Within minutes of ingestion, there is an onset of intraoral and perioral paresthesia, particularly of the tongue and gums similar to ciguatera but slower in onset. Paresthesias are rapidly followed by weakness, dysarthria, dysphagia, and other symptoms. The mortality rate is estimated at 25% or higher in children*.
Pufferfish (Fugu) Poisoning	Caused by ingestion of pufferfish contaminated with tetrodotoxins	Paresthesia of the face and extremities, nausea, dizziness, loss of reflexes, weakness, and paralysis. The marked weakness and paralysis of pufferfish poisoning are not seen in CFP.
Neurotoxic Shellfish Poisoning	Caused by ingestion of molluscan shellfish contaminated with brevetoxins	Nausea and vomiting, paresthesia of the mouth, lips, tongue, and extremities, ataxia, slurred speech, and dizziness. Neurologic symptoms can progress to partial paralysis; respiratory distress may occur.
Scombrotoxin Fish Poisoning	Caused by ingestion of fresh, canned or smoked fish with high histamine levels due to improper processing or storage	Flushing, rash, hives, palpitations, headache, dizziness, sweating, and burning of the mouth and throat; abdominal cramps, nausea, vomiting and diarrhea; bronchospasm, respiratory distress, and vasodilatory shock may occur.
Botulism	Caused by ingestion of canned foods contaminated with botulinum toxin.	Vomiting, diarrhea, abdominal pain, extraocular muscle weakness, dysphagia, and respiratory paralysis and unless promptly treated in the intensive care setting may result in death. Unlike in CFP, there are no sensory symptoms.
Guillain–Barré Syndrome	Cause unknown. Believed to be an autoimmune reaction in response to a viral or bacterial infection.	Acute inflammatory demyelinating polyradiculoneuropathy, which may present with paresthesia followed by weakness of the extremities, loss of reflexes, and in severe cases dysphagia and respiratory failure. The early onset paresthesia may resemble ciguatera, especially if there is a history of gastrointestinal symptoms before the onset of paresthesia and a history of having eaten fish associated with CFP.
Acute Arsenic poisoning	Caused by the intentional or unintentional ingestion of arsenic.	May present with gastrointestinal symptoms and subsequent peripheral neuropathy. Unless there is suspicion of arsenic ingestion, the diagnosis of arsenical neuropathy may be overlooked, and CFP may be wrongly diagnosed.
Organophosphate poisoning	Caused by dermal, inhalational or oral exposure to organophosphate compounds, usually pesticides.	Initial symptoms of vomiting, diarrhea, and abdominal pain may resemble CFP. It may cause delayed sensory and motor peripheral neuropathy. Unlike CFP, it has cholinergic symptoms of salivation, bronchorrhea, and bronchospasm.
Acute bacterial or viral gastroenteritis	Caused by ingestion of contaminated food or exposure to infectious individuals	Nausea, vomiting, and diarrhea alone or combined, with or without neurologic symptoms (enterotoxigenic E. coli, Shigella) in patients with a history of exposure.

1.4. Toxin source and compound description

The compounds principally responsible for CP are ladder-shaped lipophilic polyether marine toxins, described in a class called Ciguatoxin (CTXs) of which there are over 30 currently described. These marine biotoxins are high molecular weight molecules (1000-1200 Da) produced by naturally occurring marine benthic and epi-benthic microalgae, specifically, dinoflagellates in the genera *Gambierdiscus* spp. and *Fukuyoa* spp. (here after *Gambierdiscus* and *Fukuyoa*, due to our genus-level descriptions unless specifically mentioned) (Figure 4, Figure 5). *Gambierdiscus* are particularly slow-growing, with growth rates of 0.13-0.55 divisions day-1 [37,71]. This slow-growth is thought to be due to the extremely large size of the nuclear genome 1.85 to 112 Gigabase pairs [72]. Due to the inter- and intra- specific toxin production (differences up to factor 100 reported [73]), co-occurrence of species, and the complex population dynamics controlling their natural occurrence, ciguatoxin variability in benthic food webs and their optimal conditions in which the cells thrive in among diverse ecological niches is currently poorly characterized.

Monitoring programs for harmful algal species have been established in many endemic areas for CP to better understand the population dynamics of CTX producing species [74-84]. To collect benthic and epibenthic dinoflagellates the simplest method is by macroalgal collection, the host habitat, to which *Gambierdiscus* is commonly attached. If macroalgae (i.e., *Dictyota* spp., *Lobophora* spp., *Halimeda* spp.) is collected underwater and placed into a bag, it can be brought to the surface for processing. Local seawater is used as a medium where the macroalgae (to which the dinoflagellates are attached) are shaken in the presence of seawater and through the use of a series of filters (>200 nm screen size, and <20 nm) the dinoflagellate component can be collected. From this <20 nm sieve filter component, trained scientists can identify and enumerate *Gambierdiscus* to the genus level with a light microscope (10-100x), and isolation and cultures can be developed (Figure 5). From a successful culture, genetic information can identify which species is in the culture and experiments can be conducted to elucidate growth conditions, toxin production, etc.

Historically, species identification was performed by highly trained phycologists specialized in morphological identification methods. Typically, an isolate was grown to create a culture of clones and these were described in exquisite detail so that the shapes of the pore plates could be used for species identification (Figure 4 and Figure 5). This method was greatly enhanced with the advent of Scanning Electron Microscopes (Figure 5 [85]). These techniques are still in use, for documentation and as a

standard practice, used alone or as a complementary method for physical identification, however, currently, molecular confirmation and targeted molecular approaches (e.g., fluorescence in-situ hybridization probes, restriction fragment length polymorphism typing, high throughput sequencing metabarcoding, or polymerase chain reaction) are reliably used [86-98]. To generate sufficient reference material for method development and compound investigations, cultures of the toxin-producing algae need to be created. It can be difficult to adapt a field-collected species to culture conditions. Once a culture has been created *Gambierdiscus* has shown inter- and intra-species variability for growth, morphology, and quali-quantitative toxin profile [99,100]. This variation can be dependent on the location of collection, culture (growth) parameters [101], and the amount of time spent in culture (phycological drift) [71,81,99,102-107]. Furthermore, interactions have been described for *Gambierdiscus* with bacterial or other microbial co-inhabitants that have shown antagonism, competition, or benefits with impacts on fitness and bi-directional regulation in compound production [108-110]. Therefore, if the culture is axenic or not can be a factor capable of altering various production values (growth/toxin) of the selected algae

Species in these genera are found globally (Figure 6) and represent the biogenic origin of the primary CTXs, which are CTX4A, CTX4B, and CTX3C. Species of *Gambierdiscus* have also been known to produce other bioactive ladder-shaped polyether compounds; such as gambierol [111], gambieroxide [112], gambieric acids [113], gambierone [114] (Figure 7), 44-methyl gambierone [115-117], or maitotoxins (MTXs) [118-123] (Figure 8), as summarized by the FAO and WHO [37]. Of these compounds, CTXs and MTXs, are among the most toxic natural substances known (CTX1B LD_{50} = 0.25 µg kg^{-1}, mice, i.p. [40]) (MTX-1 LD_{50} = 50 ng kg^{-1}, mice, i.p. [118]), and no specific antidotes for the illnesses caused by them exist [124,125]. Maitotoxins are water-soluble, extracellular calcium channel activators and are one of the largest and most complex non-protein, non-polysaccharide molecules produced by any organism (Figure 8). The direct role of 44-methylgambierone and maitotoxin in ciguatera poisoning is believed to be low (due to their solubility in water and thus lack of accumulation in tissue) [115,126]. However, these compounds seem to be ubiquitous among *Gambierdiscus* species (spp.) and are indicative of conserved and therefore likely important polyketide synthases pathways for compound production in this genus [127]. Polyketide synthases are important sources of natural products, as two-thirds of all drugs in use by humans are derived from natural sources. The purpose of these biologically active structures is still unknown; however, many polyketide synthase products have antifungal, antibiotic, and predator-defense properties; therefore it is possible *Gambierdiscus* and *Fukuyoa* could produce compounds of human interest [128]. Furthermore, 63.5% of the proteins produced by *Gambierdiscus* caribbaeus' are unknown, which was rated among the highest amount of unknown proteins among studied dinoflagellates [72]. This level of unknown cellular production means their functional capacities remain unknown. The role and function of these unknown compounds in

this species' growth, compound production, environmental adaption will be elucidated when these compounds and their purpose can be better understood.

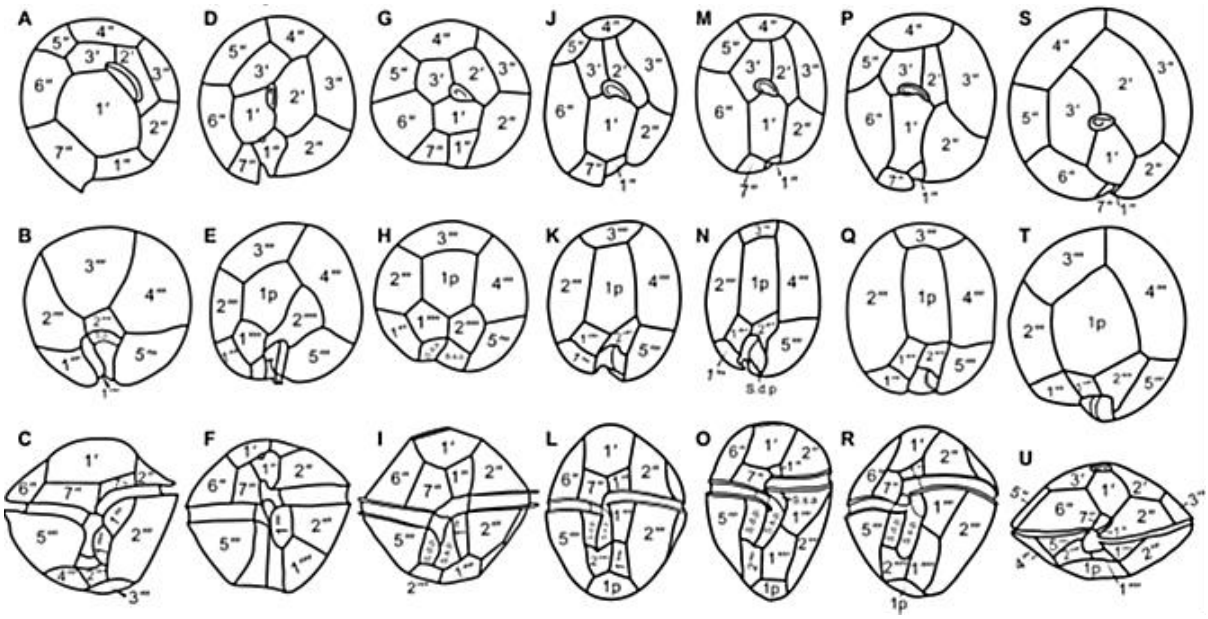
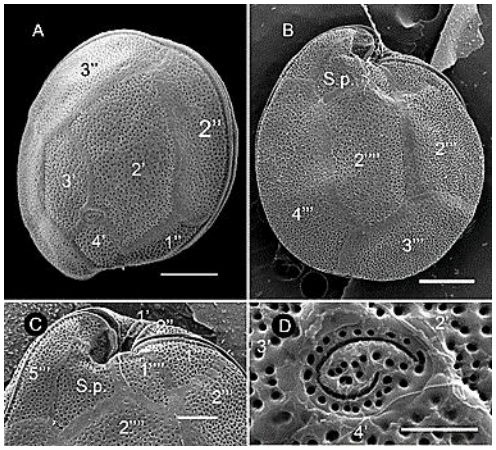


Figure 4. Line drawings of the external cell plates used for categorizing and identifying dinoflagellates. Drawings are of members of the family *Goniodomataceae* in apical (top), antapical (bottom), and ventral views (straight forward). (A-C) *Coolia canariensis* redrawn from Fraga et al. [27]. (D-F). *Alexandrium pseudogoniaulax* redrawn from Balech [25*]. (G-I) *Goniodoma polyedricum* redrawn from Balech [25*]. (J-L) *Fukuyoa paulensis* gen. et sp. nov. (M-O) *F. ruetzleri* comb. nov. was redrawn from Litaker et al. [11*]. (P-R) *F. yasumotoi* comb. nov. was redrawn from Litaker et al. [11*]. (S-U) *G. toxicus* redrawn from Litaker et al. [11*]. These species represent possible species with similar morphology and with possible environmental overlap. From [94] and references therein. *refers to references in the article.

5.1



5.2

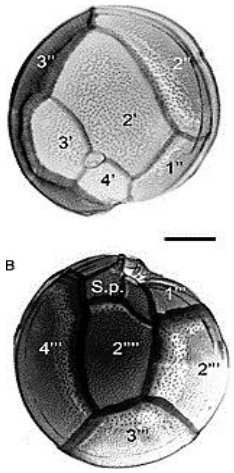


Figure 5. Example of species identification through phycological processes from Fraga et al. 2011 [85] (5.1) *Gambierdiscus excentricus* viewed under scanning electron microscope images show (a) Apical view (b) Antapical view (c) Sulcal area (d) Po plate. Scale bars: (a, b) 20 μm, (c) 10 μm, (d) 5 μm. (5.2) confocal microscope image of *G. excentricus* after calcofluor staining in both apical view and antapical view. Scale bar. 20 μm. Permission granted for re-use under license number 5154150800696 obtained from Harmful Algae (licensed content publisher), license date 22.09.2021.

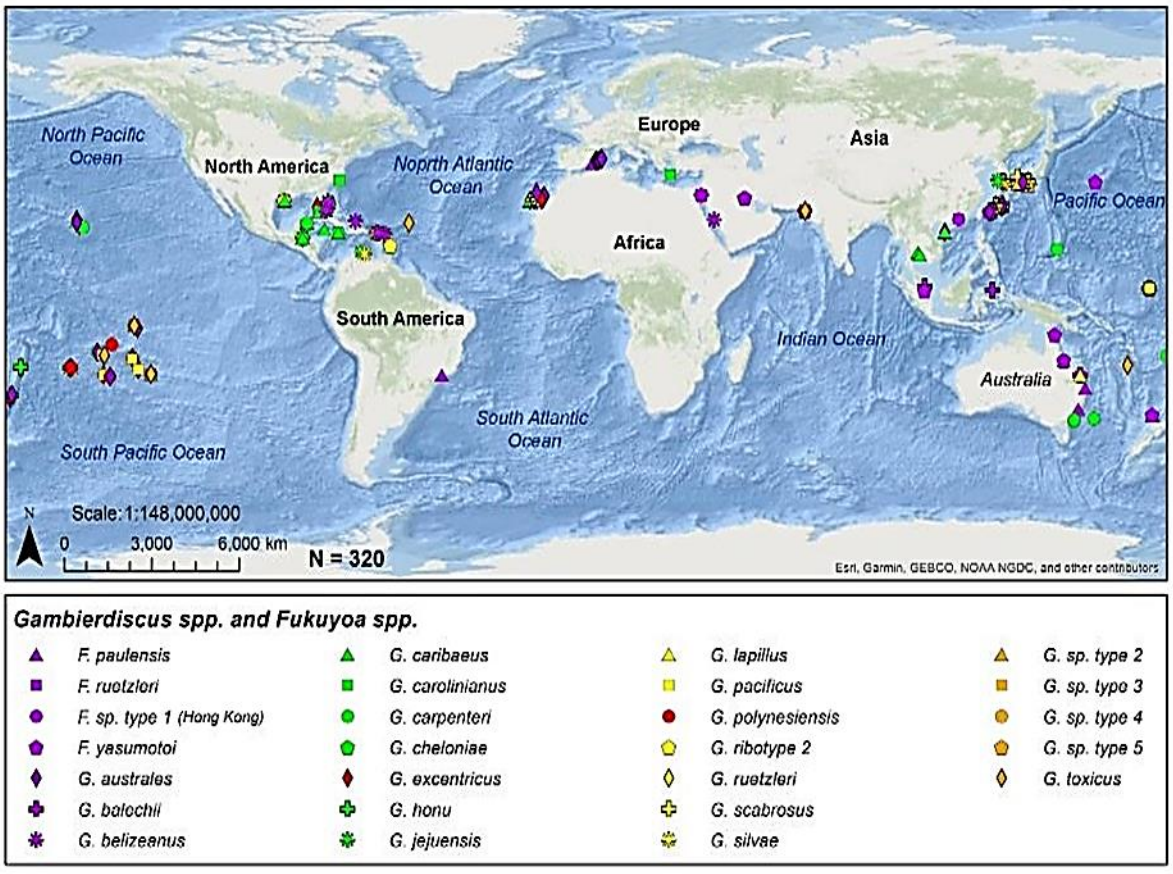


Figure 6. Global distribution of the genera *Gambierdiscus* and *Fukuyoa*. The publisher and rights holder of this work is the Intergovernmental Oceanographic Commission Harmful Algal Bloom Programme (Map from Tester et al. 2020 [77]). To the extent possible under the law, the publisher has waived all rights to these data and has dedicated them to the Public Domain (CC 1.0). Users may copy, modify, distribute and use the work, including for commercial purposes, without restriction.

Within the European Union's (EU) commercial market, all the >30 congeners (including epimers) so far described in the CTX class to date [37], are recognized as a human health hazard and therefore any foods containing CTXs must not be placed on the market [45]. Pacific Ocean (P-CTXs) [129,130], Caribbean Sea (C-CTXs) [131-133], and Indian Ocean (I-CTXs) [134] are summarized and reviewed in several articles [135,136], and the references therein [37]. Some of the distinct CTX analogs are shown in Figure 10 and include epimers such as CTX4A and its C-52 epimer CTX4B or oxidized algal metabolites like 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B. CTX analogs differ in several ways including the number of trans-fused (contiguous ether) rings (e.g., 14 for C-CTX-1 vs 13 for CTX1B) and the presence or absence of side chains [37]. The analogs and congeners all fall within four distinct groups of CTXs, categorized specifically as CTX3C, CTX4A (both Pacific CTX and P-CTX), C-CTX (Caribbean ciguatoxin e.g., $[M + H]^+$ m/z 1141.6 Da, molecular formula $C_{62}H_{92}O_{19}$), and I-CTX (Indian Ocean ciguatoxin) (Table 3). CTX4A and CTX3C group compounds have been geographically restricted to the Pacific Ocean basin, whereas C-CTXs have been found in animals of the Caribbean, Atlantic, and Gulf of Mexico basins; this is believed to be due to the geographic restriction of the CTX producing algae (Figure 6). However, several questions remain unresolved concerning the structural elucidation of I-CTXs and the microalgal origins of I- and C-CTXs; so, while the compounds

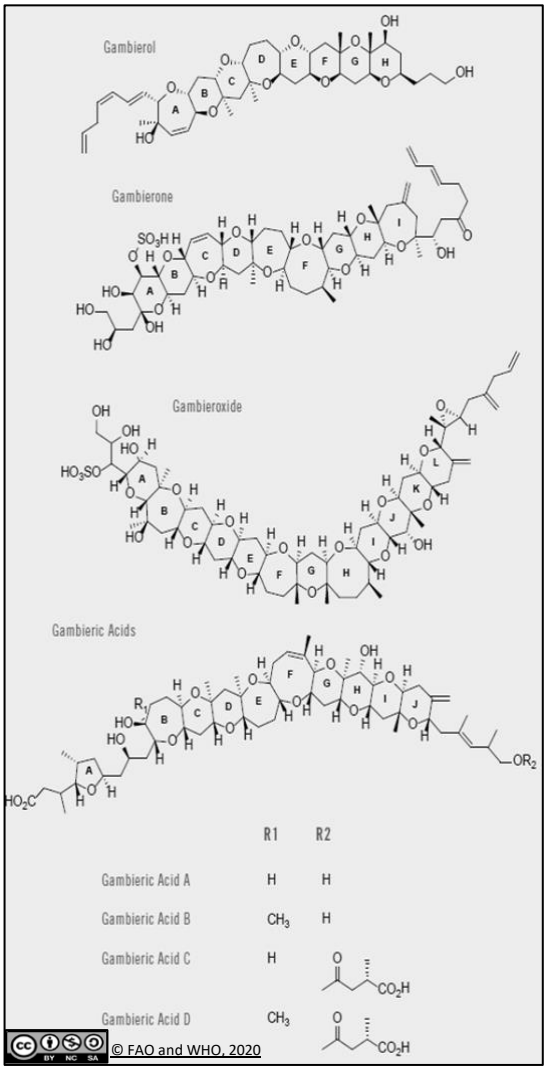


Figure 7. Skeletal structures (line -angle formula) of Gambierol, Gambierone, Gambieroxide, and Gambieric acids from FAO and WHO 2020.

are known to be specific to certain ocean basins, the algae that produce the toxins have not been identified for all source (or precursor) CTXs.

Gambierdiscus and *Fukuyoa* epitomize the introductory point in the food web, through which animals consume and incorporate CTXs. *Gambierdiscus* and *Fukuyoa*, in general, can be found in shallow, warm water, marine habitats (<200 m, down to <1% of ambient surface irradiance, >16 °C, and >15 salinity) but occur with minor abundance in the benthic and epi-benthic dinoflagellate community [137,138]. Several factors constrain their growth and distribution including physical oceanographic properties (e.g., irradiance, wave power, depth, salinity, and temperature), biological (predation, direct and indirect disturbances), and anthropogenic drivers (habitat modification, pollution, and fishing practices). All these factors can play an influential role in the spatial distribution of these species and affect their cellular abundance, both on spatial and temporal scales, as identified in both field and culture studies [38,75,139-144]. Globally, among the two genera of microalgae toxin producers, eighteen species have been described for *Gambierdiscus* and three for *Fukuyoa* [37,145] (Figure 6). Considerable differences within- and among-species exist concerning the concentration of toxin per cell, toxin production rate, conditions in which they produce or do not produce toxins, and the types of compounds they have been documented to produce [81,98,102,105,119,146-150]. Of these species, only a few (i.e., *F. paulensis* [151]*, G. australes* [100]*, G. toxicus* [100,152,153]*, G. polynesiensis* [71,148,150,154]) have demonstrated a sustained production of significant amounts of CTXs (CTX3B, -3C, -4A, -4B, 2-OHCTX3C, M-seco-CTX3C). There are, however, many other species that have been documented as capable of producing compounds believed to be CTXs (due to their similar selective action on voltage-gated sodium channels (Na_v) channels) [74,81,102,146,155], although confirmation of the causative compound in these other cases have not yet been demonstrated and therefore remain undefined.

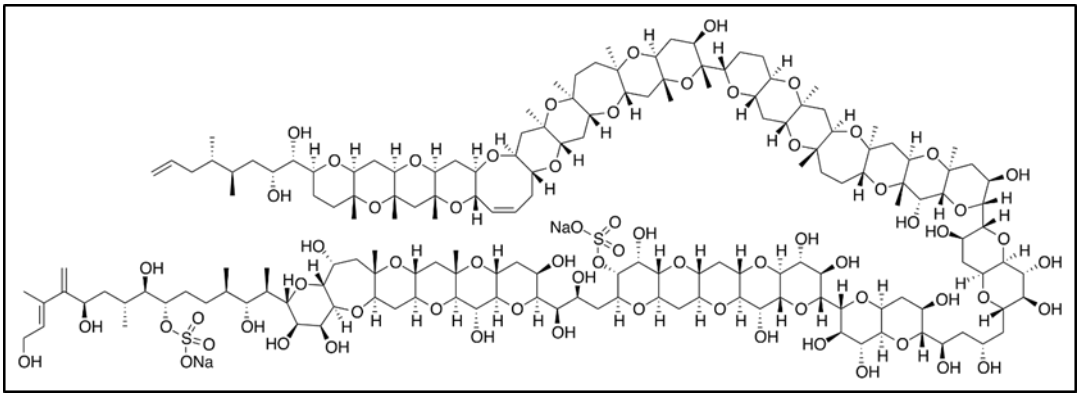


Figure 8. Skeletal structure of maitotoxins: Molecular formula $C_{164}H_{256}Na_2O_{68}S_2$, CAS Number 59392-53-9, Molar mass 3.422 g mol-1 (Wikipedia creative commons).

Table 3. Formulas and (high-resolution) mass to charge ratio (*m*/*z*) of precursor and product ions of ciguatoxins (CTX) congeners considered in [156].

CTX Congener	Formula	$[M + H - 3H_2O]^+$	$[M + H - 2H_2O]^+$	$[M + H - H_2O]^+$	$[M + H]^+$	$[M + NH_4]^+$	$[M + Na]^+$
CTX4A group [1]							
CTX4A /B	$C_{60}H_{84}O_{16}$	1007.55152	1025.56208	1043.57265	1061.58321	1078.60976	1083.56516
M-*seco*-CTX4A /B	$C_{60}H_{86}O_{17}$	1025.56208	1043.57265	1061.58321	1079.59378	1096.62033	1101.57572
52-*epi*-54-deoxyCTX1B 54-deoxyCTX1B	$C_{60}H_{86}O_{18}$	1041.55700	1059.56756	1077.57813	1095.58869	1112.61524	1117.57064
CTX1B 52-/54-*epi*CTX1B 52-*epi*-54-*epi*CTX1B 54-deoxy-50-hydroxyCTX1B	$C_{60}H_{86}O_{19}$	1057.55191	1075.56248	1093.57304	1111.58361	1128.61016	1133.56555
7-oxoCTX1B	$C_{60}H_{86}O_{20}$	1073.54683	1091.55739	1109.56796	1127.57852	1144.60507	1149.56047
7-hydroxyCTX1B	$C_{60}H_{88}O_{20}$	1075.56248	1093.57304	1111.58361	1129.59417	1146.62072	1151.57612
4-hydroxy-7-oxoCTX1B	$C_{60}H_{88}O_{21}$	1091.55739	1109.56796	1127.57852	1145.58909	1162.61564	1167.57103
CTX3C group [1]							
CTX3C/B	$C_{57}H_{82}O_{16}$	969.53587	987.54643	1005.55700	1023.56756	1040.59411	1045.54951
51-hydroxyCTX3C	$C_{57}H_{82}O_{17}$	985.53078	1003.54135	1021.55191	1039.56248	1056.58903	1061.54442
M-*seco*-CTX3C 2-hydroxyCTX3C	$C_{57}H_{84}O_{17}$	987.54643	1005.55700	1023.56756	1041.57813	1058.60468	1063.56007
M-*seco*-CTX3C methyl acetal	$C_{58}H_{86}O_{17}$	1001.56208	1019.57265	1037.58321	1055.59378	1072.62033	1077.57572
51-hydroxy-2-oxoCTX3C	$C_{57}H_{82}O_{18}$	1001.52570	1019.53626	1037.54683	1055.55739	1072.58394	1077.53934
2,3-dihydroxyCTX3C	$C_{57}H_{84}O_{18}$	1003.54135	1021.55191	1039.56248	1057.57304	1074.59959	1079.55499
A-*seco*-51-hydroxyCTX3C	$C_{57}H_{86}O_{18}$	1005.55700	1023.56756	1041.57813	1059.58869	1076.61524	1081.57064
2,3,51-trihydroxyCTX3C	$C_{57}H_{84}O_{19}$	1019.53626	1037.54683	1055.55739	1073.56796	1090.59451	1095.54990
C-CTX group [2]							
C-CTX-1/2	$C_{62}H_{92}O_{19}$	1087.59886	1105.60943	1123.61999	1141.63056	1158.65711	1163.61250
C-CTX-3/4	$C_{62}H_{94}O_{19}$	1089.61451	1107.62508	1125.63564	1143.64621	1160.67276	1165.62815
C-CTX reaction product 8	$C_{61}H_{88}O_{18}$	1055.57265	1073.58321	1091.59378	1109.60434	1126.63089	1131.58629
C-CTX reaction product 9	$C_{61}H_{90}O_{18}$	1057.58830	1075.59886	1093.60943	1111.61999	1128.64654	1133.60194
C-CTX-1127	$C_{61}H_{90}O_{19}$?	1073.6	1091.6	1109.6	1127.6	1144.6	1149.6
C-CTX-1157	$C_{62}H_{92}O_{20}$?	1103.6	1121.6	1139.6	1157.6	1174.6	1179.6
I-CTX group [3]							
I-CTX-1/2	$C_{62}H_{92}O_{19}$	1087.59886	1105.60943	1123.61999	1141.63056	1158.65711	1163.61250
I-CTX-3/4	$C_{62}H_{92}O_{20}$	1103.59378	1121.60434	1139.61491	1157.62547	1174.65202	1179.60742
I-CTX-5	$C_{62}H_{90}O_{19}$	1085.58321	1103.59378	1121.60434	1139.61491	1156.64146	1161.59685
I-CTX-6	$C_{62}H_{90}O_{20}$	1101.57813	1119.58869	1137.59926	1155.60982	1172.63637	1177.59177

? – formula is unknown or not yet confirmed. Information of a (potential) formula of congeners from 1 [157], 2 [133,158,159], 3 [26].

1.5. Toxin vectors: food chain from algae to fish to fork

The CTX producing microalgae are positioned at the base of the food web (Figure 9). A food web is a description of the interconnection of all food chains within a single ecosystem, describing the interactions of the consumer-resource system of autotrophs (primary producer) and heterotrophs (consumer). CTXs contained within the microalgae are transferred into the food web after being consumed by animals. Due to their lipid-soluble bio-accumulative properties CTXs are capable of accumulating in all animals that consume them, including major seafood products like fish and shellfish [10,12,124,141,160,161].

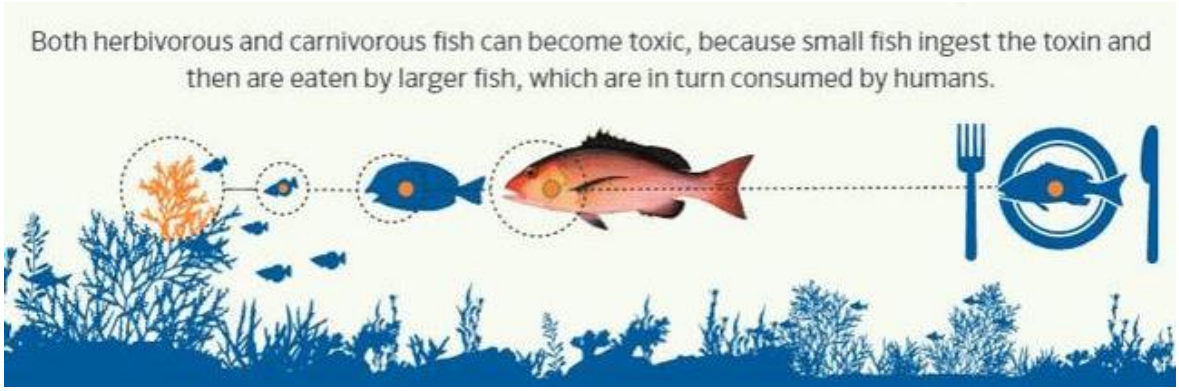
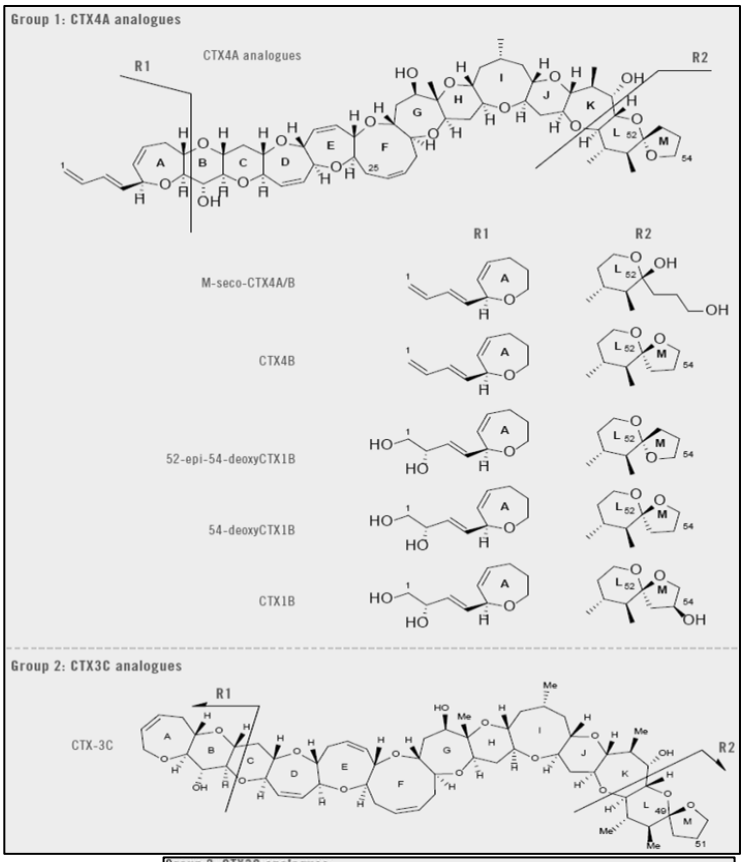
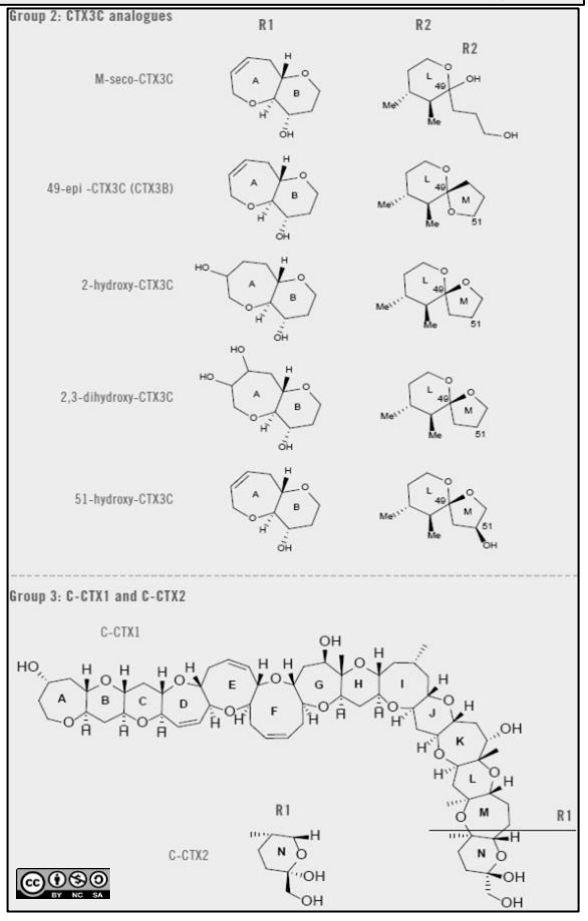


Figure 9. How ciguatoxins enter the food web adapted from [162] ©FAO.

Over 400 marine species were originally mentioned in early incidences of CP [163], and 425 species are suspected to contain CTXs and therefore potentially cause CP as listed in the 2020 report of ciguatera experts meeting by the FAO and WHO [37]. Because benthic microalgae producing CTXs are more or less ubiquitous and due to the interconnectedness of food webs it has therefore been hypothesized that all species entangled in the food web of a hyperendemic CP region can accumulate CTXs, at some point in their life to some degree (thus the high number of CP implicated species). However, to confirm this hypothesis, improvements in detection sensitivity to further identify species containing low levels of CTXs and better insight into within species variability in CTXs are needed. Depending on their food source preference animals are grouped into various categories such as detritivore, herbivore, planktivore, omnivore, invertivore, and piscivores. The uptake of CTXs from





Gambierdiscus into the fish food web starts with animals that consume the algae and microalgae that *Gambierdiscus* are attached to, such functional animal groups are detritivores, herbivores, omnivores, and planktivores for un-attached free swimming *Gambierdiscus* or *Fukuyoa* cells. Within these primary consumer groups there is variability in the feeding mechanisms used (Table 4). The feeding mechanism used by an animal determines the substrate they feed on, and *Gambierdiscus* have substrate and algal host preferences that can be species specific or can influence cell abundance [164-166]. Therefore, the individual species' feeding preference and mechanism puts the consumer species on a specific pathway of CTX accumulation, based on what they are consuming and if *Gambierdiscus* is associated with that food source. In addition to the toxin ingestion pathway individual fish have a metabolism that is species-specific, this individual metabolism can transform and modify the CTXs ingested. This was observed with certain herbivores such as grazers or lower trophic level feeders that tend to accumulate the less oxidized algal metabolites (e.g., CTX3C, CTX4A/4B), which were then consumed by piscivores that accumulate and metabolize the less oxidized compounds contained in the grazers into the highly oxidized analogs. To this date, available data suggest that the

highly oxidized analogs (e.g., CTX4A/B into CTX1B) are more potent compared to the less oxidized analogs [124]. This is relevant for human health as top order predators (e.g., barracuda) are targeted for human consumption and have therefore been implicated in severe cases of CP. The different feeding mechanisms, food source selection, and metabolic pathways of aquatic species can result in varying CTX concentrations and the CTX analog patterns found in different animals.

Figure 10. Structures of CTXs from FAO and WHO 2020

Table 4. List of common names and proposed functional groups of parrotfishes on Caribbean coral reefs. Table adapted from [167].

Latin name	Common name	Functional Group
Scarus coelestinus	Midnight parrotfish	Excavating and bioeroding grazer
Scarus coeruleus	Blue parrotfish	Scraping grazer
Scarus guacamaia	Rainbow parrotfish	Excavating and bioeroding grazer
Scarus iseri	Striped parrotfish	Scraping grazer
Scarus taeniopterus	Princess parrotfish	Scraping grazer
Scarus vetula	Queen parrotfish	Scraping grazer
Sparisoma aurofrenatum	Redband parrotfish	Macroalgal browser
Sparisoma chrysopterum	Redtail parrotfish	Macroalgal browser
Sparisoma rubripinne	Yellowtail/Redfin	Macroalgal browser
Sparisoma viride	Stoplight parrotfish	Excavating and bioeroding grazer

The CTX profile of the animal can also be influenced by behavior and subsequent changes in feeding patterns [161,168,169]. Feeding behavior can be species-specific and can be subjected to lifecycle changes [170-172], seasonal changes in home range utilization, distances traveled for feeding, prey selection feeding preference for size or type [173,174] or prey availability [175]; all of which can influence the animals CTX profile. As a working example, low trophic level fish and shellfish feed directly on or close to *Gambierdiscus* and *Fukuyoa*. This consumption pattern is reflected in an algal-specific CTX profile (the less oxidized forms/lower toxic potency) [169,176]. Some fish change their food according to their life cycle. Nursery-type habitats (e.g., mangroves or seagrass beds) are used in the juvenile life phase and this habitat contains specific prey items that are associated with the habitat type and not found elsewhere. In addition to the habitat selected for feeding, their juvenile size class restricts their hunting to prey smaller than themselves like crustaceans and plankton. Small prey are more likely to have a low trophic level algal CTX compound association [177]. When the fish grow, mature, and undergo an ontogenetic diet shift, they can move offshore towards more suitable locations or habitats such as deep shelf areas, or coral reefs. There, they change their diet to hunt larger animals, these larger prey items themselves can have accumulated a higher total body CTX content of the higher oxidized fish metabolite CTXs, and this higher CTX metabolite found in the diet will be reflected in the larger piscivores CTX profile.

Geographically, the *Gambierdiscus* species complex is regionally restricted (Figure 6) and which *Gambierdiscus* species are present can influence the CTXs profile of marine species consumed by humans. The CTXs, which have been isolated from fish and shellfish have been found to be structurally different (see Figure 7 and Figure 9) and these differences are geographically restricted such as from the Pacific Ocean (P-CTXs) [129,130], Caribbean Sea (C-CTXs) [131-133], and Indian Ocean (I-CTXs) [134].

The mating behavior can also play a role in diet and spatial habitat utilization, as fish move inter-annually or seasonally to spawning areas, or on migration routes that have staging areas, courtship areas, or temporary locations where food is sourced [178-181]. Thus, CTX accumulation can occur along these routes. Some species implicated in CP can be described in a wide geographical range, such as the Nassau grouper (*Epinephelus striatus*), which can transcend forty-three nations or territories jurisdictions [182]. Inversely, some species have small spatial home and hunting ranges such as *Cephalopholis argus*, the focus of Chapter 2, which can have occasional sporadic movements for spawning aggregations and migrations. For resource managers these movements can complicate prediction efforts for instance, a fish's behavior and feeding grounds may be stable and therefore predictable throughout the year but during the mating or spawning season the movement and feeding behaviors can change dramatically to novel prey and novel catch regions tens to hundreds of kilometers away. This CTX accumulation process of accumulation via different seasonal, movement, or life history related events which can introduce CTXs in food webs sporadically may play a role in the difficulties in predicting CTXs [183-185]. Fish are independent in these behaviors and not all fish within a species, reef, or common catch area contain CTXs, fish captured within a single harvest location can vary greatly in their CTX content. This constitutes a problem for temporal and spatial CTX prediction [38,50,183,184,186].

Animals have distinct biochemical, biological, physiological processes for metabolism and this is reflected in the enzyme-mediated biotransformation of CTXs and generation of metabolites [124,187]. Generally, after an animal has ingested CTXs, they are first taken up and detected in the gastrointestinal tract and liver, followed by detection in muscle tissue, and eventually throughout the body and skin [188-191]. The absorption efficiency of CTXs from fish feed is approximately 1–6%, and half-life depuration rates were tissue-related, lasting from several days for the liver to several months in the skin [188]. After the accumulation of CTXs, both behavioral abnormalities and the absence of their impacts have been documented in fish after feeding studies [189,191].

To predict, or at least spatially and temporally constrain areas of CP concern, investigating vectors for CTXs is imperative. Available information regarding their behavior, lifecycle, and movement patterns can help provide novel insight into the trophic transfer of CTXs to better protect consumers. The investigation of vectors is as important as investigating why certain fish species do not accumulate CTXs. Understanding their feeding habits and habitat usage, and metabolism would provide new understandings of CTX metabolism and fish feeding behavior and could provide evidence that some species that live and feed in CP endemic regions can be locally considered ‘safe for consumption’ [192]. However, environments change, and constructing a risk assessment on previous experience with a species and CP can lead to false confidence if the information is not kept up to date. This is particularly true not just locally but globally as the world’s oceans have absorbed heat and CO_2 associated with

climate change. These changes have been documented to affect the behavior and life cycles of fish; resulting in new and unforeseen consequences [193-197]. Ocean acidification is one byproduct of rising CO_2 concentration in the ocean and because calcium carbonate dissolves in acidic conditions ocean acidification can lead to the dissolution of calcium carbonate-based organisms or structures (i.e., coral reefs). Therefore, if the pH in the ocean is reduced this will impede shell-forming organisms (e.g., shellfish, corals) from undertaking the calcification process which is a major benthic habitat disrupter. If there is a state shift in the coral ecosystem through the loss of coral or shellfish bed structural habitat, turf or macroalgae would occupy the newly available space, and these algae are the preferred habitat for CP producing microalgae and food source for herbivores. The movement of animals is also influenced by climate [171,198,199], and better insight into the spaces utilized, movement, and connectivity [200] is needed for making the most accurate decisions regarding the risk management of CP and subsequent policies. Excess heat absorbed by the oceans due to global warming (Figure 11) affects the oxygen content of water, lower oxygen content in water increases the metabolic costs associated with breathing, this requires a change in the animal to offset this additional metabolic cost which occurs through changes in metabolism, respiration, body size, and the life history of fish [197,201-206]. In a hyperendemic region for CP in the Caribbean (the US Virgin Islands) herbivorous fish have been observed feeding at 20,000-156,000 bites m^2 d^{-1} on macroalgae and palatable surfaces [207]. *Gambierdiscus* can have a 1000-fold difference between the least and most toxic species [81] and the abundance on cells on algae can range from 1-1,000,000 cells g^{-1} wet weight of algae [208]. Therefore, any increase in metabolism for herbivorous fish, due to a lower oxygen content or higher temperature, that requires ingesting more algae to maintain growth could in turn result in a higher cell (toxin) ingestion rate. Furthermore, as waters warm tropical species will expand their range towards the global poleward as sub-tropical and temperate regions become warmer, this allows tropical species including *Gambierdiscus* to expand their range and enter new regions [77,209]. A rise in global sea level (approx. 1.7-3.2 mm yr^{-1}) is submerging low lying land area and creating new shallow water habitat, and a significant acceleration in the rate of sea-level rise is predicted for the 21st century [210,211]. *Gambierdiscus* can tolerate a wide range of light intensities and depths (<1 to 150 m) [212], therefore, these environmental alterations due to climate change are forecast to benefit *Gambierdiscus* spp. for habitat expansion [213-215].

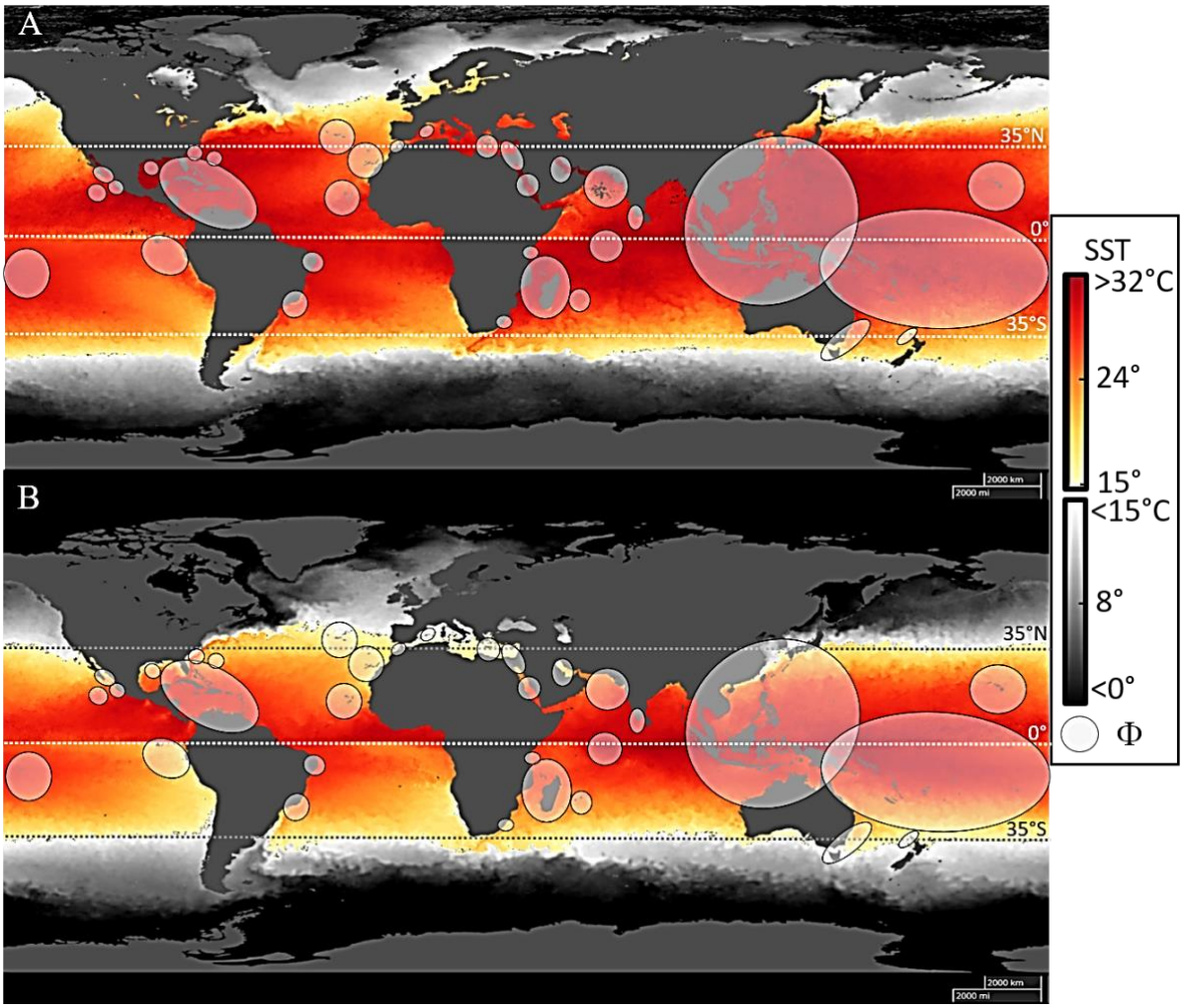


Figure 11. Global seasonal (a) maximum and (b) minimum in temperature for 2020 with bubble areas (ϕ) indicating known CP endemic regions. Heat maps with color show sea surface temperatures measured by satellite, temperatures >15 °C can indicate conditions exceeding the minimum for *Gambierdiscus* survival (by temperature) base map adapted from [59].

In Figure 12 a selection of global maps for families of fish implicated in CP are displayed as a function of where the fish could occur, based on habitat suitability. This visual interpretation is an extension of the current range of the fish but also considers the range of the wider family of fish (i.e., all barracuda spp.). By displaying where the fish can occur, as a function of habitat suitability, this also shows where the fish could have the potential to be found if inadvertently introduced into a new habitat. The invasion of a non-native species into a new habitat has occurred throughout natural history through events such as algal mats that float on oceanic currents, landmass changes, and floating debris (for algae, eggs, and animals). In the modern history of interoceanic travel by humans the introduction of invasive species has been deliberate and inadvertent. The introduced "alien" invasive species can be problematic if it becomes established gaining an ecological foothold in the new

environment. When a species is established, it requires natural resources that would otherwise be utilized by native species, therefore, their presence negatively impacts the survival of native species. Invasive species can also present an unrecognized risk to prey species, sometimes the predatory species are capable of a novel camouflage or mechanism of predation that prey species are incapable of defending against. Species co-evolve together, sometimes in what is described as an "arms race" where the predator evolves a mechanism to ensure greater hunting success and the prey forced into an evolution of successful evasion or survival strategies. This is also the case with algae and plants, where certain species are better adapted to a climate or habitat by outgrowing competitors or slowing their competitors down through the use of inhibitory compounds to outcompete other species. When species become invasive, they can cost local economies billions of dollars annually, in terms of lost fisheries production, direct cost combating the species, disruption to ecosystem services, and extinctions of native species. In the Gulf of Naples around the island of Ischia, an area considered a biogeographic boundary for the distribution limit of the temperature limited South Mediterranean thermophilous species at a 14 °C winter surface temperature isotherm, due to global warming this line has moved north and the island is now within the 15 °C isotherm divide (Figure 11). A total of 21 alien species have now been documented in the region however *Gambierdiscus* has not yet been observed [216]. The focus of Chapter 2 is on an invasive species and how it became a problematic species for CP, and how the lack of harvest due to CP can result in the invasive species becoming the dominant predator disrupting the native ecosystem. Therefore, in the context of modern society, several studies have provided evidence that humans can impact CP on a local scale, and on a regional scale, humans have facilitated the spreading of species to new regions and these invasive species are presenting novel food CP risks (as well as environmental and ecological problems). Humans have also altered the global carbon cycle resulting in a series of cascading effects due to climate change on a global scale. Therefore, while CP has been historically present, anthropogenic influences can exacerbate the problem. However, adequate monitoring or regulatory tools for CTXs are not in place that are sufficient to understand the conditions leading up to a CP event and the food chain trophic transfer of CTXs needs further elucidation.

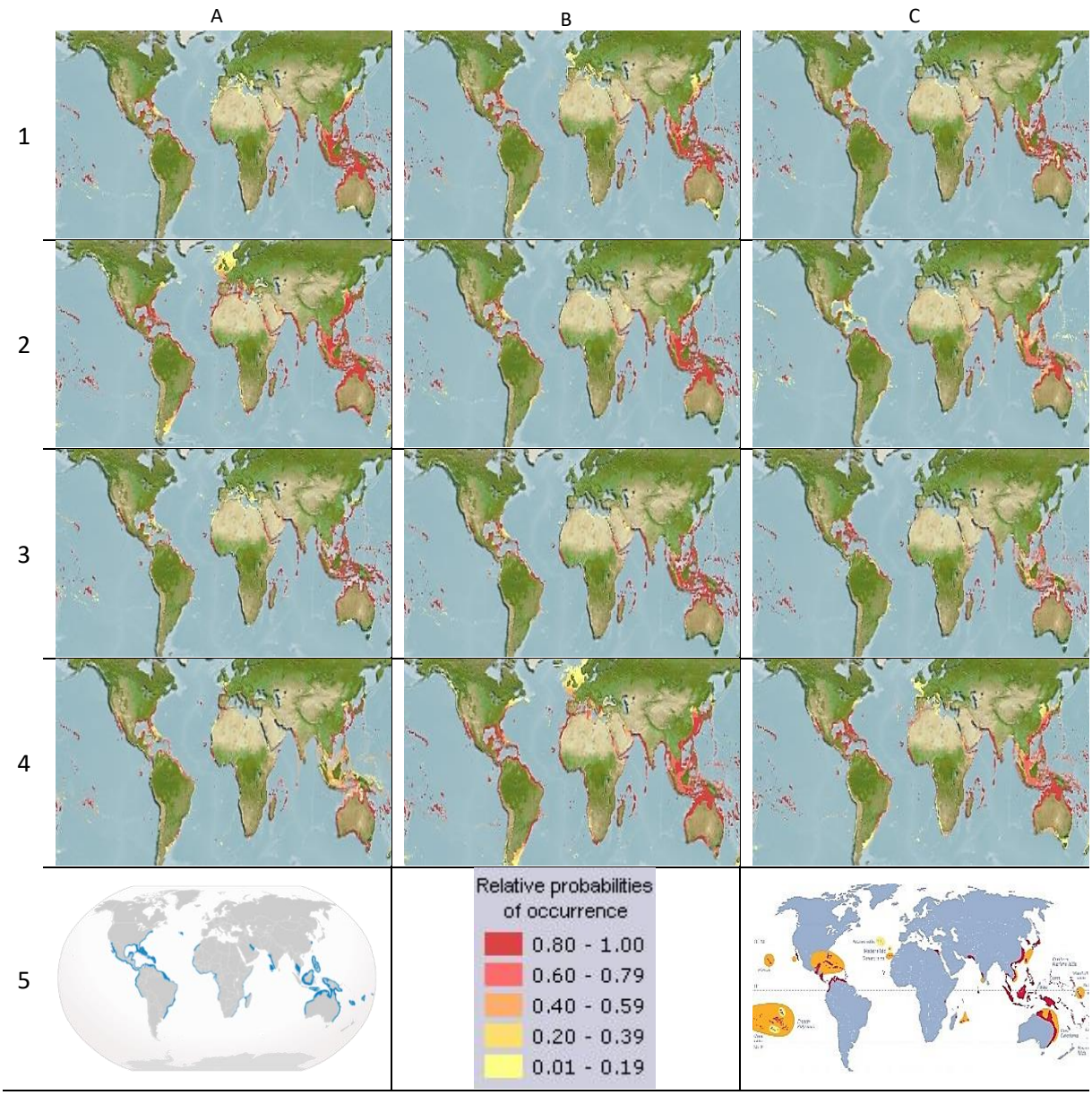


Figure 12. Examples of common fish families implicated in ciguatera poisoning events with global maps of suitable habitats for the species in yellow-red scale (see 5b in figure) [192] (Common name of the family in English (and Latin); also, some genera (g.) are cited as examples.) Note: A more exhaustive list of species and specific locations can be found in Table 4 within the FAO and WHO expert meeting 2020 document [37]. A.1. Moray eel (Muraenidae), B.1. Barracuda (Sphyraenidae), C.1. Grouper (Serranidae), A.2. Jacks/Amberjack (Carangidae, g. Seriola), B.2. Snapper (Lutjanidae), C.2. Surgeonfish (Acanthuridae). A.3. Parrotfish (Scaridae), B.3. Wrasses (Labridae), C.3. Hogfish (Labridae, g. *Lachnolaimus*). A.4. Narrow barred mackerel, Spanish mackerel (Scombridae, g. Scomberomorus) 4, B.4. Trevally *Caranx hippos* (Crevalle jack) (Carangidae, g. Caranx), C.4. Triggerfish (Balistidae). A.5. Shark (Carcharhinidae)[1] (blue color represents occurrence), B.5. Color key for all maps based on relative probabilities of occurrence for global distribution, color represents a heat color map 1=100%[4]. C.5. CP incidence map from Figure 1 (Re-use license number 5117730058978 obtained). Colors in the map indicate: Areas with at least one local CP case reported (red), Areas where CTXs have been found in locally caught fish (orange), and areas where CTXs have been found in locally caught marine invertebrates (yellow).

[1] By www.iucn.org, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=18120668

[2] AquaMaps (2019, October). Computer-generated distribution maps for Moray eel, Barracuda, Grouper, Amberjack, Snapper, Surgeonfish, Parrotfish, Wrasse, Hogfish, *Balistes vetula* (Queen triggerfish), modeled by an 'All suitable habitat' map based on IPCC RCP8.5 emissions scenario. Retrieved from https://www.aquamaps.org.

[3]NetCDF files have been produced and are hosted by the D4Science e-Infrastructure www.d4science.org, services.d4science.org. Citation: Scarponi, P., G. Coro, and P. Pagano. A collection of Aquamaps native layers in NetCDF format. Data in brief 17 (2018): 292-296.

[4] Maps were generated from [192]

1.6. Toxin detection and approaches to methodology

The methods utilized for investigating the presence of CTXs, from the algal source (toxin generation) to products targeted for human consumption, are tailored according to the user's intents, purposes, and infrastructure. The methods with the lowest cost, effort, least complexity, and highest accessibility (low infrastructure) are crude bioassays using domesticated or semi-wild animals. Historically, toxicity screening was conducted using *in-vivo*-based methods, which were not compound-specific, but was widely used because they are sensitive and capable of providing basic information about the wholesomeness of a seafood product. The first methods, including local folk methods [217,218], that were used for CP detection were based on cats [219], chicks [220], mongoose [12], mosquito, mouse assay, rat assay, as well as other methods used by communities living in endemic regions for CP as described in [37]. All methods depend upon feeding or exposing the animal with potentially toxic food. There are many different methods, that have been previously implemented for the detection of CTXs when other more specific methods are lacking, as recently reviewed by Pasinszki et al. [221]. A more controlled approach than a crude bioassay is the mouse bioassay [160,222]. Methods developed using mice have been in use for over 30 years, and entail administering mice CTXs either orally or by IP. These mouse bioassay studies have identified a set of symptoms that were considered the hallmark of CTX induced CP symptoms: loss of activity, piloerection, hypothermia, profuse diarrhea, hypersalivation, lachrymation cyanosis, motility disorders, dyspnea with gasping, possible hind-limb paralysis, and death due to respiratory distress. These symptoms were common for all tested CTX analogs, irrespective of their geographic origin or whether they are pure congeners isolated from microalgae or carnivorous fish as described in the report of FAO and WHO [37] and references therein. Differences in potency among analogs of CTXs, determined by the mouse bioassay, have been described as toxicity equivalent factors (TEFs). Based on those data, the TEF of CTX1B was set to 1.0, and the potency of other congeners was related to this compound and value. TEFs of other CTXs are as follows; 52-*epi*-54-deoxyCTX1B at 0.2, 54-deoxyCTX1B at 0.2, CTX3C at 0.2, 51-hydroxyCTX3C at 1.3, 2,3-dihydroxyCTX3C at 0.1, CTX4A at 0.2, CTX4B at 0.05, C-CTX1 at 0.1, and

C-CTX2 at 0.3 as described in FAO and WHO [37]. However, EU recommendation 2010/63/EU directs the replacement of the mouse bioassay with the use of a scientifically satisfactory method or testing strategy in routine analyses [223]. Furthermore, several questions remain to be answered before this TEF estimate based on the mouse assay can become a settled issue. Currently, of all the known or described analogs, there are only two commercially available standards (CTX1B, CTX3C, both from the Pacific CTX-group; manufacturer: FUJIFILM Wako Chemicals, as of September 2021) and no certified reference standards exist. A controlled investigation into the differences among the analogs in terms of potency and binding efficiency to voltage gated sodium channels (Na_v) has yet to be fully completed, but for the principal compound CTX1B sodium channel activity has been investigated [224]. Moreover, the class of analogs identified as I-CTXs remain to be structurally elucidated, and questions remain for which species of *Gambierdiscus* produces the family of CTXs originating in the Caribbean, Gulf of Mexico, and the Atlantic Ocean. The general information currently available regarding a comparison of potencies these results should be challenged until the evidence is grounded firmly using validated methods and certified reference standards.

Human health guidance levels for C-CTX1 and CTX1B (CTX4A derivative), of 0.10 and 0.01 ng g^{-1} equivalent toxicity in fish, respectively have been established by the U.S. Food and Drug Administration (FDA). Because these levels are contained within complex matrices (e.g., fish tissue) their detection at these concentrations, with recoveries between 50–100%, necessitates suitable facilities and advanced sensitive analytical methodologies [25,38,39,225-229]. The first step in the analysis process is toxin extraction. These extraction procedures, before analysis, are variable and can be time-consuming (up to 3 days), and to date there is no validated chemical protocol for CTX extraction in a biological matrix. This is an analytical challenge that has been also discussed in more detail in the FAO and WHO report [37]. Existing protocols designed specifically for the testing of CTXs include various bioassays (*in-vivo* and *in-vitro*), biochemical assays, and chemical assays, where the design, application, and suitability of its use are tailored to the user's capabilities, requirements, or intentions [37,230]. When a meal remnant from a CP outbreak is available the analysis methods deemed suitable for supporting a clinical diagnosis for CP generally include the authentication of the (food) species involved (e.g., by DNA barcoding) and the chemical analysis for CTXs which is recommended to be a two-tiered approach (e.g., FDA method outlined in [23,133,136]). The two-tiered approach for CTX analysis (generally) includes (i) a screening method capable of semi-quantitatively measuring combined toxicity of the sample in an action-specific or dose-response manner [136,231-239] and (ii) the confirmation of CTXs, identified either by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or LC coupled to High-Resolution MS (LC-HRMS) [32,133,134,136,154,161,226,240-243]. The carbon backbone of CTXs are stable, and because CTX + Na^+ ions produce almost no fragment ions, even under high collision energy, in many LC-MS investigative

approaches these are often set for both precursor and product ions ($[M+Na]^+ > [M+Na]^+$). Ammonium adducts can be easily fragmented, following the cleavage of ammonium the resulting $[M + H]^+$ ion can be followed by one or several water molecule losses.

The two-tiered methods and existing protocols (i.e., semi-quantification paired with confirmation) are commonly used not just for clinical analysis support, but also for the monitoring of micro-algae, fish, marine invertebrates, solid-phase adsorption toxin tracking filters, or other artificial surfaces and material with the potential to contain *Gambierdiscus* and or *Fukuyoa* for CTXs. The monitoring of various potential vectors concerning CP in the frame of surveillance programs for samples or regions of interest is in use by numerous research and human health-oriented competent authorities. As with the extraction protocols, there is currently no validated or accredited method for the routine analysis of CTXs. Methods that can satisfy the first tier 'screening type' approach are usually capable of detecting toxins below the guidance levels and have over time showed a drastic improvement in reliability and sensitivity. Because the *in vitro* cell-based assays or similar methods (e.g., enzyme-linked immunosorbent assay, receptor binding assay, etc.) are used as a 'screening-type' method they are mostly reported as semi-quantitative in their detection capabilities [244,245].

In-vitro-based methods are designed to investigate CTXs by their mechanisms of action and effects on various cell types (mouse, guinea pig, rat, human, etc., as reviewed by L'Herondelle et al. [246], and references therein). A mouse (*Mus musculus*) neuroblastoma cell line, Neuro-2a (N2a), was the stock cell line used for the development of the N2a-assay. The N2a-assay was designed to be a pharmacologically based cytotoxicity method specific for detecting compounds acting on the Na_V. Toxins that activate (e.g. CTXs, brevetoxins (PbTxs)) or block (e.g. tetrodotoxins, saxitoxins) the target Na_V channels can be differentiated by using the N2a-assay, enabling the identification of sodium channel-specific toxins among the presence of other toxins with other (non-specific) modes of action [25,32,136,231,232,247,248]. CTXs can cause persistent activation of the Na_v, increasing neuronal excitability and neurotransmitter release, impairing synaptic vesicle recycling, and causing cellular swelling [249]. This swelling can lead to osmoregulatory dysfunction and cell death. The advent of the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT (a tetrazolium salt) test as an endpoint for the N2a-assay enabled the detection of living cells and is used to measure cytotoxicity, proliferation, or activation. The use of MTT, which requires metabolically active cells to reduce a tetrazolium compound into a blue-colored formazan product, enhanced the end-point assessment and processing speed of the N2a-assay method [232]. With an appropriately designed experiment, the detection of CTXs at sub-picogram levels in complex matrices (e.g., fish extracts), capable of qualitative detection and semi-quantitative estimates of toxicity is possible. Therefore, when functioning within the prescribed parameters, the N2a-assay, is recognized as a useful, highly sensitive, and specific *in vitro* detection method for CTX screening purposes. However, the N2a-assay and other in vitro screening

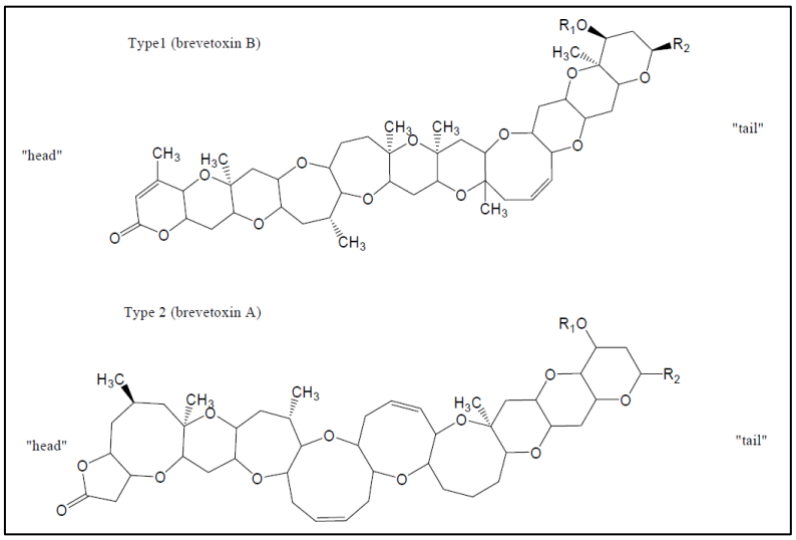
methods have not yet been validated, and without an established validated method there are many different approaches in use for conducting these methods in various laboratories around the world [229,250,251]. Because the N2a-assay is more sensitive than the traditional mouse bioassay, as well as other current analytical methods, it is none-the-less an important tool for the analysis of toxins in both food and environmental samples, and therefore the performance of the N2a-assay is the focus of Chapter 3 [83,185,229,251-255].

When selecting a method for CTX analysis sustainability and ethical concerns are often considered when compared to the mouse bioassay. However, most cell lines require protein-based serums and the use of essential supplements in cell culture, and these supplements are mainly animal-based (e.g., fetal bovine serum). Therefore, the use of animal-based products for *in vitro* cell culture raises the same scientific reliability, repeatability, and animal-based ethical concerns of *in-vivo* methods. To reduce or eliminate these animal-based products for CTX analysis the design of cell lines that are serum-free or reduced [256] and appropriate for an assortment of applications are preferred, and the substitution of critical supplements are currently available for some cell lines and in certain instances [257-260].

If a sample is deemed positive for 'CTX-like toxicity' using a mode of action specific screening method then to identify the active compound responsible for the observed toxicity an additional confirmation step is required. In step-two of the CTX analysis approach a targeted method is used to identify any CTX congener from among the four CTX groups (CTX4A, CTX3C, C-CTX, and I-CTX) making up the currently known 'CTX family' [37]. To achieve the high selectivity and sensitivity required for detecting CTXs in complex matrices at sub-ppb levels the most utilized method for the qualitative (and at times quantitative) determination of CTX analogs is High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS or LC-MS) [221,225]. These methods require sufficient materials such as toxins in natural matrices, reference materials, and analytical standards to fully implement the physical separation capabilities of the HPLC with the mass analysis capabilities of the MS. These materials are required to (i) optimize analyte ionization and fragmentation settings, to increase sensitivity for reaching the sub-ppb detection levels required for CTX analysis, (ii) to chromatographically resolve different structural analogs (of which there are >30), and (iii) to test the applicability of the method on naturally contaminated and/or spiked samples for method validation (a point that also applies to the tier-one screening methods). There are many challenges for the development of universally applicable LC-MS methods, these are related to the laboratory's LC-MS instrument platforms utilized, including different electrospray ionization source geometries, which affect the ionization behavior and ultimately detection. Because standards are not readily available for the majority of CTXs, to take a conservative standpoint in methods of detection, all known CTXs should be considered as a part of potential contaminants for the investigation to prevent a false negative result (missing CTXs and

declaring the sample negative). The remote possibility also remains that other undetermined CTXs exist and may yet be identified as methods and research coverage (qualitative and geographic) continues to improve. The European Food Safety Authority (EFSA) recommended that methods should be developed to test for CTXs in products (and to provide some recommendations). However, official test methods for CTX contaminant analysis are not described in the current legislation [261]. However, regardless of the method type (tier-one or two), a major problem for the successful development of universally applicable methods is the lack of CTX certified standards or reference materials. To make progress on the lack of material several CTX reference type stock materials have been reported in the literature which was the product of large-scale semi-purified standard derived from larger amounts of naturally incurred material. However, these are private stocks of material and selectively shared among researchers only as gifts or private purchases, a barrier to most laboratories if they are new to the field or unaffiliated with established research groups. Detection and sensitivity capabilities for most of the aforementioned methods will be aided by the availability of authentic analytical standards [262,263], and these advances will be a direct benefit to consumers of seafood who may be at risk of chronic low dose exposure to CTXs.

The detection of CTXs and PbTxs (Figure 13) has historically been conducted using the mouse bioassay [264,265], however to replace the mouse assay, various pharmacological, analytical, and immunological methods have been developed for detecting PbTxs in different matrices, including cytotoxicity and receptor-binding assays [266,267], liquid chromatography tandem-mass spectrometry LC-MS/MS [268-270], and immune assays [271]. A combination of an ELISA screening method with a confirmatory LC-MS/MS method is the approach currently employed at the US FDA [272]. CTX detection for CP outbreak response as well as for research purposes can be performed routinely in an analytical laboratory setting [32,41,133,136]. The two-tier protocol is the commonly applied method workflow and often the most appropriate for providing the information required to make informed decisions of public health and economic importance regarding CTXs and PbTxs.



Toxin	Type	R1	R2	Nominal mass
PbTx-1	2	H	$CH_2C(CH_2)CHO$	866
PbTx-2	1	H	$CH_2C(CH_2)CHO$	894
PbTx-3	1	H	$CH_2C(CH_2)CH_2OH$	896
PbTx-5	1	COCH3	K-ring acetate PbTx-2	936
PbTx-6	1	H	H-ring epoxide PbTx-2	910
PbTx-7	2	H	$CH_2C(CH_2)CH_2OH$	868
PbTx-8	1	H	CH_2COCH_2Cl	916
PbTx-9	1	H	$CH_2CH(CH_3)CH_2OH$	898
PbTx-10	2	H	$CH_2CH(CH_3)CH_2OH$	870

Figure 13. Structure and species of neurotoxic shellfish poisoning toxins from marine dinoflagellates [21]. This is an open access article distributed under the Creative Commons Attribution License.

1.7. Long term risk from CP and CTXs

In addition to acute exposure events for CP in humans, the health risks posed by CTXs over the long–term, at levels below the threshold capable of causing symptoms, are currently unknown or vaguely understood. In nature, CTXs have bioaccumulative properties (e.g., bioaccumulation in fish). Therefore, humans exposed to CTXs at low levels over time may also bioaccumulate CTXs, a situation that could present a potential for long-term human health risks. Studies on the subject of CTX bioaccumulation in humans have reported that patients who describe having a previous history (or prior episode) of CP experienced significantly worse or more severe symptoms when compared to patients' descriptions of symptoms who experienced having CP for the first time [273]. This same study also identified an increase in severity associated with age, therefore, repetitive exposure along with age-associated severity suggested that CTXs in humans were indeed bioaccumulating [273]. In a

telephone survey and medical record review of ciguatera cases in the United States Virgin Islands (USVI), an endemic region with an intoxication rate for the general population (1.2%) among the highest in the world, researchers identified evidence that patients who have had at least one previous episode of CP within the last five years were associated with an increased likelihood of the individual having another episode in the next five years [274]. Patients with CP do not build a tolerance to CTXs, they become more susceptible and show increased severity in symptomology. Because CTXs are fat-soluble it is hypothesized, based on observations of repeated exposures to CTXs, that they can accumulate in humans and lower the tolerance threshold for repeated exposure to CTXs at a concentration capable of resulting in CP [273,275]. An investigation, focusing on locally consumed species in the USVI, found that CTX-like activity was detected in 51% of all fish sampled (which included a sampling site where no CTXs were detectable) and 4% showed CTX-like activity above the US FDA guidance level (0.1 ng C-CTX-1 g^{-1}) [185]. It remains unknown what or if any human health implications will result from chronic exposures to CTXs at low doses over a long period of time, but the study in the USVI demonstrates that low-dose exposure is a consumer reality [185]. Three out of the four species investigated in the USVI fish survey are commonly consumed, representing 23% of the total artisanal fishery. Furthermore, those species are sought after in part because they are locally perceived as 'safe' for CP. Fresh wholesome local fish (perceived as safe) can net a higher market value for fishers which will lead to the fishers targeting these species. This could contribute to chronic low dose exposure in hyperendemic regions for CP.

In rats, chronic CTX exposure has been linked to emotional and cognitive dysfunction, while reports of anxiety, depression, and memory loss have also been reported in humans [276]. In other studies using rodents for the investigation of absorption, distribution, metabolism, and excretion, mice and rats were administered CTX1B by oral route (gavage) and intravenously (iv). CTX1B was found to have rapid absorption of twenty-five minutes by IP and two hours for oral exposure [277,278]. Analysis by an *in vitro* bioassay (Neuro-2a neuroblastoma cell-based assay, N2a-assay) for toxin levels in the blood, excreta, and tissue indicated similar toxin bioavailability irrespective of the route of administration. Both the parent CTX1B was detected but also two less polar metabolites [277] with a similar mode of action to CTX1B as determined by the N2a-assay. Upon conclusion of the study, 96% of the recovered total CTX activity (CTX1B and active metabolites) were found in the muscle and ~2.5% in various other tissues. Excretion of CTX was through feces and to a lesser extent in the urine. Post day 7 of the study CTX1B was undetectable in the single administration treatment study [279]. In a study of repetitive administration of CTX1B, higher concentrations in the brain were detected and the study suggested that the toxin can accumulate in the brain and blood of rats after repeated low-dose exposures to CTX1B treatments [276]. A consolidation of available data performed by the WHO and FAO experts on Ciguatera concluded that CTXs are efficiently absorbed and rapidly distributed to

various tissues after ingestion in laboratory animal models [37]. Ikehara et al. used human CYP3A5, fish liver S9 fractions, and microsomal fractions to create an *in vitro* environment for CTXs to undergo oxidative modification leading to the generation of several analogs [124]. Specifically, they used CTX4A, CTX4B, and CTX3C, which are compounds produced by the algae. These studies demonstrate the complexity faced with the detection of CTXs in patients, as CTXs can enter the body in one form and can be metabolized and moved throughout the body in different forms. In most cases, extremely sensitive methods are needed to detect the presence of CTXs at human health-relevant concentrations. Furthermore, it is difficult to diagnose low dose exposure in patients.

Some CP symptoms have a prolonged effect, with certain symptoms described as chronic fatigue syndrome (CFS) or chronic ciguatera fish poisoning lasting for years in some patients [280-284]. In reports and descriptions from local knowledge regarding CP experiences, a phenomenon is frequently described in which the symptoms of CP can spontaneously reappear, at times following the consumption of alcohol, caffeine, nuts, any fish (which is also inclusive of freshwater species), or physical exertion. The sensation of a recurrence of symptoms is postulated to be due to neurological sensitization (due to potential damage caused by CTXs), cross-sensitization, cumulative exposure, or if the CTXs are stored in fatty tissue, metabolic remobilization of this stored toxin reservoir, which would appear to support a theory of long term impacts due to CP [23,249,285]. However, there is a paucity of long-term human health studies. Therefore, additional studies are required before potentially confounding influential conditions (i.e., other medical, behavior, allergies, or psychological conditions) can be ruled out, which will result in a better understanding of the reports of the year's long-term course of illness. Therefore, to approach the answer of long-term effects of CTXs, the research field must turn to a related compound, PbTxs (Figure 13).

The potential for long-term damage has been identified concerning PbTxs [286,287]. PbTxs are a class of similarly structured ladder-shaped cyclic polyether marine biotoxins and generally similar to CTXs. Both PbTxs and CTXs share a binding site (receptor-site 5) of the α-subunit of Na_v [288,289], thereby both compounds have a similar mode of action [247], albeit the affinity of CTXs to site 5 on the α-subunit of neuronal sodium channels is 30x higher than PbTx. PbTx is created by the marine microalgae *Karenia brevis* and when this species forms a phenomenon in marine environments, it can colloquially be known as a 'red tide' (due to the red/brown discoloration of the water when the algae are in a high rate of growth or bloom formation). PbTxs is an increasing public human health concern, currently, ten subtypes of PbTxs have been described (Figure 13). Based on *in vitro* exposure of rat liver cells to PbTx B (PbTx-2), these compounds can bind to nucleic acid forming DNA adducts and damaging the DNA in the lungs of laboratory rats [290]. CTXs (CTX1B and CTX3C) have also been shown to induce DNA damage in both human primary neuronal cells [291] and a mouse neuroblastoma (N2a) cell line [292]. Neurological, respiratory, and gastrointestinal illnesses in humans can result from

exposure to brevetoxins. A recent study by Diaz et al. (2019) found significant increases in emergency department visits correlated with coastal blooms of *K. brevis*, where a headache was a primary diagnosis [293]. This risk was higher for older individuals (≥ 55 years). Both brevetoxins and CTXs can cross the blood-brain barrier. Brevetoxins can also aerosolize which can lead to potential long-term health effects, which are currently being investigated. The toxin-producing algae have a relatively weak cell wall, and simple water motion or agitation can cause the cell wall to lose its integrity and fail, thus releasing the internal contents of the cell externally, and if the conditions are conducive then the toxins can become aerosolized with the accompanying sea-spray. The detection of brevetoxins was a focus of Chapter 3, and because of these efforts, the most sensitive assay for detecting brevetoxins to date was successfully described herein.

1.8. Management, guidance, and prevention

The United States Food and Drug Administration (FDA) has established a guidance level for Caribbean CTXs at 0.1 ng C-CTX1 equivalents per gram of tissue and Pacific CTXs at 0.01 ng CTX1B eq. per gram of tissue [39]; these guidance levels, which are based on adverse effects, have included a 10x safety factor to address individual human risk factors and inherent uncertainties [32]. In the European Union (EU) seafood products containing CTX-group toxins are forbidden from being placed on the market, therefore detection levels need to be as low as possible [261]. PbTx-group toxins in shellfish or fish in Europe currently, do not have regulatory limits [264], although both PbTxs and CTXs are considered emerging toxins of concern for EU waters [45,264,272,294]. The FDA regulatory action level for shellfish NSP toxins is 0.8 ppm (20 mouse units per 100 g^{-1}) brevetoxin-2 equivalents [39], and other countries have control and monitoring programs for NSP producing algae and fishery products [230].

Communities in different regions have applied various efforts focused on the prevention of CP, and generally, prevention measures can encompass action or inaction, local and regional, government-based, or personal responsibilities. Concisely, most recommendations and advice are against the consumption of certain species of fish (mainly large predatory fish) and the avoidance of consuming or purchasing fish from areas with an established history of CP as well as emerging areas of concern [39,295]. Domestic policies such as regulating the harvest or sale of products which have a history of being implicated in CP outbreaks either by size, species, and region have had varying degrees of success [38,39,50,296-299]. These guidance efforts are not without warrant, in endemic regions such as the U.S., the number of cases reported by the Centers for Disease Control and Prevention makes CP a leading cause of the finfish-associated food-borne disease [300]. The simplest and most effective form of personal protection for prevention is abstinence from consuming fish from tropical and sub-tropical waters where CP has been documented. When choosing to consume seafood products, certain

measures of self-protection can be taken, such as knowing one's local food source and asking questions regarding incidences of CP in the region and for the product in question. Traditionally, fishers are the most knowledgeable resource regarding CP risk (i.e., species and regions of concern), and they knew or learned which areas and species carried a risk for CP. Artisanal fisheries also have a vested interest in understanding the topic to protect their customers and they spend the most time in direct contact with the local environment making observations to corroborate their toxic fish knowledge. However, with more aggressive marketing practices and selling surplus production to bulk processors, fisher to customer direct interactions can be reduced as well as the familiarity of knowing which fish seller harvested only from low-risk areas. If local knowledge is abundant and well documented it can lead to local or regional action by the government to prevent or restrict harvesting fish in certain areas or determining what species and sizes of fish are less likely to cause CP. Currently, there are no known treatments for CP, however, several traditional remedies have been described as well as their efficacy and pharmacological studies in support of their use [301-305]. CTXs are mostly unaffected by common food preparation techniques such as cooking (CTXs are temperature stable), freezing, canning, acidic or basic conditions. CP is not an issue that can be easily resolved with hazard critical control point actions, which are typically standard actions applied for other seafood-related illnesses (e.g., scombroid poisoning, bacterial or viral gastroenteritis, see Table 1 similar illnesses to CP). Potential process-related hazards are associated with finished fishery products, their packaging type, storage conditions (temperature control), and methods of distribution [39]; whereas CTXs are inherent in the product at the time of harvest.

1.9. Bibliography

1. FAO. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. **2020**.
2. Gudger, E.W. Poisonous fishes and fish poisonings, with special reference to ciguatera in the West Indies. *The American Journal of Tropical Medicine and Hygiene* **1930**, *1*, 43-55.
3. Gillespie, N.C.; Lewis, R.J.; Pearn, J.H.; Bourke, A.T.; Holmes, M.J.; Bourke, J.B.; Shields, W.J. Ciguatera in Australia. Occurrence, clinical features, pathophysiology and management. *Med J Aust* **1986**, *145*, 584-590.
4. Doherty, M.J. Captain Cook on poison fish. *Neurology* **2005**, *65*, 1788-1791, doi:10.1212/01.wnl.0000187128.80551.1b.
5. Pearn, J. Ciguatera - an Early Report. *Medical Journal of Australia* **1989**, *151*, 724-725.
6. Lewis, N.D. Ciguatera—parameters of a tropical health problem. *Human Ecology* **1984**, *12*, 253-273.
7. Lewis, N.D. Ciguatera in the Pacific - Incidence and Implications for Marine Resource Development. *Acs Symposium Series* **1984**, *262*, 289-306.
8. Ansdell, V. 49 - Seafood Poisoning. In *Travel Medicine (Fourth Edition)*, Keystone, J.S., Kozarsky, P.E., Connor, B.A., Nothdurft, H.D., Mendelson, M., Leder, K., Eds. Elsevier: London, 2019; https://doi.org/10.1016/B978-0-323-54696-6.00049-5pp. 449-456.

9. Rongo, T.; Bush, M.; van Woesik, R. Did ciguatera prompt the late Holocene Polynesian voyages of discovery? *Journal of Biogeography* 2009, pp 1423-1432.
10. Randall, J.E. A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause. *Bulletin of Marine Science* **1958**, *8*, 236-267.
11. Chinain, M.; Gatti, C.M.i.; Darius, H.T.; Quod, J.P.; Tester, P.A. Ciguatera poisonings: A global review of occurrences and trends. *Harmful Algae* **2020**, https://doi.org/10.1016/j.hal.2020.101873, 101873, doi:https://doi.org/10.1016/j.hal.2020.101873.
12. Scheuer, P.J.; Takahashi, W.; Tsutsumi, J.; Yoshida, T. Ciguatoxin: isolation and chemical nature. *Science* **1967**, *155*, 1267-1268.
13. Yasumoto, T.; Hashimot.Y; Bagnis, R.; Randall, J.E.; Banner, A.H. Toxicity of Surgeonfish. *Bulletin of the Japanese Society of Scientific Fisheries* **1971**, *37*, 724-&.
14. Yasumoto, T.; Bagnis, R.; P.Vernoux, J. Toxicity of the Surgeonfishes-II Properties of the Principal Water-soluble Toxin. *Nippon Suisan Gakkaishi* **1976**, *42*, 359-365.
15. Yasumoto, T.; Nakajima, I.; Chungue, E.; Bagnis, R. Toxins in the Gut Contents of Parrotfish. *NIPPON SUISAN GAKKAISHI* **1977**, *43*, 69-74, doi:10.2331/suisan.43.69.
16. Adachi, R.; Fukuyo, Y. Thecal Structure of a Marine Toxic Dinoflagellate Gambierdiscus-Toxicus Gen Et Sp-Nov Collected in a Ciguatera-Endemic Area. *Bulletin of the Japanese Society of Scientific Fisheries* **1979**, *45*, 67-71.
17. Yasumoto, T.; Nakajima, I.; Bagnis, R.; Adachi, R. Finding of a Dinoflagellate as a Likely Culprit of Ciguatera. *Bulletin of the Japanese Society of Scientific Fisheries* **1977**, *43*, 1021-1026.
18. Bagnis, R.; Hurtel, J.M.; Chanteau, S.; Chungue, E.; Inoue, A.; Yasumoto, T. Dinoflagellae Gambierdiscus-Toxicus Adachi Et Fukuyo - Probable Cause of Ciguatera. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D* **1979**, *289*, 671-674.
19. Bagnis, R.; Chanteau, S.; Chungue, E.; Hurtel, J.M.; Yasumoto, T.; Inoue, A. Origins of Ciguatera Fish Poisoning - a New Dinoflagellate, Gambierdiscus-Toxicus Adachi and Fukuyo, Definitively Involved as a Causal Agent. *Toxicon* **1980**, *18*, 199-208, doi:Doi 10.1016/0041-0101(80)90074-4.
20. Institute, W.H.O. Pilot Study Examines Ciguatera Fish Poisoning. Availabe online: (https://www.whoi.edu/oceanus/feature/pilot-study-examines-ciguatera-fish-poisoning/) (accessed on October).
21. Wang, D.-Z. Neurotoxins from marine dinoflagellates: a brief review. *Marine drugs* **2008**, *6*, 349-371, doi:10.3390/md20080016.
22. Lewis, R.J. Ciguatera management. *SPC Live Reef Fish Information Bulletin* **2000**, *7*, 11-13.
23. Friedman, M.A.; Fernandez, M.; Backer, L.C.; Dickey, R.W.; Bernstein, J.; Schrank, K.; Kibler, S.; Stephan, W.; Gribble, M.O.; Bienfang, P., et al. An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. *Marine Drugs* **2017**, *15*, 72, doi:10.3390/md15030072.
24. Morrison, K.; Prieto, P.; Dominguez, A.; Waltner-Toews, D.; FitzGibbon, J. Ciguatera Fish Poisoning in La Habana, Cuba: A Study of Local Social-Ecological Resilience. *Ecohealth* **2008**, *5*, 346-359, doi:DOI 10.1007/s10393-008-0188-7.
25. Caillaud, A.; de la Iglesia, P.; Darius, H.T.; Pauillac, S.; Aligizaki, K.; Fraga, S.; Chinain, M.; Diogene, J. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of ciguatera fish poisoning in Europe. *Marine Drugs* **2010**, *8*, 1838-1907, doi:Doi 10.3390/Md8061838.
26. Diogène, J.; Reverté, L.; Rambla-Alegre, M.; del Río, V.; de la Iglesia, P.; Campàs, M.; Palacios, O.; Flores, C.; Caixach, J.; Ralijaona, C., et al. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. *Scientific Reports* **2017**, *7*, 8240, doi:10.1038/s41598-017-08682-8.
27. Bagnis, R. Concerning a Fatal Case of Ciguatera Poisoning in Tuamotu-Islands. *Clinical Toxicology* **1970**, *3*, 579-&.

28. Rabenjarison, F.; Ramarolahy, A.; Velomora, A.; Rahajaniaina, M.; Raveloson, N. Intoxication à la ciguatoxine après consommation de requin à Fenerive-Est: profil épidémio-clinique et résultats de laboratoire. Ciguatera poisoning after consumption of shark in Fenerive-Est: epidemiological and clinical aspects and laboratory results. *Revue d'Anesthésie-réanimation, Médecine d'Urgence et Toxicologie* **2016**, *8*, 9-12.
29. Defusco, D.J.; Odowd, P.; Hokama, Y.; Ott, B.R. Coma Due to Ciguatera Poisoning in Rhode-Island. *American Journal of Medicine* **1993**, *95*, 240-243, doi:Doi 10.1016/0002-9343(93)90268-T.
30. Lange, W.R. Ciguatera Toxicity. *American Family Physician* **1987**, *35*, 177-182.
31. Habermehl, G.G.; Krebs, H.C.; Rasoanaivo, P.; Ramialiharisoa, A. Severe ciguatera poisoning in Madagascar: A case report. *Toxicon* **1994**, *32*, 1539-1542, doi:http://dx.doi.org/10.1016/0041-0101(94)90312-3.
32. Dickey, R.W.; Plakas, S.M. Ciguatera: A public health perspective. *Toxicon* **2010**, *56*, 123-136, doi:DOI 10.1016/j.toxicon.2009.09.008.
33. Lewis, R.J. The changing face of ciguatera. *Toxicon* **2001**, *39*, 97-106, doi:http://dx.doi.org/10.1016/S0041-0101(00)00161-6.
34. Chateau-Degat, M.L.; Huin-Blondey, M.O.; Chinain, M.; Darius, T.; Legrand, A.M.; Nguyen, N.L.; Laudon, F.; Chansin, R.; Dewailly, E. Prevalence of chronic symptoms of Ciguatera disease in french polynesian adults. *American Journal of Tropical Medicine and Hygiene* **2007**, *77*, 842-846.
35. Hamilton, B.; Whittle, N.; Shaw, G.; Eaglesham, G.; Moore, M.R.; Lewis, R.J. Human fatality associated with Pacific ciguatoxin contaminated fish. *Toxicon* **2010**, *56*, 668-673, doi:http://dx.doi.org/10.1016/j.toxicon.2009.06.007.
36. Saraya, A.; Sintunawa, C.; Wacharapluesadee, S.; Swangpun, K.; Dumrongchua, S.; Wilde, H.; Hemachudha, T. Marine Fish Toxins in Thailand: Report of 6 Suspected Ciguatera Cases. *Case Reports in Clinical Medicine* **2014**, *2014*.
37. FAO and WHO. Report of the expert meeting on ciguatera poisoning. Rome, 19–23 November 2018 *Food Safety and Quality No. 9. Rome* **2020**, *https://doi.org/10.4060/ca8817en*, doi:https://doi.org/10.4060/ca8817en.
38. U.S. Food Drug Administration. *Guidance for Industry: Purchasing Reef Fish Species Associated with the Hazard of Ciguatera Fish Poisoning*; https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-purchasing-reef-fish-species-associated-hazard-ciguatera-fish-poisoning (Accessed 15 October 2020), 2013.
39. U.S. Food Drug Administration. *Fish and Fishery Products Hazards and Controls Guidance Fourth Edition - March 2020*; https://www.fda.gov/media/80637/download (Accessed 15 October 2020), 2020; p 498.
40. Lewis, R.J.; Sellin, M.; Poli, M.A.; Norton, R.S.; MacLeod, J.K.; Sheil, M.M. Purification and characterization of ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae). *Toxicon* **1991**, *29*, 1115-1127, doi:http://dx.doi.org/10.1016/0041-0101(91)90209-A.
41. Lewis, R.J.; Jones, A.; Vernoux, J.P. HPLC/tandem electrospray mass spectrometry for the determination of Sub-ppb levels of Pacific and Caribbean ciguatoxins in crude extracts of fish. *Anal Chem* **1999**, *71*, 247-250.
42. Lehane, L.; Lewis, R.J. Ciguatera: recent advances but the risk remains. *International Journal of Food Microbiology* **2000**, *61*, 91-125, doi:http://dx.doi.org/10.1016/S0168-1605(00)00382-2.
43. Pierce, R.H.; Kirkpatrick, G.J. Innovative techniques for harmful algal toxin analysis. *Environmental Toxicology and Chemistry: An International Journal* **2001**, *20*, 107-114.
44. Lewis, R.J. Ciguatera: Australian perspectives on a global problem. *Toxicon* **2006**, *48*, 799-809, doi:http://dx.doi.org/10.1016/j.toxicon.2006.07.019.
45. EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:https://doi.org/10.2903/j.efsa.2010.1627.
46. Yasumoto, T. Chemistry, etiology, and food chain dynamics of marine toxins. *Proceedings of the Japan Academy, Series B* **2005**, *81*, 43-51.

47. Rajisha, K.; Kishore, P.; Panda, S.K.; Harikrishnan, G.; Ajitha, K.; Suresh, M.; Chowdhury, L.M.; Ravishankar, C.; Kumar, K.A. Incidence of ciguatoxin fish poisoning in Trivandrum, India. **2017**.
48. Lewis, N.D. Ciguatera - Parameters of a Tropical Health Problem. *Human Ecology* **1984**, *12*, 253-273, doi:Doi 10.1007/Bf01540591.
49. Bagnis, R.; Chanteau, S.; Yasumoto, T. Etiological Agent, Probably Cause of Ciguatera. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D* **1977**, *285*, 105-107.
50. Quod, J.P.; Turquet, J. Ciguatera in Reunion Island (SW Indian Ocean): Epidemiology and clinical patterns. *Toxicon* **1996**, *34*, 779-785, doi:Doi 10.1016/0041-0101(95)00164-6.
51. de Haro, L.; Schmitt, C.; Sinno-Tellier, S.; Paret, N.; Boels, D.; Le Roux, G.; Langrand, J.; Delcourt, N.; Labadie, M.; Simon, N. Ciguatera fish poisoning in France: experience of the French Poison Control Centre Network from 2012 to 2019. *Clinical Toxicology* **2020**, 10.1080/15563650.2020.1788052, 1-4, doi:10.1080/15563650.2020.1788052.
52. Radke, E.; Grattan, L.; Roberts, S.; Abbott, M.; Morris, J.G. Incidence and Awareness of Ciguatera Fish Poisoning in St. Thomas, Us Virgin Islands. *American Journal of Epidemiology* **2011**, *173*, S224-S224.
53. Radke, E.G.; Grattan, L.M.; Cook, R.L.; Smith, T.B.; Anderson, D.M.; Morris, J.G. Ciguatera Incidence in the US Virgin Islands Has Not Increased over a 30-Year Time Period Despite Rising Seawater Temperatures. *The American journal of tropical medicine and hygiene* **2013**.
54. Friedemann, M. Tropical fish poisonings in Germany 2012–2017 - What is ciguatera? *International Journal of Infectious Diseases* **2019**, *79*, 130, doi:https://doi.org/10.1016/j.ijid.2018.11.318.
55. Ting, J.Y.; Brown, A.F. Ciguatera poisoning: a global issue with common management problems. *Eur J Emerg Med* **2001**, *8*, 295-300.
56. Kusche, H.; Hanel, R. Consumers of mislabeled tropical fish exhibit increased risks of ciguatera intoxication: A report on substitution patterns in fish imported at Frankfurt Airport, Germany. *Food Control* **2021**, *121*, 107647, doi:https://doi.org/10.1016/j.foodcont.2020.107647.
57. Schlaich, C.; Hagelstein, J.G.; Burchard, G.D.; Schmiedel, S. Outbreak of ciguatera fish poisoning on a cargo ship in the port of Hamburg. *Journal of travel medicine* **2012**, *19*, 238-242.
58. Friedemann, M. Ciguatera fish poisoning outbreaks from 2012 to 2017 in Germany caused by snappers from India, Indonesia, and Vietnam. *Journal of Consumer Protection and Food Safety* **2019**, *14*, 71-80, doi:10.1007/s00003-018-1191-8.
59. Loeffler, C.R.; Tartaglione, L.; Friedemann, M.; Spielmeyer, A.; Kappenstein, O.; Bodi, D. Ciguatera Mini Review: 21st Century Environmental Challenges and the Interdisciplinary Research Efforts Rising to Meet Them. *International Journal of Environmental Research and Public Health* **2021**, *18*, 3027.
60. Varela Martínez, C.; León Gómez, I.; Martínez Sánchez, E.V.; Carmona Alférez, R.; Nuñez Gallo, D.; Friedemann, M.; Oleastro, M.; Boziaris, I. Incidence and epidemiological characteristics of ciguatera cases in Europe. *EFSA Supporting Publications* **2021**, *18*, 6650E, doi:https://doi.org/10.2903/sp.efsa.2021.EN-6650.
61. Canals, A.; Martínez, C.V.; Diogène, J.; Gago-Martínez, A.; Cebadera-Miranda, L.; de Vasconcelos, F.M.; Gómez, I.L.; Sánchez, E.V.M.; Alférez, R.C.; Núñez, D., et al. Risk characterisation of ciguatera poisoning in Europe. *EFSA Supporting Publications* **2021**, *18*, 6647E, doi:https://doi.org/10.2903/sp.efsa.2021.EN-6647.
62. Moller, B.; Voglhuber-Slavinsky, A.; Dönitz, E.; Rosa, A. 50 trends influencing Europe's food sector by 2035. *Fraunhofer Institute for Systems and Innovation Research ISI, Karlsruhe* **2019**.
63. Saari, U.A.; Herstatt, C.; Tiwari, R.; Dedehayir, O.; Mäkinen, S.J. The vegan trend and the microfoundations of institutional change: A commentary on food producers' sustainable innovation journeys in Europe. *Trends in Food Science & Technology* **2020**, https://doi.org/10.1016/j.tifs.2020.10.003, doi:https://doi.org/10.1016/j.tifs.2020.10.003.
64. Gvion, L. Generation V: Millennial Vegans in Israel. *Journal of Contemporary Ethnography* **2020**, *49*, 564-586, doi:10.1177/0891241620917726.

65. Hopwood, C.J.; Bleidorn, W.; Schwaba, T.; Chen, S. Health, environmental, and animal rights motives for vegetarian eating. *PLOS ONE* **2020**, *15*, e0230609, doi:10.1371/journal.pone.0230609.
66. Radke, E.G.; Reich, A.; Morris, J.G. Epidemiology of Ciguatera in Florida. *The American Journal of Tropical Medicine and Hygiene* **2015**, *93*, 425-432.
67. Administration, F.a.D. *Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins*, Second ed.; US FDA: Silver Spring, MD, USA, 2012, 2012; Vol. 2.
68. Bilbao-Sieyro, A.; Pérez-González Y.; Pavón-Salas N.; De La Cruz-Modino R.; Chinea-Mederos I.; Tabares-Santos J.L.; F., C.-S. Acquired knowledge about ciguatera fish poisoning in the Canary Islands population. *Front. Mar. Sci.* **2019**, *Conference Abstract: XX Iberian Symposium on Marine Biology Studies (SIEBM XX)* doi:10.3389/conf.fmars.2019.08.00034.
69. Wang, Z.; Fuquay, J.M.; Ledreux, A.; Barbieri, M.; Ramsdell, J.S. Sample preparation and liquid chromatography-tandem mass spectrometry for the analysis of selected Pacific ciguatoxins in blood samples. *Journal of Chromatography A* **2020**, 461050.
70. Bottein Dechraoui, M.-Y.; Wang, Z.; Ramsdell, J.S. Optimization of ciguatoxin extraction method from blood for Pacific ciguatoxin (P-CTX-1). *Toxicon* **2007**, *49*, 100-105, doi:http://dx.doi.org/10.1016/j.toxicon.2006.10.002.
71. Chinain, M.; Darius, H.T.; Ung, A.; Cruchet, P.; Wang, Z.; Ponton, D.; Laurent, D.; Pauillac, S. Growth and toxin production in the ciguatera-causing dinoflagellate Gambierdiscus polynesiensis (Dinophyceae) in culture. *Toxicon* **2010**, *56*, 739-750, doi:http://dx.doi.org/10.1016/j.toxicon.2009.06.013.
72. Stephens, T.G.; Ragan, M.A.; Bhattacharya, D.; Chan, C.X. Core genes in diverse dinoflagellate lineages include a wealth of conserved dark genes with unknown functions. *Scientific Reports* **2018**, *8*, 17175, doi:10.1038/s41598-018-35620-z.
73. Holmes, M.J.; Lewis, R.J.; Poli, M.A.; Gillespie, N.C. Strain dependent production of ciguatoxin precursors (gambiertoxins) by Gambierdiscus toxicus (Dinophyceae) in culture. *Toxicon* **1991**, *29*, 761-775, doi:http://dx.doi.org/10.1016/0041-0101(91)90068-3.
74. Liefer, J.D.; Richlen, M.L.; Smith, T.B.; DeBose, J.L.; Xu, Y.; Anderson, D.M.; Robertson, A. Asynchrony of Gambierdiscus spp. Abundance and Toxicity in the U.S. Virgin Islands: Implications for Monitoring and Management of Ciguatera. *Toxins* **2021**, *13*, 413.
75. Parsons, M.L.; Settlemier, C.J.; Bienfang, P.K. A simple model capable of simulating the population dynamics of Gambierdiscus, the benthic dinoflagellate responsible for ciguatera fish poisoning. *Harmful Algae* **2010**, *10*, 71-80, doi:http://dx.doi.org/10.1016/j.hal.2010.07.002.
76. Parsons, M.L.; Preskitt, L.B. A survey of epiphytic dinoflagellates from the coastal waters of the island of Hawai'i. *Harmful Algae* **2007**, *6*, 658-669, doi:http://dx.doi.org/10.1016/j.hal.2007.01.001.
77. Tester, P.A.; Litaker, R.W.; Berdalet, E. Climate change and harmful benthic microalgae. *Harmful Algae* **2020**, *91*, 101655, doi:https://doi.org/10.1016/j.hal.2019.101655.
78. Xu, Y.; He, X.; Lee, W.H.; Chan, L.L.; Lu, D.; Wang, P.; Tao, X.; Li, H.; Yu, K. Ciguatoxin-Producing Dinoflagellate Gambierdiscus in the Beibu Gulf: First Report of Toxic Gambierdiscus in Chinese Waters. *Toxins* **2021**, *13*, 643.
79. Chinain, M.; Gatti, C.M.; Roué, M.; Darius, H.T.; Subba Rao, D. Ciguatera-causing dinoflagellates in the genera Gambierdiscus and Fukuyoa: Distribution, ecophysiology and toxicology. *Dinoflagellates: morphology, life history and ecological significance. New-York: Nova Science Publishers. Forthcoming* **2020**.
80. Tester, P.; Wickliffe, L.; Jossart, J.; Rhodes, L.; Enevoldsen, H.; Adachi, M.; Nishimura, T.; Rodriguez, F.; Chinain, M.; Litaker, W. Global distribution of the genera Gambierdiscus and Fukuyoa. *HARMFUL ALGAE 2018–FROM ECOSYSTEMS TO SOCIO-ECOSYSTEMS* **2018**, 138.
81. Litaker, R.W.; Holland, W.C.; Hardison, D.R.; Pisapia, F.; Hess, P.; Kibler, S.R.; Tester, P.A. Ciguatoxicity of Gambierdiscus and Fukuyoa species from the Caribbean and Gulf of Mexico. *PLOS ONE* **2017**, *12*, e0185776, doi:10.1371/journal.pone.0185776.

82. Caillaud, A.; de la Iglesia, P.; Barber, E.; Eixarch, H.; Mohammad-Noor, N.; Yasumoto, T.; Diogène, J. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to Gambierdiscus pacificus in culture. *Harmful Algae* **2011**, *10*, 433-446, doi:http://dx.doi.org/10.1016/j.hal.2011.02.004.
83. Roué, M.; Darius, H.T.; Viallon, J.; Ung, A.; Gatti, C.; Harwood, D.T.; Chinain, M. Application of solid phase adsorption toxin tracking (SPATT) devices for the field detection of Gambierdiscus toxins. *Harmful Algae* **2018**, *71*, 40-49, doi:https://doi.org/10.1016/j.hal.2017.11.006.
84. Díaz-Asencio, L.; Vandersea, M.; Chomérat, N.; Fraga, S.; Clausing, R.J.; Litaker, R.W.; Chamero-Lago, D.; Gómez-Batista, M.; Moreira-González, A.; Tester, P., et al. Morphology, toxicity and molecular characterization of Gambierdiscus spp. towards risk assessment of ciguatera in south central Cuba. *Harmful Algae* **2019**, *86*, 119-127, doi:https://doi.org/10.1016/j.hal.2019.05.007.
85. Fraga, S.; Rodríguez, F.; Caillaud, A.; Diogène, J.; Raho, N.; Zapata, M. Gambierdiscus excentricus sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). *Harmful Algae* **2011**, *11*, 10-22, doi:http://dx.doi.org/10.1016/j.hal.2011.06.013.
86. Kretzschmar, A.L.; Verma, A.; Kohli, G.; Murray, S. Development of a quantitative PCR assay for the detection and enumeration of a potentially ciguatoxin-producing dinoflagellate, Gambierdiscus lapillus (Gonyaulacales, Dinophyceae). *PLOS ONE* **2019**, *14*, e0224664, doi:10.1371/journal.pone.0224664.
87. Kretzschmar, A.L.; Verma, A.; Harwood, T.; Hoppenrath, M.; Murray, S. Characterization of *Gambierdiscus lapillus* sp. nov. (Gonyaulacales, Dinophyceae): A new toxic dinoflagellate from the Great Barrier Reef (Australia). *J Phycol* **2017**, *53*, 283-297, doi:10.1111/jpy.12496.
88. Smith, K.F.; Biessy, L.; Argyle, P.A.; Trnski, T.; Halafihi, T.; Rhodes, L.L. Molecular Identification of Gambierdiscus and Fukuyoa (Dinophyceae) from Environmental Samples. *Marine Drugs* **2017**, *15*, 243.
89. Lyu, Y.; Richlen, M.L.; Sehein, T.R.; Chinain, M.; Adachi, M.; Nishimura, T.; Xu, Y.; Parsons, M.L.; Smith, T.B.; Zheng, T., et al. LSU rDNA based RFLP assays for the routine identification of Gambierdiscus species. *Harmful Algae* **2017**, *66*, 20-28, doi:https://doi.org/10.1016/j.hal.2017.04.009.
90. Sassenhagen, I.; Erdner, D.L. Microsatellite markers for the dinoflagellate Gambierdiscus caribaeus from high-throughput sequencing data. *Journal of Applied Phycology* **2017**, *29*, 1927-1932, doi:10.1007/s10811-017-1076-8.
91. Nishimura, T.; Hariganeya, N.; Tawong, W.; Sakanari, H.; Yamaguchi, H.; Adachi, M. Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate Gambierdiscus spp. (Gonyaulacales) in coastal areas of Japan. *Harmful Algae* **2016**, *52*, 11-22, doi:http://dx.doi.org/10.1016/j.hal.2015.11.018.
92. Nishimura, T.; Sato, S.; Tawong, W.; Sakanari, H.; Uehara, K.; Shah, M.M.R.; Suda, S.; Yasumoto, T.; Taira, Y.; Yamaguchi, H., et al. Genetic diversity and distribution of the ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in coastal areas of Japan. *PLoS ONE* **2013**, *8*, e60882, doi:10.1371/journal.pone.0060882.
93. Leung, P.T.Y.; Yan, M.; Lam, V.T.T.; Yiu, S.K.F.; Chen, C.-Y.; Murray, J.S.; Harwood, D.T.; Rhodes, L.L.; Lam, P.K.S.; Wai, T.-C. Phylogeny, morphology and toxicity of benthic dinoflagellates of the genus Fukuyoa (Goniodomataceae, Dinophyceae) from a subtropical reef ecosystem in the South China Sea. *Harmful Algae* **2018**, *74*, 78-97, doi:https://doi.org/10.1016/j.hal.2018.03.003.
94. Gómez, F.; Qiu, D.; Lopes, R.M.; Lin, S. *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *PLOS ONE* **2015**, *10*, e0119676, doi:10.1371/journal.pone.0119676.
95. Vandersea, M.W.; Kibler, S.R.; Holland, W.C.; Tester, P.A.; Schultz, T.F.; Faust, M.A.; Holmes, M.J.; Chinain, M.; Wayne Litaker, R. Development of semi-quantitative pcr assays for the

detection and enumeration of gambierdiscus species (gonyaulacales, dinophyceae) 1. *Journal of phycology* **2012**, *48*, 902-915.

96. Fraga, S.; Rodríguez, F. Genus Gambierdiscus in the Canary Islands (NE Atlantic Ocean) with Description of Gambierdiscus silvae sp. nov., a New Potentially Toxic Epiphytic Benthic Dinoflagellate. *Protist* **2014**, *165*, 839-853, doi:https://doi.org/10.1016/j.protis.2014.09.003.
97. Richlen, M.L.; Barber, P.H. A technique for the rapid extraction of microalgal DNA from single live and preserved cells. *Molecular Ecology Notes* **2005**, *5*, 688-691, doi:10.1111/j.1471-8286.2005.01032.x.
98. Chinain, M.; Faust, M.A.; Pauillac, S. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. *Journal of Phycology* **1999**, *35*, 1282-1296, doi:10.1046/j.1529-8817.1999.3561282.x.
99. Reverté, L.; Toldrà, A.; Andree, K.B.; Fraga, S.; de Falco, G.; Campàs, M.; Diogène, J. Assessment of cytotoxicity in ten strains of Gambierdiscus australes from Macaronesian Islands by neuro-2a cell-based assays. *Journal of Applied Phycology* **2018**, *30*, 2447-2461, doi:10.1007/s10811-018-1456-8.
100. Roeder, K.; Erler, K.; Kibler, S.; Tester, P.; Ho, V.T.; Lam, N.N.; Gerdts, G.; Luckas, B. Characteristic profiles of Ciguatera toxins in different strains of Gambierdiscus spp. *Toxicon* **2010**, *56*, 731-738, doi:DOI 10.1016/j.toxicon.2009.07.039.
101. Morton, S.; Bomber, J.; Tindall, D.; Aikman, K. Response of Gambierdiscus toxicus to light: Cell physiology and toxicity. *DEV. MAR. BIOL. 1993.* **1993**.
102. Pisapia, F.; Holland, W.C.; Hardison, D.R.; Litaker, R.W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Séchet, V.; Amzil, Z., et al. Toxicity screening of 13 Gambierdiscus strains using neuro-2a and erythrocyte lysis bioassays. *Harmful Algae* **2017**, *63*, 173-183, doi:http://dx.doi.org/10.1016/j.hal.2017.02.005.
103. Wu, Z.; Luo, H.; Yu, L.; Lee, W.H.; Li, L.; Mak, Y.L.; Lin, S.; Lam, P.K. Characterizing ciguatoxin (CTX)-and Non-CTX-producing strains of Gambierdiscus balechii using comparative transcriptomics. *Science of the Total Environment* **2020**, *717*, 137184.
104. Rossignoli, A.E.; Tudó, A.; Bravo, I.; Díaz, P.A.; Diogène, J.; Riobó, P. Toxicity Characterisation of Gambierdiscus Species from the Canary Islands. *Toxins* **2020**, *12*, 134.
105. Tudó, À.; Toldrà, A.; Rey, M.; Todolí, I.; Andree, K.B.; Fernández-Tejedor, M.; Campàs, M.; Sureda, F.X.; Diogène, J. Gambierdiscus and fukuyoa as potential indicators of ciguatera risk in the balearic islands. *Harmful Algae* **2020**, *99*, 101913, doi:https://doi.org/10.1016/j.hal.2020.101913.
106. Rhodes, L.; Harwood, T.; Smith, K.; Argyle, P.; Munday, R. Production of ciguatoxin and maitotoxin by strains of Gambierdiscus australes, G. pacificus and G. polynesiensis (Dinophyceae) isolated from Rarotonga, Cook Islands. *Harmful Algae* **2014**, *39*, 185-190, doi:https://doi.org/10.1016/j.hal.2014.07.018.
107. Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Tudó, A.; Rambla-Alegre, M.; Aligizaki, K.; Diogène, J.; Gago-Martinez, A.; Hess, P. Use of Mass Spectrometry to Determine the Diversity of Toxins Produced by Gambierdiscus and Fukuyoa Species from Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic). *Toxins* **2020**, *12*, 305.
108. Rambo, I.M.; Dombrowski, N.; Constant, L.; Erdner, D.; Baker, B.J. Metabolic relationships of uncultured bacteria associated with the microalgae *Gambierdiscus*. *Environmental Microbiology* **2020**, *22*, 1764-1783.
109. Sakami, T.; Nakahara, H.; Chinain, M.; Ishida, Y. Effects of epiphytic bacteria on the growth of the toxic dinoflagellate Gambierdiscus toxicus (Dinophyceae). *Journal of Experimental Marine Biology and Ecology* **1999**, *233*, 231-246, doi:http://dx.doi.org/10.1016/S0022-0981(98)00130-0.
110. Wang, B.; Yao, M.; Zhou, J.; Tan, S.; Jin, H.; Zhang, F.; Mak, Y.L.; Wu, J.; Lai Chan, L.; Cai, Z. Growth and toxin production of *Gambierdiscus* spp. can be regulated by quorum-sensing bacteria. *Toxins* **2018**, *10*, 257.

111. Satake, M.; Murata, M.; Yasumoto, T. Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate Gambierdiscus toxicus. *Journal of the American Chemical Society* **1993**, *115*, 361-362, doi:10.1021/ja00054a061.
112. Watanabe, R.; Uchida, H.; Suzuki, T.; Matsushima, R.; Nagae, M.; Toyohara, Y.; Satake, M.; Oshima, Y.; Inoue, A.; Yasumoto, T. Gambieroxide, a novel epoxy polyether compound from the dinoflagellate Gambierdiscus toxicus GTP2 strain. *Tetrahedron* **2013**, *69*, 10299-10303, doi:https://doi.org/10.1016/j.tet.2013.10.022.
113. Morohashi, A.; Satake, M.; Nagai, H.; Oshima, Y.; Yasumoto, T. The Absolute Configuration of Gambieric Acids A–D, Potent Antifungal Polyethers, Isolated from the Marine Dinoflagellate Gambierdiscus toxicus. *Tetrahedron* **2000**, *56*, 8995-9001, doi:https://doi.org/10.1016/S0040-4020(00)00753-5.
114. Rodríguez, I.; Genta-Jouve, G.; Alfonso, C.; Calabro, K.; Alonso, E.; Sánchez, J.A.; Alfonso, A.; Thomas, O.P.; Botana, L.M. Gambierone, a Ladder-Shaped Polyether from the Dinoflagellate Gambierdiscus belizeanus. *Organic Letters* **2015**, *17*, 2392-2395, doi:10.1021/acs.orglett.5b00902.
115. Murray, J.S.; Nishimura, T.; Finch, S.C.; Rhodes, L.L.; Puddick, J.; Harwood, D.T.; Larsson, M.E.; Doblin, M.A.; Leung, P.; Yan, M., et al. The role of 44-methylgambierone in ciguatera fish poisoning: Acute toxicity, production by marine microalgae and its potential as a biomarker for Gambierdiscus spp. *Harmful Algae* **2020**, *97*, 101853, doi:https://doi.org/10.1016/j.hal.2020.101853.
116. Holmes, M.J.; Lewis, R.J. Purification and characterisation of large and small maitotoxins from cultured Gambierdiscus toxicus. *Natural Toxins* **1994**, *2*, 64-72.
117. Boente-Juncal, A.; Álvarez, M.; Antelo, Á.; Rodríguez, I.; Calabro, K.; Vale, C.; Thomas, O.P.; Botana, L.M. Structure Elucidation and Biological Evaluation of Maitotoxin-3, a Homologue of Gambierone, from Gambierdiscus belizeanus. *Toxins* **2019**, *11*, 79.
118. Murata, M.; Naoki, H.; Iwashita, T.; Matsunaga, S.; Sasaki, M.; Yokoyama, A.; Yasumoto, T. Structure of maitotoxin. *Journal of the American Chemical Society* **1993**, *115*, 2060-2062, doi:10.1021/ja00058a075.
119. Pisapia, F.; Sibat, M.; Herrenknecht, C.; Lhaute, K.; Gaiani, G.; Ferron, P.-J.; Fessard, V.; Fraga, S.; Nascimento, S.M.; Litaker, R.W., et al. Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus excentricus*. *Marine Drugs* **2017**, *15*, 220.
120. Murata, M.; Naoki, H.; Matsunaga, S.; Satake, M.; Yasumoto, T. Structure and Partial Stereochemical Assignments for Maitotoxin, the Most Toxic and Largest Natural Non-Biopolymer. *Journal of the American Chemical Society* **1994**, *116*, 7098-7107, doi:10.1021/ja00095a013.
121. Yokoyama, A.; Murata, M.; Oshima, Y.; Iwashita, T.; Yasumoto, T. Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. *J Biochem* **1988**, *104*, 184-187, doi:10.1093/oxfordjournals.jbchem.a122438.
122. Holmes, M.J.; Lewis, R.J.; Gillespie, N.C. Toxicity of Australian and French Polynesian strains of Gambierdiscus Toxicus (Dinophyceae) grown in culture: Characterization of a new type of maitotoxin. *Toxicon* **1990**, *28*, 1159-1172, doi:http://dx.doi.org/10.1016/0041-0101(90)90116-O.
123. Holmes, M.; Lewis, R. Purification and characterisation of large and small maitotoxins from cultured Gambierdiscus toxicus. *Nat Toxins* **1994**, *2*, 64 - 72.
124. Ikehara, T.; Kuniyoshi, K.; Oshiro, N.; Yasumoto, T. Biooxidation of Ciguatoxins Leads to Species-Specific Toxin Profiles. *Toxins* **2017**, *9*, 205.
125. Fusetani, N. Marine toxins: An overview. In *Marine toxins as research tools*, Springer: Berlin, Heidelberg, 2009; pp. 1-44.
126. Kohli, G.; Papiol, G.; Rhodes, L.; Harwood, D.; Selwood, A.; Jerrett, A. A feeding study to probe the uptake of Maitotoxin by snapper (Pagrus auratus). *Harmful Algae* **2014**, *37*, 125 - 132.

127. Gallimore, A.R.; Spencer, J.B. Stereochemical uniformity in marine polyether ladders—implications for the biosynthesis and structure of maitotoxin. *Angewandte Chemie International Edition* **2006**, *45*, 4406-4413.
128. Ridley, C.P.; Lee, H.Y.; Khosla, C. Evolution of polyketide synthases in bacteria. *Proceedings of the National Academy of Sciences* **2008**, *105*, 4595-4600, doi:10.1073/pnas.0710107105.
129. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Yasumoto, T. Structures of ciguatoxin and its congener. *Journal of the American Chemical Society* **1989**, *111*, 8929-8931, doi:10.1021/ja00206a032.
130. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. Structures and configurations of ciguatoxin from the moray eel Gymnothorax javanicus and its likely precursor from the dinoflagellate Gambierdiscus toxicus. *Journal of the American Chemical Society* **1990**, *112*, 4380-4386, doi:10.1021/ja00167a040.
131. Vernoux, J.-P.; Lewis, R.J. Isolation and characterisation of Caribbean ciguatoxins from the horse-eye jack (Caranx latus). *Toxicon* **1997**, *35*, 889-900, doi:http://dx.doi.org/10.1016/S0041-0101(96)00191-2.
132. Lewis, R.J.; Vernoux, J.-P.; Brereton, I.M. Structure of Caribbean Ciguatoxin Isolated from Caranx latus. *Journal of the American Chemical Society* **1998**, *120*, 5914-5920, doi:10.1021/ja980389e.
133. Abraham, A.; Jester, E.L.E.; Granade, H.R.; Plakas, S.M.; Dickey, R.W. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chem* **2012**, *131*, 192-198, doi:https://doi.org/10.1016/j.foodchem.2011.08.059.
134. Hamilton, B.; Hurbungs, M.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Isolation and characterisation of Indian Ocean ciguatoxin. *Toxicon* **2002**, *40*, 685-693.
135. Yasumoto, T.; Murata, M. Marine toxins. *Chemical Reviews* **1993**, *93*, 1897-1909, doi:10.1021/cr00021a011.
136. Dickey, R.W. *Ciguatera toxins: chemistry, toxicology, and detection*, Second ed.; New York., 2008; Vol. 173, pp. 22.
137. Xu, Y.; Richlen, M.L.; Liefer, J.D.; Robertson, A.; Kulis, D.; Smith, T.B.; Parsons, M.L.; Anderson, D.M. Influence of Environmental Variables on Gambierdiscus spp.(Dinophyceae) Growth and Distribution. *PloS one* **2016**, *11*, e0153197.
138. Kibler, S.R.; Davenport, E.D.; Tester, P.A.; Hardison, D.R.; Holland, W.C.; Litaker, R.W. Gambierdiscus and Fukuyoa species in the greater Caribbean: Regional growth projections for ciguatera-associated dinoflagellates. *Ecological Modelling* **2017**, *360*, 204-218, doi:http://dx.doi.org/10.1016/j.ecolmodel.2017.07.007.
139. Loeffler, C.R.; Richlen, M.L.; Brandt, M.E.; Smith, T.B. Effects of grazing, nutrients, and depth on the ciguatera-causing dinoflagellate Gambierdiscus in the US Virgin Islands. *Marine Ecology Progress Series* **2015**, *531*, 91-104, doi:10.3354/meps11310.
140. Yasumoto, T.; Inoue, A.; Ochi, T.; Fujimoto, K.; Oshima, Y.; Fukuyo, Y.; Adachi, R.; Bagnis, R. Environmental-studies on a toxic dinoflagellate responsible for ciguatera. *Bulletin of the Japanese Society of Scientific Fisheries* **1980**, *46*, 1397-1404.
141. Chateau-Degat, M.L.; Chinain, M.; Cerf, N.; Gingras, S.; Hubert, B.; Dewailly, E. Seawater temperature, Gambierdiscus spp. variability and incidence of ciguatera poisoning in French Polynesia. *Harmful Algae* **2005**, *4*, 1053-1062, doi:DOI 10.1016/j.hal.2005.03.003.
142. Richlen, M.L.; Lobel, P.S. Effects of depth, habitat, and water motion on the abundance and distribution of ciguatera dinoflagellates at Johnston Atoll, Pacific Ocean. *Marine ecology progress series* **2011**, *421*, 51-66.
143. McCubbin, S.G.; Pearce, T.; Ford, J.D.; Smit, B. Social-ecological change and implications for food security in Funafuti, Tuvalu. *Ecology and Society* **2017**, *22*, doi:10.5751/ES-09129-220153.
144. Wedding, L.M.; Lecky, J.; Gove, J.M.; Walecka, H.R.; Donovan, M.K.; Williams, G.J.; Jouffray, J.-B.; Crowder, L.B.; Erickson, A.; Falinski, K. Advancing the integration of spatial data to map human and natural drivers on coral reefs. *PLoS One* **2018**, *13*, e0189792.

145. Guiry, M.D.; Guiry, G. AlgaeBase. Availabe online: http://www.algaebase.org/search/genus/detail/?genus_id=45535 (accessed on 06 August 2020).
146. Tudó, À.; Gaiani, G.; Varela, M.R.; Tsumuraya, T.; Andree, K.B.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. Further advance of Gambierdiscus Species in the Canary Islands, with the First Report of Gambierdiscus belizeanus. *Toxins* **2020**, *12*, 692.
147. Larsson, M.; Laczka, O.; Harwood, D.; Lewis, R.; Himaya, S.; Murray, S.; Doblin, M. Toxicology of Gambierdiscus spp. (Dinophyceae) from Tropical and Temperate Australian Waters. *Marine Drugs* **2018**, *16*, 7.
148. Munday, R.; Murray, S.; Rhodes, L.L.; Larsson, M.E.; Harwood, D.T. Ciguatoxins and Maitotoxins in Extracts of Sixteen Gambierdiscus Isolates and One Fukuyoa Isolate from the South Pacific and Their Toxicity to Mice by Intraperitoneal and Oral Administration. *Marine Drugs* **2017**, *15*, 208, doi:10.3390/md15070208.
149. Chinain, M.; Gatti, C.M.; Roué, M.; Darius, H.T. Ciguatera-causing dinoflagellates in the genera Gambierdiscus and Fukuyoa: Distribution, ecophysiology and toxicology. *Dinoflagellates: morphology, life history and ecological significance. New-York: Nova Science Publishers. Forthcoming* **2020**.
150. Longo, S.; Sibat, M.; Viallon, J.; Darius, H.T.; Hess, P.; Chinain, M. Intraspecific Variability in the Toxin Production and Toxin Profiles of In Vitro Cultures of Gambierdiscus polynesiensis (Dinophyceae) from French Polynesia. *Toxins* **2019**, *11*, 735.
151. Laza-Martinez, A.; David, H.; Riobo, P.; Miguel, I.; Orive, E. Characterization of a Strain of Fukuyoa paulensis (Dinophyceae) from the Western Mediterranean Sea. *The Journal of eukaryotic microbiology* **2016**, *63*, 481-497, doi:10.1111/jeu.12292.
152. Satake, M.; Murata, M.; Yasumoto, T. The structure of CTX3C, a ciguatoxin congener isolated from cultured Gambierdiscus toxicus. *Tetrahedron Letters* **1993**, *34*, 1975-1978, doi:http://dx.doi.org/10.1016/S0040-4039(00)91978-6.
153. Satake, M.; Ishibashi, Y.; Legrand, A.-M.; Yasumoto, T. Isolation and Structure of Ciguatoxin-4A, a New Ciguatoxin Precursor, from Cultures of Dinoflagellate Gambierdiscus toxicus and Parrotfish Scarus gibbus. *Bioscience, Biotechnology, and Biochemistry* **1996**, *60*, 2103-2105, doi:10.1271/bbb.60.2103.
154. Sibat, M.; Herrenknecht, C.; Darius, H.T.; Roue, M.; Chinain, M.; Hess, P. Detection of pacific ciguatoxins using liquid chromatography coupled to either low or high resolution mass spectrometry (LC-MS/MS). *Journal of chromatography. A* **2018**, 10.1016/j.chroma.2018.08.008, doi:10.1016/j.chroma.2018.08.008.
155. Rodríguez, F.; Fraga, S.; Ramilo, I.; Rial, P.; Figueroa, R.I.; Riobó, P.; Bravo, I. Canary Islands (NE Atlantic) as a biodiversity 'hotspot' of Gambierdiscus: Implications for future trends of ciguatera in the area. *Harmful Algae* **2017**, *67*, 131-143, doi:https://doi.org/10.1016/j.hal.2017.06.009.
156. Spielmeyer, A.; Loeffler, C.R.; Bodi, D. Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. *Toxins* **2021**, *13*, 630.
157. Yasumoto, T.; Igarashi, T.; Legrand, A.-M.; Cruchet, P.; Chinain, M.; Fujita, T.; Naoki, H. Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. *Journal of the American Chemical Society* **2000**, *122*, 4988-4989.
158. Kryuchkov, F.; Robertson, A.; Miles, C.O.; Mudge, E.M.; Uhlig, S. LC–HRMS and Chemical Derivatization Strategies for the Structure Elucidation of Caribbean Ciguatoxins: Identification of C-CTX-3 and -4. *Marine Drugs* **2020**, *18*, 182.
159. Pottier, I.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in Guadeloupe; French West Indies *Food Additives & Contaminants* **2002**, *19*, 1034-1042, doi:https://doi.org/10.1080/02652030210155378.
160. Banner, A.H.; Scheuer, P.J.; Sasaki, S.; Helfrich, P.; Alender, C.B. Observations on Ciguatera-Type Toxin in Fish. *Annals of the New York Academy of Sciences* **1960**, *90*, 770-787, doi:DOI 10.1111/j.1749-6632.1960.tb26421.x.

161. Yogi, K.; Oshiro, N.; Inafuku, Y.; Hirama, M.; Yasumoto, T. Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from the Pacific. *Analytical Chemistry* **2011**, *83*, 8886-8891, doi:10.1021/ac200799j.
162. FAO. Alerts and rejections - Toxic causes. Availabe online: https://www.fao.org/in-action/globefish/border-rejections/border-rejections-eu/2016-alerts-rejections/toxic-causes/en/ (accessed on October).
163. Bagnis, R.; Berglund, F.; Elias, P.e.; Van Esch, G.; Halstead, B.; Kojima, K. Problems of toxicants in marine food products: 1. Marine biotoxins. *Bulletin of the World Health Organization* **1970**, *42*, 69.
164. Mustapa, N.I.; Yong, H.L.; Lee, L.K.; Lim, Z.F.; Lim, H.C.; Teng, S.T.; Luo, Z.; Gu, H.; Leaw, C.P.; Lim, P.T. Growth and epiphytic behavior of three Gambierdiscus species (Dinophyceae) associated with various macroalgal substrates. *Harmful Algae* **2019**, *89*, 101671, doi:https://doi.org/10.1016/j.hal.2019.101671.
165. Rains, L.K.; Parsons, M.L. Gambierdiscus species exhibit different epiphytic behaviors toward a variety of macroalgal hosts. *Harmful Algae* **2015**, *49*, 29-39, doi:http://dx.doi.org/10.1016/j.hal.2015.08.005.
166. Cruz-Rivera, E.; Villareal, T.A. Macroalgal palatability and the flux of ciguatera toxins through marine food webs. *Harmful Algae* **2006**, *5*, 497-525, doi:DOI 10.1016/j.hal.2005.09.003.
167. Adam, T.C.; Burkepile, D.E.; Ruttenberg, B.I.; Paddack, M.J. Managing herbivores for their impacts on Caribbean coral reef ecosystems: A summary report for managers and practitioners. **2015**.
168. Lewis, R.J.; Holmes, M.J. Origin and Transfer of Toxins Involved in Ciguatera. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **1993**, *106*, 615-628, doi:Doi 10.1016/0742-8413(93)90217-9.
169. Yogi, K.; Sakugawa, S.; Oshiro, N.; Ikehara, T.; Sugiyama, K.; Yasumoto, T. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. *Journal of AOAC INTERNATIONAL* **2014**, *97*, 398-402.
170. Clifton, K.E. Asynchronous food availability on neighboring Caribbean coral reefs determines seasonal patterns of growth and reproduction for the herbivorous parrotfish Scarus iserti. *Marine Ecology Progress Series* **1995**, *116*, 39-46.
171. Holland, M.M.; Smith, J.A.; Everett, J.D.; Vergés, A.; Suthers, I.M. Latitudinal patterns in trophic structure of temperate reef-associated fishes and predicted consequences of climate change. *Fish and Fisheries* **2020**, *21*, 1092-1108, doi:https://doi.org/10.1111/faf.12488.
172. Szedlmayer, S.T.; Lee, J.D. Diet shifts of juvenile red snapper (Lutjanus campechanus) with changes in habitat and fish size. *Fishery Bulletin* **2004**, *102*, 366-375.
173. Munsterman, K.S.; Allgeier, J.E.; Peters, J.R.; Burkepile, D.E. A View From Both Ends: Shifts in Herbivore Assemblages Impact Top-Down and Bottom-Up Processes on Coral Reefs. *Ecosystems* **2021**, 10.1007/s10021-021-00612-0, doi:10.1007/s10021-021-00612-0.
174. Collins, A.B.; Motta, P.J. A kinematic investigation into the feeding behavior of the Goliath grouper Epinephelus itajara. *Environ Biol Fish* **2017**, *100*, 309-323, doi:10.1007/s10641-016-0543-4.
175. Zamora-Terol, S.; Novotny, A.; Winder, M. Reconstructing marine plankton food web interactions using DNA metabarcoding. *Molecular ecology* **2020**, *29*, 3380-3395, doi:https://doi.org/10.1111/mec.15555.
176. Darius, H.; Roué, M.; Sibat, M.; Viallon, J.; Gatti, C.; Vandersea, M.; Tester, P.; Litaker, R.; Amzil, Z.; Hess, P., et al. Tectus niloticus (Tegulidae, Gastropod) as a Novel Vector of Ciguatera Poisoning: Detection of Pacific Ciguatoxins in Toxic Samples from Nuku Hiva Island (French Polynesia). *Toxins* **2018**, *10*, 2.
177. Randall, J.E. *Food habits of reef fishes of the West Indies*; Institute of Marine Sciences, University of Miami: 1967.
178. Pittman, S.J.; Monaco, M.E.; Friedlander, A.M.; Legare, B.; Nemeth, R.S.; Kendall, M.S.; Poti, M.; Clark, R.D.; Wedding, L.M.; Caldow, C. Fish with Chips: Tracking Reef Fish Movements to

Evaluate Size and Connectivity of Caribbean Marine Protected Areas. *PLoS ONE* **2014**, *9*, e96028, doi:10.1371/journal.pone.0096028.
179. Nemeth, R.S. Population characteristics of a recovering US Virgin Islands red hind spawning aggregation following protection. *Mar Ecol Prog Ser* **2005**, *286*, 81-97.
180. Friedlander, A.M.; Monaco, M.E.; Clark, R.; Pittman, S.J.; Beets, J.; Boulon, R.; Callender, R.; Christensen, J.; Hile, S.D.; Kendall, M.S. Fish movement patterns in Virgin Islands national park, Virgin Islands coral reef national monument and adjacent waters. *Silver Spring: NOAA Technical Memorandum NOS NCCOS* **2013**, 172.
181. Nemeth, R.S. Ecosystem Aspects of Species That Aggregate to Spawn. In *Reef Fish Spawning Aggregations: Biology, Research and Management*, Sadovy de Mitcheson, Y., Colin, P.L., Eds. Springer Netherlands: Dordrecht, 2012; 10.1007/978-94-007-1980-4_2pp. 21-55.
182. Taylor J.C.; Karnauskas M.; Cherubin L.; Schärer-Umpierre M.; Michaels W.L.; Caillouet R. ; Campbell M.; Demer D.; Erisman B.; Fulton S., et al. Emerging Science and Technology to Improve Monitoring and Assessments of Fish Spawning Aggregations. Report from the 2019 Gulf and Caribbean Fisheries Institute Workshop *Report from the 2019 Gulf and Caribbean Fisheries Institute Workshop* **2020**, *NMFS-F/SPO-207*, 74.
183. Chinain, M.; Gatti, C.M.i.; Ung, A.; Cruchet, P.; Revel, T.; Viallon, J.; Sibat, M.; Varney, P.; Laurent, V.; Hess, P., et al. Evidence for the Range Expansion of Ciguatera in French Polynesia: A Revisit of the 2009 Mass-Poisoning Outbreak in Rapa Island (Australes Archipelago). *Toxins* **2020**, *12*, 759.
184. Oshiro, N.; Tomikawa, T.; Kuniyoshi, K.; Ishikawa, A.; Toyofuku, H.; Kojima, T.; Asakura, H. LC–MS/MS Analysis of Ciguatoxins Revealing the Regional and Species Distinction of Fish in the Tropical Western Pacific. *Journal of Marine Science and Engineering* **2021**, *9*, 299.
185. Loeffler, C.R.; Robertson, A.; Flores Quintana, H.A.; Silander, M.C.; Smith, T.B.; Olsen, D. Ciguatoxin prevalence in 4 commercial fish species along an oceanic exposure gradient in the US Virgin Islands. *Environmental toxicology and chemistry* **2018**, *37*, 1852-1863, doi:10.1002/etc.4137.
186. Olsen, D.A.; Nellis, D.W.; Wood, R.S. Ciguatera in the Eastern Caribbean. *Marine Fisheries Review* **1984**, *46*, 13-18.
187. Gwinn, J.K.; Uhlig, S.; Ivanova, L.; Fæste, C.K.; Kryuchkov, F.; Robertson, A. In Vitro Glucuronidation of Caribbean Ciguatoxins in Fish: First Report of Conjugative Ciguatoxin Metabolites. *Chemical Research in Toxicology* **2021**, *34*, 1910-1925, doi:10.1021/acs.chemrestox.1c00181.
188. Li, J.; Mak, Y.L.; Chang, Y.-H.; Xiao, C.; Chen, Y.-M.; Shen, J.; Wang, Q.; Ruan, Y.; Lam, P.K.S. Uptake and Depuration Kinetics of Pacific Ciguatoxins in Orange-Spotted Grouper (*Epinephelus coioides*). *Environmental Science & Technology* **2020**, 10.1021/acs.est.9b07888, doi:10.1021/acs.est.9b07888.
189. Clausing, R.J.; Losen, B.; Oberhaensli, F.R.; Darius, H.T.; Sibat, M.; Hess, P.; Swarzenski, P.W.; Chinain, M.; Dechraoui Bottein, M.-Y. Experimental evidence of dietary ciguatoxin accumulation in an herbivorous coral reef fish. *Aquatic Toxicology* **2018**, *200*, 257-265, doi:https://doi.org/10.1016/j.aquatox.2018.05.007.
190. Roué, M.; Darius, H.; Ung, A.; Viallon, J.; Sibat, M.; Hess, P.; Amzil, Z.; Chinain, M. Tissue Distribution and Elimination of Ciguatoxins in Tridacna maxima (Tridacnidae, Bivalvia) Fed Gambierdiscus polynesiensis. *Toxins* **2018**, *10*, 189.
191. Ledreux, A.; Brand, H.; Chinain, M.; Bottein, M.-Y.D.; Ramsdell, J.S. Dynamics of ciguatoxins from Gambierdiscus polynesiensis in the benthic herbivore Mugil cephalus: Trophic transfer implications. *Harmful Algae* **2014**, *39*, 165-174, doi:https://doi.org/10.1016/j.hal.2014.07.009.
192. Froese, R.; Pauly, D. FishBase. Availabe online: www.fishbase.org (accessed on 11.20.2020).
193. Bernal, M.A.; Schunter, C.; Lehmann, R.; Lightfoot, D.J.; Allan, B.J.M.; Veilleux, H.D.; Rummer, J.L.; Munday, P.L.; Ravasi, T. Species-specific molecular responses of wild coral reef fishes during a marine heatwave. *Science Advances* **2020**, *6*, eaay3423, doi:10.1126/sciadv.aay3423.

194. Sunday, J.M.; Pecl, G.T.; Frusher, S.; Hobday, A.J.; Hill, N.; Holbrook, N.J.; Edgar, G.J.; Stuart-Smith, R.; Barrett, N.; Wernberg, T. Species traits and climate velocity explain geographic range shifts in an ocean-warming hotspot. *Ecology letters* **2015**, *18*, 944-953.
195. Petitgas, P.; Rijnsdorp, A.D.; Dickey-Collas, M.; Engelhard, G.H.; Peck, M.A.; Pinnegar, J.K.; Drinkwater, K.; Huret, M.; Nash, R.D.M. Impacts of climate change on the complex life cycles of fish. *Fisheries Oceanography* **2013**, *22*, 121-139, doi:https://doi.org/10.1111/fog.12010.
196. Oliver, E.C.; Donat, M.G.; Burrows, M.T.; Moore, P.J.; Smale, D.A.; Alexander, L.V.; Benthuysen, J.A.; Feng, M.; Gupta, A.S.; Hobday, A.J. Longer and more frequent marine heatwaves over the past century. *Nature communications* **2018**, *9*, 1-12.
197. Holt, R.E.; Jørgensen, C. Climate change in fish: effects of respiratory constraints on optimal life history and behaviour. *Biology letters* **2015**, *11*, 20141032-20141032, doi:10.1098/rsbl.2014.1032.
198. Davidson, S.C.; Bohrer, G.; Gurarie, E.; LaPoint, S.; Mahoney, P.J.; Boelman, N.T.; Eitel, J.U.H.; Prugh, L.R.; Vierling, L.A.; Jennewein, J., et al. Ecological insights from three decades of animal movement tracking across a changing Arctic. *Science* **2020**, *370*, 712-715, doi:10.1126/science.abb7080.
199. Pecl, G.T.; Araújo, M.B.; Bell, J.D.; Blanchard, J.; Bonebrake, T.C.; Chen, I.-C.; Clark, T.D.; Colwell, R.K.; Danielsen, F.; Evengård, B., et al. Biodiversity redistribution under climate change: Impacts on ecosystems and human well-being. *Science* **2017**, *355*, eaai9214, doi:10.1126/science.aai9214.
200. O'Dor, R.K.; Stokesbury, M.J.W. The Ocean Tracking Network – Adding Marine Animal Movements to the Global Ocean Observing System. In *Tagging and Tracking of Marine Animals with Electronic Devices*, Nielsen, J.L., Arrizabalaga, H., Fragoso, N., Hobday, A., Lutcavage, M., Sibert, J., Eds. Springer Netherlands: Dordrecht, 2009; 10.1007/978-1-4020-9640-2_6pp. 91-100.
201. Alva-Basurto, J.C.; Arias-González, J.E. Modelling the effects of climate change on a Caribbean coral reef food web. *Ecological Modelling* **2014**, *289*, 1-14, doi:https://doi.org/10.1016/j.ecolmodel.2014.06.014.
202. Nilsson, G.E.; Östlund-Nilsson, S.; Munday, P.L. Effects of elevated temperature on coral reef fishes: loss of hypoxia tolerance and inability to acclimate. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **2010**, *156*, 389-393.
203. Rummer, J.L.; Couturier, C.S.; Stecyk, J.A.W.; Gardiner, N.M.; Kinch, J.P.; Nilsson, G.E.; Munday, P.L. Life on the edge: thermal optima for aerobic scope of equatorial reef fishes are close to current day temperatures. *Global Change Biology* **2013**, 10.1111/gcb.12455, n/a-n/a, doi:10.1111/gcb.12455.
204. Sheridan, J.A.; Bickford, D. Shrinking body size as an ecological response to climate change. *Nature climate change* **2011**, *1*, 401-406.
205. Cheung, W.W.L.; Sarmiento, J.L.; Dunne, J.; Frolicher, T.L.; Lam, V.W.Y.; Deng Palomares, M.L.; Watson, R.; Pauly, D. Shrinking of fishes exacerbates impacts of global ocean changes on marine ecosystems. *Nature Clim. Change* **2013**, *3*, 254-258, doi:http://www.nature.com/nclimate/journal/v3/n3/abs/nclimate1691.html#supplementary-information.
206. Doney, S.C.; Ruckelshaus, M.; Duffy, J.E.; Barry, J.P.; Chan, F.; English, C.A.; Galindo, H.M.; Grebmeier, J.M.; Hollowed, A.B.; Knowlton, N., et al. Climate Change Impacts on Marine Ecosystems. *Annual Review of Marine Science* **2012**, *4*, 11-37, doi:10.1146/annurev-marine-041911-111611.
207. Carpenter, R.C. Partitioning herbivory and its effects on coral reef algal communities. *Ecological Monographs* **1986**, 345-363.
208. Litaker, R.W.; Vandersea, M.W.; Faust, M.A.; Kibler, S.R.; Nau, A.W.; Holland, W.C.; Chinain, M.; Holmes, M.J.; Tester, P.A. Global distribution of ciguatera causing dinoflagellates in the genus Gambierdiscus. *Toxicon* **2010**, *56*, 711-730, doi:http://dx.doi.org/10.1016/j.toxicon.2010.05.017.

209. Hastings, R.A.; Rutterford, L.A.; Freer, J.J.; Collins, R.A.; Simpson, S.D.; Genner, M.J. Climate change drives poleward increases and equatorward declines in marine species. *Current Biology* **2020**.
210. Nicholls, R.J.; Cazenave, A. Sea-Level Rise and Its Impact on Coastal Zones. *Science* **2010**, *328*, 1517-1520, doi:10.1126/science.1185782.
211. Church, J.A.; White, N.J. Sea-Level Rise from the Late 19th to the Early 21st Century. *Surveys in Geophysics* **2011**, *32*, 585-602, doi:10.1007/s10712-011-9119-1.
212. Kibler, S.R.; Litaker, R.W.; Holland, W.C.; Vandersea, M.W.; Tester, P.A. Growth of eight Gambierdiscus (Dinophyceae) species: Effects of temperature, salinity and irradiance. *Harmful Algae* **2012**, *19*, 1-14, doi:10.1016/j.hal.2012.04.007.
213. Tester, P.A.; Feldman, R.L.; Nau, A.W.; Kibler, S.R.; Wayne Litaker, R. Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. *Toxicon* **2010**, *56*, 698-710, doi:http://dx.doi.org/10.1016/j.toxicon.2010.02.026.
214. Hales, S.; Weinstein, P.; Woodward, A. Ciguatera (Fish Poisoning), El Niño, and Pacific Sea Surface Temperatures. *Ecosystem Health* **1999**, *5*, 20-25, doi:10.1046/j.1526-0992.1999.09903.x.
215. Rhodes, L.L.; Smith, K.F.; Murray, J.S.; Nishimura, T.; Finch, S.C. Ciguatera Fish Poisoning: The Risk from an Aotearoa/New Zealand Perspective. *Toxins* **2020**, *12*, 50.
216. Gambi, M.C.; Lorenti, M.; Patti, F.P.; Zupo, V. An annotated list of alien marine species of the Ischia Island (Gulf of Naples). *Notiziario della Società Italiana di Biologia Marina* **2016**, *70*, 64-68.
217. Darius, H.T.; Drescher, O.; Ponton, D.; Pawlowiez, R.; Laurent, D.; Dewailly, E.; Chinain, M. Use of folk tests to detect ciguateric fish: A scientific evaluation of their effectiveness in Raivavae Island (Australes, French Polynesia). *Food Additives & Contaminants: Part A* **2012**, *30*, 550-566, doi:10.1080/19440049.2012.752581.
218. Chinain, M.; Darius, H.T.; Ung, A.; Fouc, M.T.; Revel, T.; Cruchet, P.; Pauillac, S.; Laurent, D. Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). *Toxicon* **2010**, *56*, 674-690, doi:DOI 10.1016/j.toxicon.2009.05.032.
219. Hessel, D.W.; Halstead, B.W. Marine Biotoxins .1. Ciguatera Poison - Some Biological and Chemical Aspects. *Annals of the New York Academy of Sciences* **1960**, *90*, 788-797, doi:DOI 10.1111/j.1749-6632.1960.tb26422.x.
220. Kosaki, T.I.; Stephens, B.J.; Anderson, H.H. Marine toxins from the pacific. 3. Comparative bio-assay of ciguatoxin (s) in the mouse and chicken. *Proc West Pharmacol Soc* **1968**, *11*, 126-128.
221. Pasinszki, T.; Lako, J.; Dennis, T.E. Advances in Detecting Ciguatoxins in Fish. *Toxins* **2020**, *12*, 494.
222. Hoffman, P.A.; Granade, H.R.; McMillan, J.P. The mouse ciguatoxin bioassay: A dose - response curve and symptomatology analysis. *Toxicon* **1983**, *21*, 363-369, doi:http://dx.doi.org/10.1016/0041-0101(83)90092-2.
223. European Parliament (EU). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance. Official Journal of the European Union: 2010; Vol. OJ L 276 20.10.2010, p. 33.
224. Inserra, M.C.; Israel, M.R.; Caldwell, A.; Castro, J.; Deuis, J.R.; Harrington, A.M.; Keramidas, A.; Garcia-Caraballo, S.; Maddern, J.; Erickson, A., et al. Multiple sodium channel isoforms mediate the pathological effects of Pacific ciguatoxin-1. *Scientific Reports* **2017**, *7*, 42810, doi:10.1038/srep42810.
225. Estevez, P.; Castro, D.; Manuel Leao, J.; Yasumoto, T.; Dickey, R.; Gago-Martinez, A. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. *Food chemistry* **2019**, *280*, 8-14, doi:10.1016/j.foodchem.2018.12.038.
226. Murray, J.S.; Boundy, M.J.; Selwood, A.I.; Harwood, D.T. Development of an LC–MS/MS method to simultaneously monitor maitotoxins and selected ciguatoxins in algal cultures and

P-CTX-1B in fish. *Harmful Algae* **2018**, *80*, 80-87, doi:https://doi.org/10.1016/j.hal.2018.09.001.

227. Harwood, D.T.; Murray, S.; Boundy, M.J. Sample preparation prior to marine toxin analysis. In *Recent Advances in the Analysis of Marine Toxins*, Elsevier: 2017; Vol. 78.
228. Hardison, D.R.; Holland, W.C.; McCall, J.R.; Bourdelais, A.J.; Baden, D.G.; Darius, H.T.; Chinain, M.; Tester, P.A.; Shea, D.; Flores Quintana, H.A., et al. Fluorescent Receptor Binding Assay for Detecting Ciguatoxins in Fish. *PLoS ONE* **2016**, *11*, e0153348, doi:10.1371/journal.pone.0153348.
229. Pawlowiez, R.; Darius, H.T.; Cruchet, P.; Rossi, F.; Caillaud, A.; Laurent, D.; Chinain, M. Evaluation of seafood toxicity in the Australes archipelago (French Polynesia) using the neuroblastoma cell-based assay. *Food Additives & Contaminants: Part A* **2013**, *30*, 567-586, doi:10.1080/19440049.2012.755644.
230. FAO. Marine biotoxins FAO food and nutrition paper 80. Food and Agriculture Organization of the United Nations: Rome, Italy, 2004.
231. Viallon, J.; Chinain, M.; Darius, H.T. Revisiting the Neuroblastoma Cell-Based Assay (CBA-N2a) for the Improved Detection of Marine Toxins Active on Voltage Gated Sodium Channels (VGSCs). *Toxins* **2020**, *12*, 281.
232. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC INTERNATIONAL* **1995**, *78*, 521-527, doi:https://doi.org/10.1093/jaoac/78.2.521.
233. Caillaud, A.; Cañete, E.; de la Iglesia, P.; Giménez, G.; Diogène, J. Cell-based assay coupled with chromatographic fractioning: A strategy for marine toxins detection in natural samples. *Toxicology in Vitro* **2009**, *23*, 1591-1596, doi:http://dx.doi.org/10.1016/j.tiv.2009.08.013.
234. Campora, C.E.; Dierking, J.; Tamaru, C.S.; Hokama, Y.; Vincent, D. Detection of ciguatoxin in fish tissue using sandwich ELISA and neuroblastoma cell bioassay. *Journal of Clinical Laboratory Analysis* **2008**, *22*, 246-253, doi:10.1002/jcla.20250.
235. Tsumuraya, T.; Sato, T.; Hirama, M.; Fujii, I. Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. *Analytical Chemistry* **2018**, *90*, 7318-7324, doi:10.1021/acs.analchem.8b00519.
236. Leonardo, S.; Gaiani, G.; Tsumuraya, T.; Hirama, M.; Turquet, J.; Sagristà, N.; Rambla-Alegre, M.; Flores, C.; Caixach, J.; Diogene, J., et al. Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. *Analytical Chemistry* **2020**, 10.1021/acs.analchem.9b04499, doi:10.1021/acs.analchem.9b04499.
237. Díaz-Asencio, L.; Clausing, R.J.; Rañada, M.L.; Alonso-Hernández, C.M.; Dechraoui Bottein, M.-Y. A radioligand receptor binding assay for ciguatoxin monitoring in environmental samples: Method development and determination of quality control criteria. *Journal of Environmental Radioactivity* **2018**, *192*, 289-294, doi:https://doi.org/10.1016/j.jenvrad.2018.06.019.
238. Dechraoui Bottein, M.-Y.; Clausing, R.J. Chapter Eight - Receptor-Binding Assay for the Analysis of Marine Toxins: Detection and Mode of Action. In *Comprehensive Analytical Chemistry*, Diogène, J., Campàs, M., Eds. Elsevier: 2017; Vol. 78, pp. 277-301.
239. Loeffler, C.R.; Bodi, D.; Tartaglione, L.; Dell'Aversano, C.; Preiss-Weigert, A. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). *Harmful Algae* **2021**, *103*, 101994, doi:https://doi.org/10.1016/j.hal.2021.101994.
240. Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Reis Costa, P.; Gago-Martínez, A.; Hess, P. Liquid chromatography coupled to high-resolution mass spectrometry for the confirmation of Caribbean ciguatoxin-1 as the main toxin responsible for ciguatera poisoning caused by fish from European Atlantic coasts. *Toxins* **2020**, *12*, 267.

241. Lewis, R.J.; Jones, A.; Vernoux, J.-P. HPLC/Tandem Electrospray Mass Spectrometry for the Determination of Sub-ppb Levels of Pacific and Caribbean Ciguatoxins in Crude Extracts of Fish. *Analytical Chemistry* **1999**, *71*, 247-250, doi:10.1021/ac980598h.
242. Wong, C.-K.; Hung, P.; Lo, J.Y. Ciguatera fish poisoning in Hong Kong–A 10-year perspective on the class of ciguatoxins. *Toxicon* **2014**.
243. Lewis, R.J.; Yang, A.; Jones, A. Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh. *Toxicon* **2009**, *54*, 62-66, doi:http://dx.doi.org/10.1016/j.toxicon.2009.03.013.
244. Bodero, M.; Gerssen, A.; Portier, L.; Klijnstra, M.; Hoogenboom, R.; Guzmán, L.; Hendriksen, P.; Bovee, T. A Strategy to Replace the Mouse Bioassay for Detecting and Identifying Lipophilic Marine Biotoxins by Combining the Neuro-2a Bioassay and LC-MS/MS Analysis. *Marine Drugs* **2018**, *16*, 501.
245. EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:10.2903/j.efsa.2010.1627.
246. L'Herondelle, K.; Talagas, M.; Mignen, O.; Misery, L.; Le Garrec, R. Neurological Disturbances of Ciguatera Poisoning: Clinical Features and Pathophysiological Basis. *Cells* **2020**, *9*, 2291.
247. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Wekell, M.M. Tetrazolium-Based Cell Bioassay for Neurotoxins Active on Voltage-Sensitive Sodium Channels: Semiautomated Assay for Saxitoxins, Brevetoxins, and Ciguatoxins. *Analytical Biochemistry* **1993**, *214*, 190-194, doi:http://dx.doi.org/10.1006/abio.1993.1476.
248. Manger, R.; Leja, L.; Lee, S.; Hungerford, J.; Hokama, Y.; Dickey, R.; Granade, H.; Lewis, R.; Yasumoto, T.; Wekell, M. Detection of sodium channel toxins: directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC International* **1994**, *78*, 521-527.
249. Nicholson, G.M.; Lewis, R.J. Ciguatoxins: Cyclic polyether modulators of voltage-gated Iion channel function. *Marine Drugs* **2006**, *4*, 82-118.
250. Dickey, R.W.; Plakas, S.M.; Jester, E.L.; El Said, K.R.; Johannessen, J.N.; Flewelling, L.J.; Scott, P.; Hammond, D.G.; Van Dolah, F.M.; Leighfield, T.A., et al. Multi-Laboratory Study of Five Methods for the Determination of Brevetoxins in Shellfish Tissue Extracts. *Harmful algae 2002 : proceedings of the Xth International Conference on Harmful Algae, St. Pete Beach, Florida, USA, October 21-25, 2002. International Conference on Harmful Algae (10th : 2002 : St. Pete Beach, Florida)* **2004**, *10*, 300-302.
251. Caillaud, A.; Eixarch, H.; de la Iglesia, P.; Rodriguez, M.; Dominguez, L.; Andree, K.B.; Diogène, J. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. *Food Additives & Contaminants: Part A* **2012**, *29*, 1000-1010, doi:10.1080/19440049.2012.660707.
252. Dechraoui, M.Y.B.; Tiedeken, J.A.; Persad, R.; Wang, Z.; Granade, H.R.; Dickey, R.W.; Ramsdell, J.S. Use of two detection methods to discriminate ciguatoxins from brevetoxins: Application to great barracuda from Florida Keys. *Toxicon* **2005**, *46*, 261-270, doi:http://dx.doi.org/10.1016/j.toxicon.2005.04.006.
253. Soliño, L.; Widgy, S.; Pautonnier, A.; Turquet, J.; Loeffler, C.R.; Quintana, H.A.F.; Diogène, J. Prevalence of ciguatoxins in lionfish (Pterois spp.) from Guadeloupe, Saint Martin, and Saint Barthélmy Islands (Caribbean). *Toxicon* **2015**, *102*, 62-68.
254. Darius, H.T.; Roue, M.; Sibat, M.; Viallon, J.; Gatti, C.; Vandersea, M.W.; Tester, P.A.; Litaker, R.W.; Amzil, Z.; Hess, P., et al. Toxicological Investigations on the Sea Urchin Tripneustes gratilla (Toxopneustidae, Echinoid) from Anaho Bay (Nuku Hiva, French Polynesia): Evidence for the Presence of Pacific Ciguatoxins. *Mar Drugs* **2018**, *16*, doi:10.3390/md16040122.
255. Humpage, A.R.; Ledreux, A.; Fanok, S.; Bernard, C.; Briand, J.F.; Eaglesham, G.; Papageorgiou, J.; Nicholson, B.; Steffensen, D. Application of the neuroblastoma assay for paralytic shellfish poisons to neurotoxic freshwater cyanobacteria: interlaboratory calibration and comparison with other methods of analysis. *Environmental toxicology and chemistry* **2007**, *26*, 1512-1519.

256. Freshney, I.R. Serum-Free Media. In *Culture of Animal Cells*, Freshney, R.I., Ed. 2005; https://doi.org/10.1002/0471747599.cac010pp. 129-143.
257. Andrée, B.; Ichanti, H.; Kalies, S.; Heisterkamp, A.; Strauß, S.; Vogt, P.-M.; Haverich, A.; Hilfiker, A. Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels. *Scientific reports* **2019**, *9*, 1-11.
258. Brunner, D.; Frank, J.; Appl, H.; Schöffl, H.; Pfaller, W.; Gstraunthaler, G. The serum-free media interactive online database. *ALTEX-Alternatives to animal experimentation* **2010**, *27*, 53-62.
259. Bodero, M.; Bovee, T.F.H.; Wang, S.; Hoogenboom, R.L.A.P.; Klijnstra, M.D.; Portier, L.; Hendriksen, P.J.M.; Gerssen, A. Screening for the presence of lipophilic marine biotoxins in shellfish samples using the neuro-2a bioassay. *Food Additives & Contaminants: Part A* **2018**, *35*, 351-365, doi:10.1080/19440049.2017.1368720.
260. Gstraunthaler, G. Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX-Alternatives to animal experimentation* **2003**, *20*, 275-281.
261. EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish–Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:10.2903/j.efsa.2010.1627.
262. Inoue, M.; Hirama, M. Total synthesis of ciguatoxin CTX3C, a causative toxin of ciguatera seafood poisoning. *Synlett* **2004**, DOI 10.1055/s-2004-817767, 577-595, doi:DOI 10.1055/s-2004-817767.
263. Hirama, M.; Oishi, T.; Uehara, H.; Inoue, M.; Maruyama, M.; Oguri, H.; Satake, M. Total synthesis of ciguatoxin CTX3C. *Science* **2001**, *294*, 1904-1907.
264. EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Brevetoxin group. *EFSA Journal* **2010**, *8*, doi:10.2903/j.efsa.2010.1677.
265. EFSA. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. *The EFSA Journal* **2009**, *1306*, 1-23.
266. Poli, M.A.; Mende, T.J.; Baden, D.G. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Molecular Pharmacology* **1986**, *30*, 129-135.
267. Sharkey, R.G.; Jover, E.; Couraud, F.; Baden, D.G.; Catterall, W.A. Allosteric modulation of neurotoxin binding to voltage-sensitive sodium channels by Ptychodiscus brevis toxin 2. *Mol Pharmacol* **1987**, *31*, 273-278.
268. Hua, Y.; Lu, W.; Henry, M.S.; Pierce, R.H.; Cole, R.B. Online high-performance liquid chromatography-electrospray ionization mass spectrometry for the determination of brevetoxins in" Red Tide" algae. *Analytical chemistry* **1995**, *67*, 1815-1823.
269. Hua, Y.; Lu, W.; Henry, M.S.; Pierce, R.H.; Cole, R.B. On-line liquid chromatography-electrospray ionization mass spectrometry for determination of the brevetoxin profile in natural “red tide” algae blooms. *Journal of Chromatography A* **1996**, *750*, 115-125.
270. Dickey, R.; Jester, E.; Granade, R.; Mowdy, D.; Moncreiff, C.; Rebarchik, D.; Robl, M.; Musser, S.; Poli, M. Monitoring brevetoxins during a Gymnodinium breve red tide: Comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Natural Toxins* **1999**, *7*, 157-165, doi:10.1002/(sici)1522-7189(199907/08)7:4<157::aid-nt52>3.3.co;2-r.
271. Baden, D.G.; Mende, T.J.; Szmant, A.M.; Trainer, V.L.; Edwards, R.A.; Roszell, L.E. Brevetoxin binding: molecular pharmacology versus immunoassay. *Toxicon* **1988**, *26*, 97-103.
272. Turner, A.D.; Higgins, C.; Davidson, K.; Veszelovszki, A.; Payne, D.; Hungerford, J.; Higman, W. Potential threats posed by new or emerging marine biotoxins in UK waters and examination of detection methodology used in their control: brevetoxins. *Marine drugs* **2015**, *13*, 1224-1254, doi:10.3390/md13031224.

273. Glaziou, P.; Martin, P.M.V. Study of factors that influence the clinical response to ciguatera fish poisoning. *Toxicon* **1993**, *31*, 1151-1154, doi:http://dx.doi.org/10.1016/0041-0101(93)90130-B.
274. Radke, E.G.; Grattan, L.M.; Cook, R.L.; Smith, T.B.; Anderson, D.M.; Morris, J.G., Jr. Ciguatera Incidence in the US Virgin Islands Has Not Increased over a 30-Year Time Period Despite Rising Seawater Temperatures. *Am J Trop Med Hyg* **2013**, *88*, 908-913, doi:10.4269/ajtmh.12-0676.
275. Bagnis, R.; Kuberski, T.; Laugier, S. Clinical Observations on 3009 Cases of Ciguatera (Fish Poisoning) in the South-Pacific. *American Journal of Tropical Medicine and Hygiene* **1979**, *28*, 1067-1073.
276. Wang, J.; Cao, B.; Yang, X.; Wu, J.; Chan, L.L.; Li, Y. Chronic ciguatoxin poisoning causes emotional and cognitive dysfunctions in rats. *Toxicology research* **2017**, *6*, 179-187.
277. Bottein, M.-Y.D.; Kashinsky, L.; Wang, Z.; Littnan, C.; Ramsdell, J.S. Identification of Ciguatoxins in Hawaiian Monk Seals Monachus schauinslandi from the Northwestern and Main Hawaiian Islands. *Environmental Science & Technology* **2011**, *45*, 5403-5409, doi:10.1021/es2002887.
278. Ledreux, A.; Ramsdell, J.S. Bioavailability and intravenous toxicokinetic parameters for Pacific ciguatoxin P-CTX-1 in rats. *Toxicon* **2013**, *64*, 81-86, doi:http://dx.doi.org/10.1016/j.toxicon.2012.12.026.
279. Zhang, X.; Cao, B.; Wang, J.; Liu, J.; Tung, V.O.; Lam, P.K.; Chan, L.L.; Li, Y. Neurotoxicity and reactive astrogliosis in the anterior cingulate cortex in acute ciguatera poisoning. *Neuromolecular Med* **2013**, *15*, 310-323, doi:10.1007/s12017-013-8220-7.
280. Hokama, Y.; Siu, N.; Kuribayashi, T.; Higa, N.; Suma, C.; Empey-Campora, C.; Chun, K.; Yabusaki, K. Antigenic similarity of abnormal lipids in chronic fatigue syndrome (CFS) and chronic Ciguatera fish poisoning (CCFP) sera and a homologous antibody to the lipids resembling an epitope of ciguatoxin. *Faseb Journal* **2006**, *20*, A1102-A1102.
281. Hara, C.; Empey-Campora, C.; Yabusaki, K.; Kuribayashi, T.; Siu, N.; Hokama, Y. "Acute Phase Lipids" associated with phospholipids of Cardiolipins in sera of patients with Chronic Fatigue Syndrome (CFS) and other diseases. *The FASEB Journal* **2007**, *21*, A767-A767, doi:https://doi.org/10.1096/fasebj.21.6.A767-c.
282. Campora, C.E.; Hara, C.; Kuribayashi, T.; Le Huynh, D.; Pires, A.; Imada, B.; Hokama, Y.; Yabusaki, K. The effects of acute phase phosphoplipids from patients with chronic fatigue syndrome on the sodium channel in tissue culture. *The FASEB Journal* **2008**, *22*, 897.813-897.813, doi:https://doi.org/10.1096/fasebj.22.1_supplement.897.13.
283. Lawrence, D.N.; Enriquez, M.B.; Lumish, R.M.; Maceo, A. Ciguatera Fish Poisoning in Miami. *Jama-Journal of the American Medical Association* **1980**, *244*, 254-258, doi:DOI 10.1001/jama.244.3.254.
284. Blythe, D.G.; de Sylva, D.P. Mother's Milk Turns Toxic Following Fish Feast. *JAMA* **1990**, *264*, 2074-2074, doi:10.1001/jama.1990.03450160042019.
285. Bagnis, R.A.; Bronstein, J.A.; Jouffe, G.; Forestier, R.; Meunier, J.L.; Lejan, J.; Brulefer, D.; Parc, F.; Tetaria, C. Neurologic Complications of Ciguatera. *Bulletin De La Societe De Pathologie Exotique* **1977**, *70*, 89-93.
286. Walsh, C.J.; Leggett, S.R.; Strohbehn, K.; Pierce, R.H.; Sleasman, J.W. Effects of in vitro brevetoxin exposure on apoptosis and cellular metabolism in a leukemic T cell line (Jurkat). *Marine drugs* **2008**, *6*, 291-307, doi:10.3390/md20080014.
287. Kirkpatrick, B.; Fleming, L.E.; Bean, J.A.; Nierenberg, K.; Backer, L.C.; Cheng, Y.S.; Pierce, R.; Reich, A.; Naar, J.; Wanner, A., et al. Aerosolized red tide toxins (brevetoxins) and asthma: Continued health effects after 1h beach exposure. *Harmful Algae* **2011**, *10*, 138-143, doi:https://doi.org/10.1016/j.hal.2010.08.005.
288. Gawley, R.E.; Rein, K.S.; Kinoshita, M.; Baden, D.G. Binding of brevetoxins and ciguatoxin to the voltage-sensitive sodium channel and conformational analysis of brevetoxin B. *Toxicon* **1992**, *30*, 780-785.

289. Dechraoui, M.-Y.; Naar, J.; Pauillac, S.; Legrand, A.-M. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon* **1999**, *37*, 125-143, doi:http://dx.doi.org/10.1016/S0041-0101(98)00169-X.
290. Radwan, F.F.Y.; Ramsdell, J.S. Brevetoxin Forms Covalent DNA Adducts in Rat Lung Following Intratracheal Exposure. *Environmental Health Perspectives* **2008**, *116*, 930-936, doi:doi:10.1289/ehp.11068.
291. Braidy, N.; Matin, A.; Rossi, F.; Chinain, M.; Laurent, D.; Guillemin, G.J. Neuroprotective effects of rosmarinic acid on ciguatoxin in primary human neurons. *Neurotox Res* **2014**, *25*, 226-234, doi:10.1007/s12640-013-9429-9.
292. Ghaffari, H.; Venkataramana, M.; Jalali Ghassam, B.; Chandra Nayaka, S.; Nataraju, A.; Geetha, N.P.; Prakash, H.S. Rosmarinic acid mediated neuroprotective effects against H2O2-induced neuronal cell damage in N2A cells. *Life Sciences* **2014**, *113*, 7-13, doi:https://doi.org/10.1016/j.lfs.2014.07.010.
293. Diaz, R.E.; Friedman, M.A.; Jin, D.; Beet, A.; Kirkpatrick, B.; Reich, A.; Kirkpatrick, G.; Ullmann, S.G.; Fleming, L.E.; Hoagland, P. Neurological illnesses associated with Florida red tide (Karenia brevis) blooms. *Harmful Algae* **2019**, *82*, 73-81, doi:https://doi.org/10.1016/j.hal.2018.07.002.
294. Vlamis, A.; Katikou, P. Human impact in Mediterranean coastal ecosystems and climate change: Emerging toxins. *Climate Change and Marine and Freshwater Toxins; Botana, L., Louzao, C., Natalia, V., Eds* **2015**, 239-272.
295. *Discards in the world's marine fisheries. An update.* Kelleher, K., Ed.; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS: Rome, Italy, 2005 Vol. 470, p 131.
296. Sydney Fish Market. Sydney fish market seafood handing guidelines Availabe online: https://www.sydneyfishmarket.com.au/Seafood-Trading/Quality/Food-Safety (accessed on 06.10.2020).
297. Chan, T.Y.K. Ciguatoxic potential of Brown-Marbled Grouper in relation to fish size and geographical origin. *The American Journal of Tropical Medicine and Hygiene* **2015**, *93*, 1117-1121, doi:https://doi.org/10.4269/ajtmh.15-0434.
298. Lewis, R.J. Socioeconomic impacts and management ciguatera in the Pacific. *Bulletin de la Societe de pathologie exotique (1990)* **1992**, *85*, 427-434.
299. Díaz-Asencio, L.; Clausing, R.J.; Vandersea, M.; Chamero-Lago, D.; Gómez-Batista, M.; Hernández-Albernas, J.I.; Chomérat, N.; Rojas-Abrahantes, G.; Litaker, R.W.; Tester, P., et al. Ciguatoxin Occurrence in Food-Web Components of a Cuban Coral Reef Ecosystem: Risk-Assessment Implications. *Toxins* **2019**, *11*, 722.
300. Pennotti, R.; Scallan, E.; Backer, L.; Thomas, J.; Angulo, F.J. Ciguatera and scombroid fish poisoning in the United States. *Foodborne pathogens and disease* **2013**, *10*, 1059-1066.
301. Kumar-Roine, S.; Darius, H.T.; Matsui, M.; Fabre, N.; Haddad, M.; Chinain, M.; Pauillac, S.; Laurent, D. A Review of Traditional Remedies of Ciguatera Fish Poisoning in the Pacific. *Phytotherapy Research* **2011**, *25*, 947-958, doi:Doi 10.1002/Ptr.3396.
302. Bourdy, G.; Cabalion, P.; Amade, P.; Laurent, D. Traditional Remedies Used in the Western Pacific for the Treatment of Ciguatera Poisoning. *Journal of ethnopharmacology* **1992**, *36*, 163-174, doi:Doi 10.1016/0378-8741(92)90017-L.
303. Kumar-Roiné, S.; Matsui, M.; Reybier, K.; Darius, H.T.; Chinain, M.; Pauillac, S.; Laurent, D. Ability of certain plant extracts traditionally used to treat ciguatera fish poisoning to inhibit nitric oxide production in RAW 264.7 macrophages. *Journal of ethnopharmacology* **2009**, *123*, 369-377, doi:http://dx.doi.org/10.1016/j.jep.2009.03.039.
304. Boydron-Le Garrec, R.; Benoit, E.; Sauviat, M.-P.; Lewis, R.J.; Molgó, J.; Laurent, D. Ability of some plant extracts, traditionally used to treat ciguatera fish poisoning, to prevent the in vitro neurotoxicity produced by sodium channel activators. *Toxicon* **2005**, *46*, 625-634, doi:http://dx.doi.org/10.1016/j.toxicon.2005.07.002.
305. Chinain, M.; Gatti, C.M.; Roué, M.; Darius, H.T. Ciguatera poisoning in French Polynesia: insights into the novel trends of an ancient disease. *New Microbes and New Infections* **2019**, *31*, 100565, doi:https://doi.org/10.1016/j.nmni.2019.100565.

Chapter 2. Ciguatoxin in Hawai'i: using geospatial and environmental analyses to forecast CP risk from the invasive *Cephalopholis argus* (Epinephelidae)

Significance Statement

Invasive species can precede far-reaching environmental and economic consequences. In the Hawai'ian Archipelago *Cephalopholis argus* (*C. argus*) of the family Serranidae is an established invasive fish species, now recognized as the dominant local reef predator. This has a negative impact on the native ecosystem and local fishery. In this region, no fishery for *C. argus* exists, due to its association with ciguatera poisoning (CP); a severe intoxication in humans occurring after eating (primarily) fish contaminated with ciguatoxins (CTXs). Pre-harvest prediction of CP is currently not possible; partly due to the ubiquitous nature of the microalgae producing CTXs and the diverse bioaccumulation pathways of the toxins. Fish from two geographically discrete regions (Leeward and Windward) around the main island of Hawai'i were tested for their CTX content. *C. argus* was collected and investigated for CTXs using a two-tiered CTX testing protocol. Overall, 76% of fish (87/113) exceeded the U.S. Food and Drug Administration (FDA) guidance value for CTX1B (0.01 ng g^{-1}). Results were determined by the mouse neuroblastoma (N2a) cytotoxicity assay and the maximum CTXs levels were ≅2x different by location. At the Leeward site 95% (64/67) of all fish tested were positive for CTX-like activity and within the Windward site 54% (25/46) of fish were positive. This study provides additional evidence that predictors for CP can be accurate. The local designation of *C. argus* as a risk for CP was confirmed and within a hyperendemic region for CP, ocean exposure (wave power as an early predictor) may be an explanatory factor for the differences in CTX content in the *C. argus* of this study.

Ciguatera seafood poisoning (CP), the most reported seafood toxin-related illness worldwide, is an extreme problem for small island-nations through CP-derived food insecurity, and a challenge to food and health safety organizations. Globally, the livelihoods of over a billion people are affected by changes to marine ecosystems and due to impacts from climate change and CP incidences are expected to rise. Compounding this seafood-insecurity problem is the threat to native fisheries posed by invasive species. This study used an invasive species, un-targeted by the local fishery due to ciguatera, to investigate the CTX content of fish and provided evidence that remotely observable predictors (wave action) can be used to forecast CTXs in fish.

2.1. Introduction

CP outbreaks seem to occur at random, making this illness notoriously difficult to predict and prevent. Fish containing CTXs are indistinguishable from those without CTXs and predicting the CTX content of a fish is not possible without strict laboratory testing. Therefore, the capability to accurately predict CTXs in fish (or shellfish products) before reaching the market is a desirable tool for fishers, consumers, resource managers, and researchers. In the previous chapter, some of the human health aspects of incidences of CP were discussed. The complexity regarding the path to CTX accumulation for CP vectors and why it is intricate and vaguely understood was discussed. Globally, these factors and situations are diverse and dependent upon the region of focus (i.e., CTX profile, toxic fish, symptoms all vary by region). A highly impacted endemic region for CP is the island of Hawai'i and this was the focus of study in this chapter. A naturally occurring environmental border was utilized to represent two geographically distinct regions (windward and leeward, here in lowercase to indicate a direction) within the same island. In these regions the actual and perceived risk of CP in the invasive fish *C. argus*, a species highly cited for causing CP in the region, was investigated. The primary compound responsible for CP in the Hawai'ian archipelago is CTX1B, which is internationally regarded as one of the most severe and highly potent forms of CTXs, and responsible for mortalities.

Over 1000 outbreaks of CP (a reportable foodborne illness) have occurred throughout the Hawai'ian islands between the years 1990 – 2018 [306-308]. The species most commonly associated with CP outbreaks, throughout 1999 – 2003, was *C. argus*, implicated in 18% of outbreaks and 22% of illnesses in Hawai'i. *C. argus* was introduced to Hawai'ian waters in the 1950s intended to create a commercial fishery (origin of introduction, the Society Islands of French Polynesia). In its native territory, *C. argus* is also recognized as a risk for CP [218,309,310]. Due to concerns that 20% of *C. argus* could cause CP, a fishery for *C. argus* is virtually absent [311-313], and the original goal of introducing *C. argus* to create a fishery has failed. In Hawai'i, *C. argus* is also regarded as an invasive (problematic) species, cited frequently for its negative impact on native species [314-316]. Due to several factors related to its invasive status (e.g., reduced competition, not recognized as a predator by native species) it is now the most abundant large predator in the main Hawai'ian Islands with total biomass exceeding all other nearshore reef fish predators combined [311]. A study investigating growth differences between its native and invasive range found that *C. argus* was larger, heavier, and consumed a wider variety of fish (by almost 2x at the family taxa level) in the invasive territory [317]. The success of this invasive species harms local finfish harvesters, as competition predation pressures created by *C. argus* have been attributed to the decline in local native species which are valued food fishes. To combat the

negative environmental impacts of this invasive species, a sustained effort was started in 2008 to remove *C. argus* from reefs throughout Hawai'i. However, this method of population control has been considered feasible only on the small patch reef scale [318].

Several factors that are important to the growth and distribution of *Gambierdiscus* align along a gradient from the windward side with higher wave energy, lower salinity, and cooler temperatures to create conditions that are less conducive to *Gambierdiscus* growth and distribution. Opposed to that is the leeward side, which has lower wave energy, higher salinities, and higher temperatures. For simplicity, the sampling sites are named Windward and Leeward and will be used as names from here-on. If correct, these conditions should result in a difference in *Gambierdiscus* populations (by either abundance, species composition, or toxin production) and subsequent CTX in the food web. Because the target fish species is a site-attached predator with a small home range (< 3km) this life history factor should limit movement between these two environments and maintain significantly different sampling populations. The majority of fish implicated in CP outbreaks in the island of Hawai'i has been from the Leeward (west) coast [319] and consequently people are less likely to harvest resources from this region. The local knowledge and experiences of local fishers, concerning the species and regions of risk for CP are often relied upon by consumers as guidance. In CP endemic areas around the world, regional predictive fishing guidance for CP has been linked with environmental drivers or prevailing conditions [33,185,186,320-323], indicating a potential for forecasting CP risk. *Gambierdiscus* is a benthic and epi-benthic dinoflagellate, their population abundance and distribution are determined by biological and environmental conditions. To understand or predict CP the conditions that favor (or inhibit) the growth, distribution, or restriction of CTX-producing dinoflagellates and their toxin vectors needs further elucidation.

In some of the earliest reports of CP, interviews were conducted with fishers on the Turk Islands in the Bahamas who described a situation where fish from one side of the island was more dangerous than those on the other. Additionally, in certain places fish were more poisonous than in others [2]. The Turk Island described is only 2 km wide and 9 km long. Therefore, the widespread opinion (among distinct islands and regions) that experienced fishers can predict regional differences in CP risk hints that CP can be predicted. Based on generational observations for the endemic issue of CP, fishers around Hawai'i have described a similar situation where fish are of a higher risk for CP when harvested from one side of the island and of a lower risk when harvested from the other side of the island. This colloquial description is a common reoccurrence among endemic communities, where location disparities regarding CP risk are described among fishing areas within a region. In a prior investigation conducted by Loeffler et al. [185] set in the Caribbean, four commercially available fish species were sampled for CTXs among divergent environmental regimes within one region. In that study, regional differences that were predicted by the local fishers were correlated with wave power

(e.g., disturbance). This led to the conclusion that wave energy was a possible determinant environmental factor that could be indicative of the CTX content of fish, and therefore be useful as a potential CP risk predictor. Because CTX producing microalgae are predominantly benthic associated, wave energy as a disturbance to their attachment can be a major determining factor influencing their abundance and distribution. This previous study and the description of Turk Island fishers demonstrates that experience and knowledge of fishers can contribute to identifying CP risk factors or regions of concern. The goal here was to determine whether this was also the case in Hawai'i and whether the environmental factor leading to the high CTX content in fish can be identified. Fundamental differences exist between the endemic regions of the Caribbean and Pacific (e.g., *Gambierdiscus* species complex, toxin skeletal structures and polarities, food web, and trophic transfer processes). However, among these differences wave energy is a power factor and is not a biological factor and thus remains relatively the same overtime while most all biological factors (food web, species, etc.) are regionally and fundamentally variable.

The motivation behind this chapter comes from the understanding that communities impacted by CP need tangible and immediate relief from this issue. Information is needed that can help inform resource managers, harvesting efforts, purchasing choices, and consumption decisions with regard to species of potential risk for CP. Therefore, the first question investigated for this chapter was, whether the CP risk of *C. argus* does exist, and if so if it differs between the two distinct locations around the big island of Hawai'i. To investigate the CTX content of sampled fish a two-tiered testing approach was employed (see description in 1.6), capable of providing both the qualitative and quantitative information required for investigating CTXs. Because the study site is within the jurisdiction of the USFDA their established guidance level for CTX1B was the value utilized for making the determination regarding the wholesomeness of the fish for consumption. The second major question addressed in this chapter was whether *C. argus* could act as an indicator for CP and, as such, be used to predict CP associated risk for human health. The question of prediction, in addition to accurate CTX analysis, requires information with regards to the environmental, or causative conditions that may be underlying the accumulation of the measured CTXs in a fish.

2.2. Materials and Methods

2.2.1. Sample Location and Collection

Sampling for this study was conducted at two distinct sites based on their wind energy conditions. The Windward site represented a site with stronger wind conditions and the Leeward site was representative of a lower wind energy location. Both sites have different historical associations with CP [313,324] and different physical oceanographic properties. The sampling site called Windward

was in the northeast facing coast of Hawai'i Island at Hilo Bay (Latitude: 19.736140, Longitude: -155.078435, in Decimal Degrees) (Figure 14). Here a rubble reef just inside the breakwater was used as the location where fish were captured [325]. The interior part of Hilo bay is partially enclosed by a 3 -km breakwater with a 1.5 km wide opening to the North Pacific Ocean. Rainfall at the Hilo airport station is 330.3 cm annually [326] and is consistent with the isohyets in Figure 14 [327]. Freshwater input from the Wailuku and Wailoa rivers create estuarine characteristics (i.e., lower salinity), as haloclines hold more of this fresh water at the surface nearshore and inside the breakwater. Inside the breakwater, water depths range from 0 to 15 m. Average annual water temperatures of Hilo Bay range between 22-24 °C [328]. Benthic biological cover inside the Hilo breakwater is predominantly 'uncolonized' with small areas of '10 - <50% macroalgae' and '10 - <50% coral' along the inside of the breakwater [325]. The geomorphology of Hilo Bay is characterized as predominantly 'mud' inside the breakwater with an area of 'spur and groove' immediately adjacent to the breakwater itself.

The other sampling location called 'Leeward' is located in the northwest facing coast of Hawai'i (Kohala region) at Kapa'a beach park (Latitude: 20.203884, Longitude: -155.901945) (Figure 14). The benthic habitat is mostly described as coral-covered, predominantly '10 - <50% coral' within a primarily 'rock/boulder' and 'aggregate reef' geomorphology [325]. The ocean at Kapa'a is not enclosed and has no significant surface water inputs. Rainfall measurements made in the region of Kapa'a (Mahukona station) average approximately 47.0 cm annually (46), rainfall is significantly lower than at the windward site, estimated at 2.5-5 cm per year (see Figure 14) [327]. Depth was 10-20 m in the areas where fish were collected. Temperature ranges between 25-27 °C over the annual cycle and is typically 3-4 °C warmer than the Windward sampling location during any given month [328].

Long-term geospatial analyses of the mean environmental conditions alongside the Hawai'ian shoreline by Wedding et al. [329] show that sea surface temperature (SST) and daily insolation are higher, and wave power and Chlorophyll-a are lower at the Kapa'a beach park site (Leeward sampling site) relative to Hilo Bay (Windward sampling site). Buoys simultaneously deployed on the northeast-facing Hilo Bay and the west/southwest facing Kona side recorded water quality measurements that support these observations and show some contrasts in ocean conditions between these sites.

Differences among the two sampling sites' physical properties such as wave (average wave power and direction), salinity, temperature, and rainfall were recorded to investigate the potential relationship between CTX levels in fish and the sites' respective dominant environmental conditions. Figure 14 shows the average wave power (in kW m^{-1}, according to the color scale) and the bearings denote the mean wave direction based on data from a 10-year hindcast (2000-2009). This hindcast dataset was not captured during sample collection, however, it is representative of the prevailing annual conditions of the region, providing a stable long-term outlook to understand the prevailing conditions. The wave model created by J. Stopa, consists of a 3-layered 2-way nested grid made up of

a global (55 km), regional (5.5 km), and island (0.56 km) scale which utilized the spectral wave model WAVEWATCH3 [330]. The global model was forced by modified climate forecast system winds [331] and mesoscale features important for the region which was captured by the Weather and Forecasting Model (WRF) (e.g. [332,333]).

C. argus samples (n = 113 total, 46 from Leeward, 67 from Windward) were collected via spearfishing by the publication co-author J. La Pinta. Samples were collected opportunistically (i.e., when available), each *C. argus* encountered was attempted to be collected, irrespective of size or behavior (e.g., active swimming or resting). Following collection, whole fish were placed immediately on ice until they could be processed. Measurements on the size (Total length (TL)) and wet weight were recorded. Collecting measurements were missed on one fish from the Leeward side, which tested negative for CTXs; the sample was included in the CTX analysis but omitted from any test requiring morphometric data. Following the measurements, the fish were stored at -20 °C then shipped and received hard frozen at the FDA's Gulf Coast Seafood Laboratory in Dauphin Island, Alabama for CTX analyses.

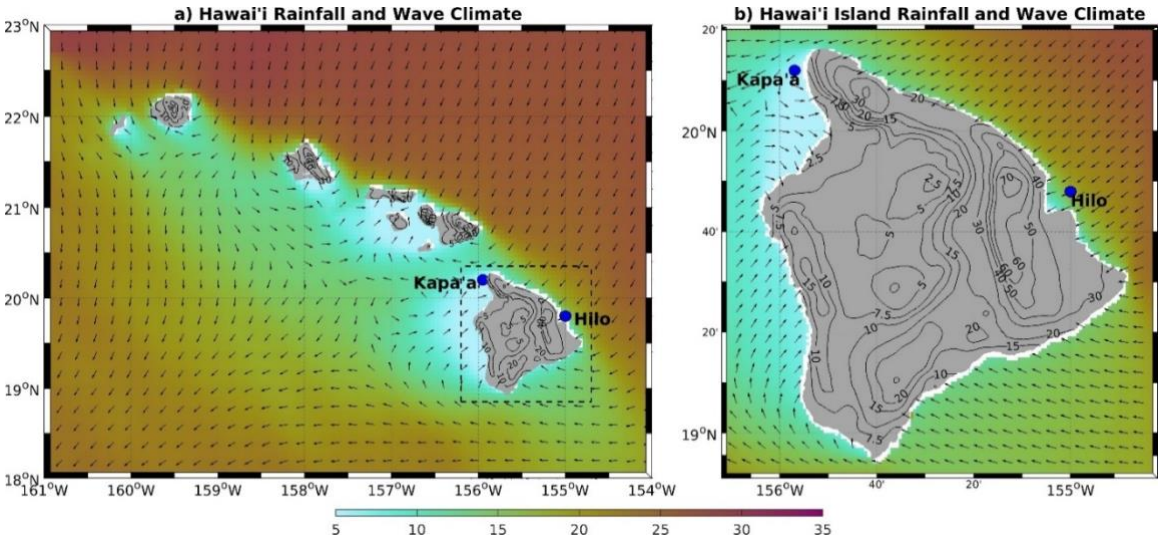


Figure 14. Environmental conditions (wave and rainfall) of (a) Hawai'ian region and (b) Hawai'i Island. The colors represent the average wave power (in kW m^{-1}) and arrows symbolize direction for the period 2000-2009. The black contours on the island designate the average rainfall in cm. The two study sites: 1) Windward (Hilo) and 2) Leeward (Kapa'a) are denoted with blue makers. Note that the representative wave conditions at both locations were taken at a water depth of 200 m offshore of the sampled sites. Map created by co-author J. Stopa. Image copyright information: This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, *Environmental Research*, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

2.2.2. Sample preparation for toxin extraction

Muscle tissue (100 g) was excised from all fish and the skin was removed to facilitate the extraction process. From a random selection of 16 fish (Windward n = 3, Leeward n = 13) viscera were excised for CTX analysis. The tissue samples were subjected to a series of extraction processes using the method outlined in Dickey 2008 [136] and reported in [334]. Briefly, the tissue went through a series of liquid-liquid partition extraction methods designed to remove potential interferences such as nonpolar lipids, proteins, and hydrophilic compounds. The final stage of the series of liquid-liquid partition extractions resulted in a final chloroform extract containing CTXs. Extracts then underwent an additional cleanup step using a Bond Elute Silica solid phase extraction cartridge. The method was conducted as follows: 100 g of fish tissue was excised and homogenized for 2 min with 200 mL (2 mL g^{-1} tissue) HPLC grade acetone, using a one-liter explosion proof blender. The fish tissue was separated from the acetone by vacuum filtering through Whatman #4 filter paper in a 500 mL filter flask. The tissue residue was re-homogenized with another 200 mL of HPLC grade acetone and separated, collecting the acetone in the same filter flask. The acetone was transferred to a 500 mL Erlenmeyer flask and chilled at -20 °C for a least 12 hr. The cold acetone extract was filtered through Whatman #5 filter paper, and the precipitate discarded. The filtrate was transferred to a 1000 mL round bottom flask and dried down at 45 °C using a rotary evaporator. To the residue 100 mL of 80% methanol in water was used to dissolve the residue and this was transferred to a 250 mL glass separatory funnel. A defatting process was conducted two times by partition the solution with 100 mL (1 mL g-1 tissue) of hexane. The hexane phases (top layer) were discarded after each partition. The aqueous methanol was collected and dried down at 45 °C by rotary evaporation. Next 100 mL (1 mL g-1 tissue) of MilliQ water was added to the residue and the solution transferred to a separatory funnel. 50 mL (0.5 mL g-1 tissue) of chloroform was added to the round bottom flask and using a gentle swirling motion added to a separatory funnel, then and shaken gently 3 times for 15 seconds, being sure to release the pressure in between the gentle shaking steps. The phases were allowed to separate, and the chloroform phase was collected in a 125 mL round bottom flask, this was dried down at 40 °C by rotary evaporator. The chloroform partition steps were repeated two more times. The dry residue then underwent a solid Phase Extraction (SPE). To prepare the SPE column first a 2g Silica Bond Elut (Agilent, Santa Clara, CA, USA) solid phase extraction cartridge was conditioned by successively passing 10 mL of 5% water in methanol, 20 mL methanol, and 20 mL chloroform. Sometimes it was necessary to pass additional volumes of chloroform until the sorbent was uniformly translucent. After the column was prepared the sample residue was dissolved in 1.0 mL of chloroform. This was applied to cartridge and allowed to pass through and into the sorbent bed by gravity. The sample vessel was rinsed three times with 1.0 mL chloroform and applied to the cartridge allowing it to pass into the bed. The cartridge was washed by slowly passing 10 mL of chloroform, and the chloroform wash was discarded. Elute cartridge by

passing 20 mL of 10% methanol in chloroform and collecting it in 13 x 100 mm glass tube and remove solvent using nitrogen gas. The final product was stored at -20 °C.

2.2.3. Toxin content estimate by N2a-assay

Toxicity screening and assessment of the sample extracts was based on the method for a semi-quantitative *in vitro* neuro-2a cytotoxicity assay described in Dickey 2008 [136] and Manger 1995 [232]. In short, mouse (*Mus musculus*) neuroblastoma cells Neuro-2a (N2a) (ATCC CCL-131, American Type Culture Collection, Rockville, MD) were propagated and maintained in RPMI media supplemented with antibiotics (50 µg mL^{-1} streptomycin, 50 units mL^{-1} penicillin), glutamine (2 mM), sodium pyruvate (1 mM), and heat-inactivated FBS (10% *v/v*) as previously described by Dickey [136]. Cells were harvested for the assay when cultures were ≅85–90% confluent, and seeded at 4×10^4 cells $well^{-1}$ (200 µL volume) into sterile 96-well polystyrene microplates (Corning™ 3596). The sensitivity and specificity of the assay to compounds activating the Na_V (i.e. CTXs) is achieved through the addition of the pharmaceuticals ouabain (O) and veratridine (V). These compounds are used to sensitize the cells for the detection of Na_V specific effects. Conversely, cytotoxicity caused by any other sample constituent during the microplate experiment can be evaluated using the non-sensitized 'control' cells. N2a cells used in this study had a survival rate of 60-80% when exposed to 0.22/0.022 mM O/V. Cell line passage numbers for utilization were between 192-230 (original passage number 185 from manufacturer). Range of raw absorbance values recorded after the MTT-colorimetric assay evaluation was between 0.10-1.52 nm (minimum survival to maximal). Full dose-response curves (8-dilutions) of sample extracts were prepared with sensitized and non-sensitized cells to determine the concentration at which cell viability was reduced by 50% (EC_{50}) compared with a CTX1B standard (CAS Number 11050-21-8 at 50 pg, provided by Dr. Richard J. Lewis, The Queensland University, Australia) [136]. All samples, standards, and relevant controls were assayed in triplicate. Results were expressed as ppb CTX1B eq. wet-weight. The limit of detection (LOD at LC_{30}) for the assay in the *C. argus* fish matrix was determined to be 0.001 ppb CTX1B eq. and the limit of quantification was 0.005 ppb CTX1B eq. All samples of toxin concentrations > 0.01 ppb CTX1B eq. as determined by N2a assay were analyzed by LC-MS/MS.

2.2.4. CTX1B confirmation by LC-MS/MS

The applied qualitative LC-MS/MS method for the confirmation of CTX1B in the fish extracts was performed by H. Flores Quintana with help from C. R. Loeffler and conducted as described in Solino et al. 2015 [253] and with modifications as published in [334]. The LC-MS/MS system utilized was an Agilent 1260 LC system (Agilent Inc., Palo Alto, CA) coupled to a QTRAP 4000 triple quadrupole/linear

ion trap hybrid mass spectrometer (Applied Biosystems, Inc., Foster City, CA). A quadrupole is an analyzer that stabilizes the trajectory of an ion based on the ion's mass to charge ratio (*m/z*). The m/z where m = ionized molecular weight of the molecule and z = charge. Analytes were separated on a Kinetex C8, 75 x 2.1 mm; 2.6 µm column (Phenomenex, Torrance, CA, USA) with a Phenomenex KrudKatcher Ultra HPLC in-line filter (0.5-µm depth x 0.004-in inner diameter). Column temperature was set at 40- °C with a flow rate of 300 µL min^{-1} was used to achieve toxin separation. LC mobile phase was water with 0.1% formic acid (A) and acetonitrile:water (95:5) with 0.1% formic acid (B). Injection volumes were 10 µL, and analyses were performed with the following gradient program: 10% B for 1 min, 95% B at 1.5 min and hold until 6 min, then back to the initial conditions, 10% B, at 6.5 min and with a re-equilibration hold until minute 9.5. The mass spectrometer was operated in positive electrospray ionization mode and the data was acquired using a multiple reaction monitoring (MRM) method. MRM is a mass spectrometry technique in which quadrupoles one (Q1) and three (Q3) are locked into a specific mass transition. In the triple quadrupole MS ions are fragmented by accelerating them into a gas, the precursor ion selected in Q1 will enter quadrupole 2 (Q2) where the Q1 ion is accelerated into a collision gas and undergoes fragmentation into ions of lesser molecular weight. The fragments, or daughter ions, exit Q2 and the collision cell exit potential focuses and accelerates the ions out of Q2 and into Q3 for scanning and mass determination. The MRM method therefore underwent a specific process where the Q1 Scan, the initial survey scan to verify the molecular weight of the compound, was used to verify the precursor ion, also known as the parent ion. The principal tune settings were: Ion Spray (IS) 5500 V, Declustering Potential (DP) 120 V, Entrance Potential (EP) 10 V, Collision Cell Exit Potential (CXP) 15 V; Collision Energy (CE) 35 eV; Temperature (TEM) 400 °C; curtain gas 20 psi, Nebulizing gas (GS1), and drying gas (GS2) both at 50 psi and Collision gas (CAD) medium. The nebulizing gas helps generate small droplets that rapidly desolvate in the ion source and GS2 helps to evaporate the spray droplets. Samples were reported as confirmed when the retention time was within 2% of the CTX1B standard and when the sample contained all three ion transitions at an S/N > 3.

2.2.5. Statistical analyses

Statistical analyses were performed using JMP software (v9, SAS Institute, Cary NC, USA). The differences between CTX1B equivalents (eq.) toxin concentration and total toxin content in edible flesh and location were investigated with a one-way ANOVA using ranked data (Friedman's Rank test). Ranked data were used because the toxin data did not meet the parametric assumptions of normality and heterogeneity, due to a high proportion of 0 values (<limit of detection, LOD), skewing the dataset. A Spearman Rank Order Correlation was performed to investigate the relationship between toxin concentration, total toxin content, total fish weight, and standard fish length. A weight-to-length ratio

was calculated and the differences between the weight-to-length ratios, by location, were investigated using a one-way ANOVA. The results of all tests were considered significant at $p \leq 0.01$. Location differences in fish length were investigated using a student's t-test ($p \leq 0.01$).

2.2.6. Species confirmation of *C. argus*

For each sampling location, one whole specimen was shipped frozen to the FDA Center for Food Safety and Applied Nutrition to authenticate the identification of *C. argus* and serve as a DNA reference standard for this species from this invasive region for the future identification of meal remnants involved in outbreaks of CP (Figure 15). Genetic identification using DNA barcoding: is based on a 655 basepair DNA fragment starting near the 5' end of the cytochrome c oxidase subunit 1 mitochondrial gene (CO1). This fragment can be obtained from all fish species, yet is unique enough to allow species identification. This was conducted by J. Deeds. *C. argus* is locally known as 'peacock grouper' or by its Tahitian name 'roi' [335], but for labeling fish sold in interstate commerce, the FDA recommends the market name Purplespotted Grouper [336].

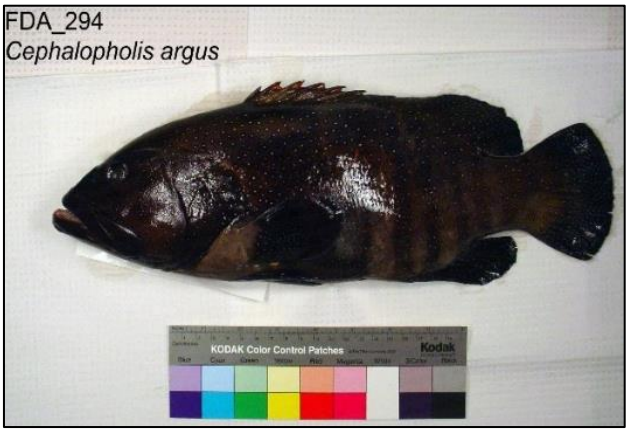


Figure 15. Photograph of *Cephalopholis argus* (Purplespotted Grouper). This fish (after a portion of tissue was removed for toxin analysis) was entered into the Smithsonian National Fish Collection (under the following identifier NMNH 437313 [337], this fish was collected at the Leeward collection site (Kapa'a)). Seafood Identification barcode data can be found under sequences FDA 294 [338], which was generated from reference specimen NMNH 437313. Photo credit: J. Deeds. Image copyright information: This image is part of an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). C. R. Loeffler, *Environmental Research*, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

Both fish were thawed and photographed, muscle tissue was sampled from the left filet and CO1 reference sequences were generated according to Handy et al. 2011 [339]. The remaining specimens were authenticated, preserved, and vouchered, according to Deeds et al. 2014 [340], for

inclusion in the FDA's Reference Standard Sequence Library (RSSL) for seafood identification [341]. The RSSL database contains barcode sequences and nomenclature information for more than 1040 seafood specimens of vertebrates and invertebrates.

2.3. Results

2.3.1. Environmental Differences between Locations

Sea surface temperatures, measured by the buoy deployments, were on average 1.6 ± 0.60 °C warmer at the Leeward location. This region of the island of Hawai'i is classified as ´arid´ or ´hot desert´, where annual evaporation exceeds precipitation (i.e., lower freshwater input and nutrient runoff) and the water column is warmer with less wind-driven wave action, lower wave power, and lower wave height (i.e., lower energy) (Table 5). Salinity was lower and more variable at the Windward location because this region receives 6x more rainfall (on average) than the Leeward region, 3000 mm and 500 mm, respectively (Figure 14). Chlorophyll a, an indicator for phytoplankton and plant biomass, was higher here than at the Leeward location. The wave and precipitation climate of the region and island, the contours located on the land denote the average rainfall [327] are shown in Figure 14. The wider region of the Windward sampling location's annual wave energy averages 25 kW m^{-1}, this energy is generated through a combination of the wind-driven seas forced by the trade winds and remote swells generated in the North Pacific [332]. The Leeward region experiences approximately 2.5x less wave energy (10 kW m^{-1}) than the Windward region. Offshore of the Windward side, the average wave energy is 18 kW m^{-1} and at Kapa'a (Leeward) the average is 5.6 kW m^{-1} (Table 5). Table 5 shows the site-specific information demonstrating the sea state near Hilo is more active (e.g., taller waves, longer period, and larger wave energy) than Kapa'a throughout the year.

Table 5. Sampling locations and the dominant physical oceanographic properties present at each location from 2000-2009. Including the average toxin concentration (ng CTX1B eq. g^{-1}) and total toxin content (measured in this study) in CTX1B equivalent for fish filet. Long-term mean values from Pacific Islands Observing System [342].

	Sampling Location			
	Leeward (West/Kohala)		Windward (East/Hilo)	
Statistic	Mean±SD	Maximum	Mean±SD	Maximum
Wave power (kW m^{-1})	5.61 ± 4.52	114	18.2 ± 13.5	203
Wave height (m)	1.12 ± 0.03	4.08	2.00 ± 0.52	5.38
Peak period (s)	12.7 ± 2.89	24.0	10.1 ± 2.56	24.0
SST (°C)	25.5 ± 0.89	-	25.2 ± 0.87	-
Chlorophyll *a*[*]	0.08 ± 0.03	-	0.09 ± 0.06	-
PAR[*](mol m^2 d^{-1})	44.4 ± 10.6	-	41.8 ± 9.57	-
Concentration (ng g^{-1} CTX1B eq.)	0.17 ± 0.03	0.75	0.06 ± 0.01	0.4

Total toxin (ng CTX1B eq.)	84.7 ± 14.5	492	18.4 ± 4.59	115

*Values for Chlorophyll a and Photosynthetically Active Radiation (PAR) from 2002-2013. SST= sea surface temperature. Table copyright information: Available online 8 October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, Environmental Research, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

2.3.2. Species confirmation of *C. argus*

The identity of *C. argus* was confirmed by J. Deeds, for both specimens by taxonomists at the Smithsonian National Museum of Natural History (Washington D.C., USA) and both selected fish were accessioned into the Smithsonian National Fish Collection (NMNH 437313 Figure 15 and NMNH 437314). CO1 DNA sequences were generated for both specimens and were included in the FDA RSSL for Seafood Identification (sequences for the specimen NMNH 437313 can be found under FDA 294 [338], Figure 15, and reference specimen NMNH 437314's [337] sequences can be found under FDA 295 [343]). Because isolated invasive populations will eventually drift genetically from the original native population (unless there is genetic re-introduction), over time this will result in genetically divergent populations; therefore, a set of references from this invasive region can be used to track this drift.

2.3.3. Concentration of CTX-like compounds and total CTX content by location and size

A total of 113 fish were collected, 46 from the Windward location and 67 from the Leeward location; for fish morphometrics and size distribution see Table 6 and Figure 16. The toxin concentration expressed as CTX1B equivalents (eq.) in edible flesh (ng CTX1B eq. g^{-1} fish tissue) of *C. argus* from Hawai'i was significantly different by capture location (Table 7). Levels were significantly higher in the Leeward location (0.17 ± 0.03 ng g^{-1}) than in the Windward side (0.06 ± 0.01 ng g^{-1}). However, the average toxin concentration for both locations (0.12 ± 0.02 ng g^{-1}; mean ± standard error) was above the FDA's guidance level (0.01 ng g^{-1} CTX1B eq.) for CTX1B concentration in the flesh of edible tissue. For the Leeward side, the percentage of fish exhibiting CTX-like activity (>LOD) following the N2a-assay (Figure 17) was 96% (64 of 67) and in the Windward location 54% (25 of 46). Combined, 79% (89 of 113) of all *C. argus* were positive for CTX-like activity by the N2a-assay. The ratio of CTX eq. determined in the liver versus the flesh (CTXs eq. ppb viscera / CTXs eq. ppb flesh) from the Leeward side was 0.8-436 (mean = 58) (Table 8). From the Windward location, the ratio of CTX-like toxicity in the liver versus the flesh was <LOD to 8x higher toxin concentration per gram of tissue in the viscera, the mean among fish measured was 4x higher concentration of CTX1B-like toxicity in the viscera among

samples tested (Table 8). A ratio >1 indicates a higher toxin concentration in the viscera (per g tissue) and a ratio <1 indicates a higher proportion of CTXs in the flesh.

Table 6. Range of fish morphometrics, CTX concentration, and total toxin content determined by the N2a-assay for *C. argus* sampled around the island of Hawai'i.

Location	Weight (g)	Edible flesh (g)	Total length (cm)	Age° (yr)	Concentration (ng g^{-1})	Total toxin (ng)
Windward (East/Hilo) (n = 46)	126-2100 ($\bar{x}$=885.9)	44.1-733 ($\bar{x}$=310.1)	16.5-52.2 ($\bar{x}$=35.2)	2-25 ($\bar{x}$=10.3)	<LOD-0.40 ($\bar{x}$=0.06)	<LOD-115 ($\bar{x}$=18.4)
Leeward (West/Kapa'a) (n = 67)	125-3020 ($\bar{x}$=1257)	43.8-1060 ($\bar{x}$=440.0)	18.5-53.0 ($\bar{x}$=39.8)	2-25 ($\bar{x}$=14.9)	<LOD-0.75 ($\bar{x}$=0.17)	<LOD-492 ($\bar{x}$=84.7)

°Estimated based on morphometric measurements, conversion calculations based on measurements converted into age from Donovan et al. [344]. Table copyright information: Available online October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). C. R. Loeffler, *Environmental Research*, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

The total toxin amounts, estimated from the CTX1B eq. g^{-1} of tissue and the amount of edible flesh (filet) per fish, around Hawai'i were significantly different among sites ($F(1,111) = 13.03$, p = <0.001). The mean total toxin content at the Leeward site was significantly higher than at the Windward site 84.7 ± 14.5 and 18.4 ± 4.59 ng CTX1B eq. per fish, respectively (Table 6). The total amount of toxin per fish (per gram toxin concentration x total grams of flesh) was <LOD - 492 ng CTX1B eq. with a mean 84.7, n = 67 for the Leeward location and ranged from <LOD - 115 ng CTX1B eq. with a mean = 18.4, n = 47 for the Windward location.

Table 7. Results of a one-way analysis of variance (Friedman's rank test)[a]. Concentration of toxin per gram of flesh by length and by location. Table copyright information: Available online October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, Environmental Research, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

	CTX1B concentration		
Effect test	*df*	*F*	*P*
Whole Model	3	9.097	<0.0001***
Total Length	1	2.748	0.1003
Location	1	14.87	<0.0002***
Total Length X Location	1	3.116	0.080

a To assess the effects of total length, location, and the interaction of total length and location on the concentration of CTX1B pg eq. from *C. argus*.

* Indicate significance at $p < 0.001$ probability level.

[1]CTX1B = Pacific ciguatoxin.

Table 8. Fish morphometrics and CTX analysis for *C. argus* tissue, viscera and liver samples from around the island of Hawai'i. CTX concentration (ng CTX1B eq.) as a tissue-equivalent (TE) in viscera and flesh, and the range of ratio's (ng CTX1B eq. g^{-1} TE viscera) / (ng CTX1B eq. g^{-1} TE flesh) of CTX concentration in the viscera vs flesh of the same sample. Table copyright information: 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). C. R. Loeffler, *Environmental Research*, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

Location	Weight (g)	Total length (cm)	ng CTX1B eq. g^{-1} flesh	ng CTX1B eq. g^{-1} viscera	Viscera Flesh^{-1} range
Windward (n = 3)	380.0-1430.0 ($\bar{x}$ = 918.3)	26.5-39.0 ($\bar{x}$ = 34.8)	<LOD-0.209 ($\bar{x}$ = 0.089)	<LOD-0.769 ($\bar{x}$ = 0.410)	<LOD-8.154 ($\bar{x}$ = 3.944)
Leeward (n = 13)	396.0-1637 ($\bar{x}$ = 1077)	29.0-45.0 ($\bar{x}$ = 38.6)	0.002-0.594 ($\bar{x}$ = 0.071)	0.200-3.333 ($\bar{x}$ = 0.871)	0.842-435.9 ($\bar{x}$ = 57.59)

$\bar{x}$ = mean value

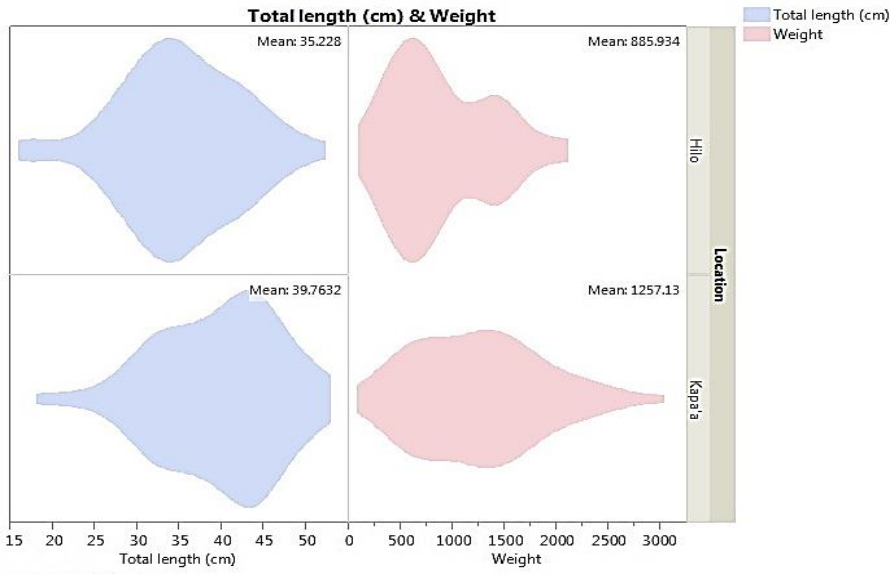


Figure 16. Size distribution of all *C. argus* samples by location (Hilo = Windward, Kapa'a = Leeward) for total length (in cm) and total weight (in grams). Image copyright information: Available online October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, Environmental Research, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

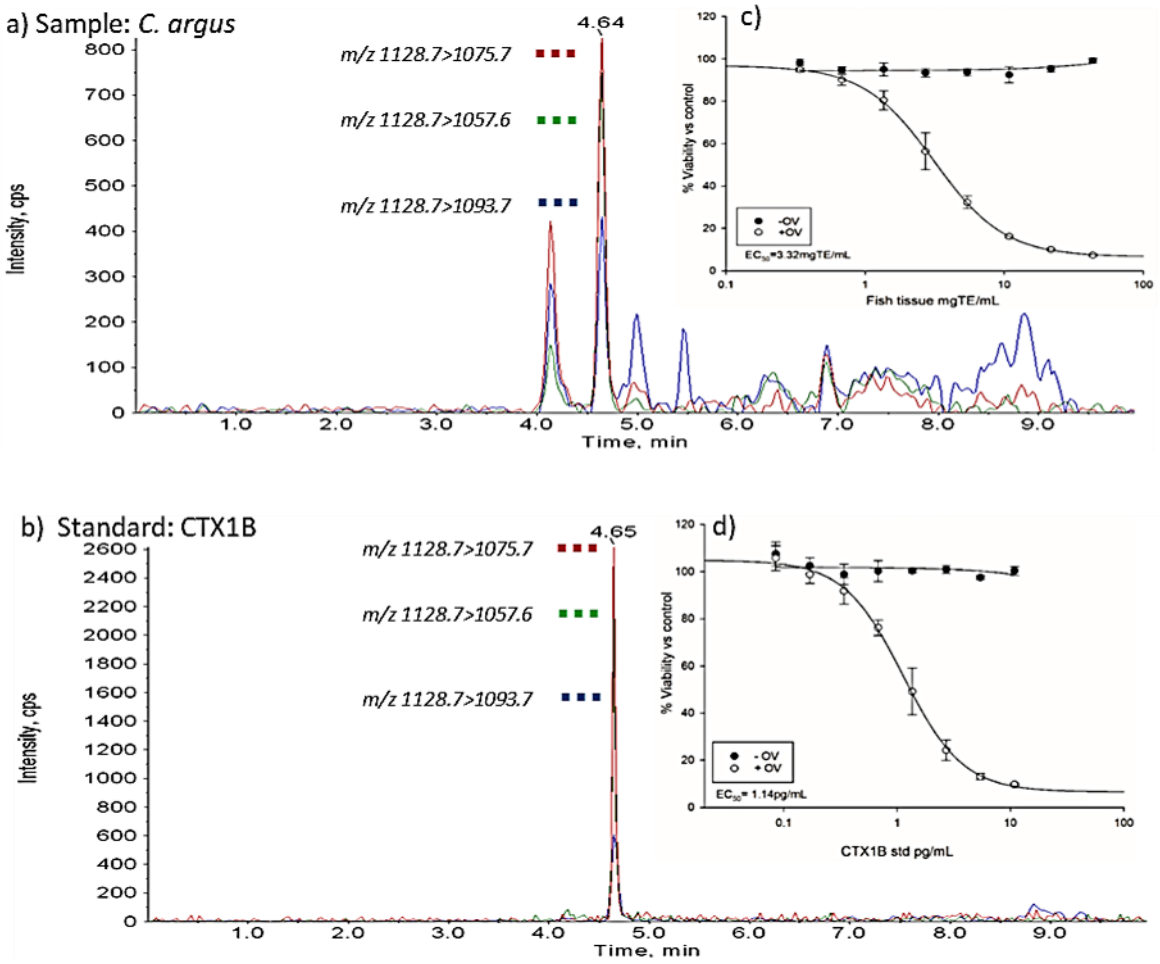


Figure 17. Representative sample toxin analysis figures for *C. argus*. Figures were generated using the US FDA two-tiered CTX testing protocol comparing samples and standards. Panels (a,b) are LC-MS/MS (MRM acquisition) representative chromatograms provided by H. Flores Quintana of (a) *C. argus* sample and (b) CTX1B standard. Panels (c,d) are N2a-assay derived concentration-response curves for (c) fish tissue extract (TE) equivalents (in mgTE mL^{-1}), the 8-pt concentration-response curve was applied starting at 43 mgTE mL^{-1} (EC50 = 3.29 mgTE mL^{-1}), and (d) CTX1B reference standard in pg mL^{-1}, the 8-pt concentration-response curve was applied starting at 10.9 pg mL^{-1} (EC50 = 1.14 pg mL^{-1}). In addition to the samples, +OV wells received 20 µL O and V solution, -OV wells received 20 µL buffer solution (PBS). Image copyright information: Available online October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, Environmental Research, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

A series of Spearman rank-order correlations were conducted to investigate any potential relationships between the toxin content (total CTX per fish and CTX per gram) and fish morphometrics (weight and length). There was a moderate monotonic positive relationship between the total CTX content of a fish and morphometrics (both weight and length). The longer the fish the higher the total

toxin content r_s (111) = 0.45, p <0.001 (Figure 18a), for the Leeward (west) site this relationship was strongest at r_s (112) = 0.58, p < 0.001 (Figure 18c). The heavier the fish, the higher the total toxin content r_s (112) = 0.46, p = <0.001 (Figure 18b). There was a weak monotonic positive relationship between the concentration of CTXs per g of tissue and fish morphometrics (weight and length). The heavier the fish, the higher the predicted concentration of CTXs r_s (112) = 0.25, p = 0.007. The longer the fish the higher the predicted concentration of CTXs r_s (111) = 0.25, p = 0.007. There was a very strong, positive monotonic correlation between the total toxin content and CTX concentration r_s (112) = 0.96, p <0.001. A very strong positive monotonic correlation between the total weight and the standard-length measurement r_s (111) = 0.97, p <0.001 was also observed, as expected.

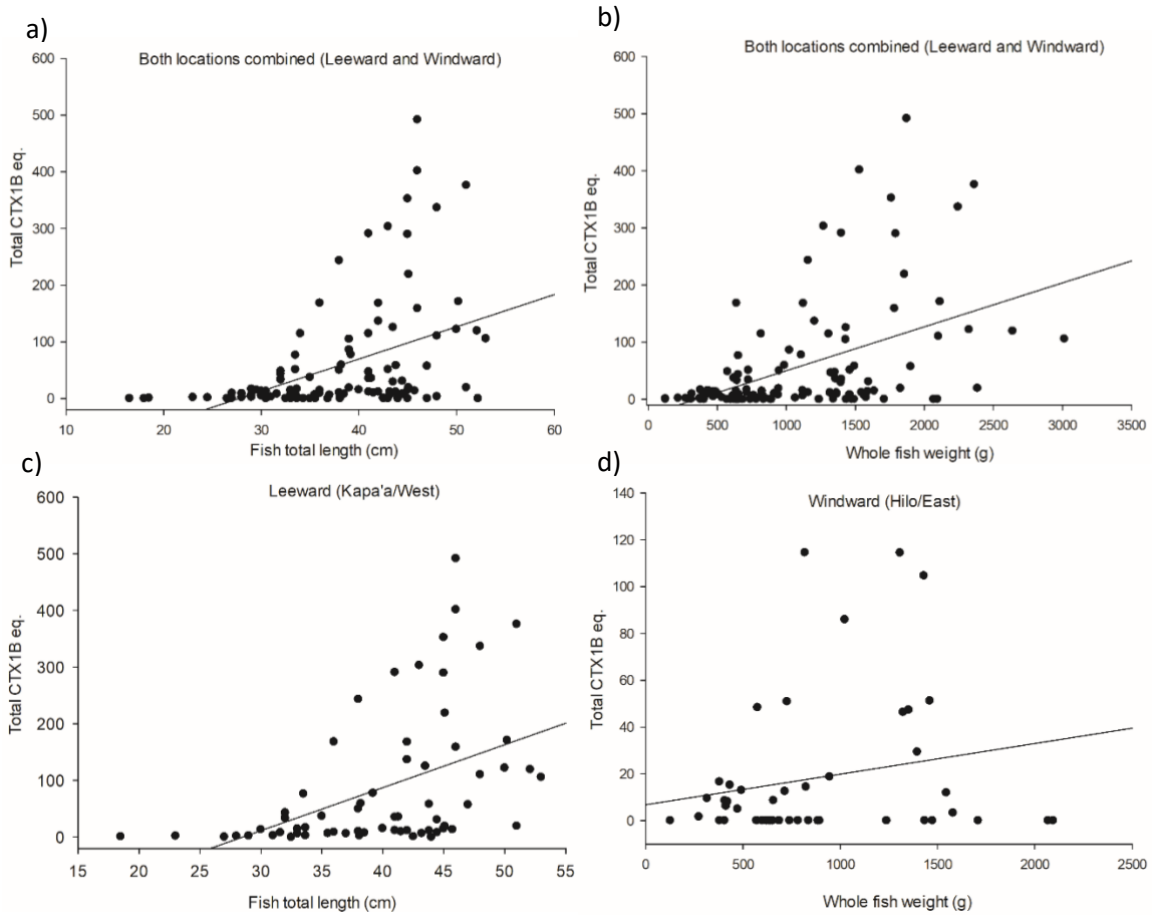


Figure 18. Regression lines for total toxin content (CTX1B eq.) per *C. argus* fish sample on the y-axis and fish morphometrics on the x-axis. a) total toxin content by fish length (cm) for all sites combined, b) total toxin content by fish weight (grams) for both locations combined. c) total toxin content by fish length (cm) for only the Leeward location, d) total toxin content by whole fish weight only for the Windward location**.** Image copyright information: Available online 8 October 2021 0013-9351/Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). Christopher R. Loeffler, *Environmental Research*, https://doi.org/10.1016/j.envres.2021.112164 referenced in this thesis as [334].

2.3.4. Confirmation of CTX1B by LC-MS/MS

The detection of CTX1B by MS/MS was performed using three precursor/product transition pairs, used to monitor for CTX1B in the form of its ammonium adduct of $[M+NH_4]^+$ *m/z* 1128.7 where characteristic ions resulting from successive waters losses in Q3 at *m/z* 1093.7, 1075.7, and 1057.6 were monitored and response summed (Figure 17 and Table 3). The transitions for CTX3C precursor ion (Q1) $[M+H]^+$ *m/z* 1023.7 and Q3 water losses were: *m/z* > 1005.7, 987.7, and 969.7. Among all the samples deemed positive by the N2a-assay (n = 89), structural confirmation of the regional CTX biomarker (CTX1B) was achieved in 33% (29/89) of samples (Figure 17, provided by H. Flores Quintana). Regionally, of the confirmed samples 38 % (n = 24) were from the Leeward site and 20 % (n = 5) were from the Windward site. There was 100% agreement between the N2a-assay and LC-MS/MS regarding the 24 samples that were deemed negative (<LOD); i.e., no false positives were reported by the N2a-assay. CTX3C was not confirmed in any sample.

2.4. Discussion

Historically one of the most accessible methods of harvesting finfish globally has been nearshore fishing; and in the Hawai'i archipelago, fishing is both a traditionally important food source and a deeply rooted custom of the local culture (50, 51). The nearshore coastal environment also generates substantial economic welfare for the wider economy, with an estimated USD 360 million in annual contributions from coral reefs (52). Fishing areas are strictly regulated (e.g., by season, species, size, harvest number) among the many potential harvest areas for the 428 km coastline of the Island of Hawai'i, however, *C. argus* is seldom protected (53). Because *C. argus* is a problematic invasive species, demonstrated to harm the fragile native species of the region, it has been the subject of various population management strategies and human consumption has been considered. However, *C. argus* has been a highly cited species responsible for CP and because of this seafood poisoning risk, there is no fishery for this species. A better understanding of the CP risk through a sampling effort and an investigation into the underlying environmental conditions were investigated here. Currently, no predictive capabilities exist to prevent CP, the most prominent seafood intoxication reported worldwide. Two sampling sites were investigated, representing distinct Windward and Leeward environmental conditions, to identify if these conditions have an impact on the accumulation of CTXs in *C. argus.*

2.4.1. Location as a factor for CTX1B in *C. argus*

Environmental tolerance restricts many diseases, sources of illness, and their vectors to strict geographic regions, or regions of endemicity. CP is endemic in tropical and subtropical shallow marine

environments with the ideal conditions for CTX-producing microalgae (e.g., temperature [345], salinity [346], light [347], water energy [142]), which control toxin production and distribution [71,81,150]) and their toxin vectors. These environmental conditions are determinants of CTX production and extend to the CTX content of benthic habitats and eventually accumulate in animals targeted for human consumption [77,81,104,150,208,348,349]. Areas that are consistently linked with CP are identified by fishers and colloquially described as 'hot-spots', or geographic areas of CTX production. *C. argus* is a site-attached predator, with a home range under three kilometers (sometimes <100 m^2) [350]. Site attached predators can therefore be used as an indicator of the underlying local CTX production in the food web (i.e., useful for identifying 'hot-spots'). CTX-levels in *C. argus* were significantly different among sites, supporting the fishers described understanding of the existence of micro-regional differences influencing CTX's production and accumulation. The identification of 'hot spots' can help focus sampling efforts to study the environmental/biological drivers underlying CTX production (e.g., what is driving CTXs at a site).

Hawai'i encompasses a diverse range of climatic, geological, and oceanographic conditions, ideal for studying distinct ecological and biological processes [351,352], which are capable of benefiting or inhibiting *Gambierdiscus* and *Fukuyoa* and or their vectors. The sites investigated here were geographically close in proximity yet dissimilar for oceanic environmental drivers (e.g., temperature (±3-4 °C), wave energy (3.2x), freshwater input (7x), nutrient runoff, benthic habitat structure, water quality, and fishing pressures [353]). Conditions at the Leeward location were presumed to be more conducive to the growth and distribution of CTX-producing species by having lower wave energy [142,354], warmer temperatures, and a typical oceanic salinity [137,142]). And indeed, studies have shown that *Gambierdiscus* abundance per gram of algae was found to be significantly higher at the Leeward side [76]. Cell abundance may not directly translate into CTXs per gram of fish tissue, however, in this study CTX levels in the fish viscera were observed to be higher at the Leeward location (where cell counts were demonstrably higher). CTXs are metabolized after toxin ingestion and detected first in the liver (often at higher concentrations), then moved throughout the body leading to tissue sequestration and finally, depuration [188,355]. The higher ratios of toxin in the viscera (e.g., by 435x) observed herein could indicate recently consumed prey with CTXs (indicative of local CTX production) and a potential future progression of CTXs into the flesh [59]; a latent risk for the portion of fish typically targeted for human consumption. Conversely, the low salinity [137,212,345], higher wave energy [142], and a moderately lower temperature [345] of the Windward site constitutes environmental conditions that are worse for *Gambierdiscus*; and the Windward site had lower CTX levels in fish, by all measurements.

CTX1B was confirmed by LC-MS/MS (Figure 17) in 33% (29/89) of N2a-assay positive samples (n = 89), or 26% of all samples, reaffirming the designation of *C. argus* as a species of concern for CP

and Hawai'i as a hyperendemic region for CP. The toxin concentration per gram of flesh in samples from the Leeward location was significantly higher than in samples from the Windward location (p <0.001), in support of the local CP knowledge. The maximum concentration measured was 75-fold above the FDA guidance level for Pacific CTXs (0.75 ng CTX1B eq. g^{-1}), a concern for the severity of a potential acute CP outbreak. Taking the fish's size into account, the total toxin content in edible muscle tissue per fish was also significantly higher (p <0.001) in the Leeward location, a relevant concern among the two locations if larger portion sizes are consumed [46].

The pattern of geographic predictability for CP risk has been discussed by fish harvesters in a variety of regions where CP is prevalent [33,183,185,186,313,320]. Recently Oshiro et al. (2021) identified variability for CTXs (qualitatively and quantitatively) among fish species and capture locations in the tropical West Pacific [184]. Regarding geographic predictors observed in this study that could be applied to theirs, the prevailing current for the island (i.e., proximity to higher water movement) was closest to their 'Zone-1' which had no detectable CTXs [184,356]. Furthermore, in the Caribbean, sampling areas with a stronger wave power coincided with less CTX content in commercial fish (e.g., CTXs were absent where wave energy was highest) [185]. The Caribbean study sites and this study have completely different CTX producing species complexes, CTX chemical/skeletal structures, biological food webs, and environmental regions, yet draw similar conclusions: 1. Confirmation of the knowledge of local fishers regarding predictability for regional differences in CP risk (i.e., 'hot-spots), and 2. Geographic predictors, within a hyperendemic region for CP, based on wave energy (as a dominant factor) can translate into quantifiable differences in the CTX content of fish, in a predictable manner (higher energy resulted in lower toxicity in two direct distinct studies and one indirect study). However, most fish in the Windward region (predicted by fishers to have a lower risk for CP) still exceeded the guidance for CP, which may be an unfortunate hallmark of the inherent risk of CP in a hyperendemic region.

2.4.2. Fish size as a factor for CTX1B in *C. argus*

Fish size is a commonly applied CP risk predictor (in addition to location) used by various fisheries resource managers [309,324,357]. The relationship of CTXs and size have been previously shown [39,296,358] and here additional evidence of this relationship is provided. The utility of an observable fish morphometric for resource managers is important because accurate testing for CTXs can only be conducted by a limited number of analytical laboratories using cost-prohibitive and time-intensive methods. Toxin accumulation for a fish can be continuous but masked by somatic growth dilution throughout its life [189], a description of total toxin content based on the amount of edible flesh can help provide a more holistic toxin profile approach to CTXs. This information is also relevant for epidemiological and consumption data [185,359].

As *C. argus* grow and mature, they undergo an ontogenetic shift, consuming larger prey of a higher trophic position [317], and in hyperendemic regions, fish size and CTX content can be associated [185]. Diet studies of *C. argus* found prey items consisted of 97% reef fish and 2% crustaceans with a positive relationship between body size and the size of prey consumed [317,360]. Therefore, larger *C. argus* consume larger prey and if the prey also has a CTX to body size relationship, then this can result in the observed larger fish's higher CTX content, i.e., fish size (as an indicator of prey selection/hunting success) and CTX content being correlated. Fish collected from the Leeward side of the island were in general larger than those collected on the Windward side, both for length and weight (Figure 18); a similar finding to that of Friedlander et al. [353]. This difference may be due to several factors including physical (e.g., habitat complexity), biological, anthropogenic (e.g., marine reserve), true size class differences among locations, or behavioral differences between these communities. On the Leeward side there is a fear of harvesting toxic fish due to CP and a reduction in fishing pressure leads to a fish stock consisting of larger and older fish, a potential reason why fish were larger at the Leeward location. A reduced fishing pressure can also desensitize larger fish from perceiving human divers as a threat, resulting in greater fishing success for capturing larger individuals. Growth to length ratios was significantly higher at the Leeward location (p = 0.004, Leeward mean 29.7 g cm^{-1}, Windward 24.0 g cm^{-1}), these measurements were substantiated by Donovan et al. [361] who also found that *C. argus* collected at the Windward side (Hilo) weighed significantly less at a given length than the Leeward region (among other sampling locations). It is unknown if this is due to the aforementioned release from fishing pressure or a resource availability factor. The stomach contents of *C. argus* from both locations were examined in this study and all Windward site specimens contained either various crustaceans or detritivore and invertivore fish species. Nine watersheds, most with impaired waters, drain into Hilo Bay, where steep-sloped land and high rainfall create a high risk for soil erosion that can smother near-shore corals and macroalgae, creating an ideal habitat for invertebrates. Because *C. argus* may not be feeding primarily on herbivores, this prey selection could be distancing Windward fish from a trophic dynamic containing the higher oxidized CTX1B, which was less prevalent in the Windward location. The Leeward location has more benthic algae and subsequently more herbivorous fishes. Therefore, as a region, the food web is expected to contain the oxidized CTX1B, and indeed more fish from this region contained CTX1B in their muscle tissueflesh. Gut contents of the Leeward specimens contained herbivorous fishes in the families *Scaridae, Pomocentridae,* and *Acanthuridae*; additionally, they had also consumed crustaceans and invertivore fishes (a commonality with the Windward site). While this was a small sample size, it may still indicate a situation similar to that described by Rongo et al. [362] where the availability or presence of high densities of herbivorous fish were strong predictors of CP; an indicator of the successful transfer of benthic algal toxins into the food web. The Leeward site had significantly higher amounts of toxins and contained more fish with

CTX1B. If the presence of herbivores is an indicator of successful CTX transfer from microalgae into the food web, the toxin detected in the flesh and the gut contents with more herbivores on the leeward site are in support of Rongo's observations.

Fish size is a commonly utilized screening method as an indicator for a lower risk for CP in small fish, however individual fish in this study below 25 cm TL, from both sites, were found to exceed the FDA guidance limit. Smaller fish may lack the total amount of tissue to result in a CP outbreak, as Yasumoto 2005 recommends not exceeding 70 ng of CTX1B in one meal [46]. The smallest fish measured only had 44 g of available tissue and at the concentrations of toxin measured this 70 ng level would not have been reached. It has been shown that larger fish can contain more CTXs, they have the size to reach a higher maximum body burden of CTXs, but this does not preclude smaller individuals from the capability for accumulating sufficient CTXs to exceed the guidance limit; particularly in regions hyperendemic for CP. Furthermore, consumers could eat more fish to make up for this size limitation, negating the small fish tissue limitation. However, low-dose long-term exposure to CTXs and the potential for human health impacts are unknown.

2.4.3. Sampling challenges and analysis considerations

Several questions, spanning several topics, remain to be addressed in future studies. Previous studies investigating the influence of CTXs on the piscivorous consumer raised the question of whether CTXs influence behavior of fish. If CTXs are a behavior modifier then it is important to understand if this is imparting bias in the effort to collect specimens, because if a fishes avoidance behavior is reduced than that could result in fish with higher levels of CTXs being captured more frequently when spearfishing, as conducted in this study. Fortunately, this bias (if true) is accurately represented by the selection process an actual fisher experiences or may encounter.

The sampling of *Gambierdiscus* in both regions both for species diversity and abundance would help provide a CTX production context into the observations of this study. However benthic cell counts do not translate directly into the CTX content measured in fish found in the same location. There is a lot of uncertainty with regards to CTX production within species of Gambierdiscus as well as among species. Trophic transfer mechanisms, sources and sinks of CTXs are all in need of further elucidation and a wider variety of species have been identified for future investigations. Therefore, while this study was limited in its species selection the observations made can help guide future investigations and sampling efforts to identify the underlying biological responses to the driving environmental conditions observed among locations.

On the CTX analysis side, several issues remain. Yogi et al. in 2011 [161] and 2014 [169] investigated the composite toxin profile of fish from the Pacific and successfully identified 16 distinct CTX type compounds, however, standards are not commercially available for these compounds. In

samples from Hawai'i, they identified CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, 2,3,51-trihydroxyCTX3C, 2,3-dihydroxyCTX3C, 2-hydroxyCTX3C, and CTX3C. Therefore, the possibility exists here for the presence of other structurally related toxins (i.e., CTX isomers and congeners) in the samples of this study, without or concurrently with CTX1B, contributing to the toxicity measured in the N2a assay. The N2a MTT-assay method is indiscriminate for sodium channel (Na_v) activating compounds and the results are interpreted as a composite response resulting from all compounds contained within the sample causing an impact on the Na_v of the cells. In future studies, bioassay-guided fractionation could be used to help determine the retention time of other compounds that are active Na_v modifiers that are contained within the samples. However, unless a substantial amount of these fractions is collected and investigated, they will remain broadly categorized as CTX-like compounds. CTX3C was not detected by the LC-MS/MS analysis in any of the samples. This could be due to a low concentration of this toxin in these samples, or due to another factor such as the extraction protocol, which differed from that used in other studies, identifying additional congeners [156,161,169]. However, to justify the focus on CTX1B: previous reports [32] describe CTX1B as serving as the primary and most abundant [363] regional biomarker, with a history of structural confirmation of CTX1B in other fish species, and their corresponding meal remnants associated with human illnesses [133,169] and in fatalities [35].

Because CTXs accumulate throughout the body of a fish each part of the fish can be a useful biological data point that can be used to provide insight into the pathways of CTX accumulation. These data points could be age (through otolith analysis), fish size (by standard morphometrics), analysis of gut contents [140] for fish prey information and dietary shifts due to prey availability or ontogeny (stable isotopic analysis to determine trophic feeding level, location, or habitat utilization type), fecundity by egg production/weight (impact of CTXs on fish reproduction), and the liver, viscera, brain for bioaccumulation and metabolism data. Using these data points and correlating them with CTXs provides insights into CTX sequestration, metabolism, and CP-related fish morphometrics (fish size and toxin content). Monitoring efforts that account for these data sources could investigate various portions of the fish and apply a hindcast approach to the environmental conditions leading up to the CTX content observed. Analysis of the skin could provide insight into the local CTX burden of the environment in the past (as CTXs are sequestered in the skin last), toxin content of the flesh would indicate current CP risk (as the targeted portion for human consumption), and the liver is indicative of the fishes most recent meal and the CTX content of that meal thus representing the future toxin burden that will be metabolized and moved throughout the body of the fish. Furthermore, these measurements when correlated with monitoring *Gambierdiscus* for CTXs/cell, cells/area could provide insight into the movement of CTXs in a food web.

2.5. Conclusions

Invasive species can cause broad economic, ecological, and human health impacts, particularly in regions where the invasive species can carry a risk for CP [253,364]. The invasive species *C. argus* has been implicated in CP outbreaks in Hawai'i and the findings of this study support the conclusion that this species is a significant risk for CP in the region. The cooperative approach working with local fishers successfully determined a significant difference in the content of CTXs in *C. argus* based on a historically recognized geographic predictor which was determined to be based on an oceanic exposure gradient. These findings are supported by prior studies investigating oceanic exposure as a predictor for CTXs in the Caribbean, providing additional evidence that this may be a potentially significant factor for researching the CTX content in food fish species, as well as the importance of local fishing knowledge. Most fish sampled exceeded the FDA guidance level for CTXs, however, these levels are not monitored for pre-harvest screening controls. The current understanding of CP, in general, is that differences in life history and diet result in only a limited number of fish, within a defined region, becoming contaminated at levels capable of causing illness. However, this study found a high number of *C. argus* containing CTXs or a significant hot-spot. The current control available in the US is a requirement that primary seafood processors recognize and avoid purchasing fish from known or emerging areas of concern. Therefore, this information fills an existing knowledge gap for the regional severity and frequency of CTXs in *C. argus* and provides resource managers with a remotely observable method of prediction for sampling for CTXs here or in other CP endemic regions. The results of this study add to the growing body of evidence for indirect CTX predictors applicable in a hyperendemic region. The top-down sampling approach provides immediate feedback for CP in specific harvest locations and fish morphometrics can be a useful tool for categorizing rough estimates of CP risk. Current CTX and CP management strategies are methods of prohibition and prevention (species, area). However, predictive capabilities are desirable for fishery stakeholders. The observations described herein regarding the accuracy of geographic predictors, mainly proximity to an oceanic exposure gradient when harvesting fish, will therefore help refine future sampling protocols to focus their efforts to enhance the understanding and underlying conditions of areas prone to fish capable of CP. Additionally, a set of reference samples of *C. argus* is an invasive territory were cataloged for future reference and comparisons. The results of this study support the status of *C. argus* as a CP risk species CP in Hawai'i and that geographical differences for CTX content exist.

2.6. Bibliography

2. Gudger, E.W. Poisonous fishes and fish poisonings, with special reference to ciguatera in the West Indies. *The American Journal of Tropical Medicine and Hygiene* **1930**, *1*, 43-55.
32. Dickey, R.W.; Plakas, S.M. Ciguatera: A public health perspective. *Toxicon* **2010**, *56*, 123-136, doi:DOI 10.1016/j.toxicon.2009.09.008.
33. Lewis, R.J. The changing face of ciguatera. *Toxicon* **2001**, *39*, 97-106, doi:http://dx.doi.org/10.1016/S0041-0101(00)00161-6.
35. Hamilton, B.; Whittle, N.; Shaw, G.; Eaglesham, G.; Moore, M.R.; Lewis, R.J. Human fatality associated with Pacific ciguatoxin contaminated fish. *Toxicon* **2010**, *56*, 668-673, doi:http://dx.doi.org/10.1016/j.toxicon.2009.06.007.
39. U.S. Food Drug Administration. *Fish and Fishery Products Hazards and Controls Guidance Fourth Edition - March 2020*; https://www.fda.gov/media/80637/download (Accessed 15 October 2020), 2020; p 498.
46. Yasumoto, T. Chemistry, etiology, and food chain dynamics of marine toxins. *Proceedings of the Japan Academy, Series B* **2005**, *81*, 43-51.
59. Loeffler, C.R.; Tartaglione, L.; Friedemann, M.; Spielmeyer, A.; Kappenstein, O.; Bodi, D. Ciguatera Mini Review: 21st Century Environmental Challenges and the Interdisciplinary Research Efforts Rising to Meet Them. *International Journal of Environmental Research and Public Health* **2021**, *18*, 3027.
71. Chinain, M.; Darius, H.T.; Ung, A.; Cruchet, P.; Wang, Z.; Ponton, D.; Laurent, D.; Pauillac, S. Growth and toxin production in the ciguatera-causing dinoflagellate Gambierdiscus polynesiensis (Dinophyceae) in culture. *Toxicon* **2010**, *56*, 739-750, doi:http://dx.doi.org/10.1016/j.toxicon.2009.06.013.
76. Parsons, M.L.; Preskitt, L.B. A survey of epiphytic dinoflagellates from the coastal waters of the island of Hawai'i. *Harmful Algae* **2007**, *6*, 658-669, doi:http://dx.doi.org/10.1016/j.hal.2007.01.001.
77. Tester, P.A.; Litaker, R.W.; Berdalet, E. Climate change and harmful benthic microalgae. *Harmful Algae* **2020**, *91*, 101655, doi:https://doi.org/10.1016/j.hal.2019.101655.
81. Litaker, R.W.; Holland, W.C.; Hardison, D.R.; Pisapia, F.; Hess, P.; Kibler, S.R.; Tester, P.A. Ciguatoxicity of Gambierdiscus and Fukuyoa species from the Caribbean and Gulf of Mexico. *PLOS ONE* **2017**, *12*, e0185776, doi:10.1371/journal.pone.0185776.
104. Rossignoli, A.E.; Tudó, A.; Bravo, I.; Díaz, P.A.; Diogène, J.; Riobó, P. Toxicity Characterisation of Gambierdiscus Species from the Canary Islands. *Toxins* **2020**, *12*, 134.
133. Abraham, A.; Jester, E.L.E.; Granade, H.R.; Plakas, S.M.; Dickey, R.W. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chem* **2012**, *131*, 192-198, doi:https://doi.org/10.1016/j.foodchem.2011.08.059.
136. Dickey, R.W. *Ciguatera toxins: chemistry, toxicology, and detection*, Second ed.; New York., 2008; Vol. 173, pp. 22.
137. Xu, Y.; Richlen, M.L.; Liefer, J.D.; Robertson, A.; Kulis, D.; Smith, T.B.; Parsons, M.L.; Anderson, D.M. Influence of Environmental Variables on Gambierdiscus spp.(Dinophyceae) Growth and Distribution. *PloS one* **2016**, *11*, e0153197.
142. Richlen, M.L.; Lobel, P.S. Effects of depth, habitat, and water motion on the abundance and distribution of ciguatera dinoflagellates at Johnston Atoll, Pacific Ocean. *Marine ecology progress series* **2011**, *421*, 51-66.
150. Longo, S.; Sibat, M.; Viallon, J.; Darius, H.T.; Hess, P.; Chinain, M. Intraspecific Variability in the Toxin Production and Toxin Profiles of In Vitro Cultures of Gambierdiscus polynesiensis (Dinophyceae) from French Polynesia. *Toxins* **2019**, *11*, 735.

156. Spielmeyer, A.; Loeffler, C.R.; Bodi, D. Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. *Toxins* **2021**, *13*, 630.
161. Yogi, K.; Oshiro, N.; Inafuku, Y.; Hirama, M.; Yasumoto, T. Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from the Pacific. *Analytical Chemistry* **2011**, *83*, 8886-8891, doi:10.1021/ac200799j.
169. Yogi, K.; Sakugawa, S.; Oshiro, N.; Ikehara, T.; Sugiyama, K.; Yasumoto, T. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. *Journal of AOAC INTERNATIONAL* **2014**, *97*, 398-402.
183. Chinain, M.; Gatti, C.M.i.; Ung, A.; Cruchet, P.; Revel, T.; Viallon, J.; Sibat, M.; Varney, P.; Laurent, V.; Hess, P., et al. Evidence for the Range Expansion of Ciguatera in French Polynesia: A Revisit of the 2009 Mass-Poisoning Outbreak in Rapa Island (Australes Archipelago). *Toxins* **2020**, *12*, 759.
184. Oshiro, N.; Tomikawa, T.; Kuniyoshi, K.; Ishikawa, A.; Toyofuku, H.; Kojima, T.; Asakura, H. LC–MS/MS Analysis of Ciguatoxins Revealing the Regional and Species Distinction of Fish in the Tropical Western Pacific. *Journal of Marine Science and Engineering* **2021**, *9*, 299.
185. Loeffler, C.R.; Robertson, A.; Flores Quintana, H.A.; Silander, M.C.; Smith, T.B.; Olsen, D. Ciguatoxin prevalence in 4 commercial fish species along an oceanic exposure gradient in the US Virgin Islands. *Environmental toxicology and chemistry* **2018**, *37*, 1852-1863, doi:10.1002/etc.4137.
186. Olsen, D.A.; Nellis, D.W.; Wood, R.S. Ciguatera in the Eastern Caribbean. *Marine Fisheries Review* **1984**, *46*, 13-18.
188. Li, J.; Mak, Y.L.; Chang, Y.-H.; Xiao, C.; Chen, Y.-M.; Shen, J.; Wang, Q.; Ruan, Y.; Lam, P.K.S. Uptake and Depuration Kinetics of Pacific Ciguatoxins in Orange-Spotted Grouper (*Epinephelus coioides*). *Environmental Science & Technology* **2020**, 10.1021/acs.est.9b07888, doi:10.1021/acs.est.9b07888.
189. Clausing, R.J.; Losen, B.; Oberhaensli, F.R.; Darius, H.T.; Sibat, M.; Hess, P.; Swarzenski, P.W.; Chinain, M.; Dechraoui Bottein, M.-Y. Experimental evidence of dietary ciguatoxin accumulation in an herbivorous coral reef fish. *Aquatic Toxicology* **2018**, *200*, 257-265, doi:https://doi.org/10.1016/j.aquatox.2018.05.007.
208. Litaker, R.W.; Vandersea, M.W.; Faust, M.A.; Kibler, S.R.; Nau, A.W.; Holland, W.C.; Chinain, M.; Holmes, M.J.; Tester, P.A. Global distribution of ciguatera causing dinoflagellates in the genus Gambierdiscus. *Toxicon* **2010**, *56*, 711-730, doi:http://dx.doi.org/10.1016/j.toxicon.2010.05.017.
212. Kibler, S.R.; Litaker, R.W.; Holland, W.C.; Vandersea, M.W.; Tester, P.A. Growth of eight Gambierdiscus (Dinophyceae) species: Effects of temperature, salinity and irradiance. *Harmful Algae* **2012**, *19*, 1-14, doi:10.1016/j.hal.2012.04.007.
218. Chinain, M.; Darius, H.T.; Ung, A.; Fouc, M.T.; Revel, T.; Cruchet, P.; Pauillac, S.; Laurent, D. Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). *Toxicon* **2010**, *56*, 674-690, doi:DOI 10.1016/j.toxicon.2009.05.032.
232. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC INTERNATIONAL* **1995**, *78*, 521-527, doi:https://doi.org/10.1093/jaoac/78.2.521.
253. Soliño, L.; Widgy, S.; Pautonnier, A.; Turquet, J.; Loeffler, C.R.; Quintana, H.A.F.; Diogène, J. Prevalence of ciguatoxins in lionfish (Pterois spp.) from Guadeloupe, Saint Martin, and Saint Barthélmy Islands (Caribbean). *Toxicon* **2015**, *102*, 62-68.
296. Sydney Fish Market. Sydney fish market seafood handing guidelines Availabe online: https://www.sydneyfishmarket.com.au/Seafood-Trading/Quality/Food-Safety (accessed on 06.10.2020).

306. Disease Outbreak Control Division. Historical Data of Reported Cases of Notifiable Diseases for State of Hawaii, Honolulu County, Hawaii County, Kauai County, and Maui County (1990-2014). State of Hawaii, D.o.H., Ed. 2014.
307. Disease Outbreak Control Division. 2015 Summary of Reported Cases of Notifiable Diseases by County, State of Hawaii. State of Hawaii, D.o.H., Ed. 2015.
308. Disease Outbreak Control Division. 2018 Summary of reported cases of notifiable diseases by county, State of Hawaii. Hawaii, D.O.H.S.o., Ed. The WEB SITE Copyright © 1995-2020 State of Hawai'i: 2018.
309. Chan, W.H.; Mak, Y.L.; Wu, J.J.; Jin, L.; Sit, W.H.; Lam, J.C.W.; Sadovy de Mitcheson, Y.; Chan, L.L.; Lam, P.K.S.; Murphy, M.B. Spatial distribution of ciguateric fish in the Republic of Kiribati. *Chemosphere* **2011**, *84*, 117-123, doi:http://dx.doi.org/10.1016/j.chemosphere.2011.02.036.
310. Mak, Y.L.; Wai, T.-C.; Murphy, M.B.; Chan, W.H.; Wu, J.J.; Lam, J.C.; Chan, L.L.; Lam, P.K. Pacific Ciguatoxins in Food Web Components of Coral Reef Systems in the Republic of Kiribati. *Environmental science & technology* **2013**, *47*, 14070-14079.
311. Dierking, J.; Campora, C.E. Ciguatera in the Introduced Fish Cephalopholis argus (Serranidae) in Hawai'i and Implications for Fishery Management. *Pacific Science* **2009**, *63*, 193-204.
312. Courtenay, W.; Collette, B.; Essington, T.E.; Hilborn, R.; Orr, J.W.; Pauly, D.; Randall, J.E.; Smith-Vaniz, W.F. Risks of introductions of marine fishes: reply to Briggs. **2009**.
313. Bienfang, P.; DeFelice, S.; Laws, E.; Wallsgrove, N.; Caldwell, P. Ciguatoxicity in the main Hawaiian Islands: spatial and temporal variability in the introduced reef carnivore Cephalopholis Argus. *J Res Environ Sci Toxicol* **2012**, *1*, 47-57.
314. Randall, J.E. Introductions of marine fishes to the Hawaiian Islands. *Bulletin of Marine Science* **1987**, *41*, 490-502.
315. Dierking, J.; Birkeland, C.; Walsh, W. Feeding biology of the introduced fish roi (Cephalopholis argus), and its impact on Hawaiian reef fishes and fisheries. *NOAA Final report* **2005**.
316. Dierking, J. Effects of the introduced predatory fish Cephalopholis argus on native reef fish populations in Hawaii. UNIVERSITY OF HAWAII, 2007.
317. Meyer, A.L.; Dierking, J. Elevated size and body condition and altered feeding ecology of the grouper Cephalopholis argus in non-native habitats. *Marine Ecology Progress Series* **2011**, *439*, 203-212.
318. Giddens, J.; Friedlander, A.M.; Conklin, E.; Wiggins, C.; Stamoulis, K.; Donovan, M.K. Experimental removal of the invasive peacock hind (roi) Cephalopholis argus, in Puakō, Hawai 'i: methods for assessing and managing marine invasive species. *Marine Ecology Progress Series* **2014**, *511*, 209-221.
319. Anderson, B.; Sims, J.; Wiebenga, N.; Sugi, M. The epidemiology of ciguatera fish poisoning in Hawaii, 1975-1981. *Hawaii medical journal* **1983**, *42*, 326-334.
320. Xu, Y.; Richlen, M.L.; Morton, S.L.; Mak, Y.L.; Chan, L.L.; Tekiau, A.; Anderson, D.M. Distribution, abundance and diversity of Gambierdiscus spp. from a ciguatera-endemic area in Marakei, Republic of Kiribati. *Harmful algae* **2014**, *34*, 56-68, doi:https://doi.org/10.1016/j.hal.2014.02.007.
321. Soliño, L.; Costa, P.R. Global impact of ciguatoxins and ciguatera fish poisoning on fish, fisheries and consumers. *Environmental research* **2020**, *182*, 109111.
322. Yong, H.L.; Mustapa, N.I.; Lee, L.K.; Lim, Z.F.; Tan, T.H.; Usup, G.; Gu, H.; Litaker, R.W.; Tester, P.A.; Lim, P.T., et al. Habitat complexity affects benthic harmful dinoflagellate assemblages in the fringing reef of Rawa Island, Malaysia. *Harmful Algae* **2018**, *78*, 56-68, doi:https://doi.org/10.1016/j.hal.2018.07.009.
323. Kibler, S.R.; Tester, P.A.; Kunkel, K.E.; Moore, S.K.; Litaker, R.W. Effects of ocean warming on growth and distribution of dinoflagellates associated with ciguatera fish poisoning in the Caribbean. *Ecological Modelling* **2015**, *316*, 194-210.
324. Dierking, J.; Campora, C.E. Ciguatera in the introduced fish *Cephalopholis argus* (Serranidae) in Hawai'i and implications for fishery management. *Pacific Science* **2009**, *63*, 193-204, doi:10.2984/049.063.0203.

325. NOAA. Mapping of Benthic Habitats for The Main Eight Hawaiian Islands. U.S. Dept. of Commerce, N.O.a.A.A., National Ocean Serice, National Centers for Coastal Ocean Science, Center for Coastal Monitoring and Assessment, Ed. U.S. Dept. of Comm.: 1305 East West Highway N/SCI1, 9th Floor Silver Spring, MD 20910, 2007; p 107.
326. Giambelluca, T.W., Q. Chen, A.G. Frazier, J.P. Price, Y.-L. Chen, P.-S. Chu, J.K. Eischeid, and D.M. Delparte,. Online Rainfall Atlas of Hawai'i. *Bull. Amer. Meteor. Soc.* **2013**, *94*, 313-316, doi:10.1175/BAMS-D-11-00228.1.
327. Frazier, A.G.; Giambelluca, T.W.; Diaz, H.F.; Needham, H.L. Comparison of geostatistical approaches to spatially interpolate month-year rainfall for the Hawaiian Islands. *International Journal of Climatology* **2016**, *36*, 1459-1470, doi:10.1002/joc.4437.
328. NOAA. Coastal Water Temperature Guide. Availabe online: https://www.nodc.noaa.gov/access/ (accessed on April 20).
329. Wedding, L.M.; Lecky, J.; Gove, J.M.; Walecka, H.R.; Donovan, M.K.; Williams, G.J.; Jouffray, J.-B.; Crowder, L.B.; Erickson, A.; Falinski, K., et al. Advancing the integration of spatial data to map human and natural drivers on coral reefs. *PLOS ONE* **2018**, *13*, e0189792, doi:10.1371/journal.pone.0189792.
330. The WAVEWATCH III Development Group. User manual and system documentation of WAVEWATCH III version 5.16. *Tech. Note 329, NOAA/NWS/NCEP/MMAB* **2016**, 326.
331. Stopa, J.; Ardhuin, F.; Stutzmann, E.; Lecocq, T. Sea State Trends and Variability: Consistency Between Models, Altimeters, Buoys, and Seismic Data (1979-2016). *Journal of Geophysical Research. Oceans* **2019**, 10.1029/2018jc014607, doi:10.1029/2018jc014607.
332. Stopa, J.E.; Filipot, J.-F.; Li, N.; Cheung, K.F.; Chen, Y.-L.; Vega, L. Wave energy resources along the Hawaiian Island chain. *Renewable Energy* **2013**, *55*, 305-321.
333. Li, N.; Cheung, K.F.; Stopa, J.E.; Hsiao, F.; Chen, Y.-L.; Vega, L.; Cross, P. Thirty-four years of Hawaii wave hindcast from downscaling of climate forecast system reanalysis. *Ocean Modelling* **2016**, *100*, 78-95.
334. Loeffler, C.R.; Abraham, A.; Stopa, J.E.; Flores Quintana, H.A.; Jester, E.L.E.; La Pinta, J.; Deeds, J.; Benner, R.A.; Adolf, J. Ciguatoxin in Hawai'i: Fisheries forecasting using geospatial and environmental analyses for the invasive Cephalopholis argus (Epinephelidae). *Environmental Research* **2021**, https://doi.org/10.1016/j.envres.2021.112164, 112164, doi:https://doi.org/10.1016/j.envres.2021.112164.
335. Kaneko, J.; Takenaka, B.; Bartram, P. Keeping Hawaii Seafood Safe to Eat. NOAA, Ed. PacMar, Inc.: Honolulu, Hawaii, 2005; p 28.
336. U.S. Food and Drug Administration. The Seafood List: Scientific Name Cephalopholis argus. Availabe online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=SeafoodList&id=Cephalopholis_argus (accessed on
337. Smithsonian National Fish Collection. Fish Collections Data at National Museum of Natural History. Availabe online: https://collections.nmnh.si.edu/search/fishes/ (accessed on April 11th).
338. Administration, U.S.F.D. Reference Standard Sequence Library (RSSL) for Seafood Identification for Cephalopholis argus. Availabe online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=RSSL_seafood_barcode_data&id=FDA_294&sort=&order=ASC&startrow=1&type=basic&search=294 (accessed on 11.04.2021).
339. Handy, S.M.; Deeds, J.R.; Ivanova, N.V.; Hebert, P.D.; Hanner, R.H.; Ormos, A.; Weigt, L.A.; Moore, M.M.; Yancy, H.F. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *Journal of AOAC International* **2011**, *94*, 201-210.
340. Deeds, J.R.; Handy, S.M.; Fry, F., Jr.; Granade, H.; Williams, J.T.; Powers, M.; Shipp, R.; Weigt, L.A. Protocol for building a reference standard sequence library for DNA-based seafood identification. *Journal of AOAC International* **2014**, *97*, 1626-1633, doi:10.5740/jaoacint.14-111.

341. FDA. Reference Standard Sequence Library for Seafood Identification (RSSL). Availabe online: https://www.fda.gov/food/dna-based-seafood-identification/reference-standard-sequence-library-seafood-identification-rssl (accessed on April 11 2021).
342. Pacific Islands Ocean Observing System. Projects : Ocean Tipping Points: Hawai'i Case Study. Availabe online: http://www.pacioos.hawaii.edu/projects/oceantippingpoints/#data (accessed on April 20).
343. Administration, U.S.F.D. Reference Standard Sequence Library (RSSL) for Seafood Identification of Cephalopholis argus sample FDA 295. Availabe online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=RSSL_seafood_barcode_data&id=FDA_295&sort=&order=ASC&startrow=1&type=basic&search=295 (accessed on 11.04.2021).
344. Donovan, M.; Friedlander, A.; DeMartini, E.; Donahue, M.; Williams, I. Demographic patterns in the peacock grouper (Cephalopholis argus), an introduced Hawaiian reef fish. *Environ Biol Fish* **2013**, *96*, 981-994, doi:10.1007/s10641-012-0095-1.
345. Yoshimatsu, T.; Yamaguchi, H.; Iwamoto, H.; Nishimura, T.; Adachi, M. Effects of temperature, salinity and their interaction on growth of Japanese Gambierdiscus spp. (Dinophyceae). *Harmful Algae* **2014**, *35*, 29-37, doi:https://doi.org/10.1016/j.hal.2014.03.007.
346. Tawong, W.; Yoshimatsu, T.; Yamaguchi, H.; Adachi, M. Temperature and salinity effects and toxicity of Gambierdiscus caribaeus (Dinophyceae) from Thailand. *Phycologia* **2016**, *55*, 274-278, doi:10.2216/15-111.1.
347. Yoshimatsu, T.; Tie, C.; Yamaguchi, H.; Funaki, H.; Honma, C.; Tanaka, K.; Adachi, M. The effects of light intensity on the growth of Japanese Gambierdiscus spp. (Dinophyceae). *Harmful Algae* **2016**, *60*, 107-115, doi:https://doi.org/10.1016/j.hal.2016.10.009.
348. Pisapia, F.; Holland, W.C.; Hardison, D.R.; Litaker, R.W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Sechet, V.; Amzil, Z., et al. Toxicity screening of 13 Gambierdiscus strains using neuro-2a and erythrocyte lysis bioassays. *Harmful algae* **2017**, *63*, 173-183, doi:10.1016/j.hal.2017.02.005.
349. Bravo, I.; Rodriguez, F.; Ramilo, I.; Rial, P.; Fraga, S. Ciguatera-Causing Dinoflagellate Gambierdiscus spp.(Dinophyceae) in a Subtropical Region of North Atlantic Ocean (Canary Islands): Morphological Characterization and Biogeography. *Toxins* **2019**, *11*, 423.
350. Meyer, A.L. *An ecological comparison of Cephalopholis argus between native and introduced populations*; 2008.
351. Friedlander, A.M.; Parrish, J.D. Habitat characteristics affecting fish assemblages on a Hawaiian coral reef. *Journal of Experimental Marine Biology and Ecology* **1998**, *224*, 1-30, doi:https://doi.org/10.1016/S0022-0981(97)00164-0.
352. Grigg, R.W. Holocene coral reef accretion in Hawaii: a function of wave exposure and sea level history. *Coral Reefs* **1998**, *17*, 263-272, doi:10.1007/s003380050127.
353. Friedlander, A.M.; Brown, E.K.; Jokiel, P.L.; Smith, W.R.; Rodgers, K.S. Effects of habitat, wave exposure, and marine protected area status on coral reef fish assemblages in the Hawaiian archipelago. *Coral Reefs* **2003**, *22*, 291-305, doi:10.1007/s00338-003-0317-2.
354. Tindall, D.R.; Morton, S.L. Community dynamics and physiology of epiphytic/benthic dinoflagellates associated with ciguatera. *NATO ASI SERIES G ECOLOGICAL SCIENCES* **1998**, *41*, 293-314.
355. Kohli, G.S.; Haslauer, K.; Sarowar, C.; Kretzschmar, A.L.; Boulter, M.; Harwood, D.T.; Laczka, O.; Murray, S.A. Qualitative and quantitative assessment of the presence of ciguatoxin, P-CTX-1B, in Spanish Mackerel (Scomberomorus commerson) from waters in New South Wales (Australia). *Toxicology Reports* **2017**, *4*, 328-334, doi:https://doi.org/10.1016/j.toxrep.2017.06.006.
356. Eastwood, E.K.; López, E.H.; Drew, J.A. Population Connectivity Measures of Fishery-Targeted Coral Reef Species to Inform Marine Reserve Network Design in Fiji. *Scientific Reports* **2016**, *6*, 19318, doi:10.1038/srep19318.
357. Sanchez-Henao, A.; García-Álvarez, N.; Silva Sergent, F.; Estévez, P.; Gago-Martínez, A.; Martín, F.; Ramos-Sosa, M.; Fernández, A.; Diogène, J.; Real, F. Presence of CTXs in moray eels and

dusky groupers in the marine environment of the Canary Islands. *Aquatic Toxicology* **2020**, *221*, 105427, doi:https://doi.org/10.1016/j.aquatox.2020.105427.

358. Chan, T.Y.K. Ciguatoxic Potential of Brown-Marbled Grouper in Relation to Fish Size and Geographical Origin. *The American Journal of Tropical Medicine and Hygiene* **2015**, 10.4269/ajtmh.15-0434, doi:10.4269/ajtmh.15-0434.
359. Hossen, V.; Soliño, L.; Leroy, P.; David, E.; Velge, P.; Dragacci, S.; Krys, S.; Quintana, H.F.; Diogène, J. Contribution to the risk characterization of ciguatoxins: LOAEL estimated from eight ciguatera fish poisoning events in Guadeloupe (French West Indies). *Environmental research* **2015**, *143*, 100-108.
360. Dierking, J.; Williams, I.D.; Walsh, W.J. Diet composition and prey selection of the introduced grouper species peacock hind (Cephalopholis argus) in Hawaii. *Fishery Bulletin* **2009**, *107*, 464-476.
361. Donovan, M.K.; Friedlander, A.M.; DeMartini, E.E.; Donahue, M.J.; Williams, I.D. Demographic patterns in the peacock grouper (Cephalopholis argus), an introduced Hawaiian reef fish. *Environ Biol Fish* **2013**, *96*, 981-994, doi:10.1007/s10641-012-0095-1.
362. Rongo, T.; van Woesik, R. The effects of natural disturbances, reef state, and herbivorous fish densities on ciguatera poisoning in Rarotonga, southern Cook Islands. *Toxicon* **2013**, *64*, 87-95, doi:http://dx.doi.org/10.1016/j.toxicon.2012.12.018.
363. Legrand, A.M.; Fukui, M.; Cruchet, P.; Yasumoto, T. Progress on chemical knowledge of ciguatoxins. *Bull Soc Pathol Exot* **1992**, *85*, 467-469.
364. Hardison, D.R.; Holland, W.C.; Darius, H.T.; Chinain, M.; Tester, P.A.; Shea, D.; Bogdanoff, A.K.; Morris, J.A., Jr.; Flores Quintana, H.A.; Loeffler, C.R., et al. Investigation of ciguatoxins in invasive lionfish from the greater Caribbean region: Implications for fishery development. *PLOS ONE* **2018**, *13*, e0198358, doi:10.1371/journal.pone.0198358.

Chapter 3. Improving in vitro ciguatoxin and brevetoxin detection by selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS)

Significance Statement

The detection of CTXs is paramount to understanding their presence in outbreaks and tracking their movement through the food web. However, because CTXs are biologically relevant (impactful) at levels that are difficult to detect, even the most advanced and sensitive analytical methods (e.g., LC-MS/MS) can fail to detect CTXs when they are present in seafood. Therefore, there is a need to improve CTX detection to provide adequate consumer protection by CTX detection in food and the environment. Furthermore, low dose chronic exposure to CTXs can go undetected due to a lack of acute impacts, however, CTX long-term human health implications cannot be excluded. Additionally, within the European Union's (EU) commercial market, the regulation regarding health standards for fish products (EC No 853/2004) states that food business operators must ensure that fishery products placed on the market for human consumption do not contain CTXs. Furthermore EC No. 2019/627 Ch. I.G.3 states that controls are to take place to ensure fishery products containing 'ciguatera' are not placed on the market [45,365-367]. This regulation is irrespective of a concentration, therefore the most sensitive detection methods possible should be employed for CTX detection, which currently is the N2a-MTT assay. Therefore, the motivation for this chapter was to improve upon the most sensitive method yet described for CTX detection. To accomplish this goal the current method was established at both the German Federal Institute for Risk Assessment (BfR) and the University of Napoli Federico II and then this method was modified with a cell selection process for a lower sensitivity to ouabain (O) and veratridine (V) to improve the sensitivity of detection.

3.1. Introduction

Marine neurotoxins of the class ciguatoxin (CTX) and brevetoxin (PbTx) are natural products produced by various phytoplankton (dinoflagellates) from the genera *Gambierdiscus*, *Fukuyoa*, and *Karenia*, respectively [17,94,368] . CTXs and PbTxs are two groups of potent toxins that share a chemical feature, both being lipid-soluble polycyclic polyether compounds with a high affinity and specificity for the Na_V [369]. The impact of these toxins on the Na_V is characterized pharmacologically by persistent activation, blocking inactivation, increasing

neuronal excitability, and neurotransmitter release, impairing synaptic vesicle recycling, and causes cellular swelling [224,370]. Human poisonings due to the ingestion of CTX or PbTx contaminated seafood can be severe, and the clinical presentations can include, but are not limited to, gastrointestinal, neurological, and cardiovascular symptoms [275,371,372]; PbTxs can be aerosolized, leading to respiratory symptoms such as cough, dyspnea, and bronchospasm [373]. Human illness due to the ingestion of CTX contaminated seafood products (primarily finfish) is classified as CP and is the most frequently reported seafood-toxin illness worldwide [23] (see section 1.3). PbTxs are transmitted in shellfish, therefore the clinical description of the illness is Neurotoxic Shellfish Poisoning (NSP), however, the epidemiology of NSP is not well documented and remains a relatively rare disease [374].

NSP management and prevention are achieved mainly through environmental monitoring for algal blooms and controls before the harvest of shellfish [375]. Blooms of PbTx producing *K. brevis* are usually large, causing discoloration of the water, and are easily visible from shore or through aerial monitoring; these events are known as Red Tides. CTX producing dinoflagellates do not cause visible blooms and the toxins produced undergo metabolic modifications as explained in Chapters 1 and 2. Therefore CP prevention and management is more difficult, prevention efforts are reliant on local CTX knowledge regarding the location of capture (e.g., harvest location [39,185,218,309,313,376], species-specific association with illness in the region [52,186,296,324,377]), the reporting and outreach of CP events to human health agencies, as well as efforts for public education, and awareness[23,378-380]. The management of these toxin classes is important for Small Island Developing States (SIDS) and their artisanal fisheries, which are vulnerable to seafood insecurity and depend on a secure local seafood supply [381,382].

To detect CTXs and PbTxs, biological assays such as the N2a-assay (see section 1.6) based on the common mode of action for these groups of compounds are often used to serve as a screening method. The N2a assay relies on exposure to the pharmaceuticals ouabain (O) and veratridine (V) for its sensitivity and specificity to Na_v activating compounds such as CTXs and PbTxs, and in the absence of O and V the cells are insensitive to CTXs and BTXs. Neuro-2a cells rely on primary active transport to maintain a low intracellular Na^+ concentration (against the concentration gradient) in order to create a membrane potential (or membrane voltage). The difference in electric potential between the inside and outside the cell is used to transmit a signal, where the increase in Na^+ permeability is responsible for the rising, depolarizing, phase of the action potential spike; allowing intercellular signaling. Neuro-2a cells maintain this gradient through the actions of a sodium-potassium pump (Na^+ K^+ activated ATPase). The pretreatment of Neuro-2a cells with V initiates the Na_v into an open state allowing Na^+ uptake into the cell which causes repetitive electrical signal firing and persistent activation of sodium channels, the addition of O inhibits the ion transport activity of the plasma membrane Na^+/K^+ -ATPase via binding to its catalytic α-subunit (inhibiting the sodium-potassium pump) [383,384].

Without the actions of the sodium-potassium pump cells cannot exchange internal Na^+ for external K^+ against the concentration gradient in order to re-establish an action potential. BTX and CTX group toxins bind to the V activated Na_v channels and increase the ion flux; further exacerbating the intracellular sodium concentration, resulting in dose dependent cell mortality, and hence the assays specificity [249]. The N2a-assay, when functioning within the prescribed parameters, is useful, highly sensitive, and specific for screening purposes, but has yet to be validated [229,250,251].When performing the N2a-assay as it is described in the literature, for the detecting CTXs and PbTxs, consistent reductions in cell survival were observed between 10-80% in O/V control samples, the survival rate was dependent on the O/V concentration described [251,304,385,386]. In addition to the importance of O/V, there is a range of other parameters applied in the N2a-assay that can also influence cell survival as outlined in Viallon, Chinain and Darius [231]. Therefore, cell mortality due to the design of the assay was found to be a confounding factor for distinguishing cellular toxicity sources, which required further elucidation.

The various instrumental, biological, and chemical methods used for the detection of marine biotoxins can encounter problems regarding reliability, ease of use, transferability of analytical procedures, and ultimately detection at human health-relevant concentrations. Descriptions of how to perform the N2a-assay in the literature face a wide range of conditions (e.g. number of cells, amount of O/V, media components), because this method has yet to be validated [228,387,388], and a consensus protocol for all other confounding factors have not been identified. Therefore, the basic conditions of performing the N2a-assay were standardized across all the experiments described herein (i.e., cell line maintenance, assay, and development conditions) to optimize assay sensitivity the focus of this investigation was restricted only to the effects of O and V. Here, a strategy is investigated for overcoming an observed problem of cell oversensitivity to O and V through a selection process designed to reduce the sensitivity of the N2a cell line. An a *priori* target of using 0.22/0.022 mM O/V during the cytotoxicity assay [102,133,136,228,389,390] was used. This concentration is roughly double the rate most commonly reported to induce 0-20% mortality (0.13-0.08 mM O and 0.013-0.005 mM V) [26,81,176,183,190,231,244,251,253,289,299,357,359,385,387,391-397], but at approximately half the higher concentrations reported to induce 50-80% mortality (0.5/0.05 mM O/V) [229,252,313,376]. A cell survival range was used as a guideline to identify which conditions were best suited for evaluating sensitivity and detection performance factors. The method and process described herein use newly purchased N2a cells with the intention that this method and process applies to both; laboratories that have no prior experience or infrastructure, as well as laboratories experienced in the use of the N2a-assay method (i.e., novice or expert laboratories for the N2a-assay). The technique's specificity and sensitivity were tested using CTX3C (pure and in a matrix) and Brevetoxin-3 (*K. brevis*) standard solutions, as well as CTX1B, purified from naturally incurred fish tissue. These three compounds

represent a range of sourced compounds that are commercially available, CTX3C was synthesized, PbTx-3 was purified from algae, and CTX1B was extracted from fish tissue and is representative of a bio-oxidation-end-product derived from its analog CTX4A, produced by *Gambierdiscus* [129]. This method for pharmacological modification of a newly purchased, commercially available, Neuro-2a cell line will allow laboratories to overcome issues with sensitivity when using O and V and to improve the performance of this assay with the intention for the routine analysis for detecting CTX3C, CTX1B, and Brevetoxin-3 (PbTx-3) (and other Na_v activating compounds). Therefore, to accomplish these goals several approaches were used: I) Establishing a range of acceptable cell survival as a quality assurance parameter to ensure the suitability of a cell line to the concentration of O and V used in the N2a-assay, II) Modifying the cell line to obtain a cell line with lower sensitivity to O/V (this modified cell line will be called OV-LS hereafter) to match the quality assurance parameters, III) Assessing the performance of the OV-LS cell line for the detection of sodium channel activating toxins, IV) Comparing the OV-LS and N2a cell lines based on the performance of detecting CTXs and PbTx-3.

3.2 Materials and Methods

3.2.1 Materials and reagents

All cell line work was performed in a Class II microbiological safety cabinet (model Claire® B-3-160, Berner International GmbH, Elmshorn, Germany). Two CB-60 incubators from BINDER GmbH (Tuttlingen, Germany) were used during this study; one solely for culture maintenance, and one for the assay. Both incubator conditions (37 °C and 5% CO_2 atmosphere) were the same. Consumables including serological pipettes, C-Chip disposable Hemacytometer, filter capped culture flasks, ninety-six-well polystyrene plates (Corning™ 3596), methanol, hexane, chloroform, and water were purchased from Fisher Scientific GmbH (Schwerte, Germany). Bond elut silica solid-phase extraction (SPE) cartridges were obtained from Agilent Technologies (California, USA). Mouse (*Mus musculus*) neuroblast type cells, cell line Neuro-2a (ATCC® CCL-131™) were purchased from the American Type Culture Collection LGC Standards GmbH (Wesel, Germany) from the lot numbered: 63649750, which was frozen 24 February 2016 at passage number 184. Heat-inactivated fetal bovine serum (FBS), Roswell Park Memorial Institute media(RPMI-1640), glutamine, sodium pyruvate, penicillin-streptomycin, 10X Trypsin-EDTA and reagents for N2a-assay, specifically, ouabainoctahydrate, veratridine hydrochloride, phosphate-buffered saline, dimethyl sulfoxide, HPLC grade water, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Phosphate Buffered Saline (PBS) was prepared fresh as follows: 16 g NaCl, 400 mg KCl, 2.3g Na_2HPO_4, and 400 mg KH_2PO_4 were dissolved in 2 liters of HPLC grade water; the solution was autoclaved and stored at 4°C. Trypsin working solution (2.0 mL in 38 mL PBS) was

used to remove the cells from the culture flask (e.g. for passaging or plating). MTT stock solution was prepared by dissolving 500 mg MTT in 100 ml PBS. CTX3C standard (Lot #TWJ6482, CAS Number 148471-85-6) was purchased from FUJIFILM Wako Chemicals Europe GmbH (Neuss, Germany) and dissolved in MeOH at 1 pg μL^{-1}. Brevetoxin PbTx-3, (*Karenia brevis* CAS-no: 85079-48-7, Cat. No. 203734-100 µg) was purchased from Merck Chemicals GmbH (Darmstadt, Germany) and dissolved in MeOH at 10 ng μL^{-1}. The CTX1B standard (provided by Professor R.J. Lewis, The Queensland University, Australia, prepared November 2005) was used as a reference toxin for the assessment of CTX-like toxicity and dissolved in MeOH at 1 pg μL^{-1}. All standards were maintained at -20 °C.

3.2.2 Cell handling procedure

Cell line handling procedures for frozen cells, subculturing procedure (1:4), and cryopreservation medium were followed per the manufacturer's guidelines [398] and specific product data sheet for Neuro-2a (ATCC® CCL131™). To prevent passage-specific cell line effects, both Neuro-2a and those with OV-LS were maintained for a maximum of 14 weeks or roughly 50 passages, from the start of the experiments. Culture growth maintenance media was supplemented with streptomycin (50 µg mL^{-1}), penicillin (50 units mL^{-1}), glutamine (2 mM), sodium pyruvate (1 mM), and FBS (10% *v/v*) [232]. Dosing assay media for cytotoxicity assays was made with RPMI prepared in the same manner as the culture growth media but with 5% FBS (*v/v)*.

Growth curves for cell lines were conducted by harvesting cells using trypsin to dislodge the cells, then a dilution factor (3-11x) was applied. A 10 µL aliquot of the dilution was used for enumeration using a C-Chip disposable Hemacytometer. Cells were counted under an inverted microscope at 10x. Cells mL^{-1} were then converted to cells per area by multiplying (volume in mL $flask^{-1}$ area in cm^2).

3.2.3 *In vitro* cytotoxicity assay

For the assay, cells were harvested when cultures were approximately 85 to 90% confluent (observed under an inverted microscope at 10x magnification), counted on a C-Chip disposable hemocytometer, and seeded at 4.0 x 10^4 cells $well^{-1}$ (200 mL volume) into sterile 96-well microplates and allowed to adhere overnight in the incubator. After the 24 h incubation period, the microplate cultures were examined microscopically to ensure confluent monolayers of cells were present before use. Microplate layout used for the *in vitro* cytotoxicity assay was as described in Dickey [136] Figure 19.

Aliquots of CTX3C, CTX1B, and PbTx-3 dissolved in methanol were dried by nitrogen stream in the safety cabinet, re-dissolved in 5% FBS-RPMI and vortexed to ensure the complete reconstitution for delivery to the culture wells. One-half of the interior wells (Figure 19, Rows E, F, G) received 10 µL

each of O (2.5 or 5 mM) and V (0.25 or 0.5 mM). Six of the 30 +O/V wells (column 2 and 3 rows E, F, G) received an additional 10 µL PBS and were used as the +O/V controls with no further treatment (column 2 and 3 rows B, C, D) as described in Dickey [136]. The remaining 30 interior wells received 20 µL PBS (-O/V wells) (Figure 19, Rows E, F, G). From each standard sample concentration 10 µL was added in triplicate to 24 wells of sensitized (0.1/0.01 or 0.22/0.022 mM O/V) (+O/V) and 24 wells of non-sensitized (–O/V) cells (Columns 4-11, Figure 19). Each well on the assay plate had a total final volume (including all additions) of 230 µL as described in Dickey [136]. The range of concentrations tested (in well) for CTX3C and CTX1B was 0.1-124 pg mL^{-1} and 0.1-124 pg mL^{-1}. The range of concentrations tested (in well) for PbTx-3 was 0.12-145 ng mL^{-1}. The microplate cultures were then incubated for 22-24 h. Samples were tested over a range of cell passage numbers, with a maximum cell passage number of 45 (post-OV-LS) to reduce cell passage number effects.

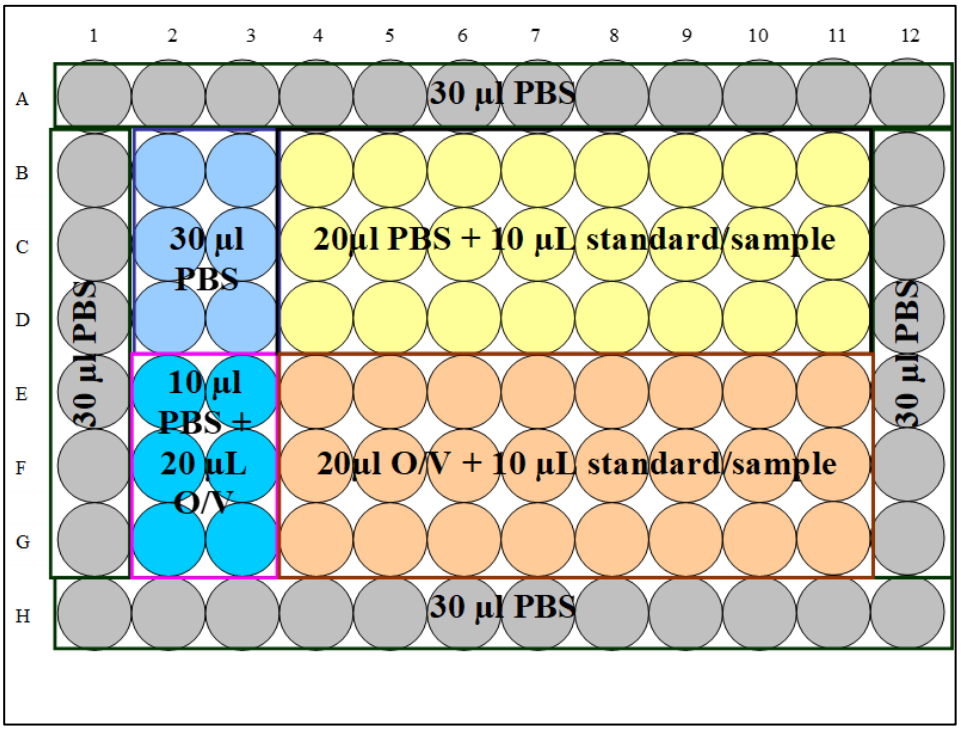


Figure 19. 96-well plate layout for compound additions for performing the *in vitro* cytotoxicity assay, adapted from Dickey 2008 [136].

3.2.4 Procedure for lowering the sensitivity of Neuro-2a cells to ouabain and veratridine

3.2.4.1 Initial tests and experimental approaches

Initial tests of sensitivity for two commonly described concentrations of O and V for the N2a cell line (0.1/0.01 or 0.22/0.022 mM O/V) were performed and various experimental approaches (e.g., O/V concentration and exposure duration) were attempted to lower the sensitivity of N2a cells to O

and V, as follows. During the cell line maintenance procedure, a subculture of original un-modified Neuro-2a (O/V-un) cells was transferred into a new flask containing RPMI growth medium supplemented with 10% FBS *v/v* and 0.33mM ouabain and 0.033mM veratridine, labeled OV-R (ouabain/veratridine-resistant), given a continuous passage number, and then returned to the incubator. Depending upon the treatment, after a 24-hr or a 72-hr exposure to +O/V conditions (cell line knockdown near 99%), the medium was removed, the cells were washed with PBS, and fresh growth medium without O/V was added to the flask (no trypsin step). The flask was then returned to the incubator. When the OV-R cell line reached near confluence (approximately five days for the seventy-two hour exposure) then the normal cell line maintenance procedure was resumed. The newly selected cell line was tested for OV-R performance at 0.22 mM ouabain and 0.022mM veratridine concentrations in a cytotoxicity assay format. After the resistance performance assessment, for the seventy-two hour cell line the OV-R selection process was repeated and the new line labeled OV-R 72hr-2x. Multiple OV-R modified lines (e.g., OV-R: 24-hr 1x, 72-hr-1x, and 72-hr-2x) were created to test the selection method for O/V resistance, and one original line was maintained, serving as an unmodified control cell line (0x or OV-un).

3.2.4.2 Final modification procedure for making the OV-LS cell line

The process of lowering the sensitivity of N2a cells to O and V was further refined to achieve an N2a-assay sensitivity of 60-80% at 0.22/0.022 mM O/V in a two-step process presented here. This final modification was labeled as OV-LS. In step one (using a new cell line undergoing normal passage and splitting procedures) the cells were passaged into a new flask containing growth media supplemented with a final concentration of 0.33 mM O and 0.033 mM V. The O/V concentration was achieved by adding 1 mL of 10 mM O solution and 1 mL of 1 mM V solution to a 75 cm^2 flask containing 28 mL of regular growth media with 1ml passaged cells, and maintained in the incubator for 72 hours. After 72 h, the growth medium with O/V was removed, the cells were washed with PBS (10 mL), and a fresh growth medium was added. The cells were allowed to recover for up to 120 h, or until confluence. In step two the cells were subjected to an additional 72 h 0.33 mM O and 0.033 mM V treatment period with the same recovery as step one (washing, replacing of the media, and 120 h recovery to confluence). The OV-LS process was replicated, in two independent laboratories, to determine OV-LS method repeatability with three reproductions of the process. OV-LS cells were cryogenically frozen to test durability, survival to the freezing process, and revived to test for maintaining OV-LS performance.

3.2.5 MTT-colorimetric assay evaluation

Following the procedure outlined in section 3.2.3, after the cytotoxicity assay incubation period, the treatment medium was removed by hand-flick, taking care to avoid dislodging the adherent cells. MTT plate development solution (0.83 mg mL^{-1} RPMI medium; 60 µL $well^{-1}$) was added, and the plates were returned to the incubator for 35 minutes, where viable cells with active metabolism converted the MTT into a purple-colored formazan product, which was accumulated as an intracellular and extracellular insoluble precipitate [399]. After the incubation period, the MTT plate development solution was removed by hand-flick and the formazan was solubilized in DMSO (100 µL $well^{-1}$) [136,247]. The plates were agitated (back and forth, side to side motion) to equally distribute the solubilized precipitate before measuring the absorbance in each well (Figure 20), including the wells without any additions (Figure 19, wells in grey color on the outside edge that boarder the plate), at 570 nm by a microplate spectrophotometer (Thermo Scientific Multiskan FC cat. No. 5119100). Cellular response to treatments was determined by measuring the intensity of formazan color development in viable +O/V cells following exposure to CTX3C, CTX1B, and PbTx-3. Absorbance in treated wells was expressed as a % of survival obtained in CBA-N2a assays as described and calculated in [136,396] Nonspecific cell activity was assessed in the wells where the sample was added without O and V (-O/V sample) [136]. To evaluate the outcome of the toxin addition, the effective concentration at 50% (EC_{50}) was defined by the Four Parameter Logistic Equation of SigmaPlot v14.0 as 'where the x value for the curve point that is midway between the max and min parameters'. Results were expressed in dry toxin standard weight per mL (e.g. pg mL^{-1}).

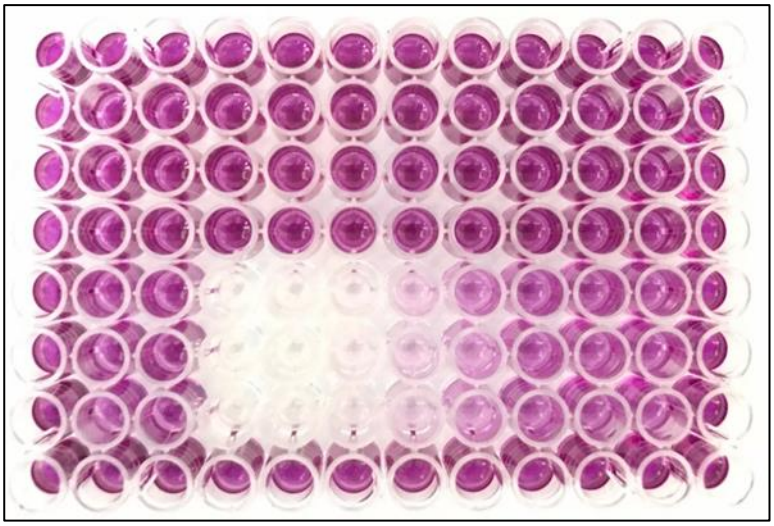


Figure 20. Photo of a 96-well plate post MTT development (image perspective looking down from above). Example plate shows a dilution dose-response for a typical Na_v active compound. Clear wells in the lower half of the plate indicate that no active metabolism was available in the cells to convert the MTT into a purple-colored formazan, indicating cell death. Purple color indicates active metabolism and normal function (Photo credit C. Loeffler).

3.2.6 Matrix evaluation for OV-LS

A whole snapper (*Lutjanus malabaricus*) was purchased, and a subsample of muscle tissue was excised without skin or bone. A 5.0 g aliquot (wet weight) of the fish muscle sample was extracted as previously described [136]. The fish tissue was homogenized with HPLC grade acetone (2 mL g^{-1} tissue) in 50 mL polypropylene centrifuge tube for one minute, using an ultra-turrax. The homogenate was centrifuged at 3000 rpm for 5 min and the acetone was collected with a pasteur pipette and transferred into a 20 mL glass tube. Using a spatula or dissecting needle, the tissue pellet was broken up for a second homogenization step. The homogenization step with acetone (2 mL g^{-1} tissue) was repeated for 1 min followed by a repeat of the centrifugation step. The acetone extracts were combined and chilled at -20 °C for at least 12 hours. After the 12 hours step, the extracts were centrifuged at 4 °C for 5 min at 3500 rpm. The supernatant was transferred into a clean glass tube using a Pasteur pipette. The acetone was removed by drying under nitrogen gas at 45 °C. Then 80% methanol in water (1 mL g^{-1} tissue) was added and the vessel vortexed for 15 seconds. The sample was defatted twice by washing with n-hexane (HPLC grade, 1.0 mL g-1 tissue), followed by vortexing for 30 seconds, and centrifugation for 5 min at 3500 rpm each time. The hexane phases (top layer) were discarded. The aqueous methanol was removed by drying with nitrogen at 45 °C. Water (1.0 mL g^{-1} tissue) was then added to the vessel. Chloroform (1.0 mL g^{-1} tissue) was then added, and the vessel was vortexed for 15 seconds, followed by centrifugation for a 10 min, 4 °C at 3500 rpm. The bottom chloroform layer was collected with a Pasteur pipette and transferred to a new glass test tube (e.g., 13 x 100 mm). The water phase was re-extracted two more times; by repeating the chloroform addition followed by vortex-centrifugation steps. The chloroform phases were combined and dried under nitrogen at 45 °C. A 500 mg Si-SPE cartridge was conditioned by first passing: 1 column volume of 5% water in methanol, 1 column volume of methanol and a least 1 column volume of chloroform (until it turns 80% translucent). The sample was dissolved in 50 µL of chloroform and applied to the column. The sample vessel was rinsed three times with the same volume of chloroform and each wash was applied to the column. The column was allowed to absorb the sample and then the column was washed with one column volume of chloroform. The chloroform wash was discarded. The sample was eluted with 2 column volumes of 10% methanol in chloroform and collected into a 13 x 100 mm glass test tube. The collected column elution was dried by nitrogen gas at 45 °C. The final product was capped, labeled, and the residue stored at -20 °C.

Final tissue residue was re-dissolved in 1 mL MeOH (5g TE mL^{-1}) and stored at -20 °C until analysis by the MTT-colorimetric assay. Screening for CTXs was performed and the sample was found to be negative for the presence of CTX-like activity. Therefore, this material was used as a CTX-blank matrix representative. The tissue equivalent (TE) mL^{-1} range used for analysis was 5.52-707 mgTE mL^{--1}. To this tissue matrix range of concentrations, 3.4 pg mL^{-1} of CTX3C was added, presenting a range of

detection for 0.0048 – 0.6182 pg CTX3C $mgTE^{-1}$ to evaluate the toxin response recognition (a constant) while in the presence of a serial dilution of matrix concentrations. This test was repeated in three independent 96-well microplates.

3.2.7 Statistical analyses

The differences in cell survival between OV-LS, N2a, and a control (no O/V exposure) when exposed to 0.1/0.01 mM O/V, respectively, during the assay, was investigated using a Kruskal-Wallis One-Way Analysis of Variance (ANOVA) on Ranked Data, post hoc comparisons were performed using Dunn's Multiple Comparison Test. A one-way ANOVA model was used to investigate the effects of the OV-LS selection process, the N2a, and the control cell line (no O/V exposure) on the survival of cells exposed to 0.22/0.022 mM O/V administered during the cytotoxicity assay. Data were first tested for homogeneity of variance and normality. Post hoc comparisons were performed using Tukey HSD tests. A one-way ANOVA model was used to investigate the repeatability of the OV-LS selection process based on cell survival to the application of 0.22/0.022 mM O/V administered during the cytotoxicity assay, data were first tested for homogeneity of variance and normality. An independent-samples t-test was conducted to compare the EC_{50} values for CTX3C, CTX1B, and PbTx-3 in N2a and OV-LS cell line conditions. Values for CTX3C, CTX1B, and PbTx-3 met the assumptions for independence, normality, and homogeneity of variance. For all experiments, results are presented as the mean ± SD and a value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SigmaPlot v14.0 (Systat Software, San Jose, CA).

3.3 Results

3.3.1 Induction of resistance (lower sensitivity) to ouabain and veratridine (OV-LS) in Neuro-2a cells

Growth curves of N2a cells and N2a OV-LS cells in RPMI containing 10% FBS were conducted. N2a and OV-LS cells were initially seeded at a density of 1.7×10^4 ($\pm 4.7 \times 10^3$) and 1.9×10^4 ($\pm 7.9 \times 10^3$), cells, respectively, per cm^2 and grown up to a density of 2.1×10^5 ($\pm 5.7 \times 10^4$) and 2.3×10^5 ($\pm 6.8 \times 10^4$) cells per cm^2. Data points represent mean ± standard deviation for Neuro-2a measurements at time point 0 (n = 3), 2 (n = 8), and 3 (n = 4), and for OV-LS N2a cells at time point 0 (n = 8), 1 (n = 3), 2 (n = 4), 3 (n = 4), and 4 (n = 3) where each n represents a separate growth flask serving as a replicate experiment. Experiments were conducted between passage numbers 217-235 and data can be found in Figure 21.

A series of tests were conducted to investigate what exposure conditions elicited the desired de-sensitization response in the cells when exposed to OV in the assay conditions. This testing and

optimization study can be found in Figure 22, Figure 23, Figure 24, Figure 25, Table 9, and Table 10. These conditions represent different OV exposure durations and concentration conditions as well as tests among the different conditions. Following this trial process, the OV-LS method was established as the ideal conditions capable of rapidly altering the cell line in a two-step process while achieving the desired sensitivity, the following results are based on this refined method deemed OV-LS.

Results are shown in Figure 22, Figure 23, Table 9, Table 10 (comparison of all test conditions). Concentration-response curves for Neuro-2a cells, when exposed to CTX3C, are represented by -OV (black symbols) and +OV (open symbols), conditions are presented in (a) percent survival vs. control and (b) raw absorbance values. Control at % survival lines represents the percent survival of the +OV/-OV control wells without the addition of CTX3C when the cell line was exposed to different starting cell viability background physiological conditions (100, 90, 70, 40, and 20% survival) created when exposed to 0.0/0.0, 0.05/0.005, 0.1/0.01, 0.15/0.015, and 0.22/0.022 mM O/V, respectively Figure 24. Figure 25 shows cell viability of controls when exposed to +OV (0.1/0.01 or 0.22/0.022 mM O/V) conditions compared to –OV control wells for each the N2a, OV-LS cell lines, as well as an unexposed control cell line representing full survival. Raw absorbance data was between the ranges of 0.1-1.4. Differences in the number of microplates shown (n values) are representative of the utility of the plates under those conditions.

The Neuro-2a cell lines that underwent the OV-LS process were irrevocably changed. Cells maintained their OV-LS characteristic (lower sensitivity to OV, increased CTX, and PbTx-3 detection sensitivity) over the utility of the cell line (proactively terminated when utility passage number reached a maximum 50, beyond the original manufactured starting number). The OV-LS process was reproducible, and the OV-LS characteristic was maintained through cryogenic freezing and revival process. The OV-LS line was demonstrated to detect CTX3C at 5x below the human health-relevant guidance level (0.01 ng CTX eq. g^{-1} TE) in a complex fish matrix.

Cell survival, when exposed to O/V, was used for defining quality control parameters, first for resistance (OV-LS) induction as well as for assay performance. Cell survival that is too low or high can lead to poor assay results. Poor assay results can stem from two opposing conditions: i) insufficient +O/V response (+O/V survival >80%), this can lead to an insensitive response, where no discernible changes in cell response were evident (only suggestive of toxicity) after the addition of a known standard (e.g. response at 90% survival Figure 24); ii) excessive +O/V response (+O/V survival <60%) where the addition of O/V in excess was found to cause a high cell death (e.g. control at 20% survival Figure 24), which was found to obscure a potential signal attributable to a known positive sample through oversaturation of the concentration-response curve. Therefore, for the O/V conditions used it is important to report the percentage of cell survival to ensure assay conditions and results can be replicated among cell lines, laboratories, and users. When cell lines were tested at their ideal O/V

conditions (where cell survival was acceptable), occasionally assays performed unfavorably with O/V control survival rates falling outside the 60-80% cutoff points. This occurred more frequently for the N2a line (13%, 8/64) than the OV-LS (7.7%, 15/232). The number of rejected assays should be kept as low as possible as they result in loss of material, resources, and time.

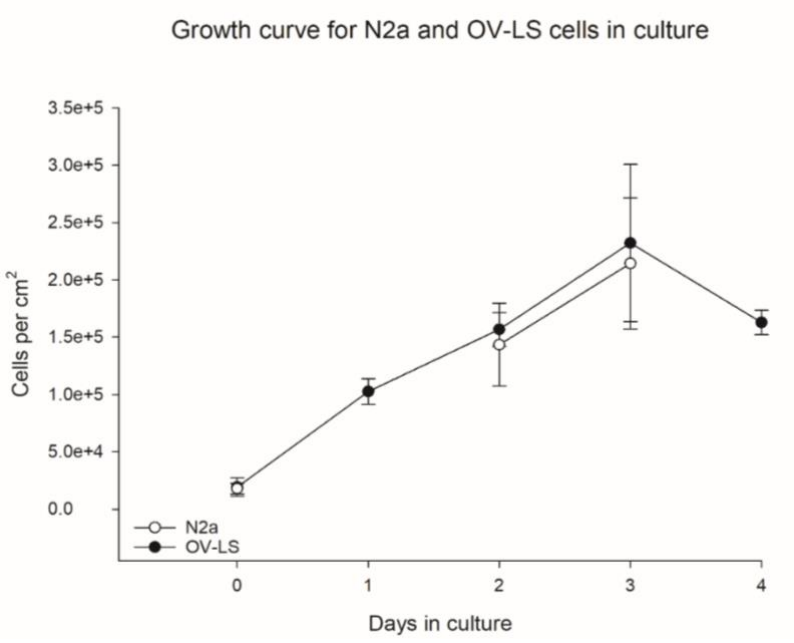


Figure 21. Growth curve of N2a cells (open circles) and OV-LS N2a cells (full circles) in RPMI containing 10% FBS. Cells were seeded at a density of 1.7 x 10^4 (±4.7x10^3) and 1.9 x 104 (±7.9x10^3), cells, respectively, per cm2 and grown up to a density of 2.1 x 10^5 (±5.7x10^4) and 2.3 x 105 (±6.8x104) cells per cm^2. Cells were harvested and enumerated. Points represent mean ± standard deviation for Neuro-2a measurements at time point 0 (n = 3), 2 (n = 8), and 3 (n = 4), and for OV-LS N2a cells at time point 0 (n = 8), 1 (n = 3), 2 (n = 4), 3 (n = 4), and 4 (n = 3) where each 'n' represents a separate growth flask serving as a replicate experiment from a starting time point of cell seeding. igure copyright information: Available online February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

To test sensitivity to O/V, newly purchased N2a cells and OV-LS N2a cell lines were exposed to 0.1/0.01 mM O/V, respectively, and mean cell survival was significantly different among the OV-LS, N2a, and control (unexposed to O/V) ($\chi^2(2) = 27.26$, $p < 0.001$). There was a high degree of difference between ranks for both the N2a vs control and the N2a vs OV-LS (Figure 25). The OV-LS cell line survival to 0.1/0.01 mM O/V was high (98% ±4.0 (n = 7)), indicating a lack of sensitivity to that concentration, compared to N2a cells (66% ±10.1 (n = 56)) [mean % survival ± SD and n = number of individual microplates], all in percent survival compared to –O/V control cells (Table 11, Figure 25). OV-LS cell

survival performance at 0.1/0.01 mM O/V was indistinguishable from cells unexposed to O/V (control 102% ±2.5 (n = 6)) and not significantly different (p = 1), indicating successful induction of resistance when exposed to the pharmaceuticals O/V (Figure 25).

The OV-LS cell line was unaffected by the addition of O/V at 0.1/0.01 mM. Therefore, the N2a cell line, the OV-LS, and an intermediate pre-OV-LS line (after 1-step of desensitization treatment) were exposed to 0.22/0.022 mM O/V to test their response. Percent cell survival, compared to their -O/V control cells, by modification method type was N2a 20.4 ±9.0 (n = 17), control (unexposed to OV) 102 ±2.5 (n = 6), pre-OV-LS 1-step 67 ±8.1 (n = 49), and OV-LS 71 ±7.9 (n = 91) (n = number of individual 96-well microplates) (Table 11, Figure 22, Figure 25, Figure 22). An ANOVA was conducted on the percent survival for cell lines exposed to 0.22/0.022 mM O/V and a strongly significant effect due to the cell line selection process was observed at the p <0.05 level (F(3,155) = 220.7, p = <0.001). A post hoc Tukey test showed that all groups differed significantly (p < 0.05), with the greatest difference in means (81.74) found between the control (without O/V) and the N2a line (highly sensitive to O/V at 0.22/0.022 mM). The lowest difference among average percent survival (3.98) was found for the pre-OV-LS tested after 1-step and OV-LS tested after completing the two-step selection process. However, this difference was still significant (p = 0.006). The difference of means between N2a and OV-LS (in this case labeled as 2x-R 72hr) was 50.4 (p < 0.001) as shown in Table 9, indicating a strong difference between the OV-LS modified cell line to the original N2a line. To investigate the stability of the OV-LS performance, N2a and pre-OV-LS (1-step) were mixed (1:1) and seeded in individual 96-well microplates at 40,000 cells per well and exposed to 0.22/0.022 mM O/V. The blended cell line had an average survival rate of 45% ±8.0, n = 5, a midway point between survival performance at this O/V concentration for the pre-OV-LS (1-step) (66.8%) and N2a line (20%). Cell line cryogenic freeze/thaw stability was tested over three years. Average percent survival for replicates revived was 70.1 ±5.5 (n = 89), 72.1 ±5.7 (n = 93), and 70.8 ±6.6 (n = 50), for 2018, 2019, and 2020, respectively (n = individual 96-well microplate assays). An ANOVA was conducted on the percent survival for these replicates of the OV-LS cell line exposed to 0.22/0.022 mM O/V and the differences in the mean values among the treatment replicates were not significantly different (F(2,229) = 2.43, p = 0.09).

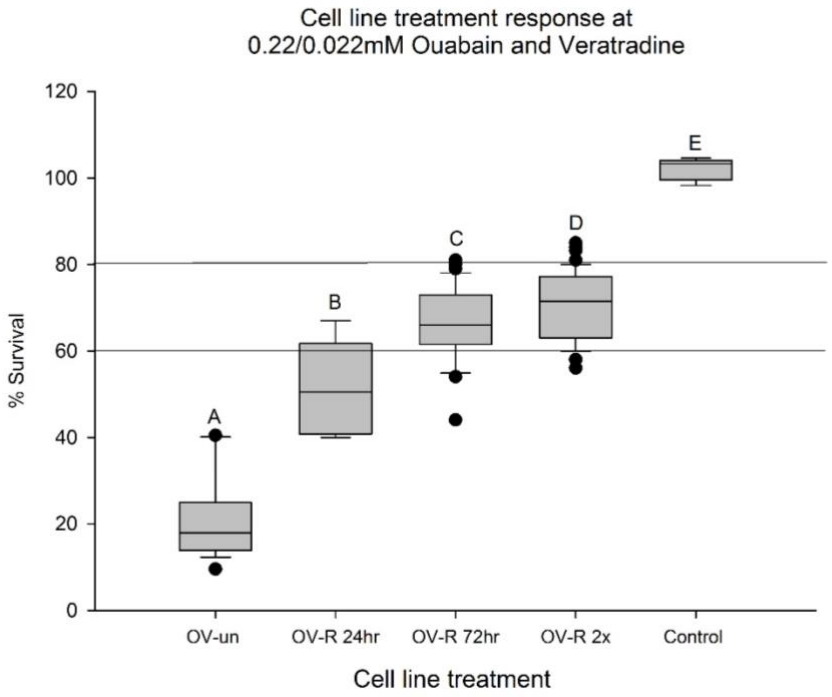


Figure 22. Neuro-2a cell response to a concentration of 0.22 mM ouabain (O) and 0.022 mM veratridine (V) administered during the assay, reported as 'percent survival' as indicated by the MTT test by cell line treatment (see section 3.2.5 for details). Control' was un-modified and un-exposed to O/V. Boxes indicate median and quartile ranges, whiskers delineate full ranges, black dots are maximum and minimum values Groups not sharing an uppercase letter are significantly different (Tukey's HSD; $p < 0.05$). Original (un = unmodified) N2a cell cultures underwent O and V sensitivity testing. N2a cells were exposed to 0.033 mM V and 0.33 mM O in their growth medium in 3 experimental conditions i) for 24hr (OV-R 24hr) ii) for 72hr (OV-R 72hr), iii) taking an OV-R 72hr cell line and exposing it for an additional 72hr (OV-R 2x), and iv) a control unexposed to OV. Cell lines were analyzed for cell sensitivity for exposure to 0.22/0.022 mM OV using the methods outlined in section 3.2.3 and 3.2.4 and results are displayed here in a box and whiskers plot. Different letters indicate significantly different sensitivity to 0.22/0.022 mM OV (Table 9).

Table 9. Results of all pairwise multiple comparison procedures (Tukey Test) , for the survival of cell lines to the concentration of O/V at 0.22/0.022 mM , respectively. All OV resistance test conditions are indicated with 'R', the 'OV-LS' nomenclature is only used for the final revised end process. Table copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

All Pairwise Multiple Comparison Procedures (Tukey Test)

Comparison	Diff of Means	p	q	P
R 72hr vs 2x-R 72hr	3.98	5	3.91	0.045
R 72hr vs R 24hr	15.3	5	6.21	<0.001
R 24hr vs. 2x-R 72hr	19.3	5	8.04	<0.001
un vs R 24hr	31.1	5	11.4	<0.001
Control vs. 2x-R 72hr	31.3	5	12.0	<0.001
Control vs. R 72hr	35.3	5	13.2	<0.001
R 72hr vs un	46.5	5	28.3	<0.001
un vs. 2x-R 72hr	50.4	5	32.6	<0.001
Control vs. R 24hr	50.6	5	14.7	<0.001
Control vs. un	81.7	5	28.0	<0.001

R = OV-Resistant cell line, un = OV-unmodified cell line, #x=number of O/V resistance selection procedures.

Table 10. Pre-OV-LS cell line modification comparisons tests. Cell response when attempting to desensitize N2a cells to OV. Resistance (R) steps were conducted as described in section 3.2.3. Each step, 1R-6R, represents a successive exposure of cells to the OV+ culture conditions. 0-R served as the control, was un-modified without any steps for resistance to OV. Descriptive statistics for treatment by desensitization to O and V. Mean % cell survival. Recovery post desensitization was 7-14 days. Cell confluency was achieved before re-testing survival rates. The sample size represents independent 96-well microplate assays. Table copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Resistance step	Mean % cell survival (±Std. Dev)	Std. error	C.I. of mean	Sample size
0R-1R	16 (±3.7)	1.0	2.2	13
2R-3R	31 (±6.3)	2.1	4.9	9
4R-5R	53 (±16)	3.9	8.4	16
6R	68 (±6.2)	0.6	1.2	101

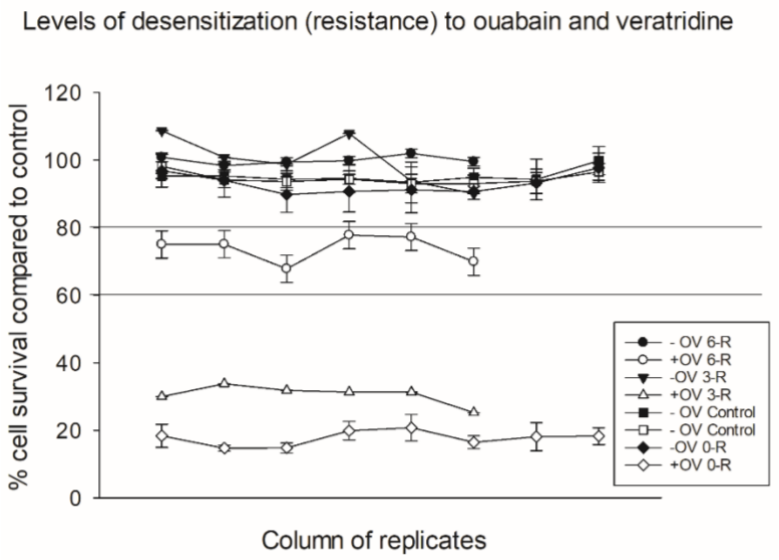
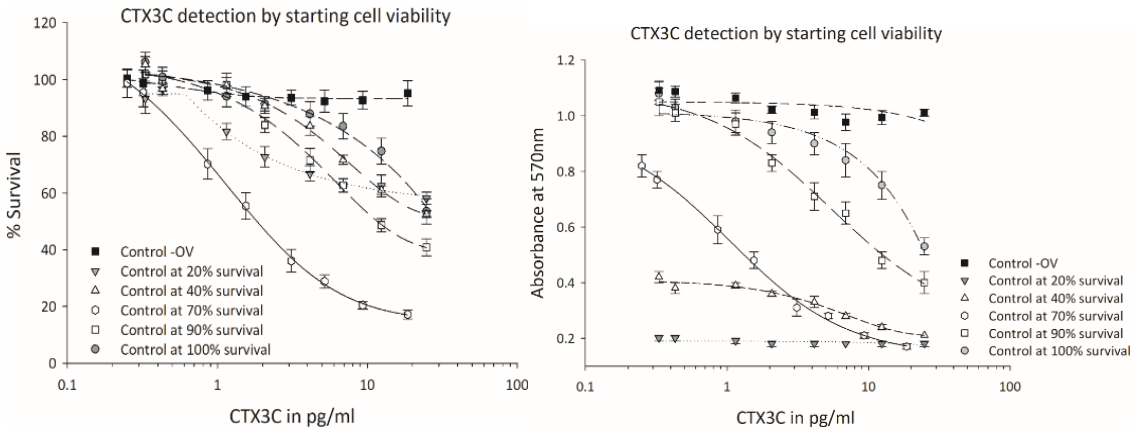


Figure 23. Cell survival to +O/V for 0-R, 3-R, and 6-R treatments. -O/V includes controls and cell survival control. Resistance (R) steps were conducted by subjecting N2a cells to 0.1 mM ouabain (O) and 0.01 mM veratridine (V) in culture for 72 hrs. Each step, 1R-6R, represents a successive exposure of cells to the OV+ culture conditions. After each exposure cells were exposed to a concentration of 0.22 mM O and 0.022mM V administered during the assay, with absorbance data reported as 'percent survival' as indicated by the MTT test by cell line treatment (see 'Materials and methods: 2.4 Cytotoxicity evaluation' for details). Figure copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

OV-LS survival after a two-step treatment process had an average survival of 71% and a standard deviation within the 60-80% cell survival with over 232 individual assays performed (Figure 25) which includes independent OV-LS cell line inductions. The repeatability of the OV-LS process was tested and an ANOVA was conducted on the percent survival when exposed to 0.22/0.022 mM O/V. The differences in the mean values among the treatment replicate (individual 96-well microplate assays) were not significantly different ($F(3, 55) = 1.5$, $p = 0.22$). Therefore, this process for the OV-LS line was selected as the best adapted to 0.22/0.022 mM exposure and further tested for its performance for detecting CTX3C, CTX1B, and PbTx-3s (Section 3.2.3). Additionally, the 72-hour selection process was repeated for the third time and the average percent survival rate was 75 ±5.4, n = 9, a rate where exceeding the established maximum survival rate (80%) became more likely and was decided to be excluded from further 0.22/0.022 mM OV assay tests.

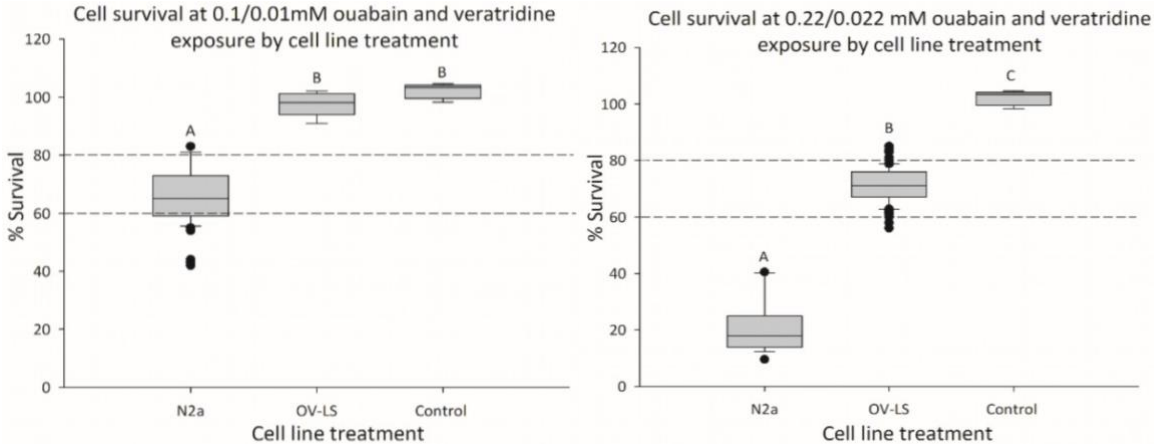
3.3.2 N2a-assay performance for detecting CTX3C, CTX1B, and PbTx-3; N2a vs. OV-LS cell lines

To determine the utility of the OV-LS cell line, two commercially available standards representing two distinct toxins (CTX3C and PbTx-3), and a naturally incurred purified standard commonly used in routine analyses, prepared by Professor R. Lewis (CTX1B), were tested against both the N2a and the OV-LS cell lines. The N2a cell line was exposed to 0.1/0.01 mM O/V and the OV-LS line was exposed to 0.22/0.022 mM O/V, both O/V conditions were selected due to their cell survival rate during the line modification and selection process (Table 9, Figure 25). The differences between the EC_{50} values obtained using the different cell lines were most evident for CTX3C where the O/V-LS cell line average was 1.35 pg mL^{-1} ±0.28 (mean ± SD, n = 25) and the N2a cell line was 3.52 pg mL^{-1} ± 0.27 (n = 5), with a greater than 2-fold increase in mean sensitivity ($t(28) = 15.56$, $p = <0.001$) (Table 12, Figure 26). There was a significant difference in the EC_{50} values for CTX1B between the OV-LS at 2.06 pg mL^{-1} ±0.65 (n = 36) and the N2a cell line 2.64 pg mL^{-1} ±1.0 (n = 14), a difference in means of 1.3, $t(48)= -2.4$, $p = 0.02$. There was a strongly significant difference in the EC_{50} values observed for PbTx-3, with the OV-LS at 3.04 ng mL^{-1} ± 0.90 (n = 22), which was 1.9 times more sensitive than the N2a cell line 5.88 ng mL^{-1} ± 1.48 (n = 8) ($t(28)= -5.1$, $p <0.001$). The number of replicates was variable due to two factors, the primary reason being that the reference material is restricted due to cost and limitation, and secondly that the regular N2a cell line performance has been replicated and investigated in various international studies while the OV-LS needed higher replicates to investigate the performance. Inter- and intra-assay variability was assessed for the concentration-response curves (EC_{50}) of N2a and OV-LS cells exposed to CTX3C, CTX1B, and PbTx-3 (Table 13). The OV-LS cells are capable of detecting 0.002 ng CTX3C per g^{-1} TE, this level of detection is at a concentration 5x below the recommended guidance limit of 0.01 ng CTX1B g^{-1} (Figure 27).



(a) (b)

Figure 24. Concentration-response of N2a cells to a CTX3C standard in -OV (black symbols) and +OV (open symbols) conditions presented in (a) percent survival vs. control and (b) raw absorbance values. Percent survival values are calculated from the raw absorbance values for each well. For control wells at percent survival (+OV/-OV)*100. For standards (e.g., CTX3C) where raw absorbance values for each well in +OV conditions are divided by –OV wells exposed to the same CTX3C concentration. Raw absorbance values are presented without normalization to control wells CTX3C (+OV/-OV)*100 (Figure 19). Symbols represent microplate conditions where Control at % survival lines represent the percent survival of the +OV/-OV control wells without the addition of CTX3C when the cell line was exposed to different background physiological conditions created by exposure to a concentration of +OV capable of reducing the physiological conditions of the cells to 100, 90, 70, 40, and 20 % survival, which was 0.0/0.0, 0.05/0.005, 0.1/0.01, 0.15/0.015, and 0.22/0.022 mM O/V respectively. Cells at these conditions were then exposed to increasing concentrations of CTX3C to investigate their performance for detecting concentrations of CTX3C. Data represent the mean ±SD of five independent 96-well microplate experiments (including three replicates per data point per experiment) for control survival at 20 (passage number 206-208), 40 (passage number 206-208), 90 (passage number 207-209), and 100% (passage number 214-216) and nine experiments for control at 70% survival (passage numbers 205-207, 215). Figure copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].



(a) (b)

Figure 25. Cell response, to the concentration of ouabain (O) and veratridine (V) administered during the N2a-assay , reported as 'percent survival' as indicated by the MTT test by cell line treatment (see section 3.2.5 for details). n= number of true replicates (independent 96-well microplate assays or 'plates'), 'Control' was un-modified and un-exposed to O/V. (a) cell lines exposed to 0.10/0.01 mM O/V, N2a (n = 56), OV-LS (n = 7), and Control (n = 6). (b) cell lines exposed to 0.22/0.022 mM O/V. N2a (n = 16), OV-LS (n = 232), Control (n = 5). Boxes indicate median and quartile ranges, whiskers delineate full ranges, black dots are maximum and minimum values. Raw absorbance values recorded in the MTT-assay before conversion to % survival were in the range from 0.12-1.4. Groups not sharing an uppercase letter are significantly different (Tukey's HSD; $p < 0.05$). Black dotted horizontal lines, set here at 60 and 80% survival, represent the *a priori* accepted range of cell line O/V sensitivity performance (see Figure 24 for performance examples). Figure copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Table 11. MTT colorimetric assay based mean cell survival represented in percent (% ± SD) and the number of individual samples (n = number of individual microplates) by cell line type when exposed to 0.10/0.01 mM or 0.22/0.022 mM ouabain (O) and veratridine (V), respectively. The 'control' cell line was un-modified and unexposed to O/V. The range of absorbance unit values from which the cell survival in percent was generated was 0.18-1.14. Table copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Applied concentration									
0.10/0.01 mM O/V					0.22/0.022 mM O/V				
Cell line	n	Mean % survival	Std Dev	Mean Absorb.	Cell line	n	Mean % survival	Std Dev	Mean Absorb.
N2a	56	65.8	10.1	0.743	N2a	16	20.4	8.99	0.18
OV-LS	7	97.5	4.04	1.09	OV-LS	232	71.1	7.85	0.795
Control	6	102	2.53	1.12	Control	5	102	2.53	1.14

Table 12. Results of an independent-samples t-test for cell line treatments (OV-LS and N2a) for the detection of various marine biotoxins using the N2a-assay. Mean (±SD) effective concentration at 50% survival (EC_{50}), n = number of individual microplate experiments. Ouabain (O)/Veratridine (V) lower sensitivity (OV-LS) cell line exposed to 0.22/0.022 mM O/V and N2a cell line exposed to 0.10/0.01 mM O/V. Table copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Cell line performance for marine biotoxin detection

Standard	EC_{50} OV-LS	Passage # range	EC_{50} N2a	Passage # range	Df	T	*p*
CTX3C (pg mL^{-1})	1.35±0.28 (n = 25)	195-231	3.52±0.27 (n = 5)	200-236	28	15.6	<0.001
CTX1B (pg mL^{-1})	2.06±0.65 (n = 36)	195-231	2.64±1.03 (n = 14)	200-236	48	-2.4	0.02
PbTx-3 (ng mL^{-1})	3.04±0.90 (n = 22)	211-239	5.88±1.48 (n = 8)	217-244	28	-6.4	<0.001

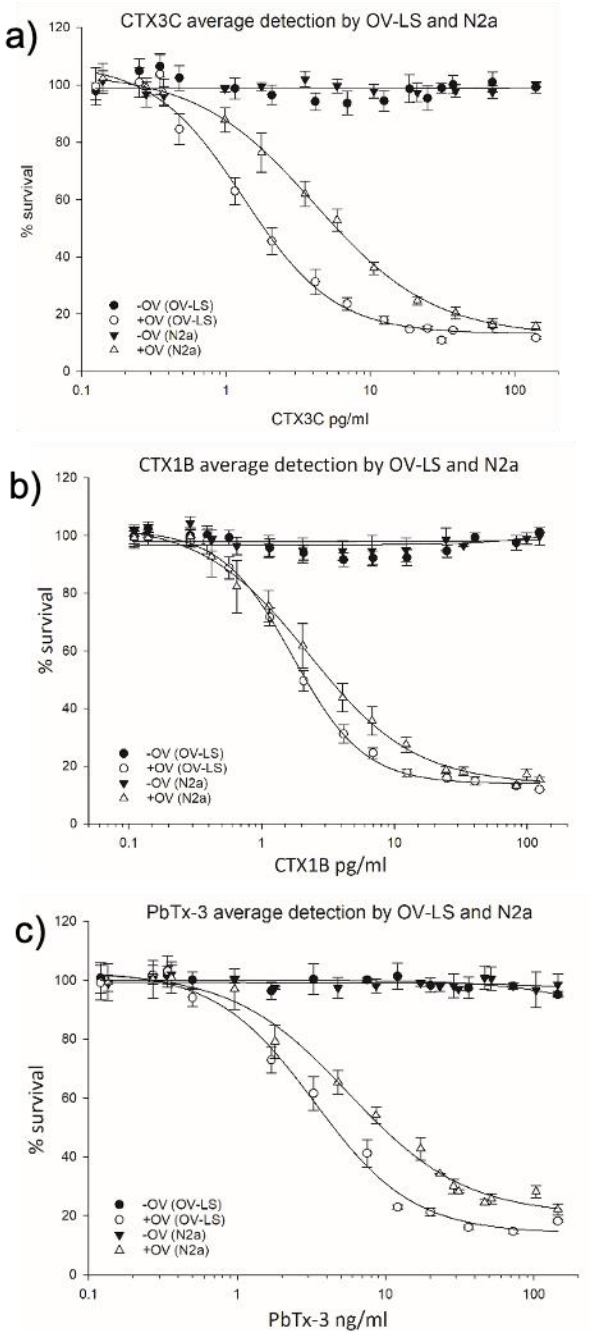


Figure 26. Combined concentration-response curves of OV-LS (circle) and N2a cells (triangle) in —OV (solid symbols) and +OV (open symbols) conditions, when exposed to increasing concentrations of (a) CTX3C, (b) CTX1B, (c) PbTx-3 following the cytotoxicity evaluation procedure. OV-LS cells were exposed to 0.22/0.022 mM O/V and N2a cells were exposed to 0.1/0.01 mM O/V. Data represent the mean ± SD of multiple microplate experiments for OV-LS (n = 25(a), 36(b), 22(c)) and N2a (n = 5(a), 14(b), 8(c)) cell lines. OV-LS concentration-response curve hillslope, EC_{50}, passage number range, raw absorbance range, percent survival minimum and maximum values, and R^2 are (a) -1.4, 1.39 pg mL^{-1}, 195-231, 0.11-1.3, 13, 108, and R^2 = 0.99 (b) -1.6, 1.74 pg mL^{-1}, 195-231, 0.12-1.45, 14, 102, and R^2 = 0.99 (c) -1.3, 3.3 ng mL^{-1}, 211-239, 0.16-1.2, 14, 103, and R^2 = 0.99. N2a concentration-response curve hillslope, EC_{50}, passage number range, raw absorbance range, percent survival minimum and maximum values, and R^2 are (a) -1.1, 4.2 pg mL^{-1}, 200-236, 0.18 -1.2, 12, 104, and R^2 = 0.99 (b) -11, 2.4 pg mL^{-1}, 200-236, 0.17-1.1, 14, 104, and R^2 = 0.99

(c) -1.1, 5.6 ng mL^{-1}, 217-244, 0.22-1.1, 20, 102, and R^2 = 0.99. Figure copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

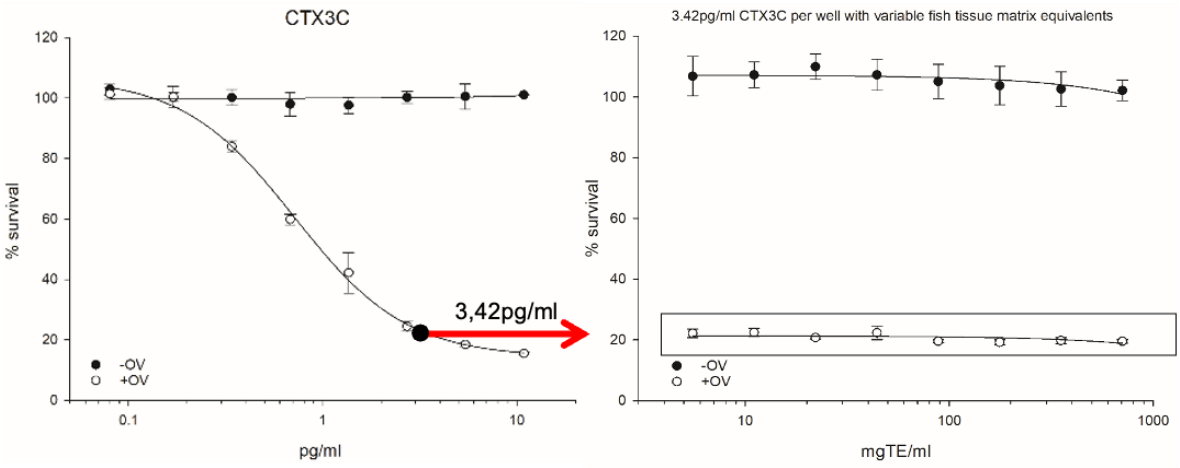


Figure 27. Concentration-response curves of CTX3C and matrix influence on CTX3C detection, conducted using the OV-LS cells as outlined in sections 3.2.2 and 3.2.3. Cells are in -OV (solid symbols) and +OV (open symbols) conditions. a) CTX3C at a concentration range from 0.085 -10.9 pg mL-1 without any matrix. OV-LS concentration-response curve hillslope, percent survival minimum, and maximum values for the CTX3C standard are -1.5, 14, and 107, R^2 = 0.99, respectively. b) CTX3C at a concentration of 3.42 pg mL^{-1} applied to each well with a variable matrix concentration from 5.52 - 707 mg TE mL^{-1}. The black circle in b) highlights the concentration-response applied in the variable matrix effect test. Figure copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Table 13. *In vitro* cytotoxicity inter-and intra-assay variability assessment for concentration-response curves (EC_{50}) of N2a and OV-LS cells exposed to CTX3C, CTX1B, and PbTx-3. Table copyright information: Available online 2 February 2021 1568-9883/© 2021 The Author(s) Loeffler et al. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/) referenced in this thesis as [239].

Variability	Sample (n)	Mean EC_{50} (±SD)	CV (%)	Passage number
N2a within-day	CTX3C (3)	3.4 (±0.25)	7.4	216
	CTX1B (3)	3.1 (±0.35)	11	206
	PbTx-3 (3)	5.3 (±1.70)	32	226
OV-LS within-day	CTX3C (4)	1.4 (±0.17)	12	207
	CTX1B (5)	1.9 (±0.25)	13	202
	PbTx-3 (3)	2.8 (±0.06)	2	215
N2a between-day	CTX3C (3)	3.5 (±0.37)	11	211, 216, 221
	CTX1B (3)	2.6 (±0.32)	12	203, 206, 210
	PbTx-3 (3)	5.8 (±1.70)	29	220, 231, 235
OV-LS between-day	CTX3C (4)	1.4 (±0.18)	13	211, 212, 217, 220
	CTX1B (3)	1.9 (±0.23)	12	202, 211, 220
	PbTx-3 (4)	3.0 (±0.17)	5.6	211, 215, 217, 220

n = number of independent 96-well microplates. CV = Coefficient of variation.

3.4 Discussion

The purpose of this study was to establish and conduct the N2a-assay as described in the literature for the analysis of material for CTXs and PbTxs. However, when attempting to conduct the assay as described, several issues were encountered including high cell mortality due to the prescribed cell assay conditions and high variability among replicates. Therefore, corrective measures were investigated and demonstrated herein for overcoming these issues. To ensure these efforts could be replicated this study was conducted in two independent laboratories, both setting up and establishing the N2a-assay for the first time; therefore no proprietary cell lines or equipment was used. The N2a cells used were newly purchased from a commercial cell line provider, therefore widely available and accessible for use to any prospective or existing facility. Because this was a novel setup for these labs and legacy cell lines were not used, the first issue was that the N2a cells were too sensitive (unacceptably high mortality rates) when the pharmaceuticals ouabain (O) and veratridine (V) were applied according to existing protocols. Secondary to this main problem were issues with reliability

among replicates. The goal of this study was to enhance the sensitivity for CTX and PbTx detection using the N2a-assay, therefore these drawbacks needed to be overcome, which was achieved through a novel modification to the cell line to achieve an OV-LS function.

The first issue demonstrated that cell survival, when exposed to O/V during the N2a-assay, can create a problem for target compound detection when the survival of cells falls outside a pre-defined performance range (Figure 26). When absorbance or percent cell survival values are low, this is an indicator that the physiological condition of the N2a cells is poor, as shown in Figure 24, e.g., when viability is at 20%. When this occurs, the cells can be saturated by adverse conditions, which can obscure the possibility of observing a response. The goal of the method is to observe a concentration-response curve over a wide spectrum of possible responses (e.g., 8-pt dilution) for Na_V activating compounds, however, detection cannot be achieved when the background conditions inhibit survival. When background cell mortality levels are high, this makes it difficult to observe or to achieve the sensitivity required for target compound detection (to observe an effect). Therefore, testing the cells using the basic prescribed conditions of the N2a assay can be important for establishing the native sensitivity of the cell line to these conditions so they can be optimized for compound detection. This will ensure the best conditions are used that can fit between the desired method and the behavior of the cell line. O/V survival (sensitivity) in the control wells (without sample application) was used as a performance indicator and when used within an optimal range can result in an improvement in target compound detection [400]. The OV-LS process simplifies designing an optimal survival range and this process is variable and dependent upon the concentration of O/V and time administered (Figure 22, Figure 23, Figure 25). Therefore, this process can be tailored to achieve a specific sensitivity goal depending upon the user's desired results. Specifically, the desired OV-LS properties could be achieved gradually, with repeated low O/V concentration and short exposure periods [400], or rapidly through the use of higher O/V concentrations for longer exposure periods, as demonstrated in this study. This flexible process (of rapid or gradual cell modification) is useful in the event of a CP outbreak, where a rapid response is required to prevent the product from circulating in commerce and a new cell line is procured. The modification process outlined here was designed to quickly create an OV-LS cell line with a significant shift in sensitivity to O/V capable of withstanding twice the O/V concentration during the N2a-assay in comparison with the original N2a cell line and with a significant increase in sensitivity for CTXs and PbTx-3 detection.

In addition to sensitivity improvements, the stability of the cell line is an important factor for its use and utility in quantitative CTX analysis. The OV-LS modification was sustained, unchanged, through the life of the cell line (terminated at ~50 passages) and this modification was maintained through the process of cryopreservation and replication, enabling the shipping and trade of modified cell lines or to modify a cell line and freeze it for revival as needed. There was no difference between

the OV-LS and N2a cell lines for their growth rate and passage rate, furthermore, the standard guidance from the manufacturer for the original cell line maintenance could be followed with the OV-LS cells simplifying handling. Because there was no difference between the modified and original line, this indicates that the OV-LS process did not inhibit the growth or function of the cell line. Additionally, a combination test was performed where the N2a and OV-LS lines were added in equal parts and tested in the N2a-assay. This combined cell line resulted in an average of sensitivity comparable to each cell line's performance. This indicated that no particular line was growth dominant when the two cell lines were mixed. Furthermore, none of the marine biotoxins tested using the OV-LS cells was cytotoxic in the absence of O/V, a result consistent with the sodium channel specificity of the assay, and in agreement with other studies and previous descriptions of the N2a assay [136,232,247]. The OV-LS line was suitable for use with complex matrices, no adverse matrix effects were detected when subjected to 707 mg^{-1} TE/ml of extracted fish material, or 0.0048 pg CTX3C mg^{-1} TE, demonstrating its utility for CTX detection in complex matrices. However, to preserve materials a maximum in tissue equivalency was not assessed, the goal for the matrix test was to achieve a value that was sufficient for achieving detection below the strict CTX1B FDA guidance limit of 0.01pg CTX1B eq. g^{-1} tissue equivalents, which was accomplished.

The OV-LS line sensitivity performance was tested using several marine biotoxins. These standards were derived from an algal source (PbTx-3), synthesized (CTX3C), and the principle ichthyotoxin (CTX1B) involved in CP, isolated from a naturally contaminated product. Doubling the amount of O/V administered during the assay from 0.10/0.01 mM to 0.22/0.022 mM O/V led to a significant increase in the sensitivity for detecting CTXs and PbTx-3. CTXs and PbTxs are similar in structure and have been identified as selective activators of the voltage-gated sodium channels (Na_V), and both bind to site 5 on neuronal Na_V with a high affinity [288,369]. These three compounds have physical differences in their molecular structure which results in different binding affinities to site 5 of the Na_V, with CTXs being more affine than PbTxs [252]. Despite these structural differences in the compounds, the OV-LS line outperformed the N2a line in detection sensitivity for all compounds tested. This indicates a conserved response performance (sensitivity) determined by the concentration of O/V applied. The use of more OV could be considered a tradeoff in terms of an increase in detection sensitivity for PbTxs and CTXs and increased use of O/V per microplate assay. However, the price per mg of O and V is less than 0.001% of the price of CTX3C. Increasing less expensive compounds by factor 2, which is offset by an approximately twofold reduction in the use of a rare and significantly more expensive compound, represents an important component in the realization of a reduction in operational costs for this assay.

Many studies have been conducted that use the N2a-assay to investigate the presence of CTXs and PbTx-3 in different matrices and provide a semi-quantification of their concentrations therein

[25,37,99,136,385]. The original method description by Manger et al. [232] is the most often cited by current studies using the N2a-assay studies, however, the concentrations of O (0.1-0.5 mM) and V (0.005-0.05 mM) utilized can vary, within, and among authors. Other parameters for cell line maintenance, growth, and microplate assay set-up also vary, further complicating comparisons among studies [231,252,386]. Cell survival range of 60-100% have been described for OV conditions ranging from 0.8-0.13/0.005-0.013 mM OV [231,244,289,388,395,397]. In this study, 66% (±10.1) survival was achieved at 0.1/0.01mM O/V administered to the cells, therefore this study falls on the lower end of the survival scale for this method. When authors describe higher O/V conditions used such as 0.25-0.3/0.025-0.03 mM O/V, the survival ranges have been reported at 10-80% [102,231,364,401], whereas without the OV-LS modification cell survival rates described herein were on the order of 20% (±9) when exposed to 0.22/0.022 mM O/V. Differences in cell survival reported in the literature can include discrepancies in method applications and can include but are not limited to differences in cell count, media renewal, media volume, cell line handling, MTT incubation time, and cell line age.

A range of EC_{50}'s described for CTX1B and CTX3C were provided in the FAO and WHO [37] report (Table 7 in the report and the references therein), which listed CTX1B quantification values of 0.053-19.0 pg CTX1B mL^{-1}, 0.57-3.1 pg CTX3C mL^{-1}. A range of EC_{50} for PbTx-3 has been reported in the literature of 5.8-66 ng PbTx-3 mL^{-1} [231,252,402]. The variability reported (factor >10) is likely due to the use of non-validated methods that compare values generated using different methods that were based on standards that are not controlled or commercially available for inter-laboratory comparisons. The studies that describe using a high level of O/V (0.5/0.05 mM O/V) also report the highest levels of sensitivity for CTX detection. The study presented here supported these observations, where the detection capabilities increased by an average of approximately factor 2 when increasing O/V concentrations 2x (with the OV-LS method) while maintaining consistency across all cell assay factors. Other groups have also shown that moderately increasing their OV concentration by 15-20% resulted in a 15-60% increase in sensitivity for the detection of CTX3C [83,190,231,396,397]. Additionally, Lewis, Inserra, Vetter, Holland, Hardison, Tester and Litaker [394] in a study assessing the bioactivity of CTXs and MTXs using an SH-SY5Y cell-based Fluorescent Imaging Plate Reader (FLIPR) assay, described this synergistic effect of CTXs on veratridine responses in SH-SY5Y cells, where increasing amounts of veratridine enhanced detection sensitivity. Further OV-LS modifications to withstand higher O/V is possible, demonstrated herein with additional OV-LS treatment, and it is therefore likely that a maximum sensitivity for this assay has yet to be identified. CTX1B has historically been described as the most toxic among the known CTXs (based on the mouse bioassay), whereas in the results of this set of experiments CTX3C was determined to be slightly more potent for the N2a assay. However, the CTX1B used in this study was not a synthesized compound, but instead generated from semi-purified naturally incurred material and a non-purified standard may contain background matrix interference

compounds. Only recently has CTX1B been synthesized and is now (October 2021) available for commercial sale and will be the subject of future studies. Therefore, as new commercial sources of certified reference material (CRM) become available from certified producers with verified purity, accurate toxicity equivalency should be reassessed [26,124,134,161,321,403].

The selection method for an OV-LS cell line described herein, designed for marine biotoxin detection, presents a path towards understanding an important performance factor for the N2a-assay method. In the initial efforts to establish this assay, based on following existing protocols, sensitivity issues were encountered when attempting to apply the O/V concentrations reported in the literature. Oversensitivity compromised the interpretation of data which required the identification of a solution to this sensitivity and variability. The conditions best suited for the OV-LS selection process were determined through a growth and exposure procedure followed by quality assurance testing to ensure the sensitivity problems to O/V was overcome. This was followed by a refined process to induce the OV-LS parameters faster, as presented here. Currently, laboratories investigating CTXs or PbTxs with the N2a-assay are using a wide range of O/V concentrations, which has resulted in a high level of inter-study variability and subsequent toxin estimation in samples. Here the importance of O/V on the detection process was demonstrated. Because of the importance of O/V for assay sensitivity and data interpretation among studies, a description of the concentration of O/V employed in the assay in tandem with the cellular response provided in studies that use the N2a-assay would provide the research community with the necessary information to interpret potential influences on the performance of the assay. A standardized description of the parameters used would enable better comparability among laboratory methods. Although certified reference material was not commercially available for all the toxins used in this study, non-certified reference material is now available, and the prospect of a harmonized N2a-assay approach as well as its validation and standardization across working groups using these available standards is desirable. To avoid confusion in studies employing an OV-LS modification, a detailed description should include: i) the procedure of modification (O/V exposure concentration and time); ii) standard cell line properties such as cell seeding density per well, total well volume including all additions (to ensure accurate compound per liquid volume calculations); iii) cell survival of the OV-LS line at the concentration of O/V administered, and performance in the presence of a standard or commercially available reference material; iv) original cell line passage number and modified cell line passage number (to account for the age of the cell line). This information should help ensure that the results generated can be replicated and compared among studies. The OV-LS procedure is permanent; therefore, sufficient amounts of the original line should be cryopreserved before the OV-LS procedure if it is of interest for the laboratory to maintain an original stock. Having an original and modified cell line will ensure that maximum utility for the detection of both Na_V

blockers and activators can still be maintained from the original cell line, as the OV-LS procedure was not designed or tested for the detection of Na_V blocking compounds.

Many critical challenges for addressing the future of harmful algae science and food web processes remain, as outlined by Wells et al. [404], from primary producers to human consumption, and ultimately health risks. The investigation described herein worked towards meeting the challenges set forth for improving method development for toxin detection. These efforts can provide more information on the emergent occurrence of CTXs in fish due to the increase in sensitivity which can identify CTXs in trophic level fish or cases of low dose exposure. This method also made advances toward the goal of optimizing a CP method other than the mouse bioassay; which is a stated goal of the European Food Safety Authority [261]. The results of this study demonstrate that an increase in detection capabilities for both CTXs and PbTx-3 was possible while also maintaining a high throughput capability. Indeed, the description of the OV-LS line appears to be the most sensitive detection of PbTx-3 yet described by the N2a-assay. This detection value was remaining remarkably consistent with the detection of the previous most sensitive description EC_{50} 5.8 ng mL^{-1} by N2a cells when 0.10/0.01 mM O/V was used; therefore with this method description, the same level of sensitivity could be achieved, providing confidence in the eventuality of a unified consistent method among laboratories [231]. The N2a-assay is an established method with over twenty-five years of utility and is a widely used detection method for supporting the clinical diagnosis of CP, and any improvement to its utility is a benefit for many stakeholders. International collaborative studies providing a consensus protocol for the detection of CTX's and PbTx's using the N2a-assay method such as the IOC-IAEA-FAO-WHO 'Global Ciguatera Strategy' may yet present a turning point towards a sought after international standardization of this method [37]. Monitoring programs have documented the expansion of harmful algal species to new geographical regions, and in this perspective, the sensitivity of the methods for marine biotoxin detection will play a key role in filling the data gaps required to describe the early warning indicators that can lead to human health and environmental impacts. Temperature has been identified as the most important environmental impact factor on ocean plankton communities, and the continuance of ocean warming through the 21st century is predicted to promote the intensification and redistribution of harmful algae [405,406]. Indeed the geographic range of CP has expanded, along with the risk of importing CTX contaminated fish [54,407]. In areas where toxin-producing microalgae are rare, or seldom encountered, the occurrence and toxin concentrations will challenge the prevailing limits of detection. In these emerging areas of concern, methods with the lowest level of detection will enable early warning and detection capabilities. In conclusion, this study serves as a description of a novel strategy to address a vexing sensitivity problem for the N2a-assay that needed elucidation. The N2a-assay is utilized by several human health organizations, but it still needs refinements [23], and this

investigation approaches a better understanding of the source of seemingly inherent variability while approaching the ultimate goal of increasing the sensitivity for detecting CTXs and PbTx-3.

3.5. Bibliography

17. Yasumoto, T.; Nakajima, I.; Bagnis, R.; Adachi, R. Finding of a Dinoflagellate as a Likely Culprit of Ciguatera. *Bulletin of the Japanese Society of Scientific Fisheries* **1977**, *43*, 1021-1026.
23. Friedman, M.A.; Fernandez, M.; Backer, L.C.; Dickey, R.W.; Bernstein, J.; Schrank, K.; Kibler, S.; Stephan, W.; Gribble, M.O.; Bienfang, P., et al. An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. *Marine Drugs* **2017**, *15*, 72, doi:10.3390/md15030072.
25. Caillaud, A.; de la Iglesia, P.; Darius, H.T.; Pauillac, S.; Aligizaki, K.; Fraga, S.; Chinain, M.; Diogene, J. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of ciguatera fish poisoning in Europe. *Marine Drugs* **2010**, *8*, 1838-1907, doi:Doi 10.3390/Md8061838.
26. Diogène, J.; Reverté, L.; Rambla-Alegre, M.; del Río, V.; de la Iglesia, P.; Campàs, M.; Palacios, O.; Flores, C.; Caixach, J.; Ralijaona, C., et al. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. *Scientific Reports* **2017**, *7*, 8240, doi:10.1038/s41598-017-08682-8.
37. FAO and WHO. Report of the expert meeting on ciguatera poisoning. Rome, 19–23 November 2018 *Food Safety and Quality No. 9. Rome* **2020**, *https://doi.org/10.4060/ca8817en*, doi:https://doi.org/10.4060/ca8817en.
39. U.S. Food Drug Administration. *Fish and Fishery Products Hazards and Controls Guidance Fourth Edition - March 2020*; https://www.fda.gov/media/80637/download (Accessed 15 October 2020), 2020; p 498.
45. EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:https://doi.org/10.2903/j.efsa.2010.1627.
52. Radke, E.; Grattan, L.; Roberts, S.; Abbott, M.; Morris, J.G. Incidence and Awareness of Ciguatera Fish Poisoning in St. Thomas, Us Virgin Islands. *American Journal of Epidemiology* **2011**, *173*, S224-S224.
54. Friedemann, M. Tropical fish poisonings in Germany 2012–2017 - What is ciguatera? *International Journal of Infectious Diseases* **2019**, *79*, 130, doi:https://doi.org/10.1016/j.ijid.2018.11.318.
81. Litaker, R.W.; Holland, W.C.; Hardison, D.R.; Pisapia, F.; Hess, P.; Kibler, S.R.; Tester, P.A. Ciguatoxicity of Gambierdiscus and Fukuyoa species from the Caribbean and Gulf of Mexico. *PLOS ONE* **2017**, *12*, e0185776, doi:10.1371/journal.pone.0185776.
83. Roué, M.; Darius, H.T.; Viallon, J.; Ung, A.; Gatti, C.; Harwood, D.T.; Chinain, M. Application of solid phase adsorption toxin tracking (SPATT) devices for the field detection of Gambierdiscus toxins. *Harmful Algae* **2018**, *71*, 40-49, doi:https://doi.org/10.1016/j.hal.2017.11.006.
94. Gómez, F.; Qiu, D.; Lopes, R.M.; Lin, S. *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *PLOS ONE* **2015**, *10*, e0119676, doi:10.1371/journal.pone.0119676.
99. Reverté, L.; Toldrà, A.; Andree, K.B.; Fraga, S.; de Falco, G.; Campàs, M.; Diogène, J. Assessment of cytotoxicity in ten strains of Gambierdiscus australes from Macaronesian Islands by neuro-2a cell-based assays. *Journal of Applied Phycology* **2018**, *30*, 2447-2461, doi:10.1007/s10811-018-1456-8.
102. Pisapia, F.; Holland, W.C.; Hardison, D.R.; Litaker, R.W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Séchet, V.; Amzil, Z., et al. Toxicity screening of 13 Gambierdiscus strains using neuro-2a and erythrocyte lysis bioassays. *Harmful Algae* **2017**, *63*, 173-183, doi:http://dx.doi.org/10.1016/j.hal.2017.02.005.

124. Ikehara, T.; Kuniyoshi, K.; Oshiro, N.; Yasumoto, T. Biooxidation of Ciguatoxins Leads to Species-Specific Toxin Profiles. *Toxins* **2017**, *9*, 205.
129. Murata, M.; Legrand, A.M.; Ishibashi, Y.; Yasumoto, T. Structures of ciguatoxin and its congener. *Journal of the American Chemical Society* **1989**, *111*, 8929-8931, doi:10.1021/ja00206a032.
133. Abraham, A.; Jester, E.L.E.; Granade, H.R.; Plakas, S.M.; Dickey, R.W. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chem* **2012**, *131*, 192-198, doi:https://doi.org/10.1016/j.foodchem.2011.08.059.
134. Hamilton, B.; Hurbungs, M.; Vernoux, J.P.; Jones, A.; Lewis, R.J. Isolation and characterisation of Indian Ocean ciguatoxin. *Toxicon* **2002**, *40*, 685-693.
136. Dickey, R.W. *Ciguatera toxins: chemistry, toxicology, and detection*, Second ed.; New York., 2008; Vol. 173, pp. 22.
161. Yogi, K.; Oshiro, N.; Inafuku, Y.; Hirama, M.; Yasumoto, T. Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from the Pacific. *Analytical Chemistry* **2011**, *83*, 8886-8891, doi:10.1021/ac200799j.
176. Darius, H.; Roué, M.; Sibat, M.; Viallon, J.; Gatti, C.; Vandersea, M.; Tester, P.; Litaker, R.; Amzil, Z.; Hess, P., et al. Tectus niloticus (Tegulidae, Gastropod) as a Novel Vector of Ciguatera Poisoning: Detection of Pacific Ciguatoxins in Toxic Samples from Nuku Hiva Island (French Polynesia). *Toxins* **2018**, *10*, 2.
183. Chinain, M.; Gatti, C.M.i.; Ung, A.; Cruchet, P.; Revel, T.; Viallon, J.; Sibat, M.; Varney, P.; Laurent, V.; Hess, P., et al. Evidence for the Range Expansion of Ciguatera in French Polynesia: A Revisit of the 2009 Mass-Poisoning Outbreak in Rapa Island (Australes Archipelago). *Toxins* **2020**, *12*, 759.
185. Loeffler, C.R.; Robertson, A.; Flores Quintana, H.A.; Silander, M.C.; Smith, T.B.; Olsen, D. Ciguatoxin prevalence in 4 commercial fish species along an oceanic exposure gradient in the US Virgin Islands. *Environmental toxicology and chemistry* **2018**, *37*, 1852-1863, doi:10.1002/etc.4137.
186. Olsen, D.A.; Nellis, D.W.; Wood, R.S. Ciguatera in the Eastern Caribbean. *Marine Fisheries Review* **1984**, *46*, 13-18.
190. Roué, M.; Darius, H.; Ung, A.; Viallon, J.; Sibat, M.; Hess, P.; Amzil, Z.; Chinain, M. Tissue Distribution and Elimination of Ciguatoxins in Tridacna maxima (Tridacnidae, Bivalvia) Fed Gambierdiscus polynesiensis. *Toxins* **2018**, *10*, 189.
218. Chinain, M.; Darius, H.T.; Ung, A.; Fouc, M.T.; Revel, T.; Cruchet, P.; Pauillac, S.; Laurent, D. Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). *Toxicon* **2010**, *56*, 674-690, doi:DOI 10.1016/j.toxicon.2009.05.032.
224. Inserra, M.C.; Israel, M.R.; Caldwell, A.; Castro, J.; Deuis, J.R.; Harrington, A.M.; Keramidas, A.; Garcia-Caraballo, S.; Maddern, J.; Erickson, A., et al. Multiple sodium channel isoforms mediate the pathological effects of Pacific ciguatoxin-1. *Scientific Reports* **2017**, *7*, 42810, doi:10.1038/srep42810.
228. Hardison, D.R.; Holland, W.C.; McCall, J.R.; Bourdelais, A.J.; Baden, D.G.; Darius, H.T.; Chinain, M.; Tester, P.A.; Shea, D.; Flores Quintana, H.A., et al. Fluorescent Receptor Binding Assay for Detecting Ciguatoxins in Fish. *PLoS ONE* **2016**, *11*, e0153348, doi:10.1371/journal.pone.0153348.
229. Pawlowiez, R.; Darius, H.T.; Cruchet, P.; Rossi, F.; Caillaud, A.; Laurent, D.; Chinain, M. Evaluation of seafood toxicity in the Australes archipelago (French Polynesia) using the neuroblastoma cell-based assay. *Food Additives & Contaminants: Part A* **2013**, *30*, 567-586, doi:10.1080/19440049.2012.755644.
231. Viallon, J.; Chinain, M.; Darius, H.T. Revisiting the Neuroblastoma Cell-Based Assay (CBA-N2a) for the Improved Detection of Marine Toxins Active on Voltage Gated Sodium Channels (VGSCs). *Toxins* **2020**, *12*, 281.
232. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium channel toxins: Directed

cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC INTERNATIONAL* **1995**, *78*, 521-527, doi:https://doi.org/10.1093/jaoac/78.2.521.

239. Loeffler, C.R.; Bodi, D.; Tartaglione, L.; Dell'Aversano, C.; Preiss-Weigert, A. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). *Harmful Algae* **2021**, *103*, 101994, doi:https://doi.org/10.1016/j.hal.2021.101994.
244. Bodero, M.; Gerssen, A.; Portier, L.; Klijnstra, M.; Hoogenboom, R.; Guzmán, L.; Hendriksen, P.; Bovee, T. A Strategy to Replace the Mouse Bioassay for Detecting and Identifying Lipophilic Marine Biotoxins by Combining the Neuro-2a Bioassay and LC-MS/MS Analysis. *Marine Drugs* **2018**, *16*, 501.
247. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Wekell, M.M. Tetrazolium-Based Cell Bioassay for Neurotoxins Active on Voltage-Sensitive Sodium Channels: Semiautomated Assay for Saxitoxins, Brevetoxins, and Ciguatoxins. *Analytical Biochemistry* **1993**, *214*, 190-194, doi:http://dx.doi.org/10.1006/abio.1993.1476.
249. Nicholson, G.M.; Lewis, R.J. Ciguatoxins: Cyclic polyether modulators of voltage-gated Iion channel function. *Marine Drugs* **2006**, *4*, 82-118.
250. Dickey, R.W.; Plakas, S.M.; Jester, E.L.; El Said, K.R.; Johannessen, J.N.; Flewelling, L.J.; Scott, P.; Hammond, D.G.; Van Dolah, F.M.; Leighfield, T.A., et al. Multi-Laboratory Study of Five Methods for the Determination of Brevetoxins in Shellfish Tissue Extracts. *Harmful algae 2002 : proceedings of the Xth International Conference on Harmful Algae, St. Pete Beach, Florida, USA, October 21-25, 2002. International Conference on Harmful Algae (10th : 2002 : St. Pete Beach, Florida)* **2004**, *10*, 300-302.
251. Caillaud, A.; Eixarch, H.; de la Iglesia, P.; Rodriguez, M.; Dominguez, L.; Andree, K.B.; Diogène, J. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. *Food Additives & Contaminants: Part A* **2012**, *29*, 1000-1010, doi:10.1080/19440049.2012.660707.
252. Dechraoui, M.Y.B.; Tiedeken, J.A.; Persad, R.; Wang, Z.; Granade, H.R.; Dickey, R.W.; Ramsdell, J.S. Use of two detection methods to discriminate ciguatoxins from brevetoxins: Application to great barracuda from Florida Keys. *Toxicon* **2005**, *46*, 261-270, doi:http://dx.doi.org/10.1016/j.toxicon.2005.04.006.
253. Soliño, L.; Widgy, S.; Pautonnier, A.; Turquet, J.; Loeffler, C.R.; Quintana, H.A.F.; Diogène, J. Prevalence of ciguatoxins in lionfish (Pterois spp.) from Guadeloupe, Saint Martin, and Saint Barthélmy Islands (Caribbean). *Toxicon* **2015**, *102*, 62-68.
261. EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish–Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:10.2903/j.efsa.2010.1627.
275. Bagnis, R.; Kuberski, T.; Laugier, S. Clinical Observations on 3009 Cases of Ciguatera (Fish Poisoning) in the South-Pacific. *American Journal of Tropical Medicine and Hygiene* **1979**, *28*, 1067-1073.
288. Gawley, R.E.; Rein, K.S.; Kinoshita, M.; Baden, D.G. Binding of brevetoxins and ciguatoxin to the voltage-sensitive sodium channel and conformational analysis of brevetoxin B. *Toxicon* **1992**, *30*, 780-785.
289. Dechraoui, M.-Y.; Naar, J.; Pauillac, S.; Legrand, A.-M. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon* **1999**, *37*, 125-143, doi:http://dx.doi.org/10.1016/S0041-0101(98)00169-X.
296. Sydney Fish Market. Sydney fish market seafood handing guidelines Availabe online: https://www.sydneyfishmarket.com.au/Seafood-Trading/Quality/Food-Safety (accessed on 06.10.2020).
299. Díaz-Asencio, L.; Clausing, R.J.; Vandersea, M.; Chamero-Lago, D.; Gómez-Batista, M.; Hernández-Albernas, J.I.; Chomérat, N.; Rojas-Abrahantes, G.; Litaker, R.W.; Tester, P., et al.

Ciguatoxin Occurrence in Food-Web Components of a Cuban Coral Reef Ecosystem: Risk-Assessment Implications. *Toxins* **2019**, *11*, 722.

304. Boydron-Le Garrec, R.; Benoit, E.; Sauviat, M.-P.; Lewis, R.J.; Molgó, J.; Laurent, D. Ability of some plant extracts, traditionally used to treat ciguatera fish poisoning, to prevent the in vitro neurotoxicity produced by sodium channel activators. *Toxicon* **2005**, *46*, 625-634, doi:http://dx.doi.org/10.1016/j.toxicon.2005.07.002.
309. Chan, W.H.; Mak, Y.L.; Wu, J.J.; Jin, L.; Sit, W.H.; Lam, J.C.W.; Sadovy de Mitcheson, Y.; Chan, L.L.; Lam, P.K.S.; Murphy, M.B. Spatial distribution of ciguateric fish in the Republic of Kiribati. *Chemosphere* **2011**, *84*, 117-123, doi:http://dx.doi.org/10.1016/j.chemosphere.2011.02.036.
313. Bienfang, P.; DeFelice, S.; Laws, E.; Wallsgrove, N.; Caldwell, P. Ciguatoxicity in the main Hawaiian Islands: spatial and temporal variability in the introduced reef carnivore Cephalopholis Argus. *J Res Environ Sci Toxicol* **2012**, *1*, 47-57.
321. Soliño, L.; Costa, P.R. Global impact of ciguatoxins and ciguatera fish poisoning on fish, fisheries and consumers. *Environmental research* **2020**, *182*, 109111.
324. Dierking, J.; Campora, C.E. Ciguatera in the introduced fish *Cephalopholis argus* (Serranidae) in Hawai'i and implications for fishery management. *Pacific Science* **2009**, *63*, 193-204, doi:10.2984/049.063.0203.
357. Sanchez-Henao, A.; García-Álvarez, N.; Silva Sergent, F.; Estévez, P.; Gago-Martínez, A.; Martín, F.; Ramos-Sosa, M.; Fernández, A.; Diogène, J.; Real, F. Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands. *Aquatic Toxicology* **2020**, *221*, 105427, doi:https://doi.org/10.1016/j.aquatox.2020.105427.
359. Hossen, V.; Soliño, L.; Leroy, P.; David, E.; Velge, P.; Dragacci, S.; Krys, S.; Quintana, H.F.; Diogène, J. Contribution to the risk characterization of ciguatoxins: LOAEL estimated from eight ciguatera fish poisoning events in Guadeloupe (French West Indies). *Environmental research* **2015**, *143*, 100-108.
364. Hardison, D.R.; Holland, W.C.; Darius, H.T.; Chinain, M.; Tester, P.A.; Shea, D.; Bogdanoff, A.K.; Morris, J.A., Jr.; Flores Quintana, H.A.; Loeffler, C.R., et al. Investigation of ciguatoxins in invasive lionfish from the greater Caribbean region: Implications for fishery development. *PLOS ONE* **2018**, *13*, e0198358, doi:10.1371/journal.pone.0198358.
365. (EC), R. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. 02004R0854 — EN — 01.01.2019 — 013.001 — 1 ed.; Council, E.P.a.o.t., Ed. 2004; Vol. Regulation (EC) No 854/2004.
366. Regulation (EC). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. In *Official Journal of the European Union. L 139/55*, 2004; Vol. 47.
367. (EC), R. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019. In *02019R0627 — EN — 01.01.2021 — 001.001 — 1*, European Parlament, C.o.t.E.U., Ed. 2019.
368. Lin, Y.-Y.; Risk, M.; Ray, S.M.; Van Engen, D.; Clardy, J.; Golik, J.; James, J.C.; Nakanishi, K. Isolation and structure of brevetoxin B from the" red tide" dinoflagellate Ptychodiscus brevis (Gymnodinium breve). *Journal of the American Chemical Society* **1981**, *103*, 6773-6775.
369. Lombet, A.; Bidard, J.-N.; Lazdunski, M. Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na+ channel. *FEBS Letters* **1987**, *219*, 355-359, doi:http://dx.doi.org/10.1016/0014-5793(87)80252-1.
370. Hidalgo, J.; Liberona, J.L.; Molgó, J.; Jaimovich, E. Pacific ciguatoxin-1b effect over Na+ and K+ currents, inositol 1, 4, 5-triphosphate content and intracellular Ca2+ signals in cultured rat myotubes. *British journal of pharmacology* **2002**, *137*, 1055-1062.
371. Calvert, C.M.; Hryhorczuk, D.O.; Leiken, J.B. Treatment of Ciguatera Fish Poisoning with Amitriptyline and Nifedipine. *Veterinary and Human Toxicology* **1987**, *29*, 489-489.
372. Withers, N.W. Ciguatera Fish Poisoning. *Annual Review of Medicine* **1982**, *33*, 97-111, doi:DOI 10.1146/annurev.me.33.020182.000525.

373. Fleming, L.E.; Kirkpatrick, B.; Backer, L.C.; Bean, J.A.; Wanner, A.; Dalpra, D.; Tamer, R.; Zaias, J.; Cheng, Y.S.; Pierce, R. Initial evaluation of the effects of aerosolized Florida red tide toxins (brevetoxins) in persons with asthma. *Environmental Health Perspectives* **2005**, *113*, 650-657.
374. Watkins, S.M.; Reich, A.; Fleming, L.E.; Hammond, R. Neurotoxic shellfish poisoning. *Marine drugs* **2008**, *6*, 431-455, doi:10.3390/md20080021.
375. Plakas, S.M.; Dickey, R.W. Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. *Toxicon* **2010**, *56*, 137-149, doi:10.1016/j.toxicon.2009.11.007.
376. O'Toole, A.C.; Bottein, M.Y.D.; Danylchuk, A.J.; Ramsdell, J.S.; Cooke, S.J. Linking ciguatera poisoning to spatial ecology of fish: A novel approach to examining the distribution of biotoxin levels in the great barracuda by combining non-lethal blood sampling and biotelemetry. *Science of the Total Environment* **2012**, *427*, 98-105, doi:DOI 10.1016/j.scitotenv.2011.11.053.
377. Darius, H.T.; Ponton, D.; Revel, T.; Cruchet, P.; Ung, A.; Fouc, M.T.; Chinain, M. Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. *Toxicon* **2007**, *50*, 612-626, doi:DOI 10.1016/j.toxicon.2007.05.007.
378. Lewis, R.J.; Vetter, I. Ciguatoxin and ciguatera. *Marine and Freshwater Toxins, Toxinology; Gopalakrishnakone, P., Haddad, V., Jr., Tubaro, A., Kim, E., Kem, WR, Eds* **2016**, 71-92.
379. Morin, E.; Gatti, C.; Bambridge, T.; Chinain, M. Ciguatera fish poisoning: Incidence, health costs and risk perception on Moorea Island (Society archipelago, French Polynesia). *Harmful Algae* **2016**, *60*, 1-10, doi:https://doi.org/10.1016/j.hal.2016.10.003.
380. Chinain, M.; Gatti, C.M.; Martin-Yken, H.; Roué, M.; Darius, H.T. 10 Ciguatera poisoning: an increasing burden for Pacific island communities in light of climate change? *Climate Change and Marine and Freshwater Toxins* **2020**, 369.
381. Connell, J.; Lowitt, K. *Food security in small island states*; Springer Nature Singapore Pte Ltd. 2020: Springer, Singapore, 2020; https://doi.org/10.1007/978-981-13-8256-7.
382. Barnett, J. Dangerous climate change in the Pacific Islands: food production and food security. *Regional Environmental Change* **2011**, *11*, 229-237, doi:10.1007/s10113-010-0160-2.
383. Skou, J.C. ENZYMATIC BASIS FOR ACTIVE TRANSPORT OF NA+ AND K+ ACROSS CELL MEMBRANE. *Physiol Rev* **1965**, *45*, 596-617, doi:10.1152/physrev.1965.45.3.596.
384. Cestèle, S.; Catterall, W.A. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* **2000**, *82*, 883-892, doi:https://doi.org/10.1016/S0300-9084(00)01174-3.
385. Cañete, E.; Diogène, J. Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma x glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. *Toxicon* **2008**, *52*, 541-550, doi:http://dx.doi.org/10.1016/j.toxicon.2008.06.028.
386. McCall, J.R.; Elliott, E.A.; Bourdelais, A.J. A new cytotoxicity assay for brevetoxins using fluorescence microscopy. *Marine drugs* **2014**, *12*, 4868-4882, doi:10.3390/md12094868.
387. Nicolas, J.; Hendriksen, P.J.M.; Gerssen, A.; Bovee, T.F.H.; Rietjens, I.M.C.M. Marine neurotoxins: State of the art, bottlenecks, and perspectives for mode of action based methods of detection in seafood. *Molecular Nutrition & Food Research* **2014**, *58*, 87-100, doi:doi:10.1002/mnfr.201300520.
388. Caillaud, A.; Eixarch, H.; de la Iglesia, P.; Rodriguez, M.; Dominguez, L.; Andree, K.; Diogene, J. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. *Food Additives & Contaminants: Part A* **2012**, *29*, 1000-1010.
389. Robertson, A.; Garcia, A.C.; Quintana, H.A.F.; Smith, T.B.; II, B.F.C.; Reale-Munroe, K.; Gulli, J.A.; Olsen, D.A.; Hooe-Rollman, J.I.; Jester, E.L. Invasive lionfish (Pterois volitans): a potential human health threat for ciguatera fish poisoning in tropical waters. *Marine drugs* **2013**, *12*, 88-97.
390. Boada, L.D.; Zumbado, M.; Luzardo, O.P.; Almeida-González, M.; Plakas, S.M.; Granade, H.R.; Abraham, A.; Jester, E.L.; Dickey, R.W. Ciguatera fish poisoning on the West Africa Coast: An emerging risk in the Canary Islands (Spain). *Toxicon* **2010**, *56*, 1516-1519.

391. Catania, D.; Richlen, M.L.; Mak, Y.L.; Morton, S.L.; Laban, E.H.; Xu, Y.; Anderson, D.M.; Chan, L.L.; Berumen, M.L. The prevalence of benthic dinoflagellates associated with ciguatera fish poisoning in the central Red Sea. *Harmful Algae* **2017**, *68*, 206-216, doi:https://doi.org/10.1016/j.hal.2017.08.005.
392. Wu, J.; Mak, Y.; Murphy, M.; Lam, J.W.; Chan, W.; Wang, M.; Chan, L.; Lam, P.S. Validation of an accelerated solvent extraction liquid chromatography–tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma assay. *Analytical and bioanalytical chemistry* **2011**, *400*, 3165-3175, doi:10.1007/s00216-011-4977-4.
393. Cañete, E.; Diogène, J. Improvements in the use of neuroblastomaxglioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. *Toxicon* **2010**, *55*, 381-389, doi:https://doi.org/10.1016/j.toxicon.2009.08.017.
394. Lewis, R.J.; Inserra, M.; Vetter, I.; Holland, W.C.; Hardison, D.R.; Tester, P.A.; Litaker, R.W. Rapid Extraction and Identification of Maitotoxin and Ciguatoxin-Like Toxins from Caribbean and Pacific Gambierdiscus Using a New Functional Bioassay. *PLOS ONE* **2016**, *11*, e0160006, doi:10.1371/journal.pone.0160006.
395. Castro, D.; Manger, R.; Vilariño, O.; Gago-Martínez, A. Evaluation of Matrix Issues in the Applicability of the Neuro-2a Cell Based Assay on the Detection of CTX in Fish Samples. *Toxins* **2020**, *12*, 308.
396. Roué, M.; Darius, H.T.; Picot, S.; Ung, A.; Viallon, J.; Gaertner-Mazouni, N.; Sibat, M.; Amzil, Z.; Chinain, M. Evidence of the bioaccumulation of ciguatoxins in giant clams (Tridacna maxima) exposed to Gambierdiscus spp. cells. *Harmful Algae* **2016**, *57, Part A*, 78-87, doi:http://dx.doi.org/10.1016/j.hal.2016.05.007.
397. Roué, M.; Smith, K.F.; Sibat, M.; Viallon, J.; Henry, K.; Ung, A.; Biessy, L.; Hess, P.; Darius, H.T.; Chinain, M. Assessment of Ciguatera and Other Phycotoxin-Related Risks in Anaho Bay (Nuku Hiva Island, French Polynesia): Molecular, Toxicological, and Chemical Analyses of Passive Samplers. *Toxins* **2020**, *12*, 321.
398. Collection, A.T.C. Animal Cell Culture Guide. ©2020 American Type Culture Collection: Manassas, Virginia, 2020; p 32.
399. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **1983**, *65*, 55-63, doi:http://dx.doi.org/10.1016/0022-1759(83)90303-4.
400. Loeffler, C.R.; Bodi, D.; Preiss-Weigert, A. Neuroblastoma cell-based assay optimization for harmonized marine biotoxin detection: reducing ouabain and veratradine oversensitivity. *Proceedings of the 18th International Conference on Harmful Algae* **2018**, 413.
401. Bienfang, P.; Oben, B.; DeFelice, S.; Moeller, P.; Huncik, K.; Oben, P.; Toonen, R.; Daly-Engel, T.; Bowen, B. Ciguatera: the detection of neurotoxins in carnivorous reef fish from the coast of Cameroon, West Africa. *African Journal of Marine Science* **2008**, *30*, 533-540, doi:10.2989/ajms.2008.30.3.8.64.
402. Rhodes, L.L.; Smith, K.F.; Munday, R.; Selwood, A.I.; McNabb, P.S.; Holland, P.T.; Bottein, M.-Y. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. *Toxicon* **2010**, *56*, 751-758.
403. Soliño, L.; Costa, P.R. Differential toxin profiles of ciguatoxins in marine organisms: Chemistry, fate and global distribution. *Toxicon* **2018**, *150*, 124-143, doi:https://doi.org/10.1016/j.toxicon.2018.05.005.
404. Wells, M.L.; Karlson, B.; Wulff, A.; Kudela, R.; Trick, C.; Asnaghi, V.; Berdalet, E.; Cochlan, W.; Davidson, K.; De Rijcke, M., et al. Future HAB science: Directions and challenges in a changing climate. *Harmful Algae* **2019**, https://doi.org/10.1016/j.hal.2019.101632, 101632, doi:https://doi.org/10.1016/j.hal.2019.101632.
405. Gobler, C.J.; Doherty, O.M.; Hattenrath-Lehmann, T.K.; Griffith, A.W.; Kang, Y.; Litaker, R.W. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. *Proceedings of the National Academy of Sciences* **2017**, *114*, 4975-4980, doi:10.1073/pnas.1619575114.

406. Glibert, P.M.; Allen, J.I.; Artioli, Y.; Beusen, A.; Bouwman, L.; Harle, J.; Holmes, R.; Holt, J. Vulnerability of coastal ecosystems to changes in harmful algal bloom distribution in response to climate change: projections based on model analysis. *Global Change Biology* **2014**, *20*, 3845-3858, doi:10.1111/gcb.12662.
407. Bravo, J.; Suárez, F.; Ramírez, A.; Acosta, F. Ciguatera, an emerging human poisoning in Europe. *J. Aquac. Mar. Biol.* **2015**, *3*, 00053.

Chapter 4. Ciguatera food safety risk in Germany: outbreak trace-back, toxin elucidation, and public health implications from imported mislabeled fish

Significance Statement

CP risks to human health can be described as roughly fitting a spectrum, ranging from mild self-resolving cases that went unreported (only to be identified after, through surveys and outreach) and the end of the spectrum (the worst-case scenario) at which CTXs have been attributed to mortality and even mass mortality events. Sometimes an outbreak can lead to chronic illnesses that can be life-changing, and at the onset of an outbreak, the nature of the illness, the severity, and duration or extent of the illness is unknown. Therefore, outbreaks should be treated seriously, and additional seafood related to the CP implicated product investigated thoroughly both to stop an ongoing outbreak and to prevent future outbreaks. This should include efforts to exhaust all the possible resources of knowledge surrounding the outbreak from the catch location (and regional CTX information) through the international food trade (rules and regulations) to the fork of the consumer (packaging and proper labeling) as well as the epidemiological response identifying the illness and setting in motion the trace-back efforts. The consumer who falls ill to CP should be entitled to know the events that occurred leading up to the outbreak, as well as what food and health organizations have learned and what their plans are for ensuring the prevention of CP moving forward. This chapter is a culmination of the previous chapters put into practice. Chapter 1 provided background information for the current knowledge surrounding the issue of ciguatera, Chapter 2 investigated predictive factors of potential use for predicting and preventing the harvest of fish with CTXs. Chapter 3 took the existing methods for Chapter 2 and improved their reliability and sensitivity. Here in Chapter 4, these previous efforts will be combined to investigate a real-world outbreak affecting a larger number of people over a wide geographic area. The motivation behind this chapter was to provide the first demonstration in Germany of an established method for toxicity evaluation of CTXs (Chapter 2 along with modifications from Chapter 3) in suspected and outbreak-related fish. The goals of this chapter were i) to classify and quantify the compounds responsible for the outbreak, ii) to authenticate the species responsible, iii) to trace back the fish to their likely source of origin, iv) to describe the outbreak event in as much detail as possible, and v) to identify what changes or actions can be made to prevent a future outbreak. Outbreaks of CP are often limited in the information available to fully describe the event (from source

to consumption, to analysis), therefore this will be a significant contribution towards presenting a close to complete CP outbreak.

4.1. Introduction

It is the role of public health and human safety organizations not just to respond to events affecting human health but also to anticipate and predict outbreaks to prevent their future occurrences. To accomplish this set of tasks requires a thorough understanding of the conditions that create the event, and in the case of CP, this involves an extremely complex and yet not fully defined or understood set of environmental, biological, abiotic, physical, spatial, and toxicological conditions that take place through time. This information once realized needs to be understood in the context of how those events and conditions dampen or exacerbate the issue of CP.

The intake of 0.1 µg of CTX orally can be enough to result in human illness [21]. CTXs are essentially organoleptically undetectable and the consumers often have described the piece of fish responsible for the CP outbreak as delicious and otherwise tasteful. Therefore, in many cases, the entire portion of fish was consumed, leaving no meal remnant. When a consumer experiences adverse health effects after consuming the fish and believing the meal to be responsible for the illness, the remaining product is often discarded. Consequently, a meal remnant for confirmation is rarely available for analysis when an outbreak occurs. In self-caught products, at times an uncooked portion of the fish may remain, and this product can be treated as a meal remnant, as the distribution of CTXs throughout the flesh of the fish is similar in concentration [408], providing a comparative estimate of the toxin content in the consumed product. Although recent work by Varriale [409] reported CTX variability in a piece of fish (steak type cut), therefore, this assumption of homogeneity of CTXs in fillets may need to be revisited. What is more likely and common in the event of an outbreak when the actual responsible piece is no longer available, is that there are associated fish, either from the same bag, same lot, or same batch available for testing. Ciguatoxins among fish have historically been temporally, and spatially sporadic; meaning that fish from the same area can have significantly different concentrations of CTXs, to the point of being from highly toxic to absent. Fish processing practices also play a role in the handling of products, adding a layer of complexity and uncertainty to the efforts at investigating fisheries products (or attempting to draw conclusions). Hundreds of kilometers can separate fishing grounds and fish processing plants can accept and combine products from multiple fishing vessels. This essentially randomizes the catch location of the fish at the processing plant, and this becomes a source of uncertainty for trace-back efforts. Furthermore, a single fish can be divided into multiple pieces that can be divided into several different bags, and bags of fish can consist of pieces from different fish. Selling bags of mixed fish from different locations can create a situation

where a single packet of fish can consist of multiple pieces from different fish from different fishing grounds. And if CTXs are a byproduct of local benthic algae this site to CTX association is essentially obscured due to this form of processing randomization. It is with this reality in mind that testing a bag, consignment, or an entire lot of products related to a meal remnant can provide a wide range of possible results and thus severely limit the conclusions that can be determined from testing a random bag of fish. CTXs are not always detected in parallel samples taken from the same lot of a meal remnant from an outbreak, which is consistent with the perceived sporadic occurrence of CP and the observation that not all fish of a given species or from a given location is toxic [32]. For this reason, Klekamp et al. (2015) concluded that food recalls are likely to be inefficient public health tools in response to CP outbreaks [410]. Consequently, conclusions based on the CTX content of the meal remnant or extrapolated from tests conducted on the wider lot can be inconclusive. Testing the wider lot or related product can still provide coverage information about the implicated species from the processor or region of capture. Currently, basic information for most species and their habitats regarding CTXs (qualitatively and quantitatively) is lacking and in need of further investigation. Therefore, in the least, investigating associated lots can fill research data gaps but if more fish in the lot are toxic, then a meal remnant can also serve as a warning for hazards contained within the larger lot. Varriale [409] described an outbreak that occurred in the United Kingdom involving a family (n = 3) where one uncooked frozen fillet was recovered. A preliminary positive toxin analysis by LC-MS/MS and the N2a-MTT assay for CTXs on this sample led to the consignment of the whole production batch of the product (total weight of entire withdrawn production batch 1230 kg). Subsequent testing by LC-MS/MS and the N2a-MTT assay on 24% (467 packets) of the entire lot (99 boxes that contained 1973 packets and each packet contained 550 g of 'Red Snapper') revealed that all samples tested contained CTXs with chromatographic peaks attributable to C/I-CTX 1 and 2 [409]. The fish samples were from India and reportedly caught in the Indian Ocean Food and Agriculture Organization (FAO) 51. This case proves that, especially because CP is chronically underreported, when a victim or patient comes forward with a legitimate complaint the event should be taken seriously. A fast recall effort can remove the additional product from the market before the remaining product is sold, potentially removing any remaining product which could pose a serious and significant health threat to the fish consuming public, as was demonstrated in Varriale [409].

In Germany, a series of six outbreaks of CP beginning in 2012, has been documented. The source of the outbreaks in each case was identified as imported snappers (Lutjanidae) [58]. In this investigation, information is presented on an outbreak of CP that occurred in 2017. This information is regarding the cases (clinical aspects), clusters (geographical aspects), causes (CTX analysis), and food authentication (DNA-barcoding). The information generated from sample material was from portions of the commercial product and available CP-related meal remnants. The initial case(s) of CP in this

outbreak were reported from the south of Germany in March 2017. The event became widely known, beyond the impacted region, when the competent authorities in Germany notified the European Commission via the Rapid Alert System for Food and Feed (RASFF). This notification was in response to the potential presence of the food contaminant 'ciguatoxin' in a seafood product described as 'Red Snapper' (RASFF report notification reference number 2017.0345) [411]. The implicated product was linked to two independent lots of seafood from the same processors and due to the RASFF alert, other Member States of the EU identified products from the lot within their respective jurisdictions, indicating the product was distributed in a wider region beyond Germany, where CP cases were reported. Customers were informed by the product distributor that there was a recall on the two implicated seafood lots, and the distributor provided investigators with a client distribution list inside Germany. In answer to the notification from the EU, the Vietnamese Agro-Forestry-Fisheries Quality Assurance Department issued an official letter (Ref.# 451/QLCL-CL1, 28th March 2017) to the seafood processing plant and required the establishment to investigate the cause of the contamination and to apply corrective actions. However, no further information regarding any findings or actions was shared. The outstanding product available to customers on the commercial market (or in the distribution chain) was recalled from the marketplace by the wholesaler, voluntarily, due to the concern that it could also contain CTXs. All information available from this outbreak including CTX analysis data were used to elucidate the origin, distribution, and potential impact of the contaminated product. Furthermore, necessary steps of potentially standardizing an outbreak response and CP risk assessment were discussed in this study.

4.1.1. Environmental and biological background

A multitude of complex species and region-specific, biotic, abiotic, temporal, and geographic factors determine how and when CTXs are ingested by fish. CTX buildup, as it relates to snappers, therefore necessitates attention at numerous ecological levels to account for the intrinsic variability among this large class of fish (e.g., seasonal movements, food selection, region, size, or behavior). Subsequently, the categorically wide-ranging fish product labeled as Red Snapper, if it is not ascribed to a specific species or location, is inadequate for making a ciguatera risk assessment. However, even if the species is accurately labeled and the catch region is described, this information at best can only provide minimal confidence for risk. When attempting to make an informed decision on CP risk, risk assessors require data about the region of captures' detailed historic information of CP [32,39,185,186,218,296,412,413], which itself is subject to the aforementioned variability. At its worst, the fish could be mislabeled, leading to a situation where the consumer decides based on a false belief in the reputability of a species. The family Lutjanidae, or snappers, are an important food fish that also have a high level of non-compliance in labeling [414,415]. Mislabeled fish, or illegitimate species

switching of one species for another, may constitute economic fraud and/or misbranding violations contingent upon the local rules and regulations. Furthermore, species substitution may introduce species-related food safety hazards. Some species have a known association with a hazard but if the species is mislabeled, it can potentially avoid the normal checks or scrutiny by food processors or consumers. Species substitution is a specific concern for CP [56,415], where a fish (or invertebrate) is known to carry CTXs is falsely labeled as a species without a recognized risk for CP. In these circumstances, DNA-testing is a suitable method for authenticating a product for verification. If action is taken to inform the supply chain that created the inaccurate label, it may help discourage operators from falsely labeling catches. Correct labeling on products can raise consumer awareness, especially in the situation where a species is suspected of causing CP, authenticating CP involved fish allows future risk assessments to have a verified source of information from which to make a risk assessment and outbreaks to be attributed to the correct species [23,39,58,416-421].

The exclusive economic fishing zone for Germany does not border a CP endemic oceanic area [422], therefore the risk to Germany (as well as other non-CP endemic territories) is either travel-related or import/trade induced. Because CTXs would be entering from abroad, the product could theoretically be from any CP endemic region. The EU (where trade is unrestricted among members, e.g., Germany) does have endemic regions where the causative algae, related toxins, and CP intoxications have been identified, particularly in the Canary Islands and Madeira [85,104,225,423-427]. These areas now contain CP-induced fishing restrictions and have monitoring protocols in place to protect consumers [428,429]. However, because fish and the algae are free to move to whatever environment is suitable, as waters have warmed, sub-tropical territories that were once too cold are now thermally suitable, resulting in an expansion of CTX producers and vectors to new territories previously free of CP and historically perceived as safe [23,146,183,430]. Expanded habitat suitability of CTX vectors now coincide with documented CP outbreaks in the areas of emerging concern (e.g., the Canary Islands and Madeira) and this territorial expansion is expected to continue further to encompass yet more territories and fish habitats [183,323,431,432]. The closing of fishing grounds or restricting of species for harvest is harmful to fisheries-dependent communities. Currently, the EU regulations regarding health standards for fisher products (EC No 853/2004) states that food business operators must ensure that fishery products placed on the market for human consumption do not contain CTXs. Furthermore EC No. 2019/627 Ch. I.G.3 states that controls are to take place to ensure fishery products containing ‘ciguatera’ are not placed on the market [45,365-367]. Actively managing fisheries resources by providing a guidance level for CP is the result of a global recognition that CP is a significant public health problem.

Precautionary consumer protection, through the prevention of food products containing hazardous contaminants from reaching the consumer market, is a priority for health and food safety

organizations, and these efforts include the prevention of CP. Various countries, both where tropical fish are endemic and imported, have implemented a wide range of domestic policies for combating CP based on local knowledge. These include outright bans, varying levels of restrictions, or non-actionable warnings. Prohibitions, restrictions, and warnings usually are based on, or among, three primary factors: species, size, and location. These are based on the experience that certain regions are endemic to CTXs (by way of the toxin-producing algae and the food web that perpetuates the compound), that certain species (based on their life history) are more prone to accumulate CTXs, and sometimes ontogenetic migrations or behavior can allow the sale of certain species based on size. Naturally, with such a wide range of approaches, there are varying degrees of success [38,39,50,296-299]. Consumer avoidance techniques that rely on species recognition can be less effective when identifiable characteristics like the head or skin are removed during processing [51,58] or when species hybridize [413], or when species are mislabeled [56]. Consumer protection measures based on regional harvest restrictions or locations can be subverted by falsifying capture location information [415]. The European Union's fisheries and aquaculture products market is among the most valuable worldwide [245,433]. However, currently, there is no reliable, cost-effective, commercially available preemptive fish-testing commodity for CTXs [434] to ensure a product is CTX free before reaching the market. To highlight some of the different approaches, the Sydney Fish Market, the Canary Islands (Spain), and French Polynesia, all provide guidance and restrictions according to an evolving list of species, size, and regional restrictions. In the United States of America, there is federal guidance on regional restrictions for fishing areas of concern, and local ordinances such as Hawai'i have their state guidance and some counties within a state banning certain species like Barracuda (as in Miami Dade County), which are frequently locally implicated in CP cases. In Hong Kong food poisoning by ciguatera is a statutory notifiable disease, allowing the investigation by the Center for Health Protection and the Center for Food Safety. Currently, the Center for Food Safety advises against the trade and for the avoidance of sourcing fishes from high-risk regions [435]. In the live fish market of Hong Kong, current laws prohibit the sale of fish with ciguatoxins, however, if the fish is alive then it is not classified as food, therefore the government has no authority to prevent their sale, however, they recommend avoiding fish larger than 1.8 kg [436].

4.1.2. Justification and study overview

In response to the RASFF notification [411] and the recall by the importer of the product from the market, the Bavarian State Office for Health and Food Safety (LGL) sent 10 kg (total gross weight) from one of the implicated lots to the German Federal Institute for Risk Assessment (BfR) for CTX analysis. Additionally, they collected two meal remnants, and at the request of the BfR, the US Food and Drug Administration (FDA) conducted an independent CTX sample analysis on the two meal

remnants. DNA-barcoding was performed on both meal remnants and a subset of samples from both implicated lots to authenticate the species indicated on the product label and attribute the actual species implicated to the outbreak. Descriptions therein are for the recalled suspicious product and meal remnants, regarding the results of their sample analysis for CTXs (semi-quantitatively and qualitatively), their epidemiological impact, and estimation for their assumed catch region. A two-tiered analysis approach was used and adapted for the investigation of CTXs in the recalled product samples at the BfR. It was adapted based on the original approach to CTX analysis methodology applied by the US FDA for official CP case sample analysis which has been in use since 1999 [136]. The FDA method was designed for the investigation of CTXs in the cooked or raw product involved in a CP outbreak [133]. Because fish can be mislabeled and therefore could be an unknown species from any CTX endemic region around the globe, the samples tested could theoretically contain any of the CTX-group compounds. Therefore, no a *priori* assumption regarding the region of sample origin (i.e., targeting a region-specific CTX-group) was utilized in this study. This unbiased approach for LC-MS/MS analysis incorporated the known and currently described CTXs to prevent false negatives during the investigation of CTXs in samples of imported fish (i.e., with an assumption bearing unknown origin). Concurrently, this is the first demonstration in Germany of an established method of toxicity assessment for CTXs in suspected and outbreak-related fish.

4.2. Methods:

4.2.1. Extraction and bio-assay analysis: toxicity evaluation by in vitro Neuro-2a cytotoxicity MTT-assay

Acetonitrile, methanol, formic acid, ammonium acetate (all LC-MS grade), chloroform, ethyl acetate (LC grade), *n*-hexane (GC-MS grade), acetone were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Muscle tissue (5 g) was excised, without bones or skin, to facilitate the extraction. The tissue samples were processed for toxin extraction using previously published methods [136]. The muscle tissue went through a series of liquid-liquid partition extraction methods to remove potential interferences (e.g., nonpolar lipids, proteins, and hydrophilic compounds) and enrich potential CTXs in a final chloroform extract. Solid-phase extraction (Bond Elut SI, Agilent, Waldbronn, Germany) was then performed to further clean extracts before toxicity assessment.

The method used to determine the composite toxicity in the sample extracts from the processed fish was based on the method for a semi-quantitative *in vitro* neuro-2a cytotoxicity assay described in [136,232], and with modifications, as described in [239]. In short, mouse (*Mus musculus*) neuroblastoma cells (N2a CCL-131, American Type Culture Collection, Rockville, MD, passage number

185) were propagated and subjected to the O and V lower sensitivity (OV-LS) method as described in section 3.2.3 [239]. Cells were harvested for assay, when cultures were approximately 80–90% confluent, and seeded at 4×10^4 cells $well^{-1}$ (200 µL volume) into sterile 96-well polystyrene plates (Corning™ 3596). Sodium channel activity in N2a cells is dependent on O and V, and these compounds are used to sensitize cells for the detection of sodium channel-specific effects. Conversely, N2a cytotoxicity caused by any other sample constituent can be evaluated using non-sensitized cells. N2a cells used in this study were OV-LS modified and had a survival of 60-80% when exposed to 0.22/0.022 mM O/V. Cell line passage numbers for utilization were between 190-245 (original passage number 185 from manufacturer). The range of raw absorbance values recorded after the MTT-colorimetric assay evaluation was between 0.08-1.43 nm (minimum to maximal survival). Full dose-response curves (8-dilutions) of sample extracts were prepared with sensitized and non-sensitized cells to determine the concentration at which cell viability was reduced by 50% (EC_{50}) compared with a CTX3C standard (Lot #TWJ6482, Wako chemicals GmbH, Neuss, Germany) [136]. All samples, standards, and relevant controls were assayed in triplicate. Results were expressed as ng CTX3C per gram TE (tissue equivalent), a wet-weight measurement.

The effective concentration causing a reduction in cell viability at 50% (EC_{50}) for CTX3C standard, calculated from the dose-response curve with a regression factor ($R^2 = 0.99$), was 1.35 ±0.28 pg mL^{-1} (n = 25), as described in [239]. The fish TE mL^{-1} range used for analysis among all samples was 0.0085-130.0 mg TE mL^{-1}, determined by the individual samples' assay response.

4.2.2. Fractionation method

Bioassay-guided fractionation was performed for one sample showing CTX-like activity in the N2a-assay. The tissue extract (See section 4.2.1) was directly utilized for HPLC fractionation (performed by A. Spielmeyer), using the same setup as for LC-MS/MS analysis (See Section 4.2.3). A total volume of 20 µL was applied, split into three consecutive runs consisting of two times 7 µL and one time 6 µL per injection, respectively. Higher injection volumes were avoided due to increasing peak broadening. Fractions were collected in glass vials in intervals of 30 seconds over a run time of 11 min (0 to 11 min), resulting in 22 fractions. Collected fractions were reduced to dryness in a stream of nitrogen and reconstituted in 50 µL 5% FBS RPMI and utilized for the N2a-assay (20 mg wet TE applied per well). Reconstituted samples were utilized undiluted and after 1:2 dilution with 5% FBS RPMI.

4.2.3. LC-MS/MS

Analyses were conducted by A. Spielmeyer using a UHPLC-MS/MS system consisting of an Agilent 1290 Infinity II UHPLC (Agilent, Waldbronn, Germany) and a Sciex QTrap 6500+ (Sciex,

Darmstadt, Germany). High-resolution measurements were performed with an Agilent 1260 Infinity II LC connected to a Sciex TripleTOF 6600+.

Details regarding chromatographic setups are provided in [156]. The LC-MS/MS method for analysis of the sodium adducts covered over 30 congeners by applying twenty different ion transitions (multiple reaction monitoring, MRM). Both in Q1 and Q3, the *m/z* of the sodium adduct was selected. Product ions of the ammonium adducts were analyzed using two different methods. For low-resolution analysis (UHPLC-QTrap system), four MRM transitions were monitored for each congener, with fragments corresponding to the $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, and $[M+H-3H_2O]^+$ of the respective congener; formulas and m/z of precursor and product ions of CTX congeners can be found in Table 3. For the high-resolution analysis (UHPLC-TripleTOF system), product ion scans were performed and the aforementioned fragment ions were extracted from the chromatograms with an extraction window of ±25 mDa. All details about the *m/z* and MS-parameters are provided in [156].

4.2.4. FDA analysis

In addition to the investigation at the BfR, the FDA Gulf Coast Seafood Laboratory (USA) was provided two meal remnants, pieces of cooked fish with the skin on, for bio-toxicity assessments and LC-MS/MS analysis. The two samples were shipped on dry ice on the 18th of July 2017 and were received frozen on dry ice and in good condition. The sample weights were 22.6 g (FDA 1) and 28 g (FDA 2). The skin was removed, and the tissue of each piece was homogenized separately. For each sample, homogenates were extracted with acetone, and the acetone extracts were subjected to solvent partitioning and cleaned up by solid-phase extraction, using the same procedure as described in section 2.2.2 (with solvent volume adjustments for tissue weight). Sample analysis was performed by the N2a-MTT assay (C.R. Loeffler) with the C-CTX-1 and P-CTX-1 (CTX1B) reference standards used for CTX-like activity comparison. LC-MS/MS analysis was performed by A. Abraham with the following method [133], and extracts were examined for the presence of C-CTX-1 and P-CTX-1 using C-CTX-1 and P-CTX-1 reference materials. The LC-MS/MS system used was an Agilent 1260 LC and ABSciex 4000 QTrap mass spectrometer.

4.2.5. DNA-barcoding

The DNA-barcoding performed for this study was done in part by LGL Oberschleißheim, Germany (n = 10). The other DNA-barcoding analyses on a fillet from the samples received for toxin investigation (lot #124) were performed by the Unit of Effect-based Analytics and Toxicogenomics, Department of Food Safety at the BfR (n = 1).

4.3. Results and Discussion

4.3.1. CP outbreak in Germany, 2017

Based on information contained within the RASFF-notification 2017.0345, the first case happened on the 3rd of March 2017 in Garmisch-Partenkirchen (Figure 28), located in the South of Germany, the patient was hospitalized locally. According to the supplier distribution list, 20 kg was delivered to this region (Table 14). An additional ten cases in the same region of Bavaria (south Germany) were also reported. Because the CP outbreak initially occurred in a geographically restricted region it was believed at the time that the outbreak was isolated to this region of Bavaria. However, because the outbreak initially involved a high number of cases a public recall campaign was conducted by the wholesaler, and due to this effort additional people who were also affected, self-reported their symptoms to the German Public Health Authorities. Patients of three separate clusters self-reported to a specifically established hotline at the German Federal Office of Consumer Protection and Food Safety (BVL). Clusters were defined following Friedemann 2019 and were named according to the geographic places where the fish were acquired by the people who were poisoned [58]. Clusters interconnected by a common batch of imported fish define the geographical dimension of a CP outbreak. Sixteen CP patients in total suffered symptoms consistent with the clinical descriptions of CP following their ingesting of fish labeled as `Red Snapper` in March 2017 (here the name Red Snapper within quotation marks ‘ ‘ is to indicate a false or mislabeled name). Geographically, affected people were from four districts in Germany which fall within four German Federal States, namely Bavaria, Baden-Württemberg, North Rhine-Westphalia, and Mecklenburg-Western Pomeranian. The locations where the ingestion and subsequent CP intoxication took place were four restaurants and two households, which in terms of clusters resulted in seven distinct outbreak clusters (in one cluster (postal code 82275) the fish was purchased at a restaurant but consumed at home), based on the purchase location of the CP implicated products (Table 14). Additionally, an unidentified number of people were known to be similarly affected with CP symptoms from fish in at least two hotels (personal communication); therefore, this outbreak could be considered as another illustration of the underreporting issue facing the accurate accounting of CP patients, clusters, and outbreaks.

Table 14. Ciguatera poisoning outbreak clusters that occurred in Germany in 2017 from imported fish labeled as 'Red Snapper'. Postal code, city, and federal region provide locational details for each cluster. Delivery date and amount (in kilograms with glazing / gross weight) of product delivered were provided by the wholesaler according to postal codes. Sample type references the material collected, meal (M) are leftovers or meal remnants from a CP outbreak and suspected (S) are related products collected from the same point of sale of where a CP case acquired the product, (P) is a parallel sample, (DNA) = molecular species confirmation, CTX = ciguatoxin analysis by N2a-assay and LC-MS/MS. Home (H) or Restaurant (R) is the location of consumption, WS= Wholesale. BW= Baden-Württemberg, BY= Bayern, NW= North Rhine-Westphalia, and MW= Mecklenburg-Western Pomeranian. Lot # was VN/385/III/124 or VN/385/III/122, only the final number is indicated in the table for simplicity. The date of symptoms are the same as the date of consumption and are based on the most complete information available, the dates/information listed are as they were reported. UNK= unknown. Data collated by M. Friedemann, K. Kapp, D. Bodi, and staff at the LGL.

Postal code	City, Federal region	Delivery date in 2017	Number of cases	Sample type/ analysis	Lot VN/385/III /#	Home (H) or Restaurant (R)	Date of symptoms in 2017
82275	*Emmering, BY	February (10 kg)	8	S DNA CTX	124	R/H	Early March
82216	Maisach, BY	February (10 kg)	0	S DNA CTX	124	(R)	-
87466	Oy-Mittelberg, BY	-	2	M DNA CTX	122	H	20th March
82467	Garmisch, BY	2nd March (15 kg)	1&	-	124	R	3rd March
63739	Aschaffen-burg, BY	7th March (5 kg)	1	-	124	R	10th March
87435	Kempten, BY	-	0	P DNA	122	WS	-
50670	Köln, NW	-	2	-	UNK	H	25th March
18609	Binz, MW	3rd March (5 kg)	1&	-	124	R	4th March
70734	Fellbach, BW	-	1	-	UNK	R	March

*Cases were reported from the common location of Fürstenfeldbruck, the product was purchased at a restaurant but consumed at home. & Other cases reported colloquially.

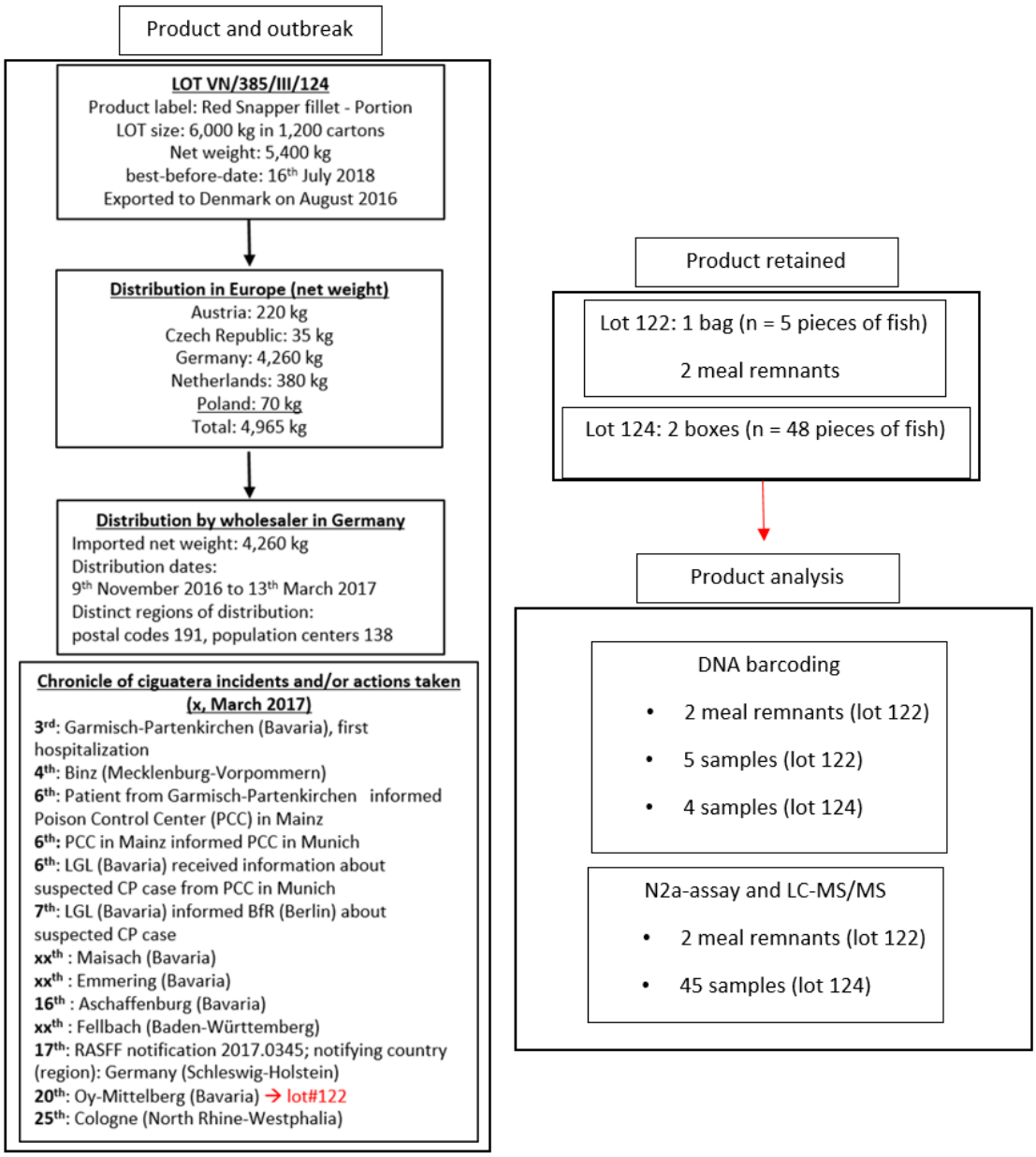


Figure 28. Flow chart describing the arrival and description of lot VN/385/III/124, its distribution in Europe and Germany, and a chronicle of the CP incidences and/or actions are taken.

Data on two medical reports were collected during the investigation by M. Friedemann, which was provided by the German Poisoning Information Centers to the local Public Health Authorities and the BfR to facilitate a coordinated response effort. These reports were provided following section §16e of the German Chemicals Act (ChemG), guiding physicians to report poisonings. Three additional patients returned questionnaires directly to the BfR, Department Exposure. In total, detailed information is available for nine patients of the outbreak, regarding their sex (5 female / 4 male), age (median 49 years, min 26 years, max 60 years), and interval time after ingestion of the fish before the

onset of symptoms became evident (median 2 h, minimum 1 h, maximum 4 h). Five patients (4 female / 1 male) with a mean age of 42.5 showed a medium degree of severity, three of whom were hospitalized, and one woman (37-year-old) was treated by an emergency physician.

In the acute phase, symptoms began with gastrointestinal (GI) disorders (nausea, abdominal pain, vomiting, and diarrhea). Strong vomiting was reported in some cases, this was different from the previous CP outbreaks that occurred in Germany and the fish were from the same region of origin (Indian Ocean/Pacific Ocean region). All patients reported cold allodynia, i.e., the reversal of cold feeling. Other neurological symptoms were pruritus mainly of the extremities, numbness of oral skin and mucosa, muscle pain, and headache. The enduring burning pain of genital mucosa was reported by three patients. A 48-year-old woman showed respiratory and cardiovascular disorders, high sensitivity to pain, and a burning sensation of the oral mucosa lasting for several weeks. One patient reported taste disorders and that the symptoms became aggravated during times of stress.

A laboratory staff individual (age: 60 years, sex: male) conducted (out of his own volition) a self-administered dab sensory test with a 90 mg piece from one of the meal remnants (personal communication). To conduct the test, he dabbed the portion on the left side of the lip, tongue, and incisors and held it in place for two minutes. During the placement, a ‘mild sunburn’ sensation was felt by the subject, within one hour the sensation spread to the skin around the mouth, cheeks, and nose. Directly after the brief perioral dermal and mucosal contact, tingling and numbness developed in the mouth. After one hour a sensation of ‘coolness’ was perceptible in the mucous membranes of both nasal passages, described as a sensation like inhalation after peppermint oil. These sensations were perceptible daily for several months. This sensation of ‘coolness’ was reported by a patient involved in a CP outbreak involving *L. bohar*, from the same region of export (as this study). This method of ‘contact reaction’, where a small piece of suspected fish is rubbed on the mouth or skin, has been reportedly used in folk tests for CP in endemic regions [437].

4.3.2. Source-food trace-back sample collection and product description

In 2017, a RASFF report notification reference 2017.0345 indicated that a ciguatera outbreak occurred in Germany and was connected with the ingestion of imported ‘Snapper product’ associated with lot numbers VN/385/III/122 and VN/385/III/124. Lot VN/385/III/122 was tropical fish exported from Vietnam to Denmark in July 2016 and according to the commercial invoice, two hundred and fifty cartons were distributed (total lot weight reported 2,500 kg divided into cartons, ten bags each with a net weight of 800 g fish (listed at 20% glazing)). Lot VN/385/III/124 was comprised of 5,400 kg (net weight without glazing, 6,000 kg gross weight with 10% glazing), this was the main set of products available for the CTX laboratory investigation described herein. Lot number VN/385/III/124 was

exported in August 2016 from the Cam Lam district of the Khanh Hoa province, Vietnam, and imported into Denmark in September 2016 with a content listed at 6,000 kg split into 1200 cartons of the product (listed at 5 kg per box). Deliveries to regional wholesalers throughout Germany were made between the 9th of November 2016 and the 13th of March 2017. The wholesaler circulated 4,260 kg (net after recalled product) of the frozen fish product among 191 unique postal codes and 138 population centers throughout Germany. The batch was also circulated to Austria (220 kg – product returned or destroyed), Czech Republic (35 kg – all returned), Netherlands (380 kg – none was distributed), and Poland (70 kg – some consumed, remaining product withdrawn, 'any complaints were reported'). The dates for product expiration (i.e., best before/expiration dates) for lot #122 and #124 were the 31st of December 2018 and the 16th of July 2018, respectively.

Following the report of the first CP cases that occurred in March 2017, two meal remnants (22.6 g and 28.9 g) and one package from the implicated lot (#122) containing 5 pieces of fish (800 g net weight) were collected by the LGL (Figure 29). Two boxes of frozen fish from the other implicated lot number (#124, 5 kg each) (Figure 29) were collected for DNA-barcoding and toxin examination. The two boxes each contained twenty-four individual pieces of snapper fillets of variable sizes and the product label described the pieces as weighting 170-230 g (Figure 29) estimated at 10 kg; thus, representing roughly 0.2% of the net 4,260 kg (minus returned) of product circulated by the wholesaler throughout Germany. From the original forty-eight fillets, three were retained for records (DNA-barcoding), and forty-five were sent (frozen) to the BfR for contaminant analysis.

Fillets weighed between 141-209 g (wet weight) with a median of 175 ± 16.1 g. Before the analysis, the fillets displayed minimal loss of water content (observation). The difference between the sample weight recorded, and package description (170-230 g) may have been due to water loss while at the BfR, as, frozen water i.e., 'glazing' was removed from the surface of the samples before weighing to obtain a more accurate tissue weight.

Fish pieces were cut in three- to four-cornered fillets, the skin color was partly orange, partly carmine to blood-colored skin, trending from a lighter color near the underside of the fish towards dark brown towards the back (Figure 29). Based on the cuts of fish, six fillet pieces were obtained per fish (Figure 29), which during handling, processing, and packaging were distributed unequally among the individual boxes during product packaging (observation from the collected material). The original catch weight of each fish was estimated to be approximately 3.5 kg, based on an assumed filleting yield of 35%. To estimate the original (whole fish) catch landing size for lot number VN/385/III/124, based on the lot net weight and yield per fish, approximately 15,500 kg (i.e., 4,428 fish), would have been required.

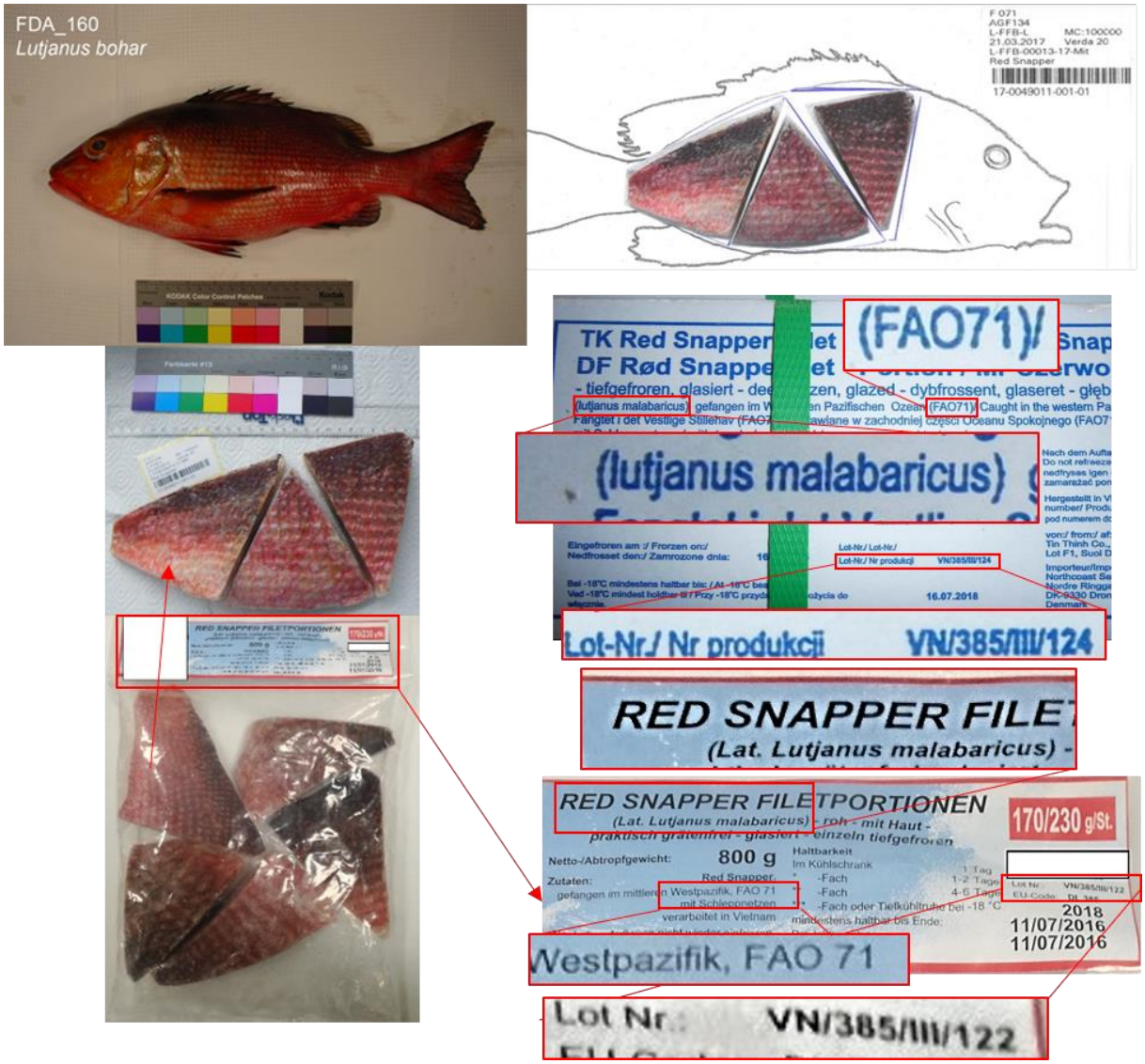


Figure 29. Photographs of the packaging, cartons, and fish with relevant trace-back information are highlighted. Highlighted information includes catch region (FAO 71), lot numbers VN/385/III/122 and 124, and species name on the packaging. Images of the fish in original packaging and sample description images provided by LGL staff authenticated reference fish photo for *L. bohar* from the US Smithsonian National Fish Collection (USNMNH 395518) and US Food and Drug Administration (FDA) reference standard sequence library for seafood identification, specimen ID (FDA 160) [337]. The contents of the FDA website (www.fda.gov) — both text and graphics — are not copyrighted. They are in the public domain and may be republished, reprinted, and otherwise used freely by anyone without the need to obtain permission from FDA.

4.3.3. DNA-barcoding and labeling of the imported fishes

Consumption habits in Germany indicate that 61% and 15% of respondents eat fishery and aquaculture products ≥1x per month at home and outside the home, respectively and 64% thought the name and species should be included on the label [438]. Identifying and authenticating fish species is a major challenge for the international fish trade, as 25-41% of seafood in commerce is estimated to

be mislabeled by species [414,439-441]. Inaccuracy and non-compliance negatively impact global trade, species substitution can also present a direct risk to consumers via foodborne illnesses [56]. The labeling of products accurately helps to ensure that compliance with existing regulations created for protecting fish stocks (i.e., sustainability), product quality, and enabling the accurate valuation of fish products [414,442,443]. In the outbreak described herein, the documents describing the implicated fishes designated the product as 'Red Snapper fillet' (*Lutjanus malabaricus*). Red Snapper as a general market name commands a high commercial value for fish with a conserved morphology and coloration, however, this description covers many international species and is non-specific [444,445]. Globally, the association of CP to fish sold as Red Snapper is as broad as the potential species the name encompasses; from 'high-risk' species banned in certain markets (e.g., *L. bohar* [296,310,446]) to species with 'no known risk' (e.g., *L. malabaricus*), and many have overlapping habitats. Therefore, to control this issue, the German seafood market only allows the trade name Red Snapper to be for *L. malabaricus*, specifically originating from the Indo-Pacific region [56,447].

In Germany from 2012-2017, all CP outbreaks were from imported snappers (Lutjanidae) [58]. Three outbreak-involved cluster regions in Germany which included ten samples were authenticated by DNA-barcoding analyses (one was from the related samples investigated at the BfR). All independent molecular biological examinations of individual fish fillets were in agreement and the species identified in both lots to be *L. bohar*: base pair agreement of 100% in nine samples and 99% in two samples, lot number #124 n=4, lot #122 n=7 (this included both meal remnant samples).

The sale of the raw fish fillets was under the agreement of Annex 1 (a) Regulation (EU) 1379/2013 (CN code 0304) [448] which allows the 'sale to final consumers or caterers if the commercial designation of the species and its scientific name is indicated' and in conjunction with national law [449,450]. However, because the name Red Snapper may be used only for *L. malabaricus* and the fish was identified as *L. bohar* this product was inaccurately labeled [447]. *L. bohar* can be sold in Germany under the trade names 'Snapper', *'Lutjanus* spp.', or 'Doppelfleckschnapper'. According to European legislation (Regulation (EU) No 1169/2011; Article 7, 1(a); Regulation (EU) No 178/2002; Article 16), the *L. bohar* being sold as *L. malabaricus* was judged as misleading. *L. bohar* is recognized by the US FDA (FDA fish/fisheries handbook [39]) as a species-related risk for CP and throughout its native range (Food and Agricultural Organization of the United Nations fishing zones 51, 57, 61, 71, 77, 81) *L. bohar* is recognized as a 'high-risk' species for CP. *L. malabaricus* is not recognized as a risk species for CP even though both species co-inhabit the same Food and Agricultural Organization of the United Nations (FAO) zones.

Species occupying the same habitats can have different CP risk associations. This is a function of the species feeding behavior or food web association, as CTXs are accumulated through diet. In NW Australia, Takahashi et al. [451] identified and distinguished cryptic *Lutjanus* species based on their

diet. Diet composition and habitat utilization are strongly linked and diet patterns can help identify what habitats are being used by a species, and ultimately the fine-scale partitioning of habitats between similar species hunting different prey in the same space [451]. Adult *L. malabaricus* was found to be unique in comparison to three other studied groups [451] having the highest prey richness (19 prey taxa) with 68% of the prey taxa being malacostracans (crustaceans). *L. bohar* feeds mostly on fish (70-80% of diet) [452,453] hunting coral reef-associated prey in shallow water (4-180 m depth) [192]. This hunting environment coincides with the preferred habitat of *Gambierdiscus* and *Fukuyoa* spp. Therefore, even fish that share the same habitat can have divergent CP risks due to their niche prey selection (i.e., diet). The life history of *L. bohar* in an endemic region for CTXs and associated feeding behavior at a high trophic level have resulted in this species being widely regarded as a high-risk species for CP [47,169,454-457].

For a consumer to make an appropriate purchase decision food must be labeled accurately. Substitution with an inferior product (e.g., price or quality) or one with an unequal risk constitutes inaccurate labeling which can be misleading to the consumer. The latter was the case here, *L. bohar* a species with a known CP risk was labeled and placed onto the market as a product that does not carry a known risk [56]. Therefore, multi-disciplinary and inter-agency recognition and coordination for regular surveillance regarding the accurate labeling of a species of Lutjanidae could have helped strengthen CP prevention efforts through a reduction of mislabeled products entering the market (and in this case could have prevented a major CP outbreak if identified before the product reached consumers) [37,59,61].

4.3.4. Catch area and source location

According to the product packaging (case and packaging, Figure 29), the harvest region was the Food and Agricultural Organization of the United Nations (FAO) region 71. It is described as Pacific, Western Central, which comprises the Western Pacific Ocean and includes Oceania and Australia. Fish that are discarded at sea in this catch region, due to the explanation of the fish being a potential risk for 'ciguatera' (among other non-desirable species) is considered 'insignificant' (0.5 %) [295]. Based on FAO reports, this zone practices CP self-governance, meaning that in areas that are known for 'ciguatera' fishing is prohibited or avoided [295]. FAO 71 has a high number of small-scale fisheries; this indicates shallow water catch efforts that are conducted nearshore [458-460]. The fishery in Vietnam (product origin) consists of medium-sized modern-style boats that fish 9-13 km offshore and larger-sized boats that can reach fishing grounds 110-240 km away [461]. Because of these fishing fleet maximum distance descriptions, the catch region of the two implicated lots was more likely located within the western portion of FAO 71, within the exclusive economic zone of the exporting country (Figure 30). The export item description indicates the Western Central Pacific as the catch region and

trawl fishing as the method. Based on these assumptions, as well as local CP records, biological, and ecological information a specific location of likelihood was encircled to represent a 'best guess' for the collection source of the implicated fish (Figure 30). A distance of 220 and 330 km from the coastal region of the CP implicated processing plant (black square, Figure 30 are represented by the smaller and larger pink circles in Figure 30, respectively). The distance to shore was restricted by several factors including the fishing vessel distance limit (e.g., larger local fishing vessels travel distance plus a conservative 90 km additional distance buffer), the FAO 71 fishing zone (described on the packaging), and the exclusive economic zone of Vietnam (adhering to international fishing rights) [462].

Vietnam has 3,260 km of coastline, 3,000 islands and islets, and 1,270 km^2 of coral reef area (red areas on Figure 30) [463]. In the coastal region of Vietnam, three species of *Gambierdiscus* have been identified (*G. toxicus*, *G. pacificus*, and *G. polynesiensis*) [464]. Cau Island, Binh Thuan, Vietnam (Figure 30, black triangle) was the collection site for *G. toxicus*, and in culture, it was described as producing CTXs [465]. The habitat around the processing plant for both lots of fish implicated in the CP outbreak is suitable for CTX producing source algae (black triangle) and the preferred habitat of the implicated vector (*L. bohar*) (coral reefs, red areas Figure 30); all within the encircled area.

Khanh Hoa Cam Lam, Vietnam (Black square, Figure 30) was the location of export identified in the RASFF report. In this region, several CP outbreaks from *L. bohar* were described in 2014 and on the 22nd of June, 2016 in Phan Thiet City (left gray square, Figure 30) and Nha Trang City (right gray square, Figure 30) [454]. Ten cases of CP were reported from *L. bohar* fish weighing 2-5 kg (a similar size range estimated for the product investigated here, see Section 4.3.2). From the meal remnants of these outbreaks CTX1B, 54-deoxyCTX1B, and 52-*epi*-54-deoxyCTX1B were identified as the responsible CTXs [454]. While no connection could be confirmed, similarities were noted between lot #122 and a CP outbreak in Vietnam [454]; patients also experienced the 'coolness' described in the dab-sensory test, both sets of fish were from the same processing region, involved the same species, and both the CP outbreak and the processing/packaging for lot #122 occurring in June 2016. Since 2007 red snappers have been increasingly reported in CP outbreaks in Vietnam [454]. If CP events are increasing in occurrence the local environment may be experiencing a shift towards higher CTX levels. In neighboring Thailand, red snapper (unidentified) fish curry was implicated in the poisoning of four individuals [466].

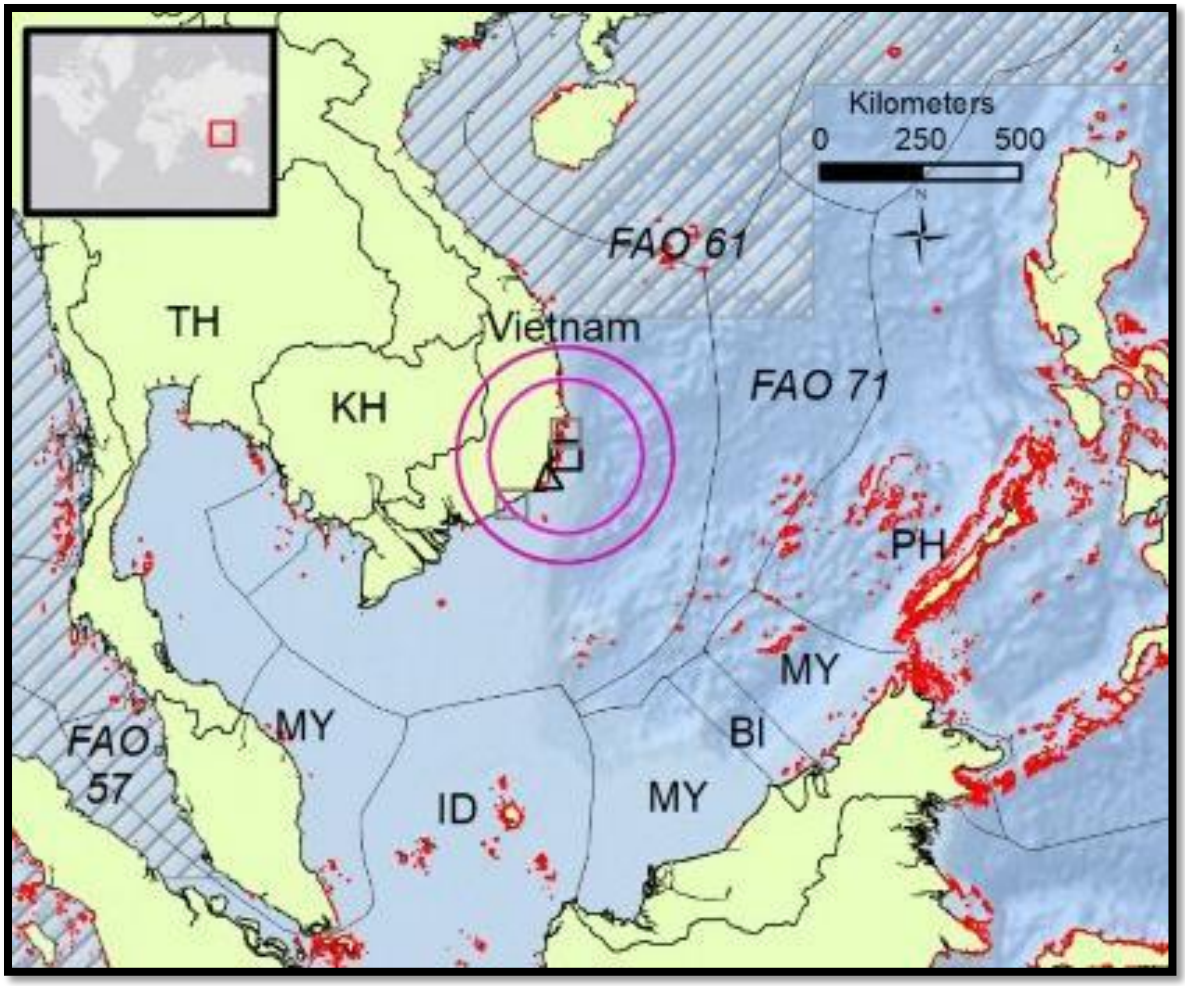


Figure 30. Map displaying the country of export (Vietnam) and surrounding waters [467], exclusive economic zones (EEZ) of neighboring countries [468], FAO recognized fishing zones [469], known coral reefs [470], and symbols representing locations of interest. The World map in the upper left corner contains a red square that indicates the regional area depicted in the main map figure. FAO major fishing zones are indicated with italicized font and dashed horizontal gray bars for zone 57 and 61, whereas FAO fishing zone 71 (catch region for the implicated product) is without horizontal hashing. The estimated catch area (based on the factors discussed in Section 4.3.4) for the product implicated in CP is within the two pink rings; inner and outer pink rings represent approximately 220 and 330 km distance from the coastal area of the processing plant for the exported product (black square). Pink circles represent a 'best guess' area for where the implicated fish were likely harvested from based on the EEZ (black dotted line) surrounding the exporting country, the ecology of L. bohar, and distance restrictions of the modern-large class local fishing vessels [463]. Gray squares are locations of CP outbreaks attributed to L. bohar in 2014 and 2016, black triangle is the region of Cau Island Binh Thuan, Vietnam, where *Gambierdiscus* toxicus was collected [465]. Country codes (BI = Burundi, ID = Indonesia, KH = Cambodia, MY = Malaysia, PH = Philippines, TH = Thailand) were based on the International Organization for Standardization [471]. Map created by C. R. Loeffler.

Beyond the biological and ecological uncertainty associated with CP (based on CTX algal source and vector species variability), the processor can impart significant uncertainty in the CP prediction and trace-back process. For example, a processor can package fish for sale either by mixing parts from different individual fish into one bag or mixing different fish from different locations into one bag. The fish in this study (within one bag) were variable in size and shape (Figure 29), therefore a random mixing type processing (either, mixed-fish, mixed-location, or both) was likely used [32]. Lot #124 was large, estimated at 15,500 kg total original fish weight (see Section 4.3.2), requiring approximately 4,400 fish to fill the lot. Therefore, this lot was likely sourced from multiple catch locations, as many red snapper-type resources in the source region have been effectively exploited [472]. Mixed-batch and/or mixed-source processing reduces the confidence in a CP trace-back regarding the origin of the product, whether it is made up from a regional-wide or representative of a specific reef. The fish could have been captured at a spawning aggregation site, potentially simplifying the date of capture, but complicating the origin trace-back information for fish. Spawning aggregations (as reported for *L. bohar* [473]) are made up of individual fish that arrive at a central point from many areas, leaving uncertainty around the location of capture. Regarding fisheries management practices, harvesting at spawning aggregation events has historically resulted in overfishing and the collapse of various fisheries and is therefore forbidden in many regions [473,474]. Vietnam/Cambodia are recognized as areas under heavy exploitation for reef fish spawning aggregations [475]. Locations are distinct in their food web associated CP risk because CTX producing algae are site attached. Therefore, whether the fish in the larger lot is harvested from one or multiple locations should be considered (i.e., is the fish indicative of one reef or is it a random selection from a region?), when drawing conclusions from an individual fish implicated in an outbreak to assume a similar conclusion for the larger lot [32]. This inherent uncertainty is a complication for the trace-back method; while it is useful and informative (when accurate) regarding the species and location, it can still fall short of providing information about the wider lot. For instance, the implicated fish in a CP outbreak may be the only fish contaminated, or all the fish in the lot may have some level of contamination (a potential 'hot-spot' harvest). Therefore, while a completely contaminated lot may be rarer [32,136], both scenarios (one fish or all) are theoretically plausible and therefore should be considered [418]. The safest method for the consumer is to assume all fish in the related lot are potentially a risk for CP if one is found.

4.3.5. Evaluation of samples by *in vitro* cytotoxicity assay

All samples tested exceeded the determined limit of detection (>0.001 ng CTX3C eq. g^{-1}) and therefore required less tissue than the 150 mg (wet TE) mL^{-1} applicability previously demonstrated [239]. All samples were determined to contain a toxin concentration > 0.01 ng CTX3C eq. g^{-1} (N2a-assay) and were investigated by LC-MS/MS. All forty-five fish samples tested were determined to be 'positive'

by the N2a-MTT assay for the presence of CTX-like activity. Toxin estimates per gram of fish wet tissue equivalents (eq.) ranged from 0.23-11.4 ng CTX3C eq. g^{-1} (n = 45) (Table 15, Figure 31). The total amount of toxin per fish portion (toxin concentration x portion weight) ranged from 39 to 2148 ng CTX3C eq. (n = 45) (Table 15). Two meal remnants were analyzed at the US FDA Gulf Coast Seafood Laboratory and both tested positive for CTX-like activity by the N2a-MTT assay (performed by C. R. Loeffler, Figure 31b, samples indicated in the figure with * symbol). Composite cytotoxicity was 3.67 ng and 2.88 ng CTX3C eq. g^{-1} wet tissue equivalent

Table 15. Toxin content equivalent (eq.) description for fish fillet samples (n = 45)

	Mean	Median	Std dev.	Min	Max
The concentration of CTX3C in ng eq. g^{-1} wet tissue eq.	2.84	1.88	2.34	0.23	11.4
Total ng CTX3C eq. per fish piece	506	357	437	39	2148

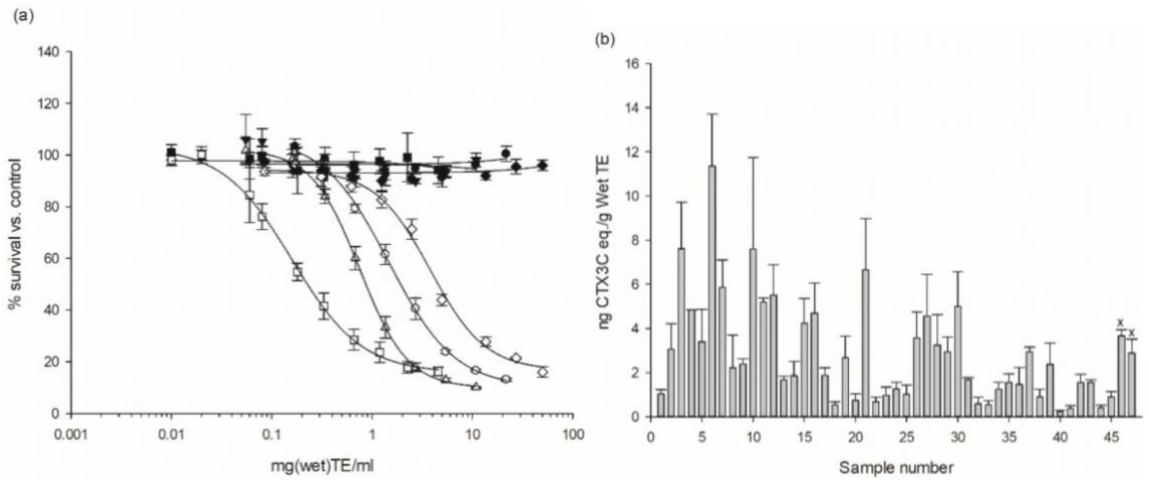


Figure 31. Toxicity assessment results for all samples involved in the CP outbreak in Germany. a) Combined concentration-response curves of N2a cells without (-) or with (+) ouabain (O) and veratridine (V) (– OV, solid symbols and +OV, open symbols) when exposed to various concentrations of semi-purified extracts of fish. Lines represent high (square, sample #21), medium (triangle, sample #35), medium-low (circle, sample #1), and lowest (diamond, sample #40) dose-response curves representing high to low toxin concentration estimates among the sampled lot. OV-LS N2a cells were exposed to 0.22/0.022 mM O/V. Dose-response curves were used to measure the effective concentration (EC) for a 50% reduction in cell survival. b) Bar graphs with error bars represent the toxin concentration (based on the mean EC50's measured) expressed as ng CTX3C eq. per gram wet tissue eq., generated from the dose-response curves: data display all samples tested and all replicates. Symbol 'x' above the error bar

indicates samples from meal remnants from lot # 122 (samples # 46 and 47). Error bars represent the standard deviation from all independent 96-well plate analyses performed for each sample (minimum three independent assays, each assay includes three replicate points).

The results of toxin quantitation presented in this chapter refer to wet weight tissue equivalents, as is commonly the case for reporting. However, biological tissue contains variable amounts of water weight depending on the condition and type of tissue. The fish processing method can also introduce additional water weight due to glazing (i.e., net weight versus gross weight) and subsequent freeze-thaw cycles can result in water loss (e.g., due to freeze-drying during prolonged storage). The process of cooking also manipulates the weight of a sample, cooking can reduce water content and meal preparations could add weight (e.g., breading, seasoning, salting, oil, etc.) or subtract weight (grilling, baking, etc.). Therefore, these water-weight manipulation factors (addition of weight or subtraction) will alter the weight of the fish or meal remnant. Because the final estimate is presented as toxin eq. and this refers to the weight initially used for extraction, these weight changes (+/-) can alter the estimated toxin content. A variable original weight becomes problematic when attempting to reconcile the toxin eq. in the sample to an established guidance level or when comparing a current CTX result with a legal limit or guideline (Figure 32), as errors in original weight can result in an inaccurate conclusion about the suitability of the fish product for consumption when below or above a given limit.

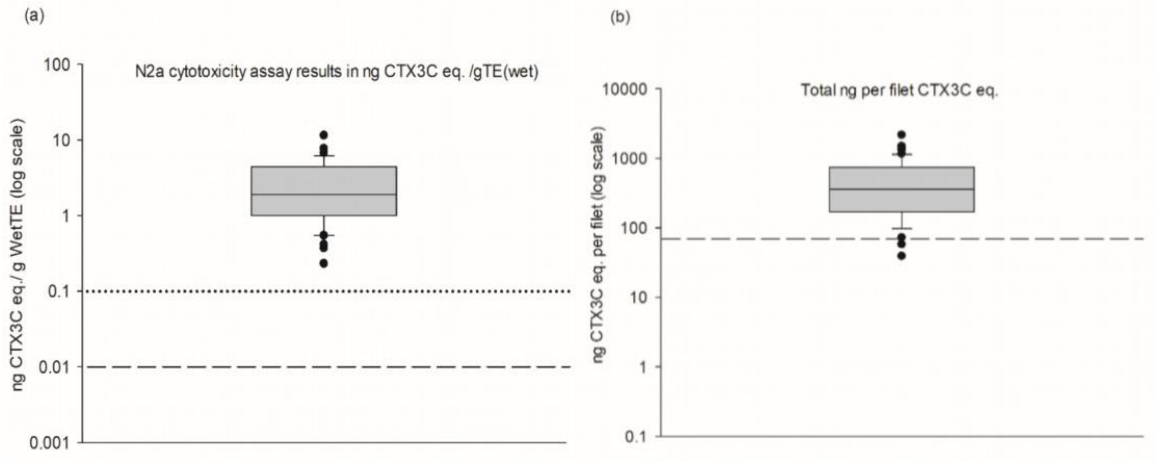


Figure 32. Toxicity assessment by N2a-assay combined results displayed as a box-and-whisker plot. a) box-and-whisker plot of the toxin concentration of CTX3C eq. per gram of fish tissue eq. for the forty-five fish samples, the box represents the upper to lower quartile range, the median is marked by the horizontal line in the box, and whiskers indicate variability outside the upper and lower quartiles, outside points, are outliers. Health guidance levels for CTX1B eq. (dashed line at 0.01 ng g^{-1}) and C-CTX-1 eq. (dotted line at 0.1 ng g^{-1}) are indicated for the context of human health seafood-consumption safety levels used by the US Food and Drug Administration. b) box-and-whisker plot of the total toxin content per fillet (ng CTX3C eq.), with a 70 ng health guidance level (dashed horizontal line), based on the Yasumoto 2005 recommendation regarding a total ng CTX intake for human health seafood-consumption safety [46].

When reporting a samples toxin eq. content, a sample weight description consensus would help normalize methods, reports, and comparisons among studies. Specifically, whether to report dry tissue or wet tissue equivalents. In this study, wet tissue equivalents are reported because this is the most reported sample description and value. However, the condition of the product (i.e., cooked, raw/frozen, or dry) the water content will change (by up to 80%, raw filet versus freeze-dried material) and therefore should be interpreted accordingly when reporting a final toxin per tissue-equivalent value. By starting with a dry product via removing the water weight (or simply reporting the wet versus dry weight of the sample) this unification would simplify inter-laboratory toxin equivalency comparisons. In the EU, products that test positive for CTXs (irrespective of toxin concentration) are not permitted for sale. This issue of reporting values based on an original tissue weight could be a confounding factor in regions where guidance levels represent actionable values (which can indicate the suitability of a seafood product's wholesomeness for sale or consumption).

4.3.6. Evaluation of samples for ciguatoxins by LC-MS/MS

All forty-five samples and two meal remnants were investigated by LC-MS/MS by A. Spielmeyer at the BfR. The two meal-remnants were investigated by LC-MS/MS at the USFDA Gulf Coast Seafood Laboratory FDA for the presence of the confirmatory compounds for Caribbean and Pacific ciguatoxins (C-CTX-1 and P-CTX-1 (CTX1B), respectively). The in-source collision-induced dissociation of C-CTX-1 $[M+H^+]$ *m/z* 1141.6 resulted in the loss of water $[M+H-H_2O]^+$ and major fragment ion of *m/z* 1123.6. This ion was used as the precursor for CID in the collision cell, and the three precursor/product ion transitions for SRM monitoring were *m/z* 1123.6 (*m/z* 1105.6, 1087.6, and 1069.6). For the detection of CTX1B three precursor/product transition pairs of $(M+H)^+$ *m/z* 1111.6 (*m/z* 1093.6, 1075.6, and 1057.6) and $[M+NH_4]^+$ 1128.6 (*m/z* 1093.6, 1075.6, and 1057.6) were monitored and the response summed [136]. However, both samples were determined to be negative for these compounds. The FDA provided the following summary statement in a sample analysis report provided to the BfR*: '…Based on the species of the fish samples reported by the German Federal Institute for Risk Assessment* (L. bohar) *and the presence of sodium channel-specific cytotoxicity, the samples analyzed may contain uncharacterized ciguatera-like toxins.'*.

Given these conclusions, an HPLC fractionation of a sample extract followed by the N2a-MTT analysis was conducted (i.e., bio-assay guided fractionation) to investigate the possible presence of other CTXs, analogs, or isomers. To accomplish this, sample #21 was selected based on its demonstrated high degree of measured toxicity (Figure 31b). Based on the bio-assay guided fractionation of the extract multiple fractions (5-7, 11-13, 19 for undiluted samples) showed a high degree of sodium channel (Na_v) specific activity demonstrating the presence of several distinct Na_v activating compounds (CTX-like toxicity) (Figure 33a). Because this method indicated that within a

single extract multiple Na_v activating compounds were present, the dose-response curves observed in the N2a-MTT assay (Figure 31a) were the result of a combined toxicity effect caused by a mixture of CTX-like congeners.

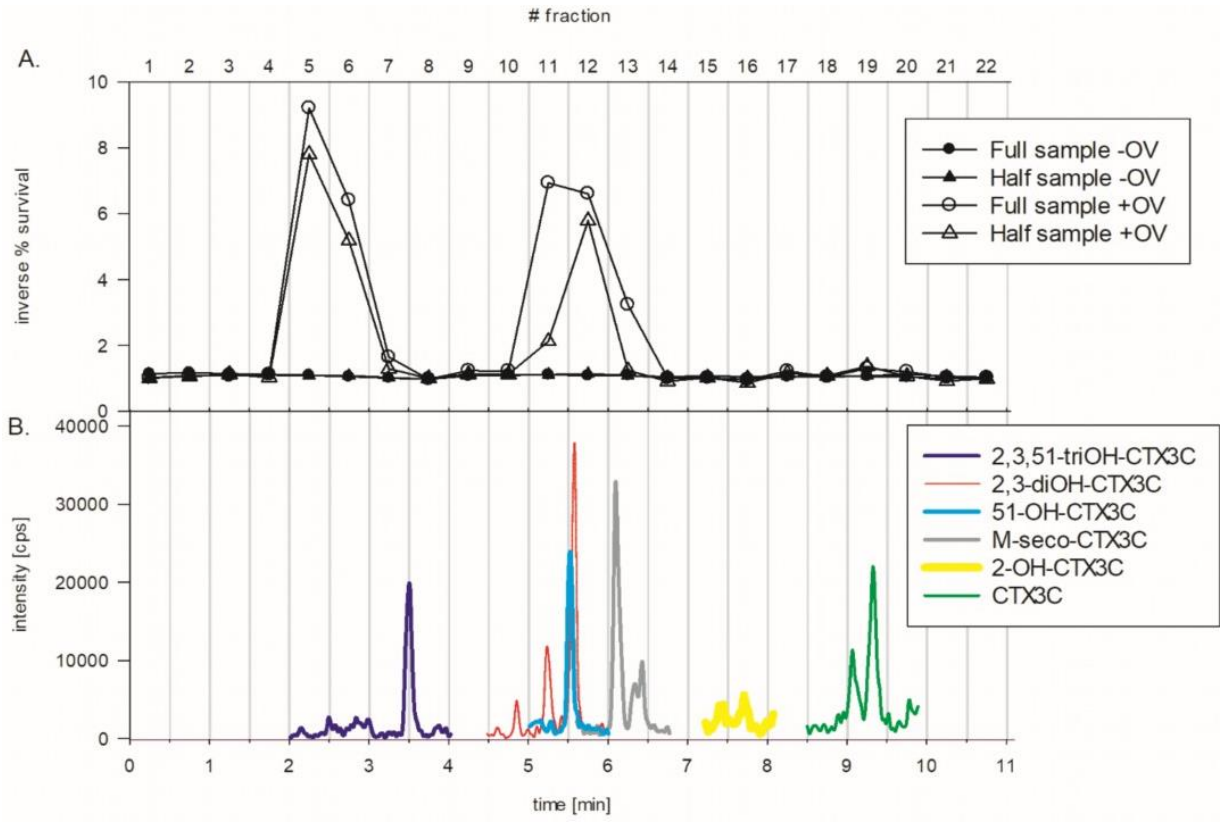


Figure 33. Bioassay-guided fractionation showing CTX-like toxicity and LC-MS/MS analysis of sample extract #21. a) results of the N2a-MTT assay performed with fractions collected according to Section 4.2.2; circles and triangles represent undiluted and 1:2 diluted samples, respectively; black symbols correspond to control wells (-OV), open symbols correspond to +OV conditions (0.22/0.022 mM O/V) (See Figure 31a for details); b) Extracted Ion Chromatograms of potential CTX congeners detected in the sample extract; colored lines represent the *m/z* of the sodium adducts of 2,3,51-trihydroxyCTX3C (dark blue), 2,3-dihydroxyCTX3C (red), 51-hydroxyCTX3C (light blue), M-*seco*-CTX3C (gray), 2-hydroxyCTX3C (bold yellow), and CTX3C (green); OH – hydroxyl. Vertical lines indicate the collection period of each fraction. The exact masses of the ions (MRM transitions) can be found below in Table 16.

The extract of sample #21 was used for LC-MS/MS analysis and the analytical method included the >30 CTX congeners reported in the literature [37,156]. Based on this broad compound approach, several potential CTX congeners of the CTX3C group were identified using their sodium adducts ($[M+Na]^+$, left column in Figure 34). To further support the compound identification, ammonium adducts' fragmentation (middle and right column in Figure 34) by low- and high-resolution analyses was used. This process detected six potential congeners plus their putative epimeric forms, which

included 2,3,51-trihydroxyCTX3C (3.50 min), 2,3-dihydroxyCTX3C (5.23 and 5.57 min), 51-hydroxyCTX3C (5.54 min), M-*seco*-CTX3C (6.13 and 6.46 min), 2-hydroxyCTX3C (7.47 and 7.74 min), and CTX3C (9.05 and 9.33 min) (additional details regarding compound assignment are provided in [156]). *L. bohar* and other species caught in the Pacific have been described as containing congeners of the CTX3C group (2,3,51-trihydroxyCTX3C, 2,3-dihydroxyCTX3C, 51-hydroxyCTX3C, 2-hydroxyCTX3C) [161,169,476], supporting the peak annotation identified here. The conclusion that cytotoxicity in the N2a-MTT assay was the result of a composite mixture of CTX congeners was therefore further supported by the identification of multiple CTX candidates by LC-MS/MS. However, none of the congeners labeled within the samples have an attributed specific guidance value, and no regulatory limits for CTX3C-group toxins are established in the EU. The official controls of fishery products in the EU states that 'fishery products containing biotoxins such as *ciguatera* [*sic*] or other toxins dangerous to human health are not placed on the market' [365].

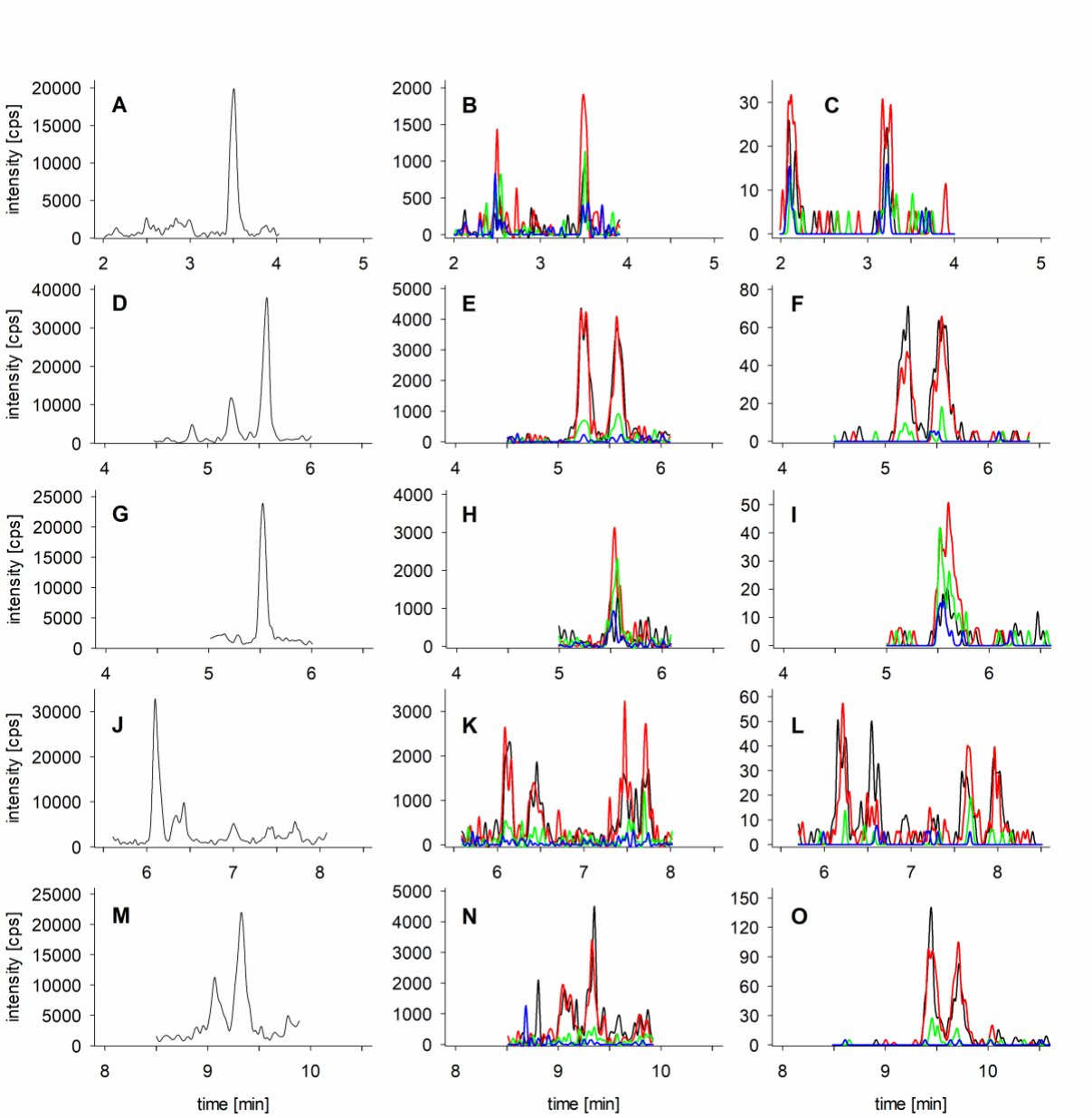


Figure 34. Extracted Ion Chromatograms (EIC) of the LC-MS/MS analyses of the CTX-like positive sample #21 (determined by the N2a-MTT-assay, section 4.3.5); panels show the EIC of the *m/z* of (A-C) 2,3,51-trihydroxyCTX3C, (D-F) 2,3,-dihydroxyCTX3C, (G-I) 51-hydroxyCTX3C, (J-L) M-*seco*-CTX3C and 2-hydroxyCTX3C, and (M-O) CTX3C. Analyses were performed for the sodium adducts (left column) as well as the low-resolution ion transitions of ammonium adducts to characteristic fragment ions (middle column) and high-resolution analyses (right column) of the fragments of the respective ammonium adducts; for ammonium adducts, fragments corresponding to $[M+H]^+$ (black), $[M+H-H_2O]^+$ (red), $[M+H-2H_2O]^+$ (green), and $[M+H-3H_2O]^+$ (blue). The exact masses of the ions are provided in Table 16. Low and high-resolution analysis was performed with two different HPLC setups, leading to slightly different retention times, setup methods details are provided in [156]. Analysis conducted by A. Spielmeyer.

Table 16. Excerpt of the relevant ions m/z of precursor and product ions of CTXs from Table 3 of relevance for Chapter 4.

CTX Congener	Formula	$[M + H - 3H_2O]^+$	$[M + H - 2H_2O]^+$	$[M + H - H_2O]^+$	$[M + H]^+$	$[M + NH_4]^+$	$[M + Na]^+$
CTX3C group							
CTX3C/B	$C_{57}H_{82}O_{16}$	969.53587	987.54643	1005.55700	1023.56756	1040.59411	1045.54951
51-hydroxyCTX3C	$C_{57}H_{82}O_{17}$	985.53078	1003.54135	1021.55191	1039.56248	1056.58903	1061.54442
M-*seco*-CTX3C 2-hydroxyCTX3C	$C_{57}H_{84}O_{17}$	987.54643	1005.55700	1023.56756	1041.57813	1058.60468	1063.56007
M-*seco*-CTX3C methyl acetal	$C_{58}H_{86}O_{17}$	1001.56208	1019.57265	1037.58321	1055.59378	1072.62033	1077.57572
51-hydroxy-2-oxoCTX3C	$C_{57}H_{82}O_{18}$	1001.52570	1019.53626	1037.54683	1055.55739	1072.58394	1077.53934
2,3-dihydroxyCTX3C	$C_{57}H_{84}O_{18}$	1003.54135	1021.55191	1039.56248	1057.57304	1074.59959	1079.55499
A-*seco*-51-hydroxyCTX3C	$C_{57}H_{86}O_{18}$	1005.55700	1023.56756	1041.57813	1059.58869	1076.61524	1081.57064
2,3,51-trihydroxyCTX3C	$C_{57}H_{84}O_{19}$	1019.53626	1037.54683	1055.55739	1073.56796	1090.59451	1095.54990

The results of the bioassay-guided fractionation and LC-MS/MS analyses were compared, to further aid the identification of compounds. In fractions 11-13 the toxicity may be ascribed to 51-hydroxyCTX3C, 2,3,-dihydroxyCTX3C and/or M-*seco*-CTX3C and in fraction 19 attributed to CTX3C. CTX3C and its potential epimer, 49-*epi*CTX3C (CTX3B), showed an intense signal in the LC-MS/MS chromatogram, although only trace toxicity was observed in fraction 19 (Figure 33). Enhanced cell growth that can obscure results has previously been described due to unidentified matrix component effects on the cell assay. However, for the control (-OV) portion of the assay, no growth was observed. The intensity of the peak for CTX3C/B was comparable to other congeners present in the sample (Figure 33b). Variability in ionization efficiencies can lead to significant differences in peak intensities of equimolar compounds. It remains possible that the concentration of the isolated fraction corresponding to CTX3C was too low to deliver a strong response in the N2a-assay. Alternatively, while CTX3C/B and the other congeners may possess similar concentrations, they may differ in their affinity to the Na_v; resulting in a lower effect on the cells (as a form of function at a specific concentration). Similarly structured ladder-shaped cyclic polyether marine toxins (e.g., brevetoxin) have shown varying affinities (differential impact) on the N2a-MTT assay. Because analytical standards are lacking, single compound toxicity has not been systematically evaluated among the known compounds. Mouse bioassay-based toxicity equivalent factors (TEFs) are available for some compounds. Based on those data, the TEF of CTX1B was set to 1.0, and the potency of other congeners was correlated to this compound and value. TEFs of other CTXs described therein are as follows; CTX3C 0.2, 51-hydroxyCTX3C 1.3, 2,3-dihydroxyCTX3C 0.1 [37]. Based on the TEFs, 51-hydroxyCTX3C is six times more toxic than CTX3C and over ten times more toxic than 2,3-dihydroxyCTX3C. That would help explain the higher

toxicity observed in fractions 11 and 12 compared to fraction 19 (Figure 33a). A better understanding of the function of these toxins by their form (i.e., Na_v affinity) will enhance the interpretation of bioassay-guided fractionation results as well as present an opportunity for pharmaceutical investigations.

Fractions 15 and 16 produced no reaction in the N2a-MTT assay at the concentrations tested, although peaks of potential 2-hydroxyCTX3C were detected in that time window (Figure 34, J-L). 2-hydroxyCTX3C is expected to possess Na_V activity, but the application may have been at a concentration that was too low for detection in the N2a-MTT assay. This congener was not evaluated concerning its TEF, and no statement was provided regarding the toxicity of this specific congener in comparison with the others [45]. Fractions 11 and 13 exhibited a strong reduction or no observable toxicity after a factor 2 dilution (Figure 33a). Therefore, concentrations of toxins collected in the fractions may be near the limit of detection for the N2a-MTT assay. In future studies, collections can be made with additional sample material to increase the availability of the compounds, or by reducing the number of fractions to ensure an accurate collection window (e.g., collection of one congener per fraction versus one congener spread/diluted over two fractions).

The toxicity observed for fraction 7 is assumed to be caused by 2,3,51-trihydroxyCTX3C. However, the high toxicity in fractions 5 and 6 cannot be explained so far. At 2.51 min, a putative CTX congener was observed presenting the same *m/z* as 2,3,51-trihydroxyCTX3C (Figure 34, B-C). This may be described as an epimeric form, however, epimers of the other congeners were found to elute close to the parent compound (time window <0.5 min, Figure 34). Whereas the retention times of the two peaks differed by 1 min for 2,3,51-trihydroxyCTX3C. Because of the observed fragmentation (like 2,3,51-trihydroxyCTX3C, Figure 34 b, and c) and the high toxicity of the respective fractions, the observed peak is a potential CTX congener. Based on the MS data it might be an epimer or isomer of 2,3,51-trihydroxyCTX3C which is currently un-described. Besides this compound, the observed toxicity in fractions 5 and 6 (both undiluted and diluted) could have been due to other unknown CTX congeners. Unidentified matrix compounds, eluting at the beginning of the chromatographic run contributing to the toxicity, cannot be ruled out; however, this would be highly unusual based on the sodium channel-specific mode of action for the assay.

4.3.7. Public Health Implications

CP incidence rates in the EU and European Economic Area countries are low (0.0054 per 100,000 inhabitants per year, excluding travel-related cases). However, it is assumed that only 10-20% of cases are reported in endemic countries [429], percentages of cases that are underreported have never been estimated for non-endemic countries. This is the case for the outbreak described here as additional people were involved who suffered adverse effects but because they did not seek medical

advice, medical care, or self-report to a Public Health institution (see Section 4.3.1) they were therefore not counted. In non-endemic countries (e.g., Germany), the public and even health care providers are largely unaware of CP. However, in 2017, CP was part of a pilot monitoring program bringing together institutions responsible in the field of poisonings (e.g., German Association of Clinical Toxicologists, the German Poisoning Information Centers, and the German Federal Institute for Risk Assessment). Now, with the methods for CTX analysis established at the BfR and a CP awareness program coordinating competent authorities, awareness of the diagnosis of CP will hopefully increase in Germany.

The CTX-like activity in the assay was detected in all samples and several (potential) congeners from the CTX3C-group were identified by LC-MS/MS, together this provides additional evidence for the risk *L. bohar* can pose for CP. Specific guidance levels for congeners of the CTX3C-group do not yet exist, but the demonstration here of this potential CTX hazard in seafood products provides further evidence that this toxin group should be included as part of future risk assessment efforts for CTXs. The findings of this study agree with the literature regarding CTXs identified in fish from the tropical western Pacific catch region [184] and specifically within *L. bohar* [161].

To attempt quantifying the product measured compared to the wider lot, using the package description of 170 g minimum weight per filet, the sixteen portions from the CP cases reported would be estimated at 2.7 kg. This 2.7 kg represents 0.032% of the total two original lots (2,500 kg lot #122 and 6,000 kg lot # 124), irrespective of glazing weight or recalled product (as originally distributed). Samples measured in this study represented 0.14% (7.5 kg net weight measured from lot # 124 with a net weight of 5,400 kg), and 100% of these samples contained CTX toxin equivalents that exceeded all multi-national product legal requirements and recommended guidelines for CTXs. Therefore, forty-five potential CP cases (counting one potential case per fillet investigated) were demonstrably avoided through this recall effort. Generally, not all fish of the same species or from a given area is toxic. Therefore, samples taken from the same lot of the CP outbreak described by Klekamp et al. 2015 have been found non-toxic [410], and this result is consistent with the known sporadic nature of CP. Due to these findings, potential food recalls were considered inefficient public health tools in response to CP outbreaks, and that testing an entire commercial lot may not identify any additional toxic fish [32,410]. This was not the case here, Table 14 illustrates that in the context of CTX outbreaks it may be a necessary precautionary consumer protection effort to employ a 'batch suspicion' under Regulation (EC) No 178/2002; Art. 14 (6) (20) which states that if any part of a batch, lot, or consignment of food is found unsafe then it shall be presumed that all the food in that batch, lot or consignment is also unsafe unless following a detailed assessment that finds no evidence that the rest of the batch, lot or consignment is unsafe. The two boxes of the product tested here and demonstrated to contain CTXs was a successful and clear protectionary recall measure.

It remains unknown whether the 100% positive rate for the samples investigated in this study are anomalous to the larger lot, whether the toxin content of the other portions was below an adverse effect level, or more cases went unreported. Because the lots could be comprised of fish from different locations with different concentrations of various CTX congeners it is unknown if the samples represent the average sample in the larger lot. This highlights the complications from mixed lots, with different fish from potentially different locations, large volume fish imports will therefore remain difficult to predict for risk.

The approach for traceback examinations based on a case- and cluster- description and included the combination of CTX and fish-DNA analysis, was proven here as an important complete workflow for identifying and authenticating the key characteristics of a CP outbreak. In addition to data about patients and their symptoms, the species of fish and the CTX content should be determined in meal remnants (when available) in addition to the analysis of lot-related products, which was achieved here for the first time in Germany.

4.4. Conclusions

Many factors are involved leading up to CP outbreaks, which can make them seem sporadic or unpredictable. There are many variables in each stage of the process from CTX production, fish to fork, followed by diagnosis and medical advice, traceback, and reporting (also confounded by un-reported illnesses); and not all are understood. In non-endemic regions the global seafood trade is responsible for many CP events, however, this trade also provides nearly 20% of the animal protein intake for more than 3.3 billion people; and nearly 40% of total fisheries and aquaculture production were traded internationally [1] demonstrating the dependence of society on the global seafood trade. The fish involved in this CP outbreak was a product of the international seafood trade, the species was mislabeled, and contained a health risk that is unfamiliar to non-endemic consumers. The distribution footprint of the product was large (being widely available), and the CP risk was temporally persistent as well due to the frozen preserved condition of the product and CTXs temperature stability. Based on the high percentage of fish from these lots that contained CTXs over both the EU restrictions and FDA guidance for CTXs in seafood products the likelihood of randomly selecting a fillet portion with a CP risk was ostensibly high. If the seafood trade and food safety authorities do not flag a product as a risk, then it is up to the consumer to educate themselves about potential health hazards. In this outbreak, the responsible species (*L. bohar*) has a known history of causing CP, and guidance on this species is available [39]. However, because the species was mislabeled consumers lacked adequate information to apply the known self-protection measures against CP. Regarding efforts at stopping CTX contaminated products from reaching the market, the region of harvest lacks adequate information about CTXs in territorial waters and CP outbreak documentation is sparse (a similarity with many CP

endemic regions). Specifically, regional accounting of CTX producing algal species, the local environmental factors driving their growth/distribution, an assessment of the frequency of CTXs in commercially targeted species, and the identification of regions of concern (i.e., hot-spots). Environmental changes and adaptations to climate changes [477,478] should be taken into account in current and future CP risk assessments. The accounting of this information is needed by local consumers and those vulnerable to CP via the international seafood trade to make informed decisions about their consumption habits. Regarding the human health response, the lots distributed were large (combined ~8,500 kg) and the results obtained in this small sampling size suggest the health impact of these lots in Germany was potentially underestimated, also supported by data that CP events, in general, are classically underreported. Consumers and restaurants could benefit from public outreach and education, to inform the public to help identify patients and to retain samples for analyses. For example, several of the cases would have been missed in this outbreak without the public outreach efforts following the recall, highlighting the importance of awareness campaigns. Additionally, in non-endemic regions, outbreaks are rare and complex; therefore, a coordinated procedure could aid the various competent authorities responding to the CP outbreak. Particularly, that fish species and CTX content should be determined, and a resource is available for physicians and patients to contact if CP is suspected (i.e., the regional Poisoning Information Centers and the regional Public Health authorities, or directly to the BfR when in Germany). Taken together, this investigation provides additional data towards the response efforts which are important for a CP outbreak: i) immediate action for consumer health protection and legal aspects (symptom recognition, reporting the outbreak to the competent authorities, initializing a traceback effort, recalling the product from the market) and ii) follow up action (product authentication, CTX analysis, and the location of toxin source). The recognition of CP symptoms by the physicians of the German Poisoning Centers and the competent Food Safety Authorities to create the RASFF reporting process was critical to the awareness of this outbreak. Without this recognition, both lots of fish (if not reclaimed) demonstrably would have resulted in more outbreaks.

Standard substances covering the range of CTXs are not available, therefore the detection process based on the two-tiered semi-targeted CTX analysis approach utilized herein was necessary for the successful identification of CTXs. By using this tandem approach, a false negative sample analysis conclusion can be prevented by identifying CTXs based on their cellular function (mode of action). In this study, a high level of mislabeling in these lots was identified, which indicates that the seafood global market (e.g., source, exporters, fish-importers, wholesalers, as well as inspectors and governments) may not recognize CP as being related to mislabeled products. Reg. (EC) No 178/2002; art. 14 (6) applies to these outbreak-associated lots ‘where any part of a batch, lot, or consignment of food is found unsafe then it shall be presumed that all the food in that batch, lot, or consignment is

also unsafe unless following a detailed assessment there is no evidence that the rest of the batch, lot or consignment is unsafe' [418]. Therefore, the regulatory framework exists for labeling a product with sufficient information to protect consumers. However, if the existing rules are not followed then that may indicate a need for a revised enforcement or labeling approach that can effectively inform consumers in the EU (or globally in a CP 'at risk' consumer population). A direct connection between CP mislabeled fish, and the detection of the causative substance therein was made in this study, which corresponds with the first demonstration of this CP connection in Germany. This situation is not unique to Germany, it is a global issue in need of refreshed attention.

4.5. Bibliography

1. FAO. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. **2020**.
21. Wang, D.-Z. Neurotoxins from marine dinoflagellates: a brief review. *Marine drugs* **2008**, *6*, 349-371, doi:10.3390/md20080016.
23. Friedman, M.A.; Fernandez, M.; Backer, L.C.; Dickey, R.W.; Bernstein, J.; Schrank, K.; Kibler, S.; Stephan, W.; Gribble, M.O.; Bienfang, P., et al. An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. *Marine Drugs* **2017**, *15*, 72, doi:10.3390/md15030072.
32. Dickey, R.W.; Plakas, S.M. Ciguatera: A public health perspective. *Toxicon* **2010**, *56*, 123-136, doi:DOI 10.1016/j.toxicon.2009.09.008.
37. FAO and WHO. Report of the expert meeting on ciguatera poisoning. Rome, 19–23 November 2018 *Food Safety and Quality No. 9. Rome* **2020**, *https://doi.org/10.4060/ca8817en*, doi:https://doi.org/10.4060/ca8817en.
38. U.S. Food Drug Administration. *Guidance for Industry: Purchasing Reef Fish Species Associated with the Hazard of Ciguatera Fish Poisoning*; https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-purchasing-reef-fish-species-associated-hazard-ciguatera-fish-poisoning (Accessed 15 October 2020), 2013.
39. U.S. Food Drug Administration. *Fish and Fishery Products Hazards and Controls Guidance Fourth Edition - March 2020*; https://www.fda.gov/media/80637/download (Accessed 15 October 2020), 2020; p 498.
45. EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:https://doi.org/10.2903/j.efsa.2010.1627.
46. Yasumoto, T. Chemistry, etiology, and food chain dynamics of marine toxins. *Proceedings of the Japan Academy, Series B* **2005**, *81*, 43-51.
47. Rajisha, K.; Kishore, P.; Panda, S.K.; Harikrishnan, G.; Ajitha, K.; Suresh, M.; Chowdhury, L.M.; Ravishankar, C.; Kumar, K.A. Incidence of ciguatoxin fish poisoning in Trivandrum, India. **2017**.
50. Quod, J.P.; Turquet, J. Ciguatera in Reunion Island (SW Indian Ocean): Epidemiology and clinical patterns. *Toxicon* **1996**, *34*, 779-785, doi:Doi 10.1016/0041-0101(95)00164-6.
51. de Haro, L.; Schmitt, C.; Sinno-Tellier, S.; Paret, N.; Boels, D.; Le Roux, G.; Langrand, J.; Delcourt, N.; Labadie, M.; Simon, N. Ciguatera fish poisoning in France: experience of the French Poison Control Centre Network from 2012 to 2019. *Clinical Toxicology* **2020**, 10.1080/15563650.2020.1788052, 1-4, doi:10.1080/15563650.2020.1788052.
56. Kusche, H.; Hanel, R. Consumers of mislabeled tropical fish exhibit increased risks of ciguatera intoxication: A report on substitution patterns in fish imported at Frankfurt Airport, Germany. *Food Control* **2021**, *121*, 107647, doi:https://doi.org/10.1016/j.foodcont.2020.107647.
58. Friedemann, M. Ciguatera fish poisoning outbreaks from 2012 to 2017 in Germany caused by snappers from India, Indonesia, and Vietnam. *Journal of Consumer Protection and Food Safety* **2019**, *14*, 71-80, doi:10.1007/s00003-018-1191-8.

59. Loeffler, C.R.; Tartaglione, L.; Friedemann, M.; Spielmeyer, A.; Kappenstein, O.; Bodi, D. Ciguatera Mini Review: 21st Century Environmental Challenges and the Interdisciplinary Research Efforts Rising to Meet Them. *International Journal of Environmental Research and Public Health* **2021**, *18*, 3027.
61. Canals, A.; Martínez, C.V.; Diogène, J.; Gago-Martínez, A.; Cebadera-Miranda, L.; de Vasconcelos, F.M.; Gómez, I.L.; Sánchez, E.V.M.; Alférez, R.C.; Núñez, D., et al. Risk characterisation of ciguatera poisoning in Europe. *EFSA Supporting Publications* **2021**, *18*, 6647E, doi:https://doi.org/10.2903/sp.efsa.2021.EN-6647.
85. Fraga, S.; Rodríguez, F.; Caillaud, A.; Diogène, J.; Raho, N.; Zapata, M. Gambierdiscus excentricus sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). *Harmful Algae* **2011**, *11*, 10-22, doi:http://dx.doi.org/10.1016/j.hal.2011.06.013.
104. Rossignoli, A.E.; Tudó, A.; Bravo, I.; Díaz, P.A.; Diogène, J.; Riobó, P. Toxicity Characterisation of Gambierdiscus Species from the Canary Islands. *Toxins* **2020**, *12*, 134.
133. Abraham, A.; Jester, E.L.E.; Granade, H.R.; Plakas, S.M.; Dickey, R.W. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chem* **2012**, *131*, 192-198, doi:https://doi.org/10.1016/j.foodchem.2011.08.059.
136. Dickey, R.W. *Ciguatera toxins: chemistry, toxicology, and detection*, Second ed.; New York., 2008; Vol. 173, pp. 22.
146. Tudó, À.; Gaiani, G.; Varela, M.R.; Tsumuraya, T.; Andree, K.B.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. Further advance of Gambierdiscus Species in the Canary Islands, with the First Report of Gambierdiscus belizeanus. *Toxins* **2020**, *12*, 692.
156. Spielmeyer, A.; Loeffler, C.R.; Bodi, D. Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. *Toxins* **2021**, *13*, 630.
161. Yogi, K.; Oshiro, N.; Inafuku, Y.; Hirama, M.; Yasumoto, T. Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from the Pacific. *Analytical Chemistry* **2011**, *83*, 8886-8891, doi:10.1021/ac200799j.
169. Yogi, K.; Sakugawa, S.; Oshiro, N.; Ikehara, T.; Sugiyama, K.; Yasumoto, T. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. *Journal of AOAC INTERNATIONAL* **2014**, *97*, 398-402.
183. Chinain, M.; Gatti, C.M.i.; Ung, A.; Cruchet, P.; Revel, T.; Viallon, J.; Sibat, M.; Varney, P.; Laurent, V.; Hess, P., et al. Evidence for the Range Expansion of Ciguatera in French Polynesia: A Revisit of the 2009 Mass-Poisoning Outbreak in Rapa Island (Australes Archipelago). *Toxins* **2020**, *12*, 759.
184. Oshiro, N.; Tomikawa, T.; Kuniyoshi, K.; Ishikawa, A.; Toyofuku, H.; Kojima, T.; Asakura, H. LC–MS/MS Analysis of Ciguatoxins Revealing the Regional and Species Distinction of Fish in the Tropical Western Pacific. *Journal of Marine Science and Engineering* **2021**, *9*, 299.
185. Loeffler, C.R.; Robertson, A.; Flores Quintana, H.A.; Silander, M.C.; Smith, T.B.; Olsen, D. Ciguatoxin prevalence in 4 commercial fish species along an oceanic exposure gradient in the US Virgin Islands. *Environmental toxicology and chemistry* **2018**, *37*, 1852-1863, doi:10.1002/etc.4137.
186. Olsen, D.A.; Nellis, D.W.; Wood, R.S. Ciguatera in the Eastern Caribbean. *Marine Fisheries Review* **1984**, *46*, 13-18.
192. Froese, R.; Pauly, D. FishBase. Availabe online: www.fishbase.org (accessed on 11.20.2020).
218. Chinain, M.; Darius, H.T.; Ung, A.; Fouc, M.T.; Revel, T.; Cruchet, P.; Pauillac, S.; Laurent, D. Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). *Toxicon* **2010**, *56*, 674-690, doi:DOI 10.1016/j.toxicon.2009.05.032.
225. Estevez, P.; Castro, D.; Manuel Leao, J.; Yasumoto, T.; Dickey, R.; Gago-Martinez, A. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. *Food chemistry* **2019**, *280*, 8-14, doi:10.1016/j.foodchem.2018.12.038.

232. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC INTERNATIONAL* **1995**, *78*, 521-527, doi:https://doi.org/10.1093/jaoac/78.2.521.

239. Loeffler, C.R.; Bodi, D.; Tartaglione, L.; Dell'Aversano, C.; Preiss-Weigert, A. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). *Harmful Algae* **2021**, *103*, 101994, doi:https://doi.org/10.1016/j.hal.2021.101994.

245. EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:10.2903/j.efsa.2010.1627.

295. *Discards in the world's marine fisheries. An update.* Kelleher, K., Ed.; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS: Rome, Italy, 2005 Vol. 470, p 131.

296. Sydney Fish Market. Sydney fish market seafood handing guidelines Availabe online: https://www.sydneyfishmarket.com.au/Seafood-Trading/Quality/Food-Safety (accessed on 06.10.2020).

297. Chan, T.Y.K. Ciguatoxic potential of Brown-Marbled Grouper in relation to fish size and geographical origin. *The American Journal of Tropical Medicine and Hygiene* **2015**, *93*, 1117-1121, doi:https://doi.org/10.4269/ajtmh.15-0434.

298. Lewis, R.J. Socioeconomic impacts and management ciguatera in the Pacific. *Bulletin de la Societe de pathologie exotique (1990)* **1992**, *85*, 427-434.

299. Díaz-Asencio, L.; Clausing, R.J.; Vandersea, M.; Chamero-Lago, D.; Gómez-Batista, M.; Hernández-Albernas, J.I.; Chomérat, N.; Rojas-Abrahantes, G.; Litaker, R.W.; Tester, P., et al. Ciguatoxin Occurrence in Food-Web Components of a Cuban Coral Reef Ecosystem: Risk-Assessment Implications. *Toxins* **2019**, *11*, 722.

310. Mak, Y.L.; Wai, T.-C.; Murphy, M.B.; Chan, W.H.; Wu, J.J.; Lam, J.C.; Chan, L.L.; Lam, P.K. Pacific Ciguatoxins in Food Web Components of Coral Reef Systems in the Republic of Kiribati. *Environmental science & technology* **2013**, *47*, 14070-14079.

323. Kibler, S.R.; Tester, P.A.; Kunkel, K.E.; Moore, S.K.; Litaker, R.W. Effects of ocean warming on growth and distribution of dinoflagellates associated with ciguatera fish poisoning in the Caribbean. *Ecological Modelling* **2015**, *316*, 194-210.

337. Smithsonian National Fish Collection. Fish Collections Data at National Museum of Natural History. Availabe online: https://collections.nmnh.si.edu/search/fishes/ (accessed on April 11th).

365. (EC), R. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. 02004R0854 — EN — 01.01.2019 — 013.001 — 1 ed.; Council, E.P.a.o.t., Ed. 2004; Vol. Regulation (EC) No 854/2004.

366. Regulation (EC). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. In *Official Journal of the European Union. L 139/55*, 2004; Vol. 47.

367. (EC), R. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019. In *02019R0627 — EN — 01.01.2021 — 001.001 — 1*, European Parlament, C.o.t.E.U., Ed. 2019.

408. Oshiro, N.; Nagasawa, H.; Kuniyoshi, K.; Kobayashi, N.; Sugita-Konishi, Y.; Asakura, H.; Yasumoto, T. Characteristic Distribution of Ciguatoxins in the Edible Parts of a Grouper, Variola louti. *Toxins* **2021**, *13*, 218.

409. Varriale, F. Emerging toxins of European concern: identification, development of reference material and methods for their detection. Ph.D., University of Naples Federico II, Naples, Italy, 2021.

410. Klekamp, B.G.; Bodager, D.; Matthews, S.D. Use of Surveillance Systems in Detection of a Ciguatera Fish Poisoning Outbreak - Orange County, Florida, 2014. *MMWR Morb Mortal Wkly Rep* **2015**, *64*, 1142-1144, doi:10.15585/mmwr.mm6440a3.
411. European Commission. RASFF Window Version 2.0.5 No. 2017.0345. Availabe online: https://webgate.ec.europa.eu/rasff-window/screen/search (accessed on August 2021).
412. André, L.V.; Van Wynsberge, S.; Chinain, M.; Gatti, C.M.I.; Dempsey, A.; Andréfouët, S. A framework for mapping local knowledge on ciguatera and artisanal fisheries to inform systematic conservation planning. *ICES Journal of Marine Science* **2021**.
413. Loeffler, C.R.; Handy, S.M.; Flores Quintana, H.A.; Deeds, J.R. Fish hybridization leads to uncertainty regarding ciguatera fish poisoning risk: Confirmation of hybridization and ciguatoxin accumulation with implications for stakeholders. *Journal of Marine Science and Engineering* **2019**, *7*, 105.
414. Di Muri, C.; Vandamme, S.G.; Peace, C.; Barnes, W.; Mariani, S. Biodiversity defrosted: unveiling non-compliant fish trade in ethnic food stores. *Biological Conservation* **2018**, *217*, 419-427, doi:https://doi.org/10.1016/j.biocon.2017.11.028.
415. Kappel, K.; Schröder, U. Difficulties in DNA barcoding-based authentication of snapper products due to ambiguous nucleotide sequences in public databases. *Food Control* **2020**, *118*, 107348, doi:https://doi.org/10.1016/j.foodcont.2020.107348.
416. (EU), R. Regulation (EU) No 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy, amending Council Regulations (EC) No 1954/2003 and (EC) No 1224/2009 and repealing Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC. OJ L 354 28.12.2013, p., Ed. 2013.
417. (EU), R. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 Text with EEA relevance. OJ L 304, p. 18–63 Ed. 2011.
418. (EC), R. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *OJ L 031 1.2.2002, p. 1* **2002**.
419. Deeds, J.R.; Handy, S.M.; Fry Jr, F.; Granade, H.; Williams, J.T.; Powers, M.; Shipp, R.; Weigt, L.A. Protocol for building a reference standard sequence library for DNA-based seafood identification. *Journal of AOAC International* **2014**, *97*, 1626-1633.
420. Handy, S.M.; Deeds, J.R.; Ivanova, N.V.; Hebert, P.D.; Hanner, R.H.; Ormos, A.; Weigt, L.A.; Moore, M.M.; Yancy, H.F. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *Journal of AOAC International* **2011**, *94*, 201-210.
421. (EU), R. Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy, amending Council Regulations (EC) No 1954/2003 and (EC) No 1224/2009 and repealing Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC. 354/1, O.J.o.t.E.U.L., Ed. 2013.
422. Strehlow, H.V.; Schultz, N.; Zimmermann, C.; Hammer, C. Cod catches taken by the German recreational fishery in the western Baltic Sea, 2005–2010: implications for stock assessment and management. *ICES Journal of Marine Science* **2012**, *69*, 1769-1780, doi:10.1093/icesjms/fss152.
423. Otero, P.; Pérez, S.; Alfonso, A.; Vale, C.; Rodríguez, P.; Gouveia, N.N.; Gouveia, N.; Delgado, J.o.; Vale, P.; Hirama, M., et al. First Toxin Profile of Ciguateric Fish in Madeira Arquipelago (Europe). *Analytical Chemistry* **2010**, *82*, 6032-6039, doi:10.1021/ac100516q.

424. Aligizaki, K.; Nikolaidis, G. Morphological identification of two tropical dinoflagellates of the genera Gambierdiscus and Sinophysis in the Mediterranean Sea. *J. Biol. Res* **2008**, *9*, 75-82.
425. Estevez, P.; Castro, D.; Pequeño-Valtierra, A.; Leao, J.M.; Vilariño, O.; Diogène, J.; Gago-Martínez, A. An Attempt to Characterize the Ciguatoxin Profile in Seriola fasciata Causing Ciguatera Fish Poisoning in Macaronesia. *Toxins* **2019**, *11*, 221.
426. Gago-Martinez, A.; Leão, J.M.; Estevez, P.; Castro, D.; Barrios, C.; Hess, P.; Sibat, M. Characterisation of ciguatoxins. *EFSA Supporting Publications* **2021**, *18*, 6649E, doi:https://doi.org/10.2903/sp.efsa.2021.EN-6649.
427. Diogène, J.; Rambla, M.; Campàs, M.; Fernández, M.; Andree, K.; Tudó, A.; Rey, M.; Sagristà, N.; Aguayo, P.; Leonardo, S., et al. Evaluation of ciguatoxins in seafood and the environment in Europe. *EFSA Supporting Publications* **2021**, *18*, 6648E, doi:https://doi.org/10.2903/sp.efsa.2021.EN-6648.
428. Sanchez-Henao, J.A.; García-Álvarez, N.; Fernández, A.; Saavedra, P.; Silva Sergent, F.; Padilla, D.; Acosta-Hernández, B.; Martel Suárez, M.; Diogène, J.; Real, F. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. *Science of The Total Environment* **2019**, *673*, 576-584, doi:https://doi.org/10.1016/j.scitotenv.2019.03.445.
429. Canals, A.; Martínez, C.V.; Diogène, J.; Gago-Martínez, A.; Cebadera-Miranda, L.; de Vasconcelos, F.M.; Gómez, I.L.; Sánchez, E.V.M.; Alférez, R.C.; Núñez, D. Risk characterisation of ciguatera poisoning in Europe. *EFSA Supporting Publications* **2021**, *18*, 6647E.
430. Tudó, À.; Toldrà, A.; Andree, K.B.; Rey, M.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. First report of Gambierdiscus in the Western Mediterranean Sea (Balearic Islands). *Harmful Algae News* **2018**.
431. Kohli, G.S.; Farrell, H.; Murray, S.A. Climate Change and Marine and Freshwater Toxins. In *9. Gambierdiscus, the cause of ciguatera fish poisoning: an increased human health threat influenced by climate change*, Luis, M.B., Carmen, L., Natalia, V., Eds. De Gruyter: 2015; doi:10.1515/9783110333596-011pp. 273-312.
432. Intergovernmental Panel on Climate Change. Special report on the ocean and cryosphere in a changing climate. **2019**.
433. EUMOFA. The EU fish market 2019 edition. European Market Observatory for Fisheries and Aquaculture Products, Ed. European Union: Luxembourg: Publications Office of the European Union, 2019, 2019; 10.2771/168390p 107.
434. Bienfang, P.; DeFelice, S.; Dowling, A. Quantitative evaluation of commercially available test kit for ciguatera in fish. *Food and Nutrition Sciences* **2011**, *2011*.
435. Chan, T.Y.K. Epidemiology and Clinical Features of Ciguatera Fish Poisoning in Hong Kong. *Toxins* **2014**, *6*, 2989-2997, doi:10.3390/toxins6102989.
436. Sadovy, Y. Ciguatera: a continuing problem for Hong Kong's consumers, live reef fish traders and high-value target species. *Live Reef Fish Information Bulletin* **1999**.
437. Darius, H.T.; Drescher, O.; Ponton, D.; Pawlowiez, R.; Laurent, D.; Dewailly, E.; Chinain, M. Use of folk tests to detect ciguateric fish: a scientific evaluation of their effectiveness in Raivavae Island (Australes, French Polynesia). *Food Additives & Contaminants: Part A* **2013**, *30*, 550-566, doi:10.1080/19440049.2012.752581.
438. Commission, E. *EU consumer habits regarding fishery and aquaculture products*; Maritime Affairs and Fisheries: 2021; p 147.
439. Khaksar, R.; Carlson, T.; Schaffner, D.W.; Ghorashi, M.; Best, D.; Jandhyala, S.; Traverso, J.; Amini, S. Unmasking seafood mislabeling in US markets: DNA barcoding as a unique technology for food authentication and quality control. *Food Control* **2015**, *56*, 71-76.
440. Miller, D.; Jessel, A.; Mariani, S. Seafood mislabelling: comparisons of two western European case studies assist in defining influencing factors, mechanisms and motives. *Fish and fisheries* **2012**, *13*, 345-358.
441. Aung, M.M.; Chang, Y.S. Traceability in a food supply chain: Safety and quality perspectives. *Food control* **2014**, *39*, 172-184.

442. Di Pinto, A.; Marchetti, P.; Mottola, A.; Bozzo, G.; Bonerba, E.; Ceci, E.; Bottaro, M.; Tantillo, G. Species identification in fish fillet products using DNA barcoding. *Fisheries Research* **2015**, *170*, 9-13, doi:https://doi.org/10.1016/j.fishres.2015.05.006.
443. Ardura, A.; Linde, A.R.; Moreira, J.C.; Garcia-Vazquez, E. DNA barcoding for conservation and management of Amazonian commercial fish. *Biological Conservation* **2010**, *143*, 1438-1443.
444. Gold, J.R.; Willis, S.C.; Renshaw, M.A.; Buentello, A.; Walker, H.J.; Puritz, J.B.; Hollenbeck, C.M.; Voelker, G. Phylogenetic relationships of tropical eastern Pacific snappers (Lutjanidae) inferred from mitochondrial DNA sequences. *Systematics and Biodiversity* **2015**, *13*, 596-607, doi:10.1080/14772000.2015.1078857.
445. O'Neill, M.F.; Leigh, G.M.; Martin, J.M.; Newman, S.J.; Chambers, M.; Dichmont, C.M.; Buckworth, R.C. Sustaining productivity of tropical red snappers using new monitoring and reference points. **2011**.
446. Darius, H.T.; Ponton, D.; Revel, T.; Cruchet, P.; Ung, A.; Tchou Fouc, M.; Chinain, M. Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. *Toxicon* **2007**, *50*, 612-626, doi:http://dx.doi.org/10.1016/j.toxicon.2007.05.007.
447. Federal Agency for Agriculture and Food. List of commercial designations for fishery and aquaculture products Fisheries, Ed. 2021.
448. (EU), R. Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy, amending Council Regulations (EC) No 1954/2003 and (EC)

No 1224/2009 and repealing Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC. 354/1, O.J.o.t.E.U.L., Ed. 2013.
449. Gesetz zur Durchführung der Rechtsakte der Europäischen Gemeinschaft über die Etikettierung von Fischen und Fischereierzeugnissen (Fischetikettierungsgesetz - FischEtikettG) FischEtikettG Ausfertigungsdatum: 01.08.2002 Vollzitat:"Fischetikettierungsgesetz vom 1. August 2002 (BGBl. I S. 2980), das zuletzt durch Artikel 1 des Gesetzes vom 20. Oktober 2015 (BGBl. I S. 1736) geändert worden ist". *Bundesgesetzblatt* **2015**, *40*, 3.
450. Verordnung zur Durchführung des Fischetikettierungsgesetzes. (Fischetikettierungsverordnung - FischEtikettV) Fisch Etikett V Ausfertigungsdatum: 15.08.2002Vollzitat:"Fischetikettierungsverordnung vom 15. August 2002 (BGBl. I S. 3363), die zuletzt durch Artikel 1 derVerordnung vom 5. November 2015 (BGBl. I S. 1926) geändert worden ist". *Bundesgesetzblatt* **2015**, *44*.
451. Takahashi, M.; DiBattista, J.D.; Jarman, S.; Newman, S.J.; Wakefield, C.B.; Harvey, E.S.; Bunce, M. Partitioning of diet between species and life history stages of sympatric and cryptic snappers (Lutjanidae) based on DNA metabarcoding. *Scientific reports* **2020**, *10*, 1-13.
452. Ali, M.K.H.; Belluscio, A.; Ventura, D.; Ardizzone, G. Feeding ecology of some fish species occurring in artisanal fishery of Socotra Island (Yemen). *Marine pollution bulletin* **2016**, *105*, 613-628.
453. Randall, J.E. *Coastal fishes of Oman*; University of Hawaii Press: 1995.
454. Ha, D.V.; Uesugi, A.; Uchida, H.; Ky, P.X.; Minh, D.Q.; Watanabe, R.; Matsushima, R.; Oikawa, H.; Nagai, S.; Iwataki, M., et al. Identification of Causative Ciguatoxins in Red Snappers Lutjanus bohar Implicated in Ciguatera Fish Poisonings in Vietnam. *Toxins* **2018**, *10*, 420.
455. Banner, A.H.; Helfrich, P.; Piyakarnchana, T. Retention of Ciguatera Toxin by the Red Snapper, Lutjanus bohar. *Copeia* **1966**, *1966*, 297-301, doi:10.2307/1441137.
456. Oshiro, N.; Yogi, K.; Asato, S.; Sasaki, T.; Tamanaha, K.; Hirama, M.; Yasumoto, T.; Inafuku, Y. Ciguatera incidence and fish toxicity in Okinawa, Japan. *Toxicon* **2010**, *56*, 656-661, doi:http://dx.doi.org/10.1016/j.toxicon.2009.05.036.
457. Gaboriau, M.; Ponton, D.; Darius, H.T.; Chinain, M. Ciguatera fish toxicity in French Polynesia: size does not always matter. *Toxicon* **2014**, *84*, 41-50, doi:10.1016/j.toxicon.2014.03.006.

458. Teh, L.C.L.; Pauly, D. Who Brings in the Fish? The Relative Contribution of Small-Scale and Industrial Fisheries to Food Security in Southeast Asia. *Frontiers in Marine Science* **2018**, *5*, doi:10.3389/fmars.2018.00044.
459. Zeller, D.; Harper, S.; Zylich, K.; Pauly, D. Synthesis of underreported small-scale fisheries catch in Pacific island waters. *Coral Reefs* **2015**, *34*, 25-39.
460. Pomeroy, R.; Thi Nguyen, K.A.; Thong, H.X. Small-scale marine fisheries policy in Vietnam. *Marine Policy* **2009**, *33*, 419-428, doi:https://doi.org/10.1016/j.marpol.2008.10.001.
461. Pham, C.M.H. Boats of Vietnam. *Encyclopaedia of the History of Science, Technology, and Medicine in Non-Western Cultures* **2015**.
462. Pho Hoang Han, M.M.M. Fisheries development in Vietnam: A case study in the exclusive economic zone. *Ocean & Coastal Management* **2007**, *50*, 699-712, doi:https://doi.org/10.1016/j.ocecoaman.2002.06.001.
463. Tun, K.; Chou, L.M.; Cabanban, A.; Tuan, V.; Suharsono, T.Y.; Sour, K.; Lane, D. Status of coral reefs, coral reef monitoring and management in Southeast Asia 2004. 2005.
464. Rhodes, L.L.; Smith, K.F.; Murray, S.; Harwood, D.T.; Trnski, T.; Munday, R. The epiphytic genus Gambierdiscus (Dinophyceae) in the Kermadec Islands and Zealandia regions of the southwestern Pacific and the associated risk of ciguatera fish poisoning. *Marine drugs* **2017**, *15*, 219.
465. Roeder, K.; Erler, K.; Kibler, S.; Tester, P.; Van The, H.; Nguyen-Ngoc, L.; Gerdts, G.; Luckas, B. Characteristic profiles of Ciguatera toxins in different strains of Gambierdiscus spp. *Toxicon* **2010**, *56*, 731-738, doi:http://dx.doi.org/10.1016/j.toxicon.2009.07.039.
466. Saraya, A.; Sintunawa, C.; Wacharapluesadee, S.; Swangpun, K.; Dumrongchua, S.; Wilde, H.; Hemachudha, T. Marine Fish Toxins in Thailand: Report of 6 Suspected Ciguatera Cases. *Case Reports in Clinical Medicine* **2014**, *Vol.03No.05*, 7, doi:10.4236/crcm.2014.35065.
467. Flanders Marine Institute. MarineRegions.org Available online at www.marineregions.org. , 2021.
468. (VLIZ), F.M.I. Maritime Boundaries Geodatabase, version 8 in conjunction with NOAA. NOAA, Ed. 2014.
469. FAO. FAO Statistical Areas for Fishery Purposes. In: FAO Fisheries and Aquaculture Department [online]. FAO, Ed. Rome, 2014.
470. Institute for Marine Remote Sensing, U.o.S.F.I.U., Institut de Recherche pour le Développement (IRD), UNEPWCMC, The WorldFish Center and WRI,. Global Coral Reefs composite dataset compiled from multiple sources for use in the Reefs at Risk Revisited project incorporating products from the Millennium Coral Reef Mapping Project prepared by IMaRS/USF and IRD. . 2011.
471. Patrias, K.; Wendling, D. Citing medicine: the NLM style guide for authors, editors, and publishers National Library of Medicine (US): Bethesda, Maryland, 2007.
472. Badrudin, B.; Aisyah, A. Separate stocks of red snapper exploitation and management in the Indonesian sector of the Arafura Sea. *Indonesian Fisheries Research Journal* **2017**, *15*, 81-88.
473. De Mitcheson; Yvonne Sadovy; Cornish, A.; Domeier, M.; Colin, P.L.; Russell, M.; Lindeman, K.C. A Global Baseline for Spawning Aggregations of Reef Fishes. *Conservation Biology* **2008**, *22*, 1233-1244, doi:https://doi.org/10.1111/j.1523-1739.2008.01020.x.
474. Chollett, I.; Priest, M.; Fulton, S.; Heyman, W.D. Should we protect extirpated fish spawning aggregation sites? *Biological Conservation* **2020**, *241*, 108395, doi:https://doi.org/10.1016/j.biocon.2019.108395.
475. Domeier, M.L.; Colin, P.L.; Donaldson, T.J.; Heyman, W.D.; Pet, J.S.; Russell, M.; Sadovy, Y.; Samoilys, M.A.; Smith, A.; Yeeting, B.M. Transforming coral reef conservation: reef fish spawning aggregations component. *Spawning Aggregation Working Group Report, The Nature Conservancy, Hawaii* **2002**, 85.
476. Oshiro, N.; Tomikawa, T.; Kuniyoshi, K.; Kimura, K.; Kojima, T.; Yasumoto, T.; Asakura, H. [Detection of Ciguatoxins from Fish Introduced into a Wholesale Market in Japan]. *Shokuhin Eiseigaku Zasshi* **2021**, *62*, 8-13, doi:10.3358/shokueishi.62.8.

477. Dung, L.D. The status of coral reefs in central Vietnam's coastal water under climate change. *Aquatic Ecosystem Health & Management* **2020**, *23*, 323-331, doi:10.1080/14634988.2020.1819715.
478. Hung, N.Q.; Chieu, H.D.; Dung, D.T.; Duc, V.T. Climate Change Impacts on Marine Ecosystems in Vietnam. In *Environmental Management of Marine Ecosystems*, Md. Nazrul Islam, S.E.J., Ed. CRC Press: Boca Raton, 2017; pp. 209-235.

Chapter 5. Extraction modification

5.1. Introduction

The extraction of CTXs from a biological matrix is typically the initial step towards an eventual CTX detection. This is an essential process worth careful consideration, as the development of a method, depending on its design, will selectively target specific compounds that are enriched in the final semi-purified sample and extract. Because CTXs are tissue bound, current sample extraction processes typically begin with breaking down cellular walls in a fish tissue matrix by a mechanical means to free up and facilitate the separation of tissue bound components. Following this crude breakdown of tissue is often a series of steps of separation to remove unwanted components, for instance, initial steps involve protein precipitation, then several steps of liquid-liquid partitioning, e.g., for defatting, drying/evaporation steps, and one or more solid phase extraction(s) (SPE; reviews, e.g., provided by [25,479]). These methods, together, can be time-intensive, consisting of overnight protein precipitation, temperature-controlled drying steps for aqueous phases [169,480,481], and often include the use of large volumes of solvents (e.g., 19 mL [481] or 6 mL [169,482] per gram tissue for initial extraction). These large solvent volumes can be difficult to handle, hazardous, expensive, and a problem for chemical waste disposal. Rapid extraction methods have been developed to overcome extraction based time disadvantages, however, these methods often have speed-applicability tradeoffs, where they are often designed with a focus towards specific compounds (CTX1B in case of [243]), medium polar congeners [483], or they are coupled with LC-MS/MS methods with an equally specific target of C-CTX or P-CTX [169,243,481-483]. However, the potential pitfall of a narrow-focused approach is the possibility of a false-negative outcome when the principal compound is absent (or below the LOD) and other known congeners are overlooked. Analyses by LC-MS/MS also require a low matrix interference (noise) and some rapid extraction methods can result in an increasing baseline as the runtime increases, this implies that unpolar matrix compounds/constituents are present in the final extract. Therefore, some of these methods can be deemed unsuitable to investigate a broad range of CTX congeners, which represent a range of polarities and have been shown to elute at time points representative of the entire chromatographic run (depending upon the conditions of the method). A method capable of detecting a broad range of CTXs is required for CTX detection in the EU [45,365-367,484,485]. *A priori* user assumptions about the region of origin of a sample can lead to a false-negative conclusion if a region-specific CTX group is targeted and not present in the sample. As discussed in Chapter 4, species substitution, mislabeling, and invasive species (CTX-producing or potential CP-vector) are all problems facing the global fish trade

which can reduce product label certainty and question the perceived region that a targeted approach would be based upon [59]. This study was therefore designed to address these known issues: (1) extraction efficiency, (2) speed of use, (3) waste stream, and (4) reduced likelihood of a false negative result.

Because CTXs can be protein-bound [486-488], extraction protocols using mechanical treatment can result in an incomplete breakdown of the tissue. Therefore, to solve issue 1, the approach described herein utilized a new approach for the degradation of this protein matrix, founded on enzymatic hydrolysis via papain (described as suitable for fish protein hydrolysis [489]). Following digestion, extraction, defatting, and solid-phase extraction (SPE), further enhancements in solvent volumes were improved (issue 3) and this process was demonstrated to reduce the number and duration of evaporation steps, allowing the complete extraction process to be completed in one working day (7–8 h for 4–6 samples, treated by one person) (issue 2). An extraction product suitability test was performed based on the two-tiered approach for CTX analysis (N2a-assay and Liquid chromatography-mass spectrometry LC-MS/MS). To avoid issue number 4, the LC-MS/MS method included >30 congeners reported in the literature [37]. This method is considered as a semi-targeted workflow because reference standards are still lacking for most compounds which would normally be required to provide confirmative information such as retention time or fragmentation pattern [490]. Because this congener approach is inclusive of the known spectrum of CTXs, it is applicable for both *a priori* assumed CTX groups as well as blind, unbiased sample analysis. Furthermore, a major impediment towards method development is the availability of standards and reference materials, the exchange of naturally incurred fish tissue can be costly and logistically prohibitive [59]. Therefore, the method described herein was designed for (freeze-)dried fish tissue and applicable to both raw (wet) and dry tissue. The dried material is temperature stable, thus cutting shipping logistics costs and issues (costs associated with weight, express (fast), and temperature-controlled shipping). This incorporation into the method design allows for a range of sample conditions and material transfer capabilities.

5.2. Materials and Methods

5.2.1. Materials and reagents

Standard solutions of CTX1B (4 µg L^{-1}), 52-epi-54-deoxyCTX1B (P-CTX-2, 1 µg L^{-1}), and 54-deoxyCTX1B (P-CTX-3, 2 µg L^{-1}) in methanol were purchased from Professor R. J. Lewis (The Queensland University, Australia, prepared 17.11.2005). CTX3C (100 ng, lot APK4222) was purchased from FUJIFILM Wako Chemicals Europe GmbH (Neuss, Germany) and dissolved in 1 mL methanol. All

solutions were stored in glass vials at −20 °C. Mixed standard solutions were prepared in methanol and stored in glass vials at −20 °C.

Acetonitrile, methanol, formic acid, ammonium acetate (all LC-MS grade), chloroform, ethyl acetate (LC grade), *n*-hexane (GC-MS grade), acetone, acetic acid, citric acid monohydrate, anhydrous sodium carbonate, and sodium chloride (all p.a. grade) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Papain (>30,000 USP-U mg^{-1}, for biochemistry) was purchased from Carl Roth (Karlsruhe, Germany). Deionized water was prepared using a Milli-Q Reference A+ system (Merck Millipore, Darmstadt, Germany). Bond Elut SI (silica), 500 mg, 3 mL cartridges for solid-phase extraction (SPE) (Agilent, Waldbronn, Germany) and Chromabond Easy, 3 mL, 200 mg (Macherey-Nagel, Düren, Germany) were used according to the manufacturer, Chromabond Easy sorbent consists of a polystyrene-divinylbenzene copolymer modified with a weak anion exchanger.

Naturally contaminated snapper samples (*L. bohar*, previously verified by DNA barcoding, [58], and the subject of Chapter 4) were obtained during a CP incident in Germany in 2017. The fish of the respective lot was caught in the FAO mayor fishing area 71. An overview concerning sample data (catchment area, capture time) and previously conducted sample analyses is provided in [58]. Fillets were utilized without skin. Lyophilization was performed in a freeze-dryer (Lyovac GT2, Amsco/Finn-Aqua, Hürth, Germany) over 36 h for unskinned fillets. Freeze-dried material was ground to a fine powder, transferred to 50 mL polypropylene tubes, and stored at 20 °C before usage. Water contents were 80% for snapper (determined for fillet without skin, glaze water was removed before freeze-drying).

Details concerning chemicals, materials, and the cell line utilized for the N2a-assay are provided in [239] and Section 3.2.

5.2.2 Sample Preparation—Enzyme Protocol

A flow chart of the sample preparation is provided in Figure 35. Aqueous solutions were prepared with deionized water. Due to limited stability, the papain solution, as well as solvent mixtures for silica gel SPE (SiOH SPE), were prepared immediately before use. Acidified ethyl acetate (ethyl acetate + 0.1 vol% acetic acid) was freshly prepared daily to prevent storage-related ester hydrolysis. To avoid the possible sorption of CTX to plastic surfaces, all sample preparation was conducted in glass vessels (except SPE cartridges) [243]. In the following, vortex steps were performed for 30 s. Centrifugation was conducted at 1900× g for 3 min.

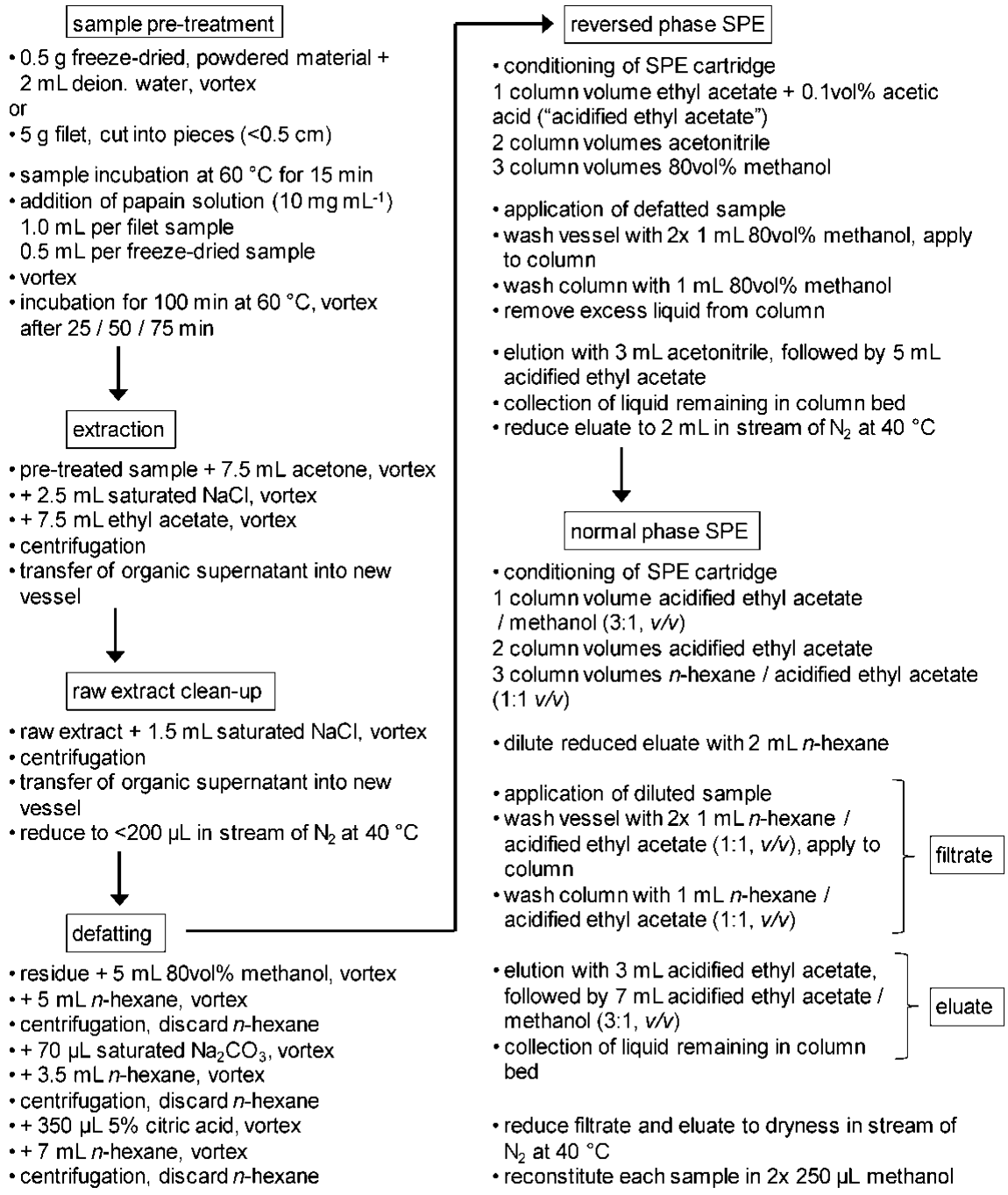


Figure 35. Flow chart of the sample preparation ; vortex steps were performed for 30 s each, centrifugation was conducted for 3 min at 1900× g. Image Copyright: © 2021 by the authors of Spielmeyer, Loeffler and Bodi [156] Licensee MDPI, Basel, Switzerland. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license by Spielmeyer et al. 2021 [156].

5.2.3 Sample Pre-Treatment and Extraction

Frozen or thawed fish fillets were cut into pieces (length ≤0.5 cm) and 5.00 ± 0.01 g weighed and placed into 50 mL glass vessels each with a screw cap. Freeze-dried sample material 0.50 ± 0.01 g was mixed with 2 mL deionized water before use.

Incubation of fish samples with water at 60 °C was conducted for 15 min. Following incubation, 1 mL of papain solution (10 mg mL^{-1}) per fillet sample and 0.5 mL per freeze-dried sample were added. All samples were incubated at 60 °C for 100 min to facilitate protein hydrolysis. Samples were vortexed at time points 25, 50, and 75 min to enhance papain-induced matrix decomposition.

CTXs extraction was performed by A. Spielmeyer over three consecutive steps. In step one, 7.5 mL acetone was added to the hydrolyzed sample, and the mixture was vortexed. Secondly, 2.5 mL of a saturated sodium chloride solution was added, then vortexed. Third, 7.5 mL ethyl acetate was added, followed by vortexing and centrifugation.

The organic supernatant (raw extract) was transferred to a new glass vessel (50 mL). Then mixed with 1.5 mL saturated sodium chloride solution and vortexed, then centrifuged. The supernatant was then transferred into a new 50 mL glass vessel and reduced to ≤200 µL in a stream of nitrogen at 40 °C. In the case where a sample has a high-fat content (e.g., grouper matrix), a liquid fat residue may be present; however, the presence of this residue will not impair the proceeding steps.

5.2.4 Defatting

The defatting procedure was performed by A. Spielmeyer and adapted from [481] and modified using reduced solvent volumes (82.5%) to permit handling in glass centrifuge tubes. Defatting with *n*-hexane was conducted in three steps. In step one, the residue product of the extraction steps of Section 5.2.3. was reconstituted in 5 mL 80 vol% methanol, and to this, 5 mL *n*-hexane was added. The sample was then vortexed, centrifuged, and the *n*-hexane phase (upper layer) was discarded.

In step two, 70 µL of a saturated sodium carbonate solution was added to the methanol phase, followed by brief (5s) vortexing. Then, 3.5 mL *n*-hexane was added, followed by vortexing, centrifugation, and the *n*-hexane phase was discarded. In step three, 350 µL of 5% citric acid solution was added, and the sample vortexed. Then, 7 mL *n*-hexane was added, the sample was vortexed, centrifuged, and the *n*-hexane phase was discarded. The remaining methanol phase was directly purified by SPE. Removal of precipitated salt residues was not necessary.

5.2.5 Reversed-Phase (RP) SPE

Chromabond Easy cartridges were selected for reversed-phase SPE, under reduced pressure conditions (ca. 960 mbar). The column was conditioned with one column volume of acidified ethyl acetate, two-column volumes of acetonitrile, and three column volumes of 80 vol% methanol.

The defatted product from Section 5.2.4. was applied directly to the column with a flow rate of approximately 2 mL min^{-1}. The glass vessel was washed twice with 1 mL 80 vol% methanol, and each wash was applied to the column. The column itself was then washed with 1 mL 80 vol% methanol, then the column was allowed to run dry to ensure excess liquid was removed.

A 10 mL glass tube was placed under the column and a reduced pressure of 960 mbar was applied to the column. 3 mL of acetonitrile was added and after the first approximately 300 µL acetonitrile elution passed through the column was returned to atmospheric pressure and 5 mL acidified ethyl acetate was added to the column. The eluate was collected and positive pressure at the column inlet was added to collect the excess liquid remaining in the column bed. The eluate was reduced to 2 mL in a stream of nitrogen at 40 °C.

5.2.6 Normal-phase (NP) SPE

Normal-phase SPE was conducted by A. Spielmeyer using Bond Elut SI cartridges at atmospheric pressure. This step provides two fractions (“filtrate” and “eluate”) which are distinct for CTX congeners and matrix load. Consequently, the fractions are recommended to be stored separately (as was done here).

The column was initially conditioned using one column void volume of acidified ethyl acetate/methanol (3:1, *v/v*), two-column volumes of acidified ethyl acetate, and three column volumes of acidified ethyl acetate/*n*-hexane (1:1, *v/v*), allowed to pass to waste. After column conditioning, a 10 mL glass tube was placed under the column outlet for “filtrate” sample collection.

The reduced eluate of Section 5.2.5. was diluted with 2 mL *n*-hexane and applied to the column with a flow rate of approximately 2 mL min^{-1}. The glass vessel was rinsed twice with 1 mL acidified ethyl acetate/*n*-hexane (1:1, *v/v*), and the rinse solvent was applied to the column. The column was washed with 1 mL acidified ethyl acetate/*n*-hexane (1:1, *v/v*). The glass tube under the column outlet was removed afterward. It contained the fraction “filtrate”.

For the elution, an additional separate 10 mL glass tube was placed under the column outlet for collection. Elution was performed with 3 mL of acidified ethyl acetate, followed by 7 mL of acidified ethyl acetate/methanol (3:1, *v/v*). In the end, excess liquid remaining in the column bed was collected by applying positive pressure at the column inlet to obtain the second fraction “eluate”. Both fractions

were reduced to dryness in a stream of nitrogen at 40 °C. For sample reconstitution, the vessels were rinsed twice with 250 µL methanol. Samples were stored in glass vials at −20 °C.

5.2.7 Extraction comparison

To compare the performance of the method described herein and a commonly cited mechanical extraction-based protocol described by [480] and used in Chapters 2, 3, and 4, the same sample material was extracted using the mechanical extraction protocol (with slight modifications) as follows. Briefly, 5.00 ± 0.01 g fish fillets were homogenized by ultra turrax and subsequently extracted twice, with 15- and 10-mL acetone. The aqueous acetone extract was evaporated to dryness in a stream of nitrogen at 40 °C, and the residue was reconstituted in 5 mL 80 vol% methanol. Defatting was performed twice with 5 mL *n*-hexane each. The aqueous methanol phase was reduced to dryness. The dry residue was reconstituted in 5 mL water and CTXs were extracted twice with chloroform (two times 5 mL). The chloroform extract was reduced to dryness and reconstituted in 50 µL chloroform for a concentrated transfer. A Bond Elut SI cartridge was pre-conditioned with 5 vol% water in methanol, 100% methanol, and chloroform. Following column conditioning, the concentrated sample was applied to the column. The sample vessel was rinsed three times with 200 µL chloroform (to ensure compound transfer from the vessel to the column), and the combined rinse solvent was applied to the column and allowed to pass through (but not run dry, a minimum meniscus was maintained above the bed). The cartridge was then washed with one column volume of chloroform, this was allowed to pass to waste. A 10 mL glass tube was placed under the column for collection and the elution was then performed with two column volumes of 10 vol% methanol in chloroform. The eluate was reduced to dryness and reconstituted in 1 mL methanol and stored in a glass vial at −20 °C until usage. The additional SPE step described by [480] using an amino phase was not conducted as the purity of extracts was sufficient for N2a-assay.

5.2.8 Extract Performance in the N2a-assay

The extract products resulting from Section 5.2.6, were investigated using the N2a-MTT-assay. Cell line maintenance and dosing procedures are described in [232,247,480], with cell line modifications described in Chapter 3 and published in [239]. For each test sample, an eight-point dose-response curve was performed, and cellular responses were compared among replicate sample analysis.

To investigate potential matrix components inherent in the samples that could cause interference in the N2a-assay (i.e., growth or non-specific cell death), blank matrix extracts of snapper (fillet and freeze-dried) were also prepared according to Figure 35 and Section 5.2 and investigated via

the N2a-assay. Based on the starting tissue type extract, samples were evaluated for any interfering matrix effects on cell performance when compared to non-exposed control cells. The tissue equivalent (TE) concentration range applied was between 0.24–31.25 mg wet TE (i.e., fillet) and 0.024–3.13 mg dry TE (i.e., freeze-dried), respectively. Eluate and filtrate samples were utilized in combination and independently to evaluate the individual contributions or potential impacts on the cell assay.

In addition to the CTX-blank matrix evaluation, naturally contaminated snapper samples (*L. bohar*) were investigated to test the performance. This naturally incurred sample material was obtained during a product recall after a CP incident in Germany in 2017 [58]. The material was tested in Chapter 4 and deemed CTX-positive. Three independent fillet samples were extracted using the method described herein. These samples were representative of a low, medium, and high toxicity among the spectrum of samples investigated in that lot. Eluate and filtrate portions of the extracts were evaluated separately and in combination. The range of mg TE applied among all samples was between 0.005 and 30 mg wet TE per well.

5.3 Results and Discussion

5.3.1. Development of the revised extraction protocol

Existing CTX extraction methods that use acetone or aqueous methanol require a time-intensive drying step after tissue filtration and often also overnight protein precipitation [480]. The enzyme digestion method included a first extraction step using acetone for CTX extraction from the matrix. Saturated sodium chloride solution was added afterward to increase the polarity of the aqueous phase. This enabled the partitioning of the water content containing the vast of the polar matrix load. It also reduced the need for drying aqueous acetone as commonly described in acetone based CTX extraction methods, thus advancing the method's speed. The raw extract was then washed with aqueous saturated sodium chloride for additional extraction of polar matrix components from the organic phase. Additional extraction steps from the hydrolyzed fish matrix resulted in a diminishing non-productive yield, an observation further supported by the results of other studies [481].

The removal of lipid components from the residue of the primary extract was based on the method by [481] and modified through a solvent volume reduction. The residue was reconstituted in 80% methanol and primary defatting was conducted with *n*-hexane. Then saturated sodium carbonate and 5% citric acid were added to remove basic and acidic matrix constituents using a second and third *n*-hexane extraction, respectively.

To speed up the sample preparation procedure, the reversed-phase (RP) SPE was conducted first, followed by a normal phase (NP) SPE (see also [243]). By switching the order of SPEs, a drying step

was avoided. Applications of NP SPE are often conducted after the defatting step [481,482], and this normally requires drying the aqueous methanol to reconstitute the sample in a suitable solvent for NP SPE. Here, the defatted sample could be applied directly to an RP SPE. A quality control step check determined that no elution of CTXs was observed with 80 vol% methanol, and therefore, no water content adjustment was needed. Because ethyl acetate and water are not miscible, two solvents were used for elution from the RP column. To remove the remaining water from the column material, acetonitrile was used before the application of acidified ethyl acetate. Adding acetic acid was essential as recovery rates were below 25% for CTX1B (20%), 52-*epi*-54-deoxyCTX1B (21%), and 54-deoxyCTX1B (23%) without acidification. On the contrary, CTX3C was less affected. The effect of acetic acid on the CTX4A group congeners might be due to the hydroxylated side chain of these compounds. The RP SPE material contains a weak anion exchanger, which might influence the retention of the congeners.

The NP SPE delivered two fractions, "filtrate" and "eluate" (see Sections 5.2 and 5.2.6 for details), and both contained CTX congeners. The partition was influenced by the volume applied to the NP SPE. If excess volumes are used CTX3C was demonstrated to transfer fully into the filtrate fraction (a portion containing unwanted matrix components and was demonstrably less appropriate for sensitive LC-MS/MS analysis). Other congeners (i.e., 52-*epi*-54-deoxyCTX1B or 54-deoxyCTX1B) were (partly) found in the filtrate fraction as well, but to a lesser degree. This partition into eluate and filtrate negatively influences parameters such as the limit of detection. Therefore, within the final protocol, the RP SPE eluate is reduced and diluted with *n*-hexane (here: final volume 4 mL) before being applied to the NP SPE. This allows a complete transfer of more polar congeners into the eluate only. This observation demonstrates that caution should be exercised when developing methods for optimizing SPE conditions based on specific CTXs, i.e., what may work for one congener might not apply to all. The approach used in Chapter 2 was a specific method only looking for CTX1B, it turned out to be the case that only 33% (n = 29) of samples that were positive by the N2a-assay (n = 89) were positive for CTX1B (i.e., 29/89), indicating that other Na_v active compounds were present, but not identified. As further demonstrated in Chapter 4 it can often be the case that naturally incurred fish contain a suite of CTXs, which are responsible for their CP risk, not just one.

The NP SPE enabled a fractionation of CTX congeners and matrix compounds with the majority of matrix constituents being transferred into the "filtrate" sample. The detection of CTX3C in the "filtrate" was not impaired by those compounds. The separation of matrix and analyte peaks is especially beneficial for evaluating (potential) CTXs for which no standards exist. Investigating potential CTXs without standards is complicated, thus, the sample preparation protocol was applied to blank matrix samples. LC-MS/MS analysis revealed no confounding peaks, additional data provided in Spielmeyer (2021) [156]. This allowed the focus to remain on potential CTX signals observed in fractions

from suspected (N2a-assay positive) samples and their consideration and evaluation as being more likely to be authentic CTX congeners instead of false matrix components.

Investigation into the observation of CTX3C splitting up (at a ratio of 2:3) among the eluate and filtrate (Figure 36), irrespective of the matrix, was not conducted further. CTX3C was still present in the target eluate and the LODs and LOQs were comparable to the congeners detected in the eluate only [156]. A positive confirmation is currently sufficient for target method development, as no specific guidance concerning CTX3C exists. Therefore, with the broad ban on all CTXs in the EU, a confirmation satisfies the testing requirements [484,485].

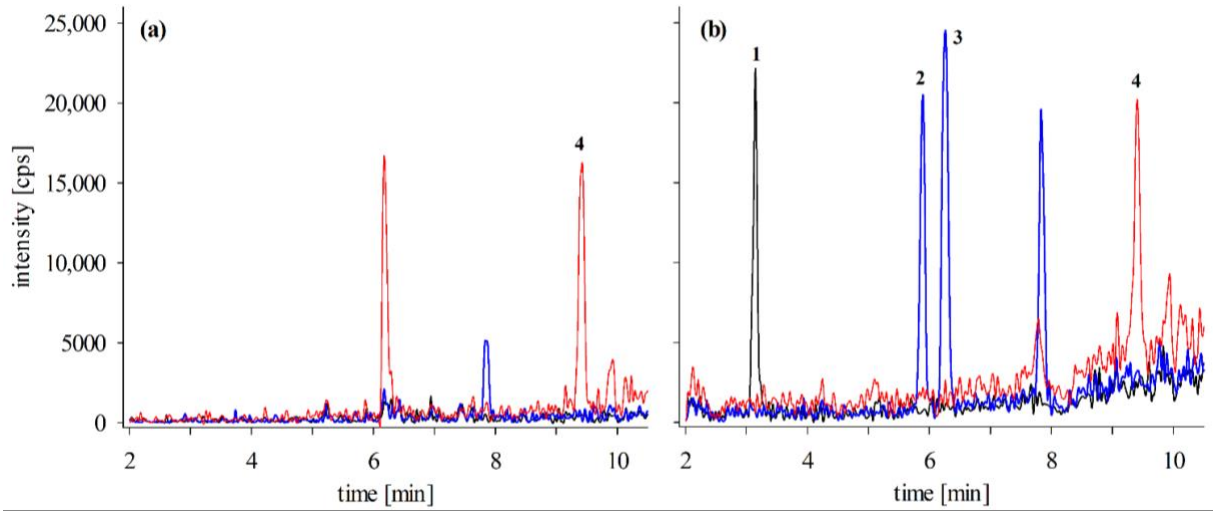


Figure 36. Extracted Ion Chromatograms of an extract of snapper fillet fortified prior to sample preparation with 0.2 μg kg−1 CTX1B [M + H]+ m/z 1111.58361, (1, black line), 52-epi-54-deoxyCTX1B [M + H]+ m/z 1095.58869 (2, blue line), 54-deoxyCTX1B [M + H]+ m/z 1095.58869 (3, blue line), and 0.4 μg kg−1 CTX3C [M + H]+ m/z 1023.56756 (4, red line) with (a) filtrate and (b) eluate fraction; m/z are provided in Table 3 in Chapter 1. Image is Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license by Spielmeyer et al. 2021 [156].

5.3.2. Extract Suitability for *in vitro* Assay (N2a-assay) Analysis

The enzyme protocol was designed with a focus on an optimized method for LC-MS/MS analysis. However, because the two-tiered CTX analysis approach is used for CP outbreak response and prevention programs (e.g., on the Canary Islands, [428]), and the screening method typically used is the *in vitro* N2a-assay, the extracts were investigated for their suitability for being applied to the N2a-assay.

A snapper fillet extracted from raw material and an extract prepared from freeze-dried snapper was applied to the assay at a dosage of up to 15.62 mg wet TE and 1.56 mg dry TE; irrespective of the extract final product used (eluate, filtrate, or combination). There was no observable adverse effect on the viability of the cells (either growth or death) when a comparison between unexposed control cells (with or without the addition of O/V) in the presence of a blank matrix extract was conducted. If these concentrations were exceeded an observed increase in cell viability (growth of approximately +15% above the control) was recorded starting at a tissue-equivalent (TE) dose application of 31.25 mg wet TE and 3.13 mg dry TE. Other studies investigating matrix interference effects on the N2a-assay recommended a maximum matrix load of 4.6 mg TE (20 mg TE mL^{-1}) or 10 mg dry TE mL^{-1} extract to avoid potential matrix interferences [491]. However, as noted elsewhere [231,395] the optimum maximum tissue dose equivalent (MTDE) was found to be species-dependent, and the lipid content was considered as a relevant factor. For fish with a low- and medium-lipid content, an MTDE of 50 mg TE was proposed, whereas, for high-lipid content fish, a limit of 5 mg TE was applied [395]. In agreement with those results, no matrix effect was observed in the naturally incurred sample extracts when 30 mg wet TE was used (Figure 37c). Because the matrix contained within the type of tissue and species of fish sampled is variable, so too can these differences impact the response observed in the assay, in species-specific (i.e., matrix) ways. A blank reference fish (*L. malabaricus*) was tested and found to be less suitable for the assay (lower MDTE) in these conditions when compared with the naturally incurred species (*L. bohar*) utilized here, providing additional evidence that species-specific differences in the MDTE exist.

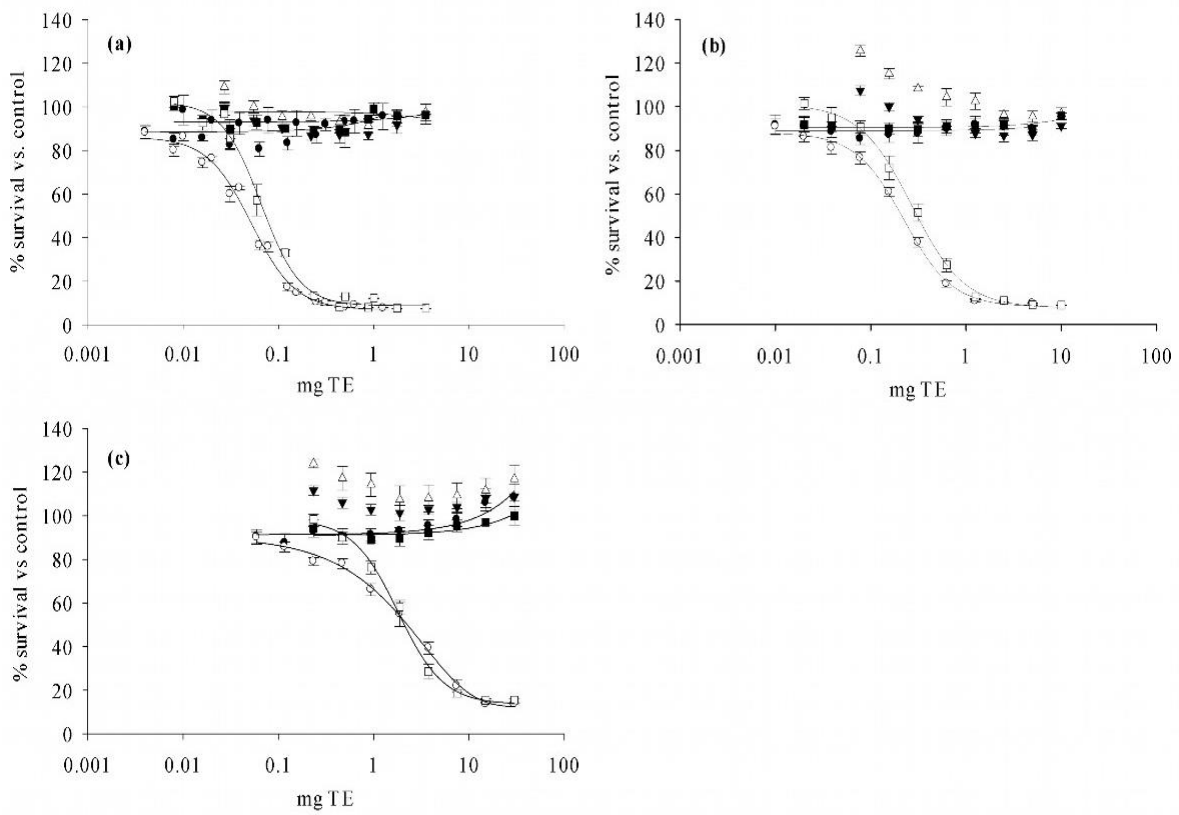


Figure 37. Dose-response curves of extracts obtained for naturally contaminated *L. bohar* samples of a) high, b) medium, and c) low toxicity with filtrate (triangles), eluate (squares), and combination of filtrate and eluate (circles) tested; open and black symbols show samples with and without ouabain/veratridine (+OV/-OV) addition, respectively; data points and error bars are based on a minimum of three independent 96-well plate analyses and triplicate wells per data point per plate/sample.

In the presence of CTXs, extracts derived from naturally contaminated material resulted in dose-response curves that were deemed suitable for the calculation of an EC_{50} (Figure 37). Between the two SPE extract, final products (Eluate and Filtrate) assay analysis results were comparable for either the eluate or the combination of eluate and filtrate with an EC_{50} of 0.066 and 0.052 mg wet TE (high toxicity), 0.285 and 0.278 mg wet TE (medium), and 1.83 and 2.26 mg wet TE (low), respectively. To compare the performance of the extraction products of the current method presented here, the same material was extracted with a previously described and commonly used established extraction method [480]. The data were similar for the EC_{50} values achieved (0.055, 0.268, and 2.17 mg wet TE, respectively). Because both methods of extraction resulted in similar quantification estimates, this implies that the extraction efficiencies of the established method and the new enzyme protocol presented here are comparable. Therefore, the enzyme treatment before extraction is a suitable

alternative to a method based on mechanical treatment. Additionally, the treatment of a sample by enzymatic digestion does not increase the number of matrix peaks in the investigated fish species. Because this method does not introduce any additional matrix peaks it does not deter the identification of CTX congeners when investigating samples without reference standards [156]. Therefore, this protocol using an enzyme treatment results in extracts deemed suitable, for analysis by both a functional bioassay (N2a-assay) and an instrumental analytical method (LC-MS/MS). Together these detection methods represent a commonly applied for CP response analysis and diagnostic support. Therefore, the consolidation of an extraction protocol into one that is suitable for two divergent analysis methods maintains a simplified workflow for the commonly used two-tier approach for CTX analysis when providing analytical confirmation of CP events.

5.4 Conclusions

In this study, a novel sample preparation protocol for CTX analysis in fish was developed [156]. The purpose was to improve several drawbacks to existing extraction methods that are based on mechanical break-down of tissue, which can result in an incomplete extraction of CTXs, be time-intensive, and create a large waste stream. To overcome these issues enzymatic digestion of the fish tissue was performed, followed by extraction, defatting, RP, and NP SPE. The suitability of the enzyme method was demonstrated using various CP-associated fish species, consisting of both fillet and freeze-dried matrices, and the method was proven to be applicable for the two-tiered CTX analysis protocol workflow. Specifically, extracts were appropriate for application in the N2a-assay which can detect unknown CTXs based on their mode of action, and without uncharacteristically high matrix effects which can inhibit detection. Samples with an unknown CTX profile can be investigated with the inclusion of a broad range of congeners, described in the workflow. Future studies will investigate samples involved in CP outbreaks, as well as environmental samples from global CP endemic regions using this new method. To approach the necessary elucidation of the trophic transfer mechanisms and metabolic pathways of CTXs in biological food webs of endemic regions this method will be applied in support of ongoing CP risk assessment efforts.

5.5 Bibliography

25. Caillaud, A.; de la Iglesia, P.; Darius, H.T.; Pauillac, S.; Aligizaki, K.; Fraga, S.; Chinain, M.; Diogene, J. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of ciguatera fish poisoning in Europe. *Marine Drugs* **2010**, *8*, 1838-1907, doi:Doi 10.3390/Md8061838.

37. FAO and WHO. Report of the expert meeting on ciguatera poisoning. Rome, 19–23 November 2018 *Food Safety and Quality No. 9. Rome* **2020**, *https://doi.org/10.4060/ca8817en*, doi:https://doi.org/10.4060/ca8817en.
45. EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:https://doi.org/10.2903/j.efsa.2010.1627.
58. Friedemann, M. Ciguatera fish poisoning outbreaks from 2012 to 2017 in Germany caused by snappers from India, Indonesia, and Vietnam. *Journal of Consumer Protection and Food Safety* **2019**, *14*, 71-80, doi:10.1007/s00003-018-1191-8.
59. Loeffler, C.R.; Tartaglione, L.; Friedemann, M.; Spielmeyer, A.; Kappenstein, O.; Bodi, D. Ciguatera Mini Review: 21st Century Environmental Challenges and the Interdisciplinary Research Efforts Rising to Meet Them. *International Journal of Environmental Research and Public Health* **2021**, *18*, 3027.
156. Spielmeyer, A.; Loeffler, C.R.; Bodi, D. Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. *Toxins* **2021**, *13*, 630.
169. Yogi, K.; Sakugawa, S.; Oshiro, N.; Ikehara, T.; Sugiyama, K.; Yasumoto, T. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. *Journal of AOAC INTERNATIONAL* **2014**, *97*, 398-402.
231. Viallon, J.; Chinain, M.; Darius, H.T. Revisiting the Neuroblastoma Cell-Based Assay (CBA-N2a) for the Improved Detection of Marine Toxins Active on Voltage Gated Sodium Channels (VGSCs). *Toxins* **2020**, *12*, 281.
232. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Hokama, Y.; Dickey, R.W.; Granade, H.R.; Lewis, R.; Yasumoto, T.; Wekell, M.M. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC INTERNATIONAL* **1995**, *78*, 521-527, doi:https://doi.org/10.1093/jaoac/78.2.521.
239. Loeffler, C.R.; Bodi, D.; Tartaglione, L.; Dell'Aversano, C.; Preiss-Weigert, A. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). *Harmful Algae* **2021**, *103*, 101994, doi:https://doi.org/10.1016/j.hal.2021.101994.
243. Lewis, R.J.; Yang, A.; Jones, A. Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh. *Toxicon* **2009**, *54*, 62-66, doi:http://dx.doi.org/10.1016/j.toxicon.2009.03.013.
247. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerford, J.M.; Wekell, M.M. Tetrazolium-Based Cell Bioassay for Neurotoxins Active on Voltage-Sensitive Sodium Channels: Semiautomated Assay for Saxitoxins, Brevetoxins, and Ciguatoxins. *Analytical Biochemistry* **1993**, *214*, 190-194, doi:http://dx.doi.org/10.1006/abio.1993.1476.
365. (EC), R. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. 02004R0854 — EN — 01.01.2019 — 013.001 — 1 ed.; Council, E.P.a.o.t., Ed. 2004; Vol. Regulation (EC) No 854/2004.
366. Regulation (EC). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. In *Official Journal of the European Union. L 139/55*, 2004; Vol. 47.
367. (EC), R. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019. In *02019R0627 — EN — 01.01.2021 — 001.001 — 1*, European Parlament, C.o.t.E.U., Ed. 2019.
395. Castro, D.; Manger, R.; Vilariño, O.; Gago-Martínez, A. Evaluation of Matrix Issues in the Applicability of the Neuro-2a Cell Based Assay on the Detection of CTX in Fish Samples. *Toxins* **2020**, *12*, 308.
428. Sanchez-Henao, J.A.; García-Álvarez, N.; Fernández, A.; Saavedra, P.; Silva Sergent, F.; Padilla, D.; Acosta-Hernández, B.; Martel Suárez, M.; Diogène, J.; Real, F. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the

Canary Islands. *Science of The Total Environment* **2019**, *673*, 576-584, doi:https://doi.org/10.1016/j.scitotenv.2019.03.445.

479. Harwood, D.T.; Murray, S.; Boundy, M.J. Sample Preparation Prior to Marine Toxin Analysis. *Comp Anal C* **2017**, *78*, 89-136, doi:https://doi.org/10.1016/bs.coac.2017.07.003.
480. Dickey, R.W. Ciguatera Toxins: Chemistry, Toxicology, and Detection. In *Seafood and Freshwater Toxins. Boca Raton: CRC Press* Botana, L.M., Ed. CRC Press: 2008; Vol. Second Edition, pp. 479-500.
481. Nagae, M.; Igarashi, T.; Mizukoshi, K.; Yasumoto, T.; Kuniyoshi, K.; Oshiro, N. Development and validation of an LC-MS/MS method for the ultra-trace analysis of Pacific ciguatoxins in fish. *Journal of AOAC INTERNATIONAL* **2021**, https://doi.org/10.1093/jaoacint/qsab052, 1-10, doi:https://doi.org/10.1093/jaoacint/qsab052.
482. Estevez, P.; Castro, D.; Manuel Leao, J.; Yasumoto, T.; Dickey, R.; Gago-Martinez, A. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. *Food Chem* **2019**, *280*, 8-14, doi:https://doi.org/10.1016/j.foodchem.2018.12.038.
483. Stewart, I.; Eaglesham, G.K.; Poole, S.; Graham, G.; Paulo, C.; Wickramasinghe, W.; Sadler, R.; Shaw, G.R. Establishing a public health analytical service based on chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples. *Toxicon* **2010**, *56*, 804-812, doi:https://doi.org/10.1016/j.toxicon.2009.07.028.
484. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.
485. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls.
486. Dechraoui Bottein, M.-Y.; Wang, Z.; Ramsdell, J.S. Toxicokinetics of the ciguatoxin P-CTX-1 in rats after intraperitoneal or oral administration. *Toxicology* **2011**, *284*, 1-6, doi:http://dx.doi.org/10.1016/j.tox.2011.02.005.
487. Hahn, S.T.; Capra, M.F.; Walsh, T.P. Ciguatoxin-protein association in skeletal muscle of Spanish mackerel (Scomberomorus commersoni). *Toxicon* **1992**, *30*, 843-852, doi:http://dx.doi.org/10.1016/0041-0101(92)90382-F.
488. Vernoux, J.P.; Lahlou, N.; Abbad el Andaloussi, S.; Riyeche, N.; Magras, L.P. A study of the distribution of ciguatoxin in individual Caribbean fish. *Acta Trop* **1985**, *42*, 225-233.
489. Kristinsson, H.G.; Rasco, B.A. Fish protein hydrolysates: Production, biochemical, and functional properties. *Crit Rev Food Sci* **2000**, *40*, 43-81, doi:https://doi.org/10.1080/10408690091189266.
490. Kaufmann, A.; Butcher, P.; Maden, K.; Walker, S.; Widmer, M. Semi-targeted residue screening in complex matrices with liquid chromatography coupled to high resolution mass spectrometry: current possibilities and limitations. *Analyst* **2011**, *136*, 1898-1909, doi:10.1039/C0AN00902D.
491. Caillaud, A.; Eixarch, H.; de la Iglesia, P.; Rodriguez, M.; Dominguez, L.; Andree, K.B.; Diogene, J. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2012**, *29*, 1000-1010, doi:10.1080/19440049.2012.660707.

Chapter 6. General Summary and Conclusion

The focus of this thesis was on the issue of CP in general and it was therefore designed to investigate the conditions leading up to ciguatoxin acquisition in fish and finally to a CP outbreak. To accomplish these investigative efforts three main areas of focus were identified, 1. Forecasting risk (to prevent illnesses from occurring), 2. The development of methods to help investigate the causative compound (improving detection capabilities), and 3. The application of a holistic approach to an actual ciguatera outbreak event (proof of concept). In Chapter 2 modern human health food safety methods which were designed for investigating ciguatera outbreaks were used in the region of Hawai'i to investigate the area for ciguatoxins. Herein the status of this region was confirmed as being a hyperendemic area for CP. DNA barcoding was used to confirm the invasive species status of the target CP vector (*Cephalopholis argus*) investigated and representative specimens were cataloged and entered into the Smithsonian National Fish Collection in the United States with the corresponding data freely available and openly accessible. Of all the fish that were tested around the island of Hawai'i 76% were found to exceed the FDA guidance value for CTX1B (0.01 ng g^{-1}). The analysis was conducted using the N2a-assay and CTX1B confirmation was performed by LC-MS. Regional wave action was used as a remotely observable predictor and found to correlate with CTXs in fish and this was used as a forecaster for CP risk in *C. argus*. Specifically, lower wave energy was found to be associated with a higher CTX content in fish. Additionally, local fisher knowledge describing areas known to be distinct for CTXs was used, and this information was found to be accurate; providing evidence that local fishers have an understanding that can generally recognize areas of higher risk for CP. The content of Chapter 2 was selected for publication in the journal Environmental Research and the FDA provided funding to ensure the data is openly accessible.

The focus of Chapter 3 was the evaluation of an influential performance factor for the *in vitro* N2a-assay method of detection for CTXs and PbTxs, with the stated goal of improving the usability and toxin detection sensitivity. A new cell line modification was developed and described as (OV-LS), then tested for cryo-preservation and thaw suitability (for long-term transport and use), growth rate, and compound detection in the fish tissue matrix. Detection performance for CTX1B, CTX3C, and PbTx-3 was evaluated for non-modified N2a and modified OV-LS N2a cells. OV-LS N2a cells were 1.3–2.6-fold more sensitive for detecting CTX3C, CTX1B, and PbTx-3 compared to the standard N2a cells. Furthermore, the detection quantification sensitivity of PbTx-3 reported herein is now the lowest yet described by the N2a-assay. Therefore, the results of Chapter 3 help to meet the challenges of

improving method development for toxin detection, which in turn can provide more information on the emergent occurrence of CTXs in fish, through an increase in detection limit sensitivity and working toward the goal of optimizing a CP method other than the mouse bioassay; which are stated goals of the European Food Safety Authority [45]. Chapter 3 was selected for publication in the journal Harmful Algae and funding was provided by the BfR to ensure the publication is openly accessible.

In Chapter 4 an investigation into a major CP outbreak that occurred In Germany was conducted. The outbreak was caused by imported snapper fish labeled as *Lutjanus malabaricus* and a trace-back study was described. DNA barcoding identified the responsible species as *L. bohar* and therefore, revealed product mislabeling. CTX analysis was conducted by the OV-LS N2a-assay (developed in Chapter 3) and LC-MS/MS. All samples revealed CTX-like toxicity and the presence of CTXs of the CTX3C-group. Together this constituted the first demonstration in Germany of an established method for toxicity evaluation of CTXs in suspected and outbreak-related fish (a major stated goal for the BfR). Chapter 4 has been prepared and will be submitted for publication in the journal Food and Chemical Toxicology.

Chapter 5 describes the development of a novel single-day CTX extraction (sample preparation) and analysis protocol for fish, with variable matrices and variable tissue conditions (i.e., fillet, freeze-dried material). The extracts were found to be suitable for application to the N2a-assay as well as by LC-MS/MS; and therefore is appropriate for use in the two-tiered workflow for CTX analysis investigations (the preferred workflow for CTX analysis). Several additional benefits were realized with this method including a reduction in the use of hazardous solvents during extraction and by using freeze-dried material shipping and transfer costs for material transfer agreements can be greatly reduced. Chapter 5 was the subject of a publication in the journal Toxins and is openly accessible from funding from the BfR.

Taken together, the complete works presented here constitute preventative measures that can be applied to preempt the harvest of fish containing CTXs from reaching the market (a major goal of fisheries stakeholders and consumers). Furthermore, a novel methodology was developed to improve the workflow for researchers and the demonstration of a lower LOD and LOQ to ensure that food safety measures can be followed for consumer protection. And finally, the work exhibits a combined method workflow for a complete CP trace-back effort from source to fork to showcase a model response effort for responding to CP.

Bibliography

45. EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. *EFSA Journal* **2010**, *8*, 1627, doi:https://doi.org/10.2903/j.efsa.2010.1627.

Abbreviations

©- Copyright
®- Registered trademark symbol
ANOVA – Analysis of Variance ATPase
Art. – Article
ATCC – American Type Culture Collection
BfR- German Federal Institute for Risk Assessment
CAS- Chemical Abstracts Service
Cat. No. – Catalogue number
CBA- Cell based assay
CC – Creative Commons
CC-BY- Creative Commons with Attribution
CC-BY-NC- Creative Commons By Author for Non-commercial purposes
CCL – Proprietary name for Neuro-2a Cell line by ATCC (CCL-131)
C-CTX – Caribbean Sea Ciguatoxin
CDC – Centers for Disease Control and Prevention (United States)
CFP- Ciguatera Fish Poisoning
CFS – Chronic fatigue syndrome
cm – Centimeter
CN- Combined nomenclature (a customs/terrif designation required in the EU)
CP – Ciguatera poisoning
CTX- Ciguatoxin
CRM- Certified reference material
CYP3A5 – Cytochrome P450 Family 3 Subfamily A Member 5 (a Protein coding gene)
Da -Dalton (unified atomic mass unit)
DMSO – Dimethyl Sulfoxide
DNA - Deoxyribonucleic acid
e.g. – For example
$EC_{\#}$ – Effective concentration
EC – European Commission
EFSA – European Food Safety Authority
EIC- Extracted Ion Chromatogram
epi - Epimer
Eq. – Equivalents
ESI – Electrospray Ionization
Et al. – Latin meaning 'and others'
EU – European Union
FAO – Food and Agricultural Organization
FBS- Fetal bovine serum
FDA- United States Food and Drug Administration
FLIPR- Flourescent Imaging Plate Reader
Gbp – Gigabase pairs
Gen.- Genera
GI – Gastrointestinal symptoms
GmbH- Gesellschaft mit beschränkter Haftung (Company with limited liability)
HPLC- High-performance liquid chromatography
hr – Hour
HRMS- High resolution Mass Spectrometry
HSD- Honestly significant difference test
i.e. – In other words
IAEA- International Atomic Energy Agency
I-CTX – Indian Ocean Ciguatoxin
ID- identification
In-situ – Latin: on site (sampling conducted in place)
In-vitro – Latin: *in glass* (to study in culture)
In-vivo- Latin: within the living (experimentation done in a whole organism)
IOC- Intergovernmental Oceanographic Commission
IP - Intraperitoneal injection
K^+ - Potassium ion
kg – Kilograms
km – Kilometer
kw – Kilowatts
LC- Liquid Chromatography
LD -Lethal Dose
LGL – Bavarian State Office for Health and Food Safety
LOD – Limit of detection
LOQ – limit of quantification
m – Meter
m/z- Mass-to-charge ratio
MDPI- Multidisciplinary Digital Publishing Institute
MDTE – Maximum tissue dose equivalent
mM – millimolar
MRM – Multiple Reaction Monitoring
MS – Mass Spectrometry
MS/MS- Tandem Mass spectrometry
MTT- Yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MTX – Maitotoxin
n – Number
N2a -Neuro-2a
Na^+- Sodium ion
Na_v – Voltage-gated sodium channel

ng – Nanograms
nm – Nanometer
NMNH – Smithsonian National Museum of Natural History
NOAA - National Oceanic and Atmospheric Administration (United States)
NP – Normal-phase
NSP- Neurotoxic shellfish poisoning
O- Ouabain
OV-**LS** – Ouabain and veratridine lower sensitivity
PAR- Photosynthetically active radiation
PBS- Phosphate-Buffered Saline
PbTx – Brevetoxin
P-CTX- Pacific Ocean Ciguatoxin (CTX1B)
pH- Potential of hydrogen
ppb – parts per billion
ppm – parts per million
R – Resistant
RASFF- the Rapid Alert System for Food and Feed
RP – Reverse-phase
RPMI- Roswell Park Comprehensive Cancer Center (formerly Roswell Park Memorial Institute)
RSSL- Reference Standard Sequence Library
S9- Supernatant fraction obtained from liver homogenate
SD- Standard deviation
SEM – Scanning Electron Microscopy
SH-**SY5Y** – Human derived cell line (neuroblastoma)
SIDS- Small Island Developing States
SPE – Solid-phase extraction cartridge
spp. Plural form for 'Species'
SST- Sea surface temperature
TEF – Toxicity equivalent factor
TL- Total length
™ - Trade mark
TOF – Time of flight
v - voltage
V- Veratridine
v/v – volume per volume
WHO – World Health Organization
ww- wet weight
Yr- year
µg – microgram

End of document